Benzophenone-containing cholesterol surrogates: synthesis and biological evaluation

Thomas A. Spencer,[1,*] Pingzhen Wang,[*] Dansu Li,[*] Jonathon S. Russel,[*] David H. Blank,[*] Jarkko Huuskonen,[§] Phoebe E. Fielding,[†] and Christopher J. Fielding[§]

Department of Chemistry,[*] Dartmouth College, Hanover, NH 03755; Cardiovascular Research Institute and Department of Medicine,[†] University of California, San Francisco, CA 94143; and Cardiovascular Research Institute and Department of Physiology,[§] University of California, San Francisco, CA 94143

Abstract Eight analogs of cholesterol (1) containing a benzophenone group have been synthesized as prospective photoaffinity labels for studies in cellular sterol efflux and HDL formation. Six of these compounds (4–9) have the photophore replacing different portions of the cholesterol alkyl side chain, and two (10 and 11) have it attached via nitrogen at carbon 3. The suitability of these analogs as cholesterol surrogates was determined by examining their ability to replace [^{3}H]1 in fibroblasts preequilibrated with [^{3}H]1. All eight analogs were effective in replacing natural 1 in competition with [^{3}H]1 for apolipoprotein A-I-induced efflux. These are the first compounds shown to replace cholesterol successfully in a complex pathway of multiple intracellular steps. The results suggest an unexpected tolerance of biological membranes regarding the incorporation of sterols of differing chemical structure.—Spencer, T. A., P. Wang, D. Li, J. S. Russel, D. H. Blank, J. Huuskonen, P. E. Fielding, and C. J. Fielding. **Benzophenone-containing cholesterol surrogates: synthesis and biological evaluation. *J. Lipid Res.* 2004.** 45: **1510–1518.**

Supplementary key words cholesterol • cellular sterol efflux • apolipoprotein A-I • photoactivable sterols • photoaffinity labeling

As part of a study of cellular cholesterol efflux and HDL formation, we are investigating how cholesterol [free cholesterol (FC)], **1**, **Fig. 1**] interacts with the proteins caveolin and apolipoprotein A-I (apoA-I) in these processes. Photoactivable analogs of FC could provide information about transient complexes between FC and these proteins by forming permanent covalent links that can serve to identify their binding sites (1–3). Recently, two analogs of FC (**2** and **3**, Fig. 1) containing diazirine groups have been prepared (4, 5) and used in photolabeling experiments (4–8). Analog **2** ("photocholesterol") has been used in a variety of biochemical media to photolabel synaptophysin (4), proteolipid protein (6), and proteins in *Caenorhabditis elegans* (7). Evidence has been presented that **2** can serve as a substitute for FC in model membranes (9). Analog **3** and its linoleate ester were shown in Chinese hamster ovary cells to effect labeling of several proteins, including caveolin (5).

In the present work, the benzophenone group was chosen as photophore because it offers several distinct advantages (1, 10–12). It is readily activated, like the diazirine group, by light at a wavelength (ca. 350 nm) that is relatively nondestructive to proteins. It is hydrophobic and, therefore, well suited for incorporation into most portions of the cholesterol structure. It is very stable and will survive intact and be traceable through complex cellular processes. And, finally, unlike diazirines, benzophenones are efficient and selective for labeling amino acid α carbon atoms.

This paper describes the synthesis of four pairs of analogs of FC containing benzophenone groups (compounds **4–11**, **Fig. 2A**). In three of these pairs (**4–9**) the benzophenone moiety extends, or replaces most of, the sterol alkyl side chain; in the fourth pair (**10**, **11**), the photophore is attached at carbon 3 (C3) via an amide linkage. The schematic of Fig. 2B shows the approximate area that the incorporated photophore occupies in each of the eight analogs, giving an idea of the extension at either end of the sterol tetracycle that exists in the cases of **4–9** or **10** and **11**.

One of these analogs, compound **4**, designated FC benzophenone (FCBP), has already been used to substitute successfully for 47% of cellular FC without perturbing smooth muscle cell function, to photolabel caveolin effectively, and to obtain evidence that FC transferred to apoA-I was mainly derived from caveolin-rich domains

Manuscript received 26 February 2004, in revised form 18 May 2004, and in re-revised form 21 May 2004.

Published, JLR Papers in Press, May 16, 2004.
DOI 10.1194/jlr.M400081-JLR200

Abbreviations: apoA-I, apolipoprotein A-I; C3, carbon 3; DEAD, diethyl azodicarboxylate; DMF, dimethylformamide; EI-HRMS, electron-impact high resolution mass spectrometry; FC, free cholesterol; FCBP, FC benzophenone; THF, Tetrahydrofuran.

[1] To whom correspondence should be addressed.
e-mail: taspen@dartmouth.edu

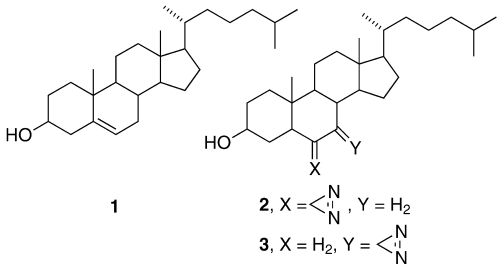

Fig. 1. The structures of cholesterol and two recently reported photoactivable analogs are shown.

(13). In the present work, isotope dilution was used to test the ability of each of the analogs **4–11** to substitute for FC in cells labeled with [^{3}H]FC, which is known to equilibrate completely with cellular FC pools (14). Cellular phospholipids and FC are transferred from [^{3}H]FC-labeled cultured fibroblast monolayers or other peripheral cells to the extracellular medium in a process facilitated by the presence of apoA-I. If an unlabeled analog of FC is introduced into such cells, successful substitution for FC would lead to proportional dilution of the label and reduction of the radioactivity returned to the medium by efflux. Those photoactivable analogs of FC that meet this demanding standard could provide a novel approach to determining the organization of lipids and proteins in the membranes of living cells.

MATERIALS AND METHODS

Chemical synthesis

General methods. ^{1}H and ^{13}C NMR spectra, unless otherwise noted, were taken in $CDCl_3$ at 300 MHz and 75 MHz, respectively. The chemical shifts are reported in units of δ. Melting points are uncorrected. Thin-layer chromatography (tlc) was carried out on polyester sheets precoated with silica gel 60 F-254 (Whatman). Visualization was obtained by exposure to 5% phosphomolybdic acid in ethanol or by a UV_{254} light source. Flash chromatography was carried out on EM silica gel 60 (230–400 mesh) unless otherwise specified. $MgSO_4$ was used as a drying agent unless otherwise noted. Tetrahydrofuran (THF) and ether were distilled from sodium/benzophenone. Acetone was distilled from $CaCO_3$ onto 3 Å molecular sieves. CH_2Cl_2 was distilled from calcium hydride. Dimethylformamide (DMF) and pyridine were distilled from calcium hydride onto 3 Å molecular sieves. Benzene was dried over 4 Å molecular sieves for 6 h, then distilled onto 4 Å molecular sieves. Methanol was distilled from magnesium and iodine onto 3 Å molecular sieves. Acetic acid was distilled from acetic anhydride. All reactions were magnetically stirred. All reagents, unless otherwise noted, were obtained from Aldrich Chemical Co.

*3α,5-Cyclo-22-(p-benzoylphenoxy)-5α-23,24-bisnorcholan-6β-ol 6-methyl ether(**16**).* In Procedure A, according to modification of a procedure by Marecak et al. (15), a solution of 221 mg (0.64 mmol) of **12** in 1 ml of THF was added dropwise to a solution of 190 mg (0.96 mmol) of **14** and 201 mg (0.77 mmol) of triphenylphosphine in 1 ml of THF with stirring at room temperature (rt) under nitrogen. This solution was cooled to 0°C, treated with 134 mg (0.77 mmol) of diethyl azodicarboxylate (DEAD), warmed to rt over 2 h,

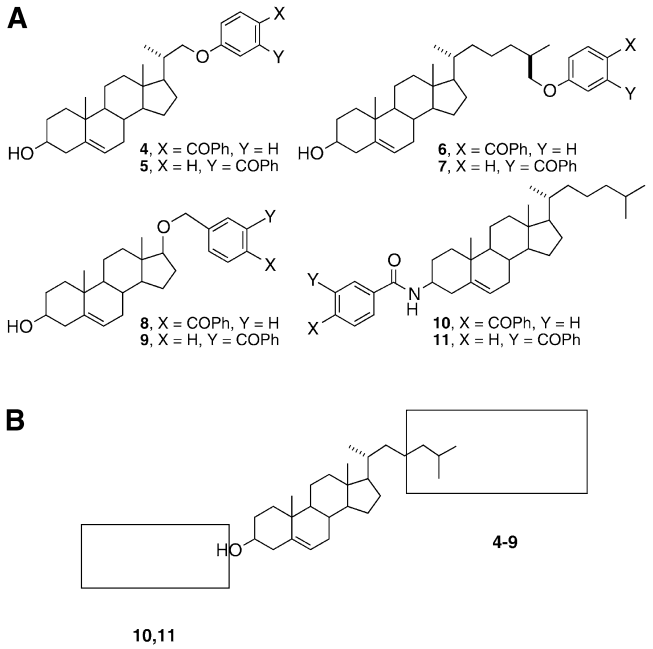

Fig. 2. A: The structures of the eight benzophenone-containing cholesterol analogs described in this paper. B: Schematic depiction of the elongating effect on the cholesterol structure when converted to compounds **4–9** or **10** and **11**.

and stirred for an additional 22 h. The THF was evaporated and, according to modification of a procedure by Mitsunobu (16), the residue was treated with 3 × 10 ml of ether and filtered. The ether was evaporated and the residue (232 mg) was chromatographed on silica gel (1:19; EtOAc:hexane) to afford 145 mg (43%) of yellow oily **16** containing trace amounts of reduced DEAD, as judged by ^{1}H NMR.

In Procedure B, a solution of 590 mg (3.0 mmol) of **14**, 96 mg (0.30 mmol) of tetra-*n*-butylammonium bromide, and 119 mg (2.98 mmol) of NaOH in 6 ml of water was treated with 388 mg (0.85 mmol) of **13** in 6 ml of CH_2Cl_2, and the resulting mixture was heated at 40°C for 4 days. The aqueous layer was removed, and the CH_2Cl_2 layer was washed with 2 × 10 ml of water, dried, filtered, and evaporated to afford 760 mg of residue that was chromatographed (1:19; EtOAc:hexane) to afford 357 mg (81%) of yellow oily **16**: ^{1}H NMR 7.81–7.71 (m, 4H), 7.56–7.50 (m, 1H), 7.47–7.41 (m, 2H), 6.94–6.89 (m, 2H), 3.99–3.95 (dd, J = 9.0, 3.5 Hz, 1H), 3.76–3.71 (dd, J = 9.0, 7.0 Hz, 1H), 3.30 (s, 3H), 2.75 (br, 1H), 2.05–0.85 (m, 20H), 1.12 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 0.76 m (s, 3H), 0.64 (m, 1H), 0.42 (m, 1H); ^{13}C NMR 195.8, 163.4, 138.6, 132.8, 132.0, 130.0, 129.9, 128.4, 114.2, 82.6, 73.5, 56.8, 56.4, 53.0, 48.2, 43.6, 43.2, 40.3, 36.6, 35.4, 35.3, 33.6, 30.8, 28.1, 25.2, 24.5, 23.0, 21.7, 19.5, 17.6, 13.3, 12.6. Electron-impact high resolution mass spectrometry (EI-HRMS) (M^+) calculated (calcd) for $C_{36}H_{46}O_3$: 526.3477. Found: 526.3451.

*22-(p-Benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol (**4**).* According to modification of a procedure by Partridge, Faber, and Uskokovic (17), a solution of 280 mg (0.53 mmol) of **16** and 25 mg (0.13 mmol) of *p*-toluenesulfonic acid monohydrate in 6 ml of 25% aqueous dioxane was heated (oil bath temperature 79–80°C) for 5 h. The mixture was cooled to rt, quenched with 10 ml of saturated $NaHCO_3$ solution, and extracted with 3 × 25 ml of CH_2Cl_2. The CH_2Cl_2 layers were dried, filtered, and evaporated to afford 265 mg of residue that was chromatographed (1:4; EtOAc:hexane) to afford 185 mg (68%) of colorless **4**: melting point (mp)

135–138°C. Recrystallization from 2:1 pentane:acetone gave 145 mg of **4**: mp 158.5–160.2°C; ^{1}H NMR (500 MHz) 7.82–7.70 (m, 4H), 7.57–7.50 (m, 1H), 7.48–7.40 (m, 2H), 6.95–6.89 (m, 2H), 5.26 (br, 1H), 3.99–3.95 (dd, J = 9.0, 3.5 Hz, 1H), 3.76–3.71 (dd, J = 9.0, 7.0 Hz, 1H), 3.55–3.45 (m, 1H), 2.05–0.85 (m, 22H), 1.13 (d, J = 6.5 Hz, 3H), 0.99 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (125 MHz) 195.8, 163.4, 141.0, 138.5, 132.8, 132.0, 130.0, 129.9, 128.3, 121.8, 114.2, 73.5, 71.9, 56.6, 52.8, 50.3, 42.8, 42.5, 39.8, 37.5, 36.7, 36.6, 32.1, 32.1, 31.8, 28.0, 24.6, 21.3, 19.6, 17.6, 12.2. EI-HRMS (M^+) calcd for $C_{35}H_{44}O_3$: 512.3290. Found: 512.3293. Analysis (anal.) calcd for $C_{35}H_{44}O_3$: C, 81.99; H, 8.65. Found: C, 82.03; H, 8.75.

*3α,5-Cyclo-22-(m-benzoylphenoxy)-5α-23,24-bisnorcholan-6β-ol 6-methyl ether (**17**).* In Procedure A for **17**, as in Procedure A for the preparation of **16**, 400 mg (1.16 mmol) of **12** and 345 mg (1.74 mmol) of **15** gave 265 mg (43%) of yellow oily **17** containing trace amounts of reduced DEAD, as judged by ^{1}H NMR.

In Procedure B for **17**, similar to Procedure B for the preparation of **16**, a solution of 456 mg (2.30 mmol) of **15**, 74 mg (0.23 mmol) of tetra-*n*-butylammonium bromide, and 92 mg (2.30 mmol) of NaOH in 5 ml of water was treated with 300 mg (0.66 mmol) of **13** in 5 ml of CH_2Cl_2, and the resulting mixture was heated at 40°C for 5 days. The aqueous layer was extracted with CH_2Cl_2, and the combined extracts were washed with brine, dried, filtered, and evaporated to afford 380 mg of residue that was chromatographed (1:30; EtOAc:hexane) to afford 287 mg (83%) of colorless oily **17**: ^{1}H NMR 7.81–7.76 (m, 2H), 7.59–7.53 (m, 1H), 7.49–7.42 (m, 2H), 7.36–7.26 (m, 3H), 7.12–7.07 (m, 1H), 3.97–3.92 (dd, J = 9.0, 3.5 Hz, 1H), 3.74–3.67 (dd, J = 9.0, 7.0 Hz, 1H), 3.31 (s, 3H), 2.75 (br, 1H), 2.03–1.07 (m, 16H), 1.12 (d, J = 6.6 Hz, 3H), 1.10 (s, 3H), 0.95–0.85 (m, 4H), 0.95 (s, 3H), 0.69 (m, 1H), 0.48 (m, 1H); ^{13}C NMR 196.7, 159.6, 139.0, 137.8, 132.5, 130.2, 129.3, 128.4, 122.7, 119.5, 115.2, 82.5, 73.4, 56.7, 56.4, 53.0, 48.2, 43.5, 43.1, 40.3, 36.6, 35.4, 35.2, 33.5, 30.7, 28.1, 25.1, 24.4, 22.9, 21.6, 19.5, 17.6, 13.3, 12.5. EI-HRMS (M^+) calcd for $C_{36}H_{46}O_3$: 526.3447. Found: 526.3443.

*22-(m-Benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol (**5**).* As in the preparation of **4**, 165 mg (0.31 mmol) of **17** gave 138 mg (86%) of colorless **5**: mp 87–90°C. Recrystallization from 2:1 pentane:acetone gave 60 mg of **5**: mp 104.2–106.6°C; ^{1}H NMR 7.81–7.76 (m, 2H), 7.60–7.53 (m, 1H), 7.49–7.42 (m, 2H), 7.36–7.26 (m, 3H), 7.12–7.07 (m, 1H), 5.35 (br, 1H), 3.97–3.92 (dd, J = 9.0, 3.5 Hz, 1H), 3.74–3.68 (dd, J = 9.0, 7.0 Hz, 1H), 3.56–3.46 (m, 1H), 2.30 (m, 2H), 2.06–0.88 (m, 20H), 1.12 (d, J = 6.6 Hz, 3H), 1.00 (s, 3H), 0.72 (s, 3H); ^{13}C NMR 196.9, 159.6, 141.0, 139.0, 137.9, 132.6, 130.3, 129.3, 128.5, 122.8, 121.8, 119.6, 115.2, 73.5, 72.0, 56.7, 52.6, 50.3, 42.8, 42.5, 39.8, 37.5, 36.7, 32.2, 32.1, 31.9, 28.0, 24.6, 21.3, 19.6, 17.7, 12.2. EI-HRMS (M^+) calcd for $C_{35}H_{44}O_3$: 512.3290. Found: 512.3289. Anal. calcd for $C_{35}H_{44}O_3$: C, 81.99; H, 8.65. Found: C, 81.92; H, 8.85.

*(25R)26-(p-Benzoylphenoxy)-cholest-5-ene-3β,26-diol (**6**).* According to procedures by Marecak et al. (15) and Mitsunobu (16), to a mixture of 99.9 mg (0.249 mmol) of 27-hydroxycholesterol, prepared by the method of Kim et al. (18), 79.9 mg (0.32 mmol) of triphenylphosphine and 73.8 mg (0.372 mmol) of **14** in 2 ml of dry THF at 0°C was added dropwise 0.05 ml (0.318 mmol) of diethyl azodicarboxylate under N_2. The resulting mixture was stirred at 0°C for 2 h, warmed to rt, and stirred for 20 h. The THF was evaporated, and the residue was dissolved in 10 ml of ether and filtered. The ether filtrate was washed with 2 × 10 ml of 2N NaOH solution and 10 ml of water, dried, filtered, and evaporated to give 328 mg of residue, which was chromatographed (1:4; EtOAc:hexane) to give 106 mg (74%) of colorless solid **6**. Recrystallization from CH_2Cl_2:hexane gave **6**: mp 128.4–129.6°C; ^{1}H NMR (500 MHz) 7.83 (m, 2H), 7.76 (m, 2H), 7.58 (m, 1H), 7.49 (m, 2H), 6.96 (m, 2H), 5.37 (m, 1H), 3.90 (m, 1H), 3.82 (m, 1H), 3.54 (m, 1H), 2.30 (m, 2H), 2.00 (m, 3H), 1.84 (m, 3H), 1.62–0.87 (m, 21H), 1.06 (d, J = 7.0 Hz, 3H), 1.04 (s, 3H), 0.95 (d, J = 7.0 Hz, 3H), 0.70 (s, 3H); ^{13}C NMR (125 MHz) 195.9, 163.3, 141.0, 138.6, 132.8, 132.1, 130.1, 129.9, 128.4, 121.9, 114.3, 73.8, 72.0, 57.0, 56.4, 50.4, 42.6, 42.5, 40.0, 37.5, 36.7, 36.3, 35.9, 34.0, 33.3, 32.1, 32.1, 31.9, 28.5, 24.5, 23.6, 21.3, 19.6, 18.9, 17.2, 12.1. Anal. calcd for $C_{40}H_{54}O_3$: C, 82.43; H, 9.34. Found: C, 82.14; H, 9.45.

*(25R)26-(m-Benzoylphenoxy)-cholest-5-ene-3β,26-diol (**7**).* As in the preparation of **6**, 175 mg (0.44 mmol) of 27-hydroxycholesterol and 130 mg (0.65 mmol) of **15** gave 131 mg (52%) of colorless **7**: mp 75.1–77.4°C. Recrystallization from ether:petroleum ether gave **7**: mp 77.9–78.8°C; ^{1}H NMR (500 MHz) 7.82 (m, 2H), 7.60 (m, 1H), 7.49 (m, 2H), 7.34 (m, 3H), 7.13 (m, 1H), 5.36 (m, 1H), 3.87 (m, 1H), 3.78 (m, 1H), 3.52 (m, 1H), 2.30 (m, 2H), 2.00 (m, 3H), 1.84 (m, 4H), 1.57–1.38 (m, 10H), 1.29–0.93 (m, 10H), 1.04 (d, J = 6.5 Hz, 3H), 1.02 (s, 3H), 0.94 (d, J = 6.5 Hz, 3H), 0.70 (s, 3H); ^{13}C NMR (125 MHz) 196.9, 159.6, 141.0, 139.1, 137.9, 132.6, 130.3, 129.4, 128.5, 122.9, 121.9, 119.6, 115.2, 73.8, 72.0, 57.0, 56.4, 50.3, 42.6, 42.5, 40.0, 37.5, 36.7, 36.3, 35.9, 34.1, 33.4, 32.2, 32.1, 31.9, 28.5, 24.5, 23.6, 21.3, 19.7, 18.9, 17.2, 12.1. Anal. calcd for $C_{40}H_{54}O_3$: C, 82.43; H, 9.34. Found: C, 82.15; H, 9.40.

*3β-t-Butyldimethylsilanyloxy-17β-(4-benzoylbenzyloxy)-androst-5-ene (**22**).* According to the procedure of Takaku, Kamaike, and Tsuchiya (19), to a solution of 710 mg (1.76 mmol) of **19**, prepared according to the procedure of Rychnovsky and Mickus (20), in 10 ml of dry CH_2Cl_2 were added 967 mg (3.51 mmol) of **20**, mp 109–111°C [literature (21), mp 110–112°C], prepared according to the procedure of Zhao et al. (21), 813 mg (3.51 mmol) of Ag_2O, and 710 mg of 3Å molecule sieves. After the mixture was heated at 50°C for 2 days, the same amounts of **20**, Ag_2O, and 3 Å molecule sieves were added again, and the resulting mixture was heated at 50°C for another 5 days. Insoluble material was removed by passing the mixture through a short pad of Celite, and the solvent was evaporated to give 2.48 g of residue, which was chromatographed (1:40–1:20; EtOAc:hexane) to give 1.37 g of **22** as a white solid that was used directly in the next step. Further purification by similar chromatography gave **22**: mp 112.7–114.9°C; ^{1}H NMR 7.82 (m, 4H), 7.61 (m, 1H), 7.50 (m, 4H), 5.35 (m, 1H), 4.67 (s, 2H), 3.50 (m, 2H), 2.23 (m, 2H), 2.02 (m, 2H), 1.85 (m, 2H), 1.65-1.29 (m, 8H), 1.08–0.80 (m, 5H), 1.06 (s, 3H), 0.92 (s, 9H), 0.90 (s, 3H), 0.08 (s, 6H); ^{13}C NMR 196.7, 144.5, 141.8, 137.9, 136.7, 132.5, 130.4, 130.2, 128.4, 127.0, 121.1, 89.0, 72.8, 71.3, 51.8, 50.5, 43.2, 43.0, 38.1, 37.6, 36.9, 32.3, 32.0, 31.7, 28.1, 26.1, 23.7, 20.9, 19.7, 18.5, 11.9, −4.4. EI-HRMS (M-H_2^+) calcd for $C_{39}H_{54}SiO_3$-H_2^+: 596.3684. Found: 596.3685.

*3β-Hydroxy-17β-(4-benzoylbenzyloxy)-androst-5-ene (**8**).* To a solution of 1.36 g of **22** prepared as described above in 10 ml of dry THF was added 22.7 ml (22.7 mmol) of 1.0 M tetrabutylammonium fluoride in THF. The resulting mixture was stirred at rt overnight, quenched by addition of 20 ml of water, extracted with 3 × 60 ml of ethyl acetate, washed with 2 × 15 ml of brine, dried, filtered, and evaporated to afford 4.28 g of residue, which was chromatographed (1:4–1:2; EtOAc:hexane) to give 595 mg (70% from **19**) of colorless **8**: mp 169.8–172.3°C. Recrystallization from CH_2Cl_2:hexane gave **8**: mp 181.7–182.7°C; ^{1}H NMR (500 MHz) 7.82 (m, 4H), 7.60 (m, 1H), 7.48 (m, 4H), 5.36 (m, 1H), 4.65 (s, 2H), 3.53 (m, 1H), 3.47 (t, J = 8.5 Hz, 1H), 2.30 (m, 2H), 2.02 (m, 3H), 1.87 (m, 2H), 1.65-1.48 (m, 8H), 1.33 (m, 1H), 1.21 (m, 1H), 1.12–0.96 (m, 3H), 1.05 (s, 3H), 0.89 (s, 3H); ^{13}C NMR (125 MHz) 196.7, 144.5, 141.1, 138.0, 136.8, 132.6, 130.5, 130.3, 128.5, 127.0, 121.6, 89.0, 72.0, 71.3, 51.8, 50.5, 43.2, 42.5, 38.1, 37.5, 36.8, 32.0, 31.9, 31.7, 28.1, 23.7, 21.0, 19.7, 12.0. Anal. calcd for $C_{33}H_{40}O_3$: C, 81.78; H, 8.32. Found: C, 81.55; H, 8.30.

*3β-t-Butyldimethylsilanyloxy-17β-(3-benzoylbenzyloxy)androst-5-ene **(23)**.* As in the preparation of **22**, 995 mg (2.46 mmol) of **19** and 1.35 g (4.92 mmol) of **21**, mp 66–68°C, prepared according to the procedure of Zhao et al. (21), gave 4.57 g of residue, which was chromatographed (1:30–1:20; EtOAc:hexane) to give 1.93 g of **23** as a white solid that was used directly in the next step. Purification by similar chromatography gave **23**: mp 73.2–74.8°C; ^{1}H NMR (500 MHz) 7.83 (m, 3H), 7.71 (m, 1H), 7.62 (m, 2H), 7.52 (m, 3H), 5.33 (m, 1H), 4.62 (s, 2H), 3.47 (m, 2H), 2.29 (m, 1H), 2.20 (m, 1H), 2.00–1.72 (m, 4H), 1.62-1.27 (m, 8H), 1.18-0.87 (m, 5H), 1.03 (s, 3H), 0.91 (s, 9H), 0.85 (s, 3H), 0.08 (s, 6H); ^{13}C NMR (125 MHz) 197.0, 141.9, 140.0, 137.9, 137.8, 132.6, 131.5, 130.3, 129.4, 129.0, 128.5, 128.5, 121.1, 88.9, 72.8, 71.4, 51.8, 50.6, 43.2, 43.0, 38.1, 37.6, 36.9, 32.3, 32.0, 31.8, 28.2, 26.2, 23.7, 21.0, 19.7, 18.5, 12.0, −4.4. EI-HRMS (M-H_2^+) calcd for $C_{39}H_{54}SiO_3$-H_2^+: 596.3684. Found: 596.3687.

*3β-Hydroxy-17β-(3-benzoylbenzyloxy)-androst-5-ene **(9)**.* As in the preparation of **8**, 1.93 g of **23** afforded 6.1 g of residue, which was chromatographed (1:4–1:1; EtOAc:hexane) to give 782 mg (66% from **19**) of **9**: mp 103.8–105.7°C. Recrystallization from ether: pentane gave **9**: mp 107.0–109.2°C; ^{1}H NMR (500 MHz) 7.82 (m, 3H), 7.71 (m, 1H), 7.61 (m, 2H), 7.49 (m, 3H), 5.36 (m, 1H), 4.62 (s, 2H), 3.53 (m, 1H), 3.45 (t, J = 8.5 Hz, 1H), 2.32 (m, 2H), 2.02 (m, 3H), 1.87 (m, 2H), 1.64-1.48 (m, 8H), 1.32-0.95 (m, 5H), 1.04 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (125 MHz) 197.0, 141.1, 140.0, 137.9, 137.8, 132.7, 131.5, 130.3, 129.4, 129.0, 128.5, 128.5, 121.6, 88.9, 72.0, 71.4, 51.8, 50.5, 43.1, 42.5, 38.0, 37.5, 36.8, 32.0, 31.9, 31.7, 28.1, 23.7, 21.0, 19.7, 12.0. Anal. calcd for $C_{33}H_{40}O_3$: C, 81.78; H, 8.32. Found: C, 81.72; H, 8.35.

*3β-Aminocholest-5-ene **(24)**.* 3α,5-Cyclo-6β-methoxy-5α-cholestane, mp 77–79°C, was prepared via cholesteryl tosylate by the procedure of Barton and Morgan (22). The *i*-steroid was converted to 3β-azidocholest-5-ene by the following modification of the procedure of Jarreau, Khung-Huu, and Goutarel (23). A solution of HN_3 in benzene was prepared according to a procedure by Wolff (24) by adding 9.0 ml of benzene to a paste of 1.45 g (22.3 mmol) of NaN_3 in 1.5 ml of water. After the mixture was cooled to 0°C, 0.8 ml of concentrated H_2SO_4 was added dropwise, and the resulting mixture was stirred for 15 min. The benzene layer was decanted, dried 2× over Na_2SO_4, and added to 529 mg (1.32 mmol) of 3α,5-cyclo-6β-methoxy-5α-cholestane. To the resulting solution, 0.25 ml of $BF_3{\cdot}Et_2O$ was added, the mixture was stirred at rt overnight, 15 ml of concentrated NH_4OH was added, and the aqueous layer was extracted with 3 × 15 ml of hexane. The combined organic layers were washed with 15 ml of brine, dried, filtered, and evaporated to give 484 mg of yellow solid, which was chromatographed (1:99; EtOAc:hexane) to give 328 mg (60%) of colorless azide: mp 85–86.5°C (literature (25), mp 84–85°C). The azide was converted to **24**, mp 88–91°C, by the procedure of Bose, Kistner, and Farber (26).

*3β-(4-Benzoylbenzoyl)amino-cholest-5-ene **(10)**.* According to procedures by Firestone et al. (27) and Cope and Ciganek (28), to a solution of 1.09 g (4.85 mmol) of **25**, prepared according to the procedure of Wertheim (29), in 12 ml of dry CH_2Cl_2 was added 1.3 ml (14.5 mmol) of $(COCl)_2$ and five drops of DMF. The mixture was stirred at 0°C for 2 h. The solvent was evaporated to give a light yellow solid, which was dissolved in 10 ml of dry ether. To this solution, a mixture of 210 mg (0.55 mmol) of **24** in 10 ml of dry ether and 3.0 ml of dry pyridine was added at −78°C. The mixture was stirred at −78°C for 1 h, allowed to stir at rt overnight, and 20 ml of water was added. The resulting mixture was extracted with 3 × 40 ml of CH_2Cl_2, washed with 2 × 20 ml of 2N NaOH solution and 2 × 10 ml of brine, dried, filtered, and evaporated to give 401 mg of residue, which was chromatographed (1:9–1:4; EtOAc:hexane) to give 190 mg (59%) of **10**: mp 218–220°C. Recrystallization from ethanol gave **10**: mp 219–220°C; ^{1}H NMR (500 MHz) 7.87 (m, 4H), 7.80 (m, 2H), 7.62 (m, 1H), 7.50 (m, 2H), 6.04 (d, J = 8.0 Hz, 1H), 5.45 (m, 1H), 3.95 (m, 1H), 2.48 (m, 1H), 2.27 (m, 1H), 2.04–1.84 (m, 6H), 1.60-0.83 (m, 20H), 1.02 (s, 3H), 0.93 (d, J = 6.3 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H), 0.69 (s, 3H); ^{13}C NMR (125 MHz) 196.2, 166.2, 140.3, 140.2, 138.5, 137.3, 133.1, 130.3, 130.3, 128.7, 127.2, 122.5, 56.9, 56.4, 50.7, 50.3, 42.5, 40.0, 39.8, 39.5, 38.1, 36.8, 36.4, 36.1, 32.1, 29.4, 28.5, 28.3, 24.5, 24.1, 23.1, 22.8, 21.2, 19.6, 19.0, 12.1. Anal. calcd for $C_{41}H_{55}NO_2$: C, 82.92; H, 9.33; N, 2.36. Found: C, 82.71; H, 9.35; N, 2.35.

*3β-(3-Benzoylbenzoyl)amino-cholest-5-ene**(11)**.* As in the preparation of **10**, 250 mg (1.10 mmol) of **26**, prepared according to the procedure of Wertheim (29), and 46.5 mg (0.121 mmol) of **24** gave 176 mg of yellow residue, which was chromatographed (neutral alumina, 1:9; EtOAc:hexane) to give 55.0 mg (77%) of colorless **11**. Recrystallization from CH_2Cl_2:hexane twice gave **11**: mp 218–219°C; ^{1}H NMR 7.84 (m, 5H), 7.61 (m, 1H), 7.50 (m, 2H), 7.27 (s, 1H), 6.15 (d, J = 7.8 Hz, 1H), 5.42 (m, 1H), 3.93 (m, 1H), 2.44–0.86 (m, 28H), 1.04 (s, 3H), 0.93 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 6H), 0.69 (s, 3H); ^{13}C NMR 196.3, 165.9, 140.3, 138.0, 137.3, 135.5, 133.0, 131.3, 130.2, 128.8, 128.7, 127.9, 122.4, 56.9, 56.3, 50.6, 50.3, 42.5, 39.9, 39.7, 39.5, 38.1, 36.8, 36.4, 36.0, 32.1, 29.3, 28.4, 28.2, 24.5, 24.5, 23.0, 22.8, 21.2, 19.6, 18.9, 12.1. Anal. calcd for $C_{41}H_{55}O_2$: C, 82.92; H, 9.33; N. 2.36. Found: C, 82.66; H, 9.41; N, 2.35.

*3α,5-Cyclo-22-(3′,5′-dibromo-p-benzoylphenoxy)5α-23,24-bisnorcholan-6β-ol 6-methyl ether **(28)**.* A solution of 680 mg (1.9 mmol) of 3,5-dibromo-4-hydroxybenzophenone (**27**), mp 155–156°C, prepared by the method of Blakey, Jones, and Scarborough (30), and 61 mg (0.19 mmol) of tetrabutylammonium bromide in 2 ml of 2 M NaOH was treated with 365 mg (0.80 mmol) of **13** in 5 ml of EtOAc and heated at reflux for 4 days. The aqueous layer was removed and the EtOAc layer was washed with 2 × 10 ml of water, dried, filtered, and evaporated to afford 1.1 g of residue, which was treated with 2 × 10 ml of 1:4 EtOAc:hexane to precipitate excess **27** that was collected by filtration. The filtrate was evaporated and the residue (550 mg) was chromatographed (1:4; EtOAc:hexane) to afford 442 mg (80%) of colorless, waxy **28**: mp: soften 72–76°C; ^{1}H NMR 7.99 (s, 2H), 7.79 (m, 2H), 7.67 (m, 1H), 7.54 (m, 2H), 4.03 (dd, J = 9.0, 3.5 Hz, 1H), 3.88 (m, 1H), 3.37 (s, 3H), 2.82 (br, 1H), 2.11-1.12 (m, 16H), 1.35 (d, J = 6.6 Hz, 3H), 1.08 (s, 3H), 0.95-0.89 (m, 4H), 0.85 (s, 3H), 0.70 (m, 1H), 0.48 (m, 1H); ^{13}C NMR 193.4, 157.3, 136.8, 135.4, 134.7, 133.1, 130.1, 128.8, 118.7, 82.6, 78.8, 56.8, 56.5, 52.8, 48.2, 43.6, 43.3, 40.3, 38.1, 35.4, 35.3, 33.6, 30.8, 28.3, 25.2, 24.6, 23.0, 21.6, 19.5, 17.6, 13.3, 12.5. EI-HRMS (M^+) calcd for $C_{36}H_{44}Br_2O_3$: 682.1657. Found: 681.1650. Anal. calcd for $C_{36}H_{44}Br_2O_3{\cdot}H_2O$: C, 61.55; H, 6.60. Found: C, 61.27; H, 6.74.

*Reduction of **28** over 20% Pd(OH)$_2$ on carbon.* According to a modification of a procedure by Dorman et al. (31), a solution of 38 mg (0.056 mmol) of **28** and 50 μl (0.36 mmol) of triethylamine in 5 ml of EtOAc was added to 38 mg of 20% Pd(OH)$_2$ on carbon (Degussa type). The reaction mixture was flushed 3× with H_2 and stirred under a balloon of H_2 at rt. Hydrodebromination went to completion within 5 min as indicated by tlc analysis (1:4; EtOAc:hexane), with only trace amounts of ketone reduction. Reduction of the ketone went nearly to completion within 25 min as indicated by tlc. The product was obtained from the 5 min reduction by filtration through a pad of Celite, washing the filtrate with saturated NH_4Cl solution, water, and brine, drying, filtering, and evaporating to afford **16** with ^{1}H NMR and IR spectra essentially identical with those for **16** prepared using **14**. The product similarly obtained from the 25 min reduction was the alcohol formed by reduction of the carbonyl group: ^{1}H NMR 7.38–7.21 (m, 7H), 6.84–6.76 (m, 2H), 5.79 and 5.75 (2s, 1H), 3.96–3.85 (s, 1H), 3.72–3.59 (m, 1H), 3.31 (s, 3H), 2.75 (br, 1H),

2.02–0.82 (m, 21H), 1.10–1.08 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 0.74 (s, 3H), 0.63 (m, 1H), 0.42 (m, 1H).

Biochemical experiments

Human apoA-I was obtained from centrifugally isolated human HDL delipidated with ethanol and diethyl ether, and dissolved in 6 M urea, 0.01 M , tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.1. apoA-I, which was about 60% of the total protein in HDL, was purified by molecular sieve column chromatography over Sephadex G-150 in the same buffer, based on the procedure of Brewer, Ronan, and Bishop (32). Fractions containing apoA-I (>99%) were dialyzed against 1 mM Na-phosphate buffer, pH 8.1 (0.8–1 mg protein/ml), flash frozen, stored at −70°C, and thawed immediately before use.

[^{3}H][1,2]FC (50–55 mCi/mmole) was from Perkin Elmer/NEN. FBS from the cell culture facility contained 65 μg ml^{-1} of FC, determined with cholesterol oxidase (33). Human skin fibroblasts were grown to near confluence in 10% v/v of FBS in DMEM (2.25 μg ml^{-1} FC) in 12-well plastic dishes (1 ml medium per well). Under these conditions, the cell FC content was 2.2 ± 0.2 μg FC per dish. For the final 48 h, 5 μCi of [^{3}H]FC was included for each milliliter of medium for control incubations. In the other incubations, 5 μCi of [^{3}H]FC per milliliter of medium was included, together with an amount of unlabeled FC or photoactivable analog (one of **4–11**) representing the molar equivalent of the sum of cellular and medium FC. These mixtures of labeled and unlabeled sterol were dissolved in DMSO and added with stirring to a final solvent concentration of 1–3% v/v. DMSO alone was added to control incubations.

To assay apoA-I-dependent efflux, the cells were washed (3×) with phosphate-buffered saline, then incubated overnight in DMEM containing 1 mg ml^{-1} of high molecular weight dextran (Pharmacia, T-500) as oncotic agent. The cells were then washed with the same medium and incubated (3 h) in the presence or absence of 10 μg ml^{-1} apoA-I. At the end of incubation, the medium was collected, centrifuged (5000 *g*, 10 min, 4°C) to pellet any unattached cells, and assayed for ^{3}H-label. Efflux of FC was linear over this incubation period. apoA-I-dependent FC efflux was expressed as [efflux in the presence of apoA-I] − [efflux in the absence of apoA-I] under the same experimental conditions. Both total and apoA-I-dependent efflux were linear with time over the period of the assay. apoA-I-independent efflux was <20% of total efflux under the conditions of these experiments.

ABCA1 mRNA levels in control fibroblasts, or fibroblasts equilibrated with FC or one of **4–11**, were determined by RT-PCR as previously described (34). In brief, RNA was reverse transcribed with Superscript II enzyme. PCR was carried out with Taq polymerase (Qiagen) using SYBR Green as fluophor. Glyceraldehyde 3-phosphate dehydrogenase mRNA was the control.

RESULTS

Synthesis

Compound **4**, in which the benzophenone group is attached to C22 of a truncated cholesterol framework via an ether linkage, was prepared either by Mitsunobu coupling of the C22 alcohol **12** (17, 35, 36) with 4-hydroxybenzophenone (**14**) to give 43% yield of **16**, or, preferably, by phase transfer catalyzed alkylation of C22 iodide **13** (17, 35) with **14** to afford **16** in 81% yield (**Fig. 3**). Acidic hydrolysis of *i*-steroid **16** gave 68% of **4**. Isomeric analog **5** was prepared by the same two methods via coupling with 3-hydroxybenzophenone (**15**) to give **17** in 43% yield

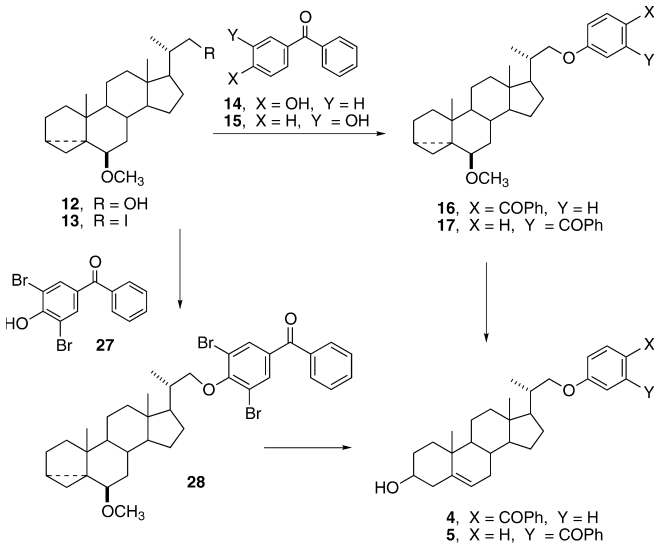

Fig. 3. The synthetic pathway to cholesterol analogs **4** and **5** via **16** and **17** and the pathway for synthesis of [^{3}H]**4** via **28** are shown.

from **12** and in 83% yield from **13**, followed by acidic hydrolysis to afford 86% of **5**.

Analogs **6** and **7**, having the benzophenone group at the end of the cholesterol side chain, were synthesized in 74% and 52% yield, respectively, by selective Mitsunobu reaction at the primary hydroxyl group of 27-hydroxycholesterol, which had been prepared by the method of Kim et al. (18), with **14** or **15**, respectively. Analog **8** was prepared in 70% overall yield from the known (20) 3β-*t*-butyldimethylsilyl derivative **19** of androst-5-en-3β,17-diol (**18**) by Ag_2O promoted alkylation (19) with 4-bromomethylbenzophenone (**20**), prepared by the method of Zhao et al. (21), to produce **22**, followed by deprotection of the 3β-hydroxyl group (**Fig. 4A**). Analog **9** was comparably prepared in 66% overall yield from **19** and 3-bromomethylbenzophenone (**21**) via **23**.

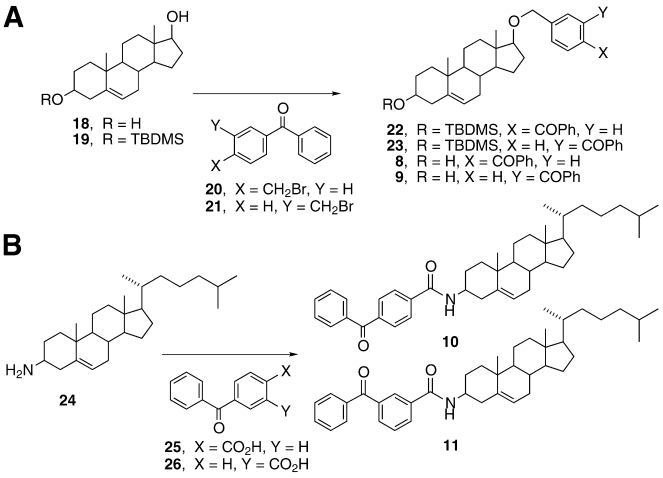

Fig. 4. A: The synthetic pathway to cholesterol analogs **8** and **9**. B: The synthetic pathway to cholesterol analogs **10** and **11**.

We desired also to prepare cholesterol analogs having the benzophenone moiety at the opposite end of the structure from its location in **4–9**. Because it was considered potentially important to retain the hydrogen bonding capability of the C3 hydroxyl group of cholesterol itself, amides **10** and **11** were selected as candidates of this type. For synthesis of **10** and **11**, cholesterol was converted to 3-amino-cholest-5-ene (**24**) by modifications of procedures for reactions developed by Barton and Morgan (22) and Jarreau, Khung-Huu, and Goutarel (23), and this amine was acylated with the acid chlorides prepared in situ from benzophenone carboxylic acids **25**, to give 59% of **10**, and **26**, to give 77% of **11** (Fig. 4B).

As described in the next section, all eight analogs show promise as biochemical surrogates for cholesterol. For the initial photoaffinity labeling experiments, it was decided to use the first analog prepared, FCBP (**4**) (13). In order to facilitate analysis of the photoaffinity labeling products and for use in additional types of experiments, isotopically-labeled FCBP was needed. A procedure for preparing tritiated **4** was developed via the dibromo analog **28** of intermediate **16**. Compound **28** was prepared in 80% yield by use of the known (30) dibromohydroxybenzophenone **27** for alkylation by **13** (Fig. 3). Careful hydrogenation using Degussa $Pd(OH)_2/C$ catalyst (31) effected debromination to **16** without carbonyl group reduction, and this procedure, followed by acid hydrolysis of the *i*-steroid moiety, has been used with commercial catalytic tritiation to afford [^{3}H]**4**. As noted above, FCBP (**4**) and [^{3}H]FCBP have already proved to be excellent biochemical research tools (13).

Cellular equilibration of sterols

As described in Materials and Methods, the extracellular medium of cultured fibroblasts was enriched with [^{3}H]FC alone, with the same level of [^{3}H]FC + unlabeled FC in a mass equivalent to total FC in cells plus medium, or with [^{3}H]FC + unlabeled photoactivable analog to the same molar concentration. The cells and medium were incubated at 37°C for 48 h to equilibrate sterol across the cell membrane. The labeled medium was removed and replaced by DME medium containing 1 mg ml^{-1} of high molecular weight dextran as an oncotic agent, in the presence or absence of apoA-I (10 μg ml^{-1}). As shown in **Fig. 5**, there was approximately a 50% decrease in apoA-I-dependent medium radioactivity if the specific activity of [^{3}H]FC was reduced by ca. 50% by inclusion of unlabeled FC in the culture medium. As also shown in Fig. 5, there was a similar decrease when each of the benzophenone-modified FC analogs replaced unlabeled FC.

Cells incubated with [^{3}H]FC only had a sterol content of 2.2 ± 0.1 μg, while cells incubated with medium supplemented with unlabeled FC or analog had a sterol content of 2.1 ± 0.2 μg. These data indicate that equilibration with either FC or one of **4–11,** under the conditions described in Materials and Methods, reflected sterol exchange and not simply sterol loading of the cells. To obtain further evidence that the results in Fig. 5 reflect sterol exchange rather than loading, the mRNA levels for the

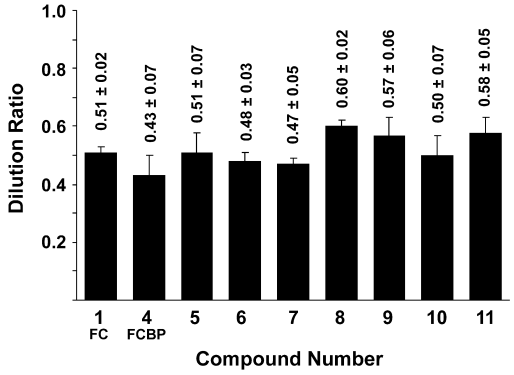

Fig. 5. The dilution of [^{3}H]free cholesterol (FC) label in fibroblast monolayers by FC or FC analogs **4–11**. The dilution ratio is the reduction in [^{3}H]FC efflux to the cellular medium when fibroblast monolayers were equilibrated (48 h, 37°C) with 10 μCi [^{3}H]FC plus unlabeled FC or FC analog equal to the total sterol content of cells and medium (10% plasma v/v) compared with cells labeled with the same level of tracer [^{3}H]FC only. Complete equilibration between sterol pools is indicated by a dilution ratio of 0:5. Values shown represent means ± 1 SD of three independent experiments, each including triplicate dishes of fibroblasts incubated as described in Materials and Methods.

ABCA1 gene were measured for the cells incubated with sterol-supplemented medium. The ABCA1 gene, whose product mediates HDL genesis, is highly sensitive to sterol loading, and it has been reported (37, 38) that mRNA levels were upregulated 4- to 10-fold by a FC-albumin suspension that promotes FC uptake, but that FC efflux was not upregulated. In contrast, the mRNA levels in the cells incubated with FC or **4–11** supplemented medium in 10% FBS in the current investigation were essentially unchanged from control (**Fig. 6**). These data confirm the

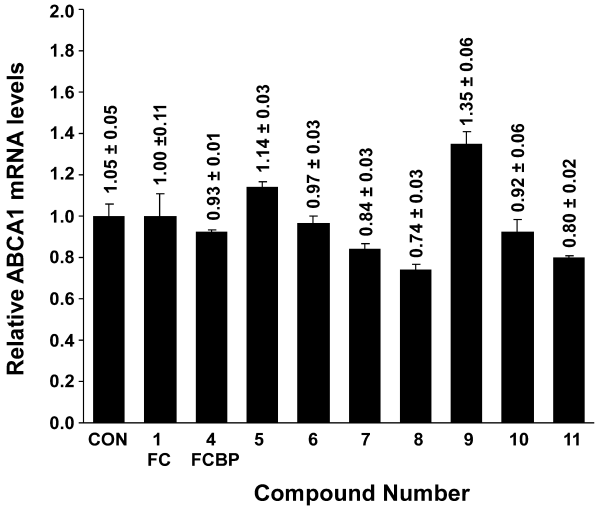

Fig. 6. ABCA1 mRNA levels in control fibroblasts, or fibroblasts equilibrated with FC or with one of **4–11**, determined as described in Materials and Methods. Representative data from one of three separate experiments are shown. Each data point is the mean of triplicate samples. Differences from the control did not reach statistical significance ($P > 0.05$).

conclusion that sterol analogs **4–11** were each effective in substituting for FC in the cellular pools contributing to apoA-I-dependent sterol efflux.

DISCUSSION

Photoactivable FC analogs capable of substituting successfully for FC in the membranes of living cells could provide novel data on membrane lipid-protein and lipid-lipid associations at the molecular level, including those complexes too transient or unstable to be isolated by classical fractionation techniques. FC plays several different roles in mammalian cells (39). It promotes the condensation of phospholipid acyl chains in synthetic or natural membrane bilayers and facilitates the separation of FC/sphingolipid-rich, liquid-ordered microdomains (lipid rafts) (40–42). In addition, single molecules of FC form stoichiometric complexes with recognition sites on sterol-binding proteins, including caveolin, Niemann-Pick type C2, steroidogenic acute regulatory, and the benzodiazepine receptor protein (13, 43–45). These recognition sites include both hydrophobic and positively charged amino acid residues and often conform to the motif $V/L_{1-4}\,Y\,x_{1-4}\,R/K$, where x is any amino acid (46). It seems likely that most sterol-dependent biological pathways include both bulk phase and monomolecular effects, so a successful FC analog must substitute for FC in both types of interaction. For this reason, we chose as the test for acceptability as an FC surrogate a complex pathway, apoA-I-dependent sterol efflux from recycling low density lipoprotein FC (47), the major source of free sterol in FBS (48). The initial uptake of FC depends mainly on the membrane properties of coated pits (49, 50). Intracellular recycling is vesicular, possibly dependent on both membrane properties and specific FC-protein interactions (51). The binding of intracellular FC to cell-surface caveolae depends on FC-caveolin binding (13). Successful replacement of FC in apoA-I-dependent sterol efflux thus represents a challenging standard for a prospective cholesterol surrogate.

In the present investigation, seven analogs of FC (compounds **5–11**) have been prepared in addition to FCBP (**4**). Five of these, like FCBP, have the benzophenone moiety replacing or extending the ring D side chain, occupying together the approximate area relative to the sterol tetracyclic core shown schematically in Fig. 2B. The other two (**10** and **11**) have the photophore appended via an amide linkage to C3, extending the opposite end of the structure roughly by the area also shown in Fig. 2B, while maintaining the possibility of hydrogen bonding by the 3β-substituent. Each of these eight analogs was tested to see if it could compete with FC in apoA-I-induced cellular sterol efflux. The dilution of [^{3}H]FC by analogs **4–11** was essentially the same in all cases as that by unlabeled FC (Fig. 5). These eight benzophenone-containing FC analogs are the first shown to replace FC successfully in the major pools involved in a complex pathway of multiple intracellular steps.

At first sight, these results appear to conflict with the high degree of specificity reported for sterol incorporation into biological membranes (52). Such physical specificity had been thought responsible for the normally low levels in mammalian plasma and tissues of plant sterols, which differ in structure from FC primarily in the alkyl side chain (53). However, the mechanism of this effect was recently shown to be mediated retroactively via the ABCG5/ABCG8 transporter complex (54, 55), which promotes the selective efflux of plant sterols from intestinal cell membranes. A number of recent observations support the idea that for incorporation into membranes, a sterol must have a sufficiently large and quite hydrophobic side chain, but that structural specificity is not required. Cholestatrienol is an example of a sterol with the side chain of FC, but with an otherwise slightly modified structure, which can replace FC in membranes (56). It has been reported that tracer levels of 7,7-azocholestanol (**3**) mimic FC in its distribution between intracellular membrane compartments (5), and that 6,6-azocholestanol (**2**) is incorporated at high levels into model membranes (9). Sterols with modified side chains that act like FC in association with phospholipids include dehydroergosterol (57). Stigmasterol, sitosterol, and a synthetic C10 side-chain analog, each also containing a somewhat larger side chain than FC, were all found to mimic FC in lipid raft formation (58). FCBP (**4**) is incorporated at high levels in human aortic smooth muscle cells, despite replacement of a major portion of the side chain by the likewise hydrophobic benzophenone moiety (13). In contrast, 22 and 25(7-nitrobenz-2-oxa-1,3-diazol-4-yl)cholesterol, with even larger and distinctly more polar side chains, are packed in membranes quite differently from FC (59). A synthetic analog lacking six carbons of the side chain (58) and several similarly truncated, variously substituted steroids (60) did not promote lipid raft formation. The success in the current work of analogs **4–9** as replacements for FC (**1**) is consistent with the requirement for a large hydrophobic side chain, which can be somewhat bigger than that in FC. However, even in the most extended side-chain analogs (**6** and **7**), the overall length, as judged from examination of Dreiding molecular models, is only ca. 2 Å longer than the fully extended C18 saturated fatty acid component of the phospholipid, with which it might compete for packing.

All natural sterols and all the synthetic analogs discussed above, with the exception of **10** and **11**, retain the 3β-hydroxyl group of FC. There has been general agreement that this functional group is important in the orientation of FC in biological membranes (59, 61). It was, therefore, of interest and somewhat unexpected to find that replacement of up to 50% of cellular FC with either **10** or **11**, in which the 3β-hydroxyl group had been replaced with a large amidobenzophenone group, was without effect on the ability of human skin cells to efflux sterol to apoA-I. Although the amide linker had been selected specifically because it retains the hydrogen bond donor and receptor capabilities of the hydroxyl group, the data in Fig. 5 suggest that the 3β-hydroxyl group per se is not

essential for biological activity, at least as assayed by promotion of apoA-I-dependent sterol efflux. The polarity of the amide link is probably sufficient to maintain an "upright" orientation of the molecule in biological membranes (59), despite the attached benzophenone group. The precise positioning of this hydrophobic appendage is not revealed by the current investigation.

There is, in principle, no reason why a sterol analog that can replace FC in binding to proteins should also mimic FC in its condensing function in membranes. For example, 3β-doxylcholestane displaces FC from intestinal proteins (62), but does not replace FC in synthetic membrane bilayers (59). The progestin promegestone displaces FC from several membrane proteins, including the benzodiazepine receptor (45) and the HIV-1 nef protein (63), but does not condense membrane phospholipids or promote raft formation (60). In contrast, FCBP (**4**) mimics FC (**1**) in both protein binding and in the delivery of cellular sterol to apoA-I (13), and the present research shows that each of seven additional benzophenone derivatives can also substitute successfully in the major roles of sterol in the cell.

In summary, the present data suggest that a variety of cholesterol analogs containing benzophenone groups at either end of the sterol structure can serve as satisfactory substitutes for FC in studying lipid-protein and lipid-lipid complexes in living cells. Further studies are in progress to determine whether replacement of part of the sterol tetracyclic nucleus with a benzophenone moiety can also afford potentially useful cholesterol surrogates.

This research was supported by National Institutes of Health Grants HL-67294 and HL-57976.

REFERENCES

1. Dorman, G., and G. D. Prestwich. 1994. Benzophenone photophores in biochemistry. *Biochemistry.* **33:** 5661–5673.
2. Kym, P. R., K. E. Carlson, and J. A. Katzenellenbogen. 1993. Progestin 16α,17α-dioxolane ketals as molecular probes for the progesterone receptor: synthesis, binding affinity, and photochemical evaluation. *J. Med. Chem.* **36:** 1111–1119.
3. Fra, A. M., M. Masserini, P. Palestini, S. Sonnino, and K. Simons. 1995. A photoreactive derivative of ganglioside GM1 specifically crosslinks VIP21-caveolin on the cell surface. *FEBS Lett.* **375:** 11–14.
4. Thiele, C., M. J. Hannah, F. Fahrenholz, and W. B. Huttner. 2000. Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nat. Cell Biol.* **2:** 42–49.
5. Cruz, J. C., M. Thomas, E. Wong, N. Ohgami, S. Sugii, T. Curphey, C. C. Y. Chang, and T-Y. Chang. 2002. Synthesis and biochemical properties of a new photoactivatable cholesterol analog 7,7-azocholestanol and its linoleate ester in Chinese hamster ovary cell lines. *J. Lipid Res.* **43:** 1341–1347.
6. Simons, M., E-M. Krämer, C. Thiele, W. Stoffel, and J. Trotter. 2000. Assembly of myelin by association of proteolipid protein with cholesterol- and galactosylceramide-rich membrane domains. *J. Cell Biol.* **151:** 143–153.
7. Matyash, V., C. Geier, A. Henske, S. Mukherjee, D. Hirsh, C. Thiele, B. Grant, F. R. Maxfield, and T. V. Kurzchalia. 2001. Distribution and transport of cholesterol in *Caenorhabditis elegans*. *Mol. Biol. Cell.* **12:** 1725–1736.
8. Wang, N., D. L. Silver, C. Thiele, and A. R. Tall. 2001. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J. Biol. Chem.* **276:** 23742–23747.
9. Mintzer, E. A., B-L. Waarts, J. Wilschut, and R. Bittman. 2002. Behavior of a photoactivable analog of cholesterol, 6-photocholesterol, in model membranes. *FEBS Lett.* **510:** 181–184.
10. Prestwich, G. D., G. Dorman, J. T. Elliott, D. M. Marecak, and A. Chaudhary. 1997. Benzophenone photophores in biochemistry. *Photochem. Photobiol.* **65:** 222–234.
11. Nakatani, Y., M. Yamamoto, Y. Diyizou, W. Warnock, V. Dolle, W. Hahn, A. Milon, and G. Ourisson. 1996. Studies on the topography of biomembranes: regioselective photolabeling in vesicles with the tandem use of cholesterol and a photoactivable phospholipidic probe. *Chem. Eur. J.* **2:** 129–138.
12. Weber, P. J. A., and A. G. Beck-Sickinger. 1997. Comparison of the photochemical behavior of four different photoactivable probes. *J. Pept. Res.* **49:** 375–383.
13. Fielding, P. E., J. S. Russel, T. A. Spencer, H. Hakamata, K. Nagao, and C. J. Fielding. 2002. Sterol efflux to apolipoprotein A-I originates from caveolin-rich microdomains and potentiates PDGF-dependent protein kinase activity. *Biochemistry.* **41:** 4929–4937.
14. Johnson, W. J., F. H. Mahlberg, G. H. Rothblat, and M. C. Phillips. 1991. Cholesterol transport between cells and high-density lipoproteins. *Biochim. Biophys. Acta.* **1085:** 273–298.
15. Marecak, D. M., Y. Horiuchi, H. Arai, M. Shimonaga, Y. Maki, T. Koyama, K. Ogura, and G. D. Prestwich. 1997. Benzoylphenoxy analogs of isoprenoid diphosphates as photoactivatable substrates for bacterial prenyltransferases. *Bioorg. Med. Chem. Lett.* **7:** 1973–1978.
16. Mitsunobu, O. 1981. The use of diethyl azodicarboxylate and triphenylphosphine in synthesis and transformation of natural products. *Synthesis.* 1–28.
17. Partridge, J. J., S. Faber, and M. R. Uskokovic. 1974. Vitamin D3 metabolites I. Synthesis of 25-hydroxycholesterol. *Helv. Chim. Acta.* **57:** 764–771.
18. Kim, H-S., W. K. Wilson, D. H. Needleman, F. D. Pinkerton, D. K. Wilson, F. A. Quiocho, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chemical synthesis, structure, and biological activities of (25*R*)-3β-26-dihydroxy-5α-cholest-8(14)-en-15-one, a metabolite of 3β-hydroxy-5α-cholest-8(14)-en-15-one. *J. Lipid Res.* **30:** 247–261.
19. Takaku, H., K. Kamaike, and H. Tsuchiya. 1984. Synthesis of riboligonucleotides using the 4-methoxybenzyl group as a new protecting group for the 2'-hydroxyl group. *J. Org. Chem.* **49:** 51–56.
20. Rychnovsky, S. D., and D. E. Mickus. 1992. Synthesis of *ent*-cholesterol, the unnatural enantiomer. *J. Org. Chem.* **57:** 2732–2736.
21. Zhao, H., N. Neamati, Y. Pommier, and T. R. Burke, Jr. 1997. Design and synthesis of photoactivatable coumarin-containing HIV-1 integrase inhibitors. *Heterocycles.* **45:** 2277–2282.
22. Barton, D. H. R., and L. R. Morgan, Jr. 1962. Photochemical transformations. XII. Photolysis of azides. *J. Chem. Soc.* 622–631.
23. Jarreau, F. X., Q. Khung-Huu, and R. Goutarel. 1963. Steroid alkaloids. XIX. New method of synthesis of 3β-amino-Δ-5-steroids. *Bull. Soc. Chim. Fr.* **8–9:** 1861–1865.
24. Wolff, H. 1946. Schmidt reaction. *Org. React.* **III:** 307–336.
25. Barnett, J., B. E. Ryman, and F. Smith. 1946. Aminosteroids. II. Preparation of 3,7-diketocholestene. *J. Chem. Soc.* 526–528.
26. Bose, A. K., J. F. Kistner, and L. Farber. 1962. Convenient synthesis of axial amines. *J. Org. Chem.* **27:** 2925–2927.
27. Firestone, R. A., J. M. Pisano, J. R. Falck, M. M. McPhaul, and M. M. Krieger. 1984. Selective delivery of cytotoxic compounds to cells by the LDL pathway. *J. Med. Chem.* **27:** 1037–1043.
28. Cope, A. C., and E. Ciganek. 1963. *N,N*-Dimethylcyclohexylmethylamine. *Org. Synth. Coll. Vol.* **4:** 339–342.
29. Wertheim, E. 1933. Benzylbenzaldoxime. *J. Am. Chem. Soc.* **55:** 2540–2543.
30. Blakey, W., W. I. Jones, and H. A. Scarborough. 1927. Substitution products of 4-hydroxybenzophenone and of its methyl ether. *J. Chem. Soc. Abstracts.* 2865–2872.
31. Dorman, G., J. D. Olszewski, G. D. Prestwich, Y. Hong, and D. G. Ahern. 1995. Synthesis of highly tritiated 4-benzoyl-L-phenylalanine, a photoactivatable amino acid. *J. Org. Chem.* **60:** 2292–2297.
32. Brewer, H. B., Jr., R. Ronan, and C. Bishop. 1986. Isolation and characterization of apolipoproteins A-I, A-II, and A-IV. *Methods Enzymol.* **128:** 223–246.
33. Fielding, C. J. 1985. Lecithin-cholesterol acyltransferase and cholesterol transport. *Methods Enzymol.* **111:** 267–274.
34. Huuskonen, J., M. Vishnu, C. R. Pullinger, P. E. Fielding, and C. J. Fielding. 2004. Regulation of ATP-binding cassette transporter A1

transcription by thyroid hormone receptor. *Biochemistry.* **43:** 1626–1632.
35. Tomkinson, N. C. O., T. M. Willson, J. S. Russel, and T. A. Spencer. 1998. Efficient stereoselective synthesis of 24(*S*),25-epoxycholesterol. *J. Org. Chem.* **63:** 9919–9923.
36. Spencer, T. A., D. Li, J. S. Russel, N. C. O. Tomkinson, and T. M. Willson. 2000. Further studies on the synthesis of 24(*S*),25-epoxycholesterol. A new efficient synthesis of desmosterol. *J. Org. Chem.* **65:** 1919–1923.
37. Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan, and J. F. Oram. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
38. Huuskonen, J., M. Abedin, M. Vishnu, C. R. Pullinger, S. E. Baranzini, J. P. Kane, P. E. Fielding, and C. J. Fielding. 2003. Dynamic regulation of alternative ATP-binding cassette transporter A1 transcripts. *Biochem. Biophys. Res. Commun.* **306:** 463–468.
39. Yeagle, P. L. 1991. Modulation of membrane function by cholesterol. *Biochimie.* **73:** 1303–1310.
40. McConnell, H. M., and A. Radhakrishnan. 2003. Condensed complexes of cholesterol and phospholipids. *Biochim. Biophys. Acta.* **1610:** 159–173.
41. Cao, H., N. Tokutake, and S. L. Regen. 2003. Unraveling the mystery surrounding cholesterol's condensing effect. *J. Am. Chem. Soc.* **125:** 16182–16183.
42. Wang, J., Megha, and E. London. 2004. Relationship between sterol/steroid structure and participation in ordered lipid domains (lipid rafts): implications for lipid raft structure and function. *Biochemistry.* **43:** 1010–1018.
43. Friedland, N., H-L. Liou, P. Lobel, and A. M. Stock. 2003. Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. *Proc. Natl. Acad. Sci. USA.* **100:** 2512–2517.
44. Mathieu, A. P., P. Lavigne, and J-G. LeHoux. 2002. Molecular modeling and structure-based thermodynamic analyses of the StAR protein. *Endocr. Res.* **28:** 419–423.
45. Li, H., Z. Yao, B. Degenhardt, G. Teper, and V. Papadopoulos. 2001. Cholesterol binding and the cholesterol recognition/interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. *Proc. Natl. Acad. Sci. USA.* **98:** 1267–1272.
46. Li, H., and V. Papadopoulos. 1998. Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. *Endocrinology.* **139:** 4991–4997.
47. Fielding, C. J., A. Bist, and P. E. Fielding. 1998. Intracellular cholesterol transport in synchronized human skin fibroblasts. *In* Intracellular Cholesterol Trafficking. T-Y. Chang, and D. A. Freeman, editors. Kluwer Academic Publishing, Boston, MA. 273–288.
48. Forte, T. M., J. J. Bell-Quint, and F. Cheng. 1981. Lipoproteins of fetal and newborn calves and adult steer: a study of developmental changes. *Lipids.* **16:** 240–245.
49. Fielding, P. E., and C. J. Fielding. 1996. Intracellular transport of low density lipoprotein derived free cholesterol begins at clathrin coated-pits and terminates at cell surface caveolae. *Biochemistry.* **35:** 14932–14938.
50. Subtil, A., I. Gaidarov, K. Kobylarz, M. A. Lampson, J. H. Keen, and T. E. McGraw. 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. USA.* **96:** 6775–6780.
51. Gagescu, R., N. Demaurex, R. G. Parton, W. Hunziker, L. A. Huber, and J. Gruenberg. 2000. The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. *Mol. Biol. Cell.* **11:** 2775–2791.
52. Wilson, M. D., and L. L. Rudel. 1994. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *J. Lipid Res.* **35:** 943–955.
53. Salen, G., S. Shefer, L. Nguyen, G. C. Ness, G. S. Tint, and V. Shore. 1992. Sitosterolemia. *J. Lipid Res.* **33:** 945–955.
54. Lee, M-H., K. Lu, S. Hazard, H. Yu, S. Shulenin, H. Hidaka, H. Kojima, R. Allikmets, N. Sakuma, R. Pegoraro, A. K. Srivastava, G. Salen, M. Dean, and S. B. Patel. 2001. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat. Genet.* **27:** 79–83.
55. Lu, K., M. H. Lee, S. Hazard, A. Brooks-Wilson, H. Hidaka, H. Kojima, L. Ose, A. F. H. Stalenhoef, T. Mietennen, I. Bjorkhem, E. Bruckert, A. Pandya, H. B. Brewer, Jr., G. Salen, M. Dean, A. Srivastava, and S. B. Patel. 2001. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. *Am. J. Hum. Genet.* **69:** 278–290.
56. Schroeder, F., G. Nemecz, E. Gratton, Y. Barenholtz, and T. E. Thompson. 1988. Fluorescence properties of cholestatrienol in phosphatidylcholine bilayer vesicles. *Biophys. Chem.* **32:** 57–72.
57. Mukherjee, S., X. Zha, I. Tabas, and F. R. Maxfield. 1998. Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys. J.* **75:** 1915–1925.
58. Xu, X., R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, and E. London. 2001. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). *J. Biol. Chem.* **276:** 33540–33546.
59. Scheidt, H. A., P. Müller, A. Herrmann, and D. Huster. 2003. The potential of fluorescent and spin-labeled steroid analogs to mimic natural cholesterol. *J. Biol. Chem.* **278:** 45563–45569.
60. Wenz, J. J., and F. J. Barrantes. 2003. Steroid structural requirements for stabilizing or disrupting lipid domains. *Biochemistry.* **42:** 14267–14276.
61. Forslind, E., and R. Kjellander. 1975. Structure model for the lecithin-cholesterol-water membrane. *J. Theor. Biol.* **51:** 97–109.
62. Thurnhofer, H., and H. Hauser. 1990. Uptake of cholesterol by small intestinal brush border membrane is protein-mediated. *Biochemistry.* **29:** 2142–2148.
63. Zheng, Y-H., A. Plemenitas, C. J. Fielding, B. Peterlin, and B. Matija. 2003. Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions. *Proc. Natl. Acad. Sci. USA.* **100:** 8460–8465.