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

Electronic Supplementary Information (ESI)

Cyclotrimerization of phenylacetylene catalyzed by a cobalt half-sandwich complex embedded in an engineered variant of transmembrane protein FhuA

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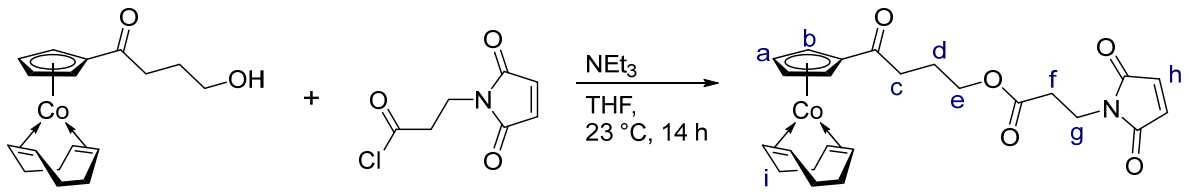
1. General remarks

All experiments were performed under an inert atmosphere of argon or nitrogen using standard Schlenk or glovebox technique if not mentioned otherwise. Water and organic solvents were degassed using “freeze-pump thaw” technique prior to use. THF was obtained dry and degassed from a SPS 800 solvent purification system from *MBraun*. THF-d_8, C_6D_6, Chloroform-d_1 were dried over calcium hydride, distilled, degassed and stored under inert atmosphere. NMR measurements were recorded on a *Bruker Avance II 400* or *Bruker Avance III HD 400* at ambient temperatures if not mentioned otherwise. The chemical shifts (δ ppm) in ^{1}H and ^{13}C NMR spectra were referenced to the residual signal of the deuterated solvents.[1] IR spectra were measured on KBr pellets using an *AVATAR 360 FT-IR* spectrometer. Elemental analysis was performed using an *elementar vario EL* machine. GC MS spectra were recorded on a *Shimadzu GCMS-GP2010 Plus* machine. Helium was used as the carrier gas at 58 kPa. The column used was an *FS-Supreme-5ms* with 30 m length. The method used was as follows: initial oven temperature 60 °C; initial hold time 2 min; rate 10 °C/min; final oven temperature 270 °C; final time 5 min. Circular dichroism (CD) spectra were recorded on a *JASCO J1100* at ambient temperatures. MALDI–TOF MS spectra were measured on an Ultraflex III TOF/TOF mass spectrometer from *Bruker* using 2,5-dihydroxybenzoic acid (DHB) as matrix.
Complex **2**[2] and **3**[3] were synthesized as reported. Phenylacetylene, methyl propiolate, ethyl propiolate, *tert*-butyl propiolate, 2-propyn-1-ol, *N,N*-dimethyl-2-propynylamine, propiolamide were used as received from Sigma Aldrich. Protein FhuA was prepared and purified as published.[4]

2. Synthesis

Synthesis of cobalt complex 4.



2 **3** **4**
89%

*Scheme S1: Synthesis of metal complex **4***

Triethylamine (150 mg, 1.48 mmol) was added to a solution of cobalt complex **2** (83 mg, 0.26 mmol) dissolved in THF/Et_2O (5 mL/5 mL). While stirring at 23 °C a solution of acyl chloride **3** (49 mg, 0.26 mmol) in THF (1 mL) was added dropwise and the reaction mixture stirred for 14 h. The brown suspension was filtered through Celite® and the precipitate was washed with Et_2O (2x5 mL). All volatiles were removed in vacuo and the red waxy product was recrystallized from THF/*n*-pentane to give **4** as an orange solid (108 mg, 0.23 mmol); yield, 89%.

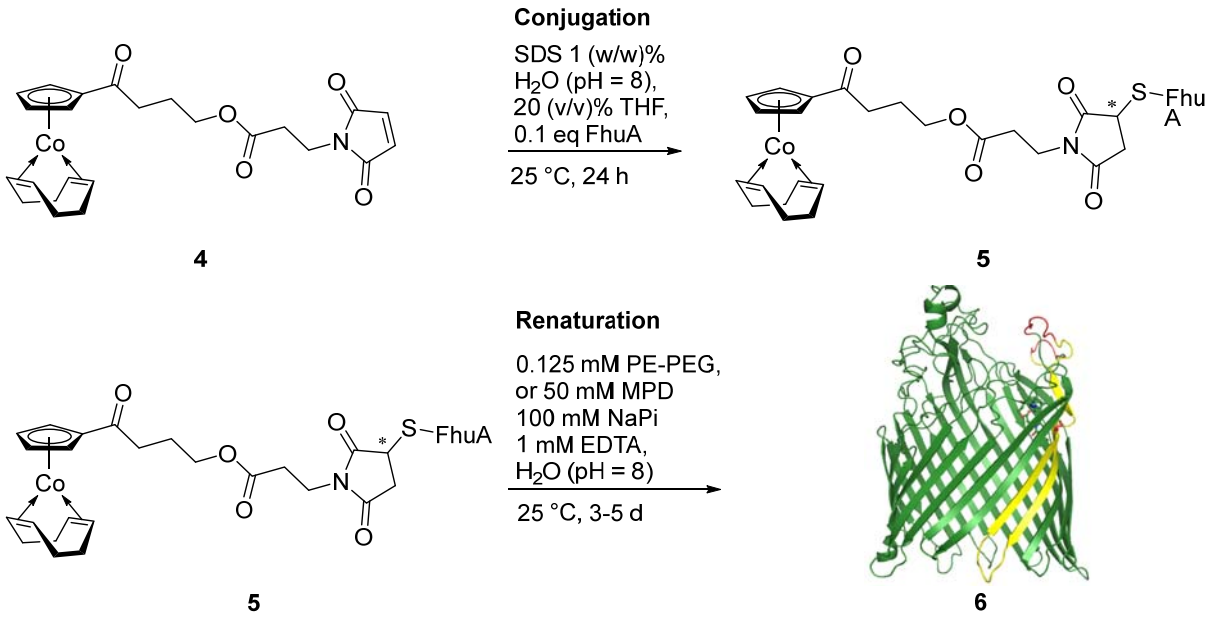
^{1}H NMR (400MHz, THF-d_8): δ = 6.79 (s, 2 H, H_h), 5.18 (t, $^3J_{CH}$ = 2.3 Hz, 2 H, H_b), 4.29 (t, $^3J_{CH}$ = 2.3 Hz, 2 H, H_a), 4.23 (t, $^3J_{CH}$ = 6.5 Hz, 2 H, H_g), 3.76 (t, $^3J_{CH}$ = 7.5 Hz, 2 H, H_f), 3.53 (br, 4 H, H_i), 3.15 (t, $^3J_{CH}$ = 7.3, 2 H, H_c), 2.61 (t, $^3J_{CH}$ = 7.3 Hz, 2 H, H_e), 2.37 (m, 4 H, H_i), 2.16 (p, $^3J_{CH}$ = 7.3 Hz, 2 H, H_d), 1.64 (q, $^3J_{CH}$ = 8.0 Hz, 4 H, H_i) ppm.

^{13}C NMR (101 MHz, THF-d_8): δ = 195.5, 171.2, 171.3, 135.3, 98.3, 89.3, 84.0, 69.2, 65.2, 36.2, 34.5, 33.6, 32.7, 24.6 ppm.

IR (KBr): $\tilde{\nu}$ = 2934, 2870, 2826, 1654, 1470, 1442, 1396, 1320, 1259, 1187, 1072, 994, 855, 826, 696 cm^{-1}.

Elemental Analysis: calculated for $C_{24}H_{28}CoNO_5$: C: 61.89, H: 6.08, N: 4.29; found: C: 61.41, H: 6.01, N: 2.98.

Conjugation and refolding to give biohybrid catalyst 6.



*Scheme S2: Conjugation and renaturation to metalloprotein **6.***

Under anaerobic conditions, FhuA ΔCVF^{tev} was dissolved in degassed water (10 mg/mL). 10 equiv. of catalyst **4** dissolved in degassed THF (10 (v/v)%) was added dropwise and stirred for 16 h. After the solvent was removed under reduced pressure, the residue was washed with degassed THF (3 x 10 mL). The residue was taken up in degassed water and transferred into a dialysis tube (MWCO = 12-14 kDa). The dialysis proceeded against a buffered solution containing 100 fold volume, either polyethylene-*block*-poly(ethylene glycol) (PE-PEG, 0.125 mM, average Mn = 2250 g/mol) or 2-Methyl-2,4-pentanediol (MPD, 50 mM), sodium phosphate buffer (100 mM, pH 8.0), and water. The dialysis solution was changed every 24 h.

The protein concentration (up to 10 mg/mL) was determined using BCA assay.[5] Coupling efficiency (up to 91%) was determined by ThioGlo® fluorescence titration.[6] Structural integrity an refolding to *β*-barrel refolding was confirmed by CD spectroscopy. MALDI-TOF MS was measured after digestion with TEV proteases as previously reported.[4]

Synthesis of *N,N,N*-trimethyl-2-propynylammonium iodide[7]

N,N-Dimethyl-2-propynylamine (428 mg, 5.15 mmol) was dissolved in Et_2O (15 mL) and iodomethane (0.32 mL, 5.15 mmol) was added dropwise. The reaction mixture was stirred for 22 h, during which time a precipitate formed. The precipitate was filtered off, washed with Et_2O (3x10 mL) and dried in vacuum. The salt was isolated as a colorless solid (779 mg, 3.46 mmol), yield, 67%.

^{1}H NMR (400MHz, D_2O): δ = 4.24 (s, 2 H) and 3.21 (s, 10 H) ppm.

3. Characterization of **4**

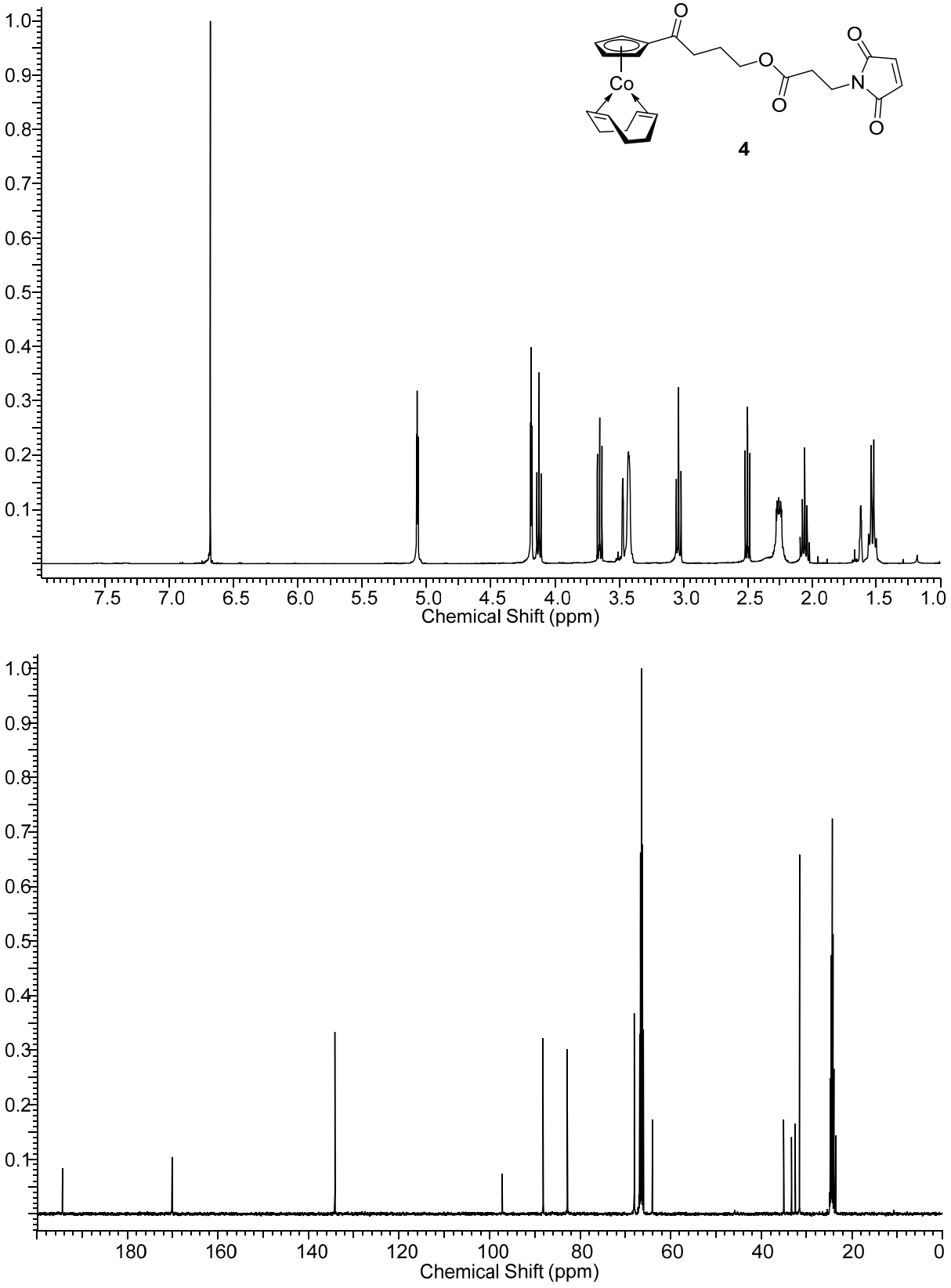


*Figure S1: ^{1}H NMR spectrum (top) and ^{13}C NMR spectrum (bottom) of complex **4** (THF-d_8, 23 °C).*

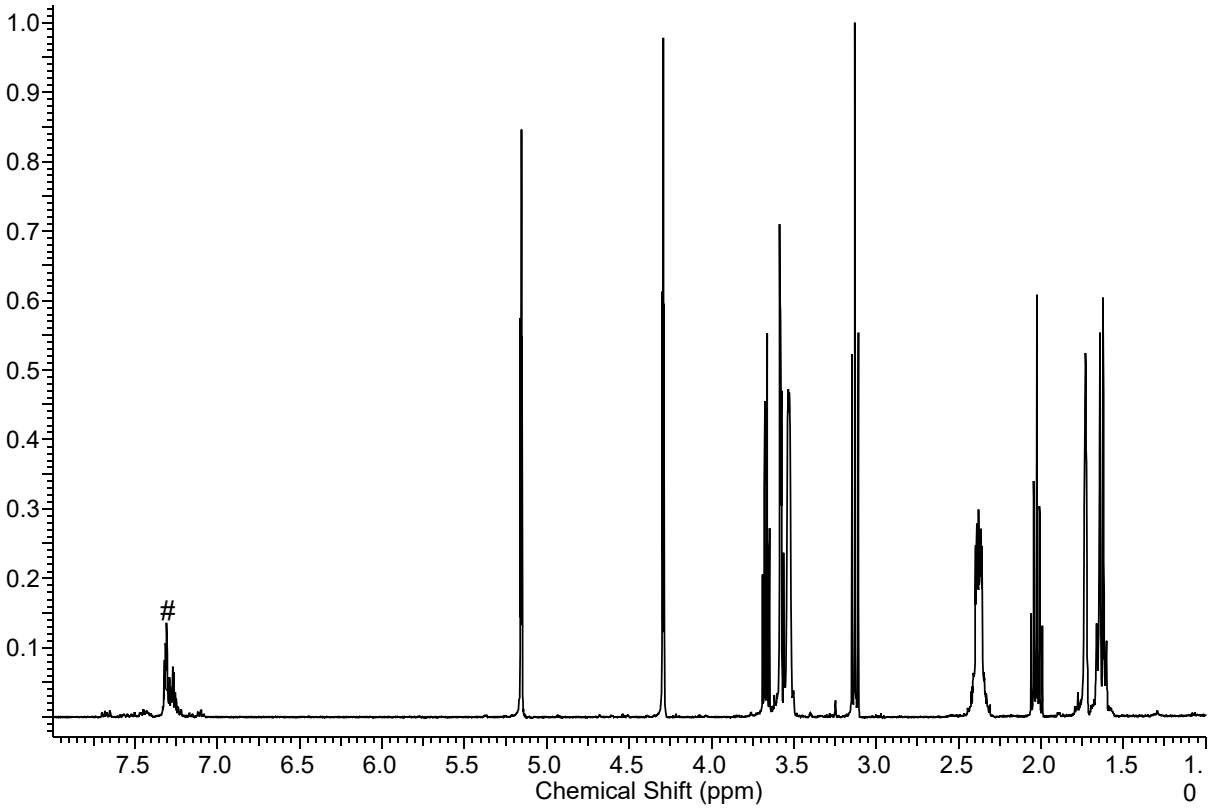


*Figure S1a: ^{1}H NMR spectrum of complex **2** (THF-d_8, 23 °C). # = impurity. Impuritiy was removed during recrystallizing compound **4**.*

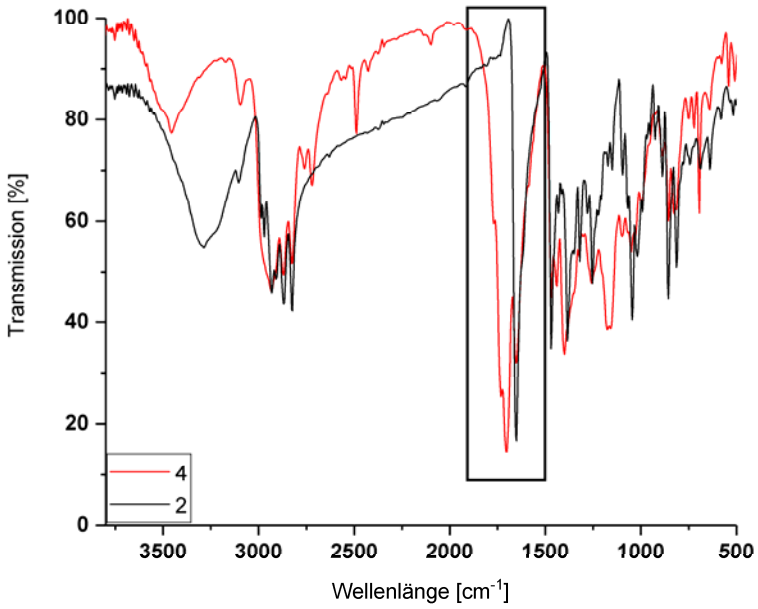


*Figure S2: IR spectrum of complex **2** and **4**. Carbonyl bands are marked in the black box.*

4. Characterization of metalloprotein **6**

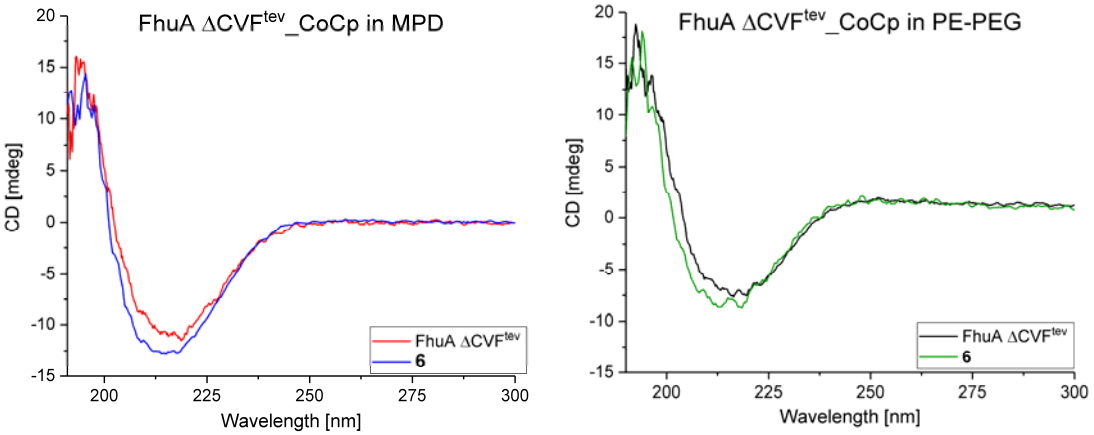


*Figure S3: CD spectrum comparing metal-free FhuA protein refolded with biohybrid catalyst **6** in MPD buffer solution (left). CD spectrum comparing metal-free FhuA protein refolded with biohybrid catalyst **6** und PE-PEG buffer solution (right).*

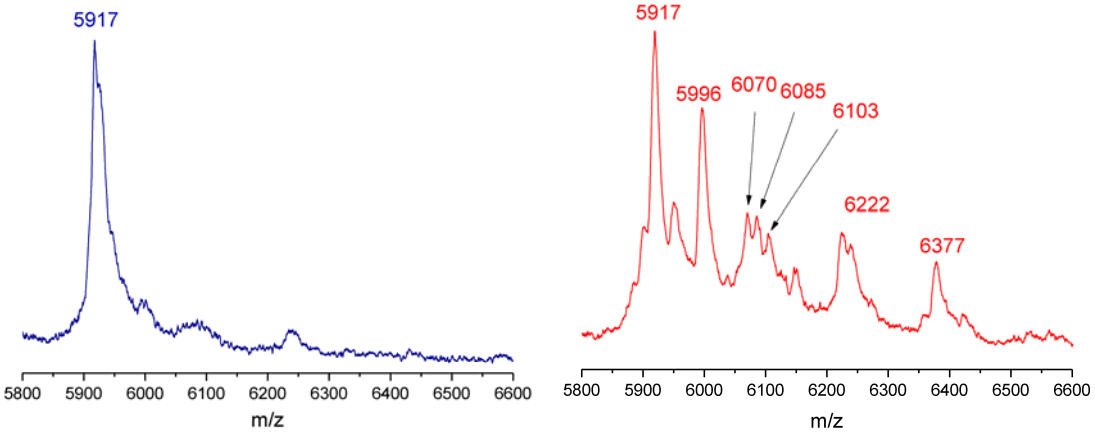
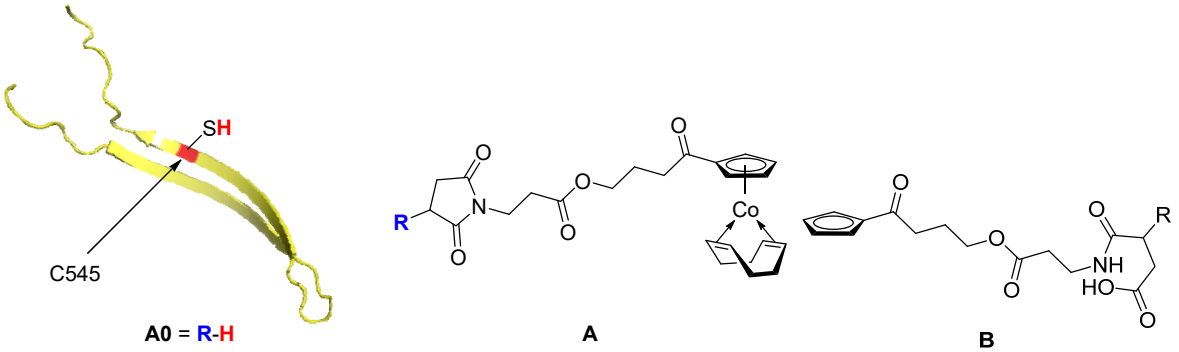


*Figure S4: MALDI-TOF MS spectra. FhuA without metal co-factor (left). FhuA with conjugated Co complex **4** (right). It should be noted that further signals can be related to fragments of A0 (Figure S5) and these could stem from partial saponification during the digestion (e.g. **B**, Figure S5). The matrix used to measure MALDI-TOF mass spectrum overlaps with signals up to m/z = 6150. Due to these overlaps we did not assign further signals.*



A0 = **R**-H
calculated: m/z = 5902 Da
found: m/z = 5917 Da

A
calculated: m/z = 6371 Da
found: m/z = 6377 Da

B
calculated: m/z = 6221 Da
found: m/z = 6222 Da

Figure S5: A0: Fragment of FhuA after TEV protease without metal co-factor; A: fragment after TEV protease of metalloprotein 6 with cobalt co-factor attached; B: fragment after TEV protease with hydrolyzed maleimide moiety and cleaved "Co(cod)" residue.

5. Catalysis

For catalysis a catalyst stock solution was prepared and used: 0.1M in THF (1mg/20µL).

NMR procedure

Catalyst stock solution (3.197 µmol, 0.1 M in THF) was added to a J. Young-type NMR tube. THF was removed under reduced pressure and dissolved in THF-d_8 (50 µL). Degassed D_2O (450 µL), acetone (4 µL, int. std.) and substrate (63.94 µmol) was added. The reaction was heated at 60 °C and analyzed every 24 h by ^{1}H NMR spectroscopy.

GC MS procedure

Catalyst stock solution (0.1 M in THF) was transferred to the reaction vessel. Further degassed THF was added until 50 µL (10 (v/v)%) was achieved. 450 µL (90 (v/v)%) aqueous solution and substrate were added. The reaction mixture was heated at 60 °C. Internal standard (1 mL, mesitylene, 15 mM in THF) was added and the homogeneous reaction mixture was analyzed by GC MS.

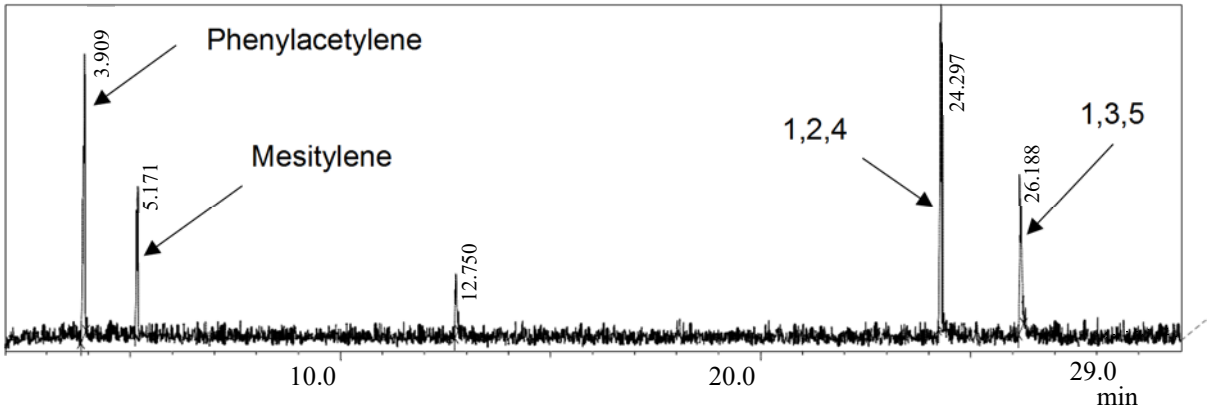


Figure S6: Gas chromatogram displaying phenylacetylene, mesitylene and both benzene regioisomers (here: 1,2,4-triphenylbenzene after 24.297 min. and 1,3,5-triphenylbenzene after 26.188 min.) 2,6-Di-tert-butyl-4-methylphenol (BHT), inhibitor in THF, was observed at 12.750 min.

Solvent screening

Table S1: Solvent screening

#[a]	Cat (mol%)	Solvent	Time [h]	A/B[b]	Conv.[c] [%]	TON
1	**4** (5)	D_2O/THF 10 (v/v)%	48	67/33	39	8
2	**4** (5)	D_2O/EtOH 10 (v/v)%	48	72/28	11	2
3	**4** (5)	D_2O/^{i}PrOH 10 (v/v)%	48	70/30	29	6
4	**4** (5)	D_2O/^{t}BuOH 10 (v/v)%	48	n.d.	n.d.	-

a) V = 0,5 mL, 60 °C, **4** was dissolved in organic co-solvent and added to D_2O, 3.2 µmol (0.1 M in THF), 16 µmol phenylacetylene; b) determined by GC MS, int. std.: mesitylene (1.0 mL, 15mM in THF); c) determined by ^{1}H NMR, int. std.: acetone (4.0 µL)

Note: catalyst **4** was insoluble in ^{t}BuOH and weakly soluble in EtOH and ^{i}PrOH.

Variation of catalyst loadings

Table S2: Cyclotrimerization of phenylacetylene with variation of catalyst loadings

Entry[a]	Cat (mol%)	Solvent	Time [h]	A/B[e]	Conv.[e] [%]	TON
1[b]	**4** (1)	H_2O/THF	72	67/33	28	28
2[b]	**4** (1)	H_2O/THF	504	63/37	>99	99
3[c]	**4** (2.5)	H_2O/THF	72	65/35	36	15
4[d]	**4** (7.5)	H_2O/THF	72	66/34	62	12

a) V = 0,5 mL, 60 °C, a stock solution (0.1 M in THF) was used; b) 106.5 mM phenylacetylene; c) 63.5 mM phenylacetylene; d) 35.5 mM phenylacetylene; e) determined by GC MS, int. std.: mesitylene (1.0 mL, 15 mM in THF).

Organocatalyzed reaction of methyl propiolate

Table S3: Amine-catalyzed reaction of methyl propiolate

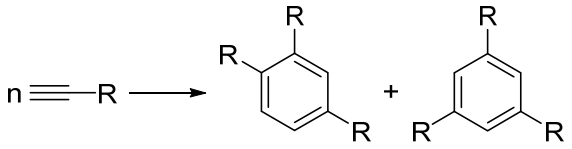
Entry[a]	Cat (mol%)	Time [h]	A / B[f]	Conv.[g] [%]	TON
1[b]	**4** (5)	72	64/36	51	10
2[c]	L-histidine (5)	72	<1 / >99	22	4
3[d]	L-lysine (5)	72	<1 / >99	7	1
3[e]	Tris-HCl (5)	72	<1 / >99	0	0
4[e]	Tris-base (5)	48	<1 / >99	0	0
5[e]	FhuA (5)	72	<1 / >99	40	8

a) V = 0,5 mL, D_2O/THF-d_8 (9:1), 60 °C, b) catalyst **4**: stock solution (0.1 M in THF), transferred to reaction vessel and THF removed under reduced pressure; c) 157 mM methyl propiolate; d) 200 mM methyl propiolate; e) 124 mM methyl propiolate; f) determined by GC MS, int. std.: mesitylene (1.0 mL, 15 mM in THF); g) determined by ^{1}H NMR, int. std.: acetone (4.0 µL).

Substrate scope

Reaction was performed in 90% D_2O and 10% THF-d_8. Conversion was determined by following the consumption of the alkyne proton signal using acetone (4 µL) as internal standard. Observed products were compared to literature referred to (Table S4).

Table S4: Substrate Scope



Entry[a]	Cat (mol%)	Solvent	R	Time [h]	Conv.[b] [%]
1	**4** (5)	D_2O/THF	Ph	72	51[8]
2	**4** (5)	D_2O/THF	COOMe	24	10[2]
3	**4** (5)	D_2O/THF	COOEt	72	21[8]
4	**4** (5)	D_2O/THF	COO^{t}Bu	72	32[9]
5	**4** (5)	D_2O/THF	CH_2OH	72	0[10]
6	**4** (5)	D_2O/THF	CH_2NMe_2	72	0[2]
7	**4** (5)	D_2O/THF	$CH_2NMe_3^+ I^-$	48	0
8	**4** (5)	D_2O/THF	$CONH_2$	48	0
9	**4** (5)	D_2O/THF	$(CH_2)_3CN$	48	0[10]

a) V = 0,5 mL, D_2O/THF-d_8 (9:1), 60 °C, catalyst **4**: stock solution (0.1 M in THF, 30 µL), transferred to reaction vessel and THF removed under reduced pressure; b) determined by ^{1}H NMR, int. std.: acetone (4.0 µmol).

6. References

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