Electronic Supplementary Material (ESI) for Green Chemistry.

Supplementary information

Batch and continuous flow asymmetric synthesis of anabolic-androgenic steroids via single-cell biocatalytic Δ^1-dehydrogenation and C17β-carbonyl reduction cascade

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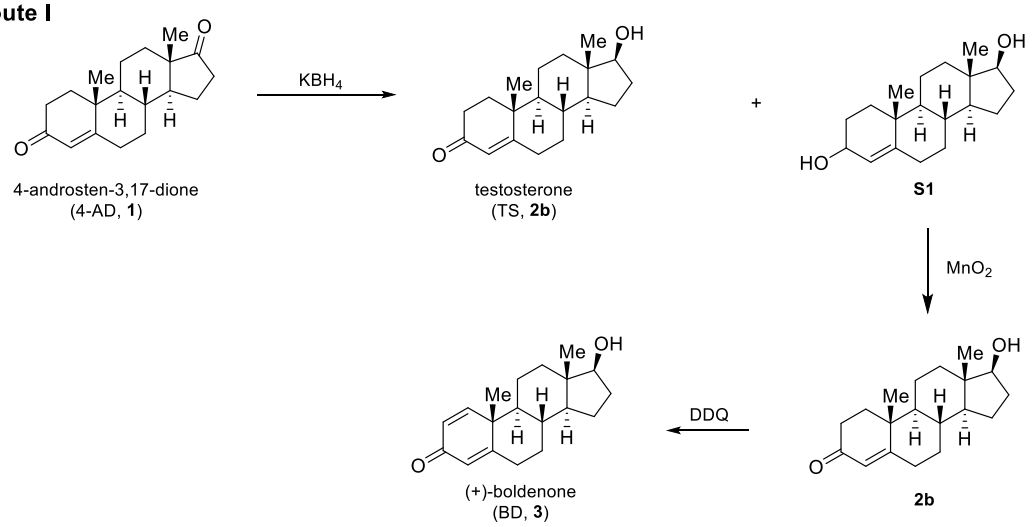
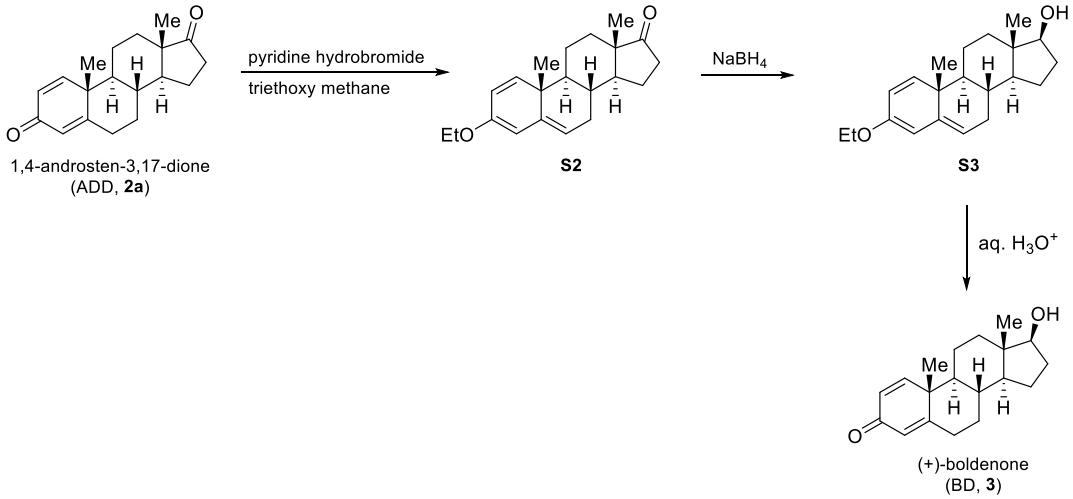
Content

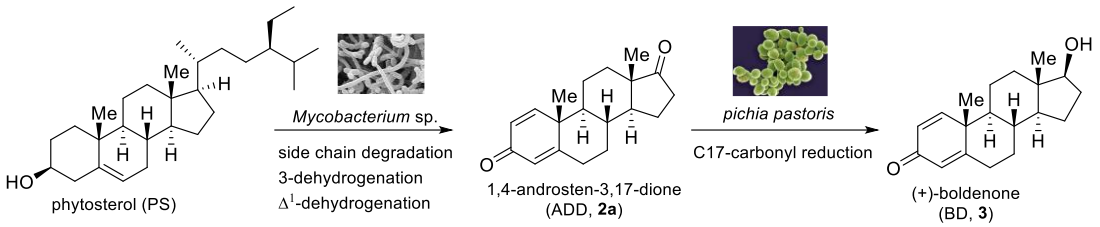
route I



route II



Scheme S1. Chemical approaches for the synthesis of (+)-boldenone.



Scheme S2 The stepwise transformations from PS to (+)-boldenone.

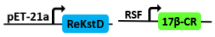
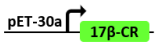
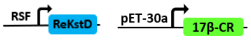
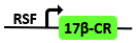
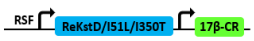
Tab. S1 Primers for mutagenesis of ReKstD

Primer	5′ to 3′ Sequence
ReKstD-F	CATATGCAGGATTGGACGAGTGAA (*Nde* I)
ReKstD-R	AAGCTTTTATTTAGCCATATCCTGA (*Hind* III)
G48/A49-F	ACAAGTGCGTATAGCNDTNDTAGTATTTGGTTACCGGGCACACAGGT
G48/A49-R	CGGTAACCAAATACTAHNAHNGCTATACGCACTTGTACCGCCAAA
S50/I51-F	GCGTATAGCGGTGCGNDTNDTTGGTTACCGGGCACACAGGTTCAG
S50/I51-R	TGTGCCCGGTAACCAAHNAHNCGCACCGCTATACGCACTTGTACC
P488-F	AGGTTTTATCCAGGCNDTGGTGTTCCGTTAGGTACAGCGATGGTG
P488-R	ACCTAACGGAACACCAHNGCCTGGATAAAACCTCCCCGACAGTGA
F114-F	TTTGAATTTCGTGCCNDTCCGGATTATTATAAAGCAGAAGGTCGC
F114-R	TTTATAATAATCCGGAHNGGCACGAAATTCAAATTCGATATTTGG
L445-F	ATTGTGCTGAGCGATNDTGGGACAAAAGGTGGTTTAGTGACCGAT
L445-R	ACCACCTTTTGTCCCAHNATCGCTCAGCACAATGCGTGCAGCATA
F416-F	CCTTACGATGCCTTTNDTTGTCCTCCTAATGGCGGTCCTAATGC
F416-R	GCCATTAGGAGGACAAHNAAAGGCATCGTAAGGATCTTCACCGCG
F292/V294-F	GATGGCTCTGCTGCCNDTATGNDTGGTGTTCGTGGTGGCTTAGTT
F292/V294-R	GCCACCACGAACACCAHNCATAHNGGCAGCAGAGCCATCTGGCTG
I350/I352-F	GGCGGCTTACCAGCCNDTTGTNDTCCAAATACAGCTCCTGCCAAA
I350/I352-R	AGGAGCTGTATTTGGAHNACAAHNGGCTGGTAAGCCGCCACCTTC
I51G-F	GCGAGTGGCTGGTTACCGGGCAC
I51G-R	TAACCAGCCACTCGCACCGCTAT
I51A-F	GCGAGTGCGTGGTTACCGGGCAC
I51A-R	TAACCACGCACTCGCACCGCTAT
I51L-F	GCGAGTCTGTGGTTACCGGGCAC
I51L-R	TAACCACAGACTCGCACCGCTAT
I51V-F	GCGAGTGTTTGGTTACCGGGCAC
I51V-R	TAACCAAACACTCGCACCGCTAT
I51P-F	GCGAGTCCATGGTTACCGGGCAC
I51P-R	TAACCATGGACTCGCACCGCTAT
I51F-F	GCGAGTTTTTGGTTACCGGGCAC
I51F-R	TAACCAAAAACTCGCACCGCTAT
I51M-F	GCGAGTATGTGGTTACCGGGCAC
I51M-R	TAACCACATACTCGCACCGCTAT
I51W-F	GCGAGTTGGTGGTTACCGGGCAC
I51W-R	TAACCACCAACTCGCACCGCTAT
I51S-F	GCGAGTAGCTGGTTACCGGGCAC
I51S-R	TAACCAGCTACTCGCACCGCTAT
I51Q-F	GCGAGTCAGTGGTTACCGGGCAC
I51Q-R	TAACCACTGACTCGCACCGCTAT
I51T-F	GCGAGTACCTGGTTACCGGGCAC
I51T-R	TAACCAGGTACTCGCACCGCTAT
I51C-F	GCGAGTTGTTGGTTACCGGGCAC
I51C-R	TAACCAACAACTCGCACCGCTAT
I51N-F	GCGAGTAATTGGTTACCGGGCAC
I51N-R	TAACCAATTACTCGCACCGCTAT
I51Y-F	GCGAGTTATTGGTTACCGGGCAC
I51Y-R	TAACCAATAACTCGCACCGCTAT
I51D-F	GCGAGTGATTGGTTACCGGGCAC
I51D-R	TAACCAATCACTCGCACCGCTAT
I51E-F	GCGAGTGAATGGTTACCGGGCAC
I51E-R	TAACCATTCACTCGCACCGCTAT
I51K-F	GCGAGTAAATGGTTACCGGGCAC

I51K-R	TAACCATTTACTCGCACCGCTAT
I51R-F	GCGAGTAGGTGGTTACCGGGCAC
I51R-R	TAACCACCTACTCGCACCGCTAT
I51H-F	GCGAGTCATTGGTTACCGGGCAC
I51H-R	TAACCAATGACTCGCACCGCTAT
I350G-F	CCAGCCGGCTGTATCCCAAATACAGC
I350G-R	GATACAGCCGGCTGGTAAGCCGCC
I350A-F	CCAGCCGCCTGTATCCCAAATACAGC
I350A-R	GATACAGGCGGCTGGTAAGCCGCC
I350L-F	CCAGCCGCGTGTATCCCAAATACAGC
I350L-R	GATACACGCGGCTGGTAAGCCGCC
I350V-F	CCAGCCGTTTGTATCCCAAATACAGC
I350V-R	GATACAAACGGCTGGTAAGCCGCC
I350P-F	CCAGCCCCGTGTATCCCAAATACAGC
I350P-R	GATACACGGGGCTGGTAAGCCGCC
I350F-F	CCAGCCTTTTGTATCCCAAATACAGC
I350F-R	GATACAAAAGGCTGGTAAGCCGCC
I350M-F	CCAGCCATGTGTATCCCAAATACAGC
I350M-R	GATACACATGGCTGGTAAGCCGCC
I350W-F	CCAGCCTGGTGTATCCCAAATACAGC
I350W-R	GATACACCAGGCTGGTAAGCCGCC
I350S-F	CCAGCCAGCTGTATCCCAAATACAGC
I350S-R	GATACAGCTGGCTGGTAAGCCGCC
I350Q-F	CCAGCCCAGTGTATCCCAAATACAGC
I350Q-R	GATACACTGGGCTGGTAAGCCGCC
I350T-F	CCAGCCACCTGTATCCCAAATACAGC
I350T-R	GATACAGGTGGCTGGTAAGCCGCC
I350C-F	CCAGCCTGTTGTATCCCAAATACAGC
I350C-R	GATACAACAGGCTGGTAAGCCGCC
I350N-F	CCAGCCAATTGTATCCCAAATACAGC
I350N-R	GATACAATTGGCTGGTAAGCCGCC
I350Y-F	CCAGCCTATTGTATCCCAAATACAGC
I350Y-R	GATACAATAGGCTGGTAAGCCGCC
I350D-F	CCAGCCGATTGTATCCCAAATACAGC
I350D-R	GATACAATCGGCTGGTAAGCCGCC
I350E-F	CCAGCCGAATGTATCCCAAATACAGC
I350E-R	GATACATTCGGCTGGTAAGCCGCC
I350K-F	CCAGCCAAATGTATCCCAAATACAGC
I350K-R	GATACATTTGGCTGGTAAGCCGCC
I350R-F	CCAGCCAGGTGTATCCCAAATACAGC
I350R-R	GATACACCTGGCTGGTAAGCCGCC
I350H-F	CCAGCCCATTGTATCC CAAATACAGC
I350H-R	GATACAATGGGCTGGTAAGCCGCC

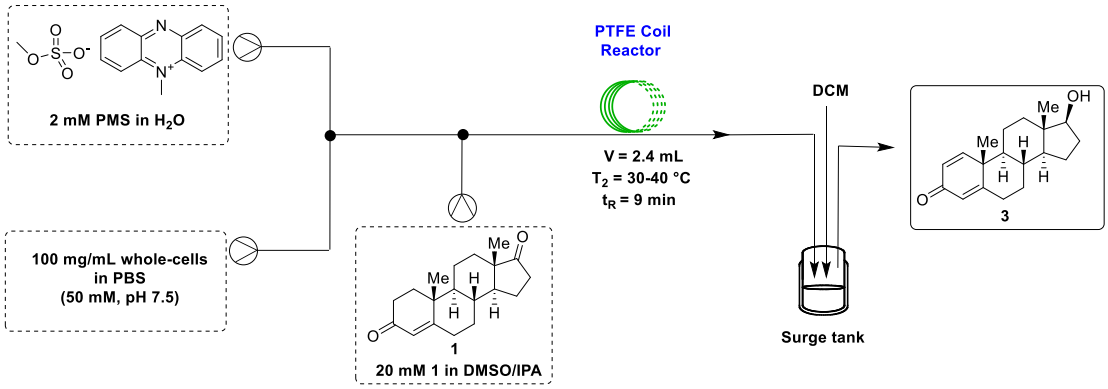
*The sequences in this table were designed based on wild type ReKstD. The primer sequence was adjusted when the template is variant.

Tab. S2 Construction of each *E. coli* cells based on different plasmid compositions.

Entry	*E. coli* strains	Plasmids[a]	Entry	*E. coli* strains	Plasmids[a]
1	*E. coli* (Re-21a)		7	*E. coli* (ReM2)	
2	*E. coli* (Re-RSF)		8	*E. coli* (Re-21a+CR-RSF)	
3	*E. coli* (CR-30a)		9	*E. coli* (Re-RSF+CR-30a)	
4	*E. coli* (CR-RSF)		10	*E. coli* (RSF-ReM2_CR)	
5	*E. coli* (Re-I51L)		11	*E. coli* (RSF-CR_ReM2)	
6	*E. coli* (Re-I350T)				

[a] RSF: pRSFDuet-1; arrow: T7 promoter; "+": Different but compatible plasmids; underline: Tandem arrangement of different genes.

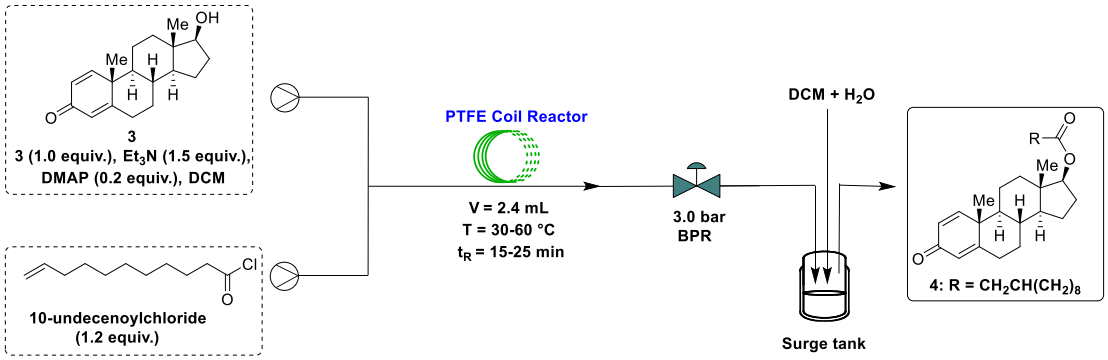
Tab. S3 Continuous flow synthesis of (+)-boldenone (3) through biocatalytic Δ[1]-dehydrogenation and C17β-carbonyl reduction cascade in a PTFE coil reactor.



Entry	Temp. (°C)	DMSO (%, v/v)	t_R (min)	Yield (%)[a]
1	30	30	9	79.7
2	30	40	9	99.2
3	30	50	9	70.7
4	40	40	9	45.4

[a]Yield was determined by LC-MS.

Tab.S4 Continuous flow synthesis of (+)-boldenone undecylenate (4) starting from purified (+)-boldenone.



Entry	Temp. (°C)	t_R (min)	Yield (%)[a]	Entry	Temp. (°C)	t_R (min)	Yield (%)[a]
1	30	20	87.6	5	60	10	61.3
2	40	20	92.6	6	60	15	68.2
3	50	20	92.1	7	60	25	98.9
4	60	20	93.9				

[a]Yield was determined by LC-MS.

Tab. S5 NMR information of steroid compounds

Compounds	^{1}H NMR
(+)-Boldenone (**3**)	(500 MHz, Chloroform-*d*) δ = 7.03 (d, *J* = 10.2 Hz, 1H), 6.19 (dd, *J* = 10.2, 2.0 Hz, 1H), 6.03 (d, *J* = 1.8 Hz, 1H), 3.60 (t, *J* = 8.5 Hz, 1H), 2.43 (tdd, *J* = 13.5, 5.2, 1.6 Hz, 1H), 2.32 (ddd, *J* = 13.3, 4.5, 2.6 Hz, 1H), 2.12-1.97 (m, 2H), 1.96-1.88 (m, 1H), 1.88-1.80 (m, 1H), 1.77-1.69 (m, 1H), 1.69-1.52 (m, 3H), 1.44 (dddd, *J* = 13.5, 11.7, 8.1, 3.4 Hz, 1H), 1.29 (qd, *J* = 12.1, 5.7 Hz, 1H), 1.20 (s, 3H), 1.09-0.95 (m, 3H), 0.91 (ddd, *J* = 12.2, 10.7, 7.1 Hz, 1H), 0.78 (s, 3H).
(+)-boldenone undecylenate (**4**)	(500 MHz, Chloroform-*d*) δ = 7.04 (d, *J* = 10.2 Hz, 1H), 6.22 (dd, *J* = 10.1, 1.9 Hz, 1H), 6.06 (t, *J* = 1.7 Hz, 1H), 5.79 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1H), 5.02-4.88 (m, 2H), 4.58 (dd, *J* = 9.2, 7.8 Hz, 1H), 2.46 (tdd, *J* = 13.5, 5.1, 1.6 Hz, 1H), 2.36 (ddd, *J* = 13.3, 4.4, 2.5 Hz, 1H), 2.28 (t, *J* = 7.5 Hz, 2H), 2.16 (dtd, *J* = 13.6, 9.4, 6.2 Hz, 1H), 2.02 (tdd, *J* = 6.6, 5.3, 1.4 Hz, 2H), 1.97-1.91 (m, 1H), 1.80-1.69 (m, 2H), 1.69-1.56 (m, 4H), 1.53-1.44 (m, 1H), 1.37 (dt, *J* = 12.2, 9.3 Hz, 3H), 1.28 (hepta, *J* = 5.0, 4.1 Hz, 9H), 1.25-1.15 (m, 4H), 1.04 (tdd, *J* = 12.4, 10.4, 5.7 Hz, 3H), 0.85 (s, 3H).

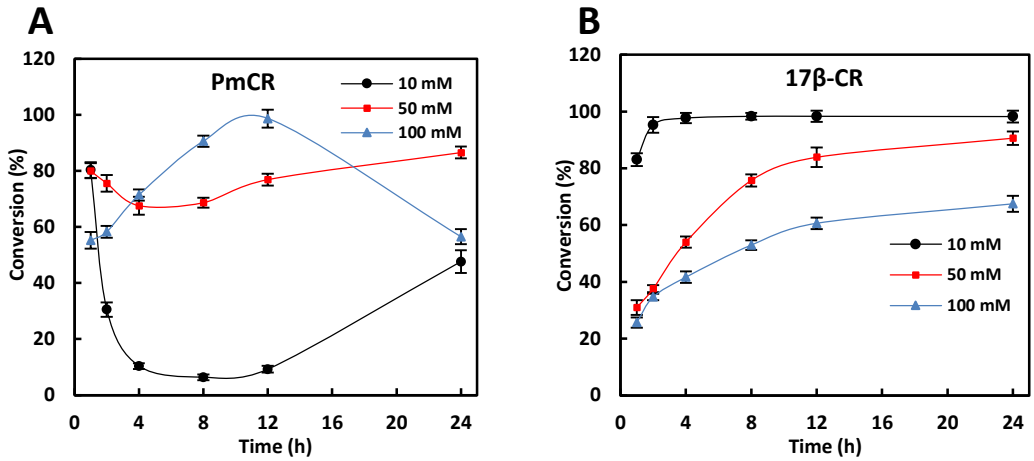


Fig. S1 A Time course for *E. coli*-PmCR catalyzed conversion of 1 to 2b with different substrate concentrations. Reaction conditions: the reaction mixtures performed with substrate **1** (10, 50 and 100 mM), 100 mg/mL expressed *E. coli*-PmCR wet cells, 20 mg/mL lytic cells expressing *Bst*FDH, 10.2 mg/mL sodium formate, 0.3 mM $NADP^+$, 4% (v/v) Tween 80, PBS (50 mM, pH 7.5) until the total system was 1 mL, 37 °C, 200 rpm for 0-24 h. **B Time course for *E. coli*-17β-CR catalyzed conversion of 1 to 2b with different substrate concentrations.** Reaction conditions: the reaction mixtures performed with 10% (v/v) IPA, substrate **1** (10, 50 and 100 mM), 100 mg/mL expressed *E. coli*-17β-CR wet cells, PBS (50 mM, pH 7.5) until the total system was 1 mL, 37 °C, 200 rpm for 0-24 h.

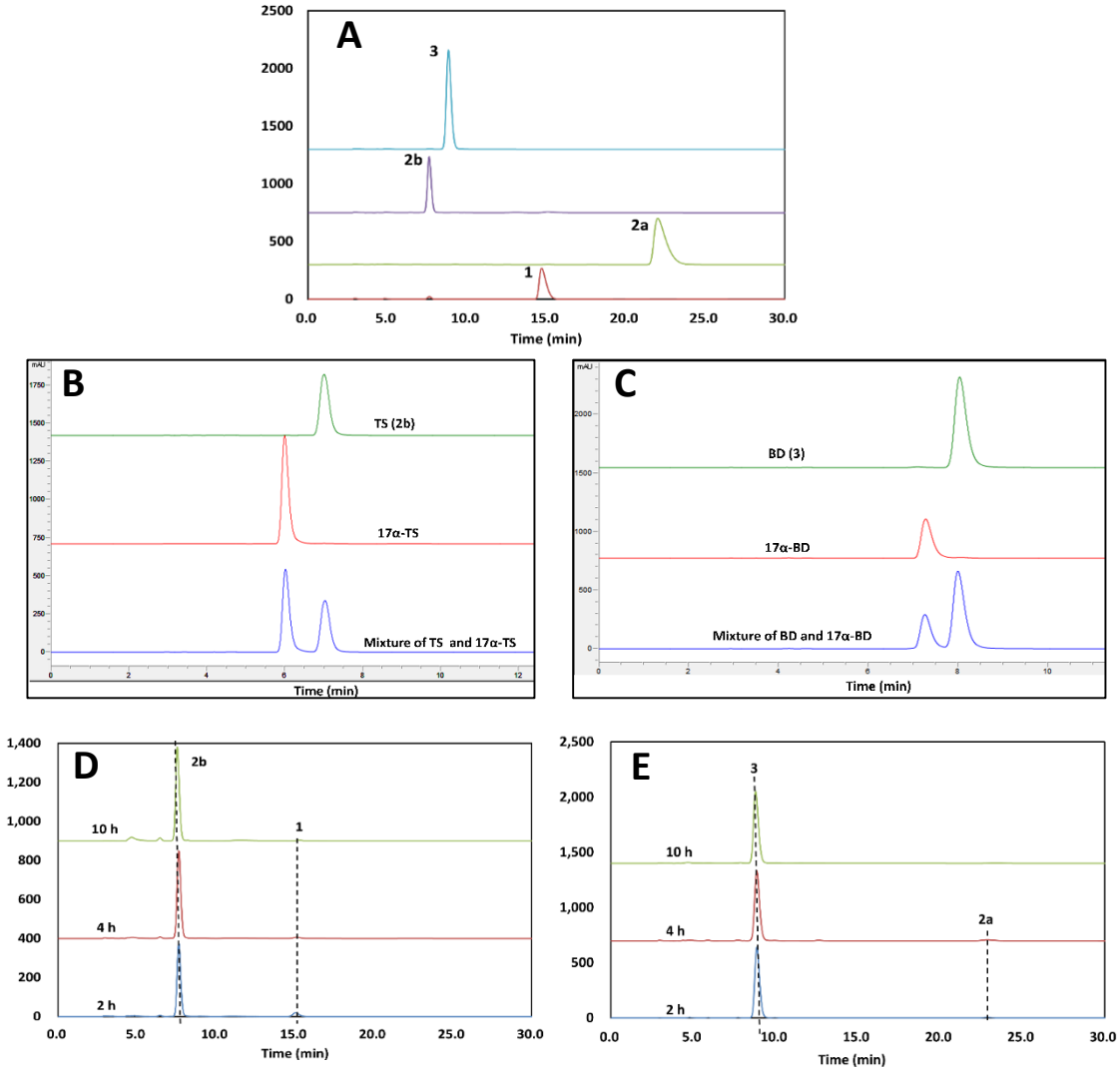


Fig. S2 HPLC analysis of 4-AD (1), ADD (2a), TS (2b), and BD (3) equipped with chiral OD-H column. A, B and **C** The standard samples were dissolved in isopropanol, and used for diastereomeric excess (*de*) determination with high-performance liquid chromatography (HPLC) equipped with a Chiralcel OD-H column. HPLC was performed at 254 nm and 30 °C, hexane/isopropanol (80:20, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The retention times of 4-AD, ADD, TS, 17α-testosterone (17α-TS), BD and 17α-boldenone (17α-BD) were 14.70, 22.01, 7.63, 6.23, 8.84 and 7.21 min, respectively. **D** Time course for *E. coli* (17β-CR)-catalyzed conversion of **1** to **2b**. **E** Time course for *E. coli* (17β-CR)-catalyzed conversion of **2a** to **3**. Reaction conditions: 25 mM **1** or **2a**, 50 mg wet cells expressing 17β-CR, 10% (v/v) IPA, PBS (50 mM, pH 7.5), 30 °C, 200 rpm, 2, 4 and 10 h, respectively. The reaction mixtures were extracted with ethyl acetate, evaporated under vacuum, dried over anhydrous Na_2SO_4, and used for diastereomeric excess value (*de* value) determination with the test method of standards.

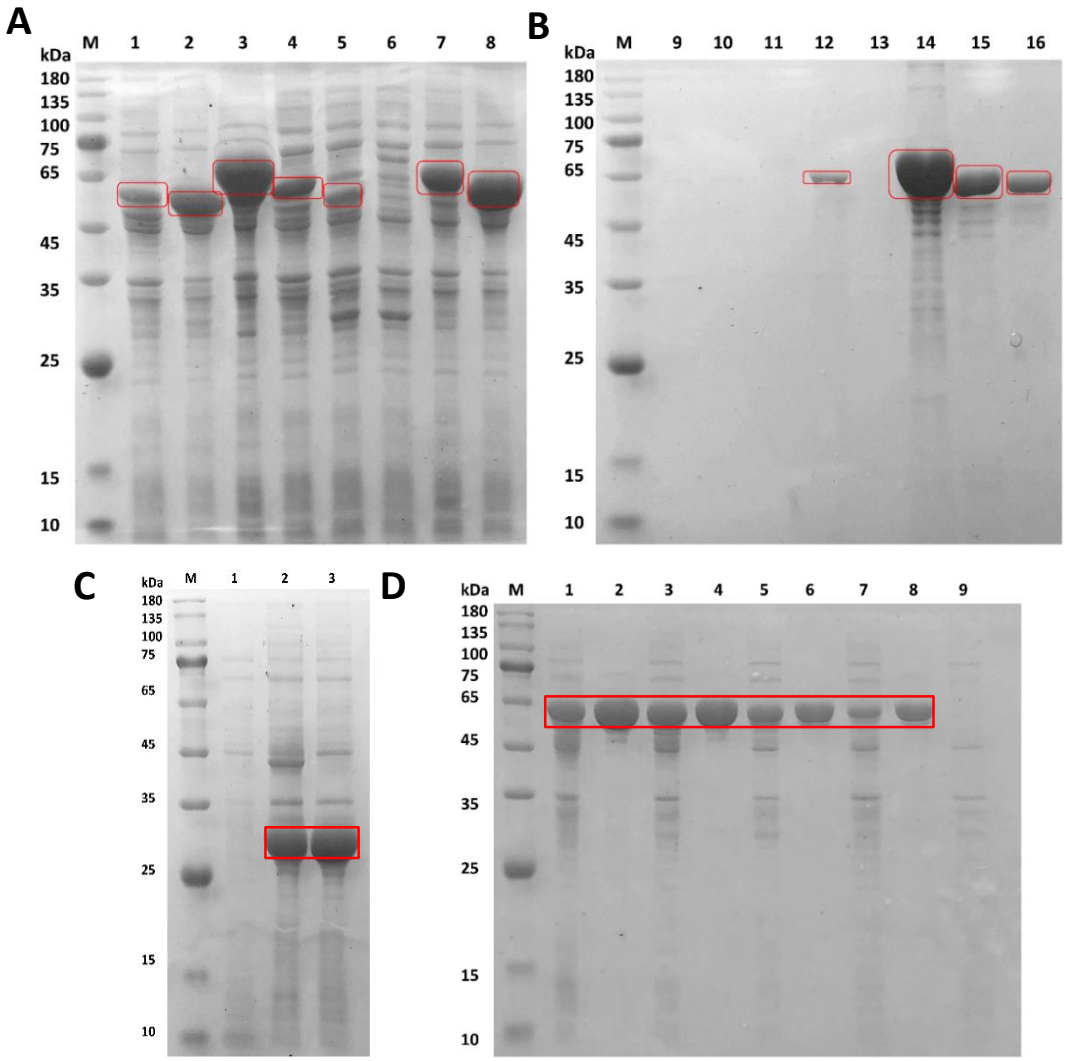


Fig. S3 A, B Protein expression of the selected Δ[1]-KstDs. After ultrasonic crushing centrifugal, SDS-PAGE analysis of different Δ[1]-KstDs in supernatant. SDS-PAGE analysis of different Δ[1]-KstDs in precipitation. Lane **M**, molecular weight standards; lane **1** and **9**, *E. coli*-MsKstD1 supernatant and precipitation; lane **2** and **10**, *E. coli*-ReKstD supernatant and precipitation; lane **3** and **11**, *E. coli*-RgKstD supernatant and precipitation; lane **4** and **12**, *E. coli*-NocKstD supernatant and precipitation; lane **5** and **13**, *E. coli*-SQ1KstD supernatant and precipitation; lane **6** and **14**, *E. coli*-LnKstD supernatant and precipitation; lane **7** and **15**, *E. coli*-MtKstD supernatant and precipitation; lane **8** and **16**, *E. coli*-MrKstD supernatant and precipitation. SDS-PAGE analysis illustrated that most of protein mainly existed in the supernatant, indicating a soluble expression, with an exception, LnKstD protein was expressed in form of inclusion bodies (IB) entirely with no activity. **C Protein expression of the selected carbonyl reductases.** Lane **M**, molecular weight standards; lane **1**, *E. coli*-pET-30a; lane **2**, *E. coli*-PmCR; lane **3**, *E. coli*-17β-CR. **D Protein expression and purification of ReKstD and its variants.** SDS-PAGE analysis of the supernatant of the lysates and the purified proteins. Lane **M**, molecular weight standards; Lane **1**, **3**, **5**, **7** and **9** are respectively the supernatants of the *E. coli* strains expressing ReKstD, Re-I51L, Re-I350T, ReM2 and empty vector pET-21a; Lane **2**, **4**, **6** and **8** are respectively the purified protein of ReKstD, Re-I51L, Re-I350T and ReM2.

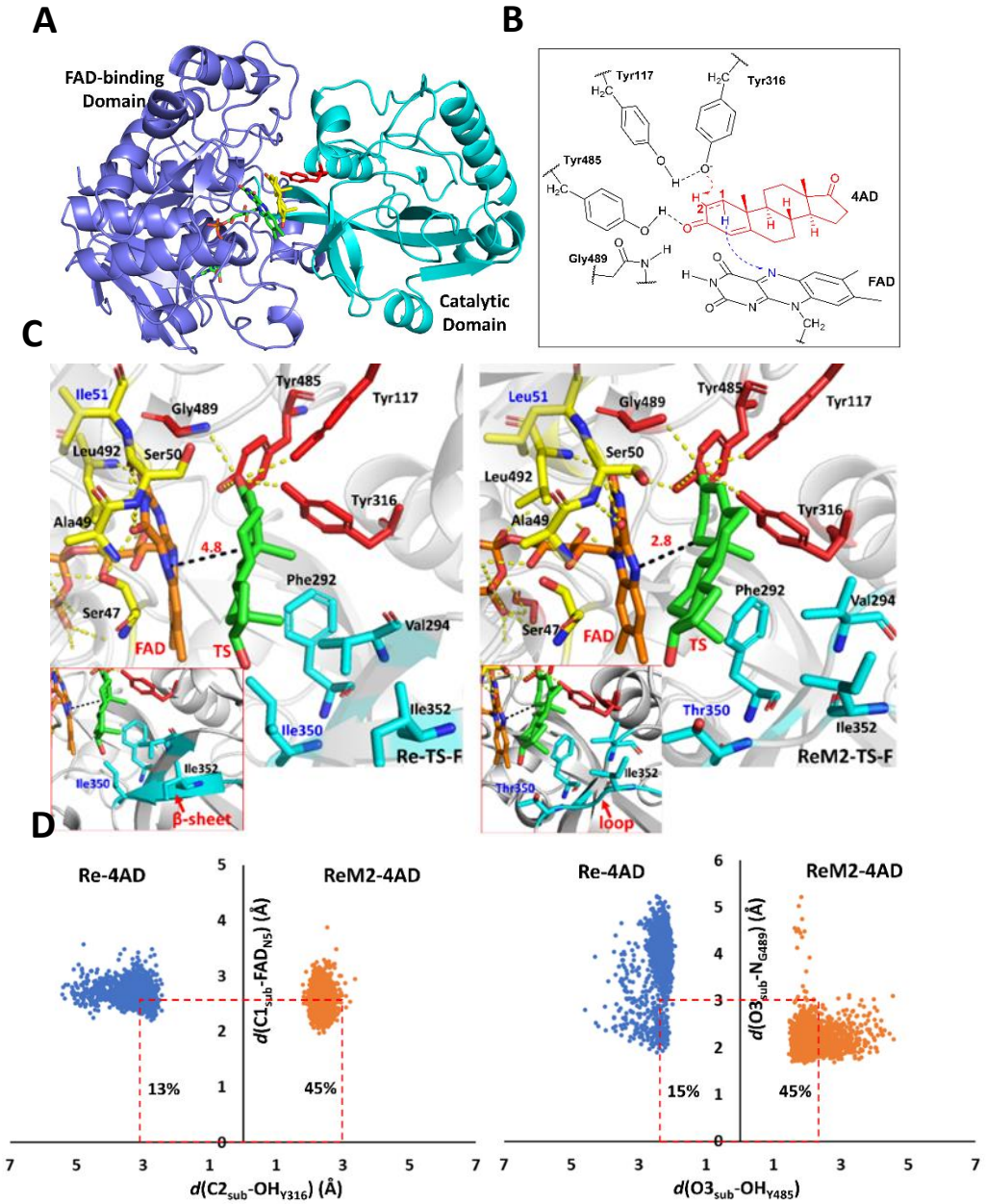


Fig. S4 A Ribbon diagram of ReKstD assembled with FAD and 4-AD. Green, yellow, and red sticks were FAD, 4-AD, and Tyr316, respectively, and FAD-binding domain was slate blue, the catalytic domain was in cyan. **B Reaction scheme of Δ^1-dehydrogenation of 4-AD.** The substrate is marked in red, and dashed lines represent hydrogen bond interactions. The first step corresponds to the abstraction of the 2β proton at the C2 position of 3-ketosteroid by tyrosine anion Tyr316 and the second step is the 1α hydride transfer from the C1 position of the 3-ketosteroid to the N5 of the FAD. **C Comparison of binding modes and hydrogen bonding of Re-TS and ReM2-TS in free-state simulation.** The amino acid residues are shown in sticks, the four key residues which involved in Δ^1-dehydrogenation reaction are shown red sticks. TS and FAD are depicted with green and orange carbon atoms, respectively. Hydrogen bonds are shown in yellow dashed lines, and the distances are shown with black dashes and value (Å). **D Conformation maps of Re-4AD (blue) and ReM2-4AD (orange).** The limits of "catalytic distances" colored by red dashes were $d(OH_{Y316}\text{-}C2_{sub}) \leq 3.0$ Å, $d(N5_{FAD}\text{-}C1_{sub}) \leq 2.6$ Å, $d(O3_{sub}\text{-}OH_{Y485}) \leq 2.4$ Å, and $d(O3_{sub}\text{-}OH_{G489}) \leq 3.0$ Å.

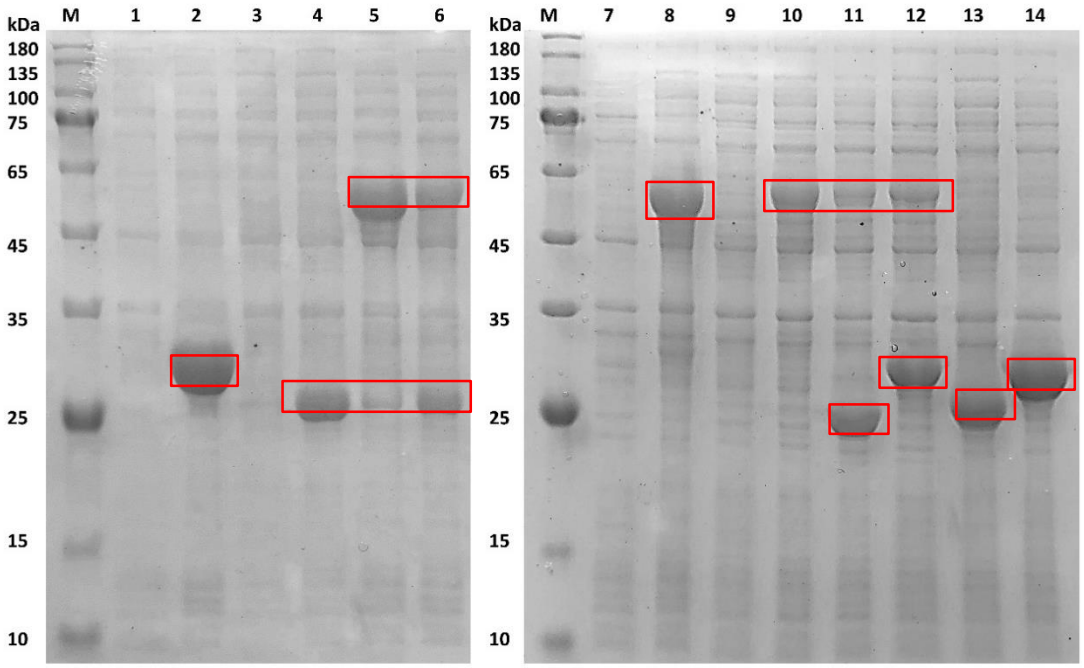


Fig. S5 Protein expression and co-expression of ReM2 and 17β-CR. After ultrasonic crushing centrifugal, SDS-PAGE analysis of different samples in supernatant. Lane **M**, molecular weight standards; lane **1**, *E. coli*-pET-30a; lane **2**, *E. coli*-pET-30a-17β-CR; lane **3**, *E. coli*-pRSFDuet-1; lane **4**, *E. coli*-pRSFDuet-1-17β-CR; lane **5**, *E. coli* (pRSFDuet-1-17β-CR+pET-21a-ReM2); lane **6**, *E. coli*-pRSFDuet-1-17β-CR-ReM2; lane **7**, *E. coli*-pET-21a; lane **8**, *E. coli*-pET-21a-ReM2; lane **9**, *E. coli*-pRSFDuet-1; lane **10**, *E. coli*-pRSFDuet-1-ReM2; lane **11**, *E. coli*-pRSFDuet-1-ReM2-17β-CR; lane **12**, *E. coli* (pRSFDuet-*1*-ReM2+ pET-30a-17β-CR); lane **13**, *E. coli*-pRSFDuet-1-17β-CR; lane **14**, *E. coli*-pET-30a-17β-CR.

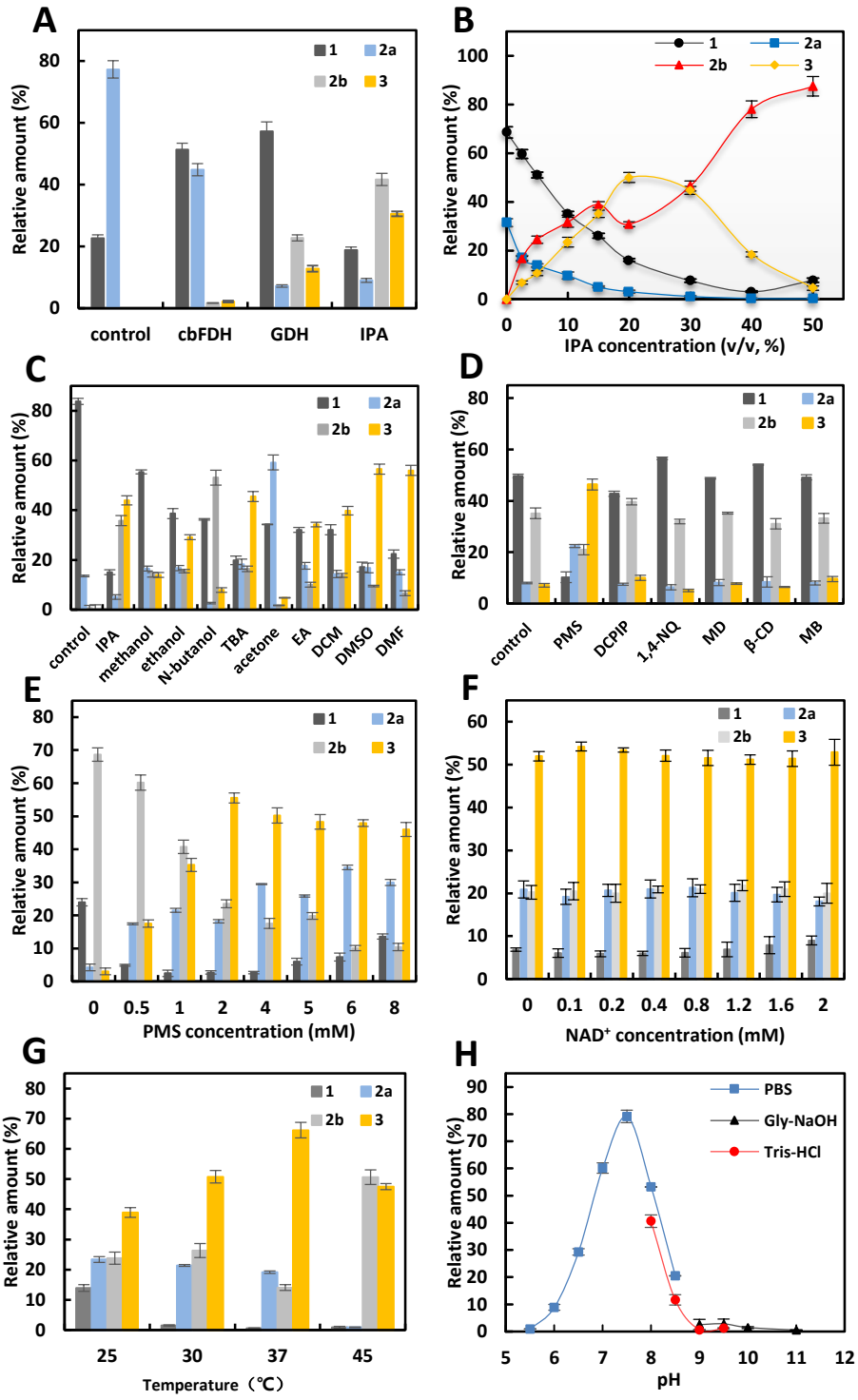


Fig. S6 Reaction condition optimization for the enzymatic cascade synthesis of 3. A Cofactor regeneration system. Reaction conditions (1 mL): the reaction mixtures performed with 4-AD (20 mM), 100 mg/mL co-expressed *E. coli* wet cells, 1 mM PMS, 10% (v/v) DMSO, PBS (50 mM, pH 7.5), 30 °C, 200 rpm for 2 h. I: 35 mM sodium formate, and 0.2 mM NAD^+, 50 μL *cb*FDH (100 mg/mL wet cells) for *cb*FDH regeneration system; II: 35 mM glucose, and 0.2 mM NAD^+, 50 μL *Bt*GDH (100 mg/mL wet cells) for GDH regeneration system; III: 0.2 mM NAD^+, 10% (v/v) IPA for IPA regeneration system,). **B** IPA concentration. The reaction mixtures (1 mL) performed with 0-50% (v/v) IPA, 20 mM 4-AD, 100 mg/mL co-expressed *E. coli* wet cells, 1 mM PMS, and PBS (50 mM, pH 7.5) at 30 °C, 200 rpm for 2 h. **C**

Cosolvents. The reaction mixtures (1 mL) contained 0.5% (v/v) IPA, 20% (v/v) cosolvents, 20 mM 4-AD, 100 g/L co-expressed *E. coli* wet cells, 1 mM PMS, and PBS (50 mM, pH 7.5) at 30 °C, 200 rpm for 2 h. **D** Electron acceptors. The reaction mixtures (1 mL) performed with 0.5% (v/v) IPA, 20% (v/v) DMSO, 20 mM 4-AD, 100 g/L co-expressed *E. coli* wet cells, 6 mM electron acceptor, and PBS (50 mM, pH 7.5) at 30 °C, 200 rpm for 2 h. **E** PMS concentration. The reaction mixtures (1 mL) performed with 0.5% (v/v) IPA, 20% (v/v) DMSO, 20 mM 4-AD, 100 g/L co-expressed *E. coli* wet cells, 0-8 mM PMS, and PBS (50 mM, pH 7.5) at 30 °C, 200 rpm for 2 h. **F** NAD^+ concentration. The reaction mixtures (1 mL) performed with 0.5% (v/v) IPA, 20% (v/v) DMSO, 20 mM 4-AD, 100 g/L co-expressed *E. coli* wet cells, 2 mM PMS, and PBS (50 mM, pH 7.5) at 30 °C, 200 rpm for 2 h. **G** Temperature effect. The reaction mixtures (1 mL) performed with 0.5% (v/v) IPA, 20% (v/v) DMSO, 20 mM 4-AD, 100 g/L co-expressed *E. coli* wet cells, 2 mM PMS, and PBS (50 mM, pH 7.5) at 25-45 °C, 200 rpm for 2 h. **H** pH effect. The reaction mixtures (1 mL) performed with 0.5% (v/v) IPA, 20% (v/v) DMSO, 20 mM 4-AD, 100 g/L co-expressed *E. coli* wet cells, 2 mM PMS, and 50 mM PBS pH 5.5-8.5, 50 mM Gly-NaOH buffer pH 8.0-9.5, 50 mM Tris-HCl buffer pH 9.0-11.0, at 37 °C, 200 rpm for 2 h.

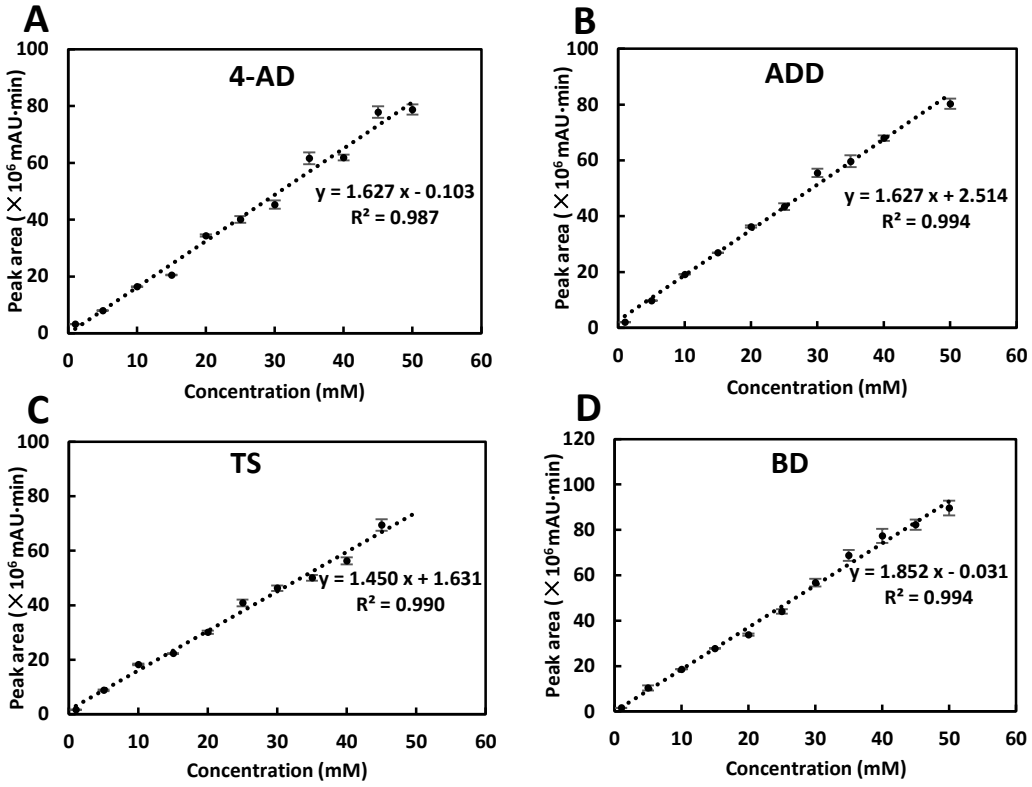


Fig. S7 HPLC standard curve of 4-AD (A), ADD (B), TS (C) and BD (D). The sample was dissolved in isopropanol alcohol (IPA), and was analyzed by high-performance liquid chromatography (HPLC) equipped with C18 column (SHIMADZU Shimpack, 5 μm particles, 150 mm × 4.6 mm), and 35% acetonitrile and 65% water (v/v) as the mobile phase at a flow rate of 0.8 mL/min. The column oven temperature was set as 35 ℃. The UV absorbance was determined at 254 nm. The retention time of 4-AD, ADD, TS and BD were 28.95 min, 18.56 min, 20.72 min and 13.65 min, respectively.

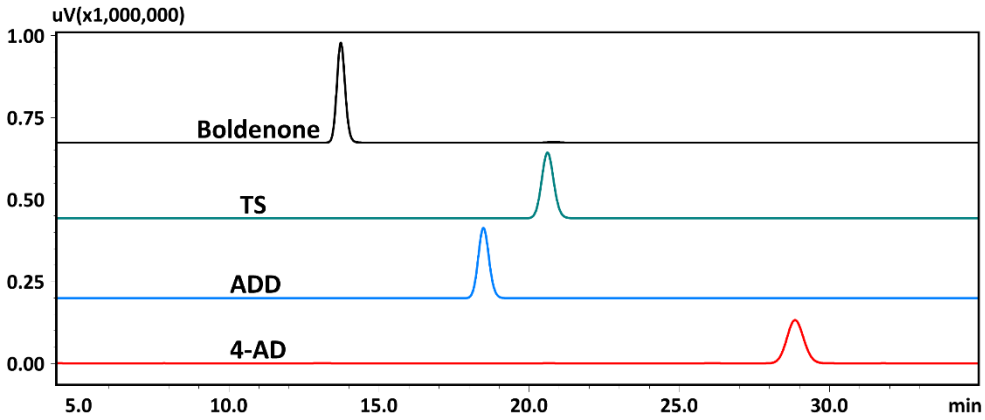


Fig. S8 HPLC analysis of 4-AD, ADD, TS and BD standards. The sample was dissolved in isopropanol alcohol (IPA), and was analyzed by high-performance liquid chromatography (HPLC) equipped with C18 column (SHIMADZU Shimpack, 5 µm particles, 150 mm × 4.6 mm), and 35% acetonitrile and 65% water (v/v) as the mobile phase at a flow rate of 0.8 mL/min. The column oven temperature was set as 35 °C. The UV absorbance was determined at 254 nm. The retention time of 4-AD, ADD, TS and BD were 28.95 min, 18.56 min, 20.72 min and 13.65 min, respectively.

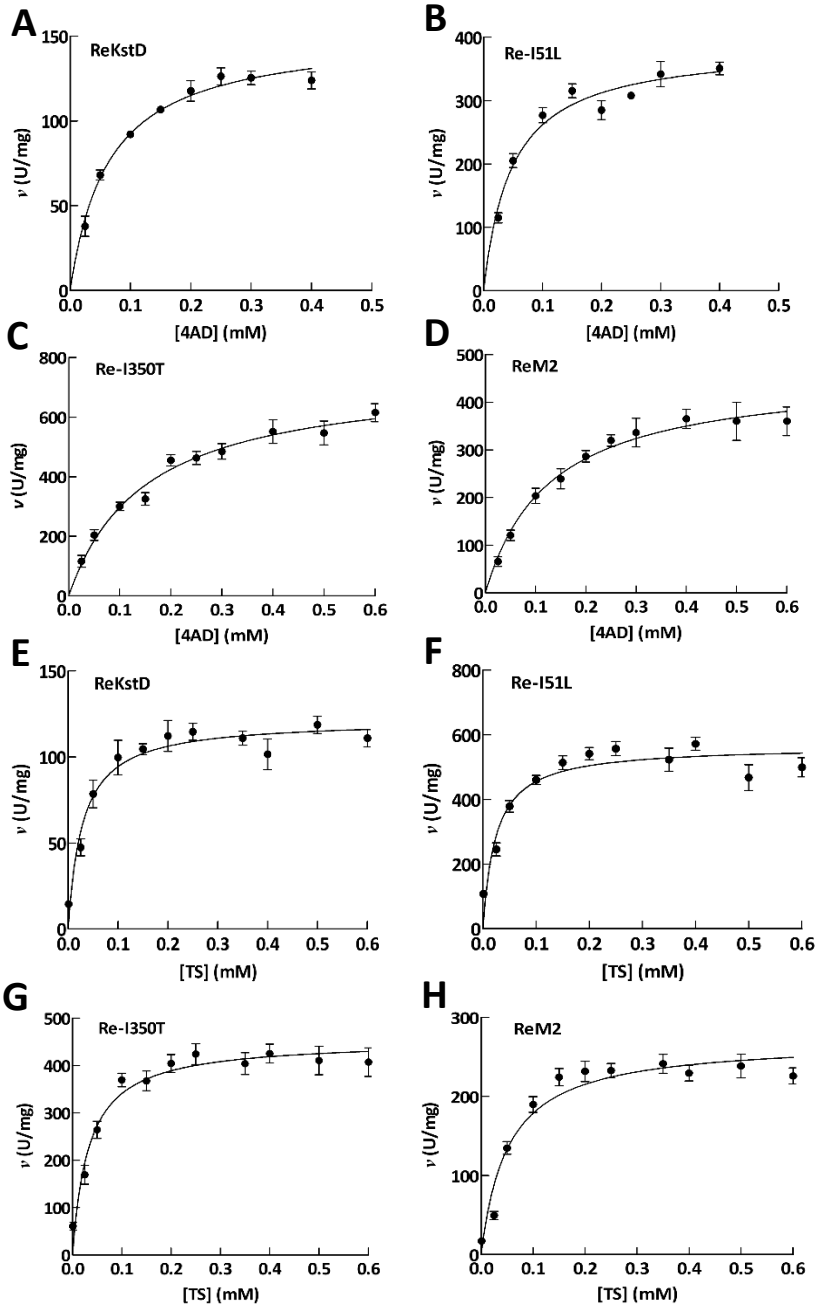


Fig. S9 A plot of the reaction velocity as a function of the substrate concentration as described by the Michaelis-Menten equation. A-D, the reaction velocity toward various concentration of 4-AD catalyzed by ReKstD, Re-I51L, Re-I350T, and ReM2, respectively. **E-H**, the reaction velocity toward various concentration of TS catalyzed by ReKstD, Re-I51L, Re-I350T, and ReM2, respectively. The rate of reaction was experimentally measured at several steroid substrate concentration values (4-AD: 0.025-0.60 mM; TS: 0.025-0.60 mM). *V*max is the maximum reaction rate that is observed at saturating substrate concentrations, *K*m is referred to as the Michaelis constant and is the substrate concentration at which the reaction rate is exactly half of *V*max. Reaction conditions: The reaction mixtures (200 μL) contained 1.5 mM PMS, 40 μM DCPIP, 20 μL of purified enzyme with an appropriate concentration, and 0-600 μM steroidal substrate (20 μL solution in DMSO) in Tris-HCl buffer (50 mM, pH 8.0). The reaction rates were determined by measuring the absorption of DCPIP at 600 nm (ε_{600nm} = 18.7 ×10^3 /cm/M) with microplate reader at 30 °C. One unit of enzyme activity (U) is defined as the reduction of 1 μmol DCPIP per minute.