Supporting Information

Switching the chemoselectivity of perakine reductase for selective reduction of α,β-unsaturated ketones by Arg127 mutation

Zehao Zhang,‡[a,b] Yun Zhou,‡[b] Hong Zhao,[b] Yihang Wu,*[a] and Lianli Sun*[b,c]

[a.] *Department of Pharmacy, Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine, College of Life Sciences, China Jiliang University, Hangzhou 310018, China. E-mail: yihangwu@cjlu.edu.cn*

[b.] *Institute of Drug Metabolism and Pharmaceutical Analysis, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, 310058, China. E-mail: sunlianli@zju.edu.cn.*

[c.] *Jinhua Institute of Zhejiang University, Jinhua 321299, China.*

‡ Both authors contributed equally.

Table of Contents

Materials and methods

1.General information

The general chemicals used in this study were purchased from Aladdin (Shanghai, China). The cofactor NADPH was purchased from Yeasen (Shanghai, China). Ampicillin, kanamycin, and RNAaseA were purchased from SangonBiotech Company (Shanghai, China). Substrate, (*E*)-benzalacetone (**1**), standards of enzymatic reaction products 4-phenyl-3-buten-2-ol (**1a**) and benzylacetone (**1b**) were purchased from Sigma Aldrich (St. Louis, MO, USA). The three (*E*)-benzalacetone derivates (**2-4**) were purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China).

^{1}H NMR spectra were recorded at 500 MHz. The spectra data were reported as follows: chemical shift (δ ppm), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), and coupling constant (Hz).

2.Mutation of PR

Site-directed mutagenesis of PR was performed using the previously constructed His$_6$-PR-pQE-2 as the template. The following oligonucleotides were used as primers to introduce the mutation: forward primer for His126Glu mutation, 5'-d-AT CTC TTC TAC ATA GAG CGC ATA GAT ACA ACA GTG CCT A-3'; reverse primer for His126Glu mutation, 5'-d- AGG CAC TGT TGT ATC TAT GCG CTC TAT GTA GAA GAG ATC GA -3'; forward primer for saturated mutation of Arg127, 5'-d-TC GAT CTC TTC TAC ATA CAT **NNK** ATA GAT ACA ACA GTG CCT AT -3', and reverse primer for saturated mutation of Arg127, 5'-d-AT AGG CAC TGT TGT ATC TAT **NNK** ATG TAT GTA GAA GAG ATC GA -3'. NNK represents the codon for all the amino acids.

3.Protein Expression and Purification

Expression and purification of recombinant His$_6$-PR wild type and mutants was performed as previously reported.[1] Briefly, *E. coli* M15 transformed with His$_6$-PR-pQE-2 plasmid were cultured in LB medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin up to a cell density of OD$_{600}$ 0.6-0.7 at 37 °C. Subsequently, protein expression was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 23 °C for 36 h. The bacteria were collected by centrifugation at the speed of 4000 rpm for 25 min at 4 °C and stored under -20 °C.

Typically, 3 g of PR expressing bacteria were used for one protein purification. The bacteria were resuspended in extract buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 1 mg/mL lysozyme. After sonication (4 °C) and centrifugation (12000 ×g 25 min, 4 °C), the supernatant was loaded into a column with 2 ml Ni-nitrilo-tri-acetic acid (Ni-NTA) superflow (Qiagen, Hilden, Germany). The column was then washed with 20 ml extract buffer and 20 ml wash buffer containing 20 mM imidazole. PR enzyme was eluted with elution buffer containing 250 mM imidazole. The collected protein fraction was dialyzed against 5 L Kpi buffer (50 mM KH_2PO_4, 50 mM K_2HPO_4, 10 mM EDTA, pH 7.0) for 12 h for further enzymatic reaction.

4.Enzyme assay

For determination of the chemoselectivity and catalytic efficiency of PR Arg127-mutants, we used α,β-unsaturated aryl ketone (*E*)-benzalacetone (**1**) as a substrate. The enzymatic reaction contains 1.5 mg/ml purified PR enzyme, 1.2 mM NADPH, and 0.8 mM substrate in 50 mM Kpi buffer (pH 7.0) in a total volume of 200 μL. The reaction was performed at 30 °C for 10 h. Same amount of NADPH was freshly added at 2, 4, 6, 8 h during the reaction.

The reaction was terminated by adding the equal volume of methanol. Subsequently, the mixture was centrifugated at 12000×g for 30 min, and 10 μl of the supernatant was analyzed by HPLC. Agilent 1260 HPLC system was employed for HPLC analyses. The samples were analyzed under the following conditions: using Agilent HC-C18 column (5 μm, 250*4.6 mm) as a separation column, 214 nm as detection wavelength, 1 ml/min flow. To monitor the enzymatic reaction of substrates (**1**) and (**3**), we used 55% acetonitrile and 45% water as mobile phase; for substrate (**2**), we used 40% acetonitrile and 60% water as mobile phase; for substrate (**4**), we used 45% acetonitrile and 55% water as mobile phase. We used 4'-nitroacetophenone as an internal standard for quantification of substrate (**1**, **3**, **4**) and their products, and used phenylethanol as an internal standard for quantification of substrate (**2**) and its product. The compounds (**1a**) and (**1b**) were used as product standards for C=O selective reduction and C=C selective reduction, respectively. All assays were performed in triplicate.

Except the enzyme amount of PR Arg127Met mutant was reduced to 0.75 mg/ml, the relative activity of the three (*E*)-benzalacetone derivates (**2-4**) by PR Arg127Met were determined using

the same method as compound (**1**).

5.Products preparation for NMR analysis

For NMR analysis, the enzymatic reactions were carried out in 25 ml volume under the same conditions as described above, containing 2.0 mg/ml of enzyme, 1 mM substrate and 1.5 mM NADPH. Three times of 25 ml reaction mixtures were combined, and the reaction was terminated by adding the equal volume of MeOH. Following centrifugation at 10000 rpm for 5 min, the supernatant was evaporated under reduced pressure. The residue was dissolved in MeOH and purified by reverse-phase preparative HPLC (Agilent Prostar 410), equipping with a preparative column (Elite, SinoChrom ODS-BP 10 μm, 20.0 * 250 mm).

6.Determination of kinetic parameters

Kinetic parameters of PR wild type and Arg127Met catalyzing the reduction of (*E*)-benzalacetone (**1**) were determined by monitoring product formation rates. The reactions were carried out in the same incubation system used for enzyme assay with varying concentrations of substrate (1-12 mM) and excess NADPH (20 mM). The reaction was carried out under 30 ℃ for 55 min. The reaction mixtures were further processed with the same procedure that used for enzyme assay. K_m value were calculated using Lineweaver-Burk plot.

7.Synthesis of 4-phenyl-2-butanol (1c) by stepwise reaction

A stepwise reaction was performed to synthesize 4-phenyl-2-butanol, which is the product of benzalacetone (**1**) with both C=O and C=C bonds reduced. Wild type PR and Arg127Met mutant showed the best activity on C=O reduction and C=C reduction, respectively, therefore were selected for the stepwise reaction. PR Arg127Met mutant were only active on the C=C bond conjugated with C=O bond, while the conjugation of C=O with C=C is not required to be a substrate for wild type PR, thus the C=C bond of substrate benzalacetone (**1**) was first reduced by Arg127Met mutant, followed with C=O reduction by WT PR. Briefly, a mixture of 0.8 mM substrate, 1.2 mM NADPH, and 400 μg PR Arg127Met mutant in 50 mM Kpi buffer (pH 7.0) were incubated for 10 h under same conditions as described in "Enzyme assay". Next, 400 μg of wild type PR was added into the reaction mixture and continued incubating under the same conditions for 10 h. The reaction was terminated by adding the same volume of MeOH. The products of the reaction were detected by HPLC, using 40% acetonitrile and 60% water as

mobile phase.

8.Molecular Docking

In this study, we used AutoDock Vina software for the molecular docking.[2] The initial crystal structure of PR wild type (WT) in complex with NADPH was downloaded from PDB data bank (PDB ID: 3V0S), however, nicotinamide and ribosyl moiety of NADPH was disordered in the crystal structure. The missing part was then docked into the complex structure. To illustrate the binding mode of substrate in PR, we further performed the molecular docking of substrate into the complex. All water molecules were removed from the original PDB file, and polar hydrogen atoms were added. Gasteiger charge, atomic solvation parameters, and fragmental volumes were assigned to the protein. The cofactor NADPH and substrate (*E*)-benzalacetone (**1**) were preprocessed by AutoDock Tools. From a catalytic reaction point of view, it is reasonable to assume that the substrate binding pocket of PR connects with the active site comprising with the AKR tetrad Asp52, Lys84, Tyr57 and His126. The grid box dimensions and center were set as follows: center x, y, z = 41.9, 41.35, 37.1, size x, y, z = 10.85, 13.4, 10.4. The distances between C-4 atom the pyridine ring of NADPH and the reactive groups of the substrate were measured with Pymol software. Based on the crystal structure of PR WT, Arg127 was mutated to methionine (Arg127Met), tryptophan (Arg127Trp) and glutamine (Arg127Gln). The structures of above three variants were modeled by Swiss-Model Server.[3]

9. Molecular dynamics (MD) simulations

Molecular simulations of PR wild type complexed with NADPH was performed in Gromacs (4.5.6)[4] and the Amber 14SB force field.[5] Solvation effects were simulated with SPCE water model.[6] The system was inserted in an octahedron water box in which the edges were about 5 nm. Eight Na^+ ions were added to neutralize the negative charge of the system. A thorough energy minimization was performed before MD simulations, consisting of: 2000 steps using the steepest descent algorithm followed by 2000 steps of conjugate gradient minimization of the whole system. The minimized system was equilibrated for 1ns at 303.15 K, then was subsequently submitted to 20 ns MD simulation, and snapshot structures were extracted every 20 ps. During the equilibration and MD simulation, the C-α atoms of purine ring part of NADPH and its nearby 5 Å residues were set as restraints. The system temperature and pressure

were maintained at 303.15 K and 1 bar using V-rescale[7] thermostat and the isotropic Parrinello–Rahman[8] barostat. The LINCS algorithm was implemented to constraint all bonds, angles and dihedrals.[9] For the electrostatic calculations, we applied Particle mesh Ewald (PME) method.[10] GROMACS suite of tools was used for all analyses. The hydrogen bonds between the protein and ligand were calculated by setting cutoff bond distance of 3.5 Å and angle of 30°.

Table S1. Chemo-selectivity and activity of PR variants toward (*E*)-benzalacetone (**1**)

Chemo-selectivity	PR variant	C=O Reduction		C=C Reduction	
		Yield (%)	Relative Activity (%)	Yield (%)	Relative Activity (%)
	Wild type (Arg127-His126)	87.9 ± 15.6	100.0	N.D.	N.D.
	Arg127Leu	5.0 ± 0.7	5.7	N.D.	N.D.
	Arg127Trp	4.5 ± 0.1	5.1	N.D.	N.D.
	Arg127Val	1.6 ± 0.3	1.9	N.D.	N.D.
	Arg127Thr	1.6 ± 0.2	1.9	N.D.	N.D.
	Arg127Tyr	1.1 ± 0.2	1.2	N.D.	N.D.
	Arg127Ala	0.7 ± 0.01	0.8	N.D.	N.D.
	Arg127Glu	0.3 ± 0.01	0.4	N.D.	N.D.
	Arg127Asp	N.D.	N.D.	N.D.	N.D.
	Arg127Ile	7.6 ± 0.3	8.6	1.5 ± 0.1	16.7
	Arg127His	3.1 ± 0.5	3.5	0.9 ± 0.1	10.1
	Arg127Gly	2.1 ± 0.7	2.4	6.4 ± 0.5	73.1
	Arg127Cys	1.3 ± 0.4	1.5	10.2± 0.04	115.9
	Arg127Gln	3.1 ± 0.6	3.6	35.8 ± 5.9	406.8
	Arg127Lys	N.D.	N.D.	7.7 ± 0.01	87.5
	Arg127Phe	N.D.	N.D.	17.7 ± 1.7	201.1
	Arg127Ser	N.D.	N.D.	35.6 ± 6.0	404.5
	Arg127Asn	N.D.	N.D.	**67.3 ± 0.6**	**764.8**
	Arg127Pro	N.D.	N.D.	**74.9 ± 0.1**	**851.1**
	Arg127Met	N.D.	N.D.	**79.4 ± 2.0**	**902.3**
	His126Glu	N.D.	N.D.	8.8 ± 1.7	100.0

C=O Reduction (top, orange arrow) → C=C Reduction (bottom, green arrow)

[a] Yields were calculated using 0.8 mM substrate and 1.5 mg/ml enzyme. [b] Relative activities of wild type and His126Glu variants were set at 100% for C=O and C=C bond reduction, respectively. Data represent the mean ± SD of values from three measurements.

Table S2. Kinetic parameters of PR wild-type and Arg127Met variant toward (*E*)-benzalacetone (**1**)

Enzyme	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1}·mM^{-1}]
Wild type	3.5	1.9×10^{-3}	0.54×10^{-3}
Arg127Met	5.4	7.9×10^{-3}	1.46×10^{-3}

**Table S3.** C=O reduction yields and relative activities of wild type PR toward benzalacetone derivates.

Entry	Yield (%)	Relative activity (%)
(**1**)	45.7 ± 2.2	100
(**2**)	12.9 ± 7.2	28.2

As C=O reduction activity of wild type PR toward substrate (**3**) and (**4**) has already been determined in our previously study[1], we did not repeat in this study.

Table S4. C=C reduction yields and relative activities of PR Arg127Met variant toward benzalacetone derivates.

Entry	Yield (%)	Relative Activity (%)
(**1**)	43.3 ± 9.0	100
(**2**)	66.5 ± 19.2	153.4
(**3**)	76.2 ± 5.9	175.7
(**4**)	64.6 ± 4.3	149.1

[a] Yields were calculated using 0.8 mM substances and 0.75 mg/ml enzyme. [b] Relative activities of (*E*)-benzalacetone (**1**) was set at 100%. Data represent the mean ± SD of values from three measurements. N.D.: not detectable.
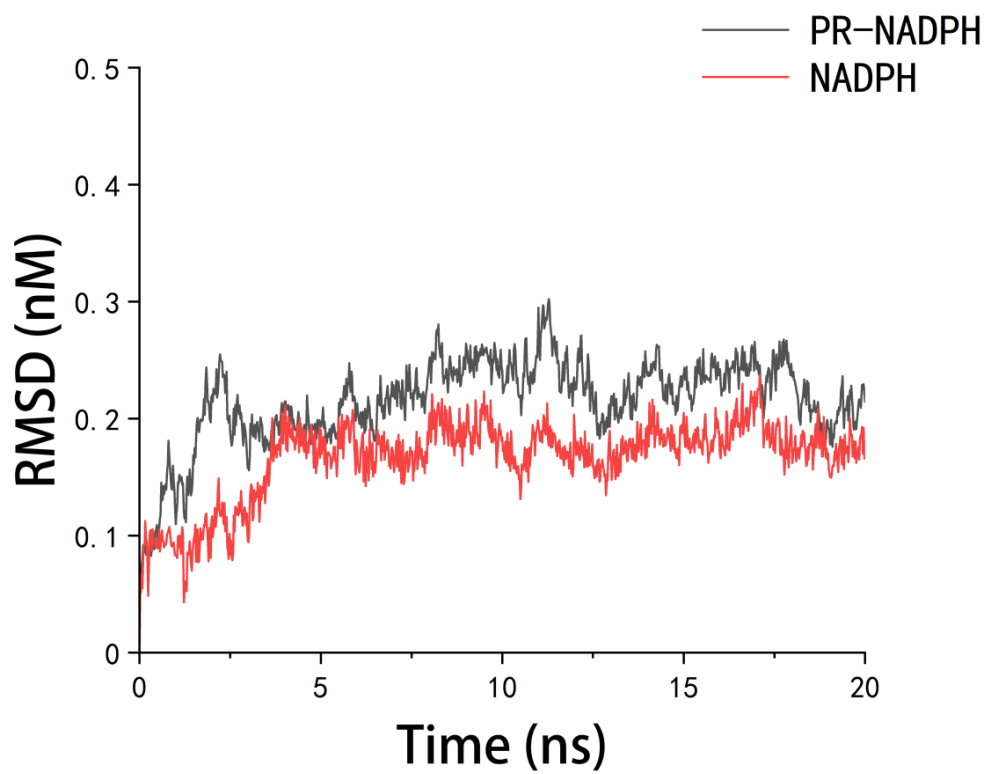
Fig. S1 The root mean square deviations (RMSD) of the entire complex of PR wild type with NADPH and NADPH molecule in the complex.
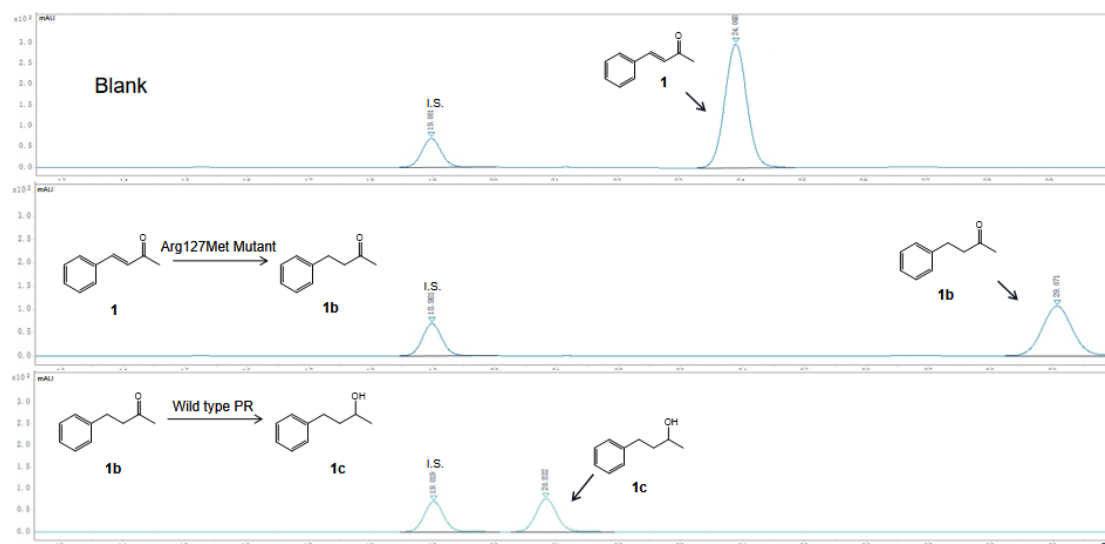
Fig. S2 Product detection of the stepwise reaction for synthesis of 4-phenyl-2-butanol (**1c**) by HPLC. I.S.: internal standard.
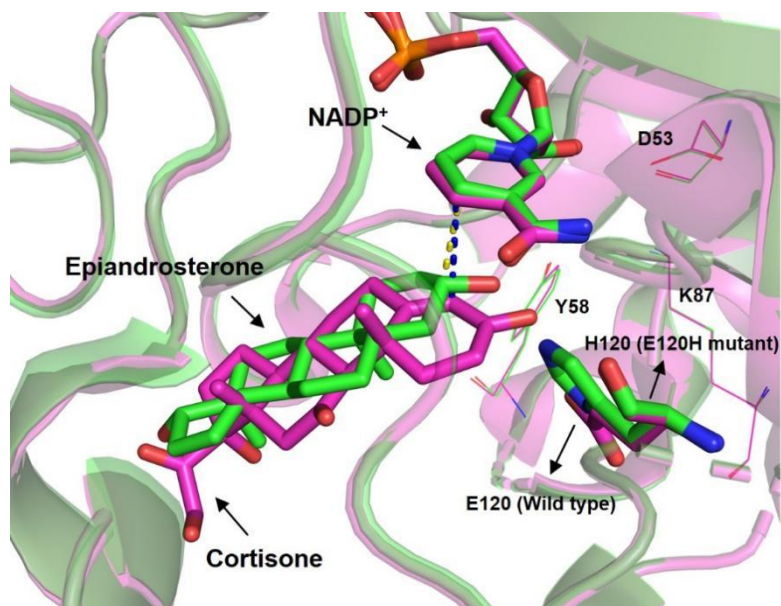
Fig. S3 Comparison of hydride transfer trajectory in AKR1D1 (C=C bond reductase) and its E120H (C=O bond reductase). The blue dashed line represents the hydride transfer trajectory for C=C bond reduction in wild type AKR1D1; The yellow dashed line represents the hydride transfer trajectory for C=O bond reduction in AKR1D1 E120H mutant. The main chain and carbon atoms of AKR1D1 is shown in purple, and AKR1D1 E120H mutant is shown in green.
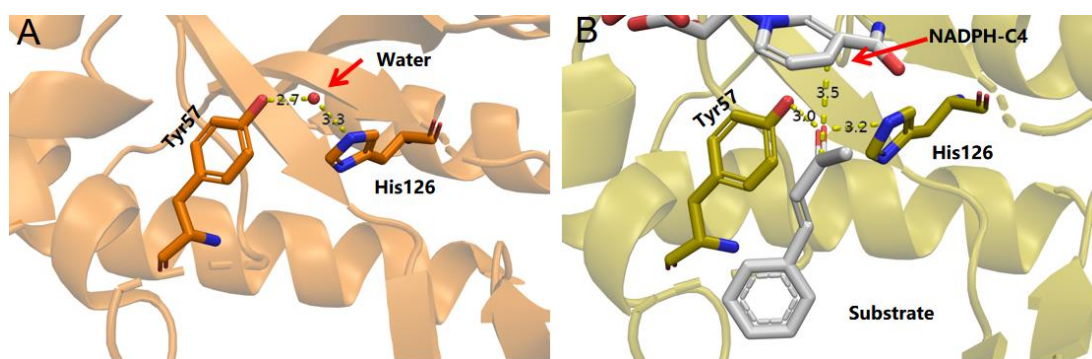
Fig. S4 (A) The spatial arrangement of the two key residues Tyr57, His126 in the crystal structure. The water molecule is supposed to be replaced by the carbonyl group of the substrate during the reaction. (B) Prediction of spatial arrangement of Tyr57, His126, NADPH and the substrate by molecular modelling.
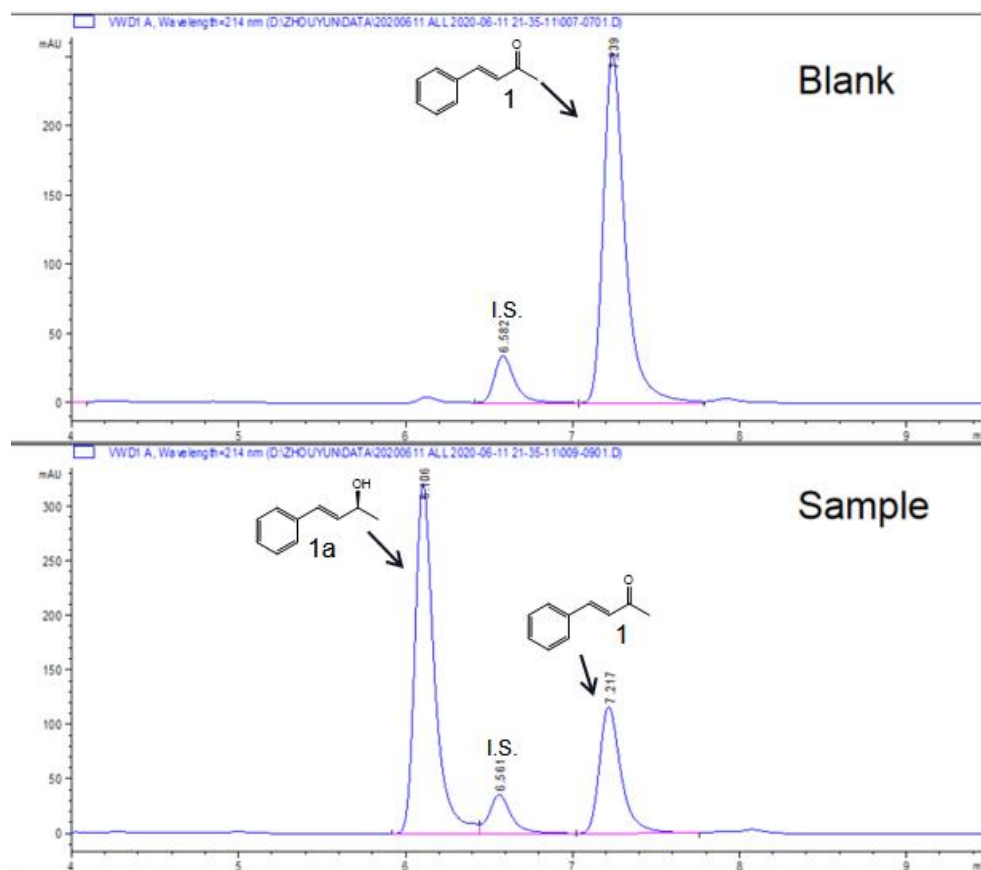
HPLC analysis of wild type PR catalyzed enzymatic reaction using (**1**) as the substrate. I.S.: internal standard.
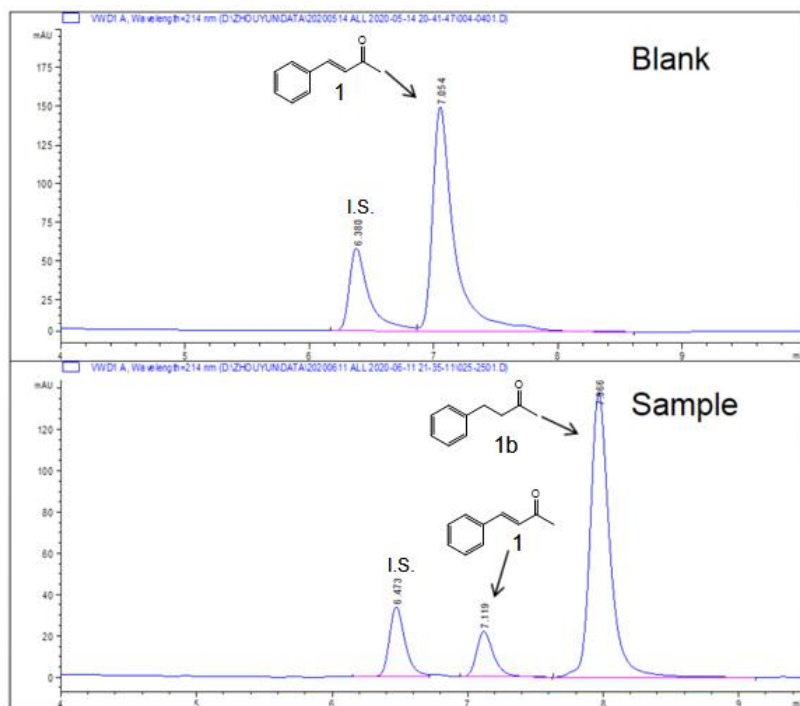
HPLC analysis of Arg127Met mutant catalyzed enzymatic reaction using (**1**) as the substrate.
I.S.: internal standard.
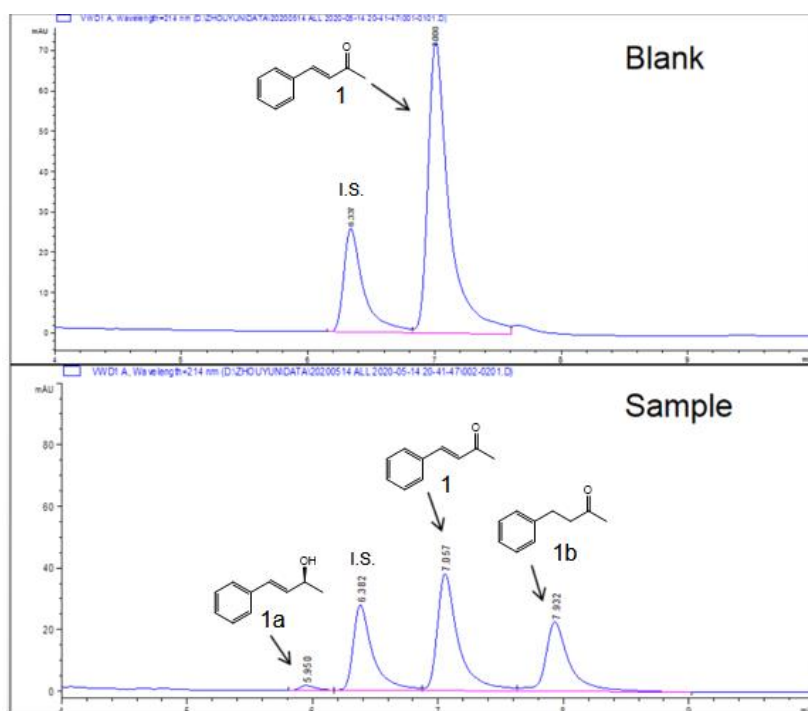
HPLC analysis of Arg127Gln mutant catalyzed enzymatic reaction using (**1**) as the substrate. I.S.: internal standard.
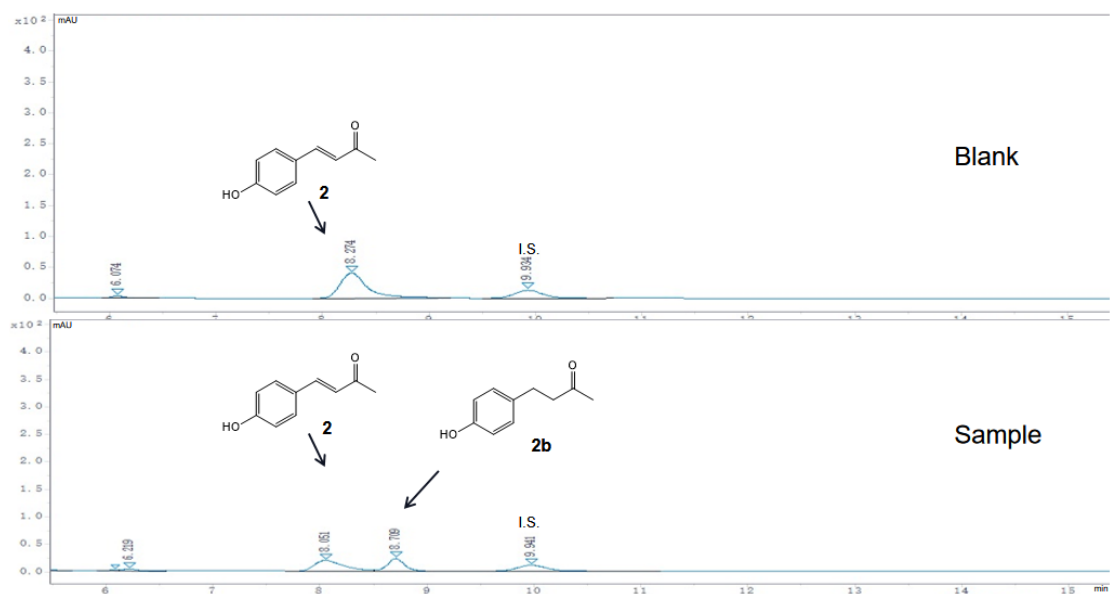
HPLC analysis of Arg127Met mutant catalyzed enzymatic reaction using (2) as the substrate. I.S.: internal standard.
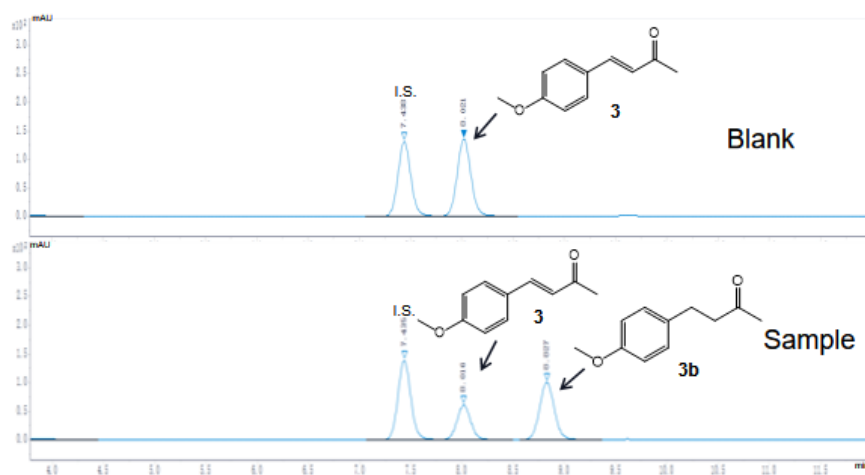
HPLC analysis of Arg127Met mutant catalyzed enzymatic reaction using (**3**) as the substrate.
I.S.: internal standard.
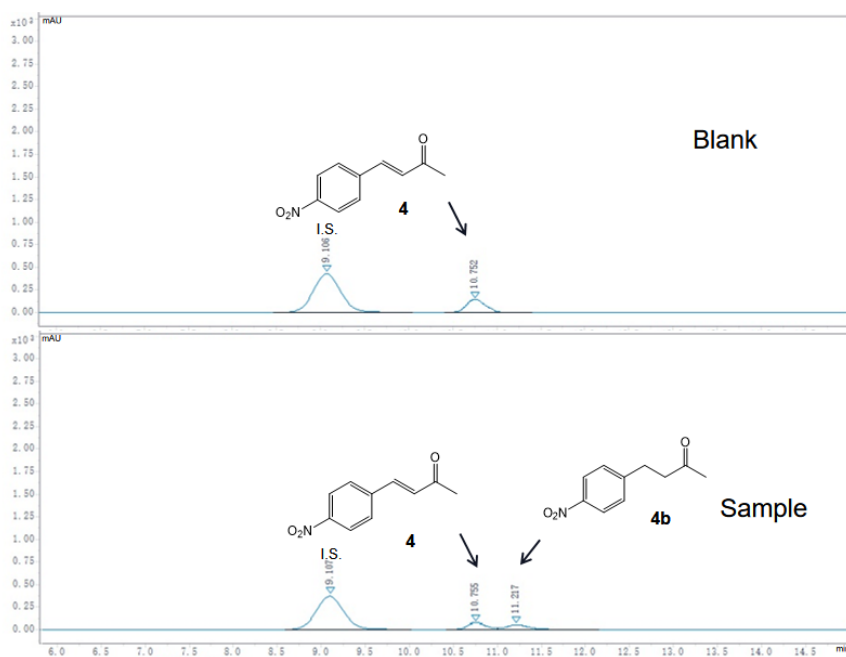
HPLC analysis of Arg127Met mutant catalyzed enzymatic reaction using (**4**) as the substrate. I.S.: internal standard.

^{1}H-NMR data
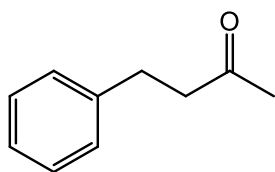
Benzylacetone (1b), colorless oil (7.8 mg)
^{1}H NMR (500 MHz, Chloroform-d) δ 7.28 (t, J = 7.4 Hz, 2H), 7.19 (t, J = 8.0 Hz, 3H), 2.90 (t, J = 7.6 Hz, 2H), 2.76 (t, J = 7.6 Hz, 2H), 2.14 (s, 3H).
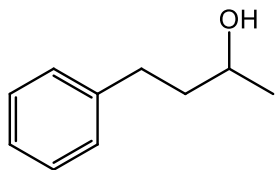
4-Phenyl-2-butanol (1c), colorless oil (6.4 mg)
^{1}H NMR (500 MHz, Methanol-d4) δ 7.24 (t, J = 7.5 Hz, 2H), 7.19 (d, J = 6.7 Hz, 2H), 7.14 (t, J = 7.2 Hz, 1H), 3.72 (dt, J = 12.4, 6.2 Hz, 1H), 2.72 (ddd, J = 13.5, 9.9, 5.9 Hz, 1H), 2.63 (ddd, J = 13.7, 9.7, 6.7 Hz, 1H), 1.78 – 1.65 (m, 2H), 1.18 (d, J = 6.2 Hz, 3H).
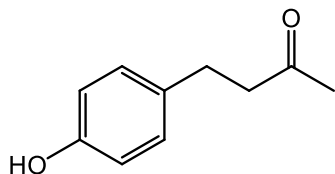
4-(4-hydroxyphenyl)-2-butanone (2b), white solid (5.2 mg)
^{1}H NMR (500 MHz, Methanol-d_4) δ 7.00 (d, *J* = 8.5 Hz, 2H), 6.68 (d, *J* = 8.5 Hz, 2H), 2.76 – 2.72 (m, 4H), 2.10 (s,3H).
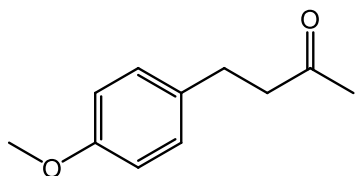
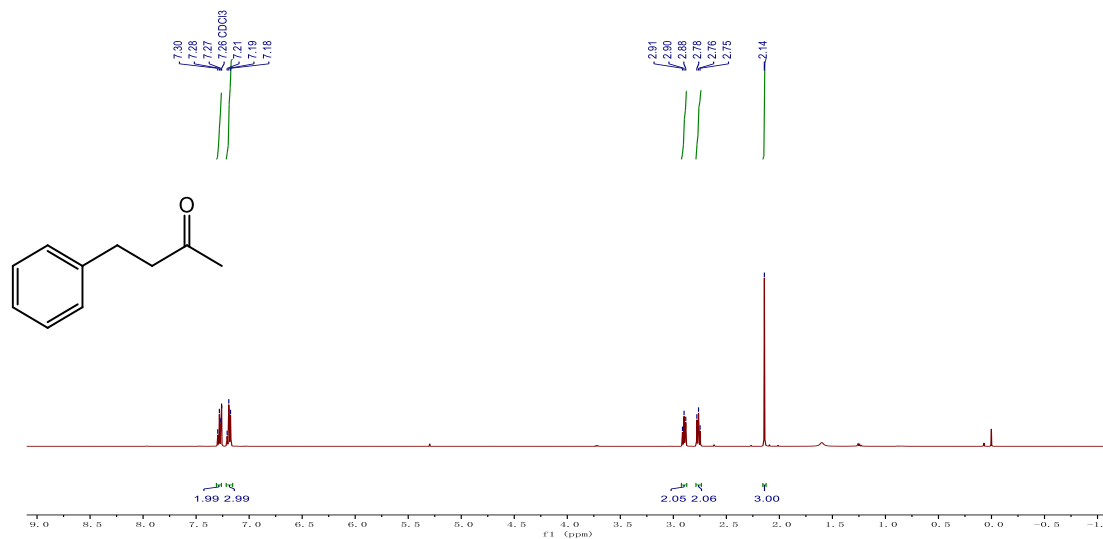

4-(4-Methoxyphenyl)-2-butanone (3b), yellow solid (6.5 mg)
^{1}H NMR (500 MHz, Chloroform-d) δ 7.10 (d, J = 8.7 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 3.78 (s, 3H), 2.84 (t, 2H), 2.72 (t, 2H), 2.13 (s, 3H).
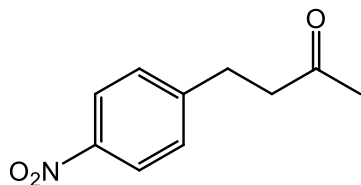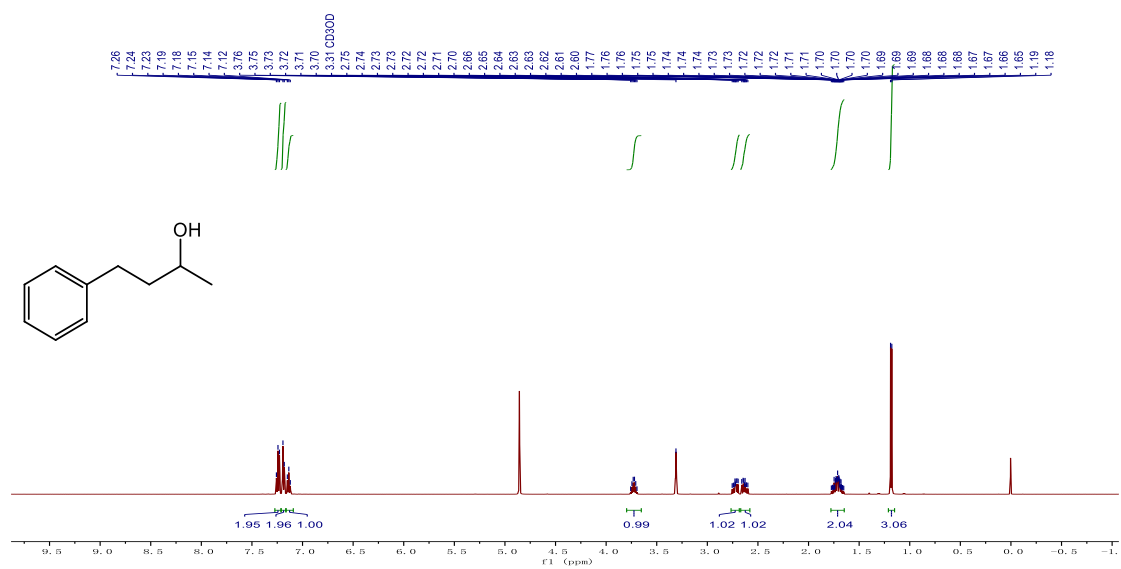

4-(4-nitrophenyl)butan-2-one (4b), white solid (5.5 mg)
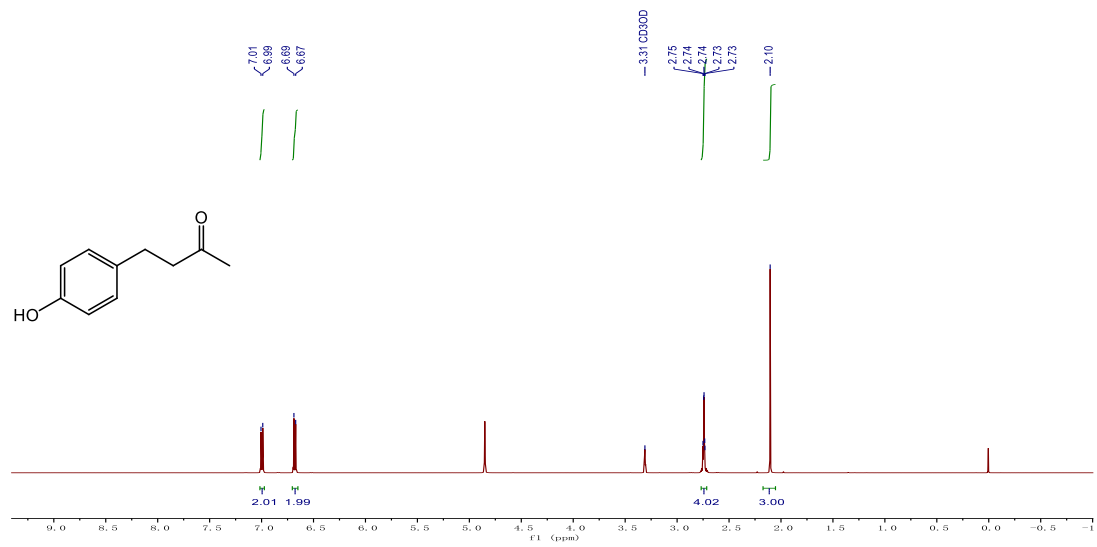^{1}H NMR (500 MHz, Chloroform-d) δ 8.14 (d, J = 8.7 Hz, 2H), 7.35 (d, J = 8.7 Hz, 2H), 3.00 (t, J = 7.4 Hz, 2H), 2.81 (t, J = 7.4 Hz, 2H), 2.16 (s, 3H).
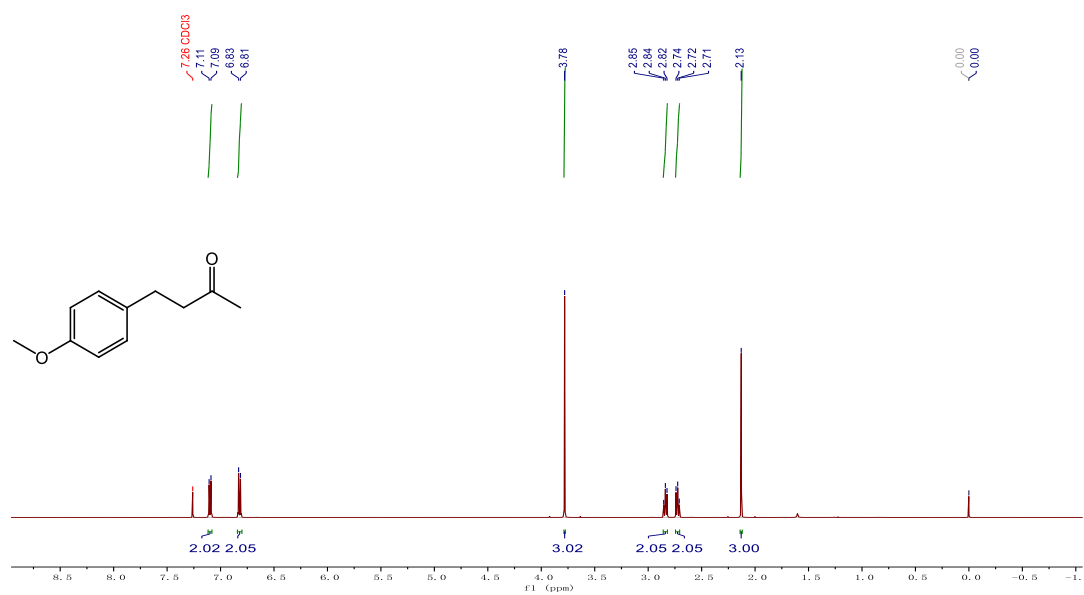
^{1}H-NMR spectrum of benzylacetone (**1b**) in CDCl$_3$.

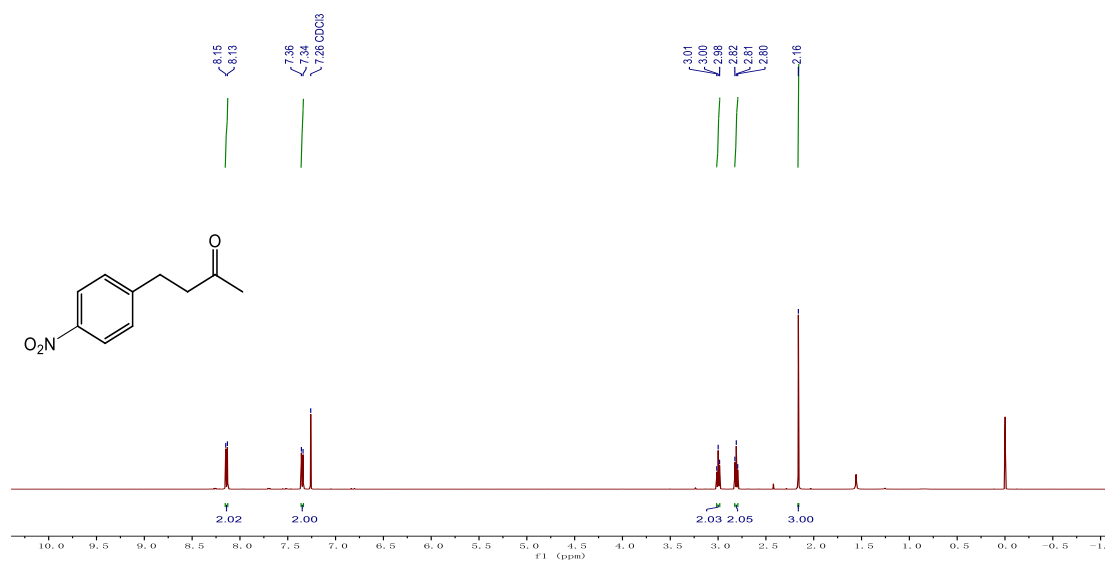^{1}H-NMR spectrum of 4-phenyl-2-butanol (**1c**) in methanol-D4.

^{1}H-NMR spectrum of 4-(4-hydroxyphenyl)-2-butanone (**2b**) in methanol-D4.

^{1}H-NMR spectrum of 4-(4-Methoxyphenyl)-2-butanone (**3b**) in CDCl$_3$.

^{1}H-NMR spectrum of 4-(4-nitrophenyl) butan-2-one (**4b**) in CDCl$_3$.

References

1. S. Cai, N. Shao, Y. Chen, A. Li, J. Pan, H. Zhu, H. Zou, S. Zeng, L. Sun and J. Zhao, *Org Lett*, 2019, **21**, 4411-4414.

2. O. Trott and A. J. Olson, *J Comput Chem*, 2010, **31**, 455-461.

3. L. Bordoli and T. Schwede, *Methods Mol Biol*, 2012, **857**, 107-136.

4. M. J. Abraham, T. Murtola, R. Schulz, S. Páll, J. C. Smith, B. Hess and E. Lindahl, *SoftwareX*, 2015, **1-2**, 19-25.

5. L. F. Song, T.-S. Lee, C. Zhu, D. M. York and K. M. Merz, Jr., *Journal of Chemical Information and Modeling*, 2019, **59**, 3128-3135.

6. H. J. C. Berendsen, J. R. Grigera and T. P. Straatsma, *The Journal of Physical Chemistry*, 1987, **91**, 6269-6271.

7. G. Bussi, D. Donadio and M. Parrinello, *J Chem Phys*, 2007, **126**, 014101.

8. M. Parrinello and A. Rahman, *Physical Review Letters*, 1980, **45**, 1196-1199.

9. B. Hess, H. Bekker, H. J. C. Berendsen and J. G. E. M. Fraaije, *Journal of Computational Chemistry*, 1997, **18**, 1463-1472.

10. T. Darden, D. York and L. Pedersen, *Journal of Chemical Physics*, 1993, **98**, 10089.