Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

In Vivo Assembly of Artificial Metalloenzymes and Application in Whole-Cell Biocatalysis**

*Shreyans Chordia, Siddarth Narasimhan, Alessandra Lucini Paioni, Marc Baldus, and Gerard Roelfes**

anie_202014771_sm_miscellaneous_information.pdf

Supporting Information

Table of contents

1. Supplementary tables

Table S1: Effect of mutations in LmrR on *in vivo* and *in vitro* catalysis of the Friedel-Crafts reaction between 1 and 2b

Cu(II)-Phen⊂LmrR	***in vivo* catalysis[a]**	***in vitro* catalysis[b]**	
Protein variant	**ee (%)**	**yield (%)**	**ee (%)**
LmrR	34±5	51±11	91±0
LmrR_M8A	41±1	85±7	93±0
LmrR_L18A	20±8	39±0	90±0
LmrR_N88A	26±1	14±1	82±0
LmrR_M89A	14±2	14±0	67±0
LmrR_A92E	63±1	Full conversion	94±0
LmrR_F93A	3±1	3±0	30±1
LmrR_W96A	2±1	8±1	18±4
LmrR_D100A	5±2	18±2	72±0

[a] F-C Alkylation of **1** (1 mM) and **2b** (1 mM), cell suspension 500 µL, Cu(II)-Phen 90 µM, reaction time 16 h at 4 ^{o}C.

[b] F-C Alkylation of **1** (1 mM) and **2b** (1 mM), purified protein 120 µM, Cu(II)-Phen 90 µM, reaction time 24 h at 4 ^{o}C.

Table S2: *in vivo* catalysis results from Alanine scanning of LmrR for the Friedel-Crafts reaction between 1 and 2b.

LmrR⊂ Cu(II)-Phen	***in vivo* catalysis**	
Protein variant	**yield (%)**	**ee (%)**
LmrR	6±4	34±5
LmrR_E7A	6±2	27±2
LmrR_M8A	8±0	41±1
LmrR_R10A	2±0	32±8
LmrR_Q12A	6±0	21±1
LmrR_N14A	5±1	35±10
LmrR_V15A	6±1	25±7
LmrR_L18A	7±5	20±8
LmrR_N88A	2±1	26±1
LmrR_M89A	2±0	14±2
LmrR_A92E	17±5	63±1
LmrR_F93A	4±1	3±1
LmrR_S95A	2±0	37±10
LmrR_W96A	5±1	2±1
LmrR_S97A	2±0	22±8
LmrR_V99A	4±2	13±7
LmrR_D100A	5±3	5±2

Conditions: **1** (1 mM) and **2b** (1 mM), cell suspension 500 µL, Cu(II)-Phen 90 µM, reaction time 16 h at 4 ^{o}C.

Table S3: Influence of Glutathione on in vitro Cu(II)-ArM catalysis of the reaction of 1 with 2a.[a]

catalyst	Glutathione (mM)	yield (%)	ee (%)
LmrR_A92E_M8D[b]	0	37±10	98±0
Cu(II)-Phen[b]	0	3±0	0
LmrR_A92E_M8D[c]	1	40±21	97±1
Cu(II)-Phen[c]	1	1±0	0
LmrR_A92E_M8D[d]	10	7±2	93±1
Cu(II)-Phen[d]	10	13±1	<5

[a] **1** (1 mM), **2a** (1 mM). [b] Catalyst loading 0.9%, reaction time 30 min at 4 ^{o}C. [c] Catalyst loading 9%, reaction time 30 min at 4 ^{o}C. [d] Catalyst loading 9%, reaction time 48h at 4 ^{o}C.

Diels Alder reaction:

Table S4: *in vivo* catalysis results from Alanine scanning of LmrR for the Diels-Alder reaction between 4 and 5 to give the Diels-Alder product 6.

LmrR⊂ Cu(II)-Phen	***in vivo* catalysis**		***In vitro catalysis***	
Protein variant	**yield (%)[a]**	**ee (%)[a,b]**	**yield (%)[a]**	**ee (%)[a,b]**
LmrR	24	-17	22	-58
LmrR_A92E	24	-31	33	-68
LmrR_A92E_M8A	20	-16	11	-36
LmrR_R10A	21	-11	11	-59
LmrR_A92E_N14A	23	-22	8	-51
LmrR_A92E_V15A	42	-65	52	-84
LmrR_L18A	12	<5	7	-36
LmrR_N88A	18	-5	8	-40
LmrR_A92E_M89A	18	-11	7	-41
LmrR_A92E_F93A	16	6	5	-11
LmrR_W96A	17	<5	7	<5
LmrR_S97A	15	-12[c]	5[c]	-21
LmrR_V99A	24	-15	12	-60
LmrR_A92E_D100A[c]	20	5	4	-22

Conditions in vivo catalysis *E. coli* C43(DE3) cell suspension overexpressing LmrR_X 2 mL (OD_{600nm} = 4), Cu(II)-Phen 360 µM, : **4** (1 mM) and **5** (33 mM), reaction time 72 h at 4 ^{o}C; conditions in vitro catalysis: Cu(II)-Phen 90 µM, LmrR_X 120 µM, **4** (1 mM) and **5** (33 mM), reaction time 48 h at 4 ^{o}C. Results are the average of duplicate experiments. a) of the endo isomer of **6**. b) + or – signs indicate which is the major enantiomer peak based on order of elution from the hplc, first and second, respectively. c) data from single experiments.

Table S5: *in vivo* catalysis of Diels-Alder by LmrR_V15A_A92E/Cu(II)-Phen and control experiments

	yield %[a]	ee %[a]
Whole cell_cytoplasmic SUMO/Cu(II)-Phen	14±1	0
Whole cell_cytoplasmic Cu(II)-Phen⊂LmrR	12±4	-8±1
Whole cell_cytoplasmic Cu(II)-Phen⊂LmrR_A92E	15±6	-17±1
Whole cell_cytoplasmic Cu(II)-Phen⊂LmrR_V15A_A92E	20±3	-45±0
Whole cell_cytoplasmic LmrR_V15A_A92E No Cu(II)-Phen added	7±3	<-5 (-1±0)
Supernatant after Cu(II)-Phen incubation_cytoplasmic LmrR_V15A_A92E	4±0	<-5 (-1±0)
Whole cell_cytoplasmic Cu(II)-Phen⊂LmrR_V15A_A92E After 48 hours *in vivo* catalysis, Recycled cell fraction + substrates	46±11	-46±2
Whole cell_cytoplasmic Cu(II)-Phen ⊂LmrR_V15A_A92E After 48 hours *in vivo* catalysis, Supernatant + substrates	19±1	<-5 (-4±1)

Conditions: Cu(II)-Phen (180 µM), *E. coli* cell suspension 1 mL**, 4** (1 mM) and **5** (33 mM) in 20 mM MOPS, 150 mM NaCl at pH 7, reaction time 48 h at 4 ^{o}C. a. Of the endo isomer. Values represent the average of duplicate experiments, each performed *in duplo*. Errors are given as standard deviations. + or – signs indicate which is the major enantiomer peak based on order of elution from the hplc, first and second, respectively.

Table S6: Results of catalysis of the Diels-Alder reaction of 4 with 5 by isolated LmrR/Cu(II)-phen and LmrR mutants.

Catalyst:	**yield %[a]**	**ee %[a,b]**
Cu-Phen	17±2	-
LmrR/Cu(II)-Phen	22±5	-58±6
LmrR-A92E/Cu(II)-Phen	33±10	-68±2
LmrR-V15A/Cu(II)-Phen	41±5	-78±1
LmrR-A92E-V15A/Cu(II)-Phen	52±7	-84±3
LmrR-A92E-M8D/Cu(II)-Phen	25±2	-55±2

Conditions: Cu(II)-Phen (90 µM), LmrR variant (120 µM), **4** (1 mM) and **5** (33 mM) in 20 mM MOPS, 150 mM NaCl at pH 7, reaction time 48 h at 4 ^{o}C; a. Of the endo isomer. Values represent the average of duplicate experiments, each performed *in duplo*. Errors are given as standard deviations. b) + or – signs indicate which is the major enantiomer peak based on order of elution from the hplc, first and second, respectively.

2. General remarks

Chemicals and reagents were purchased from Sigma Aldrich and used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian 400 MHz or a Bruker 600 MHz in $CDCl_3$. *E. coli* strains NEB5-alpha, NEB10-beta and C43(DE3) (New England Biolabs and Lucigen, USA) were used for cloning and expression of proteins. Primers were synthesized and DNA sequencing carried out by Eurofins Genomics (Germany). All the enzymes for cloning and mutagenesis were purchased from New England Biolabs (USA). Plasmid Purification Kits were obtained from QIAGEN (Germany). The DNA and protein concentrations in solutions were derived from the absorption at 260 nm or 280 nm on a Thermo Fisher Scientific Nanodrop 2000 UV-Vis spectrophotometer, respectively. Strep-tactin columns (Strep-Tactin® Superflow® high capacity) were purchased from IBA-Lifesciences (Germany). Mass spectra (HRMS) were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. mode). Fluorescence experiments were recorded using a JASCO FP-6200 spectrometer and a TECAN Spark10M fluorimeter.

3. *Chemical synthesis*

Copper complex:

Cu(1,10-phenanthroline)$(NO_3)_2$ **(Cu(II)-Phen)** was prepared by following the published procedure.[1]

Elemental analysis calcd. for $C_{12}H8CuN_4O_6$: C, 39.19; H, 2.19; N, 15.23. Found C, 38.95; H, 2.13 ; N, 15.13.

Substrate 1:

(E)-1-(1-Methyl-1H-imidazole-2-yl)-but-2-en-1-one (**1**) was prepared following a literature procedure**.**[2]

Friedel-Crafts Alkylation products:

1-(1-methyl-1H-imidazol-2-yl)-3-(2-methyl-1H-indol-3-yl)butan-1-one (**3a**), 3-(5-methoxy-1H-indol-3-yl)-1-(1-methyl-1H-imidazol-2-yl)butan-1-one (**3b**), 3-(1H-indol-3-yl)-1-(1-methyl-1H-imidazol-2-yl)butan-1-one (**3c**) and 3-(5-chloro-1H-indol-3-yl)-1-(1-methyl-1H-imidazol-2-yl)butan-1-one (**3d**) were prepared following a literature procedure.[2, 3] The NMR data match those reported in the literature.

Dienophile Substrate 4:

(E)-3-phenyl-1-(pyridin-2-yl)prop-2-en-1-one was prepared following a literature procedure.[4, 5]

Diels-Alder product 6:

3-phenylbicyclo[2.2.1]hept-5-en-2-yl)(pyridin-2-yl)methanone was prepared following a literature procedure. The NMR data match those reported in the literature.[4, 5]

4. Molecular biology

Site-Directed mutagenesis of LmrR

The template used for making the mutants was either pET17b_LmrR or pET17b_LmrR_A92E, which contain mutations at 2 lysines (K55D and K59Q) that negate the natural DNA-binding ability of the protein and was codon optimized for expression in *E. coli*.[6, 7] The Quikchange Site Directed Mutagenesis Protocol (Agilent Technologies) was employed, using oligos described in (Table S5). The protocol was as follows: (1) initial denaturation at 95 ^{o}C for 1 min, (2) 25 cycles of denaturation at 98 ^{o}C for 30 s, annealing at 52-60 ^{o}C for 30 s (depending on the Tm of the primers) and extension at 72 ^{o}C for 4 min 30 s; (3) final extension at 72 ^{o}C for 10 min. The PCR product was digested with 1 µL DpnI for 2 h at 37 ^{o}C and chemically competent *E. coli* NEB5-alpha cells were transformed with 5 µL reaction mixture. A single colony picked from LB agar plates supplemented with 100 µg/µL ampicillin was transferred to LB media containing 100 µg/µL ampicillin. The overnight cell culture was used to make a glycerol stock, isolate the plasmid DNA and DNA sequencing confirmed the sequence of the mutant. The glycerol stock was used to make overnight starter cultures and subsequent day culture for protein expression and purification.

Shake flask cultivation and LmrR expression

Freshly transformed single colonies of *E. coli* C43(DE3) containing plasmid pET17b_LmrR were picked and used to inoculate a starter culture of 5 mL of fresh LB medium containing 100 µg/mL ampicillin. The entire starter culture was used to inoculate 500 mL of fresh LB medium containing 100 µg/mL ampicillin in a 2 L flask. When the culture reached an OD_{600nm} between 0.8–0.9, isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentration of 1 mM was added to induce the expression of LmrR. Overnight expression was carried out at 30 °C and the cells were used for *in vivo* catalysis, cell fractionation or protein purification.

Protein purification

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant was centrifuged (6000 rpm, JA10, 20 min, 4 °C, Beckman). The cell pellet was re-suspended in 15-20 mL of wash buffer (50 mM NaH_2PO_4, pH 8.0, 150 mM NaCl) along with half a tablet of mini complete EDTA-free protease inhibitor cocktail (Roche) and PMSF. Next, DnaseI (final concentration, 0.1 mg/mL) and $MgCl_2$ (final concentration, 10mM) were added. The cells were sonicated (sonication tip diameter 6 mm, 10 min, 10 s ON, 15 s OFF, Amplitude 75%). Additional sheer forcing with a syringe and a long needle was applied at least 2 times. Cell debris was centrifuged (16000 rpm, JA-17, 45 min, 4 °C, Beckman). The supernatant was filtered (cell-

free extract) and equilibrated with 5 mL of pre-equilibrated Strep-tag Tactin column material for 1 h (mixed at 200 rpm on a rotary shaker) at 4 °C. The column was washed with 3 x 2 CV (column volume) of wash buffer and eluted multiple times with 0.5 CV (6-7 times) of elution buffer (wash buffer containing 5 mM desthiobiotin). Fractions containing protein (excluding the first elution) were concentrated in centrifugal filters and re-buffered to 20 mM MOPS, pH 7.0, 150 mM NaCl by dialysis. The concentration of purified protein was determined by using the calculated extinction coefficient obtained from Protparam on the Expasy server (for example, LmrR monomer has ε280 = 25440 M^{-1} cm^{-1}). Expression yields typically were 30-40 mg of protein L^{-1} media.

Analysis of purified protein

The fractions of purified protein samples were compared against a protein ladder (PageRuler™ Unstained Broad Range) on a 12% polyacrylamide SDS-Tris Tricine gel followed by Coomassie Blue staining. The protein samples were also analyzed by LC-MS. They were separated/desalted on a Kinetex 2.6u EVO C18 column using a Shimadzu Prominence UFLC (Shimadzu) at a flow rate of 0.3 mL/min. First an isocratic flow with 10% eluent B (B = 100% AcN/0.1% formic acid) for 1.5 min was maintained. Then, a linear gradient of 4.5 min from 10–90% buffer A (A = H_2O/0.1% formic acid) was applied. MS data were acquired from m/z 500 to 1500. Protein masses were determined by deconvolution.

Table S7. List of Primers

Primer name	DNA Sequence (5’ -> 3’)
LmrR_M8_F	ATCCCGAAAGAAGCGCTGCGTGCTCAA
LmrR_M8A_R	TTGAGCACGCAGCGCTTCTTTCGGGAT
LmrR_N14A_F	CGTGCTCAAACCGCGGTCATCCTGCTG
LmrR_N14A_R	CAGCAGGATGACCGCGGTTTGAGCACG
LmrR_N88A_F	GGCCATGAAGCCATGCGCCTG
LmrR_N88A_R	CAGGCGCATGGCTTCATGGCC
LmrR_M89A_F	CATGAAAACGCGCGCCTGGCG
LmrR_M89A_R	CGCCAGGCGCGCGTTTTCATG

Primer name	DNA Sequence (5’ -> 3’)
LmrR_A92E_F	AACATGCGCCTGGAATTCGAATCCTGG
LmrR_A92E_R	CCAGGATTCGAATTCCAGGCGCATGTT
LmrR_F93A_F	CGCCTGGCGGCGGAATCCTGG
LmrR_F93A_R	CCAGGATTCCGCCGCCAGGCG
LmrR_W96A_F	GCGTTCGAATCCGCGAGTCGTGTGGAC
LmrR_W96A_R	GTCCACACGACTCGCGGATTCGAACGC
LmrR_D100A_F	TGGAGTCGTGTGGCGAAAATCATTGAA
LmrR_D100A_R	TTCAATGATTTTCGCCACACGACTCCA
LmrR_R10A_F	AAAGAAATGCTGGCGGCTCAAACCAAT
LmrR_R10A_R	ATTGGTTTGAGCCGCCAGCATTTCTTT
LmrR_S95A_F	CTGGCGTTCGAAGCCTGGAGTCGTGTG
LmrR_S95A_R	CACACGACTCCAGGCTTCGAACGCCAG
LmrR_A92H_F	AACATGCGCCTGCATTTCGAATCCTGG
LmrR_A92H_R	CCAGGATTCGAAATGCAGGCGCATGTT
LmrR_L18A_F	AATGTCATCCTGGCGAATGTCCTGAAA
LmrR_L18A_R	TTTCAGGACATTCGCCAGGATGACATT
LmrR_A92D_F	AACATGCGCCTGGATTTCGAATCCTGG
LmrR_A92D_R	CCAGGATTCGAAATCCAGGCGCATGTT
LmrR_V99A_F	TCCTGGAGTCGTGCGGACAAAATCATT
LmrR_V99A_R	AATGATTTTGTCCGCACGACTCCAGGA
LmrR_E7A_F	GAAATCCCGAAAGCGATGCTGCGTGCT
LmrR_E7A_R	AGCACGCAGCATCGCTTTCGGGATTTC
LmrR_S97A_F	TTCGAATCCTGGGCGCGTGTGGACAAA
LmrR_S97A_R	TTTGTCCACACGCGCCCAGGATTCGAA
LmrR_ A92Q_F	AACATGCGCCTGCAGTTCGAATCCTGG
LmrR_ A92Q_R	CCAGGATTCGAACTGCAGGCGCATGTT
LmrR_V15A_F	GCTCAAACCAATGCGATCCTGCTGAAT
LmrR_V15A_R	ATTCAGCAGGATCGCATTGGTTTGAGC
LmrR_Q12A_F	CTGCGTGCTGCGACCAATGTCA
LmrR_Q12A_R	TGACATTGGTCGCAGCACGCAG
SRP-LmrR_F	GAGCATATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAGCGTTTAGCGCATCGGCGGCGCAGATGGGTGCCGAAATCCCGAA
SRP-LmrR_R	CTCAAGCTTTTATTTTTCGAACTGCGGGTGGC

Table S8. List of Primers used for A92E_X mutants

Primer name	DNA Sequence (5' -> 3')
LmrR_A92E_M8NDT_F	ATCCCGAAAGAANDTCTGCGTGCTCAA
LmrR_A92E_M8NDT _R	TTGAGCACGCAGAHNTTCTTTCGGGAT
LmrR_A92E_N14NDT _F	CGTGCTCAAACCNDTGTCATCCTGCTG
LmrR_A92E_N14NDT _R	CAGCAGGATGACAHNGGTTTGAGCACG
LmrR_A92E_N88NDT _F	ATCGGCCATGAANDTATGCGCCTGGAA
LmrR_A92E_N88NDT _R	TTCCAGGCGCATAHNTTCATGGCCGAT
LmrR_A92E_M89NDT _F	GGCCATGAAAACNDTCGCCTGGAATTC
LmrR_A92E_M89NDT _R	GAATTCCAGGCGAHNGTTTTCATGGCC
LmrR_A92E_F93NDT _F	ATGCGCCTGGAANDTGAATCCTGGAGT
LmrR_A92E_F93NDT _R	ACTCCAGGATTCAHNTTCCAGGCGCAT
LmrR_A92E_D100NDT _F	TCCTGGAGTCGTGTGNDTAAAATCATTGCGAAT
LmrR_A92E_D100NDT _R	ATTCGCAATGATTTTAHNCACACGACTCCAGGA
LmrR_A92E_M89A_F	ATGCGCCTGGAAGCCGAATCCTGGAGT
LmrR_A92E_M89A_R	ACTCCAGGATTCGGCTTCCAGGCGCAT
LmrR_A92E_F93A_F	GGCCATGAAAACGCGCGCCTGGAATTC
LmrR_A92E_F93A_R	GAATTCCAGGCGCGCGTTTTCATGGCC
LmrR-V15A-A92E-M8NDT_F	CGAAAGAANDTCTGCGTGCTCAA
LmrR-V15A-A92E-M8NDT_R	GCACGCAGAHNTTCTTTCGGGAT
LmrR-V15A-A92E- N14NDT_F	CTCAAACCNDTGCGATCCTGCTG
LmrR-V15A-A92E-N14NDT_R	AGGATCGCAHNGGTTTGAGCAC
LmrR-V15A-A92E-A15NDT_F	CAAACCAATNDTATCCTGCTGAAT
LmrR-V15A-A92E-A15NDT_R	AGCAGGATAHNATTGGTTTGAGC
LmrR-V15A-A92E-M89NDT_F	TGAAAACNDTCGCCTGGAATTC
LmrR-V15A-A92E-M89NDT_R	GGTACTTTTGNHAGCGGACCTT
LmrR-V15A-A92E-F93NDT_F	GCCTGGAANDTGAATCCTGGAGT
LmrR-V15A-A92E-F93NDT_R	CAGGATTCAHNTTCCAGGCGCAT
LmrR-V15A-A92E-V99NDT_F	GGAGTCGTNDTGACAAAATCATT
LmrR-V15A-A92E-V99NDT_R	ATTTTGTCAHNACGACTCCAGG
LmrR-V15A-A92E-D100NDT_F	GAGTCGTGTGNDTAAAATCATTGA
LmrR-V15A-A92E-D100NDT_R	ATGATTTTAHNCACACGACTCCAG

5. Catalysis

Friedel-Crafts alkylation reactions

Representative procedure for in vitro ArM catalysis

The purified protein LmrR variant (12 or 120 µM) was incubated in catalysis buffer (20 mM MOPS, 150 mM NaCl, pH 7) containing 9 or 90 µM Cu(II)-Phen at 4 ^{o}C for 30 min. Next, substrates were added to a concentration of 1 mM. The reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C. 50 µL of internal standard solution (2-phenylquinoline, 2 mM in CH_3CN) was added to the reaction mixture and the crude product was isolated by liquid-liquid extraction using diethyl ether (3 x 0.5 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude reaction mixture was re-dissolved in 150 µL of a heptane:propan-2-ol mixture (9:1) and analyzed by chiral separation on np-HPLC (Daicel Chiralpak AD) to determine yield and ee.

Representative procedure for ArM catalysis in cell-free extract

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant (1 mL, OD_{600nm} = 4) was washed and re-suspended in 500 µL of catalysis buffer. The cells were sonicated (sonication tip diameter 2 mm, 5 min, 5 s ON, 10 s OFF, Amplitude 40%) and the cell debris pelleted by centrifugation (13000 rpm, 45 min, 4 ^{o}C). 250 µL of the cell free extract was incubated with 90 µM Cu(II)-Phen at 4 ^{o}C for 1 h and continuously mixed on a rotator. The substrates were added to a concentration of 1 mM and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C. 50 µL of internal standard (2-phenylquinoline, 2 mM in CH_3CN) was added to the reaction mixture and the crude product was isolated by liquid-liquid extraction using diethyl ether (3 x 0.5 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude reaction mixture was re-dissolved in 150 µL of a heptane:propan-2-ol mixture (9:1), filtered with a 0.2 µ filter and analyzed by chiral separation on np-HPLC to determine yield and ee.

Representative procedure for in vivo ArM catalysis of Friedel-Crafts alkylation reactions

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant (0.5 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in catalysis buffer containing 90 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The cells were washed and re-suspended in catalysis buffer. The substrates were added to a concentration of 1 mM and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

Comparing catalytic activity of cell and supernatant fractions

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant (0.5 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in catalysis buffer containing 90 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The sample was centrifuged and the supernatant and cell pellet were separated. To both the supernatant and cell fraction we added substrates **1** and **2b** to a concentration of 1 mM and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

For some samples we did not perform the extraction. Instead, the samples were centrifuged and the supernatant and cell pellet again separated. To both the supernatant and the recycled cell fraction we added substrates **1** and **2a** to a concentration of 1 mM and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

Influence of Glutathione on ArM catalysis

The purified protein LmrR_A92E_M8D (120 µM) was incubated in catalysis buffer containing 1 or 10 mM glutathione at 4 ^{o}C for 30 min and continuously mixed on a rotator. Next, Cu(II)-Phen was added to a concentration of 90 µM and the sample mixed by continuous inversion at 4 ^{o}C for 30 min. Then the substrates 1 and 2 were added to a concentration of 1 mM and the reaction was mixed by continuous inversion at 4 ^{o}C.

Diels-Alder reactions

Representative procedure for in vitro ArM catalysis

The purified protein LmrR variant (120 µM) was incubated in catalysis buffer (20 mM MOPS, 150 mM NaCl, pH 7) containing 90 µM Cu(II)-Phen at 4 ^{o}C for 30 min. Substrates, aza-chalcone (**4**, final concentration, 1 mM) and cyclopentadiene (**5**, final concentration, 33 mM) were added and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C for 48 hours. 50 µL of internal standard (2-phenylquinoline, 2 mM in CH_3CN) was added to the reaction mixture and the crude product was isolated by liquid-liquid extraction using diethyl ether (3 x 0.5 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude reaction mixture was re-dissolved in 150 µL of a heptane:propan-2-ol mixture (9:1)

and analyzed by chiral separation on np-HPLC (Daicel Chiralpak AD-H) to determine yield and ee of the Diels-Alder product **6**.

Representative procedure for setting in vivo ArM catalysis of Diels-Alder reactions for Alanine scan

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant (2 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in catalysis buffer containing 360 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The cells were washed and re-suspended in catalysis buffer. The substrates, aza-chalcone (**4**, final concentration, 1 mM) and cyclopentadiene (**5**, final concentration, 33 mM) were added and and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C for 72 hours. 50 µL of internal standard (2-phenylquinoline, 2 mM in CH_3CN) was added to the reaction mixture and the crude product was isolated by liquid-liquid extraction using diethyl ether (3 x 0.5 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude reaction mixture was re-dissolved in 150 µL of a heptane:propan-2-ol mixture (9:1), filtered with a 0.2 µ filter and analyzed by chiral separation on np-HPLC to determine yield and ee.

Representative procedure for setting in vivo ArM catalysis of Diels-Alder reactions

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant (2 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in catalysis buffer containing 360 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The cells were washed and re-suspended in catalysis buffer. The substrates were added to a concentration of 1 mM and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

Comparing catalytic activity of cell and supernatant fractions

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR variant (1 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in catalysis buffer containing 180 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The sample was centrifuged and the supernatant and cell pellet were separated. To both the supernatant and cell fraction the substrates, aza-chalcone (final concentration, 1 mM) and cyclopentadiene (final concentration, 33 mM) were added and and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C for 48 hours. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

For some samples we did not perform the extraction. Instead, the samples were centrifuged and the supernatant and cell pellet again separated. To both the supernatant and the recycled cell fraction the substrates, aza-chalcone (final concentration, 1 mM) and cyclopentadiene (final concentration, 33 mM) were added and and the reaction (300 µL) was mixed by continuous inversion at 4 ^{o}C for 48 hours. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

6. ICP-OES quantification of cellular copper content

An overnight induced *E. coli* C43(DE3) cell culture over-expressing LmrR (10 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in buffer containing 90 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The sample was centrifuged, and the supernatant and cell pellet were separated. The cell pellet was washed 10 mL of catalysis buffer and the supernatant from the current and previous step was pooled. The wet cell pellet and the pooled supernatant were transferred to pre-weighed pressure vials and dried in an oven at 80 ^{o}C for 48 h. The dry weight of the cell pellet was measured. The dried contents of the pressure vials were dissolved in 2.5 mL of 5% HNO_3 (in double distilled water). The samples were boiled at 115 ^{o}C for 1-2 days, after which the sample was filtered and analyzed by ICP-OES to determine the amount of Cu. The Cu content of the prepared 90 µM Cu(II)-Phen solution, used to incubate the cells with, was also measured by ICP-OES by Mr. J. van de Velde at the analytical department of the Stratingh Institute for Chemistry.

7. *Inhibitor studies*

In vitro and in vivo ArM catalysis with inhibitors

The purified protein LmrR (120 µM) was incubated in catalysis buffer containing 360 µM of dye (4 equivalents with respect to Cu(II)-Phen) at 4 ^{o}C for 30 min and continuously mixed on a rotator. Next, Cu(II)-Phen was added to a concentration of 90 µM and the sample incubated at 4 ^{o}C for 30 min and continuously mixed. Then the substrates **1** and **2b** were added to a concentration of 1 mM and the reaction was mixed by continuous inversion at 4 ^{o}C. The workup of the crude reaction mixture was performed as described above and analyzed by np-HPLC.

An overnight induced *E. coli* C43 (DE3) cell culture over-expressing LmrR (2 mL, OD_{600nm} = 4) was washed with catalysis buffer and re-suspended in catalysis buffer containing 180, 360 or 720 µM dye (0.5, 1 or 2 equivalents with respect to Cu(II)-Phen). The sample was incubated at 4 ^{o}C for 30 min and continuously mixed on a rotator. The cells were washed with catalysis buffer and re-suspended in catalysis buffer containing 360 µM Cu(II)-Phen. The sample was incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. The cells were washed and re-suspended in catalysis buffer. The substrates **1** and **2b** were added to a concentration of 1 mM and the reaction was mixed by continuous inversion at 4 ^{o}C. The workup of the crude reaction mixture was performed as described above, filtered with a 0.2 µ filter and analyzed by np-HPLC.

Live cell confocal microscopy

E. coli BW25113 was initially grown overnight from a glycerol stock in LB (lysogeny broth) at 30 °C at 180 rpm. The overnight culture was used to inoculate 3 mL of fresh LB next day and incubated at 30 °C at 180 rpm until the OD reached 0.8. At this stage, 30 µL of sterile 10 % arabinose was added and the cells were grown overnight at 30 °C at 180 rpm. The overnight culture was used to inoculate 3 mL of fresh LB containing 0.1 % arabinose next day and incubated at 30 °C at 180 rpm till OD reached 0.6. 500 µL of culture was then centrifuged at 11000 × *g* for 1 min. The supernatant was removed and the cells were re-suspended in 250 µL LB medium. The cells were now incubated with 3.5 µL of 25.36 mM EtBr stock solution (final concentration 360 µM) and 2.3 µL of 1.62 mM Hoechst 33342 stock solution (final concentration 15 µM) for 5 min. The cells were now washed in 1000 µL LB medium thrice and then re-suspended in 100 µL LB in the final step of washing. All procedures were carried out at 30 °C. Confocal imaging was performed on (3-aminopropyl)triethoxysilane (APTES)-treated cover slides(APTES) coated

slides on a LSM710 Zeiss confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The slides were prepared by first sonicating for 1 h in 5 M KOH, rinsing 10 times with MQ and blowing off the remaining MQ with pressurized nitrogen. The slides were then incubated in acetone that contained 2 % v/v (3-aminopropyl)triethoxysilane for 15 min at room temperature. Thereafter, the acetone and APTES was removed and the slides 10 times were rinsed with milliQ water. The remaining milliQ water was blown off with pressurized nitrogen. For imaging 20 µL of the cell suspension was used on the cover-slide and an object slide was placed on top for stability. The stage temperature was maintained at 30 °C. The slide was used for no longer than 20 min after depositing the cells.

Fluorescence spectra

The emission spectra of EtBr and Hoechst 33342 with and without LmrR were recorded on a TECAN Spark10M fluorimeter at 30 °C. In a typical measurement, a final concentration of 25 µM of LmrR and 1 µM of either dye was mixed in 20 mM MOPS, 150 mM NaCl buffered at pH 7.0 and incubated for 5 min at 30 °C. Both EtBr and Hoechst 33342 were excited at 360 nm and the emission spectra were recorded from 540-750 nm and 380-650 nm (in 2 nm intervals) respectively. Both excitation and emission bandwidths were kept constant at 5 nm. The data was measured in triplicates over time to ascertain binding and effect on fluorescence emission was temporally stable.

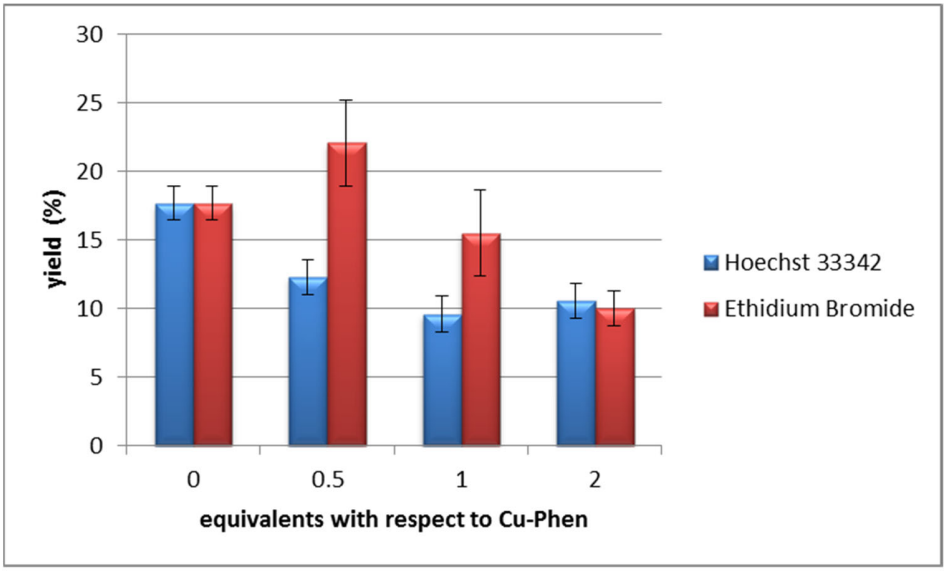
Fluorescence titration experiment

Titrations were performed with 3 µM solutions of protein dimer (2 mL) titrated with 80 µM solution of compound in the appropriate buffer solution. 30 µM protein conjugates solutions (dimer) in buffer solution (absorbance checked by Nanodrop 2000, Fisher Scientific) was prepared and then diluted tenfold. Emission spectra were recorded at room temperature after 2 min incubation. The sample was excited at 295 nm and emission spectra were obtained from 310 nm to 450 nm. The excitation and emission slit widths were 5 nm. All the experiments were performed in duplicate with independent batches of the corresponding compound and protein dimer. The intensity of fluorescence at the maximum of the emission spectra of the tryptophan (345 nm) was plotted against the concentration ratio between Cu(II) complex and protein dimer.

The data obtained from the titration of the corresponding compound with LmrR were fitted using non-linear regression analysis (program OriginPro 8.5), employing equation 1 [3].

$$\mathrm{FQ} = \frac{k}{2}\left(C_P + C_L + K_d - \sqrt{\left(C_P + C_L + K_d\right)^2 - 4C_P C_L} \right) \qquad (1)$$

where FQ is the tryptophan fluorescence quenching (ΔQF) by the compound (expressed as $(I_{max}-I)/(I_{max}-I_0)$, with I_{max} the intensity of fluorescence in the absence of quencher, I the intensity of fluorescence upon addition of quencher and I_0 the intensity of fluorescence at saturation). C_p and C_L are the total concentrations of protein and compound, respectively, and k is a constant.



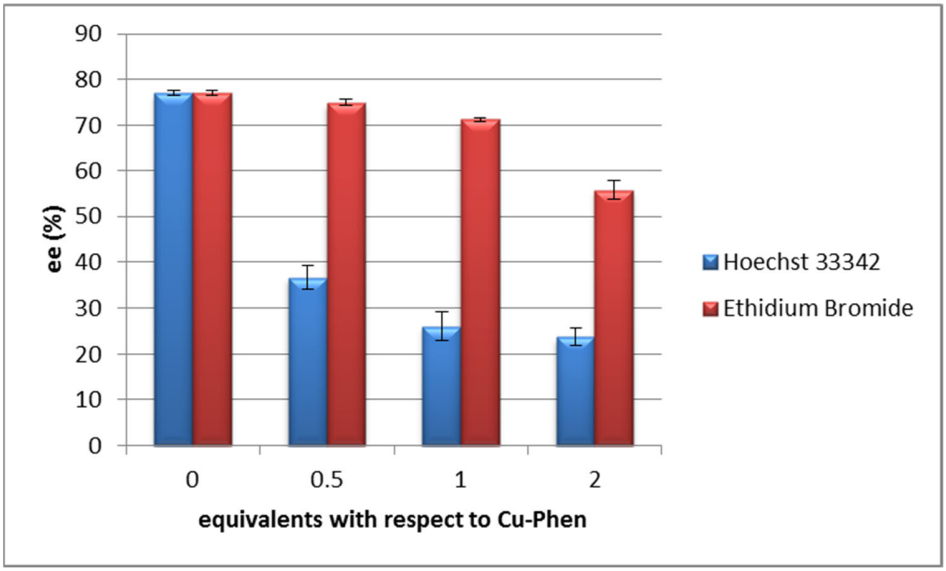
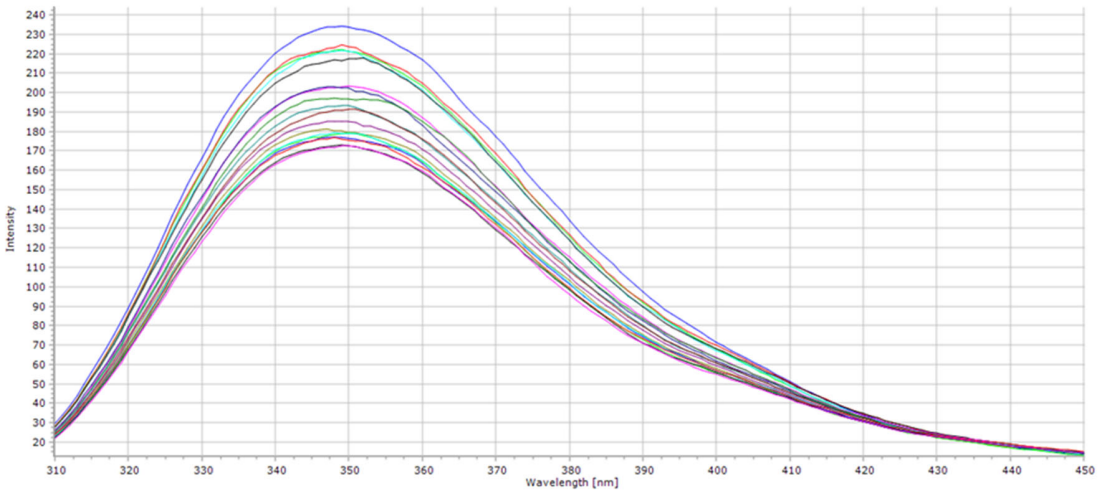


Figure S1. Effect of dyes on in vivo F-C Alkylation of 1 and 2a.

Determining K_d values with Fluorescence titration experiments



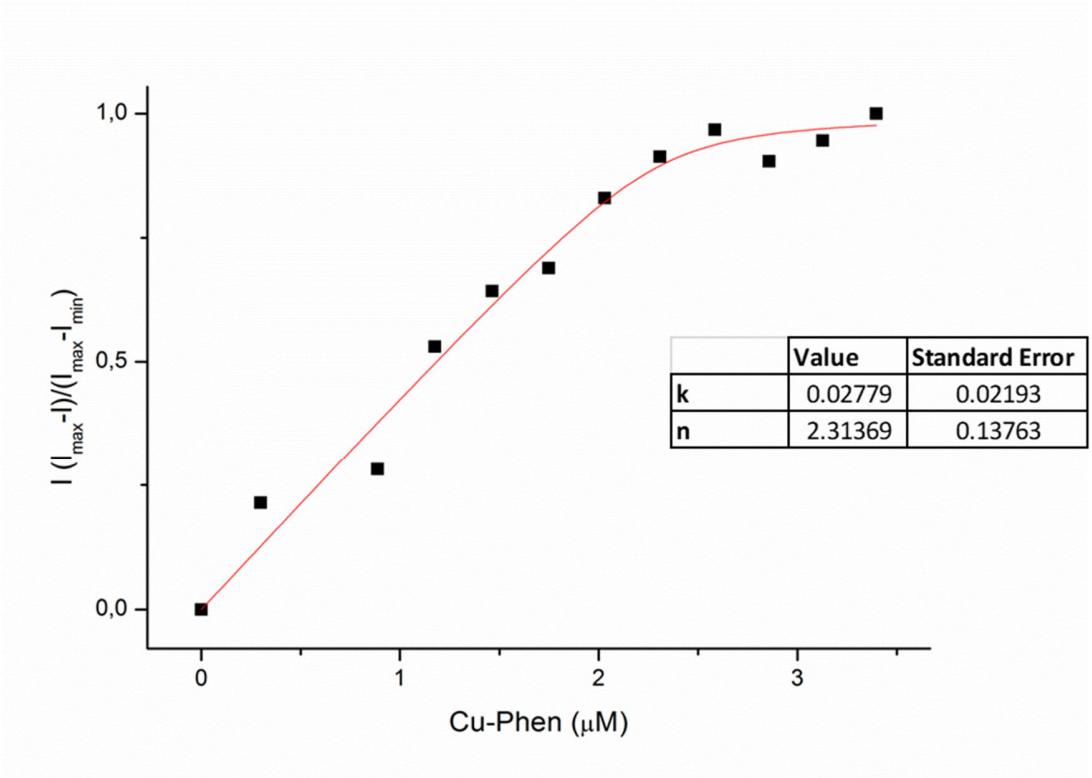


Figure S2. Titration of Cu(II)-Phen with LmrR_A92E_M8D

Binding constant of Cu(II)-Phen with LmrR_A92E_M8D

Binding constant K_d (nM)
16.30 ± 16.26

Determining K_d values by Fluorescence titration experiments

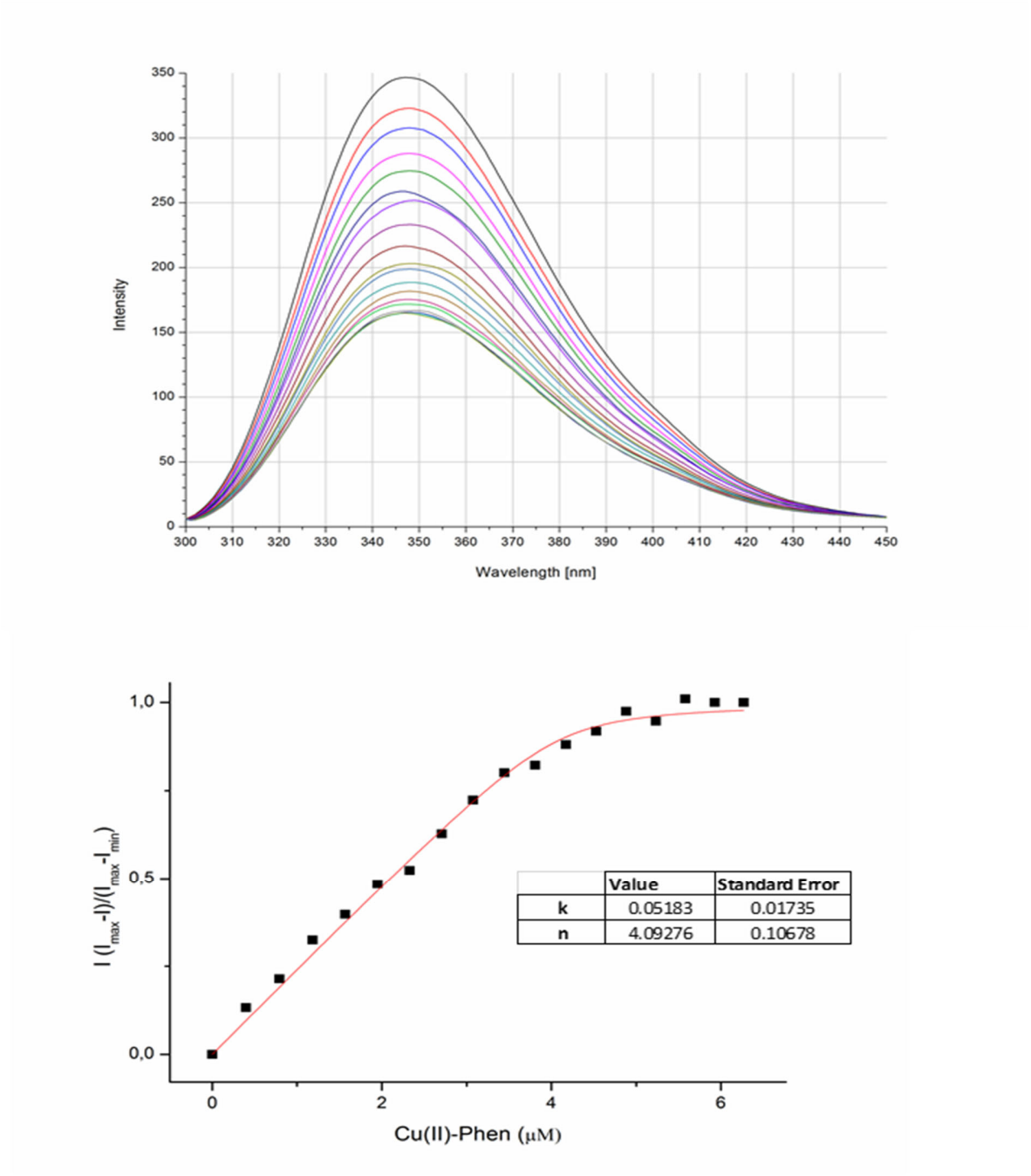


Figure S3. Titration of Cu(II)-Phen with LmrR_ V15A _A92E

Binding constant of Cu(II)-Phen with LmrR_ V15A _A92E

Binding constant K_d (nM)
56 ± 5

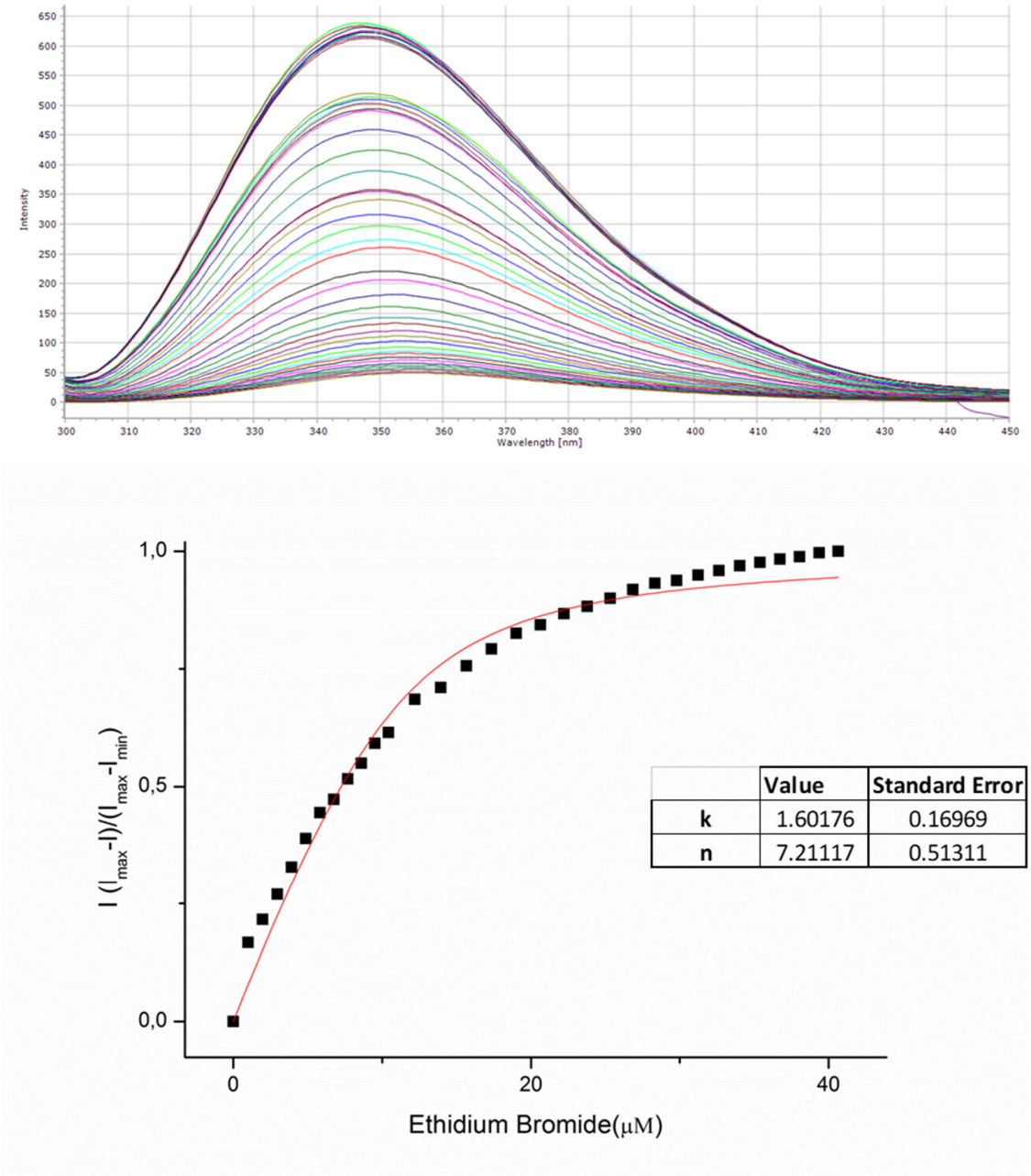


Figure S4. Titration of Ethidium Bromide with LmrR

Binding constant of EtBr with LmrR

Binding constant K_d (μM)
1.70 ± 0.13

Confocal Microscopy of stained live *E. coli* cells

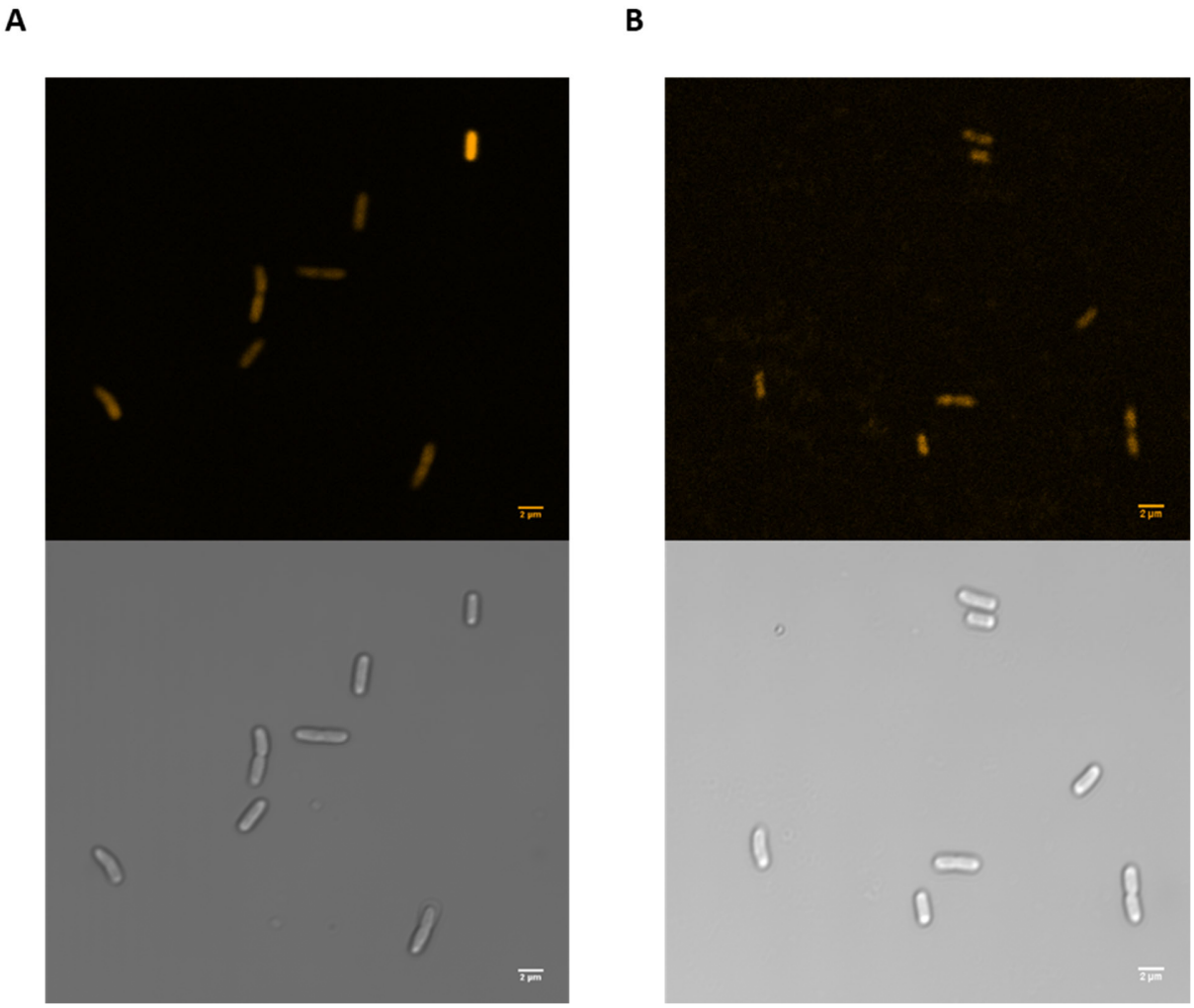


Figure S5. Confocal microscopy of live *E. coli* cells incubated with Hoechst 33342. A) Induced LmrR gene expression in *E. coli*. B) Uninduced LmrR gene expression in *E. coli*.

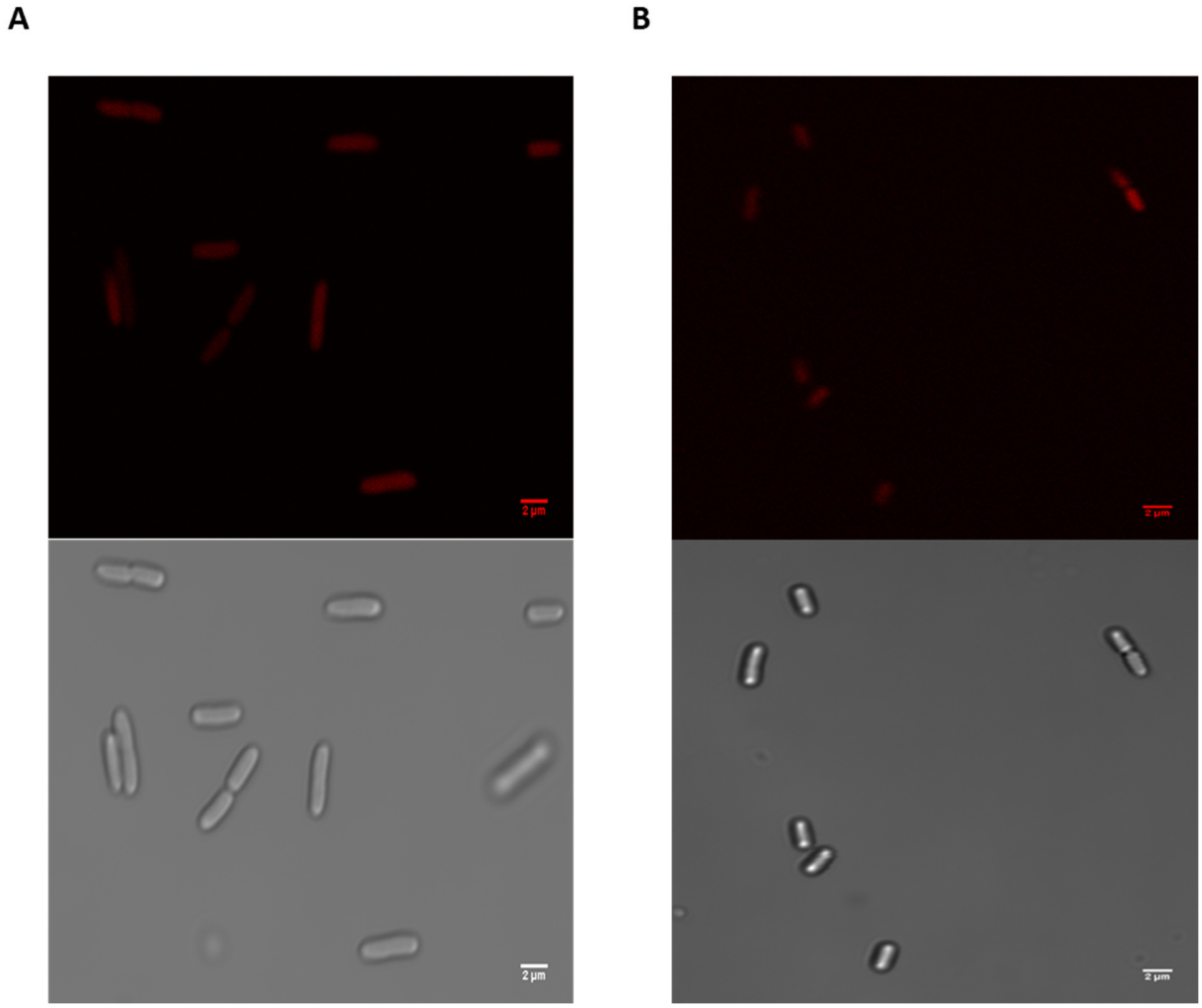


Figure S6. Confocal microscopy of live *E. coli* cells incubated with ethidium bromide. A) Induced LmrR gene expression in *E. coli*. B) Uninduced LmrR gene expression in *E. coli*.

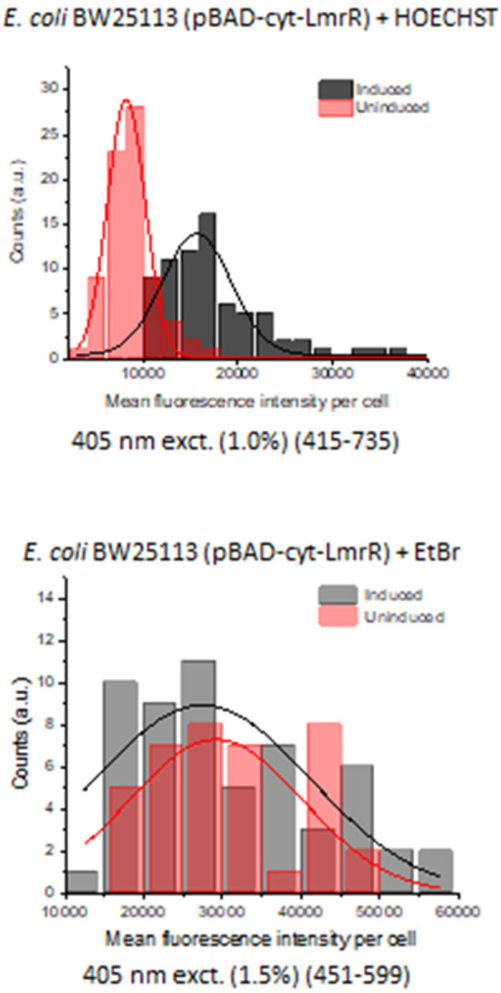


Figure S7. Comparing results from live cell confocal imaging of *E. coli* incubated with dyes.

From this comparison it can be inferred that Ethidium Bromide has difficulties crossing the double membrane barrier of *E. coli* and binding LmrR expressed in the cytoplasm. In contrast, Hoechst 33342 is permeable through the cell membranes and shows doubling in fluorescence intensity upon staining *E. coli* cells expressing LmrR in the cytoplasm.

Fluorescence measurement of free and LmrR bound dye

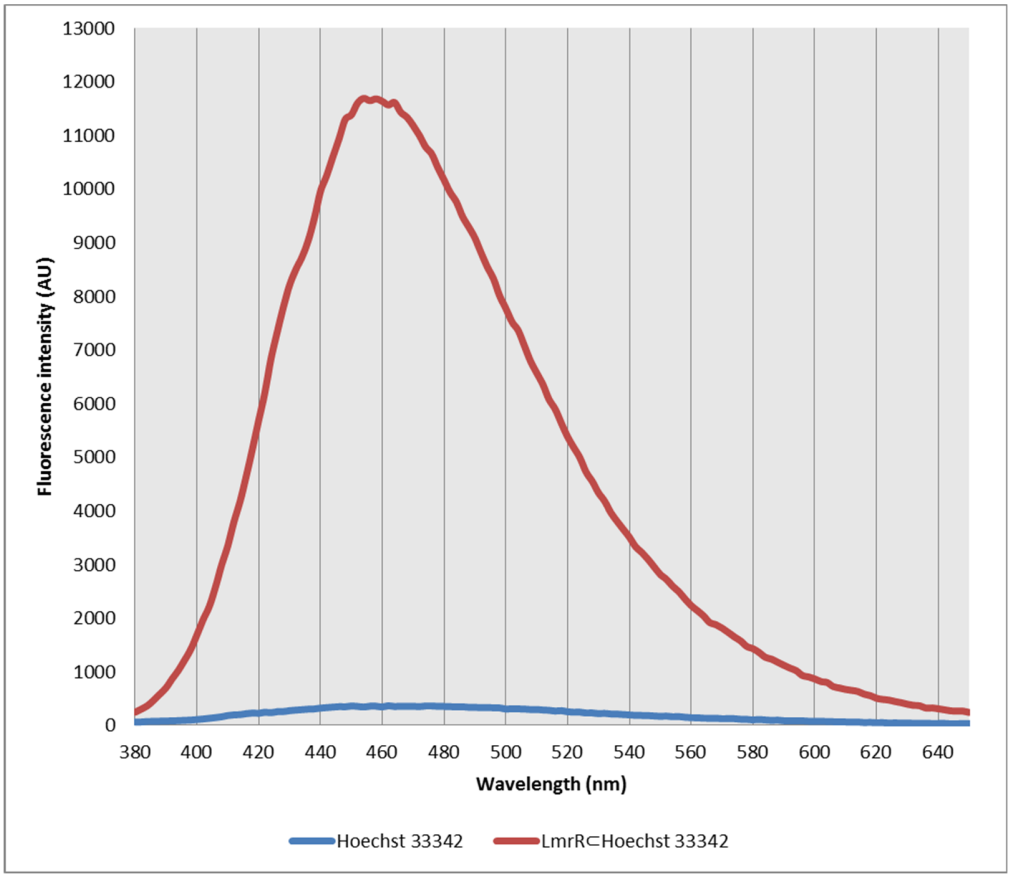


Figure S8. Fluorescence measurement of free and LmrR bound Hoechst 33342.

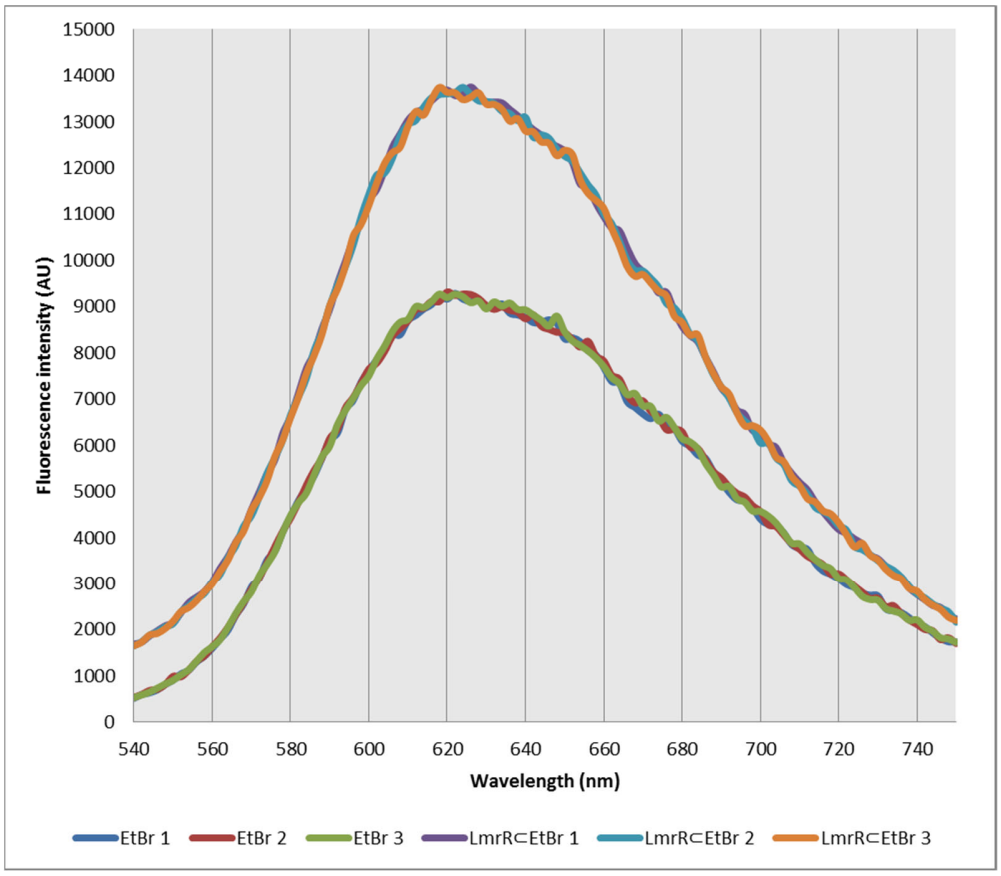


Figure S9. Fluorescence measurement of free and LmrR bound ethidium bromide.

8. *Cell fractionation*

To identify the localization of LmrR and SRP-LmrR over-expressed in the *E. coli* NEB10beta, periplasmic extraction and cell lysis was performed for SDS-PAGE analysis. Cell suspension (1mL, OD_{600nm} = 4) was spun down in an Eppendorf tube and the supernatant discarded. For some samples the cells were re-suspended in catalysis buffer containing 90 µM Cu(II)-Phen. These samples were incubated at 4 ^{o}C for 1 h and continuously mixed on a rotator. All the cell samples were washed and re-suspended in catalysis buffer. Then the cells were re-suspended in 250 µL of osmotic shock solution (200 mM Tris, 20% w/v sucrose, 1 mM EDTA, pH 7.5). The samples were slowly shaken in a Thermo mixer (23 ^{o}C, 400 rpm, 10 min). Next, the cells were spun down (4000 rpm, 5 min, 4 ^{o}C) and the supernatant discarded. The Eppendorf tubes were transferred to ice and the cell pellets carefully re-suspended with 250 µL of ice-cold 5 mM $MgSO_4$ solution. The sample was incubated on ice for 10 min, centrifuged and the supernatant (periplasmic fraction) collected. The remaining cell pellet was re-suspended in MQ to an OD_{600nm} of 6. Transfer 15 µL of the cell suspension to a new Eppendorf tube and add 5 µL of 5x SDS-PAGE loading dye. Boil the sample at 95 ^{o}C for 15 minutes and centrifuge (13000 rpm, 10 min, 25 ^{o}C) the cell debris to obtain the cytoplasmic fraction.

9. NMR studies

Table S9. Backbone assignments of LmrR used in this work, continued in Table S10.

Residue	N	H	C_α	C_β
A2	-	-	52	18.64
E3	120.47	8.3	55.37	29.91
P5	-	-	63.04	29.85
K6	126.35	7.92	56.92	29.85
L21	118.87	7.67	55.75	40.65
K22	123.28	8.11	58.41	-
Q23	115.3	7.35	55.61	27.01
G24	107.23	7.35	44.32	-
D25	122.56	8.45	54.48	39.16
N26	118.9	8.39	51.34	41.92
Y27	119.86	9.36	53.71	40.22
V28	122.29	9.34	68.56	30.04
Y29	117.18	7.7	61.1	36.84
G30	107.43	5.71	46.05	-
I31	123.28	7.83	64.95	37
I32	119.1	7.72	65.7	37.28
K33	120.44	7.61	59.16	31.66
Q34	118.85	7.97	58.33	27.63
V35	119.97	8.33	66.7	30.02
K36	124.24	8.1	59.64	31.44
E37	118.52	8.08	58.9	28.41
A38	121.62	8.3	53.58	18.6
S39	109.53	7.66	57.02	64.23
N40	-	-	54.11	36.73
G41	104.11	8.74	44.57	-
E42	118.97	7.42	57.53	29.78
M43	118.53	8.16	54.59	32.48

E44	128.94	8.37	53.86	29.32
L45	126.07	8.37	53.7	42.26
N46	118.12	7.92	51.44	40
E47	124.82	9.02	59.88	28.51
A48	119.92	8.06	54.72	17.14
T49	114.82	7.65	65.23	67.99
L50	121.93	7.47	57.94	39.42
Y51	116.73	8.1	61.52	36.43

Table S10. Backbone assignments of LmrR used in this work, continued from Table S9.

Residue	N	H	C_α	C_β
T52	114.31	7.48	65.72	68.43
I53	123.89	7.57	64.65	37.34
F54	119.45	8.86	58.12	35.66
R56	120.17	7.04	57.83	28.97
L57	118.26	8.28	57.72	41.53
E58	121.88	8.78	58.51	28.81
D60	117.03	7.79	54.75	40.66
G61	107.46	7.8	45.23	-
I62	122.47	8.1	61.67	37.34
I63	108.5	6.8	57.37	40.59
S64	116.88	9.29	56.37	65.56
S65	114.31	8.13	56.13	67.2
Y66	117.07	8.32	55.82	38.54
W67	120.19	8.66	56	30.02
G68	110.86	9.33	44.1	-
D69	120.14	8.35	53.86	41.14
E70	122.23	8.5	56.37	-
S71	118.11	8.38	59.3	-
G73	109.1	8.12	44.77	-

R75	121.04	8.22	55.45	-
R76	124.01	8.63	54.3	-
K77	126.51	8.22	54.74	-
Y78	125.95	8.44	56.62	-
Y79	115.22	8.49	56.25	-
R80	117.44	8.52	52.6	-
L81	124.99	8.52	54.81	-
T82	115.86	7.87	60.24	-
E83	121.12	9.02	60.32	28.17
I84	120.3	7.89	63.7	36.78
G85	111.42	8.05	48.46	-
H86	120.95	8.55	60.71	30.42
E107	118.98	7.79	58.7	-
A108	122.67	7.94	53.88	17.66
N109	116.85	8.13	53.81	37.49
K110	121.38	7.65	56.86	31.38
K111	120.88	8.01	56.91	31.57
S112	116.24	8.08	58.89	63.3
E113	122.94	8.16	56.68	29.23
A114	124.08	8	52.51	18.19
I115	120.36	7.87	61.22	37.45

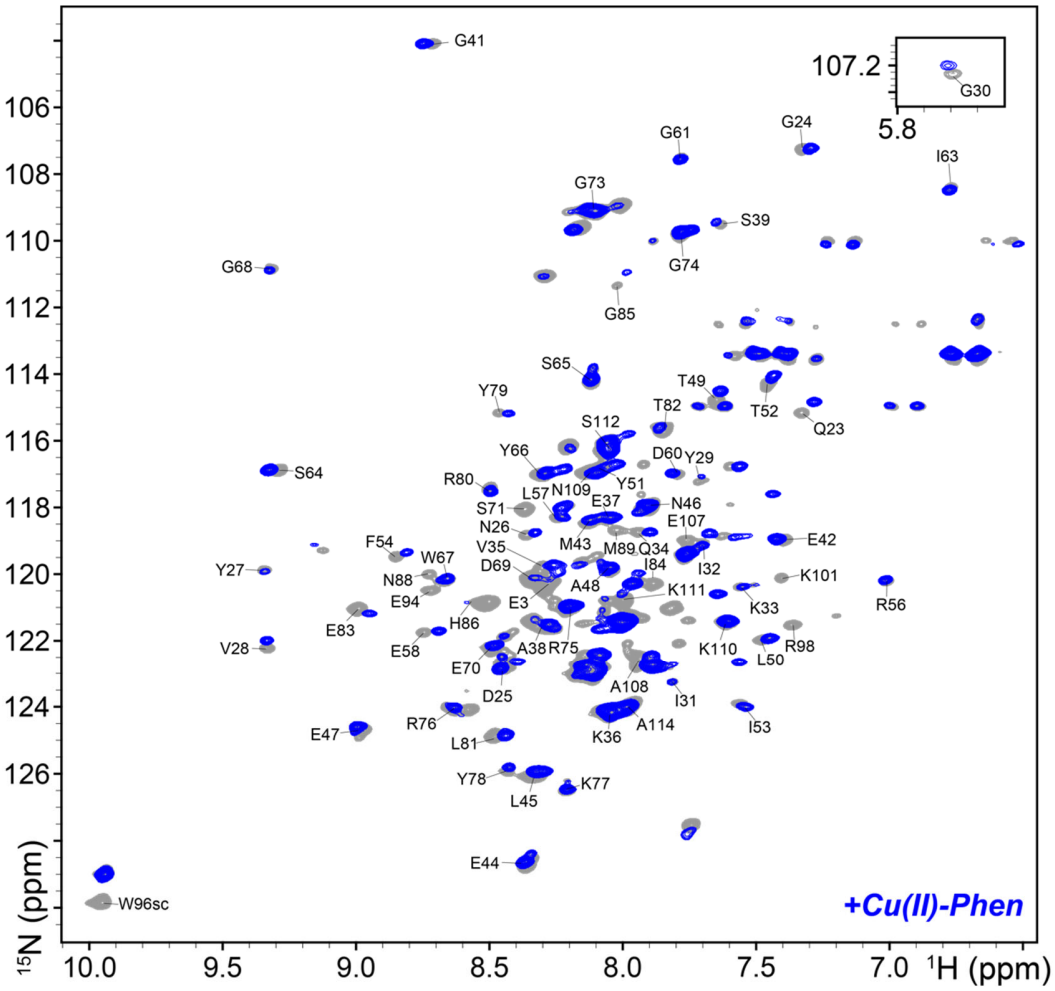


Figure S10. Solution state ^{15}N- TROSY experiment on perdeuterated LmrR with and without Cu(II)-Phen. Note that W96 side chain is a tentative assignment on the basis of strong signal loss after the addition of Cu(II)-Phen.

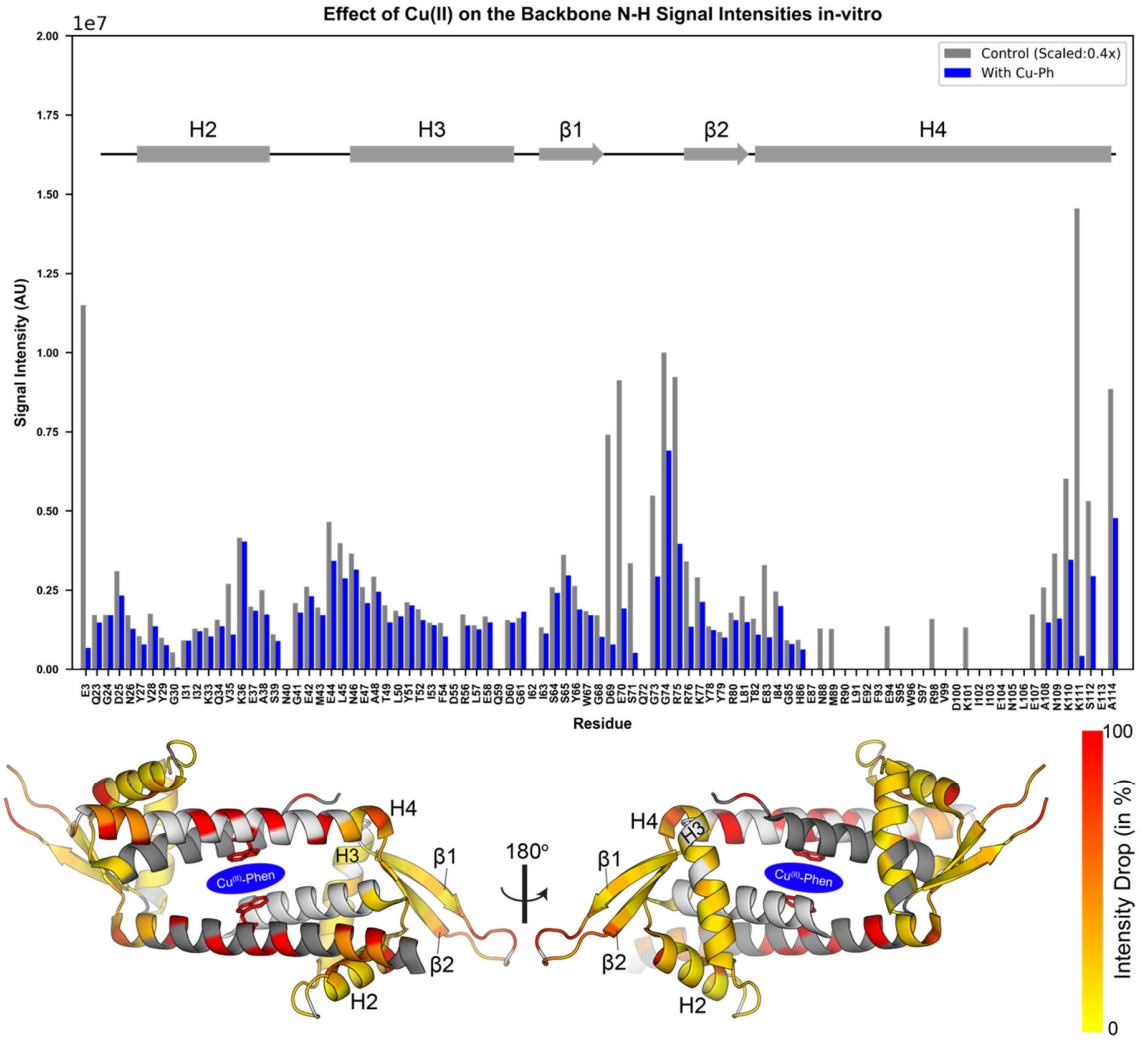


Figure S11. Residue-specific drop in NMR signal intensity of the backbone amides in LmrR due to paramagnetic quenching effects (in solution-state NMR). Data are plotted on the crystal structure of LmrR (PDB ID: 3F8F). Residues that are not assigned are shown in gray on the structure, with the exception of W96, where we observed quenching of side-chain resonance (See Figure S10 for the 2D spectrum).

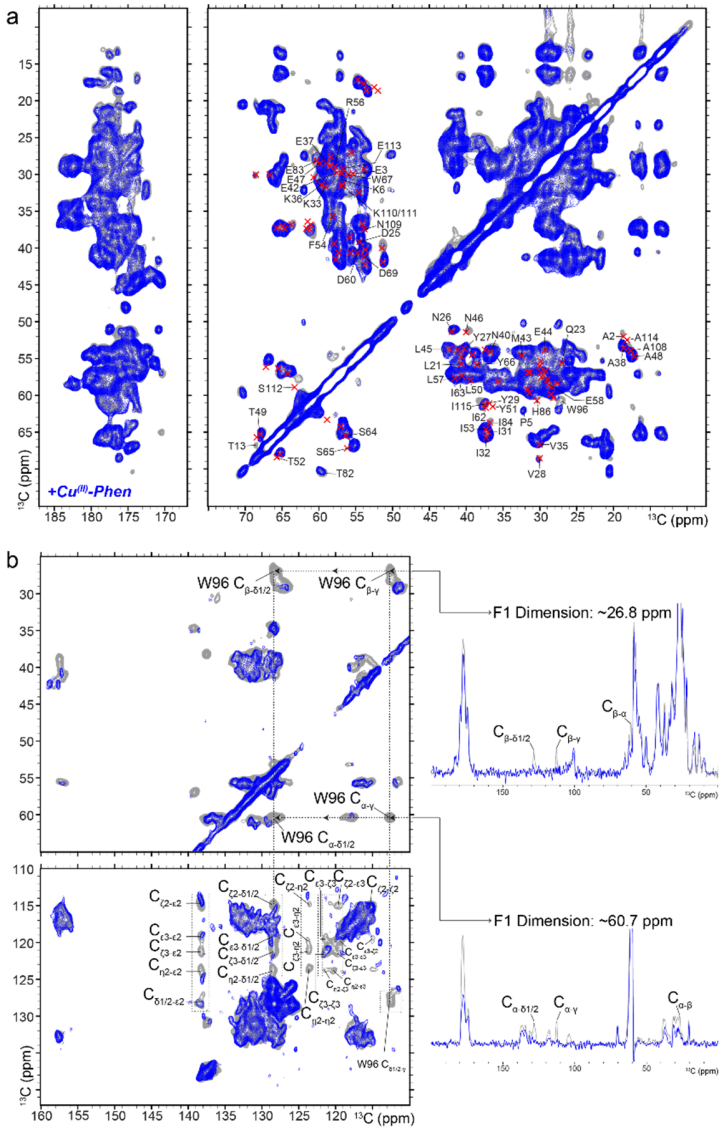


Figure S12. a) 2D ^{13}C,^{13}C correlated PARIS spectrum of LmrR in the microcrystalline state with (blue) and without (grey) Cu(II)-Phen. Red crosses are *in-vitro* solution NMR derrived assignments of the C_α-C_β correlations (Table S9-10). The residues tentatively assigned in the ssNMR spectra are indicated without crosses. b) Analysis of the aromatic region of the spectrum shows selective and partial quenching of aromatic side chains of Trp, with the complete quenching of the backbone resonances (of W96: alpha-helical Trp) correlating with the aromatics.

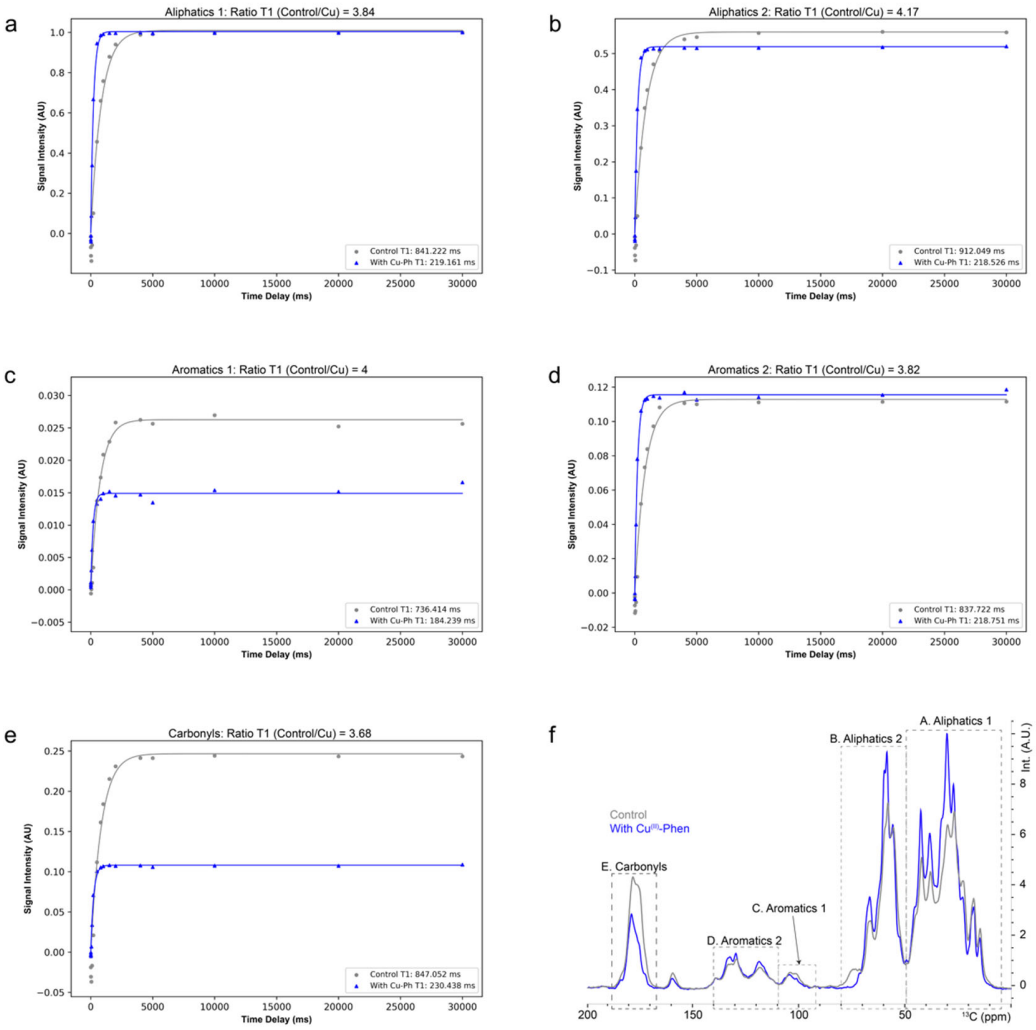


Figure S13. Relaxation profiles (a-e) of different spectral regions of LmrR in a ^{1}H-^{13}C cross-polarized saturation recovery experiment (f), before and after addition of Cu(II)-Phen. In the presence of Cu(II), the relaxation times are between 3.68-4.17 times faster.

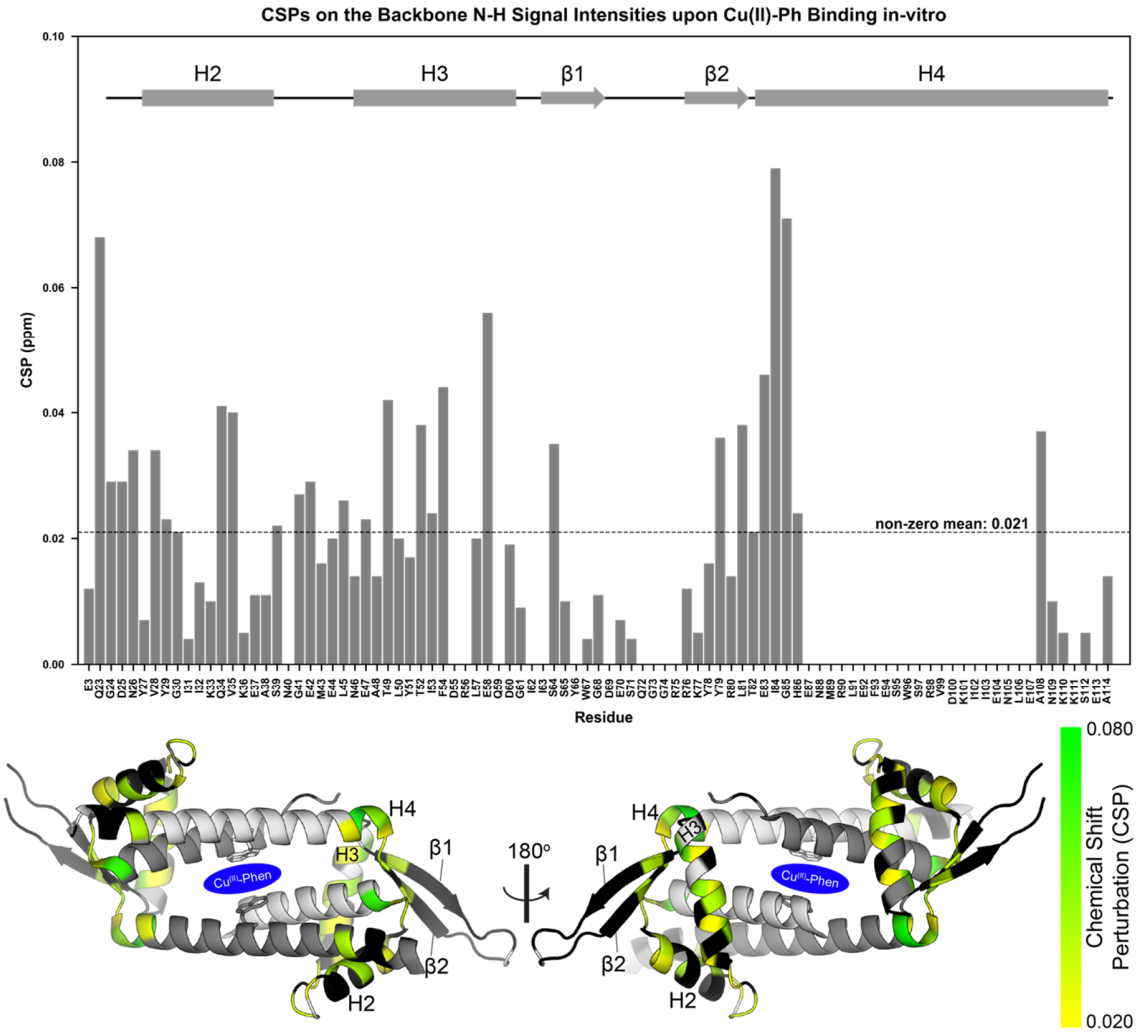


Figure S14. CSPs per residue of LmrR due to the binding of Cu(II)-Phen (in solution-state NMR). Residues in black exhibit CSPs of less than 0.02 ppm on the backbone amide. Data are plotted on the crystal structure of LmrR (PDB ID: 3F8F). See Figure S10 for the 2D spectrum.

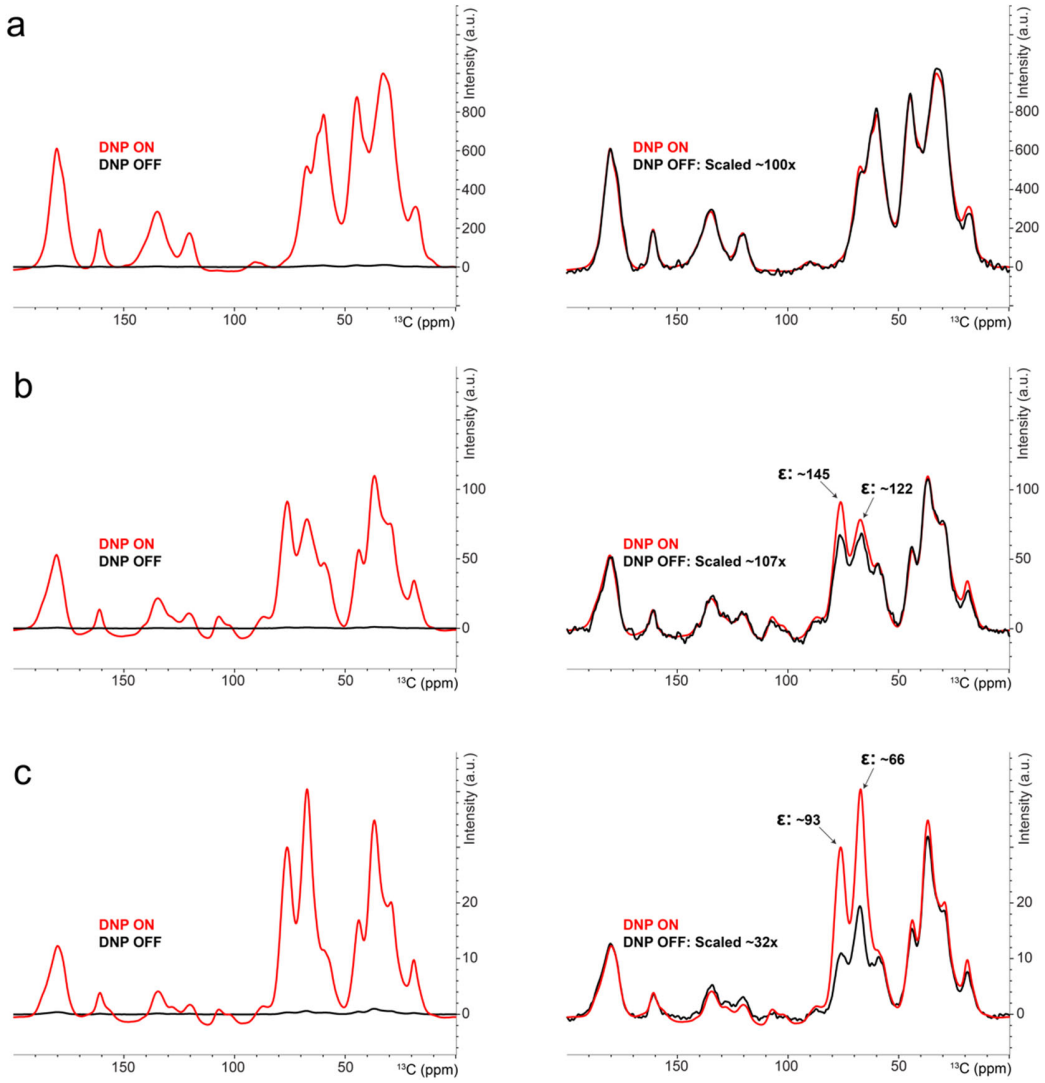


Figure S15 DNP enhancements, scaled on protein signals in the in-cell DNP samples referred to in the main text. a) Measured on deuterated cells where an algal amino acid mixture was used as the labelling source. b) & c) Measured on deuterated cells where ^{13}C Glucose and ^{15}N Ammonium Chloride were used as an isotope labelling source. In addition, when Cu(II)-Phen was introduced to the cells (c), enhancement dropped by a factor ~3 on the proteins and a factor ~2 on the non-protein background.

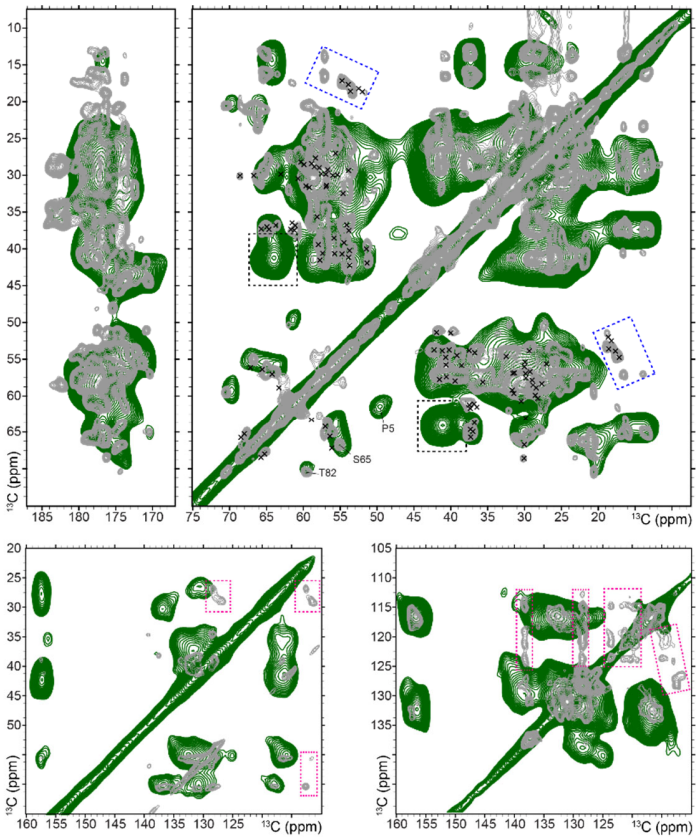


Figure S16. 2D ^{13}C-^{13}C, proton driven spin-diffusion experiment on LmrR in *E. coli* (green) overlaid with the 2D ^{13}C-^{13}C spectrum of microcrystalline LmrR (grey). C_α-C_β assignments obtained from solution NMR are superimposed (black crosses). Overall, the spectra are in good overall agreement with each other, and the most resolved peaks are indicated. Trp resonances were missing (red dashed boxes) because the algal mixture does not contain labeled Trp (see Materials and Methods). As often seen in DNP data sets, Ala methyl correlations were strongly reduced due to methyl group rotation at low temperatures (blue dashed boxes). Also, background labelling in the cells is strongly suppressed, except for a correlation marked using black dashed box, corresponding to the head group C_α-C_β correlation of phosphatidylethanolamine (PE).[8]

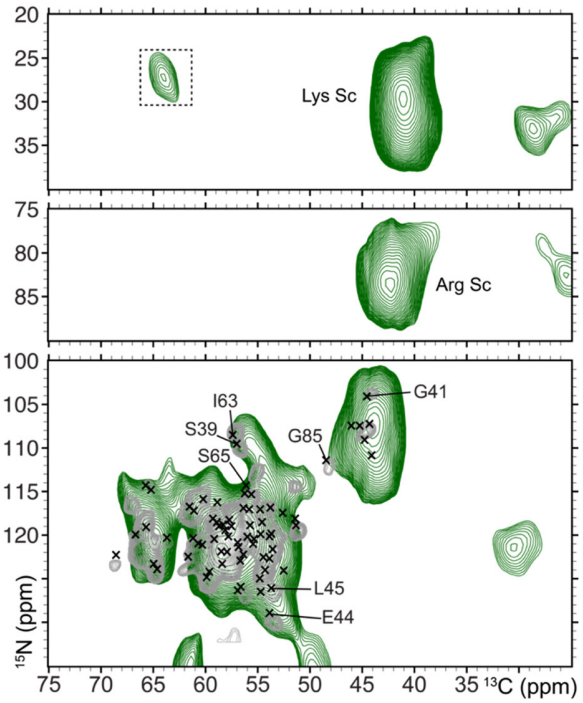


Figure S17. 2D aliphatic ^{15}N-^{13}C correlation experiment on LmrR in *E. coli* labelled using the ^{13}C-, ^{15}N- labelled algae sourced amino acid mixture (green). N-C_α assignments obtained from solution NMR are overlaid (black crosses). The overlaid spectrum in grey corresponds to the analogous in-vitro microcrystalline sample (only backbone region shown). The spectra are in good agreement with each other, strongly suggesting that the dominant species of LmrR in cells which is observed in NMR is LmrR that is properly folded. As with Figure S16, background labelling or free isotope labelled amino acids is strongly suppressed with the exception of a correlation that may stem from the lipid head group of phosphatidylethanolamine[8] (PE) marked by a black dashed box.

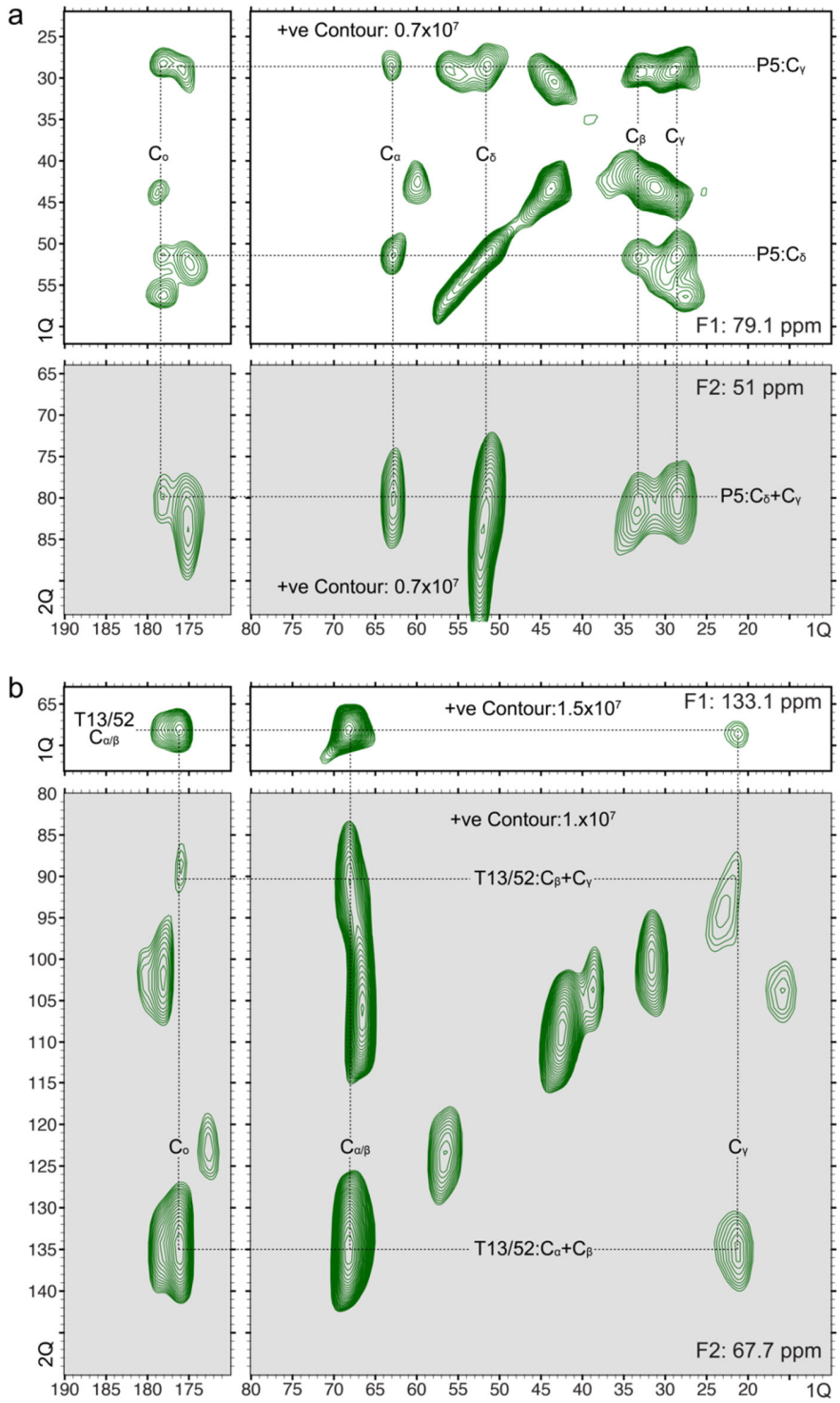


Figure S18. Signals in F_1-F_3 dimension (grey background) correlating with signals in the F_2-F_3 dimension (white background) for strips corresponding to P5, & T13/T52 shown in Figure 3b.

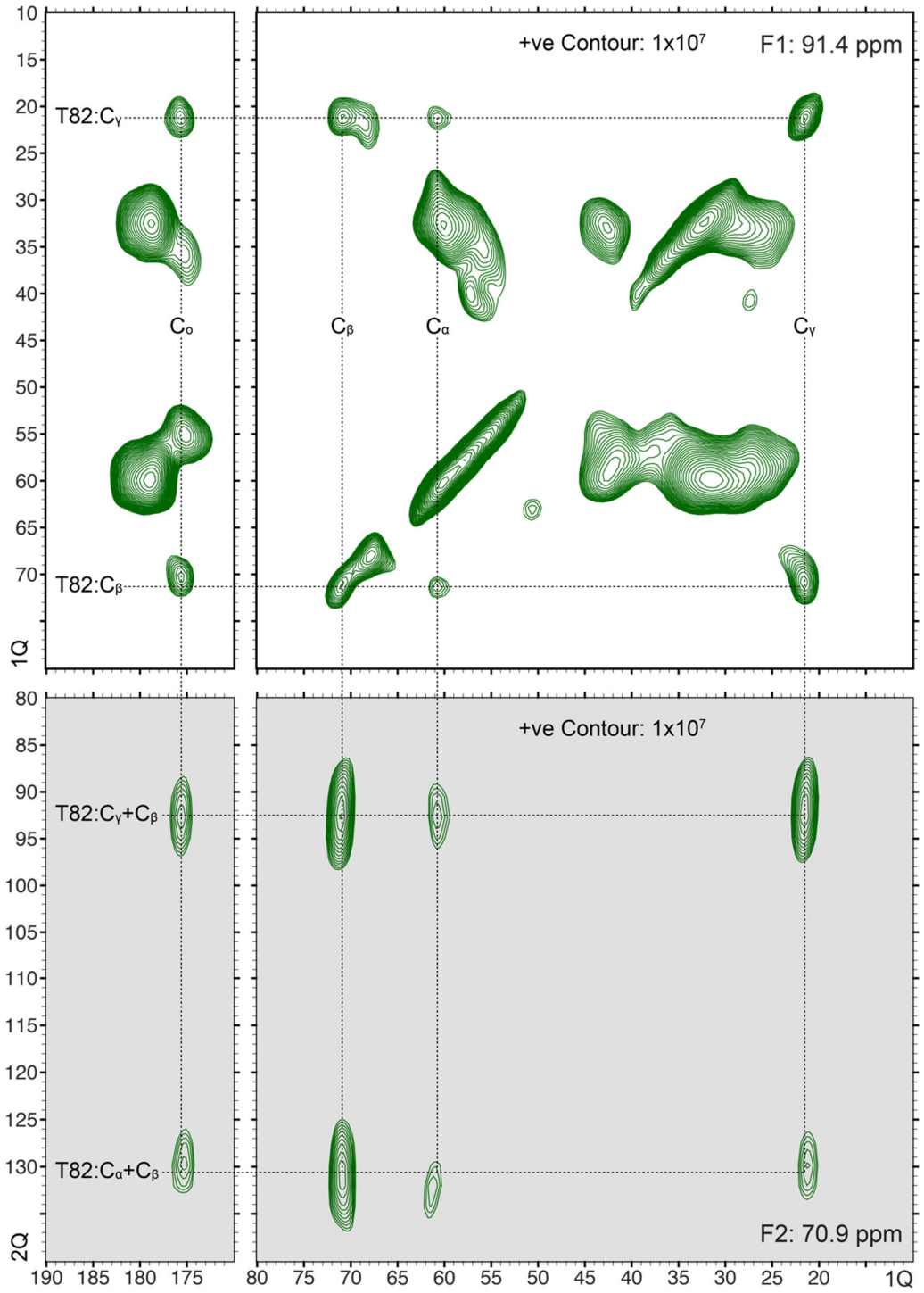


Figure S19. Signals in F_1-F_3 dimension (grey background) correlating with signals in the F_2-F_3 dimension (white background) for strips corresponding to T82 shown in Figure 3b.

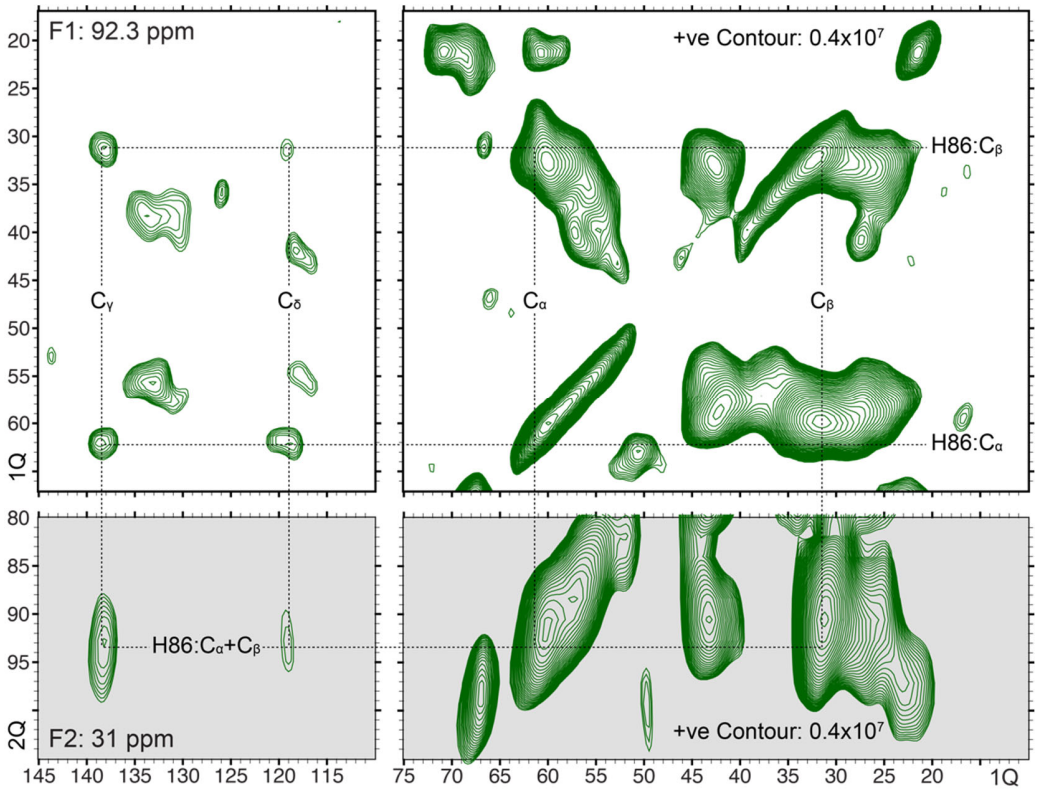


Figure S20. Signals in F_1-F_3 dimension (grey background) correlating with signals in the F_2-F_3 dimension (white background) for strips corresponding to H86 shown in Figure 3b.

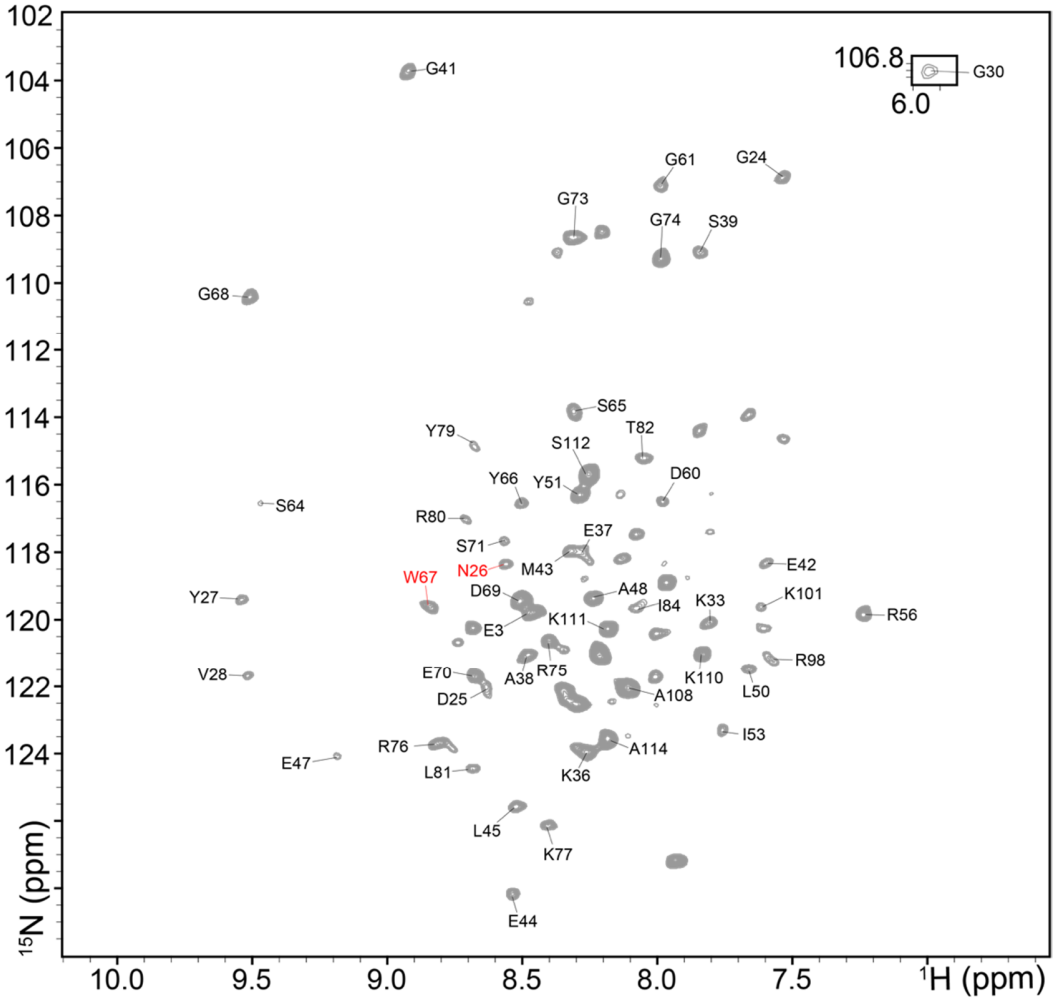


Figure S21 ^{15}N-^{1}H HSQC Experiment on cell lysates containing LmrR in-vivo labelled using the ^{13}C-, ^{15}N-labelled algae sourced amino acid mixture. The lysates were prepared from identical cells used in DNP-ssNMR experiments. All Asn, Gln and Trp resonances except for the W67 and N26 backbone amide are absent. Labelling of W67 and N26 (shown in red) can be attributed to scrambling, in specific transamination reactions from Glu and Asp that have been reported to occur.[9]

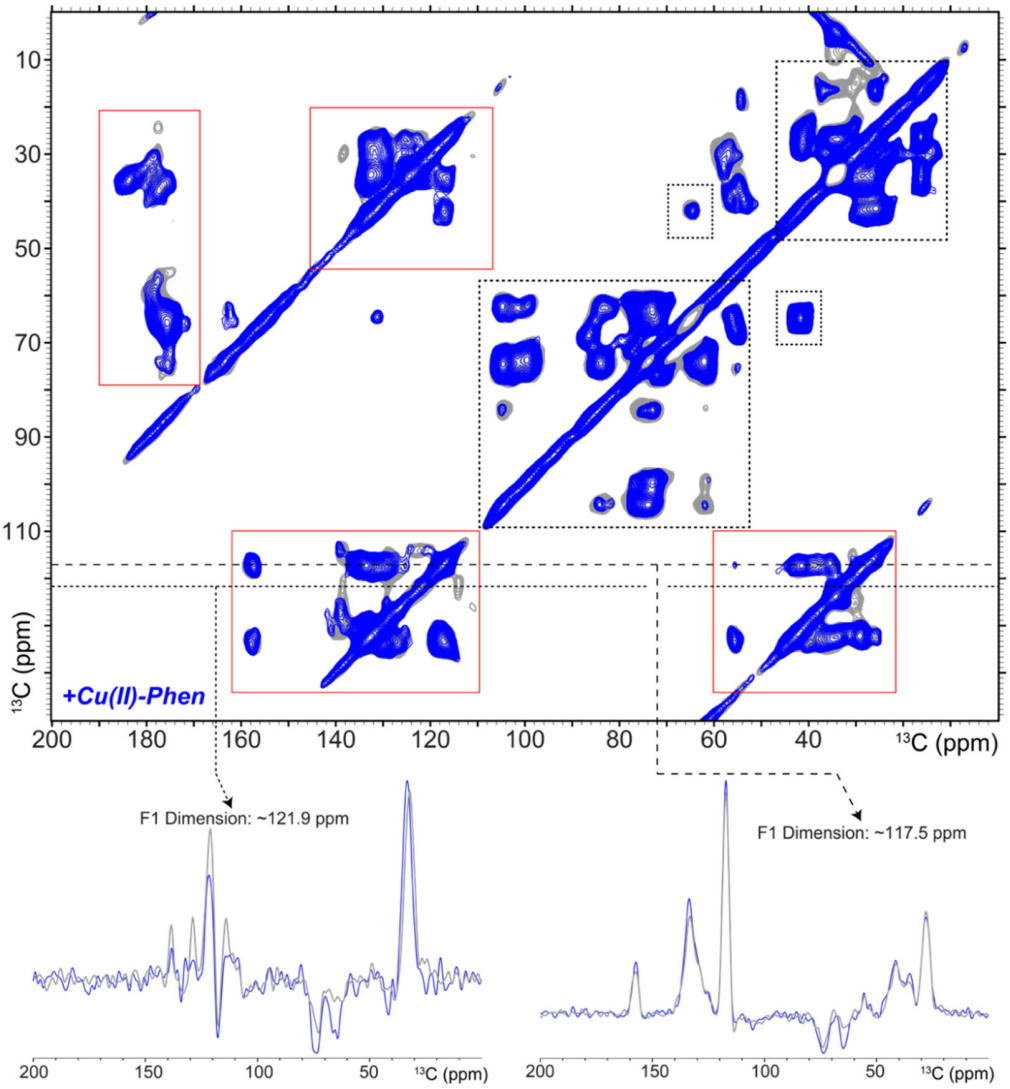


Figure S22 ^{13}C-^{13}C spin diffusion (PDSD) spectra on ^{13}C, ^{15}N labelled LmrR in *E. coli* cells. The aliphatic region was heavily dominated by lipid and sugar resonances (dashed boxes). The aromatic region between 110-160 ppm and the carbonyl region, solely correspond to protein resonances (shown in red boxes). 1D slices from purely Trp aromatic resonances (bottom left) and other aromatic resonances (bottom right) are scaled and show the selective quenching of Trp signal intensities compared to other aromatic residues (see also Figure 3c).

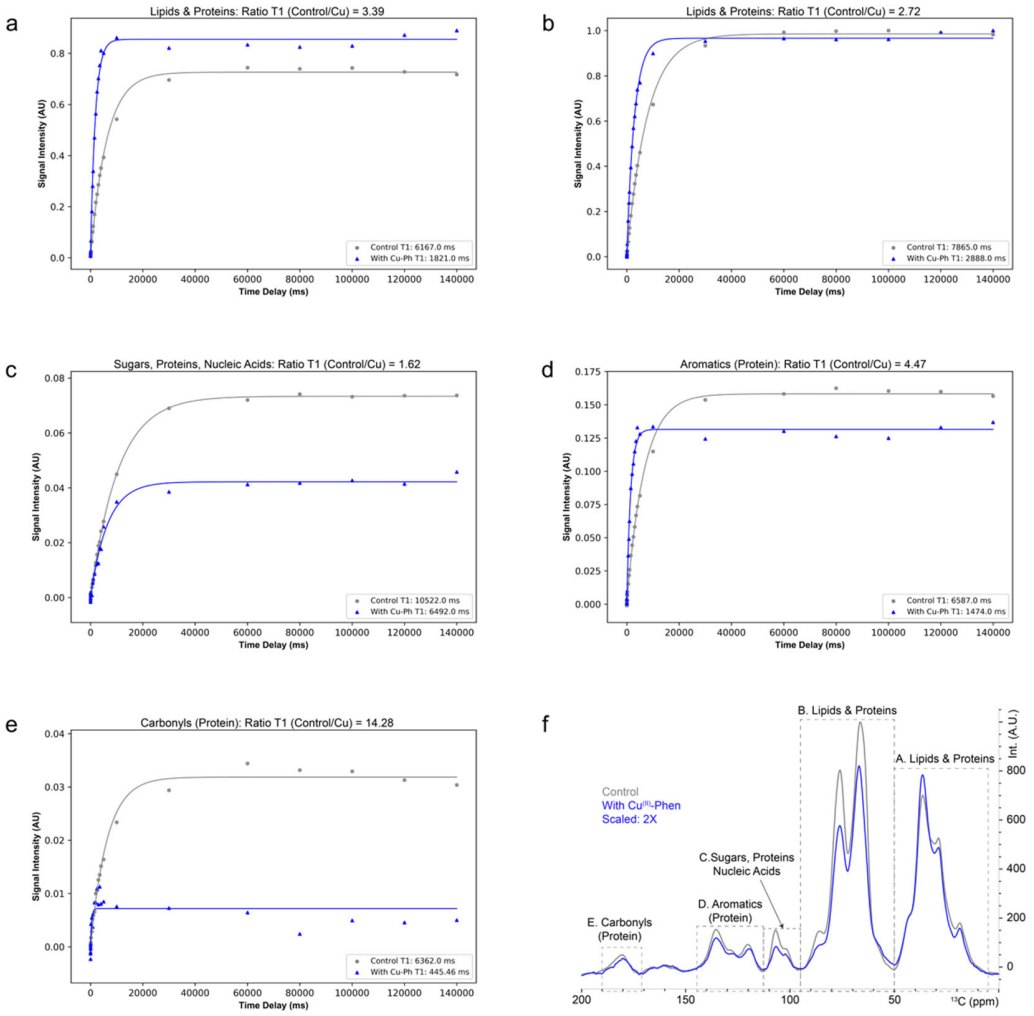


Figure S23 Relaxation profiles (a-e) of different spectral regions of the cellular samples in a ^{1}H-^{13}C cross-polarized saturation recovery experiment (f), before and after addition of Cu(II)-Phen. In the presence of Cu(II), the relaxation times are fastest in the protein rich signals such as aromatics & carbonyls.

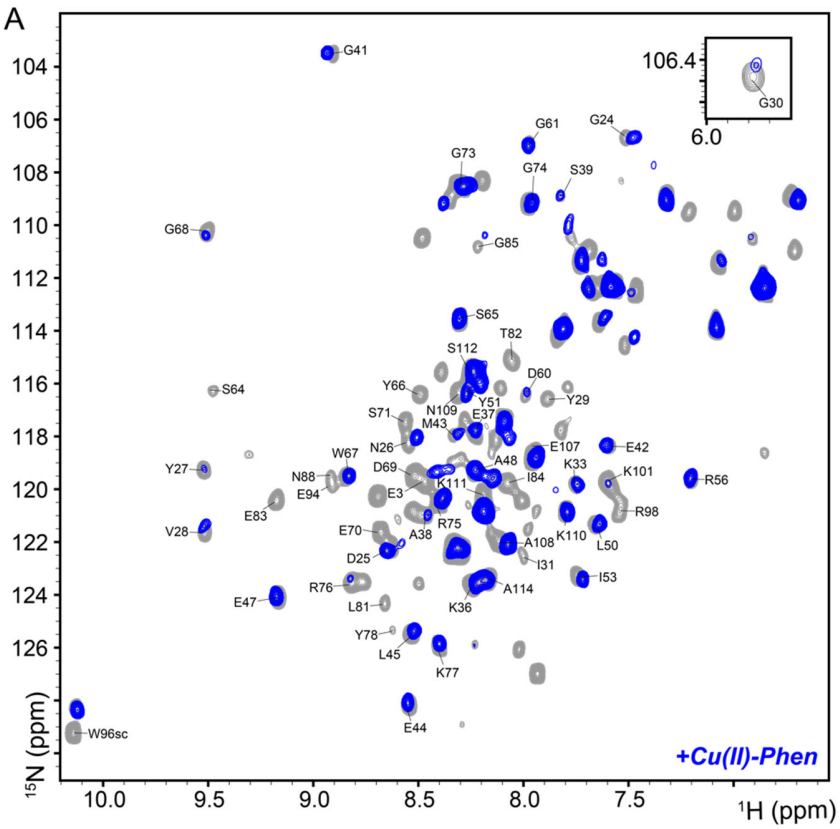


Figure S24. ^{15}N- HSQC spectra of cell lysate containing LmrR with and without Cu(II)-Phen. Note that W96 side chain is a tentative assignment on the basis of strong signal loss after the addition of Cu(II)-Phen, as also seen in the in-vitro spectrum (Figure S10).

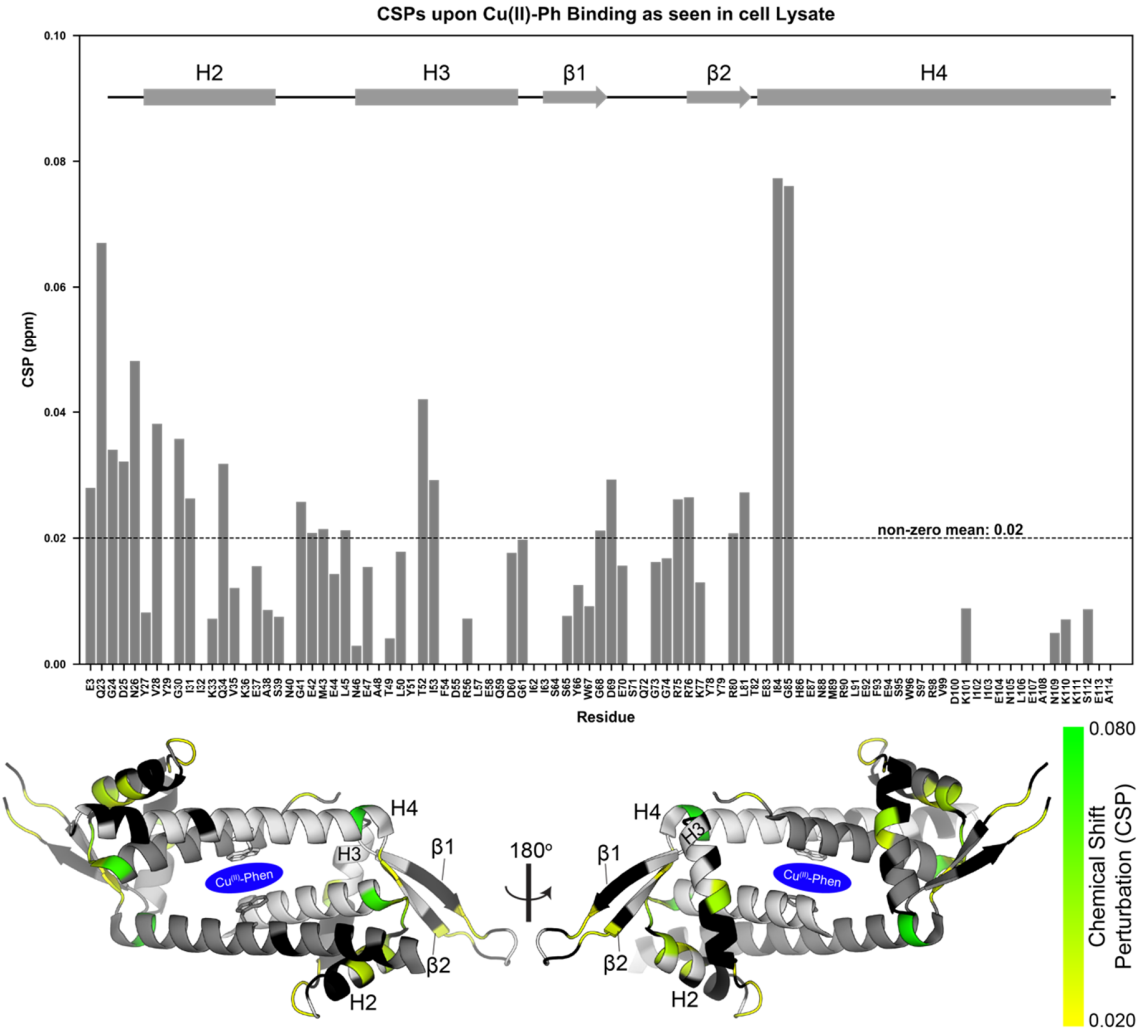


Figure S25. CSPs per residue of LmrR due to the Cu(II)-Phen binding in lysates (in solution-state NMR). Residues in black exhibit CSP's of less than 0.02 ppm on the backbone amide. Data are plotted on the crystal structure of LmrR (PDB ID: 3F8F). See Figure S24 for the 2D spectrum.

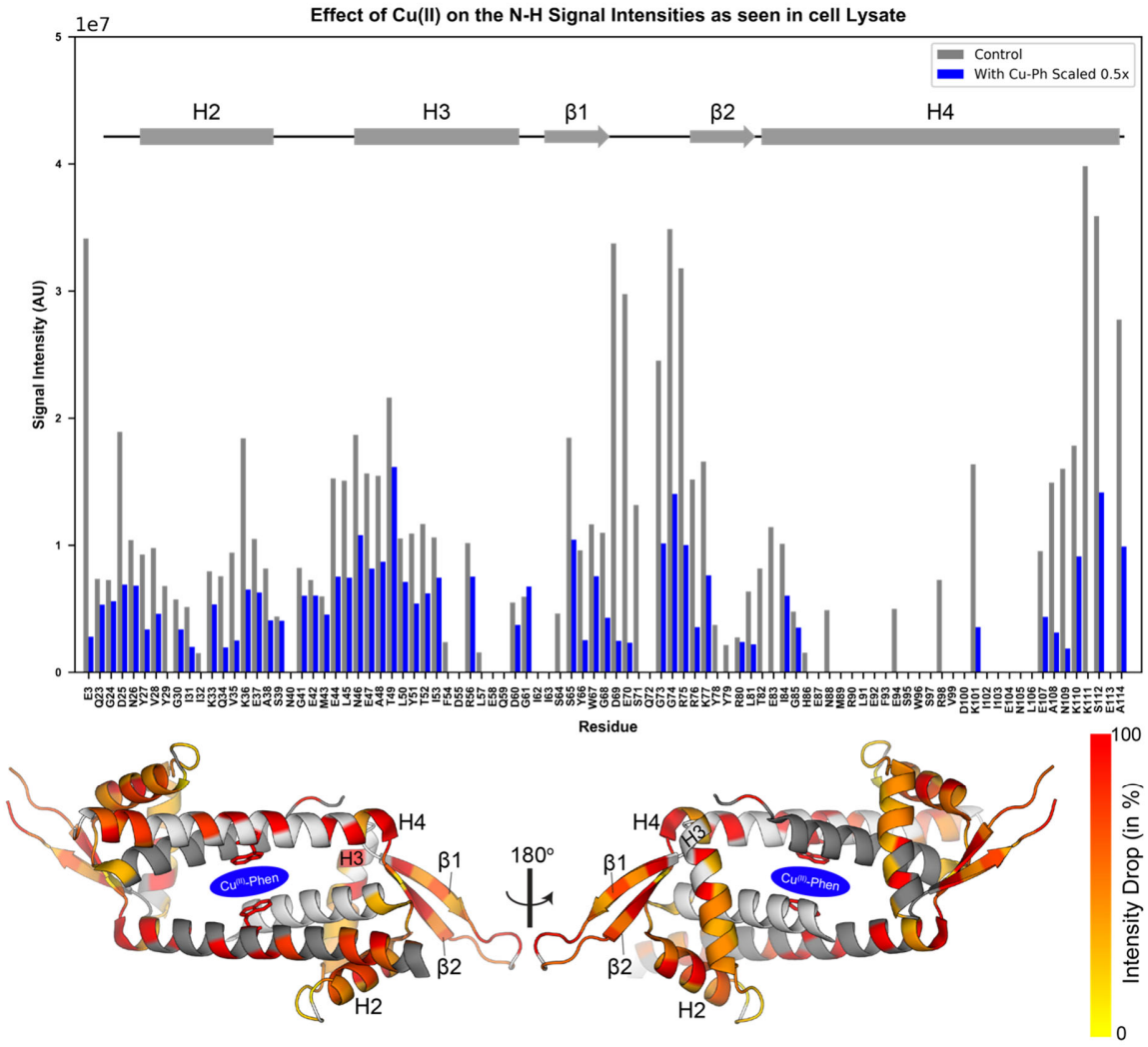


Figure S26. Residue-specific drop in NMR signal intensity of the backbone amides in LmrR in cell lysates due to Cu(II)-Phen (in solution-state NMR). Data are plotted on the crystal structure of LmrR (PDB ID: 3F8F). Residues that are not assigned are shown in gray on the structure, except for W96, where we observed complete quenching of the side-chain resonance. See Figure S24 for the 2D spectrum.

Materials and Methods NMR experiments

Expression and purification of isotope labelled LmrR

E. coli Lemo21 cells were transformed with pET17b_LmrR_LM_A92E including a C-terminal Strep-Tag where LM stands for "Lysine mutant", which is a variant of LmrR with the K55D and K59Q mutations to reduce DNA binding,[6] and A92E mutation that enables stronger binding to Cu(II)-Phen.[10] This variant will be referred to as LmrR in the following, for simplicity. A single colony of the fresh transformant was incubated in LB supplemented with 100 mg/L ampicillin. The culture was scaled up to 50 mL in LB and transferred to 1 L isotope labelled M9 medium when an OD600 of 0.5 was reached. For the samples used for in-vitro ssNMR studies, the M9 medium consisted of ^{13}C- glucose and ^{15}N- ammonium chloride. For the samples used to perform in-vitro solution-state NMR experiments we produced perdeuterated proteins using an M9 medium containing D_2O with ^{13}C- deuterated (d_7) glucose and ^{15}N- ammonium chloride. The cultures were induced at OD600 of 0.8-0.9 using 1 mM IPTG and expression was done overnight at 30^oC. Cells were harvested by pelleting and resuspending in the lysis buffer containing 50 mM $NaHPO_4$ pH 8.0, 150 mM NaCl along with a tablet of EDTA-free protease inhibitor cocktail (Roche).

For purification, the cell suspension was first freeze-thawed and subjected to sonication. Sonication was done 10 times, on ice, using 10-15 s long 13 kHz pulses with a 30 s gap to prevent heating. The lysates were then passed through a 19G syringe multiple times and spun down at 20,000 G for 30 mins. The clear lysate was then loaded on to a 5 ml Strep-Tactin Superflow cartridge pre-equilibrated with the lysis/ wash buffer- 50 mM $NaHPO_4$ pH 8.0, 150 mM NaCl. The column was washed with the wash buffer until no protein was seen in the UV-Vis output. The protein was eluted using 5 mM desthiobiotin in 50 mM $NaHPO_4$ pH 8.0, 150 mM NaCl. Prior to any subsequent experiments, the protein was buffer exchanged in 20 mM MOPS pH 7.0 with 150 mM NaCl by dialysis and gel filtration was performed using a Superdex 75pg column. Concentration of the yield was determined using a Nanodrop by measuring the absorbance at 280 nm (ε_{280} = 25440 M^{-1} cm^{-1}).

Assembling the metalloenzyme in-vitro

DMSO stocks of 100-120 mM Cu(II)-Phen (synthesized using an approach reported previously)[1] was freshly prepared. To assemble the metalloenzyme, a 1:10 molar ratio of LmrR:Cu(II)-Phen mixture was prepared and incubated for an hour at room temperature. The excess Cu(II)-Phen was subsequently washed away using multiple steps of concentrating and diluting in 20 mM MOPS pH 7.0 with 150 mM NaCl to 10 times the original volume using 15,000 Da centrifugal filters.

Protein precipitation for in-vitro ssNMR studies

Upto 20 mL of 0.5-1.5 mg/mL LmrR (with/ without Cu(II)-Phen) was added to an equal volume of 50% w/v Polyethylene Glycol (PEG) 2000 Monomethyl Ether (MME) prepared in 20 mM MOPS pH 7.0 with 150 mM NaCl. After overnight incubation (16-18 hours) at 4^{o}C, the microcrystalline LmrR (precipitated) was spun down, resuspended in 1 mL of fresh 25% PEG 2000 MME in 20 mM MOPS pH 7.0 with 150 mM NaCl and spun into 3.2 mm zirconia rotors for ssNMR experiments at ambient temperatures.

Targeted labelling of LmrR in E. coli cells with background deuteration

A single colony of freshly transformed *E. coli* Lemo21(DE3) cells harboring the plasmid containing LmrR was isolated and grown in an overnight LB culture with 100 mg/L ampicillin. 50 µL of the overnight culture was incubated in 50 mL of LB and grown up to OD600 of 0.05. The cells were then switched to an equal volume (50 mL) of unlabelled M9 medium prepared in D_2O using deuterated d_7 D- glucose and grown to an OD600 of 1.0. Expression was induced by adding 1 mM IPTG (final concentration) and the culture was incubated at 30^{o}C for 30 minutes. The cells were then moved to an 18^{o}C incubator and incubated for 5 mins to cool them down. Subsequently, 100 mg/L rifampicin (from a 1000x DMSO stock) was added and the flasks were incubated for another 15 mins. 500 mg/L of ^{13}C-, ^{15}N- labelled algal amino acid mixture (devoid of N, Q, C & W amino acids) was then added to the culture medium to achieve protein selective labelling. Alternatively, for the sample preparations that required labelling of Tryptophan (W), the cells were switched to precooled (18^{o}C) fully labelled M9 medium prepared in D_2O (supplemented with 100 mg/L rifampicin, 2 g/L of ^{13}C-D-glucose and 0.5 g/L of $^{15}NH_4Cl$). After 16h of expression at 18^{o}C, the cells were switched to a precooled, unlabelled and deuterated M9 medium containing a reduced concentration of rifampicin (50 mg/L) for 4h. The final step involved transferring the cells to unlabelled and deuterated M9 medium devoid of rifampicin for the last 2h of expression. The rifampicin concentration was thus gradually reduced, to remove bulk rifampicin from the cells which might otherwise interfere with Cu(II)-Phen.

Cu(II)- Phen incubation in cells for DNP-ssNMR

After expression, the 50 mL culture was spun down and resuspended in 10 mL of precooled (4^{o}C) 20 mM MOPS pH 7.0 with 150 mM NaCl prepared in D_2O with 10 µL of Cu(II)-Phen stock (350 mM in deuterated d_6 DMSO). 10 µL DMSO was used instead of a Cu(II)-Phen stock solution for the control samples. The incubation was carried out for 2h at 4^{o}C. The cells were then pelleted, washed twice using ice cold 20 mM MOPS pH 7.0 with 150 mM NaCl prepared in D_2O. Half the pellet was frozen for solution state NMR analysis of lysates and the other half was resuspended in 50 µL of deuterated DNP juice (60% deuterated d_8- and ^{12}C enriched glycerol, in D_2O) comprising of 15 mM AMUPol[11] in 20 mM MOPS pH 7.0 with 150 mM NaCl. Washing buffers were prepared in D_2O, to prevent back-exchange of deuterated amide protons, thereby maximizing cell deuteration.

Lysates for solution-state NMR

The other half of the harvested cell pellet, which was frozen during DNP sample preparation, was thawed on ice. This was followed by the addition of ice-cold buffer of 20 mM MOPS pH 7.0 with 150 mM NaCl prepared in 10% D_2O. Subsequently, cells were resuspended and sonicated on ice at 13 kHz with 10-15 s long pulses with a gap of 30 s to avoid excessive heating. After sonication, the cell debris was passed through a 19G syringe multiple times and finally spun down at 20,000 G for 30 mins. The pellet was discarded, and the clear lysate was spun down for another 30 mins to remove insoluble components.

DNP solid-state NMR experiments

DNP ssNMR experiments were conducted under low temperature (100 K) DNP conditions using a 3.2 mm triple-resonance (^{1}H, ^{13}C, and ^{15}N) magic-angle-spinning (MAS) probe head in a static magnetic field of 9.4 T, corresponding to proton/electron resonance frequencies of 400 MHz/263 GHz. All spectra were recorded at a MAS rate of 9 kHz using SPINAL-64 proton decoupling[12] at an r.f. field strength of 84 kHz.

Solid-state NMR experiments on in-vitro LmrR

We conducted in-vitro solid-state NMR experiments using a 3.2 mm triple-resonance (^{1}H, ^{13}C, and ^{15}N) E-free flip-angle MAS probe head in a static magnetic field of 16.4 T, corresponding to a proton frequency of 700 MHz. The set temperature was 253K and all spectra were recorded at a MAS rate of 13 kHz using a 77 kHz SPINAL-64 proton decoupling.[12]

1D- ^{1}H-^{13}C Cross-Polarization experiments

The 1D H-C (CP) spectra were recorded using a 2 s recycle delay and 10 ms acquisition time. Cross-polarization was achieved using contact times of 300-800 µs for in-vitro ssNMR experiments and 900-1200 µs for DNP-ssNMR experiments. 100 Hz line-broadening was applied prior to Fourier transformation.

2D- Proton-driven spin diffusion (PDSD) experiments in cells (DNP)

For experiments using cells labelled with the algal amino acid mixture, a H-C CP contact time of 1.2 ms and a mixing time of 30 ms was used. Acquisition times were 15 ms (760 points used- 405.3 ppm spectral width) and 3.5 ms (200 data points- 280 ppm spectral width) for direct and indirect dimensions respectively. FIDs from three different experiments with 192, 64 & 64 scans were added.

For all the other cellular samples, the following experimental conditions were employed. A short H-C CP contact time of 100 µs was used to avoid excitation of background lipids/ sugars. In addition, a mixing time of 60 ms was employed. Acquisition times were set to 15 ms (600 data points used- 405.3 ppm spectral width) and 2.8 ms (160 data points- 280 ppm spectral width) for the direct and indirect dimensions, respectively. 128 scans were recorded.

All in-cell spindiffusion spectra were processed using a 0.25 π shifted sine squared window function on both dimensions.

2D- ^{13}C-^{13}C PARIS experiments for microcrystalline LmrR

A 300 µs contact time was used for H-C CP, followed by PARIS[13] recoupling for a mixing time of 30 ms using a ^{1}H radio frequency amplitude of 7 kHz. Acquisition times were set to 10 ms (1302 points used- 354.98 ppm spectral width) and 5.4 ms (380 data points- 200 ppm spectral width) for direct and indirect dimensions respectively. 64 scans were recorded and a 0.33 π shifted sine squared window function was applied on both dimensions.

Calculating T_1 relaxation times

T_1 relaxation rates for ^{1}H were calculated from the data obtained from a ^{1}H-^{13}C CP Saturation-Recovery experiment. 100 Hz line-broadening was applied prior to Fourier transformation of the series of 1D spectra. Peaks were manually selected and integrated, and the T_1 relaxation tool in TOPSPIN 4.0.7 was used to estimate the T_1 relaxation rates.

2D- ^{15}N-^{13}C correlation experiments

Conventional ssNMR on samples prepared in-vitro involved the following experimental parameters. A ^{1}H-^{15}N CP step of 1.1 ms and a SPECIFIC-CP[14] ^{15}N-^{13}C transfer time of 1.5 ms were employed. On-resonance frequencies were set to 115 ppm and 56 ppm for ^{15}N and ^{13}C dimensions, respectively to select for N-C_α transfer. A recycle delay of 2 s was used, and 512 scans were acquired. Acquisition times were set to 10 ms (800 data points used- 338 ppm spectral width) and 9.2 ms (42 data points- 32 ppm spectral width) for the direct and indirect dimensions, respectively. In addition, linear forward prediction using 4 points and 4 coefficients were employed in the indirect dimension. The 2D spectra were processed using a 0.33 π shifted sine squared window function on both dimensions.

For DNP-ssNMR experiment on cells labelled with the algal amino acid mixture, the following experimental conditions were used. A ^{1}H-^{15}N CP step of 800 µs and a SPECIFIC-CP[15] ^{15}N-^{13}C transfer time of 2 ms were employed. On-resonance frequencies were set to 75 ppm and 40 ppm for ^{15}N and ^{13}C dimensions, respectively. A recycle delay of 2 s was used, and 256 scans were acquired. Acquisition times were set to 10 ms (512 data points used- 497 ppm spectral width) and 2.5 ms (36 data points- 180 ppm spectral width) for the direct and indirect dimensions, respectively. Linear forward prediction using 8 points and 8 coefficients was applied in the indirect dimension. The 2D spectra were processed using a 0.25 π shifted sine squared window function on both dimensions.

3D- ^{13}C DQ-SQ-SQ correlation experiment

The ^{13}C 3D 2Q-1Q-1Q correlation spectrum, was obtained using 10 blocks of SPC5[15] mixing (2.5 ms) for generation and subsequent reconversion of double quantum coherences. A contact time of 300 µs was used for H-C cross polarization and continuous wave (CW) ^{1}H decoupling was applied during the SPC5 sequence with a field strength of 85 kHz. Acquisition times were 10 ms (460 data points used- 307 ppm spectral width) in the F_3 1Q direct dimension, 2.7 ms (100 data points- 185 ppm spectral width) in the F_2 1Q indirect dimension and 0.9 ms (50 data points- 280 ppm spectral width) for the F_1 2Q indirect dimension. 64 scans were acquired. The spectrum was processed using a 0.28 π shifted sine squared window function in the direct dimension and a 0.25 π shifted sine squared window function in the two indirect dimensions.

Solution-state NMR experiments

We conducted solution-state NMR experiments at ambient temperatures (293-298 K) using triple channel (^{1}H, ^{13}C, and ^{15}N) probes. A static magnetic field of 21.1 T corresponding to a proton frequency of

900 MHz, with cryogenic probe was used to record 2D spectra. A static magnetic field of 17.6 T corresponding to a proton frequency of 750 MHz was used to record 3D experiments serving to obtain backbone assignments. We acquired 3D HNCA & HNCACB (TROSY) spectra of perdeuterated LmrR. We used the spectral assignments for ^{15}N and amide ^{1}H for wild type LmrR[16, 17] to assign the ^{13}C chemical shifts in our spectra. The ^{15}N-^{1}H chemical shifts were kindly provided to us by the Shimada group (University of Tokyo).

For the 2D in-vitro ^{15}N TROSY experiments, a total of 32 and 64 scans were recorded for control and Cu(II)-Phen treated samples respectively with a recycle delay of 0.3 s. On-resonance frequencies were set to 8.2 and 118.508 ppm for ^{1}H and ^{15}N respectively. Acquisition times were set to 80 ms (1024 data points used- 14 ppm spectral width) and 70 ms (512 data points used- 40 ppm spectral width) in the indirect and direct dimensions respectively. The 2D spectra were processed using a cosine squared window function on both dimensions.

For the 2D ^{15}N HSQC experiments, 32 scans were recorded in lysate samples, with a recycle delay of 1 s. On-resonance frequencies were set to 4.714 and 118.508 ppm for ^{1}H and ^{15}N respectively. Acquisition times were set to 71 ms (800 data points used- 16 ppm spectral width) and 20 ms (128 data points used- 35 ppm spectral width) in the indirect and direct dimensions respectively. The 2D spectra were processed using 0.33 π shifted sine squared window function on both dimensions.

Spectral visualization and analysis

All 2D and 3D spectra were processed using TOPSPIN 4.0.7 except the solution-state NMR 3D spectra used for assignments (Tables S9-10), which was processed using NMRPipe[18]. Spectra were visualized and analyzed using the NMRFAM-SPARKY[19] software, and the peaks pertaining to every experiment was generated using FANDAS 2.0[20] software package. Weighted chemical shift perturbations (CSPs) were calculated using the following formula:

$$\boldsymbol{CSP} = \sqrt{(\Delta\boldsymbol{\delta_N}/\mathbf{6.51})^{\mathbf{2}} + \Delta\boldsymbol{\delta_H}^{\mathbf{2}}}$$

where $\Delta\delta_N$ and $\Delta\delta_H$ are the differences in ^{15}N and ^{1}H chemical shifts, respectively.

10. Directed Evolution

Site-saturation mutagenesis of LmrR

LmrR_A92E was selected as the starting point for the directed evolution study. We employed the simplified NDT codon degeneracy to make libraries that explore a diverse array of 12 amino acids at 6 positions individually.[21] Randomization was introduced by application of the Quikchange Site Directed Mutagenesis Protocol (Agilent Technologies) using degenerate oligos described in (Table S7). The pooled plasmid DNA or PCR product was checked by Sanger Sequencing and a Q value was calculated.[22] The library was considered of good quality if the Q value was greater than 0.7 (see Table S11). To get about 95 % library coverage we analyzed 35 single colonies from each library.

Table S11 Site-saturation mutagenesis library quality

Number	Position	Degeneracy	Q (> 0.70)
1	8	NDT	0.71[1]
2	14	NDT	0.72[2]
3	88	NDT	0.74[1]
4	89	NDT	0.88[1]
5	93	NDT	0.86[2]
6	100	NDT	0.83[2]

[1] DNA sequencing of PCR product. [2] DNA sequencing of pooled plasmid DNA.

Culturing and protein expression in well plates

The 35 single colonies picked from each library, freshly transformed single colonies of LmrR_A92E (positive control) and LmrR_W96A (negative control) were cultivated overnight in a 96-well plate (sealed with a sealing film) containing 1500 µL of LB media and 100 µg/mL ampicillin at 37 ^{o}C, 1050 rpm. The next morning, 100 µL of the overnight culture was transferred to 24-well plates containing 4900 µL of LB media and 100 µg/mL ampicillin and incubated at 37 ^{o}C, 1050 rpm. After 4.5 h of growth, 50 µL of 1 mM IPTG was added to induce protein production and the cells were cultured at 30 ^{o}C, 1050 rpm overnight.

In vivo catalysis in well plates

The overnight induced cell cultures over-expressing LmrR variants (2 mL, OD_{600nm} = 4) were transferred to a 96-well reaction plate (sealed with a sealing film), washed with catalysis buffer and re-suspended in catalysis buffer containing 360 µM Cu(II)-Phen. The cells are incubated in a plate shaker (4 ^{o}C, 1050 rpm, 1 h). The cells are then again washed (to remove unbound copper complex) and re-suspended in catalysis

buffer. The substrates **1** and **2b** were added to a concentration of 1 mM and the reaction plate was kept in a plate shaker (4 ^{o}C, 1050 rpm, 24 h).

Extraction and HPLC

Internal standard, 2-phenylquinoline, was added to determine yield and ee values. The crude product was isolated by liquid-liquid extraction using diethyl ether (3 x 0.4 mL) in a 96-well plate using a manual multi-channel pipette. The organic phase was dried over Na_2SO_4 and filtered using a 96-well filter plate. The dried organic phase was evaporated under reduced pressure. The crude product was re-suspended in HPLC solvent and analyzed by chiral separation on np-HPLC to determine yield and ee.

Reliability of enantioselectivity based enzyme screening

Table S12: Reproducibility of catalysis data for various mutants screened from a single plate

F-C Alkylation of 1 and 2b to yield product 3b. Cell suspension 500 µL and OD_{600nm}=4, Cu(II)-Phen 90 µM, reaction time 24 h.						
Replicate no.	**LmrR_W96A**		**LmrR**		**LmrR_A92E**	
	yield (%)	**ee (%)**	**yield (%)**	**ee (%)**	**yield (%)**	**ee (%)**
1	1	6	5	64	17	85
2	1	7	4	73	14	80
3	1	6	3	74	16	86
4	1	5	4	74	15	85
5	2	8	5	58	15	86
6	1	13	4	71	16	86
	1±0	8±2	4±1	69±5	16±0	85±2
F-C Alkylation of 1 and 2b to yield product 3b. Cell suspension 2 mL and OD_{600nm}=4, Cu(II)-Phen 360 µM, reaction time 24 h.						
Replicate no.	**LmrR_W96A**		**LmrR**		**LmrR_A92E**	
	yield (%)	**ee (%)**	**yield (%)**	**ee (%)**	**yield (%)**	**ee (%)**
1	1	19	11	76	34	86
2	5	16	13	69	28	83
3	3	18	12	77	25	85
	3±1	18±1	12±1	74±3	29±4	85±1

The value of yield (%) and ee (%) is reproducible between replicates for various mutants of LmrR screened from a single plate.

Table S13. Reproducibility of positive and negative controls across all the in vivo screening experiments

	LmrR_W96A (Negative control)		LmrR_A92E (Positive control/Parent enzyme)	
	Yield of 3b (%)	**ee (%)**	**yield of 3b (%)**	**ee (%)**
Average	6	13	32	79
SD	4	6	9	7
CV %[23]	65	47	28	8

The trends in yield and ee values for the controls are reproducible throughout the screening assays. The catalysis data for the negative control are noisy as expected because of the low yields. The catalysis data for positive control/parent enzyme are reproducible across all the samples and reaction plates screened. The screening assay is therefore reliable to find a mutant that performs better than the positive control/parent enzyme.

Selecting clones from in vivo screening of libraries

Representative procedure (M8NDT library). 34 single colonies from this library were screened. Enantioselectivity was used as selection criterion. The top three colonies whose ee values were higher than the parent enzyme (LmrR_A92E) were selected. It was checked if these mutant containing cells gave a yield of at least 50 % as much as the cells expressing the parent enzyme (LmrR_A92E) on the same reaction plate. Thus, from the screening of the library M8NDT (See Table S12) LmrR_A92E_M8S, LmrR_A92E_M8D and LmrR_A92E_M8Y were selected for *in vitro* catalysis. The colonies that matched the selection criteria were sequenced, cultured on a larger scale, the protein variant was purified and *in vitro* catalysis performed to check yield and ee values.

Table S14. Comparing in vivo and in vitro activity of clones that match selection criteria from plate screening

Plate 1				
	Whole cell catalysis[1]		Purified protein catalysis[2]	
Protein variant	**Yield %**	**ee%**	**Yield %**	**ee%**
LmrR_A92E	32±1	80±0	11±1	91±1
LmrR_A92E_M8D	26±4	85±0	23±1	93±2
LmrR_A92E_M8S	24±1	86±0	17±0	92±1
LmrR_A92E_M8Y	24±1	85±0	17±0	93±0
Plate 2				
LmrR_A92E	39±3	86±0	11±1	91±1
LmrR_A92E_M8A	41±2	90±0	20±0	94±0
Plate 3				
LmrR_A92E	36±8	78±0	11±1	91±1
LmrR_A92E_N14I	56±19	79±2	12±1	88±1
LmrR_A92E_N14V (CFU 1)	73±16	81±2	15±0	87±2
LmrR_A92E_N14V (CFU 2)	75±5	78±1		
Plate 4				
LmrR_A92E	44±3	86±0	11±1	91±1
LmrR_A92E_N14A	42±9	87±0	17±2	90±1
LmrR_A92E_M89N (CFU 1)	55±1	84±0	7±1	85±2
LmrR_A92E_M89N (CFU 2)	60±1	84±0		
Plate 5				
LmrR_A92E	37±3	82±0	11±1	91±1
LmrR_A92E_N88D	63±5	83±0	16±2	90±1
LmrR_A92E_N88G	49±7	82±0	13±0	89±1
LmrR_A92E_N88I	33±0	86±0	15±1	87±2

F-C Alkylation of 1 and 3. [1]Cell suspension 2 mL and OD_{600nm}=4, Cu(II)-Phen 360 µM, reaction time 24 h. [2]Protein 12 µM, Cu(II)-Phen 9 µM, reaction time 6 h.

Most mutants that show increased yield *in vitro* correspond to increased enantioselectivity observed during *in vivo* screening. There is no corresponding trend with increased yield observed during *in vivo* screening. Thus, this justifies and confirms the use of enantioselectivity as the reliable parameter for the screening assay.

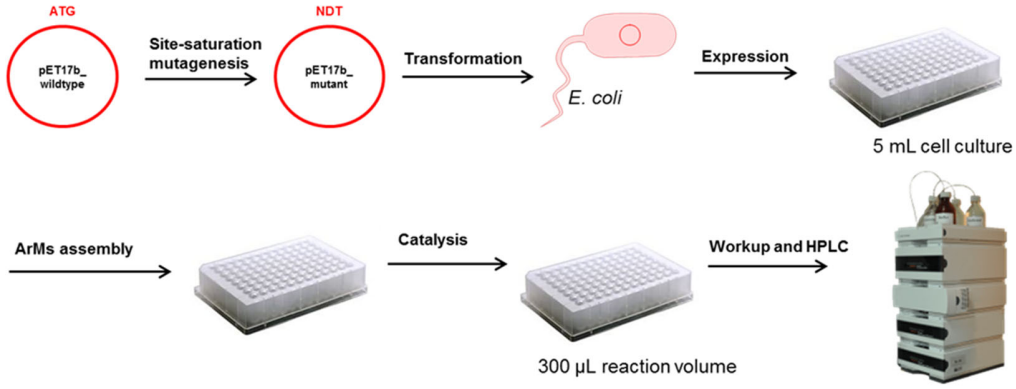


Figure S27. Directed Evolution workflow

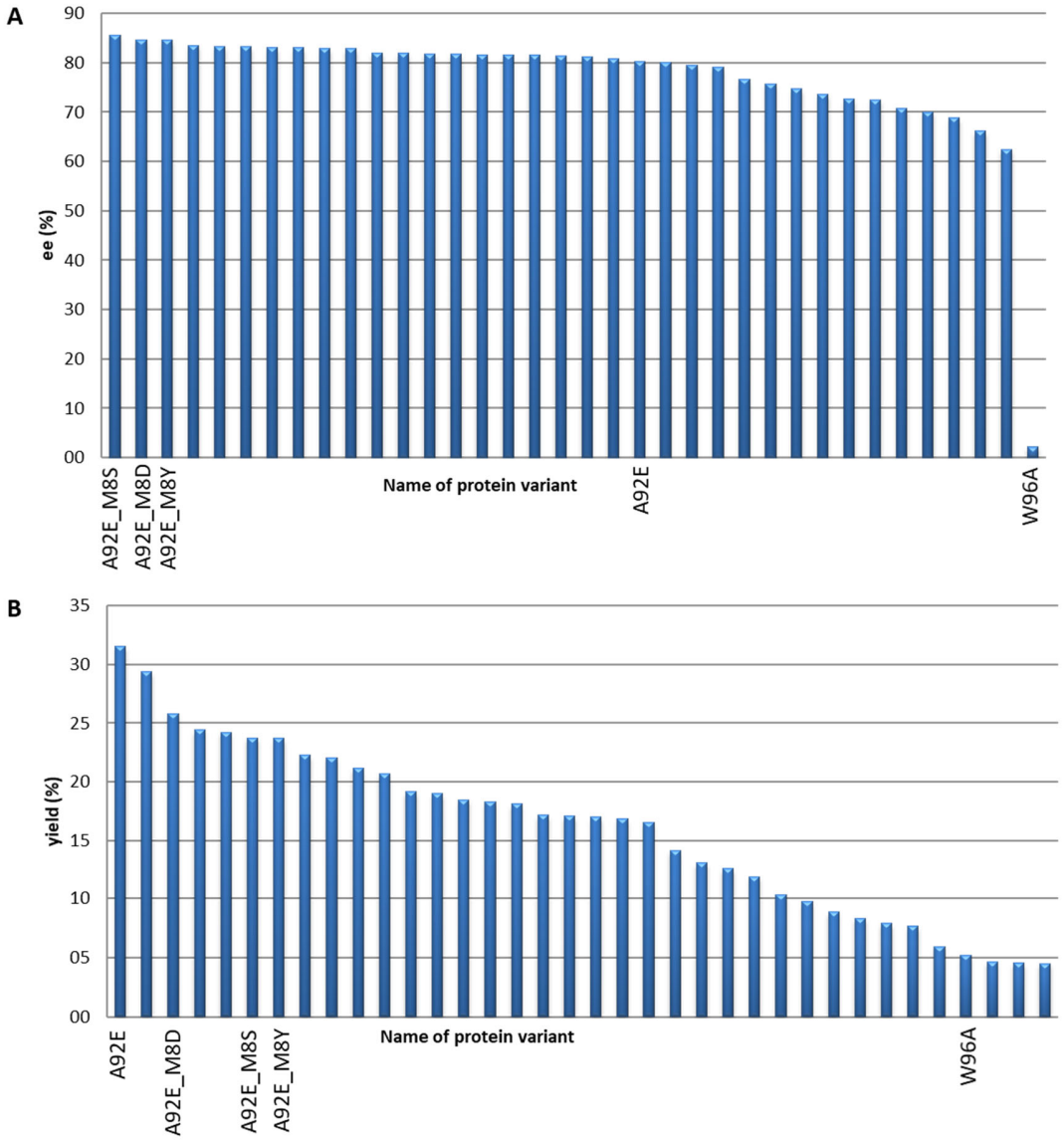


Figure S28. HPLC based screening of NDT library on position M8 A) Comparing ee % of product **3b** for the different colonies tested. B) Comparing yield % product **3b** for the different colonies tested.

11. Kinetic Studies

A solution of purified protein variants of LmrR (6.7 µM) was mixed with Cu(II)-Phen (5 µM) in a 2 mL HPLC vial. After 30 min the substrates **1** and **2b** were added and the vial loaded into the sample tray of a rp-HPLC, maintained at 4 ^{o}C. A sample was injected every 30 min onto a Chiralpak AD column, protected by a guard column from possible precipitation in samples. The product separated from the reaction mixture on the HPLC column and the yield was calculated. For the kinetics assay we could not go higher than 5 mM final substrate concentration due to solubility issues. Since substrate saturation was not reached and the V_{obs} was still in the linear part of the Michaelis-Menten curve ($S << K_M$), only the k_{cat}/K_M values could be calculated by the following equation $V_{obs}/[E] = K_{cat}/K_M * [S]$.

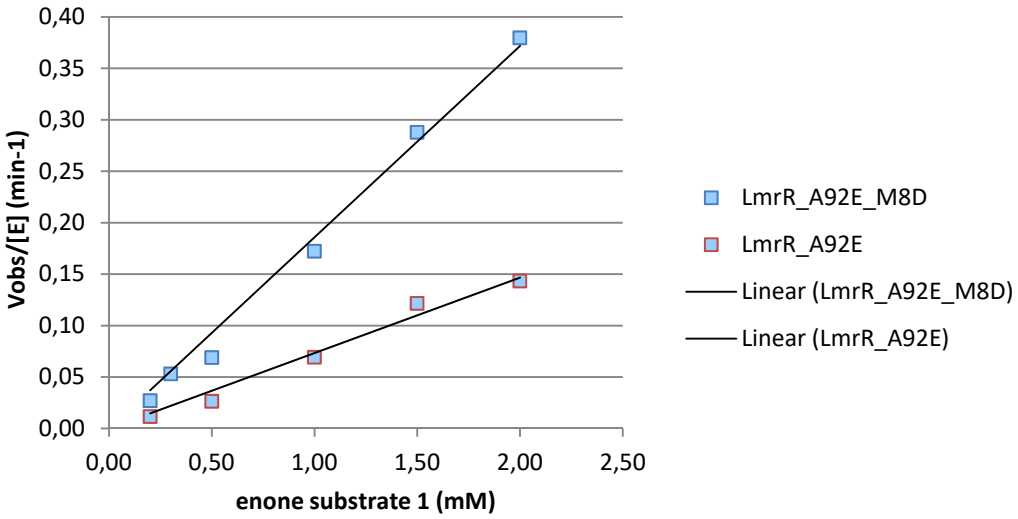
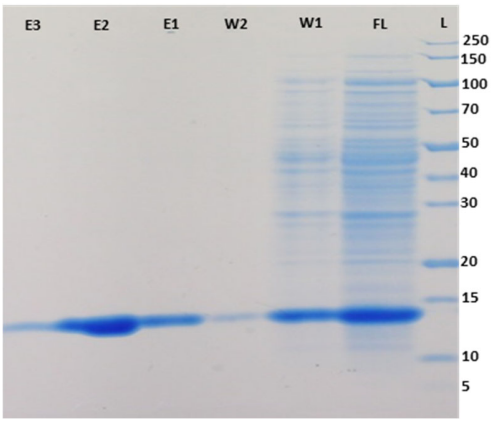


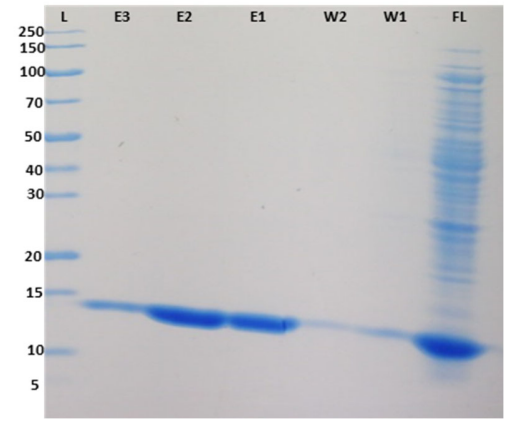
Figure S29. Kinetics of ArM catalyzed Friedel-Crafts alkylation reaction of **1** with **2b.**

12. SDS-PAGE gels of the LmrR mutants after Strep-Tag purification

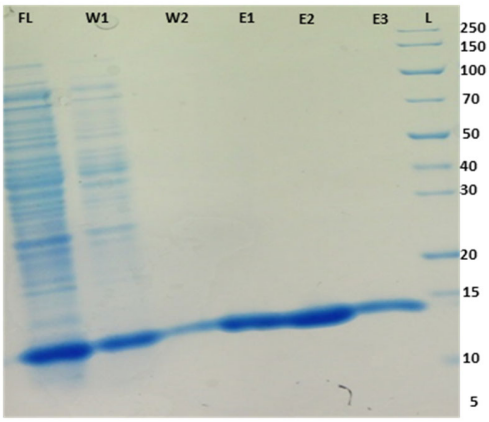
LmrR_N14A



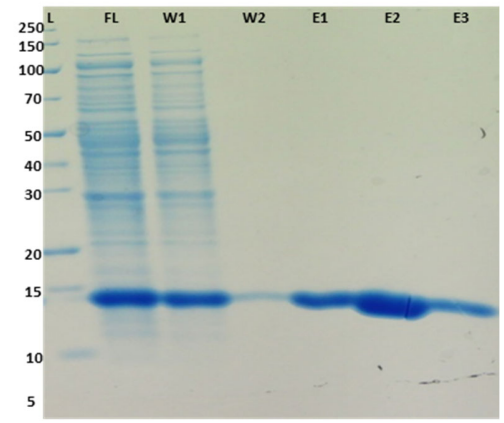
LmrR_N88A



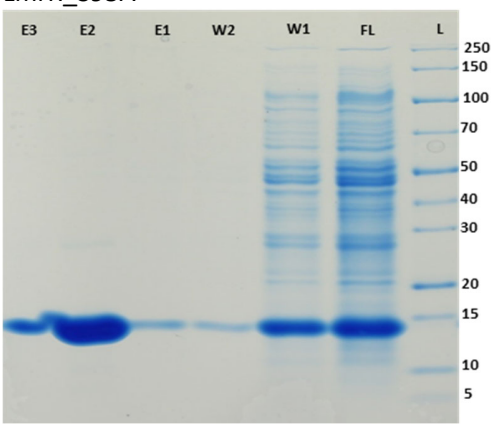
LmrR_M89A



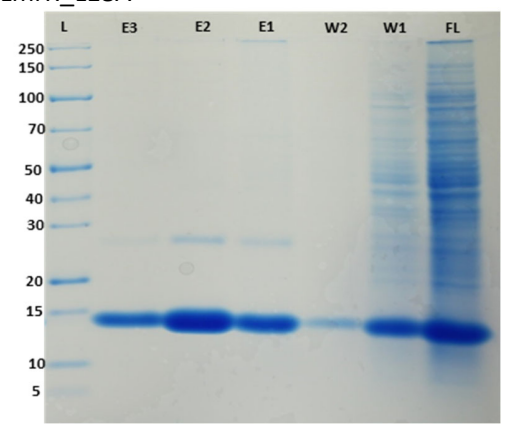
LmrR_R10A



LmrR_S95A

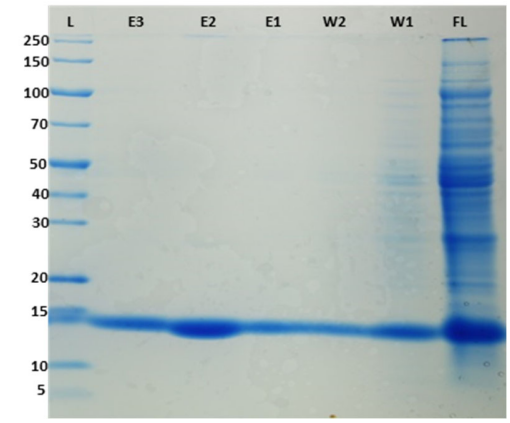


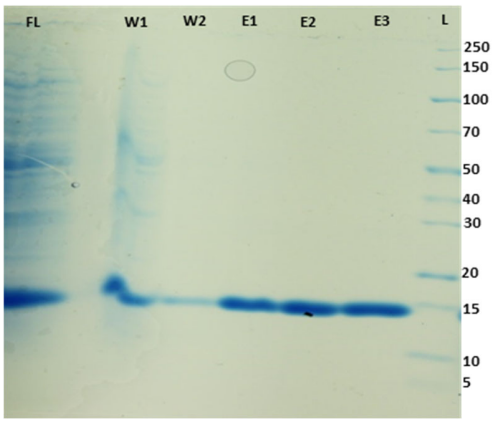
LmrR_L18A



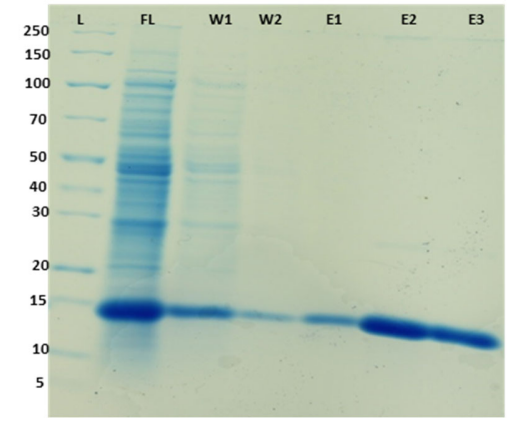
LmrR_S97A

LmrR_A92E_M8D

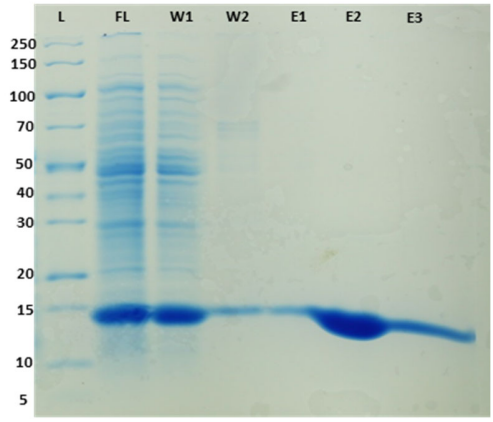




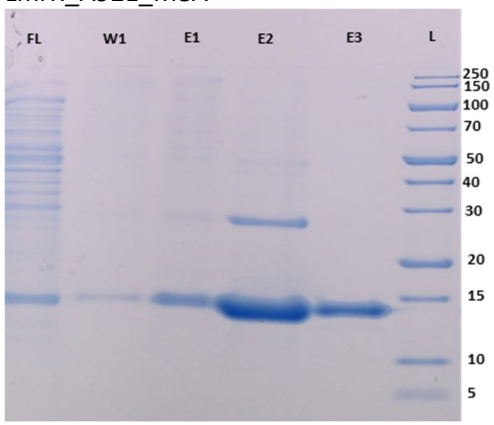
LmrR_A92E_M8S



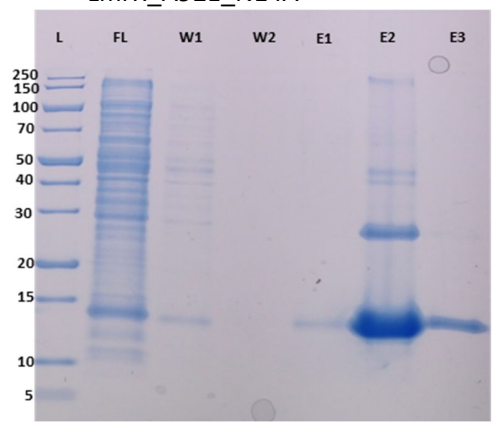
LmrR_A92E_M8Y



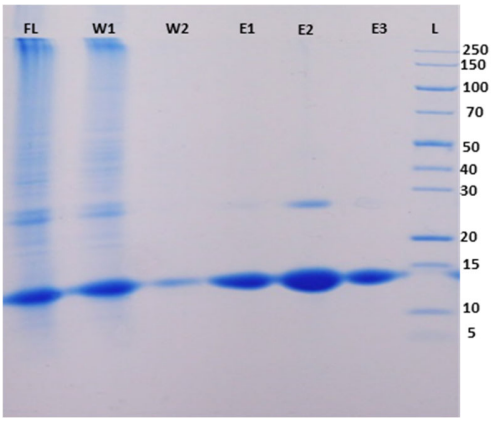
LmrR_A92E_M8A



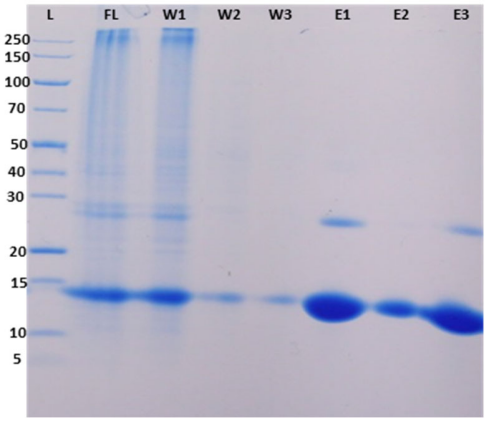
LmrR_A92E_N14A



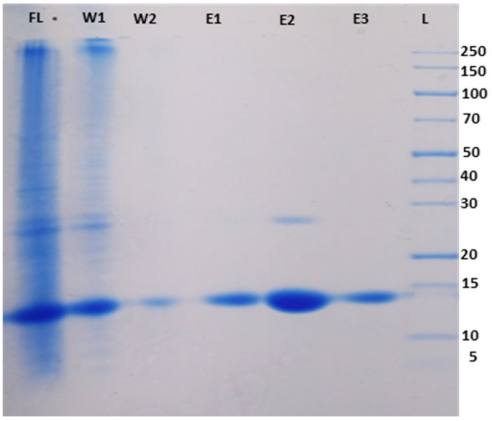
LmrR_A92E_N88D



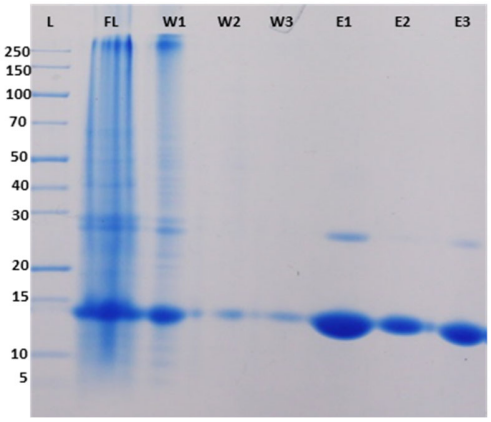
LmrR_A92E_N88G



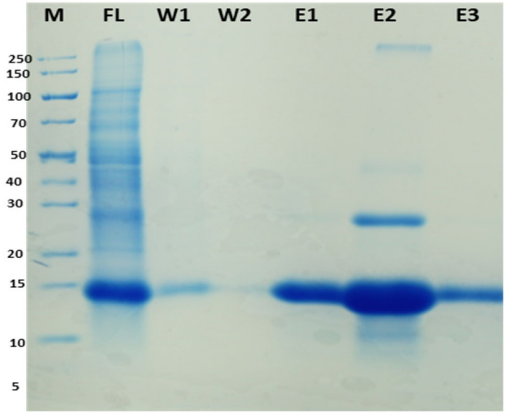
LmrR_A92E_N14I



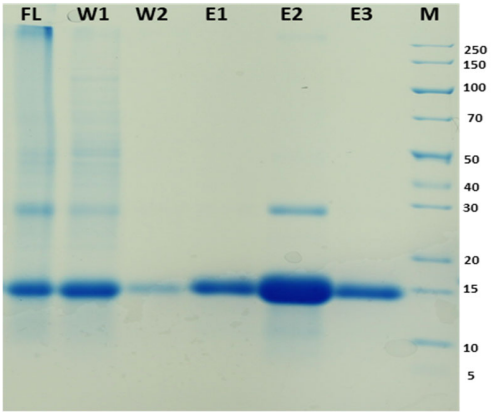
LmrR_A92E_N14V



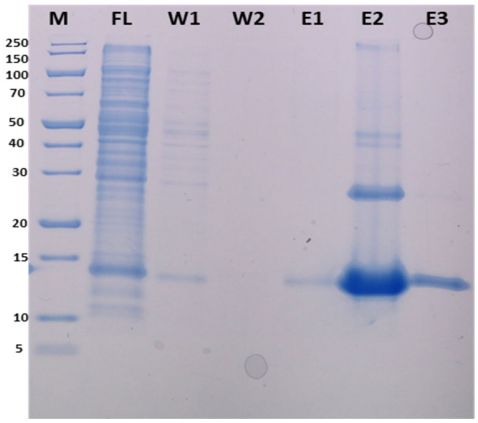
LmrR_A92E_N88I



LmrR_A92E_M89N



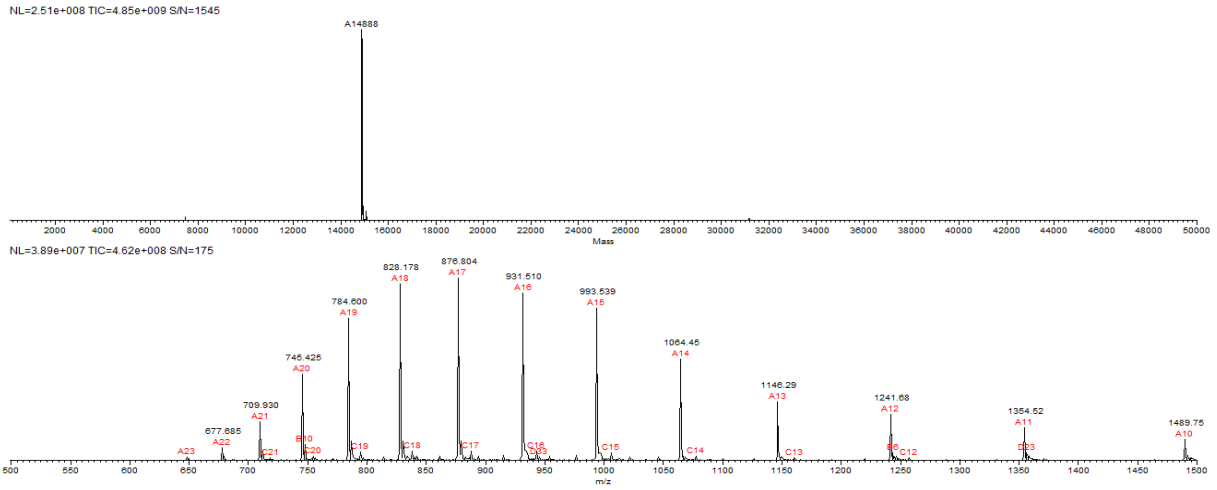
LmrR_A92E_V15A



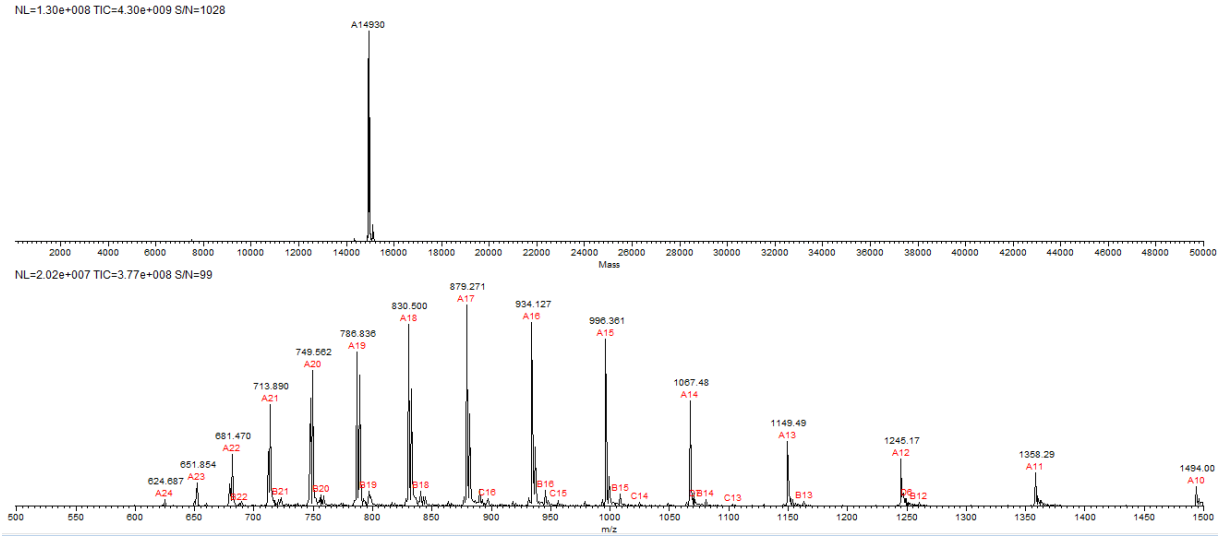
Legend: L- Ladder (PageRuler™ Unstained Broad Range, mol wt 5 to 250kDa). FL – Flow-through column. W - Wash fraction. E - Elution fraction. Gels were stained with InstantBlueTM (Expedeon).

13. Mass spectra

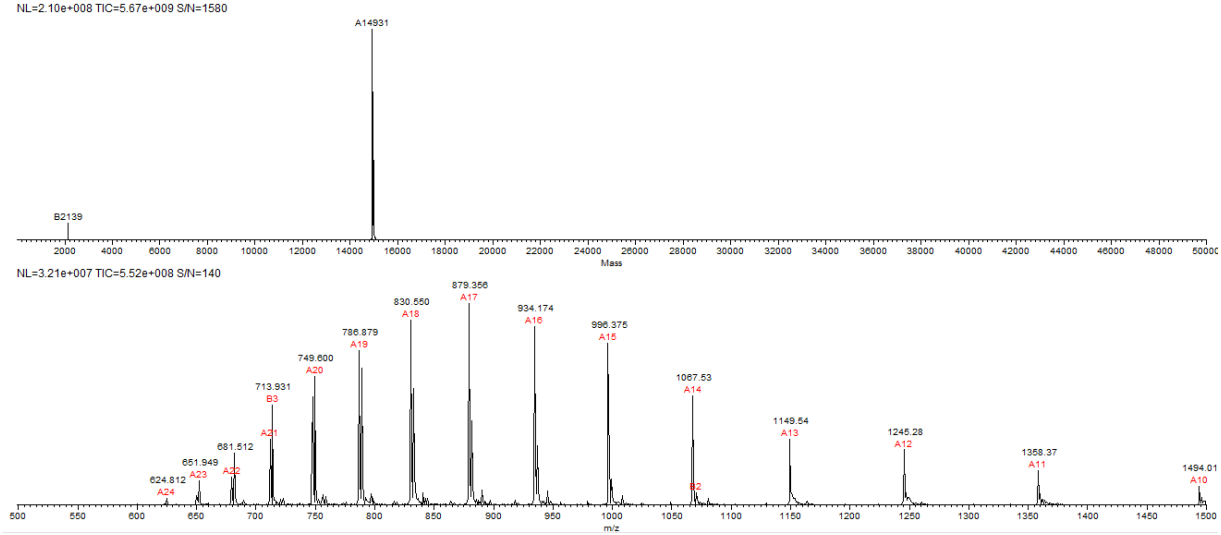
LmrR_R10A calculated mass (-Met) 14885.7



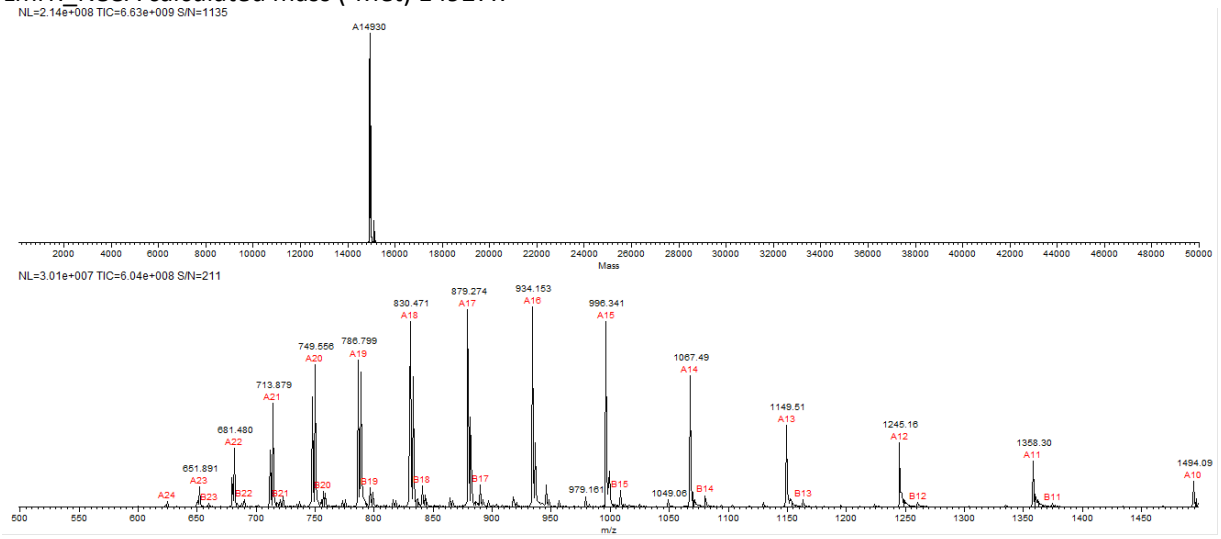
LmrR_N14A calculated mass (-Met) 14927.7



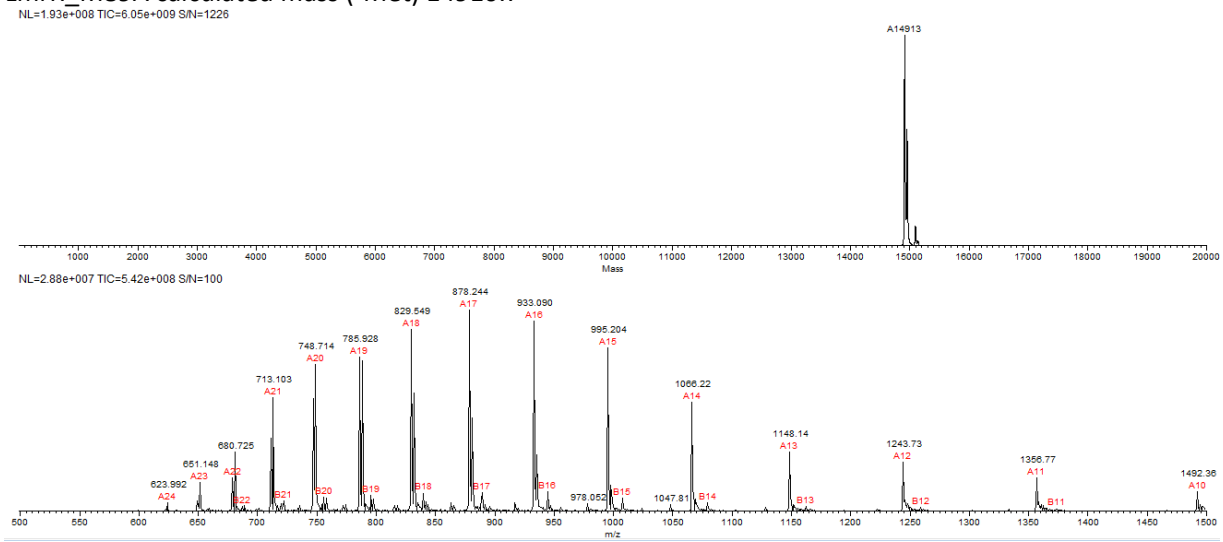
LmrR_L18A calculated mass (-Met) 14928.7



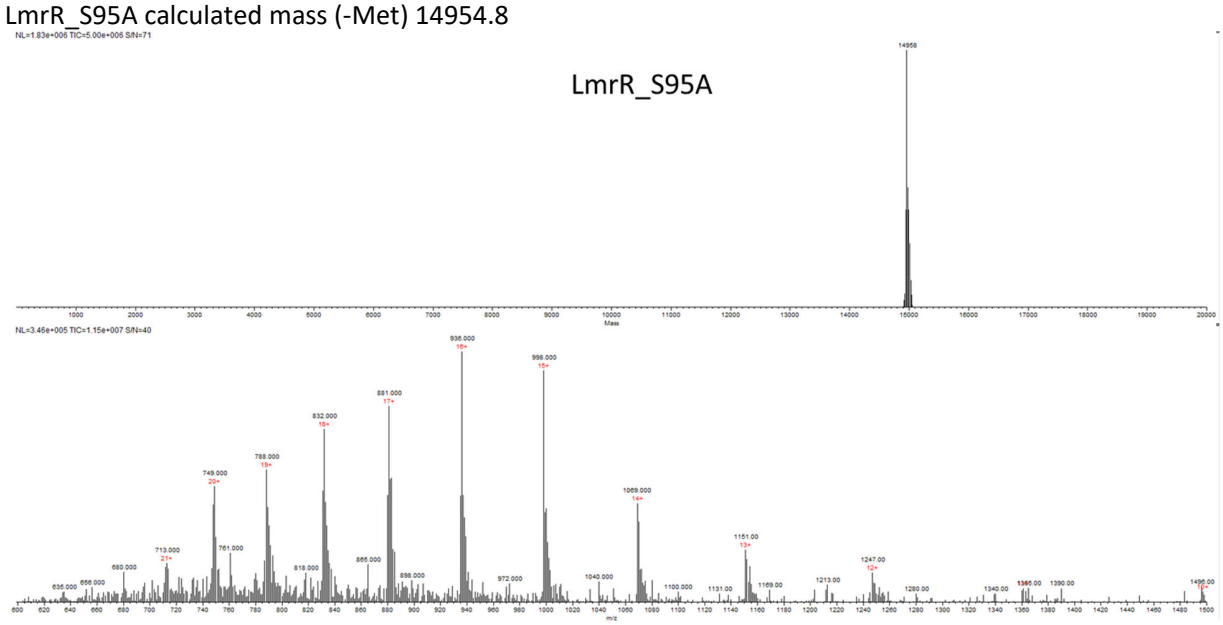
LmrR_N88A calculated mass (-Met) 14927.7



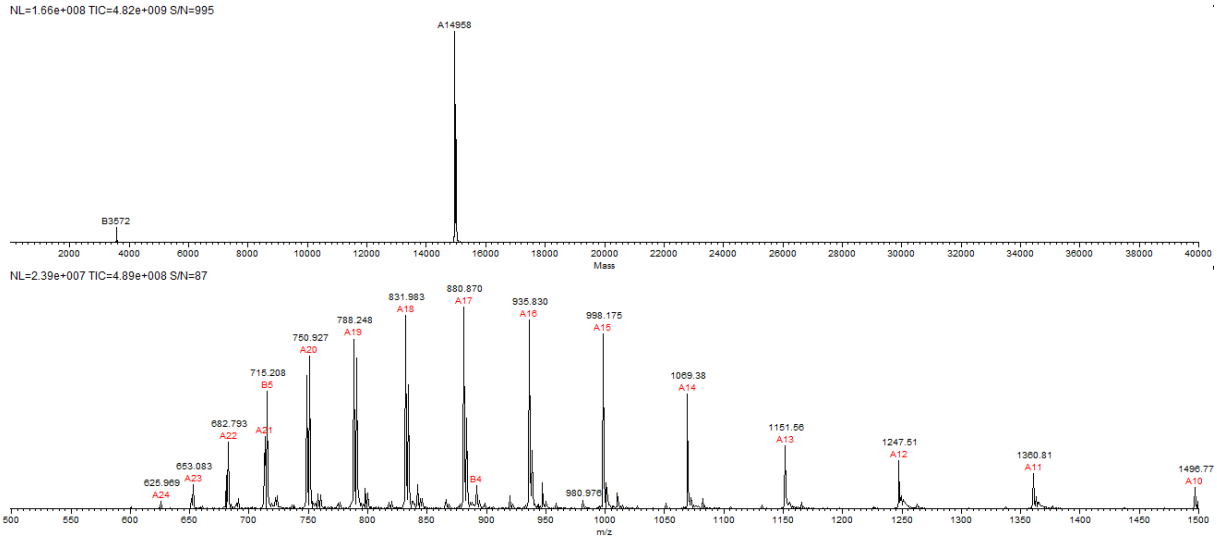
LmrR_M89A calculated mass (-Met) 14910.7



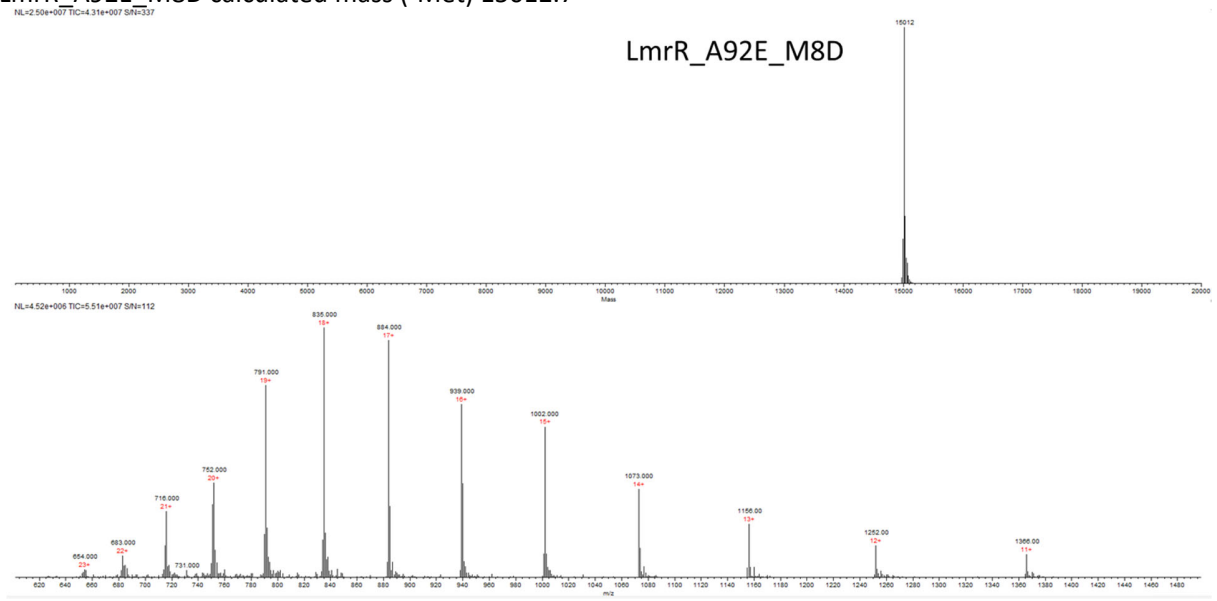
LmrR_S95A calculated mass (-Met) 14954.8



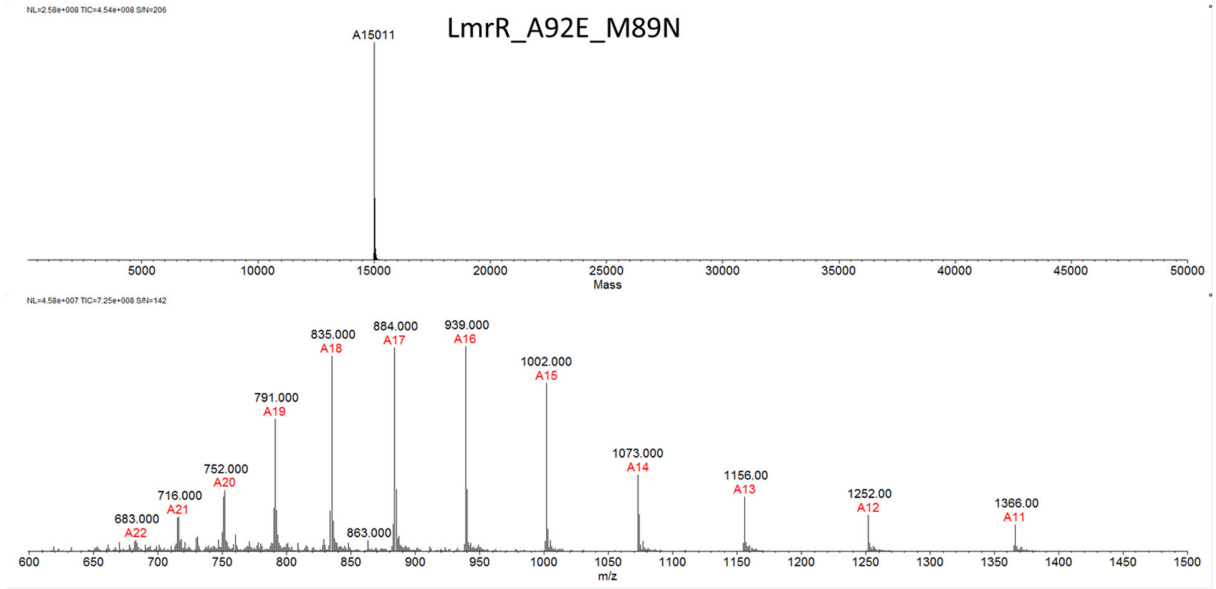
LmrR_S97A calculated mass (-Met) 14954.8



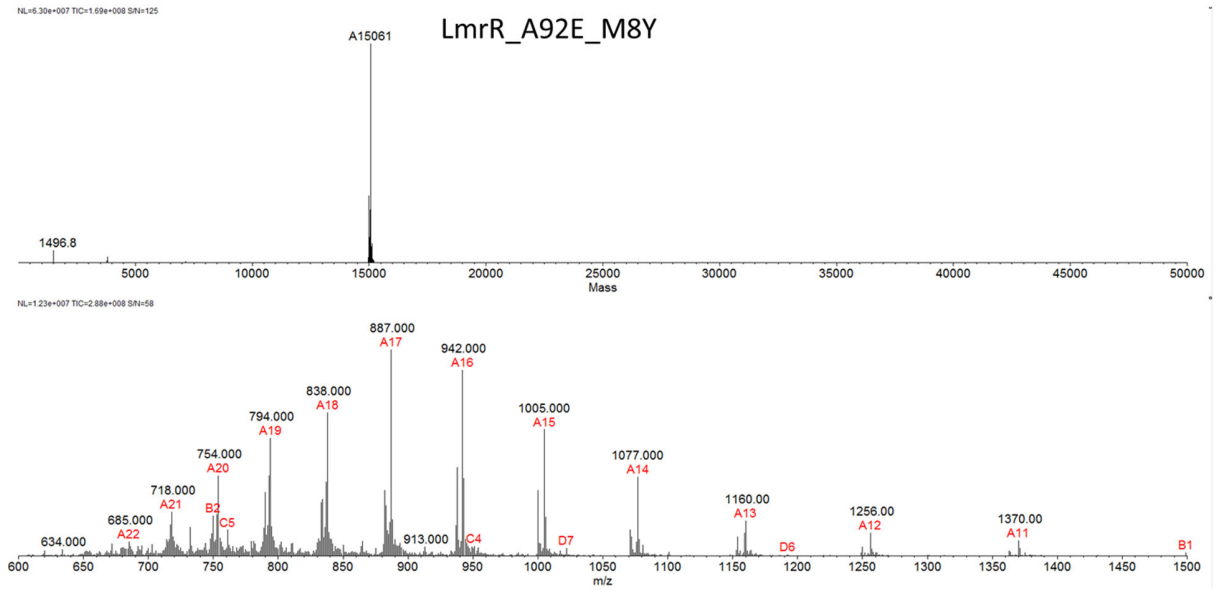
LmrR_A92E_M8D calculated mass (-Met) 15012.7



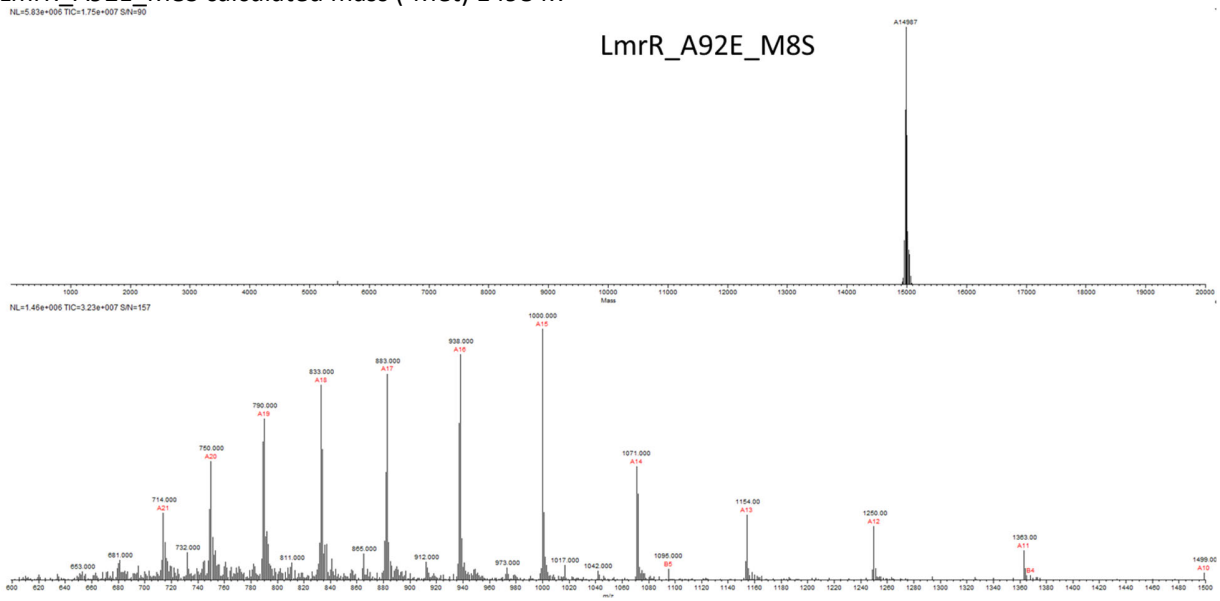
LmrR_A92E_M89N calculated mass (-Met) 15011.8



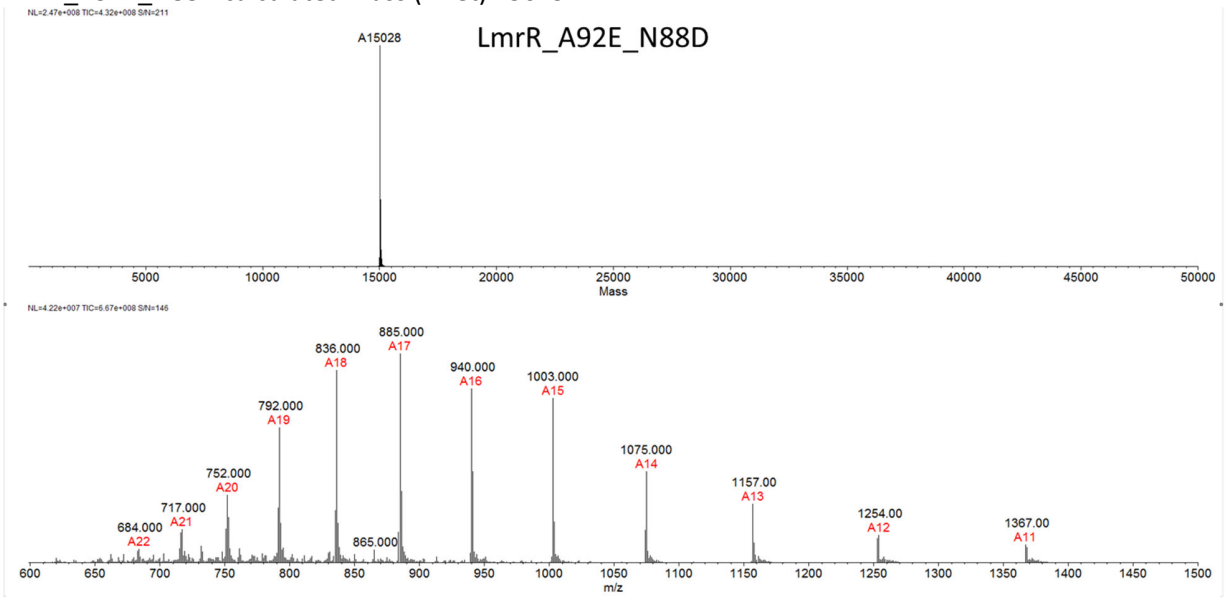
LmrR_A92E_M8Y calculated mass (-Met) 15060.8



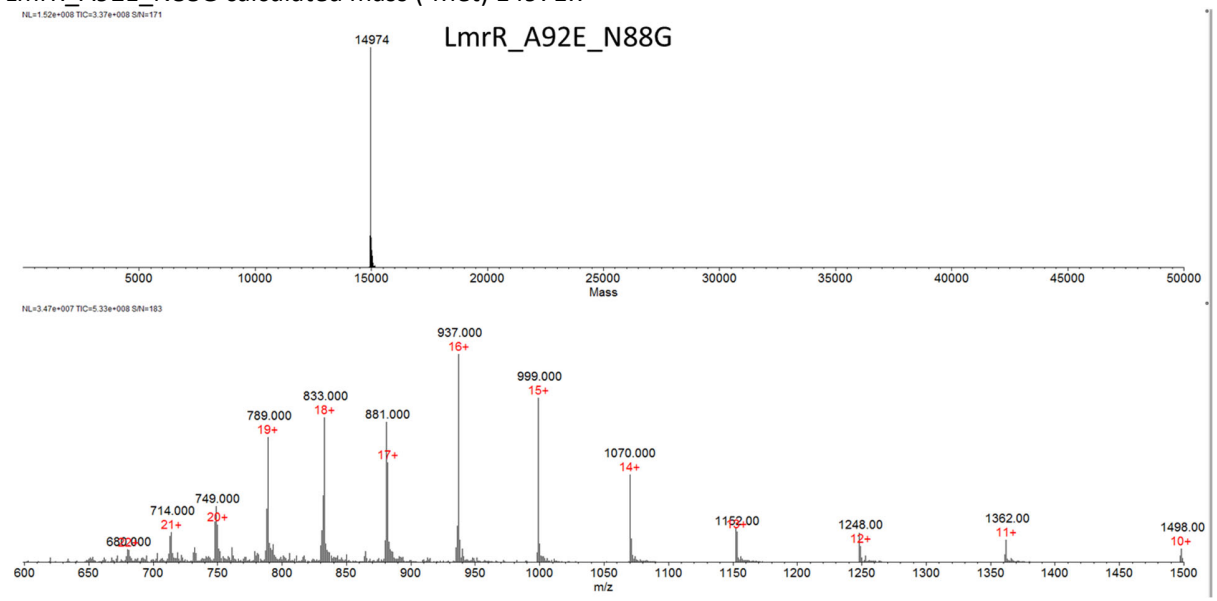
LmrR_A92E_M8S calculated mass (-Met) 14984.7



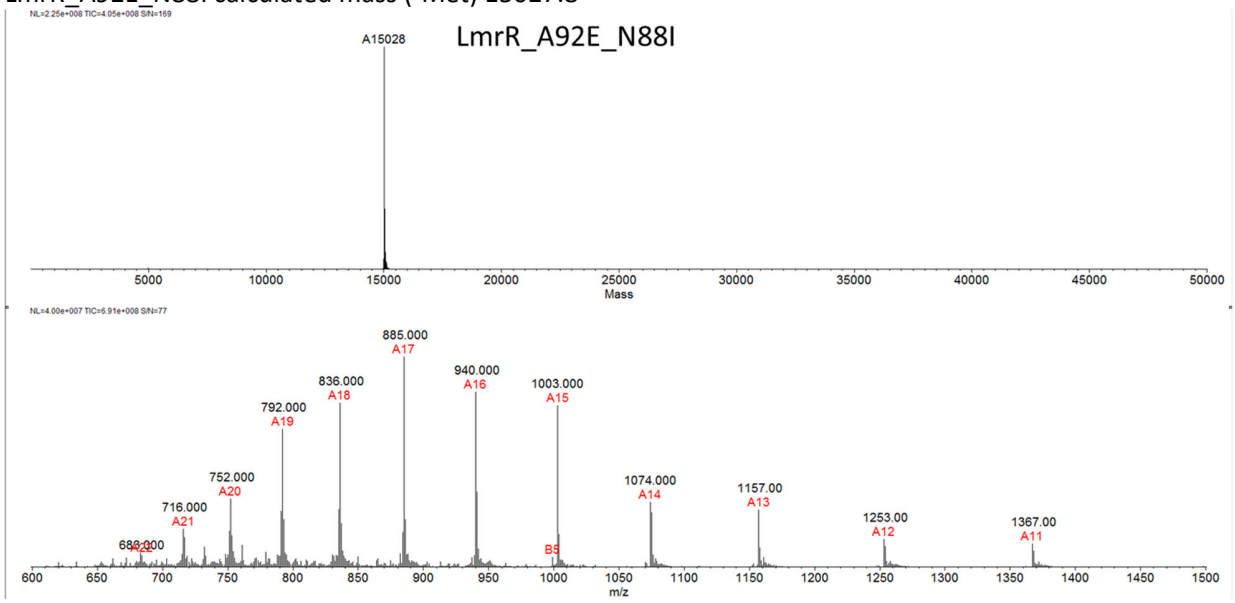
LmrR_A92E_N88D calculated mass (-Met) 15029.7



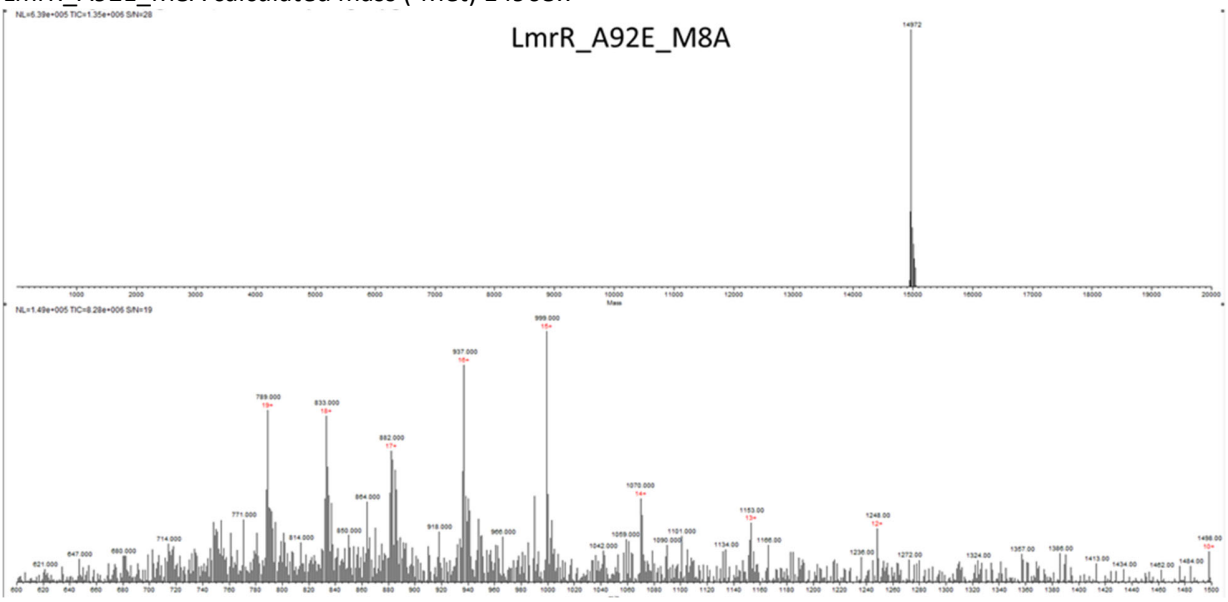
LmrR_A92E_N88G calculated mass (-Met) 14971.7



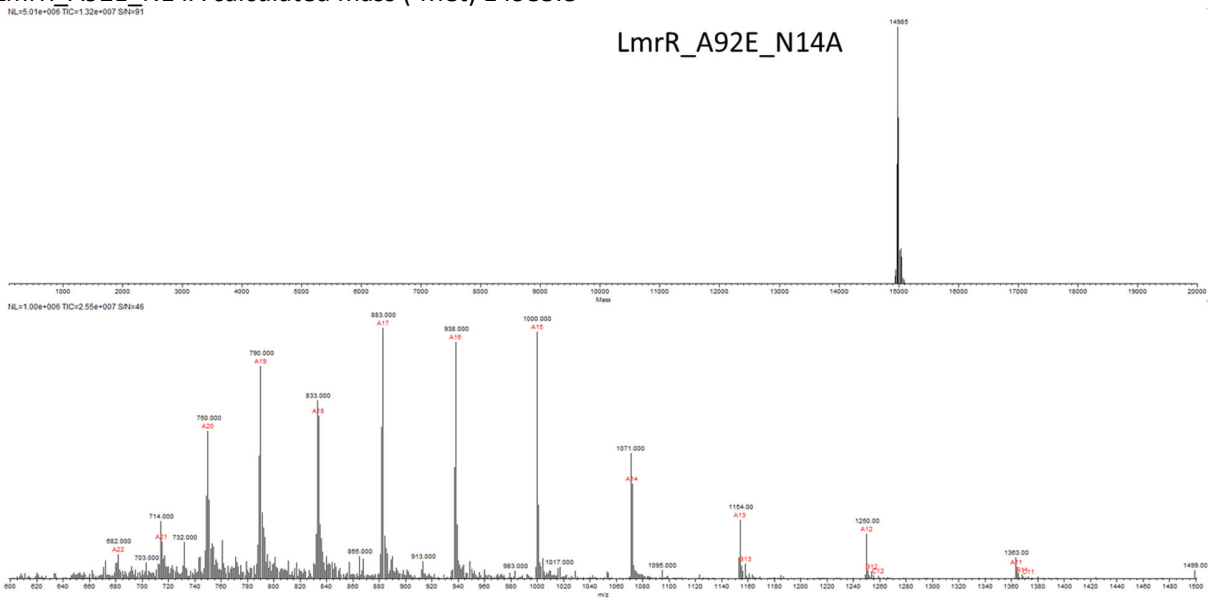
LmrR_A92E_N88I calculated mass (-Met) 15027.8



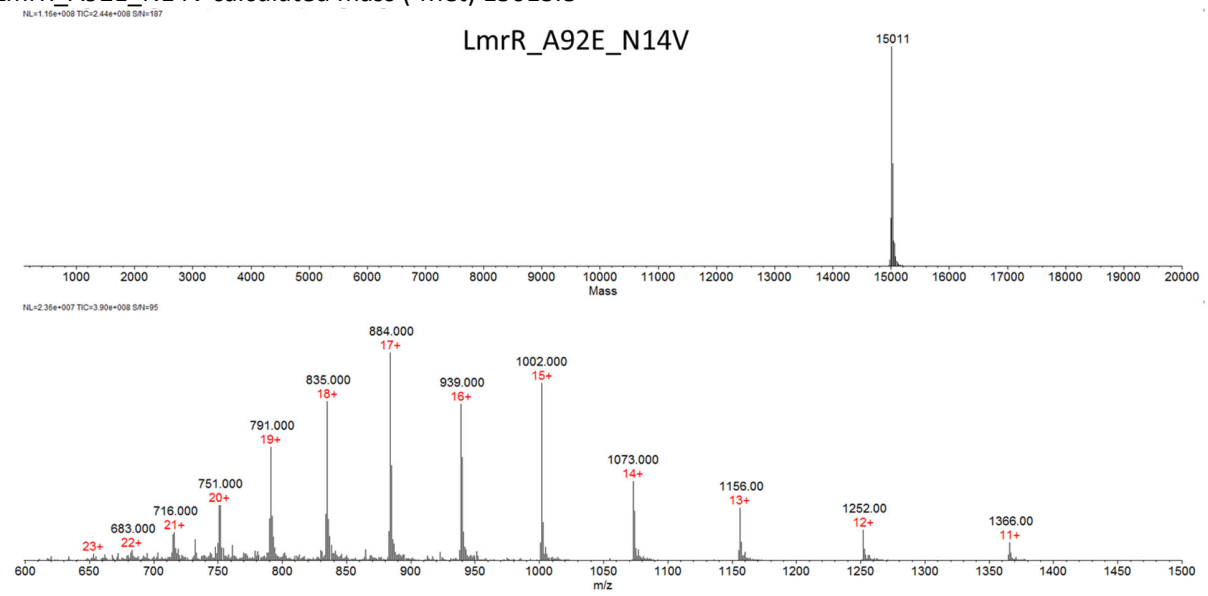
LmrR_A92E_M8A calculated mass (-Met) 14968.7



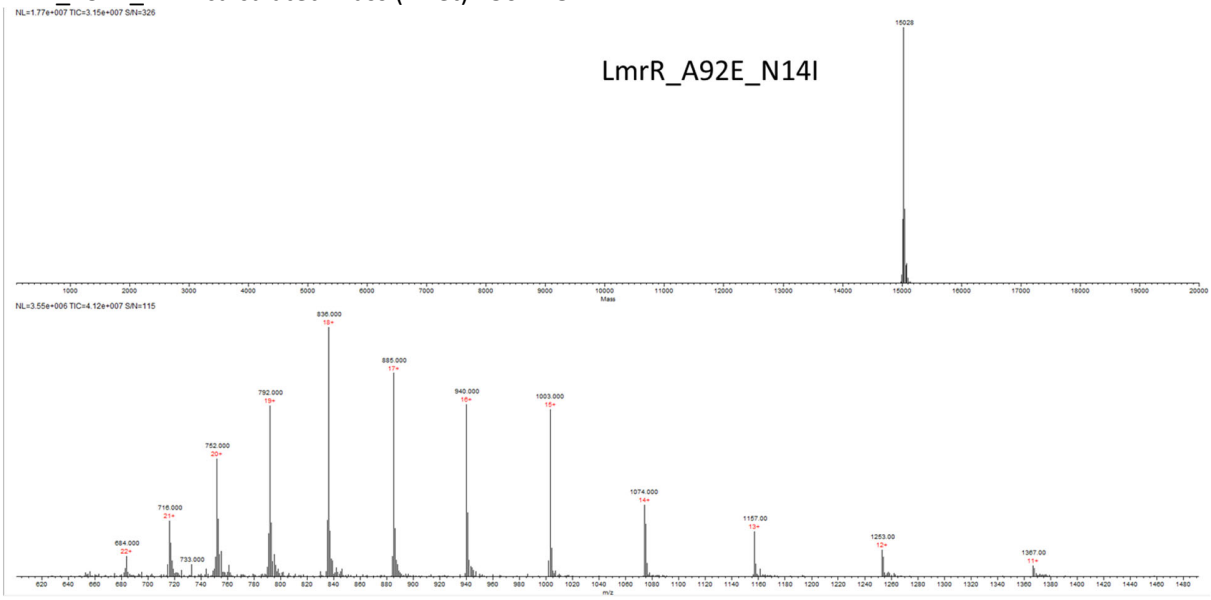
LmrR_A92E_N14A calculated mass (-Met) 14985.8



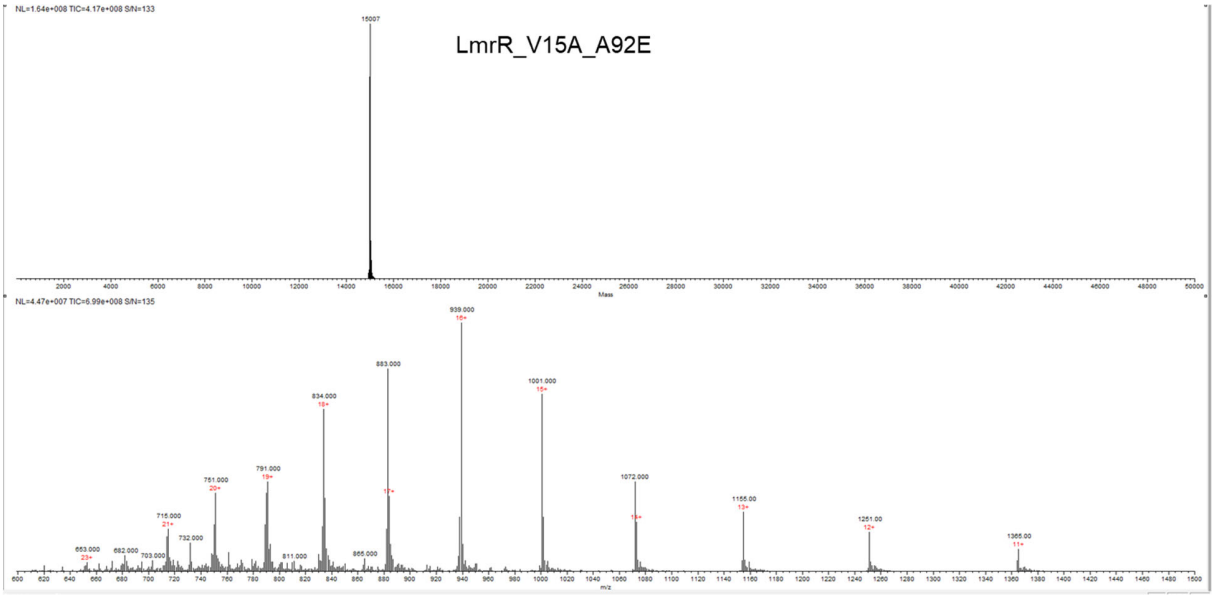
LmrR_A92E_N14V calculated mass (-Met) 15013.8



LmrR_A92E_N14I calculated mass (-Met) 15027.8



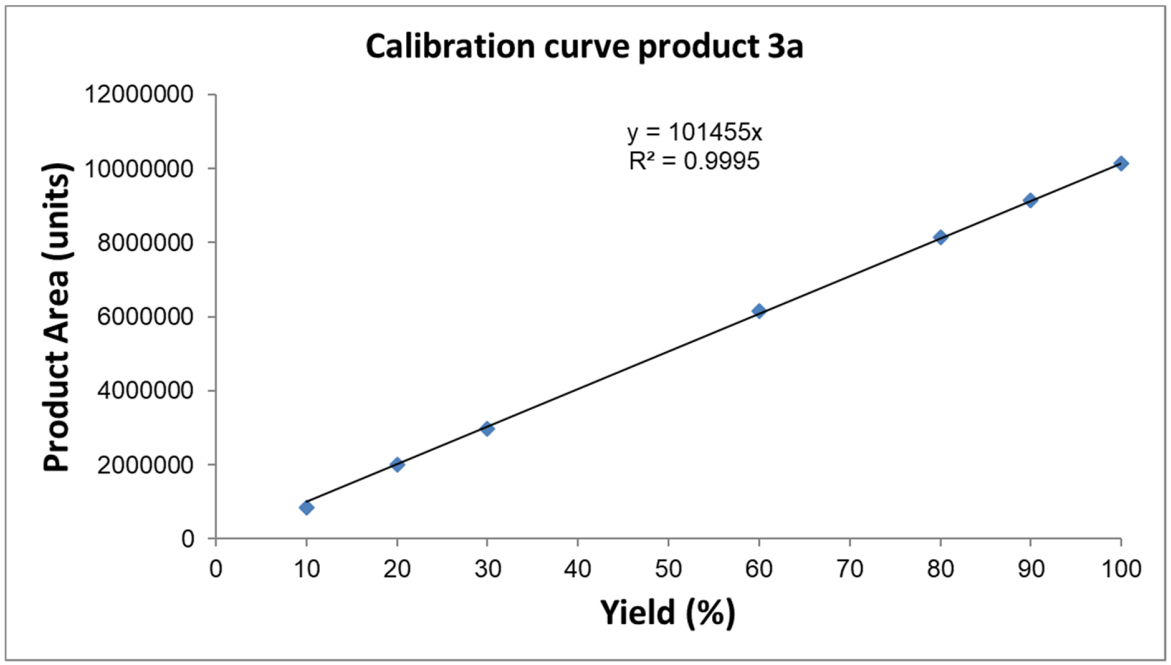
LmrR_V15A_A92E calculated mass (-Met) 15000.8



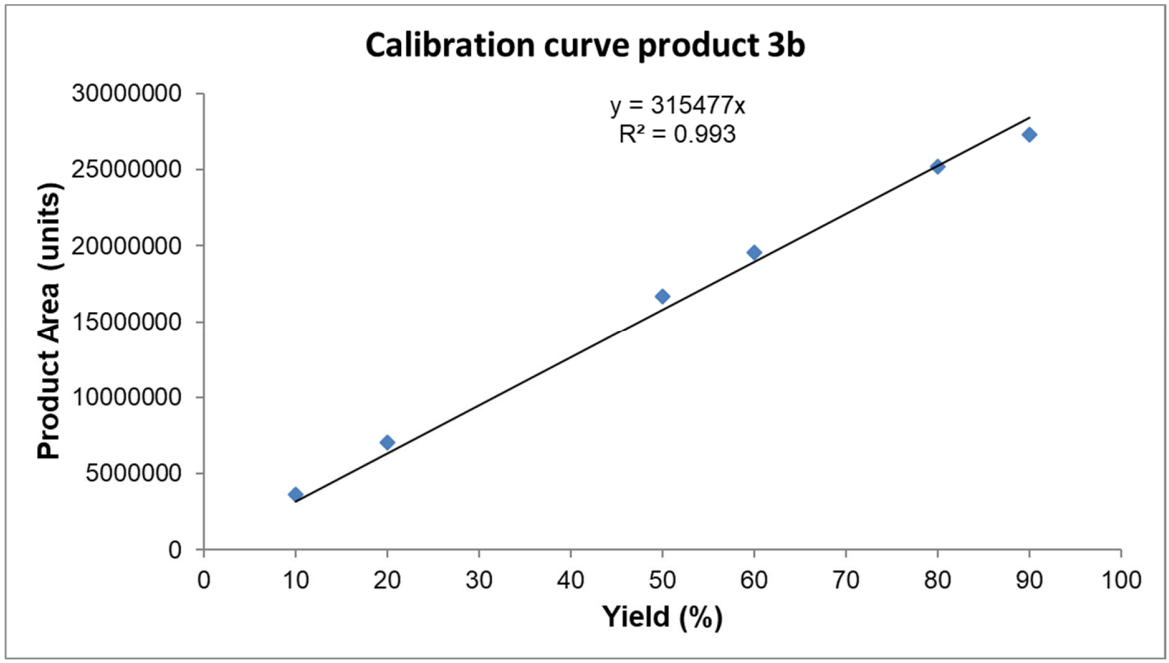
14. HPLC analysis and chromatograms

The reactions were analyzed by adding 50 µL internal standard (2-phenylquinoline, 2 mM in CH_3CN) to the reaction mixture, followed by extraction with diethyl ether (3 x 500 µL) and separated organic layer were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Subsequently, the samples were re-dissolved in 150 µL of a heptane:propan-2-ol mixture (9:1) and the yields and enantiomeric excess were determined by HPLC. Calibration curves for quantification of the different products were constructed using pure reference compounds produced synthetically as described in section "Chemical synthesis".

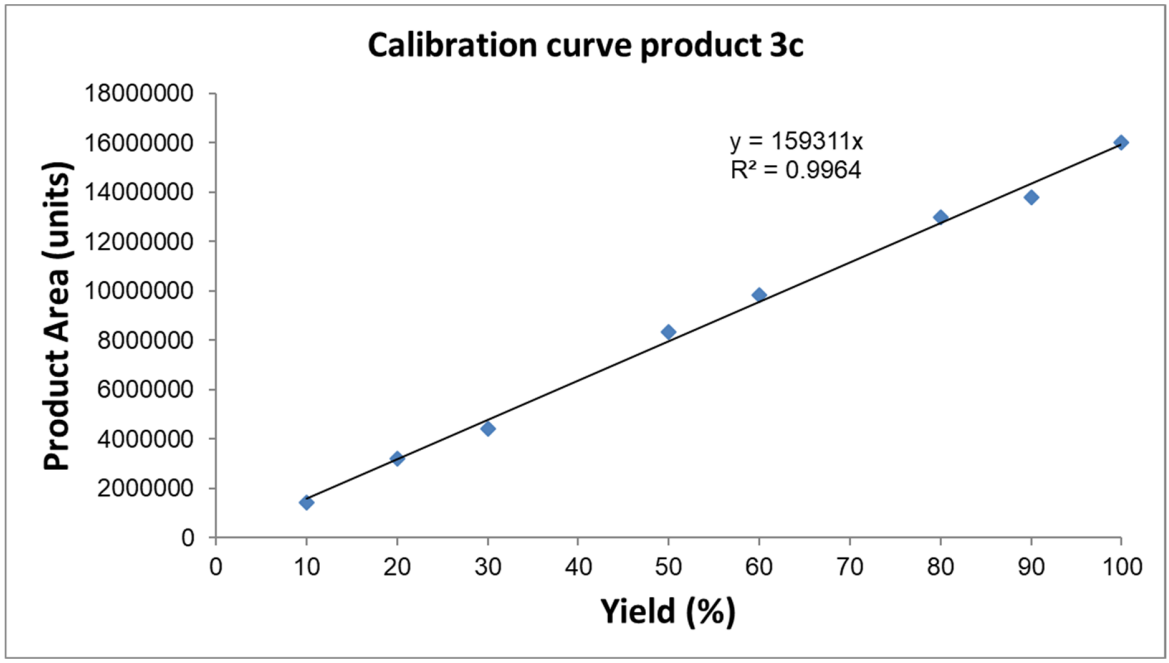
Calibration curve for product 3a (F-C Alkylation of 1 and 2a)



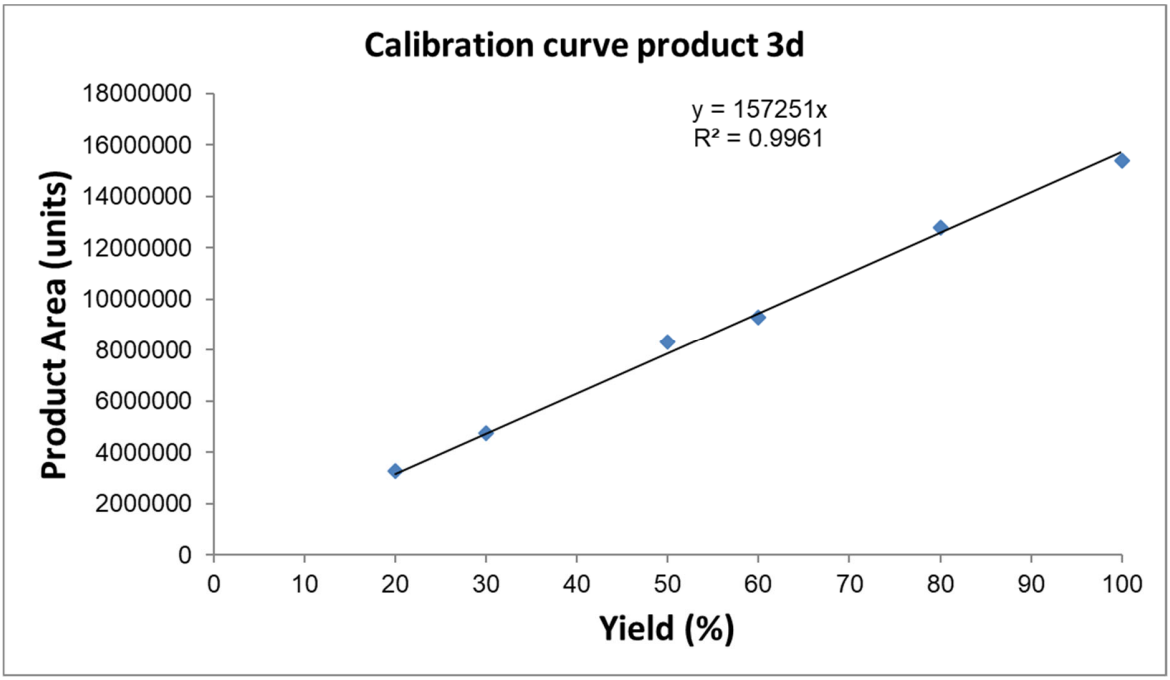
Calibration curve for product 3b (F-C Alkylation of 1 and 2b)



Calibration curve for product 3c (F-C Alkylation of 1 and 2c)



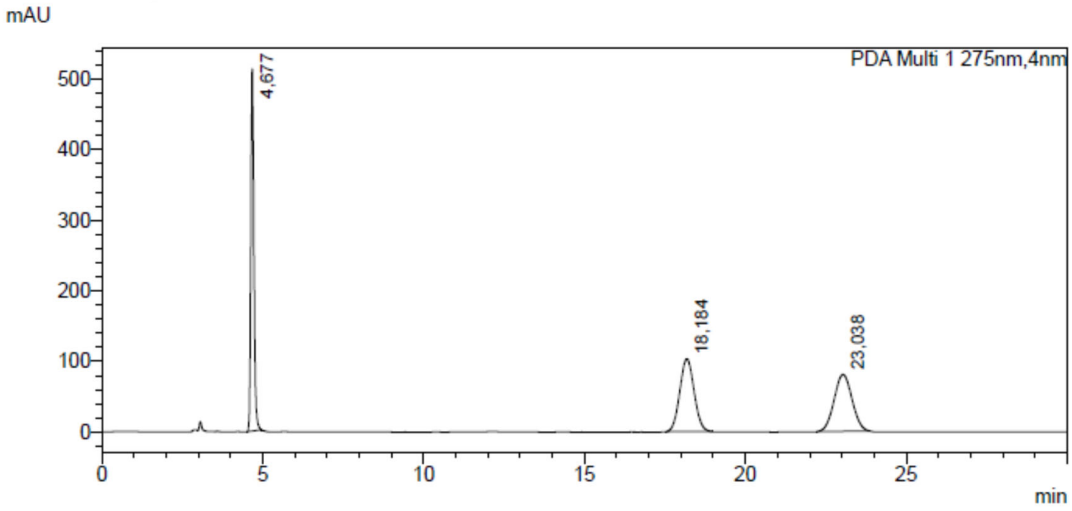
Calibration curve for product 3d (F-C Alkylation of 1 and 2d)



HPLC Chromatograms

Racemate of product 3a (F-C Alkylation of 1 and 2a)

<Chromatogram>



<Peak Table>

ELSD-LT2

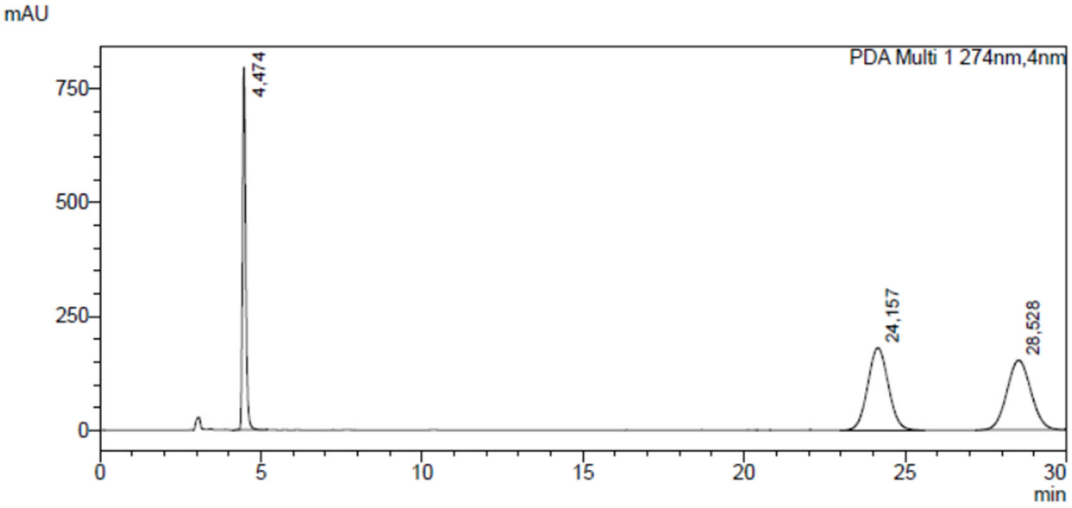
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
Total							

PDA Ch1 275nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	4,677	3374020	512640	0,000		M	
2	18,184	3188948	103186	0,000		M	
3	23,038	3125206	80539	0,000		M	
Total		9688174	696365				

Racemate of product 3b (F-C Alkylation of 1 and 2b)

<Chromatogram>



<Peak Table>

ELSD-LT2

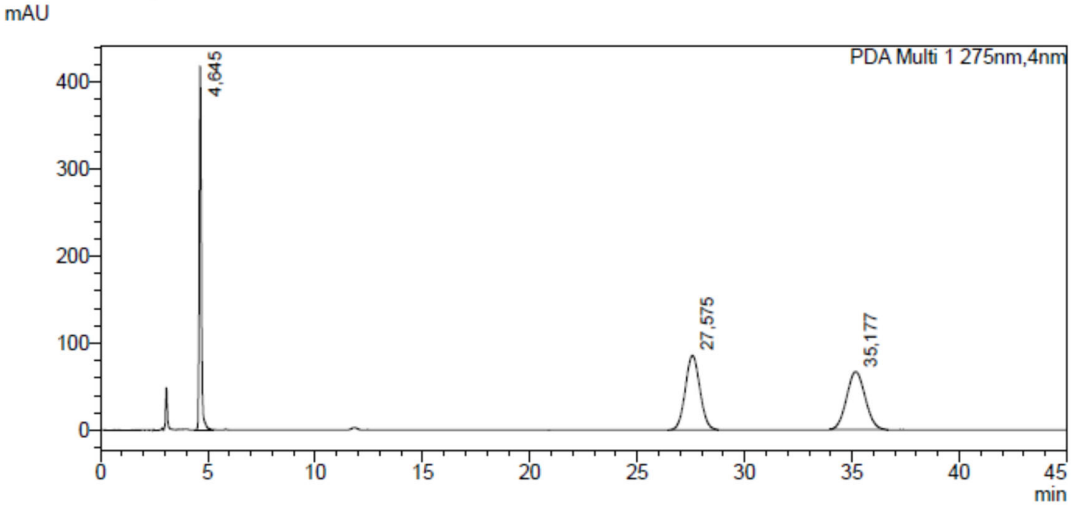
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
Total							

PDA Ch1 274nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	4,474	5439200	794737	0,000			
2	24,157	8005070	180778	0,000			
3	28,528	7975424	153295	0,000			
Total		21419695	1128810				

Racemate of product 3c (F-C Alkylation of 1 and 2c)

<Chromatogram>



<Peak Table>

ELSD-LT2

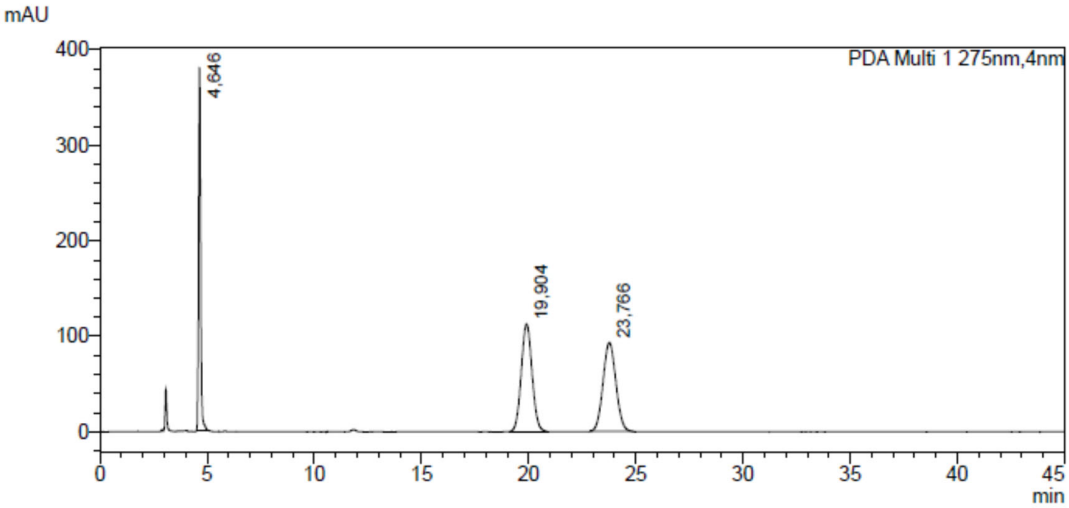
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
Total							

PDA Ch1 275nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	4,645	2832578	416490	0,000		M	
2	27,575	4026717	85372	0,000		M	
3	35,177	3994004	66398	0,000		M	
Total		10853299	568260				

Racemate of product 3d (F-C Alkylation of 1 and 2d)

<Chromatogram>



<Peak Table>

ELSD-LT2

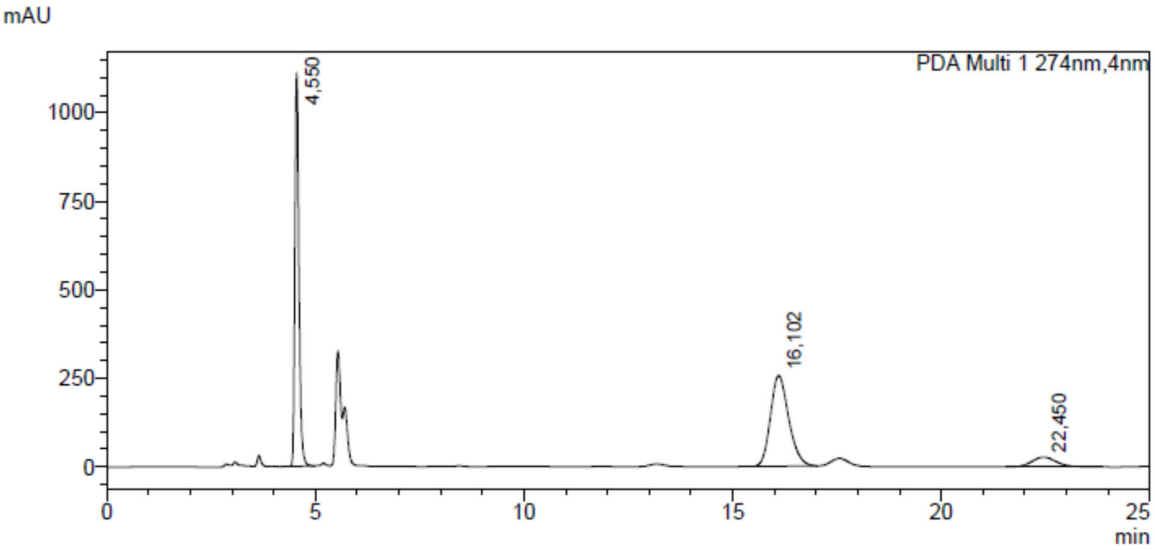
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
Total							

PDA Ch1 275nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	4,646	2555892	379114	0,000		M	
2	19,904	3948001	112463	0,000		M	
3	23,766	3873609	92885	0,000		M	
Total		10377502	584462				

Cell free extract_LmrR ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2a)

<Chromatogram>



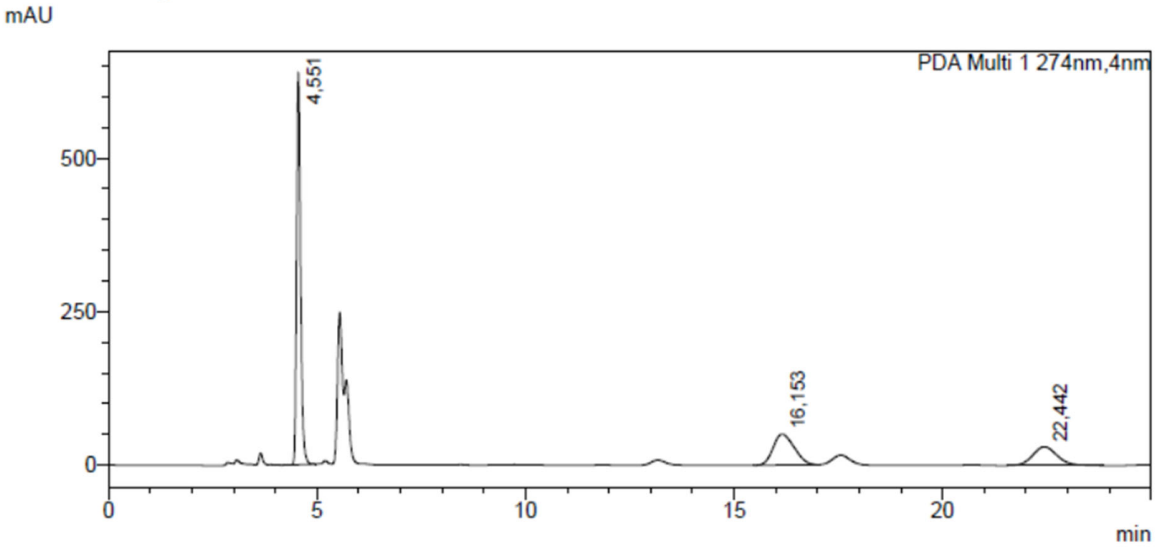
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,550	7483280	45,592
	16,102	7805759	47,557
	22,450	1124614	6,852
		16413653	100,000

Cell free extract_SUMO_Cu(II)-Phen (F-C Alkylation of 1 and 2a)

<Chromatogram>



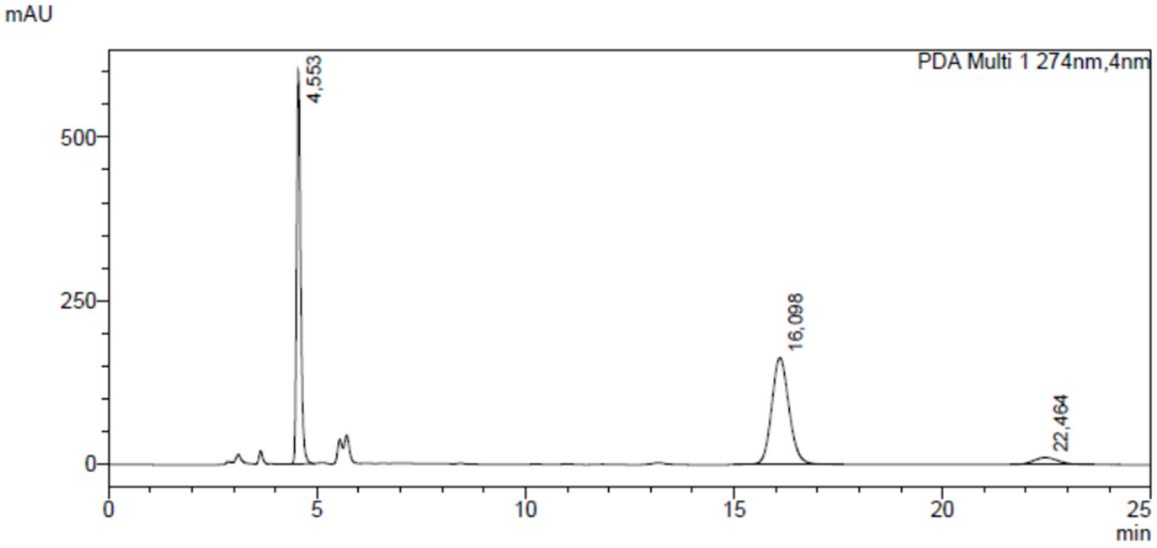
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,551	4260191	58,680
	16,153	1758852	24,226
	22,442	1241031	17,094
		7260074	100,000

Whole cell_LmrR ⊂ Cu(II)-Phen (cytoplasm) (F-C Alkylation of 1 and 2a)

<Chromatogram>



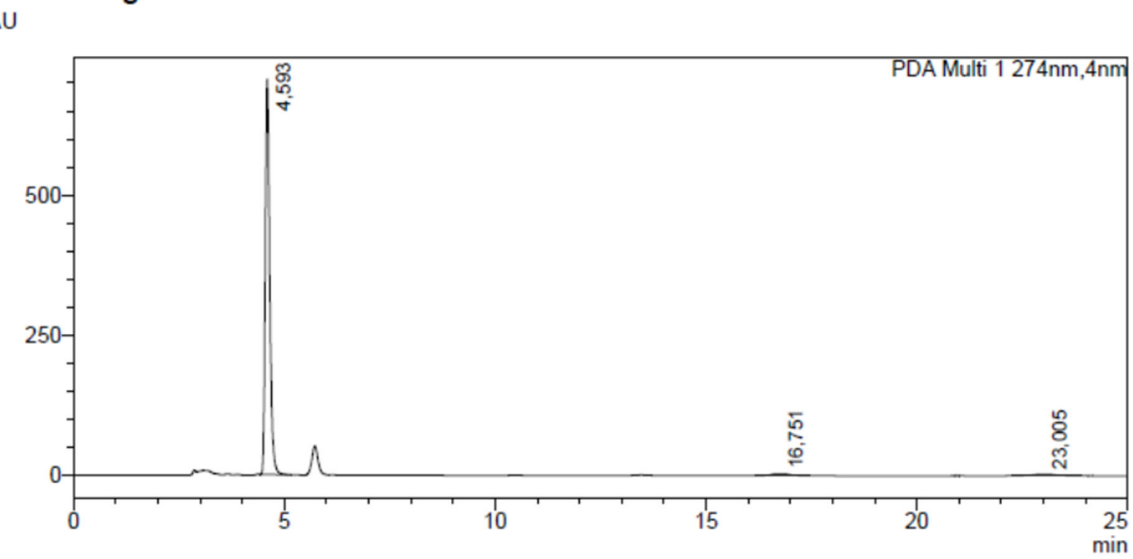
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,553	4013928	43,713
	16,098	4731596	51,528
	22,464	437002	4,759
		9182526	100,000

Whole cell_LmrR_no Cu(II)-Phen added (cytoplasm) (F-C Alkylation of 1 and 2a)

<Chromatogram>



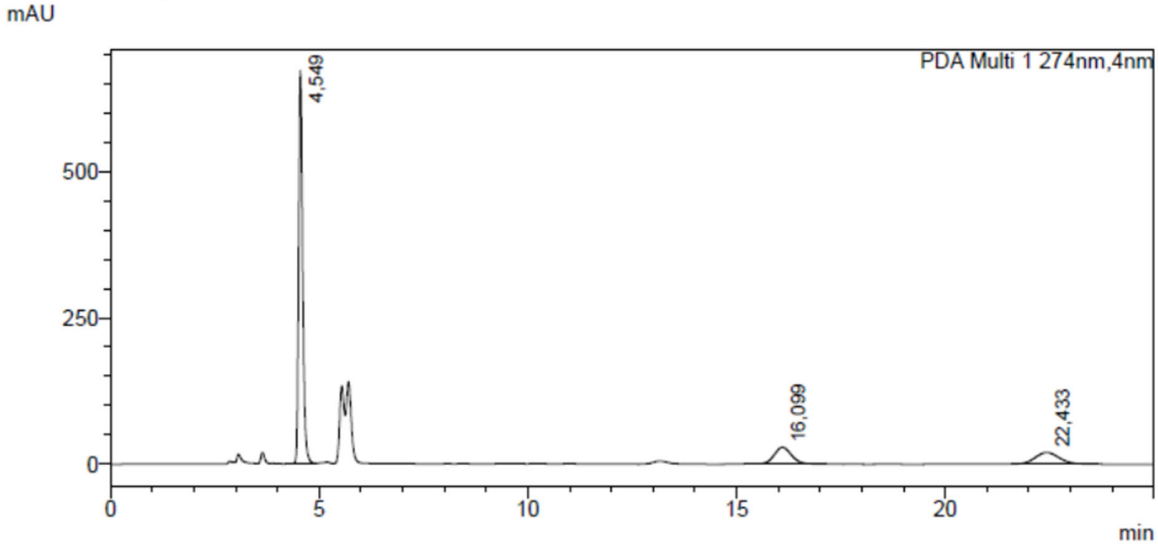
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
	4,593	5194041	96,221
	16,751	101202	1,875
	23,005	102768	1,904
		5398010	100,000

Whole cell_SUMO_Cu(II)-Phen (cytoplasm) (F-C Alkylation of 1 and 2a)

<Chromatogram>



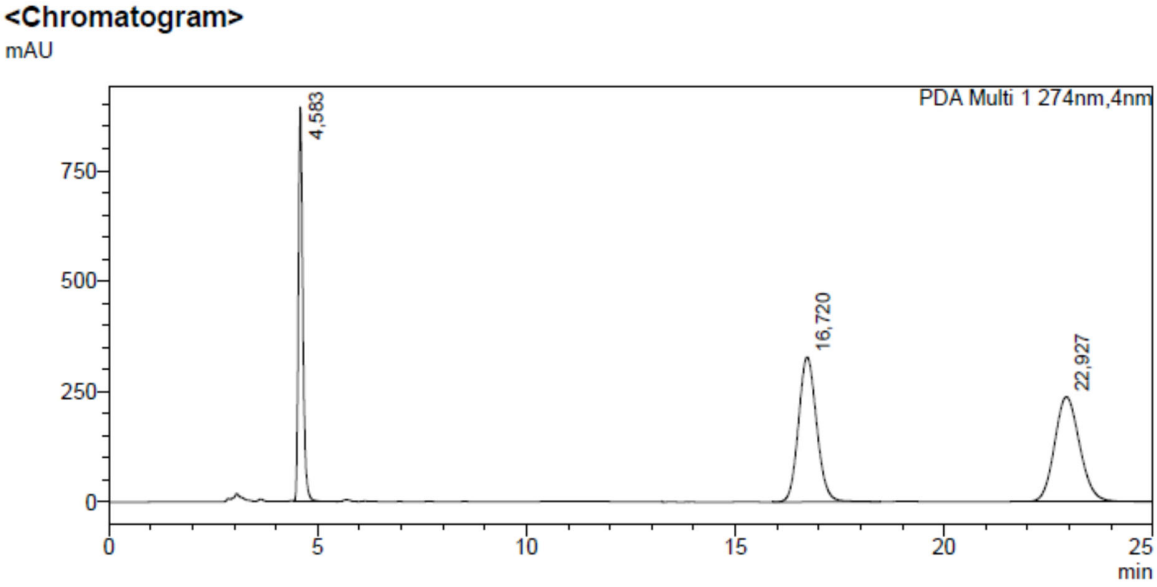
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,549	4492950	73,397
	16,099	830104	13,561
	22,433	798349	13,042
		6121403	100,000

Whole cell_LmrR_product 3a added (F-C Alkylation of 1 and 2a)

<Chromatogram>



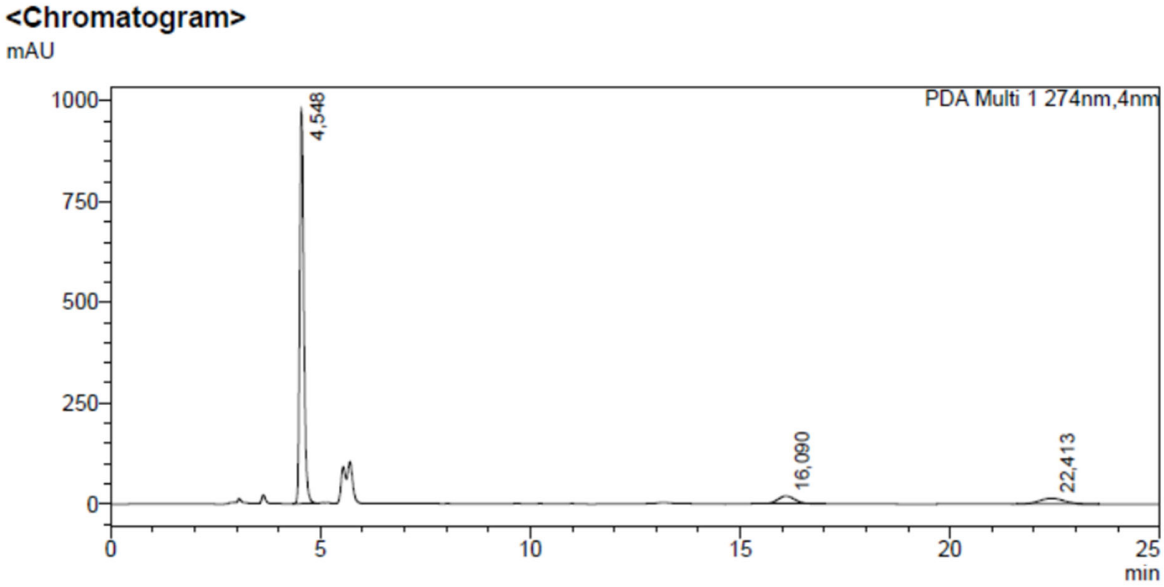
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,583	6538392	24,708
	16,720	9954867	37,618
	22,927	9969510	37,674
		26462769	100,000

Cu(II)-Phen catalyzed reaction (F-C Alkylation of 1 and 2a)

<Chromatogram>



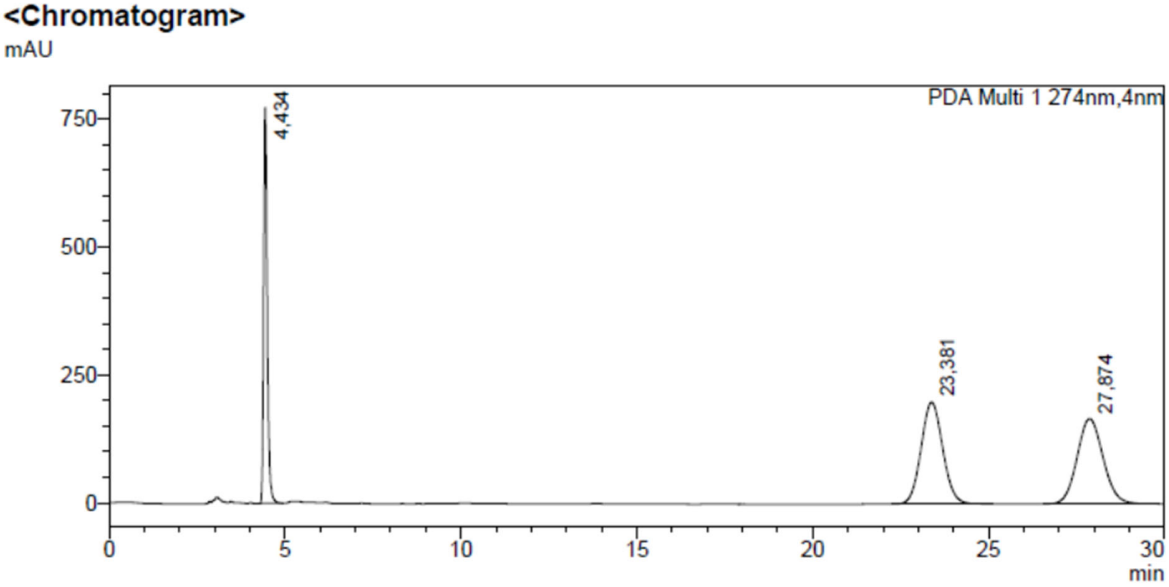
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,548	6595110	85,213
	16,090	570273	7,368
	22,413	574211	7,419
		7739595	100,000

Whole cell_LmrR_product 3b added (F-C Alkylation of 1 and 2b)

<Chromatogram>



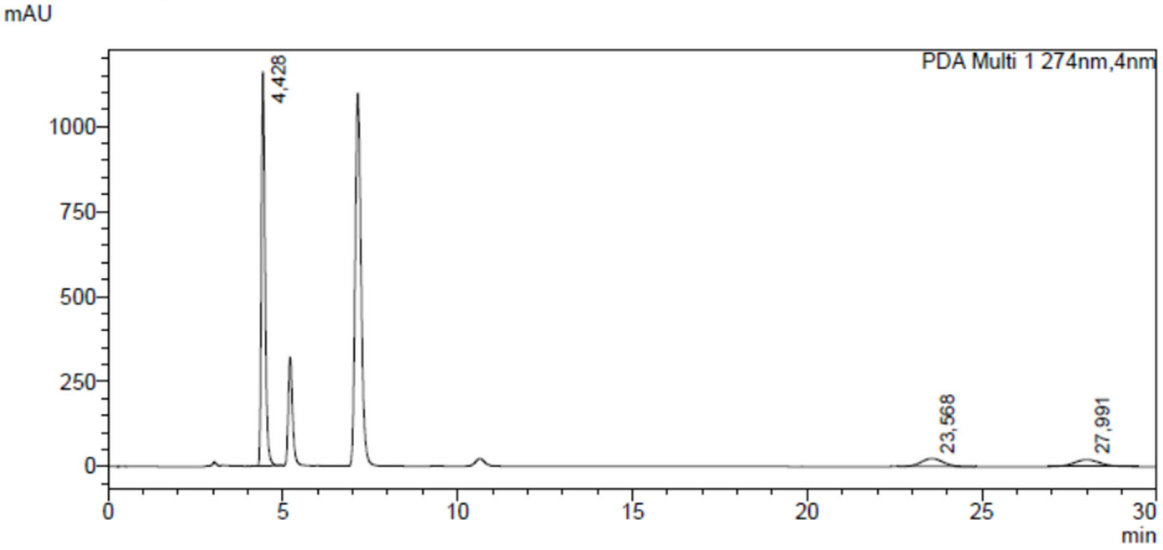
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,434	5482058	24,296
product ee1	23,381	8521597	37,767
product ee2	27,874	8559990	37,937
		22563645	100,000

Cu(II)-Phen catalyzed reaction (F-C Alkylation of 1 and 2b)

<Chromatogram>



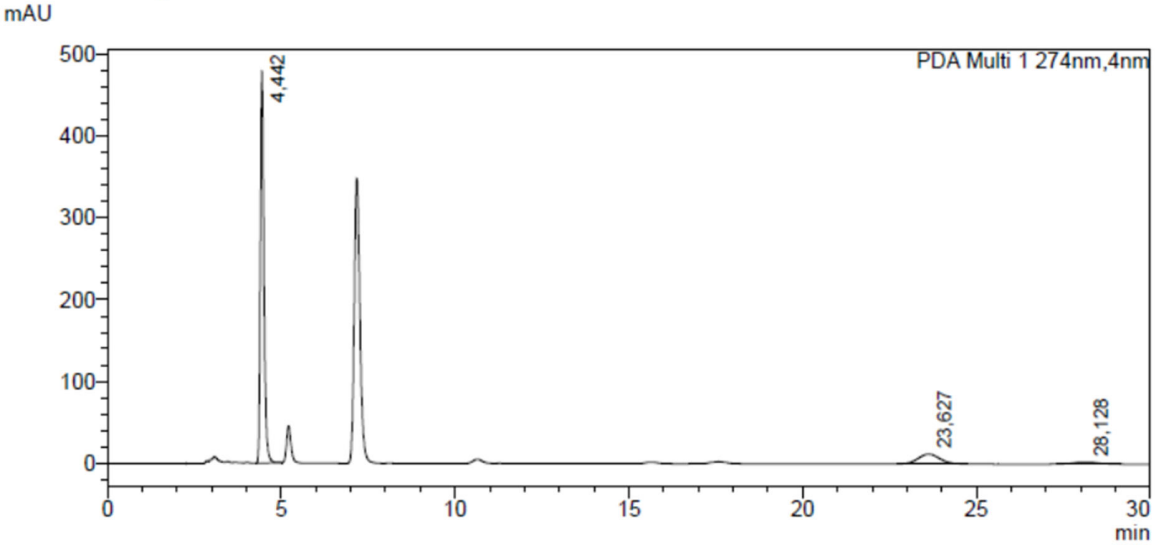
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,428	8517994	80,649
product ee1	23,568	1020952	9,666
product ee2	27,991	1022828	9,684
		10561774	100,000

Whole cell_LmrR ⊂Cu(II)-Phen (cell fraction) (F-C Alkylation of 1 and 2b)

<Chromatogram>



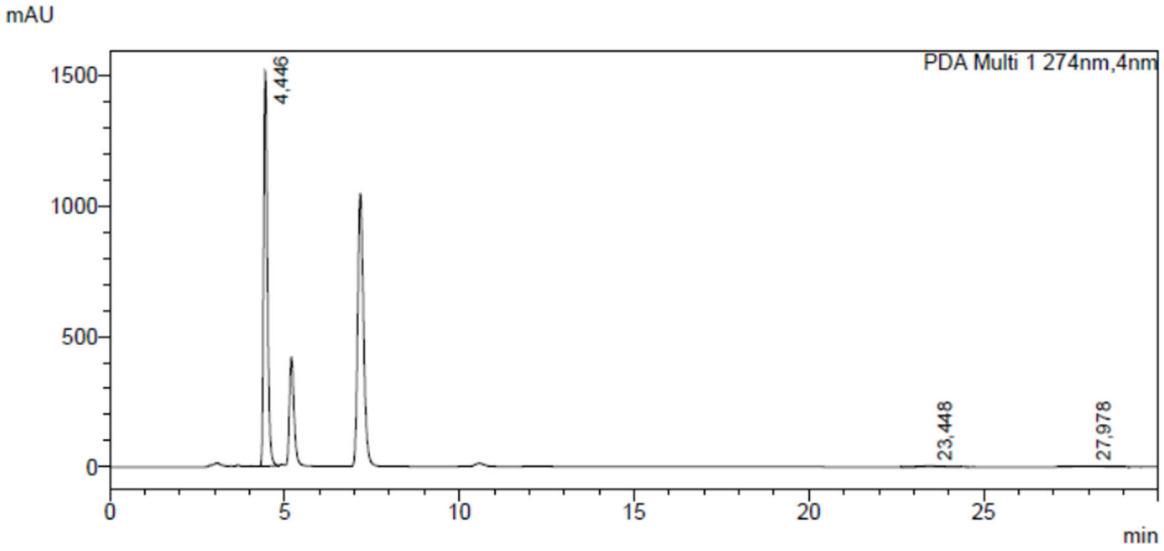
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,442	3440413	84,638
product ee1	23,627	516573	12,708
product ee2	28,128	107892	2,654
		4064878	100,000

Whole cell_LmrR ⊂ Cu(II)-Phen (supernatant fraction) (F-C Alkylation of 1 and 2b)

<Chromatogram>



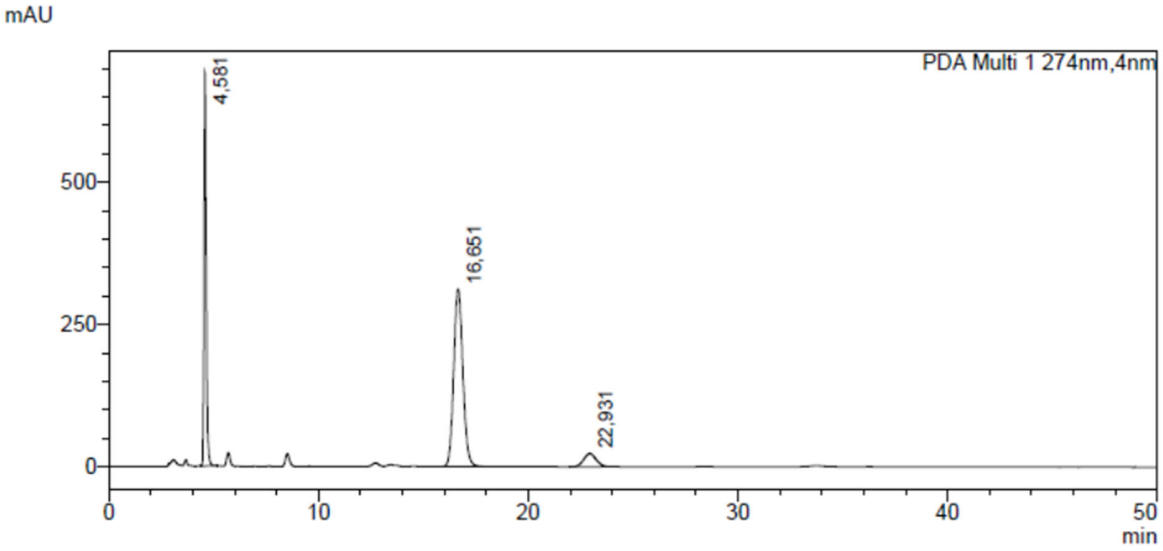
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,446	11376503	97,358
product ee1	23,448	156254	1,337
product ee2	27,978	152513	1,305
		11685270	100,000

Recycled whole cell_LmrR ⊂Cu(II)-Phen (cell fraction) (F-C Alkylation of 1 and 2a)

<Chromatogram>



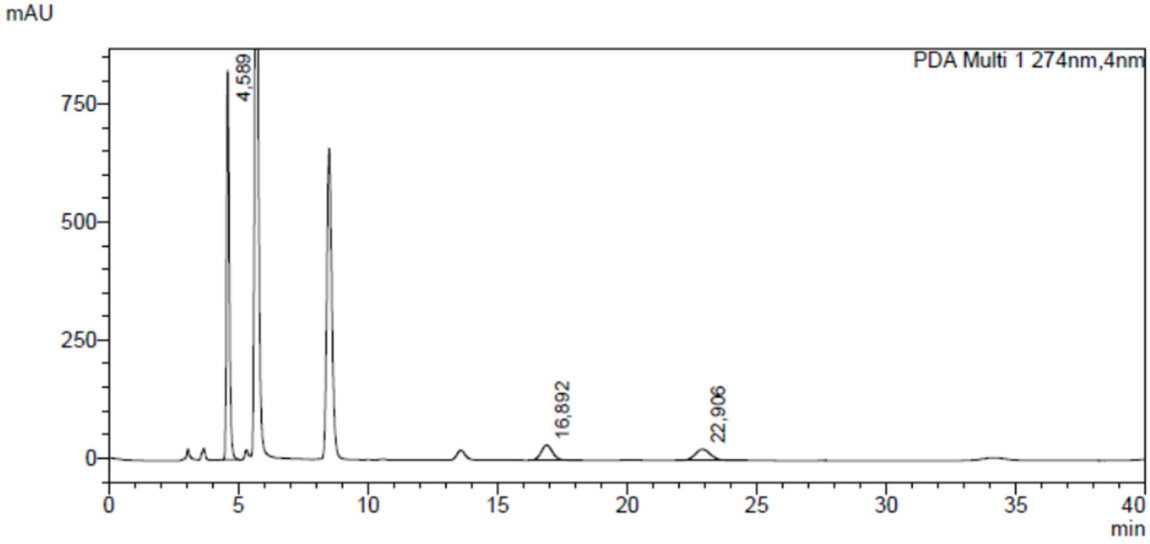
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,581	5170811	33,137
	16,651	9465699	60,660
	22,931	967993	6,203
		15604503	100,000

Recycled whole cell_LmrR⊂Cu(II)-Phen(supernatant fraction)(F-C Alkylation of 1 and 2a)

<Chromatogram>



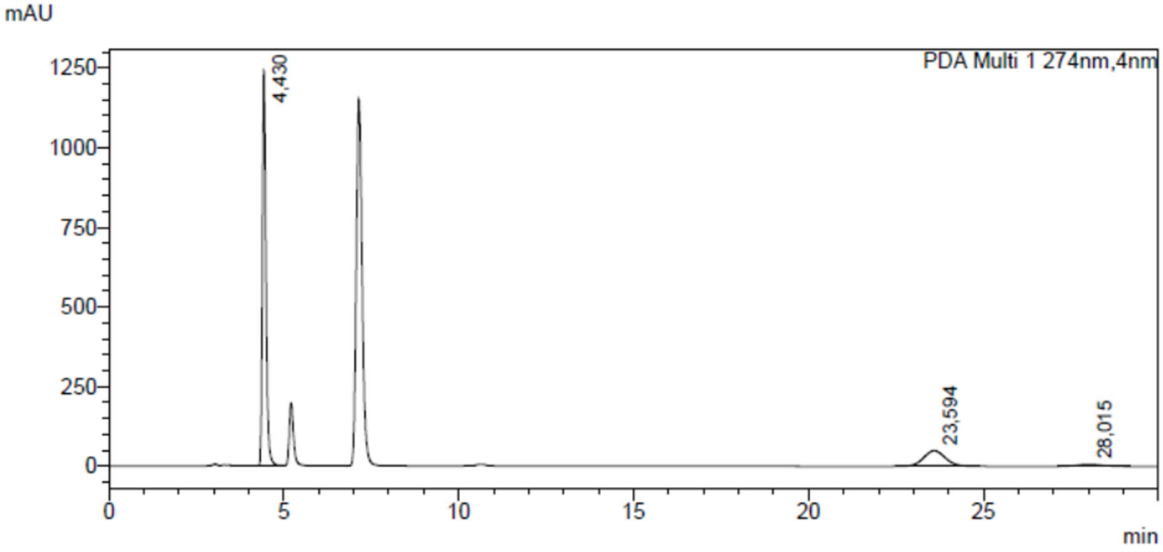
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,589	6181500	75,951
	16,892	980468	12,047
	22,906	976867	12,003
		8138834	100,000

Purified protein LmrR ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2b)

<Chromatogram>



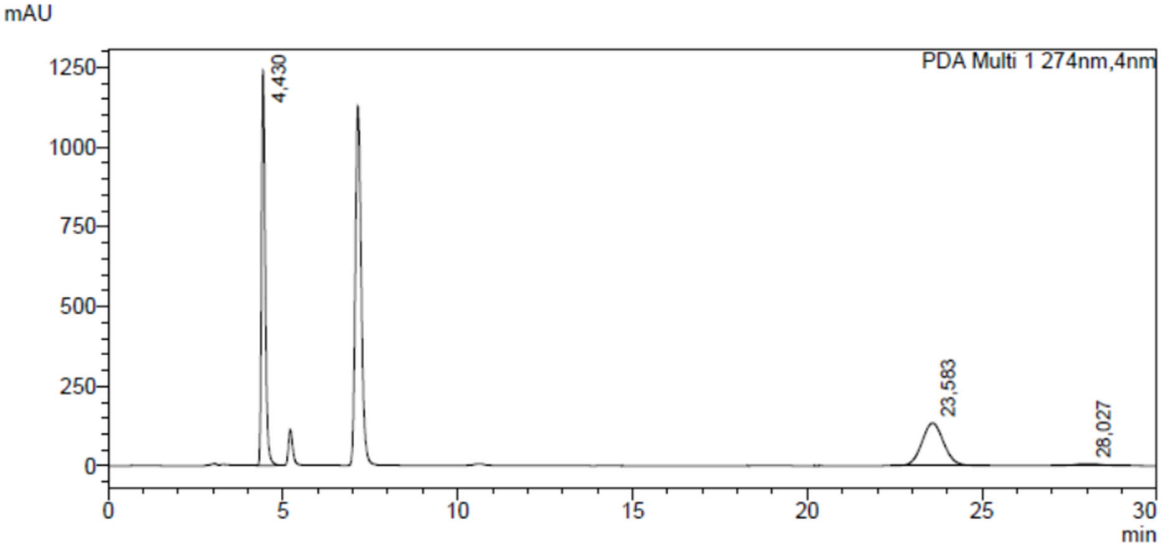
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,430	8852688	78,949
product ee1	23,594	2086504	18,608
product ee2	28,015	274004	2,444
		11213196	100,000

Purified protein LmrR_A92E ⊂Cu(II)-Phen (F-C Alkylation of 1 and 2b)

<Chromatogram>



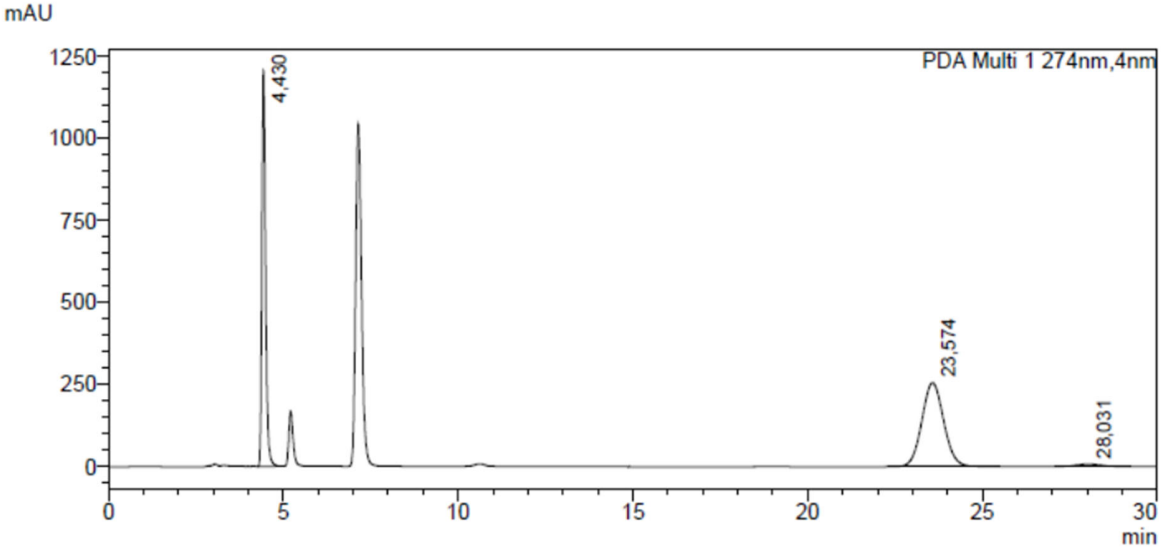
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,430	9107589	59,967
product ee1	23,583	5775183	38,025
product ee2	28,027	304982	2,008
		15187754	100,000

Purified protein LmrR_A92E_M8D ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2b)

<Chromatogram>



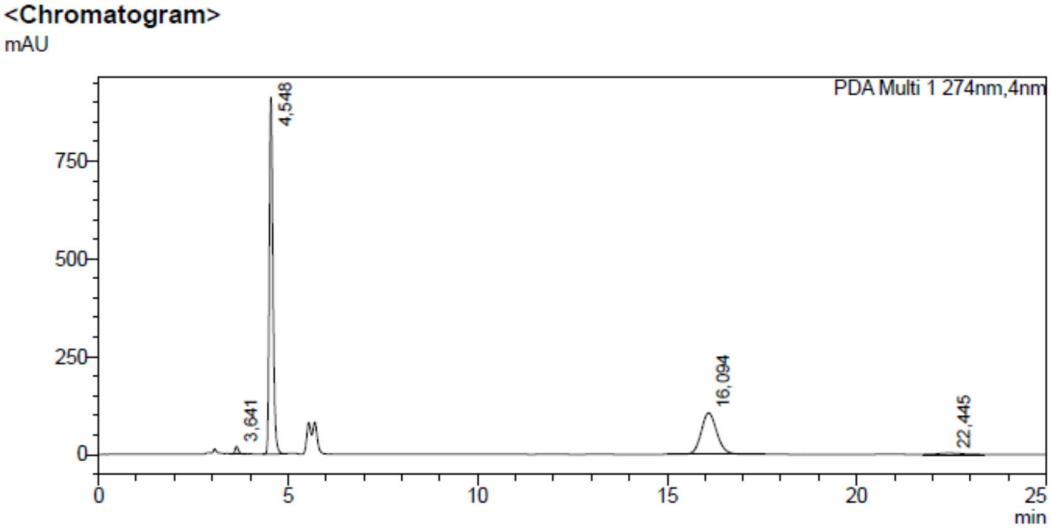
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,430	8885152	43,606
product ee1	23,574	11107541	54,513
product ee2	28,031	383068	1,880
		20375761	100,000

Purified protein LmrR ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2a)

<Chromatogram>



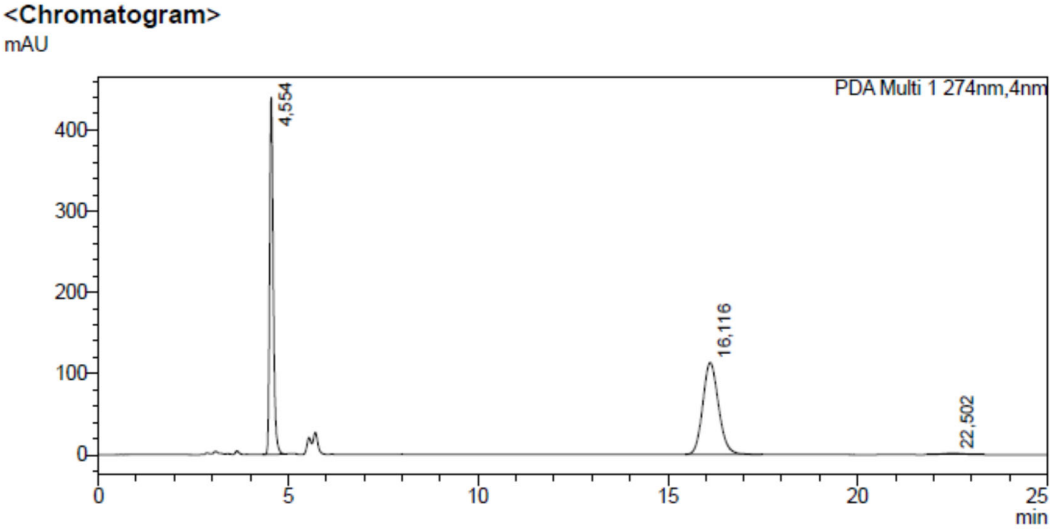
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
	3,641	131143	1,389
2-phenylqui	4,548	6091423	64,538
	16,094	3073856	32,567
	22,445	142141	1,506
		9438563	100,000

Purified protein LmrR_A92E ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2a)

<Chromatogram>



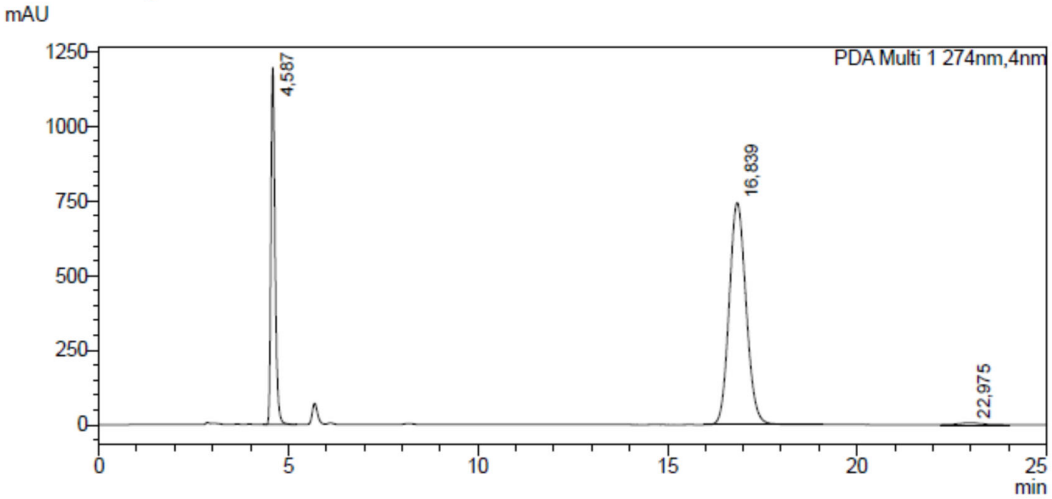
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,554	2923629	46,554
	16,116	3283394	52,283
	22,502	73040	1,163
		6280064	100,000

Purified protein LmrR_A92E_M8D ⊂Cu(II)-Phen (F-C Alkylation of 1 and 2a)

<Chromatogram>



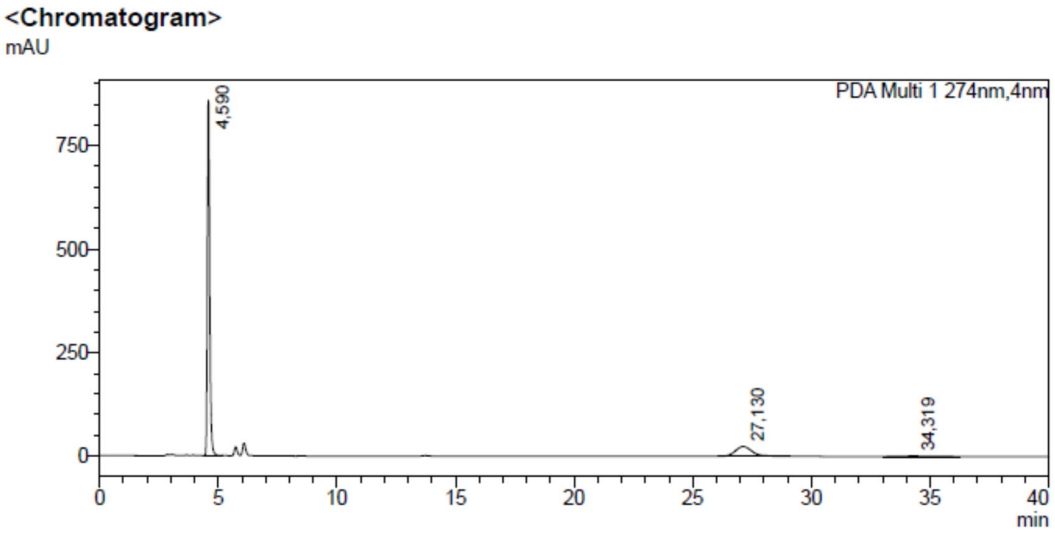
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,587	9080724	28,013
	16,839	23074989	71,184
	22,975	260199	0,803
		32415912	100,000

Purified protein LmrR ⊂Cu(II)-Phen (F-C Alkylation of 1 and 2c)

<Chromatogram>



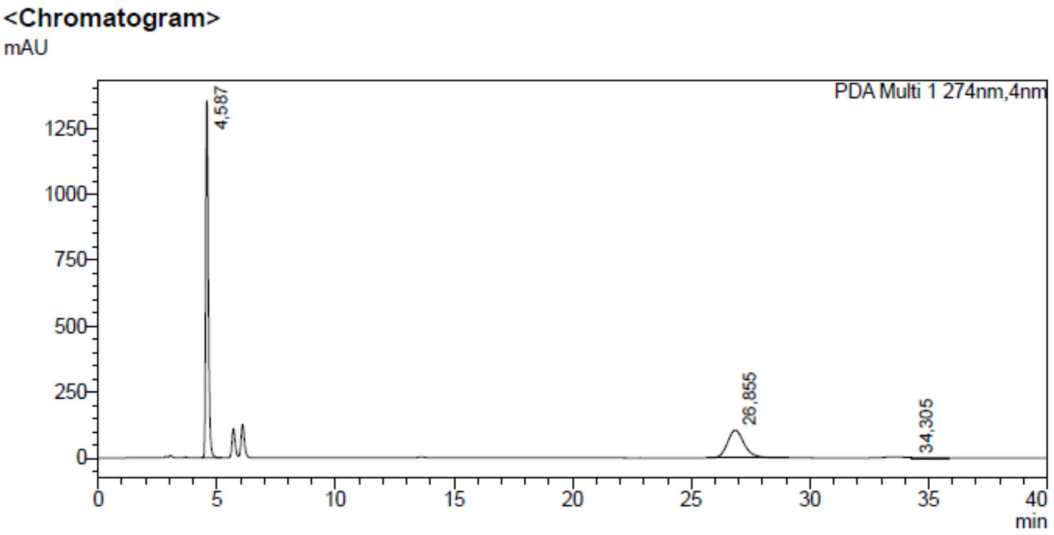
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,590	5957982	81,840
	27,130	1209410	16,613
	34,319	112682	1,548
		7280074	100,000

Purified protein LmrR_A92E ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2c)

<Chromatogram>



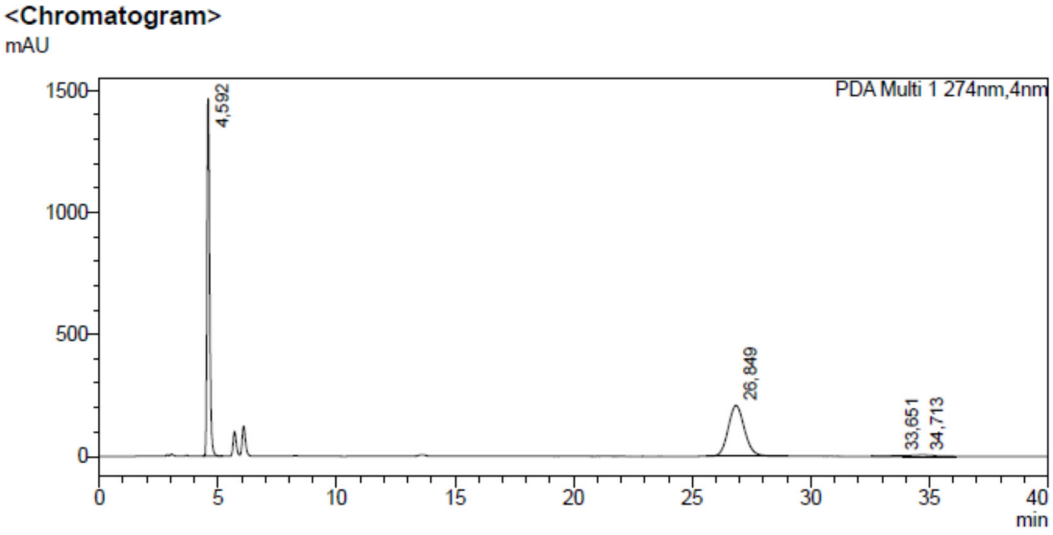
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,587	10407838	65,976
	26,855	5231343	33,162
	34,305	136043	0,862
		15775224	100,000

Purified protein LmrR_A92E_M8D ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2c)

<Chromatogram>



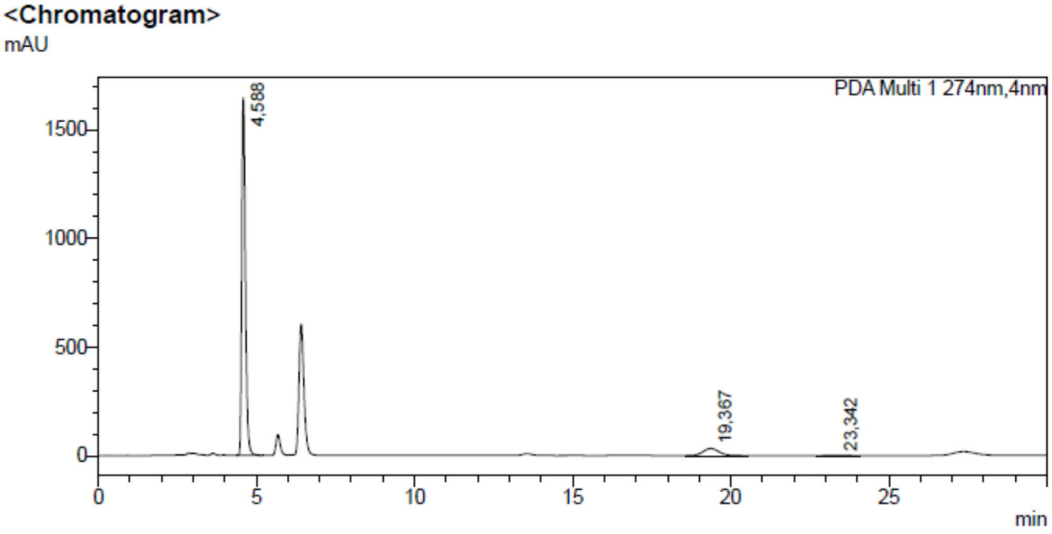
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,592	11389697	52,046
	26,849	10013569	45,758
	33,651	109052	0,498
	34,713	371512	1,698
		21883831	100,000

Purified protein LmrR_A92E ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2d)

<Chromatogram>



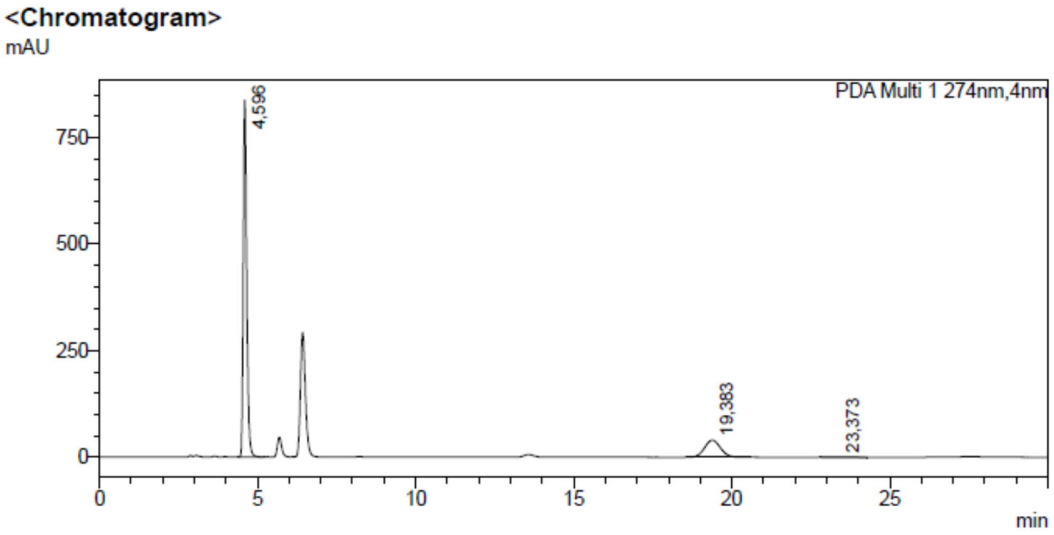
<Peak Table>

PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,588	13215013	91,756
	19,367	1155051	8,020
product ee1	23,342	32347	0,225
		14402411	100,000

Purified protein LmrR_A92E_M8D ⊂ Cu(II)-Phen (F-C Alkylation of 1 and 2d)

<Chromatogram>



<Peak Table>

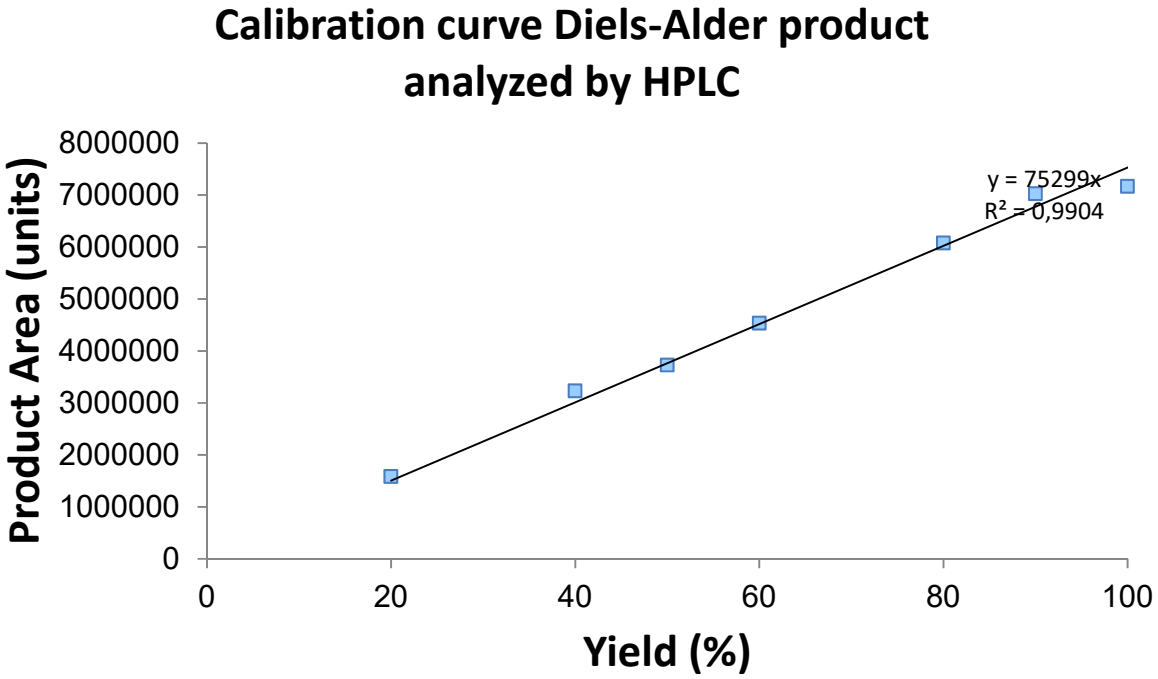
PDA Ch1 274nm

Name	Ret. Time	Area	Area%
2-phenylqui	4,596	6370063	81,345
	19,383	1417054	18,096
product ee1	23,373	43779	0,559
		7830896	100,000

Diels-Alder reaction

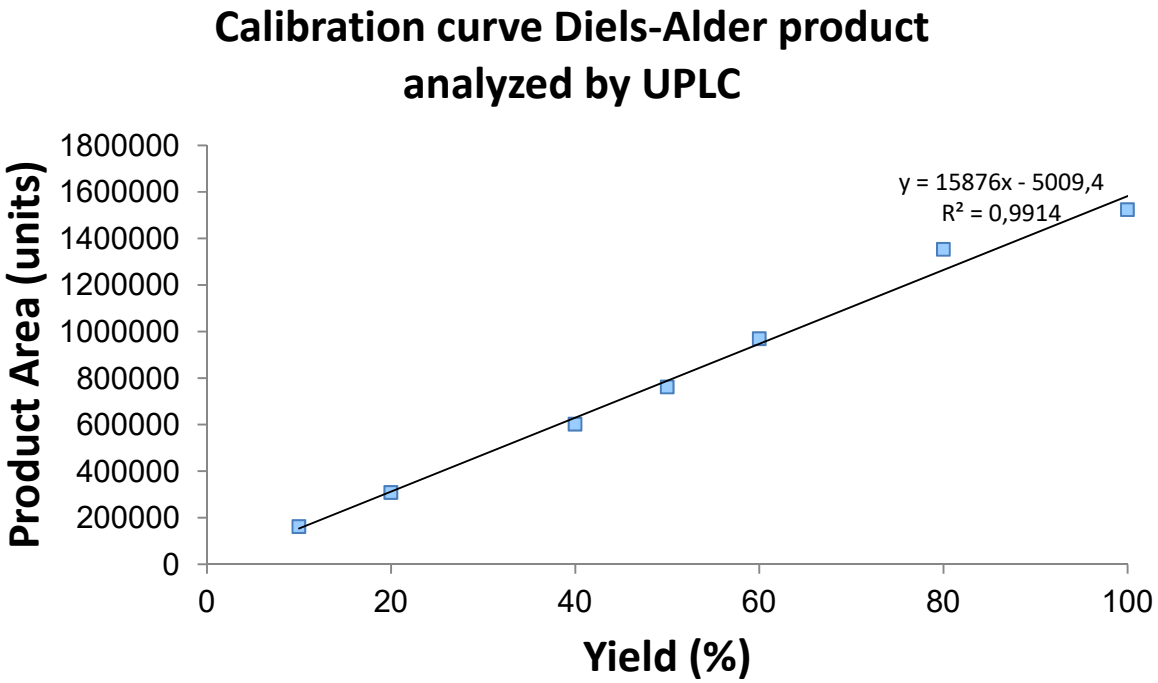
The reactions were analyzed by adding 50 µL internal standard (2-phenylquinoline, 2 mM in CH_3CN) to the reaction mixture, followed by extraction with diethyl ether (3 x 500 µL) and separated organic layer were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Subsequently, the samples were re-dissolved in 150 µL of a heptane:propan-2-ol mixture (9:1) and the yields and enantiomeric excess were determined by HPLC (Daicel chiralpak AD-H). Calibration curves for quantification of the different products were constructed using pure reference compounds produced synthetically as described in section "Chemical synthesis".

Calibration curve for product analyzed by HPLC (Diels-Alder reaction of aza-chalcone and cyclopentadiene)



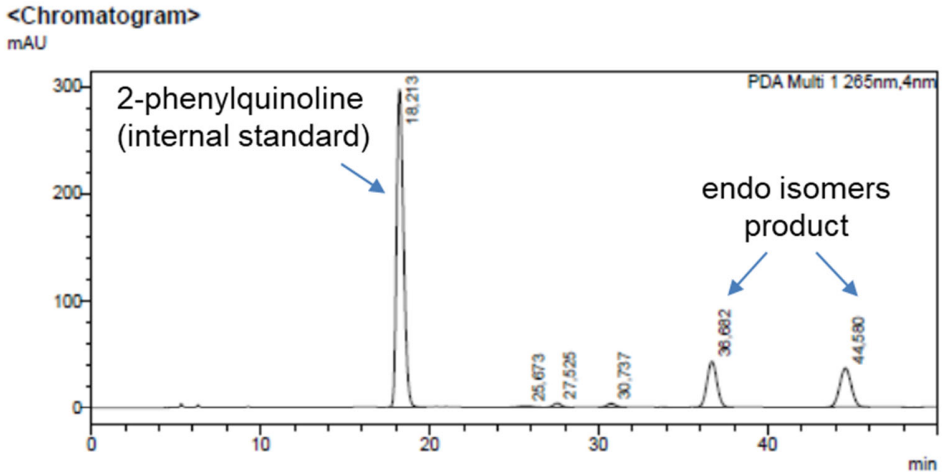
The reactions were analyzed by adding 50 µL internal standard (2-phenylquinoline, 2 mM in CH_3CN) to the reaction mixture, followed by liquid-liquid extraction using diethyl ether (2 x 0.75 mL). The organic phase was filtered and then evaporated under reduced pressure. Subsequently, the samples were re-dissolved in 150 µL of a water:methanol mixture (1:1) and the yield was determined by UPLC. Calibration curves for quantification of the different products were constructed using pure reference compounds produced synthetically as described in section "Chemical synthesis".

Calibration curve for product analyzed by UPLC (Diels-Alder reaction of aza-chalcone and cyclopentadiene)



HPLC Chromatograms

Racemate of Diels-Alder product 6 (with internal standard)



<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	8466571	18,213	68,777	296164
	65921	25,673	0,536	1206
	125137	27,525	1,017	3931
	124138	30,737	1,008	3476
	1762507	36,682	14,317	42972
	1765923	44,580	14,345	36717
	12310197		100,000	384466

18.8 min – 2-phenylquinoline (internal standard)

26.7 min – exo product enantiomer 1

29.7 min – exo product enantiomer 2

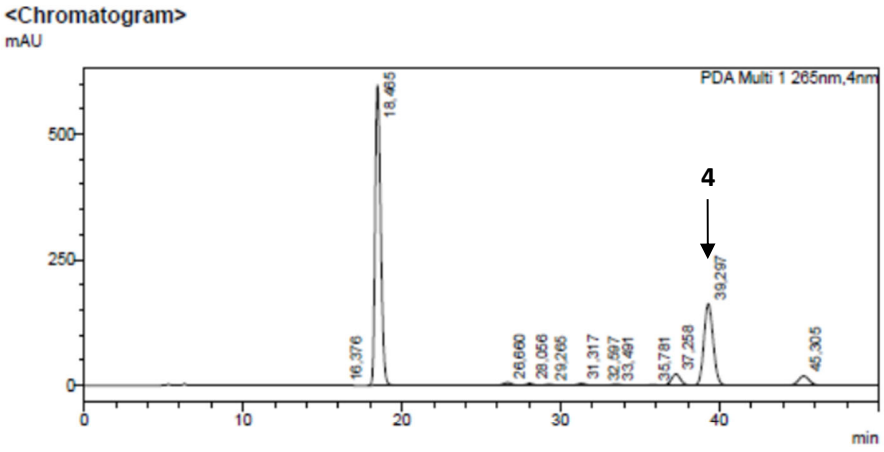
35.4 min – endo product enantiomer 1

42.5 min – endo product enantiomer 2

Unreacted azachalcone (**4**): 39.0 min (not present in this chromatogram).

Cu-Phen catalyzed Diels-Alder reaction

<Chromatogram>



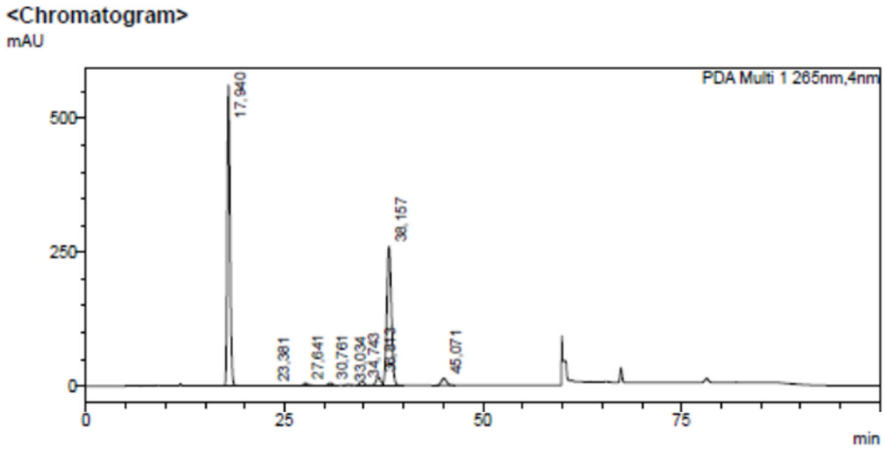
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	4712	16,376	0,020	220
	14489385	18,465	62,127	597231
	182237	26,660	0,781	5204
	104694	28,056	0,449	3515
	65807	29,265	0,282	1883
	118198	31,317	0,507	3400
	11301	32,597	0,048	389
	66119	33,491	0,284	1923
	39803	35,781	0,171	1196
	864138	37,258	3,705	22124
	6518062	39,297	27,948	161935
	857642	45,305	3,677	18356
	23322096		100,000	817377

Whole cell_cytoplasmic SUMO_Cu(II)-Phen added

<Chromatogram>



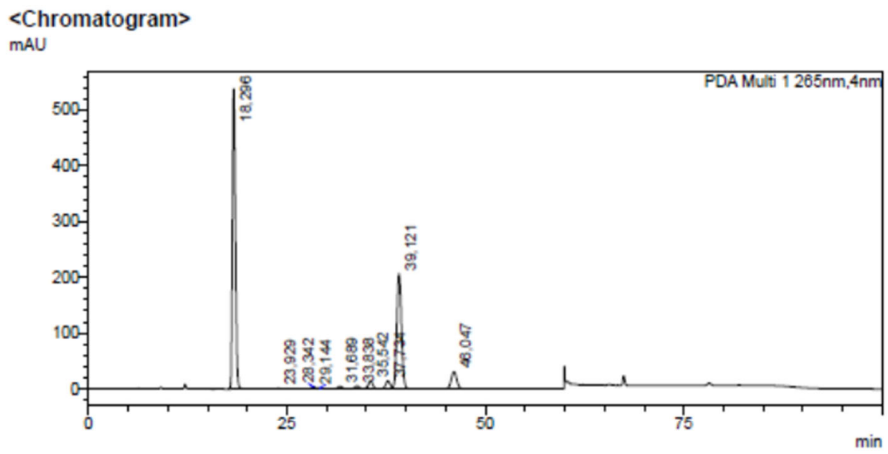
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	13968867	17,940	52,988	561922
	6953	23,381	0,026	315
	184256	27,641	0,699	4928
	168543	30,761	0,639	4686
	29341	33,034	0,111	915
	298013	34,743	1,130	8461
	617221	36,813	2,341	15799
	10462028	38,157	39,685	259773
	627206	45,071	2,379	13115
	26362429		100,000	869914

Whole cell_cytoplasmic LmrR_V15A_A92E⊂Cu(II)-Phen

<Chromatogram>



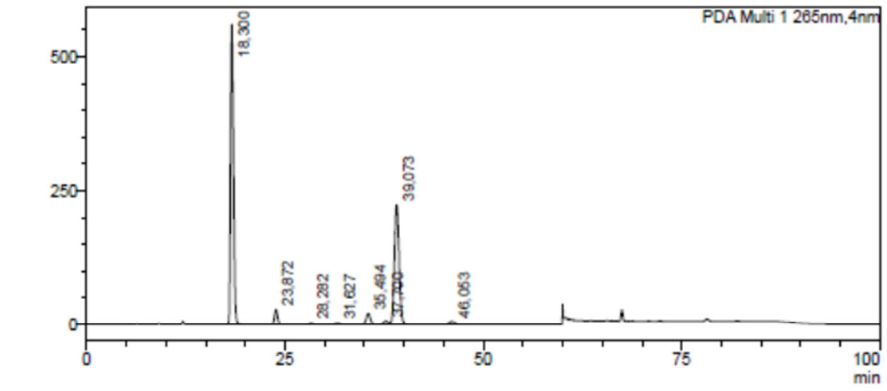
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	13837809	18,296	54,760	537779
	22471	23,929	0,089	914
	113119	28,342	0,448	3702
	44639	29,144	0,177	1360
	158233	31,689	0,626	4274
	175181	33,838	0,693	4976
	530335	35,542	2,099	14117
	545333	37,734	2,158	13905
	8392822	39,121	33,213	205985
	1450078	46,047	5,738	30429
	25270020		100,000	817441

Whole cell_cytoplasmic LmrR_V15A_A92E no Cu(II)-Phen added

<Chromatogram>



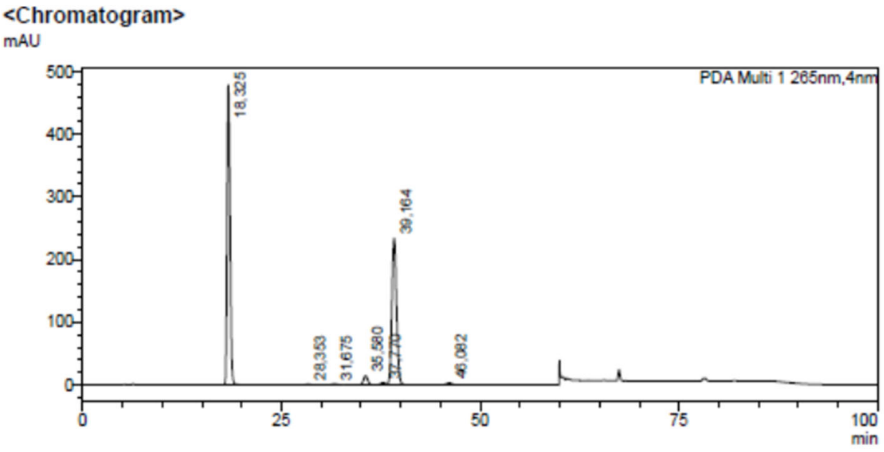
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	14267297	18,300	55,826	560701
	722057	23,872	2,825	28268
	71642	28,282	0,280	2458
	101882	31,627	0,399	2560
	752624	35,494	2,945	20846
	249876	37,700	0,978	6440
	9135872	39,073	35,748	223302
	255268	46,053	0,999	5389
	25556517		100,000	849964

Supernatant after Cu(II)-Phen incubation_cytoplasmic LmrR_V15A_A92E

<Chromatogram>



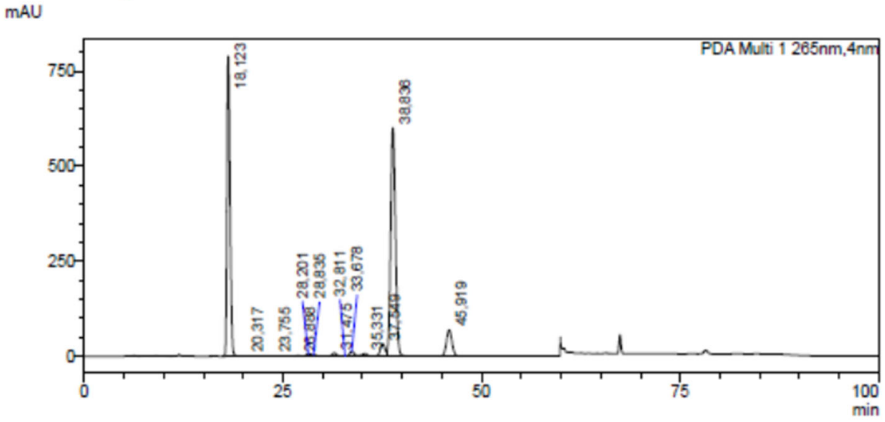
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	12216067	18,325	54,103	478250
	30833	28,353	0,137	1078
	51994	31,675	0,230	1205
	512154	35,580	2,268	14235
	120783	37,770	0,535	3170
	9522526	39,164	42,174	233337
	124858	46,082	0,553	2671
	22579215		100,000	733948

Whole cell_cytoplasmic LmrR_V15A_A92E⊂Cu(II)-Phen After 48 hours in vivo catalysis, Recycled cell fraction + substrates

<Chromatogram>



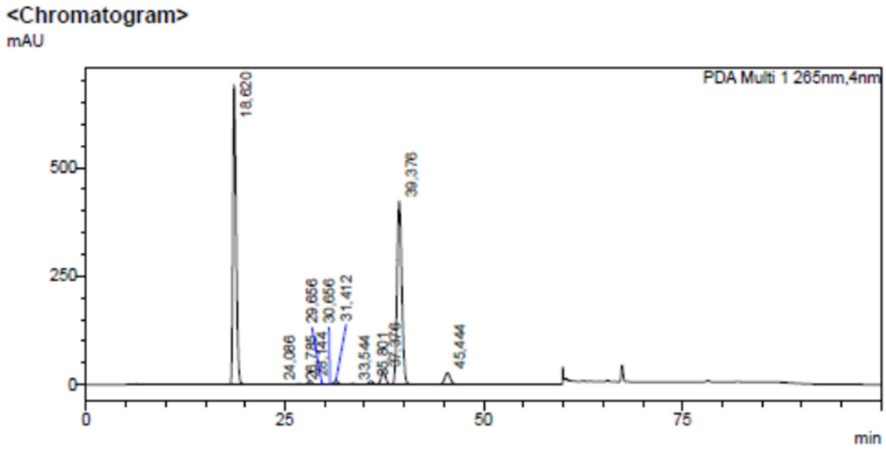
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	21109340	18,123	40,267	790018
	10389	20,317	0,020	264
	14787	23,755	0,028	606
	47644	26,888	0,091	1143
	209550	28,201	0,400	6624
	145825	28,835	0,278	4287
	292275	31,475	0,558	7938
	10874	32,811	0,021	456
	479647	33,678	0,915	12867
	294081	35,331	0,561	6391
	1275471	37,549	2,433	32362
	25234731	38,836	48,136	600994
	3299129	45,919	6,293	68308
	52423743		100,000	1532258

Whole cell_cytoplasmic LmrR_V15A_A92E⊂Cu(II)-Phen After 48 hours in vivo catalysis, Supernatant + substrates

<Chromatogram>



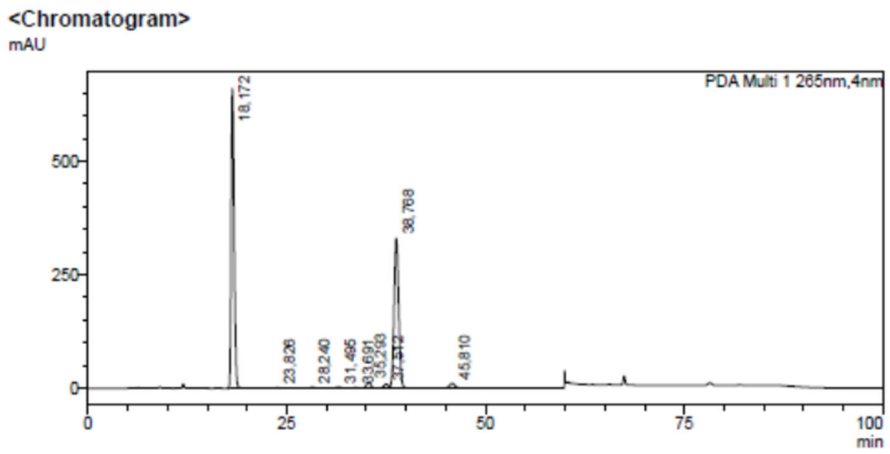
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	19623588	18,620	48,160	690828
	5901	24,086	0,014	256
	49314	26,785	0,121	1269
	270582	28,144	0,664	8691
	39440	29,656	0,097	1146
	20484	30,656	0,050	807
	287045	31,412	0,704	8030
	58251	33,544	0,143	1738
	275329	35,801	0,676	7689
	1157212	37,376	2,840	28863
	17688489	39,376	43,411	422746
	1271373	45,444	3,120	26788
	40747007		100,000	1198850

Whole cell_cytoplasmic LmrR_A92E⊂Cu(II)-Phen

<Chromatogram>



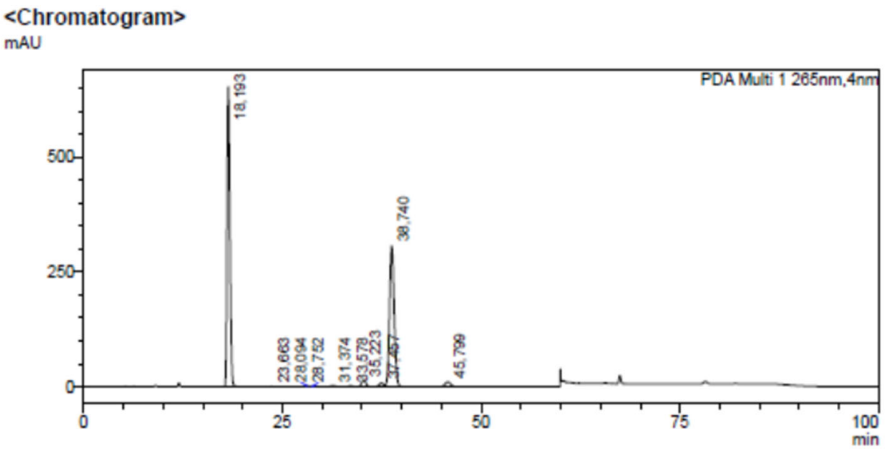
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	17034175	18,172	53,558	659731
	26971	23,826	0,085	1076
	44511	28,240	0,140	1714
	92875	31,495	0,292	2493
	41710	33,691	0,131	1299
	482252	35,293	1,516	13502
	323899	37,512	1,018	8645
	13287172	38,768	41,777	329630
	471509	45,810	1,482	9964
	31805074		100,000	1028054

Whole cell_cytoplasmic LmrR_V15A⊂Cu(II)-Phen

<Chromatogram>



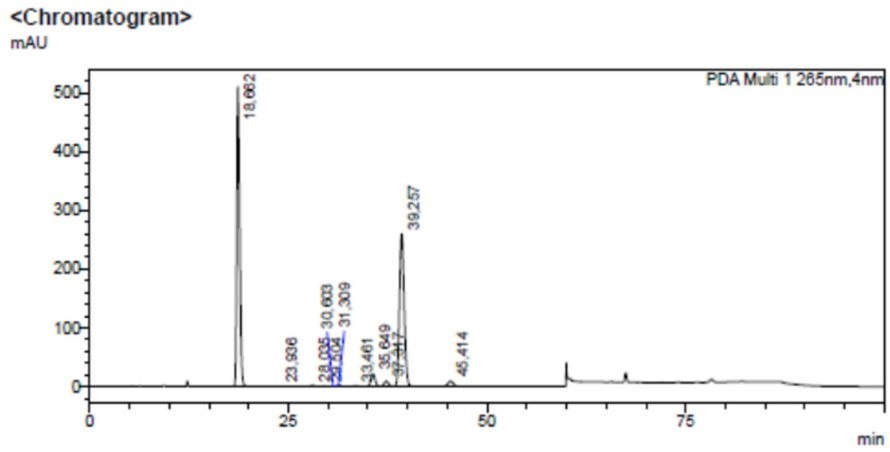
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	16369770	18,193	53,707	653088
	16816	23,663	0,055	688
	57658	28,094	0,189	1839
	24768	28,752	0,081	811
	89404	31,374	0,293	2287
	56707	33,578	0,186	1694
	530692	35,223	1,741	14661
	289294	37,457	0,949	7639
	12550625	38,740	41,177	306711
	493809	45,799	1,620	10386
	30479543		100,000	999803

Whole cell_cytoplasmic LmrR⊂Cu(II)-Phen

<Chromatogram>



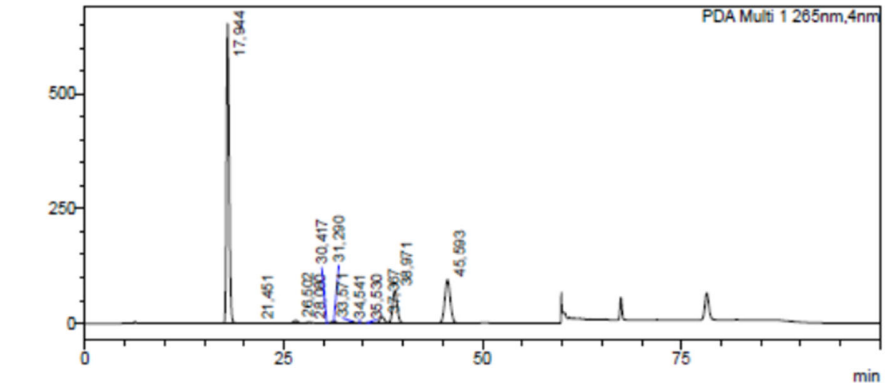
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	13143791	18,662	51,530	509737
	13264	23,936	0,052	558
	59336	28,035	0,233	2011
	17642	29,504	0,069	581
	14698	30,603	0,058	499
	68666	31,309	0,269	1970
	34557	33,461	0,135	1057
	767406	35,649	3,009	21381
	344698	37,317	1,351	8751
	10628330	39,257	41,668	259153
	414614	45,414	1,625	8824
	25507001		100,000	814524

Purified protein LmrR_V15A_A92E⊂Cu-Phen

<Chromatogram>



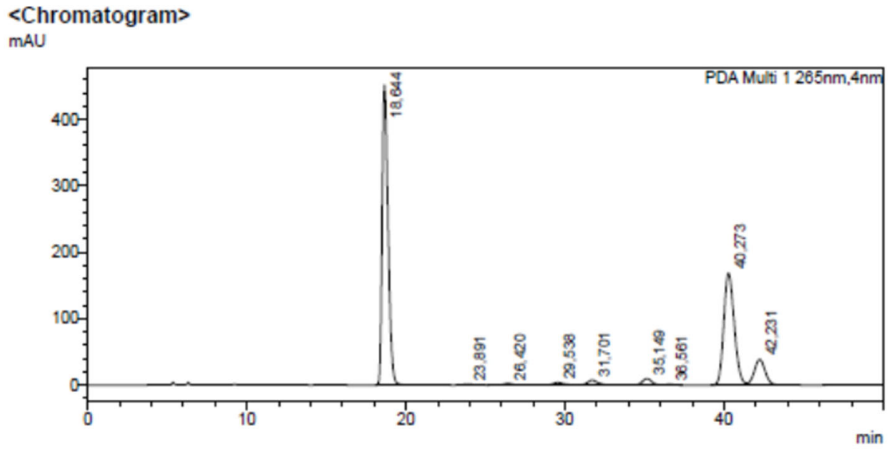
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	15671535	17,944	64,313	652357
	5861	21,451	0,024	300
	224527	26,502	0,921	6131
	46626	28,080	0,191	1739
	26978	30,417	0,111	826
	183914	31,290	0,755	5172
	150492	33,571	0,618	4241
	55733	34,541	0,229	1551
	25015	35,530	0,103	728
	553246	37,367	2,270	13948
	2882962	38,971	11,831	71205
	4540623	45,593	18,634	94668
	24367513		100,000	852866

Purified protein LmrR_A92E⊂Cu-Phen

<Chromatogram>



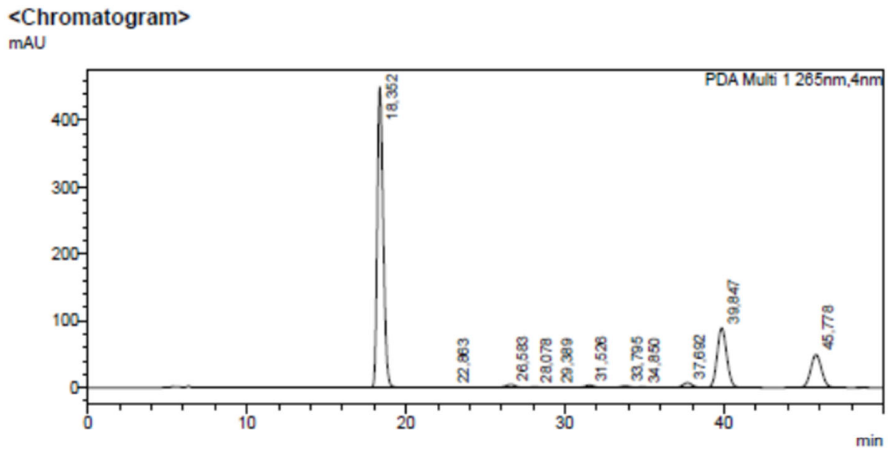
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	11649144	18,644	52,840	451169
	38930	23,891	0,177	738
	61236	26,420	0,278	2013
	129750	29,538	0,589	3226
	282701	31,701	1,282	6832
	371920	35,149	1,687	9459
	31597	36,561	0,143	781
	7683110	40,273	34,851	168113
	1797498	42,231	8,153	38307
	22045886		100,000	680637

Purified protein LmrR_V15A⊂Cu-Phen

<Chromatogram>



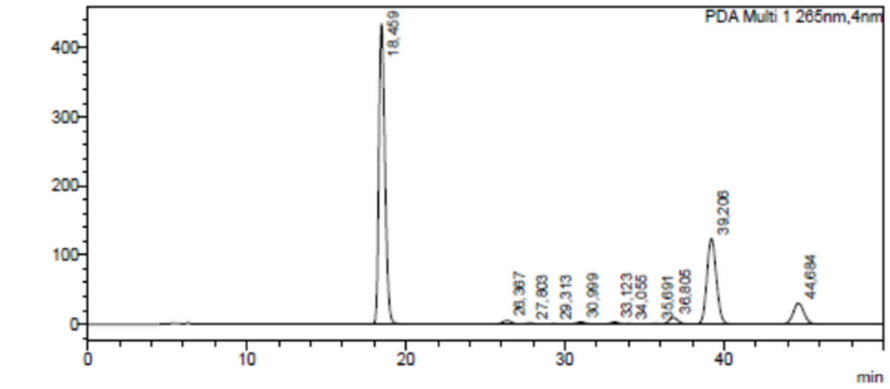
<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	11644277	18,352	63,188	449524
	3245	22,863	0,018	157
	176725	26,583	0,959	4476
	32478	28,078	0,176	1090
	12087	29,389	0,066	354
	124666	31,526	0,677	3354
	89272	33,795	0,484	2413
	29067	34,850	0,158	796
	278758	37,692	1,513	6928
	3712268	39,847	20,145	88978
	2325166	45,778	12,618	49154
	18428008		100,000	607223

Purified protein LmrR⊂Cu-Phen

<Chromatogram>



<Peak Table>

PDA Ch1 265nm

Name	Area	Ret. Time	Area%	Height
	11414999	18,459	60,848	434189
	180840	26,367	0,964	4844
	43959	27,803	0,234	1468
	18476	29,313	0,098	565
	102374	30,999	0,546	2815
	94271	33,123	0,503	2693
	19506	34,055	0,104	593
	26900	35,691	0,143	822
	367243	36,805	1,958	9280
	5078492	39,206	27,071	123643
	1412830	44,684	7,531	30080
	18759891		100,000	610992

15. References

(1) Roelfes, G.; Boersma, A.; Feringa, B. Highly enantioselective DNA-based catalysis. *Chem. Commun.* **2006**, 635-637.

(2) Evans, D.; Fandrick, K.; Song, H. Enantioselective Friedel-Crafts alkylations of alpha-beta-unsaturated 2-acyl imidazoles catalyzed by bis(oxazolinyl)pyridine-scandium(III) triflate complexes. *J. Am. Chem. Soc.* **2005**, 127, 8942-8943.

(3) Bos, J.; Browne, W. R.; Driessen, A. J. M.; Roelfes, G. Supramolecular Assembly of Artificial Metalloenzymes Based on the Dimeric Protein LmrR as Promiscuous Scaffold. *J. Am. Chem. Soc.* **2015**, 137, 9796-9799.

(4) Otto, S.; Bertoncin, F.; Engberts, J. Lewis acid catalysis of a Diels-Alder reaction in water. *J. Am. Chem. Soc.* **1996**, 118, 7702-7707.

(5) Roelfes, G.; Feringa, B. DNA-based asymmetric catalysis. *Angew. Chem. Int. Ed.* **2005**, 44, 3230-3232.

(6) Bos, J.; García-Herraiz, A.; Roelfes, G. An enantioselective artificial metallo-hydratase. *Chem. Sci.* **2013**, 4, 3578-3582.

(7) Drienovská, I.; Rioz-Martinez, A.; Draksharapu, A.; Roelfes, G. Novel artificial metalloenzymes by in vivo incorporation of metal-binding unnatural amino acids. *Chem. Sci.* **2015**, 6, 770-776.

(8) Renault, M.; Pawsey, S.; Bos, M. P.; Koers, E. J.; Nand, D.; Tommassen-van Boxtel, R.; Rosay, M.; Tommassen, J.; Maas, W. E.; Baldus, M. Solid-State NMR Spectroscopy on Cellular Preparations Enhanced by Dynamic Nuclear Polarization. *Angew. Chem. Int. Ed.* **2012**, 51, 2998-3001.

(9) Lacabanne, D.; Meier, B. H.; Bockmann, A. Selective labeling and unlabeling strategies in protein solid-state NMR spectroscopy. *J. Biomol. NMR* **2018**, 71, 141-150.

(10) Villarino, L.; Chordia, S.; Alonso-Cotchico, L.; Reddem, E. R.; Zhou, Z.; Thunnissen, A.-M.; Maréchal, J.-D.; Roelfes, G. Cofactor binding dynamics influence the catalytic activity and selectivity of an artificial metalloenzyme. ACS Catal. **2020**. DOI: 10.1021/acscatal.0c01619.

(11) Sauvee, C.; Rosay, M.; Casano, G.; Aussenac, F.; Weber, R. T.; Ouari, O.; Tordo, P. Highly Efficient, Water-Soluble Polarizing Agents for Dynamic Nuclear Polarization at High Frequency. *Angew. Chem. Int. Ed.* **2013**, 52, 10858-10861.

(12) Fung, B.; Khitrin, A.; Ermolaev, K. An improved broadband decoupling sequence for liquid crystals and solids. *J. Magn. Res.* **2000**, 142, 97-101.

(13) Weingarth, M.; Demco, D. E.; Bodenhausen, G.; Tekely, P. Improved magnetization transfer in solid-state NMR with fast magic angle spinning. *Chem. Phys. Lett.* **2009**, 469, 342-348.

(14) Baldus, M.; Petkova, A.; Herzfeld, J.; Griffin, R. Cross polarization in the tilted frame: assignment and spectral simplification in heteronuclear spin systems. *Mol. Phys.* **1998**, 95, 1197-1207.

(15) Hohwy, M.; Rienstra, C.; Jaroniec, C.; Griffin, R. Fivefold symmetric homonuclear dipolar recoupling in rotating solids: Application to double quantum spectroscopy. *J. Chem. Phys.* **1999**, 110, 7983-7992.

(16) Takeuchi, K.; Imai, M.; Shimada, I. Dynamic equilibrium on DNA defines transcriptional regulation of a multidrug binding transcriptional repressor, LmrR. *Sci. Rep.* **2017**, 7, 267.

(17) Takeuchi, K.; Tokunaga, Y.; Imai, M.; Takahashi, H.; Shimada, I. Dynamic multidrug recognition by multidrug transcriptional repressor LmrR. *Sci. Rep.* **2014**, 4, 6922.

(18) Delaglio, F.; Grzesiek, S.; Vuister, G.; Zhu, G.; Pfeifer, J.; Bax, A. NMRpipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *J. Biomol. NMR* **1995**, 6, 277-293.

(19) Lee, W.; Tonelli, M.; Markley, J. L. NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. *Bioinformatics* **2015**, 31, 1325-1327.

(20) Narasimhan S., Mance D., Pinto C., Weingarth M., Bonvin A.M.J.J., Baldus M. (2018) Rapid Prediction of Multi-dimensional NMR Data Sets Using FANDAS. In: Ghose R. (eds) Protein NMR. Methods in Molecular Biology, vol 1688. Humana Press, New York, NY.

(21) Reetz, M. T.; Kahakeaw, D.; Lohmer, R. Addressing the numbers problem in directed evolution. *ChemBioChem* **2008**, 9, 1797-1804.

(22) Sullivan, B.; Walton, A. Z.; Stewart, J. D. Library construction and evaluation for site saturation mutagenesis. *Enzyme Microb. Technol.* **2013**, 53, 70-77.

(23) Arnold F.H., Georgiou G. (2003) Directed enzyme evolution: screening and selection methods, vol 230. Humana Press, Totowa. DOI: 10.1385/1592593968