Supporting Information for

Conservation of Progesterone Hormone Function in Invertebrate Reproduction

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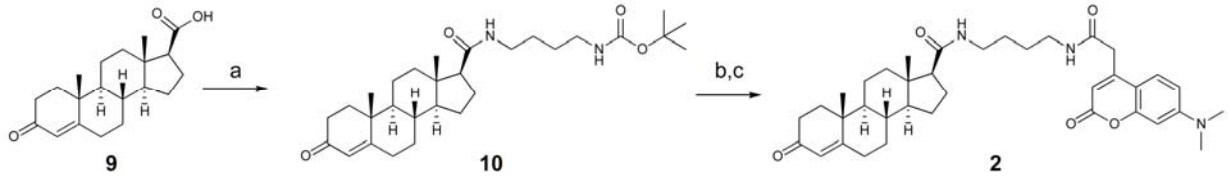
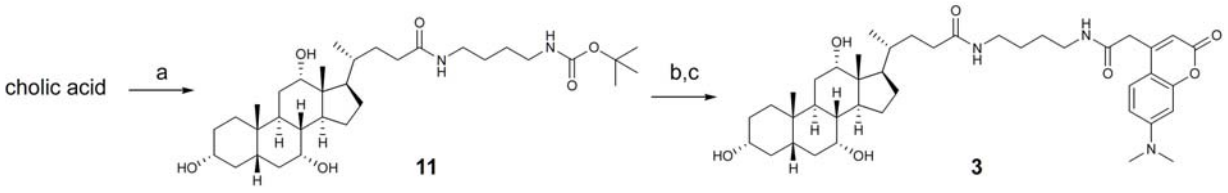
Contents:

I. Supporting schemes and figures

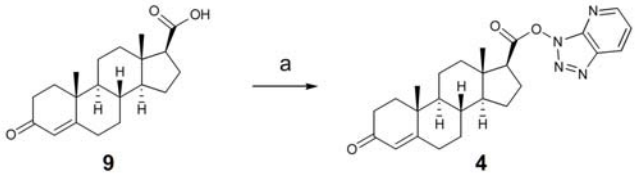
Supporting schemes



Scheme S1. Synthesis of IAF labeled progesterone probe **2**. Reagents and conditions: **a)** *N*-boc-1,4,-butyldiamine, HATU, DMF, 89%; **b)** TFA, CH_2Cl_2, rt; **c)** 7-dimethylamino-4-coumarinacetic acid, Et*i*Pr_2, HATU, DMF, 67% over two steps.



Scheme S2. Synthesis of IAF labeled control probe **3**. Reagents and conditions: **a)** *N*-boc-1,4-butanediamine, Et_3N, HATU, DMF, 80%; **b)** TFA, CH_2Cl_2, rt; **c)** 7-dimethylamino-4-coumarinacetic acid, Et_3N, HATU, DMF, 52% over two steps.



Scheme S3. Synthesis of reactive ester **4**. Reagents and conditions: **a)** HATU, DMF, 94%.

Supporting figures

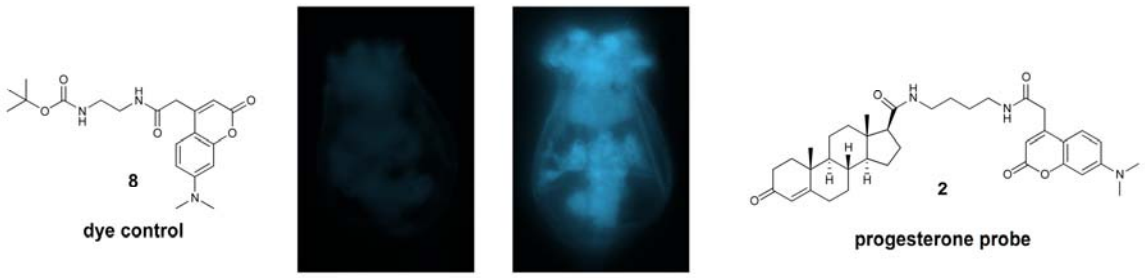


Figure S1. *Brachionus manjavacas* hatchlings incubated with the IAF-labeled progesterone probe **2** (right) and a fluorescent control **8** (left) at 10 μg/mL. Uptake of **8** was not detected under the conditions used to image hatchlings incubated with **2**.

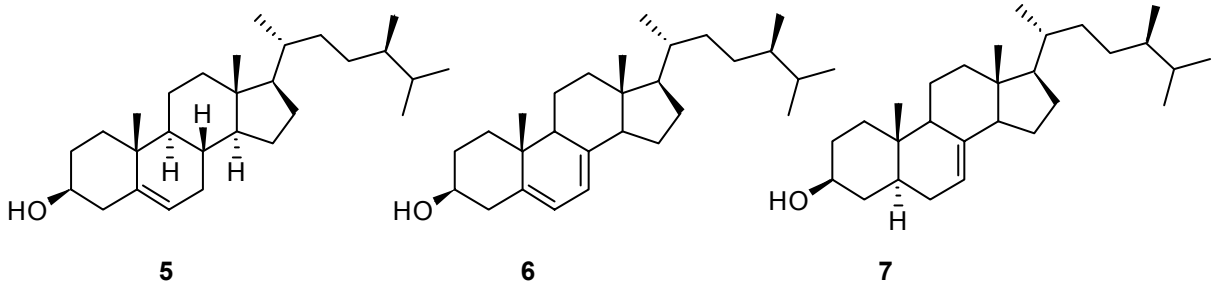


Figure S2. Steroids isolated from *Brachionus manjavacas* including campesterol (24*R*-ergost-5-en-3β-ol, **5**), campesta-5,7-dien-3-β-ol (**6**), and 5-α-campest-7-en-3-β-ol (**7**). Authentic standards of **5** and **7** were purchased from Sigma-Aldrich for verification of structures. Campesterol (Sigma-Aldrich product number C5157) was purchased as a 65:35 mixture of crystalline 24*R*-ergost-5-en-3β-ol and 24*S*-ergost-5-en-3β-ol, distinguishable via ^{13}C NMR spectroscopy. Carbon-13 NMR spectral data was acquired for both the authentic standard and the isolated rotifer steroid. ^{13}C NMR spectral data for the isolated rotifer steroid **5** were identical to 24*R*-ergost-5-en-3β-ol ^{13}C NMR chemical shifts, consistent with the hypothesis that **5-7** were of dietary (algal) origin. Sterol **6** was identified by comparing spectrometric data to reported literature values.

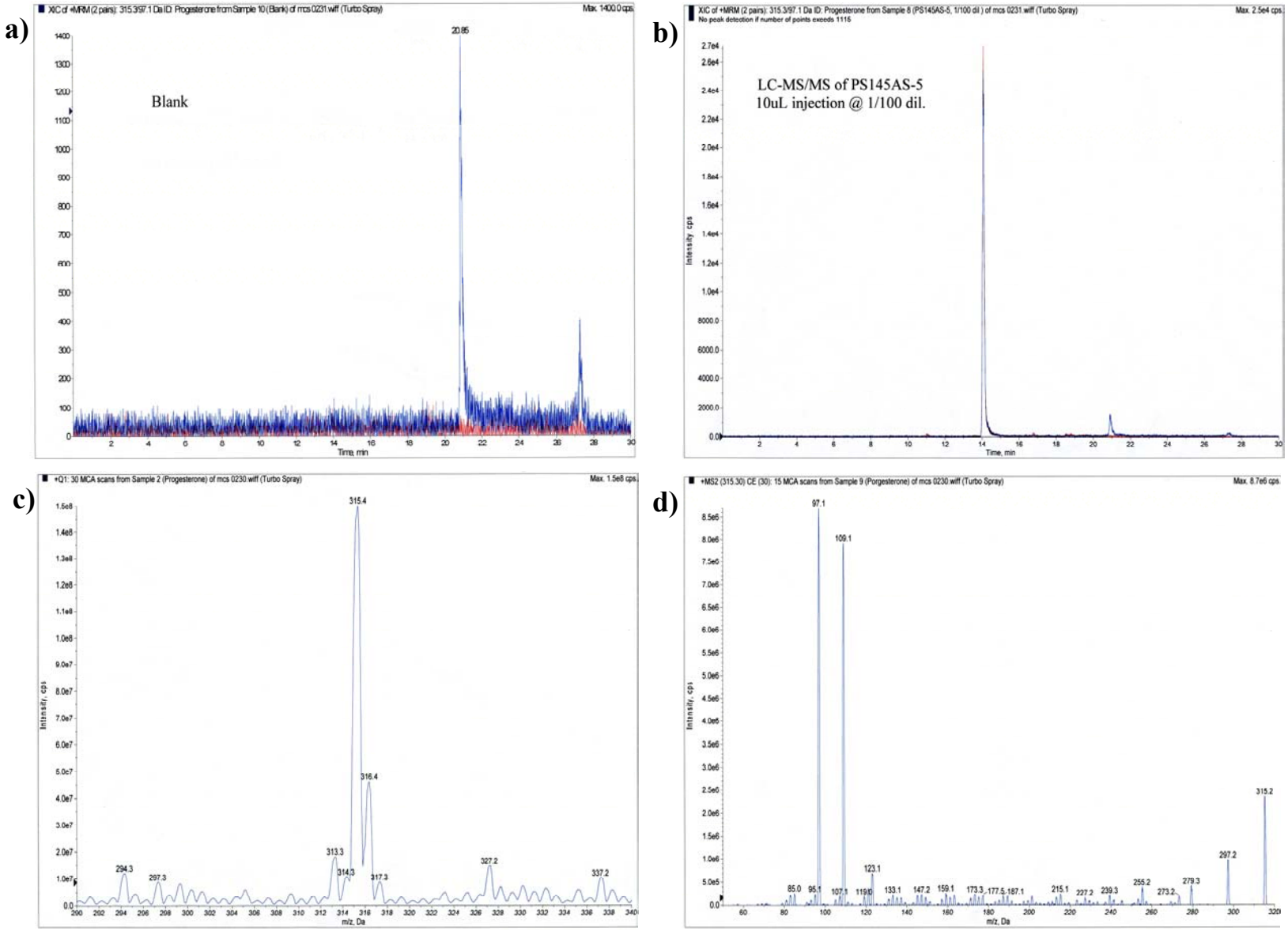


Figure S3. LC-MS/MS data operating in multiple reaction monitoring mode for analysis of progesterone in a partially purified rotifer steroid-containing fraction; **a)** blank injection prior to sample analysis; **b)** LC chromatogram of rotifer steroid sample, where progesterone elutes at 14 min.; **c)** mass spectrum of LC peak at 14 min corresponding to progesterone $[M+H]^+$ *m/z* 315.4; **d)** MS/MS daughter ions of *m/z* 109.1 and 97.1, corresponding to known progesterone fragmentation.

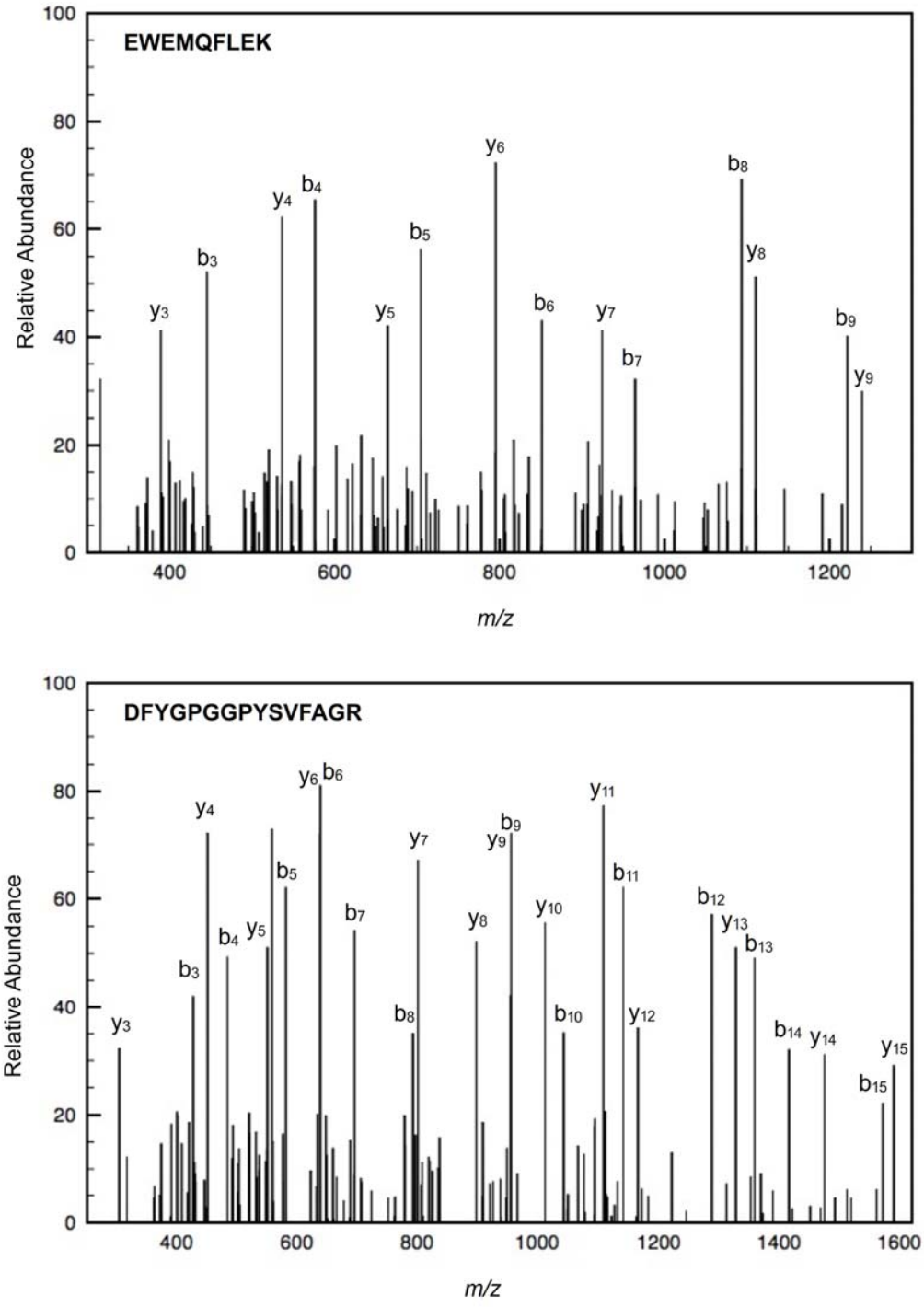


Figure S4. MS/MS detection of peptides: (top) daughter ions of *m/z* 1239.57 corresponding to EWEMQFLEK and (bottom) daughter ions of *m/z* 1589.74 corresponding to DFYGPGGPYSVFAGR.

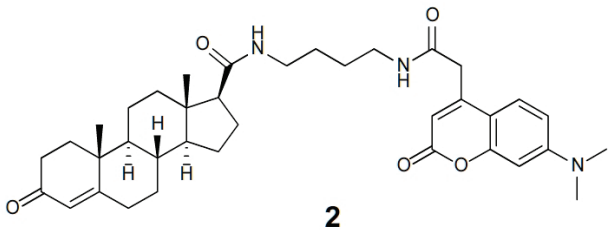
P1:	1	EWEMQFLEK	9	P2:	2	FYGPGGPYSVFAGR	15
A1:	160	EWEMQFMEK	168	A2:	103	FYGPGGPYSAFAGR	116
B1:	137	EWEMQFMEK	145	B2:	64	FYGPGGPYEVFAGR	77
C1:	163	EWEMQFMEK	171	C2:	91	FYGPGGPYSNFAGR	105

Figure S5. Blast searches on the respective peptides EWEMQFLEK (P1) and DFYGPGGPYSVFAGR (P2). Peptide A1 is found in the progestin membrane receptor component 2 from *Oncorhynchus mykiss* (gb|ABD58973.1|, 88% identities); B1 is found in the progesterone receptor membrane component 1 from *Strongylocentrotus purpuratus* (ref|XP_783332.1|, 88% identities); and C1 is found in the progesterone receptor membrane component 2 from *Danio rerio* (gb|AAH53415.1|, 88% identities). Peptide A2 is found in the progestin membrane receptor component 1 from *Penaeus monodon* (gb|ACC62174.1|, 92% identities); B2 is found in the steroid binding protein 1 from *Thalassiosira pseudonana* (gb|EED96317.1|, 92% identities); and C2 is found in the hypothetical protein FG02758.1 from *Gibberella zeae* PH-1 (ref|XP_382934.1|, 86% identities).

ATGGCC**CGACGGTTCTTTGACGATGT**CATATCATCTCCGGTCAATATTTTTCTAGTAGGTTTGATCT
GCTACTTCTCGTACAAATTGATCAAAAAAGATAGCACCAAATCGCCGGCGAGAAAAAACTCAAAAT
CAGAAAATGATTTGGCTAAAATGCCAAAACAAGATTTTACTTTGGAAGAGCTAAAGCAATATGACG
GGATTAAATCCGATGGACGTATTTTAATTGGGGTACTTGGCAAAGTTTTTGATGTTTCAAAAGCGAA
AGATTTCTATGGACCGGGCGGTCCGTATTCCGTATTTGCTGGTCGCGATGCATCCAGAGCGTTGGGC
ACTTTTTCTGTGGACAAATCCCAATTTAAAGATGAATACGATGATCTAAGCGATCTGAAGAGTTCTC
AGATGGAAAGTATCAAAGAATGGGAGATGCAATTTTTGGAAAAATATCCACTTGTTGGTAATTTGCT
AAGGCCTGGTGAAGAGCCCACTGTTT**ACGAAGAAGAGTCAGCCGAA**GTTAAAAAACAACTTTAA

Figure S6. ORF within the rotifer progesterone gene (GenBank accession FJ829246) from the *Brachionus manjavacas* cDNA database. This is the reverse complement of the contig sequence. Bold and underlined bases indicate primer sequences; green indicates area amplified for the dsRNA used in RNAi knockdown experiments.

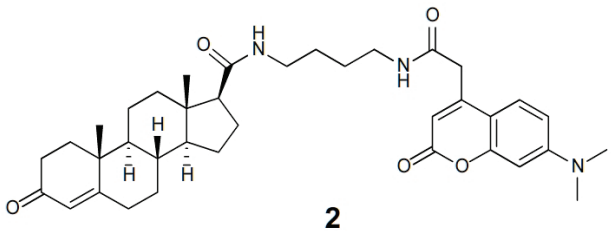
Figure S7a



2

^{1}H-NMR
500 MHz
acetone-d_6

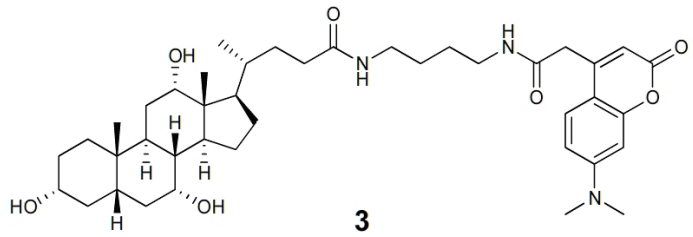
Figure S7b



2

^{13}C-NMR
125 MHz
acetone-d_6

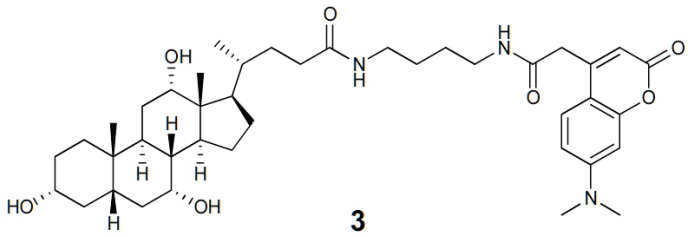
Figure S7c



3

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

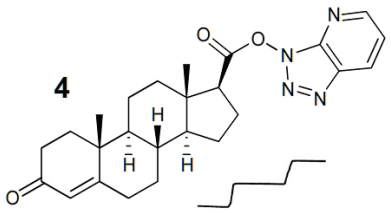
Figure S7d



3

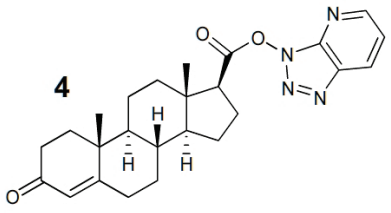
^{13}C-NMR
125 MHz
$CDCl_3$

Figure S7e



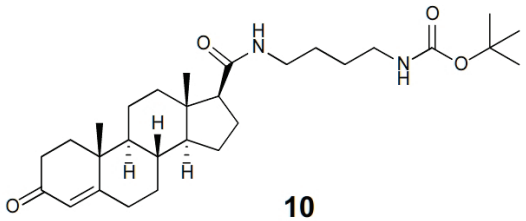
^{1}H-NMR
500 MHz
$CDCl_3$

Figure S7f



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

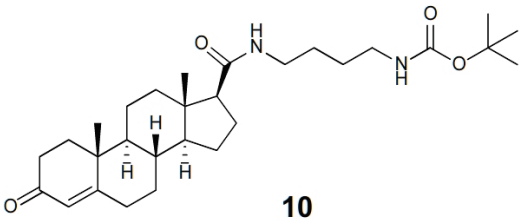
Figure S7g



10

^{1}H-NMR
500 MHz
acetone-d_6

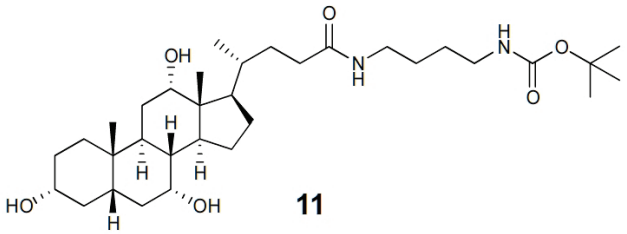
Figure S7h



10

^{13}C-NMR
125 MHz
acetone-d_6

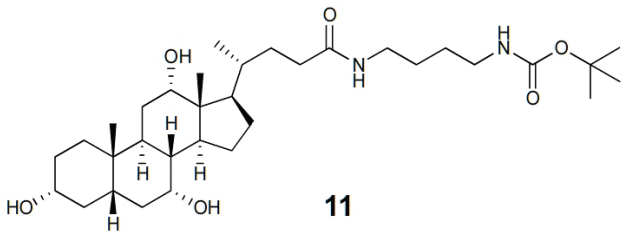
Figure S7i



11

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

Figure S7j



11

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

II. Supporting Western Blot methods

Western blotting studies using reactive probe 4

The method was first tested by screening for PR expression in a MCF-7 cell lysates. Whole MCF-7 cell lysate was prepared by homogenization in modified RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml pepstatin-A, 2 μg/ml lLeupeptin, 1 mM Na_3VO_4, 1 mM NaF). The debris was removed by centrifugation and the protein concentration was concentrated to 1 mg/mL using a 3 kDa cutoff filter. An aliquot of this lysate (500 μL) was treated with 20 μL of a 1 mg/mL stock of **4** in DMSO at 4 °C. A 50 μL sample was taken at 4 h and boiled for 5 min in SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% SDS, 0.01% bromophenol blue containing 5% β-mercaptoethanol). SDS-PAGE gel analysis was conducted on a Novex X-cell station using NuPage 4-12% Bis-Tris gels and MOPS SDS running buffer. Western blotting was conducted by transfer to Hybond-P PVDF membrane by GE Healthcare (Piscataway, NJ) followed by blocking for 2 h with a solution of 0.1% Tween-20 with 5% w/v nonfat dry milk 20 in tris-buffered saline (TBS, pH 7.6). One lane (lane L5, **Fig. 3c**) was stained with a mouse anti-progesterone receptor 00005241-M01 Anti-PGR (1-110) mAb (anti-PR) from Abnova (Ann Arbor, MI), in 0.1% Tween-20 with 5% BSA in TBS, and a Goat anti-mouse IgG HRP mAb conjugate from Promega (Madison, WI) in 0.1% Tween-20 with 5% BSA in TBS. A second lane (lane L6, **Fig. 3c**) was stained a mouse anti-progesterone IgG mAb (anti-P) from Assay Designs (Tapei City, Taiwan), in 0.1% Tween-20 with 5% BSA in TBS, and a Goat anti-mouse IgG HRP mAb conjugate from Promega (Madison, WI) in 0.1% Tween-20 with 5% BSA in TBS. For both experiments, the primary and secondary antibodies were applied at 1:100 and 1:2,000 dilution, respectively, from their manufactures preparation. The total protein content in each gel was determined by staining with ponceau S from Promega (Madison, WI).

III. Synthetic procedures and compound characterization.

Copies of ^{1}H and ^{13}C NMR spectra on each compound are provided in Figure S7.

***tert*-butyl 4-((8S,9S,10R,13S,14S,17S)-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro-1H-cyclopenta[α]phenanthrene-17-carboxamido)butylcarbamate (10).** HATU (157 mg, 0.41 mmol) was added to a mixture of 3-keto-4-etiocholenic acid **9** (65 mg, 0.021 mmol), *N*-boc-1,4-butyldiamine (53.5 mg, 0.28 mmol), and $EtN^{i}Pr_2$ (108 μL, 0.62 mmol) in anhydrous DMF (5 mL). The reaction was allowed to stir for 12 h at rt and then dried under vacuum. Any remaining HATU was precipitated with CH_2Cl_2 and removed via filtration. Compound **10** (94 mg) was obtained as a colorless oil, 89% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.98 (bs, 1H); 6.99 (bs, 1H); 6.51 (s, 1H); 3.63 (m, 1H); 3.35 (m 3H); 2.90 (s, 6H); 2.66-2.41 (m, 2H); 2.36-2.22 (m, 1H); 2.27-2.16 (m, 3H); 2.11-2.01 (m, 2H); 2.00-1.88 (m, 1H); 1.87-1.73 (m, 1H); 1.67-1.42 (m, 6H); 1.41-1.25 (m, 5H); 1.23 (s, 3H); 1.19-1.05 (m, 3H); 1.02 (s, 3H); 0.94-0.58 (m, 4H); 0.39 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 197.5; 171.7; 170.3; 155.9; 123.5; 77.5; 56.4; 55.6; 54.1; 43.6; 39.9; 38.6; 38.5; 38.2; 37.9; 35.8; 35.6; 33.7; 32.5; 32.1; 27.9; 27.5; 27.2; 24.4; 23.5; 20.8; 16.8; 13.0. HR-ESI-MS (*m/z*): Calcd for $C_{31}H_{51}N_2O_4$ [M + H], 515.3771; Found 515.3784.

(*R*)-*N*-(4-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)butyl)-4-((3*R*,5*S*,7*R*,8*R*, 9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta-[α]]phenanthren-17-yl)pentanamide (2). Intermediate **10** (54 mg, 0.10 mmol) was dissolved in CH_2Cl_2 (1 mL). Trifluoroacetic acid (0.020 mL, 0.285 mmol) was added dropwise and the reaction was allowed to stir at room temperature for 1 h. The reaction was then diluted with CH_2Cl_2 (10 mL) and washed with 0.001 N NaOH (1 mL) and water. The organic layer was concentrated *in vacuo*, and crude amine was used without further purification. HATU (80 mg, 0.20 mmol) was added to a solution of the crude amine, 7-dimethylamino-4-coumarinacetic acid (31 mg, 0.13 mmol), and $EtN^{i}Pr_2$ (55 μL, 0.31 mmol) in anhydrous DMF (1.5 mL). The reaction was allowed to stir for 16 h and then dried. Remaining HATU was precipitated with MeOH and removed via filtration. The crude product was subjected to flash chromatography (2:1 hexanes/EtOAc to 1:5 MeOH/EtOAc) with **2**, eluting with 1:10 MeOH/EtOAc, collected as a yellow wax (45 mg, 67% yield). ^{1}H NMR (acetone-d_6, 500 MHz) δ: 9.44 (s, 2H); 8.99 (d, 1H, *J* = 11.3Hz) ;8.94 (bm, 1H); 8.00 (bm, 1H); 7.91 (dd, 1H, *J* = 3.2, 11.2 Hz); 7.72 (d, 1H, *J* = 3.2 Hz); 7.01 (s, 1H); 6.50 (s, 1H); 4.35 (m, 2H); 4.21 (dt, 1H, *J* = 8.0, 8.0, 16.1 Hz); 4.06 (s, 2H); 3.70 (m, 2H); 3.59 (m, 1H); 3.51 (m, 2H); 3.39 (m, 1H); 3.34 (s, 2H); 2.90 (s, 6H); 2.61-2.42 (m, 3H); 2.33 (ddd, 1H, *J* = 3.0, 5.0, 18.1 Hz); 2.26-2.17 (m, 2H); 2.09-1.97 (m, 1H); 1.95-1.78 (m, 3H); 1.62-1.42 (m, 4H); 1.41-1.22 (m, 2H); 1.28 (s, 3H); 1.27 (s, 3H); 1.24 (d, 3H, *J* = 9.1 Hz); 1.17-1.04 (m, 4H); 1.01 (s, 3H); 0.90-0.63 (m, 4H); 0.35 (s, 3H). HR-ESI-MS (*m/z*): Calcd for $C_{37}H_{50}N_3O_5$ [M + H], 616.3672; Found 616.3618.

***t*-butyl 4-((*R*)-4-((3*R*,5*S*,7*R*,8*R*,9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,7,12-trihydroxy-10,13-dimethyl-hexadecahydro-1H-cyclopenta[α]phenanthren-17yl)pentanamido)butylcarbamate (11).** HATU (175 mg, 0.46 mmol) was added to a solution of cholic acid (100 mg, 0.24 mmol), *N*-boc-1,4-butanediamine (95 mL, 0.49 mmol), and Et_3N (0.15 mL, 1.1 mmol) in anhydrous DMF (1.5 mL). The reaction was allowed to stir for 16 h, then diluted with deionized H_2O (20 mL) and extracted with

EtOAc (20 mL). The organics were washed three times with deionized H_2O (15 mL), dried over $MgSO_4$, and concentrated *in vacuo.* Any remaining HATU was precipitated with CH_2Cl_2 and removed via filtration. Compound **11** (114 mg) was obtained as a clear oil, 80% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 6.58 (br s, 1H); 4.92 (br s, 1H); 3.90 (s, 1H); 3.78 (s, 1H); 3.41 (s, 9H); 3.17 (br d, 2H, *J* = 5.4 Hz); 3.06 (br d, 2H, *J* = 4.6 Hz); 2.27 (br s, 2H); 2.15 (t, 2H, *J* = 13.2 Hz); 1.14 (m, 1H); 1.88-1.75 (m, 2H); 1.70 (t, 3H, *J* = 13.7 Hz); 1.65-1.49 (m, 2H); 1.47 (br s, 6H); 1.38 (s, 12H); 1.2 (m, 1H); 1.02 (m, 1H); 0.94 (d, 3H, *J* = 5.5 Hz); 0.83 (s, 3H); 0.62 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 174.5; 156.3; 79.1; 73.1; 71.8; 68.4; 50.4; 46.3; 41.5; 41.3; 40.1; 39.5; 39.3; 39.0; 35.3; 35.2; 34.7; 34.6; 32.9; 31.7; 30.2; 28.3 (3C); 28.0; 27.5; 27.4; 26.6; 26.2; 23.2; 22.3; 17.4; 12.4. HR-ESI-MS (*m/z*): Calcd for $C_{33}H_{58}N_2O_6Na$ [M + Na], 601.4192; Found 601.4176.

(*R*)-*N*-(4-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)butyl)-4-((3*R*,5*S*,7*R*,8*R*, 9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta-[α]-phenanthren-17-yl)pentanamide (3). Intermediate **11** (55 mg, 0.095 mmol) was dissolved in CH_2Cl_2 (1 mL). Trifluoroacetic acid (0.020 mL, 0.285 mmol) was added dropwise and the reaction was allowed to stir at room temperature for 1 h. The reaction was then diluted with CH_2Cl_2 (10 mL) and washed with deionized H_2O (10 mL). The organic layer was concentrated *in vacuo*, and crude amine was used without further purification. HATU (63 mg, 0.167 mmol) was added to a solution of the crude amine, 7-dimethylamino-4-coumarinacetic acid (21 mg, 0.084 mmol), and Et_3N (0.035 mL, 0.25 mmol) in anhydrous DMF (1.5 mL). The reaction was allowed to stir for 16 h, then diluted with deionized H_2O (25 mL) and extracted with EtOAc (25 mL). The organics were washed 3× with deionized H_2O (20 mL), dried over $MgSO_4$, and concentrated *in vacuo.* Remaining HATU was precipitated with MeOH and removed via filtration. The organics were subjected to reversed-phase HPLC, and **2**, eluting with 58% aqueous acetonitrile, was collected as a yellow powder (35 mg, 52% yield). ^{1}H NMR ($CDCl_3$, 500 MHz) d: 7.49 (d, 1H, *J* = 9.0 Hz); 7.00 (t, 1H, *J* = 5.2 Hz); 6.57 (dd, 1H, *J* = 9.0, 2.4 Hz); 6.45 (t, 1H, *J* = 5.2 Hz); 6.39 (d, 1H, *J* = 2.4 Hz); 6.05 (s, 1H); 3.92 (br m, 1H); 3.8 (br s, 1H); 3.61 (s, 2H); 3.46 (d, 1H, *J* = 5.5 Hz); 3.40 (br m, 1H); 3.25-3.10 (m, 4H); 3.00 (s, 6H); 2.65 (s, 1H); 2.18 (t, 2H, *J* = 11.7 Hz); 2.10-1.95 (m, 4H); 1.79 (m, 2H); 1.82-1.53 (m, 8H); 1.48 (br m, 9H); 1.36 (m, 2H); 1.21 (m, 1H); 1.07 (m, 1H); 0.96 (d, 3H, *J* = 5.8 Hz); 0.85 (s, 3H); 0.63 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) d: 174.5; 168.5; 162.3; 155.9; 153.0; 150.7; 125.9; 109.9; 109.2; 108.6; 98.0; 73.0; 71.9; 68.4; 46.4; 46.2; 41.8; 41.5; 40.3; 40.1 (3C); 39.7; 39.6; 39.5; 38.9; 35.3; 34.7 (2C); 32.7; 31.7; 30.5; 28.2; 27.6; 27.0; 26.4; 26.3; 23.2; 22.5; 17.5; 12.5. HR-ESI-MS (*m/z*): Calcd for $C_{41}H_{62}N_3O_7$ [M + H], 708.4582; Found 708.4583.

(*R*)-*N*-(4-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)butyl)-4-((3*R*,5*S*,7*R*,8*R*, 9*S*,10*S*,12*S*,13*R*,14*S*,17*R*)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta-[a]phenanthren-17-yl)pentanamide (4). HATU (157 mg, 0.41 mmol) was added to a mixture of 3-keto-4-etiocholenic acid **9** (65 mg, 0.021 mmol), and EtN^iPr_2 (108 µL, 0.62 mmol) in anhydrous DMF (5 mL). The reaction was allowed to stir for 12 h at rt and then dried under vacuum. Compound **4** (84 mg) was obtained as a clear wax, 94% yield after flash chromatography (2:1 hexanes/EtOAc to 1:10 MeOH/EtOAc). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.70 (d, 1H, *J* = 4.5 Hz); 8.40 (d, 1H J = 8.4 Hz); 7.48 (dd, 1H, *J* = 4.5, 8.4 Hz); 5.73 (s, 1H); 4.09 (dd, 1J, *J* = 7.1, 7.1 Hz); 2.89 (t, 1H, *J* = 9.3 Hz); 2.49-2.16

(m, 6H); 2.14-2.03 (m, 2H); 1.95-1.77 (m, 5H); 1.76-1.37 (m, 2H); 1.35-1.22 (m, 1H); 1.21 (s, 3H); 1.18-1.00 (m, 2H); 1.00 (s, 3H). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 199.7; 170.8; 170.2; 151.8; 140.8; 135.2; 129.7; 124.3; 121; 60.6; 55.8; 53.8; 52.9; 45.6; 38.8; 38; 36.1; 36; 34.2; 32.9; 32.1; 24.8; 24.2; 21.3; 17.6; 14.4; 13.8. HR-ESI-MS (*m/z*): Calcd for $C_{25}H_{31}N_4O_3$ [M + H], 435.2318; Found 435.2389.