Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Multidrug resistance regulators (MDRs) as scaffold for the design of artificial metalloenzymes

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Supporting Information

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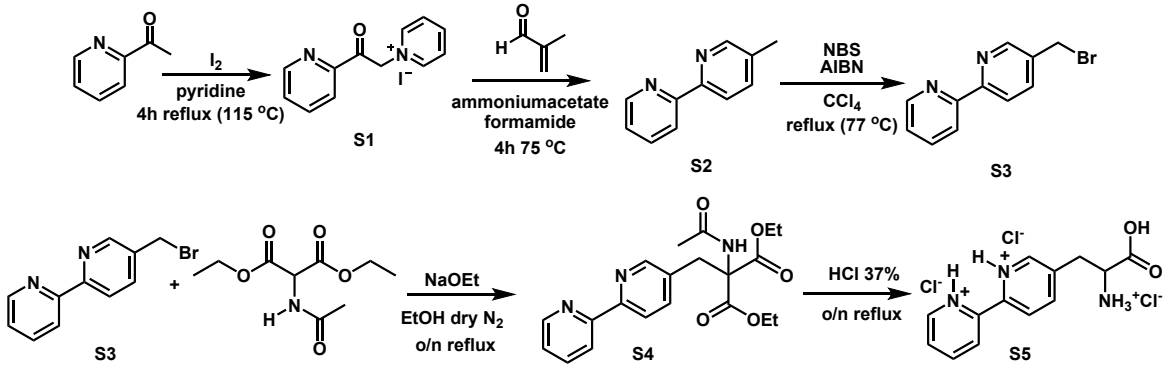
1. General remarks

Chemicals were purchased from Sigma Aldrich or Acros and used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian 400 MHz in $CDCl_3$ or DMSO-d6. Chemical shifts (δ) are denoted in ppm using residual solvent peaks as internal standard. Enantiomeric excess determinations were performed by HPLC analysis using UV-detection (Shimadzu SCL-10Avp) on Chiralpak AD, n-heptane:iPrOH 90:10, 1.0 ml/min. UPLC-MS on protein samples was performed on a Acquity TQ Detector (ESITQD- MS) coupled to Waters Acquity Ultra Performance LC using a Acquity BEH C8 (1.7 µm 2.1 x 150 mm). Water (solvent A) and acetonitrile (solvent B) containing 0.1% formic acid by volume, were used as the mobile phase at a flow rate of 0.3 mL/min. Gradient: 90% A for 2 min, linear gradient to 50% A in 2 min, linear gradient to 20% A in 5 min, followed by 2 min at 5% A. Re-equilibration of the column with 2 min at 90% A. Markers used for SDS-PAGE: PageRuler™ prestained 10-1180 KDa (Thermo Scientific). UPLC-MS chromatograms were analyzed with MassLynx V4.1 and deconvolution of spectra was obtained with the algorithm MagTran.[1] Fluorescence and UV-visible spectra were recorded on Jasco FP-6200 Spectrofluorometer and Jasco V-660 Spectrophotometer, respectively. E. coli strains NEB5α and BL21 (DE3)C43 (Stratagene) were used for routine cloning and protein expression, respectively. PCR reactions were carried out using an Eppendorf Mastercycler Personal apparatus. DNA sequencing was carried out by GATC-Biotech. Primers were synthesized by Eurofins. Pfu Turbo polymerase was purchased from Agilent and DpnI was purchased from New England Biolabs. Plasmid Puriying Kit was purchased from Qiagen. FPLC columns were purchased from GE Healthcare. Concentration of the proteins was measured with Nanodrop 2000 (Thermo Scientific). Extinction coefficients of protein (ε^{280}) have been calculated by the Protparam tool on the Expasy server (contribution of bipyridine moiety was accounted for by addition of the measured extinction coefficient obtained for 2,2'-bipyridine (ε=14800 $M^{-1}cm^{-1}$) to the extinction coefficient of the proteins.

2. Synthetic procedures and characterization



N-([2,2'-Bipyridin]-5-ylmethyl)-2-bromoacetamide was synthesized according to literature procedures.

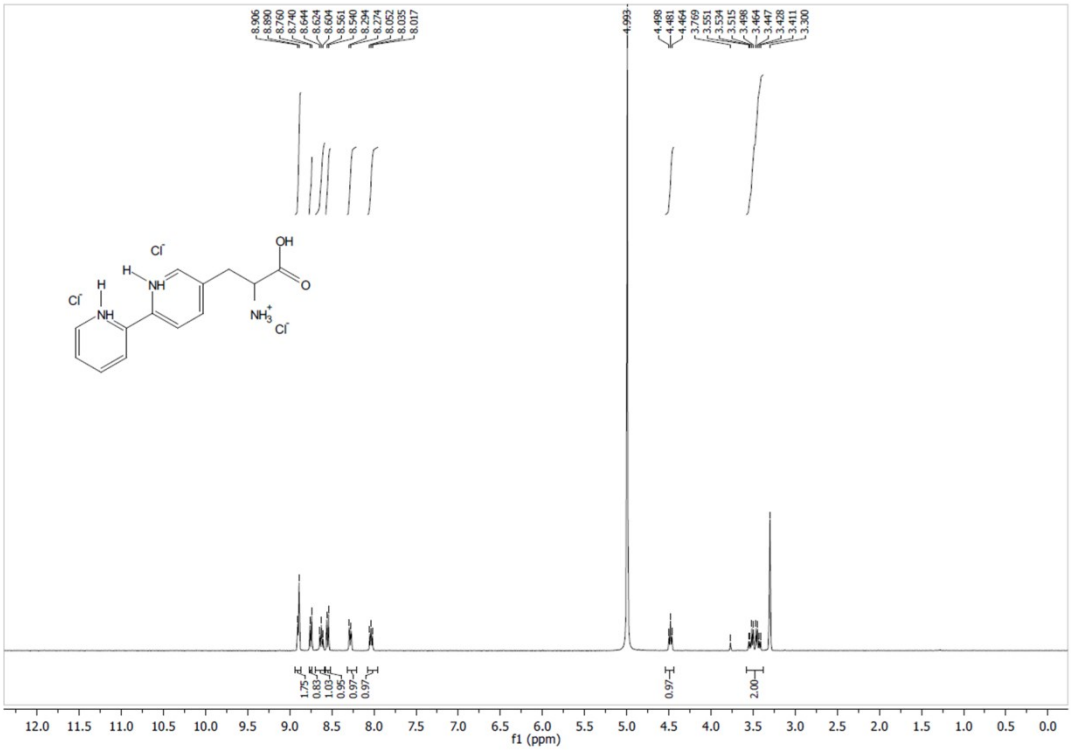
1-[2-Oxo-(2-pyridin-2-yl-)ethyl]pyridinium iodide (**S1**)[2] ^{1}H NMR (400 MHz, DMSO): 6.51 (s, 2H), 7.82-7.85 (m, 1H), 8.06-8.09 (m, 1H), 8.12-8.17 (m,1H), 8.26-8.30 (m, 2H), 8.71-8.75 (m, 1H), 8.87-8.88(m, 1H), 9.02-9.04 (m, 2H)

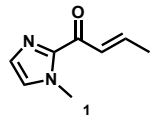
5-(methyl) 2,2´- bipyridine (**S2**)[2] ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.34 (s, 3H), 7.22-7.26 (m, 1H), 7.58 (d, 1H, J=8.1 Hz), 7.74- 7.80 (m, 1H), 8.25 (d, 1H, J=8.1 Hz), 8.32(d, 1H, J=8.1 Hz), 8.47 (s, 1H), 8.63-8.65 (m, 1H);

5-(bromomethyl) 2,2´- bipyridine (**S3**)[2] ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 4.54 (s, 2H), 7.31-7.34 (m, 1H), 7.80-7.87 (m, 2H), 8.40-8.46 (m, 2H), 8.69 (s, 2H).

Diethyl 2-(2,2_-bipyridin-5-ylmethyl)-2-acetamidomalonate (**S4**)[3] ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.30 (t, 6H, J=7.12 Hz), 2.06 (s, 3H), 3.73 (s, 2H), 4.27-4.30 (m, 4H), 6.60 (s, 1H), 7.28-7.31 (m, 1H), 7.47 (dd, 1H, J_1 8.0 Hz, J_2 2.32 Hz), 7.80 (td, 1H, J_1=8.0 Hz, J_2 =2.32 Hz), 8.27-8.34 (m, 3H), 8.66 (m, 1H).

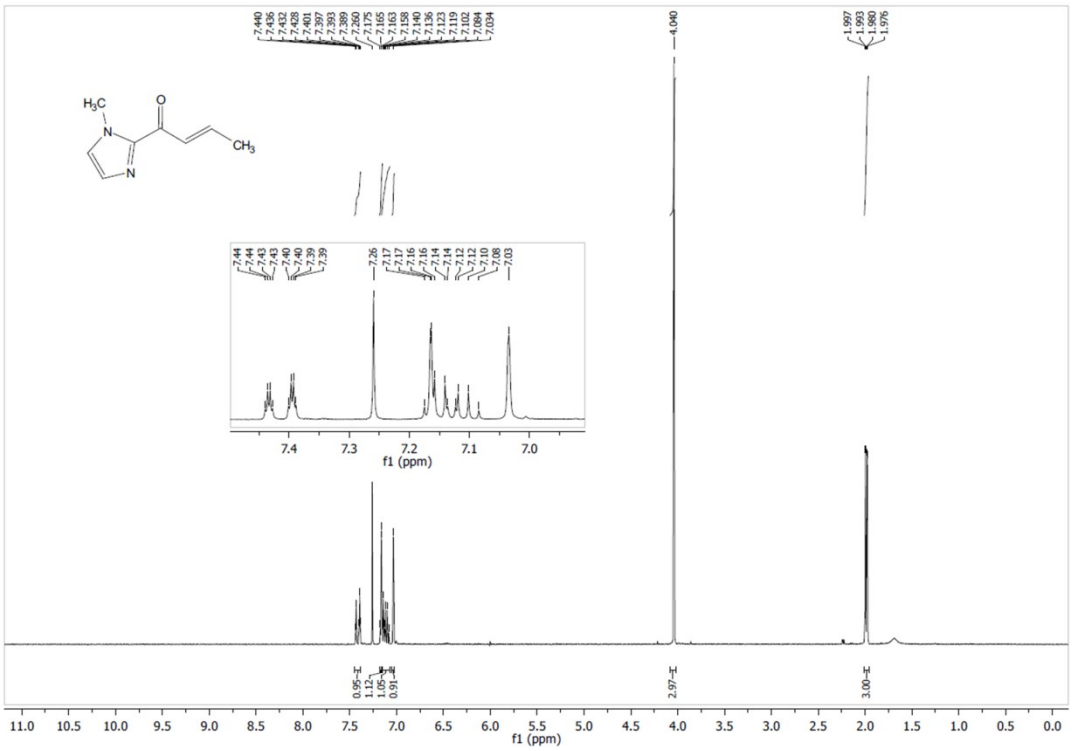
3-(2,2-bipyridin-5-yl)-2-aminopropanoic acid (**S5**)[3] ^{1}H-NMR (D_2O, 400 MHz): δ= 3.31-3.39 (m, 2H), 4.30 (t, 1H, J_1 6.8 Hz), 7.87-7.91 (m, 1H), 8.03 (d, 1H, $3J_2$ 8.2 Hz), 8.19 (d, 1H, $3J_2$ 8.4 Hz), 8.42-8.48 (m, 1H) and 8.46-8.70 (m, 2H).

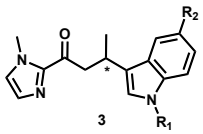




1

(E)-1-(1-Methyl-1H-imidazole-2-yl)-but-2-en-1-one (**1**) was synthesized according to literature procedure with a yield of 24%.[4] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 (m, 1H), 7.12 (m, 1H), 7.09-7.04 (m, 1H), 7.01 (s, 1H), 4.00 (s, 3H), 1.95 (d, J=6.9, 3H).



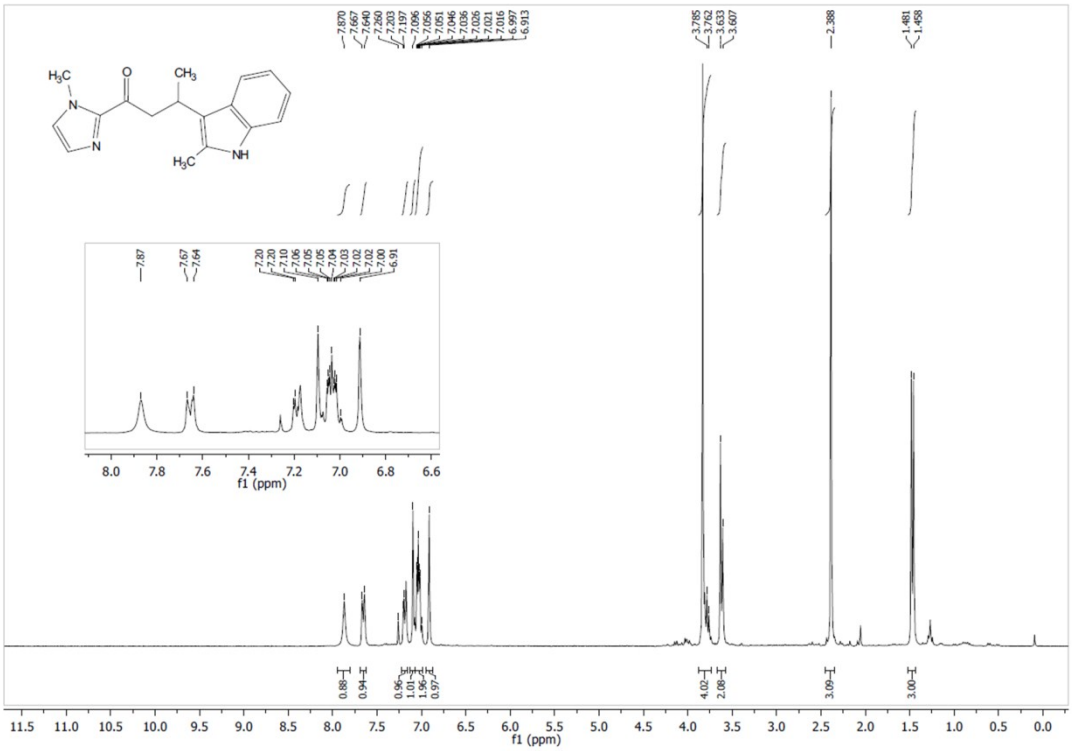


3

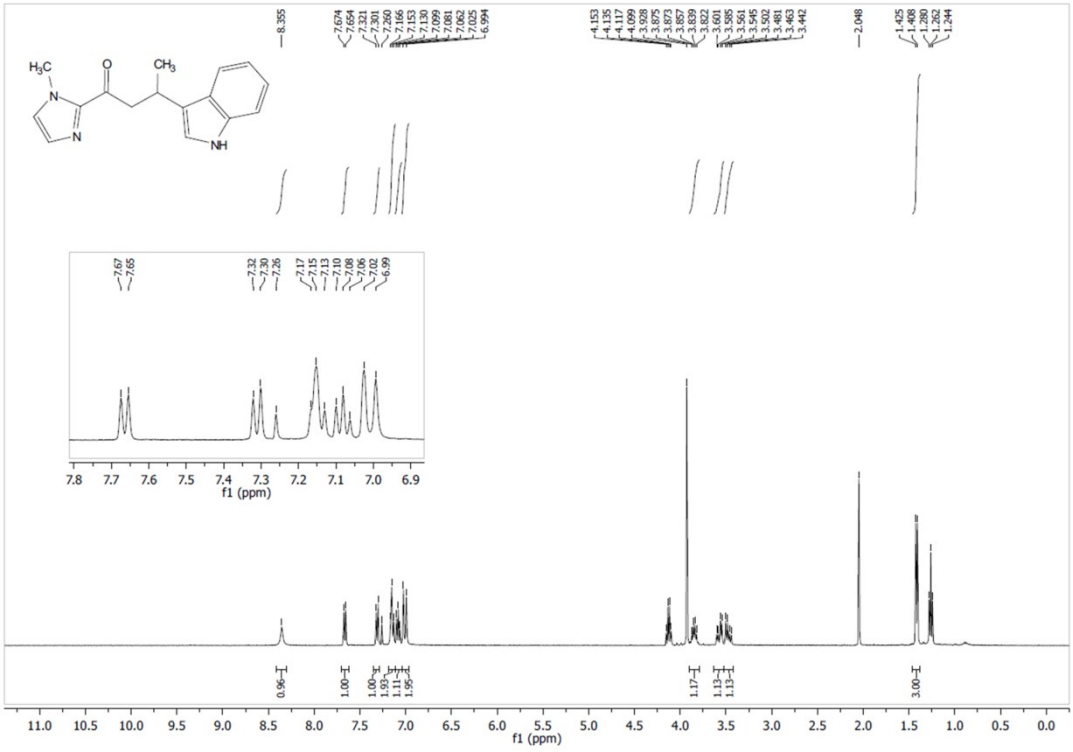
a R_1= H R_2=H
b R_1= Me R_2=H
c R_1= H R_2= OMe
d R_1= H R_2= Cl

General procedure for the preparation of the Friedel-Crafts products as reference material (adapted from literature[5,6]): 50 mg of the corresponding α,β-unsaturated-2-acyl imidazole and 2 equivalents of the corresponding indole, predissolved in 1 mL acetonitrile, were added to 500 mL of water containing 1.2 g sodium dodecyl sulfate (8 mM end concentration) and 15 mol% $Cu(NO_3)_2.3H_2O$.The reaction was stirred at room temperature for 16 hours. 5 g of NaCl was added and the aqueous phase was extracted 3 times with 100 mL diethyl ether. The combined organic phases were washed with 100 mL brine and dried over Na_2SO_4. The product was purified by column chromatography (ethylacetate: heptane 1:1).

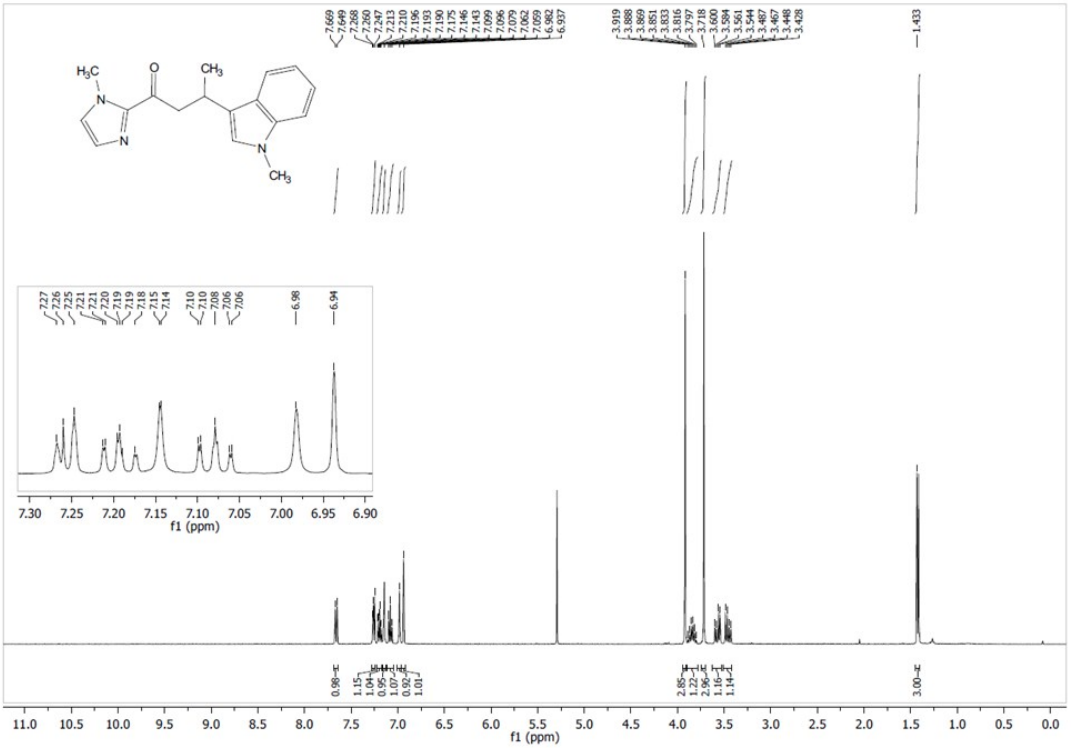
3 ^{1}H NMR (400 MHz, $CDCl_3$): (δ, ppm) 1.47 (d, 3H, J = 9.2 Hz), 2.39 (s, 3H), 3.62 (d, 2H, J=10.4 Hz), 3.76 – 3.78 (m, 1H), 3.7 (s, 3H), 6.91 (s, 1H), 7.00-7.06 (m, 1H), 7.10 (s, 1H), 7.19-7.20 (m, 1H), 7.64-7.67 (m, 1H), 7.72 (s, 1H).



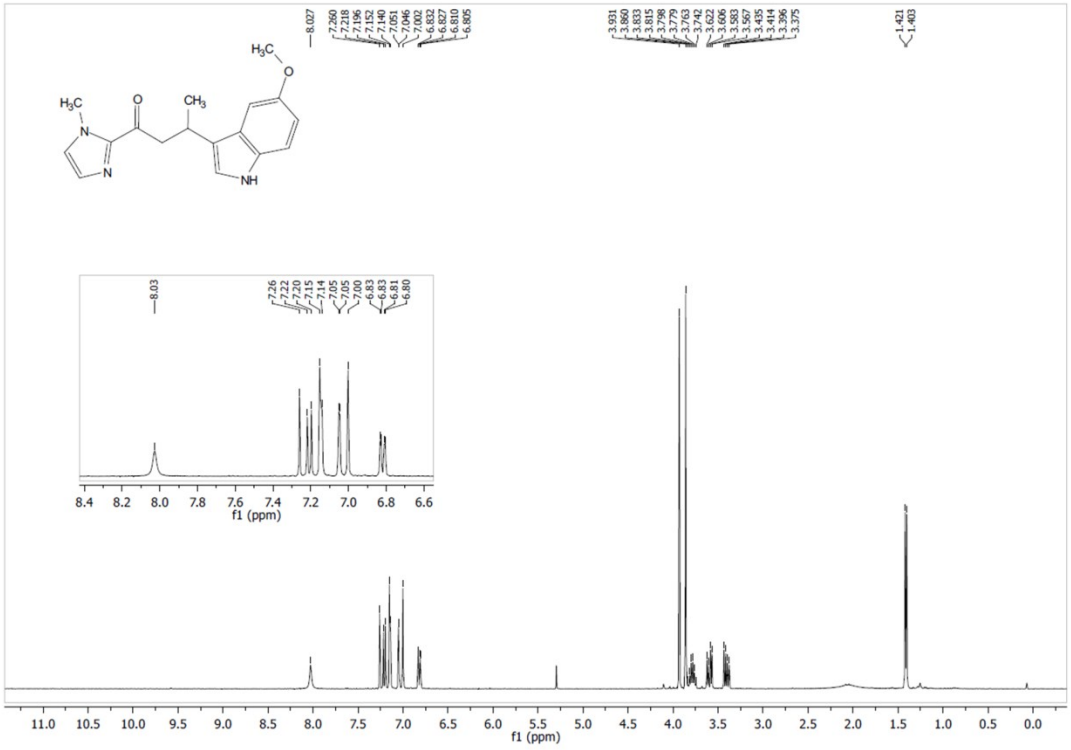
3a (R_1=R_2=H) ^{1}H NMR (400 MHz, $CDCl_3$): (δ, ppm) 1.41 (d, 3H, J = 6.9), 3.4-3.5 (m, 2H), 3.8-3.8 (m, 1H), 3.92 (s, 3H), 7.00 (d, 2H, J = 12.2 Hz), 7.06 (t, 1H, J = 8.0 Hz), 7.1-7.2 (m, 2H), 7.30 (d, 1H, J = 8.0 Hz), 7.65 (d, 1H, J=8.0), 8.34 (s, 1H).



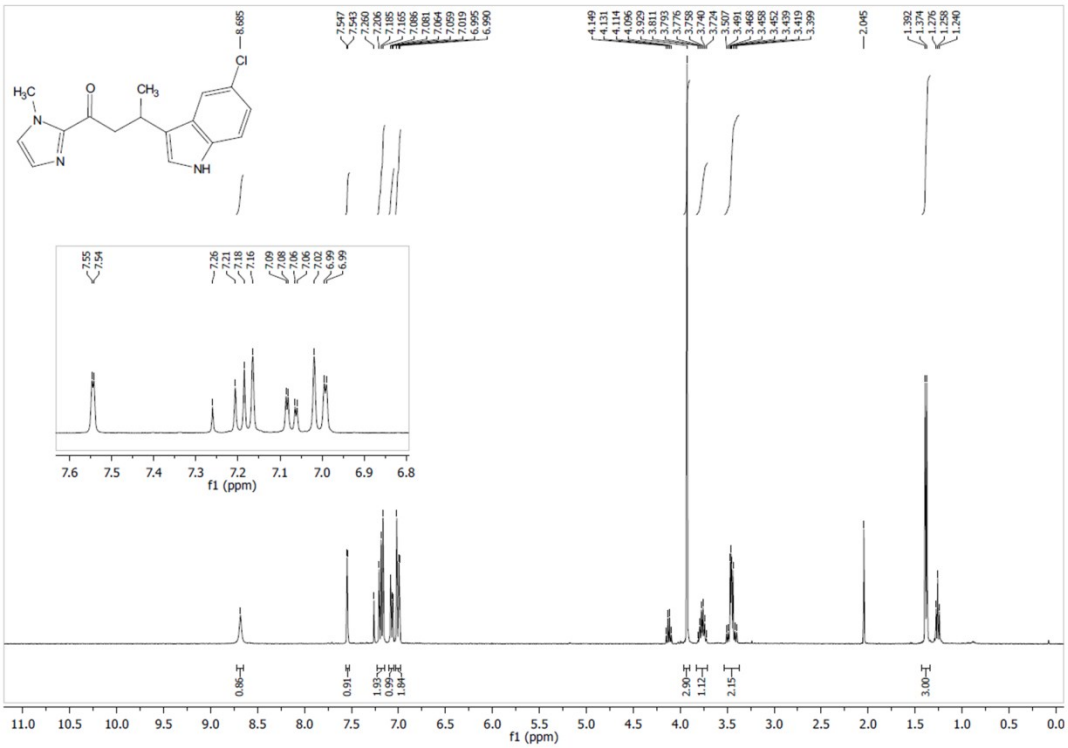
3b (R_1=Me R_2=H) ^{1}H NMR (400 MHz, $CDCl_3$): (δ, ppm) 1.43 (d, 3H, J = 6.9 Hz), 3.45 (dd, 1H, J = 9.2 Hz, J = 15.7 Hz), 3.56 (dd, 1H, J = 6.4, J = 15.7 Hz), 3.72 (s, 3H), 3.81 – 3.86 (m, 1H), 3.92 (s, 3H), 6.94 (s, 1H), 6.98 (s, 1H), 7.06-7.10 (m, 1H), 7.14 (s, 1H), 7.19-7.21 (m, 1H), 7.25-7.27 (m, 1H), 7.65 (d, 1H, J = 8.0).



3c (R_1=H R_2=OMe) ^{1}H NMR (400 MHz, $CDCl_3$): (δ, ppm) 1.41 (d, 3H, J = 6.9), 3.4-3.5 (m, 2H), 3.8-3.8 (m, 1H), 3.92 (s, 3H), 7.00 (d, 2H, J = 12.2 Hz), 7.06 (t, 1H, J = 8.0 Hz), 7.1-7.2 (m, 2H), 7.30 (d, 1H, J = 8.0 Hz), 7.65 (d, 1H, J=8.0), 8.34 (s, 1H).



3d (R_1=H R_2=Cl) ^{1}H NMR (400 MHz, $CDCl_3$): (δ, ppm) 1.38 (d, 3H, J =6.9), 3.39-3.51 (m, 2H), 3.72-3.81 (m, 1H), 3.93 (s, 3H), 7.00 (dd, 2H, J = 9.4 Hz, J = 2.0 Hz), 7.00 (dd, 1H, J = 8.3 Hz, J = 2.0 Hz), 7.1-7.2 (m, 2H), 7.53 (d, 1H, J= 2.0 Hz), 8.67 (s, 1H).



3. Molecular biology

Gene sequences

RamR, Codon optimized: Host expression organism: Escherichia coli, Length: 624 bp,
CATATGGTGGCGCGTCCGAAGAGCGAGGACAAGAAACAAGCGCTGCTGGAAGCGGCGACCCAGGCGATTGCGCAAAGCGGTATTGCGGCGAGCAC
CGCGGTGATTGCGCGTAACGCGGGTGTTGCGGAGGGTACCCTGTTCCGTTACTTTGCGACCAAGGACGAACTGATTAACACCCTGTATCTGCACCTGA
AACAGGATCTGAGCCAAAGCATGATCATGGAGCTGGACCGTAGCATTACCGATGCGAAAATGATGACCCGTTTCATCTGGAACAGCTACATTAGCTGG
GGCCTGAACCATCCGGCGCGTCACCGTGCGATCCGTCAGCTGGCGGTTAGCGAGAAGCTGACCAAAGAAACCGAACAACGTGCGGACGATATGTTCC
CGGAACTGCGTGATCTGAGCCACCGTAGCGTGCTGATGGTTTTTATGAGCGACGAGTACCGTGCGTTCGGTGATGGCCTGTTTCTGGCGCTGGCGGAA
ACCACCATGGATTTTGCGGCGCGTGATCCGGCGCGTGCGGGCGAGTATATTGCGCTGGGCTTTGAAGCGATGTGGCGTGCGCTGACCCGTGAGGAAC
AGGCGGCGTGGAGCCACCCGCAATTTGAAAAGTAACTCGAG,

QacR, Codon optimized: Host expression organism: Escherichia coli, Length: 606 bp,
CATATGAACCTGAAGGACAAAATCCTGGGTGTGGCGAAGGAGCTGTTCATTAAAAACGGTTATAACGCGACCACCACCGGCGAGATCGTTAAGCTGA
GCGAAAGCAGCAAAGGCAACCTGTACTATCACTTCAAGACCAAAGAGAACCTGTTTCTGGAAATCCTGAACATTGAGGAAAGCAAGTGGCAGGAGCA
ATGGAAAAGCGAACAGATTAAGTGCAAAACCAACCGTGAGAAGTTCTATCTGTACAACGAACTGAGCCTGACCACCCAGTACTATTACCCGCTGCAAA
ACGCGATCATCGAGTTCTACACCGAATACTACAAGACCAACAGCATCAACGAGAAGATGAACAAACTGGAAAACAAGTATATCGACGCGTACCACGTG
ATTTTCAAAGAGGGTAACCTGAACGGCGAATGGTGCATTAACGATGTGAACGCGGTTAGCAAGATCGCGGCGAACGCGGTGAACGGTATTGTTACCTT
TACCCACGAGCAGAACATCAACGAACGTATTAAGCTGATGAACAAATTCAGCCAAATCTTTCTGAACGGCCTGAGCAAGGCGGCGTGGAGCCACCCGC
AATTTGAAAAATAACTCGAG

CgmR, Codon optimized: Host expression organism: Escherichia coli, Length: 573 bp,
CATATGCGTACCAGCAAGAAAGAGATGATCCTGCGTACCGCGATCGACTACATTGGCGAGTATAGCCTGGAAACCCTGAGCTACGATAGCCTGGCGGA
AGCGACCGGTCTGAGCAAGAGCGGCCTGATCTATCACTTTCCGAGCCGTCATGCGCTGCTGCTGGGTATGCACGAGCTGCTGGCGGACGATTGGGACA
AAGAACTGCGTGATATTACCCGTGACCCGGAGGATCCGCTGGAACGTCTGCGTGCGGTGGTTGTGACCCTGGCGGAGAACGTTAGCCGTCCGGAACT
GCTGCTGCTGATTGATGCGCCGAGCCACCCGGATTTCCTGAACGCGTGGCGTACCGTGAACCACCAGTGGATTCCGGACACCGACGATCTGGAGAACG
ATGCGCACAAGCGTGCGGTTTACCTGGTGCAACTGGCGGCGGACGGTCTGTTTGTTCACGATTATATTCACGACGATGTGCTGAGCAAGAGCAAACGT
CAGGCGATGCTGGAAACCATCCTGGAACTGATTCCGAGCGAGCTGGCGGCGTGGAGCCACCCGCAATTCGAAAAATAACTCGAG

Site-directed mutagenesis

pET 17b plasmids encoding for wt-QacR , wt-CgmR and wt-RamR were purchased from Genescript (USA) as codon optimized sequences for *E.Coli* expression and included a C-terminal Strep-tag for purification purposes. Site directed mutagenesis (Quick Change) was performed to remove cysteines from the QacR gene (C72A and C141S) and to introduce the TAG codon for all the mutants prepared in this work. The primers used for the mutagenesis are listed in Table S1. Standard Pfu Turbo DNA polymerase (Agilent technologies) protocol was used with an initial denaturation at 95°C for 1 min. The following cycle was repeated 16 times: denaturation at 95°C for 30 s, annealing at 52°C for 1 min, elongation at 72°C for 4 minutes. Final elongation was performed at 72°C for 10 min. The resulting PCR product was digested with restriction endonuclease DpnI for 1 h at 37°C and 5 µL of the mixture were directly transformed into the *E.Coli* NEB5α. Cells were spread onto an agar plate containing 100 µg/mL of ampicillin. Single colonies were selected after overnight growth and used to inoculate 5 mL LB medium containing the same antibiotic. Plasmids were isolated using a plasmid purification kit (Qiagen) and the sequence was confirmed by Sanger sequencing (GATC Biotech, T7 sequencing primers).

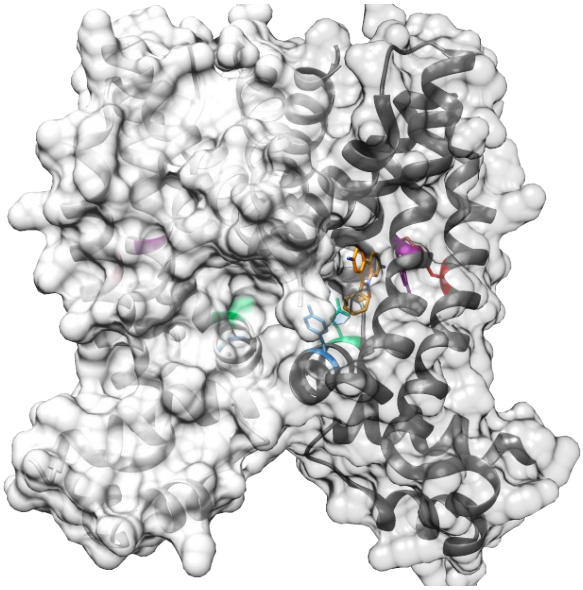
Table S1 Primers used for site directed mutagenesis (fw: forward primer, rv: reverse primer). Single point mutations in bold.

Primer	Sequence (5' → 3')
QacR C72A_fw	GAA CAG ATT AAG **GCG** AAA ACC AAC CGT
QacR C72A_rv	ACG GTT GGT TTT **CGC** CTT AAT CTG TTC
QacR C141S_fw	AAC GGC GAA TGG **TCT** ATT AAC GAT GTG
QacR C141S_rv	CAC ATC GTT AAT **AGA** CCA TTC GCC GTT
QacR W61X_fw	GAG GAA AGC AAG **TAG** CAG GAG CAA TGG
QacR W61X_rv	CCA TTG CTC CTG **CTA** CTT GCT TTC CTC
QacR Q96X_fw	TAT TAC CCG CTG **TAG** AAC GCG ATC ATC
QacR Q96X _rv	GAT GAT CGC GTT **CTA** CAG CGG GTA ATA
QacR Y103X_fw	ATC ATC GAG TTC **TAG** ACC GAA TAC TAC
QacR Y103X_rv	GTA GTA TTC GGT **CTA** GAA CTC GAT GAT
QacR Y123X_fw	CTG GAA AAC AAG **TAG** ATC GAC GCG TAC
QacR Y123X_rv	GTA CGC GTC GAT **CTA** CTT GTT TTC CAG
CgmR W63X_fw	CTT GCA GAT GAT **TAG** GAC AAA GAA CTT
CgmR W63X_rv	CAG TTC TTT GTC **CTA** ATC GTC CGC CAG
CgmR L100X_fw	CCG GAA CTG CTG CTG **TAG** ATT GAT GCG CCG AG C
CgmR L100X_rv	GCT CGG CGC ATC AAT **CTA** CAG CAG CAG TTC CGG
CgmR W113X_fw	TTC CTG AAC GCG **TAG** CGT ACC GTG AAC
CgmR W113X_rv	GTT CAC GGT ACG **CTA** CGC GTT CAG GAA
CgmR F147X_fw	GCG GAC GGT CTG **TAG** GTT CAC GAT TAT
CgmR F147X_rv	ATA ATC GTG AAC **CTA** CAG ACC GTC CGC
RamR Y59X_fw	ATT AAC ACC CTG **TAG** CTG CAC CTG AAA
RamR Y59X_rv	TTT CAG GTG CAG **CTA** CAG GGT GTT AAT
RamR W89X_fw	ACC CGT TTC ATC **TAG** AAC AGC TAC ATT
RamR W89X_rv	AAT GTA GCT GTT **CTA** GAT GAA ACG GGT
RamR Y92X_fw	ATC TGG AAC AGC **TAG** ATT AGC TGG GGC
RamR Y92X_rv	GCC CCA GCT AAT **CTA** GCT GTT CCA GAT
RamR Y155X_fw	GAT GGC CTG **TAG** CTG GCG CTG
RamR Y155X_rv	CAG CGC CAG **CTA** CAG GCC ATC

4. Surface representation of QacR, CgmR and RamR binding pockets

Figure S1: a) Surface representation of QacR with ethidium bromide bound within the hydrophobic pocket (PDB 3PM1). Positions selected for the incorporation of the unnatural amino acid are highlighted: Y103 (light blue), Q96 (green), Y123 (purple), W61 (red). b) Zoom in of the hydrophobic pockets, front and back view

a)



b)

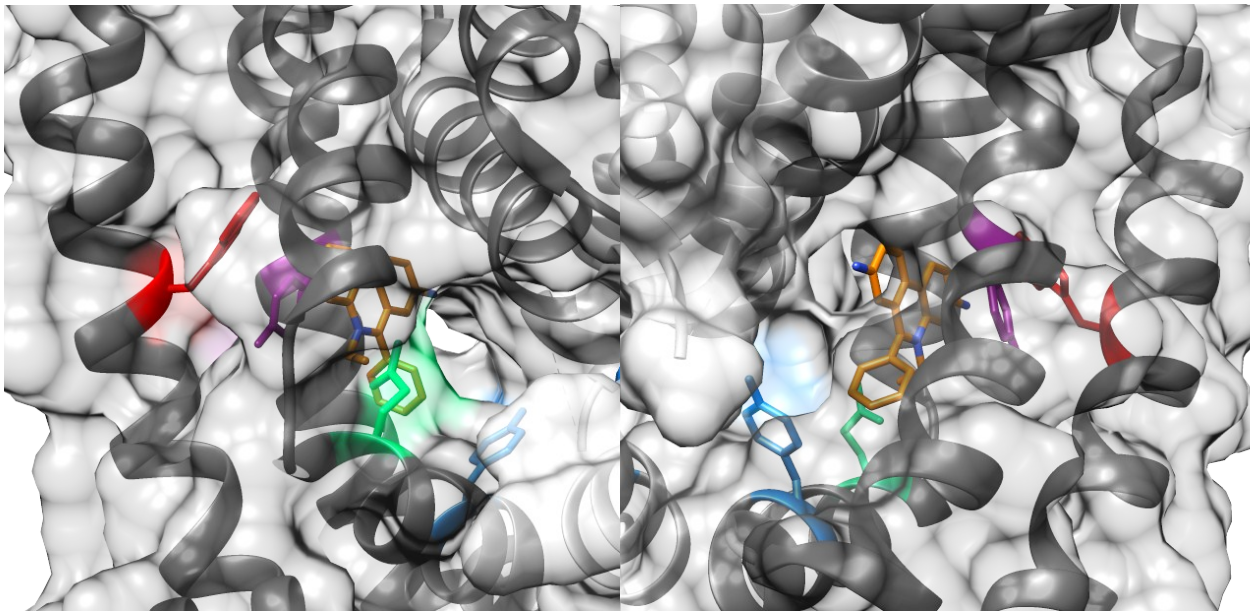
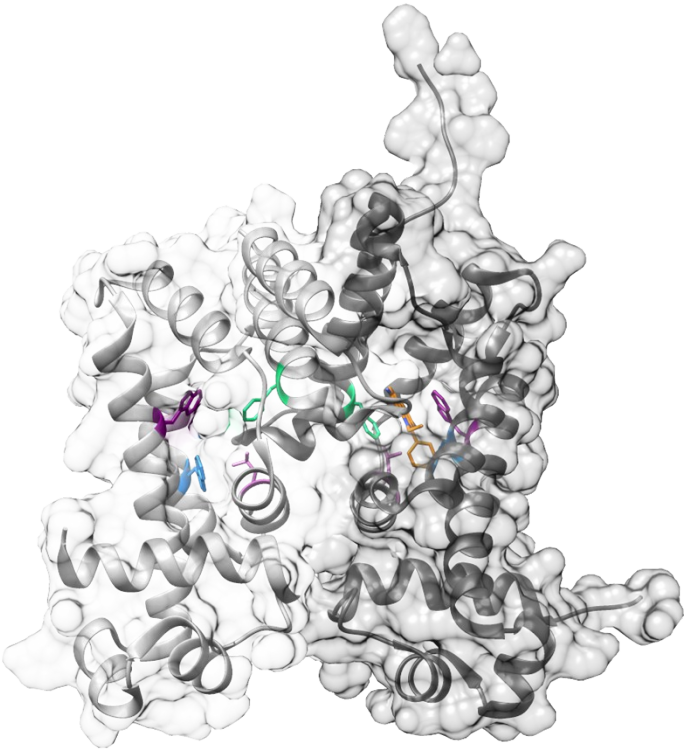


Figure S2: a) Surface representation of CgmR with ethidium bromide bound within the hydrophobic pocket (PDB 2ZOZ). Positions selected for the incorporation of the unnatural amino acid are highlighted: W63 (light blue), L100 (pink), W113 (purple), F147 (green). b) Zoom in of the hydrophobic pockets, front and back view

a)



b)

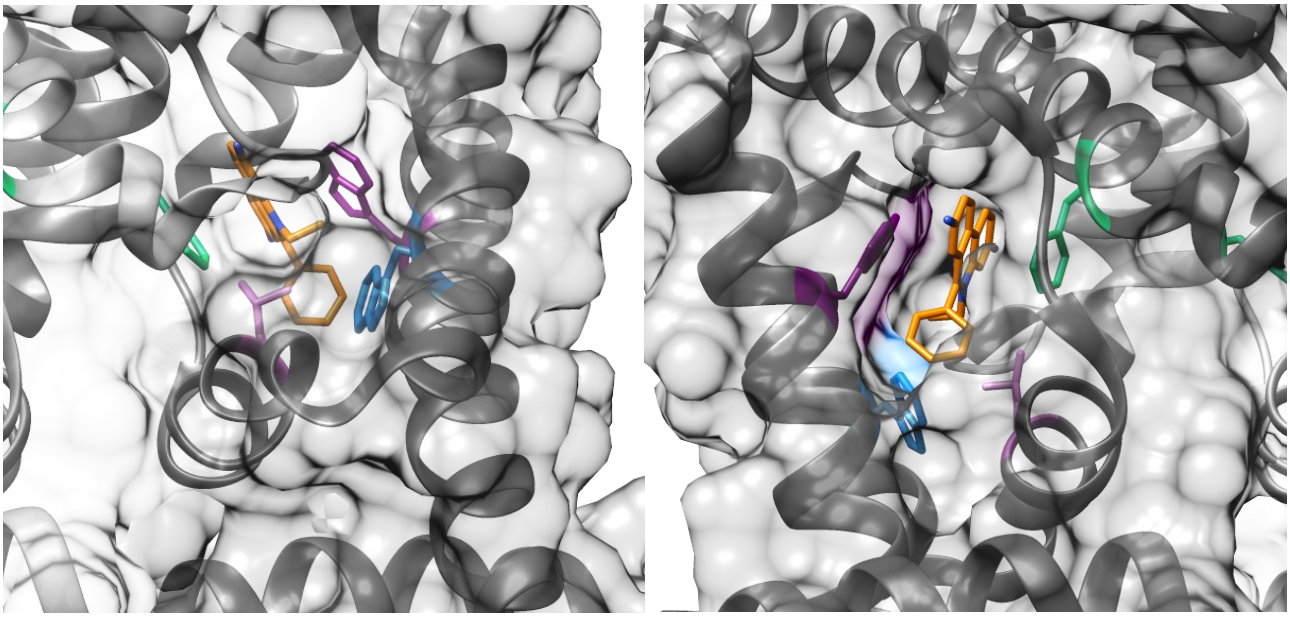
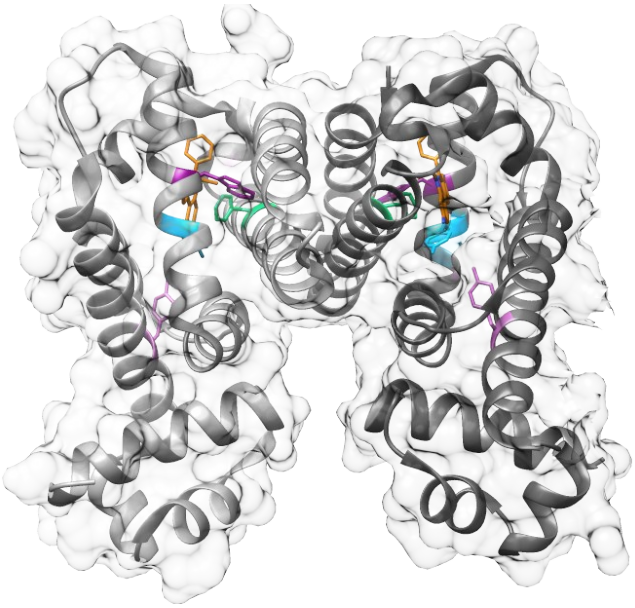


Figure S3: a) Surface representation of RamR with ethidium bromide bound within the hydrophobic pocket (PDB 3VVY). Positions selected for the incorporation of the unnatural amino acid are highlighted: Y59 (pink), W89 (purple), Y92 (light blue), F155 (green). b) Zoom in of the hydrophobic pockets, front and back view

a)



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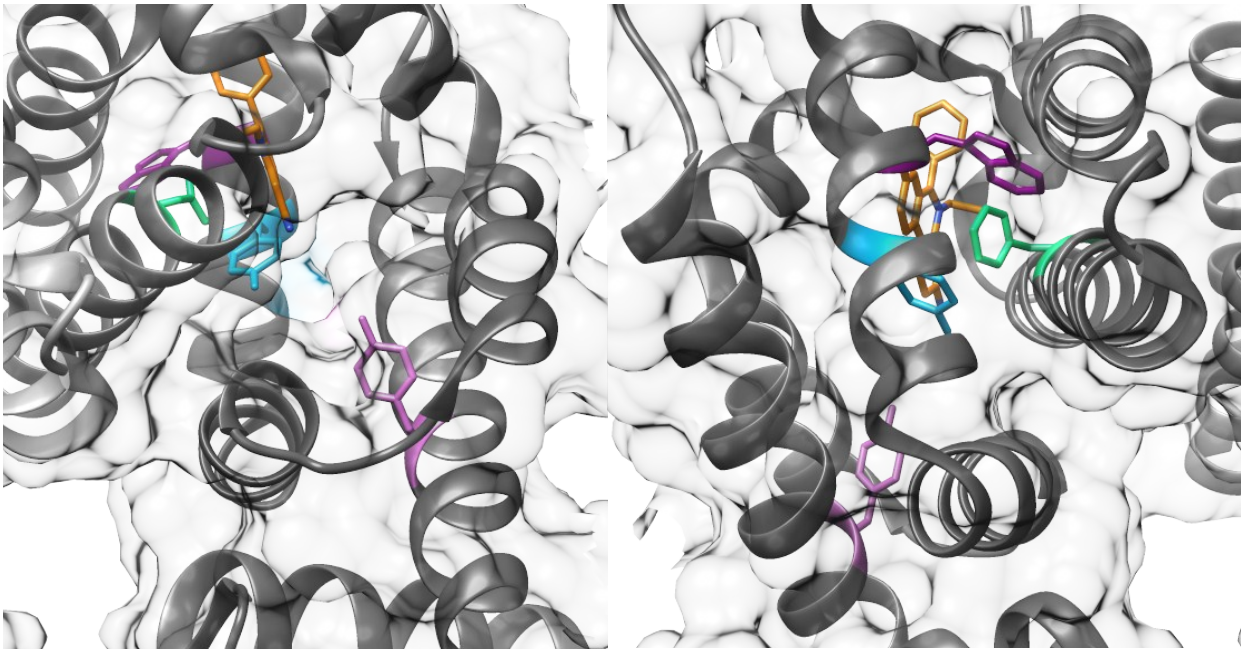
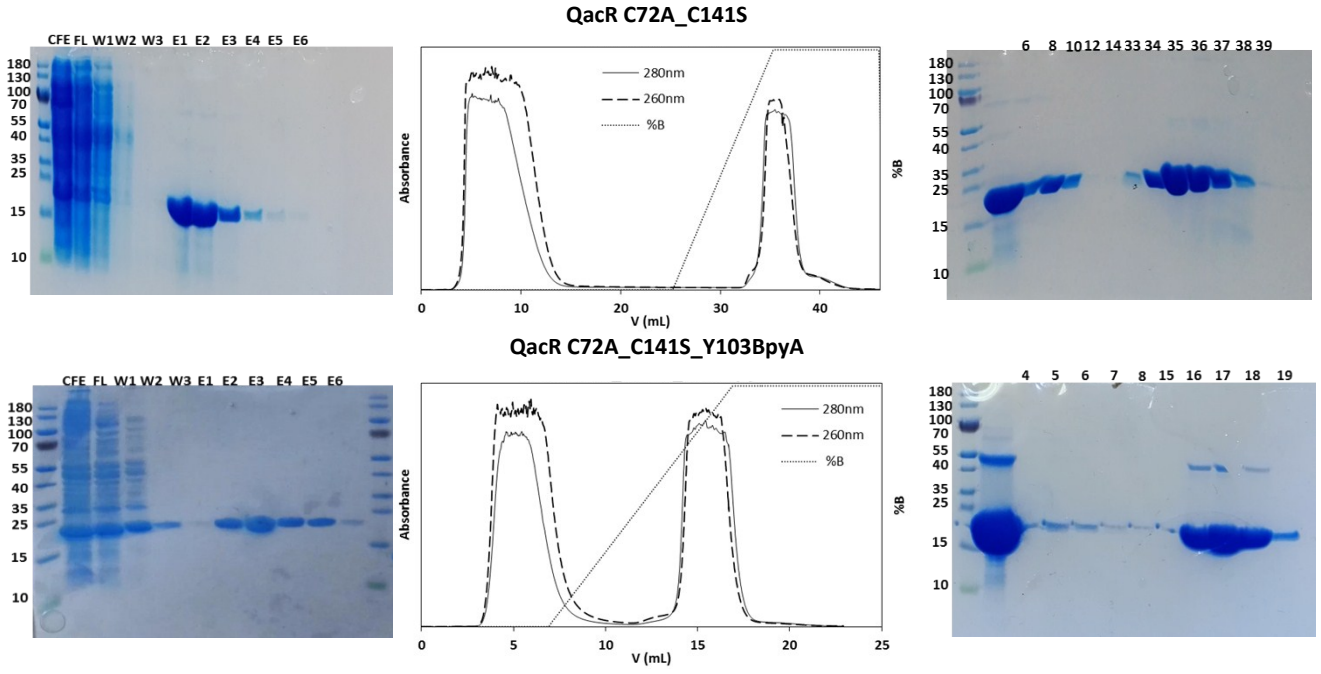


5. Expression and purification

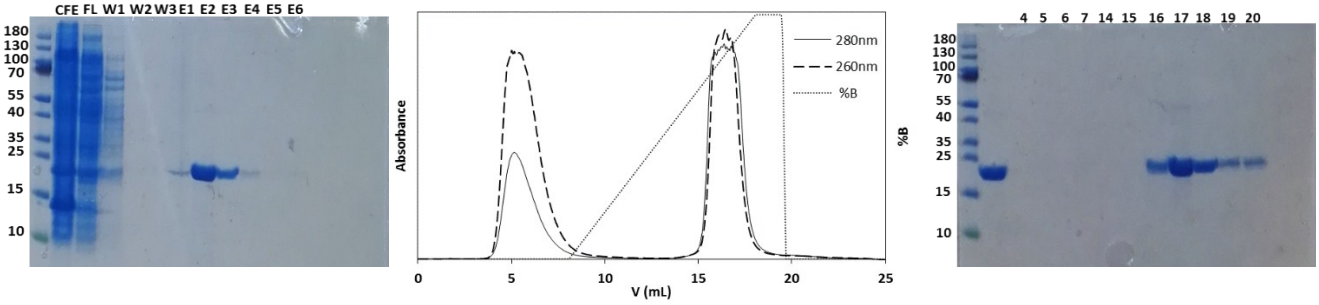
pET17b plasmids encoding for wt-QacR, wt-CgmR and wt-RamR were transformed into E. coli BL21 (DE3) C43 which were spread onto an agar plate containing 100 µg/mL of ampicillin. Single colonies were selected after overnight growth and used to inoculate 5 mL LB medium containing the same antibiotic. This starter culture was grown at 37 °C overnight and used to inoculate 500 mL fresh LB medium with the same antibiotic. The culture was grown at 37 °C until OD^{600} = 0.8 (approximately 3 h) and then isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentration of 1 mM was added to induce the expression of target proteins. pET17b plasmids encoding for QacR, CgmR and RamR mutants were co-transformed with pEVOL BpyA into BL21 (DE3) C43 were spread onto an agar plate containing 100 µg/mL of ampicillin and 34 µg/mL chloramphenicol. Single colonies were selected after overnight growth and used to inoculate 5 mL LB medium containing the same antibiotics. This starter culture was grown at 37 °C overnight and used to inoculate 500 mL fresh LB medium with the same antibiotics. The culture was grown at 37 °C and when OD^{600} reached 0.8. BpyA (final concentration of 0.5 mM), L-arabinose (1 mM) and IPTG (final concentration of 1 mM) were added to induce the expression of target proteins. Protein expression was performed at 30 °C overnight. Cells were harvested by centrifugation (6000 rpm, JA-10, 20 min, 4 °C, Beckman) and the pellet was resuspended in 50 mM NaH_2PO_4, pH 8.0, 150 mM NaCl and protease inhibitor cocktail (Complete, Roche, 1 tablet, for 50 mL of resuspension buffer). Cells were sonicated (70% (200W) for 10 min (10 sec on, 15 sec off) after which PMSF solution (final concentration 0.1 mM) and DNaseI (0.1 mg/mL, containing 10 mM $MgCl_2$) were added. The cell free extract obtained after centrifugation (10000 rpm, 45 min, 4 °C, Eppendorf) was loaded onto columns containing 3 mL of pre-equilibrated slurry of Strep-tag Tactin column material (50% high capacity Strep-tag Tactin in storage buffer) for 1 h at 4 °C (mixed at 200 rpm on a rotary shaker). Columns were washed three times with 1 CV (column volume) of resuspension buffer and eluted with seven fractions of 0.5 CV of resuspension buffer containing 5 mM desthiobiotin. Elution fractions were analyzed on a 12% polyacrylamide SDS-Tris Tricine gel followed by Coumassie staining (InstantBlue, Expedeon). Fractions containing protein were pulled concentrated using Vivaspin Turbo (5000 MWCO, Sartorius) centrifugation filters. When A^{260}/A^{280} was between 0.9-1.0, cation exchange chromatography was performed on a Hitrap Heparin HP column by a gradient of NaCl concentration from 0 to 1 M in 5 min with a flow of 1 mL min^{-1} (heparin column not necessary for wt-LmrR_LM). Elution fractions were analyzed on a 12% polyacrylamide Tricine-SDS-PAGE, followed by Coumassie staining (InstantBlue, Expedeon). Fractions containing protein were pooled and concentrated using ultracentrifugation filters (Vivaspin turbo 15, Sartorius). Concentration of the proteins was measured on a Nanodrop 2000 (Thermo Scientific), using the calculated extinction coefficient for monomer (Protparam, Expasy server). Typical expression yields were between 15 and 30 mg/L.

6. SDS-PAGE

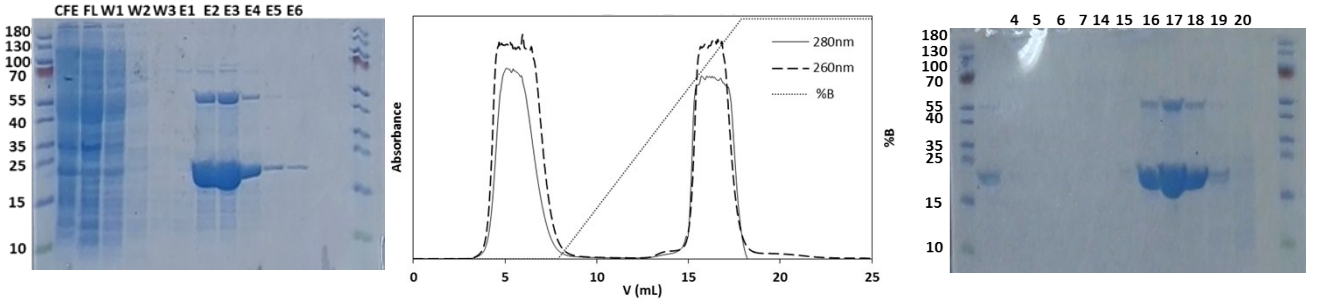
Figure S4: 12% Tricine SDS-PAGE after Strep-Tag purification, heparin purification chromatography trace, Tricine SDS-PAGE after heparin purification (when applicable). *CFE: Cell Free Extract; FL: flow-through column; W: wash fraction; E: elution fraction*



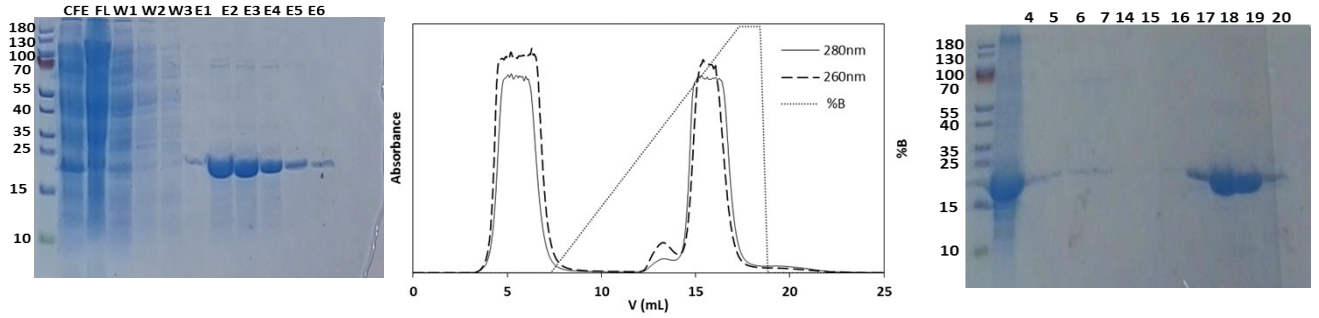
QacR C72A_C141S_Y123BpyA



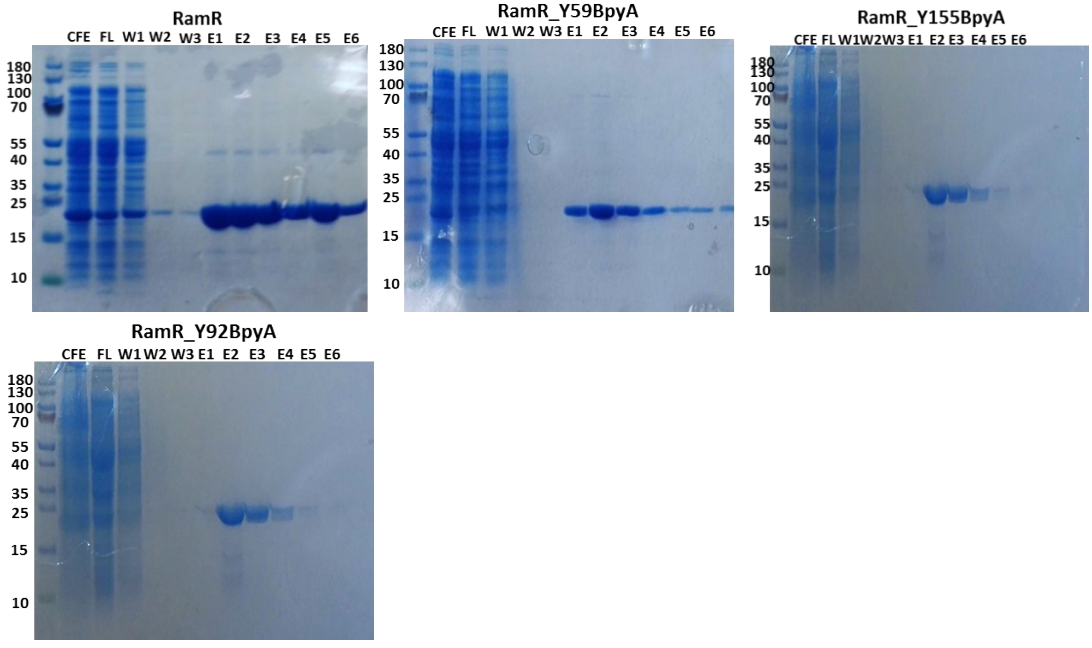
QacR C72A_C141S_Q96BpyA



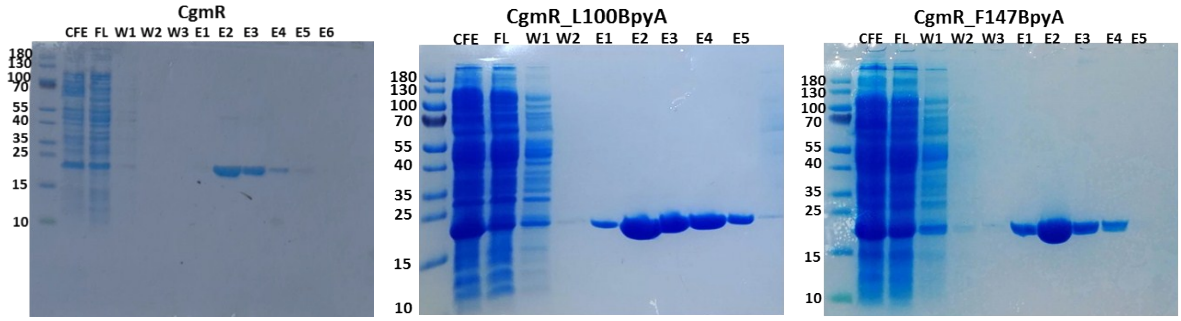
QacR C72A_C141S_W61BpyA



RamR mutants



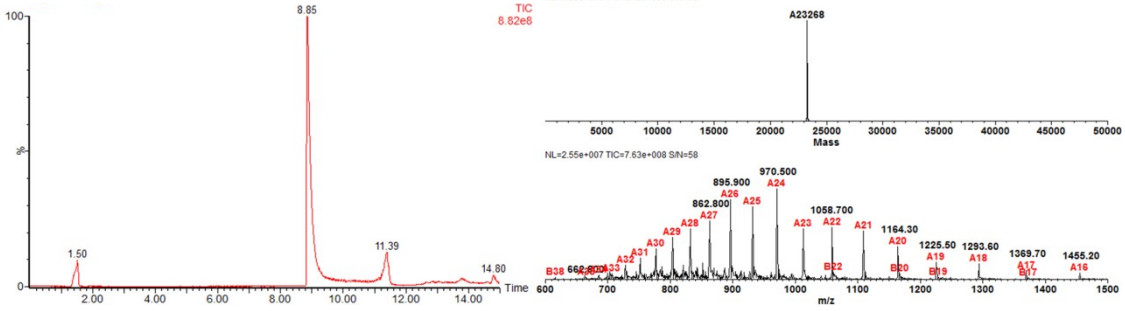
CgmR mutants



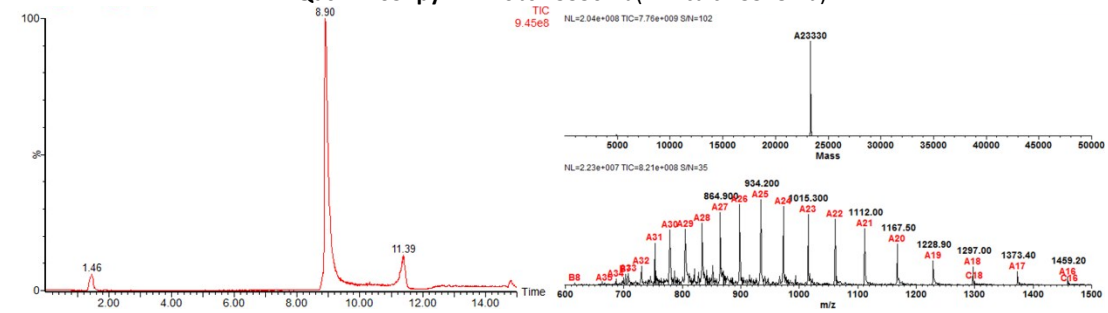
7. UPLC-MS chromatograms and ESI(+) mass traces

Figure S5: UPLC-MS chromatograms and ESI(+) mass traces

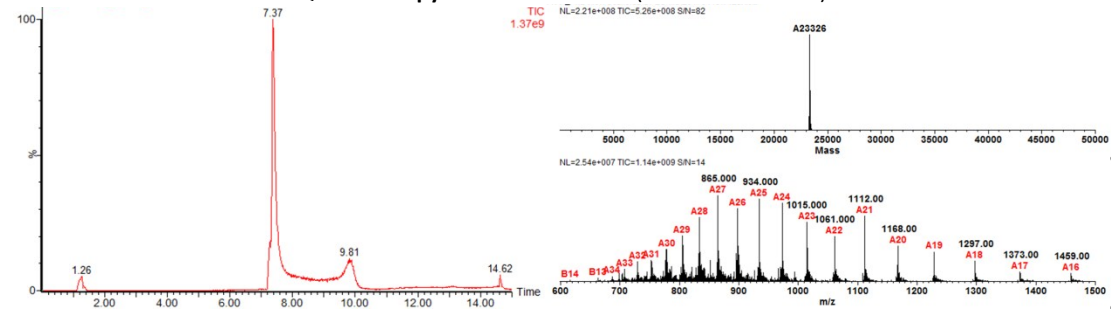
wt QacR (C72A, C141S) MWobs 23268 Da(MWcalc 23266 Da)



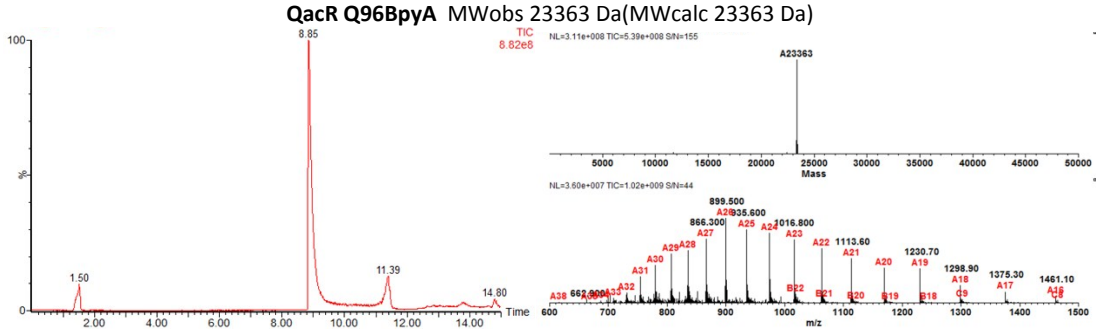
QacR Y103BpyA MWobs 23330 Da(MWcalc 23328 Da)



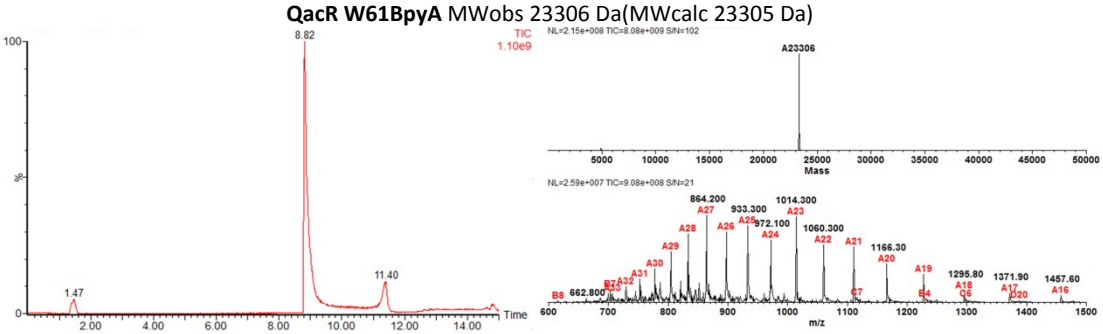
QacR Y123BpyA MWobs 23326 Da(MWcalc 23328 Da)



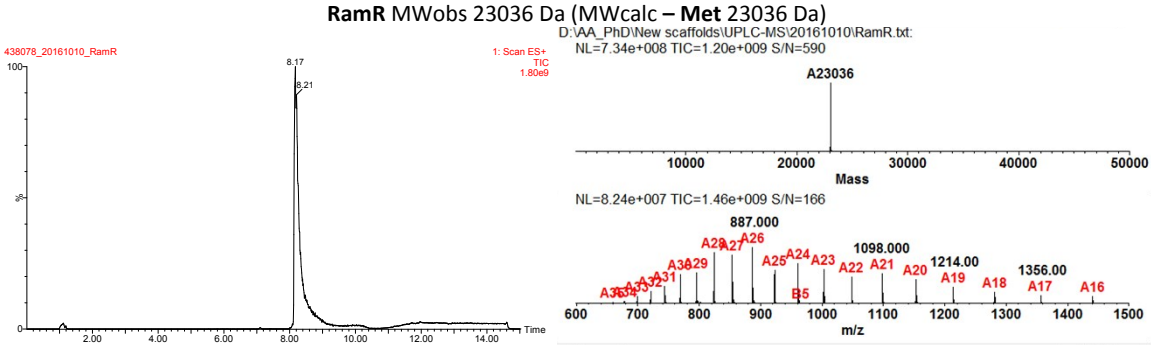
QacR Q96BpyA MWobs 23363 Da(MWcalc 23363 Da)



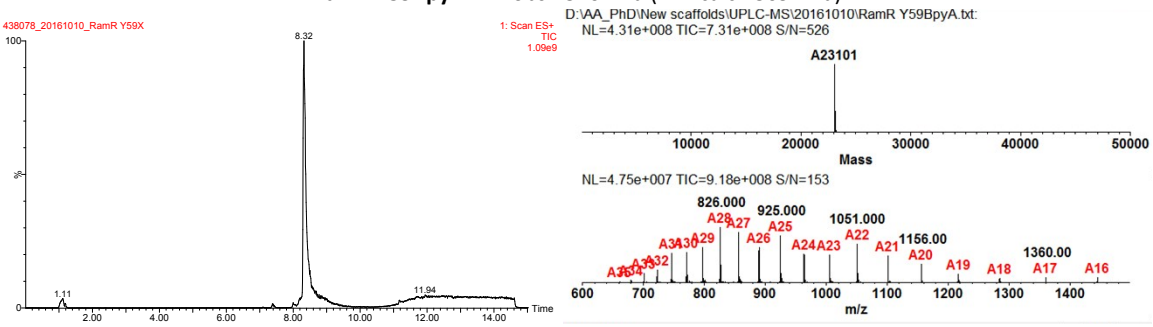
QacR W61BpyA MWobs 23306 Da(MWcalc 23305 Da)



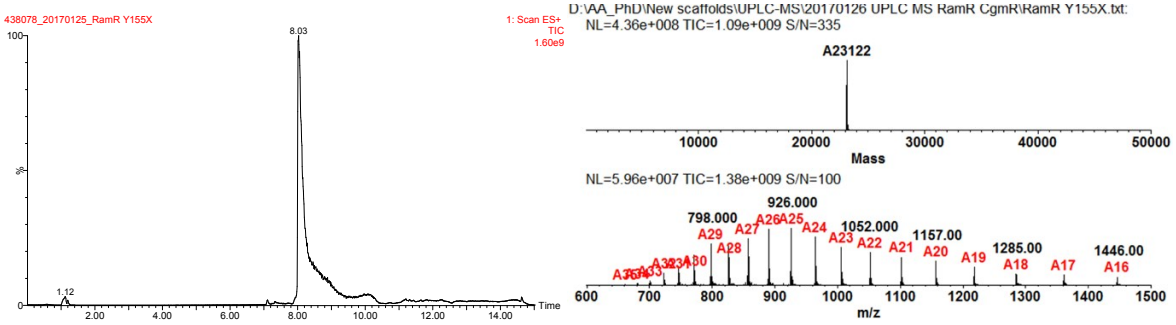
RamR MWobs 23036 Da (MWcalc – **Met** 23036 Da)



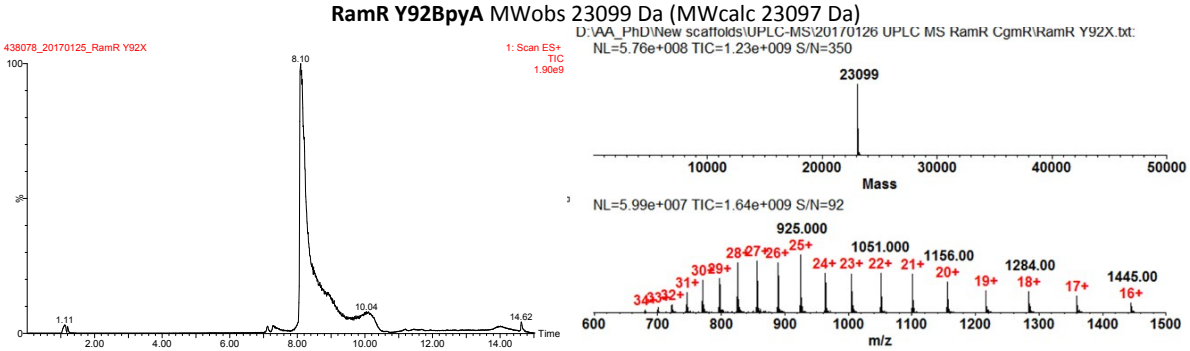
RamR Y59BpyA MWobs 23101 Da (MWcalc 23097 Da)



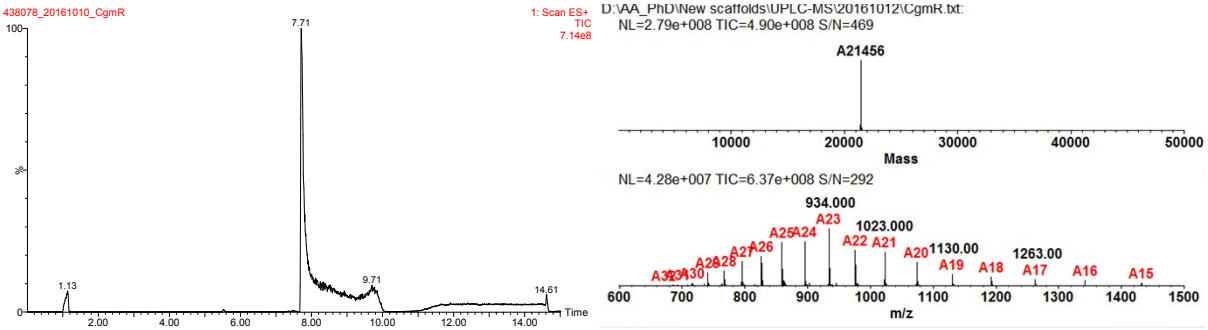
RamR F155BpyA MWobs 23122 Da (MWcalc 23113 Da)



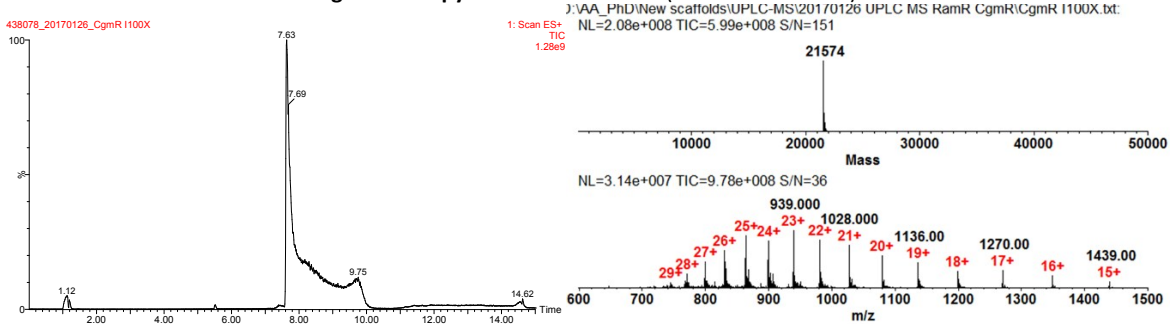
RamR Y92BpyA MWobs 23099 Da (MWcalc 23097 Da)



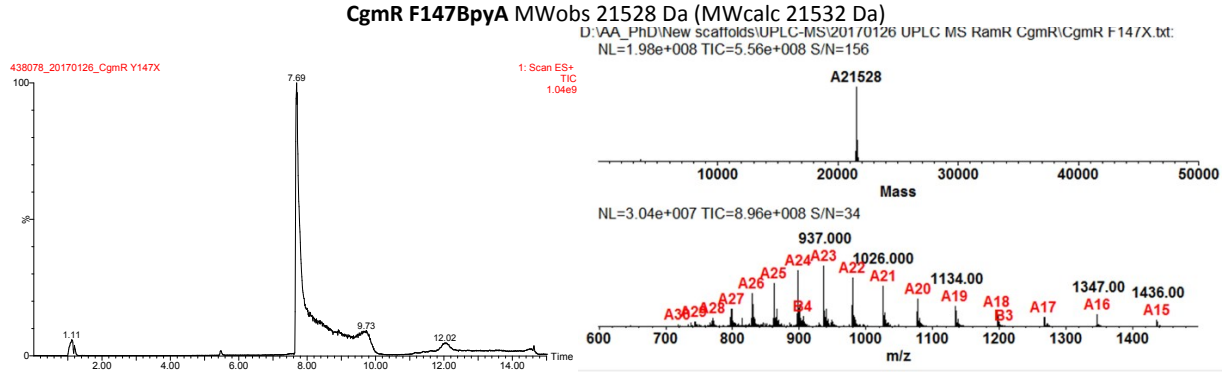
CgmR MWobs 21456 Da (MWcalc 21455 Da)



CgmR L100BpyA MWobs 21574 Da (MWcalc 21566 Da)



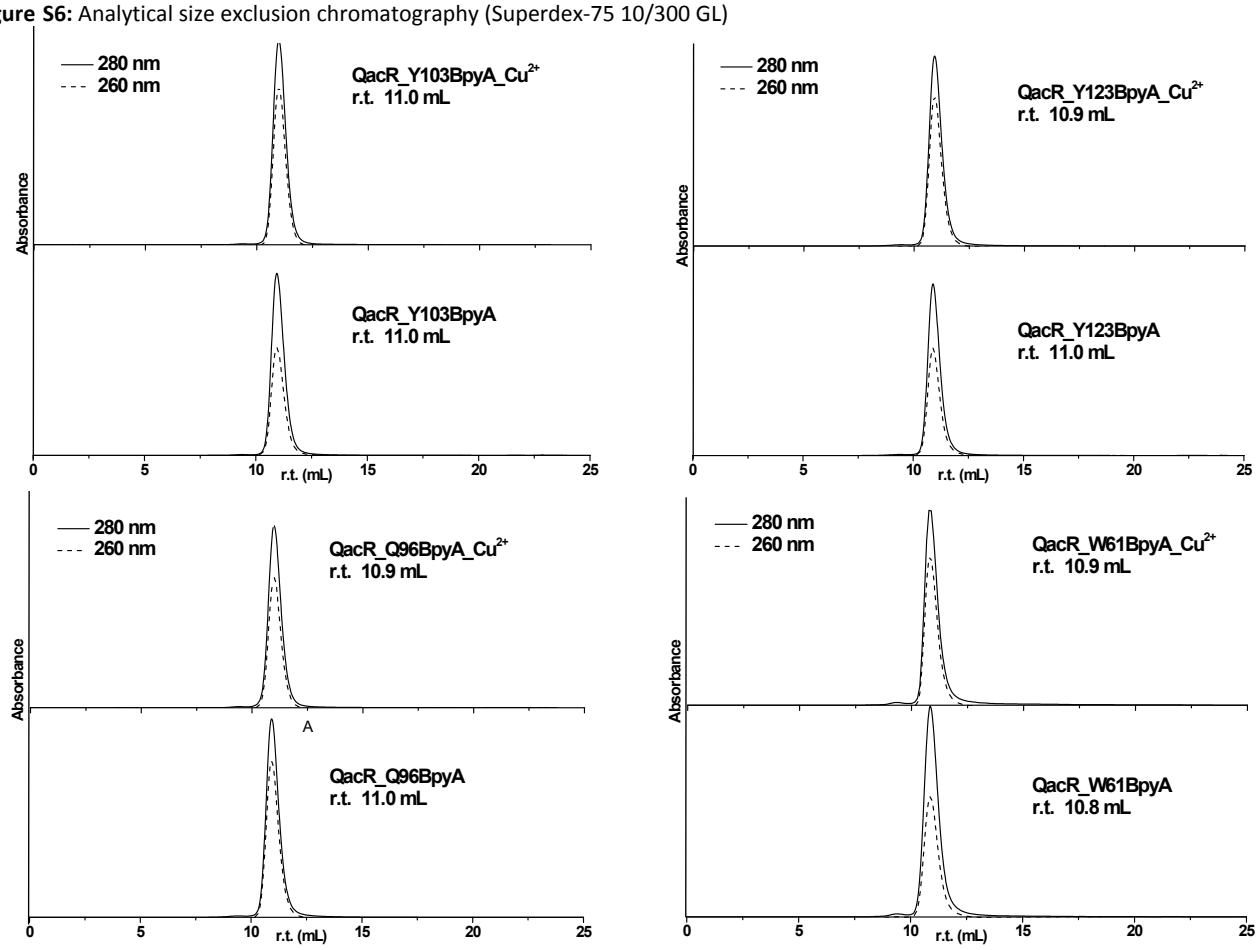
CgmR F147BpyA MWobs 21528 Da (MWcalc 21532 Da)

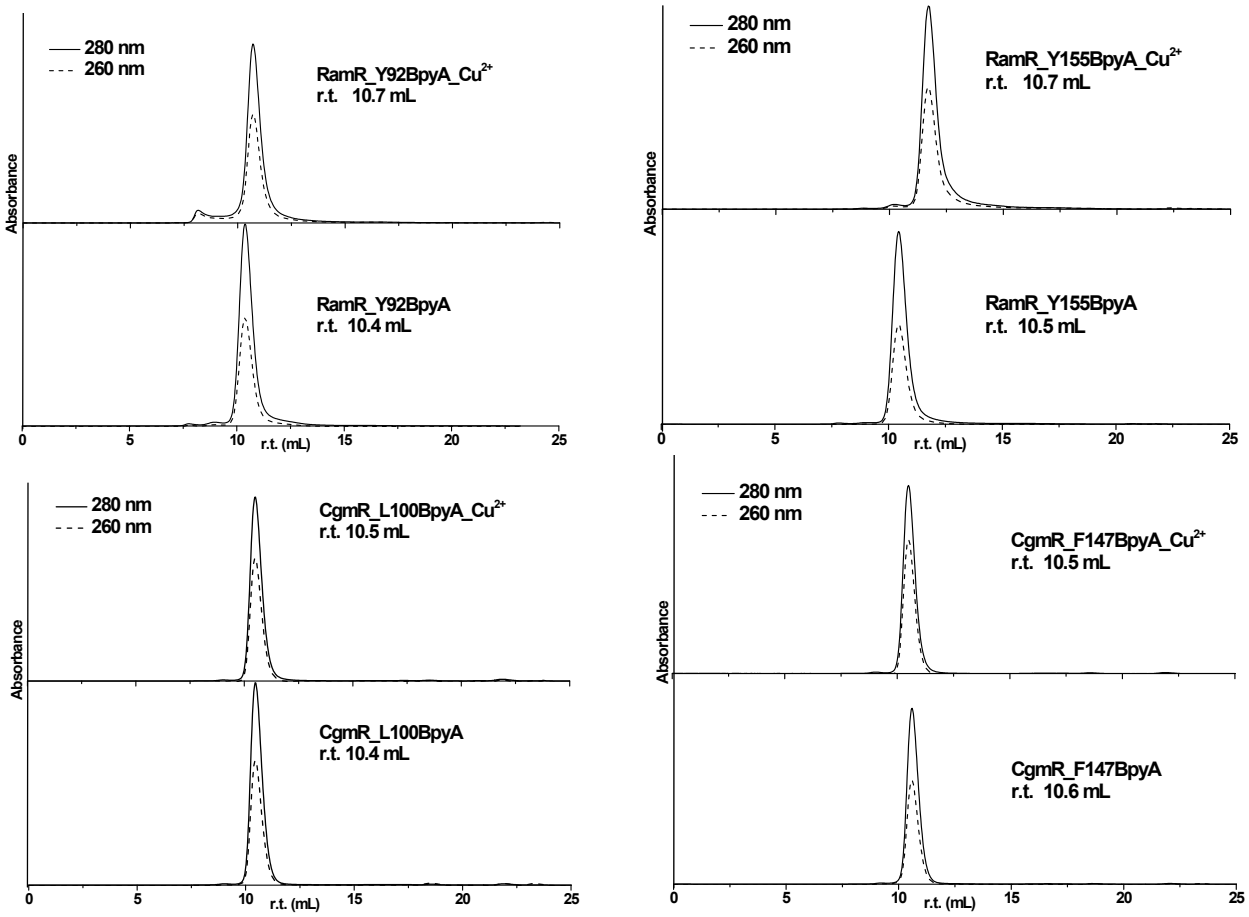


8. Size exclusion chromatography

Analytical size exclusion chromatography was performed on a Superdex 75 10/300 GL (GE Healthcare). 100 µL of the sample was injected using 20 mM MOPS, pH 7.0, 500 mM NaCl as buffer (flow 0.5 mL min^{-1}). Analytical size exclusion chromatograms were recorded for the proteins and of the protein after incubation with 1 equivalent of $Cu(NO_3)_2$ per monomer. No significant deviations from the dimeric structure of the wild type proteins were observed upon introduction of BpyAla, as well as after coordination to Cu^{2+}.

Figure S6: Analytical size exclusion chromatography (Superdex-75 10/300 GL)



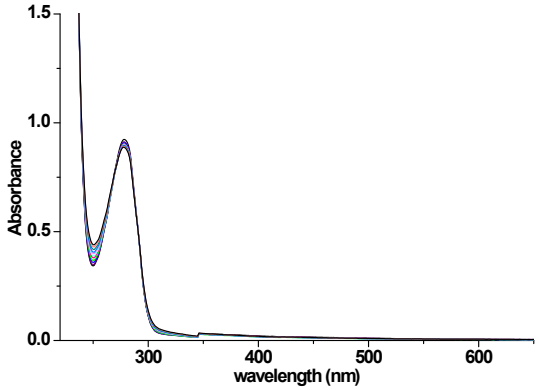


9. UV-visible titrations and dissociation constants

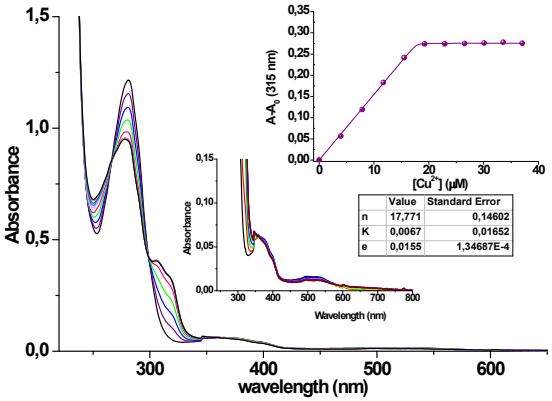
0.5 mM solution of $Cu(NO_3)_2 \cdot 3H_2O$ in milliQ water was prepared by dilution of a 5 mM stock solution. Protein solutions (20 µM, 200 µL) in 50 mM NaH_2PO_4 pH 7.0, 150 mM NaCl were added to a 0.5 mL cuvette and titrated with 0.5 mM working solutions of metal salts $Cu(NO_3)_2 \cdot 3H_2O$ (2 µL each addition, 0.2 eq). UV-visible spectra were recorded at 25°C from 220 nm to 800 nm.

Figure S7: UV-visible titrations

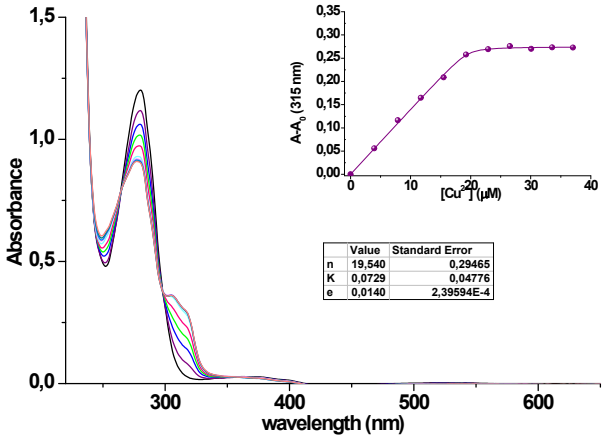
QacR (C72A, C141S): MW=23266.41 Da ε=41370 $M^{-1}cm^{-1}$



QacR Y103BpyA: MW=23328.49 Da ε=54680 $M^{-1}cm^{-1}$ (-Tyr+BpyA)

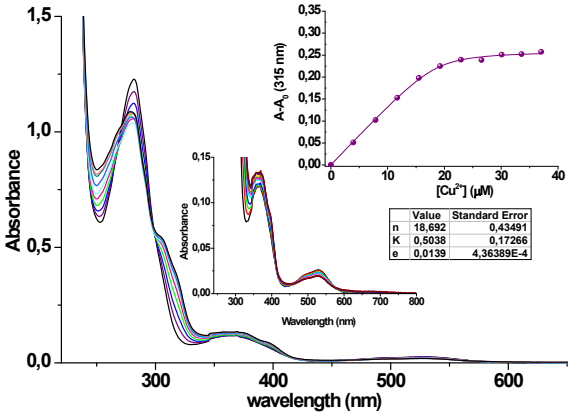


	Value	Standard Error
n	17,771	0,14602
K	0,0067	0,01652
e	0,0155	1,34687E-4

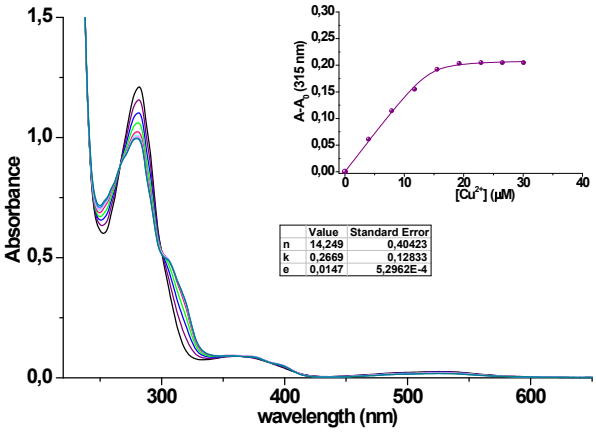


	Value	Standard Error
n	19,540	0,29465
K	0,0729	0,04776
e	0,0140	2,39594E-4

QacR Y123BpyA: MW=23328.49 Da ε=54680 $M^{-1}cm^{-1}$ (-Tyr+BpyA)

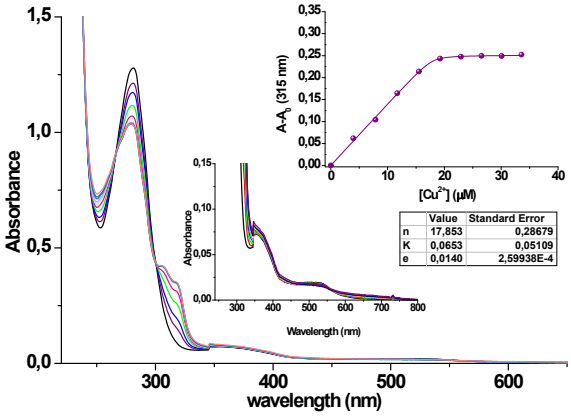


	Value	Standard Error
n	18,692	0,43491
K	0,5038	0,17266
e	0,0139	4,36389E-4

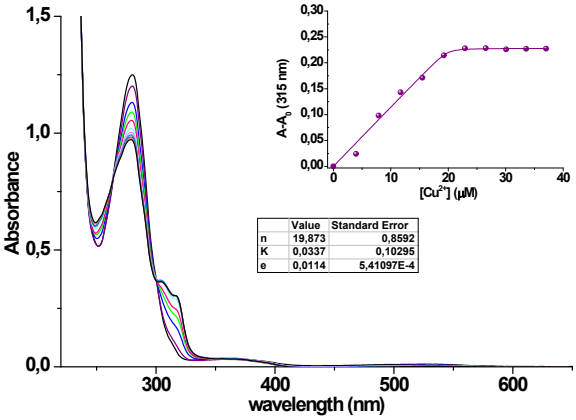


	Value	Standard Error
n	14,249	0,40423
k	0,2669	0,12833
e	0,0147	5,2962E-4

QacR Q96BpyA: MW=23363.53 Da ε=56170 $M^{-1}cm^{-1}$ (+BpyA)

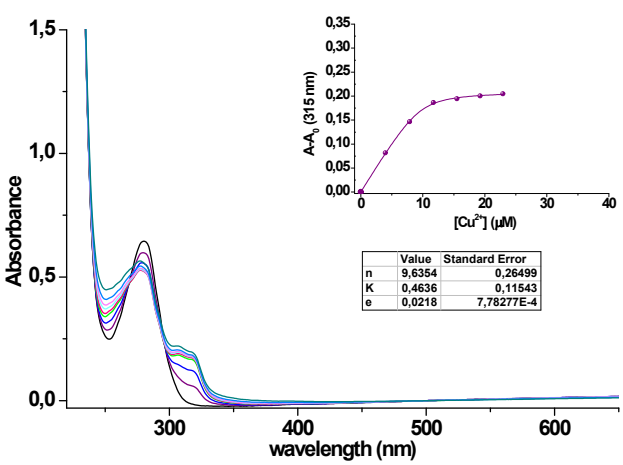


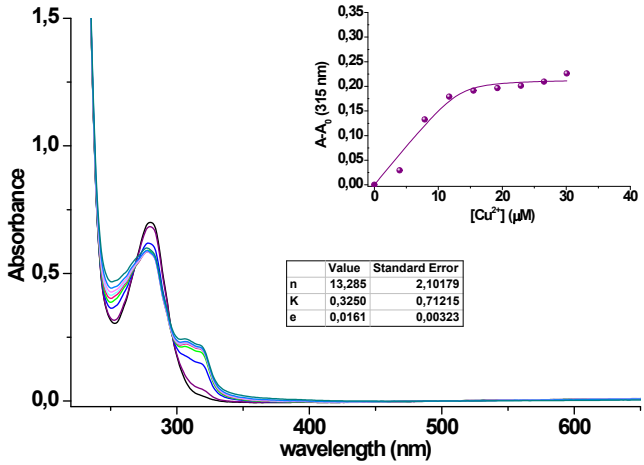
	Value	Standard Error
n	17,853	0,28679
K	0,0653	0,05109
e	0,0140	2,59938E-4



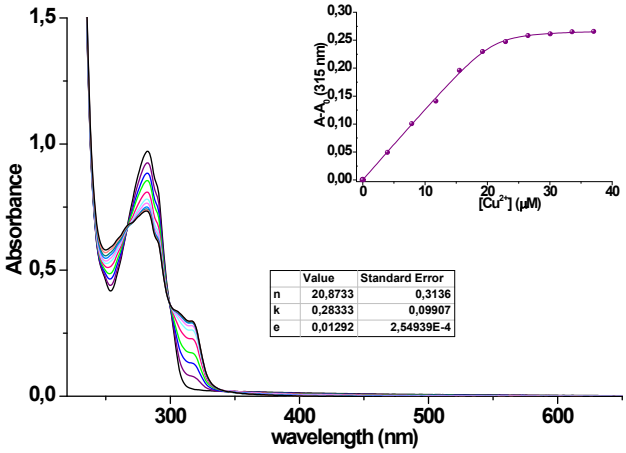
	Value	Standard Error
n	19,873	0,8592
K	0,0337	0,10295
e	0,0114	5,41097E-4

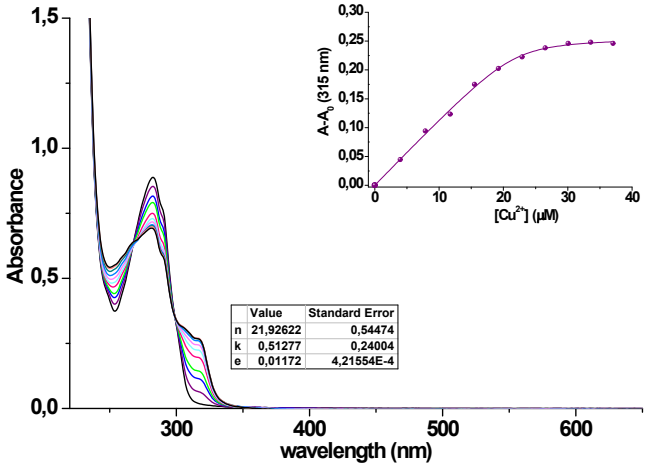
QacR W61BpyA: MW=23305.45 Da ε=50670 $M^{-1}cm^{-1}$ (-Trp+BpyA)



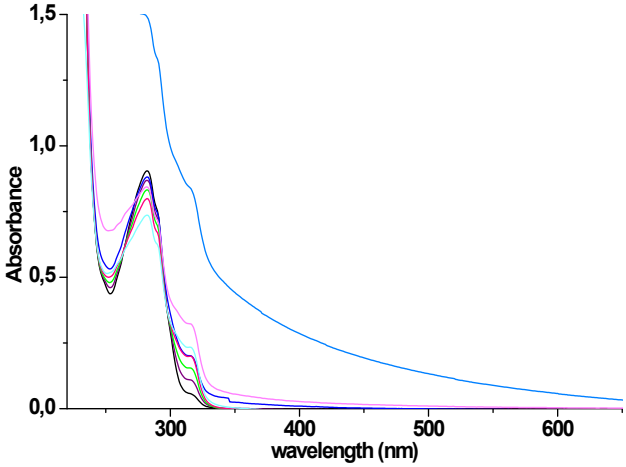


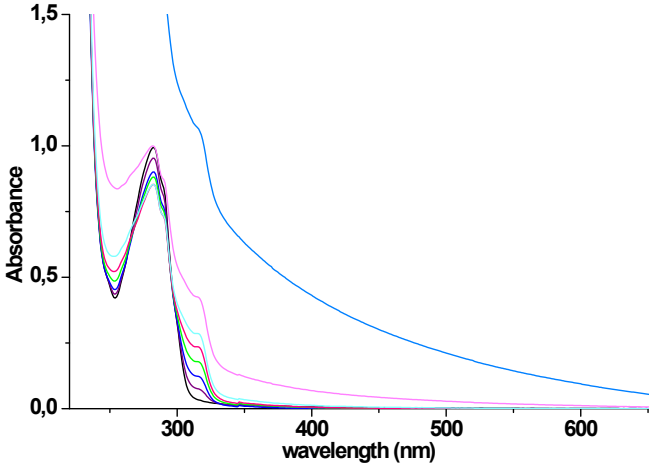
RamR F155BpyA: MW=23113 Da ε=44250 $M^{-1}cm^{-1}$ (+BpyA)





RamR Y92BpyA MW= 23097 Da ε=42760 $M^{-1}cm^{-1}$ (-Tyr+BpyA)





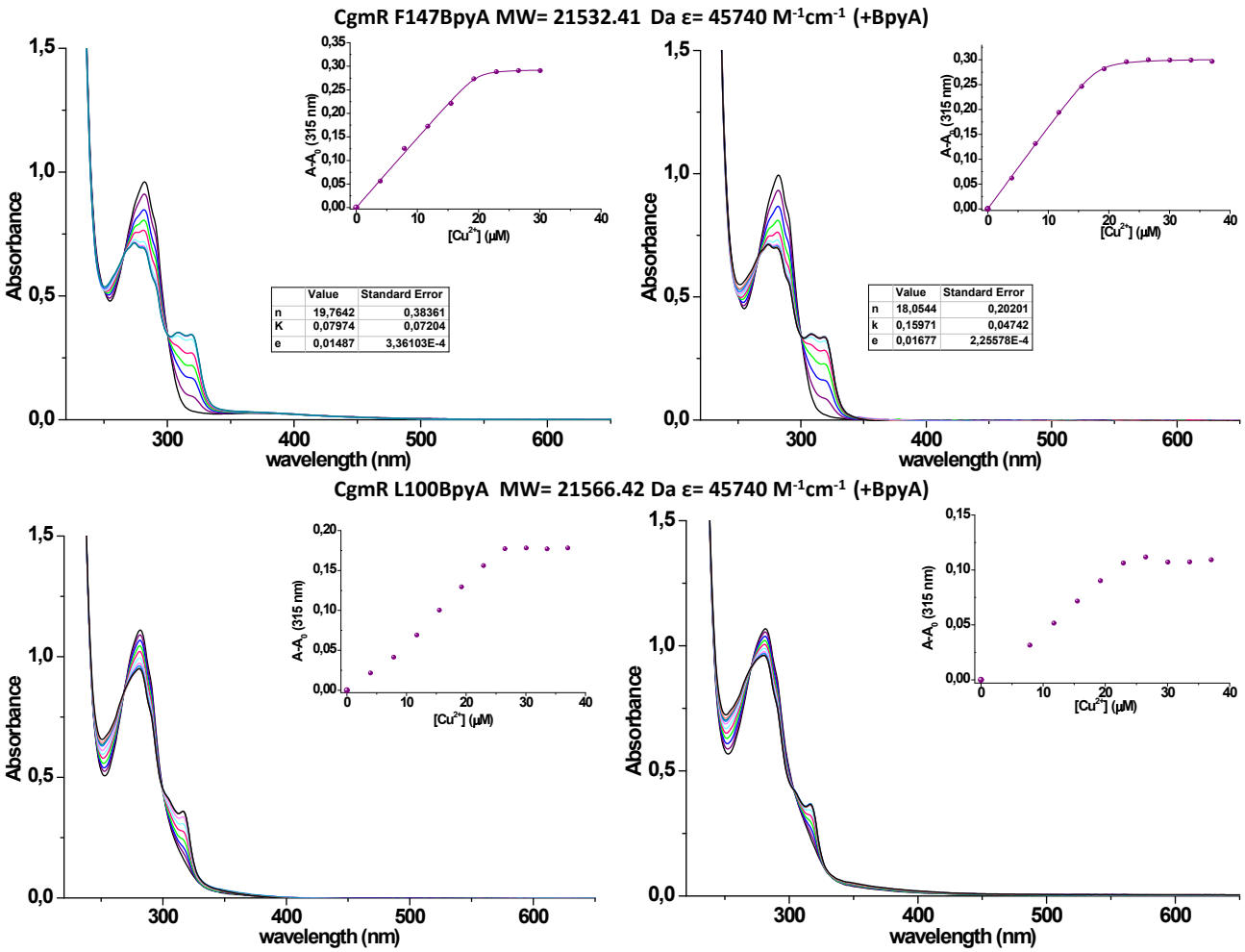


Table S2 Dissociation constants for QacR_bipyridine mutants with Cu^{2+} determined by UV-visible titrations.

Entry	Protein	K_D (μM)
1	QacR W61ByA	0.39±0.07
2	QacR Q96ByA	0.05±0.02
3	QacR Y103ByA	0.04±0.03
4	QacR Y123ByA	0.4±0.1
5 [a]	RamR Y92BpyA	-
6	RamR F155BpyA	0.4±0.1
7 [a]	CgmR L100BpyA	-
8	CgmR F147BpyA	0.12±0.04

Apparent dissociation constants (K_D) were obtained assuming a 1:1 binding stoichiometry using non-linear regression analysis (Origin) employing the following equation:

$$y(Absorbance) = \frac{\left(C_{protein} + K_D + [M^{2+}] - \sqrt{(C_{protein} + K_D + [M^{2+}])^2 - 4C_{protein}x[M^{2+}]}\right)}{2\varepsilon}$$

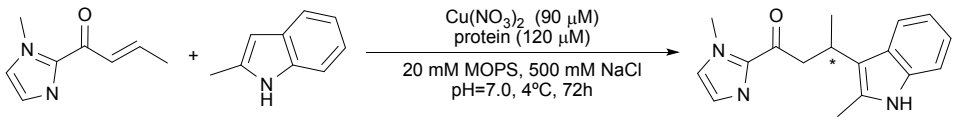
The reported apparent dissociation constants are the average of two independent experiments performed with two independent batches of protein and the error bars correspond to the standard deviation.

[a] K_D for mutants **RamR Y92BpyA** and **CgmR L100BpyA** were not determined due to precipitation of the protein after addition of more than 1 eq of Cu^{2+} and impossibility of fitting the data, respectively

10. Catalysis

Catalytic reactions were performed in 150 µL total volume containing 90 µM $Cu(NO_3)_2$ (9 mol%) and 120 µM proteins (monomer, 1.3 equivalents), 1 mM of substrate **1** and **2** in 20 mM MOPS buffer pH 7.0, 500 mM NaCl. Reactions were incubated under continuous inversion at 4 °C for 72 h after which 50 µL of a 1 mM solution of 2-phenylquinoline in 20 mM MOPS buffer pH 7.0, 500 mM NaCl, 20% CH_3CN were added. Reactions were extracted 3 times with 500 µL diethylether and the organic layers were dried over Na_2SO_4 and evaporated in vacuo. The resulting products were redissolved in 150 µL heptane:isopropanol 9:1 and analyzed by chiral HPLC (Chiralpak ADH). Yields of all the catalytic reactions are based on based on peak areas at 275 nm using 2-phenylquinoline as internal standard.[7]

Table S3 Control experiments for catalytic vinylogous Friedel–Crafts alkylation reactions

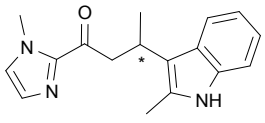


1 **2** **3**

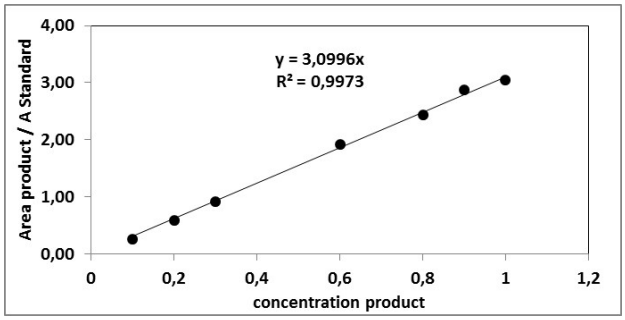
Entry	Catalyst	Yield (%) [a]	ee (%)[b]
1	QacR Y103BpyA_Cu^{2+}(EDTA) [c]	36±22	35±6 (-)
2	QacR Y103BpyA	<5	n.d.
3	QacR Y103BpyA (EDTA) [c]	<5	n.d.
4	QacR Y123BpyA	59±5	70±7 (+)

Typical conditions: 9 mol% $Cu(NO_3)_2$ (90 µM) loading with 1.3 eq of protein (120 µM), 1 mM of substrate **1** and **2** in 20 mM MOPS buffer pH 7.0, 500 mM NaCl, for 72 h at 4 °C. All the results listed correspond to the average of two independent experiments, each carried out in duplicate. Errors listed are standard deviations [a] Yields were determined by HPLC and using 2-phenylquinoline as internal standard. [b] Sign of rotation was assigned by comparison with elution order in chiral HPLC based on previous reports[33, 32], For yields <5% ee's were not determined. [c] Protein sample incubated with 50 mM EDTA for 16h followed by dialysis in 20 mM MOPS buffer pH 7.0, 500 mM NaCl

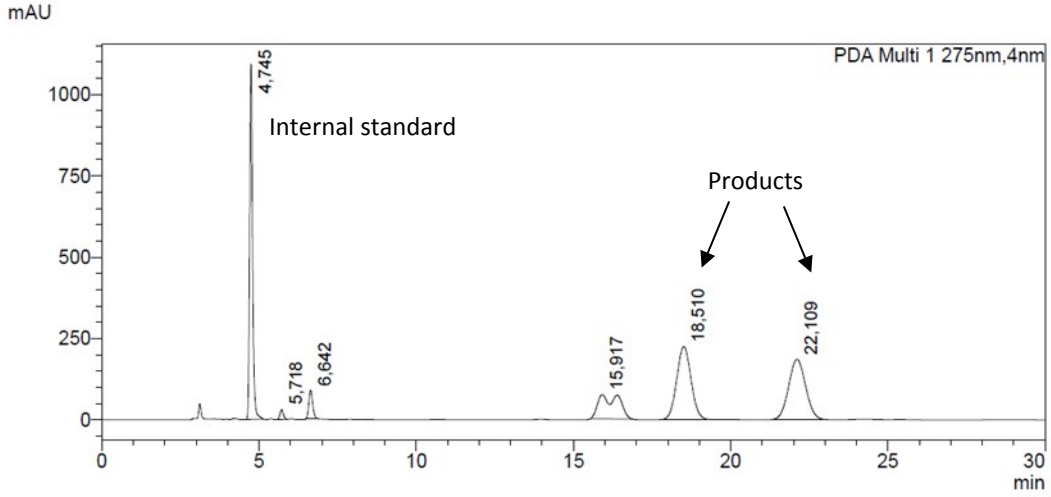
Calibration curve for 3



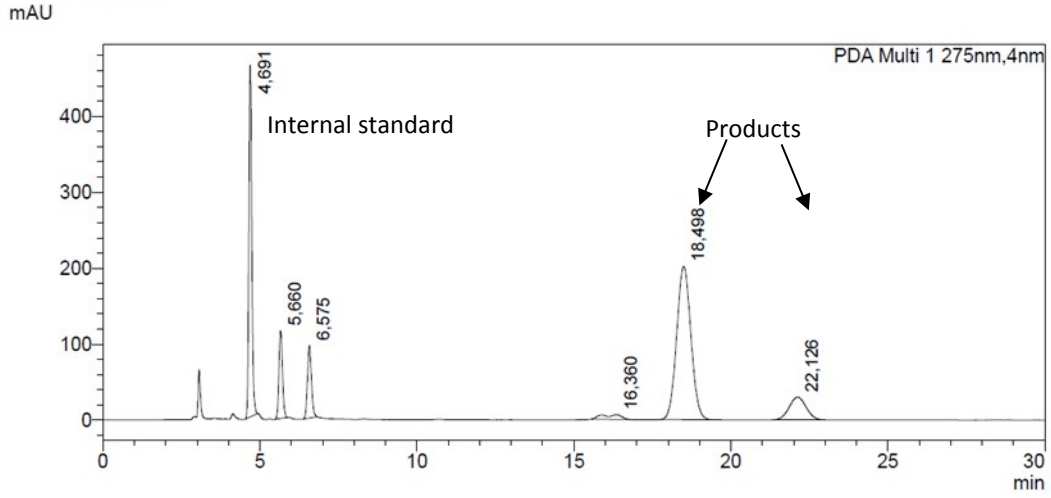
3



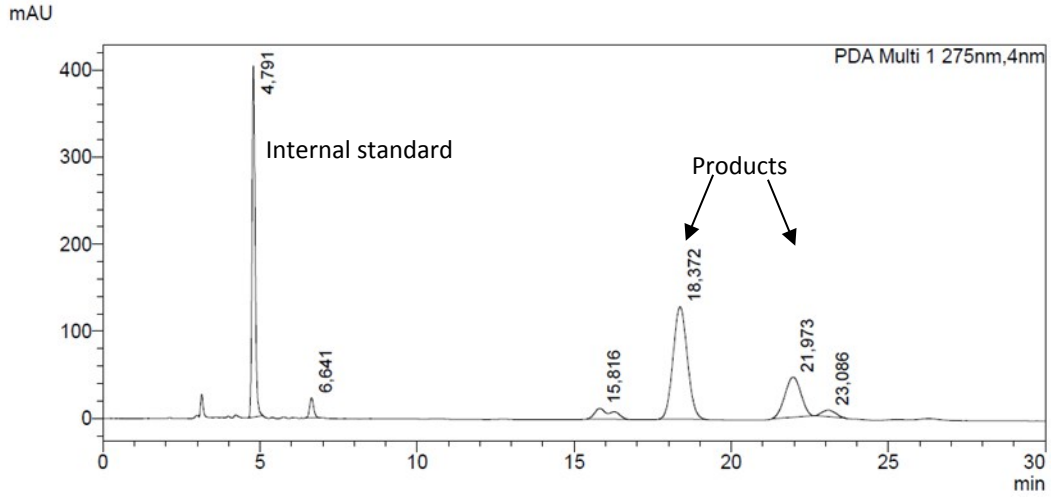
3 – Catalyst: $Cu(NO_3)_2$



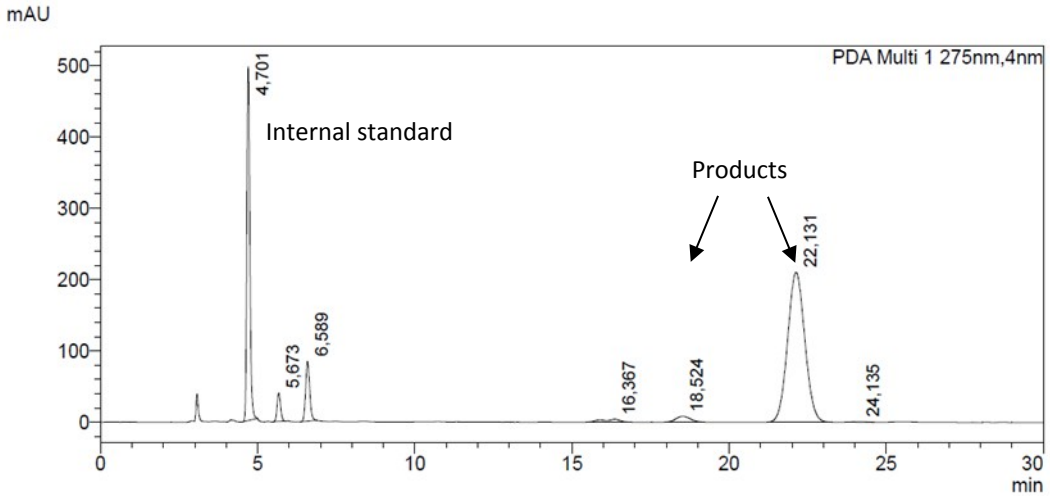
3 – Catalyst: LmrR M89BpyA_Cu^{2+}



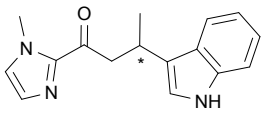
3 – Catalyst wt-QacR_Cu^{2+}



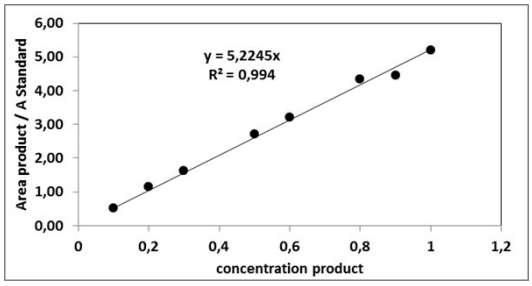
3 – Catalyst: QacR Y123BpyA_Cu^{2+}



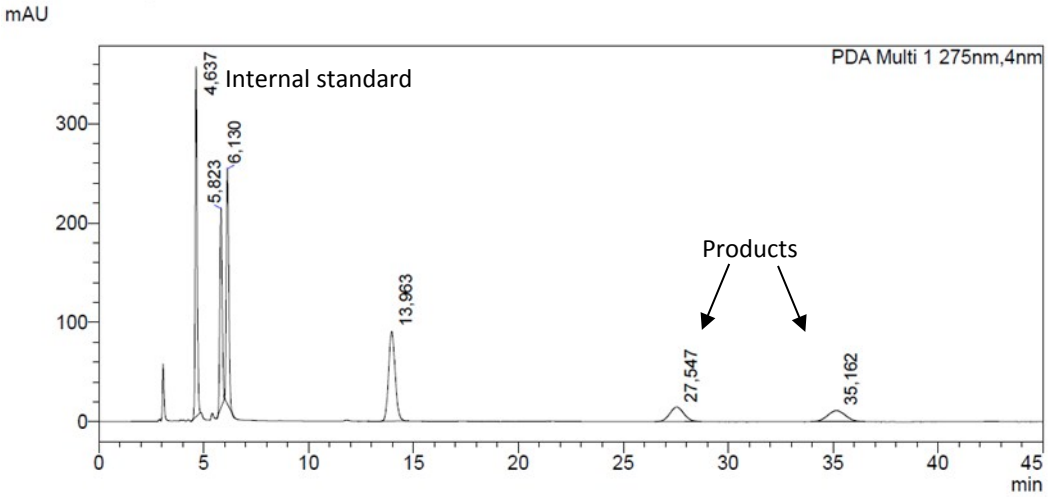
Calibration curve for 3a



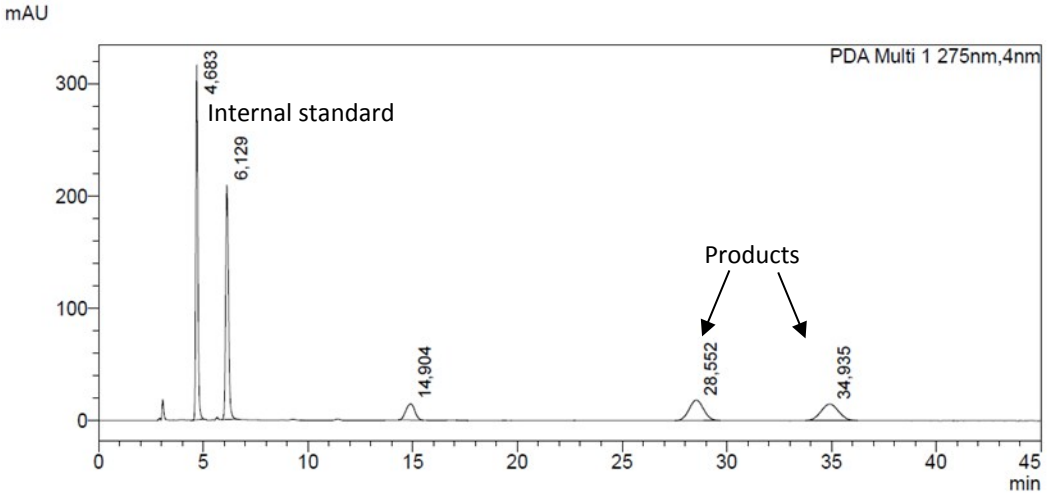
3a



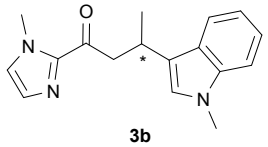
3a – Catalyst: $Cu(NO_3)_2$



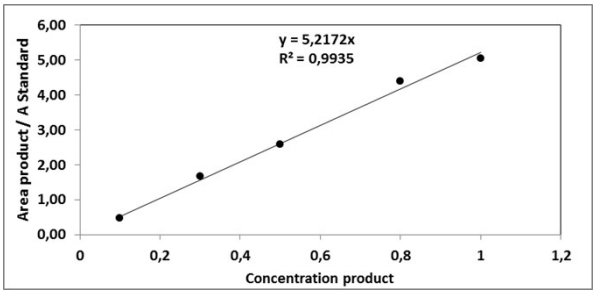
3a– Catalyst: QacR Y123BpyA_Cu^{2+}



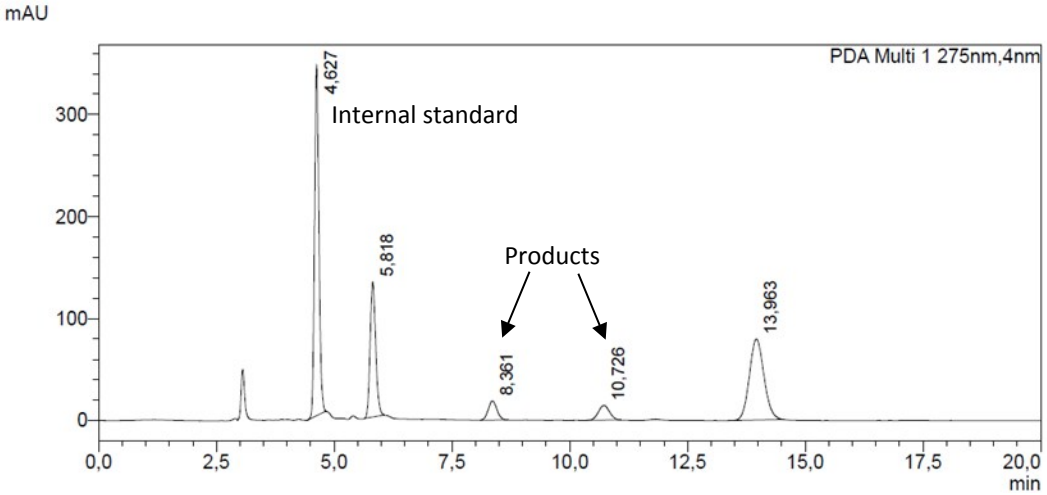
Calibration curve for 3b



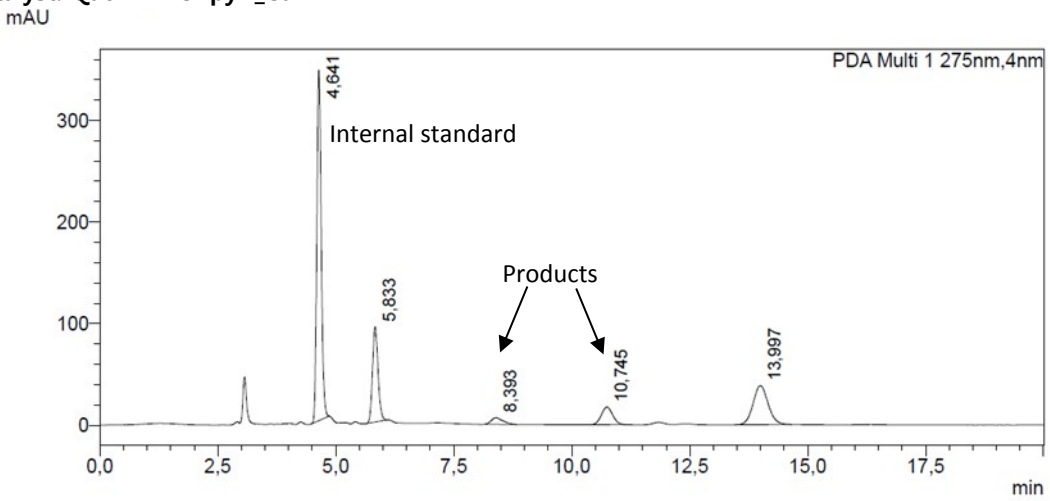
3b



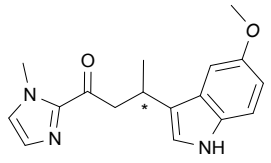
3b – Catalyst: $Cu(NO_3)_2$



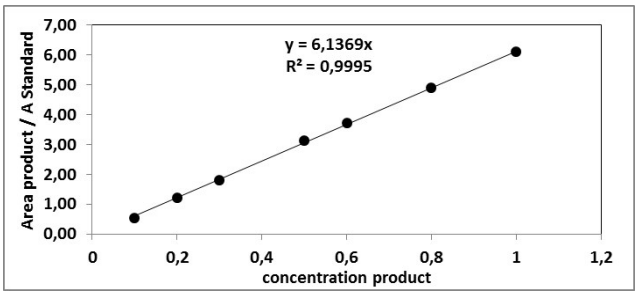
3b– Catalyst: QacR Y123BpyA_Cu^{2+}



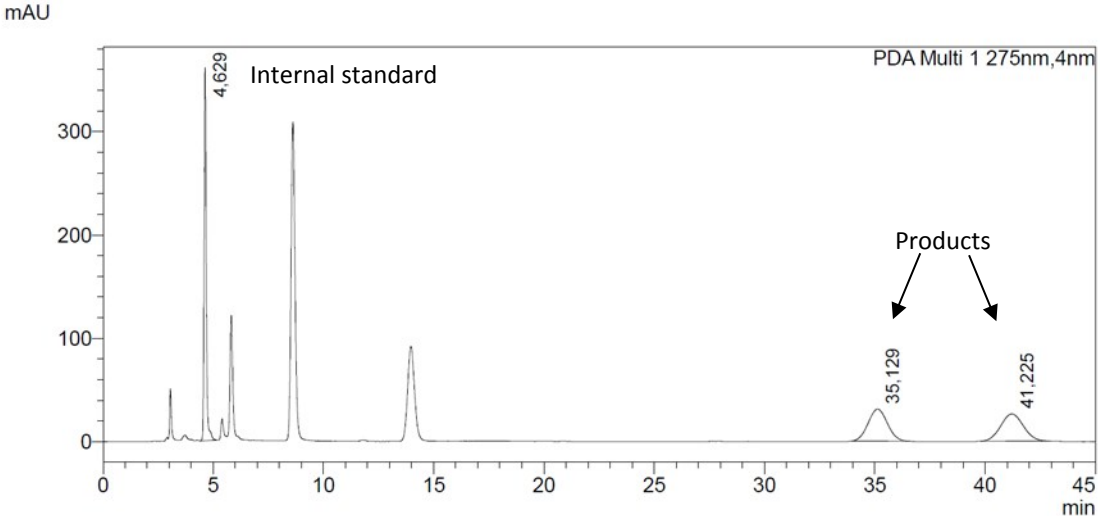
Calibration curve for 3c



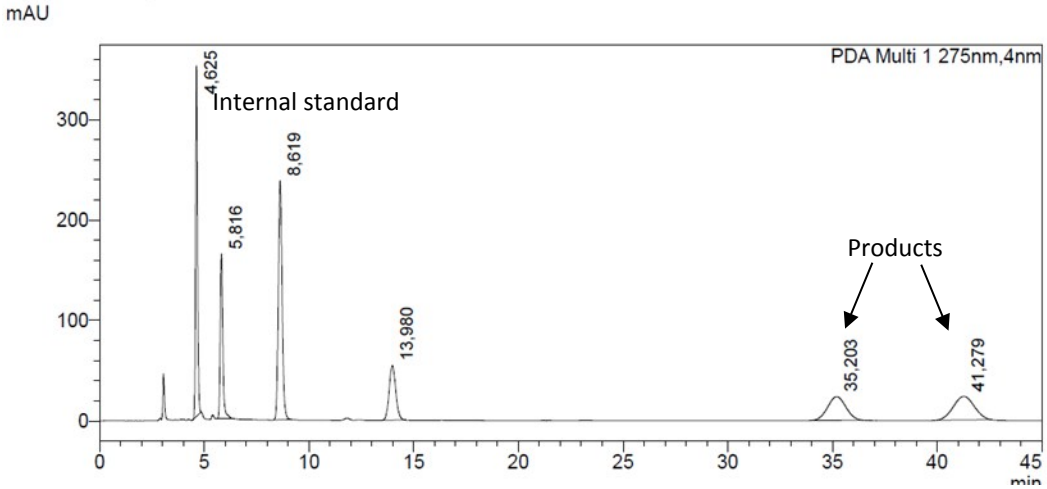
3c



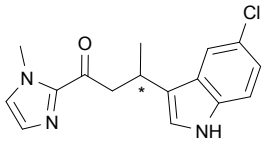
3c – Catalyst: $Cu(NO_3)_2$



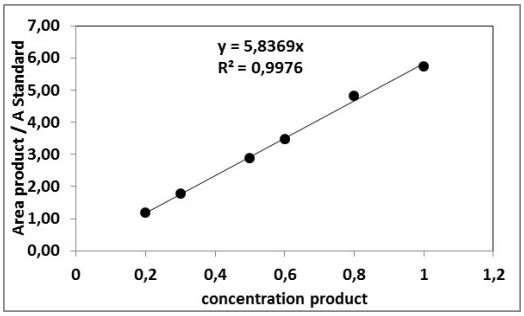
3c– Catalyst: QacR Y123BpyA_Cu^{2+}



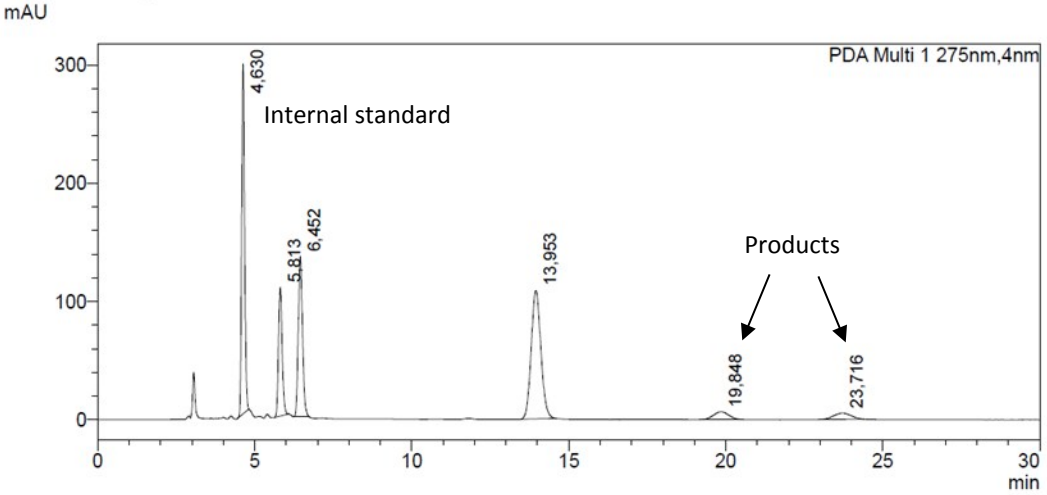
Calibration curve for 3d



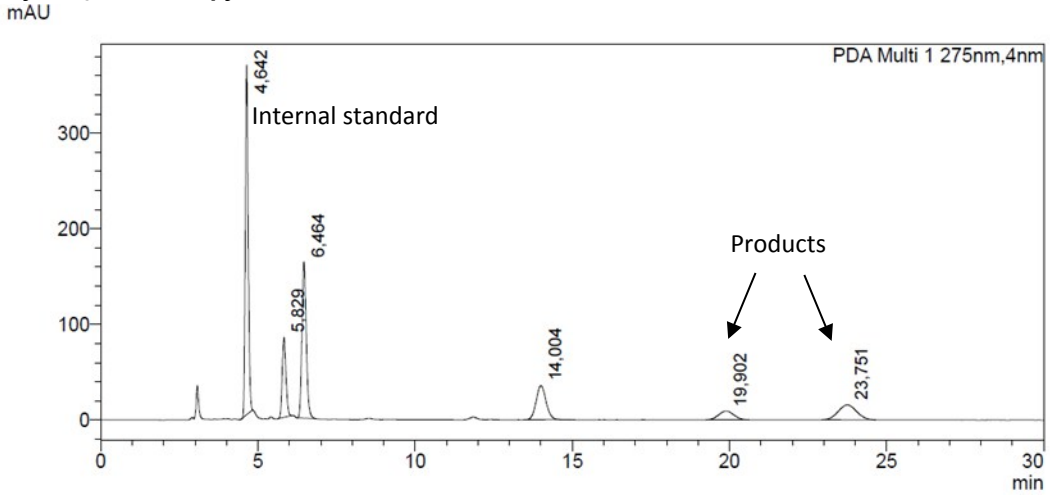
3d



3d – Catalyst: $Cu(NO_3)_2$



3d– Catalyst: QacR Y123BpyA_Cu^{2+}



11. References

1 Z. Zhang and A. G. Marshall, *J. Am. Soc. Mass Spectrom.*, 1998, **9**, 225–233.

2 R. Ballardini, V. Balzani, M. Clemente-León, A. Credi, M. T. Gandolfi, E. Ishow, J. Perkins, J. F. Stoddart, H.-R. Tseng and S. Wenger, *J. Am. Chem. Soc.*, 2002, **124**, 12786–12795.

3 H. S. Lee and P. G. Schultz, *J. Am. Chem. Soc.*, 2008, **130**, 13194–13195.

4 D. A. Evans, K. R. Fandrick and H.-J. Song, *J. Am. Chem. Soc.*, 2005, **127**, 8942–8943.

5 F. Rosati, J. Oelerich and G. Roelfes, *Chem. Commun.*, 2010, **46**, 7804–7806.

6 J. Bos, W. R. Browne, A. J. M. Driessen and G. Roelfes, *J. Am. Chem. Soc.*, 2015, **137**, 9796–9799.

7 A. J. Boersma, B. L. Feringa and G. Roelfes, *Angew. Chem. Int. Ed.*, 2009, **48**, 3346–3348.