Supplementary Information for:

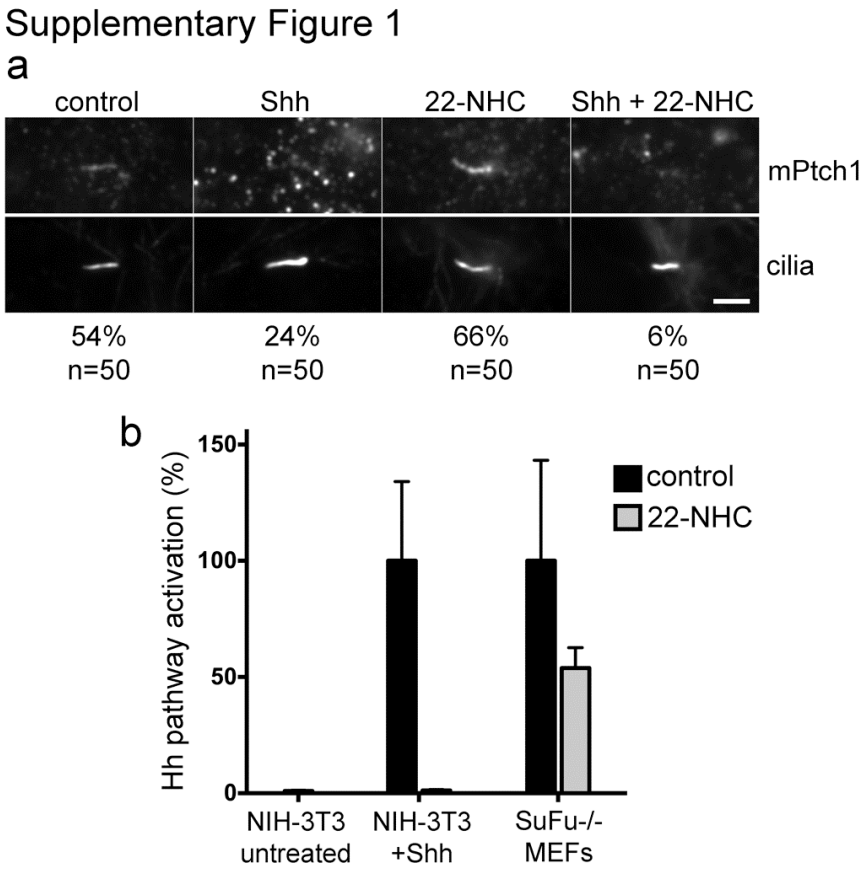
Oxysterol binding to the extracellular domain of Smoothened in Hedgehog signaling

Daniel Nedelcu, Jing Liu, Yangqing Xu, Cindy Jao and Adrian Salic*

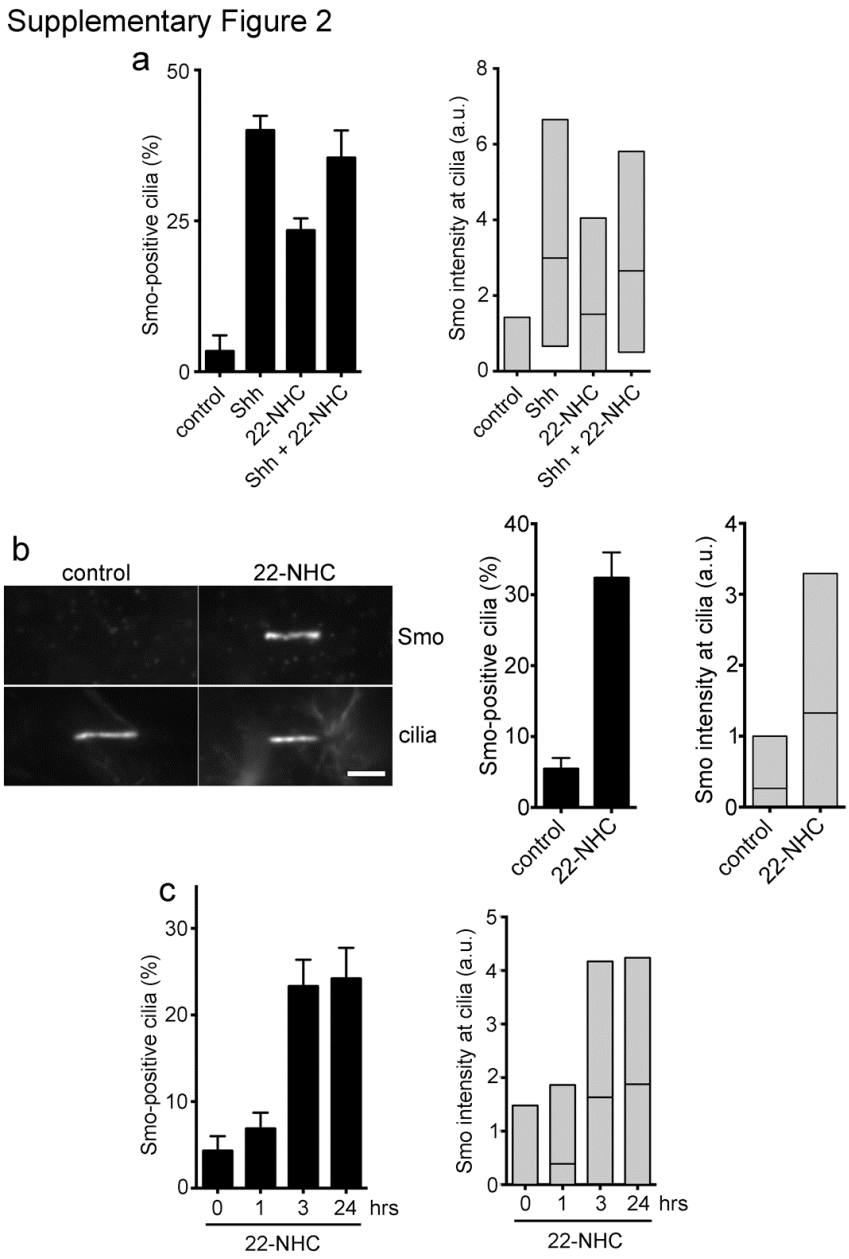
Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115

* Correspondence: asalic@hms.harvard.edu

Supplementary results

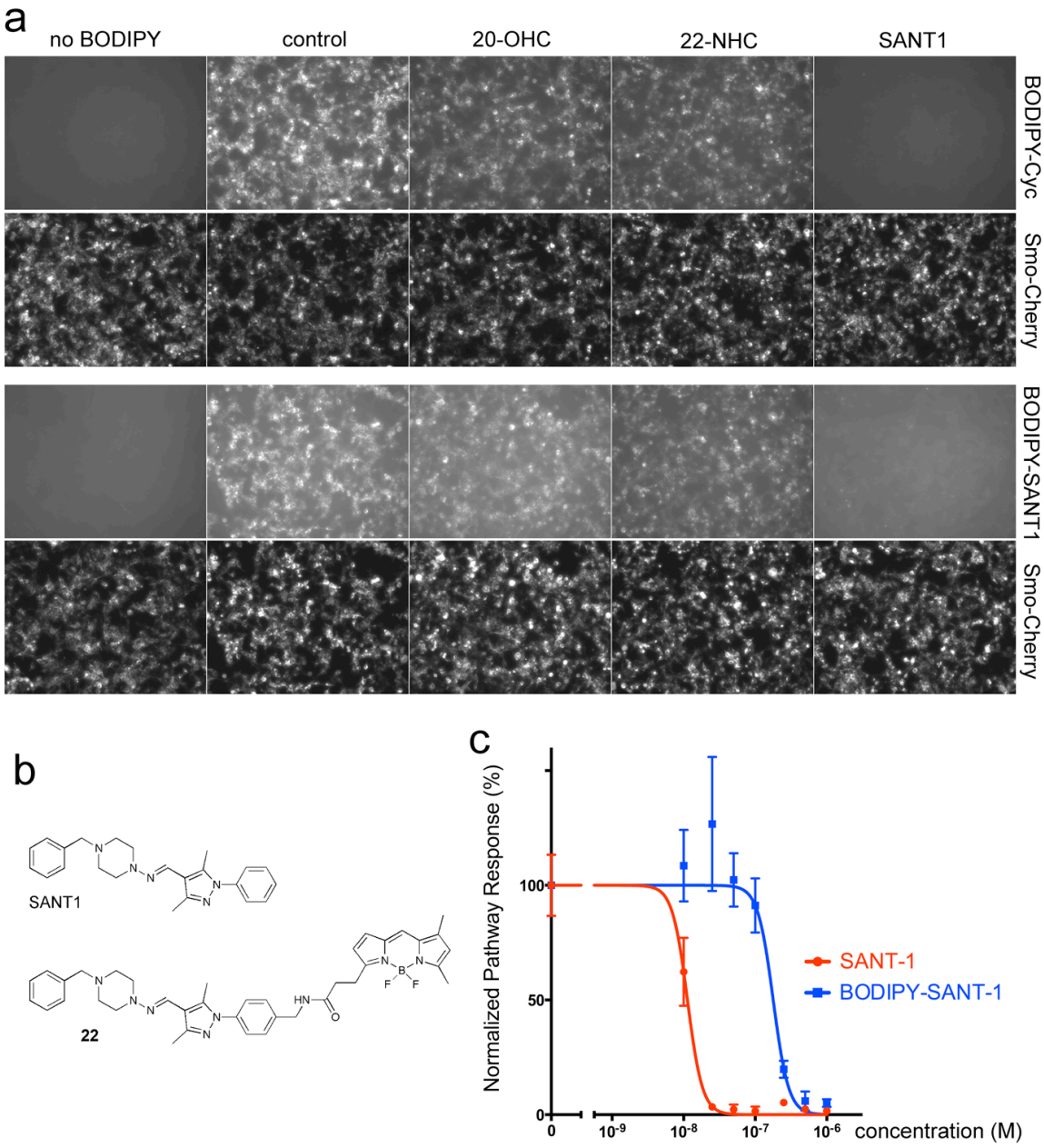


Supplementary Figure 1: 22-NHC has no effect on Ptch1 removal from the cilium, and does not inhibit Hh signaling due to loss of SuFu. (**a**) Ptch1-/- MEFs stably expressing Cherry-tagged mPtch1 were incubated with Shh, in the presence or absence of 22-NHC (10 μM). Cells were stained with rabbit anti-Cherry antibodies (to visualize mPtch1) and mouse anti-acetylated tubulin antibodies (to visualize primary cilia). The micrographs show representative images of cilia, with Ptch1-positive cilia counts shown below the panels. Scale bar = 2 μm. 22-NHC does not block the disappearance of mPtch1 from primary cilia in response to Shh. (**b**) SuFu-/- MEFs were incubated in the absence or presence of 22-NHC (10 μM), and transcription of the target gene Gli1 was measured by Q-PCR. The same concentration of 22-NHC blocks Gli1 transcription in NIH-3T3 cells stimulated with Shh. Error bars indicate standard deviation (n=3). 22-NHC does not block constitutive Hh signaling in SuFu-/- MEFs.



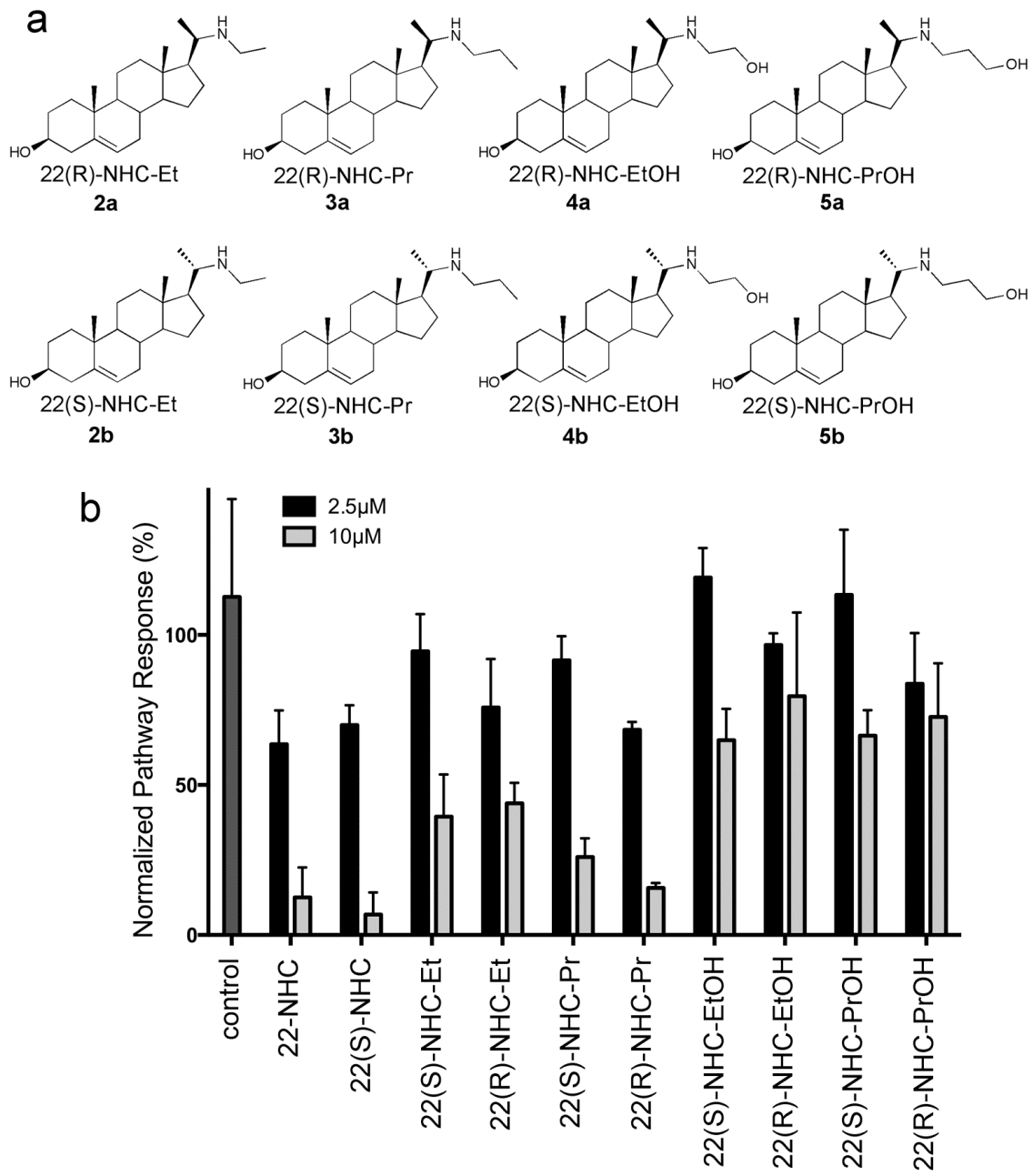
Supplementary Figure 2: 22-NHC causes Smo to accumulate at primary cilia. (**a**) NIH-3T3 cells were treated for 24 hours with Shh, in the presence or absence of 22(S)-NHC (10 μM), after which localization of endogenous Smo was determined by immunofluorescence microscopy. Left: graph showing the percentage of Smo-positive cilia. Error bars represent the sub-sampling standard deviation of the fraction of positive cilia (see Online Methods). Between 446-595 cilia were analyzed per condition. Right: box plot showing fluorescence intensity of ciliary Smo; the lower and upper bounds of each box represent the 25th and 75th percentile of the intensity distribution, while the horizontal line represents the median intensity. In cells treated with Shh and 22(S)-NHC, Smo recruitment to cilia is very similar to cells treated with Shh alone. 22-NHC does not inhibit Smo accumulation at primary cilia in response to Shh stimulation. (**b**) As in (a), but with incubation with 22(S)-NHC (10 μM) for 24 hours. Left: representative micrographs of cilia (scale bar = 2 μm). Middle: graph showing percentage of Smo-positive cilia. Right: box plot showing fluorescence intensity of ciliary Smo. Between 396-525 cilia were analyzed per condition. 22-NHC causes Smo accumulation at primary cilia. (**c**) Time course of Smo accumulation at primary cilia in response to 22-NHC. As in (b), but cells were treated with 22(S)-NHC (10 μM) for the indicated amount of time. Between 281-611 cilia were analyzed per condition.

Supplementary Figure 3



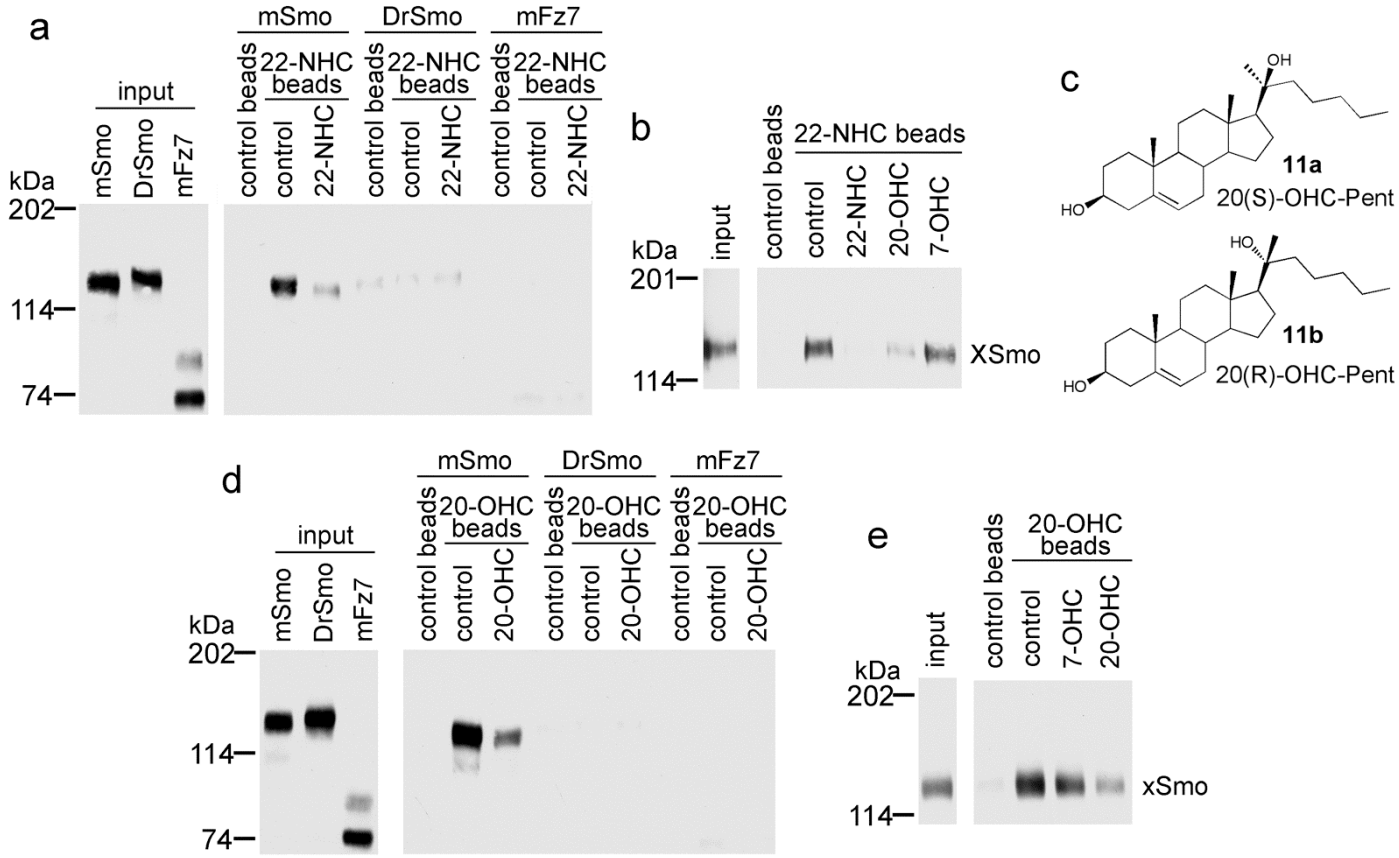
Supplementary Figure 3: 22-NHC does not compete with BODIPY-cyclopamine and BODIPY-SANT1 for binding to Smo. (**a**) Human 293T cells expressing mSmo-Cherry were incubated with 10 nM BODIPY derivative, in the presence or absence of 10 μM 20-OHC, 10 μM 22-NHC or 4 μM SANT1. Cells were fixed, washed, and imaged by fluorescence microscopy, to detect mSmo-Cherry and the BODIPY conjugate. 22-NHC and 20-OHC do not compete binding of BODIPY-cyclopamine (BODIPY-Cyc) or BODIPY-SANT1 (compound **22**), in contrast to SANT1. (**b**) Structures of SANT1 and BODIPY-SANT1. (**c**) Shh Light II cells were stimulated with Shh in the presence of increasing concentrations of SANT1 and BODIPY-SANT1, and Hh pathway activity was measured by luciferase assay. Both SANT1 and BODIPY-SANT1 inhibit Hh signaling in a dose-dependent manner. Error bars represent standard deviation (n=4). Hh pathway activity was normalized to the activity of Shh alone (100%).

Supplementary Figure 4



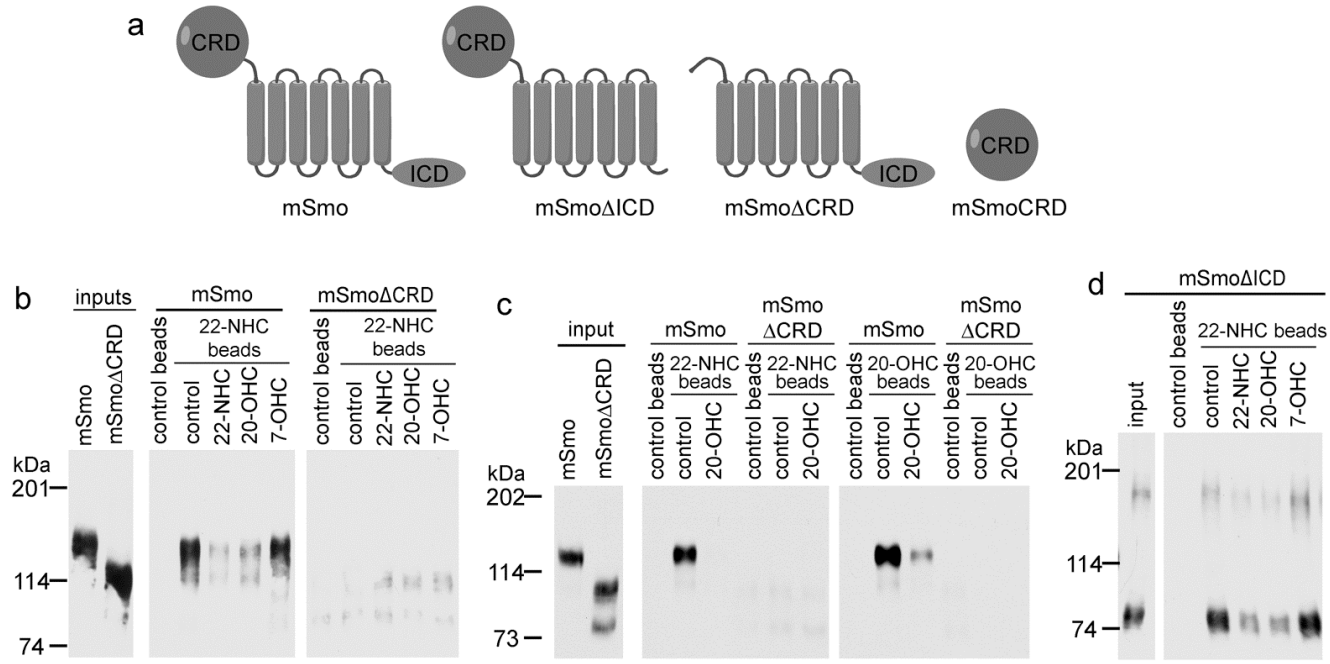
Supplementary Figure 4: Structure-activity analysis of 22-NHC derivatives. (**a**) Structures of the 22-NHC analogs, 22-NHC-Et (**2**), 22-NHC-Pr (**3**), 22-NHC-EtOH (**4**), and 22-NHC-PrOH (**5**). The two diastereomers resulting from the two C-20 configurations (R and S) are shown for each analog. (**b**) Shh Light II cells were stimulated with Shh in the presence of the indicated concentrations of 22(S)-NHC, 22(S)-NHC-Et, 22(R)-NHC-Et, 22(S)-NHC-Pr, 22(R)-NHC-Pr, 22(S)-NHC-EtOH, 22(R)-NHC-EtOH, 22(S)-NHC-PrOH, and 22(R)-NHC-PrOH. Both R and S diastereomers of 22-NHC-Et and 22-NHC-Pr inhibit Hh signaling. 22(S)-NHC-EtOH and 22(S)-NHC-PrOH have reduced inhibitory activity, while 22(R)-NHC-EtOH and 22(R)-NHC-PrOH are inactive. Error bars represent standard deviation (n=4).

Supplementary Figure 5



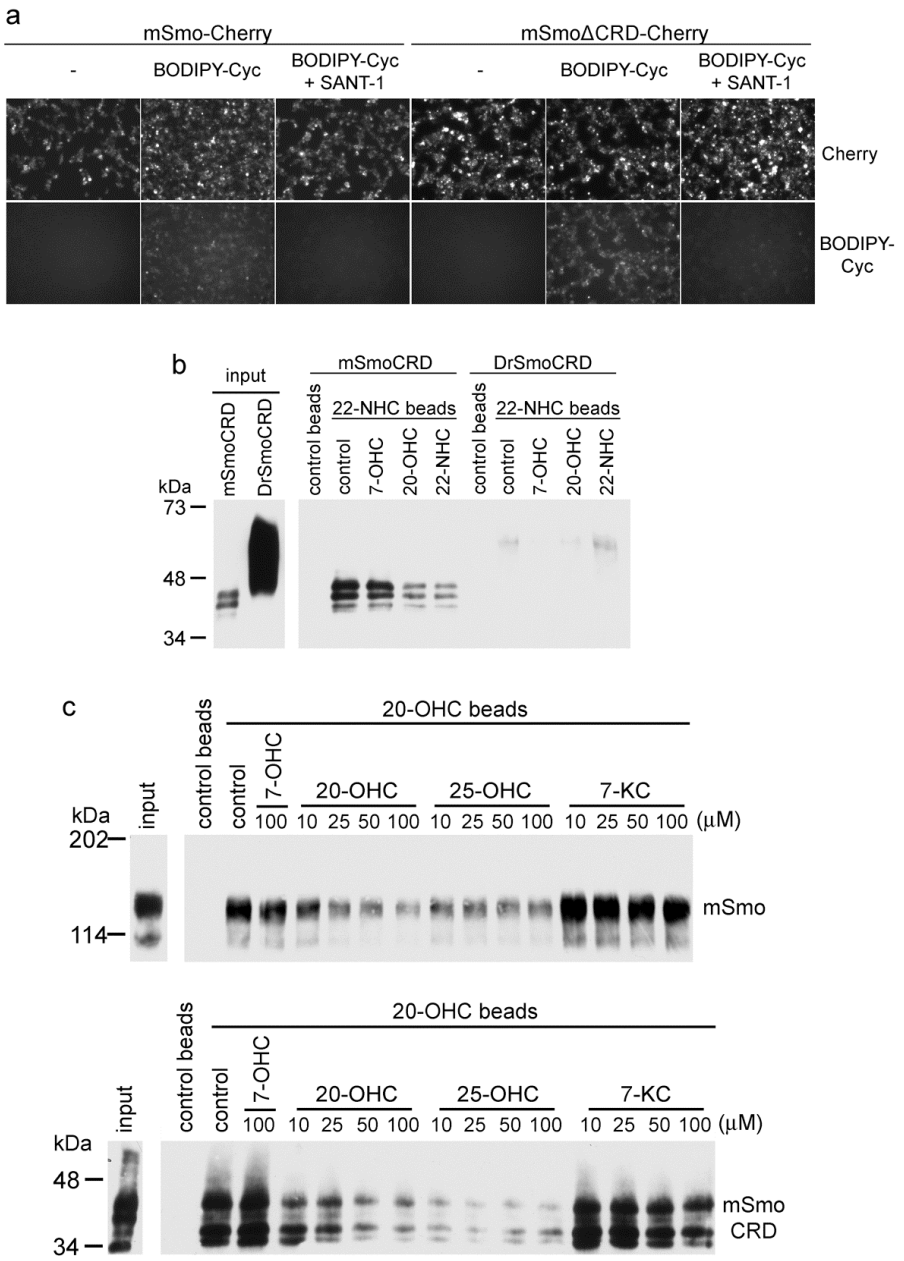
Supplementary Figure 5: Specific binding of vertebrate Smo to 22-NHC and 20-OHC beads. (**a**) 22-NHC beads were incubated with detergent extracts of 293T cells expressing Cherry-tagged mSmo, DrSmo or mFz7, in the absence or presence of free 22-NHC (100 μM). The beads were washed and bound protein was analyzed by SDS-PAGE and immunoblotting with anti-Cherry antibodies. A portion of the extract was analyzed in parallel, to show input. DrSmo and mFz7 do not bind 22-NHC beads, in contrast to mSmo. (**b**) As in (a), but with eGFP-tagged xSmo (expressed in Sf9 cells) and with addition of 22-NHC, 20-OHC or 7-OHC (all used at 100 μM). Protein bound to beads was detected by immunoblotting with anti-GFP antibodies. Binding of xSmo to 22-NHC beads is competed by free 22-NHC and 20-OHC but not by 7-OHC. (**c**) Structures of the two diastereomers of the active oxysterol 20-OHC-Pent (**11**). (**d**) As in (a) but with 20-OHC beads, and with addition of free 20-OHC competitor (100 μM). DrSmo and mFz7 do not bind 20-OHC beads, in contrast to mSmo. Binding of mSmo was competed by free 20-OHC. (**e**) As in (b) but with 20-OHC beads, and with addition of free 20-OHC (100 μM) or 7-OHC (100 μM). XSmo binds to 20-OHC beads and is competed by 20-OHC but not 7-OHC. The full immunoblots for this figure are shown in Supplementary figure 12.

Supplementary Figure 6



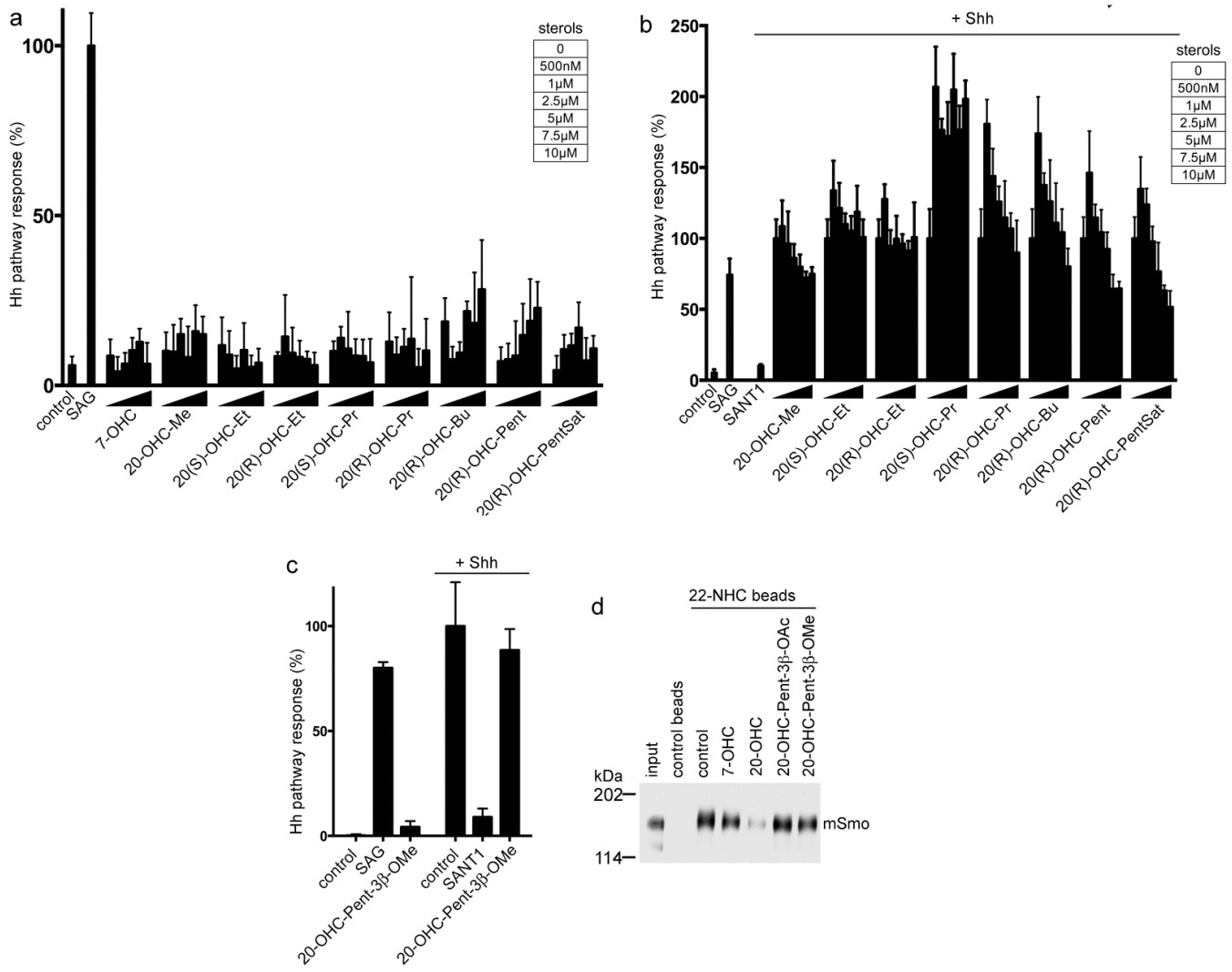
Supplementary Figure 6: Deletion analysis of mSmo binding to 22-NHC and 20-OHC beads. (**a**) Schematic of mSmo and of the mSmo deletion mutants used to map the oxysterol-binding site. (**b**) Binding of Cherry-tagged mSmo or mSmoΔCRD to 22-NHC beads was tested in the presence of 100 μM of the indicated compounds. The CRD of mSmo is required for binding to 22-NHC beads. (**c**) As in (b) but with 22-NHC or 20-OHC beads, and with addition of free 20-OHC competitor (100 μM). The CRD of mSmo is required for binding to 20-OHC beads. (**d**) As in (b) but with mSmoΔICD-Cherry. The ICD of mSmo is not required for binding to 22-NHC beads. The full immunoblots for this figure are shown in Supplementary figure 12.

Supplementary Figure 7



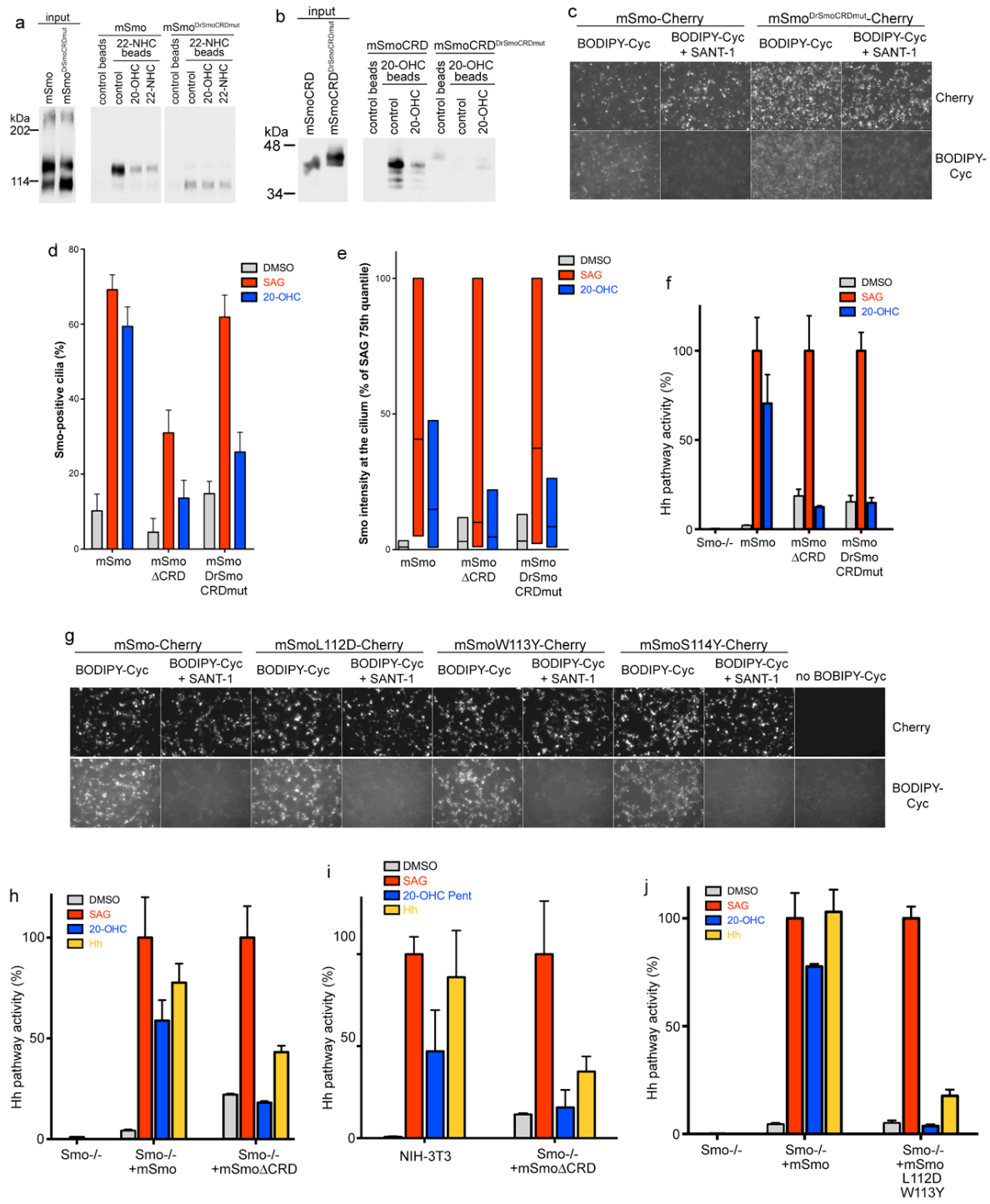
Supplementary Figure 7: Oxysterols and 22-NHC bind the CRD of vertebrate Smo. (**a**) Human 293T cells expressing mSmo-Cherry or SmoΔCRD-Cherry were incubated with 10 nM BODIPY-Cyc, in the presence or absence of 2 μM SANT1. Cells were fixed, washed, and imaged by fluorescence microscopy, to detect the Cherry fusions and the BODIPY conjugate. Both mSmo and mSmoΔCRD bind BODIPY-Cyc. (**b**) Secreted HA-tagged mSmoCRD and DrSmoCRD were tested for binding to 22-NHC beads, in the presence of free 7-OHC, 20-OHC or 22-NHC (each at 100 μM). DrSmoCRD does not bind 22-NHC beads, in contrast to mSmoCRD. (**c**) Detergent extracts of 293T cells expressing mSmo-Cherry, or supernatants containing HA-tagged mSmoCRD were incubated with 20-OHC beads, in the presence of 7-OHC (negative control), or the active oxysterols 20-OHC and 25-OHC, or the inactive 7-keto-cholesterol (7-KC). Bound mSmo-Cherry and mSmoΔCRD-HA were analyzed by SDS-PAGE and immunoblotting. Binding of mSmo and mSmoCRD to 20-OHC beads is competed by 20-OHC and 25-OHC, but not by 7-KC. The full immunoblots for this figure are shown in Supplementary figure 12.

Supplementary Figure 8



Supplementary Figure 8: Structure-activity analysis of C-20 oxysterol compounds. (**a**) Shh Light II cells were treated for 30 hours with the oxysterols 20-OHC-Me, 20(S)-OHC-Et, 20(R)-OHC-Et, 20(S)-OHC-Pr, 20(R)-OHC-Pr, 20(R)-OHC-Bu, 20(R)-OHC-Pent and 20(R)-OHC-PentSat, followed by luciferase assay to measure Hh pathway activity. 7-OHC and SAG (1 μM) were used as negative and positive controls, respectively. Error bars represent standard deviation (n=4). (**b**) As in (a) but with addition of Shh, to test if the oxysterols have inhibitory activity on Hh signaling. The Smo inhibitor, SANT1 (1 μM) was used as positive control for Shh inhibition. (**c**) As in (a) but with addition of 20-OHC-Pent-3β–OMe, in the absence or presence of Shh. SAG1 (1 μM) and SANT1 (1 μM) were used as positive controls for pathway activation and for Shh inhibition, respectively. The ether derivative 20-OHC-Pent-3β–OMe does not inhibit or activate Hh signaling. (**d**) Detergent extracts of 293T cells expressing mSmo-Cherry were incubated with 22-NHC beads, in the presence of free 7-OHC, 20-OHC, 20-OHC-Pent-3β–OAc, or 20-OHC-Pent-3β–OMe (100 μM each). After washing, protein bound to beads was analyzed SDS-PAGE and immunoblotting with anti-Cherry antibodies. 20-OHC-Pent-3β–OMe and 20-OHC-Pent-3β–OAc do not compete binding of mSmo to 22-NHC beads. The full immunoblot is shown in Supplementary figure 12.

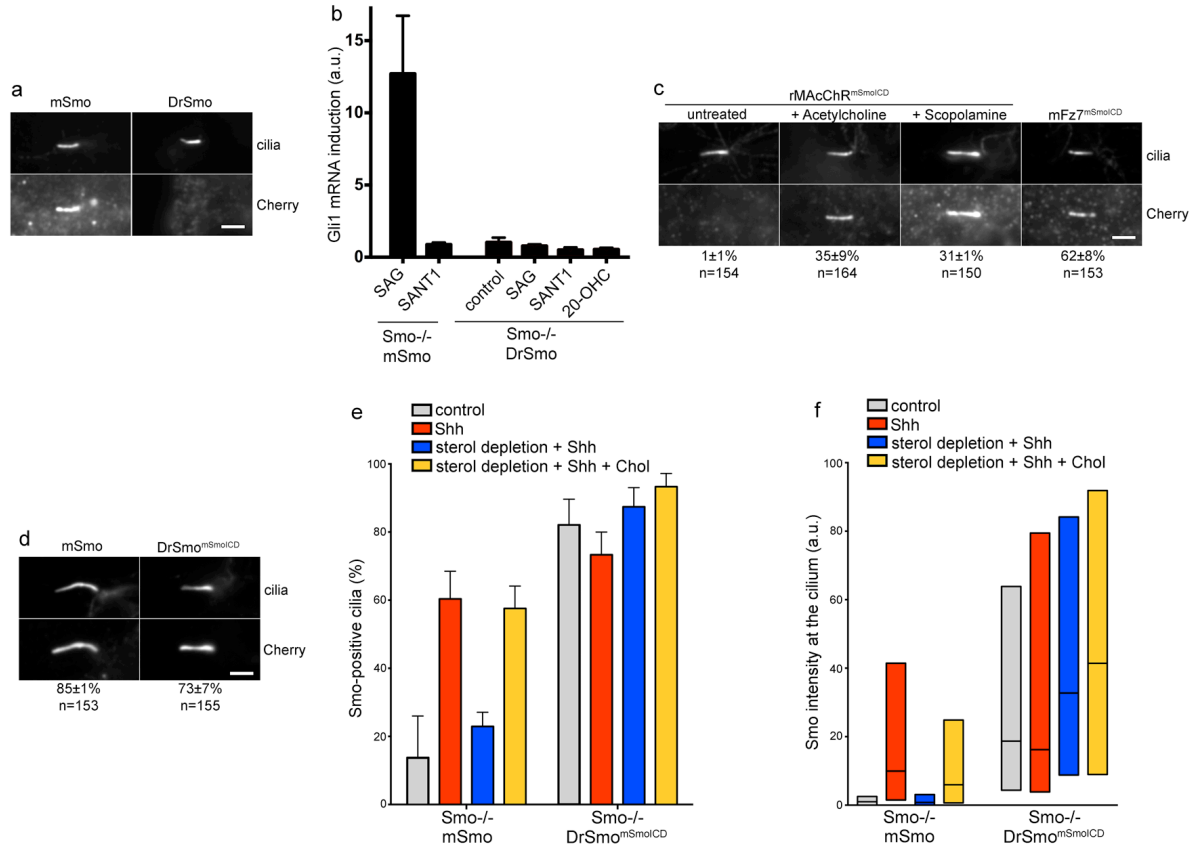
Supplementary Figure 9



Supplementary Figure 9: The role of oxysterol binding to Smo in Hh signaling. (**a**) Cherry-tagged mSmo or mSmo^{DrSmoCRDmut} were incubated with 22-NHC beads, in the presence of 22-NHC or 20-OHC (100 μM each). MSmo^{DrSmoCRDmut} does not bind 22-NHC beads, in contrast to mSmo. The full immunoblot is shown in Supplementary figure 12. (**b**) Secreted HA-tagged mSmoCRD or mSmoCRD^{DrSmoCRDmut} were incubated with 20-OHC beads, in the presence of free 20-OHC (100 μM). MSmoCRD^{DrSmoCRDmut} does not bind 20-OHC beads, in contrast to mSmoCRD. The full immunoblot is shown in Supplementary figure 12. (**c**) Human 293T cells expressing Cherry-tagged mSmo or mSmo^{DrSmoCRDmut} were incubated with 10 nM BODIPY-Cyc, in the presence or absence of 2 μM SANT1. Cells were fixed, washed, and imaged by fluorescence microscopy, to detect the Cherry fusions and the BODIPY conjugate. Both proteins bind

BODIPY-Cyc specifically. (**d**) Smo-/- MEFs, stably expressing low levels of Cherry-tagged mSmo, mSmoΔCRD or $mSmo^{DrSmoCRDmut}$ were incubated overnight in the presence of DMSO control, SAG (1 μM) or 20-OHC (10 μM). The cells were processed for immunofluorescence with anti-Cherry antibodies (to detect mSmo) and anti-acetylated tubulin antibodies (to visualize cilia). The graph shows percentage of cells with Smo-positive cilia. Error bars represent the sub-sampling standard deviation of the fraction of positive cilia (see Online Methods). Between 141-286 cilia were analyzed per condition. MSmoΔCRD and $mSmo^{DrSmoCRDmut}$ respond to SAG but have a defective response to 20-OHC, compared to mSmo. (**e**) As in (d), but with box plots showing the fluorescence intensity of Cherry-tagged proteins at cilia. For each condition, the Cherry signal was normalized to the intensity of the SAG treatment for the respective cell line. The lower and upper bounds of each box represent the 25th and 75th percentile of the distribution of ciliary fluorescence intensity, while the horizontal line represents the median intensity across the entire population of cilia. (**f**) As in (d), but cells were processed for Q-PCR, to measure Gli1 transcription. Gli1 mRNA levels were normalized to the level induced by SAG treatment in the respective cell line. Smo-/- MEFs were included as negative control. Error bars represent standard deviation (n=3). MSmoΔCRD and $mSmo^{DrSmoCRDmut}$ respond to SAG but not to 20-OHC, while mSmo responds to both. (**g**) As in (c) but with expression of Cherry-tagged mSmo, mSmoL112D, mSmoW113Y or mSmoS114Y. All 4 proteins bind BODIPY-Cyc specifically. (**h**) Smo-/- MEFs, stably expressing low levels of Cherry-tagged mSmo or mSmoΔCRD were incubated overnight with DMSO control, SAG (1 μM), 20-OHC (10 μM) or Shh. Smo-/- MEFs were included as negative control. Gli1 mRNA was measured as in (f). Error bars represent standard deviation (n=3). MSmoΔCRD does not respond to 20-OHC and has a reduced responsiveness to Shh. (**i**) NIH-3T3 cells or Smo-/- MEFs stably expressing low levels of mSmoΔCRD-Cherry were incubated overnight with DMSO control, SAG (1 μM), 20(S)-OHC-Pent (10 μM) or Shh. Gli1 mRNA was measured as in (f). Error bars represent standard deviation (n=3). MSmoΔCRD does not respond to 20-OHC-Pent. (**j**) As in (h), but with Smo-/- MEFs stably expressing low levels of Cherry-tagged mSmo or the double point mutant mSmoL112D/W113Y. MSmoL112D/W113Y does not respond to 20-OHC and has a reduced responsiveness to Shh.

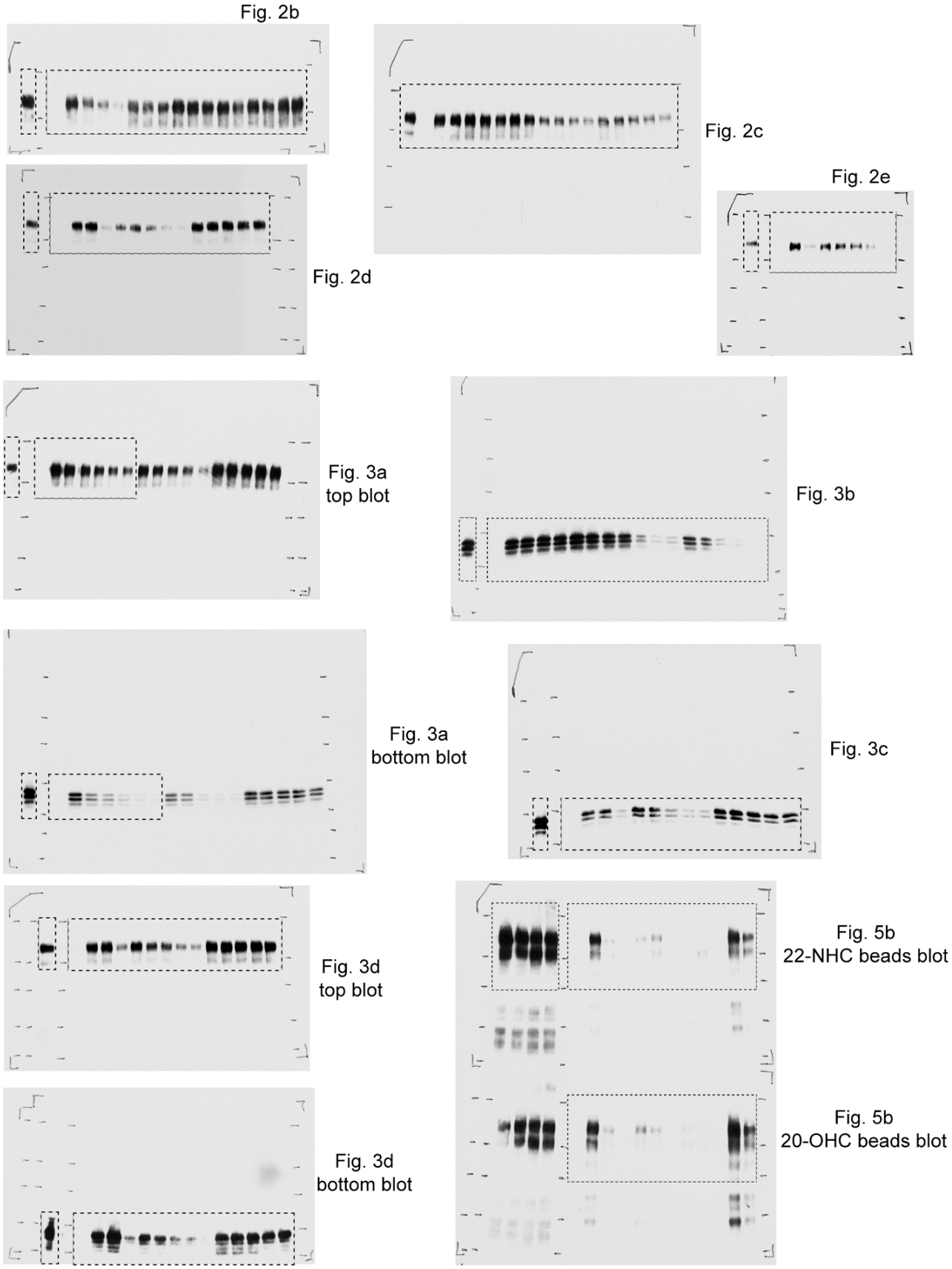
Supplementary Figure 10



Supplementary Figure 10: The intracellular domain of mSmo is sufficient for ciliary localization and confers constitutive activity to DrSmo in mammalian cells. (**a**) Cherry-tagged Drosophila Smoothened (DrSmo) or mSmo were stably expressed in Smo-/- MEFs. The cells were processed for immunofluorescence with antibodies against Cherry and acetylated tubulin (cilia marker). The micrographs show representative images of cilia. DrSmo does not localize to cilia, in contrast to mSmo. Scale bar = 2 μm. (**b**) As in (a) but with overnight incubation in the presence of DMSO control, SAG (1 μM), SANT1 (1 μM), or 20-OHC (10 μM). Gli1 transcription was measured by Q-PCR. Error bars represent standard deviation (n=3). DrSmo does not rescue Hh signaling in Smo-/- MEFs, in contrast to mSmo. (**c**) The cytoplasmic tail of the rat muscarinic acetylcholine receptor M2 (rMAcChR) or of the mouse Frizzled7 protein (mFz7) was replaced with the intracellular domain of mSmo (mSmoICD), to generate the chimeras rMAcChR^{mSmoICD} and mFz7^{mSmoICD}. These chimeras were C-terminally tagged with Cherry, and were stably expressed in Smo-/- MEFs. Cells expressing rMAcChR^{mSmoICD} were incubated overnight with acetylcholine (100 μM) or scopolamine (100 μM), while cells expressing mFz7^{mSmoICD} were incubated in DMEM. The cells were processed for immunofluorescence as in (a). Ciliary localization of the fusion protein was scored manually. MFz7^{mSmoICD} localizes to cilia constitutively, while rMAcChR^{mSmoICD} localizes to cilia only upon treatment with agonist (acetylcholine) or antagonist (scopolamine). Scale bar = 2 μm. (**d**) As in (c), but with the ICD of DrSmo replaced with the ICD of mSmo, to generate DrSmo^{mSmoICD}. Smo-/- MEFs expressing high levels of mSmo-Cherry were used as positive control. DrSmo^{mSmoICD} localizes to cilia, like mSmo. Scale bar = 2 μm. (**e**) Smo-/- MEFs stably expressing low levels of Cherry-tagged DrSmo^{mSmoICD} or mSmo were isolated by FACS. The cells were depleted of sterols by treatment with methyl-β-cyclodextrin (MCD), followed by incubation with pravastatin (40 μM). Cholesterol was added back as MCD-cholesterol (Chol) complexes (100 μM in DMEM), after which the cultures were incubated in the presence or absence of Shh. The cells were processed for immunofluorescence as in (a). The graph shows the percentage of cells with Smo-positive cilia. Error bars represent the sub-sampling standard deviation of the fraction of positive cilia (see

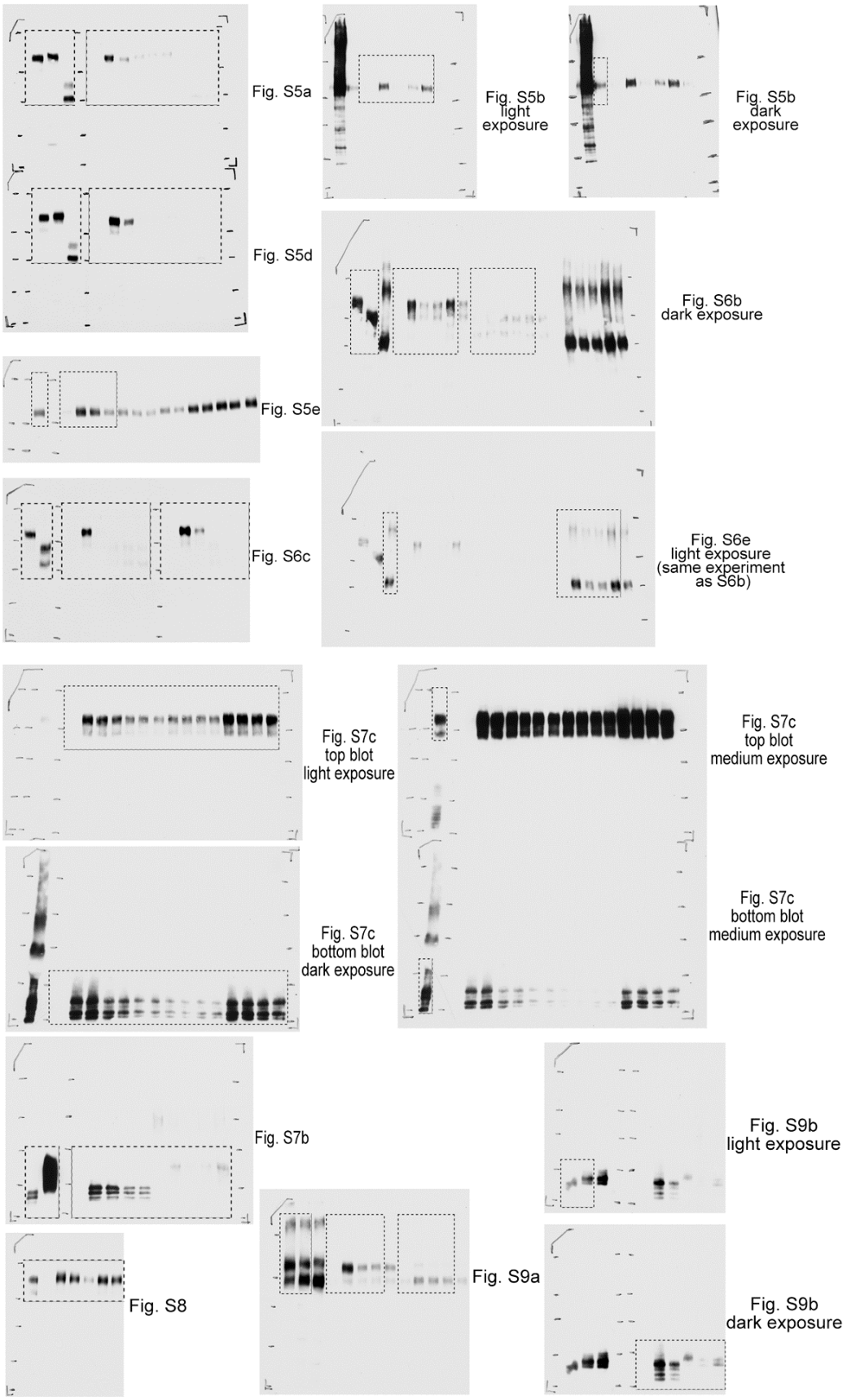
Online Methods). Between 151-217 cilia were analyzed per condition. DrSmo^{mSmoICD} localizes to cilia, with or without sterol depletion; in contrast, mSmo accumulates in cilia upon Shh stimulation, and this accumulation is blocked by sterol depletion. (**f**) As in (e), but with box plots showing fluorescence intensity of Smo at cilia. The lower and upper bounds of each box represent the 25th and 75th percentile of the distribution of ciliary fluorescence intensity, while the horizontal line represents the median intensity across the entire population of cilia.

Supplementary figure 11



Supplementary Figure 11: Full immunoblots for cropped gels in the main text figures. The boxed areas represent the regions of the blot used in the figures.

Supplementary figure 12



Supplementary Figure 12: Full immunoblots for cropped gels in Supplementary figures. The boxed areas represent the regions of the blot used in the figures.

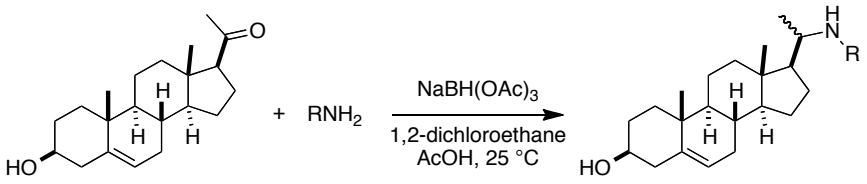
Supplementary note. Synthesis and characterization of sterol derivatives and BODIPY-SANT1

General methods for synthesis

All solvents and reagents were obtained from commercial sources and were used without further purification. NMR spectra were recorded on a Varian 400 MHz NMR spectrometer or a Varian Oxford 600 MHz NMR spectrometer. NMR chemical shifts were expressed in ppm relative to internal solvent peaks, and coupling constants were measured in Hz. High-resolution mass spectra were obtained at the Harvard University Mass Spectrometry Facility, or were obtained in-house on a Bruker microTOF-QII instrument, using an ESI source. LC/MS was performed on a Waters Micromass ZQ instrument using an ESI source coupled to a Waters 2525 HPLC system operating in reverse mode with a Waters Sunfire™ C185 uM 4.6×50 mm column. Flash chromatography was performed on silica gel columns using a Biotage Isolera One flash purification system. Diastereomers of 20-OHC analogs were purified on a chiral RegisCell (25 cm×21.1 mm) column using a preparative HPLC system composed of a Waters 1525 binary pump and Waters 2467 UV absorbance detector.

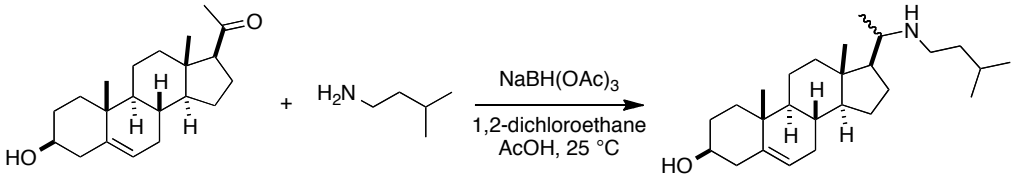
Synthesis of 22-azacholesterol (22-NHC) derivatives (1-6)

General procedure for reductive amination of pregnenolone



Pregnenolone 22-azacholesterol (**1-6**)

Following a reductive amination protocol[1], pregnenolone (5-pregnen-3β-ol-20-one, 1.0 eq) and amine (1.0 eq) were mixed in 1,2-dichloroethane (c = 0.2 mol/L) and were then treated with sodium triacetoxyborohydride (1.5 eq) and AcOH (1.0 eq). The mixture was stirred at room temperature under nitrogen for 16 h. The reaction was quenched with 1M NaOH, and the product was extracted 3 times with diethyl ether. The ether layer was washed with brine and dried over Na_2SO_4. The solvent was evaporated to give the crude free base, which was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH, gradient elution) to provide the 22-NHC analog as a mixture of diastereomers. The ratio between the diastereomers in the mixture was estimated by LC/MS. This mixture was further purified by flash chromatography (silica gel, CH_2Cl_2/$CHCl_3$: MeOH:NH_4OH(89:10:1), gradient elution) to yield the pure C20 diastereomers.

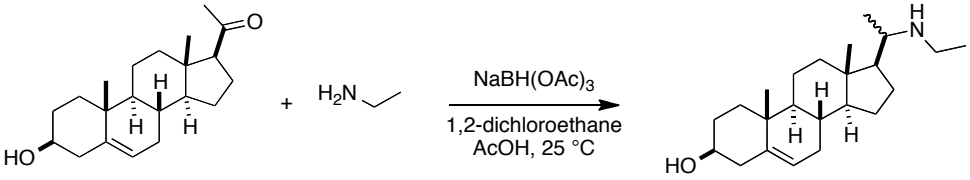


1

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(1-(isopentylamino)ethyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol (22-NHC, 1). Following the general procedure, compound **1** was obtained from pregnenolone (1.01 g, 3.2 mmol) and 3-methylbutan-1-amine (279 mg, 3.2 mmol) as a mixture of diastereomers (20*R*:20*S*; 1:11.8) (152 mg, 12%), from which the major diastereomer **1**(20*S*) was purified as a white solid. This diastereomer was assigned the 20*S*/α configuration based on the reported properties of 22-azacholesterol diasteromers (mp of 20α: 128–129°C, mp of 20β: 151-152°C in reference[2], and mp of 20α: 120–121°C in reference[3]). In the case of the

other analogs of 22-azacholesterol (**2**-**6**), configuration of diastereomers was assigned based on similarity to the diastereomers of **1** in retention time (LC/MS, reverse-phase) and in chemical shift of H_3-20 (doublet). For all 22-azacholesterol analogs described here, the 20*R* diastereomer has a smaller retention time than 20*S*, and the H_3-20 chemical shift of the 20*R* diastereomer is smaller than that of 20*S* by 0.05-0.12 ppm.

1(20*S*/α): mp 128–129°C; $[\alpha]_D^{24}$ = -25.2 (*c* = 0.38, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.36 (m, 1H), 3.46-3.56 (m, 1H), 2.66-2.74 (m, 1H), 2.38-2.54 (m, 2H), 2.18-2.32 (m, 2H), 1.79-2.02 (m, 6H), 1.40-1.68 (m, 8H), 1.28-1.40 (m, 4H), 1.12-1.24 (m, 2H), 1.02-1.12 (m, 5H), 1.00 (s, 3H), 0.91-0.99 (m, 1H), 0.90 (d, *J* = 2.0 Hz, 3H), 0.89 (d, *J* = 2.0 Hz, 3H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.5, 71.6, 56.6, 56.3, 50.0, 44.9, 42.3, 41.9, 39.4, 39.3, 37.2, 36.5, 31.8, 31.7, 31.6, 27.1, 26.2, 24.2, 22.8, 22.5, 20.9, 19.4, 19.3, 12.2; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{26}H_{45}NO$: 388.3574, found 388.3582.

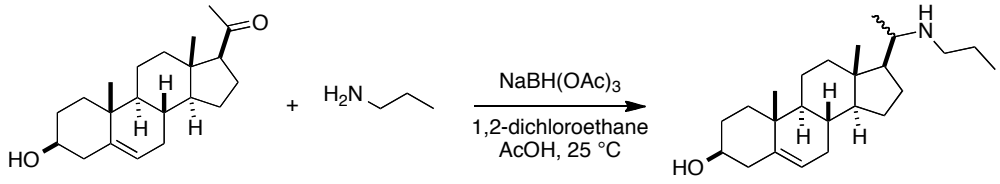


2

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(1-(ethylamino)ethyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[α]phenanthren-3-ol (22-NHC-Et, 2). Following the general procedure, compound **2** was obtained from pregnenolone (1.01 g, 3.2 mmol) and ethylamine (144 mg, 3.2 mmol) as a mixture of diastereomers (20*R*:20*S*; 1.5:1) (500 mg, 45%), which was further separated to yield each pure diastereomer (**2a** and **2b)** as a white solid.

2a(20*R*): $[\alpha]_D^{24}$ = -68.5 (*c* = 0.55, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.35 (m, 1H), 3.47-3.57 (m, 1H), 2.69-2.80 (m, 1H), 2.57-2.66 (m, 1H), 2.44-2.54 (m, 1H), 2.18-2.32 (m, 2H), 1.92-2.02 (m, 2H), 1.71-1.87 (m, 3H), 1.41-1.65 (m, 7H), 1.20-1.40 (m, 3H), 1.02-1.15 (m, 6H), 0.91-1.02 (m, 8H), 0.72 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.9, 121.3, 71.3, 56.3, 55.9, 55.7, 49.9, 42.3, 41.9, 40.9, 40.2, 37.2, 36.4, 31.7, 31.7, 31.6, 26.6, 24.1, 21.0, 19.3, 19.1, 15.5, 12.3; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{23}H_{39}NO$: 346.3104, found 346.3105.

2b(20*S*): $[\alpha]_D^{24}$ = -29.0 (*c* = 0.34, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.36 (m, 1H), 3.46-3.56 (m, 1H), 2.70-2.80 (m, 1H), 2.44-2.58 (m, 2H), 2.18-2.32 (m, 2H), 1.70-2.02 (m, 5H), 1.42-1.68 (m, 7H), 1.14-1.41 (m, 4H), 1.06-1.14 (m, 8H), 0.90-1.06 (m, 5H), 0.70 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.5, 71.6, 56.6, 56.4, 56.2, 49.9, 42.3, 41.9, 40.8, 39.3, 37.2, 36.5, 31.8, 31.7, 31.6, 27.1, 24.1, 20.9, 19.4, 19.2, 15.4, 12.2; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{23}H_{39}NO$: 346.3104, found 346.3108.

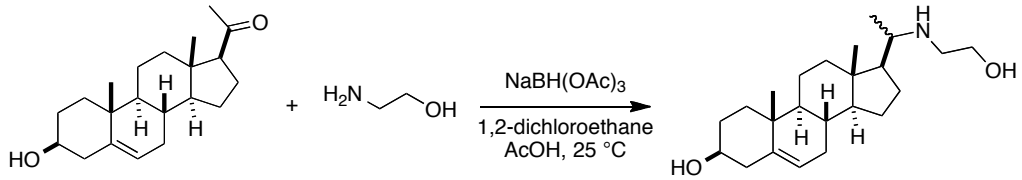


3

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-10,13-dimethyl-17-(1-(propylamino)ethyl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (22-NHC-Pr, 3). Following the general procedure, compound **3** was obtained from pregnenolone (1.01 g, 3.2 mmol) and propylamine (189 mg, 3.2 mmol) as a mixture of diastereomers (20R:20S; 1:1.3) (504 mg, 44%), which was further separated to yield each pure diastereomer (**3a** and **3b)** as a white solid.

3a(20*R*): $[\alpha]_D^{24}$ = -58.0 (*c* = 0.43, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.35 (m, 1H), 3.46-3.56 (m, 1H), 2.65-2.75 (m, 1H), 2.53-2.62 (m, 1H), 2.34-2.42 (m, 1H), 2.18-2.22 (m, 2H), 1.93-2.02 (m, 2H), 1.71-1.87 (m, 3H), 1.41-1.65 (m, 9H), 1.20-1.38 (m, 3H), 1.02-1.17 (m, 3H), 0.95-1.02 (m, 8H), 0.93 (t, *J* = 7.2 Hz, 3H), 0.72 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.5, 71.5, 56.4, 56.2, 56.0, 50.0, 49.0, 42.3, 42.0, 40.2, 37.2, 36.5, 31.8, 31.8, 31.6, 26.7, 24.2, 23.5, 21.1, 19.4, 19.2, 12.3, 12.0; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{24}H_{41}NO$: 360.3261, found 360.3257.

3b(20*S*): $[\alpha]_D^{24}$ = -27.3 (*c* = 0.39, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.36 (m, 1H), 3.46-3.56 (m, 1H), 2.61-2.69 (m, 1H), 2.46-2.54 (m, 1H), 2.36-2.44 (m, 1H), 2.18-2.32 (m, 2H), 1.78-2.02 (m, 5H), 1.40-1.68 (m, 9H), 1.28-1.40 (m, 2H), 1.05-1.24 (m, 6H), 0.94-1.05 (m, 5H), 0.88-0.96 (m, 4H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.4, 71.5, 56.6, 56.3, 56.3, 49.9, 48.7, 42.3, 41.8, 39.3, 37.2, 36.5, 31.8, 31.7, 31.6, 27.0, 24.1, 23.4, 20.9, 19.3, 19.2, 12.2, 11.9; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{24}H_{41}NO$: 360.3261, found 360.3262.

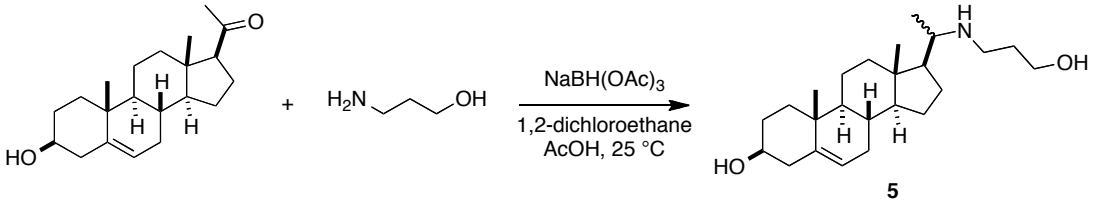


4

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(1-((2-hydroxyethyl)amino)ethyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (4). Following the general procedure, compound **4** was obtained from pregnenolone (1.01 g, 3.2 mmol) and ethanolamine (196 mg, 3.2 mmol) as a mixture of diastereomers (20R:20S; 1.5:1) (160 mg, 14%), which was further separated to yield each pure diastereomer (**4a** and **4b)** as a white solid.

4a(20*R*): $[\alpha]_D^{24}$ = -46.0 (*c* = 0.39, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.60-3.68 (m, 1H), 3.47-3.60 (m, 2H), 2.85-2.93 (m, 1H), 2.54-2.66 (m, 2H), 2.18-2.33 (m, 2H), 2.04-2.11 (m, 1H), 1.93-2.02 (m, 1H), 1.72-1.88 (m, 4H), 1.41-1.66 (m, 8H), 1.20-1.32 (m, 3H), 0.88-1.17 (m, 10H), 0.74 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.5, 71.7, 61.3, 56.8, 56.4, 55.7, 50.1, 48.1, 42.3, 42.2, 40.4, 37.2, 36.5, 31.8, 31.8, 31.6, 27.0, 24.2, 21.1, 19.7, 19.4, 12.4; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{23}H_{39}NO_2$: 362.3054, found 362.3052.

4b(20*S*): $[\alpha]_D^{24}$ = -24.7 (*c* = 0.25, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.48-3.65 (m, 3H), 2.82-2.89 (m, 1H), 2.62-2.70 (m, 1H), 2.46-2.55 (m, 1H), 2.18-2.34 (m, 2H), 1.90-2.04 (m, 3H), 1.70-1.90 (m, 6H), 1.38-1.68 (m, 8H), 0.80-1.34 (m, 10H), 0.69 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.7, 121.6, 71.7, 61.0, 56.8, 56.7, 55.9, 50.0, 47.5, 42.3, 41.8, 39.3, 37.2, 36.5, 31.8, 31.7, 31.6, 27.4, 24.1, 20.9, 19.9, 19.4, 12.2; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{23}H_{39}NO_2$: 362.3054, found 362.3052.



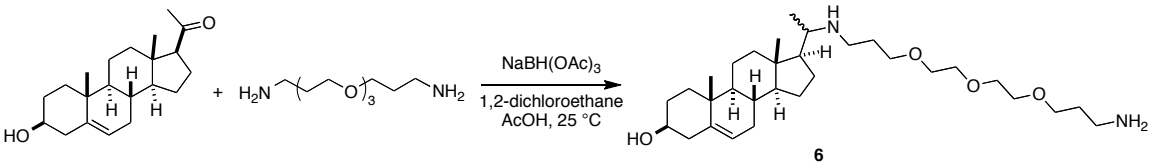
5

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(1-((3-hydroxypropyl)amino)ethyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (5). Following the general procedure, compound **5** was obtained from pregnenolone (1.01 g, 3.2 mmol) and 3-amino-1-

propanol (240 mg, 3.2 mmol) as a mixture of diastereomers (20R:20S; 1:1.4) (61 mg, 5%), which was further separated to yield each pure diastereomer (**5a** and **5b)** as a white solid.

5a(20*R*): $[\alpha]_D^{24}$ = -55.7 (*c* = 0.49, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.36 (m, 1H), 3.80 (t, *J* = 5.2 Hz, 2H), 3.46-3.56 (m, 1H), 2.92-3.00 (m, 1H), 2.65-2.76 (m, 1H), 2.53-2.63 (m, 1H), 2.17-2.33 (m, 2H), 1.92-2.04 (m, 2H), 1.40-1.88 (m, 14H), 1.19-1.34 (m, 3H), 0.89-1.19 (m, 10H), 0.73 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.4, 71.6, 64.4, 56.4, 56.3, 56.1, 50.0, 46.8, 42.2, 42.0, 40.0, 37.2, 36.5, 31.8, 31.8, 31.6, 31.5, 26.9, 24.1, 21.0, 19.4, 19.2, 12.3; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for C24H41NO2: 376.3210, found 376.3206.

5b(20*S*): $[\alpha]_D^{24}$ = -23.3 (*c* = 0.31, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.32-5.36 (m, 1H), 3.81 (t, *J* = 5.2 Hz, 2H), 3.46-3.56 (m, 1H), 3.00-3.07 (m, 1H), 2.66-2.74 (m, 1H), 2.46-2.55 (m, 1H), 2.18-2.33 (m, 2H), 1.78-2.02 (m, 6H), 1.35-1.74 (m, 10H), 0.80-1.32 (m, 13H), 0.68 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.5, 71.6, 64.6, 56.8, 56.6, 56.5, 50.0, 46.9, 42.2, 41.8, 39.2, 37.2, 36.5, 31.8, 31.7, 31.6, 30.9, 27.4, 24.1, 20.9, 19.4, 19.0, 12.1; HRMS: (ESI, m/z) calcd **[M+H]$^+$** for C24H41NO2: 376.3210, found 376.3210.



(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(16-amino-7,10,13-trioxa-3-azahexadecan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol (22-NHC-PEG-NH$_2$, 6). Following the general procedure, compound **6** was obtained from pregnenolone (1.01 g, 3.2 mmol) and 4,7,10-trioxa-1,13-tridecanediamine (2.2 g, 10 mmol) after silica gel column chromatography as a mixture of diastereomers (20*R*:20*S*; 2.6:1) as a yellow oil (749 mg, 45%) and was used in the next step without further purification.

6: ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.33-5.36 (m, 1H), 3.47-3.67 (m, 13H), 2.77-2.89 (m, 3H), 2.45-2.70 (m, 6H), 2.20-2.32 (m, 2H), 1.91-2.05 (m, 3H), 1.69-1.89 (m, 7H), 1.39-1.69 (m, 7H), 0.90-1.39 (m, 11H), 0.72, 0.70 (2.6:1; s, 3H); HRMS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{31}H_{56}N_2O_4$: 521.4313, found 521.4311.

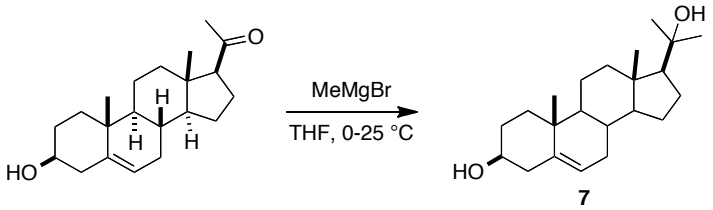
Synthesis of 20-hydroxycholesterol (20-OHC) analogs (7-16)

General procedure for Grignard reactions of pregnenolone

Pregnenolone — 20-hydroxycholesterol (**7-11**)

Pregnenolone (1.0 eq.) was dissolved in dry THF (0.16 mol/L) and the resulting solution was cooled to 0 °C under nitrogen. A solution of alkylmagnesium bromide or chloride (3.0 eq.) was added drop-wise over 10 min. The reaction mixture was stirred at 25 °C for 16 h and was then cooled to 0 °C. A saturated solution of NH_4Cl was added and the mixture was stirred for 30 min, after which it was extracted 3 times with EtOAc. The organic phase was washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a residue, which was purified by flash chromatography on silica gel (step-wise gradient elution, 0-70%

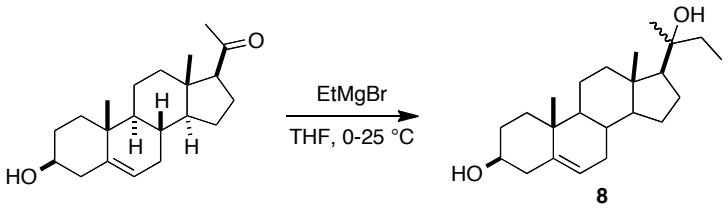
EtOAc/hexane) to provide the 20-hydroxycholesterol analog as a mixture of diasteromers. This mixture was subjected to normal-phase chiral HPLC purification (6% *i*-PrOH/Hexane), to yield the pure diastereomers. The configuration of the diastereomers of compounds **8-12** was assigned by comparison with the reported NMR characterization of *nat*-20(*R*)-hydroxycholesterol and related compounds[4], based on the trend in the chemical shifts of C-20 and H_3-21 (singlet). For all 20-hydroxycholesterol analogs examined here, the C-20 chemical shift of the 20*S* diastereomer is around 75.2 ppm while it is 75.8 ppm for 20*R*, and the H_3-21 chemical shift of the 20*S* diastereomer is around 1.26 ppm while it is 1.12 ppm for 20*R*. This assignment is consistent with results of biological activity assays, as the 20*R* diastereomers of **10-12** activate the Hedgehog pathway in cells and bind Smoothened, while the 20*S* diastereomers of **10-12** are inactive (see text). We also observed that the 20*S* diastereomers have a smaller retention time during the chiral HPLC separation.



7

(3*S*,10*R*,13*S*,17*S*)-17-(2-Hydroxypropan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (20-OHC-Me, 7). Following the general procedure, compound **7** was obtained from pregnenolone (1.0 g) and methylmagnesium bromide (3.2 mL of 3.0 M solution in Et_2O) in dry THF (20 mL), as a white solid (117 mg, 11% yield after HPLC purification).

7: $[\alpha]_D^{24}$ =-43.7 (*c* = 0.21, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.47-3.57 (m, 1H), 2.19-2.34 (m, 2H), 2.07-2.14 (m, 1H), 1.94-2.03 (m, 1H), 1.80-1.88 (m, 2H), 1.70-1.78 (m, 2H), 1.60-1.70 (m, 2H), 1.41-1.59 (m, 6H), 1.31 (s, 3H), 1.20-1.28 (m, 2H), 1.20 (s, 3H), 1.03-1.18 (m, 2H), 1.01 (s, 3H), 0.88-1.00 (m, 2H), 0.85 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.6, 73.5, 71.8, 60.2, 56.8, 50.0, 42.7, 42.3, 40.1, 37.2, 36.5, 31.8, 31.6, 31.3, 31.0, 30.1, 23.8, 23.1, 20.9, 19.4, 13.5; HRMS: (ESI, m/z) calcd **[M+Na]$^+$** for $C_{22}H_{36}O_2$, 355.2613; found, 355.2630.

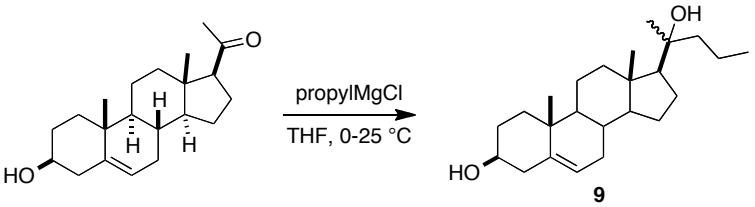


8

(3*S*,10*R*,13*S*,17*S*)-17-(2-Hydroxybutan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (20-OHC-Et, 8). Following the general procedure, compound **8** was obtained from pregnenolone (1.0 g) and ethylmagnesium bromide (3.2 mL of 3.0 M solution in Et_2O) in dry THF (20 mL) as a white solid (136 mg, 12%). Further preparative chiral HPLC separation yielded the pure diastereomers (**8a** and **8b)** as white solids.

8a(20*S*): $[\alpha]_D^{24}$ = -40.3 (*c* = 0.18, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.34-5.37 (m, 1H), 3.49-3.55 (m, 1H), 2.28-2.32 (m, 1H), 2.20-2.27 (m, 1H), 2.07-2.12 (m, 1H), 1.95-2.01 (m, 1H), 1.82-1.87 (m, 2H), 1.70-1.78 (m, 1H), 1.57-1.70 (m, 2H), 1.44-1.57 (m, 8H), 1.36-1.44 (m, 1H), 1.25 (s, 3H), 1.02-1.24 (m, 4H), 1.01 (s, 3H), 0.90-1.00 (m, 2H), 0.87 (s, 3H), 0.86 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 140.8, 121.6, 75.4, 71.8, 57.2, 56.9, 50.0, 42.6, 42.3, 40.1, 37.2, 36.5, 36.1, 31.8, 31.6, 31.3, 25.7, 23.8, 22.3, 20.9, 19.4, 13.6, 8.5; HRMS: (ESI, m/z) calcd **[M+Na]$^+$** for $C_{23}H_{38}O_2$, 369.2770; found, 369.2771.

8b(20*R*): $[\alpha]_D^{24}$ = -24.4 (*c* = 0.14, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.34-5.37 (m, 1H), 3.48-3.56 (m, 1H), 2.27-2.32 (m, 1H), 2.22-2.27 (m, 1H), 2.08-2.13 (m, 1H), 1.95-2.01 (m, 1H), 1.82-1.87 (m, 2H), 1.55-1.78 (m, 3H), 1.43-1.55 (m, 7H), 1.20-1.28 (m, 2H), 1.11-1.18 (m, 2H), 1.11 (s, 3H), 1.02-1.10 (m, 2H), 1.01 (s, 3H), 0.93-1.00 (m, 2H), 0.92 (t, *J* = 7.2 Hz, 3H), 0.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 140.8, 121.6, 75.9, 71.8, 58.0, 56.9, 50.0, 42.8, 42.3, 40.2, 37.2, 36.5, 34.9, 31.8, 31.6, 31.3, 26.3, 23.8, 23.2, 20.9, 19.4, 13.7, 8.4; HRMS: (ESI, m/z) calcd **[M+**H-2H_2O**]$^+$** for $C_{23}H_{38}O_2$, 311.2739; found, 311.2746.

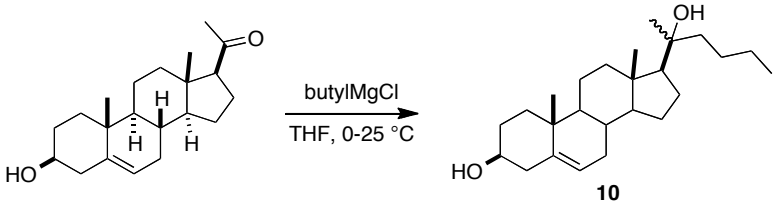


9

(3*S*,10*R*,13*S*, 17S)-17-(2-Hydroxypentan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol (20-OHC-Pr, 9). Following the general procedure, compound **9** was obtained from pregnenolone and (1.0 g) and propylmagnesium chloride (4.7 mL of a 2.0 M solution in Et_2O) in dry THF (20 mL) as a white solid (81 mg, 7%). Further preparative chiral HPLC separation yielded the pure diastereomers (**9a** and **9b)** as white solids.

9a(20*S*): $[\alpha]_D^{24}$ = -39.7 (*c* = 0.20, MeOH); ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.34-5.37 (m, 1H), 3.48-3.55 (m, 1H), 2.27-2.32 (m, 1H), 2.20-2.27 (m, 1H), 2.07-2.12 (m, 1H), 1.95-2.00 (m, 1H), 1.81-1.87 (m, 2H), 1.70-1.80 (m, 1H), 1.57-1.70 (m, 2H), 1.40-1.57 (m, 8H), 1.24-1.35 (m, 6H), 1.04-1.23 (m, 4H), 1.01 (s, 3H), 0.87-1.00 (m, 5H), 0.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 140.8, 121.6, 75.2, 71.8, 57.7, 56.9, 50.0, 46.4, 42.6, 42.3, 40.1, 37.2, 36.5, 31.8, 31.6, 31.3, 26.4, 23.8, 22.4, 20.9, 19.4, 17.5, 14.7, 13.6; HRMS: (ESI, m/z) calcd **[M+**Na**]$^+$** for $C_{24}H_{40}O_2$, 383.2926; found, 383.2936.

9b(20*R*): $[\alpha]_D^{24}$ = -38.5 (*c* = 0.60, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.47-3.57 (m, 1H), 2.18-2.33 (m, 2H), 2.06-2.12 (m, 1H), 1.93-2.02 (m, 1H), 1.79-1.87 (m, 2H), 1.44-1.79 (m, 12H), 1.33-1.44 (m, 2H), 1.21-1.30 (m, 2H), 1.05-1.20 (m, 5H), 0.97-1.05 (m, 4H), 0.88-0.97 (m, 4H), 0.87 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.6, 75.8, 71.7, 58.2, 56.9, 50.0, 45.1, 42.9, 42.3, 40.1, 37.2, 36.5, 31.8, 31.6, 31.3, 27.0, 23.8, 23.1, 20.9, 19.4, 17.3, 14.7, 13.7; HRMS: (ESI, m/z) calcd **[M+**Na**]$^+$** for $C_{24}H_{40}O_2$, 383.2926; found, 383.2925.



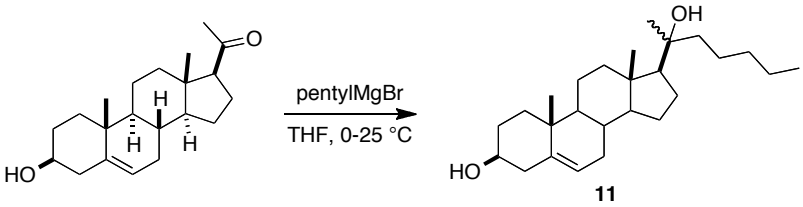
10

(3*S*,10*R*,13*S*,17*S*)-17-(2-hydroxyhexan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol (20-OHC-Bu, 10). Following the general procedure, compound **10** was obtained from pregnenolone (1.0 g) and butylmagnesium chloride (4.7 mL of a 2.0 M solution in THF) in dry THF (20 mL) as a white solid (415 mg, 35%). Further preparative chiral HPLC separation yielded the pure diastereomers (**10a** and **10b)** as white solids.

10a(20*S*): $[\alpha]_D^{24}$ = -54.7 (*c* = 0.42, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.47-3.57 (m, 1H), 2.18-2.33 (m, 2H), 2.06-2.13 (m, 1H), 1.93-2.03 (m, 1H), 1.78-1.87 (m, 2H), 1.70-1.78 (m, 1H), 1.41-

1.70 (m, 11H), 1.05-1.36 (m, 11H), 0.97-1.05 (m, 4H), 0.87-0.96 (m, 4H), 0.87 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.6, 75.2, 71.8, 57.6, 56.9, 50.0, 43.7, 42.6, 42.3, 40.1, 37.2, 36.5, 31.8, 31.6, 31.3, 26.5, 26.4, 23.8, 23.3, 22.3, 20.9, 19.4, 14.1, 13.6; HRMS: (ESI, m/z) calcd **[M+Na]$^+$** for $C_{25}H_{42}O_2$, 397.3083; found, 397.3083.

10b(20*R*): $[\alpha]_D^{24}$ = -49.4 (*c* = 0.16, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.48-3.57 (m, 1H), 2.18-2.33 (m, 2H), 2.06-2.12 (m, 1H), 1.93-2.03 (m, 1H), 1.78-1.88 (m, 2H), 1.60-1.78 (m, 2H), 1.42-1.60 (m, 10H), 1.21-1.38 (m, 6H), 1.09-1.18 (m, 4H), 0.97-1.09 (m, 5H), 0.88-0.97 (m, 4H), 0.87 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.6, 75.8, 71.8, 58.2, 56.9, 50.0, 42.9, 42.6, 42.3, 40.1, 37.2, 36.5, 31.8, 31.6, 31.3, 27.0, 26.3, 23.8, 23.4, 23.2, 20.9, 19.4, 14.2, 13.7; HRMS: (ESI, m/z) calcd **[M+Na]$^+$** for $C_{25}H_{42}O_2$, 397.3083; found, 397.3079.

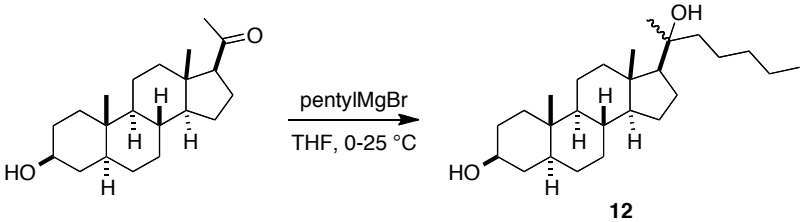


11

(3*S*,10*R*,13*S*,17*S*)-17-(2-Hydroxyheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol (20-OHC-Pent, 11). Following the general procedure, compound **5** was obtained from pregnenolone (1.0 g) and pentylmagnesium bromide (4.7 mL of a 2.0 M solution in Et_2O) in dry THF (20 mL) as a white solid (553 mg, 45%). Further preparative chiral HPLC separation yielded the pure diastereomers (**11a** and **11b)** as white solids.

11a(20*S*): $[\alpha]_D^{24}$ = -54.5 (*c* = 0.31, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34-5.37 (m, 1H), 3.47-3.57 (m, 1H), 2.19-2.34 (m, 2H), 2.06-2.13 (m, 1H), 1.93-2.02 (m, 1H), 1.79-1.88 (m, 2H), 1.40-1.78 (m, 12H), 1.05-1.37 (m, 13H), 0.97-1.05 (m, 4H), 0.85-0.97 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.6, 75.2, 71.7, 57.6, 56.9, 50.0, 44.0, 42.6, 42.3, 40.1, 37.2, 36.5, 32.5, 31.8, 31.6, 31.3, 26.4, 24.0, 23.8, 22.7, 22.3, 20.9, 19.4, 14.1, 13.6; HRMS: (ESI, m/z) calcd **[M+Na]$^+$** for $C_{26}H_{44}O_2$, 411.3239; found, 411.3235.

11b(20*R*): $[\alpha]_D^{24}$ = -49.7 (*c* = 0.26, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33-5.37 (m, 1H), 3.48-3.57 (m, 1H), 2.18-2.34 (m, 2H), 2.06-2.12 (m, 1H), 1.94-2.02 (m, 1H), 1.79-1.88 (m, 2H), 1.42-1.78 (m, 12H), 1.20-1.40 (m, 8H), 1.09-1.18 (m, 4H), 0.97-1.09 (m, 5H), 0.85-0.97 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 140.8, 121.6, 75.8, 71.8, 58.2, 56.9, 50.0, 42.9, 42.8, 42.3, 40.1, 37.2, 36.5, 32.5, 31.8, 31.6, 31.3, 27.0, 23.8, 23.7, 23.1, 22.7, 20.9, 19.4, 14.1, 13.7; HRMS: (ESI, m/z) calcd **[M+Na]$^+$** for $C_{26}H_{44}O_2$, 411.3239; found, 411.3240.

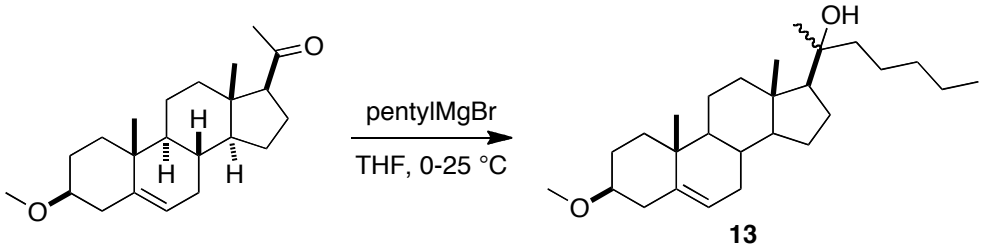


12

(3*S*,5*S*,10*S*,13*S*, 17S)-17-(2-Hydroxyheptan-2-yl)-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol (20-OHC-PentSat, 12). Following the general procedure, compound **12** was obtained from 5α-pregnan-3β-ol-20-one (1.0 g) and pentylmagnesium bromide (4.7 mL of a 2.0 M solution in Et_2O) in dry THF (20 mL) as a white solid (354 mg, 29%). Further chiral HPLC separation yielded the pure diastereomers (**12a** and **12b)** as white solids.

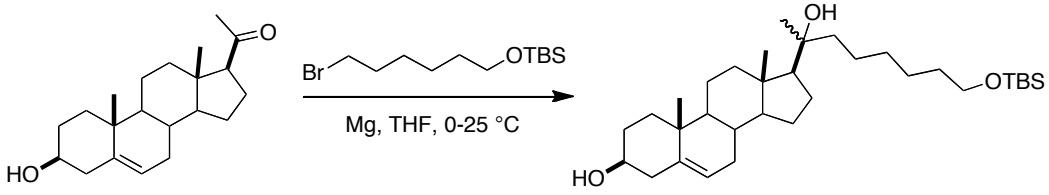
12a(20*S*): $[\alpha]_D^{24}$ = 2.6 (*c* = 0.32, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.54-3.63 (m, 1H), 2.01-2.07 (m, 1H), 1.20-1.83 (m, 27H), 1.03-1.20 (m, 4H), 0.94-1.03 (m, 2H), 0.85-0.94 (m, 4H), 0.83 (s, 3H), 0.80 (s, 3H), 0.57-0.65 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 75.2, 71.3, 57.7, 56.6, 54.3, 44.8, 43.9, 42.9, 40.4, 38.1, 37.0, 35.4, 34.8, 32.5, 31.9, 31.5, 28.7, 26.3, 23.9, 23.7, 22.6, 22.3, 21.1, 14.1, 13.8, 12.3; HRMS: (ESI, m/z) calcd **[M+**H-H_2O**]**$^+$ for $C_{26}H_{46}O_2$, 373.3470; found, 373.3464.

12b(20*R*): $[\alpha]_D^{24}$ = 10.9 (*c* = 0.34, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.54-3.63 (m, 1H), 2.01-2.08 (m, 1H), 1.43-1.84 (m, 12H), 1.15-1.43 (m, 15H), 1.03-1.15 (m, 4H), 0.94-1.03 (m, 2H), 0.85-0.94 (m, 4H), 0.84 (s, 3H), 0.80 (s, 3H), 0.57-0.65 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 75.8, 71.3, 58.4, 56.6, 54.3, 44.8, 43.2, 42.7, 40.4, 38.2, 37.0, 35.4, 34.9, 32.5, 31.9, 31.5, 28.7, 27.0, 23.8, 23.7, 23.1, 22.7, 21.1, 14.1, 13.9, 12.3; HRMS: (ESI, m/z) calcd **[M+**H-H_2O**]**$^+$ for $C_{26}H_{46}O_2$, 373.3470; found, 373.3482.



13

2-((3*S*,10*R*,13*S*,17*S*)-3-methoxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)heptan-2-ol (20-OHC-Pent-3β−OMe, 13). To a dry 250 mL flask equipped with a magnetic stir bar was added pregnenolone methyl ether (960 mg, 2.9 mmol, from Steraloids) and 5 mL anhydrous tetrahydrofuran. The solution was stirred under argon while 14.5 mL (29 mmol) of 2M pentyl magnesium bromide was added dropwise. The reaction was allowed to proceed overnight under argon. The reaction mix was diluted with 100 mL diethyl ether and quenched by the addition of 1% aqueous acetic acid. The organic phase was washed with water and brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude product (TLC: 30% ethyl acetate/hexanes, starting material Rf = 0.33, product Rf = 0.38) was dissolved in a small amount of dichloromethane and purified by column chromatography using a gradient of 5-20% ethyl acetate in hexanes, yielding the product **13** as a colorless oil (806 mg, 69%). ^{1}H NMR (400 MHz) δ 5.35 (m, 1H), 3.35 (s, 3H), 3.06 (m, 1H), 2.37 (m, 1H), 1.27 (s, 3H), 1.00 (s, 3H), 0.87 (s, 3H). ^{13}C NMR (400 MHz) 140.84, 121.46, 80.28, 75.09, 57.64, 56.90, 55.54, 50.10, 43.97, 42.61, 40.11, 38.65, 37.15, 36.85, 32.50, 31.79, 31.29, 27.96, 26.39, 23.93, 23.76, 22.65, 22.32, 20.91, 19.33, 14.07, 13.57.

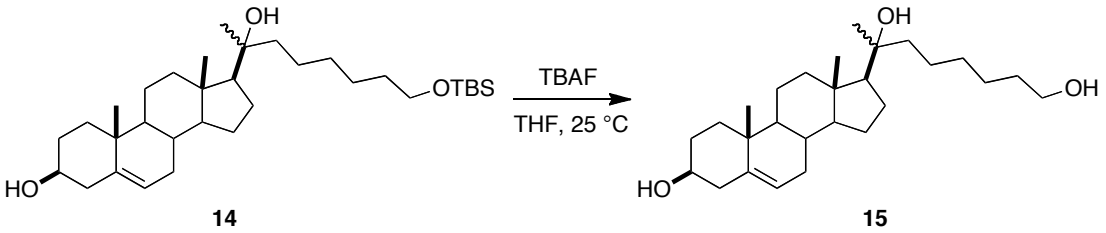


Pregnenolone **14**

(3*S*,10*R*,13*S*,17*S*)-17-(8-((*tert*-butyldimethylsilyl)oxy)-2-hydroxyoctan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol (14). In a 2-necked round bottom flask equipped with a condenser, magnesium turnings (2.2 g, 89.1 mmol) were stirred in anhydrous THF (2 mL) under nitrogen. (6-Bromohexyloxy)-*tert*-butyldimethylsilane (5.3 g, 17.8 mmol) was dissolved in anhydrous THF (18 mL) and 1/10 of this solution was added to the flask, followed by a few drops of 1,2-dibromoethane. The mixture was warmed and stirred vigorously until cloudiness and bubbling was observed. The rest of the THF solution of (6-bromohexyloxy)-*tert*-butyldimethylsilane was slowly

added to the flask to keep the reaction going. The reaction mixture was heated to reflux for an additional hour, and then cooled to room temperature. The Grignard reagent thus prepared was transferred drop-wise via syringe to a solution of pregnenolone (1.9 g, 5.9 mmol) in anhydrous THF (10 mL) at 0°C. The reaction was stirred at room temperature for 12 h under nitrogen and was quenched with aqueous NH_4Cl, followed by extraction with EtOAc. The organic extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel (step-wise gradient elution, 0-30% EtOAc/Hexanes) to yield compound **14** (1.4 g, 46%) as a colorless, amorphous solid (mixture of diastereomers).

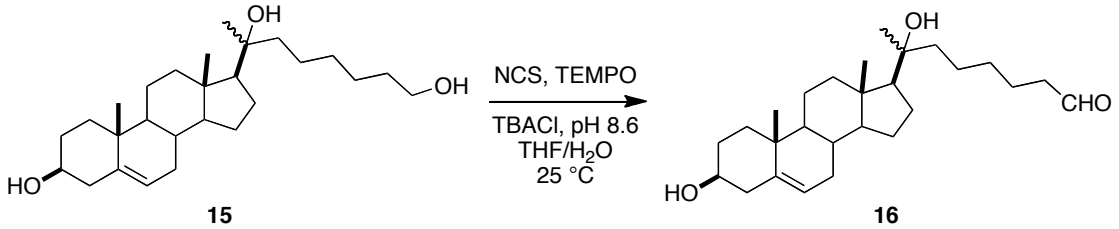
14: ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.29-5.32 (m, 1H), 3.53-3.62 (m, 3H), 3.44-3.51 (m, 1H), 2.22-2.27 (m, 1H), 2.16-2.22 (m, 1H), 2.02-2.06 (m, 1H), 1.90-1.96 (m, 1H), 1.76-1.82 (m, 2H), 1.55-1.74 (m, 4H), 1.38-1.55 (m, 8H), 1.18-1.34 (m, 8H), 0.99-1.18 (m, 4H), 0.91-0.99 (m, 4H), 0.83-0.91 (m, 12H), 0.82 (s, 3H), 0.00 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) (as the major diastereomer): δ 140.8, 121.6, 75.2, 71.7, 63.3, 57.6, 56.9, 50.0, 43.9, 42.6, 42.3, 40.1, 37.2, 36.5, 32.8, 31.8, 31.6, 31.3, 30.1, 26.4, 26.0, 25.8, 24.3, 23.8, 22.3, 20.9, 19.4, 18.4, 13.6, -5.3.



14 **15**

7-((3*S*,10*R*,13*S*,17*S*)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-17-yl)octane-1,7-diol (15). To compound **13** (1.1g, 2.1 mmol) in dry THF (15 mL) was added tetrabutylammonium fluoride (6.3 mL of a 1.0 M solution in THF, 6.3 mmol), and the reaction was stirred for 4 h. Upon reaction completion, water was added and most of the product was precipitated by addition of a large excess of Et_2O. The organic phase was separated and the solvent was removed by rotary evaporation. The resulting residue was washed with water and CH_2Cl_2, and was combined with the solid obtained by ether precipitation. The combined solids were dried *in vacuo* to yield the crude **15** (0.72 g, 82 %) as a white solid (mixture of diastereomers).

15: ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.34-5.37 (m, 1H), 3.62-3.67 (m, 2H), 3.48-3.56 (m, 1H), 2.27-2.32 (m, 1H), 2.20-2.26 (m, 1H), 2.06-2.11 (m, 1H), 1.95-2.01 (m, 1H), 1.81-1.87 (m, 2H), 1.62-1.78 (m, 3H), 1.43-1.62 (m, 10H), 1.24-1.41 (m, 9H), 1.04-1.24 (m, 6H), 0.96-1.04 (m, 4H), 0.90-0.96 (m, 1H), 0.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) (as a mixture of diastereomers): δ 140.8, 121.6, 75.8, 75.2, 71.8, 63.0(x2), 58.3, 57.7, 56.9(x2), 50.0, 43.8, 42.9, 42.7, 42.6, 42.3, 40.1(x2), 37.2, 36.5, 32.8, 32.7, 31.8, 31.6, 31.3(x2), 30.1, 30.0, 27.0, 26.4, 25.8, 25.7, 24.2, 24.0, 23.8(x2), 23.2, 22.4, 20.9, 19.4, 13.7, 13.6.

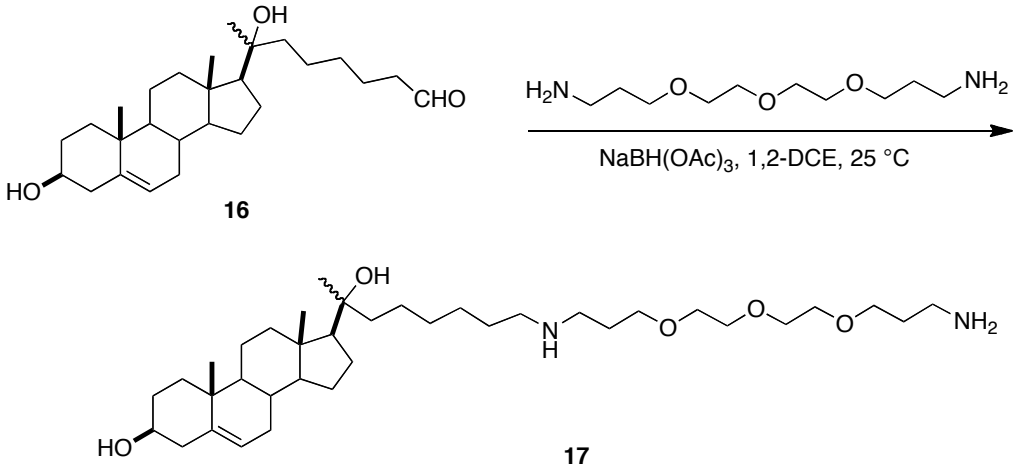


15 **16**

7-hydroxy-7-((3*S*,10*R*,13*S*,17*S*)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-17-yl)octanal (16). Compound **15** was obtained by a modification of a procedure to selectively oxidize primary alcohols to aldehydes[5]. A solution of triol **14** (711 mg, 1.7 mmol), 2,2,6,6-tetramethyl-1-piperidinyloxy (26.6 mg, 0.17 mmol), tetrabutylammonium chloride

(47.2 mg, 0.17 mmol) in THF (15 mL) and 15 mL of an aqueous solution of $NaHCO_3$ (0.5 M) and K_2CO_3 (0.05 M) were vigorously stirred at room temperature. *N*-chlorosuccinimide (340.5 mg, 2.6 mmol) was then added, and stirring was maintained for 16 h. The organic phase was separated, and the aqueous phase was diluted with brine and extracted 3 times with CH_2Cl_2 (some precipitate might form and just filter to collect the solid and filtrate). The combined organic phases were concentrated *in vacuo*, the residue was dissolved in a small amount of CH_2Cl_2/MeOH and was purified by flash chromatography on silica gel (step-wise gradient elution, 0-40% EtOAc/hexanes) to yield the aldehyde **16** (443 mg, 54%) as a white solid (mixture of diastereomers).

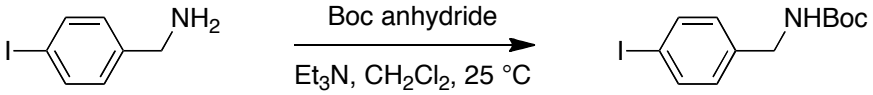
16: ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.76-9.77 (m, 1H), 5.34-5.37 (m, 1H), 3.48-3.56 (m, 1H), 2.41-2.46 (m, 2H), 2.27-2.32 (m, 1H), 2.20-2.27 (m, 1H), 2.05-2.11 (m, 1H), 1.95-2.01 (m, 1H), 1.81-1.87 (m, 2H), 1.59-1.79 (m, 6H), 1.42-1.58 (m, 8H), 1.24-1.38 (m, 7H), 1.04-1.24 (m, 4H), 0.96-1.03 (m, 4H), 0.90-0.96 (m, 1H), 0.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) (as the major diastereomer): δ 202.8, 140.8, 121.5, 75.1, 71.7, 57.8, 56.9, 50.0, 43.8, 43.6, 42.6, 42.2, 40.1, 37.2, 36.5, 31.7, 31.6, 31.3, 29.7, 26.3, 23.9, 23.7, 22.3, 22.0, 20.9, 19.4, 13.6.



1-amino-21-((3*S*,10*R*,13*S*,17*S*)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-17-yl)-4,7,10-trioxa-14-azadocosan-21-ol (20-OHC-PEG-NH$_2$, 17). Compound **15** (208 mg, 0.5 mmol) and 4,7,10-trioxa-1,13-tridecanediamine (220 mg, 1 mmol) were mixed in 1,2-dichloroethane (5 mL) and then treated with sodium triacetoxyborohydride (159 mg, 0.75 mmol). The mixture was stirred at room temperature for 16 h until the reactants were consumed, as determined by TLC. The reaction mix was concentrated *in vacuo*, the residue was dissolved in DMSO and was purified by preparative reverse-phase HPLC on a Waters Symmetry C18 column (19 x 50 mm, 5μM). Elution was with a gradient of 15-80% MeOH in water with 0.035% trifluoroacetic acid, over 15 min and at a flow rate of 20 mL/min. The product **17** (mixture of diastereomers) was obtained as the di(trifluoroacetate) salt (yellow oil, 154 mg, 36%).

17: ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.16 (br, 2H), 7.99 (br, 3H), 5.33-5.37 (m, 1H), 3.74 (t, J = 5.2 Hz, 2H), 3.56-3.66 (m, 10H), 3.47-3.56 (m, 3H), 3.04-3.22 (m, 4H), 2.72-2.98 (m, 6H), 2.18-2.33 (m, 2H), 1.93-2.11 (m, 4H), 1.79-1.88 (m, 2H), 1.58-1.79 (m, 4H), 1.38-1.54 (m, 6H), 1.22-1.38 (m, 9H), 1.06-1.22 (m, 4H), 0.87-1.06 (m, 5H), 0.85 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) (as the major diastereomer): δ 140.7, 121.6, 75.2, 71.8, 71.0, 70.0, 69.8, 69.5, 69.4, 68.4, 57.8, 56.8, 50.0, 48.3, 46.1, 43.6, 42.6, 42.2, 40.3, 40.0, 37.2, 36.5, 31.7, 31.6, 31.3, 29.5, 26.5, 26.1, 25.8, 25.7, 25.5, 23.8, 23.7, 22.3, 20.9, 19.4, 13.6. LC/MS: (ESI, m/z) calcd **[M+H]$^+$** for $C_{37}H_{68}N_2O_5$: 621.5, found 621.4.

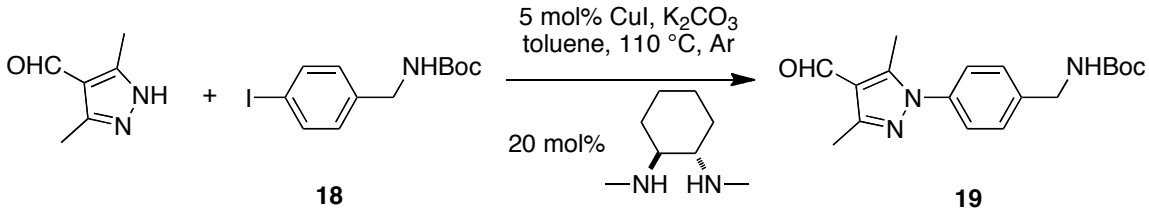
Synthesis of BODIPY-SANT1 (17-21)



18

***tert*-butyl 4-iodobenzylcarbamate (18)**. 4-Iodobenzylamine (349.6 mg, 1.5 mmol) and triethylamine (0.44 mL, 3.15 mmol) were dissolved in dichloromethane (6 mL), and Boc anhydride (343.7 mg, 1.6 mmol) in dichloromethane (6 mL) was added drop-wise. After stirring for 16 h at room temperature, the reaction mixture was washed with 1N HCl, saturated aqueous $NaHCO_3$, and brine, then dried over anhydrous Na_2SO_4 and concentrated to yield compound **18** as a white solid (499 mg, 100 %).

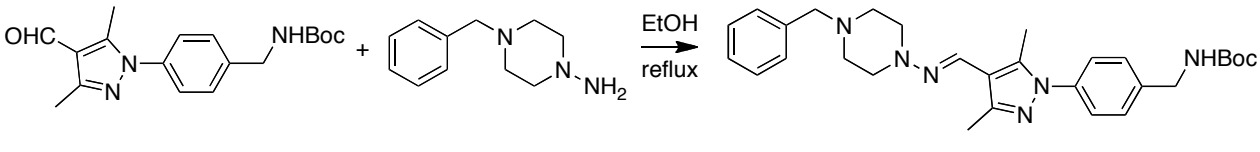
18: ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.65 (d, *J* = 8.4 Hz, 2H), 7.03 (d, *J* = 7.8 Hz, 2H), 4.83 (br, 1H), 4.25(d, *J* = 4.2 Hz, 2H), 1.45 (s, 9H). Characterization matched the data reported for compound **18**[6].



18 **19**

***tert*-butyl 4-(4-formyl-3,5-dimethyl-1*H*-pyrazol-1-yl)benzylcarbamate (19).** Compound **19** was obtained by *N*-arylation following a reported protocol[7]. To a screw cap glass vial were added CuI (4.6 mg, 0.024 mmol, 5 mol%), 3,5-dimethyl-1*H*-pyrazole-4-carbaldehyde (59.5 mg, 0.48 mmol), K_2CO_3 (139.3 mg, 1.0 mmol), and a stir bar. The reaction vessel was fitted with a rubber septum, was evacuated and back-filled with argon, and this sequence was repeated twice. Aryl iodide **18** (191.8 mg, 0.58 mmol), (1*S*,2*S*)-N^1,N^2-dimethylcyclohexane-1,2-diamine (13.6 mg, 0.96 mmol, 20 mol%) and toluene (2 mL) were then added successively under a stream of argon. The reaction vial was sealed and immersed in an oil bath preheated to 110 °C, and the reaction was stirred for 24 h. The reaction mixture was cooled to room temperature, diluted with EtOAc and filtered through Celite. The filtrate was washed with aqueous NH_4OH (v/v, 1/1) and brine, then was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by flash chromatography on silica gel (step-wise gradient from 9:1 to 2:3 hexane/EtOAc) to provide compound **19** as a yellow oil (17.0 mg, 11 %).

19: ^{1}H NMR (600 MHz, $CDCl_3$): δ 10.22 (s, 1H), 7.62 (d, *J* = 7.2 Hz, 2H), 7.57 (d, *J* = 7.8 Hz, 2H), 5.12 (br, 1H), 4.59 (br, 2H), 2.74 (s, 3H), 2.72 (s, 3H), 1.67 (s, 9H); LC/MS: (ESI, m/z) calcd $[M+H]^+$ for $C_{18}H_{23}N_3O_3$, 330.2, found 330.2.

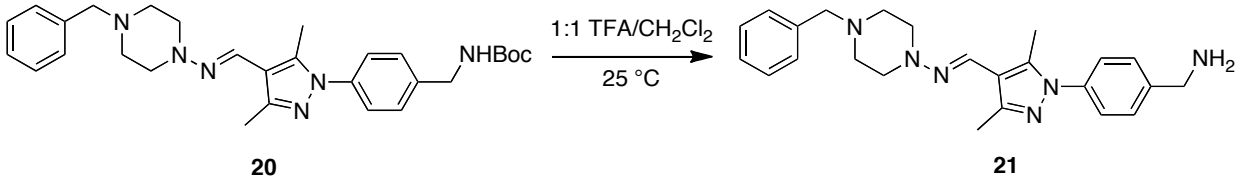


19 **20**

***(E)-tert*-butyl 4-(4-(((4-benzylpiperazin-1-yl)imino)methyl)-3,5-dimethyl-1*H*-pyrazol-1-yl)benzylcarbamate (20).** Following a reported protocol [8], an equimolar mixture of **18** (14.8 mg, 45.0 μmol) and 1-amino-4-benzylpiperazine (8.6 mg, 45.0 μmol) in absolute ethanol (0.5 mL) was heated under

reflux for 12 h. After concentration, the resulting residue was purified by flash chromatography on silica gel (step-wise gradient elution from 100:1 to 9:1 CH_2Cl_2/MeOH) to provide compound **20** as a yellow oil (22.5 mg, 100 %).

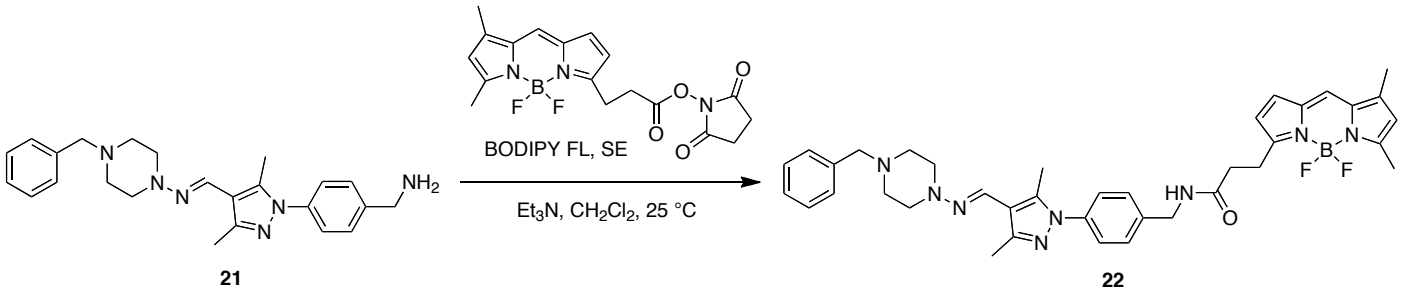
20: ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.53 (s, 1H), 7.17-7.31 (m, 9H), 4.88 (br, 1H), 4.28 (d, *J* = 5.4 Hz, 2H), 3.53 (s, 2H), 3.08-3.11 (m, 4H), 2.57-2.63 (m, 4H), 2.33 (s, 3H), 2.32 (s, 3H), 1.39 (s, 9H); LC/MS: (ESI, m/z) calcd $[M+H]^+$ for $C_{29}H_{38}N_6O_2$: 503.3, found 503.1.



20 **21**

***(E)-N*-((1-(4-(aminomethyl)phenyl)-3,5-dimethyl-1*H*-pyrazol-4-yl)methylene)-4-benzylpiperazin-1-amine (21).** Compound **20** (7.5 mg, 15.0 μmol) was dissolved in a 1:1 mixture of TFA (0.8 mL) and CH_2Cl_2 (0.8 mL,) and was stirred at room temperature for 50 min. Volatiles were evaporated *in vacuo* and the resulting residue was used in the next step without further purification.

21: LC/MS: (ESI, m/z) calcd $[M+H]^+$ for $C_{24}H_{30}N_6$: 403.20, found 403.1.



21 **22**

(*E*)-3-(3-((4-(4-(((4-benzylpiperazin-1-yl)imino)methyl)-3,5-dimethyl-1*H*-pyrazol-1-yl)benzyl)amino)-3-oxopropyl)-5,5-difluoro-7,9-dimethyl-5*H*-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-4-ium-5-uide (BODIPY-SANT1, 22). Triethylamine (9.1 μL, 65.0 μmol) and BODIPY-FL *N*-hydroxysuccinimide ester (5.0 mg, 13.0 μmol) were added to a solution of amine **21** (6.0 mg, 15.0 μmol) in dichloromethane (800 μL). The reaction mixture was stirred at room temperature for 18 h and then evaporated to dryness under a stream of nitrogen gas. Purification by flash chromatography on silica gel (step-wise gradient from 100:1 to 85:15 CH_2Cl_2/MeOH) yielded the fluorescent amide **22** as a dark red solid (7.8 mg, 89 %).

22: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (s, 1H), 7.13-7.35 (m, 9H), 7.02 (s, 1H), 6.79 (d, *J* = 4.0 Hz, 1H), 6.22 (d, *J* = 4.0 Hz, 1H), 6.12 (t, *J* = 5.2 Hz, 1H), 6.03 (s, 1H), 4.36 (d, *J* = 6.0 Hz, 2H), 3.56 (d, *J* = 7.6 Hz, 1H), 3.52 (d, *J* = 7.2 Hz, 1H), 3.23 (t, *J* = 7.2 Hz, 2H), 3.04-3.16 (m, 4H), 2.59-2.71 (m, 6H), 2.47 (s, 3H), 2.31 (s, 6H), 2.17 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.6, 160.4, 156.9, 148.1, 144.1, 138.4, 137.9, 135.2, 133.4, 129.3, 128.4, 128.2, 127.3, 125.1, 123.9, 120.5, 117.5, 63.5, 62.5, 53.0, 52.1, 51.4, 45.8, 42.9, 35.9, 24.8, 19.0, 14.9, 13.0, 11.9, 11.3, 8.1; LC/MS: (ESI, m/z) calcd $[M+H]^+$ for $C_{38}H_{43}BF_2N_8O$: 677.4, found 677.1.

Supplementary references

1 Abdel-Magid, A. F., Carson, K. G., Harris, B. D., Maryanoff, C. A. & Shah, R. D. Reductive Amination of Aldehydes and Ketones with Sodium Triacetoxyborohydride. Studies on Direct and Indirect Reductive Amination Procedures(1). *The Journal of organic chemistry* **61**, 3849-3862 (1996).

2 Lu, M. C., Afiatpour, P., Sullivan, C. B. & Counsell, R. E. Inhibition of cholesterol side-chain cleavage. 2. Synthesis of epimeric azacholesterols. *Journal of medicinal chemistry* **15**, 1284-1287 (1972).

3 Counsell, R. E., Klimstra, P. D., Nysted, L. N. & Ranney, R. E. Hypocholesterolemic Agents. V. Isomeric Azacholesterols. *Journal of medicinal chemistry* **8**, 45-48 (1965).

4 Nachtergaele, S. *et al*. Oxysterols are allosteric activators of the oncoprotein Smoothened. *Nature chemical biology* **8**, 211-220, doi:10.1038/nchembio.765 (2012).

5 Gunbas, D. D. & Brouwer, A. M. Degenerate molecular shuttles with flexible and rigid spacers. *The Journal of organic chemistry* **77**, 5724-5735, doi:10.1021/jo300907r (2012).

6 Antilla, J. C., Baskin, J. M., Barder, T. E. & Buchwald, S. L. Copper-diamine-catalyzed N-arylation of pyrroles, pyrazoles, indazoles, imidazoles, and triazoles. *The Journal of organic chemistry* **69**, 5578-5587, doi:10.1021/jo049658b (2004).

7 Fahmy, H. T., Rostom, S. A. & Bekhit, A. A. Synthesis and antitumor evaluation of new polysubstituted thiazole and derived thiazolo[4, 5-d]pyrimidine systems. *Archiv der Pharmazie* **335**, 213-222, doi:10.1002/1521-4184(200205)335:5<213::AID-ARDP213>3.0.CO;2-H (2002).