Supporting Information

The Cytochrome P450-Catalyzed Oxidative Rearrangement in the Final Step of Pentalenolactone Biosynthesis: Substrate Structure Determines Mechanism

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	PntM	PntM + 2	PntM + 7	PntM + 1
PDB entry	5L1R	5L1O	5L1Q	5L1P
Data collection ¹				
Space group	P 2 ₁ 2 ₁ 2			
Cell dimensions				
a, b, c (Å)	44.46, 164.59, 83.30	44.75, 164.48, 83.29	44.53, 164.37, 82.04	44.33, 163.76, 81.34
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å) ²	45.65 - 2.00 (2.05 -	45.79 - 2.03 (2.08 -	45.56 - 2.03 (2.08 -	45.33 - 2.28 (2.36 -
	2.00)	2.03)	2.03)	2.28)
CC 1/2	0.999 (0.935)	0.996 (0.893)	0.992 (0.624)	0.983 (0.647)
Ι/γΙ	20.2 (6.0)	15.5 (4.7)	8.7 (2.2)	6.4 (2.0)
Completeness (%)	99.1 (90.9)	98.8 (91.5)	99.8 (97.2)	100.0 (100.0)
Redundancy	6.9 (4.6)	7.1 (5.4)	6.9 (5.0)	7.2 (7.2)
Refinement				
Resolution (Å)	45.65 – 2.00 (2.071	43.18 – 2.03 (2.103	45.56 – 2.03 (2.103	42.79 – 2.28 (2.361
	-2.00)	-2.03)	-2.03)	-2.28)
No. reflections	41436 (3736)	40100 (3663)	39781 (3818)	27902 (2748)
R _{work} / R _{free}	0.1441 (0.1716)/	0.1430 (0.1711)/	0.1598 (0.2261)	0.1776 (0.2372)
	0.1731 (0.2201)	0.1776 (0.2175)	/0.1903 (0.2710)	/0.2221 (0.3191)
No. atoms	3790	3848	3663	3476
macromolecules	3117	3146	3119	3130
Ligands	54	63	63	63
ions/water	619	639	481	283
B-factors	20.32	20.03	22.45	27.13
macromolecules	17.56	17.55	20.69	26.55
Ligands	16.40	10.64	13.79	20.13
lons/water	34.55	33.16	35.01	35.10
R.m.s deviation				
Bond lengths (Å)	0.007	0.008	0.008	0.008
Bond angles (°)	1.24	1.20	1.20	1.28
Ramachandran plot				
Favored (%)	98	99	98	97
Outliers (%)	0	0	0	0

Table S1. Data collection and refinement statistics for substrate-free PntM and PntM complexed with **2**, **7**, or **1**.

1. One crystal was used for this dataset. 2. Highest resolution shell is shown in parenthesis.

	PntM-F232L	PntM-M77S	PntM-M81A	PntM-M81C	PntM-M81C-BME
PDB entry	5L1S	5L1T	5L1U	5L1V	5L1W
Data collection ¹					
Space group	P 2 ₁ 2 ₁ 2				
Cell dimensions					
a, b, c (Å)	44.60, 164.15,	44.68, 164.60,	44.62, 163.86,	44.56, 164.23,	44.57, 163.23,
	82.54	83.51	82.22	82.97	83.29
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å) ²	45.60 - 2.08	45.85 – 2.08	45.50 - 2.07	45.69 - 2.12	44.57 - 2.06 (2.12
	(2.13 - 2.08)	(2.14 – 2.08)	(2.13 - 2.07)	(2.18 - 2.12)	- 2.06)
CC 1/2	0.993 (0.724)	0.982 (0.618)	0.995 (0.809)	0.987 (0.702)	0.995 (0.821)
[]γ]	11.0 (2.9)	9.7 (2.1)	12.2 (3.5)	7.5 (2.2)	13.6 (4.4)
Completeness	98.6 (92.1)	98.2 (90.4)	98.7 (91.3)	100.0 (100.0)	97.2 (92.0)
(%)					
Redundancy	7.1 (4.8)	7.0 (4.8)	7.1 (4.7)	7.2 (7.1)	7.3 (6.5)
Refinement					
Resolution (Å)	41.27 – 2.076	41.75 – 2.082	43.06 - 2.074	43.01 – 2.12	41.64 – 2.06
	(2.15 -2.076)	(2.157 -2.081)	(2.148 -2.074)	(2.196 -2.12)	(2.134 -2.06)
No. reflections	36872 (3507)	37605 (3436)	36997 (3426)	35477 (3450)	37401 (3510)
R _{work} / R _{free}	0.1540 (0.2072) /	0.1599 (0.2417) /	0.1541 (0.1972) /	0.1604 (0.2271) /	0.1613 (0.1953) /
	0.1924 (0.2644)	0.1951 (0.2796)	0.1845 (0.2416)	0.1956 (0.2731)	0.1855 (0.2203)
No. atoms	3689	3711	3700	3733	3822
macromolecules	3143	3136	3146	3119	3128
ligands	63	63	63	63	63
ions/water	483	502	491	551	631
B-factors	21.14	20.77	19.42	22.93	18.89
macromolecules	19.79	19.36	17.83	20.90	16.49
ligands	12.05	12.82	10.90	15.11	10.62
ions/water	31.13	30.38	30.70	35.30	31.61
R.m.s deviations					
Bond lengths (Å)	0.009	0.009	0.008	0.008	0.006
Bond angles (°)	1.24	1.24	1.24	1.23	1.15
Ramachandran					
plot					
Favored (%)	98	98	98	98	98
Outliers (%)	0	0	0	0	0

 Table S2. Data collection and refinement statistics PntM mutants complexed with 2.

1. One crystal was used for this dataset. 2. Highest resolution shell is shown in parenthesis.

	Forward Primer (5' - 3')	Reverse Primer $(5' - 3')$
50000	GCC GTG CTG TTG GGC GGC	GGT CGT TTC ATA GCC GCC
F232G	TAT GAA ACG ACC	CAA CAG CAC GGC
F000A	GCC GTG CTG TTG GCC GGC	GGT CGT TTC ATA GCC GGC
FZ3ZA	TAT GAA ACG ACC	CAA CAG CAC GGC
	GCC GTG CTG TTG <u>TAC</u> GGC	GGT CGT TTC ATA GCC GTA
FZ3Z I	TAT GAA ACG ACC	CAA CAG CAC GGC
Faaa	GCC GTG CTG TTG <u>CTG</u> GGC	GGT CGT TTC ATA GCC <u>CAG</u>
FZJZL	TAT GAA ACG ACC	CAA CAG CAC GGC
EJJJU	GCC GTG CTG TTG GAT GGC	GGT CGT TTC ATA GCC <u>ATC</u> CAA
F23211	TAT GAA ACG ACC	CAG CAC GGC
	GTA GTG GCT CTG <u>GCC</u> GCC	GTC ATC ACC GGC GGC CAG
MOTA	GGT GAT GAC	AGC CAC CAT
M91C	GTA GTG GCT CTG <u>TGC</u> GCC	GTC ATC ACC GGC <u>GCA</u> CAG
	GGT GAT GAC	AGC CAC CAT
M779	GCT GCG CGT GCT TTC AGC	GGC CAT CAG AGC CAC TCG
11/13	GTG GCT CTG ATG GCC	GAA AGC ACG CGC AGC

 Table S3. Primer pairs for site-directed mutagenesis

Table S4. Predicted MW and HR-LC-ESI-MS	observed $M_{\rm D}$ of PntM and mutants
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Protein	Observed <i>M</i> _D (MH ⁺)	Calculated MW
PntM	44445.27	44444.87
F232A	44369.80	44368.77
F232G	44355.86	44354.74
F232L	44412.67	44410.85
F232H	44436.51	44434.83
M81C	44418.39	44416.18
M81C-BME	44494.37	44492.31
M77S	44401.20	44400.75

The calculated MW is based on protein with an N-terminal Gly-Ser-His remaining after thrombin cleavage of the N-terminal His₆-tag.



Figure S1. SDS-PAGE analysis of PntM and mutants after removal of N-terminal His_6 -tag.



Figure S2. 6,7-Dihydropentalenolactone F methyl ester (**7-Me**). a) ¹H NMR (400 MHz, CDCl₃); b) ¹³C NMR (100 MHz, CDCl₃).



Figure S3. 6,7-Dihydropentalenolactone F methyl ester (7-Me), EI-MS



Figure S4. Titration of PntM, UV difference spectra. a) Pentalenolactone F (2); b) 6,7-Dihydropentalenolactone F (7).



Figure S5. Titration of PntM mutants with pentalenolactone F (2), UV difference spectra.



Figure S6. Plots of UV difference (A_{390}) vs concentration of **2** for wild-type PntM and mutants.



Figure S7. GC-MS calibration plot for pentalenolactone methyl ester (1-Me).



Figure S8. Steady-state kinetic analysis of conversion of 2 to 1 catalyzed by PntM and mutants.

_		55	74	77 81		104
Α	PntM-p450	DRRERESNPN	PQPSAKSAAR	AFMVALMAGD	DHETEPARHA	QMRSLLIPRE
	WP 061917787	DRRESESNPN	POPSAKSAAR		DHETEAVRHA	RMRALLVPRE
	WP 014182237	DRRLGLSNPN	PEOSAKSAAR		DYDTEATRHA	OMRALLVPRE
	WP 044385543	DRRLALSNPY	PERTIKSAAR		DHHTEAPRHA	OMRALLVPRE
	WP 029385653	DRRLALSNPY	PERTIKSAAR			
	WP_031092484		PKPSAKSAAR			
	DENM STREY					
	WP 028428038		RERAAOSAAR			
	WP_020420030					
	WF_002770033					
	WP_019336326					
	WP_055532571					
	WP_055701367					
	VVP_025355028	DERLGRSHPK	PERAARVINS	ATLGG - ASGD		
	VVP_043718171	DPREGRSHPN	PQEAARTINS			
	KVVX01965	DRRLGRSHPD	PDQAARFSNS	AFLGG - PTGN		RERREMIRSE
	KVVX00091	DRRLGRSHPD	PDQAARESNS	AFLGG - PTGN	Y - I IEEEDHA	RERREMIRSE
	WP_010239567	DDRLGRSHPE	PERAARISGS	MVFGG - PSGD	SPETERVNHT	QMRILLAPAF
	WP_060573398	DDRLGRSHPE	PERAARISGS		SPETERVNHI	QMRILLAPAF
	WP_055507803	DDRLGRSHPD	PPSAARTGDS	ALFGG - PVGD	F - DTERVDHA	
	WP_030505981	DDREGRSHPT	PETAARLSNN	ALLGG - PTGE	- YAKERSVHK	EMRRLLTPAF
	WP_013132979	DPREGRSHPV	PEDAPRLSNS	ALIGG - PVGD		KLRRLLAPAF
		206		232	240	253
в	PntM-p450					
в	PntM-p450 WP 061917787		- GALSDADIA - GALSDAFIA	232 HLGNAVLLFG QLGNAVLLFG	240 YETTIVRIDL YETTIVRIDL	253 GTLLLRNPV GTLLLRNPD
в	PntM-p450 WP_061917787 WP_014182237	206 SELIAEKD - SELIAAED - SELIAAED -	- GALSDADIA - GALSDADIA - SALSDADIA	232 H L G N A V L L F G Q L G N A V L L F G H L G N A V L L F G	240 YETTIVRIDL YETTIVRIDL YETTIVRIDL	253 GTLLLLRNPV GTLLLLRNPD GTLLLLRNPA
В	PntM-p450 WP_061917787 WP_014182237 WP_044385543	206 SELIAEKD - SELIAAED - SELIAAED - SELIAHKD -	- GALSDADIA - GALSDADIA - SALSDADIA - HTLSDAFIA	232 H L G N A V L L F G Q L G N A V L L F G H L G N A V L L F G H L G N A V L L F G	240 YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL	253 GTLLLLRNPV GTLLLLRNPD GTLLLLRNPA
В	PntM-p450 WP_061917787 WP_014182237 WP_044385543 WP_029385653	206 SELIAEKD - SELIAAED - SELIAAHKD - SELIADKD - SELIADKD -	- GALSDADIA - GALSDADIA - SALSDADIA - HTLSDATA	232 H L G N A V L L F G Q L G N A V L L F G H L G N A V L L F G H L G N A V L L F G	240 YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL	253 GTLLLRNPV GTLLLRNPA GTLLLRNPA GTLLLRNPA GTLLLRNPA
В	PntM-p450 WP_061917787 WP_014182237 WP_044385543 WP_029385653 WP_031092484	206 SELIAEKD - SELIAAED - SELIAAKD - SELIAEKD - SELIAEKD - SELIAEKD -	- GALSDADIA - GALSDADIA - SALSDADIA - HILSDAEIA - HALSDAEIA	232 H G NAVI FG Q G NAVI FG H G NAVI FG H G NAVI FG H G NAVI FG	240 YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL	253 GTLLLRNPV GTLLLRNPD GTLLLRNPA GTLLLRNPA GTLLLRNPA
В	PntM-p450 WP_061917787 WP_014182237 WP_044385543 WP_029385653 WP_031092484 PENM_STREX	206 SELIAE SELIAE <th>- GALSDADIA - GALSDADIA - SALSDADIA - HTLSDACIA - HTLSDACIA - GTLSDACIA</th> <th>232 H G NAV L FG Q G NAV L FG H G NAV L FG H G NAV L FG G G NAV L FG H G NAV L FG</th> <th>240 YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDM YETTIVRIDM</th> <th>253 GTLLLRNPU GTLLLRNPD GTLLLRNPA GTLLLRNPA GTLLLRNPA GTLLLRNPA GVLLLRNPA</th>	- GALSDADIA - GALSDADIA - SALSDADIA - HTLSDACIA - HTLSDACIA - GTLSDACIA	232 H G NAV L FG Q G NAV L FG H G NAV L FG H G NAV L FG G G NAV L FG H G NAV L FG	240 YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDM YETTIVRIDM	253 GTLLLRNPU GTLLLRNPD GTLLLRNPA GTLLLRNPA GTLLLRNPA GTLLLRNPA GVLLLRNPA
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В	PntM-p450 WP_061917787 WP_014182237 WP_044385543 WP_029385653 WP_031092484 PENM_STREX WP_028428038 WP_062770835	206 SELIAECD SELIAECD SELIAECD SELIAECD SELIAECD SELIAECD SELIAECD SELIAECD SELIAECD	- GA SDADA - GA SDADA - SA SDADA - HT SDA - HT SDA - HT SDA - A - GT SDA - A - GA SDA - A - GA SDA - A - GA SDA - A	232 H G NAVILFG Q G NAVILFG H G NAVILFG H G NAVILFG Q G NAVILFG H G C AVILFG H G NAVILFG G G AVILFG	240 YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDL YETTIVRIDM YETTIVRIDM YETTIVRIDL YETTIVRIDL	253 GTLLLRNPM GTLLLRNPA GTLLLRNPA GTLLLRNPA GTLLLRNPA GVLLRNPA GVLLRNPA GTLLLRNPA GTLLLRNPA
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Figure S9. BLAST comparison of selected regions of PntM with top 20 matches. A) D55-F104, including key active site residues R74, M77, and M81. B) L206-V253, including key active site residues F232, T236, and R240









Figure S10. Comparison of the structures of PntM with bound ligands. **A**, Alignment and comparison of the overview structures of PntM with bound bicine and PntM with bound substrate **2**. PntM with bound bicine is shown in light pink and PntM with bound **2** is shown in green, heme is shown as, heme Fe⁺³ is shown as a sphere and ligands are shown as sticks. **B**, comparison of the active site regions of PntM with bound bicine and PntM with bound 2. The relevant residues and water molecules of PntM with bound bicine are shown in light pink, while the relevant residues and water molecules of PntM with bound bicine are shown in green. **C**, comparison of the overview structures of PntM with bound product **1** (shown in slate) and PntM with bound substrate **2**. **D**, comparison of the overview structures of PntM with bound **1** and PntM with bound **2**. **E**, comparison of the overview structures of PntM with bound substrate **2**. **F**, comparison of the active site region of PntM with bound **7** and PntM with bound **2**.













Figure S11. Structures of PntM mutants. **A**, overview structure of F232L with bound substrate **2**. Heme is shown as sticks with carbon atoms in green, heme Fe³⁺ is shown as a green sphere. The mutated residue L232 is shown as sticks with carbon atoms in green. **B**, comparison of the active site region of F232L with bound **2** and PntM with bound **2**. The relevant residues and water molecules of F232L with bound **2** are shown in green, while those for PntM with bound **2** are shown in white blue. The mutated residue in F232L is labeled as L232'. **C**, overview structure of M77S with bound substrate **2**. The mutated residue S77 is shown as sticks with carbon atoms in green. **D**, comparison of the active site region of M77S with bound **2**. The mutated residue in M77S is labeled as S77'. **E**, overview showing the structure of M81A with bound

substrate 2. The mutated residue A81 is shown as sticks with carbon atoms in green. **F**, comparison of the active site region of M81A with bound 2 and PntM with bound 2. Mutated residue in M81A is labeled as A81'. **G**, overview showing the structure of M81C with bound substrate 2. The mutated residue C81 is shown as sticks with carbon atoms in green. **H**, comparison of the active site region of M81A with bound 2 and PntM with bound 2. The mutated residue in M81C is labeled as C81'. **I**, overview showing the structure of M81C-BME with bound substrate 2. The mutated residue as C81'. **I**, overview showing the structure of M81C-BME with bound substrate 2. The mutated residue C-BME81 is shown as sticks with carbon atoms in green. **J**, comparison of the active site region of M81C-BME with bound 2 and PntM with bound 2. The mutated residue in M81C-BME with bound 2 and PntM with bound 2. The mutated residue in M81C-BME with bound 2 and PntM with bound 2. The mutated residue in M81C-BME is labeled as C81'. **I** and PntM with bound 2 and PntM with bound 2. The mutated residue C-BME81 is shown as sticks with carbon atoms in green. **J**, comparison of the active site region of M81C-BME with bound 2 and PntM with bound 2. The mutated residue in M81C-BME is labeled as C-BME81'.