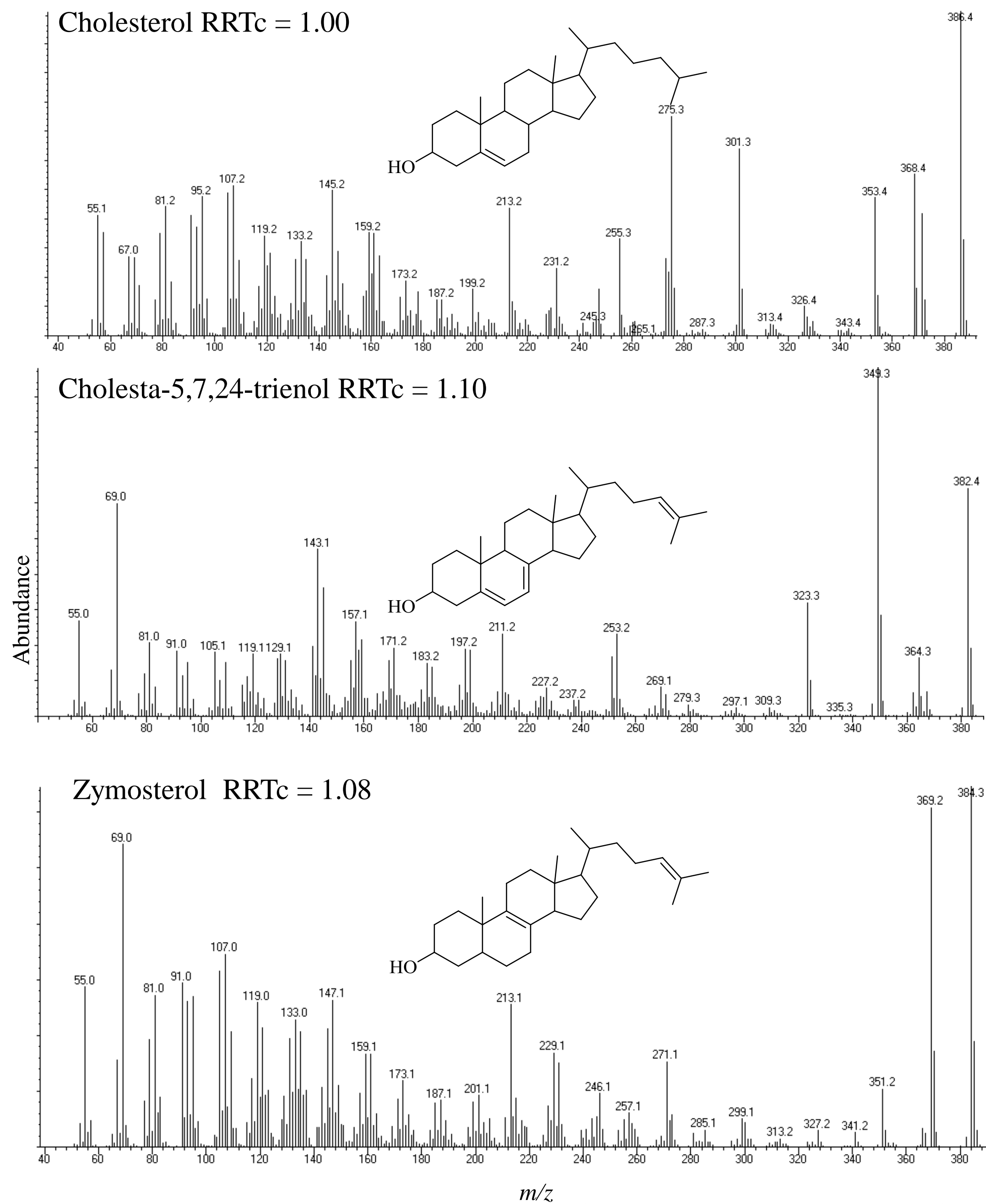
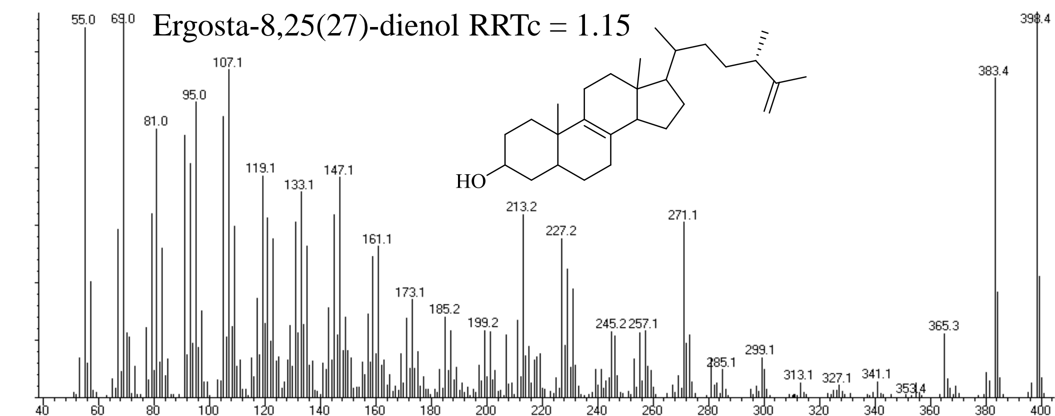
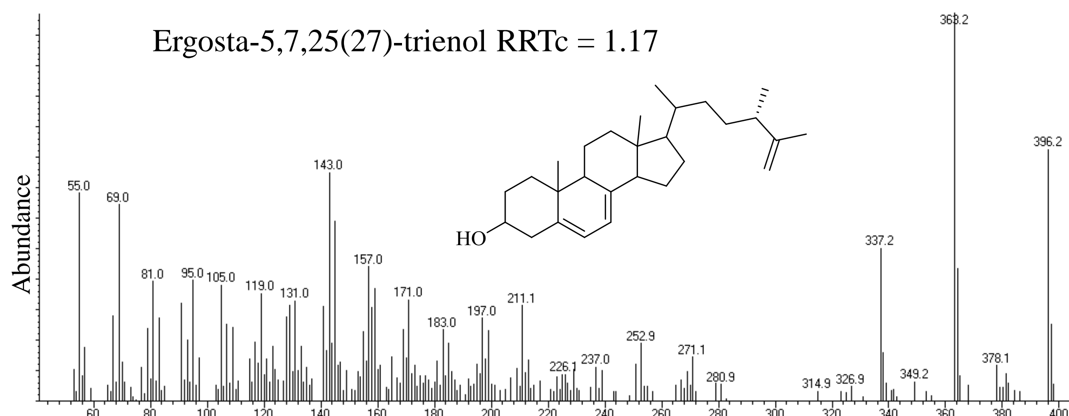


Figure S1A, related to Figure 3: Mass spectra of sterol standards isolated from *Tb* PCF.





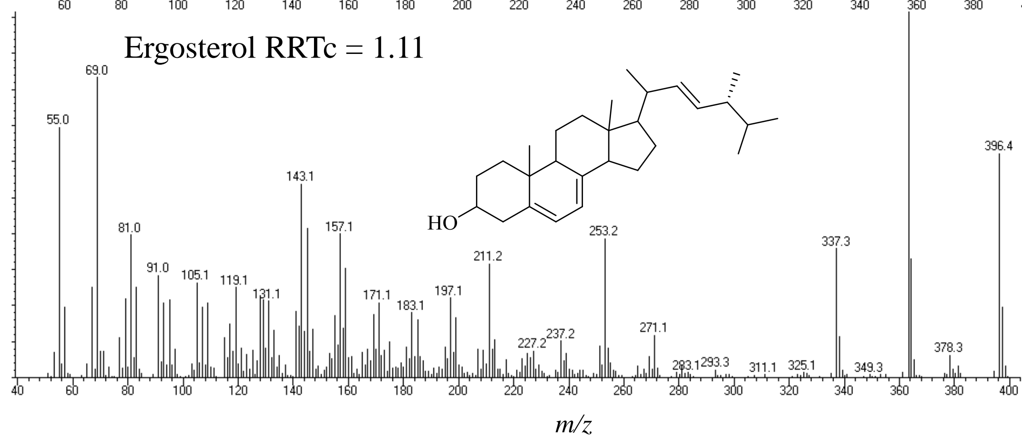
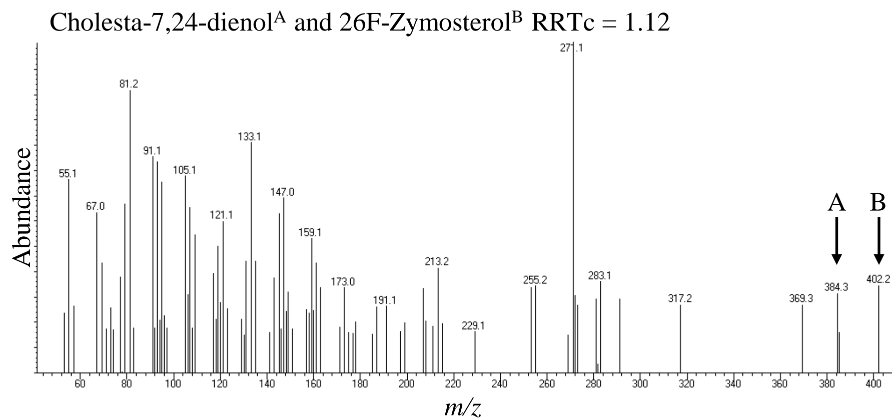
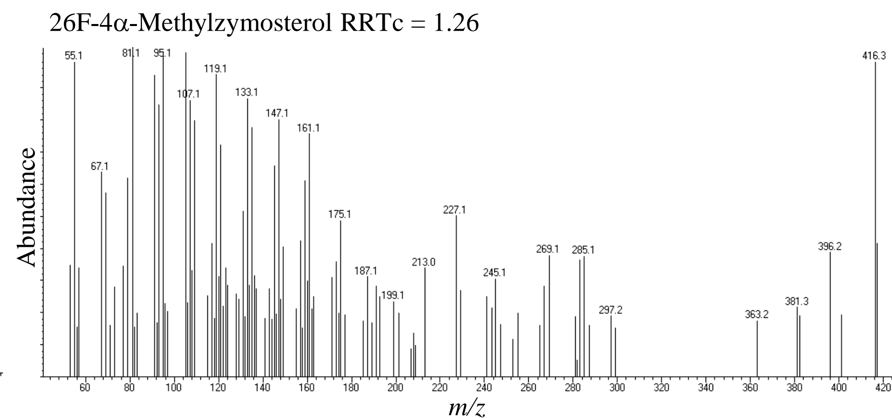
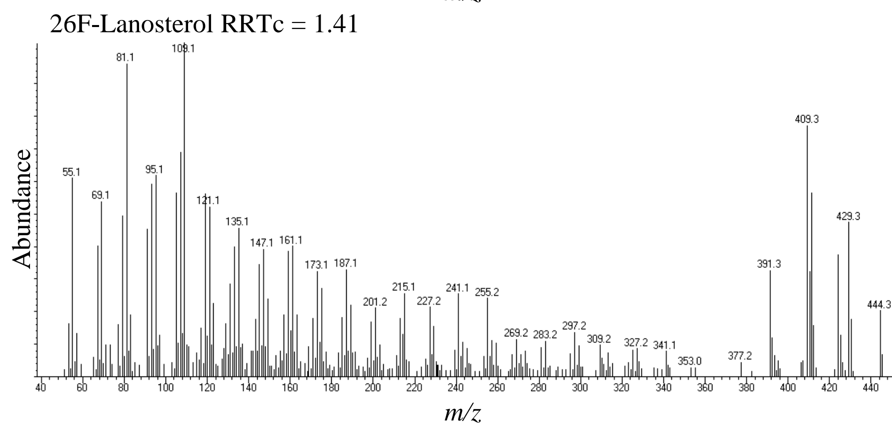


Figure S1B, related to Figure 3: Mass spectra of sterol standards isolated from *Tb* PCF.







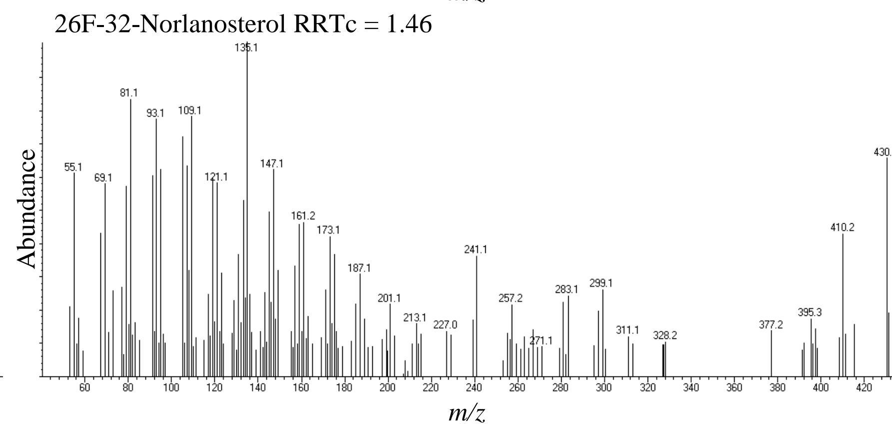
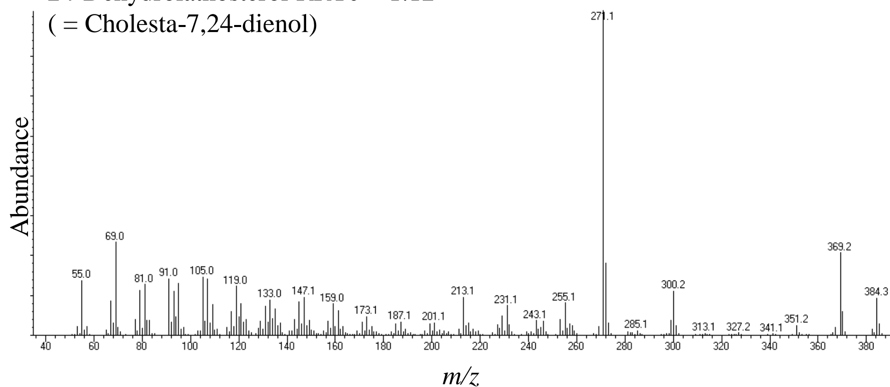
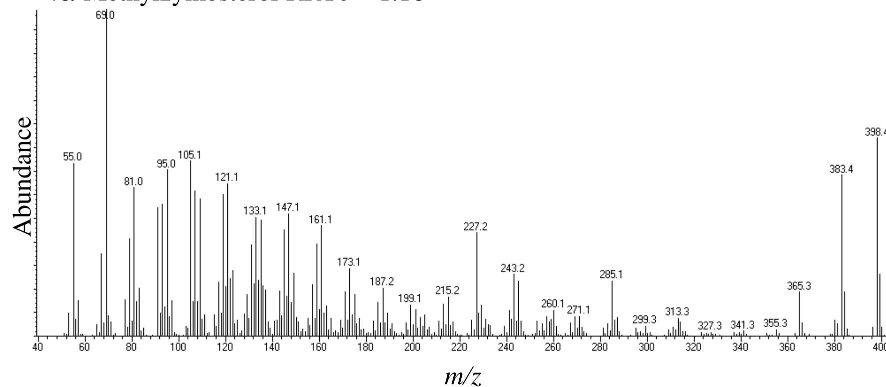


Figure S2A, related to Figure 4: Mass spectra of fluorinated sterols isolated from 26-FL treated HEK cells.

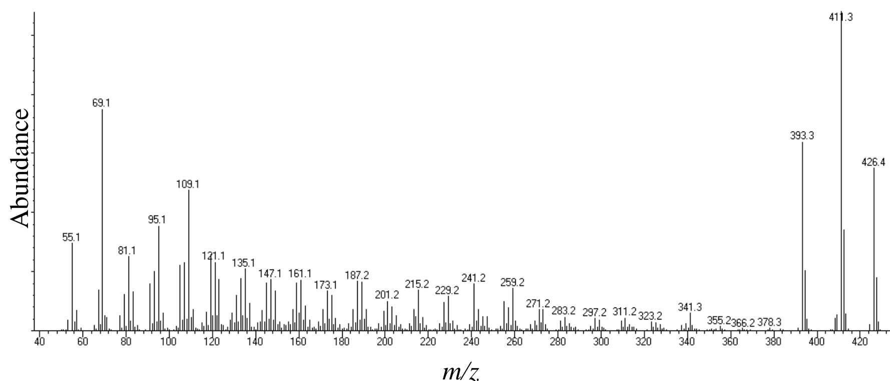
24-Dehydrolathosterol RRTc = 1.12
(= Cholesta-7,24-dienol)



4α-Methylzymosterol RRTc = 1.16



Lanosterol RRTc = 1.31



32-Norlanosterol RRTc = 1.34

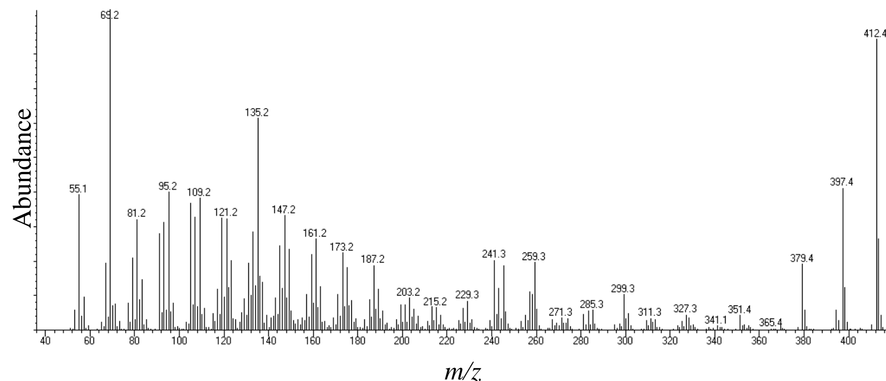


Figure S2B, related to Figure 4: Mass spectra of sterols typically synthesized in animal cell cultures.

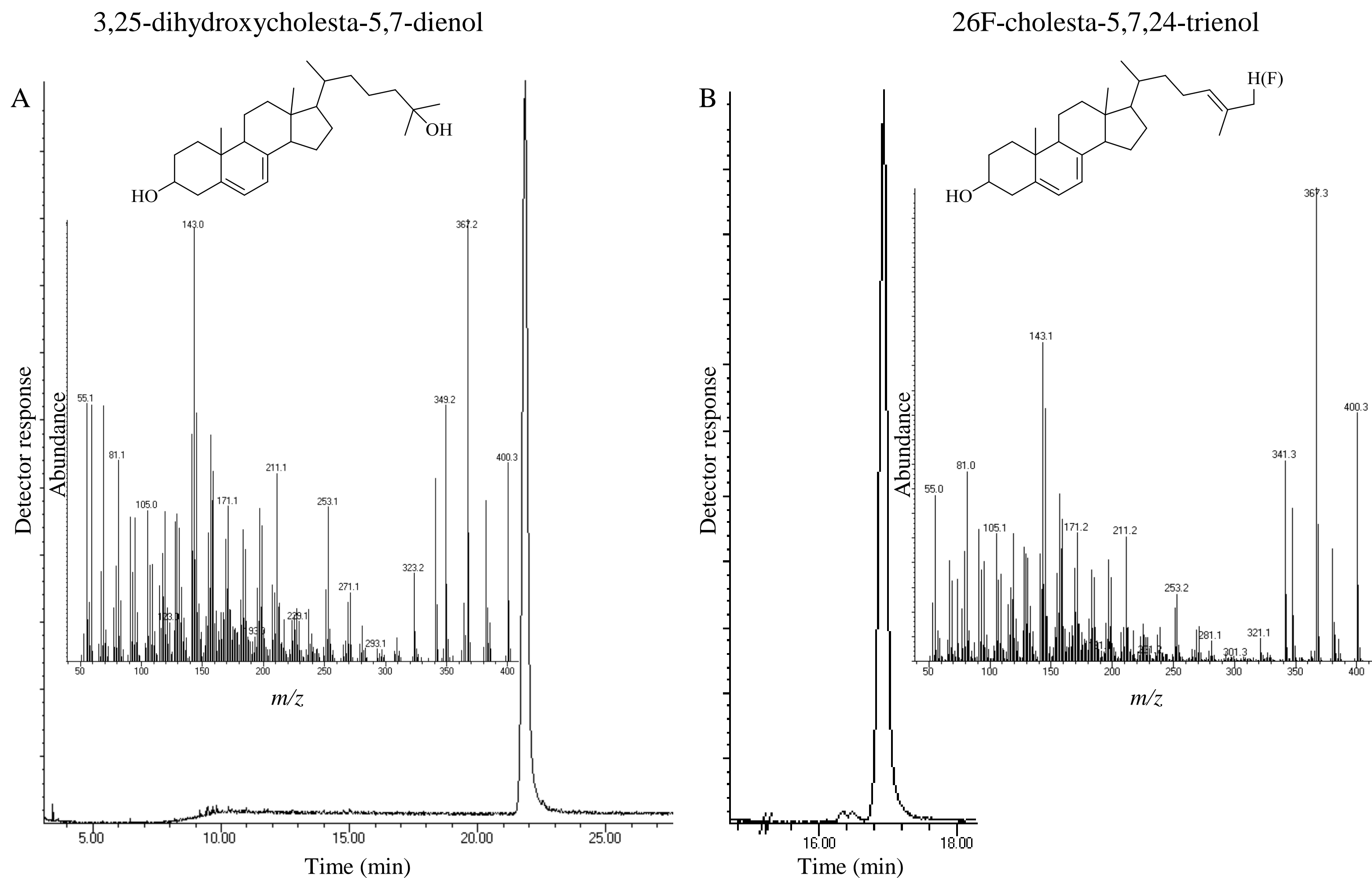


Figure S3, related to Figure 4: GC and mass spectrum of relevant intermediate in the synthesis of 26-fluoroholesta-5,7,24-trienol (26FCT) (left panel) and GC and mass spectrum of synthetically produced 26FCT (right panel).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials. Chemicals and reagents were purchased from Sigma and used without further purification or treatment unless otherwise noted. SAM was the iodide salt, [*methyl*-$^3H_3$]SAM was purchased from PerkinElmer and diluted with non-radioactive SAM to specific activity of 10 µCi/µmol. DAST (diethylammoniumsulfur trifluoride) was purchased from Alfa Aesar. Zymosterol was isolated from yeast and lanosterol was purified from commercial "lanosterol" (Sigma) derived from lanolin-sheep's wool by a combination of flash chromatography and argentation chromatography (Xu et al., 1988). Ergosterol was purchased from Sigma. Sterol standards, including cholesta-5,7,24-trienol, isolated from the *ERG*6 mutant yeast (Xu and Nes, 1988), were from our sterol collection. All substrates were of 95% or greater purity by capillary GC analysis.

Proton and carbon NMR spectra were recorded at room temperature using a Varian Unity Inova 500 MHz NMR spectrometer (^1H at 499.71 and ^{13}C at 125.66 MHz, respectively) or JEOL 400 MHz NMR spectrometer (^1H at 399.78 and ^{13}C at 100.53 MHZ, respectively). Chemical shifts (δ, ppm) are referenced to $CDCl_3$ (δ, 7.260) for ^1H and $CDCl_3$ (δ, 77.00) for ^{13}C. GC-MS was obtained on a Hewlett-Packard 6890 GC-interfaced to a Hewlett-Packard 5973 MSD [70 eVEI (electron impact), scan range 50-550 atomic mass units] equipped with 30 m length x 0.25 mm inner diameter fused silica column coated with Zebron ZB-5, film thickness 0.25 µm (Phenomenex) with He as carrier at a flow rate of 1.2 ml/min. The oven was programed to be isothermal at 170 °C for the first minute, and then ramped to the maximum temperature of 280 °C at 20 °C per minute. The source temperature was 230 °C. Cholesterol used as reference standard for calculating retention factors ($RRT_{cholesterol}$) under these conditions elutes at 13.8 min (old column) or 14.6 min (new column).

Flash chromatography was performed on silica gel 60 using eluents of diethyl ether graded into hexanes or dichloromethane graded into methanol. Analytical TLC was carried out on commercially prepared aluminum backed plates (silica gel F-254, Sigma) with visualization by fluorescence quenching (254 nM UV light) or by slow heating on a hot plate following light spray of 2 N sulfuric acid solutions. High performance liquid chromatography (HPLC) was carried out using TSK or C18-Luna columns at room temperature or 35 °C with acetonitrile/isopropanol (6/4, v/v) 100 % methanol as solvent. Diode array multiple wavelength detector system (Agilent 1100 HPLC system) monitored over the range 200 to 340 nm or individually at 210, 240 or 282 nm was used to determine the purity and UV maximum absorbance (λmax) of sterol.

Synthesis of 26-fluorolanosterol (3). Reagents and solvents were purified and dried by standard protocols (Lepesheva and Waterman, 2011) and non-aqueous reactions were performed under an atmosphere of nitrogen. 3-Acetoxylanosta-8,24-dienol-26-al (2, Figure S4), generated from lanosterol (1, Figure S4) by C3-acetylation followed by selective C26-side chain oxidation using selenium dioxide (Patkar et al., 2013), served as the starting material for this synthesis. In the general procedure for the fluorination of sterols at C26 using DAST, the C3-protected C26-aldehyde of lanosterol was prepared first as shown in Figure S4. The C26-aldehyde was reacted with sodium borohydride in methanol at room temperature to yield the corresponding C26-alcohol followed by fluorination at C26 under nitrogen using DAST in dichloromethane and the reaction quenched with deionized water.

The C3-acetate group was removed by saponification in methanolic KOH to afford 26-fluorolanosterol in 70% yield; after work-up and HPLC to remove the C25(27)-isomer side product afforded overall yield of approximately 50%. ^{1}HNMR: ($CDCL_3$, 400 MHz) δ 4.96 (H_1- 26, m), 4.91 (H_1- 26, m), 4.75 (H_1 - 24, dq, J = 48.6, 6.4 Hz) 3.23 (H_1 -3, dd, J = 11.9), 1.72 (H_3 -27, s), 1.00 (H_3- 19, s), 0.98 (H_3- 30, s), 0.93 (H_3 -21, d = 6.5), 0.88 (H_3- 32, d = 6.5), 0.81 (H_3, - 31, s), 0.69 (H_3- 18, s); EI-MS: 444 (M^+), 429 (M^+- CH_3), 424 (M^+-HF), 411 (M^+-CH_3-$H_2$0), 409 (M^+-HF-CH_3), 391 (M^+- CH_3-HF-$H_2$0), 377, 341, 327, 299, 283, 269, 255, 241, 227, 215 (see Figure S3 for mass spectrum); λmax, end absorption. RRT_c, 1.43 (compared to lanosterol = RRT_c of 1.32); and in HPLC (Luna, analytical, methanol) elutes with a retention factor relative to cholesterol retention time of 0.66.

Synthesis of 26-fluorocholesta-5,7,24-trienol (8). 3β,25-Hydroxycholest-5,7-dienol (25-hydroxyprovitamin D, 1, M^+ 402. Figure S3), prepared from commercial ergosterol as described (Fuse et al., 2012), served as starting material for these syntheses. The convergent synthesis of 26-fluorocholesta-5,7,24-trienol (8) involved the selective C3-acetylation of diol 4 (Figure S4) in acetic anhydride and pyridine followed by protection of the $\Delta^{5,7}$-group through formation of the 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) yielding adduct 5 which was then dehydrated in mesyl chloride and N-triethylamine at 0 ^{o}C to afford intermediate 6 in 90% yield. Compound 6 was oxidized by the known method using selenium dioxide in conjunction with t-butylhydroperoxide in ethanol (Patkar et al., 2013) and then reacted with sodium borohydride in ethanol at 0 ^{o}C to room temperature to give the C26-alcohol (7). To complete the preparation of the fluoroolefin, the PTAD adduct 7 was treated with DAST (1 ml, 75 ^{o}C for 2 hr) and the product reduced with lithium aluminium hydride in THF from 0 to 50 ^{o}C to give fluoroalcohol 8 after quenching with water, extractive work-up and column chromatography. ^{1}HNMR: ($CDCL_3$, 500 MHz) δ 5.55 (H_1- 6, dd, J = 5.6, 2.3), 5.37 (H_1- 6, t, J = 2.69), 4.96 (H_1 - 26, m), 4.90 (H_1 -26, m), 4.77 (H_1 -24, dq, J = 48, 6.7 Hz), 3.61 (H_1- 3, m), 1.04 (H_3- 21, d, J = 6.6), 0.95 (H_3-19, s), 0.69 (H_3- 18, s); ^{13}C NMR ($CDCl_3$, 125 MHz) of relevant side chain chemical shifts at C24 (112.68), C25 (112.79), C26 (30.93) and C27 (14.12) [for reference note compilation of ^{13}C NMR chemical shifts of sterols in reference 10]; EI-MS, 400 (M^+), 385 (M^+ - 15), 380 (M^+ - HF), 367 (M^+ - CH_3- $H_2$0), 347 (M^+- CH_3- $H_2$0-HF), 341, 321, 271, 253, 211 and relevant ion cluster at 143, 157, 158 and 159 diagnostic of the $\Delta^{5,7}$-system (Goad and Akihisa, 1997) [see Figure S3 for mass spectrum], λ_{max} = 282 nm, RRT_c of 1.16 compared to a RRTc of 1.10 for cholesta-5,7,24-trienol. In HPLC, 8 eluted at α_c 0.39 to the retention time of cholesterol (TSK C_{18}-column eluted with ACN/i-PrOH at 1 ml/min, at 40 ^{0}C).

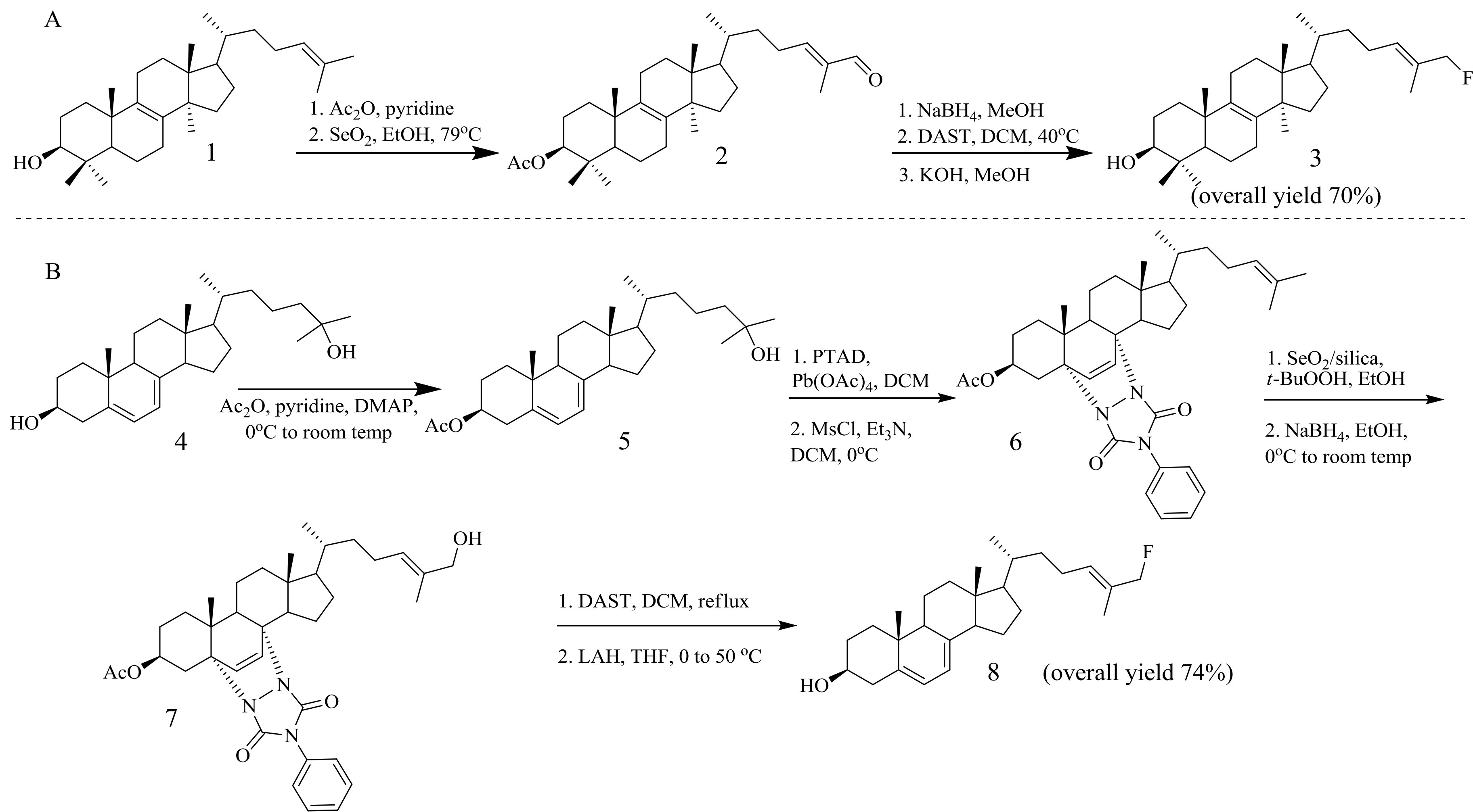


Figure S4, related to Figure 4: Scheme for the synthesis of 26-fluorolansoterol (Panel A) and the synthesis of 26-fluorocholesta-5,7,24-trienol (Panel B).

Kinetic and chemical evaluation of TbSMT

Cloned *Tb*SMT activity was monitored as described previously by incubating sterol substrate or analog in 8 ml test tubes containing 600 µl of 20 mM phosphate buffer (pH, 75), 5% glycerol, 2- 3 mg lysate protein (75 µg *Tb*SMT) and zymosterol or fluorinated compound emulsified in Tween 80 (final concentration of 1.2 g/l). The reaction was initiated by addition of the radiolabeled co-substrate [$^3H_3$-*methyl*]SAM, 0.6 µCi per reaction tube, followed by gentle swirling at 35 ^{0}C for 45 min and then terminated by the addition of a solution (600 µl) of 10% methanolic KOH (Zhou et al., 2006). The enzyme-generated products were extracted in hexanes, dried and the neutral lipid analyzed by aliquot counting with ^{3}H = 30%; all samples were quench-corrected by internal standardization and were counted to a standard error of <1%. Control experiments of [*methyl*-$^3H_3$]SAM without sterol were conducted with each enzyme preparation to determine the radioactive background, and in all cases non-enzymatic product formation was negligible (less than 500 dpm in the hexane-extractable lipids). Steady-state kinetic parameters, in the presence and absence of inhibitor, were determined by computer-assisted least squares-linear regression analysis in conjunction with the graphical procedures of Lineweaver-Burk using "Enzyme Kinetics" or SigmaPlot 2001 plus the enzyme kinetics module software. K_m and K_i values were initially determined by varying the substrate concentration in the presence or absence of inhibitor at fixed concentrations, followed by another series of assay in which the concentration of inhibitor varied while substrate concentrations were held constant. Data for K_m and V_{max} values typically had r-values > 0.98 and the curve fit data had coefficient of determination values (r2) <-.90. Protein was determined by the Bio-Rad dye binding assay according the manufacturer's direction employing bovine serum albumin as a standard. *Tb*SMT concentration was estimated in total protein from the SDS-PAGE gels and related to the previously determined amount of recombinant protein in the soluble 100,000 *g* fraction of 15 µg *Tb*SMT in 2 mg total soluble protein; for zymosterol incubation with soluble protein the specific activity (V_{max}) is 901 pm/min/mg and following purification to homogeneity the k_{cat} is 0.06 min^{-1} (Zhou et al., 2006). Total sterol was determined by GC analysis against a curve of increasing concentrations (0.3 mg to 3.0 mg) of cholesterol (prepared from the dibromide to remove oxidation contaminants). For preparative-scale incubations, lysate preparations of 3 mg total protein were incubated with saturating amounts of sterol (100 µM) and SAM (100 µM) for 12-16 hr. The enzyme-generated products in the neutral lipids were analyzed by GC-MS.

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Goad, L. J. and Akihisa, T. (1997). Analysis of Sterols. Blackie, Academic, London.