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THE PREPARATION OF 3-CHOLESTERYL 6-(GLYCOSYLTHIO)HEXYL ETHERS AND THEIR INCORPORATION INTO LIPOSOMES

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ABSTRACT

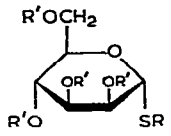
Four 3-cholesteryl 6-(glycosylthio)hexyl ether glycolipids were prepared and incorporated at a concentration of 15–18% into dipalmitoylphosphatidylcholine liposomes This incorporation suppressed the phospholipid phase transition in all cases With a D-mannose derivative, this effect was shown to be a regular function of increasing amounts of incorporated glycolipid Liposomes containing the D-mannosyl ether gave electron micrographs characteristic of liposome populations heterogeneous in size

INTRODUCTION

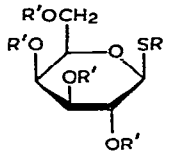
The use of liposomes as drug delivery vehicles has received increasing attention during the past few years, with reports of their application to the delivery of a variety of proteins[1–3], drugs[4–7], and ions[8] appearing regularly Particularly interesting are the observations that the *in vivo* tissue distribution of liposomes varies with the size and charge of the vesicle[9–11] Such behavior represents the first steps toward inducing tissue selectivity Since carbohydrates have been implicated in both cell-cell and cell-hormone interactions, we felt that the incorporation of synthetic glycolipid analogs into liposomes might provide the basis of a practical drug-delivery system Although gangliosides[12 13] and erythrocyte sialoglycoprotein[14] have been incorporated into liposomes no systematic study of the effects of external carbohydrate determinants on liposome distribution has appeared Such an investigation requires access to a variety of catabolically stable glycolipids that can be easily introduced into phospholipid vesicles As a beginning, we wish to report the synthesis of four such glycolipids (**1.4**, **7**, and **11**), their incorporation into dipalmitoyllecithin vesicles and some physical characteristics of such systems

RESULTS AND DISCUSSION

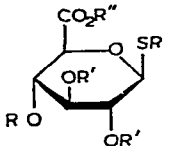
3-Cholesteryl 6-iodohexyl ether (**21**) was prepared, in three steps *via* **19** and **20**, in a 26% overall yield from cholesteryl *p*-toluenesulfonate (**18**) It was coupled with



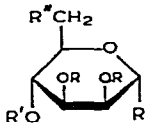
1 R = X, R′ = H
2 R = H, R′ = Ac
3 R = X, R′ = Ac



4 R = X, R′ = H
5 R = H, R′ = Ac
6 R = X R′ = Ac

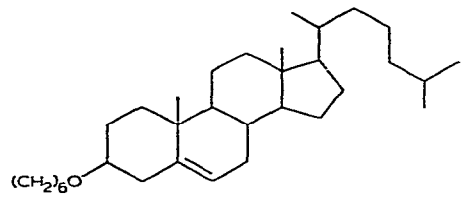


7 R = X, R′ = R″ = H
8 R = H R′ = Ac R″ = CH_3
9 R = $C(=NH_2^+)NH_2$ Br^-
R′ = Ac R″ = CH_3
10 R = X, R′ = Ac R″ = CH_3

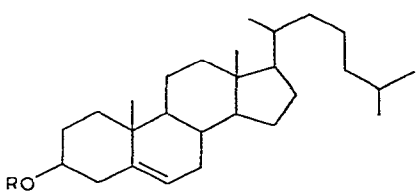


11 R = SX R′ = H R = NH_2
12 R = Br, R′ = Ac, R″ = OMs
13 R = SH R = Ac R″ = OMs
14 R = SX R = Ac, R″ = OMs
15 R = SX, R = Ac R″ = N_3
16 R = SX R = H, R″ = N_3
17 R = $SC(=NH_2^+)NH_2$ Br^-,
R′ = Ac, R″ = OMs

X =



the 1-thioaldoses **2**, **5**, and **8**, and the resulting ethers were deprotected to give the neutral and acidic glycolipids **1**, **4**, and **7** The preparation of aminodeoxyglycolipid **11** required a modification of this route, wherein the glycosyl bromide **12** was converted into the thiol **13**, which was condensed with iodide **21** The resulting methanesulfonate **14** was converted into the azide **15** by reaction with sodium azide Deprotection of **15** to give **16**, followed by reduction with hydrogen sulfide, provided the desired amine **11**



18 R = Ts
19 R = $HO(CH_2)_6$
20 R = $TsO(CH_2)_6$
21 R = $I(CH_2)_6$

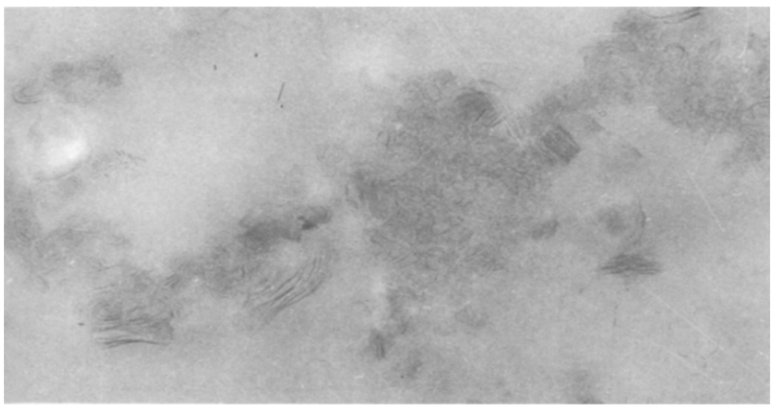


Fig 1 Electron micrograph of dipalmitoylphosphatidylcholine liposomes containing 13% (by wt) of glycolipid **1** Magnification 9500 ×

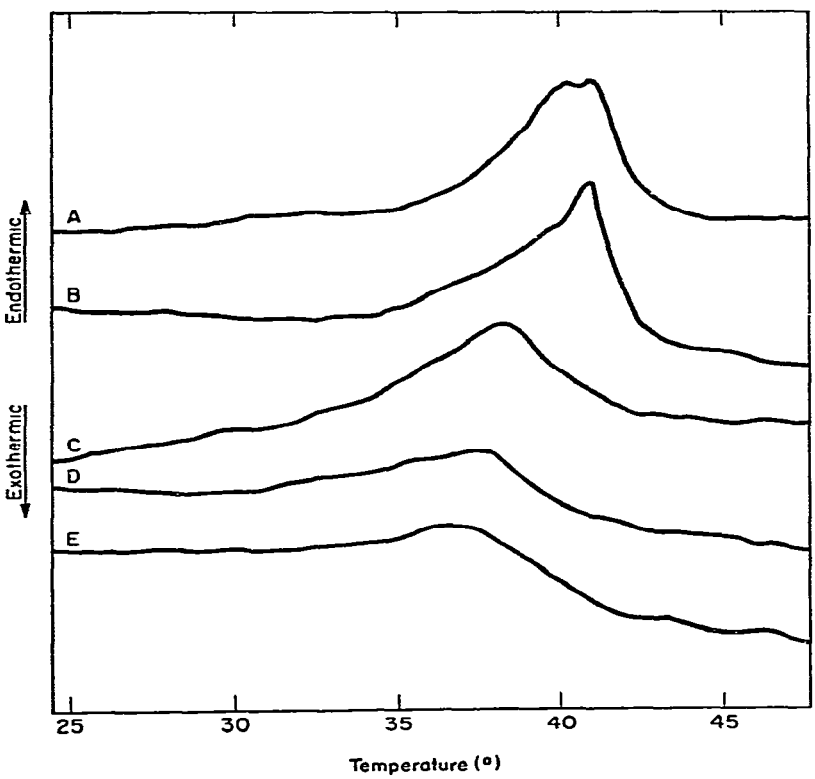


Fig 2 Effect of glycolipids **1**, **4**, **7**, and **11** on the dipalmitoylphosphatidylcholine (DPPC) phase-transition (A) Pure DPPC (9 4% by wt of total lipid), (B) 4 5% by wt of **1** 20 5% by wt of DPPC, (C) 3 8% by wt of **4**, 21 2% by wt of DPPC, (D) 1 3% by wt of **7**, 8 7% by wt of DPPC, and (E) 1 5% by wt of **11**, 8 5% by wt of DPPC

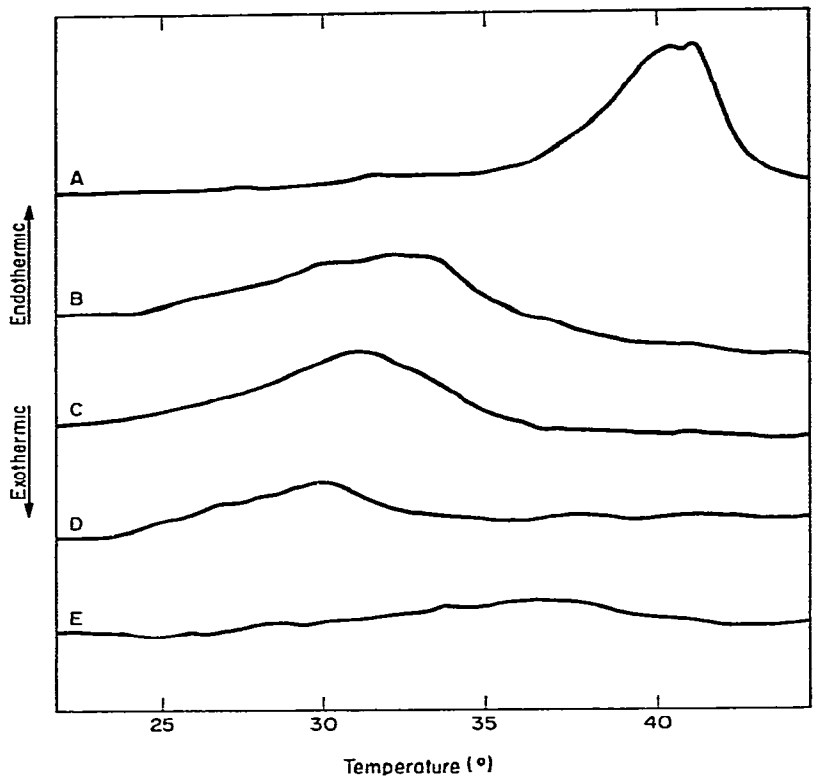


Fig 3 Effect of various amounts of **1** on the dipalmitoylphosphatidylcholine (DPPC) phase-transition (A) 0 0% of **1** 9 4% by wt of DPPC (0% of **1** in DPPC), (B) 0 19% by wt of **1**, 12 4% by wt of DPPC (1 5% of **1** in DPPC), (C) 0 31% by wt of **1**, 8 9% by wt of DPPC (3 4% of **1** in DPPC), (D) 0 63% by wt of **1** 8 6% by wt of DPPC (6 9% of **1** in DPPC), and (E) 0 89% by wt of **1** 8 3% by wt of DPPC (9 7% of **1** in DPPC)

Each glycolipid was incorporated at a concentration of 15–18% (by weight) into dipalmitoylphosphatidylcholine liposomes by probe sonication of lipid mixtures in aqueous 0 1M KCl and 10mM Tris buffer at pH 8 0 Electron-microscopic examination of DPPC liposomes containing 13% (wt) of mannolipid **1** (Fig 1) revealed the lamellar nature as well as size heterogeneity of the sample Phospholipid was required to effect solubilization of **1** in aqueous media for, although mixtures of **1** and DPPC could be rapidly sonicated to clarity, aqueous suspensions of **1** alone could not Low-speed (2000 r p m) centrifugation of sonicated **1** recovered the glycolipid quantitatively, while analysis of the supernate indicated that the concentration of **1** was 10–30nM If more than 18% (wt.) of glycolipid was added, the lipid mixtures could not be sonicated to clarity

The inclusion of **1**, **4**, **7**, and **11** in the lipid bilayer had a pronounced effect upon the phospholipid phase-transition (Fig 2) In each case, the transition was displaced to lower temperatures and suppressed, although the shapes of the curves prohibited quantification of the transition energy The effect of varying the amount of incor-

porated glycolipid is shown in Fig 3 As the percentage (by weight) of **1** was increased, the endothermic absorption shifted regularly to lower temperatures The energy of the transition shows a qualitative decrease, but the nonlinearity of the baseline again precluded quantification

Taken together, these results indicate that synthetic glycolipids can indeed become intercalated into phospholipid vesicle-membranes, and experiments to investigate the *in vivo* distribution of such liposomes are in progress

EXPERIMENTAL

General — Melting points were determined with a Thomas–Hoover melting point apparatus and are uncorrected I r spectra were recorded with a Perkin–Elmer 137 or 267 spectrophotometer on neat films or Nujol mulls P m r spectra were obtained with a Varian T-60 instrument with tetramethylsilane as an internal standard Optical rotations, mass spectra, and combustion analyses were provided by the physical measurements and microanalytical facilities of Merck Sharp & Dohme

Methods — Lipids were weighed into 0 5-ml Microflex tubes (obtained from Kontes, Vineland, N J 08360) and dissolved in 3 1 (v/v) $CHCl_3$–tetrahydrofuran Solvents were evaporated under a stream of N_2, and the residue kept for 20 min at 0 3 torr An aliquot of 0 1M KCl and 0 01M Tris buffer (pH 8 0) was added, and the mixture sonicated (until clear) at 35 W for 30–60 min, in 15-min pulses, with a Branson Model W185 sonifier equipped with a special microtip Samples were cooled with an ambient temperature water-bath Differential scanning calorimetry (d s c) was performed with a Perkin–Elmer DSC-1B Samples were weighed before calorimetry and after drying for 20 h *in vacuo* Weight of lipid was corrected for weight of solutes Analysis of sonicated **1** was performed with a modification of a published procedure[15] Compound **1** was sonicated for 1 h in 0 1M KCl and 0 01M Tris, and then centrifuged at 2000 r p m for 20 min The supernatant solution was treated with nitric acid and analyzed turbidimetrically as $BaSO_4$ at 620 nm with a spectrophotometer Correction was made for absorbance of reagents treated in the same manner

Materials — Dipalmitoylphosphatidylcholine was purchased from Calbiochem (La Jolla, CA 92032) and used without further purification Other materials were of reagent or equivalent grade and used as received

Cholest-5-en-3β-yl 6-hydroxyhexyl ether (**19**) — By use of the procedure of Kosower and Winstein[16], as described by Davis[17], cholesteryl *p*-toluenesulfonate (**18**) and 1,6-hexanediol were condensed in boiling *p*-dioxane to give **19** (52%) as colorless plates (from hexane), m p 75 9–81°, $[\alpha]_D^{25}$ −28 1±0 5° (*c* 1 03, chloroform) ν_{max}^{mull} 3500–3200 (OH), 1100 and 1080 (ether) cm^{-1}, n m r ($CDCl_3$) δ 2 8–3 3 (broad, 1 H, H-3), 3 3–3 75 (2 t, 4 H, H-1 and H-6 of the hexyl chain) and 5 4 (m, 1 H, H-6)

Anal Calc for $C_{33}H_{58}O_2$ C 81 42, H, 12 01 Found C, 81 74, H, 11 78

Cholest-5-en-3β-yl 6-iodohexyl ether (**21**) — Alcohol **19** (16 0 g 32 9 mmol) in dry benzene (650 ml) was treated with *p*-toluenesulfonic anhydride[18] (11 9 g,

36 3 mmol) and 2,4,6-trimethylpyridine (5 8 ml, 4 4 g, 36 mmol), and stirred for 1 h at room temperature with the exclusion of moisture The mixture was filtered through Florisil and concentrated to 16 0 g (76%) waxy cholest-5-en-3β-yl 6-(*p*-tolylsulfonyloxy)hexyl ether (**20**) homogeneous on t l c (silica gel, 17 3, v/v, benzene–ethyl acetate), ν_{max}^{mull} 1189 and 1175 (sulfonate), 1092 and 1087 (ether) cm^{-1}, n m r ($CDCl_3$) δ 2 43 (s, 3 H, PhCH_3), 2 7–3 3 (m, H-3, overlapping t, H-1 of the hexyl chain, 3 H), 3 90 (t. 2 H, H-6 of the hexyl chain), 5 3 (m, 1 H, H-6), 7 24 and 7 70 (d of d, 4 H, aromatic H) A solution of *p*-toluenesulfonate **20** (14 2 g, 22 2 mmol) and NaI (7 0 g, 47. mmol) in acetone (120 ml) was boiled under reflux for 4 h The solvent was removed under reduced pressure The residue was treated with ether (75 ml), filtered, and the collected salts washed well with ether The filtrate was evaporated and the residual yellow oil boiled with hexane (200 ml) The solution was decanted, concentrated to 100 ml, and refrigerated for 2 days, depositing white needles (9 85 g) A second crop afforded 2 90 g (total yield 12 75 g, 97%) m p 103 5–104 5°. $[\alpha]_D^{25}$ $-22\ 6\pm0\ 5°$ (*c* 1 02, $CHCl_3$) ν_{max}^{mull} 1105 (ether) cm^{-1}

Anal Calc for $C_{33}H_{57}IO$ C, 66 42, H 9 63 Found C, 66 32 H 9 55

1-Thioglycoses — 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-galactopyranose[19] (**5**) and 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranose[20] (**2**) were prepared by known procedures Methyl 2,3,4-tri-*O*-acetyl-1-thio-β-D-glucopyranuronate (**8**) was prepared from methyl (2 3 4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide)uronate[21] by known methods[19]. requiring the use of butanone for the preparation of the intermediate isothiouronium bromide (**9**), needles, m p 129 5–130°, $[\alpha]_D^{25}$ $-4\ 2\pm0\ 5°$ (*c* 1 0 chloroform) ν_{max}^{mull} 2550 (thiol) and 1740 (acetate) cm^{-1} n m r ($CDCl_3$) δ 2 03 (s, 6 H), and 2 10 (s 3 H. acetate CH_3) 2 33 (d *J* 10 Hz, 1 H SH) and 3 78 (s, 3 H. CO_2CH_3)

Anal Calc for $C_{13}H_{18}O_9S$ C 44 57 H, 5 18 S, 9 15 Found C, 44 41 H, 4 97. S 8 94

Preparation of protected glycolipids **3**, **6** *and* **10** — To a solution of 1-thioaldoses **2** **5** or **8** (1 equiv), in dichloromethane (20 ml per g thiol) was added **21** (1 equiv) and triethylamine (1 equiv) After being stirred under N_2 overnight at room temperature, the mixture was chromatographed on silica gel by use of a gradient elution with 5–25% of ethyl acetate in benzene

6-(Cholest-5-en-3β-yloxy)hexyl 2,3,4,6-tetra-O-acetyl-1-thio-α-D-mannopyranoside (**3**) — By use of the foregoing general procedure, thiol **2** (0 287 g, 0 788 mmol) and iodide **21** (0 470 g, 0 788 mmol) were condensed to provide **3** (0 361 g, 55%), m p 103–103 5°, $[\alpha]_D^{25}$ $+34\ 3\pm0\ 5°$ (*c* 1 00 chloroform), ν_{max}^{mull} 1730 (acetate) cm^{-1}

Anal Calc for $C_{47}H_{76}O_{10}S$ C, 67 75, H, 9 19, S, 3 85 Found C, 67 92, H, 9 19, S, 3 85

6-(Cholest-5-en-3β-yloxy)hexyl 2,3,4,6-tetra-O-acetyl-1-thio-α-D-galactopyranoside (**6**) — Thiol **5** (3 10 g, 8 51 mmol) and iodide **21** (4 15 g, 7 46 mmol) were condensed as just described to give **6** (5 78 g, 93%) as a wax, $[\alpha]_D^{25}$ $-23\ 0\pm0\ 5°$ (*c* 1 06, $CHCl_3$), ν_{max}^{mull} 1740 (acetate) cm^{-1}

Anal Calc for $C_{47}H_{76}O_{10}S$ C, 67 75, H, 9 19, S, 3 85 Found C, 67 89 H, 8 89, S, 3 77

Methyl [6-(cholest-5-en-3β-yloxyl)hexyl 2,3,4-tri-O-acetyl-1-thio-β-D-glucopyranosid]uronate (**10**) — Thiol **8** (0 102 g, 0 291 mmol) and iodide **21** (0 174 g, 0 291 mmol) were condensed as just described to give **10** (0 115 g, 48%) as needles (from 3 17, v/v, benzene–hexane), m p 144 5–145°, $[\alpha]_D^{25}$ −42 9±0 5° (*c* 1 01, $CHCl_3$), ν_{max}^{mull} 1735 (ester) cm^{-1}

Anal Calc for $C_{47}H_{74}O_{10}S$ C, 67 45, H, 9 11, S, 3 91 Found C, 67 68, H, 9 44, S, 4 20

Deblocking of glycolipids **3**, **6**, *and* **15** — A solution of glycolipid **3**, **6**, or **15** (1 equiv) in 1 1 (v/v) ethanol–tetrahydrofuran (33 ml per g of glycolipid) was treated with 2 5–3 fold excess of Bio-Rad AG 1-X2 OH^- ion-exchange resin (Bio-Rad Laboratories Richmond, CA 94804) suspended in ethanol (16 ml per g of glycolipid) and stirred for 45 min at room temperature The resin was filtered off and washed with warm tetrahydrofuran (3 × 16 ml per g of glycolipid), and the combined filtrates evaporated

6-(Cholest-5-en-3β-yloxyl)hexyl 1-thio-α-D-mannopyranoside (**1**) — Deblocking of **3** (2 54 g, 2 94 mmol) by the procedure just described gave **1** (1 77 g, 91%) as needles (from tetrahydrofuran) d s c endothermic transitions 64–65, 81–82, and 226–227° $[\alpha]_D^{25}$ +77 9±0 9° (*c* 1 11 tetrahydrofuran), ν_{max}^{mull} 3600–3100 (OH) cm^{-1}, m s 665 (M^-), 501, 368

Anal Calc for $C_{39}H_{68}O_6S$ C, 70 44, H, 10 31, S 4 82 Found C, 70 20, H 10 22 S, 4 80

6-(Cholest-5-en-3β-yloxyl)hexyl 1-thio-β-D-galactopyranoside (**4**) — Deblocking of **6** (3 00 g 3 60 mmol) by the procedure just described gave **4** (1 58 g, 66%) as an amorphous powder m p 104–106° (to liquid crystal) and 219–223° (to isotropic liquid) ν_{max}^{mull} 3600–3100 (OH) cm^{-1}

Anal Calc for $C_{39}H_{68}O_6S$ C, 70 44, H, 10 31 S 4 82 Found C, 70 16 H 10 03 S, 4 79

[6-(Cholest-5-en-3β-yloxyl)hexyl 1-thio-β-D-glucopyranosid]uronic acid (**7**) — A solution of esterified glycolipid **10** (30 2 mg, 29 1 μmol) in 1 1 (v/v) methanol–tetrahydrofuran (2 0 ml) containing water (20 μl) was treated with sodium methoxide (7 4 mg, 137 μmol) and stirred for 3 h at 25° The mixture was treated with 2 5M HCl (60 μl, 10% excess), evaporated, and the residue extracted with tetrahydrofuran Evaporation of tetrahydrofuran left 13 7 mg (52%) of white powder, m p 104–107° ν_{max}^{mull} 3600–2800 (RCO*OH*), 1745 and 1728 (R*C*OOH) cm^{-1}, field desorption m s 701 (M^+, Na^+ salt) A sample (1 mg) was per-*O*-trimethylsilylated with trifluoro *N*,*N*-bis(trimethylsilyl)acetamide at 25° in *N*,*N*-dimethylformamide and analyzed by m s 967 (M^-) 942, 874, 859, 501, 465 High resolution m s gave 501 4126 (calc for $C_{33}H_{57}OS^-$ 501 4126) and 465 1965 (calc for $C_{18}H_{41}O_6Si_4^+$ 465 1982)

Anal Calc for $C_{39}H_{66}O_7S$ C, 68 99, H 9 79, S, 4 72 Found C, 69 48, H, 9 59, S, 4 65

2,3,4-Tri-O-acetyl-6-O-methylsulfonyl-α-D-mannopyranosyl bromide (**12**) — An ice-cold solution of 1,2,3,4-tetra-*O*-acetyl-6-*O*-methylsulfonyl-β-D-mannopyranose[22,23] (14 9 g, 34 9 mmol) in dry dichloromethane (60 ml) was treated with 30–32% HBr in glacial acetic acid (21 ml), and kept for 2 5 h at 25° The mixture was poured onto stirred ice–water (400 ml), separated, and the aqueous phase washed with dichloromethane (3 × 20 ml) The combined organic layers were washed with water and saturated $NaHCO_3$, dried (Na_2SO_4), and evaporated The residue was triturated with petroleum ether (b p 30–60°), filtered off, and air dried to leave 14 6 g (93%) of **12**, m p 167 5–168 5° (dec), $[\alpha]_D^{25}$ +120 0±0 5° (*c* 1 01, $CHCl_3$), ν_{max}^{mull} 1740 and 1725 (acetate) cm^{-1}, n m r ($CDCl_3$) δ 2 01, 2 08, and 2 15 (3 s, 3 H each, acetate CH_3), 3 03 (s, 3 H, SO_2CH_3), and 6 22 (d, *J* 1 Hz, 1 H, H-1)

Anal Calc for $C_{13}H_{19}BrO_{10}S$ C, 34 91, H, 4 28, Br, 17 87, S, 7 17 Found C, 35 04, H, 4 18, Br, 17 61, S, 7 27

6-(Cholest-5-en-3β-yloxy)hexyl 2,3,4-tri-O-acetyl-6-azido-6-deoxy-1-thio-α-D-mannopyranoside (**15**) — Mannosyl bromide **12** was converted *via* the isothiouronium bromide **17** [85%, white powder, m p 87–90° (dec), ν_{max}^{mull} 3600–3100 (N–H), 1740 (acetate), and 1640 (isothiouronium) cm^{-1}] to 2,3,4-tri-*O*-acetyl-6-*O*-methylsulfonyl-1-thio-α-D-mannopyranose (**13**) [89%, colorless glass, ν_{max}^{mull} 2560 (thiol) and 1750–1730 (acetate) cm^{-1}] by use of a known method[19] Thiol **13** was coupled with **21** by the general procedure just described to give 61% of 6-(cholest-5-en-3β-yloxy)hexyl 2,3,4-tri-*O*-acetyl-6-*O*-methylsulfonyl-1-thio-α-D-mannopyranoside (**14**), a white glass, ν_{max}^{mull} 1740 (acetate) cm^{-1} Finally methanesulfonate **14** (1 14 g, 1 32 mmol), NaN_3 (0 49 g 7 5 mmol), and dry *N*,*N*-dimethylformamide (40 ml) were stirred for 4 5 h at 70–75° under N_2 After evaporation of the solvent the residue was dissolved in ether (20 ml) The solution was washed with three 10-ml portions of water, dried ($MgSO_4$), and evaporated to leave 1 05 g (97%) of **15** as a colorless glass, homogeneous on t l c (silica gel 3 22, v v ethyl acetate–benzene) $[\alpha]_D^{25}$ +12 6±0 5° (*c* 1 02, $CHCl_3$), ν_{max}^{mull} 2095 (azide) and 1755 (acetate) cm^{-1} A sample (0 54 g) was further purified by column chromatography (silica gel, 3 22, v/v, ethylacetate–benzene) to give 0 417 g (75% yield) of pure **15** as a colorless glass, which solidified on prolonged standing, m p 68–70°

Anal Calc for $C_{45}H_{73}N_3O_8S$ C, 66 22, H, 9 02 N, 5 15, S, 3 93 Found C, 66 53, H, 8 98, N 5 04, S, 3 98

6-(Cholest-5-en-3β-yloxy)hexyl 6-azido-6-deoxy-1-thio-α-D-mannopyranoside (**16**) — Compound **15** was deblocked by the resin procedure just described to give **16** (62%) as a glass, ν_{max}^{mull} 3600–3100 (OH) and 2095 (azide) cm^{-1}

Anal Calc for $C_{39}H_{67}N_3O_5S$ C, 67 88, H, 9 79, S, 4 65 Found C, 67 80, H, 9 55, S, 4 54

6-(Cholest-5-en-3β-yloxy)hexyl 6-amino-6-deoxy-1-thio-α-D-mannopyranoside (**11**) — A solution of **16** (0 163 g, 0 236 mmol) in chloroform (4 0 ml) containing triethylamine (3 0 ml) was treated with dry gaseous H_2S for 4 5 h at room temperature The volatile components were removed by rotary evaporation and the product was isolated by preparative t l c (silica gel, 7 2 1, v/v, $CHCl_3$–CH_3OH–conc aqueous

NH_3) to yield 65.0 mg (41%) of white powder, m.p. indef., ν_{max}^{mull} 3650 (NH_2) and 3600–3100 (OH) cm^{-1}, m/s: 635 ($M^+ - CH_2{=}NH^+$), 503, 470, 386, 368, 353. A sample (1 mg) was per-*O*-trimethylsilylated with trifluoro-*N*,*N*-bis(trimethylsilyl)-acetamide in *N*,*N*-dimethylformamide for 15 min at 65–70°, m/s: 1024 (M^+), 1009, 951 ($M^+ - Me_3Si$), 936, 921, 850 [$M^+ - N(SiMe_3)_2$], 523, 501, 368, 174 (base peak). A high resolution m/s of the per-*O*-trimethylsilylated saccharide fragment gave 522.2721 (calc. for $C_{21}H_{52}NO_4Si^+$ 522.2744).

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