Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting information

Mechanistic investigation of B12-independent glycerol dehydratase and its activating enzyme GD-AE

Yaoyang Li, Yadi Yao, Lu Yu, Changlin Tian and Min Dong*

Materials and methods

Reagents and Methods:

Reagents

SAM was purchased from SolarBio (Shanghai, China) and neutralized to pH 7.5 before use. The vector pTrc-HisA was purchased from Hedgehogbio Science and Technology Ltd (Shanghai, China).

Expression and purification of GD

The codon-optimized gene fragment of GD was synthesized by GENEWIZ (Suzhou, China) and inserted into vector pET28a. The plasmid was used to transform the *E. coli* Rosetta (DE3) strain. Cells were grown in 1 liter LB medium with 20 μg/mL chloramphenicol and 30 μg/mL kanamycin at 37°C and 200 rpm. When the OD_{600} reached 0.6, the cultures were cooled to 16°C. Protein expression was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested after incubation at 16°C and 200 rpm for 20 h**.**

The cell pellet was suspended in 25 mL lysis buffer (150 mM NaCl, 50 mM Tris at pH 7.5). Lysozyme (100 mg), DNase I (1 mg) and 1 mM phenylmethanesulfonyl fluoride (PMSF) were added and incubated at room temperature (RT) for 30 min. The mixture was lysed using a cell disruptor (Scientz China). Cell debris was removed by centrifugation at 12000 g (Thermo, Germany) for 35 min. The supernatant was incubated for 50 min with 2 mL Ni-NTA resin (Genscript) pre-equilibrated with the lysis buffer. The Ni-NTA resin was loaded onto a polypropylene column and washed with 20 column volumes (CVs) of lysis buffer, followed by 20 mM and 30 mM imidazole in lysis buffer. GD was eluted from the column with elution buffer (500 mM imidazole in lysis buffer). The elution fractions were buffer-exchanged to 125 mM KCl, 500 mM Tris-HCl at pH 7.5 and 10% glycerol using a PD-10 desalting column (GE). The purified proteins were concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore). The protein concentration was determined by the Bradford assay. The purified protein was then examined by 16% SDS–polyacrylamide gel electrophoresis. Aerobically purified GD proteins were degassed by the Schlenk line before use.

Cloning and expression of GD-AE

The codon-optimized gene fragment of GD-AE was synthesized by GENEWIZ (Suzhou, China), and inserted into the vector pTrc-HisA. This vector incorporates a six-poly-His tail at the N-terminal end of GD-AE. Plasmid pDB1282, which contains the *Azotobacter vinelandii isc* operon under the control of an arabinose-inducible promoter, can assist in the reconstitution of iron-sulfur clusters. To co-transform the pDB1282 plasmid with an ampicillin selective marker, we changed the antibiotic resistance of the GD-AE plasmid pTrc-HisA from ampicillin to kanamycin using the SE Seamless Cloning and Assembly protocol. *E. coli* BL21 (DE3) cells were co-transformed with plasmids GD-AE and pDB1282. Cells were grown in 1 liter TB

medium with 50 μg/mL ampicillin and 30 μg/mL kanamycin at 37°C and 200 rpm. When the OD_{600} reached 0.3, solid arabinose was added to each flask at a final concentration of 0.1% (w/v). When the OD_{600} reached 0.6, the cultures were cooled to 16°C and supplemented with $FeCl_3$, $Fe(NH_4)_2(SO_4)_2$ and L-cysteine to final concentrations of 50 μM, 50 μM and 400 μM, respectively.[1] Protein expression was induced by 1 mM IPTG, at which point the culture flasks were sealed to limit the amount of oxygen in the system. Cells were incubated in a shaker at 16°C and 200 rpm for 20 h before being harvested.

Anaerobic purification of GD-AE

Purification of GD-AE was performed in an anaerobic chamber (Mikrouna Ltd, China). Cells from 1 liter cultures were suspended in 25 mL degassed lysis buffer (50 mM Na_3PO_4, 300 mM NaCl, pH 7.4, 1 mM PMSF, 120 mg lysozyme, 0.03% Triton X-100, and 1 mg of DNase I). The cell suspension was frozen in a -80°C freezer and then thawed and incubated at RT for 30 min to allow lysis. The cells were further lysed by sonication on ice. Cell debris was removed by centrifugation at 12000 g for 35 min. The supernatant was incubated for 40 min with 2 mL Talon metal affinity resin (Clonetech, Palo Alto, CA) pre-equilibrated with the binding buffer (same as lysis buffer, without the enzymes or protease inhibitors). The Talon metal affinity resin was loaded onto a polypropylene column and washed with 20 CVs of binding buffer, followed by binding buffer supplemented with a final concentration of 5 mM imidazole and eluted with buffer containing 150 mM imidazole. The brown-colored elution fractions were buffer-exchanged to a desalting buffer comprising 125 mM KCl, 500 mM Tris-HCl at pH 7.5 and 10% glycerol using a PD-10 desalting column (GE). The purified proteins were concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore). The protein concentration was determined by the Bradford assay. The purified protein was then examined by 16% SDS–polyacrylamide gel electrophoresis.

In vitro Fe-S cluster reconstitution of GD-AE

Reconstitution of [4Fe-4S] clusters was carried out in an anaerobic chamber at 4°C in a cooling-heating block (Dry Bath H2O3-100C; Coyote Bioscience, Beijing, China). Dithiothreitol (DTT) was added to the purified protein fraction (50 μM) at a final concentration of 10 mM. The mixture was incubated for 1 h before 12 equivalents of Fe $(NH_4)_2$ $(SO_4)_2$ solution was added carefully. After incubation at 4°C for 30 min, 3 equivalents of Na_2S solution was added every 20 min 4 times (12 equivalents total). The mixture was incubated overnight at 4°C.[2] The excess iron and sulfide were removed by repeated concentration with a centrifugal filter unit and diluted with desalting buffer containing 125 mM KCl, 500 mM Tris-HCl at pH 7.5 and 10% glycerol. The resulting blackish solution was subjected to a PD-10 desalting column (GE) pre-equilibrated with desalting buffer. The protein concentration was determined by the Bradford assay.

Cloning, expression and purification of YqhD

The NADPH-dependent aldehyde reductase YqhD was amplified from the genomic DNA of *E. coli* by PCR, and the primers used were YqhD-F (5'-CGCGGATCCATGAACAACTTTAATCTGCACACCCCAACC-3') and YqhD-R (5'-CCGCTCGAGTTAGCGGGCGGCTTCGTATATACG-3'). The gene segment of YqhD was inserted into pET28a and the plasmid was used to transform the *E. coli* BL21 (DE3) strain. Cells were grown in 1 liter LB medium with 30 μg/mL kanamycin at 37°C and 200 rpm. When the OD_{600} reached 0.6, the cultures were cooled to 16°C. Protein expression was induced by 0.25 mM IPTG. Cells were harvested after incubation at 16°C and 200 rpm for 20 h**.** The cell pellet was suspended in 25 mL lysis buffer (500 mM NaCl, 5 mM imidazole, 20 mM Tris at pH 7.5). Cells were lysed using a cell disruptor. Cell debris was removed by centrifugation at 12000 g for 35 min.

YqhD was purified through Ni-NTA affinity chromatography following the same protocol as that used for the purification of GD. YqhD was eluted from the column with elution buffer (200 mM imidazole in lysis buffer). The elution fractions were buffer-exchanged to 125 mM KCl, 500 mM Tris-HCl at pH 7.5 and 10% glycerol using a PD-10 desalting column (GE). The purified proteins were concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore). The protein concentration was determined by the Bradford assay. The purified protein was then examined by 16% SDS–polyacrylamide gel electrophoresis. Aerobically purified YqhD proteins were degassed by the Schlenk line before use.

UV-Vis spectroscopy of reconstituted GD-AE

GD-AE (50 μM) was prepared anaerobically in 125 mM KCl and 500 mM Tris-HCl at pH 7.5 and 10% glycerol. To obtain the absorption spectrum of reduced GD-AE, the dithionite-treated sample was allowed to incubate for 10 min after adding the reducing agent at a final concentration of 1 mM before the absorbance measurement[2]. A control assay omitting dithionite was also performed. The absorption spectra were acquired in the 200-850 nm range using a Q9000 microvolume spectrophotometer and the baseline was corrected with the buffer used to prepare the samples. The UV-Vis absorption spectra exhibited the 410 nm feature of $[4Fe\text{-}4S]^{2+}$ clusters and disappeared upon reduction.

Activation of GD by GD-AE.

To stabilize the proteins, 10% glycerol was always used in the purification buffer. However, glycerol is the substrate of GD. To prevent the reaction from producing too much aldehyde to inactivate the enzyme, glycerol-free desalting buffer was used for buffer exchange to remove the glycerol in GD and GD-AE before the experiments. It should be noted that the GD and GD-AE used in the following experiments were all treated as this before the assay unless otherwise specified.

To test the ability of GD-AE to activate GD, we used a coupled assay that

explored the use of the aldehyde reductase YqhD to catalyze the NADPH-dependent conversion of 3-hydroxypropionaldehyde to 1,3-PD. The reaction was monitored at 340 nm by UV-Vis spectroscopy for the consumption of NADPH. The assay was performed under strictly anaerobic conditions in sealed quartz cuvettes at 25°C, and the final assay volume was 150 µL.[3] The assay solution contained 125 mM KCl, 500 mM Tris-HCl (pH 7.5), 2 mM glycerol, 5 µM GD, 150 µM YqhD, 200 µM NADPH, and 1 mM dithionite. This solution was prepared with and without 5 mM SAM. Before the assay was initiated, the spectrophotometer was blanked against the buffer containing 125 mM KCl, 500 mM Tris-HCl (pH 7.5). The assay was initiated by the addition of 20 µM of GD-AE, and the absorbance at 340 nm was monitored. A control experiment without sodium dithionite was also performed.

Analyzing the SAM cleavage activity of GD-AE with high-performance liquid chromatography (HPLC)

Under anaerobic conditions, the reactions were assembled with 80 µM GD-AE, 1 mM SAM, 5 mM dithionite, 125 mM KCl, 1 mM DTT, and 500 mM Tris-HCl at pH 7.5 in a final volume of 50 µL. The reactions were carried out with or without GD (80 µM). The control samples (without GD-AE or dithionite) were set up similarly by replacing the corresponding component with an equal volume of buffer containing 500 mM Tris/HCl, pH 7.5 and 125 mM KCl. The reaction was incubated at 25°C in a glovebox for 6 h and quenched by adding 50 µL of 10% trifluoroacetic acid (TFA). Proteins were precipitated by centrifugation and the supernatant was analyzed by high-performance liquid chromatography (Shimadzu) on a C18 column (5 µm, 4.6 mm×150 mm) monitored at 260 nm absorbance. A gradient from 5 to 25% buffer B over 13 min was followed by a gradient from 25 to 90% buffer B over 3 min and then kept with 90% buffer B for 3 min. The flow rate was constant at 1 mL/min. (solvent A: 0.1% aqueous TFA and solvent B: 0.1% TFA in acetonitrile). Standards of 5′-deoxy-5′-methylthioadenosine (MTA) and 5′-deoxyadenosine (5′-dA) were analyzed by HPLC using the same protocol.

LC-MS analysis of 5′-dA produced by GD-AE

HPLC fractions for MS were dried under a vacuum freeze dryer and then redissolved in water. A 5 µL portion of the supernatant was analyzed by a miorOTOF-QII LC/MS instrument (Bruker Daltonics Inc., USA) on a C18 column (5 µm, 4.6 mm×150 mm).

Sample preparation for EPR experiments

X band EPR spectra were recorded on a Bruker X-band EMX plus 10/12 spectrometer at a frequency of 9.4 GHz in quartz EPR tubes (Wilmad, 707-SQ-250 M, 4 mm OD). EPR measurements were carried out at 12 K or 50 K using an ESR910 continuous liquid-helium cryostat (Oxford Instruments). The spectrometer settings were as follows: microwave power 20 µW, modulation frequency 100 kHz,

modulation amplitude 5 G, scan rate 52 s/scan, magnetic field scanning range 2850-4150 G. Usually 9 scans were accumulated to obtain a good S/N ratio. EPR samples were all prepared in an anaerobic chamber (Coy Laboratory Products).

An EPR sample of the reduced GD-AE (600 μM GD-AE, 10 mM dithionite in 500 mM Tris-HCl with 125 mM KCl, pH 7.5) was set up with a volume of 50 μL. The reaction was transferred to an EPR tube and sealed in the anaerobic chamber. Then, the sample was taken out and frozen in liquid N_2 for EPR analysis.

EPR experiments of the time-dependent detection of glycyl radical on GD (600 μM GD-AE and GD, 10 mM dithionite, and 5 mM SAM in 500 mM Tris-HCl with 125 mM KCl, pH 7.5) were set up with a volume of 50 μL for each time point at room temperature. The reactions were transferred to EPR tubes and sealed in the anaerobic chamber. Then the sample was taken out and frozen in liquid N_2 at different time points: 20 min and 50 min for EPR analysis.

Synthesis of 2-deoxy-2-fluoroglycerol

Sodium borohydride (0.90 g, 2.4 mM) was added to a stirred solution of tetrahydrofuran (THF) and 50% ethanol (aq) (10 mL) at 0°C.[4] The mixture was allowed to warm to RT and stirred for 1 h. The solution was then cooled to 0°C again and diethyl 2-fluoromalonate (1.00 g, 0.6 mM) was added slowly. The reaction was allowed to warm to RT and stirred for 1 h and an additional 5 h at 40°C. The reaction was cooled to 0°C and HCl was added slowly. The reaction was filtered and the solvent was removed by rotary evaporation. The residue was dissolved in water and extracted with ethyl acetate. After removal of the solvent, 2-deoxy-2-fluoroglycerol was obtained as colorless oil and analyzed by NMR. ^{1}H NMR (600 MHz, DMSO-d6) δ 4.79 (t, 5.7 Hz, 2H, OH), 4.41-4.33 (m, 1H, CHF), 3.60-3.37(m, 4H, 2*CH_2). ^{19}F NMR (564 MHz, DMSO-d6) δ -192.64(m, 1F).

Detection of the GD product 3-hydroxypropionaldehyde by derivative reaction

To avoid the interference of glycerol introduced during the protein purification, especially for the experiments with 2-deoxy-2-fluoroglycerol, we used buffer without adding glycerol during the purification of GD, GD-AE and the reconstitution of GD-AE. At the same time, we used Tris-HCl (pH 7.0) to improve the stability of GD-AE in the reconstitution step without glycerol.

Under anaerobic conditions, reactions were assembled with 30 μM GD-AE, 30 μM GD, 2 mM glycerol or 2-deoxy-2-fluoroglycerol, 5 mM dithionite, 125 mM KCl, 1 mM DTT and 500 mM Tris-HCl at pH 7.5, in a final volume of 100 μL. The mixtures were incubated for 15 min at 4℃ in a glovebox to allow the reduction of GD-AE. SAM (1 mM) was added to initiate the cleavage reaction. The control samples (without GD-AE or dithionite) were set up similarly by replacing the corresponding component with an equal volume of buffer. The reactions were incubated at 25℃ in the glovebox for 6 h, then 50 μL of 4-nitrobenzylhydroxylamine (NBHA) solution in pyridine (9 mM) was added and reacted at 25°C for 3 h. [5]

The reaction was quenched with 150 μL methanol, followed by centrifugation to separate the precipitated proteins and the supernatant. The supernatant was dried under a vacuum freeze dryer and then redissolved in 100 μL of water. An aliquot of the solution (5 μL) was injected into an HPLC (Shimadzu) on a C18 column monitored at 254 nm absorbance. A gradient from 3 to 20% buffer B over 5 min was followed by a gradient from 20 to 40% buffer B over 12 min. Then, the gradient was increased to 100% immediately and kept constant for 5 min. The flow rate was constant at 1 mL/min. (solvent A: 0.1% aqueous TFA and solvent B: 0.1% TFA in acetonitrile). Standards of MTA, 5′-dA, and NBHA were analyzed by HPLC using the same protocol.

LC-MS assays for derivative products of 3-hydroxypropionaldehyde

Fractions were collected from the HPLC experiments of the derivative products of 3-hydroxypropionaldehyde in the total reactions with glycerol and 2-deoxy-2-fluoroglycerol as substrates. The samples for MS were dried under a vacuum freeze dryer and then redissolved in water. A 5 μL portion of the supernatant was analyzed by LC-MS.

Supplementary Figures:

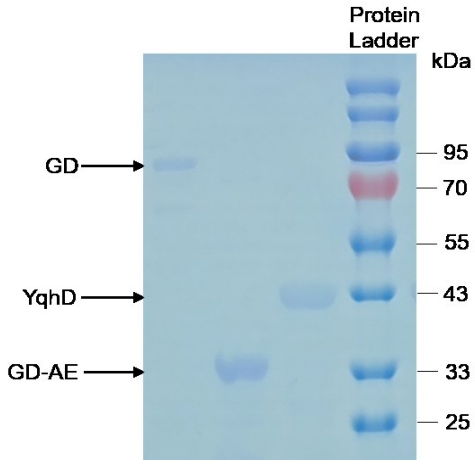

Figure S1. Coomassie blue-stained SDS-PAGE showing purified proteins of GD, GD-AE and YqhD.

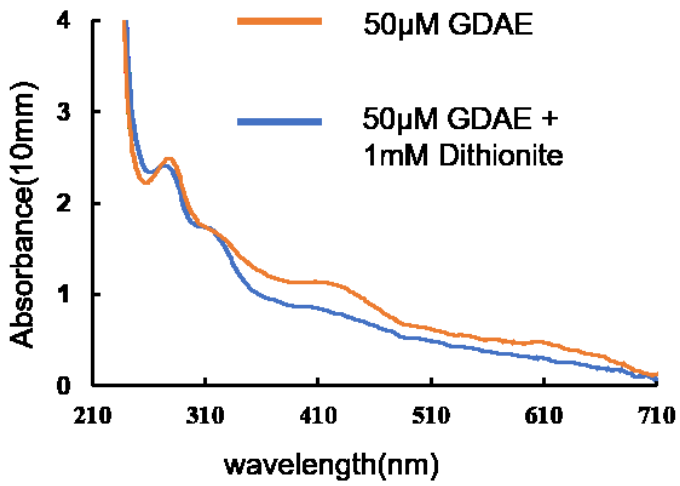

Figure S2. UV-Vis absorption spectra of reconstituted GD-AE.

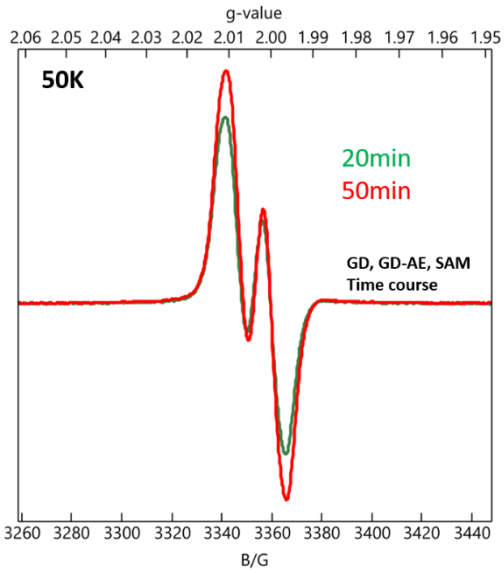

Figure S3. X-band EPR spectrum of the glycyl radical produced by GD-AE on GD at different incubation time. 20 min (Green); 50 min (Red). Conditions: modulation amplitude, 5 G; T = 50 K.

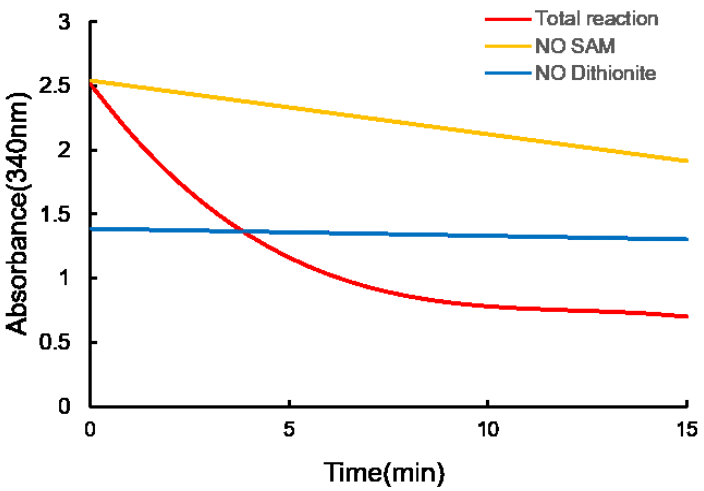

Figure S4. SAM-dependent activation of GD by GD-AE monitored on NADPH consumption at 340 nm. The full reaction (red) and the control reactions without SAM (yellow) or dithionite (blue).

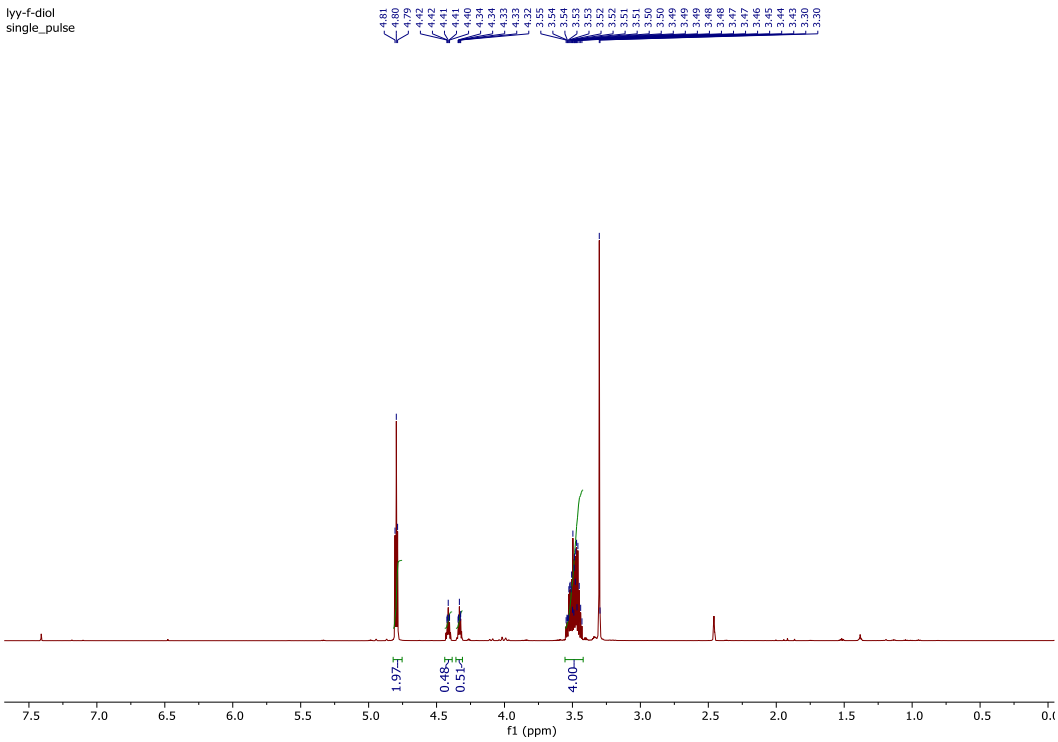

Figure S5. ^{1}H-NMR spectrum of 2-deoxy-2-fluoroglycerol.

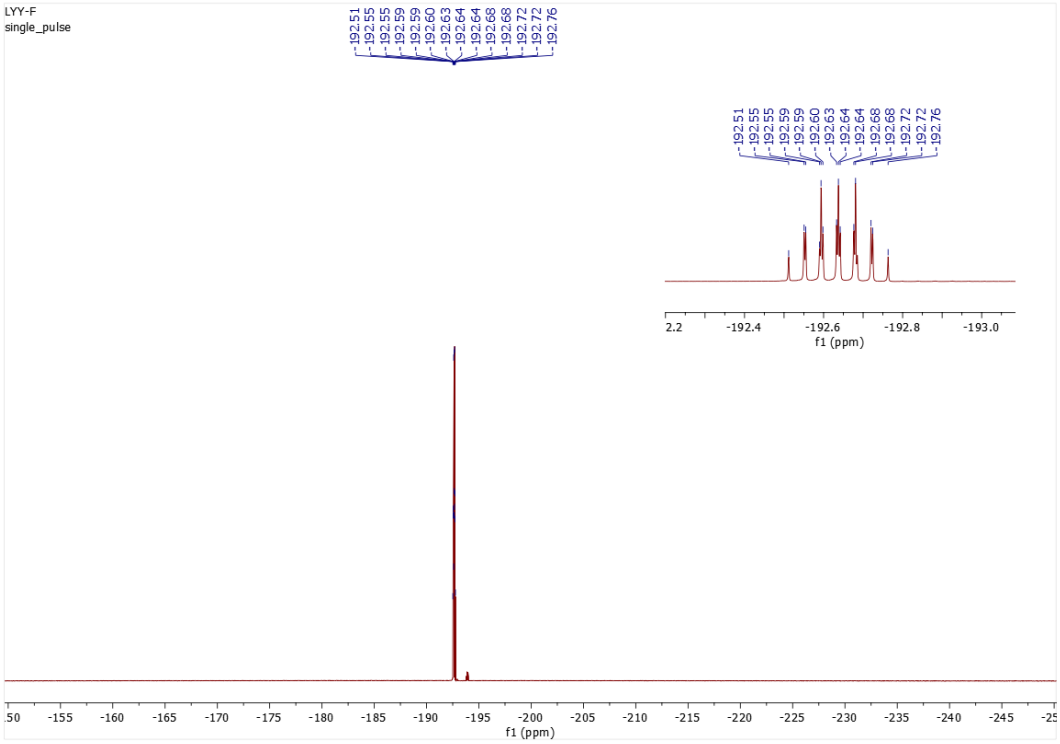

Figure S6. ^{19}F NMR spectrum of 2-deoxy-2-fluoroglycerol.

References:

(1) M. Dong, V. Kathiresan, M. K. Fenwick, A. T. Torelli, Y. Zhang, J. D. Caranto, B. Dzikovski, A. Sharma, K. M. Lancaster, J. H. Freed, S. E. Ealick, B. M. Hoffman and H. Lin, *Science*, 2018, **359,** 1247-1250.

(2) M. Xing, Y. Wei, Y. Zhou, J. Zhang, L. Lin, Y. Hu, G. Hua, A. N. N. Urs, D. Liu, F. Wang, C. Guo, Y. Tong, M. Li, Y. Liu, E. L. Ang, H. Zhao, Z. Yuchi and Y. Zhang, *Nat. Comm.*, 2019, 10, 1609.

(3) J. R. O'Brien, C. Raynaud, C. Croux, L. Girbal, P. Soucaille and W. N. Lanzilotta, *Biochemistry*, 2004, **43,** 4635-4645.

(4) T. Toba, R. Suzuki, J. Futamura-Takahashi, Y. Kawamoto, S. Tamura, M. Kuroda, Y. Shimmyo, M. Kadokura, K. Goto, T. Inoue, T. Muto and H. Annoura, *Bioorg. Med. Chem. Lett.,* 2018, **28,** 2528-2532.

(5) F. Kudo, Y. Yamamoto, K. Yokoyama, T. Eguchi and K. Kakinuma, *J. Antibiot.*, 2005, **58,** 766-774.