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Synthesis of Novel Thiol-Reactive Amphiphilic Lipids Based on Cholesterol for Protein-Liposome Coupling

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Summary. The synthesis of a series of coupling lipids designed for covalently linking proteins to liposomes is described. The new compounds have in common a cholesterylsuccinyl unit as a lipid anchor and a thiol-reactive maleimidobenzoyl unit which are linked by alkyl or (poly)ethylene glycol spacers that differ in length and polarity.

Keywords. Maleimides; Polyethylene glycols; Cholesterol derivatives; Synthesis of; Liposomes.

Synthese neuartiger thiol-reaktiver amphiphiler Lipide auf Cholesterolbasis für die Kopplung von Proteinen an Liposomen

Zusammenfassung. Die Synthese einer Serie von Kopplungslipiden für die kovalente Bindung von Proteinen an Liposomen wird beschrieben. Die neuen Verbindungen haben eine Cholesterylsuccinyl-Einheit als Lipidanker und eine thiol-reaktive Maleimidobenzoylgruppe gemeinsam, die durch Alkyl- oder (Poly)ethylenglykol-Spacer verbunden sind, welche sich in Kettenlänge und Polarität unterscheiden.

Introduction

For many applications liposomes have proven to be the vehicle of choice for drug delivery [1, 2]. *In vitro* and *in vivo* studies have revealed their potential for the therapy of cancer, HIV infection, and other applications [3–11]. Liposomes can be selectively targeted to different tissues by covalently linking antibodies or other proteins to the liposome surface [1, 7, 11–14]. This linking is usually achieved by reacting the proteins with thiol- or amino-reactive lipid components of the preformed liposomes [15–17]. Coupling lipids with thiol-reactive maleimido groups are widely used because they selectively form stable thioether linkages to proteins bearing thiol groups. Almost all coupling lipids reported until now are based on phosphatidylethanolamine as the lipid anchor [5, 13, 18–20]. These compounds are prepared from heterobifunctional coupling agents and phosphatidylethanolamine, thus forming phospholipid derivatives with reactive groups for

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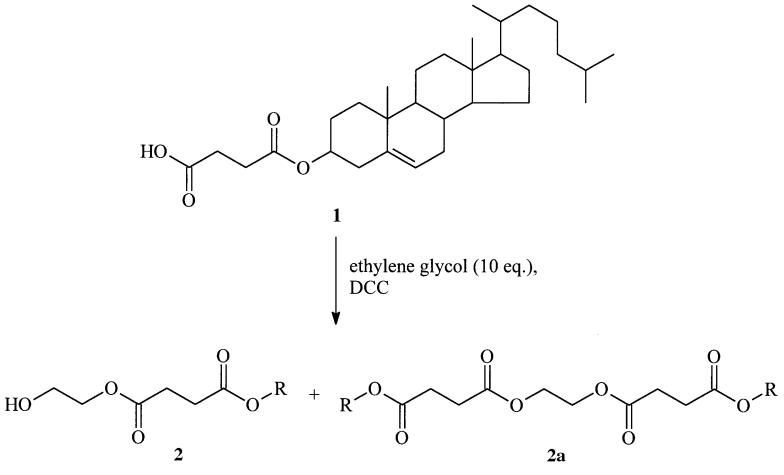
protein linkage. In contrast, however, little is reported on coupling lipids based on cholesterol anchors [10, 21]. This may be due to poor coupling efficiencies observed when using coupling lipids bearing maleimido groups linked to a cholesterol moiety by short apolar spacer units [21]. We now report the synthesis of a series of novel coupling lipids with spacers varying in both length and polarity. The use of long hydrophilic spacers is essential for coupling proteins to sterically stabilized liposomes with lipid bilayers containing glycolipids or lipid conjugates with polyethylene glycols [1, 5, 12, 13, 16, 17].

Results and Discussion

To synthesize the desired coupling lipids **7–12**, α,ω-diol spacer units (ethylene glycol, 1, 12-octadecanediol, tetraethylene glycol, polyethylene glycol 400, or polyethylene glycol 1000) were first esterified with cholesterol hemisuccinate (**1**) as the lipid anchor at one end and next with a maleimidobenzoic acid as the thiol reactive group at the other end. Being sensitive to reactions with nucleophiles, maleimidobenzoates were introduced in the last step.

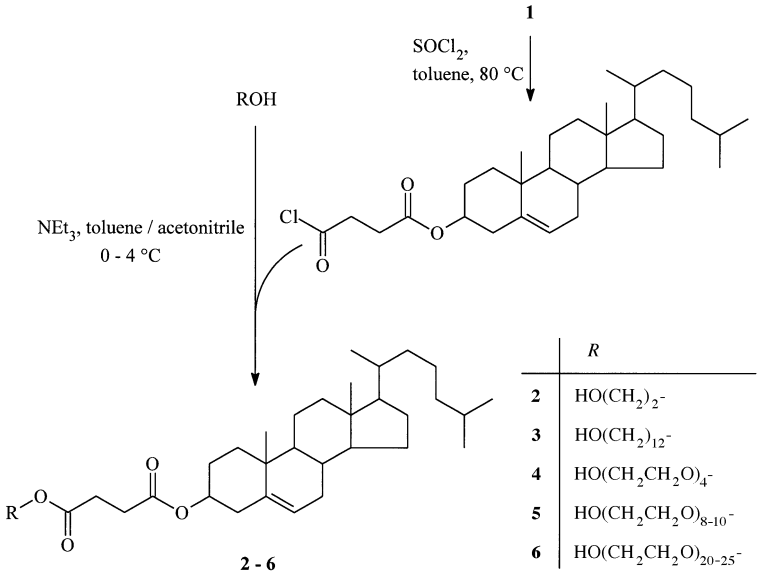
7–12 were prepared by a linear reaction sequence starting with cholesterol hemisuccinate (**1**) obtained from cholesterol and succinic anhydride by a simplified version of the synthesis described by *ElKihel et al.* [22].

The lipid anchor **1** was esterified with an excess of the α,ω-diol spacer units to achieve monoacylation (see Scheme 2). Interestingly, when exploring suitable reaction conditions we found a non-statistical product distribution with 60% of the diester **2a** and only 34% of the desired monoester **2** for the reaction of **1** with a



Solvent	Yield	
	2	**2a**
THF	34%	60%
toluene/acetonitrile 1:1	84%	9%

Scheme 1. Esterification of cholesterol hemisuccinate (**1**) with ethylene glycol: product distribution depending on the solvent

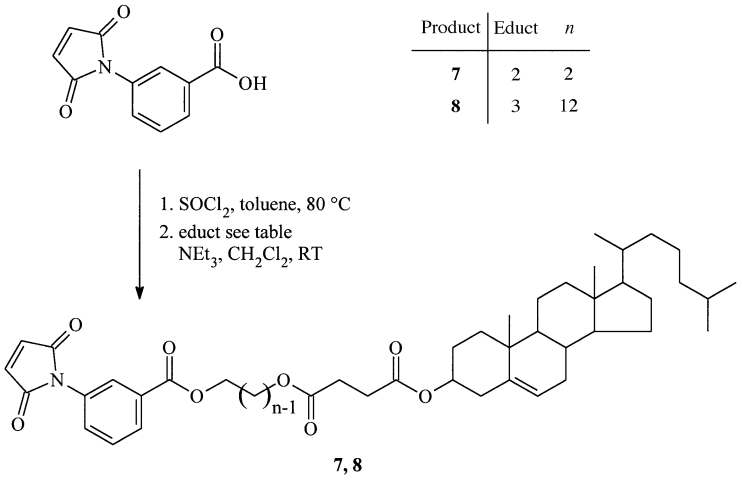


Scheme 2. Synthesis of the diol monoesters **2–6**

tenfold excess of ethylene glycol and *DCC* as condensing agent if *THF* was used as solvent (see Scheme 1). In contrast, a statistical product distribution was observed when using toluene/acetonitrile 1:1 as solvent. This could be explained by aggregate formation of **1** and **2** in *THF*. In this case, **1** would preferably react with **2** due to high local concentrations. The high bulk concentration of the diol which would not be present in the superstructure would be ineffective. This explanation is in accordance with observations that amphiphilic lipids tending to aggregate formation in one-component solvents can be monomolecularly dissolved by the use of appropriate solvent mixtures [23]. Therefore, the synthesis of **2–6** was performed in toluene/acetonitrile. For an easier workup of the upscaled preparations of **2–6**, we avoided the need of separating dicyclohexyl urea by converting **1** into the respective acid chloride using thionyl chloride in toluene instead of *DCC* as condensing agent (Scheme 2). After removal of the excess thionyl chloride, the resulting solution was added to the respective diol dissolved in acetonitrile, obtaining the proper solvent mixture (see above). After chromatographic purification, **2–5** were obtained in 56–64% yield. The lower yield (23%) of **6** which contains a *PEG* 1000 moiety is due to the chromatographic workup and corresponds to our experience of other long chain polyethylene glycol derivatives.

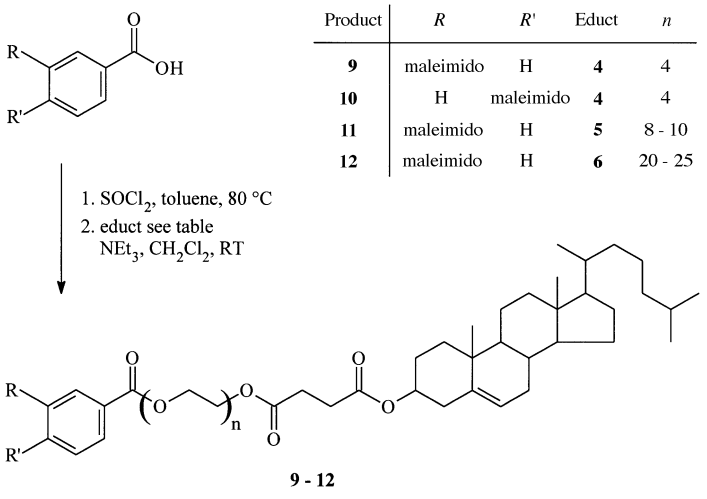
Esterification of **2–6** with *meta*- or *para*-maleimidobenzoic acid chloride was performed in dichloromethane with triethylamine as base at ambient temperature. The acid chlorides were freshly prepared from the respective maleimido benzoic acids (see Schemes 3 and 4). The products were purified by column chromatography without aqueous workup in order to avoid reaction of maleimide with hydroxide ions.

Our results show that the reaction sequence outlined above allows the preparation of thiol reactive lipids with variations in (*i*) polarity, (*ii*) chain length of the spacer unit, and (*iii*) the constitution of the thiol-reactive moiety. The influence



Product	Educt	*n*
7	2	2
8	3	12

Scheme 3. Synthesis of the coupling lipids **7** and **8** bearing α,ω-alkanediol spacer units



Product	*R*	*R'*	Educt	*n*
9	maleimido	H	**4**	4
10	H	maleimido	**4**	4
11	maleimido	H	**5**	8 - 10
12	maleimido	H	**6**	20 - 25

Scheme 4. Synthesis of the coupling lipids **9–12** bearing hydrophilic tetra- or polyethylene glycol spacer units

of these three parameters on the efficiency of coupling proteins to liposomes are currently under investigation in our group.

Experimental

Cholesterol (from lanolin) was purchased from Fluka (Buchs, CH). (Poly)ethylene glycols were of reagent grade and purchased from Merck (Darmstadt, FRG). Solvents used for the acylations were dried with sodium sulfate (ethyl acetate), over molecular sieve 0.4 nm (toluene), or by distillation from *di*-phosphorus pentoxide (acetonitrile). Triethylamine was distilled from calcium oxide. Removal of solvents was carried out in a rotatory evaporator under reduced pressure. Residual water was removed by azeotropic distillation with ethyl acetate. Chromatographic separations were performed in glass columns (diameter 4 cm or 2 cm, fraction size 20 ml). Silica gel (Kieselgel 60, 0.063–0.100 mm) and TLC plates were obtained from Merck (Darmstadt, FRG). ^{1}H NMR spectra were recorded with a Bruker AM 400 spectrometer (400 MHz, δ in ppm, *TMS* = 0 ppm). Infrared

spectra were measured using a Perkin Elmer 16 PC FT-IR spectrometer. Peaks are given in $cm^{-1}$and labelled 'ss' (very strong), 's' (strong), and 'm' (medium).

Cholesterol hemisuccinate (**1**)

A solution of 38.7 g (100 mmol) Cholesterol, 20.0 g (200 mmol) succinic anhydride, 0.6 g (5 mmol) *DMAP* and 51.2 ml (400 mmol) triethylamine in 500 ml ethyl acetate was refluxed for 12 h. 100 ml ethyl acetate and 100 ml methanol were added, and the mixture was extracted with 200 ml 2 *N* hydrochloric acid. After addition of further 100 ml ethyl acetate, the organic layer was extracted twice with 150 ml 0.2 *N* hydrochloric acid/methanol 2:1. The solvent of the organic layer was removed and 300 ml methanol were added. The resulting suspension was stirred for 15 min; then 300 ml water were added. The crude product was filtered off with suction, washed twice with water, dried *in vacuo*, and recrystallized from 200 ml diisopropyl ether to yield 43.4 g (89.2 mmol, 89%) **1** as a colorless powder.

$R_f = 0.38$ (ethyl acetate); IR (KBr): $\nu = 2950$ (ss) -CH_{2}-, 2870 (m) -CH_{2}-, 1730 (ss) C=O ester, 1710 (ss) C=O acid, 1175 (s) C-O; ^{1}H NMR ($CDCI_3$, 400 MHz): $\delta = 0.68$ (s, 3H, CH_3-18), 0.86 and 0.87 (2d, $^3J = 6.6$ Hz, 6H, diastereotopic CH_3-26/27), 0.92 (d, $^3J = 6.4$ Hz, 3H, CH_3-21), 0.94–1.62 (m, 21H) 1.02 (s, 3H, CH_3-19), 1.78–1.89 (m, 3H), 1.93–2.05 (m, 2H), 2.31 and 2.33 (2sb, 2H, allyl. CH_2-4), 2.58–2.70 (m, 4H, -OCO-$(CH_2)_2$-COO-), 4.59–4.68 (m, 1H, CH-3), 5.35–5.39 (m, 1H, vinyl. CH-6).

Compounds **2–6** (*Esterification of* **1**)

9.73 g (20 mmol) **1** and 3.63 ml (50 mmol) thionyl chloride in 100 ml toluene were stirred at 80°C for 1 h. The excess of thionyl chloride was distilled off, and the resulting solution was added dropwise to an ice-cold solution of the corresponding diol and 6.93 ml (50 mmol) triethylamine in 100 ml (for the preparation of **2**, **3**, and **4** or 150 ml (for the preparation of **5** and **6**) acetonitrile. The mixture was stirred for further 4 h at ambient temperature and then worked up as follows.

Workup of cholesteryl-(2-hydroxyethyl)-succinate (**2**)

Diol employed: 11.2 ml (200 mmol) ethylene glycol. After addition of 200 ml 0.25 *N* hydrochloric acid, the mixture was extracted twice with 80 ml ethyl acetate. The organic layers were evaporated to dryness, and the residue was purified on silica gel (150 g) using first diisopropyl ether, then diisopropyl ether/*tert*-butylmethyl ether 2:1 as eluent. Yield: 6.42 g (12.1 mmol, 60%) **2** as a wax-like solid.

$R_f = 0.49$ (*tert*-butylmethyl ether); IR (KBr): $\nu = 2950$ (ss) -CH_{2}-, 2905 (m) -CH_{2}-, 2870 (m) -CH_{2}-, 1735 (ss) C=O, 1165 (s) C–O; ^{1}H NMR ($CDCI_3$, 400 MHz): $\delta = 0.68$ (s, 3H, CH_3-18), 0.86 and 0.87 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.91 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21 H), 1.02 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.16 (t, $^3J_{OH,CH} = 6.2$ Hz, 1H, OH), 2.31 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.60–2.68 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.82 (dt, $^3J_{OH,CH} = 6.2$ Hz, $^3J_{CH,CH} = 4.7$ Hz, -CH_2OH), 4.23–4.27 (m, 2H, diastereotopic -CH_2-O-CO), 4.57–4.67 (m, 1H, CH-3), 5.35–5.39 (m, 1 H, vinyl. CH-6).

Workup of cholesteryl-(12-hydroxydodecyl)-succinate (**3**)

Diol employed: 40.5 g (200 mmol) 1,12-dodecanediol. After addition of 200 ml 0.25 *N* hydrochloric acid, the mixture was extracted with 80 ml ethyl acetate. The organic layer was evaporated to dryness, and the residue was stirred with 80 ml diisopropyl ether at 45°C for 1 h. The undissolved diol was filtered off with suction and the filtrate again evaporated to dryness. Purification of the

residue on silica gel (150 g) using cyclohexane/diisopropyl ether 1:1 as eluent yielded 7.45 g (11.1 mmol, 56%) **2** as a wax-like solid.

$R_f = 0.31$ (diisopropyl ether; IR (KBr): $\nu = 2930$ (ss) -CH_2-, 2850 (s) -CH_2-, 1735 (s) and 1725 (s) C=O, 1170 (s) C-O; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.68$ (s, 3H, CH_3-18), 0.86 and 0.87 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.92 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.71 (m, 25H), 1.02 (s, 3H, CH_3-19), 1.27 (s, 16H, O-$(CH_2)_2$-(CH_2)$_8$-$(CH_2)_2$-O), 1.78–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.56–2.64 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.60–3.67 (m, 2H, -CH_2-OH), 4.08 (t, $^3J = 6.7$ Hz, 2H, -CH_2-O-CO), 4.57–4.67 (m, 1H, CH-3), 5.35–5.38 (m, 1H, vinyl. CH-6).

Workup of mono-O-(3-cholesteryloxycarbonyl)propionyl tetraethylene glycol (**4**)

Diol employed: 34.5 mmol (200 mmol) tetraethylene glycol. Workup as described for **2** using ethyl acetate as eluent yielded 8.10 g (12.2 mmol, 61%) **4** as a wax-like solid.

$R_f = 0.27$ (ethyl acetate); IR (KBr): $\nu = 2940$ (ss) -CH_2-, 2905 (m)-CH_2-, 2870 (m) -CH_2-, 1730 (ss) C=O, 1160 (m) C–O; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.68$ (s, 3H, CH_3-18), 0.86 and 0.87 (2d, $^3J = 6.6$ Hz, 6H, diastereotopic CH_3-26/27), 0.91 (d, $^2J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21H), 1.02 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.47–2.54 (m, 1H, OH), 2.57–2.68 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.59–3.75 (m, 14H, HO-(C_2H_4O)$_3$-CH_2-), 4.25 (t, $^3J = 4.8$ Hz, 2H, -CH_2-O-CO), 4.57–4.67 (m, 1H, CH-3), 5.35–5.38 (m, 1H, vinyl. CH-6).

Workup of mono-O-(3-cholesteryloxycarbonyl)propionyl polyethylene glycol 400 (**5**)

Diol employed: 80.0 g (200 mmol) polyethylene glycol 400. Workup as described for **2** using first ethyl acetate, then ethyl acetate/methanol 9:1 as eluent yielded 11.2 g (12.8 mmol, 64%) **5** as a soft slime.

$R_f = 0.1$–0.5 (ethyl acetate/methanol 8:1); IR (KBr): $\nu = 2935$ (ss) -CH_2-, 2905 (m) -CH_2-, 2870 (m)-CH_2-, 1735 (ss) C=O, 1165 (m) C-O, 1115 (s) C–O; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.68$ (s, 3H, CH_3-18), 0.86 and 0.87 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.92 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21H), 1.02 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.57–2.68 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.59–3.73 (m, approx. 34H, HO-(C_2H_4O)$_{8(\text{on average})}$-CH_2-), 4.25 (t, $^3J = 4.8$ Hz, 2H, -CH_2-O-CO), 4.57–4.67 (m, 1H, CH-3), 5.35–5.38 (m, 1H, vinyl. CH-6).

Workup of mono-O-(3-cholesteryloxycarbonyl)propionyl polyethylene glycol 1000 (**6**)

Diol employed: 100 g (100 mmol) polyethylene glycol 1000. After addition of 200 ml 0.25 *N* hydrochloric acid the mixture was extracted twice with 120 ml ethyl acetate/methanol 2:1. The combined organic layers were evaporated to dryness. Purification of the residue on Silica gel (150 g) using first diisopropyl ether/methanol 2:1 to eluate the apolar by-products, then ethyl acetate/methanol 2:1 as eluent yielded 6.86 g (4.67 mmol, 23%) **6** as an amorphous solid.

$R_f = 0.2$–0.3 (ethyl acetate/tetrahydrofurane/methanol 1:1:1); IR (KBr): $\nu = 2940$ (s) -CH_2-, 2890 (ss) -CH_2-, 1730 (s) C=O, 1115 (ss) C-O; ^{1}H NMR ($CDCl_3$/CD_3OD/D_2O (1:4:1), 400 MHz): $\delta = 0.71$ (s, 3H, CH_3-18), 0.87 and 0.88 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.94 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.95–1.69 (m, 21H), 1.05 (s, 3H, CH_3-19), 1.81–1.94 (m, 3H), 1.95–2.08 (m, 2H), 2.32 and 2.34 (2sb, 2H, allyl. CH_2-4), 2.61–2.70 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.67 (s, approx. 90H, HO-(C_2H_4O)$_{22(\text{on average})}$-CH_2-), 4.24–4.28 (m, 2H, -CH_2-O-CO), 4.56–4.65 (m, 1H, CH-3), 5.38–5.41 (m, 1H, vinyl. CH-6).

O-(3-Cholesteryloxycarbonyl)propionyl-O′-m-maleimidobenzoyl ethylene glycol (**7**)

236 mg (1.0 mmol) *m*-maleimidobenzoic acid and 218 µl (3.0 mmol) thionyl chloride in 5 ml toluene were stirred at 80°C for 1 h. The volatile components were distilled off to obtain crude *m*-maleimidobenzoyl chloride as a light yellow solid. 637 mg (1.2 mmol) **2** and 139 µl (1.0 mmol) triethylamine dissolved in 5 ml dichloromethane were added, and the mixture was stirred at ambient temperature for 2 h. After evaporation to dryness the residue was purified on silica gel (35 g) using cyclohexane/ethyl acetate 4:1 as eluent. Yield: 431 mg (0.59 mmol, 59%) **7** as a solid.

$R_f = 0.40$ (cyclohexane/ethyl acetate 2:1); IR (KBr): $\nu = 2950$ (ss) -CH_2-, 2905 (m) -CH_2-, 2865 (m) -CH_2-, 2850 (m) -CH_2-, 1725 (ss) C=O, 1590 (m) arom, 1490 (m) arom, 1165 (ss) C-O, 755 (m) arom; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.67$ (s, 3H, CH_3-18), 0.85 and 0.88 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.91 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21H), 1.02 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.56–2.72 (m, 4H, -OCO- $(CH_2)_2$-COO-), 4.40–4.48 (m, 2H, -CH_2-O-CO-CH_2), 4.50–4.56 (m, 2H,-CH_2-O-CO-aryl), 4.55–4.70 (m, 1H CH-3), 5.32–5.40 (m, 1H, vinyl. CH-6), 6.89 (s, 2H, OC-CH=CH-CO), 7.50–7.62 (m, 2H, arom. H), 8.00–8.11 (m, 2H, arom. H).

O-(3-Cholesteryloxycarbonyl)propionyl-O′-m-maleimidobenzoyl 1,12-dodecanediol (**8**)

204 mg (0.87 mmol) *m*-maleimidobenzoic acid were converted into *m*-maleimidobenzoyl chloride as described in the preparation of **7**. 487 mg (0.73 mmol) **3** and 100 µl (0.73 mmol) triethylamine dissolved in 5 ml dichloromethane were added, and the mixture was stirred at ambient temperature for 2 h. After evaporation to dryness the residue was purified on silica gel (30 g) using cyclohexane/ethyl acetate 6:1 as eluent. Yield: 290 mg (0.33 mmol, 46%) **8** as a solid.

$R_f = 0.22$ (cyclohexane/ethyl acetate 4:1); IR (KBr): $\nu = 2935$ (ss) -CH_2-, 2870 (m) -CH_2-, 2850(m) -CH_2-, 1725 (ss) C=O, 1165 (ss) C-O, 755 (m) arom; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.67$ (s, 3H, CH_3-18), 0.85 and 0.87 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.92 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.71 (m, 25H), 1.01 (s, 3H, CH_3-19), 1.20 (m, 16H, O-$(CH_2)_2$-(CH_2)$_8$-$(CH_2)_2$-O), 1.40 (m, 4H, O-CH_2-CH_2-$(CH_2)_8$-CH_2-CH_2-O), 1.78–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.56–2.64 (m, 4H, -OCO-$(CH_2)_2$-COO-), 4.08 (t, $^3J = 6.7$ Hz, 2H, -CH_2-O-CO-CH_2), 4.32 (t,$^3J = 6.7$ Hz, 2H, -CH_2-O-CO-aryl), 4.54–4.70 (m, 1H, CH-3), 5.33–5.40 (m, 1H, vinyl. CH-6), 6.89 (s, 2H, OC-CH=CH-CO), 7.51–7.58 (m, 2H, arom. H), 8.00–8.09 (m, 2H, arom. H).

O-(3-Cholesteryloxycarbonyl)propionyl-O′-m-maleimidobenzoyl tetraethylene glycol (**9**)

200 mg (0.92 mmol) *m*-maleimidobenzoic acid were converted into *m*-maleimidobenzoyl chloride as described in the preparation of **7**. 502 mg (0.74 mmol) **4** and 127 µl (0.92 mmol) triethylamine dissolved in 5 ml dichloromethane were added, and the mixture was stirred at ambient temperature for 2 h. After evaporation to dryness the residue was purified on silica gel (20 g) using cyclohexane/ethyl acetate 1:1 as eluent. Yield: 432 mg (0.50 mmol, 68%) **9** as a wax-like solid.

$R_f = 0.65$ (ethyl acetate); IR (KBr): $\nu = 2945$ (ss) -CH_2-, 2905 (m) -CH_2-, 2870 (m) -CH_2-, 1720 (ss) C=O, 1490 (m) arom, 1165 (m) C-O, 1145(m) C-O, 755 (m) arom; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.67$ (s, 3H, CH_3-18), 0.85 and 0.88 (2d, $^3J = 6.6$ Hz, 6H, diastereotopic CH_3-26/27), 0.91 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21H), 1.01 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.55–2.69 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.59–3.75 (m, 10H,-OCH_2CH_2O-$(C_2H_4O)_2$-C_2H_4O-CO-aryl), 3.84 (t, $^3J = 4.8$ Hz, 2H, -CH_2-CH_2-O-CO-aryl), 4.24 (t, $^3J = 4.8$ Hz, 2H, -CH_2-O-CO-CH_2), 4.50 (t, $^3J = 4.8$ Hz, 2H, -CH_2-O-CO-aryl), 4.55–4.70 (m, 1H, CH-3), 5.35–5.40 (m, 1H, vinyl. CH-6), 6.89 (s, 2H, OC-CH=CH-CO), 7.61–7.53 (m, 2H, arom. H), 8.03–8.05 (m, 2H, arom. H).

O-(3-Cholesteryloxycarbonyl)propionyl-O′-p-maleimidobenzoyl tetraethylene glycol (**10**)

200 mg (0.92 mmol) *p*-maleimidobenzoic acid were converted into *p*-maleimidobenzoyl chloride according to the procedure described in the preparation of **7**. 502 mg (0.74 mmol) **4** and 127 µl (0.92 mmol) triethylamine dissolved in 5 ml dichloromethane were added, and the mixture was stirred at ambient temperature for 2 h. After evaporation to dryness the residue was purified on silica gel (20 g) using cyclohexane/ethyl acetate 1:1 as eluent. Yield: 448 mg (0.52 mmol, 71%) **10** as a wax-like solid.

$R_f = 0.67$ (ethyl acetate); IR (KBr): $\nu = 2945$ (ss) -CH_2-, 2905 (m) -CH_2-, 2870 (m)-CH_2-, 1720 (ss) C=O, 1610 (m) arom, 1515 (m), arom, 1145 (m), C-O, 755 (m) arom; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.67$ (s, 3H, CH_3-18), 0.85 and 0.87 (2d, $^3J = 6.6$ Hz, 6H, diastereotopic CH_3-26/27), 0.91 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21H), 1.01 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.56–2.67 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.61–3.72 (m, 10H, -OCH_2CH_2O-$(C_2H_4O)_2$-C_2H_4O-CO-aryl), 3.84 (t, $^3J = 4.8$ Hz, 2H, -CH_2-CH_2-O-CO-aryl), 4.23 (t, $^3J = 4.8$ Hz, 2H, -CH_2-O-CO-CH_2), 4.49 (t, $^3J = 4.8$ Hz, 2H, -CH_2-O-CO-aryl), 4.55–4.70 (m, 1H, CH-3), 5.35–5.40 (m, 1H, vinyl. CH-6), 6.88 (s, 2H, OC-CH=CH-CO), 7.49–7.52 (m, 2H, arom. H), 8.13–8.17 (m, 2H, arom. H).

O-(3-Cholesteryloxycarbonyl)propionyl-O′-m-maleimidobenzoyl polyethylene glycol 400 (**11**)

326 mg (1.5 mmol) *m*-maleimidobenzoic acid were converted into *m*-maleimidobenzoyl chloride as described in the preparation of **7**. 870 mg (1.0 mmol) **5** and 139 µl (1.0 mmol) triethylamine dissolved in 10 ml dichloromethane were added, and the mixture was stirred at ambient temperature for 2 h. After evaporation to dryness the residue was purified on silica gel (20 g) using first cyclohexane/ethyl acetate 1:1, then ethyl acetate as eluent. Yield: 357 mg (0.33 mmol, 33%) **11** as a wax-like solid.

$R_f = 0.22$–0.6 (ethyl acetate/tetrahydrofurane 4:1); IR (KBr): $\nu = 2940$ (ss) -CH_2-, 2905 (m) -CH_2-, 2870 (m) -CH_2-, 1720 (ss) C=O, 1590 (m) arom, 1490 (m) arom, 1145 (m) C-O, 755 (m) arom; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.69$ (s, 3H, CH_3-18), 0.85 and 0.88 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.92 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.93–1.64 (m, 21H), 1.03 (s, 3H, CH_3-19), 1.77–1.89 (m, 3H), 1.92–2.04 (m, 2H), 2.30 and 2.32 (2sb, 2H, allyl. CH_2-4), 2.57–2.70 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.60–3.75 (m, approx. 30H, -OCH_2CH_2O-$(C_2H_4O)_{7(\text{on average})}$-$C_2H_4O$-CO-aryl), 3.86 (t, $^3J = 4.8$ Hz, 2H,-CH_2-CH_2-O-CO-aryl), 4.23 (t, $^3J = 4.9$ Hz, 2H, -CH_2-O-CO-CH_2), 4.50 (t, $^3J = 4.9$ Hz, 2H, -CH_2-O-CO-aryl), 4.53–4.69 (m, 1H, CH-3), 5.35–5.40 (m, 1H, vinyl. CH-6), 6.90 (s, 2H, OC-CH=CH-CO), 7.55–7.61 (m, 2H, arom. H), 8.04–8.12 (m, 2H, arom. H).

O-(3-Cholesteryloxycarbonyl)propionyl-O′-m-maleimidobenzoyl polyethylene glycol 1000 (**12**)

195 mg (0.9 mmol) *m*-maleimidobenzoic acid were converted into *m*-maleimidobenzoyl chloride as described in the preparation of **7**. 930 mg (0.6 mmol) **6** and 83 µl (0.6 mmol) triethylamine dissolved in 10 ml dichloromethane were added, and the mixture was stirred at ambient temperature for 2 h. After evaporation to dryness the residue was purified on silica gel (20 g) using first ethyl acetate, then ethyl acetate/tetrahydrofurane 1:1 as eluent. Yield: 420 mg (0.24 mmol, 40%) **12** as a solid.

$R_f = 0.43$–0.67 (ethyl acetate/tetrahydrofurane/methanol 1:1:1); IR (KBr):$\nu = 2950$ (ss) -CH_2-, 2890 (m) -CH_2-, 1720 (ss) C=O, 1145 (m) C-O, 755 (m) arom; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.68$ (s, 3H, CH_3-18), 0.85 and 0.87 (2d, $^3J = 6.7$ Hz, 6H, diastereotopic CH_3-26/27), 0.92 (d, $^3J = 6.7$ Hz, 3H, CH_3-21), 0.95–1.69 (m, 21H), 1.01 (s, 3H, CH_3-19), 1.80–1.93 (m, 3H), 1.95–2.09 (m, 2H), 2.32 and 2.34 (2sb, 2H, allyl. CH_2-4), 2.55–2.70 (m, 4H, -OCO-$(CH_2)_2$-COO-), 3.55–3.75 (m, approx. 86H, -OCH_2CH_2O-$(C_2H_4O)_{21(\text{on average})}$-$C_2H_4O$-CO-aryl), 3.83 (t, $^3J = 4.9$ Hz, 2H, -CH_2-CH_2-O-CO-aryl), 4.25 (t, $^3J = 4.9$ Hz, 2H, -CH_2-O-CO-CH_2), 4.49 (t, $^3J = 4.9$ Hz, 2H, -CH_2-O-CO-aryl), 4.56–4.70 (m, 1H, CH-3), 5.34–5.40 (m, 1H, vinyl. CH-6), 6.90 (s, 2H, OC-CH=CH-CO), 7.54–7.58 (m, 2H, arom. H), 8.04–8.09 (m, 2H, arom. H).

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