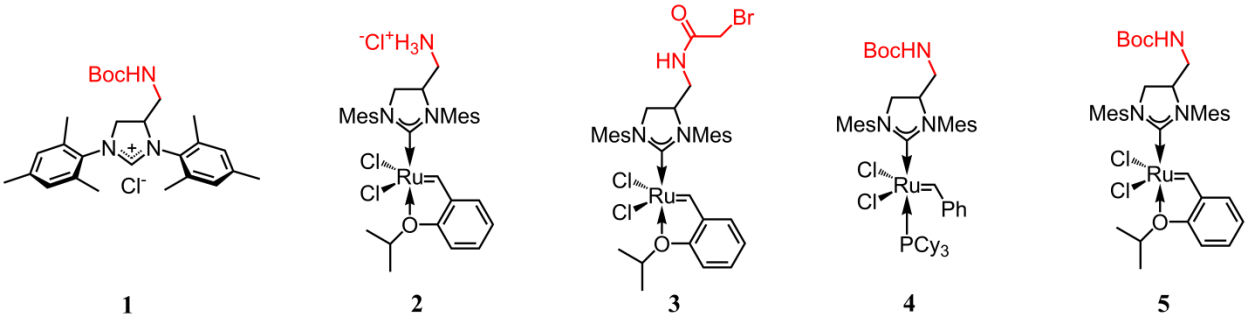
Supporting Information: An Artificial Metalloenzyme for Olefin Metathesis

Materials and Methods: All reactions were conducted in oven-dried (135 °C) or flame-dried glassware under an inert atmosphere of dry N_2 unless otherwise stated. All solvents and buffers were degassed prior to use. NMR spectra were recorded on Bruker AV600 (^{1}H 600 MHz, ^{13}C 150.9 MHz), ARX 300 (^{1}H 300 MHz, ^{13}C 75 MHz), or Varian Gemini 300 (^{1}H 300 MHz, ^{13}C 75 MHz) NMR spectrometers. All ^{13}C-NMR spectra are ^{1}H-broadband decoupled and were measured at room temperature; the ^{13}C resonance of chloroform was used as an internal standard ($^{13}CDCl_3$: 77.16). Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance resulting from incomplete deuteration as the internal standard ($CDCl_3$: 7.26). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet or combinations thereof), coupling constants, and integration. Multiplicity was assigned according to a method described by Hoye.[1] **1**, **4**, and N,N-diallyl-4-toluenesulfonamide were synthesized as previously described in the literature.[2,3] For anaerobic silica gel chromatography, columns were first purged with N_2, and all eluants degassed with a generous N_2 sparge (at least 30 min). Product was then eluted under N_2 and collected in a round-bottom flask already purged with N_2. Solvents were then removed *in vacuo* (not by rotary evaporation). Analytical RP-HPLC runs were performed with a Luna C18 3µm 50x2.00 mm using a linear gradient from 5% to 60% ACN containing 0.08% TFA in 0.1% aqueous TFA for 6 min with a flow rate of 700 µL/min. SEC-HPLC were carried out with a Phenomenex BioSep-SEC-S 4000 300x7.8 mm, employing an isocratic flow of 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer pH 7.0 with a flow rate of 1 ml/min.



1 **2** **3** **4** **5**

Synthesis of 2: A flame dried round-bottom flask equipped with a magnetic stir-bar, a septum and purged with N_2 was charged with **S1** (138 mg, 0.141 mmol), 2-isopropoxystyrene (34.2 mg, 0.211 mmol) and CuCl (27.9 mg, 0.282 mmol). The seal of the flask was reinforced with Teflon tape and dry degassed dichloromethane (5 ml) was added. The reaction mixture was heated to 40 °C for 90 min. In the

course of the reaction the magenta suspension turned dark green. Conversion was followed by TLC (15% EtOAc in hexane), monitoring the disappearance of **4**. After complete conversion, the reaction mixture was concentrated to 1 ml and loaded on an anaerobic silica gel column (elution with 15% EtOAc in hexane). The dark green band was collected and the solvent was removed *in vacuo* to obtain the *t*-Boc protected ruthenium complex **5** as a dark green solid. This material was subsequently dissolved in 5 ml degassed methanol and hydrochloride acid (37% in water, 2 ml) added dropwise. The reaction mixture was stirred for 30 min at room temperature and after complete deprotection of the Boc-group (judged by TLC) the solvent was removed *in vacuo.* The green residue was taken up in 5 ml degassed water and frozen. Lyophilization yielded ruthenium complex **2** as a green solid (65 mg, 0.086 mmol, 61% yield over 2 steps). An NMR sample of **2** was prepared under N_2 in degassed methanol.

^{1}H-NMR (300 MHz, MeOH): δ 16.46 (s, 1H), 7.59 (t, J= 7.5 Hz, 1H), 7.13 (bs, 4H), 7.00 (d, J= 8.4 Hz, 1H), 6.88 (m, 2H), 4.95 (m, 1H), 4.77 (m, 1H), 4.46 (t, J= 10.8 Hz, 1H), 4.13 (t, J= 6.6 Hz, 1H), 3.21 (m, 1H), 2.70 – 2.28 (bd, 18H), 1.21 (d, J= 5.7 Hz, 6H); **^{13}C-NMR (75 MHz, MeOH):** 299.3, 217.1, 153.4, 146.3, 140.7, 140.4, 140.1, 131.2, 130.6, 130.5, 130.3, 123.2, 122.9, 114.3, 76.6, 62.7, 57.9, 43.5, 21.9, 21.5, 20.4. **HRMS (MALDI, -HCl):** calc. 655.1660, found 655.1659

Synthesis of 3: A dry round-bottom flask equipped with a magnetic stir-bar and purged with N_2 was charged with PyBOP (108 mg, 0.29 mmol), bromoacetic acid (40.2 mg, 0.29 mmol) and 5 ml dry degassed dichloromethane. Hünig's base (55 µL, 41.1 mg, 0.318 mmol) was added at 0 °C and the reaction mixture was allowed to stir for 10 min to form the activated ester. A solution of **2** (20 mg, 0.029 mmol) in 2 ml dry degassed dichloromethane was added dropwise over a time period of 20 min with stirring. The reaction was allowed to warm to room temperature and was stirred for a further 15 min. The resulting dark green solution was concentrated to 1 ml by evaporating the solvent under a constant N_2 flow. The dark green mixture was subjected to anaerobic silica-gel chromatography (elution with 20% EtOAc in hexane) and the green band was collected. The solvent was removed *in vacuo* to afford **3** (6.4 mg, 8.2 µmol, 29% yield) as a green solid. An NMR sample of **3** was prepared under N_2 in degassed chloroform.

^{1}H NMR (600 MHz, $CDCl_3$): 7.51 – 7.47 (m, 1H), 7.14 – 7.04 (m, 4H), 6.95 – 6.81 (m, 3H), 6.81 (d, *J* = 8.3 Hz, 1H), 4.97 – 4.89 (m, 1H), 4.60 (m, 1H), 4.29 (t, *J* = 10.9 Hz, 2H), 3.94 – 3.88 (m, 2H), 3.81 (m, 2H), 3.62 (bs, 3H), 2.91 – 2.28 (m, 18H), 1.40 – 1.27 (m, 6H); **^{13}C NMR (151 MHz, $CDCl_3$):** δ 298.3, 216.3, 190.2, 152.4, 145.2, 139.4, 139.2, 135.8, 130.6, 130.1, 130.0, 129.7, 128.3, 122.9, 122.4, 120.4, 114.0, 112.9, 75.1, 63.2, 55.6, 42.7, 28.7, 22.0, 21.2, 21.1; **HRMS (MALDI, MH^+):** calc. 777.0864, found 777.0865

Molecular Cloning of G41C MjHSP

The gene encoding the small heat shock protein 16.5 (MjHSP) was amplified from *Methanocaldococcus jannaschii* genomic DNA with the following primers (restriction sites for NdeI and SpeI underlined): forward: 5-GGA GAT ATA CAT ATG TTC GGA AGA GAC CC-3'; reverse: 5'-CAG CTG ACT AGT CAT TAT TAT TCA ATG TTG ATT CCT TTC-3'. Polymerase chain reaction (PCR)-mediated site directed mutagenesis was employed to introduce the desired G41C muation into MjHSP as described earlier.[4] The resulting insert encoding G41C MjHSP was cloned into the SpeI / NdeI restriction site of the pMG211 vector, resulting in the pMG211-G41C-MjHSP plasmid. After transformation into XL1 Blue *E. coli*, cells were grown on LB_{Amp} (containing 200 mg/L ampicillin) plates overnight at 37 °C, plasmid DNA isolated (Eppendorf Mini Prep Kit), and the DNA insert sequenced. After sequence verification, the pMG211-G41C-MjHSP was subcloned into BL21(DE3) *E. coli* for protein production.

Protein Production and Purification

The G41C MjHSP gene was expressed from plasmid pMG211-G41C-MjHSP in BL21(DE3) *E. coli* in LB_{Amp}. A densely grown overnight culture (750 µL) was used to inoculate 750 mL LB_{Amp}. After incubating at 37 °C and 250 rpm for 4 hrs an OD of 0.6 was reached and protein production was induced with IPTG (250 µM final concentration). After 6 hours of induction, cells were harvested by centrifugation at 4000 g at 4 °C for 5 min. The cell pellet was resuspended in ice-cold cell lysis buffer (20 ml, 50 mM HEPES, pH 8.0) and subsequently lysed by sonication (0.5 s cycles, amplitude 60, 5x 1 min with a 1 min break between cycles). Cell debris was removed by centrifugation (14,000 g at 4 °C for 30 min) and the supernatant was heated for 15 min to 60 °C, thereby denaturing many heat labile *E. coli* proteins. The suspension was centrifuged at 14,000 g at 4 °C for 30 min and Tris(2-chlorethyl)phosphat (TCEP, final concentration 1 mM) was added to the supernatant to fully reduce G41C MjHSP. The crude protein solution was further purified by anion exchange chromatography (MonoQ, Biorad Duoflow, 20 mM potassium phosphate buffer, pH 7.0 with a linear gradient from 0 to 1 M NaCl). The purity of G41C MjHSP was estimated by SDS poly-acrylamide gel electrophoresis (SDS-PAGE) to be >95 % (data not shown) and the mass was confirmed by LC/MS analysis (found 16,498 Da, calc. 16,498 Da). The yield of G41C MjHSP was determined to be 25 mg/L culture.

Alkylation of G41C MjHSP

All steps were carried out in degassed buffers under an N_2 atmosphere. 400 µL *t*-BuOH was added to 3 ml of freshly purified G41C MjHSP (3 mg/mL, 182 µM, in 50 mM potassium phosphate buffer pH 7.0). The reaction mixture was stirred for 10 min at room temperature prior to dropwise addition of a solution of **3** (1.27 mg, 3.0 eq.) in 600 µL *t*-BuOH. After complete addition, the green

solution was allowed to stand for another 4 hours at room temperature. Reaction progress was monitored by titrating free thiols in the reaction mixture with 5,5'-dithiobis-(2-nitrobenzoic acid).[5] After more than 95% of cysteine thiols had reacted, 10 mL of nanopure water was added and the protein mixture was concentrated employing Amicon Ultra-15 centrifugal filters (10 kDa cutoff). Two further cycles of dilution with nanopure water followed by concentration were performed until a 2 mL [Ru]MjHSP solution was obtained. Subsequently, 13 mL of 50 mM potassium phosphate buffer (pH 2.2) was added and the reaction mixture was allowed to stand for 15 min after mixing on ice. Amicon Ultra-15 Centrifugal filters (3 kDa cutoff) were used to concentrate the protein solution to 3 mL, followed by another addition of 12 mL potassium phosphate buffer pH 2.2 and concentrating to 3 mL. The green solution was transferred to a Slide-A-Lyzer dialysis chamber (7 kDa cutoff) and dialyzed against 50 mM potassium phosphate buffer pH 2.2 overnight to remove loosely associated **3** from the protein solution. After dialysis an analytical sample (100 µL) was removed for analytical HPLC, SEC-HPLC, and ESI-MS analyses (**Figure S2**, **S3**, and **S4**). Nanopure water (12 mL) was added to the remaining green solution and Amicron Ultra-15 Centrifugal filters (3 kDa cutoff) were used to concentrate the [Ru]MjHSP solution to 1.5 mL. The protein concentration of the solution was determined by Bradford assay in which a G41C MjHSP stock solution of known concentration (determined by UV, ε = 9,322 $M^{-1}cm^{-1}$)[4] was used for the calibration. The concentration of the ready-to-use [Ru]MjHSP solution was typically between 3.5 and 4.5 mg/mL.

Representative Activity Assay with [Ru]MjHSP

A freshly prepared [Ru]MjHSP solution (100 µL, 4.29 mg/mL, 260 µM) was mixed with 20 µL of 325 mM buffer stock or 65 mM HCl solution. After mixing, 10 µL of a N,N-diallyl-4-toluenesulfonamide solution (65 mM in *t*-BuOH) was added and the reaction vessels were incubated for 12 hrs at 45 °C. After the reaction was complete 20 µL p-bromophenol (internal standard, 65 mM in *t*-BuOH) was added and the reaction mixture extracted 3 times with 700 µL diethyl ether. The solvent was removed *in vacuo* and the residue was redissolved in 520 µL acetonitrile by vortexing. A 10 µL aliquot of the resulting solution was analyzed by HPLC, and conversion was determined according to a previously performed calibration (**Figure S4**, N-(p-toluenesulfonyl)-3-pyrroline, **P**, was obtained from ABCR-Chemicals). Turnover numbers (TONs) in **Table 1** represent averages from two separate experiments, each carried out in triplicate.

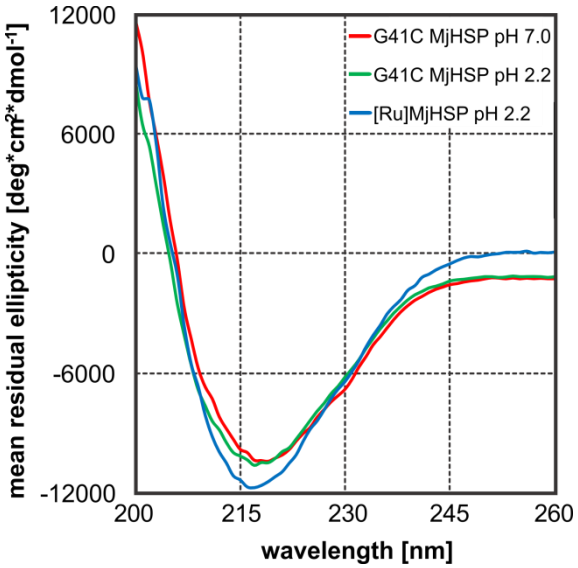


Figure S1: Comparison of CD spectra of G41 MjHSP and [Ru]MjHSP at 10 µM in 10 mM potassium phosphate buffer pH 7.0 and 2.2, respectively.

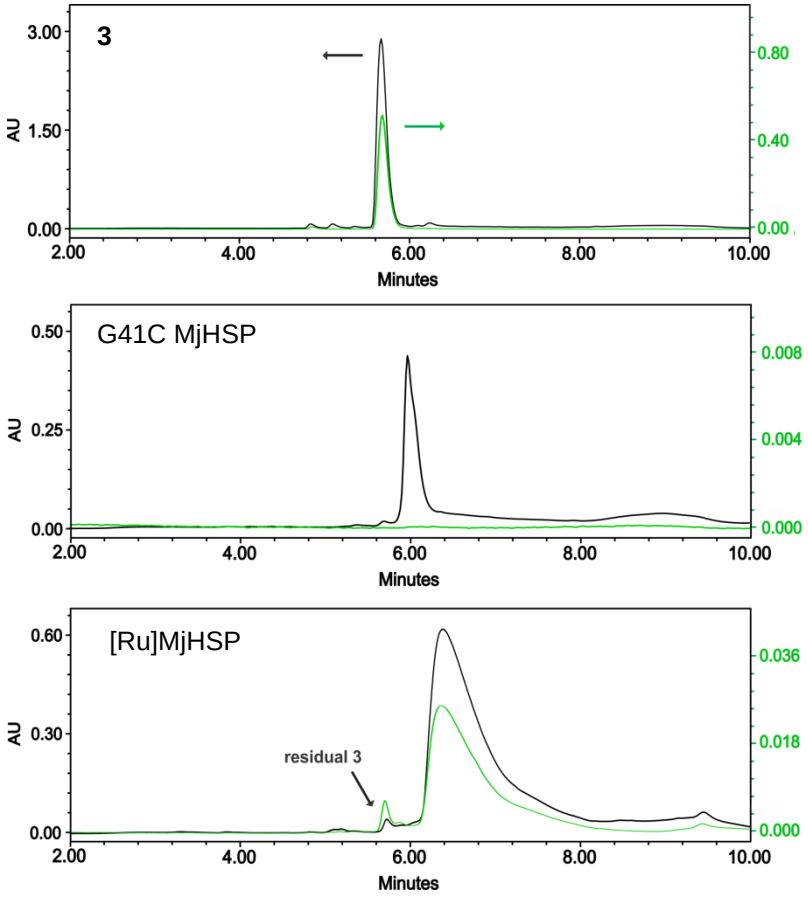


Figure S2: Comparison of analytical HPLC runs of G41C MjHSP, **3** and [Ru]MjHSP at 220 nm (black traces) and 370 nm (green traces). The amount of residual catalyst (integration of peaks at 370 nm) was found to be < 2 %. (for the HPLC conditions see the "Materials and Methods" section)

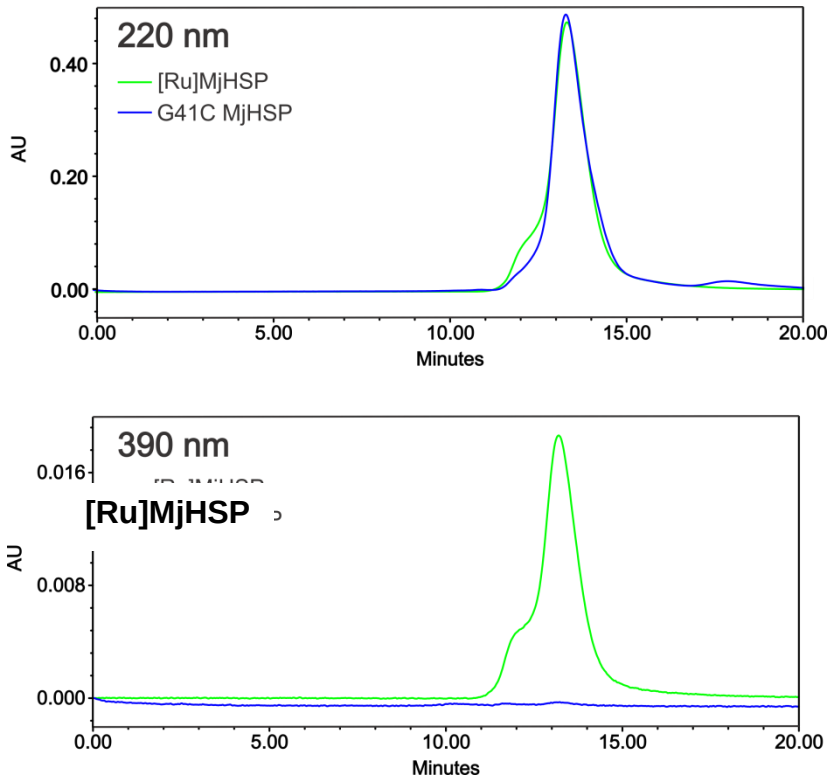


Figure S3: Comparison of SEC-HPLC chromatograms of G41C MjHSP (blue traces) and [Ru]MjHSP (green traces) at 220 nm and 390 nm. [Ru]MjHSP elutes in 20 mM MOPS buffer, pH 7.0 predominantly as a capsid with a leading peak corresponding to an uncharacterized aggregate.

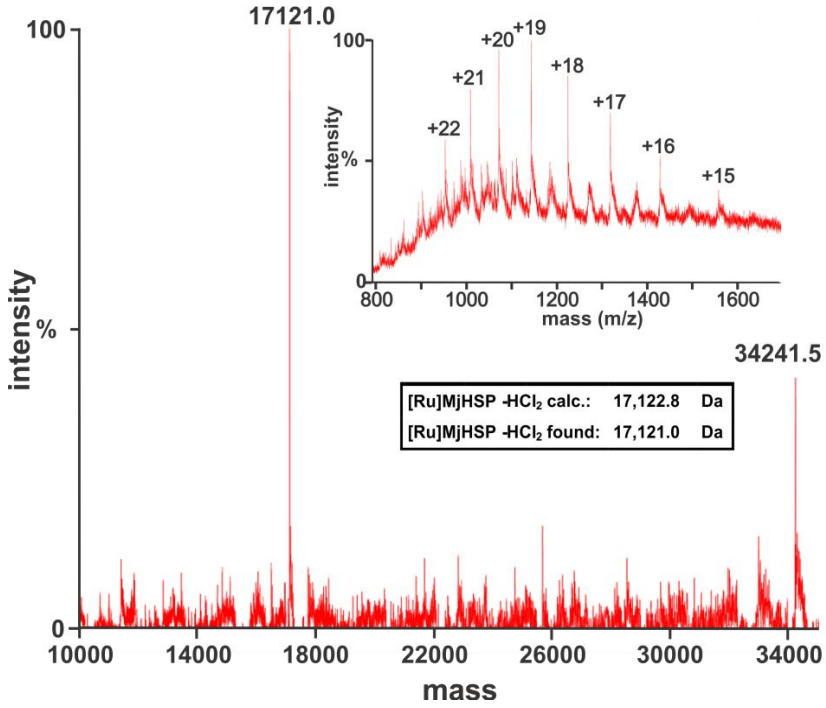


Figure S4: ESI-MS analysis of [Ru]MjHSP, raw data inserted. Mass calculated [Ru]MjHSP -HCl_2: 17,122.8 Da, mass found 17,121.0 Da. A dimer peak is observed at 32,241.5 Da (calc. 34245.6 Da).

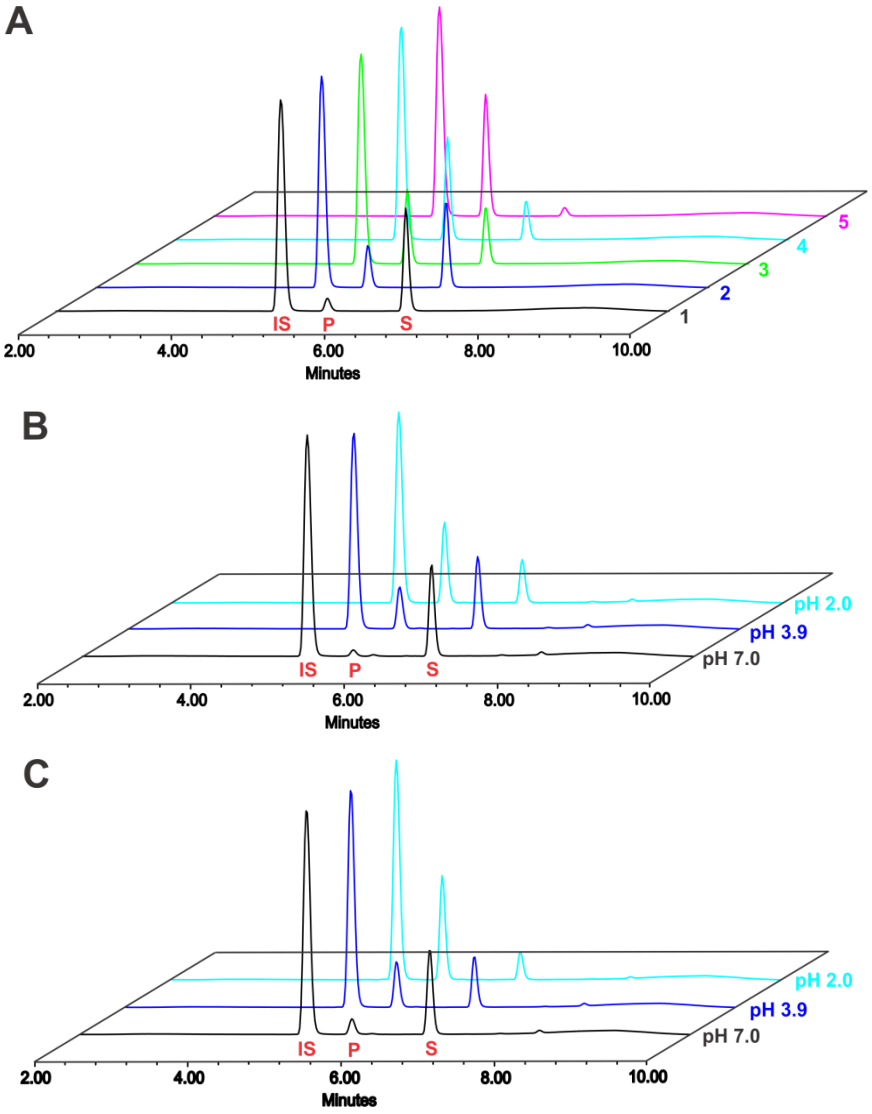


Figure S5: RP-HPLC traces for the activity assay shown in **Figure 3** in the main text. **A**: Calibration standards (1-5) containing 10 mM p-bromophenol (**IS**), 0.5-4.5 mM N-(p-toluenesulfonyl)-3-pyrroline (**P**) , 0.5-4.5 mM N,N-diallyl-4-toluenesulfonamide (**S**). **B**: Activity assay with **3**; 5 mM substrate, 2mol% catalyst loading, 45 °C for 10 hrs, in 20% *t*-BuOH; 50 mM potassium phosphate buffer (pH 7.0), 50 mM MES buffer (pH 3.9), and 10 mM HCl (pH 2.0). **C**: Activity assay with [Ru]MjHSP; 5 mM substrate; 4mol% catalyst loading, 45 °C for 12 hrs; 50 mM potassium phosphate buffer (pH 7.0), 50 mM MES buffer (pH 3.9), and 10 mM HCl (pH 2.0).

References

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