Supporting Information

Strategic Enzymatic Enantioselective Desymmetrization of Prochiral Cyclohexa-2,5-dienones

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I. General details

***General information*:** Unless otherwise noted, all reagents were used as received from commercial suppliers. Surespin plasmid mini kit from Genetixbiotech and GeneJET Gel Extraction Kit from Thermo Fisher Scientific were purchased. Oligonucleotides were synthesized and purchased from Sahagene (India). Nickel(II)-nitrilotriacetic acid (Ni-NTA) agarose was purchased from Merck. All reactions were performed under inert atmosphere and in a flame-dried or oven-dried glassware with magnetic stirring. All solvents were dried before use following the standard procedures. Reactions were monitored using thin-layer chromatography (SiO_2). TLC plates were visualized with UV light (254 nm), iodine treatment or using *p*-anisaldehyde stain or *β*-napthol stain. Column chromatography was carried out using silica gel (100-200 mesh) packed in glass columns. NMR spectra were recorded at 400, 500 MHz (H) and at 100, 125 MHz (C), respectively. Chemical shifts (δ) are reported in ppm, using the residual solvent peak in $CDCl_3$ (H: δ = 7.26 and C: δ = 77.16 ppm) as internal standard, and coupling constants (*J*) are given in Hz. HRMS were recorded using ESI-TOF techniques.

II. Detailed experimental procedures

IIA. Recombinant *B. subtilis* YqjM production

The nucleotide sequence of yqjM gene of B. *subtilis* was obtained from NCBI (NC_000964.3). The yqjM gene was PCR amplified by using genomic DNA isolated from *Bacillus subtilis* strain 168 (ATCC 23857) as DNA template while incorporating appropriate restriction sites, and cloned into NdeI and HindIII restriction sites of pET-28a (+) vector system using conventional restriction-based cloning. The construct was verified by sequence analysis with a N-terminal 6× His-tagged protein, which was transformed into *Escherichia coli* BL21 (DE3) expression system. This allowed over-expression of confirmed clone *B. subtilis* YqjM under the control of isopropyl-1-thio-D-galactopyranoside-inducible (IPTG) T7 promoter. [1]

Gene sequence of *Bacillus subtilis* NADPH dehydrogenase (YqjM), uniprot ID: P54550

ATGGCCAGAAAATTATTTACACCTATTACAATTAAAGATATGACGTTAAAAAACCGCATTG
TCATGTCGCCAATGTGCATGTATTCTTCTCATGAAAAGGACGGAAAATTAACACCGTTCCA
CATGGCACATTACATATCGCGCGCAATCGGCCAGGTCGGACTGATTATTGTAGAGGCGTCA
GCGGTTAACCCTCAAGGACGAATCACTGACCAAGACTTAGGCATTTGGAGCGACGAGCATA

TTGAAGGCTTTGCAAAACTGACTGAGCAGGTCAAAGAACAAGGTTCAAAAATCGGCATTCA
GCTTGCCCATGCCGGACGTAAAGCTGAGCTTGAAGGAGATATCTTCGCTCCATCGGCGATT
GCGTTTGACGAACAATCAGCAACACCTGTAGAAATGTCAGCAGAAAAAGTAAAAGAAACG
GTCCAGGAGTTCAAGCAAGCGGCTGCCCGCGCAAAAGAAGCCGGCTTTGATGTGATTGAA
ATTCATGCGGCGCACGGATATTTAATTCATGAATTTTTGTCTCCGCTTTCCAACCATCGAAC
AGATGAATATGGCGGCTCACCTGAAAACCGCTATCGTTTCTTGAGAGAGATCATTGATGAA
GTCAAACAAGTATGGGACGGTCCTTTATTTGTCCGTGTATCTGCTTCTGACTACACTGATAA
AGGCTTAGACATTGCCGATCACATCGGTTTTGCAAAATGGATGAAGGAGCAGGGTGTTGAC
TTAATTGACTGCAGCTCAGGCGCCCTTGTTCACGCAGACATTAACGTATTCCCTGGCTATCA
GGTCAGCTTCGCTGAGAAAATCCGTGAACAGGCGGACATGGCTACTGGTGCCGTCGGCATG
ATTACAGACGGTTCAATGGCTGAAGAAATTCTGCAAAACGGACGTGCCGACCTCATCTTTA
TCGGCAGAGAGCTTTTGCGGGATCCATTTTTTGCAAGAACTGCTGCGAAACAGCTCAATAC
AGAGATTCCGGCCCCTGTTCAATACGAAAGAGGCTGGTAA

IIB. Expression and purification of *B. subtilis* YqjM (WT)

Recombinant protein was over-expressed and cultured in 2 L Luria-Bertani (LB) media. 0.5 mM IPTG was added for induction and grown at 37 °C for 4 hours in baffled shake flasks. The cell pellet (10 grams) was preserved at -80 °C until further use.

For Ni-NTA affinity chromatography purification, harvested cells were thawed and re-suspended in the lysis buffer A (50 mM Tris, 200 mM NaCl [pH 7.5] in the presence of 1 mg/mL lysozyme, 1% protease cocktail inhibitor). Bacterial cells were lysed by sonication (pulse of 2 sec ON, 5 sec OFF) and 0.01 mg/mL streptomycin sulphate was added, then centrifuged at 38,724 g for 40 minutes at 4 °C. 2M urea was added to the supernatant before loading on to a 5 mL His Ni-NTA column (Sigma). The column was equilibrated with lysis buffer A prior to loading the supernatant. Washed the column with buffer A to remove impurities and eluted with a step gradient of 2%, 5%, 10%, 20%, 50%, and 100% of buffer B (50 mM Tris, 200 mM NaCl [pH 7.5], 500 mM imidazole, pH 8.0). Extra FMN (5mM concentration, 30mM stock) was added to the pure fraction, as determined by monitoring the absorbance of the column eluent at 280 nm followed by SDS-PAGE analysis and were dialyzed against the buffer C (20 mM Tris [pH 7.5], 100 mM NaCl) to remove imidazole and unbound FMN. Pure concentrated proteins were

analyzed on SDS-PAGE for purity (>95% pure). Eluted fractions were pooled and concentrated up to 2 mg/mL, aliquoted 100 µL volumes, flash-frozen in liquid nitrogen and stored at -80 °C. Total yield of the protein after affinity-based purification was 92 mg.[1,2]

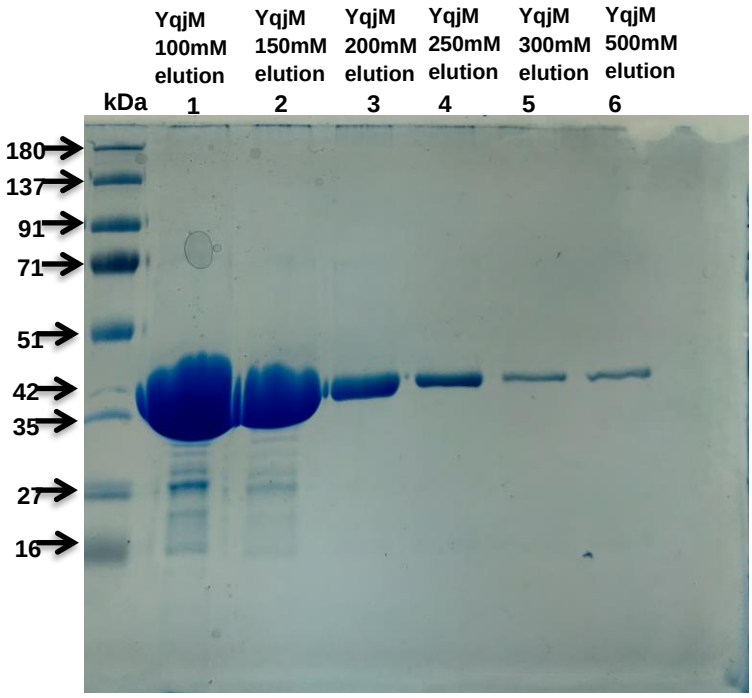
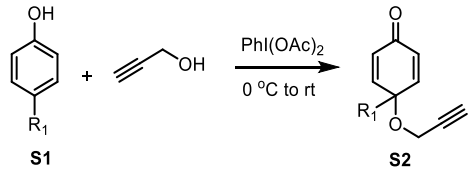


Fig S1. SDS-PAGE analysis of pure *Bs* Yqjm (WT) after Ni-NTA affinity chromatography.

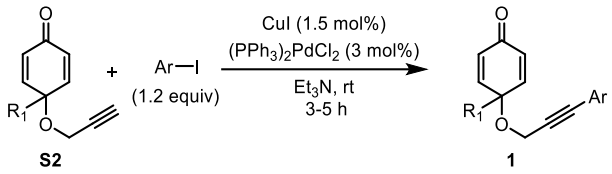
III. Analytical data of substrates

IIIa. General procedure for the dearomatization of phenols:



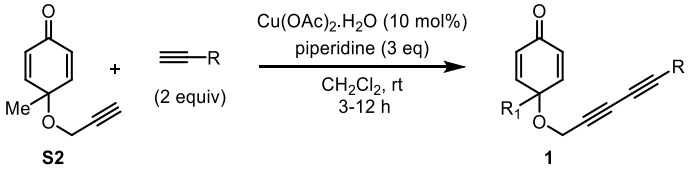
To a solution of the phenol **S1** (1.0 mmol) in 1 mL of propargyl alcohol was added phenyliodine (III) diacetate (1.5 mmol) in many portions at 0 °C. The resulting reaction mixture was stirred at room temperature for overnight. Then the reaction mixture was diluted with water (10 mL) and extracted with ethyl acetate (15 mL × 3). The combined organic solvent was washed with brine (15 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The crude reaction mixture was purified by column chromatography (EtOAc/hexane) to give the desired product **S2**.[3-6]

IIIb. General procedure for the Sonogashira coupling for the synthesis of Cyclohexadienone-tethered alkynes 1:



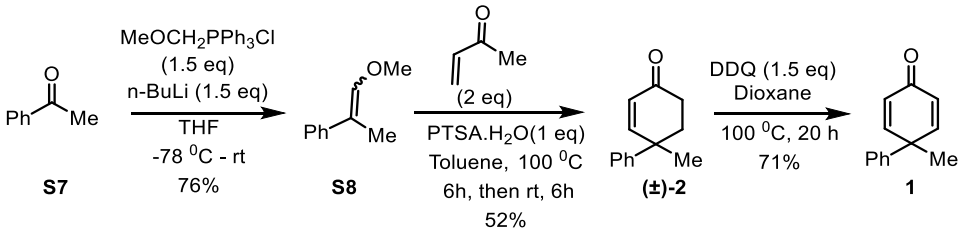
To a solution of *O*-tethered alkyne **S2** (3.0 mmol) in degassed Et_3N (0.5 M, 6 mL) was added $Pd(PPh_3)_2Cl_2$ (21 mg, 1 mol%), CuI (2.8 mg, 0.5 mol%) and aryl iodide (3.6 mmol). The mixture was stirred at room temperature for 3-5 hours. The reaction was cooled to room temperature, water (15 mL) was added, and the mixture was extracted with EtOAc (3 × 10 mL). The combined organic solvent was washed with 10% aqueous HCl (6 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The mixture was purified by column chromatography (EtOAc/hexane) to give aryl substituted alkynes **1** high yields.[7]

IIIc. General procedure for the synthesis of 1,3-diyne-tethered cyclohexadienone 1:



A mixture of alkyne-tethered cyclohexadienone **S2** (3 mmol), terminal alkyne (6 mmol), piperidine (890 µL, 9 mmol), and $Cu(OAc)_2 \cdot H_2O$ (60 mg, 10 mol%) in CH_2Cl_2 (0.5 M, 6 mL) was stirred under open atmospheric air at 25°C for 3–12 h. After completion of reaction (monitored by TLC), the mixture was concentrated in *vacuo* and the residue was purified by flash column chromatography on silica gel to afford 1,3-diyne-tethered cyclohexadienone **1**.[8]

General method for preparation of γ,γ- disubstituted cyclohexadienone (1): [9]



Step-1:

A suspension of (methoxymethyl)triphenylphosphonium chloride (5.14 g, 15 mmol) in THF (100 mL) was cooled to –78 °C and n-BuLi (1.6 M in hexane, 9.4 mL, 15 mmol) was added dropwise. The resulting red suspension was stirred for 30 min at –78 °C, and then for the additional 30 min at room temperature. After re-cooling to –78 °C, a solution of ketone **S7** (10 mmol) in THF (20 mL) was added dropwise and the mixture was stirred at this temperature for 30 min and then warmed up to room temperature. After stirring overnight, the reaction mixture was poured into water and the aqueous phase was extracted with Et_2O. The organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on silica gel afforded the enol-methyl ether compound **S8** in 76% yield.

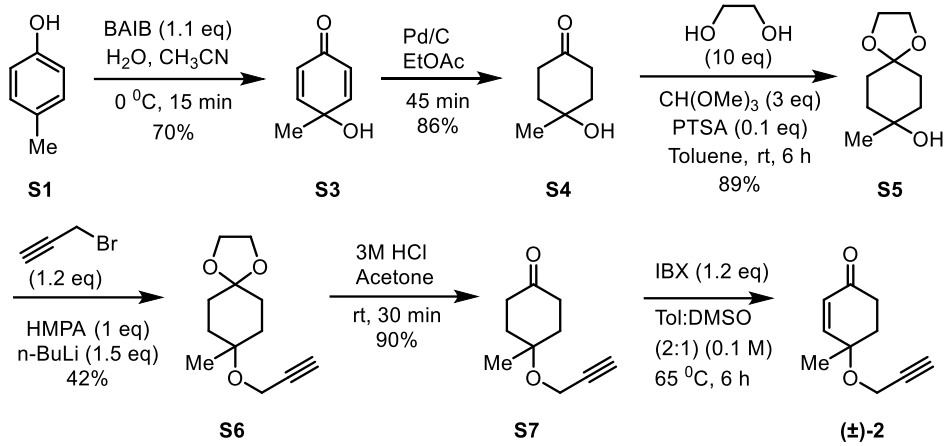
Step-2:

Methyl vinyl ketone (2 eq.) was added to a stirred mixture of compound **S8** (1 eq.) and p-toluenesulfonic acid monohydrate (1 eq.) in toluene, and the resulting mixture was stirred at 100 °C for 6 h and then allowed to cool to room temperature overnight. The mixture was poured into saturated $NaHCO_3$, extracted with EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on silica gel afforded γ,γ-disubstituted cyclohexenone **2**.

Step-3:

The mixture of compound A and DDQ (2 eq.) in 1,4-dioxane was heated at 100 °C for 20 h. The cooled resulting mixture was poured into a 1:1 mixture of ether / hexane and washed with 5% aq NaOH and water. The organic phase dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography on silica gel afforded γ,γ-disubstituted cyclohexadienone **1**.

Preparation of racemic compounds:



Step-1:

A round bottom flask was charged with cresol (5 g, 46.3 mmol, 1 equiv), in CH_3CN (300 mL) and H_2O (126 mL). The reaction mixture was cooled to 0 ^oC and treated portion wise with $PhI(OAc)_2$ (16.4 g, 1.1 equiv). The reaction mixture was stirred at same temperature for another 10 minutes until consumption of starting material **2a** monitored by thin layer chromatography (TLC). Then, diluted with EtOAc (50 mL), washed with 1M $NaHCO_3$ (25 mL). The combined organic solvent was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Then residue was purified by flash column chromatography on silica gel (40% EtOAc/hexanes; R_f = 0.4) to afford product **S3** in 70% yield (4 g) yellowish oils.[10]

Step-2:

A round bottom flask was charged with enone **S3** (3 g, 24.2 mmol, 1 equiv), in EtOAc (30 mL) under inert atmosphere then added 10% Pd-C catalyst (300 mg). The reaction mixture was stirred at room temperature under hydrogen atmosphere for 1 h until consumption of starting material **S3** monitored by thin layer chromatography (TLC). The reaction mixture was filtered through pad of celite, concentrated on reduced pressure, and the residue was directly subjected to flash column chromatography on silica gel (40% EtOAc/hexanes; R_f = 0.4) to afford the desired product **S4** with 86% yield (2.7 g) as colourless oil.[11]

Step-3:

A mixture of ketone **S4** (3 g, 23.4 mmol, 1 equiv), trimethyl orthoformate (7.7 mL, 3 equiv), p-TsOH (403 mg, 0.1 equiv) in ethylene glycol (13.5 mL, 10 equiv) was stirred at r.t. under Ar overnight. The mixture was diluted with sat. $NaHCO_3$ (15 mL), extracted with EtOAc (3X15 mL). The combined

organic layers were washed with brine, dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by chromatography on silica gel (EtOAc/hexanes, 1:9) to afford ketal **S5** in 89% yield (3.5 g) as a colorless oil.

Step-4:

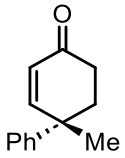
1,4-Dioxaspiro[4.5]decan-8-ol **S5** (3.5 g, 20.3 mmol, 1 equiv) and hexamethylphosphoramide (3.5 mL, 1 equiv) were dissolved in anhydrous THF, and the mixture was stirred at 0 ^oC under argon atmosphere. And then n-BuLi (19 mL, 1.6 M, 1.5 equiv) was added slowly into the reaction mixture over 10 minutes. 3-Bromopropyne (2.3 mL, 1.2 equiv, 80% in toluene) was added after 30 minutes. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was quenched with brine. The phases were separated and the aqueous phase was extracted with EtOAc (3×40 mL). The combined organic layers were dried over Na_2SO_4, filtered, evaporated, the residue was purified by column chromatography to afford **S6** as yellow oil (1.9 g, 46% yield).[12]

Step-5:

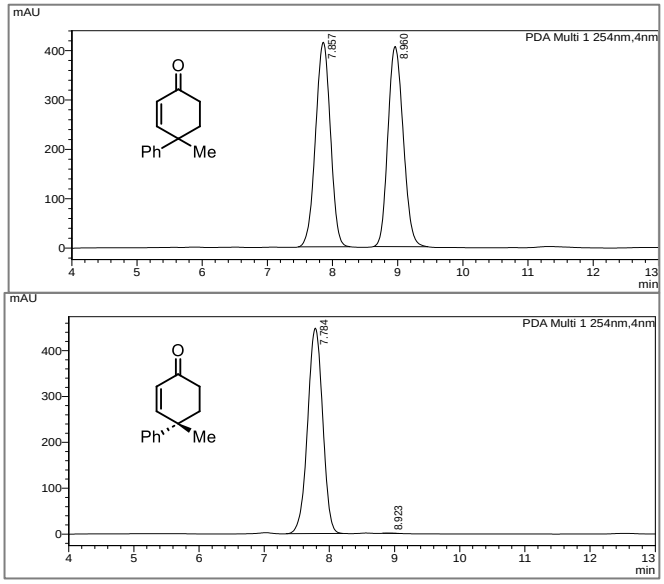
8-Methyl-1,4-dioxaspiro[4.5]decane-8-ol **S6** (1.9 mg, 9 mmol, 1 equiv) was dissolved in THF (20 ml), followed by addition of 3M aqueous HCl solution (5 ml), and then the resulting mixture was stirred at room temperature for 1 hour. The resulting reaction liquid was concentrated under reduced pressure, and then extracted with 10% MeOH/CH_2Cl_2 (20 ml x 5). The organic layer was dried over anhydrous sodium sulfate, followed by filtration and concentration, and then the residue was subjected to column chromatography to afford **S7** as yellow oil (1.35 g, 90% yield).

Compounds **1g-1i** was prepared according to a previously reported procedure.[13]

(*S*)-1-methyl-2,3-dihydro-[1,1'-biphenyl]-4(1*H*)-one (2a):



Prepared according to the general procedure as described above in 92% yield. It was purified by flash chromatography (10% EtOAc/hexanes; R_f = 0.4) to afford a yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.26 (m, 4H), 7.24 – 7.16 (m, 1H), 6.87 (dd, *J* = 10.2, 0.9 Hz, 1H), 6.06 (d, *J* = 10.1 Hz, 1H), 2.40 – 2.30 (m, 1H), 2.28 – 2.16 (m, 2H), 2.12 – 2.03 (m, 1H), 1.50 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.6, 157.2, 145.4, 128.7, 128.7, 126.9, 126.3, 40.7, 38.2, 34.7, 27.7; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{13}H_{15}O$ 187.1123; Found 187.1118; $[\alpha]^{20}_D$ = -70.74° (*c* 0.45, EtOH); >99.7% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak AD-H 250X4.6 mm 5µcolumn; hexane/2-propanol = 97:03, detected at 254 nm, Flow rate = 1 mL/min, Retention times: 7.78 min (major), 8.92 min (minor).



<Peak Table>

PDA Ch1 254nm

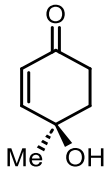
Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.857	6560502	414374	49.784	50.548
2	8.960	6617392	405384	50.216	49.452
Total		13177893	819758	100.000	100.000

<Peak Table>

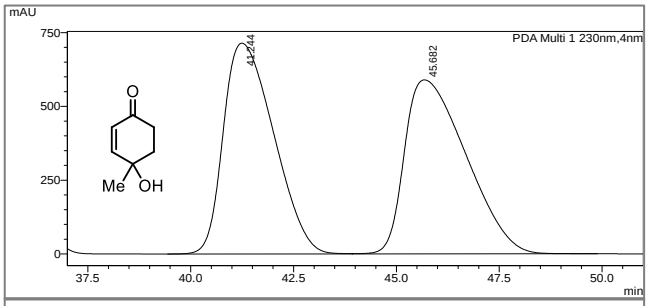
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.784	7183046	447660	99.914	99.867
2	8.923	6190	598	0.086	0.133
Total		7189236	448258	100.000	100.000

4-Hydroxy-4-methylcyclohex-2-en-1-one (2b):



Prepared according to the general procedure as described above in 84% yield. It was purified by flash chromatography (30% EtOAc in hexanes; R_f = 0.5) to afford a dark yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.75 (d, *J* = 10.1 Hz, 1H), 5.84 (d, *J* = 10.2 Hz, 1H), 2.79 (s, 1H), 2.59 (ddd, *J* = 17.2, 6.3, 5.3 Hz, 1H), 2.39 (ddd, *J* = 17.2, 9.3, 5.7 Hz, 1H), 2.15 – 2.04 (m, 2H), 1.43 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 199.5, 155.2, 127.9, 68.5, 37.3, 34.9, 27.2; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_7H_{11}O_2$ 127.0759; Found 127.0750; $[\alpha]^{20}_D$ = -3.33° (*c* 0.1, $CHCl_3$); 40% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µ column; hexane/2-propanol = 96:04, detected at 230 nm, Flow rate = 1 mL/min, Retention times: 41.35 min (major), 46.25 min (minor).



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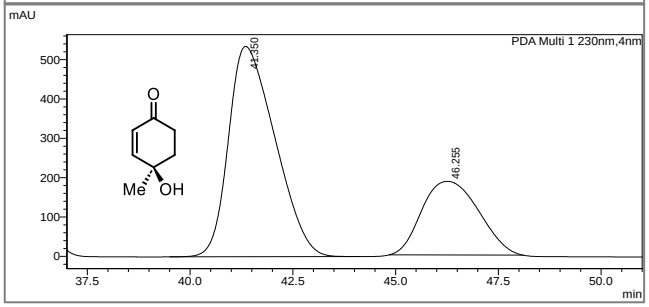
PDA Ch1 230nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	41.244	60768395	714254	49.795	54.777
2	45.682	61269111	589680	50.205	45.223
Total		122037506	1303934	100.000	100.000

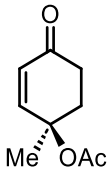


<Peak Table>

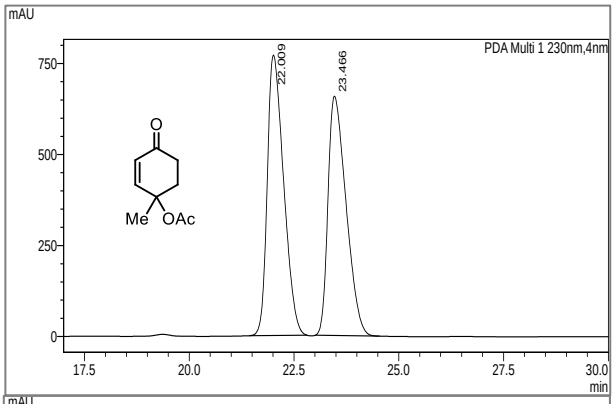
PDA Ch1 230nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	41.350	42076112	534383	70.011	74.056
2	46.255	18022887	187211	29.989	25.944
Total		60098999	721594	100.000	100.000

1-Methyl-4-oxocyclohex-2-en-1-yl acetate (2d):



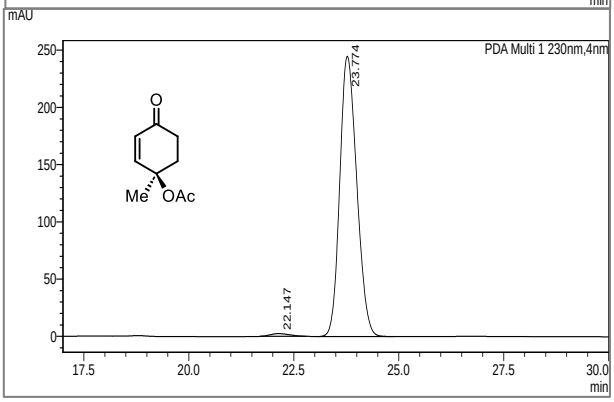
Prepared according to the general procedure as described above in 77% yield. It was purified by flash chromatography (20% EtOAc in hexanes; R_f = 0.4) to afford a dark yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (d, *J* = 10.2 Hz, 1H), 5.92 (d, *J* = 10.2 Hz, 1H), 2.71 – 2.63 (m, 1H), 2.53 – 2.39 (m, 2H), 2.18 – 2.09 (m, 1H), 2.04 – 2.02 (m, 3H), 1.65 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.4, 170.3, 151.1, 128.6, 77.4, 35.1, 34.2, 24.7, 21.9; $[\alpha]^{20}_D$ = -2.67° (*c* 0.15, $CHCl_3$); ~98% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µcolumn; hexane/2-propanol = 90:10, detected at 230 nm, Flow rate = 1 mL/min, Retention times: 22.14 min (minor), 23.77 min (major).



<Peak Table>

PDA Ch1 230nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	22.009	20903015	770404	51.433	53.952
2	23.466	19737957	657538	48.567	46.048
Total		40640972	1427941	100.000	100.000



<Peak Table>

PDA Ch1 230nm

Peak#	Ret. Time	Area	Height	Area%	Height%
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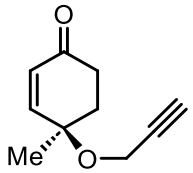
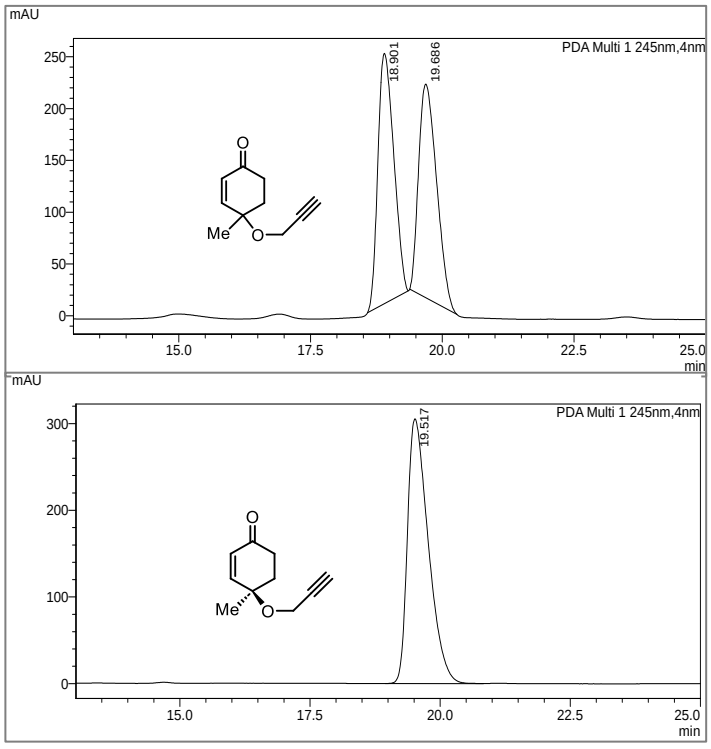
2	23.774	6883194	244739	98.937	99.051
Total		6957156	247083	100.000	100.000

4-Methyl-4-(prop-2-yn-1-yloxy)cyclohex-2-en-1-one (2e):



Prepared according to the general procedure as described above in 89% yield. It was purified by flash chromatography (15% EtOAc/hexanes; R_f = 0.4) to afford a yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.81 (dd, *J* = 10.3, 1.2 Hz, 1H), 5.98 (d, *J* = 10.2 Hz, 1H), 4.16 (d, *J* = 2.4 Hz, 2H), 2.65 (ddd, *J* = 9.7, 6.4, 4.1 Hz, 1H), 2.43 (t, *J* = 2.4 Hz, 1H), 2.45 – 2.32 (m, 2H), 2.02 – 1.96 (m, 1H), 1.46 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 198.6, 152.8, 130.2, 80.8, 74.4, 74.3, 51.6, 34.9, 33.5, 24.4; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{13}O_2$ 165.0916; Found 165.0874; $[\alpha]^{20}_D$ = -47.00° (*c* 0.6, $CHCl_3$); ~100% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µcolumn; hexane/2-propanol = 95:05, detected at 245 nm, Flow rate = 1 mL/min, Retention times: 19.51 min (major).



<Peak Table>

PDA Ch1 245nm

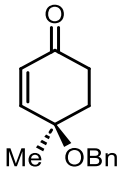
Peak#	Ret. Time	Area	Height	Area%	Height%
1	18.901	5071424	241588	50.767	53.960
2	19.686	4918135	206126	49.233	46.040
Total		9989559	447715	100.000	100.000

<Peak Table>

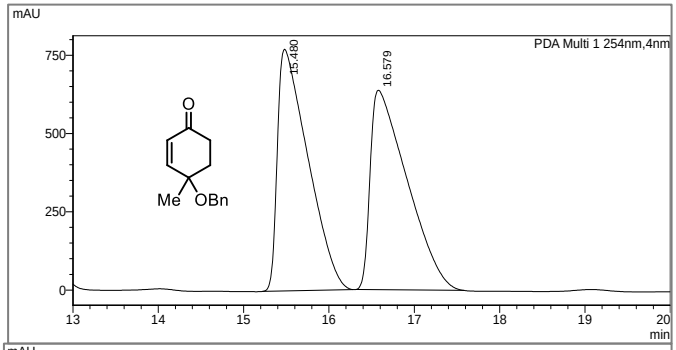
PDA Ch1 245nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	19.517	8515178	305312	100.000	100.000
Total		8515178	305312	100.000	100.000

4-(Benzyloxy)-4-methylcyclohex-2-en-1-one (2f):



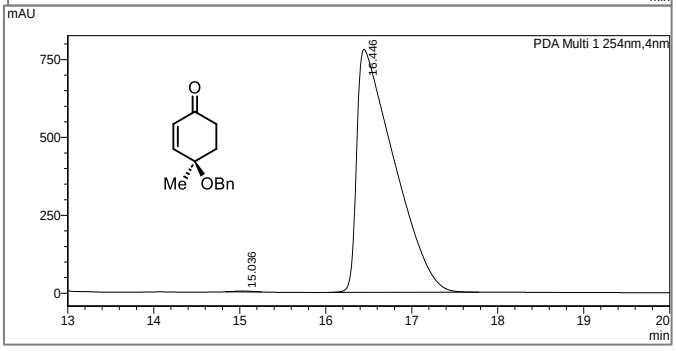
Prepared according to the general procedure as described above in 74% yield. It was purified by flash chromatography (10% EtOAc/hexanes; R_f = 0.4) to afford a yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.27 (m, 5H), 6.88 (dd, *J* = 10.3, 1.2 Hz, 1H), 6.00 (d, *J* = 10.3 Hz, 1H), 4.58 – 4.49 (m, 2H), 2.75 – 2.62 (m, 1H), 2.51 – 2.37 (m, 2H), 2.10 – 2.00 (m, 1H), 1.52 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.0, 154.0, 138.8, 129.9, 128.6, 127.7, 127.5, 73.5, 65.4, 35.0, 33.7, 24.5; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{14}H_{17}O_2$ 217.1229; Found 217.1212; $[\alpha]^{20}_D$ = -72.49° (*c* 0.2, $CHCl_3$); >99.5% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µcolumn; hexane/2-propanol = 95:05, detected at 254 nm, Flow rate = 1 mL/min, Retention times: 15.03 min (minor), 16.44 min (major).



<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	15.480	19164131	772073	49.998	54.805
2	16.579	19165327	636700	50.002	45.195
Total		38329458	1408773	100.000	100.000

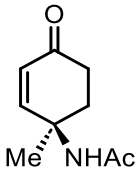


<Peak Table>

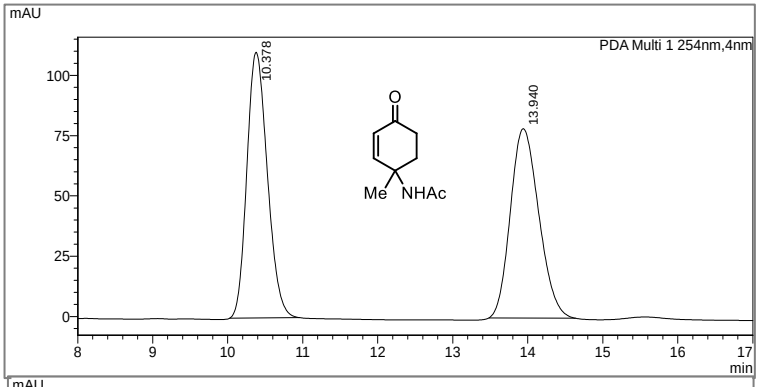
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	15.036	39010	2705	0.165	0.345
2	16.446	23537356	780167	99.835	99.655
Total		23576366	782871	100.000	100.000

N-(1-Methyl-4-oxocyclohex-2-en-1-yl) acetamide (2g):



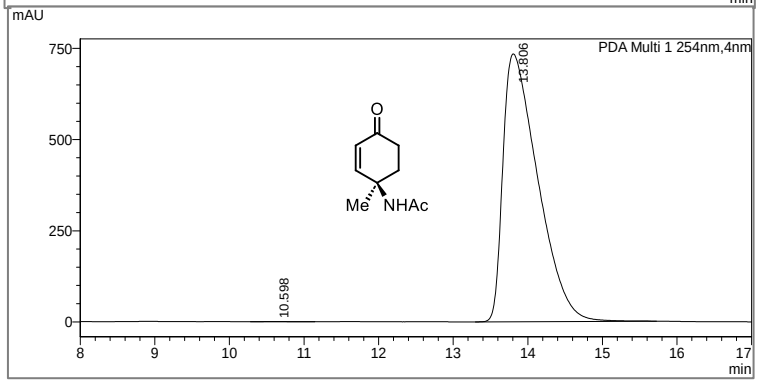
Prepared according to the general procedure as described above in 70% yield. It was purified by flash chromatography (100% EtOAc; R_f = 0.4) to afford a dark yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (dd, *J* = 10.2, 1.2 Hz, 1H), 5.92 (d, *J* = 10.2 Hz, 1H), 5.57 (s, 1H), 2.73 – 2.40 (m, 4H), 1.98 (s, 3H), 1.53 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.1, 169.8, 154.7, 127.7, 52.6, 34.5, 34.4, 25.1, 24.1; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_9H_{13}NO_2Na$ 190.0844; Found 190.0839; $[\alpha]^{20}_D$ = -1° (*c* 0.1, $CHCl_3$); >99.8% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µcolumn; hexane/2-propanol = 60:40, detected at 254 nm, Flow rate = 1 mL/min, Retention times: 10.59 min (minor), 13.80 min (major).



<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	10.378	2092988	110198	50.286	58.395
2	13.940	2069209	78515	49.714	41.605
Total		4162198	188713	100.000	100.000

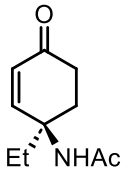


<Peak Table>

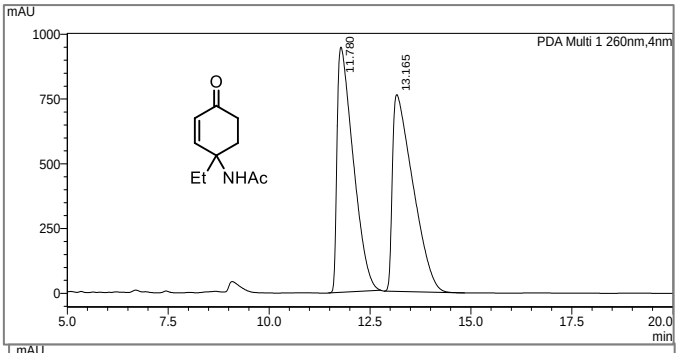
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	10.598	13500	680	0.057	0.092
2	13.806	23849020	734290	99.943	99.908
Total		23862519	734970	100.000	100.000

N-(1-Ethyl-4-oxocyclohex-2-en-1-yl)acetamide (2h):



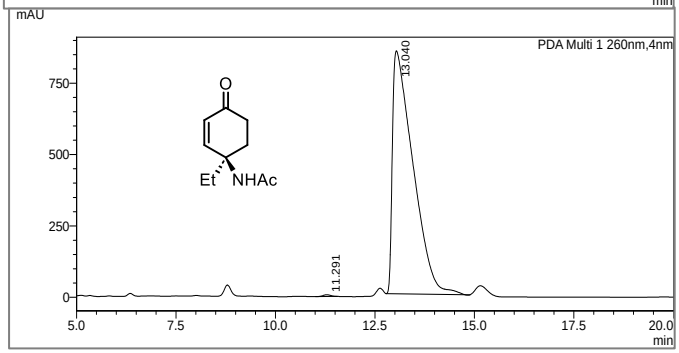
Prepared according to the general procedure as described above in 88% yield. It was purified by flash chromatography (100% EtOAc; R_f = 0.4) to afford a dark yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (d, *J* = 10.3 Hz, 1H), 5.94 (d, *J* = 10.3 Hz, 1H), 5.56 (s, 1H), 2.58 – 2.39 (m, 3H), 2.11 – 2.03 (m, 2H), 1.98 (s, 3H), 1.78 (td, *J* = 14.8, 7.4 Hz, 1H), 0.95 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.4, 170.0, 154.0, 128.1, 55.2, 34.0, 31.7, 29.9, 24.0, 8.2; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{15}NO_2Na$ 204.1000; Found 204.0999; $[\alpha]^{20}_D$ = -30.75° (*c* 0.8, $CHCl_3$); >99.5% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5μ column; hexane/2-propanol = 65:35, detected at 254 nm, Flow rate = 1 mL/min, Retention times: 11.29 min (minor), 13.04 min (major).



<Peak Table>

PDA Ch1 260nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	11.780	26404125	946856	49.521	55.485
2	13.165	26915213	759637	50.479	44.515
Total		53319338	1706493	100.000	100.000

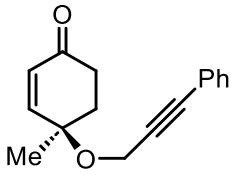


<Peak Table>

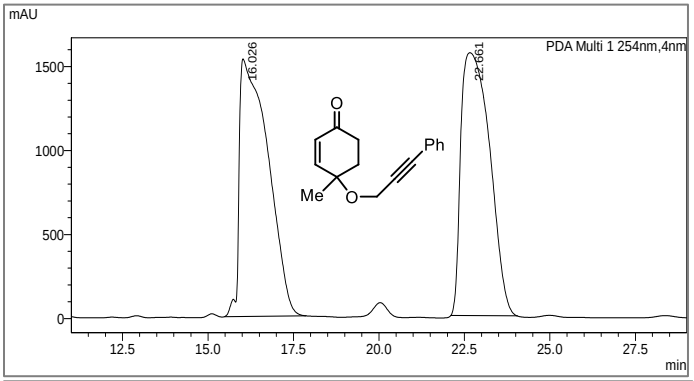
PDA Ch1 260nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	11.291	82668	6327	0.270	0.738
2	13.040	30567043	850670	99.730	99.262
Total		30649711	856997	100.000	100.000

4-Methyl-4-((3-phenylprop-2-yn-1-yl)oxy)cyclohex-2-en-1-one (2j):



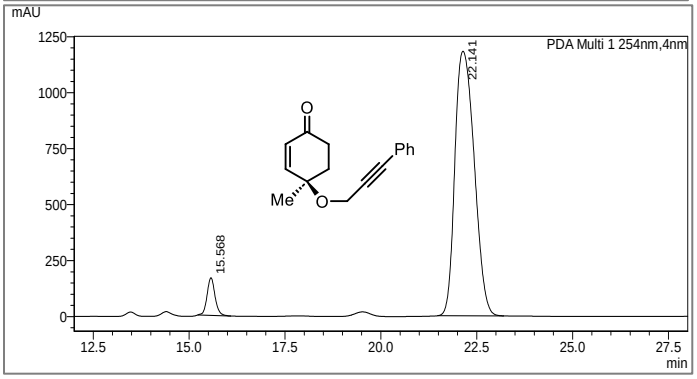
Prepared according to the general procedure as described above in 87% yield. It was purified by flash chromatography (15% EtOAc/hexanes; R_f = 0.4) to afford a yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.40 (m, 2H), 7.34 – 7.27 (m, 3H), 6.89 (dd, *J* = 10.3, 1.2 Hz, 1H), 6.01 (d, *J* = 10.3 Hz, 1H), 4.40 (s, 2H), 2.74 – 2.64 (m, 1H), 2.50 – 2.39 (m, 2H), 2.06 – 2.00 (m, 1H), 1.51 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.8, 153.2, 131.8, 130.1, 128.7, 128.4, 122.6, 86.1, 86.0, 74.3, 52.4, 35.0, 33.5, 24.5; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{16}H_{17}O_2$ 241.1229; Found 241.1220; $[\alpha]^{20}_D$ = -96.57° (*c* 1.2, $CHCl_3$); 90% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µcolumn; hexane/2-propanol = 95:05, detected at 254 nm, Flow rate = 1 mL/min, Retention times: 16.29 min (minor), 22.49 min (major).



<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	16.026	89506064	1533538	49.335	49.479
2	22.661	91919664	1565834	50.665	50.521
Total		181425729	3099372	100.000	100.000

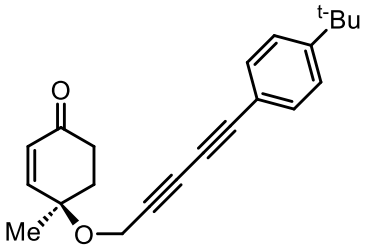


<Peak Table>

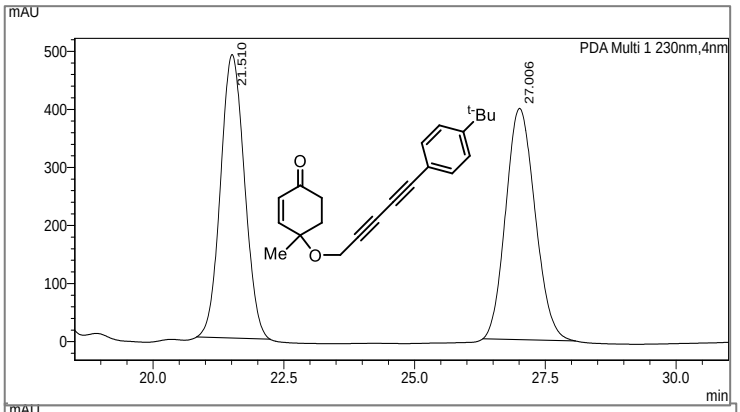
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	15.568	2409542	167260	5.489	12.402
2	22.141	41484152	1181413	94.511	87.598
Total		43893694	1348673	100.000	100.000

4-((5-(4-(Tert-butyl)phenyl)penta-2,4-diyn-1-yl)oxy)-4-methylcyclohex-2-en-1-one (2n):



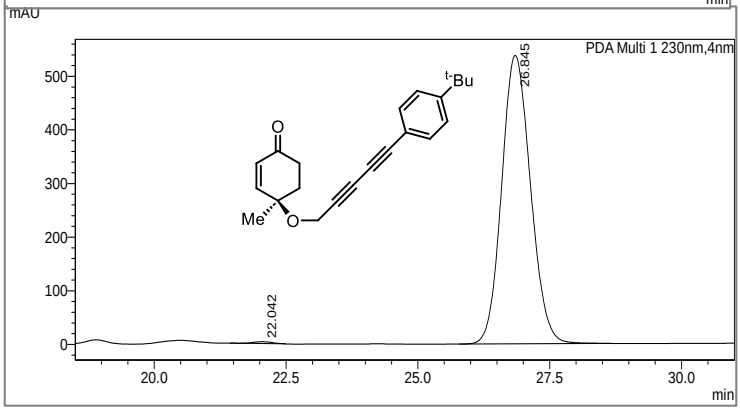
Prepared according to the general procedure as described above in 94% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 – 7.41 (m, 2H), 7.36 – 7.32 (m, 2H), 6.83 (dd, *J* = 10.3, 1.2 Hz, 1H), 6.01 (d, *J* = 10.3 Hz, 1H), 4.31 (s, 2H), 2.68 (ddd, *J* = 9.8, 6.4, 3.9 Hz, 1H), 2.48 – 2.36 (m, 2H), 2.04 – 2.00 (m, 1H), 1.48 (s, 3H), 1.30 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 198.6, 153.0, 152.7, 132.5, 130.3, 125.6, 118.4, 79.1, 78.9, 74.6, 72.8, 71.0, 52.4, 35.1, 34.9, 33.6, 31.2, 24.4; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{22}H_{25}O_2$ 321.1855; Found 321.1855; $[\alpha]^{20}_D$ = -19.20° (*c* 0.5, $CHCl_3$); ~99% *ee*; Chiral HPLC analysis of the product: Daicel Chiralpak IC 250X4.6 mm 5µcolumn; hexane/2-propanol = 93:07, detected at 230 nm, Flow rate = 1 mL/min, Retention times: 22.04 min (minor), 26.84 min (major).



<Peak Table>

PDA Ch1 230nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	21.510	15544099	488309	49.942	55.063
2	27.006	15580215	398513	50.058	44.937
Total		31124315	886821	100.000	100.000

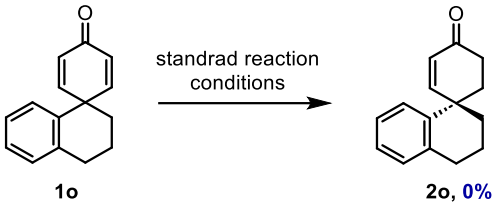


<Peak Table>

PDA Ch1 230nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	22.042	88462	3418	0.430	0.631
2	26.845	20477672	537946	99.570	99.369
Total		20566134	541364	100.000	100.000

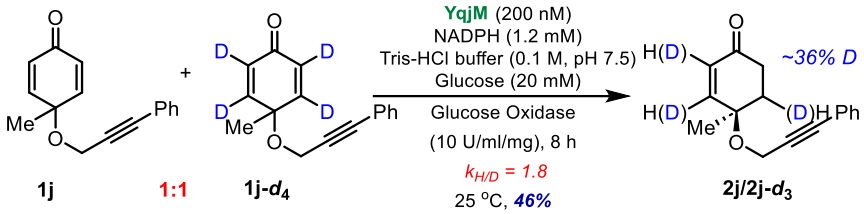
3',4'-Dihydro-2'H-spiro[cyclohexane-1,1'-naphthalen]-2-en-4-one (2o):



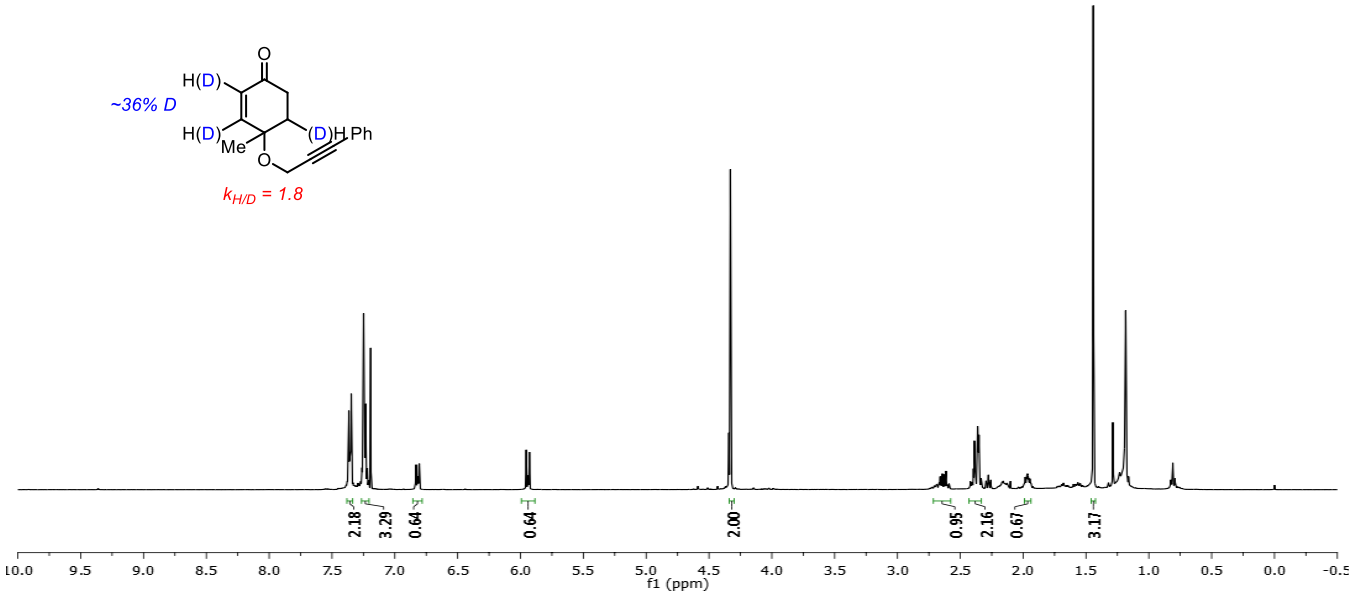
We carried out experiment on compound **1o** in the presence YqjM (WT) enzyme. The assay reaction was performed by dissolving the substrate in ethanol (1 mM) and added to a Tris buffer solution (0.1 M, pH 7.5). To this, we have added glucose (20 mM), NADPH (1.2 mM), glucose oxidase (10 $Umg^{-1}mL^{-1}$). The reaction was initiated after addition of purified enzymes at 200 nM. The reaction mix was incubated for 12 h in an orbital shaker (100 rpm, 25°C). However, The reaction did not proceed and most of the starting material was recovered.

IV. Deuterium Labeling Experiments.

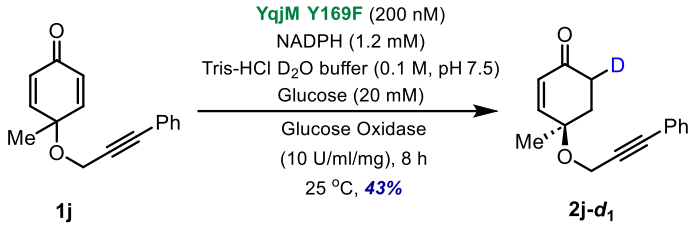
IVa. Intermolecular competition experiment between compound 1j and 1j-*d*$_4$:



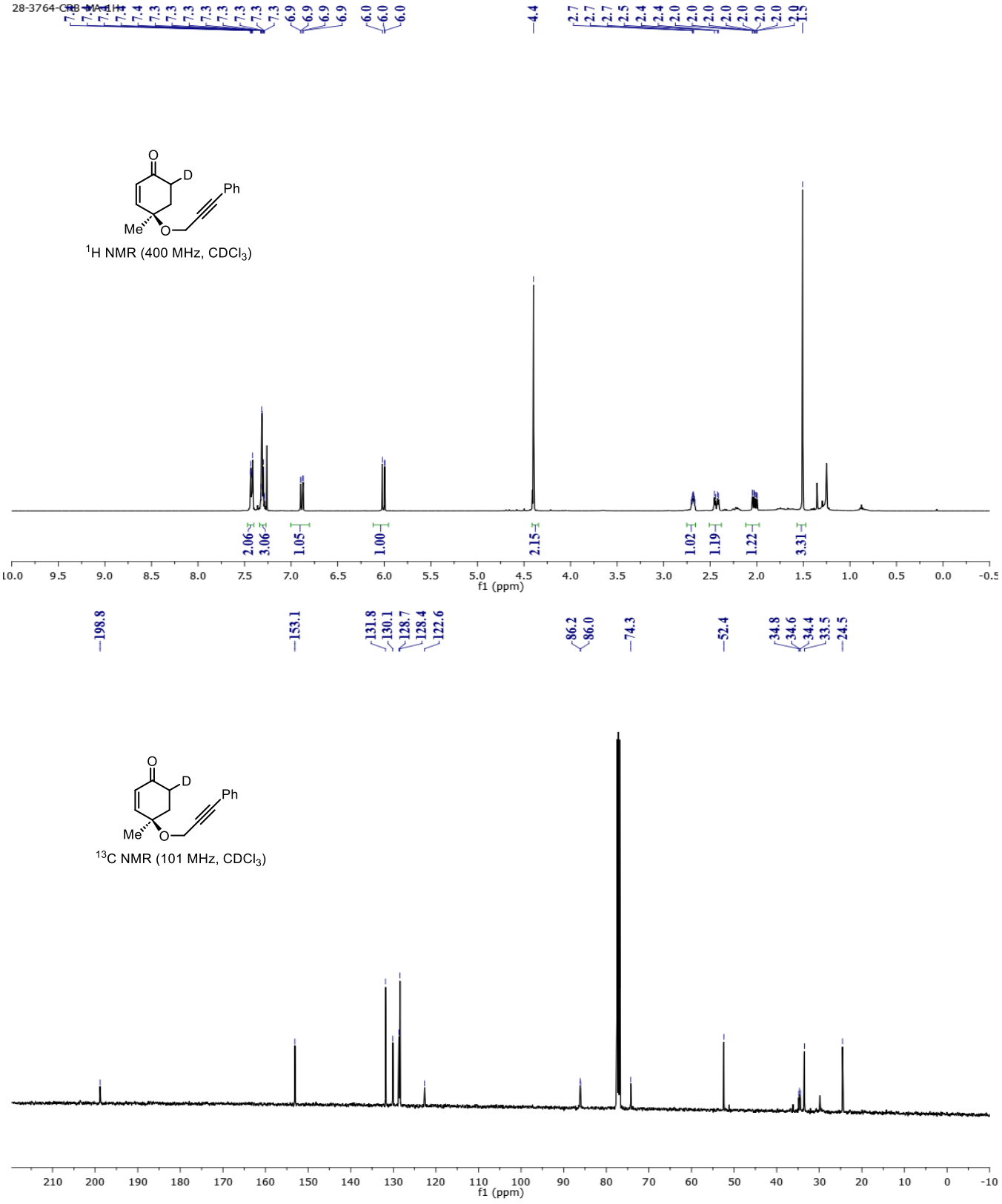
We conducted intermolecular competition experiment between compound **1j** and **1j-d$_4$** by using both the substrates in 1:1 ratio. The chosen substrates were dissolved in a suitable solvent (1 mM) and added to a Tris buffer solution (0.1 M, pH 7.5) containing glucose (20 mM), NADPH (1.2 mM), glucose oxidase (10 $Umg^{-1}mL^{-1}$) and purified YqjM was added at 200 nM. The assay mixture was incubated for 8 h in an orbital shaker (100 rpm, 25°C), to give **2j/2j-d$_3$** in 46% yield with ~36% deuterium incorporation.



IVb. Labeling experiment on compound 1j:



We carried out deuterium labeling experiment on compound **1j** in the presence YqjM Y169F mutant enzyme. The assay reaction was performed by dissolving the substrate in a suitable solvent (1 mM) and added to a Tris-D_2O buffer (0.1 M, pH 7.5). To this, we added glucose (20 mM), NADPH (1.2 mM), glucose oxidase (10 $Umg^{-1}mL^{-1}$) and reaction was initiated after addition of purified Y169F at 200 nM. The reaction mix was incubated for 8 h in an orbital shaker (100 rpm, 25°C), to give **2j-d$_1$** in 43% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.41 (m, 2H), 7.34 – 7.28 (m, 3H), 6.88 (dd, *J* = 10.3, 0.9 Hz, 1H), 6.00 (dd, *J* = 10.2, 0.4 Hz, 1H), 4.40 (s, 2H), 2.73 – 2.65 (m, 1H), 2.43 (dd, *J* = 13.3, 4.8 Hz, 1H), 2.02 (ddd, *J* = 13.5, 6.8, 1.0 Hz, 1H), 1.51 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.8, 153.1, 131.8, 130.1, 128.7, 128.4, 122.6, 86.1, 86.0, 74.3, 52.4, 34.6 (t, J_{CD} = 20.1 Hz, 1D), 33.5, 24.5; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{16}{}^1H_{16}{}^2HO_2$ 242.1291; Found 242.1244.


28-3764-CRB-MA-1H-1
7.7
7.7
7.7
7.4
7.4
7.3
7.3
7.3
7.3
7.3
7.3
7.3
7.3
6.9
6.9
6.9
6.9
6.0
6.0
6.0
4.4
2.7
2.7
2.7
2.5
2.4
2.4
2.0
2.0
2.0
2.0
2.0
2.0
2.0
1.5
O
D
Ph
Me
O
1H NMR (400 MHz, CDCl3)
2.06
3.06
1.05
1.00
2.15
1.02
1.19
1.22
3.31
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)
198.8
153.1
131.8
130.1
128.7
128.4
122.6
86.2
86.0
74.3
52.4
34.8
34.6
34.4
33.5
24.5
O
D
Ph
Me
O
13C NMR (101 MHz, CDCl3)
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

V. Steady-state kinetic parameters.

Activity was measured by the consumption of NADPH [$Abs_{340\ nm}$, ε_{340nm} = 6.2 mM^{-1} cm^{-1}] including an oxygen consuming system photometrically. Reaction condition included: 58 µL Tris-HCl buffer (100 mM, pH 7.5); 10 µL substrate in suitable solvent (1 mM); 10 µL NADPH (1.2 mM); 2 µL glucose (20 mM); 10 µL glucose oxidase (10 U/mg/mL); 10 µL YqjM WT (0.2 µM) to make final reaction volume of 100 µL at 25ºC. The change in absorption values at 340 nm was monitored for 30 min using a microtiter plate reader (TECAN infinite M200 PRO, Austria).[1]

Every measurement was performed in triplicate and corrected by a blank in the absence of substrate. The Km, Vmax and kcat values, were obtained from the non-linear regression of Michaelis–Menten plots (using GraphPad Prism 8.0 software). Error bars represent the standard deviations; data expressed as the mean ± SEM.

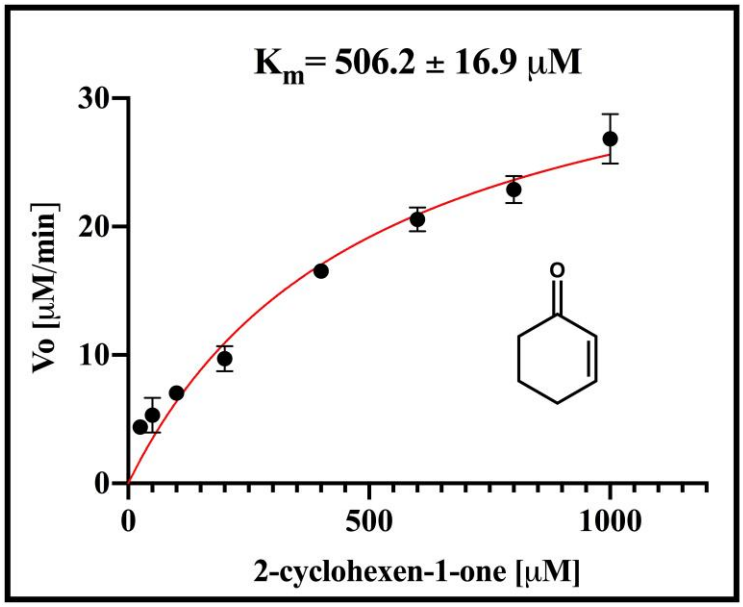


Figure S2. Michaelis-Menten plot for the reduction of 2-**cyclohexen-1-one** by YqjM (WT).

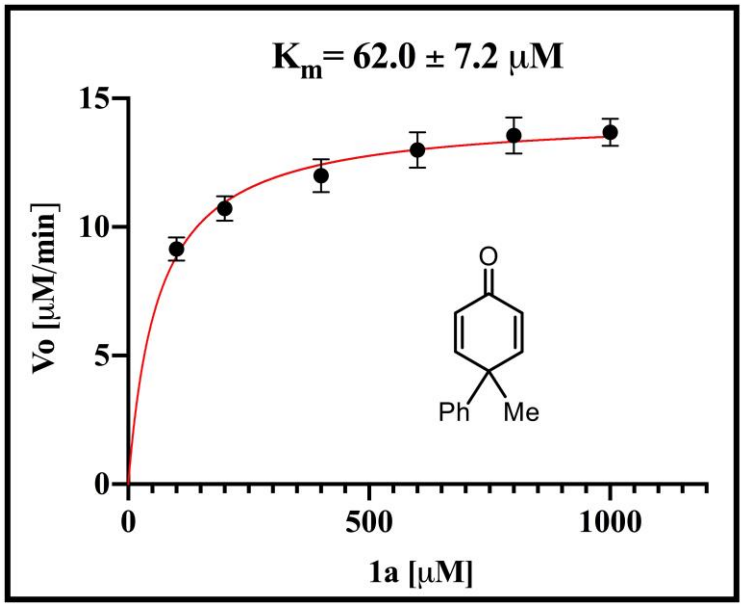


Figure S3. Michaelis-Menten plot for the reduction of **1a** by YqjM (WT).

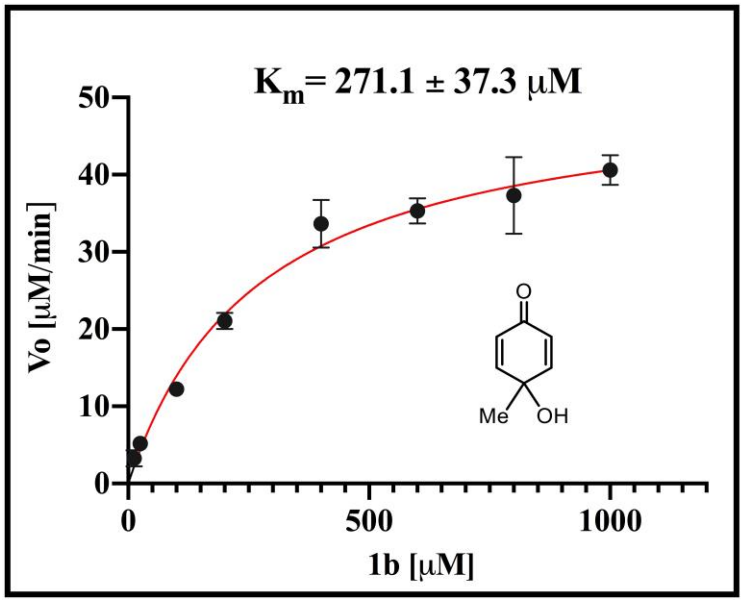


Figure S4. Michaelis-Menten plot for the reduction of **1b** by YqjM (WT).

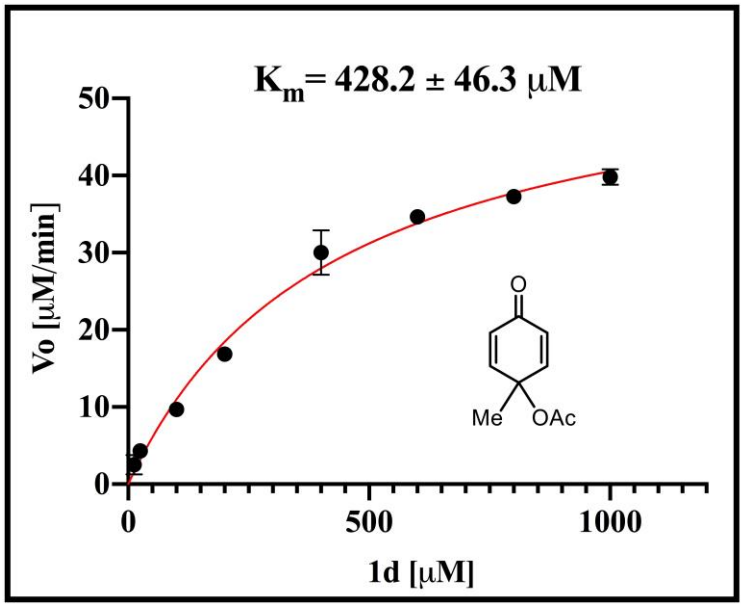


Figure S5. Michaelis-Menten plot for the reduction of **1d** by YqjM (WT).

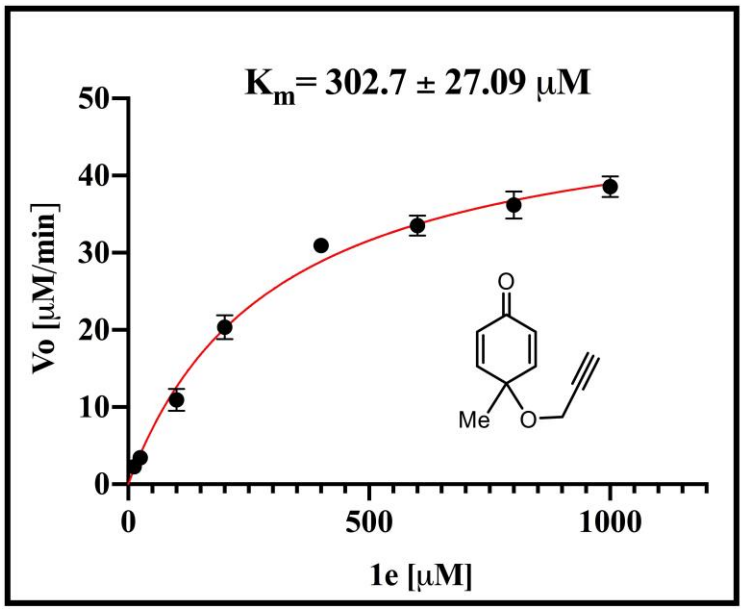


Figure S6. Michaelis-Menten plot for the reduction of **1e** by YqjM (WT).

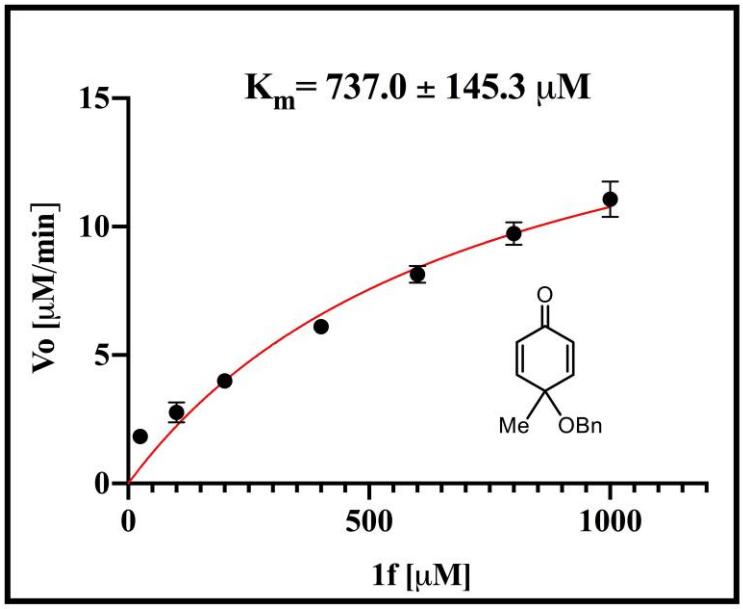


Figure S7. Michaelis-Menten plot for the reduction of **1f** by YqjM (WT).

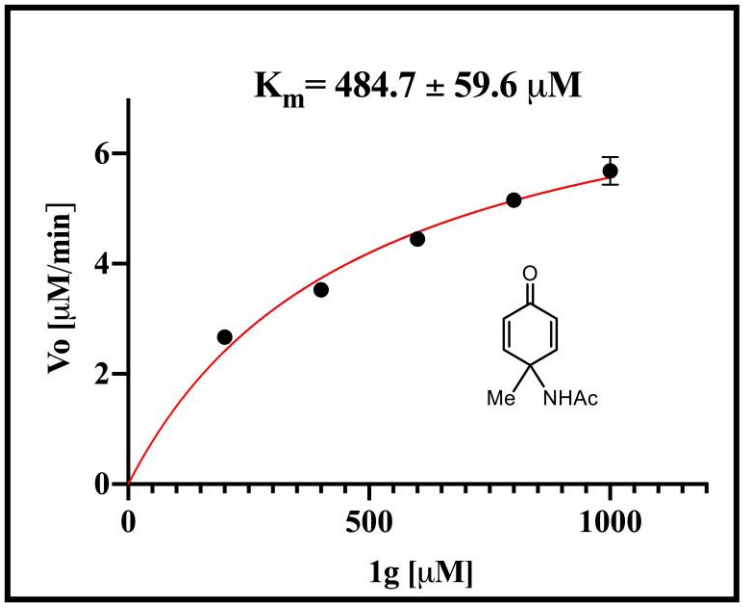


Figure S8. Michaelis-Menten plot for the reduction of **1g** by YqjM (WT).

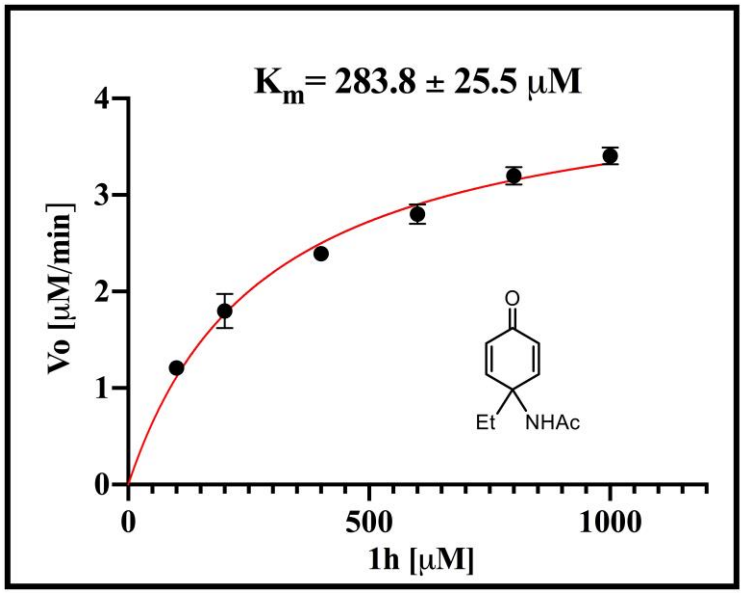


Figure S9. Michaelis-Menten plot for the reduction of **1h** by YqjM (WT).

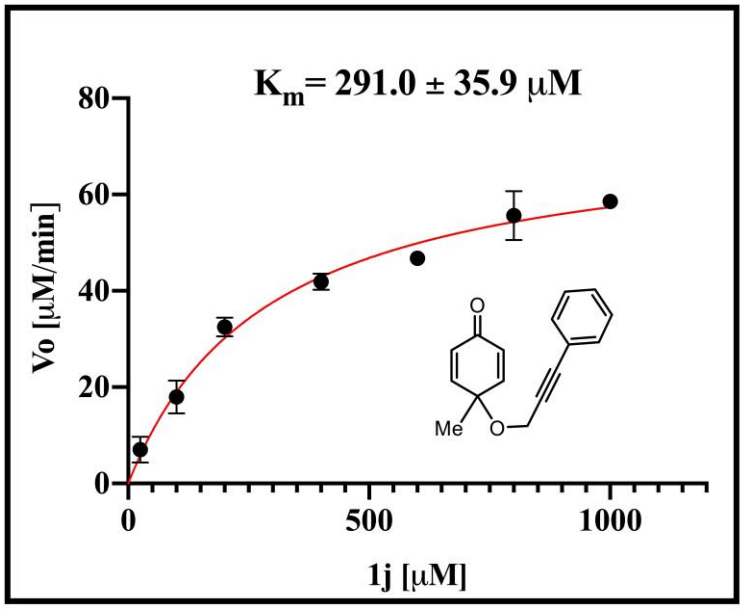


Figure S10. Michaelis-Menten plot for the reduction of **1j** by YqjM (WT).

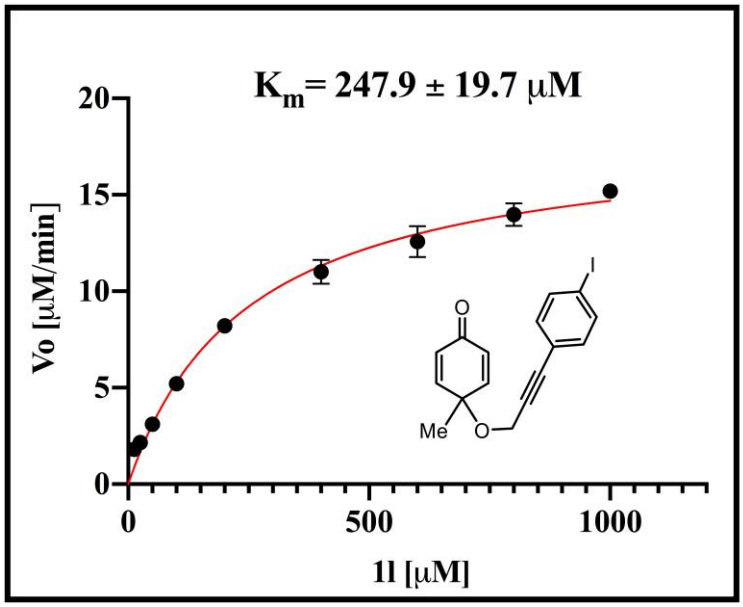


Figure S11. Michaelis-Menten plot for the reduction of **1l** by YqjM (WT).

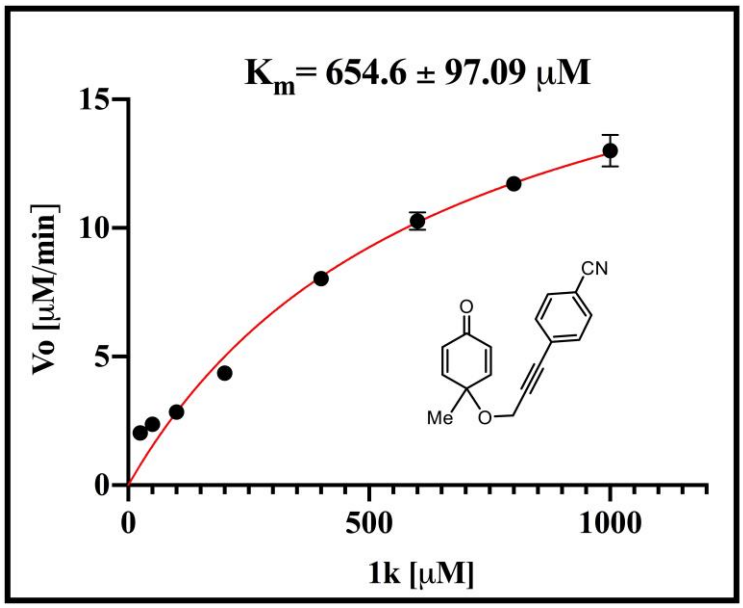


Figure S12. Michaelis-Menten plot for the reduction of **1k** by YqjM (WT).

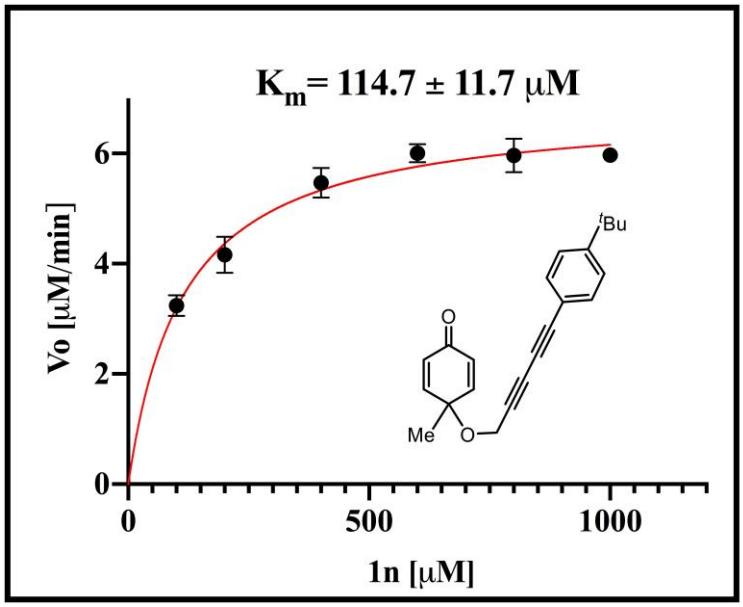


Figure S13. Michaelis-Menten plot for the reduction of **1n** by YqjM (WT)

VI. Bio-reduction of α, β-unsaturated substrates

The chosen substrate dissolved in a suitable solvent (1 mM) was added to a Tris buffer solution (0.1 M, pH 7.5) containing glucose (20 mM), NADPH (1.2 mM), glucose oxidase (10U mg-1mL−1) and finally purified YqjM was added (200 nM). The reaction mixture was incubated for 12 h in an orbital shaker (100 rpm, 25°C). The solution was extracted with EtOAc and the combined organic solutions were dried over anhydrous Na2SO4 for further analysis. [2]

VII. NADPH recycling by GDH coupled assay

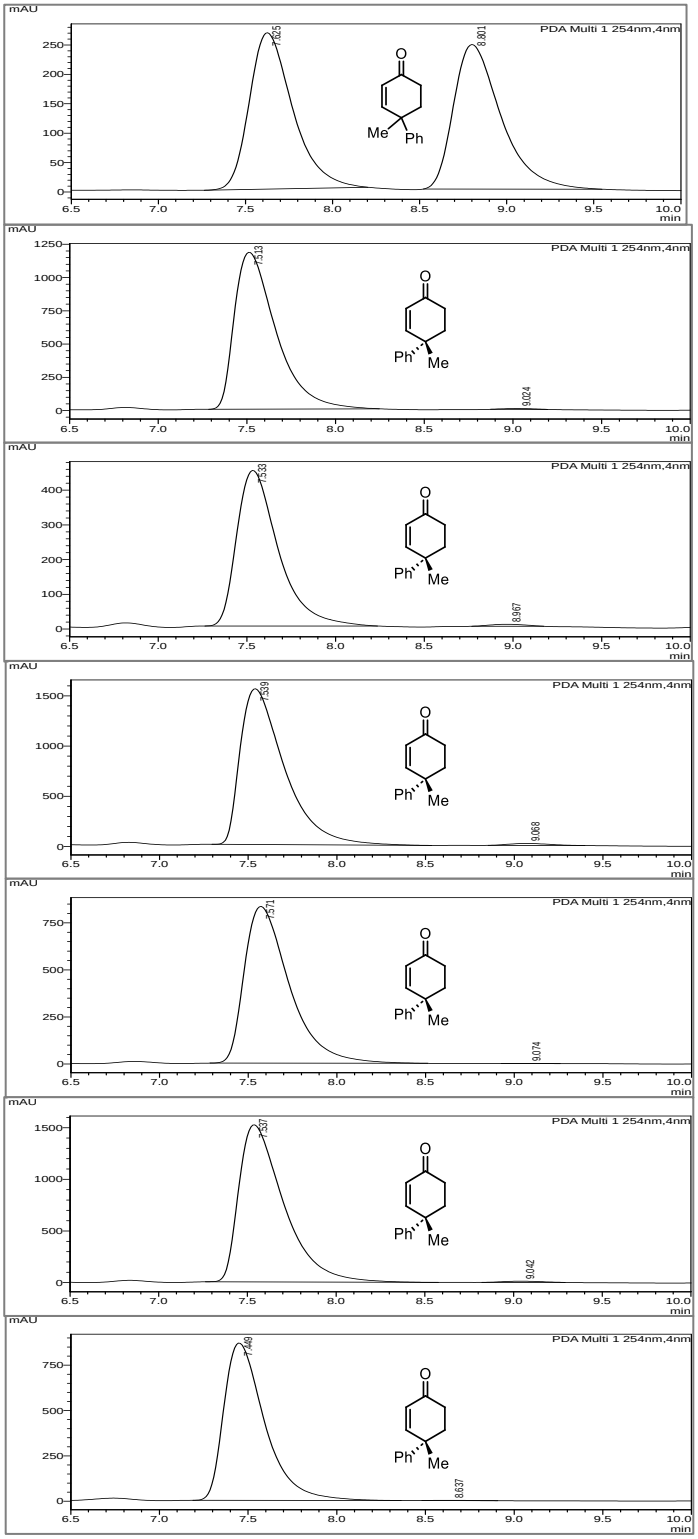
For the reduction of substrates, the following conditions were used- the reaction mixture contained substrate (1 mM), $NADP^+$ (0.1 mM), glucose (20 mM), glucose dehydrogenase (0.5 U/mL/mg) in a Tris buffer (0.1 M, pH 8.0). Finally purified enzyme was added to a final concentration of 200nM and incubated for 12 h at 30◦C in an orbital shaker at 100 rpm. Adding EtOAc terminated the solution and product was purified for further analysis. [14]

VIII. YqjM Site Directed Mutagenesis

Primers used for mutagenesis:

List	Mutants	Primers
1	C26A- FP	TCGCCAATGGCGATGTATTCTT
	C26A- RP	AAGAATACATCGCCATTGGCG
2	C26S- FP	TCGCCAATGAGCATGTATTCTT
	C26S- RP	AAGAATACATGCTCATTGGCG
3	I69A- FP	GACGAGCGACTGACCAAGACTTAGGCATTTGGAGCGACGAG
	I69A- RP	CTAAGTCTTGGTCAGTCGCTCGTCCTTGAGGGTTAACCGCTGA
4	R336A- FP	GCCCCTGTTCAATACGAAGCGGGCTGGTAA
	R336A- RP	CGCTTCGTATTGAACAGGGGCCGGAATCTCTG
5	Y28A- FP	CATGGCGTCTTCTCATGAAAAGGACGGAAAATTAACACCGTTC
	Y28A- RP	CTTTTCATGAGAAGACGCCATGCACATTGGCGACATGACAAT
6	Y28F- FP	ATGTGCATGTTTTCTTCTCATG
	Y28F- RP	CATGAGAAGAAAACATGCACAT
7	Y169F- FP	GCGCACGGATTTTTAATTCAT
	Y169F- RP	ATGAATTAAAAATCCGTGCGC

IX. Stereochemical outcomes of YqjM mutants



RACEMIC

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.625	4617381	265756	49.727	51.957
2	8.801	4668016	245740	50.273	48.043
Total		9285397	511496	100.000	100.000

YqjM WT

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.513	19238123	1179313	99.637	99.493
2	9.024	70073	6011	0.363	0.507
Total		19308196	1185324	100.000	100.000

YqjM C26A

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.533	7324269	448041	98.943	98.756
2	8.967	78252	5646	1.057	1.244
Total		7402521	453686	100.000	100.000

YqjM C26S

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.539	26999010	1550146	98.869	98.760
2	9.068	308797	19469	1.131	1.240
Total		27307807	1569615	100.000	100.000

YqjM R336A

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.571	14760136	832762	99.952	99.924
2	9.074	7027	629	0.048	0.076
Total		14767163	833391	100.000	100.000

YqjM Y28A

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.537	26847818	1520433	99.331	99.216
2	9.042	180711	12010	0.669	0.784
Total		27028529	1532443	100.000	100.000

YqjM Y28F

<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Area%	Height%
1	7.449	13915648	867000	99.877	99.872
2	8.637	17092	1112	0.123	0.128
Total		13932741	868113	100.000	100.000

Fig S14. Chiral traces of mutagenesis

X. References

(1) M. Pesic, E. Fernández-Fueyo, F. Hollmann, *ChemistrySelect*, 2017, **2**, 3866-3871.

(2) K. Kitzing, T. B. Fitzpatrick, C. Wilken, J. Sawa, G. P. Bourenkov, P. Macheroux, T. Clausen, *J Biol Chem.*, 2005, **280**, 27904-27913.

(3) J. K. Hexum, R.Tello-Aburto, N. B. Struntz, A. M. Harned, D. A. Harki, *ACS Med. Chem. Lett.*, 2012, **3**, 459-464.

(4) K. Juliane, L. Mark, *Org. Lett.* 2013, **15**, 1148–1151.

(5) Y. Fukui, P. Liu, Q. Liu, Z.T. He, N.Y. Wu, P. Tian, G.Q. Lin, *J. Am. Chem. Soc.*, 2014, **136**, 15607-15614.

(6) Z. T. He, X. Q. Tang, L. B. Xie, M. Cheng, P. Tian, G. Q. Lin, *Angew. Chem., Int. Ed.*, 2015, **54,** 14815-14818.

(7) C. Christopher, L. H. Wai, *J. Am. Chem. Soc.*, 2016, **138,** 8068–8071.

(8) K. Balaraman, V. Kesavan, *Synthesis*, 2010, 3461-3466, DOI: 10.1055/s-0030-1258199

(9) Y. Naganawa, M. Kawagishi, J. I. Ito, H. Nishiyama, *Angew. Chem.*, 2016, **128**, 6987-6990.

(10) A. Presser, G. Lainer, N. Kretschmer, W. Schuehly, R. Saf, M. Kaiser, M.M. Kalt, *Molecules*, 2018, **23**, 2902.

(11) K. Matsunaga, N. Saito, H.;Kogen, K. Takatori, *Org. Lett.*, 2019, **21**, 6054-6057.

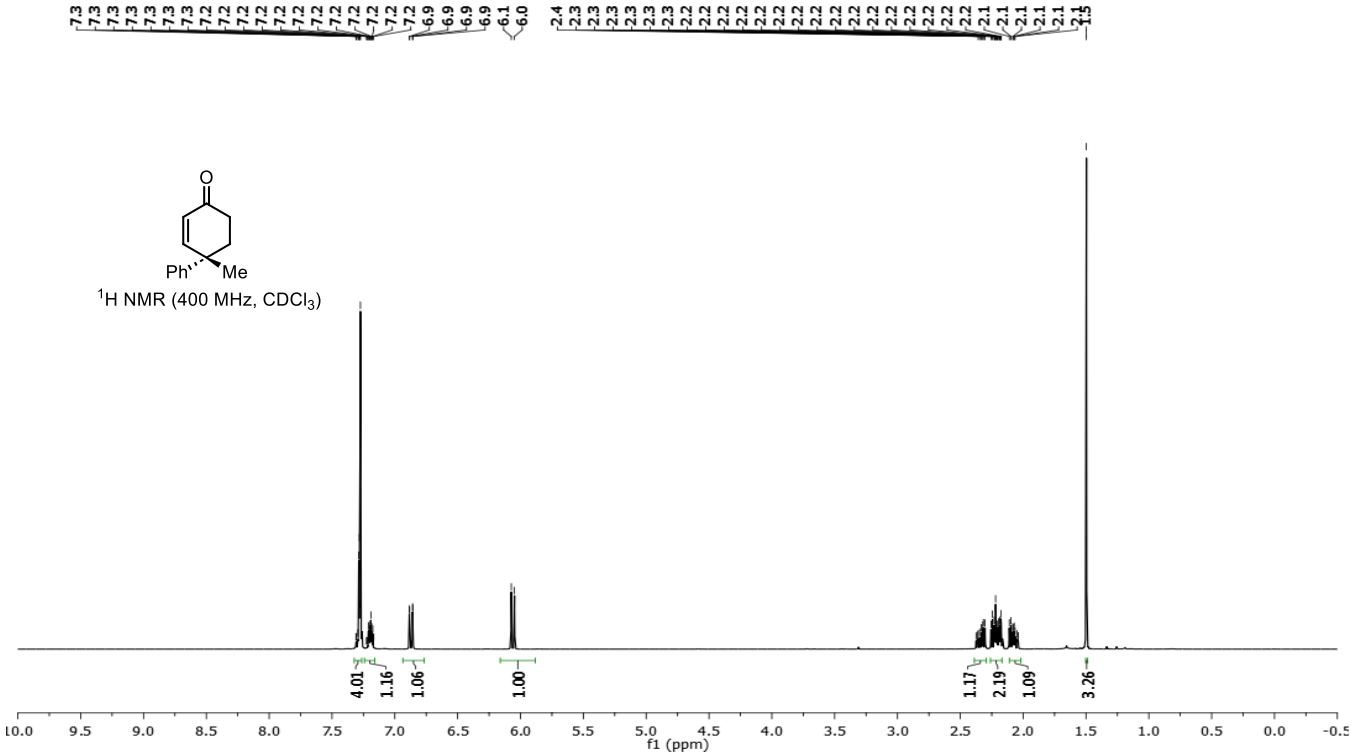
(12) A. Victoria, *Chem. Sci.*, 2020, **11**, 7444-7450.

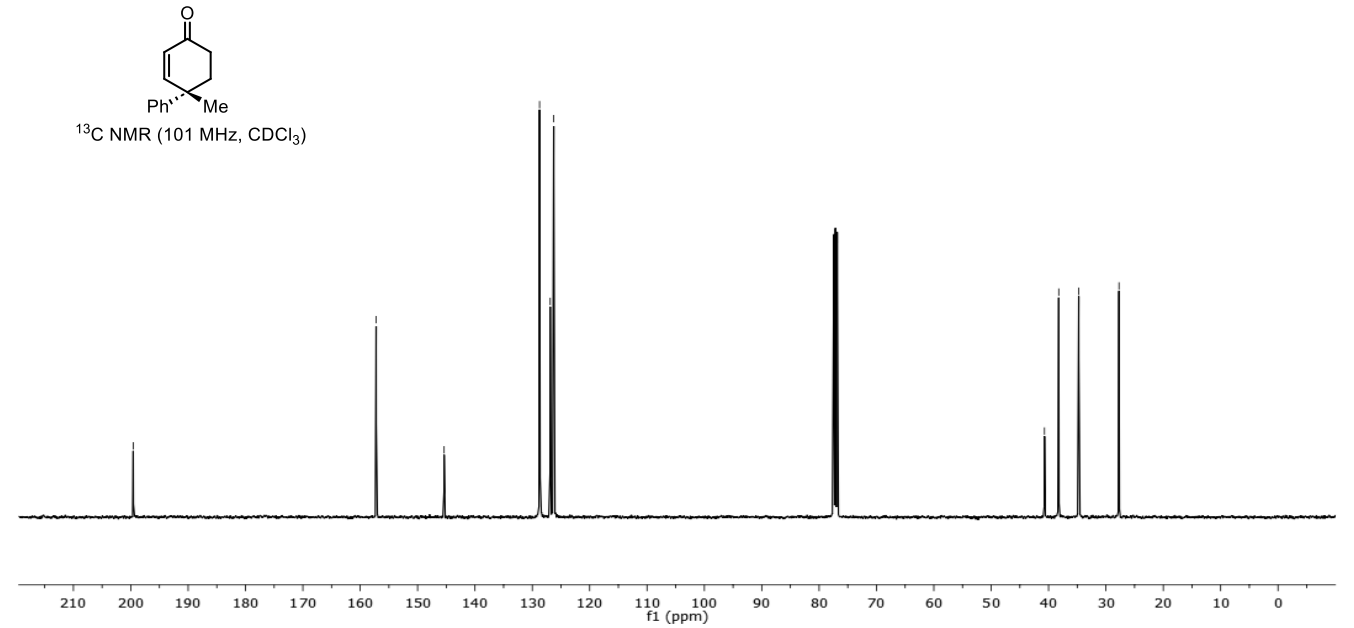
(13) H. Liang, M.A. Ciufolini, *J. Org. Chem.*, **2008**, *73*, 4299–4301.

(14) E. Rüthlein, T. Classen, L. Dobnikar, M. Schölzel, J. Pietruszka, *Adv. Synth. Catal.*, 2015, **357,** 1775-1786.

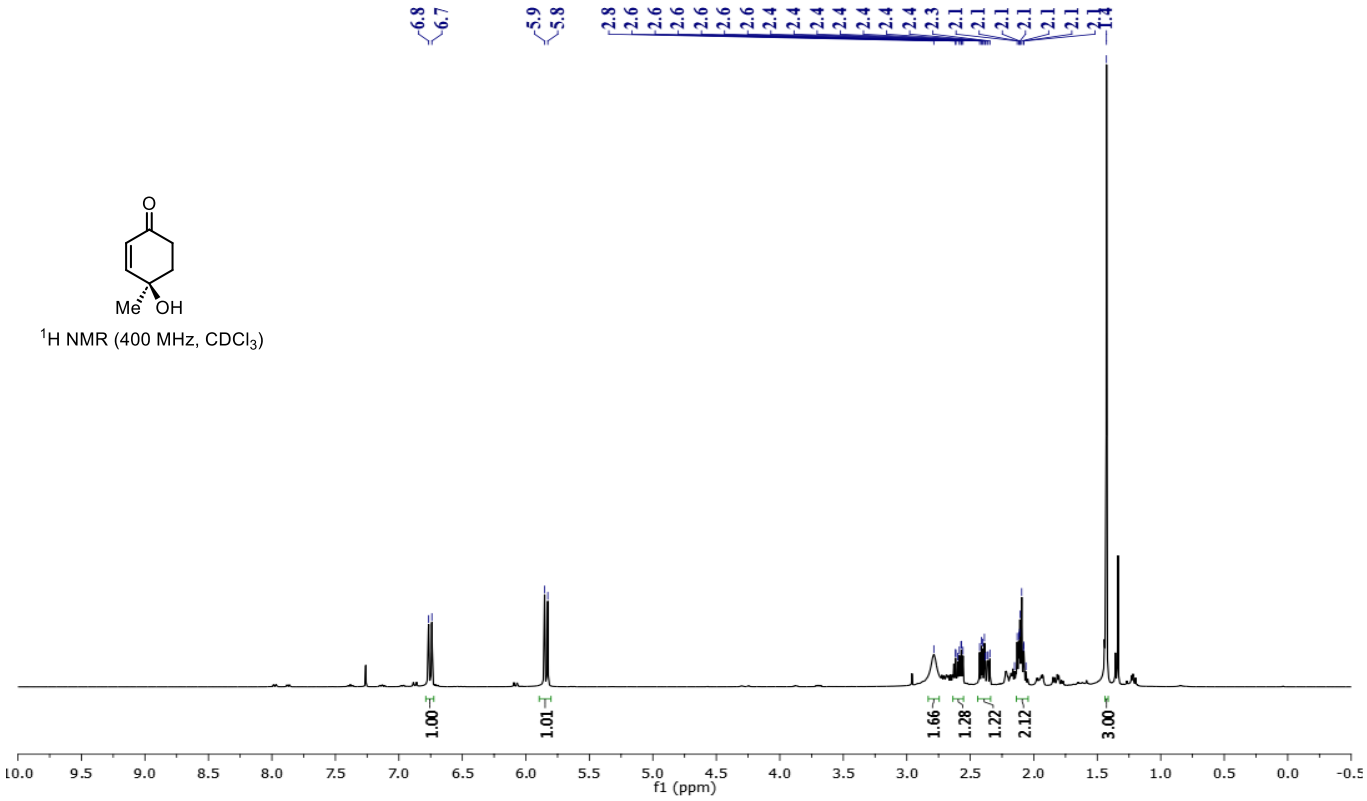
XI. ^{1}H &^{13}C NMR Spectra

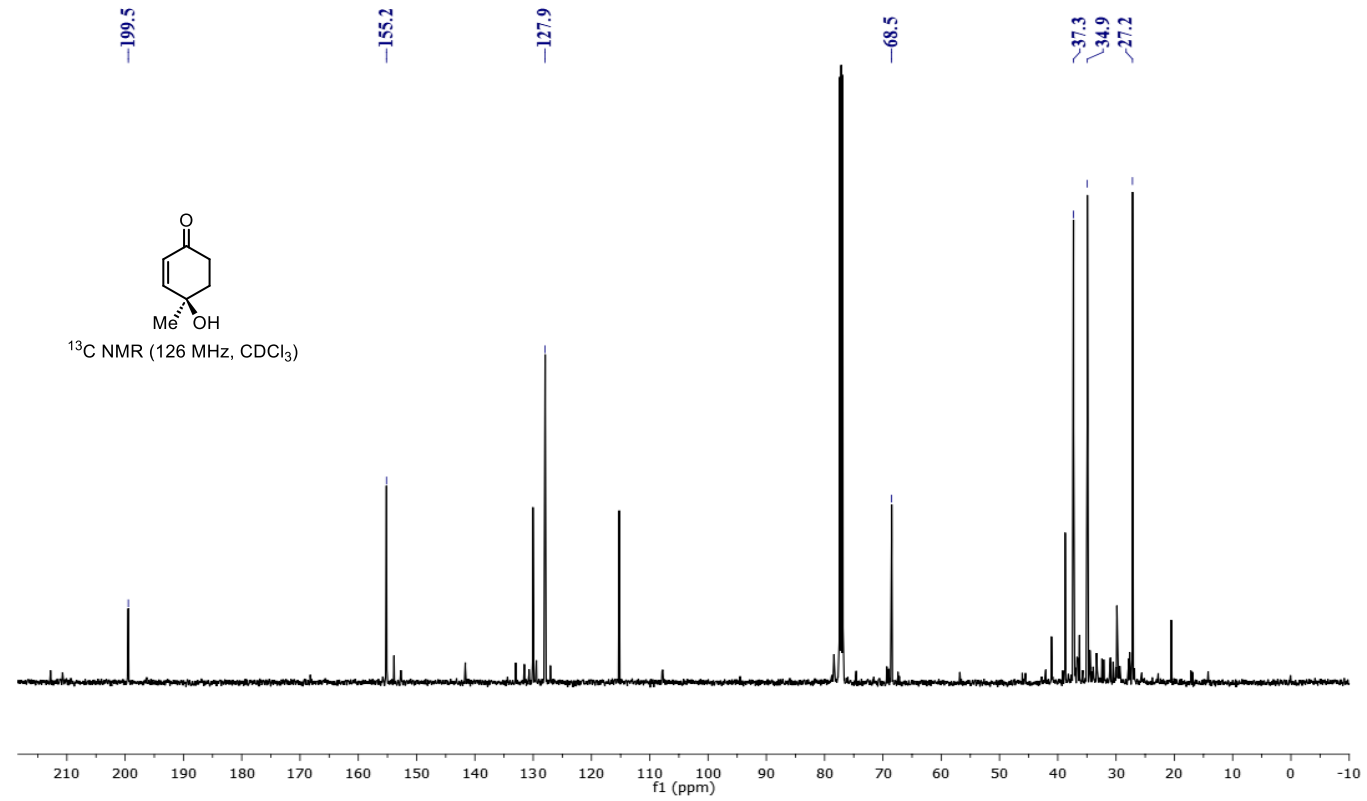
(*S*)-1-methyl-2,3-dihydro-[1,1'-biphenyl]-4(1*H*)-one (2a):



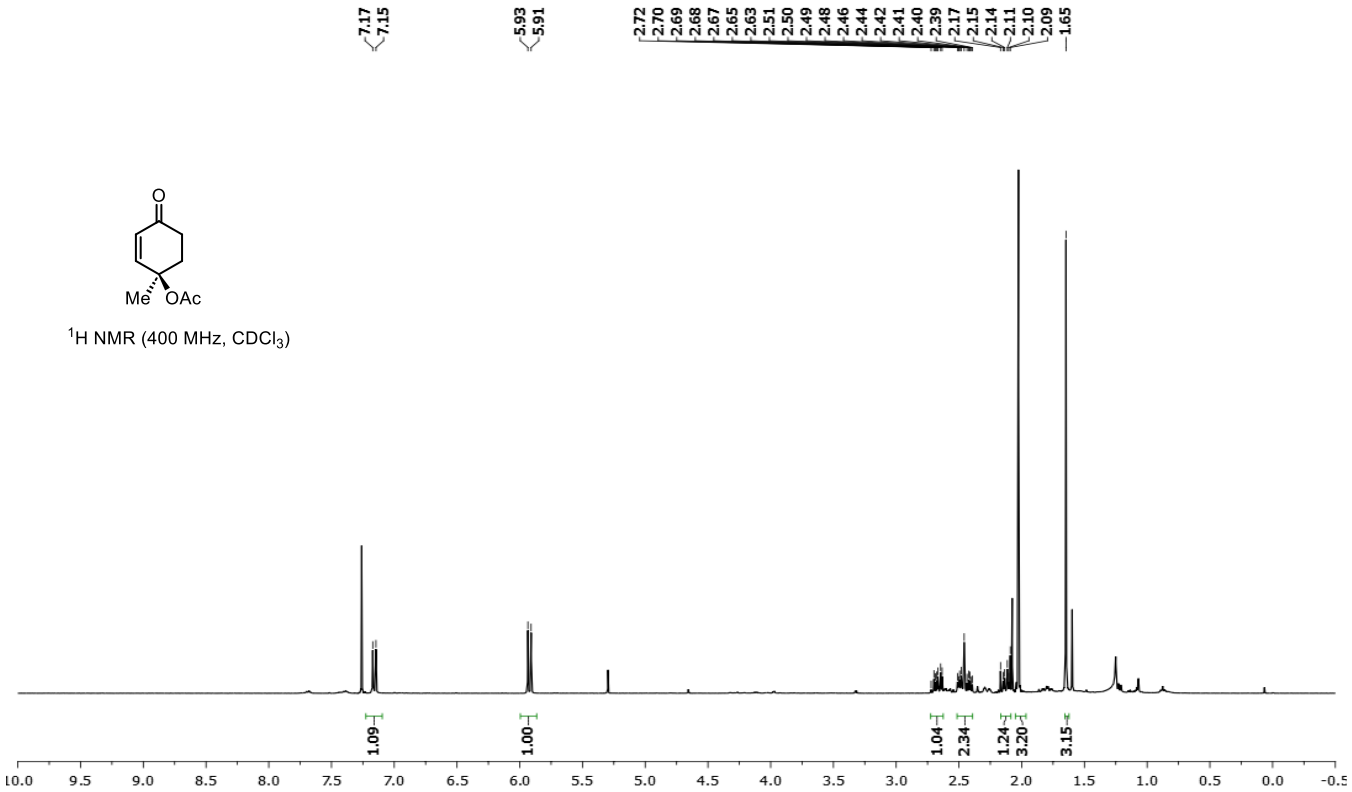


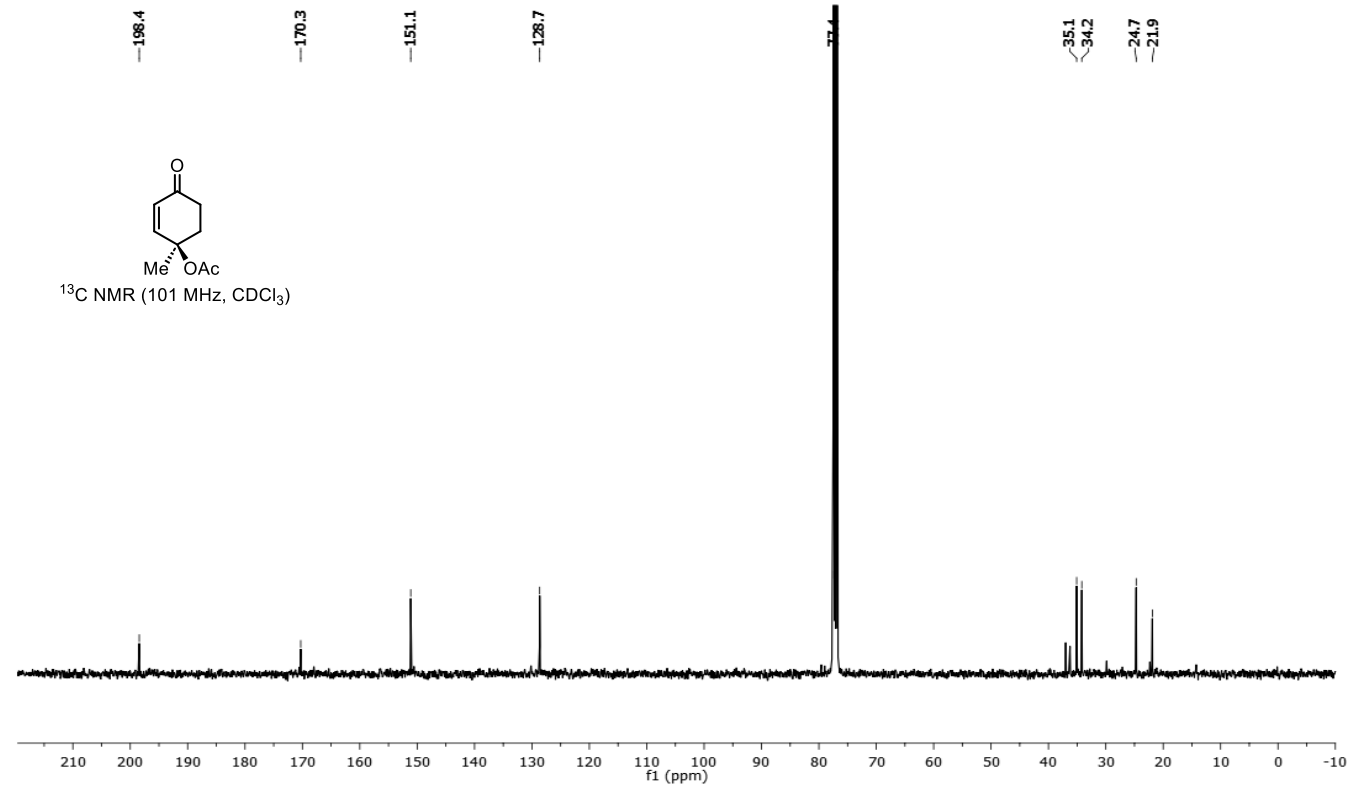
4-Hydroxy-4-methylcyclohex-2-en-1-one (2b):



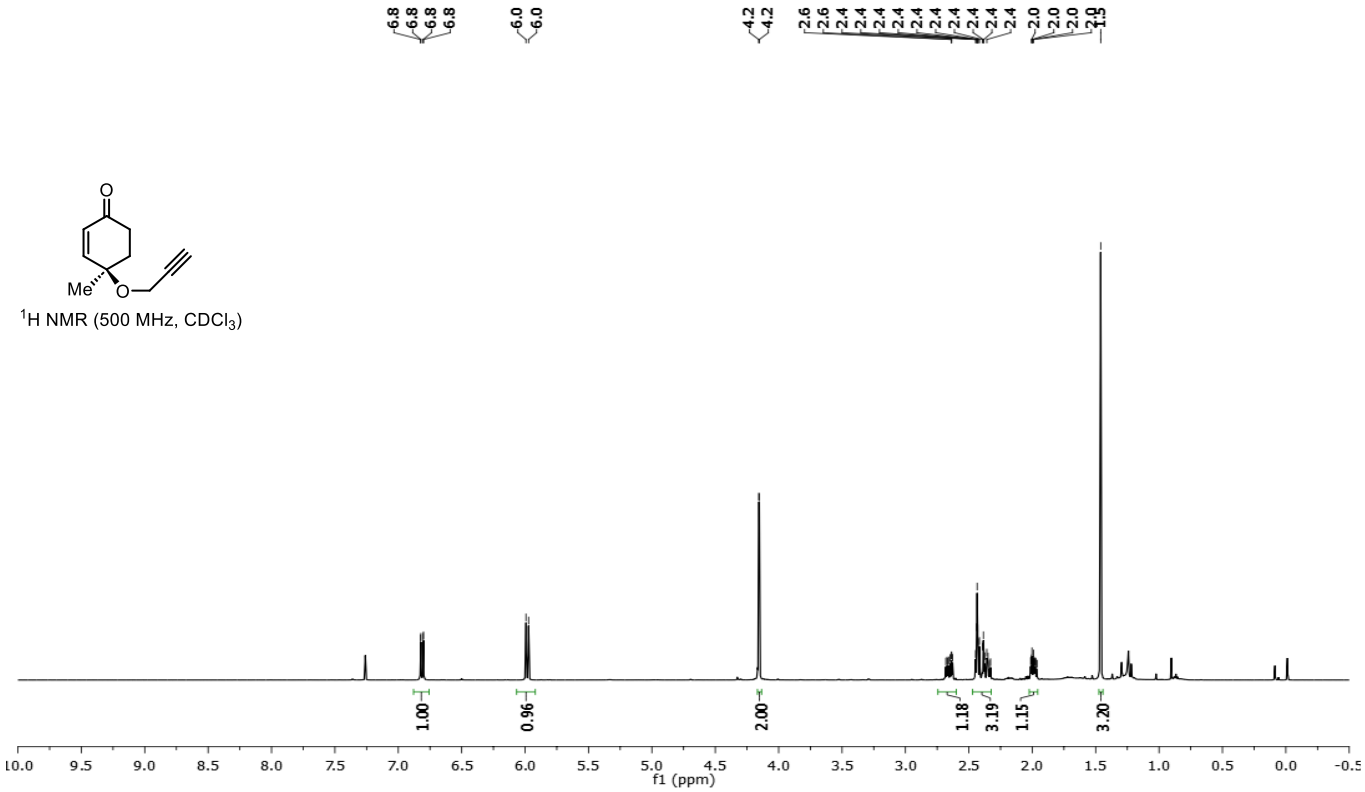


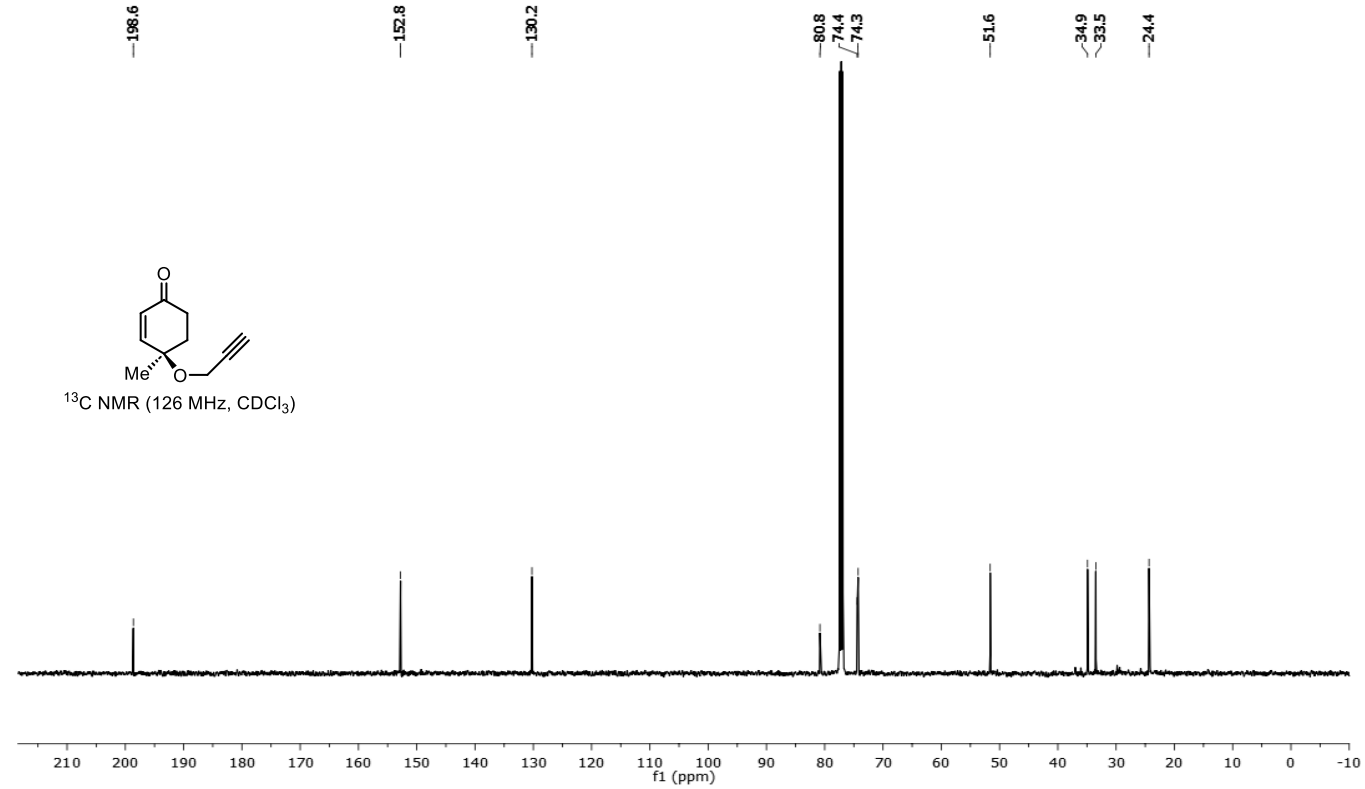
1-Methyl-4-oxocyclohex-2-en-1-yl acetate (2d):



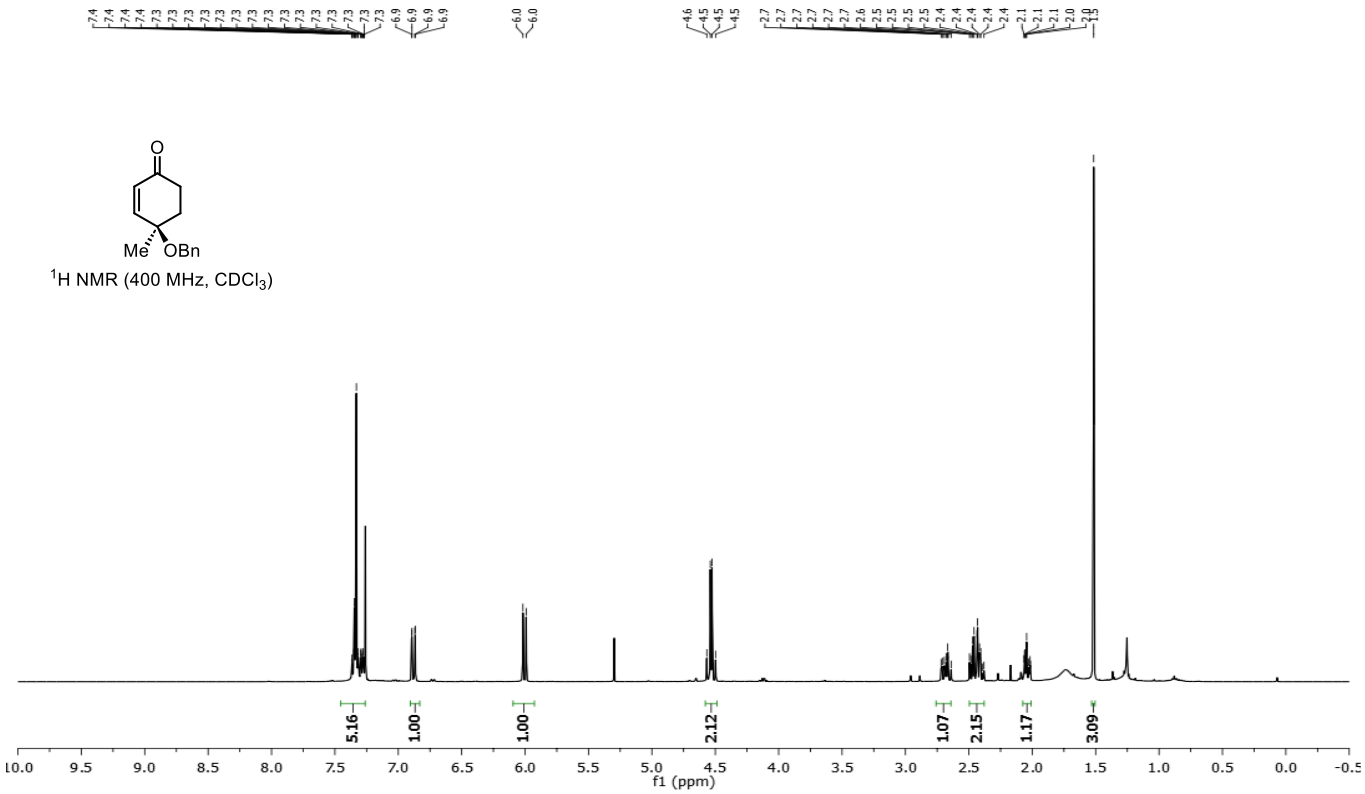


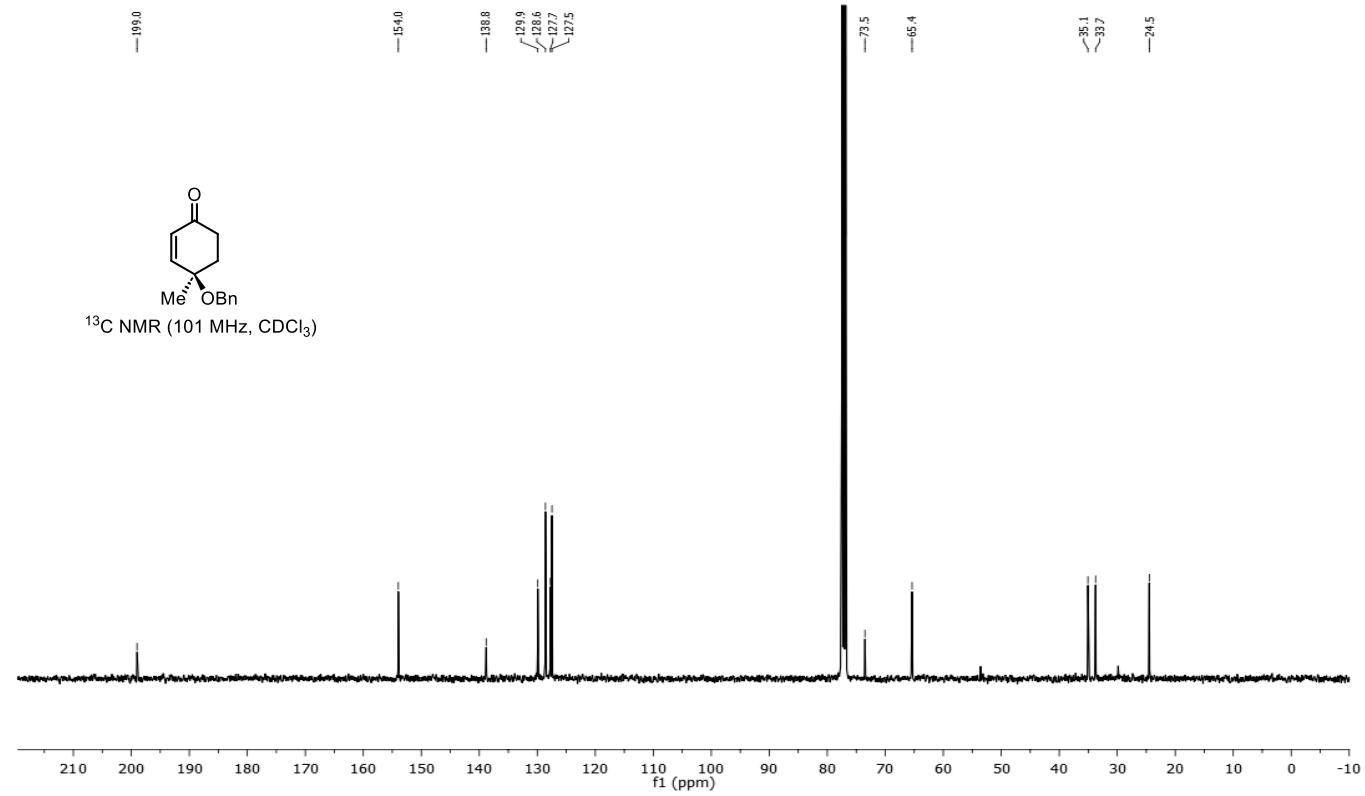
4-Methyl-4-(prop-2-yn-1-yloxy)cyclohex-2-en-1-one (2e):



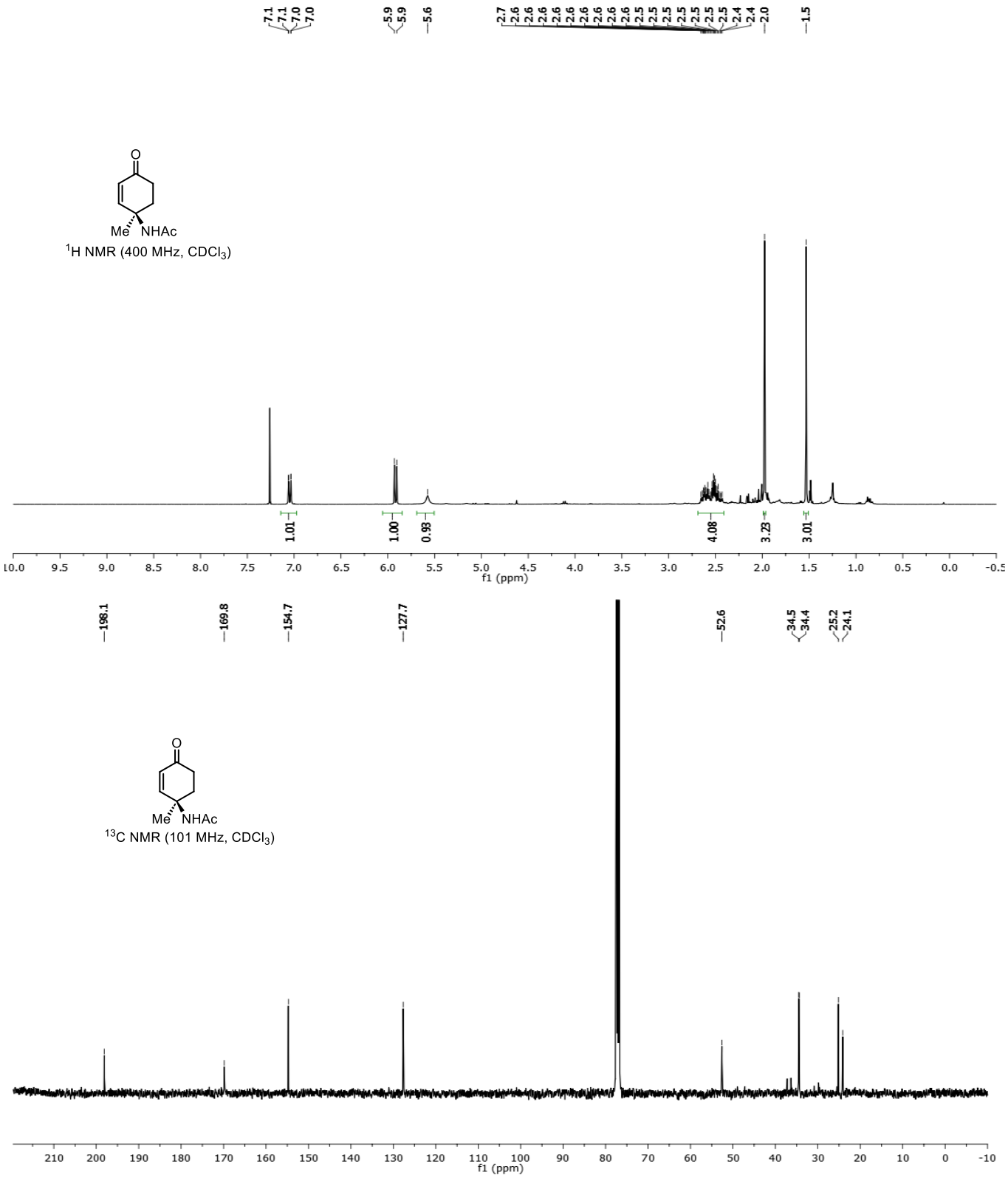


4-(Benzyloxy)-4-methylcyclohex-2-en-1-one (2f):

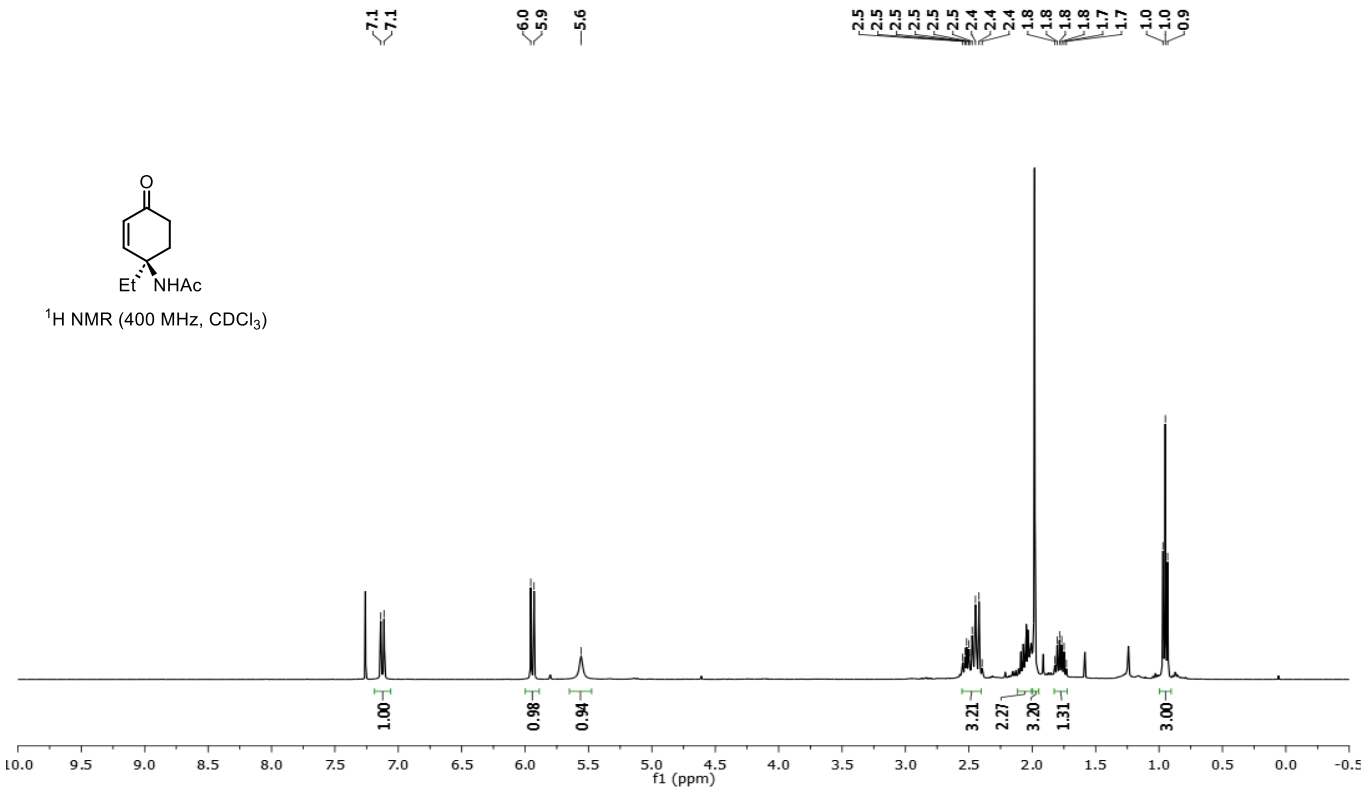


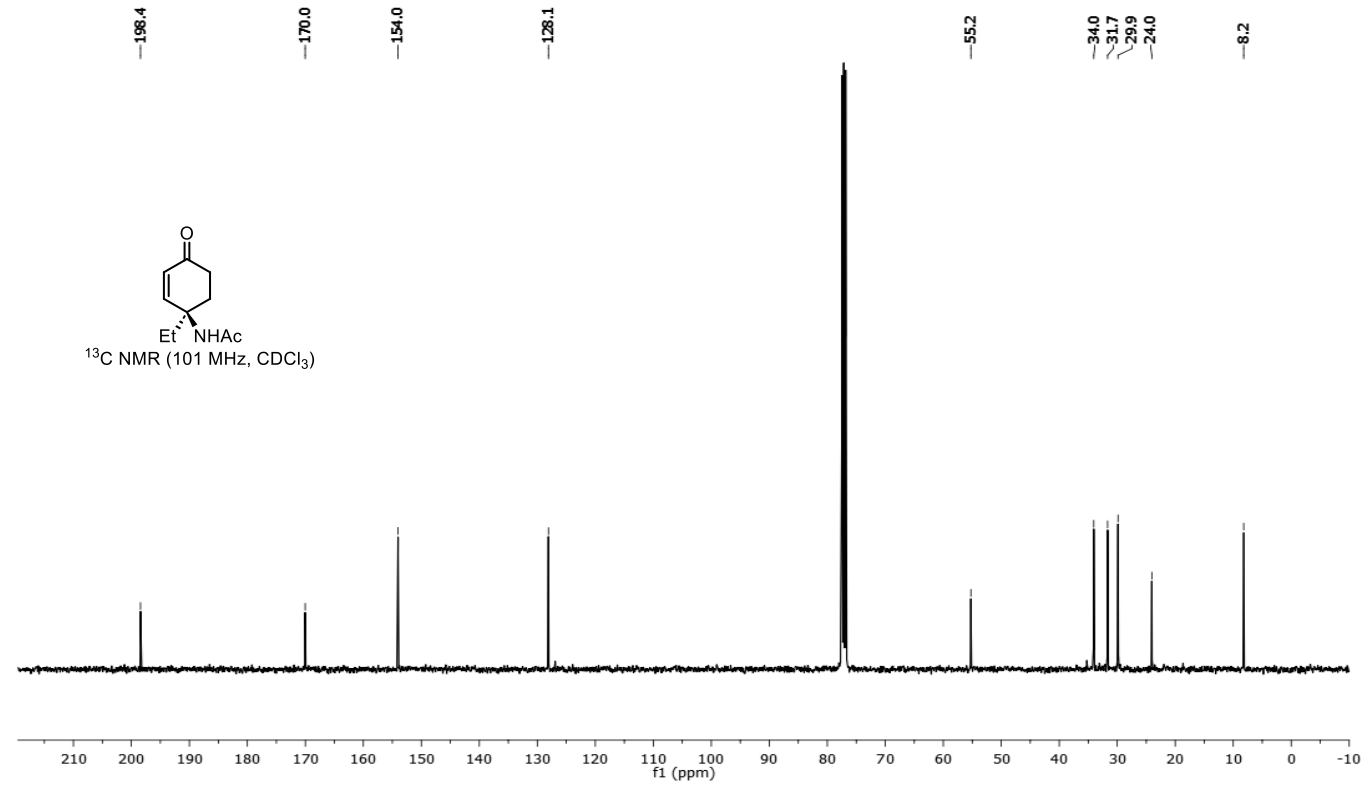


N-(1-Methyl-4-oxocyclohex-2-en-1-yl)acetamide (2g):

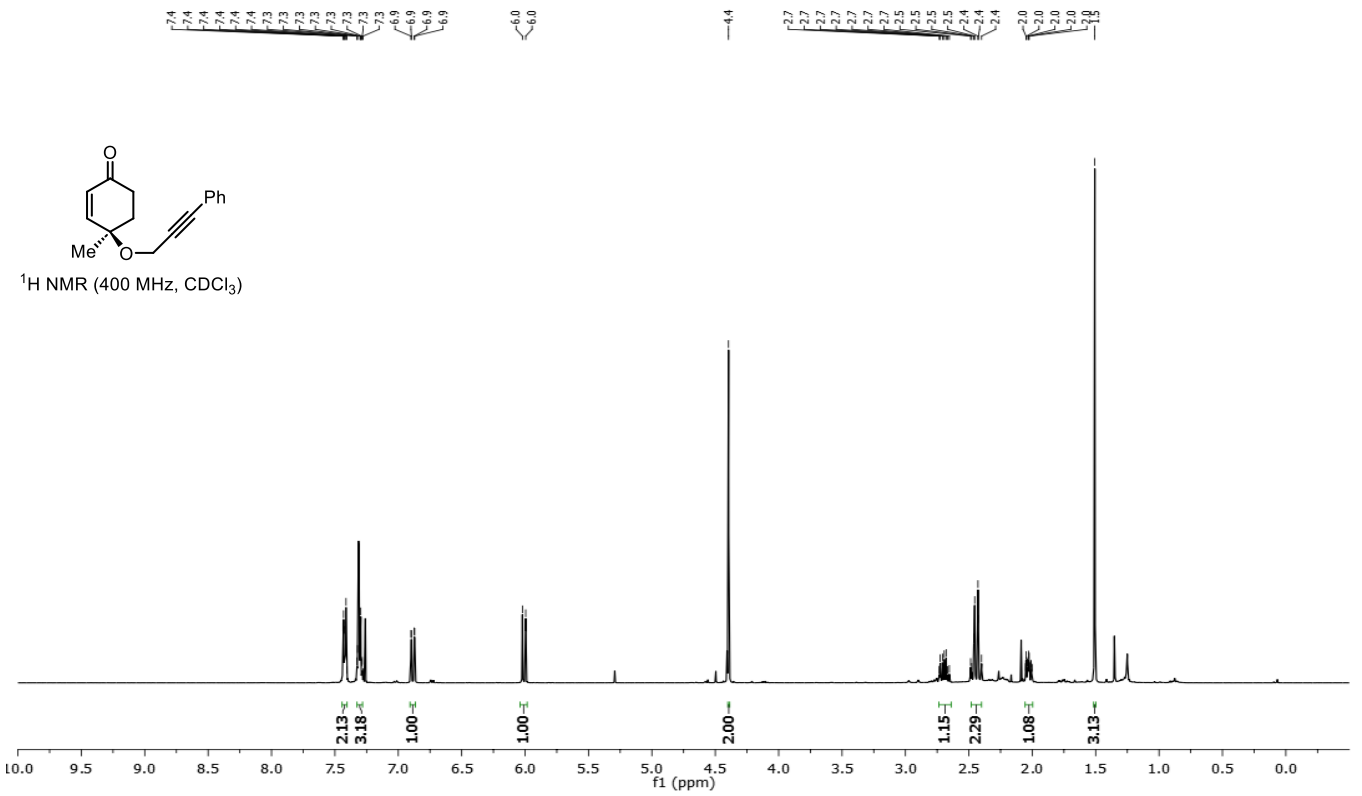


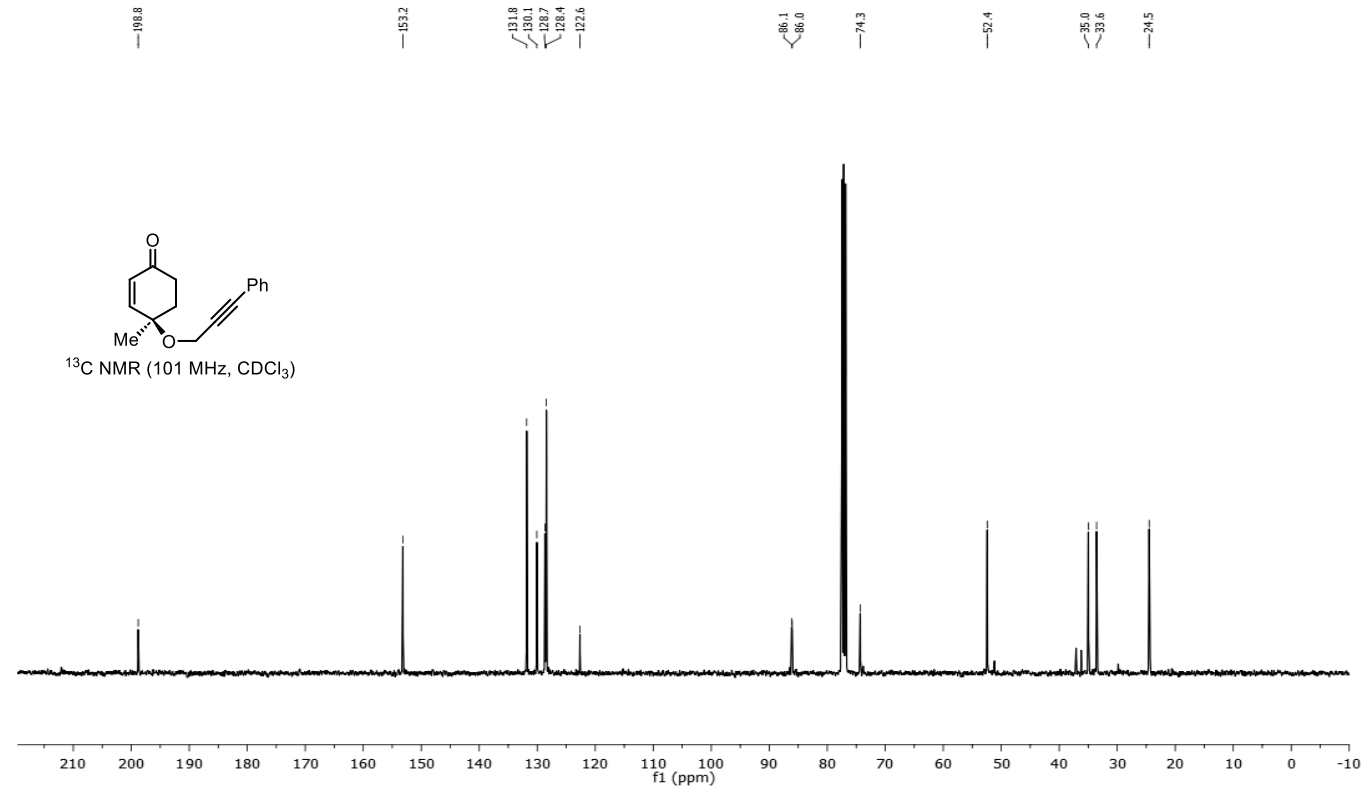
N-(1-Ethyl-4-oxocyclohex-2-en-1-yl)acetamide (2h):



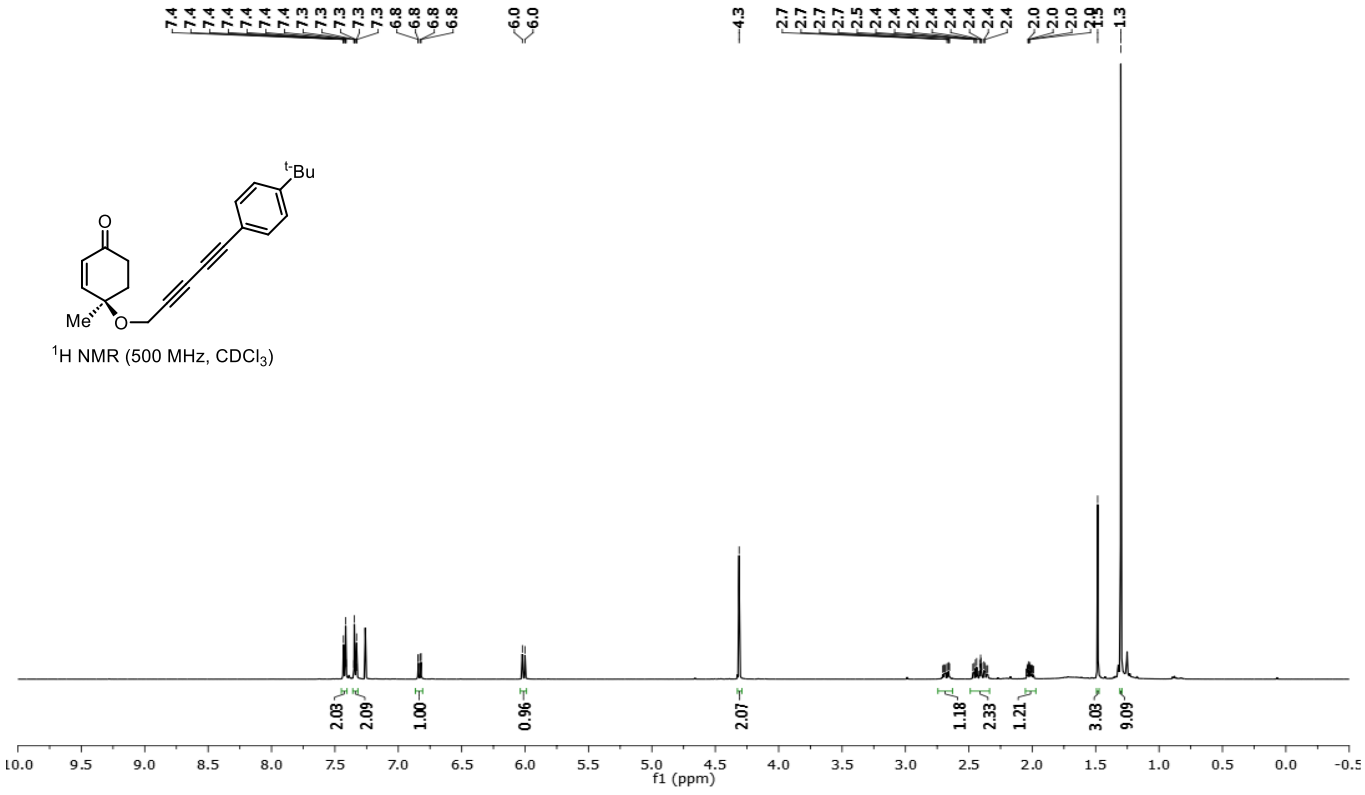


4-Methyl-4-((3-phenylprop-2-yn-1-yl)oxy)cyclohex-2-en-1-one (2j):

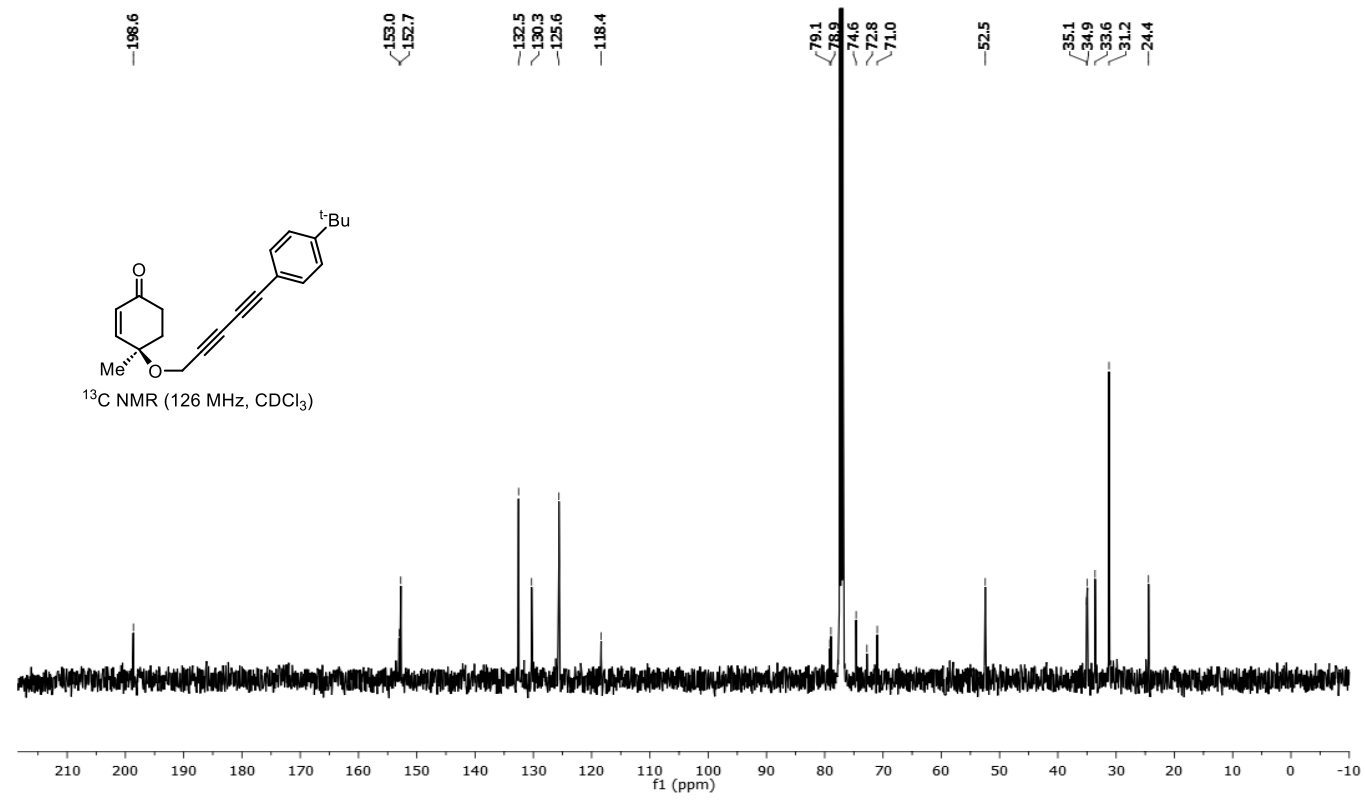




4-((5-(4-(Tert-butyl)phenyl)penta-2,4-diyn-1-yl)oxy)-4-methylcyclohex-2-en-1-one (2n):



^{1}H NMR (500 MHz, $CDCl_3$)



^{13}C NMR (126 MHz, $CDCl_3$)