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Short communication

# Synthesis of cholesterol analogs having varying length alkyl side chains including cholesterol-23, 23, 24, 24, 25, 26, 26, 26, 27, 27, 27-$d_{11}$ as probes of cholesterol's functions and properties

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ABSTRACT

Cholesterol-23, 23, 24, 24, 25, 26, 26, 26, 27, 27, 27-$d_{11}$ and nondeuterated long-chain analogs of cholesterol were prepared by alkylation of cyano-containing sterols with isopentyl-$d_{11}$ 4-methylbenzenesulfonate (1.0 equiv.) or with isoalkyl bromides, followed by reductive decyanation with excess potassium metal and a crown ether in toluene. The products are potentially useful probes of the role of the side-chain of cholesterol in the sterol's interactions with membrane lipids and proteins.



## 1. Introduction

The role of the isooctyl side chain of cholesterol in cholesterol's ability to influence membrane properties and interactions with phospholipids and proteins has been examined by using a variety of biophysical methods (Chia et al., 1993; McMullen et al., 1995; Vilchèze et al., 1996; Jaikishan and Slotte, 2011; Mattjus et al., 1995; Scheidt et al., 2013; for a review, see Bittman, 1997). Alteration of the hydrophobic lengths of lipids and proteins affects both lipid–lipid and lipid–protein interactions, thereby modifying the organization of lateral domains in bilayer membranes. In addition to the use of short side-chain and long side-chain analogs of cholesterol to examine the hydrophobic mismatch effect in membranes, deuterium has been introduced in the isooctyl chain of cholesterol for use in $^2$H NMR and neutron diffraction studies to examine the location of cholesterol in lipid model systems. For these studies, "methyl-tail" deuterated cholesterol analogs bearing 6 deuterium atoms (at C26 and C27 in cholesterol) or 7 deuterium atoms (at C25, C26, and C27) have been used frequently (e.g., Mihailescu et al., 2011; Lütjohann et al., 1993). In this communication, we report a new synthesis of alkyl side-chain analogs of cholesterol and of cholesterol-$d_{11}$, which bears deuterium atoms at C23, C23, C24, C24, C25, C26, C26, C26, C27, C27, and C27. Our synthesis of cholesterol bearing various isoalkyl chains is more practical than the syntheses reported previously.

Our synthesis of cholesterol-$d_{11}$ reported here represents the first deuterium-labeled cholesterol with more than seven deuterium atoms. Visualization of the uptake and trafficking of individual biomolecules in living cells with stable isotope labels (to avoid the use of bulky fluorescent probes) is under intensive current investigation by Raman spectroscopy, especially with lipids and probes or metabolites added exogenously (for a recent review, see Pezacki et al., 2011). Replacing C—H bonds with C—D bonds in biomolecules shifts the stretching frequency to a spectral region in which no other biomolecules have Raman peaks, giving distinctive, narrow signals in minimally perturbed molecules. The symmetric C—D stretching frequency (~2097–2120 $cm^{-1}$ for $CD_2$ groups and at ~2194 $cm^{-1}$ for $CD_3$ groups at the chain terminus) has been utilized to monitor various lipids containing perdeuterated fatty acyl chains such as palmitic acid-$d_{31}$ and dipalmitoylphosphatidylcholine-$d_{62}$ (Li et al., 2005; Weeks and Huser, 2010) and the A ring of cholesterol-$d_6$ (Matthaus et al., 2012). Cholesterol-$d_{11}$ may be useful in imaging of side-chain deuterated cholesterol by coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) microscopy since it is expected to give higher signal-to-noise ratios than the commercially available methyl-tail deuterium-labeled ($d_6$ and $d_7$) cholesterol probes.

## 2. Results and discussion

### 2.1. Previous syntheses of sterols bearing a deuterated alkyl side chain

Cholesterol-26, 26, 26, 27, 27, 27-$d_6$ was synthesized from pregnenolone via a Wittig olefination reaction with

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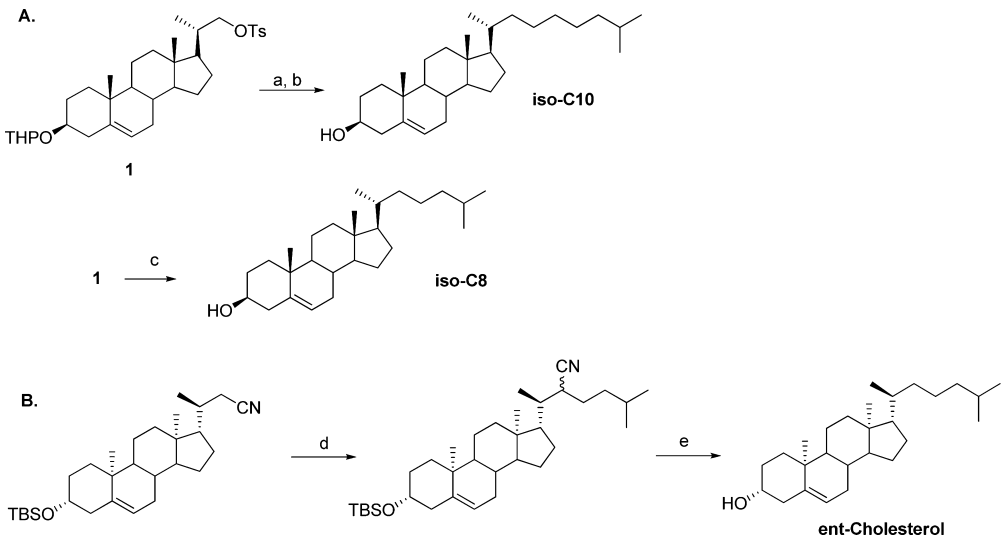



**Scheme 1.** Known synthetic schemes for the preparation of (**A**) isoalkyl-chain analogs of cholesterol and (**B**) *ent*-cholesterol. *Reagents and conditions:* (a) iso-$C_7H_{15}MgBr$, $Li_2CuCl_4$, THF; (b) $H^+$, MeOH, 52% (2 steps); (c) (i) iso-$C_5H_{11}MgBr$ (30 equiv.), $Li_2CuCl_4$, THF; (ii) conc. HCl, MeOH, 55%; (d) 1-bromo-3-methylbutane (10 equiv.), LDA, THF, 82%; (e) (i) K, dicyclohexano-18-crown-6, toluene; (ii) 1 M HCl, THF, 83% (2 steps).

4-methylpentyl-$d_6$-triphenylphosphonium bromide (Holm and Crossland, 1996). Cholesterol-25, 26, 26, 26, 27, 27, 27-$d_7$ was prepared by addition of isopropyl-$d_7$-magnesium bromide to a steroid bearing a formyl group at C-23 (6β-methoxy-3α,5-cyclo-5α-cholan-24-al) (Chu and Li, 2000). Deuterioalkyl groups have also been introduced by nucleophilic displacement reactions of sterol derivatives with α-lithiated deuterioalkyl phenylsulfones (Kirk et al., 1983; Ciuffreda et al., 2003). Many of these synthetic strategies proceeded in low overall yields. The present work describes the synthesis of such compounds more expeditiously.

### 2.2. Previous synthetic approaches to side-chain analogs of cholesterol and installation of the side chain into ent-cholesterol

Scheme 1A shows the synthetic route we and others employed previously to prepare isoalkyl-chain modified cholesterol analogs. The copper-catalyzed Grignard reaction of a hydroxyl-protected sterol-bearing tosylate (**1**) with an alkyl halide affords the products, such as cholesterol (iso-C8) and its homologue, iso-C10, but the reaction gives low yields, requires careful drying to secure the generation of the Grignard reagent, and consumes a large excess (as much as 30 equiv.) of the halide compound (Morisaki et al., 1980; Slotte et al., 1994).

Primary nitriles undergo α-alkylation reactions via formation of α-carbanion intermediates, which undergo substitution reactions with alkylating agents (Rojas et al., 2007). Reductive cleavage of a carbon-cyano bond has been achieved by using a variety of reductive methodologies with hydride ion or hydrogen atom sources, affording a carbon–hydrogen bond in moderate to good yield (Savoia et al., 1980; Mattalia et al., 2006; Patra et al., 2013). The use of a crown ether and a benzyl radical anion generated in a one-electron transfer reaction from potassium metal in toluene was shown to be effective for reductive cyanation of primary, secondary, and tertiary nitriles (Ohsawa et al., 1985). Scheme 1B shows a key step in the synthesis of the unnatural enantiomer of cholesterol, *ent*-cholesterol, in which the isooctyl chain was installed via alkylation of a nitrile-bearing aliphatic chain with 10 equiv. of an alkyl bromide, followed by reductive decyanation with potassium and a crown ether in toluene (Rychnovsky and Mickus, 1992).

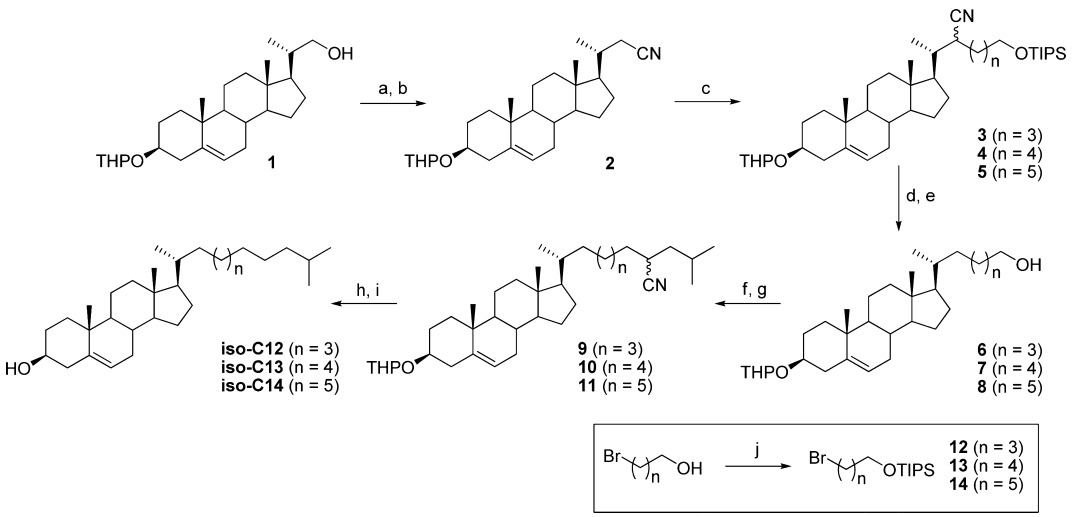
### 2.3. Coupling reactions via LDA treatment of cyano-bearing sterol intermediates

Scheme 2 shows the preparation of iso-C12, iso-C13, and iso-C14 from THP-protected alcohol **1** in nine steps and overall yields of ~39%. Alcohol **1** was prepared from commercially available bisnorcholenic acid as previously reported (Li et al., 2006). The reaction steps in Scheme 2 are easily controlled. It is noteworthy that alkylation of the α-carbanion generated by LDA treatment of sterol nitrile **2** with the silyl ether of an alkyl bromide (**12**, **13**, and **14**; step c in Scheme 2) required only 1 equiv. of the halide. Reaction of the α-carbanion of isocapronitrile with mesylates **6**, **7**, and **8** afforded the diastereomeric isoalkyl sterols **9**, **10**, and **11** in ~80% yield. The use of isocapronitrile in this synthetic strategy is a convenient route to isoalkyl chain analogs, since many isoalkyl halides are not available commercially. Finally, decyanation with potassium and dicyclohexano-18-crown-6, followed by quenching of the excess potassium with methanol, and hydrolysis of the protected alcohol afforded iso-C12, iso-C13, and iso-C14.

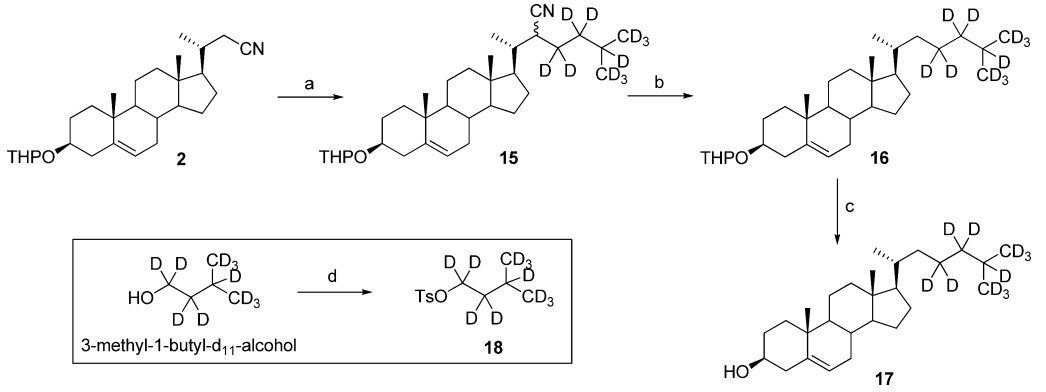
### 2.4. Preparation of cholesterol-$d_{11}$ (17)

We used nitrile **2** as the starting material for the synthesis of the target product **17**, as depicted in Scheme 3. Alkylation of **2** with 1 equiv. of tosylate **18**, which was prepared from commercially available 3-methyl-1-butanol-$d_{11}$, afforded **15** (epimeric at C-22) in 89% yield. We used tosylate **18** (1 equiv.) for alkylation rather than the corresponding bromide (as in Scheme 2) because 1-bromo-3-methylbutane is a volatile liquid (bp, 121 °C). Reductive decyanation with potassium metal and dicyclohexano-18-crown-6 in toluene, followed by acid-mediated hydrolysis of the tetrahydropyranyl ether, afforded target product **17**. Thus, cholesterol-$d_{11}$ was prepared in only four steps and in an overall yield of 71% from compound **2**.

The synthetic strategy described here has the potential of being readily scaled up to prepare gram quantities of cholesterol-$d_{11}$ and isoalkyl analogs of cholesterol with shorter or longer chains than the iso-C8 chain of natural cholesterol.




**Scheme 2.** Synthesis of non-deuterated sterol analogs with varying isoalkyl side-chain lengths. *Reagents and conditions:* (a) MsCl (3 equiv.), $Et_3N$, $CH_2Cl_2$; (b) KCN, DMSO, 82% for 2 steps; (c) **12**, **13**, or **14**, LDA, THF, −78 °C, 90% for **3**, 92% for **4**, 88% for **5**; (d) K, dicyclohexano-18-crown-6, toluene; (e) TBAF, THF, overall yields (2 steps) 74% for **6**, 76% for **7**, 77% for **8**; (f) MsCl, $Et_3N$, $CH_2Cl_2$; (g) isocapronitrile (1 equiv.), LDA, THF, −78 °C, overall yields (2 steps) 81% for **9**, 78% for **10**, 79% for **11**; (h) K, dicyclohexano-18-crown-6, toluene; (i) 1 M HCl, THF, overall yields (2 steps) 88% for **iso-C12**, 84% for **iso-C13**, and 85% for **isoC14**; (j) TIPSCl (1 equiv.), imidazole (2 equiv.), $CH_2Cl_2$, 85% for **12**, 88% for **13**, 79% for **14**.




**Scheme 3.** Synthesis of cholesterol-$d_{11}$ (**17**). *Reagents and conditions:* (a) **18**, LDA, THF, 89%; (b) K, dicyclohexano-18-crown-6, toluene, 92%; (c) 1 M HCl, THF, 87%; (d) TsCl, DMAP, $Et_3N$, $CH_2Cl_2$, 98%.

## 3. Experimental procedures

### 3.1. General synthetic methods

For a description of the general methods, see Byun and Bittman (2012).

### 3.2. 22-Cyanochol-5-en-3β-ol THP ether (2)

A solution of alcohol **1** (300 mg, 0.72 mmol), which was prepared from bisnorcholenic acid by a known procedure (Li et al., 2006), and $NEt_3$ (1.0 mL, 7.2 mmol) in $CH_2Cl_2$ (10 mL) was cooled to 0 °C. Methanesulfonyl chloride (0.28 mL, 3.6 mmol) was added dropwise. After the addition was complete, the mixture was stirred at room temperature overnight. The solution was washed with water, dried, and concentrated. To a solution of the resulting mesylate in DMSO (10 mL) was added potassium cyanide (234 mg, 3.6 mmol). The mixture was heated at 100 °C for 1 d, and then was diluted with EtOAc and water. The organic layer was separated, washed with brine, dried ($Na_2SO_4$), and concentrated. The residue was purified by chromatography (hexane/EtOAc 8:1) to give 251 mg (82%) of nitrile **2** as a white solid: mp 180–182 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.70 (s, 3H), 1.01 (s, 3H), 1.02–1.65 (m, 19H), 1.17 (d, $J$ = 6.6 Hz, 3H), 1.78–1.87 (m, 4H), 1.95–1.99 (m, 2H), 2.20–2.29 (m, 2H), 2.32–2.39 (m, 2H), 3.47–3.57 (m, 2H), 3.89–3.95 (m, 1H), 4.70–4.72 (m, 1H), 5.34–5.35 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 14.7, 19.3, 19.4, 20.0, 20.1, 21.0, 21.8, 24.2, 24.8, 25.5, 28.1, 31.3, 31.8, 33.6, 36.5, 36.7, 36.8, 37.2, 38.7, 39.4, 40.2, 42.7, 50.0, 54.7, 54.8, 56.5, 62.9, 63.0, 71.7, 76.0, 96.9, 97.0, 119.1, 121.3, 121.4; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{28}H_{43}D_{43}NO_2$ 425.3294, found 425.3289.

### 3.3. Synthesis of compounds 3, 4, and 5

After a solution of **2** (100 mg, 0.23 mmol) in THF (4 mL) was cooled to −78 °C, 0.63 mL (0.94 mmol) of LDA (1.5 M solution in cyclohexane) was added dropwise, followed by for 30 min. The halide-containing compound (**12**, **13**, or **14**) (1.0 equiv.) was added, and stirring was continued for another 4 h. The reaction was quenched with aqueous $NH_4Cl$ solution. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were dried ($Na_2SO_4$), filtered, and concentrated under reduced pressure. The product was purified by chromatography (hexane/EtOAc 10:1) to give compounds **3**, **4**, and **5** as colorless oils.

Compound **3**: 90%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.69 (s, 3H), 0.97–1.11 (m, 30H), 1.22–1.30 (m, 4H), 1.41–1.57 (m, 15H), 1.63–1.75 (m, 3H), 1.83–2.00 (m, 6H), 2.17–2.22 (m, 1H), 2.33–2.37 (m, 1H), 2.60–2.65 (m, 1H), 3.47–3.56 (m, 2H), 3.65–3.71 (m, 2H),

3.89–3.93 (m, 1H), 4.70–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.7, 11.8, 11.9, 12.3, 12.5, 13.4, 14.1, 14.5, 15.5, 17.1, 17.7, 18.0, 18.8, 19.4, 20.1, 20.7, 20.1, 22.7, 23.7, 24.1, 24.2, 24.8, 25.3, 25.5, 27.6, 28.0, 28.1, 29.1, 29.7, 29.8, 30.7, 31.3, 31.6, 31.8, 31.9, 32.4, 32.7, 34.5, 34.7, 36.1, 36.5, 36.7, 36.8, 37.2, 37.4, 38.1, 38.8, 39.5, 40.2, 42.4, 42.5, 43.8, 49.9, 50.0, 52.4, 54.1, 56.5, 56.7, 62.9, 63.0, 63.1, 76.0, 96.9, 97.0, 121.1, 121.3, 121.4, 122.9, 140.9, 141.1; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{41}H_{72}NO_3Si$ 654.5281, found 654.5277.

Compound **4**: 92%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.69 (s, 3H), 1.00–1.13 (m, 30H), 1.15–1.30 (m, 4H), 1.40–1.48 (m, 7H), 1.50–1.58 (m, 10H), 1.63–1.75 (m, 3H), 1.80–2.01 (m, 6H), 2.16–2.38 (m, 2H), 2.60–2.64 (m, 1H), 3.47–3.56 (m, 2H), 3.69 (t, $J$ = 6.3 Hz, 2H), 3.89–3.94 (m, 1H), 4.70–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.8, 11.9, 14.6, 18.0, 18.7, 18.9, 19.4, 20.1, 21.0, 23.9, 24.1, 25.5, 25.6, 27.6, 28.0, 28.1, 29.7, 29.8, 30.9, 31.3, 31.8, 31.9, 32.7, 32.8, 35.9, 36.4, 36.7, 36.8, 37.2, 37.4, 38.0, 38.2, 38.8, 39.5, 40.2, 42.4, 42.5, 43.7, 49.9, 50.0, 54.2, 56.5, 62.9, 63.2, 63.3, 76.0, 96.9, 97.0, 121.2, 121.3, 121.4, 122.9, 140.9, 141.0, 141.1; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{42}H_{74}NO_3Si$ 668.5438, found 668.5437.

Compound **5**: 88%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.69 (s, 3H), 1.00–1.11 (m, 30H), 1.16–1.1.30 (m, 9H), 1.35–1.46 (m, 4H), 1.48–1.57 (m, 10H), 1.61–1.75 (m, 3H), 1.83–2.00 (m, 6H), 2.20–2.43 (m, 2H), 2.59–2.64 (m, 1H), 3.45–3.57 (m, 2H), 3.64–3.69 (m, 2H), 3.89–3.94 (m, 1H), 4.70–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.8, 11.9, 12.0, 12.6, 14.6, 17.7, 18.0, 18.8, 18.9, 19.4, 20.1, 21.0, 23.8, 24.1, 25.5, 25.6, 25.7, 27.8, 28.1, 29.0, 29.3, 29.7, 30.9, 31.3, 31.8, 31.9, 32.9, 33.0, 36.5, 36.7, 36.8, 37.2, 37.4, 38.3, 38.8, 39.5, 40.3, 42.4, 50.0, 54.2, 56.5, 63.0, 63.3, 63.4, 76.0, 97.0, 97.1, 121.2, 121.4, 122.8, 141.1; ESI-HRMS $(M+H)^+$ $m/z$ calcd for $C_{43}H_{76}NO_3Si$ 682.5594, found 682.5591.

### 3.4. Synthesis of compounds *6*, *7*, and *8*

To 70 mg of compound **3**, **4**, or **5** in 4 mL of toluene was added excess (>6 equiv.) potassium metal and dicyclohexyl-18-crown-6 (1.2 equiv.) at room temperature. After 8 h, methanol was added carefully to quench the excess potassium. The mixture was diluted with EtOAc, and the organic layer was separated, washed with brine, dried ($Na_2SO_4$), and concentrated under reduced pressure. To a solution of the residue in THF (4 mL) was added 3 equiv. of TBAF (tetra-*n*-butylammonium fluoride, 1 M solution in THF). After being stirred at room temperature for 12 h, the reaction mixture was diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. Purification by silica gel chromatography, eluting with hexane/EtOAc (2:1), gave compounds (**6**, **7**, and **8**) as white solids.

Compound **6**: 74%; mp 118–120 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.90–0.98 (m, 4H), 1.01–1.12 (m, 8H), 1.15–1.38 (m, 12H), 1.42–1.62 (m, 7H), 1.71–1.75 (m, 2H), 1.83–1.86 (m, 4H), 1.92–2.01 (m, 2H), 2.17–2.36 (m, 2H), 3.47–3.52 (m, 2H), 3.62 (t, $J$ = 6.6 Hz, 2H), 3.89–3.93 (m, 1H), 4.70–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 14.1, 18.7, 19.4, 20.0, 20.1, 21.1, 24.3, 25.5, 25.9, 26.2, 28.0, 28.3, 29.7, 31.3, 31.9, 32.2, 32.9, 35.7, 35.9, 36.8, 37.2, 37.4, 38.8, 39.8, 40.2, 42.3, 50.1, 50.2, 56.1, 62.8, 62.9, 63.0, 76.0, 96.8, 96.9, 121.5, 121.6, 140.9, 141.0; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{31}H_{53}O_3$ 473.3995, found 473.3991.

Compound **7**: 76%; mp 142–144 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.90–0.95 (m, 4H), 1.00–1.11 (m, 8H), 1.14–1.1.32 (m, 12H), 1.42–1.58 (m, 9H), 1.68–1.73 (m, 2H), 1.78–1.87 (m, 4H), 1.92–2.01 (m, 2H), 2.18–2.36 (m, 2H), 3.45–3.54 (m, 2H), 3.62 (t, $J$ = 6.6 Hz, 2H), 3.87–3.94 (m, 1H), 4.70–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.7, 19.4, 20.0, 21.0, 24.3, 25.5, 25.8, 26.1, 28.0, 28.2, 29.7, 29.9, 31.2, 31.3, 31.9, 32.8, 35.7, 35.9, 36.7, 36.8, 37.2, 37.4, 38.8, 39.8, 40.3, 42.3, 50.1, 50.2, 56.1, 56.7, 62.8, 62.9, 76.0, 96.8, 96.9, 121.5, 121.6, 140.8, 141.0, ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{32}H_{55}O_3$ 487.4151, found 487.4143.

Compound **8**: 77%; mp 124–125 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.90–0.94 (m, 4H), 0.97–1.11 (m, 8H), 1.14–1.33 (m, 12H), 1.42–1.56 (m, 11H), 1.69–1.74 (m, 2H), 1.78–1.86 (m, 4H), 1.94–2.01 (m, 2H), 2.17–2.36 (m, 2H), 3.46–3.54 (m, 2H), 3.62 (t, $J$ = 6.6 Hz, 2H), 3.89–3.94 (m, 1H), 4.71–4.72 (m, 1H), 5.32–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 14.1, 18.7, 19.4, 20.0, 20.1, 21.1, 22.7, 24.3, 25.5, 25.6, 25.8, 26.0, 28.0, 28.2, 29.3, 29.4, 29.5, 29.7, 30.1, 31.3, 31.9, 32.8, 35.7, 35.9, 36.8, 37.2, 37.5, 38.8, 39.8, 40.2, 42.3, 50.2, 53.8, 56.1, 56.8, 62.8, 62.9, 63.0, 76.0, 96.8, 97.0, 121.5, 121.6, 140.9, 141.1; ESI-HRMS $(M+Na)^+$ $m/z$ calcd. for $C_{33}H_{56}O_3Na$ 523.4127, found 523.4123.

### 3.5. Synthesis of compounds *9*, *10*, and *11*

To a solution of **6**, **7** or **8** (0.32, 0.42, or 0.25 mmol, respectively) and $NEt_3$ (10 equiv.) in $CH_2Cl_2$ (3 mL) was added methanesulfonyl chloride (3 equiv.). After being stirred at room temperature for 3 h, the reaction mixture was evaporated under reduced pressure, diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. After a solution of isocapronitrile (1 equiv.) in THF (3 mL) was cooled to −78 °C, LDA (1.5 M solution in cyclohexane, 4 equiv.) was added dropwise, and the resulting solution was stirred for 30 min. Mesylated compound (1 equiv.) was added, and stirring was continued for another 4 h. The reaction was quenched with aqueous $NH_4Cl$ solution. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were dried ($Na_2SO_4$), filtered, and concentrated under reduced pressure. The product was purified by chromatography (hexane/EtOAc 6:1) to give compounds **9**, **10**, and **11** as waxy solids.

Compound **9**: 81%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.86–0.96 (m, 12H), 0.98–1.02 (m, 6H), 1.03–1.11 (m, 4H), 1.15–1.28 (m, 8H), 1.33–1.38 (m, 4H), 1.43–1.63 (m, 9H), 1.68–1.71 (m, 2H), 1.77–1.86 (m, 4H), 1.93–2.04 (m, 2H), 2.18–2.37 (m, 2H), 3.47–3.53 (m, 2H), 3.91–3.93 (m, 1H), 4.72–4.73 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 14.1, 14.2, 18.7, 19.4, 20.0, 20.1, 21.1, 21.3, 21.5, 21.7, 22.3, 22.4, 22.7, 22.9, 24.2, 24.3, 25.5, 25.8, 26.1, 26.2, 27.2, 28.0, 28.3, 29.4, 29.7, 30.0, 30.9, 31.1, 31.3, 31.9, 32.7, 35.7, 35.8, 36.8, 37.2, 37.4, 37.5, 38.8, 39.8, 40.3, 41.3, 42.3, 42.8, 49.7, 50.2, 56.0, 56.8, 60.4, 62.8, 62.9, 64.3, 76.0, 96.8, 97.0, 121.5, 121.6, 122.5, 141.1; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{37}H_{62}NO_2$ 552.4781, found 552.4765.

Compound **10**: 78%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.87–0.96 (m, 12H), 0.98–1.02 (m, 6H), 1.04–1.12 (m, 4H), 1.15–1.29 (m, 8H), 1.31–1.38 (m, 4H), 1.40–1.62 (m, 11H), 1.64–1.79 (m, 2H), 1.81–1.88 (m, 4H), 1.95–2.05 (m, 2H), 2.15–2.38 (m, 2H), 3.47–3.55 (m, 2H), 3.91–3.93 (m, 1H), 4.72–4.74 (m, 1H), 5.35–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 14.1, 17.2, 18.7, 19.4, 20.1, 21.1, 22.4, 22.7, 24.3, 25.5, 25.8, 25.9, 26.0, 27.0, 28.0, 28.3, 28.7, 29.4, 29.5, 29.7, 30.0, 31.1, 31.3, 31.9, 32.7, 35.7, 35.8, 36.8, 37.2, 37.5, 38.8, 39.8, 40.3, 42.3, 43.6, 45.2, 50.2, 56.1, 56.8, 60.4, 62.8, 62.9, 64.3, 76.0, 96.8, 97.0, 121.5, 121.6, 122.4, 141.1; ESI-HRMS $(M+Na)^+$ $m/z$ calcd. for $C_{38}H_{63}NO_2Na$ 588.4756, found 588.4791.

Compound **11**: 79%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.87–0.97 (m, 12H), 0.98–1.03 (m, 6H), 1.04–1.19 (m, 4H), 1.21–1.40 (m, 12H), 1.43–1.62 (m, 13H), 1.66–1.74 (m, 2H), 1.77–1.89 (m, 4H), 1.91–2.05 (m, 2H), 2.17–2.37 (m, 2H), 3.47–3.55 (m, 2H), 3.89–3.94 (m, 1H), 4.71–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 14.2, 18.7, 19.4, 20.0, 20.2, 21.1, 21.6, 22.4, 22.7, 22.9, 23.3, 24.3, 25.5, 25.8, 26.0, 26.2, 27.2, 27.5, 28.0, 28.3, 28.7, 29.2, 29.3, 29.4, 29.7, 29.8, 30.0, 30.3, 31.3, 31.9, 32.7, 34.3, 35.6, 35.8, 36.8, 37.2, 37.5, 38.5, 38.8, 39.3, 39.9, 41.5, 42.4, 50.2, 56.1, 56.8, 60.6, 62.8, 62.9, 76.1, 96.8, 97.0, 121.5, 121.6, 122.5, 140.9, 141.1;

ESI-HRMS $(M+Na)^+$ $m/z$ calcd. for $C_{39}H_{65}NO_2Na$ 602.4913, found 602.4910.

### 3.6. Synthesis of compounds **12**, **13**, and **14**

To a solution of 100 mg of alcohol (4-bromo-1-butanol, 5-bromo-1-pentanol, or 6-bromo-1-hexanol) in 5 mL of $CH_2Cl_2$ were added 2 equiv. of imidazole and 1.0 equiv. of TIPSCl (triisopropylsilyl chloride). After being stirred at room temperature for 4 h, the reaction mixture was diluted with water, and the product was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. The product was purified by chromatography (hexane/EtOAc 20:1) to give compounds **12**, **13**, and **14** as colorless oils.

Compound **12**: 85%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.03–1.10 (m, 21H), 1.68 (quin, $J$ = 6.1 Hz, 2H), 1.98 (quin, $J$ = 7.0 Hz, 2H), 3.46 (t, $J$ = 6.8 Hz, 2H), 3.72 (t, $J$ = 6.1 Hz, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.0, 29.6, 31.5, 34.0, 62.4; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{13}H_{30}BrOSi$ 309.1249, found 309.1248.

Compound **13**: 88%; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.05–1.11 (m, 21H), 1.49–1.58 (m, 4H), 1.89 (t, $J$ = 7.6 Hz, 2H), 3.41 (t, $J$ = 6.9 Hz, 2H), 3.69 (t, $J$ = 6.1 Hz, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 12.0, 18.0, 24.6, 32.1, 32.7, 33.8; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{14}H_{32}BrOSi$ 323.1406, found 323.1401.

Compound **14**: 79%: $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.00–1.12 (m, 21H), 1.33–1.50 (m, 4H), 1.55 (quin, $J$ = 6.8 Hz, 2H), 1.87 (quin, $J$ = 7.2 Hz, 2H), 3.41 (t, $J$ = 6.9 Hz, 2H), 3.68 (t, $J$ = 6.4 Hz, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 12.0, 18.0, 25.1, 28.0, 32.8, 32.9, 33.9, 63.2; ESI-HRMS $(M+H)^+$ $m/z$ calcd for $C_{15}H_{34}BrOSi$ 337.1562, found 337.1557.

### 3.7. Synthesis of iso-C12, iso-C13, and iso-C14

To a solution of cyano-sterol **9**, **10**, or **11** (0.030 mmol) in 4 mL of toluene were added excess potassium metal and 1.2 equiv. of dicyclohexyl-18-crown-6. After 8 h, methanol was added to quench the excess potassium. The mixture was diluted with EtOAc, the organic layer was separated, washed with brine, dried ($Na_2SO_4$), and concentrated. The residue was dissolved in THF (3 mL), and 1 M HCl (0.5 mL) was added. The mixture was stirred at room temperature for 24 h. The solution was concentrated, and the residue was purified by chromatography (hexane/EtOAc 3:1) to give the desired cholesterol analogs as white solids.

Compound **iso-C12**: 88%; mp 112–114 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.68 (s, 3H), 0.87 (d, $J$ = 6.6 Hz, 6H), 0.91 (d, $J$ = 6.6 Hz, 3H), 0.93–1.01 (m, 5H), 1.05–1.22 (m, 8H), 1.23–1.40 (m, 12H), 1.43–1.59 (m, 6H), 1.80–1.88 (m, 4H), 1.94–2.02 (m, 2H), 2.23–2.29 (m, 2H), 3.52 (tt, $J$ = 4.8, 11.6 Hz, 1H), 5.35 (d, $J$ = 5.2 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.7, 19.4, 21.1, 22.7, 24.3, 26.1, 27.5, 28.0, 28.2, 29.8, 30.0, 30.2, 31.7, 31.9, 35.8, 36.0, 36.5, 37.3, 39.1, 39.8, 42.3, 50.1, 56.1, 56.8, 71.8, 121.8, 140.8; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{31}H_{55}O$ 443.4253, found 443.4233.

Compound **iso-C13**: 84%; mp 111–112 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.68 (s, 3H), 0.86 (d, $J$ = 6.6 Hz, 6H), 0.90 (d, $J$ = 6.6 Hz, 3H), 0.93–0.1.01 (m, 5H), 1.03–1.19 (m, 8H), 1.22–1.40 (m, 12H), 1.45–1.61 (m, 8H), 1.80–1.87 (m, 4H), 1.95–2.05 (m, 2H), 2.23–2.29 (m, 2H), 3.53 (tt, $J$ = 4.5, 11.5 Hz, 1H), 5.35 (d, $J$ = 5.2 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.7, 19.4, 21.1, 22.7, 24.3, 26.1, 27.5, 28.0, 28.3, 29.8, 30.0, 30.2, 31.7, 31.9, 35.8, 36.0, 36.5, 37.3, 39.1, 39.8, 42.3, 50.1, 56.1, 56.8, 71.8, 121.8, 140.8; ESI-HRMS $(M+H)^+$ $m/z$ calcd for $C_{32}H_{57}O$ 457.4409, found 457.4385.

Compound **iso-C14**: 85%; mp 108–110 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.68 (s, 3H), 0.86 (d, $J$ = 6.6 Hz, 6H), 0.90 (d, $J$ = 6.6 Hz, 3H), 0.92–0.1.01 (m, 5H), 1.03–1.19 (m, 8H), 1.21–1.36 (m, 12H), 1.40–1.59 (m, 10H), 1.78–1.87 (m, 4H), 1.94–2.05 (m, 2H), 2.19–2.32 (m, 2H), 3.53 (tt, $J$ = 4.6, 11.1 Hz, 1H), 5.35 (d, $J$ = 5.2 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.7, 19.4, 21.1, 22.7, 24.3, 27.5, 28.0, 28.3, 29.7, 30.0, 30.2, 31.9, 35.8, 36.0, 36.5, 37.3, 39.1, 39.8, 42.3, 50.1, 56.1, 56.8, 71.8, 121.7, 140.8; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{33}H_{59}O$ 471.4566, found 471.4559.

### 3.8. Isopentyl-$d_{11}$ 4-methylbenzenesulfonate (18)

To a solution of 3-methyl-1-butanol-$d_{11}$ (100 mg, 1.0 mmol) in $CH_2Cl_2$ (3 mL) were added 0.7 mL (5.0 mmol) of $NEt_3$, 12 mg (0.10 mmol) of DMAP, and 384 mg (2.0 mmol) of $p$-toluenesulfonyl chloride. After being stirred at room temperature for 5 h, the reaction mixture was diluted with EtOAc and water. The organic layer was separated, washed with brine, dried ($Na_2SO_4$), and concentrated. The residue was purified by chromatography (hexane/EtOAc 10:1) to give 250 mg (98%) of tosylate **18** as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 2.45 (s, 3H), 7.34 (d, $J$ = 8.0 Hz, 2H), 7.79 (d, $J$ = 8.3 Hz, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 20.6, 20.8, 21.0, 21.1, 21.3, 21.6, 23.1, 23.3, 23.5, 35.8, 36.0, 36.2, 36.4, 36.5, 68.0, 68.3, 68.5, 68.7, 69.0, 127.9, 129.8, 133.2, 144.7; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{12}H_7D_{11}O_3S$ 253.1656, found 253.1667.

### 3.9. 22-Cyanochol-23, 23, 24, 24, 25, 26, 26, 26, 27, 27, 27-$d_{11}$-5-en-3$\beta$-ol THP ether (15)

After a solution of **2** (220 mg, 0.52 mmol) in THF (8 mL) was cooled to −78 °C, 1.4 mL (2.07 mmol) of LDA (1.5 M solution in cyclohexane) was added dropwise. The reaction mixture was stirred for 30 min. Deuterated tosylate **18** (131 mg, 0.52 mmo1) was added, and stirring was continued for another 4 h. The reaction was quenched with aqueous $NH_4Cl$ solution. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were dried ($Na_2SO_4$), filtered, and concentrated under reduced pressure. The product was purified by chromatography (hexane/EtOAc 3:1) to give 235 mg (89%) of alkylated product **15** as a white solid: mp 189–190 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.69 (s, 3H), 0.98 (s, 3H), 1.07 (d, $J$ = 6.5 Hz, 3H), 1.09–1.75 (m, 19H), 1.83–2.05 (m, 6H), 2.17–2.37 (m, 2H), 2.56 (d, $J$ = 2.6 Hz, 1H), 3.47–3.55 (m, 2H), 3.90–3.93 (m, 1H), 4.70–471 (m, 1H), 5.33–5.35 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 12.0, 14.6, 19.4, 20.2, 21.0, 21.2, 21.4, 21.5, 21.6, 21.7, 22.1, 22.2, 22.3, 22.4, 24.1, 25.5, 26.4, 26.6, 26.8, 27.7, 28.0, 28.1, 29.7, 31.3, 31.8, 31.9, 36.2, 26.4, 36.6, 36.7, 36.8, 37.2, 37.4, 38.0, 38.2, 38.3, 38.8, 39.5, 40.2, 42.4, 42.5, 49.9, 50.0, 52.4, 54.2, 56.5, 56.6, 63.0, 67.1, 76.0, 97.0, 97.1, 121.3, 121.4, 140.9, 141.1; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{33}H_{42}D_{11}NO_2$ 506.4756, found 506.4768.

### 3.10. Chol-23, 23, 24, 24, 25, 26, 26, 26, 27, 27, 27-$d_{11}$-5-en-3$\beta$-ol THP ether (16)

To 200 mg (0.39 mmol) of 22-cyanocholesterol THP ether **15** in 6 mL of toluene were added 100 mg (2.56 mmol) of potassium metal and 294 mg (0.79 mmol) of dicyclohexyl-18-crown-6. After 8 h, methanol was added carefully to quench the excess potassium. The mixture was diluted with EtOAc, and the organic layer was separated, washed with brine, dried ($Na_2SO_4$), and concentrated. The residue was purified by chromatography (hexane/EtOAc 20:1) to give 175 mg (92%) of **16** as a white solid: mp 148–150 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.67 (s, 3H), 0.91 (d, $J$ = 6.5 Hz, 3H), 1.01 (s, 3H), 0.94–1.74 (m, 21H), 1.80–1.86 (m, 4H), 1.94–2.02 (m, 2H), 2.17–2.37 (m, 2H), 3.46–3.57 (m, 2H), 3.91–3.94 (m, 1H), 4.70–4.72 (m, 1H), 5.33–5.36 (m, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.7, 19.4, 20.0, 20.1, 21.1, 21.2, 21.4, 21.6, 21.8, 24.3, 25.5, 26.7, 26.8, 27.0, 28.0, 28.3, 29.7, 31.3, 31.9, 32.0, 35.4, 35.5, 35.7, 35.8, 36.0, 36.8, 36.9, 37.2, 37.4, 37.8, 38.0, 38.2, 38.4, 38.5, 38.8, 39.8, 40.3, 42.3, 50.2, 56.2, 56.8, 62.9, 76.0, 96.8, 97.0, 121.5, 121.6, 140.9, 141.1; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{32}H_{43}D_{11}O_2$ 481.4803, found 481.4818.

### 3.11. Cholesterol-23, 23, 24, 24, 25, 26, 26, 26, 27, 27, 27-$d_{11}$ (17)

To a solution of ether **16** (50 mg, 0.10 mmol) in THF (2 mL) was added 1 M HCl (0.5 mL). The mixture was stirred at room temperature for 2 d. The solution was concentrated, and the residue was purified by chromatography (hexane/EtOAc 3:1) to give 36 mg (87%) of product **17** as a white solid: mp 138–139 °C; $[\alpha]_D^{23}$ −41.3° (*c* 0.75, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.68 (s, 3H), 0.91 (d, $J$ = 6.5 Hz, 3H), 1.01 (s, 3H), 0.93–1.61 (m, 16H), 1.78–1.87 (m, 3H), 1.94–2.05 (m, 2H), 2.19–2.32 (m, 2H), 3.48–3.56 (m, 1H), 5.35 (d, $J$ = 5.2 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 11.9, 18.7, 19.4, 21.1, 21.3, 21.4, 21.7, 21.9, 22.5, 22.7, 22.8, 23.0, 23.2, 23.3, 24.3, 26.6, 26.8, 27.0, 28.3, 31.7, 31.9, 35.4, 35.5, 35.7, 35.8, 36.0, 37.3, 37.8, 38.0, 38.2, 38.4, 38.5, 39.8, 42.4, 50.1, 53.4, 56.1, 56.2, 56.8, 71.8, 121.7, 140.8; ESI-HRMS $(M+H)^+$ $m/z$ calcd. for $C_{27}H_{35}D_{11}O$ 397.4228, found 397.4242.

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