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

Supporting Information for

Discovery of Dimethylsulfoxonium Propionate Lyases – A Missing Enzyme Relevant to the Global Sulfur Cycle

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Strains and culture conditions

Celeribacter halophilus DSM 26270, *Dinoroseobacter shibae* DSM 16493 and *Ferrimonas balearica* DSM 9799 were obtained from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) and grown in marine broth (20 g L^{-1}, MB2216, Roth, Karlsruhe, Germany) for 2 – 3 days at 28 °C. *E. coli* BL21 (DE3) was purchased from Thermo Scientific (Waltham, Massachusetts, USA), and cultured in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.2, 1 L water) at 37 °C. *Saccharomyces cerevisiae* FY834 was cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C. For cultivations on agar plates 1.5% agar was added to the medium.

Gene cloning

Cloning of the genes coding for DddW, DddQ and DddP from *Ruegeria pomeroyi* DSS-3 was reported previously and followed the same method as described here.[1] *Celeribacter halophilus* DSM 26270, *Dinoroseobacter shibae* DSM 16493 and *Ferrimonas balearica* DSM 9799 were grown in marine broth for 2 – 3 days at 28 °C. The cells were collected by centrifugation and gDNA was isolated by application of the phenol/chloroform method.[2] The coding genes for DddK from *C. halophilus* (WP_231730497), DddL from *D. shibae* (WP_012179973) and DddY from *F. balearica* (accession number WP_013344058) were amplified from genomic DNA by PCR using Q5 polymerase (New England Biolabs, Ipswich, MA, USA) and the short primers listed in Table S1. The standard protocol for PCR was: initial denaturation at 98 °C for 40 sec, followed by 30 cycles of a 3 steps program (denaturation at 98 °C for 10 sec, annealing at 62 – 67 °C for 30 sec, elongation at 72 °C for 45 sec) and final elongation at 72 °C for 2 min. The long primers were used for a second PCR in which the product of the first PCR was used as a template to attach homology arms (bold underlined, Table S1) for homologous recombination in yeast with the linearised pYE-Express vector (EcoRI and HindIII digestion).[3] Homologous recombination was performed by the PEG/LiOAc method.[4] After culturing of *Saccharomyces cerevisiae* FY834 in YPD medium for 3 days, the plasmid containing the integrated gene was isolated by using the Zymoprep Yeast Plasmid Miniprep II kit (Zymoresearch, Irvine, CA, USA), followed by introduction into *E. coli* BL21 (DE3) through electroporation. The transformants were cultured on LB agar plates with kanamycin (50 μg/mL) at 37 °C overnight. Single colonies were selected and grown in liquid LB medium overnight for plasmid DNA isolation by using the PureYield Plasmid Miniprep System (Promega, Madison, WI, USA). The sequences of the cloned genes were verified by DNA sequencing.

Table S1. Primers used in this study.

Primer name	Nucleotide sequence
DddK	
AC11_Fw	ATGCAATGCCGGAAAAGAGATGC
AC11_Rv	TTATGTGGCATTATCGGAGAACCTGTAG
AC12_Fw	**GGCAGCCATATGGCTAGCATGACTGGTGGA**ATGCAATGCCGGAAAAGAGATGC
AC12_Rv	**TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTG**TTATGTGGCATTATCGGAGAACCTGTAG
DddL	
AC13_Fw	ATGCCGGACCTGCCAAACC
AC13_Rv	CTAGACCCCCGCGCCC
AC14_Fw	**GGCAGCCATATGGCTAGCATGACTGGTGGA**ATGCCGGACCTGCCAAACC
AC14_Rv	**TCTCAGTGGTGGTGGTGGTGGTGCTCGAGTG**CTAGACCCCCGCGCCC
DddY	
IB011_Fw	ATGAAACCCATTGCCCTATT
IB011_Rv	TCAGTACTTGGTCTTGGTCA
IB012_Fw	**GGCAGCCATATGGCTAGCATGACTGGTGGA**ATGAAACCCATTGCCCTATT
IB012_Rv	**TCTCAGTGGTGGTGGTGGTGGTGCTCGAGT**TCAGTACTTGGTCTTGGTCA

Gene expression and protein purification

E. coli BL21 cells carrying the respective expression plasmid were precultured in LB medium (10 mL) containing kanamycin (50 μg/mL) at 37 °C and 160 rpm overnight. Pre-cultures were used to inoculate expression cultures in LB medium (1 L) containing kanamycin (50 μg/mL). The expression cultures were grown to OD_{600} = 0.5–0.6 at 37 °C at 160 rpm and then cooled to 18 °C before addition of a solution of IPTG (0.4 mM in water, 1 mL L^{-1}). The expression cultures were incubated at 18 °C for 18 h at 160 rpm and cells were collected by centrifugation (3500 rpm, 1 h, 4 °C). The cells pellets were taken up in lysis buffer (For DddW and DddQ: 10 mM Tris, 200 mM NaCl, 20 mM imidazole, pH 8; for DddP: 20 mM MES, 50 mM NaCl, 20 mM imidazole, pH 6; DddK: 50 mM HEPES, 300 mM NaCl, 5 mM imidazole, pH 7.5; DddL and DddY: 100 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 10 mg lysozyme, pH 8; 15 mL for 1 L of cell culture) and ultra-sonicated (DddW, DddQ, DddP, DddL and DddY for 5x 30 sec intervals, DddK for 5x 4 sec interval). The cell debris was removed by centrifugation (10000 x g, 10 min, 4 °C). The supernatant was passed through a cellulose filter (20 μm) and loaded onto a Ni^{2+}-NTA affinity chromatography column. The column was washed using lysis buffer (2x 10 mL), followed by elution of the target protein using elution buffer (DddW: 10 mM Tris, 200 mM NaCl, 500 mM imidazole, pH 8, 10 mL; DddQ: 10 mM Tris, 200 mM NaCl, 100 mM imidazole, pH 8, 10 mL; DddP: 20 mM MES, 50 mM NaCl, pH 6, 10 mL; DddK: 50 mM HEPES, 300 mM NaCl, 500 mM imidazole, pH 7.5, 10 mL; DddY: 100 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, 10 mg lysozyme, 150 mM imidazole, pH 8, 10 mL). DddL was used without purification, because this enzyme lost activity during purification. The enzyme concentrations were calculated through Bradford assay.[5] The enzyme solutions were stored at –80 °C with glycerol (10% v/v) added.

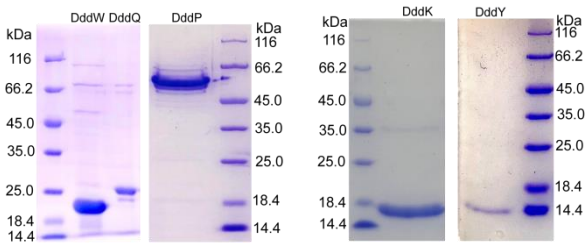
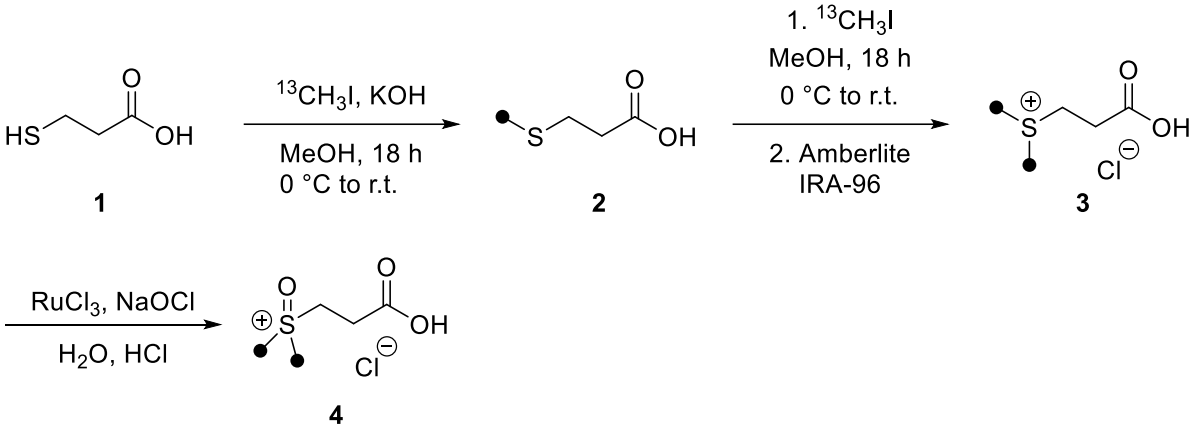


Figure S1. SDS-PAGE analysis of DddW, DddQ, DddP, DddK and DddY.

General synthetic and analytical methods

All chemicals were purchased from TCI Deutschland or Sigma Aldrich and used without purification. Solvents were dried according to standard procedures and purified by distillation. Silica gel 60 (100 – 200 mesh) was used for column chromatography. Thin-layer chromatography was performed with 0.2 mm pre-coated plastic sheets Polygram Sil G/UV254 (Machery-Nagel). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AV I (400 MHz) or AV III HD Prodigy (500 MHz) spectrometer in $CDCl_3$ or D_2O. Spectra were referenced against solvent signals of $CDCl_3$ (δ = 7.26 ppm) and D_2O (δ = 4.79 ppm) for ^{1}H-NMR and $CDCl_3$ (δ = 77.01 ppm) for ^{13}C-NMR.[6] IR spectra were recorded on a Bruker α infrared spectrometer with a diamond ATR probehead. Peak intensities are given as s (strong), m (medium), w (weak) and br (broad). Mass was recorded on LTQ Orbitrap XL (ESI-NEG, Fisher Scientific) using acetonitrile as solvent.



Scheme S1. Synthesis of (*methyl*-$^{13}C_2$)DMSOP.

Synthesis of 3-((^13^C)methylthio)propanoic acid (2)

To a solution of 3-mercaptopropanoic acid (**1**, 2.00 g, 18.8 mmol) in methanol (8 mL) KOH (2.64 g, 45.5 mmol, 2.5 equiv. in 6 mL MeOH) was added dropwise at 0 °C. Then $^{13}CH_3I$ (7.3 g, 50.9 mmol, 2.7 equiv.) was added dropwise over 15 min at the same temperature. The reaction mixture was allowed to stir for 18 h at room temperature. After completion the reaction was quenched by the addition of water and adjusted to pH 3 using HCl (6 N). The aqueous layer was extracted with EtOAc (3x). The combined organic phases were dried over $MgSO_4$, filtered and then concentrated in vacuo to give **2** (1.83 g, 15.3 mmol, 81%) as a yellowish oil.

TLC (cyclohexane/EtOAc = 1:1): R_f = 0.62. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 2.77 (m, 2H), 2.68 (m, 2H), 2.27 (s, 1H), 1.99 (s, 1H) ppm. ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 178.17 (C), 34.34 (CH_2), 28.84 (CH_2), 15.67 (CH_3) ppm. IR (diamond-ATR): $\tilde{\nu}$ = 3032 (w), 2964 (w), 2915 (w), 2663 (w), 2565 (w), 1706 (s), 1427 (m), 1336 (w), 1244 (w), 1199 (w), 1148 (w), 934 (w), 809 (w), 652 (w).

Synthesis of (*methyl*-^13^C~2~)dimethylsulfoniopropionate (3)

Compound **2** (1.7 g, 14.0 mmol) was dissolved in nitromethane (5 mL) and treated with $^{13}CH_3I$ (5.41 g, 37.9 mmol, 2.7 equiv.). The reaction mixture was stirred for 24 h at

room temperature. After completion the reaction was quenched by the addition of water (20 mL), followed by extraction with EtOAc (3x). The aqueous phase was concentrated in vacuo to 10 mL and subjected to anion exchange chromatography. For this purpose, the OH^- form of the ion exchange resin (Amberlite IRA-96) in a glas column was washed with two column volumes of HCl (1 M) and then with water until pH 6 was reached. The aqueous solution of the salt was loaded onto the column, followed by slow elution with 2 column volumes of distilled water. Lyophilisation of elution fraction yielded **3** (2.04 g, 11.6 mmol, 83%) as a white solid.
^{1}H-NMR (D_2O, 500 MHz): δ = 3.56 (tt, *J* = 6.8, 2.9 Hz, 2H), 3.10 (d, *J* = 3.6 Hz, 3H), 3.02 (t, *J* = 6.9 Hz, 2H), 2.81 (d, *J* = 3.6 Hz, 3H) ppm. ^{13}C-NMR (D_2O, 125 MHz): δ = 173.63 (C), 38.73 (CH_2), 28.47 (CH_2), 25.17(2xCH_3) ppm. IR (diamond-ATR): $\tilde{\nu}$ = 2997 (w), 2693 (w), 2607 (w), 2547 (w), 2474 (w), 2421 (w), 1783 (w), 1723 (w), 1692 (m), 1411 (w), 1395 (w), 1323 (w), 1323 (w), 1246 (w), 1185 (m), 1041 (w), 1002 (w), 904 (w), 753 (w), 638 (w). HRMS (EI, 70 eV): calculated for $[C_3{}^{13}C_2H_{11}SO_2]^+$ *m*/*z* 137.0541, found *m*/*z* 137.0542.

Synthesis of (*methyl*-$^{13}C_2$)dimethylsulfoxonium propionate (4)
Compound **3** (100 mg, 0.58 mmol) was dissolved in H_2O (2 mL), followed by the addition of $RuCl_3$ (0.24 mL, 0.24 M). After 5 min NaOCl (12% in H_2O) was added dropwise until the color changed from black to light yellow. After a few minutes of stirring, the colour of the reaction changed to brown. More NaOCl was added dropwise until the colour again changed to light yellow. The reaction mixture was adjusted to pH 5 by the addition of HCl (1 N). The reaction mixture was stirred for 30 min and then water was evaporated to obtain a white solid. The solid was dissolved in the minimum amount of MeOH (ca. 1 mL). The desired product was precipitated by the dropwise addition of diethyl ether to yield pure **4** (78 mg, 4.1 mmol, 71%) as a white solid.
^{1}H-NMR (D_2O, 500 MHz): δ = 4.21 (t, *J* = 7.8 Hz, 2H), 3.99 (d, 3H), 3.70 (d, 3H), 2.93 (t, *J* = 7.0 Hz, 2H) ppm. ^{13}C-NMR (D_2O, 125 MHz): δ = 175.62 (C), 50.01 (CH_2), 38.40 (2xCH_3), 27.85 (CH_2) ppm. IR (diamond-ATR): $\tilde{\nu}$ = 3010 (w), 2970 (w), 2950 (w), 2156 (w), 1954 (w), 1728 (m), 1437 (w), 1365 (w), 1228 (w), 1216 (w), 1216 (w), 1206 (w), 894 (w), 536 (w). HRMS (EI, 70 eV): calculated for $[C_3{}^{13}C_2H_{11}SO_3]^+$ *m*/*z* 153.0491, found *m*/*z* 153.0490.

Enzyme activity assay with DMSOP

In order to test the enzyme activities with the substrate DMSOP, enzyme reactions with DddW, DddQ, DddP, DddK, DddL and DddY were carried out. In a 1.5 mL tube the enzyme (3 µM final concentration) was added to an incubation buffer (10 mM sodium citrate dihydrate, 50 mM Tris, pH 8; for DddP: 10 mM sodium citrate dihydrate, 10 mM citric acid, pH 6), followed by incubation for 30 min at 30 °C. Then (*methyl*-$^{13}C_2$)DMSOP (5 mM final concentration) was added and incubation was continued for 30 min at 30 °C. The reaction was stopped by heating to 90 °C for 5 min. The reaction product was measured immediately by ^{13}C-NMR spectroscopy. A control experiment was performed in the absence of enzyme by incubating (*methyl*-$^{13}C_2$)DMSOP (5 mM).

Determination of pH optima

In order to determine the pH optima of the enyzmes, enzyme reactions with DddW, DddQ, DddP, DddK, DddL and DddY were carried out at variable pH. In a 1.5 mL tube the enzyme (0.5 µM final concentration; for DddW: 0.1 µM) was added to a buffer of variable pH (10 mM sodium citrate dihydrate, 10 mM citric acid, pH 3, pH 4, pH 5 or pH 6; 100 mM sodium phosphate, pH 7; 50 mM Tris, pH 8 or pH 9; 50 mM sodium hydrogen carbonate, pH 10), followed by incubation for 30 min at 30 °C. Then (*methyl*-$^{13}C_2$)DMSOP (5 mM final concentration) was added and the incubation was continued for 30 min at 30 °C. The reaction was stopped by heating to 90 °C for 5 min. The reaction product was measured immediately by ^{13}C-NMR spectroscopy.

Determination of optimal temperature

In order to determine the temperature optima of the enzymes, the enzyme reactions with DddW, DddQ, DddP, DddK, DddL and DddY were carried out at variable temperatures. In a 1.5 mL tube the enzyme (0.5 µM final concentration; for DddW: 0.1 µM) was added to a specific enzyme buffer (for DddW, DddQ, DddK, DddL and DddY: 50 mM Tris, pH 8; for DddP: 10 mM sodium citrate dihydrate, 10 mM citric acid, pH 6), followed by incubation for 30 min at variable temperature (10 °C to 80 °C in steps of 10 °C). Then (*methyl*-$^{13}C_2$)DMSOP (5 mM final concentration) was added and incubation was continued for 30 min at the same temperature. The reaction was stopped by heating to 90 °C for 5 min. The reaction product was measured immediately by ^{13}C-NMR spectroscopy.

Determination of enzyme kinetic parameters (Michaelis-Menten)

Solutions of DMSOP or DMSPin suitable kinetic assay buffers (for DddW, DddQ, DddK, and DddY: 50 mM HEPES, 50 mM NaCl, pH 8; for DddP: 20 mM MES, 50 mM NaCl, pH 6; substrate concentration 30mM) were prepared freshly before the assay. Kinetic measurements were performed at room temperature by mixing different concentrations of DMSOP or DMSP with enzyme (initial enzyme concentration $[E]_0$ = 0.9 µM) in a UV cuvette (1 mL reaction volume). Formation of the product acrylic acid was monitored at λ = 232 nm (HEPES, pH 8) or at λ = 229 (MES, pH 6) for 5 min. The used wavelengths for absorption measurements of the formed product acrylic acid were chosen higher than the λ_{max} in the corresponding buffer (Figures S2 and S3) to prevent exceeding the measurement range of the UV-spectrometer at high substrate concentrations. Substrate concentrations of DMSOP and DMSP ranged from 0.5 mM up to 100 mM until saturation of the kinetic plot was observed. Only for DddP that showed substrate inhibition with DMSOP at high concentrations of this substrate the DMSOP concentrations ranged from 0.5 mM to 2.5 mM.

Linear regression of the absorption plot recorded by the Cary WinUV kinetics application (Agilent) was used to calculate the initial speed of the reaction (v_0). Correlation factors ($F_{abs->[c]}$) to convert measured absorptions into product concentrations were determined by measuring absorptions of different concentrations at the chosen wavelength in a concentration range for the product of 0.2 – 1.0mM in the respective buffer. Absorptions were plotted against concentrations and the slope was determined via linear regression, giving $F_{abs->[c]}$ (Table S2).

Table S2. Correlation factors to determine product concentrations.

product	buffer	λ [nm]	$F_{abs->[c]}$ [L mol^{-1}]
acrylic acid	MES (pH 6)	229	2.20±0.001
acrylic acid	HEPES (pH 8)	232	2.38±0.01

For each enzyme-substrate combination the kinetic measurements were performed in triplicates for each substrate concentration. From these replicates average and standard deviation for each data point in Figures S4 – S13, reaction rates (v_0) versus substrate concentration ([S]), were determined. The Hill function

$$v_0 = v_{max} \frac{([S]^n)}{K^n + [S]^n} \quad (1)$$

was fitted to the data using OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA), defining n = 1 to give the Michaelis-Menten equation. From these plots the Michaelis-Menten constant (K_M) and the maximum reaction rate (v_{max}) were determined using the implemented OriginPro 8.5 functions. The turnover number (k_{cat}) was then calculated using the equation

$$k_{cat} = \frac{v_{max}}{E_0} \quad (2).$$

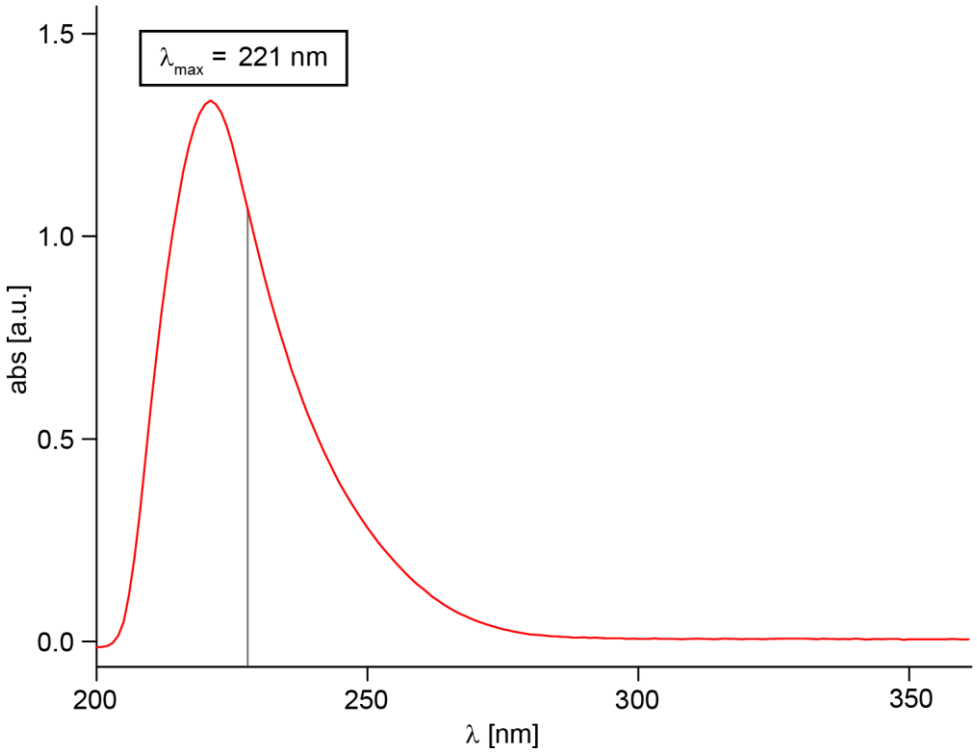


Figure S2. UV spectrum of acrylic acid in 50 mM HEPES buffer at pH 8. The vertical line indicates the wavelength chosen for kinetic measurements (λ = 232 nm).

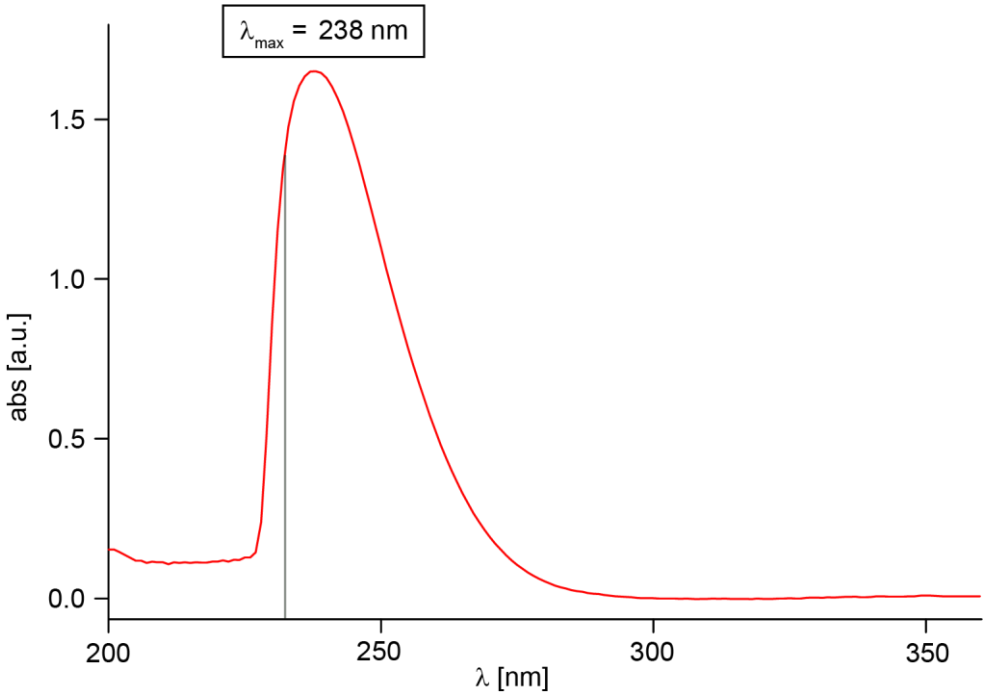


Figure S3. UV spectrum of acrylic acid in 20 mM MES buffer at pH 6. The vertical line indicates the wavelength chosen for kinetic measurements (λ = 229 nm).

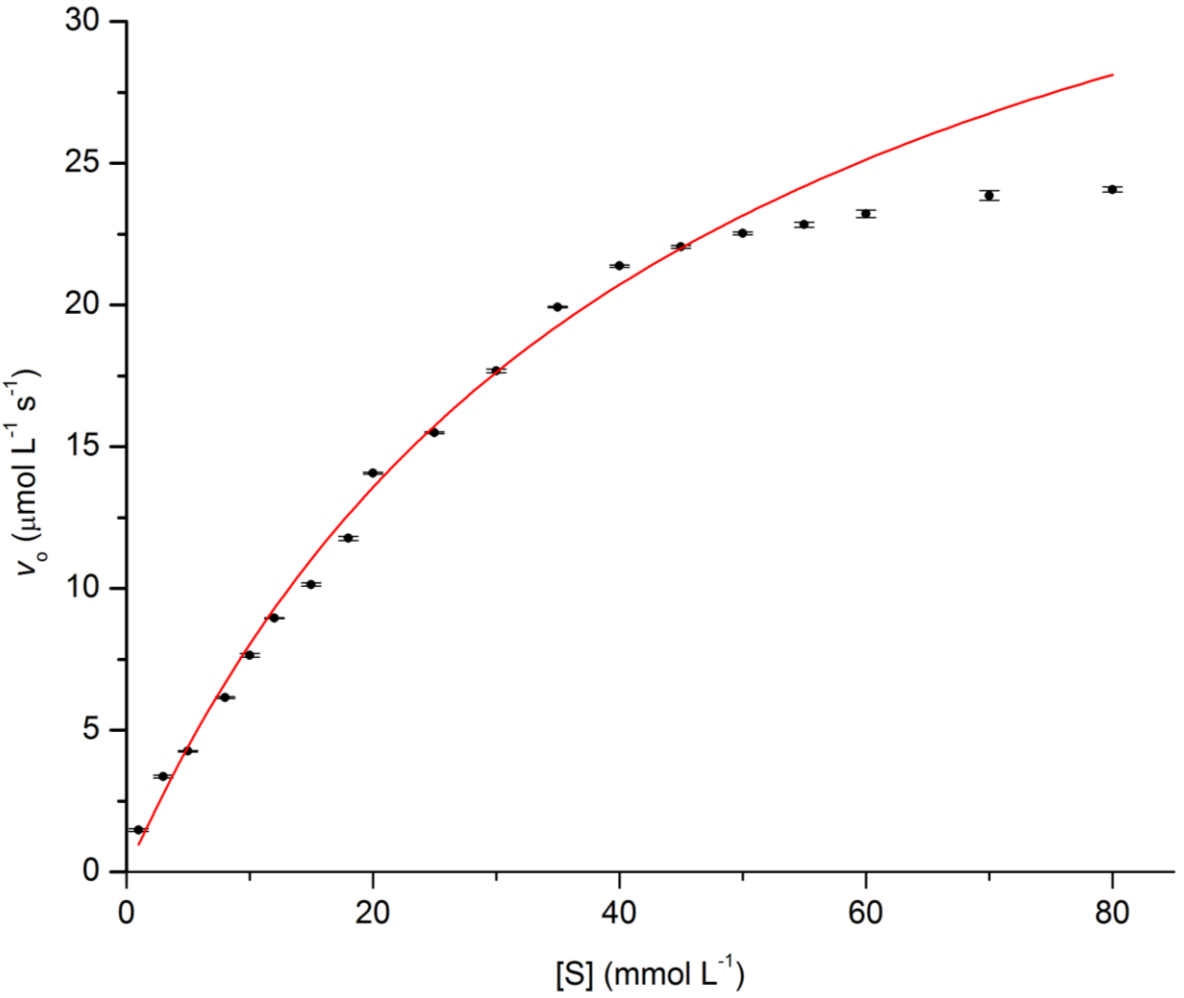


Figure S4. Michaelis-Menten plot for DddW and the substrate DMSOP. Dots and error bars indicate mean and standard deviations from triplicates.

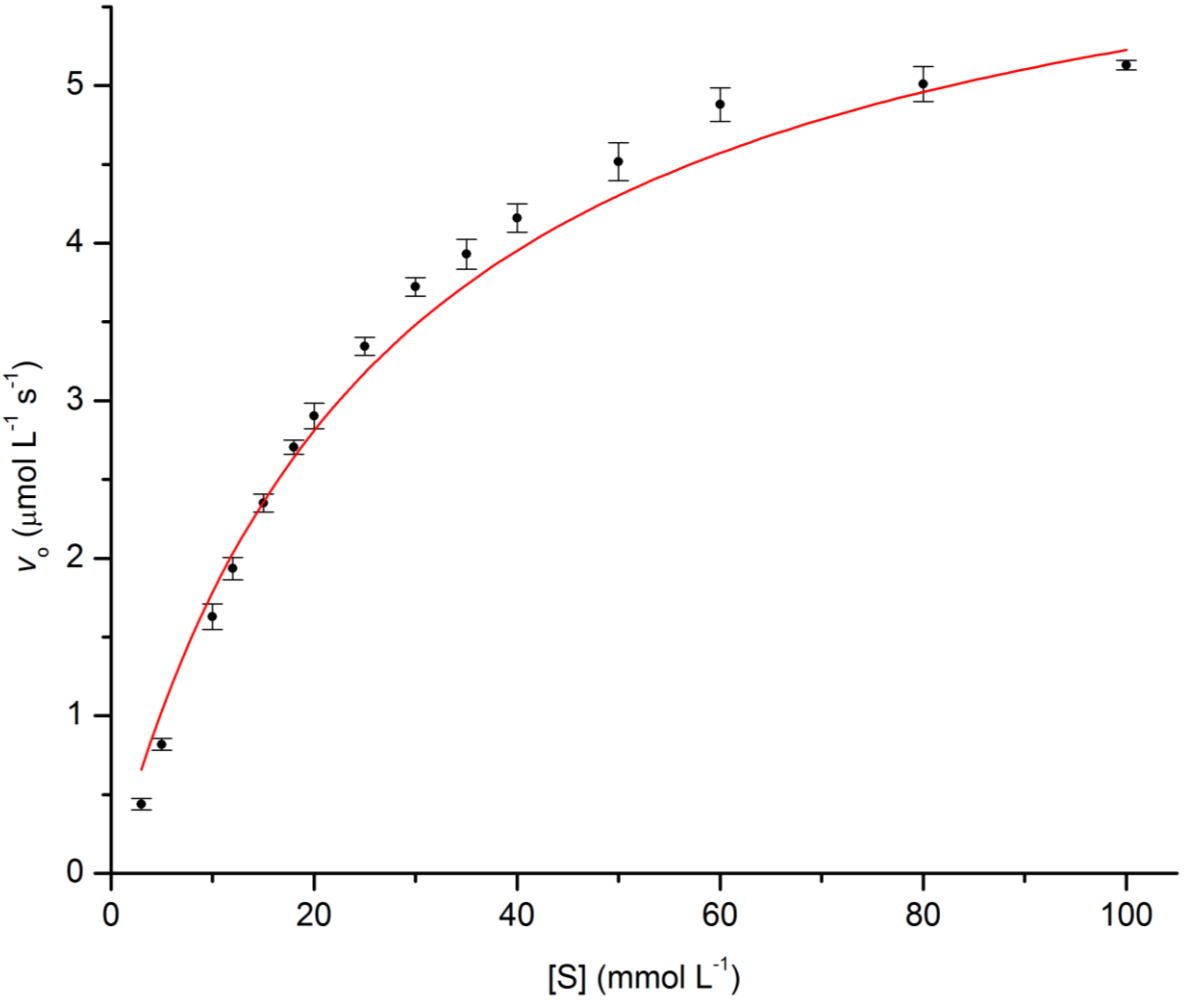


Figure S5. Michaelis-Menten plot for DddQ and the substrate DMSOP. Dots and error bars indicate mean and standard deviations from triplicates.

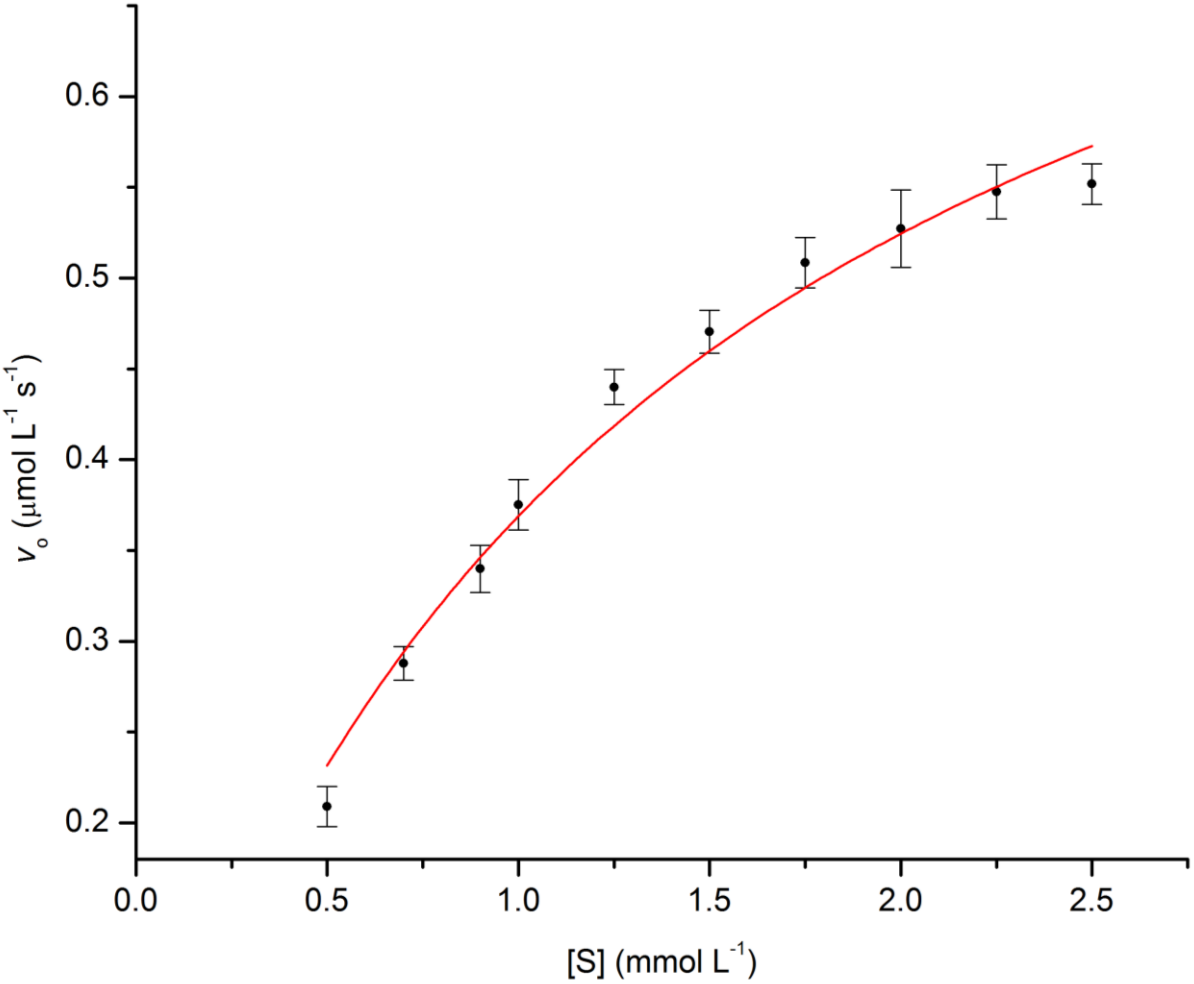


Figure S6. Michaelis-Menten plot for DddP and the substrate DMSOP. This enzyme shows substrate inhibition at concentrations higher than 2.5 mM. Dots and error bars indicate mean and standard deviations from triplicates.

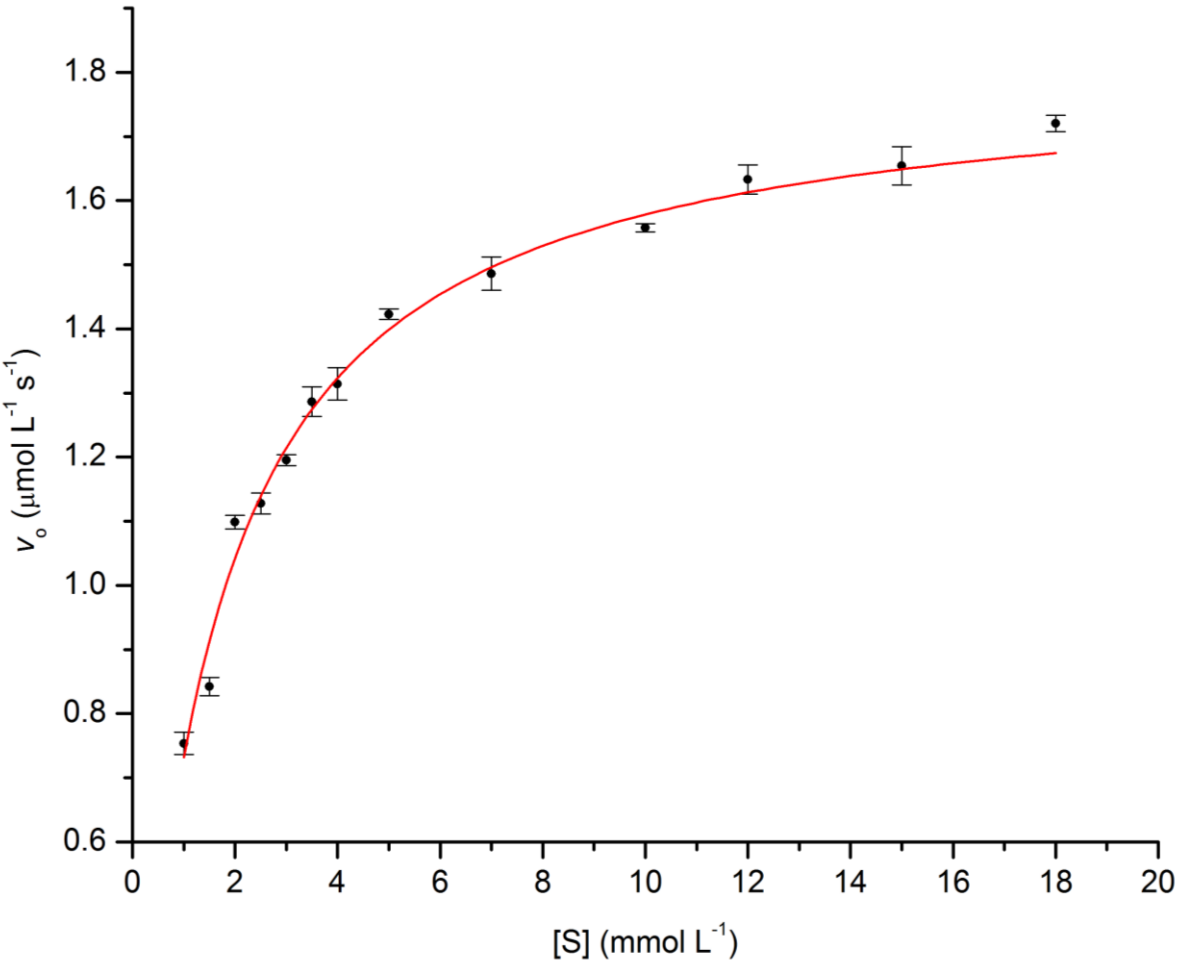


Figure S7. Michaelis-Menten plot for DddK and the substrate DMSOP. Dots and error bars indicate mean and standard deviations from triplicates.

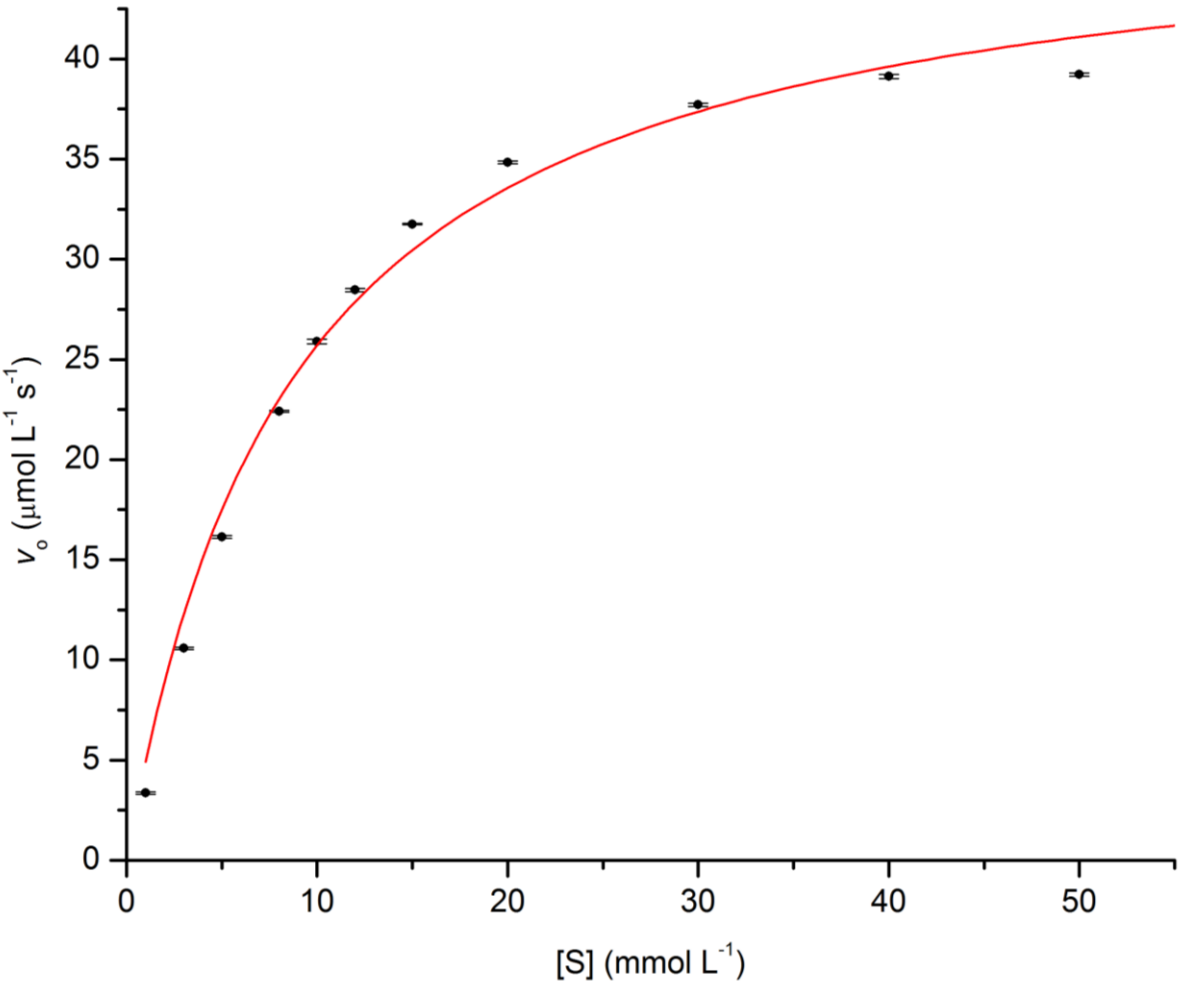


Figure S8. Michaelis-Menten plot for DddY and the substrate DMSOP. Dots and error bars indicate mean and standard deviations from triplicates.

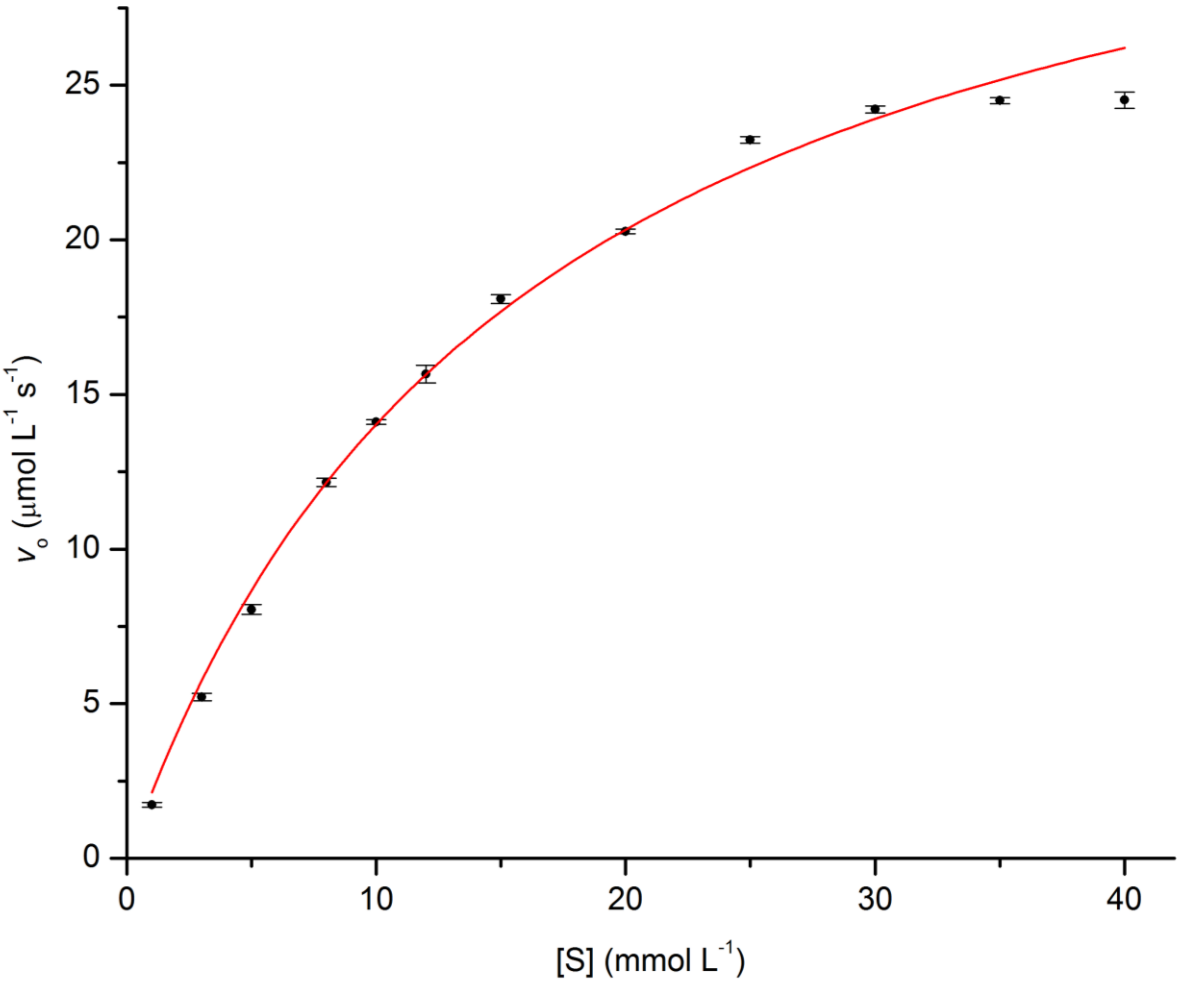


Figure S9. Michaelis-Menten plot for DddW and the substrate DMSP. Dots and error bars indicate mean and standard deviations from triplicates.

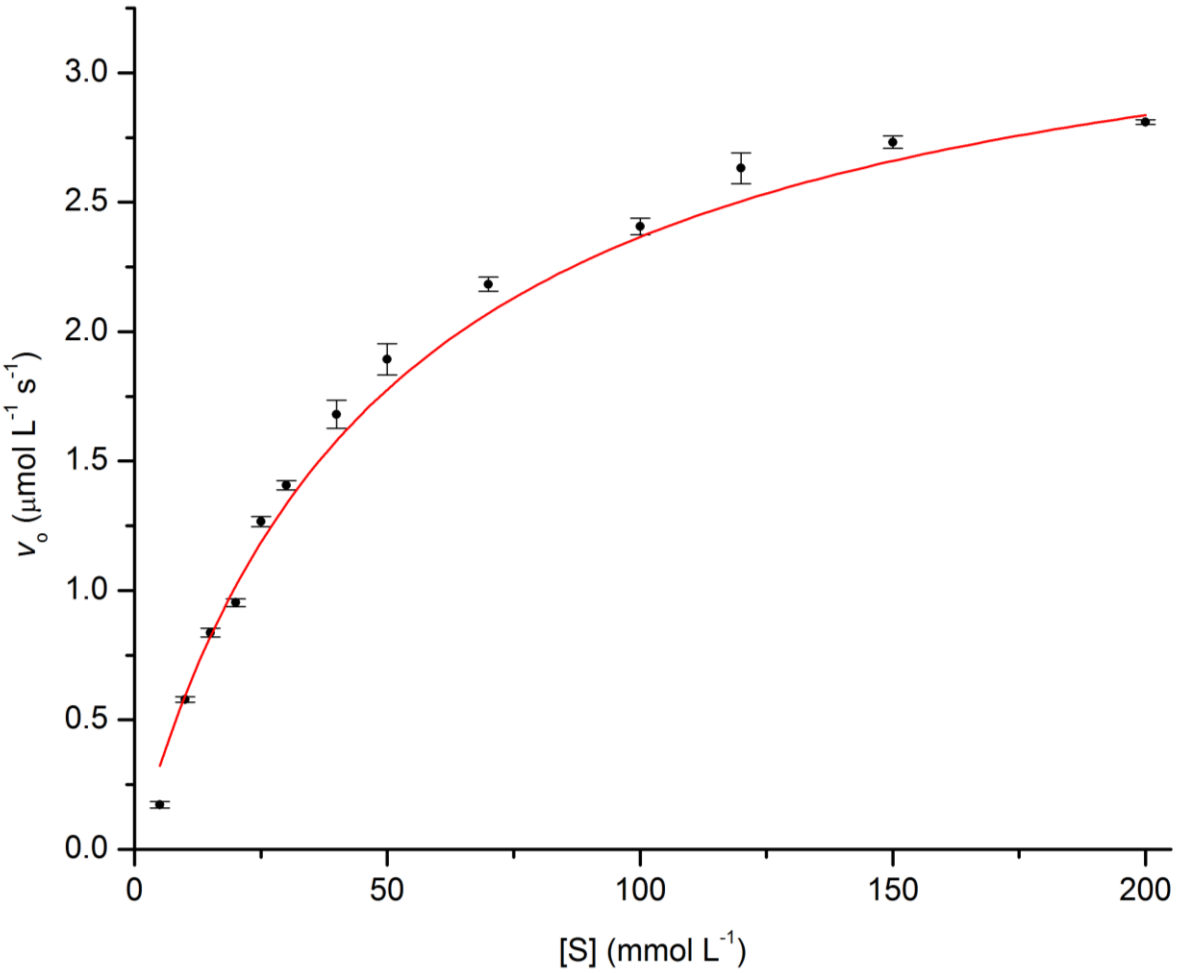


Figure S10. Michaelis-Menten plot for DddQ and the substrate DMSP. Dots and error bars indicate mean and standard deviations from triplicates.

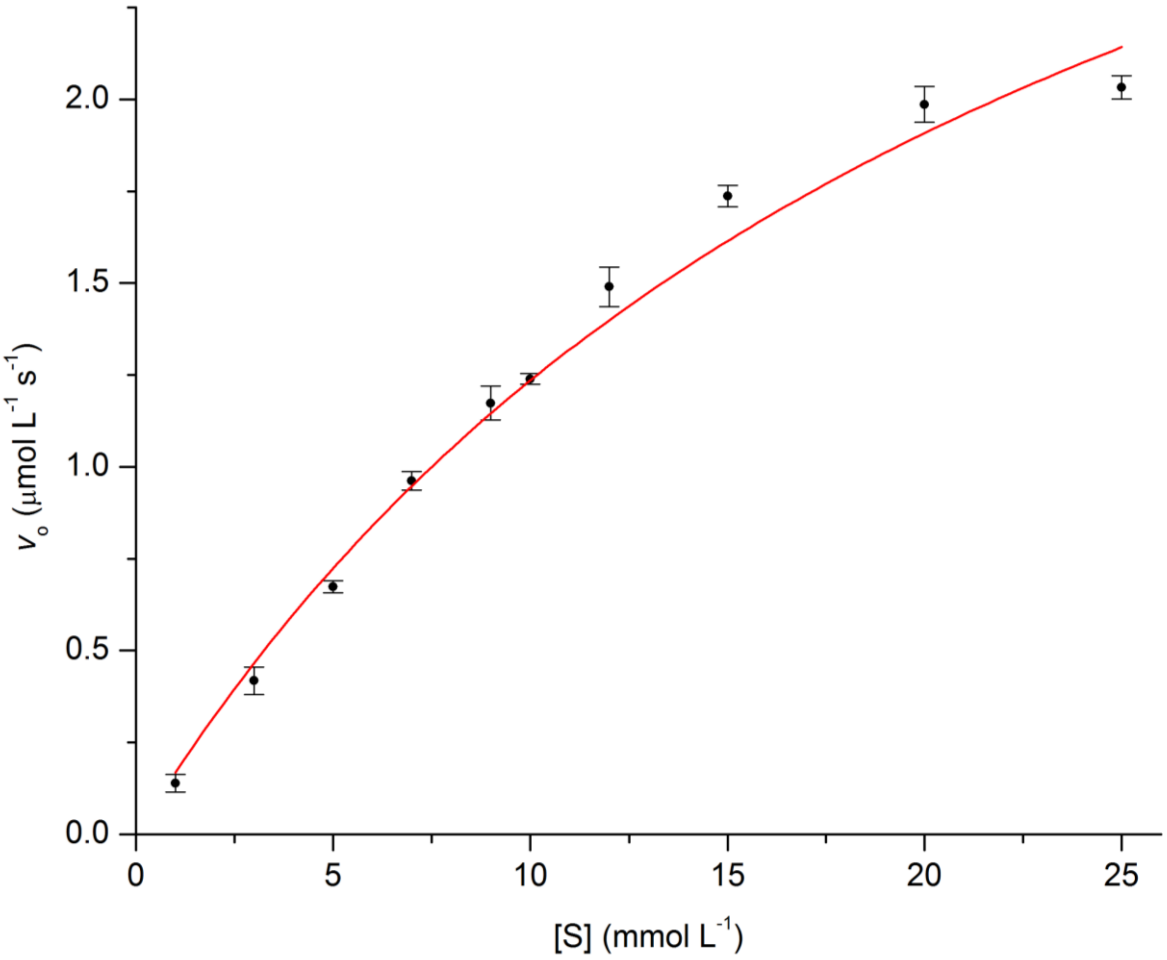


Figure S11. Michaelis-Menten plot for DddP and the substrate DMSP. Dots and error bars indicate mean and standard deviations from triplicates.

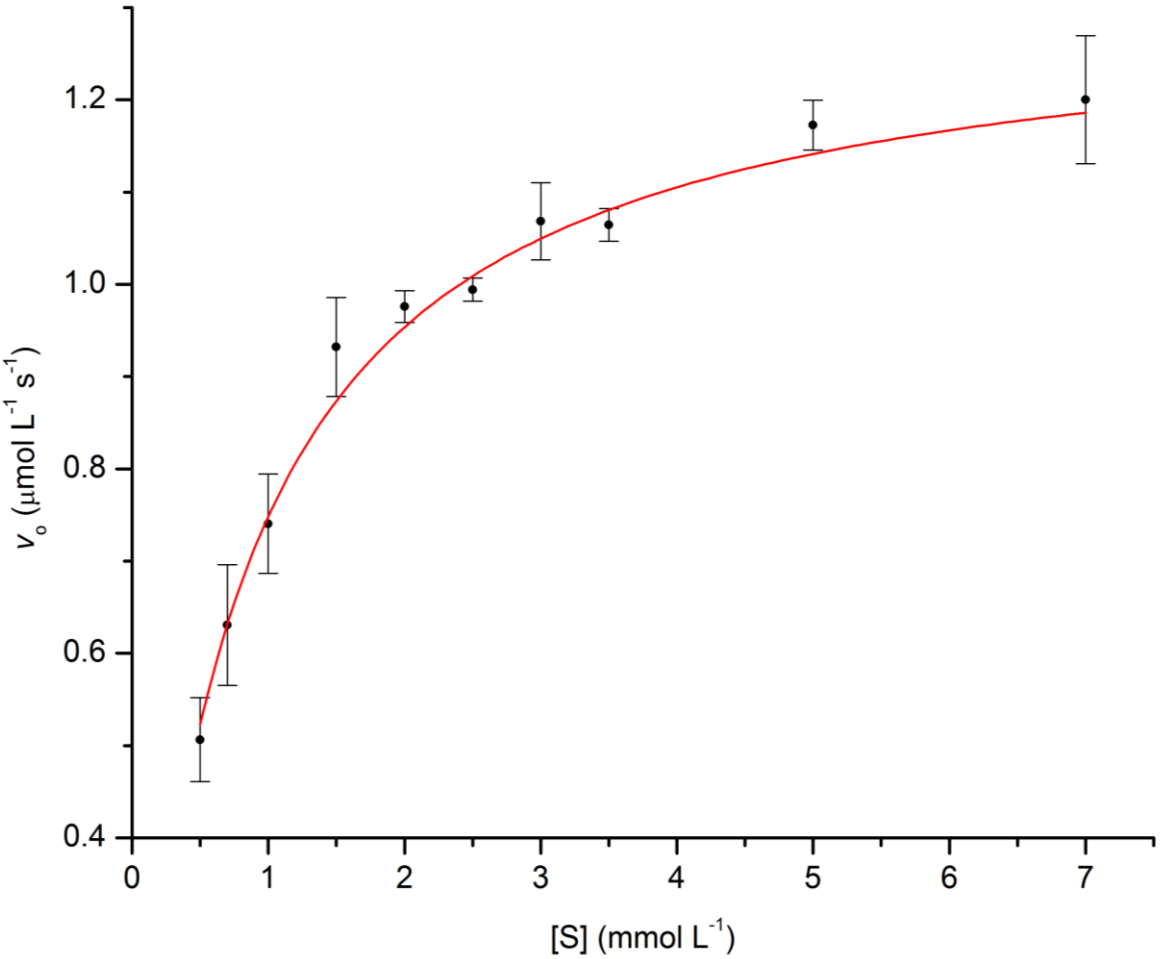


Figure S12. Michaelis-Menten plot for DddK and the substrate DMSP. Dots and error bars indicate mean and standard deviations from triplicates.

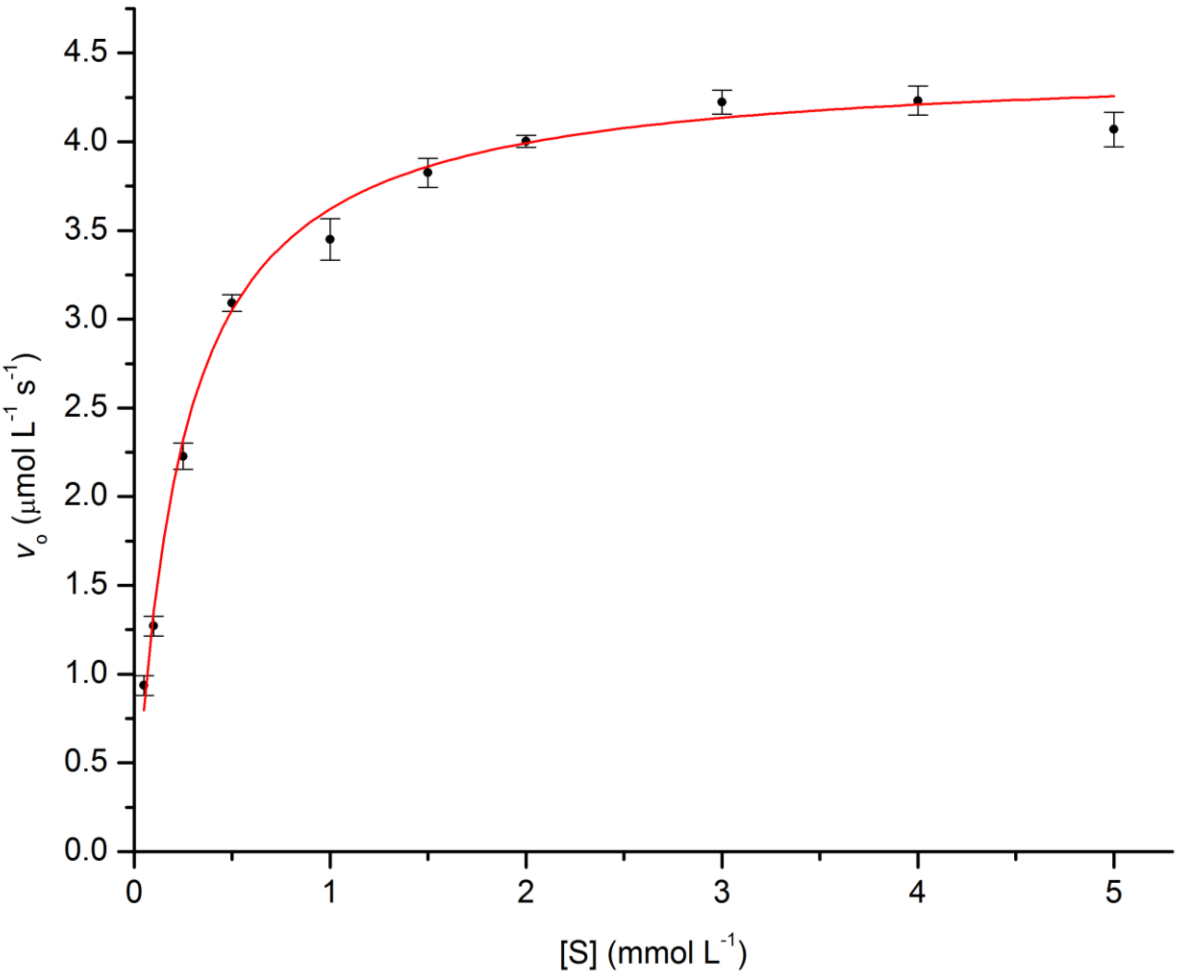


Figure S13. Michaelis-Menten plot for DddY and the substrate DMSP. Dots and error bars indicate mean and standard deviations from triplicates.

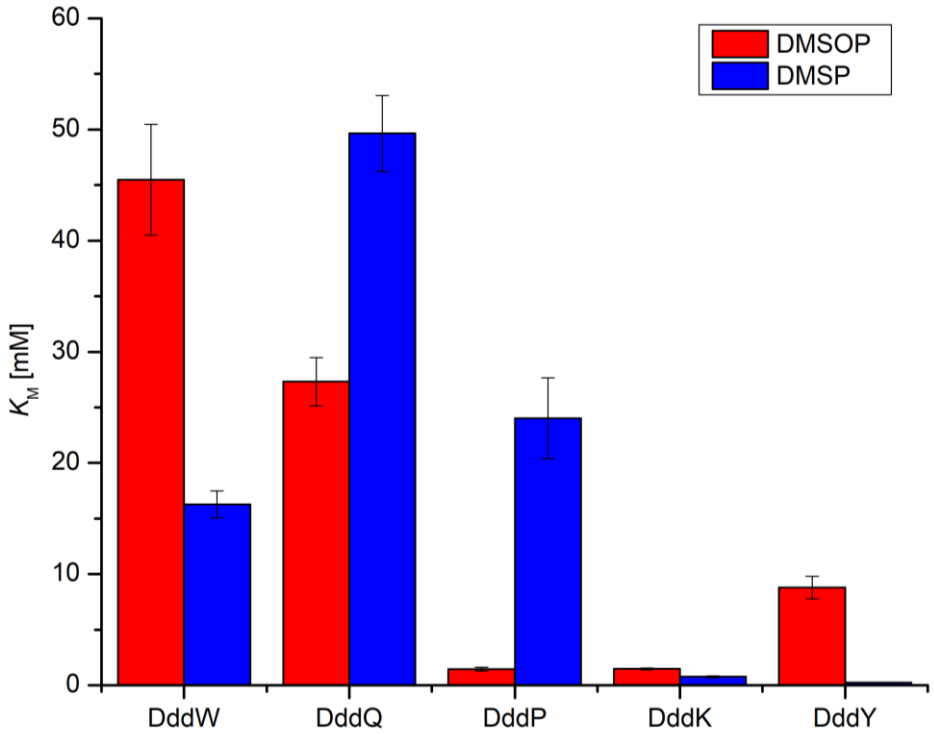


Figure S14. Michaelis constants (K_M) of DMSP lyases towards DMSOP. Error bars indicate mean and standard deviations from triplicates (some error bars are small, data are also summarised in Table 2 of main text).

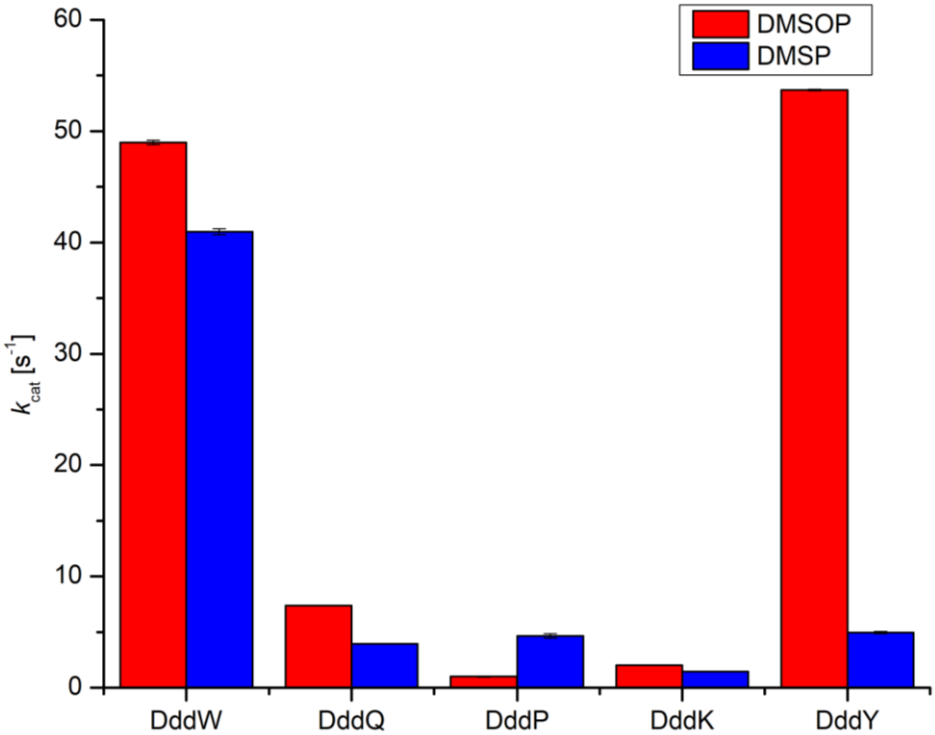


Figure S15. Turnover numbers (k_{cat}) of DMSP lyases towards DMSOP. Error bars indicate mean and standard deviations from triplicates (some error bars are small, data are also summarised in Table 2 of main text).

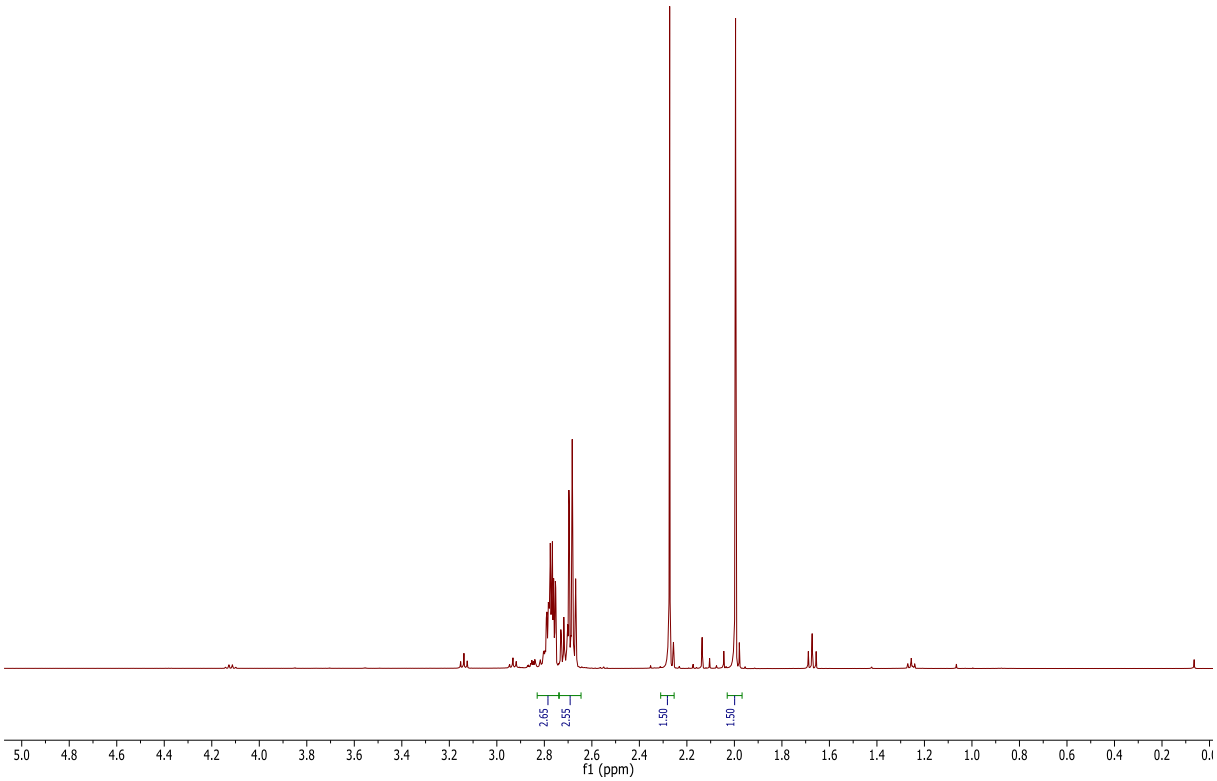


Figure S16. ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of **2**.

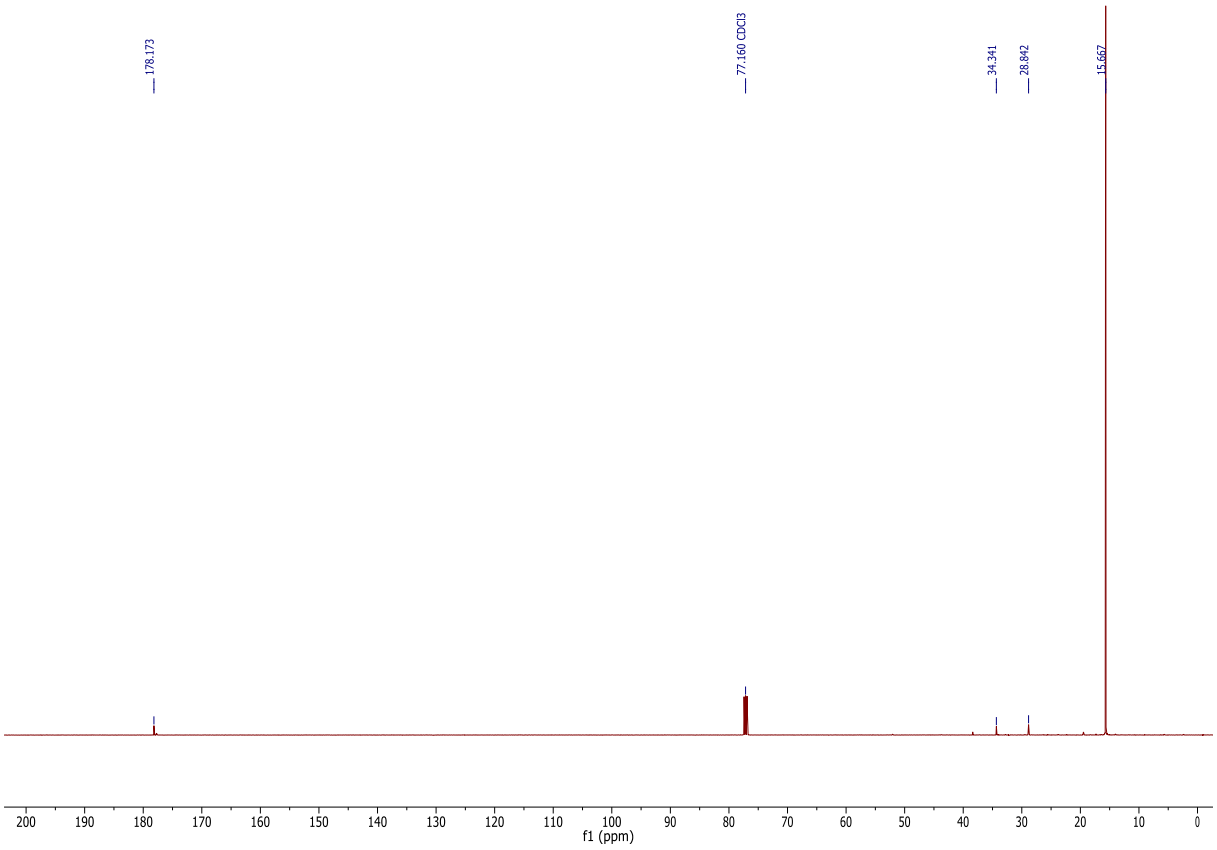


Figure S17. ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of **2**.

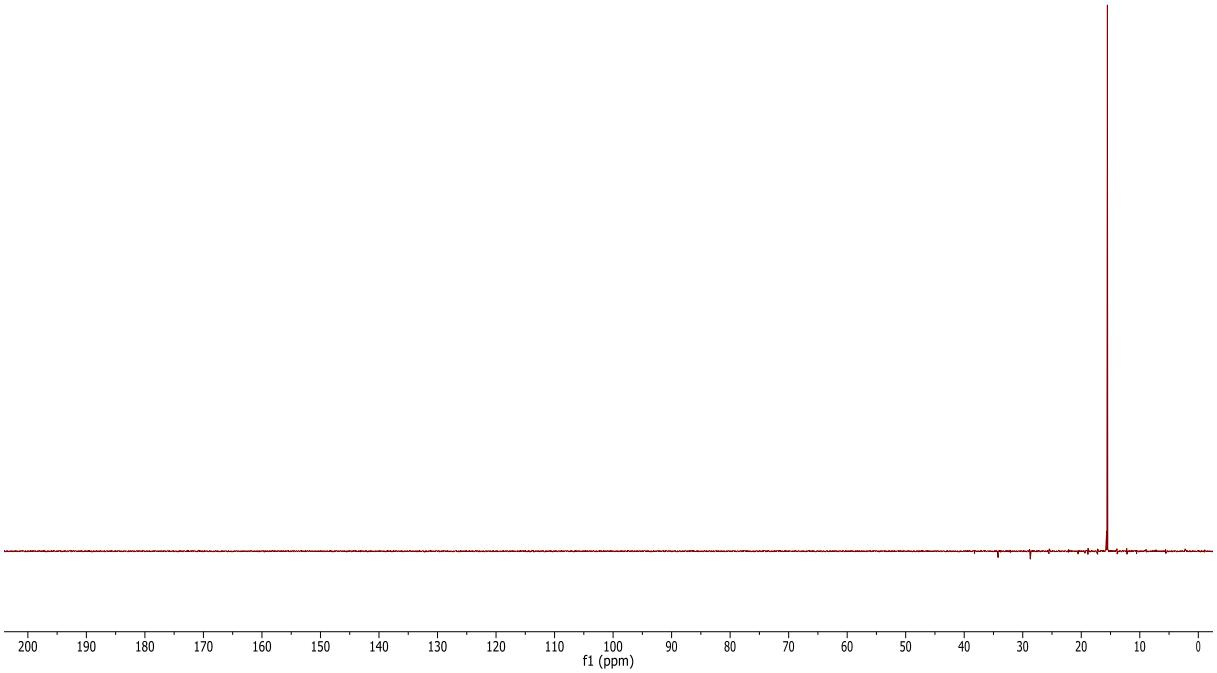


Figure S18. ^{13}C-DEPT135 spectrum ($CDCl_3$, 500 MHz) of **2**.

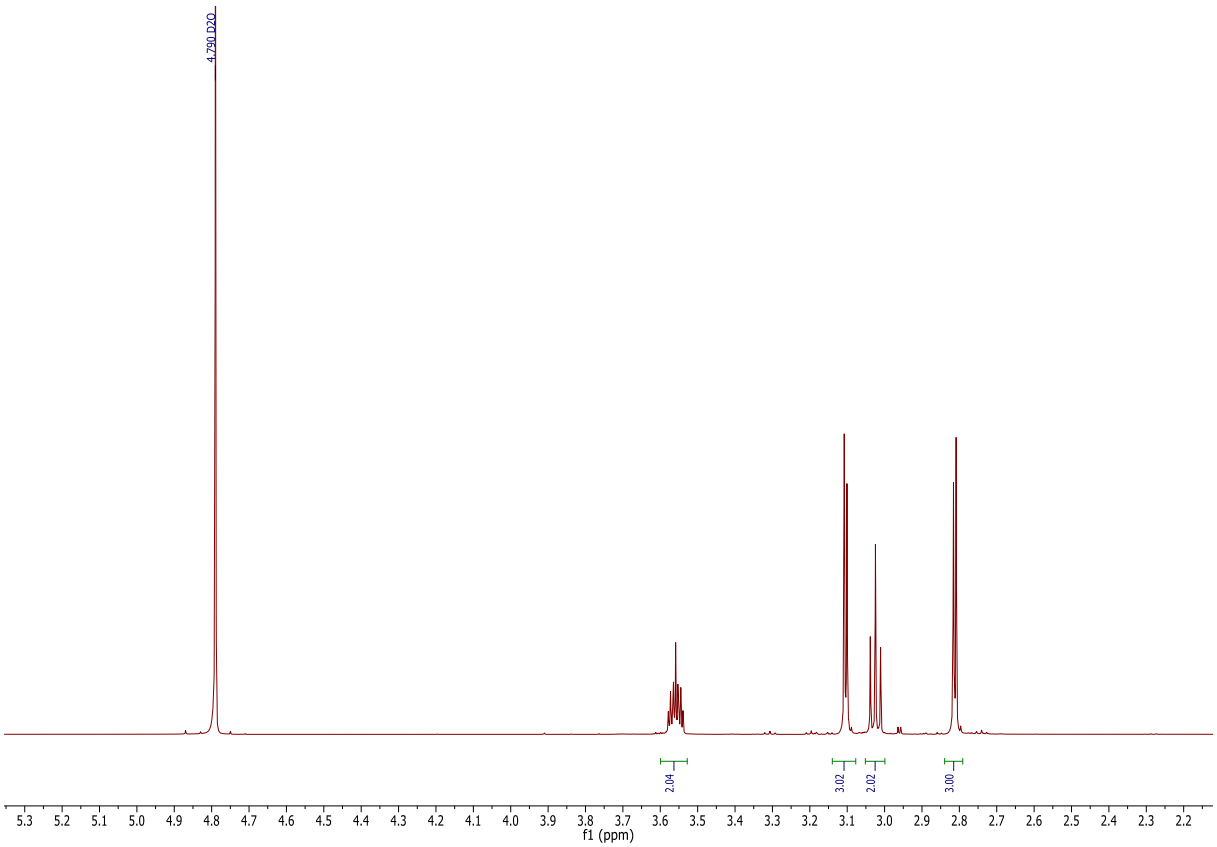


Figure S19. ^{1}H-NMR spectrum (D_2O, 500 MHz) of **3**.

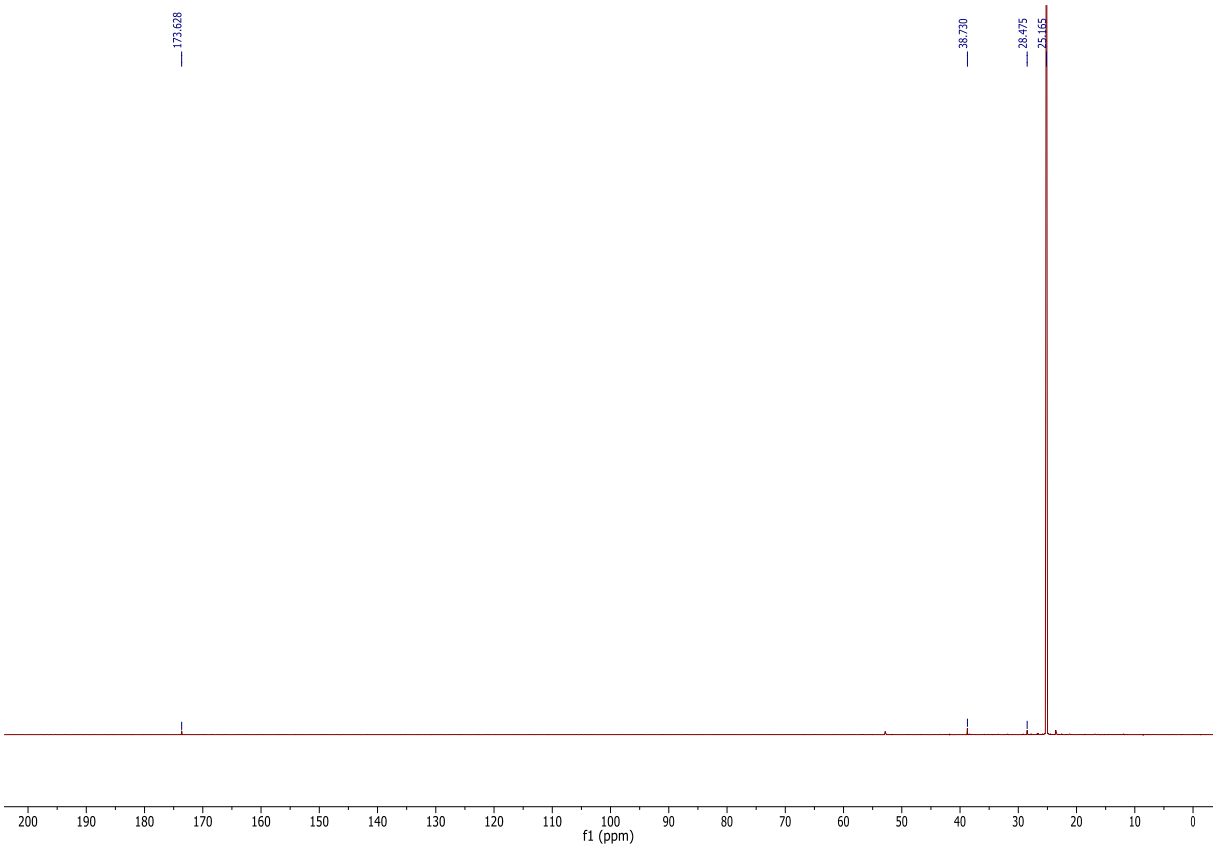


Figure S20. ^{13}C-NMR spectrum (D_2O, 125 MHz) of **3**.

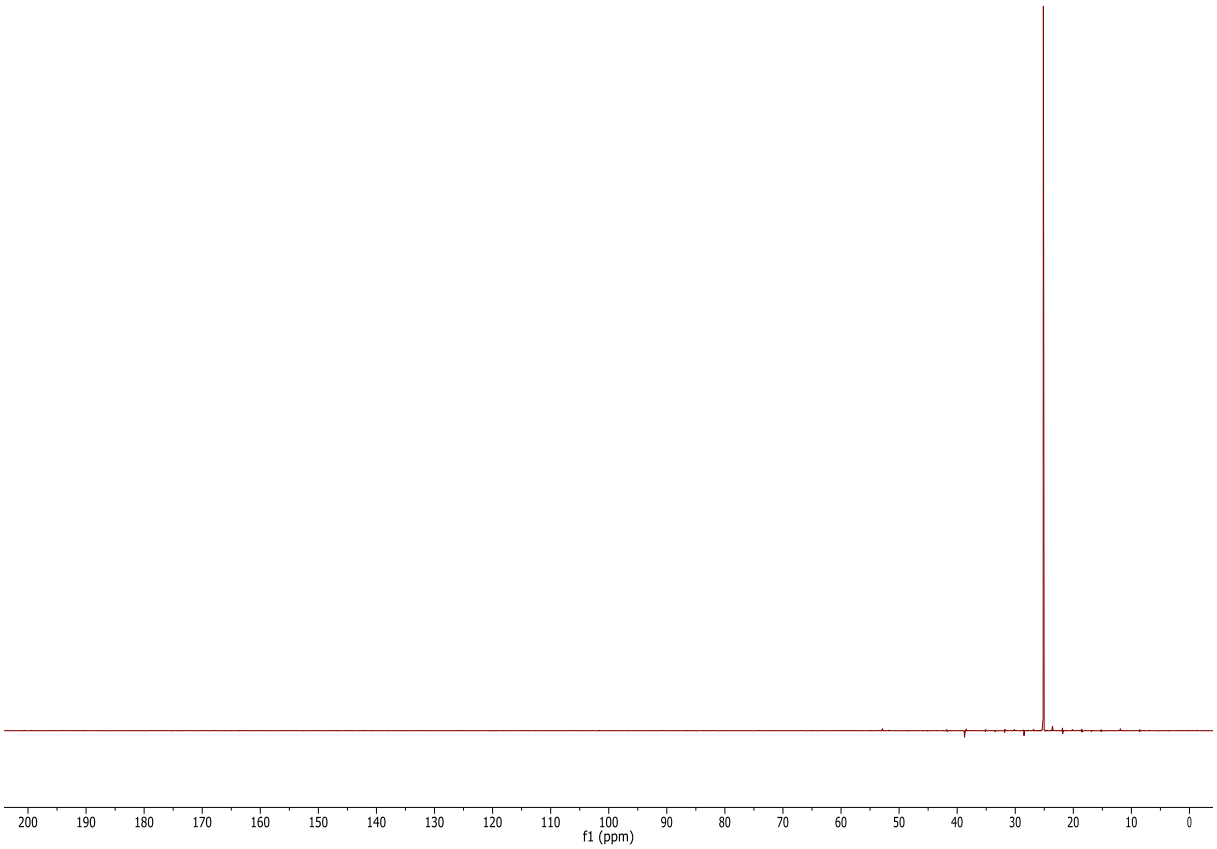


Figure S21. ^{13}C-DEPT135 spectrum (D_2O, 500 MHz) of **3**.

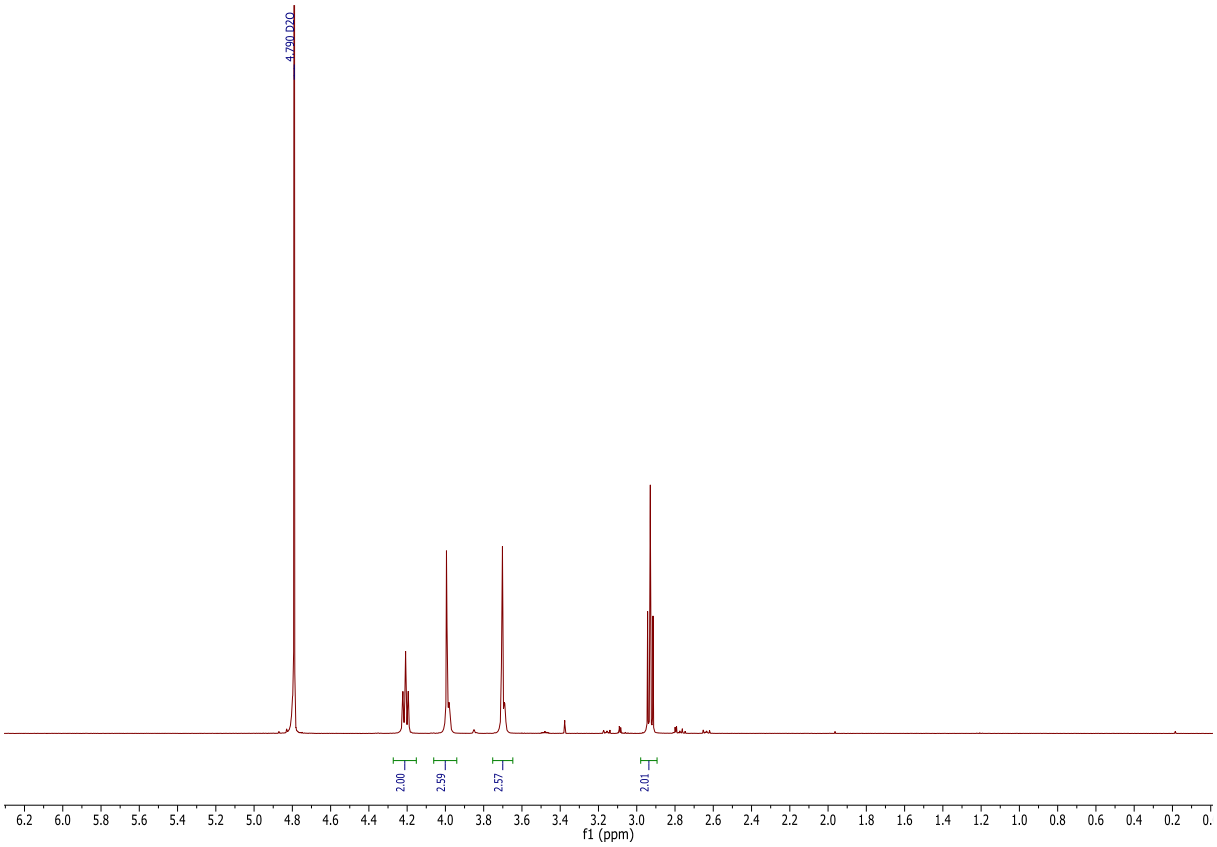


Figure S22. ^{1}H-NMR spectrum (D_2O, 500 MHz) of **4**.

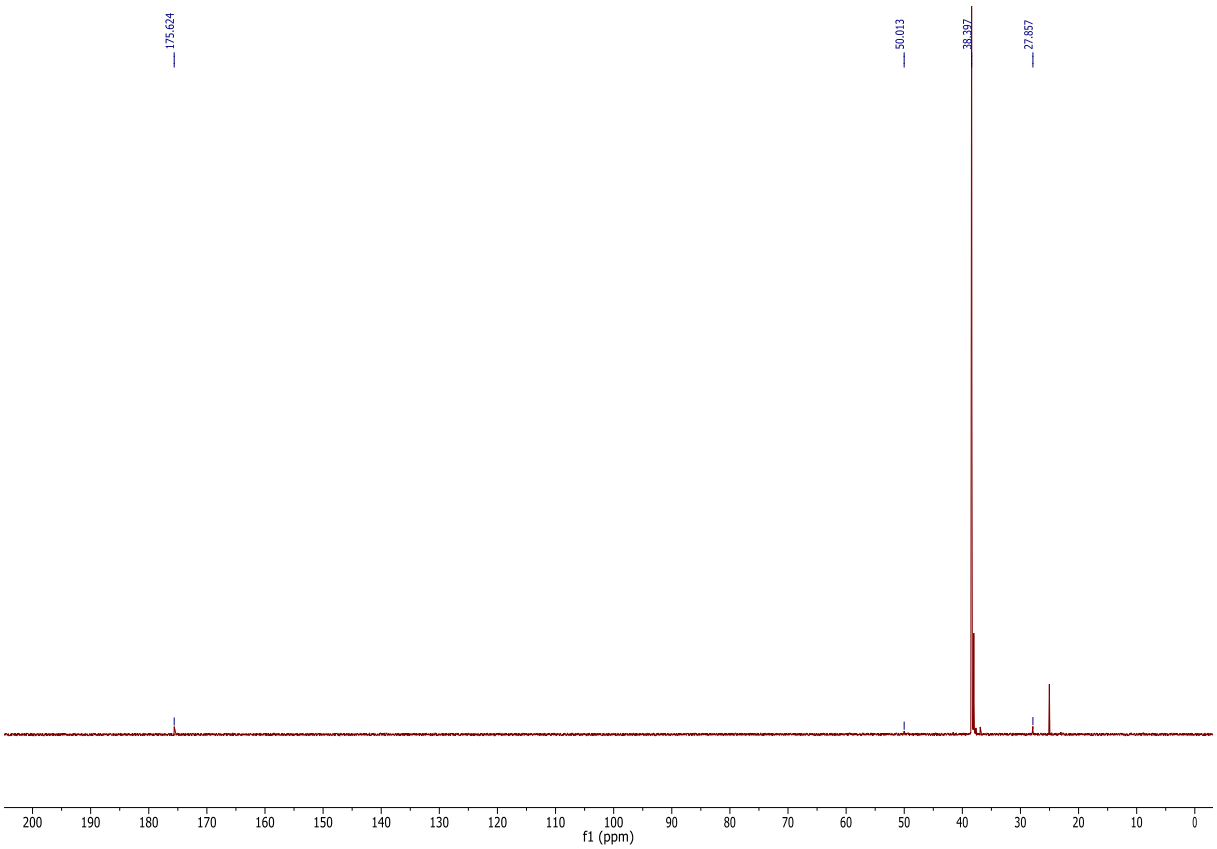


Figure S23. ^{13}C-NMR spectrum (D_2O, 125 MHz) of **4**.

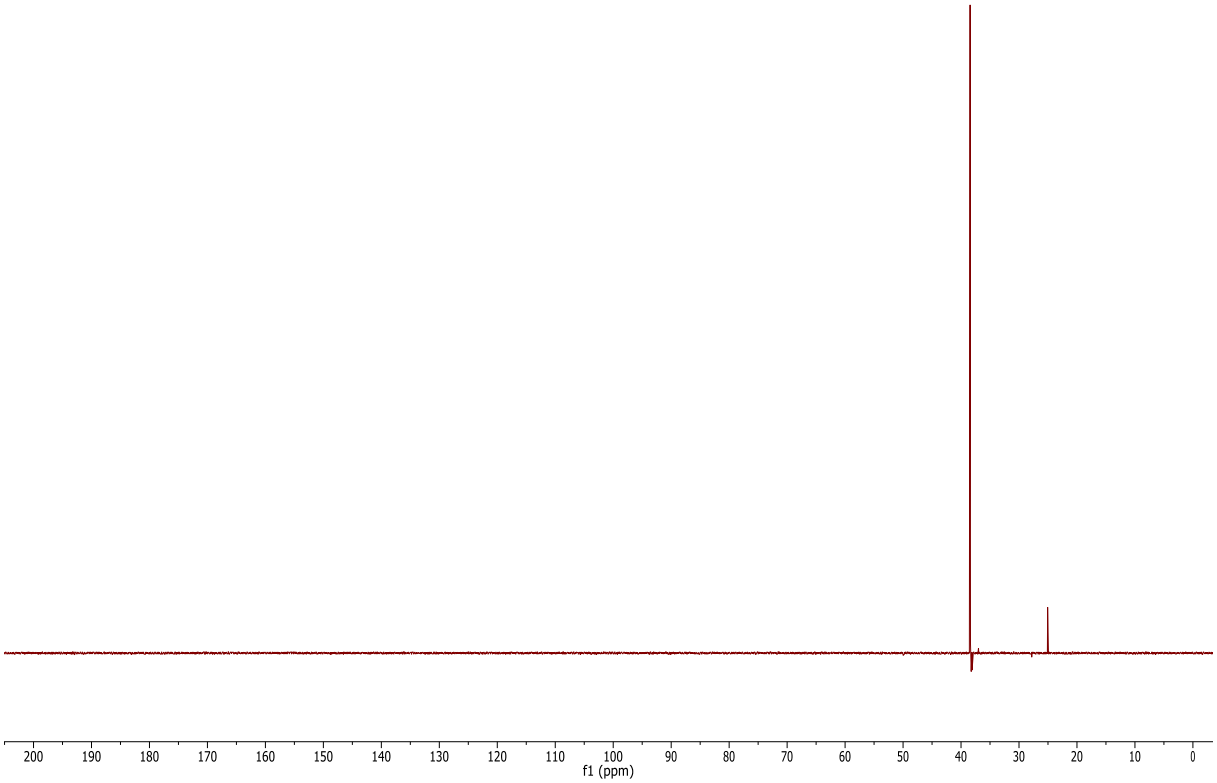


Figure S24. ^{13}C-DEPT135 spectrum (D_2O, 500 MHz) of **4**.

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