**Supporting Information**

# Enzyme-Mediated Two-Step Regio- and Stereoselective Synthesis of Potential Rapid Acting Antidepressant (2*S*,6*S*)-Hydroxynorketamine

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## OLIGONUCLEOTIDES

Table S1. Oligonucleotides for the cloning and the quickchange mutagenesis. The mutated codon is marked by color and underline. Primer of for variants that were already available in our institute are not listed below. Fw: forward primer; Rev: reverse Primer.

| **Primer QuickChange** | **Sequence (5'→ 3')** |
|---|---|
| Fw: CYP154E1 T178F | CAGACGCTGGTGACGCGCTTCCTCTCCGGCACGGACCCTGAG |
| Rev: CYP154E1 T178F | CTCAGGGTCCGTGCCGGAGAGGAAGCGCGTCACCAGCGTCTG |
| Fw: CYP154E1 T178I | CAGACGCTGGTGACGCGCATCCTCTCCGGCACGGACCCTGAG |
| Rev: CYP154E1 T178I | CTCAGGGTCCGTGCCGGAGAGGATGCGCGTCACCAGCGTCTG |
| Fw: CYP154E1 L179F | GTGACGCGCACCTTTTCCGGCACGGACCCTGAG |
| Rev: CYP154E1 L179F | CTCAGGGTCCGTGCCGGAAAAGGTGCGCGTCAC |
| Fw: CYP154E1 L179K | GTGACGCGCACCAAATCCGGCACGGACCCTGAG |
| Rev: CYP154E1 L179K | CTCAGGGTCCGTGCCGGATTTGGTGCGCGTCAC |
| Fw: CYP154E1 L179R | GTGACGCGCACCCGCTCCGGCACGGACCCTGAG |
| Rev: CYP154E1 L179R | CTCAGGGTCCGTGCCGGAGCGGGTGCGCGTCAC |
| Fw: CYP154E1 L234Q | CTGATCCACAACACGCAGCTGCTCATCCAGGCG |
| Rev: CYP154E1 L234Q | CGCCTGGATGAGCAGCTGCGTGTTGTGGATCAG |
| Fw: CYP154E1 L235D | GAGCTGATCCACAACACGCTGGACCTCATCATCGG |
| Rev: CYP154E1 L235D | CCGATGATGAGGTCCAGCGTGTTGTGGATCAGCTC |
| Fw: CYP154E1 L235E | GAGCTGATCCACAACACGCTGGAACTCATCATCGG |
| Rev: CYP154E1 L235E | CCGATGATGAGTTCCAGCGTGTTGTGGATCAGCTC |
| Fw: CYP154E1 L235N | GAGCTGATCCACAACACGCTGAACCTCATCATCGG |
| Rev: CYP154E1 L235N | CCGATGATGAGGTTCAGCGTGTTGTGGATCAGCTC |
| Fw: CYP154E1 L235Q | GAGCTGATCCACAACACGCTGCAGCTCATCATCGG |
| Rev: CYP154E1 L235Q | CCGATGATGAGCTGCAGCGTGTTGTGGATCAGCTC |
| Fw: CYP154E1 L235T | GAGCTGATCCACAACACGCTGACCCTCATCATCGG |
| Rev: CYP154E1 L235T | CCGATGATGAGGGTCAGCGTGTTGTGGATCAGCTC |
| Fw: CYP154E1 I238D | GCTGCTGCTCATCGACGGCGGGTTCGAAACCAC |
| Rev: CYP154E1 I238D | GTGGTTTCGAACCCGCCGTCGATGAGCAGCAGC |
| Fw: CYP154E1 I238E | GCTGCTGCTCATCGAGGGCGGGTTCGAAACCAC |
| Rev: CYP154E1 I238E | GTGGTTTCGAACCCGCCCTCGATGAGCAGCAGC |
| Fw: CYP154E1 I238N | GCTGCTGCTCATCAACGGCGGGTTCGAAACCAC |
| Rev: CYP154E1 I238N | GTGGTTTCGAACCCGCCGTTGATGAGCAGCAGC |
| Fw: CYP154E1 I238Q | GCTGCTGCTCATCCAGGGCGGGTTCGAAACCAC |
| Rev: CYP154E1 I238Q | GTGGTTTCGAACCCGCCCTGGATGAGCAGCAGC |
| Fw: CYP154E1 I238T | GCTGCTGCTCATCACCGGCGGGTTCGAAACCAC |
| Rev: CYP154E1 I238T | GTGGTTTCGAACCCGCCGGTGATGAGCAGCAGC |
| Fw: CYP154E1 T243D | CAGGCGGGGTTCGAAGACACCATGGGCATGATC |
| Rev: CYP154E1 T243D | GATCATGCCCATGGTGTCTTCGAACCCCGCCTG |
| Fw: CYP154E1 T243N | CAGGCGGGGTTCGAAAACACCATGGGCATGATC |
| Rev: CYP154E1 T243N | GATCATGCCCATGGTGTTTTCGAACCCCGCCTG |
| Fw: CYP154E1 T243S | CAGGCGGGGTTCGAAAGCACCATGGGCATGATC |
| Rev: CYP154E1 T243S | GATCATGCCCATGGTGCTTTCGAACCCCGCCTG |

| | |
|---|---|
| Fw: CYP154E1 V286I | CGAATCAGCGATCGTCATGCTGCCGTTCC |
| Rev: CYP154E1 V286I | GGAACGGCAGCATGACGATCGCTGATTCG |
| Fw: CYP154E1 V286N | CGAATCAGCGAACGTCATGCTGCCGTTCC |
| Rev: CYP154E1 V286N | GGAACGGCAGCATGACGTTCGCTGATTCG |
| Fw: CYP154E1 M388A | GACGCCGACCGTGTTCGCGAACCATCCGCTGAG |
| Rev: CYP154E1 M388A | CTCAGCGGATGGTTCGCGAACACGGTCGGCGTC |
| Fw: CYP154E1 M388G | GACGCCGACCGTGTTCGGTAACCATCCGCTGAG |
| Rev: CYP154E1 M388G | CTCAGCGGATGGTTACCGAACACGGTCGGCGTC |
| **Primer Cloning** | |
| Fw: CYP154E1 QAA in pET22b (*Nde*I) | CAGCCATATGGGACAGTCCCGCCGACCC |
| Rev: CYP154E1 QAA in pET22b (*Eco*RI) | GCTCGAATTCTCAGGGTTTCGGGCGCAAG |
| Fw: YkuN in pCOLA Duet MCS I (*Nco*I) | TATACCATGGCTAAAGCCTTGATTACATATGC |
| Rev: YkuN in pCOLA Duet MCS I (*Bam*HI) | GATCGGATCCTCATGAAACATGGATTTTTTCCTTG |
| Fw: FdR in pCOLA Duet MCS II (*Bgl*II) | TATAAGATCTCATGGCTGATTGGGTAACAGGCAAAG |
| Rev: FdR in pCOLA Duet MCS II (*Xho*I) | GATCCTCGAGTTACCAGTAATGCTCCGCTGTC |
| **Sequencing primer (pET vectors)** | |
| T7 | TAATACGACTCACTATAGGG |
| pET-RP | CTAGTTATTGCTCAGCGG |
| **Sequencing primer (pCOLA Duet vector)** | |
| ACYC_Duet_Up1 | GGATCTCGACGCTCTCCCT |
| Duet_DOWN1 | GATTATGCGGCCGTGTACAA |
| Duet_Up2 | TTGTACACGGCCGCATAATC |

## CLONING, EXPRESSION AND PURIFICATION

Construction of CYP154E1 mutants was carried out according to a modified quik-change mutagenesis protocol of Edelheit, Hanukoglu, and Hanukoglu (2009) using two separated single-primer reactions.[1]

Expression of the redox partner FdR from *Escherichia coli*, and the NADPH regenerating glucose dehydrogenase (GDH) from *Bacillus megaterium* was performed as described elsewhere except for the expression vector pET11a for FdR.[2-3] For screening, the genes coding for CYP154E1 wild type and mutants integrated into the pET28a+ vector were heterologously expressed in *E. coli* BL21(DE3). Expression was carried out in 50 ml TB medium in a 0.5 liter flask, initially at 37°C and 180 rpm until $OD_{600}$ of 0.6-0.8 was reached. After induction with 0.1 mM IPTG, 0.1 mM $FeSO_4$ and 0.5 mM 5-aminolevulinic acid, cultures were stirred for another 20 hours at 25°C and 140 rpm before cells were harvested. For protein purification, CYP154E1 wild type and mutants were expressed in 400 ml scale in 2 liter flasks under the same conditions as described above.

Purification was performed on an ÄKTApurifier (GE Healthcare) on a HisTrap FF crude 5 ml column (immobilized nickel affinity chromatography). Proteins were eluted using a buffer with 150 mM (FdR), 100 mM (CYP154E1) and 200 mM (YkuN) imidazole as the eluent. After subsequent desalting via size exclusion chromatography on a PD-10 column (GE Healthcare) proteins were stored in buffers containing 50 mM Tris-HCl, 50 mM NaCl, 5% (w/v) glycerol, pH 7.5 (YkuN) and 50 mM KPi, 50 mM NaCl, 5% (w/v) glycerol pH 7.5 (FdR and CYP154E1 variants). For CYP154E1 variants an additional heat precipitation step at 60°C for 15 min was included after sample desalting. After heat precipitation, target CYP154E1 enzymes were incubated on ice for another 20 min and precipitated proteins were removed via centrifugation.

## EXPRESSION OF YKUN IN A 7.5 L BIOREACTOR

The vector pET16b carrying the gene for the electron transfer partner YkuN from *Bacillus subtilis* was already available in our laboratory. 10 ml LB pre-cultures containing *E. coli* transformants, hosting the YkuN-gene encoded vector pET16b, were grown over night at 37°C and 180 rpm. These pre-cultures were used to inoculate 500 ml TB medium starter cultures to an $OD_{600}$ of 0.05. After growth for several hours at 30°C and 180 rpm, the starter cultures were used onwards to inoculate a 7.5 l bioreactor (Infors, Bottmingen, Switzerland) containing 3.5 l fermentation basal salt medium (19.13 g $l^{-1}$ $K_2HPO_4 \cdot 3\ H_2O$, 4 g $l^{-1}$ $NaH_2PO_4 \cdot 2\ H_2O$, 2.47 g $l^{-1}$ $(NH_4)_2SO_4$, 2 g $l^{-1}$ $Na_2SO_4$, 0.5 g $l^{-1}$ $NH_4Cl$, 1 g $l^{-1}$ $(NH_4)_2$ citrate, 20 g $l^{-1}$ glycerol, 2 ml $l^{-1}$ 1 M $MgSO_4$, 1 ml $l^{-1}$ thiamine (100 mg $ml^{-1}$), 1 ml $l^{-1}$ ampicillin (100 mg $ml^{-1}$), 3.5 ml $l^{-1}$ trace metals (0.5 g $l^{-1}$ $CaCl_2$, 0.18 g $l^{-1}$ $ZnSO_4 \cdot 7\ H_2O$, 0.18 g $l^{-1}$ $CoCl_2 \cdot 6\ H_2O$, 0.16 g $l^{-1}$ $CuSO_4 \cdot 5\ H_2O$, 0.1 g $l^{-1}$ $MnSO_4 \cdot H_2O$, 22.9 g $l^{-1}$ $Na_2EDTA \cdot 2\ H_2O$, 16.7 g $l^{-1}$ $FeCl_3 \cdot 6\ H_2O$) to an $OD_{600}$ of 0.5. Temperature was maintained at 30°C until initial glycerol was exhausted (measurable by $pO_2$ increase). Afterwards expression was induced via addition of 0.1 mM IPTG. Temperature was decreased to 20°C and control of cell growth was maintained automatically by glycerol (50%) addition whenever the carbon source was consumed (again measurable by $pO_2$ increase).

## ENZYME ASSAYS

Concentrations of cytochrome P450s (*E. coli* cell lysates and purified enzymes) were calculated via CO difference spectra using the extinction coefficient $\varepsilon_{450-490}$ = 91 $mM^{-1}cm^{-1}$ as described elsewhere.[4] The concentration of the purified redox proteins YkuN and FdR were determined spectroscopically as described elsewhere.[5-8] The $NADP^+$ reduction activity of the GDH was measured by increase of absorbance at 340 nm over time ($\varepsilon_{340}$ = 6.22 $mM^{-1}cm^{-1}$). The reaction was set up with 0.1 M glucose, 100 µl of diluted GDH solution in 50 mM potassium phosphate buffer pH 7.5. The reaction was started by adding 0.1 mM $NADP^+$.

## GC/MS SCREENING DATA

Table S2. (*S*)-ketamine oxidation catalyzed by CYP154E1 variants. NK: norketamine; (2S,6)-HNK: (2S,6)-hydroxynorketamine. Conversion was estimated from the ratio of product peak areas only, compared to the sum of all peak areas (including the substrate peak area). *: CYP154E1 variants of our in-house collection.

| | | Product distribution [%] | | |
|---|---|---|---|---|
| CYP154E1 variant | Conversion [%] | NK | (2*S*,6)-HNK | Others |
| Wild Type | 1.8 | 100 | - | - |
| M87V | 0.0 | - | - | - |
| L94F* | 0.2 | 100 | - | - |
| T178F* | 1.1 | 100 | - | - |
| T178I* | 1.6 | 100 | - | - |
| L179F* | 1.7 | 100 | - | - |
| L179K* | 0.2 | 100 | - | - |
| L179R* | 0.6 | 100 | - | - |
| L235D | 0.0 | - | - | - |
| L235E | 0.0 | - | - | - |
| L235N | 0.0 | - | - | - |
| L235Q | 0.0 | - | - | - |
| L235T | 0.8 | 100 | - | - |
| I238D | 0.0 | - | - | - |
| I238E | 0.2 | 100 | - | - |
| I238N | 0.0 | - | - | - |
| I238Q | 21.9 | 58.1 | 7.1 | 34.8 |
| I238T | 2.4 | 100 | - | - |
| G239A* | 12.2 | 96.7 | 3.3 | - |
| V286A* | 0.3 | 100 | - | - |
| V286I | 0.6 | 100 | - | - |
| V286L* | 0.0 | - | - | - |
| V286N | 0.2 | 100 | - | - |
| M388A | 6.1 | 90.6 | 9.4 | - |
| M388G | 1.4 | 100 | - | - |
| M388Q* | 0.0 | - | - | - |

## RIGID DOCKING OF (*S*)-KETAMINE AND (*S*)-NORKETAMINE

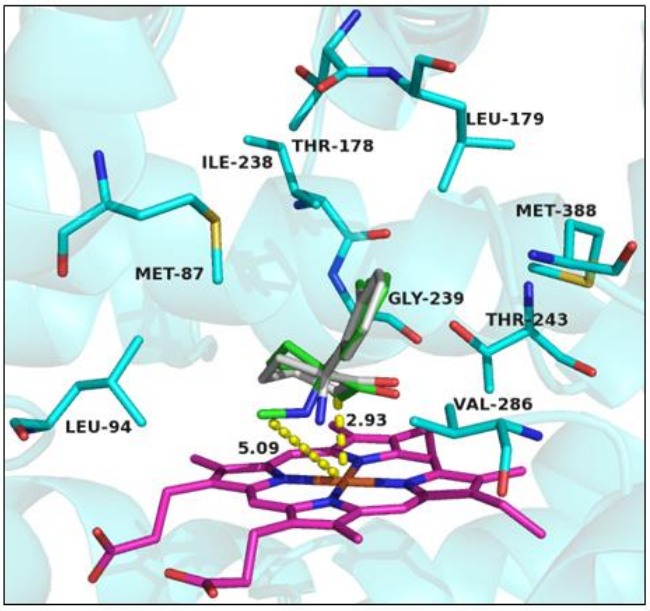


Figure S1. Best scored rigid docking poses of **(*S*)-ketamine** (green) and **(*S*)-norketamine** (grey) in the active site of CYP154E1 wild type. Dashed yellow lines indicate distances between atoms (5.09 Å between heme iron and aminomethyl group of (*S*)-ketamine and 2.93 Å between heme iron and C6 of (*S*)-norketamine).

## FURTHER MUTAGENESIS

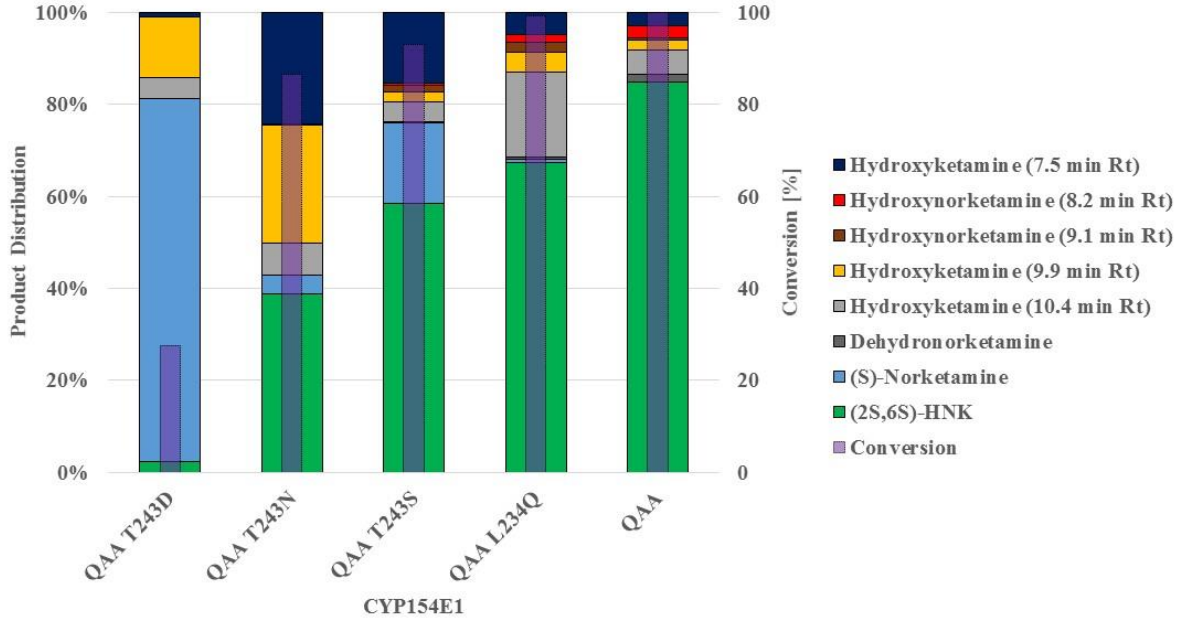


Figure S2. Product distribution and conversion of (*S*)-ketamine with CYP154E1 quadruple mutants in comparison to triple mutant QAA. Green columns represent desired products of (2*S*,6*S*)-hydroxynorketamine isomers. Mean values are calculated from three separate experiments.

## HPLC DATA

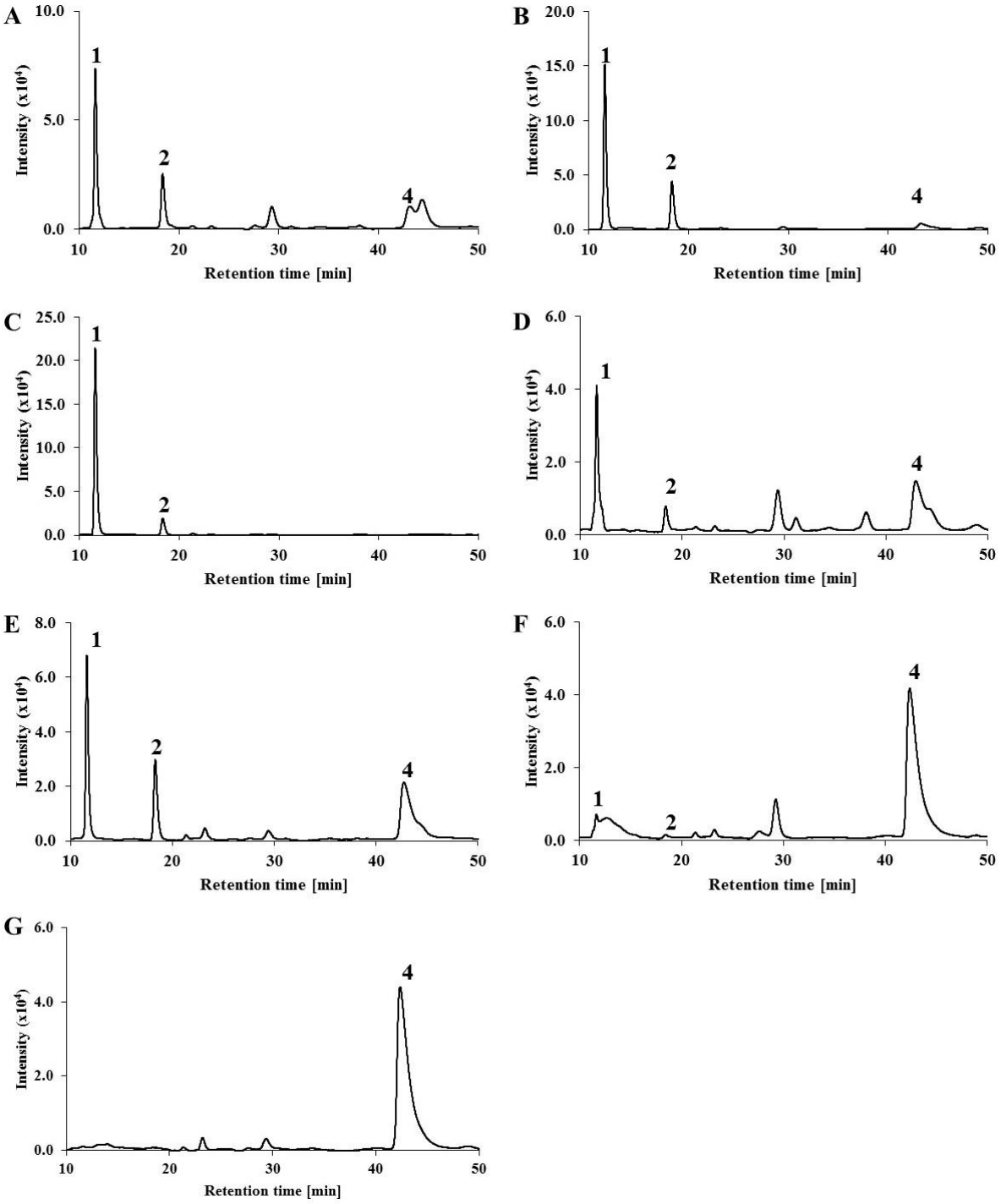


Figure S3. HPLC chromatograms of (*S*)-ketamine oxidation catalyzed by CYP154E1 I238Q (**A**), G239A (**B**), M388A (**C**), I238Q G239A (**D**), G239A M388A (**E**), I238Q M388A (**F**) and QAA (**G**) analyzed on a chiral Chiralpak IB (0.46 cm Ø x 25 cm, Chiral Technologies Europe) column. 1: (*S*)-ketamine, 2: (*S*)-norketamine, 4: (2*S*,6*S*)-hydroxynorketamine (identified by comparing with authentic standard).

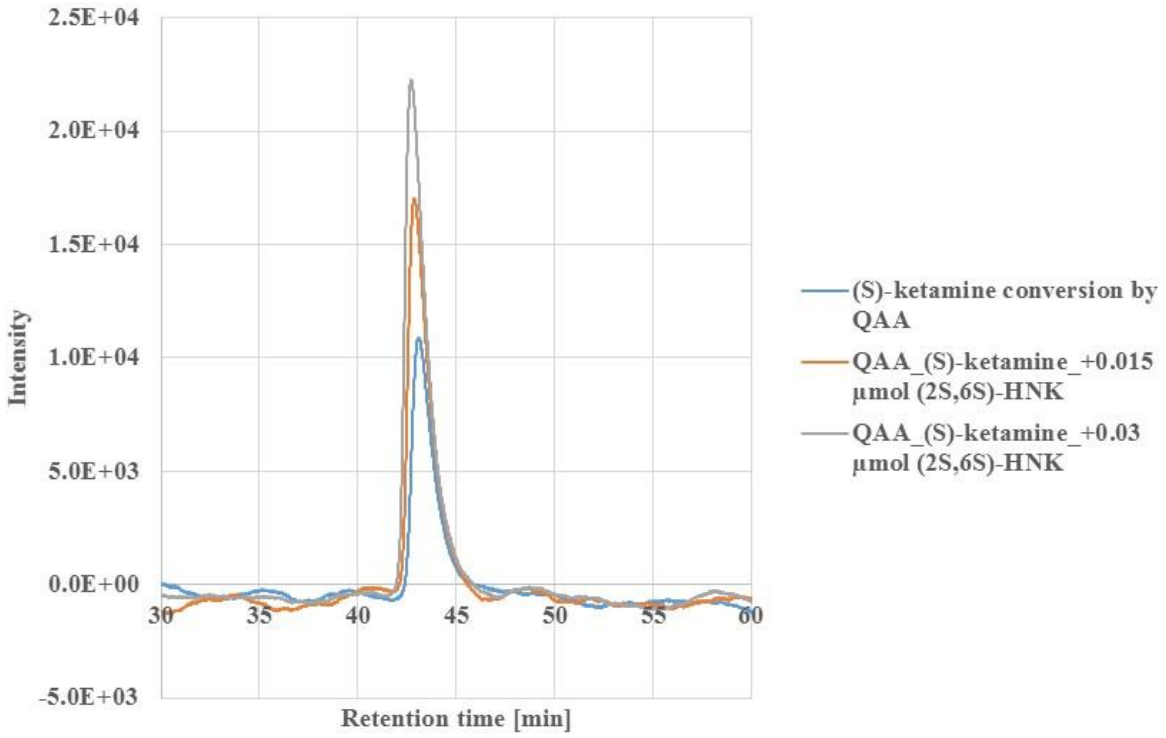


Figure S4. (2*S*,6*S*)-HNK product identification by spiking. Blue: Conversion of 500 µM (*S*)-ketamine by CYP154E1 QAA; orange: blue + 0.015 µmol (2*S*,6*S*)-HNK standard; grey: blue + 0.03 µmol (2S,6S)-HNK standard. Analytics were carried out with chiral column Chiralpak IB (0.46 cm Ø x 25 cm, Chiral Technologies Europe) on HPLC.

## CYP154E1 QAA CHARACTERIZATION

## SUBSTRATE BINDING SPECTRA FOR CYP154E1 QAA WITH (*S*)-KETAMINE

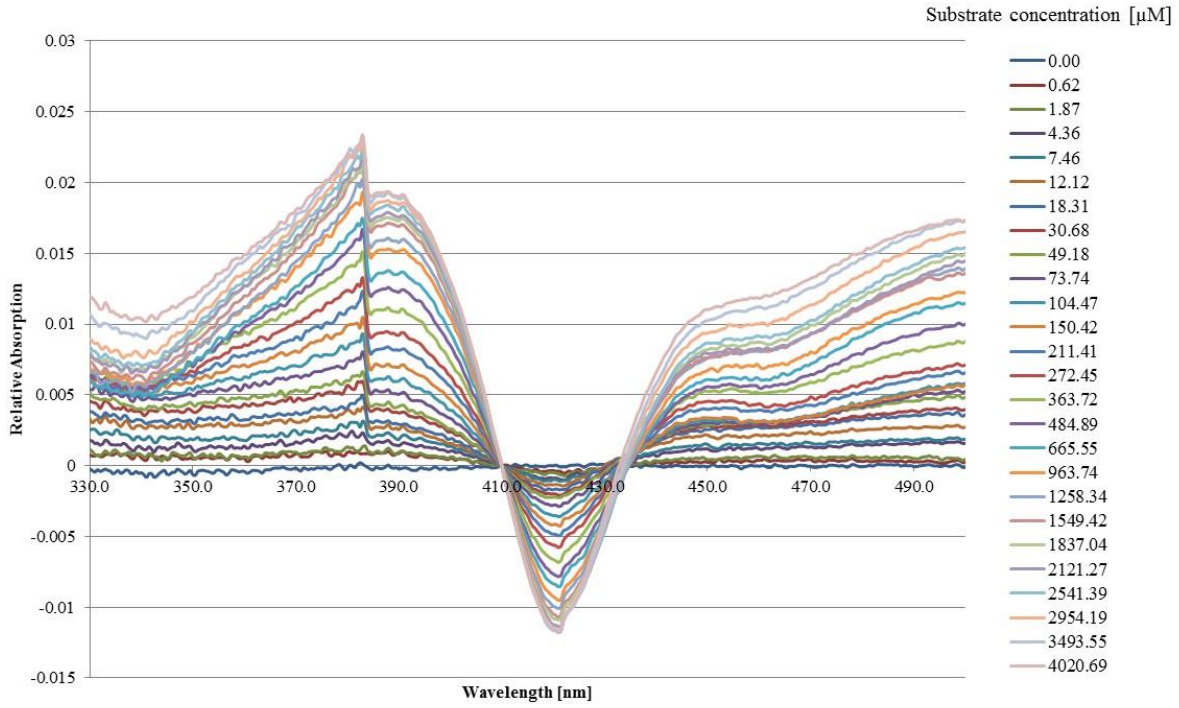


Figure S5. Substrate binding spectrum of CYP154E1 QAA with (*S*)-ketamine.

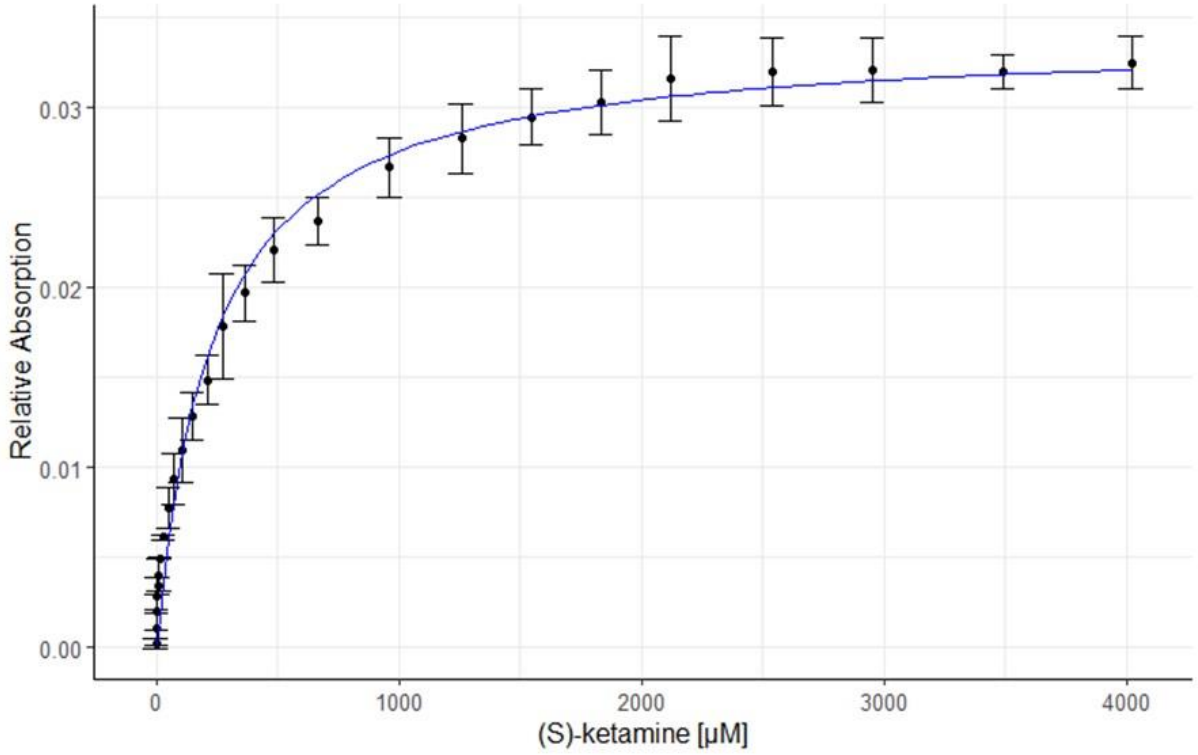


Figure S6. Kd-constant estimation. Plotting was done by using a Michaelis-Menten-like equation (see MM) using RStudio software (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/).

**KINETICS**

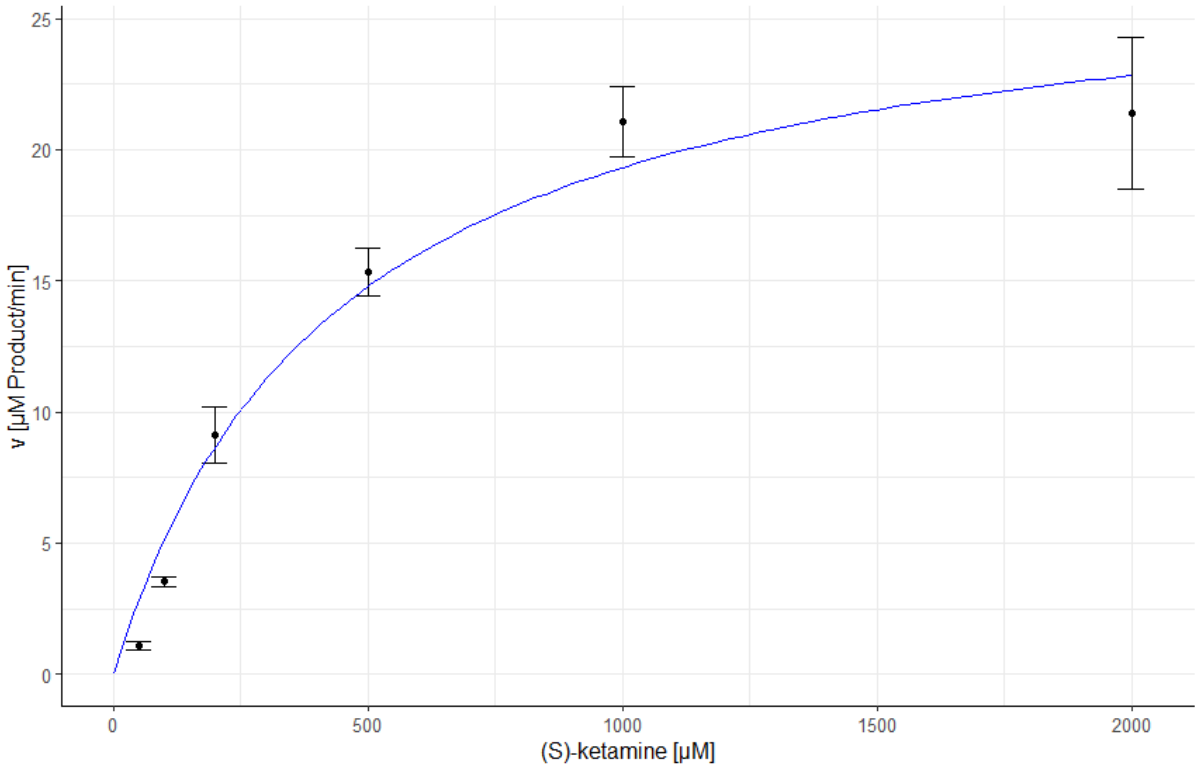


Figure S7. Reaction velocity depending on the (*S*)-ketamine concentration. Plotting was done by using the Michaelis-Menten equation using RStudio software (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/).

## RIGID DOCKING OF (2*S*,6*S*)-HYDROXYKETAMINE

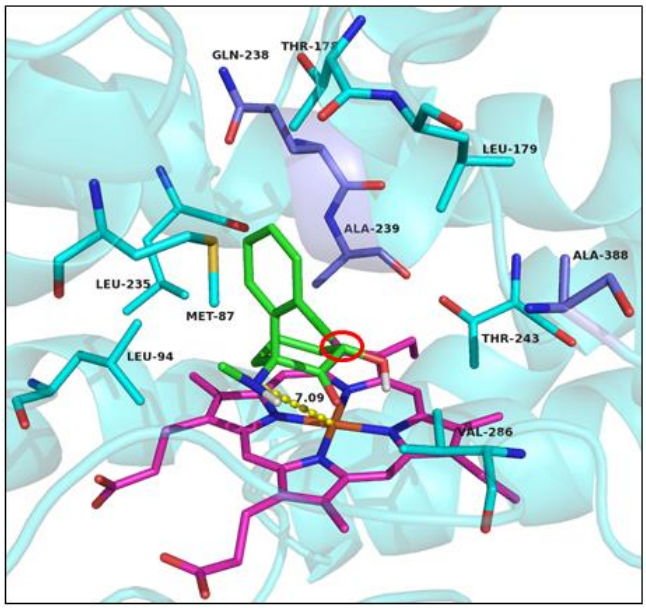


Figure S8. Best scored rigid docking poses of (2*S*,6*S*)-hydroxyketamine in the active site of CYP154E1 QAA. Position C6 of the cyclohexanone ring is marked by red circle. Dashed yellow lines indicate distances between atoms. The numerical distance is given in Å.

**PREPARATIVE SCALE CONVERSION**

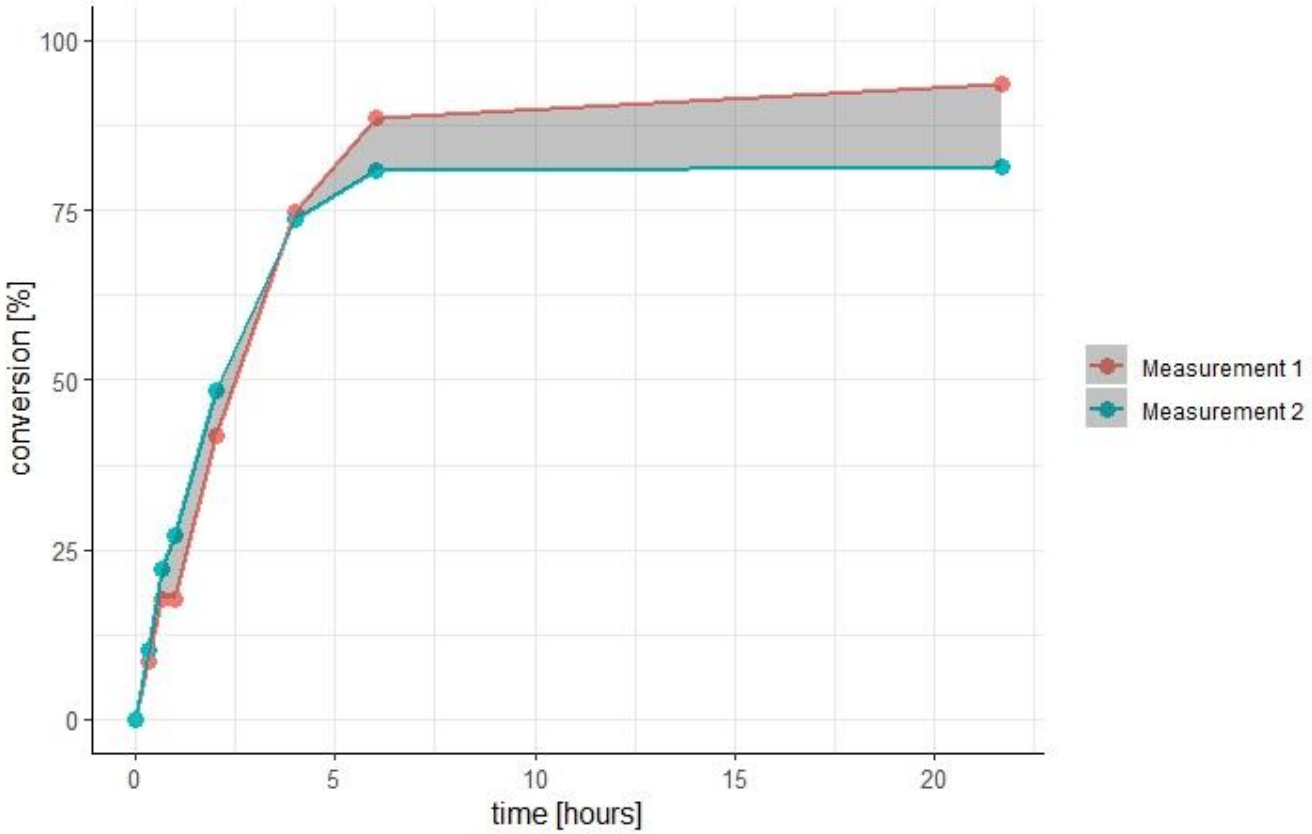


Figure S9. (*S*)-ketamine (5 mM) conversion in 10 ml whole cell biotransformation approach over a period of 21.67 hours in biological duplicate. Plotting was done using RStudio software (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/).

## NMR ANALYSIS

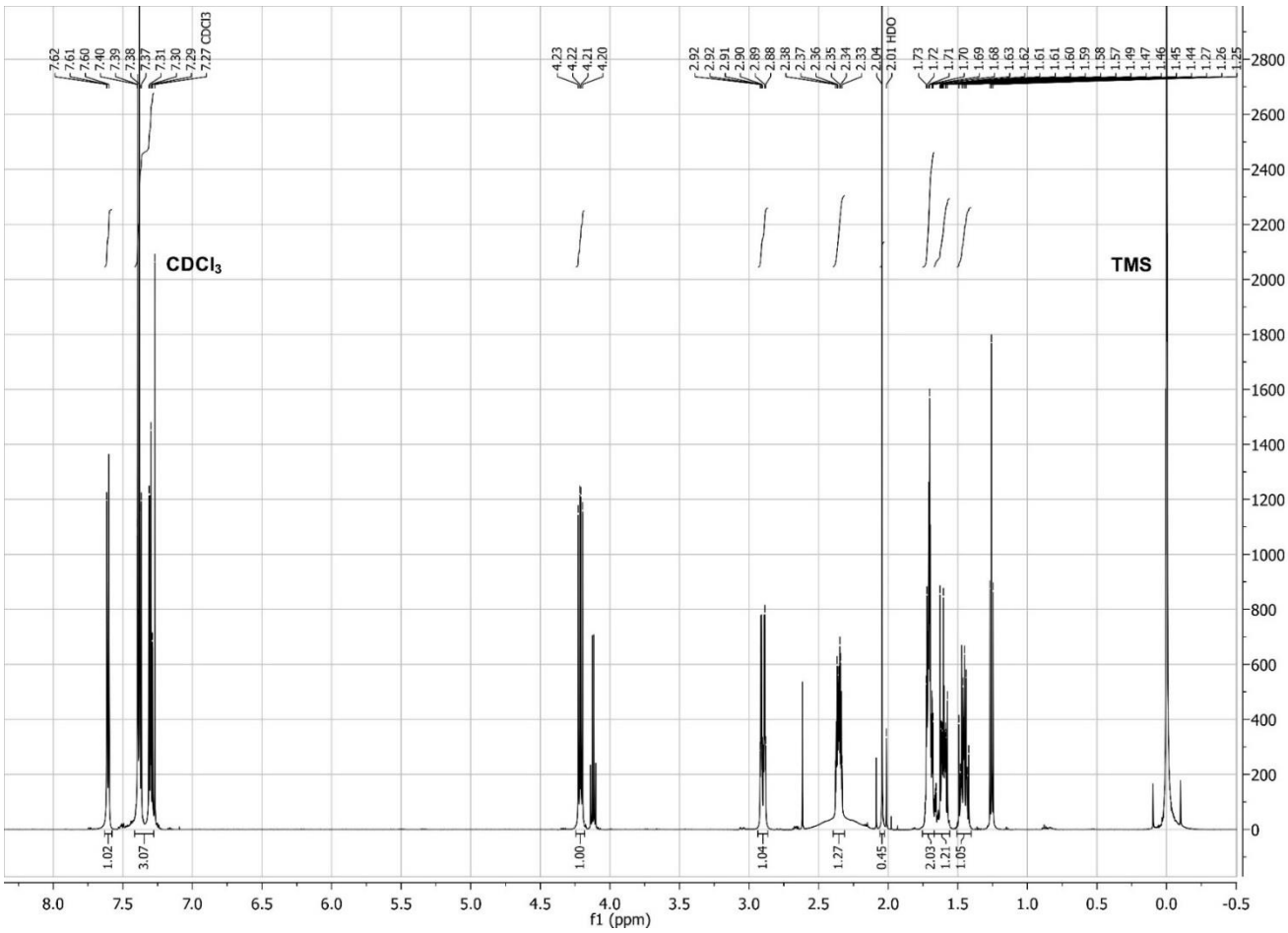


Figure S10. $^{1}$H NMR of (2*S*,6*S*)-hydroxynorketamine in $CDCl_3$.

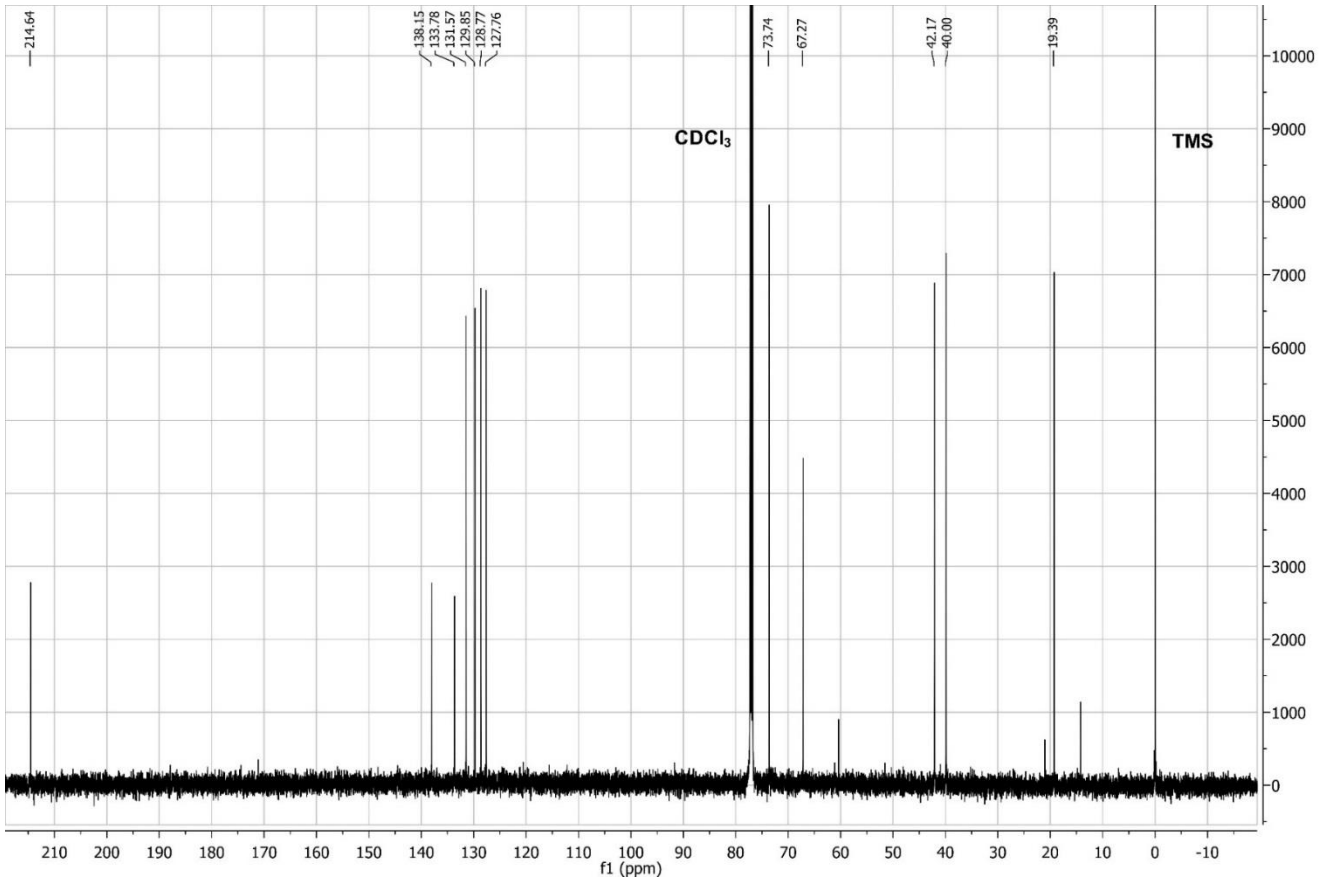


Figure S11. $^{13}C$ NMR of (2*S*,6*S*)-hydroxynorketamine in $CDCl_3$.

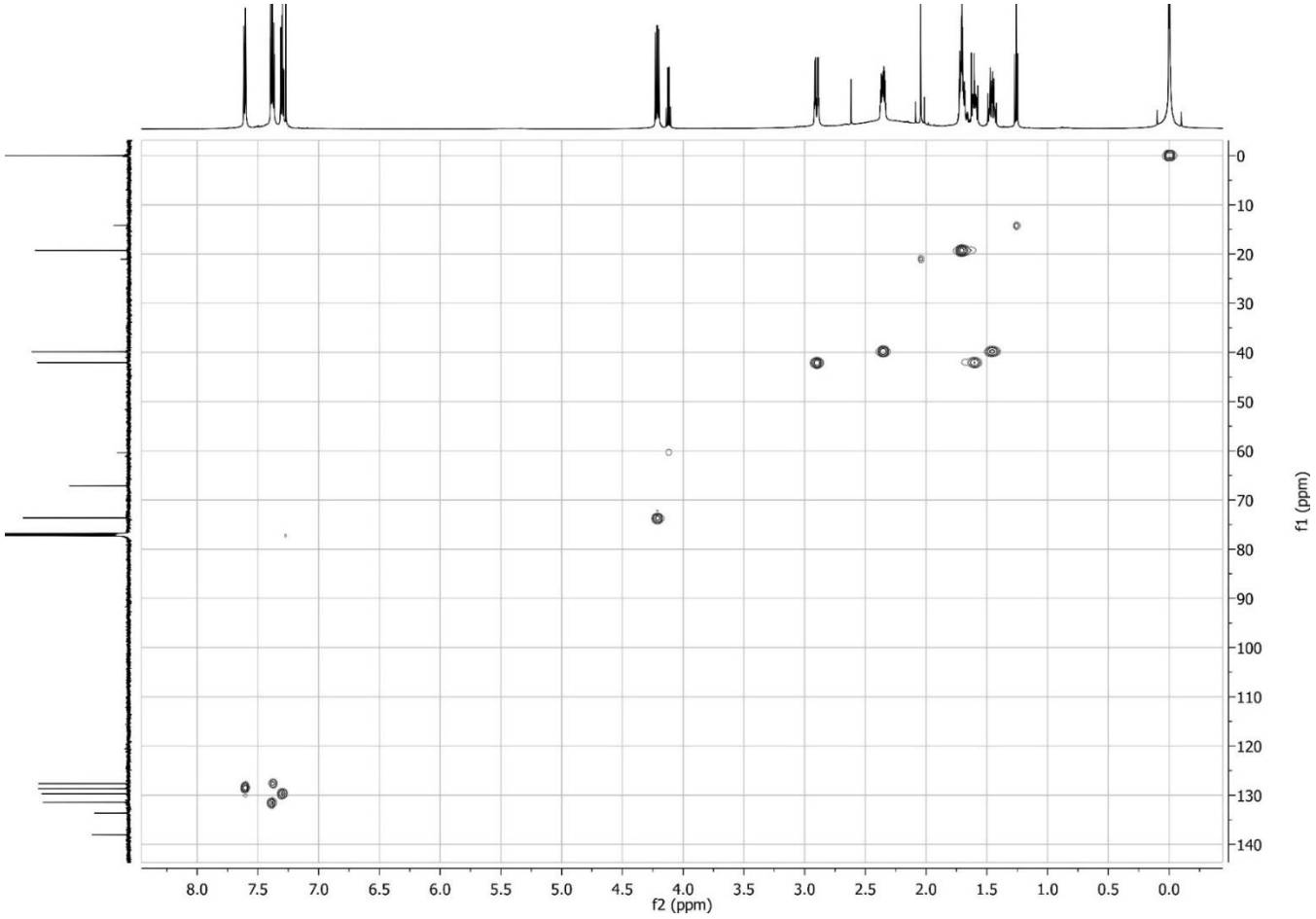


Figure S12. $^{1}H$-$^{13}C$ HSQC spectrum of (2*S*,6*S*)-hydroxynorketamine in $CDCl_3$.

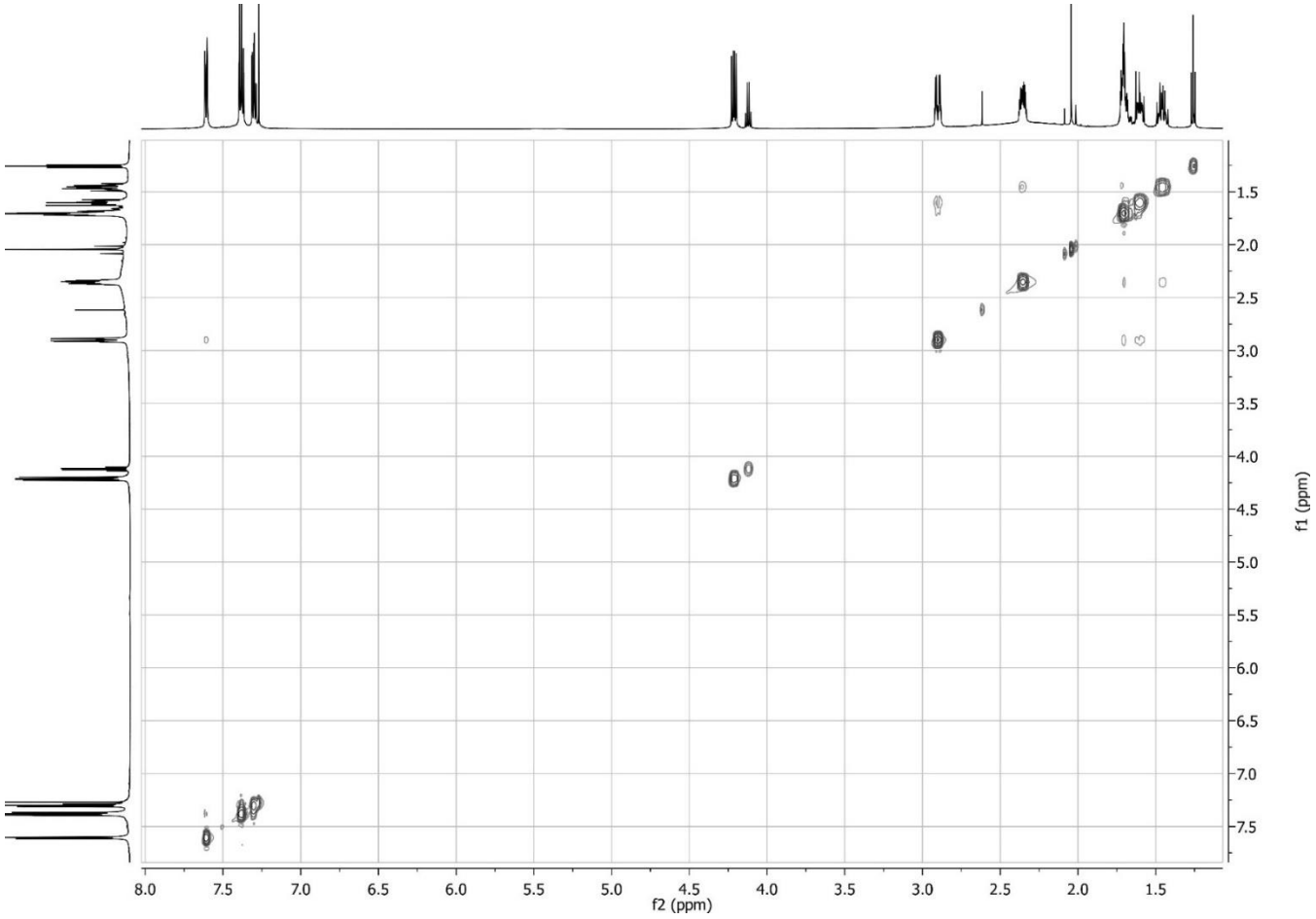


Figure S13. $^{1}H$-$^{1}H$ NOESY spectrum of (2*S*,6*S*)-hydroxynorketamine in $CDCl_3$.

## REFERENCES


(1) Edelheit, O.; Hanukoglu, A.; Hanukoglu, I. Simple and Efficient Site-Directed Mutagenesis Using Two Single-Primer Reactions in Parallel to Generate Mutants for Protein Structure-Function Studies. *BMC Biotechnol.* **2009**, *9*.

(2) Girhard, M.; Klaus, T.; Khatri, Y.; Bernhardt, R.; Urlacher, V. B. Characterization of the Versatile Monooxygenase Cyp109b1 from *Bacillus Subtilis*. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 595-607.

(3) Le-Huu, P.; Heidt, T.; Claasen, B.; Laschat, S.; Urlacher, V. B. Chemo-, Regio-, and Stereoselective Oxidation of the Monocyclic Diterpenoid B-Cembrenediol by P450 BM3. *ACS Catalysis* **2015**, *5*, 1772-1780.

(4) Omura, T.; Sato, R. The Carbon Monoxide-Binding Pigment of Liver Microsomes. *J. Biol. Chem.* **1964**, *239*, 2379-2385.

(5) Fujii, K.; Huennekens, F. M. Activation of Methionine Synthetase by a Reduced Triphosphopyridine Nucleotide-Dependent Flavoprotein System. *The Journal of Biological Chemistry* **1974**, *249*, 6745-6753.

(6) McIver, L.; Leadbeater, C.; Campopiano, D. J.; Baxter, R. L.; Daff, S. N.; Chapman, S. K.; Munro, A. W. Characterisation of Flavodoxin $Nadp^{+}$ Oxidoreductase and Flavodoxin; Key Components of Electron Transfer in *Escherichia Coli*. *Eur. J. Biochem.* **1998**, *257*, 577-585.

(7) Wang, Z.-Q.; Lawson, R. J.; Buddha, M. R.; Wei, C.-C.; Crane, B. R.; Munro, A. W.; Stuehr, D. J. Bacterial Flavodoxins Support Nitric Oxide Production by *Bacillus Subtilis* Nitric-Oxide Synthase. *J. Biol. Chem.* **2007**, *282*, 2196-2202.

(8) Lawson, R. J.; von Wachenfeldt, C.; Haq, I.; Perkins, J.; Munro, A. W. Expression and Characterization of the Two Flavodoxin Proteins of *Bacillus Subtilis*, Ykun and Ykup: Biophysical Properties and Interactions with Cytochrome P450 Bioi. *Biochemistry* **2004**, *43*, 12390-12409.