ChemPubSoc Europe
DOI: 10.1002/cmdc.201500550
CHEMMEDCHEM Full Papers

Synthesis and Evaluation of Osteogenic Oxysterols as Hedgehog Pathway Activators

Chad A. Maschinot, Audrey R. Corman, Albert M. DeBerardinis, and M. Kyle Hadden*[a]

Oxysterols (OHCs) are metabolic byproducts of cholesterol that are known to function as agonists of the Hedgehog (Hh) signaling pathway. Previously, we reported 23(*S*)-hydroxycholesterol [23(*S*)-OHC, **4**] as a potent activator of Hh signaling with the ability to functionally differentiate mouse embryonic fibroblasts to an osteogenic fate. To obtain 23(*S*)-OHC in quantities suitable for in vivo evaluation, we developed a revised synthetic route that decreases the number of steps and chromatographic purifications, and which also enhances the stereoselective nature of the synthesis. This new route also allows access to the C21 methyl group of the OHC scaffold, and several new analogues with varying stereochemistry at this location were evaluated for their ability to up-regulate the Hh pathway.

Introduction

The Hedgehog (Hh) signaling pathway is a developmental signaling pathway that is essential for proper differentiation of a variety of tissues during embryonic development.[1] Aberrant activation of Hh signaling has been linked to several human malignancies, and multiple small-molecule inhibitors of Hh signal transmission are in preclinical or clinical trials as anticancer chemotherapeutics. In contrast, the development of pathway agonists as therapeutic agents has received less attention. The therapeutic potential of Hh agonists exists primarily with neurological disorders[2] such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), as well as osteodegenerative disorders[3] in which de novo bone formation is of interest, such as bone fracture repair and osteoporosis. Among the Hh agonists that have been studied, oxysterols (OHCs) have shown promise with the activation of Hh signaling, resulting in osteo-inductive effects in vitro and bone formation in vivo.[4–7]

Naturally occurring OHCs are formed as metabolic byproducts of cholesterol oxidation and exert a range of physiological effects through multiple cellular receptors.[8] One of the most potent naturally occurring OHCs, 20(*S*)-OHC (**1**, Figure 1) has been used to characterize the OHC class of sterols as activators of Hh signaling that function through direct binding to Smoothened (Smo), a seven-transmembrane G-protein-like receptor that serves as a key regulator of the Hh signaling cascade. These studies have demonstrated that OHCs bind to the extracellular cysteine-rich domain (CRD) of Smo.[9,10]

Previous structure–activity relationship (SAR) studies for OHC agonists of Hh signaling have continually identified the importance of incorporating a hydroxy group in the alkyl side chain.[11,12] Potent activation of the Hh pathway also depends on the location and stereochemical orientation of the hydroxy moiety. Whereas 20(*S*)-OHC is a potent Hh agonist, 20(*R*)-OHC is significantly less active. In addition, the most active synthetic OHC agonists of the Hh pathway disclosed to date (represented by Oxy34) all incorporate the 20(*S*)-hydroxy stereochemistry and the C21 methyl group.

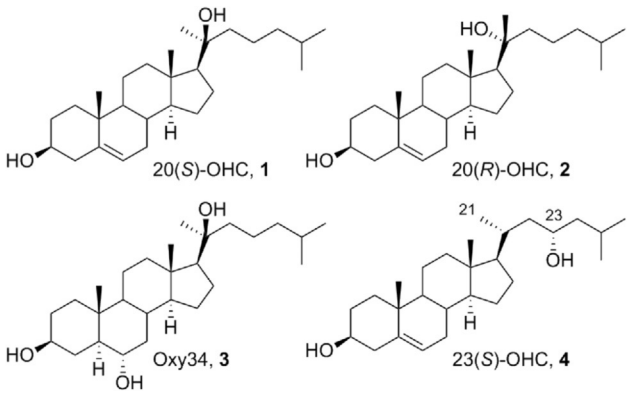


Figure 1. Natural and synthetic OHC agonists of Hh signaling.

We previously performed a series of SAR studies focused on the OHC side chain to further explore the stereo- and regioselective specificity of Hh activation for this region of the scaffold.[11] The most active compound to emerge from our study, 23(*S*)-OHC (**4**), was found to possess potency similar to that of 20(*S*)-OHC (EC_{50}: 0.57 and 0.52 μM, respectively). Compared with 20(*S*)-OHC, 23(*S*)-OHC also demonstrated greater selectivity for Hh agonism relative to the liver X receptor (LXR), which has been identified as a negative regulator of the Hh pathway and which maintains affinity for many endogenous OHC agonists of Hh signaling.[13] OHC **4** was also found to induce osteogenic differentiation and osteoblast formation in cultured M2-

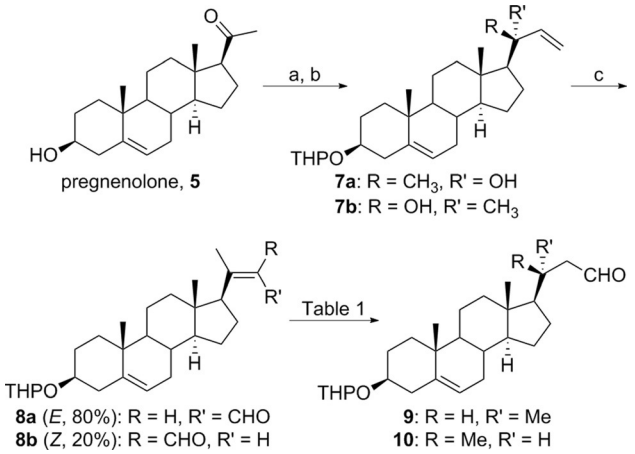
[a] C. A. Maschinot, Dr. A. R. Corman, Dr. A. M. DeBerardinis, Prof. M. K. Hadden
Department of Pharmaceutical Sciences, University of Connecticut
69 North Eagleville Road, Unit 3092, Storrs, CT, 06269-3092 (USA)
E-mail: kyle.hadden@uconn.edu

Supporting information for this article can be found under http://dx.doi.org/10.1002/cmdc.201500550.

10B4 cells, highlighting its therapeutic potential; however, the initial synthesis of **4** was low yielding (1.8%) and required twelve steps from commercially available hyodeoxycholic acid.[11] For this reason, we sought to develop a more direct and higher-yielding procedure for the synthesis of 23(*S*)-OHC. In addition, we used both our revised route and a slightly modified procedure to synthesize and evaluate OHC analogues that incorporate modifications to the C21 methyl group as a means to explore the importance of this functionality and its stereochemical orientation.

Results and Discussion

Our revised synthetic route began with protection of commercially available pregnenolone as the tetrahydropyran (THP) ether (Scheme 1). Notably, the THP protecting group was



Scheme 1. Asymmetric synthesis of aldehydes **9** and **10**. *Reagents and conditions*: a) 3,4-dihydro-2*H*-pyran, *p*-TsOH, 12 h, RT, 96%; b) vinyl MgBr, −78 °C→RT, 12 h, 97%; c) PCC, NaOAc, RT, 12 h, 64%.

chosen because it had previously resulted in a more straightforward chromatographic separation of the 23(*S*)- and 23(*R*)-OHC stereoisomers. Following a previously reported procedure,[14] Grignard addition of vinyl magnesium bromide gave a mixture (1:9) of the tertiary allylic alcohols **7a** and **7b**. Treatment of the mixture with pyridinium chlorchromate resulted in oxidative rearrangement of the allylic alcohol to yield an 80:20 *E*/*Z* mixture of β-substituted-α,β-unsaturated aldehydes, **8a** and **8b**.

A key requirement of this synthetic route was the ability to reduce the α,β-unsaturation in **8a** and/or **8b** in a stereoselective fashion to provide both the *R* and *S* configurations of the C21 methyl moiety. Initial attempts using standard palladium or platinum catalysts resulted in nonselective reduction of the side chain olefin or complete saturation of the OHC scaffold (Table 1, Entries 1–3). Neither Stryker's reagent (Entry 4) nor the ruthenium BINAP complex (Entry 5) effected reduction of either alkene in the scaffold. Following these unsuccessful attempts, we turned to the iminium catalysts previously developed in the MacMillan research group, a reaction of particular

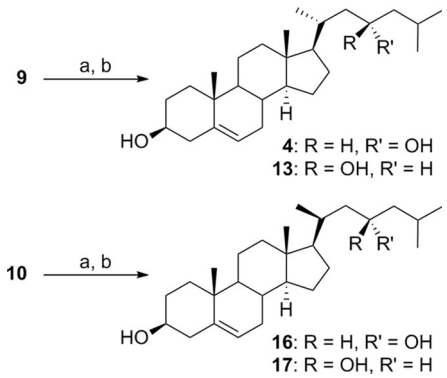
Table 1. Reduction of α,β-unsaturated aldehyde to provide compounds **9** and **10**.

Entry	Reagent(s)	Results
1	5% Pd/C, H_2	Nonselective olefin reduction
2	10% Pd/C, H_2	Complete saturation
3	PtO_2	Complete saturation and deprotection
4	$[(PPh_3)CuH]_6$	No reaction
5	(*R*)-$Ru(OAc)_2$(BINAP)	No reaction
6	(*S*)-(+)-2-(*tert*-butyl)-3-methyl-4-imidazolidinone TFA, Hantzsch ester	**9**, 60%
7	(*R*)-(+)-2-(*tert*-butyl)-3-methyl-4-imidazolidinone TFA, Hantzsch ester	**10**, 60%

interest, given its ability to convert both *E* and *Z* alkenes into a single β-stereogenic aldehyde.[15] Using the Hantzsch ester as hydride source and (*S*)-imidazolidinone, the **8a**/**8b** mixture was converted into aldehyde **9** [20(*R*) configuration] in good overall yield with no formation of stereoisomer **10**. Performing the same protocol on the **8a**/**8b** mixture with (*R*)-imidazolidinone provided **10** [20(*S*) configuration] in good yield as the singular reduction product.

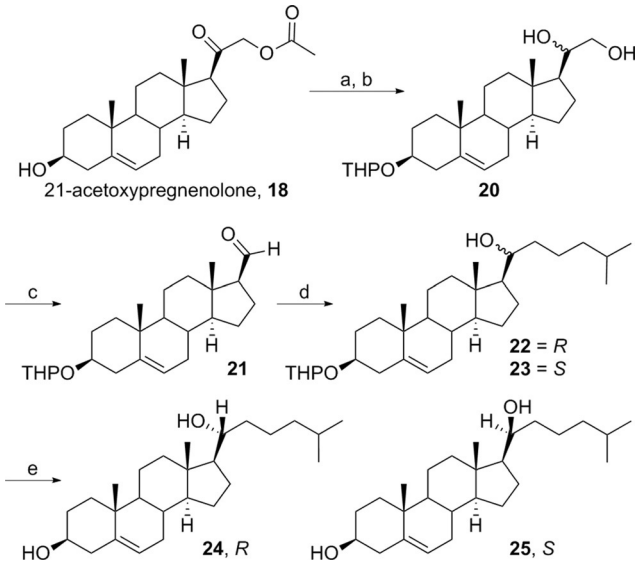
We next looked to perform an enantioselective Grignard addition to aldehyde **9** to selectively obtain **4**. Multiple attempts were taken (see Supporting Information) following known procedures. The first series of asymmetric Grignard attempts used $Ti(OiPr)_4$, chiral BINOL, and a chelating agent (BDMAEE, bis[2-(*N*,*N*-dimethylamino)ethyl] ether).[16] After multiple alterations to the procedure with no success, we thought the Grignard reagent may be too deactivated with the use of the chelating BDMAEE, making the reaction unsuccessful even with heating due to the deactivated nature of the alkyl-aldehyde starting material. Several other attempts were taken without the use of BDMAEE following a second procedure.[17] With up to 10 equivalents of Grignard used and no reaction observed for either method, we believe that the enolizable proton of **9** may result in its enolization by $Ti(OiPr)_4$, preventing the Grignard addition from occurring.

Following our unsuccessful attempts to prepare 23(*S*)-OHC from aldehyde **9** in a diasteroselective fashion using enantiopure reagents, we used standard Grignard addition of isobutyl magnesium bromide to either **9** or **10** to provide the corresponding 23(*S*)- and 23(*R*)-OHC analogues as a 1:1 mixture that was easily separable via standard silica gel chromatography (Scheme 2). Final removal of the THP protecting group afforded 23-hydroxylated OHCs **4**, **13**, and **16**–**17** in excellent yield. This revised synthetic route afforded 23(*S*)-OHC (**4**) in an overall yield of 14.2% in six steps from commercially available pregnenolone, **5**, 28.3% overall for both isomers. Previous assignments of the stereochemistry at C23 were based on a combination of thin-layer chromatography (TLC), ^1H and ^{13}C NMR spectroscopy, and circular dichroism analysis.[11] We used crystal structure analysis of **4** and **13** to both unambiguously assign the stereochemistry of the C23 hydroxy group and for use as a base for assigning the stereochemistry of **16**–**17** and other OHC analogues (Supporting Information).



Scheme 2. Synthesis of 23-OHC analogues. *Reagents and conditions*: a) *i*BuMgBr, THF, −78 °C→RT, 72–80%; b) 2 N HCl, MeOH/THF, 96–97%.

To complete our analysis of the importance of the C21 methyl functionality for Hh agonism, we prepared two OHC analogues that maintain the C20 hydroxy moiety, but do not incorporate the C21 methyl group. Preparation of these analogues began with THP protection of commercially available 21-acetoxypregnenolone followed by lithium aluminum hydride mediated reduction of the ester and ketone functionalities to afford **20** (Scheme 3). Oxidative cleavage of the 1,2-diol



Scheme 3. Synthesis of 21-desmethyl OHC analogues. *Reagents and conditions*: a) 3,4-dihydro-2*H*-pyran, *p*-TsOH, 12 h, RT, 96%; b) LAH, THF, 0 °C→RT, 3 h, 95%; c) $NaIO_4$, RT, 1 h, 93%; d) Mg, isopentyl bromide, THF, 50 °C, 55%; e) 2 N HCl, MeOH/THF, RT, 96%.

with sodium periodate provided aldehyde **21**, which underwent Grignard addition with in situ generated isopentyl magnesium bromide to provide a mixture of C20 secondary alcohols, **22** and **23**. Similar to our previous OHCs, use of the THP protecting group proved advantageous for the separation of the *R* and *S* isomers, **24** and **25**, respectively. Assignment of the stereochemical orientation of the C20 hydroxy moiety for these compounds was performed through Mosher ester analysis of THP-protected intermediates **22** and **23** (Supporting Information).

OHC analogues **4**, **13**, **16**–**17**, and **24**–**25** were initially evaluated for their ability to selectively activate Hh signaling in M2-10B4 cells, a multipotent murine bone stromal cell line previously used as a model to evaluate small-molecule Hh agonists. The ability of each analogue to up-regulate known Hh (GLI1) or LXR (ABCA1) target genes was measured. For these studies, DMSO was used as a control (set at 1.0), and all OHC analogues were evaluated at 5 μM. Values in Table 2 represent the

Table 2. Activity of OHC analogues in M2-10B4 cells.

OHC[a]	GLI1[b]	ABCA1[b]	Hh Selectivity[c]
DMSO	1.0	1.0	–
1	19.9 ± 0.7	17.9 ± 0.2	1.1
4	18.2 ± 0.6	6.2 ± 0.3	2.9
13	8.7 ± 0.1	3.6 ± 0.1	2.4
16	1.3 ± 0.3	1.0 ± 0.3	1.3
17	0.7 ± 0.01	0.7 ± 0.2	–
24	24.3 ± 0.8	2.4 ± 0.1	10.1
25	1.3 ± 0.01	1.3 ± 0.4	–

[a] All OHCs tested at 5 μM. [b] Values are the mean fold mRNA up-regulation ± SEM. [c] Hh selectivity determined as the ratio of GLI1/ABCA1. Data are the average of three separate experiments performed in triplicate.

fold increase in mRNA expression over DMSO levels. The ability of both **4** and **13** to up-regulate GLI1 mRNA closely mirrored that reported from OHCs prepared through our previous synthetic route.[11] Interestingly, inversion of the C21 methyl group on the 23-hydroxylated OHCs (**16** and **17**) completely abolished their ability to up-regulate GLI1 expression. This was particularly interesting for analogue **16**, which loses all Hh agonism compared to 23(*S*)-OHC, when the C21 methyl is inverted. These results suggest that inversion of the C21 methyl group actively prevents the OHC scaffold from binding the Smo CRD. This result is supported by a previous study demonstrating that inversion of the C20 hydroxy group to 20(*R*)-OHC resulted in complete loss of Hh agonism relative to natural 20(*S*)-OHC.[9]

Interestingly, removal of the C21 methyl moiety of 20(*S*)-OHC, as in OHC **25**, completely abolished the ability of the scaffold to up-regulate GLI1 expression. Removal of the C21 methyl group for 20(*R*)-OHC resulted in OHC **24**, which shows potent agonism of Hh signaling, an unexpected result considering that 22(*R*)-OHC is a poor activator of Hh signaling.[11] Taken together, these results strongly suggest stereoselective interactions between the side chain of the OHC scaffold and its binding site in the Smo CRD are essential for its ability to activate Hh signaling.

Based on its comparable potency and improved selectivity, we evaluated OHC **24** in a series of secondary assays to further probe its ability to activate the Hh signaling cascade. To confirm that GLI1 up-regulation with **24** is mediated through the Hh pathway, a competition study was performed with cyclopamine (Cyc), a potent Hh-selective inhibitor known to attenuate GLI1 expression induced by OHCs.[5,9,11] As expected, co-administration with Cyc (5 μM) abolished GLI1 up-regulation, indicat-

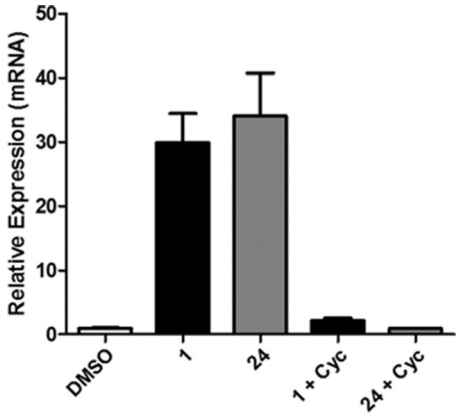


Figure 2. Attenuation of **24**-mediated GLI1 up-regulation with Cyc co-administration in M2-10B4 cells. OHC and Cyc evaluated at 5 μM. Data are the mean ± SEM from a representative experiment performed in triplicate.

Table 3. Dose-dependent pathway activation in M2-10B4 cells.

OHC	EC_{50} [μM] GLI1[a]	PTCH1[a]	ABCA1[a]
1	0.52 ± 0.04	0.72 ± 0.2	4.67 ± 0.9
4	0.57 ± 0.1	0.65 ± 0.06	1.53 ± 0.2
24	1.33 ± 0.1	1.29 ± 0.01	2.76 ± 0.1

[a] Values are the average ± SEM of three separate experiments performed in triplicate.

ing OHC **24** functions through Hh activation (Figure 2). Dose-dependent evaluation of OHC **24** demonstrated that its ability to up-regulate the key Hh target genes GLI1 and PTCH1 was approximately twofold less than both **1** and **4** (Table 3). In addition, the EC_{50} value for up-regulation of ABCA1 by **24** was similar to that obtained for the Hh pathway target genes, suggesting removal of the C21 methyl group also affects its ability to selectively activate Hh signaling.

When the Hh pathway is functionally activated in M2-10B4 cells, osteogenic differentiation is promoted, resulting in mature osteoblastic cells.[3,8] This differentiation process can be followed by measuring the early- (osterox, OSX) and late-stage (alkaline phosphatase, ALP) transcriptional markers of osteogenesis.[3,8] Previously, OHCs **1** and **3**–**4** demonstrated the ability to promote differentiation of M2-10B4 cells, and we evaluated **24** for its activity in this assay. Cells were treated with either **1** or **24** (5 μM) for 24, 48, or 96 h to compare their ability to both up-regulate GLI1 and induce cellular differentiation over this time course (Figure 3). Both compounds demonstrated similar induction of GLI1 expression after 24 and 48 h; however, OHC **1** continued to increase GLI1 expression up to 96 h post-treatment, whereas no additional increase in GLI1 expression was observed after 48 h for **24**. After 24 h, both compounds induced a modest increase in both OSX and ALP expression. Interestingly, while OHC **1** significantly increased OSX expression up to 96 h, OSX expression was not up-regulated by compound **24**. In addition, increased expression of ALP, denoting mature osteoblasts, was demonstrated at both 48 and

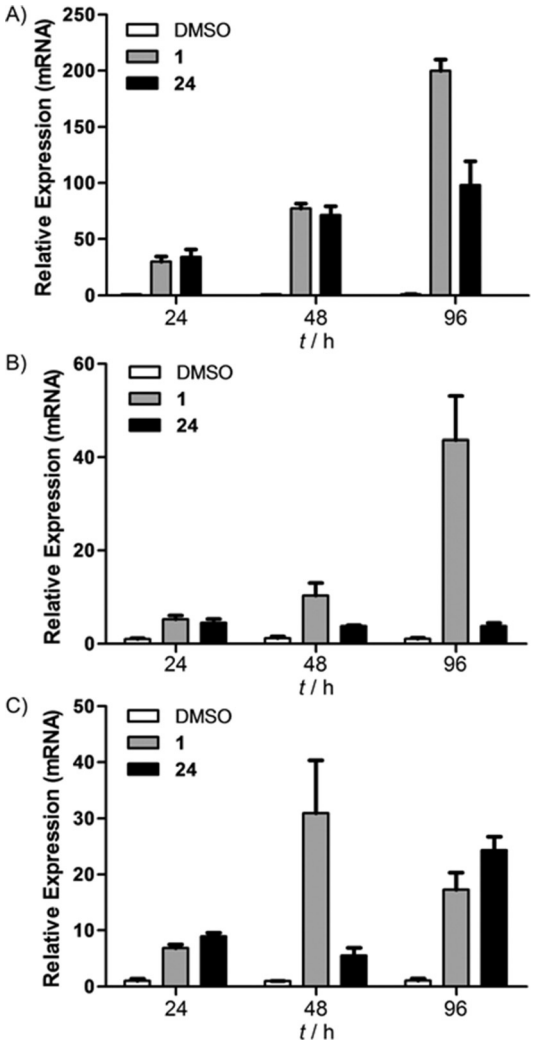


Figure 3. Induction of osteogenic differentiation and osteoblast maturation by OHCs at 24, 48, or 96 h; up-regulation of A) GLI1, B) OSX, and C) ALP. Data are the mean ± SEM from a representative experiment performed in triplicate.

96 h for OHC **1**, but not until 96 h for OHC **24**. Taken together, these data support the ability of both OHCs to terminally differentiate precursor cells via Hh activation, while also suggesting that OHC **24** mediates its osteogenic action through a mechanism distinct from OSX.

Conclusions

In summary, we have developed a new synthetic approach to obtain 23(*S*)-OHC (**4**) from inexpensive commercially available starting materials. This new route uses half the number of steps previously required (six down from twelve), with only two required chromatographic purifications (following the Grignard coupling and the final deprotection), and a tenfold increase in overall yield. The new approach also allowed the synthesis and evaluation of two new diastereomers of **4**, analogues that did not activate Hh signaling. Based on the known importance of the stereochemistry of the hydroxy moiety at C20, we wanted to further explore SAR at this region of the

scaffold by synthesizing and evaluating analogues of 20(*S*)-OHC (**1**) and 20(*R*)-OHC (**2**) in which the C21 methyl group has been removed. Interestingly, only **24** demonstrated the ability to up-regulate Hh target genes at a level similar to **1** at 5 μM; however, the EC_{50} value, selectivity, and capacity to functionally differentiate mouse embryonic fibroblasts of **24** was significantly decreased relative to both **1** and **4**. Finally, our results support the previous findings from our research group and others that the proper stereochemical orientation of functional groups on the OHC side chain is essential for Smo binding and Hh agonism. Continued work detailing OHC binding to the Smo CRD will provide further insight into the exact molecular interactions that govern OHC-mediated Hh activation and provide for a structure-based approach to designing more potent compounds.

Experimental Section

General information. Pregnenolone (**5**) and 21-acetoxypregnenolone (**18**) were purchased from Sigma–Aldrich. All other reagents were purchased from commercial sources (Fisher Scientific or Sigma–Aldrich) unless otherwise stated. Column chromatography was performed with silica gel purchased from Sorbtech (Sorbent Technologies). All ^{1}H and ^{13}C NMR data were collected on a Bruker AVANCE 500 spectrometer and analyzed with MestReNova software (ver. 9.1.0). HRMS data were gathered at the Mass Spectrometry Facility at the University of Connecticut, performed by Dr. You-Jun Fu. FTIR analysis was performed on a Bruker Alpha Platinum ATR instrument using OPUS software (ver. 7.2).

1-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)ethanone, THP-pregnenolone (6). To a solution of pregnenolone (**5**, 10.0 g, 31.6 mmol) in anhydrous CH_2Cl_2 (150 mL) under argon, 3,4-dihydro-2*H*-pyran (39.5 mmol, 3.6 mL) and a catalytic amount of *p*-toluenesulfonic acid were added. The mixture was stirred for 12 h at RT, washed with saturated $NaHCO_3$ (2×75 mL), saturated NaCl (2×75 mL), dried over Na_2SO_4, and concentrated. The mixture was purified by column chromatography (SiO_2, 10% EtOAc in hexanes) to give **6** as a white solid (12.2 g, 96%). Characterization matched that previously described.[16] ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.37 (m, 1H), 4.75 (m, 1H), 3.93 (m, 1H), 352 (m, 2H), 2.56 (m, 1H), 2.39 (m, 1H), 2.21 (m, 1H), 2.15 (s, 3H), 2.08–2.00 (2H), 1.88 (m, 3H), 1.77–1.64 (5H), 1.59–1.47 (11H), 1.29–1.09 (3H), 1.04 (s, 3H), 0.66 ppm (s, 3H).

(2*R*)-2-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)but-3-en-2-ol (7a) and **(2*S*)-2-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)but-3-en-2-ol (7b)**. A solution of **6** (5.00 g, 12.5 mmol) in THF (100 mL) was cooled to −78 °C under argon. Vinyl magnesium bromide (0.7 M in THF, 44.8 mL, 31.4 mmol) was added dropwise. Upon complete addition, the mixture was stirred for 1 h at −78 °C and then warmed to RT over 12 h. The mixture was re-cooled to 0 °C and quenched with saturated aqueous NH_4Cl and washed with Et_2O (3×60 mL). The organic layers were combined and washed with saturated NaCl (1×100 mL), dried with Na_2SO_4 and concentrated. Purification by flash chromatography (SiO_2, 12% EtOAc in hexanes) afforded a 1:9 mixture of **7a**/**7b** as a white solid (5.2 g, 97%) that was used without additional separation. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.01 (dd, 1H), 5.37 (s, 1H), 5.17 (d, 1H), 4.99 (d, 1H), 4.75 (s, 1H), 3.94 (s, 1H), 3.53 (m, 2H) 2.30 (m, 2H), 2.13 (m, 1H), 1.99 (m, 1H), 1.89 (m, 3H), 1.76–1.64 (m, 5H), 1.58–1.39 (m, 14H) 1.36 (s, 3H), 1.28 (m, 1H), 1.13 (m, 2H), 1.04 (s, 3H), 0.96 (m, 1H), 0.86 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 146.14, 140.98, 121.43, 121.27, 110.22, 96.99, 96.85, 75.92, 75.73, 63.76, 62.90, 62.81, 59.48, 56.95, 56.83, 50.13, 44.03, 42.85, 40.21, 38.89, 37.47, 37.23, 36.84, 31.89, 31.55, 31.37, 29.71, 28.78, 28.01, 25.53, 24.51, 23.84, 23.22, 22.86, 20.90, 20.09, 19.40, 13.82, 13.24 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3516, 2933, 1467, 1453, 1336, 1260, 1200, 1140, 1111, 1055, 1021, 976, 911, 866 cm^{-1}; HRMS: $[M+NH_4]^+$, calcd: 445.3634, obsd: 446.3667.

(*Z*)-3-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-2-methylbut-2-enal (8a) and **(*E*)-3-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-2-methylbut-2-enal (8b)**. To a suspension of pyridinium chlorochromate (5.2 g, 24.1 mmol) and NaOAc (4.9 g, 59.7 mmol) in CH_2Cl_2 (45 mL) under argon, was added the mixture of **7a**/**7b** (5.2 g, 12.13 mmol). The mixture was stirred at RT for 12 h. The mixture was filtered through Celite, followed by copious rinsing with CH_2Cl_2 and concentrated. Purification by flash chromatography (SiO_2, 10% EtOAc in hexanes) provided an 80:20 *E*/*Z* mixture of **8a**/**8b** as a white solid (3.3 g, 64%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.10 (d, 1H), 10.00 (d, 0.2H), 6.06 (d, 0.2H), 5.95 (d, 1H), 5.39 (m, 1H), 4.92 (m, 0.2H), 4.75 (m, 1H), 4.05 (m, 0.2H), 3.95 (m, 1H) 3.53 (m, 2H), 3.25(m, 0.2H), 2.4–2.26 (m, 2H), 2.23 (s, 3H), 2.01 (m, 2H), 1.87 (m, 6H), 1.75 (m, 4H), 1.61–1.40 (m, 15H), 1.39–1.28 (m, 3H), 1.24–1.08 (m, 3H), 1.04 (s, 3H), 0.72 (s, 0.6H) 0.64 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 191.3, 190.7, 164.3, 140.9, 131.5, 127.9, 121.2, 96.9, 75.9, 62.8, 60.2, 56.8, 56.2, 51.4, 50.2, 46.3, 45.1, 40.2, 38.7, 38.6, 37.4, 37.2, 36.8, 32.2, 31.8, 31.3, 29.6, 27.9, 25.5, 24.9, 24.4, 21.0, 20.0, 19.4, 19.3, 13.9, 13.1 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 2930, 1714, 1656, 1434, 1376, 1354, 1254, 1230, 1197, 1130, 1112, 1060, 1022, 972, 910, 864 cm^{-1}; HRMS: $[M+H]^+$, calcd: 427.3112, obsd: 427.3211.

(3*R*)-3-((3*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)butanal (9). A solution of **8a**/**8b** (0.300 g, 0.706 mmol) in $CHCl_3$ (10 mL) was cooled to 0 °C. The trichloroacetic acid salt of (*S*)-(+)-2-(*tert*-butyl)-3-methylimidazolin-4-one (0.038 g, 0.14 mmol) and Hantzsch ester (0.215 g, 0.85 mmol) were added, and the mixture was stirred at 0 °C until the starting material was consumed as evidenced by continuous TLC analysis (~16 h). The solution was concentrated and directly purified by flash chromatography (SiO_2, 10% EtOAc in hexanes) to yield **9** as a white solid (0.195 g, 64%). Characterization matched that previously reported by our research group.[11]

(3*S*)-3-((3*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)butanal (10). A solution of **8a**/**8b** (0.300 g, 0.706 mmol) in $CHCl_3$ (10 mL) was cooled to 0 °C. The trichloroacetic acid salt of (*R*)-2-(*tert*-butyl)-3-methylimidazolin-4-one (0.038 g, 0.14 mmol) and Hantzsch ester (0.215 g, 0.85 mmol) were added and the mixture was stirred at 0 °C until the starting material was consumed as evidenced by continuous TLC analysis (~16 h). The solution was concentrated and directly purified by flash chromatography (SiO_2, 10% EtOAc in hexanes) to yield **10** as a white solid (0.195 g, 64%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.78 (s, 1H), 5.37 (m, 1H), 4.76 (m, 1H), 3.85 (m, 1H), 3.55 (m, 2H), 2.72 (m, 1H), 2.41–2.26 (m, 3H), 2.04 (m, 2H), 1.91 (m, 5H), 1.75 (m, 1H),

1.64 (m, 2H), 1.57 (m, 6H), 1.51 (m, 2H), 1.37 (m, 1H), 1.27- 1.12 (m, 5H), 1.05 (s, 3H), 0.97 (d, 3H), 0.76 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 203.3, 140.9, 121.4, 96.9, 75.9, 62.9, 56.6, 55.6, 49.9, 42.4, 40.2, 39.7, 38.8, 37.4, 37.2, 36.8, 31.8, 31.3, 30.6, 29.7, 27.8, 25.5, 24.0, 21.0, 20.1, 19.9, 19.4, 12.3 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 2930, 1715, 1439, 1372, 1258, 1199, 1135, 1112, 1056, 1021, 974, 912, 867 cm^{-1}; HRMS: $[M+H]^+$, calcd: 429.3368, obsd: 429.3360.

(2*S*,4*S*)-2-((3*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-6-methylheptan-4-ol (11) and **(2*S*,4*R*)-2-((3*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-6-methylheptan-4-ol (12)**. A solution of isobutyl magnesium chloride (2.0 M in THF, 0.84 mL, 1.68 mmol) was cooled to 0 °C in THF (5 mL) under argon. A solution of **9** (0.090 g, 0.210 mmol) in THF (5 mL) was added dropwise, and the mixture was stirred at 0 °C for 90 min. The reaction was quenched with saturated aqueous NH_4Cl and washed with Et_2O (2×25 mL). The organic layers were dried (Na_2SO_4) and concentrated. Purification by flash chromatography (SiO_2, 8% EtOAc in hexanes) provided two separable isomers as white solids (80% overall total yield of both isomers). Characterization of fraction 1 [**11**, THP-23(*S*)-OHC, R_f = 0.39 in 8:1 hexanes/EtOAc] and fraction 2 [**12**, THP-23(*R*)-OHC, R_f = 0.29 in 8:1 hexanes/EtOAc] were as previously described.[11]

23(*S*)-hydroxycholesterol (4). OHC **11** (0.050 g, 0.103 mmol) was dissolved in 5:1 MeOH/THF (6 mL), to which 2 N HCl (2 mL) was added dropwise. The mixture was stirred at RT for 3 h. The reaction was quenched with H_2O (5 mL) and washed with EtOAc (2× 20 mL). The organic layers were dried over Na_2SO_4, concentrated, and purified as previously described to afford **4** as a white solid (0.040 g, 97%). Characterization matched that previously published.[11] ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.37 (m, 1H), 3.79 (m, 1H), 3.54 (m, 1H), 2.27 (m, 2H), 2.02 (m, 2H), 1.85 (m, 4H), 1.61–1.43 (11H), 1.28 (m, 4H), 1.20–1.07 (4H), 1.03 (s, 3H), 0.98 (d, 3H), 0.94 (dd, 6H), 0.71 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.8, 121.7, 71.8, 68.7, 56.9, 56.7, 50.2, 46.5, 45.2, 42.4, 42.3, 39.8, 37.3, 36.5, 33.9, 31.9, 31.6, 28.5, 24.5, 24.3, 23.9, 21.7, 21.1, 19.43, 19.42, 11.9 ppm.

23(*R*)-hydroxycholesterol (13). The THP group was removed from OHC **12** as described above for the preparation of **4**. Purification by flash chromatography (SiO_2, 20% EtOAc afforded) **13** as a white solid in excellent yield (0.025 g, 94%). Characterization matched that previously published.[11] ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.37 (m, 1H), 3.80 (m, 1H), 3.54 (m, 1H), 2.28 (m, 2H), 2.04 (m, 1H), 2.00 (m, 1H), 1.86 (m, 3H), 1.77 (m, 1H), 1.67 (m, 1H), 1.61–1.39 (11H), 1.28 (m, 2H), 1.20 (m, 2H), 1.12–1.05 (4H), 1.03 (s, 3H), 0.99 (d, 3H), 0.93 (dd, 6H), 0.74 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.77, 121.67, 71.8, 67.0, 56.84, 56.78, 50.1, 48.0, 44.5, 42.4, 42.3, 39.8, 37.3, 36.5, 32.5, 31.9, 31.7, 28.5, 24.7, 24.3, 23.3, 22.3, 21.1, 19.4, 18.7, 11.9 ppm.

Preparation of 14 and 15. Grignard addition of isobutyl magnesium chloride to **10** was analogous to that described above for addition to **9**. Purification by flash chromatography (SiO_2, 8% EtOAc in hexanes) provided two separable isomers as white solids (72% overall total yield of both isomers).

(2*S*,4*S*)-2-((3*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-6-methylheptan-4-ol (14). R_f = 0.41 in 8:1 hexanes/EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.39 (m, 1H), 4.76 (m, 1H), 3.96 (m, 1H), 3.81 (m, 1H), 3.55 (m, 2H), 2.41 (m, 1H), 2.24 (m, 1H), 2.01 (m, 2H), 1.93–1.73 (7H), 1.66–1.45 (11H), 1.30 (m, 5H), 1.19–1.07 (5H), 1.05 (s, 3H), 0.97(dd, 6H), 0.90 (d, 3H), 0.73 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 141.1, 121.5, 97.0, 76.0, 68.6, 62.9, 56.5, 50.2, 46.1, 44.8, 42.4, 40.3, 39.9, 38.8, 37.5, 37.2, 36.8, 32.6, 31.9, 31.3, 29.7, 28.0, 27.5, 25.5, 24.5, 24.9, 21.7, 21.0, 20.1, 19.4, 18.9, 12.3 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3519, 2931, 1465, 1375, 1260, 1113, 1075, 1055, 1019, 909, 868 cm^{-1}; HRMS: $[M+H]^+$, calcd: 487.4151, obsd: 487.4145; $[M-OH]^+$, calcd: 469.4046, obsd: 469.4045.

(2*S*,4*R*)-2-((3*S*,10*R*,13*R*,17*R*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-6-methylheptan-4-ol (15). R_f = 0.30 in 8:1 hexanes/EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.38 (m, 1H), 4.75 (m, 1H), 3.96 (m, 1H), 3.83 (m, 1H), 3.52 (m, 2H), 2.39–2.32 (2H), 2.01 (m, 2H), 1.88 (m, 4H), 1.76 (m, 4H), 1.63–1.35 (11H), 1.29–1.07 (9H), 1.04 (s, 3H), 0.96 (d, 6H), 0.92 (d, 3H), 0.74 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.9, 121.5, 97.0, 76.01, 67.4, 62.8, 56.8, 50.2, 48.1, 44.0, 42.4, 40.1, 38.8, 37.5, 37.2, 36.8, 31.9, 31.3, 29.7, 28.1, 28.0, 25.5, 24.7, 24.2, 23.4, 22.3, 21.1, 20.1, 19.4, 18.5, 12.2 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3491, 2931, 1465, 1374, 1261, 1140, 1110, 1056, 1018, 978, 864 cm^{-1}; HRMS: $[M+H]^+$, calcd: 487.4151, obsd: 487.4082; $[M-OH]^+$, calcd: 469.4046, obsd: 469.4035.

(3*S*,10*R*,13*R*,17*R*)-17-((2*S*,4*S*)-4-hydroxy-6-methylheptan-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 (16). The THP group was removed from OHC **14** as described above for the preparation of **4**. Purification by flash chromatography (SiO_2, 20% EtOAc afforded) **16** as a white solid in excellent yield (0.035 g, 95%). ^{1}H NMR (500 MHz, $CDCl_3$: δ = 5.39 (m, 1H), 3.82 (m, 1H), 3.57 (m, 1H), 2.30 (m, 2H), 2.03 (m, 2H), 1.88 (m, 3H), 1.79 (m, 1H), 1.62 (m, 3H), 1.58–1.48 (6H), 1.34 (m, 3H), 1.30 (s, 2H), 1.24–1.09 (4H), 1.05 (s, 3H), 0.97 (dd, 6H), 0.91 (d, 3H), 0.73 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.80, 121.65, 71.81, 68.61, 56.76, 56.54, 50.17, 46.09, 44.83, 42.41, 42.32, 39.89, 37.27, 36.54, 32.57, 31.91, 31.69, 29.72, 27.46, 24.50, 24.13, 23.97, 21.69, 21.06, 19.41, 18.92, 12.30 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3247, 2928, 1463, 1435, 1373, 1140, 1112, 1056, 1019 cm^{-1}; HRMS: $[M+NH_4]^+$, calcd: 420.3842, obsd: 420.3801; $[M-OH]^+$, calcd: 385.3470, obsd: 385.3434.

(3*S*,10*R*,13*R*,17*R*)-17-((2*S*,4*R*)-4-hydroxy-6-methylheptan-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 (17). The THP group was removed from OHC **15** as described above for the preparation of **4**. Purification by flash chromatography (SiO_2, 20% EtOAc in hexanes) afforded **17** as a white solid in excellent yield (0.034 g, 96%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.40 (m, 1H), 3.84 (m, 1H), 3.56 (m, 1H), 2.30 (m, 2H), 2.05 (m, 2H), 1.88 (m, 3H), 1.76 (m, 3H), 1.64–1.32 (m, 11H), 1.24–1.12 (m, 7H), 1.05 (s, 3H), 0.96 (d, 6H), 0.93 (d, 3H), 0.76 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.8, 121.7, 71.8, 67.4, 56.9, 56.8, 50.1, 48.0, 44.0, 42.4, 42.3, 40.1, 37.3, 36.5, 31.9, 31.8, 31.7, 28.1, 24.7, 24.2, 23.4, 22.3, 21.1, 19.4, 18.5, 12.2 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3332, 2936, 1460, 1433, 1382, 1305, 1055, 952 cm^{-1}; HRMS: $[M+NH_4]^+$, calcd: 420.3842, obsd: 420.3839; $[M-OH]^+$, calcd: 385.3470, obsd: 385.3434.

3-THP-21-acetoxypregnenolone (19). THP protection of 21-acetoxypregnenolone (**18**) was carried out as described above for the preparation of **6**. Purification by flash chromatography (10% EtOAc in hexanes) afforded **19** as a white solid in excellent yield (1.16 g, 95%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.37 (m, 1H), 4.73 (m, 2H), 4.56 (d, 2H), 3.94 (m, 1H), 3.54 (m, 2H), 2.53 (m, 1H), 2.38 (m, 1H), 2.24 (m, 1H), 2.19 (s, 3H), 2.06 (m, 2H), 1.88 (m, 3H), 1.73 (m, 3H),

1.64 (m, 1H), 1.58 (m, 7H), 1.49 M, 1H), 1.42 (m, 1H), 1.31 (m, 1H), 1.21–1.07 (2H), 1.03 (s, 3H), 0.99 (m, 1H), 0.69 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 203.7, 170.2, 141.1, 121.1, 96.9, 75.9, 69.2, 62.8, 59.3, 57.1, 49.9, 44.7, 40.2, 38.6, 37.4, 37.2, 36.8, 31.9, 31.3, 29.6, 27.9, 25.5, 24.6, 22.8, 21.0, 20.5, 20.0, 19.4, 13.1 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 2936, 1745, 1722, 1417, 1370, 1229, 1198, 1112, 1074, 1056, 1029, 975, 904, 837 cm^{-1}; HRMS: $[M+H]^+$, calcd: 459.3111, obsd: 459.3186.

1-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)ethane-1,2-diol (20). A solution of **19** (1.0 g, 2.18 mmol) in anhydrous THF (20 mL) under argon was cooled to 0 °C. $LiAlH_4$ (1.0 M in THF, 8.8 mmol) was added dropwise, and the mixture was stirred for 4 h at 0 °C. The reaction was quenched using the Fieser method[18] and concentrated. Purification by flash chromatography (SiO_2, 30% EtOAc in hexanes) afforded **20** as a white solid in excellent yield (1.02 g, 96%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.36 (m, 1H), 4.74 (s, 1H), 3.94 (m, 1H), 3.67 (d, 2H), 3.51 (m, 2H), 3.38 (m, 1H), 2.38 (m, 1H), 2.22–2.11 (m, 3H), 2.00 (m, 1H), 1.87 (m, 3H), 1.74 (m, 2H), 1.66 (m, 2H), 1.55–1.47 (m, 10H), 1.27–1.11 (4H), 1.04 (s, 3H), 0.81 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 141.2, 121.3, 96.9, 75.9, 74.6, 66.5, 62.8, 55.9, 52.4, 50.2, 42.5, 40.2, 39.7, 38.8, 37.2, 36.8, 31.9, 31.8, 31.3, 29.7, 27.9, 25.5, 24.6, 20.9, 20.1, 19.4, 12.4 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3338, 2931, 1438, 1351, 1305, 1199, 1135, 1114, 1060, 1027, 972, 909, 868 cm^{-1}; HRMS: $[M+H]^+$, calcd: 419.3161, obsd: 419.3139.

(3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthrene-17-carbaldehyde (21). To a solution of **20** (0.75 g, 1.79 mmol) in 2:1 THF/H_2O (45 mL) was added $NaIO_4$ (1.2 g, 5.61 mmol), and the mixture was stirred at RT for 1 h. The mixture was extracted with EtOAc (3×50 mL), and the organic layer was dried over Na_2SO_4 and concentrated. Purification by flash chromatography (10% EtOAc in hexanes) afforded **21** as a white solid (0.64 g, 92%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.37 (s, 1H), 4.74 (s, 1H), 3.94 (m, 1H), 3.54 (m, 2H), 3.38 (m, 1H), 2.35 (m, 2H), 2.04 (m, 2H), 1.88 (m, 3H), 1.76 (m, 3H), 1.29 (m, 1H), 1.15 (m, 2H), 1.04 (s, 3H), 0.79 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 204.9, 141.1, 121.2, 96.9, 75.9, 62.84, 56.5, 50.2, 44.7, 40.2, 38.3, 37.2, 36.8, 31.8, 31.5, 29.7, 27.9, 25.5, 24.9, 21.1, 20.6, 20.1, 19.4, 13.7 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 2938, 2695, 1719, 1438, 1352, 1260, 1198, 1112, 1054, 1021, 973, 911, 866 cm^{-1}; HRMS: $[M-H]^+$, calcd: 385.2743, obsd: 385.2742; $[M+H]^+$, calcd: 387.2899, obsd: 387.2867.

Grignard addition to 21. To a suspension of acid-activated Mg turnings (0.360 g, 14.8 mmol) in anhydrous THF (5 mL) under argon at RT was added a catalytic amount of Br_2Et (3 drops) and heated at 35 °C. The suspension was stirred until bubbling of the Mg turnings was observed. A solution of bromo-4-methylpentane (1.6 mL, 10.9 mmol) in anhydrous THF (6 mL) was added dropwise over 20 min to the Mg suspension and heated at 55 °C. After stirring for 2 h, the mixture was cooled to 0 °C. A solution of **21** (0.700 g, 1.81 mmol) in anhydrous THF (6 mL) was added and stirred at 0 °C for 2 h. The reaction was quenched with saturated NH_4Cl (15 mL) and washed with Et_2O (2×25 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. Purification by flash chromatography (10% EtOAc in hexanes) afforded **22** and **23** as easily separable white solids (55% overall conversion).

(1*R*)-1-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-5-methylhexan-1-ol (22). R_f = 0.28 in 10:1 hexanes/EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.40 (s, 1H), 4.76 (s, 1H), 3.97 (m, 1H), 3.58 (m, 3H), 2.41 (m, 2H), 2.13 (m, 1H), 2.02 (m, 1H), 1.91 (m, 3H), 1.76 (m, 1H), 1.68 (m, 2H), 1.57 (m, 13H), 1.40–1.20 (m, 12H), 1.06 (s, 3H), 1.00 (m, 1H), 0.92 (d, J = 7.5 Hz, 6H), 0.82 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 141.2, 121.3, 96.8, 76.0, 74.4, 62.8, 56.7, 56.2, 50.2, 42.4, 40.3, 40.0, 39.1, 37.3, 37.2, 36.8, 31.9, 31.8, 31.3, 28.03, 25.54, 25.50, 24.6, 22.9, 22.7, 22.5, 21.0, 20.1, 19.4, 12.4 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3466, 2933, 1464, 1376, 1364, 1259, 1134, 1112, 1093, 1077, 1057, 1022, 973, 910, 867 cm^{-1}; HRMS: $[M+H]^+$, calcd: 473.3995, obsd: 473.4140. $[M-OH]^+$, calcd: 455.3889, obsd: 455.3848.

(1*S*)-1-((3*S*,10*R*,13*S*,17*S*)-10,13-dimethyl-3-((tetrahydro-2*H*-pyran-2-yl)oxy)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-5-methylhexan-1-ol (23). R_f = 0.23 in 10:1 hexanes/EtOAc. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.39 (s, 1H), 4.76 (s, 1H), 3.96 (m, 1H), 3.58 (m, 3H), 2.38–2.24 (2H), 2.13 (m, 1H), 2.02 (m, 1H), 1.91 (m, 5H), 1.75–1.19 (30H), 1.05 (s, 3H), 0.92 (d, 6H), 0.74 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.9, 121.5, 97.0, 76.0, 73.2, 62.9, 56.6, 56.3, 50.3, 41.7, 40.3, 39.1, 38.8, 37.5, 37.2, 36.9, 31.9, 31.6, 31.3, 29.7, 28.1, 25.5, 24.8, 24.2, 23.2, 22.7, 22.6, 20.8, 20.1, 19.4, 12.7 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3500, 2923, 1460, 1384, 1353, 1260, 1199, 1109, 1053, 1020, 973, 911, 864 cm^{-1}; HRMS: $[M+H]^+$, calcd: 473.3995, obsd: 473.4028; $[M-OH]^+$, calcd: 455.3889, obsd: 455.3873.

(3*S*,10*R*,13*S*,17*S*)-17-((*R*)-1-hydroxy-5-methylhexyl)-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 (24). The THP group was removed from OHC **22** as described above for the preparation of **4**. Purification by flash chromatography (SiO_2, 20% EtOAc in hexanes) afforded **24** as a white solid in excellent yield (0.063 g, 96%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.40 (s, 1H), 3.60 (m, 2H), 2.30 (m, 2H), 2.15 (m, 1H), 2.02 (m, 1H), 1.89 (m, 2H), 1.69 (m, 2H), 1.56 (m, 8H), 1.43 (m, 2H), 1.30–1.14 (12H), 1.07 (s, 3H), 0.92 (d, 6H), 0.82 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.9, 121.6, 74.4, 71.8, 56.7, 56.2, 50.2, 42.4, 42.3, 40.0, 39.1, 37.3, 37.2, 36.6, 31.9, 31.8, 31.7, 28.0, 25.5, 24.6, 22.9, 22.7, 22.5, 21.0, 19.4, 12.4 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3298, 2925, 1729, 1463, 1373, 1351, 1260, 1056, 1023, 954, 912 cm^{-1}; HRMS: $[M+NH_4]^+$, calcd: 406.3685, obsd: 406.3661; $[M-OH]^+$, calcd: 371.3314, obsd: 371.3302.

(3*S*,10*R*,13*S*,17*S*)-17-((*S*)-1-hydroxy-5-methylhexyl)-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 (25). The THP group was removed from OHC **23** as described above for the preparation of **4**. Purification by flash chromatography (SiO_2, 20% EtOAc in hexanes) afforded **25** as a white solid in excellent yield (0.031 g, 95%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.40 (s, 1H), 3.60 (m, 2H), 2.31 (m, 2H), 2.04 (m, 1H), 1.90 (m, 4H), 1.68 (m, 2H), 1.56 (m, 9H), 1.42 (m, 4H), 1.30–1.12 (8H), 1.06 (s, 3H), 0.92 (d, 6H), 0.75 ppm (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 140.8, 121.6, 73.2, 71.8, 56.6, 56.2, 50.2, 42.3, 41.7, 39.1, 38.8, 37.3, 37.2, 36.6, 31.9, 31.7, 31.6, 28.1, 24.8, 24.2, 23.2, 22.7, 22.6, 20.8, 19.4, 12.8 ppm; IR (ATR FTIR): $\tilde{\nu}$ = 3290, 2926, 1715, 14622, 1378, 1365, 1320, 1238, 1058, 1028, 953, 936 cm^{-1}; HRMS: $[M+NH_4]^+$, calcd: 406.3685, obsd: 406.3658; $[M-OH]^+$, calcd: 371.3314, obsd: 371.3300.

Biological assays

Cell culture and reagents: The murine cell line, M2-10B4, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). M2-10B4 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained using the media described above (denoted "growth" media). Media denoted as "low

FBS" contained 0.5% FBS and the same percentage of other supplements as specified for growth media. Cells were grown in Corning Cell Culture, canted neck T75 or T150 flasks (Fisher Scientific) in an Autoflow IR water-jacket CO_2 incubator. Experiments were performed in BD Falcon sterile 24-well tissue culture treated plates. Dimethyl sulfoxide (DMSO) was used as solvent to prepare all drug solutions, and the final DMSO concentration did not exceed 0.3%. 20(*S*)-Hydroxycholesterol was purchased from Sigma–Aldrich. Cyclopamine was purchased from LC Labs.

General procedure for analysis of Hh target gene regulation: Cells were grown to confluence in T75 flasks. Once cells reached confluence, they were plated into 24-well plates at concentration of 50000 cells in 500 μL growth media per well. After 24 h, the growth media was removed and replaced with low FBS media (500 μL per well). Cells were treated with corresponding compound: DMSO, OHC, or OHC and cyclopamine. Cells were incubated (37 °C, 5% CO_2) for the indicated time period, at which time mRNA was isolated and evaluated as described previously.[11]

General procedure for RT-PCR analysis: Following treatment and incubation, total RNA was extracted using Ambion® by Life Technologies Taqman® Fast Cells-to-CT™ kit following the manufacturer's instructions. Synthesis of cDNA was performed using a BioRad MyCycler. Quantitative PCR was performed on an ABI 7500 system using the following Taqman Gene Expression Primer/Probe solutions (ABI): mouse ActB (Mm00607939s1), mouse GLI1 (Mm00494645m1), mouse PTCH1 (Mm00436926m1), mouse ABCA1 (Mm00442646m1), mouse Alp1 (Mm00475834m1), mouse sp7 (Mm04209856m1). Relative gene expression levels were computed by the ΔΔCt method. Values represent mRNA expression relative to DMSO control (vehicle, set at 1.00). Data were analyzed using GraphPad Prism 5, and EC_{50} values are the mean ± SEM for at least two separate experiments performed in triplicate.

Acknowledgements

The authors thank Victor W. Day (University of Kansas, USA) for crystallography work, as well as the US National Science Foundation (NSF) Major Research Instrumentation (MRI) Program (CHE-0923449) for financial support of the X-ray diffractometer and software used in this study.

Keywords: Hedgehog · oxysterols · signal transduction · stereochemistry · structure–activity relationships

[1] A. P. McMahon, P. W. Ingham, C. J. Tabin, *Curr. Top. Dev. Biol.* **2003**, *53*, 1 – 114.
[2] T. Dellovade, J. T. Romer, T. Curran, L. L. Rubin, *Annu. Rev. Neurosci.* **2006**, *29*, 539 – 563.
[3] P. V. Giannoudis, H. Dinopoulos, E. Tsirisdis, *Injury* **2005**, *36*, S20 – S27.
[4] R. B. Corcoran, M. P. Scott, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 8408 – 8413.
[5] J. R. Dwyer, N. Sever, M. Carlson, S. F. Nelson, P. A. Beachy, F. Parhami, *J. Biol. Chem.* **2007**, *282*, 8959 – 8968.
[6] H. T. Kha, B. Basseri, D. Shouhed, J. Richardson, S. Tetradis, T. J. Hahn, F. Parhami, *J. Bone Miner. Res.* **2004**, *19*, 830 – 840.
[7] T. L. Aghaloo, C. M. Amantea, C. M. Cowanm, J. A. Richardson, B. M. Wu, F. Parhami, S. Tetradis, *J. Orthop. Res.* **2007**, *25*, 1488 – 1497.
[8] V. A. Olkkonen, O. Beaslas, E. Nissila, *Biomolecules* **2012**, *2*, 76 – 103.
[9] S. Nachtergaele, L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, F. D. Covey, R. Rohatgi, *Nat. Chem. Biol.* **2012**, *8*, 211 – 220.
[10] D. Nedelcu, J. Liu, Y. Xu, C. Jao, A. Salic, *Nat. Chem. Biol.* **2013**, *9*, 557 – 564.
[11] A. Corman, A. M. DeBerardinis, M. K. Hadden, *ACS Med. Chem. Lett.* **2012**, *3*, 828 – 833.
[12] M. K. Hadden, *ChemMedChem* **2014**, *9*, 27 – 37.
[13] W. K. Kim, V. Meliton, K. W. Park, C. H. Hong, P. Tontonoz, P. Niewiadomski, J. A. Waschek, S. Tetradis, F. Parhami, *Mol. Endocrinol.* **2009**, *23*, 1532 – 1543.
[14] J. Drew, M. Letellier, P. Morand, A. G. Szabo, *J. Org. Chem.* **1987**, *52*, 4047 – 4052.
[15] S. G. Ouellet, J. B. Tuttle, D. W. C. MacMillan, *J. Am. Chem. Soc.* **2005**, *127*, 32 – 33.
[16] R. Katoch, S. S. Korde, K. D. Deodhar, G. K. Trivedi, *Tetrahedron* **1999**, *55*, 1741 – 1754.
[17] E. Fernández-Mateous, B. Maciá, M. Yus, *Adv. Synth. Catal.* **2013**, *355*, 1249 – 1254.
[18] L. F. Fieser, M. Fieser, *Reagents for Organic Synthesis Vol. 1*, Wiley, New York, **1967**, p. 564.

Received: November 23, 2015
Revised: February 12, 2016
Published online on February 25, 2016