Supporting Information: Identification of PDE6D as a molecular target of anecortave acetate via a methotrexate-anchored yeast three-hybrid screen.

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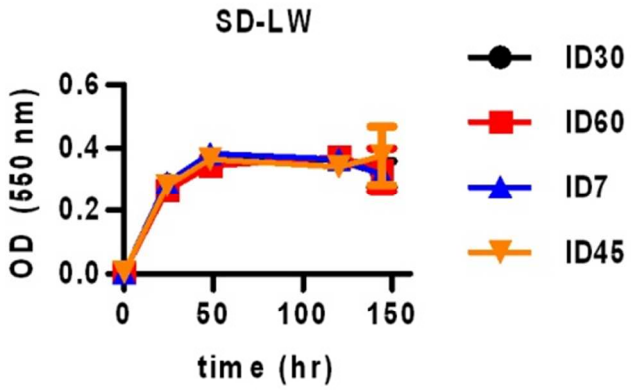
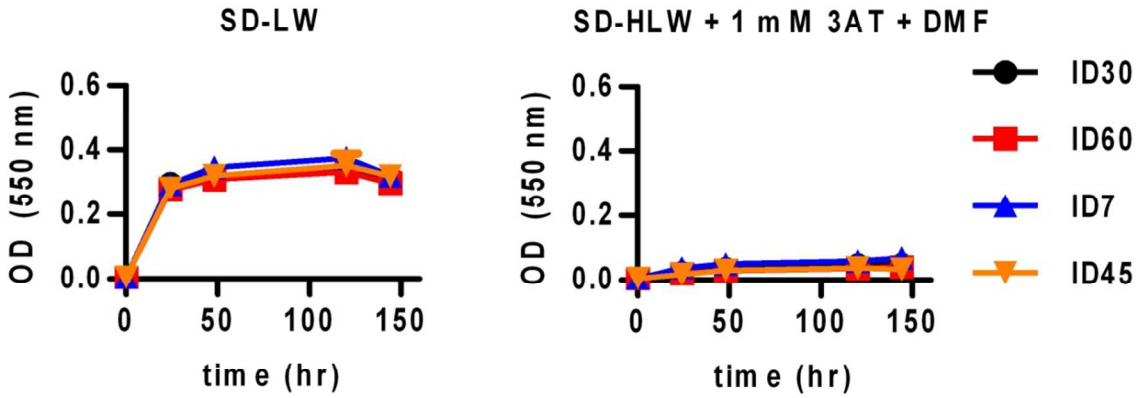
Supplementary Figure 1, related to Figure 4b.



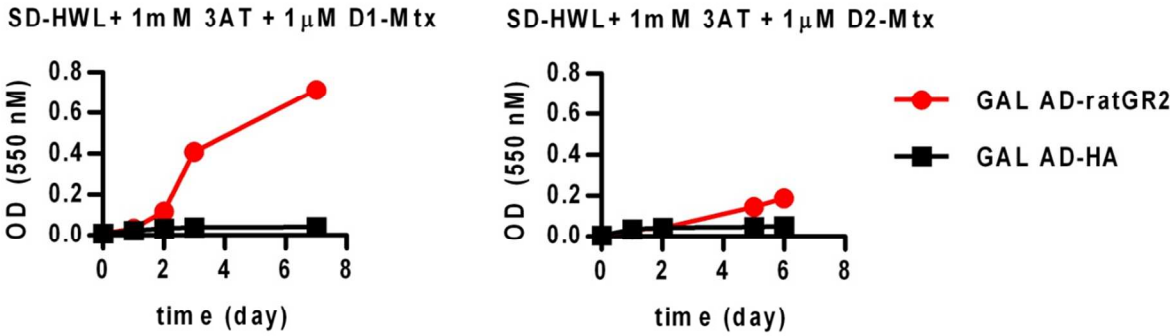
Control experiments measuring growth of selected hit clones from the A1-Mtx Y3H screen in non-selective media (SD with leucine and tryptophan) after retransformation demonstrated that expression of the clones was not toxic to yeast.

Supplementary Figure 2, related to Figure 4d:



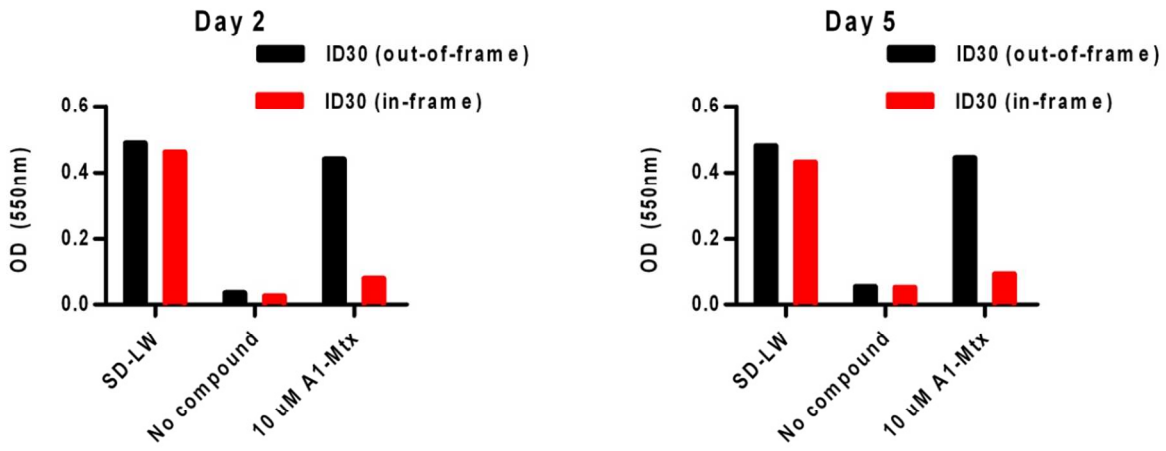
Control experiments measuring growth of selected hit clones from the A1-Mtx Y3H screen in non-selective media (SD with leucine and tryptophan) and selective media (SD with histine, tryptophan and leucine) after retransformation verified the veracity of the system.

Supplementary Figure 3.



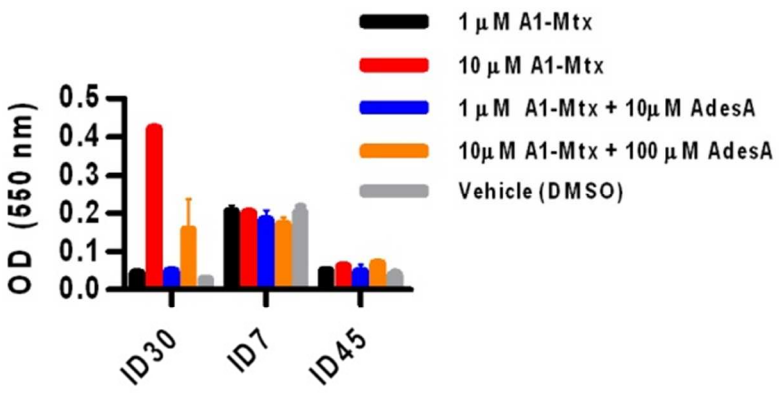
Growth of the positive control strain, GAL AD-ratGR2 (expresses a fusion between the GAL4 activation domain and the rat glucocorticoid receptor), and the negative control strain, GAL AD-HA (expresses a fusion between the GAL4 activation domain and HA) were grown under selective conditions with either 1 μM D1-Mtx or 1 μM D2-Mtx.

Supplementary Figure 4.



Interaction of in-frame and out-of-frame ID30 hits with A1-Mtx. Yeast expressing either the original out-of-frame ID30 hit or the recloned in-frame ID30 were grown in non-selective medium (SD-WL) or selective medium in the presence (10 μM A1-Mtx) or absence (No compound) of A1-Mtx and cell growth was measured after 2 and 5 days. Only the out-of-frame ID30 hit originally identified in the screen displayed A1-Mtx dependent growth.

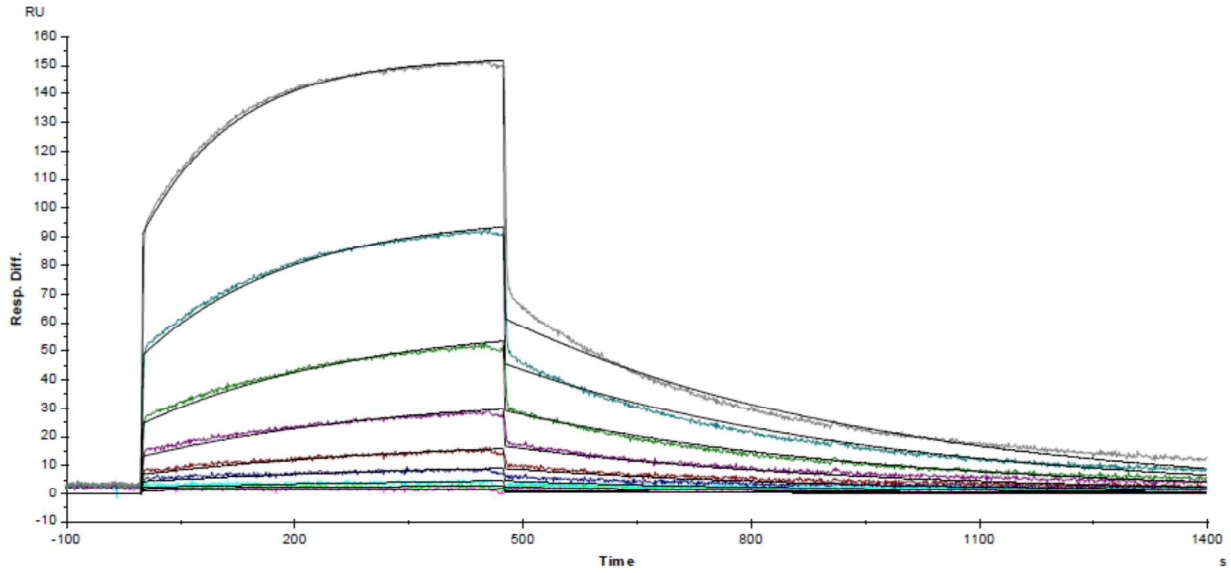
Supplementary Figure 5.



Y3H competition assays with ID30, ID7 and ID45. Clones were grown in the presence of A1-Mtx and 10x AdesA to determine whether Y3H interactions could be competed off by excess AdesA. Only the Y3H interaction between ID30 and A1-Mtx at 10µM could be competed off with 10X excess AdesA. No competition was observed with ID7 and ID45.

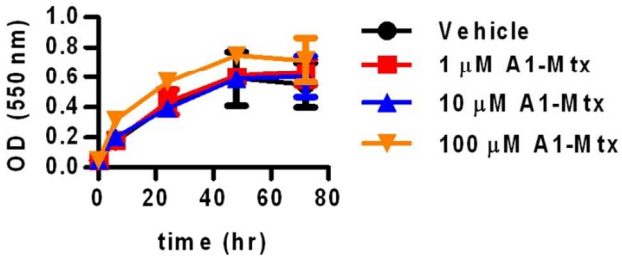
Supplementary Figure 6.

Kinetic mode		
k_{diss} in s^{-1}	k_{ass} in M^{-1} s^{-1}	K_D in µM
2.1×10^{-3}	7.6×10^{2}	2.7



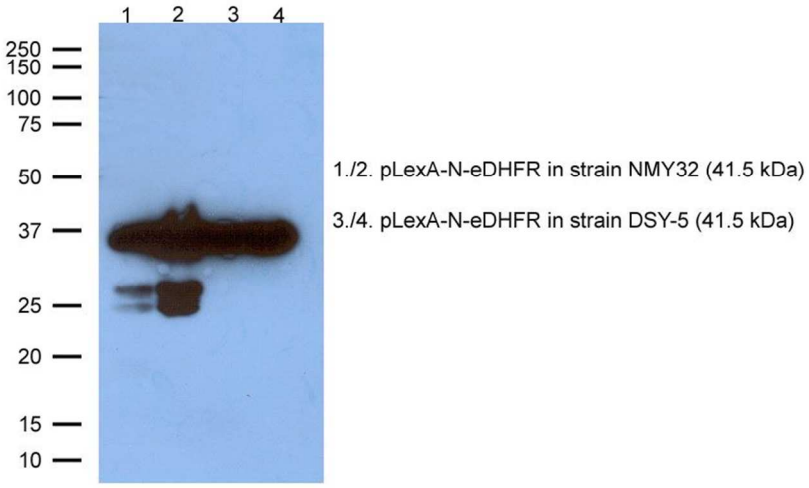
Dissociation constants of A1-Mtx interaction with PDE6D measured by surface plasmon resonance. PDE6D (31.3 nM - 8µM) binding to A1-Mtx captured on DHFR (30 µl/min, 25°C).

Supplementary Figure 7.



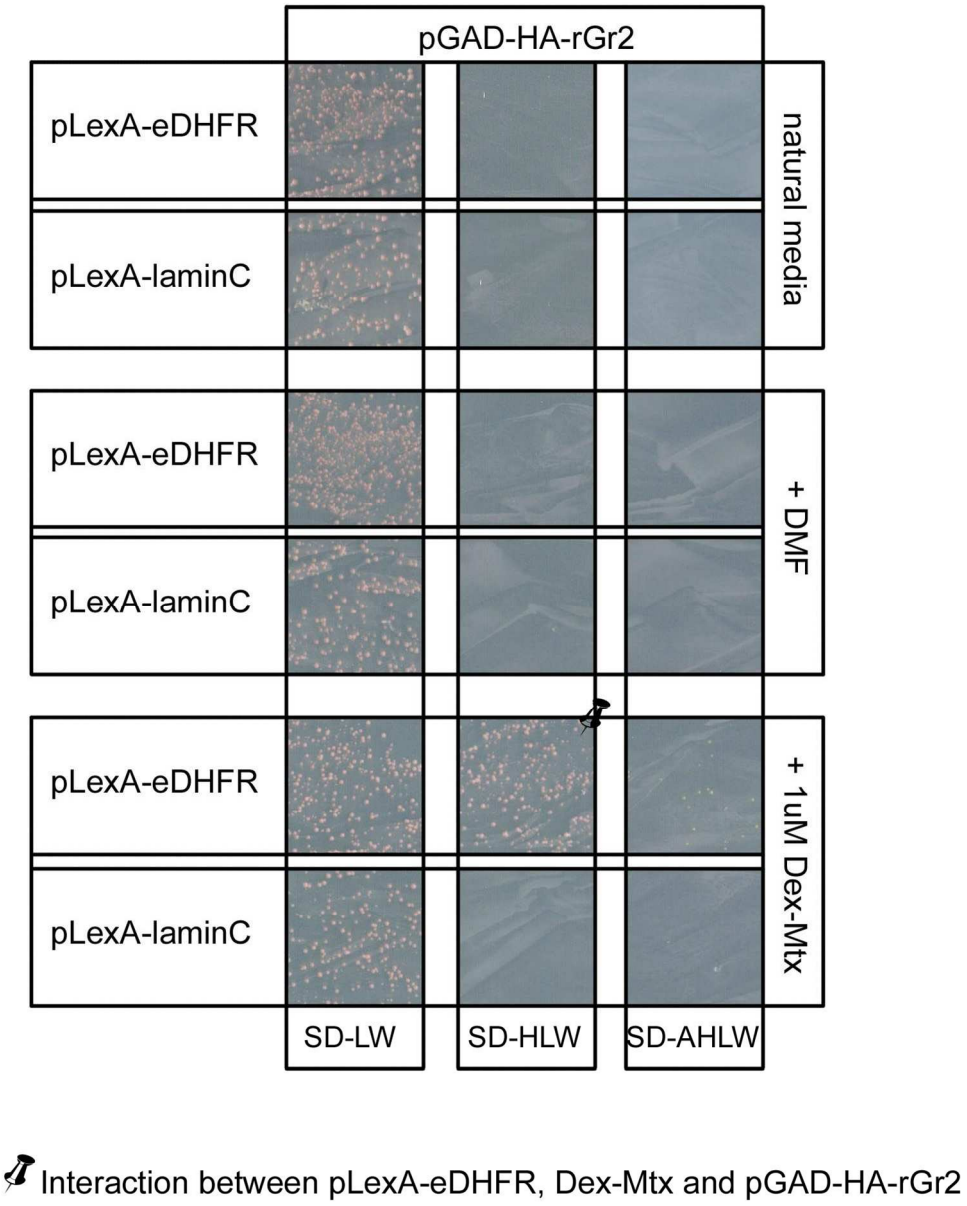
Effect of A1-Mtx (1, 10, 100 μM) or vehicle on time-dependent growth of yeast strain NMY51-C3 containing pLexA-DHFR. The A1-Mtx CID was shown to be non-toxic to yeast cell growth up to 100 μM.

Supplementary Figure 8.



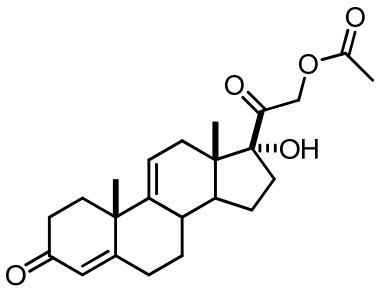
Western blot analysis of cellular supernatant (lanes 1 and 3) and pellet (lanes 2 and 4) from yeast (strains NMY32 or DSY-5) transformed with either hook (pLexA-N-eDHFR) or fish (pGAD-HA-rGR2) vectors.

Supplementary Figure 9.



Pairwise combinations of hook vectors pLexA-eDHFR or pLexA-laminC and fish vector pGAD-HA-rGR2 transformed into yeast and checked for growth in minimal medium lacking amino acids tryptophan, leucine, and histidine (selective medium) with bait (D1-Mtx) or vehicle (DMF).

Chemical synthesis and analysis

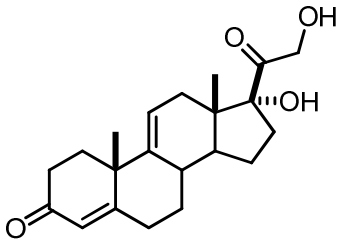


Anecortave acetate (AA)

Anecortave acetate (17α,21-dihydroxy-4,9(11)-pregnadien-3,20-dione-21-acetate). A method of D. R. Buss, US Patent 3,441,559, was adapted (Note 1). Under Ar, a mixture of cortisol 21-acetate (200 g) and dry pyridine (0.85 L) was stirred and heated to 50 °C to give a clear solution, then cooled in ice. To this solution was added a solution of SO_2 (42 g) in dry pyridine (0.30 L) at <10 °C and then a solution of Br_2 (26 mL) in CH_2Cl_2 (0.18 L) at <10 °C. After a further 2 h at <10 °C the product was collected by filtration and air dried, then slurried with MeOH (2 L) and filtered. This slurry/filtration procedure was repeated twice. A final wash with 1 L of MeOH was followed by air drying to give 187 g of a solid. Crystallization from 98:2 (v/v) glyme/water (Note 2) followed by drying at 110 °C/0.1 Torr afforded anecortave acetate (91%) as fine spars, mp 238-240 °C, $[\alpha]_D$ (*c* 1.0, $CHCl_3$) +126°, lit. mp 239.5-241 °C, $[\alpha]_D$ +120°: S. Bernstein, R. Littell and J. H. Williams, *J. Am. Chem. Soc.* **1953**, *75(19),* 4830-4832; Anal. Calcd for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.44, 71.54; H, 7.85, 7.85. IR (KBr) ν 3436 (br), 3054, 1746, 1722, 1654, 1615 (w), 1415, 1240 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.66 (s, 3H), 1.34 (s, 3H), 1.0-2.9 (m, 17H), 2.18 (s, 3H), 4.97 (AB, *J* = 18, Δν = 42 Hz, 2H), 5.56 (br d, 1H), 5.74 (d, *J* = 1.6, 1H); ^{13}C NMR ($CDCl_3$) δ 14.3, 20.5, 24.3, 26.2, 31.9, 32.2, 32.8, 33.8, 34.0, 34.7, 37.6, 41.0, 46.5, 47.9, 67.8, 89.6, 118.7, 124.0, 144.1, 169.8, 170.6, 199.4, 204.9.

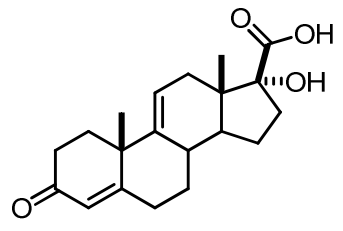
Notes: (1) Other methods of synthesis: J. G. Reid and T. Debiak-Krook, *Tetrahedron Lett.* **1990**, *31(26),* 3669-3772; D. A. Livingston, J. E. Petre and C. L. Bergh, *J. Am. Chem. Soc.* **1990**, *112(17),* 6449-6450; R. Breslow, M. Brandl, J. Hunger and A. D. Adams, *J. Am. Chem.*

Soc. **1987**, *109(12),* 3799-3801; V. Van Rheenen and K. P. Shephard, *J. Org. Chem.* **1979**, *44(9),* 1582-1584. (2) *n*-Propyl acetate is also a useful crystallization solvent.



Anecortave desacetate (AdesA)

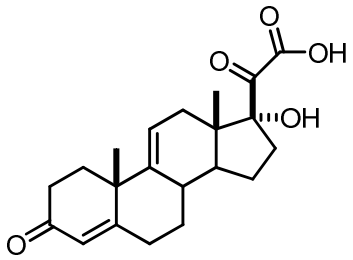
Anecortave desacetate (17α,21-dihydroxy-4,9(11)-pregnadien-3,20-dione). Under N_2, a mixture of anecortave acetate (270 g), potassium acetate (270 g) and MeOH (17 L) was stirred and heated to reflux over 2 h, held at reflux for 4.5 h, then cooled to RT over 64 h. The crystalline product was collected by filtration, washed with MeOH (4 x 0.4 L) and MTBE (4 x 1 L), then dried in vacuo at 80 °C to give 202 g (84%) of anecortave desacetate, mp 247-250 °C, lit. mp 243-249 °C: US Patent 4,921,638 ex. 62; 259-260 °C: Bernstein, *op. cit.* Anal. Calcd for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 73.14; H, 8.20. IR (KBr) ν 3518, 3461 (br), 2923, 1711, 1662 (br), 1612 (w), 1232, 1102 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.49 (s, 3H), 1.02 (q, 1H, *J* = 12), 1.30 (s, 3H), 1.32 (m, 1H), 1.6 (m, 2H), 1.8 (m, 2H), 2.0 (m, 3H), 2.3 (m, 3H), 2.5 (m, 1H), 2.6 (m, 3H), 4.3 (AB, 2H, *J* = 19, Δν = 82.5 Hz, further split (*J* = 5) by OH), 4.6 (t, *J* = 5, 1H, OH), 5.28 (s, 1H, OH), 5.52 (d, 1H, *J* = 5), 5.66 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 14.4, 23.9, 25.8, 31.9, 32.0, 32.1, 33.2, 33.4, 33.9, 36.9, 40.5, 45.4, 47.5, 65.8, 88.0, 118.3, 123.2, 144.2, 169.5, 197.7, 211.7.



A1

A1 (17α-hydroxy-4,9(11)-androstadien-3-one-17β-carboxylic acid). To a stirred suspension of anecortave desacetate (15.0 g) in 1.0 L of EtOH was added a solution of

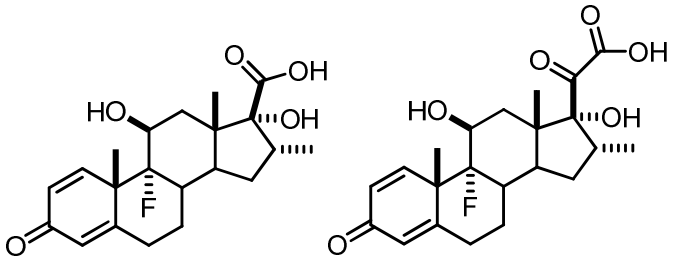
$NaIO_4$ (9.8 g) in 0.25 L of water, followed by careful addition of 27 mL of H_2SO_4. After 3 h the mixture was poured into 10 L of water and the precipitated solid was collected by filtration. This material was dissolved in 0.5 L of 5% aq NaOH plus 0.4 L of water followed by filtration through Celite. The filtrate was acidified with 50 mL of conc aq HCl to pH 2. The precipitated product was collected by filtration, washed with water and dried in vacuo at 50 °C to give 12.5 g (87%) of **A1**, mp 248-252 °C (dec), lit. mp 267-268 °C: US Patent 4,252,729; TLC (silica plate, 9:1 CH_2Cl_2/*i*-PrOH): Rf 0.5; HPLC (C18) purity 96%; Anal. Calcd for $C_{20}H_{26}O_4 \cdot 0.2H_2O$: C, 71.91; H, 7.97. Found: C, 72.04; H, 7.79. APCI MS $(M+H)^+$ = 331; ^{1}H NMR (DMSO-d_6) δ 0.63 (s, 3H), 1.00 (br q, *J*=13, 1H), 1.31 (s, 3H), 1.33 (br s, 1H), 1.60-1.85 (m, 4H), 1.95-2.13 (m, 3H), 2.18-2.37 (m, 3H), 2.44 -2.65 (m, 4H), 4.9 (v br, 1H, OH), 5.53 (d, J=5.5, 1H), 5.66 (s, 1H), 12.2 (v br, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 14.9, 23.9, 25.8, 31.9, 32.0, 32.1, 33.3, 33.4, 33.9, 37.0, 40.6, 45.2, 46.5, 84.2, 118.7, 123.2, 144.1, 169.7, 174.9, 197.8.



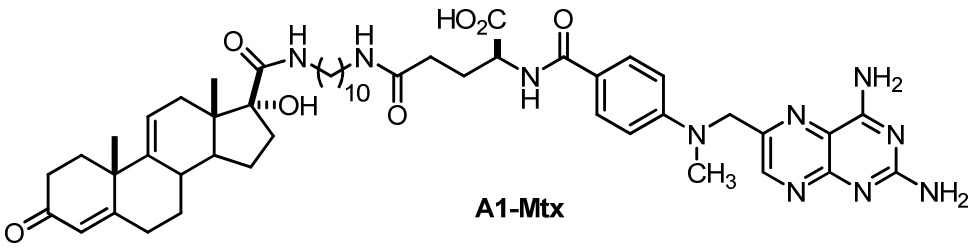
A2

A2 (17α-hydroxy-4,9(11)-pregnadien-3,20-dione-21-oic acid). A solution of SO_3–pyridine complex (1.9 g, 12 mmol) in 10 mL of dry DMSO was prepared and allowed to stand at RT for 0.7 h. This solution was then added to a stirred solution of anecortave desacetate (1.38 g, 4.00 mmol) and Et_3N (7.0 mL, 50 mmol) in 10 mL of dry DMSO. After 1 h the mixture was poured into sat aq KH_2PO_4 and extracted with EtOAc. The organic solution was washed with sat aq KH_2PO_4 (to pH 5), water and brine, dried ($MgSO_4$), filtered and concentrated to give 1.45 g of a semisolid. To a solution of this aldehyde in 15 mL of *t*-BuOH was added a 2.0 M solution of 2-methyl-2-butene in THF (15 mL, 30 mmol) and 10 mL of sat aq KH_2PO_4. The mixture was stirred in an RT water bath and a solution of $NaClO_2$ (80%, 0.52 g, 4.5 mmol) in 5 mL of water was added. After 18 h, the mixture was diluted with EtOAc, the phases were separated and the organic phase was extracted twice with water.

The combined aqueous extract (pH 5) was acidified to pH 2 with aq $NaHSO_4$ to give a precipitate that was extracted into EtOAc. This EtOAc solution was dried ($MgSO_4$), filtered and concentrated to give 0.22 g of a solid, which was slurried with 20 mL of sat aq KH_2PO_4 and 20 mL of Et_2O to give a clear biphasic mixture. The ether was allowed to evaporate over 3 days. The solid was removed by filtration and the filtrate was adjusted to pH 2 with aq $NaHSO_4$ to give a precipitate that was extracted into EtOAc. This EtOAc solution was dried ($MgSO_4$), filtered and concentrated to give 0.07 g of a solid. Trituration with 2 mL of Et_2O followed by drying in vacuo at 50 °C afforded 0.05 g (4%) of **A2**, mp 176-177.5 °C (dec), TLC (silica plate, 9:1 CH_2Cl_2/*i*-PrOH): Rf 0.05; Anal. Calcd for $C_{21}H_{26}O_5 \cdot 0.75H_2O$: C, 67.81; H, 7.40. Found: C, 67.88; H, 7.38. APCI MS $(M+H)^+$ = 359; ^{1}H NMR (DMSO-d_6) δ 0.58 (s, 3H), 1.00 (br q, J=13, 1H), 1.30 (s, 3H), 1.35 (m, 1H), 1.65-1.85 (m, 4H), 1.90-2.10 (m, 3H), 2.20-2.35 (m, 3H), 2.45-2.65 (m, 4H), 3.3 (br s, 1H, OH), 5.52 (d, J=5.5, 1H), 5.65 (s, 1H), 13.5 (v br, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 14.3, 24.0, 25.7, 32.0, 32.1(2C), 33.3, 33.9, 34.0, 37.0, 40.5, 47.2, 48.0, 87.2, 118.7, 123.2, 143.7, 168.0, 169.6, 197.8, 201.6.

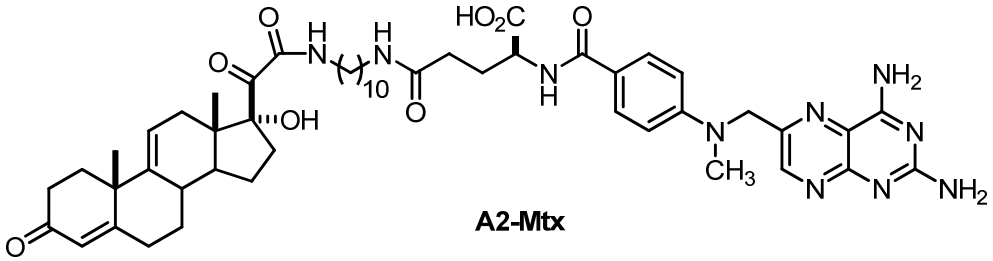


D1 (11β,17α-dihydroxy-9α-fluoro-16α-methyl-1,4-androstadien-3-one-17β-carboxylic acid) and D2 (11β,17α-dihydroxy-9α-fluoro-16α-methyl-1,4-pregnadien-3,20-dione-21-oic acid). Detailed synthetic procedures with complete analytical data (^{1}H and ^{13}C NMR, IR, MS, $[\alpha]_D$, mp, elemental analysis) are recorded in Conrow, Dillow et al., *J. Org. Chem.* **2002**, *67(19),* 6835-6836.



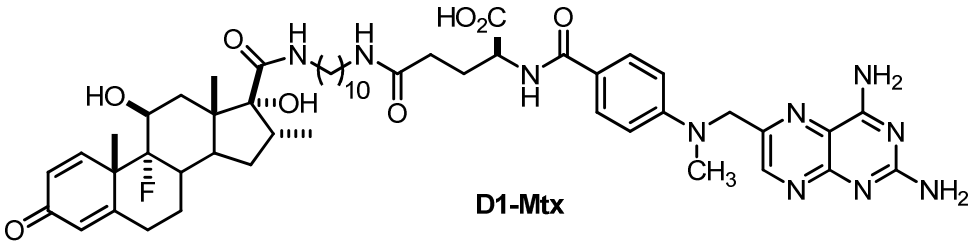
A1-Mtx

A1-Mtx. Orange solid, mp 204-207 °C; MW ($C_{50}H_{68}N_{10}O_7$) 921.14, APCI MS $(M-H)^-$ = 920; TLC (silica plate, 80:18:2 $CHCl_3$/MeOH/NH_4OH): Rf 0.33; HPLC (C18, MeCN-H_2O, 254 nm) >99%; ^1H NMR (CD_3OD) δ 0.67 (s, 3H), 1.12 (br q, *J* = 15, 1H), 1.2-2.8 (m), 1.35 (s, 3H), 3.10 (br t, *J* = 5, 4H), 3.23 (s, 3H), 4.50 (br s, 1H), 5.58 (br s, 1H), 5.71 (s, 1H), 6.85 (d, *J* = 9, 2H), 7.74 (d, *J* = 9, 2H), 8.58 (s, 1H).



A2-Mtx

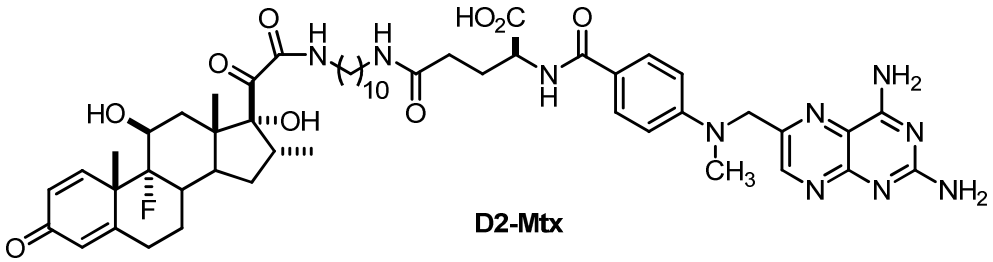
A2-Mtx. Orange solid, mp 197-200 °C; MW ($C_{51}H_{68}N_{10}O_8$) 949.15, APCI MS $(M-H)^-$ = 948; TLC (silica plate, 80:18:2 $CHCl_3$/MeOH/NH_4OH): Rf 0.32; HPLC (C18, MeCN-H_2O, 254 nm) >99%; ^1H NMR (CD_3OD+$CDCl_3$) δ 0.60 (s, 3H), 0.89 (br s, 1H), 1.12 (br q, *J* = 15, 1H), 1.2-2.8 (m), 1.29 (s, 3H), 3.09 (br t, *J* = 5, 2H), 3.2 (m, 2H), 3.23 (s, 1H), 4.50 (br s, 1H), 5.58 (br s, 1H), 5.73 (s, 1H), 6.84 (d, *J* = 9, 2H), 7.74 (d, *J* = 9, 2H), 8.58 (s, 1H).



D1-Mtx

D1-Mtx. Orange solid, mp 210-213 °C; MW ($C_{51}H_{69}FN_{10}O_8$) 969.15, ESI MS $(M+H)^+$ = 970; TLC (silica plate, 80:18:2 $CHCl_3$/MeOH/NH_4OH): Rf 0.24; HPLC (C18, MeCN-H_2O-0.05% TFA, 254 nm) >99%; ^1H NMR (CD_3OD) δ 0.88 (d, *J* = 7, 3H), 1.08 (s, 3H), 1.0-1.5 (m), 1.57 (s, 3H), 1.70 (q, *J* = 12, 1H), 1.75 (br s, 1H), 2.0-2.5 (m), 2.67 (br t, J = 13, 1H), 3.10 (br t, *J* = 5,

4H), 3.24 (s, 3H), 4.21 (br d, *J* = 10, 1H), 4.50 (br s, 1H), 4.93 (s, 2H), 6.06 (s, 1H), 6.26 (d, *J* = 10, 1H), 6.86 (d, *J* = 9, 2H), 7.40 (d, *J* = 10, 1H), 7.51 (br t, *J* = 3, 1H), 7.75 (d, *J* = 9, 2H), 8.59 (s, 1H). Known compound: V. W. Cornish, US Patent 7,419,780; D. C. Henthorn et al., *Biochem. Pharmacol.* **2002**, *63(9),* 1619-1628.



D2-Mtx

D2-Mtx. Orange solid, mp 199-202 °C; MW ($C_{52}H_{69}FN_{10}O_9$) 997.16, ESI MS $(M-H)^-$ = 996; TLC (silica plate, 80:18:2 $CHCl_3$/MeOH/NH_4OH): Rf 0.23; HPLC (C18, MeCN-H_2O, 254 nm) 96.7%; ^1H NMR (CD_3OD+$CDCl_3$) δ 0.85 (d, *J* = 7, 3H), 0.95 (s, 3H), 1.0-1.8 (m), 1.54 (s, 3H), 1.85 (br s, 1H), 2.0-2.5 (m), 2.67 (br t, J = 13, 1H), 3.07 (br t, *J* = 5, 2H), 3.2 (m, 2H), 3.21 (s, 1H), 4.24 (br d, *J* = 10, 1H), 4.46 (br s, 1H), 6.07 (s, 1H), 6.27 (d, *J* = 10, 1H), 6.78 (d, *J* = 9, 2H), 7.37 (d, *J* = 10, 1H), 7.71 (d, *J* = 9, 2H), 8.53 (s, 1H).

Notes: (1) The synthesis of the four hybrids was performed according to known methods as described in the text. (2) For ^1H NMR spectra of methotrexate and related compounds, with proton assignments, see: Y. Zhang et al., *Bioconjugate Chem.* **2010**, *21,* 489-495.