A proteome-wide map of 20(*S*)-hydroxycholesterol interactors in cell membranes

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Supplementary information

Supplementary Table 1. GSEA analysis of **2**-enriched proteins using the oncogenic and immunologic signature gene set collections.

NAME	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
C6 - Oncogenic gene sets								
TBK1.DF_UP	28	0.34581167	2.126574	0.00828157	0.07074088	0.168	399	tags=57%, list=23%, signal=73%
MTOR_UP.V1_DN	16	0.43844807	2.140364	0	0.09935021	0.157	545	tags=75%, list=32%, signal=108%
SNF5_DN.V1_DN	8	0.554007	1.942313	0.00596422	0.13944581	0.449	775	tags=100%, list=45%, signal=180%
MEL18_DN.V1_UP	20	0.37163743	1.9779348	0.00392927	0.1401781	0.386	661	tags=75%, list=38%, signal=120%
ESC_J1_UP_LATE.V1_UP	23	0.3325692	1.9000235	0.004	0.14836091	0.532	710	tags=74%, list=41%, signal=124%
KRAS.600.LUNG.BREAST_UP.V1_DN	10	0.5761628	2.164868	0.00194553	0.1702008	0.135	565	tags=90%, list=33%, signal=133%
C7 - Immunologic gene sets								
GSE23505_IL6_IL1_VS_IL6_IL1_IL23_TREATED_ CD4_TCELL_DN	28	0.4710425	2.9283345	0	0.00928697	0.01	433	tags=71%, list=25%, signal=94%
GSE22140_GERMFREE_VS_SPF_ARTHRITIC_ MOUSE_CD4_TCELL_UP	38	0.34300736	2.5023267	0	0.1808601	0.32	921	tags=87%, list=53%, signal=182%

Supplementary Table 2. Oligonucleotide sequences.

Primers used for site-directed mutagenesis of Tmem97-Myc-FLAG			
Construct	**Plasmid template**	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**
Tmem97 (Y150A)	pCMV6-Tmem97-Myc-DDK	GTGTCTACGCCCCCgcTTTAATAATCCCCC	GGGGGATTATTAAAgcGGGGGCGTAGACAC
Tmem97 (F88A)	pCMV6-Tmem97-Myc-DDK	GCGGCATATGCCgcCTTCAAAGGAAGCTG	CAGCTTCCTTTGAAGgcGGCATATGCCGC
Tmem97 (E61A)	pCMV6-Tmem97-Myc-DDK	GACCCTCTGATGCAGGcGCCCCCAGTGTG	CACACTGGGGGCgCCTGCATCAGAGGGTC
Tmem97 (W65A)	pCMV6-Tmem97-Myc-DDK	CCCAGTGgcGTTCAAGTCCTTCCTGCTCTGTGAGC	ACTTGAACgcCACTGGGGGCTCCTGCATCAGAGGG
Tmem97 (W95A)	pCMV6-Tmem97-Myc-DDK	CTGCCGGgcGATCCGAATCCCTGCAATCATCTATG	TTCGGATCgcCCGGCAGCTTCCTTTGAAGAAGGC
Tmem97 (Y150F)	pCMV6-Tmem97-Myc-DDK	GCCCCCTtTTTAATAATCCCCCTCATACTCCTC	TTATTAAAaAGGGGGCGTAGACACCTACGAGGG
Tmem97 (Y150S)	pCMV6-Tmem97-Myc-DDK	GCCCCCTcTTTAATAATCCCCCTCATACTCCTCC	TTATTAAAgAGGGGGCGTAGACACCTACGAGGGTC

sgRNA guides for CRISPR/Cas9 knockout of Tmem97 in NIH-3T3 cells			
Name	**sgRNA sequence (5' to 3')**	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**
gTmem97	TCGTAGCCCAACGGACAGGA	caccgTCGTAGCCCAACGGACAGGA	aaacTCCTGTCCGTTGGGCTACGAc
gNT (non-targeted)	GATCGCGATCGAAGTACGTA	caccGATCGCGATCGAAGTACGTA	aaacTACGTACTTCGATCGCGATC

Primers used for TIDE analysis			
Name	**Product size (bp)**	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**
Tide_gTmem97	810	TCTCGGCTTACACTGGACTT	GCGAATAAAACACTCGTGGC

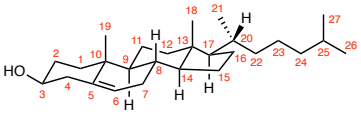
Supplementary Note 1. Chemical synthesis and characterization data.

General procedures
All reactions were performed in oven-dried or flame-dried round-bottomed flasks. The flasks were fitted with rubber septa and reactions were conducted under a positive pressure of nitrogen unless otherwise indicated. Stainless steel syringes or cannulae were used to transfer air- and moisture-sensitive liquids. Flash column chromatography was performed either as described by Still et al. using pre-packaged RediSep Rf columns on a CombiFlash Rf system (Teledyne ISCO Inc., Lincoln NE). Analytical thin-layer chromatography was performed using glass plates pre-coated with 0.25 mm 230-400 mesh silica gel impregnated with a fluorescent indicator (254 nm) and were visualized by UV, *p*-anisaldehyde, $KMnO_4$, CAM (ceric ammonium molybdate) or PMA (phosphomolybdic acid) staining.

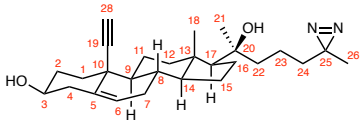
Materials
Tetrahydrofuran (THF), methylene chloride (CH_2Cl_2), toluene, and dimethylformamide (DMF) were dried by passing through activated alumina columns in a solvent purification system (Pure Process Technologies, Inc., Nashua, NH). Commercial reagents and solvents were used as received unless otherwise indicated.

Instrumentation
^1H and ^{13}C NMR spectra were recorded on a Bruker 400 with Prodigy broadband cryoprobe (at 400 MHz and 100 MHz, respectively) or a Varian Inova 500 (at 500 MHz and 125 MHz, respectively), and are reported relative to internal chloroform (^1H, δ = 7.26 ppm, ^{13}C, δ = 77.0 ppm). NMR assignments are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration, assignment). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sept = septuplet, m = multiplet, br s = broad singlet, br d = broad doublet.

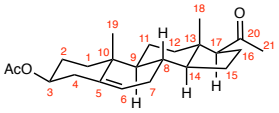
Compound numbering



20(*S*)-hydroxycholesterol (20(*S*)-OHC, **1**)

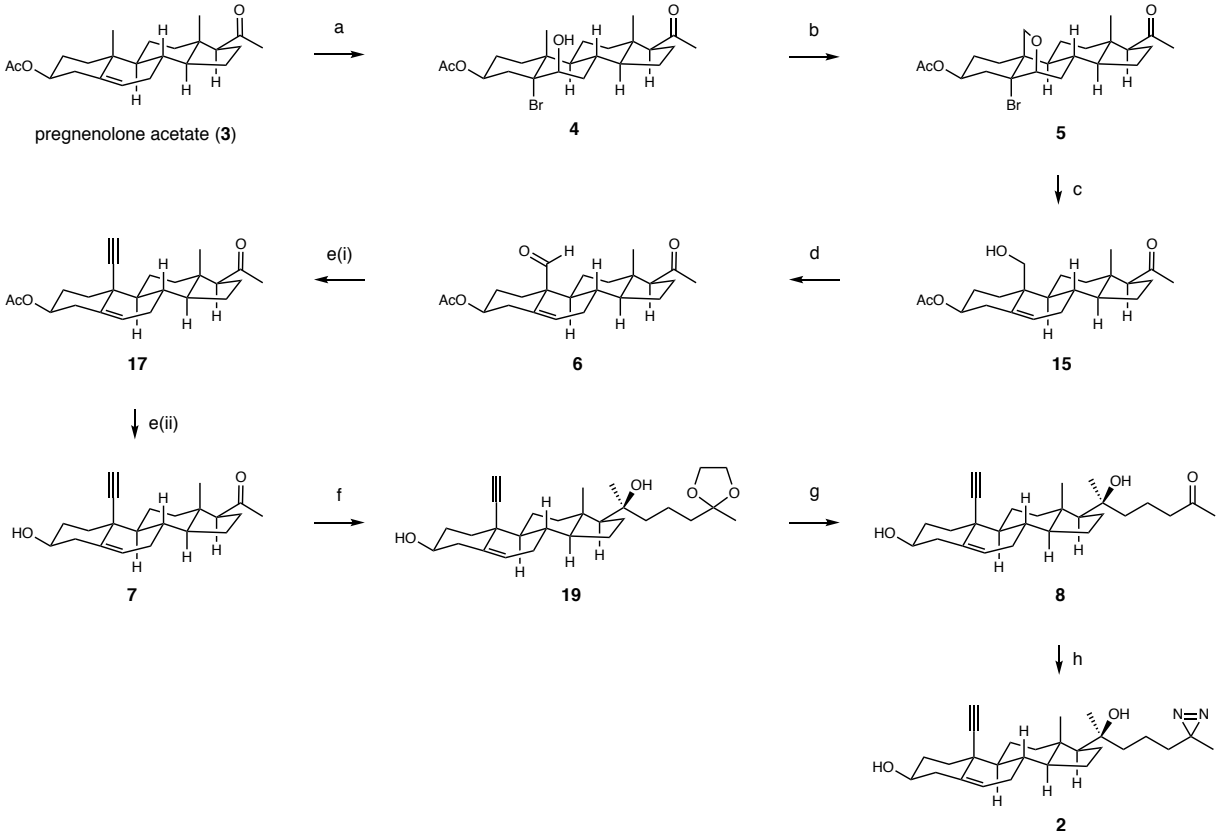


20(*S*)-OHC probe (**2**)

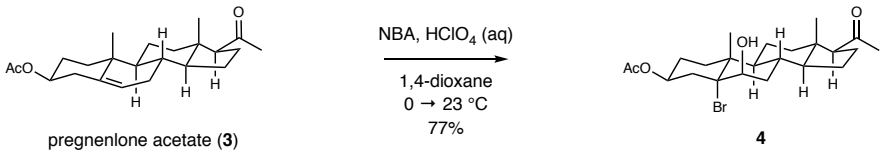


pregnenolone acetate (**3**)

Synthetic scheme for probe 2



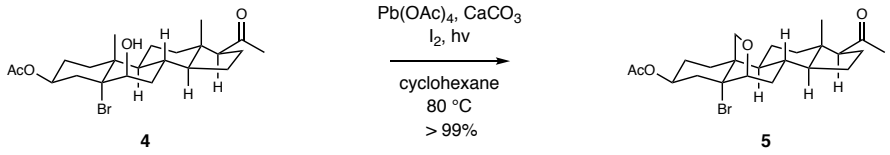
a. NBA, $HClO_4$ (aq), 1,4-dioxane, 0 → 23 °C, 91%;
b. $Pb(OAc)_4$, $CaCO_3$, I_2, h*v*, cyclohexane, 80 °C, > 99%;
c. Zn, AcOH-H_2O, 45 °C, 88%;
d. PCC, Celite, CH_2Cl_2, 23 °C, 87%;
e. (i) Seyferth-Gilbert reagent (**16**), t-BuOK, THF, –78 °C, 80%; (ii) Cs_2CO_3, MeOH, 23 °C, >99%;
f. 2-(3-bromopropyl)-2-methyl-1,3-dioxolane (**18**), Mg, THF, 0 → 23 °C, 61%;
g. HCl, THF, 23 °C, 94%;
h. (i) NH_3, MeOH, 0 °C; (ii) NH_2HSO_3, 0 → 23 °C; (iii) I_2, Et_3N, THF, 23 °C, 46%.



3β-Acetoxy-5-bromo-6β-hydroxy-5α-pregnan-20-one (4)

To a solution of pregnenolone acetate (**3**) (7.39 g, 20.6 mmol, 1.0 equiv.) in 117 mL 1,4-dioxane cooled using an ice-water bath was added 4.33 mL 10% perchloric acid (0.62 mL 70% perchloric acid and 3.71 mL water) dropwise via syringe. *N*-bromoacetamide (4.49 g, 31.5 mmol, 1.5 equiv) was then added in six portions over 10 min and the mixture was stirred for 30 min at the same temperature. The mixture was allowed to warm to room temperature and stirred for an additional 20 min. The reaction flask was cooled in ice-water bath again before quenching with 100 mL sodium thiosulfate solution (9:1 water: saturated $Na_2S_2O_3$ (aq)) and diluted with 100 mL Et_2O. The aqueous layer was separated and extracted with Et_2O (2 × 100 mL). The combined organic layers were washed with a mixture of brine, saturated $NaHCO_3$ (aq) and saturated $Na_2S_2O_3$(aq) (8:1:1), dried over Na_2SO_4, filtered, and concentrated in vacuo to give **4** as a yellow solid (10.4 g, 23 mmol, 91% yield, 82% purity). The crude can be further purified by recrystallization (EtOAc/hexanes).

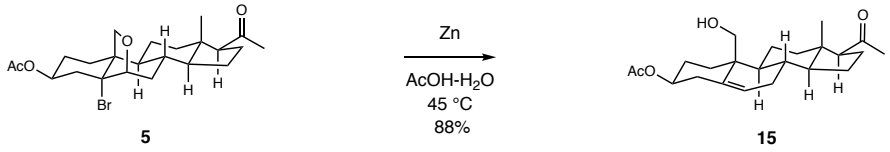
^{1}H NMR (500 MHz, $CDCl_3$):	5.48 (tt, *J* = 11.0, 5.6 Hz, 1H, C_3**H**), 4.19 (dt, *J* = 4.2, 2.4 Hz 1H, C_6**H**), 2.57 (t, *J* = 8.9 Hz, 1H, C_{17}**H**), 2.51 (dd, *J* = 13.5, 10.5 Hz, 1H, C_4**H**$_a$), 2.29 (ddd, *J* = 14.5, 12.5, 3.6 Hz, 1H, C_1**H**$_a$), 2.19 (ddd, *J* = 13.7, 5.4, 1.7 Hz, 1H, C_4**H**$_b$), 2.20 – 2.10 (m, 1H, C_{16}**H**$_a$), 2.13 (s, 3H, C_{21}**H**), 2.05–2.01 (m, 1H, C_{12}**H**$_a$), 2.05 (s, 3H, OC(O)C**H**$_3$), 2.01–1.95 (m, 1H, C_2**H**$_a$), 1.85–1.75 (m, 2H, C_8**H**, C_7**H**$_a$), 1.74–1.53 (m, 8H, C_1**H**$_b$, C_{15}**H**$_a$, C_{16}**H**$_b$, C_9**H**, C_2**H**$_b$, C_7**H**$_b$, C_{11}**H**$_a$), 1.50 (td, *J* = 12.4, 4.1 Hz, 1H, C_{12}**H**$_b$), 1.39–1.30 (m, 2H, C_{11}**H**$_b$, C_{14}**H**), 1.33 (s, 3H, C_{19}**H**), 1.24 (ddd, *J* = 18.2, 11.7, 5.8 Hz, 1H, C_{15}**H**$_b$), 0.64 (s, 3H, C_{18}**H**)
^{13}C NMR (100 MHz, $CDCl_3$):	209.68 (**C**$_{20}$), 170.60 (O**C**(O)CH_3), 86.67 (**C**$_5$), 75.70 (**C**$_6$), 72.15 (**C**$_3$), 63.73 (**C**$_{17}$), 56.08 (**C**$_{14}$), 47.50 (**C**$_9$), 44.41 (**C**$_{13}$), 40.53 (**C**$_{10}$), 38.86 (**C**$_{12}$), 38.52 (**C**$_4$), 35.25 (**C**$_7$), 34.65 (**C**$_1$), 31.70 (**C**$_{21}$), 30.78 (**C**$_8$), 26.46 (**C**$_2$), 24.40 (**C**$_{15}$), 22.96 (**C**$_{16}$), 21.51 (**C**$_{11}$), 21.45 (OC(O)**C**H_3), 18.10 (**C**$_{19}$), 13.72 (**C**$_{18}$)
TLC (SiO_2, 35% EtOAc–hexanes), R_f :	0.37 (CAM, *p*-anisaldehyde, UV)



3β-Acetoxy-5α-bromo-6β,19-epoxypregnan-20-one (5)

An oven-dried 500 mL round-bottom flask was charged with lead(IV) acetate (3.99 g, 8.54 mmol, 3.9 equiv), $CaCO_3$ (2.78 g, 27.5 mmol, 12.6 equiv), and 143 mL cyclohexane. After the white suspension was heated to 80 °C in an oil bath, iodine (1.12 g, 4.41 mmol, 2.0 equiv) and bromohydrin **4** (990 mg, 2.17 mmol, 1.0 equiv) were added and the mixture was stirred vigorously for an additional 5 min. The oil bath was removed and the uncapped reaction mixture was exposed to a 500 W long-wavelength UV lamp for 1.5 h with vigorous stirring. The mixture was then diluted with Et_2O, filtered, washed with 10% sodium thiosulfate, water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give a white solid (1.1 g, 2.4 mmol, >99% yield). The crude product was submitted to the next reaction without further purification. A small aliquot of sample was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) for characterization purposes.

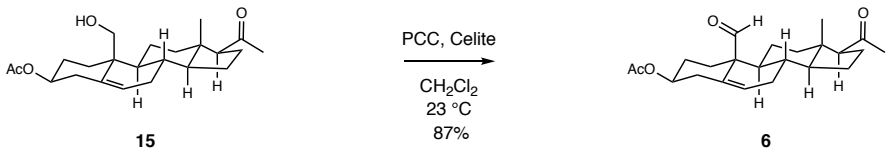
^{1}H NMR (400 MHz, $CDCl_3$):	5.20 (dddd, J = 7.0, 6.0, 4.0, 3.0 Hz, 1H, C_3**H**), 4.07 (d, J = 4.6 Hz, 1H, C_6**H**), 3.94 (d, J = 8.4 Hz, 1H, C_{19}**H**$_a$), 3.73 (d, J = 8.4 Hz, 1H, C_{19}**H**$_b$), 2.55 (t, J = 8.9 Hz, 1H, C_{17}**H**), 2.34 (ddd, J = 13.9, 4.9, 2.0 Hz, 1H, C_4**H**$_a$), 2.27 (dd, J = 14.0, 11.1 Hz, 1H, C_4**H**$_b$), 2.21–2.08 (m, 2H, C_1**H**$_a$, C_{16}**H**$_a$), 2.11 (s, 3H, C_{21}**H**), 2.07–1.97 (m, 2H, C_{12}**H**$_a$, C_2**H**$_a$), 2.03 (s, 3H, OC(O)C**H**$_3$), 1.76–1.60 (m, 7H, C_1**H**$_b$, C_2**H**$_b$, C_7**H**$_a$, C_{15}**H**$_a$, C_{16}**H**$_b$, C_8**H**, C_9**H**), 1.61–1.45 (m, 3H, C_7**H**$_b$, C_{12}**H**$_b$, C_{11}**H**$_a$), 1.38 (ddd, J = 12.4, 10.6, 6.5 Hz, 1H, C_{11}**H**$_b$), 1.29–1.14 (m, 2H, C_{15}**H**$_b$, C_{14}**H**), 0.65 (s, 3H, C_{18}**H**)
^{13}C NMR (100 MHz, $CDCl_3$):	209.53 (**C**$_{20}$), 170.51 (O**C**(O)CH$_3$), 82.29 (**C**$_6$), 74.54 (**C**$_5$), 70.00 (**C**$_{19}$), 67.63 (**C**$_3$), 63.52 (**C**$_{17}$), 54.73 (**C**$_{14}$), 48.74 (**C**$_{10}$), 46.00 (**C**$_9$), 44.76 (**C**$_{13}$), 41.41 (**C**$_{12}$), 38.88 (**C**$_4$), 33.44 (**C**$_8$), 32.87 (**C**$_7$), 31.61 (**C**$_{21}$), 26.98 (**C**$_1$), 23.83 (**C**$_2$), 23.44 (**C**$_{15}$), 23.03 (**C**$_{11}$), 22.74 (**C**$_{16}$), 21.47 (OC(O)**C**H$_3$), 13.95 (**C**$_{18}$)
TLC (SiO_2, 30% EtOAc–hexanes), R_f :	0.26 (CAM, *p*-anisaldehyde)



3β-Acetoxy-19-hydroxy-5-pregnen-20-one (15)

To a solution of bromoether **5** (1.60 g, 2.82 mmol, 1.0 equiv) in 26 mL AcOH and 1.2 mL H_2O in a 100 mL round-bottom flask was added Zn (1.38 g, 24.5 mmol, 8.7 equiv) in one portion. The flask was sealed with glass stopper and the grey suspension was stirred vigorously at 45 °C for 2 h until no bromoether **5** was detected by TLC. The mixture was allowed to cool to room temperature, filtered, and concentrated on a rotary evaporator equipped with a KOH trap. The residue was then suspended in 50 mL EtOAc and 50 mL water, and saturated $NaHCO_3$ (aq) was added dropwise until the pH of the solution was approximately 7. The aqueous layer was separated and extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (CombiFlash, 0–100% EtOAc/hexanes) to yield **15** as a white solid (814 mg, 2.47 mmol, 88% yield).

^{1}H NMR (400 MHz, $CDCl_3$):	5.79–5.73 (m, 1H, C_6**H**), 4.63 (tt, *J* = 11.5, 4.8 Hz, 1H, C_3**H**), 3.84 (d, *J* = 11.5 Hz, 1H, C_{19}**H**$_a$), 3.60 (d, *J* = 11.5 Hz, 1H, C_{19}**H**$_b$), 2.51 (t, *J* = 8.9 Hz, 1H, C_{17}**H**), 2.42 (ddd, *J* = 13.0, 5.0, 2.3 Hz, 1H, C_4**H**$_a$), 2.26 (tdt, *J* = 11.6, 4.9, 2.3 Hz, 1H, C_4**H**$_b$), 2.21–2.10 (m, 2H, C_1**H**$_a$, C_{16}**H**$_a$), 2.11 (s, 3H, C_{21}**H**), 2.10–2.03 (m, 1H, C_2**H**$_a$), 2.02 (s, 3H, OC(O)C**H**$_3$), 1.96 (dt, *J* = 13.9, 3.7 Hz, 1H, C_{12}**H**$_a$), 1.92–1.81 (m, 2H, C_2**H**$_b$, C_7**H**$_a$), 1.73–1.38 (m, 7H, C_7**H**$_b$, C_{12}**H**$_b$, C_{11}**H**, C_{15}**H**$_a$, C_{16}**H**$_b$, C_8**H**), 1.23 (tdd, *J* = 11.2, 5.0, 1.8 Hz, 1H, C_{15}**H**$_b$), 1.16 (dd, *J* = 14.1, 4.2 Hz, 1H, C_{14}**H**), 1.12–1.01 (m, 1H, C_1**H**$_b$), 1.01–0.91 (m, 1H, C_9**H**), 0.67 (s, 3H, C_{18}**H**)
^{13}C NMR (100 MHz, $CDCl_3$):	209.56 (**C**$_{20}$), 170.53 (O**C**(O)CH_3), 134.53 (**C**$_5$), 128.00 (**C**$_6$), 73.29 (**C**$_3$), 63.61 (**C**$_{17}$), 62.70 (**C**$_{19}$), 57.69 (**C**$_{14}$), 50.14 (**C**$_9$), 44.18 (**C**$_{13}$), 41.58 (**C**$_{10}$), 39.04 (**C**$_{12}$), 38.14 (**C**$_4$), 33.27 (**C**$_1$), 33.16 (**C**$_8$), 31.52 (**C**$_7$), 31.09 (**C**$_{21}$), 28.05 (**C**$_2$), 24.28 (**C**$_{15}$), 22.79 (**C**$_{16}$), 21.69 (**C**$_{11}$), 21.40 (OC(O)**C**H_3), 13.58 (**C**$_{18}$)
TLC (SiO_2, 40% EtOAc–hexanes), R_f :	0.30 (CAM, *p*-anisaldehyde, $KMnO_4$)

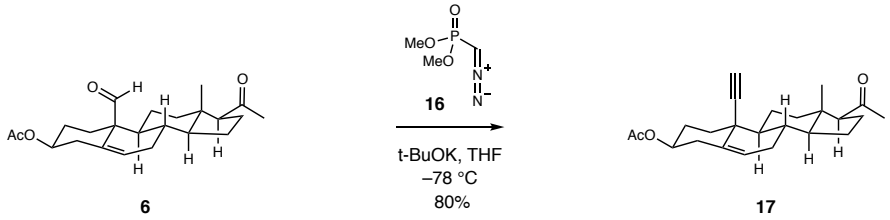


15 **6**

3β-Acetoxy-10-formyl-5-pregnen-20-one (6)

An oven-dried 50 mL round-bottom flask was charged with alcohol **15** (300 mg, 0.8 mmol, 1.0 equiv), Celite (260 mg), and 9 mL dry CH_2Cl_2. Pyridinium chlorochromate (PCC) (260 mg, 1.2 mmol, 1.5 equiv) was added in portions over 5 min and the mixture was stirred at room temperature for 3 h until no alcohol **11** was detected by TLC. The mixture was then diluted with 10 mL Et_2O, sonicated briefly, and filtered through a pad of Celite. The filter cake was washed with Et_2O (3 × 10 mL). The combined filtrates were dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (CombiFlash, 0–100% EtOAc/hexanes) to yield **6** as an off-white solid (260 mg, 0.7 mmol, 87% yield).

^{1}H NMR (400 MHz, $CDCl_3$):	9.65 (d, J = 1.4 Hz, 1H, C_{19}**H**), 5.87 (dt, J = 5.8, 1.8 Hz, 1H, C_6**H**), 4.59 (tt, J = 11.6, 4.5 Hz, 1H, C_3**H**), 2.57 (ddd, J = 13.7, 4.2, 3.1 Hz, 1H, C_4**H**$_a$), 2.51 (t, J = 8.9 Hz, 1H, C_{17}**H**), 2.49 (ddd, J = 13.0, 4.6, 2.6 Hz, 1H, C_4**H**$_b$), 2.26–2.12 (m, 2H, C_{16}**H**$_a$, C_1**H**$_a$), 2.09 (s, 3H, C_{21}**H**), 2.05 (d, J = 2.9 Hz, 1H, C_2**H**$_a$), 2.00 (s, 3H, OC(O)C**H**$_3$), 1.98–1.90 (m, 1H, C_{12}**H**$_a$), 1.87–1.75 (m, 2H, C_2**H**$_b$, C_7**H**$_a$), 1.73–1.62 (m, 5H, C_7**H**$_b$, C_{15}**H**$_a$, C_{16}**H**$_b$, C_{11}**H**$_a$, C_8**H**), 1.57 (dddd, J = 14.0, 12.8, 11.4, 4.1 Hz, 1H, C_{12}**H**$_b$), 1.40 (td, J = 12.5, 4.0 Hz, 1H, C_{11}**H**$_b$), 1.32–1.17 (m, 2H, C_{15}**H**$_b$, C_{14}**H**), 1.17–1.04 (m, 2H, C_1**H**$_b$, C_9**H**), 0.57 (s, 3H, C_{18}**H**)
TLC (SiO_2, 30% EtOAc–hexanes), R_f:	0.34 (*p*-anisaldehyde)



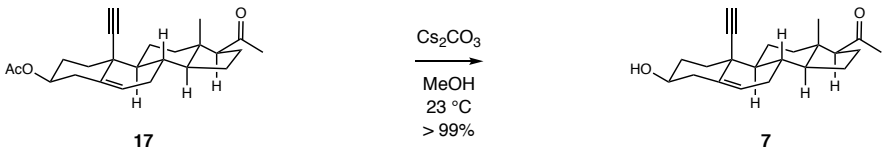
6 17

3β-Acetoxy-19-ethynyl-5-pregnen-20-one (17)

An oven-dried 25 mL round-bottom flask was charged with potassium *tert*-butoxide (179 mg, 1.59 mmol, 9.9 equiv) and 3 mL dry THF. After the suspension was cooled to –78 °C in a dry ice-acetone bath, a solution of dimethyl (1-diazo-2-oxopropyl)phosphonate (Gilbert-Seyferth reagent, **16**) (218 mg, 1.45 mmol, 9.0 equiv) in 2 mL of THF was added dropwise via syringe over a period of 10 min. The mixture was stirred for 5 min, and a solution of aldehyde **6** (60 mg, 0.161 mmol, 1.0 equiv) in 2 mL THF was added via syringe over a period of 10 min. The resulting solution was stirred at –78 °C for 24 h before quenching with water. The mixture was extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to yield **17** as an off-white solid (47.4 mg, 0.13 mmol, 80% yield).

^{1}H NMR (400 MHz, $CDCl_3$): 5.52 (d, J = 5.5 Hz, 1H, C_6**H**), 4.63 (tt, J = 10.9, 5.0 Hz, 1H, C_3**H**), 2.57 (t, J = 8.9 Hz, 1H, C_{17}**H**), 2.55–2.42 (m, 2H, C_4**H**), 2.30 (s, 1H, C_{28}**H**), 2.26–2.15 (m, 2H, C_{16}**H**$_a$, C_1**H**$_a$), 2.16 (s, 3H, C_{21}**H**), 2.14–2.07 (m, 2H, C_2**H**$_a$, C_{12}**H**$_a$), 2.07 (s, 3H, OC(O)C**H**$_3$), 2.02–1.84 (m, 3H, C_2**H**$_b$, C_{11}**H**$_a$, C_7**H**$_a$), 1.82–1.58 (m, 5H, C_{11}**H**$_b$, C_7**H**$_b$, C_8**H**, C_{15}**H**$_a$, C_{16}**H**$_b$), 1.51 (td, J = 12.9, 4.5 Hz, 1H, C_{12}**H**$_b$), 1.34–1.14 (m, 3H, C_{15}**H**$_b$, C_{14}**H**, C_1**H**$_b$), 1.08 (td, J = 11.6, 5.1 Hz, 1H, C_9**H**), 0.70 (s, 3H, C_{18}**H**)

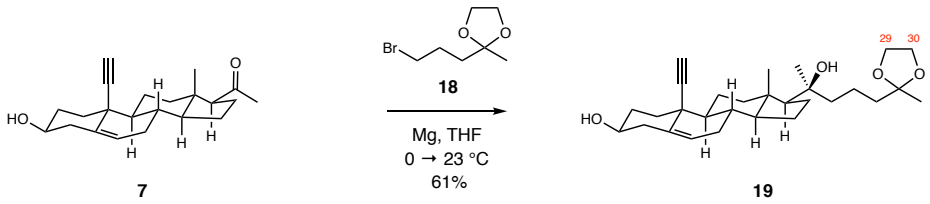
TLC (SiO_2, 50% EtOAc–hexanes), R_f: 0.57 (CAM)



19-Ethynyl-3β-hydroxy-5-pregnen-20-one (7)

To a solution of acetate **17** (21.7 mg, 0.059 mmol, 1.0 equiv) in 1.6 mL dry MeOH and 0.22 mL dry THF was added anhydrous Cs_2CO_3 (79 mg, 0.241 mmol, 4.0 equiv) under Ar. The mixture was stirred for 2 h at room temperature and 1.5 mL water was added. The mixture was extracted with EtOAc (3 × 3 mL), washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to yield **7** as a white solid (19.2 mg, 0.059 mmol, >99% yield).

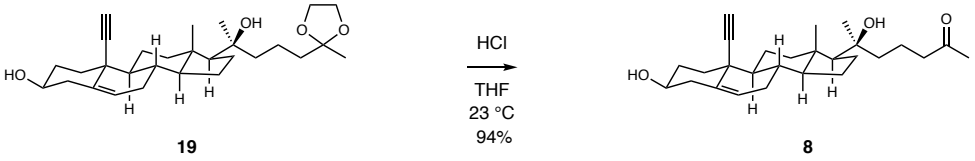
^{1}H NMR (400 MHz, $CDCl_3$):	5.48 (d, J = 4.6 Hz, 1H, C_6**H**), 3.52 (m, 1H, C_3**H**), 2.54 (d, J = 8.9 Hz, 1H, C_{17}**H**), 2.42 (d, J = 7.5 Hz, 2H, C_4**H**), 2.26 (s, 1H, C_{28}**H**), 2.26–2.17 (m, 1H, C_{16}**H**$_a$), 2.22–2.13 (m, 1H, C_1**H**$_a$), 2.16 (s, 3H, C_{21}**H**), 2.14–2.06 (m, 1H, C_{12}**H**$_a$), 2.13–2.05 (m, 1H, C_2**H**$_a$), 1.99–1.91 (m, 1H, C_7**H**$_a$), 1.97–1.86 (m, 1H, C_{11}**H**$_a$), 1.86–1.74 (m, 1H, C_7**H**$_b$), 1.81–1.73 (m, 1H, C_{11}**H**$_b$), 1.73–1.63 (m, 3H, C_8**H**, C_{15}**H**$_a$, C_{16}**H**$_b$), 1.66–1.56 (m, 1H, C_2**H**$_b$), 1.48 (td, J = 12.9, 4.4 Hz, 1H, C_{12}**H**$_b$), 1.31–1.23 (m, 1H, C_{15}**H**$_b$), 1.22–1.15 (m, 1H, C_{14}**H**), 1.20–1.11 (m, 1H, C_1**H**$_b$), 1.04 (td, J = 11.6, 5.2 Hz, 1H, C_9**H**), 0.68 (s, 3H, C_{18}**H**)
^{13}C NMR (100 MHz, $CDCl_3$):	209.50 (**C**$_{20}$), 136.82 (**C**$_5$), 122.96 (**C**$_6$), 86.73 (**C**$_{19}$), 72.28 (**C**$_{28}$), 70.82 (**C**$_3$), 63.66 (**C**$_{17}$), 55.94 (**C**$_{14}$), 48.04 (**C**$_9$), 44.11 (**C**$_{10}$), 42.37 (**C**$_4$), 40.02 (**C**$_{13}$), 38.43 (**C**$_{12}$), 35.75 (**C**$_1$), 33.21 (**C**$_8$), 32.27 (**C**$_7$), 31.57 (**C**$_{21}$), 31.55 (**C**$_2$), 24.44 (**C**$_{15}$), 22.83 (**C**$_{16}$), 22.68 (**C**$_{11}$), 13.12 (**C**$_{18}$)
TLC (SiO_2, 50% EtOAc–hexanes), R_f :	0.34 (CAM, *p*-anisaldehyde)



3β,20α-Dihydroxy-19-ethynyl-27-norcholest-5-ene-25-one ethylene ketal (19)

2-(3-Bromopropyl)-2-methyl-1,3-dioxolane (**18**) (214 mg, 0.92 mmol, 3.0 equiv) was dissolved in 15 mL dry THF in a flame-dried 50 mL round-bottom flask. One-third (5 mL) of the solution was transferred to a flame-dried 100 mL round-bottom flask via syringe and Mg tunings (25 mg, 1 mmol, 3.4 equiv) and a small crystal of I_2 were added under argon. The light brown mixture was sonicated to initiate the reaction, whereupon the color changed to colorless. The remaining two-thirds of the ketal solution (10 mL) was then added via syringe at 50 °C. The mixture was stirred at for 1 h at the same temperature, whereupon complete consumption of Mg was observed. The resulting Grignard reagent was cooled in an ice-water bath, then a solution of alkyne **7** (100 mg, 0.31 mmol, 1.0 equiv) in 15 mL dry THF was added dropwise via cannula over a period of 10 min. The mixture was stirred for additional 2 h at the same temperature before quenching with ice-water (10 mL) dropwise. The solution was diluted with EtOAc (50 mL) and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to yield **19** as a white solid (85 mg, 0.19 mmol, 61% yield).

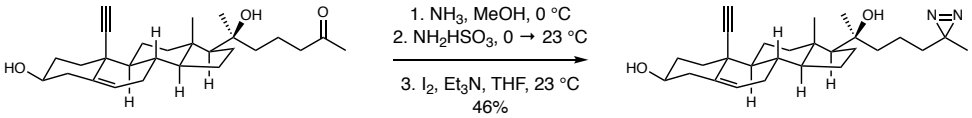
^{1}H NMR (400 MHz, $CDCl_3$):	5.46 (d, J = 4.3 Hz, 1H, C_6**H**), 3.97–3.88 (m, 4H, C_{29}**H**, C_{30}**H**), 3.51 (dtd, J = 12.0, 8.3, 4.4 Hz, 1H, C_3**H**), 2.40 (d, J = 7.3 Hz, 2H, C_4**H**), 2.25 (s, 1H, C_{28}**H**), 2.16–2.08 (m, 1H, C_1**H**$_a$), 2.14–2.06 (m, 1H, C_{12}**H**$_a$), 2.04 (dt, J = 17.1, 4.9 Hz, 1H, C_7**H**$_a$), 1.95–1.86 (m, 1H, C_2**H**$_a$), 1.92–1.84 (m, 1H, C_{11}**H**$_a$), 1.82–1.71 (m, 2H, C_2**H**$_b$, C_{11}**H**$_b$), 1.70 – 1.56 (m, 3H, C_{15}**H**$_a$, C_{16}**H**), 1.69–1.61 (m, 1H, C_8**H**), 1.63–1.54 (m, 2H, C_{24}**H**), 1.55–1.47 (m, 1H, C_7**H**$_b$), 1.49–1.41 (m, 1H, C_{17}**H**), 1.47–1.40 (m, 1H, C_{22}**H**$_a$), 1.43–1.31 (m, 2H, C_{23}**H**), 1.37–1.29 (m, 1H, C_{22}**H**$_b$), 1.32 (s, 3H, C_{26}**H**), 1.28 (s, 3H, C_{21}**H**), 1.28–1.17 (m, 1H, C_{12}**H**$_b$), 1.15–1.04 (m, 1H, C_1**H**$_b$), 1.14–1.11 (m, 1H, C_{15}**H**$_b$), 1.04–0.93 (m, 1H, C_{14}**H**), 1.00–0.89 (m, 1H, C_9**H**), 0.89 (s, 3H, C_{18}**H**)
^{13}C NMR (100 MHz, $CDCl_3$):	136.73 (**C**$_5$), 123.06 (**C**$_6$), 109.95 (**C**$_{25}$), 86.89 (**C**$_{19}$), 75.02 (**C**$_{20}$), 72.01 (**C**$_{28}$), 70.72 (**C**$_3$), 64.53 (**C**$_{29}$), 64.50 (**C**$_{30}$), 57.55 (**C**$_{17}$), 55.81 (**C**$_{14}$), 48.00 (**C**$_9$), 43.84 (**C**$_{22}$), 42.67 (**C**$_{13}$), 42.30 (**C**$_4$), 39.90 (**C**$_{10}$), 39.59 (**C**$_{12}$), 39.57 (**C**$_{24}$), 35.64 (**C**$_1$), 32.59 (**C**$_8$), 32.18 (**C**$_2$), 31.43 (**C**$_7$), 26.28 (**C**$_{21}$), 23.67 (**C**$_{26}$), 23.62 (**C**$_{15}$), 22.46 (**C**$_{16}$), 22.28 (**C**$_{11}$), 18.74 (**C**$_{23}$), 13.40 (**C**$_{18}$)
TLC (SiO_2, 50% EtOAc–hexanes), R_f:	0.29 (*p*-anisaldehyde)



19-Ethynyl-27-nor-25-ketocholest-5-ene-3β,20α-diol (8)

To a solution of ketal **19** (80 mg, 0.175mmol, 1.0 equiv) in 5 mL THF was added 0.5 mL 4 M HCl dropwise via Pasteur pipette at room temperature. The mixture was stirred for 2 h until no ketal **19** was detected by TLC. 5 mL saturated $NaHCO_3$ (aq) was added to the mixture dropwise until the pH of the solution was approximately 7. The mixture was extracted by EtOAc (3 × 20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to give **8** as an off-white solid (68 mg, 0.165 mmol, 94% yield).

^{1}H NMR (400 MHz, $CDCl_3$):	5.47 (d, *J* = 4.2 Hz, 1H, C_6**H**), 3.52 (dtd, *J* = 12.0, 8.3, 4.4 Hz, C_3**H**), 2.49–2.35 (m, 4H, C_4**H**, C_{24}**H**), 2.26 (s, 1H, C_{28}**H**), 2.13 (s, 3H, C_{26}**H**), 2.17–2.10 (m, 1H, C_1**H**$_a$), 2.14–2.07 (m, 1H, C_{12}**H**$_a$), 2.10–2.00 (m, 1H, C_7**H**$_a$), 1.96–1.89 (m, 1H, C_2**H**$_a$), 1.92–1.84 (m, 1H, C_{11}**H**$_a$), 1.83–1.72 (m, 1H, C_2**H**$_b$), 1.77–1.70 (m, 1H, C_{11}**H**$_b$), 1.70–1.60 (m, 3H, C_{15}**H**$_a$, C_{16}**H**), 1.70–1.64 (m, 1H, C_8**H**), 1.60–1.55 (m, 2H, C_{23}**H**), 1.59–1.50 (m, 1H, C_7**H**$_b$), 1.51–1.43 (m, 1H, C_{17}**H**), 1.43–1.41 (m, 1H, C_{22}**H**$_a$), 1.34–1.27 (m, 1H, C_{22}**H**$_b$), 1.29 (s, 3H, C_{21}**H**), 1.29–1.19 (m, 1H, C_{12}**H**$_b$), 1.17–1.11 (m, 1H, C_{15}**H**$_b$), 1.16–1.06 (m, 1H, C_1**H**$_b$), 1.05–0.95 (m, 1H, C_{14}**H**), 1.01–0.93 (m, 1H, C_9**H**), 0.90 (s, 3H, C_{18}**H**)
^{13}C NMR (100 MHz, $CDCl_3$):	209.06 (**C**$_{25}$), 136.72 (**C**$_5$), 123.10 (**C**$_6$), 86.91 (**C**$_{19}$), 74.95 (**C**$_{20}$), 72.02 (**C**$_{28}$), 70.78 (**C**$_3$), 57.47 (**C**$_{17}$), 55.83 (**C**$_{14}$), 47.99 (**C**$_9$), 43.96 (**C**$_{24}$), 42.94 (**C**$_{22}$), 42.71 (**C**$_{13}$), 42.30 (**C**$_4$), 39.92 (**C**$_{10}$), 39.57 (**C**$_{12}$), 35.64 (**C**$_1$), 32.60 (**C**$_8$), 32.20 (**C**$_2$), 31.44 (**C**$_7$), 29.96 (**C**$_{26}$), 26.27 (**C**$_{21}$), 23.62 (**C**$_{15}$), 22.46 (**C**$_{16}$), 22.25 (**C**$_{11}$), 18.40 (**C**$_{23}$), 13.44 (**C**$_{18}$)
TLC (SiO_2, 50% EtOAc–hexanes), R_f :	0.19 (*p*-anisaldehyde)



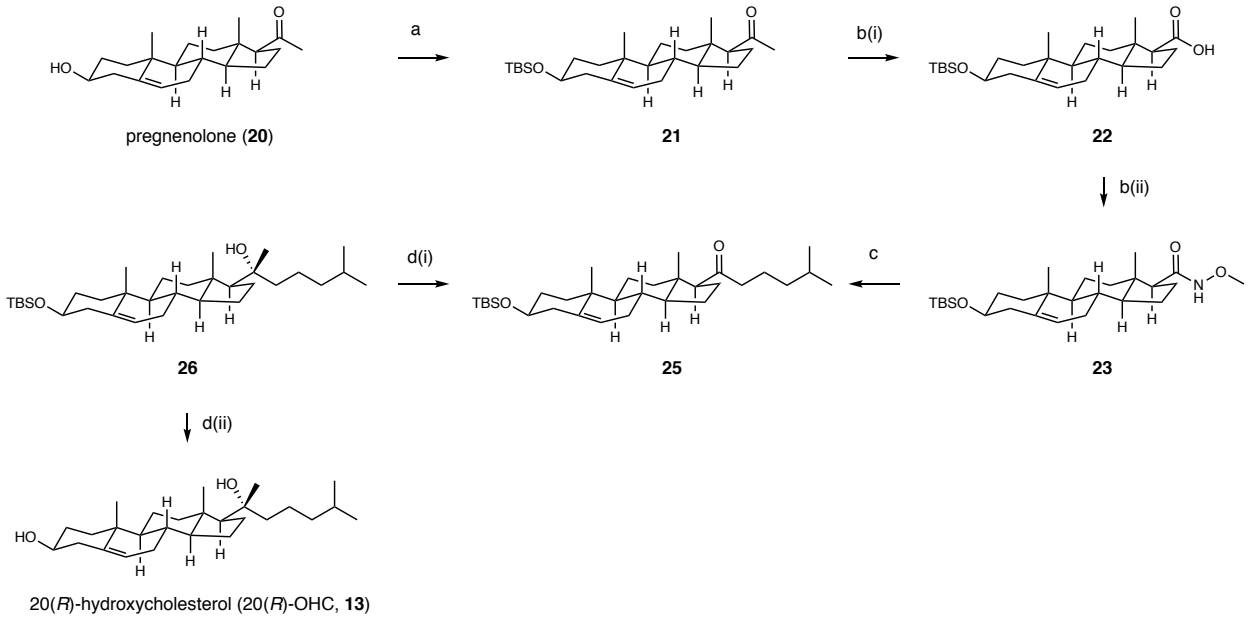
8 **2**

25-Diazirinyl-19-ethynyl-27-norcholest-5-ene-3β,20*α*-diol (20(*S*)-OHC probe, 2)

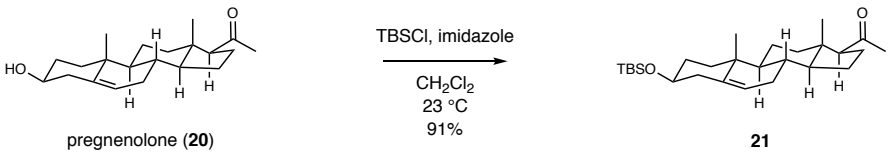
A solution of ketone **8** (34 mg, 0.0824 mmol, 1.0 equiv) in 2 mL anhydrous MeOH in an ice-water bath was bubbled with NH_3 gas for 3 h under continuous stirring. A solution of hydroxylamine-*O*-sulfonic acid (32 mg, 0.284 mmol, 3.5 equiv) in 1 mL anhydrous methanol was added dropwise via syringe and the mixture was stirred for 16 h at room temperature. The crude reaction mixture was filtered and the filter cake was washed with MeOH. The combined filtrate was supplemented with 0.1 ml triethylamine and concentrated under reduced pressure. The residue was dissolved in 2 mL MeOH and 0.2 mL triethylamine, and 10 % iodine in methanol was added dropwise until the yellow color persisted. After excess iodine was removed by addition of sodium thiosulfate, the solution was concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to give **2** as a clear oil (16 mg, 0.0377 mmol, 46% yield).

^{1}H NMR (500 MHz, $CDCl_3$):	δ 5.47 (dd, *J* = 5.3, 1.6 Hz, 1H, C_6**H**), 3.52 (dtd, *J* = 11.8, 8.1, 4.4 Hz, C_3**H**), 2.41 (d, *J* = 7.0 Hz, 2H, C_4**H**), 2.26 (s, 1H, C_{28}**H**), 2.16–2.12 (m, 1H, C_1**H**$_a$), 2.14–2.08 (m, 1H, C_{12}**H**$_a$), 2.04 (dt, *J* = 17.2, 5.2 Hz, 1H, C_7**H**$_a$), 1.95–1.90 (m, 1H, C_2**H**$_a$), 1.92–1.84 (m, 1H, C_{11}**H**$_a$), 1.82–1.73 (m, 1H, C_2**H**$_b$), 1.78–1.71 (m, 1H, C_{11}**H**$_b$),1.71 – 1.59 (m, 3H, C_{15}**H**$_a$, C_{16}**H**), 1.70–1.62 (m, 1H, C_8**H**), 1.59–1.51 (m, 1H, C_7**H**$_b$), 1.45–1.40 (m, 1H, C_{17}**H**), 1.43–1.36 (m, 1H, C_{22}**H**$_a$), 1.42–1.35 (m, 1H, C_{24}**H**$_a$), 1.34–1.26 (m, 1H, C_{24}**H**$_b$), 1.32–1.25 (m, 1H, C_{22}**H**$_b$), 1.29–1.20 (m, 1H, C_{12}**H**$_b$), 1.26 (s, 3H, C_{21}**H**), 1.19–1.13 (m, 1H, C_{15}**H**$_b$), 1.19–1.08 (m, 2H, C_{23}**H**), 1.15–1.08 (m, 1H, C_1**H**$_b$), 1.04–0.97 (m, 1H, C_{14}**H**), 1.01 (s, 3H, C_{26}**H**), 1.01–0.95 (m, 1H, C_9**H**), 0.90 (s, 3H, C_{18}**H**)
^{13}C NMR (125 MHz, $CDCl_3$):	δ 136.73 (**C**$_5$), 123.08 (**C**$_6$), 86.89 (**C**$_{19}$), 74.90 (**C**$_{20}$), 72.03 (**C**$_{28}$), 70.77 (**C**$_3$), 57.64 (**C**$_{17}$), 55.82 (**C**$_{14}$), 47.99 (**C**$_9$), 43.08 (**C**$_{22}$), 42.70 (**C**$_{13}$), 42.31 (**C**$_4$), 39.92 (**C**$_{10}$), 39.58 (**C**$_{12}$), 35.64 (**C**$_1$), 34.69 (**C**$_{24}$), 32.60 (**C**$_8$), 32.20 (**C**$_2$), 31.44 (**C**$_7$), 26.22 (**C**$_{21}$), 25.79 (**C**$_{25}$), 23.61 (**C**$_{15}$), 22.45 (**C**$_{16}$), 22.29 (**C**$_{11}$), 19.94 (**C**$_{26}$), 18.72 (**C**$_{23}$), 13.42 (**C**$_{18}$)
TLC (SiO_2, 50% EtOAc–hexanes), R_f :	0.41 (*p*-anisaldehyde, PMA)

Synthetic scheme for 20(*R*)-OHC (13)



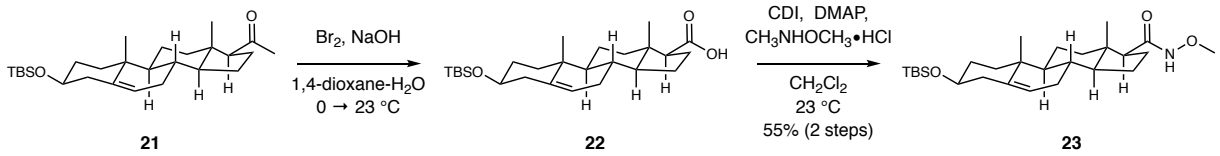
a. TBSCl, imidazole, CH_2Cl_2, 23 °C, 91%;
b. (i) Br_2, NaOH, 1,4- dioxane/H_2O, 0 → 23 °C, 80%; (ii) CDI, DMAP, $CH_3NHOCH_3 \cdot HCl$, CH_2Cl_2, 23 °C, 55% (2 steps);
c. 1-bromo-4-methylpentane (**24**), Mg, THF, 0 → 23 °C, 48%;
d. (i) CH_3MgBr, THF, 0 → 23 °C, 63%; (ii) TBAF, MeOH, THF, 0 → 23 °C, 57%.



3β-(tert-Butyldimethylsilyloxy)-5-pregnen-20-one (21)

To a solution of pregnenolone (**20**) (5.00 g, 15.8 mmol, 1.0 equiv) in 150 mL dry CH_2Cl_2 was added *tert*-butyldimethylsilyl chloride (3.61 g, 23.7 mmol, 1.5 equiv) and imidazole (2.69 g, 39.5 mmol, 2.5 equiv). The mixture was stirred at the room temperature for 2 h until no starting material was detected by TLC. The mixture was then diluted with 50 mL CH_2Cl_2 and quenched with 100 mL water. The aqueous layer was separated and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to give **21** as a white solid (6.21 g, 14.4 mmol, 91% yield).

^{1}H NMR (400 MHz, $CDCl_3$):	5.32 (dt, J = 5.5, 2.0 Hz, 1H, C_6**H**), 3.48 (tt, J = 11.0, 4.8 Hz, 1H, C_3**H**), 2.53 (t, J = 8.9 Hz, 1H, C_{17}**H**), 2.32 – 2.12 (m, 3H, C_4**H**, C_{16}**H**$_a$), 2.12 (s, 3H, C_{21}**H**), 2.07 – 1.95 (m, 2H, C_7**H**$_a$, C_{12}**H**$_a$), 1.82 (dt, J = 13.2, 3.5 Hz, 1H, C_1**H**$_a$), 1.76 – 1.42 (m, 9H, C_2**H**, C_7**H**$_b$, C_{11}**H**, C_{12}**H**$_b$, C_{15}**H**$_a$, C_{16}**H**$_b$, C_8**H**), 1.29 – 1.12 (m, 2H, C_{15}**H**$_b$, C_{14}**H**), 1.10 – 1.02 (m, 1H, C_1**H**$_b$), 1.00 (s, 3H, C_{19}**H**), 0.97 – 0.93 (m, 1H, C_9**H**), 0.89 (s, 9H, SiC(C**H**$_3$)$_3$), 0.62 (s, 3H, C_{18}**H**), 0.06 (s, 6H, Si(C**H**$_3$)$_2$)
TLC (SiO_2, 20% EtOAc–hexanes), R_f:	0.53 (CAM)

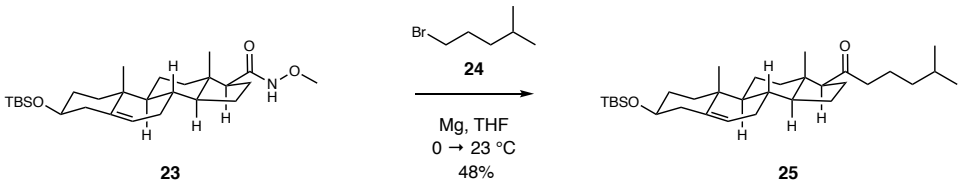


21 **22** **23**

3β-(tert-Butyldimethylsilyloxy)-N-methoxy-N-methyl-5-androstene-17β-carboxamide (23)
To a vigorously stirred solution of NaOH (608 mg, 15.2 mmol, 13.1 equiv) in 5.2 mL water was added Br_2 (198 µL, 613 mg, 3.82 mmol, 3.3 equiv) dropwise via syringe at 0 °C, followed by 3.5 mL ice-cold 1,4-dioxane. The resulting hypobromite solution was added slowly to a solution of compound **21** (500 mg, 1.16 mmol, 1.0 equiv) in 16 mL 1,4-dioxane and 4.6 mL water at 0 °C. The mixture was allowed to warm to room temperature and stirred overnight. The excess sodium hypobromite was quenched by the addition of 10% Na_2SO_3(aq) and the mixture was acidified to pH 2 with 1 N HCl. The white precipitate was collected by vacuum filtration, washed with ice-water, and dried in vacuo to give the crude acid **22**.

1,1'-carbonyldiimidazole (237 mg, 1.46 mmol, 1.3 equiv) was added in portions to a solution of crude acid **22** (528 mg) and 4-dimethylaminopyridine (181 mg, 1.46 mmol, 1.3 equiv) in 10.6 mL dry CH_2Cl_2 and the mixture was stirred for 1 h at room temperature. *N*,*O*-dimethylhydroxylamine hydrochloride (143 mg, 1.46 mmol, 1.3 equiv) was added and the reaction mixture was stirred overnight at room temperature. The mixture was diluted with CH_2Cl_2 and water and the aqueous layer was separated and extracted with 2 × CH_2Cl_2. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to give Weinreb amide **23** (305 mg, 0.64 mmol, 55% yield over two steps).

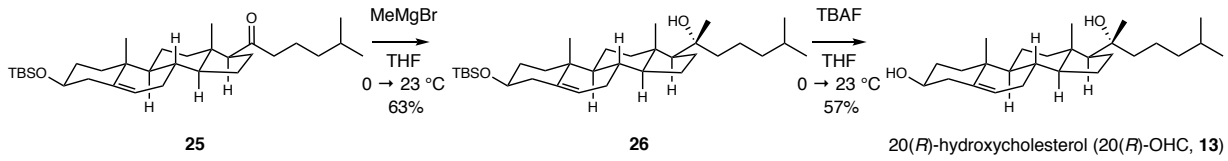
^{1}H NMR (400 MHz, $CDCl_3$):	5.31 (dt, *J* = 5.7, 1.7 Hz, 1H, C_6**H**), 3.64 (s, 3H, NC**H**$_3$), 3.47 (tt, *J* = 10.9, 4.7 Hz, 1H, C_3**H**), 3.19 (s, 3H, NOC**H**$_3$), 2.78 (s, 1H, C_{17}**H**), 2.25 (dtd, J = 12.7, 10.6, 2.0 Hz, 1H, C_4**H**$_a$), 2.19 – 2.12 (m, 2H, C_4**H**$_b$, C_{16}**H**$_a$), 2.08 – 1.94 (m, 1H, C_7**H**$_a$), 1.88 – 1.77 (m, 2H, C_1**H**$_a$, C_{12}**H**$_a$), 1.76 – 1.66 (m, 3H, C_2**H**, C_{15}**H**$_a$), 1.61 – 1.39 (m, 5H , C_7**H**$_b$, C_{11}**H**, C_{16}**H**$_b$, C_8**H**), 1.37 – 1.22 (m, 2H, C_{12}**H**$_b$, C_{15}**H**$_b$), 1.21 – 1.08 (m, 1H, C_{14}**H**), 1.07 – 1.00 (m, 1H, C_1**H**$_b$), 0.99 (s, 3H, C_{19}**H**), 0.94 (ddd, J = 11.9, 10.6, 5.1 Hz, 1H, C_9**H**), 0.88 (s, 9H, SiC(C**H**$_3$)$_3$), 0.74 (s, 3H, C_{18}**H**), 0.05 (s, 6H, Si(C**H**$_3$)$_2$)
^{13}C NMR (100 MHz, $CDCl_3$):	175.01 (**C**$_{20}$), 141.73 (**C**$_5$), 121.05 (**C**$_6$), 72.69 (**C**$_3$), 61.05 (NO**C**H$_3$), 56.87 (**C**$_{14}$), 51.01 (**C**$_{17}$), 50.35 (**C**$_9$), 45.32 (**C**$_{13}$), 42.91 (**C**$_4$), 39.03 (**C**$_{12}$), 37.52 (**C**$_1$), 36.78 (**C**$_{10}$), 32.18 (**C**$_8$), 32.12 (**C**$_7$), 32.11 (N**C**H$_3$), 32.10 (**C**$_2$), 26.07 (SiC(**C**H$_3$)$_3$), 25.07 (**C**$_{15}$), 24.99 (**C**$_{16}$), 21.11 (**C**$_{11}$), 19.57 (**C**$_{19}$), 18.40 (Si**C**(CH$_3$)$_3$), 13.92 (**C**$_{18}$), -4.44 (Si(**C**H$_3$)$_2$)
TLC (SiO_2, 20% EtOAc–hexanes), R_f :	0.58 (**S5**), 0.29 (**S4**) (CAM, ninhydrin, $KMnO_4$)



3β-(tert-Butyldimethylsilyloxy)-21-norcholest-5-en-20-one (25)

A flame-dried 25 mL round-bottom flask was charged with Mg (22 mg, 0.9 mmol, 4.3 equiv), a small crystal of I_2 and 2 mL of dry THF. One portion of 1-bromo-4-methylpentane (**24**) (41 µL, 46 mg, 0.28 mmol, 1.3 equiv) was added dropwise via syringe. The light brown mixture was sonicated to initiate the reaction, whereupon the color changed to colorless. Another portion of 1-bromo-4-methylpentane (84 µL, 96 mg, 0.56 mmol, 2.7 equiv) was then added via syringe at 50 °C. The mixture was stirred at for 1 h at the same temperature until complete consumption of Mg was observed. The resulting Grignard reagent was cooled on an ice-water bath, then a solution of Weinreb amide **23** (100 mg, 0.21 mmol, 1.0 equiv) in 3 mL dry THF was added dropwise via syringe over a period of 10 min. The mixture was allowed to warm to room temperature and stirred for additional 2 h before quenching by dropwise addition of 5 mL saturated NH_4Cl(aq). The aqueous layer was separated and extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with water, brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) give **25** as a white solid (51 mg, 0.102 mmol, 48% yield).

^{1}H NMR (400 MHz, $CDCl_3$):	5.31–5.18 (m, 1H, C_6**H**), 3.52–3.32 (m, 1H, C_3**H**), 2.46 (t, *J* = 9.0 Hz, 1H, C_{17}**H**), 2.29 (t, *J* = 7.4 Hz, 2H, C_{22}**H**), 2.26–2.07 (m, 3H, C_4**H**, C_{16}**H**$_a$), 2.00–1.87 (m, 2H, C_{12}**H**$_a$, C_7**H**$_a$), 1.76 (dt, *J* = 13.2, 3.5 Hz, 1H, C_1**H**$_a$), 1.71–1.31 (m, 11H, C_2**H**, C_7**H**$_b$, C_{11}**H**, C_{15}**H**$_a$, C_{16}**H**$_b$, C_{23}**H**, C_{25}**H**, C_8**H**), 1.25–1.05 (m, 5H, C_{12}**H**$_b$, C_{15}**H**$_b$, C_{24}**H**, C_{14}**H**), 1.04–0.95 (m, 1H, C_1**H**$_b$), 0.94 (s, 3H, C_{19}**H**), 0.94–0.87 (m, 1H, C_9**H**), 0.83 (s, 9H, SiC(C**H**$_3$)$_3$), 0.82 (d, *J* = 6.6 Hz, 6H, C_{26}**H**, C_{27}**H**), 0.55 (s, 3H, C_{18}**H**), 0.00 (s, 6H, Si(C**H**$_3$)$_2$)
^{13}C NMR (100 MHz, $CDCl_3$):	211.87 (**C**$_{20}$), 141.65 (**C**$_5$), 121.02 (**C**$_6$), 72.67 (**C**$_3$), 62.99 (**C**$_{17}$), 57.15 (**C**$_{14}$), 50.22 (**C**$_9$), 44.72 (**C**$_{22}$), 44.31 (**C**$_{13}$), 42.90 (**C**$_4$), 39.11 (**C**$_{12}$), 38.75 (**C**$_{24}$), 37.52 (**C**$_1$), 36.73 (**C**$_{10}$), 32.18 (**C**$_8$), 32.00 (**C**$_7$), 31.97 (**C**$_2$), 28.08 (**C**$_{25}$), 26.07 (SiC(**C**H$_3$)$_3$), 24.68 (**C**$_{15}$), 23.09 (**C**$_{16}$), 22.68 (**C**$_{26}$), 22.65 (**C**$_{27}$), 21.72 (**C**$_{23}$), 21.22 (**C**$_{11}$), 19.56 (**C**$_{19}$), 18.40 (Si**C**(CH$_3$)$_3$), 13.54 (**C**$_{18}$), -4.44 (Si(**C**H$_3$)$_2$)
TLC (SiO_2, 10% EtOAc–hexanes), R_f :	0.47 (CAM, *p*-anisaldehyde, $KMnO_4$)



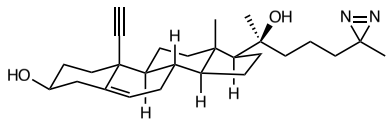
20(*R*)-Hydroxycholesterol (20(*R*)-OHC, 13)

A solution of compound **25** (18 mg, 0.036 mmol, 1.0 equiv) in 1 mL dry THF was added dropwise via syringe over a period of 5 min to a solution of methylmagnesium bromide (3.0 M in Et_2O, 80 µL, 0.24 mmol, 6.7 equiv) in an flame-dried 25 mL round-bottom flask at 0 °C under argon. The mixture was stirred for 2 h before quenching dropwise with 2 mL saturated NH_4Cl(aq) (2 mL). The aqueous layer was separated and extracted with EtOAc (3 × 2 mL). The combined organic layers were washed with water, brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude compound **26** (12 mg, 0.023 mmol).

To a solution of crude TBS-ether **26** (12 mg, 0.023 mmol, 1.0 equiv) in 1 mL THF was added tetrabutylammonium fluoride (1 M in THF, 60 µL, 0.06 mmol, 2.6 equiv) at 0 °C. The mixture was allowed to warm to room temperature and stirred overnight. The mixture was then quenched with saturated NH_4Cl (aq) and the aqueous layer was separated and extracted with EtOAc (3 × 2 mL). The combined organic layers were washed with water, brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography (CombiFlash, 0–100% EtOAc/hexanes) to give 20(*R*)-OHC (**13**) as a white solid (5.2 mg, 0.013 mmol, 57% yield).

^{1}H NMR (500 MHz, $CDCl_3$):	5.35 (dt, *J* = 5.5, 1.8 Hz, 1H, C_6**H**), 3.52 (tq, *J* = 9.4, 4.7 Hz, 1H, C_3**H**), 2.34 – 2.19 (m, 2H, C_4**H**), 2.08 (dt, *J* = 12.4, 3.5 Hz, 1H, C_{12}**H**$_a$), 1.97 (dtd, *J* = 16.3, 4.5, 2.4 Hz, 1H, C_7**H**$_a$), 1.89–1.80 (m, 2H, C_1**H**$_a$, C_2**H**$_a$), 1.79–1.64 (m, 2H, C_{22}**H**), 1.65–1.42 (m, 9H, C_{25}**H**, C_{11}**H**, C_{23}**H**, C_2**H**$_b$, C_7**H**$_b$, C_{17}**H**, C_8**H**), 1.40–1.30 (m, 2H, C_{16}**H**), 1.29–1.21 (m, 2H, C_{24}**H**), 1.21–1.12 (m, 1H, C_{12}**H**$_b$), 1.20–1.07 (m, 2H, C_{15}**H**), 1.14–1.04 (m, 1H, C_1**H**$_b$), 1.12 (s, 3H, C_{21}**H**), 1.06–0.95 (m, 1H, C_{14}**H**), 1.01 (s, 3H, C_{19}**H**), 0.98–0.88 (m, 1H, C_9**H**), 0.89 (s, 3H, C_{18}**H**), 0.87 (d, *J* = 3.0 Hz, 6H, C_{26}**H**, C_{27}**H**)
^{13}C NMR (125 MHz, $CDCl_3$):	140.92 (**C**$_5$), 121.76 (**C**$_6$), 75.97 (**C**$_{20}$), 71.93 (**C**$_3$), 58.38 (**C**$_{17}$), 57.01 (**C**$_{14}$), 50.16 (**C**$_9$), 43.17 (**C**$_{23}$), 43.02 (**C**$_{13}$), 42.42 (**C**$_4$), 40.26 (**C**$_{12}$), 39.79 (**C**$_{24}$), 37.39 (**C**$_1$), 36.65 (**C**$_{10}$), 31.93 (**C**$_2$), 31.78 (**C**$_7$), 31.48 (**C**$_8$), 28.20 (**C**$_{25}$), 27.20 (**C**$_{21}$), 23.98 (**C**$_{15}$), 23.28 (**C**$_{22}$), 22.87 (**C**$_{26}$), 22.77 (**C**$_{27}$), 21.98 (**C**$_{16}$), 21.06 (**C**$_{11}$), 19.54 (**C**$_{19}$), 13.86 (**C**$_{18}$)
TLC (SiO_2, 20% EtOAc–hexanes), R_f :	0.18 (**20(*R*)-OHC**), 0.47 (**S7**) (CAM)

^{1}H NMR, 500 MHz, $CDCl_3$



2

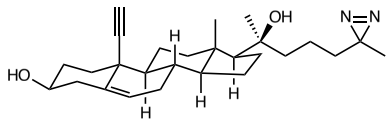
21

1.00 1.03 1.91 0.90 1.94 1.07 2.02 2.27 4.16 1.84 2.76 6.15 4.12 4.62 2.71

.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.

f1 (ppm)

^{13}C NMR, 125 MHz, $CDCl_3$



2

136.73
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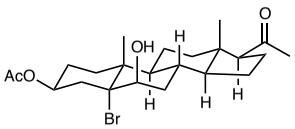
86.89
74.90
72.03
70.77
57.64
55.82
47.99
43.08
42.70
42.31
39.92
39.58
35.64
34.69
32.60
32.20
31.44
26.22
25.79
23.61
22.45
22.29
19.94
18.72
13.42

22

220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

f1 (ppm)

^{1}H NMR, 400 MHz, $CDCl_3$



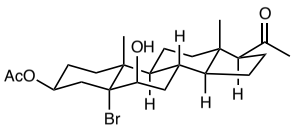
4

23

1.00 1.01 0.97 1.03 0.97 2.03 2.98 3.98 0.97 2.75 5.47 3.52 1.07 4.92 1.11 2.80

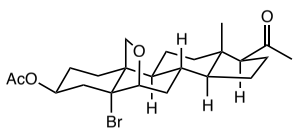
f1 (ppm)

^{13}C NMR, 100 MHz, $CDCl_3$



4

^{1}H NMR, 400 MHz, $CDCl_3$



5

25

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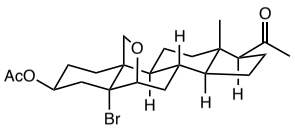
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.

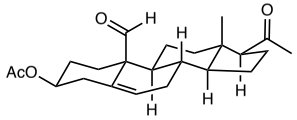
f1 (ppm)

^{13}C NMR, 100 MHz, $CDCl_3$



5

^{1}H NMR, 400 MHz, $CDCl_3$



6

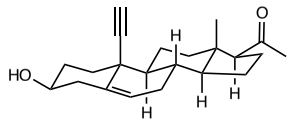
27

0.99 1.00 1.00 1.00 1.98 2.18 2.98 0.97 3.00 1.05 2.00 4.84 1.11 1.00 3.23 2.10 2.97

9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5

f1 (ppm)

^{1}H NMR, 400 MHz, $CDCl_3$

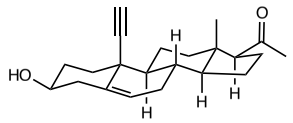


7

28

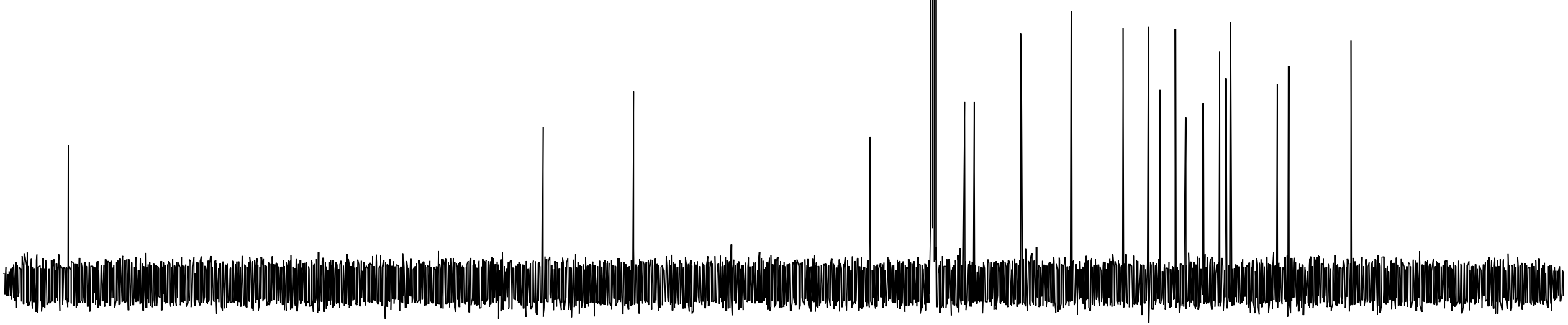
^{13}C NMR, 100 MHz, $CDCl_3$

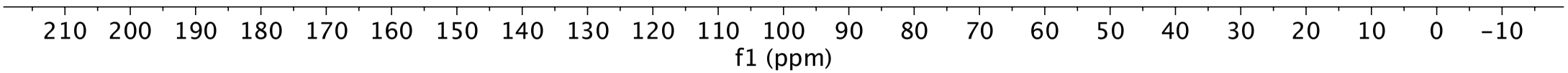
209.20
136.82
122.96
86.73
72.28
70.82
63.66
55.94
48.04
44.11
42.37
40.02
38.43
35.75
33.21
32.27
31.57
31.55
24.44
22.83
22.68
13.12



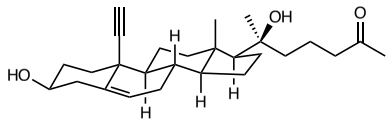
7

29





^{1}H NMR, 400 MHz, $CDCl_3$



8

30

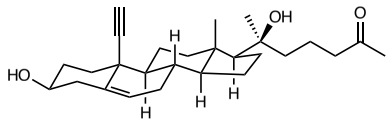
1.00 1.00 3.96 0.99 5.97 2.04 2.51 9.96 2.19 6.50 2.50 2.13 2.95

.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.

f1 (ppm)

^{13}C NMR, 100 MHz, $CDCl_3$

209.06
136.72
123.10
86.91
74.95
72.02
70.78
57.47
55.83
47.99
43.96
42.94
42.71
42.30
39.92
39.57
35.64
32.60
32.20
31.44
29.96
26.27
23.62
22.46
22.25
18.40
13.44



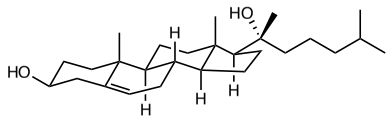
8

31

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

f1 (ppm)

^{1}H NMR, 400 MHz, $CDCl_3$



20(*R*)-OHC (**13**)

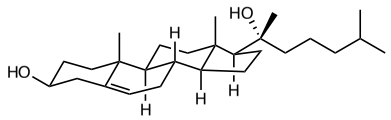
32

1.00 1.00 2.07 0.98 1.01 2.08 1.98 11.91 1.10 2.07 1.90 2.76 3.08 1.35 3.14 0.76 1.03 3.05 6.22

.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.

f1 (ppm)

^{13}C NMR, 100 MHz, $CDCl_3$



20(*R*)-OHC (**13**)

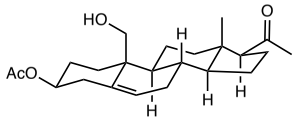
140.92
121.76
75.97
71.93
58.38
57.01
50.16
43.17
42.42
40.26
39.79
37.39
36.65
31.93
31.78
31.48
28.20
27.20
23.98
23.28
22.87
22.77
21.98
21.06
19.54

33

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

f1 (ppm)

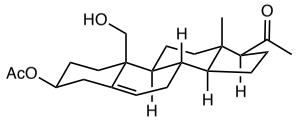
^{1}H NMR, 400 MHz, $CDCl_3$



15

^{13}C NMR, 100 MHz, $CDCl_3$

209.56
170.53
134.53
128.00
73.29
63.61
62.70
57.69
50.14
44.18
41.58
39.04
38.14
33.27
33.16
31.52
31.09
28.05
24.28
22.79
21.69
21.40
13.58

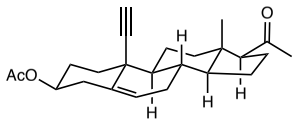


15

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 −10

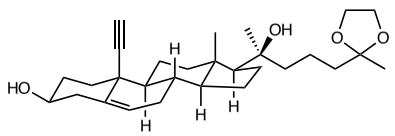
f1 (ppm)

^{1}H NMR, 400 MHz, $CDCl_3$



17

^{1}H NMR, 400 MHz, $CDCl_3$



19

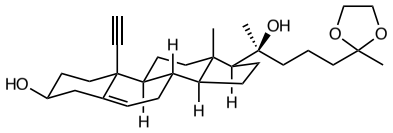
37

1.01 4.19 1.00 2.02 1.02 3.07 1.92 3.12 6.10 5.30 7.12 3.70 2.27 3.08

.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.

f1 (ppm)

^{13}C NMR, 100 MHz, $CDCl_3$



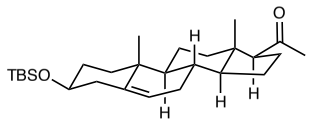
19

136.73
123.06
109.95
86.89
75.02
72.01
70.72
64.53
64.50
57.55
55.81
48.00
43.84
42.67
42.30
39.90
39.59
39.57
35.64
32.59
32.18
31.43
26.28
23.67
23.62
22.46
22.28
18.74
13.40

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

f1 (ppm)

^{1}H NMR, 400 MHz, $CDCl_3$



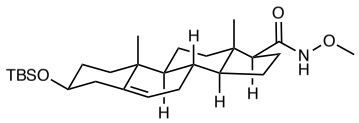
21

1.00 0.98 0.98 1.14 1.90 3.07 1.99 1.00 5.67 3.87 1.13 0.96 1.23 3.38 0.78 9.12 2.99 6.06

.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1

f1 (ppm)

^{1}H NMR, 400 MHz, $CDCl_3$



23

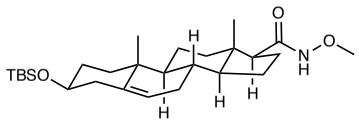
40

1.00 3.14 1.02 3.08 0.89 3.12 1.09 2.26 3.17 5.40 2.31 1.12 1.18 4.22 1.03 9.97 3.24 6.47

.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1

f1 (ppm)

^{13}C NMR, 100 MHz, $CDCl_3$



23

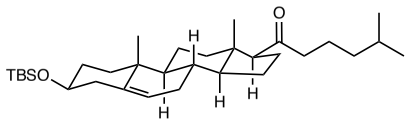
175.01
141.73
121.05
72.69
61.05
56.87
51.01
50.35
45.32
42.91
39.03
37.52
36.78
32.18
32.12
32.10
26.07
25.07
24.99
21.11
19.57
18.40
13.92
-4.16
-4.44
-4.72

41

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

f1 (ppm)

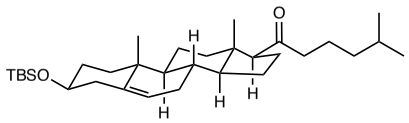
^{1}H NMR, 400 MHz, $CDCl_3$



25

42

^{13}C NMR, 100 MHz, $CDCl_3$



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Supplementary Note 2. Additional mass spectrometry procedures.

LC-MS/MS analysis of tryptic digests

Label-free quantification. The dried, desalted sample was resuspended in 10 µL buffer A (98% H_2O, 2% acetonitrile, 0.2% formic acid). 2 µL of the resuspended sample was subjected to LC-MS/MS analysis on an EASY-nLC 1200 (ThermoFisher Scientific, San Jose, CA) coupled to an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanospray Flex ion source. Samples were directly loaded onto a C18 Aurora column (25 cm x 50 µm ID, 1.6 µm, Ion Opticks, Parkville, Australia) maintained at 50 °C and separated over 75 min at a flow rate of 350 nL/min with the following gradient: 2–6% Solvent B (3.5 min), 6–25% B (42 min), 25–40% B (14.5 min), 40–98% B (1 min), and 98% B (14 min). Solvent A consisted of 2% acetonitrile, 97.8% H_2O and 0.2% formic acid and solvent B consisted of 80% acetonitrile, 19.8% H_2O, and 0.2% formic acid. The QExactive HF was operated in data dependent mode with Tune (version 2.8) instrument control software. Spray voltage was set to 1.5 kV, with S-lens RF level at 60 and heated capillary at 275 °C. MS1 spectra were acquired at 60K resolution with a scan range from 400–1650 m/z, an AGC target of 3e6, and a maximum injection time of 15 ms in Profile mode. A Top 12 DDA analysis was then performed in which features were filtered for monoisotopic peaks with a charge state of 2–5, a minimum intensity of 1e5, and a minimum AGC target of 4.5e3, with dynamic exclusion set to exclude features after 1 time for 45 seconds and exclude isotopes turned on. HCD fragmentation was performed with normalized collision energy of 28 after quadrupole isolation of features using an isolation window of 1.2 m/z, an AGC target of 1e5, and a maximum injection time of 45 ms. MS2 scans were then acquired at 30K resolution in Centroid mode with the first mass fixed at 100 and a scan range of 200–2000 m/z.

Isobaric mass tag quantification. The dried, desalted sample was resuspended in 30 µL of buffer A (98% H_2O, 2% acetonitrile, 0.2% formic acid). A preliminary run was conducted for generating "targeted database" in which 7 µL of the 1:10 diluted resuspended sample was subjected to LC-MS/MS analysis on an EASY-nLC 1200 (ThermoFisher Scientific, San Jose, CA) coupled to an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanospray Flex ion source. Samples were directly loaded onto a C18 Aurora column (25 cm x 50 µm ID, 1.6 µm, Ion Opticks, Parkville, Australia) maintained at 50 °C and separated over 136 min at a flow rate of 350 nL/min with the following gradient: 2–6% solvent B (7.5 min), 6–25% B (82.5 min), 25–40% B (30 min), 40–98% B (1 min), and 98% B (15 min). Solvent A consisted of 2% acetonitrile, 97.8% H_2O and 0.2% formic acid and solvent B consisted of 80% acetonitrile, 19.8% H_2O, and 0.2% formic acid. The QExactive HF was operated in data dependent mode with Tune (version 2.8) instrument control software. Spray voltage was

set to 1.5 kV, S-lens RF level at 60, and heated capillary at 275 °C. MS1 spectra were acquired at 120K resolution with a scan range from 380–1500 m/z, an AGC target of 3e6, and a maximum injection time of 50 ms in Profile mode. A Top 15 DDA analysis was then performed in which features were filtered for monoisotopic peaks with a charge state of 2–6, a minimum intensity of 3.8e4, and a minimum AGC target of 4e3, with dynamic exclusion set to exclude features after 1 time for 45 seconds and exclude isotopes turned on. HCD fragmentation was performed with normalized collision energy of 28 after quadrupole isolation of features using an isolation window of 1.2 m/z, an AGC target of 1e5, and a maximum injection time of 106 ms. MS2 scans were then acquired at 60K resolution in Centroid mode with the first mass fixed at 100 and a scan range of 200–2000 m/z.

Data analysis. SEQUEST HT search parameters were as follows: fully tryptic peptides with no more than 2 missed cleavages, precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.6 Da, and a maximum of 3 equal modifications and 4 dynamic modifications per peptide. Static modifications were carbamidomethylation of cysteine (+57.021464 Da) and TMT6plex or TMT12plex addition to lysines and peptide N-termini (+229.162932 Da). Oxidation on methionine residues (+15.994915 Da), methionine loss on protein N-termini (−131.040485 Da), methionine loss + acetylation on protein N-termini (−89.02992 Da), acetylation on protein N-termini (+42.010565 Da), and phosphorylation of serine, threonine, and tyrosine (+79.966331 Da) were dynamic modifications. Percolator FDRs were set at 0.01 (strict) and 0.05 (relaxed). Spectrum file retention time calibration was used with TMT6plex or TMT12plex addition to peptide N-termini and lysines and carbamidomethylation of cysteine as static modifications. Reporter ion quantification used a co-isolation threshold of 100, average reporter S/N threshold of 5, and SPS mass match threshold of 70%. Peptide FDRs were set at 0.001 (strict) and 0.01 (relaxed), with peptide confidence at least medium, lower confidence peptides excluded, and minimum peptide length set at 6. All proteins identified by SEQUEST HT or Byonic and additional potential 20(*S*)-OHC binding proteins were included in the "targeted database".

RTS-SPS-MS^3 analysis. After generating the targeted database, 2 µL of the resuspended sample was subjected to LC-MS/MS analysis on an EASY-nLC 1000 coupled to an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Samples were directly loaded onto a UHPLC monolithic column (Capillary EX-Nano MonoCap C18 HighResolution 2000, 0.1 x 2000 mm, Merck, Darmstadt, Germany) fitted with a silica coated PicoTip emitter (New Objective, FS360-20-10-D) and separated over 218 min at a flow rate of 0.4 µL/min with the following gradient: 2–8% solvent B (10 min), 8–44% B (170 min), 44–98% B (1 min), and 98% B (37 min). Solvent A consisted of 97.8% H_2O,

2% acetonitrile, and 0.2% formic acid, and solvent B consisted of 19.8% H_2O, 80% acetonitrile, and 0.2% formic acid.

MS1 spectra were acquired in the Orbitrap at 120K resolution with a scan range from 350–2000 m/z, an AGC target of 1e6, and the Auto maximum injection time mode in Profile mode. Features were filtered for monoisotopic peaks with a charge state of 2–7 and a minimum intensity of 1e4, with dynamic exclusion set to exclude features after 1 time for 45 seconds with a 5 ppm mass tolerance. CID fragmentation was performed with collision energy of 35%, activation time of 10 ms, and activation Q of 0.25 following quadrupole isolation of features using an isolation window of 0.4 m/z, an AGC target of 1e4, and a maximum injection time of 45 ms. MS2 scans were then acquired in the Ion Trap using Rapid scan rate in Centroid mode. Real-time search parameters were set for fully tryptic peptides with a maximum of 1 missed cleavage and up to two variable modifications. Static modifications were cysteine carbamidomethylation and TMT6plex or TMT12plex addition to lysines and peptide N-termini. Variable modifications were methionine oxidation and serine, threonine, and tyrosine phosphorylation.

The custom targeted database generated from the preliminary search was used for the RTS search. Scoring thresholds were Xcorr = 1, dCn = 0, precursor mass error of 10 ppm, and charge state not 1. Maximum search time was set to 35 ms. Up to 20 MS2 features matching identified peptide b- and y- ions were selected by SPS after filtering for mass range of 300–2000 m/z, TMT6plex or TMT12plex tag addition, and a precursor ion exclusion window between –50 m/z and +5 m/z. HCD fragmentation of SPS ions was performed with a fixed collision energy of 55%. MS3 quadrupole isolation used an isolation window of 0.7 and an MS2 notch isolation window of 3 m/z, an AGC target of 2.5e5, and the Auto maximum injection time mode. MS3 spectra were then acquired in the Orbitrap at 50K resolution in Centroid mode with a scan range of 100–500 m/z. Cycle time was set for 3 seconds.