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The Synthesis of Substituted Piperazine-cholesterol Conjugates for use as Components of Nucleic Acid Transfection Lipoplexes†

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ABSTRACT

A small library of cholesterol-piperazine conjugates were synthesized by the reaction of cholesteryl chloroformate with a set of substituted piperazines in dichloromethane at room temperature. The conjugates, all obtained in good to excellent yields, were synthesized to be key components of nucleic acid transfection lipoplexes.

KEYWORDS

Piperazine, cholesterol, conjugates, transfection, cationic lipoplexes.

1. Introduction

In recent years lipoplexes have been utilized as nucleic acid transfer agents in attempts to transfect cells with DNA and RNA, with varying degrees of success. This area has been the focus of much research activity and a number of reviews have summarized the highlights in the area.[1] In terms of the lipoplex nanostructures utilized to perform the cellular transfection, one of their major components usually consists of a lipophillic 'tail' portion (fatty acid, steroid etc.) with a positively charged polar 'head' group to facilitate charged complexation with the negatively charged nucleic acid sequence to be transported. One of the most popular lipophillic groups utilized in this field has been cholesterol and representative examples are shown in Fig. 1 (compounds **1**–**4**).

The examples shown in Fig. 1 include cholesterol conjugated to a linear polyamine, such as spermine, using a carbamate linker, arranged in a linear (compound **1**[2]) or a 'T-shaped' (compound **2**[3]) manner. Other researchers have opted for the use of cyclic amines to provide the cationic head group under physiological conditions. For instance, Gao and Hui utilized a piperazine head group as shown in compounds **3** and **4**.[4] Intrigued by these latter results, our research group decided to do a systematic study into the ability of cholesterol-piperazine conjugates to perform as components of gene delivery vehicles.[5] The synthetic aspects of this work are described in this paper.[6]

2. Results and Discussion

As illustrated in Fig. 1, the use of a piperazine head group to form cholesterol-piperazine conjugates has been investigated.[4] However, the strategy used by these researchers was not

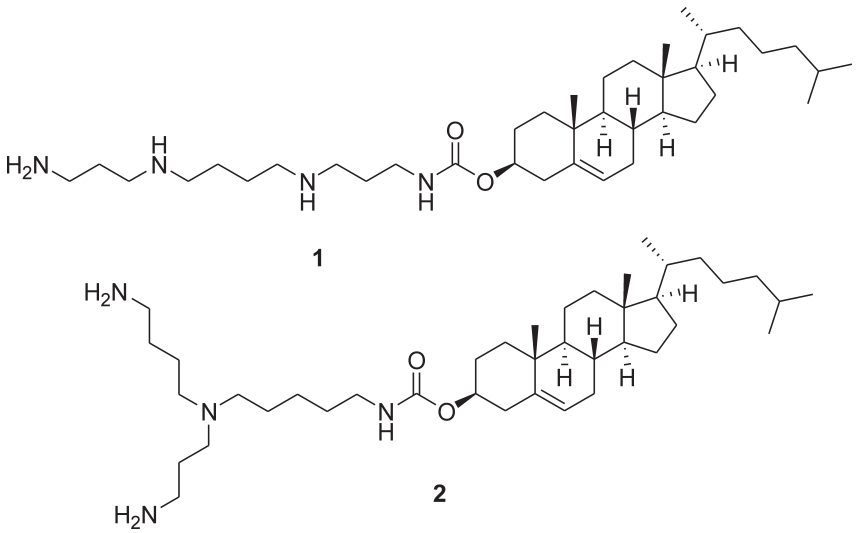

Figure 1a Examples of previously synthesized polyamine-and piperazine-cholesterol conjugates.

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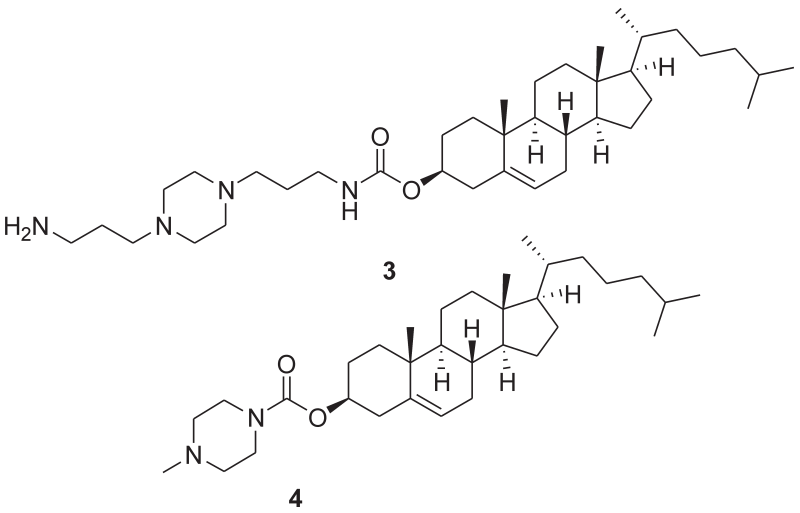


Figure 1b Examples of previously synthesized polyamine-and piperazine-cholesterol conjugates.

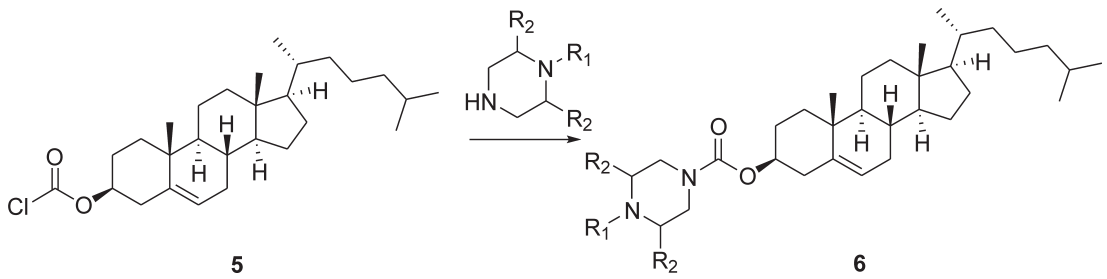
systematic, nor was a diverse set of differentially substituted piperazines utilized. It was thus decided to synthesize a diversified library of substituted piperazines, covalently attached via a carbamate unit to cholesterol. The synthetic strategy involved reaction of the commercially-available cholesteryl chloroformate with the desired substituted piperazines in dichloromethane at room temperature (Scheme 1). After the reactions were complete (monitored by tlc), the desired compounds were purified by silica gel column chromatography. All the compounds were characterized by ^{1}H, ^{13}C NMR and IR spectroscopy and the molecular masses confirmed by mass spectroscopy.

The first set of compounds that were synthesized contained simple piperazines. These piperazines were unsubstituted or bore only simple alkyl, allyl, amine, cyano and ester substituents (for piperazine structures and reaction yields see Table 1). The ease with which the compounds were synthesized is demonstrated by the fact that all the products were obtained in good yields (>75 %). Compounds synthesized included those with a piperazine (**6a**), dimethyl- (**6b**), *N*-methyl- (**6c**) or *N*-ethylpiperazine (**6d**) moiety. In addition, piperazines bearing short alkyl amine pendant chains were also synthesized, namely the *N,N*-dimethylpropyl and *N,N*-dimethylethyl derivatives (**6e**) and (**6f**), respectively (Table 1). Both these compounds have the advantage in that they bear an additional amine group which can be protonated under physiological conditions. This should increase the cationic charge required for the lipoplexes to transfect the negatively charged RNA or DNA sequences. Other piperazine derivatives were also obtained which included piperazines bearing groups such as *N*-allyl (**6i**) and (**6j**), ester (**6h**) and cyano (**6g**) functional groups. Compounds (**6i**) and (**6j**) are of interest because they are amenable to further synthetic transformations using, in particular, an envisaged cross metathesis reaction[7] (Table 1). Finally, the *N*-amino-piperazine-cholesterol conjugate (**7**) was also synthesized by the reaction of (**5**) with 4-methylpiperazin-1-amine (**8**),[4] utilizing the simple synthetic methodology shown in Scheme 2.

NMR spectral analysis confirmed that the compounds (**6a–j**) and **7** had been efficiently synthesized. Apart from the characteristic signals for cholesterol in the ^{1}H and ^{13}C NMR spectra, signals for the methylene protons of the piperazine ring at about δ 2.5 and δ 3.5 were evident for most new compounds. In addition, the ^{13}C NMR spectra displayed a characteristic signal at about δ 155 for the newly formed carbamate carbonyl.

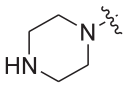
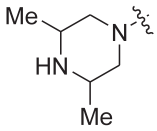
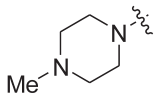
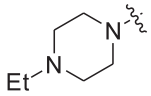
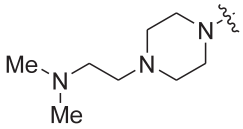
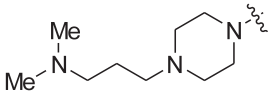
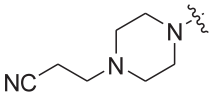
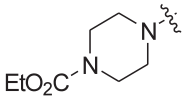
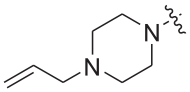
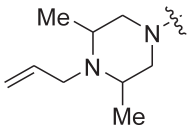
Interestingly, during the synthesis of the cholest-5-en-3-yl 1-piperazinecarboxylate (**6a**) a reasonable amount (16 %) of the symmetrical *bis*-cholesterol (**9**) was obtained (Scheme 3). The fact that double addition of the cholesteryl moiety had occurred was confirmed by the NMR spectrum, as well as the high-resolution mass spectrum. A double addition also occurred when 2-(piperazin-1-yl)ethanamine (**10**) was utilized to obtain compound (**11**) as shown in Scheme 3. This compound was isolated in a yield of 78 % based on cholesteryl chloroformate, indicating that the primary amine was successfully competing with the secondary amine for the cholesteryl chloroformate substrate. It is important to note that *bis*-cholesterol-substituted lipoplex components have previously been utilized for gene transfection with varying results.[4,8] The isolated products (**9**) and (**11**) require further evaluation for their ability to function as transfection agents in lipoplexes and this will be performed in future bioactivity studies.

Finally, a set of cholesteryl derivatives, containing piperazines substituted with additional aromatic groups, were synthesized (Table 2). These included the pyridine substituted piperazines (**6k**) and (**6l**), as well as the 2-pyrimidine (**6m**). Of note is that other pyridinium-based cationic lipid conjugates have been successfully used as gene transfer agents.[9] Finally, a number of



Scheme 1
General reaction scheme for synthesis of piperazine-cholesterol conjugates. For yields see Tables 1 and 2.

Table 1 Cholesterol conjugated to piperazines substituted with alkyl side chains (refer to Scheme 1 for reaction scheme).

Entry	(6)	Piperazine	Yield
1	**a**		84 %
2	**b**		89 %
3	**c**/(**4**)[4]		79 %
4	**d**		81 %
5	**e**		89 %
6	**f**		83 %
7	**g**		78 %
8	**h**		80 %
9	**i**		83 %
10	**j**		87 %

simple aromatic-piperazine cholesterol derivatives were obtained to complete the small library; these included the phenyl (**6n**), 2-fluorophenyl (**6o**), 4-fluorophenyl (**6p**) and the oxygenated phenyl (**6q**)-substituted piperazine head groups and once again the products were all obtained in excellent yields (Table 2).

3. Conclusion

In this paper we have disclosed the synthesis of a diverse library of cholesterol derivatives conjugated to substituted piperazines by way of a carbamate linker. An advantage of the strategy utilized was that it afforded the desired compounds in high yields. Moreover, the approach allowed for incorporation of functional diversity on the piperazine head group because of the significant variety of commercially available substituted piperazines. Initial studies[5] have shown that a number of the synthesized piperazine-cholesterol conjugates have potential as gene transfer agents but further investigations need to be performed, including the use of *in vivo* mouse models.

4. Experimental

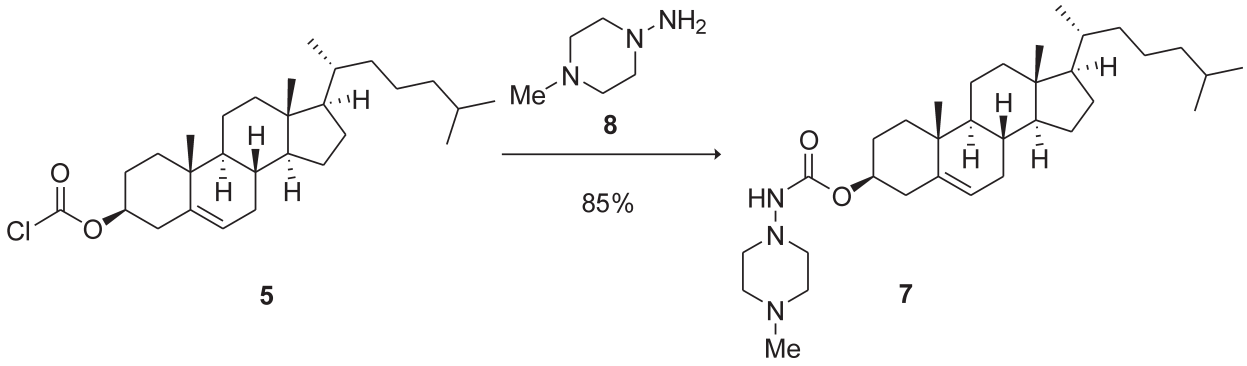
^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 300 spectrometer at the frequency indicated. *J*-Values are given in Hz. Infrared spectra were recorded on a Bruker Vector 22 Fourier Transform spectrometer. High resolution electrospray ionization mass spectra (ESI-FTMS) were recorded at the Technical University Dortmund, Germany, using a Thermo LTQ Orbitrap (high resolution mass spectrometer from Thermo Electron) coupled to an 'Accela' HPLC System supplied with a 'Hypersil GOLD' column (Thermo Electron). Macherey-Nagel Kieselgel 60 (particle size 0.063–0.200 mm) was used for conventional silica gel chromatography. All solvents used for reactions and chromatography were distilled prior to use to remove residual non-volatiles. Removal or concentration of solvent *in vacuo* implies the evaporation of solvent at 20–25 Torr utilizing a rotary evaporator. Melting points are reported as uncorrected.

General procedure for the synthesis of compounds 6a–q: The substituted piperazines (1 mmol) and NEt_3 (1.5 mmol) were dissolved in CH_2Cl_2 (10 mL). The solution was cooled to 0°C and cholesteryl chloroformate (1 mmol) in CH_2Cl_2 (10 mL) was then added over 10 min. Then the solution was stirred for 10 min. The reaction mixture was then stirred 4–8 h at rt under N_2. The reaction mixture was then washed with water (3 × 10 mL) and the organic phase dried ($MgSO_4$) before being removed under reduced pressure to give a crude solid. This solid was then purified by silica gel chromatography (2–10 % MeOH/CH_2Cl_2) to afford the desired compounds in good to excellent yields.

Cholest-5-en-3-yl 1-piperazinecarboxylate 6a. Yield: 84 %; mp: 140–142 °C IR: 2945, 2863, 1687, 1427 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.85–1.59 (m, 23H), 1.83–2.03 (m, 5H), 2.28–2.35 (m, 2H), 2.80–2.84 (m, 4H, NCH_2), 3.41–3.45 (m, 4H, NCH_2), 4.46–4.57 (m, 1H), 5.37 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.3, 21.0, 22.5, 22.7, 23.8, 24.2, 27.9, 28.2, 31.8, 31.8, 35.7, 36.1, 36.5, 36.9, 38.6, 39.4, 39.7, 42.2, 44.7, 45.8, 49.9, 56.1, 56.6, 74.7, 122.3, 139.8, 155.0; MS 499 (M^+, 100 %), 369 (20), HRMS: $(M + H)^+$ $C_{32}H_{56}O_2N_2$ Calculated 499.4258, Found 499.4250.

Cholest-5-en-3-yl 3,5-dimethyl-1-piperazinecarboxylate 6b. Yield: 89 %; mp: 174–176 °C IR: 2932, 2898, 1691, 1682, 1531 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.87 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 1.34 (d, 6H, J = 6.0, 2 × CH_3), 0.85–1.64 (m, 22H), 1.77–2.04 (m, 5H), 2.25–2.40 (m, 2H), 2.70–2.86 (m, 2H), 2.95–3.09 (m, 2H), 4.08–4.16 (m, 2H), 4.47–4.58 (m, 2H), 5.37–5.38 (d, 1H, J = 4); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 17.2, 18.6, 19.3, 21.0, 22.5, 22.7, 23.8, 24.2, 27.9, 28.1, 31.8, 31.9, 35.7, 36.1, 36.5, 36.9, 38.5, 39.4, 39.7, 42.2, 48.0 br, 49.9, 51.7, 56.1, 56.6, 75.4, 122.6, 139.6, 154.4; MS: 600 (15 %), 527 (100); HRMS: $(M + H)^+$ $C_{34}H_{59}N_2O_2$ Calculated 527.4571, Found 527.4563.

Cholest-5-en-3-yl 4-methyl-1-piperazinecarboxylate 6c. Yield: 79 %; mp: 135–137 °C; IR: 2932, 2864, 1697, 1430 cm^{-1} ; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.85–1.58 (m, 23H), 1.83–2.03 (m, 5H), 2.29 (s, 4H, NCH_2), 2.35 (s, 4H, 2 × NCH_2), 3.48 (s, 3H, NCH_3), 4.48–4.55 (m, 1H), 5.37 (s, 1H); ^{13}C NMR (75 MHz,



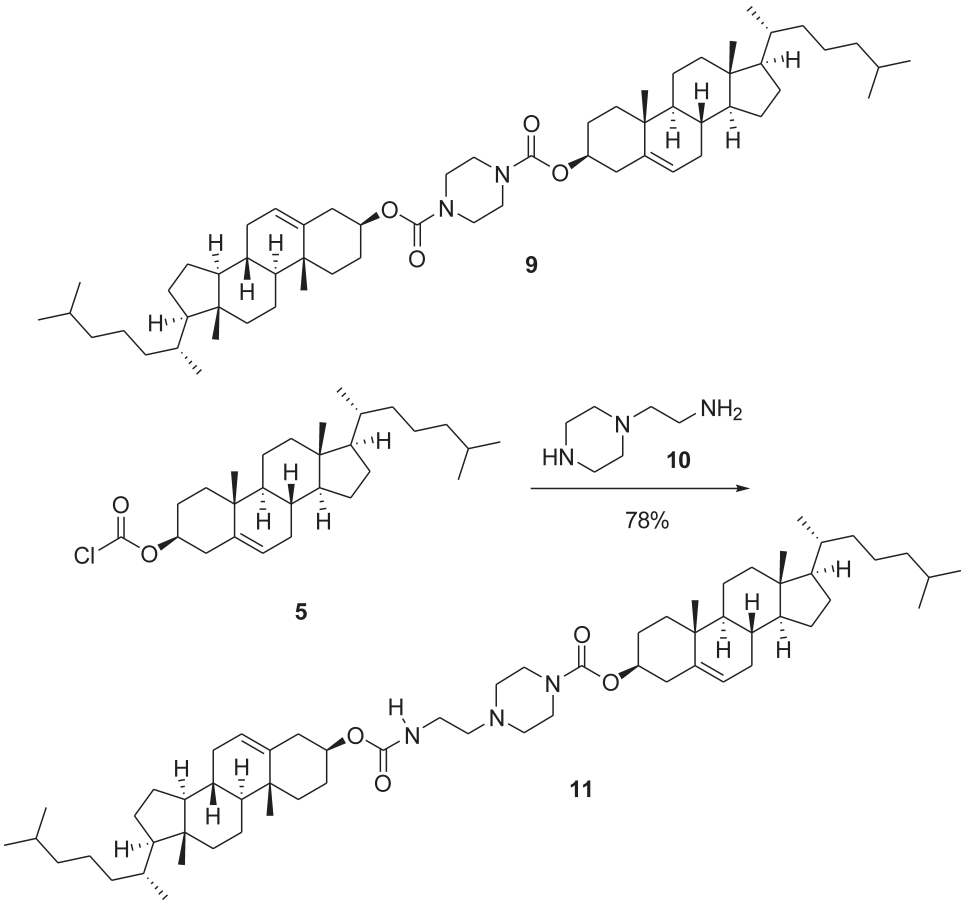
Scheme 2
Synthesis of *N*-amino-piperazine-cholesterol conjugate (**7**).

$CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.7, 23.8, 24.2, 28.0, 28.2, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.5, 39.7, 42.3, 43.5 br, 46.0, 50.0, 54.7, 56.1, 56.6, 74.8, 122.4, 139.8, 155.0; MS 586 (30 %), 513 (M^+, 100); HRMS: $(M + H)^+$ $C_{33}H_{57}O_2N_2$. Calculated 513.4415, Found 513.4407.

Cholest-5-en-3-yl 4-ethyl-1-piperazinecarboxylate 6d. Yield: 81 %; mp: 164–166 °C IR: 2933, 2898, 1694, 1684, 1455 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.87 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 1.08–1.68 (m, 25H), 1.83–2.03 (m, 5H), 2.24–2.46 (m, 8H), 3.47–3.51 (m, 4H, 2 × NCH_2), 4.46–4.55 (m, 1H), 5.36–5.37 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 11.8, 18.7, 19.4, 21.0, 22.5, 22.7, 22.9, 23.8, 24.2, 27.9, 28.2, 31.8, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.5, 39.7, 42.3, 50.0, 52.5, 56.1, 56.6, 74.8, 76.5, 122.4, 139.8, 154.9; MS: 527 (100 %), HRMS: $(M + H)^+$ $C_{34}H_{59}N_2O_2$ Calculated 527.4571, Found 527.4562.

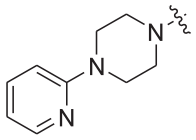
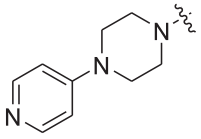
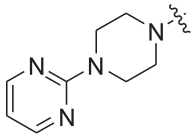
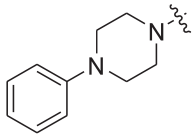
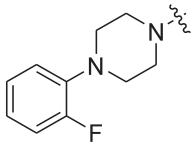
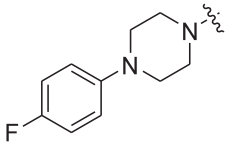
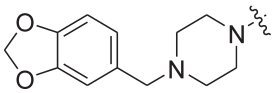
Cholest-5-en-3-yl 4-[2-(dimethylamino)ethyl]-1-piperazinecarboxylate 6e. Yield: 89 %; mp: 194–196 °C IR: 2943, 2890, 1738, 1727, 1458, 1423 cm^{-1}; ^1H NMR (300 MHz): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.85–1.68 (m, 21H), 1.82–2.04 (m, 5H), 2.24–2.35 (m, 2H), 2.27–2.49 (m, 4H), 2.79 (s, 6H, 2 × $N(CH_3)_2$), 2.79–2.83 (m, 2H), 2.97–3.01 (m, 2H), 3.49–3.52 (m, 4H), 4.46–4.54 (m, 1H), 5.37 (brs, 1H); ^{13}C NMR (75 MHz): 11.7, 18.6, 19.2, 20.9, 22.4, 22.7, 23.7, 24.2, 27.9, 28.1, 31.8, 31.8, 35.7, 36.1, 36.5, 36.9, 38.5, 39.4, 39.6, 42.2, 44.1, 49.9 (× 2), 52.8, 56.0, 56.6, 74.9, 122.3, 139.7, 154.8 (some C overlapping/not observed); MS: 618 (15 %), 570 (100), HRMS: $(M + H)^+$ $C_{36}H_{64}N_3O_2$ Calculated 570.4993, Found 570.4986.

Cholest-5-en-3-yl 4-[3-(dimethylamino)propyl]-1-piperazinecarboxylate 6f. Yield: 83 %; mp: 113–115 °C; IR: 2943, 2888, 1738, 1699, 1457, 1431 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H,



Scheme 3
Synthesis of *bis*-cholesterol piperazine conjugates.

Table 2 Cholesterol conjugated to piperazines substituted with pyridine- and aromatic-bearing side chains (refer to Scheme 1 for reaction scheme).

Entry	(6)	Piperazine	Yield
12	**k**		91%
13	**l**		91 %
14	**m**		93 %
15	**n**		89 %
16	**o**		92 %
17	**p**		90 %
18	**q**		92 %

CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.95–1.71 (m, 23H), 1.83–2.03 (m, 5H), 2.22 (brs, 6H), 2.29 (t, 2H, J = 5.5), 2.35–2.41 (m, 8H), 3.46–3.49 (m, 4H), 4.46–4.56 (m, 1H), 5.37 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.3, 21.0, 22.5, 22.7, 23.8, 24.2, 25.0, 27.9, 28.2, 31.8, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.5, 39.7, 42.2, 43.6 br, 45.4 (× 2), 49.9, 52.9, 56.1, 56.6, 57.8, 74.7, 122.3, 139.8, 154.9; MS: 584 (85 %), 369 (100), 216 (70), HRMS: $(M + H)^+$ $C_{37}H_{66}N_3O_2$ Calculated 584.5150, Found 584.5146.

Cholest-5-en-3-yl 4-(2-cyanoethyl)-1-piperazinecarboxylate 6g. Yield: 78 %; mp: 172–174 °C; IR: 3316, 2931, 2897, 1692, 1682, 1454 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.67 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.95–1.62 (m, 22H), 1.79–2.03 (m, 5H), 2.24–2.52 (m, 8H), 2.70 (t, 2H, J = 6.6), 3.47–3.50 (m, 4H), 4.45–4.58 (m, 1H), 5.36 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 15.9, 18.7, 19.3, 20.9, 22.5, 22.8, 23.7, 24.2, 27.9, 28.1, 31.8, 35.7, 36.1, 36.5, 36.9, 38.5, 39.4, 39.7, 42.3, 43.4 br, 49.9, 52.3, 53.3, 56.0, 56.6, 75.0, 118.5, 122.5, 139.8, 154.9 (CN); MS: 625 (50 %), 552 (100), 369 (40), HRMS: $(M + H)^+$ $C_{35}H_{58}N_3O_2$ Calculated 552.4524, Found 552.4516.

1-Cholest-5-en-3-yl 4-ethyl 1,4-piperazinedicarboxylate 6h. Yield: 80 %; mp: 126–128 °C; IR: 2932, 2861, 1682, 1454 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 1.27 (t, 3H, J = 7.1, OCH_2CH_3), 0.95–1.66 (m, 21H), 1.77–2.03 (m, 5H), 2.25–2.41 (m, 2H), 3.45 (brs, 8H), 4.15 (q, 2H, J = 7.1, OCH_2CH_3), 4.48–4.58 (m, 1H), 5.36–5.38 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 14.5, 18.5, 19.2, 20.8, 22.4, 22.6, 23.7, 24.2, 27.8, 28.0, 28.2, 31.8, 35.6, 36.1, 36.4, 36.9, 38.4, 39.4, 39.6, 42.1, 43.3 br, 49.8, 55.9, 56.5, 61.5, 76.4, 122.5, 139.6, 154.9, 155.3; MS: 1141 (30%), 414 (20), 372 (100), 299 (60), 166 (40); HRMS: $(2M+H)^+$ $C_{70}H_{117}O_8N_4$ Calculated 1141.8866, Found 1141.8877.

Cholest-5-en-3-yl 4-allyl-1-piperazinecarboxylate 6i. Yield: 83 %; mp: 210–212 °C; IR: 2940, 1697, 1419 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 1.06–1.59 (m, 22H), 1.83–2.03 (m, 5H), 2.25–2.40 (m, 2H), 2.97 (brs, 4H), 3.52 (d, 2H, J = 6.9), 3.88 (brs, 4H), 4.47–4.57 (m, 1H), 5.33–5.37 (m, 3H), 6.07–6.20 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.2, 20.9, 22.4, 22.7, 23.7, 24.2, 27.9, 28.0, 28.1, 31.8, 31.8, 35.7, 36.1, 36.4, 36.8, 38.4, 39.4, 39.6, 41.0 br, 42.2, 49.9, 51.3, 56.0, 56.6, 60.1, 75.7, 122.7, 124.9 br, 127.4 br, 139.4, 154.3; MS: 539 (100 %), HRMS: $(M + H)^+$ $C_{35}H_{59}N_2O_2$ Calculated 539.4571, Found 539.4563.

Cholest-5-en-3-yl 4-allyl-3,5-dimethyl-1-piperazinecarboxylate 6j. Yield: 87 %; mp: 88–90 °C; IR: 3316, 2932, 2865, 1738, 1692, 1455 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 1.25 (d, 6H, J = 6.0, 2 × CH_3), 0.92–1.62 (m, 20H), 1.78–2.11 (m, 5H), 2.22–2.39 (m, 3H), 2.60–2.65 (m, 1H), 2.90–2.97 (m, 2H), 3.04–3.10 (m, 2H), 3.28–3.33 (m, 1H), 3.70 (d, 1H, J = 12.9), 4.24–4.27 (m, 1H), 4.47–4.58 (m, 1H), 5.12 (d, 1H, J = 10.2), 5.18 (dd, 1H, J = 1.2, 18.9), 5.36–5.37 (brs, 1H), 5.74–5.87 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 7.4, 11.8, 16.2, 18.7, 19.3, 21.0, 22.5, 22.7, 23.9, 24.2, 27.9, 28.2, 28.3, 31.8, 31.9, 35.7, 36.1, 36.5, 36.9, 38.5, 38.6, 39.4, 39.6, 42.2, 44.4, 46.9, 48.8, 49.9, 52.2, 56.0, 56.6, 57.8, 74.5, 116.9, 122.3, 135.9, 139.8, 139.9, 155.5; MS: 567 (100 %), HRMS: $(M + H)^+$ $C_{37}H_{63}N_2O_2$ Calculated 567.4884, Found 567.4876.

Cholest-5-en-3-yl 4-(2-pyridinyl)-1-piperazinecarboxylate 6k. Yield: 91 %; mp: 95–97 °C; IR: 2943, 1727, 1705, 1459 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.00 (s, 3H, CH_3), 0.90–1.75 (m, 21H), 1.82–1.99 (m, 5H), 2.32–2.39 (m, 2H), 3.53–3.59 (m, 8H), 4.50–4.61 (m, 1H), 5.38 (brs, 1H), 6.62–6.66 (m, 2H, ArH), 7.46–7.49 (m, 1H, ArH), 8.18–8.20 (m, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 27.9, 28.2, 31.8, 31.9, 35.7, 36.1, 36.5, 36.9, 38.6, 39.4, 39.7, 42.3, 43.4 br, 45.0, 49.9, 56.1, 56.6, 75.1, 107.2, 113.6, 122.5, 137.5, 139.8, 147.9, 155.0, 159.2; MS: 576 (100 %), HRMS: $(M + H)^+$ $C_{37}H_{58}N_3O_2$576.4524, Found 576.4514.

Cholest-5-en-3-yl 4-(4-pyridinyl)-1-piperazinecarboxylate 6l. Yield: 91 %; mp: 188–190 °C; IR: 2941, 2868, 1738, 1699, 1597, 1542 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.08 (s, 3H, CH_3), 1.07–1.56 (m, 21H), 1.79–1.99 (m, 5H), 2.31–2.38 (m, 2H), 3.31–3.35 (m, 4H), 3.59–3.63 (m, 4H), 4.51–4.61 (m, 1H), 5.38 (brs, 1H), 6.66 (d, 2H, J = 6.6, ArH), 8.29 (d, 2H, J = 6.4, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.3, 21.0, 22.5, 22.7, 22.9, 23.8, 24.3, 27.9, 28.2, 31.9, 35.8, 36.2, 36.6, 36.9, 38.6, 39.5, 39.7, 42.3, 42.9, 45.7, 49.9, 56.1, 56.7, 75.2, 108.5 (× 2), 122.6, 139.7, 150.4 (× 2), 154.7, 154.9; MS: 576 (100 %), HRMS: $(M + H)^+$ $C_{37}H_{58}N_3O_2$ Calculated 576.4524, Found 576.4513

Cholest-5-en-3-yl 4-(2-pyrimidinyl)-1-piperazinecarboxylate 6m. Yield: 93 %; mp: 183–185 °C; IR: 2969, 2865, 1738, 1698, 1544, 1457 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.03 (s, 3H, CH_3), 0.95–1.63 (m, 21H), 1.79–1.94 (m, 5H), 2.28–2.43 (m, 2H), 3.52–3.56 (m, 4H), 3.80–3.84 (m, 4H), 4.51–4.61 (m, 1H), 5.39 (brs, 1H), 6.50–6.53 (m, 1H, ArH), 8.32 (d, 2H, J = 4.8, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 27.9, 28.2, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.5, 39.7, 42.3, 43.4 (× 2), 49.9, 56.1, 56.6, 75.0,110.2, 122.5, 139.8, 155.1, 157.7 (× 2), 161.6; MS: 577 (100 %), 369 (10), HRMS: $(M + H)^+$ $C_{36}H_{57}N_4O_2$ Calculated 577.4476, Found 577.4471.

Cholest-5-en-3-yl 4-phenyl-1-piperazinecarboxylate 6n. Yield: 89 %; mp: 169–171 °C; IR: 2946, 1775, 1683, 1598, 1433 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.87 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.03 (s, 3H, CH_3), 0.95–1.65 (m, 21H), 1.81–2.03 (m, 5H), 2.27–2.47 (m, 2H), 3.14 (brs, 4H), 3.63 (brs, 4H), 4.51–4.59 (m, 1H), 5.38 (brs, 1H), 6.87–6.98 (m, 3H, ArH), 7.26–7.31 (m, 2H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.7, 28.0, 28.2, 31.8, 31.9, 35.7, 36.1, 36.5, 36.9, 38.6, 39.4, 39.7, 42.3, 43.6, 49.4, 49.9, 56.0, 56.6, 75.0, 116.6 (× 2), 120.3, 122.5, 129.1 (× 2), 139.7, 151.2, 154.9; MS: 575 (100 %), HRMS: $(M + H)^+$ $C_{38}H_{59}N_2O_2$ Calculated 575.4571, Found 575.4565.

Cholest-5-en-3-yl 4-(2-fluorophenyl)-1-piperazinecarboxylate 6o. Yield: 92 %; mp: 123–125 °C IR: 2938, 1980, 1691, 1543 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 3H, CH_3), 0.87 (d, 6H, J = 6.0, 2 × CH_3), 0.92 (d, 3H, J = 6.0, CH_3), 1.03 (s, 3H, CH_3), 0.95–1.65 (m, 21H), 1.85–2.03 (m, 5H), 2.27–2.47 (m, 2H), 3.04 (brs, 4H), 3.64 (brs, 4H), 4.51–4.59 (m, 1H), 5.39 (brs, 1H), 6.91–7.09 (m, 4H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 27.9, 28.2, 31.8, 35.7, 36.1, 36.5, 36.9, 38.6, 39.4, 39.7, 42.2, 43.7 br, 49.9, 50.4, 56.0, 56.6, 75.0, 116.2 (d, J = 20.8), 119.1 (d, J = 2.7), 122.4, 122.9 (d, J = 3.7), 124.4 (d, J = 7.9), 139.8, 139.9 154.9, 155.8 (d, J = 246.2); MS: 1186 (20 %), 593 (100), 369 (20), HRMS: $(M + H)^+$ $C_{38}H_{58}N_2O_2F$ Calculated 593.4477, Found 593.4472.

Cholest-5-en-3-yl 4-(4-fluorophenyl)-1-piperazinecarboxylate 6p. Yield: 90 %; mp: 152–154 °C; IR: 2928, 2864, 1979, 1688, 1512, 1443 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.67 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.96–1.67 (m, 21H), 1.84–2.02 (m, 5H), 2.27–2.42 (m, 2H), 3.04 (brs, 4H), 3.60–3.63 (m, 4H), 4.50–4.58 (m, 1H), 5.37–5.38 (brs, 1H), 6.85–7.00 (m, 4H, ArH); 13C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 27.9, 28.2, 31.8, 31.9, 35.7, 36.1, 36.5, 36.9, 38.6, 39.4, 39.6, 42.2, 43.6 br, 50.0, 50.4, 56.0, 56.7, 75.1, 115.6 (d, J = 22.1), 118.5 (d, J = 7.8), 122.5, 139.6, 147.8, 155.0, 157.4 (d, J = 239.6); MS: 593 (100 %), 369 (15). HRMS: $(M + H)^+$ $C_{38}H_{58}N_2O_2F$ Calculated 593.4477, Found 593.4471.

Cholest-5-en-3-yl 4-(1,3-benzodioxol-5-ylmethyl)-1-piperazine carboxylate 6q. Yield: 92 %; mp: 115–117 °C; IR: 2940, 2866, 1738, 1698, 1597, 1542 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.67 (s, 3H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.02 (s, 3H, CH_3), 0.90–1.58 (m, 22H), 1.73–2.04 (m, 5H), 2.23–2.37 (m, 6H), 3.41 (s, 2H), 3.45 (s, 4H), 4.45–4.54 (m, 1H), 5.36–5.37 (brs, 1H), 5.94 (s, 2H, OCH_2O), 6.70–6.76 (m, 2H, ArH), 6.84 (brs, 1H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.3, 21.0, 22.5, 22.8, 23.7, 24.2, 27.9, 28.1, 31.8, 35.7, 36.1, 36.5, 36.9, 38.6, 39.4, 39.6, 42.2, 43.6 br, 49.9, 52.6, 56.0, 56.6, 62.7, 74.7, 100.8, 107.8, 109.3, 122.1, 122.3, 131.6, 139.8, 146.6, 147.8, 154.9; MS: 633 (100 %), HRMS: $(M + H)^+$ $C_{40}H_{61}N_2O_4$ Calculated 633.46258, Found 633.46170.

Cholest-5-en-3-yl 4-methyl-1-piperazinylcarbamate 7. Yield: 85 %; mp: 166–168 °C; IR: 2937, 2864, 1736, 1717, 1456, 1441 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.67 (s, 6H, CH_3), 0.86 (d, 6H, J = 6.0, 2 × CH_3), 0.91 (d, 3H, J = 6.0, CH_3), 1.00 (s, 3H, CH_3), 0.90–1.68 (m, 21H), 1.75–2.05 (m, 5H), 2.20–2.40 (m, 4H), 2.29 (s, 3H, NCH_3), 2.55 (brs, 4H), 2.84 (brs, 4H), 4.49–4.58 (m, 1H), 5.37 (brs, 1H), 5.54 (s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.6, 19.2, 21.0, 22.5, 22.7, 23.7, 24.2, 27.9, 28.1, 31.8, 35.7, 36.1, 36.5, 36.9, 38.5, 39.4, 39.7, 42.2, 45.6, 49.9, 54.3 (× 2), 55.8, 56.1, 56.6, 74.6, 122.5, 139.6, 154.9 br; MS: 528 (100 %), HRMS: $(M + H)^+$ $C_{33}H_{58}N_3O_2$ Calculated 528.4524, Found 528.4517.

Dicholest-5-en-3-yl 1,4-piperazinedicarboxylate 9. Yield: 16 %; mp: 164–166 °C; IR: 2935, 1696, 1567, 1467 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 6H, 2 × CH_3), 0.86 (2 × d, 12H, J = 6.0, 4 × CH_3), 0.92 (d, 6H, J = 6.0, 2 × CH_3), 1.02 (s, 6H, 2 × CH_3), 0.94–1.64 (m, 42H), 1.77–2.03 (m, 10H), 2.25–2.39 (m, 4H), 3.44 (brs, 8H, NCH_2), 4.48–4.58 (m, 2H), 5.38 (brs, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 28.0, 28.2, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.5, 39.7, 42.3, 43.4 br, 50.0, 56.1, 56.6, 75.2, 122.5, 139.7, 154.9.

Cholest-5-en-3-yl 4-(2-{[(cholest-5-en-3-yloxy)carbonyl] amino}ethyl)-1-piperazinecarboxylate 11. Yield: 78 % (based on cholesteryl chloroformate); mp: 140–142 °C; IR: 2943, 2866, 1720, 1703, 1687, 1516, 1464 cm^{-1} .^1H NMR (300 MHz, $CDCl_3$): 0.68 (s, 6H, 2 × CH_3), 0.86 (d, 12H, J = 6.0, 4 × CH_3), 0.91 (d, 6H, J = 6.0, 2 × CH_3), 1.01 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.95–1.59 (m, 42H), 1.71–2.03 (m, 10H), 2.24–2.49 (m, 10H), 3.27–3.28 (m, 2H), 3.45–3.47 (m, 4H), 4.47–4.55 (m, 2H), 5.07 (brs, 1H, NH), 5.37 (brs, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 28.0, 28.2, 31.8, 35.7, 36.1, 36.5, 37.0, 37.3 br, 38.5, 38.6, 39.5, 39.7, 42.3, 43.6 br, 50.0, 52.6, 56.1, 56.6, 57.1, 74.3, 74.9, 122.4, 139.8, 139.8, 154.9, 156.0; MS: 954 (100 %), 663 (20), 413 (40), 282 (70); HRMS: $(M + H)^+$ $C_{62}H_{104}O_4N_3$ Calculated 954.8021, Found 954.8026.

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