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Supporting information for article:

Swit_4259, an acetoacetate decarboxylase-like enzyme from *Sphingomonas wittichii* RW1

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S1. Additional Materials and Methods

S1.1. Size exclusion chromatography

The solution molecular weight of Swit_4259 was estimated by gel filtration chromatography using a BioBasic SEC-300 (250 × 4.6 mm) column (Thermo Scientific, USA) on an Agilent 1220 Compact LC. The mobile phase consisted of 25 mM TRIS, pH 8.0, 300 mM NaCl at a flow rate of 0.5 ml/min with UV-Vis detection at 280 nm. The column was calibrated using standard proteins including equine heart cytochrome C (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), and sweet potato *β*–amylase (200 kDa). To define the standard curve, each protein was dissolved at 10 mg/mL in the running buffer and the separation was performed on 250 µL of the standard mixture at 25 °C. A sample of Swit_4259 (6 mg/mL) was run immediately afterward using the same column and identical conditions.

S1.2. Acetoacetate decarboxylase activity assay

Swit_4259 was assayed for acetoacetate decarboxylase activity by monitoring the disappearance of the enolate form of acetoacetate spectrophotometrically at 270 nm ($\boldsymbol{\Delta\varepsilon}$ = 44.0 M^{-1} cm^{-1}) as described (Highbarger *et al.*, 1996). Reactions were run at 25 °C in 50 mM phosphate buffer, pH 6.5, 2 mM EDTA, on a Thermo Scientific Evolution 300 spectrophotometer. The reaction mix containing 20 mM lithium acetoacetate in the assay buffer was used as the blank. The reaction was initiated by the addition of 10 µM Swit_4259 in the same buffer.

S1.3. Determination of binding constants

There is one tryptophan (Trp24) in the active-site pocket close to Lys122 (about 7 Å between Nε of Lys122 and the indole ring of Trp24), which is sensitive to the binding of small molecules in the active site. Potential substrates can be titrated to determine the dissociation constant (K_D) of each compound. The dissociation constants were measured for the α-keto-acids pyruvate, α-keto glutarate, and 2-oxoadipate binding to Swit_4259. Alanine was used as a control compound since it cannot react with the active site lysine. The fluorescence signal of Trp excited at 280nm was monitored using a TgK Scientific stopped flow with 20 µM Swit_4259 or Hitachi F-4500 fluorimeter with 5 µM Swit_4259 and varied substrate concentrations ranging from 1 mM to 1 M, all in 100 mM BIS-TRIS pH 6.5. K_D was determined by nonlinear regression to fractional occupancy, as described by Equation 1:

$$\boldsymbol{Fractional\ Occupancy} = \frac{[\boldsymbol{Ligand}]}{\boldsymbol{K_D}+[\boldsymbol{Ligand}]} \qquad [1]$$

The error bars in Figure 4B are from the standard deviation of three independent measurements.

S1.4. Production of 2-hydroxymuconic acid (3)

The synthesis of 2-hydroxymuconic acid was based on that of Metanis (Metanis *et al.*, 2005). A solution of t-BuOK was prepared by dissolving potassium metal (1.92 g) in t-BuOH (20 mL) and refluxing until homogenous. Then, an 8 mL excess of the t-BuOK solution was combined with 2 mL of ether and stirred at 0° C for 15 minutes. A solution of diethyl oxalate was made by mixing 1.35 mL of diethyl oxalate and 1 mL of ether. This solution was cooled to 0 °C and added to the t-BuOK solution via syringe. The reaction mixture was allowed to stir for another 15 minutes. A solution of ethyl crotonate was made by mixing 1.2 mL of ethyl crotonate with 1 mL ether. This solution was cooled to 0° C and added via syringe. The mixture was stirred for 30 minutes, then stored at 4° C overnight to precipitate the potassium salt. After the potassium salt was filtered, it was rinsed with hexanes and dried under vacuum to yield 1.907 g of the potassium salt. The potassium salt was dissolved in 25 mL DI H_2O and 1 mL of glacial acetic acid. The resulting precipitate was collected by filtration and dried under vacuum to yield 1.3209 g of the diester. The diester was dissolved in a NaOH solution (2 M, 20 mL) and left at room temperature overnight. The solution was then acidified to pH 2.0 with HCl (12 M) and placed on an ice bath for 1 hour. The resulting precipitate was collected by filtration and dried under vacuum to yield 339.5 mg of diacid (22% isolated yield based on ethyl crotonate). The product was verified by ESI-LCMS and ^{1}H NMR (Figure S3).

S1.5. Preparation of 3-(2-furyl)acrolenepyruvate (11)

6-(furan-2-yl)-2-oxohexa-3,5-dieneoate synthesis began with 0.4243 g (3.47 mmol) of 3-(furan-2-yl)acrylaldehyde. This was combined with 0.420 g (3.86 mmol) of sodium pyruvate and 0.1300g (3.25 mmol) of sodium hydroxide in a dry round bottom flask. 50 mL of methanol was added to the reaction mixture and the flask was attached to a reflux condenser, then flushed with nitrogen. The reaction mixture refluxed for 23 hours, at which point TLC had indicated the total consumption of the starting aldehyde and the reflux was stopped. The reaction mixture cooled to room temperature, then chilled to precipitate the product. The precipitate was filtered and acidified by bubbling CO_2 through until a pH of 8.0 was attained. The filtrate was concentrated via rotary evaporation and dried in a desiccator for several days, affording 0.8824 g of a bright yellow solid (124%).

The product was placed into an Erlenmeyer flask where upon 20 mL of dichloromethane, 4 mL of trimethylamine and 8 mL of distilled H_2O. CO_2 was bubbled through the mixture, with stirring, until the trimethylamine and aqueous phases became homogenous. This was transferred to a separatory funnel and the product was further extracted by two 20 mL additions of dichloromethane. The organic phases were combined, dried over $MgSO_4$ and concentrated by rotary evaporation to afford a dark amber oil. Once dried, the sodium salt was formed by suspending the oil in ethyl acetate, and washing with 1 M HCl until the organic phase indicated a pH of 2.0. The organic layer was then separated and a 1 M methanolic solution of sodium hydroxide was added until a pH of 8.0. This was concentrated

by rotary evaporation and dried in a desiccator for two days to afford 0.5430 g (69% isolated yield) of the sodium salt verified by ESI-LCMS and ^{1}H NMR (Figure S4).

S1.6. Steady-state kinetics and product identification.

Based on our hypothesis that Swit_4259 is an aldolase-dehydratase acting in an uncharacterized hydrocarbon degradation pathway, the activity of Swit_4259 was tested with potential substrates including 2-oxo-hex-3-enedioate, and 3-(2-furyl)acrolein and pyruvate. The assays were performed with 0.5 – 1 μM Swit_4259 in 50 mM BIS-TRIS pH 6.5, and various substrate concentrations. In aldol condensation reactions, sodium pyruvate was saturating (50 mM).

The enzyme 4-oxalocrotonate tautomerase was used to produce 2-oxo-hex-3-enedioate from 2-hydroxymuconic acid. Reactions contained 50 mM sodium phosphate buffer pH 6.5, 1 μM 4-oxalocrotonate tautomerase, and 100 μM 2-hydroxymuconic acid in a final volume of 0.5 ml. The production of 2-oxo-hex-3-enedioate was monitored at 236 nm (ε = 6580 M^{-1} cm^{-1}) or in UV-Vis spectra from 220 to 340 nm. Each assay was incubated without Swit_4259 until the absorbance at 236 nm reached a maximum. At this point, Swit_4259 was added to the mixture and the loss of absorbance at 236 nm was monitored. The assay was performed with 100 μM 2-hydroxymuconic acid and different concentrations of Swit_4259 (1, 2, or 4 μM). The assay also was performed using 5 μM Swit_4259 and concentrations of 2-hydroxymuconic acid ranging from 0 to 200 μM. Swit_4259 Asn118Glu steady state kinetics were performed in the same manner as Swit_4259 WT.

S1.7. Reverse phase HPLC analysis of reaction mixtures

All reactions and standards were prepared in 10mM BIS-TRIS pH 6.5 and allowed to stand for 2h at RT, after which the enzyme was removed by filtering the samples through 3000 Da molecular weight cut-off concentrators. The filtrate from each sample (5 μl) was loaded onto a 2 x 50 mm Kinetex XB-C18 column (Phenomenex) at a flow rate of 0.5 ml/min. Analytes were separated using a linear gradient from 2% acetonitrile in 0.1% TFA/water to 50% acetonitrile in 8 min. Between each run the column was flushed with 100% acetonitrile for 3 minutes, followed by re-equilibration at 2% acetonitrile. All separations were performed on an Agilent 1220 Infinity LC and monitored at 330 nm.

S1.8. Swit_4259 Asn118Glu macromolecule production

Swit_4259 WT plasmid with four different sets of primers and varied PCR conditions did not take up the Asn118Glu mutant with the QuikChange Site-Directed Mutagenesis Kit (Agilent). Thus, Swit_4259 Asn118Glu was produced in the same manner as Swit_4259 WT, including being synthesized by GenScript (Piscataway, NJ). Asn118Glu followed expression and purification in the same manner as Swit_4259 WT described in this paper. Numerous crystallization endeavors of Swit_4259 Asn118Glu were done to collect data from unliganded and soaked (**7**, **11**) crystals, in a

range of conditions based on optimization from Hampton Research Index HT, PEG/Ion HT, and Salt Rx screens, none of which obtained a high enough resolution for structure determination.

S1.9. Proton NMR

The ^{1}H NMR in Figures S5 and S6 were performed on a Bruker DRX 500 MHz NMR spectrometer, with a 5 mm inverse BBI probe at room temperature, for 256 scans. The samples were carried out in 600 µL of 50 mM sodium phosphate pH 7.4 (H_2O), with 10 µM Swit_4259 for reaction in Figure S5. After overnight incubation at 4 ºC of Swit_4259 and **4**, the samples were evaporated to dryness in a CentriFan centrifugal evaporator and resuspended in 600 µL of D_2O.

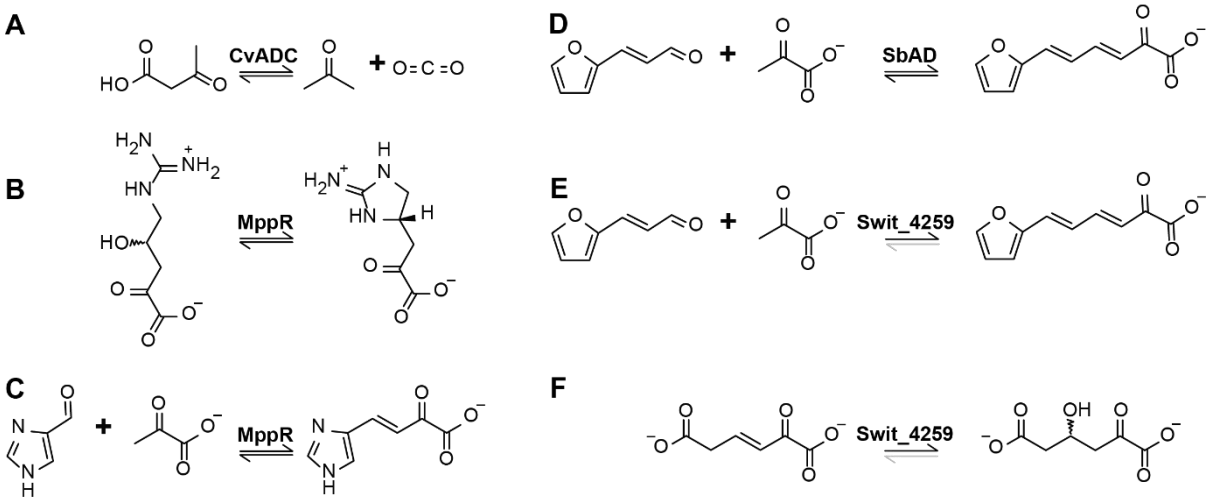

Figure S1 Reactions of the ADCSF. A prototypical acetoacetate decarboxylase reaction is shown in (A), as represented by *Chromobacterium violaceum* ADC (CvADC). The remaining reactions are catalyzed by Family V of the ADCSF. (B) and (C) illustrate reactions from *Streptomyces hygroscopicus* MppR (ShMppR), (D) indicates the reversibility of *Streptomyces bingchenggensis* SbAD, and (E) and (F) represent the known catalysis of *Sphingomonas wittichii* Swit_4259 as indicated in the paper.

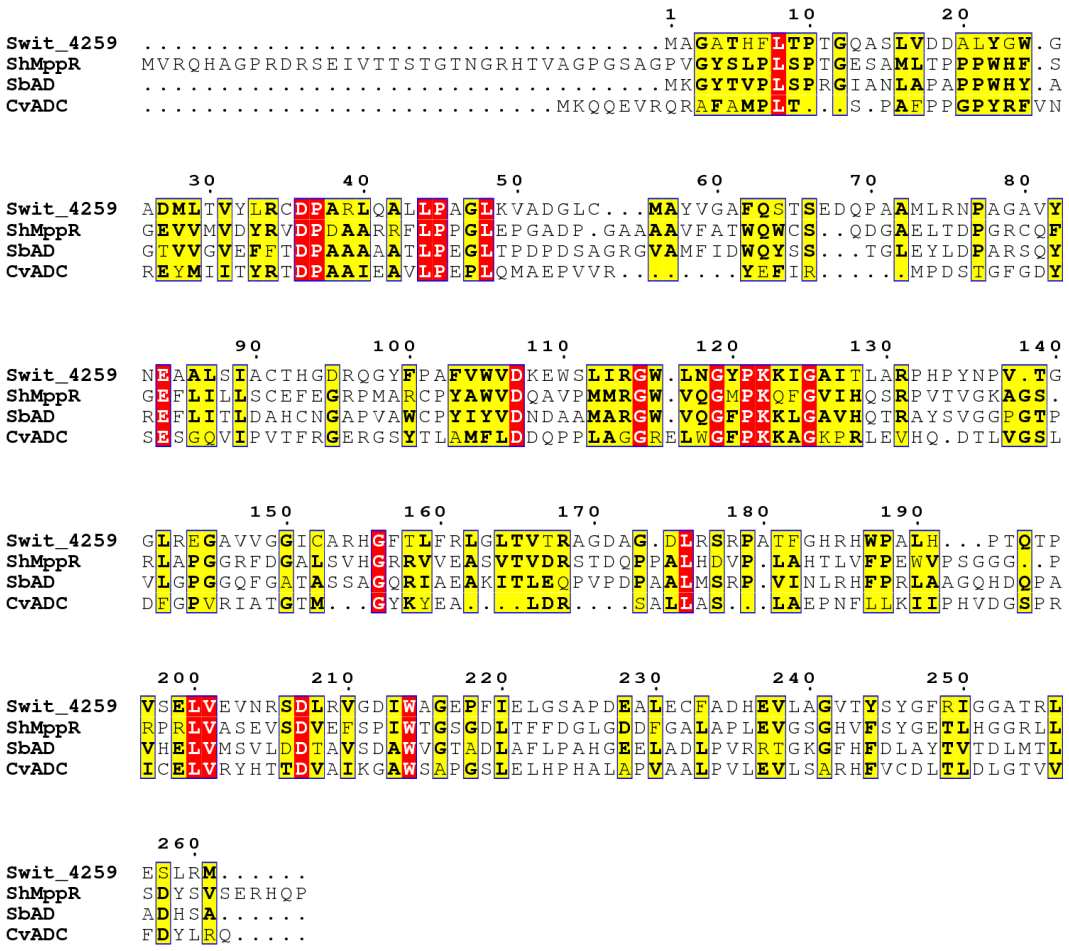

Figure S2 Alignment of Family V ADCSF Swit_4259, ShMppR, SbAD, and Family I CvADC. The Schiff-base forming residues, E84 and K122 as per Swit_4259 annotation, are conserved across both families. The figure was produced in ESPript (Robert & Gouet, 2014).

Table S1 Percent sequence identity matrix based on Clustal Omega multiple sequence alignment (Sievers *et al.*, 2011), where Swit_4259, ShMppR, and SbAD are Family V ADCSF, and CvADC is a canonical Family I acetoacetate decarboxylase.

	Percent Identical			
ADCSF Enzyme	Swit_4259	ShMppR	SbAD	CvADC
Swit_4259	100	26	25	20
ShMppR	26	100	39	23
SbAD	25	39	100	22
CvADC	20	23	23	100

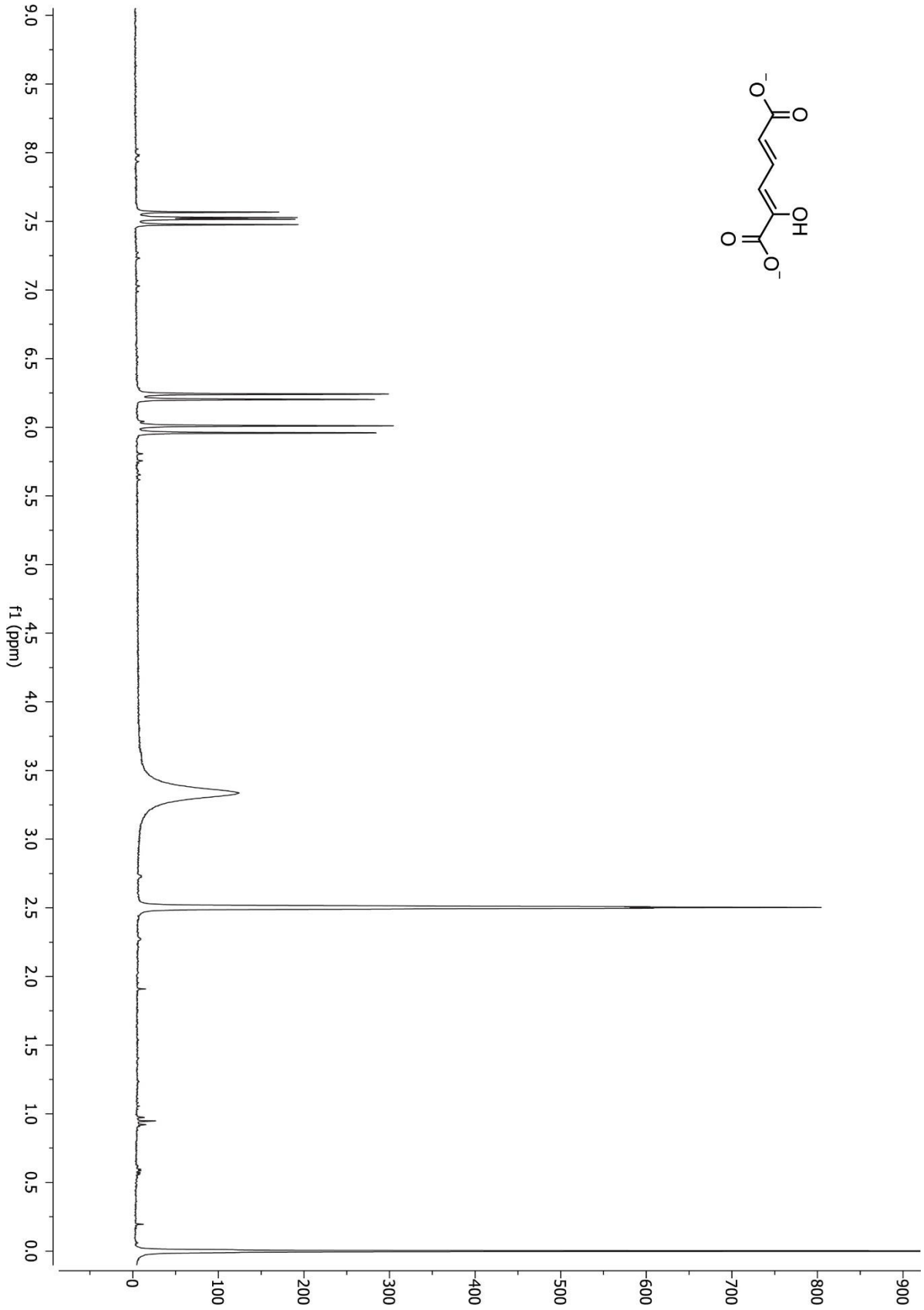

Figure S3 Proton NMR spectrum of **3** in DMSO.

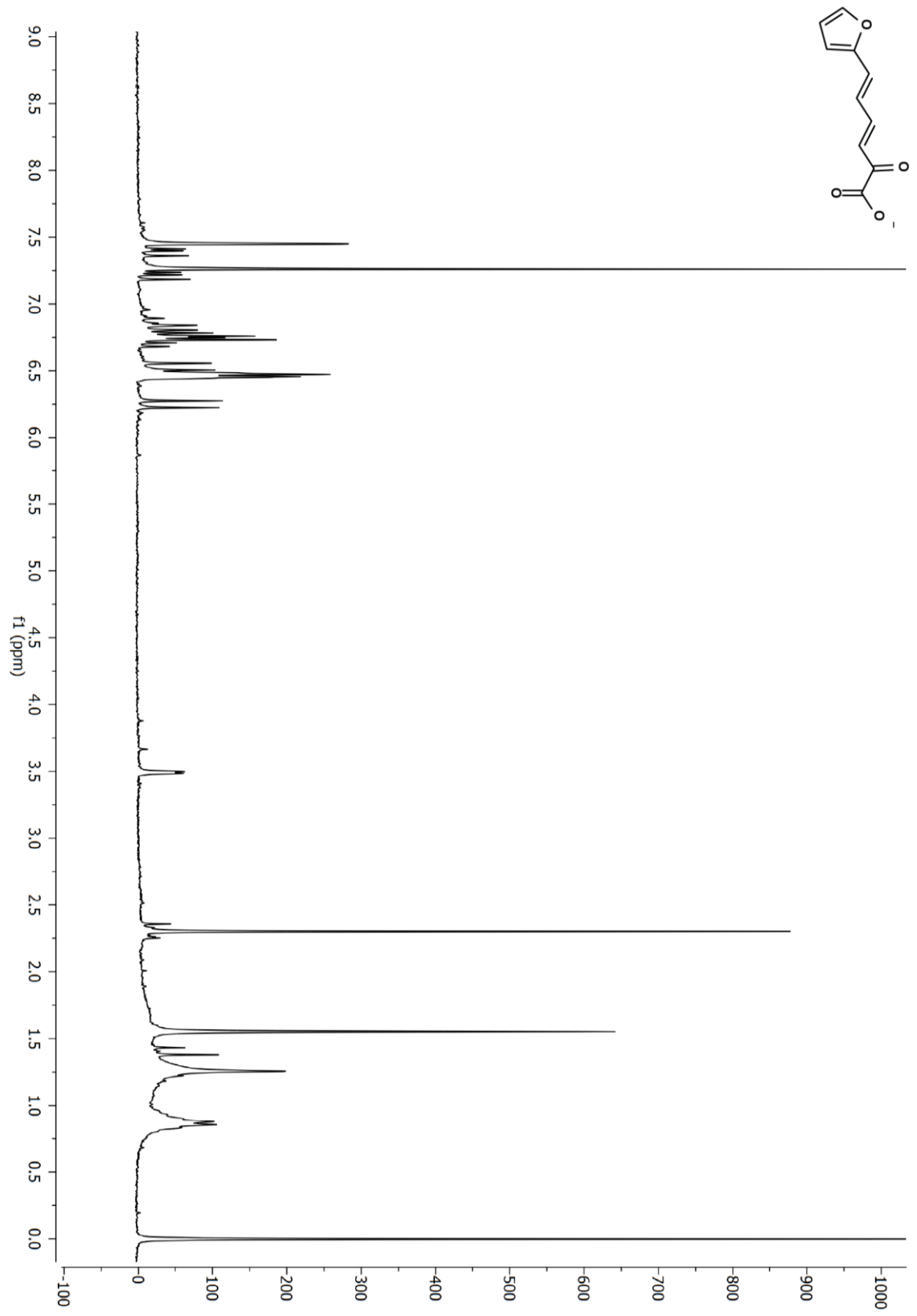

Figure S4 Proton NMR spectrum of **11** in deuterated chloroform.

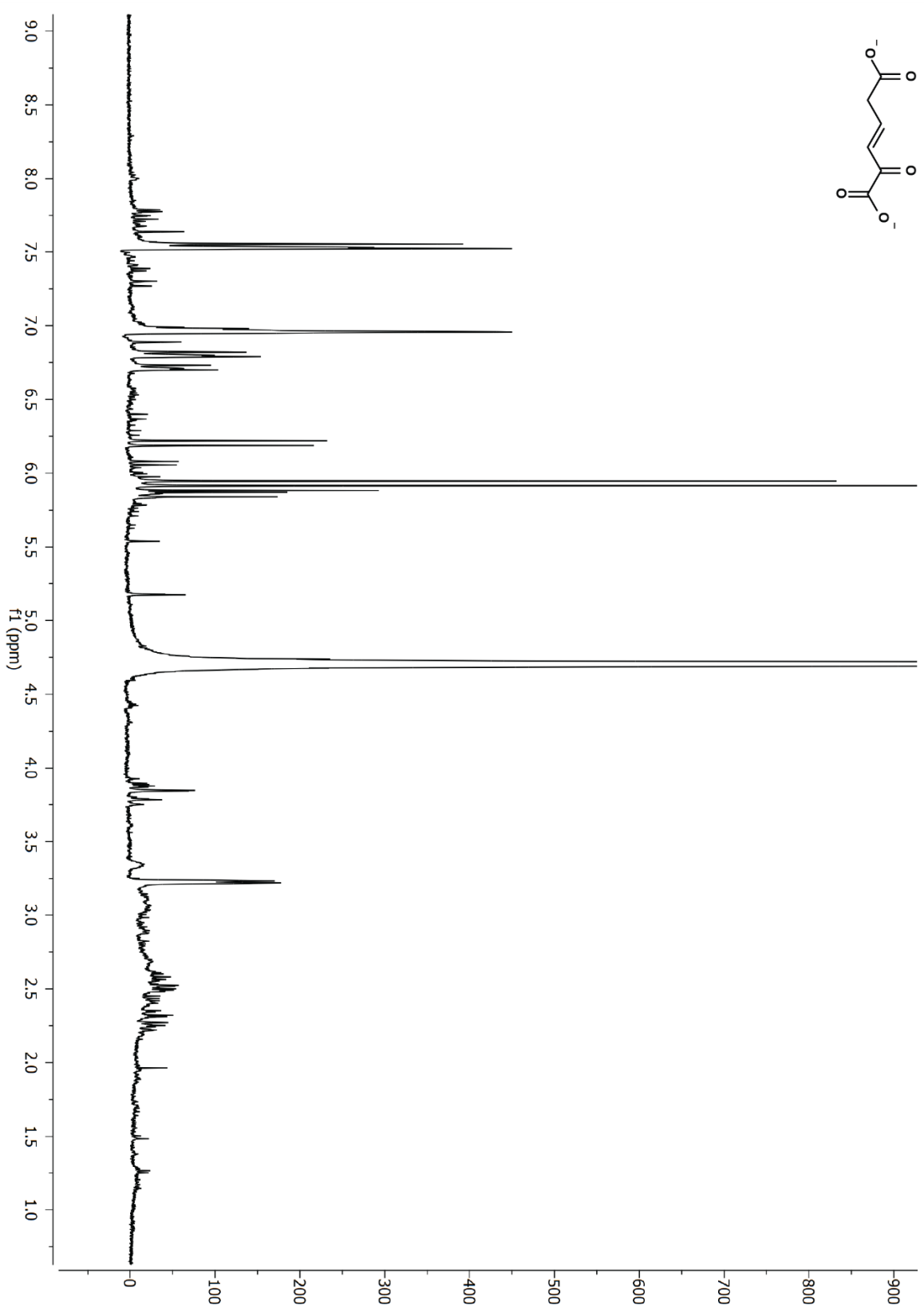

Figure S5 Proton NMR spectrum containing 15 mM **4** in 50 mM sodium phosphate buffer (D_2O) pH 7.4.

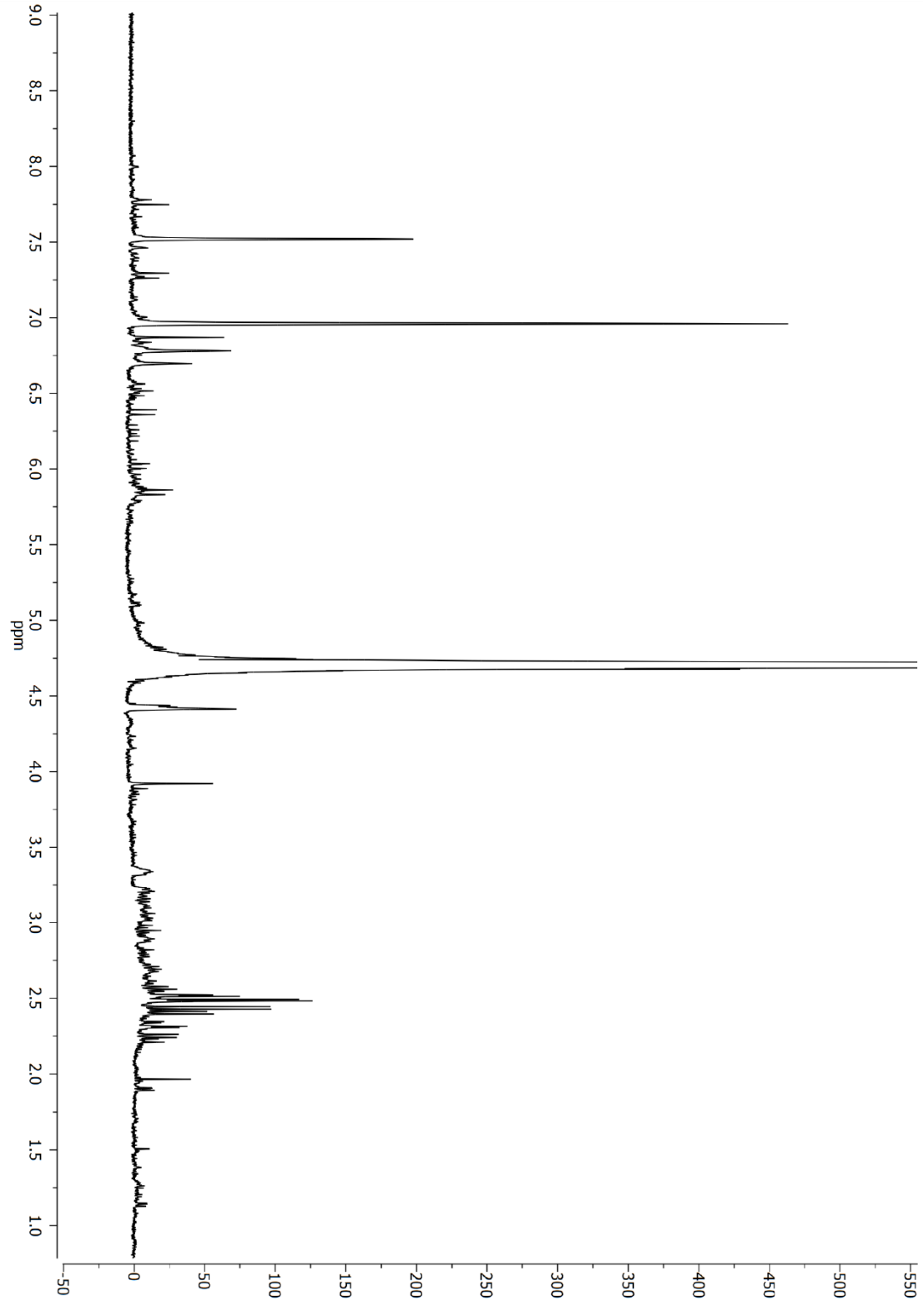

Figure S6 Proton NMR spectrum of a solution containg 15 mM **4** and Swit_4259 in 50 mM sodium phosphate buffer (D2O) pH 7.4.