Functional Cholesteryl Binding Agents: Synthesis, Characterization, and Evaluation of Antibody Binding to Modified Phospholipid Vesicles

Timothy R. Carroll,† Alan Davison,*,† and Alun G. Jones‡

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Department of Radiology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115.
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A series of functionalized cholesteryl derivatives were synthesized. The various lipophilic protein modification agents were analyzed for their protein binding ability. The binding of human IgG, labeled with ^{125}I, to modified phospholipid vesicles was ascertained. Two agents performed well. These were cholest-5-en-3β-yl 5-carboxypentyl ether succinimido ester and cholest-5-en-3β-yl 6-carboxyhexyl ether succinimido ester.

There has been considerable interest in modifying liposomes by conjugation with sugars, proteins, etc., in an effort to utilize such liposomes for a variety of purposes. Much of this work has been directed toward inceasing the stability of liposomes in vivo,[1-4] specific site direction of such liposomes,[5-9] and their use as drug delivery systems.[10-11]

Compounds have been prepared in which modified sugars are attached to the cholesterol oxygen through a six-carbon side chain.[12] These thioglycosyl groups direct the liposomes formed with the cholesterol derivative in vivo. The sugars alter the surface characteristics so that leukocytes recognize the vesicles very readily.[8] Cholesterol derivatives are also useful because they have been shown to stabilize liposomes in vivo.[9]

This study details the synthesis of a range of possible lipophilic protein modification agents using this cholesterol derivative chemistry. Liposomes were prepared incorporating these cholesterol derivatives. Labeled human IgG was incubated with the liposomes, the binding was determined, and the cholesterol derivatives were then evaluated in terms of their binding efficiency.

Experimental Section

Proton NMR measurements were made on a Hitachi Perkin-Elmer R-24B or Bruker 250- or 270-MHz spectrometer with $CDCl_3$ as solvent and tetramethylsilane as the internal calibrant. Melting points were taken on a Mel-Temp apparatus and are uncorrected. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA.

The vesicles were made by use of a Branson Sonifier Model W200P equipped with a titanium microtip. The ^{125}I activity was counted on a Packard Auto-Gamma 500 counter. Centrifugations were carried out on either a tabletop Fisher Safety Centrifuge (400*g*) or a refrigerated International Centrifuge Model PR-2 (1000*g*).

Cholesterol was purchased from the J. T. Baker Co., Phillipsburg, NJ. Iodogen[13] was obtained from Pierce Chemical Co., Rockford, IL. Human IgG, Sepharose 4B, and Sephadex G-50-80 were obtained from the Sigma Chemical Co., St. Louis, MO. Columns were preconditioned with human IgG in the appropriate buffer to block any nonspecific binding sites. Distearoylphosphatidylcholine was obtained from Calbiochem, LaJolla, CA. ^{125}I as NaI was obtained from Amersham, Arlington Heights, IL.

Distilled water was passed through a Barnstead Ultrapure demineralizing cartridge and distilled by use of a Corning MegaPure still prior to use. The isotonic buffers (borate-buffered saline (BBS) at pH 8.0 and phosphate-buffered saline (PBS) at pH 6.7) were prepared fresh periodically from reagent grade chemicals. All other reagents were used as received except where noted.

All manipulations were carried out in laboratories licensed for the use of ^{125}I.

Cholest-5-en-3β-yl 5-Carboxypentyl Ether (IV). Cholest-5-en-3β-yl 6-hydroxyhexyl ether[12] (5.0 g, 10.3 mmol) was dissolved in acetone (250 mL). Jones reagent (prepared by literature methods[14]) was slowly dripped into the stirred alcohol solution until the yellow color persisted (4.9 mL, 96% of theoretical). The small excess of Jones reagent was quenched with 2-propanol (2 mL). The solid was filtered off, and the solution was concentrated under reduced pressure. The rest of the green chromium salts were filtered off, and the solvent was removed. The white solid was passed down a silica gel 60H column (eluant 5% MeOH/CH_2Cl_2) and the product recrystallized from hexane. The yield was 3.1 g (60%): mp 109–111 °C; ^1H NMR ($CDCl_3$) δ 2.1–3.6 (m, 5 H, H3, H1, and H6 of hexyl chain), 5.3 (m, 1 H, H6), 10.7 (br s, 1 H, acid H); $[\alpha]^{25}_D$ −27.8 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{33}H_{56}O_3$) C, H.

Cholest-5-en-3β-yl 6-[(*p*-Tolylsulfonyl)oxy]hexyl Ether (VI). Cholest-5-en-3β-yl 6-hydroxyhexyl ether[12] (22 g, 45.2 mmol) and *p*-toluenesulfonyl chloride (25 g, 131 mmol) were dissolved in pyridine (100 mL). The solution was stirred (4 h, 25 °C) under argon. The reaction mixture was poured into ice water (300 mL), forming a milky suspension, which was carefully extracted with small portions of CH_2Cl_2 (6 × 50 mL). The combined CH_2Cl_2 fractions were washed with water (2 × 100 mL) and dried over Na_2SO_4. The CH_2Cl_2 was removed, leaving a gum, which was crystallized from hot ethanol (100 mL) on standing (−5 °C, 18 h). The crude yield was 25 g (85%). The product was recrystallized from hexane: mp 63.0–64.0 °C; $[\alpha]^{25}_D$ −21.2 ± 0.2° (*c* 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.41 (s, 3 H, $PhCH_3$), 2.8–3.4 (m, 3 H, H3 and H1 of hexyl chain), 3.97 (t, 2 H, H6 of hexyl chain), 5.3 (m, 1 H, H6), 7.25 and 7.72 (d of d, 4 H, aromatic H). Anal. ($C_{40}H_{64}O_4S$) C, H, S.

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† Massachusetts Institute of Technology.
‡ Harvard Medical School and Brigham and Women's Hospital.

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Cholest-5-en-3β-yl 6-Nitrilohexyl Ether (VII). Cholest-5-en-3β-yl 6-[(*p*-tolylsulfonyl)oxy]hexyl ether (3.0 g, 4.7 mmol) was dissolved in dimethyl sulfoxide (125 mL). Sodium cyanide (2.26 g, 47 mmol) and sodium iodide (0.06 g, 0.47 mmol) were added, and the solution was stirred at 50 °C for 17 h. The solution was cooled, and ether (200 mL) and water (200 mL) were added. The aqueous layer was separated and extracted with ether (200 mL). The combined ether extracts were washed with water (200 mL) and dried over Na_2SO_4. The ether was then removed, and the product was crystallized from hexane, yielding 2.09 g (90.0%) of pure product: mp 89–90.5 °C; ^1H NMR ($CDCl_3$) δ 2.8–3.6 (m, 5 H, H1 and H6 of hexyl chain, H3), 5.3 (m, 1 H, H6); $[\alpha]^{25}_D$ −28.3 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{34}H_{57}ON$) C, H, N.

Cholest-5-en-3β-yl 6-Carboxyhexyl Ether (VIII). Cholest-5-en-3β-yl 6-nitrilohexyl ether (1.927 g, 3.89 mmol) was added to potassium hydroxide in ethanol (20% w/v, 50 mL). Water (3 mL) was added and the suspension refluxed under argon.[15] After 120 h, the solution was cooled and slowly added to aqueous HCl (4 M, 75 mL). The resulting white suspension was extracted with ether (2 × 50 mL). The combined ether fractions were washed with water (50 mL) and dried over Na_2SO_4.

The solvent was removed to leave a white solid. Chromatography on silica gel 60H (eluant 1% MeOH in CH_2Cl_2) yielded a pure product (0.80 g, 40%): mp 90–91 °C; ^1H NMR ($CDCl_3$) δ 3.4 (t, 2 H, H1 of hexyl chain), 5.3 (m, 1 H, H6), 9.7 (br, 1 H, acid proton); $[\alpha]^{25}_D$ −28.2 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{34}H_{58}O_3$) C, H.

Cholest-5-en-3β-yl 6-Mercaptohexyl Ether (XI). Cholest-5-en-3β-yl 6-iodohexyl ether[12] (5.0 g, 8.4 mmol) and thiourea (0.76 g, 10.0 mmol) were added to ethanol (250 mL) and refluxed for 8 h.[16] NaOH (1 M) in 50% ethanol/water v/v (25 mL) was added, and the cloudy solution was refluxed for 14 h. The solution was cooled, 1 M HCl (aqueous (25 mL) was added, and the solution was concentrated to 100 mL. Water (100 mL) and ether (100 mL) were added, and the ether layer was collected. The water layer was extracted with ether (100 mL). The combined ether fractions were washed with water (100 mL) and dried over Na_2SO_4. The solvent was removed and the product purified by column chromatography (silica gel 60H, 40 g, eluant CH_2Cl_2). The yield was 3.012 g (71.5%): mp 89–91 °C; ^1H NMR ($CDCl_3$) δ 2.8–3.6 (overlapping t and m, 4 H, H1 of hexyl chain, H3, thiol H), 5.3 (m, 1 H, H6); $[\alpha]^{25}_D$ −27.8 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{33}H_{58}OS$) C, H, S.

Cholest-5-en-3β-yl 6-[(*p*-Carboxybenzyl)thio]hexyl Ether (XII). Cholest-5-en-3β-yl 6-mercaptohexyl ether (500 mg, 0.99 mmol) was dissolved in benzene (50 mL), and α-bromo-*p*-toluylic acid (1.00 g, 4.6 mmol) was partially dissolved in benzene (200 mL), and 0.086 M NaOMe in methanol (65.6 mL, 5.6 mmol) was added as the other solutions were mixed. The cloudy solution was stirred at room temperatue for 45 min.

The solution was poured into water (250 mL) containing HCl (10 mmol). The benzene layer was separated, washed with water (250 mL), and dried over Na_2SO_4. The benzene was removed, and the compound was passed down a silica gel 60H column (20 g, eluant 1% MeOH/CH_2Cl_2). The pure fractions were isolated, and the impure fractions passed down another silica gel 60H column (10 g, eluant 1% MeOH/CH_2Cl_2). The pure fractions from both columns were combined to yield 0.327 g of product (51.6%): mp 146–148 °C; ^1H NMR ($CDCl_3$) δ 2.9–3.3 (m, 1 H, H3), 3.4 (t, 2 H, H1 of hexyl chain), 3.7 (s, 2 H, SCH_2Ph), 5.3 (m, 1 H, H6), 7.5 and 8.1 (d of d, 4 H, phenyl H); $[\alpha]^{25}_D$ −21.6 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{41}H_{64}O_3S$) C, H, S.

Cholest-5-en-3β-yl 6-[(*p*-Carboxybenzyl)thio]hexyl Ether, Succinimido Ester (XIII). Cholest-5-en-3β-yl 6-[(*p*-carboxybenzyl)thio]hexyl ether (150 mg, 0.24 mmol) and *N*-hydroxysuccinimide (30 mg, 0.26 mmol) were dissolved in CH_2Cl_2 (25 mL) and cooled to 0 °C. Dicyclohexylcarbodiimide (60 mg, 0.28 mmol) was dissolved in CH_2Cl_2 (20 mL) and added to the cooled solution. The resulting solution was allowed to stand at 0 °C for 2 h. The precipitate was filtered off and the solvent removed from the filtrate. The product was passed down a silica gel 60H column (3 g, eluant 1% MeOH/CH_2Cl_2). The yield of pure product was 0.167 g (96.6%): mp 102–105 °C; 270-MHz ^1H NMR ($CDCl_3$) δ 2.37 (t, 2 H, H6 of hexyl chain), 2.91 (s, 4 H, succinimide), 3.12 (m, 1 H, H3), 3.39 (t, 2 H, H1 of hexyl chain), 3.74 (s, 2 H, SCH_2Ph), 5.34 (m, 1 H, H6), 8.08 and 7.46 (d of d, 4 H, phenyl H); $[\alpha]^{25}_D$ −22.0 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{45}H_{67}O_5NS$) C, H, N, S.

Cholest-5-en-3β-yl 5-Carboxypentyl Ether, Succinimido Ester (V). This was prepared similarly to cholest-5-en-3β-yl 6-[(*p*-carboxybenzyl)thio]hexyl ether, succinimido ester (vide supra): mp 97–99 °C; 270-MHz ^1H NMR ($CDCl_3$) δ 2.62 (t, 2 H, H6 of hexyl chain), 2.84 (s, 4 H, succinimide H), 3.13 (m, 1 H, H3), 3.47 (t, 2 H, H1 of hexyl chain), 5.34 (m, 1 H, H6); $[\alpha]^{25}_D$ −24.2 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{37}H_{59}O_5N$) C, H, N.

Cholest-5-en-3β-yl 6-Carboxyhexyl Ether, Succinimido Ester (IX). This was prepared similarly to cholest-5-en-3β-yl 6-[(*p*-carboxybenzyl)thio]hexyl ether, succinimido ester: mp 116–119 °C; 250-MHz ^1H NMR ($CDCl_3$) δ 5.34 (m, 1 H, H6), 3.45 (t, 2 H, H1 of hexyl chain), 3.12 (m, 1 H, H3), 2.84 (s, 4 H, succinimide H), 2.61 (t, 2 H, H6 of hexyl chain); $[\alpha]^{25}_D$ −22.7 ± 0.2° (*c* 0.80, $CHCl_3$). Anal. ($C_{38}H_{61}O_5N$) C, H, N.

Cholest-5-en-3β-yl 6-Phthalimidohexyl Ether (XIV). The method of Landini and Rolla[17] for the preparation of alkylphthalimides was used in this preparation.

Cholest-5-en-3β-yl 6-[(*p*-tolylsulfonyl)oxy]hexyl ether (30 g, 46.8 mmol) was dissolved in toluene (100 mL). Potassium phthalimide (10.8 g, 58.3 mmol) was added but did not dissolve. Hexadecyltri-*n*-butylphosphonium bromide (3.0 g, 5.9 mmol) was added as a phase-transfer catalyst. The suspension was stirred and heated at 95 °C for 4 h. The suspension was cooled and filtered and the precipitate washed with ether (50 mL). The resulting solution was passed down a silica gel column (gel 60H, 50 g, eluant ether). The eluant was washed with 10% sodium hydroxide (100 mL) and water (100 mL) and dried over sodium sulfate. The ether was removed, leaving a gum, which was then crystallized from ethanol. The yield was 21.5 g (74.6%): mp 76–78 °C; ^1H NMR ($CDCl_3$) δ 2.8–3.3 (m, 1 H, H3), 3.4 and 3.55 (overlapping t, 4 H, H1 and H6 of hexyl chain), 5.3 (m, 1 H, H6), 7.67 (m, 4 H, aromatic H); $[\alpha]^{25}_D$ −22.7 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. $C_{41}H_{61}O_3N$) C, H, N.

Cholest-5-en-3β-yl 6-Aminohexyl Ether Hydrochloride (XV). Cholest-5-en-3β-yl 6-phthalimidohexyl ether (21.5 g, 34.9 mmol) was dissolved in hot ethanol (200 mL). The solution was heated to reflux and hydrazine hydrate (2.2 mL, 44.9 mmol) was added. The solution was refluxed under argon for 4 h.

Dichloromethane (200 mL) and sodium hydroxide (1.0 M (aqueous), 150 mL) were added to the cooled suspension. The dichloromethane layer was collected, and the aqueous phase was extracted with dichloromethane (100 mL). The combined dichloromethane layers were washed with water (2 × 100 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, ether was added to dissolve the gum, and an excess of HCl(g) was bubbled through the solution. The solvent was removed and the resulting gum crystallized from ether. The yield was 14.4 g (78.8%): mp 165 °C dec: $[\alpha]^{25}_D$ −24.4 ± 0.2° (*c* 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 2.8–3.6 (m, 5 H, H3, H1 and H6 of hexyl chain), 5.3 (m, 1 H, H6), 7.5 (m, 3 H, RNH_3^+). Anal. ($C_{33}H_{60}ONCl$) C, H, N, Cl.

Cholest-5-en-3β-yl 6-Isothiocyanatohexyl Ether (XVI). A modification of the Staab and Walther[18] synthesis for alkyl isothiocyanates was used in this preparation. Cholest-5-en-3β-yl 6-aminohexyl ether hydrochloride (4.0 g, 7.6 mmol) was added to ether (25 mL). Thiocarbonyldiimidazole (1.36 g, 7.6 mmol) and diisopropylethylamine (0.98 g, 7.6 mmol) were added. The reaction mixture was stirred (18 h, 25 °C). The solution was washed with water and dried over sodium sulfate and then evaporated to an orange oil (25 mmHg, 40 °C). Repeated silica gel (60H) column chromatography yielded a colorless oil. A small amount of the oil was taken up in acetone (3 mL) and water (3 mL) added. Scratching the vessel with a glass rod crystallized

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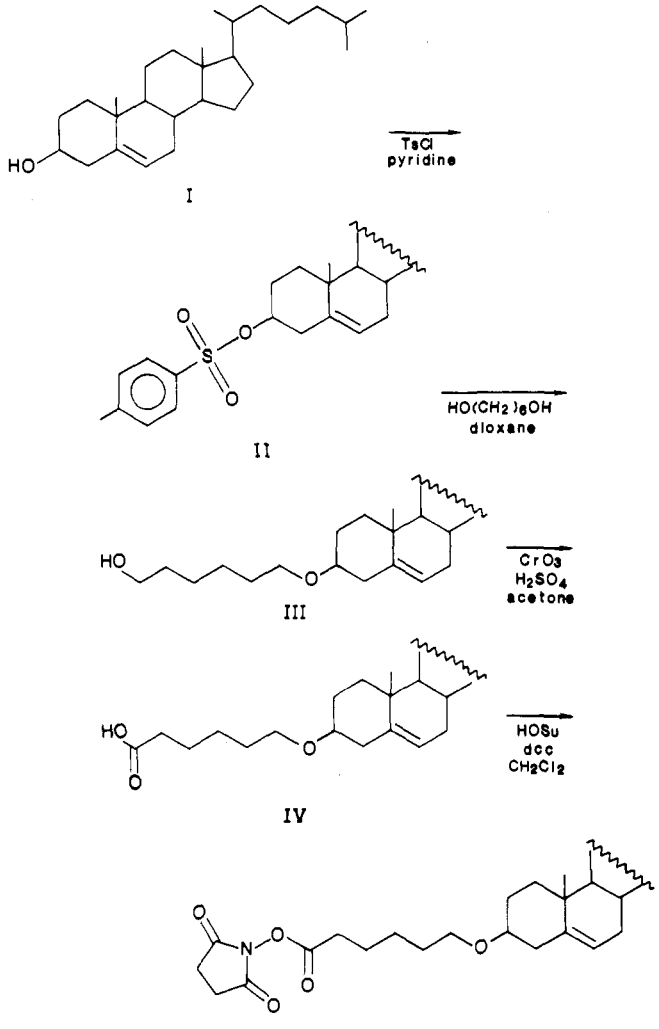
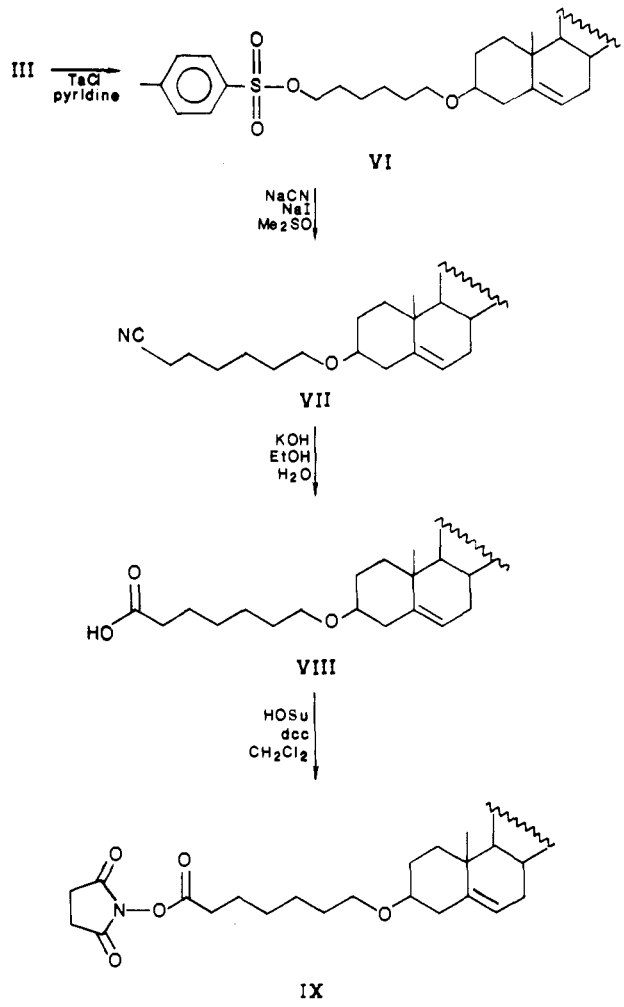
Scheme I. Synthesis of Cholesteryl 5-Carboxypentyl Ether, Succinimido Ester



Scheme II. Synthesis of Cholesteryl 6-Carboxyhexyl Ether, Succinimido Ester



the product. The resulting crystals were used as seed crystals for the entire reaction. The mixture precipitated on standing at 5 °C. The yield was 2.1 g (50%): mp 40–42 °C; 270-MHz ^{1}H NMR ($CDCl_3$) showed the expected pattern, δ 5.30 (br, 1 H, H6), 3.44 (d of t, 4 H, H1 and H6 of hexyl chain), 3.07 (m, 1 H, H3); $[\alpha]^{25}_D$ −26.4 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{34}H_{57}ONS$) C, H, N, S.

Cholest-5-en-3β-yl 6-(*m*-Maleimidobenzamido)hexyl Ether (XVII). Cholest-5-en-3β-yl 6-aminohexyl ether hydrochloride (1.0 g, 1.9 mmol), *m*-maleimidobenzoic acid succinimido ester[19] (0.8 g, 2.5 mmol), and diisopropylethylamine (0.3 mL, 2.2 mmol) were dissolved in 10% CH_2Cl_2/THF (50 mL). The yellow solution was stirred at room temperature for 1 h. The solvent was removed, and the resulting orange oil was passed down silica gel 60H (5 g, eluant ether). The yellow product was crystallized from acetone, crude yield 1.1 g (84%). The compound was recrystallized from acetone, crude yield 1.1 g (84%). The compound was recrystallized from acetone to yield a white product: mp 149–152 °C; 270-MHz ^{1}H NMR ($CDCl_3$) δ 3.12 (m, 1 H, H3), 3.43 (overlapping t, 4 H, H1 and H6 of hexyl chain), 5.34 (m, 1 H, H6), 6.43 (t, 1 H, amide), 6.85 (s, 2 H, maleimide), 7.78–7.44 (m, 4 H, phenyl H); $[\alpha]^{25}_D$ −20.6 ± 0.2° (*c* 1.00, $CHCl_3$). Anal. ($C_{44}H_{64}O_4N_2$) C, H, N.

Iodination Procedure for Proteins. Iodogen[13] (1 mg) was dissolved in CH_2Cl_2 (10 mL) and 100 μL of this solution placed in a small vial. The CH_2Cl_2 was evaporated under a stream of argon.

The iodogen tube was placed in an ice bath, and 100 μL of a 2.5-mg/mL solution of protein was added, followed by 10 μL of $Na^{125}I$, and the solution was mixed. The reaction was allowed to proceed for 10 min. The solution was removed from the vial and run down a preconditioned Sephadex G-50-80 column (1 × 15 cm, eluant BBS). Fractions were collected, and the first radioactive peak contained the protein. Unbound $^{125}I^-$ came out later and was discarded.

The iodinated protein was stored at 2 °C.

Preparation of Unilamellar Vesicles. The cholesterol derivative (2 mg) and distearoylphosphatidylcholine (DSPC) (16 mg) were combined and dissolved in $CHCl_3$ (1.50 mL). This was then distributed to three 3-mL Pyrex centrifuge tubes (0.50 mL each). The $CHCl_3$ was evaporated under a stream of argon while in a warm water bath. The tubes were dried further by storage under vacuum overnight.

An aliquot of borate-buffered saline (BBS) (0.75 mL) was added to each tube, and the solutions were sonicated at 100 W for 5 min. The tubes were cooled by immersion in glycerol during the sonication. The tubes were then incubated for 10 min at 60 °C to "anneal" the vesicles.[20] The tubes were centrifuged (400*g*) at room temperature for 30 min. The supernatant was then used for incubation.

Binding of Proteins to Vesicles. The supernatant solution from the vesicle preparation was placed in a 15- × 85-mm culture tube. To this was added the protin solution (0.50 mL), and the tube was agitated to mix the contents. The tube was incubated at 37 °C for 1 h. The resulting mixture was subjected to size-exclusion gel chromatography on a preconditioned Sepharose 4B column (1 × 15 cm, eluant phosphate-buffered saline). The vesicles appeared at the void volume of the column, the protein somewhat later. When the proteins were iodinated, the fractions were counted in a gamma counter to determine the binding ef-

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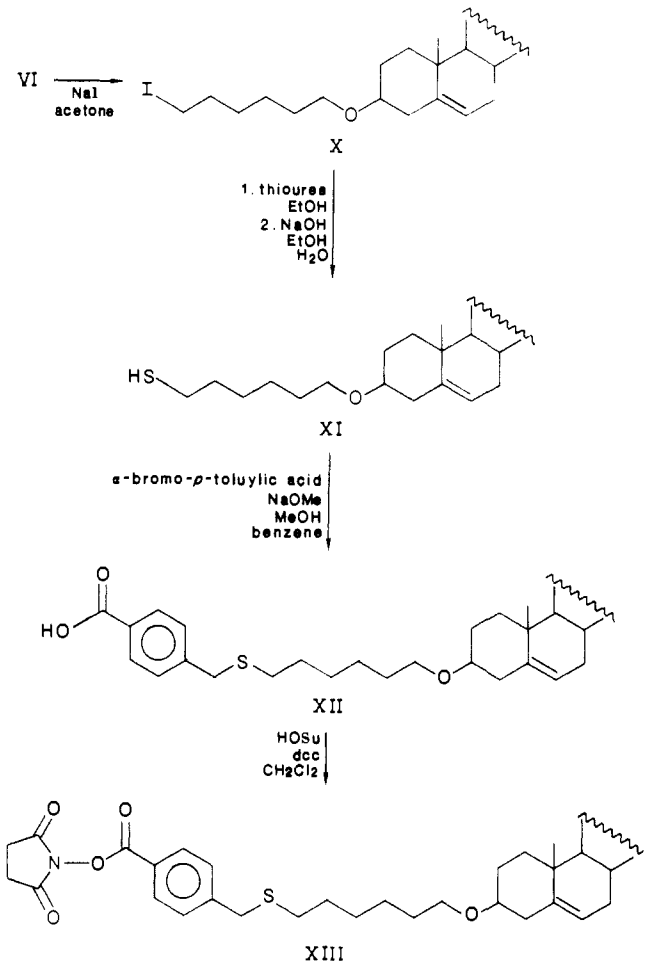
Scheme III. Synthesis of Cholesteryl 6-[(*p*-Carboxybenzyl)thio]hexyl Ether Succinimido Ester



ficiencies. Usually, 90% of the radioactivity would elute from the column.

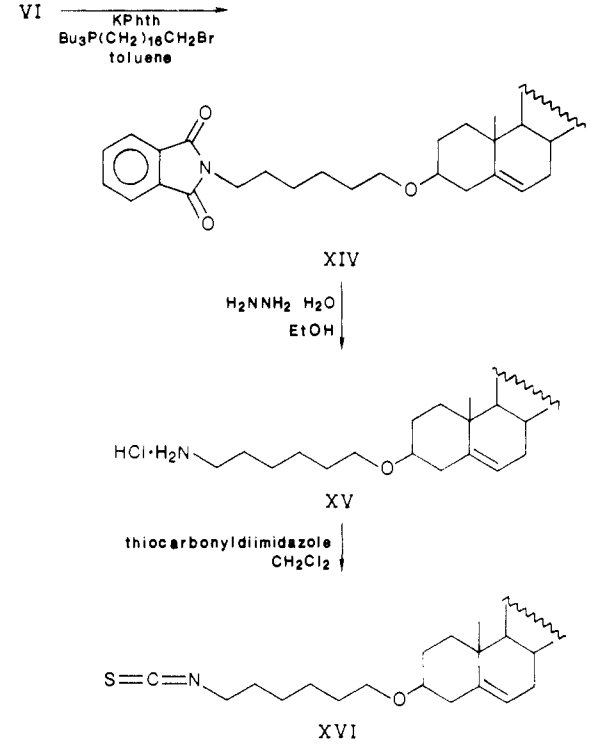
Chemical Results

The various syntheses were performed following the Schemes I–V. The 5-carboxypentyl cholesteryl ether succinimide (V, Scheme I) was prepared with cholesterol (I) as the starting material. The cholesterol was tosylated in pyridine to yield cholesteryl tosylate (II). This was reacted with 1,6-hexanediol to form the 6-hydroxyhexyl cholesteryl ether (III), which was oxidized to the carboxylic acid (IV) with use of Jones reagent. The final compound (V) was prepared as the *N*-hydroxysuccinimide ester by use of dicyclohexylcarbodiimide (dcc).

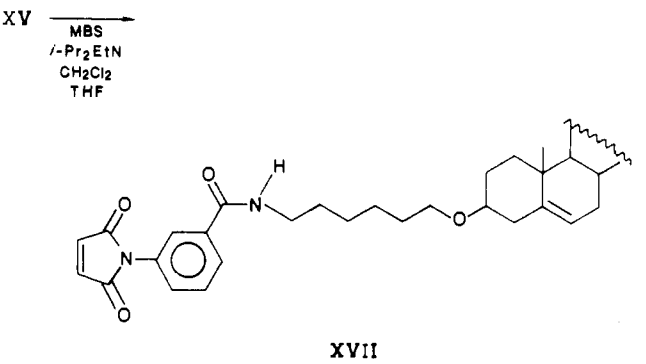
The closely related compound 6-carboxyhexyl cholesteryl ether succinimide (IX, Scheme II) was synthesized by a different route. The 6-tosylhexyl cholesteryl ether (VI) was prepared from 6-hydroxyhexyl cholesteryl ether (III) with *p*-toluenesulfonyl chloride in pyridine. The isolation of this compound was difficult and is discussed later. The tosyl compound (VI) was converted to the nitrile (VII) by use of sodium cyanide and a catalytic amount of sodium iodide. The nitrile was hydrolyzed completely in aqueous ethanolic base to the carboxylic acid (VIII). It was then converted to the succinimido ester (IX) by use of dcc as before.

The aromatic ester (XIII, Scheme III) was prepared by converting the tosylate (VI) to an iodohexyl cholesterol ether (X) by use of sodium iodide in acetone. The iodo complex was converted to the thiol via a two-step procedure involving thiourea as a nucleophile to form the thiuronium iodide, followed by attack by hydroxide ion to

Scheme IV. Synthesis of Cholesteryl 6-Isothiocyanatohexyl Ether



Scheme V. Synthesis of Cholesteryl 6-(*m*-Maleimidobenzamido)hexyl Ether



form urea, iodide, and the thiolate (XI). This reacted with methanolic sodium methoxide in benzene and α-bromo-*p*-toluylic acid to make the aryl acid thioether compound (XII). The succinimido ester (XIII) was synthesized in the standard manner by use of dcc.

The isothiocyanatohexyl cholesteryl ether (XVI, Scheme IV) was formed by conversion of the hexyl tosylate (VI) to the phthalimide (XIV) by use of potassium phthalimide and hexadecyltri-*n*-butylphosphonium bromide. The phosphonium salt is a phase-transfer catalyst that solubilized the phthalimide in the toluene solvent. The phthalimide compound (XIV) was hydrazinolyzed to form the amine, which was best handled as the hydrochloride salt (XV). The amine was then reacted with thiocarbonyldiimidazole (a thiophosgene replacement) to form the isothiocyanate (XVI).

Finally, the (*m*-maleimidobenzamido)hexyl cholesteryl ether (XVII, Scheme V) was formed by reaction of the amine (XV) with *m*-maleimidobenzoic acid succinimido ester. This succinimide is a heterobifunctional protein modification agent that binds amines on one end and thiols on the other.[19]

In the syntheses, common intermediates were used whenever possible. The final products all have an active

group that has an affinity for proteins. The only difference between XIII and XVII is one methylene group. This was an artifact of the synthetic procedure but made a rough determination of the sensitivity of protein binding to chain length possible.

Many of the compounds are liquid crystals; III and IV have large mesomorphic states, existing over a 15 °C temperature range. The other compounds have mesomorphic ranges from essentially 0 to 10 °C. The separation during the synthesis of IV is very sensitive, and great care must be taken to ensure that emulsions do not form. Once formed, they do not clear for many hours, even with traditional deemulsification techniques such as saturated brine, gentle agitation with a glass rod, or filtration. The pyridine and the soapy cholesterol derivative combine to form an easily emulsified solution. Methylene chloride must be simply poured through the solution for the first few portions. Then gentle swirling should be used. Not until the last portion can the mixture be shaken safely. The water wash that follows should be handled carefully as well; gentle swirling is all that can be safely used.

The final products all have relatively low melting points, which is an indication of their fatty liquid crystal properties. The characteristic proton NMR was used to determine the authenticity of the compounds prior to further analysis. Thin-layer chromatography was used extensively throughout the syntheses to determine the purity and identity of the various derivatives.

The compounds were essentially insoluble in polar solvents, and this created problems in synthesis. The succinimido esters are hydrolytically unstable, so they must be protected from moisture. All of the compounds will slowly oxidize in air, especially in the presence of ultraviolet light, so they are all stored under argon and protected from light in brown jars when applicable.

The compounds can be divided into three groups: the succinimido esters, the isothiocyanate, and the maleimide. Succinimido esters have been used in peptide synthesis and protein binding.[19,21] In aqueous solution and mild base (pH 8), the active esters react quickly and cleanly with amines to form an amide bond. A protein usually contains a large number of free amines, giving a wide range of coupling sites. The isothiocyanate group was originally used for N-terminal protein analysis.[22] Free amines react to form thiourea linkages in mild conditions (pH 8–9, 0 °C) Of course, not only the N-terminal amines will react but so will any other amine, giving the same versatility as the succinimido esters. The maleimide group reacts with thiols to form a thioether linkage. This reaction is very fast and selective.[19] Immunoglobulins are held together by disulfide bonds, a situation that tends to tie up the available thiol groups. However, there are some free thiols, and these will react.

Each protein modification agent forms a different linkage to the protein and has a different reactivity. The combination of NMR and IR spectra together with elemental analysis and optical rotation assures that the various compounds have been correctly synthesized and identified.

Biological Results

The protein binding efficiencies of the cholesteryl derivatives (V, IX, XIII, XVI, and XVII) were determined. Iodinated human IgG was used as the protein throughout in order to eliminate variations due to protein differences.

Table I. Human IgG Binding by 8:1 DSPC/Cholesterol Derivative Vesicles after 1-h Incubation in BBS at 37 °C

binding agent	1 mg/mL: binding, %	100 μg/mL	
		binding, %	stability, %
V	40.3	77.5	94.1
IX	46.0	69.5	91.0
XIII	43.3	60.4	91.8
XVI	31.9	55.9	86.5
XVII	39.5	61.1	86.0
III (nonspecific)	31.9	58.7	86.4

Typically, the protein (1 mg/mL and 100 μg/mL) was incubated with the liposomes containing a single protein modification agent for 1 h in BBS at 37 °C. The unbound protein was separated from the liposomes by gel exclusion chromatography, by use of freshly prepared, preconditioned Sepharose 4B columns. Human IgG showed nonspecific binding when incubated with vesicles that did not contain a protein agent. The vesicles were prepared with the cholesteryl derivative and DSPC in the same manner as Mauk and Gamble.[10] This method has been shown to reproducibly prepare small, unilamellar vesicles. The small size is important for biological applications because the vesicle–antibody complex must be able to pass through the capillary beds of the body.

The results are shown in Table I. A 10-fold difference in protein concentration made a significant variation in binding efficiency. This was probably due to a kinetic effect, as there were approximately equivalent numbers of vesicles and antibody molecules at the 100-μg/mL concentration, and there was on the order of 10 times the number of antibody molecules as vesicles at the 1-mg/mL antibody concentration. The protein modification agents, especially the succinimido esters, have a limited half-life in basic saline and will not have a chance to interact with as high a percentage of the antibody molecules at the higher concentration. The 24-h stability of the vesicle–antibody complex was generally good for all of the cholesterol derivatives (even for the nonspecific case). The greatest stability was found for the best binding agents. This may be due to the antibodies actually stabilizing the vesicles.

The results were ambiguous in the determination of which of the cholesterol derivatives was the best binding agent. Both cholesteryl 6-carboxyhexyl ether succinimido ester and cholesteryl 5-carboxypentyl ether succinimide ester performed well. The other agents did not bind protein so effectively, and the cholesteryl 6-isothiocyanatohexyl ether binding was no higher than nonspecific binding. It presumably was so lipophilic that the NCS group stayed within the phospholipid bilayer and never penetrated into the aqueous phase to interact with the protein.

The actual binding efficiencies varied from assay to assay, depending on the history of the human IgG. Different lots of antibody gave different results, but the ordering remained essentially constant. The succinimido esters always outperformed the other agents.

The high nonspecific binding seemed to be due to protein aggregates or otherwise denatured protein. When human IgG was preincubated with vesicles, it showed a lower nonspecific binding. The 32% nonspecific binding at 1 mg/mL dropped to approximately 12% with a single 1-h preincubation. Monoclonal antibodies showed low nonspecific binding, presumably due to the better handling and higher purity of the protein.[23] The human IgG was

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used as a model because of cost and availability factors.

The results in Table I clearly show that the vesicles incubated with the IgG at the 1-mg/mL concentrated level contain significantly greater quantities of bound protein than vesicles incubated with 100 μg/mL of protein. For example, with the derivative IX, the vesicles had 230 μg of bound protein at the higher concentration but only 35 μg at the lower concentration. Thus the method enables the labeling of ca. 6 mg of lipid vesicles with a significant quantity of antibody. This method can be extended 23 to the conjugation of monoclonal antibodies to vesicles. Such conjugates have potential utility as immunodirected therapeutic and diagnostic agents.

Registry No. III, 68354-84-7; IV, 103024-61-9; V, 103003-22-1; VI, 68354-85-8; VII, 103003-23-2; VIII, 103003-24-3; IX, 103003-25-4; X, 68354-86-9; XI, 73960-67-5; XII, 103003-26-5; XIII, 103003-27-6; XIV, 79360-09-1; XV, 103003-28-7; XVI, 103003-29-8; XVII, 103003-30-1; α-bromo-*p*-toluylic acid, 6232-88-8; *N*-hydroxysuccinimide, 6066-82-6; *m*-maleimidobenzoic acid succinimido ester, 58626-38-3.

Structure–Activity Studies of 16-Methoxy-16-methyl Prostaglandins

Umberto Guzzi,† Romeo Ciabatti,* Giovanna Padova, Franco Battaglia, Mario Cellentani, Adele Depaoli, Giulio Galliani, Pierfranco Schiatti, and Giuseppe Spina

Gruppo Lepetit S.p.A. Research Laboratories, Via Durando, 38-Milano, Italy. *Received November 13, 1985*

The synthesis of the pure diastereoisomer of 16-methoxy-16-methyl-$PGF_{2\alpha}$, -PGE_2, and -PGE_1 is described. The absolute configuration of C-16 was established by chemical methods, while the absolute C-15 configurations of the diastereoisomers were assigned tentatively on the basis of their chromatographic behavior and NMR spectra. The synthetic prostaglandin analogues were evaluated for antisecretory, antifertility, and diarrheogenic effects. Both the C-15 and C-16 configurations were found to be critical for the biological activities. These studies indicate that the introduction of the methyl and methoxy groups at C-16 into the prostaglandin analogues markedly increases the ratio of antisecretory to diarrheogenic action. One of the PGE_1 derivatives, **9f**(15α,16*R*) (MDL 646, mexiprostil), was selected for further pharmacological evaluation and is currently under clinical investigation.

In a previous paper[1] we reported the synthesis of 16-methoxy prostaglandins and that some of the 16-methoxy-PGE_2 analogues, in preliminary screening, were shown to have antisecretory effects in the Heidenhain gastric pouch test in dogs.[2] These compounds had weak activity after parenteral administration and no effect when administered orally. An additional methyl group was introduced on C-16 to make the molecule more resistant to metabolic oxidation of the C-15 hydroxyl group, following a strategy reported by many investigators (e.g., 16,16-dimethyl-PGs).[3] The synthesis and structure–activity relationship of 16-methoxy-16-methyl-$PGF_{2\alpha}$, -PGE_2, and -PGE_1 analogues are the subject of this paper.

Chemistry. Wittig reaction of the aldehydes **1a,b**[4] (Scheme I) with the racemic phosphonate **2a** gave the enones **4a–d**, and the diastereoisomers were separated by preparative layer chromatography.

The absolute C-16 configuration of enones **4a–d** was established by resolving the acid **10** with (*S*)-(+)-amphetamine into the (+) isomer **11a** and the partially resolved (−) isomer **11b** (Scheme II) and by comparing the circular dichroism (CD) curves of **11a** and **11b** with that of **3b**[5] [CD (c = 13.1 g L^{-1}, cyclohexane), θ_{226} +330; $[\alpha]_D$ +32.4° (c 1, $CHCl_3$)], a similar compound of known configuration *R* (Figure 1). The latter was prepared from resolved (*R*)-(−)-atrolactic acid[6,7] by treating it with NaH/CH_3I in THF and hydrogenation with 5% Rh/Al_2O_3 as catalyst according to Stocker[8] (Scheme III). From the *R*-(+) isomer **11a** the optically active phosphonate **2b** was prepared (Scheme II). Reaction of **2b** with **1a** and **1b** gave (Scheme I) **4a** and **4c**, thus establishing the configuration for **4a–d**. Reduction of **4a–d** with $NaBH_4$ gave the four pairs of the epimeric diols **5e,f**, **5g,h**, **6e,f**, and **6g,h**. The epimeric diols were separated by column chromatography, and in each case the less polar diols (TLC, Et_2O) (**5e,g**, **6e,g**) were obtained in greater amounts than the more polar compounds (**5f,h**, **6f,h**), the relative rates varying from 2:1 to 4:1, depending on the C-16 configurations and on the presence or absence of the double bond in the upper side chain.

Deacetylation of **5e–h** with K_2CO_3 in dry MeOH gave the related $PGF_{2\alpha}$ analogues **7e–h**. The PGE_2 and PGE_1 analogues **8e–h** and **9e–h** were produced by protecting **5e–h** and **6e–h** with dihydropyran (i), deacetylating with K_2CO_3 in dry MeOH (ii), oxidizing the C-9 hydroxy derivative with Collins reagent (iii), and acid hydrolysis of the tetrahydropyranyl ethers (iv).

The configurations of the C-15 isomers were assigned tentatively on the basis of their chromatographic behavior and NMR spectra, and both methods resulted in the same conclusions. With the first technique, the β(*S*) configurations were assigned to the less polar epimers **5e,g–9e,g**, and the α(*R*) to the more polar epimers, **5f,h–9f,h**, in analogy with the chromatographic behavior of the natural prostaglandins, following a rule accepted by many investigators.[9] With the second method, the chemical shift of

†Present address: Midy S.p.A., Research Center, Via Piranesi 38, 20137 Milan, Italy.

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