Supplementary Information

Oxysterols are allosteric activators of the oncoprotein Smoothened

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Supplementary methods

General methods for synthesis and characterization of nat-20(R)-OHC, ent-20(S)-OHC, nat-20(S)-yne, and nat-20(S)-COOH.

Full synthetic procedures are provided in the Supplementary Information (nat-20(R)-OHC: Supplementary Scheme 1; ent-20(S)-OHC: Supplementary Scheme 2; nat-20(S)-yne: Supplementary Scheme 3; nat-20(S)-COOH: Supplementary Scheme 4). Melting points were determined on a Kofler micro hot stage and were uncorrected. NMR spectra were recorded in CDCl$_3$, at 300MHz ($^1$H) or 75MHz ($^{13}$C). Chemical shifts ($\delta$) were reported downfield from internal Me$_4$Si ($\delta$: 0.00). HR FAB-MS determinations were made with the use of JEOL MStation (JMS-700) Mass Spectrometer, matrix m-nitrobenzyl alcohol, with NaI as necessary, using mass spectrometry facilities located at the University of Missouri – St. Louis. HRESI-MS determinations were made with the use of Thermo Orbitrap Velos Mass Spectrometer, using the facilities located at Washington University in St. Louis. IR spectra were recorded as films on a NaCl plate or in KBr. Elemental analyses were carried out by M–H–W laboratories. Chromatography was performed using flash chromatography grade silica gel (32–63 µm; Scientific Adsorbents). Dichloromethane was distilled over CaH prior to application. Tetrahydrofuran was distilled over Na/benzophenone just prior to application. All other chemicals were used as purchased without further purification. Organic extracts were dried over anhydrous Na$_2$SO$_4$.

Immunofluorescence

Cells were fixed with cold 4% paraformaldehyde (10 min, room temperature (RT)), washed with phosphate buffered saline (PBS) (3 times, 5 min each), placed in blocking solution (PBS, 1% (v/v) normal goat serum, 0.1% (v/v) Triton X-100, 10 mg/mL bovine serum albumin) for 30 minutes at RT and then stained with primary antibodies (2 h, RT): anti-acetylated tubulin (Sigma, #T6793) at 1:3000 (v/v) dilution and anti-Smo$^+$ at 1:500 (v/v) dilution in blocking solution. After washing (3 times, 5 min in PBS + 0.1% Triton X-100), Alexa-coupled secondary antibodies (Invitrogen) were applied (1:500 (v/v) dilution, 1 h, RT). Finally, stained cells were washed in PBS (3 times, 5 min) and mounted onto glass slides with DAPI-containing Mowiol solution (2 g Mowiol [Sigma], 6 g glycerol, 6 mL water, 12 mL Tris-HCl pH 8.5, 1 mg/mL 4',6-diamidino-2-phenylindole [DAPI], 25 mg/mL 1,4 diazabicyclo[2.2.2]octane [DABCO]).

Microscopy and image analysis

Fixed cells were imaged with a Leica DMIRE2 laser scanning confocal microscope, using a 63x oil objective (NA 1.40) and 2x zoom. For the quantitative analysis of Smo levels in cilia, all images used for comparisons were taken with identical gain, offset, and laser power settings on the microscope. Non-manipulated images were used for quantitation (ImageJ). A mask was constructed by manually outlining cilia in the acetylated tubulin image and then applied to the corresponding Smo channel image to measure Smo fluorescence at cilia. Local background correction was performed by moving the mask to measure fluorescence at a nearby region, and this value was subtracted from the ciliary Smo fluorescence.

Immunoblotting

Cells were scraped into ice-cold PBS containing Protease Inhibitor Cocktail with EDTA (Roche) and collected as a pellet by centrifugation (1000xg, 10 min, 4°C). Cells were lysed (1 h,
4°C) by agitation in modified RIPA buffer (50 mM sodium-Tris pH 7.4, 150 mM sodium chloride, 2% NP-40, 0.5% deoxycholate, 0.1% sodium-dodecyl sulfate [SDS], 1 mM dithiothreitol, 1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride [AEBSF], 10 µg/mL leupeptin-pepsstatin-chymostatin protease inhibitor mix and the Roche Complete Protease Inhibitor EDTA-Free cocktail). After clarification (20,000xg, 45 min, 4°C), lysate protein concentrations were measured using the bicinchoninic acid assay (Pierce). Samples containing equal total protein were fractionated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane for immunoblotting with anti-Gli1 antibody (Cell Signaling, #L42B10, 1:500) and anti-p38 antibody (Abcam, #ab31828, 1:2000). In figures, vertical gray lines represent non-contiguous lanes from the same immunoblot juxtaposed for clarity.

**Ligand affinity chromatography with receptors transfected into 293T cells**

70% confluent 6 cm dishes of 293T cells were transfected with 100 ng of DNA encoding YFP-Smo, SSTR3-GFP or HTR6-GFP, and grown to confluence. After harvesting in SEAT buffer, membranes were isolated and a detergent extract prepared and used for ligand affinity chromatography exactly as described in the methods included with the main text.

**Liposome expansion assay**

10 mg of dioleyl-phosphatidylcholine (DOPC) lipid was lyophilized and reconstituted using 1 ml of a solution of 5 mM CF in EB buffer. The lipid mixture was subjected to 10 freeze-thaw cycles by repetitive immersion in liquid N2 and room temperature water. Next the lipid mixture was extruded through a 200 nm filter and run over a column containing Sephadex G-100-120. The second, third and fourth fluorescent fractions (3 drops per fraction) were collected and used in the assay. For both the nat- and ent-20(S)-OHC, solutions of 10 µM were made by diluting pure compound in isopropanol.

At the beginning of the assay, approximately 100 ng of liposomes containing CF was added to a spectrofluorometric cuvette containing 1 ml of EB buffer. Then, 10 µl of 10 µM oxysterol was added and vesicle swelling was monitored for 60 minutes. Fluorescence was normalized to a maximal dequenching value as determined by addition of 10 µl of 20% Triton X-100 at the end of each time series. nat- and ent-20(S)-OHC at 10 µM were tested in triplicate, and for each set of trials an equivalent volume (10 µl) of isopropanol was used as a control.
Supplementary scheme 1. Synthesis and characterization of nat-20(R)-OHC (2)

To a stirred solution of the commercially available (3β)-21-(acetyloxy)-3-hydroxypregn-5-en-20-one (1.0 g, 2.67 mmol), in CH₂Cl₂ (20 mL) under N₂, was added diisopropylethylamine (0.93 mL, 5.34 mmol). The reaction mixture was cooled to 0°C, and methoxymethyl chloride (0.358 mL, 5.07 mmol) was added dropwise. After 15 min, the reaction was brought to rt, and allowed to stir for 16 h. The reaction was monitored by TLC, and upon completion was cooled to 0°C, and quenched with MeOH (~0.5 mL). The mixture was then diluted with CH₂Cl₂ (20 mL), and washed sequentially with sat. aqueous NaHCO₃, (10 mL) and brine (2 x 10 mL). The organic phase was then dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc–hexanes, gradient elution), to yield compound 6 as a white solid (1.07 g, 97%). Compound 6: R =$= 0.49$ (EtOAc–hexanes, 4/6, v/v); mp 153–155°C
\( \text{(Et}_2\text{O-hexanes); } [\alpha]_D^{20} = +27.7 \quad (c = 0.17, \text{CHCl}_3) \); IR: 2960, 2936, 2903, 2882, 1745, 1727, 1375, 1229, 1033 cm\(^{-1}\); \(^1^H\) NMR (CDCl\(_3\)) \( \delta \) 5.35 (m, 1H, H-6), 4.72 (d, 1H, \( J = 17.1 \) Hz, H-21), 4.69 (s, 2H, CH\(_3\)OC\(_2\)H\(_2\)O), 4.54 (d, 1H, \( J = 16.8 \) Hz, H-21), 3.37-3.48 (m, 1H, H-3), 3.37 (s, 3H, CH\(_2\)OCH\(_2\)O), 2.51 (t, 1H, \( J = 8.7 \) Hz, H-17), 2.17 (s, 3H, OAc), 1.01 (s, 3H, H-19), 0.67 (s, 3H, H-18); \(^{13}C\) NMR (CDCl\(_3\)) \( \delta \) 204.0, 170.5, 141.0, 121.6, 94.9, 77.0, 69.4, 59.5, 57.3, 55.4, 50.2, 44.9, 39.7, 38.8, 37.4, 37.0, 32.1, 29.1, 24.8, 23.0, 21.2, 20.7, 19.6, 13.3. Anal. Calcd. for C\(_{25}\)H\(_{38}\)O\(_5\): C, 71.74; H, 9.15. Obsd. C, 71.89; H, 9.15.

Based upon known literature procedures\(^{2,3}\), compound 6 (0.78 g, 1.86 mmol) was dissolved in anhydrous Et\(_2\)O (60 mL) at 0°C, under N\(_2\). LAH (0.28 g, 7.45 mmol) was then added portionwise, and the resulting mixture was brought to rt and allowed to stir for 3 h. Upon completion, the reaction was quenched by the sequential addition of water (0.3 mL) and 15% aq NaOH (0.3 mL), and allowed to stir for 30 min. Additional water (0.9 mL) was then added, and the reaction was stirred another 5 min until white aluminum salts were precipitated. The solids were filtered and rinsed several times with Et\(_2\)O, and the filtrate was then dried over Na\(_2\)SO\(_4\), and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc–hexanes, gradient elution), to yield compound 7 as a white solid (0.64 g, 91%). Compound 7: R\(_f\) = 0.25 (EtOAc–hexanes, 6/4, v/v); mp 134–137°C (CH\(_2\)Cl\(_2\)-hexanes); [\( \alpha \)]\(_D\)^{20} = –53.9 \( (c = 0.22, \text{CHCl}_3) \); IR: 3339, 2963, 2940, 2906, 1438, 1367, 1151, 1102, 1042, 1032 cm\(^{-1}\); \(^1^H\) NMR (CDCl\(_3\)) \( \delta \) 5.34 (m, 1H, H-6), 4.67 (s, 3H, CH\(_3\)OC\(_2\)H\(_2\)O), 3.62 (br d, 2H, \( J = 5.4 \) Hz, H-21), 3.40-3.45 (m, 5H, H-3, H-20, C\(_3\)H\(_3\)OCH\(_2\)O), 2.98 (br s, 1H, CH\(_2\)OH), 2.69 (br s, 1H, CHO\(_2\)H), 1.01 (s, 3H, H-19), 0.77 (s, 3H, H-18); \(^{13}C\) NMR (CDCl\(_3\)) \( \delta \) 140.9, 121.7, 94.8, 77.1, 74.8, 66.6, 56.1, 55.3, 52.5, 50.3, 42.6, 39.8, 39.7, 37.4, 36.9, 32.1, 31.9, 29.0, 24.8, 24.8, 21.1, 19.5, 12.5. Anal. Calcd. for C\(_{23}\)H\(_{38}\)O\(_4\): C, 72.98; H, 10.12. Obsd. C, 72.79; H, 10.10.

\((3\beta, 20R,S)-3\text{-Methoxymethoxypregn-5-ene-20,21-diol (7)}\)

\[(\text{3}\beta, \text{20R, S})-3\text{-Methoxymethoxypregn-5-ene-20,21-diol (7)}\]

Using a known literature procedure\(^2\), compound 7 (0.64 g, 1.69 mmol) was dissolved in THF (25 mL), followed by the addition of water (15 mL). Sodium periodate (1.08 g, 5.07 mmol) was then added and the reaction was stirred for 45 min. Upon completion, the reaction mixture was
extracted with Et₂O (4 x 10 mL). The organic fractions were then combined, dried over Na₂SO₄, and concentrated in vacuo. The white solid was recrystallized from hexanes, to yield pure compound 8 (0.58 g, 99%). Compound 8: R = 0.74 (EtOAc–hexanes, 6/4, v/v); mp 134–137°C (hexanes); [α]D20 = −11.8 (c = 0.28, CHCl₃); IR: 2938, 2903, 1709, 1451, 1436, 1384, 1369, 1147, 1100, 1048, 1035 cm⁻¹; ¹H NMR (CDCl₃) δ 9.76 (d, 1H, J = 2.1 Hz, OCH), 5.33-5.35 (m, 1H, H-6), 4.67 (s, 2H, CH₂CH₂O), 3.35-3.48 (m, 1H, H-3), 3.35 (s, 3H, CH₃CH₂O), 1.00 (s, 3H, H-19), 0.75 (s, 3H, H-18); ¹³C NMR (CDCl₃) δ 205.1, 140.9, 121.5, 94.8, 77.0, 63.0, 56.6, 55.3, 50.3, 44.8, 39.7, 38.4, 37.4, 36.9, 31.9, 31.6, 29.0, 25.1, 21.2, 20.7, 19.5, 13.9. Anal. Calcd. for C₂₂H₄₄O₅: C, 76.26; H, 9.89. Obsd. C, 76.41; H, 9.91.

(3β, 20R,5)-3-Methoxymethoxy-21-norcholest-5-en-20-ol (9)

In a 2-necked flask equipped with a condenser, was stirred magnesium metal turnings (0.21 g, 8.66 mmol) in anhydrous Et₂O (20 mL) under N₂. 1-Bromo-4-methylpentane (1.26 mL, 8.66 mmol) was added, followed by a few drops of 1,2-dibromoethane. The reaction mixture was warmed slightly to 30°C, and stirred vigorously until cloudiness was observed (~1-3 min). The reaction mixture was stirred an additional hr at rt, until the magnesium turnings were consumed, indicating complete formation of the bromo(4-methylpentyl)magnesium reagent. A solution of compound 8 (0.3 g, 0.87 mmol) in anhydrous Et₂O (6 mL), was then added dropwise to the reaction. The reaction was monitored by TLC, whereupon two new spots were formed. Upon completion, the reaction was quenched with aqueous NH₄Cl (10 mL). The phases were separated, and the aqueous phase was extracted with Et₂O (3 x 5 mL). The combined organic fractions were then washed with brine (1 x 10 mL), dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (acetone–hexane, gradient elution), to yield compound 9 as a separable mixture of diastereomers (20R:20S; 1:2.7) in 98%. Although it is not necessary to separate the diastereomers, ¹H NMR and ¹³C NMR data was collected on each pure diastereomer. Compound 9: R = 0.50(20R), 0.57(20S) (acetone–hexanes, 2/8, v/v); IR (mixture of diastereomers): 3468, 2933, 2905, 2868, 1464, 1382, 1367, 1149, 1105, 1049, 1040 cm⁻¹; ¹H NMR (CDCl₃) 9(20R) δ 5.35-5.37 (m, 1H, H-6), 4.70 (s, 2H, CH₂CH₂O), 3.51-3.61 (m, 1H, H-20), 3.38-3.50 (m, 1H, H-3), 3.38 (s, 3H, CH₃CH₂O), 1.03 (s, 3H, H-19), 0.88 (overlapping d, 6H, J = 6.6 Hz, H-26, H-27), 0.78 (s, 3H, H-18); ¹³C NMR (CDCl₃) 9(20R) δ 141.0, 121.8, 94.9, 77.1, 74.6, 56.9, 56.4, 55.4, 50.4, 42.5, 40.2, 39.8, 39.3, 37.5, 37.3, 37.0, 32.1, 31.9, 29.1, 28.2, 25.7, 24.8, 23.2, 22.9, 22.8, 21.2, 19.6, 12.6; ¹H NMR (CDCl₃) 9(20S) δ 5.35-5.37 (m, 1H, H-6), 4.70 (s, 2H, CH₂CH₂O), 3.51-3.61 (m, 1H, H-20), 3.38-3.50 (m, 1H, H-3), 3.38 (s, 3H, CH₃CH₂O), 1.03 (s, 3H, H-19), 0.88 (overlapping d, 6H, J = 6.6 Hz, H-26, H-27), 0.78 (s, 3H, H-18); ¹³C NMR (CDCl₃) 9(20S) δ 141.0, 121.8, 94.9, 77.1, 74.6, 56.9, 56.4, 55.4, 42.5, 40.2, 39.8, 39.3, 37.5, 37.3, 37.0, 32.1, 31.9, 29.1, 28.2, 25.7, 24.8, 23.2, 22.9, 22.8, 21.2, 19.6, 12.6. Anal. Calcd. for C₂₈H₄₈O₃ (mixture of diastereomers): C, 77.72; H, 11.18. Obsd. C, 77.98; H, 11.42.
(3β)-3-Hydroxy-21-norcholesterol-5-en-20-one (10)

To a stirred solution of compound 9 (0.24 g, 0.54 mmol) in acetone (7 mL), was added dropwise freshly prepared Jones Reagent (~0.2 mL; 30% CrO₃·30% H₂SO₄·40% H₂O) until the reaction solution turned from green to yellow (~0.2 mL). Monitoring by TLC showed the two diastereomer spots, corresponding to 9, to be converted into one product spot. The reaction was subsequently quenched with isopropanol (3 mL) and the resulting blue-green solution was extracted with CH₂Cl₂ (4 x 3 mL). The organic layers were filtered through a pad of silica and then concentrated in vacuo. The resulting solid was then dissolved in MeOH (15 mL), whereupon acetyl chloride (0.8 mL) was added dropwise over 15 min, and the reaction was stirred for 2 h. Upon completion, the reaction flask was cooled to 0°C, and was neutralized with sat. aqueous NaHCO₃ (~10 mL). The reaction mixture was extracted with CH₂Cl₂ (4 x 10 mL), and the organic fractions were combined, dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (acetone–hexane, gradient elution), to yield compound 10 as a white solid (0.16 g, 76% over 2 steps). Compound 10: Rf = 0.42 (acetone–hexanes, 5/15, v/v); [α]D²⁰ = +17.6 (c = 0.30, CHCl₃); IR: 3219, 2940, 2924, 2901, 2872, 2849, 1705, 1464, 1383, 1365, 1062 cm⁻¹; ¹H NMR δ 5.36 (m, 1H, H-6), 3.48–3.59 (m, 1H, H-3), 2.52 (t, 1H, J = 8.7 Hz, H-17), 1.01 (s, 3H, H-19), 0.88 (overlapping d, 6H, J = 6.6, H-26, H-27), 0.62 (s, 3H, H-18); ¹³C NMR δ 212.0, 141.0, 121.6, 71.9, 63.1, 57.2, 50.2, 44.8, 44.4, 42.5, 39.2, 38.8, 37.5, 36.7, 32.1, 32.0, 31.8, 28.1, 24.7, 23.2, 22.7 (x2), 21.8, 21.3, 19.6, 13.6. Anal. Calcd. for C₂₆H₄₂O₂: C, 80.77; H, 10.95. Obsd. C, 80.65; H, 10.82.

(3β,20R)-Cholest-5-ene-3,20-diol (nat-20(R)-OHC)

Following a reported protocol⁴ compound 10 (0.14 g, 0.36 mmol), was dissolved in anhydrous Et₂O (25 mL) under N₂, and cooled to 0°C. Methylmagnesium bromide; 3M in Et₂O (0.60 mL, 1.81 mmol) was added dropwise, and the reaction mixture was allowed to stir for 30 min. Upon completion, the reaction was diluted with Et₂O (15 mL), and quenched with aqueous NH₄Cl (15 mL). The phases were separated, and the aqueous phase was extracted with Et₂O (3 x 10 mL). The combined organic fractions were then washed with brine (1 x 15 mL), dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by column chromatography on silica gel.
(acetone–hexane, gradient elution), to yield the desired **nat-20(R)-OHC** as the major diastereomer (R:S, 20:1) as a white solid in (0.13 g, 98%). Recrystallization from acetone–MeOH, yielded the pure **nat-20(R)-OHC**. **nat-20(R)-OHC**: R = 0.39 (acetone–hexanes, 5/15, v/v); mp 104–106°C (acetone–hexanes); \([\alpha]_D^{20} = -48.1\) (c = 0.90, CHCl₃); IR: 3392, 2950, 2935, 2902, 2869, 1465, 1375, 1054 cm⁻¹; \(^1^H\) NMR δ 5.35–5.36 (m, 1H, H-6), 3.46–3.60 (m, 1H, H-3), 1.13 (s, 3H, H-21), 1.01 (s, 3H, H-19), 0.88 (overlapping d, 6H, J = 6.9 Hz, H-26, H-27), 0.87 (s, 3H, H-18); \(^{13}C\) NMR δ 141.0, 121.8, 76.0, 72.0, 58.5, 57.1, 50.2, 43.2, 43.1, 42.4, 40.3, 39.9, 37.4, 36.7, 32.0, 31.8, 31.5, 28.2, 27.2, 24.0, 23.3, 22.9, 22.8, 22.0, 21.1, 19.6, 13.9; HR-FAB MS [M+Na]⁺, Anal. calcd. for C₂₇H₄₆O₂Na⁺ 425.3396, found 425.3397.
Supplementary scheme 2. Synthesis and characterization of \textit{ent-20(S)-OHC} (3)

\textbf{ent-\([3\beta,20S]\text{-Cholest-5-ene-3,20-diol}\) (\textit{ent-20(S)-OHC})

1-Bromo-4-methylpentane (0.29 mL, 2 mmol) was added to magnesium metal turnings (48.6 mg, 2 mmol) in Et\(_2\)O (10 mL) in a 50-mL round-bottom flask fitted with a condenser and a \textsubscript{N\_2} filled balloon and the mixture was stirred vigorously. A drop of 1,2-dibromoethane was added to this solution and the mixture was heated at reflux using a hot water bath for few min to initiate reaction of the 1-bromo-4-methylpentane with the magnesium to form bromo(4-methylpentyl)magnesium. The hot water bath was removed and the reaction mixture was stirred at rt for 1 h until the solution became homogenous. \textit{ent}-Pregnenolone (60 mg, 0.19 mmol), prepared as described previously\textsuperscript{5}, was then added as a solid and the cloudy solution was allowed to stir at rt for 18 h under a \textsubscript{N\_2} atmosphere. The reaction was quenched with sat. aqueous NH\(_4\)Cl solution and extracted with EtOAc (3 x 30 mL). The combined organic extracts were dried over anhydrous Na\(_2\)(SO\(_4\)) and concentrated to give a white solid. The crude product was purified by silica gel column chromatography using 20-40\% EtOAc–hexanes. The desired \textit{ent-20(S)-OHC} was isolated (45 mg, 59\%) and the \textsuperscript{13}C NMR spectrum of this product showed that it contained \textasciitilde20\% of the undesired and inseparable \textit{ent-20(R)-OHC} epimer. Attempts to remove the \textit{ent-20(R)-OHC} epimer by recrystallization using various solvent conditions were not successful and the following derivatization procedure was required for diastereomer separation.

\textbf{Purification:} The mixture of epimers (45 mg, 0.11 mmol) and 4-nitrobenzoyl chloride (41 mg, 0.22 mmol) in pyridine in the presence of a catalytic amount of 4-dimethylaminopyridine was stirred at rt for 5 hr. The reaction was quenched with sat. aqueous NaHCO\(_3\) and extracted with CH\(_2\)Cl\(_2\). The combined CH\(_2\)Cl\(_2\) extracts were dried over anhydrous Na\(_2\)(SO\(_4\)) and concentrated to give a yellow solid, which was purified by silica gel column chromatography using a gradient of 15-30\% EtOAc–hexanes, to give an epimeric mixture of the 3-(4-nitrobenzoate) derivatives (50 mg, 82\%). The mixture was then crystallized (five times) from MeOH-acetone to obtain the pure enantiomer of the 3-(4-nitrobenzoate) of \textit{ent-20(S)-OHC} (25 mg): mp 197–200 °C; IR: 3573, 2947, 2883, 1710, 1607, 1530, 1350, 1317, 1285 cm\(^{-1}\); \textsuperscript{1}H NMR (CDCl\(_3\)) \(\delta\) 8.29 (d, 2H, J = 9.1 Hz, Ar-H), 8.22 (d, 2H, J = 9.1Hz, Ar-H), 5.46 (b s, 1H, H-6), 4.91 (m, 1H, H-3), 2.49 (d, 1H, J = 7.4 Hz, H-17), 1.29 (s, 3H, H-21), 1.09 (s, 3H, H-19), 0.88 (overlapping d, 6H, J = 6 Hz, H-26, H-27), 0.87 (s, 3H, H-18); \textsuperscript{13}C NMR (CDCl\(_3\)) \(\delta\) 164.08, 150.42, 139.25, 136.18, 130.64 (x2), 123.45 (x2), 123.08, 75.73, 75.22, 57.62, 56.78, 49.92, 44.20, 42.62, 40.02, 39.58, 38.05, 36.93, 36.61, 31.77, 31.25, 27.91, 27.78, 26.42, 23.76, 22.71, 22.55, 22.34, 22.01, 20.88, 19.34, 13.58.
The 3-(4-nitrobenzoate) of pure ent-20(S)-OHC (23 mg, 0.04 mmol) was hydrolyzed back to ent-20(S)-OHC using LiOH monohydrate (200 mg, 4.76 mmol) in a stirred solution of water (2 mL), MeOH (5 mL) and THF (2 mL) at 80°C for 1 h. The reaction was cooled and the MeOH and THF were removed under reduced pressure. The resulting residue was diluted with water and extracted with CH₂Cl₂. The CH₂Cl₂ extracts were dried with anhydrous Na₂SO₄ and concentrated to give a white solid, which was purified by on a short column of silica gel using 40% ethyl acetate in hexanes as eluents, yielding pure ent-20(S)-OHC (15 mg, 91%). ent-20(S)-OHC: Rₚ = 0.39 (acetone–hexanes, 5/15, v/v); mp 125–128 °C; [α]D²⁰ = +56 (c = 0.06, CHCl₃); IR: 3369, 2935, 2869, 1665, 1465, 1377 cm⁻¹; ¹H NMR (CDCl₃) δ 5.36 (m, 1H, H-6), 3.53 (m, 1H, H-3), 1.28 (s, 3H, H-21), 1.02 (s, 3H, H-19) 0.88 (overlapping d, 6H, J = 6 Hz, H-26, H-27), 0.87 (s, 3H, H-18); ¹³C NMR (CDCl₃): δ 141.0, 121.8, 75.5, 72.0, 57.9, 57.1, 50.2, 44.3, 42.9, 42.5, 40.3, 39.8, 37.5, 36.7, 32.0, 31.8, 31.5, 28.1, 26.6, 24.0, 22.9, 22.8, 22.6, 22.2, 21.1, 19.6, 13.8; HR-ESI MS [M+Na]⁺, Anal. calcd. for C₂₇H₄₆O₂Na⁺ 425.3396, found 425.3395.
Supplementary scheme 3. Synthesis and characterization of *nat-20*(S)-yne (4)

![Scheme 3](image)

4-Bromo-1-methylsilyl-1-butyne (11)

Compound 11 was prepared according to a known literature procedure. To a solution of commercially available 4-trimethylsilyl-3-butyn-1-ol (2.0 g, 14.06 mmol) in dry dichloromethane (40 mL) at -30°C, was added CBr₄ (8.5 g, 25.6 mmol). The mixture was stirred vigorously for 10 min, until the CBr₄ was completely dissolved, whereupon a solution of PPh₃ (5.53 g, 21.09 mmol) in dry dichloromethane (12 mL) was added dropwise. The reaction mixture was stirred until -30°C for 2 h, after which the temperature was raised to 0°C and was allowed to slowly warm to rt over the next 2 h. Upon completion, the reaction mixture was filtered through a pad of silica, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (100% hexane elution), to yield compound 11 as a colorless liquid (2.16 g, 75%). Analytical data for 11 is as previously reported.

(3β, 17β)-17-(1-hydroxy-1-methylpent-4-ynyl)-androst-5-en-3-ol (*nat-20*(S)-yne)

*nat-20*(S)-yne was synthesized following a protocol similar to the synthesis of compound 9. In a 2-necked flask equipped with a condenser, was stirred magnesium turnings (0.45 g, 18.5 mmol) in anhydrous diethyl ether (100 mL) under nitrogen. Compound 11 (3.8 g, 18.5 mmol) was added, followed by a few drops of 1,2-dibromoethane. The reaction mixture was warmed slightly to 30°C, and stirred vigorously until cloudiness was observed. The reaction mixture was stirred an additional hour at rt, until the magnesium turnings were consumed. A solution of pregnenolone (1.0 g, 3.16 mmol) in anhydrous diethyl ether (45 mL), was then added dropwise to the reaction at 0°C. The reaction was then allowed to stir at rt for 1 h. Upon completion, the reaction was cooled to 0°C, diluted with diethyl ether (50 mL), and quenched with aq NH₄Cl (50 mL). The phases were separated, and the aqueous phase was extracted with Et₂O (4 x 20 mL). The combined organic fractions were then washed with brine (50 mL), dried over Na₂SO₄, and concentrated in vacuo. The residue was passed through a short column of silica gel to remove residual Grignard byproducts, and the crude reaction mixture was then concentrated in vacuo. The reaction mixture was then redissolved in dry THF (60 mL), and cooled to 0°C. Dropwise was added tetrabutylammonium bromide; 1M in THF (5 mL, 5.0 mmol), and the reaction was stirred for 15 min. Remaining at 0°C, the reaction was then quenched with H₂O (5 mL), and extracted with diethyl ether (2 x 50 mL). The combined organic fractions were then washed with...
brine, dried over Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (acetone–hexane gradient elution), to yield almost exclusively the desired S-diastereomer. The pure S-diastereomer was obtained by recrystallizing from methanol-dichloromethane. As methanol was unable to be fully evaporated from the crystals, even after extensive drying, the compound was redissolved in dichloromethane and re-evaporated to yield nat-20(S)-yne (0.65 g, 56%). nat-20(S)-yne: $R_f = 0.48$ (acetone–hexanes, 3/7, v/v); mp=170-172°C (DCM); [$\alpha$]$_{D}^{20} = -55.8$ (c = 0.17, CHCl$_3$); IR: 3293, 2933, 2900, 2867, 1464, 1435, 1376, 1058 cm$^{-1}$; $^1$H NMR (CDCl$_3$): $\delta$ 5.37 (m, 1H, H-6), 3.46–3.60 (m, 1H, H-3), 1.95 (t, 1H, J=2.4 Hz, C=CH), 1.29 (s, 3H, H-21), 1.01 (s, 3H, H-19), 0.86 (s, 3H, H-18); $^{13}$C NMR (CDCl$_3$): $\delta$ 141.0, 121.8, 85.2, 75.0, 72.0, 68.5, 58.9, 57.1, 50.2, 43.0, 42.5, 41.8, 40.3, 37.4, 36.7, 32.0, 31.8, 31.5, 25.8, 24.0, 22.7, 21.1, 19.6, 13.8, 13.5; Anal. Calcd. for C$_{25}$H$_{38}$O$_2$: C, 81.03; H, 10.34. Obsd. C, 80.77; H, 10.16. HR-FAB MS [M+Na]$^+$, Anal. calcd. for C$_{25}$H$_{38}$O$_7$Na$^+$ 393.2770, found 393.2778.
Supplementary scheme 4. Synthesis and characterization of \textit{nat-20(S)-COOH} (5)

(3β, 17β)-17-(1-Hydroxy-1-methylpent-4-ynyl)-androstan-5-en-3-ol-PEG4-carboxylic acid-triazole adduct (\textit{nat-20(S)-COOH})

Following known literature procedure\textsuperscript{7,8}, \textit{nat-20(S)-yne} (10.0 mg, 27.0 µmol) and the commercially available PEG4-NHS-ester azide (10.0 mg, 25.7 µmol) were dissolved in a 1:1 mixture of THF:H\textsubscript{2}O (0.6 mL) at 30°C. To this was added dropwise sodium ascorbate (0.64 mg, 2.6 µmol, dissolved in 0.1 mL H\textsubscript{2}O) followed by CuSO\textsubscript{4} (1.02 mg, 5.2 µmol, dissolved in 0.1 mL H\textsubscript{2}O). The reaction mixture was allowed to stir for 5 h at 30°C. Upon completion, the reaction mixture was extracted first with DCM (1 mL), followed by extraction with DCM:MeOH (4:1) (3 x 1 mL). The organic layers were then combined, dried over Na\textsubscript{2}SO\textsubscript{4}, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (ethylacetate–hexane → methanol:H\textsubscript{2}O (50:1)-dichloromethane gradient elution) to yield \textit{nat-20(S)-COOH} as a white solid (14.3 mg, 84%). In employing these initial reaction conditions, it was found that we had inadvertently hydrolyzed the labile N-hydroxysuccinimide protecting group, forming instead the carboxylic acid. However, the loss of this protecting group was of no consequence, as this product was still able to be utilized in the subsequent coupling reaction (although it should be noted that in a later refinement of this procedure, it was found that a reaction time of 30 min formed the triazole product with the NHS protecting group intact; in this case, extraction during workup should be done with DCM only, taking care to avoid exposure to protic solvents). \textit{nat-20(S)-COOH}: \(R = 0.45\) (dichloromethane–methanol:H\textsubscript{2}O (30:1), 2/8, v/v); IR: 3401, 2931, 2900, 2870, 1708, 1103, 1062 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \(\delta 7.53\) (s, 1H, H-N-C\textsubscript{6}H), 5.34–5.36 (m, 1H, H-6), 4.53 (br s, 2H), 3.88 (br s, 2H), 3.76 (br s, 2H), 3.46-3.71 (m, 13H), 2.76 (br s, 2H), 2.59 (br s, 2H), 1.35 (s, 3H, H-21), 1.01 (s, 3H, H-19), 0.88 (s, 3H, H-18); \textsuperscript{13}C NMR ((CD\textsubscript{3})\textsubscript{2}SO): \(\delta 147.3, 141.2, 121.8, 120.4, 72.6, 69.9, 69.7(x4), 69.5(x3), 68.7, 57.8, 56.4, 49.6, 49.1, 43.2, 42.2, 42.0, 36.9, 36.0, 31.4, 31.3, 30.9, 25.6, 23.4, 22.0, 20.5, 20.1, 19.1, 13.3\); HR-ESI MS [M+Na]\textsuperscript{+}: Calcd. for C\textsubscript{36}H\textsubscript{59}N\textsubscript{3}O\textsubscript{8}Na\textsuperscript{+}: 684.4200, found 684.4193.
Supplementary Scheme 5. Coupling of nat-20(S)-COOH (5) to SiMAG amine beads

5 mg of SiMag-Amine magnetic beads (Chemicell) per coupling reaction were washed into dimethylformamide (DMF). Stock solutions of hydroxybenzotriazole (HOBt, 100 mM), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, 100 mM) and nat-20(S)-COOH (10 mM) were prepared in DMF. 5 mg SiMAG amine beads were combined with 2 mM HOBt, 2 mM EDC and 0.35 µmol nat-20(S)-COOH in DMF (1 mL reaction volume). Control beads were prepared in parallel by preparing a duplicate reaction lacking nat-20(S)-COOH. Reactions were monitored over time by briefly separating the beads against the side of the tube with a magnet and removing 20 µL aliquots of the supernatant to analyze by TLC. Prior to TLC, these aliquots were diluted with 60 µL of water and then extracted with 40 µL methyl tert-butyl-ether. Plates were developed using a methanol/dichloromethane (2/8, v/v) mixture and visualized by charring with 5% v/v sulfuric acid and heating. After 4 hours, the reaction was stopped by magnetically collecting the beads and removing the supernatant. The beads were then incubated for 3 hours in an acetic anhydride/DMF (2/8, v/v) solution to block any remaining free amino groups.
Supplementary Results

Supplementary Figure 1. Oxysterols do not activate Wnt signaling. To ensure that the activity of nat-20(S)OHC is not due to activation of multiple pathways, WntL cells carrying a Wnt-responsive luciferase gene were treated with the indicated oxysterols overnight. Each point represents the mean of triplicate wells (± SEM). Wnt-conditioned media (WntCM), as a positive control, yielded a 17-fold increase in reporter activity. However, neither oxysterols nor the Smo agonist SAG (100 nM) induced Wnt reporter activity.

Supplementary Figure 2. nat- and ent-20(S)-OHC have identical effects on liposome swelling. Using a previously described carboxyfluorescein dequenching assay, we established that nat- and ent-20(S)-OHC can expand DOPC-containing unilamellar vesicles to very similar degrees (see also Fig. 2d, where only the average curves are shown). For each compound, the fractional dequenching measured in three independent trials as a function of time. Three individual trials as well as the average of the three trials, along with a solvent (isopropanol) control, are plotted on the same graph for comparison.
Supplementary Figure 3. *nat*-20(S)-OHC enantioselectively induces Smo translocation to the primary cilium. These are representative images corresponding to the data shown in Figure 2f. NIH 3T3 cells were treated with a solvent control or 10 µM of the indicated oxysterols and stained with DAPI to show nuclei (blue) and antibodies against acetylated tubulin (cilia marker; red) and Smo (green). Insets show shifted overlays (red and green channels) of a single cilium. Scale bar: 5 µm.

Supplementary Figure 4. *nat*-20(S)-OHC and *ent*-20(S) do not affect cell viability. The ATP-based Cell-Titer Glo cell viability assay (Promega) was used to assay ATP levels, a measure of viability, in cells treated with *nat-* or *ent*-20(S)-OHC. 10 nM, 100 nM and 1 µM ATP were added to media as positive controls. Each bar represents the mean of triplicate wells (± SEM) in arbitrary units (AU) of luminescence.
Supplementary Figure 5. *nat-* and *ent*-20(S)-OHC cannot further increase the high level of Gli1 transcription in *ptc1/*- cells. Serum starved *Ptc1/*- cells were treated with various concentrations of *nat-* and *ent*-20(S)-OHC for 6 hours. Gli1 and GAPDH transcript abundance were then quantified by q-RT-PCR. Neither *nat-* or *ent*-20(S)-OHC had a significant effect on Hh pathway activation as measured by Gli1 transcription, consistent with previous findings. As a control, cyclopamine does suppress target gene transcription.

Supplementary Figure 6. A competitive interaction between SAG and cyclopamine. NIH 3T3 cells bearing a Hh-responsive luciferase gene were treated with increasing doses of cyclopamine in the presence of the indicated concentrations of SAG or *nat*-20(S)-OHC. The IC50 (concentration at which 50% inhibition is achieved) of cyclopamine increases with increasing concentrations of SAG, indicative of a competitive interaction. In contrast, the IC50 of cyclopamine is unaffected by increasing amounts of *nat*-20(S)-OHC.
Supplementary Figure 7. SAG and nat-20(S)-OHC synergistically induce Smo translocation to the primary cilium. These are representative images corresponding to the data shown in Figure 4d. Combining low concentrations of SAG and nat-20(S)-OHC can fully activate Hh signaling and drive accumulation of Smo in cilia. NIH 3T3 cells were treated with a solvent control or the indicated oxysterol/SAG combination and stained with DAPI to show nuclei (blue) and antibodies against acetylated tubulin (cilia marker; red) and Smo (green). Insets show shifted overlays (red and green channel) of a single cilium. Scale bar: 5 µm.
Supplementary Figure 8. Supporting data for Figure 5. (a) Confocal images of NIH 3T3 cells treated for 12 hours with solvent (control), nat-20(S)-OHC or nat-20(S)-yne. The cilia marker acetylated tubulin (red) and Smo (green) were detected by immunofluorescence, and nuclei were detected by DAPI (blue). The inset is a zoomed image of a single cilium where the green channel is shifted relative to the red channel. Scale bar: 5 µm. (b) An immunoblot (inset) and corresponding graph show the amount of YFP-Smo precipitated when increasing amounts of YFP-Smo containing extract are added to a fixed amount of nat-20(S)-yne beads. (c) The immunoblot corresponding to the data shown in Figure 5d. Control beads or nat-20(S)-yne beads were incubated with membrane extracts made from 293T cells expressing YFP-Smo, a GFP-tagged somatostatin receptor (SSTR3-GFP) or a GFP-tagged serotonin receptor (HTR6-GFP). Similar to Smo, these two receptors have seven transmembrane segments and localize in primary cilia. Since the antibody used to detect these fusion proteins recognizes YFP and GFP equally well, the signals on the blot are directly comparable. (d) The immunoblot corresponding to Figure 5f, showing that YFP-Smo captured on nat-20(S)-yne beads can be eluted by nat-20(S)-yne and nat-20(S)-OHC but not by ent-20(S)-OHC or 22(S)-OHC (500 µM each).
**Supplementary Figure 9. Smo antagonists do not compete with YFP-Smo for binding to nat-20(S)-yne beads.** The interaction of YFP-Smo with nat-20(S)-yne beads was tested in the presence of a series of concentrations of the Smo antagonists cyclopamine, SANT-1 and itraconazole. We chose competitor concentrations equal to 1/2x, 1x, 2x, and 5x the IC50 for each inhibitor determined in the Hh reporter assays shown in Figure 3, main text. From Figure 3, the IC50s for oxysterol-induced Hh signaling are 10 nM for SANT-1, 50 nM for cyclopamine, and 1 µM for itraconazole. While SANT-1 does have a small effect on YFP-Smo binding, this effect is partial even at a concentration 5-fold higher than its IC50.
Supplementary Figure 10. Full blot scans for cropped gels. Boxed regions correspond to the regions of the gel used in figures.
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