

Supplementary Information

Gap-free near-T2T genome assembly of *Digitalis lanata* enables complete enzymatic production of digoxigenin

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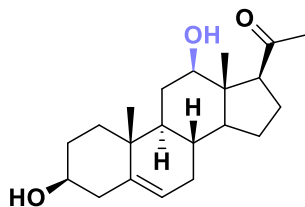
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Supplementary Notes

HRMS (ESI), ^1H and ^{13}C NMR spectral data.

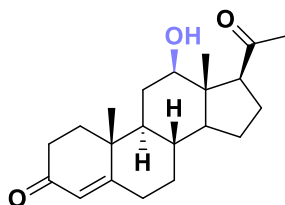


12 β -hydroxypregnenolone (2b)

^1H -NMR (600 MHz, CDCl_3): δ 5.35 (1H, m, H-1), 4.94 (1H, s, HO-3), 3.52 (1H, s, HO-3), 3.48 (1H, dd, J = 10.7, 4.2 Hz, H-17), 2.22 (3H, s, H₃-21), 1.02 (3H, s, H₃-19), 0.74 (3H, s, H₃-18)

^{13}C -NMR (150 MHz, CDCl_3): δ 214.4 (C-20), 141.0 (C-5), 121.1 (C-6), 77.6 (C-12), 71.7 (C-3), 64.0 (C-17), 54.2 (C-14), 49.0 (C-13), 47.8 (C-9), 42.1 (C-4), 37.1 (C-10), 36.6 (C-8), 31.6 (C-21), 31.2 (C-2), 30.7 (C-7), 30.3 (C-11), 29.5 (C-11), 25.0 (C-15), 23.7 (C-16), 19.4 (C-19), 7.8 (C-18)

(+)-ESI-HRMS calculated for $\text{C}_{21}\text{H}_{32}\text{O}_3$ $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ m/z 315.2385, found m/z 315.2337.



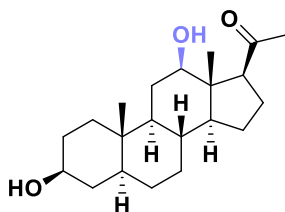
12 β -hydroxyprogesterone (3b)

^1H -NMR (600 MHz, CDCl_3): δ 5.73 (1H, s, H-1), 3.45 (1H, dd, J = 10.9, 4.6 Hz, H-12), 2.43 (1H, m, H-17), 2.39 (2H, m, H₂-2), 2.39 (1H, m, H-7a), 2.29 (1H, m, H-7b), 2.22 (3H, s, H₃-21), 2.20 (1H, m, H-16a), 2.06 (1H, m, H-1a), 2.00 (1H, m, H-16b), 1.86 (1H, m, H-6a), 1.84 (1H, m, H-15a), 1.84 (1H, m, H-11a), 1.70 (1H, dt, J = 14.3, 4.7 Hz, H-1b), 1.50 (1H, m, H-8), 1.44 (1H, m, H-15b), 1.39 (1H, m, H-11b), 1.19 (3H, s, H₃-19), 1.06 (1H, m, H-14), 1.04 (1H, m, H-9), 1.02 (1H, m, H-6b), 0.77 (3H, s, H₃-18)

^{13}C -NMR (150 MHz, CDCl_3): δ 214.2 (C-20), 199.4 (C-3), 170.2 (C-5), 124.2 (C-4),

63.9 (C-17), 53.2 (C-14), 52.1 (C-9), 47.7 (C-13), 38.5 (C-10), 35.6 (C-1), 34.3 (C-8), 33.9 (C-2), 32.7 (C-7), 31.2 (C-6), 30.3 (C-21), 29.2 (C-11), 24.9 (C-16), 23.7 (C-15), 17.3 (C-19), 7.9 (C-18)

(+)-ESI-HRMS calculated for $C_{21}H_{30}O_3$ $[M+H-H_2O]^+$ m/z 313.2228, found m/z 313.2176.

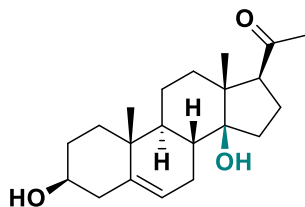


3β,12β-dihydroxy-5α-pregnan-20-one (12b)

1H -NMR (800 MHz, $CDCl_3$): δ 3.61 (1H, m, H-3), 3.44 (1H, dd, J = 10.9, 4.6 Hz, H-12), 2.43 (1H, m, H-17), 2.23 (3H, s, H_3 -21), 2.18 (1H, m, H-15a), 1.99 (1H, m, H-15b), 1.84 (1H, m, H-11a), 1.81 (1H, m, H-16a), 1.76 (1H, m, H-1a), 1.61 (1H, m, H-4a), 1.39 (1H, m, H-16b), 1.31 (1H, m, H-4b), 1.28 (1H, m, H-11b), 1.33 (1H, m, H-8), 1.13 (1H, m, H-5), 1.05 (1H, m, H-14), 1.05 (1H, m, H-1b), 0.96 (1H, m, H-2a), 0.88 (1H, m, H-2b), 0.83 (3H, s, H_3 -19), 0.73 (3H, s, H_3 -18)

^{13}C -NMR (200 MHz, $CDCl_3$): δ 214.4 (C-20), 78.1 (C-12), 71.3 (C-3), 64.1 (C-17), 53.9 (C-14), 53.9 (C-14), 52.6 (C-9), 48.1 (C-13), 44.8 (C-5), 38.0 (C-4), 36.9 (C-1), 35.6 (C-10), 34.3 (C-8), 31.4 (C-7), 30.3 (C-21), 29.6 (C-11), 28.6 (C-6), 25.1 (C-15), 23.7 (C-16), 12.2 (C-19), 8.0 (C-18)

(+)-ESI-HRMS calculated for $C_{21}H_{34}O_3$ $[M+H-H_2O]^+$ m/z 317.2541, found m/z 317.2498.



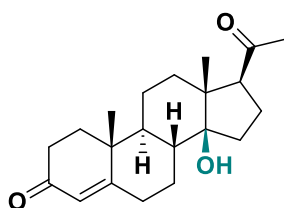
14β-hydroxypregnenolone (2a)

1H -NMR (600 MHz, $DMSO-d_6$): δ 5.30 (1H, m, H-6), 4.64 (1H, d, J = 4.58 Hz, HO-3), 4.16 (1H, s, HO-14), 3.24 (1H, m, H-3), 2.72 (1H, dd, J = 9.1, 5.2 Hz, H-17), 2.19

(3H, s, H₃-21), 0.91 (3H, s, H₃-19), 0.86 (3H, s, H₃-18)

¹³C-NMR (150 MHz, DMSO-*d*₆): δ 215.7 (C-20), 140.4 (C-5), 121.4 (C-6), 84.5 (C-14), 70.4 (C-3), 63.0 (C-17), 49.2 (C-13), 45.9 (C-9), 42.5 (C-4), 37.3 (C-1), 37.0 (C-10), 37.0 (C-8), 33.8 (C-7), 32.0 (C-21), 31.8 (C-2), 27.3 (C-15), 23.8 (C-16), 20.8 (C-11), 19.7 (C-19), 15.7 (C-18)

(+)-ESI-HRMS calculated for C₂₁H₃₂O₃ [M+H-H₂O]⁺ *m/z* 315.2385, found *m/z* 315.2339.



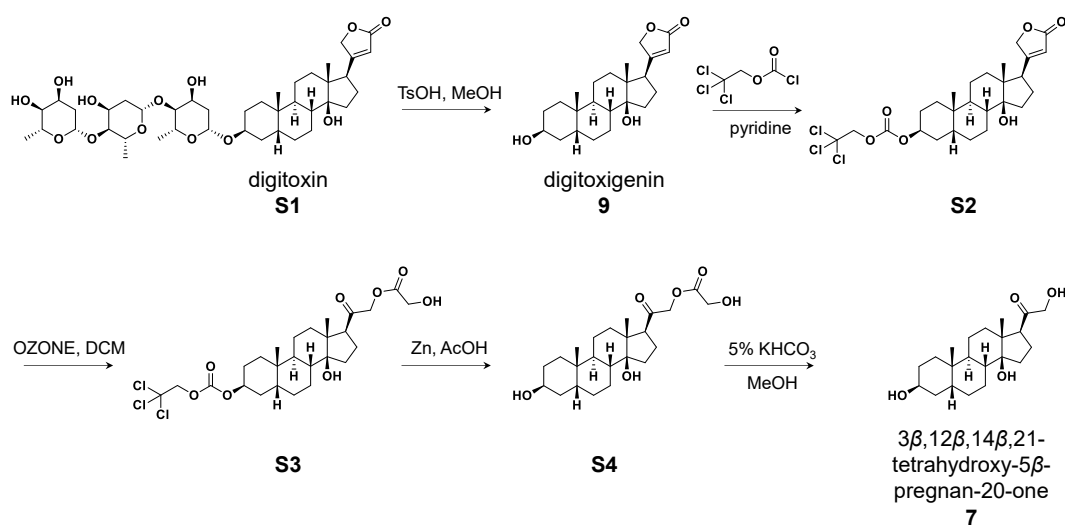
14β-hydroxyprogesterone (3a)

¹H-NMR (600 MHz, CDCl₃): δ 5.74 (1H, s, H-4), 4.56 (1H, s, HO-14), 2.93 (1H, m, H-17), 2.26 (3H, s, H₃-21), 1.18 (3H, s, H₃-19), 1.02 (3H, s, H₃-18)

¹³C-NMR (150 MHz, CDCl₃): δ 214.7 (C-20), 171.2 (C-5), 123.9 (C-4), 84.6 (C-14), 62.1 (C-17), 49.3 (C-9), 49.1 (C-13), 38.8 (C-10), 35.9 (C-1), 34.1 (C-8), 34.0 (C-2), 33.6 (C-7), 33.2 (C-21), 28.0 (C-11), 25.0 (C-16), 20.8 (C-15), 17.7 (C-19), 15.3 (C-18)

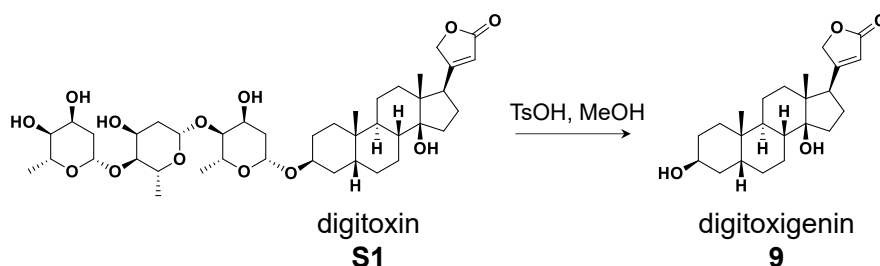
(+)-ESI-HRMS calculated for C₂₁H₃₀O₃ [M+H-H₂O]⁺ *m/z* 331.2228, found *m/z* 331.2284.

Chemical synthesis



Experimental procedure

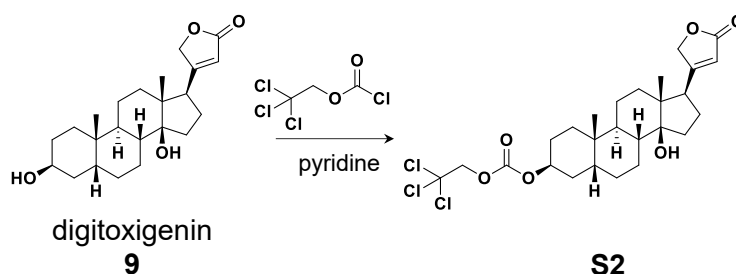
Step 1



To a solution of **S1** (2.50 g, 3.27 mmol, 1.0 equiv) in MeOH (75 mL) was added TsOH·H₂O (62.17 mg, 326.82 μmol, 0.10 equiv). The mixture was stirred at 25°C for 16 h. The mixture was then concentrated and purified by silica gel column chromatography (gradient elution of 0% to 70% EtOAc in hexane) to afford **9** (1.20 g, 3.20 mmol, 98% yield) as a yellow solid.

¹H-NMR (400 MHz, CDCl₃): δ 5.86 (1H, s), 5.01 (1H, m), 4.81 (1H, m), 4.13 (1H, m), 2.79 (1H, m), 2.17 (2H, m), 1.87 (3H, m), 1.71 (4H, m), 1.57 (5H, m), 1.31 (7H, m), 0.95 (3H, s), 0.86 (3H, s).

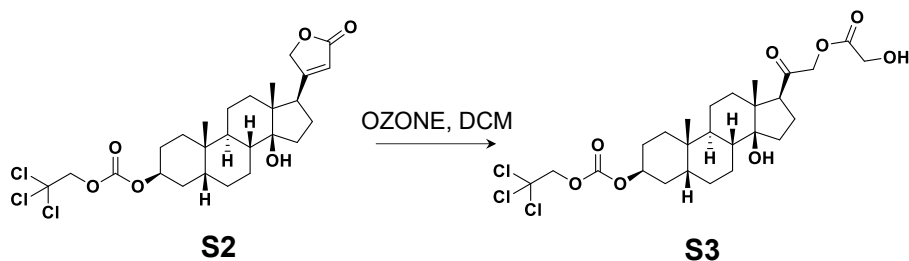
Step 2



To a solution of **9** (1.10 g, 2.94 mmol, 1.0 equiv) in pyridine (12 mL) was added 2,2,2-trichloroethyl chloroformate (2.20 g, 1.39 mL, 10.40 mmol, 3.54 equiv). The mixture was stirred at 25°C for 16 h. The mixture was then poured into cold water (50 mL) and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (gradient elution of 0% to 50% EtOAc in hexane) to afford **S2** (1.30 g, 2.36 mmol, 80% yield) as a white solid.

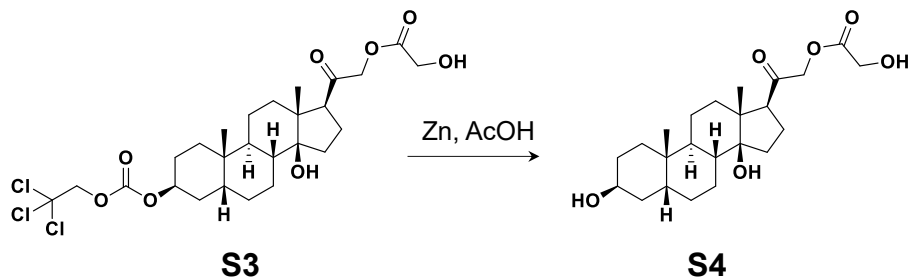
¹H-NMR (400 MHz, CDCl₃): δ 5.87 (1H, s), 4.99 (2H, m), 4.79 (3H, m), 2.77 (1H, m), 2.15 (2H, m), 1.92 (3H, m), 1.74 (4H, m), 1.53 (8H, m), 1.42 (4H, m), 0.98 (3H, s), 0.87 (3H, s).

Step 3



Ozone was bubbled into a solution of **S2** (1.30 g, 2.36 mmol, 1.0 equiv) in MeOH (100 mL) at -70°C for 50 min. The excess ozone was removed by purging with a stream of nitrogen for 5-10 min, and then dimethyl sulfide (0.5 mL, 6.8 mmol) was added dropwise at -70°C. The mixture was allowed to warm to room temperature and stirred for an additional 2 h. The solvent was removed under reduced pressure, and the crude residue was used directly for the next step without further purification.

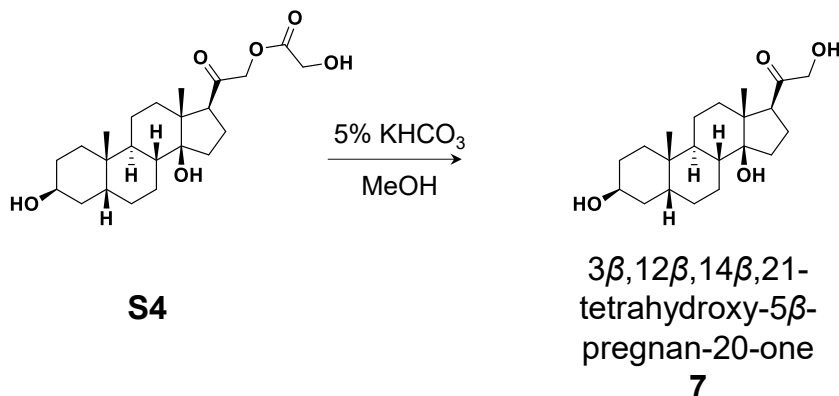
Step 4



To a mixture of **S3** (1.30 g, 2.23 mmol, 1.0 equiv) in AcOH (280 mL) was added Zn dust (83.0 g, 1.27 mol, 570 equiv). The mixture was stirred at 25°C under a N₂ atmosphere for 5 min. The mixture was then filtered, and the zinc residue was rinsed with cold MeOH and DCM. The combined filtrate and washings were carefully poured into ice-water (500 mL) and extracted with DCM (3 × 150 mL). The combined organic layers were washed with water, saturated aqueous NaHCO₃, and brine, dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure to afford **S4** (850 mg, crude) as a white solid.

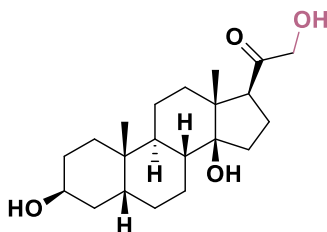
¹H-NMR (400 MHz, CDCl₃): δ 4.85 (2H, m), 4.32 (2H, s), 4.12 (1H, s), 2.78 (1H, m), 2.14 (2H, m), 1.90 (7H, m), 1.57 (11H, m), 1.37 (5H, m), 1.22 (4H, m), 0.95 (6H, s).

Step 5



To a solution of **S4** (850 mg, 2.08 mmol, 1.0 equiv) in MeOH (102 mL) and H₂O (34 mL) was added KHCO₃ (1.56 g, 15.60 mmol, 7.5 equiv). The mixture was stirred at 25°C for 16 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash column chromatography on silica gel (gradient elution of 0% to 60% EtOAc in hexane) followed by recrystallization from MeCN (2 mL) to afford **7** (310 mg, 0.884 mmol, 42% yield) as a white solid.

HRMS (ESI), ¹H and ¹³C NMR spectral data.



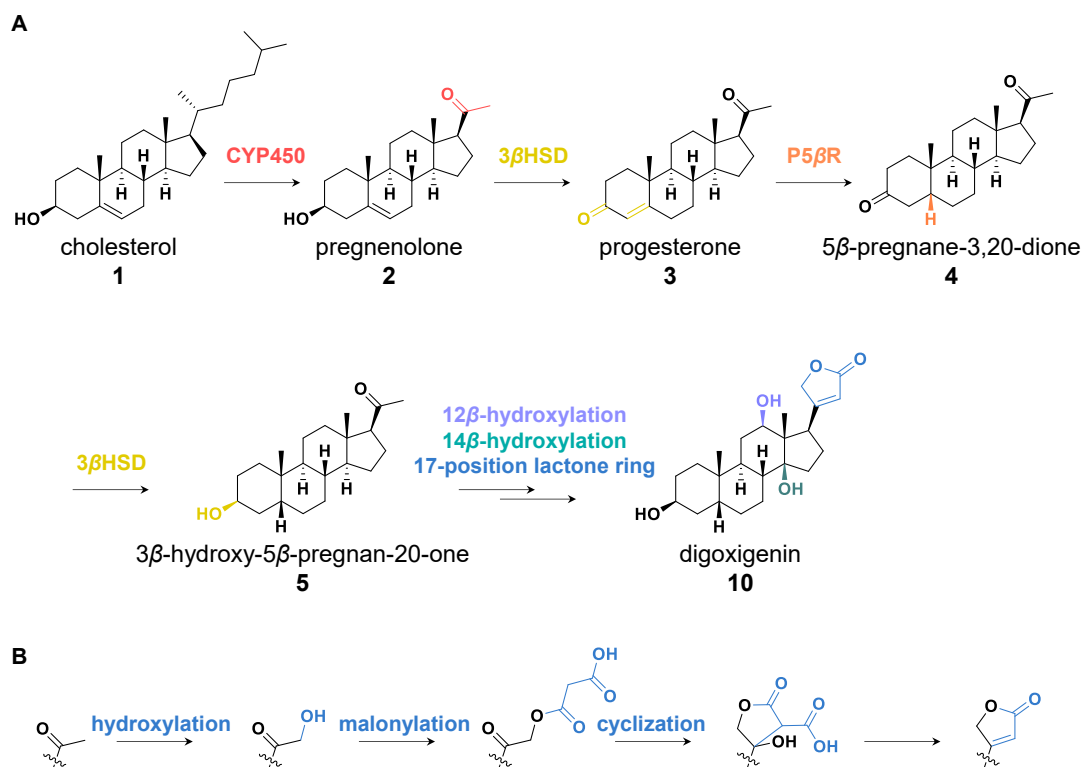
3 β ,14 β ,21-trihydroxy-5 β -pregnan-20-one (7)

¹H-NMR (400 MHz, CDCl₃): δ 4.28 (2H, d, J = 7.67 Hz, H₂-21), 4.12 (1H, brs, H-3), 2.72 (1H, dd, J = 9.72, 3.24 Hz, H-17), 2.19 (1H, m, H-15a), 2.05 (1H, m, H-16a), 1.94 (1H, m, H-1a), 1.91 (1H, m, H-2a), 1.91 (1H, m, H-16b), 1.90 (1H, m, H-6a), 1.86 (1H, m, H-15b), 1.74 (1H, m, H-5), 1.63 (1H, m, H-8), 1.60 (1H, m, H-9), 1.57 (1H, m, H-11a), 1.50 (1H, m, H-11b), 1.50 (1H, m, H-4a), 1.44 (1H, m, H-4b), 1.40 (1H, m, H-6a), 1.35 (1H, m, H-1b), 1.22 (1H, m, H-2b), 1.22 (1H, m, H-6b), 0.96 (3H, s, H₃-19), 0.91 (3H, s, H₃-18)

¹³C-NMR (100 MHz, CDCl₃): δ 217.4 (C-20), 85.1 (C-14), 70.3 (C-21), 67.1 (C-3), 58.0 (C-17), 49.9 (C-13), 40.2 (C-8), 39.3 (C-12), 36.2 (C-5), 35.5 (C-10), 35.1 (C-8), 34.1 (C-15), 33.5 (C-1), 29.8 (C-4), 28.0 (C-11), 26.6 (C-2), 25.0 (C-16), 23.9 (C-19), 21.7 (C-7), 21.0 (C-6), 15.4 (C-18)

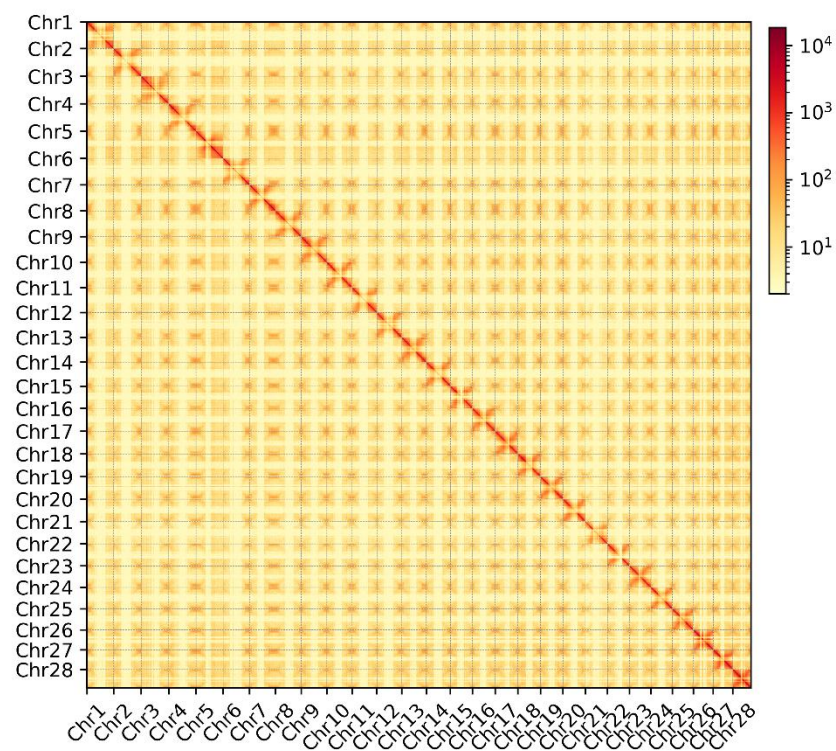
(+)-ESI-HRMS calculated for C₂₁H₃₄O₄ [M+H-2H₂O]⁺ *m/z* 315.2491, found *m/z* 315.2335.

Supplementary Figures



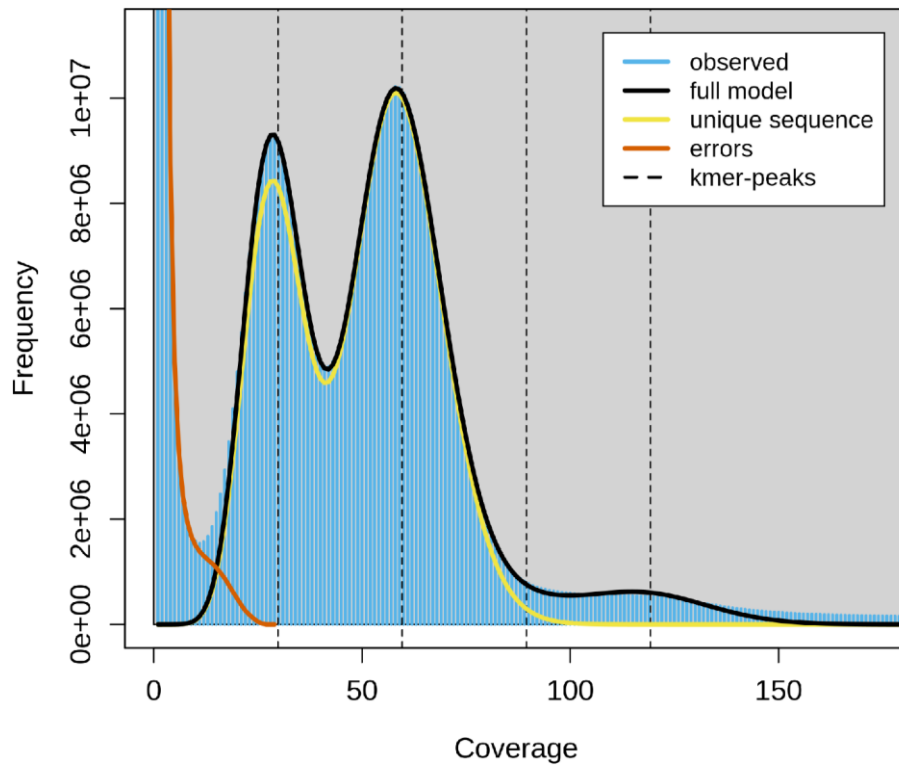
Supplementary Figure 1.

Proposed biosynthetic pathway of digoxigenin. (A) Proposed biosynthetic pathway of digoxigenin starting from cholesterol. (B) Putative formation process of the unsaturated lactone ring at position 17.



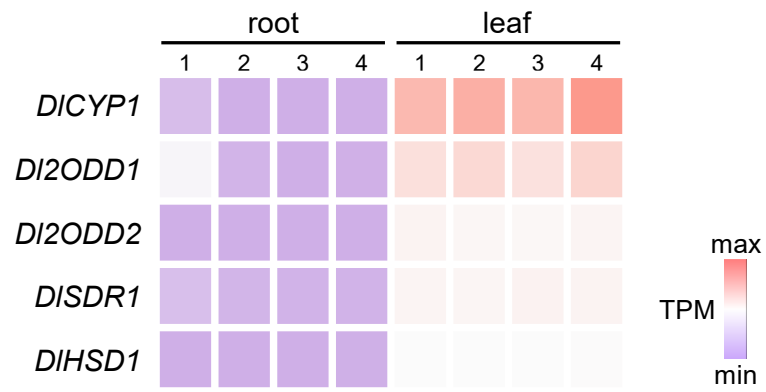
Supplementary Figure 2.

Genome-wide Hi-C interaction heatmap of the *Digitalis lanata* genome (500 Kb resolution). Redder colors indicate more intense interactions.



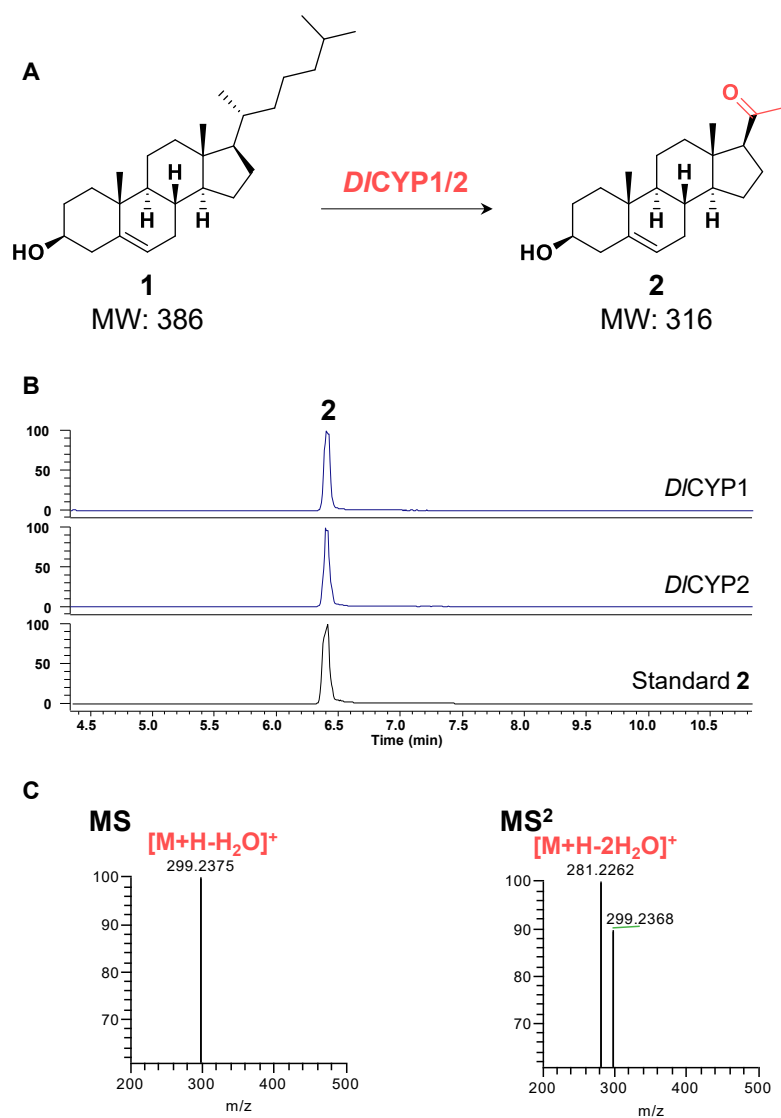
Supplementary Figure 3.

K-mer distribution profile of the *Digitalis lanata* genome.



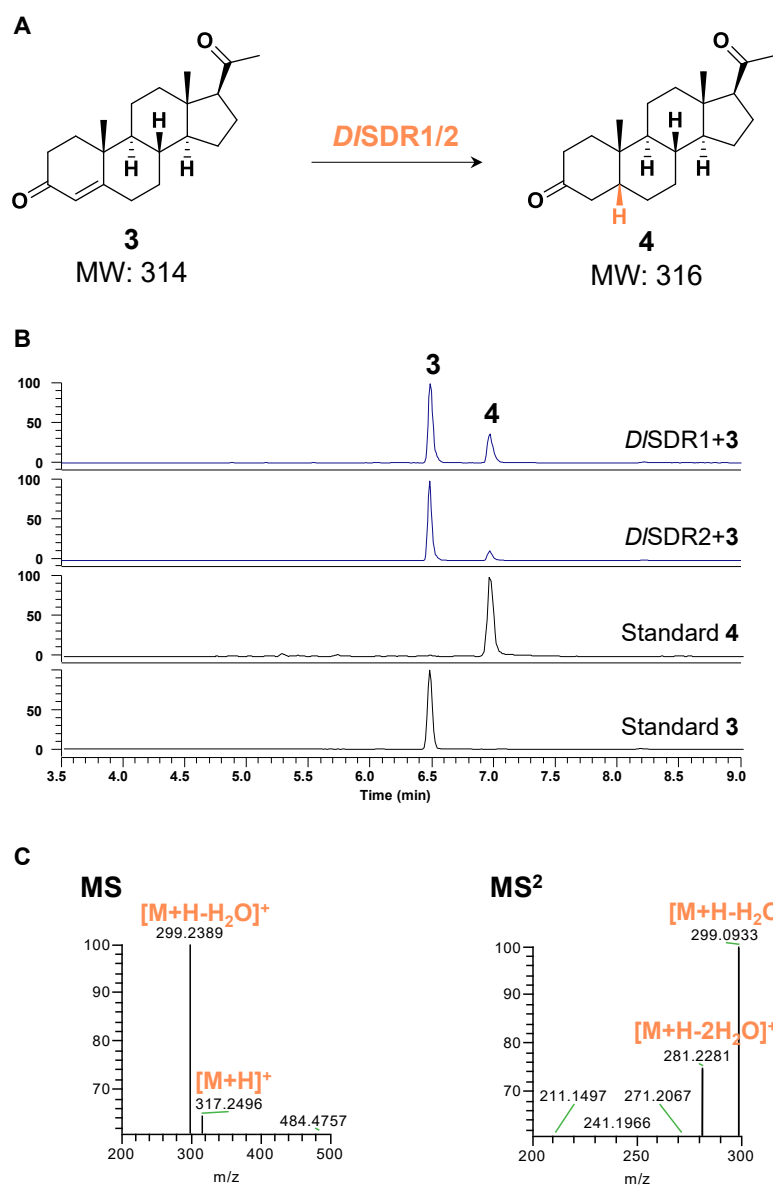
Supplementary Figure 4.

Expression levels of functional genes in the transcriptomes of different plant parts of *D. lanata*. Data represent four biological replicates ($n = 4$).



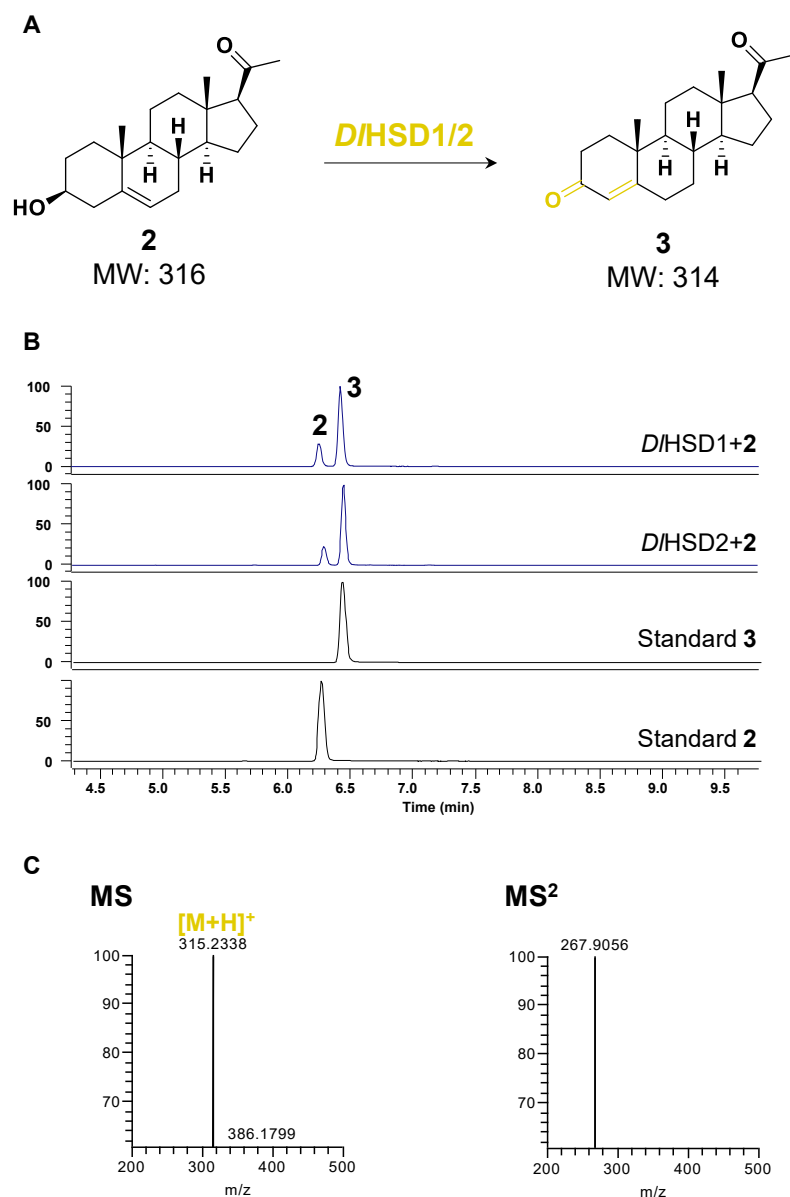
Supplementary Figure 5.

Functional characterization of by *D1CYP1* and *D1CYP2* in *N. benthamiana*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *D1CYP1* and *D1CYP2*, using **1** as substrate. (C) (+)-ESI-MS and MS/MS spectra of product **2**.



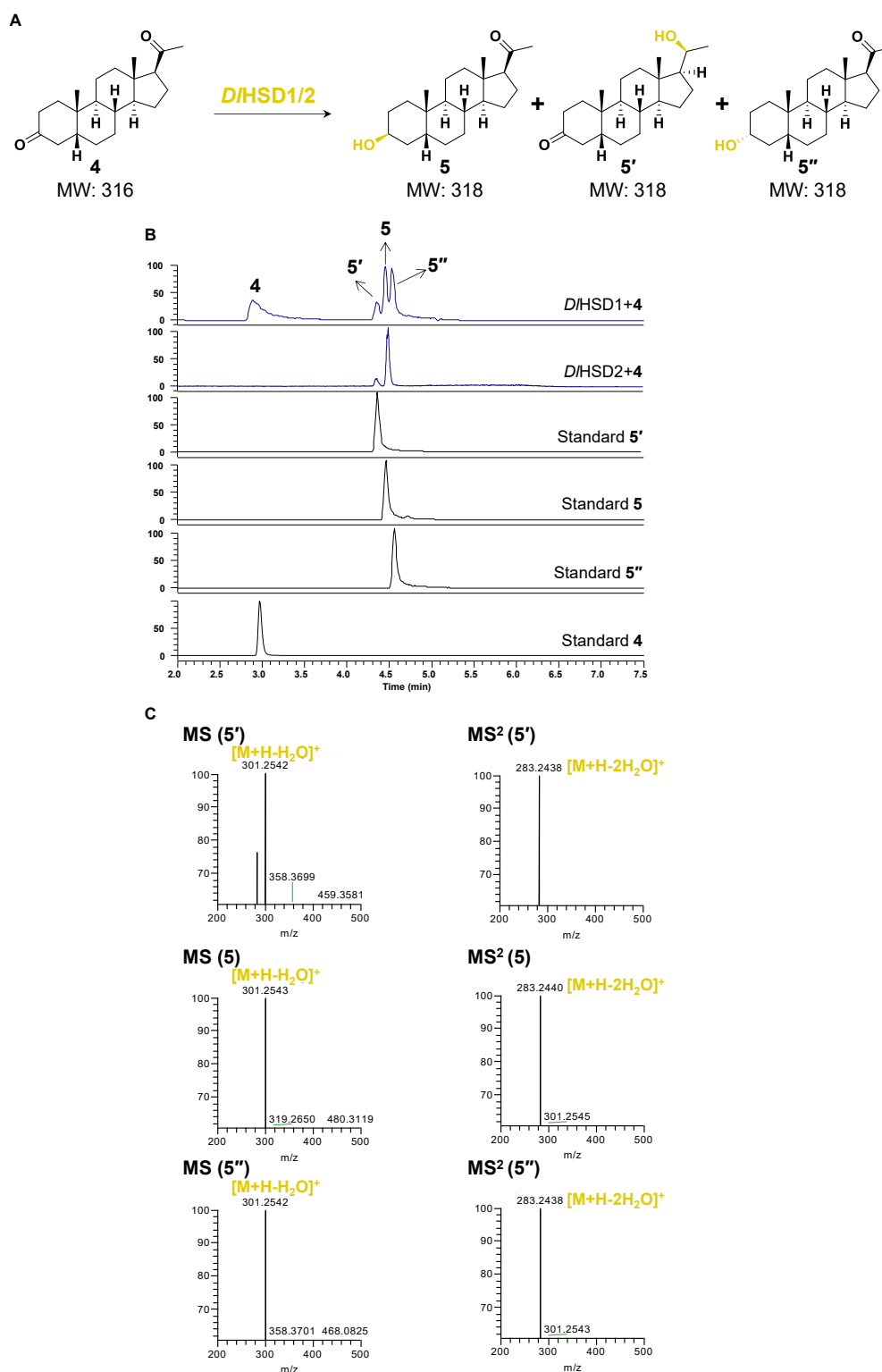
Supplementary Figure 6.

Enzymatic catalysis of **3** by *D/SDR1* and *D/SDR2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/SDR1* and *D/SDR2* with substrate **3**. (C) (+)-ESI-MS and MS/MS spectra of product **4**.



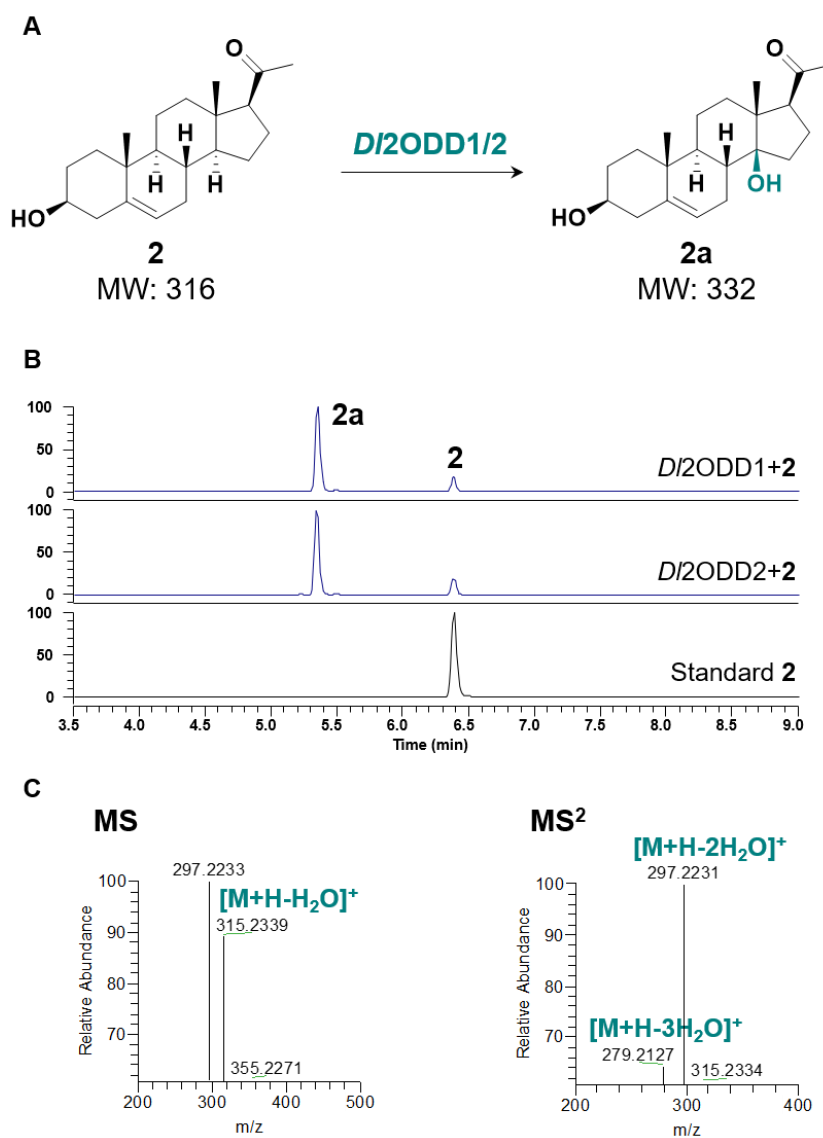
Supplementary Figure 7.

Enzymatic catalysis of **2** by *D/HSD1* and *D/HSD2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/HSD1* and *D/HSD2* with substrate **2**. (C) (+)-ESI-MS and MS/MS spectra of product **3**.



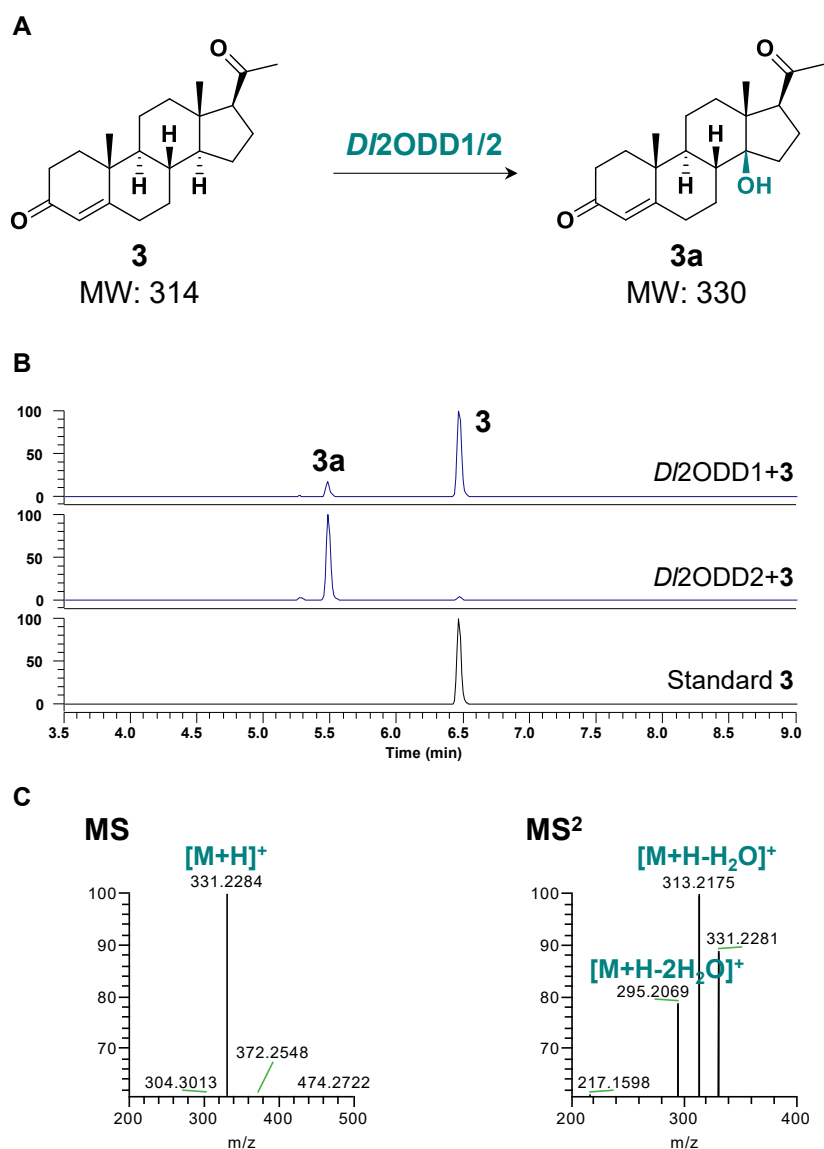
Supplementary Figure 8.

Enzymatic catalysis of **4** by *D/HSD1* and *D/HSD2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/HSD1* and *D/HSD2* with substrate **4**. (C) (+)-ESI-MS and MS/MS spectra of products **5**, **5'**, and **5''**.



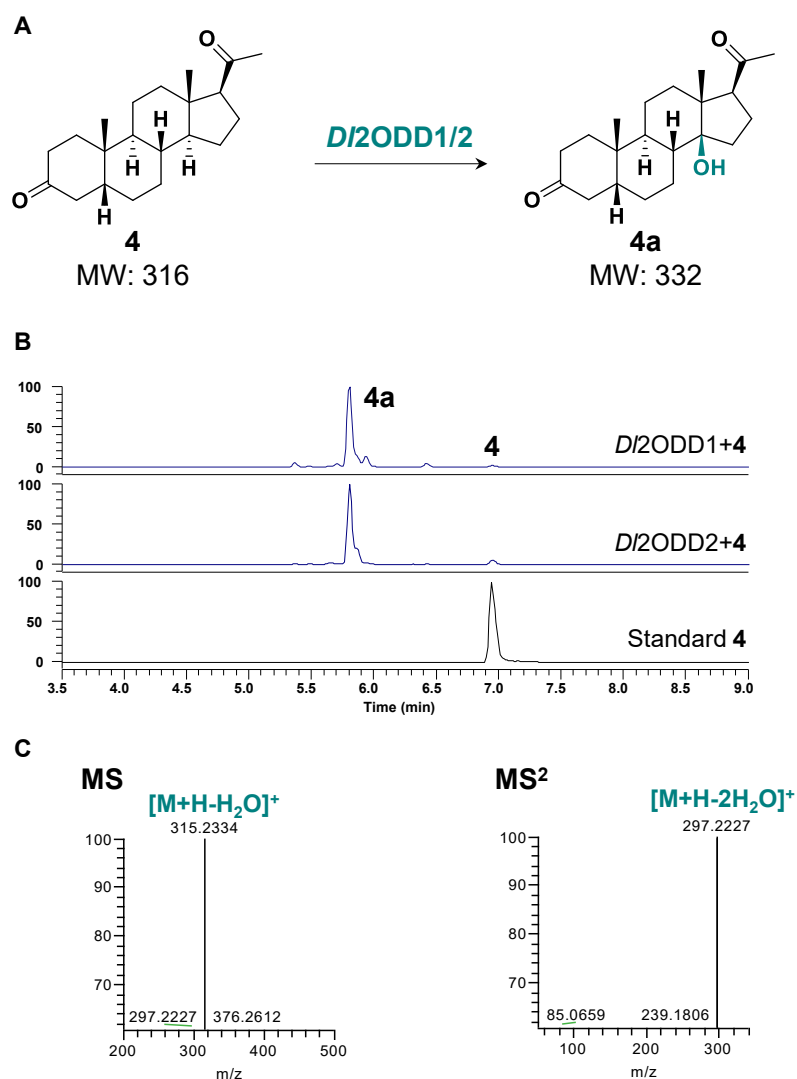
Supplementary Figure 9.

Enzymatic catalysis of **2** by *D/I2ODD1* and *D/I2ODD2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/I2ODD1* and *D/I2ODD2* with substrate **2**. (C) (+)-ESI-MS and MS/MS spectra of product **2a**.



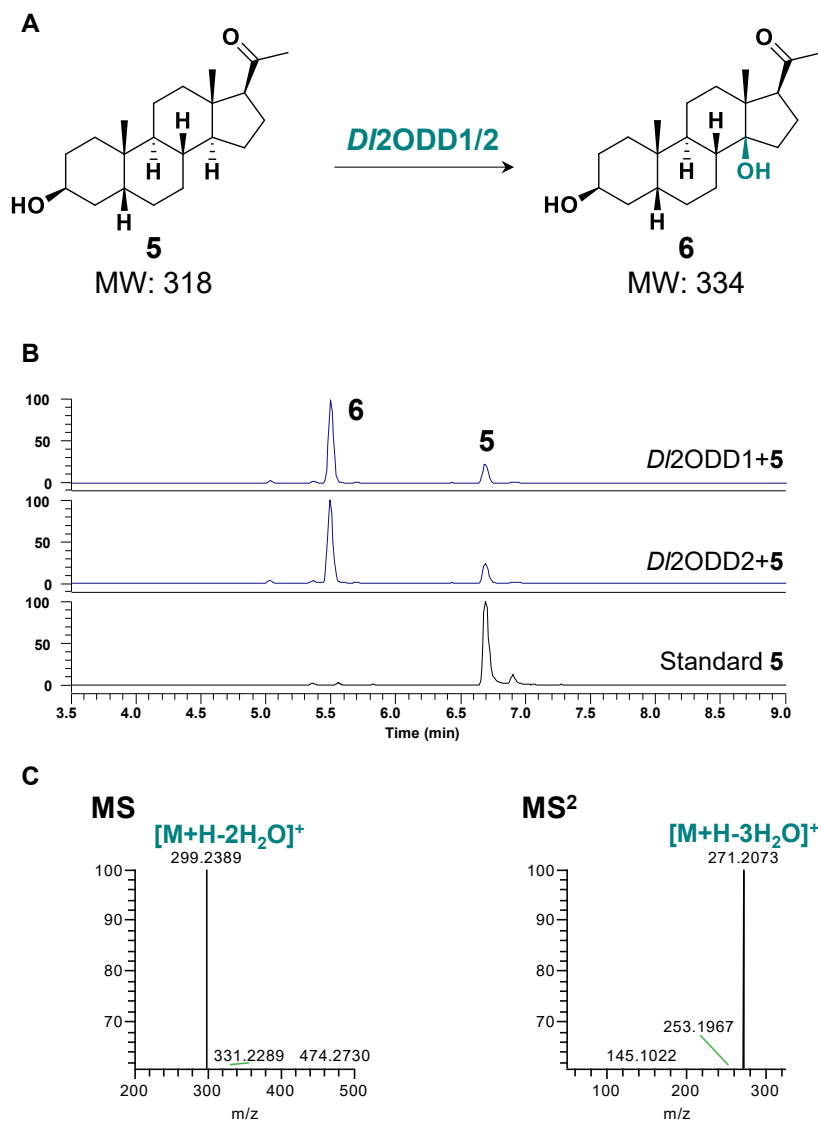
Supplementary Figure 10.

Enzymatic catalysis of **3** by *D12ODD1* and *D12ODD2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD1* and *D12ODD2* with substrate **3**. (C) (+)-ESI-MS and MS/MS spectra of product **3a**.



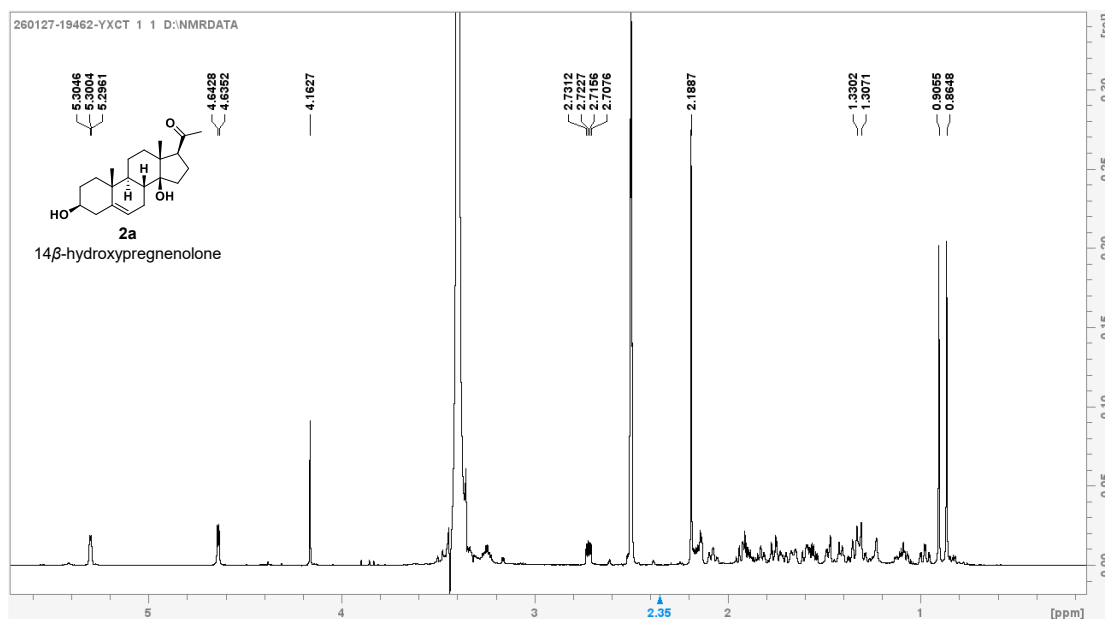
Supplementary Figure 11.

Enzymatic catalysis of **4** by *D12ODD1* and *D12ODD2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD1* and *D12ODD2* with substrate **4**. (C) (+)-ESI-MS and MS/MS spectra of product **4a**.

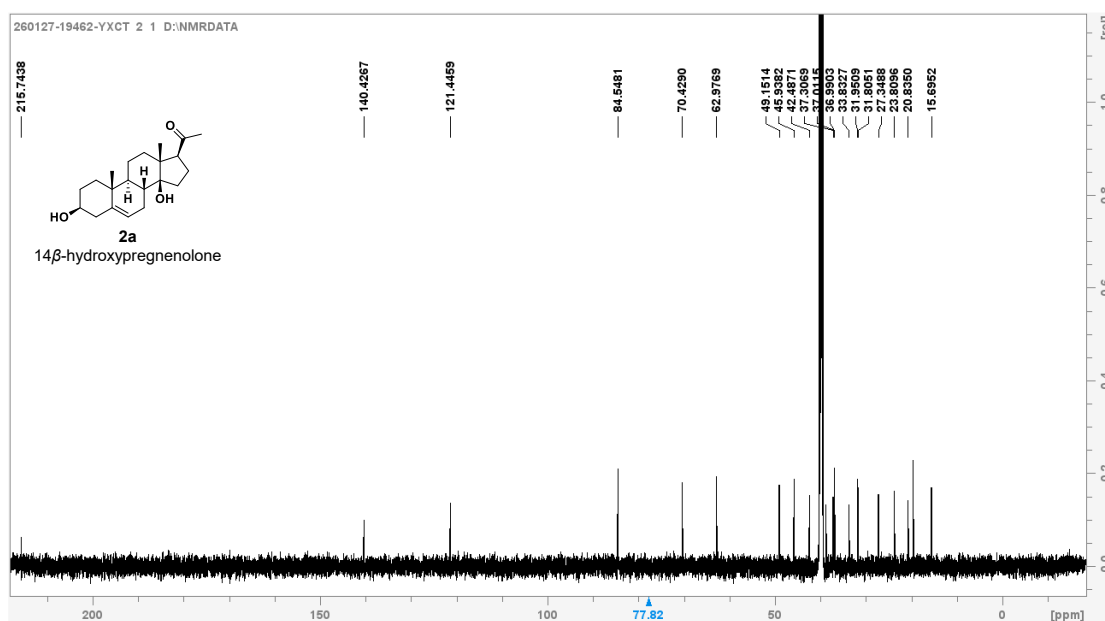


Supplementary Figure 12.

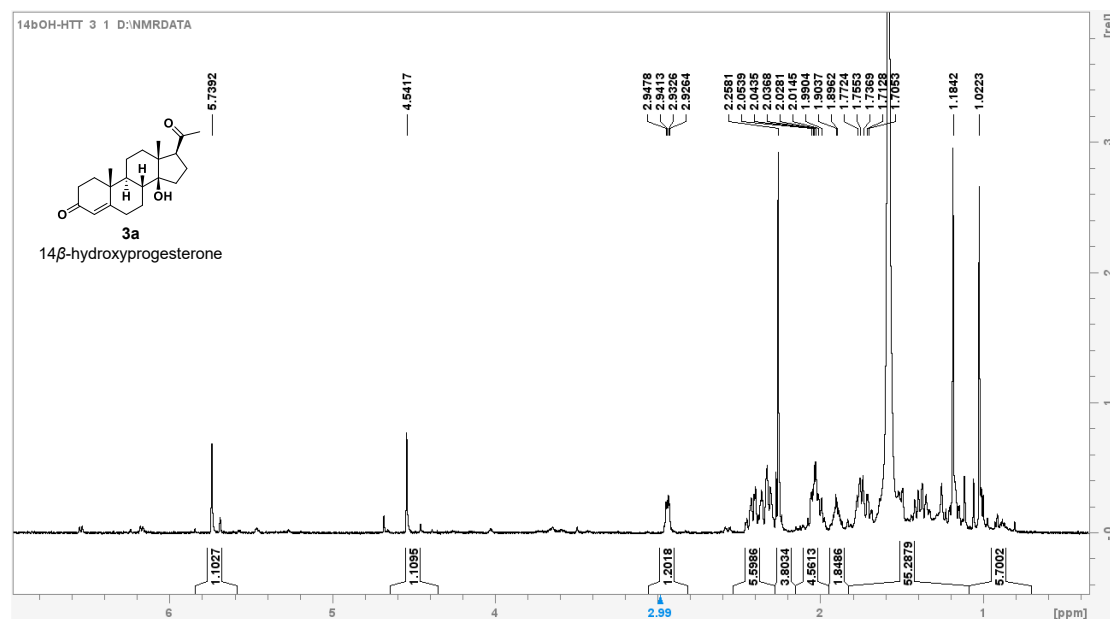
Enzymatic catalysis of **5** by *D/2ODD1* and *D/2ODD2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/2ODD1* and *D/2ODD2* with substrate **5**. (C) (+)-ESI-MS and MS/MS spectra of product **6**.



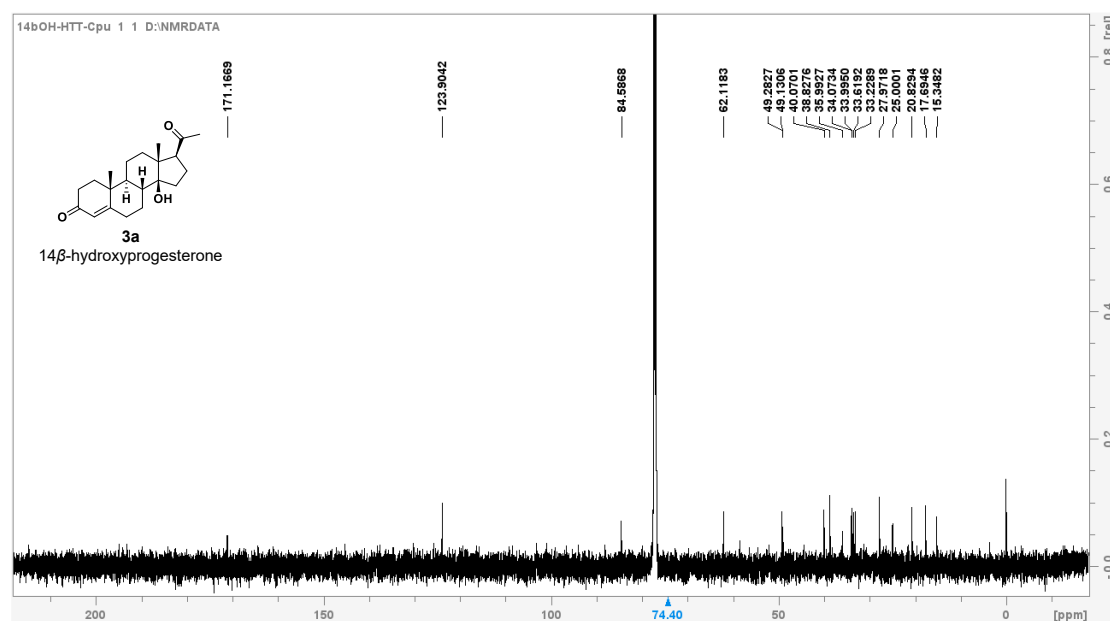
Supplementary Figure 13. The ^1H NMR spectrum of **2a** in $\text{DMSO}-d_6$ (600 MHz).



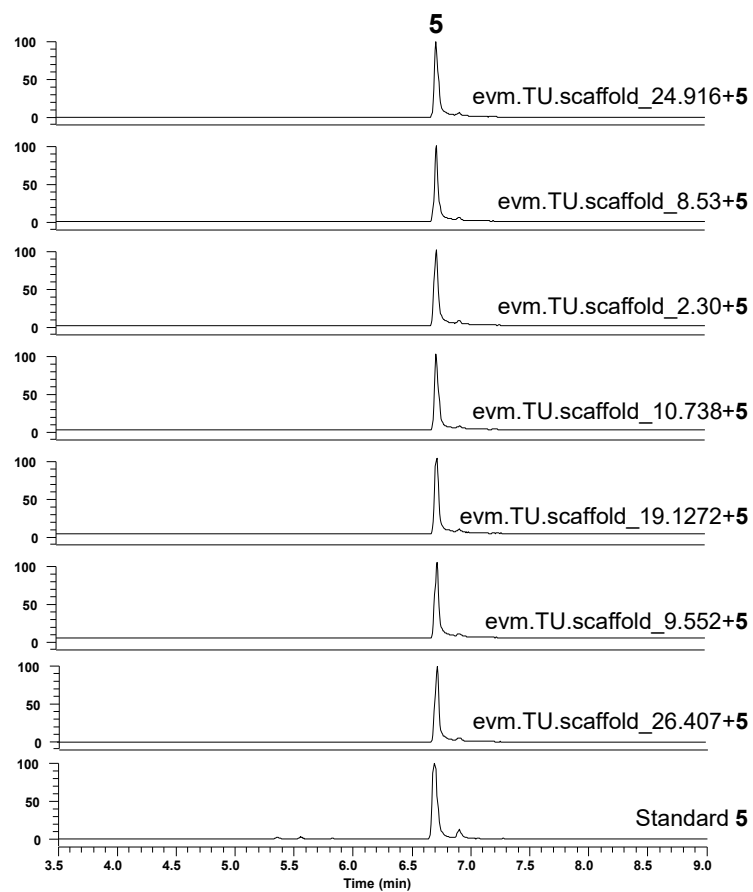
Supplementary Figure 14. The ^{13}C NMR spectrum of **2a** in $\text{DMSO}-d_6$ (150 MHz).



Supplementary Figure 15. The ^1H NMR spectrum of **3a** in CDCl_3 (600 MHz).

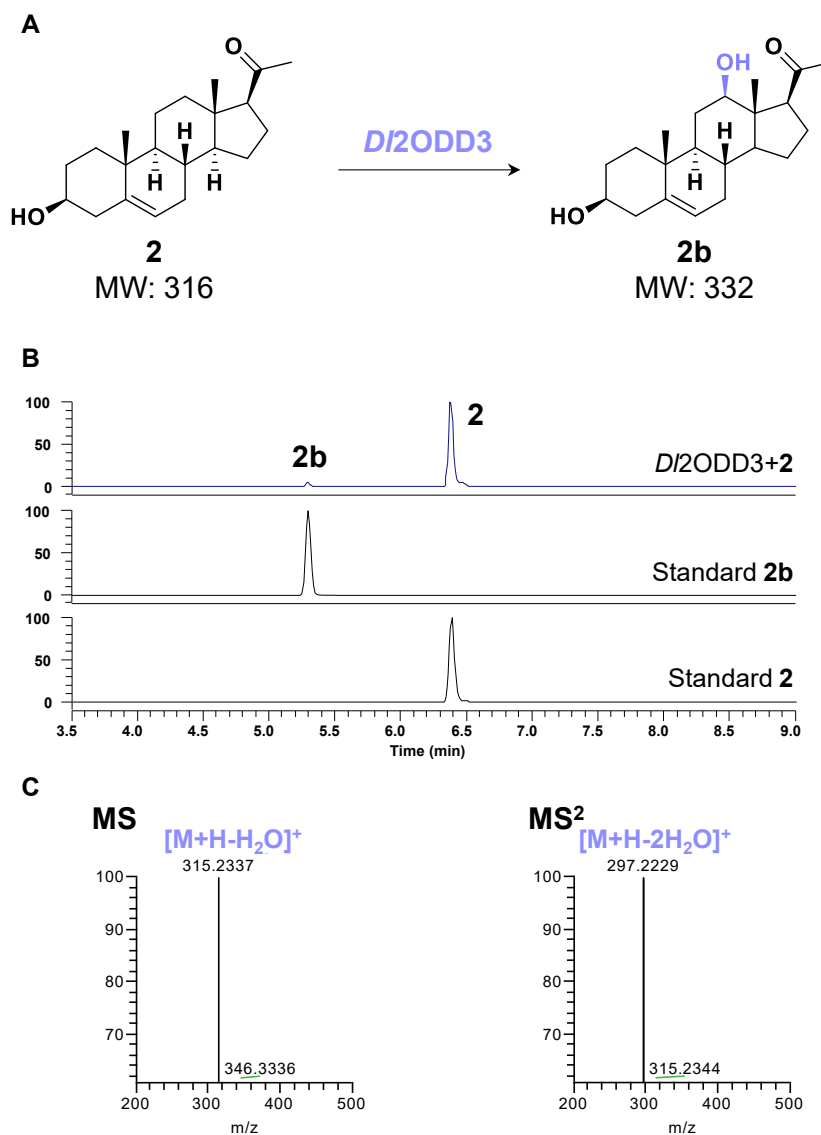


Supplementary Figure 16. The ^{13}C NMR spectrum of **3a** in CDCl_3 (150 MHz).



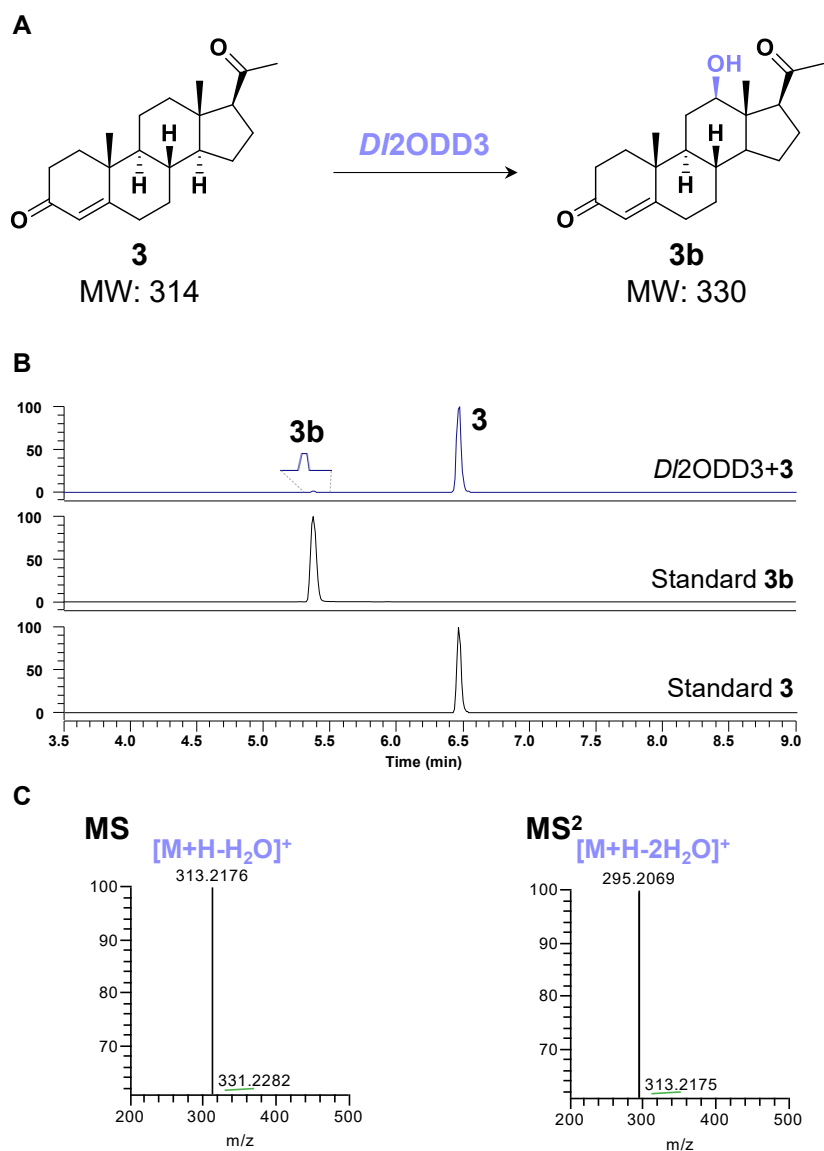
Supplementary Figure 17.

UPLC-HRMS chromatograms of seven candidate P450s, using **5** as substrate.



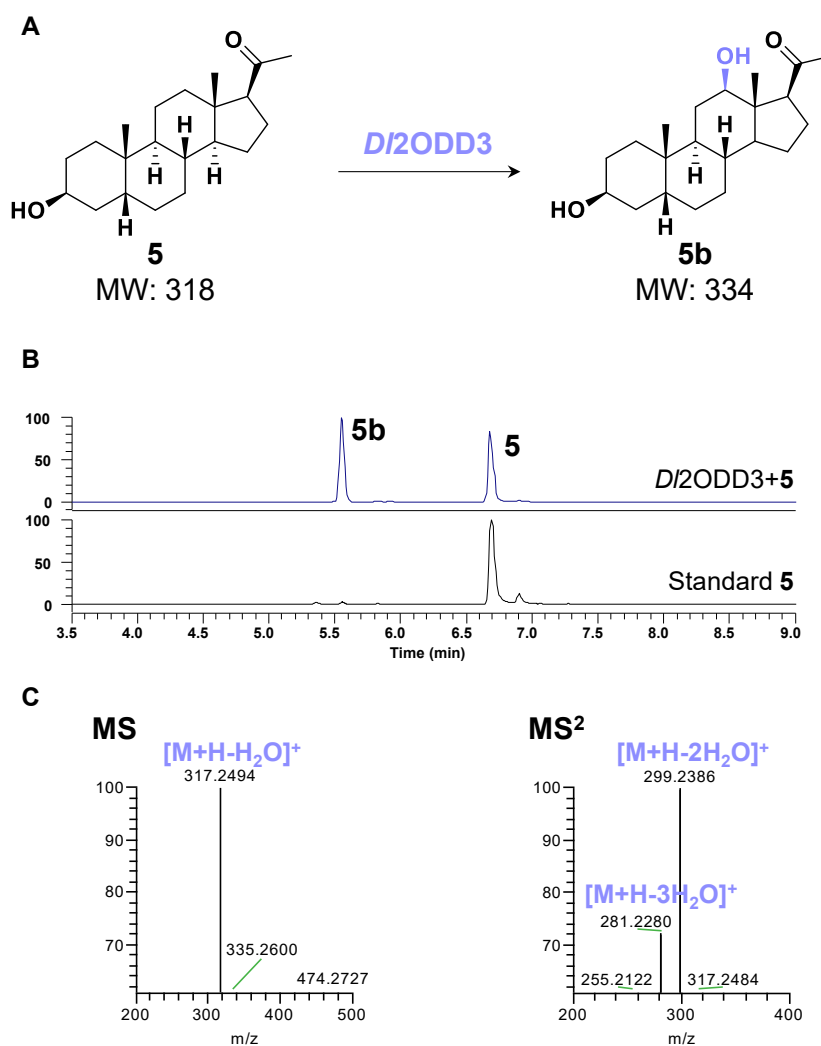
Supplementary Figure 18.

Enzymatic catalysis of **2** by *D12ODD3*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3* with substrate **2**. (C) (+)-ESI-MS and MS/MS spectra of product **2b**.



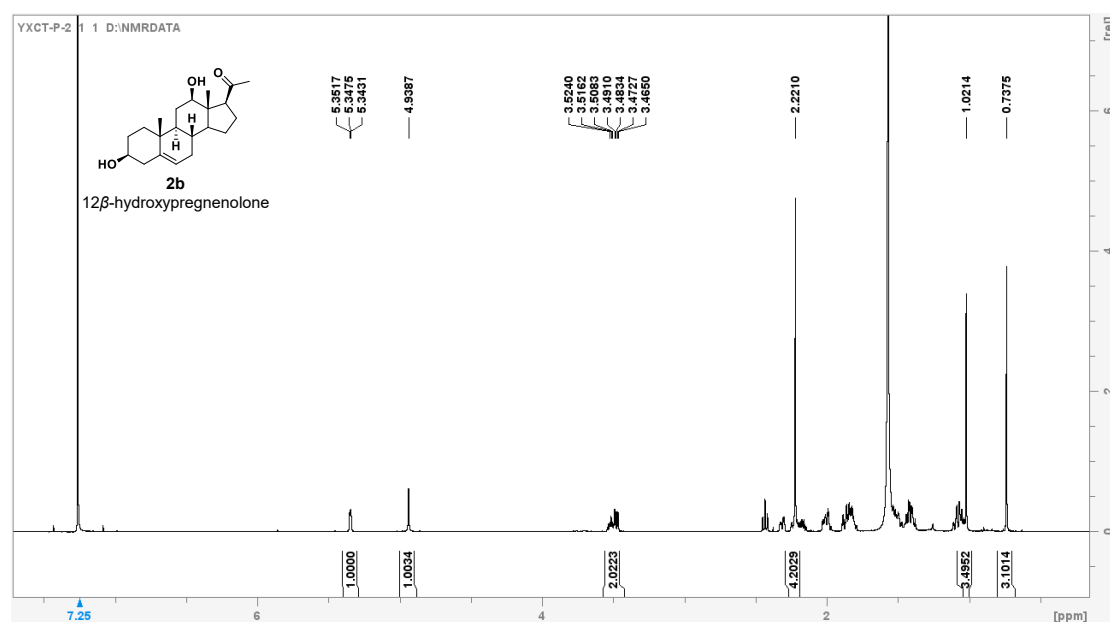
Supplementary Figure 19.

Enzymatic catalysis of **3** by *D/I2ODD3*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/I2ODD3* with substrate **3**. (C) (+)-ESI-MS and MS/MS spectra of product **3b**.

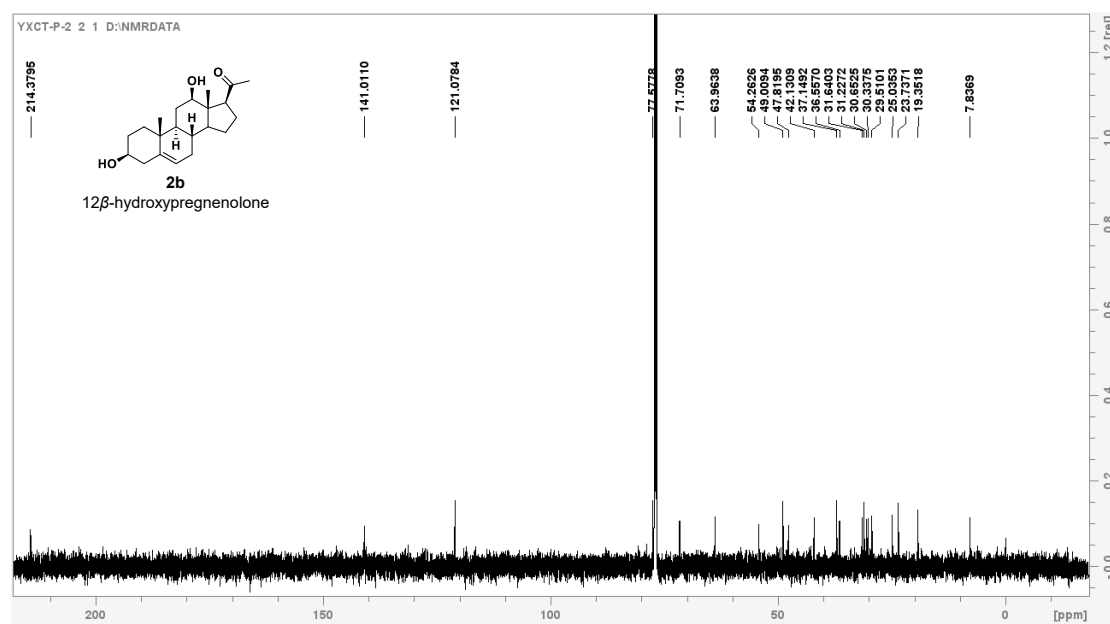


Supplementary Figure 20.

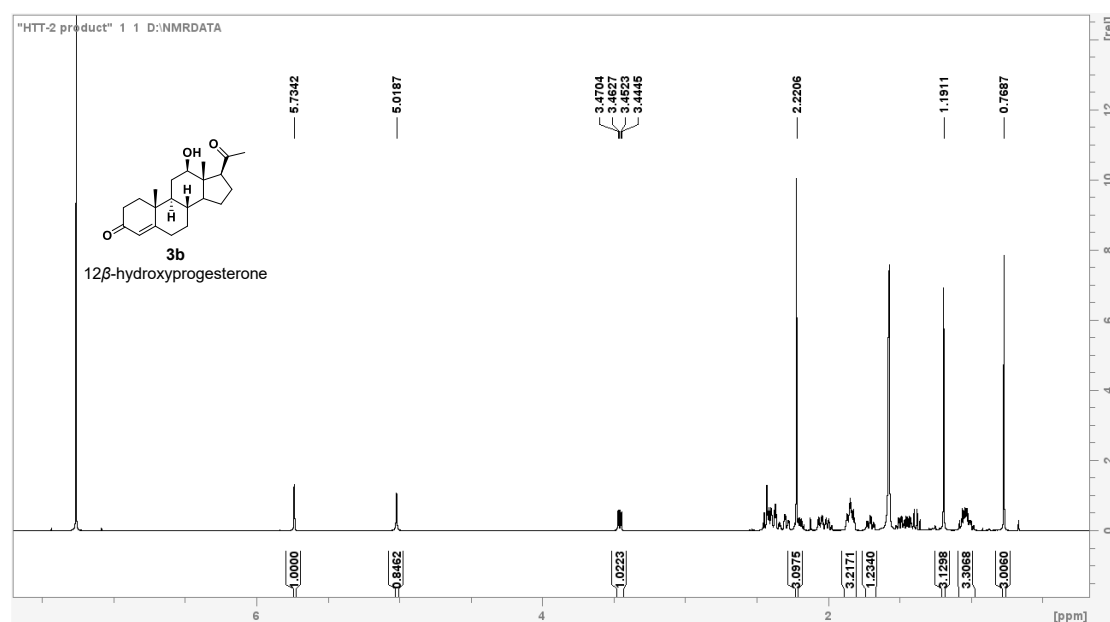
Enzymatic catalysis of **5** by *D/I2ODD3*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/I2ODD3* with substrate **5**. (C) (+)-ESI-MS and MS/MS spectra of product **5b**.



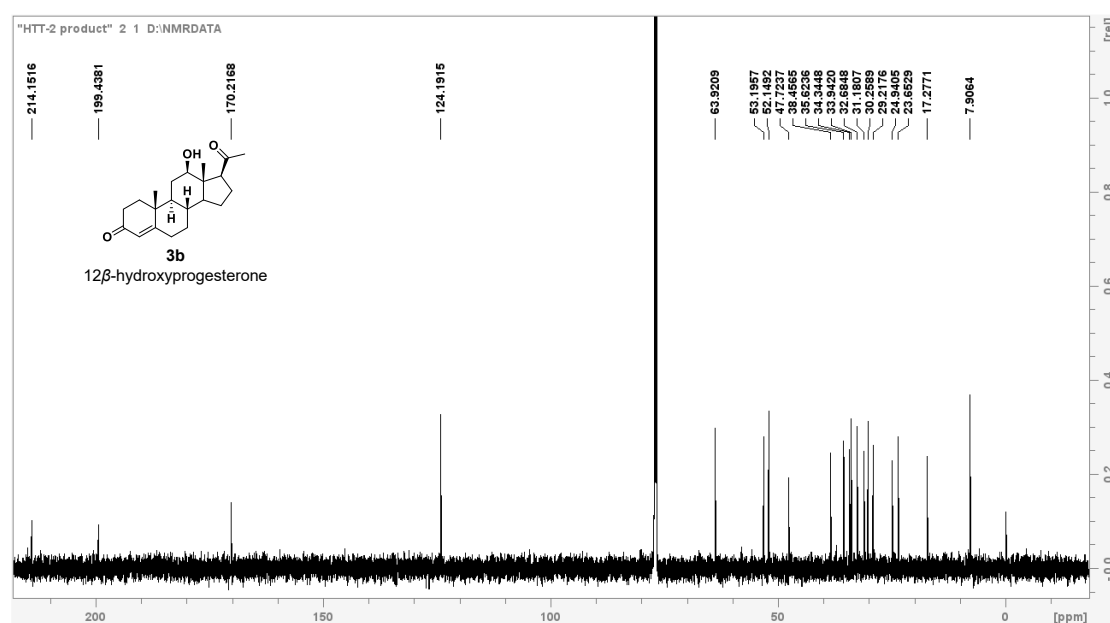
Supplementary Figure 21. The ^1H NMR spectrum of **2b** in CDCl_3 (600 MHz).



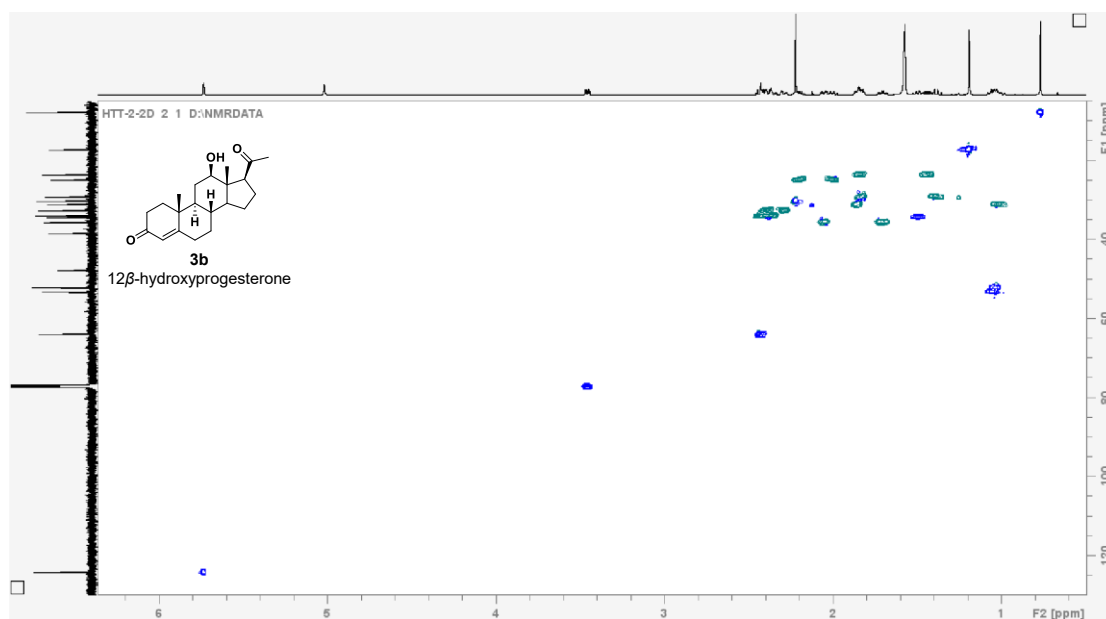
Supplementary Figure 22. The ^{13}C NMR spectrum of **2b** in CDCl_3 (150 MHz).



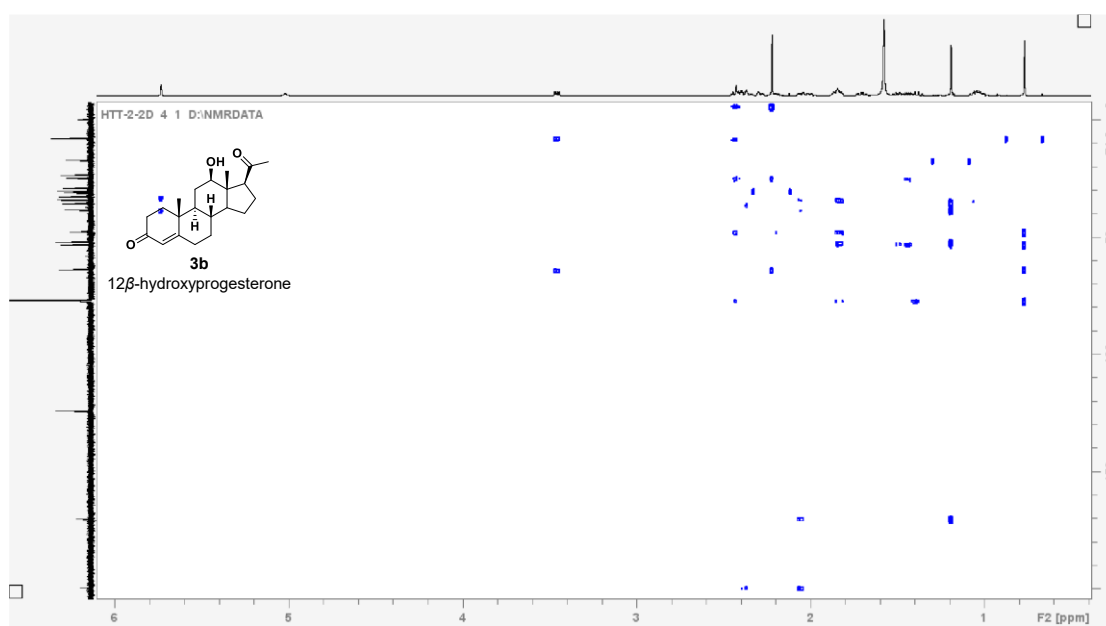
Supplementary Figure 23. The ^1H NMR spectrum of **3b** in CDCl_3 (600 MHz).



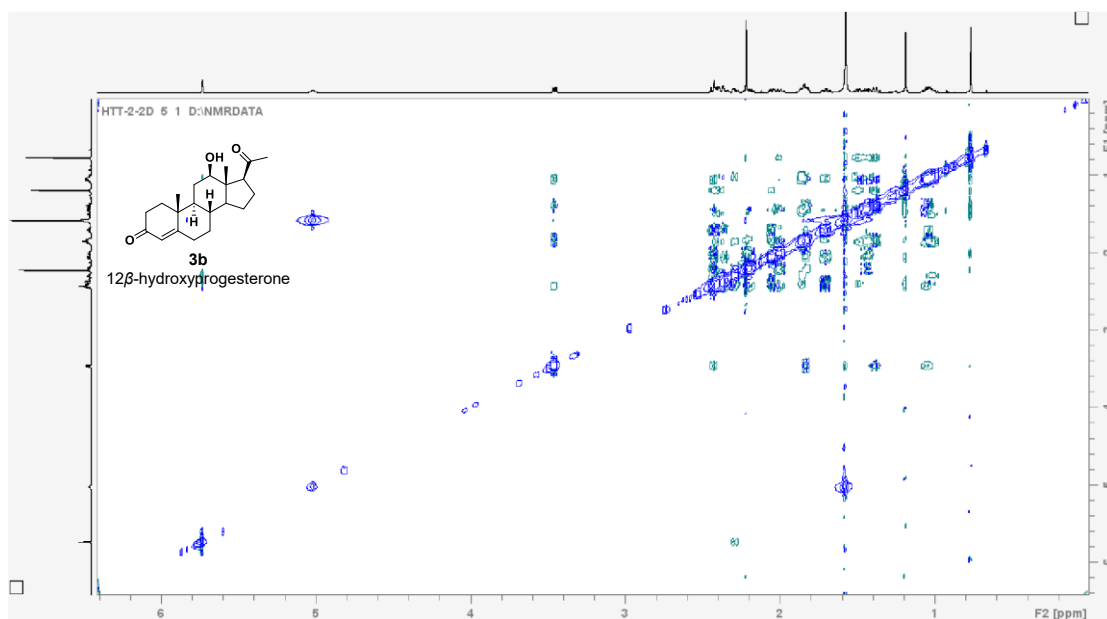
Supplementary Figure 24. The ^{13}C NMR spectrum of **3b** in CDCl_3 (150 MHz).



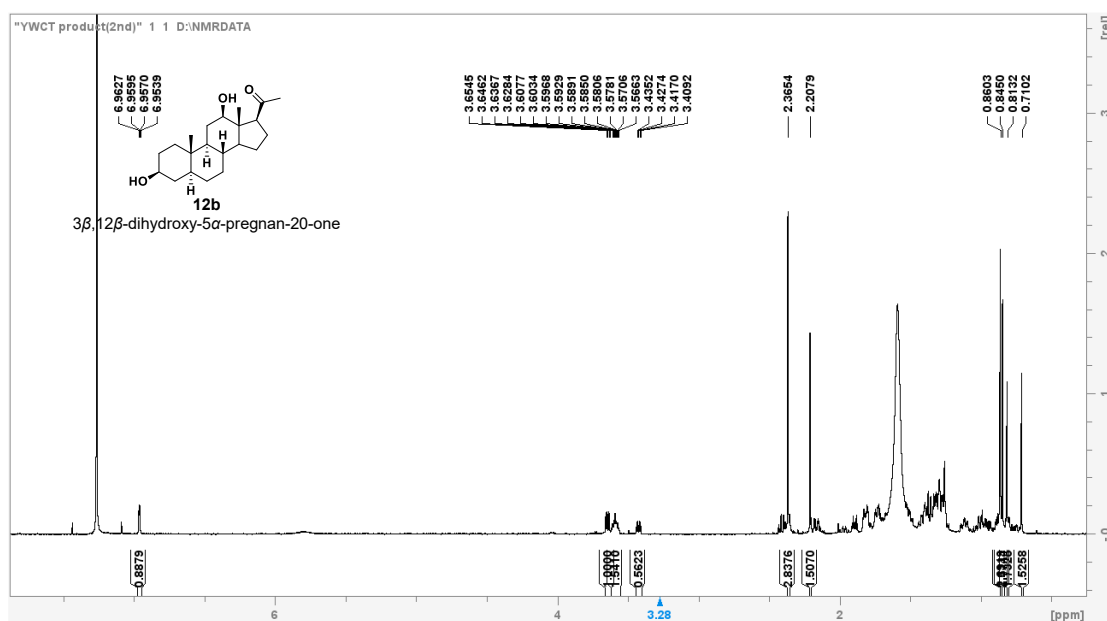
Supplementary Figure 25. The HSQC spectrum of **3b** in CDCl₃ (600 MHz).



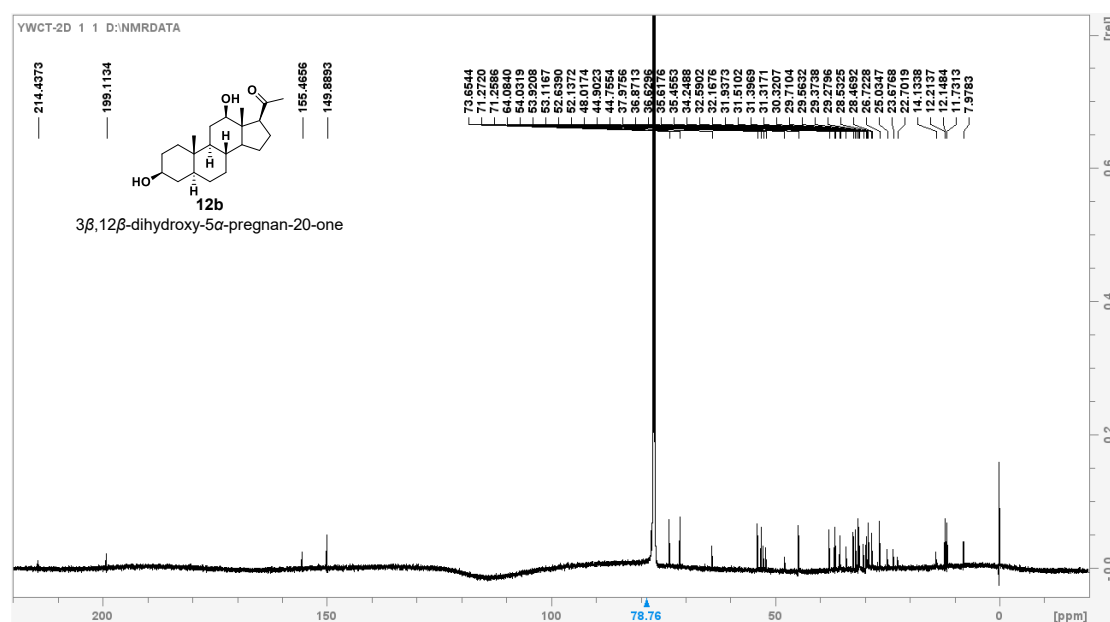
Supplementary Figure 26. The HMBC spectrum of **3b** in CDCl₃ (600 MHz).



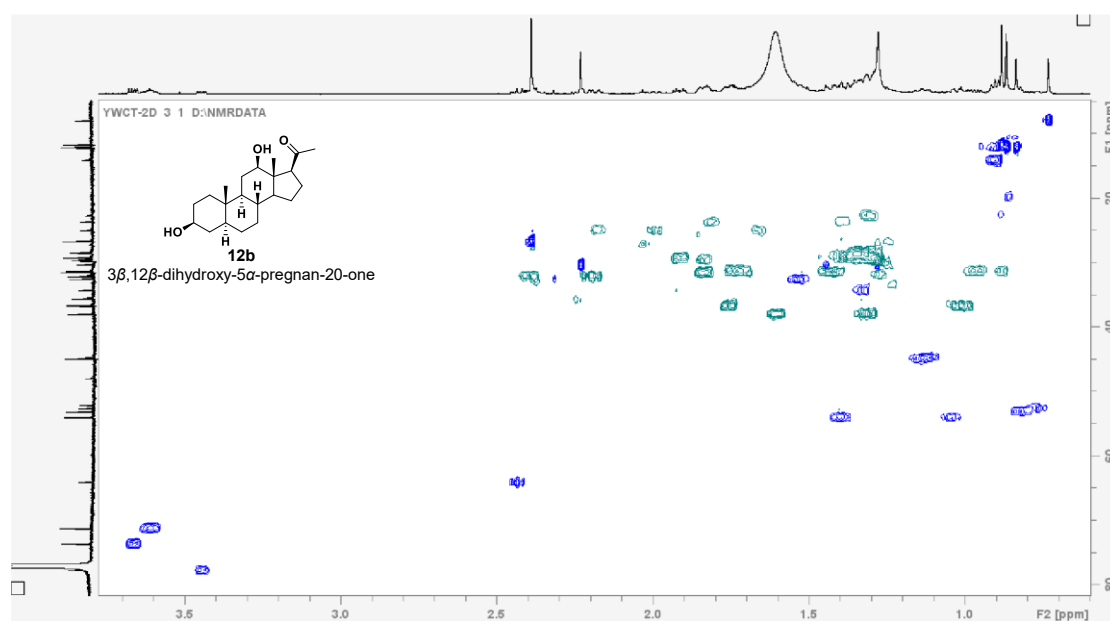
Supplementary Figure 27. The ^1H - ^1H COSY spectrum of **3b** in CDCl_3 (600 MHz).



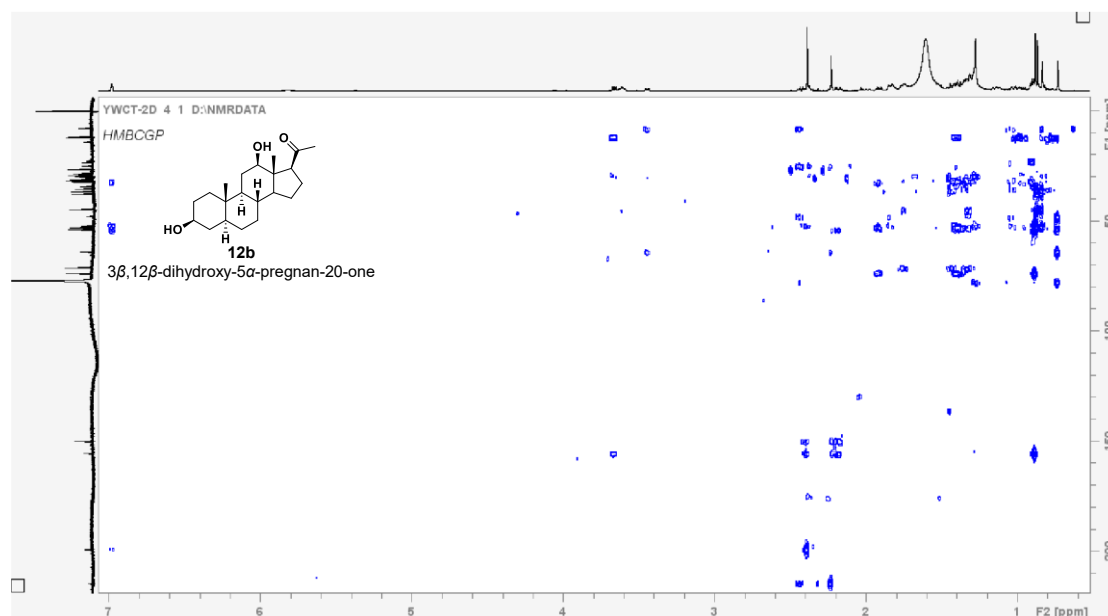
Supplementary Figure 28. The ^1H NMR spectrum of **12b** in CDCl_3 (800 MHz).



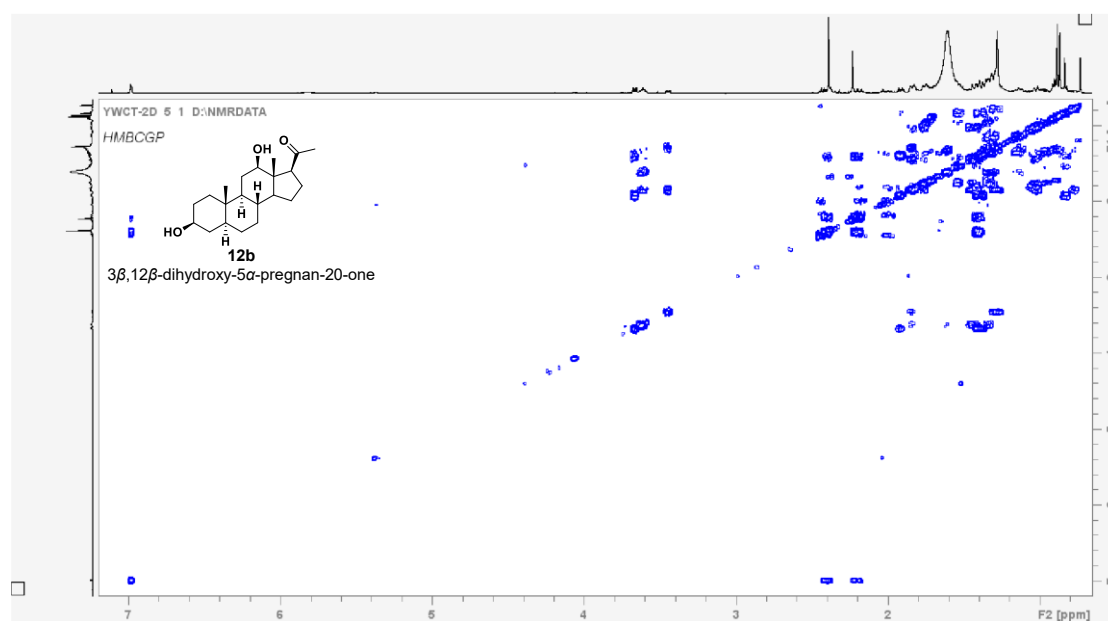
Supplementary Figure 29. The ^{13}C NMR spectrum of **12b** in CDCl_3 (200 MHz).



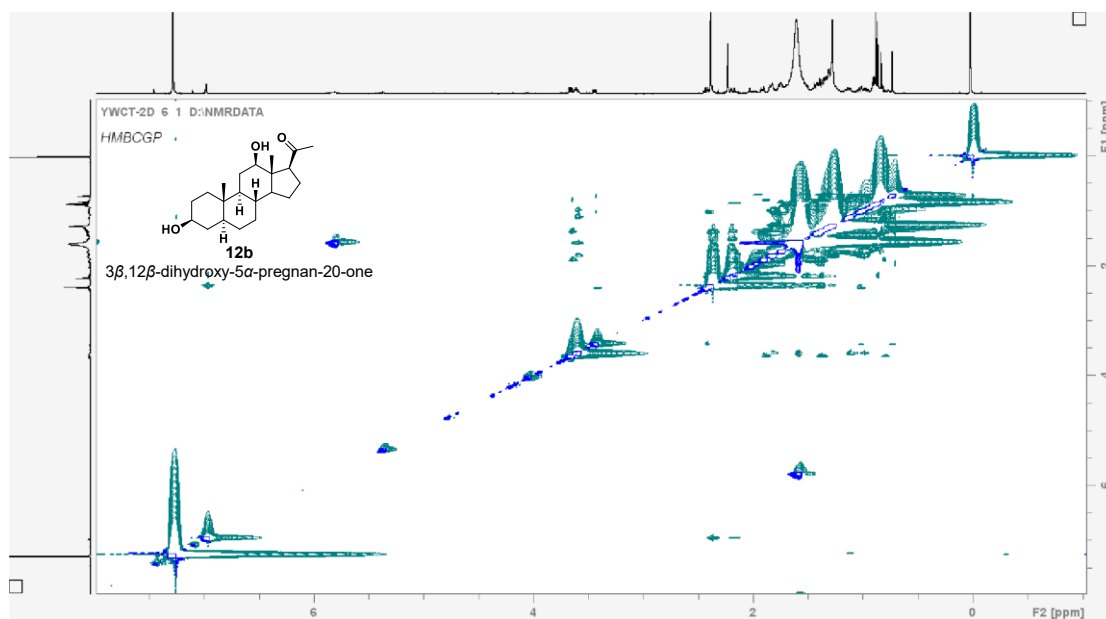
Supplementary Figure 30. The HSQC spectrum of **12b** in CDCl_3 (800 MHz).



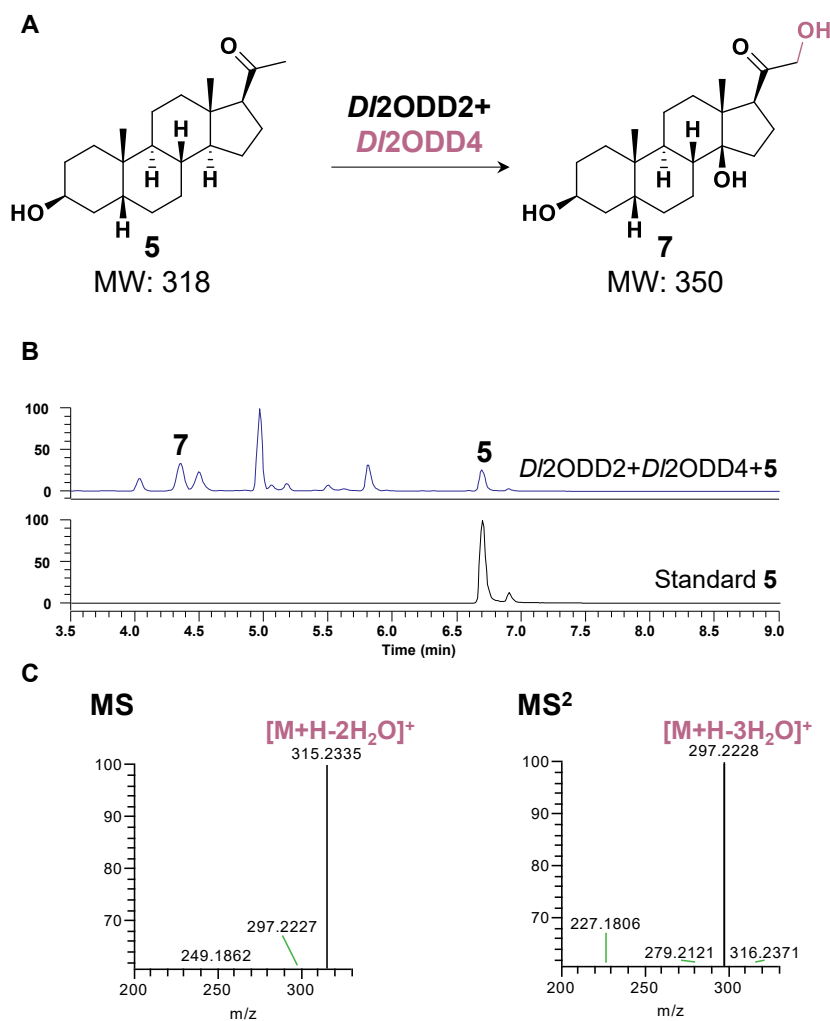
Supplementary Figure 31. The HMBC spectrum of **12b** in CDCl₃ (800 MHz).



Supplementary Figure 32. The ¹H-¹H COSY spectrum of **12b** in CDCl₃ (800 MHz).

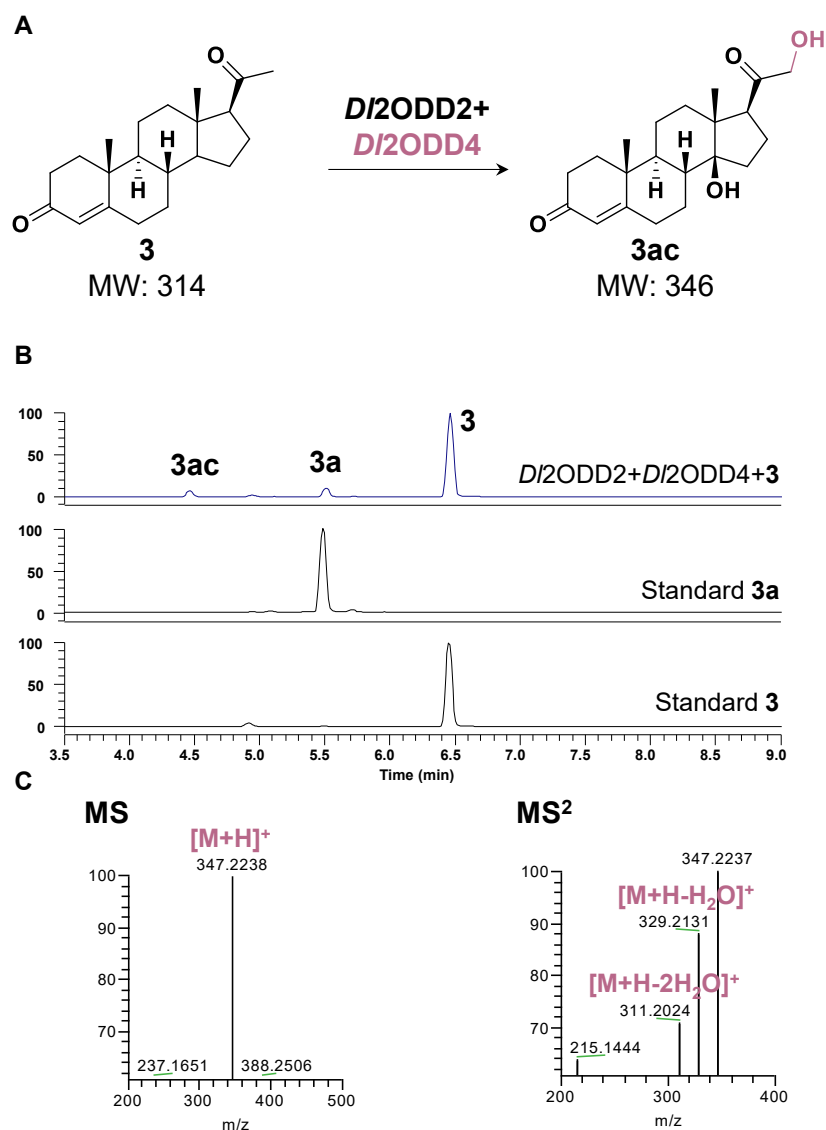


Supplementary Figure 33. The NOESY spectrum of **12b** in CDCl_3 (800 MHz).



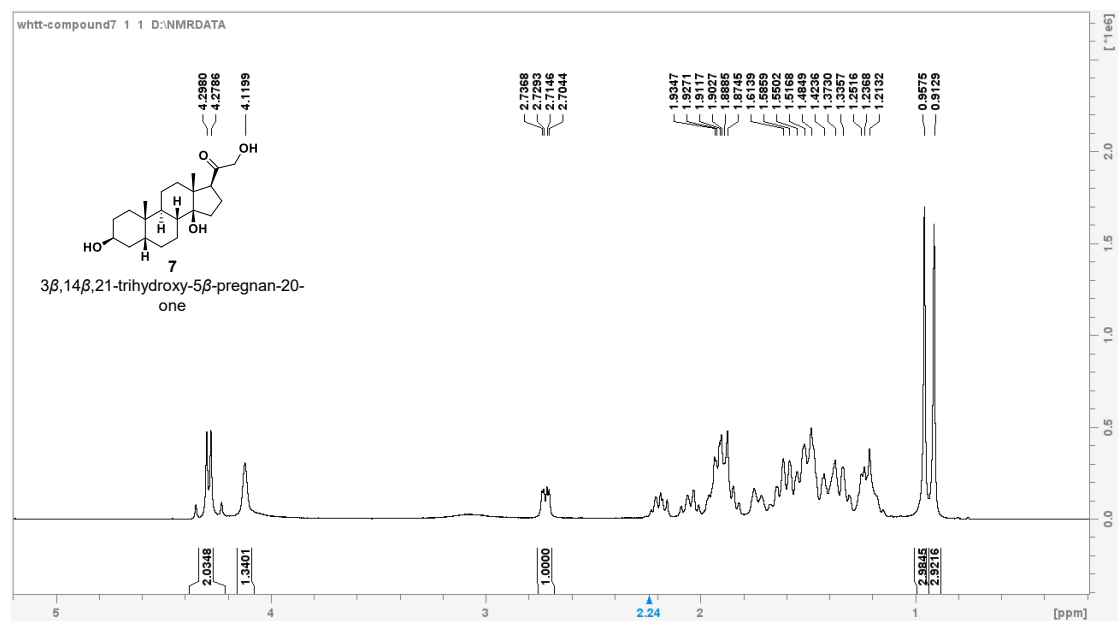
Supplementary Figure 34.

Two-step enzymatic catalysis of substrate **5** by *D/I2ODD2* and *D/I2ODD4* in a coupled assay. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of two-step enzymatic catalysis, using **5** as substrate. (C) (+)-ESI-MS and MS/MS spectra of product **7**.

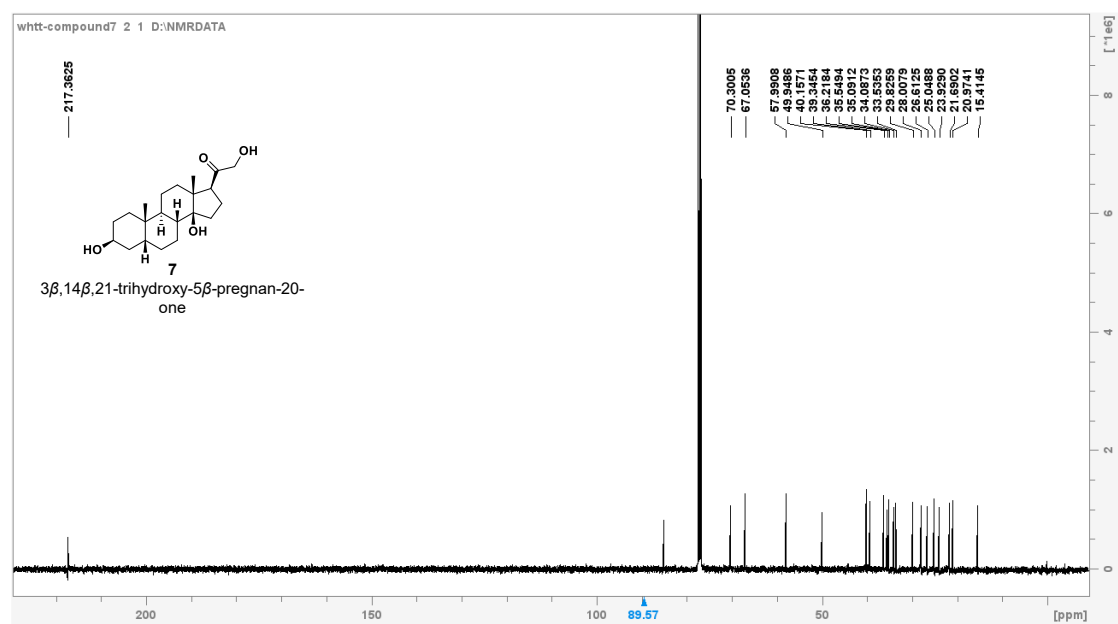


Supplementary Figure 35.

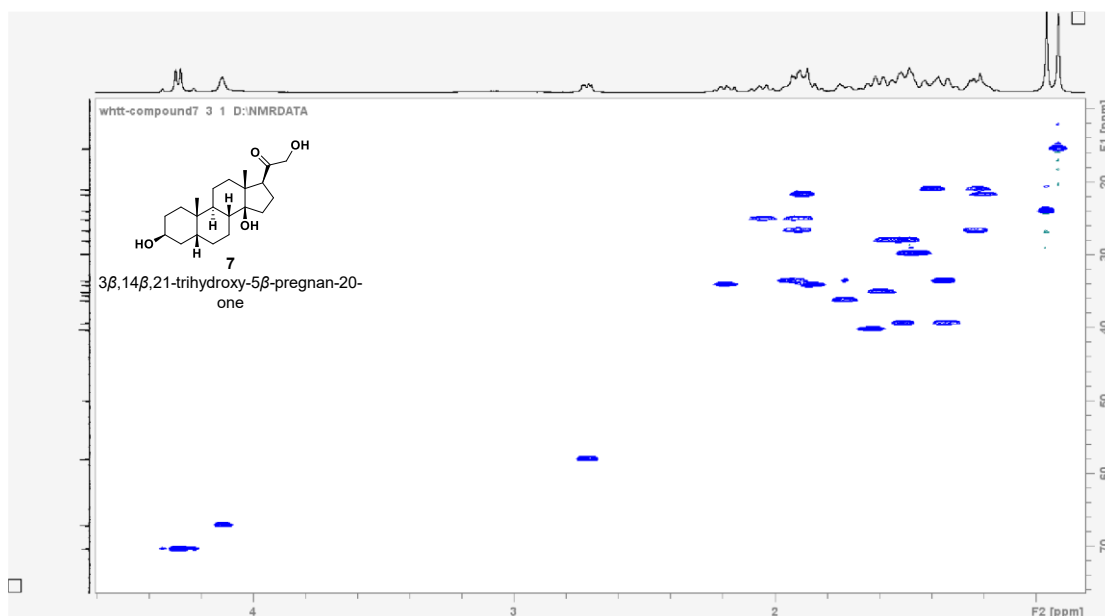
Two-step enzymatic catalysis of substrate **3** by *D12ODD2* and *D12ODD4* in a coupled assay. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of two-step enzymatic catalysis, using **3** as substrate. (C) (+)-ESI-MS and MS/MS spectra of product **3ac**.



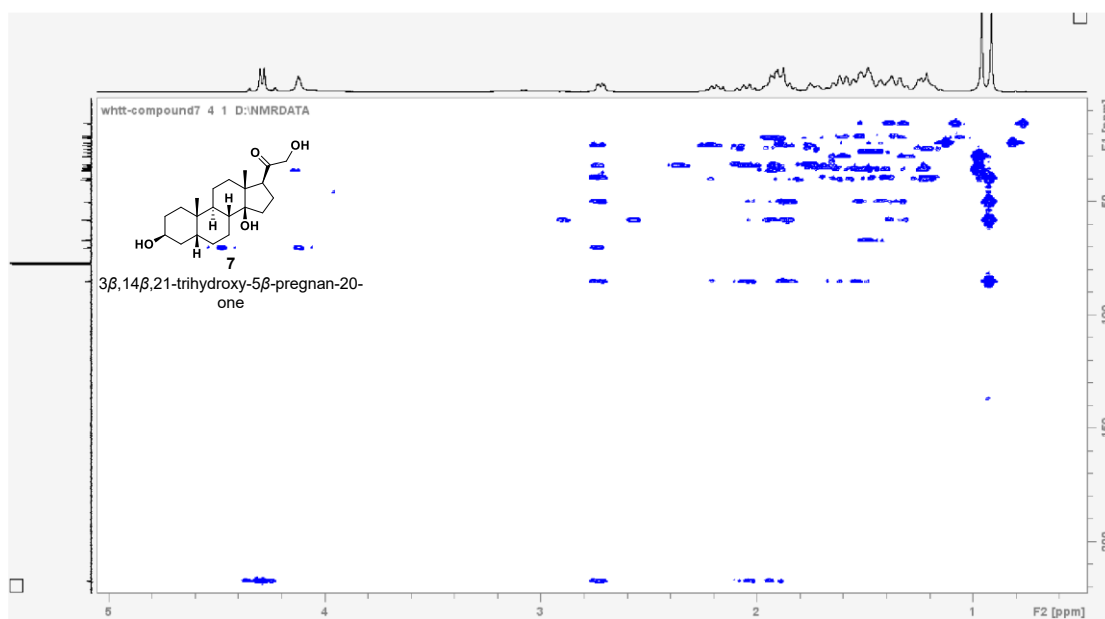
Supplementary Figure 36. The ^1H NMR spectrum of 7 in CDCl_3 (400 MHz).



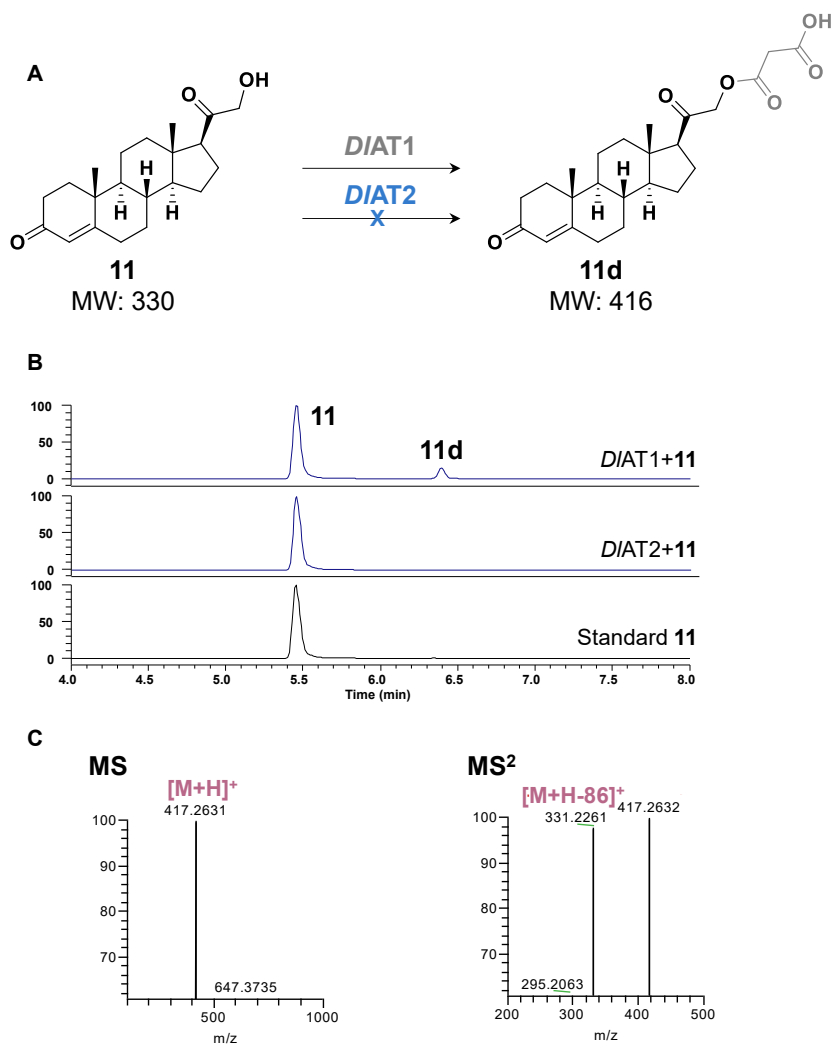
Supplementary Figure 37. The ^{13}C NMR spectrum of 7 in CDCl_3 (100 MHz).



Supplementary Figure 38. The HSQC spectrum of **7** in CDCl₃ (400 MHz).

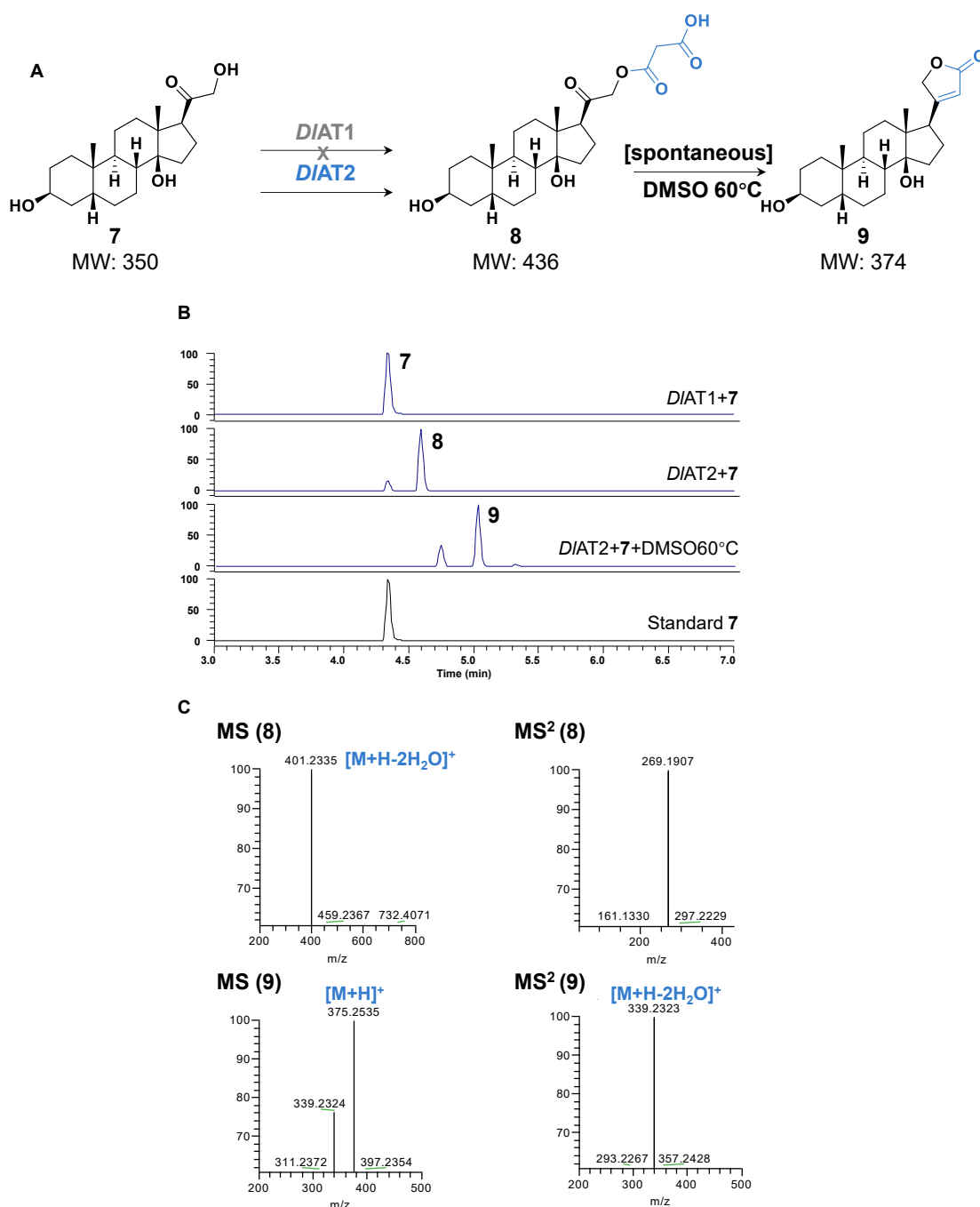


Supplementary Figure 39. The HMBC spectrum of **7** in CDCl₃ (400 MHz).



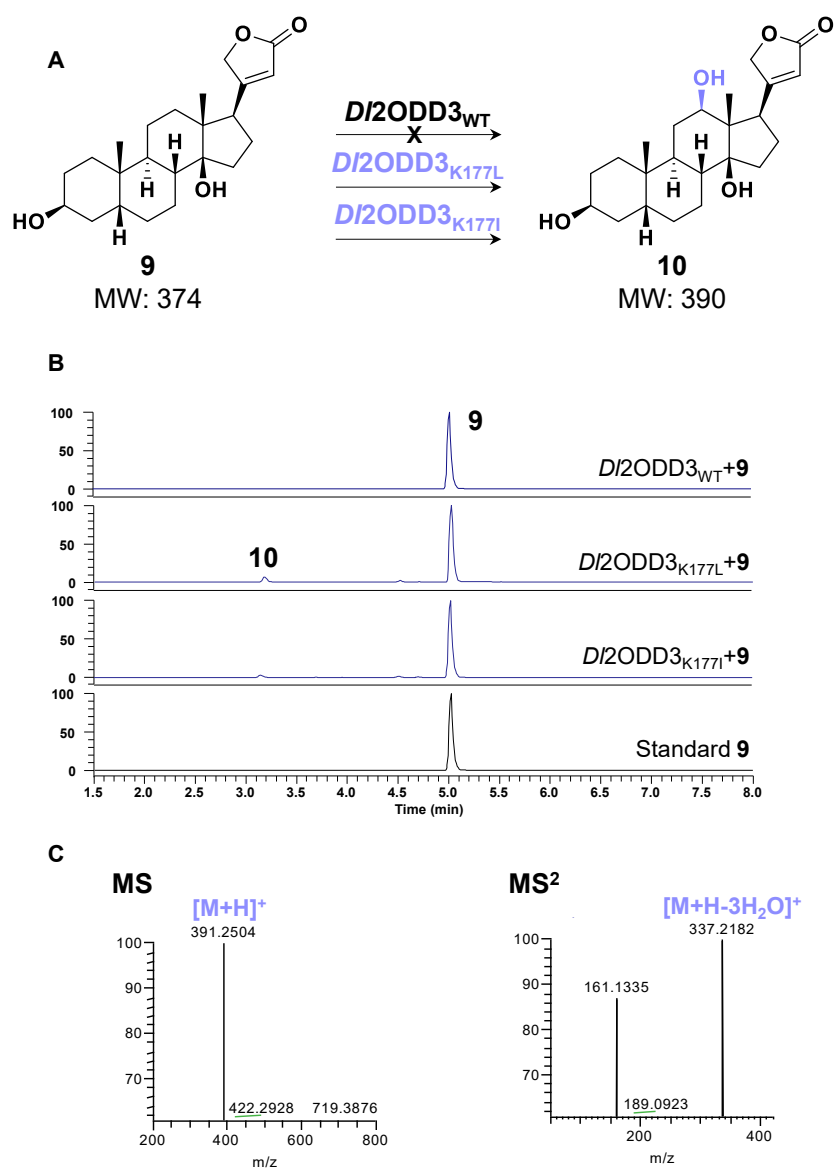
Supplementary Figure 40.

Enzymatic catalysis of **11** by *DIAT1*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *DIAT1* and *DIAT2* with substrate **11**. (C) (+)-ESI-MS and MS/MS spectra of product **11d**.



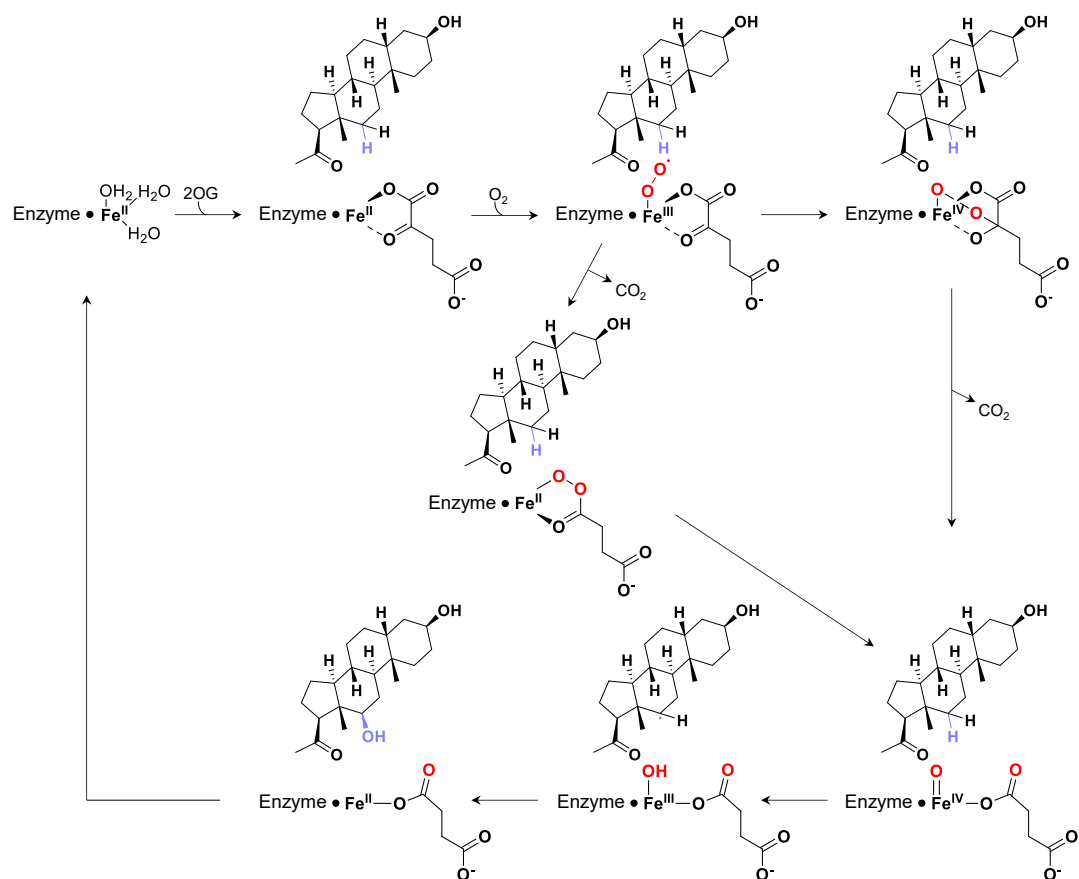
Supplementary Figure 41.

Enzymatic catalysis of **7** by *DIAT2*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *DIAT1* and *DIAT2* with substrate **7**. (C) (+)-ESI-MS and MS/MS spectra of products **8** and **9**. Product **8** was concentrated to dryness by centrifugation, dissolved in DMSO, and heated at 60°C for 2 h. After extraction with dichloromethane and evaporation of the solvent, the residue was reconstituted in 50% (v/v) methanol, and **9** was detected.



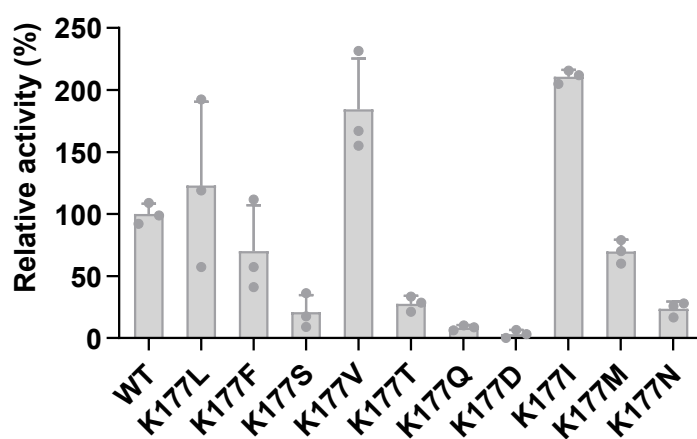
Supplementary Figure 42.

Enzymatic catalysis of **9** by *D/2ODD3*_{WT}, *D/2ODD3*_{K177L}, and *D/2ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/2ODD3*_{WT}, *D/2ODD3*_{K177L}, and *D/2ODD3*_{K177I} with substrate **9**. (C) (+)-ESI-MS and MS/MS spectra of product **10**.



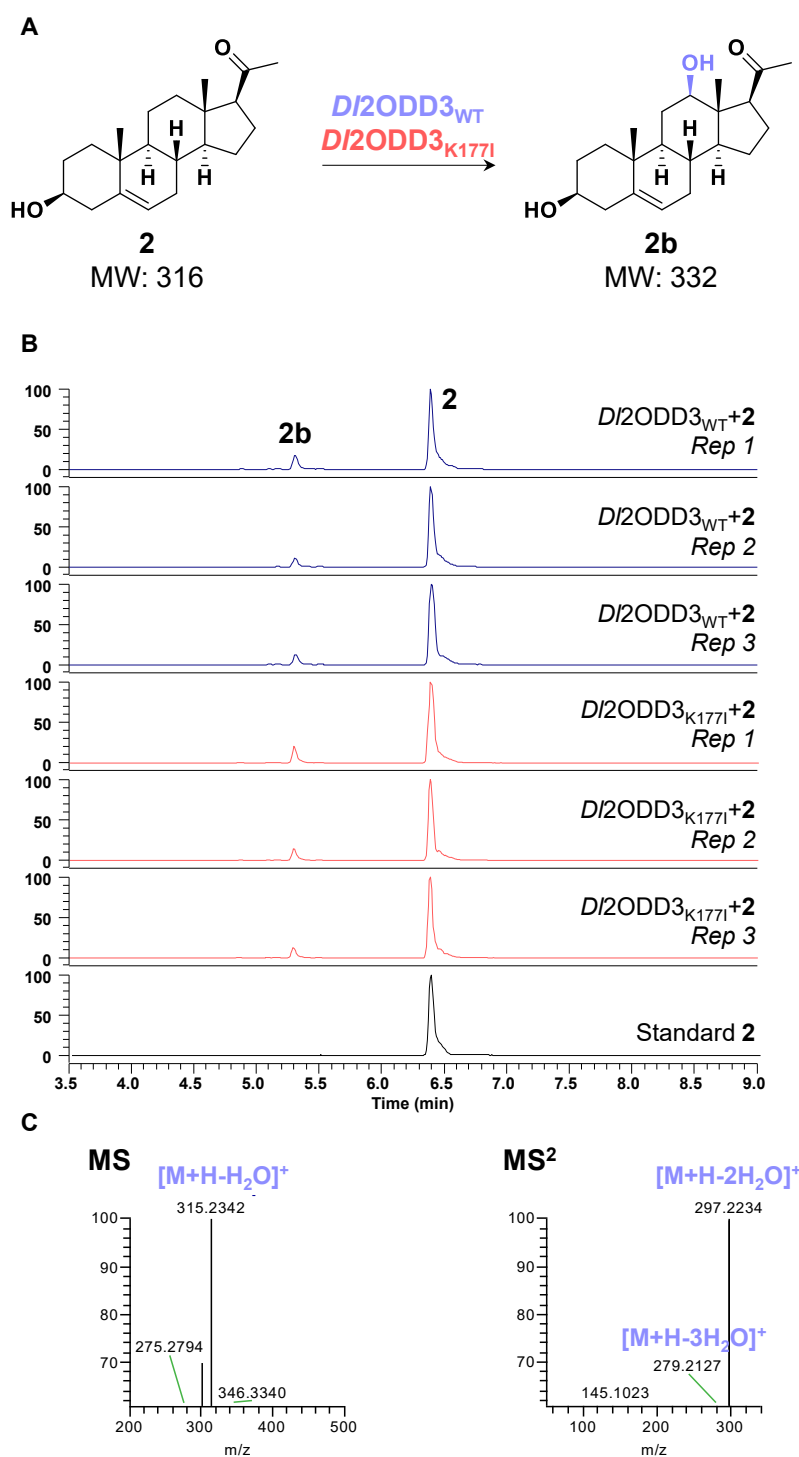
Supplementary Figure 43.

Proposed mechanism of *D12ODD3*-catalyzed hydroxylation.



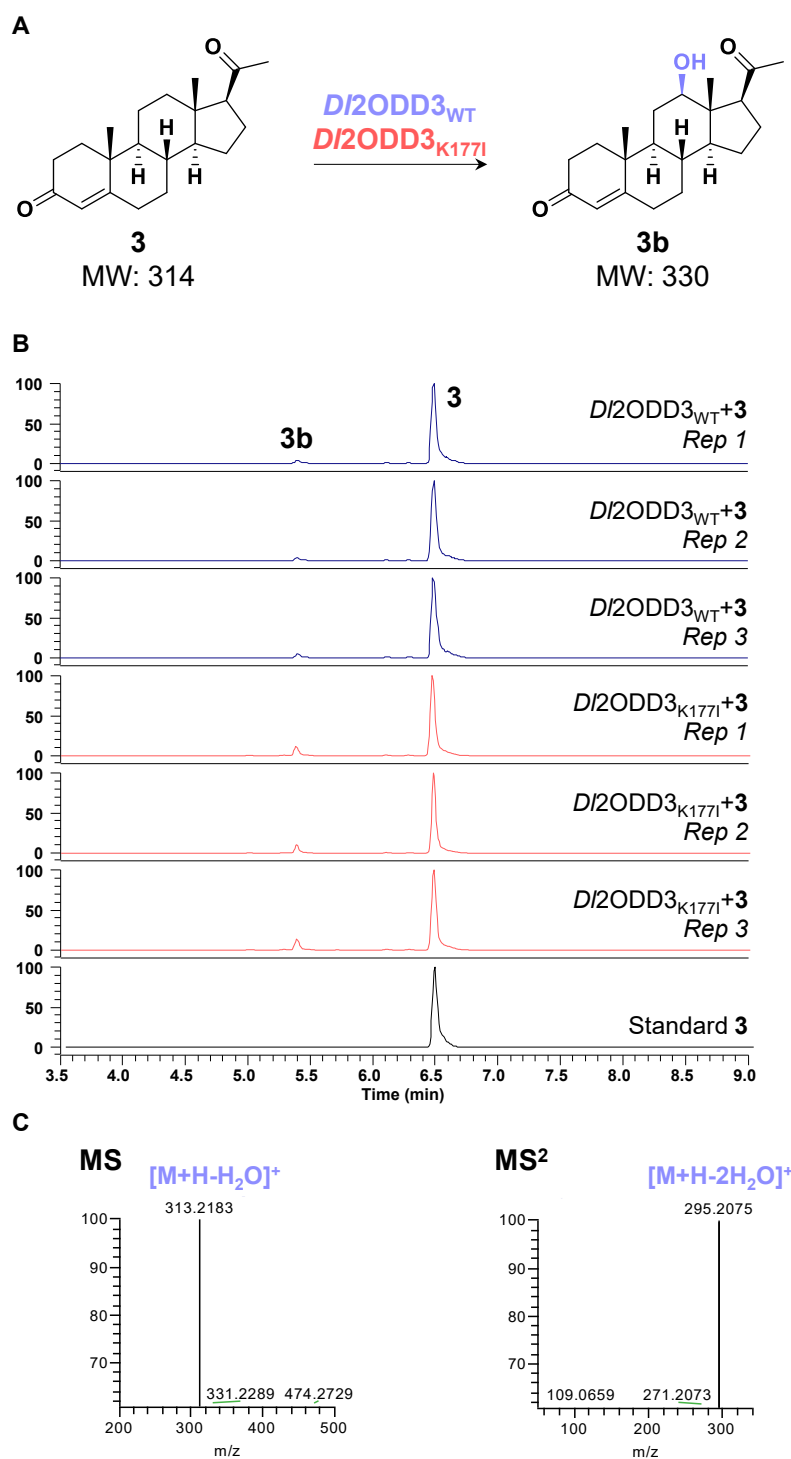
Supplementary Figure 44.

Relative activity of *D/2ODD3* mutants at residue K177, using **5** as substrate. The conversion rates are presented as the mean $\pm$ SD of three independent samples ($n = 3$).



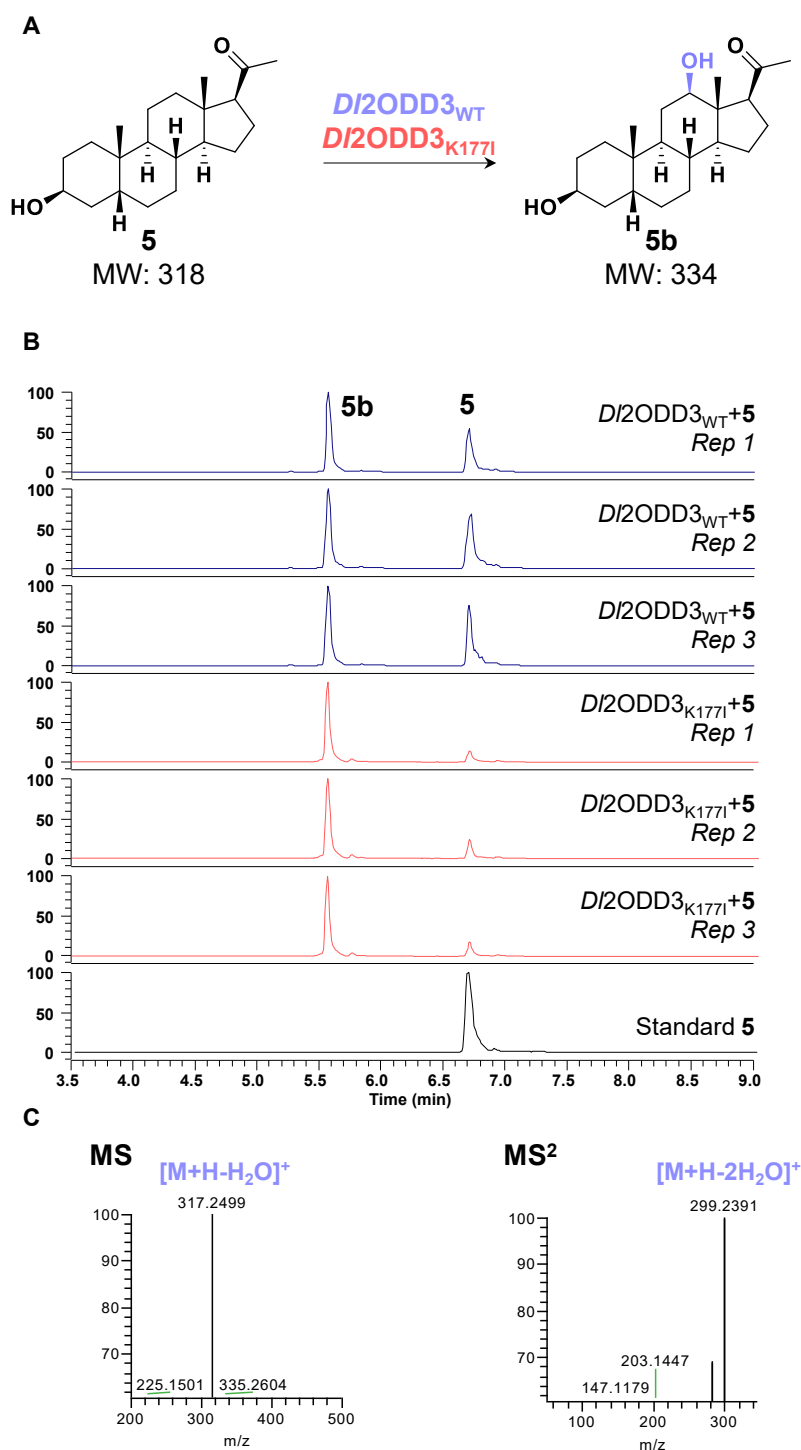
Supplementary Figure 45.

Enzymatic catalysis of **2** by *D12ODD3_{WT}* and *D12ODD3_{K177I}*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3_{WT}* and *D12ODD3_{K177I}* with substrate **2**. (C) (+)-ESI-MS and MS/MS spectra of product **2b**.



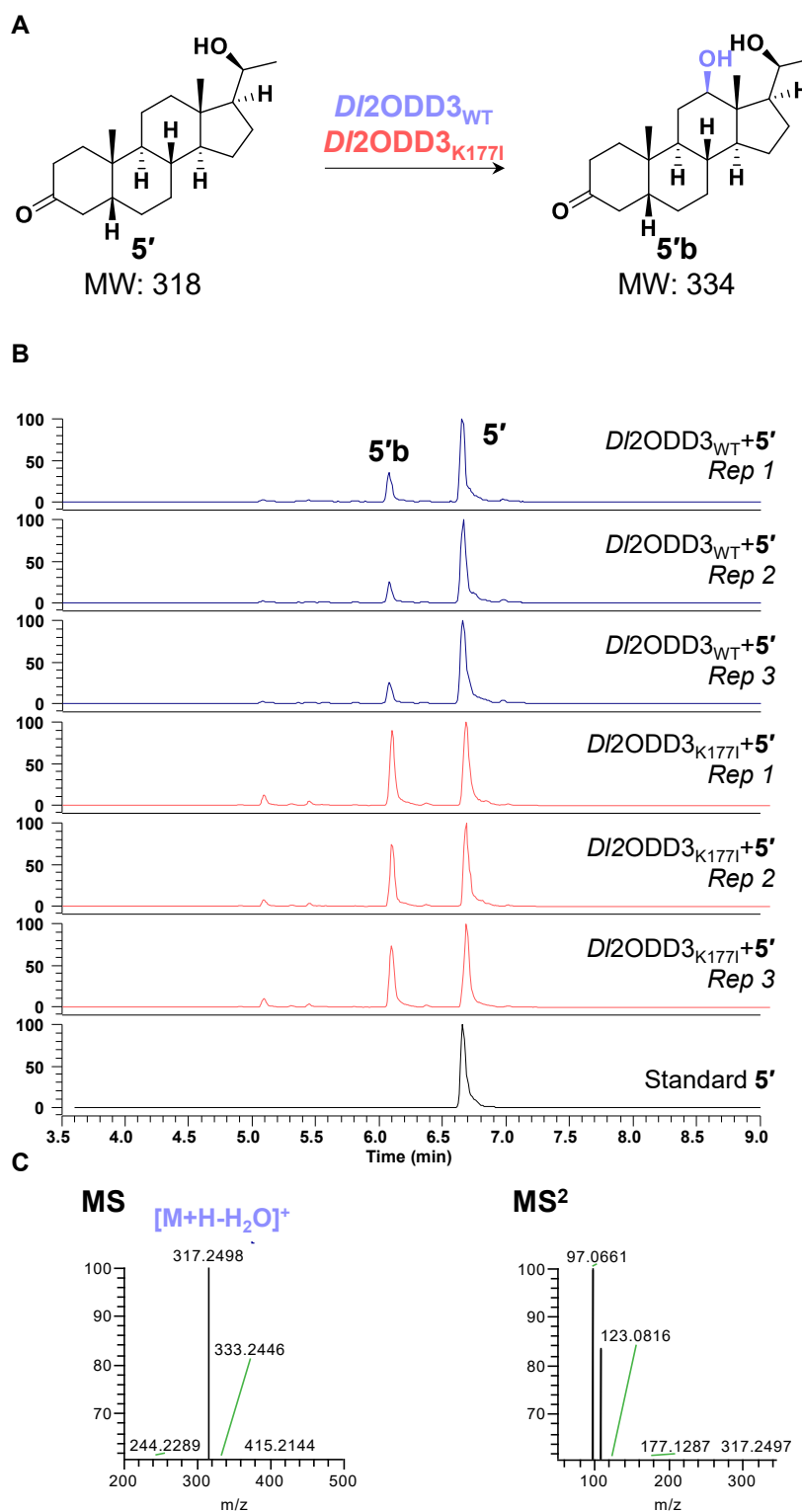
Supplementary Figure 46.

Enzymatic catalysis of **3** by $D12ODD3_{WT}$ and $D12ODD3_{K177I}$. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by $D12ODD3_{WT}$ and $D12ODD3_{K177I}$ with substrate **3**. (C) (+)-ESI-MS and MS/MS spectra of product **3b**.



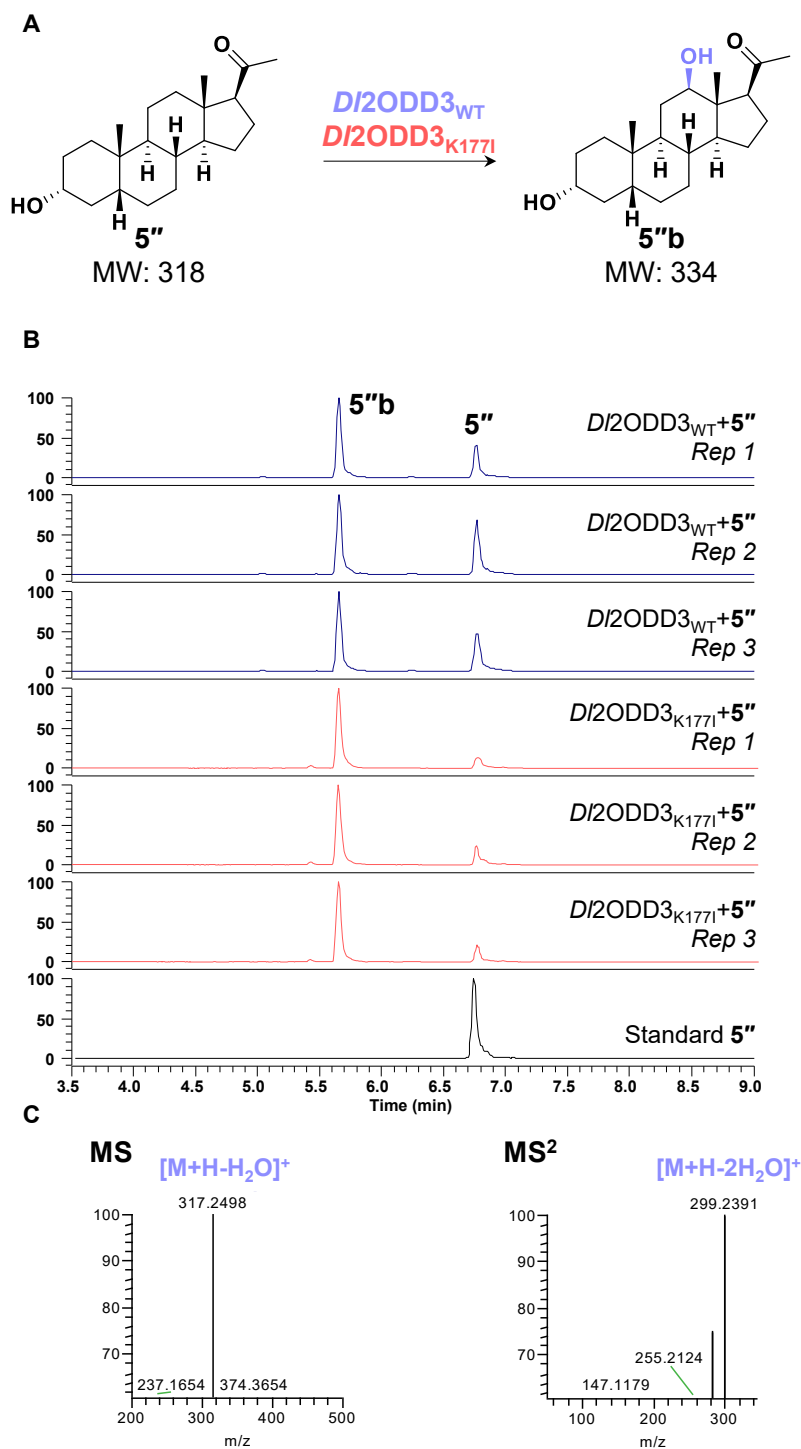
Supplementary Figure 47.

Enzymatic catalysis of **5** by *D12ODD3*_{WT} and *D12ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **5**. (C) (+)-ESI-MS and MS/MS spectra of product **5b**.



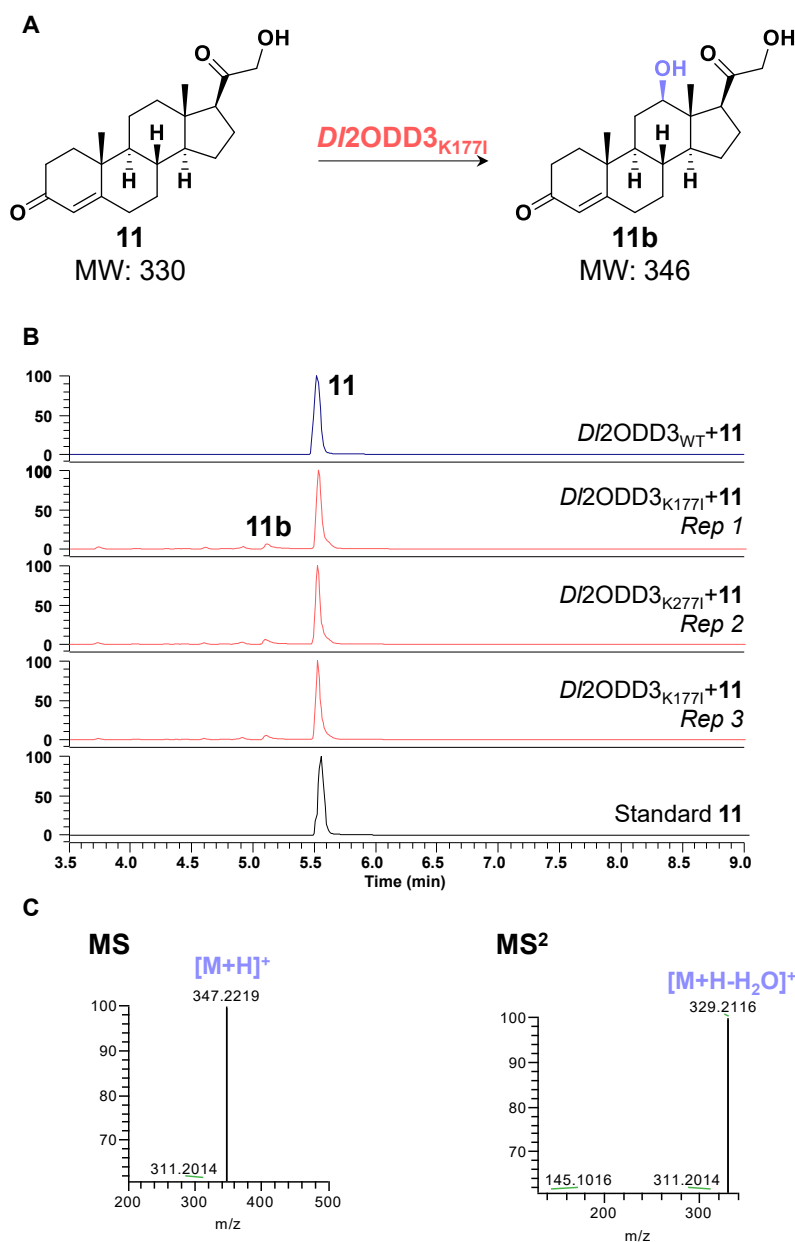
Supplementary Figure 48.

Enzymatic catalysis of **5'** by *D/2ODD3*_{WT} and *D/2ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/2ODD3*_{WT} and *D/2ODD3*_{K177I} with substrate **5'**. (C) (+)-ESI-MS and MS/MS spectra of product **5'b**.



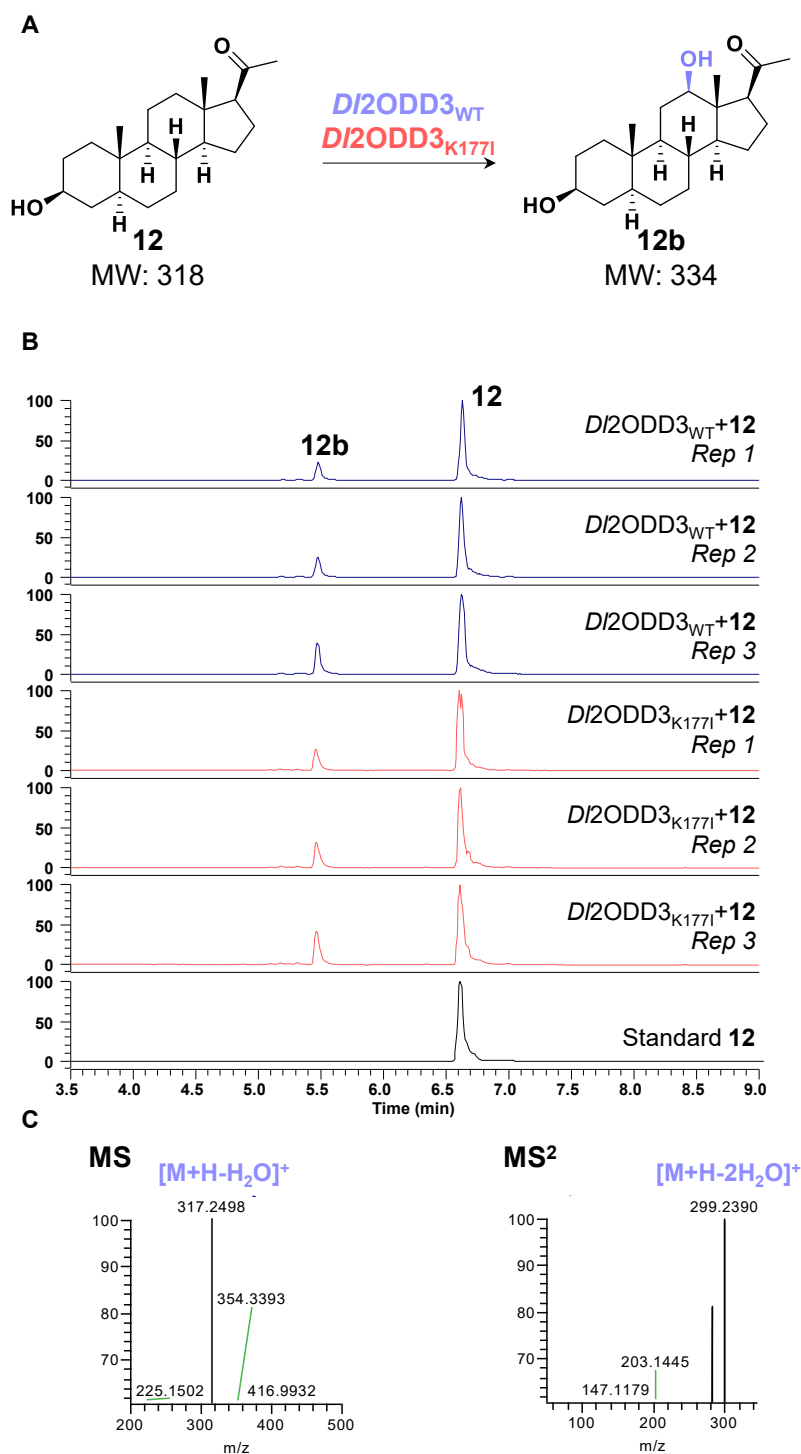
Supplementary Figure 49.

Enzymatic catalysis of **5''** by *D12ODD3*_{WT} and *D12ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **5''**. (C) (+)-ESI-MS and MS/MS spectra of product **5''b**.



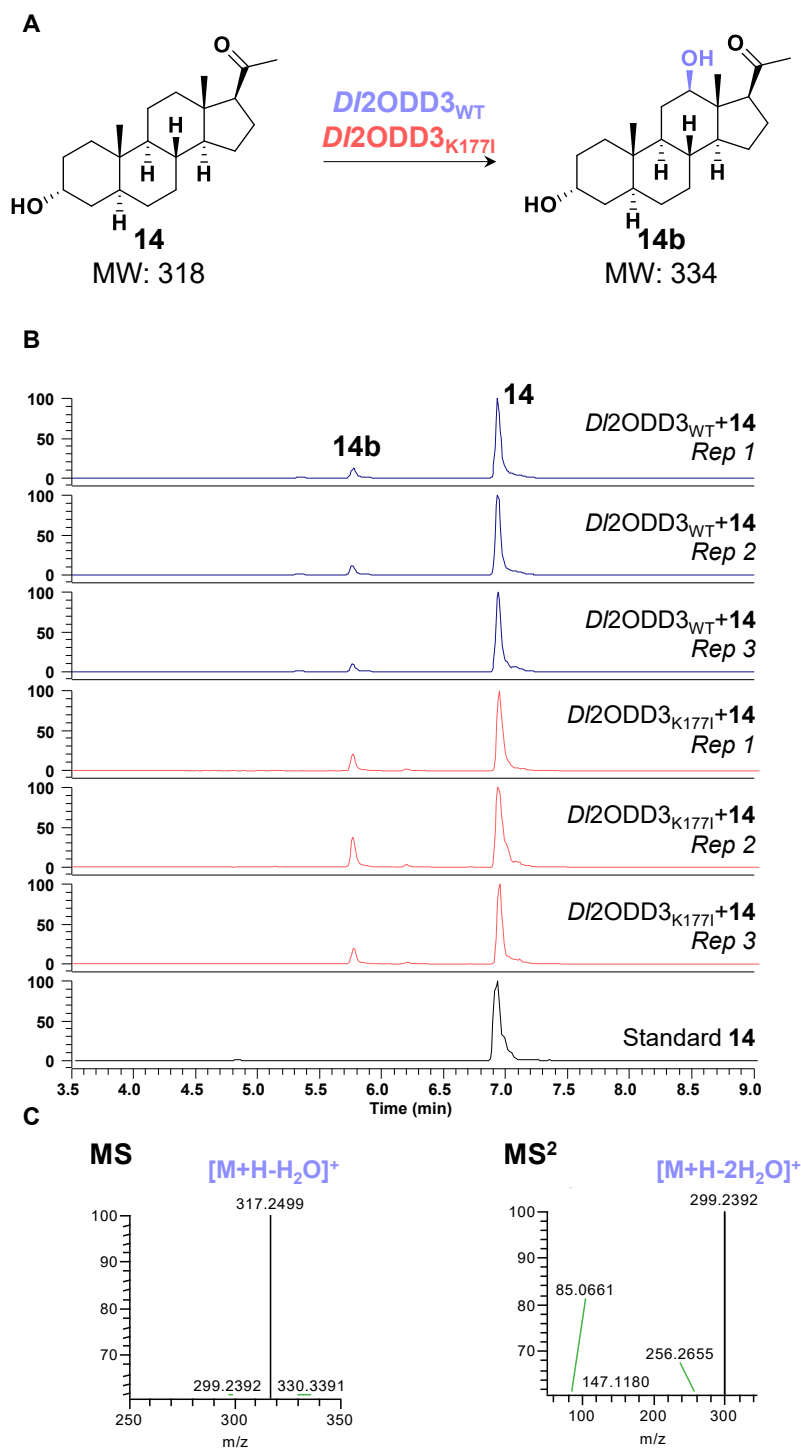
Supplementary Figure 50.

Enzymatic catalysis of **11** by *D12ODD3_{WT}* and *D12ODD3_{K177I}*. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3_{WT}* and *D12ODD3_{K177I}* with substrate **11**. (C) (+)-ESI-MS and MS/MS spectra of product **11b**.



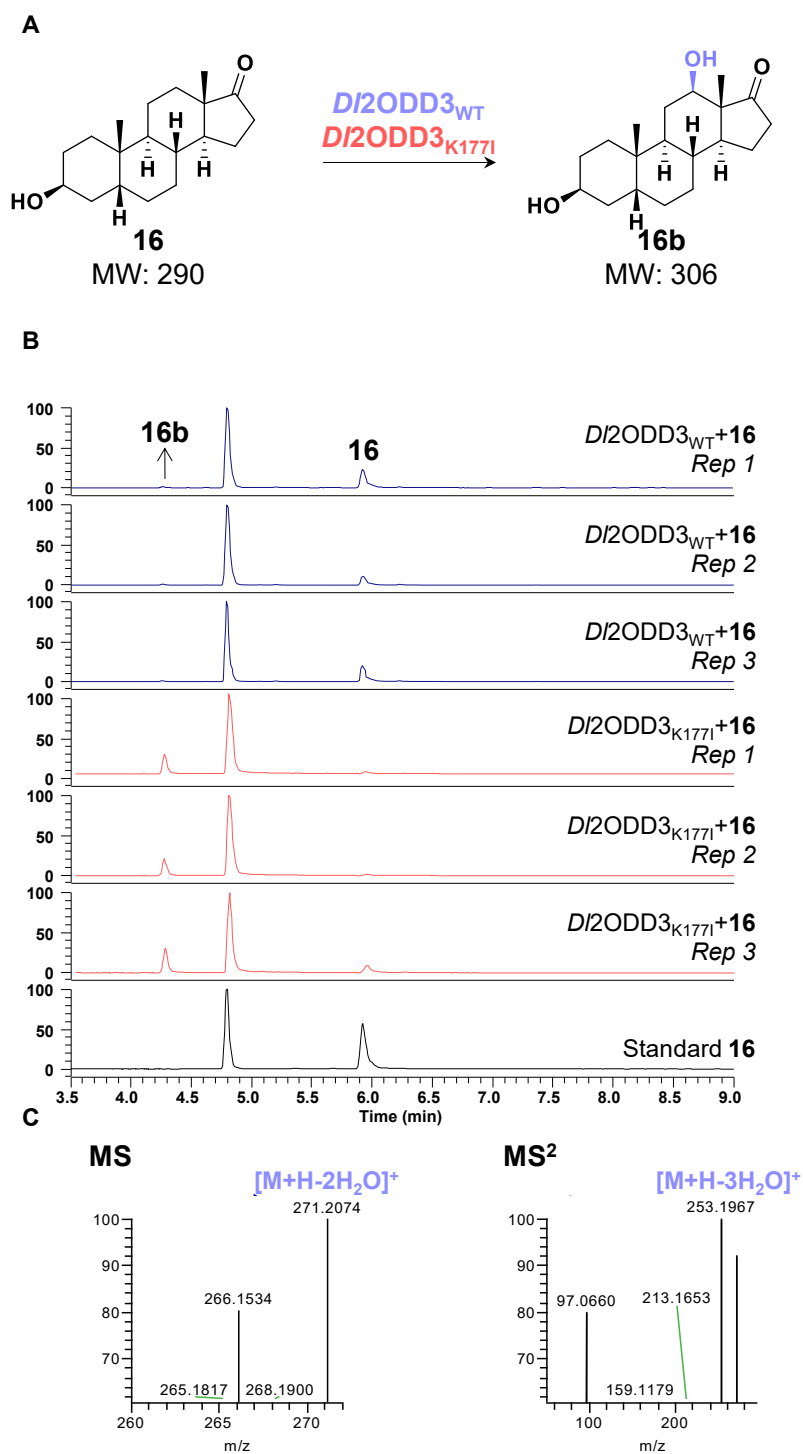
Supplementary Figure 51.

Enzymatic catalysis of **12** by *D12ODD3*_{WT} and *D12ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **12**. (C) (+)-ESI-MS and MS/MS spectra of product **12b**.



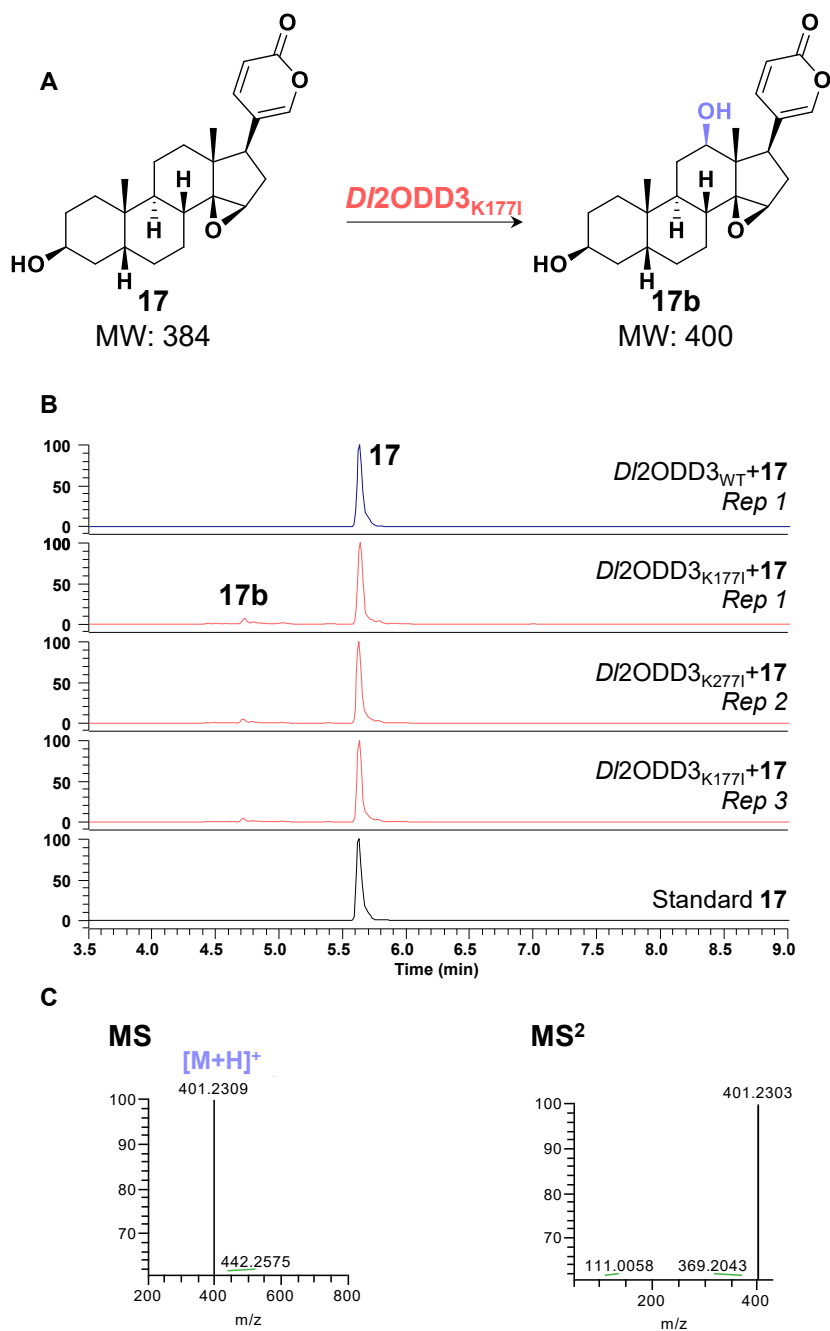
Supplementary Figure 52.

Enzymatic catalysis of **14** by *D12ODD3*_{WT} and *D12ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **14**. (C) (+)-ESI-MS and MS/MS spectra of product **14b**.



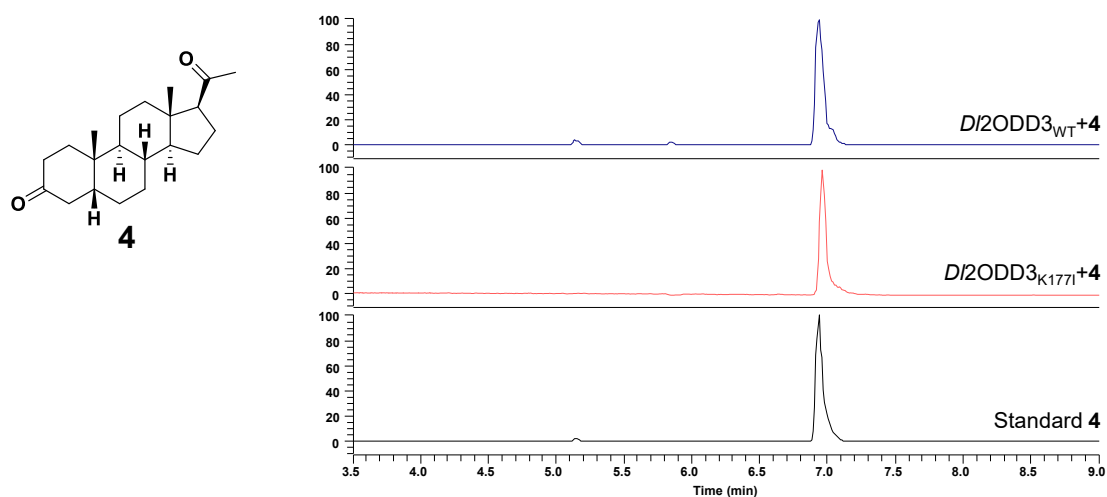
Supplementary Figure 53.

Enzymatic catalysis of **16** by *D12ODD3*_{WT} and *D12ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **16**. (C) (+)-ESI-MS and MS/MS spectra of product **16b**.



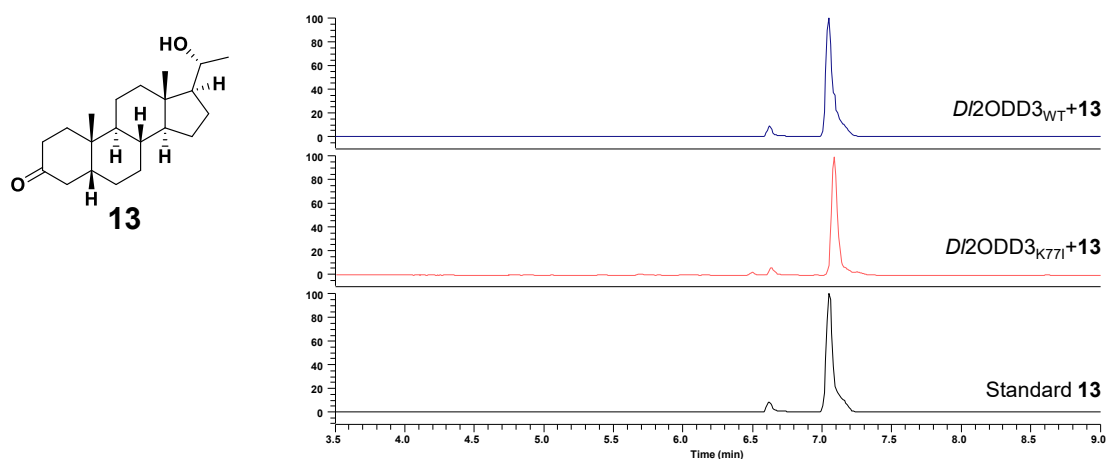
Supplementary Figure 54.

Enzymatic catalysis of **17** by *D12ODD3*_{WT} and *D12ODD3*_{K177I}. (A) The catalytic reaction. (B) UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **17**. (C) (+)-ESI-MS and MS/MS spectra of product **17b**.



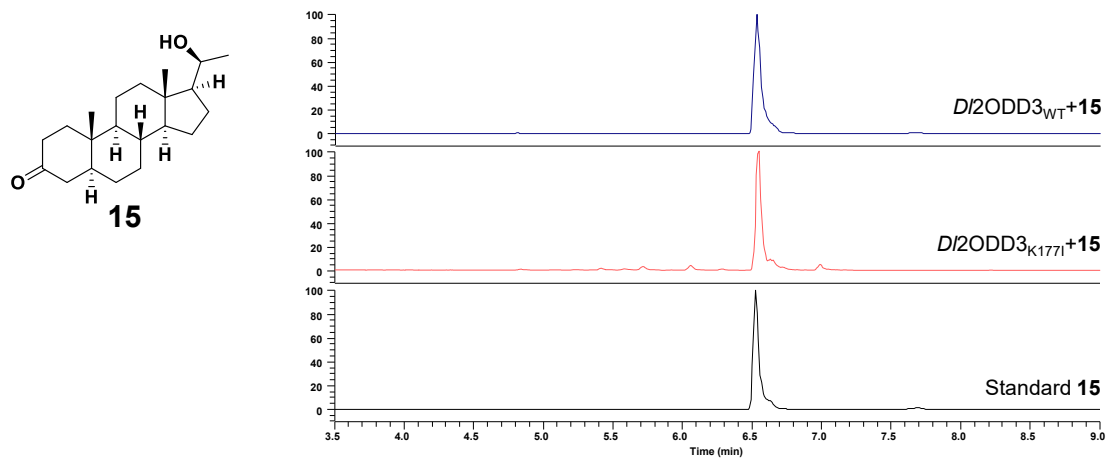
Supplementary Figure 55.

UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D/l2ODD3*_{WT} and *D/l2ODD3*_{K177I} with substrate 4.



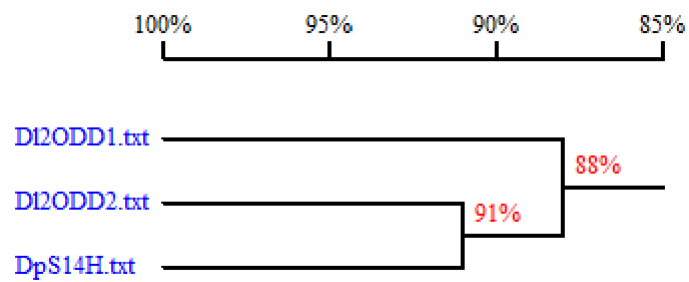
Supplementary Figure 56.

UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **13**.



Supplementary Figure 57.

UPLC-HRMS chromatograms of *in vitro* enzyme assays showing the reactions catalyzed by *D12ODD3*_{WT} and *D12ODD3*_{K177I} with substrate **15**.



Supplementary Figure 58.

The homology tree of *Dl2ODD1*, *Dl2ODD2*, and *DpS14 β H*. It was visualized by DNAMAN.

Dl2ODD1.txt	MATKTI ¹ CKLPVIDLS ² DLKRGTL ³ AWESVKTQVRKA ⁴ FE ⁵ EYGC	40
Dl2ODD2.txt	MATKTMCKLPVIDLSN ¹ LKRGTV ² EWESVRTQVRNA ³ FE ⁴ EYGC	40
DpS14βH.txt	MATKTMCKLPVIDLS ¹ DLKRGTA ² EWESLRTQVRKA ³ FE ⁴ EYGC	40
Consensus	matkt cklpvidls lkrgt wes tqvr afeeygc	
Dl2ODD1.txt	FEAF ¹ CDDISE ² SLRNSVFNGVQQLF ³ DLPLETKERNKSD ⁴ KPA	80
Dl2ODD2.txt	FEAF ¹ CDDITETLRS ² SVFNGVQQLFNLPSE ³ TKERNKSD ⁴ MPA	80
DpS14βH.txt	FEAF ¹ SADITETLRT ² SVFNGVQQLF ³ DLPLETKERNKSD ⁴ MPA	80
Consensus	feaf di e lr svfngvqqflf lp etk rnksd pa	
Dl2ODD1.txt	RGYAKINLYESMGIGDVL ¹ TRETIE ² NFTNCLWPEGNPTV ³ SK	120
Dl2ODD2.txt	RGYAKINLYESMGIGDVLN ¹ RETIES ² SFTNCLWPQGNPTV ³ CE	120
DpS14βH.txt	RGYRKITLYESMGIGDVLN ¹ RETIES ² SFTNCLWPEGNPTV ³ CE	120
Consensus	rgy ki lyesmgigdvl retie ftnclwp gnptv	
Dl2ODD1.txt	NVLSFS ¹ DKICE ² VEQNVRMVVESYEL ³ DKH ⁴ MEDEFMNS ⁵ TVYL	160
Dl2ODD2.txt	NVLSFS ¹ DKICELEQNVRMVVESYEL ² EK ³ HVDEFMNS ⁴ SIYL	160
DpS14βH.txt	NLLSFS ¹ DKICELEQNVRMVVESYEL ² EE ³ H ⁴ LDEFMNS ⁵ SIYL	160
Consensus	n lsfsdkice eqnvrrmvvesyel h defmns yl	
Dl2ODD1.txt	LRFQKYDAPLN ¹ DEGEV ² GLK ³ PHTD ⁴ DFVTLLFPSEVAGLEI	200
Dl2ODD2.txt	LRFQKYDAPHNSKGEI ¹ GLK ² PHTD ³ DFVTLLFPSEVAGLEI	200
DpS14βH.txt	LRFQKYNAPHNDEGEL ¹ GLN ² PHTD ³ DFVTLLFPSEVAGLEI	200
Consensus	lrfqky ap n ge gl phtdtdfvtllfpsevaglei	
Dl2ODD1.txt	LTRDGRWLDANPSPNHFIVFAGDTF ¹ HAWTNGRLHSVEHRV	240
Dl2ODD2.txt	LTKDGO ¹ WMDANPSPNHFIVFAGDTF ² HAWTNGRLHSVEHRV	240
DpS14βH.txt	LTRDGE ¹ WMNANPSPNHFIVFAGDTF ² HAWTNGRLHSVEHKV	240
Consensus	lt dg w anpspnhfivfagdtfhawtngrlhsveh v	
Dl2ODD1.txt	VMKGDVSRYSIGLFS ¹ IPKIGYV ² IKAPE ³ EMVDEEHPVLFKP	280
Dl2ODD2.txt	VMKGDVARYSIGLFS ¹ IPKIGYT ² IKAPE ³ EMVDEEHPVLFKP	280
DpS14βH.txt	VMKGDVARYSIGLFS ¹ F ² PKIGYI ³ IKAPE ⁴ KMVDEEHPVLFKP	280
Consensus	vmkgdv rysiglfs pkigy ikape mvdeehpvlfkp	
Dl2ODD1.txt	YEH ¹ SKFMEYYFSR ² PQLGNPHAVLKEFCG	308
Dl2ODD2.txt	YEH ¹ SKFMEYYFSRTELGNPYAVLKEFCG	308
DpS14βH.txt	YEHT ¹ KFMEYYFSRTELGNPYAVLKEFCG	308
Consensus	yeh kfmeyyfsr lgnp avlkefcg	

Supplementary Figure 59.

Sequence alignment of *Dl2ODD1*, *Dl2ODD2*, and *DpS14βH*. It was visualized by DNAMAN.



D/2ODD3/Fe²⁺

Supplementary Figure 60.

Picture of crystals of *D/2ODD3/Fe²⁺*.

Supplementary Tables

Supplementary Table 1. Information of compounds in this study.

No	Name	CAS number	Molecular formula
1	cholesterol	57-88-5	C ₂₇ H ₄₆ O
2	pregnenolone	145-13-1	C ₂₁ H ₃₂ O ₂
2a	14 β -hydroxypregnenolone	17059-32-4	C ₂₁ H ₃₂ O ₃
2b	12 β -hydroxypregnenolone	-	C ₂₁ H ₃₂ O ₃
3	progesterone	57-83-0	C ₂₁ H ₃₀ O ₂
3a	14 β -hydroxyprogesterone	24853-94-9	C ₂₁ H ₃₀ O ₃
3b	12 β -hydroxyprogesterone	29332-92-1	C ₂₁ H ₃₀ O ₃
3ac	14 β ,21-dihydroxy-progesterone	-	C ₂₁ H ₃₀ O ₄
4	5 β -pregnane-3,20-dione	128-23-4	C ₂₁ H ₃₂ O ₂
4a	14 β -hydroxy-5 β -pregnane-3,20-dione	80373-61-1	C ₂₁ H ₃₂ O ₃
5	3 β -hydroxy-5 β -pregnan-20-one	128-21-2	C ₂₁ H ₃₄ O ₂
5b	3 β ,12 β -dihydroxy-5 β -pregnan-20-one	26932-41-2	C ₂₁ H ₃₄ O ₃
5bc	3 β ,12 β ,21-trihydroxy-5 β -pregnan-20-one	-	C ₂₁ H ₃₄ O ₄
5'	20 α -hydroxy-5 β -pregnan-3-one	54353-11-6	C ₂₁ H ₃₄ O ₂
5'b	12 β ,20 α -dihydroxy-5 β -pregnan-3-one	-	C ₂₁ H ₃₄ O ₃
5''	3 α -hydroxy-5 β -pregnan-20-one	128-20-1	C ₂₁ H ₃₄ O ₂
5''b	3 α ,12 β -dihydroxy-5 β -pregnan-20-one	214191-95-4	C ₂₁ H ₃₄ O ₃
6	3 β ,14 β -dihydroxy-5 β -pregnan-20-one	14443-32-4	C ₂₁ H ₃₄ O ₃
7	3 β ,14 β ,21-trihydroxy-5 β -pregnan-20-one	3946-61-0	C ₂₁ H ₃₄ O ₄
8	3 β ,14 β ,21-trihydroxy-5 β -pregnane-20-one-21- <i>O</i> -malonylhemiester	-	C ₂₄ H ₃₆ O ₇
9	digitoxigenin	143-62-4	C ₂₃ H ₃₄ O ₄
10	digoxigenin	1672-46-4	C ₂₃ H ₃₄ O ₅
11	21-hydroxyprogesterone	64-85-7	C ₂₁ H ₃₀ O ₃
11b	12 β ,21-dihydroxy-progesterone	880765-57-1	C ₂₁ H ₃₀ O ₄
11d	21-hydroxyprogesterone-21- <i>O</i> -malonylhemiester	22040-73-9	C ₂₄ H ₃₂ O ₆
12	3 β -hydroxy-5 α -pregnan-20-one	516-55-2	C ₂₁ H ₃₄ O ₂
12b	3 β ,12 β -dihydroxy-5 α -pregnan-20-one	13914-66-4	C ₂₁ H ₃₄ O ₃
13	20 β -hydroxy-5 β -pregnan-3-one	80-93-3	C ₂₁ H ₃₄ O ₂
14	3 α -hydroxy-5 α -pregnan-20-one	516-54-1	C ₂₁ H ₃₄ O ₂
14b	3 α ,12 β -dihydroxy-5 α -pregnan-20-one	1426309-54-7	C ₂₁ H ₃₄ O ₃
15	20 α -hydroxy-5 α -pregnan-3-one	516-59-6	C ₂₁ H ₃₄ O ₂
16	3 β -hydroxy-5 β -androstan-17-one	571-31-3	C ₁₉ H ₃₀ O ₂
16b	3 β ,12 β -dihydroxy-5 β -androstan-17-one	88378-71-6	C ₁₉ H ₃₀ O ₃

17	respigon	465-39-4	$C_{24}H_{32}O_4$
17b	12 β -hydroxyrespigon	4026-95-3	$C_{24}H_{32}O_5$

Supplementary Table 2. Statistics for assembly and annotation of *Digitalis lanata*.

Assembly	
Estimated genome size	1,124,009,190
total assembly size (bp)	1,219,128,363
Number of contigs	103
contig N90	37558580
contig N50	44131463
L50	13
L90	25
Number of scaffolds	103
Scaffold N90	37558580
Scaffold N50	44131463
Complete BUSCOs (C)	97.72%
Annotation	
Number of genes	44769
Mean gene length	4461
Mean CDS length	1474
Exons per transcript	5.9
Complete BUSCOs (C)	97.12

Supplementary Table 3. PCR primers used in this study.

Prime	Sequence(5'-3')
pEAQ-DICYP1-F	CTGCCCAAATTCGCGCTCGAGATGTCGTTGGTAGCTATTAGCGCC
pEAQ-DICYP1-R	GAAACCAGAGTTAACTCGAGTTTGCATGCAGCCTCCCTCTCAGTC
pEAQ-DICYP2-F	CTGCCCAAATTCGCGCTCGAGATGTCGTTAGTAGCTATGAGCG
pEAQ-DICYP2-R	GAAACCAGAGTTAACTCGAGGCCTCTCTCAGTCATGTAAACGTG
pEAQ-DISDR1-F	CTGCCCAAATTCGCGCTCGAGATGAGCAGCTGGTGGGCGGCTG
pEAQ-DISDR1-R	GAAACCAGAGTTAACTCGAGTTAGGGCACAATCTTGTAAGATC
pEAQ-DISDR2-F	CTGCCCAAATTCGCGCTCGAGATGAGCTGGTGGTGGGCTGGAG
pEAQ-DISDR2-R	GAAACCAGAGTTAACTCGAGAGGAACAATCTTGTAAGCTTTTG
pEAQ-DIHSD1-F	CTGCCCAAATTCGCGCTCGAGATGTCAAAGCCAAGATTGGATGG
pEAQ-DIHSD1-R	GAAACCAGAGTTAACTCGAGGCGTACGACGGAGAAGCCGCC
pEAQ-DIHSD2-F	CTGCCCAAATTCGCGCTCGAGATGTCGTCAAAGCCAAGGTTG
pEAQ-DIHSD2-R	GAAACCAGAGTTAACTCGAGCACGACGGTGAAGCCGCCGTC
pET28a(+)-DISDR1-F	GGACAGCAAATGGGTGCGCCGGATGAGCAGCTGGTGGGCGGCTG
pET28a(+)-DISDR1-R	GTCGACGGAGCTCGAATTCGGTTAGGGCACAATCTTGTAAGATC
pET28a(+)-DISDR2-F	GGACAGCAAATGGGTGCGCCGGATGAGCTGGTGGTGGGCTGGAG
pET28a(+)-DISDR2-R	GTCGACGGAGCTCGAATTCGGAGGAACAATCTTGTAAGCTTTTG
pET28a(+)-DIHSD1-F	GACAGCAAATGGGTGCGCCGGATGTCAAAGCCAAGATTGGATGG
pET28a(+)-DIHSD1-R	GTCGACGGAGCTCGAATTCGGGCGTACGACGGAGAAGCCGCC
pET28a(+)-DIHSD2-F	GACAGCAAATGGGTGCGCCGGATGTCGTCAAAGCCAAGGTTG
pET28a(+)-DIHSD2-R	GTCGACGGAGCTCGAATTCGGCACGACGGTGAAGCCGCCGTC
pET28a(+)-DI2ODD1-F	GGACAGCAAATGGGTGCGCCGGATGGCGACTAAAACCATCTGC
pET28a(+)-DI2ODD1-R	GTCGACGGAGCTCGAATTCGGCTATGCACCACAAAATTCCTTG
pET28a(+)-DI2ODD2-F	GGACAGCAAATGGGTGCGCCGGATGGCGACTAAAACCATGTGCAAAC
pET28a(+)-DI2ODD2-R	GTCGACGGAGCTCGAATTCGGTTATGCACCACAGAATTCCTTC
pET28a(+)-DI2ODD3-F	GGACAGCAAATGGGTGCGCCGGATGGGTTGAGAAGGAATGAC
pET28a(+)-DI2ODD3-R	GTCGACGGAGCTCGAATTCGGTTAGACACCACAATAATCTTTTAG
pET28a(+)-DI2ODD4-F	GGACAGCAAATGGGTGCGCCGGATGGCAGCTGGTAAAACCATGC
pET28a(+)-DI2ODD4-R	GTCGACGGAGCTCGAATTCGGTTAATAAGCACCACAATATTCCTTC
pET28a(+)-DIAT1-F	GGACAGCAAATGGGTGCGCCGGATGGCCGGTGAAGTAATCACAAATG
pET28a(+)-DIAT1-R	GTCGACGGAGCTCGAATTCGGTTAATTCTCCATAAGGAACTCC
pET28a(+)-DIAT2-F	GGACAGCAAATGGGTGCGCCGGATGGAAGTTATATCTACATGTTC
pET28a(+)-DIAT2-R	GTCGACGGAGCTCGAATTCGGTCAGAGATCGACATGTTCCATGAAC
pET28a(+)-DI2ODD3 _{Q90A} -F	GATgCGCGTCCCCTGTTTGAAAGCCTTGCC
pET28a(+)-DI2ODD3 _{Q90A} -R	CAAACAGGGGACGCGcATCCACATAGGCAAG
pET28a(+)-DI2ODD3 _{F94A} -F	CCTGgcaGAAAGCCTTGCCGTTGATGACTC
pET28a(+)-DI2ODD3 _{F94A} -R	CGGCAAGGCTTTCtgcCAGGGGACGCTGATC

pET28a(+)-DI2ODD3 _{R160A} -F	GgcATTTCAGAAATATGCAGGAACTAAAAG
pET28a(+)-DI2ODD3 _{R160A} -R	CATATTTCTGAAATgcCATGGTGTACTTCATAC
pET28a(+)-DI2ODD3 _{P172A} -F	GTTAgCACCCCACACAGACAAGGGCTTTCTC
pET28a(+)-DI2ODD3 _{P172A} -R	GTCTGTGTGGGGTGcTAACTCACTTTTAGTTC
pET28a(+)-DI2ODD3 _{K177A} -F	CgcGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177A} -R	GTGAGAAAGCCCgcGTCTGTGTGGGGTGG
pET28a(+)-DI2ODD3 _{K177L} -F	CctGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177L} -R	GTGAGAAAGCCCagGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177I} -F	CAtGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177I} -R	GAGAAAGCCaaTGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177F} -F	CtttGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177F} -R	GAGAAAGCCaaaGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177S} -F	CtctGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177S} -R	GAGAAAGCCagaGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177V} -F	CgttGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177V} -R	GAGAAAGCCaacGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177T} -F	CaccGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177T} -R	GAGAAAGCCggTGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177Q} -F	CcAaGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177Q} -R	GAGAAAGCCtTgGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177D} -F	CgAtGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177D} -R	GAGAAAGCCaTcGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177M} -F	CAtGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177M} -R	GAGAAAGCCCCaTGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{K177N} -F	CAAiGGGCTTTCTCACAATACTTTGGCAAAATC
pET28a(+)-DI2ODD3 _{K177N} -R	GAGAAAGCCaTTGTCTGTGTGGGGTGGTAAC
pET28a(+)-DI2ODD3 _{F247A} -F	CgcaGGGTTTCCTAGAGATGGATATGTGG
pET28a(+)-DI2ODD3 _{F247A} -R	CTAGGAAACCCtgcGATTCCGATTGTGTAGCG
pET28a(+)-DI2ODD3 _{Y283A} -F	GTTTgcCGTCACTGAAGCTGGATTGAAC
pET28a(+)-DI2ODD3 _{Y283A} -R	GCTTCAGTGACGgcAAACGCAACGAACTTAGCG

Note: The vector sequences and the mutation sites are labeled in blue.

Supplementary Table 4. Accession numbers of genes in this study.

No	Name	Genbank accession number
1	DICYP1	PZ683672
2	DICYP2	PZ683673
3	DIHSD1	PZ683674
4	DIHSD2	PZ683675
5	DISDR1	PZ683676
6	DISDR2	PZ683677
7	DI2ODD1	PZ683678
8	DI2ODD2	PZ683679
9	DI2ODD3	PZ683680
10	DI2ODD4	PZ683681
11	DIAT1	PZ683682
12	DIAT2	PZ683683

Supplementary Table 5. Co-expression analysis of *D. lanata* transcriptome using *D1CYP1/2*, *D1HSD1*, *D1SDR1*, and *D12ODD1/2* as bait genes for the identification of 2ODDs.

gene	Pearson correlation coefficient					
	D1CYP1	D1CYP2	D12ODD1	D12ODD2	D1SDR1	D1HSD1
evm.TU.scaffold_15.1187 (D12ODD4)	0.99	0.98	0.99	0.98	1.00	0.97
evm.TU.scaffold_4.1461 (D12ODD3)	0.99	0.98	0.98	0.98	0.99	0.97
evm.TU.scaffold_21.1335	0.98	1.00	0.94	0.99	0.98	1.00
evm.TU.scaffold_4.475	0.98	0.98	0.96	0.98	0.99	0.98
evm.TU.scaffold_22.299	0.98	0.98	0.94	0.98	0.98	1.00
evm.TU.scaffold_15.2	0.99	0.96	0.99	0.96	0.99	0.95
evm.TU.scaffold_7.1289	0.97	0.99	0.95	0.98	0.98	0.97
evm.TU.scaffold_6.1260	0.96	0.98	0.92	0.98	0.96	0.98
evm.TU.scaffold_8.1634	0.97	0.93	0.97	0.92	0.96	0.93
evm.TU.scaffold_9.399	0.93	0.97	0.88	0.97	0.95	0.97
evm.TU.scaffold_5.1632	0.91	0.96	0.84	0.96	0.92	0.96
evm.TU.scaffold_1.1016	0.94	0.89	0.98	0.89	0.94	0.87
evm.TU.scaffold_13.1348	0.90	0.95	0.83	0.95	0.91	0.94
evm.TU.scaffold_4.1361	0.90	0.84	0.95	0.85	0.90	0.84

Supplementary Table 6. Co-expression analysis of *D. lanata* transcriptome using *D1CYP1/2*, *DIHSD1*, *DISDR1*, and *DI2ODD1/2* as bait genes for the identification of P450s.

gene	Pearson correlation coefficient					
	D1CYP1	D1CYP2	DI2ODD1	DI2ODD2	DISDR1	DIHSD1
evm.TU.scaffold_10.738	0.99	0.99	0.96	0.99	0.99	0.99
evm.TU.scaffold_19.1272	0.99	0.99	0.96	0.98	0.99	0.99
evm.TU.scaffold_8.53	0.99	0.99	0.96	0.98	0.99	0.99
evm.TU.scaffold_2.30	0.98	0.99	0.94	0.99	0.98	0.99
evm.TU.scaffold_25.725	0.98	0.98	0.96	0.98	0.98	0.97
evm.TU.scaffold_9.1243	0.97	0.99	0.93	0.99	0.98	1.00
evm.TU.scaffold_10.76	0.97	0.98	0.94	0.99	0.98	0.97
evm.TU.scaffold_1.706	0.97	0.98	0.93	0.97	0.97	0.98
evm.TU.scaffold_15.488	0.96	0.98	0.93	0.97	0.97	0.99
evm.TU.scaffold_6.1732	0.96	0.98	0.92	0.97	0.97	0.99
evm.TU.scaffold_21.192	0.97	0.98	0.96	0.97	0.97	0.95
evm.TU.scaffold_24.916	0.96	0.98	0.92	0.97	0.96	0.98
evm.TU.scaffold_10.503	0.96	0.98	0.93	0.97	0.97	0.96
evm.TU.scaffold_22.1097	0.96	0.98	0.92	0.98	0.96	0.96
evm.TU.scaffold_26.407	0.95	0.98	0.89	0.98	0.94	0.96
evm.TU.scaffold_25.726	0.95	0.97	0.93	0.96	0.95	0.94
evm.TU.scaffold_6.830	0.95	0.96	0.90	0.96	0.96	0.97
evm.TU.scaffold_9.552	0.96	0.94	0.95	0.92	0.95	0.94
evm.TU.scaffold_15.829	0.92	0.97	0.85	0.97	0.93	0.96
evm.TU.scaffold_21.193	0.93	0.91	0.96	0.90	0.94	0.89
evm.TU.scaffold_18.237	0.91	0.95	0.83	0.95	0.91	0.96
evm.TU.scaffold_27.839	0.91	0.93	0.83	0.95	0.92	0.95

Supplementary Table 7. The expression levels of 14 2ODD candidate genes screened by co-expression analysis.

gene	root				leaf			
	1	2	3	4	1	2	3	4
evm.TU.scaffold_15.1187 (DI2ODD4)	16.53	8.80	2.57	3.05	1196.27	887.66	1026.50	1147.39
evm.TU.scaffold_4.1461 (DI2ODD3)	15.72	7.57	6.94	6.47	136.58	117.41	94.45	169.30
evm.TU.scaffold_21.1335	0.00	0.07	0.00	0.42	52.66	73.14	72.51	69.57
evm.TU.scaffold_4.475	2.08	1.41	0.80	0.86	40.01	20.31	25.36	48.67
evm.TU.scaffold_22.299	0.00	0.00	0.00	0.00	3.93	3.37	5.44	6.40
evm.TU.scaffold_15.2	14.67	0.21	0.20	0.29	843.61	407.19	685.94	695.88
evm.TU.scaffold_7.1289	31.82	39.84	32.38	20.83	200.37	288.54	229.99	249.25
evm.TU.scaffold_6.1260	16.11	23.95	19.45	16.25	61.23	79.48	70.11	84.11
evm.TU.scaffold_8.1634	3.62	0.23	0.44	1.28	9.84	11.82	12.91	16.15
evm.TU.scaffold_9.399	7.79	11.75	12.19	8.67	32.50	36.38	43.17	33.56
evm.TU.scaffold_5.1632	51.83	93.16	89.27	77.24	280.73	230.21	238.04	275.52
evm.TU.scaffold_1.1016	40.65	18.39	7.46	10.24	88.51	80.52	79.74	99.75
evm.TU.scaffold_13.1348	30.88	64.13	83.09	58.23	500.49	670.70	398.60	312.37
evm.TU.scaffold_4.1361	19.76	4.20	0.11	0.21	72.41	30.22	47.11	121.50

Note: Gray shading indicates genes with leaf TPM < 50.

Supplementary Table 8. The expression levels of 22 P450 candidate genes screened by co-expression analysis.

gene	root				leaf			
	1	2	3	4	1	2	3	4
evm.TU.scaffold_10.738	5.78	2.60	3.50	3.60	109.65	88.89	130.12	180.14
evm.TU.scaffold_19.1272	4.23	2.96	2.35	2.30	60.94	74.04	113.93	129.58
evm.TU.scaffold_8.53	0.91	0.04	0.00	0.15	68.33	175.35	198.52	211.32
evm.TU.scaffold_2.30	0.13	0.12	0.14	0.17	69.08	181.18	197.18	188.08
evm.TU.scaffold_25.725	2.49	1.49	1.00	2.10	40.05	53.07	44.23	25.55
evm.TU.scaffold_9.1243	0.00	0.00	0.12	0.00	1.99	1.87	2.28	2.94
evm.TU.scaffold_10.76	0.06	0.06	0.02	0.04	6.79	5.89	6.29	3.71
evm.TU.scaffold_1.706	3.19	4.32	3.61	3.09	22.29	40.64	44.09	44.01
evm.TU.scaffold_15.488	0.39	0.87	0.09	1.42	18.32	31.78	40.01	45.03
evm.TU.scaffold_6.1732	1.73	2.17	1.96	1.55	10.87	14.19	21.58	20.30
evm.TU.scaffold_21.192	1.09	1.19	0.23	0.12	19.08	49.32	15.41	30.73
evm.TU.scaffold_24.916	5.81	4.64	7.11	7.29	69.08	185.97	181.62	227.69
evm.TU.scaffold_10.503	1.95	3.36	2.01	1.42	26.23	58.60	46.84	30.66
evm.TU.scaffold_22.1097	0.03	0.14	0.17	0.12	4.02	6.15	3.90	3.03
evm.TU.scaffold_26.407	0.47	0.86	2.09	1.19	49.87	97.79	32.11	36.89
evm.TU.scaffold_25.726	0.43	0.48	0.17	0.33	3.68	6.41	4.00	2.75
evm.TU.scaffold_6.830	0.00	0.00	0.09	0.00	0.78	0.81	1.43	1.10
evm.TU.scaffold_9.552	14.27	6.86	6.00	9.41	30.80	75.87	90.07	83.59
evm.TU.scaffold_15.829	0.00	0.70	0.95	0.96	8.48	8.95	7.00	6.75

evm.TU.scaffold_21.193	2.85	2.93	0.00	0.03	11.10	14.96	15.60	16.77
evm.TU.scaffold_18.237	0.43	0.87	2.96	2.43	13.39	21.06	18.33	23.16
evm.TU.scaffold_27.839	0.51	0.64	1.91	1.05	9.63	4.47	8.80	8.13

Note: Gray shading indicates genes with leaf TPM < 50.

Supplementary Table 9. Co-expression analysis of *D. lanata* transcriptome using *DICYP1/2*, *DIHSD1*, *DISDR1*, and *DI2ODD1/2* as bait genes for the identification of ATs.

gene	Pearson correlation coefficient					
	DICYP1	DICYP2	DI2ODD1	DI2ODD2	DISDR1	DIHSD1
evm.TU.scaffold_10.562	0.99	0.96	1.00	1.00	0.99	0.99
evm.TU.scaffold_19.670 (DIAT1)	0.98	0.94	0.99	1.00	0.98	1.00
evm.TU.scaffold_20.554	0.98	0.94	0.99	0.99	0.98	0.98
evm.TU.scaffold_18.1374	0.99	0.97	0.97	0.98	0.99	0.97
evm.TU.scaffold_28.211	0.98	0.95	0.98	0.99	0.97	0.98
evm.TU.scaffold_11.761	0.98	0.96	0.97	0.98	0.98	0.97
evm.TU.scaffold_27.497	0.98	0.94	0.97	0.98	0.98	0.99
evm.TU.scaffold_11.547	0.98	0.94	0.97	0.98	0.97	0.99
evm.TU.scaffold_18.611	0.98	0.96	0.96	0.97	0.97	0.96
evm.TU.scaffold_5.1489 (DIAT2)	0.97	0.95	0.97	0.97	0.98	0.96
evm.TU.scaffold_7.240	0.98	0.98	0.95	0.95	0.98	0.96
evm.TU.scaffold_11.922	0.95	0.99	0.90	0.91	0.96	0.90

Supplementary Table 10. The expression levels of 12 AT candidate genes screened by co-expression analysis.

gene	root				leaf			
	1	2	3	4	1	2	3	4
evm.TU.scaffold_10.562	6.13	4.80	3.88	4.31	235.64	269.38	263.07	316.13
evm.TU.scaffold_19.670 (DIAT1)	0.50	0.37	0.87	0.92	228.44	327.74	368.87	383.26
evm.TU.scaffold_20.554	0.62	0.80	1.07	0.52	100.58	129.41	94.42	69.48
evm.TU.scaffold_18.1374	13.54	5.24	8.25	6.93	67.02	106.93	103.08	105.83
evm.TU.scaffold_28.211	0.17	0.00	0.00	0.00	4.55	8.96	5.12	8.40
evm.TU.scaffold_11.761	3.02	1.26	2.18	1.19	21.24	43.06	28.71	43.51
evm.TU.scaffold_27.497	1.72	0.54	0.79	2.15	86.07	300.07	312.06	353.57
evm.TU.scaffold_11.547	0.73	0.37	0.56	0.89	3.60	4.35	4.44	5.97
evm.TU.scaffold_18.611	1.31	0.06	0.00	0.06	22.53	110.60	41.81	91.54
evm.TU.scaffold_5.1489 (DIAT2)	15.96	20.87	13.27	4.86	263.35	232.04	391.88	310.72
evm.TU.scaffold_7.240	16.06	11.43	6.99	9.42	39.62	42.58	59.73	65.52
evm.TU.scaffold_11.922	8.27	4.88	2.25	3.26	14.65	16.66	17.70	18.12

Note: Gray shading indicates genes with leaf TPM < 50.

Supplementary Table 11. Data collection and refinement statistics of *D/2ODD3/Fe²⁺* crystal (PDB 43SG).

	<i>D/2ODD3/Fe²⁺</i>
Datacollection	
Spacegroup	<i>P2₁2₁2₁</i>
Cell dimensions	
<i>a,b,c</i> (Å)	47.06,89.11,97.95
α,β,γ (°)	90,90,90
Resolution(Å)	48.97-2.88(3.04-2.88)
<i>R</i> _{merge} (%)	34.1(94.6)
<i>I</i> / σ <i>I</i>	5.5(3.1)
Completeness(%)	99.5(100)
Redundancy	8.3(9.2)
Refinement	
Resolution(Å)	48.98-2.88(3.17-2.88)
No.reflections	9724
<i>R</i> _{work} / <i>R</i> _{free} (%)	20.90(25.68)/ 27.92(35.17)
No.atoms	2291
Protein	2290
Ligand	1
Water	-
Average B-factor(Å ²)	34.07
Protein	34.07
Ligand	27.81
Water	-
R.m.s.deviation	
Bond lengths(Å)	0.0090
Bond angles(°)	1.057