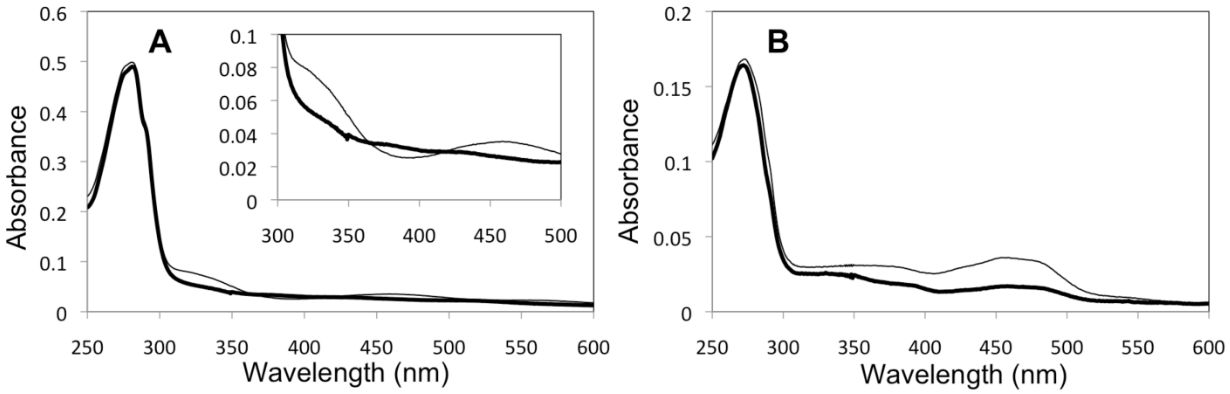
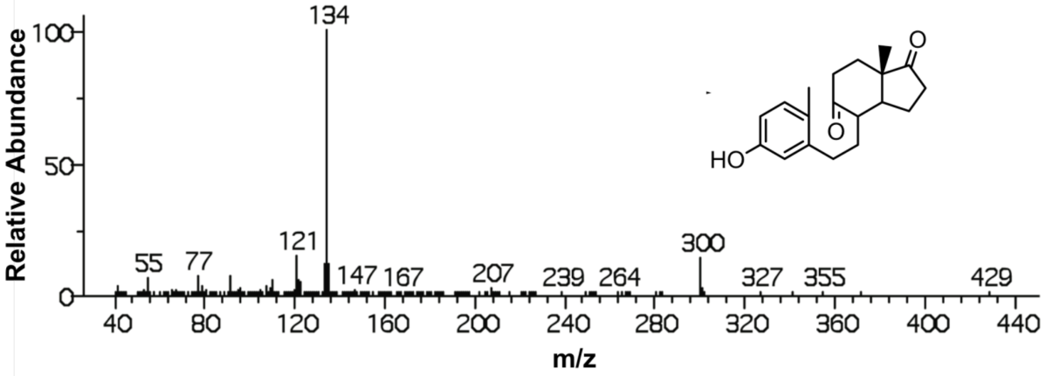
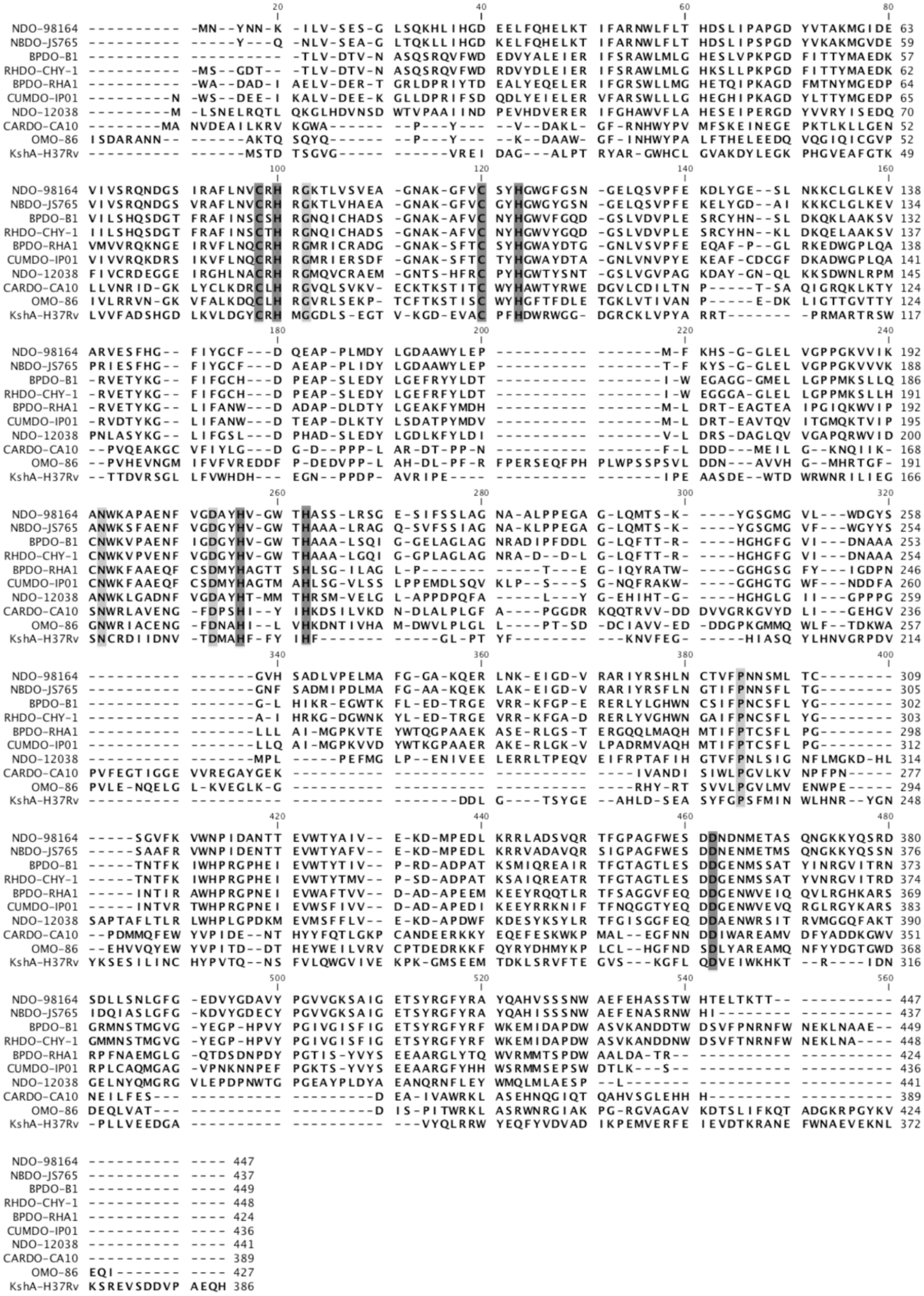


Supplemental Figure 1. Spectra of reduced and oxidized KshA and KshB. UV-vis spectra of KshA (A) and KshB (B) in reduced and oxidized forms are shown as heavy and light lines, respectively. All spectra were recorded in 0.1M potassium phosphate, pH 7.0. Reduced spectra were collected anaerobically. Oxidized KshA and reduced KshB were prepared by adding an excess of potassium ferricyanide or NADH respectively before buffer exchange by gel filtration chromatography.



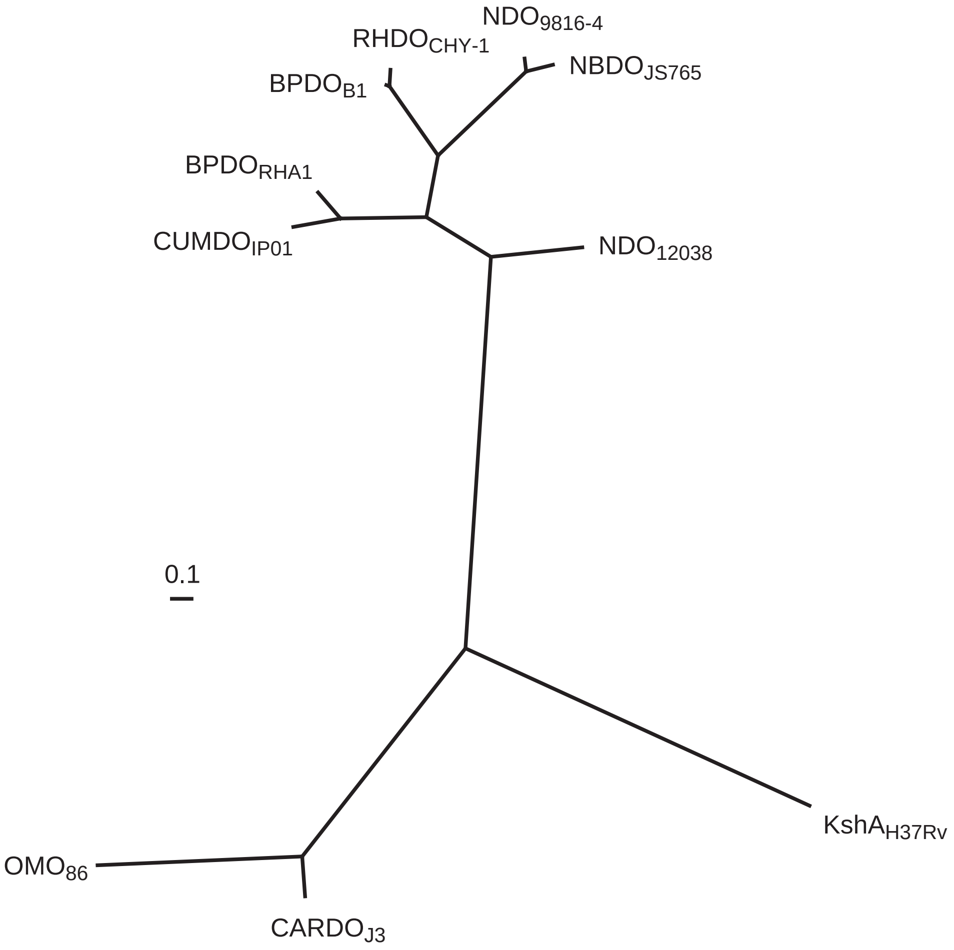
Supplemental Figure 2. Fragmentation pattern of KshA-catalyzed transformation of ADD. GC-MS R_t = 15.32 min; EI-MS (70 eV, EI); m/z: 300 ([M^+], 15%), 134 (100%), 121 (15%), 77 (8%), 55 (6%). This retention time and fragmentation pattern correspond to those of authentic 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (HSA).



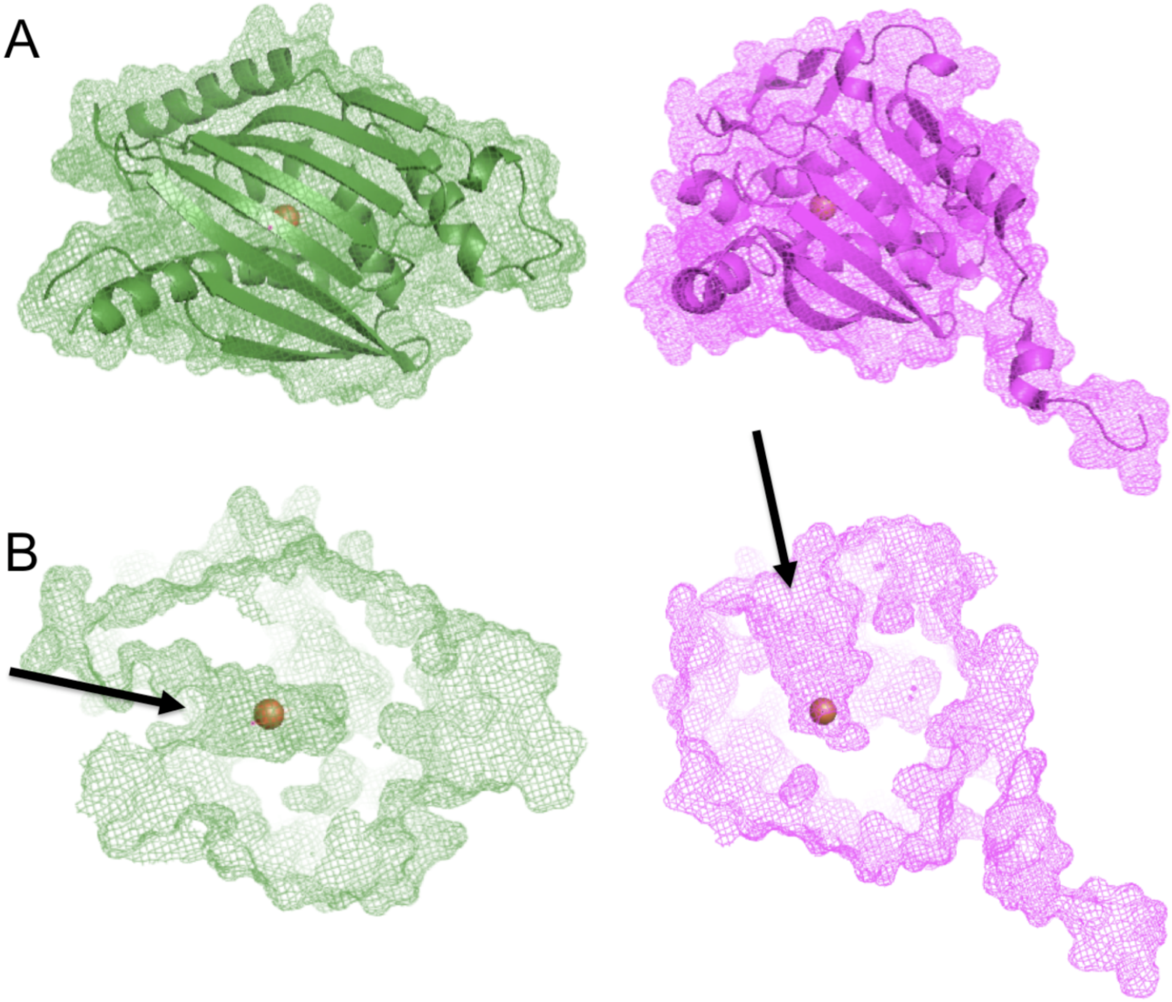
```
                              20                    40                    60                    80
                               |                     |                     |                     |
 NDO-98164 ---------- -MN--YNN-K -ILV-SES-G LSQKHLIHGD EELFQHELKT IFARNWLFLT HDSLIPAPGD YVTAKMGIDE 63
NBDO-JS765 ---------- -----Y---Q -NLV-SEA-G LTQKLLIHGD KELFQHELKT IFARNWLFLT HDSLIPSPGD YVKAKMGVDE 59
   BPDO-B1 ---------- ---------- -TLV-DTV-N ASQSRQVFWD EDVYALEIER IFSRAWLMLG HESLVPKPGD FITTYMAEDK 57
RHDO-CHY-1 ---------- -MS--GDT-- -TLV-DTV-N ASQSRQVFWD RDVYDLEIER IFSRAWLMLG HKSLLPKPGD FITTYMAEDK 62
 BPDO-RHA1 ---------- -WA--DAD-I AELV-DER-T GRLDPRIYTD EALYEQELER IFGRSWLLMG HETQIPKAGD FMTNYMGEDP 64
CUMDO-IP01 ---------N -WS--DEE-I KALV-DEE-K GLLDPRIFSD QDLYEIELER VFARSWLLLG HEGHIPKAGD YLTTYMGEDP 65
 NDO-12038 ---------M -LSNELRQTL QKGLHDVNSD WTVPAAIIND PEVHDVERER IFGHAWVFLA HESEIPERGD YVVRYISEDQ 70
CARDO-CA10 --------MA NVDEAILKRV KGWA------ --P---Y--- --V--DAKL- GF-RNHWYPV MFSKEINEGE PKTLKLLGEN 52
    OMO-86 ISDARANN-- ------AKTQ SQYQ------ --P---Y--- --K--DAAW- GF-INHWYPA LFTHELEEDQ VQGIQICGVP 52
KshA-H37Rv ---------- ------MSTD TSGVG----- ------VREI DAG---ALPT RYAR-GWHCL GVAKDYLEGK PHGVEAFGTK 49
                             100                   120                   140                   160
                               |                     |                     |                     |
 NDO-98164 VIVSRQNDGS IRAFLNVCRH RGKTLVSVEA -GNAK-GFVC SYHGWGFGSN -GELQSVPFE KDLYGE--SL NKKCLGLKEV 138
NBDO-JS765 VIVSRQNDGS VRAFLNVCRH RGKTLVHAEA -GNAK-GFVC GYHGWGYGSN -GELQSVPFE KELYGD--AI KKKCLGLKEV 134
   BPDO-B1 VILSHQSDGT FRAFINSCSH RGNQICHADS -GNAK-AFVC NYHGWVFGQD -GSLVDVPLE SRCYHN--SL DKQKLAAKSV 132
RHDO-CHY-1 IILSHQSDGT FRAFINSCTH RGNQICHADS -GNAK-AFVC NYHGWVYGQD -GSLVDVPLE SRCYHN--KL DKQELAAKSV 137
 BPDO-RHA1 VMVVRQKNGE IRVFLNQCRH RGMRICRADG -GNAK-SFTC SYHGWAYDTG -GNLVSVPFE EQAF-P--GL RKEDWGPLQA 138
CUMDO-IP01 VIVVRQKDRS IKVFLNQCRH RGMRIERSDF -GNAK-SFTC TYHGWAYDTA -GNLVNVPYE KEAF-CDCGF DKADWGPLQA 141
 NDO-12038 FIVCRDEGGE IRGHLNACRH RGMQVCRAEM -GNTS-HFRC PYHGWTYSNT -GSLVGVPAG KDAY-GN-QL KKSDWNLRPM 145
CARDO-CA10 LLVNRID-GK LYCLKDRCLH RGVQLSVKV- ECKTKSTITC WYHAWTYRWE DGVLCDILTN P-----T-SA QIGRQKLKTY 124
    OMO-86 IVLRRVN-GK VFALKDQCLH RGVRLSEKP- TCFTKSTISC WYHGFTFDLE TGKLVTIVAN P-----E-DK LIGTTGVTTY 124
KshA-H37Rv LVVFADSHGD LKVLDGYCRH MGGDLS-EGT V-KGD-EVAC PFHDWRWGG- DGRCKLVPYA RRT------- -PRMARTRSW 117
                             180                   200                   220                   240
                               |                     |                     |                     |
 NDO-98164 ARVESFHG-- FIYGCF---D QEAP-PLMDY LGDAAWYLEP ---------- -------M-F KHS-G-GLEL VGPPGKVVIK 192
NBDO-JS765 PRIESFHG-- FIYGCF---D AEAP-PLIDY LGDAAWYLEP ---------- -------T-F KYS-G-GLEL VGPPGKVVVK 188
   BPDO-B1 -RVETYKG-- FIFGCH---D PEAP-SLEDY LGEFRYYLDT ---------- -------I-W EGAGG-GMEL LGPPMKSLLQ 186
RHDO-CHY-1 -RVETYKG-- FIFGCH---D PEAP-SLEDY LGEFRFYLDT ---------- -------I-W EGGGA-GLEL LGPPMKSLLH 191
 BPDO-RHA1 -RVETYKG-- LIFANW---D ADAP-DLDTY LGEAKFYMDH ---------- -------M-L DRT-EAGTEA IPGIQKWVIP 192
CUMDO-IP01 -RVDTYKG-- LIFANW---D TEAP-DLKTY LSDATPYMDV ---------- -------M-L DRT-EAVTQV ITGMQKTVIP 195
 NDO-12038 PNLASYKG-- LIFGSL---D PHAD-SLEDY LGDLKFYLDI ---------- -------V-L DRS-DAGLQV VGAPQRWVID 200
CARDO-CA10 --PVQEAKGC VFIYLG---D G-D--PPP-L AR-DT-PP-N ---------- -------F-L DDD---MEIL G--KNQIIK- 168
    OMO-86 --PVHEVNGM IFVFVREDDF P-DEDVPP-L AH-DL-PF-R FPERSEQFPH PLWPSSPSVL DDN---AVVH G--MHRTGF- 191
KshA-H37Rv --TTDVRSGL LFVWHDH--- EGN--PPDP- AVRIPE---- ---------- -------IPE AASDE--WTD WRWNRILIEG 166
                             260                   280                   300                   320
                               |                     |                     |                     |
 NDO-98164 ANWKAPAENF VGDAYHV-GW THASS-LRSG E-SIFSSLAG NA-ALPPEGA G-LQMTS-K- ----YGSGMG VL---WDGYS 258
NBDO-JS765 ANWKSFAENF VGDGYHV-GW THAAA-LRAG Q-SVFSSIAG NA-KLPPEGA G-LQMTS-K- ----YGSGMG VF---WGYYS 254
   BPDO-B1 CNWKVPAENF IGDGYHV-GW THAAA-LSQI G-GELAGLAG NRADIPFDDL G-LQFTT-R- ----HGHGFG VI---DNAAA 253
RHDO-CHY-1 CNWKVPVENF VGDGYHV-GW THAAA-LGQI G-GPLAGLAG NRA-D--D-L G-LQFTT-R- ----HGHGFG VI---DNAAA 254
 BPDO-RHA1 CNWKFAAEQF CSDMYHAGTT SHLSG-ILAG L-P------- -----T---E G-IQYRATW- ----GGHGSG FY---IGDPN 246
CUMDO-IP01 CNWKFAAEQF CSDMYHAGTM AHLSG-VLSS LPPEMDLSQV KLP--S---S G-NQFRAKW- ----GGHGTG WF---NDDFA 260
 NDO-12038 ANWKLGADNF VGDAYHT-MM THRSM-VELG L-APPDPQFA -----L---Y G-EHIHT-G- ----HGHGLG II---GPPPG 259
CARDO-CA10 SNWRLAVENG -FDPSHI--Y IHKDSILVKD N-DLALPLGF A----PGGDR KQQTRVV-DD DVVGRKGVYD LI---GEHGV 236
    OMO-86 GNWRIACENG -FDNAHI--L VHKDNTIVHA M-DWVLPLGL L----PT-SD -DCIAVV-ED -DDGPKGMMQ WLF--TDKWA 257
KshA-H37Rv SNCRDIIDNV -TDMAHF-FY IHF------- -----GL-PT YF-------- ---KNVFEG- -----HIASQ YLHNVGRPDV 214
                             340                   360                   380                   400
                               |                     |                     |                     |
 NDO-98164 ---------- -------GVH SADLVPELMA FG-GA-KQER LNK-EIGD-V RARIYRSHLN CTVFPNNSML TC-------- 309
NBDO-JS765 ---------- -------GNF SADMIPDLMA FG-AA-KQEK LAK-EIGD-V RARIYRSFLN GTIFPNNSFL TG-------- 305
   BPDO-B1 ---------- -------G-L HIKR-EGWTK FL-ED-TRGE VRR-KFGP-E RERLYLGHWN CSIFPNCSFL YG-------- 302
RHDO-CHY-1 ---------- -------A-I HRKG-DGWNK YL-ED-TRGE VRR-KFGA-D RERLYVGHWN GAIFPNCSFL YG-------- 303
 BPDO-RHA1 ---------- -------LLL AI-MGPKVTE YWTQGPAAEK ASE-RLGS-T ERGQQLMAQH MTIFPTCSFL PG-------- 298
CUMDO-IP01 ---------- -------LLQ AI-MGPKVVD YWTKGPAAER AKE-RLGK-V LPADRMVAQH MTIFPTCSFL PG-------- 312
 NDO-12038 ---------- -------MPL -----PEFMG LP--ENIVEE LERRLTPEQV EIFRPTAFIH GTVFPNLSIG NFLMGKD-HL 314
CARDO-CA10 PVFEGTIGGE VVREGAYGEK ---------- ---------- ---------- ----IVANDI SIWLPGVLKV NPFPN----- 277
    OMO-86 PVLE-NQELG L-KVEGLK-G ---------- ---------- ---------- ----RHY-RT SVVLPGVLMV ENWPE----- 294
KshA-H37Rv ---------- ---------- ---------- -------DDL G----TSYGE --AHLD-SEA SYFGPSFMIN WLHNR--YGN 248
                             420                   440                   460                   480
                               |                     |                     |                     |
 NDO-98164 -----SGVFK VWNPIDANTT EVWTYAIV-- E-KD-MPEDL KRRLADSVQR TFGPAGFWES DDNDNMETAS QNGKKYQSRD 380
NBDO-JS765 -----SAAFR VWNPIDENTT EVWTYAFV-- E-KD-MPEDL KRRVADAVQR SIGPAGFWES DDNENMETMS QNGKKYQSSN 376
   BPDO-B1 -----TNTFK IWHPRGPHEI EVWTYTIV-- P-RD-ADPAT KSMIQREAIR TFGTAGTLES DDGENMSSAT YINRGVITRN 373
RHDO-CHY-1 -----TNTFK IWHPRGPHEI EVWTYTMV-- P-SD-ADPAT KSAIQREATR TFGTAGTLES DDGENMSSAT YVNRGVITRD 374
 BPDO-RHA1 -----INTIR AWHPRGPNEI EVWAFTVV-- D-AD-APEEM KEEYRQQTLR TFSAGGVFEQ DDGENWVEIQ QVLRGHKARS 369
CUMDO-IP01 -----INTVR TWHPRGPNEI EVWSFIVV-- D-AD-APEDI KEEYRRKNIF TFNQGGTYEQ DDGENWVEVQ RGLRGYKARS 383
 NDO-12038 SAPTAFLTLR LWHPLGPDKM EVMSFFLV-- E-KD-APDWF KDESYKSYLR TFGISGGFEQ DDAENWRSIT RVMGGQFAKT 390
CARDO-CA10 --PDMMQFEW YVPIDE--NT HYYFQTLGKP CANDEERKKY EQEFESKWKP MAL--EGFNN DDIWAREAMV DFYADDKGWV 351
    OMO-86 --EHVVQYEW YVPITD--DT HEYWEILVRV CPTDEDRKKF QYRYDHMYKP LCL--HGFND SDLYAREAMQ NFYYDGTGWD 368
KshA-H37Rv YKSESILINC HYPVTQ--NS FVLQWGVIVE KPK-GMSEEM TDKLSRVFTE GVS---KGFL QDVEIWKHKT --R----IDN 316
                             500                   520                   540                   560
                               |                     |                     |                     |
 NDO-98164 SDLLSNLGFG -EDVYGDAVY PGVVGKSAIG ETSYRGFYRA YQAHVSSSNW AEFEHASSTW HTELTKTT-- ---------- 447
NBDO-JS765 IDQIASLGFG -KDVYGDECY PGVVGKSAIG ETSYRGFYRA YQAHISSSNW AEFENASRNW HI-------- ---------- 437
   BPDO-B1 GRMNSTMGVG -YEGP-HPVY PGIVGISFIG ETSYRGFYRF WKEMIDAPDW ASVKANDDTW DSVFPNRNFW NEKLNAAE-- 449
RHDO-CHY-1 GMMNSTMGVG -YEGP-HPVY PGIVGISFIG ETSYRGFYRF WKEMIDAPDW ASVKANDDNW DSVFTNRNFW NEKLNA---- 448
 BPDO-RHA1 RPFNAEMGLG -QTDSDNPDY PGTIS-YVYS EEAARGLYTQ WVRMMTSPDW AALDA-TR-- ---------- ---------- 424
CUMDO-IP01 RPLCAQMGAG -VPNKNNPEF PGKTS-YVYS EEAARGFYHH WSRMMSEPSW DTLK---S-- ---------- ---------- 436
 NDO-12038 GELNYQMGRG VLEPDPNWTG PGEAYPLDYA EANQRNFLEY WMQLMLAESP --L------- ---------- ---------- 441
CARDO-CA10 NEILFES--- ---------- ---------D EA-IVAWRKL ASEHNQGIQT QAHVSGLEHH H--------- ---------- 389
    OMO-86 DEQLVAT--- ---------- ---------D IS-PITWRKL ASRWNRGIAK PG-RGVAGAV KDTSLIFKQT ADGKRPGYKV 424
KshA-H37Rv -PLLVEEDGA ---------- ---------- ---VYQLRRW YEQFYVDVAD IKPEMVERFE IEVDTKRANE FWNAEVEKNL 372

 NDO-98164 ---------- ---- 447
NBDO-JS765 ---------- ---- 437
   BPDO-B1 ---------- ---- 449
RHDO-CHY-1 ---------- ---- 448
 BPDO-RHA1 ---------- ---- 424
CUMDO-IP01 ---------- ---- 436
 NDO-12038 ---------- ---- 441
CARDO-CA10 ---------- ---- 389
    OMO-86 EQI------- ---- 427
KshA-H37Rv KSREVSDDVP AEQH 386
```

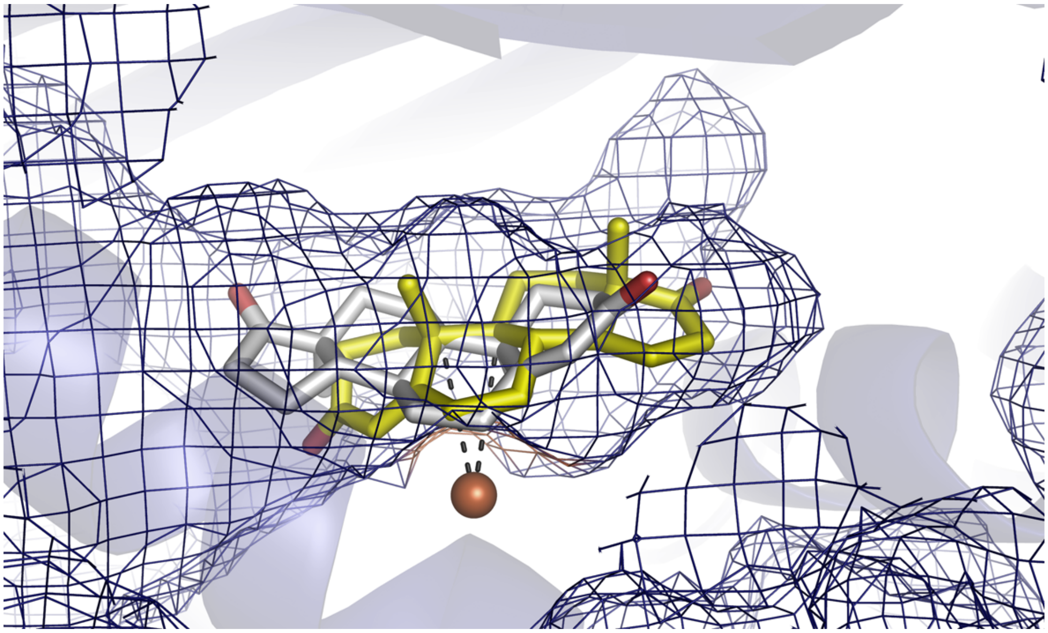
Supplemental Figure 3. Structure-based sequence alignment of ROs of known structure. The ROs are identified in the legend of Supplemental Figure 4. Metal ligands are highlighted in dark grey. Other conserved residues are highlighted in light grey.



Supplemental Figure 4. Radial phylogram of structurally characterized ROs. The α_3 and $\alpha_3\beta_3$ enzymes are clustered at the bottom and top of the tree, respectively. Structure-based sequence alignments were performed using: $\text{KshA}_{\text{H37Rv}}$ from *M. tuberculosis* H37Rv (PDB accession code 2ZYL); OMO_{86}, 2-oxoquinoline 8-monooxygenase from *P. putida* 86 (1Z02), CARDO_{J3}, carbazole 1,9α-dioxygenase from *Janthinobacerium* sp. strain J3 (1WW9); BPDO_{B1}, biphenyl dioxygenase from *S. yanoikuyae* B1 (2GBW); $\text{BPDO}_{\text{RHA1}}$ from *R. jostii* RHA1 (1ULI); $\text{NDO}_{9816\text{-}4}$, naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816-4 (1O7W); NDO_{12038} from *Rhodococcus* sp. strain NCIMB 12038 (2B1X); $\text{CUMDO}_{\text{IP01}}$, cumene dioxygenase from *P. fluorescens* IP01 (1WQL); $\text{NBDO}_{\text{JS765}}$ from *Comamonas* sp. strain JS765 (2BMO); and $\text{RHDO}_{\text{CHY-1}}$, ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain CHY-1 (2CKF).



Supplemental Figure 5. Orientation of the active site channel. The catalytic domains of KshA (green) and CARDO$_{J3}$ (magenta) are shown in the same orientation as A) ribbon diagrams with the Connolly surface displayed as mesh, and B) cross sections of the Connolly surface. The orientation of the active site channel is indicated with a black arrow. Figures were prepared using PYMOL.



Supplemental Figure 6. Additional solution of ADD docking simulation, from the same top rmsd cluster. Two conformations of ADD docked in the active site of KshA are shown as stick representations. Carbon atoms of the two conformations are shown in magenta and grey, oxygen atoms are shown in red. The distance between the mononuclear iron and the C9 atom is 3.8 Å and 3.7 Å for the two solutions. The mononuclear iron is represented as an orange sphere. Docking simulations were performed using AutoDock. The Connolly surface of the substrate-binding pocket is represented by blue mesh. Figure generated using PYMOL.