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Design and Synthesis of a Crosslinker for Studying Intracellular Steroid Trafficking Pathways

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Abstract

A crosslinker was designed and synthesized as a molecular tool for potential use in probing the intracellular trafficking pathways of steroids. The design was guided by computational modeling based upon a model for the transfer of cholesterol between two proteins, NPC1 and NPC2. These proteins play critical roles in the transport of low-density lipoprotein-derived cholesterol from the lumen of lysosomes to other subcellular compartments. Two modified cholesterol residues were covalently joined by a tether based on molecular modeling of the transient interaction of NPC1 and NPC2 during the transfer of cholesterol from the binding site of one of these proteins to the other. With two cholesterol molecules appropriately connected, we hypothesize that the cholesterol binding sites of both proteins will be simultaneously occupied in a manner that will stabilize the protein-protein interaction to permit detailed structural analysis of the resulting complex. A photoaffinity label has also been introduced into one of the cholesterol cores to permit covalent attachment of one of the units into its respective protein-binding pocket. The basic design of these crosslinkers should render them useful for examining interactions of the NPC1/NPC2 pair as well as other sterol transport proteins.

Introduction

Proteins, lipids, carbohydrates, and even simple metal ions are transported within and between cellular compartments by intracellular transport. Biochemical compounds produced or processed in one compartment of a cell are often transferred to another site for further

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processing or to carry out their functional roles. Transport may either be passive, by means of simple diffusion, or mediated, usually by the action of carrier proteins.

Cholesterol is an especially important example of a lipid that undergoes specific intracellular trafficking. Cells go to great lengths to sense the availability of cellular cholesterol, and use this information to regulate its biosynthesis or storage.^{1,2} Important information regarding how cells first obtain cholesterol has come from investigations of the cell biology of Niemann-Pick type C (NPC) disease, a rare lysosomal storage disorder characterized by abnormal accumulation of cholesterol and other lipids in late endosomes and lysosomes.³⁻⁵ A result of these studies was the finding that the lipid accumulation is due to a defect in the normal trafficking of cholesterol in lysosomes. Two proteins, NPC1 and NPC2, were shown to be important for the export of free cholesterol from lysosomes (Figure 1). NPC1 is a large, 1278 amino acid lysosomal protein containing 13 transmembrane domains.⁶ In contrast, NPC2 is a much smaller, 130 amino acid soluble protein.⁷ More than 250 distinct mutations in these proteins have been shown to lead to deficient cholesterol transport and the characteristics of NPC disease.

In the disease-free state, cholesterol is delivered to lysosomes by endocytosis of low-density lipoprotein (LDL) particles that are comprised primarily of cholesteryl esters surrounded by a phospholipid monolayer and free cholesterol. In lysosomes, lysosomal acid lipase (LAL) catalyzes ester hydrolysis to release free cholesterol and fatty acids.⁵ NPC2 protein binds to the released, free cholesterol via its iso-octyl side chain.⁸ NPC2 is then thought to transfer its bound cholesterol to the lumenally oriented, N-terminal domain of NPC1 protein,⁹ which binds cholesterol in the opposite orientation.⁶ After binding to NPC1, cholesterol then traverses the lysosome membrane by an unknown mechanism and is transferred to other subcellular compartments.

There are important gaps in our understanding of this trafficking pathway. First, the precise mechanisms by which free cholesterol, released by LAL, first binds NPC2 is not yet established. This could occur by simple diffusion; recent studies suggest it may be enhanced by anionic phospholipids¹⁰ and bismonoacylglycerol,¹¹ a special lipid within lysosomes.¹² The precise details of the interaction of NPC1 and NPC2 proteins during the critical cholesterol hand-off have only begun to be elucidated,¹³ and computational modeling has also been used to probe this interaction.^{14,15} Finally, how the NPC1-bound cholesterol is transported across the lysosome membrane and whether a cytosolically-oriented protein drives the transfer equilibrium by receiving cholesterol from NPC1 is not yet known.

Cholesterol-based crosslinkers represent a powerful tool to investigate these questions. They offer the potential of identifying the participants in this pathway and may stabilize the interactions between the participants, including NPC1 and NPC2 proteins.^{16,17} Here, we report our design and synthesis of crosslinkers created to aid our understanding of lysosomal cholesterol trafficking. The crosslinkers described herein may also find application in other, cholesterol-mediated processes.

Results and Discussion

Design of the crosslinkers

Our basic design concept (Figure 2) was to join two individual cholesterol Units 1 and 2 by a suitable linker in a head-to-tail manner so that one unit could bind tail-end first in the NPC2 binding pocket, while the other unit could simultaneously bind head-end first in the NPC1 binding pocket. The linker would need to fulfill two requirements: it would need to be of the correct length to permit the NPC1 and NPC2 proteins to engage each other in their normal interactions (suggested by the dashed lines in Figure 2), and it would need to be of a structure such that it does not interfere with the interactions at the protein-protein interface or with the interactions of cholesterol with either of the two proteins. The resulting ternary complexes would ideally be isolatable for detailed structural studies such as X-ray crystallography. An optional feature of the design is that at least one of the cholesterol units could be equipped with a photoaffinity label to covalently anchor the unit into its respective binding site in the event that the non-covalent aggregates are not sufficiently stable to permit isolation and characterization. Photoaffinity labeled cholesterol derivatives have previously been used in studies of binding to the individual proteins.¹⁷

There have been a number of previous reports of cross-linked steroid derivatives. However, the vast majority are either head-to-head^{18,19} or tail-to-tail^{20,21} linked derivatives, and several others are linked through positions on the B or C rings,²²⁻²⁵ none of which would be suitable for our purposes. A limited number of head-to-tail linked derivatives have been reported, consisting of linked bile acid components with linkers of varying lengths and functional group patterns (Figure 3), including some that are related to the linker selected in our primary design (Figure 5).^{26,27} These compounds have been prepared primarily as potential pharmaceutical agents rather than as biochemical tools for the study of a lipid hand-off process.

We began our crosslinker design with computational modeling of the NPC1/NPC2 interactions based upon the reported protein X-ray crystal structures of the sterol binding domain of NPC1 loaded with cholesterol (PDB: 3GKI)⁶ and of NPC2 loaded with cholesterol *O*-sulfate (PDB: 2HKA)⁸. Superposition of these crystal structures onto the previously modeled^{6,14} NPC1-NPC2 cholesterol complex (Figure 6a) showed that the isooctyl tail of cholesterol in NPC1 and the hydroxyl head of cholesterol sulfate in NPC2 are separated by approximately 5 Å. In an extended conformation, this distance corresponds to five linker atoms. An extended conformation is required for simultaneous binding of NPC1 and NPC2, but this conformation might not be the most favorable in the unbound form. To ensure that there is an appropriate population of conformations available for the presumably sequential binding process, it is desirable to use a flexible linker. Finally, the region between the cholesterol units contains a small enclosed pocket of solvent-accessible volume, which facilitates the space requirements of a small covalent linker, allowing, for example, the replacement of the isopropyl group at the end of the cholesterol tail by simpler moieties.

A number of linkers between the two cholesterol units that satisfy one or more of these design elements were considered, including triazoles, polyether chains, and amides (Figure 4). Triazoles were considered due to the facility with which they can be generated by

copper-catalyzed “click” reactions between readily available alkyne and azide components,^{28,29} but modeling indicated that a triazole ring would interfere with the protein-protein interactions at the interface between NPC1 and NPC2 proteins. We therefore modeled various polyether and amide linkers with the correct linker length to span the 5-Å gap between the binding sites. The modeling studies indicated that both ether and amide linkers could fulfill the design criteria above. Although both designs were initially pursued experimentally, an amide linker was found to provide more facile synthetic accessibility, which led to the selection of amide **1** (Figure 5) for more detailed studies.

Molecular dynamics (MD) simulations of amide **1** bound to the NPC1/NPC2 complex for a total of 0.4 μs indicated a favorable fit: the linker is of the correct size to fill the small pocket between NPC1 and NPC2 shown in Figure 6a while maintaining key interactions exhibited by the crystal structures and positioning the two cholesterol units of **1** close to their crystallographic positions (for a more detailed view, see Supplementary Material, Figure S1). Figure 6b shows a representative snapshot of the binding mode from the MD simulation. Using the cholesterol backbone in Figure 6a as a reference, the binding mode of **1** in Figure 6b has an RMSD of ca. 0.3 Å throughout the MD trajectory (see Supplementary Material, Figure S2). Importantly, the NPC1-NPC2 complex bound to amide **1** remained stable during four independent 100ns MD simulations. In previous work,¹⁴ we showed that the NPC1-NPC2 complex remained stable with cholesterol loaded into NPC2, but quickly dissociated when cholesterol was bound to NPC1 or upon mutation of key residues at the protein-protein interface. Bound to amide **1**, the protein-protein contacts responsible for stabilizing the complex when cholesterol is bound to NPC2 are maintained even when the second cholesterol moiety of **1** is bound to NPC1. The modeling also supported a simplification of the design whereby the terminal isopropyl could be omitted from the tail of cholesterol unit 1 at the linker attachment site without unfavorably impacting protein binding. An advantage from a synthesis perspective is that this change avoided the need to control the configuration of a chirality center at the point of attachment of the amide nitrogen of the linker. Thus, the simulations suggest that the covalently linked cholesterol dimer **1** is indeed suitable for stabilizing the NPC1-NPC2 interaction. Based on these results as well as the robustness, synthetic accessibility, and beneficial effect on the solubility of the otherwise very lipophilic cholesterol dimer, amide **1** was selected as the first choice of a crosslinker to be synthesized.

Synthesis of the crosslinkers

Cholesterol Unit 2 was readily available as the previously reported ether-linked carboxylic acid **2** prepared in two simple steps from cholesterol itself (Figure 7).³⁰ The synthesis of Cholesterol Unit 1 was more demanding due to the need of a steroid as a starting material lacking the terminal portion of the cholesterol tail. Commercially available hydoxycholic acid served this purpose but required considerable modification of the functional group pattern of the A and B rings of its core in a manner similar to previous work of Zhou, *et al.*³¹ Conversion of hydoxycholic acid to the methyl ester **3** was followed by sulfonylation to give ditosylate **4**. A combination of substitution of the C(3) tosylate and elimination of the C(6) tosylate generated the functional group pattern of the cholesterol core as unsaturated alcohol **5**. The C(3) hydroxy group was protected as the silyl ether **6** for differentiation from

the primary alcohol **7** obtained by reduction of the side chain ester. Finally, Cholesterol Unit 1 was provided as the azide **8** by a modified Mitsunobu reaction. Staudinger reduction generated a primary amine intermediate, which was subjected to amidation with Cholesterol Unit 2 as the carboxylic acid **2** to give linked compound **9**, from which removal of the silyl protecting group gave the desired crosslinker **1**.

We have demonstrated the option of installing a photoaffinity label in one of the two cholesterol units by the incorporation of a diazirine in a modified synthesis of our crosslinker (Figure 8). As reported previously,³⁰ cholesterol was *O*-alkylated to give the *t*-butyl acetate derivative **10** (see also **2** in Figure 7). Allylic oxidation afforded enone **11**, which was subjected to catalytic hydrogenation to give saturated ketone **12**. Ester hydrolysis completed the synthesis of the modified Cholesterol Unit 2 as the free carboxylic acid **13**. The azide **8** (see Figure 7) was again subjected to a Staudinger reduction followed by direct amidation of the amine intermediate with acid **13**. The silyl protecting group of the resulting amide **14** was removed to give the alcohol **15**. A procedure that has been reported previously for incorporation of a C(7) diazirine³² provided the desired labeled crosslinker **16**, albeit in low yield. The positioning of the diazirine in **16** at C(7) of Cholesterol Unit 2 corresponds to a photoaffinity labeled derivative of cholesterol that has successfully been employed for binding to the NPC1 and NPC2 proteins individually.³³ This precedent provides strong support for the proposed anchoring of **16** in the NPC2 protein prior to entry of the linked Cholesterol Unit 1 into the binding site of a partner protein such as NPC1.

Conclusions

We have completed the synthesis of a specially designed crosslinker containing two independent cholesterol units, with or without a photoaffinity label. These compounds are now available as biochemical tools for studying the transfer of cholesterol between cholesterol binding proteins. Future *in vitro* studies will explore the interaction of these compounds with purified protein constituents and their ability to stabilize complexes of protein pairs. Although these crosslinkers were designed based upon the NPC1/NPC2 cholesterol hand-off, the strategy of molecular design based on MD studies used here should also be amenable for use with other sterol binding proteins. An extension of the strategy described here would be to anchor a photoaffinity labeled cholesterol unit (*e.g.* a diazirine derivative of a ketone related to **14**) in one protein and through use of a suitable functional group on this anchored unit, join a second photoaffinity labeled cholesterol unit for binding to a second partner to give a doubly fixed protein/linker/protein assembly.

Experimental Section

Materials and Methods

Computational model design—Crystal structures of NPC1 bound to cholesterol (PDB: 3GKI)⁶ and NPC2 bound to cholesterol sulfate (PDB: 2HKA)⁸ were aligned to a snapshot of the NPC1-NPC2 cholesterol transport complex, which we previously modeled¹⁴ using PyMOL.³⁴ Side chain overlaps and steric clashes at the NPC1-NPC2 interface were alleviated by manually positioning affected side chains to a conformation in agreement with the stabilized NPC1-NPC2 cholesterol transfer model from our previous work.¹⁴ Utilizing

the existing cholesterol scaffolds in their parent crystal structure positions, the two cholesterol units were manually linked together to form amide **1**, affording a reasonable starting guess for its binding mode. These coordinates were processed using the Protein Preparation Wizard in Schrödinger 2013³⁵ to assign bond orders, add hydrogens, and remove extraneous ligands and ions. The protonation states of protein side chains were determined using the PROPKA server set to pH 7.0.³⁶ Crystallographic water molecules were not removed except for those at the interface between NPC2 and NPC1 in the model. While freezing all protein non-hydrogen atoms, a short minimization of the hand-drawn ligand geometry in the field of the protein was performed with the OPLS forcefield in Schrödinger 2013.³⁷ These coordinates were surrounded by a periodic octahedral box of TIP3P water extending 10 Å from the protein. Sodium ions were added to neutralize the system charge with the LEaP module of AMBER 12.³⁸ The protein was parameterized with the ff03 forcefield.³⁹ Sodium ions used 'frcmod.ionsjc_tip3p' parameters in AMBER 12 for which has the atomic radius is optimized for use with TIP3P waters.⁴⁰ The ligand utilized GAFF forcefield parameters. Ligand atom types corresponding to the GAFF forcefield were assigned by the antechamber module of AMBER 12 from the OPLS-minimized conformation described above. These parameter assignments were manually checked and deemed to be correct. To find ligand charges, the isolated ligand geometry was minimized at the B3LYP/6-31g(d,p) level of theory followed by calculation of an electrostatic potential (ESP) in IEFPCM ether solvent at B3LYP/ccpVTZ. Using the QM optimized ligand geometry used to calculate the ESP, atom-centered charges were fitted to the ESP via standard RESP methodology with the antechamber module of AMBER 12. All QM work was performed in Gaussian 09, Revision D01.⁴¹ Atomic radii for all atoms were defined using the mbondi2 radius set in AMBER12.

Molecular Dynamics Protocol—Molecular dynamics (MD) simulations to equilibrate the system were performed using the pmemd.MPI module of AMBER 12 with a 1 fs timestep. Production MD simulations were performed with a 2 fs timestep using the 'pmemd.CUDA' module of AMBER12 set to SPDP hybrid arithmetic precision using an Nvidia Tesla K20 GPU. All settings remained consistent between equilibration MD and production MD except where stated during the system equilibration. The SHAKE algorithm was applied to all hydrogen-containing bonds except during minimizations. Full pairwise electrostatic calculations were cut off at 9 Å. Electrostatics at distances longer than 9 Å were approximated using Particle Mesh Ewald summation. System temperature was kept at 300 K using Langevin dynamics with a collision frequency of 1.0 ps⁻¹. and pressure was maintained at 1 atm using isotropic position scaling.

Four independent systems starting from slightly different side chain conformations at the NPC1-NPC2 interface were equilibrated first by holding all non-water atoms in place with a 50 kcal/mol harmonic restraint and subjected to a 3500 step minimization. While restraining all solute heavy atoms in place with a harmonic restraint of 10 kcal/mol, the system was slowly heated to 300 K over 50,000 1fs timesteps using Langevin dynamics with a collision frequency of 1.0 ps⁻¹, followed by pressure coupling for 400,000 timesteps to assert proper system density and water configuration. A whole-system minimization of 10,000 steps was then performed with no positional restraints, followed by slowly heating to 300 K over

50,000 timesteps with 5 kcal/mol heavy-atom restraints set on all solute heavy atoms. The restraints were then reduced to 2 kcal/mol and pressure coupling was enabled for 20,000 timesteps. With pressure and temperature coupling maintained, all harmonic restraints were then removed from the system and a short 150,000 step simulation with a 2fs timestep was performed. Production MDs for each of the 4 systems were then run for 100 ns and subsequently analyzed using the ptraj, cpptraj, and MMPBSA.py modules in AMBER 12.

Each trajectory was imaged, centered to the NPC1/NPC2 proteins, and rms fitted to the protein backbone. For each trajectory, the binding site was then determined, as defined by all ligand atoms plus any protein heavy atom that reside within 3 Å of the ligand during MD. Each trajectory was then clustered using the average linkage algorithm in ptraj, with the binding site as the atom mask. The optimal number of clusters for each simulation was chosen based on offering a relatively high pSF, low DBI and satisfying the 'elbow criteria' of the critical distance. In each trajectory, each individual cluster identified is thought to describe a different binding mode. Average binding energies of each trajectory, stripped of water and ions, were calculated with a spacing of 10 ps between data points via a single-trajectory MMPBSA approach using the MMPBSA.py module in AMBER 12. An ionic strength of 100mM was specified, and all other settings were left at their default values.

Synthetic methods—All reactions were performed in flame- or oven-dried glassware under an atmosphere of argon unless otherwise specified. THF was distilled from sodium/benzophenone ketyl radical. Alternatively, THF or toluene was processed through a commercial solvent purification system and stored over 4 Å molecular sieves. Triglyme, acetone, chloroform and 1,4-dioxane were dried over 4 Å molecular sieves for at least 3 d prior to use. Dichloromethane (DCM) and DMF were purchased as extra dry grade over molecular sieves. Anhydrous pyridine was purchased in septum-sealed bottles. Other reagents and solvents were used as received from commercial sources. Column chromatography was performed using either flash chromatography with 20-400 mesh silica gel or using a commercial automated medium pressure liquid chromatography (MPLC) system equipped with a photodiode array detector and cartridges loaded with normal phase silica gel. TLC analysis was performed with commercial glass and aluminum-backed silica gel plates with a fluorescent indicator. NMR spectra were recorded on 300, 400, 500 and 600 MHz spectrometers using CDCl₃ or DMSO as solvents. Chemical shifts were referenced to residual solvent signals. Infrared spectra were obtained on FT spectrometers operating in either transmission or attenuated total reflection (ATR) mode. High resolution mass spectra were obtained using electron impact or FAB ionization TOF instruments using either direct injection or an LC/MS interface operating with a 2.1×150 mm, 5 µm C8 column in isocratic mode with 100% acetonitrile or 2:1 acetonitrile:methylene chloride containing 0.1% formic acid. Melting points were obtained in capillaries with a commercial heated oil immersion apparatus and are uncorrected.

Methyl Hyodeoxycholate (3)⁴²: A mixture of hyodeoxycholic acid (10.0 g, 25.5 mmol) and p-toluenesulfonic acid (1.21 g, 6.38 mmol) was dissolved in methanol (100 mL) and allowed to stand unstirred for 24 h at 25 °C. Afterwards, most of the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate, washed with brine, dried over

anhyd Na₂SO₄ and concentrated under reduced pressure. The residue was dissolved in a small amount of ethyl acetate, and hexanes were added to precipitate the product, which was isolated by filtration filtered to provide 10.27 g (94%) of **3** as a white solid. ¹H NMR (CDCl₃) δ 4.05 (m, 1H), 3.67 (s, 3H), 3.63 (m, 1H), 2.42–0.98 (m, 30H), 0.92 (d, 3H, J = 6.2 Hz), 0.91 (s, 3H), 0.64 (s, 3H) (lit.⁴³ ¹H NMR).

Methyl 3α, 6α-Ditosyloxyhyodeoxycholate (4): Pyridine (65 mL) was added to a mixture of **3** (7.43 g, 18.3 mmol) and tosyl chloride (10.43 g, 54.8 mmol) at 25 °C. The mixture was stirred for 4 h at 25 °C. Ice chips were added to the reaction mixture, and a precipitate formed. The mixture was filtered, and the solid was dissolved in DCM. The solution was washed with 1M HCl, water, and brine, dried over anhyd Na₂SO₄, and concentrated under vacuum to give 11.26 g (91%) of **4** as a white amorphous solid, which was used without further purification. ¹H NMR (CDCl₃) δ 7.79 (d, 2H, J = 8.1 Hz), 7.73 (d, 2H, J = 8.1 Hz), 7.35 (m, 4H), 4.78 (m, 1H), 4.31 (m, 1H), 3.67 (s, 3H), 2.47 (s, 6H), 2.38–0.91 (m, 34H), 0.90 (d, 3H, J = 6.1 Hz), 0.81 (s, 3H), 0.59 (s, 3H) (lit.⁴⁴ ¹H NMR)

Methyl 3β-Hydroxychol-5-en-24-oate (5): A 4:1 mixture of DMF/water (120 mL) was added to a mixture of **4** (7.95 g, 11.1 mmol) and potassium acetate (1.64 g, 16.7 mmol) at 25 °C, and the cloudy mixture was heated at reflux for 12 h. Upon cooling to 25 °C, the mixture was extracted with ethyl acetate, the organic layer was washed with brine and dried over anhyd Na₂SO₄, and the solvent was evaporated under reduced pressure. The resulting residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 1.45 g (34%) of **5** as a white solid. ¹H NMR (CDCl₃) δ 5.33 (m, 1H), 3.64 (s, 3H), 3.49 (m, 1H), 2.45–1.02 (m, 32H), 0.98 (s, 3H), 0.95 (d, 3H, J = 6.3 Hz), 0.66 (s, 3H). (lit.⁴⁴ ¹H NMR)

Methyl 3β-(Dimethyl-tert-butylsilyloxy)chol-5-en-24-oate (6): Dry pyridine (0.67mL) and DMF (14.8 mL) were added to a mixture of **5** (1.10 g, 2.60 mmol), *t*-butyldimethylsilyl chloride (1.29 g, 8.58 mmol), and imidazole (2.43 g, 35.7 mmol) at 25 °C. The mixture was stirred for 1.5 h before it was diluted with ethyl acetate and washed with water and brine. The organic layer was dried over anhyd Na₂SO₄, and excess solvent was removed under reduced pressure. The crude residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 1.27 g (97%) of **6** as a white solid. ¹H NMR (CDCl₃) δ 5.33 (m, 1H), 3.67 (s, 3H), 3.49 (m, 1H), 2.40–1.04 (m, 31H), 1.00 (s, 3H), 0.94 (d, 3H, J = 6.3 Hz), 0.87 (s, 9H), 0.68 (s, 3H), 0.06 (s, 6H). (lit.⁴⁵ ¹H NMR)

3β-tert-Butyldimethylsilyloxy)chol-5-en-24-ol (7): A solution of **6** (1.00 g, 1.99 mmol) in THF (4.5 mL) was added dropwise to a solution of lithium aluminum hydride (0.151 g, 3.98 mmol) in THF (4.5 mL) at 0 °C. The mixture was stirred at 0 °C for 1.5 h before 0.1 mL of water was added to the mixture. Then 0.1 mL of 1N NaOH and 0.3 mL of water were added to the mixture. The mixture was filtered, and the filtrate was extracted with diethyl ether. The extracts were washed with brine and dried over anhyd Na₂SO₄. The solvent was removed under reduced pressure, and the residue was purified via flash chromatography (3:1 hexanes/ethyl acetate) to yield 0.633g (67%) of **7** as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 5.33 (m, 1H), 3.62 (m, 2H), 3.49 (m, 1H), 2.31–1.04 (m, 29H), 1.01 (s, 3H), 0.96 (d, 3H, J = 6.5 Hz), 0.90 (s, 9H), 0.69 (s, 3H), 0.06 (s, 6H) (lit.⁴⁶ ¹H NMR).

24-Azido-3β-(tert-butyldimethylsilyloxy)chol-5-ene (8): Diisopropylazodicarboxylate (0.33 mL, 1.7 mmol) and diphenylphosphoryl azide (0.36 mL, 1.7 mmol) were added to a solution of **7** (0.510 g, 1.07 mmol) and triphenylphosphine (0.423 g, 1.61 mmol) in THF (12 mL) at 0 °C. The mixture was stirred at 0 °C for 10 min before it was allowed to warm to 25 °C and stirred for an additional 22 h. The solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (9:1 hexanes/ethyl acetate) to yield 0.500 g (93%) of **8** as a white solid. mp 68–72 °C; ¹H NMR (300 MHz, CDCl₃), δ 5.33 (m, 1H), 3.49 (m, 1H), 3.23 (m, 2H), 2.30–1.04 (m, 44H), 1.00 (s, 3H), 0.99–0.91 (m, 10H), 0.90 (s, 9H), 0.69 (s, 3H), 0.06 (s, 6H); ¹³C NMR (125 MHz, CDCl₃), δ 141.58, 121.10, 72.63, 56.79, 55.88, 51.98, 50.20, 42.83, 42.37, 39.80, 37.40, 36.59, 35.46, 35.45, 32.94, 32.10, 31.92, 31.77, 28.74, 28.21, 26.05, 25.94, 25.51, 24.26, 21.06, 21.06, 21.06, 19.43, 18.65, 18.58, 11.86, -4.58; HRMS calcd for C₃₀H₅₃N₃NaOSi (M+Na)⁺ 522.3850, found for 522.3874; IR (solid) 2934, 2856, 2097 cm⁻¹.

OTBDMS-Protected Crosslinker (9): A 10:1 THF/water solution (2.4 mL) was added to a mixture of **9** (0.118 g, 0.236 mmol) and triphenylphosphine (0.130 g, 0.494 mmol) at 25 °C. The mixture was heated at reflux for 3 h and then allowed to cool to 25 °C. The mixture was diluted with diethyl ether, washed with brine and dried over anhyd Na₂SO₄. Excess solvent was removed under reduced pressure to give the crude amine to the same flask was added **2** (0.126 g, 0.284 mmol). Dry DCM (4 mL) was added, and the resulting solution was cooled to 0 °C. *N,N'*-Dicyclohexylcarbodiimide (0.068 g, 0.33 mmol) and 4-(*N,N*-dimethylamino)pyridine (0.003 g, 0.02 mmol) were added to the reaction flask. After being stirred for 10 min at 0 °C, the reaction mixture was allowed to warm to 25 °C and stir for 1.5 h. The mixture was diluted with DCM, and the mixture was filtered. The filtrate was concentrated at reduced pressure, and the resulting residue was purified by MPLC (3% to 10% methanol in DCM) to yield 0.169g (79% over 2 steps) of **9** as a white solid, which could be further purified by recrystallization from THF and water. mp 139–142 °C; ¹H NMR (300 MHz, CDCl₃), δ 6.62 (br s, 1H), 5.38 (m, 1H), 5.32 (m, 1H), 3.97 (s, 2H), 3.49 (m, 1H), 3.23 (m, 3H), 2.38–1.04 (m, 68H), 1.02 (s, 6H), 0.99–0.91 (m, 14H), 0.90 (s, 9H), 0.87 (app dd, 6H, J= 1.7, 4.9 Hz), 0.69 (s, 6H), 0.07 (s, 6H); ¹³C NMR (125 MHz, CDCl₃), δ 170.07, 141.58, 140.07, 122.27, 121.08, 80.17, 72.63, 67.65, 56.81, 56.75, 56.19, 55.93, 50.23, 50.15, 42.84, 42.36, 42.33, 39.77, 39.52, 39.18, 39.05, 37.40, 37.03, 36.80, 36.59, 36.20, 35.77, 35.39, 33.06, 32.10, 31.92, 31.89, 28.38, 28.20, 28.00, 26.12, 26.03, 25.93, 24.28, 23.84, 22.78, 22.54, 21.08, 19.41, 19.34, 18.72, 18.67, 11.86, -4.58; HRMS calcd for C₅₉H₁₀₂NO₃Si (M+H)⁺ 900.7620, found for 900.7583; IR (solid) 3321.8, 2927.9, 1625.2, 1572.7.

Crosslinker (1): A 1 M solution of TBAF in THF (0.059 mL, 0.0059 mmol) was added dropwise to a solution of **9** (0.053g, 0.0059 mmol) in dry THF (2 mL) at 25 °C. The mixture was stirred for 24 h at 25 °C. The reaction was quenched with water, and the mixture was extracted with chloroform. The organic extracts was washed with water, dried over anhyd Na₂SO₄, and concentrated under reduced pressure. The resulting residue was purified by MPLC (0% to 7% methanol in DCM) to yield 0.037 g (80%) of **1** as a white solid. mp 93–95 °C; ¹H NMR (300 MHz, CDCl₃), δ 6.62 (br s, 1H), 5.36 (m, 2H), 3.97 (s, 2H), 3.53 (m, 1H), 3.30 (m, 1H), 3.22 (m, 2H), 2.37–1.05 (m, 66H), 1.01 (s, 6H), 0.92 (app t, 6H, J= 6.6

Hz), 0.87 (app dd, 6H, J= 2.8, 3.8 Hz), 0.68 (s, 6H); ^{13}C NMR (125 MHz, CDCl_3), δ 170.12, 140.75, 140.03, 122.29, 121.62, 80.14, 71.75, 67.59, 56.72, 56.70, 56.12, 55.85, 50.08, 50.07, 42.31, 42.29, 42.27, 39.72, 39.49, 39.17, 39.01, 37.23, 36.99, 36.77, 36.48, 36.16, 35.77, 35.39, 33.84, 33.02, 31.90, 31.86, 31.84, 31.63, 28.34, 28.21, 28.00, 26.08, 25.56, 24.89, 24.27, 24.25, 23.81, 22.81, 22.55, 21.05, 19.39, 19.35, 18.70, 18.65, 11.85; HRMS (FAB) calcd for $\text{C}_{53}\text{H}_{88}\text{NO}_3$ (M+H) $^+$ 786.6759, found for 786.6453; IR (solid) 3416, 2934, 1661, 1542, 1464 cm^{-1} .

tert-Butyl Cholest-5-en-3 β -yloxyacetate (10): A solution of cholesterol (3.00 g, 7.76 mmol) in toluene (40 mL) was added to a mixture of potassium *tert*-butoxide (2.61 g, 23.28 mmol) in toluene (20 mL) at 25 °C. The mixture was stirred for 3 h. *tert*-Butyl bromoacetate (2.29 mL, 15.5 mmol) was added dropwise, and the mixture was stirred for 14 h. Afterwards, the mixture was diluted with toluene, washed water and brine, and dried over anhyd Na_2SO_4 . The solvent was removed under reduced pressure, and the resulting residue was purified via flash chromatography (DCM) to yield 1.60 g (41%) of **10** as a white solid. ^1H NMR (CDCl_3) δ 5.37 (m, 1H), 4.01 (s, 2H), 3.24 (m, 1H), 2.43–1.50 (m, 13H), 1.48 (s, 9H), 1.44–1.04 (m, 14H), 1.01 (s, 3H), 0.92 (d, 3H, J= 6.5 Hz), 0.87 (app dd, 6H, J=5.5, 1.1 Hz), 0.68 (s, 3H) (lit.³⁰ ^1H NMR).

tert-Butyl Cholest-7-keto-5-en-3 β -yloxyacetate (11)⁴²: A 70% aq solution of *tert*-butyl hydrogen peroxide (2.6 mL) was added slowly to a suspension of **10** (1.5 g, 3.0 mmol), pyridinium dichromate (3.49 g, 9.29 mmol), and Celite (3 g) in benzene (23 mL) at 0 °C. The mixture was warmed to 25 °C stirred for 24 h. The mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure. The resulting residue was purified by MPLC (5% to 50% ethyl acetate in hexanes) to yield 0.794 g (52%) of **11** as a light brown solid. mp 55–57°C; ^1H NMR (300 MHz, CDCl_3), δ 5.68 (m, 1H), 4.01 (s, 2H), 3.36 (m, 1H), 2.61–1.51 (m, 17H), 1.47 (s, 9H), 1.37–1.21 (m, 10H), 1.18 (s, 3H), 1.15–0.95 (m, 10H), 0.92 (d, 3H, J= 6.6 Hz), 0.85 (app dd, 6H, J= 3.8, 2.8 Hz), 0.67 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3), δ 202.22, 169.72, 164.85, 126.20, 81.69, 78.44, 66.28, 54.76, 49.93, 49.89, 45.37, 43.07, 39.45, 38.68, 38.63, 38.55, 36.16, 35.69, 28.53, 28.09, 27.98, 27.80, 26.29, 23.80, 22.80, 22.54, 21.18, 18.85, 17.25, 11.96; HRMS (FAB) calcd for $\text{C}_{33}\text{H}_{54}\text{NaO}_4$ (M+Na) $^+$ 537.3914, found for 537.3918; IR (solid) 2950, 1742, 1668 cm^{-1} .

tert-Butyl cholestan-7-keto-3 β -yloxyacetate (12): 10% Palladium on carbon (0.090 g) was added to a degassed solution of **11** (0.907 g, 1.76 mmol) in 1:1 methanol/DCM (20 mL) at 25 °C. The mixture was degassed again before it was placed under an atmosphere of hydrogen gas at ambient pressure. After being stirred for 14 h, the mixture was filtered through a pad of Celite, and the filtrate was evaporated to dryness. The resulting residue was purified by MPLC (6% to 50% ethyl acetate in hexanes) to yield 0.560 g (62%) of **12** as a light brown solid. ^1H NMR (300 MHz, CDCl_3), δ 3.95 (s, 2H), 3.28 (m, 1H), 2.36–1.67 (m, 10H), 1.50 (m, 3H), 1.44 (s, 9H), 1.42–1.08 (m, 12H), 1.04 (s, 3H), 1.03–0.90 (m, 4H), 0.88 (d, 3H, J= 6.5 Hz), 0.83 (app dd, 6H, J= 4.9, 1.8 Hz), 0.62 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3), δ 212.34, 170.23, 81.73, 78.86, 66.36, 55.48, 55.22, 50.20, 49.09, 47.04, 46.40, 42.71, 39.69, 38.96, 36.46, 36.35, 36.20, 35.87, 34.54, 28.63, 28.33, 28.22, 27.91, 25.19,

23.98, 23.03, 22.78, 22.05, 19.00, 18.88, 12.28, 12.00; HRMS (FAB) calcd for $C_{33}H_{57}O_4$ (M+H)⁺ 517.4251, found for 517.4233; IR (solid) 2933, 2869, 1749, 1709 cm^{-1} .

Cholestan-7-keto-3 β -yloxyacetic acid (13**)³⁰:** Formic acid (12 mL) was added to a solution of **12** (0.615 g, 1.20 mmol) in diethyl ether (12 mL). The mixture was heated at 65 °C for 4 h. The solvents were removed under reduced pressure to afford 0.542 g (99%) of **13** as a beige solid. mp 111–113 °C; ¹H NMR (300 MHz, CDCl₃), δ 4.14 (s, 2H), 3.41 (m, 1H), 2.40–1.11 (m, 35H), 1.10 (s, 3H), 1.08–0.94 (m, 4H), 0.92 (d, 3H, J = 6.6 Hz), 0.87 (app dd, 6H, J = 3.8, 2.8 Hz), 0.66 (s, 3H); ¹³C NMR (125 MHz, CDCl₃), δ 212.22, 173.62, 79.48, 65.47, 55.35, 55.21, 50.19, 49.05, 46.86, 46.22, 42.69, 39.67, 38.90, 36.39, 36.34, 36.04, 35.85, 34.47, 28.61, 28.21, 27.79, 25.16, 23.97, 23.02, 22.77, 22.05, 18.99, 12.27, 12.00; HRMS calcd for $C_{29}H_{49}O_4$ (M+H)⁺ 461.3625, found for 461.3605; IR (solid) 3736, 2934, 2866, 1740, 1700 cm^{-1} .

OTBDMS-Protected Keto Crosslinker (14**):** A 10:1 THF/water solution (11 mL) was added to a mixture of **8** (0.145 g, 0.290 mmol) and triphenylphosphine (0.159 g, 0.607 mmol) at 25 °C. The mixture was heated at reflux for 3 h and then allowed to cool to 25 °C. The mixture was diluted with diethyl ether, washed with brine, dried over anhyd Na₂SO₄, and concentrated under reduced pressure, resulting in the isolation of the crude amine. **13** (0.160 g, 0.349 mmol) and dry DCM (14 mL) were added to the same flask, which was then cooled to 0 °C. *N,N'*-dicyclohexylcarbodiimide (0.084 g, 0.41 mmol) and 4-(dimethylamino)pyridine (0.004 g, 0.03 mmol) were added. After being stirred for 10 min at 0 °C, the reaction mixture was warmed to 25 °C and stirred for 1.5 h. The mixture was diluted with DCM, and the mixture was filtered. The filtrate was concentrated at reduced pressure, and the resulting residue was purified BY HPLC (3% to 10% methanol in DCM) to yield 0.202 g (76% over 2 steps) of **14** as a beige solid. The compound was further purified by recrystallization in THF and water. mp 164–166 °C; ¹H NMR (300 MHz, CDCl₃), δ 6.57 (br s, 1H), 5.32 (m, 1H), 3.95 (s, 2H), 3.48 (m, 1H), 3.28 (m, 2H), 3.22 (m, 1H), 2.38–1.11 (m, 76H), 1.09 (s, 6H), 1.08–1.01 (m, 6H), 1.00 (s, 6H), 0.99–0.94 (m, 4H), 0.93 (d, 3H, J = 6.5 Hz), 0.91 (d, 3H, J = 6.6 Hz), 0.89 (s, 9H), 0.87 (app dd, 6H, J = 3.8, 2.8 Hz), 0.67 (s, 3H), 0.65 (s, 3H), 0.06 (s, 6H); ¹³C NMR (125 MHz, CDCl₃), 212.09, 170.16, 141.91, 121.44, 79.27, 72.97, 67.99, 57.13, 56.17, 55.46, 55.34, 50.50, 50.32, 49.19, 46.94, 46.39, 43.15, 42.83, 42.67, 40.11, 39.80, 39.52, 39.04, 37.71, 36.91, 36.55, 36.53, 36.47, 36.18, 35.98, 35.71, 34.97, 34.64, 34.26, 33.34, 32.41, 32.22, 28.74, 28.58, 28.33, 28.11, 26.40, 26.29, 25.31, 24.60, 24.11, 23.15, 22.90, 22.30, 22.18, 21.38, 19.77, 19.12, 19.01, 18.62, 12.40, 12.20, 12.14, -4.24; HRMS calcd for $C_{59}H_{102}NO_4Si$ (M+H)⁺ 916.7573, found for 916.7654; IR (solid) 2900, 2852, 1707, 1683, 1539 cm^{-1} .

Keto Crosslinker (15**):** A solution of HF-pyridine (0.30 mL) and **14** (0.140 g, 0.153 mmol) in THF (4 mL) was stirred at 25 °C for 2 d in a plastic container instead of glass due to the use of HF. The mixture was diluted with DCM and quenched with saturated aq NaHCO₃. The mixture was extracted with DCM, and the organic extracts were dried over anhyd Na₂SO₄ and concentrated under reduced pressure. The residue was purified by HPLC (0% to 7% methanol in DCM) to yield 0.115 g (94%) of **15** as a beige solid. mp 85–88 °C; ¹H NMR (300 MHz, CDCl₃), δ 6.57 (br s, 1H), 5.36 (s, 1H), 3.95 (s, 2H), 3.53 (m, 1H), 3.31 (m, 2H),

3.23 (m, 1H), 2.38–1.15 (m, 63H), 1.09 (s, 6H), 1.01 (s, 6H), 1.00–0.95 (m, 4H), 0.94 (d, 3H, $J = 6.5$ Hz), 0.92 (d, 3H, $J = 6.5$ Hz), 0.86 (app dd, 6H, $J = 3.8, 2.8$ Hz), 0.68 (s, 3H), 0.66 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3), δ 212.09, 170.16, 141.11, 121.98, 79.27, 72.12, 68.00, 57.09, 56.19, 55.46, 55.35, 50.42, 50.32, 49.20, 46.95, 46.40, 42.83, 42.67, 42.63, 40.09, 39.81, 39.52, 39.04, 37.58, 36.83, 36.54, 36.47, 36.18, 35.99, 35.72, 34.97, 33.34, 32.22, 31.99, 28.74, 28.58, 28.34, 28.11, 26.42, 25.31, 24.60, 24.11, 23.15, 22.90, 22.19, 21.40, 19.75, 19.13, 19.01, 12.41, 12.22, 12.14; HRMS calcd for $\text{C}_{53}\text{H}_{87}\text{NNaO}_4$ ($\text{M}+\text{Na}$) $^+$ 824.6527, found for 824.6485; IR (film) 3416, 2933, 1707, 1670, 1539 cm^{-1} .

Crosslinker (16): This procedure was performed in the dark. According to a general method,³² anhyd NH_3 was bubbled through a solution of **15** (0.0584 g, 0.0728 mmol) in 2:1 dry THF/methanol (3 mL) for 2 h at 0 °C. A solution of hydroxylamine-O-sulfonic acid (0.0387 g, 0.342 mmol) in methanol (1 mL) was added dropwise to the mixture at 0 °C over 10 min. The mixture was stirred for 1 h at 0 °C and at 25 °C for 14 h. The mixture was filtered, and the filtrate was concentrated under reduced pressure. The resulting white residue was the diaziridine intermediate, which was dissolved in dry methanol (5 mL) and triethylamine (0.20 mL). A solution of iodine (0.080 g, 0.32 mmol) in dry methanol (1 mL) was added until the reaction mixture remained light brown. Excess iodine was reduced by slow addition of sodium dithionite. The mixture was diluted with chloroform, washed with brine, dried over anhyd Na_2SO_4 , and concentrated under reduced pressure. The residue was purified by preparative HPLC (Waters xBridge Prep C18 5 μm 10 $\times$ 50-mm reverse phase column; solvent A = water, solvent B = methanol; flow rate = 4 mL/min; 5:95 solvent A:B for 10 min; solvent B increased to 100% over 1 min and held at 100% for 4 min) to yield 0.006 g (10%) of **16** as a beige solid. ^1H NMR (300 MHz, CDCl_3), δ 6.57 (m, 1H), 5.37 (m, 1H), 3.94 (s, 2H), 3.53 (m, 1H), 3.34 (m, 2H), 3.22 (m, 1H), 2.24 (m, 4H), 2.05–1.80 (m, 12H), 1.55 (s, H_2O), 1.52–1.04 (m, 39H), 1.02 (s, 3H), 0.93 (s, 3H), 0.85 (app dd, 6H, $J = 4, 2.6$ Hz), 0.68 (s, 3H), 0.59 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3), δ 170.32, 141.10, 122.01, 79.46, 76.99, 72.15, 67.95, 57.09, 56.19, 54.81, 52.69, 50.42, 50.17, 43.54, 42.68, 42.64, 40.09, 39.79, 39.50, 39.14, 37.59, 37.53, 36.84, 36.69, 36.40, 36.37, 36.32, 35.76, 35.73, 34.30, 33.35, 32.43, 32.22, 32.00, 30.06, 28.58, 28.34, 28.27, 26.42, 25.96, 25.19, 24.60, 24.08, 23.15, 22.89, 21.41, 21.35, 19.75, 19.14, 19.01, 12.31, 12.22, 11.79; HRMS (FAB) calcd for $\text{C}_{53}\text{H}_{88}\text{N}_3\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 814.6820, found for 814.6773; UV 349.5, 366.5 nm; IR (solid) 3384, 1652, 1635, 1455 cm^{-1} .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Current model for lysosomal cholesterol transport. Least understood is the mechanism of trans-membrane transport.

Figure 2. Schematic of the crosslinker design in the context of the NPC1/NPC2 interaction during the transfer of cholesterol from one binding pocket to the other. The dashed lines indicate the protein-protein interactions that occur during the transfer.

Figure 3.
Representative examples of previously reported cross-linked bile acid derivatives.

Figure 4.
Examples of possible linkers between cholesterol units

Figure 5.
Primary design for parent head-to-tail cross-linked cholesterol derivative.

Figure 6.

(a) Crystal structures of NPC2 bound to cholesterol sulfate (tan, left, PDB: 2HKA)⁸ and NPC1 bound to cholesterol (blue, right, PDB: 3GKI),⁶ which are aligned to previously modeled structures of the NPC1-NPC2 cholesterol transport complex.^{6,14} **(b)** MD-refined structure of amide **1** bound to the NPC1-NPC2 complex. Cavity-enclosed solvent accessible surface area is shown in gray.

Figure 7.
Synthesis of parent crosslinker **1**.

Figure 8.
 Synthesis of photoaffinity labeled crosslinker **16**.