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First Synthesis of Free Cholesterol−BODIPY Conjugates

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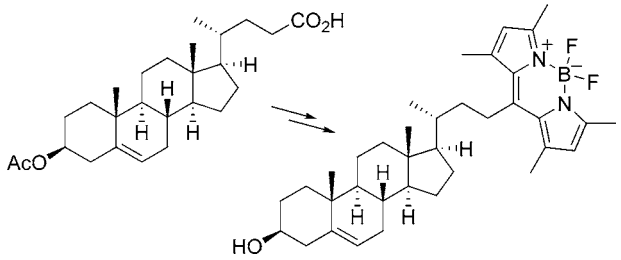

Analogues of cholesterol (compounds **1** and **2**) and coprostanol (compound **3**) containing the BODIPY fluorophore in the aliphatic tail of the free sterol have been synthesized starting with bisnorcholenic acid, cholenic acid 3β-acetate, and lithocholic acid, respectively. An ester linkage joining the fluorophore to the sterol nucleus interfered with the ability of the fluorescent sterol to pack with phospholipids in monolayers. However, an analogue in which the linker was devoid of polar atoms exhibited a substantially similar physical behavior to cholesterol in model membranes with respect to localization in raft domains.

The fluorophore consisting of a dipyrromethene boron difluoride chelate (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene), which is commonly known under the trademark BODIPY,[1] has many attractive spectral characteristics, such as high absorption coefficient, high fluorescence quantum yield, and long-wavelength emission. Other useful properties of the BODIPY fluorophore are its photochemical stability and insensitivity to changes in experimental conditions, such as the polarity, pH, and oxygen content of the medium.[1] BODIPY has been coupled covalently to all classes of biomolecules, including proteins,[1] DNA,[2] and carbohydrates.[3] In addition, BODIPY derivatives have been utilized as fluorescent switches[4] and as probes for protons,[5] mercuric ion,[6] and nitric oxide.[7]

Because the BODIPY moiety is hydrophobic, BODIPY conjugates of lipids are taken up efficiently into cell membranes and phospholipid vesicles.[1a,8] Many membrane components have been coupled covalently to BODIPY, including fatty acids,[9] triglycerides,[10] phospholipids,[8,11] and glycolipids.[12] It is surprising that the BODIPY fluorophore has not yet been coupled to free cholesterol, a major constituent of eukaryotic membranes with a critical role in membrane structure and function.[13] The only report of a BODIPY moiety coupled with a sterol molecule concerns cholesteryl esters, in which a BODIPY-containing fatty acid was esterified to the C3-hydroxy group of the sterol.

We report here the synthesis of three new BODIPY−sterol conjugates, in which different linkers were used to couple a BODIPY fluorophore to the sterol's aliphatic side chain. The linker used to couple the BODIPY moiety to the sterol in compound **1** (see Chart 1) contains an ester functionality. We also prepared compounds **2** and **3** (Chart 1), in which a short linker devoid of oxygen atoms was used. Compound **3** is an analogue of coprostanol (5β-cholestan-3β-ol); the cis A/B ring juncture gives rise to a nonplanar steroid nucleus. Therefore, the molecular area of coprostanol is larger than that of cholesterol,[14] resulting in reduced interactions of coprostanol with phospholipids compared with those of cholesterol.[15] Thus, compound **2** resembles the structure of cholesterol, which also has no polar atoms in its aliphatic side chain, to a greater extent than do compounds **1** and **3**. Our studies with monolayers and multibilayer vesicles (MLVs) reported here indicate that fluorescent analogue **2** is a faithful mimetic of cholesterol under the conditions we employed.

Synthetic Plan. Our strategy to prepare analogue **1**, in which an ester moiety is used to link the fluorophore with the sterol, involves the synthesis of the BODIPY derivative **6** bearing a short-chain carboxylic acid at the methene bridge (Scheme 1). To prepare analogues **2** and **3**, which lack a functional group in the linker, we constructed the BODIPY moiety via a condensation reaction with an excess of 2,4-dimethylpyrrole and a sterol bearing an acyl chloride in its side chain, followed by addition of $BF_3{\cdot}OEt_2$.

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(15) Compound **3** was prepared to serve as a negative control. It does not mimic cholesterol with respect to lipid−lipid interactions; therefore, it is not expected to be imbedded in the bilayer interior or enter into liquid-ordered domains.

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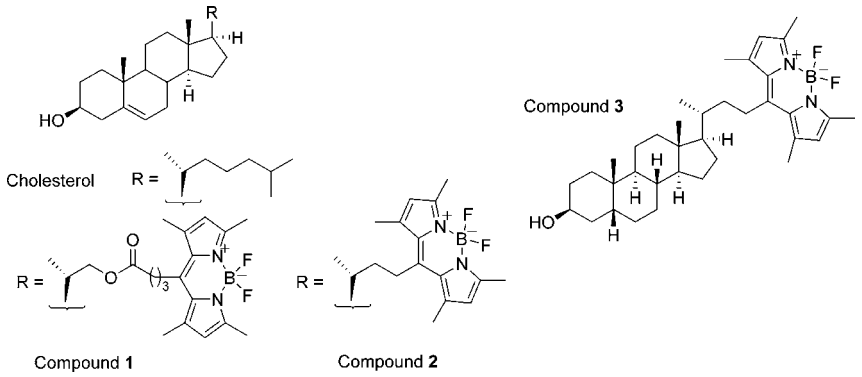
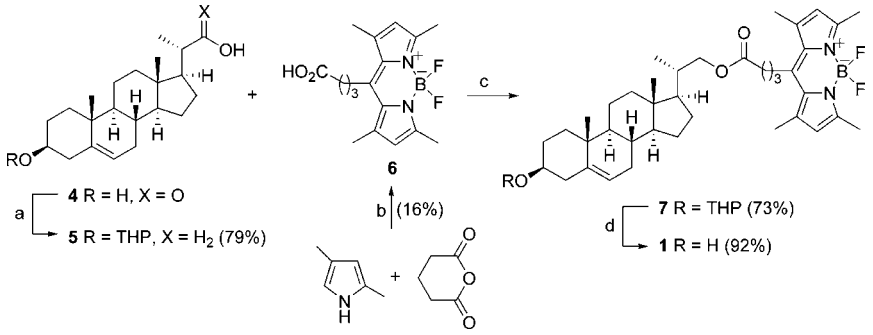
CHART 1. Structures of Cholesterol and Three BODIPY-Based Sterol Probes: BODIPY−Cholesterol Analogues 1 and 2 and BODIPY−Coprostanol Analogue 3

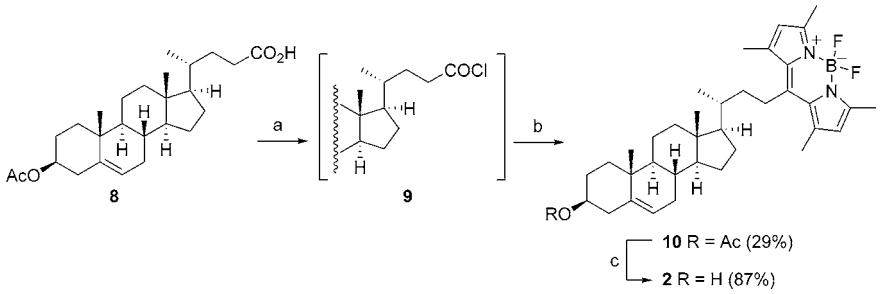


SCHEME 1. Synthesis of 1[a]



[a] Reagents and conditions: (a) (i) DHP, *p*-TsOH, THF, rt, 1 day, (ii) $LiAlH_4$, THF, rt, overnight; (b) (i) $BF_3{\cdot}OEt_2$, reflux, 5 h, (ii) $BF_3{\cdot}OEt_2$, Et_3N, rt, overnight; (c) DCC, DMAP, CH_2Cl_2, rt, 1 day; (d) PPTS, MeOH, 55 °C, 3 h.

SCHEME 2. Synthesis of 2[a]



[a] Reagents and conditions: (a) $(COCl)_2$, CH_2Cl_2, 0 °C to room temperature, overnight; (b) (i) 2,4-dimethylpyrrole, CH_2Cl_2, reflux, 4 h, (ii) $BF_3{\cdot}OEt_2$, Et_3N, rt, overnight; (c) K_2CO_3, MeOH, rt, 2 days.

Synthesis of Analogue 1−3. The synthesis of BODIPY−cholesterol ester analogue **1** is outlined in Scheme 1. The hydroxy group of bisnorcholenic acid (**4**) was protected as a THP ether, and the carboxylic acid was reduced with $LiAlH_4$ to afford alcohol **5** in good yield. A BF_3-mediated reaction of glutaric anhydride with 2,4-dimethylpyrrole afforded **6** in one pot, and a DCC-mediated condensation of **5** with **6** provided ester **7**. Deprotection of the THP ether (PPTS, MeOH, reflux) furnished BODIPY−cholesterol ester analogue **1** in 92% overall yield.

BODIPY−cholesterol analogue **2**, which has a short linker devoid of oxygen atoms, was synthesized in two steps from cholenic acid 3β-acetate (**8**; Scheme 2). Compound **10** was obtained in a 29% overall yield by the condensation of acyl chloride **9**, which was prepared in situ by treating **8** with oxalyl chloride, with 2,4-dimethylpyrrole, followed by a reaction with $BF_3{\cdot}OEt_2$ in the presence of Et_3N.[16] The hydrolysis of acetate **10** was accomplished by using potassium carbonate in methanol, providing BODIPY−cholesterol analogue **2** in 87% yield.

Scheme 3 depicts the synthesis of BODIPY−coprostanol analogue **3**. 3α-Mesylate **12**, which was prepared from lithocholic acid (**11**) in quantitative yield, was converted to 3β-acetate **13** in 74% yield by the reaction with cesium acetate in toluene in the presence of a phase-transfer catalyst, 18-crown-6.[17] The BODIPY fluorophore was then constructed in a 28% overall yield from acid **13** via the reaction of acyl chloride **14** with 2,4-dimethylpyrrole and $BF_3{\cdot}OEt_2$. BODIPY−coprostanol analogue **3** was obtained in 72% yield on the hydrolysis of acetate **15**.[18]

Biophysical Evaluation of Compounds 1−3. Mixing Behavior in Two-Component Monomolecular Films. The molecular areas of mixed monolayers comprised of varying mole fractions of sterol (cholesterol and analogues **1** and **2**) and

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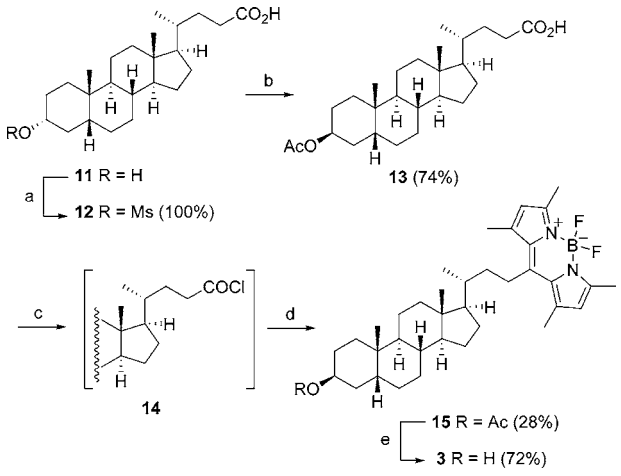
SCHEME 3. Synthesis of 3[a]



[a] Reagents and conditions: (a) MsCl, Et_3N, CH_2Cl_2, 0 °C, 1 h, then rt, 4 h; (b) CsOAc (10 equiv), 18-crown-6, toluene, reflux, 28 h; (c) $(COCl)_2$, CH_2Cl_2, rt, overnight; (d) (i) 2,4-dimethylpyrrole, CH_2Cl_2, reflux, 4 h, (ii) $BF_3{\cdot}OEt_2$, Et_3N, rt, overnight; (e) K_2CO_3, MeOH, rt, 2 days.

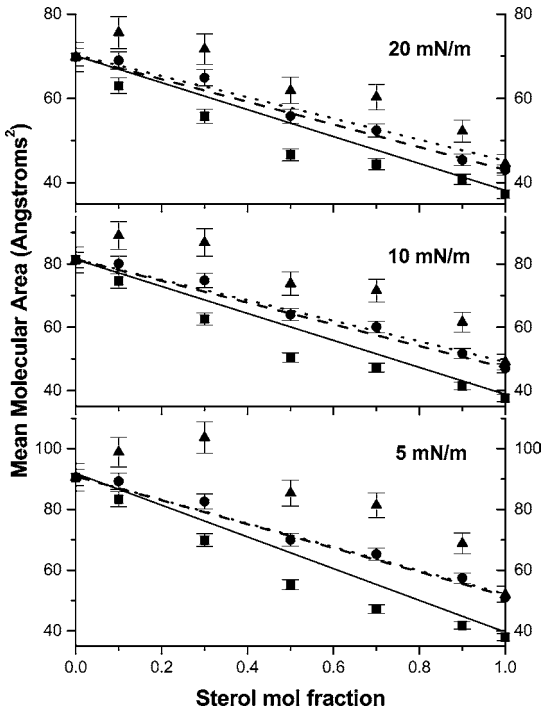


FIGURE 1. Mean-molecular-area composition plots at three surface pressures, as labeled in the panels. Surface areas are averages of at least four separate experiments at each composition. The straight lines represent surface areas at ideal additivity of the mean molecular areas of the components of the binary films. Cholesterol, squares, solid lines; analogue **1**, triangles, dotted lines; analogue **2**, circles, dashed lines.

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) at three surface pressures are shown in Figure 1. The dashed lines represent the areas expected from the ideal mixing of the two components. The smaller area of cholesterol/POPC films (squares) illustrates the "condensation effect" of cholesterol on the surface area of phospholipids. This is a result of the reduced number of gauche conformers in the fatty acyl chains of phospholipids in the presence of cholesterol; thus, the molecules pack tightly and occupy a smaller area versus the ideal isotherms.[19] The deviation from the ideal mixing observed in monomolecular films of **1** with POPC (triangles) is indicative of demixing. The oxygen atoms in the ester linkage used to conjugate the sterol with the BODIPY moiety apparently interfere with the ideal mixing of this analogue with POPC because we observed ideal mixing of **2** with POPC at the surface pressures and mole fractions we examined (circles).

Partitioning into Liquid-Ordered Domains. Lipid−lipid immiscibility in membranes results in lateral heterogeneity in the plane of the bilayer, forming microdomains.[20] Cholesterol is generally presumed to partition preferentially into ordered raft domains.[21,22] The conjugation of a bulky fluorophore with a lipid may sterically hinder the interaction of the lipid with neighboring phospho- and sphingolipid molecules in raft domains, and polar groups in the fluorophore may also interfere with the ability of the lipid to partition into liquid-ordered domains. Indeed, there is evidence from monolayer fluorescence microscopy that many fluorescent sterol probes tend to partition spontaneously into the more loosely packed, liquid-expanded domains, resulting in bright areas of fluorescence; the condensed regions of the monolayer that exclude the probe appear as dark domains and represent liquid-ordered domains.[23]

MLVs prepared from mixtures of sterols with saturated glycerophospholipids or sphingomyelin form detergent-resistant domains ("lipid rafts").[24] We compared the ability of cholesterol and the ability of analogue **2** to support detergent-resistant domains in MLVs, as this property is sensitive to sterol structure.[24] Cholesterol and analogue **2** exhibited similar capacities to support lipid raft formation in MLVs with sphingomyelin at the molar ratios examined (see Supporting Information, Figure S1). These results suggest that the BODIPY moiety in analogue **2** does not interfere with the tight lipid packing required for formation of lipid raft domains in MLVs. On the other hand, by the use of an assay in which the ratio of the fluorescence intensity of the pellet is compared to that of the supernatant after centrifugation (data not shown),[25] we found that analogues **1** and **3** did not partition as well as cholesterol into the insoluble fraction of detergent-treated MLVs.

(18) Compounds **1**−**3** exhibit a strong green fluorescence as dilute solutions in $CHCl_3$ and MeOH. In $CHCl_3$, the λ_{ex} and λ_{em} of compounds **1**−**3** are 474 and 526 nm, respectively. In MeOH, the λ_{ex} and λ_{em} of compound **1** are 492 and 512 nm, respectively.

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Cyclodextrin-Mediated Adsorption from Monolayers. We measured the rates of desorption of cholesterol and compound **2** from mixed monolayers composed of POPC and sterol at a constant surface area on injection of 2-hydroxypropyl-β-cyclodextrin (HPCD), a water-soluble cyclodextrin derivative, into the subphase. This method has been used to estimate the extent of interaction of various sterols with phospholipids in monolayers.[26] The first-order rate constants of the HPCD-induced desorption of cholesterol and **2** from the monolayers are similar (see Supporting Information, Figure S2). These results suggest similar affinities of the two sterols for POPC under the conditions employed.[27]

Other Fluorescent Probes of Cholesterol. The site of attachment of a fluorophore to the sterol molecule and the size and physical properties of the fluorophore affect how the probe molecule is partitioned in membrane domains. A nitrobenzoxadiazole (NBD) moiety has been conjugated to cholesterol at C22 and C25.[28] However, unlike cholesterol whose side chain is deeply imbedded among the fatty acyl groups of phospholipids in the nonpolar interior of the membrane,[13] these NBD-containing probes tend to be oriented (at least transiently) with their side chain near the polar milieu.[29] The probe with the NBD group at C22 distributes preferentially into the liquid-disordered phase, unlike cholesterol under similar conditions.[30] An analogue in which a dansyl group was coupled to cholestanol at C6 was reported to mimic cholesterol;[31] however, this fluorophore is more hydrophilic than BODIPY,[32] which may alter its membrane orientation compared with that of cholesterol. These findings indicate that NBD− and dansyl−cholesterol may not be suitable probes for cholesterol.[33]

In conclusion, the first syntheses of three cholesterol−BODIPY analogues were achieved. Compound **2** is fully miscible with POPC over the entire range of the sterol/phospholipid molar ratio investigated, indicating the BODIPY moiety is sufficiently hydrophobic to mix with the fatty acyl chains of phospholipids. When a biochemical method based on the resistance of lipid rafts to be extracted by a detergent was used,[24] we found that compound **2** supported the formation of liquid-ordered domains in model membranes. The rate of desorption of **2** is similar to that of cholesterol on the addition of a water-soluble cyclodextrin to mixed monolayers with sphingomyelin. Compounds **1** and **3** exhibited markedly lower affinities than cholesterol and compound **2** for liquid-ordered domains. These results indicate that the structure of the linker used to ligate BODIPY to the aliphatic side chain of the sterol, as well as the structure of the steroid ring system, are important determinants of the ability of the probe to partition into liquid-ordered versus liquid-disordered membrane domains.

Experimental Section

General Information. See Supporting Information for general experimental details. NMR spectra (400 MHz for ^{1}H, 100 MHz for ^{13}C, and 376 MHz for ^{19}F) were recorded in $CDCl_3$, unless otherwise noted.

22-[4-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)butyroxy]-23,24-bisnorchol-5-en-3β-ol (1). A solution of ester **7** (13.1 mg, 17.8 μmol) and PPTS (5 mg, 19.9 μmol) in MeOH (5 mL) was heated at 55 °C until the reaction was complete (~3 h, monitored by TLC using hexane/EtOAc, 4:1). After the solvent was removed under vacuum, 11 mg (92%) of compound **1** was obtained by chromatography (hexane/EtOAc, 4:1). HPLC: C18 column, 2.1 × 150 mm, t_R 11.9 min (50−100% MeOH gradient, 0.4 mL/min, 465 nm). ^{1}H NMR δ 6.06 (s, 2H), 5.37−5.33 (m, 1H), 4.15−4.08 (dd, 1H, J = 10.6 Hz, J = 3.3 Hz), 3.83−3.76 (dd, 1H, J = 10.6 Hz, J = 7.6 Hz), 3.57−3.47 (m, 1H), 3.03−2.98 (m, 2H), 2.51 (s, 6H), 2.49 (t, 2H, J = 7.1 Hz), 2.43 (s, 6H), 2.32−2.19 (m, 2H), 2.04−0.82 (m, 28H), 0.71 (s, 3H); ^{13}C NMR δ 172.8, 154.2, 145.0, 140.8, 140.4, 131.5, 121.8, 121.6, 71.8, 69.8, 56.5, 52.8, 50.1, 42.6, 42.3, 39.6, 37.3, 36.5, 35.9, 34.4, 34.0, 32.0, 31.7, 27.7, 26.9, 25.6, 25.0, 24.4, 21.1, 19.4, 17.2, 16.4, 14.4, 14.1, 11.9; ^{19}F NMR δ −146.4−146.9 (m). HRMS *m*/*z*: calcd for $C_{39}H_{55}BF_2N_2O_3Na$ (MNa^+), 671.4166; found, 671.4170.

23-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)-24-norchol-5-en-3β-ol (2). A mixture of acetate **10** (24 mg, 38.7 μmol) and anhydrous K_2CO_3 (10.7 mg, 77.5 μmol) in MeOH (5 mL) was stirred vigorously at room temperature until the disappearance of **10**. The solvent was removed under vacuum, and the residue was partitioned between water (10 mL) and CH_2Cl_2 (10 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic phase was dried over Na_2SO_4 and evaporated to dryness. After purification by chromatography (hexane/EtOAc, 3:1), compound **2** (19 mg, 87%) was obtained. HPLC: C18 column, 2.1 × 150 mm, t_R 12.3 min (50−100% MeOH gradient, 0.4 mL/min, 465 nm). ^{1}H NMR δ 6.04 (s, 2H), 5.37−5.32 (m, 1H), 3.57−3.47 (m, 1H), 3.19−3.10 (m, 1H), 2.76−2.66 (m, 1H), 2.51 (s, 6H), 2.42 (s, 6H), 2.32−2.17 (m, 2H), 2.08−0.86 (m, 28H), 0.72 (s, 3H); ^{13}C NMR δ 147.3, 140.7, 140.1, 131.4, 121.64, 121.56, 100.0, 71.8, 57.0, 56.6, 50.0, 42.6, 42.3, 39.8, 38.1, 38.0, 37.3, 36.5, 31.9, 31.8, 31.6, 28.6, 26.8, 24.2, 21.1, 19.4, 18.1, 16.6, 14.4, 14.2, 11.9; ^{19}F NMR δ −146.2−146.9 (m). HRMS *m*/*z*: calcd for $C_{36}H_{51}BF_2N_2ONa$ (MNa^+), 599.3955; found, 599.3954.

23-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)-24-nor-5β-cholestan-3β-ol (3). The same method used to prepare compound **2** was used to prepare compound **3** from compound **15**, with a yield of 72%. ^{1}H NMR δ 6.04 (s, 2H), 4.16−4.08 (m, 1H), 3.20−3.11 (m, 1H), 2.76−2.67 (m, 1H), 2.52 (s, 6H), 2.43 (s, 6H), 2.02−0.80 (m, 30H), 0.69 (s, 3H); ^{13}C NMR δ 147.4, 139.7, 121.5, 67.4, 57.3, 56.4, 43.1, 39.9, 38.2, 36.6, 35.6, 35.3, 33.8, 32.2, 31.8, 30.1, 29.9, 28.9, 26.4, 24.4, 24.3, 22.8, 21.2, 18.2, 16.7, 14.6, 14.4, 12.2; ^{19}F NMR δ −146.3−146.8 (m). HRMS *m*/*z*: calcd for $C_{36}H_{53}BF_2N_2ONa$ (MNa^+), 601.4111; found, 601.4092.

Acknowledgment. We thank Prof. Erwin London for the fluorescence assay of partitioning of the analogues into liquid-ordered and liquid-disordered domains.

Supporting Information Available: Experimental protocols for compounds **5**−**7**, **10**, **13**, and **15**; ^{1}H, ^{13}C, and ^{19}F NMR spectra for all new compounds; experimental methods for the biophysical studies used; and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(33) Fluorescent analogues of cholesterol without bulky groups are available (e.g., cholestatrienol and dehydroergosterol), but they possess some undesirable spectroscopic properties (low quantum yield and excitation wavelengths in the UV). Therefore, they are not appropriate for live-cell imaging studies.