Electronic Supporting Information

Enzymatic Δ^1-Dehydrogenation of 3-Ketosteroids – Reconciliation of Kinetic Isotope Effects with the Reaction Mechanism

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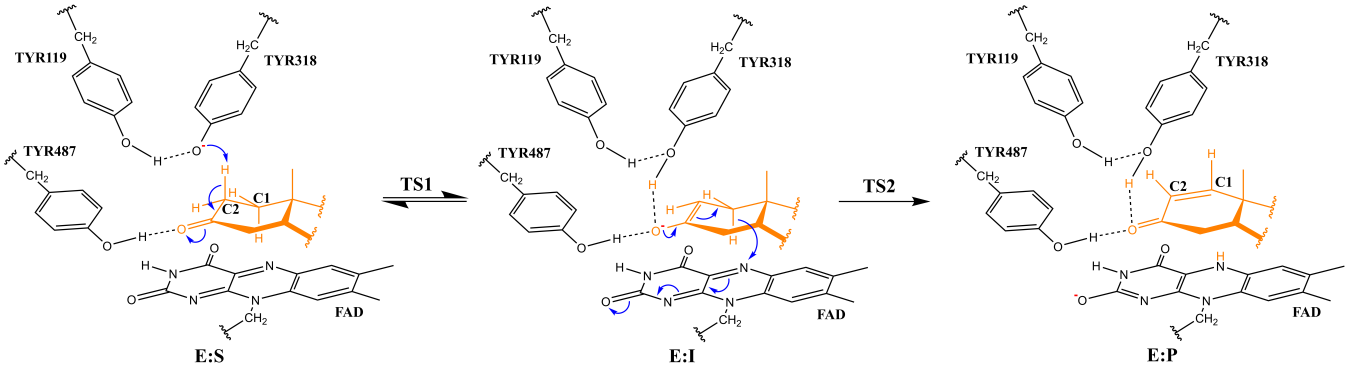


Figure S1: Scheme of main pathway mechanism

The substrate (DHT) is located in the active site pocket near the isoalloxazine ring system of FAD. The ring A of the substrate is almost parallel to the keto-pyrimidine moiety of the FAD ring system, the 3-keto group of substrate lies above the N3 atom of the FAD. DHTs position is stabilized by H-bonds between the 3-keto group and Tyr487 (d(O-H_{Tyr487}...O_{ket}) = 1.99 Å ± 0.09) as well as Gly491 (d(NH_{Gly491}...O_{ket}) = 2.25 Å ± 0.02). The initial distance between the Tyr318 anionic oxygen atom (OH_{Tyr318}) and the $2\beta H$ atom of the substrate is 1.95 Å ± 0.07. The position of Tyr318 is stabilized by H-bond interactions with Tyr119, with the distance between the hydrogen atom of Tyr119 hydroxyl group (HH_{Tyr119}) and (OH_{Tyr318}) 1.92 Å ± 0.10. The H-bond between Tyr119 and Tyr318 remains stable throughout the whole RHR.
In the first step of the reaction, the steroid is activated by the abstraction of the proton from the 2β position by the Tyr318 anion. In the transition state **TS1**, the distance between C2 and 2β-H atom is 1.46 Å ± 0.01 for DHT while the distance between 2β-H and OH atom of Tyr318 is 1.17 Å ± 0.01 (see Fig. 7 from the main text). Interestingly, there is no significant shift in the position of the proton H-bond stabilizing the 3-keto group (i.e. with Tyr487) from **E:S** to **TS1**, as the d(O-H_{Tyr487}...O_{ket}) = 1.92 ± Å 0.06). However, the transfer of a proton from the substrate toward a tyrosyl anion induces a shift of a negative charge in the system with the accumulation of the negative charge at the oxygen atom in the 3-keto group (change of charge from -0.506 to -0.628). Upon completion of the proton transfer to Tyr318, the intermediate enolate structure is formed (**E:I**) and the H-bond between the 3-keto group and Tyr487 indeed gets stronger (d(O-H_{Tyr487}...O_{ket}) = 1.82 Å ± 0.03. Additionally, a new H-bond is formed between the 3-keto group and protonated with 2β -H atom Tyr318 (d(O-H_{Tyr318}...O_{ket}) = 1.76 Å ± 0.05. Formation of intermediate enolate results with a change in ring A geometry and repositioning of the substrate in the active site which decreases the distance between 1H of DHT and $N5_{FAD}$ atom is 2.62 Å ± 0.18 in **E:S** to 1.96 Å ± 0.003 in **E:I**. This change of geometry facilitates the next step of the reaction.
In the second step of the reaction, the hydride is transferred from the 1 position of ketosteroid to N5 atom in FAD residue, resulting in a 2-electron reduced (hydroquinone) unprotonated state of flavin. In the transition state, **TS2**, the distance between H1 of a substrate and C1 atom of the substrate is 1.38 Å ± 0.01 and between 1H and $N5_{FAD}$ is 1.33 Å ± 0.01. Finally, the double bond is formed between C1 and C2 atoms of steroid and the enzyme-product is formed (E:P, Figure 6 right). At this stage of reaction, FAD is at a two-electron reduced (hydroquinone), deprotonated state.

2 Detailed description of alternative mechanism – protonated Tyr318

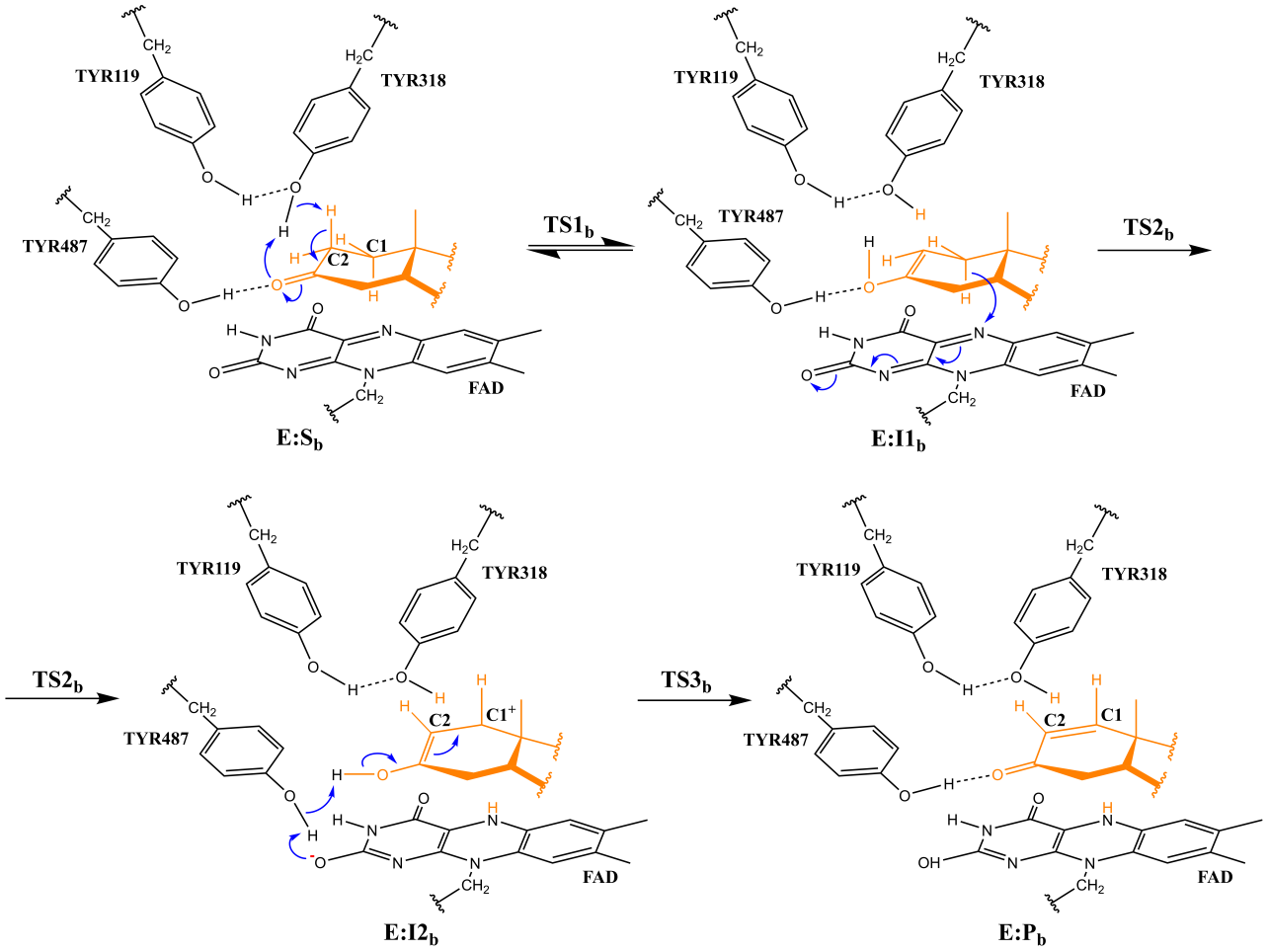


Figure S2: Scheme of alternative mechanism

In comparison to **E:S** from main pathway, no significant changes in the active site of the optimized structure of **E:Sb** were noted, except for the state of Tyr318. The distance between the oxygen atom (OH_{Tyr318}) of Tyr318 and the hydrogen atom of Tyr119 hydroxyl group (HH_{Tyr119}) is 1.98 Å. In the first step, activation of C2 atom (**TS1b**) occurs via a double hydrogen shift i.e., we observe a transfer of the 2β proton to Tyr318 with a concomitant shift of HH_{Tyr318} proton to 3-keto group of the substrate. In the optimized **TS1b**, the distance d($C2_{DHT}\ldots 2\beta H$) is 1.34 Å and d($2\beta H\ldots O_{Tyr318}$) = 1.33 Å, while d($O_{Tyr318}\ldots H_{Tyr318}$) = 1.32 Å and d($H_{Tyr318}\ldots O_{ket}$) = 1.13 Å. The obtained enol intermediate (**E:Ib**) is stabilized by hydrogen bonds with Tyr318 and Tyr487, similarly to enolate **E:I** in the main pathway. Distance between hydrogen and oxygen atoms in the enol group is 0.982 Å. In the optimized structure of **TS1b**, the hydrogen bond between substrate and Tyr318 is formed by hydrogen from the enol group, while the hydrogen atom bonded to O_{Tyr318} is not directed to steroid. In the second step, the hydride is transferred from 1α position of ketosteroid to N5 atom in FAD residue and an unprotonated hydroquinone state of flavin is formed. The distance between C1 and 1H is 1.63 Å and $N5_{FAD}$ atom is 1.24 Å away from the transferred H atom in **TS2b**. In the last step, there is another concomitant shift of two protons i.e, a proton from the enol group is transferred to Tyr487, and the proton from the OH group of Tyr487 is transferred to the $N1_{FAD}$. In the optimized geometry of **TS3b**, the hydrogen atom from the enol group is 1.30 Å away from Tyr487 OH atom and 1.15 from the oxygen atom of the substrate. The distance between HH_{Tyr487} and OH_{Tyr487} is 1.21. As a result, the reduced and protonated (i.e. uncharged) form of FADH2 is

obtained. Calculations showed that the alternative mechanism is extremely improbable, even the energy of enol intermediate is over 27 kcal/mol higher relative to the substrate. Therefore, we did not perform further analysis as this pathway is thermodynamically improbable.

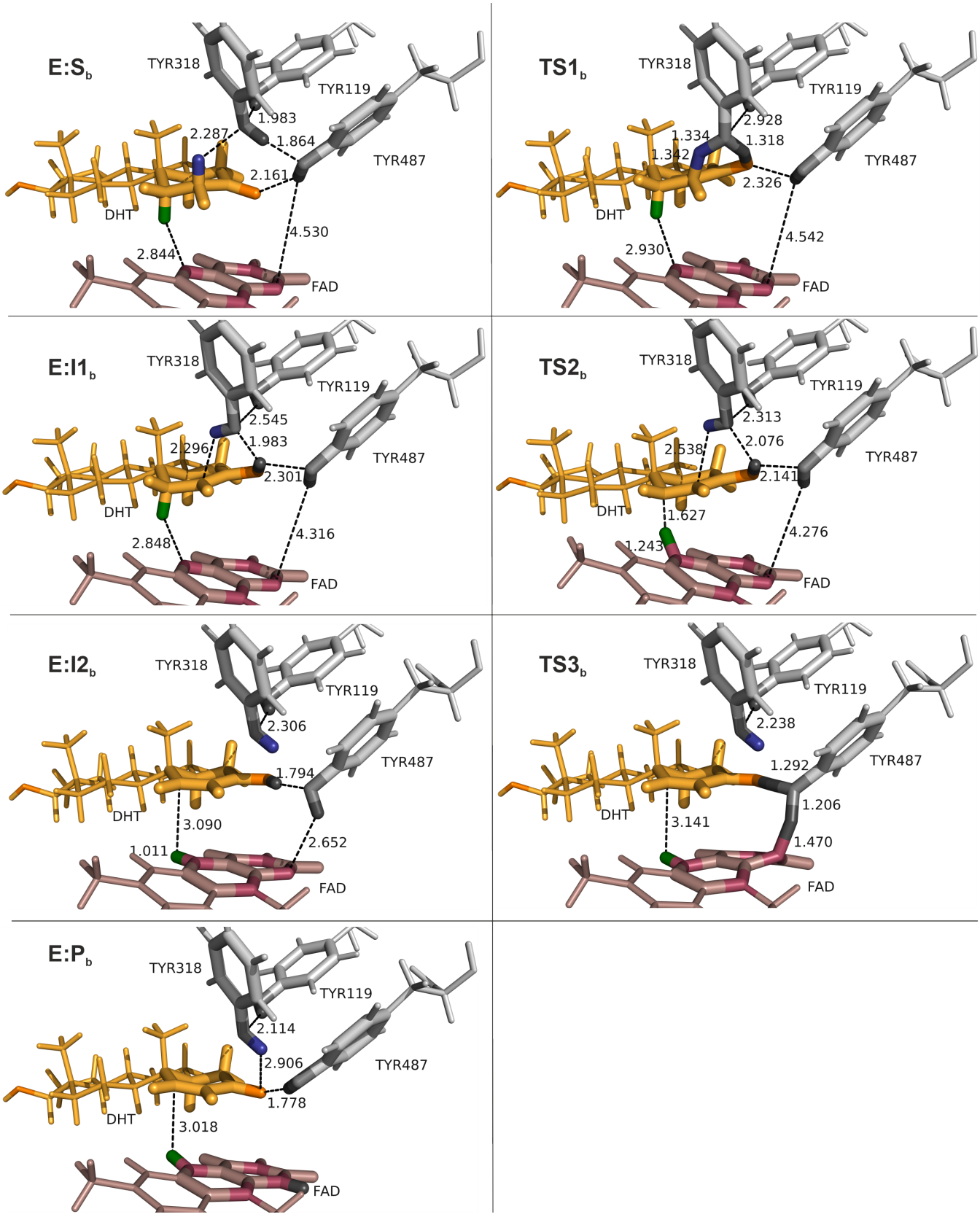


Figure S3: Structures of the stationary geometries along the alternative path. The distances are given in Å.

3 Derivation of relationship between KIE_2 and intrinsic KIEs

For two step reaction with reversible first step:

$$E:S \underset{k_{I-S}}{\overset{k_{S-I}}{\longleftrightarrow}} E:I \xrightarrow{k_{I-P}} E:P$$

we can write rate constant for the overall process:

$$k_2 = \frac{k_{S-I}k_{I-P}}{k_{S-I}+k_{I-S}+k_{I-P}}$$

For ratio between rate constant for reactants not isotopically substituted and substituted with deuterium, we use notation:

$$KIE_i = \frac{k_i^H}{k_i^D}$$

Kinetic isotope effect of the overall process can be expressed as follows:

$$\begin{aligned} KIE_2 &= \frac{k_2^H}{k_2^D} \\ &= \frac{k_{S-I}^H k_{I-P}^H}{k_{S-I}^H + k_{I-S}^H + k_{I-P}^H} \cdot \frac{k_{S-I}^D + k_{I-S}^D + k_{I-P}^D}{k_{S-I}^D k_{I-P}^D} \\ &= KIE_{S-I} \cdot KIE_{I-P} \cdot \frac{k_{S-I}^D + k_{I-S}^D + k_{I-P}^D}{k_{S-I}^H + k_{I-S}^H + k_{I-P}^H} \end{aligned}$$

Such obtained equation can be further modified to obtain:

$$\begin{aligned} KIE_2 &= KIE_{S-I} \cdot KIE_{I-P} \cdot \frac{\frac{k_{S-I}^H}{KIE_{S-I}} + \frac{k_{I-S}^H}{KIE_{I-S}} + \frac{k_{I-P}^H}{KIE_{I-P}}}{k_{S-I}^H + k_{I-S}^H + k_{I-P}^H} \\ &= KIE_{S-I} \cdot KIE_{I-P} \cdot \frac{\frac{1}{KIE_{S-I}} + \frac{1}{KIE_{I-S}}\frac{k_{I-S}^H}{k_{S-I}^H} + \frac{1}{KIE_{I-P}}\frac{k_{I-P}^H}{k_{S-I}^H}}{1 + \frac{k_{I-S}^H}{k_{S-I}^H} + \frac{k_{I-P}^H}{k_{S-I}^H}} \end{aligned}$$

4 Methodology details

4.1 Prediction of titrable aminoacids pKa

Table S 1: Predicted pKa shifts for KSTD from *Rhodococcus erythropolis*

Residue	pKa^{propka}	pKa^{H++}	Residue	pKa^{propka}	pKa^{H++}	Residue	pKa^{propka}	pKa^{H++}
GLU-7	4.04	4.182	ASP-156	3.57	<0.000	ARG-344	12.47	>12.000
CYS-8	12.04	>12.000	ARG-157	10.86	>12.000	GLU-345	4.05	3.613
ASP-9	2.89	2.162	ASP-161	2.77	2.535	CYS-353	11.83	>12.000
TYR-24	10.45	>12.000	HIS-162	5.52	5.894	LYS-361	10.72	11.133
GLU-37	3.15	<0.000	ARG-171	10.56	>12.000	HIS-362	4.3	6.239
LYS-38	10.13	>12.000	ARG-176	11.28	>12.000	GLU-364	4.38	4.193
ASP-40	3.81	2.807	LYS-186	10.38	10.803	ASP-372	3.93	3.702
ARG-41	12.89	>12.000	GLU-188	4.05	3.245	GLU-375	2.57	2.631
TYR-48	13.05	>12.000	ARG-190	12.74	>12.000	GLU-376	4.79	4.155
GLU-62	4.73	4.425	GLU-192	4.87	4.405	LYS-380	10.45	11.412
ARG-63	13.98	>12.000	GLU-201	4.67	4.575	ASP-386	3.52	3.992
ASP-68	2.55	0.301	ASP-202	3.91	3.704	ARG-389	14.35	>12.000
GLU-71	3.27	3.178	ARG-204	13.05	>12.000	GLU-393	4.68	4.562
ARG-74	15.6	>12.000	GLU-209	4.79	3.967	LYS-394	11.38	>12.000
TYR-76	13.96	>12.000	GLU-211	4.2	5.185	ASP-397	3.95	3.802
ARG-78	13.15	>12.000	GLU-215	4.65	4.727	LYS-400	10.55	11.122
ASP-83	3.87	3.942	ARG-218	12.34	>12.000	ASP-404	1.74	<0.000
GLU-85	4.06	2.323	LYS-220	10.54	>12.000	GLU-405	4.6	4.454
GLU-87	4.22	4.170	ARG-223	12.6	>12.000	GLU-406	4.02	3.599
ARG-88	11.39	>12.000	GLU-233	6.22	<0.000	HIS-408	6.84	8.407
ASP-90	2.39	0.691	GLU-237	4.28	5.044	ARG-409	11.51	>12.000
TYR-92	14.51	>12.000	ARG-239	12.77	>12.000	GLU-411	4.8	5.387
GLU-94	4.13	3.500	GLU-240	4.59	4.454	ASP-412	2.87	<0.000
GLU-104	2.84	1.269	LYS-247	10.14	11.241	TYR-414	14.02	>12.000
GLU-110	4.75	5.039	ASP-261	3.07	1.728	ASP-415	5.68	3.872
GLU-112	4.21	3.234	ASP-277	3.62	3.029	CYS-419	10.57	>12.000
ARG-114	13.08	>12.000	CYS-282	13.47	>12.000	GLU-433	4.81	4.561
ASP-118	5.33	<0.000	GLU-286	4.64	3.127	TYR-438	10.14	>12.000
TYR-119	17.57	>12.000	ASP-289	2.81	2.542	ARG-441	12.83	>12.000
TYR-120	12.7	>12.000	ARG-299	13.5	>12.000	ASP-446	4.11	<0.000
LYS-121	10.57	11.446	ASP-305	3.36	3.248	LYS-450	6.49	>12.000
GLU-123	4.68	4.546	GLU-309	5.2	6.151	ASP-456	3.64	0.561
ARG-125	13.69	>12.000	ARG-310	15.29	>12.000	ARG-460	13.64	>12.000
ASP-127	4.0	3.142	TYR-311	12.66	>12.000	ARG-463	12.33	>12.000
ARG-130	14.06	>12.000	GLU-314	3.46	<0.000	ASP-465	2.19	2.823
ASP-136	3.85	2.971	TYR-318	22.84	>12.000	ASP-470	3.04	2.862
ASP-138	3.43	3.319	ASP-319	4.46	<0.000	TYR-473	13.47	>12.000
ASP-141	4.34	4.666	ARG-323	13.07	>12.000	ARG-485	12.34	>12.000
ASP-144	3.25	3.510	ASP-326	4.94	4.012	TYR-487	14.21	>12.000
LYS-148	10.64	11.396	HIS-328	6.97	9.197	TYR-502	10.93	11.684
ARG-150	13.86	>12.000	ASP-329	3.39	2.427	ARG-503	11.77	>12.000
GLU-152	4.99	1.029	ASP-330	4.01	4.131	ASP-507	3.93	2.678
ASP-154	4.31	3.723	ASP-342	3.11	0.837	LYS-510	10.63	10.246

4.2 MM parameters of non-standard residues

Table S 2: Atom types and charges of 17-MT

Atom name	x coord	y coord	z coord	AMBER atom type	charge
C1	-47.4343	-13.4170	39.4166	c3	0.368358
C2	-48.5942	-14.3963	39.8152	c3	-0.183339
C3	-49.8146	-14.0734	38.9085	c3	-0.140011
C4	-49.2680	-13.0592	37.8837	c3	-0.025215
C5	-48.2006	-12.2597	38.6688	c3	0.182344
C6	-48.8245	-11.3306	39.7367	c3	-0.319715
C7	-47.3961	-11.4189	37.6641	c3	-0.014596
C8	-48.3175	-10.5081	36.8245	c3	-0.173206
C9	-49.4559	-11.2796	36.1126	c3	0.004675
C10	-50.2533	-12.1848	37.0952	c3	-0.030842
C11	-51.2714	-13.0327	36.3213	c3	-0.073365
C12	-52.2112	-12.1563	35.4819	c3	-0.037738
C13	-51.4624	-11.2117	34.5726	c2	0.057068
C14	-51.7908	-11.1264	33.2667	ce	-0.411307
C15	-51.2206	-10.1454	32.3280	c	0.625409
O1	-51.5354	-10.1236	31.1456	o	-0.505666
C16	-50.2722	-9.1271	32.9363	c3	-0.307608
C17	-49.4832	-9.7175	34.1084	c3	0.042530
C18	-50.3655	-10.3549	35.2152	c3	0.137544
C19	-51.0495	-9.2359	36.0451	c3	-0.299956
H1	-48.2696	-15.4358	39.7115	hc	0.054870
H2	-48.8397	-14.2609	40.8736	hc	0.054870
H3	-50.2233	-14.9676	38.4298	hc	0.053253
H4	-50.6301	-13.6302	39.4925	hc	0.053253
H5	-48.7185	-13.6405	37.1267	hc	0.032234
H6	-48.0339	-10.7840	40.2605	hc	0.070106
H7	-49.4081	-11.8793	40.4829	hc	0.070106
H8	-49.4963	-10.5877	39.3026	hc	0.070106
H9	-46.6423	-10.8108	38.1777	hc	0.008127
H10	-46.8529	-12.0826	36.9826	hc	0.008127
H11	-48.7401	-9.7272	37.4655	hc	0.058512
H12	-47.7038	-9.9880	36.0824	hc	0.058512
H13	-48.9694	-11.9739	35.4081	hc	0.010677
H14	-50.8109	-11.5535	37.8019	hc	0.044180
H15	-50.7343	-13.7277	35.6599	hc	0.032561
H16	-51.8622	-13.6463	37.0108	hc	0.032561
H17	-52.8980	-12.7709	34.8930	hc	0.041323
H18	-52.8361	-11.5619	36.1658	hc	0.041323
H19	-52.5604	-11.7719	32.8485	ha	0.151083
H20	-49.6098	-8.7534	32.1498	hc	0.090629
H21	-48.8102	-10.4911	33.7152	hc	0.012863
H22	-51.7241	-8.6456	35.4177	hc	0.081255
H23	-50.3087	-8.5522	36.4692	hc	0.081255
H24	-51.6441	-9.6373	36.8705	hc	0.081255
H25	-50.8786	-8.2713	33.2661	hc	0.090629
H26	-48.8444	-8.9427	34.5448	hc	0.012863
O2	-46.6867	-12.9675	40.5494	oh	-0.621754
H27	-47.3096	-12.6419	41.2115	ho	0.378715
C20	-46.3815	-14.1265	38.5597	c3	-0.265460
H28	-45.5578	-13.4502	38.3200	hc	0.072201
H29	-46.7975	-14.5195	37.6280	hc	0.072201
H30	-45.9695	-14.9651	39.1288	hc	0.072201

Table S 3: MM parameters for 17MT

Nonbon			Bonds			Angles			Dihedrals				Dihedrals			
c	1.908	0.086	c2-ce	547.3	1.346	c2-c3-c3	63.4	111.56	c2-ce-c-o	2.175	180.0	2	oh-c3-c3-hc	0.25	0.0	1
c2	1.908	0.086	c2-c3	326.8	1.51	c2-ce-ha	49.6	119.94	c3-c3-c3-oh	0.156	0.0	3	c3-c3-oh-ho	0.16	0.0	3
ce	1.908	0.086	c3-c2	326.8	1.51	o-c-c3	67.4	123.2	o-c-c3-hc	0.8	0.0	1	ho-oh-c3-c3	0.25	0.0	1
hc	1.487	0.0157	oh-c3	316.7	1.423	c2-c3-hc	47.0	110.36	c-c3-c3-c3	0.156	0.0	3	c3-c2-ce-c	6.65	180.0	2
o	1.6612	0.21	c-o	637.7	1.218	oh-c3-c3	67.5	110.19	ho-oh-c3-c3	0.16	0.0	3	oh-c3-c3-hc	0.0	0.0	3
ha	1.459	0.015	c3-hc	330.6	1.097	c3-c2-ce	64.0	123.15	oh-c3-c3-oh	0.144	0.0	3	c3-c3-c2-c3	0.0	0.0	2
c3	1.908	0.1094	oh-ho	371.4	0.973	c3-c3-oh	67.5	110.19	hc-c3-c3-c3	0.16	0.0	3	c3-c3-c3-c3	0.2	180.0	1
ho	0	0	c3-c3	300.9	1.538	ce-c-c3	62.9	116.44	c3-c3-c3-hc	0.16	0.0	3	o-c-ce-ha	2.175	180.0	2
oh	1.721	0.2104	ce-c	354.5	1.482	ho-oh-c3	47.4	107.26	o-c-c3-hc	0.0	0.0	2	ce-c2-c3-c3	0.0	0.0	2
			ce-ha	342.5	1.088	c2-ce-c	65.5	120.42	c2-c3-c3-c3	0.156	0.0	3	c3-c2-c3-c3	0.0	0.0	2
			c3-oh	316.7	1.423	c3-c2-c3	62.9	115.65	o-c-c3-hc	0.08	180.0	3	c3-c3-c3-c3	0.25	180.0	2
			c-c3	313.0	1.524	ce-c2-c3	64.0	123.15	ce-c-c3-hc	0.0	180.0	2	c-ce-c2-c3	6.65	180.0	2
						c3-oh-ho	47.4	107.26	o-c-c3-c3	0.0	180.0	2	oh-c3-c3-c3	0.156	0.0	3
						c3-c3-c2	63.4	111.56	hc-c3-c3-oh	0.25	0.0	1	hc-c3-c3-hc	0.15	0.0	3
						c-c3-c3	63.3	111.04	c3-c3-c3-c2	0.156	0.0	3	c2-c3-c3-hc	0.156	0.0	3
						ce-c-o	68.8	123.2	hc-c3-c3-oh	0.0	0.0	3	ce-c-c3-c3	0.0	180.0	2
						c-ce-ha	46.5	116.46	c-c3-c3-hc	0.156	0.0	3	c3-c3-c2-ce	0.0	0.0	2
						c-c3-hc	46.9	108.77	c3-c3-c3-c3	0.18	0.0	3	c2-ce-c-c3	2.175	180.0	2
						c3-c3-hc	46.3	109.8	c3-c-ce-ha	2.175	180.0	2	c3-c3-oh-ho	0.25	0.0	1
						hc-c3-hc	39.4	107.58	oh-c3-c3-oh	1.175	0.0	2	Impropers			
						c3-c3-c3	62.9	111.51	ce-c2-c3-hc	0.0	0.0	2	c3-ce-c-o	10.5	180.0	2
									c3-c2-c3-hc	0.0	0.0	2	c-c2-ce-ha	1.1	180.0	2
									c3-c2-ce-ha	6.65	180.0	2	c3-c3-c2-ce	1.1	180.0	2

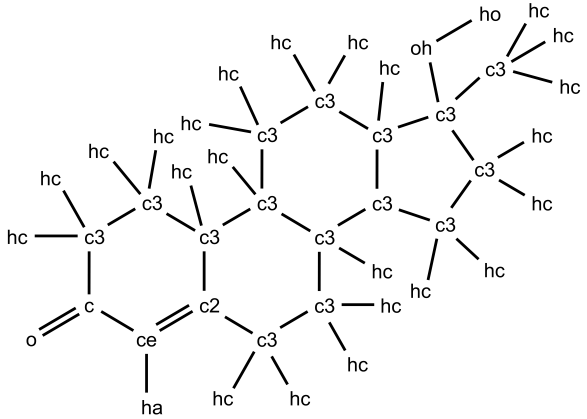


Figure S4: 17MT structure and atom types.

Table S 4: Atom types and charges of DHT

Atom name	x coord	y coord	z coord	AMBER atom type	charge
C1	74.1034	31.0552	43.1953	c3	0.257735
C2	74.8885	31.1937	44.5327	c3	-0.254944
C3	74.3486	32.4806	45.2228	c3	-0.029405
C4	73.1712	32.9164	44.3234	c3	0.010159
C5	73.5995	32.4935	42.8989	c3	0.123720
C6	74.7636	33.3293	42.3274	c3	-0.335742
C7	72.3719	32.5859	41.9817	c3	-0.062103
C8	71.7424	33.9931	42.0188	c3	-0.145315
C9	71.4055	34.4820	43.4503	c3	0.032464
C10	72.6273	34.3487	44.4112	c3	0.019151
C11	72.2449	34.7266	45.8514	c3	-0.075114
C12	71.5710	36.0990	45.9408	c3	-0.120649
C13	70.3647	36.1842	44.9968	c3	0.180131
C14	69.5793	37.4994	45.1877	c3	-0.305659
C15	68.3456	37.5683	44.3013	c	0.542985
O1	67.2575	37.9124	44.7225	o	-0.483056
C16	68.5716	37.1774	42.8490	c3	-0.216899
C17	69.4164	35.8951	42.6953	c3	0.038091
C18	70.7402	35.9034	43.5060	c3	0.131804
C19	71.6848	36.9841	42.9314	c3	-0.319106
H1	74.7684	30.3014	45.1539	hc	0.072301
H2	75.9609	31.2767	44.3201	hc	0.072301
H3	74.0350	32.2988	46.2539	hc	0.025273
H4	75.1206	33.2578	45.2610	hc	0.025273
H5	72.3262	32.2568	44.5848	hc	-0.006993
H6	75.0555	32.9313	41.3496	hc	0.081655
H7	75.6470	33.3165	42.9739	hc	0.081655
H8	74.4944	34.3774	42.1794	hc	0.081655
H9	72.6326	32.3193	40.9504	hc	0.021437
H10	71.6272	31.8489	42.3139	hc	0.021437
H11	72.4202	34.7039	41.5323	hc	0.054212
H12	70.8334	33.9820	41.4093	hc	0.054212
H13	70.6446	33.7864	43.8432	hc	-0.015720
H14	73.4176	35.0396	44.0825	hc	0.018803
H15	71.5561	33.9643	46.2467	hc	0.024321
H16	73.1334	34.7000	46.4936	hc	0.024321
H17	71.2421	36.2890	46.9703	hc	0.035255
H18	72.2919	36.8916	45.7004	hc	0.035255
H19	67.5970	37.0738	42.3641	hc	0.064777
H20	68.8131	35.0366	43.0215	hc	-0.002658
H21	71.2707	37.9907	43.0336	hc	0.073713
H22	71.8575	36.8206	41.8632	hc	0.073713
H23	72.6607	36.9897	43.4237	hc	0.073713
H24	69.0843	38.0173	42.3616	hc	0.064777
H25	69.6197	35.7422	41.6315	hc	-0.002658
H26	73.2249	30.4171	43.3516	h1	0.052841
O2	74.8201	30.4116	42.1534	oh	-0.620352
H27	75.6768	30.8500	42.0700	ho	0.387925
H28	70.2208	38.3595	44.9478	hc	0.077459
H29	69.2591	37.6179	46.2266	hc	0.077459
H30	69.6805	35.3710	45.2885	hc	-0.015611

Table S 5: MM parameters for DHT

Nonbon			Bonds			Angles			Dihedrals				Dihedrals			
oh	1.721	0.2104	oh-c3	316.7	1.423	c3-c3-c3	62.9	111.51	c3-c3-c3-c3	0.2	180.0	1	o-c-c3-hc	0.0	0.0	2
ho	0	0	c3-c3	300.9	1.538	c-c3-hc	46.9	108.77	hc-c3-c3-oh	0.0	0.0	3	ho-oh-c3-c3	0.16	0.0	3
c3	1.908	0.1094	oh-ho	371.4	0.973	c3-c3-h1	46.4	109.56	c3-c3-c3-c3	0.18	0.0	3	o-c-c3-hc	0.8	0.0	1
h1	1.387	0.0157	c-c3	313.0	1.524	c3-c3-c	63.3	111.04	oh-c3-c3-oh	1.175	0.0	2	c3-c3-c3-c	0.156	0.0	3
o	1.6612	0.21	c3-hc	330.6	1.097	c3-c-o	67.4	123.2	c3-c3-oh-ho	0.16	0.0	3	oh-c3-c3-h1	0.0	0.0	3
c	1.908	0.086	c3-oh	316.7	1.423	o-c-c3	67.4	123.2	o-c-c3-c3	0.0	180.0	2	c3-c3-c-c3	0.0	180.0	2
hc	1.487	0.0157	c3-h1	330.6	1.097	c3-c3-hc	46.3	109.8	c3-c3-c3-hc	0.16	0.0	3	oh-c3-c3-h1	0.25	0.0	1
			c-o	637.7	1.218	c3-c-c3	62.0	116.5	h1-c3-oh-ho	0.167	0.0	3	oh-c3-c3-oh	0.144	0.0	3
			c3-c	313.0	1.524	c3-oh-ho	47.4	107.26	ho-oh-c3-c3	0.25	0.0	1	oh-c3-c3-c3	0.156	0.0	3
						ho-oh-c3	47.4	107.26	hc-c3-c3-hc	0.15	0.0	3	c3-c-c3-c3	0.0	180.0	2
						h1-c3-oh	50.9	110.26	o-c-c3-hc	0.08	180.0	3	c-c3-c3-hc	0.156	0.0	3
						oh-c3-h1	50.9	110.26	h1-c3-c3-c3	0.156	0.0	3	h1-c3-c3-oh	0.0	0.0	3
						h1-c3-h1	39.2	108.46	c3-c3-c3-h1	0.156	0.0	3	h1-c3-c3-oh	0.25	0.0	1
						c3-c3-oh	67.5	110.19	h1-c3-c3-h1	0.156	0.0	3	hc-c3-c3-h1	0.156	0.0	3
						hc-c3-hc	39.4	107.58	c-c3-c3-c3	0.156	0.0	3	c3-c3-c3-c3	0.25	180.0	2
						c-c3-c3	63.3	111.04	ho-oh-c3-h1	0.167	0.0	3	hc-c3-c3-oh	0.25	0.0	1
						h1-c3-c3	46.4	109.56	c3-c3-c3-oh	0.156	0.0	3	c3-c-c3-hc	0.0	180.0	2
						oh-c3-c3	67.5	110.19	c3-c3-c-o	0.0	180.0	2	Impropers			
									c3-c3-oh-ho	0.25	0.0	1	c3-c3-c-o	1.1	180.0	2

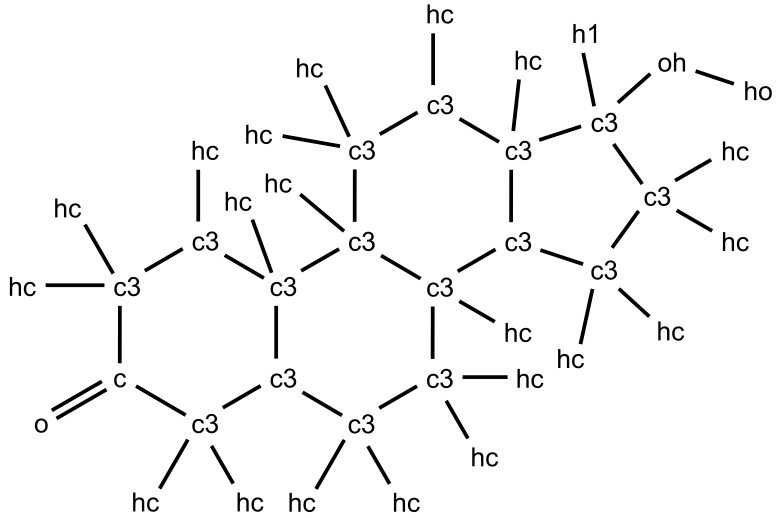


Figure S5: DHT structure and atom types.

Table S 6: Atom types and charges of FAD

Atom name	x coord	y coord	z coord	AMBER atom type	charge
O3'	-36.6980	-10.8920	22.6570	oh	-0.597900
H3T	-37.3610	-11.5690	23.1100	ho	0.422500
C1'	-36.6480	-9.3810	20.0630	c3	0.100100
H1'	-35.9420	-10.1960	19.9040	h2	0.143800
C2'	-36.5040	-8.8850	21.4370	c3	0.142600
H2'	-36.7910	-7.8370	21.5270	h1	0.052500
C3'	-37.4000	-9.7810	22.2180	c3	0.118000
H3'	-37.8690	-9.2520	23.0480	h1	0.057500
C4'	-38.4280	-10.2690	21.2200	c3	0.148400
H4'	-38.5150	-11.3540	21.2720	h1	0.074400
C5'	-39.8050	-9.6090	21.5030	c3	0.107000
H5'1	-40.3490	-9.4620	20.5700	h1	0.041700
H5'2	-39.6650	-8.6480	21.9970	h1	0.041700
O4'	-37.9530	-9.8760	19.9330	os	-0.362800
O2'	-35.1140	-9.1130	21.8370	oh	-0.606900
H2T	-35.0060	-8.9080	22.8610	ho	0.431500
N6	-34.7420	-6.4690	15.4300	nh	-0.728900
H61	-35.1530	-5.5630	15.8230	hn	0.363200
H62	-34.2240	-6.4600	14.4940	hn	0.363200
C6	-34.8730	-7.5930	16.0970	ca	0.569800
C5	-35.5400	-7.5170	17.2690	ca	0.061500
N7	-36.1390	-6.5030	17.9290	nc	-0.602900
C8	-36.6560	-6.9930	19.0500	cd	0.158100
H8	-37.2100	-6.4320	19.8030	h5	0.145100
N9	-36.3720	-8.3080	19.0870	na	-0.029100
C4	-35.6950	-8.6200	17.9920	ca	0.460700
N3	-35.2050	-9.7940	17.5600	nb	-0.753200
C2	-34.5270	-9.8970	16.3310	ca	0.550300
H2	-34.1580	-10.8580	15.9730	h5	0.038400
N1	-34.3390	-8.7240	15.5780	nb	-0.734900
O5A	-40.5280	-10.5000	22.3580	os	-0.411100
PA	-41.4820	-9.9730	23.4350	p5	1.040000
OA1	-40.7670	-8.8800	24.1350	o	-0.790800
OA2	-41.8820	-11.1440	24.2370	o	-0.790800
OP	-42.6890	-9.2030	22.5450	os	-0.445800
PB	-44.2010	-9.8180	22.1960	p5	1.040000
OB1	-44.9790	-8.6560	21.7040	o	-0.790800
OB2	-44.2000	-11.0710	21.4410	o	-0.790800
O5B	-44.6640	-10.2210	23.6080	os	-0.411100
C1	-47.0510	-8.4520	28.9110	c3	-0.045300
H11	-48.0490	-8.2150	28.5410	h1	0.116500
H12	-46.5050	-7.5280	29.1020	h1	0.116500
C2	-46.3130	-9.2800	27.8920	c3	0.191800
H2	-46.9650	-10.1040	27.6000	h1	0.057700
O2	-45.1110	-9.8470	28.4390	oh	-0.589400
HO2	-44.4750	-9.0760	28.7620	ho	0.371700
C3	-45.9650	-8.4910	26.6270	c3	0.019700
H3	-45.0420	-7.9270	26.7600	h1	0.124600
O3	-47.0450	-7.6280	26.2630	oh	-0.645000
HO3	-47.9060	-8.2010	26.0810	ho	0.426400
C4	-45.7880	-9.5810	25.6130	c3	0.113600
H4	-46.7560	-9.8560	25.1940	h1	0.134500
O4	-45.2000	-10.7320	26.3010	oh	-0.576000
HO4	-44.2730	-10.4590	26.7110	ho	0.402000
C5	-44.8610	-9.1220	24.5050	c3	-0.036900
H51	-45.3100	-8.2850	23.9710	h1	0.085700
H52	-43.9050	-8.8140	24.9270	h1	0.085700
N1	-48.7040	-10.6800	29.3680	nd	-0.518600

C2	-49.4920	-11.8140	29.6510	c	0.608100
O2	-50.2690	-12.1950	28.7860	o	-0.569600
N3	-49.5000	-12.4960	30.8300	n	-0.321300
H3	-50.1160	-13.2620	30.9300	hn	0.310500
C4	-48.7360	-12.1290	31.7620	c	0.372300
O4	-48.7680	-12.6750	32.8740	o	-0.520700
N10	-47.1450	-9.1440	30.1160	na	0.061400
C10A	-47.9410	-10.2700	30.2670	cc	0.165400
C4A	-47.9170	-10.9880	31.5170	cc	0.437200
N5	-47.2000	-10.6290	32.4720	nd	-0.554900
C5A	-46.3770	-9.6290	32.3860	ca	0.301200
C9A	-46.2580	-8.8720	31.2290	ca	-0.004200
C9	-45.2480	-7.9160	31.2020	ca	-0.295800
H9	-45.0880	-7.3270	30.3390	ha	0.165200
C8	-44.4140	-7.7450	32.3040	ca	0.116400
C7	-44.5950	-8.4810	33.4730	ca	0.065200
C6	-45.5900	-9.4260	33.4990	ca	-0.216000
H6	-45.7320	-10.0370	34.3590	ha	0.158700
C7M	-43.7450	-8.3450	34.7110	c3	-0.227400
H7M1	-43.2030	-7.3960	34.7380	hc	0.084500
H7M2	-43.0160	-9.1780	34.7160	hc	0.084500
H7M3	-44.3800	-8.4740	35.6010	hc	0.084500
C8M	-43.3410	-6.7820	32.2010	c3	-0.208300
H8M1	-43.4030	-6.2230	31.2570	hc	0.081100
H8M2	-42.3860	-7.3210	32.2650	hc	0.081100
H8M3	-43.3810	-6.1090	33.0670	hc	0.081100

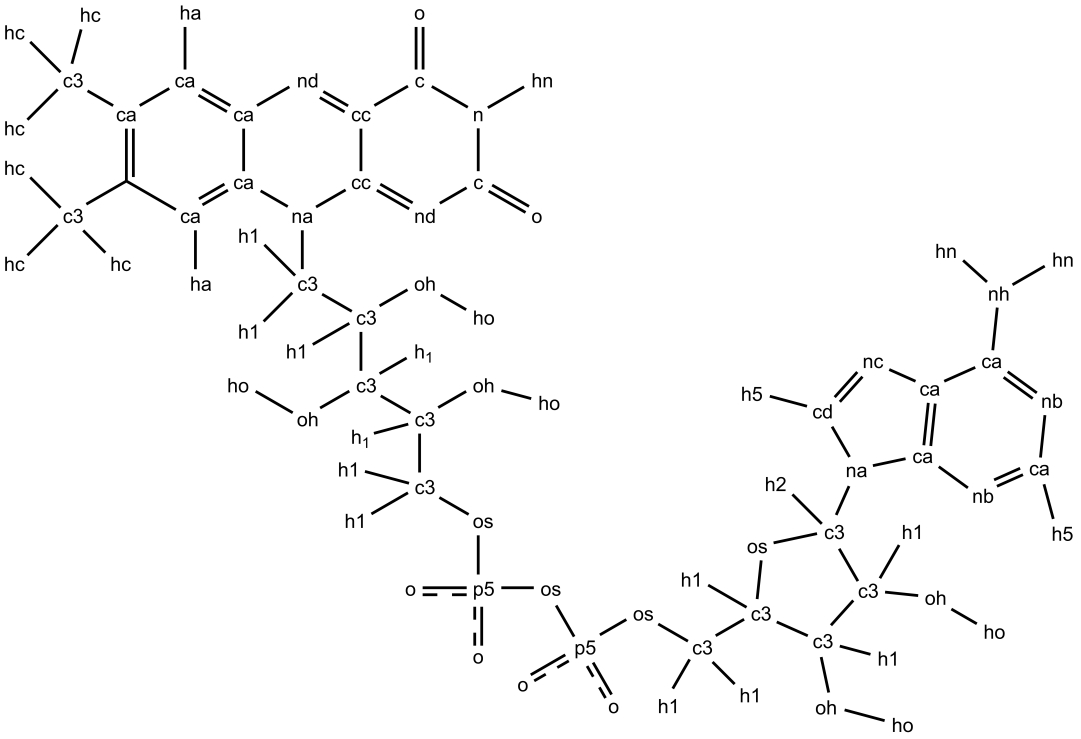


Figure S6: FAD structure and atom types.

Table S 7: MM parameters for FAD

Nonbon			Bonds			Angles			Dihedrals				Dihedrals			
nb	1.824	0.17	c-o	637.7	1.218	h1-c3-os	50.8	109.78	c3-os-c3-na	0.383	0.0	3	c3-c3-oh-ho	0.16	0.0	3
nc	1.824	0.17	oh-c3	316.7	1.423	c-n-c	63.7	127.08	os-c3-c3-oh	0.144	0.0	3	ca-nc-cd-h5	4.75	180.0	2
nh	1.824	0.17	c3-os	308.6	1.432	c-cc-cc	63.6	122.69	c3-os-p5-os	1.2	0.0	2	os-c3-c3-oh	1.175	0.0	2
oh	1.721	0.2104	cd-h5	351.8	1.082	ca-ca-c3	63.5	120.77	c3-os-c3-c3	0.1	180.0	2	o-c-cc-cc	2.875	180.0	2
p5	2.1	0.2	ca-ha	345.8	1.086	o-c-cc	69.1	123.93	o-c-cc-nd	2.875	180.0	2	h1-c3-na-cc	0.0	0.0	2
ho	0	0	ca-ca	461.1	1.398	ca-ca-nb	68.8	122.94	os-p5-os-p5	0.8	0.0	2	c3-na-cc-cc	1.7	180.0	2
hc	1.487	0.0157	nd-c	416.9	1.387	h1-c3-h1	39.2	108.46	nh-ca-nb-ca	4.8	180.0	2	na-ca-nb-ca	4.8	180.0	2
na	1.824	0.17	c3-hc	330.6	1.097	h1-c3-na	49.8	108.78	c3-c3-c3-hc	0.16	0.0	3	os-p5-os-c3	0.25	0.0	3
h5	1.359	0.015	c-n	427.6	1.379	ha-ca-ca	48.2	119.88	hn-nh-ca-ca	1.05	180.0	2	h1-c3-oh-ho	0.167	0.0	3
c	1.908	0.086	os-p5	330.6	1.615	nc-ca-ca	69.5	119.72	hc-c3-c3-oh	0.25	0.0	1	nd-cc-cc-nd	4.0	180.0	2
os	1.6837	0.17	cc-nd	525.4	1.317	c-c3-hc	46.9	108.77	h2-c3-na-cd	0.0	0.0	2	hc-c3-c3-hc	0.15	0.0	3
cc	1.908	0.086	nd-cc	525.4	1.317	o-c-c3	67.4	123.2	c3-os-p5-o	0.8	0.0	2	c3-c3-na-cc	0.0	0.0	2
c3	1.908	0.1094	ca-nb	488.0	1.339	p5-os-c3	77.7	119.54	c-nd-cc-na	4.75	180.0	2	c3-c3-c3-h1	0.156	0.0	3
cd	1.908	0.086	c3-oh	316.7	1.423	na-ca-nb	69.8	127.09	na-cc-cc-nd	4.0	180.0	2	hn-nh-ca-nb	1.05	180.0	2
n	1.824	0.17	c3-h1	330.6	1.097	c3-oh-ho	47.4	107.26	p5-os-c3-c3	0.383	0.0	3	ca-ca-ca-ca	3.625	180.0	2
hn	0.6	0.0157	nh-ca	417.9	1.386	nb-ca-h5	51.9	115.82	c3-c3-c3-os	0.156	0.0	3	ca-ca-nc-cd	4.8	180.0	2
ca	1.908	0.086	ca-h5	343.2	1.088	os-p5-os	45.0	101.84	ca-ca-ca-na	3.625	180.0	2	cd-nc-ca-ca	4.8	180.0	2
h1	1.387	0.0157	p5-os	330.6	1.615	c-nd-cc	66.7	120.49	ca-ca-nb-ca	4.8	180.0	2	ca-ca-c3-hc	0.0	0.0	2
nd	1.824	0.17	c3-h2	331.7	1.096	os-c3-h1	50.8	109.78	h1-c3-na-ca	0.0	0.0	2	ha-ca-ca-ca	3.625	180.0	2
o	1.6612	0.21	n-hn	403.2	1.013	cc-na-ca	67.4	113.15	ca-ca-ca-nb	3.625	180.0	2	ha-ca-ca-c3	3.625	180.0	2
ha	1.459	0.015	c3-na	327.7	1.463	nd-c-n	71.6	117.11	c-c3-c3-hc	0.156	0.0	3	n-c-cc-cc	2.875	180.0	2
h2	1.287	0.0157	oh-ho	371.4	0.973	na-cc-cc	68.6	117.77	os-c3-na-cd	0.0	0.0	2	na-ca-ca-ca	3.625	180.0	2
			c-c3	313.0	1.524	nd-cc-cc	71.6	112.56	n-c-cc-nd	2.875	180.0	2	oh-c3-c3-hc	0.25	0.0	1
			ca-nc	467.7	1.352	oh-c3-h1	50.9	110.26	p5-os-c3-h1	0.383	0.0	3	o-c-n-c	2.5	180.0	2
			na-ca	420.5	1.384	n-c-o	74.2	123.05	nd-ca-ca-ha	3.625	180.0	2	cc-na-ca-ca	0.3	180.0	2
			cc-cc	419.8	1.428	ca-c3-hc	46.8	110.47	c3-na-ca-nb	0.3	180.0	2	o-c-c3-c3	0.0	180.0	2
			c-cc	371.0	1.468	hn-n-c	48.3	117.55	nd-c-n-hn	2.5	180.0	2	na-ca-ca-nd	3.625	180.0	2
			os-c3	308.6	1.432	n-c-cc	69.1	112.7	ca-nb-ca-h5	4.8	180.0	2	c3-c3-c3-na	0.156	0.0	3
			c3-c3	300.9	1.538	ca-ca-na	69.1	118.34	oh-c3-c3-hc	0.0	0.0	3	oh-c3-c3-na	0.156	0.0	3
			p5-o	479.5	1.487	c3-c3-h1	46.4	109.56	c3-c3-os-p5	3.95	180.0	1	nc-ca-ca-na	3.625	180.0	2
			n-c	427.6	1.379	h2-c3-na	50.2	107.31	nd-c-n-c	2.5	180.0	2	os-c3-c3-os	1.175	0.0	2
			nc-cd	525.4	1.317	nb-ca-nb	70.9	127.26	oh-c3-c3-oh	0.144	0.0	3	o-c-nd-cc	4.0	180.0	2
			nh-hn	404.6	1.012	nd-ca-ca	69.5	119.72	cc-cc-na-ca	1.7	180.0	2	c3-na-cd-h5	1.7	180.0	2
			cd-na	425.8	1.38	p5-os-p5	98.4	126.25	ca-nc-cd-na	4.75	180.0	2	c-nd-cc-cc	4.75	180.0	2
			ca-c3	321.0	1.516	c3-na-cd	61.9	126.46	c3-os-c3-na	0.65	0.0	2	c-cc-cc-na	4.0	180.0	2
			na-cc	425.8	1.38	nc-cd-na	74.9	112.22	c3-os-c3-c3	0.383	0.0	3	os-c3-na-ca	0.0	0.0	2
			nd-ca	467.7	1.352	h1-c3-c3	46.4	109.56	c3-c3-c3-c3	0.18	0.0	3	o-c-c3-hc	0.08	180.0	3
			nb-ca	488.0	1.339	c-cc-nd	67.6	121.88	o-p5-os-c3	0.8	0.0	2	h1-c3-c3-h1	0.156	0.0	3
						h5-ca-nb	51.9	115.82	h2-c3-c3-h1	0.156	0.0	3	hc-c3-c3-c3	0.16	0.0	3
						oh-c3-c3	67.5	110.19	oh-c3-c3-os	0.144	0.0	3	c3-na-cc-nd	1.7	180.0	2
						na-ca-ca	69.1	118.34	p5-os-p5-o	0.8	0.0	2	nd-ca-ca-ca	3.625	180.0	2

ca-ca-ha	48.2	119.88	c-c3-c3-c3	0.156	0.0	3	cd-na-ca-nb	0.3	180.0	2
c3-c3-na	65.5	112.88	c3-c3-os-c3	0.1	180.0	2	c3-c3-na-ca	0.0	0.0	2
cd-na-ca	67.4	113.15	c3-os-p5-o	0.55	0.0	3	o-c-n-hn	2.5	180.0	2
ca-nb-ca	68.3	117.22	o-c-c3-hc	0.0	0.0	2	h1-c3-c3-na	0.156	0.0	3
hc-c3-hc	39.4	107.58	h1-c3-os-p5	0.383	0.0	3	oh-c3-c3-oh	1.175	0.0	2
nd-c-o	73.9	123.18	ca-nb-ca-nb	4.8	180.0	2	ho-oh-c3-h1	0.167	0.0	3
os-c3-c3	68.0	107.97	nc-cd-na-ca	1.7	180.0	2	p5-os-p5-os	0.8	0.0	2
c3-c3-oh	67.5	110.19	h5-cd-na-ca	1.7	180.0	2	hn-n-c-cc	2.5	180.0	2
ca-nc-cd	72.5	104.88	nd-cc-na-ca	1.7	180.0	2	ho-oh-c3-c3	0.25	0.0	1
c3-c3-hc	46.3	109.8	h2-c3-c3-c3	0.156	0.0	3	os-c3-c3-h1	0.0	0.0	3
o-c-n	74.2	123.05	c3-os-p5-os	0.25	0.0	3	oh-c3-c3-c3	0.156	0.0	3
c3-na-cc	61.9	126.46	h2-c3-na-ca	0.0	0.0	2	c3-na-cd-nc	1.7	180.0	2
o-p5-os	43.9	115.46	os-p5-os-c3	1.2	0.0	2	ca-ca-ca-ha	3.625	180.0	2
h2-c3-c3	46.2	110.22	oh-c3-c3-os	1.175	0.0	2	ca-ca-ca-c3	3.625	180.0	2
hn-nh-ca	48.4	116.07	nc-ca-ca-nb	3.625	180.0	2	cc-nd-ca-ca	4.8	180.0	2
ho-oh-c3	47.4	107.26	os-c3-c3-h1	0.25	0.0	1	hn-n-c-o	2.0	0.0	1
hn-nh-hn	40.1	115.12	cc-cc-nd-ca	4.75	180.0	2	p5-os-c3-c3	3.95	180.0	1
c3-os-c3	62.7	112.48	c-n-c-cc	2.5	180.0	2	ca-ca-na-cd	0.3	180.0	2
cc-cc-nd	71.6	112.56	c3-c3-os-c3	0.383	0.0	3	nh-ca-ca-nc	3.625	180.0	2
c3-c3-c3	62.9	111.51	c3-c3-os-p5	0.383	0.0	3	h1-c3-c3-oh	0.0	0.0	3
c-c3-c3	63.3	111.04	nh-ca-ca-ca	3.625	180.0	2	h2-c3-c3-oh	0.156	0.0	3
ca-ca-ca	66.6	120.02	os-c3-c3-c3	0.156	0.0	3	c-cc-nd-ca	4.75	180.0	2
cc-nd-ca	72.5	104.88	c3-ca-ca-c3	3.625	180.0	2	h1-c3-c3-c3	0.156	0.0	3
c3-na-ca	62.3	124.36	hn-n-c-o	2.5	180.0	2	h1-c3-c3-os	0.25	0.0	1
ca-ca-nc	69.5	119.72	h1-c3-c3-oh	0.25	0.0	1	oh-c3-c3-h1	0.25	0.0	1
c3-c3-os	68.0	107.97	c3-c3-na-cd	0.0	0.0	2	c3-na-ca-ca	0.3	180.0	2
c3-os-p5	77.7	119.54	c3-c3-oh-ho	0.25	0.0	1	c3-c3-c3-c3	0.25	180.0	2
nh-ca-nb	72.7	116.94	os-c3-c3-os	0.144	0.0	3	Impropers			
nh-ca-ca	68.3	120.95	h1-c3-c3-os	0.0	0.0	3	ca-ca-ca-nd	1.1	180.0	2
h1-c3-oh	50.9	110.26	nd-cc-cc-c	4.0	180.0	2	n-cc-c-o	10.5	180.0	2
c-n-hn	48.3	117.55	oh-c3-c3-h1	0.0	0.0	3	ca-nb-ca-nh	1.1	180.0	2
o-p5-o	45.8	115.8	c3-os-c3-h1	0.383	0.0	3	c3-ca-na-cd	1.1	180.0	2
h5-cd-na	49.6	121.55	ho-oh-c3-c3	0.16	0.0	3	ca-ca-ca-na	1.1	180.0	2
h2-c3-os	50.9	109.58	o-c-n-hn	2.0	0.0	1	ca-na-ca-nb	1.1	180.0	2
nc-cd-h5	50.6	125.52	c3-c3-c3-c3	0.2	180.0	1	c3-ca-na-cc	6.1	180.0	2
os-p5-o	43.9	115.46	o-p5-os-c3	0.55	0.0	3	h5-nb-ca-nb	10.5	180.0	2
nd-cc-na	74.9	112.22	o-p5-os-p5	0.8	0.0	2	ca-ca-ca-nc	1.1	180.0	2
os-c3-na	71.3	109.03	c-n-c-o	2.5	180.0	2	ca-hn-nh-hn	1.1	180.0	2
			na-ca-ca-ha	3.625	180.0	2	h5-na-cd-nc	1.1	180.0	2
			c3-c3-c3-oh	0.156	0.0	3	ca-ca-ca-ha	1.1	180.0	2
			h2-c3-os-c3	0.383	0.0	3	c-c-n-hn	1.1	180.0	2
			n-c-nd-cc	4.0	180.0	2	ca-ca-ca-c3	1.1	180.0	2
			hc-c3-c3-oh	0.0	0.0	3	nd-n-c-o	10.5	180.0	2
			o-c-c3-hc	0.8	0.0	1	c3-ca-ca-ca	1.1	180.0	2

4.3 Root Mean Square Deviation (RMSD) plots from MD simulations

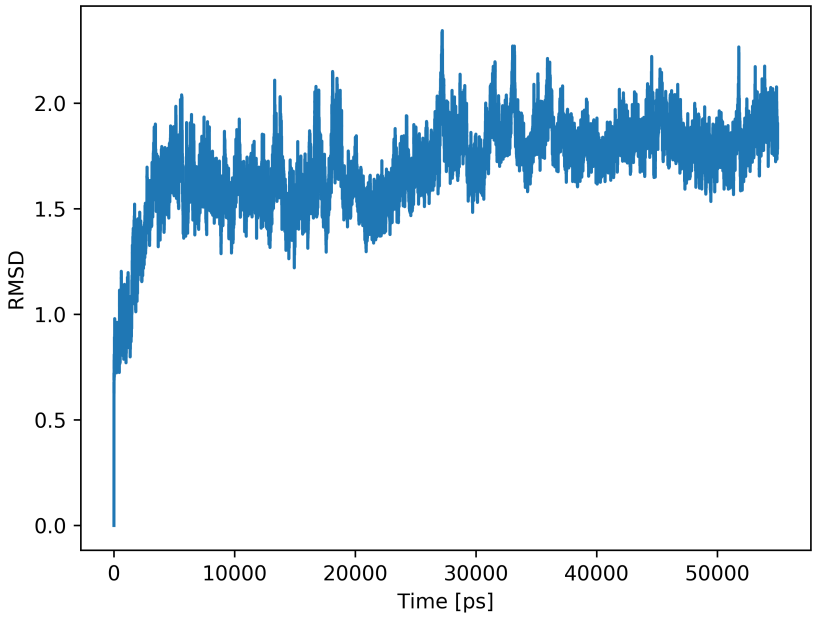


Figure S7: RMSD of KSTD with 17-MT (TYR318 is deprotonated), results from 55 ns of MD simulation.

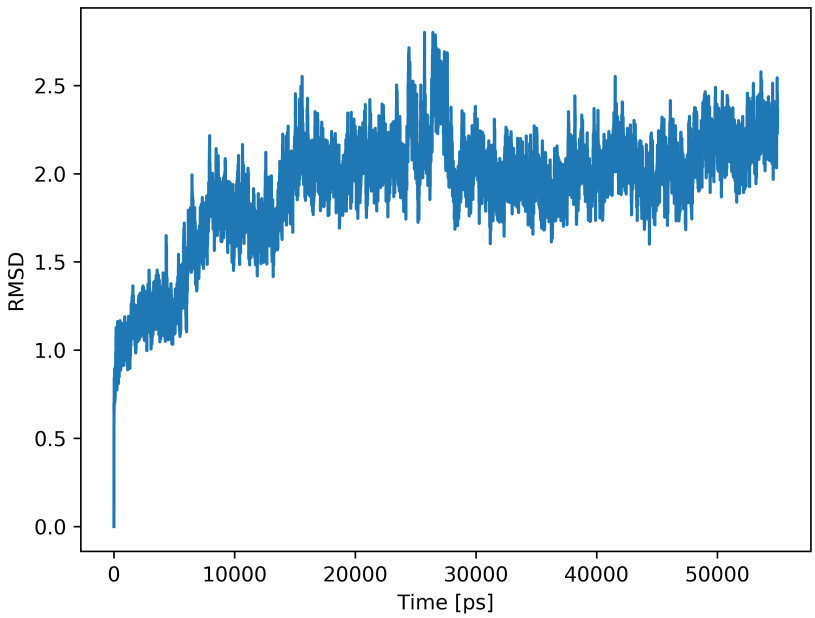


Figure S8: RMSD of KSTD with DHT (TYR318 is deprotonated), results from 55 ns of MD simulation.

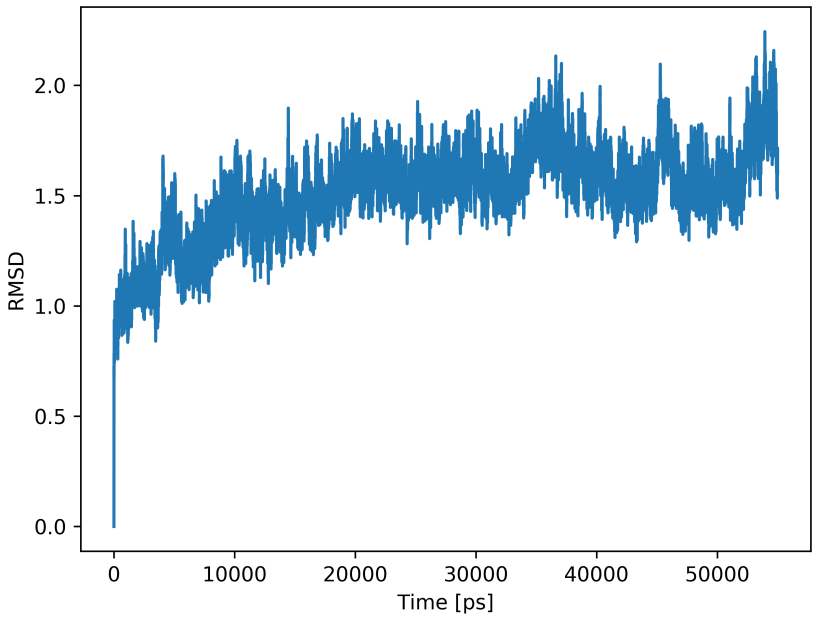


Figure S9: RMSD of KSTD with 17-MT (alternative pathway - TYR318 is protonated), results from 55 ns of MD simulation.

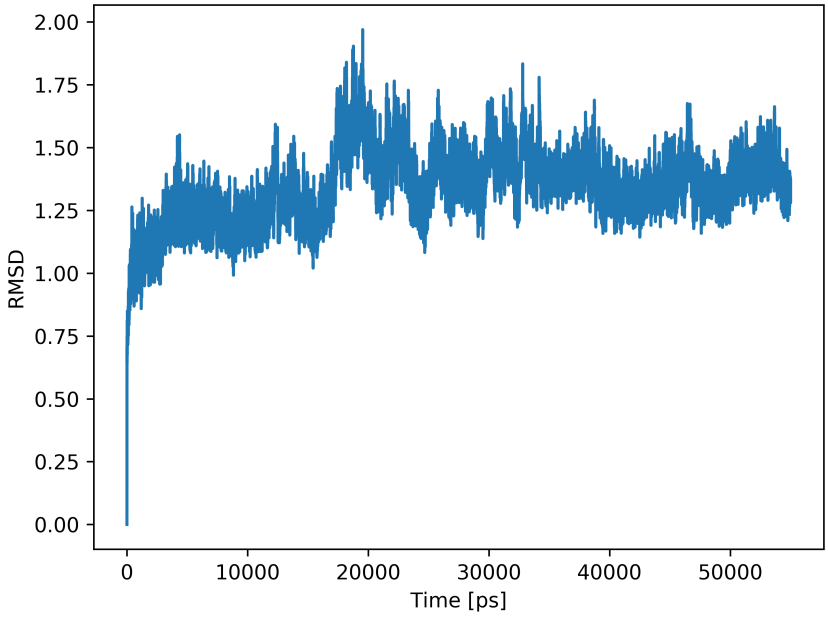


Figure S10: RMSD of KSTD with DHT (alternative pathway - TYR318 is protonated), results from 55 ns of MD simulation.

4.4 Alternative mechanism – model preparation

Model setup was identical like for the main pathway, but Tyr318 was kept in its standard protonation state. The system was neutralized by adding 32 sodium ions. Calculation was conducted for 17MT and DHT. Structure was relaxed by 55 ns of MD. QM layer was enlarged by including TYR487.

5 Results details

5.1 Two dimensional Potential Energy Surface

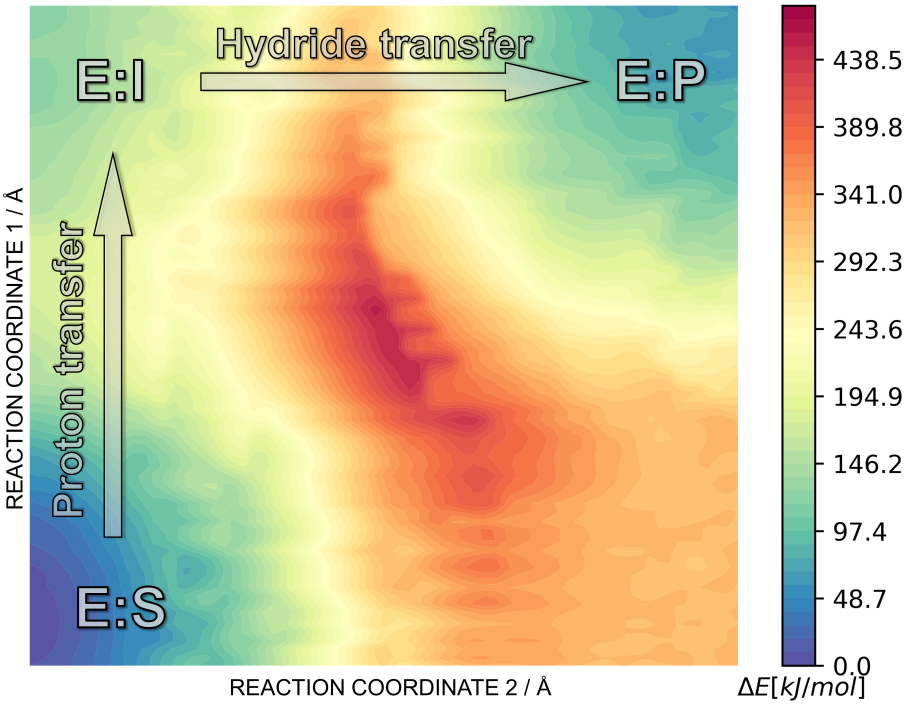


Figure S11: PES for Δ^1-dehydrogenation of 17MT at AM1/AMBER level of theory

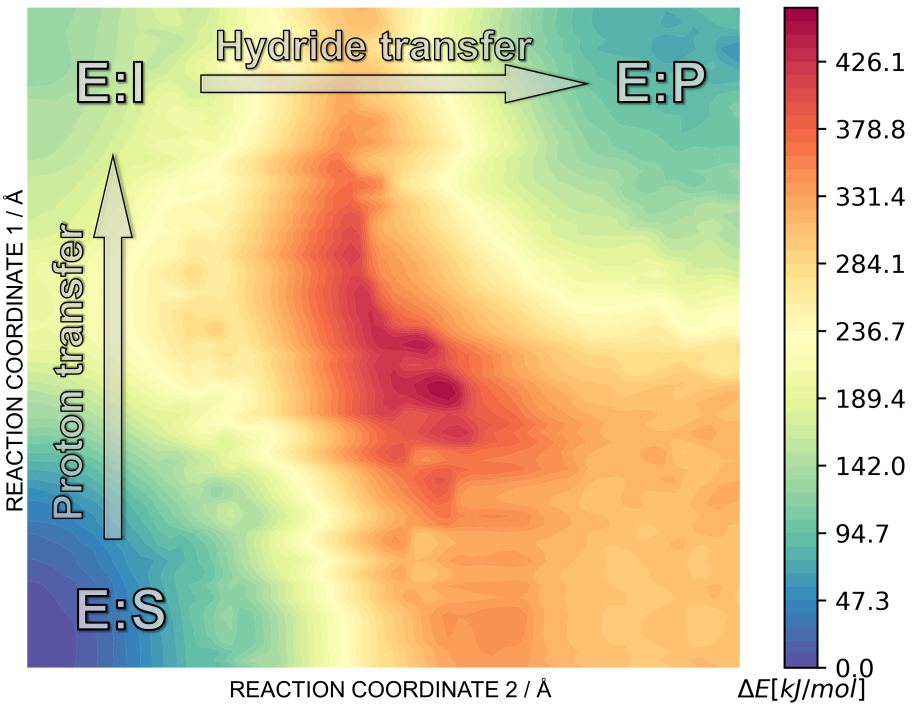


Figure S12: PES for Δ^1-dehydrogenation of DHT at AM1/AMBER level of theory

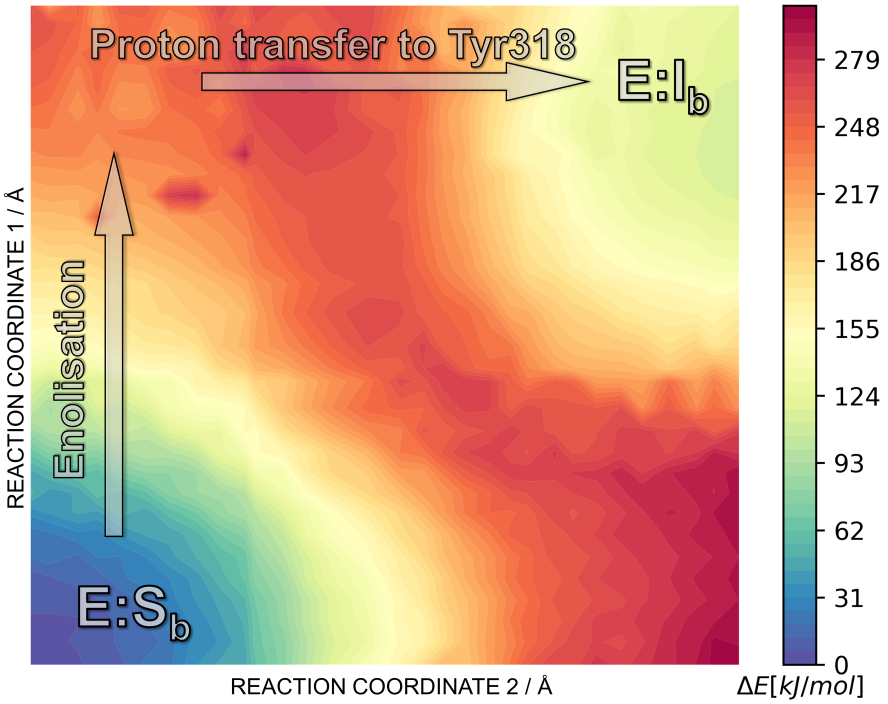


Figure S13: PES for 17MT for first step of alternative mechanism. Presented energies are obtained from single point calculations at B3LYP/AMBER level at theory. Structures were optimized at AM1/AMBER level of theory

5.2 Potential of Mean Force

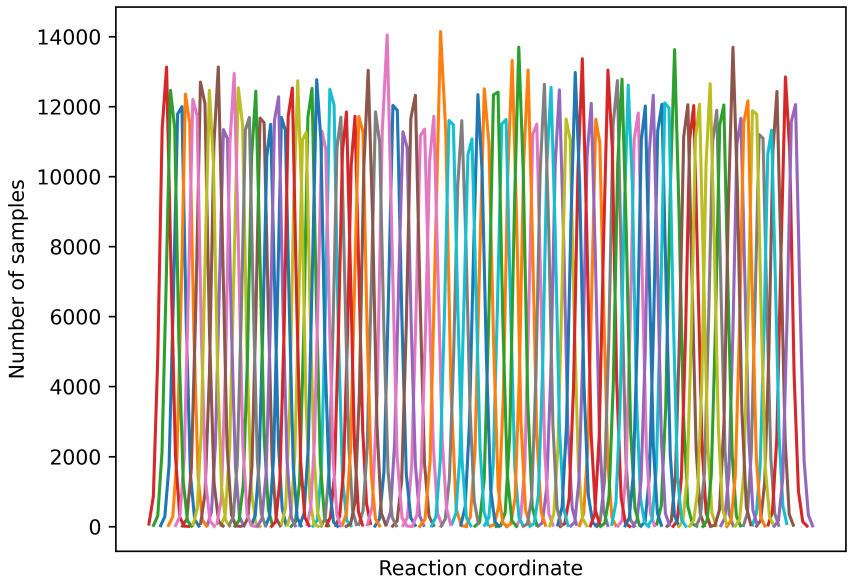


Figure S14: Reaction coordinate distribution for each window of PMF calculations of first stage for 17MT

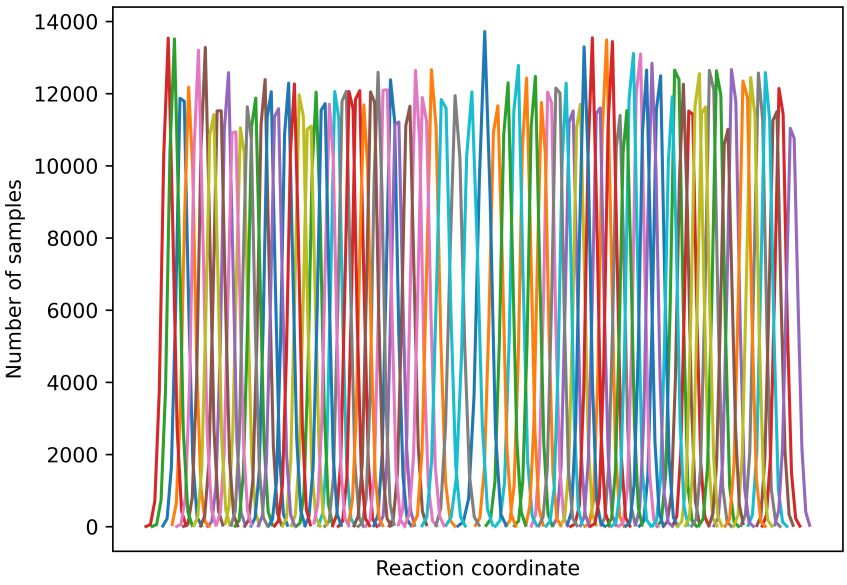


Figure S15: Reaction coordinate distribution for each window of PMF calculations of second stage for 17MT

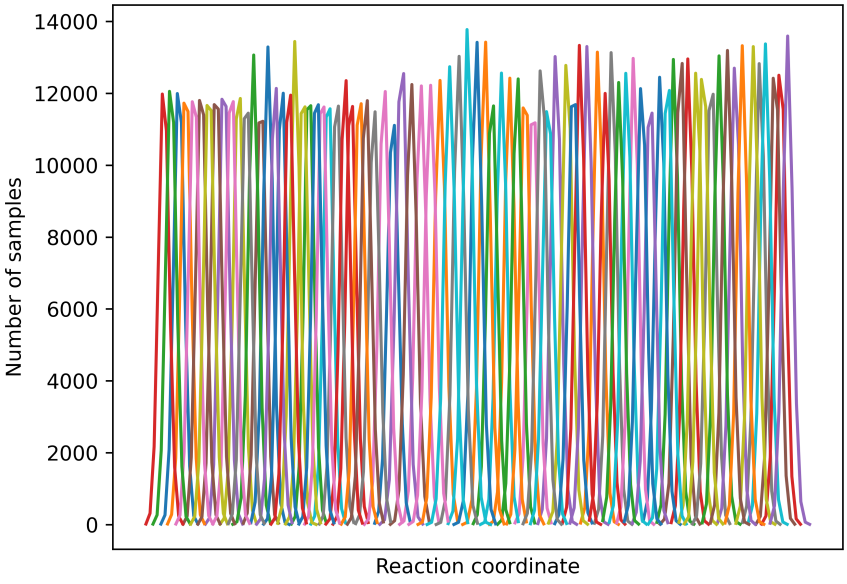


Figure S16: Reaction coordinate distribution for each window of PMF calculations of first stage for DHT

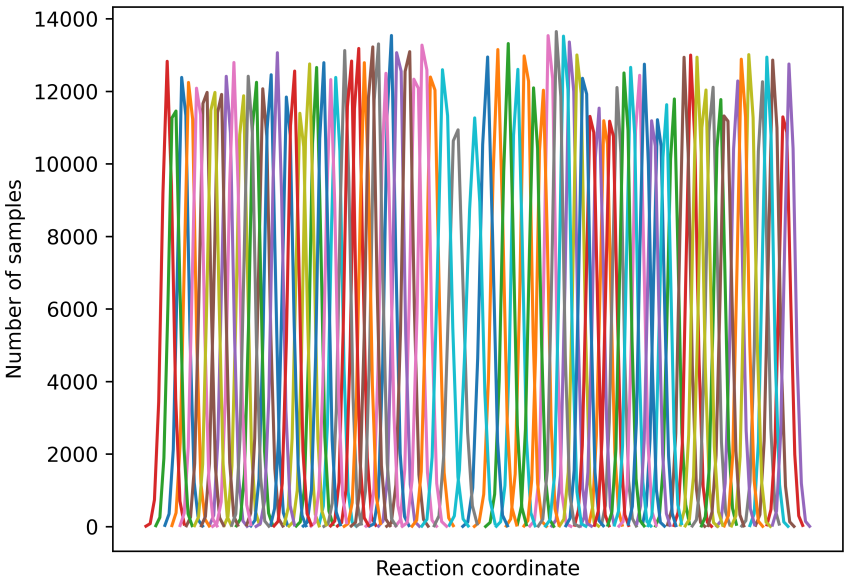


Figure S17: Reaction coordinate distribution for each window of PMF calculations of second stage for DHT

5.3 Protein identification

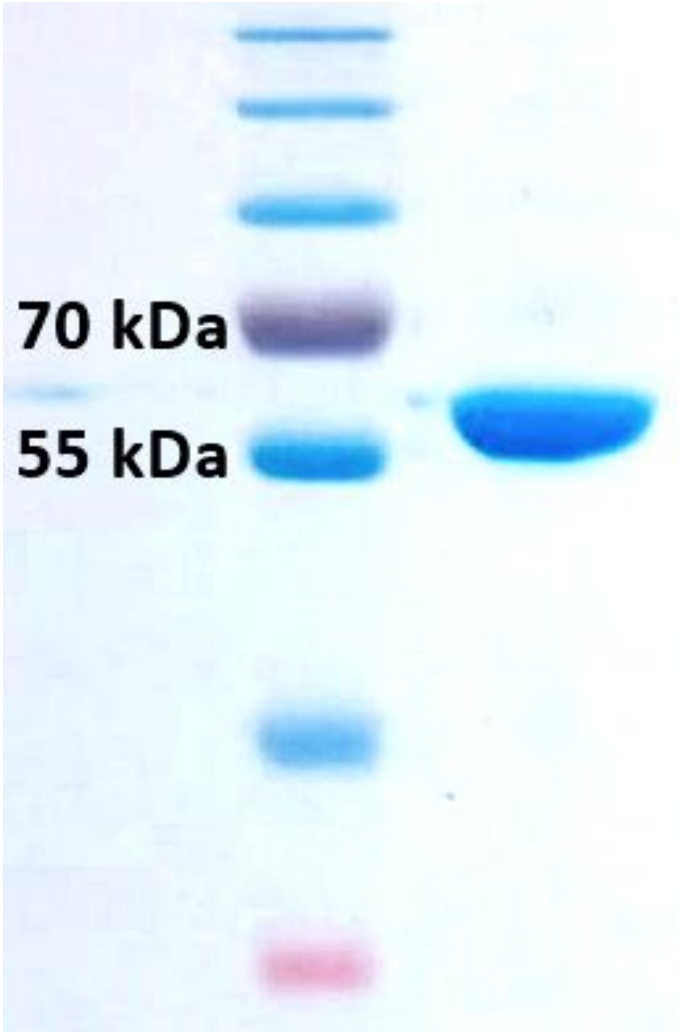


Figure S18: Polyacrylamide gel of purified his-tagged KSTD (56 kDa).

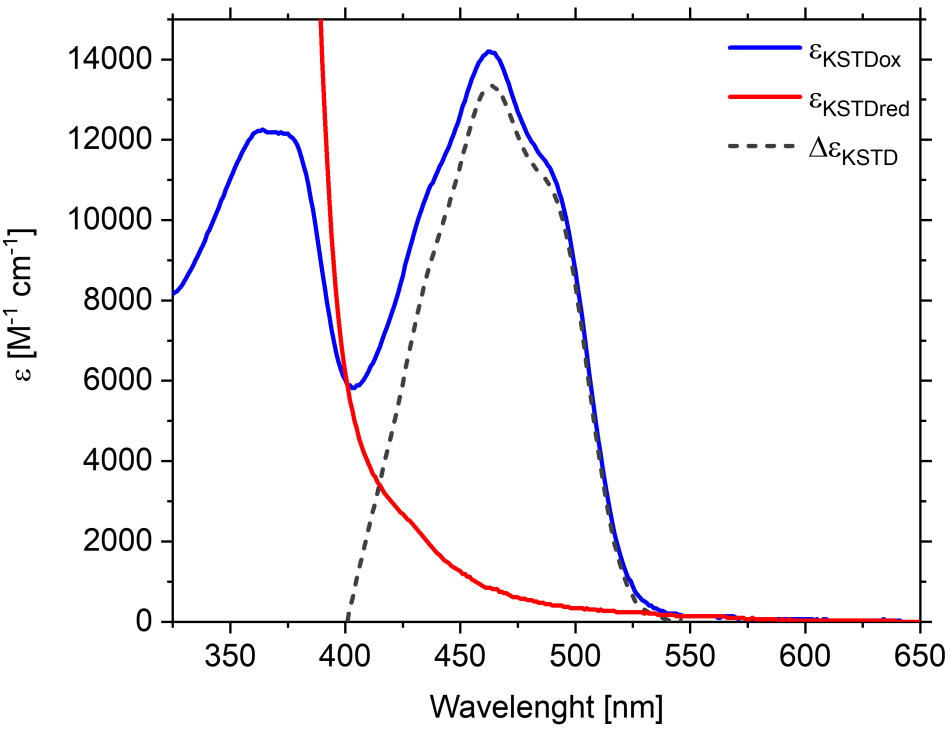


Figure S19: Plot of the molecular extinction coefficient of the oxidized form of KSTD (ε_{KSTDox}), the reduced form of KSTD ($\varepsilon_{KSTDred}$) and the difference between them ($\Delta\varepsilon_{KSTD}$).

Based on the proteomic experiment the protein was successfully identified as Chain A, Crystal Structure Of 3-ketosteroid Δ^1-dehydrogenase From Rhodococcus Erythropolis Sq1based on NCBInr protein database search with Mascot Score equal to 2170.6 and Sequence Coverage equals 53.2% (23 peptides were identified).

Table S 8: Proteins identified in the sample.

Protein	Molecular Weight [kDa]	pI	Scores	Number of peptides	Sequence Coverage [%]
Chain A, Crystal Structure Of 3-ketosteroid Delta1-dehydrogenase From Rhodococcus Erythropolis Sq1	55.2	4.6	2170.6	23	53.2
3-ketosteroid delta(1)-dehydrogenase KstD [Rhodococcus wratislaviensis NBRC 100605]	54.0	4.6	370.9	5	13.5
Chain B, Refined 1.8 Angstroms Resolution Crystal Structure Of Porcine Epsilon-Trypsin	8.8	7.7	174.9	1	24.4
3-ketosteroid delta(1)-dehydrogenase KstD1 [Rhodococcus triatomae]	52.5	4.4	125.5	2	4.5
Chain A, Crystal Structure Of The N-Domain Of Fkbp22 From Shewanella Sp. Sib1	9.4	5.8	110.2	2	19.3
transcriptional regulator [Leptolyngbya sp. PCC 7375]	16.3	5.2	64.4	1	6.8
hypothetical protein, partial [Escherichia coli]	7.8	10.2	60.8	1	16.9

5.4 Plots for KSTD reaction with *bi bi* ping-pong mechanism

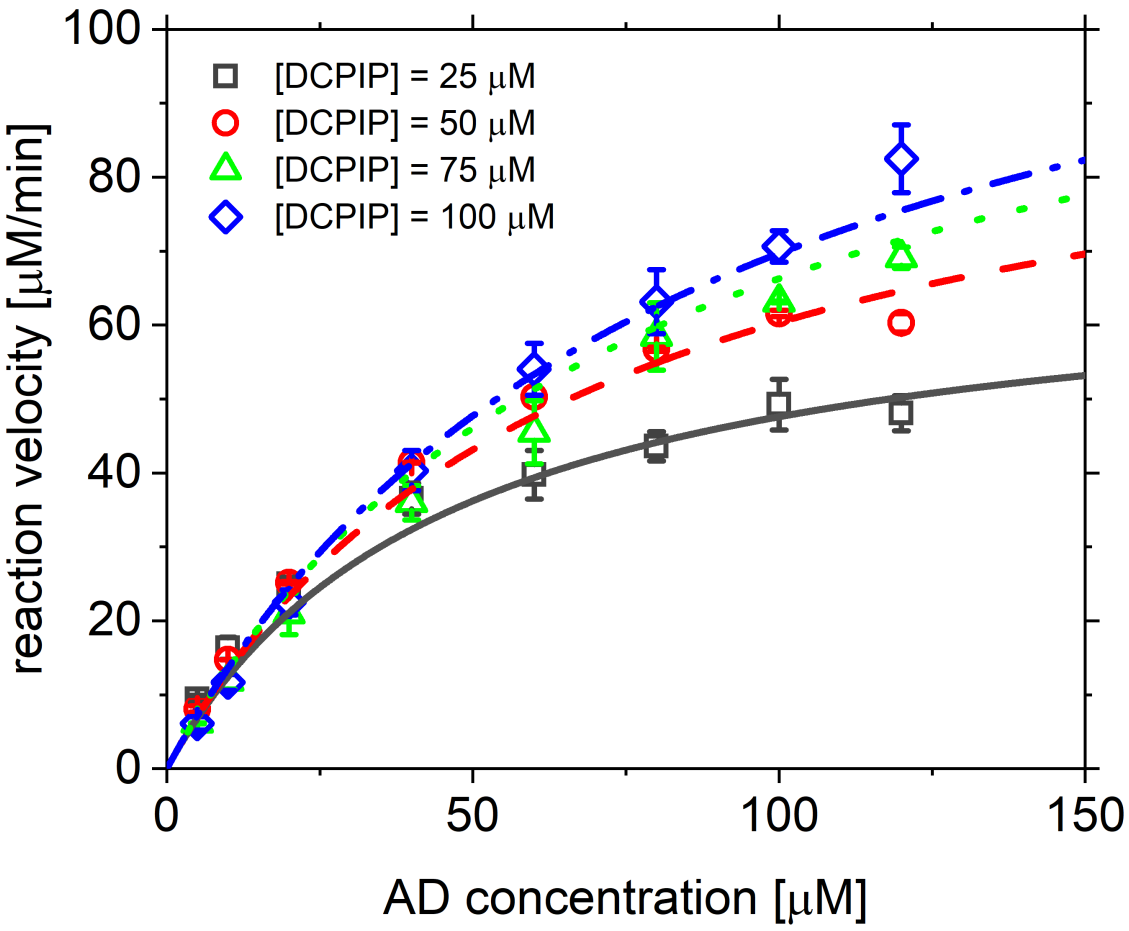


Figure S20: Plots of reaction velocity versus androst-4-en-3,17-dione concentration at various constant concentrations of DCPIP and Michaelis-Menten model fitting. Reaction conditions: 50 mM Tris-HCl buffer pH 8.0 with 7 nM of KSTD, 5 – 120 μM of AD in isopropanol (1%) and 25 μM (□), 50 μM (○), 75 μM (△) or 100 μM (◊) of DCPIP, respectively.

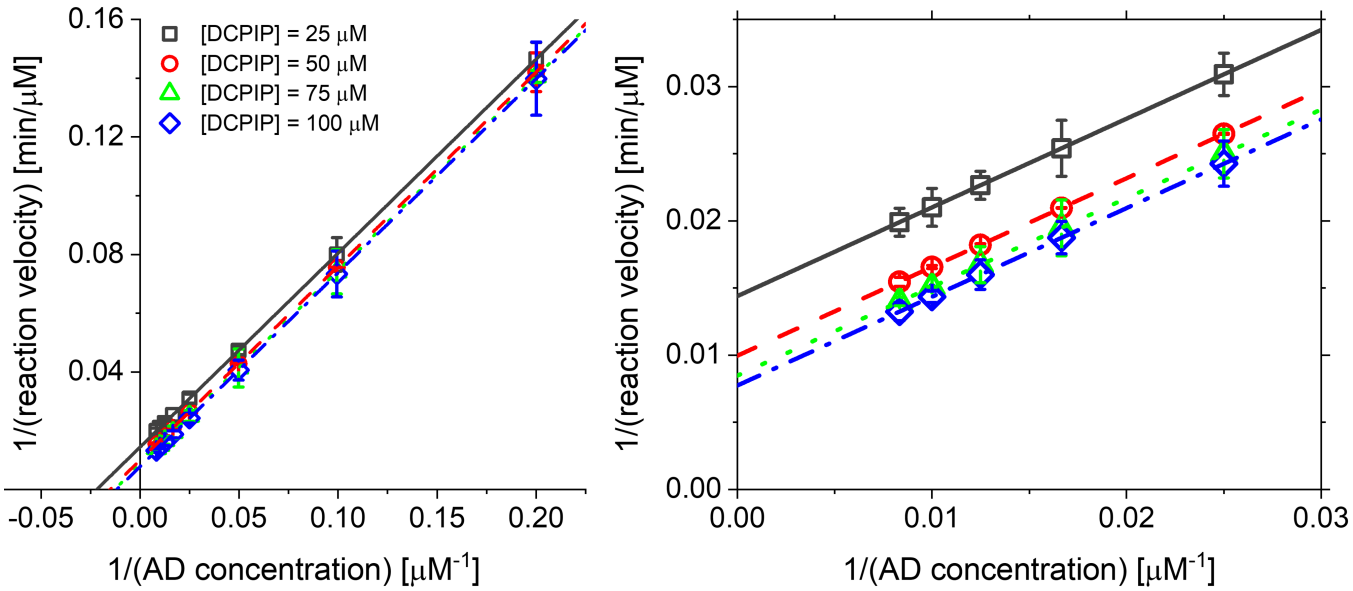


Figure S21: Plots of inverse initial rate versus inverse androst-4-en-3,17-dione concentration at various constant concentrations of DCPIP. Reaction conditions: 50 mM Tris-HCl buffer pH 8.0 with 7 nM of KSTD, 5 – 120 μM of AD in isopropanol (1%) and 25 μM (□), 50 μM (○), 75 μM (△) or 100 μM (◊) of DCPIP, respectively.

5.5 17-MT kinetic

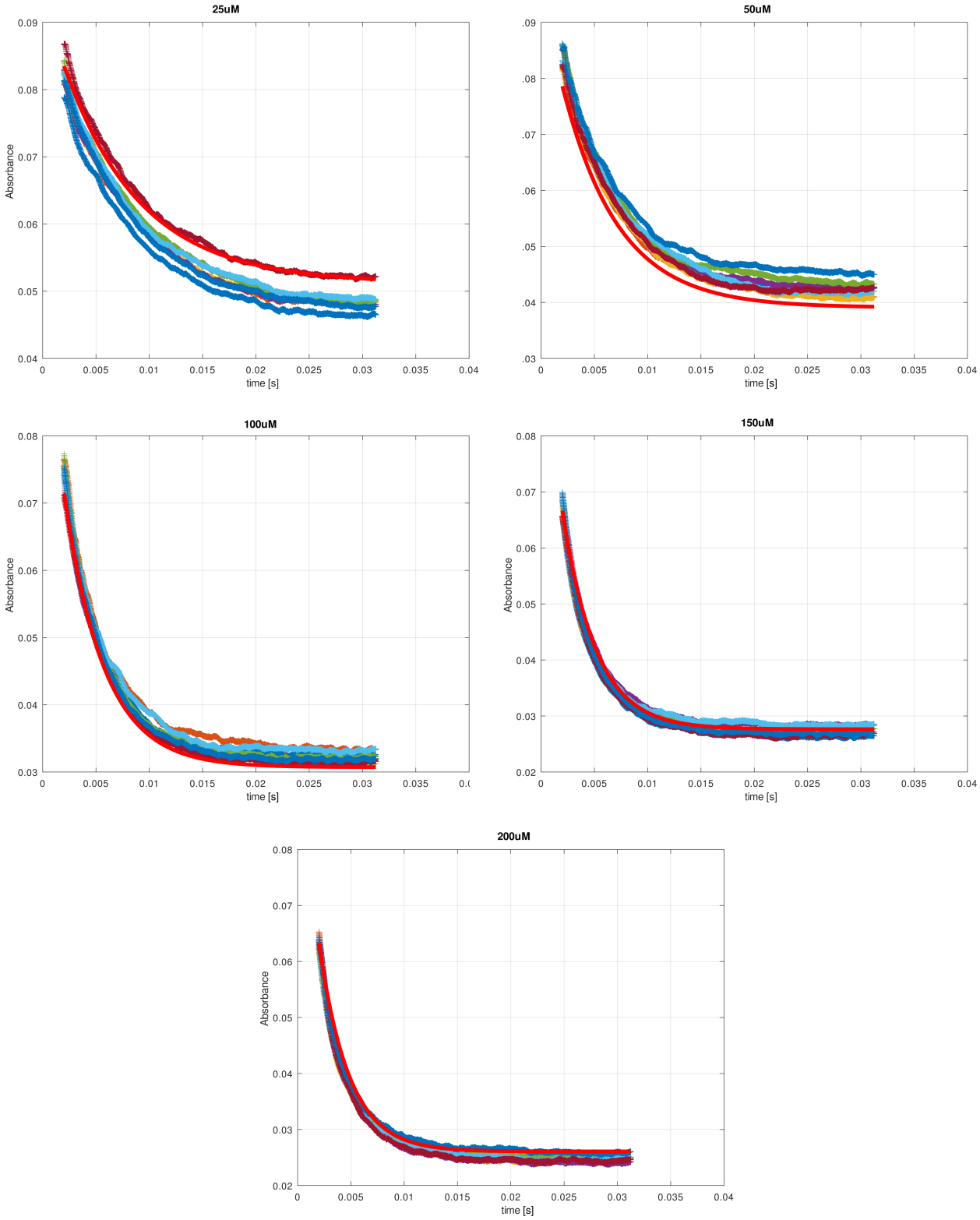


Figure S22: Experimental absorbance traces recorder for specified initial substrate concentrations. Thick red lines represent the best predictions from the two equilibrium steps kinetic model.

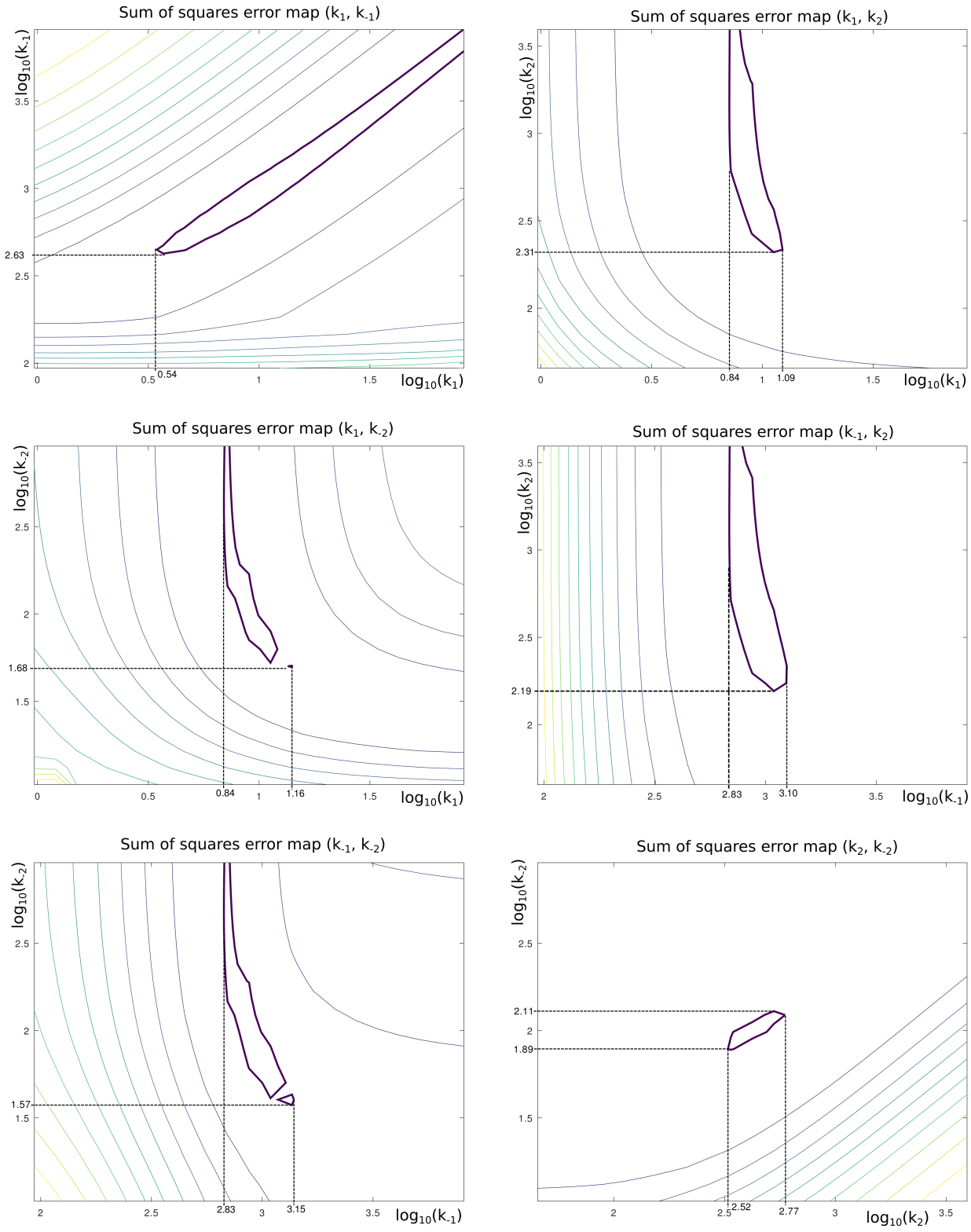


Figure S23: Maps showing dependence between sum of squares error and two scanned kinetic constants; remaining constants were optimized to minimize the error. The optimum values are always in the middle of the map. Thick violet contours mark the regions with error within 110% of the minimal value. Ranges of (log10 of) kinetic constants read from these contours (marked by dashed lines) are used to determine the confidence intervals for fitted rate constants.

Table S 9: Kinetic parameters obtained from global data fittedto the pre-steady-state experiment with 17-MT.

	Constant value	StdD	Confidence range
k_1 $[s^{-1}\mu M^{-1}]$	9.01	0.10	6.9 -12.3
k_{-1} $[s^{-1}]$	871.81	11.60	676 - 1259
k_2 $[s^{-1}]$	422.06	1.25	327 -593
k_{-2} $[s^{-1}]$	98.14	0.37	77.6 -129

5.6 KSVE details

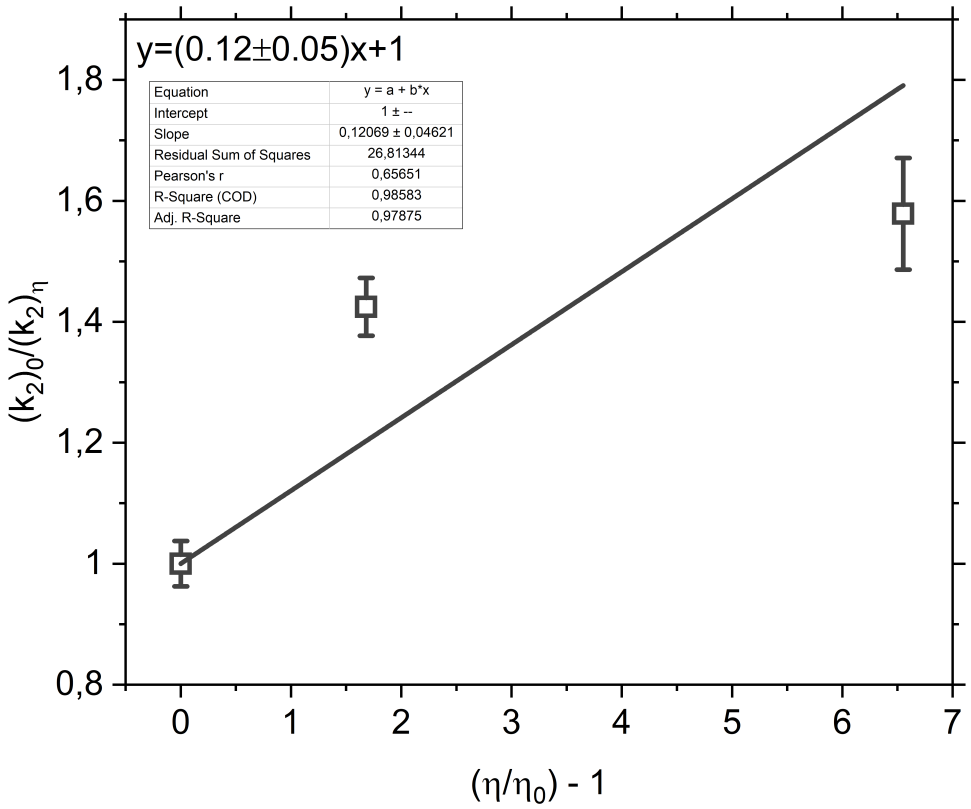


Figure S24: The effect on the k_2 value with PEG 20000.

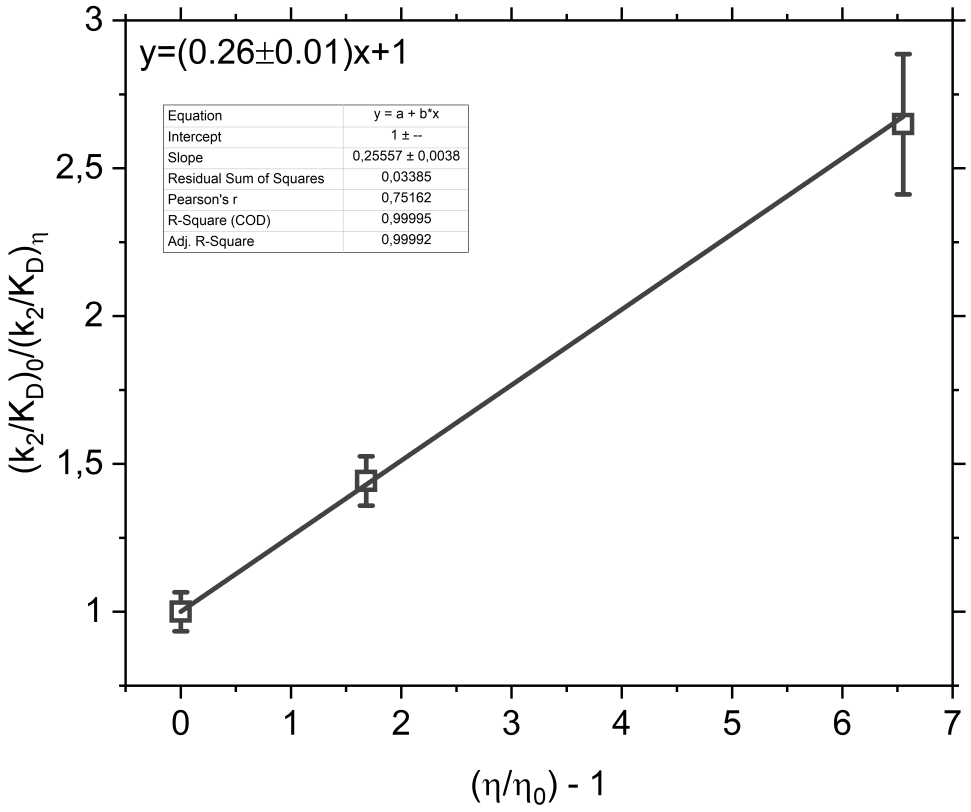


Figure S25: The effect on the k_2/K_d value with PEG 20000.

5.7 Hydrodynamic diameter of KSTD and PEG

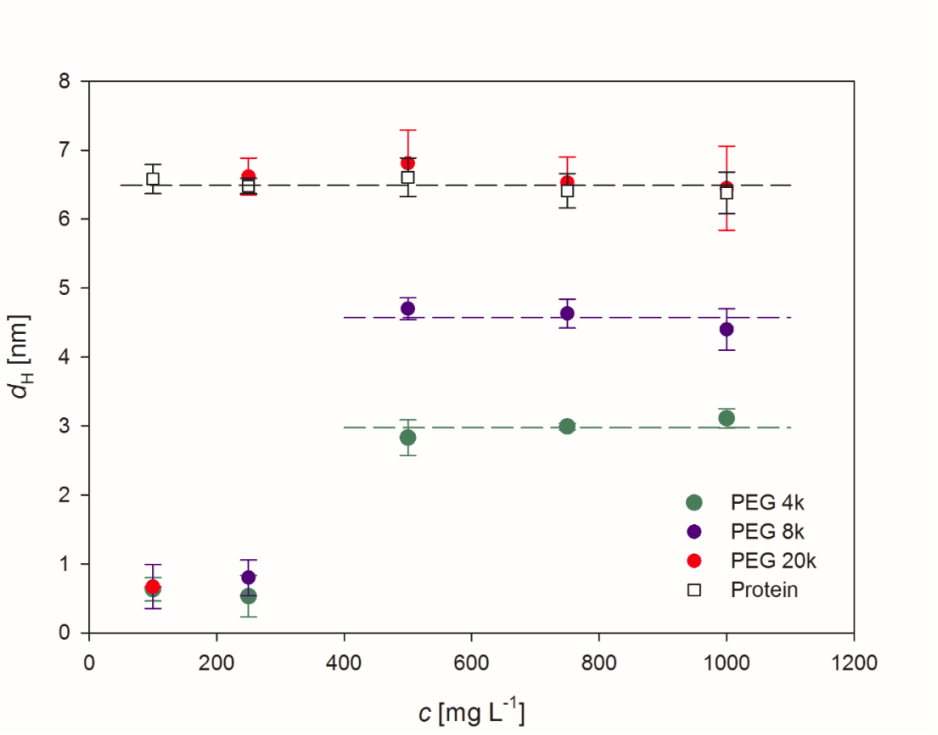


Figure S26: Dependence of the hydrodynamic diameter on the concentration of KSTD (square), PEG 4000 (green dot), PEG 8000 (purple dot) and PEG 20000 (red dot).

Table S 10: Dependence of the hydrodynamic diameter on the concentration of KSTD, PEG 4000, PEG 8000 and PEG 20000.

	PEG 4000		**PEG 8000**		**PEG 20000**		**KSTD**	
$c\ [ppm]$	$d_H\ [nm]$	SD	$d_H\ [nm]$	SD	$d_H\ [nm]$	SD	$d_H\ [nm]$	SD
100	0.63	0.17	0.67	0.32	0.67	0.01	6.58	0.21
250	0.53	0.30	0.80	0.26	6.62	0.27	6.48	0.11
500	2.83	0.26	4.70	0.16	6.81	0.48	6.61	0.28
750	2.99	0.05	4.63	0.21	6.53	0.37	6.41	0.25
1000	3.11	0.14	4.40	0.30	6.45	0.61	6.38	0.30

5.8 Competitive KIE

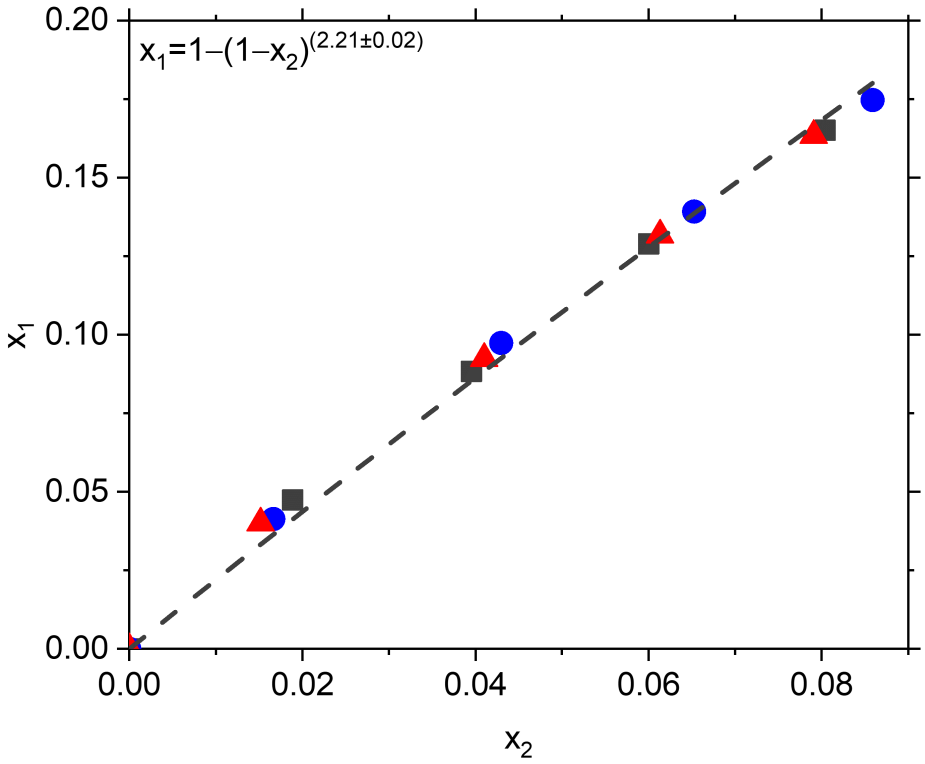


Figure S27: Results of the kinetic experiment of isotope fractionation by KSTD measured for 17-MT.

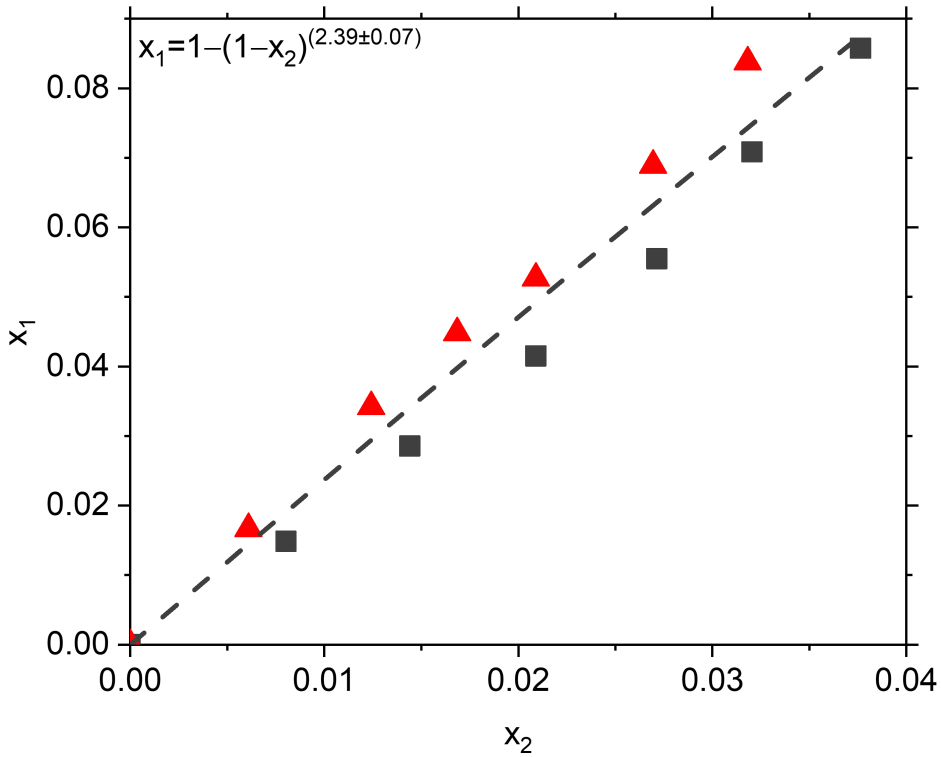


Figure S28: Results of the kinetic experiment of isotope fractionation by KSTD measured for DHT.

Table S 11: LC-ESI-MS/MS parameters of the substrates used for competitive KIE determination and their products.

MS PARAMETERS	
Ionization mode	ESI
Gas temperature	350°C
Gas flow	10 L/min
Nebulizer pressure	40 psi
Sheath gas temperature	350°C
Sheath gas flow	10 L/min
Capillary voltage	4500 V
Nozzle voltage	1000 V
Fragmentor	100
Dwell	100
17-MT METHOD PARAMETERS	
H_2:ACN:HCOOH (v:v:v)	60:40:0.1
Flow	0.4 mL/min
Total time	3 min
SIM1 $[M+H]^+$	0 – 2 min, 305.3 m/z, 301.3 m/z
SIM2 $[M+H]^+$	2 – 3 min, 308.3 m/z, 303.3 m/z
2,4,6,6-d_4-MTD retention time	2.237 min
MTD retention time	2.287 min
2,2,4,6,6-d_5-17-MT retention time	1.513 min
17-MT retention time	1.542 min
DHT METHOD PARAMETERS	
H2O:ACN:HCOOH (v:v:v)	60:40:0.1
Flow	0.4 mL/min
Total time	4 min
SIM1 $[M+H]^+$	0 – 2.9 min, 292.2 m/z, 289.3 m/z
SIM2 $[M+H]^+$	2.9 – 4 min, 295.3 m/z, 291.3 m/z
16,16,17-d_3-1-TE retention time	2.452 min
1-TE retention time	2.492 min
1,16,16,17-d_4-DHT retention time	3.185 min
DHT retention time	3.253 min

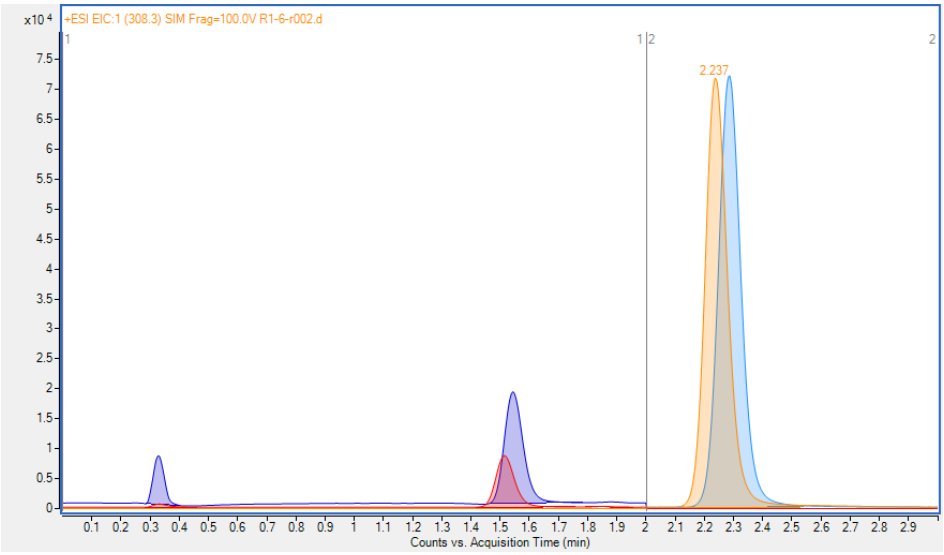


Figure S29: Chromatogram of the reaction mixture of 2,4,6,6-d_4-MTD (red), MTD (navy), 2,2,4,6,6-d_5-17-MT (orange), 17-MT (blue).

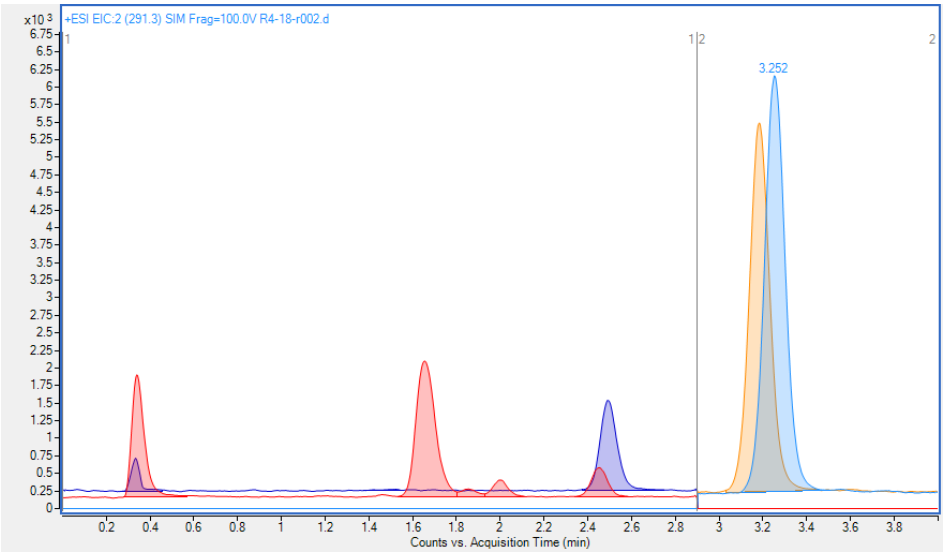


Figure S30: Chromatogram of the reaction mixture of 16,16,17-d_3-1-TE (red), 1-TE (navy), 1,16,16,17-d_4-DHT (orange), DHT (blue).

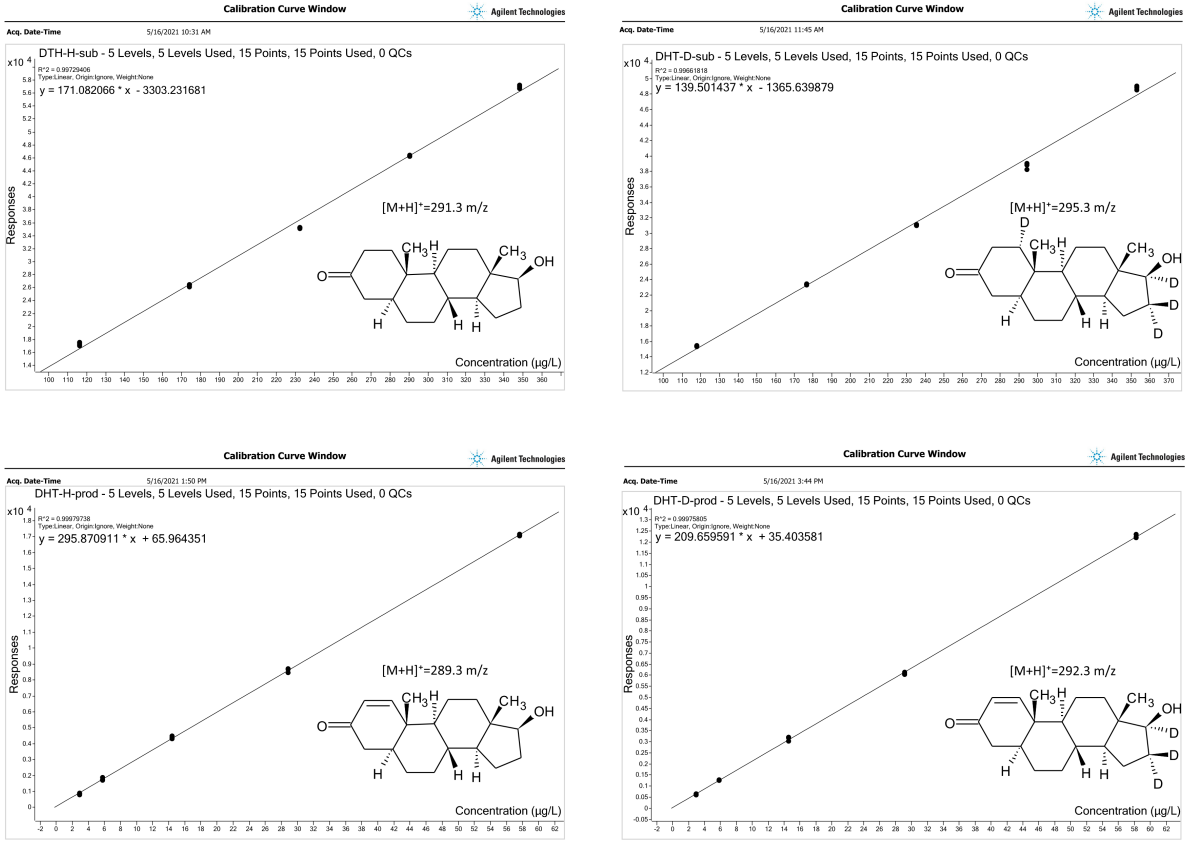


Figure S31: MS calibration curves of DHT, 1,16,16,17-d_4-DHT, 1-TE, 16,16,17-d_3-1-TE, respectively.

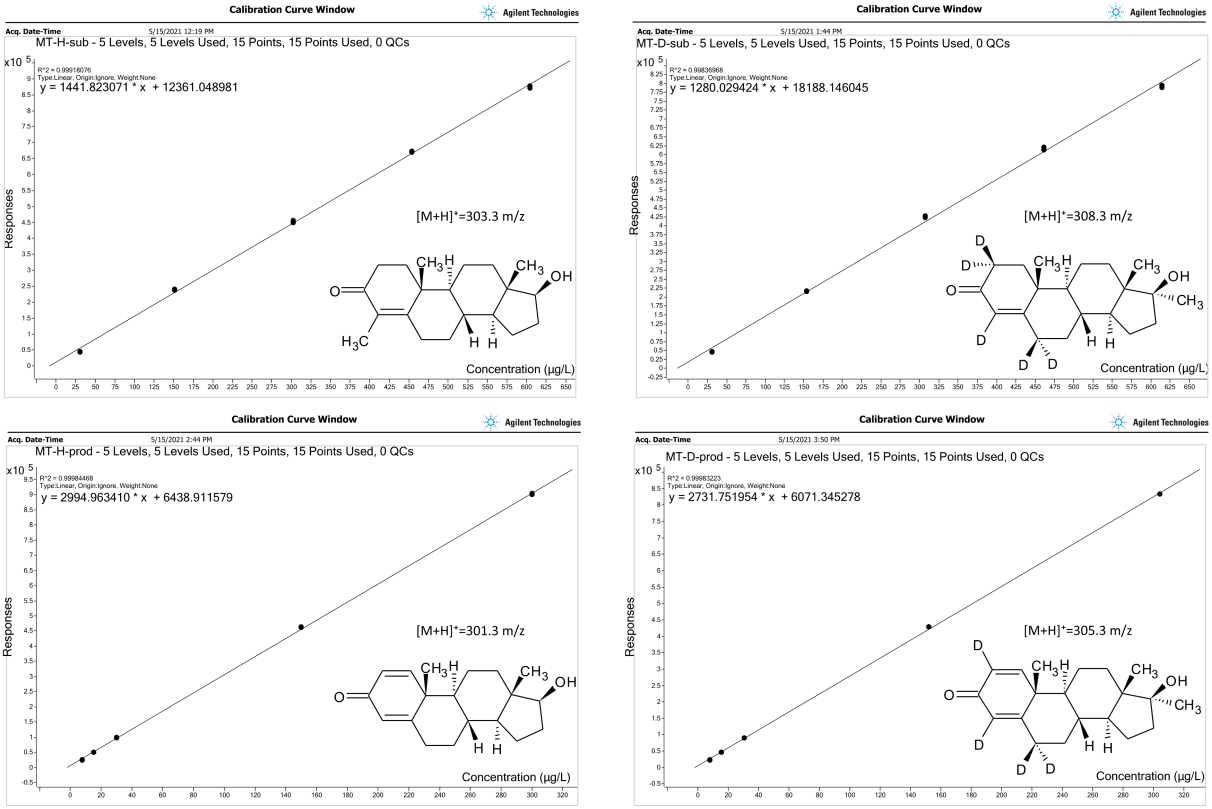
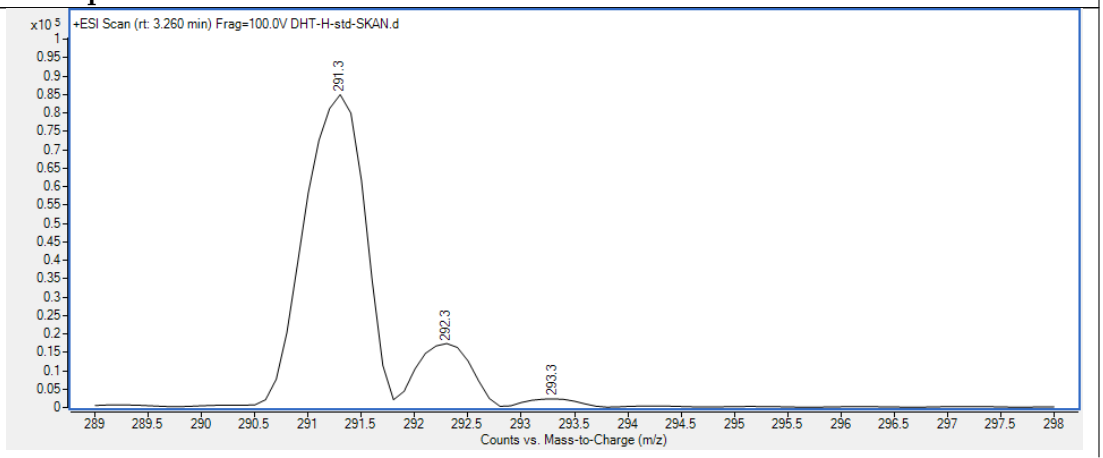
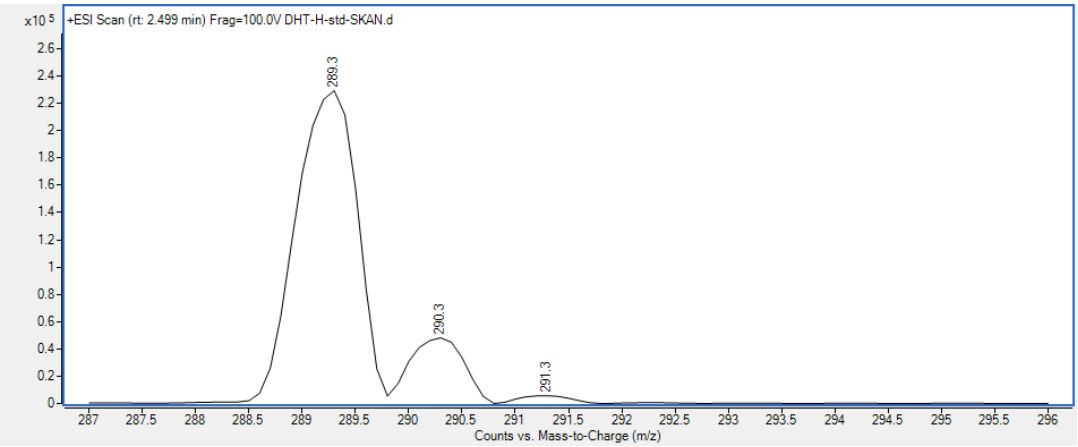


Figure S32: MS calibration curves of 17-MT, 2,2,4,6,6-d_5-17-MT, MTD, 2,4,6,6-d_4-MTD, respectively.

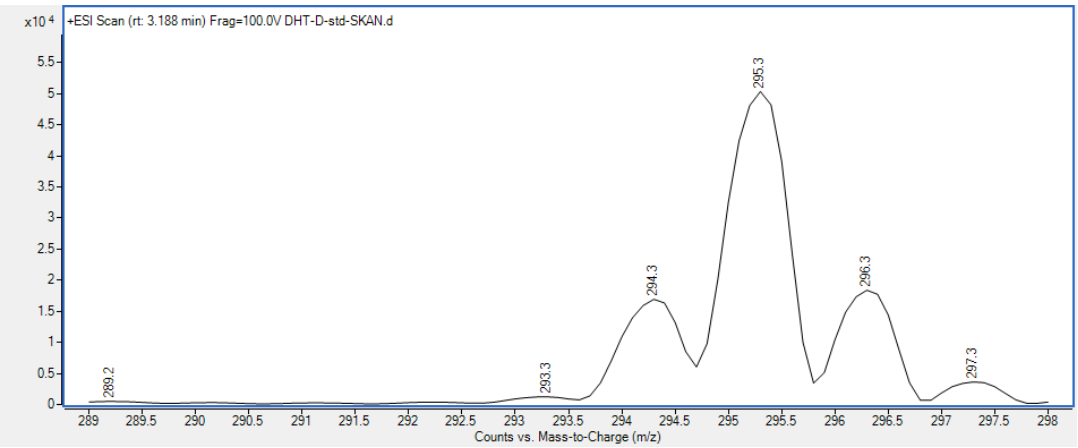
Table S 12: Mass spectrum of DHT, 17-MT and their derivates.

Name	MS spectrum of DHT derivates
DHT	

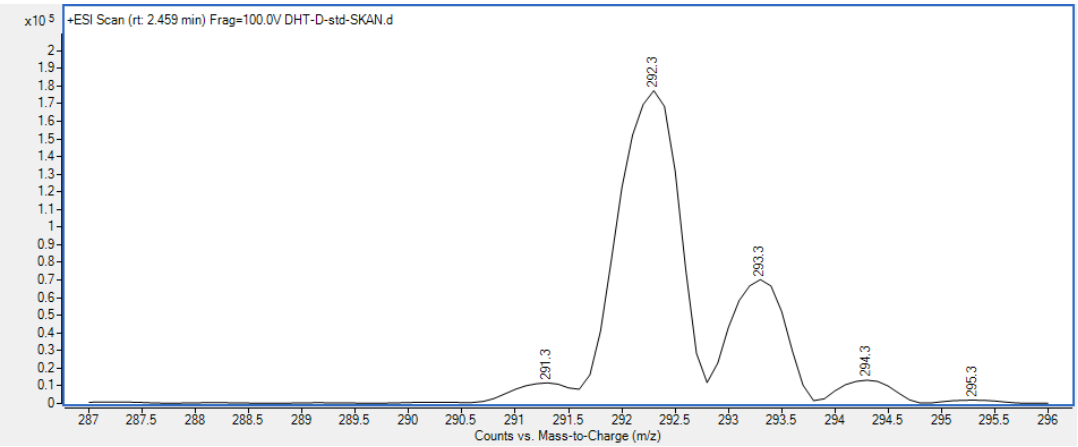
1-TE



1,16,16,17-d_4-DHT

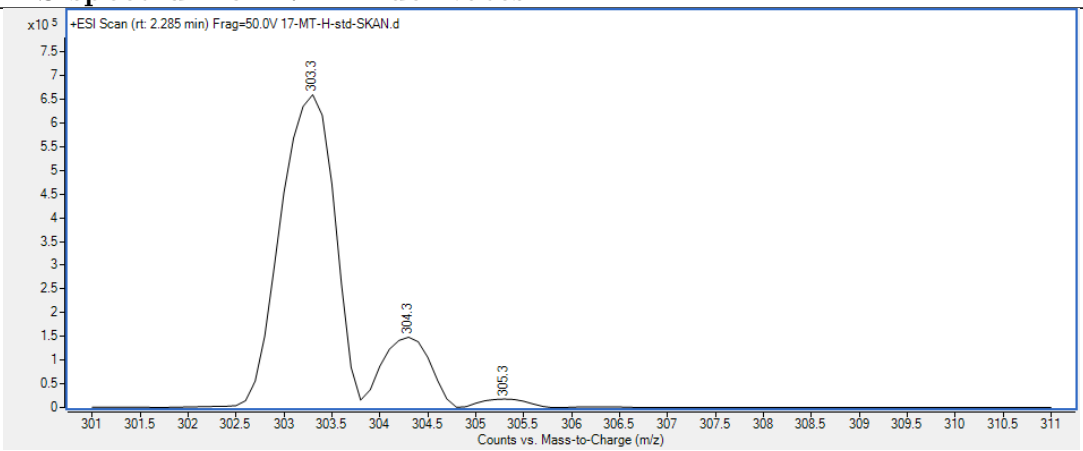


16,16,17-d_3-1-TE

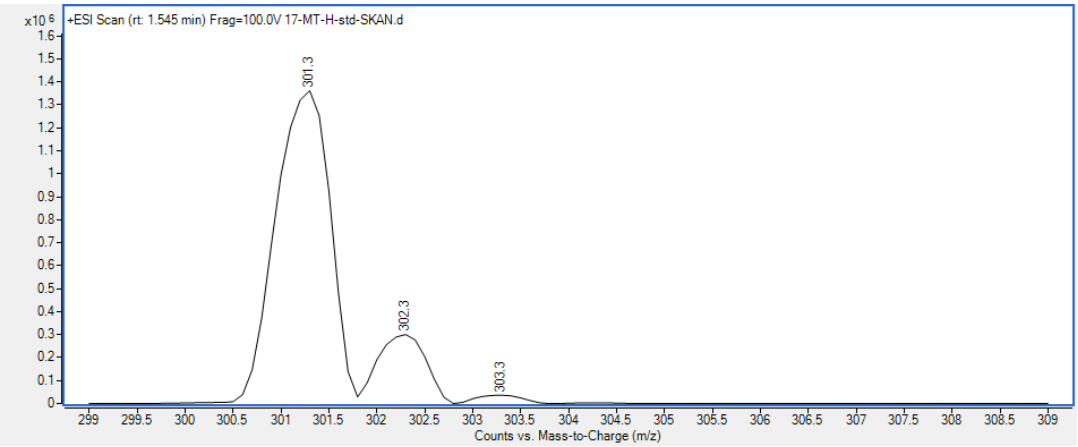


MS spectrum of 17-MT derivates

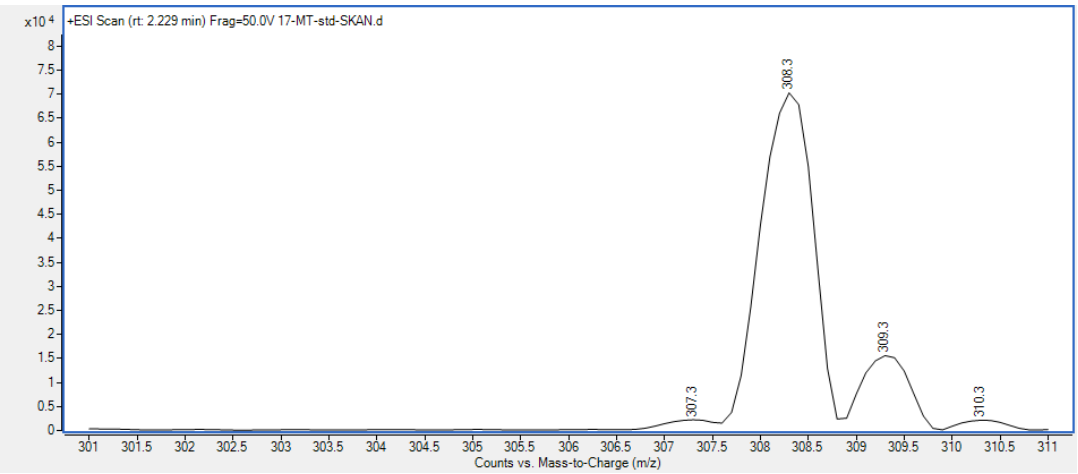
17-MT



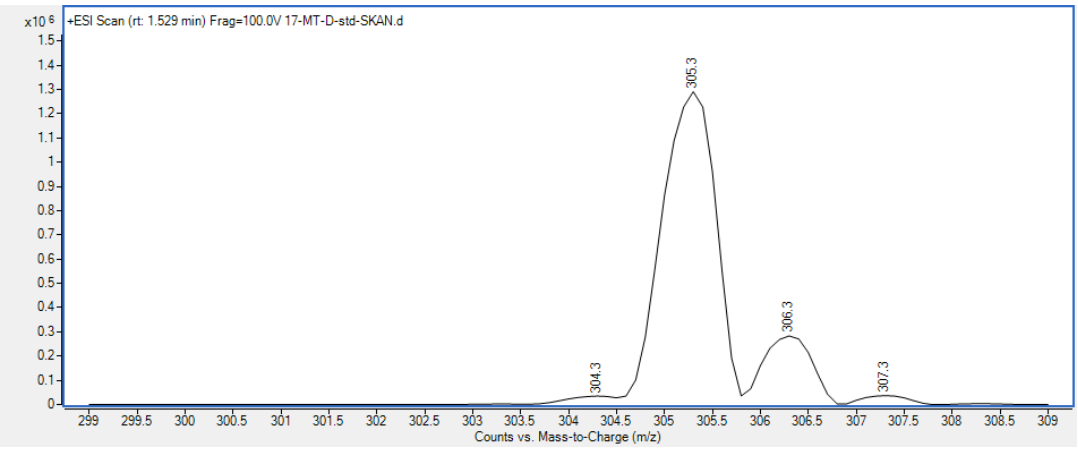
MTD



$2,2,4,6,6\text{-}d_5$-17-MT



$2,4,6,6\text{-}d_4$-MTD



5.9 Cartesian coordinates of transition states from main pathway

Table S 13: Cartesian coordinates of QM atoms for TS1 of DHT optimized at B3LYP/AMBER level of theory. ν_i = -985.386 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	58.810978	43.638094	33.969968	H	67.097403	36.955890	36.891048
O	62.491886	39.560510	32.480627	H	66.172885	35.523094	37.333083
H	58.790524	40.968177	34.637907	H	66.289858	35.829856	34.820255
C	61.588901	40.499812	32.759616	H	64.637620	35.950533	35.361921
H	58.641516	43.540595	35.050321	H	64.569888	37.535079	32.972088
C	60.487168	40.215984	33.592632	H	64.944444	41.353147	33.222372
H	59.278202	44.618993	33.832873	H	65.270122	40.712856	35.557675
C	61.714224	41.816863	32.272008	H	62.962407	37.108851	35.728599
C	59.605432	41.220482	33.962850	H	62.812916	38.062802	34.235871
H	60.950764	43.824686	32.264638	H	62.271635	38.734158	35.756892
H	62.542395	42.033180	31.610664	H	63.534146	39.918659	32.825279
C	59.751760	42.543391	33.510511	H	63.548076	40.673266	35.228245
C	60.816281	42.812552	32.644962	O	64.943538	39.029110	42.433102
H	60.367017	39.204573	33.967192	H	64.486118	39.862302	42.257147
H	66.364234	39.471492	40.993202	H	66.329401	37.337579	32.915570
C	65.634970	38.680545	41.241470	H	66.508399	38.339997	35.155895
C	66.377129	37.337480	41.412379	C	69.739161	44.455948	32.585532
C	66.392435	36.688275	39.997020	H	69.268147	44.093340	31.669752
C	65.737000	37.747363	39.087542	H	69.386244	45.469821	32.768458
C	64.734571	38.468396	40.010230	N	69.479661	41.815197	32.225230
C	63.536695	37.575708	40.395226	C	69.422300	40.455385	31.959546
C	64.261863	39.771511	39.348453	O	69.737905	39.978398	30.886311
C	63.770433	39.528873	37.912116	N	69.082218	39.597621	33.020926
C	64.800174	38.759885	37.058694	H	68.966844	38.626870	32.759262
C	65.142866	37.390525	37.721734	C	68.758840	39.935718	34.310801
C	66.088834	36.519019	36.878769	O	68.577151	39.106403	35.192826
C	65.615981	36.444342	35.425487	N	69.273612	43.595019	33.675274
C	65.560311	37.871644	34.865110	C	69.154416	42.238401	33.412728
C	65.517914	37.937668	33.341022	C	68.706774	41.407799	34.525005
C	65.657063	39.380854	32.846577	N	68.352656	41.865678	35.693938
O	66.410318	39.647457	31.893671	C	68.482925	43.212021	35.918497
C	64.786929	40.312671	33.507215	C	69.004367	44.100558	34.940528
C	64.493685	40.164966	34.992493	C	69.247265	45.432902	35.303742
C	64.442676	38.711367	35.543924	H	69.676840	46.119727	34.586187
C	63.047074	38.110728	35.301017	C	68.966964	45.904146	36.583920
H	67.383805	37.482017	41.816743	C	68.408683	45.031821	37.551287
H	65.825007	36.729501	42.134549	C	68.174547	43.712624	37.195990
H	67.406681	36.478264	39.659240	H	67.766616	43.002504	37.908431
H	65.846438	35.739685	39.986930	C	68.097615	45.510605	38.946557
H	66.536231	38.476044	38.874123	H	67.304254	46.269008	38.956221
H	63.835864	36.542539	40.595416	H	68.979898	45.956968	39.416925
H	62.779652	37.547240	39.608812	H	67.771048	44.679546	39.574030
H	63.080147	37.958264	41.310473	C	69.282056	47.340452	36.922937
H	63.472244	40.252915	39.945526	H	68.569737	47.747826	37.642142
H	65.107039	40.474750	39.314809	H	69.261755	47.972294	36.030040
H	62.821251	38.980257	37.933003	H	70.277844	47.438261	37.376223
H	63.553790	40.490822	37.439326	H	70.729209	44.444076	32.445302
H	65.737040	39.337247	37.096582	H	57.911224	43.661984	33.534224
H	64.212638	36.819746	37.865266				

Table S 14: Cartesian coordinates of QM atoms for TS2 of DHT optimized at B3LYP/AMBER level of theory. ν_i = -1455.109 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	58.861722	43.791537	33.924545	H	67.209492	36.924301	36.978613
O	62.729186	39.988049	32.130087	H	65.904876	35.829290	37.448313
H	58.985419	41.043983	34.299727	H	65.958117	36.198620	34.951561
C	61.873652	40.951610	32.541454	H	64.456517	36.780901	35.635048
H	58.697804	43.626126	34.997101	H	64.503090	38.066286	33.381526
C	60.756058	40.518134	33.267017	H	65.734954	41.916577	33.411882
H	59.261624	44.807978	33.846052	H	66.884844	41.291804	35.696745
C	62.019541	42.322328	32.300310	H	63.237266	40.326794	36.356778
C	59.812347	41.429021	33.711971	H	63.267864	38.561754	36.345966
H	61.175656	44.283075	32.513655	H	63.323745	39.469903	34.829421
H	62.906982	42.687191	31.801957	H	63.601023	40.306618	31.812858
C	59.904602	42.803003	33.426640	H	65.112951	41.719582	35.744899
C	61.039180	43.223709	32.725932	O	65.736093	38.997221	42.835082
H	60.659456	39.459875	33.484659	H	64.862470	38.595160	42.931953
H	67.290739	38.942720	41.574072	H	66.194457	37.780854	33.023354
C	66.292638	38.493280	41.625405	H	66.940047	38.483830	35.161507
C	66.426388	36.937086	41.582775	C	69.188452	44.506829	32.418414
C	66.134506	36.517377	40.117665	H	68.643716	44.243475	31.510019
C	66.155479	37.846457	39.344669	H	68.946652	45.537704	32.669829
C	65.531143	38.859443	40.326392	N	69.081225	41.890042	32.025786
C	64.020553	38.643360	40.552321	C	69.305798	40.560942	31.770481
C	65.778046	40.271298	39.783403	O	69.627081	40.111591	30.677405
C	65.267034	40.431247	38.338929	N	69.227407	39.679589	32.861096
C	65.775262	39.344552	37.367155	H	69.458064	38.715366	32.664628
C	65.549013	37.912027	37.944809	C	68.880300	39.988735	34.165272
C	66.116169	36.820776	37.028334	O	68.912121	39.129743	35.054121
C	65.540963	36.958237	35.624373	N	68.720940	43.627876	33.499742
C	65.857035	38.350884	35.073066	C	68.744371	42.255445	33.246193
C	65.518567	38.427673	33.594507	C	68.504467	41.378426	34.345739
C	65.594019	39.812559	33.001421	N	68.119924	41.776723	35.597846
O	65.404227	39.917190	31.771256	C	68.234101	43.152331	35.840000
C	65.734405	40.938326	33.882441	C	68.631528	44.073057	34.830099
C	65.637186	40.866092	35.296442	C	68.965656	45.381220	35.212254
C	65.225288	39.503009	35.912891	H	69.340875	46.078202	34.472730
C	63.676487	39.454968	35.863517	C	68.862311	45.830269	36.529814
H	67.424851	36.626208	41.908148	C	68.407999	44.940942	37.523167
H	65.717984	36.484181	42.287041	C	68.100381	43.630689	37.153372
H	66.855155	35.796467	39.730226	H	67.812669	42.908419	37.912656
H	65.149056	36.049208	40.030980	C	68.332876	45.368645	38.970034
H	67.215633	38.133926	39.240161	H	69.239660	45.902012	39.279957
H	63.442993	38.791133	39.638526	H	68.226132	44.501126	39.624969
H	63.652134	39.368683	41.286692	H	67.486678	46.042058	39.165357
H	63.785855	37.637656	40.919475	C	69.281277	47.242314	36.871174
H	65.281355	41.004146	40.425974	H	68.590234	47.713050	37.577541
H	66.856486	40.481792	39.815673	H	69.320677	47.870933	35.975933
H	64.171424	40.433182	38.348203	H	70.276434	47.274548	37.338952
H	65.563693	41.419387	37.970080	H	70.164672	44.413805	32.222606
H	66.862156	39.470170	37.271323	H	57.961653	43.780553	33.488936
H	64.467188	37.730165	38.026592				

Table S 15: Cartesian coordinates of QM atoms for TS1 of 17MT optimized at B3LYP/AMBER level of theory. ν_i = -998.663 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	30.846496	22.225471	49.459513	H	37.083917	16.632330	56.378821
O	34.716765	18.131319	50.115854	H	37.431589	15.962102	53.972719
H	30.829041	20.103890	51.215371	H	35.776133	16.454941	54.283445
C	33.738521	18.999639	49.869912	H	38.145662	16.708436	51.920710
H	30.504096	22.541553	50.452174	H	36.829350	20.406637	50.155267
C	32.569282	19.027349	50.654943	H	37.333174	20.784258	52.666134
H	31.305711	23.104799	49.001785	H	34.418388	18.158684	52.435410
C	33.889214	20.004506	48.894315	H	33.982483	19.703355	53.161019
C	31.675561	20.078742	50.537124	H	34.243886	18.306867	54.199149
H	33.117805	21.798209	48.002025	H	35.635144	18.814903	50.465603
H	34.779186	19.979044	48.286459	H	35.628269	20.956141	52.271214
C	31.848746	21.103877	49.592965	O	35.404282	21.387403	59.987408
C	32.962414	21.028521	48.755647	H	35.278679	22.270111	59.610922
H	32.415039	18.252477	51.399670	C	37.641248	21.501175	59.188285
C	36.339425	20.700010	59.147283	H	37.459086	22.513384	58.826209
C	36.537825	19.275403	59.736625	H	38.443372	21.062003	58.590602
C	36.705209	18.308205	58.534139	H	37.976069	21.579353	60.225616
C	36.684541	19.219787	57.291237	C	41.866125	22.890357	48.901719
C	35.792755	20.412964	57.701432	H	41.682188	22.054048	48.227954
C	34.299375	20.034381	57.828293	H	41.396027	23.777019	48.489952
C	35.957741	21.509621	56.630710	N	41.456960	20.333871	49.673568
C	35.645799	20.977108	55.210847	C	41.342166	18.997598	50.026297
C	36.441259	19.703840	54.834104	O	41.699175	18.086021	49.302937
C	36.312663	18.614945	55.937244	N	40.897488	18.707231	51.324930
C	37.182471	17.402683	55.602405	H	40.759440	17.722429	51.505600
C	36.809212	16.825666	54.231334	C	40.378709	19.574764	52.258610
C	36.898050	17.874180	53.152292	O	40.033196	19.221543	53.376554
C	37.603714	17.646813	52.030970	N	41.159940	22.573646	50.154826
C	37.653545	18.571184	50.860590	C	40.996132	21.238169	50.491322
O	38.366926	18.311834	49.873504	C	40.340124	20.976741	51.768379
C	36.708840	19.654096	50.936785	N	39.804235	21.890125	52.528791
C	36.443295	20.223505	52.321029	C	39.944701	23.198558	52.150418
C	36.094814	19.158448	53.397037	C	40.671001	23.577347	50.989078
C	34.591940	18.805976	53.295821	C	40.885114	24.942776	50.757476
H	37.400442	19.251565	60.410077	H	41.420020	25.271769	49.875900
H	35.657022	19.028902	60.335477	C	40.428803	25.912134	51.647954
H	37.631397	17.730098	58.591863	C	39.713718	25.540467	52.810756
H	35.883343	17.585852	58.497863	C	39.462325	24.194391	53.020644
H	37.703499	19.618117	57.171318	H	38.923817	23.848485	53.897153
H	33.735275	20.886006	58.216790	C	39.240585	26.581270	53.796418
H	34.136801	19.204862	58.522090	H	39.099976	26.136656	54.782779
H	33.865891	19.752490	56.868069	H	38.286904	27.032285	53.491082
H	35.308896	22.372219	56.845981	H	39.961993	27.396318	53.907323
H	36.991452	21.876896	56.642717	C	40.663100	27.363841	51.339259
H	34.571268	20.781872	55.125189	H	40.892569	27.501385	50.279783
H	35.863255	21.768738	54.486077	H	41.489383	27.788022	51.922493
H	37.506409	19.978667	54.797883	H	39.774065	27.951925	51.577286
H	35.269891	18.277652	55.989136	H	42.853515	23.023173	48.987864
H	38.229612	17.731513	55.592199	H	30.034366	21.980437	48.929981

Table S 16: Cartesian coordinates of QM atoms for TS2 of 17MT optimized at B3LYP/AMBER level of theory. ν_i = -1414.896 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	30.893723	22.088559	49.458067	H	37.246222	16.823570	56.301359
O	34.513247	17.682418	49.973778	H	37.473273	16.210873	53.861567
H	30.796761	19.887360	51.146725	H	35.856300	16.761702	54.262261
C	33.683379	18.741447	49.805284	H	37.597727	16.762702	51.653005
H	30.593290	22.409131	50.462781	H	37.333040	20.799304	50.226577
C	32.520231	18.786315	50.585325	H	38.333842	21.170436	52.692373
H	31.381618	22.952265	48.999589	H	34.790621	19.009939	52.134287
C	33.938093	19.799467	48.926172	H	34.493095	20.467396	53.078396
C	31.662620	19.873853	50.491749	H	34.436055	18.878729	53.855639
H	33.259404	21.659601	48.103266	H	35.420907	17.864530	49.627926
H	34.859781	19.794678	48.364540	H	36.545238	21.552612	52.419051
C	31.876741	20.928027	49.582795	O	35.240968	21.290076	60.044611
C	33.038959	20.860816	48.808441	H	35.127821	22.187109	59.701868
H	32.326477	17.977631	51.284328	C	37.539561	21.448261	59.443233
C	36.243408	20.656005	59.240856	H	37.414203	22.482196	59.114315
C	36.396095	19.190610	59.739267	H	38.396854	21.024236	58.913914
C	36.693145	18.316553	58.489794	H	37.760735	21.472656	60.512207
C	36.756925	19.318398	57.318899	C	41.805242	22.813513	48.972997
C	35.821622	20.470289	57.740574	H	41.647013	21.998300	48.267390
C	34.328409	20.067892	57.705067	H	41.333570	23.708806	48.575863
C	36.071980	21.647750	56.781293	N	41.435984	20.212888	49.677178
C	35.902107	21.233686	55.302640	C	41.375528	18.877424	49.992368
C	36.722976	19.985258	54.894099	O	41.757603	17.978553	49.249914
C	36.487389	18.819698	55.902215	N	40.907652	18.541033	51.269655
C	37.346256	17.610759	55.543476	H	40.916534	17.556034	51.493075
C	36.904874	17.090656	54.181021	C	40.323397	19.390071	52.199465
C	37.006956	18.154175	53.121169	O	39.959559	18.984207	53.308553
C	37.357904	17.796482	51.878033	N	41.064857	22.433687	50.200105
C	37.297299	18.721488	50.753619	C	40.885329	21.082902	50.502339
O	37.152093	18.266985	49.587077	C	40.204093	20.753537	51.720591
C	37.294688	20.115419	51.066954	N	39.599424	21.644611	52.562704
C	37.085933	20.598900	52.386126	C	39.761070	22.985468	52.207755
C	36.489363	19.567730	53.400661	C	40.528998	23.396582	51.074118
C	34.956508	19.479667	53.100981	C	40.755052	24.765254	50.884205
H	37.187498	19.117192	60.490062	H	41.324943	25.101192	50.026402
H	35.463609	18.893843	60.226573	C	40.268135	25.737878	51.759299
H	37.627495	17.757089	58.586189	C	39.521100	25.340782	52.882069
H	35.901605	17.576549	58.331259	C	39.274695	23.980889	53.070708
H	37.777814	19.727075	57.306971	H	38.723077	23.641940	53.943348
H	33.720132	20.856663	58.152977	C	39.011993	26.363030	53.869629
H	34.128588	19.153815	58.271845	H	39.017041	25.953957	54.881621
H	33.972140	19.904779	56.686912	H	37.987749	26.686568	53.639694
H	35.388580	22.481175	57.000299	H	39.634085	27.261365	53.887054
H	37.092429	22.020255	56.924924	C	40.504547	27.196153	51.455587
H	34.840788	21.050596	55.110800	H	40.850280	27.326612	50.426776
H	36.177170	22.081944	54.665661	H	41.248396	27.656127	52.119072
H	37.786334	20.238415	54.959123	H	39.581863	27.773128	51.569244
H	35.437142	18.498633	55.844987	H	42.790614	22.963428	49.054032
H	38.399284	17.915449	55.505565	H	30.052630	21.898956	48.951496

5.10 Cartesian coordinates of transition states from alternative pathway

Table S 17: Cartesian coordinates of QM atoms for TS1 of alternative mechanism optimized at B3LYP/AMBER level of theory. ν_i = -1047.125 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	16.567631	55.419028	76.140995	H	19.204891	46.456802	71.352146
H	17.587614	55.817559	76.194425	H	18.301869	46.136270	69.888843
H	16.562446	54.486763	76.711710	H	17.764205	48.383612	71.888488
C	16.182358	55.115459	74.705026	H	18.349349	48.538174	70.248716
C	15.482725	53.945353	74.395146	H	16.297036	47.869883	69.268194
H	15.203786	53.266824	75.197923	H	15.005428	47.268000	71.962446
C	15.146155	53.615270	73.085646	H	14.182992	46.716524	69.081724
H	14.606686	52.695689	72.886281	H	13.227507	46.324600	70.506775
C	15.516163	54.444677	72.008088	H	12.482762	48.479072	69.376361
O	15.366938	54.080230	70.733185	H	12.891681	48.645059	71.068275
H	15.803463	52.776847	70.443511	H	12.864397	50.656624	68.672313
C	16.132811	55.672785	72.329971	H	16.576835	52.570528	68.951827
H	16.358490	56.359185	71.520931	H	16.962008	50.101234	68.869817
C	16.484926	55.977345	73.639427	H	16.401871	49.920163	72.481616
H	17.013190	56.909590	73.831994	H	14.753090	49.278212	72.490580
C	11.855714	59.588855	70.062975	H	15.056891	50.949789	72.018772
H	12.538561	60.445102	70.088716	H	14.419316	53.681219	69.480147
H	11.605759	59.375949	71.109050	H	17.490328	50.663182	70.448842
C	12.631434	58.423437	69.469898	O	18.294651	43.593839	72.774379
C	12.061954	57.309849	68.842183	H	19.204071	43.903040	72.658647
H	10.986309	57.233346	68.714360	C	18.227913	43.543470	70.381837
C	12.856183	56.266928	68.358006	H	18.333313	42.456144	70.438032
H	12.405333	55.410259	67.865281	H	19.231305	43.973245	70.286319
C	14.246193	56.326483	68.466284	H	17.674912	43.788047	69.470760
O	15.070078	55.351224	67.969451	C	18.954667	54.748485	65.860471
H	14.548859	54.744746	67.424260	H	17.938302	55.080553	65.658184
C	14.837012	57.420739	69.097140	H	19.338995	55.300279	66.718496
H	15.917737	57.465707	69.176474	N	16.768668	53.237774	65.261885
C	14.029352	58.435629	69.593859	C	15.636674	52.546403	64.896023
H	14.500635	59.277943	70.089187	O	14.718098	53.055036	64.285214
C	17.527881	44.034660	71.656674	N	15.551884	51.174746	65.243402
C	16.125043	43.389666	71.824352	H	14.697814	50.708654	64.960349
C	15.064273	44.466936	71.495023	C	16.418354	50.455943	66.034489
C	15.881618	45.630333	70.920295	O	16.206734	49.307243	66.387022
C	17.227224	45.577418	71.678095	N	18.862714	53.321883	66.241174
C	17.092209	46.034660	73.149130	C	17.709707	52.625669	65.925506
C	18.199339	46.493039	70.919647	C	17.639524	51.243827	66.393974
C	17.685725	47.946950	70.887663	N	18.554762	50.665232	67.110213
C	16.236181	48.068732	70.349305	C	19.688415	51.370685	67.397455
C	15.249008	47.016108	70.922541	C	19.882742	52.702857	66.946449
C	13.953160	47.039701	70.104573	C	21.104353	53.327630	67.233525
C	13.320132	48.434827	70.078071	H	21.294511	54.337116	66.895359
C	14.289177	49.550375	69.790751	C	22.110476	52.658994	67.920501
C	13.866454	50.626498	69.081301	C	21.917013	51.329312	68.387061
C	14.618455	51.844489	69.113037	C	20.703032	50.726640	68.131092
O	13.960234	52.965444	68.910851	H	20.492205	49.717854	68.464141
C	15.971092	51.888742	69.557590	C	23.010698	50.590305	69.116052
C	16.609119	50.532416	69.813263	H	23.915335	50.508989	68.502867
C	15.651149	49.518487	70.480630	H	22.690852	49.578084	69.371575
C	15.443452	49.932405	71.956720	H	23.301320	51.096797	70.043796
H	16.043853	42.516428	71.179911	C	23.424476	53.337717	68.166555
H	16.036725	43.028724	72.847790	H	23.672931	53.274455	69.225307
H	14.311899	44.110762	70.786906	H	23.413346	54.386724	67.864971
H	14.528767	44.779117	72.399856	H	24.230702	52.831083	67.624484
H	16.104369	45.381775	69.870999	H	19.525370	54.910849	65.055527
H	16.851764	47.095066	73.228930	H	11.026609	59.888305	69.590835
H	18.022677	45.859582	73.693977	H	15.959799	56.057180	76.613543

H	16.308886	45.488305	73.678013				

Table S 18: Cartesian coordinates of QM atoms for TS2 of alternative mechanism optimized at B3LYP/AMBER level of theory. ν_i = -1341.259 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	16.602815	55.400335	76.201808	H	19.303372	46.444193	71.300285
H	17.624408	55.786342	76.280524	H	18.400140	46.093741	69.844148
H	16.561144	54.477879	76.785533	H	17.873403	48.403134	71.776720
C	16.240519	55.093285	74.766331	H	18.497517	48.516498	70.148188
C	15.500826	53.947252	74.453670	H	16.435115	47.853719	69.140620
H	15.228962	53.257143	75.247165	H	15.152050	47.348128	71.868776
C	15.101802	53.666964	73.152424	H	14.251697	46.747391	69.023656
H	14.540133	52.768463	72.928848	H	13.347383	46.355296	70.484972
C	15.429107	54.552959	72.122784	H	12.556003	48.517299	69.417138
O	15.038694	54.237923	70.854277	H	13.081977	48.680463	71.079319
H	15.385115	54.893055	70.233684	H	12.964164	50.600846	68.532554
C	16.144667	55.715878	72.416835	H	16.657010	52.719837	69.040213
H	16.366808	56.428022	71.626881	H	17.588442	50.302450	68.434746
C	16.567452	55.960716	73.719210	H	16.593306	50.041086	72.293462
H	17.140183	56.861550	73.921635	H	14.963760	49.352510	72.342798
C	11.826793	59.554592	70.098999	H	15.211628	51.018706	71.799600
H	12.508416	60.409646	70.143083	H	14.611462	53.635674	68.232927
H	11.560901	59.331077	71.138687	H	17.606235	50.830918	70.281108
C	12.615654	58.393505	69.512475	O	18.320213	43.635466	72.819033
C	12.059487	57.254527	68.910278	H	19.238714	43.918987	72.709755
H	10.983206	57.150343	68.815141	C	18.303708	43.507166	70.426884
C	12.869602	56.222387	68.425381	H	18.390930	42.420606	70.521682
H	12.445833	55.332343	67.973803	H	19.315159	43.918273	70.335936
C	14.253755	56.333546	68.515825	H	17.770899	43.727939	69.497906
O	15.098539	55.297631	68.078065	C	18.888995	54.636763	65.903970
H	15.236212	55.334630	67.116567	H	17.888120	54.993723	65.664741
C	14.834846	57.444012	69.117342	H	19.266652	55.208360	66.756822
H	15.915998	57.520002	69.177681	N	16.612236	53.227083	65.374780
C	14.011831	58.447855	69.618096	C	15.498777	52.550705	64.929926
H	14.465888	59.306448	70.099327	O	14.635892	53.075077	64.244874
C	17.586277	44.051623	71.669356	N	15.388398	51.187194	65.259874
C	16.168411	43.433041	71.825445	H	14.547199	50.727530	64.935692
C	15.131019	44.528418	71.481962	C	16.188056	50.477402	66.140009
C	15.975134	45.658729	70.875049	O	15.933452	49.326445	66.493504
C	17.312753	45.600754	71.642704	N	18.713141	53.233678	66.345851
C	17.174747	46.090301	73.103711	C	17.512050	52.581550	66.087467
C	18.300365	46.483798	70.863005	C	17.346345	51.243072	66.585669
C	17.810205	47.947436	70.783168	N	18.208187	50.612750	67.425318
C	16.375918	48.083541	70.213484	C	19.408504	51.297705	67.669170
C	15.373021	47.058945	70.832816	C	19.683375	52.574480	67.112337
C	14.056597	47.070586	70.053458	C	20.949010	53.134369	67.329926
C	13.439101	48.467566	70.060490	H	21.179661	54.109841	66.920310
C	14.407240	49.557490	69.678163	C	21.942150	52.465861	68.041280
C	13.972602	50.590255	68.929675	C	21.676835	51.188530	68.581685
C	14.771499	51.767921	68.736368	C	20.409649	50.648537	68.401766
O	14.108492	52.795079	68.201995	H	20.173478	49.665009	68.793426
C	16.093585	51.798997	69.148739	C	22.753903	50.401532	69.283975
C	16.724167	50.633044	69.667459	H	23.604519	50.209796	68.619065
C	15.805754	49.551403	70.291801	H	22.371005	49.434496	69.614448
C	15.629072	50.010864	71.779220	H	23.152859	50.927535	70.159210
H	16.075732	42.562085	71.178000	C	23.297013	53.096801	68.215356
H	16.062907	43.072121	72.847926	H	23.592103	53.084365	69.266799
H	14.363839	44.172334	70.789927	H	23.308531	54.132270	67.866589
H	14.610855	44.872094	72.384347	H	24.070260	52.545589	67.666490
H	16.196481	45.370308	69.835952	H	19.471645	54.809036	65.109715
H	16.934985	47.152308	73.161503	H	11.009678	59.856605	69.607968
H	18.102268	45.922350	73.655227	H	15.981702	56.052582	76.636310

H	16.392313	45.552255	73.643014				

Table S 19: Cartesian coordinates of QM atoms for TS2 of alternative mechanism optimized at B3LYP/AMBER level of theory. ν_i = -1427.788 cm^{-1}

Atom	x	y	z	Atom	x	y	z
C	16.633822	55.417153	76.215834	H	19.196574	46.505434	71.331586
H	17.652644	55.805116	76.314875	H	18.310668	46.174654	69.860469
H	16.588796	54.485651	76.784775	H	17.736844	48.408604	71.868113
C	16.296790	55.127128	74.767363	H	18.328618	48.582204	70.232793
C	15.587577	53.970213	74.425652	H	16.272457	47.944628	69.234155
H	15.319597	53.260763	75.203700	H	15.002678	47.232988	71.918434
C	15.208349	53.706880	73.114815	H	14.155923	46.754922	69.036883
H	14.664664	52.803603	72.868316	H	13.238829	46.277854	70.462453
C	15.515626	54.619446	72.101968	H	12.419175	48.460627	69.444116
O	15.132183	54.311953	70.831278	H	12.867323	48.569951	71.133177
H	15.340183	55.037121	70.223488	H	12.857156	50.571185	68.577202
C	16.209616	55.789158	72.422679	H	16.588236	52.629488	69.230665
H	16.431091	56.516826	71.646751	H	18.224606	49.855408	67.355911
C	16.617312	56.015543	73.734011	H	16.316764	49.951133	72.537335
H	17.171929	56.923065	73.954298	H	14.771194	49.086176	72.534007
C	11.978400	59.390393	69.873165	H	14.829247	50.807842	72.129761
H	12.693422	60.219162	69.912405	H	14.599004	53.664160	68.180108
H	11.785307	59.140940	70.924515	H	17.461841	50.691543	70.438573
C	12.746168	58.196263	69.227984	O	18.306342	43.623379	72.750325
C	12.183374	57.033615	68.670493	H	19.222534	43.909040	72.629267
H	11.102601	56.943986	68.602298	C	18.263612	43.555829	70.357600
C	12.964544	55.963356	68.194093	H	18.377185	42.469816	70.426768
H	12.484926	55.085716	67.774194	H	19.264067	43.992565	70.265612
C	14.370226	55.999920	68.227859	H	17.714715	43.783732	69.439838
O	15.158265	54.904482	67.864293	C	18.870983	54.859512	65.947679
H	15.707206	54.539701	66.851100	H	17.887359	55.229420	65.667666
C	14.951299	57.152910	68.780724	H	19.269196	55.534303	66.714006
H	16.034756	57.232493	68.824076	N	16.336420	53.628447	65.962496
C	14.153964	58.196308	69.275974	C	15.392395	52.901849	65.228625
H	14.658418	59.038647	69.738963	O	14.597713	53.407853	64.458006
C	17.548659	44.051415	71.621642	N	15.373615	51.509759	65.405559
C	16.149775	43.394050	71.776546	H	14.652740	51.015409	64.897039
C	15.082870	44.456230	71.419465	C	16.221846	50.771558	66.213123
C	15.896208	45.631001	70.858441	O	16.110983	49.547480	66.384737
C	17.229636	45.592885	71.636038	N	18.658071	53.532368	66.552642
C	17.069218	46.038754	73.108349	C	17.417163	52.901621	66.419799
C	18.194739	46.527744	70.891230	C	17.311193	51.553993	66.713305
C	17.665049	47.980200	70.863161	N	18.306647	50.862377	67.397672
C	16.216060	48.095950	70.321768	C	19.532898	51.471238	67.595481
C	15.244040	47.008853	70.872551	C	19.735482	52.804118	67.151741
C	13.936811	47.028037	70.075888	C	21.007528	53.345133	67.340865
C	13.271957	48.408722	70.124949	H	21.219892	54.347780	66.991102
C	14.218961	49.539294	69.835384	C	22.050856	52.637013	67.964087
C	13.828071	50.571567	69.056584	C	21.820814	51.338301	68.438695
C	14.635353	51.747636	68.887166	C	20.554303	50.786456	68.248795
O	14.069774	52.741756	68.291850	H	20.343735	49.781188	68.604129
C	15.975423	51.754158	69.392727	C	22.894327	50.537134	69.135683
C	16.447635	50.669409	70.054089	H	23.750807	50.341114	68.480278
C	15.574470	49.530836	70.513245	H	22.505752	49.570871	69.466446
C	15.354304	49.857003	72.030027	H	23.288892	51.056464	70.017710
H	16.089360	42.513302	71.140319	C	23.407029	53.261924	68.142409
H	16.049238	43.042818	72.802676	H	23.713865	53.184957	69.186499
H	14.350670	44.087721	70.696750	H	23.420826	54.315739	67.854627
H	14.523258	44.762940	72.311574	H	24.176032	52.739978	67.559649
H	16.133914	45.390938	69.810925	H	19.458645	54.901849	65.139682
H	16.832216	47.099110	73.196757	H	11.133369	59.760632	69.487359
H	17.988714	45.851377	73.666748	H	16.005153	56.060662	76.652493

H	16.274015	45.489601	73.616988				

5.11 17-MT – computed KIE details

Table S 20: Overview of computed KIE values for 17-MT.

Stage	AM1:AMBER	B3LYP:AMBER	B3LYP:AMBER Wigner corr.
E:S⟶TS1	**4.6931** ± 0.52063	**5.3885** ± 0.12346	**7.16** ± 0.18
E:S⟶TS2	**1.1168** ± 0.05405	**0.97662** ± 0.042572	**0.977** ± 0.043
E:I⟶TS1	**3.6687** ± 0.62819	**5.126** ± 0.068516	**6.81** ± 0.11
E:I⟶TS2	**0.97408** ± 0.051163	**0.91915** ± 0.06294	**0.920** ± 0.063
E:S⟶E:I	**1.3056** ± 0.20534	**1.0512** ± 0.020787	**1.051** ± 0.031

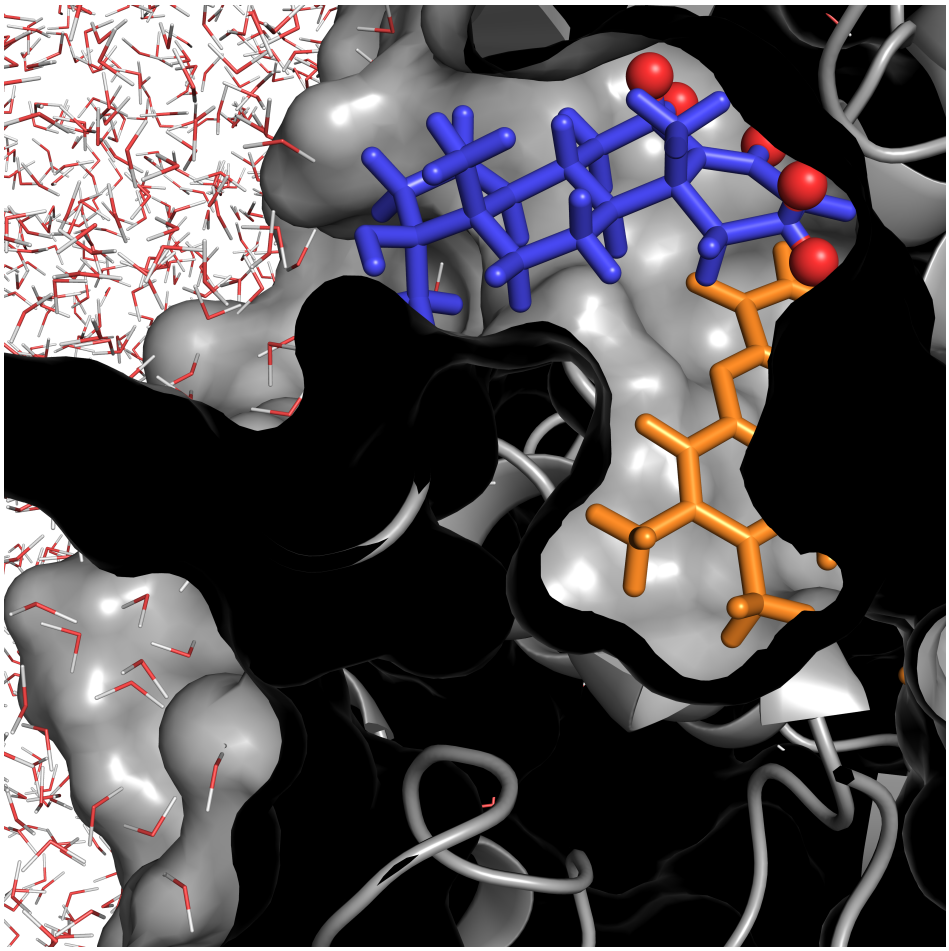


Figure S33: Representative structure of DHT (blue sticks)–enzyme (gray) complex. Atoms substituted with deuterium are marked as red spheres.

5.12 DHT – computed KIE details

Table S 21: Overview of computed KIE values for DHT.

Stage	AM1:AMBER	B3LYP:AMBER	B3LYP:AMBER Wigner corr.
E:S⟶TS1	**1.0298** ± 0.02599	**1.1241** ± 0.035588	**1.124** ± 0.036
E:S⟶TS2	**4.7988** ± 0.14875	**4.9532** ± 0.13808	**6.85** ± 0.20
E:I⟶TS1	**1.0013** ± 0.019547	**0.96991** ± 0.036058	**0.970** ± 0.036
E:I⟶TS2	**4.6291** ± 0.14308	**4.3646** ± 0.15518	**6.04** ± 0.22
E:S⟶E:I	**1.0288** ± 0.027973	**1.16** ± 0.046048	**1.159** ± 0.057

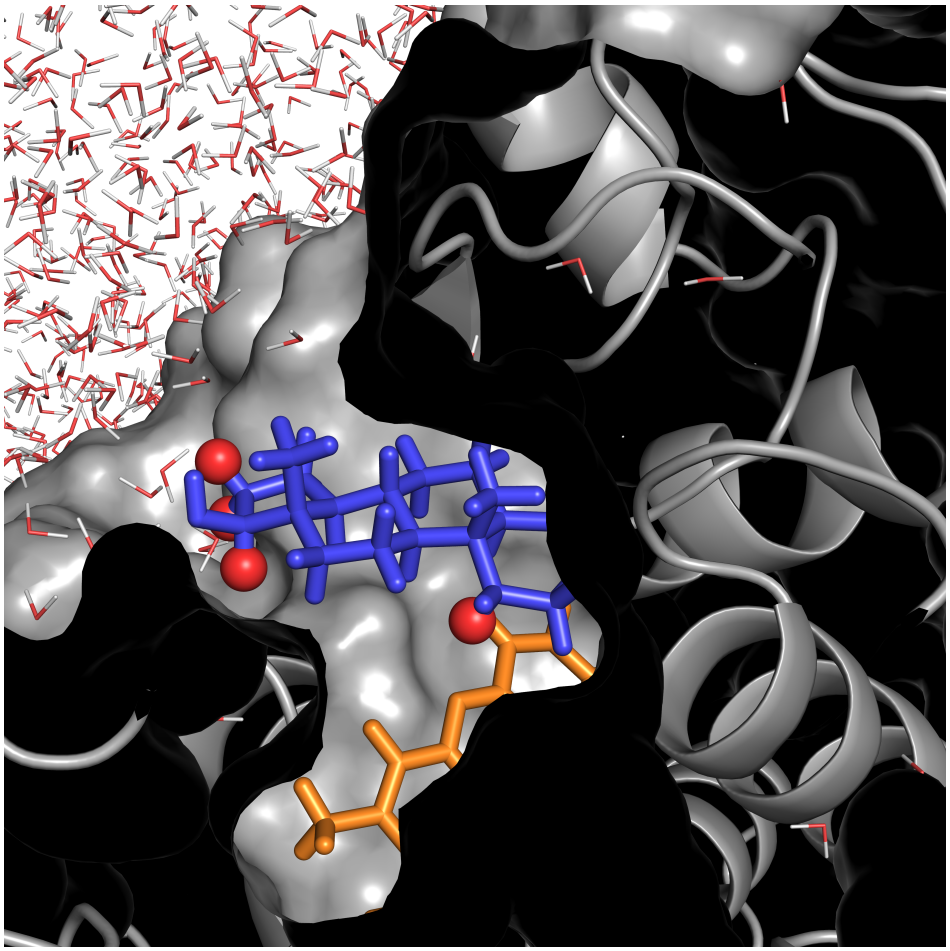


Figure S34: Representative structure of DHT (blue sticks)–enzyme (gray) complex. Atoms substituted with deuterium are marked as red spheres.

5.13 KIE contribution from individual atoms

Table S 22: KIE contribution from individual deuterium atoms for 17MT, S⟶TS1

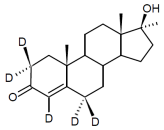
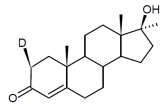
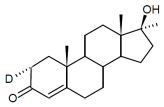
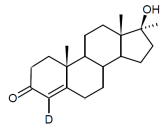
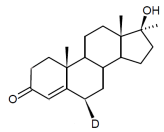
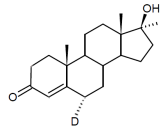
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
4.6931	0.52063	1.9084	**5.3885**	0.12346	0.37894	
4.6228	0.39968	1.3104	**4.7951**	0.043492	0.11177	
1.0407	0.023459	0.11594	**1.0863**	0.0046135	0.010637	
1.0038	0.023266	0.1025	**1.0144**	0.013856	0.04413	
0.97875	0.008086	0.034514	**0.99855**	0.0040321	0.012843	
0.98051	0.01121	0.041743	**1.0079**	0.0078959	0.025763	

Table S 23: KIE contribution from individual deuterium atoms for 17MT, I⟶TS1

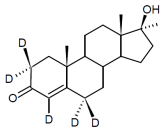
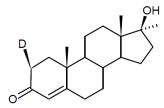
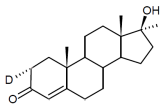
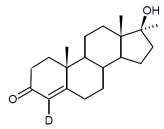
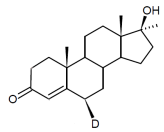
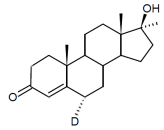
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
3.6687	0.62819	2.5582	**5.126**	0.068516	0.18964	
3.8069	0.72602	2.7384	**5.4398**	0.041293	0.11866	
0.9799	0.024778	0.11592	**0.94661**	0.0051138	0.011629	
0.99987	0.023654	0.10345	**1.0083**	0.019101	0.060745	
0.99537	0.0082126	0.038895	**0.97261**	0.0025893	0.0068353	
0.99488	0.014379	0.06276	**1.017**	0.01187	0.034772	

Table S 24: KIE contribution from individual deuterium atoms for 17MT, I⟶TS2

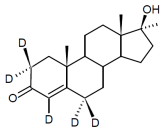
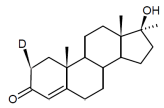
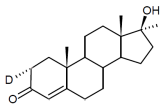
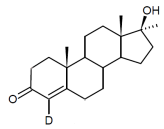
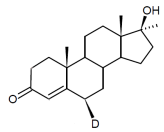
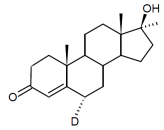
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
0.97408	0.051163	0.24457	**0.91915**	0.06294	0.17839	
0.948	0.021182	0.097175	**0.96439**	0.023296	0.069148	
1.0003	0.015425	0.075839	**0.94904**	0.0040438	0.011448	
1.0012	0.016481	0.080304	**0.99364**	0.02392	0.071782	
1.0137	0.006864	0.032632	**1.0053**	0.0075283	0.021251	
1.0114	0.0087242	0.036826	**1.004**	0.016958	0.046937	

Table S 25: KIE contribution from individual deuterium atoms for 17MT, S⟶TS2

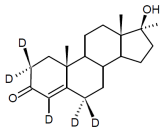
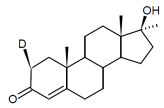
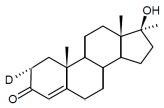
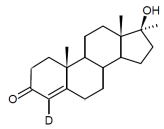
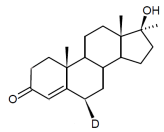
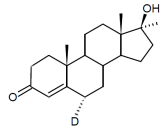
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
1.1168	0.05405	0.26734	**0.97662**	0.042572	0.1196	
0.99541	0.014175	0.064241	**0.86155**	0.014016	0.041283	
1.0779	0.024716	0.12212	**1.0761**	0.0047021	0.012027	
1.0128	0.019793	0.087102	**0.99659**	0.018107	0.053371	
1.0025	0.0081102	0.03564	**1.0313**	0.004622	0.014392	
1.0119	0.0061296	0.026297	**1.0098**	0.0099398	0.030114	

Table S 26: KIE contribution from individual deuterium atoms for DHT, S⟶TS1

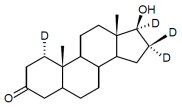
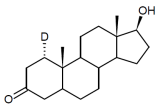
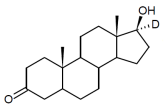
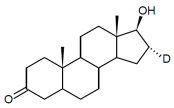
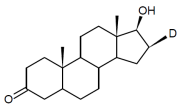
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
1.0298	0.02599	0.13132	**1.1241**	0.035588	0.11303	
1.0255	0.004388	0.020789	**1.1163**	0.017658	0.050495	
0.99818	0.019915	0.090244	**0.99982**	0.042435	0.12247	
0.99734	0.014464	0.059107	**0.99827**	0.01491	0.048042	
1.0088	0.016951	0.07317	**1.0095**	0.018289	0.0593	

Table S 27: KIE contribution from individual deuterium atoms for DHT, I⟶TS1

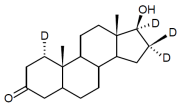
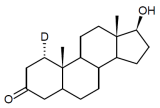
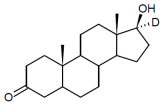
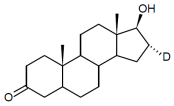
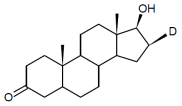
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
1.0013	0.019547	0.1041	**0.96991**	0.036058	0.10383	
0.99874	0.006973	0.028978	**0.96001**	0.0096493	0.028594	
0.99902	0.018107	0.081268	**1.0026**	0.041578	0.11882	
0.99946	0.014193	0.056181	**1.0011**	0.015128	0.048911	
1.0042	0.013827	0.05828	**1.007**	0.017792	0.054939	

Table S 28: KIE contribution from individual deuterium atoms for DHT, I⟶TS2

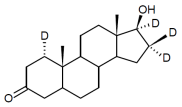
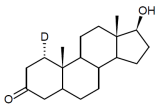
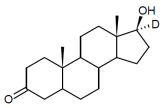
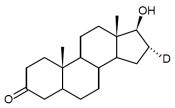
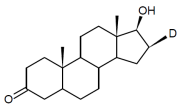
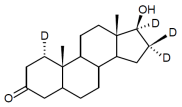
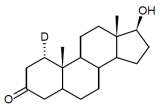
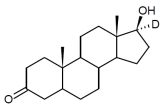
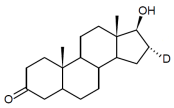
AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
4.6291	0.14308	0.71294	**4.3646**	0.15518	0.46853	
4.6182	0.06111	0.26868	**4.3422**	0.036866	0.085493	
0.9995	0.018838	0.097834	**1.0029**	0.049484	0.15634	
1.0031	0.01578	0.066468	**1.004**	0.020212	0.063215	
0.99978	0.021735	0.111	**0.99927**	0.026952	0.083667	

Table S 29: KIE contribution from individual deuterium atoms for DHT, S⟶TS2

AM1:AMBER			B3LYP:AMBER			Molecule
Mean	Stdev	max-min	Mean	Stdev	max-min	
4.7988	0.14875	0.74841	**4.9532**	0.13808	0.43339	
4.7554	0.060651	0.24244	**4.9123**	0.072691	0.17124	
0.9975	0.021063	0.11136	**0.99848**	0.044729	0.1345	
0.99945	0.015214	0.063753	**0.99797**	0.015977	0.051953	
1.0124	0.022037	0.10359	**1.0127**	0.023873	0.07295