SUPPORTING INFORMATION

Investigating *Saccharomyces cerevisiae* Alkene Reductase OYE 3 by Substrate Profiling, X-Ray Crystallography and Computational Methods

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Experimental details

Growth media. LB medium contained 5 g / L Bacto-Yeast Extract, 10 g / L Bacto-Tryptone and 10 g / L NaCl. SOC medium contained 5 g / L Bacto-Yeast Extract, 20 g / L Bacto-Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose. ZYP-5052 autoinduction medium[32] contained 10 g / L tryptone, 5 g / L yeast extract, 1 mM $MgSO_4$, 25 mM $(NH_4)_2SO_4$, 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 5 g / L glycerol, 0.5 g / L anhydrous glucose and 2 g / L α-lactose monohydrate.

OYE 3 purification. E. coli BL21 (DE3) harboring plasmid pRP4 was grown at 37° C in a 4 L fermenter containing LB medium supplemented with 200 μg/mL ampicillin. Cells were grown in the fermenter with 600 rpm stirring for 2 h to achieve mid log phase then induced by adding IPTG and glucose to final concentrations of 0.4 and 100 mM, respectively. Growth was continued at 30° C and 600 rpm stirring for an additional 3 h. The culture was chilled at 4° C for 30 min before centrifugation at 5,000 × *g* for 10 min at 4ºC.

All purification steps were carried out at 0 – 4º C. The cell pellet (22 g wet cell weight) was resuspended in 22 mL of 100 mM Tris-Cl, pH 8.0 supplemented with 10 μM PMSF. Cells were lysed by a French pressure cell (12,000 psi), then the lysate was centrifuged at 18,000 × *g* for 60 min to remove insoluble debris. Nucleic acids were precipitated from the supernatant by adding protamine sulfate to a final concentration of 1 mg / mL and stirring for 20 min at 4°C. After centrifuging at 18,000 × *g* for 20 min, proteins were precipitated from the supernatant by adding solid $(NH_4)_2SO_4$ (in 5 equal portions) to 78% saturation. Proteins were recovered by centrifuging at 18,000 × *g* for 60 min.

The ammonium sulfate pellet was resuspended in 22 mL of 100 mM Tris-Cl, 100 mM $(NH_4)_2SO_4$, 10 μM PMSF, pH 8.0, then dialyzed against 1 L of this buffer overnight. The

dialysate was then transferred to a fresh portion of the same buffer (1 L) supplemented with 10 mM sodium dithionite and dialyzed for 2 h to reduce the FMN cofactor and release any endogenous phenolic ligands present during protein overexpression. This step was repeated, then the dialysate was then transferred to a fresh portion of the same buffer lacking dithionite (1 L). After 2 h, a final dialysis was carried out overnight against the same buffer (1 L) of buffer without dithionite. During the final two dialyses, the protein solution became strongly yellow, indicating FMN re-oxidation. The dialysate was centrifuged at 18,000 × g for 30 min to remove insoluble debris, then 10 mL portions were applied to a 3 mL *N*-(4-hydroxybenzoyl)aminohexyl agarose affinity column[42] that had been equilibrated with 100 mM Tris-Cl, 100 mM $(NH_4)_2SO_4$, 10 μM PMSF, pH 8.0. OYE 3 binding turned the column green. After washing with 30 mL of starting buffer to remove unbound protein, OYE 3 was eluted by washing with 10 mL of 100 mM Tris-Cl, 100 mM $(NH_4)_2SO_4$, 10 μM PMSF, 0.5 g / L of dithionite, pH 8.0. Fractions containing OYE 3 were pooled and further purified by gel filtration with a Superdex 200 column (Pharmacia) that had been equilibrated with 50 mM Tris-Cl, 50 mM NaCl, pH 7.5.

Protein sequence alignments

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80.5% identity in 399 residues overlap; Score: 1735.0; Gap frequency: 0.0%

OYE 1          1 MSFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYTQ
OYE 3          1 MPFVKGFEPISLRDTNLFEPIKIGNTQLAHRAVMPPLTRMRATHPGNIPNKEWAAVYYGQ
                 * *** * *  * ***** ******  * **** ******** *******  **  ** *

OYE 1         61 RAQRPGTMIITEGAFISPQAGGYDNAPGVWSEEQMVEWTKIFNAIHEKKSFVWVQLWVLG
OYE 3         61 RAQRPGTMIITEGTFISPQAGGYDNAPGIWSDEQVAEWKNIFLAIHDCQSFAWVQLWSLG
                 ************* ************** ** **  **  ** ***   ** ***** **

OYE 1        121 WAAFPDNLARDGLRYDSASDNVFMDAEQEAKAKKANNPQHSLTKDEIKQYIKEYVQAAKN
OYE 3        121 WASFPDVLARDGLRYDCASDRVYMNATLQEKAKDANNLEHSLTKDDIKQYIKDYIHAAKN
                 ** *** ********* *** * * *    *** ***  ****** ****** *  ****

OYE 1        181 SIAAGADGVEIHSANGYLLNQFLDPHSNTRTDEYGGSIENRARFTLEVVDALVEAIGHEK
OYE 3        181 SIAAGADGVEIHSANGYLLNQFLDPHSNKRTDEYGGTIENRARFTLEVVDALIETIGPER
                 *************************** ******* ************** * ** *

OYE 1        241 VGLRLSPYGVFNSMSGGAETGIVAQYAYVAGELEKRAKAGKRLAFVHLVEPRVTNPFLTE
OYE 3        241 VGLRLSPYGTFNSMSGGAEPGIIAQYSYVLGELEKRAKAGKRLAFVHLVEPRVTDPSLVE
                 ********* ********* ** *** ** ************************ * * *

OYE 1        301 GEGEYEGGSNDFVYSIWKGPVIRAGNFALHPEVVREEVKDKRTLIGYGRFFISNPDLVDR
OYE 3        301 GEGEYSEGTNDFAYSIWKGPIIRAGNYALHPEVVREQVKDPRTLIGYGRFFISNPDLVYR
                 *****  * *** ******* ***** ********* *** ***************** *

OYE 1        361 LEKGLPLNKYDRDTFYQMSAHGYIDYPTYEEALKLGWDKK
OYE 3        361 LEEGLPLNKYDRSTFYTMSAEGYTDYPTYEEAVDLGWNKN
                 ** ********* *** *** ** ********  *** *
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OYE 1 versus OYE 2

92.0% identity in 399 residues overlap; Score: 1969.0; Gap frequency: 0.0%

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OYE 1          1 MSFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYTQ
OYE 2          1 MPFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRAQHPGNIPNRDWAVEYYAQ
                 * ************************************** ************** *

OYE 1         61 RAQRPGTMIITEGAFISPQAGGYDNAPGVWSEEQMVEWTKIFNAIHEKKSFVWVQLWVLG
OYE 2         61 RAQRPGTLIITEGTFPSPQSGGYDNAPGIWSEEQIKEWTKIFKAIHENKSFAWVQLWVLG
                 ******* ***** * *** ******** *****  ****** **** *** ********

OYE 1        121 WAAFPDNLARDGLRYDSASDNVFMDAEQEAKAKKANNPQHSLTKDEIKQYIKEYVQAAKN
OYE 2        121 WAAFPDTLARDGLRYDSASDNVYMNAEQEEKAKKANNPQHSITKDEIKQYVKEYVQAAKN
                 ****** *************** * **** *********** ******** *********

OYE 1        181 SIAAGADGVEIHSANGYLLNQFLDPHSNTRTDEYGGSIENRARFTLEVVDALVEAIGHEK
OYE 2        181 SIAAGADGVEIHSANGYLLNQFLDPHSNNRTDEYGGSIENRARFTLEVVDAVVDAIGPEK
                 **************************** ********************** * *** **

OYE 1        241 VGLRLSPYGVFNSMSGGAETGIVAQYAYVAGELEKRAKAGKRLAFVHLVEPRVTNPFLTE
OYE 2        241 VGLRLSPYGVFNSMSGGAETGIVAQYAYVLGELERRAKAGKRLAFVHLVEPRVTNPFLTE
                 **************************** **** ************************

OYE 1        301 GEGEYEGGSNDFVYSIWKGPVIRAGNFALHPEVVREEVKDKRTLIGYGRFFISNPDLVDR
OYE 2        301 GEGEYNGGSNKFAYSIWKGPIIRAGNFALHPEVVREEVKDPRTLIGYGRFFISNPDLVDR
                 ***** **** * ******* ******************** ********************

OYE 1        361 LEKGLPLNKYDRDTFYQMSAHGYIDYPTYEEALKLGWDKK
OYE 2        361 LEKGLPLNKYDRDTFYKMSAEGYIDYPTYEEALKLGWDKN
                 **************** *** ******************
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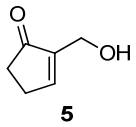
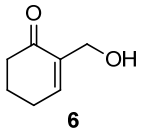
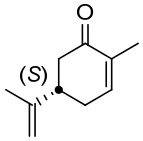
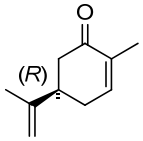
Supporting Information Table 1. Conversion data for Trp 116 site-saturation mutagenesis data for OYE 3 and OYE 1. Reactions that yield products from "flipped" substrate binding orientations are shown in red, and key results are highlighted by grey shading.

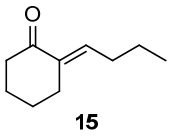
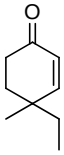
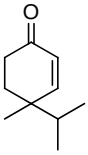
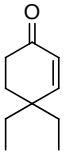
Amino acid at position 116	% ee				%de			
	 5		 6		 7		 8	
	OYE 3	OYE 1[a]	OYE 3	OYE1[a]	OYE 3	OYE 1[b]	OYE 3	OYE 1[b]
W (wt)	---[c]	51%	---	---	74%	91%	13%	94%
A	20%	>98%	16%	84%	---	93%	8% (*cis*)	77%
C	---	47%	12%	31%	18%	13%	---	---
D	---	95%	---	>98%	25%	32%	24%	54%
E	47%	96%	47%	93%	37%	15%	37%	---
F	49%	98%	33%	>98%	73%	76%	29%	92%
G	12%	>98%	12%	98%	31%	26%	16%	---
H	77%	>98%	93%	>98%	64%	20%	28%	76%
I	---	>98%	---	>98%	24%	90%	816%	79%
K	---	75%	---	60%	16%	---	15%	18%
L	40%	>98%	48%	>98%	89%	96%	20%	97%
M	29%	>98%	50%	>98%	77%	86%	25%	87%
N	53%	>98%	45%	>98%	44%	81%	20%	82%
P	---	16%	---	14%	19%	86%	38%	75%
Q	62%	>98%	76%	>98%	47%	89%	25%	78%
R	---	---	---	---	20%	---	20%	---
S	---	87%	15%	84%	14%	41%	25%	---
T	12%	44%	16%	28%	22%	32%	32%	---
V	30%	97%	13%	84%	46%	93%	17%	72%
Y	82%	>98%	34%	>98%	43%	49%	14%	93%

[a]Walton, A.Z.; Conerly, W.C.; Pompeu, Y.A.; Sullivan, B.; Stewart, J.D. *ACS Catal.* **2011**, *1*, 989-993.

[b]Pompeu, Y.A.; Sullivan, B.; Stewart, J.D. *ACS Catal.* **2013**, *3*, 2376-2390.

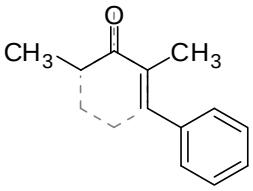
[c]≤ 10% substrate conversion after 24 hr was observed.

Supporting Information Table 2. Conversion data for substrates 15 – 19 reduced by OYE 3 Trp 116 variants.

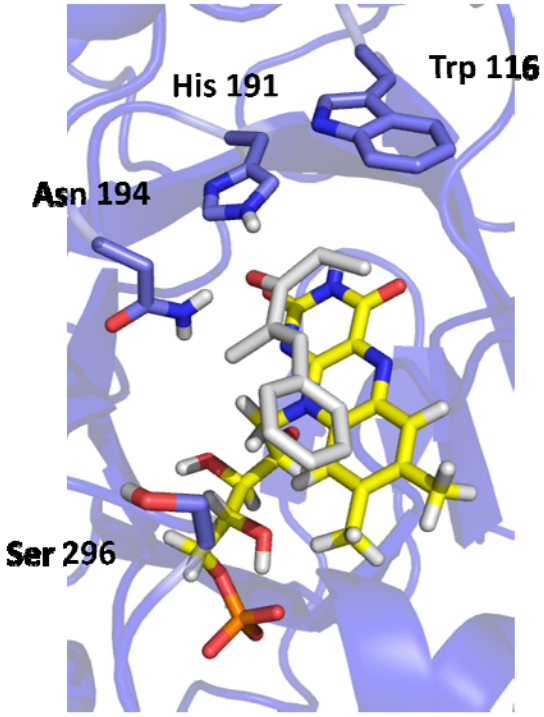
Amino acid at position 116	 15	 16	 17	 18	 19
W (wt)	47%	95%	53%	--- [a]	---
A	36%	59%	42%	---	---
C	26%	86%	37%	---	21%
D	22%	34%	39%	---	---
E	19%	72%	41%	---	---
F	29%	60%	50%	---	---
G	15%	55%	31%	---	---
H	30%	70%	37%	---	---
I	32%	67%	52%	---	---
K	44%	24%	28%	---	---
L	36%	53%	54%	---	---
M	---	88%	51%	11%	---
N	72%	91%	50%	---	---
P	45%	67%	57%	22%	---
Q	30%	73%	43%	---	---
R	28%	36%	51%	---	---
S	44%	51%	24%	---	---
T	40%	58%	47%	20%	---
V	---	65%	41%	---	---
Y	50%	58%	38%	---	---

[a] $\leq$ 10% substrate conversion after 24 hr was observed.

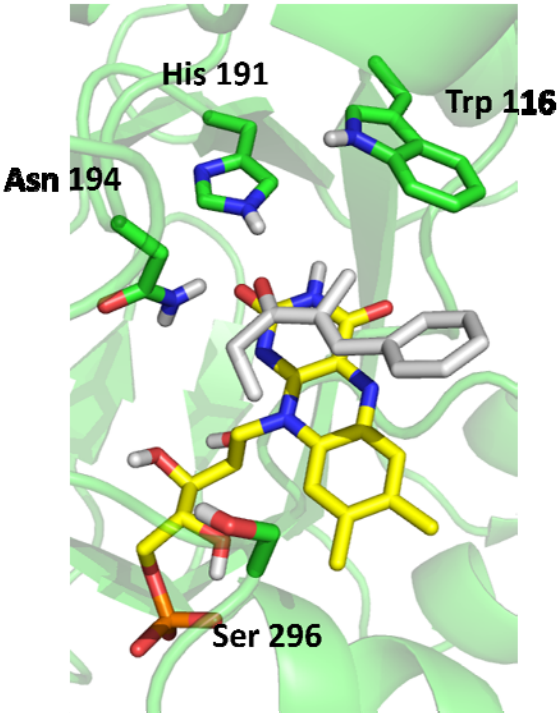
Example of modeled OYE 3 / substrate complexes in both potential binding orientations.

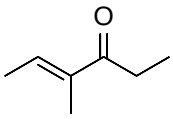


Flipped orientation

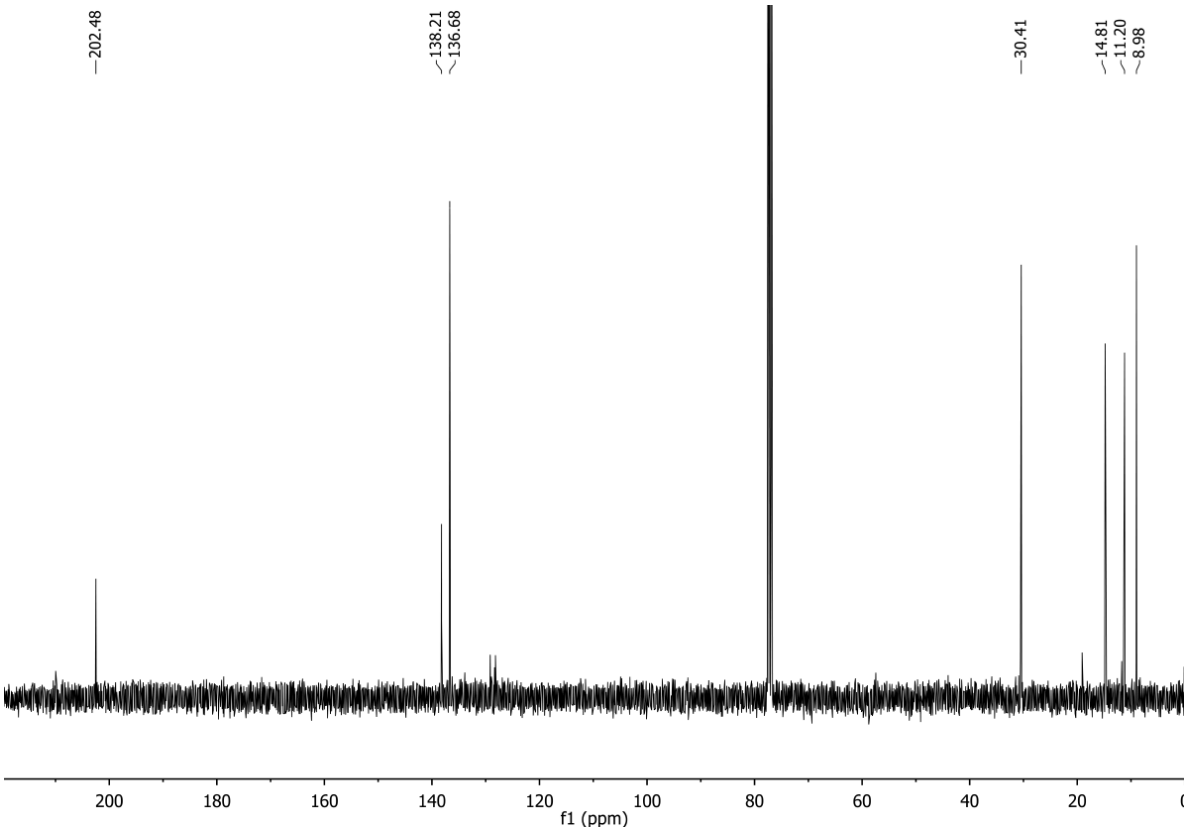


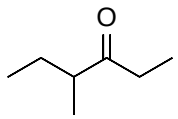
Normal orientation



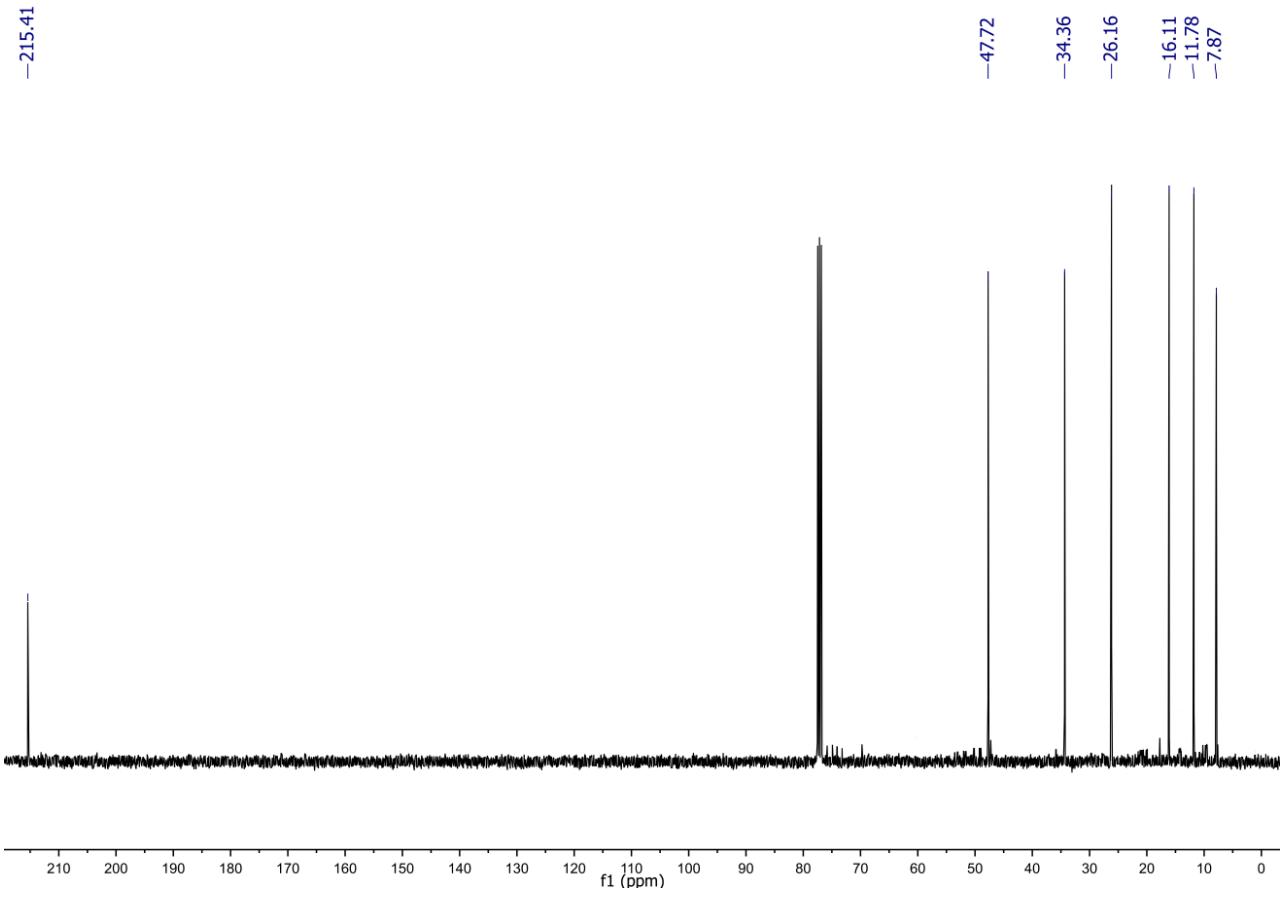

O

1.00
2.11
3.06
3.06
3.37
10
9
8
7
6
5
4
3
2
1
0
f1 (ppm)


202.48
138.21
136.68
30.41
14.81
11.20
8.98
200
180
160
140
120
100
80
60
40
20
f1 (ppm)


O

3.00
1.12
1.47
6.17
3.05
f1 (ppm)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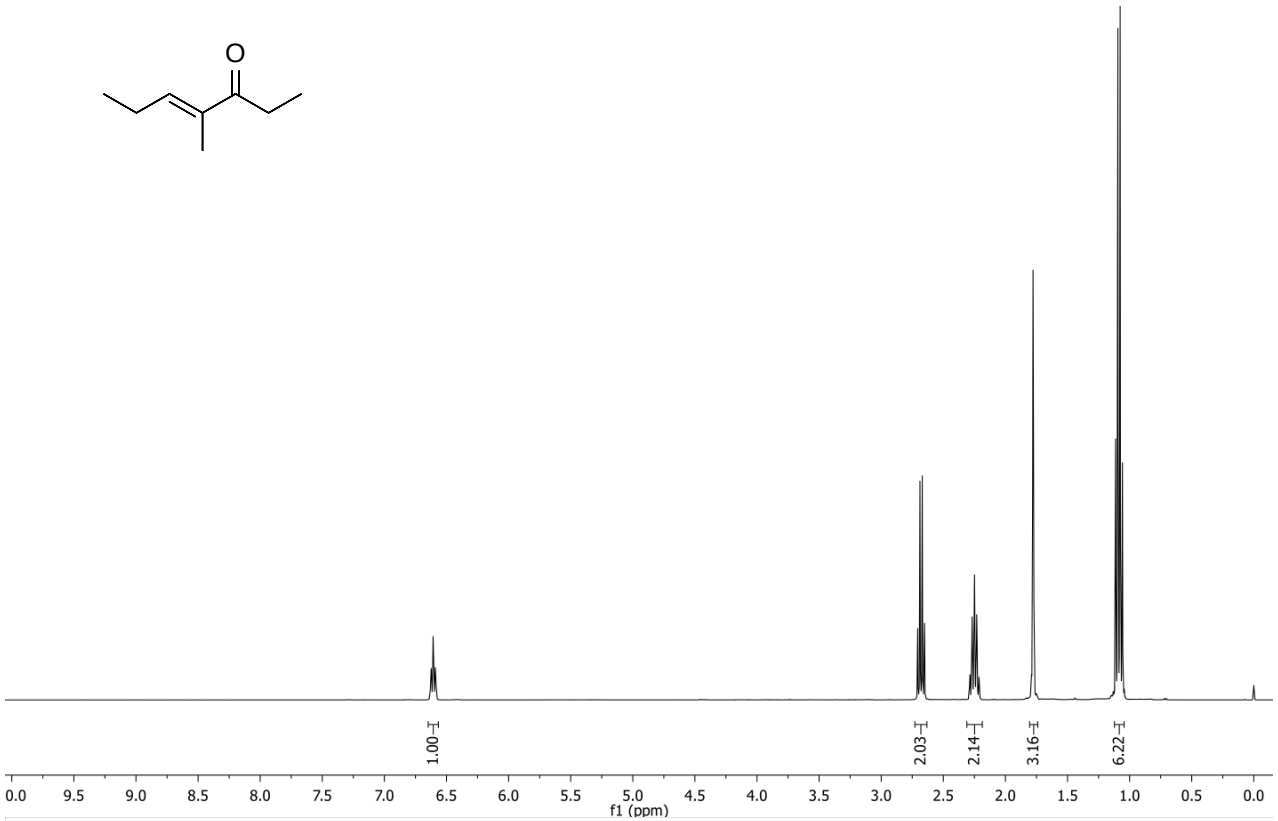
47.72
34.36
26.16
16.11
11.78
7.87
f1 (ppm)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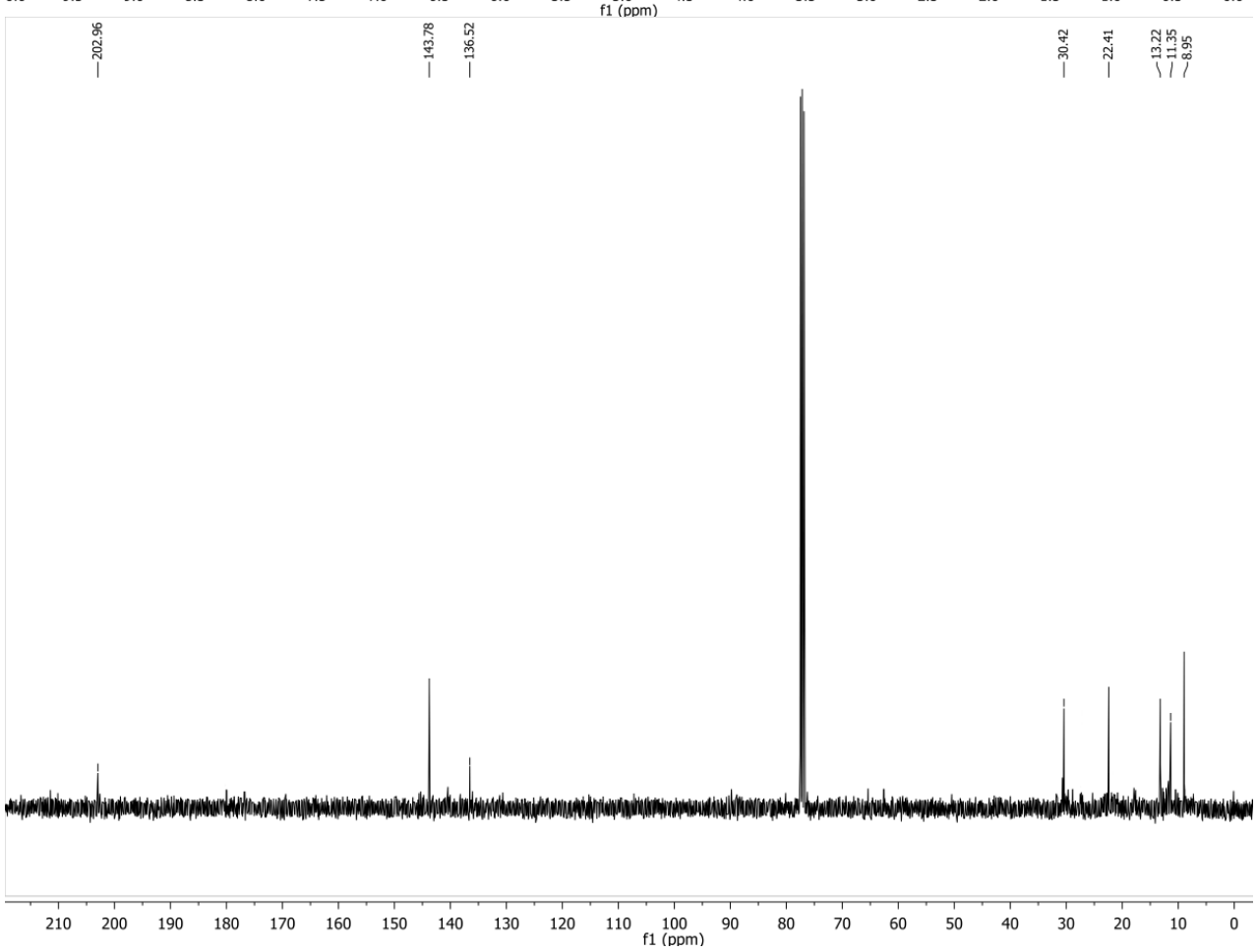
3.16
6.22
f1 (ppm)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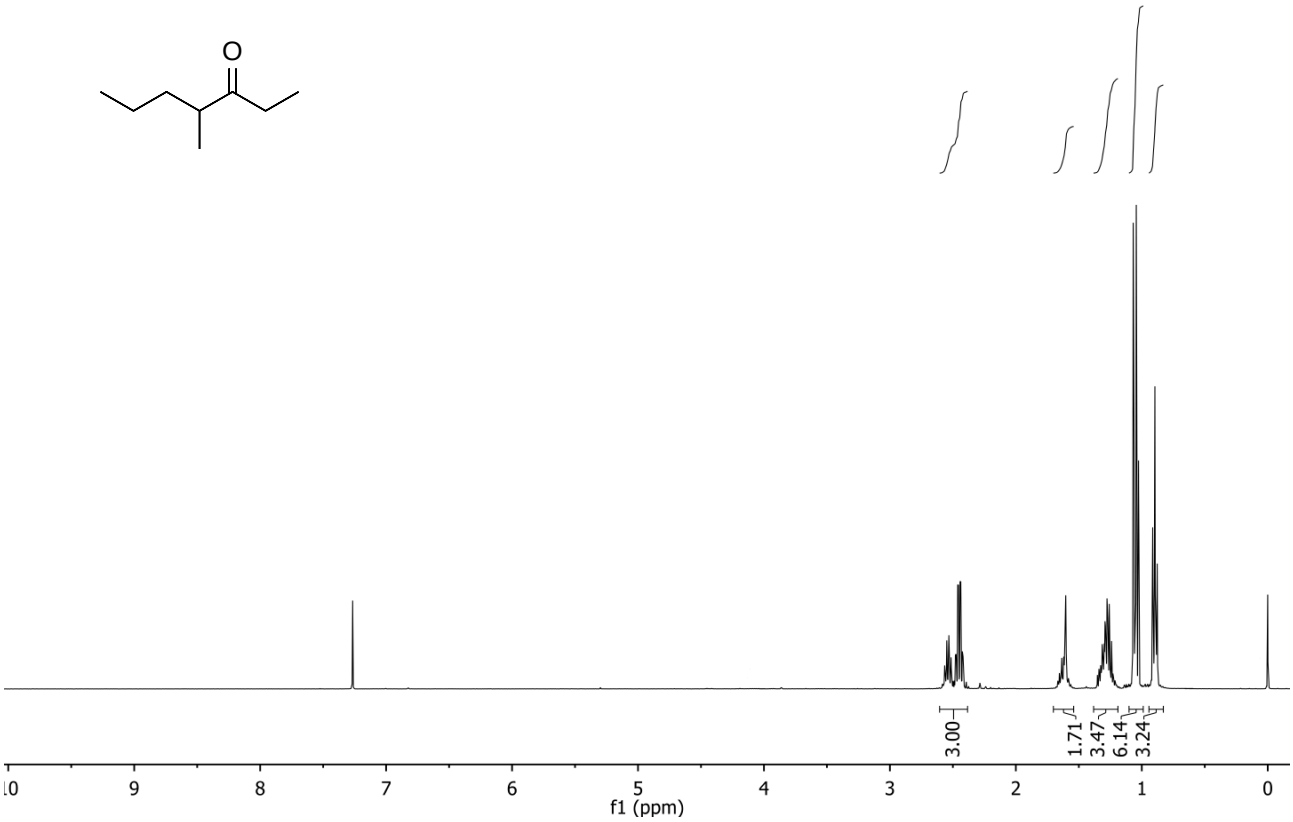
8.95
f1 (ppm)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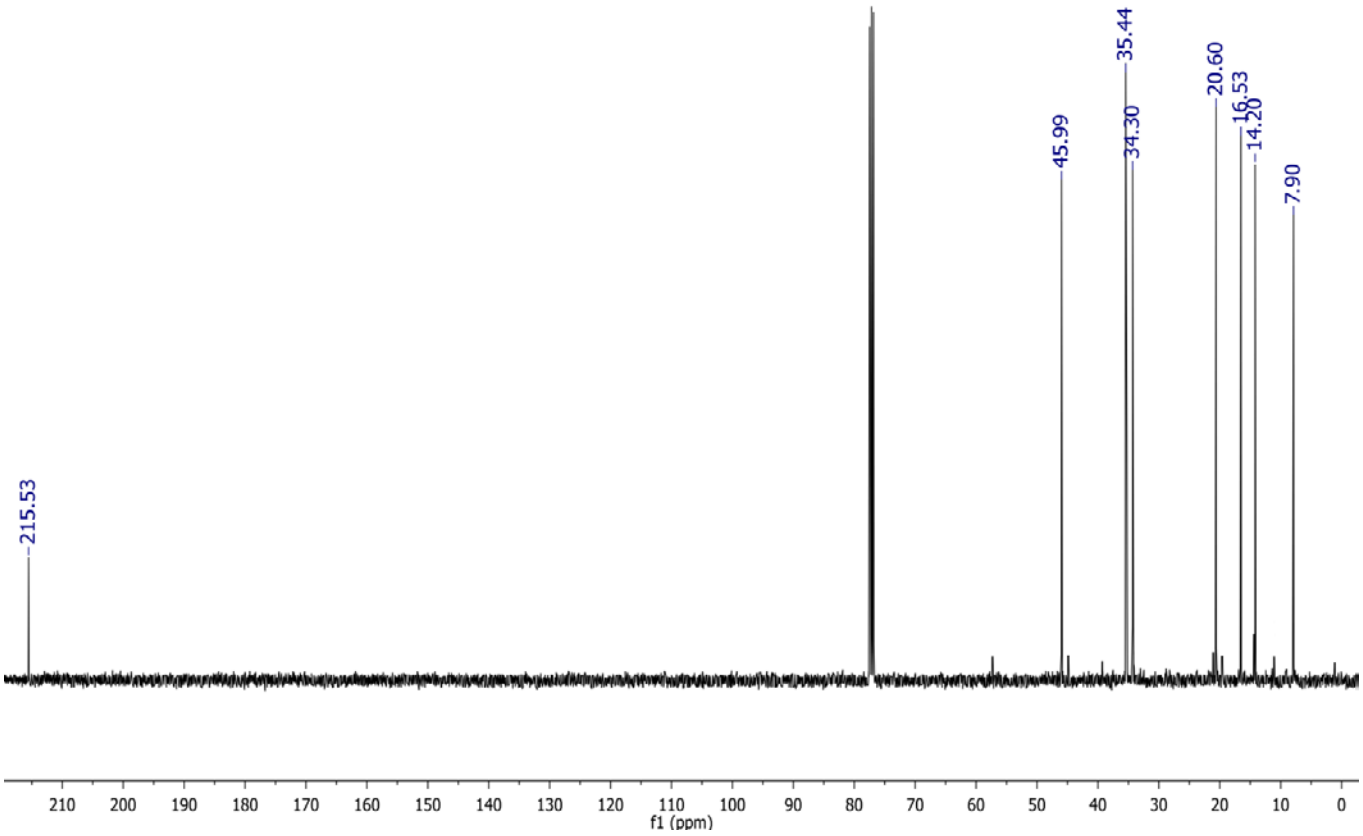
f1 (ppm)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45.99
35.44
34.30
20.60
16.53
14.20
7.90
f1 (ppm)