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The Design and Synthesis of Simple Molecular Tethers for Binding Biomembranes to a Gold Surface

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Abstract: Molecular tethers have been synthesised for fixing biomembranes to a gold surface. These are comprised of a thiol at one end to bind to the gold, a polyethylenoxy chain of defined length (two, six or twelve ethylenoxy units) in the middle and a cholesteryl residue at the other end to insert into the biomembrane. © 1997 Elsevier Science Ltd.

INTRODUCTION

The technology for tethering biomembranes to solid supports is important in a variety of areas from fundamental studies of bilayer structure and function to the creation of biosensors.[1] A number of approaches to the production of such tethered membranes have been reported. These have relied on covalently linking one of the phospholipid components of the membrane to the surface of the solid,[2,3] We have developed a simpler approach to the problem which tethers the membrane through another major biomembrane component, cholesterol.[4] Figure 1 shows a schematic representation of our method. A mixed self-assembled monolayer (SAM) is formed on a gold surface. This is made up of long chain 'tether' molecules, each of which is terminated with a cholesteryl moiety, and shorter chain polyethylenoxythiols which act as 'spacers'. When this SAM is exposed to an aqueous solution of unilamellar phospholipid vesicles the cholesteryl residues 'anchor' themselves to the biomembrane and the vesicles unroll to form a bilayer which covers the surface.

The mixed nature of the SAM and its composition in terms of the proportions of 'spacer' and 'tether' molecules is critical to allow the undulations present in the fluid lipid bilayers to be accommodated adjacent to the solid surface[5] and this fluidity is in turn essential for the insertion of biomolecules (peptides, enzymes, ion-channels *etc.*) required to impart useful functionality to the bilayer. The present paper is concerned with the design and synthesis of the tethers. The physical characterisation and application of the surface-bound bilayers is published elsewhere.[4]

The choice of thiol linked to gold as the basis for the SAM was made to produce a system that could be addressed electrochemically. Cholesterol rather than phospholipid anchors were chosen to provide tethers that were physically and chemically robust and to simplify their synthesis. Polyethylenoxy chain was chosen as the simplest aqueous mimetic/hydrophilic chains to link the thiol and cholesterol together. In addition we expected the membrane-compatible steroid to modulate the properties of some anchored bilayers favourably by controlling their fluidity and decreasing their permeability to electrolytes. Clearly the length of the polyethylenoxy chain dictates the maximum space/distance between the bound membrane and the solid surface. Since some of the proteins that we wanted to insert into the membrane project a considerable distance into the aqueous phase this presents a synthetic problem in making *long monodisperse* polyethylenoxy chains (up to twelve ethylenoxys). The other significant synthetic problem was that of choosing a suitable protecting group for the thiol residue.

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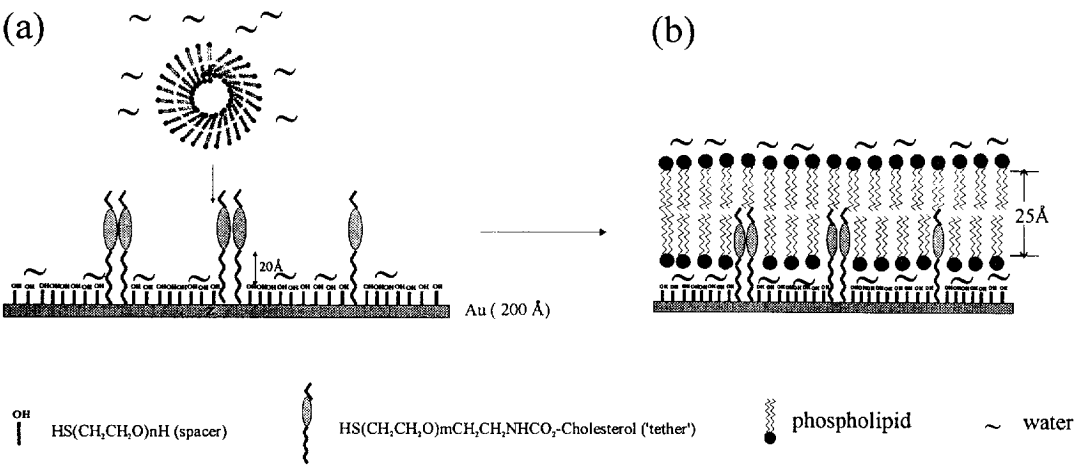
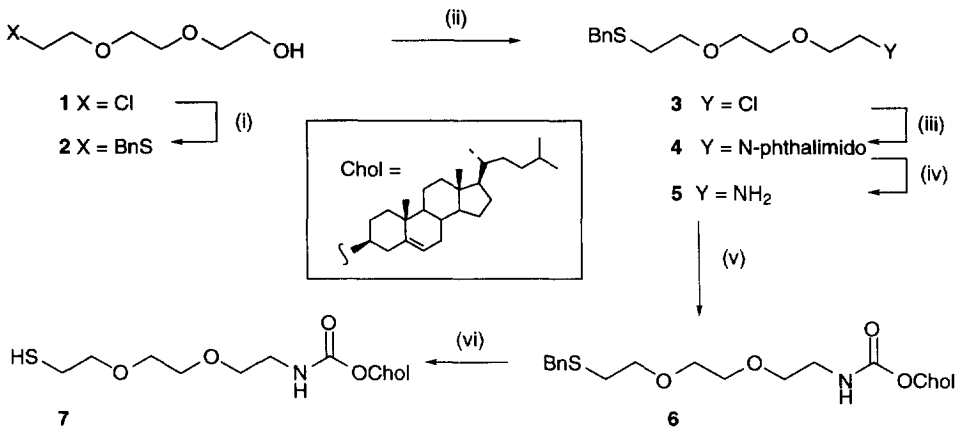


Scheme showing the tethering of a biomembrane to a gold surface: (a) A mixed SAM containing the cholesteryl terminated tether molecules is formed on a gold surface and is exposed to an aqueous solution of unilamellar vesicles; (b) The cholesteryl residues insert into the bilayer and the anchored biomembrane is formed. The cholesteryl residues are shown in a position and orientation similar to that adopted by cholesterol itself.

Figure 1

RESULTS AND DISCUSSION

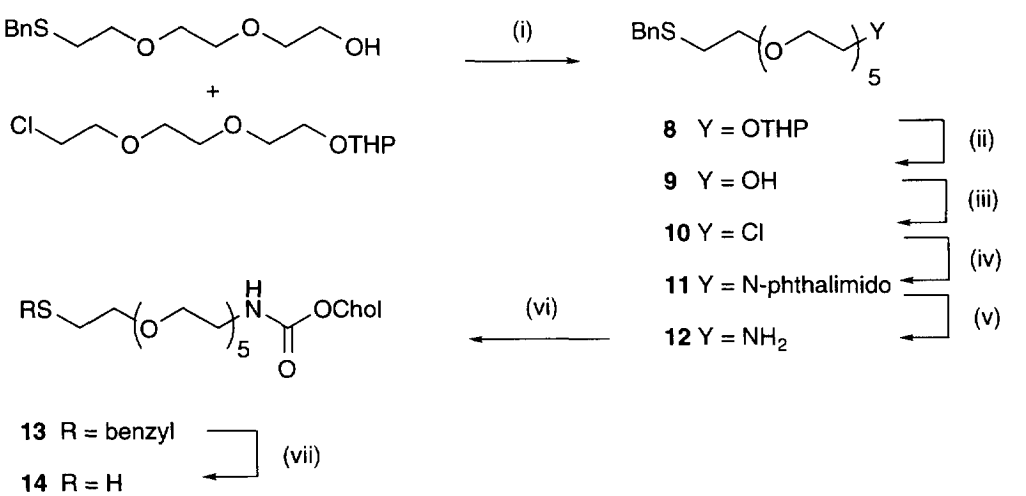
Previous syntheses of this type of molecule have used a disulfide as the thiol protecting group.[3] This was unsatisfactory for our purposes since it is incompatible with a variety of nucleophilic reagents we needed to use in the synthesis. Therefore, we chose the more robust benzyl thioether protecting group which could be readily removed at the end of the sequence using sodium in liquid ammonia reduction.[6] The synthetic route to tether molecule **7** is depicted in Scheme 1. Amino-terminated polyethylene oxides such as compound **5** have been prepared by other methods,[7] although to the best of our knowledge not with an ω-benzylthioether or thiol moiety. We were unable to obtain satisfactory microanalytical data for this compound, despite repeated purification. Complete characterisation was obtained for subsequent compounds however, indicating the veracity of the structural assignment. Coupling to cholesteryl chloroformate[8] and subsequent deprotection of compound **6** proceeded in good yield to give the thiol **7**.



Reagents and Conditions: (i) BnSH, EtONa, EtOH (78%); (ii) $SOCl_2$, pyridine (70%); (iii) potassium phthalimide, DMF, 100 °C (55%); (iv) NH_2NH_2, EtOH, reflux (85%); (v) cholesteryl chloroformate, Et_3N, CH_2Cl_2 (77%); (vi) Na-NH_3 (90%).

Scheme 1

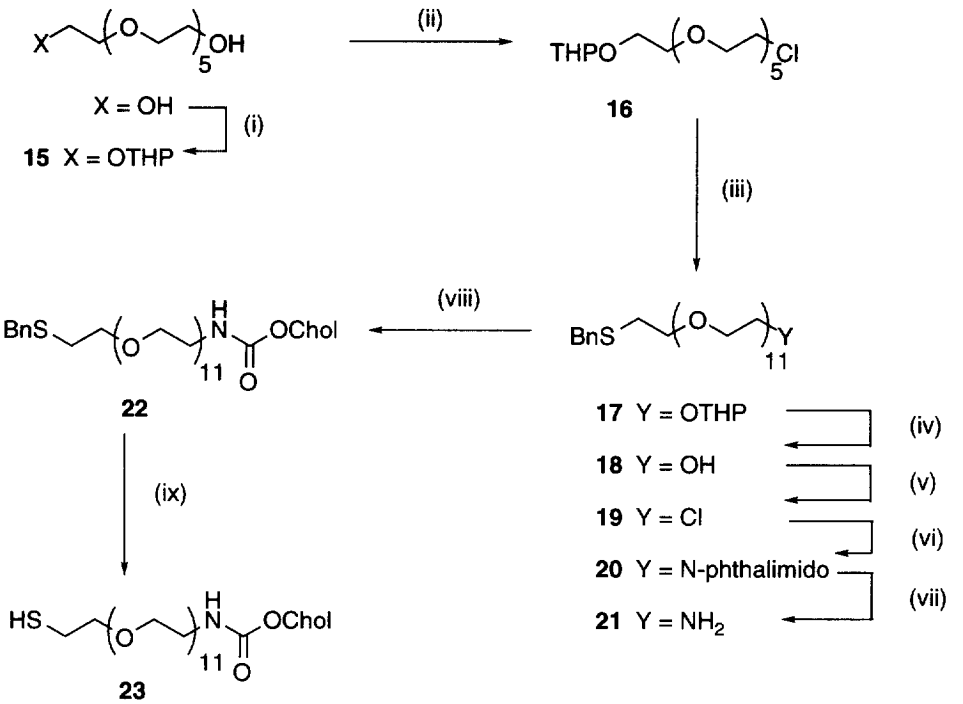
The 'EO6' homologue **14** was prepared by a similar route, beginning with a phase transfer catalysed coupling[9] of two 'EO3' subunits.[10] Conversion of the resulting tetrahydropyranyl ether **8** to the target cholesteryl carbamate **14** was achieved by a sequence similar to that for compound **7** (Scheme 2).



Reagents and Conditions: (i) KOH (50% aq. w/w), Bu_4NHSO_4, CH_2Cl_2; (ii) 10-camphorsulfonic acid, MeOH (59% over two steps); (iii) $SOCl_2$, pyridine (54%); (iv) potassium phthalimide, DMF, 100 °C (77%); (v) NH_2NH_2, EtOH, reflux (76%); (vi) cholesteryl chloroformate, Et_3N, CH_2Cl_2 (47%); (vii) Na-NH_3 (41%)

Scheme 2

The synthesis of the 'EO12' homologue **23** is outlined in Scheme 3. The ^1H NMR spectra of the thiol tethers were characterised by a quartet at 2.65δ corresponding to the -CH_2SH group. When left in the air they slowly oxidise to the disulfide, characterised by a triplet at lower field (2.80δ).

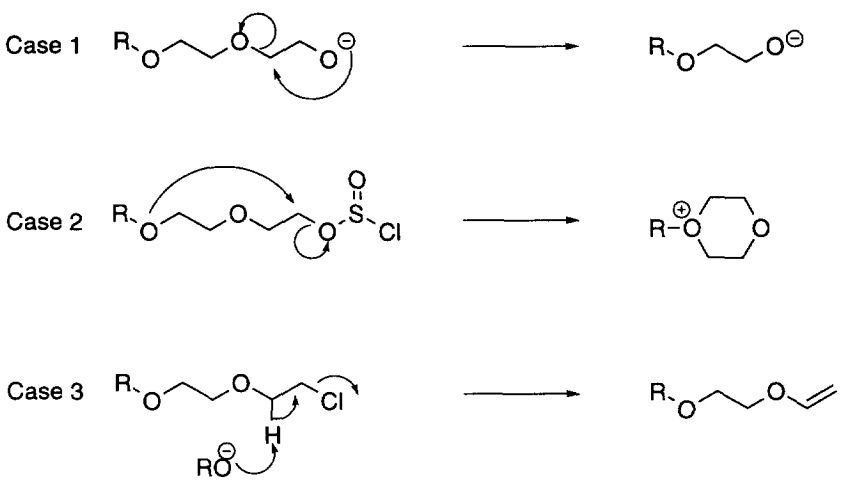


Reagents and Conditions: (i) 2,3-dihydropyran, HCl (34%); (ii) $SOCl_2$, pyridine (33%); (iii) NaOH (50% aq. w/w), compound **9** (51%); (iv) 10-camphorsulfonic acid, MeOH (86%); (v) $SOCl_2$, pyridine (50%); (vi) potassium phthalimide, DMF, 100 °C (51%); (vii) NH_2NH_2, EtOH, 50 °C (98%); (viii) cholesteryl chloroformate, Et_3N, CH_2Cl_2 (61%); (ix) Na-NH_3 (68%).

Scheme 3

Whereas the synthesis of compound **7** is based on a simple coupling of commercially available precursors, that of compound **23** involves a doubling up of two 'EO3' chains to form an 'EO6' chain, and then a doubling up of two 'EO6' chains to form the 'EO12' chain. In principle this sort of progression is simple but, in practice, great care needs to be taken. In this, and related studies[11,12] we have found that many depolymerisation processes can thwart the synthesis of monodisperse 'EOn' derivatives. In the past, supposedly monodisperse polyethylenoxy derivatives have often been made by a simple Williamson ether synthesis using the sodium salt of the alcohol [say $CH_3(OCH_2CH_2)_nOH$ or $H(OCH_2CH_2)_mONa$] and the corresponding chloride [say $CH_3(OCH_2CH_2)_nCl$ or $ClCH_2CH_2(OCH_2CH_2)_nCl$].[13] In fact the rather forcing conditions required for this reaction mean that there is almost always some base-catalysed depolymerisation (Scheme 4: case 1). Hence the reaction of $H(OCH_2CH_2)_3ONa$ with $CH_3(OCH_2CH_2)_3Cl$ requires 100°C for about 3 days and the EO6 product is contaminated with *ca.* 15% 'EO5' as well as smaller amounts of 'EO4' materials,[12] a mixture which is all but impossible to separate by fractional distillation. Another well-known depolymerisation of EOn chains involves the elimination of 'EO2' (dioxan) units in, for example, thionyl chloride chlorination of polyethylenoxy alcohols, (Scheme 4: case 2) a reaction which is suppressed in the presence of pyridine. Yet another type of depolymerisation which has caused problems is the elimination reaction illustrated as case 3 in Scheme 4. Depending on the workup procedure this leads either to the vinyl ether or the product missing one 'EO' unit.[11] Given these many ways in which the chains can depolymerise it is necessary to monitor the products by methods

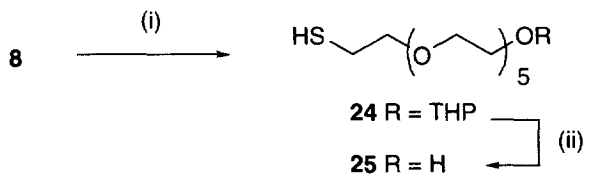
such as GLC and MS which are better suited to reveal minor contaminants of this type than, for example elemental analysis or NMR spectroscopy.



Common mechanisms of depolymerisation of polyethylenoxy derivatives (see text).

Scheme 4

The short chain 'spacer' in the mixed SAM's shown in Figure 1 was often the hydroxythiol **25.** This was prepared by a route which allowed for the unavoidable difficulties of isolation of such water-soluble materials (Scheme 5). The benzyl protection was removed first by treatment with sodium-liquid ammonia. The tetrahydropyranyl group could then be cleaved without recourse to aqueous workup by the use of methanol and 10-camphorsulfonic acid. Purification by flash column chromatography gave hydroxythiol **25** directly.



Reagents and Conditions: (i) Na-NH_3 (77%); (ii) MeOH, 10-camphorsulfonic acid (51%).

Scheme 5

CONCLUSIONS

We have developed relatively simple syntheses of novel tether molecules for inclusion in mixed SAMs and which can be used to bind biomembranes to a gold surface.[4] Such supported bilayers, that incorporate functional biomolecules, have many potential applications.

ACKNOWLEDGEMENTS

We thank Dr Heiko Wolf (University of Mainz) for open discussions and the EPSRC for funding.

EXPERIMENTAL

Materials and general methods

2-(2-(2-Chloroethoxy)ethoxy)ethanol, **1** and benzyl mercaptan were purchased from Aldrich Chemical Co., and cholesteryl chloroformate from Lancaster Synthesis. Reagents were used as received, but where appropriate solvents were purified before use according to standard procedures.[14] Thin-layer chromatography (TLC) was carried out on precoated plates (silica gel 60 F 254, Merck 5715) and the products were visualised using UV light (254 nm), iodine and either ammonium molybdate or ninhydrin dip as appropriate. Column chromatography on silica refers to the use of Merck silica gel 109385. Samples for combustion analysis were routinely dried at *ca.* 0.5mmHg but, as is common with polyethyenoxy derivatives, some compounds analysed as the corresponding hydrates. Routine ^{1}H-NMR spectra were measured in $CDCl_3$ using a General Electric 300 MHz instrument with additional experiments performed using a Bruker 400 MHz machine. Chemical shifts are relative to tetramethylsilane, coupling constants are given in Hz. Mass spectra were obtained on a VG Autospec instrument.

8-Benzylthio-3,6-dioxaoctanol 2

To an ice-cooled flask of ethanol (100 ml) fitted with a condenser was added sodium (4.4 g, 192 mmol, 1.1 eq.) in small pieces. To this flask was added benzyl mercaptan (20.4 ml, 174 mmol) *via* a syringe, followed by a solution of 2-(2-(2-chloroethoxy)ethoxy)ethanol **1** (29.4 g, 174 mmol) in ethanol (50 ml). The reaction was heated at reflux overnight then cooled and poured into saturated ammonium chloride solution (200 ml) and extracted with chloroform (3 x 200 ml). The combined organic phases were dried with magnesium sulfate, filtered and evaporated under reduced pressure to give an oil which was purified by flash chromatography on silica eluting with chloroform to 10% methanol : chloroform (gradient) to give the *title compound*, **2** as a colourless oil (31.94 g, 135 mmol, 78%) (Found: C, 60.9; H, 7.9. $C_{13}H_{20}O_3S$ requires C, 60.6; H, 8.0%); δ_H (300 MHz) 7.40 - 7.20 (5H, m, ArH), 3.80 (2H, s, benzyl CH_2), 3.76 - 3.60 (10H, m, CH_2O), 2.63 (2H, t, J 7, SCH_2), 2.58 (1H, t, OH; exch. D_2O); *m/z* (FAB) 257 (12.5%) [M+H$^+$], 255 (8.4) [M-H$^+$], 168 (9.8), 151 (89.0).

8-Benzylthio-3,6-dioxaoctyl chloride 3

To a stirred solution of alcohol, **2** (15.3 g, 59.7 mmol) in dry dichloromethane (250 ml) was added pyridine (distilled from potassium hydroxide pellets, 9.7 ml, 119.4 mmol, 2.0 eq.) and thionyl chloride (4.8 ml, 65.5 mmol, 1.1 eq.). The reaction was stirred and heated at reflux overnight under nitrogen. The mixture was cooled, poured into 2M hydrochloric acid (250 ml) and extracted with dichloromethane (2 x 250 ml). The organics were washed with water (50 ml) then twice with 2M hydrochloric acid (50 ml), dried over magnesium sulfate, filtered, evaporated under reduced pressure and purified by flash chromatography on silica eluting with 20% to 50% ether : petrol to give the *title compound*, **3** as a near-colourless oil (11.62g, 42.3 mmol, 70%) (Found: C, 56.6; H, 7.0. $C_{13}H_{19}O_2ClS$ requires C, 56.8; H, 7.0%) δ_H (300 MHz) 7.30 (5H, m, ArH), 3.80 (2H, s, benzyl CH_2), 3.75 - 3.60 (10H, m, CH_2O), 2.65 (2H, t, J $\approx$ 7, SCH_2), 2.58 (1H, t, OH; exch. D_2O); *m/z* (EI) 275 (0.12%)

[^{37}ClM - H$^+$], 274 (0.12) [^{35}ClM$^+$], 273 (0.3) [^{35}ClM - H$^+$], 238 (1), 168 (17.4), 150 (12.4), 123 (56.7), 91 (100).

8-Benzylthio-1-(*N*-phthalimido)-3,6-dioxaoctane 4

To a stirred solution of chloride **3** (5.91 g, 2.5 mmol) in dimethylformamide (50 ml) was added potassium phthalimide (5.0 g, 27.0 mmol, 1.2 eq.) and the reaction heated to 100 °C overnight. The reaction was cooled, poured into water (100 ml) and extracted with ether (3 x 75 ml). The organics were combined, washed with water, brine, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give an oil which was purified by flash chromatography on silica eluting with 30% to 50% ether : petrol to give the *title compound*, **4** as a colourless oil (3.95 g, 12.3 mmol, 55%) (Found: C, 65.3; H, 6.0; N, 3.7. $C_{21}H_{23}O_4NS$ requires C, 65.4; H, 6.0; N, 3.6%) δ_H (300 MHz) 7.85 (2H, m, phthalimido, *ortho*), 7.68 (2H, m, phthalimido, *meta*), 7.20 - 7.30 (5H, m, ArH), 3.92 (2H, t, J 5, CH_2N), 3.76 (2H, t, J 6, CH_2O), 3.72 (2H, s, benzyl, CH_2), 3.64 (2H, t, J 6), 3.58 (4H, m, OCH_2), 2.58 (2H, t, J 7.5, SCH_2).

8-Benzylthio-3,6-dioxaoctylamine 5

To a stirred solution of phthalimide **4** (3.72g, 11.57 mmol) in hot ethanol (60 ml) was added hydrazine hydrate (*ca.* 1 ml) and the reaction heated to reflux for 30 min giving a cream precipitate. More ethanol (50 ml) was added and the precipitate broken up and acidified to pH 1with conc. hydrochloric acid. The solid was filtered, washed with ethanol and the filtrate concentrated to give a white solid. The solid was dissolved in water (30 ml), basified with 1M sodium hydroxide and extracted with ether (3 x 50 ml). The combined organic extracts were washed with brine, dried with magnesium sulfate, filtered and evaporated to give the *title compound*, **5** as a pale yellow oil (1.95 g, 7.63 mmol, 66%) δ_H (300 MHz) 7.30 - 7.20 (5H, m, ArH), 3.78 (2H, s, CH_2 benzyl), 3.70 - 3.42 (8H, m, CH_2), 2.90 (2H, br. s, NH_2), 2.62 (2H, t, J 7.5, CH_2S); *m/z* (FAB) 257 (10.0%), 256 (58.9) [MH$^+$], 254 (3.6).

Cholesteryl urethane 6

To a stirred solution of amine **5** (591 mg, 2.31 mmol) in dichloromethane (10 ml) under nitrogen was added triethylamine (483 µl, 3.47 mmol, 1.5 eq.) and cholesteryl chloroformate (1.143 g, 2.54 mmol, 1.1 eq.) and the reaction stirred for 1.5 h. The solution was poured into saturated sodium bicarbonate solution (100 ml) and extracted with ether (3 x 50 ml). The combined organics were washed with brine (50 ml), dried with magnesium sulfate, filtered and evaporated to give a colourless glass. Purification by flash chromatography on silica eluting with 30% to 50% ether : hexane gave the *title compound*, **6** as a glassy solid (1.19 g, 77%) (Found: MH$^+$ 668.4758. $C_{41}H_{66}NO_4S$ requires 668.4713), δ_H (400 MHz) 7.40-7.20 (5H, m, ArH), 5.38 (1H, d, J 3, vinylic on steroid), 5.22 (1H, br. s, NH amide), 4.50 (1H, br. s, CHOR steroid), 3.70 (2H, s, CH_2 benzyl), 3.60-3.50 (8H, m, CH_2O), 3.38 (2H, m, CH_2N), 2.62 (2H, t, J 7.5, CH_2S), 2.40-2.20 (2H, m, steroid), 2.00-1.70 (4H, m, steroid), 1.60-0.85 (34H, m, steroid), 0.68 (3H, s, CH_3 steroid); *m/z* (FAB) 690 (12%) [MNa$^+$]668 (10) [M+H$^+$], 369 (100) [cholesteryl$^+$].

Thiol 7 (the EO3 'tether')

Liquid ammonia (75 ml) was condensed into a flask fitted with a dry ice condenser. Small pieces of sodium were added to obtain a permanent blue colouration (*ca.* 1g, excess). The reaction was cooled to -78°C and a solution of

benzyl thioether **6** in dry tetrahydrofuran (10 ml) was added *via* cannula. The reaction was stirred at low temperature for 1 h, warmed to reflux for 45 min then re-cooled and quenched at -78°C with wet tetrahydrofuran (50 ml). The reaction was allowed to warm slowly to 0°C whereupon ether (50 ml) and saturated ammonium chloride (25 ml) was added under an atmosphere of nitrogen and the mixture stirred overnight. The reaction was then diluted with water (100 ml) and extracted with chloroform (3 x 50ml). The combined organics were dried with magnesium sulfate, filtered and evaporated under reduced pressure to give an oil. This was purified by flash chromatography on silica eluting with 40% ether : hexane to pure ether (gradient) to give the *title compound*, **7** in a colourless oil (800 mg, 1.39 mmol, 88%) (Found: C, 71.0; H, 10.1; N, 2.4. $C_{34}H_{59}O_4NS$ requires C, 70.8; H, 10.1; N, 2.4%); δ_H (400 MHz) 5.36 (1H, m, vinyl steroid), 5.10 (1H, br. s, NH), 4.50 (1H, m, CHO-steroid), 3.61 (6H, t, J 6.5, CH_2O), 3.55 (2H, t, J 5.1, CH_2N), 3.37 (2H, q, J 5.1, CH_2 β to N), 2.70 (2H, q, J 6.5, CH_2S) 2.39 - 2.22 (2H, m, steroid), 2.10 - 1.78 (4H, m, steroid), 1.60 - 0.82 (34H, m, steroid CH_2, CH_3), 0.66 (3H, CH_3 steroid); *m/z* (FAB) 600 [M+], 369 [cholesteryl$^+$].

17-Benzylthio-3,6,9,12,15-pentaoxaheptadecanol tetrahydropyranyl ether 8

To a solution of alcohol **2** in dichloromethane was added an aqueous solution of sodium hydroxide (50% w/w, 3.1g, 156 mmol, 20 eq.), tetrabutylammonium hydrogensulfate (210 mg, 0.62 mmol, 0.08 eq.) and tetrahydropyranyl 2-(2-(2-chloroethoxy)ethanol (5.86 g, 15.6 mmol, 3 eq.) and the bi-phasic mixture stirred vigorously under nitrogen for 3 days at 65°C. The reaction was poured into water (200 ml) and extracted with dichloromethane (3 x 100 ml). The combined extracts were dried with magnesium sulfate, filtered and evaporated under reduced pressure to give an oil which was used directly in the next step. A small portion was purified by flash chromatography (on silica eluting with 50% ethyl acetate: hexane to pure ethyl acetate to 5% methanol : ethyl acetate (gradient) furnishing the *title compound*, **8** as a colourless oil, (Found: C, 60.8; H, 8.4. $C_{24}H_{40}O_7S$ requires C, 61.0; H, 8.5%); δ_H (300 MHz) 7.40-7.20 (5H, m, ArH), 4.62 (1H, m, pyran methine), 3.90 (2H, m, CH_2O), 3.80-3.60 (24H, m, CH_2O, $PhCH_2S$) 2.62 (2H, t, J 8, CH_2S), 1.80-1.40 (6H, m, CH_2 pyran); *m/z* (FAB) 472 (0.8%) [M^+], 471 (2.9), 405 (4.0), 390 (3.1), 389 (14.2), 151 (75.7).

17-Benzylthio-3,6,9,12,15-pentaoxaheptadecanol 9

To a stirred solution of tetrahydropyranyl ether **8** (*ca.* 156 mmol) in methanol (200 ml) was added a catalytic amount of 10-camphorsulfonic acid and the reaction stirred overnight. The solvent was evaporated quickly under reduced pressure and the resulting syrup dissolved in dichloromethane (200 ml), washed with saturated sodium bicarbonate solution (50 ml), dried over magnesium sulfate, filtered and evaporated. The resultant oil was purified by flash chromatography on silica eluting with 30% ethyl acetate : hexane to pure ethyl acetate (gradient) to give the *title compound*, **9** as a colourless oil (1.792 g, 4.61 mmol, 59% over two steps) (Found C, 58.6; H, 8.3. $C_{19}H_{32}O_6S$ requires C, 58.7; H, 8.2); δ_H (300 MHz) 7.30 (5H, m, ArH), 3.80 (2H, s, CH_2 benzyl), 3.78-3.60 (22H, m, CH_2O), 2.62 (2H, t, J 8, CH_2S), 2.10 (1H, br. s, exch., OH); *m/z* (EI) 388 (0.04%), 387 (0.11), 239 (9), 168 (11), 151 (18), 150 (28).

17-Benzylthio-3,6,9,12,15-pentaoxaheptadecanyl chloride 10

To a stirred solution of alcohol **9** (1.0 g, 2.57 mmol) in dichloromethane (30 ml) was added pyridine (420 mg, 5.15 mmol, 2 eq.) and thionyl chloride (207 mg, 2.83 mmol, 1.1 eq.) and the reaction refluxed for 12 h. The

mixture was cooled and poured into saturated ammonium chloride (100 ml) and extracted with dichloromethane (3 x 50 ml). The combined organics were washed with 2M hydrochloric acid (50 ml), dried with magnesium sulfate and evaporated under reduced pressure. The resultant oil was purified by flash chromatography on silica eluting with 25% ether : hexane to pure ether gradient to give the *title compound*, **10** as a colourless oil (570 mg, 1.4 mmol, 54%) (Found: C, 56.0; H, 7.9. $C_{19}H_{31}O_5SCl$ requires C, 56.1; H, 7.7%); δ_H (300 MHz) 7.30 (5H, m, ArH), 3.78 (2H, s, CH_2 benzyl), 3.70-3.58 (22H, m, CH_2O), 2.62 (2H, t, J 8, CH_2S); *m/z* (FAB) 409 (3.9%) [^{37}Cl MH^+], 407 (11.2) [^{35}Cl MH^+], 405 (3.7), 168 (5.2), 153 (5.4), 151 (100).

17-Benzylthio-1-(*N*-phthalimido)-3,6,9,12,15-pentaoxaheptadecane 11

To a stirred solution of chloride **10** (570 mg, 1.40 mmol) in dimethylformamide (10 ml) under nitrogen was added potassium phthalimide (311 mg, 1.68 mmol, 1.2 eq.) and the reaction heated to 100°C overnight. The mixture was cooled and poured into water (100 ml) and the aqueous phase extracted with dichloromethane (3 x 50 ml). The combined organic extracts were dried with magnesium sulfate, filtered and evaporated to give an oil. Purification by flash chromatography on silica eluting with 50% ethyl acetate : hexane to pure ethyl acetate gradient gave the *title compound*, **11** as a colourless oil (560 mg, 1.08 mmol, 77%) (Found: C, 62.4; H, 6.8; N, 2.6. $C_{27}H_{35}O_7NS$ requires C, 62.7; H, 6.8; N, 2.7%); δ_H (300 MHz) 7.85 (2H, m, phthalimide, *ortho*), 7.72 (2H, m, phthalimide, *para*), 7.30 (5H, m, ArH), 3.90 (2H, t, J 4, CH_2N), 3.78 (4H, m, CH_2O, $PhCH_2S$), 3.60 - 3.50 (18H, m, CH_2O x 9) 2.64 (2H, t, J 7, CH_2S) ; *m/z* (EI) 518 (14.5%) [MH^+], 369 (20.1), 368 (64.9), 324 (12.6), 306 (18.3).

17-Benzylthio-3,6,9,12,15-pentaoxaheptadecylamine 12

To a stirred solution of phthalimide **11** (530 mg, 0.918 mmol) in ethanol (8 ml) under nitrogen was added hydrazine (53 µl, 1.10 mmol, 1.2 eq.) and the reaction refluxed for 30 min. The cloudy mixture was then cooled, ethanol (50 ml) was added and acidified to pH 1 with conc. hydrochloric acid. The solid was filtered, washed with ethanol and the filtrate concentrated to give a white solid. This solid was dissolved in water (10 ml), basified with 1M sodium hydroxide and extracted with ether (3 x 20 ml). The combined organic extracts were washed with brine, dried with magnesium sulfate, filtered and evaporated to give the *title compound*, **12** as a near colourless oil (270 mg, 0.69 mmol, 76%) (Found: C, 58.8; H, 8.5; N, 3.4. $C_{19}H_{33}O_5NS$ requires C, 58.9; H, 8.6; N, 3.6%); δ_H (300 MHz) 7.33 (5H, m, ArH), 3.78 (2H, s, benzyl CH_2), 3.67-3.56 (22H, m, CH_2O), 2.89 (2H, br. s, NH_2), 2.64 (2H, t, J 6.5, CH_2S); *m/z* (FAB) 388 (0.7%) [MH^+], 387 (0.7), 297 (5), 296 (27.1), 220 (9.8), 207 (7.6).

Cholesteryl urethane 13

To a stirred solution of amine **12** (230 mg, 0.64 mmol) in dichloromethane (10 ml) under nitrogen was added triethylamine (126 µl, 0.90 mmol, 1.4 eq.) and cholesteryl chloroformate (318 mg, 0.71 mmol, 1.1 eq.). After 3h the mixture was poured into saturated sodium hydrogen carbonate (50 ml) and extracted with dichloromethane (3 x 50 ml). The combined organics were dried with magnesium sulfate, filtered and evaporated to give an oil which was purified by flash chromatography on silica eluting with 70% ethyl acetate : hexane to pure ethyl acetate (gradient) to give the *title compound*, **13** as a colourless oil (240 mg, 0.32 mmol, 47%) (Found: C, 70.3; H, 10.0; N, 1.8. $C_{47}H_{77}O_7NS$ requires C, 70.5; H, 9.7; N, 1.8%); δ_H (400 MHz) 7.40-7.20 (5H, m, ArH), 5.40 (1H, br. s, vinylic steroid), 5.20 (1H, br. s, NH amide), 4.50 (1H, br. s, CHOR steroid), 3.75 (2H, s,

$PhCH_2S$), 3.70-3.55 (18H, m, CH_2O), 3.52 (2H, m, $O\underline{CH_2}CH_2N$), 3.35 (2H, m, CH_2N), 2.62 (2H, t, J 7, CH_2S), 2.40-2.20 (2H, m, steroid), 2.10-1.80 (4H, m, steroid), 1.70-0.85 (34H, m, steroid), 0.68 (3H, s, CH_3 steroid); *m/z* (EI) 799 (0.1%) [M^+], 755 (0.2), 458 (0.9), 386 (35).

Thiol 14 (the EO6 'tether')

Liquid ammonia (50 ml) was condensed into a flask fitted with a dry ice condenser and nitrogen bubbler. Small pieces of sodium were added to obtain a permanent blue colouration (*ca.* 0.5 g, excess). The reaction was cooled to -78°C and a solution of benzyl thioether **13** (240 mg) in dry tetrahydrofuran (5 ml) was added *via* cannula. The reaction was stirred at low temperature for 1 h, warmed to reflux for 45 min then re-cooled and quenched at -78°C with wet tetrahydrofuran (25 ml). The reaction was allowed to warm slowly to 0 °C whereupon ether (50 ml) and saturated ammonium chloride (25 ml) were added under an atmosphere of nitrogen and the mixture stirred overnight. The reaction was then diluted with water (100 ml) and extracted with chloroform (3 x 50 ml). The combined organics were dried with magnesium sulfate, filtered and evaporated under reduced pressure. The resultant oil was purified by flash chromatography on silica eluting with ethyl acetate to 5% methanol : ethyl acetate to give the *title compound*, **14** as a colourless glass (86 mg, 0.13 mmol, 41%) (Found: C, 67.0; H, 10.2 ; N, 2.1. $C_{40}H_{71}O_7NS{\bullet}0.5\ H_2O$ requires C, 66.8; H, 10.1; N, 2.0 %); δ_H (400 MHz) 5.34 (1H, m, 1H, vinyl steroid), 5.18 (1H, br. s, NH), 4.48 (1H, m, $C\underline{H}O$-steroid), 3.65 - 3.59 (18H, m, CH_2O), 3.52 (2H, t, J 5.1, CH_2O), 3.33 (2H, br. s, CH_2N), 2.66 (2H, q, J 6.5, CH_2SH), 2.35 (1H, dd, steroid), 2.25 (1H, t, steroid), 1.98 (1H, dt, steroid), 1.94 - 1.78 (3H, m, steroid), 1.60 - 0.82 (34H, m, steroid), 0.66 (3H, s, CH_3); *m/z* (FAB) 732 [MNa+], 369 [cholesteryl$^+$].

3,6,9,12,15-Pentaoxahetadecan-1,17-diol monotetrahydropyranyl ether 15

Hexaethylene glycol (24.10 g, 85.5mmol) and 2,3-dihydropyran (8.63 g, 84.1 mmol) were stirred under nitrogen at room temperature as conc. hydrochloric acid (two drops) was added. Stirring was continued for 12 h. After this time the solution was diluted with ether (100 ml) and washed with saturated sodium hydrogen carbonate solution (2 x 100 ml) and water (100 ml). The organic phase was removed and the combined aqueous phases extracted with dichloromethane (3 x 50 ml). The organics were combined, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give a pale green oil. Purification by flash chromatography on silica eluting with 90% ethyl acetate : petroleum ether afforded the *title compound* **15** as a colourless oil (10.5 g, 34%) (Found: C, 56.0; H: 9.2. $C_{17}H_{34}O_8$ requires C: 55.7; H: 9.4%); δ_H (300 MHz) 4.62 (1H, m, pyran methine), 3.94 (2H, m, CH_2O), 3.50-3.80 (24H, m, CH_2O), 1.40-1.90 (6H, m, CH_2 pyran); *m/z* (FAB) 390 (24%) [M^+ + 23], 284 (60), 133 (7), 86 (100).

17-Chloro-3,6,9,12,15-pentaoxahetadecanol tetrahydropyranyl ether 16

A solution of thionyl chloride (4.10 g, 32.7 mmol) in dry dichloromethane (20 ml) was added dropwise to a stirred solution of compound **15** (10 g, 27.3 mmol) and dry pyridine (4.5 g, 54.6 mmol) in dry dichloromethane (20 ml) under nitrogen. The resulting solution was boiled under reflux for 18h. Upon cooling the reaction mass was washed with 2M hydrochloric acid (2 x 30ml) and water (50 ml). The organic phase was removed and the combined aqueous phases extracted with dichloromethane (3 x 50ml). The organics were combined, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give a dark brown oil. Purification by flash chromatography on silica using ethyl acetate as the eluent afforded the *title compound* **16** as a yellow oil (3.47 g,

33%). (Found: C, 53.1; H, 8.7; Cl, 9.2. $C_{17}H_{33}O_7Cl$ requires C, 53.1; H, 8.7; Cl, 9.2%); δ_H (300 MHz) 4.62 (1H, m, pyran methine), 3.50-4.00 (26H, m, CH_2O, CH_2Cl), 1.40-1.90 (6H, m, CH_2 pyran); *m/z* (EI) 384 (1%) [M^+], 383 [M-H^+] (2), 196 (5), 107 (19), 85 (100), 63 (34).

35-Benzylthio-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontanol tetrahydropyranyl ether 17

1-Thiobenzylpentaethylene glycol **9** (1.77 g, 4.5 mmol), tetrabutylammonium hydrogen sulfate (10 mol%) and compound **16** (3.50 g, 9.1 mmol) were stirred under nitrogen during the dropwise addition of 50% sodium hydroxide solution (3.6 ml, 91.1 mmol), the resulting two-phase mixture was stirred vigorously for 3 days at 65°C. After cooling and diluting with dichloromethane (30 ml), the organics were washed with water (2 x 30 ml) and saturated brine (30 ml). The organics were dried with magnesium sulfate, filtered and evaporated under reduced pressure to give a brown oil. Purification by flash chromatography on silica using 5% methanol : ethyl acetate as the eluent afforded the *title compound* **17** as a pale yellow oil (1.71 g, 51%) (Found: C, 58.5; H, 8.8; S, 4.4. $C_{36}H_{64}O_{13}S$ requires C, 58.7; H, 8.8; S, 4.4%); δ_H (300 MHz) 7.30 (5H, m, ArH), 4.62 (1H, m, pyran methine), 3.50-4.00 (50H, m, CH_2O, $PhCH_2S$), 2.62 (2H, t, J 7, CH_2S), 1.50-1.90 (6H, m, CH_2 pyran); *m/z* (FAB) 760 (28%) 759 [M^++ 23] (71%), 653 (27), 151 (84), 85 (100).

35-Benzylthio-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontanol 18

A mixture of compound **17** (1.70 g) and 10-camphor sulfonic acid (0.10 g) was stirred overnight in methanol. After this time the methanol was removed and the residue dissolved in dichloromethane (30 ml). The organics were washed with water (2 x 15ml) and brine (15 ml) prior to drying with magnesium sulfate, filtration and evaporation under reduced pressure to give the crude product. Purification by flash chromatography on silica using 20% methanol : ethyl acetate as the eluent afforded the *title compound* **18** as a colourless oil (1.31g, 86%) (Found: C, 56.8; H, 8.6; S, 4.9. $C_{31}H_{56}O_{12}S$ requires C, 57.0; H, 8.6; S, 4.9%); δ_H (300 MHz) 7.35 (5H, m, ArH), 3.50-3.80 (48H, m, CH_2O, $PhCH_2S$), 2.61 (2H, t, J 7, CH_2S); *m/z* (FAB) 676 (26%), 675 (72) [M^+ + 23], 653 (28), 151 (100), 136 (16), 91(90).

35-Benzylthio-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontanyl chloride 19

A solution of thionyl chloride (0.20 g,1.68 mmol) in dry dichloromethane (10 ml) was added dropwise to a stirred solution of compound **17** (1.00 g, 1.53 mmol) and dry pyridine (0.24 g, 3.06 mmol) in dry dichloromethane (20 ml) under nitrogen. The resulting solution was boiled under reflux for 18 h. Upon cooling the reaction mass was washed with 2M hydrochloric acid (2 x 30 ml) and water (50 ml). The organic phase was removed and the combined aqueous phases extracted with dichloromethane (3 x 50 ml). The organics were combined, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give the crude product. Purification by flash chromatography on silica using 10% methanol : ethyl acetate as the eluent afforded the *title compound*, **19** as a brown oil (0.51 g, 50%) (Found: C, 55.5; H, 8.5; Cl, 5.1; S, 4.9. $C_{31}H_{55}O_{11}ClS$ requires C, 55.5; H, 8.3; Cl, 5.3; S, 4.8%) δ_H (300 MHz) 7.30 (5H, m, ArH), 3.50-3.80 (48H, m, CH_2O, CH_2Cl, $PhCH_2S$), 2.61 (2H, t, J 7, CH_2S); *m/z* (FAB) 696 (14%), 695 [$^{37}ClM^+$+23] (44), 694 (34), 693 [$^{35}ClM^+$+23], (100), 151 (11), 91 (7).

35-Benzylthio-1-(*N*-phthalimido)-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontane 20

Potassium phthalimide (0.13 g, 0.72 mmol) was added portionwise to a stirred solution of compound **19** (0.37 g, 0.55 mmol) in dry dimethylformamide (10 ml) under nitrogen. The resulting mixture was heated at 100 °C overnight. Upon cooling, ether (25ml) was added to the reaction mass and the whole washed with water (4 x 30 ml). The organic phase was removed and the combined aqueous phases extracted with ether (2 x 50 ml). The organics were combined, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give the crude product. Purification by flash chromatography on silica using 10% methanol : ethyl acetate as the eluent afforded the *title compound*, **20** as a pale yellow oil (0.22 g, 51%) (Found: C, 56.5; H, 7.4; N, 1.3. $C_{39}H_{59}O_{13}NS{\bullet}2H_2O$ requires C, 56.8; H, 7.7; N, 1.6%); δ_H (300 MHz) 7.85 (2H, m, phthalimide, *ortho*), 7.72 (2H, m, phthalimide, *para*), 7.30 (5H, m, ArH), 3.90 (2H, t, J 5, CH_2N), 3.60-3.80 (46H, m, CH_2O, $PhCH_2S$), 2.61 (2H, t, J 7, CH_2S).

35-Benzylthio-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontanyl amine 21

Hydrazine monohydrate (0.02 g, 0.33 mmol) was added to a solution of compound **20** (0.20 g, 0.26 mmol) in dry ethanol (10 ml) at 50°C, under nitrogen. The solution was then boiled under reflux for 2 h before conc. hydrochloric acid (two drops) was added and heating continued for a further 2 h. The reaction mass was allowed to cool and was acidified to pH 1 with conc. hydrochloric acid resulting in the formation of a white precipitate which was removed by filtration. The precipitate was washed with ethanol and the filtrate evaporated under reduced pressure to afford a white residue. This was suspended in water (5 ml) and basified with 1M sodium hydroxide upon which it redissolved. The aqueous solution was extracted with dichloromethane (4 x 20 ml) and the organics were combined, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give the crude product, **21** (0.16 g, 98%) which was used without further purification. δ_H (300 MHz) 7.35 (5H, m, ArH), 3.50-3.80 (50H, m, benzyl CH_2, CH_2O, NH_2), 2.61 (2H, t, J 6.5, CH_2S); *m/z*: (FAB) 674 [MNa^+] (2%), 652 [MH^+] (15), 177 (13), 151 (18), 109 (25), 91 (65), 57 (100).

Cholesteryl urethane 22

A mixture of compound **21** (0.16 g, 0.24 mmol), cholesteryl chloroformate (0.16 g, 0.36 mmol) and dry triethylamine (0.04 g, 0.36 mmol) in dry dichloromethane (10 ml) was stirred under nitrogen for 3 h. After this time the reaction mass was washed with saturated sodium hydrogencarbonate solution (2 x 25 ml). The organic phase was removed and the combined aqueous phases extracted with dichloromethane (3 x 15 ml). The organics were combined, dried with magnesium sulfate, filtered and evaporated under reduced pressure to give the crude product. Purification by flash chromatography on silica using 5% methanol : ethyl acetate as the eluent afforded the *title compound*, **22** as a waxy colourless solid (0.16 g, 61%) (Found: C, 65.6; H, 9.4; N, 1.3. $C_{59}H_{101}O_{13}NS{\bullet}H_2O$ requires C, 65.5; H, 9.6; N, 1.3%); δ_H (400 MHz) 7.40-7.20 (5H, m, ArH), 5.40 (1H, br. s, vinylic steroid), 4.50 (1H, br. s, CHOR steroid), 3.75-3.78 (46H, m, $PhCH_2S$, CH_2O), 3.35 (2H, m, CH_2N) 2.62 (2H, t, J 7, CH_2S), 2.40-2.20 (2H, m, steroid), 2.10-1.80 (4H, m, steroid), 1.70-0.85 (34H, m, steroid), 0.68 (3H, s, CH_3 steroid); *m/z*: 1087 (1%), 1086 [MNa^+] (1), 652 (1), 369 (100), 147 (34), 95 (71).

Thiol 23 (The EO12 'tether')

Liquid ammonia (50 ml) was condensed into a flask fitted with a dry ice condenser and nitrogen bubbler. Small pieces of sodium were added to obtain a permanent blue colouration (*ca.* 0.5 g, excess). The reaction was cooled to -78 °C and a solution of benzyl thioether **22** (160 mg) in dry tetrahydrofuran (5 ml) was added *via* cannula. The reaction was stirred at low temperature for 1 h, warmed to reflux for 1 h then re-cooled and quenched at -78 °C with wet tetrahydrofuran (15 ml). The reaction was allowed to warm slowly to 0 °C and saturated ammonium chloride (15 ml) was added under an atmosphere of nitrogen and the mixture stirred overnight. The reaction was then diluted with water (50 ml) and extracted with chloroform (5 x 30 ml). The combined organics were washed with saturated brine solution (30 ml) and the brine re-extracted with dichloromethane (2 x 30ml). The combined organic phases were dried with magnesium sulfate, filtered and evaporated under reduced pressure. The resultant oil was purified by flash chromatography on silica eluting with ethyl acetate to 10% methanol : ethyl acetate (gradient) to give the *title compound*, **23** as a soft white wax (100 mg, 68%) (Found: C, 63.1; H, 9.5 ; N, 1.3. $C_{52}H_{95}O_{13}NS\bullet H_2O$ requires C, 62.9; H, 9.8; N, 1.4 %); δ_H (400 MHz) 5.34 (1H, m, 1H, vinyl steroid), 5.18 (1H, s, NH), 4.48 (1H, m, C<u>H</u>O-steroid), 3.65 - 3.59 (42H, m, CH_2O), 3.52 (2H, t, J 6, CH_2O), 3.33 (2H, br. s, CH_2N), 2.66 (2H, q, J 7, CH_2SH), 2.35 (1H, dd, steroid), 2.25 (1H, t, steroid), 1.98 (1H, dt, steroid), 1.94 - 1.78 (3H, m, steroid), 1.60 - 0.82 (34H, m, steroid), 0.66 (3H, s, CH_3); *m/z* (FAB) 996 [MNa^+] (4%), 369 [cholesteryl$^+$] (18).

Thiol 24

Liquid ammonia (200 ml) was condensed into a flask fitted with a dry ice condenser and nitrogen bubbler. Small pieces of sodium were added to obtain a permanent blue colouration (*ca.* 1g, excess). The reaction was cooled to -78°C and a solution of benzyl thioether **8** (5.91 g, 12.5 mmol) in dry tetrahydrofuran (20 ml) was added *via* cannula. The reaction was stirred at low temperature for 1 h, warmed to reflux for 45 min then re-cooled and quenched at -78°C with wet tetrahydrofuran (100 ml). The reaction was allowed to warm slowly to 0°C whereupon ether (200 ml) and saturated ammonium chloride (50 ml) were added under an atmosphere of nitrogen and the mixture stirred overnight. The reaction mixture was then diluted with water (100 ml) and extracted with ether (3 x 150 ml). The combined organics were washed with brine (100 ml), dried over magnesium sulfate, filtered and evaporated under reduced pressure. The resultant oil was purified by flash chromatography on silica eluting with 75% ethyl acetate : hexane to 5% methanol : ethyl acetate (gradient) to give the *title compound*, **24** as a colourless glass (870 mg, 9.7 mmol, 77%) (Found: MH^+ 383.2118. $C_{17}H_{35}O_7S$ requires 383.2103) δ_H (300 MHz) 4.62 (1H, t, J 6, pyran CH), 3.85 (2H, m, CH_2O), 3.62 (18H, m, CH_2O), 2.68 (2H, q, J 7, CH_2S), 1.85-1.40 (7H, m, pyran CH_2, SH); m/z (FAB) 383 (1.94%) [MH^+], 300 (1.60), 299 (21.4), 239 (13.5).

Hydroxythiol 25

To a stirred solution of thiol **24** (2.17 g, 5.67 mmol) in methanol (60 ml) was added 10-camphorsulfonic acid (50 mg, catalytic) and the reaction stirred overnight under nitrogen. The solvent was removed and the resulting syrup purified by flash chromatography on silica eluting with 10% methanol : chloroform to give the *title compound*, **25** as a colourless oil (860 mg, 2.88 mmol, 51%) (Found: C, 48.1; H, 9.0. $C_{12}H_{26}O_6S$ requires C, 48.3; H, 8.8%); δ_H (300 MHz, $CDCl_3$) 3.80-3.50 (22H, m, CH_2O), 2.80 (1H, br. s, OH), 2.70 (2H, q, J 6, CH_2S), 2.10 (1H, br. s, H_2O), 1.60 (1H, t, J 6, CH_2S); *m/z* (EI) 299 (0.4%) [MH^+], 239 (35), 195 (30).

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