Electronic Supplementary Information

Molecular evolution of a cytochrome P450 for the synthesis of potential antidepressant (2*R*,6*R*)-hydroxynorketamine

Ansgar Bokel,[a] Michael C. Hutter,[b] and Vlada B. Urlacher*[a]

[a] *Institute of Biochemistry, Heinrich-Heine University Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany*

[b] *Center for Bioinformatics, Saarland University, Campus E2.1, 66123 Saarbruecken, Germany*

*E-mail: Vlada.Urlacher@uni-duesseldorf.de

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Material and Methods

OLIGONUCLEOTIDES

Table S1. Oligonucleotides for cloning and site-saturation mutagenesis. The codons for mutated positions are underlined. Primers for the mutations that were already available in the lab are not listed below. Fw: forward primer; Rev: reverse primer. Sat_ultrashort: binds at the 5'-end of the gene; Sat_short: binds 354 bp upstream of the 5'-end; Sat_long: binds 4958 bp upstream of the 5' end.

Primer for MegaPrimer PCR	**Sequence (5'→ 3')**
Rev: CYP154E1 I238Q M388A **M87_NNK**	GAT TCC ACG CGC AGM NNG TTG GCG ACC GGA TG
Rev: CYP154E1 I238Q M388A **L94_NNK**	TCC GGA GCG GGC MNN CAT GGA TTC CAC G
Rev: CYP154E1 I238Q M388A **L235_NNK**	CCG CCC TGG ATG AGM NNC AGC GTG TTG TGG
Rev: CYP154E1 I238Q M388A **G239_NNK**	TGG TTT CGA ACC CMN NCT GGA TGA GCA GC
Rev: CYP154E1 I238Q M388A **T243_NNK**	GCT GAT CAT GCC CAT GGT MNN TTC GAA CCC GCC CTG GAT GAG
Rev: CYP154E1 I238Q M388A **V286_NNK**	GAA CGG CAG CAT GAC MNN CGC TGA TTC GAA G
Rev: CYP154E1 I238Q M388A **L289_NNK**	CGT GGT GTA CAG GAA CGG MNN CAT GAC CAC CGC TGA TTC G
Fw: Sat_ultrashort	GGA GAT ATA CAT ATG GGA CAG TCC CGC CGA CC
Fw: Sat_short	CCT GCA TTA GGA AGC AGC CCA GTA GTA GGT TGA GGC CGT TG
Fw: Sat_long	TGG TTC ACG TAG TGG GCC ATC GCC CTG ATA GAC GG
Primer for QuikChange	
Fw: CYP154E1 L289T	CGA ATC AGC GGT GGT CAT GAC GCC GTT CCT GTA CAC CAC G
Rev: CYP154E1 L289T	CGT GGT GTA CAG GAA CGG CGT CAT GAC CAC CGC TGA TTC G
Fw: CYP154E1 L289T (I238Q M388A **V286G**)	CGA ATC AGC GGG CGT CAT GAC GCC GTT CCT GTA CAC CAC G
Rev: CYP154E1 L289T (I238Q M388A **V286G**)	CGT GGT GTA CAG GAA CGG CGT CAT GAC GCC CGC TGA TTC G
Fw: CYP154E1 I238A	CAA CAC GCT GCT GCT CAT CGC GGG CGG GTT CGA AAC C
Rev: CYP154E1 I238A	GGT TTC GAA CCC GCC CGC GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238C	CAA CAC GCT GCT GCT CAT CTG CGG CGG GTT CGA AAC C
Rev: CYP154E1 I238C	GGT TTC GAA CCC GCC GCA GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238D	GCT GCT GCT CAT CGA CGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238D	GTG GTT TCG AAC CCG CCG TCG ATG AGC AGC AGC
Fw: CYP154E1 I238E	GCT GCT GCT CAT CGA GGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238E	GTG GTT TCG AAC CCG CCC TCG ATG AGC AGC AGC
Fw: CYP154E1 I238F	GCT GCT GCT CAT CTT CGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238F	GTG GTT TCG AAC CCG CCG AAG ATG AGC AGC AGC
Fw: CYP154E1 I238G	CAA CAC GCT GCT GCT CAT CGG CGG CGG GTT CGA AAC C
Rev: CYP154E1 I238G	GGT TTC GAA CCC GCC GCC GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238H	CAA CAC GCT GCT GCT CAT CCA CGG CGG GTT CGA AAC C
Rev: CYP154E1 I238H	GGT TTC GAA CCC GCC GTG GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238K	CAA CAC GCT GCT GCT CAT CAA AGG CGG GTT CGA AAC C
Rev: CYP154E1 I238K	GGT TTC GAA CCC GCC TTT GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238L	CAA CAC GCT GCT GCT CAT CCT GGG CGG GTT CGA AAC C

Rev: CYP154E1 I238L	GGT TTC GAA CCC GCC CAG GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238M	CAA CAC GCT GCT GCT CAT CAT GGG CGG GTT CGA AAC C
Rev: CYP154E1 I238M	GGT TTC GAA CCC GCC CAT GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238N	GCT GCT GCT CAT CAA CGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238N	GTG GTT TCG AAC CCG CCG TTG ATG AGC AGC AGC
Fw: CYP154E1 I238P	CAA CAC GCT GCT GCT CAT CCC GGG CGG GTT CGA AAC C
Rev: CYP154E1 I238P	GGT TTC GAA CCC GCC CGG GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238Q	GCT GCT GCT CAT CCA GGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238Q	GTG GTT TCG AAC CCG CCC TGG ATG AGC AGC AGC
Fw: CYP154E1 I238R	CAA CAC GCT GCT GCT CAT CCG TGG CGG GTT CGA AAC C
Rev: CYP154E1 I238R	GGT TTC GAA CCC GCC ACG GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238S	GCT GCT GCT CAT CAG TGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238S	GTG GTT TCG AAC CCG CCA CTG ATG AGC AGC AGC
Fw: CYP154E1 I238T	GCT GCT GCT CAT CAC CGG CGG GTT CGA AAC CAC
Rev: CYP154E1 I238T	GTG GTT TCG AAC CCG CCG GTG ATG AGC AGC AGC
Fw: CYP154E1 I238V	CAA CAC GCT GCT GCT CAT CGT GGG CGG GTT CGA AAC C
Rev: CYP154E1 I238V	GGT TTC GAA CCC GCC CAC GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238W	CAA CAC GCT GCT GCT CAT CTG GGG CGG GTT CGA AAC C
Rev: CYP154E1 I238W	GGT TTC GAA CCC GCC CCA GAT GAG CAG CAG CGT GTT G
Fw: CYP154E1 I238Y	CAA CAC GCT GCT GCT CAT CTA CGG CGG GTT CGA AAC C
Rev: CYP154E1 I238Y	GGT TTC GAA CCC GCC GTA GAT GAG CAG CAG CGT GTT G

ISOLATION OF (*R*)-KETAMINE FROM RACEMIC KETAMINE

Isolation of (*R*)-ketamine from racemic ketamine hydrochloride was done according to patent DE60131397T2.[1] First, the free base form of racemic ketamine was generated. To this end, 4.97 g racemic ketamine hydrochloride were dissolved in 400 ml water; 1 M sodium carbonate was added till the pH of the solution reached pH 9.0. White sediment (freebase form of racemic ketamine) was filtrated under vacuum and dried at 37°C. Yield: 2.62 g. The filtrate containing remains of racemic ketamine freebase was extracted twice with 250 ml ethyl acetate. Organic phase was dried over sodium sulfate, filtered, and fully evaporated. Yield: 1.296 g. Combined isolated yield of racemic ketamine freebase was 3.916 g. In a two-necked flask, 4.93 g of ketamine freebase (additional ketamine freebase originates from a previous attempt) were dissolved in 63.1 ml acetone under constant stirring and heating. A reflux condenser was used to condense gaseous acetone. 1.89 g L-(+)-tartaric acid and 4.2 ml water were added and the solution was heated to 55°C. Temperature was hold for 15 minutes and afterwards the solution was cooled down to room temperatrue (RT) under stirring, which hold overnight. A white sediment occurred ((*S*)-ketamine tartrate), which was filtered under vacuum. The filtered white solid was stirred for 1.5 hours in 16 ml acetone - to remove remains of (*R*)-ketamine tartrate - and filtrated again (white solid was dried and stored at 4°C). The filtrates from both filtration steps were combined and evaporated to dryness ((*R*)-ketamine tartrate). Yield: 2.816 g. 2.816

g (*R*)-ketamine tartrate were dissolved in 28 ml 1 M HCl and a solution of 25% ammonium hydroxide was added until the pH reached 12. White precipitate ((*R*)-ketamine freebase) was filtered under vacuum and the purity was analyzed via chiral HPLC (Figure S1). Yield: 2.061 g; purity: ~90%. In order to increase the purity, the product was recrystallized: 2 g (*R*)-ketamine freebase were dissolved in 30 ml *n*-hexane under constant stirring and heated to the boiling point of *n*-hexane. A reflux condenser was used to condense gaseous *n*-hexane. More *n*-hexane was added stepwise until all (*R*)-ketamine was dissolved. The final solution was kept at boiling point for another 10 min before cooling down to RT overnight. Finally, the solution was cooled on ice and the white precipitate was filtered under vacuum and dried at 37°C. Yield: 1.82 g; Purity analyzed via chiral HPLC was ~99%. Finally, (*R*)-ketamine hydrochloride was synthesized. 1.8 g (*R*)-ketamine freebase were dissolved in 50 ml ethanol (HPLC grade). 37% HCl solution was added until the pH reached 2. The solution was cooled to 15°C before 51 ml diethyl ether were added and stirred for one hour at 15°C. Again, the precipitate was filtered under vacuum and the filtrate was saturated again with 37% HCl, stirred for another hour at 15°C and the precipitate again filtered under vacuum. The precipitates were combined, dissolved in 22 ml of a 1:1 mixture of ethanol and diethyl ether, stirred for 30 min and filtered under vacuum. The white powder was dried at 37°C. Yield: 1.04 g (37% yield when starting from 4.93 g ketamine freebase).

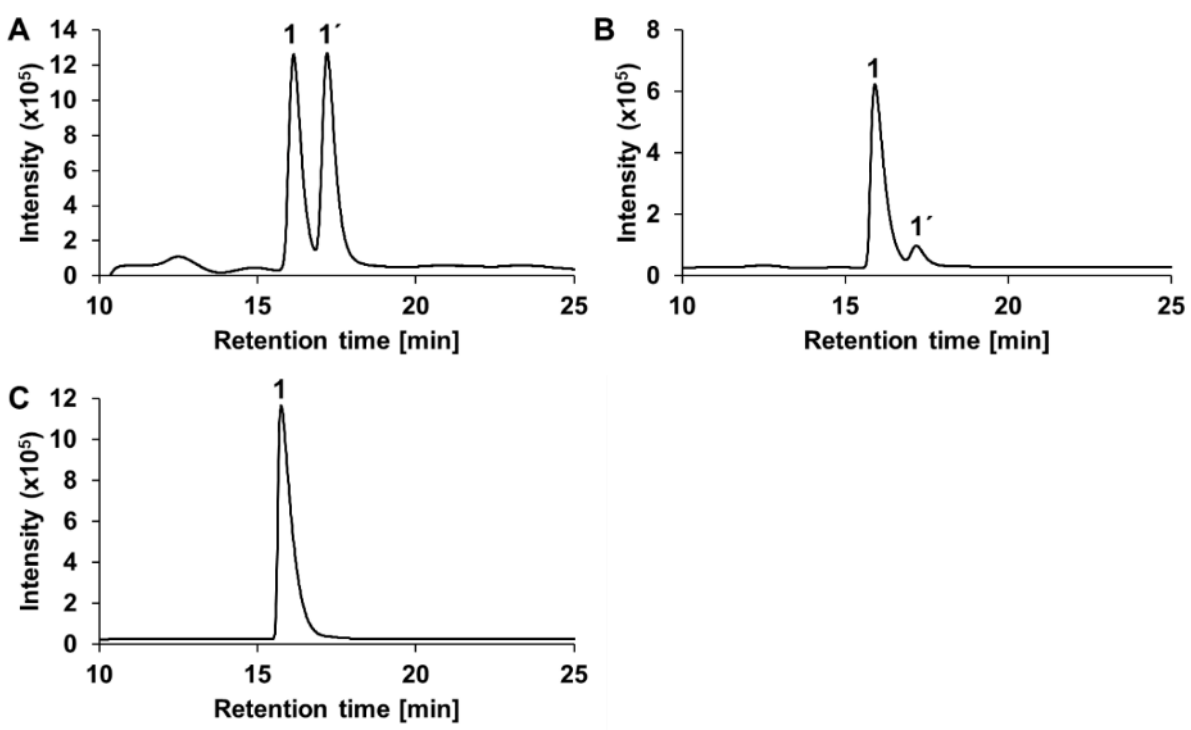

*Fig. S1. Chiral HPLC analysis of the intermediates during (R)-ketamine isolation from racemic ketamine: Racemic ketamine (**A**); (R)-ketamine free base after isolation (**B**) and after recrystallization (**C**). Analysis was carried out using HPLC on a chiral column Chiralpak IB (0.46 cm Ø x 25 cm, Chiral Technologies Europe). **1**: (R)-ketamine, **1´**: (S)-ketamine (identified by comparing with authentic standard from Sigma Aldrich).*

ENZYME EXPRESSION AND ASSAYS

Expression and purification of the redox proteins, the flavodoxin reductase FdR from *Escherichia coli* and the flavodoxin YkuN from *Bacillus subtilis*, as well as the expression of the NADPH regenerating glucose dehydrogenase (GDH) from *Bacillus megaterium* (vector pET22b) and the CYP154E1 variants (vector pET22b and pET28a) were performed as previously described.[2]

Concentrations of cytochrome P450 enzymes (in *E. coli* cell lysates) were determined based on the CO-difference spectra as previously described. Once a sample was saturated with CO and subsequently reduced with sodium dithionite (50 mM), absorption spectra were recorded between 400 and 500 nm. The P450 concentration was calculated using the extinction coefficient of $\varepsilon_{450\text{-}490\ nm} = 91\ mM^{-1}\ cm^{-1}$.[3] Concentrations of the purified redox proteins YkuN and FdR, as well as the $NADP^+$ reduction activity of the GDH were determined as described elsewhere.[4]

MUTAGENESIS AND SCREENING

Construction of the starting variant. Construction of CYP154E1 mutants was carried out according to a modified QuikChange mutagenesis protocol by Edelheit et al. (2009) using two separated single-primer reactions.[5] Each PCR reaction contained ~500 ng of the template plasmid DNA, 4% DMSO, 0.2 mM dNTPs, 1 U Phusion DNA Polymerase, and either 2 µM forward or reverse primer in a total volume of 25 µl of HF Phusion buffer. Two PCR reactions were carried out in parallel, one with the forward and the other with the reverse primer. The PCR was carried out using the following program: denaturation step at 98°C for 2 min, followed by 30 cycles of 98°C 30 s, annealing for 60 s at 60°C (independent of the primer) and elongation at 72°C for 30 s/kb and a final extension at 72°C for 6 min. Subsequently, mixtures from both PCRs were combined and reannealing of the PCR products occurred according to the program reported by Edelheit et al.[5] Template DNA was removed by *Dpn*I digestion (addition of 1 µl FastDigest *Dpn*I [Thermo Fisher Scientific] and incubation for 1 h at 37°C. Afterwards, another 1 µl FastDigest *Dpn*I was added and the mixture was incubated overnight at 37°C) prior to transformation of chemically competent *E. coli* DH5α cells.

Library construction. Construction of the site-saturation sub-libraries was carried out according to Sanchis et al 2008[6] using primer shown in Table S1. Antiprimer were designed to first generate megaprimer of different length; the antiprimer for the ultrashort megaprimer anneals to the 5'-end of the gene (generating a megaprimer of 287 - 897 bp dependent on the mutation carrying forward primer); the antiprimer for the short megaprimer anneals 354 bp

upstream of the 5'-end of the gene (generating a megaprimer of 641 - 1251 bp dependent on the mutation carrying forward primer), and the antiprimer for the long megaprimer anneals 4958 bp upstream of the 5'-gene end (generating a megaprimer of 5245 - 5855 bp dependent on the mutation carrying forward primer). The first PCR cycle was used to generate the megaprimer and comprised of the following steps: initial denaturation at 98°C for 30 seconds, followed by five cycles at 98°C for 10 s, annealing for 30 s (temperature varied at this step and for all PCRs a temperature gradient was used) and elongation at 72°C for 20 s/kb (using Phusion DNA Polymerase) dependent on the megaprimer length. In the second PCR cycle the whole plasmid was amplified and program comprised of 20 cycles at 98°C for 30 s and at 72°C for 6 min, followed by a final extension at 72°C for 12 min. Each Megaprimer-PCR was controlled via agarose gel electrophoresis. Template DNA was removed by *Dpn*I digestion (addition of 1 µl FastDigest *Dpn*I [Thermo Fisher Scientific] and incubation for 1 h at 37°C. Afterwards, another 1 µl FastDigest *Dpn*I was added and the mixture was incubated overnight at 37°C) prior to purification of the PCR sample and subsequent transformation of electrocompetent *E. coli* DH5α cells. After performing electroporation, one half of *E. coli* cells was spread on LB agar plates, whereas the other half was used to inoculate precultures, which were harvested the next day and used for plasmid preparation and subsequent sequencing. If the sequencing proved the NNK diversity at the desired positions and colony numbers were more than 100 colonies per plate, chemical competent *E. coli* BL21(DE3) pCOLA Duet YkuN (MCSI) FdR (MCSII) cells were transformed with the plasmids from the sub-libraries. Here again, the transformed cells were divided into two parts, and the quality of the library was checked again by sequencing.

Library screening. Colonies for each sub-library were toothpicked and used to inoculate 600 µl LB medium (containing 30 mg/ml kanamycin and 100 mg/ml ampicillin) in 96-deep well plates. After overnight incubation at 37°C and 600 rpm (TiMix 5 Control, Edmund Bühler GmbH), 20 µl of these precultures were used to inoculate expression cultures containing 980 µl autoinduction medium (supplemented with 30 mg/ml kanamycin, 100 mg/ml ampicillin, 100 µM $FeSO_4$ and 500 µM 5-aminolevulinic acid) per well. 300 µl 86% glycerin were added to the rest of the precultures and frozen at -80°C. The expression cultures were incubated at 37°C and 600 rpm till an OD_{600} of ~ 1.0 was reached (wells A2 -A6 contained BL21(DE) pCOLA Duet YkuN (MCSI) FdR (MCSII) pET22b CYP154E1 I238Q M388A only and wells A2-A5 were used to measure the OD_{600} whereas well A6 served as a control for the conversion of (*R*)-ketamine). After an OD_{600} of ~1.0 was reached, the plates were incubated further at 25°C and 500 rpm overnight. The plates were centrifuged at 4500 rpm for 30 min at 4°C and the cells were washed once with 1 ml per well potassium phosphate buffer pH 7.5 containing sucrose and EDTA-Na_2. Cells were resuspended in 465 µl of the same potassium phosphate buffer; 25 µl

of a nutrient solution containing glucose, lactose and citrate, and 10 µl of 25 mM (*R*)-ketamine were added. The reaction mixtures were shaken at 25°C and 1000 rpm. After 18 hours, the reactions were stopped by addition of 500 µl 1 M sodium carbonate and extracted with 400 µl ethyl acetate. After vigorous shaking and centrifugation, 100 µl of the organic supernatant were directly transferred to GC/MS glass vials. The GC/MS analysis was done on a GC/MS QP-2010 Plus (Shimadzu, Duisburg, Germany) with a FS-Supreme-5ms (30 m x 0.25 mm x 0.25 µm) column and helium as carrier gas as previously described.[2]

Verification of positive mutants. The variants performing better (higher amount of (2*R*,6)-hydroxynorketamine and middle to high conversion) than the CYP154E1 I238Q M388A control (well A6) were sequenced and their performance verified in an *in vitro* conversion of (*R*)-ketamine. To this end, positive mutants were streaked on LB plates containing ampicillin only to get rid of the pCOLA Duet YkuN (MCSI) FdR (MCSII) vector. Single colony was picked to inoculate LB precultures with ampicillin as the only antibiotic. After shaking at 37°C and 180 rpm overnight, 25 µl of the precultures were used to inoculate new precultures containing again only ampicillin. This was repeated three times to get rid of most of the pCOLA Duet YkuN (MCSI) FdR (MCSII) plasmid (which impairs the *cyp154E1* expression). Further expression of the variants was carried out as described elsewhere.[2] After expression and cell lysis, oxidation reactions were performed with *E. coli* cell lysates in 100 mM potassium phosphate buffer pH 7.5 in a total reaction volume of 125 µl at 25°C and 600 rpm. The reaction mixture contained 500 µM (*R*)-ketamine HCl (dissolved in water), 2.5 µM P450 (crude cell lysate), 2.5 µM purified FdR, 25 µM purified YkuN, 200 µM NADPH, 5 U/ml GDH in the presence of 20 mM glucose for cofactor regeneration and 600 U/ml catalase. After 18 hours, reactions were stopped by addition of 125 µl of 1 M Na_2CO_3 and 10 µl of 5 mM xylazine hydrochloride as internal standard. Reactions were extracted twice with 200 µl ethyl acetate and the combined organic phases were evaporated to dryness. Evaporated samples were resolved in a 1:1 mixture of acetonitrile and water (for LC/MS analysis) or 100 µl ethanol (for HPLC analysis) respectively.

Kinetic constants were estimated under the same reaction conditions except for enzyme and substrate concentrations and reaction time. 0.5 µM CYP, 0.5 µM FdR and 40 µM YkuN were used to convert 50 – 2000 µM substrate, and reactions were stopped after 3 – 17 min. Kinetic data were fitted to the Michaelis-Menten equation using RStudio software (RStudio Team (2015).[7]

LC/MS analysis was performed on a device consisting of a DGU-$20A_3$ Degaser, two LC-20AD modules (each for one solvent), a CBM-20A Communications Bus Module, a SPD-M20A Diode Array Detector, a SIL-20A HT Autosampler, and CTO-10AS VP column oven connected to LCMS-2020 (all from Shimadzu). A Chromolith® Performance RP-8e 100-4.6 mm column

(Merck Millipore), equipped with a Chromolith® RP-8e 5-4.6 mm guard cartridge was used. Elution occurred on a gradient between water (supplemented with 0.1% formic acid) and acetonitrile. Stereoselectivity was analyzed via HPLC (same equipment as for LC/MS except for the LCMS-2020 MS unit) equipped with the chiral column Chiralpak IB (0.46 cm Ø x 25 cm, Chiral Technologies Europe) and an isocratic elution mode consisting of 97% n-hexane and 3% ethanol.[2] Retention time and mass fragmentation patterns (GC/MS and LC/MS) compared to those of the corresponding authentic reference compounds and literature data[8] were used to identify products and substrates. Conversions were determined via substrate depletion compared to the negative control. Xylazine served as an internal standard. All experiments were performed at least in triplicate.

Spiking experiment. The reaction was carried out as described in the section above. The reaction mixture contained 500 µM (*R*)-ketamine HCl (dissolved in water), 2.5 µM CYP154E1 L289T/I2238Q/M388A, 2.5 µM purified FdR, 25 µM purified YkuN, 200 µM NADPH, 5 U/ml GDH in the presence of 20 mM glucose for cofactor regeneration and 600 U/ml catalase. After 18 hours, reactions were stopped by addition of 125 µl of 1 M Na_2CO_3. The reaction was extracted twice with 200 µl ethyl acetate and the combined organic phases were evaporated to dryness. The evaporated sample was resolved in 100 µl ethanol and analyzed via HPLC equipped with the chiral column Chiralpak IB. After the HPLC analysis, 0.0167 µmol of authentic (2*R*,6*R*)-HNK (4.5 µl 3.7 mM freebase) were added to the same HPLC vial, which was then measured again. This procedure was repeated: 0.033 µmol of (2*R*,6*R*)-HNK standard was added to the same vial and measurement again.

Oxidation of (2*R*,4*S*)-hydroxyketamine. The reaction was carried out as described in the section “Verification of positive mutants”. The reaction mixture contained 500 µM (2*R*,4*S*)-hydroxyketamine freebase (dissolved in DMSO), 2.5 µM CYP154E1 V286G/I238Q/M388A (crude cell lysate), 2.5 µM purified FdR, 25 µM purified YkuN, 200 µM NADPH, 5 U/ml GDH in the presence of 20 mM glucose for cofactor regeneration and 600 U/ml catalase. After 18 hours, reactions were stopped by addition of 125 µl of 1 M Na_2CO_3 and 10 µl of 12 mM xylazine hydrochloride as internal standard. Reactions were extracted twice with 200 µl ethyl acetate and the combined organic phases were evaporated to dryness. Evaporated samples were resolved in a 1:1 mixture of acetonitrile and water and analyzed via LC/MS equipped with a Chromolith® Performance RP-8e 100-4.6 mm (Merck Millipore) column in combination with a Chromolith® RP-8e 5-4.6 mm guard cartridge.

Reactions at higher scale and product isolation. Reactions at higher scale were performed *in vivo* with *E. coli* carrying the pCOLA Duet vector with the genes of *ykun* and *fdr* in the first and respectively second multiple cloning site and a pET22b vector containing the gene of the CYP154E1 variant. Expression, cell preparation and reactions with 1 mM (*R*)-ketamine hydrochloride with the V286G/L289T/I238Q/M388A mutant and 5 mM (*R*)-ketamine with the L289T/I238Q/M388A triple mutant were carried out according to Bokel et al. 2020.[2] Isolation of (2*R*,6*R*)-HNK and (2*R*,6)-HK was carried out using semi-preparative HPLC (see section above) on a Eurospher II 100-10-C18 column (10.0µm, 300 x 8.0mm) using water (solvent A) and acetonitrile (solvent B) as solvents. The following gradient at a flow rate of 5 ml/min was applied for product separation: 20% B to 35% B in 10 min, increase to 90% B in 1 sec, holding 90% B for 3 min, decrease to 20% B again in 1 sec and holding 20% B for 4 min. Fractions were collected automatically.

Oxidation of in total 28.53 mg (*R*)-ketamine by CYP154E1 V286G/L289T/I238Q/M388A resulted in 7.62 mg (2*R*,6*R*)-hydroxynorketamine (26.6% isolated yield) and 9.50 mg (2*R*,6)-hydroxyketamine (31.3% isolated yield), both with >99% purity (HPLC). Oxidation of 11.86 mg (*R*)-ketamine by CYP154E1 L289T/I238Q/M388A resulted in quantitative conversion with 84% product selectivity. 5.96 mg (2*R*,6*R*)-hydroxynorketamine were isolated (49.8% isolated yield) and analyzed by NMR.

Results

INITIAL SCREENING FOR CYP154E1 STARTING VARIANT

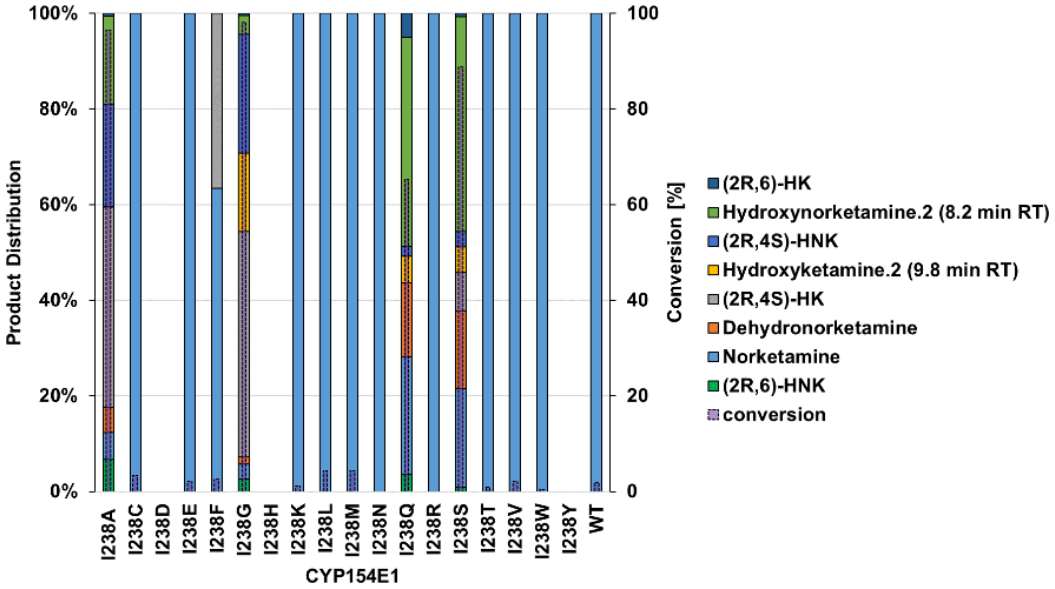

Fig. S2. Conversion of (R)-ketamine and product distribution observed with all possible I238 mutants of CYP154E1 except for I238P. Green columns represent the ratio of the desired (2R,6)-hydroxynorketamine. Conversion (secondary y-axis) is represented by transparent purple columns overlaid with the columns for the product distribution (primary y-axis). Mean values are calculated from three separate experiments. WT: wild type.

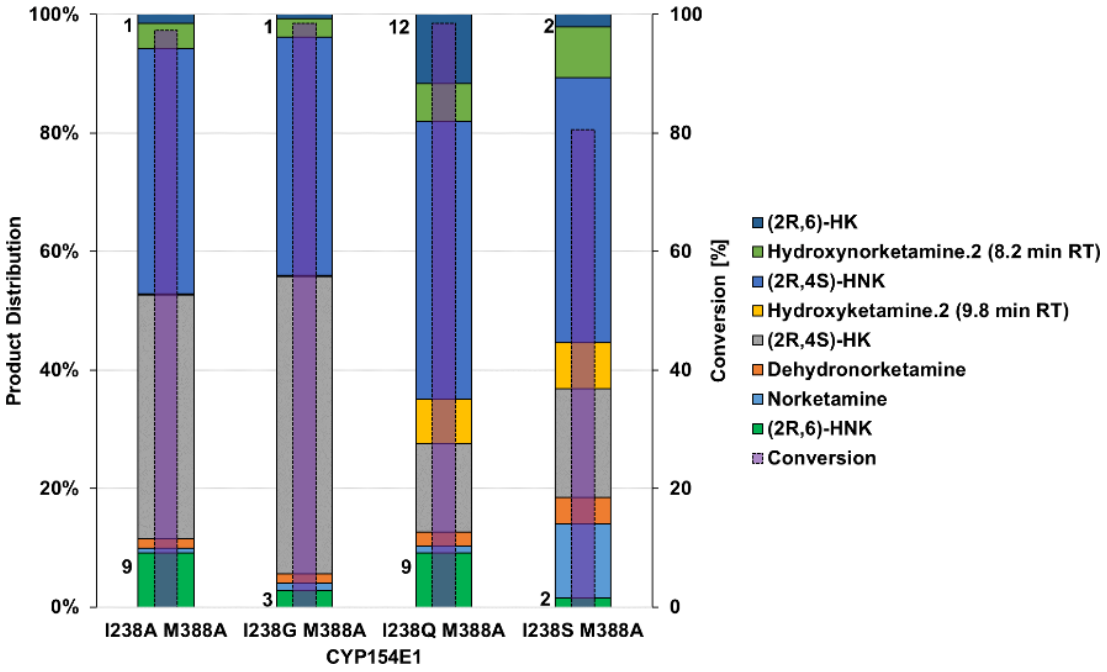

Fig. S3. Conversion of (R)-ketamine with CYP154E1 double mutants and product distribution. Mutation M388A was introduced to the best performing single mutants from Figure S1. Green columns represent (2R,6)-hydroxynorketamine and dark-blue columns represent (2R,6)-hydroxyketamine (percentage of (2R,6)-hydroxyketamine and (2R,6)--hydroxynorketamine are presented left of the respective column). Conversion (secondary y-axis) is represented by transparent purple columns overlaid with the columns for the product distribution (primary y-axis). Mean values are calculated from three separate experiments.

Table S2. Oxidation of (R)-ketamine catalyzed by CYP154E1 variants containing I238Q, L289T and M388A mutations and their combinations as well as by the quadruple mutant. (R)-NK: (R)-norketamine; (2R,6)-HNK: (2R,6)-hydroxynorketamine, (2R,6)-HK: (2R,6)-hydroxyketamine.

		Product distribution [%]					
CYP154E1 variants	Conversion [%]	(2*R*,6)-HNK	(2*R*,6)-HK	(2*R*,4*S*)-HK	(2*R*,4*S*)-HNK	(*R*)-NK	Others
L289T	11	-	-	-	-	100	-
L289T/I238Q	>99	61	30	-	-	-	9
L289T/M388A	55	14	4	-	-	76	5
I238Q/M388A	98	9	12	15	47	1	16
L289T/I238Q/M388A	>99	85	10	-	-	-	5
V286G/L289T/I238Q/M388A	>99	32	67	-	-	-	1

KINETICS

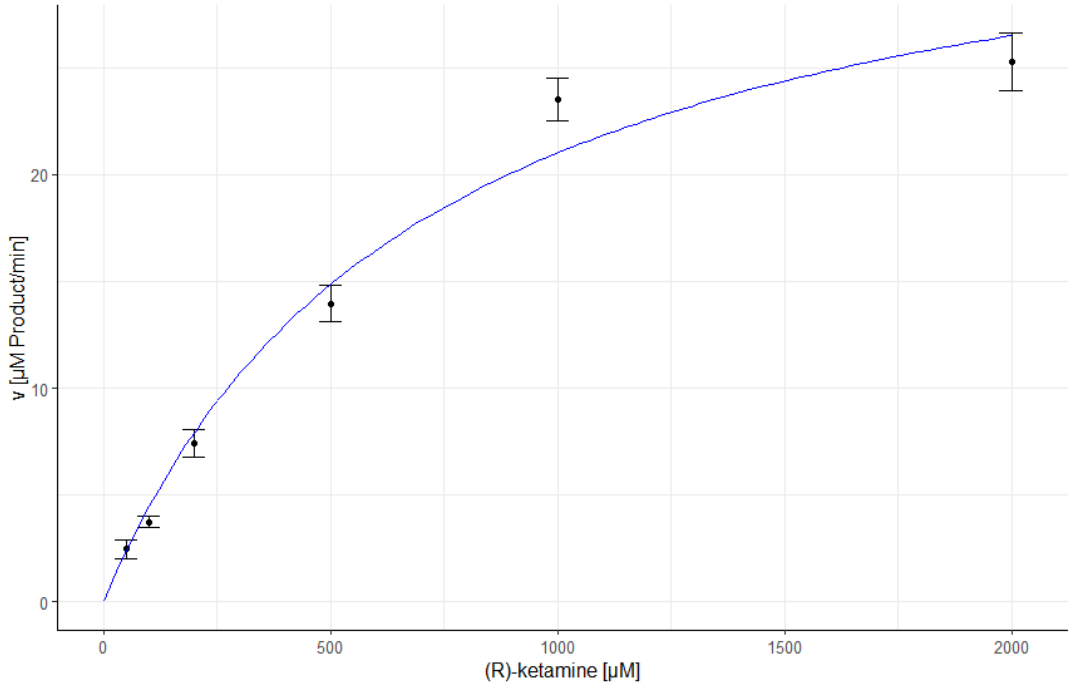

Fig. S4. Kinetics of the L289T/I238Q/M388A-catalyzed oxidation of (S)-ketamine. Reactions were carried out using 0.5 µM P450, 0.5 µM FdR and 40 µM YkuN. Reactions were stopped after 3 – 17 min. Data were plotted to the Michaelis-Menten equation.

IDENTIFICATION OF THE DESIRED PRODUCT (2*R*,6*R*)-HYDROXYNORKETAMINE BY SPIKING

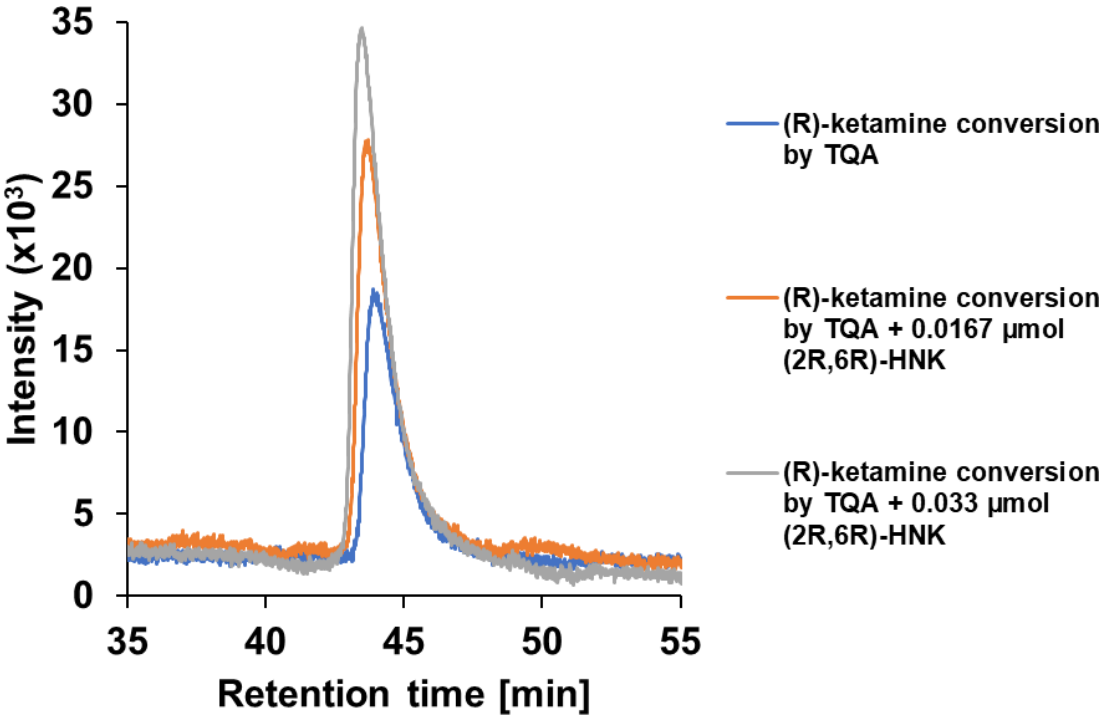

Fig. S5. HPLC analysis of the reaction catalyzed by the L289T/I238Q/M388A/ mutant (TQA). Blue: Conversion of 500 µM (R)-ketamine; orange: blue + 0.0167 µmol of authentic (2R,6R)-HNK; grey: blue + 0.033 µmol of authentic (2R,6R)-HNK. Analysis was carried out on the chiral column Chiralpak IB (0.46 cm Ø x 25 cm, Chiral Technologies Europe).

LC/MS ANALYSIS OF (2*R*,4*S*)-HYDROXYKETAMINE CONVERSION

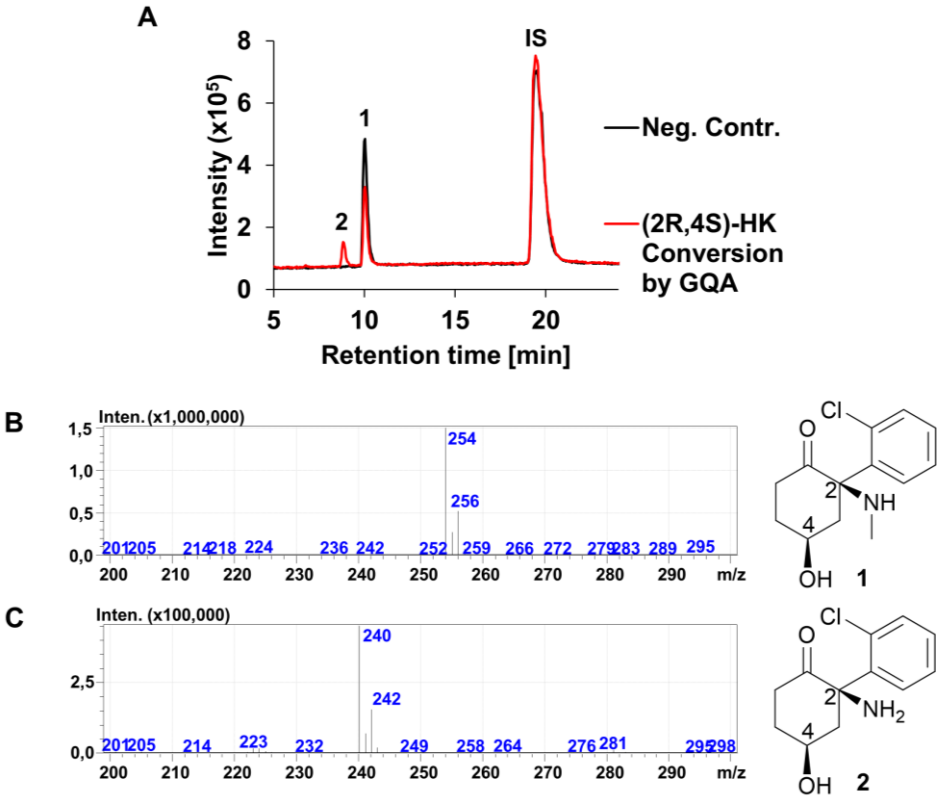

Fig. S6. LC/MS analysis of (2R,4S)-hydroxyketamine oxidation. ***A****: LC/MS chromatogram of the conversion of (2R,4S)-hydroxyketamine (1) by CYP154E1 V286G/I238Q/M388A (GQA) compared to the negative control without P450. IS: internal standard xylazine.* ***B****: ESI MS spectrum of (2R,4S)-hydroxyketamine (1). 254 m/z corresponds to $[M+H]^+$; 256 m/z corresponds to $[M+H]^+$ and results from the ^{37}Cl isotope (compared to the ^{35}Cl isotope).* ***C****: ESI MS spectrum of (2). 240 m/z results from a loss of the methyl group (Δ14 m/z). 242 m/z is again attributes to the ^{37}Cl isotope (compared to the ^{35}Cl isotope). Therefore, the product of the (2R,4S)-hydroxyketamine oxidation must be (2R,4S)-hydroxynorketamine (demethylated product).*

NMR ANALYSIS

(2*R*,6*R*)-HYDROXYNORKETAMINE

^{1}H NMR (600 MHZ, MEOD) Δ 7.77 – 7.70 (M, 1H), 7.48 – 7.41 (M, 2H), 7.39 – 7.33 (M, 1H), 4.15 (DD, J = 11.8, 6.6 HZ, 1H), 2.93 (DDD, J = 14.4, 3.1 HZ, 1H), 2.27 – 2.19 (M, 1H), 1.79 – 1.68 (M, 2H), 1.66 – 1.57 (M, 1H), 1.57 – 1.48 (M, 1H).

^{13}C NMR (151 MHZ, MEOD) Δ 214.00, 139.56, 134.80, 132.41, 131.03, 130.35, 128.97, 74.81, 68.12, 42.27, 40.36, 20.68.

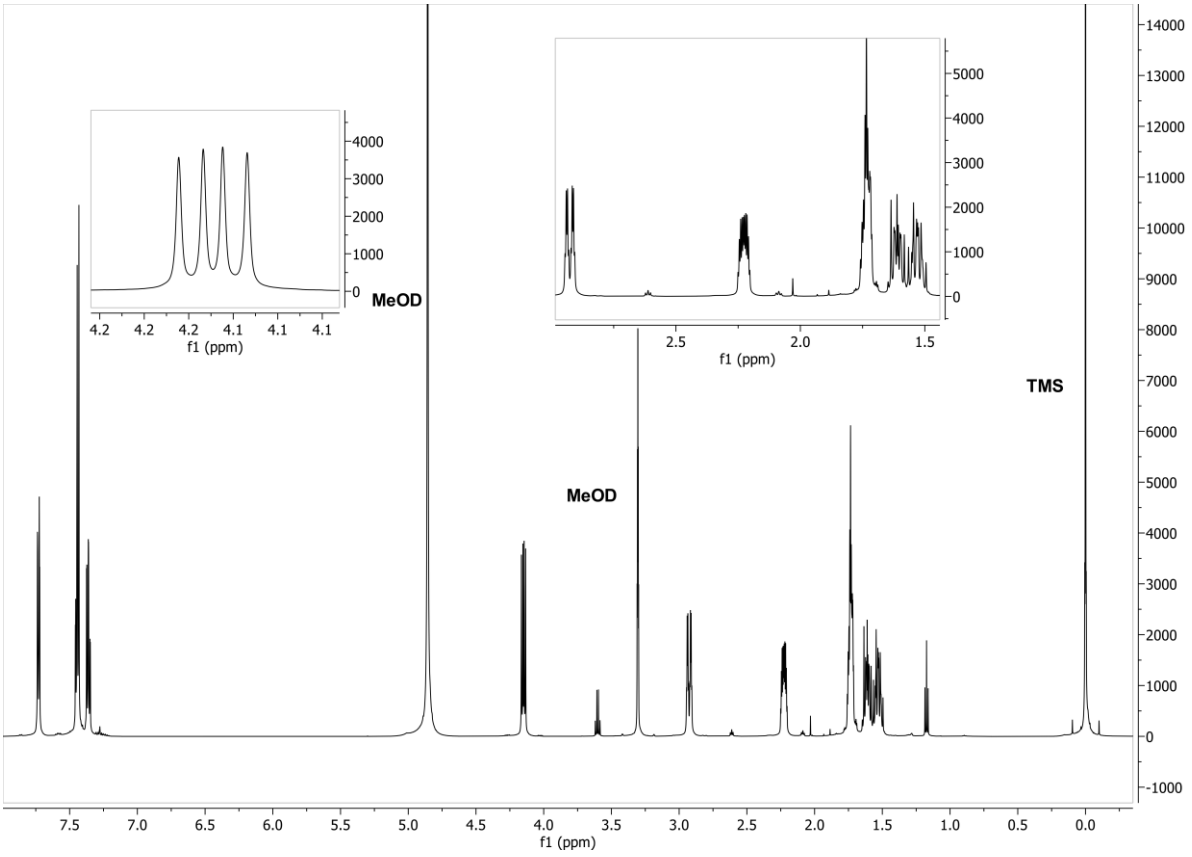

Fig. S7. ^{1}H NMR of (2R,6R)-hydroxynorketamine in MeOD. Enlargement of the doublet of doublets for the proton sitting at C6 with the hydroxyl group and the proton patterns of the cyclohexanone system.

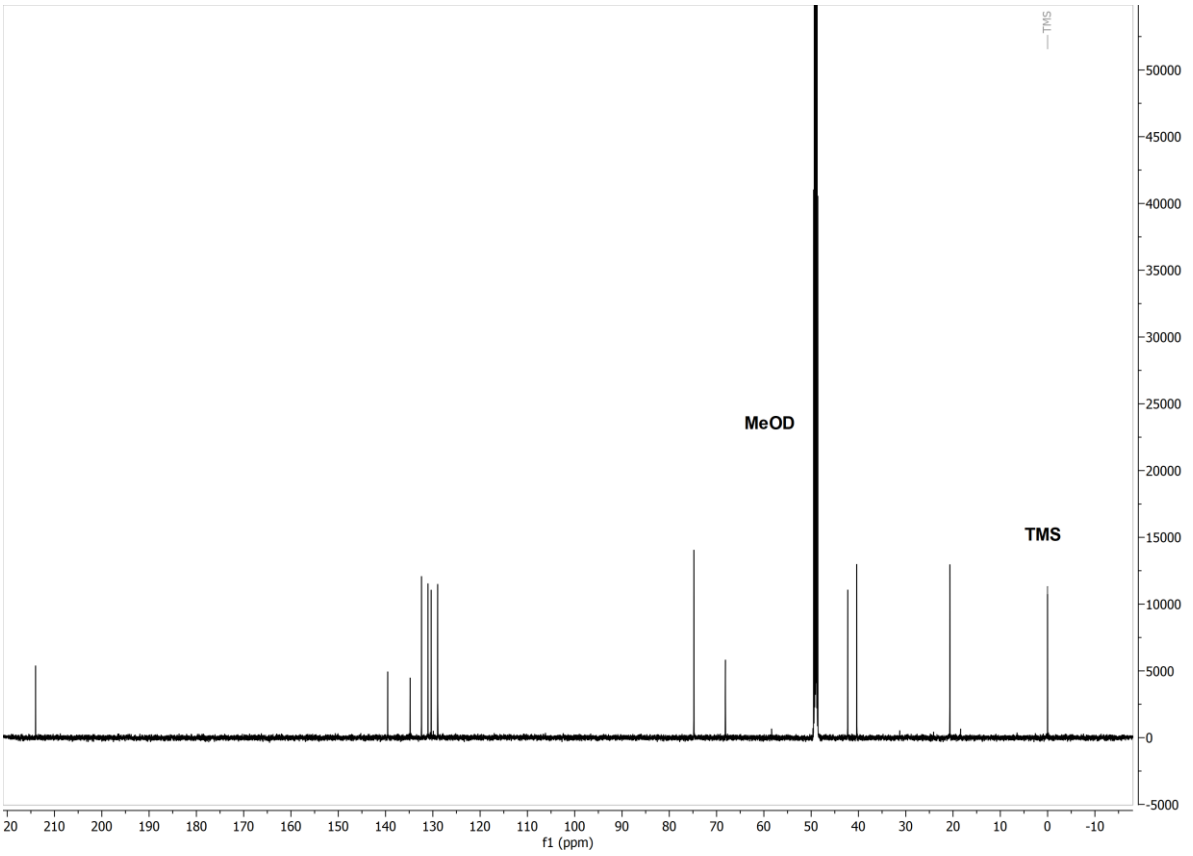

Fig.S8. ^{13}C NMR of (2R,6R)-hydroxynorketamine in MeOD.

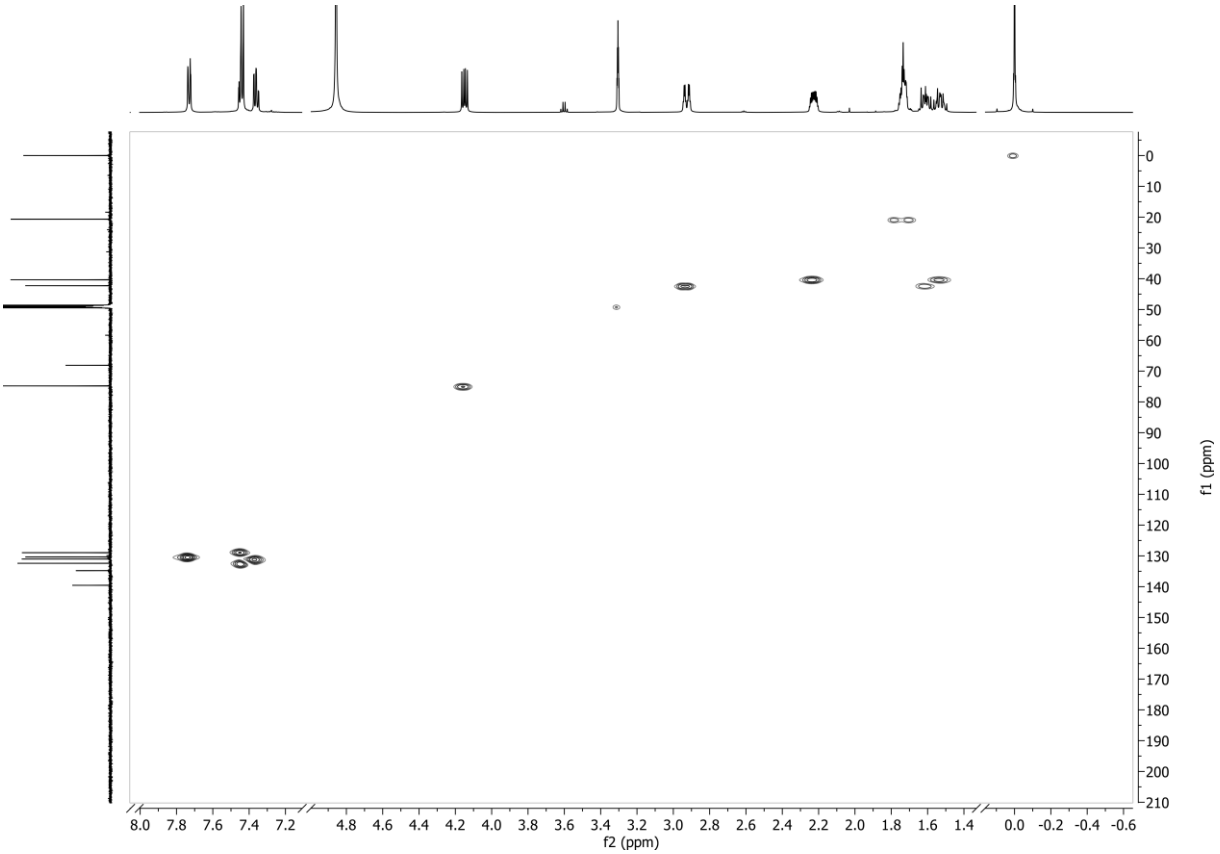

Fig. S9. ^{1}H-^{13}C HSQC spectrum of (2R,6R)-hydroxynorketamine in MeOD. Signals of impurities were cut out for simplicity.

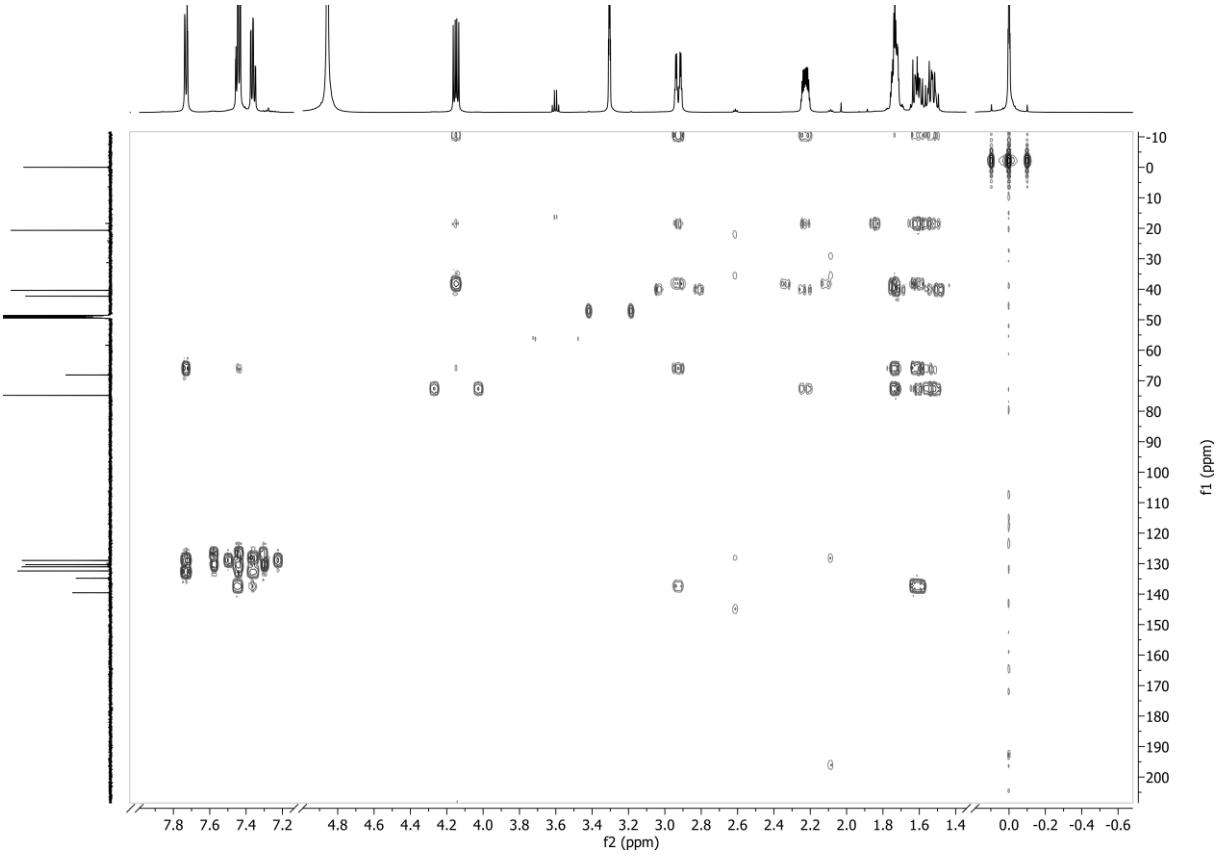

Fig. S10. ^{1}H-^{13}C HMBC spectrum of (2R,6R)-hydroxynorketamine in MeOD. Signals of impurities were cut out for simplicity.

(2*R*,6)-HYDROXYKETAMINE

^{1}H NMR (600 MHZ, MEOD) Δ = 7.69 (DD, J=8.5, 1.6, 1H), 7.50 – 7.44 (M, 2H), 7.42 – 7.37 (M, 1H), 4.17 (DD, J=11.8, 6.6, 1H), 3.12 (DDD, J=14.2, 3.0, 1H), 2.27 – 2.21 (M, 1H), 1.81 – 1.75 (M, 1H), 1.72 – 1.62 (M, 1H), 1.61 – 1.54 (M, 2H).

^{13}C NMR (151 MHZ, MEOD) Δ = 211.99, 135.67, 135.63, 132.55, 131.90, 131.34, 128.60, 75.08, 72.29, 40.35, 39.26, 28.79, 20.56.

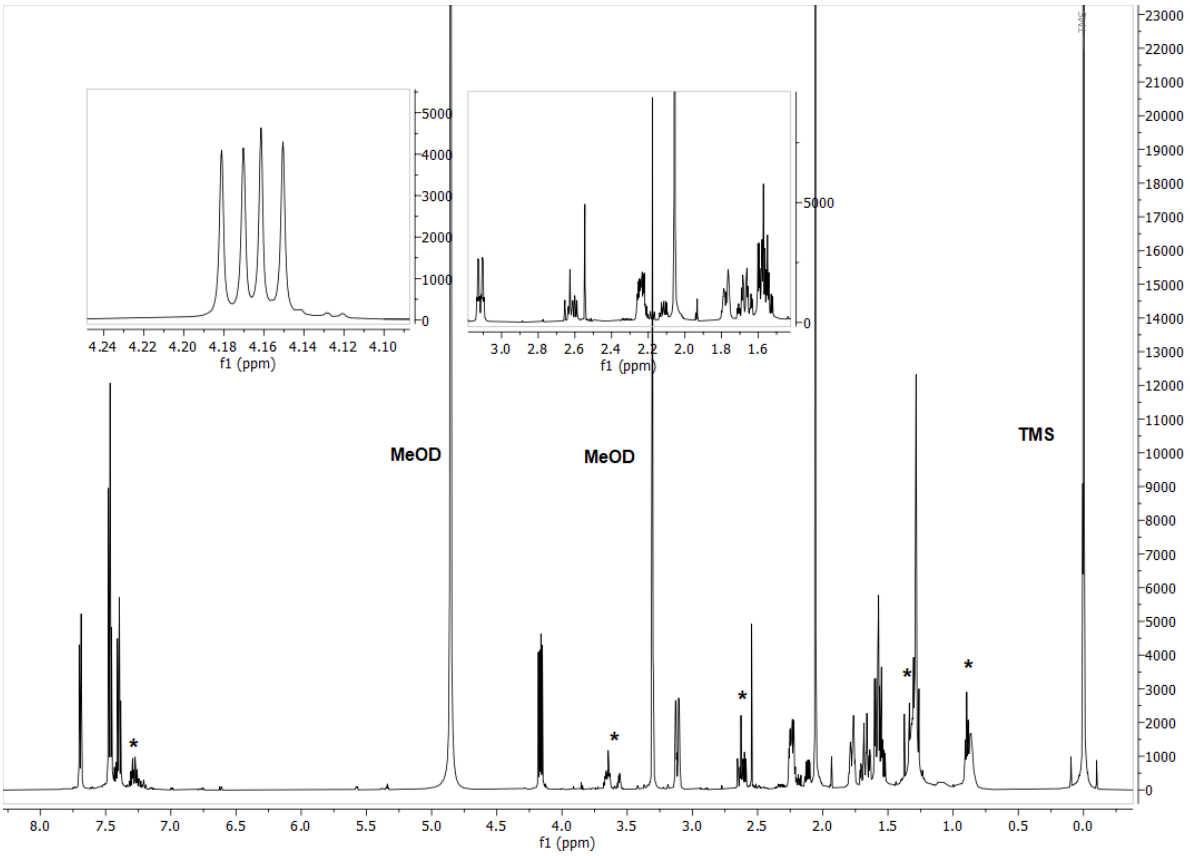

*Fig. S11. ^{1}H NMR of (2R,6)-hydroxyketamine in MeOD. *: Solvent impurities. Enlargement of the doublet of doublets for the proton sitting at C6 with the hydroxy group and the proton patterns of the cyclohexanone system.*

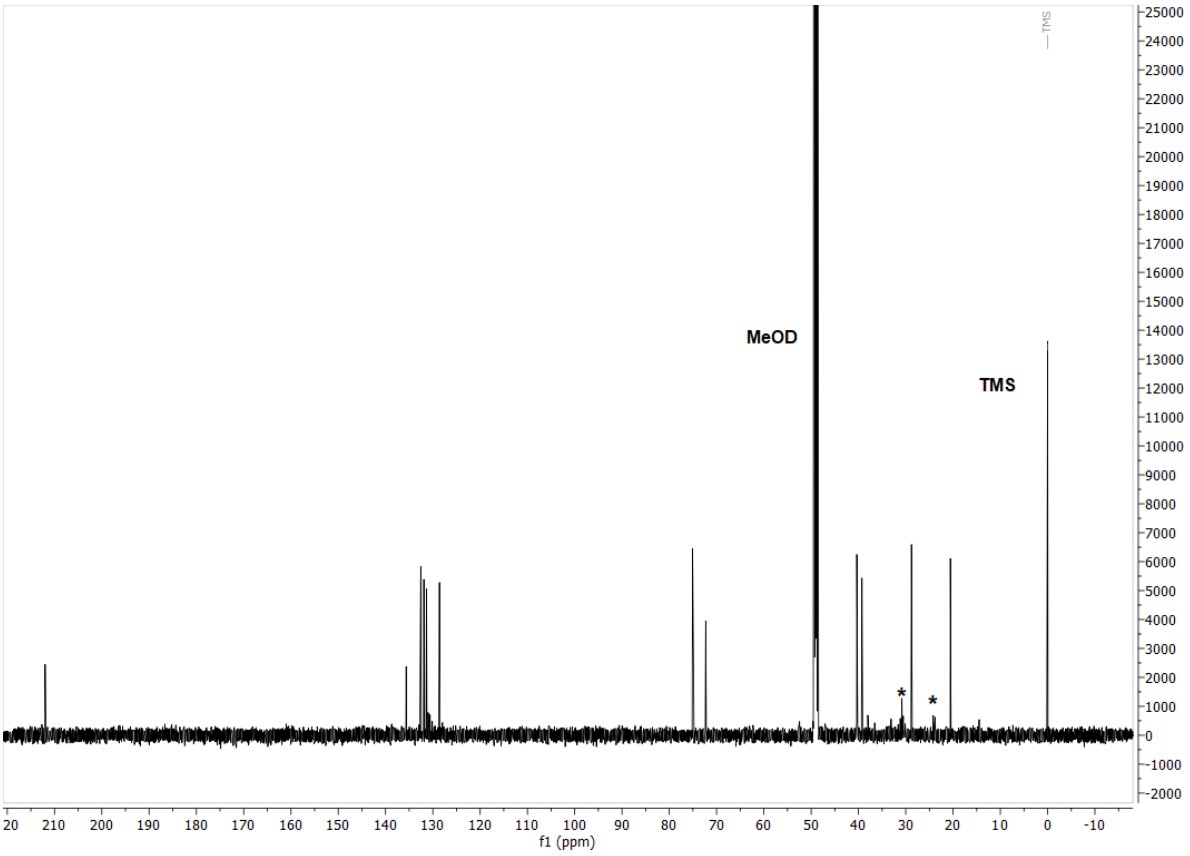

*Fig. S12. ^{13}C NMR of (2R,6)-hydroxyketamine in MeOD. *: Solvent impurities.*

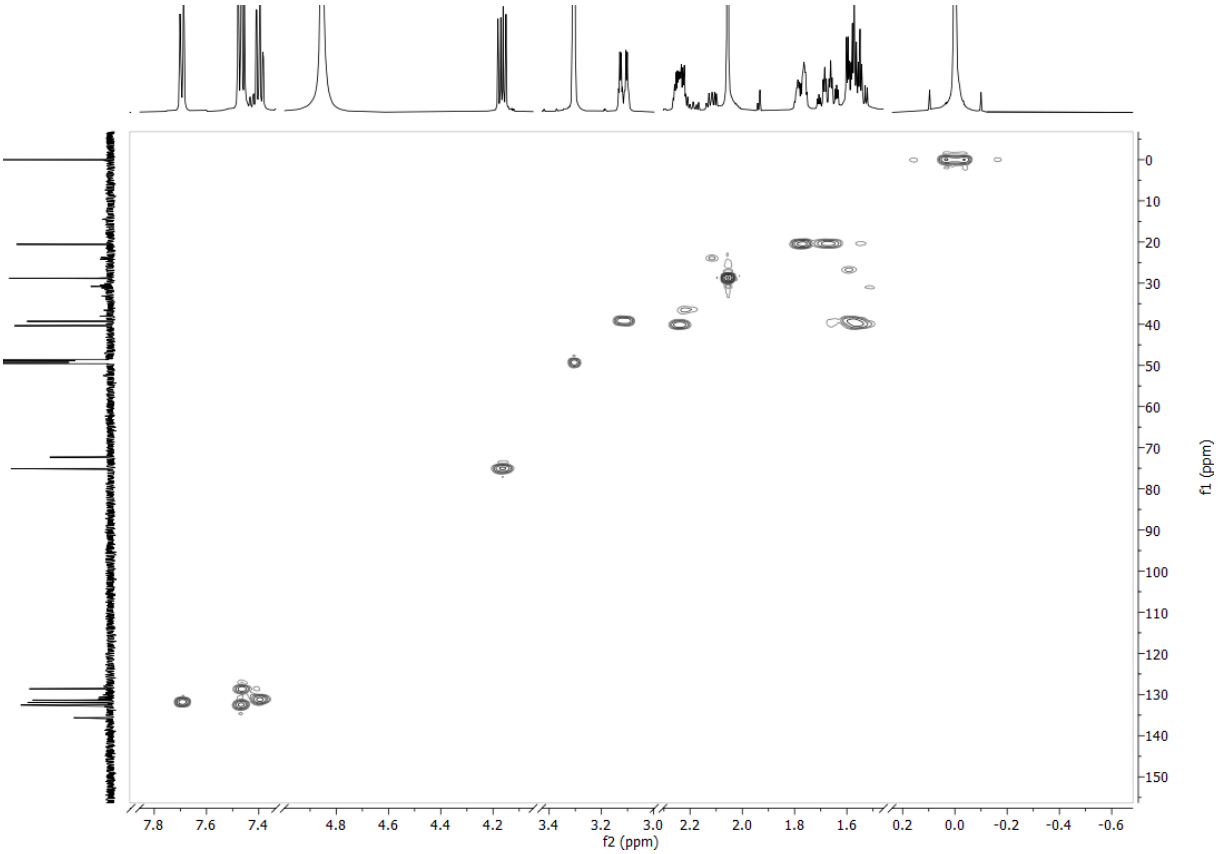

Fig. S13. ^{1}H-^{13}C HSQC spectrum of (2R,6)-hydroxyketamine in MeOD. Signals of impurities were cut out for simplicity.

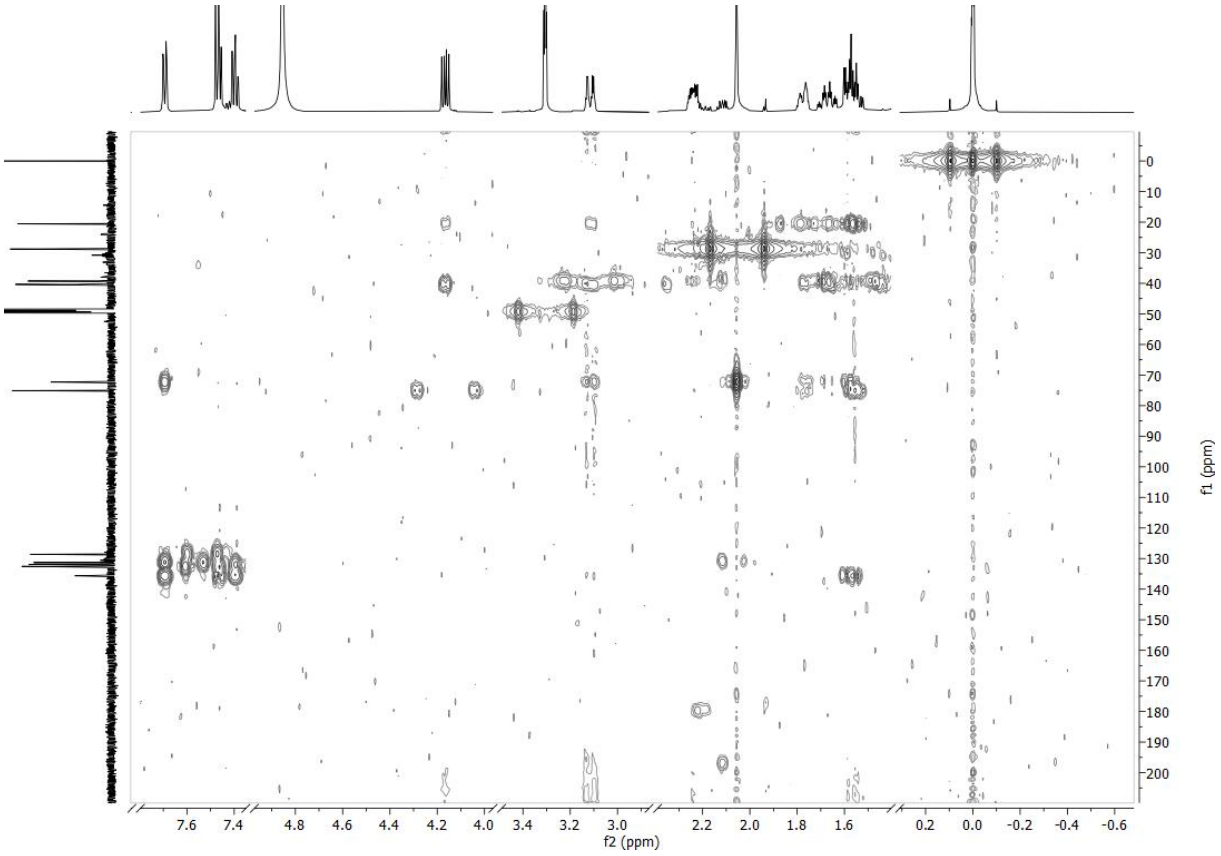

Fig. S14. ^{1}H-^{13}C HMBC spectrum of (2R,6)-hydroxyketamine in MeOD. Signals of impurities were cut out for simplicity.

DOCKING OF (*R*)-KETAMINE AND (*R*)-NORKETAMINE

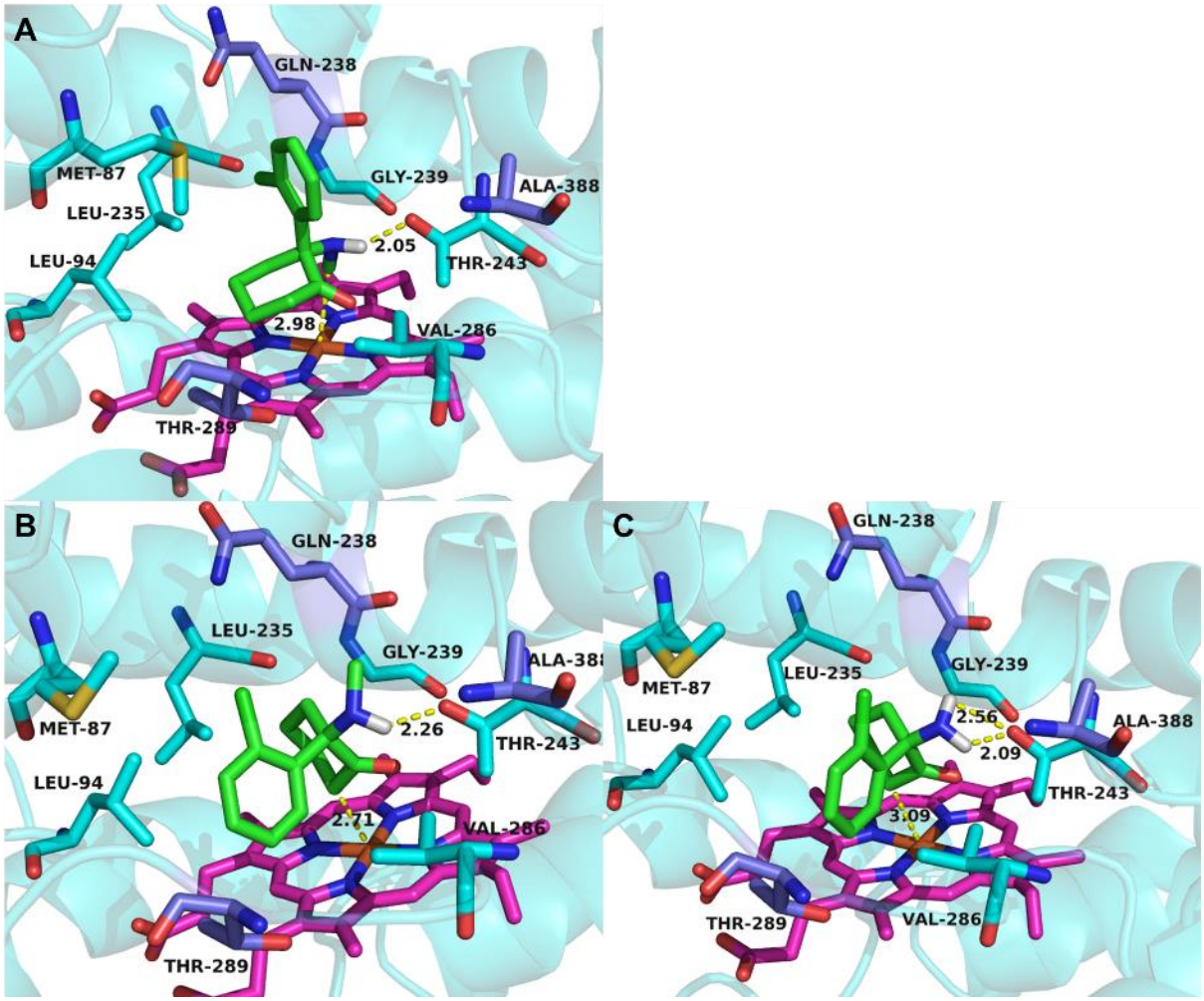

*Fig. S15. Best scored rigid docking poses of (R)-ketamine in the model A (**A**) and model B (**B**) and of (R)-norketamine in the model B (**C**) of the active site of CYP154E1 L289T/I238Q/M388A. Dashed yellow lines indicate distances between atoms. Light-blue colored residues: residues of the first-sphere; dark-blue colored residues: first-sphere mutations I238Q, L289T and M388A. The numerical distance is given in Å.*

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