Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 5 July 2011, Revised 21 September 2011, Accepted 31 October 2011 Published online 8 December 2011 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.1952

Synthesis of [D_4]- and [D_7]-4β-hydroxycholesterols for use in a novel drug–drug interaction assay

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Cytochrome P450 3A (CYP3A) enzymes are involved in the metabolism of over half of today's prescription drugs. As a result, drugs metabolized by CYP3A have a risk of drug–drug interactions (DDIs). Recent studies have shown the potential to use 4β-hydroxycholesterol as an endogenous biomarker of CYP3A activity and predictor of potential DDIs. Bristol–Myers Squibb has developed a liquid chromatography-electron ionization-tandem mass spectrometry method that accurately measures 4β-hydroxycholesterol levels in clinical plasma samples following treatment with a CYP3A inducer or inhibitor. Stable isotope labeled (SIL) [D_4]- and [D_7]-4β-hydroxycholesterols were synthesized to assist in the development of this new quantification method. The SIL analogs were prepared from the appropriate [D_4]- or [D_7]-cholesterol starting material in two steps. The labeled cholesterols were oxidized with bromine and silver acetate in pyridine to give an acetate protected hydroxy group at C4. Hydrolysis of the acetate protecting group provided [D_4]- and [D_7]-4β-hydroxycholesterols in 15%–28% overall yield. 4α-Hydroxycholesterol was also required during method development and was prepared in four steps from cholesteryl benzoate in 1% overall yield.

Keywords: 4β-hydroxycholesterol; 4α-hydroxycholesterol; deuterium; stable isotope labeling; drug–drug interactions

Introduction

Drug–drug interactions (DDIs) occur when two drugs share a common metabolic pathway.[1] These interactions may increase, decrease, or alter a drug's desired therapeutic effect. DDIs are well documented, and many drugs have been removed from the market due to their negative impact. The cytochrome P450 family of enzymes, found mainly in mitochondria, are membrane-bound hemeproteins that are vital in the phase I oxidation of organic molecules.[2] Members of the cytochrome P450 3A (CYP3A) subfamily, in particular CYP3A4 and to a lesser extent CYP3A5, are involved in the metabolism of over half of today's prescription drugs.[2,3] With CYP3A being so heavily involved in drug metabolism, the enzyme is a common target in the assessment of DDIs.[4] Current exogenous markers of CYP3A activity involve the administration and measurement of probe drugs, such as midazolam, erythromycin, alprazolam, alfentanil, nifedipine, dapsone, omeprazole, and dextromethorphan.[5] However, dosing subjects with a probe drug in addition to the drug of interest is particularly challenging in certain patient populations including pediatric and geriatric patients, transplant recipients, and cancer patients.[5,6] Measurement of the urinary 6β-hydroxycortisol-to-cortisol ratio serves as an endogenous marker of CYP3A activity eliminating the need to dose with a probe drug, but this method also suffers from several drawbacks including diurnal variation of cortisol, high inter-individual variation, and poor correlation with results obtained from a probe drug.[7]

Identification of an appropriate endogenous CYP3A substrate and development of a suitable quantification method would be an attractive alternative allowing for simplification and possibly a broader application of the phenotyping approach. Recent studies have shown the potential to use 4β-hydroxycholesterol as an endogenous biomarker of CYP3A activity.[3,8,9] 4β-Hydroxycholesterol is produced *in vivo* by the metabolism of cholesterol and it has been found to be exclusively mediated by the enzyme CYP3A.[8,10] In addition, circulating levels of 4β-hydroxycholesterol have been shown to be fairly stable in plasma, eliminating the need for urine collection and minimizing blood sample collections as the same sample could be used for both exposure and 4β-hydroxycholesterol measurements. It has been shown that subjects treated with a CYP3A inducer had an increase in 4β-hydroxycholesterol over basal levels

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and subjects treated with a CYP3A inhibitor had a decrease in 4β-hydroxycholesterol levels using an isotope-dilution Gas chromatography–mass spectrometry method.[9,11,12] The development of an liquid chromatography–mass spectrometry assay was considered more amenable to the routine measurement of 4β-hydroxycholesterol levels in clinical samples for predicting potential DDIs.

A liquid chromatography-electron ionization-tandem mass spectrometry (LC-ESI-MS/MS) method was developed for accurately measuring 4β-hydroxycholesterol levels in clinical plasma samples following treatment with a CYP3A inducer or inhibitor.[6] SIL [D_4]-4β-hydroxycholesterol **[D_4-1]**, [D_7]-4β-hydroxycholesterol **[D_7-1]**, and unlabeled 4α-hydroxycholesterol **2** were required for both method development and clinical sample analyses (Figure 1). The SIL [D_4]-4β-hydroxycholesterol **[D_4]-1** analog was utilized as the internal standard in the assay for quantification. Because of the endogenous presence of 4β-hydroxycholesterol, [D_7]-4β-hydroxycholesterol **[D_7]-1** was employed as the calibrant in the standard curve and quality control (QC) samples prepared in human plasma. The 4α-hydroxycholesterol **2** isomer is isobaric with 4β-hydroxycholesterol but is produced from the auto-oxidation of cholesterol and is not an indicator of CYP3A activity; therefore, it was necessary to ensure chromatographic resolution of the isomers for the accurate quantification of 4β-hydroxycholesterol. 4α-hydroxycholesterol **2** and [D_4]-4β-hydroxycholesterol **[D_4-1]** are not commercially available and although [D_7]-4β-hydroxycholesterol **[D_7-1]** is, it is relatively expensive in the amounts required for the method development and clinical sample analyses. As a result, samples of all three 4-hydroxycholesterol analogs needed to be synthesized. It is important to note that the success of this analytical approach was strongly influenced by the chemical and isotopic purities of [D_4]-4β-hydroxycholesterol **[D_4-1]** and [D_7]-4β-hydroxycholesterol **[D_7-1]**. This paper describes the syntheses of [D_4]-4β-hydroxycholesterol **[D_4-1]**, [D_7]-4β-hydroxycholesterol **[D_7-1]**, and unlabeled 4α-hydroxycholesterol **2**.

Results and discussion

Standards of [D_4]-4β-hydroxycholesterol **[D_4]-1**, [D_7]-4β-hydroxycholesterol **[D_7]-1**, and unlabeled 4α-hydroxycholesterol **2** needed to be synthesized in order to develop a liquid chromatography–mass spectrometry assay to quantify 4β-hydroxycholesterol in clinical samples. The SIL [D_4]-4β-hydroxycholesterol **[D_4]-1** and [D_7]-4β-hydroxycholesterol **[D_7]-1** analogs were required as either an internal standard or calibrant in the assay, respectively. 4α-hydroxycholesterol **2** was required during the development of the LC-ESI-MS/MS method to ensure chromatographic resolution of 4α-hydroxycholesterol **2** and 4β-hydroxycholesterol for accurate quantification. Our initial plan was to synthesize [D_4]-4β-hydroxycholesterol **[D_4]-1**, [D_7]-4β-hydroxycholesterol **[D_7]-1** and unlabeled 4α-hydroxycholesterol **2** following the procedure described in the literature by Shibuya.[13] In this route, cholesteryl acetate was converted in several steps to a mixture containing largely 4β-hydroxycholesterol **1** and a lesser amount of 4α-hydroxycholesterol **2** (Scheme 1). This synthetic plan appeared advantageous for several reasons. Several deuterium labeled cholesterol analogs are commercially available, which would make sourcing necessary starting materials relatively easy. More importantly, one synthetic route could be used to synthesize both 4β-hydroxycholesterols **[D_4]-1** and **[D_7]-1** and 4α-hydroxycholesterol **2**.

In Shibuya's procedure, cholesteryl acetate **3** undergoes allylic oxidation using selenium dioxide and formic acid to provide a mixture of allylic alcohols (**4** and **5**) (Scheme 1). The crude alcohols (**4** and **5**) were then treated with lithium aluminum hydride to give a 3:1 mixture of 4β- and 4α-hydroxycholesterol isomers **1** and **2** in a 57% yield. We attempted to reproduce these results in hopes of isolating pure samples of 4β- and 4α-hydroxycholesterol isomers **1** and **2**. In our hands, the oxidation step to produce the allylic alcohols (**4** and **5**) provided two major products and several minor products according to thin layer chromatography (TLC). The two major products were isolated by column chromatography and based on the literature, were assumed to be the 4β- and 4α-hydroxycholesterol acetate isomers **4** and **5**. ^{1}H NMR analysis of the isolated products revealed that the 4β-hydroxycholesterol acetate **5** and re-arrangement product **6** with the acetate protecting group on C4 were actually isolated (Scheme 2) and not the desired 4α-hydroxycholesterol acetate **5**. All of our attempts to reproduce Shibuya's results resulted in either no alpha isomer formed or the yield was so minor it could not be isolated; therefore, other literature procedures that could provide both 4β- and 4α-hydroxycholesterol isomers **1** and **2** were examined.

Marquet *et al.* detail a synthetic route that could provide both 4β- and 4α-hydroxycholesterol **1** and **2** isomers using similar chemistry (Scheme 3).[14] Cholesteryl benzoate **7** was oxidized with selenium dioxide to provide the 4β-allylic alcohol **8** in low to moderate yield. It is worth noting that none of the 4α-allylic alcohol was observed. Alcohol **8** was deprotected to give 4β-hydroxycholesterol **1** or oxidized, reduced, and deprotected to provide 4α-hydroxycholesterol **2**. This route could yield both 4β- and 4α-hydroxycholesterol isomers **1** and **2**; however, in our hands, the overall yields were relatively low and were not suitable for an isotopically labeled synthesis. Even though the yields were low, this was the only viable synthetic route that

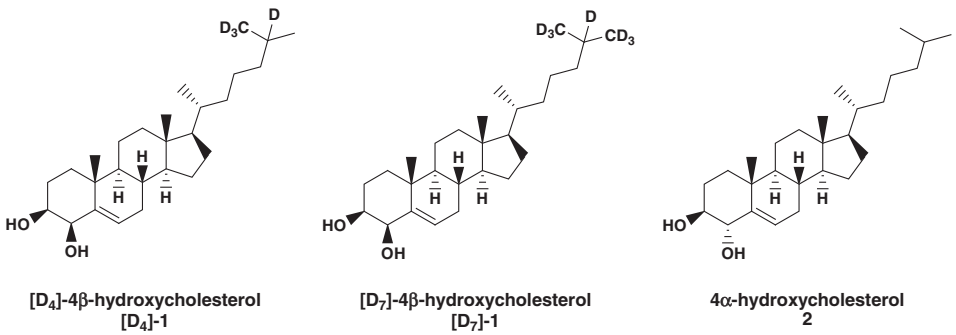
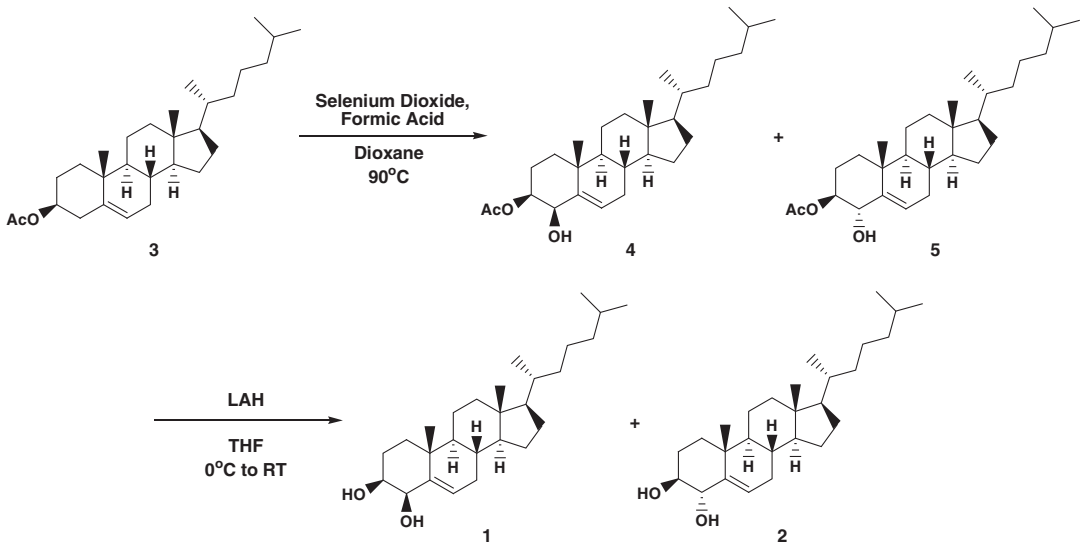
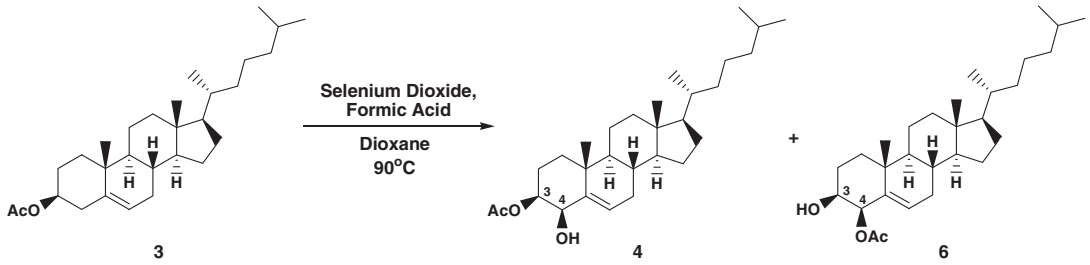


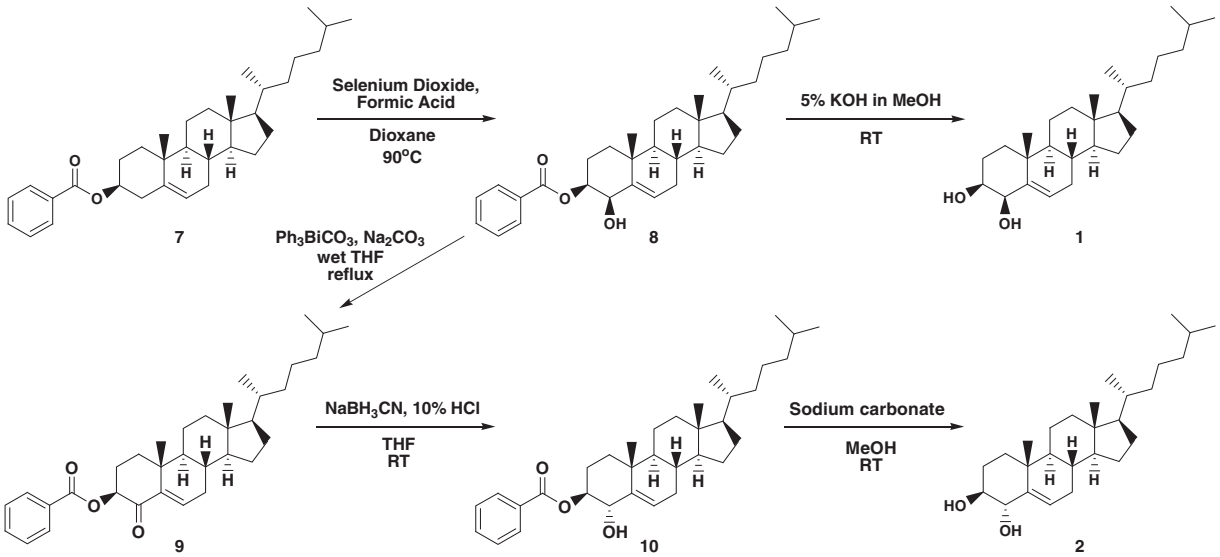
Figure 1. Stable isotope labeled 4β-hydroxycholesterols **[D4]-1** and **[D7]-1** and unlabeled 4α-hydroxycholesterol 2.



Scheme 1. Shibuya's reported synthesis of 4β- and 4α-hydroxycholestrol **1** and **2**.



Scheme 2. Observed products from oxidation reaction following Shibuya's procedure.



Scheme 3. Synthesis of unlabeled 4β- and 4α-hydroxycholesterol **1** and **2**.

was found to give 4α-hydroxycholesterol **2**. We ultimately prepared 65 mg of 4α-hydroxycholesterol **2** following Marquet's procedure (Scheme 3) in an overall yield of 1% with a chemical purity of 97%.

An additional review of the literature revealed a more direct route to 4β-hydroxycholesterol. Poza *et al.* described using Petrows' re-arrangement to incorporate a β-hydroxyl group at the C4 position of cholesterol.[15] This route was more attractive from a labeling standpoint because [D_4]-4β-hydroxycholesterol **[D_4]-1** or [D_7]- 4β-hydroxycholesterol **[D_7]-1** could be prepared from labeled cholesterol in fewer steps and in higher yield. In addition, no protection of the β-hydroxyl group at the C3 position of cholesterol would be needed. However, it should be

noted that this route leads only to 4β-hydroxycholesterol; none of the 4α-hydroxycholesterol was observed. The synthesis of deuterium labeled [D_4]-4β-hydroxycholesterol **[D_4]-1** is illustrated in Scheme 4. Commercially available [D_4]-cholesterol **[D_4]-11** was reacted with bromine in chloroform followed by treatment with silver acetate in pyridine to provide the acetate protected intermediate **[D_4]-6**. The intermediate was purified by flash column chromatography and analyzed by ^{1}H NMR. NMR results on isolated acetate intermediate **[D_4]-6** compared favorably with literature values for unlabeled compound.[11] Acetate intermediate **[D_4]-6** was then hydrolyzed with methanolic KOH to provide [D_4]-4β-hydroxycholesterol **[D_4]-1** after purification by flash column chromatography. ^{1}H NMR results compared favorably with unlabeled compound.[15] Correlation spectroscopy (COSY) experiment was conducted to unambigously assign protons C3 and C4 and the ^{1}H NMR H-H coupling constant of 3.27 Hz then confirmed the *cis* orientation at C3 and C4. [16] We ultimately prepared 158 mg of [D_4]-4β-hydroxycholesterol **[D_4]-1** in 15% overall yield with a chemical purity of 94%.

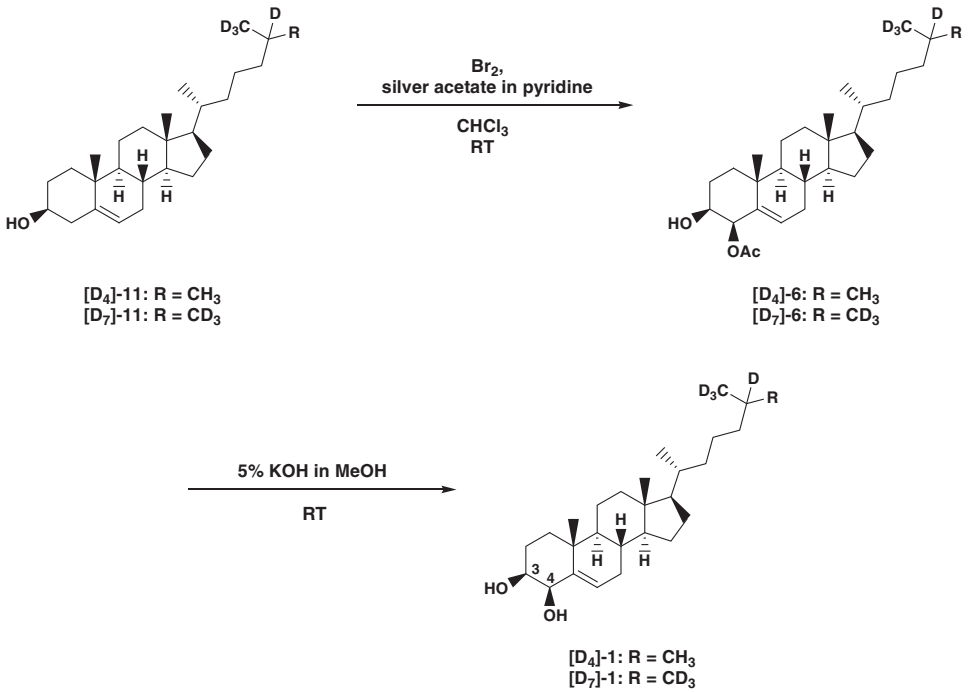
Synthesis of [D_7]-4β-hydroxycholesterol **[D_7]-1** was achieved following the same two steps to provide [D_4]-4β-hydroxycholesterol **[D_4]-1** (Scheme 4). Commercially available [D_7]-cholesterol **[D_7]-11** was converted to acetate intermediate **[D_7]-6**, then the intermediate was hydrolyzed to form the [D_7]-4β-hydroxycholesterol **[D_7]-1**. Both acetate intermediate **[D_7]-6** and [D_7]-4β-hydroxycholesterol **[D_7]-1** were purified by flash column chromatography. Each product was analyzed by ^{1}H NMR and compared with literature values for unlabeled compounds.[15] COSY experiment on and [D_7]-4β-hydroxycholesterol **[D_7]-1** assigned protons C3 and C4 and ^{1}H NMR H-H coupling constant of 3.02 Hz confirmed the *cis* orientation at C3 and C4.[16] We ultimately prepared 345 mg of [D_7]-4β-hydroxycholesterol **[D_7]-1** in 28% overall yield with a chemical purity of 93%.

Experimental procedure

Reagents were obtained from Aldrich Chemical Co., EMD Chemical Inc., and Mallinckrodt Inc. SIL [D_7]-cholesterol **[D_7]-1** was obtained from Cambridge Isotope Laboratories, Inc. SIL [D_4]-cholesterol **[D_4]-1** was obtained from CDN Isotopes. All reactions were magnetically stirred. Solvent removal under vacuum was performed using a Buchi R-124 rotary evaporator. Column chromatography was performed using a Biotage flash chromatography system. Proton NMR spectra were recorded on 400 MHz Bruker DPX400A spectrometer (Buchi, Biotage, and Bruker). Proton NMR COSY experiments were performed to determine the stereochemistry at C3 and C4. Chemical purity was assessed by HPLC/UV/Corona Charged Aerosol Detection using the following conditions: instrument = Shimadzu HPLC, column = Unison C18 (3 × 150 mm), temperature = 25 °C, mobile phase = 90/10 MeCN/water (isocratic for 25 min), and flow rate = 1 mL/min. Molecular weight was verified using GC/MS electron ionization (EI) using the following conditions: instrument = Varian 3800, column = Agilent NP-5MS (L = 30 m, ID = 250 μm, film = 0.25 μm), carrier gas = helium, oven = 200 °C for 1 min, 200–350 at 10 °C/min, 350 °C for 4 min, detection = electron impact, and flow rate = 2 mL/min.

[D_4]-4β-Acetoxycholest-5-en-3β-ol ([D_4]-6)

An aluminum foil wrapped round-bottomed flask was charged with [D_4]-cholesterol **[D_4]-11** (954 mg, 2.44 mmol) and chloroform (13.3 mL). The reaction mixture was cooled to −10 °C and stirred under nitrogen atmosphere. Bromine (0.133 mL, 2.58 mmol) was added, and the mixture was allowed to stir at −10 °C for 30 min. A suspension of silver acetate (1.77 g, 10.6 mmol) in pyridine (3.98 mL, 49.2 mmol) was added, and the reaction was warmed to room temperature. The reaction mixture was stirred at room temperature for 17 h before being filtered. The filtrate was diluted with 5% HCl (20 mL) and water



Scheme 4. Synthesis of stable isotope labeled [D_4]- and [D_7]-4β-hydroxycholesterols **[D_4]-1** and **[D_7]-1**.

(40 mL). The biphasic mixture was partitioned, and the aqueous layer was extracted with CH_2Cl_2 (4 × 30 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (100 mL) and concentrated under reduced pressure. The resulting gray solid was purified by flash column chromatography (75% hexanes/25% EtOAc; TLC conditions: 75% hexanes/25% EtOAc, stained with anisaldehyde, product $R_f = 0.28$) to provide [D_4]-4β-acetoxycholest-5-en-3β-ol **[D_4]-6** as a white solid in 20% yield (214 mg, 0.476 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.83 (1H, dd, $J = 5.0$, 2.0 Hz), 5.38 (1H, d, $J = 3.5$ Hz), 3.53–3.76 (1H, m), 2.08 (3H, s), 1.11–2.05 (23H, m), 1.09 (3H, s), 0.91 (3H, d, $J = 6.5$ Hz), 0.85 (6H, d, $J = 1.5$ Hz), 0.67 (3H, s).

[D_4]-4β-Hydroxycholesterol ([D_4]-1)

A round-bottomed flask was charged with [D_4]-4β-acetoxycholest-5-en-3β-ol **[D_4]-6** (213 mg, 0.474 mmol) and a solution of 5% KOH in methanol (12 mL). The solution was stirred at room temperature while being monitored by TLC (TLC conditions: 50% hexanes/50% EtOAc, stained with anisaldehyde, product $R_f = 0.60$). After 2.5 h of stirring, the reaction mixture was concentrated under reduced pressure to half its original volume. The remaining solution was poured into cold water (15 mL). The aqueous solution was extracted with EtOAc (3 × 15 mL). The combined organic extracts were washed with water (2 × 25 mL), saturated brine (25 mL), and concentrated under reduced pressure. The resulting light yellow solid was purified by flash column chromatography (50% hexanes/50% EtOAc) to provide [D_4]-4β-hydroxycholesterol **[D_4]-1** as a white solid in 75% yield (158 mg, 0.354 mmol) with 94.4% chemical purity. A two step overall yield of 15% starting form [D_4]-cholesterol **[D_4]-11** was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.60–5.74 (1H, m), 4.14 (1H, d, $J = 3.3$ Hz), 3.44–3.64 (1H, m), 1.18 (3H, s), 0.94–2.15 (24H, m), 0.91 (3H, d, $J = 6.5$ Hz), 0.85 (6H, d, $J = 1.5$ Hz), 0.68 (3H, s). GCMS (EI) found *m/z* of 406.22. Isotopic distribution was performed on dipicolinic ester derivatives.[6] Isotopic purity: [M + 4] 97.5%, [M + 3] 2.4%, all others below limit of detection.

[D_7]-4β-Acetoxycholest-5-en-3β-ol ([D_7]-6)

[D_7]-4β-Acetoxycholest-5-en-3β-ol **[D_7]-6** was prepared following the same chemistry for [D_4]-4β-acetoxycholest-5-en-3β-ol **[D_4]-6**. [D_7]-Cholesterol **[D_7]-11** (977 mg, 2.48 mmol) provided [D_4]-4β-acetoxycholest-5-en-3β-ol **[D_4]-6** as a white solid in 53% yield (593 mg, 1.31 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.83 (1H, dd, $J = 4.9$, 1.9 Hz), 5.38 (1H, d, $J = 3.8$ Hz), 3.54–3.71 (1H, m), 2.08 (3H, s), 1.11–2.05 (23H, m), 1.09 (3H, s), 0.91 (6H, d, $J = 6.5$ Hz), 0.67 (3H, s).

[D_7]-4β-Hydroxycholesterol ([D_7]-1)

[D_7]-4β-hydroxycholesterol **[D_7]-1** was prepared following the same chemistry for [D_4]-4β-hydroxycholesterol **[D_4]-1**. [D_4]-4β-Acetoxycholest-5-en-3β-ol **[D_7]-6** (588 mg, 1.30 mmol) gave by flash column chromatography (50% hexanes/50% EtOAc) [D_7]-4β-hydroxycholesterol **[D_7]-1** as a white solid in 55% yield (345 mg, 0.727 mmol) with 93.3% chemical purity. A two step overall yield of 28% starting form [D_7]-cholesterol **[D_7]-11** was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.68 (1H, dd), 4.14 (1H, d, $J = 3.0$ Hz), 3.56 (1H, dt, $J = 11.6$, 4.2 Hz), 1.18 (3H, s), 0.95–2.14 (21H, m), 0.91 (3H, d, $J = 6.5$ Hz), 0.84–0.89 (6H, m), 0.68 (3H, s). GCMS (EI) found *m/z* of 409.13. Isotopic distribution was performed on dipicolinic ester derivatives.[6] Isotopic purity: [M + 7] 93.8%, [M + 6] 5.9%, [M + 5] <0.5%, all others below limit of detection.

Conclusion

We successfully synthesized two SIL 4β-hydroxycholesterol (**[D_4]-1** and **[D_7]-1)** analogs to assist in the development of a new LC-ESI-MS/MS method to quantify 4β-hydroxycholesterol levels in clinical plasma samples. Starting with the appropriate commercial SIL cholesterols, [D_4]- and [D_7]-4β-hydroxycholesterols (**[D_4]-1** and **[D_7]-1)** were prepared following a two-step procedure in 15%–28% yield. Although the yields could likely be improved through additional optimization, this work provided a quick and relatively inexpensive route to [D_4]- and [D_7]-4β-hydroxycholesterols (**[D_4]-1** and **[D_7]-1**. This new MS assay has the potential to be used as a general method to assess DDIs earlier in the drug development process, potentially saving time and money compared with the current techniques used to determine DDIs.

Acknowledgements

The authors would like to thank the BMS Pharmaceutical Candidate Optimization and Discovery Analytical Sciences Groups and members of the BMS Radiochemical Synthesis Group. Special thanks to David Wang-Iverson, Christopher Poronsky, Xiaohua Huang, Yingru Zhang, Michael Witkus, and Jun Dai for all their help.

Conflict of Interest

The authors did not report any conflict of interest.

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