SUPPLEMENTAL DATA

Kinetic Analysis of the Three-step Steroid Aromatase Reaction of Human Cytochrome P450 19A1

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FIGURE S1. **cDNA optimization for P450 19A1 expression in *E. coli.*** *A*, cDNA sequence after optimization (note: a stop codon was inserted during the sequence corrections, *vide infra*). *B*, oligonucleotides used for synthon generation in polymerase cycling assembly.

A

```
ATGGCCAAAAAAACCTCTTCTAAAGGTCGTAACTACGAAGGTACTTCATC -50-
TATCCCGGGTCCTGGTTACTGCATGGGCATCGGTCCGCTGATCTCTCACG -100-
GTCGTTTCCTGTGGATGGGTATTGGTTCTGCGTGCAACTACTACAACCGT -150-
GTTTACGGCGAATTCATGCGTGTTTGGATCTCTGGCGAAGAAACCCTGAT -200-
TATCTCTAAGTCCAGCTCTATGTTCCACATCATGAAACACAACCATTACT -250-
CCTCTCGTTTCGGCTCTAAACTGGGTCTGCAGTGCATTGGTATGCACGAA -300-
AAAGGTATCATCTTCAATAACAACCCGGAGCTGTGGAAAACCACCCGTCC -350-
GTTCTTTATGAAAGCGCTGTCTGGTCCGGGTCTGGTTCGTATGGTTACCG -400-
TTTGCGCTGAATCTCTGAAAACCCACCTGGACCGTCTGGAAGAAGTCACC -450-
AATGAGTCCGGTTATGTTGACGTTCTGACCCTGCTGCGTCGTGTTATGCT -500-
GGACACCTCCAACACCCTGTTCCTGCGTATCCCGCTGGACGAATCTGCTA -550-
TCGTTGTTAAAATCCAGGGTTATTTCGACGCGTGGCAGGCGCTGCTGATC -600-
AAGCCGGACATTTTCTTCAAAATTTCCTGGCTGTACAAAAAGTACGAAAA -650-
GTCTGTTAAAGACCTGAAGGACGCGATTGAAGTTCTGATTGCGGAGAAGC -700-
GTCGTCGTATCAGCACCGAAGAAAAACTCGAAGAGTGCATGGACTTTGCG -750-
ACCGAGCTGATCCTGGCGGAAAAACGTGGTGACCTGACCCGTGAAAACGT -800-
TAACCAGTGTATCCTCGAAATGCTGATCGCGGCACCGGACACCATGAGCG -850-
TTTCTCTGTTCTTCATGCTGTTCCTCATCGCGAAACACCCGAACGTTGAG -900-
GAAGCGATCATCAAAGAAATCCAGACTGTTATTGGTGAACGTGACATCAA -950-
AATTGACGACATCCAGAAACTGAAAGTTATGGAAAACTTCATCTACGAGT -1000-
CTATGCGTTACCAGCCGGTTGTTGACCTGGTCATGCGTAAGGCGCTGGAG -1050-
GACGACGTTATTGACGGTTACCCGGTCAAAAAAGGTACCAATATCATCCT -1100-
GAACATCGGTCGTATGCACCGTCTCGAATTCTTCCCGAAACCGAACGAAT -1150-
TCACCCTCGAGAATTTTGCGAAGAATGTTCCGTACCGTTACTTCCAGCCG -1200-
TTCGGCTTCGGTCCGCGTGGTTGCGCGGGTAAATACATCGCAATGGTTAT -1250-
GATGAAGGCCATCCTGGTTACTCTGCTGCGTCGTTTCCACGTTAAAACTC -1300-
TGCAGGGTCAGTGCGTTGAATCTATTCAGAAAATCCACGACCTCTCTCTG -1350-
CACCCGGACGAAACCAAGAATATGCTGGAGATGATCTTCACCCCGCGTAA -1400-
TTCTGACCGTTGTCTGGAACACCATCATCACCACCACTAACGAAGCTTAT -1450-
```

B

Primer	PCA primer sequence
1	GGTGGTCATATGGCCAAAAAAACCTCTTCTAAAGGTCGTAACTAC
2	ACCAGGACCCGGGATAGAGGAAGTACCTTCGTAGTTACGACCTTTAGAAGAGGT
3	TCTATCCCGGGTCCTGGTTACTGCATGGGCATCGGTCCGCTGATCTCTCACGGT
4	GTTGCACGCAGAACCAATACCCATCCACAGGAAACGACCGTGAGAGATCAGCGG
5	GTATTGGTTCTGCGTGCAACTACTACAACCGTGTTTACGGCGAATTCATGCGTG
6	TAGAGATAATCAGGGTTTCTTCGCCAGAGATCCAAACACGCATGAATTCGCCGT
7	GCGAAGAAACCCTGATTATCTCTAAGTCCAGCTCTATGTTCCACATCATGAAGC
8	TTAGAGCCGAAACGAGAGGAGTAATGGTTGTGCTTCATGATGTGGAACATAGAG
9	TCCTCTCGTTTCGGCTCTAAACTGGGTCTGCAGTGCATTGGTATGCACGAAAAA
10	CCACAGCTCCGGGTTGTTATTGAAGATGATACCTTTTTCGTGCATACCAATGCA
11	ACAACCCGGAGCTGTGGAAAACCACCCGTCCGTTCTTTATGAAAGCGCTGTCTG
12	GCAAACGGTAACCATACGAACCAGACCCGGACCAGACAGCGCTTTCATAAAGAA
13	GTTCGTATGGTTACCGTTTGCGCTGAATCTCTGAAAACCCACCTGGACCGTCTG
14	GAACGTCAACATAACCGGACTCATTGGTGACTTCTTCCAGACGGTCCAGGTGGG
15	AGTCCGGTTATGTTGACGTTCTGACCCTGCTGCGTCGTGTTATGCTGGACACCT
16	TTCGTCCAGCGGGATACGCAGGAACAGGGTGTTGGAGGTGTCCAGCATAACACG
17	CGTATCCCGCTGGACGAATCTGCTATCGTTGTTAAAATCCAGGGTTATTTCGAC
18	GTCCGGCTTGATCAGCAGCGCCTGCCACGCGTCGAAATAACCCTGGATTTTAAC
19	CTGCTGATCAAGCCGGACATTTTCTTCAAAATTTCCTGGCTGTACAAGAAGTAT
20	CTTCTCATACTTCTTGTACAGCCAGGAAA
21	TCTTGGCTGTACAAAAAGTACGAAAAGTCTGTTAAAGACCTGAAGGACGCGATTG
22	GGTGCTGATACGACGACGCTTCTCCGCAATCAGAACTTCAATCGCGTCCTTCAGGTC
23	CGTCGTCGTATCAGCACCGAAGAAAAACTCGAAGAGTGCATGGACTTTGCGACCGAG
24	TCACGGGTCAGGTCACCACGTTTTTCCGCCAGGATCAGCTCGGTCGCAAAGTCCATG
25	GGTGACCTGACCCGTGAAAACGTTAACCAGCGTATCCTCGAAATGCTGATCGCGGCA
26	GGAACAGCATGAAGAACAGAGAAACGCTCATGGTGTCCGGTGCCGCGATCAGCATTT
27	CTCTGTTCTTCATGCTGTTCCTCATCGCGAAACACCCGAACGTTGAGGAAGCGATCA
28	ATGTCACGTTCACCAATAACAGTCTGGATTTCTTTGATGATCGCTTCCTCAACGTTC
29	CTGTTATTGGTGAACGTGACATCAAAATTGACGACATCCAGAAACTGAAAGTTATGG
30	GCTGGTAACGCATAGACTCGTAGATGAAGTTTTCCATAACTTTCAGTTTCTGGATGT
31	CGAGTCTATGCGTTACCAGCCGGTTGTTGACCTGGTCATGCGTAAGGCGCTGGAGGA
32	ATTGGTACCTTTTTTGACCGGGTAACCGTCAATAACGTCGTCCTCCAGCGCCTTACG
33	CCGGTCAAAAAAGGTACCAATATCATCCTGAACATCGGTCGTATGCACCGTCTCGAA
34	AAATTCTCGAGGGTGAATTCGTTCGGTTTCGGGAAGAATTCGAGACGGTGCATACGA
35	ACGAATTCACCCTCGAGAATTTTGCGAAGAATGTTCCGTACCGTTACTTCCAGCCGT
36	CGATGTATTTACCCGCGCAACCACGCGGACCGAAGCCGAACGGCTGGAAGTAACGGT
37	TGCGCGGGTAAATACATCGCAATGGTTATGATGAAGGCCATCCTGGTTACTCTGCTG
38	CGCACTGACCCTGCAGAGTTTTAACGTGGAAACGACGCAGCAGAGTAACCAGGATGG
39	TCTGCAGGGTCAGTGCGTTGAATCTATTCAGAAAATCCACGACCTCTCTCTGCACCC
40	GGGGTGAAGATCATCTCCAGCATATTCTTGGTTTCGTCCGGGTGCAGAGAGAGGTCG
41	CTGGAGATGATCTTCACCCCGCGTTCTTCTGACCGTTGTCTGGAACACCATCATCAC
42	TTTGTCATCAATAAGCTTGTGGTGGTGATGATGGTGTTCCAGACA

Note: a stop codon was inserted via site-directed mutagenesis after synthon ligation.

FIGURE S2. **Immunoblot of P450 19A1.** Four dilutions of purified P450 19A1 were loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%, w/v), along with seven dilutions of a P450 3A4 standard that also contained a hexa-histidine tag. The proteins were transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA). A primary mouse anti-his_6-peroxidase antibody was used (Roche, San Francisco, CA) to detect the presence of protein containing a hexa-histidine tag. The secondary antibody was goat anti-mouse IRD800CW, which emits infrared light at 800 nm, and was detected using an Odyssey Li-Cor instrument (Li-Cor, Lincoln, NE). Shown in the first four lanes are various dilutions of purified P450 19A1. The final seven lanes contain P450 3A4 with a hexa-histidine tag as a standard for quantitation (shown are 1, 2, 4, 8, 10, 12, and 16 pmol P450 3A4). The output from the channel detecting the infrared signal at 800 nm is shown.

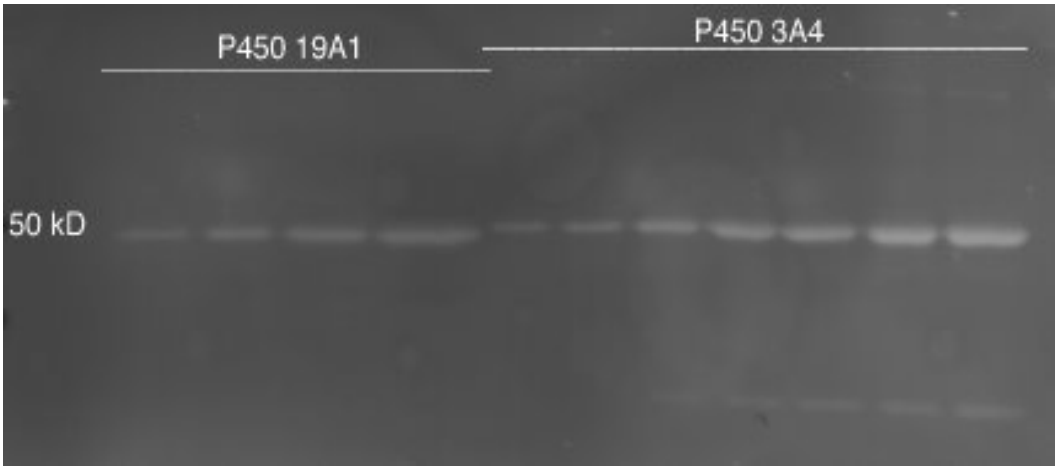


FIGURE S3. **Dynafit iterations for fitting stopped-flow spectroscopy binding data and steady-state titration data.** *A,* Dynafit script for fitting the binding of andro (Fig. 5*A*). *B,* Dynafit script for fitting the steady-state titration data (andro, Fig. 4*A*), using K_d values generated from *A*. *C,* fit resulting from script from *B*. *D,* Dynafit script for fitting the binding of 19-OH andro (Fig. 5*B*). *E,* Dynafit script for fitting the steady-state titration data (19-OH andro, Fig. 4*B*), using K_d values generated from *D*. *F,* fit resulting from script in *E*. *G,* Dynafit script for the binding of 19=O andro (Fig. 5*C*). *H,* Dynafit script for fitting the steady-state titration data (19=O andro, Fig. 4*C*), using K_d values generated from *G*. *I,* fit resulting from script in *H*.

A

```
;Christal Sohl & P450 19A1
;Kinetics of ligand binding
;All units in uM & s
;A390 SF data
;Andro
```

```
;Run 32

[task]
        data = progress
        task = fit
[mechanism]
        E + L <==> EL          :          k1        k-1
        EL <==> LE   :          k2        k-2
[constants]
        k1 = 5,  k-1 = 1.4
        k2 = 0.42, k-2 = 0.2
[concentrations]
        E = 2
        L = 2

[responses]
        EL = 0
        LE = 0.07
[sweep]
        mesh linear from 0 to 30 step 0.032
[progress]
        directory ./scripts
        extension txt
        file      2v2andro1
[output]
        directory ./projects/ANDRO0909/run32
[end]
```

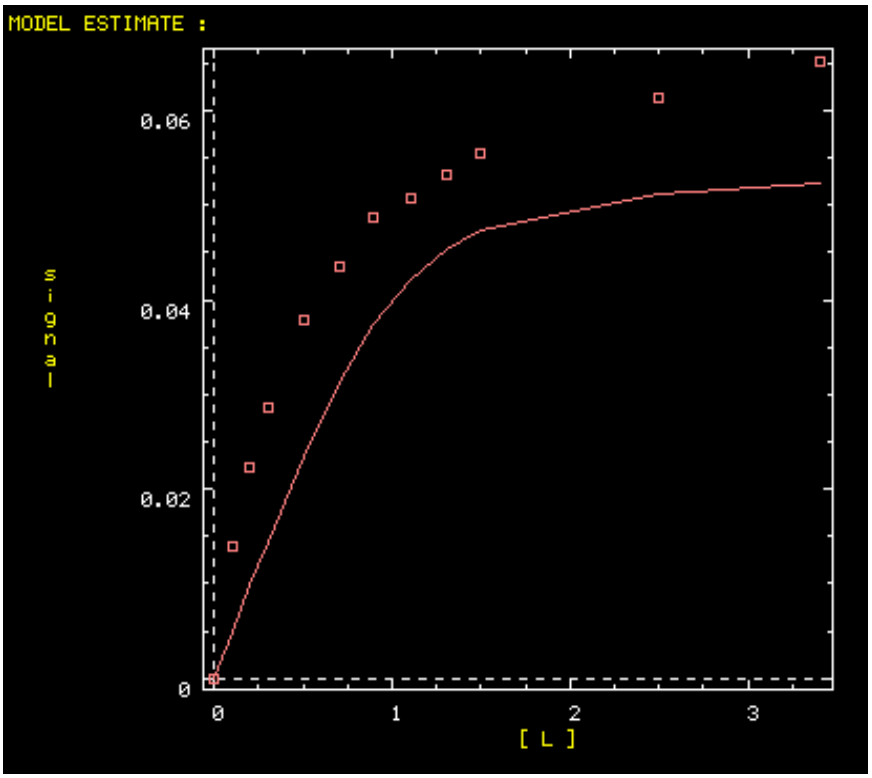
B

```
;Christal Sohl & P450 19A1
;Kinetics of ligand binding
;All units in uM & s
;Andro
;Run 31

[task]
        data = equilibria
        task = fit
[mechanism]
        E + L <==> EL :          K1 dissoc.
        EL <==> LE    :          K2 dissoc.
[constants]
        K1 = 0.28
        K2 = 2.1
[concentrations]
```

```
        E = 1
[responses]
        EL = 0
        LE = 0.08
[sweep]
[equilibria]
        variable L = 0 to 4.4 step 0.001
        file ./scripts/androquadfit1.txt
[output]
        directory ./projects/ANDRO0909/run31
[end]
```

C



D

```
;Christal Sohl & P450 19A1
;Kinetics of ligand binding
;All units in uM & s
;A390 SF data
;19-OH andro
;Run 64
```

```
[task]
        data = progress
        task = fit

[mechanism]
        E + L <==> EL          :          k1        k-1
        EL <==> LE   :          k2        k-2
[constants]
        k1 = 40,  k-1 = 240
        k2 = 0.8, k-2 = 0.15
[concentrations]
        E = 2
        L = 2
[responses]
        EL = 0
        LE = 0.065
[sweep]
        mesh linear from 0 to 30 step 0.032
[progress]
        directory ./scripts
        extension txt
        file      2v219OH1
[output]
        directory ./projects/19OH0909/run64
[end]
```

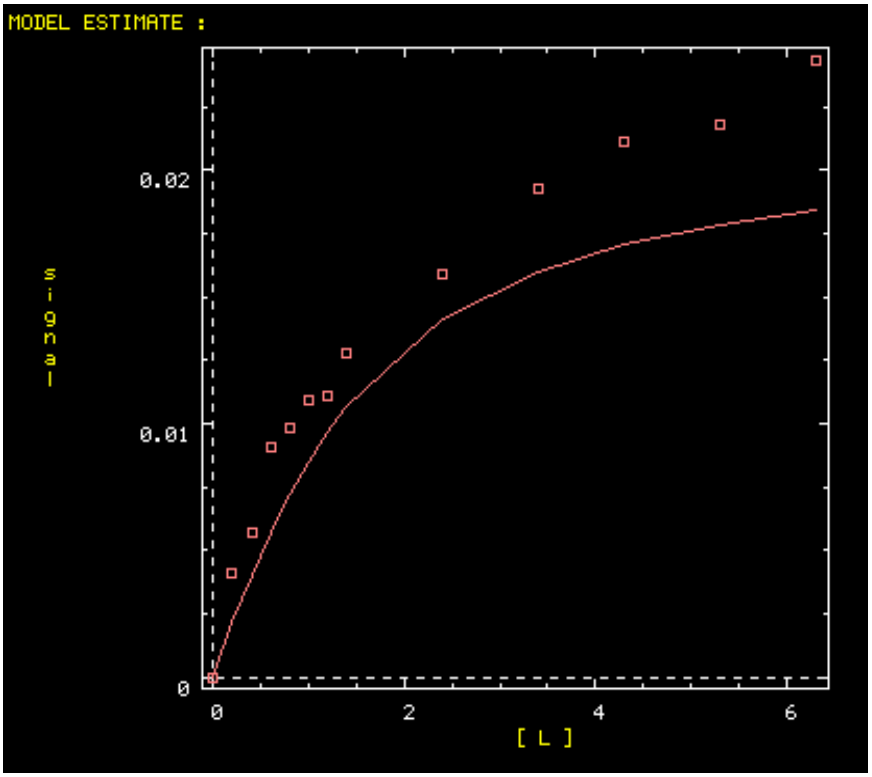
E

```
;Christal Sohl & P450 19A1
;Kinetics of ligand binding
;All units in uM & s
;titration data
;19-OH andro
;Run 61

[task]
        data = equilibria
        task = fit
[mechanism]
        E + L <==> EL          :          K1 dissoc.
        EL <==> LE   :          K2 dissoc.
[constants]
        K1 = 6
```

```
        K2 = 5.5
[concentrations]
        E = 1
[responses]
        EL = 0
        LE = 0.0255
[sweep]
[equilibria]
        variable L = 0 to 7.5 step 0.001
        file ./scripts/19OHquadfit1.txt
[output]
        directory ./projects/19OH0909/run61
[end]
```

F



G

```
;Christal Sohl & P450 19A1
;Kinetics of ligand binding
;All units in uM & s
```

```
;A390 SF data
;19=O aldo
;Run 14

[task]
        data = progress
        task = fit
[mechanism]
        E + L <==> EL          :          k1        k-1
        EL <==> LE   :          k2        k-2
[constants]
        k1 = 5,  k-1 = 300
        k2 = 2.4, k-2 = 0.13
[concentrations]
        E = 2
        L = 2
[responses]
        EL = 0
        LE = 0.06
[sweep]
        mesh linear from 0 to 30 step 0.032
[progress]
        directory ./scripts
        extension txt
        file      2v2aldo
[output]
        directory ./projects/ALDO091109/run14
[end]
```

H

```
;Christal Sohl & P450 19A1
;Kinetics of ligand binding
;All units in uM & s
;sf data
;19=O andro
;Run 16

[task]
        data = equilibria
        task = fit
[mechanism]
        E + L <==> EL          :          K1 dissoc.
        EL <==> LE   :          K2 dissoc.
```

```
[constants]
        K1 = 60
        K2 = 18
[concentrations]
        E = 1
[responses]
        EL = 0
        LE = 0.028
[sweep]
[equilibria]
        variable L = 0 to 19.4 step 1
        file ./scripts/aldoquadfit.txt
[output]
        directory ./projects/ALDO091109/run16
[end]
```

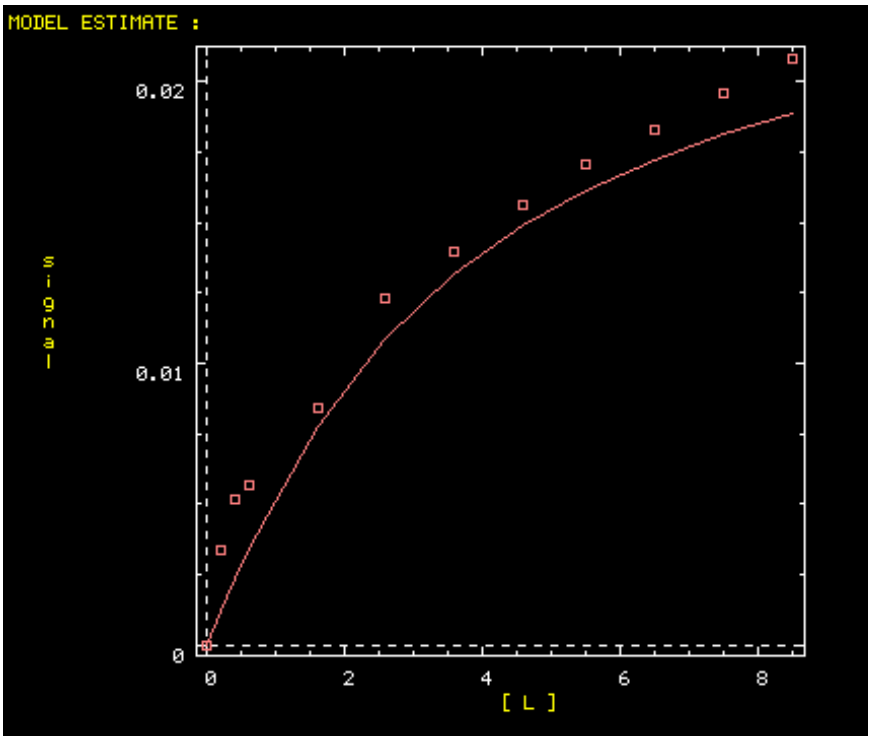


FIGURE S4. **Fe^{2+}•CO *vs.* Fe^{2+} difference spectra in the absence and presence of 19-OH andro.** P450 19A1 (1 μM) in the absence and presence of 19-OH andro (20 μM).

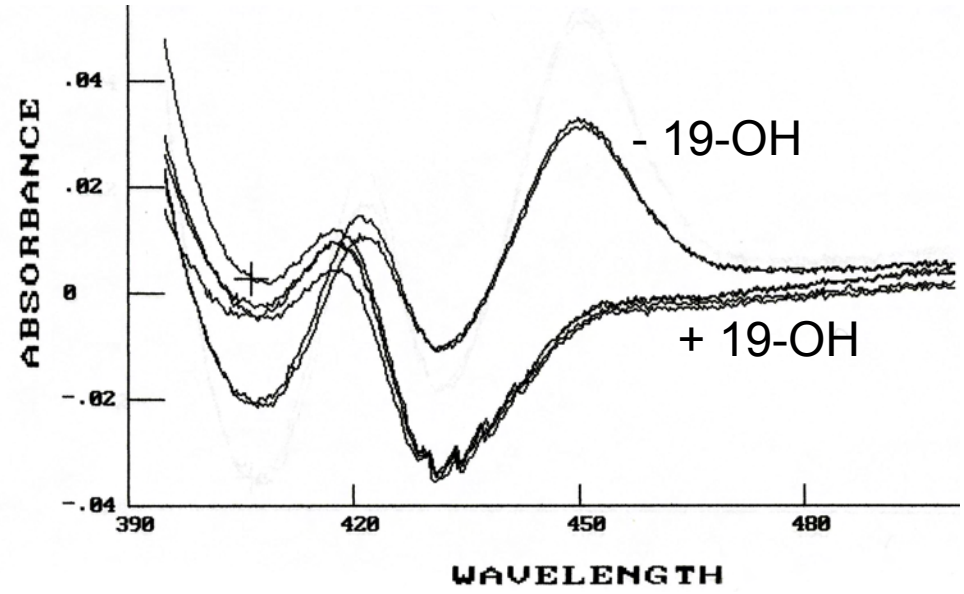


FIGURE S5. **Sample HPLC radiochromatogram following incubation of P450 19A1 with radiolabeled andro.** Shown are 19-OH andro at t_R = 3.6 min, 19=O andro at t_R = 4.5 min, andro at t_R = 5.8 min, and estrone at t_R = 7.8 min.

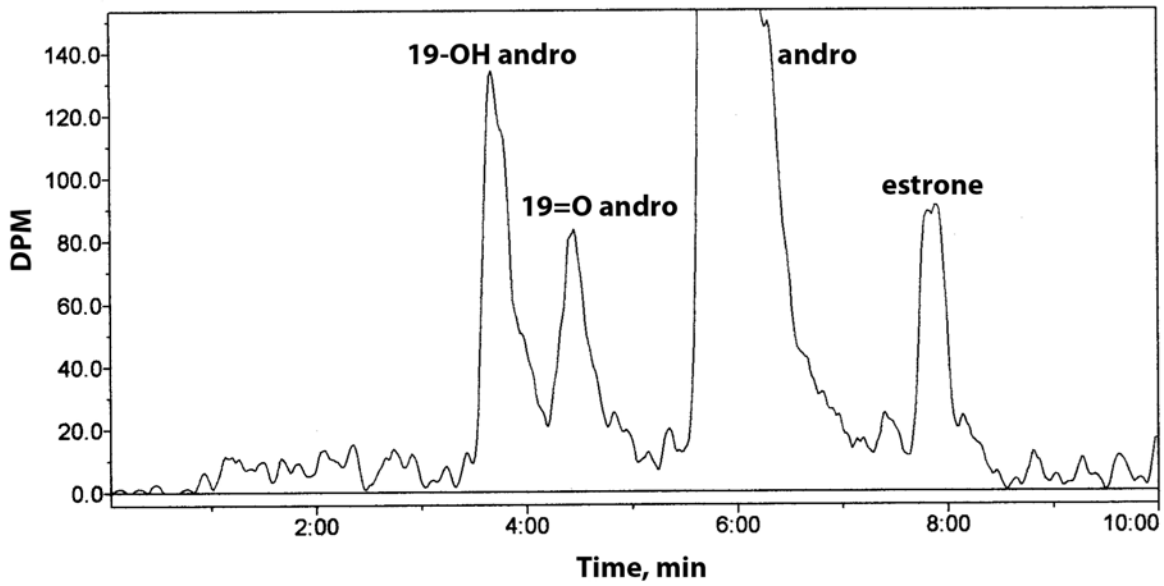


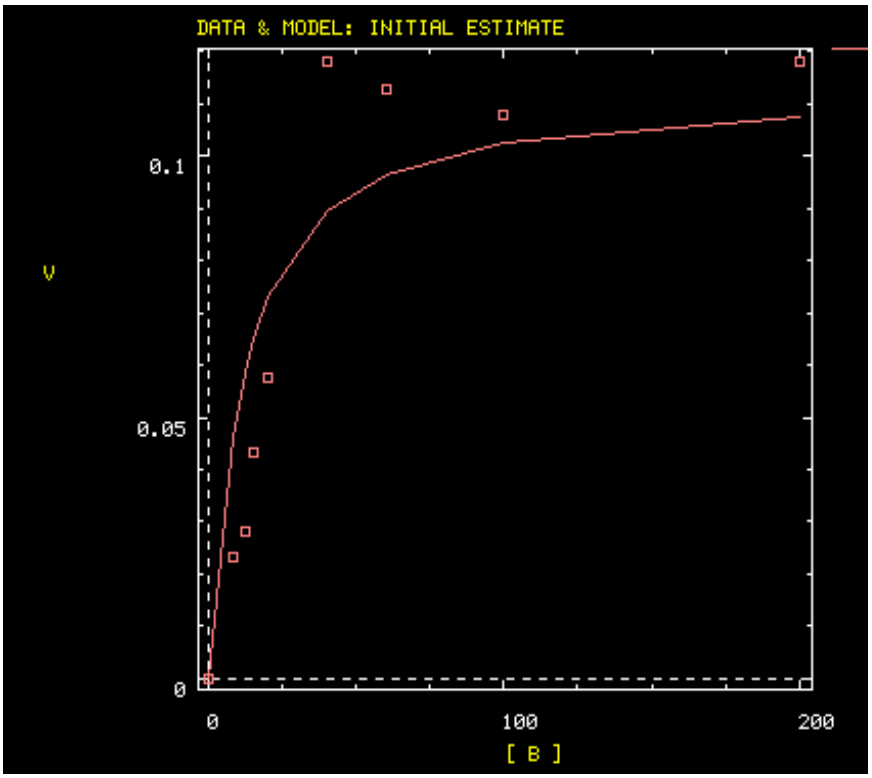
FIGURE S6. **Dynafit iterations for global fitting.** *A,* Dynafit script for fitting conversion of 19-OH andro to estrone. *B*, resulting fit for the conversion of 19-OH andro to estrone using rate constants from the single turnover experiment fit (Fig. 9, Table 3). *C,* Dynafit script for fitting conversion of 19=O andro to estrone. *D*, resulting fit for the conversion of 19=O andro to estrone, using rate constants from the single turnover experiment fit (Fig. 9, Table 3).

A

```
;Christal Sohl
;All units in uM, s
;A = andro, B = 19-OH andro, C = 19=O andro, D = estrone, E = 19A1
;19-OH v vs s

 [task]
        data = velocities
        task = fit
[mechanism]
        B + E <==> EB       :       k1      k-1
        EB <==> BE          :       k2      k-2
        BE --> EC           :       k3
        EC <==> E + C       :       k4      k-4
        EC <==> CE          :       k5      k-5
        CE --> ED           :       k6
        ED <==> E + D       :       k7      k-7
[constants]
        k1 = 70,        k-1 = 39000
        k2 = 24300,     k-2 = 320
        k3 = 7.5
        k4 = 50, k-4 = 80
        k5 = 32000, k-5 = 30000
        k6 = 5.9
        k7 = 12900, k-7 = 90
[concentrations]
        E = 0.6
[progress]
        delay = 0.01
[sweep]
[velocity]
        variable B = 0, 8, 12, 15, 20, 40, 60, 100, 200, 300
        file ./scripts/19OHinsec.txt
        responses D = 1
[output]
        directory ./projects/19A101211019OH/run215
[end]
```

B



C

```
;Christal Sohl
;All units in uM, s
;A = andro, B = 19-OH andro, C = 19=O andro, D = estrone, E = 19A1
;19=O v vs s

[task]
        data = velocities
        task = fit
[mechanism]
        E + C <==> EC       :       k1      k-1
        EC <==> CE          :       k2      k-2
        CE --> ED           :       k3
        ED <==> E + D       :       k4      k-4
[constants]
        k1 = 80, k-1 = 50
        k2 = 32000, k-2 = 30000
```

k3 = 5.9
k4 = 12900, k-4 = 90
[concentrations]
E = 0.1
[progress]
delay = 0.01
[sweep]
[velocity]
variable C = 0, 1, 5, 10, 15, 20, 40, 60, 100, 200, 300
file ./scripts/19Oinpersec.txt
responses D = 1
[output]
directory ./projects/19A101211019O/run240
[end]

D