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Structure–activity relationship studies of Niemann-Pick type C1-like 1 (NPC1L1) ligands identified by screening assay monitoring pharmacological chaperone effect

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ABSTRACT

A number of hereditary diseases are caused by defective protein trafficking due to a folding defect resulting from point mutations in proteins. Ligands that bind to the folding intermediates of such mutant proteins and rescue their trafficking defects, known as pharmacological chaperones, have promise for the treatment of certain genetic diseases, including Fabry disease, cystic fibrosis, and Niemann-Pick disease type C. Here we show that this pharmacological chaperone effect can be used for ligand screening, that is, binding of candidate ligands can be detected by monitoring the ligand-mediated correction of a localization defect caused by artificially introduced point mutations of the protein of interest. Using this method, we discovered novel steroidal ligands of Niemann-Pick type C1-like 1 (NPC1L1), an intestinal cholesterol transporter that is the target of the cholesterol absorption inhibitor ezetimibe, and conducted structure–activity relationship studies. We also present data indicating that the binding site of the new ligands is distinct from both the N-terminal sterol-binding domain and the ezetimibe-binding site.

1. Introduction

NPC1L1 is a 13-pass transmembrane protein expressed on the apical membrane of enterocytes and hepatocytes, and plays a critical role in the intestinal uptake of dietary cholesterol.[1–6] NPC1L1 has three large extracellular loops, and the first N-terminal loop, which is generally designated as the N-terminal domain (NTD), has been demonstrated to bind cholesterol and several oxysterols.[7–10] Extracellular loop 2 is important for binding of ezetimibe (Zetia), a widely used cholesterol absorption inhibitor.[11–16] Although much effort has been directed to discovery of novel NPC1L1 inhibitors, all the compounds reported so far have been discovered by means of competitive binding assay using fluorescent or radioisotope-labeled ezetimibe analogues, and all of them are considered to bind at the same site as ezetimibe.[17–21]

NPC1 (Niemann-Pick type C1) is a close homologue of NPC1L1 (42% identity, 51% similarity), and is known to be required for intracellular transport of cholesterol.[22] Loss of function of NPC1 resulting from mutations in the protein is a major cause of Niemann-Pick disease type C, a fatal progressive neurodegenerative disease. In the case of the most prevalent mutation, I1061T, the molecular mechanism of loss of function has been proposed to involve a folding and localization defect, rather than an intrinsic loss of function.[23] In such cases, an effective therapeutic strategy might be to use pharmacological chaperones, that is, small-molecular ligands that correct the trafficking defect resulting from reduced folding efficiency, presumably by binding to the folding intermediate in the endoplasmic reticulum (ER) and promoting its escape from the ER quality control system.[24–28] Recently, we reported that oxysterols and their derivatives can be used as pharmacological chaperones to correct the localization defect of NPC1^{I1061T} mutant protein.[29] We also demonstrated the existence of a second, non-NTD sterol-binding site in NPC1 and suggested that this sterol-binding site might be functionally important.

Based on these findings, we hypothesized that NPC1L1 also has a non-NTD sterol-binding site and ligands that bind at this site might be novel NPC1L1 inhibitors. To test this idea, we developed a screening system based on the pharmacological chaperone effect as a tool for detecting ligand binding to NPC1L1 protein. We artificially generated folding-defective NPC1L1 mutants, and by monitoring ligand-mediated correction of the localization defect of the mutant proteins, we were able to discover novel steroidal ligands for NPC1L1. Here, we report the identification and structure–activity relationships of these steroidal NPC1L1 ligands, and provide evidence that the binding site of this class of NPC1L1 ligands is distinct from both the NTD and the ezetimibe-binding site.

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2. Results and discussion

2.1. Cell-based ligand screening utilizing folding-defective NPC1L1 mutants

In our previous study,[29] we reported that oxysterols directly bind to the second, non-NTD sterol-binding site of NPC1, and correct the trafficking defect of folding-defective NPC1^{I1061T} mutant. Here, our strategy to identify NPC1L1 ligands directed to the putative second sterol-binding site of NPC1L1 was to screen sterols by monitoring ligand-mediated rescue of the localization defect of folding-defective NPC1L1 mutants. Based on the high homology between NPC1 and NPC1L1, we hypothesized that the second sterol-binding site of NPC1L1 would accept a subset of sterols, and we envisioned that introduction of mutation(s) at the position corresponding to the I1061T mutation of NPC1 would yield the required folding-defective NPC1L1 mutant.

First, to obtain the folding-defective NPC1L1 mutant, we introduced mutations around L1072 of NPC1L1, which corresponds to I1061 of NPC1. Although the L1072T and L1072M mutants of NPC1L1-GFP showed normal plasma membrane localization (data not shown), we obtained other mutants (including L1072S and L1072T/L1168I) that localize predominantly in the ER, a characteristic localization for folding-defective membrane proteins (Fig. 1a). To exclude terminally misfolded mutants and to select folding-defective mutants amenable to ligand-mediated defect correction, we examined the temperature sensitivity of the ER-retained mutants, because it has been demonstrated that the localization defect of folding-defective mutants can be corrected if they are expressed at reduced temperature.[30,31] As shown in Figure 1b, the localization of L1072T/L1168I mutant NPC1L1 changed from ER to plasma membrane at reduced temperature, indicating the suitability of this mutant NPC1L1 for our ligand screening assay.

2.2. Identification of oxysterols as lead compounds for further optimization

Next, we tested a variety of sterols for the ability to correct the localization defect of the L1072T/L1168I mutant, that is, for the ability to act as pharmacological chaperones. Among the oxysterols

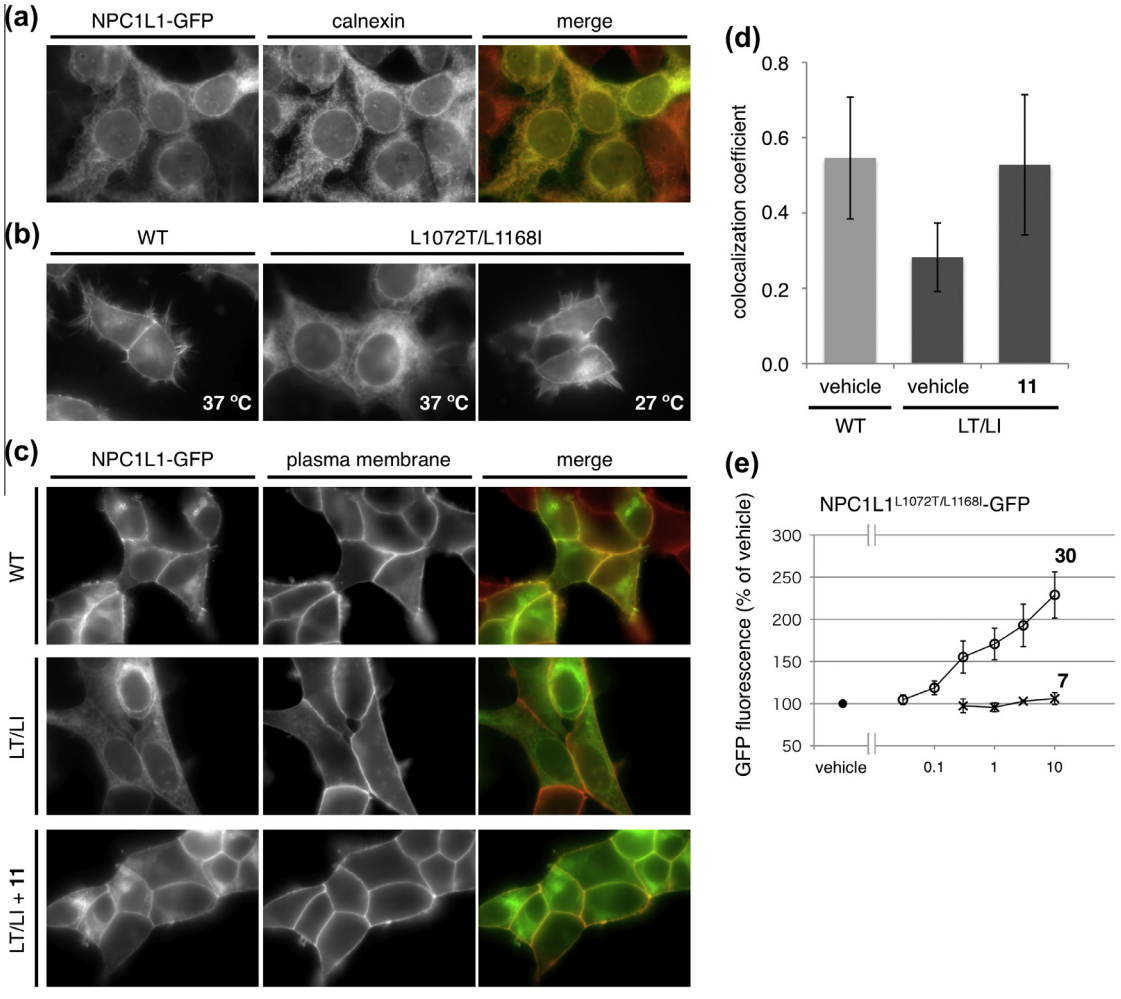


Figure 1. Correction of subcellular localization of NPC1L1$^{L1072T/L1168I}$ mutant. (a) Colocalization of L1072T/L1168I (LT/LI) mutant with a ER marker, calnexin. (b) Subcellular localization of WT or LT/LI mutant NPC1L1-GFP. The ER retention of the LT/LI mutant was corrected to plasma membrane localization when the mutant was expressed at reduced temperature. (c) Subcellular localization of WT or LT/LI mutant NPC1L1-GFP, and the effect of the steroidal carbamate **11** on the localization of LT/LI mutant. HEK293 cells stably expressing the indicated construct were incubated with **11** or vehicle for 24 h. The cells were stained with CellMask Orange plasma membrane stain and the localization of NPC1L1-GFP was examined by fluorescence microscopy. (d) The extent of colocalization was quantified, and the colocalization coefficient (thresholded Manders' coefficient) is shown with standard deviations depicted by error bars (n = 13). (e) Effects of the steroidal carbamate **30** and inactive oxysterol **7** on steady-state expression levels of LT/LI. The steady-state expression levels of NPC1L1$^{L1072T/L1168I}$-GFP were quantified by measuring GFP fluorescence in the lysate of cells treated with or without the indicated compounds. The GFP fluorescence was normalized with respect to total protein concentration determined by bicinchoninic acid assay. Data points represent the averages (n = 3) with the standard error of the means depicted by error bars.

tested, 25-hydroxycholesterol (25HC, **11**) corrected the trafficking defect of the mutant protein (Fig. 1c and d). The extent of NPC1L1 localization was quantified as described in Figure 2 and Experimental, and EC_{50} value of 25HC was calculated to be 2.3 μM (Fig. 2b, c, and Table 1). As in the case of NPC1, cholesterol itself was not active toward NPC1L1, possibly because cholesterol directly added to cell culture does not reach the ER or is not available for binding to proteins (unpublished observation).[32,33] It is well known that sufficient membrane permeability to reach the ER is a prerequisite for pharmacological chaperones, so the above result supports the view that 25HC is indeed acting as a pharmacological chaperone.[26,34]

While B-ring monohydroxylated sterols, including 6- or 7-hydroxylated sterols (**2**, **3**, **5**), were weakly active, 19-, 20-, and 22-hydroxylated sterols (**7**, **8**, **9**, **10**) proved to be completely inactive, implying that the corresponding binding site of NPC1L1 has a

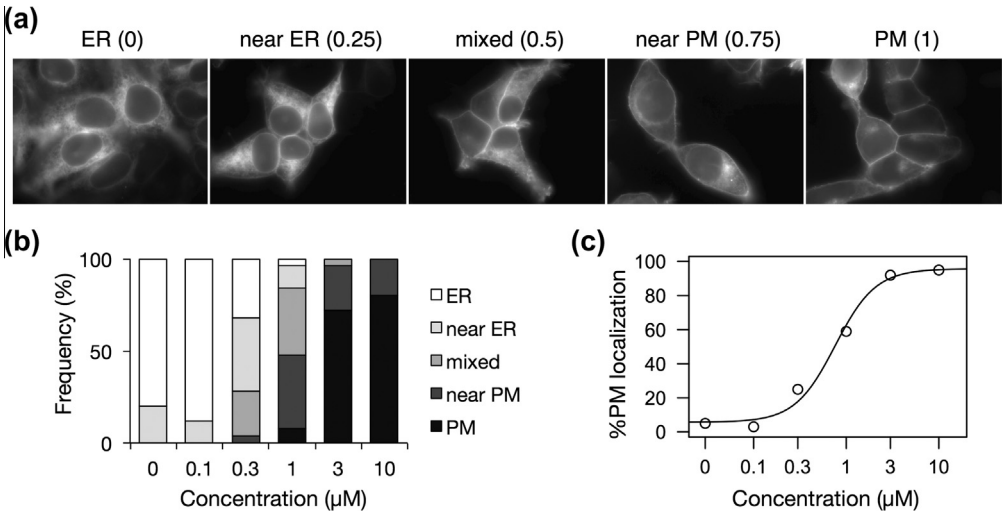
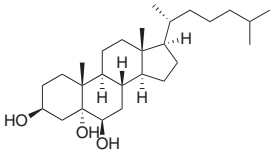
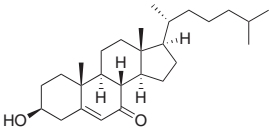
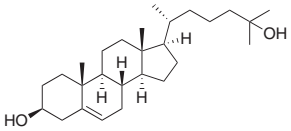
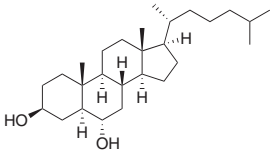
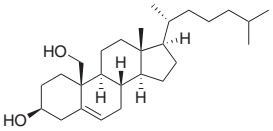
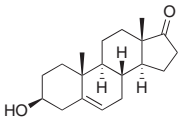
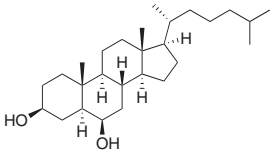
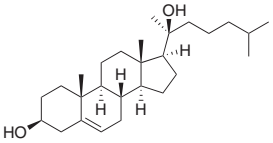
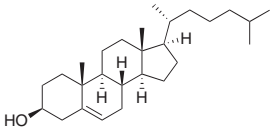
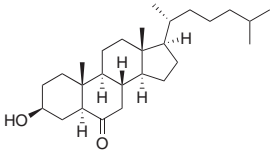
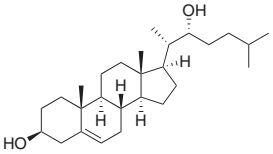
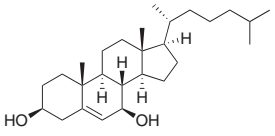
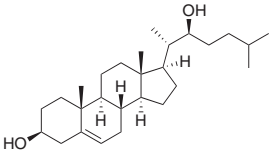


Figure 2. Quantification of localization change of NPC1L1 mutant. (a) Representative images of cells with ER, near ER, mixed, near PM, and PM localization of NPC1L1-GFP. (b and c) A representative result of quantification showing the dose-dependent correction of NPC1L1 mislocalization by steroidal carbamate **30** (Table 3). Cells expressing $NPC1L1^{L1072T/L1168I}$-GFP were treated with steroidal carbamate **30**, and images were acquired. The images were quantified as described in the Section 4.

Table 1
Pharmacological chaperone effect of oxysterols and their derivatives on $NPC1L1^{L1072T/L1168I}$ mutant

No.	Compound	EC_{50} (μM)	No.	Compound	EC_{50} (μM)	No.	Compound	EC_{50} (μM)
1		NA	**6**		9.7	**11**		2.3
2		8.5	**7**		NA	**12**		NA
3		9.7	**8**		NA	**13**		NA
4		2.8	**9**		NA			
5		10	**10**		NA			

NA: no activity (3 μM for **1** and 10 μM for the others). The EC_{50} values were determined by phenotypic analysis as described in Figure 2 and the Section 4.

hydrophobic character. Notably, 5α,6β-dihydroxylated sterol (**1**) did not correct the localization defect of the mutant NPC1L1 protein, further supporting the idea that the introduction of polar functionalities into the B ring is disfavored. These results are in marked contrast to the structure–activity relationships obtained for NPC1; that is, 22(*S*)-hydroxylated sterol (**10**) was as active as 25HC (**11**) and 5α,6β-dihydroxylated sterol (**1**) was superior to 25HC (**11**) as a pharmacological chaperone for NPC1, whereas these oxysterols are inactive in the case of NPC1L1. The implication is that compounds selective for NPC1 or NPC1L1 may be obtainable by appropriately modifying the positions of hydroxyl group(s) and/or introducing substituents at appropriate positions.

2.3. Structure–activity relationship of the isooctyl side chain moiety

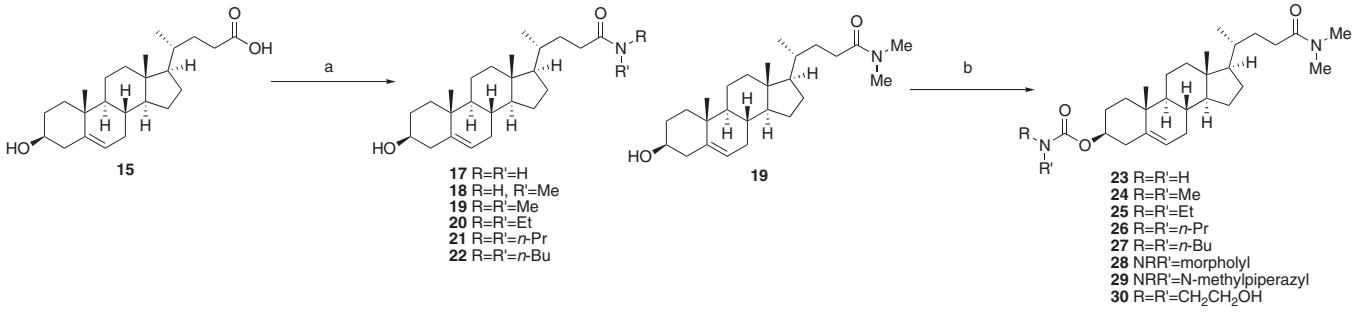
The isooctyl side chain of sterols is important for the biological activity, as demonstrated by the fact that sterol (**12**), which lacks the side chain, is inactive. In the light of this observation, we next synthesized side-chain-modified sterols, and examined the structure–activity relationship of the side-chain moiety (Scheme 1).

The one-carbon-truncated analogue of 25HC (**14**), its analogue without the dimethyl group (**16**), and the corresponding carboxylic acid (**15**) were found to be inactive, supporting the importance of side-chain structure for activity (Table 2). Among the side-chain amide derivatives tested, *N*,*N*-dimethyl amide (**19**) showed increased potency compared to 25HC, but larger substitutents on nitrogen decreased the potency, as exemplified by *N*,*N*-dibutyl amide (**22**). Combined with the finding that *N*-monomethyl amide (**18**) and N-non-substituted amide (**17**) were only marginally active, a hydrogen bond donor at this position seems to be disfavored, and this may in part explain why dimethyl amide (**19**) was superior to 25HC.

2.4. Optimization of substituents at the 3 position

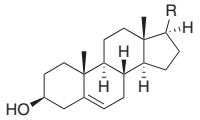
In the case of NPC1, introduction of a morpholino amide group at the 3 position improved the activity by nearly one order of magnitude.[29] Therefore, we envisioned that modification at the 3 position might result in more potent pharmacological chaperones for NPC1L1, and we synthesized a series of 3-OH-modified steroidal carbamates (Scheme 1). As shown in Table 3, N,N-dialkylated carbamates (**24**, **25**, **26**) showed increased potency compared to N-non-substituted carbamate (**23**). As the alkyl chains were elongated, however, the potency decreased and the *N*,*N*-dibutyl derivative (**27**) was completely inactive. Replacement of the terminal –CH_3 groups of (**26**) with –OH (**30**) increased the potency (Table 3 and Fig. 2), implying a favorable interaction in the binding site of NPC1L1. On the other hand, polar carbamate derivatives with a more rigid ring structure, such as morpholine or 1-methylpiperazine (**28** and **29**, respectively), were less potent than bis(2-hydroxyethyl)carbamate (**30**), indicating the importance of appropriately positioned polar groups. Although modification of the 3-position resulted in increased potency, the preferred substituent was different for NPC1L1 and NPC1. Thus, the steroidal carbamate (**30**) showed 2.5-fold selectivity for NPC1L1 (EC_{50} 0.65 μM) over NPC1 (EC_{50} 1.6 μM).

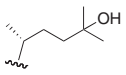
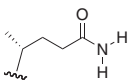
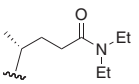
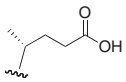
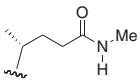
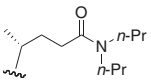
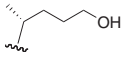
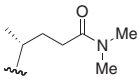
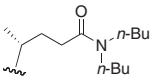
As the compound with bis(2-hydroxyethyl)carbamate at the 3-position (**30**, Table 3) was more potent than the parental non-substituted sterol (**19**, Table 2), we next tested whether this type



Scheme 1. Synthetic route to the side chain-modified sterols and their carbamate derivatives. Reagents and conditions: (a) isobutyl chloroformate, RR′NH (for **17**, 28% aqueous ammonia solution), Et_3N, THF, rt; (b) triphosgene, pyridine, RR′NH (for **23**, 28% aqueous ammonia solution), Et_3N, CH_2Cl_2, rt.

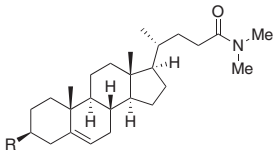
Table 2
Pharmacological chaperone effect of the side-chain-modified sterols on NPC1L1$^{L1072T/L1168I}$ mutant

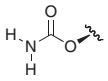
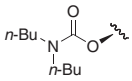
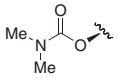
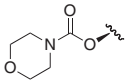
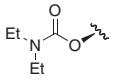
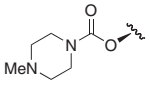
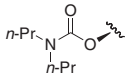
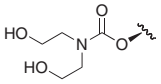


No.	R	EC_{50} (μM)	No.	R	EC_{50} (μM)	No.	R	EC_{50} (μM)
14		>10	**17**		>10	**20**		(Toxic)
15		>10	**18**		>10	**21**		(Toxic)
16		NA	**19**		0.80	**22**		>3

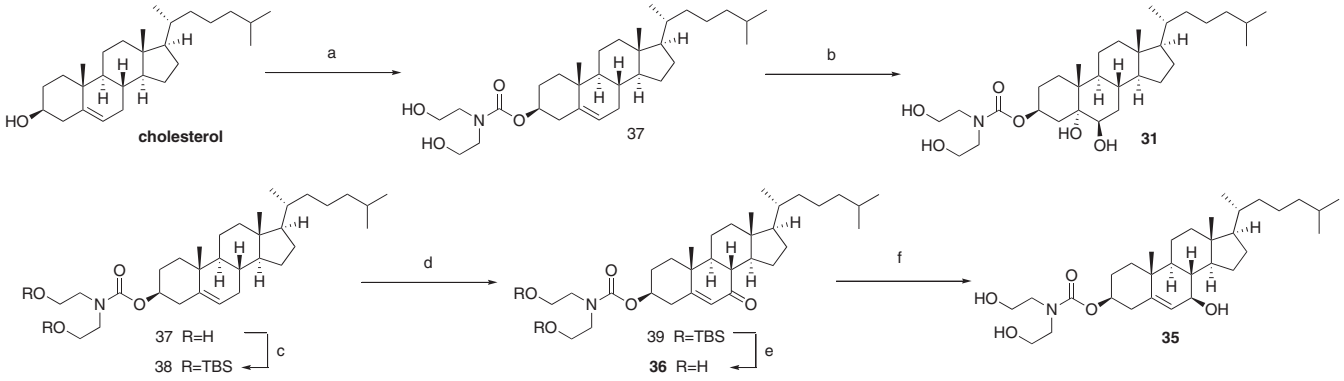
EC_{50}s of **20** and **21** could not be determined because of their cytotoxicity.

Table 3
Pharmacological chaperone effect of 3-OH-modified steroidal carbamates on LT/LI and ΔNTD-LT/LI mutants



No.	R	EC_{50} (μM)		No.	R	EC_{50} (μM)	
		LT/L1	ΔNTD-LT/L1			LT/L1	ΔNTD-LT/L1
23		8.5	>10	**27**		NA	NA
24		1.1	4.7	**28**		2.3	6.2
25		1.8	4.2	**29**		2.9	>10
26		3.0	8.0	**30**		0.65	1.7

NA: no activity (10 μM).



Scheme 2. Synthetic route to **31**, **35**, and **36**. Reagents and conditions: (a) triphosgene, pyridine, diethanolamine, THF, CH_2Cl_2, rt; (b) *m*-CPBA, CH_2Cl_2, rt then $HIO_4{\cdot}2H_2O$, THF, rt; (c) TBSCl, imidazole, DMF, CH_2Cl_2, rt; (d) $RuCl_3$, TBHP, 1,2-dichloroethane, rt; (e) TBAF, THF, rt; (f) $NaBH_4$, $CeCl_3$, MeOH, THF, rt.

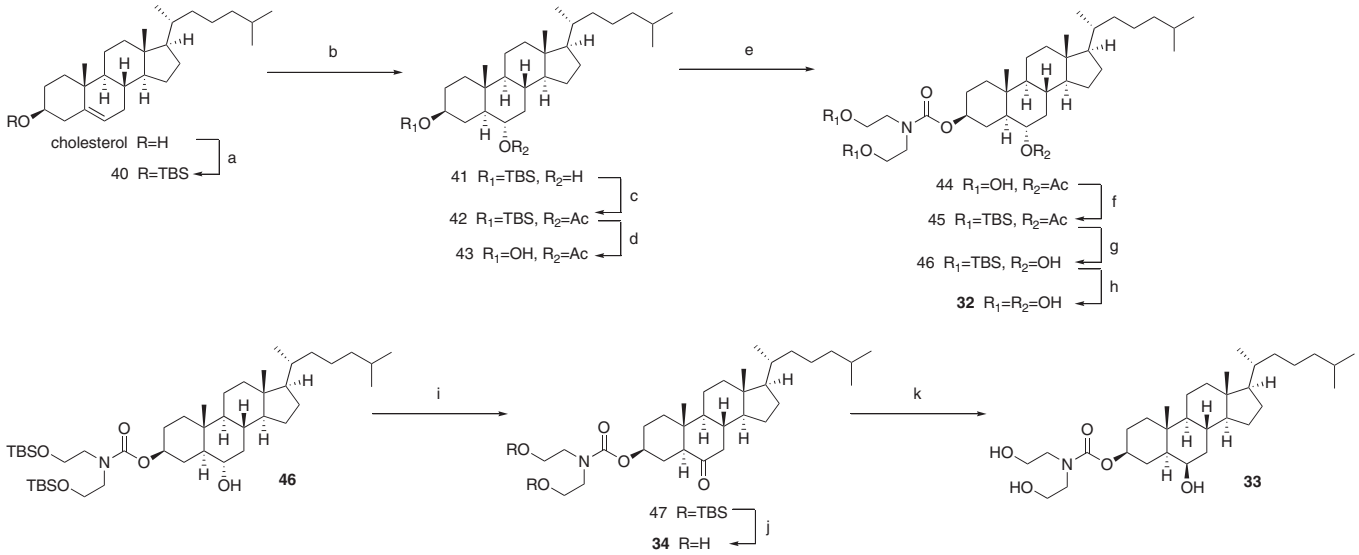
of substitution can also increase the potency of other sterols. The synthetic routes to oxysterol derivatives with bis(2-hydroxyethyl)carbamate at the 3-position are shown in Schemes 2 and 3. As shown in Table 4, all the carbamate derivatives shown here (compounds **31**–**37**, Table 4) were more potent than their non-substituted counterparts (compounds **1**–**6**, **13**, Table 1). They were, however, less potent than **30**, which is in harmony with the fact that **19** (Table 2) was more potent than B-ring hydroxylated cholesterols (**1**, **2**, **3**, **5**) and B-ring keto-cholesterols (**4**, **7**) (Table 1).

2.5. Ligand-mediated stabilization of NPC1L1 mutant

Since we had obtained compounds that could correct the localization of $NPC1L1^{L1072T/L1168I}$, we next examined whether the effect was indeed mediated through binding of the compounds to the NPC1L1 mutant. It has been shown that ligands added to cell culture can stabilize destabilized mutant proteins and increase the steady-state levels of the mutant proteins.[35–38] To test whether the steroidal carbamate (**30**) could stabilize the NPC1L1 mutant protein, we examined the steady-state level of the mutant protein by measuring the GFP fluorescence of the cell lysate. Though the inactive oxysterol (**7**) did not affect the steady-state level of the mutant protein, the most active steroidal carbamate (**30**) dose-dependently increased the level of the NPC1L1 mutant (Fig. 1e). These data support our hypothesis that the observed correction of the localization defect was mediated through binding of the sterol derivatives to the NPC1L1 mutant.

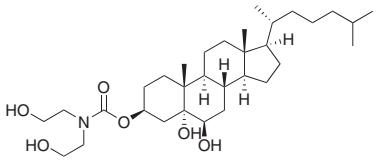
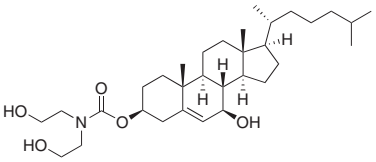
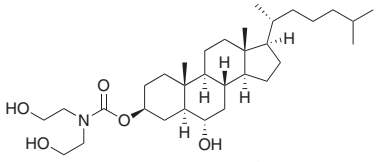
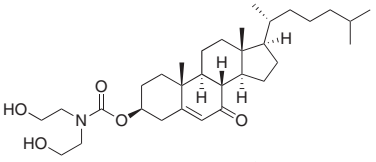
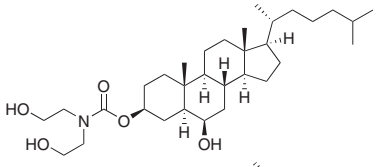
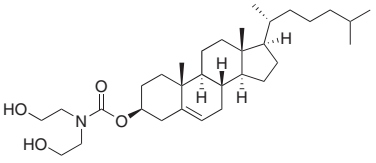
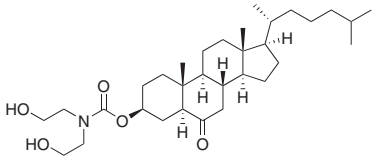
2.6. The steroidal carbamates act independently of the N-terminal sterol-binding domain

To test whether the NTD is required for ligand-mediated rescue of $NPC1L1^{L1072T/L1168I}$, we examined the effect of the sterol derivatives on NTD-deleted $NPC1L1^{L1072T/L1168I}$-GFP (ΔNTD-$NPC1L1^{L1072T/L1168I}$-GFP, Fig. 3a). While ΔNTD-$NPC1L1^{L1072T/L1168I}$ was localized predominantly in the ER in the absence of the ligands, the mutant protein showed plasma membrane localization in the presence of the ligands (Fig. 3b). Additionally, the deletion of NTD did not affect the rank-order of EC_{50} value among the sterol derivatives (Table 3). These results indicate that the NTD is not required for the effect of our sterol derivatives. Furthermore, the steroidal carbamate (**30**) also increased the steady-state level of ΔNTD-$NPC1L1^{L1072T/L1168I}$ (Fig. 3c). Therefore, it is likely that there is a second sterol-binding site on NPC1L1, as in NPC1, and that the



Scheme 3. Synthetic route to **32**, **33** and **34**. Reagents and conditions: (a) TBSCl, imidazole, DMF, CH_2Cl_2, rt; (b) BH_3·THF, THF, rt then NaOH aq and H_2O_2 aq rt; (c) Ac_2O, DMAP, pyridine, rt; (d) TBAF, THF, rt; (e) triphosgene, pyridine, diethanolamine, CH_2Cl_2, rt; (f) TBSCl, imidazole, DMF, CH_2Cl_2, rt; (g) K_2CO_3, MeOH, CH_2Cl_2, rt; (h) TBAF, THF, 45 °C; (i)PCC, AcONa, CH_2Cl_2, rt; (j) TBAF, THF, 45 °C; (k) $NaBH_4$, MeOH, rt.

Table 4
Pharmacological chaperone effect of bis(2-hydroxyethyl)carbamate derivatives of oxysterols on LT/LI

No.	Compound	EC_{50} (μM)	No.	Compound	EC_{50} (μM)
31		8.1	**35**		2.0
32		1.1	**36**		3.8
33		3.8	**37**		NA
34		2.0			

steroidal carbamates developed in this study bind to this putative second sterol-binding site.

2.7. The binding site of the steroidal carbamates is distinct from that of ezetimibe

The NPC1L1 inhibitor ezetimibe is reported to bind to the extracellular loop 2 of NPC1L1.[14] So, we next examined whether ezetimibe accesses the same binding site as our steroidal carbamates. As shown in Figure 4, ezetimibe did not correct the mislocalization of the NPC1L1 mutant, nor did it compete with the steroidal carbamate (**30**). For other steroidal carbamate derivatives, any detectable level of competition by ezetimibe was not observed either (data not shown). These results clearly indicate that the binding site of our steroidal carbamate is different from that of ezetimibe.

3. Discussion

In this study, we employed ligand-mediated correction of the localization defect of folding-defective mutant protein as a screening tool to identify novel NPC1L1 ligands. This is the first time that

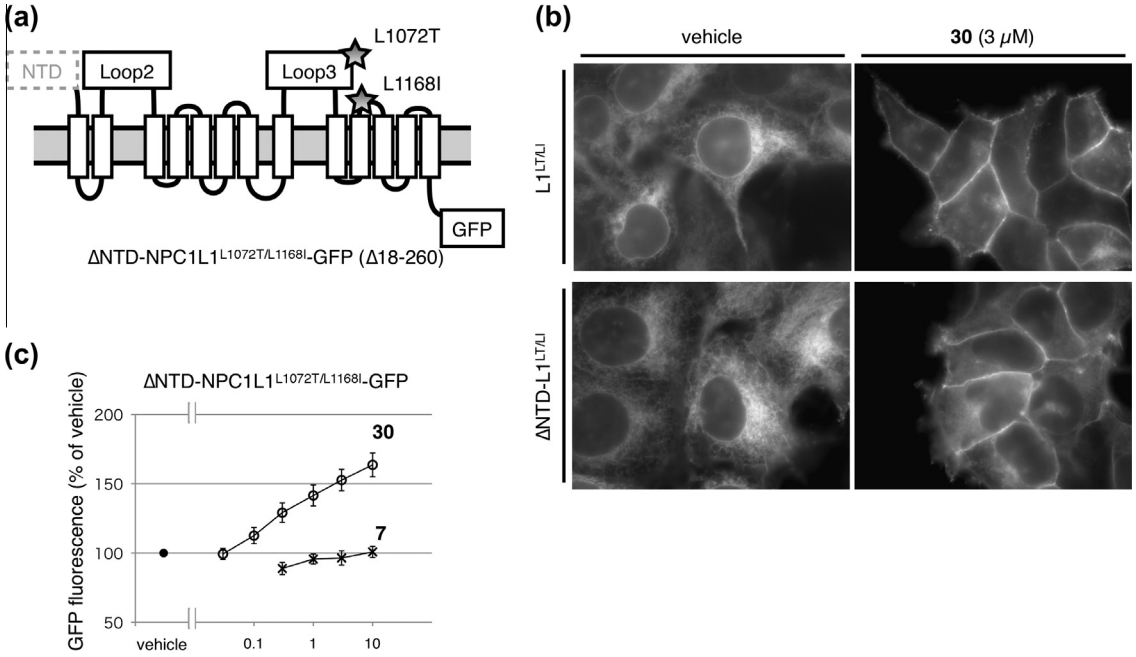


Figure 3. The steroidal carbamate acts independently of the NTD. (a) Schematic representation of NTD-deleted NPC1L1$^{L1072T/L1168I}$ mutant (ΔNTD-LT/LI). (b) Effects of **30** on full-length or NTD-deleted LT/LI mutant NPC1L1-GFP. HEK293 cells stably expressing the indicated construct were incubated with 3 μM **30** or vehicle for 24 h. The samples were fixed and the localization of NPC1L1-GFP was examined by fluorescence microscopy. (c) Effects of **30** and **7** on steady-state expression levels of ΔNTD-LT/LI.

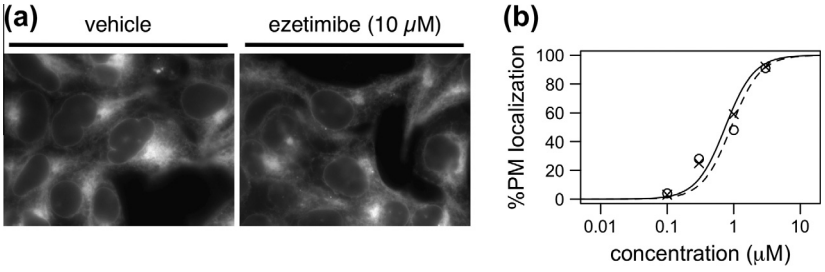


Figure 4. (a) Effect of ezetimibe on the defective localization of LT/LI mutant. Ezetimibe did not correct the trafficking defect of the mutant NPC1L1. (b) Dose-dependent correction of LT/LI localization defect by the steroidal carbamate **30**, with (dashed line) or without (solid line) 10 μM ezetimibe.

the pharmacological chaperone effect has been utilized for cell-based ligand screening, and this strategy offers several advantages. First, this assay obviates the need to purify the target protein, which is notoriously difficult for multi-pass membrane proteins. Second, this method does not require the use of a highly specific radioisotope- or fluorescent-labeled ligand. Third, this assay can be performed for proteins with unknown function or for which no functional assay suitable for ligand screening is available, since it is only necessary to know the localization of the protein of interest. Thus, this assay can be complementary to conventional ligand-binding assays or functional screening assays.

Based on our previous study on NPC1, we hypothesized the presence of a second sterol-binding site distinct from the known N-terminal sterol-binding domain on NPC1L1. In this report, we present several lines of evidence in support of this idea, indicating that NPC1L1 contains a second sterol-binding site with a different sterol specificity from that in the case of NPC1. We further show that our steroidal carbamates are likely to exert their effect through binding to this site. Thus, these compounds represent a novel class of NPC1L1 inhibitor, since their binding site on NPC1L1 is different from that of ezetimibe. Studies to examine the inhibitory activity of these compounds on NPC1L1-mediated cholesterol absorption are in progress.

4. Experimental

4.1. Biology

4.1.1. Materials

Commercially available oxysterols were from Sigma and Santa Cruz Biotechnology; Lipofectamine LTX and CellMask Orange plasma membrane stain were from Invitrogen; poly-D-lysine was from Millipore.

4.1.2. Cell culture and transfection

HEK293 cells were cultured in DMEM supplemented with 5% FBS and penicillin and streptomycin at 37 °C in a humidified incubator (5% CO_2). Cells were transfected with Lipofectamine LTX (Invitrogen). To obtain cell lines stably expressing NPC1L1 construct, G418-resistant colonies were isolated and maintained in the presence of G418 (0.4 mg/mL).

4.1.3. DNA constructs

The cDNA of human NPC1L1 (GenBank BC117178) in pCR-XL-TOPO vector was obtained from Mammalian Gene Collection (IMAGE id 40125729). The coding sequence of human NPC1L1 was amplified from this construct, and subcloned into

the pCMV6-AC-turboGFP vector (from Origene) at the SgfI and NotI sites, and the linker and turboGFP were replaced with a FLAG tag-containing linker (DYKDDDDKGGRDPPVAT) and EGFP.[39] Site-directed mutagenesis was carried out using a KOD—Plus—Mutagenesis Kit (from Toyobo).

4.1.4. CellMask Orange plasma membrane stain

At day 1, cells were seeded onto poly-D-lysine-coated glass-bottomed dishes, and treated as indicated at day 2. At day 3, CellMask Orange plasma membrane stain (0.25 μg/mL, pre-diluted with DMEM supplemented with 1% FBS) was added to the dishes and incubation was continued at 37 °C for 5 min. Then the cells were fixed (4% paraformaldehyde-PBS, 37 °C, 10 min) and washed three times with PBS. Images were immediately acquired, and the extent of colocalization was quantified as described previously.[29]

4.1.5. Immunocytochemical staining of calnexin

Cells were fixed (3.7% formaldehyde in PBS, rt, 30 min), permeabilized (0.1% Triton X-100 in PBS, rt, 10 min), and blocked in 1% BSA-PBST for 1 h. The samples were incubated with goat anti-calnexin (C-20) antibody (1:100 in blocking buffer, from Santa Cruz) for 2 h, and then washed three times with PBS. The samples were incubated with anti-goat IgG AlexaFluor 546 (1:250 in blocking buffer) for 1 h, then washed with PBS, and images were acquired.

4.1.6. NPC1L1$^{L1072T/L1168I}$ localization assay

At day 1, HEK293 cells stably expressing NPC1L1$^{L1072T/L1168I}$ under control of the CMV promoter were plated at a density of 16,000 cells/well in DMEM supplemented with 1% FBS onto a poly-D-lysine-coated glass-bottomed plate. At day 2, cells were treated with the test compounds pre-diluted with DMEM (final DMSO concentration was 0.1%). After 24 h, cells were fixed (3.7% formaldehyde-PBS, rt, 30 min) and washed with PBS. Images were obtained using an IX70 inverted fluorescence microscope (Olympus) with a ×100 objective. For quantitative assessment of NPC1L1 localization to the plasma membrane, 25 images were acquired and analyzed. Cells with ER localization, near-ER localization, mixed localization, near-PM localization, and PM localization were counted as 0, 0.25, 0.5, 0.75, and 1, respectively, and the average value was presented as a percentage (% plasma membrane localization). Images of typical cells and a representative dose–response curve obtained from the quantification procedure are shown in Figure 2.

4.1.7. Quantification of GFP fluorescence in lysates

For evaluation of steady-state NPC1L1-GFP expression levels, GFP fluorescence of lysates was measured and normalized by total protein concentration. In a 12-well plate, cells were cultured to 70% confluency in medium without phenol red and treated with vehicle or test compounds. After 24 h, the medium was removed and the cells were lysed in TNET buffer (1% Triton X-100, 25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5) for 1 h on ice. The debris was removed by centrifugation (13,500 rpm at 4 °C for 5 min), and the fluorescence of the supernatant was measured with a Wallac 1420 multilabel counter (PerkinElmer Life Science). The total protein concentrations were determined by BCA assay, and fluorescence intensities were normalized.

4.2. Chemistry

4.2.1. General

Proton and carbon nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) were recorded on a JEOL JNM-ECA 500 (500 MHz) spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard, tetramethylsilane. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMA-HX110 mass spectrometer. Steroids and reagents were purchased from Aldrich, TCI, Wako Pure Chemical Industry, and Kanto Kagaku and used without purification. Open column and flash column chromatography were performed using silica gel 60 (particle size 0.060–0.210 mm) supplied by Kanto Kagaku.

4.2.2. General procedure for synthesis of amide derivatives of 3β-hydroxy-Δ^5-cholenic acid

To a solution of 3β-hydroxy-Δ^5-cholenic acid (0.1 mmol) and Et_3N (20 μL, 0.144 mmol) in THF (8 mL) was added isobutyl chloroformate (20 μL, 0.154 mmol) at 0 °C. The mixture was stirred at 0 °C for 10 min, and then amine (200 μL) was added at 0 °C. The whole was stirred at ambient temperature for 2 h to overnight, and then diluted with AcOEt. The organic layer was washed with saturated aqueous solution of NH_4Cl and brine, dried (Na_2SO_4), filtered and evaporated. The desired compounds were purified as indicated below.

4.2.2.1. 3β-Hydroxy-Δ^5-cholenamide (17). Open column chromatography (*n*-hexane/AcOEt = 1:3 to 1:7) gave **17** (39.2 mg, 0.105 mmol, 93% yield) as colorless plates (recrystallised from CH_2Cl_2/*n*-hexane/MeOH, mp 252.5–255.0 °C). ^{1}H NMR ($CDCl_3$–CD_3OD) δ: 5.36–5.33 (1H, m), 3.51–3.41 (1H, m), 3.37–3.32 (2H, m), 1.02 (3H, s), 0.97 (3H, d, *J* = 6.71 Hz), 0.70 (3H, s). ^{13}C NMR ($CDCl_3$–CD_3OD) δ: 182.16, 144.77, 125.30, 75.10, 60.64, 59.73, 54.04, 46.24, 45.67, 43.64, 41.15, 40.36, 39.45, 36.45, 35.71 (2C), 34.96, 31.93, 28.07, 24.91, 23.06, 22.00, 15.55. ^{13}C NMR (DMSO-d_6) δ: 174.66, 141.24, 120.39, 69.98, 56.20, 55.37, 49.59, 42.21, 41.84, 36.91, 36.05, 34.92, 31.97, 31.44, 31.40, 31.34 (2C), 27.62, 23.86, 20.61, 19.13, 18.28, 11.69. FAB-MS *m*/*z*: 374 (MH^+), 356 ($[M-OH]^+$). HRMS (FAB, $[M+H]^+$) calcd for $C_{24}H_{40}NO_2$ 374.3054, found 374.3053.

4.2.2.2. *N*-Methyl-3β-hydroxy-Δ^5-cholenamide (18). Open column chromatography (*n*-hexane/AcOEt = 1:4 to 1:5) gave **18** (32.1 mg, 0.0828 mmol, 81% yield) as a colorless powder (recrystallised from CH_2Cl_2/*n*-hexane, mp 218.4–220.2 °C). ^{1}H NMR ($CDCl_3$–CD_3OD) δ: 6.05 (1H, br s), 5.36–5.32 (1H, m), 3.54–3.45 (1H, m), 2.77 (3H, s), 1.00 (3H, s), 0.93 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$–CD_3OD) δ: 175.21, 141.09, 121.83, 71.73, 56.98, 56.07, 50.35, 42.64, 42.29, 40.01, 37.51, 36.74, 35.82, 33.70, 32.13 (3C), 31.61, 28.39, 26.40, 24.50, 21.31, 19.63, 18.61, 12.10. FAB-MS *m*/*z*: 410 (MNa^+), 388 (MH^+), 370 ($[M-OH]^+$). Anal. Calcd for $C_{25}H_{41}NO_2 \cdot 1/2H_2O$: C, 75.71; H, 10.67; N, 3.53. Found: C, 75.45; H, 10.44; N, 3.20.

4.2.2.3. *N*,*N*-(Dimethyl)-3β-hydroxy-Δ^5-cholenamide (19). This reaction was performed on 0.4 mmol scale. Open column chromatography (*n*-hexane/AcOEt = 1:3 to 1:4) gave **19** (141.1 mg, 0.351 mmol, 84% yield) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 5.29–5.27 (1H, m), 3.49–3.42 (1H, m), 2.91 (6H, br s), 0.94 (3H, s), 0.88 (3H, d, *J* = 6.71 Hz), 0.62 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 173.82, 140.76, 121.61, 71.71, 56.70, 55.89, 50.07, 42.35, 42.26, 39.74, 38.69, 37.24, 36.47, 35.65, 35.60, 31.86, 31.85, 31.61, 31.23, 30.33, 28.16, 24.26, 21.05, 19.37, 18.51, 11.86. FAB-MS *m*/*z*: 402 (MH^+).

4.2.2.4. *N*,*N*-(Diethyl)-3β-hydroxy-Δ^5-cholenamide (20). Flash column chromatography (*n*-hexane/ethyl acetate 2:1 to 1:1) gave **20** (36.7 mg, 0.085 mmol, 88%) as colorless plates (recrystallised from CH_2Cl_2/*n*-hexane, mp 171.7–172.9 °C). ^{1}H NMR ($CDCl_3$) δ: 5.35 (1H, br s), 3.56–3.49 (1H, m), 3.42–3.25 (4H, m), 2.36–2.16 (4H, m), 1.18 (3H, t, *J* = 7.0 Hz), 1.10 (3H, t, *J* = 7.0 Hz), 1.01 (3H, s), 0.95 (3H, d, *J* = 6.7 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 173.59, 141.63, 122.53, 72.63, 57.60, 56.86, 50.98, 43.25, 43.16, 42.84, 40.88, 40.64, 38.13, 37.36, 36.57, 32.75 (2C), 32.52, 32.48,

31.02, 29.06, 25.16, 21.95, 20.27, 19.43, 15.32, 13.98, 12.75. FAB-MS (glycerol-mNBA 2:1 + NaCl) *m*/*z*: 430 (MH^+), 452 (MNa^+). Anal. Calcd for $C_{28}H_{47}NO_2$: C, 78.27; H, 11.03; N, 3.26. Found: C, 77.99; H, 11.05; N, 3.26.

4.2.2.5. *N,N*-(Di-*n*-propyl)-3β-hydroxy-Δ^5-cholenamide (21). Open column chromatography (***n***-hexane/AcOEt = 3:1 to 2:1) gave **21** (41.0 mg, 0.0896 mmol, 82% yield) as colorless plates (recrystallised from CH_2Cl_2/***n***-hexane, mp 117.1–118.5 °C). ^{1}H NMR ($CDCl_3$) ***δ***: 5.33–5.30 (1H, m), 3.55–3.47 (1H, m), 3.32–3.10 (4H, m), 0.99 (3H, s), 0.94 (3H, d, ***J*** = 6.9 Hz), 0.67 (3H, s). ^{13}C NMR ($CDCl_3$) ***δ***: 173.61, 140.87, 121.68, 71.78, 56.79, 56.06, 50.17, 49.83, 47.68, 42.45, 42.34, 39.83, 37.34, 36.55, 35.79, 31.94 (2C), 31.83, 31.69, 30.16, 28.26, 24.33, 22.38, 21.15, 20.99, 19.46, 18.61, 11.94, 11.38 (2C). FAB-MS ***m*/*z***: 480 (MNa^+), 458 (MH^+). Anal. Calcd for $C_{30}H_{51}NO_2$: C, 78.72; H, 11.23; N, 3.06. Found: C, 78.50; H, 11.03; N, 3.22.

4.2.2.6. *N,N*-(Di-*n*-butyl)-3β-hydroxy-Δ^5-cholenamide (22). Flash column chromatography (***n***-hexane/ethyl acetate = 3:1 to 2:1) gave **22** (35.8 mg, 0.074 mmol, 85%) as a colorless powder (recrystallised from CH_2Cl_2/***n***-hexane, mp 112.1–114.0 °C). ^{1}H NMR ($CDCl_3$) ***δ***: 5.32 (1H, d, ***J*** = 5.5 Hz), 3.53–3.46 (1H, m), 3.32–3.17 (4H, m), 0.98 (3H, s), 0.94–0.87 (total 9H, m), 0.66 (3H, s). ^{13}C NMR ($CDCl_3$) ***δ***: 173.07, 140.52, 121.43, 71.52, 56.49, 55.74, 49.86, 47.59, 45.46, 42.14, 42.05, 39.52, 37.02, 36.25, 35.47, 31.64, 31.50, 31.40, 31.05, 29.87, 29.68, 27.96, 24.04, 20.84, 20.02, 19.90, 19.16, 18.33, 13.64, 11.64. FAB-MS (glycerol-mNBA 2:1) ***m*/*z***: 486 (MH^+). Anal. Calcd for $C_{32}H_{55}NO_2$: C, 79.12: H, 11.41; N, 2.88. Found: C, 79.13; H, 11.43; N, 2.97.

4.2.3. General procedure for synthesis of carbamate derivatives of compound 19

To a solution of **19** (0.1 mmol) and pyridine (80 μL, 0.991 mmol) in CH_2Cl_2 (8 mL) was added triphosgene (33.3 mg, 0.112 mmol) at 0 °C. The mixture was stirred for 20 min at an ambient temperature. Then amine (200–500 μL) was added and stirring was continued at ambient temperature overnight. The whole was diluted with CH_2Cl_2. The organic layer was washed with water, saturated aqueous solution of NH_4Cl and brine, dried (Na_2SO_4), filtered and evaporated.

4.2.3.1. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl carbamate (23). Open column chromatography (***n***-hexane/AcOEt = 1:2) gave **23** (25.6 mg, 0.0576 mmol, 62% yield) as colorless cubes (recrystallised from CH_2Cl_2/***n***-hexane, mp 199.8–202.5 °C). ^{1}H NMR ($CDCl_3$) ***δ***: 5.41–5.36 (1H, m), 4.60 (2H, br s), 4.53–4.45 (1H, m), 3.01 (3H, s), 2.94 (3H, s), 1.01 (3H, s), 0.95 (3H, d, ***J*** = 6.10 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) ***δ***: 173.66, 156.40, 139.61, 122.56, 74.66, 56.60, 55.84, 49.95, 42.34, 39.67, 38.35, 37.31, 36.93, 36.51, 35.58, 35.38, 31.83 (2C), 31.20, 30.31, 28.12, 27.98, 24.25, 21.00, 19.29, 18.51, 11.85. FAB-MS ***m*/*z***: 467 (MNa^+), 445 (MH^+). HRMS (FAB, $[M+H]^+$) calcd for $C_{27}H_{45}N_2O_3$ 445.3425, found 445.3383.

4.2.3.2. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl *N,N*-dimethylcarbamate (24). Open column chromatography (*n*-hexane/AcOEt = 1:2) gave **24** (24.2 mg, 0.0512 mmol, 48% yield) as colorless cubes (recrystallised from CH_2Cl_2/*n*-hexane, mp 170.9–173.1 °C). ^{1}H NMR ($CDCl_3$) *δ*: 5.38–5.34 (1H, m), 4.57–4.44 (1H, m), 2.97 (6H, s), 2.88 (6H, s), 1.01 (3H, s), 0.94 (3H, d, *J* = 6.87 Hz), 0.67 (3H, s). ^{13}C NMR ($CDCl_3$) *δ*: 173.67, 156.30, 139.98, 122.33, 74.67, 56.64, 55.85, 49.96, 42.37, 39.71, 38.73, 37.34, 37.03, 36.57, 36.26, 35.85, 35.61, 35.40, 31.88 (2C), 31.22, 30.33, 28.30, 28.16, 24.28, 21.03, 19.37, 18.54, 11.88. FAB-MS *m*/*z*: 495 (MNa^+), 473 (MH^+). Anal. Calcd for $C_{29}H_{43}N_2O_3$: C, 73.68; H, 10.23; N, 5.93. Found: C, 73.98; H, 10.32; N, 5.89.

4.2.3.3. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl *N,N*-diethylcarbamate (25). Open column chromatography (*n*-hexane/AcOEt = 1:1) gave **25** (13.3 mg, 0.0266 mmol, 29% yield) as colorless cubes (recrystallised from CH_2Cl_2/*n*-hexane, mp 145.1–147.8 °C). ^{1}H NMR ($CDCl_3$) *δ*: 5.40–5.35 (1H, m), 4.56–4.47 (1H, m), 3.26 (4H, br s), 3.01 (3H, s), 2.94 (3H, s), 1.11 (6H, t, *J* = 7.02 Hz), 1.02 (3H, s), 0.95 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) *δ*: 173.64, 155.53, 140.00, 122.22, 74.22, 56.62, 55.84, 49.94, 42.35, 41.13 (2C, br s), 39.69, 38.69, 37.31, 37.03, 36.55, 35.58, 35.38, 31.85 (2C), 31.20, 30.30 (2C), 30.05, 28.29, 28.13, 24.26, 21.01, 19.36, 18.52, 11.85. FAB-MS *m*/*z*: 523 (MNa^+), 501 (MH^+). Anal. Calcd for $C_{31}H_{52}N_2O_5$: C, 74.35; H, 10.47; N, 5.59. Found: C, 74.09; H, 10.18; N, 5.49.

4.2.3.4. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl *N,N*-di-*n*-propylcarbamate (26). Open column chromatography (*n*-hexane/AcOEt = 1:2) gave **26** (30.0 mg, 0.0567 mmol, 53% yield) as colorless cubes (recrystallised from CH_2Cl_2/*n*-hexane, mp 81.4–83.2 °C). ^{1}H NMR ($CDCl_3$) *δ*: 5.37–5.32 (1H, m), 4.52–4.44 (1H, m), 3.14 (4H, s), 2.96 (6H, s), 1.00 (3H, s), 0.93 (3H, d, *J* = 6.87 Hz), 0.86 (6H, t, *J* = 7.45 Hz), 0.66 (3H, s). ^{13}C NMR ($CDCl_3$) *δ*: 173.67, 156.01, 140.02, 122.25, 74.30, 56.64, 55.85, 49.95, 49.03, 48.50, 42.36, 39.71, 38.68, 37.34, 37.05, 36.58, 35.61, 35.41, 31.88 (2C), 31.22, 30.33, 28.30, 28.16, 24.28, 21.87, 21.38, 21.04, 19.40, 18.54, 11.88, 11.30 (2C). FAB-MS *m*/*z*: 551 (MNa^+), 529 (MH^+). Anal. Calcd for $C_{33}H_{56}N_2O_3 \cdot 1/2\ H_2O$: C, 73.70; H, 10.68; N, 5.21. Found: C, 73.57; H, 10.95; N, 5.22.

4.2.3.5. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl *N,N*-di-*n*-butylcarbamate (27). Open column chromatography (*n*-hexane/AcOEt = 1:1) gave **27** (36.5 mg, 0.0656 mmol, 71% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$) *δ*: 5.39–5.34 (1H, m), 4.54–4.45 (1H, m), 3.25–3.13 (4H, m), 3.01 (3H, s), 2.94 (3H, s), 1.02 (3H, s), 0.95 (3H, d, *J* = 6.71 Hz), 0.92 (6H, t, *J* = 7.32 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) *δ*: 173.61, 155.88, 139.96, 122.19, 74.26, 56.59, 55.81, 49.91, 46.98, 46.31, 42.31, 39.66, 38.63, 37.29, 37.00, 36.53, 35.55, 35.35, 31.83, 31.17, 30.70, 30.27 (2C), 28.24, 28.11, 24.23, 20.98, 20.01(2C), 19.33, 18.49, 13.84, 11.84. FAB-MS *m*/*z*: 579 (MNa^+), 557 (MH^+). HRMS (FAB, $[M+Na]^+$) calcd for $C_{35}H_{60}N_2NaO_3$ 579.4496, found 579.4449.

4.2.3.6. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl morpholinecarbamate (28). Open column chromatography (*n*-hexane/AcOEt = 1:2 to 1:3) gave **28** (34.6 mg, 0.0672 mmol, 74% yield) as colorless cubes (recrystallised from CH_2Cl_2/*n*-hexane, mp 175.8–177.2 °C). ^{1}H NMR ($CDCl_3$) *δ*: 5.40–5.35 (1H, m), 4.58–4.49 (1H, m), 3.68–3.62 (4H, m), 3.48–3.43 (4H, m), 3.01 (3H, s), 2.94 (3H, s), 1.02 (3H, s), 0.95 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) *δ*: 173.61, 155.01, 139.70, 122.48, 75.01, 66.58 (2C), 56.58, 55.82, 49.90, 43.93 (2C, br s), 42.31, 39.65, 38.55, 37.29, 36.94, 36.51, 35.56, 35.35, 31.82 (2C), 31.17, 30.28, 28.14, 28.10, 24.23, 20.98, 19.30, 18.49, 11.83. FAB-MS *m*/*z*: 537 (MNa^+), 515 (MH^+). Anal. Calcd for $C_{31}H_{50}N_2O_4$: C, 72.33; H, 9.79; N, 5.44. Found: C, 72.07; H, 9.77; N, 5.34.

4.2.3.7. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl 4-methylpiperazinecarbamate (29). Open column chromatography ($CHCl_3$/MeOH = 100/0 to 100/3) gave **29** (30.0 mg, 0.0568 mmol, 60% yield) as as a colorless powder (recrystallised from CH_2Cl_2/*n*-hexane, mp 158.9–160.0 °C). ^{1}H NMR ($CDCl_3$) *δ*: 5.39–5.36 (1H, m), 4.55–4.47 (1H, m), 3.51–3.46 (4H, m), 3.01 (3H, s), 2.94 (3H, s), 2.37–2.35 (4H, m), 2.30 (3H, s), 1.02 (3H, s), 0.95 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) *δ*: 173.62, 154.97, 139.82, 122.39, 74.81, 56.59, 55.82, 54.72 (2C), 49.91, 46.16, 43.49 (2C, br s), 42.33, 39.66, 38.59, 37.30, 36.97, 36.53, 35.57, 35.36, 31.83 (2C), 31.18, 30.29, 28.17, 28.12, 24.24, 20.99, 19.32, 18.51, 11.84. FAB-MS *m*/

z: 550 (MNa^+), 528 (MH^+). Anal. Calcd for $C_{32}H_{53}N_3O_3 \cdot 1/3\ H_2O$: C, 72.00; H, 10.13; N, 7.87. Found: C, 72.22; H, 9.75; N, 7.98.

4.2.3.8. *N,N*-(Dimethyl)-Δ^5-cholenamid-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (30). Open column chromatography ($CHCl_3$/MeOH = 20/1) gave **30** (20.5 mg, 0.0385 mmol, 49% yield) as a colorless powder (recrystallised from CH_2Cl_2/*n*-hexane, mp 149.1–151.2 °C). ^1H NMR ($CDCl_3$) δ: 5.40–5.35 (1H, m), 4.57–4.49 (1H, m), 3.88–3.75 (4H, m), 3.57 (2H, br s), 3.52–3.42 (4H, m), 3.01 (3H, s), 2.93 (3H, s), 1.02 (3H, s), 0.95 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 173.77, 156.73, 139.62, 122.59, 75.22, 61.93 (2C), 56.60, 55.86, 52.45, 51.94, 49.93, 42.35, 39.68, 38.59, 37.35, 36.97, 36.54, 35.61, 35.42, 31.83 (2C), 31.21, 30.35, 28.23, 28.13, 24.26, 21.02, 19.34, 18.52, 11.85. FAB-MS *m*/*z*: 555 (MNa^+). Anal. Calcd for $C_{31}H_{52}N_2O_5$: C, 69.89; H, 9.84; N, 5.26. Found: C, 69.59; H, 9.97; N, 5.09.

4.2.4. Synthesis of bis(2-hydroxyethyl)carbamate derivatives of oxysterols

4.2.4.1. Cholest-5-en-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (37). To a solution of cholesterol (779.2 mg, 2.02 mmol) in CH_2Cl_2 (6 mL) and pyridine (20 mL) was added triphosgene (507.8 mg, 1.71 mmol) at 0 °C. The mixture was stirred for 20 min at 0 °C. In a separate flask, diethanolamine (4 mL, 41.5 mmol) was dissolved in THF (60 mL). The former solution was added dropwise to the latter solution at 0 °C, and the mixture was stirred overnight at ambient temperature. The reaction was quenched by adding water and the whole was extracted with CH_2Cl_2. The organic layer was washed (water, aqueous ammonium chloride, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (hexane/AcOEt = 2:1 then $CHCl_3$/MeOH = 94/6) to afford the title compound (795.8 mg, 1.54 mmol, 76%) as colorless cubes (recrystallised from hexane/CH_2Cl_2, mp 161.3–164.1 °C). ^1H NMR ($CDCl_3$) δ: 5.40–5.35(1H, m), 4.56–4.46 (1H, m), 4.45–4.16 (2H, br m), 3.83–3.73 (4H, m), 3.51–3.39 (4H, m), 1.02 (3H, s), 0.92 (3H, d, *J* = 6.71 Hz), 0.87 (3H, d, *J* = 6.71 Hz), 0.86 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 156.57, 139.58, 122.56, 75.22, 61.68, 61.54, 56.61, 56.12, 52.47, 51.99, 49.93, 42.25, 39.67, 39.44, 38.55, 36.93, 36.50, 36.13, 35.75, 31.84, 31.80, 28.17 (2C), 27.93, 24.22, 23.82, 22.75, 22.51, 20.99, 19.30, 18.66, 11.79. FAB-MS *m*/*z*: 540 (MNa^+). Anal. Calcd for $C_{32}H_{55}NO_4$: C, 74.23; H, 10.71; N, 2.71. Found: C, 74.01; H, 10.71; N, 2.68.

4.2.4.2. 5α,6β-Dihydroxy-cholestan-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (31). The title compound was prepared according to reported procedures.[29,40] To a solution of **37** (146.0 mg, 0.282 mmol) in CH_2Cl_2 (6 mL) was added mCPBA (186.0 mg, 0.753 mmol, ca. 70%). The mixture was stirred overnight at ambient temperature and then diluted with ethyl acetate. The organic layer was washed with saturated aqueous solution of $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated. The crude epoxide was subjected to the next reaction without further purification. To a solution of the epoxide in THF (8 mL) was added $HIO_4 \cdot 2H_2O$ (130.0 mg, 0.5727 mmol) at 0 °C. The solution was stirred at ambient temperature for 2 h and then diluted with ethyl acetate. The organic layer was washed with saturated aqueous solution of $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated. The residue was purified by flash column chromatography (AcOEt/CH_2Cl_2/MeOH = 10/10/1 to 10/10/2) to afford the title compound (27.0 mg, 0.049 mmol, 17%) as a colorless powder. ^1H NMR ($CDCl_3$/CD_3OD) δ: 5.04–4.97 (1H, m), 3.75 (4H, br s), 3.55–3.33 (5H, m), 2.10 (1H, dd, *J* = 12.20, 12.20 Hz), 1.98 (1H, dt, *J* = 12.41, 2.59 Hz), 1.17 (3H, s), 0.90 (3H, d, *J* = 6.71 Hz), 0.87 (3H, d, *J* = 6.71 Hz), 0.86 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$/CD_3OD) δ: 157.01, 75.80, 75.12, 72.90, 61.07, 60.96, 56.16, 55.76, 52.17, 51.62, 45.04, 42.62, 39.84, 39.39, 38.12, 37.02, 36.06, 35.72, 34.29, 32.07, 30.19, 28.12, 27.88, 27.02, 24.04, 23.79, 22.66, 22.40, 20.98, 18.53, 16.40, 11.98. FAB-MS *m*/*z*: 574 (MNa^+).

4.2.4.3. Cholest-5-en-3β-yl *N,N*-bis[2-(*tert*-butyldimethylsilyloxy)ethyl]carbamate (38). To a solution of **37** (395.3 mg, 0.764 mmol) in CH_2Cl_2 (4.5 mL) and DMF (4.5 mL) was added imidazole (423.4 mg, 6.22 mmol) and TBSCl (441.1 mg, 2.93 mmol) at 0 °C. The mixture was stirred for 16 h at ambient temperature and then diluted with AcOEt. The organic layer was washed (water, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 100/0 to 95/5) to afford the title compound (560.9 mg, 0.752 mmol, 98%) as a colorless oil. ^1H NMR ($CDCl_3$) δ: 5.34–5.30 (1H, m), 4.52–4.43 (1H, m), 3.68 (2H, t, *J* = 5.49 Hz), 3.63 (2H, t, *J* = 6.41 Hz), 3.39–3.32 (4H, m), 0.97 (3H, s), 0.86 (3H, d, *J* = 6.71 Hz), 0.84 (9H, s), 0.83 (9H, s), 0.81 (3H, d, *J* = 6.71 Hz), 0.81 (3H, d, *J* = 6.71 Hz), 0.62 (3H, s), 0.00 (6H, s), −0.01 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 155.65, 139.84, 122.38, 74.49, 61.52 (2C), 56.66, 56.12, 51.14, 50.73, 50.00, 42.29, 39.73, 39.50, 38.67, 37.02, 36.55, 36.17, 35.78, 31.87 (2C), 28.27, 28.21, 27.98, 25.88 (6C), 24.27, 23.82, 22.79, 22.54, 21.02, 19.31, 18.70, 18.21, 14.09, 11.82, −5.38 (2C), −5.44 (2C).

4.2.4.4. 7-Oxocholest-5-en-3β-yl *N,N*-bis[2-(*tert*-butyldimethylsilyloxy)ethyl]carbamate (39). The allylic oxidation was performed following a reported procedure.[41] To a stirred mixture of **38** (447.8 mg, 0.600 mmol) in 1,2-dichloroethane (3 mL) and $RuCl_3 \cdot nH_2O$ (2.2 mg) in water (200 μL) was added dropwise TBHP (800 μL), in a water bath. The mixture was stirred overnight at ambient temperature. The reaction was quenched by adding acetone (3 mL) and saturated aqueous $Na_2S_2O_3$ (3 mL). The whole was stirred for 2 h at 60 °C and then diluted with AcOEt. The organic layer was washed (water, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 10/0 to 9/1) to afford the title compound (151.1 mg, 0.199 mmol, 33%) as a colorless oil. ^1H NMR ($CDCl_3$) δ: 5.68–5.64 (1H, m), 4.65–4.55 (1H, m), 3.69 (2H, t, *J* = 5.49 Hz), 3.64 (2H, t, *J* = 6.41 Hz), 3.37 (4H, t, *J* = 5.80 Hz), 1.16 (3H, s), 0.88 (3H, d, *J* = 6.71 Hz), 0.85 (9H, s), 0.84 (9H, s), 0.82 (3H, d, *J* = 6.71 Hz), 0.82 (3H, d, *J* = 6.71 Hz), 0.64 (3H, s), 0.01 (6H, s), 0.00 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 201.88, 164.16, 155.28, 126.58, 72.80, 61.43, 61.39, 54.74, 51.08, 50.65, 49.95, 49.77, 45.36, 43.09, 39.43, 38.64, 38.28, 38.25, 36.14, 36.00, 35.67, 28.50, 27.94, 27.86, 26.27, 25.58 (6C), 23.79, 22.76, 22.51, 21.14, 18.83, 18.22, 18.17, 17.23, 11.92, -5.40 (2C), −5.45 (2C).

4.2.4.5. 7-Oxocholest-5-en-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (36). A solution of **39** (151.1 mg, 0.199 mmol) and TBAF (1 M in THF, 360 μL, 0.36 mmol) in THF (4 mL) was stirred for 15 h at ambient temperature, and then diluted with AcOEt. The organic layer was washed (water, aqueous ammonium chloride, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 1:1 then $CHCl_3$/MeOH = 9/1) to afford the title compound (58.1 mg, 0.109 mmol, 55%) as colorless plates (recrystallised from *n*-hexane/CH_2Cl_2, mp 144.5–146.8 °C). ^1H NMR ($CDCl_3$) δ: 5.71–5.69 (1H, m), 4.68–4.59 (1H, m), 4.16 (2H, br s), 3.86–3.74 (4H, m), 3.52–3.42 (4H, m), 1.21 (3H, s), 0.92 (3H, d, *J* = 6.10 Hz), 0.87 (3H, d, *J* = 6.71 Hz), 0.86 (3H, d, *J* = 6.71 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 202.12, 164.22, 156.15, 126.51, 73.40, 61.56, 61.41, 54.75, 52.41, 51.88, 49.89, 49.69, 45.35, 43.05, 39.39, 38.58, 38.26, 38.18, 36.11, 35.92, 35.66, 28.47, 27.92, 27.76, 36.23, 23.81, 22.74, 22.49, 21.12, 18.80, 17.22, 11.89. FAB-MS *m*/*z*: 554 (MNa^+). Anal. Calcd for $C_{32}H_{53}NO_5$: C, 72.28; H, 10.05; N, 2.63. Found: C, 71.98; H, 9.99; N, 2.61.

4.2.4.6. 7β-Hydroxycholest-5-en-3β-yl, *N,N*-bis(2-hydroxyethyl)carbamate (35). The title compound was prepared according to a reported reaction condition.[42,43] To a mixture of **36** (36.0 mg, 0.0677 mmol) and $CeCl_3{\cdot}7H_2O$ (76.2 mg, 0.205 mmol) in THF (2 mL) and MeOH (1 mL) was added $NaBH_4$ (16.1 mg, 0.426 mmol) at 0 °C. The mixture was stirred at ambient temperature for 14 h and then diluted with AcOEt. The organic layer was washed (water, aqueous ammonium chloride, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography ($CHCl_3$/MeOH = 10/0 to 9/1) to afford the title compound (30.7 mg, 0.0575 mmol, 85%) as a colorless powder (recrystallised from *n*-hexane/CH_2Cl_2, mp 147.8–150.2 °C). ^{1}H NMR ($CDCl_3$) δ: 5.31 (1H, s), 4.59–4.50 (1H, m), 3.85–3.72 (1H, m), 3.84–3.76 (4H, m), 3.53–3.40 (4H, m), 1.06 (3H, s), 0.92 (3H, d, J = 6.10 Hz), 0.87 (3H, d, J = 6.71 Hz), 0.86 (3H, d, J = 6.71 Hz), 0.69 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 156.62, 142.31, 126.34, 74.78, 73.17, 61.74 (2C), 55.89, 55.46, 52.41, 51.91, 48.15, 42.90, 40.68, 39.48 (2C), 38.08, 36.66, 36.48, 36.18, 35.72, 28.52, 28.18, 27.99, 26.35, 23.85, 22.79, 22.54, 21.03, 19.11, 18.76, 11.80. FAB-MS *m*/*z*: 556 (MNa^+). Anal. Calcd for $C_{32}H_{55}NO_5{\cdot}1/2\ H_2O$: C, 70.81; H, 10.40; N, 2.58. Found: C, 70.66; H, 10.32; N, 2.51.

4.2.4.7. 6α-Hydroxycholesterol *tert*-butyldimethylsilyl ether (41). The 6α-hydroxylation was conducted by employing a reported reaction condition.[43] To a solution of cholesterol (1180.0 mg, 3.052 mmol) in CH_2Cl_2 (15 mL) and DMF (15 mL) was added imidazole (803.1 mg, 11.80 mmol) and TBSCl (896.6 mg, 5.949 mmol) at 0 °C. The mixture was stirred for 4 h at ambient temperature and then concentrated to remove CH_2Cl_2. Hexane was added to the residue, and then the hexane layer was washed (water, MeOH) and concentrated. The resultant silyl ether (**40**) was used for the next reaction without further purification. A solution of the silyl ether in THF (30 mL) was treated with $BH_3{\cdot}$THF (1.03 M in THF, 4 mL, 4.12 mmol) at 0 °C. The whole was stirred for 5 h at ambient temperature, and then 2 M aqueous NaOH (5 mL) and 30% aqueous H_2O_2 (5 mL) were added to the solution at 0 °C. The whole was stirred at ambient temperature for 17 h and then diluted with AcOEt. The organic layer was washed (aqueous ammonium chloride, water, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 20/1) to afford the title compound (468.8 mg, 0.903 mmol, 30% for 2 steps) as colorless crystals. ^{1}H NMR ($CDCl_3$) δ: 3.57–3.49 (1H, m), 3.44–3.36 (1H, m), 0.91 (3H, d, J = 4.88 Hz), 0.88 (9H, s), 0.86 (3H, d, J = 6.10 Hz), 0.86 (3H, d, J = 0.86 Hz), 0.80 (3H, s), 0.64 (3H, s), 0.05 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 72.05, 69.61, 56.21 (2C), 53.89, 51.85, 42.59, 41.64, 39.87, 39.51, 37.44, 36.28, 36.14, 35.75, 34.30, 32.57, 31.67, 28.18, 28.01, 25.94 (3C), 24.20, 23.80, 22.80, 22.55, 21.15, 18.66, 18.21, 13.49, 12.03, −4.54 (2C). FAB-MS *m*/*z*: 541 (MNa^+).

4.2.4.8. Cholesterol-6α-yl acetate (43). A solution of **41** (468.8 mg, 0.903 mmol), Ac_2O (500 μL, 5.29 mmol) and DMAP (23.4 mg, 0.192 mmol) in pyridine (5 mL) was stirred at ambient temperature for 19 h and then diluted with AcOEt. The organic layer was washed (aqueous ammonium chloride, aqueous sodium bicarbonate, brine), dried (Na_2SO_4), and concentrated. The resultant acetate (**42**) was used for the next reaction without further purification. A solution of the acetate and TBAF (1 M in THF, 2 mL, 2.00 mmol) in THF (20 mL) was stirred at ambient temperature overnight and then diluted with AcOEt. The organic layer was washed (water, aqueous ammonium, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 3:1) to afford the title compound (361.5 mg, 0.809 mmol, 90% for 2 steps) as colorless crystals. ^{1}H NMR ($CDCl_3$) δ: 4.71–4.64 (1H, m), 3.56–3.48 (1H, m), 2.02 (3H, s), 0.90 (3H, d, J = 6.10 Hz), 0.88–0.84 (6H, m), 0.87 (3H, s), 0.65 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 170.71, 72.45, 70.62, 56.08, 56.05, 53.57, 48.57, 42.46, 39.63, 39.33, 37.53, 37.06, 36.42, 35.99, 35.59, 33.97, 31.99, 30.90, 28.00, 27.82, 23.94, 23.71, 22.65, 22.41, 21.07, 20.98, 18.52, 13.19, 11.86. FAB-MS *m*/*z*: 469 (MNa^+).

4.2.4.9. 6α-Acetoxy-5α-cholestan-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (44). To a solution of **43** (361.5 mg, 0.809 mmol) in CH_2Cl_2 (25 mL) and pyridine (5 mL) was added triphosgene (258.5 mg, 0.872 mmol) at 0 °C. The mixture was stirred for 20 min at 0 °C. In a separate flask, diethanolamine (2 mL, 20.7 mmol) was dissolved in CH_2Cl_2 (25 mL). The former solution was added dropwise to the latter solution at 0 °C, and the mixture was stirred overnight at ambient temperature. The reaction was quenched by adding water and the whole was extracted with CH_2Cl_2. The organic layer was washed (water, aqueous ammonium chloride, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 1:1 to 1:5) to afford the title compound (259.5 mg, 0.449 mmol, 55%) as colorless crystals. ^{1}H NMR ($CDCl_3$) δ: 4.71–4.64 (1H, m), 4.62–4.54 (1H, m), 4.10 (2H, br s), 3.86–3.72 (4H, m), 3.57–3.33 (4H, m), 2.03 (3H, s), 0.90 (3H, d, J = 6.71 Hz), 0.88 (3H, s), 0.86 (3H, d, J = 6.10 Hz), 0.86 (3H, d, J = 7.32 Hz), 0.65 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 170.99, 156.58, 74.38, 72.34, 61.71, 61.66, 56.16, 56.04, 53.45, 52.51, 51.91, 48.46, 42.54, 39.66, 39.41, 37.57, 36.83, 36.50, 36.06, 35.69, 34.04, 28.74, 28.08, 27.92, 27.59, 24.02, 23.79, 22.74, 22.49, 21.27, 21.03, 18.61, 13.24, 11.94. FAB-MS *m*/*z*: 600 (MNa^+), 578 (MH^+).

4.2.4.10. 6α-Hydroxy-5α-cholestan-3β-yl *N,N*-bis[2-(*tert*-butyldimethylsilyloxy)ethyl]carbamate (46). To a solution of **44** (198.1 mg, 0.343 mmol) in CH_2Cl_2 (1 mL) and DMF (3 mL) was added imidazole (220.2 mg, 3.234 mmol) and TBSCl (232.0 mg, 1.539 mmol) at 0 °C. The mixture was stirred overnight at ambient temperature and then diluted with AcOEt. The organic layer was washed (water, aqueous ammonium chloride, brine) and concentrated. The resultant silyl ether (**45**) was used for the next reaction without further purification. A mixture of the silyl ether and potassium carbonate (956.1 mg, 6.918 mmol) in MeOH (12 mL) and CH_2Cl_2 (2 mL) was stirred overnight at ambient temperature and then diluted with AcOEt. The organic layer was washed (aqueous ammonium chloride, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 7/1) to afford the title compound (179.8 mg, 0.235 mmol, 69% for 2 steps) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ: 4.59–4.51 (1H, m), 3.70 (2H, t, J = 5.49 Hz), 3.65 (2H, d, J = 6.41 Hz), 3.42–3.31 (4H + 1H, m), 0.89–0.88 (3H, m), 0.87 (9H, s), 0.86 (9H, s), 0.84 (3H, d, J = 6.71 Hz), 0.84 (3H, d, J = 6.10 Hz), 0.80 (3H, s), 0.63 (3H, s), 0.03 (6H, s), 0.02 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 155.70, 72.24, 69.42, 61.49 (2C), 56.16, 56.08, 53.66, 51.69, 51.11, 50.67, 42.52, 41.76, 39.75, 39.44, 37.06, 36.23, 36.08, 35.71, 34.28, 28.76, 28.12, 27.93, 27.72, 25.85 (6C), 24.14, 23.77, 22.75, 22.50, 21.07, 18.60, 18.19, 18.15, 13.32, 11.97, −5.40 (2C), −5.46 (2C).

4.2.4.11. 6α-Hydroxy-5α-cholestan-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (32). A solution of **46** (56.9 mg, 0.0745 mmol) and TBAF (1 M in THF, 200 μL, 0.200 mmol) in THF (2 mL) was stirred at 45 °C for 2 h. Then the mixture was cooled to ambient temperature and diluted with AcOEt. The organic layer was washed (aqueous ammonium, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (*n*-hexane/AcOEt = 1:5 then AcOEt/MeOH = 10/1) to afford the title compound (34.4 mg, 0.0642 mmol, 86%) as colorless needles (recrystallised from *n*-hexane/CH_2Cl_2, mp 198.3–200.1 °C). ^{1}H NMR ($CDCl_3$) δ: 4.62–4.53 (1H, m), 4.25 (2H, br s), 3.87–3.68 (4H, m), 3.58–3.52 (2H, m), 3.41–3.27 (2H + 1H, m), 0.90 (3H, d,

J = 6.10 Hz), 0.87 (3H, d, J = 6.71 Hz), 0.86 (3H, d, J = 6.71 Hz), 0.83 (3H, s), 0.65 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 156.79, 75.15, 69.37, 61.54, 61.46, 56.27, 56.16, 53.69, 52.38, 51.88, 51.69, 42.58, 41.65, 39.80, 39.47, 37.09, 36.31, 36.14, 35.78, 34.33, 28.79, 28.16, 27.98, 27.72, 24.19, 23.89, 22.78, 22.54, 21.13, 18.64, 13.39, 12.02. FAB-MS m/z: 558 (MNa^+), 536 (MH^+). Anal. Calcd for $C_{32}H_{57}NO_5$: C, 71.73; H, 10.72; N, 2.61. Found: C, 71.50; H, 10.59; N, 2.54.

4.2.4.12. 6-Oxo-5α-cholestan-3β-yl *N,N*-bis[2-(*tert*-butyldimethylsilyloxy)ethyl]carbamate (47). To a suspension of Celite (117.8 mg), sodium acetate (19.9 mg, 0.243 mmol) and PCC (68.2 mg, 0.316 mmol) in CH_2Cl_2 (1 mL) was added a solution of **46** (122.9 mg, 0.161 mmol) in CH_2Cl_2 (2 mL) at ambient temperature. The whole was stirred at ambient temperature for 13 h, filtered through a pad of Celite, and concentrated. The residue was purified by open column chromatography (n-hexane/AcOEt = 9/1) to afford the title compound (59.6 mg, 0.0782 mmol, 49%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 4.61–4.54 (1H, m), 3.71 (2H, t, J = 5.80 Hz), 3.67 (2H, t, J = 6.10 Hz), 3.42–3.36 (4H, m), 0.90 (3H, d, J = 6.71 Hz), 0.88 (9H, s), 0.87 (9H, s), 0.86 (3H, d, J = 6.71 Hz), 0.85 (3H, d, J = 6.71 Hz), 0.75 (3H, s), 0.65 (3H, s), 0.04 (6H, s), 0.03 (6H, s). ^{13}C NMR ($CDCl_3$) δ: 210.56, 155.62, 73.45, 61.62, 61.40, 56.66, 56.60, 56.11, 53.84, 51.20, 50.71, 46.66, 42.97, 40.95, 39.44 (2C), 37.98, 36.47, 36.06, 35.67, 28.00, 27.98, 27.39, 26.59, 25.87 (7C), 23.94, 23.79, 22.77, 22.52, 21.48, 18.61, 18.20, 13.04, 11.98, −5.40 (2C), −5.43 (2C).

4.2.4.13. 6-Oxo-5α-cholestan-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (34). A solution of **47** (59.6 mg, 0.0782 mmol) and TBAF (1 M in THF, 200 μL, 0.200 mmol) in THF (2 mL) was stirred at 45 °C for 3 h, then cooled to ambient temperature and diluted with AcOEt. The organic layer was washed (aqueous ammonium, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (n-hexane/AcOEt = 1:5 to 1:9) to afford the title compound (28.4 mg, 0.0532 mmol, 68%) as a colorless powder (recrystallised from n-hexane/CH_2Cl_2, mp 138.9–140.1 °C). ^{1}H NMR ($CDCl_3$) δ: 4.65–4.54 (1H, m), 3.86–3.74 (4H, m), 3.63 (2H, br s), 3.52–3.39 (4H, m), 0.91 (3H, d, J = 6.71 Hz), 0.87 (3H, d, J = 6.71 Hz), 0.86 (3H, d, J = 6.71 Hz), 0.77 (3H, s), 0.66 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 210.82, 156.59, 74.14, 61.84 (2C), 56.65, 56.52, 56.11, 53.79, 52.53, 51.92, 46.64, 42.98, 40.99, 39.43 (2C), 37.99, 36.40, 36.07, 35.67, 27.98 (2C), 27.30, 26.56, 23.94, 23.80, 22.77, 22.52, 21.48, 18.61, 13.07, 11.98. FAB-MS m/z: 556 (MNa^+), 534 (MH^+). Anal. Calcd for $C_{32}H_{55}NO_5 \cdot 1/3\ H_2O$: C, 71.20; H, 10.39; N, 2.59. Found: C, 71.34; H, 10.24; N, 2.53.

4.2.4.14. 6β-Hydroxy-5α-cholestan-3β-yl *N,N*-bis(2-hydroxyethyl)carbamate (33). Following a reported protocol, the 6-keto group was reduced to 6β-hydroxyl group.[44] To a solution of **34** (20.8 mg, 0.0390 mmol) in MeOH (1 mL) was added $NaBH_4$ (4.4 mg, 0.12 mmol) at 0 °C. The mixture was stirred at ambient temperature overnight and then diluted with AcOEt. The organic layer was washed (water, aqueous ammonium chloride, brine), dried (Na_2SO_4), and concentrated. The residue was purified by open column chromatography (n-hexane/AcOEt = 1:5 to 1:10) to afford the title compound (14.5 mg, 0.0271 mmol, 69%) as colorless plates (recrystallised from n-hexane/CH_2Cl_2/MeOH, mp 194.0–196.2 °C). ^{1}H NMR ($CDCl_3$/CD_3OD) δ: 4.69–4.60 (1H, m), 3.81–3.70 (4H + 1H, m), 3.56–3.33 (4H, m), 1.04 (3H, s), 0.91 (3H, d, J = 6.71 Hz), 0.87 (3H, d, J = 6.71 Hz), 0.86 (3H, d, J = 6.71 Hz), 0.69 (3H, s). ^{13}C NMR ($CDCl_3$) δ: 156.83, 75.31, 71.51, 61.30, 56.20, 56.03, 54.02, 52.29, 51.78, 47.08, 42.58, 39.80, 39.48, 38.43, 38.18, 36.08, 35.70, 35.30, 31.68, 30.30, 29.60, 28.11, 27.92, 27.76, 24.13, 23.75, 22.69, 22.44, 20.94, 18.58, 15.52, 11.98. FAB-MS m/z: 558 (MNa^+), 536 (MH^+). Anal. Calcd for $C_{32}H_{57}NO_5 1/4\ H_2O$:C,71.13; H, 10.73; N, 2.59. Found: C, 71.11; H, 10.35; N, 2.53.

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