Steroids 76 (2011) 1232–1240

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Synthesis of 3- and 21-monosulfates of [2,2,3β,4,4-$^2H_5$]-tetrahydrocorticosteroids in the 5β-series as internal standards for mass spectrometry

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ARTICLE INFO

Article history:
Received 12 April 2011
Received in revised form 25 May 2011
Accepted 30 May 2011
Available online 25 June 2011

Keywords:
Multiple deuteration
Hydrogen–deuterium exchange
Sodium borodeuteride
Internal standard
Electrospray ionization
Mass spectrometry

ABSTRACT

The 3- and 21-monosulfates of pentadeuterated 5β-tetrahydrocorticosteroides were synthesized, starting from cortisol and 11-deoxycotisol. The principal reactions used were (1) perdeuteration of the methylene groups adjacent to the 3-oxo group of 17,20:20,21-bismethylendioxy-5β-3-ketosteroids with NaOD in CH_3OD followed by stereoselective reduction with $NaBD_4$, (2) sulfation of hydroxy groups with sulfur trioxide–trimethylamine complex, and (3) removal of the 17,20:20,21-bismethylendioxy group with hydrogen fluoride. The labeled compounds can be used as internal standards in liquid chromatography/mass spectrometry assays for clinical and biochemical studies.

1. Introduction

Glucocorticoids (cortisol and cortisone in man) are synthesized in and secreted from the *zona fasciculata* of the adrenal grand, under the control of the adrenocorticotropic hormone (ACTH, also known as corticotropin) which is secreted from the anterior lobe of the pituitary gland [1–3]. They are metabolized by several enzymes, including irreversible inactivation by A-ring reductases (5α- and 5β-reductase). They also undergo reversible activation and inactivation mediated by 11β-hydroxysteroid dehydrogenase that converts active cortisol to inactive cortisone as well as converting tetrahydrocortisol (THF) to tetrahydrocortisone (THE) [3,4]. Further metabolism such as sulfation or glucuronidation (phase 2 biotransformation, conjugation) decreases the biological activity of hormones, decreases protein binding in plasma, and renders them substrates for organic anion transporters, thus promoting their elimination in urine and bile. At least 90% of the tetrahydro-derivatives of cortisol and cortisone metabolites are excreted into the urine as sulfate or glucuronide conjugates [5–8]. Tetrahydro-11-deoxycortisol (THS) and its 5α-stereoisomer (allo-THS) which are tetrahydro-reduced metabolites of 11-deoxycortisol, a biosynthetic precursor of cortisol, are also excreted in urine as conjugated forms. Measurement of the urinary excretion of glucocorticoids (GCs) and their metabolites provides a non-invasive, integrated evaluation of adrenocortical GC secretion, enabling the diagnosis of GC hypersecretion as occurs in Cushing's syndrome and the metabolic syndrome [9–14].

Stable isotope dilution mass spectrometry is widely accepted as the most accurate and specific method for estimation of the small amounts of endogenous and synthetic steroids in biological fluids. We have previously reported a highly sensitive and specific liquid chromatography (LC)/electrospray ionization (ESI)–linear ion trap mass spectrometry (MS) method using a deuterium-labeled internal standard for the direct measurement of 12 tetrahydrocorticosteroid glucuronides in human urine [14]. In addition, we have reported the synthesis of the 18 sulfated conjugates of tetrahydrocorticosteroids as reference standards [15]. The availability of such standards should permit the identification and measurement of all of the sulfate conjugates found in urine using LC/ESI-MS and in turn would permit assessment of the dynamics of their formation and disposal. One significant drawback of such sulfate conjugate analysis, however, is the lack of stable isotope labeled internal standards.

The present paper describes the chemical synthesis of the 3- and 21-sulfated conjugates of tetrahydrocorticosteroids (THS,

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doi:10.1016/j.steroids.2011.05.014

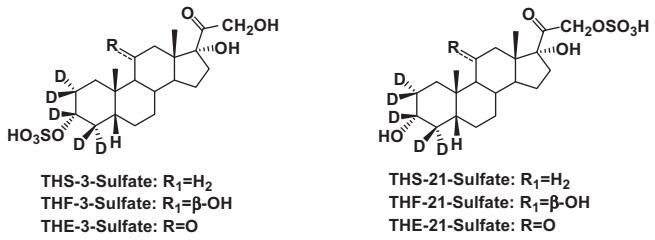

Fig. 1. Structures of 3- and 21-monosulfate conjugates of [2,2,3β,4,4-d_5]-tetrahydrocorticosteroids in the 5β-series.

THF, and THE) at C-2, C-3, and C-4 positions with deuterium atom for use of isotope dilution mass spectrometry (Fig. 1).

2. Experimental

2.1. Materials

11-Deoxycortisol (**1**) and cortisol (**7**) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Silica gel plates (Merck; F_{254}) and silica gel (Merck; 70–230 mesh) were used for analytical and column chromatography, respectively. 40% NaOD in D_2O (99.5 atom% D) and CH_3OD (99.5 atom% D) were supplied by Sigma-Aldrich Japan K. K. (Tokyo, Japan). $NaBD_4$ (99% isotopic purity) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Acetonitrile and ammonium acetate of HPLC grade were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and distilled H_2O of HPLC grade was purchased from Wako Pure Chemical Industries, Ltd. An Oasis® HLB cartridge (adsorbent weight, 1 g) was provided by Waters Co. (Milford, MS, USA) and successively conditions by washings with MeOH and H_2O prior to use. All other chemicals and solvents were analytical grade and obtained from Nacalai Tesque Inc.

2.2. Instruments

All melting points (mp) were determined on a micro hot-stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a JNM-AL400 (JEOL Ltd., Tokyo, Japan) at 400 MHz, with $CDCl_3$ or CD_3OD containing 0.1% Me_4Si as the solvent; chemical shifts were expressed as δ ppm relative to Me_4Si. The following abbreviations are used: s = singlet, d = doublet, m = multiplet. The LC/ESI-MS analyses were carried out using a Finnigan LTQ linear ion-trap mass spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, 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 were as follows: ion source voltage, −4 kV; capillary temperature, 270 °C; capillary voltage, −20 V; sheath gas (N_2 gas) flow rate, 50 arbitrary units; auxiliary gas (N_2 gas) flow rate, 5 arbitrary units; tube lens offset voltage, −100 V. For collision induced dissociation (CID) analysis, helium gas was used as the collision gas. The normalized collision energy and the activation Q value were set at 35% and 0.18, respectively. LC separations were conducted on a reversed-phase (RP) semi-micro column, TSKgel ODS-100S (5 μm, 150 × 2.0 mm I.D.) from Tosoh Co. (Tokyo, Japan) by a linear gradient elution: 15% solvent B (acetonitrile) over 5 min and then 15% solvent B to 50% B against solvent A (5 mM ammonium acetate buffer, pH 6.0) over 30 min at a flow rate of 200 μl/min.

2.3. Chemical synthesis

2.3.1. 17,20:20,21-Bismethylenedioxy-4-pregnen-3-one (*2*)

To a solution of 11-deoxycortisol (**1**, 2 g) in $CHCl_3$ (25 ml) was added 7 M HCl (40 ml) containing paraformaldehyde (4.5 g), and the mixture was stirred at room temperature for 5 h. The $CHCl_3$ was separated and the aqueous phase was extracted with $CHCl_3$. The combined extract was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the product by column chromatography on silica gel with toluene–acetone (20:1, v/v) as an eluent and recrystallization of a homogenous effluent from CH_2Cl_2–MeOH gave **2** as colorless prisms: yield, 1.6 g (71%); mp 255–257 °C (lit. [16], mp 254–255 °C). ^{1}H NMR ($CDCl_3$) δ: 0.87 (s, 3H, 18-CH_3), 1.20 (s, 3H, 19-CH_3), 3.98 and 4.01 (d, each 1H, J = 9.2 Hz, 21-CH_2), 5.04 (s, 2H, OCH_2O), 5.07 and 5.20 (s, each 1H, OCH_2O), 5.73 (s, 1H, 4-H).

2.3.2. 17,20:20,21-Bismethylenedioxy-5β-pregnan-3-one (*3*)

A solution of **2** (1 g) in pyridine (34 ml) was hydrogenated overnight at room temperature in the presence of 5% $Pd/CaCO_3$ catalyst (2 g). After filtration of the catalyst on celite, the filtrate was concentrated to dryness under reduced pressure. Recrystallization of the product from CH_2Cl_2–MeOH gave **3** as colorless plates: yield, 767 mg (76%); mp 182–185 °C (lit. [16], mp 180.5 °C). ^{1}H NMR ($CDCl_3$) δ: 0.85 (s, 3H, 18-CH_3), 1.03 (s, 3H, 19-CH_3), 3.98 and 4.01 (d, each 1H, J = 9.2 Hz, 21-CH_2), 5.05, 5.06, 5.09, and 5.20 (s, each 1H, 2× OCH_2O).

2.3.3. [2,2,3β,4,4-d_5]-17,20:20,21-Bismethylenedioxy-3α-hydroxy-5β-pregnane (*4a*)

To a solution of **3** (200 mg) in CH_3OD (10 ml) and dry dioxane (1.2 ml) was added 40% NaOD (1 ml) and D_2O (1.2 ml), and the mixture was stirred at 60 °C for 22 h under a gentle stream of N_2 gas. After being cooled in an ice bath, $NaBD_4$ (40 mg) was added to the solution and the reaction mixture was stirred at room temperature for 30 min. After addition of 10% AcOH to decompose the excess reagents, the organic solvent was evaporated under reduced pressure. The residue was diluted with EtOAc, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the crude product by column chromatography on silica gel with *n*-hexane–EtOAc (5:1, v/v) as an eluent and recrystallization of a homogeneous effluent from acetone–*n*-hexane gave **4a** as colorless plates: yield, 128 mg (64%); mp 180–181 °C. Mixed mp with the non-deuterated compound (lit. [16], mp 179–180 °C) showed no depression. ^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 3H, 18-CH_3), 0.86 (s, 3H, 19-CH_3), 3.90 and 3.93 (d, each 1H, J = 9.4 Hz, 21-CH_2), 4.97, 4.98, 5.01, and 5.12 (s, each 1H, 2× OCH_2O).

2.3.4. [2,2,3β,4,4-d_5]-17α,21-dihydroxy-3α-sulfooxy-5β-pregnan-20-one sodium salt ([2,2,3β,4,4-d_5]-THS-3-sulfate sodium salt; *5*)

To a solution of compound **4a** (112 mg) in dry pyridine (3 ml) was added sulfur trioxide–trimethylamine (SO_3–TMA) complex (60 mg) and the mixture was stirred at room temperature for 3 h. After evaporation of the solvent under reduced pressure, the residue dissolved in a small amount of H_2O was adjusted to pH 8 with 20% NaOH and loaded onto an Oasis® HLB cartridge. After being washed with H_2O, elution with MeOH gave the intermediary 3α-sulfate (**4b**), which without isolation it was treated with 46% hydrogen fluoride (3 ml) in EtOH (0.4 ml)–tetrahydrofuran (0.4 ml) and kept at ice temperature for 7 h. After addition of saturated Na_2CO_3 to adjust to pH 8, the resulting solution was diluted with H_2O and centrifuged at 3000 rpm for 10 min. The supernatant was loaded onto an Oasis® HLB cartridge. After being washed with H_2O, the desired sulfate was eluted with MeOH. Purification of the product by column chromatography on silica

gel with $CHCl_3$–MeOH (5:1, v/v) as an eluent and recrystallization of a homogenous effluent from Et_2O–MeOH gave **5** as colorless amorphous substances: yield, 75 mg (58%); mp 195 °C (dec.). Mixed mp with the non-deuterated compound [15] showed no depression. ^{1}H NMR (CD_3OD) δ: 0.60 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 4.28 and 4.64 (d, each 1H, J = 19.2 Hz, 21-CH_2). ES-MS analysis indicated d_0 (0.00%), d_1 (0.00%), d_2 (0.00%), d_3 (0.11%), d_4 (8.52%), d_5 (91.37%).

2.3.5. [2,2,3β,4,4-d_5]-3α-Acetoxy-17α,21-dihydroxy-5β-pregnan-20-one (**6a**)

The compound **4a** (123 mg) was acetylated with Ac_2O (0.25 ml) in pyridine (0.5 ml) at room temperature for 20 h. After addition of H_2O, the resulting solution was extracted with EtOAc. The organic layer was washed with 5% HCl, 5% $NaHCO_3$, and H_2O, and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure afforded the 3α-acetoxy derivative (**4c**), which without isolation it was treated with 46% hydrogen fluoride (3 ml) in EtOH (0.5 ml)–tetrahydrofuran (0.5 ml) at ice temperature for 7 h. After addition of saturated Na_2CO_3 to adjust the pH to 8, the resulting solution was extracted with EtOAc. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the crude product by column chromatography on silica gel with *n*-hexane–EtOAc (1:1, v/v) as an eluent and recrystallization of a homogenous effluent from acetone–*n*-hexane gave **6a** as colorless needles: yield, 67 mg (55%); mp 197–198 °C. Mixed mp with the non-deuterated compound [17,18] showed no depression. ^{1}H NMR ($CDCl_3$) δ: 0.65 (s, 3H, 18-CH_3), 0.93 (s, 3H, s, 19-CH_3), 2.03 (s, 3H, $OCOCH_3$), 4.30 and 4.66 (d, each 1H, J = 20.0 Hz, 21-CH_2).

2.3.6. [2,2,3β,4,4-d_5]-3α,17α-Dihydroxy-21-sulfooxy-5β-pregnan-20-one sodium salt ([2,2,3β,4,4-d_5]-THS-21-sulfate sodium salt; **6c**)

To a solution of **6a** (39 mg) in dry pyridine (0.8 ml) was added SO_3–TMA complex (20 mg), and the mixture was stirred at room temperature for 5 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in a small amount of H_2O, the pH adjusted to pH 8 with 20% NaOH, and the solution loaded onto an Oasis® HLB cartridge. After being washed with H_2O, elution with MeOH gave the intermediary 3α-acetoxy-21-sulfate (**6b**), which without isolation it was hydrolyzed with 20% NaOH (0.5 ml) in MeOH (1.5 ml) at room temperature for 1 h. After evaporation of the solvent, the residue dissolved in H_2O was loaded onto an Oasis® HLB cartridge. After being washed with H_2O, the desired sulfate was eluted with MeOH. Purification of the crude product by column chromatography on silica gel with $CHCl_3$–MeOH (4:1, v/v) as an eluent and recrystallization of a homogeneous effluent from Et_2O–MeOH gave **6c** as colorless amorphous substances: yield, 15 mg (33%); mp 190 °C (dec.). Mixed mp with the non-deuterated compound [15] showed no depression. ^{1}H NMR (CD_3OD) δ: 0.62 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 4.82 and 5.16 (d, each 1H, J = 18.0 Hz, 21-CH_2). ESI-MS analysis indicated d_0 (0.02%), d_1 (0.01%), d_2 (0.01%), d_3 (0.05%), d_4 (10.15%), d_5 (89.76%).

2.3.7. 17,20:20,21-Bismethylenedioxy-11β-hydroxy-4-pregnen-3-one (**8**)

Cortisol (**7**, 4 g) was treated with 7 M HCl (40 ml) containing paraformaldehyde (4.5 g) in $CHCl_3$ (40 ml), as described for preparation of **2**. After being processed in an analogous manner, the product was subjected to column chromatography on silica gel with toluene–acetone (5:1, v/v) as an eluent. Recrystallization of a homogenous effluent from acetone–MeOH gave **8** as colorless needles: yield, 2.91 g (65%); mp 230–232 °C (lit. [19], mp 220–223 °C from Et_2O–MeOH and lit. [20], 222–227 °C from EtOAc). ^{1}H NMR ($CDCl_3$) δ: 1.13 (s, 3H, 18-CH_3), 1.45 (s, 3H, 19-CH_3), 3.98 and 4.01 (d, each 1H, J = 9.4 Hz, 21-CH_2), 4.40–4.43 (m, 1H, 11α-H), 5.02, 5.03, 5.05 and 5.22 (s, each 1H, 2× OCH_2O), 5.68 (s, 1H, 4-H).

2.3.8. 17,20:20,21-Bismethylenedioxy-11β-hydroxy-5β-pregnan-3-one (**9**)

The compound **8** (1.14 g) was hydrogenated with 10% $Pd(OH)_2$/C (114 mg) in pyridine (10 ml), as described for preparation of **3**. After being processed in an analogous manner, the product was recrystallized from acetone–MeOH to give **9** as colorless needles: yield, 770 mg (67%); mp 239–241 °C (lit. [21], 239–240 °C). ^{1}H NMR ($CDCl_3$) δ: 1.10 (s, 3H, 18-CH_3), 1.27 (s, 3H, 19-CH_3), 3.98 and 4.02 (d, each 1H, J = 8.8 Hz, 21-CH_2), 4.33–4.38 (m, 1H, 11α-H), 5.04, 5.05, 5.06, and 5.23 (s, each 1H, 2× OCH_2O).

2.3.9. Reduction of **9** with sodium borohydride

To a solution of **9** (100 mg) in MeOH (0.3 ml)–tetrahydrofuran (0.4 ml) was added $NaBH_4$ (60 mg) and the mixture was stirred at room temperature for 30 min. After addition of 10% AcOH to decompose the excess reagent, the resulting solution was diluted with EtOAc, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the crude product by column chromatography on silica gel with *n*-hexane–EtOAc (1:1, v/v) and recrystallization of a less polar effluent from acetone–*n*-hexane gave 17,20:20,21-bismethylenedioxy-3β,11β-dihydroxy-5β-pregnane (**10a**) as colorless plates: yield, 11 mg (11%); mp 203–205 °C. ^{1}H NMR ($CDCl_3$) δ: 1.07 (s, 3H, 18-CH_3), 1.22 (s, 3H, 19-CH_3), 3.96 and 4.02 (d, each 1H, J = 8.8 Hz, 21-CH_2), 4.08–4.12 (m, 1H, 3α-H), 4.26–4.28 (m, 1H, 11α-H), 5.04, 5.05, 5.06, and 5.22 (s, each 1H, 2× OCH_2O). Recrystallization of a more polar effluent from acetone–*n*-hexane gave 17,20:20,21-bismethylenedioxy-3α,11β-dihydroxy-5β-pregnane (**10b**) as colorless plates: yield, 85 mg (85%); mp 216–218 °C. ^{1}H NMR ($CDCl_3$) δ: 1.06 (s, 3H, 18-CH_3), 1.18 (s, 3H, 19-CH_3), 3.62–3.70 (m, 1H, 3β-H), 3.96 and 4.01 (d, each 1H, J = 8.8 Hz, 21-CH_2), 4.26–4.28 (m, 1H, 11α-H), 5.04, 5.05, 5.06, and 5.22 (s, each 1H, 2× OCH_2O).

2.3.10. 3α-Acetoxy-17,20:20,21-bismethylenedioxy-11β-hydroxy-5β-pregnan (**10c**)

The compound **10b** (80 mg) was acetylated with Ac_2O (0.5 ml) in pyridine (1 ml) at room temperature for 10 h. After addition of H_2O, the resulting solution was extracted with EtOAc. The organic layer was washed with 5% HCl, 5% $NaHCO_3$, and H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Recrystallization of the product from acetone gave **10c** as colorless pillars: yield, 89 mg (100%); mp 209–211 °C. ^{1}H NMR ($CDCl_3$) δ: 1.00 (s, 3H, 18-CH_3), 1.16 (s, 3H, 19-CH_3), δ: 1.99 (s, 3H, $OCOCH_3$), 3.95–3.98 (d, each 1H, J = 9.0 Hz, 21-CH_2), 4.23–4.25 (m, 1H, 11α-H), 4.68–4.76 (m, 1H, 3β-H), 5.02 (s, 2H, OCH_2O), 5.04 and 5.20 (s, each 1H, OCH_2O).

2.3.11. 3α-Acetoxy-17,20;20,21-bismethylenedioxy-5β-pregnan-11-one (**11a**)

To a stirred solution of **10c** (258 mg) in acetone (7 ml) was added Jones' reagent (2.1 ml), and the mixture was continued to stir at room temperature for 30 min. After addition of MeOH to decompose the excess reagent, the organic solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc, washed with 5% $NaHCO_3$ and H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was purified by column chromatography on silica gel with *n*-hexane–EtOAc (1:1, v/v) as an eluent to give **11a** as colorless amorphous substances: yield, 187 mg (83%). ^{1}H NMR ($CDCl_3$) δ: 0.70 (s, 3H, 18-CH_3), 1.09 (s, 3H, 19-CH_3), 1.95 (s, 3H, $OCOCH_3$), 3.87 and 3.91 (d, each 1H, J = 9.4 Hz, 21-CH_2), 4.60–4.68 (m, 1H, 3β-H), 4.94, 5.00, 5.01, and 5.12 (s, each 1H, 2× OCH_2O).

*2.3.12. 17,20:20,21-Bismethylenedioxy-3α-hydroxy-5β-pregnan-11-one (**11b**)*

To a solution of **11a** (168 mg) in MeOH (6.5 ml) was added 20% NaOH (0.7 ml), and the mixture was stirred at room temperature for 1 h. After evaporation of the solvent, the residue was diluted with EtOAc, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the crude product by column chromatography on silica gel with *n*-hexane–EtOAc (1:1, v/v) as an eluent and recrystallization of a homogenous effluent from acetone–*n*-hexane gave **11b** as colorless needles: yield, 128 mg (84%); mp 191–193 °C. ^{1}H NMR ($CDCl_3$) δ: 0.77 (s, 3H, 18-CH_3), 1.15 (s, 3H, 19-CH_3), 3.60–3.67 (m, 1H, 3β-H), 3.94 and 3.97 (d, each 1H, J = 9.2 Hz, 21-CH_2), 5.01, 5.07, 5.08, and 5.19 (s, each 1H, 2× OCH_2O).

*2.3.13. [2,2,3β,4,4-d_5]-17,20:20,21-Bismethylenedioxy-3α,11β-dihydroxy-5β-pregnan (**12a**)*

To a solution of **9** (200 mg) in dry dioxane (1 ml)–CH_3OD (3 ml) were added 40% NaOD (1 ml) and D_2O (1.4 ml), and the solution was heated at 60 °C for 22 h under a gentle stream of N_2 gas. After cooling down, $NaBD_4$ (40 mg) was added to the mixture, and the resulting solution was stirred at room temperature for 30 min. After addition of 10% AcOH to decompose the excess reagent, the resulting solution was diluted with EtOAc, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the crude product by column chromatography on silica gel with *n*-hexane–EtOAc (2:1, v/v) as an eluent and recrystallization of a homogeneous effluent from acetone–*n*-hexane gave **12a** as colorless needles: yield, 184 mg (90%); mp 217–219 °C. Mixed mp with compound **10b** showed no depression. ^{1}H NMR ($CDCl_3$) δ: 1.06 (s, 3H, 18-CH_3), 1.17 (s, 3H, 19-CH_3), 3.98 and 4.01 (d, each 1H, J = 9.0 Hz, 21-CH_2), 4.26–4.29 (m, 1H, 11α-H), 5.04, 5.05, 5.06, and 5.22 (s, each 1H, 2× OCH_2O).

*2.3.14. [2,2,3β,4,4-d_5]-11β,17α,21-trihydroxy-3α-sulfooxy-5β-pregnan-20-one sodium salt ([2,2,3β,4,4-d_5]-THF-3-sulfate sodium salt; **13a**)*

The compound **12a** (67 mg) was treated with SO_3–TMA complex (80 mg) at room temperature, as described for preparation of **4b**. After being processed in an analogous manner, the intermediary 3-sulfate (**12b**) was treated with 46% hydrogen fluoride (2.5 ml) in EtOH (0.3 ml)–tetrahydrofuran (0.3 ml) at ice temperature for 10 h, as described for **5**. After being processed in an analogous manner, the crude product was subjected to column chromatography on silica gel with $CHCl_3$–MeOH (2:1, v/v) as an eluent. Recrystallization of a homogeneous effluent from Et_2O–MeOH gave **13a** as colorless granular: yield, 33 mg (53%); mp 198 °C (dec.). Mixed mp with the non-deuterated compound [15] showed no depression. ^{1}H NMR (CD_3OD) δ: 0.81 (s, 3H, 18-CH_3), 1.17 (s, 3H, 19-CH_3), 4.24–4.25 (m, 1H, 11α-H), 4.27 and 4.64 (d, each 1H, J = 19.0 Hz, 21-CH_2). ESI-MS analysis indicated d_0 (0.00%), d_1 (0.00%), d_2 (0.01%), d_3 (0.05%), d_4 (2.09%), d_5 (97.85%).

*2.3.15. [2,2,3β,4,4-d_5]-3α-Acetoxy-17,20:20,21-bismethylendioxy-11β-hydroxy-5β-pregnane (**12c**)*

The compound **12a** (156 mg) was acetylated at C-3 with Ac_2O (0.5 ml) in pyridine (1 ml) at room temperature for 10 h. After addition of H_2O, the resulting solution was extracted with EtOAc. The organic layer was washed with 5% HCl, 5%, $NaHCO_3$, and H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure, the product was recrystallized from acetone to give **12c** as colorless pillars: yield 171 mg (100%); mp 209–211 °C. Mixed mp with compound **10c** showed no depression. ^{1}H NMR ($CDCl_3$) δ: 1.07 (s, 3H, 18-CH_3), 1.18 (s, 3H, 19-CH_3), 2.02 (s, 3H, $OCOCH_3$), 3.97 and 4.01 (d, each 1H, J = 9.0 Hz, 21-CH_2), 4.24–4.25 (m, 1H, 11α-H), 5.05 (s, 2H, OCH_2O), 5.06 and 5.22 (s, each 1H, OCH_2O).

*2.3.16. [2,2,3β,4,4-d_5]-3α-Acetoxy-11β,17α,21-trihydroxy-5β-pregnan-20-one (**13b**)*

The compound **12c** (134 mg) was treated with 46% hydrogen fluoride (3.5 ml) in EtOH (0.8 ml)–tetrahydrofuran (0.8 ml) at ice temperature for 6 h, as described for **6a**. After being processed in an analogous manner, the crude product was purified by column chromatography on silica gel with *n*-hexane–EtOAc (3:1, v/v) as an eluent. Recrystallization of a homogeneous effluent from acetone–*n*-hexane gave **13b** as colorless needles: yield, 83 mg (68%); mp 192–194 °C. Mixed melting point with the non-deuterated compound [22] showed no depression. ^{1}H NMR ($CDCl_3$) δ: 0.81 (s, 3H, 18-CH_3), 1.12 (3H, s,19-CH_3), 1.96 (3H, s, 3-$OCOCH_3$), 4.22 and 4.58 (d, each 1H, J = 19.0 Hz, 21-CH_2), 4.23–4.24 (m, 1H, 11α-H).

*2.3.17. [2,2,3β,4,4-d_5]-3α,11β,17α-trihydroxy-21-sulfooxy-5β-pregnan-20-one sodium salt ([2,2,3β,4,4-d_5]-THF-21-sulfate sodium salt; **13d**)*

The compound **13b** (73 mg) was treated with SO_3–TMA complex (40 mg) in dry pyridine (1.5 ml) at room temperature for 5 h, as described for **5**. After being processed in an analogous manner, the intermediary 21-sulfate (**13c**) obtained was then hydrolyzed with 20% NaOH (0.1 ml) in MeOH at room temperature for 20 min, as described for **6c**. After evaporation of the solvent under reduced pressure, the residue dissolved in H_2O was loaded onto an Oasis® HLB cartridge and the cartridge was washed with H_2O (2 ml). The desired sulfate was eluted with MeOH. Purification of the crude product by column chromatography on silica gel with $CHCl_3$–MeOH (3:1, v/v) as an eluent and recrystallization of a homogenous effluent from Et_2O–MeOH gave **13d** as colorless granular: yield, 87 mg (95%); mp 186 °C. Mixed mp with the non-deuterated compound [15] showed no depression. ^{1}H NMR (CD_3OD) δ: 0.83 (s, 3H, 18-CH_3), 1.16 (s, 3H, 19-CH_3), 4.24–4.25 (m, 1H, 11α-H), 4.83 and 5.03 (each 1H, d, J = 18.0 Hz, 21-CH_2). ESI-MS analysis indicated d_0 (0.01%), d_1 (0.00%), d_2 (0.00%), d_3 (0.03%), d_4 (4.20%), d_5 (95.76%).

*2.3.18. [2,2,3β,4,4-d_5]-17,20:20,21-bismethylendioxy-3α-hydroxy-5β-pregnane-11-one (**14b**)*

To a solution of **12c** (74 mg) in CH_2Cl_2 (0.3 ml) were added pyridinium chlorochromate (44 mg) and sodium acetate trihydrate (4 mg) and celite (100 mg), and the reaction mixture was stirred at room temperature for 1.5 h. After filtration through a short pad of celite, the filtrate was evaporated to dryness. Purification of the crude product by column chromatography with *n*-hexane–EtOAc (3:1, v/v) as an eluent gave the intermediary 11-ketone **14a** (60 mg), which without isolation it was hydrolyzed with 30% NaOH (0.2 m) in MeOH (1.8 ml) at room temperature for 1 h. After work-up in the usual manner, the crude product was subjected to column chromatography on silica gel with toluene–acetone (4:1, v/v) as an eluent. Recrystallization of a homogeneous effluent from acetone–*n*-hexane gave **14b** as colorless needles: yield, 50 mg (75%); mp 193–195 °C. Mixed mp with compound **11b** showed no depression. ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 3H, 18-CH_3), 1.15 (s, 3H, 19-CH_3), 3.92 and 3.98 (d, each 1H, J = 9.2 Hz, 21-CH_2), 5.01, 5.07, 5.08, and 5.19 (s, each 1H, 2× OCH_2O).

*2.3.19. [2,2,3β,4,4-d_5]-17α,21-dihydroxy-3α-sulfooxy-5β-pregnan-11,20-dione ([2,2,3β,4,4-d_5]-THE-3-sulfate sodium salt: **15a**)*

To a solution of **14b** (40 mg) in dry pyridine (2 ml) was added SO_3–TMA complex (30 mg), and the mixture was stirred at room temperature for 5 h. After evaporation of solvent under a gentle stream of N_2 gas, the residue was dissolved in a small amount of

H_2O, the pH was adjusted to 8 with 20% NaOH, and the solution was loaded onto an Oasis® HLB cartridge. After being washed with H_2O, elution with MeOH gave the intermediary 3-sulfate (**14c**), which without isolation it was treated with 46% hydrogen fluoride (3 ml) in EtOH (0.3 ml)–tetrahydrofuran (0.3 ml) at ice temperature for 10 h, as described for **5**. After being processed in an analogous manner, the crude product was purified by column chromatography on silica gel with $CHCl_3$–MeOH (4:1, v/v) as an eluent. Recrystallization of a homogeneous effluent from MeOH gave **15a** as colorless needles: yield, 9.9 mg (22%); mp 177 °C. Mixed mp with the non-deuterated compound [15] showed no depression. ^{1}H NMR ($CDCl_3$) δ: 0.54 (s, 3H, 18-CH_3), 1.15 (s, 3H, 19-CH_3), 4.24 and 4.63 (d, each 1H, J = 19.6 Hz, 21-CH_2). ESI-MS analysis indicated d_0 (0.00%), d_1 (0.00%), d_2 (0.01%), d_3 (0.06%), d_4 (2.56%), d_5 (97.37%).

*2.3.20. [2,2,3β,4,4-d$_5$]-3α-Acetoxy-17α,21-dihydroxy-5β-pregnane-11,20-dione (**15b**)*

The compound **14a** (60 mg) was treated with 46% hydrogen fluoride (4 ml) in EtOH (0.6 ml)–tetrahydrofuran (0.6 ml) at room temperature for 8 h, as described for **5**. After being processed in an analogous manner, the crude product was subjected to column chromatography on silica gel with toluene–acetone (3:1, v/v) as an eluent. Recrystallization of a homogeneous effluent from EtOAc gave **15b** as colorless needles: yield 18 mg (47%); mp 189–191 °C. Mixed melting point with the non-deuterated compound [20] showed no depression. ^{1}H NMR ($CDCl_3$) δ: 0.60 (s, 3H, 18-CH_3), 1.15 (s, 3H, 19-CH_3), 2.03 (s, 3H, $OCOCH_3$), 4.26 and 4.64 (d, each 1H, J = 17.4 Hz, 21-CH_2).

*2.3.21. [2,2,3β,4,4-d$_5$]-3α,17α-dihydroxy-21-sulfooxy-5β-pregnane-11,20-dione sodium salt ([2,2,3β,4,4-d$_5$]-THE-21-sulfate sodium salt: **15d**)*

The C-21 sulfate ester of compound **15b** (25 mg) was prepared using SO_3–TMA complex (20 mg) in dry pyridine (1 ml) at room temperature for 3 h, followed by alkaline hydrolysis of the resulting intermediary 3α-sulfooxy derivative (**15c**) with 20% NaOH (0.15 ml) in MeOH (1.4 ml) at room temperature for 1 h, as described for preparation of **6c**. After being processed in an analogous manner, the crude product was purified by column chromatography on silica gel with $CHCl_3$–MeOH (4:1, v/v) as an eluent. Recrystallization of a homogeneous effluent from Et_2O–MeOH gave **15d** as colorless granular: yield, 13 mg (44%). mp 192–193 °C (dec.). Mixed mp with the non-deuterated compound [15] showed no depression. ^{1}H NMR (CD_3OD) δ: 0.56 (s, 3H, 18-CH_3), 1.13 (s, 3H, 19-CH_3), 4.73 and 5.02 (d, each 1H, J = 19.6 Hz, 21-CH_2). ESI-MS analysis indicated d_0 (0.00%), d_1 (0.00%), d_2 (0.00%), d_3 (0.00%), d_4 (9.17%), d_5 (90.83%).

3. Results and discussion

The use of stable isotopically labeled (^{13}C, ^{2}H, ^{15}N, ^{18}O) internal standards for the LC/MS analysis offers major advantages that they behave in an almost identical manner to the analytes through all steps in the isolation and chromatographic procedure, thereby allowing procedural losses to be compensated for. The difference between the molecular masses of the analyte and isotopically labeled IS should be at least three mass units to avoid overlapping in the monitored mass peaks. For obtaining the desired stable isotopically labeled sulfates of THF and THE, direct sulfation of commercial sources of [9,11α,12-d$_3$]-THF and [9,12,12,21,21-d$_5$]-THE with sulfating reagent such as SO_3–TMA complex can be considered. However, lack of regioselective sulfation at 3- and 21-hydroxy group of these substrates are possible problems of this synthetic approach. The other method that can be considered is to employ the known synthetic methods [15] starting from commercial sources of stable isotopically labeled cortisol and cortisone. Here, the difficulties encountered are the low yields due to the multiple reaction steps and the high cost of the stable isotopically labeled steroids. Moreover, these synthetic methods are inadequate for preparation of the multiduterated sulfates of THS due to the unavailability of the stable isotopically labeled THS and 11-deoxycortisol containing at least three deuterium atoms in their molecules. As an alternative approach toward the final goal, we undertook to introduce multiple deuterium into the active methylene adjacent to the carbonyl group of the 5β-3-keto steroid derivatives by a keto–enol exchange reaction in active solvent (D_2O and CH_3OD) under alkaline condition followed by stereoselective reduction with $NaBD_4$ leading to the 3α-hydroxy steroids.

Frequently the dihydroxyacetone side chain is unstable or inert to the reaction condition needed to modify the steroid nucleus. The bismethylenedioxy (BMD) group represents the most suitable protecting group for the dihydroxyacetone side chain of corticosteroids. This group is stable to brief heating with NaOH in alcoholic solution and Jones' oxidation, and can be hydrolyzed in a high yield by employing aqueous hydrofluoric acid at low temperature. We therefore prepared 5β-3-keto BMD derivatives (**3** and **9**) as the key compounds starting from 11-deoxycortisol (**1**) and cortisol (**7**) by treatment with paraformaldehyde as a source of alcohol-free formaldehyde in $CHCl_3$ containing HCl followed by catalytic reduction of conjugated Δ^4-3-ketosteroids into their corresponding 5β-3-ketosteroids with $Pd(OH)_2$/C and/or Pd/$CaCO_3$. Exchange reaction of the compound **3** with 20% NaOD in CH_3OD–dioxane followed by reduction with $NaBD_4$ gave [2,2,3β,4,4-d$_5$]-3α-hydroxy-5β-steroid (**4a**), which was effectively separated from the 3β-steroisomer by column chromatography on silica gel. The 3α-hydroxy group in the resulting **4a** was sulfated with SO_3–TMA complex in pyridine. The sulfation reaction proceeded cleanly and rapidly under mild experimental condition. The resulting sulfated compound (**4b**) was hydrolyzed with hydrogen fluoride to remove the BMD protecting group. To purify the crude 3-sulfate of [2,2,3β,4,4-d$_5$]-THS (**5**), it was loaded onto an Oasis® HLB cartridge for reversed-phase solid phase extraction. After the cartridge was washed with H_2O to remove excess reagents and inorganic salts, elution with MeOH yielded a homogenous effluent, which was characterized as [2,2,3β,4,4-d$_5$]-THS-3-sulfate (**5**) after chromatographic purification on silica gel.

Compound **4a** was acetylated at C-3 to obtain 3-acetate (**4c**), which in turn was hydrolyzed with hydrogen fluoride to afford [2,2,3β,4,4-d$_5$]-3α-acetoxy-17α,21-dihydroxy-20-one (**6a**). Treatment of **6a** with SO_3–TMA complex yielded [2,2,3β,4,4-d$_5$]-3α-acetoxy-17α-hydroxy-20-one-21-sulfate (**6b**). Subsequent alkaline hydrolysis at C-3 in **6b** with 20% NaOH in MeOH resulted in the formation of [2,2,3β,4,4-d$_5$]-THS-21-sulfate (**6c**) (Fig. 2).

Our next synthetic targets were focused on the 3- and 21-monosulfates of [2,2,3β,4,4-d$_5$]-THF (**13a** and **13d**) and [2,2,3β,4,4-d$_5$]-THE (**15a** and **15d**). It is well known that the reduction of 5β-3-ketosteroid with $NaBH_4$ gives a predominance of 3α-hydroxy steroids. In fact, when compound **9** was subjected to $NaBH_4$ reduction, the major isolated product was the 3α-hydroxy steroids (**10b**). The α-configuration at C-3 in **10b** was confirmed by the axial 3β-H signal appearing at 3.62–3.70 ppm as a multiplet in the ^{1}H NMR spectrum. As the non-deuterated authentic samples, compounds **10c**, **11a**, and **11b** were prepared by the selective acetylation at the 3α-hydroxy group of compound **10b** with Ac_2O in pyridine followed by Jones' oxidation of the resulting 3α-acetate (**10c**) and subsequent alkaline hydrolysis of the obtained 3α-acetoxy-11-ketone (**11a**).

Based on these findings, essentially identical procedures were used to prepare the 3- and 21-monosulfates of [2,2,3β,4,4-d$_5$]-THF (**13a** and **13d**) and [2,2,3β,4,4-d$_5$]-THE (**15a** and **15d**), starting

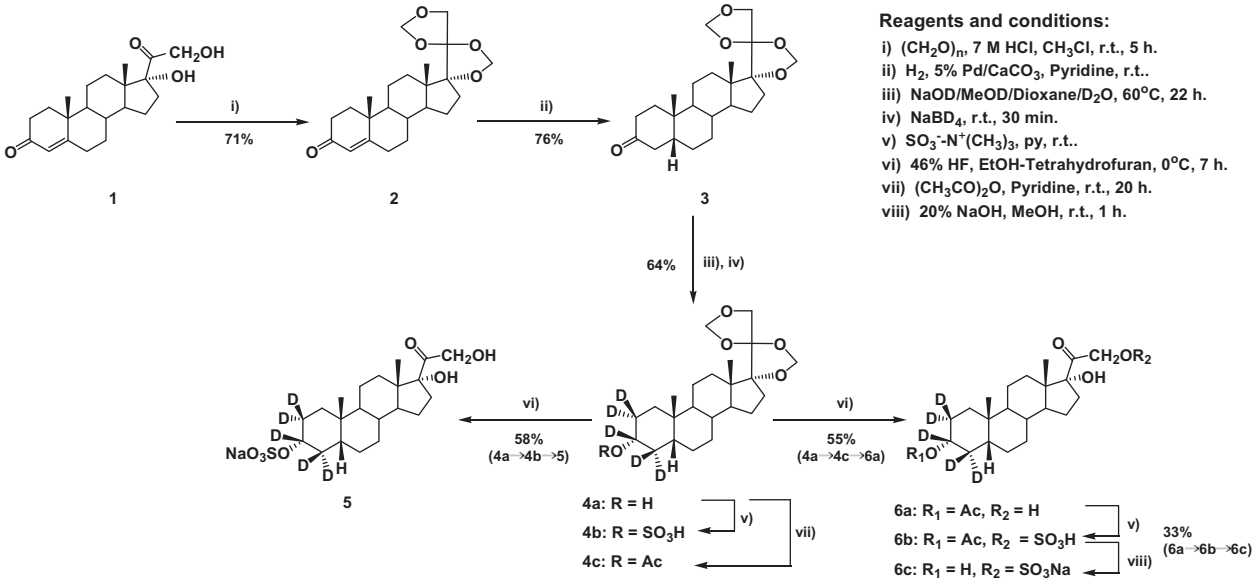

Fig. 2. Synthetic route to 3- and 21-monosulfate conjugates of [2,2,3β,4,4-d_5]-THS. Ac = –$COCH_3$.

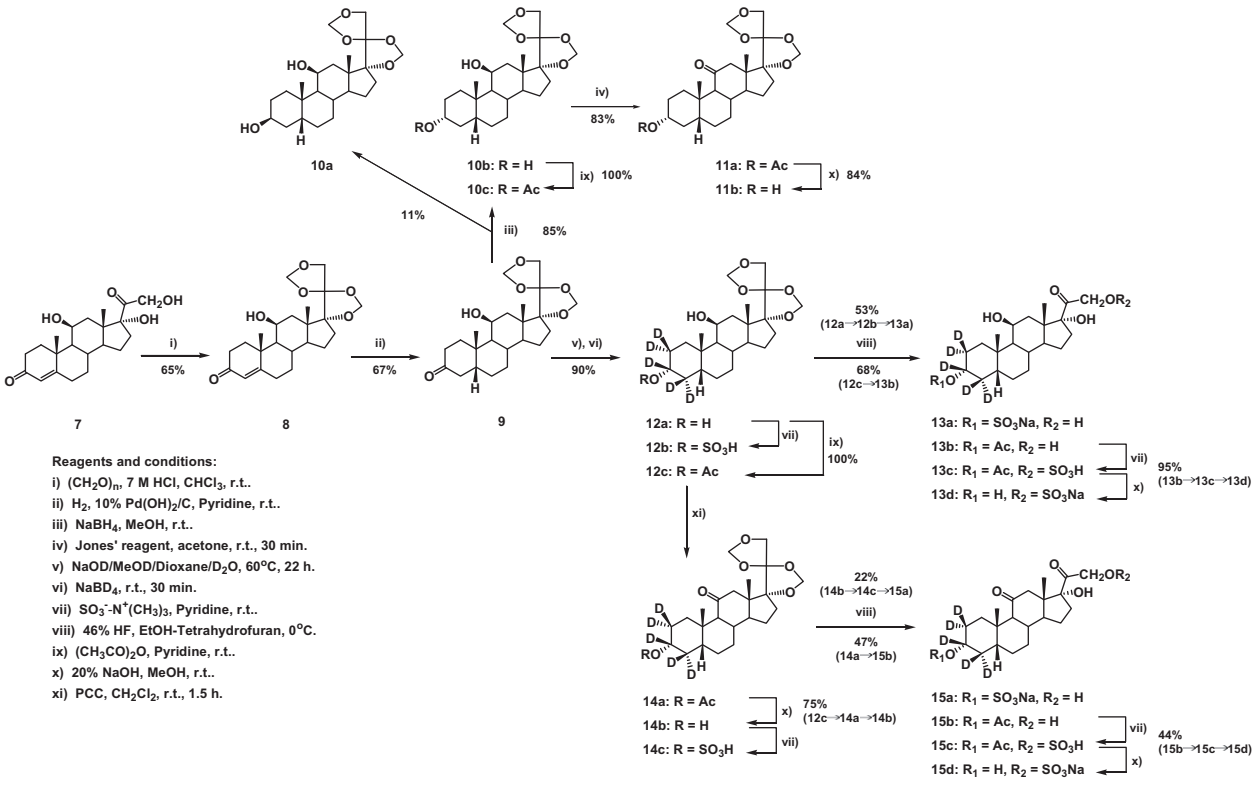

Fig. 3. Synthetic route to 3- and 21-monosulfate conjugates of [2,2,3β,4,4-d_5]-THF and [2,2,3β,4,4-d_5]-THE. Ac = –$COCH_3$.

from 17,20:20,21-bismethylendioxy-11β-hydroxy-5β-pregnan-3-one (**9**) which was prepared from cortisol (**7**) in two steps, as outlined in Fig. 3.

The structures of the obtained sulfates were confirmed by ^1H and ^{13}C NMR spectra along with ESI-MS and CID spectra. ^1H and ^{13}C NMR (data not shown) spectra were identical with those of the non-deuterated compounds except for the disappearance of 3β-proton signal. The negative-ion ESI-MS spectra showed the *m/z* values of the deprotonated molecule which were shifted to 5 Da at *m/z* 434.3 *m/z* for 3- and 21-sulfates of d_5-THS, at *m/z* 450.4 for 3- and 21-sulfates of d_5-THF, and at *m/z* 448.3 for 3- and 21-sulfates of d_5-THE, respectively (Figs. 4–6). The CID spectra of the 3-sulfates derived from the steroidal moiety by the loss of H_2O and the cleavage of dihydroxyacetone side chain were shifted 5 Da, whereby $[DSO_4]^-$ or $[M-H-CH_2O]^-$ ion as the base peak with modest peak corresponding to $[M-H-H_2O]^-$, $[M-H-CH_2O]^-$, and $[M-H-CH_2CO-H_2O]^-$ ions were observed. In contrast, the CID spectra of the 21-sulfates derived from the steroidal moiety by

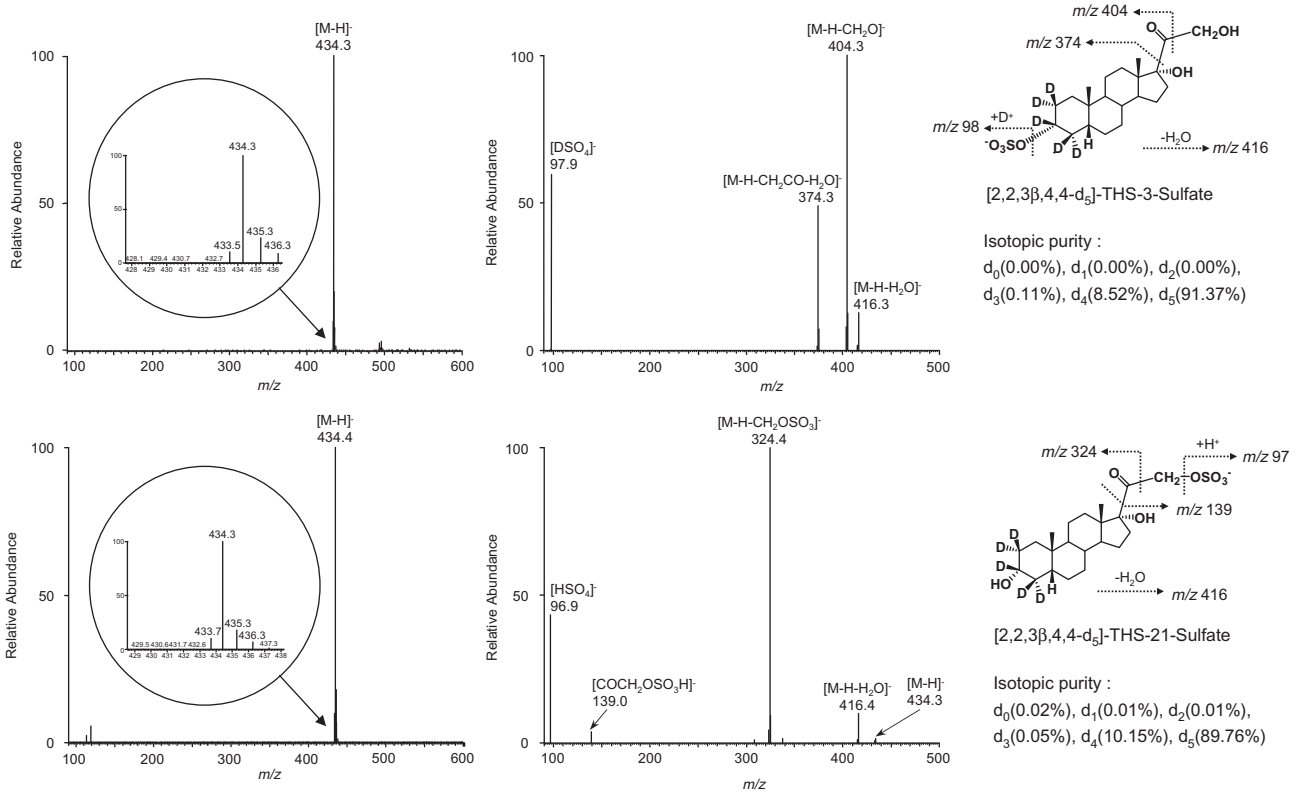

Fig. 4. ESI-MS (left) and product ion spectra obtained by CID of $[M-H]^-$ (right) of 3- and 21-monosulfate conjugates of [2,2,3β,4,4-d_5]-THF. Proposed structures of major fragments are depicted.

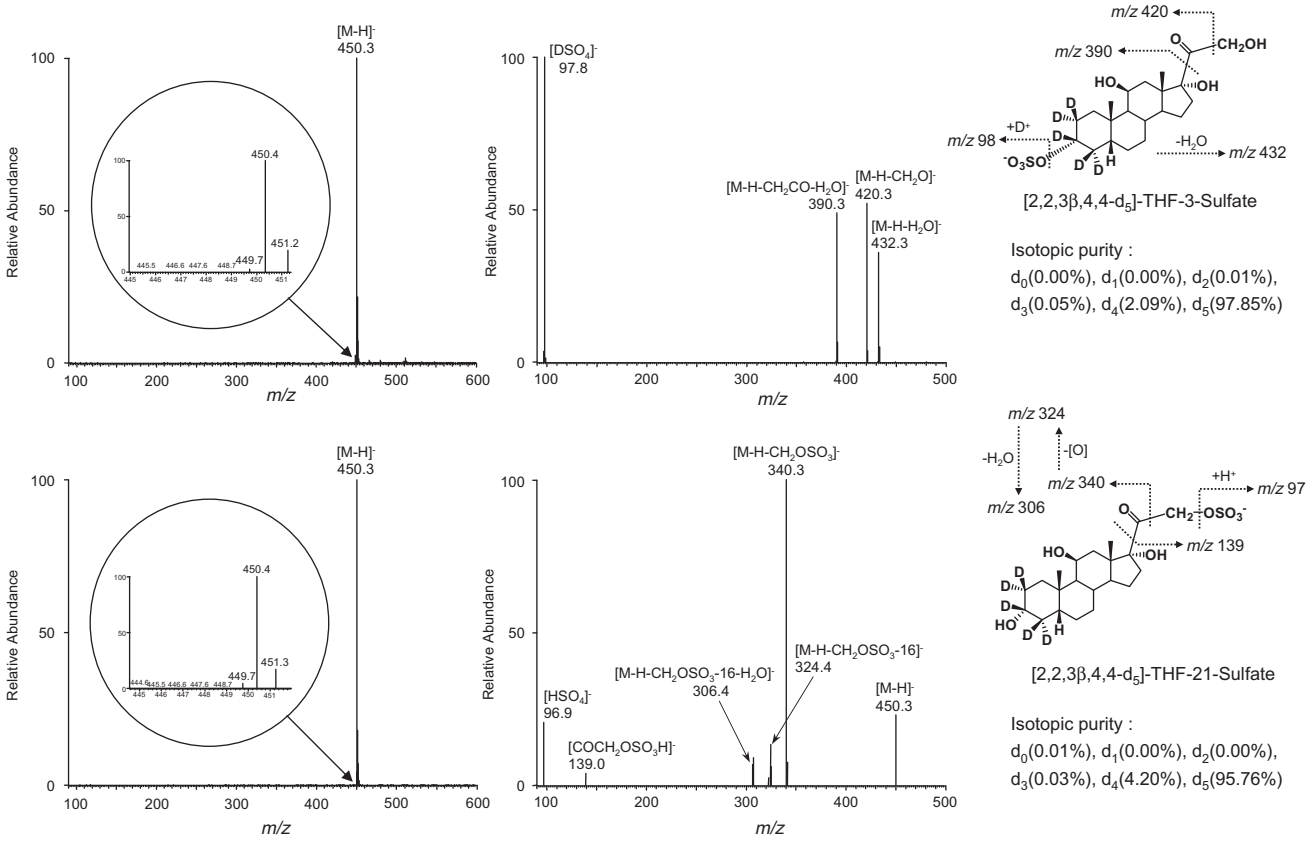

Fig. 5. ESI-MS (left) and product ion spectra obtained by CID of $[M-H]^-$ (right) of 3- and 21-monosulfate conjugates of [2,2,3β,4,4-d_5]-THE. Proposed structures of major fragments are depicted.

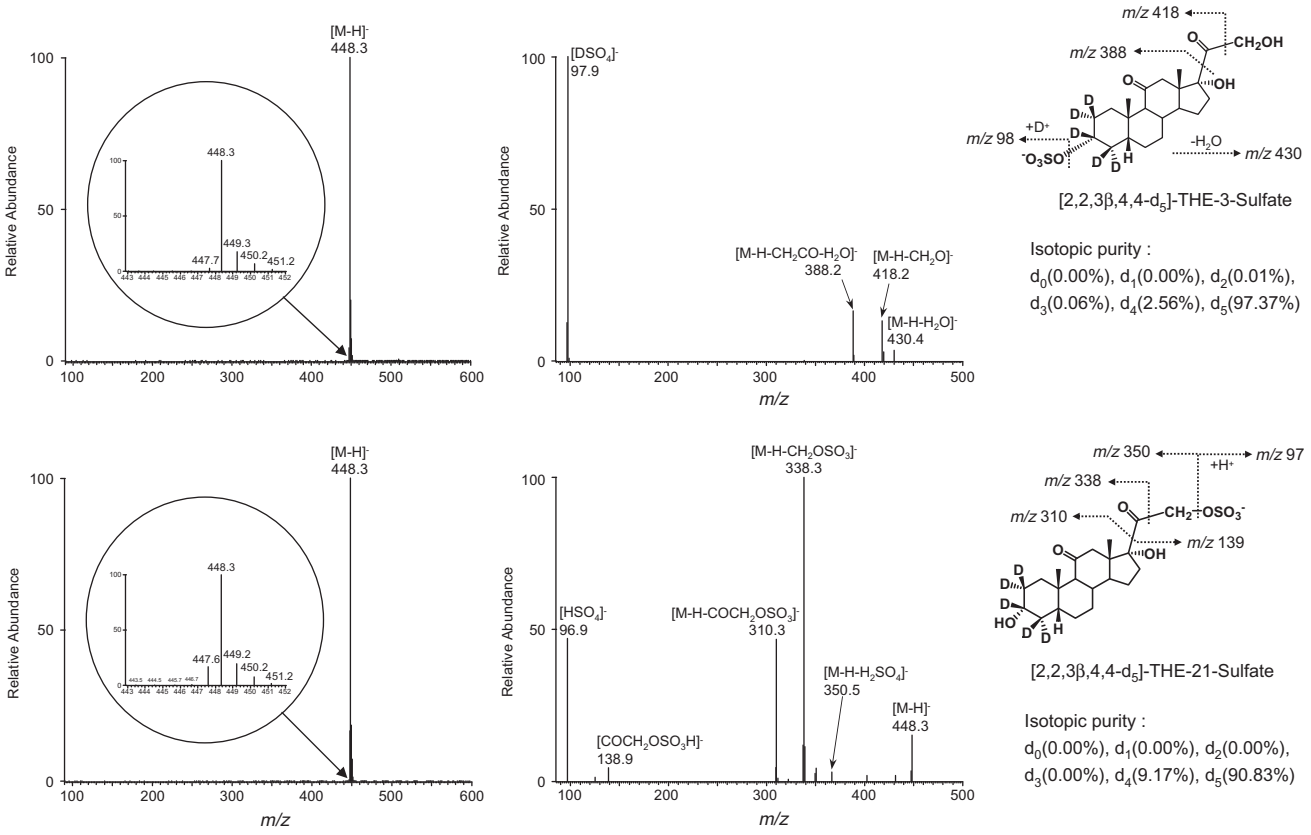

Fig. 6. ESI-MS (left) and product ion spectra obtained by CID of $[M-H]^-$ (right) of 3- and 21-monosulfate conjugates of [2,2,3β,4,4-d_5]-THS. Proposed structures of major fragments are depicted.

the cleavage of dihydroxyacetone side chain were shifted 5 Da, whereby $[M-H-CH_2OSO_3]^-$ ion as the base peak with modest or small peak corresponding to $[M-H-CH_2OSO_3-16]^-$, $[M-H-CH_2OSO_3-16-H_2O]^-$, $[COCH_2OSO_3H]^-$ and $[HSO_4]^-$ ions were observed. Thus, the results of CID analysis confirmed the structures of [2,2,3β,4,4-d_5]-THS, -THF, and -THE, which gave rise to transition $[M-H]^- \rightarrow [M-H-CH_2O]^-$ for THS-3-sulfate and $[M-H]^- \rightarrow [DSO_4]^-$ for THF- and THE-3-sulfates, and $[M-H]^- \rightarrow [M-H-CH_2OSO_3]^-$ for 21-sulfates that could be used as the monitoring ion. The isotopic purities of the labeled compounds as $[^2H_5]$-form were estimated to be more than 95 atom% D for all sulfates, based on the ion intensities in the region of deprotonated molecules of the sulfates.

In conclusion, 3- and 21-monosulfates of THS, THF, and THE labeled with multiple deuterium are now available. These compounds would be useful for the highly sensitive, selective, and accurate LC/ESI-MS/MS determination of the sulfate conjugates of tetrahydrocorticosteroids present in biological fluids. We are currently developing a stable isotope dilution LC/ESI-MS method for the measurement of sulfated tetrahydrocorticosteroids and hope to use this in clinical studies.

Acknowledgements

This work was supported in part by Grant-in-Aids for Scientific Research (C) (to. K.M., Grant 20590164) for 2008–2010, (to S.I., Grant 21590184) for 2009–2011, and (to T.I., Grant 21550091) for 2009–2011 from the Japan Society for the Promotion of Science, and Culture of Japan, and High-Tech Research Center Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology (to S.I.) for 2007–2011. We thank Dr. Alan F. Hofmann, University of California, San Diego for editing the manuscript.

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