Supporting Information

Engineering a metathesis-catalyzing artificial metalloenzyme based on HaloTag

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1. General biochemical details

All commercial enzymes were purchased from NEB, except lysozyme, which was purchased from GoldBio. All kanamycin concentrations used in cell culture were 50 µg/mL.

2. HaloTag expression and purification

A gene block for *E. coli* codon optimized HaloTag version 7 (HT) was cloned into a pET30 plasmid. The resulting plasmid expressed HT with a C-terminal His6 tag. The protein was expressed in BL21-DE3 cells using the following conditions. A pre-culture in LB supplemented with kanamycin was grown overnight. A 100-mL solution of LB/kanamycin was inoculated with the pre-culture. The expression culture wash shaken at 37 ˚C. Once the OD_{600} reached 0.5-0.6, IPTG was added to a final concentration of 0.4 mM. The cells were shaken at 37 ˚C for another 4 h. The cells were then harvested by centrifugation and frozen at -20 ˚C. The frozen cell pellets were thawed at room temperature and resuspended in B-PER (3 mL, ThermoFisher) supplemented with lysozyme (to 2-4 mg/mL), DTT (1 mM), and DNase I (to 2 U/mL). The lysates were turned end-over-end for 3 h at room temperature. The suspensions were centrifuged, and the supernatants were purified by Ni-NTA beads (1 mL) packed into plastic gravity flow columns. The proteins were eluted with 250 mM imidazole. The eluted product was buffer exchanged using centrifugal filter units (10K MWCO) until the concentration of imidazole was less than 0.5 µM. The resulting proteins were analyzed by SDS-PAGE, and activity assays with fluorescent-turn on probes.

Gene block for HT

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ATGGCAGAAATCGGTACTGGCTTTCCATTCGACCCCCATTATGTGGAAGTCCTGGGCGAGCGCATGCACTACGTCGATGTTGGTCCGCGCGAT
GGCACCCCTGTGCTGTTCCTGCACGGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCATGTTGCACCGACCCATCGCTGCATT
GCTCCAGACCTGATCGGTATGGGCAAATCCGACAAACCAGACCTGGGTTATTTCTTCGACGACCACGTCCGCTTCATGGATGCCTTCATCGAA
GCCCTGGGTCTGGAAGAGGTCGTCCTGGTCATTCACGACTGGGGCTCCGCTCTGGGTTTCCACTGGGCCAAGCGCAATCCAGAGCGCGTCAAA
GGTATTGCATTTATGGAGTTCATCCGCCCTATTCCGACCTGGGACGAATGGCCAGAATTTGCCCGTGAAACCTTCCAGGCCTTCCGTACCACC
GATGTCGGCCGCAAGCTGATCATCGACCAGAATGTTTTTATCGAGGGTACGCTGCCGATGGGTGTCGTCCGCCCGCTGACTGAAGTTGAGATG
GACCATTACCGCGAGCCGTTCCTGAATCCTGTTGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCGGTGAGCCAGCGAAC
ATCGTCGCGCTGGTCGAAGAATACATGGACTGGCTGCACCAGTCCCCTGTTCCGAAGCTGCTGTTCTGGGGCACCCCAGGCGTTCTGATCCCA
CCAGCGGAAGCGGCTCGCCTGGCCAAAAGCCTGCCAAACTGCAAAGCTGTGGACATCGGCCCGGGTCTGAATCTGCTGCAAGAAGACAATCCA
GACCTGATCGGCAGCGAGATCGCGCGCTGGCTGTCGACGCTCGAGATTTCCGGCCACCATCACCATCACCACTAA
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3. Selection of residues for mutagenesis

Residues for mutagenesis were selected based on crystallography data and homology modeling (Figure S1).

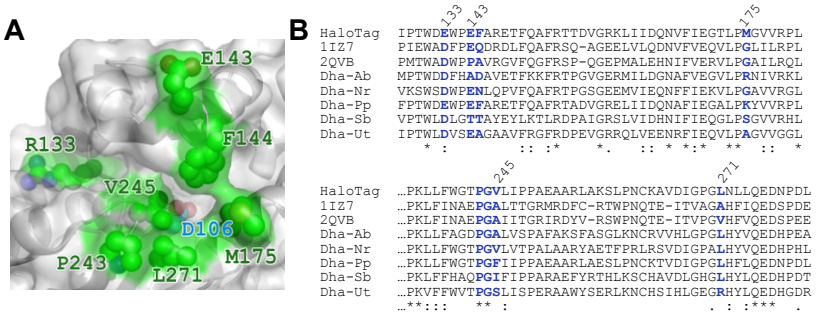


Figure S1. Crystallographic data (A) and homology modeling (B). The homology modeling is shown with blue color for the amino acid residues that are near the opening of the HT protein.

Residues that were conserved (*) within the enzyme family were not mutated because they could be important for haloalkane binding.

4. DNA Library creation

Defined libraries were created by conducting PCR in 96-well plates with primers that code for only a single mutant. The primers were either overlapping, staggered primers (traditional site-directed mutagenesis) or end-to-end primers (for NEB Q5 site-directed mutagenesis). All of the PCRs were conducted with Q5 polymerase (NEB) according to the manufacture's recommendations. The PCR products derived from overlapping, staggered primers were digested with DpnI (12-18 h, 37 ˚C). The PCR products derived from the end-to-end primers were treated with the KLD enzyme mix (5-15 min, room temperature). The DpnI and KLD reaction products were transformed into Top10 cells. The transformed cells were recovered in SOC for 1-2 h and then plated onto LB/Agar/kanamycin plates. The plates were incubated at 37 ˚C overnight, and Colonies were picked for sequencing. The correctly sequenced DNA products were used for variant expression.

Table S1. Primers for the defined DNA libraries

Name	Sequence	T_A (˚C)	Type*
R133A	GGAGTTCATC**gcc**CCTATTCCGACCTGG	68	St-Ov
R133C	GGAGTTCATC**tgc**CCTATTCCGACCTGG	68	St-Ov
R133E	GGAGTTCATC**gag**CCTATTCCGACCTGG	68	St-Ov
R133F	GGAGTTCATC**ttc**CCTATTCCGACCTGG	68	St-Ov
R133H	GGAGTTCATC**cac**CCTATTCCGACCTGG	68	St-Ov
R133I	GGAGTTCATC**atc**CCTATTCCGACCTGG	68	St-Ov
R133K	GGAGTTCATC**aag**CCTATTCCGACCTGG	68	St-Ov
R133M	GGAGTTCATC**atg**CCTATTCCGACCTGG	68	St-Ov
R133N	GGAGTTCATC**aac**CCTATTCCGACCTGG	68	St-Ov
R133P	GGAGTTCATC**cca**CCTATTCCGACCTGG	68	St-Ov
R133S	GGAGTTCATC**tca**CCTATTCCGACCTGG	68	St-Ov
R133W	GGAGTTCATC**tgg**CCTATTCCGACCTGG	68	St-Ov
R133-R	GATGAACTCCATAAATGCAATACC		St-Ov
E143F	ATGGCCA**ttc**TTTGCCCGTGAAACC	56	St-Ov
E143H	ATGGCCA**cat**TTTGCCCGTGAAACC	56	St-Ov
E143I	ATGGCCA**att**TTTGCCCGTGAAACC	56	St-Ov
E143K	ATGGCCA**aag**TTTGCCCGTGAAACC	T-up**	St-Ov
E143M	ATGGCCA**atg**TTTGCCCGTGAAACC	T-up**	St-Ov
E143R	ATGGCCA**cgt**TTTGCCCGTGAAACC	56	St-Ov
E143S	ATGGCCA**tct**TTTGCCCGTGAAACC	56	St-Ov
E143-R	TGGCCATTCGTCCCAGGTCG		St-Ov
F144A	AATGGCCAGAA**gct**GCCCGTGAAACCTTCCAGG	63	St-Ov
F144C	AATGGCCAGAA**tgt**GCCCGTGAAACCTTCCAGG	56	St-Ov
F144D	AATGGCCAGAA**gat**GCCCGTGAAACCTTCCAGG	63	St-Ov
F144H	AATGGCCAGAA**cat**GCCCGTGAAACCTTCCAGG	63	St-Ov
F144I	AATGGCCAGAA**att**GCCCGTGAAACCTTCCAGG	56	St-Ov
F144K	AATGGCCAGAA**aaa**GCCCGTGAAACCTTCCAGG	56	St-Ov
F144M	AATGGCCAGAA**atg**GCCCGTGAAACCTTCCAGG	56	St-Ov
F144N	AATGGCCAGAA**aat**GCCCGTGAAACCTTCCAGG	56	St-Ov
F144R	AATGGCCAGAA**cgt**GCCCGTGAAACCTTCCAGG	63	St-Ov
F144S	AATGGCCAGAA**tct**GCCCGTGAAACCTTCCAGG	63	St-Ov
F144W	AATGGCCAGAA**tgg**GCCCGTGAAACCTTCCAGG	63	St-Ov
F144-R	TTCTGGCCATTCGTCCCAGGTCGG		St-Ov
M175A	GGTACGCTGCCG**GCT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175C	GGTACGCTGCCG**TGT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175E	GGTACGCTGCCG**GAA**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175F	GGTACGCTGCCG**TTT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov

Name	Sequence	T_A (˚C)	Type*
M175H	GGTACGCTGCCG**CAT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175I	GGTACGCTGCCG**ATT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175K	GGTACGCTGCCG**AAA**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175Y	GGTACGCTGCCG**TAT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175N	GGTACGCTGCCG**AAT**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175R	GGTACGCTGCCG**CGC**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175S	GGTACGCTGCCG**TCA**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175W	GGTACGCTGCCG**TGG**GGTGTCGTCCGCCCGCTGAC	65	St-Ov
M175-R	CGGCAGCGTACCCTCGATAAAAACATTCTGG	65	St-Ov
V245A	GCACCCCAGGC**gct**CTGATCCCACC	T-up**	St-Ov
V245D	GCACCCCAGGC**gat**CTGATCCCACC	65	St-Ov
V245F	GCACCCCAGGC**ttt**CTGATCCCACC	65	St-Ov
V245G	GCACCCCAGGC**ggt**CTGATCCCACC	56	St-Ov
V245S	GCACCCCAGGC**tct**CTGATCCCACC	65	St-Ov
V245T	GCACCCCAGGC**act**CTGATCCCACC	T-up**	St-Ov
245-R	TGGGGTGCCCCAGAACAGCAGC		St-Ov
L271A	GGCCCGGGT**gct**AATCTGCTGCAAGAAGACAATCC	56	St-Ov
L271C	GGCCCGGGT**tgt**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271D	GGCCCGGGT**gat**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271F	GGCCCGGGT**ttt**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271H	GGCCCGGGT**cat**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271K	GGCCCGGGT**aag**AATCTGCTGCAAGAAGACAATCC	56	St-Ov
L271M	GGCCCGGGT**atg**AATCTGCTGCAAGAAGACAATCC	56	St-Ov
L271N	GGCCCGGGT**aat**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271R	GGCCCGGGT**aga**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271S	GGCCCGGGT**tct**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271G	GGCCCGGGT**ggt**AATCTGCTGCAAGAAGACAATCC	65	St-Ov
L271-R	ACCCGGGCCGATGTCCACAGCTTTGC		St-Ov
143/144KA	CGAATGGCCA**aaggcc**GCCCGTGAAACC	63	E2E
143/144KD	CGAATGGCCA**aaggat**GCCCGTGAAAC	63	E2E
143/144KH	CGAATGGCCA**aagcat**GCCCGTGAAAC	63	E2E
143/144KS	CGAATGGCCA**aagtcc**GCCCGTGAAAC	64	E2E
143/144MH	CGAATGGCCA**atgcat**GCCCGTGAAACC	64	E2E
143/144MD	CGAATGGCCA**atggat**GCCCGTGAAACC	62	E2E
143/144MI	CGAATGGCCA**atgatt**GCCCGTGAAACC	64	E2E
143/144-R	TCCCAGGTCGGAATAGGG		

*St-ov = staggered overlapping; E2E = end-to-end
**T-up = 51-72 ˚C +0.7 every step

5. Variant expression and purification

The DNA was transformed into BL21-DE3 by heat shock (50-100 ng, 15 µl cells, 42 ˚C, 20 s) in PCR tube strips. The transformation was recovered by shaking at 37 ˚C for 2 h. The transformation was directly used to inoculate solutions of LB/kanamycin in 24-well plates. The pre-cultures were shaken at 37 ˚C overnight. Fresh LB/kanamycin media (30 mL, in a 50-mL falcon tube) were inoculated with the overnight pre-culture (300 µl). The cultures were shaken at 37 ˚C. Once the OD_{600} reached 0.5-0.6 (approximately 1.5-2.5 h), IPTG was added to a final concentration of 0.4 mM. The cells were shaken at 37 ˚C for an additional 4 h. The cells were then harvested by centrifugation and frozen at -20 ˚C. The frozen cell pellets were thawed at room temperature and resuspended in B-PER (1 mL, ThermoFisher) supplemented with lysozyme (to 2-4 mg/mL) and DNase I (to 2 U/mL). The lysates were turned end-over-end for 3 h at room temperature. The suspensions were centrifuged, and the supernatants were purified by Ni-NTA beads (0.5 mL) packed into mini plastic gravity flow columns. The proteins were

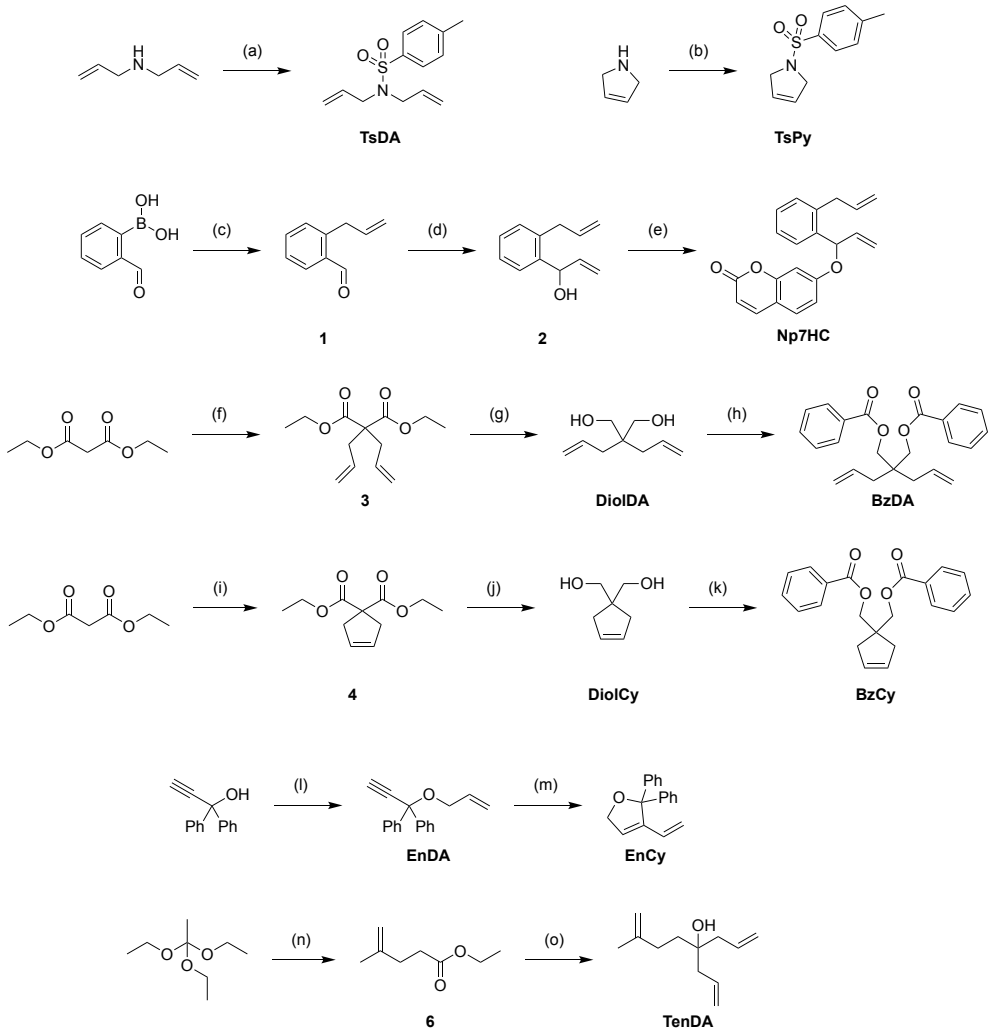
eluted with 250 mM imidazole. The eluted product was buffer exchanged using centrifugal filter units (10K MWCO) until the concentration of imidazole was less than 0.5 μM. The protein yield varied from 0.5 mg to 5 mg, depending on the protein variant. The resulting proteins were analyzed by SDS-PAGE, and activity assays with fluorescent-turn on probes.

6. General synthetic details

All starting materials and solvents were purchased from commercial sources and used without further purification if not stated else. The progress of reactions was controlled by thin layer chromatography (TLC) on pre-coated Merck silica gel 60 F_{254} plates (0.25 mm) and visualized using UV light or potassium permanganate stain. Flash column chromatography was carried out on *Silicycle* SilicaFlash P60 (230 – 400 mesh) or SNAP ULTRA C18. Concentration under reduced pressure was performed by rotary evaporation to ~7 mbar at 42 °C. NMR spectroscopy experiments were performed on an 400, 500, or 600 spectrometer by BRUKER operating at 400, 500, and 600 MHz at 298 K. The chemical shift is given in parts per million (ppm). The deuterated solvents used as references: $CDCl_3$ $\delta(^1H)$ = 7.26 ppm, DMSO-d_6 δ (^1H) = 2.50 ppm. The multiplicities were reported in Hz as: s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. NMR spectra are provided in the appendix for all previously-unpublished compounds, metathesis substrates, metathesis products, and all metathesis reactions quantified by NMR spectroscopy. For synthesis of previously-published compounds the general reaction scheme is provided.

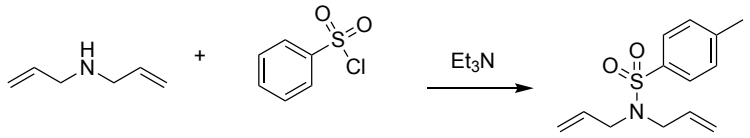
7. Synthesis of substrates and products

A general overview of the synthesis of the substrates and products are depicted in Scheme S1. The detailed methods are provided in the pages immediately following the scheme. The methods used for synthesis of **TsDA**, **Np7HC**, **DiolDA, DiolCy**, **BzDA**, **DiolCy**, **BzCy**, **EnDA**, **EnCy**, and **TenDA** were based on previously reported procedures[1–4] but are provided in detail here for clarity.



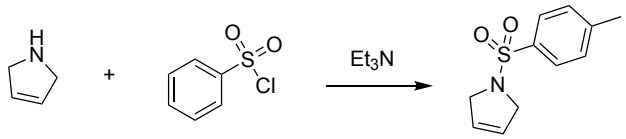
Scheme S1. Synthetic route of substrates and products: (a): p-toluenesulfonyl chloride, triethylamine, dichloromethane, 0 ˚C to RT, 18 h; (b) p-toluenesulfonyl chloride, triethylamine, DMAP, dichloromethane, RT, 18 h; (c) allyl bromide, bis(triphenylphosphine) palladium(II) chloride, sodium carbonate, THF, reflux, 18 h; (d) vinylmagnesium bromide, THF, RT, 4 h; (e) 7-hydroxycoumarin, DIAD, triphenylphosphine, THF, 0 ˚C to RT, 24 h; (f): allyl bromide, NaH, THF, RT, 18 h; (g) $LiAlH_4$, THF, 0 °C, 3.5 h; (h) benzoyl chloride, Et_3N, 4-DMAP, dichloromethane, RT, 20 h; (i) (*Z*)-1,4-dichlorobut-2-ene, NaH, THF, RT, 16 h; (j) $LiAlH_4$, THF, 0 °C, 2.5 h; (k) benzoyl chloride, Et_3N, 4-DMAP, dichloromethane, RT, 20 h; (l) allyl bromide, NaH, DMF, RT, 3.5 h; (m) Hoveyda-Grubbs 1st generation, dichloromethane, RT, 18 h; (n) 2-methylprop-2-en-1-ol, propionic acid, reflux, 38 h; (o) allyl magnesium bromide, THF, RT, 5 h.

_N_,_N_-diallyl-4-methylbenzenesulfonamide (TsDA)



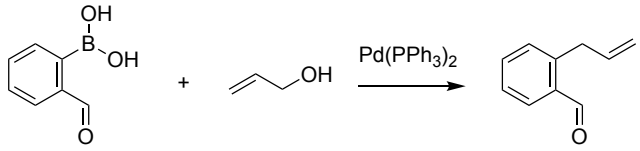
In a round-bottom flask on ice, triethylamine (843 µl, 6 mmol, 1.2 equiv.) was added dropwise to a solution of diallylamine (640 µl, 6 mmol, 1.2 equiv.) in dichloromethane (20 mL). The reaction was stirred at 0 ˚C for 30 min. To the reaction, 4-toluenesulfonyl chloride (953 mg, 5 mmol, 1 equiv.) was added slowly to the reaction. The reaction was slowly warmed to room temperature and stirred overnight. The reaction was washed with water and brine. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to yield a light yellow oil (1.2 g, 95%). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.70 (d, J = 8.2 Hz, 2H), 7.30 (d, J = 8.2 Hz, 2H), 5.68 – 5.54 (m, 2H), 5.20 – 5.07 (m, 4H), 3.80 (d, J = 6.3 Hz, 4H), 2.43 (s, 3H).

1-tosyl-2,5-dihydro-1*H*-pyrrole (TsPy)



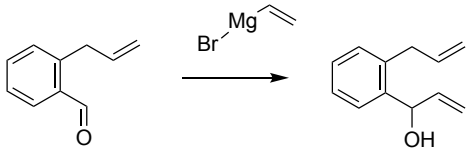
In a round-bottom flask, a solution of 3-pyrroline (380 µl, 5 mmol, 1 equiv.) and tosyl chloride (1.05 g, 5.5 mmol, 1.1 equiv.) in DCM (20 mL) was prepared. To the solution, triethylamine (2.11 mL, 15 mmol, 3 equiv.) and DMAP (60 mg, 0.5 mmol, 0.1 equiv.) were added. The solution was stirred at room temperature for 18 h. The reaction was extracted with water (50 mL). The organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was triturated with MeOH several times to yield white crystals (868 mg, 78 %). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.72 (d, J = 7.7 Hz, 2H), 7.32 (d, J = 7.7 Hz, 2H), 5.65 (s, 2H), 4.12 (s, 4H), 2.43 (s, 3H).

2-allylbenzaldehyde (1)



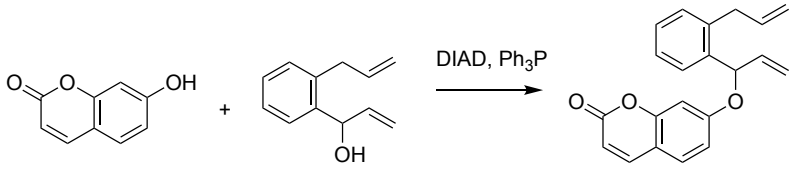
In a round-bottom flask, 2-formylphenylboronic acid (2.25 g, 15 mmol, 1 equiv.) and allyl bromide (1.56 mL, 18 mmol, 1.2 equiv.) were combined in dry THF (50 mL). Bis(triphenylphosphine)palladium(II) chloride (263 mg, 0.25 mmol, 0.025 equiv.) was added to the reaction. Sodium carbonate (20 mL, 1 M, aqueous) was added to the reaction, and the yellow suspension was heated at 71 ˚C overnight. The reaction was cooled to room temperature, and water and DCM were added. The phases were separated, and the aqueous layer was extracted twice with DCM. The combined organic layers were dried over sodium sulfate, filtered, and concentrated under reduced pressure. The brown oil was purified by flash chromatography (SiO_2, 0-10% EtOAc in cyclohexane). The product contained an impurity that could not be separated by chromatography. The crude light yellow oil (1.23 g, 56%) was used in the next reaction without further purification. R_f = 0.6 with 10% EtOAc in cyclohexane. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 10.26 (s, 1H), 7.85 (dd, J = 7.7, 1.2 Hz, 1H), 7.58 – 7.51 (m, 2H), 7.30 (d, J = 7.7 Hz, 1H), 6.04 (ddt, J = 16.4, 10.1, 6.2 Hz, 1H), 5.09 (dd, J = 10.1, 1.5 Hz, 1H), 4.99 (dd, J = 17.1, 1.5 Hz, 1H), 3.82 (d, J = 6.2 Hz, 2H).

1-(2-allylphenyl)prop-2-en-1-ol (2)



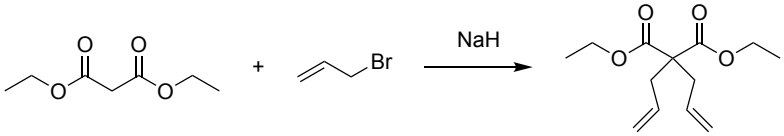
In a round-bottom flask under nitrogen atmosphere, the aldehyde (1.17 g, 8 mmol, 1 equiv.) was dissolved in dry THF (10 mL). Vinylmagnesium bromide (1 M in THF, 9.6 mmol, 1.2 equiv.) was added to the reaction slowly at room temperature. The reaction was stirred at room temperature for 4 h. When the reaction was complete as determined by TLC, the reaction was quenched with saturated ammonium chloride. The suspension was extracted with EtOAc (3 x 50 mL). The combined organic phases were dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (SiO_2, 9:1 cyclohexane:EtOAc). The resulting product was a pale yellow oil (1.02 g, 73%). R_f = 0.5; 10% EtOAc in cyclohexane. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.42 – 7.36 (m, 1H), 7.22 – 7.15 (m, 2H), 7.11 (dd, J = 7.0, 2.0 Hz, 1H), 6.04 – 5.88 (m, 2H), 5.43 – 5.37 (m, 1H), 5.27 (dt, J = 17.2, 1.4 Hz, 1H), 5.17 – 5.10 (m, 1H), 5.01 (dd, J = 10.1, 1.7 Hz, 1H), 4.93 (ddd, J = 17.2, 1.7 Hz, 1H), 3.42 (d, J = 6.2 Hz, 2H).

7-((1-(2-allylphenyl)allyl)oxy)-2*H*-chromen-2-one (Np7HC)



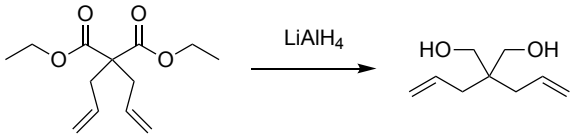
A round-bottom flask was dried under vacuum. In the round-bottom flask, the alcohol (192 mg, 1 mmol, 1 equiv), triphenylphosphine (433 mg, 1.5 mmol, 1.5 equiv.), and 7-hydroxycoumarin (214 mg, 1.2 mmol, 1.2 equiv.) were combined in dry THF (10 mL). The reaction was stirred in an ice-water bath. DIAD (311 mg, 1.4 mmol, 1.4 equiv.) was added dropwise to the reaction. After the addition was complete, the reaction was allowed to slowly come to room temperature. The reaction was stirred at room temperature for 24 and then concentrated under reduced pressure. The crude product needed to be purified by flash chromatography (SiO_2, 0-10% EtOAc in cyclohexane). The desired product was a colorless oil (53 mg, 15%). The primary product was the re-arrangement product (136 mg, 39%). After NMR, the sample was immediately frozen as a DMSO stock (100 mM, 100 µl aliquots). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.58 (d, J = 9.5 Hz, 1H), 7.44 (d, J = 7.7 Hz, 1H), 7.31 (d, J = 8.6 Hz, 1H), 7.29 – 7.18 (m, 3H), 6.87 (dd, J = 8.6, 2.3 Hz, 1H), 6.78 (d, J = 2.3 Hz, 1H), 6.20 (d, J = 9.5 Hz, 1H), 6.11 (ddd, J = 17.0, 10.3, 5.5 Hz, 1H), 6.05 – 5.88 (m, 2H), 5.39 – 5.26 (m, 2H), 5.14 (dd, J = 10.3, 1.5 Hz, 1H), 5.06 (dd, J = 17.1, 1.5 Hz, 1H), 3.49 (ddd, J = 16.0, 6.3 Hz, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 161.21, 161.04, 155.68, 143.39, 137.10, 136.61, 136.49, 136.00, 130.38, 128.71, 128.49, 127.08, 126.97, 117.74, 116.77, 114.01, 113.18, 112.73, 103.06, 77.92, 36.90.

Diethyl-2,2-diallymalonate (3)



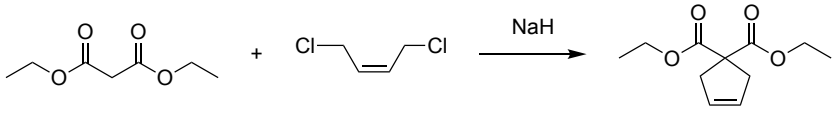
Sodium hydride (60% in mineral oil, 600 mg, 15.0 mmol, 3.2 equiv.) was suspended in THF (10 mL). Diethyl malonate (700 µL, 4.64 mmol, 1.0 equiv.) was added dropwise at 0 °C, and the suspension was stirred for 1 h. Allyl bromide (1.20 mL, 13.9 mmol, 3.0 equiv.) was added dropwise, and the reaction was stirred at room temperature for 18 h. The reaction was quenched with water (10 mL) and diluted with EtOAc (10 mL). The phases were separated, and the aqueous phase was extracted with EtOAc (2 x 20 mL). The combined organic phases were washed with brine (40 mL) and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield **1** (1.11 g, 4.64 mmol, quant.) as a colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.65 (ddt, $^3J_{HH}$ = 16.7, $^3J_{HH}$ = 10.3, $^3J_{HH}$ = 7.4 Hz, 2H), 5.13 – 5.07 (m, 4H), 4.18 (q, $^3J_{HH}$ = 7.1 Hz, 4H), 2.63 (d, $^3J_{HH}$ = 7.4 Hz, 4H), 1.24 (t, $^3J_{HH}$ = 7.1 Hz, 6H).

2,2-Diallylpropane-1,3-diol (DiolDA)



Lithium aluminum hydride (2.0 M in THF, 12.2 mL, 24.4 mmol, 3.0 equiv.) was suspended in THF (50 mL). Diethyl-2,2-diallylmalonate (1.0 M in THF, 8.00 mL, 8.11 mmol, 1.0 equiv.) was added dropwise at 0 ˚C. The reaction mixture was stirred at 0 ˚C for 3.5 h and quenched with water (2 mL). Aqueous NaOH (15%, 2 mL) and water (4 mL) were added, and the mixture was stirred for 1 hour. The precipitate was removed by filtration through Celite and was washed with THF. The solvent was removed *in vacuo*. The residue was taken up in EtOAc and dried over $MgSO_4$. The solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 1:1 (v/v) cyclohexane/EtOAc) to yield **DiolDA** (945 mg, 6.05 mmol, 75%) as a colorless oil. R_f: 0.2 (1:1 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.84 (ddt, $^3J_{HH}$ = 16.6, $^3J_{HH}$ = 10.4, $^3J_{HH}$ = 7.5 Hz, 2H), 5.14 – 5.07 (m, 4H), 3.57 (d, $^3J_{HH}$ = 4.5 Hz, 4H), 2.47 (t, $^3J_{HH}$ = 5.0 Hz, 4H), 2.08 (d, $^3J_{HH}$ = 7.5 Hz, 6H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 134.04 (s), 118.27 (s), 68.35 (s), 42.22 (s), 36.24 (s).

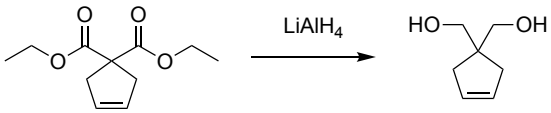
Diethyl cyclopent-3-enyl-malonate (4)



Sodium hydride (60% in mineral oil, 624 mg, 15.6 mmol, 2.5 equiv.) was suspended in THF (30 mL). Diethyl malonate (945 µL, 6.26 mmol, 1.0 equiv.) was added dropwise at 0 ˚C, and the mixture was stirred for 1 h. (*Z*)-1,4-Dichlorobut-2-ene (660 µL, 6.23 mmol, 1.0 eq) was added dropwise, and the suspension was stirred at room temperature for 16 ˚C. The reaction was quenched with water until no bubbles developed, and the solvent was evaporated under reduced pressure. The residue was taken up in dichloromethane (15 mL), the precipitate was removed by filtration, and the filtrate was washed with water (6 x 10 mL) and sat. aq. NH_4Cl (10 mL). The organic phase was dried over $MgSO_4$, and the crude product was purified by flash chromatography (silica, 20:1 (v/v) cyclohexane/EtOAc) to obtain **2** (735 mg, 3.46 mmol,

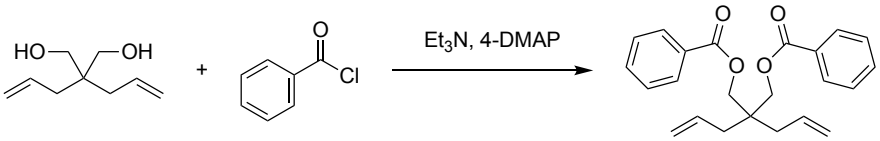
56%) as a colorless oil. R_f: 0.25 (20:1 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.61 (s, 2H), 4.20 (q, $^3J_{HH}$ = 7.1 Hz, 4H), 3.01 (s, 4H), 1.25 (t, $^3J_{HH}$ = 7.1 Hz, 6H).

Cyclopent-3-ene-1,1-diyldimethanol (DiolCy)



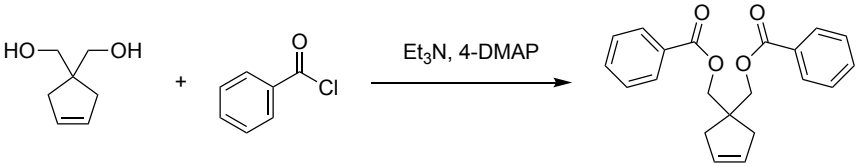
Lithium aluminium hydride (2.0 M in THF, 870 µL, 1.74 mmol, 3.0 equiv.) was suspended in THF (4 mL). Diethyl cyclopent-3-enyl-malonate (123 mg, 580 µmol, 1.0 equiv.) in THF (1 mL) was added dropwise at 0 ˚C. The reaction mixture was stirred at 0 ˚C for 2.5 h and quenched with water (1 mL). Aqueous NaOH (15%, 1 mL) and water (2 mL) was added, and the mixture was stirred for 2 hours. The precipitate was removed by filtration through Celite and washed with THF. The solvent was removed *in vacuo*; the residue was taken up in EtOAc and dried over $MgSO_4$. The solvent was removed under reduced pressure. **DiolCy** was obtained (51.0 mg, 398 µmol, 69%) as a white solid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.63 (s, 2H), 3.71 (d, $^3J_{HH}$ = 5.2 Hz, 4H), 2.22 (s, 4H), 2.20 (t, $^3J_{HH}$ = 5.2 Hz, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 128.94 (s), 70.39 (s), 47.78 (s), 38.79 (s).

2,2-Diallylpropane-1,3-diyl dibenzoate (BzDA)



Benzoyl chloride (495 µg, 4.26 mmol, 2.2 equiv.) were dissolved in dichloromethane (4 mL) and a solution of **DiolDA** (299 mg, 1.91 mmol, 1.0 equiv.), Et_3N (1.10 mL, 7.83 mmol, 4.1 equiv.) in dichloromethane (2.3 mL) was added dropwise at 0 ˚C. A small amount of DMAP was added and the yellow solution was stirred at room temperature for 20 h. The reaction was quenched with sat. aq. $NaHCO_3$ (5 mL); the layers were separated; and the organic layer was washed with water (5 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography (silica, 10:1 (v/v) cyclohexane/EtOAc) to yield **BzDA** (595 mg, 1.63 mmol, 85%) as a colorless oil. R_f: 0.3 (silica, 10:1 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.03 (dd, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 1.4 Hz, 4H), 7.56 (tt, $^3J_{HH}$ = 7.2 Hz, $^4J_{HH}$ = 1.3 Hz, 2H), 7.44 (dd, $^3J_{HH}$ = 8.0 Hz, $^3J_{HH}$ = 8.0 Hz, 4H), 5.89 (ddt, $^3J_{HH}$ = 16.6, $^3J_{HH}$ = 10.2, $^3J_{HH}$ = 7.5 Hz, 2H), 5.19 – 5.10 (m, 4H), 4.32 (s, 4H), 2.33 (d, $^3J_{HH}$ = 7.6 Hz, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 166.39 (s), 133.22 (s), 132.50 (s), 130.13 (s), 129.71 (s), 128.59 (s), 119.51 (s), 66.76 (s), 40.96 (s), 36.82 (s).

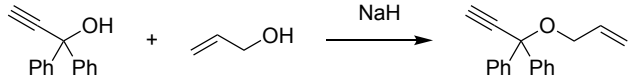
Cyclopent-3-ene-1,1-diylbis(methylene) dibenzoate (BzCy)



Benzoyl chloride (125 µL, 1.08 mmol, 2.2 equiv.) were dissolved in dichloromethane (1 mL) and a solution of **DiolCy** (63.2 mg, 493 µmol, 1.0 equiv.), Et_3N (285 µL, 2.03 mmol, 4.1 equiv.) in dichloromethane (800 µL) was added dropwise at 0 ˚C. A small amount of DMAP was

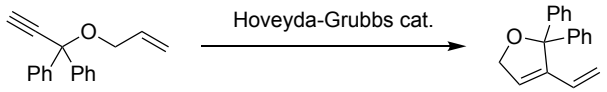
added, and the solution was stirred at room temperature for 20 h. The reaction was quenched with sat. aq. $NaHCO_3$ (2 mL), and the layers were separated. The organic layer was washed with water (2 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography (silica, 1:1 (v/v) cyclohexane/EtOAc) to yield **BzCy** (123 mg, 366 µmol, 74%) as a colorless oil. R_f: 0.8 (silica, 1:1 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.03 (dd, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 1.4 Hz, 4H), 7.56 (tt, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.5 Hz, 2H), 7.43 (dd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.8 Hz, 4H), 5.70 (s, 2H), 4.41 (s, 4H), 2.47 (s, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 166.69 (s), 133.18 (s), 130.19 (s), 129.73 (s), 128.79 (s), 128.55 (s), 68.13 (s), 45.50 (s), 39.45 (s).

(1-(Allyloxy)prop-2-yne-1,1-diyl)dibenzene (EnDA)



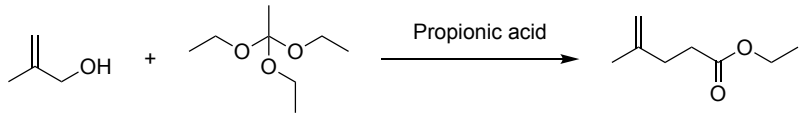
Sodium hydride (60% in mineral oil, 141 mg, 3.53 mmol, 1.1 equiv.) was suspended in DMF (30 mL). At 0 ˚C, 1,1-Diphenylprop-2-yn-1-ol (650 mg, 3.12 mmol, 1.0 equiv.) in THF (4 mL) was added. The suspension was stirred for 30 min before allyl bromide (300 µL, 3.47 mmol, 1.1 equiv.) was add at 0 ˚C. The reaction was stirred at room temperature for 3.5 h and quenched with water (5 mL). EtOAc (50 mL) was added, and the phases were separated. The organic phase was washed with sat. aq. $NaHCO_3$ (40 mL) and brine (40 mL) and then dried over $MgSO_4$. The crude product was purified by flash chromatography (silica, gradient from cyclohexane to 10:1 (v/v) cyclohexane/EtOAc) to obtain **EnDA** (450 mg, 1.81 mmol, 58%) as a colorless oil. R_f: 0.48 (silica, 20:1 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.63 – 7.59 (m, 4H), 7.34 (dd, $^3J_{HH}$ = 7.4 Hz, $^3J_{HH}$ = 7.4 Hz, 4H), 7.28 (dd, $^3J_{HH}$ = 7.6 Hz, $^3J_{HH}$ = 7.6 Hz, 2H), 6.03 (ddt, $^3J_{HH}$ = 17.2 Hz, $^3J_{HH}$ = 10.5 Hz, $^3J_{HH}$ = 5.2 Hz, 1H), 5.40 (dd, $^3J_{HH}$ = 17.2 Hz, $^4J_{HH}$ = 1.8 Hz, 1H), 5.20 (dd, $^4J_{HH}$ = 10.5 Hz, $^2J_{HH}$ = 1.7 Hz, 1H), 4.07 (ddd, $^3J_{HH}$ = 5.3 Hz, $^4J_{HH}$ = 1.6 Hz, $^4J_{HH}$ = 1.6 Hz, 2H), 2.92 (s, 1H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 143.28 (s), 134.89 (s), 128.33 (s), 127.86 (s), 126.72 (s), 116.26 (s), 83.41 (s), 80.20 (s), 77.71 (s), 66.08 (s).

2,2-Diphenyl-3-vinyl-2,5-dihydrofuran (EnCy)



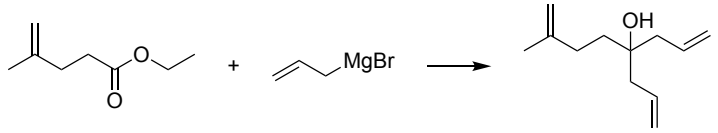
Hoveyda-Grubbs 1st generation catalyst (4.20 mg, 6.69 µmol, 2.1 mol%) was added to a solution of **EnDA** (79.9 mg, 322 µmol, 1.0 equiv.) in dichloromethane (3 mL). The reaction was stirred at room temperature for 18 h, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (silica, 20:1 (v/v) cyclohexane/EtOAc) to yield **EnCy** (69.1 mg, 278 µmol, 86%) as a dark green oil. R_f: 0.25 (silica, 20:1 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.39 – 7.27 (m, 10H), 6.29 – 6.19 (m, 2H), 5.33 (d, $^3J_{HH}$ = 17.8 Hz, 1H), 5.12 (d, $^3J_{HH}$ = 11.6 Hz, 1H), 4.80 (s, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 143.79 (s), 143.48 (s), 129.90 (s), 128.05 (s), 128.04 (s), 127.60 (s), 125.05 (s), 117.71 (s), 94.69 (s), 73.35 (s).

Ethyl 4-methylpent-4-enoate (6)



2-Methylprop-2-en-1-ol (2.30 mL, 27.1 mmol, 1.0 equiv.), triethyl orthoacetate (10.8 mL, 59.2 mmol, 2.2 equiv.) and propionic acid (1.60 mL, 21.4 mmol, 0.79 equiv.) were mixed and refluxed for 16 h. Additional propionic acid (700 µmol, 9.35 mmol, 0.35 equiv.) was added. The mixture was stirred for additional 22 h and then diluted with Et_2O (100 mL). The organic phase was washed with aq. HCl (10%, 100 mL), aq. sat. $NaHCO_3$ (100 mL) and brine (100 mL). The solution was dried over $MgSO_4$ to yield the desired product **8** in a 5:2 mixture with 2-methylallyl 4-methylpent-4-enoate (2.43 g, 16.3 mmol, 60%) as a colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 4.74 (s, 1H), 4.69 (s, 1H), 4.13 (q, $^3J_{HH}$ = 7.1 Hz, 2H), 2.47 – 2.42 (m, 2H), 2.36 – 2.31 (m, 2H), 1.74 (s, 4H), 1.25 (t, $^3J_{HH}$ = 7.1 Hz, 3H).

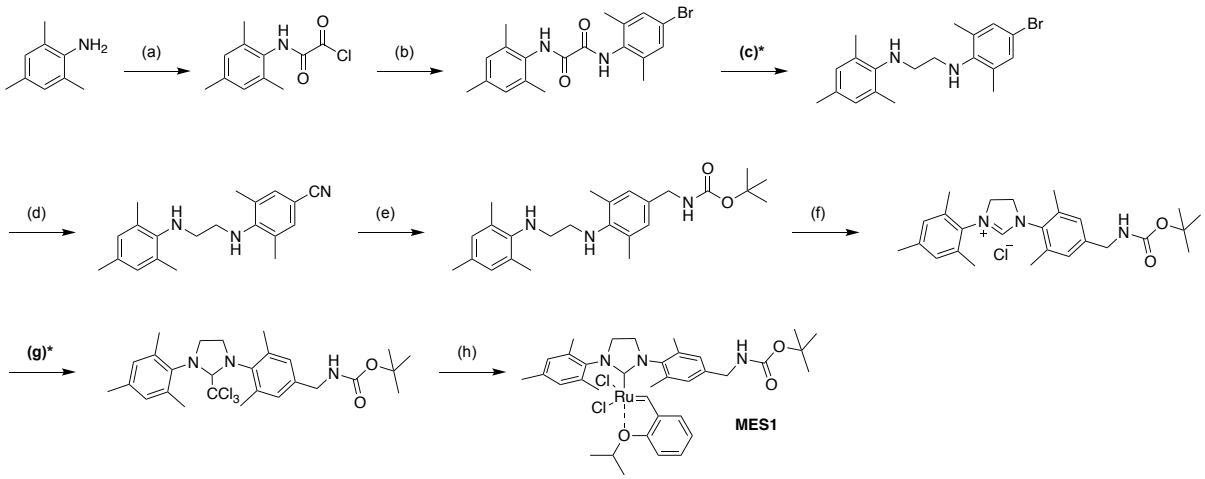
4-Allyl-7-methylocta-1,7-dien-4-ol (TenDA)



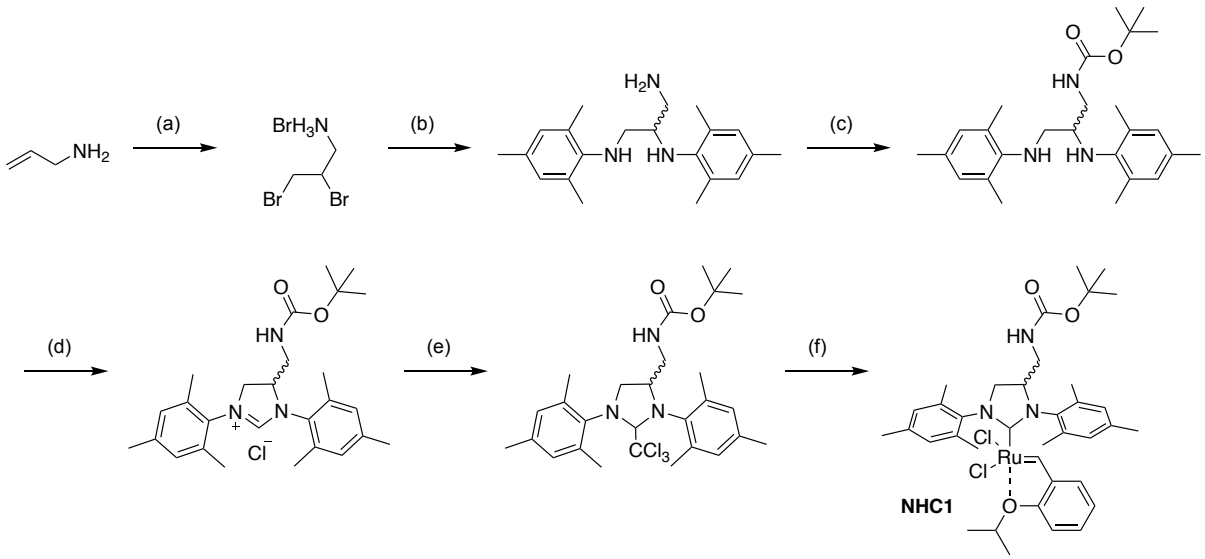
The mixture of **6** and 2-methylallyl 4-methylpent-4-enoate (5:2, 525 mg, 3.52 mmol. 1.0 equiv.) was dissolved in THF (11 mL). Allyl magnesium bromide (1.0 M in Et_2O, 7.10 mL, 7.10 mmol, 2.0 equiv.) was added dropwise at 0 ˚C. The reaction was stirred at room temperature for 5 h before it was quenched with sat. aq. NH_4Cl (5 mL). The aqueous phase was extracted with EtOAc (3 x 10 mL), the combined organic phases were washed with brine (30 mL) and dried over $MgSO_4$. The crude product was purified by flash chromatography (silica, 40:3 (v/v) cyclohexane/EtOAc) to obtain **TenDA** (300 mg, 1.66 mmol, 47%) as a colorless oil. R_f: 0.24 (silica, 40:3 (v/v) cyclohexane/EtOAc). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.86 (ddt, $^3J_{HH}$ = 17.5 Hz, $^3J_{HH}$ = 10.3 Hz, $^3J_{HH}$ = 7.4 Hz, 2H), 5.17 – 5.10 (m, 4H), 4.70 (s, 3H), 2.30 – 2.20 (m, 4H), 2.11 – 2.05 (m, 2H), 1.74 (s, 3H), 1.63 – 1.56 (m, 3H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 146.22 (s), 133.75 (s), 118.92 (s), 109.93 (s), 73.49 (s), 43.81 (s), 37.28 (s), 31.73 (s), 22.74 (s).

8. Synthesis of cofactors

The synthesis routes for the starting ruthenium catalysts (**MES1** and **NHC1**, Schemes S2 and S3) were based on previously-published reports with some changes.[5] The methods are displayed briefly in Schemes S2 and S3, and the reactions that were modified heavily are described below.

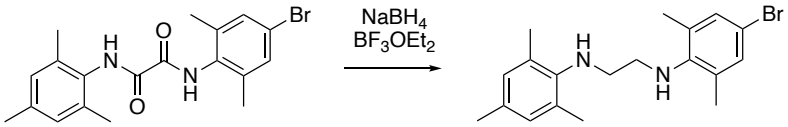


Scheme S2. Synthetic method to the mesityl-functionalized catalysts: (a) oxalyl chloride, dichloromethane, 0 ˚C to RT, 4 h, 35-40%; (b) 4-bromo-2,6-dimethylaniline, triethylamine, dichloromethane, 0 ˚C to RT, 2h , 87-95%; (c-i) sodium borohydride, boron trifluoride etherate, THF, reflux, 18 h; (c-ii) MeOH, HCl, (c-iii) sodium hydroxide 83-95%; (d) copper(I) cyanide, N-methyl-2-pyrrolidone, 160 ˚C, 20 h, 65-69%; (e-i) lithium aluminum hydride, THF, reflux, 14 h; (e-ii) di-*tert*-butyl decarbonate, DMAP, dichloromethane, 0 ˚C to RT, 3 h, 19-66%; (f) triethyl orthoformate (dry), ammonium chloride, 120 ˚C, 20 h, 47-67%; (e) chloroform, sodium hydroxide, toluene, RT, 1.5 h, 52-75%; (f) Hoveyda-Grubbs 1st generation catalyst, toluene, 70 ˚C, 1.5 h, 31-72%. The steps with asterisks were modified from previous reports.



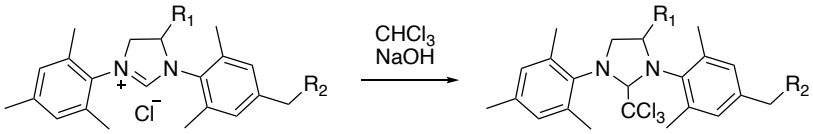
Scheme S3. Synthetic method to the NHC-core-functionalized catalysts: (a) bromine, ethanol, 0 ˚C to RT, 18 h, 61%; (b) 2,4,6-trimethylaniline, 120 ˚C, 19 h, 32%; (c) di-*tert*-butyl decarbonate, DMAP, dichloromethane, 0 ˚C to RT, 3 h, 77%; (d) triethyl orthoformate (dry), ammonium chloride, 120 ˚C, 20 h, 89%; (e) chloroform, sodium hydroxide, toluene, RT, 1.5 h, 94%; (f) Hoveyda-Grubbs 1st generation catalyst, toluene, 70 ˚C, 1.5 h, 30%.

Reduction of the oxamide to diamine



The oxamide (11.7 g, 30 mmol, 1 equiv.), sodium borohydride (6.81 g, 180 mmol, 6 equiv.), and THF (250 mL) were added to a round-bottom flask. The atmosphere was exchanged by cycling between vacuum and nitrogen several times. At room temperature, boron trifluoride diethyl etherate (30 ml, 240 mmol, 8 equiv.) was added slowly to the suspension. The reaction was placed under nitrogen and stirred at 62 ˚C for 18 h. The reaction was quenched with methanol (60 mL) and conc. HCl (6 mL, 70 mmol). The quenched suspension was stirred for 1 h. The reaction was concentrated under reduced pressure, and the resulting white residue was suspended in 2 M NaOH (300 mL). Ether (400 mL) was added, and the biphasic mixture was stirred at room temperature for 1 h. The organic layer was separated and the aqueous layer was extracted with ether (3 x 300 mL). The combined organic phases were dried over sodium sulfate, filtered, and concentrated under reduced pressure to yield a sticky, light-brown residue. The product was purified by flash chromatography (SiO_2: 10-20% EtOAc in cyclohexane; product R_f = 0.5 in 4:1 cyclohexane:EtOAc,). The yield was 8.97 g (83 %). The NMR data matched well with previously reported literature.[6]

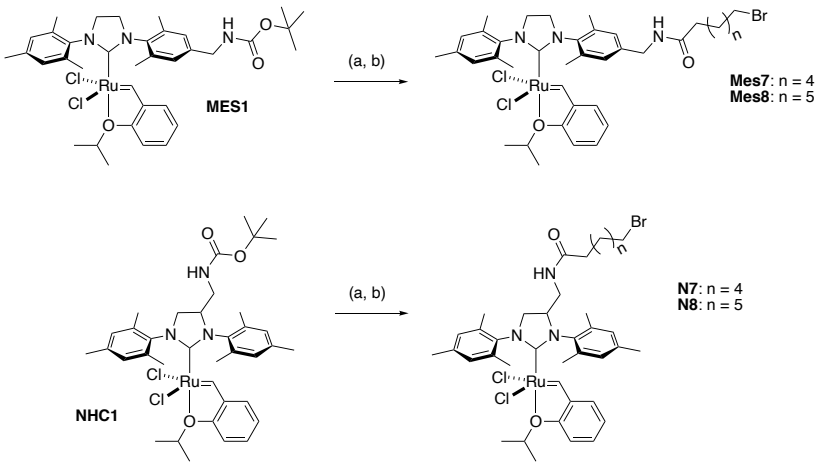
General synthesis of chloroform-NHC adducts



In a Schlenk tube, toluene (6 mL) was degassed by freeze-pump-thawing in the presence of a stir bar. In the tube, ground NaOH (800 mg, 20 mmol, 20 equiv.) was added. The suspension was cycled between vacuum and nitrogen three times. Dry chloroform (4.4 mL, 54 mmol, 54 equiv.) was added via syringe.* The reaction was stirred at room temperature for 10 min. The NHC-chloride salt (1 mmol) was then added, and the reaction turned dark brown. The suspension was stirred under nitrogen at room temperature for 1.5 h. The reaction was analyzed by TLC. The reaction was filtered through clean cotton in a glass pipette. The solids were washed with chloroform. The filtrate was concentrated under reduced pressure at 35 ˚C to yield a brown solid.† The crude material was purified by column chromatography (SiO_2: 10-20% EtOAc in cyclohexane). The product was a colorless oil that solidified to a white powder under high vacuum (52-94%). The product 1H-NMR was identical to previous literature reports.[5]

* The chloroform was from a commercial bottle dried over molecular sieves stabilized with 150 ppm amylene (Acros). The quality of chloroform greatly impacts the reaction. Ethanol stabilized chloroform should not be used, or should be washed with water, dried, and distilled prior to use.

† Care was taken to not heat the reaction or the product over 35 ◦C.



Scheme S4. Synthesis of the final cofactors: (a) $HCl_{(g)}$, dichloromethane, RT, 2 h; (b) 7-bromoheptanoyl chloride or 8-bromooctanoyl chloride, triethylamine, dichloromethane, RT, 18 h.

Mes8. In a round-bottom flask, **MES1** (19.3 mg, 0.03 mmol, 1 equiv.) was dissolved in dichloromethane (4 mL). $HCl_{(g)}$ was bubbled through the solution until the Boc protecting group was completely removed (as judged by TLC). The reaction was evaporated under reduced pressure. The crude product and 8-bromooctanoyl chloride (29 mg, 0.12 mmol, 4 equiv.) were dissolved in dichloromethane (3 mL). Triethylamine (160 µl, 1.2 mmol, 40 equiv.) was added slowly to the solution. The reaction was stirred at room temperature under nitrogen for 3 h. The reaction was concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, 50% EtOAc in cyclohexane). The bright green fractions were concentrated under reduced pressure to a yield green solid (11 mg, 43%). HRMS (ESI) M/z 776.2345 (776.2364 calcd for $C_{39}H_{53}BrN_3O_2Ru$, $[M\text{-}2Cl+e]^+$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 16.52 (s, 1H), 7.49 (ddd, J = 8.7, 7.3, 1.7 Hz, 1H), 7.15 (s, 2H), 7.07 (s, 2H), 6.92 (dd, J = 7.6, 1.7 Hz, 1H), 6.86 (t, J = 7.4 Hz, 1H), 6.80 (d, J = 8.3 Hz, 1H), 5.75 (br s, 1H), 4.90 (m, 1H), 4.49 (d, J = 5.7 Hz, 2H), 4.17 (br s, 4H), 3.40 (t, J = 6.8 Hz, 2H), 2.52 (s, 6H), 2.46 (s, 6H), 2.41 (s, 3H), 2.26 (t, J = 7.6 Hz, 2H), 1.85 (p, J = 7.0 Hz, 2H), 1.71 (p, J = 7.6 Hz, 2H), 1.38 (td, J = 10.4, 9.0, 5.8 Hz, 4H), 1.27 (d, J = 6.1 Hz, 6H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 172.76, 152.28, 146.17, 145.17, 139.22, 138.93, 129.69, 129.46, 129.44, 129.18, 127.80, 122.78, 122.33, 112.95, 74.96, 43.17, 36.74, 33.96, 32.71, 29.16, 28.52, 28.01, 25.60, 21.13.

N8. In a round-bottom flask, **NHC1** (19.7 mg, 0.03 mmol, 1 equiv.) was dissolved in dichloromethane (4 mL). $HCl_{(g)}$ was bubbled through the solution until the Boc protecting group was completely removed (as judged by TLC). The reaction was evaporated under reduced pressure. The crude product and 8-bromooctanoyl chloride (29 mg, 0.12 mmol, 4 equiv.) were dissolved in dichloromethane (3 mL). Triethylamine (169 µl, 1.2 mmol, 40 equiv.) was added slowly to the solution. The reaction was stirred at room temperature under nitrogen for 3 h. The reaction was concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, 0-50% EtOAc in cyclohexane). The bright green fractions were concentrated under reduced pressure to a yield green solid (11 mg, 42%). HRMS (ESI) M/z 834.2404 (834.2420 calcd for $C_{41}H_{55}BrN_3O_4Ru$, $[M\text{-}2Cl+CH_3COO\text{-}H]^+$). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 16.46 (s, 1H), 7.50 (t, J = 7.5 Hz, 1H), 7.05 (s, 2H), 6.88 (d, J = 7.2 Hz, 2H), 6.83 (d, J = 8.3 Hz, 1H), 6.52 (br s, 1H), 4.96 (br s, 1H), 4.51 (br s, 1H), 4.30 (t, J = 10.9 Hz, 1H), 3.88 (dd, J = 10.4, 6.5 Hz, 1H), 3.39 (t, J = 6.9 Hz, 2H), 2.54 – 2.11 (m, 20H), 1.91 – 1.77 (m, 2H), 1.57 (dd, J = 12.7, 6.0 Hz, 4H), 1.36 – 1.19 (m, 14H). ^{13}C-NMR (126 MHz,

$CDCl_3$): δ 172.51, 152.36, 145.07, 130.12, 122.86, 122.40, 112.92, 75.15, 63.45, 55.45, 36.46, 33.95, 32.74, 29.04, 28.44, 28.01, 26.93, 25.40, 21.3.

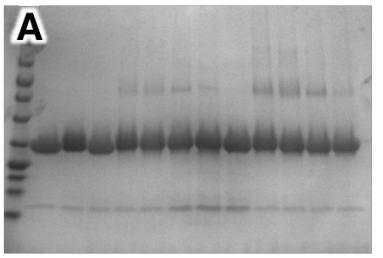
Storage of cofactors. After ^{1}H-NMR analysis, the samples were dissolved in chloroform, and aliquoted to 1 mg per vial. The samples were evaporated under reduced pressure. The dry samples were stored under nitrogen at -20 ˚C. For reactions, the samples were dissolved in acetone to 10 mM. The cofactor solutions were not stable for storage at -20 ˚C longer than 2 weeks in acetone.

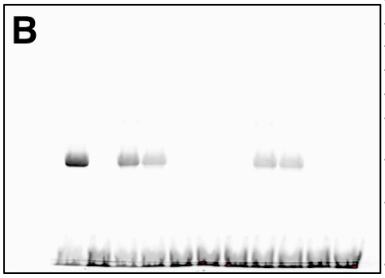
9. Cofactor bioconjugation assay

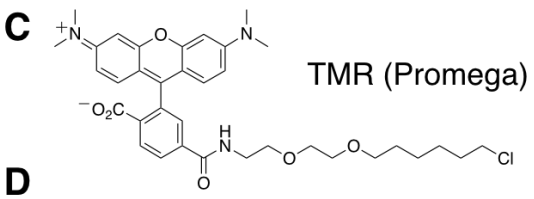
Gel-based assay: HT (30-55 μM) and the cofactor (1-4 molar equivalents) were mixed in 20 mM HEPES buffer, pH 7.5 or 40 mM MOPS, pH 7.4. The solution was incubated at 25 °C for between 10 min to 2 h. The rapidly-binding fluorescent HT probe, TMR (Promega, Rhodamine-PEG-C6-Cl) was added such that there were 1.5 equivalents of TMR to HT. After 5-10 min, protein-gel-loading dye was added to each reaction. The reaction products—HT/cofactor, HT/TMR, free cofactor, and free TMR—were separated by SDS-PAGE (15%). The resulting gel was visualized on a fluorescence-compatible gel imager and then stained with Coomassie stain for protein visualization. Both negative and positive control experiments were conducted to benchmark 0% and 100% cofactor binding. The negative control experiment contained buffer instead of the cofactor. The positive control experiment contained methyl 7-chloroheptanoate (M7CH). The fluorescence spots on the gel were integrated using ImageJ. The fraction of HT/cofactor conjugate from total HT was calculated by the following equation. Based on the binding analysis, neither **Mes7** nor **N7** were further explored for catalysis.

$$Fraction\ Bioconjugation = 1 - \left(\frac{FL_{sample} - FL_{+control}}{FL_{-control} - FL_{+control}} \right)$$

Experimental results for a representative bioconjugation assay are shown below.







		Control		10 min bioconj.					1 h bioconj.				
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
M7CH	ladder	-	-	+	-	-	-	-	+	-	-	-	-
Mes7		-	-	-	+	-	-	-	-	+	-	-	-
N7		-	-	-	-	+	-	-	-	-	+	-	-
Mes8		-	-	-	-	-	+	-	-	-	-	+	-
N8		-	-	-	-	-	-	+	-	-	-	-	+
HaloTag-TMR		-	+	+	+	+	+	+	+	+	+	+	+
Integrated FL intensity		0	1.0	0	0.469	0.279	0	0	0	0.278	0.236	0	0
Fraction bound to substrate		**-**	**0**	**1**	**0.531**	**0.721**	**1**	**1**	**1**	**0.722**	**0.764**	**1**	**1**

Figure S2. Representative data for the bioconjugation assay with wt HT (40 μM) and cofactor (160 μM). The Coomassie-stained gel (A) and the fluorescence-imaged gel (B) were used to visualize HT and TMR (C). The fluorescence gel was integrated to determine the amount of HT bound to TMR, and then the amount of HT bound to the cofactor.

Solution-based assay: Solution-based assays were used only to assess bioconjugation for the catalytic screening. These assays were more rapid, allowing for analysis of some HT variants. A solution of the cofactor (65 μM) and the HT variant (55 μM) were prepared in 10 mM HEPES buffer, pH 7.5. The bioconjugation solution was shaken at 25 °C for 2 h. To each sample a fluorescent probe for HT (the 'Chanel dye') was added to a final concentration of 60 μl (2 μl). The fluorescence was measured after 30 min. Control experiments contained either no cofactor or no protein. The fraction of bioconjugation was calculated with the same formula as above.

10. Catalytic screening assays for the purified-ArM method

The reactions were set up in three parts: i) bioconjugation with excess cofactor, ii) removal of unbound cofactor, and iii) metathesis reaction. For the bioconjugation reaction, a solution of the cofactor (180 μM) and the HT variant (55 μM) were prepared in 10 mM HEPES buffer, pH 7.5. The bioconjugation solution was shaken at 20 °C for 2 h. The samples were buffer exchanged by three cycles of dilution-concentration with 10K MWCO centrifugal filter tubes. In each round, approximately 100 μL of ArM was diluted with 350 μL of 10 mM HEPES buffer, pH 7.5. After the final round the ArMs were standardized to 55 μM by addition of reaction buffer (20 mM MOPS, 100 mM $MgCl_2$, pH 7.0). Solutions of 2 μM ArM were prepared in reaction buffer in a black 96-well plate. To initiate the reaction, Np7HC was added to a final concentration of 100 μM. The final amount of DMSO in the reaction was 5% (v/v). The formation of 7-hydroxycoumarin was determined using a fluorescence plate reader (λ_{ex} = 330 nm and λ_{em} = 450 nm). The reaction progress was monitored with these parameters for 5 h (20 min intervals) at 25 ˚C with intermittent shaking.

These reactions were not suitable for medium- or high-throughput, thus a second method was developed for screening multiple variants (ArM-screening method, below).

11. Catalytic screening assays for the ArM-screening method

The reactions were set up in two parts: i) bioconjugation and ii) metathesis reaction. For the bioconjugation reaction, a solution of the cofactor (65 μM) and the HT variant (55 μM) were prepared in 10 mM HEPES buffer, pH 7.5. The bioconjugation solution was shaken at 25 °C for 2 h. A solution-based binding assay was conducted under these conditions (Table S2), and the data were similar to expected based on the gel-based bioconjugation assay (compare with Figure 2B).

Table S2. Fraction of ArM formed after pre-catalysis bioconjugation with some HT variants

HT variant	**N8**	**Mes8**
HT (version 7, 'wt')	0.56	0.42
144A	0.66	0.38
271N	0.30	0.44
143K	0.66	n.d.
n.d. = not determined		

The reaction was diluted 10-fold with reaction buffer (20 mM MOPS, 100 mM $MgCl_2$, pH 7.0). Next, the metathesis reaction was prepared. In a 96-well plate, solutions of the ArM (2 μM), substrate (100 μM), and reaction buffer (50 μL) were prepared.

For fluorescence assessment and GC-MS analysis, the formation of 7-hydroxycoumarin was determined using a fluorescence plate reader (λ_{ex} = 330 nm and λ_{em} = 450 nm). The reactions were monitored for 3-5 h at 25 ˚C with intermittent shaking. After 5 h, the samples were extracted with ethyl acetate, and the organic fractions were analyzed by GC-MS (column: HP1-MS, 0.25 µm thickness, 30.0 m length, 0.25 mm diameter). The TON was determined from calibration curves prepared in the same manner as the samples: fluorescence, same buffer; GC-MS, extraction of the fluorescence calibration curve samples (Figure S3).

For UPLC-MS samples, the 96-well plate was shaken 25 ˚C for 21 h prior to analysis. The UPLC-MS samples were multiplexed (1:1 mixing of reactions of the same HT variant but different substrates) for more rapid analysis. An internal standard (4-amino-7-methylcoumarin, 10 µM) was added to each sample to account for variation in sample injection volume. Calibration curves of each substrate were prepared to determine the turnover number (Figure S3).

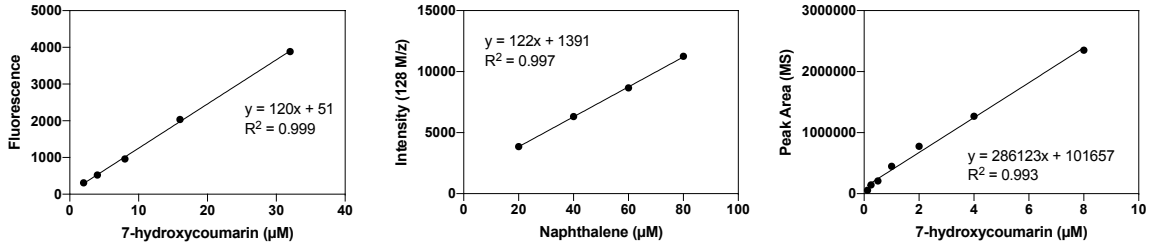


Figure S3. Calibration curves for 7-hydroxycoumarin (fluorescence), naphthalene (GC-MS), 7-hydroxycoumarin (UPLC-MS).

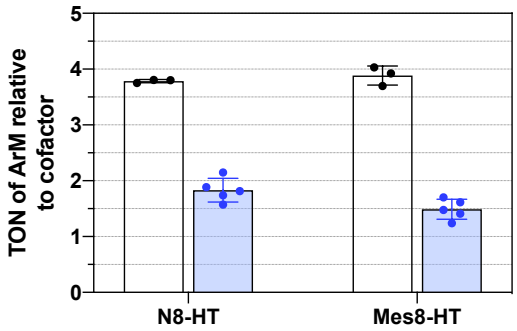


Figure S4. Comparison of different catalysis methods: Purified-ArM method (white with black outline) and ArM-screening methods (blue) for catalysis with **Np7HC**. The reactions were conducted in 20 mM MOPS, 100 mM $MgCl_2$, pH 7.0 at 25 ˚C. The data were collected at 3 h.

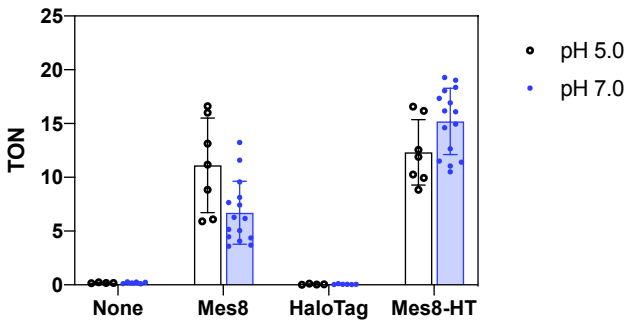


Figure S5. pH dependence of metathesis with **Np7HC** and **Mes8**. The buffers were 50 mM phosphate, 50 mM Acetate, 100 mM $MgCl_2$, pH 5.0 or 20 mM MOPS, 100 mM $MgCl_2$, pH 7.0 at room temperature. The reactions were conducted at room temperature, and the TON was determined by fluorescence assay.

The screening method described above was also used to screen the protein libraries. The results of the screening assay are depicted in Figure S6.

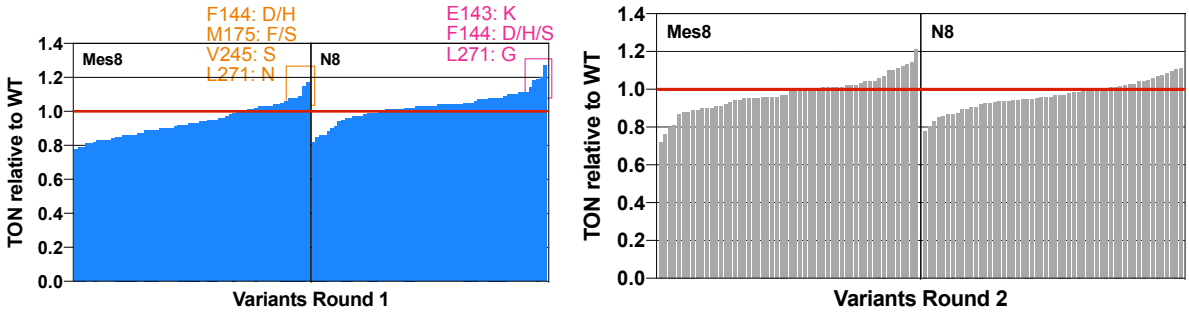


Figure S6. TON of mutants from first (left, blue) and second (right, grey) library screening.

12. Catalytic screening assays for NMR analysis

In 20 mM HEPES buffer at pH 7.5, the cofactor and HT variant were mixed in a 1.5 mL Eppendorf tube to a final concentration of 55 µM and 55 µM, respectively. The bioconjugation solution was shaken at 25 ˚C for 2 h. The bioconjugation was diluted 10-fold with reaction buffer (20 mM MOPS buffer, 100 mM $MgCl_2$, pH 7.0). The metathesis reaction was set up with ArM (2 µM), substrate (100 µM), and reaction buffer were mixed in a 50-mL Falcon tube. The final reaction volume was 15 mL. The reaction was gently shaken in an incubator at 25 ˚C for 21 h. The aqueous phase was extracted with dichloromethane (3 x 10 mL), the combined organic phases were washed with deionized water (2 x 15 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the reaction was analyzed by NMR spectroscopy in $CDCl_3$. The reactions for **BzDa**, **EnDA**, and **TenDA** were scaled up to final reaction volumes of 30 mL, 45 mL, and 150 mL, respectively. Peaks in the ^{1}H-NMR spectra were integrated to determine the TON for each reaction. The reaction spectra are depicted in Figures S7-S10. The substrate and product spectra are provided in the appendix.

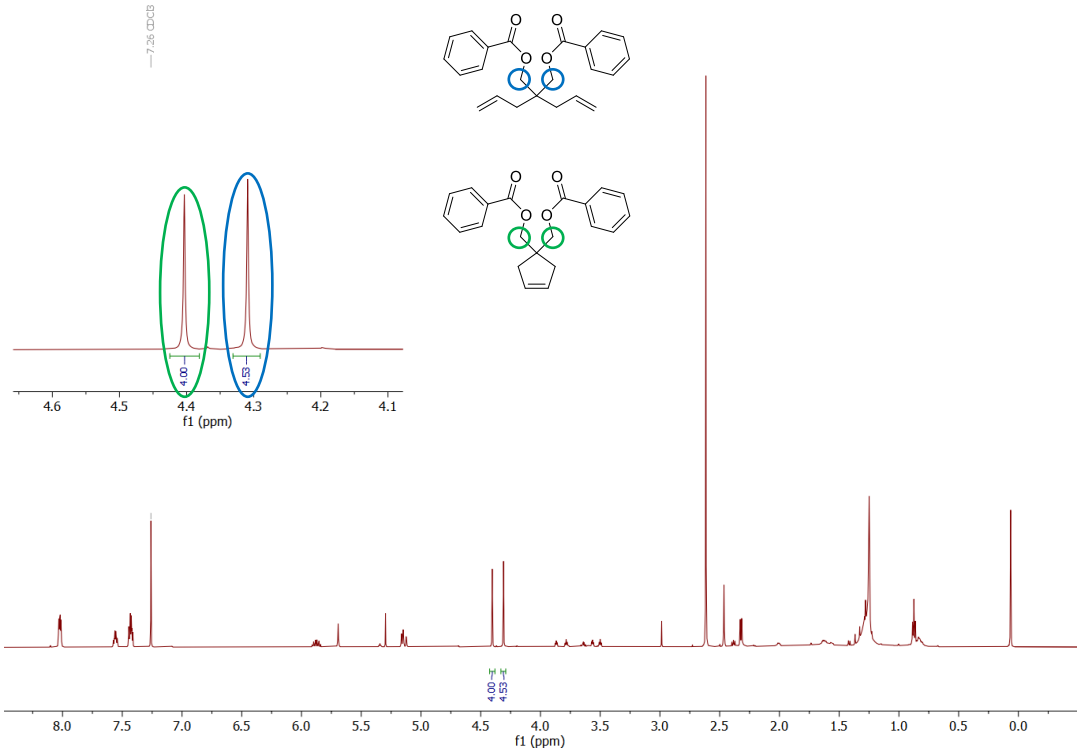


Figure S7. [1]H-NMR spectrum of the larg-scale catalysis reaction of **BzDA**.

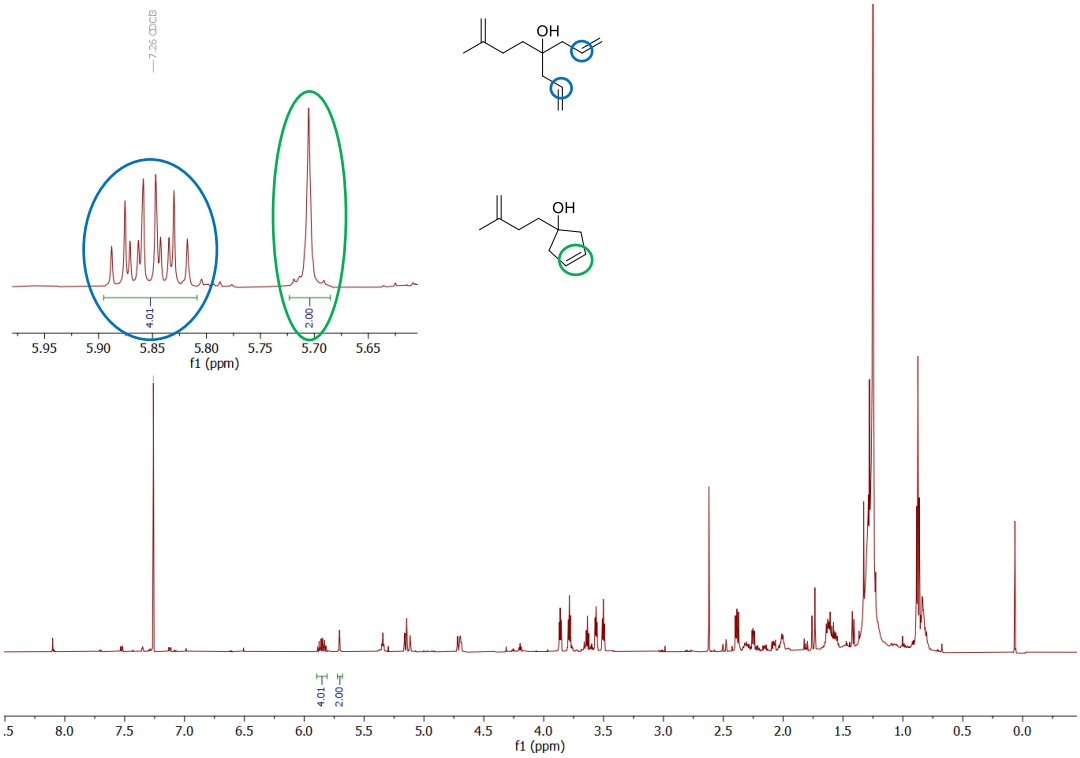


Figure S8. [1]H-NMR spectrum of the large-scale catalysis reaction of **TenDA**.

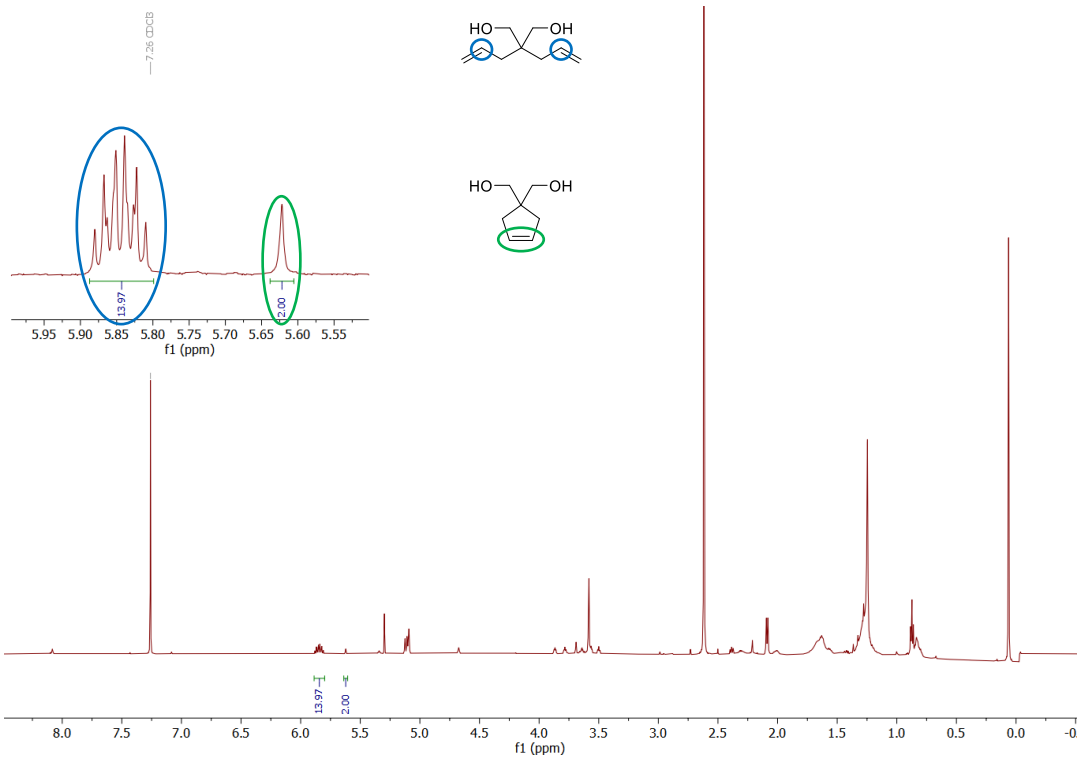


Figure S9. [1]H-NMR spectrum of the large-scale catalysis reaction of **DiolDA**.

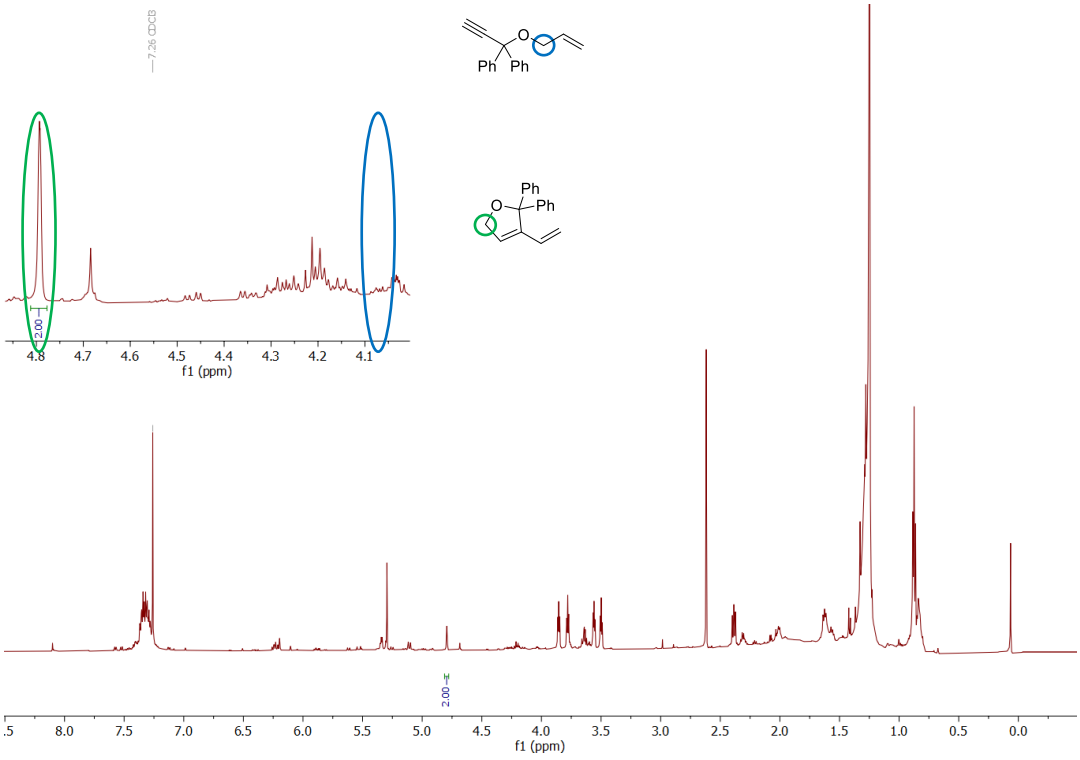


Figure S10. [1]H-NMR spectrum of the large-scale catalysis reaction of **EnDA**.

13. Citations

(1) Eda, S.; Nasibullin, I.; Vong, K.; Kudo, N.; Yoshida, M.; Kurbangalieva, A.; Tanaka, K. Biocompatibility and Therapeutic Potential of Glycosylated Albumin Artificial Metalloenzymes. *Nat. Catal.* **2019**, *2* (9), 780–792. https://doi.org/10.1038/s41929-019-0317-4.

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(3) Kikuchi, H.; Okazaki, K.; Sekiya, M.; Uryu, Y.; Ueda, K.; Katou, Y.; Kurata, S.; Oshima, Y. Synthesis and Innate Immunosuppressive Effect of 1,2-Cyclopentanediol Derivatives. *Eur. J. Med. Chem.* **2011**, *46* (4), 1263–1273. https://doi.org/https://doi.org/10.1016/j.ejmech.2011.01.049.

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(5) Kajetanowicz, A.; Chatterjee, A.; Reuter, R.; Ward, T. R. Biotinylated Metathesis Catalysts : Synthesis and Performance in Ring Closing Metathesis. *Catal. Letters* **2014**, *144*, 373–379. https://doi.org/10.1007/s10562-013-1179-z.

(6) Xu, G.; Gilbertson, S. R. Development of Building Blocks for the Synthesis of N-Heterocyclic Carbene Ligands. *Org. Lett.* **2005**, *7* (21), 4605–4608. https://doi.org/10.1021/OL0516521.

14. Appendix I: NMR spectra

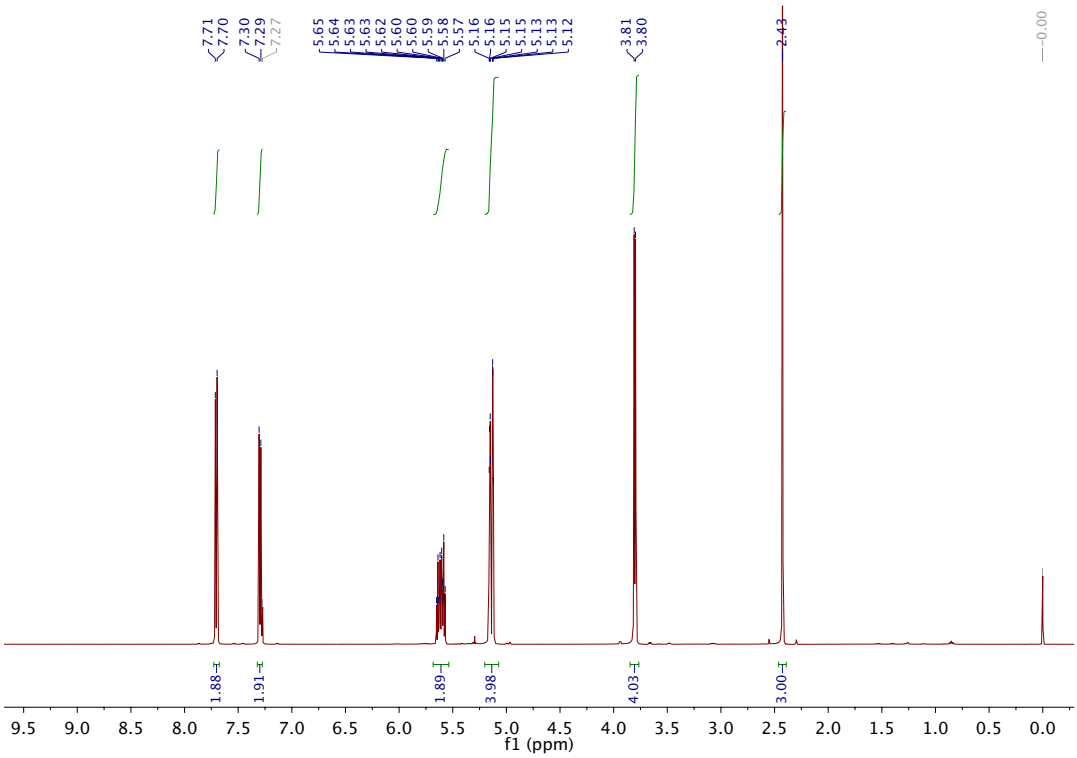

Figure S11. ^{1}H-NMR spectrum of **TsDA** in $CDCl_3$.

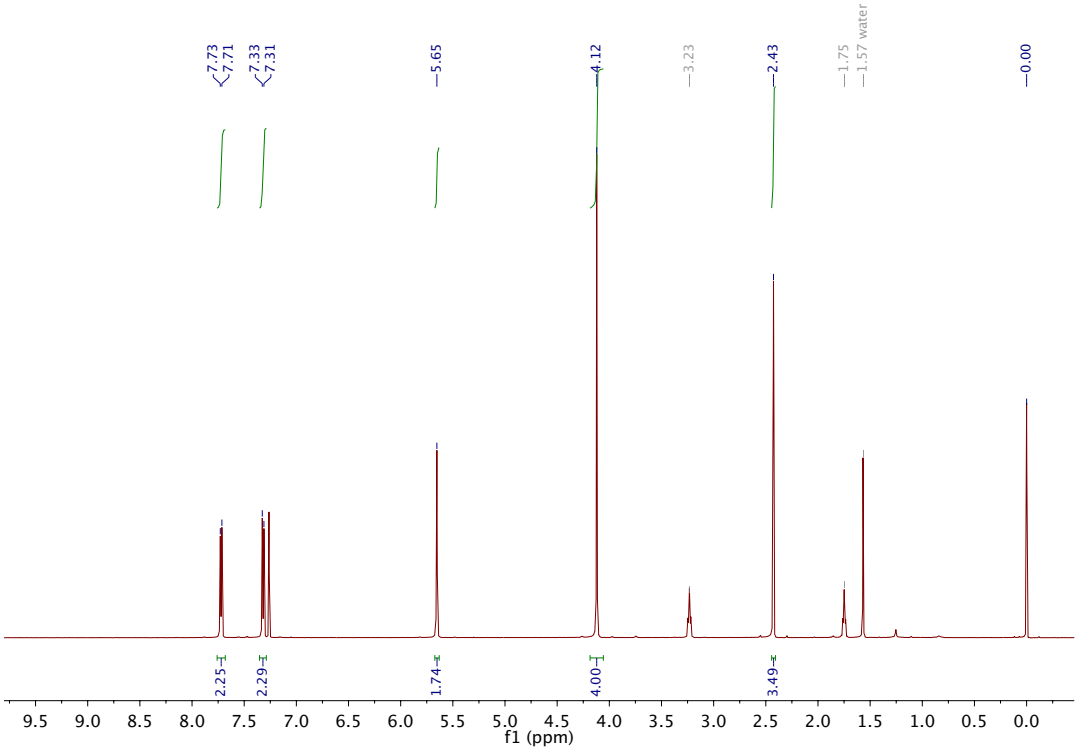

Figure S12. ^{1}H-NMR spectrum of **TsPy** in $CDCl_3$.

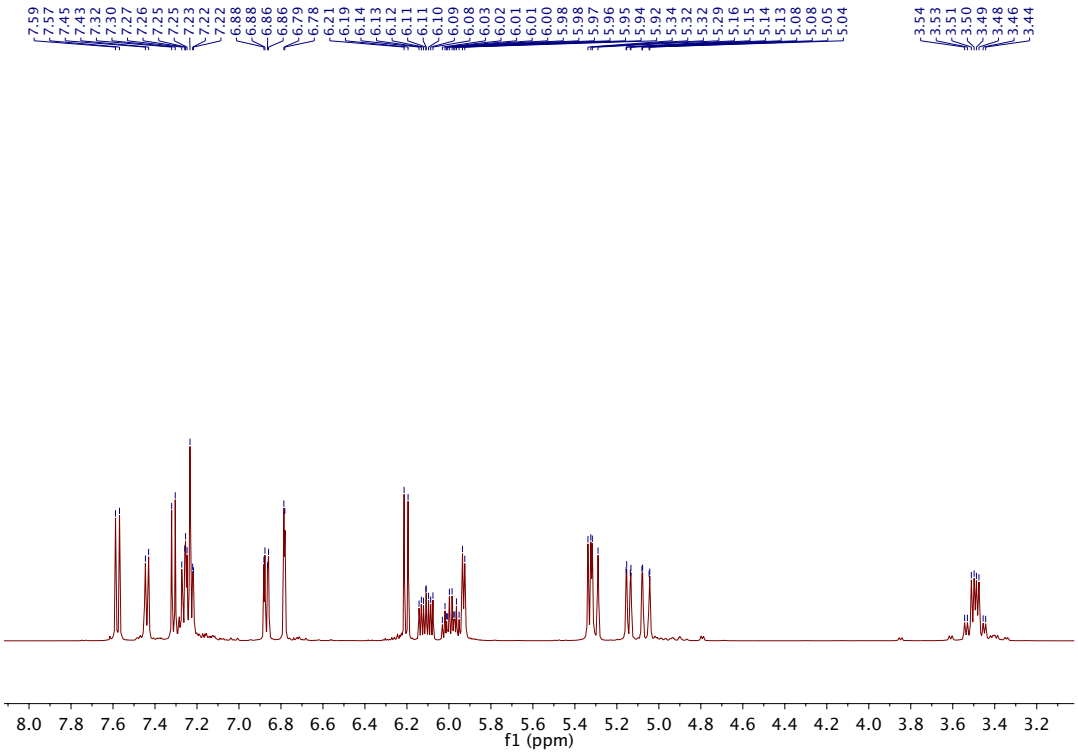

Figure S13. ^{1}H-NMR spectrum of **Np7HC** in $CDCl_3$.

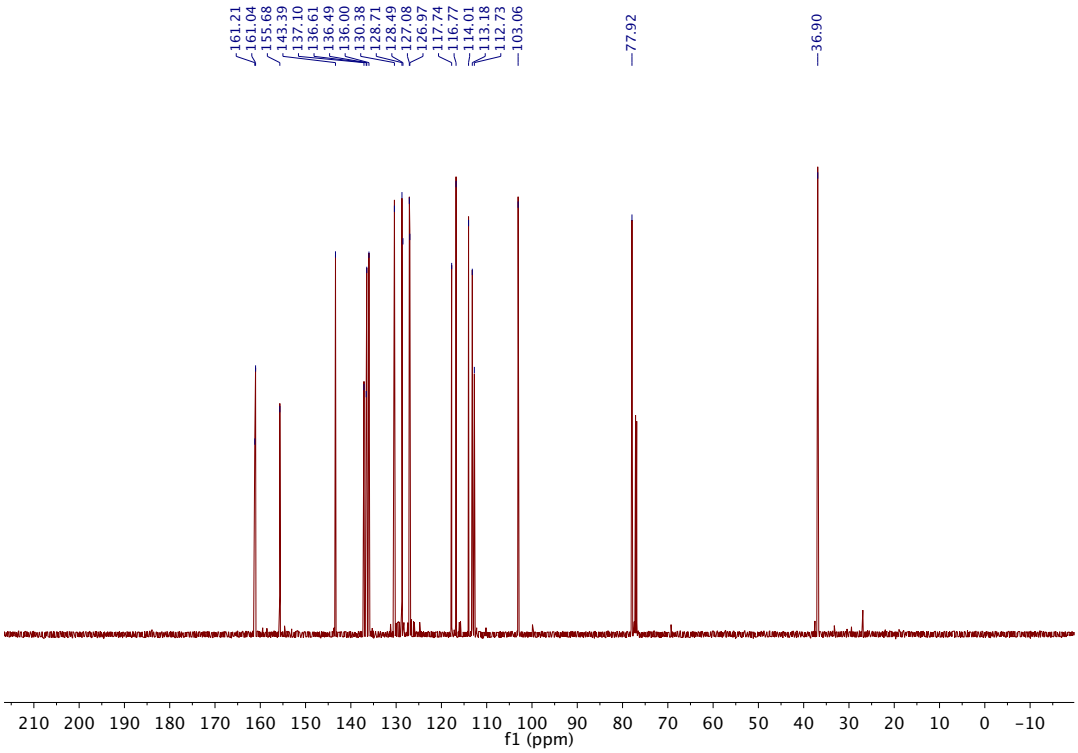

Figure S14. ^{13}C-NMR spectrum of **Np7HC** in $CDCl_3$.

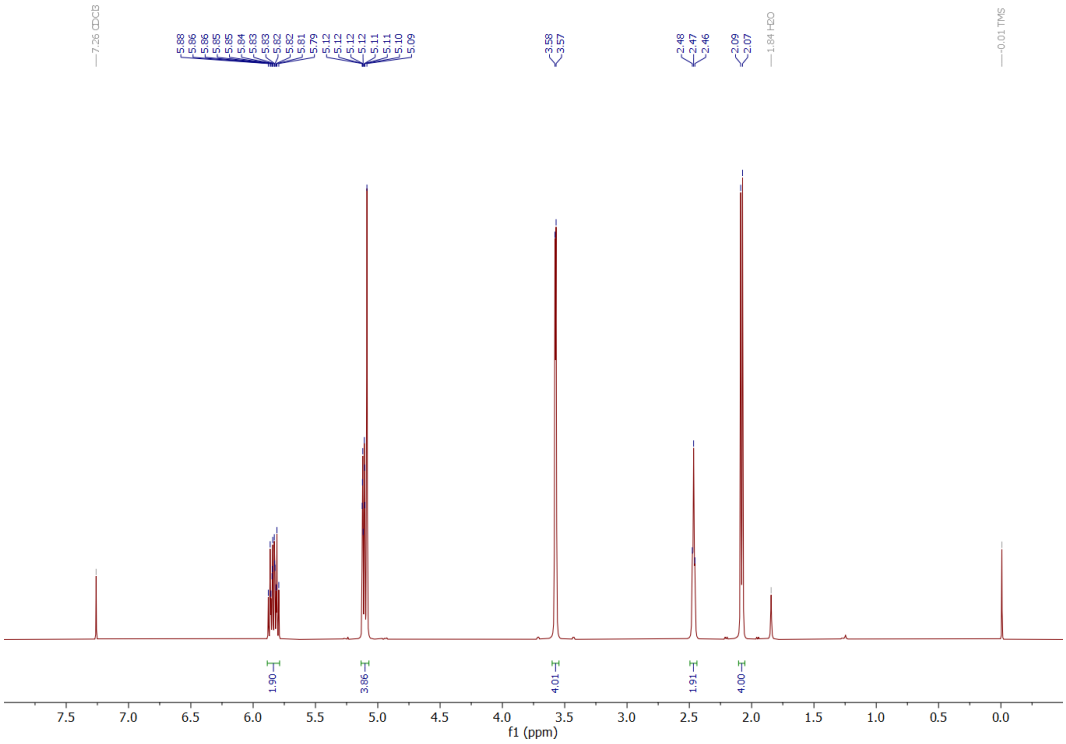

Figure S15. ^{1}H-NMR spectrum of **DiolDA** in $CDCl_3$.

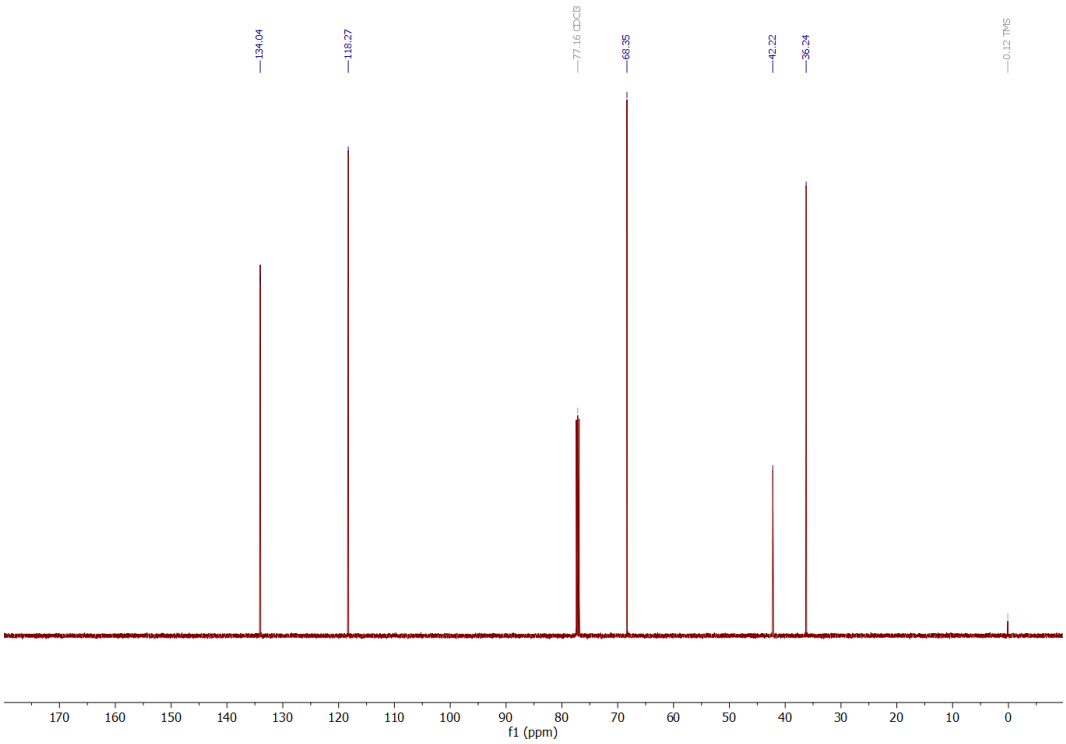

Figure S16. ^{13}C-NMR spectrum of **DiolDA** in $CDCl_3$.

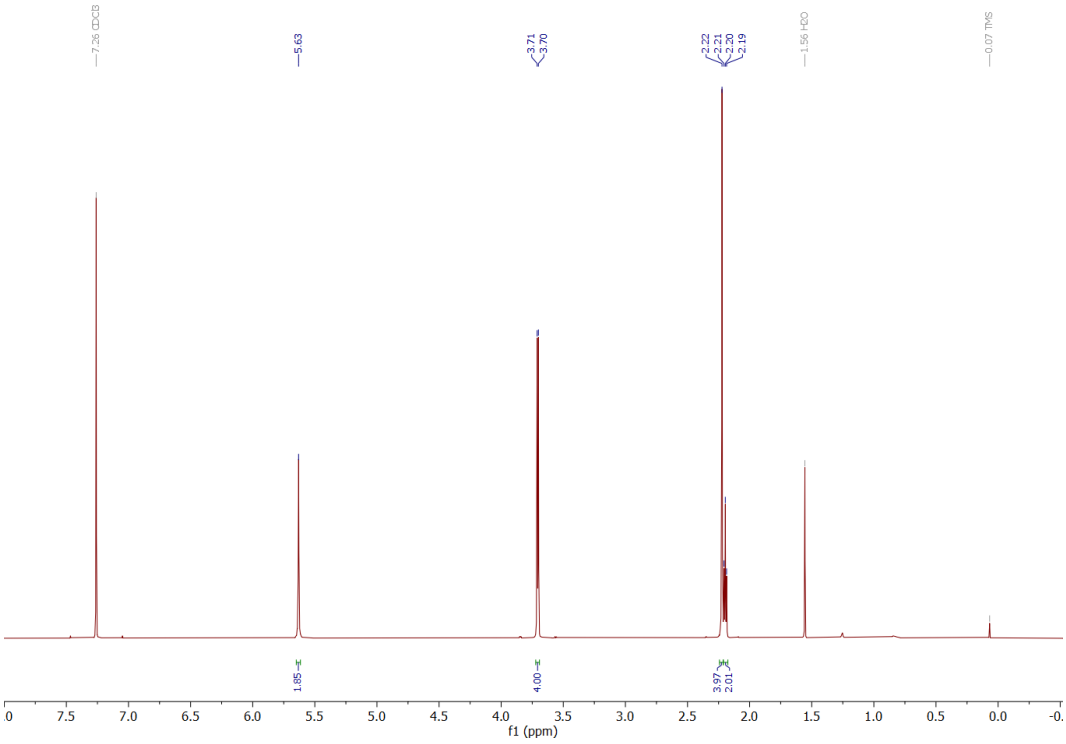

Figure S17. ^{1}H-NMR spectrum of **DiolCy** in $CDCl_3$.

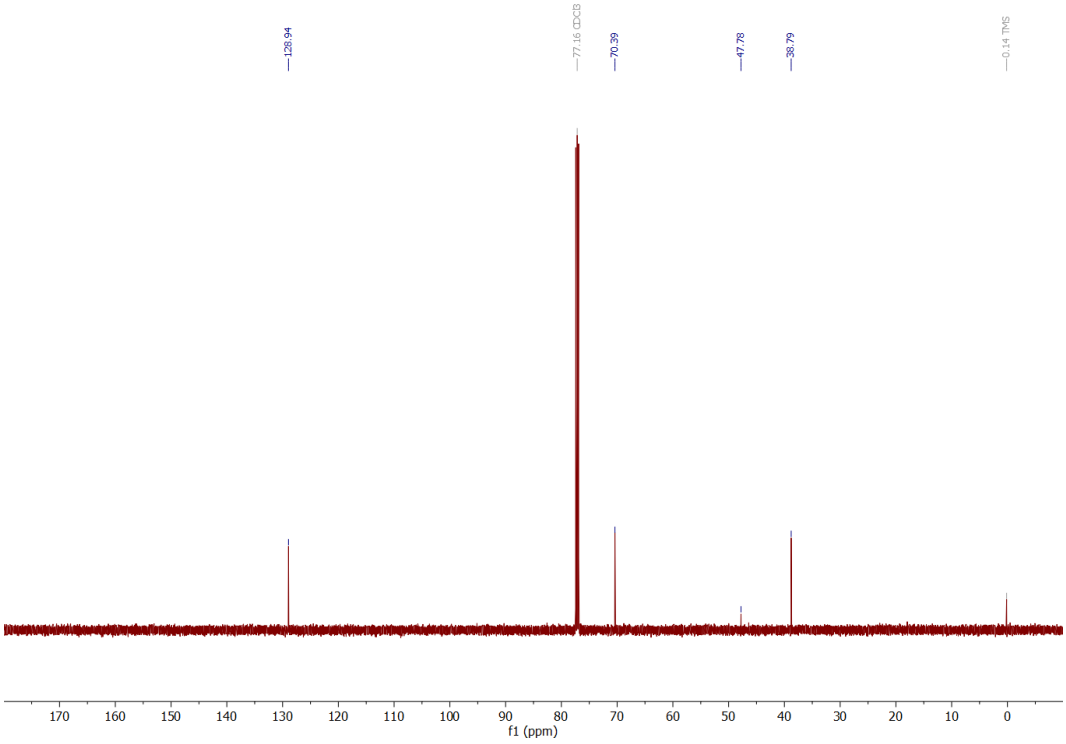

Figure S18. ^{13}C-NMR spectrum of **DiolCy** in $CDCl_3$.

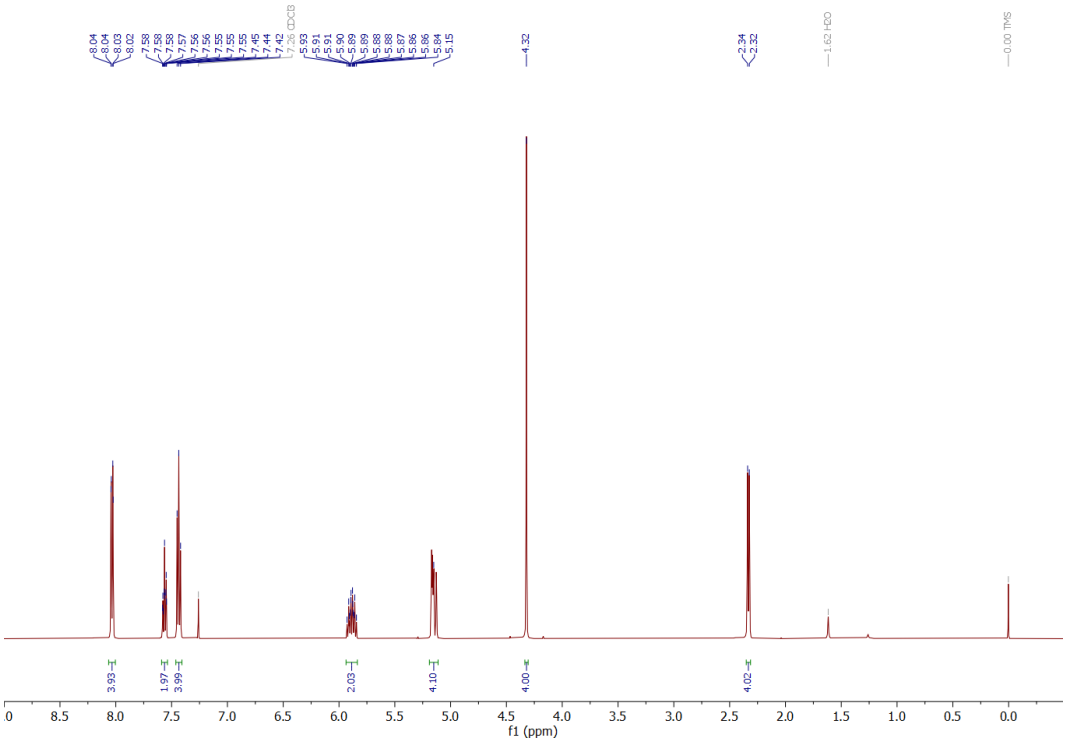

Figure S19. ^{1}H-NMR spectrum of **BzDA** in $CDCl_3$.

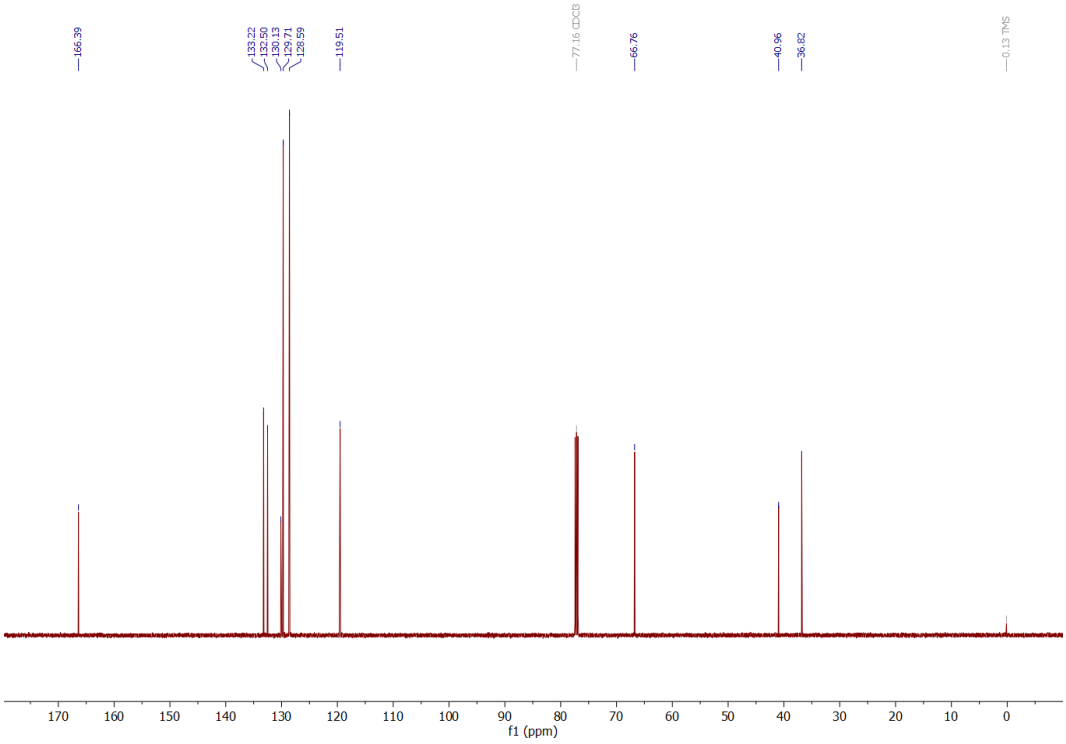

Figure S20. ^{13}C-NMR spectrum of **BzDA** in $CDCl_3$.

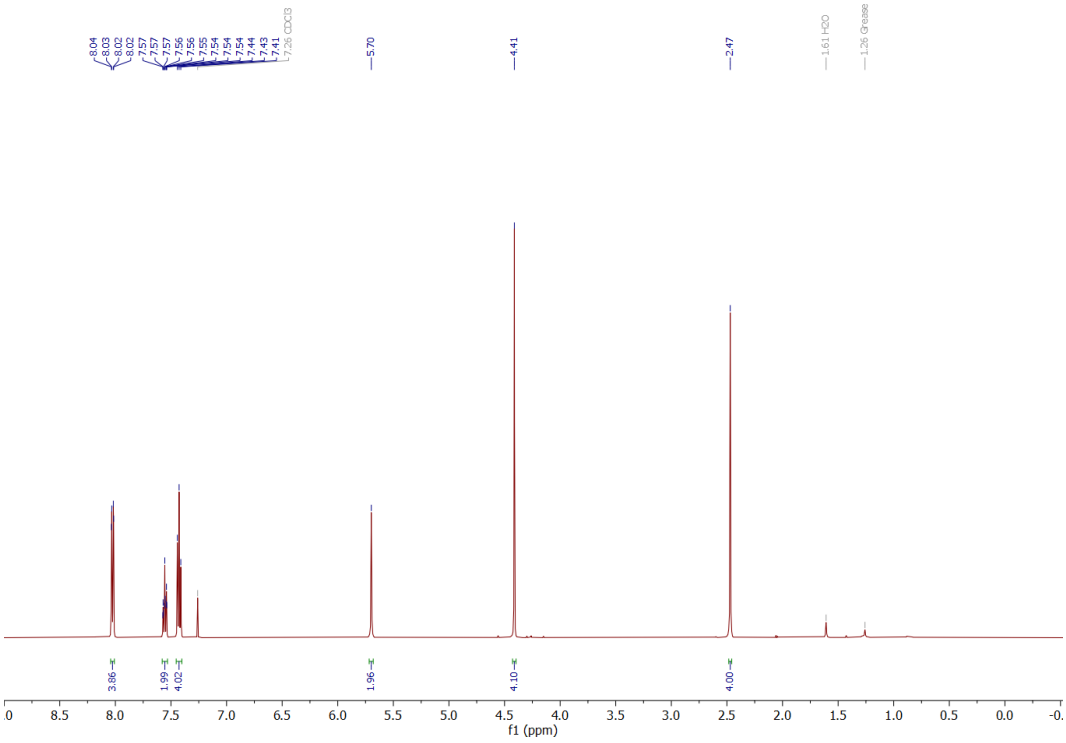

Figure S21. ^{1}H-NMR spectrum of **BzCy** in $CDCl_3$.

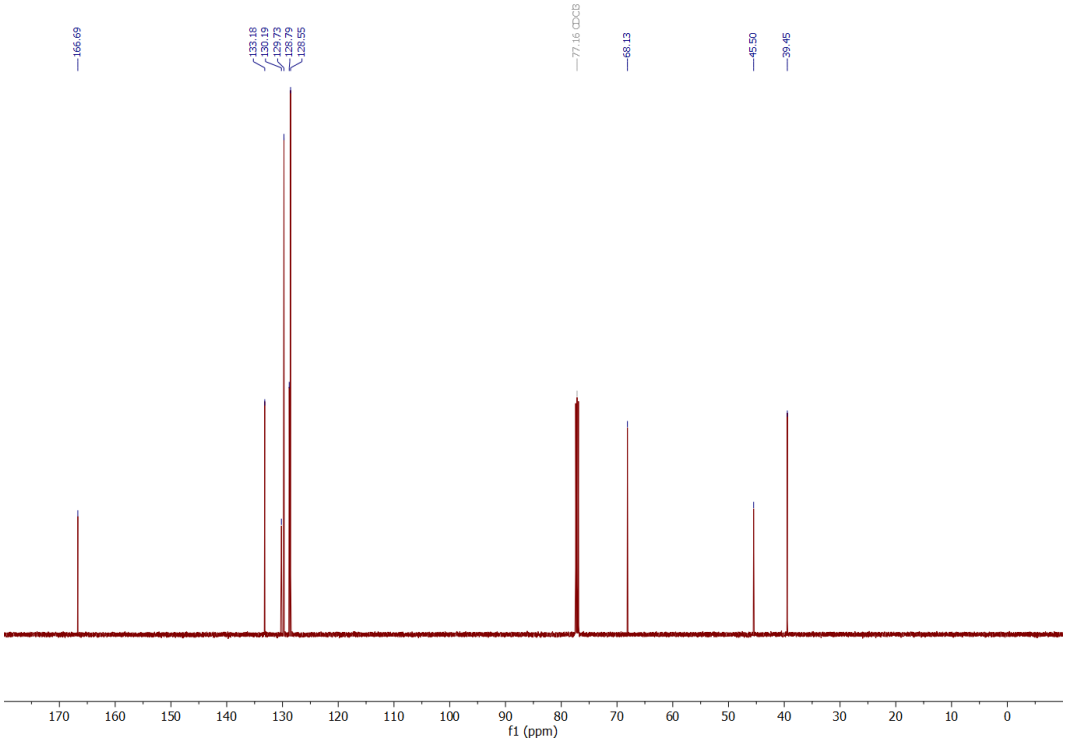

Figure S22. ^{13}C-NMR spectrum of **BzCy** in $CDCl_3$.

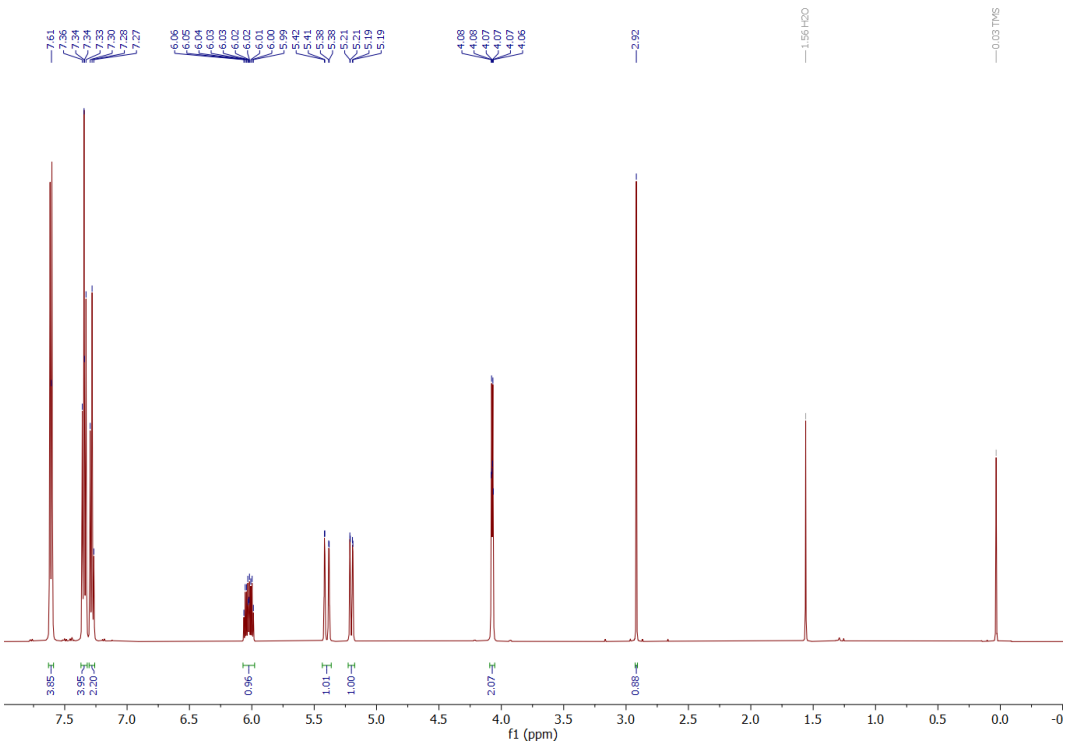

Figure S23. ^{1}H-NMR spectrum of **EnDA** in $CDCl_3$.

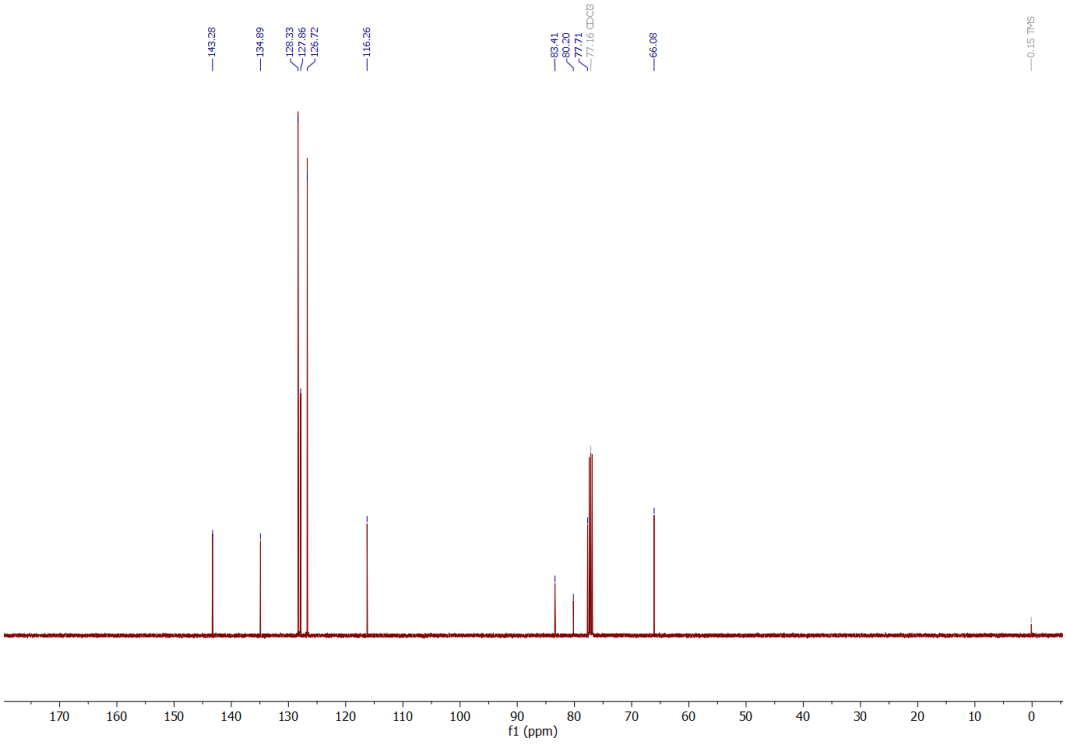

Figure S24. ^{13}C-NMR spectrum of **EnDA** in $CDCl_3$.

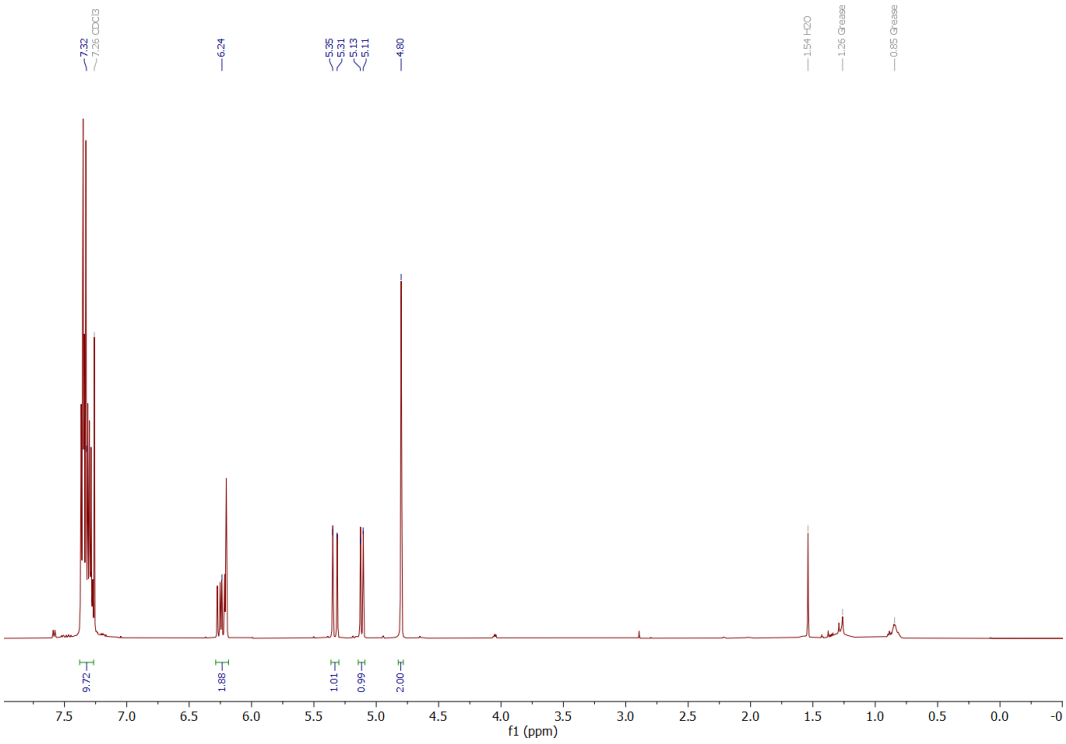

Figure S25. ^{1}H-NMR spectrum of **EnCy** in $CDCl_3$.

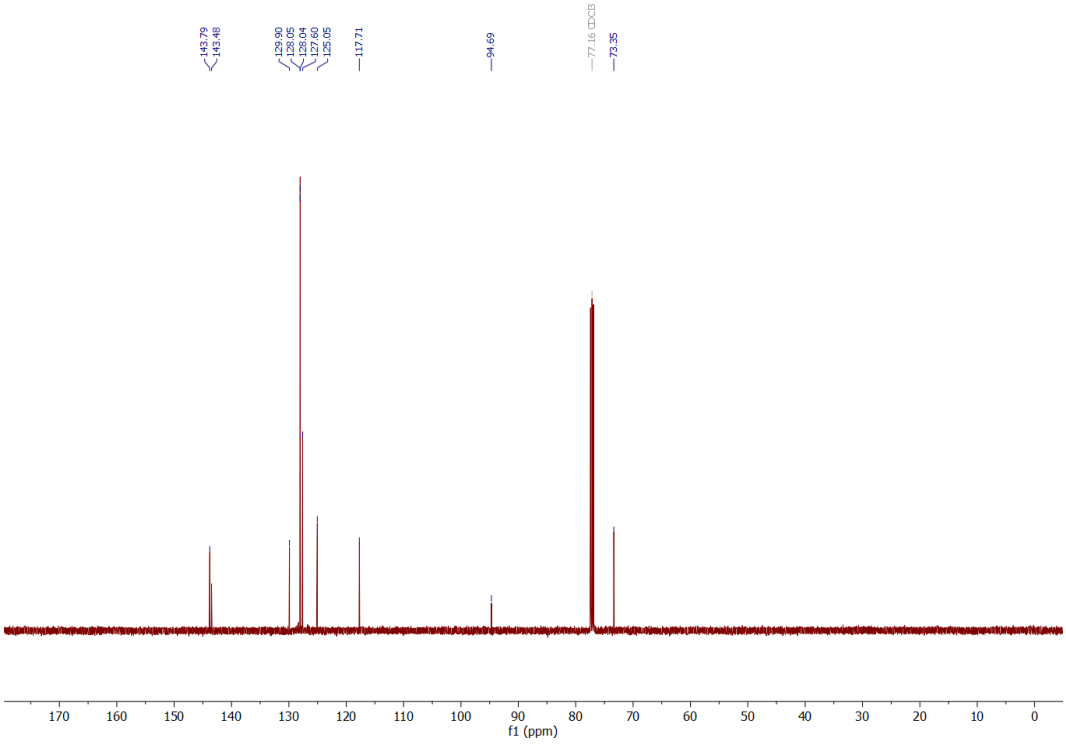

Figure S26. ^{13}C-NMR spectrum of **EnCy** in $CDCl_3$.

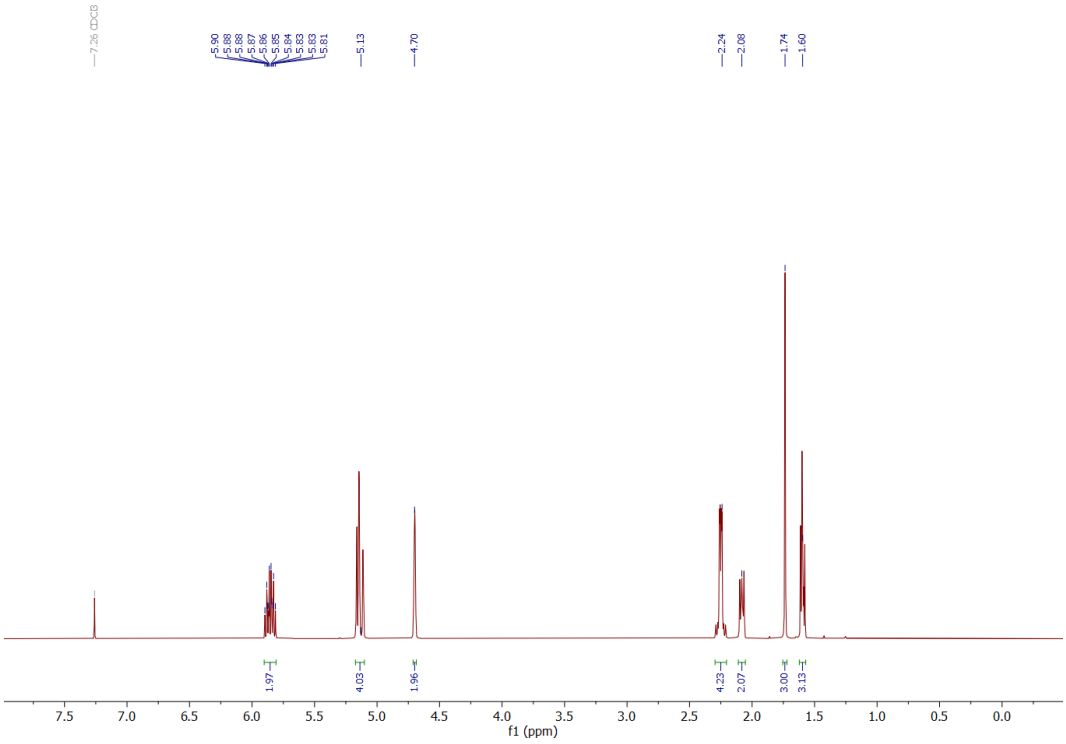

Figure S27. ^{1}H-NMR spectrum of **TenDA** in $CDCl_3$.

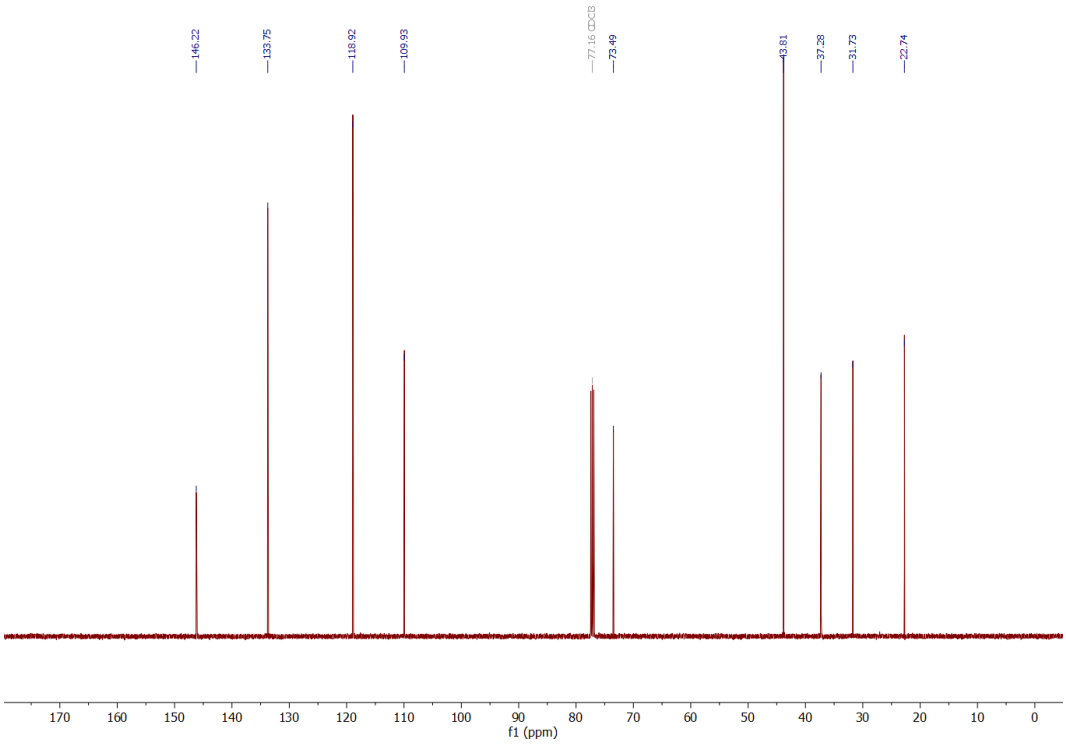

Figure S28. ^{13}C-NMR spectrum of **TenDA** in $CDCl_3$.

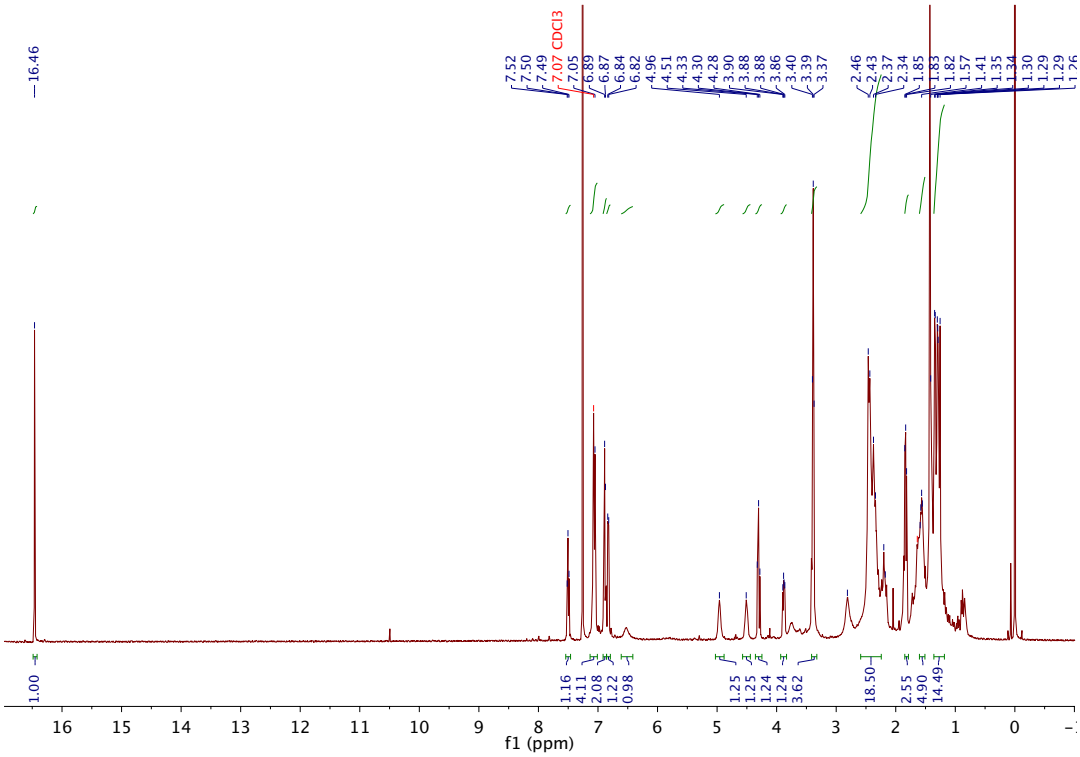

Figure S29. ^{1}H-NMR spectrum of **N8**.

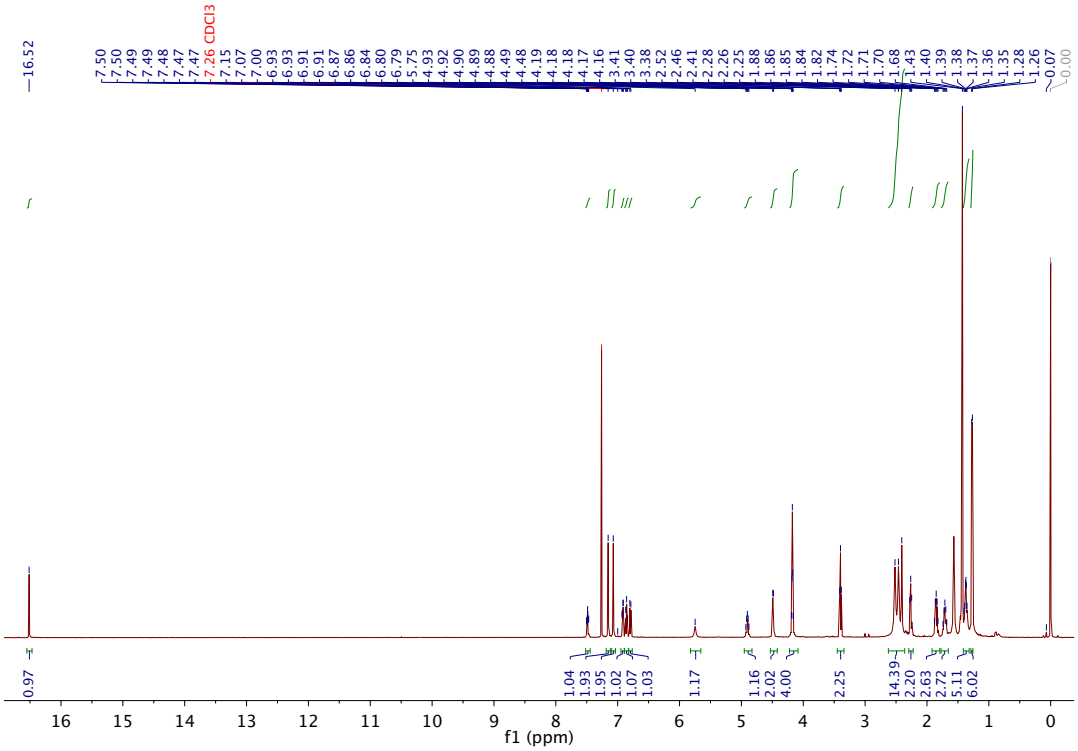

Figure S30. ^{1}H-NMR spectrum of **Mes8**.

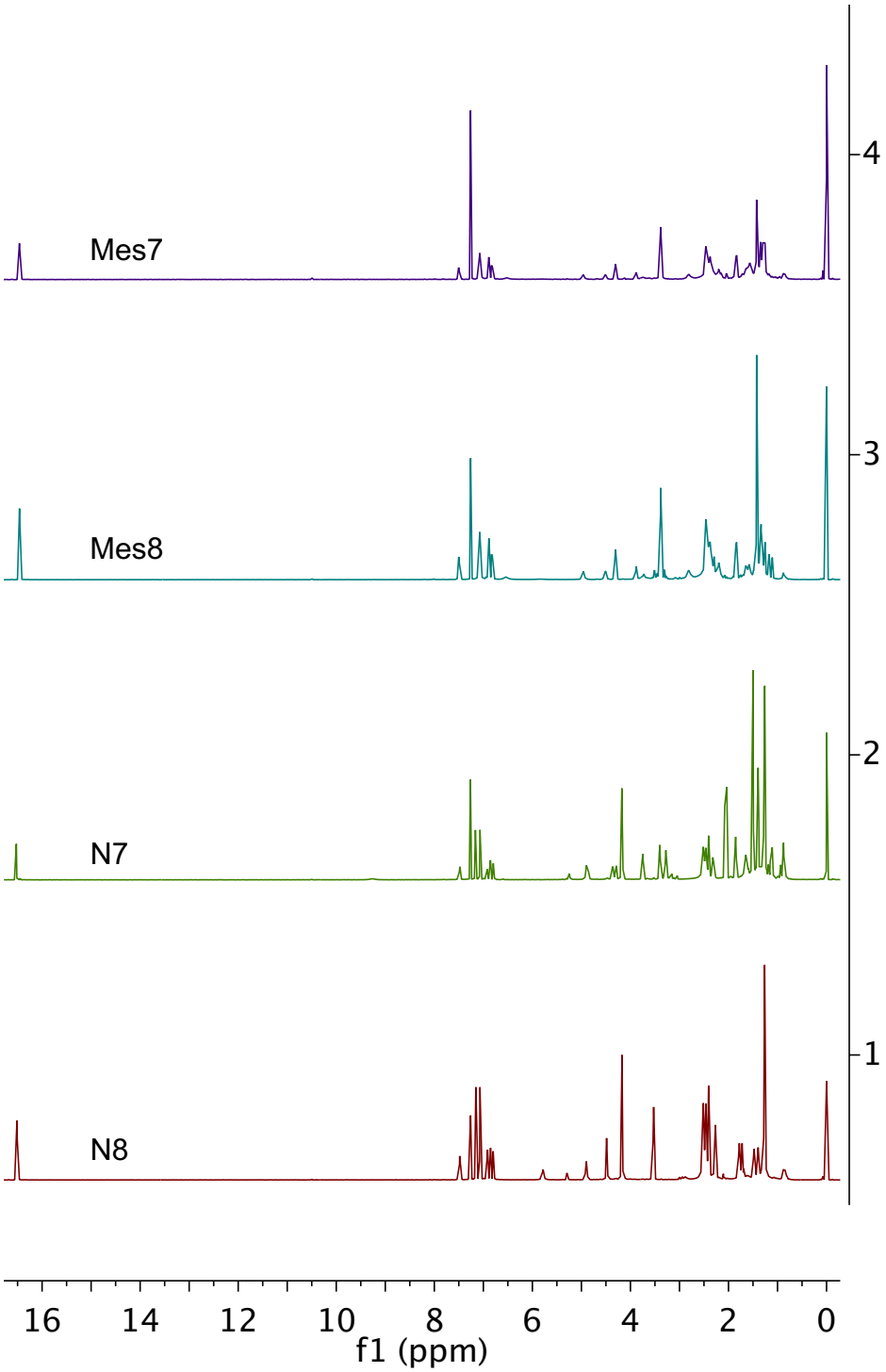

Figure S31. Comparison of ^{1}H-NMR spectra for **Mes7**, **Mes8**, **N7**, and **N8**.