Synthesis of (2β,3α,6-$^{2}H_{3}$)cholesteryl linoleate and cholesteryl oleate as internal standards for mass spectrometry

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Abbreviations

mp, melting points; ^{1}H-NMR, proton nuclear magnetic resonance; DEPT,distortionless enhancement by polarization transfer; EI, Electron ionization, LR-MS, low-resolution mass spectra; HR-MS, high-resolution mass spectra; EI, electrospray ionization; DMAP, *N*,*N'*-dimethylaminopyridine; THF, tetrahydrofuran; rDA, retro Diels-Alder; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide

ABSTRACT

The accurate analysis of trace component in complex biological matrices requires the use of reliable standards. For liquid chromatography/mass spectrometry analysis, the stable isotope-labeled derivatives of the analyte molecules are the most appropriate internal standards. We report here the synthesis of (2β,3α,6-$^{2}H_3$)cholesteryl linoleate and oleate containing three non-exchangeable deuterium in the steroid ring. The principal reactions used were: (1) trans diaxial opening of 2α,3α-epoxy-6-oxo-5α-cholestane with $LiAlD_4$ and subsequent oxidation of the resulting (2β,6α-$^{2}H_2$)-3α,6β-diol with Jones' reagent, followed by reduction of the resulting (2β-^{2}H)-3,6-dione with $NaBD_4$ leading to the (2β,3α,6α-$^{2}H_3$)-3β,6β-dihydroxy-5α-cholestane, (2) selective protection of the 3β-hydroxy group as the *tert*-butyldimethylsilyl ether, (3) dehydration of the 6β-hydroxy group with $POCl_3$ and removal of *tert*-butyldimethylsilyloxy groups with 5M HCl in acetone, and (4) esterification of the resultant (2β,3α,6-$^{2}H_3$)cholesterol with linoleic and oleic acids using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. The isotopic purity was found to be satisfactory by mass spectrometry, and nuclear magnetic resonance properties of the new compounds were tabulated. The labeled compounds can be used as internal standards in liquid chromatography/mass spectrometry assays for clinical and biochemical studies.

1. Introduction

Cholesterol is a critical component of cell membranes and lipoproteins, and is a precursor for steroid hormones, bile acids and vitamin D. Free cholesterol and cholesteryl esters in plasma are incorporated into lipoproteins containing phospholipids and other lipids as well as apolipoproteins. In recent years, attention has been paid to pharmacokinetics of cholesteryl esters since high levels of cholesteryl esters cause atherosclerosis and since the cholesterol ester transfer protein is a target of drugs aimed to increase high-density lipoprotein cholesterol.

In clinical laboratories, the serum concentration of cholesteryl ester is determined by subtracting the free cholesterol from the total cholesterol [1]. This assay measures only total and free cholesterol, instead of the cholesterol ester itself directly, and gives no information of fatty acid composition. The fatty acid profile is variable in cholesteryl esters in human serum in terms of carbon chain length, and number and location of double bond, and the degree of oxidation. It is possible that the pathophysiological significance of individual cholesterol esters is not identical. Also, it is difficult to measure trace amounts of cholesteryl esters in culture media or body fluids other than plasma because of the low sensitivity of enzymatic methods. Therefore, a sensitive and specific assay for various species of cholesteryl esters is required in lipid metabolism research.

Liquid chromatography/mass spectrometry (LC/MS) analysis with a stable

isotope-labeled internal standard is superior to the enzymatic method in molecular information and sensitivity. LC/MS is reported to be useful in the measurement of various lipid molecules at low levels [2-4]. The accurate analysis of trace lipids in complex biological matrices requires the use of reliable standards, which are often unavailable. Accordingly, synthesis of internal standards (IS) is required for constructing reliable and accurate LC/MS assays. As far as we know, there have been no reports of the chemical synthesis of deuterated cholesteryl acyl esters as IS for LC/MS. The present paper describes a chemical synthesis of (2β,3α,6-$^{2}H_{3}$)cholesteryl linoleate and cholesteryl oleate containing three deuterium atoms in the steroid ring as internal standards in LC/MS assays for clinical and biochemical studies.

2. Experimental

2.1. Materials

Cholesterol was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Silica gel plates (Merck F_{254}) and silica gel 60 (Merck; 70-230 mesh) were used for analytical and column chromatography, respectively. 2-Propanol and ammonium acetate of HPLC grade were purchased from Nacalai Tesque Inc. (Kyoto, Japan), and H_2O of HPLC grade was purchased from Wako Pure Chemical Industries, Ltd. $NaBD_4$ and $LiAlD_4$ (99% isotopic purity) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All other chemicals and solvents were analytical grade and obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan).

2.2. Instruments

All melting points (mp) were determined on a micro hot-stage apparatus and are uncorrected. Mixed melting points of the all deuterated compounds with their corresponding non-deuterated compounds showed no depression. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-AL400 (Tokyo, Japan) and a JNM-ECP

400 (400 MHz, JEOL Ltd., Tokyo, Japan) at 400 MHz with $CDCl_3$ or d_6-acetone containing 0.1 % Me_4Si as the solvent; chemical shifts are expressed as δ ppm relative to Me_4Si. The following abbreviations are used: s = singlet, d = doublet, dd = doublet of doublets, brd = broad doublet, m = multiplet. ^{13}C-NMR spectra were obtained on a NM-ECP 400 instrument at 100 MHz. The ^{13}C distortionless enhancement by polarization transfer (DEPT) spectra were also measured to determine the ^{1}H signal multiplicity and to differentiate between CH_3, CH_2, CH, and C based on their proton environments. The ^{1}H and ^{13}C NMR chemical shift assignments of deuterated cholesteryl linoleate and cholesteryl oleate are tabulated in Table 1. Electron ionization (EI) low-resolution mass spectra (LR-MS) and high-resolution mass spectra (HR-MS) were obtained by JMS-T100GCv (JEOL Ltd., Tokyo, Japan) in positive-ion mode. The LC-electrospray ionization (ESI)/MS analyses were carried out using a LTQ XL Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source and coupled to a Surveyor MS pump (Thermo Scientific, Bremen, Germany) in positive-ion detection. The mass spectra were obtained in Fourier transform mode and were calibrated with a polytyrosine as a standard. Mass spectra were acquired with a target mass resolution of R = 60,000 at *m/z* 400 under automatic gain control set to 5.0×10^5 as the target value. The ion-spray potential was set at 5.0 kV in positive-ion mode with a scan range of *m/z* 150-1,000. The trap fill-time was set at 500 ms. Nitrogen was used as sheath gas (set at 50 arbitrary units). LC separations were conducted using a reversed-phase semi-micro column, Hypersil Gold C18 (5 μm, 50 × 2.1

mm I.D.) from Thermo Fisher Scientific (Waltham, MA, USA) by a linear gradient: from 50 % solvent A (10 mM aqueous ammonium acetate, pH 6.0) against solvent B (2-propanol) for 1 min and then 100% solvent B over 8 min, and 50 % solvent A against solvent B (2-propanol) over 10 min at flow rate of 200 μl/min.

2.3. Chemical Synthesis

2.3.1. Cholest-5-ene-3β-yl p-toluenesulfonate (**1b**)

To a solution of cholesterol (**1a**, 1 g, 2.59 mmol) in dry pyridine (2 ml) and $CHCl_3$ (2 ml) were added *N,N'*-dimethylaminopyridine (DMAP, 100 mg, 0.82 mmol) and *p*-toluenesulfonyl chloride (740 mg, 3.88 mmol); the reaction mixture was stirred at ice temperature for 20 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in EtOAc (60 ml), washed with 5% HCl (1 × 30 ml), 5% $NaHCO_3$ (2 × 30 ml), and H_2O (1 × 30 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Recrystallization of the product from acetone-MeOH gave **1b** as colorless needles: yield 100% (1.4 g, 2.59 mmol); mp 131.3-131.9 ^{0}C (lit. [5], mp 132-133 ^{0}C; [6], mp 133 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-H_3), 0.857 and 0.862 (each 3H, d, *J* = 6.2 Hz, 26- and 27-H_3), 0.90 (d, *J* = 6.2 Hz, 21-H_3), 0.96 (3H, s, 19-H_3), 2.45 (3H, s, $C_6H_4C\underline{H}_3$), 4.31 - 4.33 (1H, m, 3α-H), 5.30 (1H, br.s, 6-H), 7.33 and 7.80 (each 2H, d, *J* = 8.2 Hz, $C_6\underline{H}_4CH_3$). ^{13}C-NMR ($CDCl_3$) δ: 11.80, 18.67, 19.11, 20.95, 21.61, 22.53, 22.79, 23.77,

24.21, 27.97, 28.17, 28.59, 31.71, 31.81, 35.72, 36.13, 36.31, 36.85, 38.83, 39.47, 39.61, 42.25, 49.87, 56.06, 56.61, 82.37, 123.49, 127.61, 127.84, 129.71, 134.66, 138.83, 144.36.

*2.3.2. 3β,6α-Dihydroxy-5α-cholestane-3β-yl p-toluenesulfonate (**2**)*

To a stirred solution of **1b** (3 g, 5.55 mmol) in dry tetrahydrofuran (THF, 50 ml) was added borane dimethyl sulfide (2.6 ml, 27.4 mmol) at ice temperature under a gentle stream of Ar gas, and the mixture was stirred for 2 h at ice temperature and then for 4 h at room temperature. After being cooled in ice bath, a solution of 30% H_2O_2 (25 ml)-10% NaOH (25 ml) was carefully added to the solution and the reaction mixture was stirred for 1 h at ice temperature. The resulting solution was extracted with EtOAc (1 × 120 ml). The combined extracts was washed successively with 5% $NaHSO_3$ (1 × 60 ml), 5% $NaHCO_3$ (1 × 60 ml), and saturated brine (1 × 60 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Recrystallization of the product from MeOH gave **2** as colorless needles: yield 83% (2.56 g, 4.58 mmol); mp 124.5-125.0 ^{0}C (lit. [7], mp 135-135.3 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, 18-H_3), 0.78 (3H, s 19-H_3), 0.86 and 0.87 (each 3H, d, *J* = 6.6 Hz, 26- and 27-H_3), 0.89 (d, *J* = 6.6 Hz, 21-H_3), 2.43 (3H, s, $C_6H_4C\underline{H}_3$), 3.31-3.40 (1H, m, 6β-H), 4.35-4.42 (1H, m, 3α-H), 7.31 and 7.80 (each 2H, d, *J* = 8.2 Hz, $C_6\underline{H}_4CH_3$). ^{13}C-NMR ($CDCl_3$) δ: 11.98, 13.22, 18.61, 21.04, 21.61, 22.53, 22.79, 23.76, 24.12, 27.98, 28.11, 28.23, 29.11, 34.16, 35.71, 35.97, 36.08, 36.97, 39.46, 39.66, 41.59, 42.51, 51.54, 53.49, 56.02, 56.12, 69.14, 82.48, 127.59, 129.73, 134.62, 144.36.

2.3.3. 3β-Hydroxy-5α-cholestan-6-one-3β-yl p-toluenesulfonate (**3**)

To a stirred solution of **2** (5 g, 8.95 mmol) in acetone (180 ml) was added Jones' reagent [8] (10 ml), and the reaction mixture was stirred at room temperature for 10 min. After addition of MeOH to decompose the excess reagent, the organic solvent was evaporated under reduced pressure. The residue was diluted with EtOAc (200 ml), washed with 5% $NaHCO_3$ (2 × 100 ml) and H_2O (1 × 100 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the product by column chromatography on silica gel with *n*-hexane-EtOAc (15:1, v/v) as an eluent and recrystallization of a homogeneous effluent gave **3** as colorless plates: yield 99% (4.97 g, 8.93 mmol); mp 169.9-170.2 ^{0}C (lit. [9], mp 175-177 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-H_3), 0.72 (3H, s, 19-H_3), 0.858 and 0.863 (each 3H, d, *J* = 6.6 Hz, 26- and 27-H_3), 0.99 (d, *J* = 6.6 Hz, 21-H_3), 2.44 (3H, s, $C_6H_4C\underline{H}_3$), 4.36-4.44 (1H, m, 3α-H), 7.32 and 7.78 (each d, *J* = 8.4 Hz, $C_6\underline{H}_4CH_3$). ^{13}C-NMR ($CDCl_3$) δ: 11.97, 12.91, 18.60, 21.41, 21.62, 22.52, 22.78, 23.76, 23.91, 26.87, 27.97, 28.23, 34.96, 35.64, 35.74, 36.03, 36.39, 37.79, 39.35, 39.43, 40.64, 42.93, 46.55, 53.65, 56.05, 56.17, 56.37, 69.13, 81.48, 127.55, 129.67, 134.48, 144.49, 209.56.

*2.3.4. 5α-cholest-2-en-6-one (**4**)*

A solution of **3** (500 mg, 0.900 mmol) in γ-collidine (6 ml) was refluxed for 2 h. The resulting solution was diluted with EtOAc (20 ml), washed with 5% HCl (1 × 10 ml), 5%

$NaHCO_3$ (2 × 10 ml), and H_2O (1 × 10 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. The product was subjected to column chromatography on silica gel with *n*-hexane-EtOAc (30:1, v/v) as an eluent, and recrystallization of a less polar homogeneous effluent peak from MeOH gave **4** as colorless needles: yield 83% (287 mg, 0.746 mmol); mp 105.4-105.8 ^{0}C (lit. [10], mp 97-98 ^{0}C; lit. [11], mp 99.5-100.5 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-H_3), 0.71 (3H, s, 19-H_3), 086 and 0.87 (each 3H, d, *J* = 6.6 Hz, 26- and 27-H_3), 0.92 (d, *J* = 6.6 Hz, 21-H_3), 5.58 and 5.67 and (each 1H, m, 2- and 3-H). ^{13}C-NMR ($CDCl_3$) δ: 11.91, 17.87, 18.63, 20.28, 21.28, 22.52, 22.79, 23.78, 23.94, 27.98, 33.70, 35.47, 35.68, 36.08, 37.75, 39.45, 39.47, 42.54, 46.04, 51.00, 56.01, 56.76, 132.54, 146.00, 203.50. Recrystallization a more polar homogeneous effluent from Et_2O-MeOH gave 5α-choest-4-en-6-one (**5)** as colorless plates: yield 10% (35 mg, 0.091 mmol); mp 106.2-106.5 ^{0}C (lit. [12], mp 106-107 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-H_3), 0.85 and 0.87 (each 3H, d, *J* = 6.7 Hz, 26- and 27-H_3), 0.92 (d, *J* = 6.6 Hz, 21-H_3), 0.97 (3H, s, 19-H_3), 6.09 (1H, m, 4-H). ^{13}C-NMR ($CDCl_3$) δ: 11.92, 13.49, 18.64, 21.11, 21.71, 22.54, 22.80, 23.79, 23.94, 27.99, 28.01, 35.68, 36.08, 37.71, 39.35, 39.45, 39.49, 40.03, 42.80, 47.02, 53.42, 53.83, 56.10, 56.75, 124.53, 124.94, 212.07.

*2.3.5. Epoxidation of **4** with m-chloroperbenzoic acid*

To a solution of **4** (70 mg, 0.182 mmol) in EtOAc (5 ml) was added *m*-chloroperbenzoic acid (130 mg, 0.753 mmol), and the mixture as stirred for 4 h at room temperature. The

resulting solution was diluted with EtOAc (10 ml), washed successively with 5% $NaHSO_3$ (1 × 5 ml), 5% $NaHCO_3$ (1 × 5 ml), and H_2O (1 × 5 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. The product was subjected to column chromatography on silica gel with *n*-hexane-EtOAc (15:1, v/v) as an eluent. Recrystallization of a less polar effluent peak from MeOH gave 2β,3β-epoxy-5α-cholestan-6-one (**6**) as colorless plates: yield 6% (4.5 mg, 0.011 mmol); mp 119.6-120.3 ^{0}C (lit. [13], mp 119-120 ^{0}C; lit. [14], mp 141-142 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-H_3), 0.71 (3H, s, 19-H_3), 0.862 and 0.866 (each 3H, d, *J* = 6.7 Hz, 26- and 27-H_3), 0.90 (d, *J* = 6.6 Hz, 21-H_3), 3.24 and 3.70 (each 1H, m, 2α- and 3α-H). ^{13}C-NMR ($CDCl_3$) δ: 11.91, 15.30, 18.62, 20.13, 21.03, 22.53, 22.79, 23.78, 23.94, 27.98, 35.65, 36.06, 37.08, 37.50, 39.44, 40.26, 42.76, 46.89, 50.57, 52.32, 53.17, 54.90, 56.07, 56.62, 210.92. Recrystallization of a more polar effluent peak from MeOH gave 2α,3α-epoxy-5α-cholestan-6-one (**7)** as colorless leaflets: yield 92% (67 mg, 0.167 mmol); mp 147.9-148.5 ^{0}C . ^{1}H-NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-H_3), 0.71 (3H, 19-H_3), 0.862 and 0.866 (each 3H, d, *J* = 6.7 Hz, 26- and 27-H_3), 0.91 (d, *J* = 6.6 Hz, 21-H_3), 3.12 and 3.27 and (each 1H, m, 2β- and 3β-H). ^{13}C-NMR ($CDCl_3$) δ: 11.79, 14.90, 18.55, 20.94, 22.46, 22.72, 23.68, 23.83, 27.88, 35.58, 35.97, 37.37, 37.78, 38.31, 39.28, 39.35, 42.63, 46.81, 49.77, 50.02, 52.25, 53.01, 55.94, 56.44, 211.28.

*2.3.6. Reduction of **7** with $LiAlH_4$*

A solution of **7** (20 mg, 0.050 mmol) and $LiAlH_4$ (27 mg, 0.711 mmol) in dry THF (15

ml) was refluxed for 4 h. After careful addition of H_2O to decompose the excess reagent, the resulting solution was extracted with EtOAc (1 × 10 ml). The extracts was successively washed with 5% HCl (1 × 5 ml), 5% $NaHCO_3$ (2 × 5 ml), and saturated brine (1 × 5 ml), and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure, the product was submitted to column chromatography on silica gel with *n*-hexane-EtOAc (3:1, v/v) as an eluent. Recrystallization of a homogenous effluent peak from MeOH gave 3α,6β-Dihydroxy-5α-cholestane (**8a**): yield 75% (15 mg, 0.037 mmol); mp 186-187 ^{0}C (lit. [14], mp 189.2-190.3 ^{0}C). ^{1}H-NMR (d_6-acetone) δ: 0.72 (3H, s, 18-H_3), 0.863 and 0.867 (each 3H, d, J = 6 Hz, 26- and 27-H_3), 0.94 (d, J = 6.6, 21-H_3), 1.02 (3H, s, 19-H_3), 3.62-3.67 (1H, m, 6α-H), 4.01-4.05 (1H, m, 3β-H). ^{13}C-NMR ($CDCl_3$) δ: 12.10, 14.84, 18.67, 20.59, 22.54, 22.80, 23.81, 24.17, 27.99, 28.18, 29.08, 30.28, 33.05, 33.95, 35.77, 36.07, 36.14, 39.49, 39.71, 39.91, 41.68, 42.64, 54.17, 56.20, 56.23, 66.66, 72.25.

*2.3.7. 5α-cholestane-3,6-dione (**9a**)*

To a solution of **8a** (15 mg, 0.037 mmol) in acetone (4 ml) was added Jones' reagent (0.1 ml), and the mixture was stirred at room temperature for 5 min. After addition of 2-propanol to decompose the excess reagent, the resulting solution was diluted with EtOAc (10 ml), washed with 5% $NaHCO_3$ (2 × 5 ml) and H_2O (1 × 5 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Recrystallization of the product from CH_2Cl_2-MeOH gave **9a** as colorless needles: yield 95% (14 mg, 0.035 mmol); 172.8-173.8 ^{0}C (lit. [15], mp

168.5-170 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-H_3), 0.86 and 0.88 (each 3H, d, *J* = 6 Hz), 0.92 (3H, d, *J* = 6.2 Hz, 21-H_3), 0.96 (3H, s, 19-H_3). ^{13}C-NMR ($CDCl_3$) δ: 11.98, 12.53, 18.60, 21.64, 22.52, 22.77, 23.77, 23.97, 27.97, 28.00, 35.65, 36.03, 37.00, 38.01, 39.35, 39.42, 41.23, 42.98, 46.60, 53.44, 56.08, 56.57, 57.51, 209.14, 211.38.

*2.3.8. 3β,6β-dihydroxy-5α-cholestane (**10a**)*

To a solution **9a** (20 mg, 0.050 mmol) in THF (3 ml)-MeOH (12 ml) was added $NaBH_4$ (10 mg, 0.264 mmol), and the mixture was stirred 30 min at room temperature. After careful addition of 8.3% HCl to decompose the excess reagent, the organic solvent was evaporated under reduced pressure. The residue was diluted with EtOAc (10 ml), washed successively with H_2O (1 × 5 ml), 5% $NaHCO_3$ (1 × 5 ml), and saturated brine (1 × 5 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the product by column chromatography on silica gel with *n*-hexane-EtOAc (3:1, v/v) and recrystallization of a homogeneous effluent from CH_2Cl_2-acetone gave **10a** as colorless plates: yield 84% (17 mg, 0.042 mmol); mp 190.4-191.7 ^{0}C (lit. [16], 190-191 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-H_3), 0.861 and 0.866 (each 3H, d, *J* = 6 Hz, 26- and 27-H_3), 0.90 (d, *J* = 6.6 Hz, 21-H_3), 1.03 (3H, s, 19-H_3), 3.58-3.69 (1H, m, 3α-H), 3.77-3.82 (1H, brd, *J* = 2.56 Hz, 6α-H). ^{13}C-NMR ($CDCl_3$) δ: 12.08, 15.76, 18.64, 21.02, 22.52, 22.78, 23.80, 24.19, 27.97, 28.17, 30.34, 31.42, 35.31, 35.74, 36.13, 38.47, 39.46, 39.54, 39.90, 42.63, 47.36, 54.20, 56.15, 56.25, 71.64, 71.98.

*2.3.9. 3β-tert-Butyldimethylsilyloxy-6β-hydroxy-5α-cholestane (**10b**)*

To a solution of **10a** (400 mg, 0.988 mmol) in dry *N*,*N'*-dimethylformamide (DMF, 4 ml)-dry pyridine (2 ml) were added imidazole (340 mg, 4.99 mmol) and *tert*-butyldimethylsilyl chloride (TBDMSCl, 600 mg, 3.98 mmol), and the mixture was stirred overnight at room temperature. The resulting solution was diluted with EtOAc (20 ml), washed with H_2O (2 × 10 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the product by column chromatography on silica gel with *n*-hexane-EtOAc (20:1, v/v) as an eluent and recrystallization of a homogenous effluent from Et_2O-MeOH gave **10b** as colorless leaflets: yield 100% (511 mg, 0.985 mmol); mp 167.1-167.9 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s, $Si(CH_3)_2$), 0.68 (3H, s, 18-H_3), 0.86 and 0.87 (each 3H, d, *J* = 6.6 Hz), 0.89 (9H, s, *t*-C_4H_9), 0.91 (d, *J* = 6.6, 21-H_3), 1.02 (3H, s, 19-H_3), 3.56-3.64 (1H, m, 3α-H), 3.75-3.79 (1H, m, 6α-H). ^{13}C-NMR ($CDCl_3$) δ: -4.58, 12.09, 15.82, 18.22, 18.68, 21.03, 22.55, 22.80, 23.81, 24.22, 25.91, 28.00, 28.20, 30.36, 31.90, 35.39, 35.75, 35.80, 36.15, 38.66, 39.49, 39.96, 42.66, 47.50, 54.32, 56.22, 56.28, 72.22, 72.46.

*2.3.10. Dehydration **10b** with $POCl_3$*

To a solution of **10b** (18 mg, 0.035 mmol) in dry pyridine (0.5 ml) was added $POCl_3$ (170 mg, 1.11 mmol), and the mixture was stirred overnight at room temperature. After careful addition of H_2O at ice temperature to decompose the excess reagent, the resulting

solution was diluted with EtOAc (10 ml), washed with 10% AcOH (1 × 5 ml), 5% $NaHCO_3$ (1 × 5 ml), and H_2O (1 × 5 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Recrystallization of the product from Et_2O-MeOH gave 3β-*tert*-butyldimethylsilyloxycholest-5-ene (**11a**) as colorless leaflets: yield 100% (17.5 mg, 0.035 mmol). mp 157.6-158.0 ^{0}C (lit. [16], 151-153 ^{0}C; [17], 156.6-158.5 ^{0}C). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, $Si(CH_3)_2$, 0.67 (3H, s, 18-H_3), 0.86 and 0.87 (each 3H, d, *J* =6 Hz, 26- and 27-H), 0.89 (9H, s, *t*-C_4H_9), 0.91 (d, *J* = 6.6 Hz, 21-H_3), 1.00 (3H, s, 19-H_3), 3.44-3.52 (1H, m, 3α-H), 5.29-5.32 (1H, brd, J = 5.5 Hz, 6-H). ^{13}C-NMR ($CDCl_3$) δ: -4.59, 11.85, 18.26, 18.71, 19.42, 21.06, 22.56, 22.82, 23.81, 24.29, 25.94, 28.01, 28.23, 31.90, 31.94, 32.08, 35.77, 36.19, 36.58, 37.38, 39.51, 39.80, 42.31, 42.82, 50.02, 56.14, 56.80, 72.64, 121.16, 141.56.

*2.3.11. (2β,6α-$^2H_2$)-3α,6β-Dihydroxy-5α-cholestane (**8b**)*

The compound **7** (307 mg, 0.766 mmol) was refluxed with $LiAlD_4$ (120 mg, 2.85 mmol) in dry THF (15 ml) for 6 h. After being processed in an analogous manner as described for preparation of **9a**, the product was subjected to column chromatography on silica gel with *n*-hexane-EtOAc (3:1, v/v) as an eluent. Recrystallization of a homogenous effluent from MeOH gave **8b**: yield 78% (242 mg, 0.596 mmol); mp. 172.1-174.0 ^{0}C. ^{1}H-NMR (d_6-acetone) δ: 0.72 (3H, s, 18-H_3), 0.863 and 0.867 (each 3H, d, *J* = 6 Hz, 26- and 27- H_3), 0.94 (d, *J* = 6.6, 21-H_3), 1.02 (3H, s, 19-H_3). LR-MS: *m/z* 388.34

(M-H_2O, 100%), 387.33 (M-HDO, 8.3%), 373.31 (M-H_2O-CH_3, 24.2%), 370.33 (M-$2H_2O$, 42.9%), 369.32 (M-HDO-H_2O, 10.9%), 355.30 (M-$2H_2O$-CH_3, 19.3%), (275.21 (M-H_2O-CH_3-side chain (S.C.)), 257.20 (M-$2H_2O$-CH_3- S.C., 11.5%), 234.2 (M-H_2O-ring D, 39.5%), 55.05 (C_4H_5D, 36.5%).^{13}C-NMR ($CDCl_3$) δ: 12.10, 14.83, 18.67, 20.58, 22.54, 22.80, 23.81, 24.17, 27.99, 28.18, 30.25, 33.00, 33.86, 35.77, 36.03, 36.14, 39.48, 39.58, 39.91, 41.56, 42.63, 54.17, 56.20, 56.23, 66.62.

*2.3.12. (2β,3α,6α-$^2H_3$)-3β-tert-Butyldimethylsilyloxy-6β-hydroxy-5α-cholestane (**10d**)*

The compound **8b** (228 mg, 0.561 mmol) was oxidized with Jones' reagent (2 ml) in acetone (20 ml) for 5 min at room temperature as described for preparation of **9a**. After processing in analogous manner, the resulting (2β-^{2}H)-3,6-diketone (**9b**), without purification, was reduced with $NaBD_4$ (73 mg, 1.75 mmol) in THF (8 ml)-MeOH (12 ml) for 1 h at room temperature as described for preparation of **10a.** After being processed in analogous manner, the resulting (2β,3α,6-$^2H_3$)-3β,6β-dihydroxy-5α-cholestane (**10c**), without purification,t was silylated with imidazole (340 mg, 4.99 mmol) and TBDMSCl (600 mg, 3.98 mmol) in dry DMF (4 ml)-dry pyridine (2 ml) for 12 h at room temperature as described for preparation of **10b**. After being processed in analogous manner, the crude product was subjected to column chromatography on silica gel with *n*-hexane-EtOAc (30:1, v/v) as an eluent. Recrystallization of a homogenous effluent from Et_2O-MeOH gave **10d** as colorless leaflets: yield 73% (213 mg, 0.408 mmol); mp167.3-168.1 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s,

$Si(CH_3)_2$, 0.67 (3H, s, 18-H_3), 0.86 and 0.87 (each 3H, d, *J* = 6 Hz, 26- and 27-H), 0.89 (9H, s, *t*-C_4H_9), 0.91 (d, *J* = 6.6 Hz, 21-H_3), 1.00 (3H, s, 19-H_3). ^{13}C-NMR ($CDCl_3$) δ: -4.58, 12.09, 15.82, 18.22, 18.68, 21.03, 22.55, 22.80, 23.81, 24.22, 25.91, 28.00, 28.20, 30.36, 31.90, 35.39, 35.75, 35.80, 36.15, 38.66, 39.49, 39.96, 42.66, 47.50, 54.32, 56.22, 56.28, 72.22, 72.46.

*2.3.13. (2β,3α,6-$^2H_3$)-3β-tert-Butyldimethylsilyloxycholest-5-ene (**11b**)*

The compound **10d** (1.09 g, 2.09 mmol) was treated with $POCl_3$ (845 mg, 5.51 mmol) in dry pyridine (9 ml) overnight at room temperature as described for preparation of **11a**. After being processed in an analogous manner, the product was subjected to column chromatography on silica gel with *n*-hexane-EtOAc (40:1, v/v) as an eluent. Recrystallization of a homogenous effluent from Et_2O-MeOH gave **11b** as colorless leaflets: yield 95% (997 mg, 1.98 mmol); mp 155.8-156.5 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, $Si(CH_3)_2$), 0.67 (3H, s, 18-H_3), 0.86 and 0.87 (each 3H, d, *J* = 6 Hz, 26- and 27-H), 0.89 (9H, s, *t*-C_4H_9), 0.91 (d, *J* = 6.6 Hz, 21-H_3), 1.00 (3H, s, 19-H_3). ^{13}C-NMR ($CDCl_3$) δ: -4.59, 11.85, 18.26, 18.71, 19.41, 21.05, 22.55, 22.79, 23.81, 24.28, 25.94, 28.01, 28.23, 31.81, 35.77, 36.19, 36.52, 37.28, 39.46, 39.81, 42.28, 42.60, 50.16, 56.15, 56.15, 56.76.

*2.3.14. (2β,3α,6-$^2H_3$)-3β-hydroxycholest-5-ene (**11c**)*

The compound **11b** (130 mg, 0.258 mmol) was treated with 5% HCl (0.4 ml) in acetone

(6 ml) for 45 min at room temperature. After evaporation of acetone under reduced pressure, the residue was diluted with EtOAc (10 ml), washed with 5% $NaHCO_3$ (1 × 5 ml) and H_2O (1 × 5 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Recrystallization of the product from Et_2O-MeOH gave **11c** as colorless leaflets: yield 100% (100 mg, 0.257 mmol); mp 146.7-146.9 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-H_3), 0.858 and 0.862 (each 3H, d, *J* = 6.5 Hz, 26- and 27-H), 0.91 (d, *J* = 6.7 Hz, 21-H_3), 1.00 (3H, s, 19-H_3), 2.21 and 2.29 (each 1H, dd, J = 2.0 and 13 Hz, 4α- and 4β-H). LR-EI-MS, *m/z* 389.46 (M^+, 100%), 374.43 (M-CH_3), 370.44 (M-HDO, 22.44%), 371.45 (M-H_2O, 55.7%), 356.33 (M-H_2O-CH_3), 257.28 (M-HDO-side chain (S.C.), 15.2%), 258.29 (M-H_2O-S.C., 36.5%), 162.17 (35.3%), 163.18 (47.7%), 148.16 (45.6%), 149.16 (35.7%), 109.1 (35.4%) , 95.11 (48.7%).^{13}C-NMR ($CDCl_3$) δ: 11.84, 18.70, 19.36, 21.06, 22.54, 22.80, 23.81, 24.27, 28.00, 28.22, 31.76, 31.85, 35.77, 36.16, 36.42, 37.14, 39.50, 39.76, 42.08, 42.30, 50.11, 56.12, 56.74, 140.67. HR-EI-MS, calculated for $^{12}C_{27}{}^1H\ D_3{}^{16}O_1$ $[M]^+$ 389.37370; found 389.37319. The analysis indicated d_0 0.3%, d_1 0.2 %, d_2 7.9 %, d_3 91.5 %.

*2.3.15. (2β,3α,6-$^2H_3$)-3β-acetoxycholest-5-ene (**11d**)*

The compound **11c** (10 mg, 0.026 mmol) was acetylated with acetic anhydride (0.4 ml) in dry pyridine (0.5 ml) for 17 h at room temperature. After addition of H_2O, the resulting solution was extracted with EtOAc (1 × 10 ml). The organic layer was successively washed with 5% HCl (1 × 5 ml), 5% $NaHCO_3$ (2 × 5 ml), and H_2O (1 × 5 ml), dried over anhydrous

Na_2SO_4, and evaporated to dryness. Recrystallization of the product from Et_2O-MeOH gave **11d** as colorless leaflets: yield 100% (11 mg, 0.026 mmol), mp 113-113.5 ^{0}C. ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-H_3), 0.858 and 0.863 (each 3H, d, *J* = 6.7 Hz, 26- and 27-H), 0.91 (d, *J* = 6.6 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 2.03 (3H, s, $OCOCH_3$). ^{13}C-NMR ($CDCl_3$) δ: 11.84, 18.69, 19.29, 21.01, 21.43, 22.54, 22.80, 23.81, 24.27, 27.75, 28.00, 28.21, 31.83, 31.87, 35.77, 36.16, 36.56, 36.97, 38.10, 39.50, 39.71, 42.28, 50.00, 56.11, 56.66, 73.96, 122.63, 139.62, 170.51.

*2.3.16. (2β,3α,6-$^2H_3$)cholesteryl linoleate (**12a**)*

To a solution of **11c** (210 mg, 0.539 mmol) in dry CH_2Cl_2 (5 ml) were added linoleic acid (271 mg, 0.966 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI, 210 mg, 1.350 mmol), and DMAP (20 mg, 0.164 mmol), respectively, and the mixture was stirred overnight at room temperature. After evaporation of the solvent under reduced pressure, the residue was diluted with EtOAc (10 ml), washed with 5% HCl (1 × 5 ml), 5% $NaHCO_3$ (2 × 5 ml), and H_2O (1 × 5 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification of the product by column chromatography on silica gel with *n*-hexane-benzene (2:1, v/v) gave **12a** as colorless oil: yield 91% (318 mg, 0.488 mmol). Complete assignment of ^{1}H- and ^{13}C-NMR chemical shifts are tabulated in Table 1. HR-ESI (+)-MS: calculated $^{12}C_{45}{}^{13}CH_{77}D_3{}^{16}O_2{}^{14}N$ ($M+NH_4^+$) = 670.6405; found 670.6406. MS/MS of $[M+NH_4]^+$: *m/z* 372.4 ($M-NH_4$ -NH_3-linoleic acid).

*2.3.17. (2β,3α,6-$^{2}H_3$)cholesteryl oleate (**12b**)*

The compound **11c** (100 mg, 0.257 mmol) was esterified with oleic acid (100 mg, 0.354 mmol) using EDCI (20 mg, 0.129 mmol) and DMAP (10 mg, 0.082 mmol) in anhydrous CH_2Cl_2 (10 ml) overnight at room temperature, as described for preparation of 12a. After being processed in an analogous manner, purification of the product by column chromatography on silica gel with *n*-hexane-EtOAc (30:1, v/v) gave **12b** as colorless oil: yield 87% (146 mg, 0.223 mmol). Complete assignment of ^{1}H- and ^{13}C-NMR chemical shifts are tabulated in Table 1. HR-ESI(+)-MS: calculated $^{12}C_{45}{}^{13}CH_{79}D_3{}^{16}O_2{}^{14}N$ $(M+NH_4^+)$ = 671.6528; found 671.6533. MS/MS of $[M+NH_4]^+$: m/z 372.4 $(M-NH_4-NH_3$-oleic acid).

3. Results and Discussion

The use of stable isotope (^{2}H, ^{13}C, ^{15}N and ^{18}O) labeled-internal standards offers major advantages in that they behave in a similar manner with the analytes of interest during extraction, column separation, and mass spectrometry. The difference between the molecular masses of the analytes and isotopically labeled internal standards should be at least three mass units to avoid overlapping between the natural isotope peaks and the monitored mass peaks [18]. For preparing labeled cholesteryl linoleate and cholesteryl oleate with at least three nonexchangeable stable isotopes, either esterification of cholesterol with

commercially available (9,10,12,13-$^{2}H_4$)-linoleic acid or esterification of commercially available (2,2,3α,4,4,6-$^{2}H_6$)- or (26,27-$^{2}H_6$)-cholesterol with non-labeled linoleic acid can be considered. However, these commercially available deuterides are relatively expensive. Moreover, the deuterium atoms labeled in fatty acid or cholesterol side chain are labile thorough the cholesterol metabolic process, making them unsuitable for biochemical studies. In contrast, the deuterated atoms in the steroid ring are more stable metabolically. Furthermore, too many deuterated compounds might behave a different way from the analytes for the isotope effect on HPLC separation [19, 20]. As an alternative approach toward the final goal, we undertook to introduce three nonexchangeable deuterium atoms at 2β, 3α, and C-6 position of cholesteryl linoleate and cholesteryl oleate.

As a preliminary experiment toward the final goal, we sought to establish a synthetic route by which the label could be unambiguously introduced at the desired position. Of the numerous deuteration methods so far available, the reductive cleavage of epoxide with lithium aluminum deuteride ($LiAlD_4$) and reduction of ketone with sodium borodeuteride ($NaBD_4$) or $LiAlD_4$ appeared to be favorable for the present purpose. Additionally, it is well known that opening the oxide ring of 2α,3α-epoxy-5α-steroids with $LiAlH_4$ yields predominantly 3α-hydroxy-5α-steroids [21] and reduction of 3,6-diketo-5α-steroids with sterically less demanding hydride using $NaBH_4$ yields predominantly 3β,6β-diol [22]. Moreover, it is sufficiently substantiated that dehydration of the 6β-hydroxy-5α-steroid with phosphorus oxychloride ($POCl_3$) proceeds regiospecifically to give 5-dehydrated steroid in an excellent

yield [10, 15, 23]. We therefore, prepared 2α,3α-epoxy-5α-cholestan-6-one (**7**) as the key intermediate (Fig.1).

Hydroboration of cholesteryl tosylate (**1b**), which is readily obtainable from cholesterol (**1a**), and subsequent oxidation of the organoborane with alkaline hydrogen peroxide followed by oxidation of the resulting 6α-hydroxy-3β-tosylate (**2**) with Jones' reagent furnished the 6-ketone (**3**) in a high yield. Subsequently, elimination of the oxygen function at C-3 was effected by refluxing in γ-collidine yielding the Δ^2-olefin (**4**) accompanied with a small amount of Δ^4-6-ketone (**5**) whose separation was readily attained by column chromatography on silica gel. The attack of *m*-chloroperbenzoic acid toward **4** did take place mainly from the less hindered α-side to afford the desired 2α,3α-epoxide (**7**) as a major product accompanied with a small amount of the 2β,3β-epoxide (**6**). Reductive cleavage of 2α,3α-oxido ring and reduction of the carbonyl group at C-6 position with $LiAlH_4$ was then carried out simultaneously. As expected, the *trans*-diaxial opening of the oxide ring and attack of hydride toward the carbonyl group from α-side occurred to give the 3α,6β-diol (**8a**) as the major product accompanied with a small amount of 3α,6α-diol whose separation was easily attained by column chromatography on silica. The structural assignment of the 3α,6β-diol was rationalized by inspection of the ^{1}H-NMR spectrum where multiplet signals due to the equatrial 3β-H and 6α-H at 4.01-4.04 ppm and 3.62-3.679 ppm were observed. Here, inversion of the 3α-hydroxyl to 3β-hydroxyl is required to obtain the targeted compound. Therefore, we attempted oxidation of **8a** with Jones' reagent followed by $NaBH_4$ reduction of

the resulting 3,6-diketone (**9a**) to afford 3β,6β-dihydroxy derivative (**10a**). It has previously been demonstrated that the 6β-hydroxy group is not susceptible to *tert*-butyldimethylsilylation due to steric hindrance [10,15]. Accordingly, the 3β,6β-diol was treated with *tert*-butyldimethylsilyl chloride and imidazole in the usual manner to afford the 3-monosilyl ether (**10b**) in a fairly good yield. After treating with $POCl_3$ in pyridine, **10b** was dehydrated to yield cholesterol *tert*-butyldimethylsilyl ether (**11a**), which can be readily converted to cholesterol by acid hydrolysis. The synthetic route thus established is promising to introduce the deuterium stereospecifically into the 2β, 3α, and C-6 positions of cholesterol.

The preparation of 2β-deuterated-3α-ol (**8b**) was then attained by *trans*-diaxial opening of the 2α,3α-epoxide (**7**) with $LiAlD_4$. The compound **8b** was converted to the 2β-d_1-3,6-diketone (**9b**) by oxidation with Jones' reagent. Subsequent reduction of compound **9b** with $NaBD_4$, followed by silylation of the resulting (2β,3α,6-$^2H_3$)3β,6β-diol (**10c**) afforded the (2β,3α,6-$^2H_3$)3β,6β-diol 3-monosilyl ether (**10d**). Dehydration of the 6β-hydroxyl function in **10d** with $POCl_3$ in pyridine yielded solely the Δ^5-unsaturated compound (**11b**). Being subjected to desilylation, **11b** was transformed into the desired (2β,3α,6-$^2H_3$)cholesterol. The structure of the obtained deuterated cholesterol was confirmed by ^{1}H-NMR along with positive-ion EI-MS spectrum. ^{1}H-NMR chemical shifts were identical with those of the non-deuterated cholesterol except for the disappearance of 2β-, 3α- and 6-proton signals. The positive-ion EI-MS spectrum showed $[M]^{+\bullet}$ at *m/z* 389 as the base peak which was shifted

to 3 Da, whereby ion peak of $[M\text{-}CH_3]^{+\bullet}$, $[M\text{-}HDO]^{+\bullet}$, $[M\text{-}H_2O]^{+\bullet}$, $[M\text{-}H_2O\text{-}CH_3]^{+\bullet}$, [M-HDO-side chain (S.C.)]$^{+\bullet}$, and $[M\text{-}H_2O\text{-S.C.}]^{+\bullet}$ were also observed. In addition, the most typical fragments in the MS spectrum of (2β,3α,6-$^2H_3$)cholesteryl acetate is that derived by the loss of acetic acid at *m/z* [M-60], a classical McLafferty rearrangement [24] (Fig. 2) involving the stereospecific elimination of a hydrogen atom at 2α position. One of the well-known mechanisms of ion fragmentation is the so-called retro Diels-Alder (rDA) reaction that yields a diene and an olefin. We believe that the rDA reaction produces not only the diene fragment ions but also the complementary dienophilic one. This mechanism justifies the observed fragments at *m/z* 247, *m/z* 163, and *m/z* 95. Another typical fragmentation pathway is the loss of side chain that yields ion at *m/z* 258 [M-C_8H_{17}] as well as the loss of a methyl group resulting in an ion at *m/z* 356 [M-15]. Thus the results of analysis confirmed the structure of (2β,3α,6-$^2H_3$)cholesterol. Isotopic purity of the labeled compound as ($^2H_3$)-form estimated to be more than 90 atom % $^2H_3$, based on the ion intensity in the region of molecular ion. Accordingly, deuterated cholesterol was esterified with linoleic and oleic acid using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and *N,N'*-dimethylaminopyridine in anhydrous CH_2Cl_2 to give (2β,3α,6-$^2H_3$)cholesteryl linoleate (**12a**) and (2β,3α,6-$^2H_3$)cholesteryl oleate (**12b**) in a fairly good yield. The structures of the obtained deuterated cholesteryl linoleate and oleate were confirmed by ^1H and ^{13}C NMR spectra (Table 1) along with ESI- and collision induced dissociation (CID)-MS spectra (Fig. 3). ^1H and ^{13}C NMR spectra were identical with those of the non-deuterated compound

except for the disappearance of 2β, 3α-, 6-proton and C-2, C-3 and C-3 carbon signals. The positive-ion ESI-MS spectra showed that the *m/z* values of the ammonium adduct molecules were shifted to 3 Da at *m/z* 669 for linoleate and at *m/z* 671 for oleate. The CID mass spectra of $[M+NH_4]^+$ derived from the (2β,3α,6-$^2H_3$)cholesteryl linoleate and (2β,3α,6-$^2H_3$)cholesteryl oleate by the cleavage of ester bond were shifted to 3 Da, whereby $[M+NH_4- NH_3$-linoleic acid$]^+$ and $[M+ NH_4-NH_3$-oleic acid$]^+$ as the base peak were observed. Thus the results of CID analysis confirmed the structures of (2β,3α,6-$^2H_3$)cholesteryl linoleate and cholesteryl oleate, which gave rise to transition $[M+NH_4]^+ \rightarrow [M+NH_4-NH_3$-linoleic acid$]^+$ for linoleate and $[M+ NH_4-NH_3$-oleic acid$]^+$ for oleate. These ion peaks could be used for the monitoring ion. The isotopic purities of the labeled compounds as ($^2H_3$)-form were estimated to be more than 90 atom % $^2H_3$ for these esters, based on the ion intensities in the region of ammonium adduct molecules of the esters.

In conclusion, (2β,3α,6-$^2H_3$)cholesteryl linoleate and (2β,3α,6-$^2H_3$)cholesteryl oleate are now available. These compounds should be useful for sensitive, selective, and accurate LC-MS/MS determination of cholesteryl esters present in various biological fluids. A further detailed study on a method for the analytical, clinical, and biochemical application is now progressing in our laboratory, and the result will be reported at a later date.

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Figure captions and legends

Fig. 1. Synthetic route to (2β,3α,6-$^{2}H_{3}$)cholesteryl linoleate and oleate.

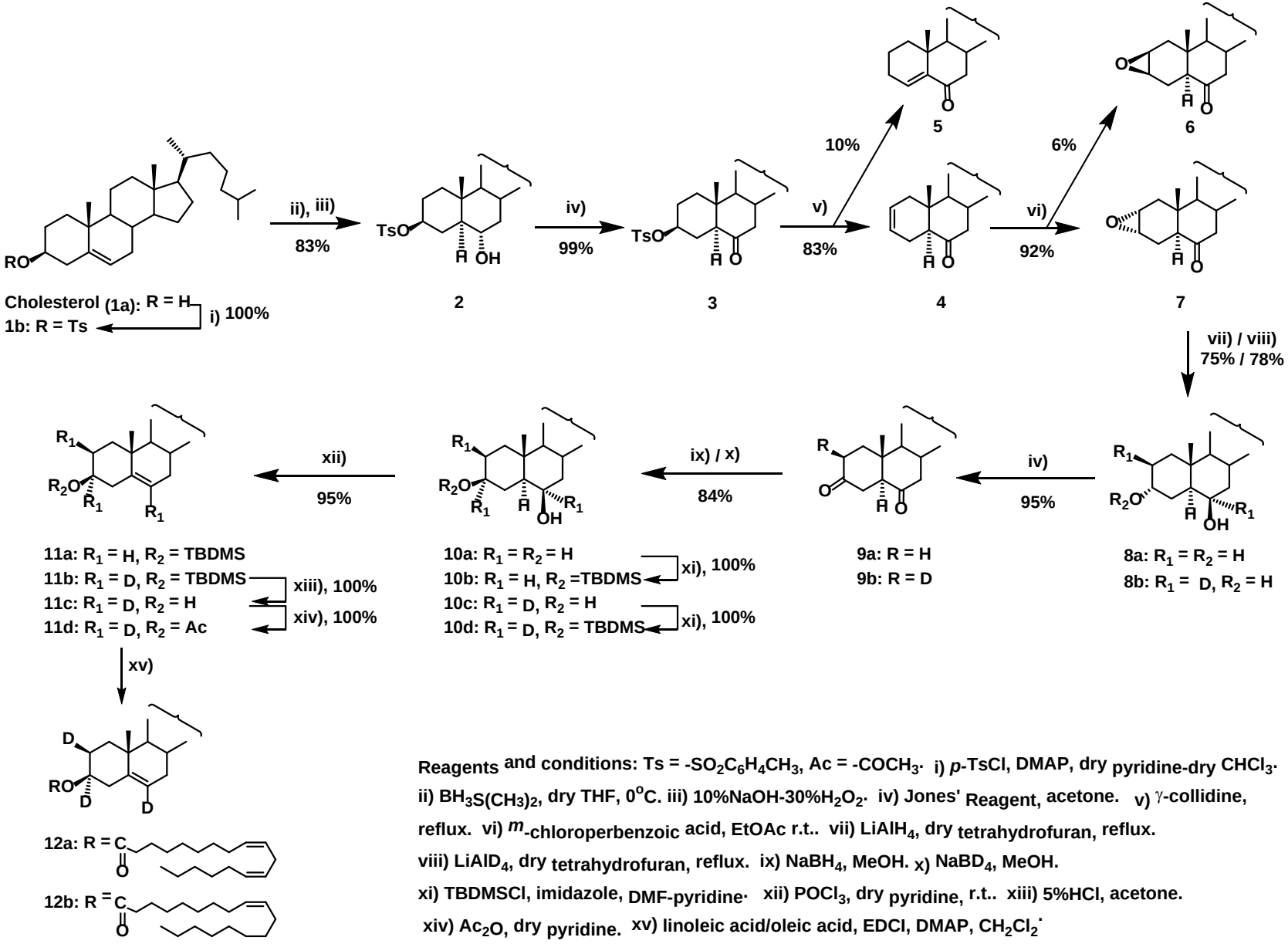
Fig. 2. EI-MS spectrum of (2β,3α,6-$^{2}H_{3}$)cholesteryl acetate and its fragmentation pathway.

Fig. 3. ESI-MS (upper) and product ion spectra (lower) obtained by CID of $[M+H]^{+}$ of (2β,3α,6-$^{2}H_{3}$)cholesteryl linoleate and oleate.

Table 1
Complete assignment of ^{1}H and ^{13}C NMR chemical shifts of $(2\beta,3\alpha,6\text{-}^{2}H_3)$cholesteryl linoleate and oleate

$(2\beta,3\alpha,6\text{-}^{2}H_3)$cholesteryl linoleate (12a)						$(2\beta,3\alpha,6\text{-}^{2}H_3)$cholesteryl oleate (12b)					
Cholesterol core			Linoleic acid core			Cholesterol core			Oleic acid core		
Carbon No. (Type)	^{13}C	^{1}H	Carbon No. (Type)	^{13}C	^{1}H	Carbon No. (Type)	^{13}C	^{1}H	Carbon No. (Type)	^{13}C	^{1}H
1 (CH_2)	23.96	1.34, 1.12 (m)	1‘(C)	173.42	----------	1 (CH_2)	23.96	1.34, 1.14 (m)	1‘(C)	173.43	----------
2 (CHD)	27.33	1.81 (m)	2‘(CH_2)	34.84	2.26 (t, *J* = 7.6)	2 (CHD)	27.36	1.82 (m)	2‘(CH_2)	34.84	2.26 (t, *J* = 7.7)
3 (CD)	----------	----------	3‘(CH_2)	25.19	1.60 (m)	3 (CD)	----------	----------	3‘(CH_2)	25.19	1.61 (m)
4 (CH_2)	38.09	2.30 (s)	**4‘(CH_2)	29.32	1.31 (m)	4 (CH_2)	38.09	2.30 (s)	**4‘(CH_2)	29.92	1.30 (m)
5 (C)	139.76	----------	**5‘(CH_2)	29.25	1.31 (m)	5 (C)	139.76	----------	**5‘(CH_2)	29.92	1.30 (m)
6 (CD)	----------	----------	**6‘(CH_2)	29.23	1.31 (m)	6 (CD)	----------	----------	**6‘(CH_2)	29.83	1.30 (m)
7 (CH_2)	31.90	1.96, 1.54 (m)	***7‘(CH_2)	29.74	1.31 (m)	7 (CH_2)	31.90	1.97, 1.44 (m)	**7‘(CH_2)	29.68	1.30 (m)
8 (CH)	31.96	1.42 (m)	8‘(CH_2)	27.33	2.04 (q, *J* = 6.6)	8 (CH)	31.96	1.56 (m)	8‘(CH_2)	27.30	2.01 (m)
9 (CH)	50.14	0.94 (m)	9‘(CH)	130.20	5.36 (m)	9 (CH)	50.14	0.95 (m)	9‘(CH)	130.12	5.34 (m)
10 (C)	36.67	----------	10‘(CH)	130.35	5.36 (m)	10 (C)	36.67	----------	10‘(CH)	129.90	5.34 (m)
11 (CH_2)	21.16	1.48 (m)	11‘(CH_2)	25.76	2.77 (dd, *J* = 6.6, 6.6)	11 (CH_2)	21.16	1.49 (m)	11‘(CH_2)	27.30	2.01 (m)
12 (CH_2)	39.85	2.01, 1.11 (m)	12'(CH)	128.04	5.33 (m)	12 (CH_2)	39.85	2.02, 1.12 (m)	**12'(CH)	29.68	1.30 (m)
13 (C)	42.43	----------	13‘(CH)	128.16	5.33 (m)	13 (C)	42.43	----------	**13‘(CH)	29.48	1.30 (m)
14 (CH)	56.81	1.01 (m)	14‘(CH_2)	27.33	2.04 (q, *J* = 6.8)	14 (CH)	56.81	1.02 (m)	**14‘(CH_2)	29.31	1.30 (m)
15 (CH_2)	24.42	1.57, 1.05 (m)	***15‘(CH_2)	29.50	1.31 (m)	15 (CH_2)	24.42	1.58, 1.04 (m)	**15‘(CH_2)	29.24	1.30 (m)
16 (CH_2)	28.37	1.84, 1.23 (m)	16‘(CH_2)	31.67	1.31 (m)	16 (CH_2)	28.38	1.85, 1.29 (m)	16‘(CH_2)	32.06	1.27 (m)
17 (CH)	56.25	1.09 (m)	17‘(CH_2)	22.72	1.29 (m)	17 (CH)	56.25	1.09 (m)	17‘(CH_2)	22.84	1.28 (m)
18 (CH_3)	11.99	0.67 (s)	18‘(CH_3)	14.24	0.89 (t, *J* = 6.3)	18 (CH_3)	11.99	0.67 (s)	18‘(CH_3)	14.27	0.88 (t, *J* = 6.3)
19 (CH_3)	19.45	1.01 (s)				19 (CH_3)	19.45	1.01 (s)			
20 (CH)	35.94	1.37 (m)				20 (CH)	35.94	1.38 (m)			
21 (CH_3)	18.84	0.91 (d, *J* = 6.3)				21 (CH_3)	18.84	0.91 (d, *J* = 6.3)			
22 (CH_2)	36.31	1.32 , 0.98 (m)				22 (CH_2)	36.31	1.33,1.00 (m)			
23 (CH_2)	39.65	1.11 (m)				23 (CH_2)	39.65	1.13 (m)			
24 (CH_2)	37.03	1.84, 1.11 (m)				24 (CH_2)	37.03	1.85, 1.12 (m)			
25 (CH)	28.16	1.51 (m)				25 (CH)	28.16	1.51 (m)			
*26 (CH_3)	22.96	0.863 (d, *J* = 6.8)				*26 (CH_3)	22.97	0.864 (d, *J* = 6.3)			
*27 (CH_3)	22.71	0.858 (d, *J* = 6.8)				*27 (CH_3)	22.71	0.859 (d, *J* = 6.3)			

Measured in $CDCl_3$ at 400 MHz in ^{1}H NMR and 100 MHz in ^{13}C NMR; chemical shifts were expressed as δ ppm relative to TMS: Values in parenthesis refer to coupling constant (*J* in Hz).
Abbreviation used: s, singlet; d, doublet; dd, doubelt of doublets; t, triplet, q, quartet, m, multiplet. *,**,***The chemical shifts are exchangeable..



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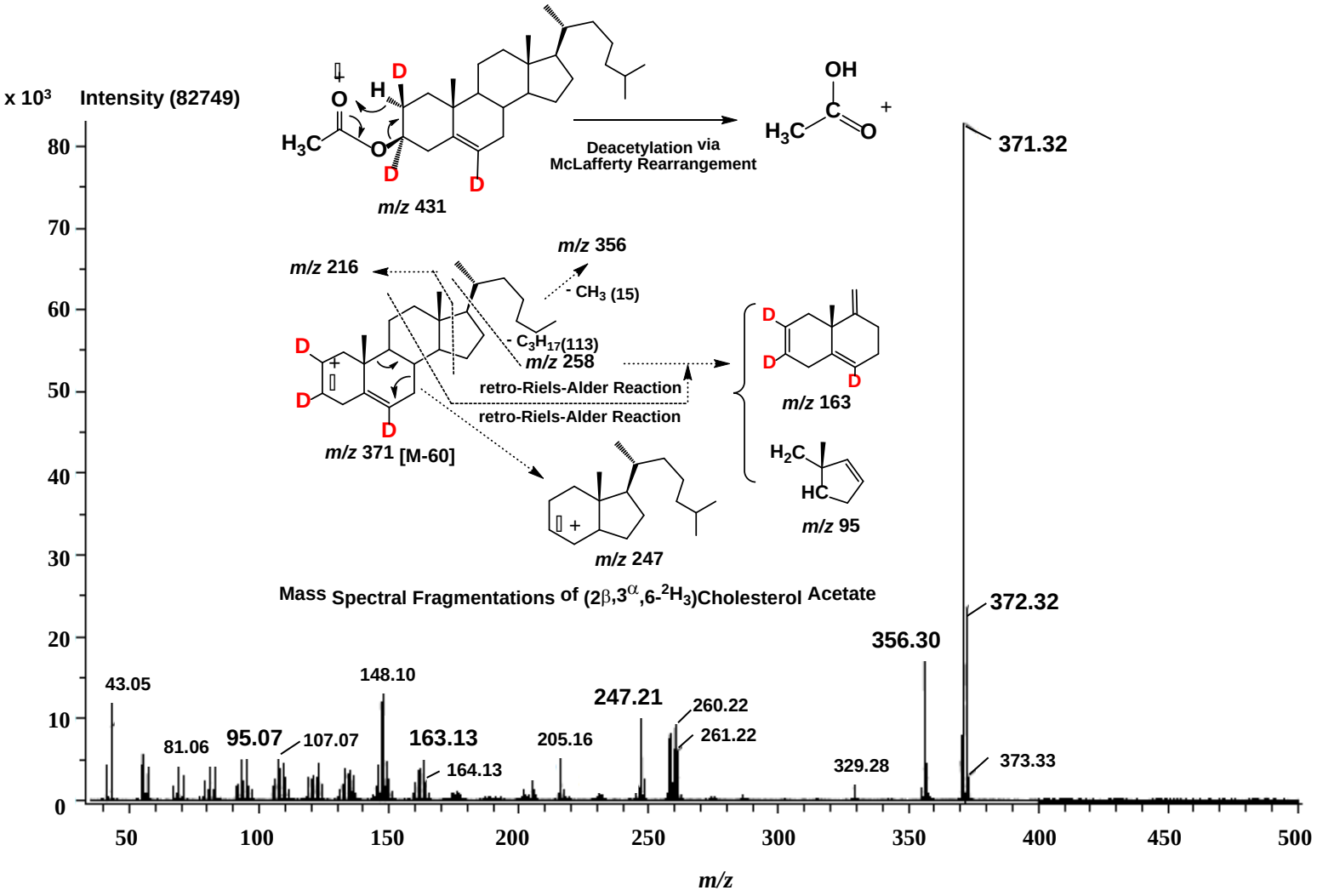

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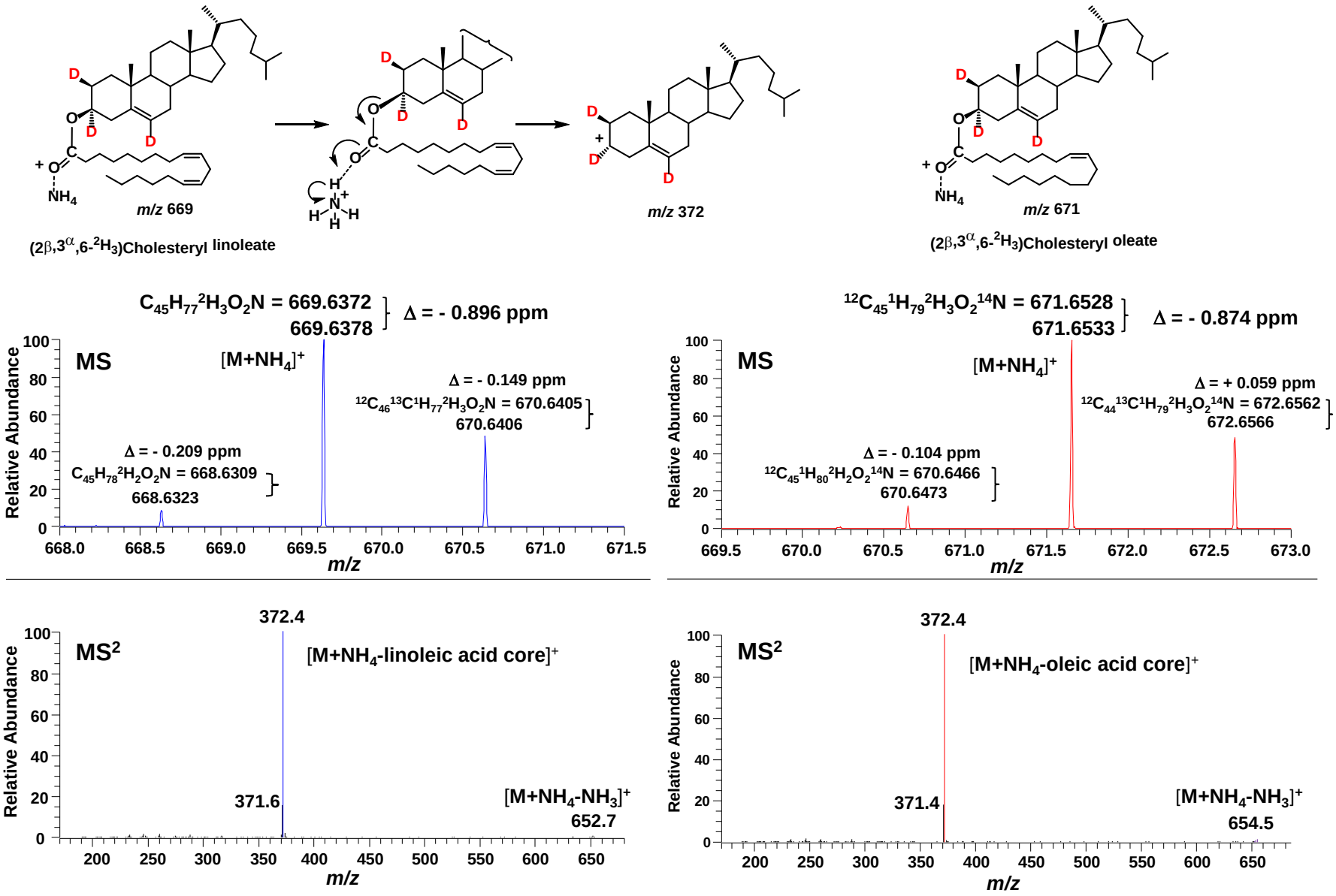

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