Supporting Information

S1. Experimental Procedures

S1.1 General

General reagents, chemicals and HPLC grade solvents were purchased from Alfa-Aesar, Fisher Scientific, Fluorochem and Sigma-Aldrich, UK. Vitamins D_2 and D_3 were from Enzo Life Sciences and Merck Life Science, UK. Media components, kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) were supplied by Melford Laboratories, UK. Hen egg white lysozyme was from Sigma-Aldrich. $NADP^+$ monosodium salt was from Prozomix, UK, and glucose dehydrogenase (GDH) was from Codexis, California, USA. Oligonucleotides were supplied by Eurofins Genetic Service, UK. Vitamin D oxidation products were purified by flash silica gel column chromatography using Geduran Silica 60, 40–63 μm (Fisher Scientific, UK) and eluting with increasingly polar mixtures of petroleum ether (bp 40–60 °C) and ethyl acetate. Analytical thin-layer chromatography was performed with petroleum ether/ethyl acetate solvent systems and bands were visualised under ultraviolet light. ^1H, ^{13}C, COSY, HSQC, HMBC and NOESY NMR spectra were acquired on Bruker AVIII-500 (500/125 MHz), Bruker AVIII-400 (400/100 MHz), or Bruker AV-400 (400/100 MHz) spectrometers. High resolution mass spectra (HRMS) were obtained on a Bruker microTOF spectrometer. UV-vis spectra were acquired on a Varian CARY50 spectrophotometer using 1 cm pathlength quartz cuvettes. Reverse-phase high performance liquid chromatography (HPLC) analyses were carried out with a C18 column (5 μm, 100 mm × 4.6 mm, Kinetex, UK) on a Shimadzu Prominence system equipped with a photodiode array detector and an autosampler. The sample injection volume was 10 μL and the column oven was set at 40 °C. Analyses of the oxidation product profiles of vitamins D_2 and D_3 were conducted with a mixture of 95% acetonitrile and 5% water at a flow rate of 1 mL/min for 20 min. Retention times for compounds were, vitamin D_3, 8.393 min; 25-hydroxy-vitamin D_3 (**1**), 2.338 min; 23,25-dihydroxy-vitamin D_3 (**2**),

1.607 min; vitamin D_2, 7.842 min; 25-hydroxy-vitamin D_2 (**3**), 2.695 min; 24,25-dihydroxy-vitamin D_2 (**4**), 1.964 min.

S1.2 Enzymes and molecular biology

Genes encoding CYP102A1 enzymes were cloned in the pET28+ vector by NcoI and BamHI restriction sites.[33] Site-directed mutagenesis was carried out by standard PCR-based protocols using KOD Hot Start DNA Polymerase toolkit from Sigma-Aldrich, UK. The presence of the target mutation(s) was confirmed by DNA sequencing. The relevant plasmid was transformed into chemically competent *E. coli* BL21 (DE3) for enzyme production and subsequent purification as described previously.[34] P450 enzyme concentrations were determined by the $Fe^{II}(CO)$ difference spectrum method of Omura and Sato using $\varepsilon_{450–490nm} = 91000\ M^{-1}\ cm^{-1}$.[35]

S1.3 Activity screening and preparative scale reactions

The vitamin D_2 and D_3 substrates were dissolved in methanol and added as a stock solution at 200 mM concentration. Enzymatic activity screening was carried out in 24-well plates. The 0.5 mL reaction mixture in each well (200 mM phosphate buffer, pH 7.9) contained 2 mM vitamin D substrate, 2 μM CYP102A1 variant, 4 U/mL GDH (4 U/μL stock) and 100 mM glucose (2 M stock). $NADP^+$ monosodium salt (40 μM, 40 mM stock) was added to initiate the reaction. Plates were shaken at 20 °C for 16 h at 120 rpm. Each reaction was then extracted with 0.3 mL ethyl acetate. After centrifugation at 14300 ×*g* to separate the phases, the organic extracts were analysed by HPLC.

Preparative scale reactions (50–1000 mL) with selected enzymes for the characterisation of vitamin D metabolites were carried out for 6–16 h under the same conditions as screening scale reactions. Progress of the reaction was monitored by analysis of organic extracts of 0.5 mL aliquots. The reaction mixture was then thrice extracted, each time with an equal volume of ethyl acetate. The combined organic extracts were washed with water and brine, dried with $Na_2(SO_4)$ and the solvent was removed by rotary evaporation. Products were purified by silica gel column chromatography.

S1.4 Molecular dynamics (MD) simulations and substrate docking

MD simulations were performed on the heme domain of CYP102A1 variants. Mutations were introduced to the crystal structure of the heme domain of the wild type enzyme with *N*-palmitoylglycine (NPG) bound within the active site (PDB code: 1jpz)[36] using Pymol. The bound molecule of NPG was removed. MD simulations were carried out within the GROMACS 2018.6 suite.[37] The protein was prepared using the Amber 99SB*-ILDN force field[38] with TIP3P water.[39] The heme was simulated in the compound I state, using parameters from Shahrokh, *et al*.[40] The protein was placed into the centre of an octahedral box, with a minimum distance of 10 nm to any box edge, followed by solvation with approximately 18000 water molecules and charge was neutralised by the addition of Na^+ ions. Steepest-descent energy minimisation was performed until the maximum force was <500 kJ mol^{-1} nm^{-2}. The system was modelled by periodic boundary conditions. Electrostatic interactions were treated by the particle mesh Ewald method[41] while bond lengths involving hydrogen atoms were constrained with the LINCS algorithm.[37b] Short-range non-bonded interactions were calculated with a 1 nm cut-off and a timestep of 2 fs. For equilibration steps, all protein backbone Cα atoms were restrained with a positional restraint force constant of 1000

kJ mol^{-1} nm^{-2}. A NVT equilibration step was performed at 298 K for 100 ps, using the modified Berendsen thermostat.[42] This was followed by an NPT equilibration step for 1250 ps utilizing the Berendsen barostat with a time constant of 1 ps.[43] All positional restraints were then removed, and 100 ns of production MD was performed in quadruplicate using the Parrinello-Rahman barostat with a time constant of 5 ps.[44] Structures were recorded every 10 ps. Stability of the simulations was confirmed by monitoring the RMSD of the backbone Cα atoms before the individual trajectories were clustered using the Daura algorithm with a cut-off of 1.2 Å. The three most populated clusters of each replica, with a population cut-off of 5% of all trajectories, were used as receptor structures for docking studies, which were performed in Autodock Vina. All water molecules were removed from the structure prior to the docking calculation and both receptor and substrate were treated as rigid entities. The docking site was defined as a 30 × 30 × 30 Å box centred on the ferryl oxygen, and poses were ranked using the Autodock Vina scoring function.

S2. Lists of $P450_{BM3}$ (CYP102A1) variants

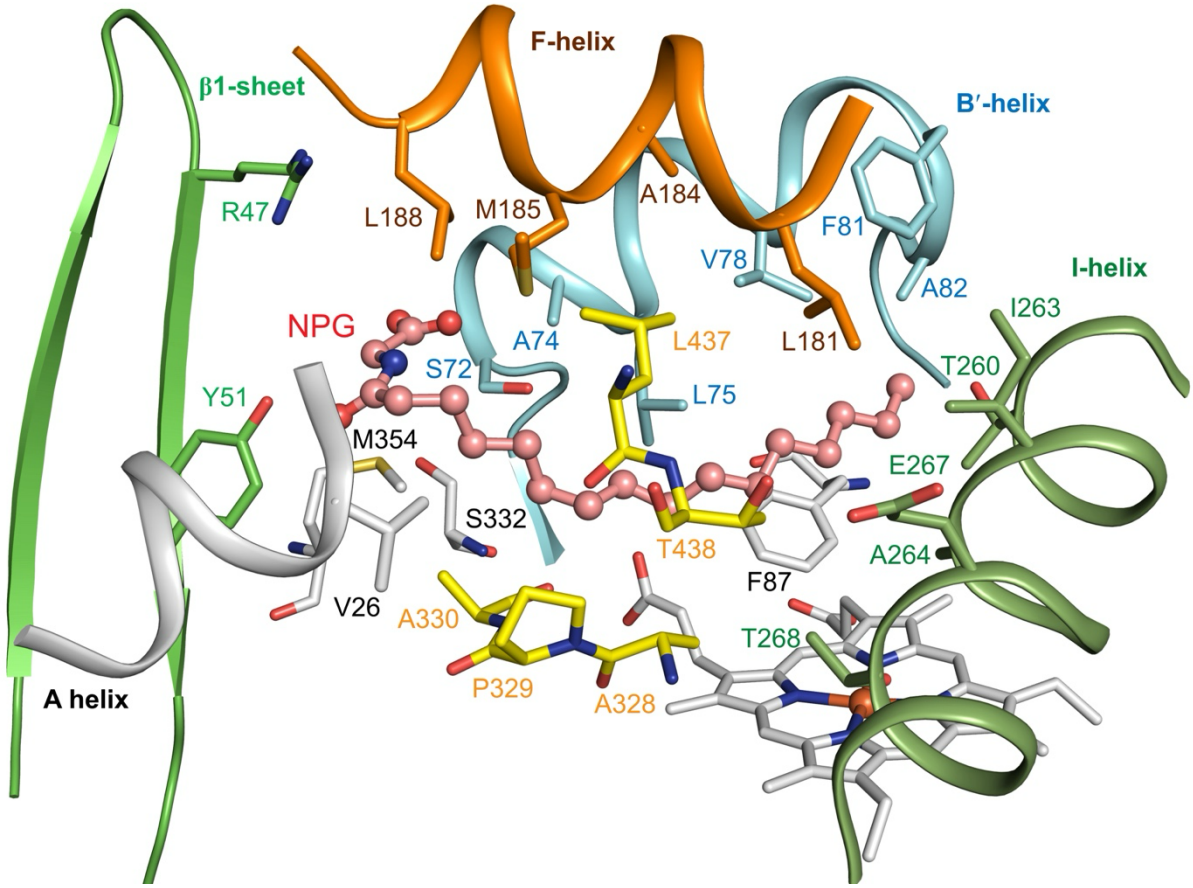


Figure S1. The active site structure of the heme domain of wild-type $P450_{BM3}$ with bound *N*-palmitoylglycine (NPG), highlighting the substrate-binding residues targeted for mutagenesis to generate the screening library of variants (pdb code: 1jpz).[34] Secondary structural elements and residues within them are highlighted by different colours.

Table S1. The initial screening library of $P450_{BM3}$ variants.

GV = A74G/F87V; GVQ = A74G/F87V/L188Q; K19 = H171L/Q307H/N319Y; R19 = R47L/Y51F/H171L/Q307H/N319Y.

Variant	Mutations	Variant	Mutations
M1	GV/A184I/A264G	**M37**	R19/F87A/A184I/I259G/I263G
M2	GV/A184I/A264G/A328G	**M38**	R19/F87A/A184I/I263G/A264G
M3	GV/A184I/A328G	**M39**	R19/F87A/A184I/I263G/A264G/A328G
M4	GV/A184I/A328G/T260G	**M40**	R19/F87A/A184I/I263G/A328G
M5	GV/A184I/I263G	**M41**	R19/F87A/A184I/I263W
M6	GV/A184I/I263G/A264G	**M42**	R19/F87A/A184I/L262G
M7	GV/A184I/I263G/A264G/A328G	**M43**	R19/F87A/A184I/S270G
M8	GV/A184I/I263G/A328G	**M44**	R19/F87A/A184I/T260G
M9	GV/A184I/I263W	**M45**	R19/F87A/A184I/T269G
M10	GV/A184I/T260G	**M46**	R19/F87A/A264G
M11	GVQ/A264G/P329G/A330P	**M47**	R19/F87A/A328G
M12	GVQ/A264G/P329G/A330W	**M48**	R19/F87A/A328G/A264G
M13	GVQ/A328G	**M49**	R19/F87A/A328G/I259G/I263G
M14	GVQ/I263G/A264G	**M50**	R19/F87A/A328G/I263G/A264G
M15	GVQ/I263G/A264G/A328G	**M51**	R19/F87A/A328G/I263W
M16	GVQ/T260G	**M52**	R19/F87A/A328G/L262G
M17	K19/F87V/A264G	**M53**	R19/F87A/A328G/P329G/A330G
M18	K19/F87V/A184I	**M54**	R19/F87A/A328G/P329G/A330G/T260G
M19	K19/F87V/I263G	**M55**	R19/F87A/A328G/S270G
M20	K19/A82M/F87A/A264G	**M56**	R19/F87A/A328G/T260G
M21	K19/A82M/F87A/A264G/A328G	**M57**	R19/F87A/A328G/T269G
M22	K19/A82M/F87A/A328G/T260G	**M58**	R19/F87A/F81W/A328G/T260G
M23	K19/A82M/F87A/A330W	**M59**	R19/F87A/G265GG
M24	K19/A82M/F87A/F261G	**M60**	R19/F87A/I259G/I263G
M25	K19/A82M/F87A/I263G/A264G	**M61**	R19/F87A/I263G/A264G
M26	K19/A82M/F87A/I263G/A264G/A328G	**M62**	R19/F87A/I263G/A328G
M27	K19/A82M/F87A/I263G/A264G/A330W	**M63**	R19/F87A/I263G/P329G/A330P
M28	K19/A82M/F87A/I263G/A328G	**M64**	R19/F87A/I263G/P329G/A330W
M29	K19/A82M/F87A/I263G/A330W	**M65**	R19/F87A/I263W
M30	K19/A82M/F87A/I263W	**M66**	R19/F87A/L262G
M31	K19/A82M/F87A/L262G	**M67**	R19/F87A/P329G/A330P
M32	K19/A82M/F87A/S270G	**M68**	R19/F87A/P329G/A330W
M33	K19/A82M/F87A/T260G	**M69**	R19/F87A/P329G/A330W/T260G
M34	R19/F87A/A184I/A264G	**M70**	R19/F87A/S270G
M35	R19/F87A/A184I/A264G/A328G	**M71**	R19/F87A/T260G
M36	R19/F87A/A184I/A328G/T260G	**M72**	R19/F87A/T269G

Table S2. $P450_{BM3}$ variants by rational design.

K19 = H171L/Q307H/N319Y; R19 = R47L/Y51F/H171L/Q307H/N319Y.

Variant	Mutations
M73	R19/F87A/T268S
M74	R19/F87A/T268S/A328I
M75	K19/F87A/A82M/T260G
M76	K19/F87A/A82M/A184I/T260G
M77	R19/F87A/A82M/T260G
M78	K19/F87A/A82M/T260G/T268S
M79	R19/F87A/A82M/T260G/A328G
M80	R19/F87A/A82M/A184I/T260G
M81	R19/F87I/A82M/A184I/T260G
M82	R19/F87V/A82M/A184I/T260G
M83	R19/F87A/A82M/A184I/A328G
M84	R19/F87A/A82M/A184I/T260G/S72L
M85	R19/F87A/A82M/A184I/T260G/A328G
M86	R19/F87A/A82M/A184I/T260G/A328G/A330I
M87	R19/F87A/A82M/A184I/T260G/A328G/A330V
M88	R19/F87A/A82M/A184I/T260G/A328G/A330L
M89	R19/F87A/A82M/A184I/T260G/A328G/S72F
M90	R19/F87A/A82M/A184I/T260G/A328G/S72W

Table S3. Variants designed by docking-guided mutagenesis.

K19 = H171L/Q307H/N319Y; R19 = R47L/Y51F/H171L/Q307H/N319Y.

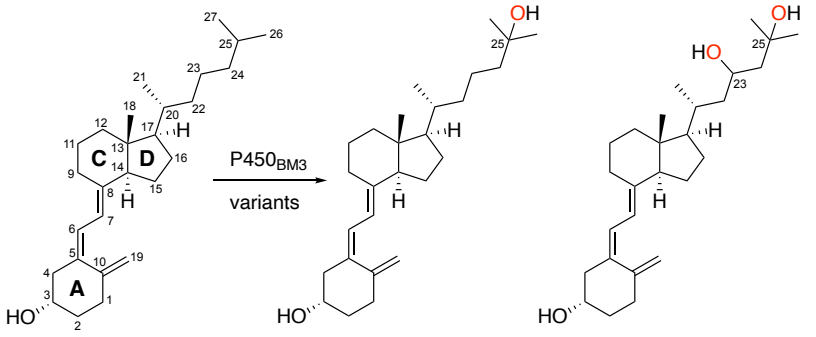
Variant	Mutations
M91	F87A
M92	F87A/A82M
M93	F87A/A82M/A184I
M94	F87A/A82M/T260G
M95	F87A/A82M/A184I/E435M
M96	F87A/A82M/A184I/T260G
M97	F87A/A82M/A184I/T260G/E435M
M98	F87A/A82M/A184I/T260G/L150P/M354G/E435M
M99	F87A/A82M/A184I/T260G/M354L/E435M
M100	F87A/A82M/A184I/T260G/S332A
M101	F87A/A82M/E435M
M102	F87A/A82M/S72A
M103	F87A/A82M/S72A/A184F/T260G/E435M
M104	F87A/A82M/S72A/A184I/T260G/E435T
M105	F87A/A82M/A184I/S72A
M106	F87A/A82M/S72A/A184I/E435M
M107	F87A/A82M/S72A/A184I/N239H/T260G/E435M
M108	F87A/A82M/S72A/A184I/T260A
M109	F87A/A82M/S72A/A184I/T260A/E435M
M110	F87A/A82M/S72A/A184I/T260G
M111	F87A/A82M/S72A/A184I/T260G/E435D
M112	F87A/A82M/S72A/A184I/T260G/E435H
M113	F87A/A82M/S72A/A184I/T260G/E435I
M114	F87A/A82M/S72A/A184I/T260G/E435I/V26H
M115	F87A/A82M/S72A/A184I/T260G/E435I/V26L
M116	F87A/A82M/S72A/A184I/T260G/E435I/V26M
M117	F87A/A82M/S72A/A184I/T260G/E435L
M118	F87A/A82M/S72A/A184I/T260G/E435M
M119	F87A/A82M/S72A/A184I/T260G/E435M/N319Y
M120	F87A/A82M/S72A/A184I/T260G/E435M/Q307H
M121	F87A/A82M/S72A/A184I/T260G/E435M/V26H
M122	F87A/A82M/S72A/A184I/T260G/E435M/V26M
M123	F87A/A82M/S72A/A184I/T260G/E435N
M124	F87A/A82M/S72A/A184I/T260G/E435Q
M125	F87A/A82M/S72A/A184I/T260G/E435R
M126	F87A/A82M/S72A/A184I/T260G/E435T/V26H
M127	F87A/A82M/S72A/A184I/T260G/E435T/V26L
M128	F87A/A82M/S72A/A184I/T260G/E435T/V26M
M129	F87A/A82M/S72A/A184I/T260G/E435W
M130	F87A/A82M/S72A/A184I/T260G/E435Y

Variant	Mutations
M131	F87A/A82M/S72A/A184I/T260G/H171L/E435M
M132	F87A/A82M/S72A/A184I/T260G/H171L/L181F/E435M
M133	F87A/A82M/S72A/A184I/T260G/H171L/N239H/E435M
M134	F87A/A82M/S72A/A184I/T260G/H171L/Q403P
M135	F87A/A82M/S72A/A184I/T260G/L181F/N239H/E435M
M136	F87A/A82M/S72A/A184I/T260G/Q403P/E435M
M137	F87A/A82M/S72A/A184I/T260G/V26L/E435M
M138	F87A/A82M/S72A/A184I/T260G/V26L/H171L
M139	F87A/A82M/S72A/A184I/T260G/V26L/H171L/E435M
M140	F87A/A82M/S72A/A184I/T260G/V26L/N239H/E435M
M141	F87A/A82M/S72A/A184I/T260G/V26L/Q403P
M142	F87A/A82M/S72A/A184I/T260G/V26L/Y51F
M143	F87A/A82M/S72A/A184I/T260G/Y51F/H171L
M144	F87A/A82M/S72A/A184I/T260G/Y51F/Q403P
M145	F87A/A82M/S72A/A184L/T260G/E435I
M146	F87A/A82M/S72A/A184M/T260G/E435I
M147	F87A/A82M/S72A/A184M/T260G/E435M
M148	F87A/A82M/S72A/A184V/T260G/E435I
M149	F87A/A82M/S72A/E435M
M150	F87A/A82M/S72A/L181F/A184G/T260G
M151	F87A/A82M/S72A/T260G
M152	F87A/A82M/S72A/T260G/E435M
M153	F87A/A82M/T260G/E435M
M154	F87A/A82M/V178F/T260G/E435M
M155	F87A/A82M/V178W/T260G/E435M
M156	F87A/S72A/A184I/T260G/E435M
M157	F87A/S72A/A184I/T260G/E435T
M158	F87I/A82M
M159	F87I/A82M/A184I/E435M
M160	F87I/A82M/A184I/T260G
M161	F87I/A82M/A184I/T260G/E435I/S72V
M162	F87I/A82M/A184I/T260G/E435I/S72W
M163	F87I/A82M/A184I/T260G/E435M
M164	F87I/A82M/A184I/T260G/E435M/S72V
M165	F87I/A82M/A184I/T260G/E435M/S72W
M166	F87I/A82M/E435M
M167	F87I/A82M/S72A
M168	F87I/A82M/S72A/A184I
M169	F87I/A82M/S72A/A184I/E435M
M170	F87I/A82M/S72A/A184I/N239H/T260G/E435I
M171	F87I/A82M/S72A/A184I/N239H/T260G/E435M
M172	F87I/A82M/S72A/A184I/T260G
M173	F87I/A82M/S72A/A184I/T260G/E435I

Variant	Mutations
M174	F87I/A82M/S72A/A184I/T260G/E435I/L75S
M175	F87I/A82M/S72A/A184I/T260G/E435I/M185T
M176	F87I/A82M/S72A/A184I/T260G/E435I/M354F
M177	F87I/A82M/S72A/A184I/T260G/E435M
M178	F87I/A82M/S72A/A184I/T260G/E435M/L188S
M179	F87I/A82M/S72A/A184I/T260G/E435M/L29A
M180	F87I/A82M/S72A/A184I/T260G/E435M/L29M
M181	F87I/A82M/S72A/A184I/T260G/E435M/L75S
M182	F87I/A82M/S72A/A184I/T260G/E435M/L75T
M183	F87I/A82M/S72A/A184I/T260G/E435M/M354F
M184	F87I/A82M/S72A/E435M
M185	F87I/A82M/S72A/T260G
M186	F87I/A82M/S72A/T260G/E435M
M187	F87I/A82M/S72A/V178F/T260G/E435I
M188	F87I/A82M/S72A/V178F/T260G/E435M
M189	F87I/A82M/S72A/V178W/T260G/E435I
M190	F87I/A82M/S72A/V178W/T260G/E435M
M191	F87I/A82M/T260G
M192	F87I/A82M/T260G/E435M
M193	F87S/A82M/S72A/A184I/T260G/E435M
M194	F87T/A82M/S72A/A184I/T260G/E435I
M195	F87T/A82M/S72A/A184I/T260G/E435M
M196	F87V/A82M/S72A/A184I/N239H/T260G/E435I
M197	F87V/A82M/S72A/A184I/N239H/T260G/E435M
M198	F87V/A82M/S72A/A184I/T260G/E435I
M199	F87V/A82M/S72A/A184I/T260G/E435M
M200	K69I/F87A/A82M/A184I/T260G/E435M
M201	K69R/F87A/A82M/A184I/T260G/E435M
M202	F87I/A82M/S72A/A184I/M185T/T260G/E435M/L29M
M203	L29M/S72A/L75S/A82M/F87T/A184I/T260G/E435I
M204	L29M/S72A/V78M/A82M/F87T/A184I/T260G/E435I
M205	R19/F87A/A82M/A184I/T260G/E435I
M206	R19/F87A/A82M/A184I/T260G/E435M
M207	S72A/A82M/F87T/A184I/M185T/T260G/E435M
M208	S72A/L75S/A82M/F87T/A184I/T260G/E435I
M209	S72A/V78M/A82M/F87T/A184I/T260G/E435I
M210	F87A/A82M/S72A/A184I/T260G/E435M/Y51F
M211	R19/F87I/A82M/A184I/T260G
M212	R19/F87T/A82M/A184I/T260G
M213	R19/F87V/A82M/A184I/T260G
M214	R19/F87A/A82M/A184I/T260G/E435T
M215	R19/F87A/A82M/A184I/T260G/S72A

S3. Vitamin D oxidation activity and selectivity

Table S4. Activity and product selectivity for the oxidation of vitamin D_3 (VD_3) catalysed by $P450_{BM3}$ variants (K19 = H171L/Q307H/N319Y. R19 = R47L/Y51F/H171L/Q307H/N319Y). The substrate-to-enzyme concentration ratio was 1000:1 (2 mM VD_3, 2 μM $P450_{BM3}$ enzyme). Conv. is the percentage of VD_3 converted to products. TON is the turnover number of the variant for the formation of 25(OH)VD_3 (**1**). All percentages are rounded to the nearest integer, while TON values are rounded to the nearest 5 or 10. All data are the average of at least two experiments which were repeated if the values differed by more than 3%. Up to four other products were observed but could not be characterised. The MS data for the two most common minor products indicated that one was a monooxygenation product (M+16) while the mass of the second (M+14) suggested a carbonyl derivative.



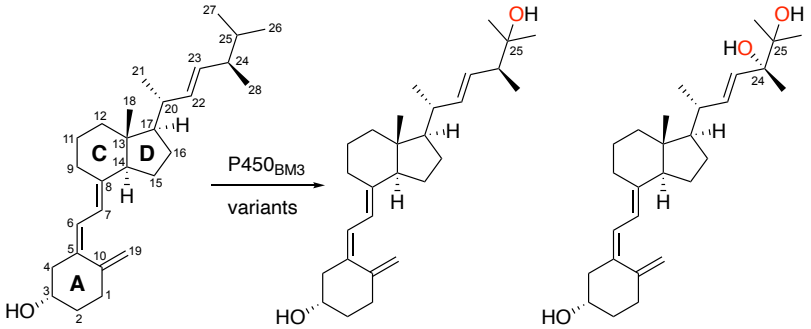
Variant	Mutations	1	2	Other	Conv.	TON
M73	R19/F87A/T268S	72%	9%	19%	39%	285
M74	R19/F87A/T268S/A328I	74%	3%	23%	36%	270
M78	K19/F87A/A82M/T260G/T268S	50%	3%	47%	18%	90
M79	R19/F87A/A82M/T260G/A328G	70%	3%	27%	37%	265
M80	R19/F87A/A82M/T260G/A184I	64%	3%	33%	32%	205
M85	R19/F87A/A82M/T260G/A184I/A328G	64%	8%	28%	30%	195
M86	R19/F87A/A82M/T260G/A184I/A328G/A330I	56%	9%	35%	30%	170
M87	R19/F87A/A82M/T260G/A184I/A328G/A330V	61%	8%	31%	25%	155
M88	R19/F87A/A82M/T260G/A184I/A328G/A330L	52%	10%	38%	29%	150
M89	R19/F87A/A82M/T260G/A184I/A328G/S72F	43%	6%	51%	19%	80
M90	R19/F87A/A82M/T260G/A184I/A328G/S72W	69%	11%	20%	54%	375
M91	F87A				<3%	
M92	F87A/A82M	72%		28%	28%	200
M93	F87A/A82M/A184I	30%		70%	26%	85
M94	F87A/A82M/T260G	38%	5%	57%	15%	60
M95	F87A/A82M/A184I/E435M	61%	21%	18%	53%	320
M96	F87A/A82M/A184I/T260G	75%	5%	20%	40%	300
M97	F87A/A82M/A184I/T260G/E435M	72%	5%	23%	50%	360
M98	F87A/A82M/A184I/T260G/L150P/M354G/E435M	19%	12%	69%	26%	50
M99	F87A/A82M/A184I/T260G/M354L/E435M	76%	4%	20%	71%	540
M100	F87A/A82M/A184I/T260G/S332A	57%	6%	37%	22%	130
M101	F87A/A82M/E435M	67%	20%	13%	66%	440
M102	F87A/A82M/S72A	65%	14%	21%	46%	300

Variant	Mutations	1	2	Other	Conv.	TON
M103	F87A/A82M/S72A/A184F/T260G/E435M	72%	5%	23%	42%	300
M104	F87A/A82M/S72A/A184I/T260G/E435T	70%	6%	24%	77%	540
M105	F87A/A82M/S72A/A184I	50%	32%	18%	52%	260
M106	F87A/A82M/S72A/A184I/E435M	71%	11%	18%	54%	390
M107	F87A/A82M/S72A/A184I/N239H/T260G/E435M	66%	28%	6%	81%	530
M108	F87A/A82M/S72A/A184I/T260A	38%	5%	57%	15%	55
M109	F87A/A82M/S72A/A184I/T260A/E435M	48%	9%	43%	20%	95
M110	F87A/A82M/S72A/A184I/T260G	63%	6%	31%	37%	235
M111	F87A/A82M/S72A/A184I/T260G/E435D	69%	7%	24%	35%	240
M112	F87A/A82M/S72A/A184I/T260G/E435H	66%	18%	16%	45%	295
M113	F87A/A82M/S72A/A184I/T260G/E435I	75%	14%	11%	83%	620
M114	F87A/A82M/S72A/A184I/T260G/E435I/V26H	30%	9%	61%	10%	30
M115	F87A/A82M/S72A/A184I/T260G/E435I/V26L	53%	5%	42%	14%	75
M116	F87A/A82M/S72A/A184I/T260G/E435I/V26M	67%	5%	28%	23%	155
M117	F87A/A82M/S72A/A184I/T260G/E435L	65%	5%	30%	29%	190
M118	F87A/A82M/S72A/A184I/T260G/E435M	73%	15%	12%	79%	570
M119	F87A/A82M/S72A/A184I/T260G/E435M/N319Y	75%	11%	14%	61%	460
M120	F87A/A82M/S72A/A184I/T260G/E435M/Q307H	15%	6%	79%	11%	15
M121	F87A/A82M/S72A/A184I/T260G/E435M/V26H	76%	5%	19%	47%	360
M122	F87A/A82M/S72A/A184I/T260G/E435M/V26M	76%	5%	19%	45%	345
M123	F87A/A82M/S72A/A184I/T260G/E435N	42%	9%	49%	17%	70
M124	F87A/A82M/S72A/A184I/T260G/E435Q	69%	12%	19%	49%	340
M125	F87A/A82M/S72A/A184I/T260G/E435R	49%	8%	43%	17%	85
M126	F87A/A82M/S72A/A184I/T260G/E435T/V26H	17%	6%	77%	9%	15
M127	F87A/A82M/S72A/A184I/T260G/E435T/V26L	9%	2%	89%	37%	35
M128	F87A/A82M/S72A/A184I/T260G/E435T/V26M	18%	10%	72%	10%	20
M129	F87A/A82M/S72A/A184I/T260G/E435W	57%	5%	38%	24%	140
M130	F87A/A82M/S72A/A184I/T260G/E435Y	57%	7%	36%	25%	140
M131	F87A/A82M/S72A/A184I/T260G/H171L/E435M	9%	4%	87%	10%	10
M132	F87A/A82M/S72A/A184I/T260G/H171L/L181F/E435M	15%	5%	80%	12%	20
M133	F87A/A82M/S72A/A184I/T260G/H171L/N239H/E435M	16%	6%	78%	12%	20
M134	F87A/A82M/S72A/A184I/T260G/H171L/Q403P	14%	6%	80%	9%	15
M135	F87A/A82M/S72A/A184I/T260G/L181F/N239H/E435M	43%	29%	28%	32%	140
M136	F87A/A82M/S72A/A184I/T260G/Q403P/E435M	23%	10%	67%	11%	25
M137	F87A/A82M/S72A/A184I/T260G/V26L/E435M	74%	5%	21%	46%	340
M138	F87A/A82M/S72A/A184I/T260G/V26L/H171L	15%	4%	81%	11%	15
M139	F87A/A82M/S72A/A184I/T260G/V26L/H171L/E435M	16%	6%	78%	12%	20
M140	F87A/A82M/S72A/A184I/T260G/V26L/N239H/E435M	77%	8%	15%	68%	530
M141	F87A/A82M/S72A/A184I/T260G/V26L/Q403P	9%	4%	87%	8%	5
M142	F87A/A82M/S72A/A184I/T260G/V26L/Y51F	69%	8%	23%	47%	325
M143	F87A/A82M/S72A/A184I/T260G/Y51F/H171L	14%	7%	79%	10%	15
M144	F87A/A82M/S72A/A184I/T260G/Y51F/Q403P	14%	8%	78%	10%	15
M145	F87A/A82M/S72A/A184L/T260G/E435I	69%	5%	26%	35%	245

Variant	Mutations	1	2	Other	Conv.	TON
M146	F87A/A82M/S72A/A184M/T260G/E435I	29%	9%	62%	12%	35
M147	F87A/A82M/S72A/A184M/T260G/E435M	64%	7%	29%	29%	185
M148	F87A/A82M/S72A/A184V/T260G/E435I	67%	5%	28%	34%	230
M149	F87A/A82M/S72A/E435M	45%	41%	14%	55%	245
M150	F87A/A82M/S72A/L181F/A184G/T260G	17%	6%	77%	11%	20
M151	F87A/A82M/S72A/T260G	47%	4%	49%	21%	100
M152	F87A/A82M/S72A/T260G/E435M	64%	10%	26%	42%	265
M153	F87A/A82M/T260G/E435M	73%	6%	21%	52%	380
M154	F87A/A82M/V178F/T260G/E435M	51%	5%	44%	31%	155
M155	F87A/A82M/V178W/T260G/E435M	51%	5%	44%	29%	145
M156	F87A/S72A/A184I/T260G/E435M	64%	7%	29%	24%	155
M157	F87A/S72A/A184I/T260G/E435T	34%	7%	59%	11%	35
M158	F87I/A82M	70%	7%	23%	32%	220
M159	F87I/A82M/A184I/E435M	61%	11%	28%	29%	175
M160	F87I/A82M/A184I/T260G	60%	6%	34%	25%	150
M161	F87I/A82M/A184I/T260G/E435I/S72V	85%		15%	54%	460
M162	F87I/A82M/A184I/T260G/E435I/S72W	62%	5%	33%	20%	125
M163	F87I/A82M/A184I/T260G/E435M	86%	4%	10%	71%	615
M164	F87I/A82M/A184I/T260G/E435M/S72V	81%	2%	17%	46%	375
M165	F87I/A82M/A184I/T260G/E435M/S72W	34%	5%	61%	11%	40
M166	F87I/A82M/E435M	71%	8%	21%	36%	255
M167	F87I/A82M/S72A	69%	9%	22%	34%	235
M168	F87I/A82M/S72A/A184I	34%	13%	53%	17%	60
M169	F87I/A82M/S72A/A184I/E435M	56%	14%	30%	25%	140
M170	F87I/A82M/S72A/A184I/N239H/T260G/E435I	82%	10%	8%	83%	680
M171	F87I/A82M/S72A/A184I/N239H/T260G/E435M	75%	13%	12%	79%	590
M172	F87I/A82M/S72A/A184I/T260G	80%	5%	15%	52%	420
M173	F87I/A82M/S72A/A184I/T260G/E435I	83%	8%	9%	83%	690
M174	F87I/A82M/S72A/A184I/T260G/E435I/L75S	84%		15%	71%	600
M175	F87I/A82M/S72A/A184I/T260G/E435I/M185T	79%		20%	61%	480
M176	F87I/A82M/S72A/A184I/T260G/E435I/M354F	13%	7%	80%	14%	20
M177	F87I/A82M/S72A/A184I/T260G/E435M	81%	10%	9%	83%	670
M178	F87I/A82M/S72A/A184I/T260G/E435M/L188S	76%	2%	22%	54%	410
M179	F87I/A82M/S72A/A184I/T260G/E435M/L29A	82%		17%	68%	555
M180	F87I/A82M/S72A/A184I/T260G/E435M/L29M	86%	4%	10%	80%	685
M181	F87I/A82M/S72A/A184I/T260G/E435M/L75S	14%	6%	80%	14%	20
M182	F87I/A82M/S72A/A184I/T260G/E435M/L75T	13%	6%	81%	15%	20
M183	F87I/A82M/S72A/A184I/T260G/E435M/M354F	85%	3%	12%	79%	670
M184	F87I/A82M/S72A/E435M	71%	16%	13%	48%	340
M185	F87I/A82M/S72A/T260G	67%	4%	29%	28%	185
M186	F87I/A82M/S72A/T260G/E435M	76%	18%	6%	75%	565
M187	F87I/A82M/S72A/V178F/T260G/E435I	70%	2%	28%	43%	295
M188	F87I/A82M/S72A/V178F/T260G/E435M	75%	6%	19%	55%	410

Variant	Mutations	1	2	Other	Conv.	TON
M189	F87I/A82M/S72A/V178W/T260G/E435I	80%	2%	18%	59%	475
M190	F87I/A82M/S72A/V178W/T260G/E435M	80%	8%	12%	75%	605
M191	F87I/A82M/T260G	36%	5%	59%	15%	55
M192	F87I/A82M/T260G/E435M	80%	6%	14%	50%	400
M193	F87S/A82M/S72A/A184I/T260G/E435M	76%	7%	17%	70%	525
M194	F87T/A82M/S72A/A184I/T260G/E435I	78%	9%	13%	70%	550
M195	F87T/A82M/S72A/A184I/T260G/E435M	74%	14%	12%	76%	560
M196	F87V/A82M/S72A/A184I/N239H/T260G/E435I	17%	9%	74%	10%	15
M197	F87V/A82M/S72A/A184I/N239H/T260G/E435M	50%	41%	9%	71%	360
M198	F87V/A82M/S72A/A184I/T260G/E435I	50%	42%	8%	83%	410
M199	F87V/A82M/S72A/A184I/T260G/E435M	49%	40%	11%	77%	380
M200	K69I/F87A/A82M/A184I/T260G/E435M	15%	8%	77%	17%	25
M201	K69R/F87A/A82M/A184I/T260G/E435M	20%	11%	69%	26%	50
M202	F87I/A82M/S72A/A184I/M185T/T260G/E435M/L29M	82%	4%	14%	53%	430
M203	L29M/S72A/L75S/A82M/F87T/A184I/T260G/E435I	22%	8%	70%	14%	30
M204	L29M/S72A/V78M/A82M/F87T/A184I/T260G/E435I	48%	6%	46%	20%	95
M205	R19/F87A/A82M/A184I/T260G/E435I	16%	6%	78%	8%	15
M206	R19/F87A/A82M/A184I/T260G/E435M	61%	5%	34%	24%	145
M207	F87T/A82M/S72A/A184I/T260G/E435M/M185T	69%	7%	24%	36%	250
M208	F87T/A82M/S72A/A184I/T260G/E435I/L75S	44%	6%	50%	19%	85
M209	F87T/A82M/S72A/A184I/T260G/E435I/V78M	78%	8%	14%	62%	480
M210	F87A/A82M/S72A/A184I/T260G/E435M/Y51F	76%	13%	11%	67%	510
M211	R19/F87I/A82M/A184I/T260G	34%	5%	61%	14%	50
M212	R19/F87T/A82M/A184I/T260G	68%	12%	20%	62%	420
M213	R19/F87V/A82M/A184I/T260G	66%	12%	22%	56%	365
M214	R19/F87A/A82M/A184I/T260G/E435T	64%	6%	30%	42%	270
M215	R19/F87A/A82M/A184I/T260G/S72A	66%	23%	11%	69%	455

Table S5. Activity and product selectivity for the oxidation of vitamin D_2 (VD_2) to 25-hydroxyVD_2 (**3**) and 24,25-dihydroxyVD_2 (**4**) catalysed by $P450_{BM3}$ variants (K19 = H171L/Q307H/N319Y. R19 = R47L/Y51F/H171L/Q307H/N319Y). The substrate-to-enzyme concentration ratio was 1000:1 (2 mM VD_2, 2 μM $P450_{BM3}$ enzyme). Conv. is the percentage of substrate converted to products. TON is the turnover number of the variant for the formation of **3**. All percentages are rounded to the nearest integer, while TON values are rounded to the nearest 5 or 10. All data are the average of at least two experiments which were repeated if the values differed by more than 3%. Up to three other products were observed but could not be characterised. The MS data (M+16) for the most common minor product indicated that it was a monooxygenation product.



Variant	Mutations	3	4	Other	Conv.	TON
M92	F87A/A82M	57%		43%	19%	105
M102	F87A/A82M/S72A	79%	5%	16%	25%	200
M101	F87A/A82M/E435M	92%	4%	4%	57%	530
M149	F87A/A82M/S72A/E435M	72%	17%	11%	55%	400
M96	F87A/A82M/A184I/T260G	62%		38%	17%	110
M110	F87A/A82M/A184I/T260G/S72A	59%	6%	35%	31%	185
M97	F87A/A82M/A184I/T260G/E435M	94%		6%	35%	330
M118	F87A/A82M/S72A/A184I/T260G/E435M	96%	2%	2%	71%	685
M113	F87A/A82M/S72A/A184I/T260G/E435I	96%	2%	2%	77%	750
M199	F87V/A82M/S72A/A184I/T260G/E435M	53%	23%	24%	75%	395
M198	F87V/A82M/S72A/A184I/T260G/E435I	50%	46%	4%	80%	400
M177	F87I/A82M/S72A/A184I/T260G/E435M	88%	12%		81%	720
M173	F87I/A82M/S72A/A184I/T260G/E435I	92%	4%	4%	88%	810
M73	R19/F87A/T268S	62%		38%	21%	130
M74	R19/F87A/T268S/A328I	66%	2%	32%	27%	180
M78	K19/F87A/A82M/T260G/T268S	53%	2%	45%	12%	60
M79	R19/F87A/A82M/T260G/A328G	79%		21%	33%	255
M80	R19/F87A/A82M/T260G/A184I	60%	4%	36%	17%	105
M85	R19/F87A/A82M/T260G/A184I/A328G	67%	2%	31%	21%	145
M86	R19/F87A/A82M/T260G/A184I/A328G/A330I	59%	3%	38%	16%	95
M87	R19/F87A/A82M/T260G/A184I/A328G/A330V	50%	3%	47%	14%	70
M88	R19/F87A/A82M/T260G/A184I/A328G/A330L	73%		27%	18%	135
M89	R19/F87A/A82M/T260G/A184I/A328G/S72F	51%		49%	17%	85

Variant	Mutations	3	4	Other	Conv.	TON
M90	R19/F87A/A82M/T260G/A184I/A328G/S72W	84%	3%	13%	49%	410
M91	F87A				<3%	
M93	F87A/A82M/A184I	55%		45%	10%	55
M94	F87A/A82M/T260G	59%		41%	4%	25
M95	F87A/A82M/A184I/E435M	82%		18%	37%	305
M98	F87A/A82M/A184I/T260G/L150P/M354G/E435M	3%		97%	6%	
M99	F87A/A82M/A184I/T260G/M354L/E435M	86%		14%	36%	315
M100	F87A/A82M/A184I/T260G/S332A	60%		39%	15%	90
M103	F87A/A82M/S72A/A184F/T260G/E435M	89%		10%	30%	270
M104	F87A/A82M/S72A/A184G/T260G/E435T	94%		5%	59%	550
M105	F87A/A82M/S72A/A184I	70%	10%	20%	23%	160
M106	F87A/A82M/S72A/A184I/E435M	93%		7%	38%	350
M107	F87A/A82M/S72A/A184I/T260G/N239H/E435M	94%	3%	3%	73%	690
M108	F87A/A82M/S72A/A184I/T260A	50%		49%	8%	40
M109	F87A/A82M/S72A/A184I/T260A/E435M	82%		17%	21%	175
M111	F87A/A82M/S72A/A184I/T260G/E435D	88%		11%	34%	300
M112	F87A/A82M/S72A/A184I/T260G/E435H	86%	3%	11%	31%	265
M114	F87A/A82M/S72A/A184I/T260G/E435I/V26H	25%	4%	71%	3%	10
M115	F87A/A82M/S72A/A184I/T260G/E435I/V26L	38%	6%	56%	5%	20
M116	F87A/A82M/S72A/A184I/T260G/E435I/V26M	77%	2%	21%	13%	100
M117	F87A/A82M/S72A/A184I/T260G/E435L	82%	2%	16%	15%	125
M119	F87A/A82M/S72A/A184I/T260G/E435M/N319Y	93%	2%	5%	56%	520
M120	F87A/A82M/S72A/A184I/T260G/E435M/Q307H	8%	4%	88%	3%	2
M121	F87A/A82M/S72A/A184I/T260G/E435M/V26H	88%		11%	32%	285
M122	F87A/A82M/S72A/A184I/T260G/E435M/V26M	90%		9%	34%	305
M123	F87A/A82M/S72A/A184I/T260G/E435N	50%		49%	8%	40
M124	F87A/A82M/S72A/A184I/T260G/E435Q	87%		12%	33%	290
M125	F87A/A82M/S72A/A184I/T260G/E435R	51%	3%	46%	7%	35
M126	F87A/A82M/S72A/A184I/T260G/E435T/V26H	7%		92%	4%	2
M127	F87A/A82M/S72A/A184I/T260G/E435T/V26L	29%	7%	64%	5%	15
M128	F87A/A82M/S72A/A184I/T260G/E435T/V26M	8%	6%	86%	4%	5
M129	F87A/A82M/S72A/A184I/T260G/E435W	81%		18%	20%	160
M130	F87A/A82M/S72A/A184I/T260G/E435Y	76%		23%	14%	105
M131	F87A/A82M/S72A/A184I/T260G/H171L/E435M	17%		83%	5%	10
M132	F87A/A82M/S72A/A184I/T260G/H171L/L181F/E435M	12%	4%	84%	5%	5
M133	F87A/A82M/S72A/A184I/T260G/H171L/N239H/E435M	6%	5%	89%	5%	5
M134	F87A/A82M/S72A/A184I/T260G/H171L/Q403P	8%		91%	4%	5
M135	F87A/A82M/S72A/A184I/T260G/L181F/N239H/E435M	64%	14%	22%	16%	100
M136	F87A/A82M/S72A/A184I/T260G/Q403P/E435M	29%		71%	3%	9
M137	F87A/A82M/S72A/A184I/T260G/V26L/E435M	86%		13%	20%	170
M138	F87A/A82M/S72A/A184I/T260G/V26L/H171L	13%		87%	5%	5

Variant	Mutations	3	4	Other	Conv.	TON
M139	F87A/A82M/S72A/A184I/T260G/V26L/H171L/E435M	23%		77%	6%	15
M140	F87A/A82M/S72A/A184I/T260G/V26L/N239H/E435M	91%		9%	73%	665
M141	F87A/A82M/S72A/A184I/T260G/V26L/Q403P	12%	2%	86%	3%	5
M142	F87A/A82M/S72A/A184I/T260G/V26L/Y51F	89%	2%	9%	36%	325
M143	F87A/A82M/S72A/A184I/T260G/Y51F/H171L	11%		89%	4%	5
M144	F87A/A82M/S72A/A184I/T260G/Y51F/Q403P	11%		89%	4%	5
M145	F87A/A82M/S72A/A184L/T260G/E435I	87%		13%	24%	215
M146	F87A/A82M/S72A/A184M/T260G/E435I	32%	4%	64%	5%	15
M147	F87A/A82M/S72A/A184M/T260G/E435M	86%	2%	12%	22%	190
M148	F87A/A82M/S72A/A184V/T260G/E435I	88%		11%	26%	230
M150	F87A/A82M/S72A/L181F/A184G/T260G	11%	7%	82%	4%	5
M151	F87A/A82M/S72A/T260G	45%		55%	16%	70
M152	F87A/A82M/S72A/T260G/E435M	93%	2%	5%	47%	435
M153	F87A/A82M/T260G/E435M	95%	2%	3%	28%	265
M154	F87A/A82M/V178F/T260G/E435M	54%		45%	12%	65
M155	F87A/A82M/V178W/T260G/E435M	70%		29%	18%	120
M156	F87A/S72A/A184I/T260G/E435M	76%	3%	21%	16%	120
M157	F87A/S72A/A184I/T260G/E435T	25%	8%	67%	6%	15
M158	F87I/A82M	76%	2%	22%	23%	175
M159	F87I/A82M/A184I/E435M	73%	5%	22%	24%	170
M160	F87I/A82M/A184I/T260G	67%	2%	31%	14%	95
M161	F87I/A82M/A184I/T260G/E435I/S72V	91%		9%	41%	375
M162	F87I/A82M/A184I/T260G/E435I/S72W	69%	2%	29%	12%	85
M163	F87I/A82M/A184I/T260G/E435M	90%	2%	8%	59%	530
M164	F87I/A82M/A184I/T260G/E435M/S72V	89%		10%	38%	340
M165	F87I/A82M/A184I/T260G/E435M/S72W	30%		70%	5%	15
M166	F87I/A82M/E435M	74%	5%	21%	25%	185
M167	F87I/A82M/S72A	78%		22%	25%	195
M168	F87I/A82M/S72A/A184I	44%	2%	54%	10%	45
M169	F87I/A82M/S72A/A184I/E435M	65%	9%	26%	22%	140
M170	F87I/A82M/S72A/A184I/N239H/T260G/E435I	88%	10%	2%	89%	780
M171	F87I/A82M/S72A/A184I/N239H/T260G/E435M	79%	14%	7%	78%	615
M172	F87I/A82M/S72A/A184I/T260G	85%		14%	37%	315
M174	F87I/A82M/S72A/A184I/T260G/E435I/L75S	89%	3%	8%	58%	515
M175	F87I/A82M/S72A/A184I/T260G/E435I/M185T	91%		9%	55%	500
M176	F87I/A82M/S72A/A184I/T260G/E435I/M354F	5%		95%	5%	2
M178	F87I/A82M/S72A/A184I/T260G/E435M/L188S	86%	2%	12%	44%	375
M179	F87I/A82M/S72A/A184I/T260G/E435M/L29A	90%		10%	37%	340
M180	F87I/A82M/S72A/A184I/T260G/E435M/L29M	94%	2%	4%	78%	740
M181	F87I/A82M/S72A/A184I/T260G/E435M/L75S	5%		95%	8%	5
M182	F87I/A82M/S72A/A184I/T260G/E435M/L75T			100%	8%	

Variant	Mutations	3	4	Other	Conv.	TON
M183	F87I/A82M/S72A/A184I/T260G/E435M/M354F	91%	2%	7%	77%	700
M184	F87I/A82M/S72A/E435M	59%	18%	23%	39%	230
M185	F87I/A82M/S72A/T260G	81%		18%	25%	205
M186	F87I/A82M/S72A/T260G/E435M	89%	3%	8%	69%	610
M187	F87I/A82M/S72A/V178F/T260G/E435I	87%		13%	39%	340
M188	F87I/A82M/S72A/V178F/T260G/E435M	90%		10%	51%	465
M189	F87I/A82M/S72A/V178W/T260G/E435I	92%		8%	58%	535
M190	F87I/A82M/S72A/V178W/T260G/E435M	86%	5%	9%	72%	620
M191	F87I/A82M/T260G	44%	3%	53%	7%	35
M192	F87I/A82M/T260G/E435M	82%	2%	16%	22%	180
M193	F87S/A82M/S72A/A184I/T260G/E435M	89%		11%	48%	430
M194	F87T/A82M/S72A/A184I/T260G/E435I	92%	2%	6%	75%	695
M195	F87T/A82M/S72A/A184I/T260G/E435M	91%		9%	70%	630
M196	F87V/A82M/S72A/A184I/N239H/T260G/E435I	4%		96%	3%	
M197	F87V/A82M/S72A/A184I/N239H/T260G/E435M	44%	28%	28%	68%	295
M200	K69I/F87A/A82M/A184I/T260G/E435M	4%	2%	94%	7%	5
M201	K69R/F87A/A82M/A184I/T260G/E435M	14%	2%	84%	6%	10
M202	F87I/A82M/S72A/A184I/M185T/T260G/E435M/L29M	88%		12%	48%	420
M203	L29M/S72A/L75S/A82M/F87T/A184I/T260G/E435I	10%	6%	84%	8%	10
M204	L29M/S72A/V78M/A82M/F87T/A184I/T260G/E435I	28%	3%	69%	6%	20
M205	R19/F87A/A82M/A184I/T260G/E435I	2%		98%	3%	
M206	R19/F87A/A82M/A184I/T260G/E435M	75%	6%	19%	17%	130
M207	F87T/A82M/S72A/A184I/T260G/E435M/M185T	86%		14%	33%	285
M208	F87T/A82M/S72A/A184I/T260G/E435I/L75S	44%	5%	51%	10%	45
M209	F87T/A82M/S72A/A184I/T260G/E435/V78M	84%	3%	13%	29%	245
M210	F87A/A82M/S72A/A184I/T260G/E435M/Y51F	93%	2%	5%	57%	525
M211	R19/F87I/A82M/A184I/T260G	38%	4%	58%	8%	30
M212	R19/F87T/A82M/A184I/T260G	73%	15%	12%	47%	340
M213	R19/F87V/A82M/A184I/T260G	75%	13%	12%	39%	295
M214	R19/F87A/A82M/A184I/T260G/E435T	72%	16%	12%	40%	285
M215	R19/F87A/A82M/A184I/T260G/S72A	87%	6%	7%	58%	505

Table S6. Activity and selectivity for the oxidation of VD_3 with the F87I/A82M/A184I/T260G/S72A/E435I variant (M173) of $P450_{BM3}$. The screening scale reaction mixture (0.5 mL) contained 2 μM enzyme, 4 U/mL GDH, and 40 μM $NADP^+$. Conv. is the percentage of substrate converted to products. TON is the turnover number for the formation of $25(OH)VD_3$ (**1**).

Entry	$[VD_3]$/mM	$[VD_3]$:$[P450_{BM3}]$	**1**	**Conv.**	**TON**
1	2	1000:1	83%	83%	690
2	5	2500:1	62%	92%	1440
3	6	3000:1	87%	86%	2230
4	7	3500:1	81%	78%	2200
5	8	4000:1	81%	76%	2440
6	9	4500:1	83%	74%	2780
7	10	5000:1	77%	72%	2750

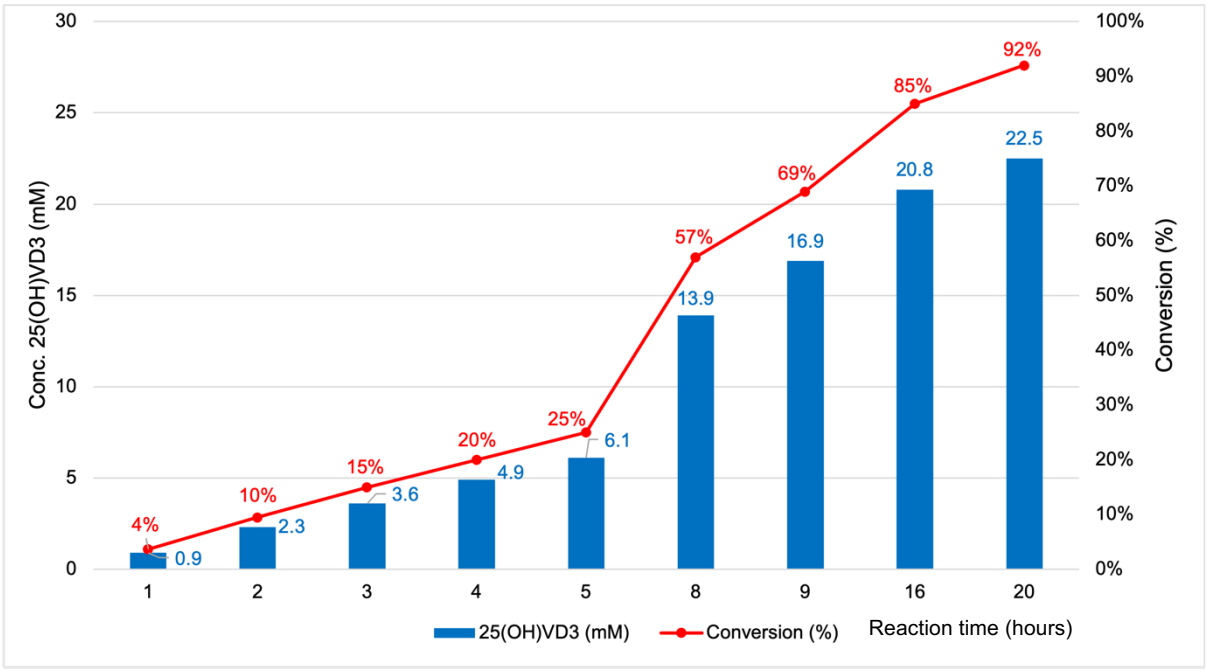
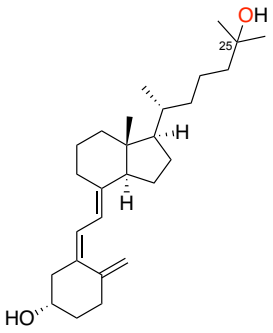


Figure S2. Time course for the conversion of vitamin D_3 and formation of 25-hydroxy-vitamin D_3 (**1**) in a 1-L scale reaction containing 10 g (26 mmol) of vitamin D_3 and 5 µmol of the F87I/A82M/A184I/T260G/S72A/E435I variant (M173) of $P450_{BM3}$. After stirring (400 rpm) with aeration (1.5 L/min) at ambient temperature for 20 hours, the reaction reached 92% conversion, from which 6.62 g of 25-hydroxy-vitamin D_3 (**1**) was isolated via silica gel column chromatography (69.1% yield based on VD_3 converted).

S4. Product characterisation

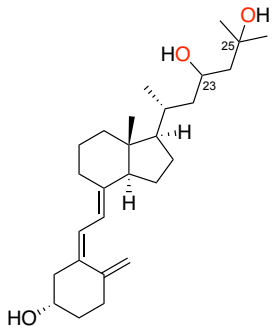
1. 25-Hydroxy-vitamin D_3



A preparative scale reaction was conducted to isolate and characterise product **1** using the variant S72A/F87A/A82M/A184I/T260G/E435I. The 100 mL reaction mixture in 200 mM potassium phosphate buffer, pH 7.9, contained 2 μM of this enzyme variant, 384 mg VD_3 (10 mM, 200 mM stock solution in methanol), 4 U/mL glucose dehydrogenase (GDH, 4 U/μL stock solution), 100 mM glucose (1 M stock solution) and 10 mM methyl-β-cyclodextrin (200 mM stock solution). $NADP^+$ monosodium salt (40 μM; 4 mM stock solution) was added to initiate the reaction. After stirring for 24 h the reaction reached 64% conversion with 87% selectivity for **1**. The reaction mixture was extracted three times with ethyl acetate. The organic extracts were combined and washed with water and then brine, dried with Na_2SO_4, and solvent was removed by rotary evaporation. The crude mixture was purified by silica gel column chromatography, eluting with mixtures of petroleum ether (bp 40–60 °C) and ethyl acetate (5:1 to 3:1 to 1:1) to give product **1** as a white solid (112 mg, 44% yield based on the amount of VD_3 converted). The assignment of **1** was consistent with literature data.[45]

^{1}H NMR (700 MHz, $CDCl_3$) δ 6.22 (d, *J* = 11.0 Hz, 1H), 6.03 (d, *J* = 11.5 Hz, 1H), 5.04 (d, *J* = 2.5 Hz, 1H), 4.81 (d, *J* = 2.5 Hz, 1H), 3.97 – 3.90 (m, 1H), 2.84 – 2.79 (m, 1H), 2.56 (dd, *J* = 13.0, 4.0 Hz, 1H), 2.39 (m, 1H), 2.28 (dd, *J* = 13.0, 7.5 Hz, 1H), 2.19 – 2.14 (m, 1H), 2.02 – 1.96 (m, 2H), 1.94 – 1.89 (m, 1H), 1.86 (m, 1H), 1.67 (m, 3H), 1.54 – 1.51 (m, 1H), 1.49 – 1.44 (m, 3H), 1.40 – 1.36 (m, 3H), 1.32 – 1.26 (m, 3H), 1.21 (s, 8H), 1.08 – 1.03 (m, 1H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.54 (s, 3H). **^{13}C NMR** (176 MHz, $CDCl_3$) δ 145.2, 142.4, 135.2, 122.6, 117.7, 112.5, 71.3, 69.3, 56.7, 56.5, 46.1, 46.0, 44.5, 40.7, 36.5, 36.2, 35.3, 32.1, 29.5, 29.4, 29.1, 27.8, 23.7, 22.4, 21.0, 18.9, 12.1. **HRMS** (ESI^+): Calc'd for $C_{27}H_{45}O_2^+$ $[M+H]^+$: 401.3414, found: 401.3414.

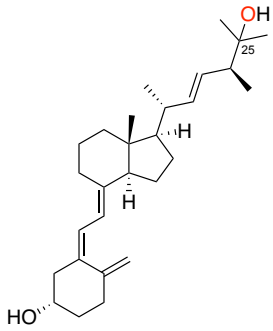
2. 23,25-Dihydroxy-vitamin D_3



A preparative scale reaction was conducted to isolate and characterise product **2** using the variant S72A/F87A/A82M/A184I/T260G/E435I. The 100 mL reaction mixture in 200 mM potassium phosphate buffer, pH 7.9, contained 2 μM of this enzyme variant, 384 mg VD_3 (10 mM, 200 mM solution stock in methanol), 4 U/mL glucose dehydrogenase (GDH, 4 U/μL stock), 100 mM glucose (1 M stock solution) and 10 mM methyl-β-cyclodextrin (200 mM stock solution). $NADP^+$ monosodium salt (40 μM, 4 mM stock solution) was added to initiate the reaction. After stirring for 24 h the reaction reached 64% conversion with 8% selectivity for **2**. The reaction mixture was extracted three times with ethyl acetate. The organic extracts were combined and washed with water and then brine, dried with Na_2SO_4, and solvent was removed by rotary evaporation. The crude mixture was purified by silica gel column chromatography, eluting with mixtures of petroleum ether (bp 40–60 °C) and ethyl acetate (5:1 to 3:1 to 1:1) to give product **2** as a white solid (23 mg, 9% yield based on the amount of VD_3 converted). The assignment of **2** was consistent with literature data.[46]

^{1}H NMR (700 MHz, $CDCl_3$) δ 6.23 (d, *J* = 11.0 Hz, 1H), 6.03 (d, *J* = 11.0,z Hz, 1H), 5.06 – 5.03 (d, *J* = 2.5 Hz, 1H), 4.81 (d, *J* = 2.5 Hz, 1H), 4.10 (m, 1H), 3.95 (m, 1H), 2.82 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.60 – 2.55 (m, 1H), 2.40 (m, 1H), 2.29 (dd, *J* = 13.5, 8.0 Hz, 1H), 2.18 (m, 1H), 2.02 – 1.97 (m, 2H), 1.95 – 1.88 (m, 2H), 1.71 – 1.65 (m, 3H), 1.57 – 1.49 (m, 6H), 1.32 – 1.27 (m, 11H), 0.97 (d, *J* = 6.0 Hz, 3H), 0.56 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 145.3, 142.1, 135.4, 122.5, 117.8, 112.5, 72.0, 69.3, 68.5, 57.4, 56.4, 47.6, 46.1, 46.0, 45.1, 40.7, 35.3, 34.1, 32.4, 32.1, 29.1, 28.1, 27.9, 23.7, 22.4, 19.5, 12.2. **HRMS** (ESI+): Calc'd for $C_{27}H_{44}NaO_3^+$ $[M+Na]^+$: 439.3183, found: 439.3183.

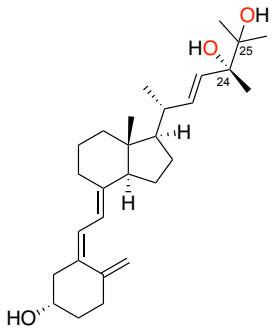
3. 25-Hydroxy-vitamin D_2



A preparative scale reaction was conducted to isolate and characterise product **3** using variant S72A/F87I/A82M/A184I/T260G/E435I which showed 88% conversion and 92% selectivity for **3** in 24 h. The 100 mL reaction mixture in 200 mM potassium phosphate buffer, pH 7.9, contained 2 μM of this enzyme variant, 159 mg VD_2 (4 mM, 200 mM solution stock in ethanol), 4 U/mL glucose dehydrogenase (GDH, 4 U/μL stock), 100 mM glucose (1 M stock) and 10 mM methyl-β-cyclodextrin (200 mM stock solution). $NADP^+$ monosodium salt (40 μM, 4 mM stock solution) was added to initiate the reaction. After stirring for 24 h the reaction mixture was extracted three times with ethyl acetate. The organic extracts were combined and washed with water and then brine, dried with Na_2SO_4, and solvent was removed by rotary evaporation. The crude mixture was purified by silica gel column chromatography, eluting with mixtures of petroleum ether (bp 40–60 °C) and ethyl acetate (5:1 to 3:1 to 1:1) to give product **3** as a white solid (95 mg, 66% yield based on the amount of VD_2 converted). The assignment of **3** was consistent with literature data.[47]

^{1}H NMR (600 MHz, CD_3OD) δ 6.22 (d, *J* = 11.0 Hz, 1H), 6.03 (d, *J* = 11.0 Hz, 1H), 5.35 (dd, *J* = 15.5, 8.0 Hz, 1H), 5.27 (dd, *J* = 15.0, 8.5 Hz, 1H), 5.03 (s, 1H), 4.77 – 4.73 (s, 1H), 3.76 (tt, *J* = 9.0, 4.0 Hz, 1H), 2.86 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.57 – 2.51 (m, 1H), 2.40 (dt, *J* = 13.5, 5.0 Hz, 1H), 2.23 – 2.16 (m, 1H), 2.15 – 1.98 (m, 6H), 1.77 – 1.66 (m, 3H), 1.59 – 1.51 (m, 2H), 1.50 – 1.42 (m, 2H), 1.39 – 1.31 (m, 3H), 1.14 (s, 3H), 1.10 (s, 3H), 1.04 (d, *J* = 6.5 Hz, 3H), 1.00 (d, *J* = 7.0 Hz, 3H), 0.57 (s, 3H). **^{13}C NMR** (151 MHz, MeOD) δ 145.6, 141.0, 137.0, 136.0, 130.0, 121.3, 117.7, 111.4, 71.9, 69.2, 56.4, 56.2, 47.8, 45.7, 45.4, 40.5, 40.4, 35.2, 32.3, 28.6, 27.6, 27.0, 24.7, 23.2, 21.9, 20.1, 14.4, 11.4. **HRMS** (ESI+): Calc'd for $C_{28}H_{45}O_2^+$ $[M+H]^+$: 413.3414, found: 413.3399.

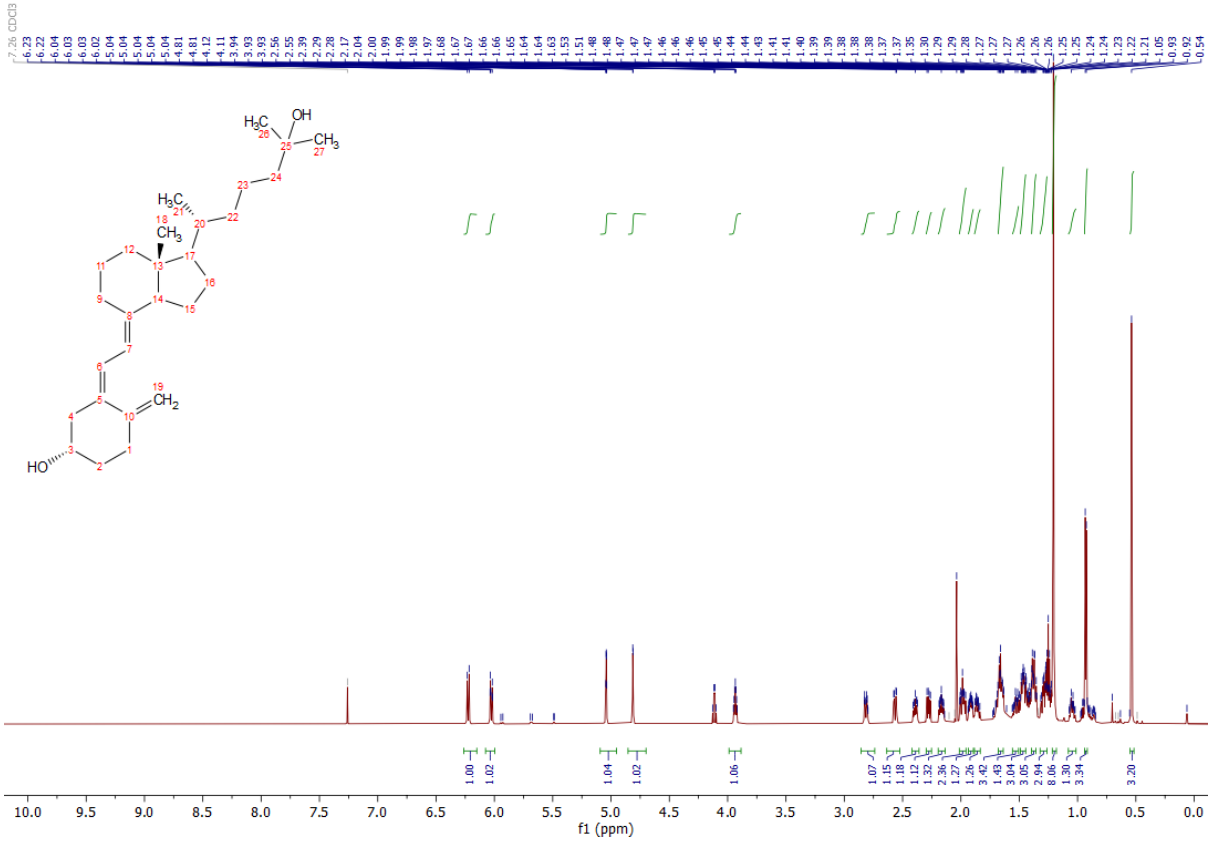
4. 24,25-Dihydroxy-vitamin D_2



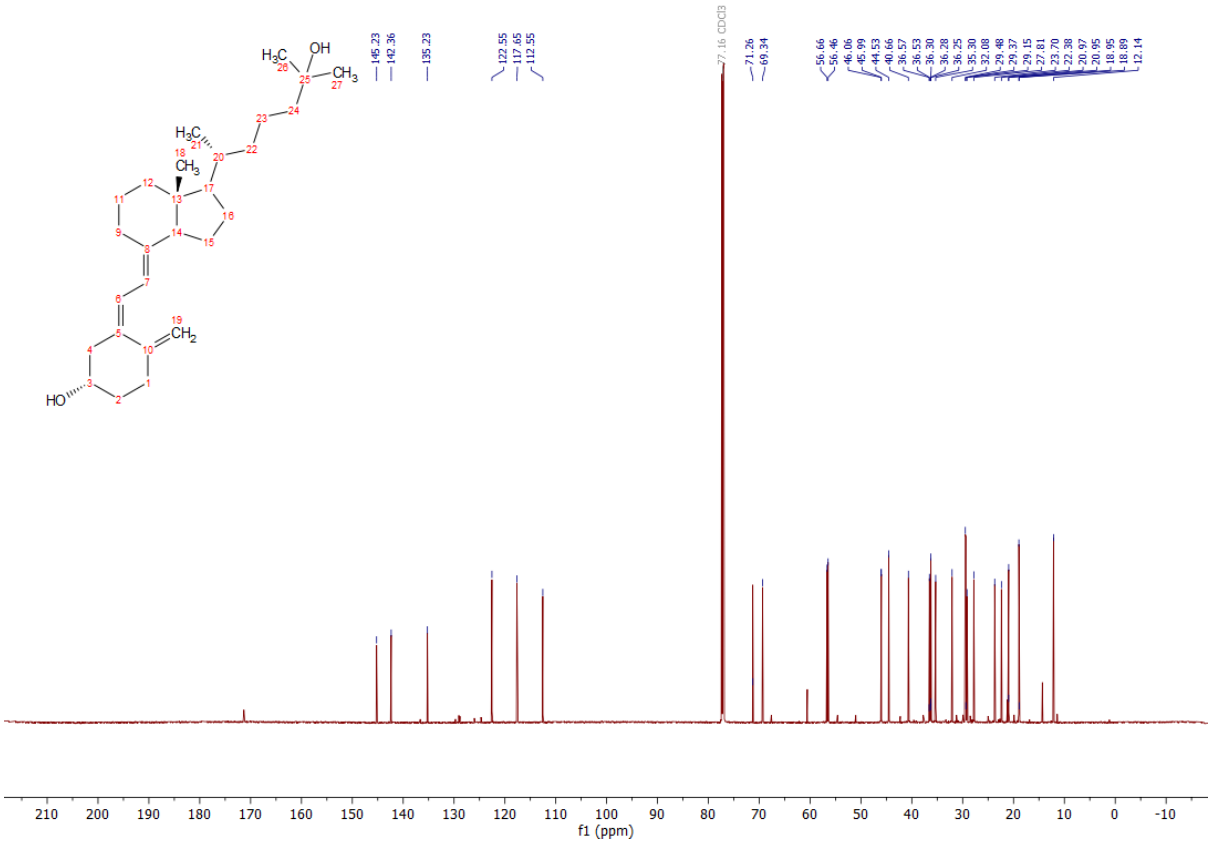
A preparative scale reaction was conducted to isolate and characterise product **4** using variant S72A/F87I/A82M/A184I/T260G/E435I which showed 88% conversion and 4% selectivity for **4** after 24 h. The 100 mL reaction mixture in 200 mM potassium phosphate buffer, pH 7.9, contained 2 μM of this variant, 159 mg vitamin D_2 (4 mM, 200 mM solution stock in ethanol), 4 U/mL glucose dehydrogenase (GDH, 4 U/μL stock solution), 100 mM glucose (1 M stock solution) and 10 mM methyl-β-cyclodextrin (200 mM stock solution). $NADP^+$ monosodium salt (40 μM, 4 mM stock solution) was added to initiate the reaction. After stirring for 24 h the reaction mixture was extracted three times with ethyl acetate. The organic extracts were combined and washed with water and then brine, dried with Na_2SO_4, and then solvent was removed by rotary evaporation. The crude mixture was purified by silica gel column chromatography, eluting with mixtures of petroleum ether (bp 40–60 °C) and ethyl acetate (5:1 to 3:1 to 1:1) to give **4** as a white solid (10 mg, 7% based on the amount of VD_2 converted). The assignment of **4** was consistent with literature data.[47,48]

^{1}H NMR (400 MHz, CD_3OD) δ 6.23 (d, *J* = 11.0 Hz, 1H), 6.04 (d, *J* = 11.0 Hz, 1H), 5.62 (d, *J* = 15.5 Hz, 1H), 5.57 – 5.50 (m, 1H), 5.07 – 5.03 (m, 1H), 4.76 (dd, *J* = 3.0, 1.0 Hz, 1H), 3.77 (tt, *J* = 9.0, 4.0 Hz, 1H), 2.88 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.58 – 2.51 (m, 1H), 2.42 (dt, *J* = 13.5, 5.0 Hz, 1H), 2.18 – 2.05 (m, 6H), 1.76 (dt, *J* = 9.0, 2.5 Hz, 3H), 1.68 – 1.31 (m, 7H), 1.25 (d, *J* = 1.0 Hz, 3H), 1.19 (s, 3H), 1.17 (s, 3H), 1.07 (d, *J* = 6.5 Hz, 3H), 0.60 (s, 3H). **^{13}C NMR** (101 MHz, MeOD) δ 147.0, 142.4, 137.4, 136.5, 133.0, 122.6, 119.0, 112.7, 77.9, 75.8, 70.6, 57.7, 57.6, 47.0, 46.9, 41.8, 36.6, 33.6, 29.9, 29.1, 25.3, 25.2, 24.5, 23.2, 23.0, 21.3, 14.5, 12.6. **HRMS** (ESI+) Calc'd for $C_{28}H_{44}O_3Na^+$ [$M+Na^+$]: 451.3183, found: 451.3180.

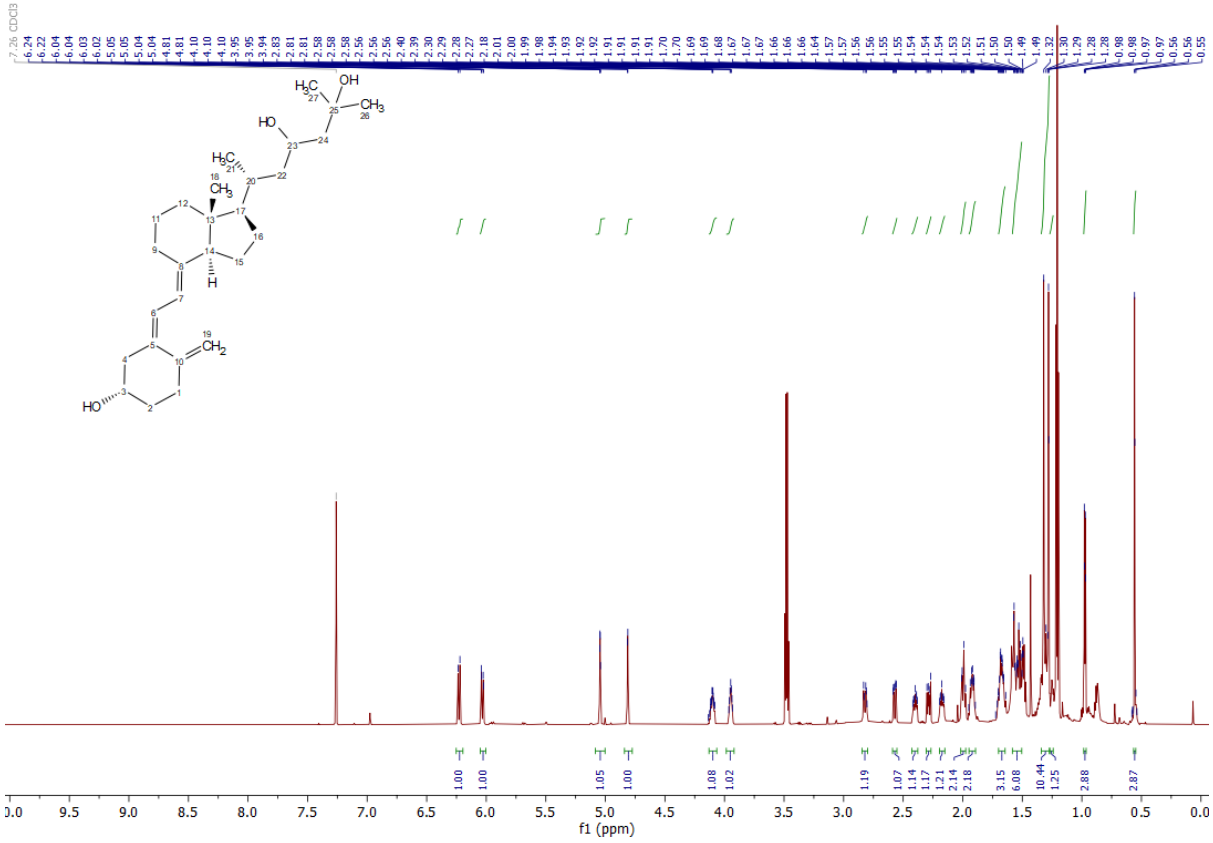
1 – ^{1}H NMR (500 MHz, $CDCl_3$)



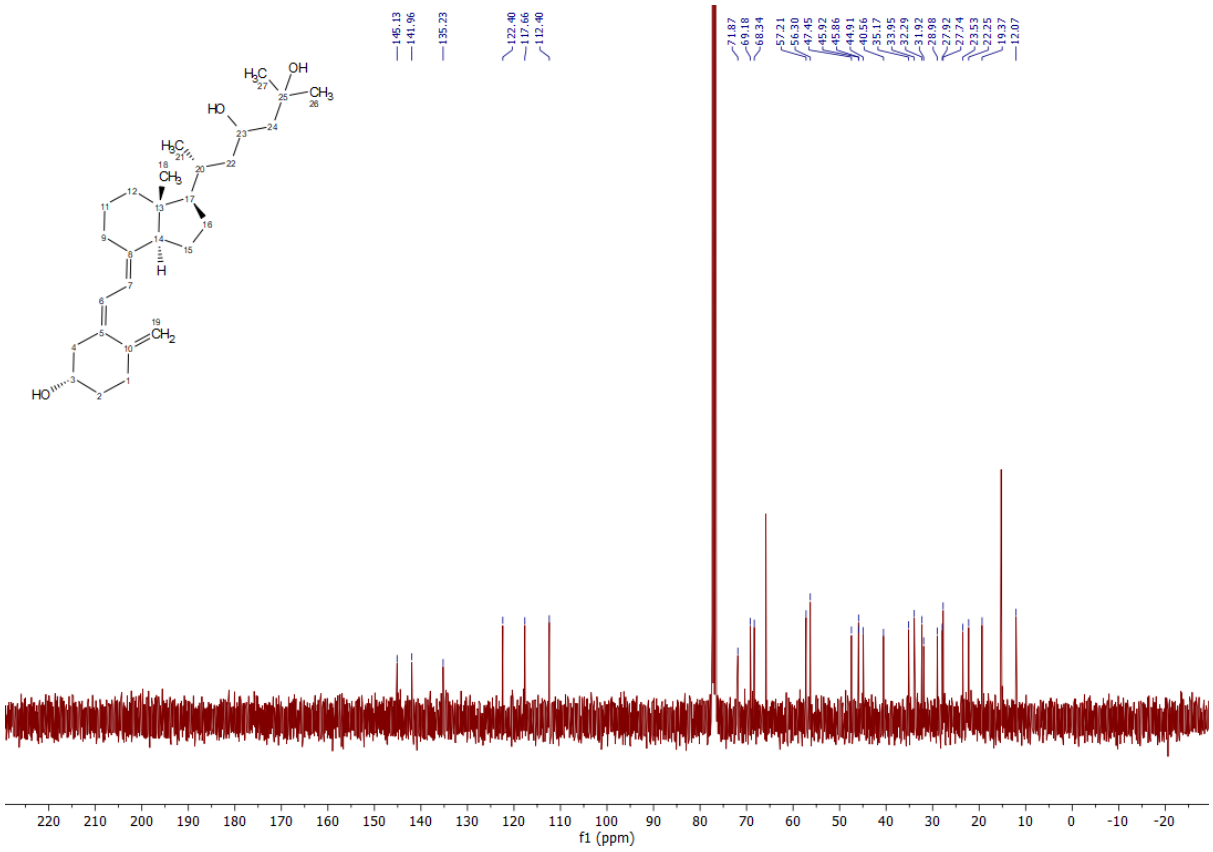
1 – ^{13}C NMR (126 MHz, $CDCl_3$)



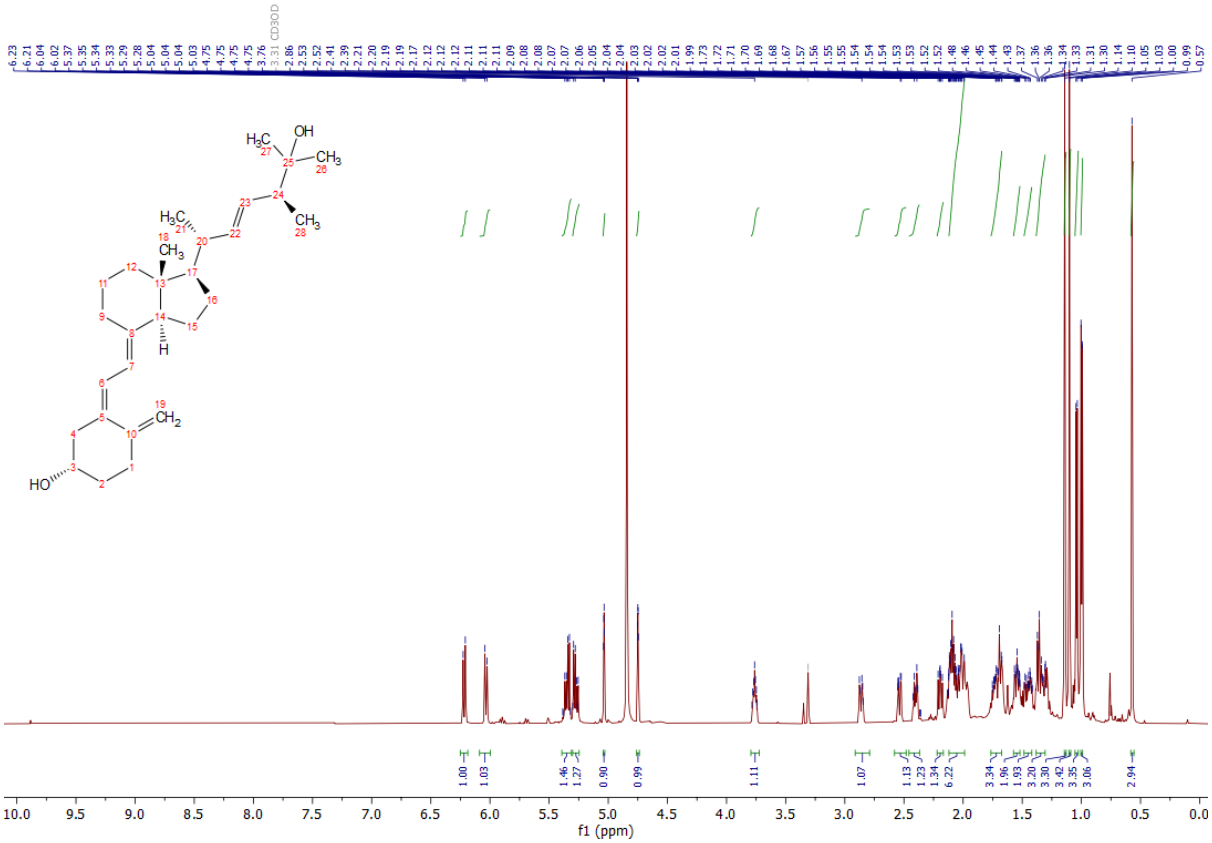
2 – ^{1}H NMR (500 MHz, $CDCl_3$)



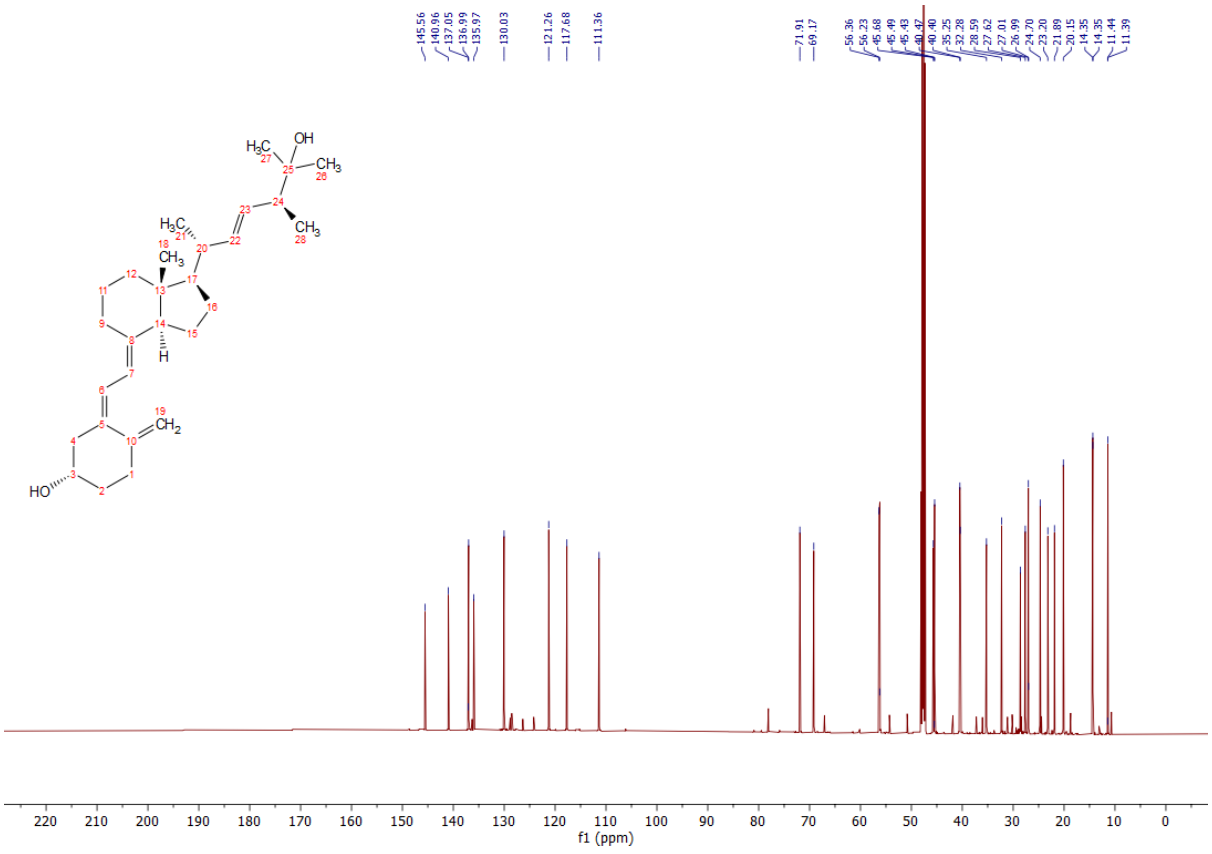
2 – ^{13}C NMR (101 MHz, $CDCl_3$)



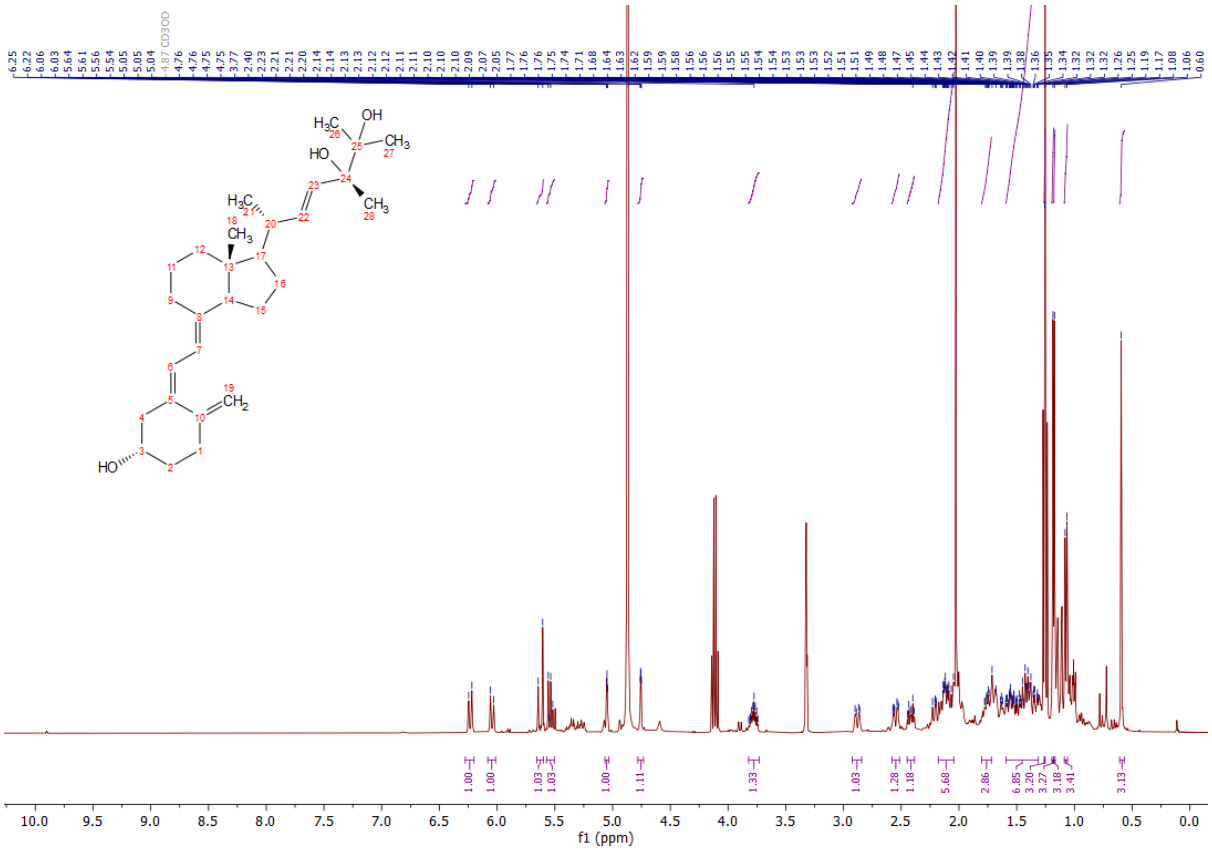
3 – ^{1}H NMR (500 MHz, CD_3OD)



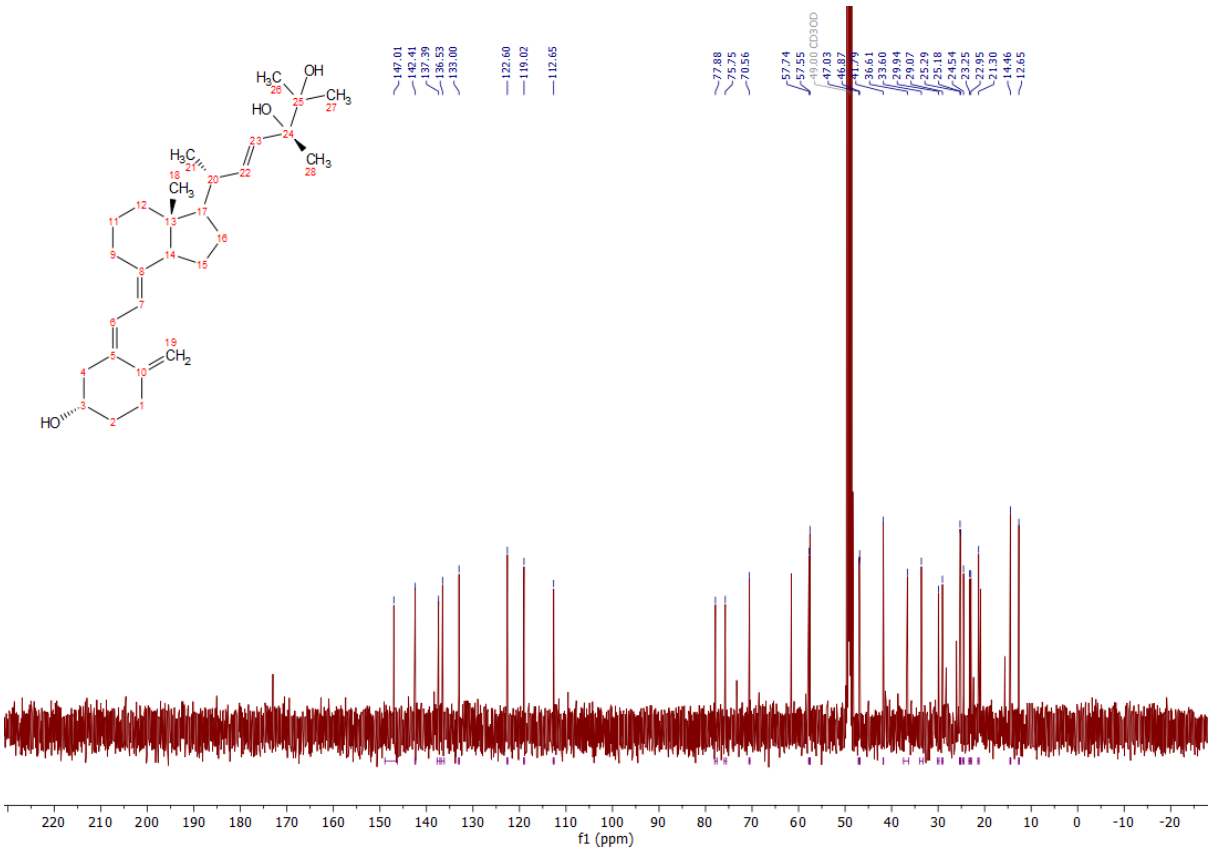
3 – ^{13}C NMR (126 MHz, CD_3OD)



4 – ^{1}H NMR (400 MHz, CD_3OD)



4 – ^{13}C NMR (101 MHz, CD_3OD)



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