Supplementary Materials:

Biosynthesis of the fungal glyceraldehyde-3-phosphate dehydrogenase inhibitor heptelidic acid and mechanism of self-resistance

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Supplementary Methods

1. General materials and methods

Biological reagents, chemicals, media and enzymes were purchased from standard commercial sources unless stated. Fungal, yeast and bacterial strains, plasmids and primers used in this study are summarized in Tables S2, S3 and S4, respectively. DNA and RNA manipulations were carried out using Zymo ZR Fungal/Bacterial DNA Microprep™ kit and Invitrogen Ribopure™ kit, respectively. DNA sequencing was performed at Laragen, Inc. The primers were synthesized by IDT, Inc.

2. Construction of *Saccharomyces cerevisiae* strains

Plasmid pXW55 (*URA3* marker) digested with *Spe*I and *Pml*I was used to introduce the *hepA* gene (XP_013949969.1).[1] A 1.2 kb fragment containing cDNA of *hepA* obtained from PCR using primers HepA-xw55-recomb-F and HepA-xw55-recomb-R was cloned into pXW55 using yeast homologous recombination to afford pHepA-xw55. The plasmid pHepA-xw55 was then transformed into *Saccharomyces cerevisiae* RC01 to generate strain TY11.

Plasmid pXW06 (*TRP1* marker) digested with *Nde*I and *Pme*I was used to introduce the *hepH* gene (XP_013949970.1).[2] A 1.6 kb fragment containing cDNA of *hepH* obtained from PCR using primers HepH-xw06-recomb-F and HepH-xw06-recomb-R were cloned into pXW06 using yeast homologous recombination to afford pHepH-xw06. Plasmid pXW55 (*URA3* marker) digested with *Nde*I and *Pme*I was used to introduce the *T. virens* NADPH-cytochrome P450 reductase gene (XP_013956415.1). A 2.1 kb fragment containing cDNA of *T. virens* cytochrome P450 reductase obtained from PCR using primers TvCPR-xw55-recomb-F and TvCPR-xw55-recomb-R was cloned into pXW55 using yeast homologous recombination to afford pTvCPR-xw55. The plasmid pHepH-xw06 and pTvCPR-XW55 was then transformed into *Saccharomyces cerevisiae* RC01 to generate strain TY12.

Plasmid pXW02 (*LEU2* marker) digested with *Nde*I and *Pme*I was used to introduce the *hepD* gene (XP_013949971.1).[2] A 1.6 kb fragment containing cDNA of *hepD* obtained from PCR using primers HepD-xw02-recomb-F and HepD-xw02-recomb-R were cloned into pXW02 using yeast homologous recombination to afford pHepD-xw02. The plasmid pHepD-xw02 and pTvCPR-XW55 was then transformed into *Saccharomyces cerevisiae* RC01 to generate strain TY13.

Plasmid pXW06 (*TRP1* marker) digested with *Nde*I and *Pme*I was used to introduce the *hepC* gene (XP_013949972.1). A 1.6 kb fragment containing cDNA of *hepC* obtained from PCR using primers HepC-xw06-recomb-F and HepC-xw06-recomb-R were cloned into pXW06 using yeast homologous recombination to afford pHepC-xw06. The plasmid pHepC-xw06 and pTvCPR-XW55 was then transformed into *Saccharomyces cerevisiae* RC01 to generate strain TY14.

Plasmid pXW02 (*LEU2* marker) digested with *Nde*I and *Pme*I was used to introduce the *hepE* gene (XP_013949974.1). A 1.6 kb fragment containing cDNA of *hepE* obtained from PCR using primers HepE-xw02-recomb-F and HepE-xw02-recomb-R were cloned into pXW02 using yeast homologous recombination to afford pHepE-xw02. The plasmid pHepE-xw02 and pTvCPR-XW55 was then transformed into *Saccharomyces cerevisiae* RC01 to generate strain TY15.

3. Characterization of HepA

Heterologous expression and purification of HepA. To express HepA, TY11 were first grown in 2 mL uracil drop-out medium at 28 °C for 1 d, the culture of TY11 was then transferred into 1 L fresh YPD medium supplemented with 2% dextrose, and the culture was shaken at 28 °C at 250 rpm for 2 d. The cells were harvested by centrifugation (5,300 rpm, 30 min, 4 °C) and resuspended in TBS buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). After sonication and centrifugation (17,000 rpm, 60 min, 4 °C), the supernatant was subjected to FLAG-tag affinity purification. Recombinant HepA was eluted by 100 μg/mL FLAG peptides. Purified proteins in buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 5% glycerol) were aliquoted, flash frozen in liquid nitrogen, and were stored at -80 °C.

Biochemical analysis of HepA. The in vitro biochemical assay was performed in the 50 μL reaction mixture (50 mM Tris-HCl pH 7.5, 1 μM HepA, 1 mM FPP and 5 mM $MgCl_2$). The reaction was incubated at 30 °C for 2 h and extracted with 50 μL hexanes. The organic phase was subjected to analysis by GC-MS. GC-MS analyses were performed using Agilent Technologies GC-MS 6890/5973 equipped with a DB-FFAP column. An inlet temperature of 240 °C and constant pressure of 4.2 psi were used. The oven temperature was initially at 60 °C and then ramped at 10 °C/min for 20 min, followed by a hold at 240 °C for 5 min.

4. Fermentation and compound analyses and isolation

Trichoderma virens was maintained on PDA (potato dextrose agar, BD) for 7 d for sporulation or in liquid PDB medium (potato dextrose broth, BD) for RNA extraction. *T. virens* was maintained in PDB medium for production of heptelidic acid and its biosynthetic intermediates at 28 °C. To isolate heptelidic acid and its biosynthetic intermediates, fermentation broth of *T. virens* was centrifuged (5000 rpm, 10 min), and supernatant was extracted three times with ethyl acetate. The organic phase was dried over sodium sulfate, concentrated to oil form, and subjected to HPLC purification.

HPLC-MS analyses were performed using a Shimadzu 2020 EVLC-MS (Phenomenex® Luna, 5 µm, 2.0 × 100 mm, C-18 column) using positive and negative mode electrospray ionization. The elution method was a linear gradient of 5-95% (v/v) acetonitrile/water in 15 min, followed by 95% (v/v) acetonitrile/water for 3 min with a flow rate of 0.3 mL/min. The HPLC buffers were supplemented with 0.05% formic acid (v/v). HPLC purifications were performed using a Shimadzu Prominence HPLC (Phenomenex® Kinetex, 5 µm, 10.0 × 250 mm, C-18 column). The elution method was a linear gradient of 65-100% (v/v) acetonitrile/water in 25 min, with a flow rate of 2.5 mL/min.

5. Structure determination of compounds

Compound **3**, colorless oil readily dissolved in ethyl acetate and chloroform, had a molecular formula $C_{15}H_{22}O_3$, as deduced from LC-MS $[M+H]^+$ *m/z* 251, $[M-H]^-$ *m/z* 249. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.14 (1H, s), 4.88 (2H, d, 35.3), 4.09 (1H, q, 9.3), 2.92 (1H, dd, 17.4, 5.8), 2.41 (1H, d, 12.0), 2.25~2.15 (2H, m), 2.10~1.85 (3H, m), 1.87 (1H, d, 12.5), 1.44 (1H, m), 1.20 (1H, m), 0.94 (3H, d, 6.8), 0.77 (3H, d, 6.9). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.9, 149.5, 141.1, 127.6, 104.8, 66.7, 51.2, 46.4, 45.9, 36.9, 32.3, 27.3, 26.8, 21.3, 15.1 (Fig. S4 and Table S5).

Compound **4**, colorless oil readily dissolved in ethyl acetate and chloroform, had a molecular formula $C_{15}H_{22}O_4$, as deduced from LC-MS $[M+H]^+$ *m/z* 267, $[M-H]^-$ *m/z* 265. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.10 (1H, s), 3.75 (1H, m), 3.21 (1H, s), 2.79 (1H, dd, 15.9, 5.4), 2.70 (1H, d, 3.4), 2.23 (1H, td, 6.9, 3.0), 2.18~1.97 (3H, m), 1.95~1.85 (2H, m), 1.43-1.23 (3H, m), 0.97 (3H, d, 6.9), 0.81 (3H, d, 6.9). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.6, 139.5, 128.2, 68.0, 64.6, 49.8, 45.9, 45.5, 42.9, 34.8, 32.6, 26.7, 23.9, 21.3, 15.1 (Fig. S4 and Table S5).

Compound **5**, colorless oil readily dissolved in ethyl acetate and chloroform, had a molecular formula $C_{15}H_{20}O_4$, as deduced from LC-MS $[M+H]^+$ *m/z* 265, $[M-H]^-$ *m/z* 263. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.31 (1H, d, 2.0), 3.89 (1H, dd, 5.5, 1.5), 3.27 (1H, dt, 20.2, 2.6), 2.96 (1H, dt, 20.1, 2.2), 2.89 (1H, d, 12.0), 2.57~2.52 (1H, m), 2.51 (1H, d, 5.7), 2.34~2.22 (1H, m), 1.95~1.80 (2H, m), 1.51 (1H, tt, 11.7, 3.1), 1.36~1.23 (2H, m), 1.03 (3H, d, 6.9), 0.83 (3H, d, 6.9). ^{13}C NMR (125 MHz, $CDCl_3$): δ 204.3, 170.1, 141.9, 127.9, 58.1, 51.4, 51.1, 46.1, 44.2, 41.1, 35.5, 26.9, 23.0, 21.5, 15.2 (Fig. S4 and Table S5).

6. Biotransformation using *Saccharomyces cerevisiae* strains

To perform biotransformation of heptelidic acid biosynthetic intermediates, strain TY12 and TY14 were first grown in 2 mL uracil and L-tryptophan double drop-out medium at 28 °C for 1 d, strain TY13 and TY15 were first grown in 2 mL uracil and L-leucine double drop-out medium at 28 °C for 1 d. 0.3 mL of the culture of each strain was then transferred into 3 mL fresh YPD medium (yeast extract 10 g/L, peptone 20 g/L supplement with 2% dextrose), and the cultures were shaken at 28 °C at 250 rpm for 2 d. 0.1 mg of heptelidic acid biosynthetic intermediate was added to each culture, and then the cultures were shaken at 28 °C at 250 rpm, with 1 d to perform biotransformation. For product detection, cell cultures were extracted with 1 mL ethyl acetate. After evaporation of the organic phase, the crude extracts were dissolved in 100 μL methanol for LC-MS analysis.

7. Microsome purification and biochemical assay

Strain TY12 and TY14 were first grown in 2 mL uracil and L-tryptophan double drop-out medium at 28 °C for 1 d, strain TY13 and TY15 were first grown in 2 mL uracil and L-leucine double drop-out medium at 28 °C for 1 d. The 2 mL culture of each strain was then transferred into 50 mL fresh YPD medium (yeast extract 10 g/L, peptone 20 g/L supplement with 2% dextrose), and the cultures were shaken at 28 °C at 250 rpm for 2 d.

The microsomes were prepared as described below. Briefly, the cells were harvested by centrifugation (3,750 rpm at 4 °C for 10 min) and the cell pellet was washed with 10 mL of TES buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.6 M sorbitol). The cells were centrifuged as above, resuspended in 5 mL of TEG-M (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20% glycerol supplemented with 1.5 mM 2-mercaptoethanol), and 25 μL protease inhibitor cocktail (Sigma) was added. Zirconia/silica beads (0.5 mm in diameter, BioSpec Products) were added to surface of the cell suspension. Cell walls were disrupted

manually by hand shaking in a cold room for 10 min at 30-s intervals separated by 30-s intervals on ice. Cell suspension were aliquoted and transferred to a 1.5 mL centrifuge tube. Finally, microsomes were obtained by differential centrifugation at 10,000 g for 5 min at 4 °C to remove cellular debris (precipitant) followed by centrifugation at 100,000 g for 30 min at 4 °C and remove the supernatant. The microsomal pellets were resuspended in 1.5 mL of TEG-M buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20% glycerol, and 1.5 mM 2-mercaptoethanol) and stored frozen at -80 °C.

Assays for microsome bioactivity with **5** in TEG-M buffer were performed at 100 μL scale with microsomal cytochrome P450 and the reducing partner, 0.4 mM NADPH, 100 μM FAD, and 100 μM FMN 28 °C for 10 h.

8. Protein expression and purification

To express and purify HepG (XP_013949968.1), primers HepG-pET-F and HepG-pET-R were used to amplify a 1.0 kb DNA fragment containing *hepG*. The PCR product was cloned into pET28a using *Nde*I and *Not*I restriction sites. The resulted plasmid HepG-pET was transformed into *E. coli* BL21(DE3) to obtain TY16. To express and purify *Tv*-GAPDH (XP_013958680.1), primers tGAPDH-pET-F and tGAPDH-pET-R were used to amplify a 1.0 kb DNA fragment containing *T. virens* GAPDH. The PCR product was cloned into pET28a using *Nde*I and *Not*I restriction sites. The resulted plasmid *Tv*-GAPDH-pET was transformed into *E. coli* BL21(DE3) to obtain TY17. To express and purify *Hs*-GAPDH (NP_001276674.1), a 1.0 kb fragment containing codon optimized *Homo sapiens gapdh* was synthesized. The fragment was cloned into pSJ2 (Amp^R marker) using *Eco*RI and *Xho*I restriction sites. The resulting plasmid *Hs*-GAPDH-pSJ2 was transformed into *E.coli* BL21 (DE3) to give TY18.

The mutant *Hs*-GAPDH-L203A-pSJ2 was generated from *Hs*-GAPDH-pSJ2 by PCR using the forward primer *Hs*-GAPDH-L203A-F and reverse primer *Hs*-GAPDH-L203A-R. The resulting plasmid *Hs*-GAPDH-L203A-pSJ2 was verified by DNA sequencing, and then transformed into *E.coli* BL21(DE3) to give TY19. The mutant HepG-A201L-pET was generated from HepG-pET by PCR using the forward primer HepG-A201L-pET-F and reverse primer HepG-A201L-pET-R. The resulting plasmid HepG-A201L-pET was verified by DNA sequencing, and then transformed into *E.coli* BL21(DE3) to give TY20. The mutant *Hs*-GAPDH-T177A-pSJ2 was generated from *Hs*-GAPDH-pSJ2 by PCR using the forward primer *Hs*-GAPDH-T177A-F and reverse primer *Hs*-GAPDH-T177A-R. The resulting plasmid *Hs*-

GAPDH-T177A-pSJ2 was verified by DNA sequencing, and then transformed into *E.coli* BL21(DE3) to give TY21. The mutant *Hs*-GAPDH-A232S-F233V-pSJ2 was generated from *Hs*-GAPDH-pSJ2 by PCR using the forward primer *Hs*-GAPDH-A232S-F233V-F and reverse primer *Hs*-GAPDH-A232S-F233V-R. The resulting plasmid *Hs*-GAPDH-A232S-F233V-pSJ2 was verified by DNA sequencing, and then transformed into *E.coli* BL21(DE3) to give TY22. The mutant *Hs*-GAPDH-I182Y-pSJ2 was generated from *Hs*-GAPDH-pSJ2 by PCR using the forward primer *Hs*-GAPDH-I182Y-F and reverse primer *Hs*-GAPDH-I182Y-R. The resulting plasmid *Hs*-GAPDH-I182Y-pSJ2 was verified by DNA sequencing, and then transformed into *E.coli* BL21(DE3) to give TY23.

All GAPDHs fused a 6 × His-tag or 8 × His-tag with a molecular weight ~ 38 kD were expressed at 16 °C 220 rpm for 20 h after 100 μM β-D-1-thiogalactopyranoside IPTG induction (IPTG was added when OD_{600} = 0.8). Cells of 1 L culture were then harvested by centrifugation at 5000 rpm at 4 °C. Cell pellet was resuspended in 15 mL Buffer A10 (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 8% glycerol, 10 mM imidazole). The cells were lysed by sonication, and the insoluble material was sedimented by centrifugation at 16000 rpm at 4 °C. The protein supernatant was then incubated with 3 mL Ni-NTA for 4 h with slow, constant rotation at 4 °C. Subsequently the Ni-NTA resin was washed with 10 column volumes of Buffer A50 (Buffer A + 50 mM imidazole). For elution of the target protein, the Ni-NTA resin was incubated for 10 min with 6 mL Buffer A250 (Buffer A + 250 mM imidazole). The supernatant from the elution step was then analyzed by SDS-PAGE together with the supernatants from the other purification steps. The elution fraction containing the recombinant protein was buffer exchanged into storage buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 10% glycerol, 5 mM dithiothreitol, 5 mM GSH).

9. Biochemical assay of GAPDHs

Steady-state kinetics measurement. In vitro activity assays were carried out in 100 μL reaction mixture containing 20 mM Tris-HCl pH 8.75, 0.2 mM EDTA, 10 mM sodium arsenate, 1 mM dithiothreitol, 1 mM NAD^+, and 0.008 to 1 mM D-glyceraldehyde-3-phosphate hydrate (GAP). After 10 min incubation at 37 ˚C, the reaction was initiated by adding 1 μL purified GAPDH enzyme to final concentration of 0.01 μM. The reaction was monitored by UV absorption at 340 nm to detect the production of NADH at 37 ˚C. The kinetic parameters K_M and k_{cat} of GAPDH for GAP were obtained by fitting the data to Michaelis-Menten equation:

$$V_0 = \frac{V_{max}[S]}{K_M + [S]}$$

Inhibition kinetics measurement. To analyze the inhibition kinetics, 2.5 μL purified GAPDH enzyme (concentration of 0.2 μM) was added to 2.5 μL of solution of 20 mM Tris-HCl pH 8.75 buffer with or without inhibitor heptelidic acid at certain concentration (6400 ~ 0.2 μM). After the mixture was incubated at 37 ˚C for three different amount of time (3 min, 6min, 12 min, 24 min or 48 min) to perform the inhibition reaction, the resulting mixture was added to 100 μL solution containing 20 mM Tris-HCl pH 8.75, 0.2 mM EDTA, 10 mM sodium arsenate, 1 mM dithiothreitol, and 1 mM NAD^+ to stop the inhibition reaction. To initiate the enzymatic reaction of remaining GAPDH, 1μL GAP was added to the resulting mixture to a final concentration of 1 mM. The reaction was monitored by UV absorption at 340 nm to detect the production of NADH at 37 ˚C. The apparent pseudo-first-order rate constant k_{app} of the inhibition reaction of GAPDH using heptelidic acid at each concentration is obtained by fitting the data to the following equation:

$$\ln\left(\frac{E}{E_0}\right) = -k_{app}t$$

where E_0 is the activity of GAPDH without inhibition, E is the remaining activity of GAPDH after inhibition reaction, which is determined by the reaction rate catalyzed by the inhibited GAPDH. Then the inhibition kinetic parameters K_I and k_{inact} of GAPDH were obtained by fitting the data to the following equation using Kitz-Wilson plot:

$$k_{app} = \frac{k_{inact}[I]}{K_I + [I]}$$

10. Structure determination of *Hs*-GAPDH-HA complex and HepG

Over-expression and purification of *Hs*-GAPDH and HepG for crystallization. The *Hs*-GAPDH cDNA was cloned into pSJ2 vector and transformed into *E*. coli strain BL21(DE3) for protein production.[3] A single colony of the strain was used to inoculate 5 mL of LB medium that was supplemented with ampicillin (50 μg/mL) and grown 6 h at 37 °C while being shaken constantly at 200 rpm. Then the culture was inoculated to 1 L of LB medium supplemented with 50 μg/mL ampicillin. After grown at 37 °C until the OD_{600} reached 0.8, the cells were cooled to 16 °C. IPTG was added into the culture to a final concentration of 0.4 mM, and incubated for 16 h to induce protein expression. The culture was harvested

at 8000 rpm for 3 min at 4 °C. The cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 8.5, 500 mM NaCl, 5 mM β-mercaptoethanol, 1mM phenylmethanesulfonyl fluoride), and lysed using high pressure cell crusher (Union Biotech, China) with 60 ~ 100 MPa at 4 °C. Cell debris was removed by centrifugation at 30000 *g*, 4 °C for 20 min. After the supernatant protein mixture was bound to Ni-NTA agarose (GE Healthcare), the unbound proteins were eluted using 100 mL of buffer A, and the *Hs*-GAPDH protein was eluted using 100 mL of buffer B (buffer A + 300 mM imidazole). To remove His-tag, the desired protein fractions obtained from the Ni-NTA purification were incubated with TEV protease in dialysis bag (14kD) for 16 ~ 18 h at 4 °C while being stirred gently in 2 L dialysate buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl). After overnight digestion, the *Hs*-GAPDH with His-tag removed was purified using a 5mL Ni-NTA column that had been equilibrated with buffer A, and the flow-through fractions were concentrated to 2 ml by Amicon® ultra filter units with a 30 kDa molecular weight cut-off (Millipore). Subsequently, the *Hs*-GAPDH protein was further purified using size exclusion chromatography (Superdex 200 16/60, GE Healthcare) in buffer C (50 mM Tris-HCl, pH 8.5, 50 mM NaCl, 2 mM dithiothreitol). The purified protein were concentrated to 30 mg/mL for crystallization. The HepG protein was purified with the same method but without removing the His-tag.

Crystallization of *Hs*-GAPDH-heptelidic acid complex and HepG. The purified *Hs*-GAPDH at concentration of 10 mg/mL in buffer C was mixed with heptelidic acid with 1:5 ratio, and the mixture was incubated on ice for half an hour. Crystallization was initially performed using crystal screening kits (Hampton Research Co. and QIAGEN Co.). The proteins were mixed in a 1:1 ratio with the reservoir solution in a 2-μL volume and equilibrated against 50 μL reservoir solution, using the sitting-drop vapor diffusion method at 16 °C. The HepG was crystallized using the same method but no complex with heptelidic acid was observed. Crystal were grown in several conditions after 3 d. After optimization, we obtained the crystal of *Hs*-GAPDH-HA diffracted to a resolution of 1.83 Å (in the condition of 0.1 M zinc acetate dihydrate, 0.1 M Sodium acetate trihydrate, pH 4.6, 12% w/v polyethylene glycol (PEG) 4000) and the HepG crystal with resolution of 2.06 Å (in the condition of 0.1 M sodium acetate, 0.1M HEPES pH 7.5, 22% (w/v) PEG 4000).

Data collection and processing. Crystals from different reservoir solution were flash-cooled in liquid nitrogen after being dipped into a solution containing 25% glycerol. The data were collected at the Beamline 18U1 in Shanghai Synchrotron Radiation Facility (SSRF). Diffraction data of *Hs*-GAPDH-

heptelidic acid complex was collected at the wavelength of 0.97853 Å. The best crystals diffracted to a resolution of 1.83 Å and belonged to space group $P2_12_12_1$. Diffraction data of HepG was collected at the wavelength of 0.97930 Å. The best crystals diffracted to a resolution of 2.06 Å and belong to space group $P12_11$. All data sets were indexed, integrated, and scaled using the HKL3000 package.[4] The statistics of the data collection are summarized in Table S6.

Structure determination and refinement. The *Hs*-GAPDH-heptelidic acid structure was solved by the molecular replacement method. The crystal structure of human placental glyceraldehyde-3-phosphate dehydrogenase (PDB:1U8F) was utilized as the search model by the Phaser embedded in the Phenix, and the resulting model were refined against the diffraction data with the Refinement embedded in the Phenix.[5-7] The protein, heptelidic acid, NAD, water molecule and so on were manually built using the program WinCoot.[8] The R_{work} and R_{free} values of the structure are 19.21% and 22.82%, respectively. The HepG structure was determined with the same method while the crystal structure of the photoreceptor glyceraldehyde 3-phosphate dehydrogenase (PDB: 4O59) was utilized as the search model.[9] The R_{work} and R_{free} values of the structure are 15.69% and 19.91%, respectively. The refinement statistics are summarized in Table S6. Structural factor and coordinate of *Hs*-GAPDH-heptelidic acid (PDB: 6M61) and HepG (PDB: 6M5X) have been deposited in the Protein Data Bank respectively. All crystal structure figures were prepared using PyMOL.[10]

Supplementary Figures

Figure S1. Phylogenetic tree of GAPDHs among bacteria, fungi and animals. The evolutionary history was inferred by using the neighbor-joining method (MEGA6). Scale-bar units represent the number of amino acid substitutions per site.

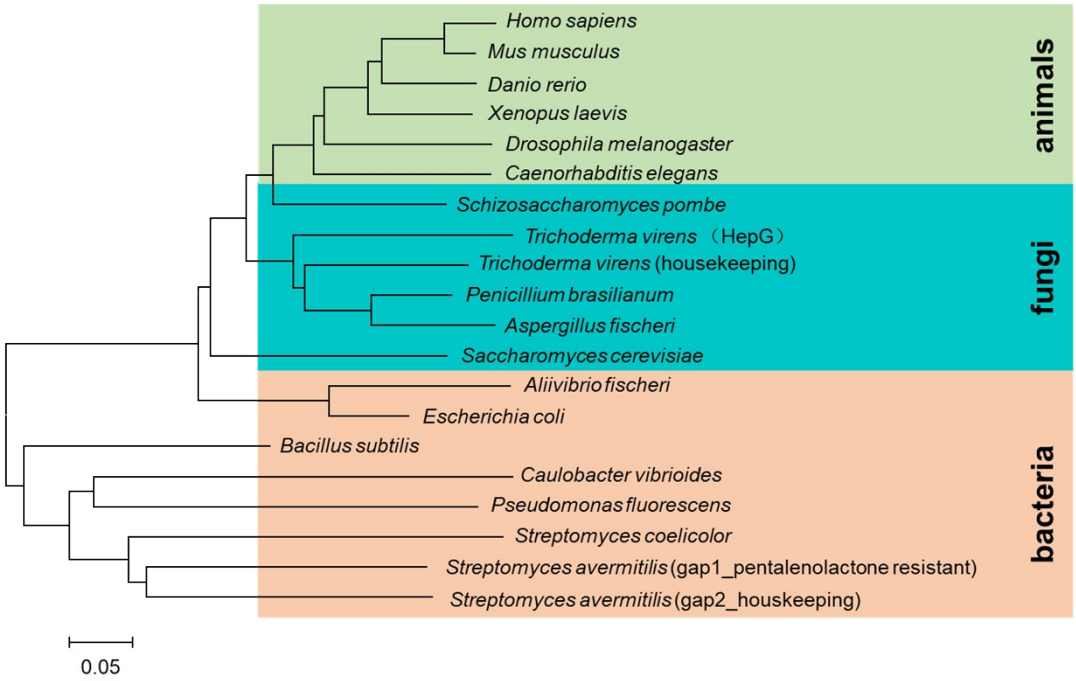


Figure S2. Characterization of HepA. **A.** SDS-PAGE analysis of purified HepA. **B.** GC trace of in vitro chemical analysis of HepA using FPP (up) and extract of *S. cerevisiae* RC01 expressing HepA. **C.** EI-MS of compound **6**. **D.** The reaction catalyzed by HepA. **E.** The biochemical analysis result is consistent with the previously reported isotopic acetate incorporation in biosynthesis of heptelidic acid.

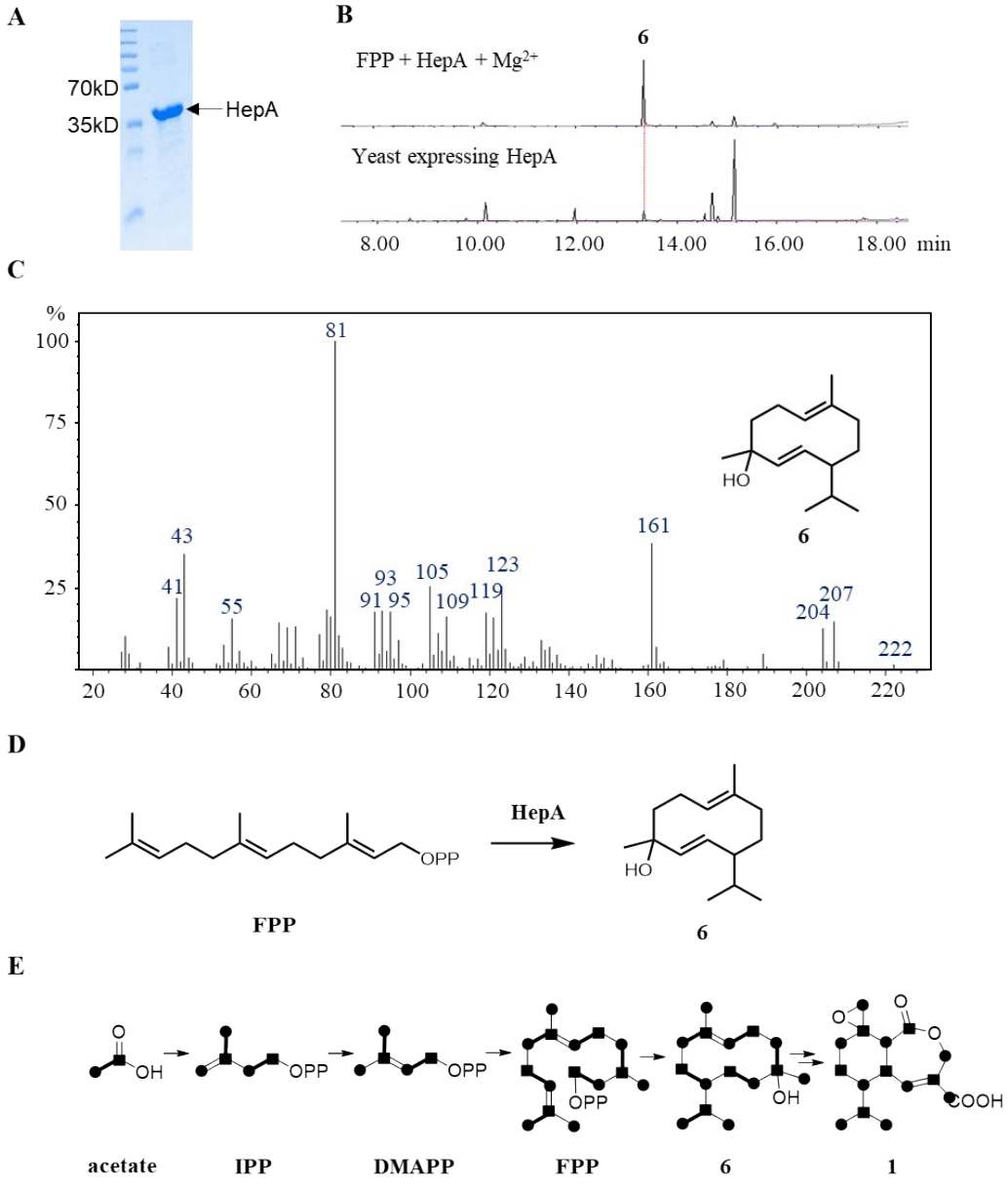
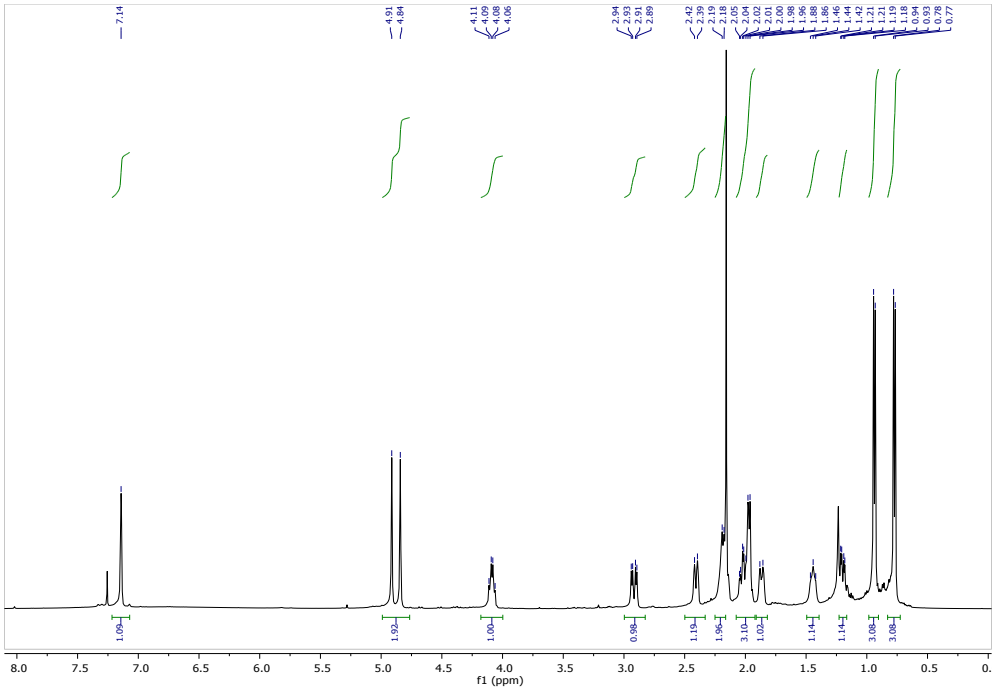
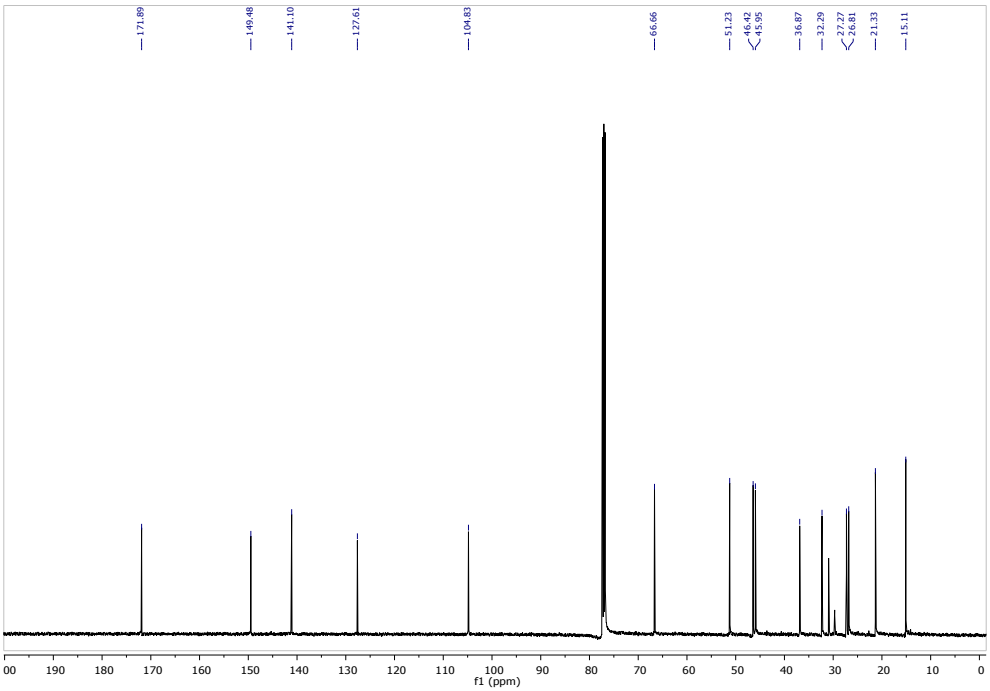


Figure S3. NMR analyses of the compounds in this study

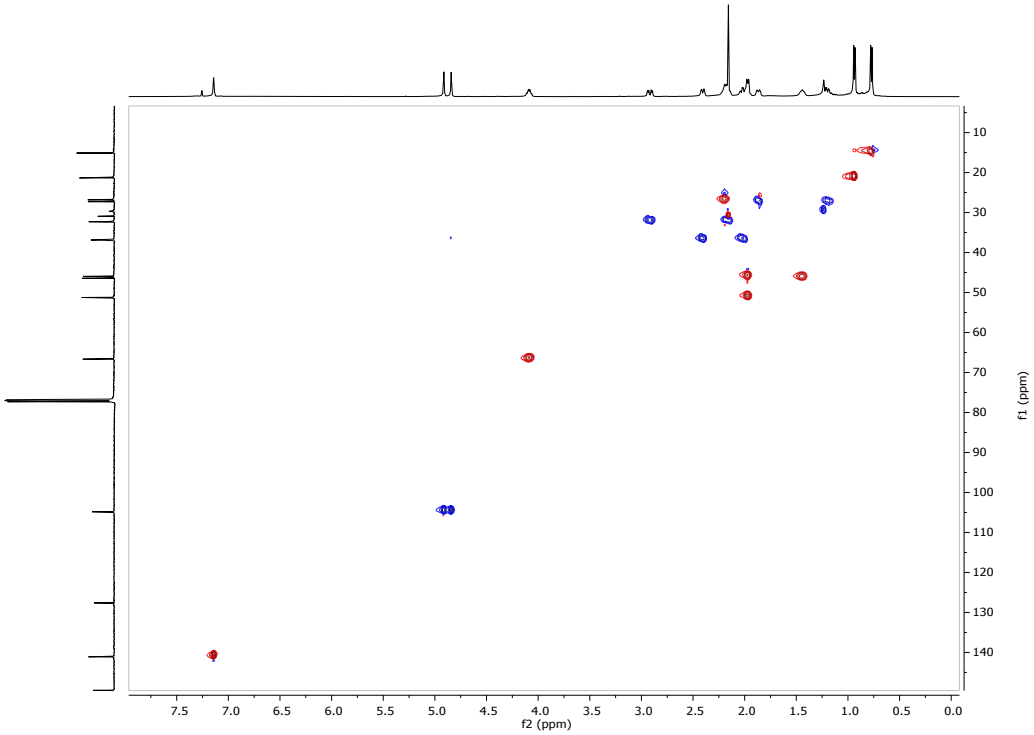
^{1}H NMR of compound 3 (500 MHz, $CDCl_3$):



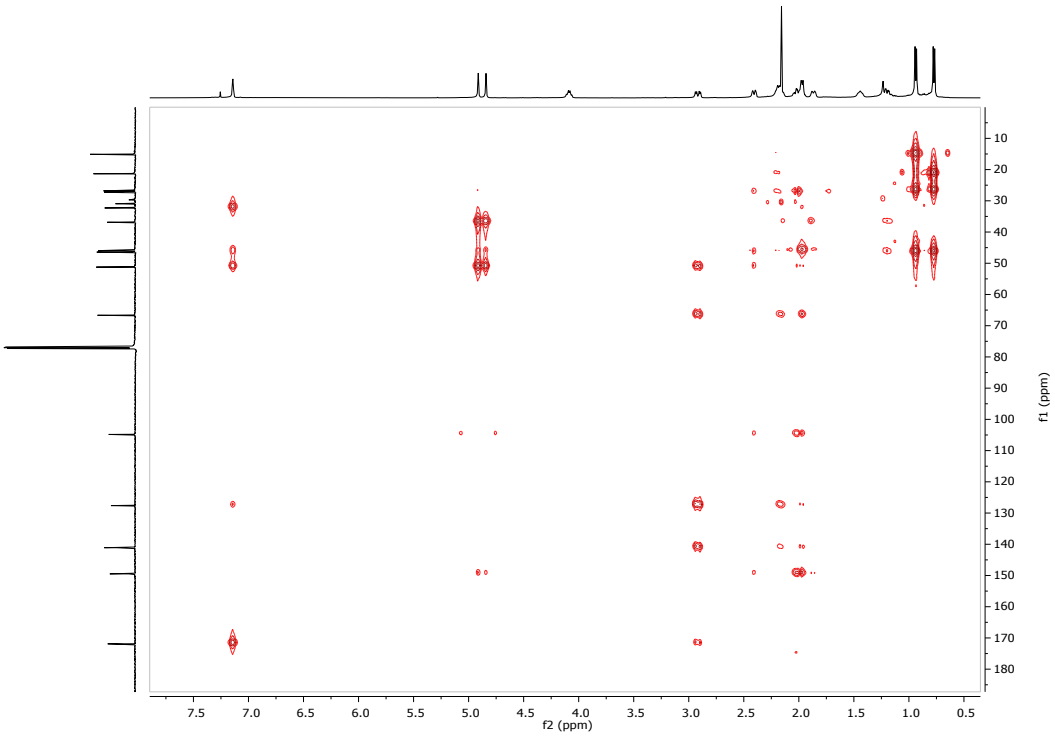
^{13}C NMR of compound 3 (125 MHz, $CDCl_3$):



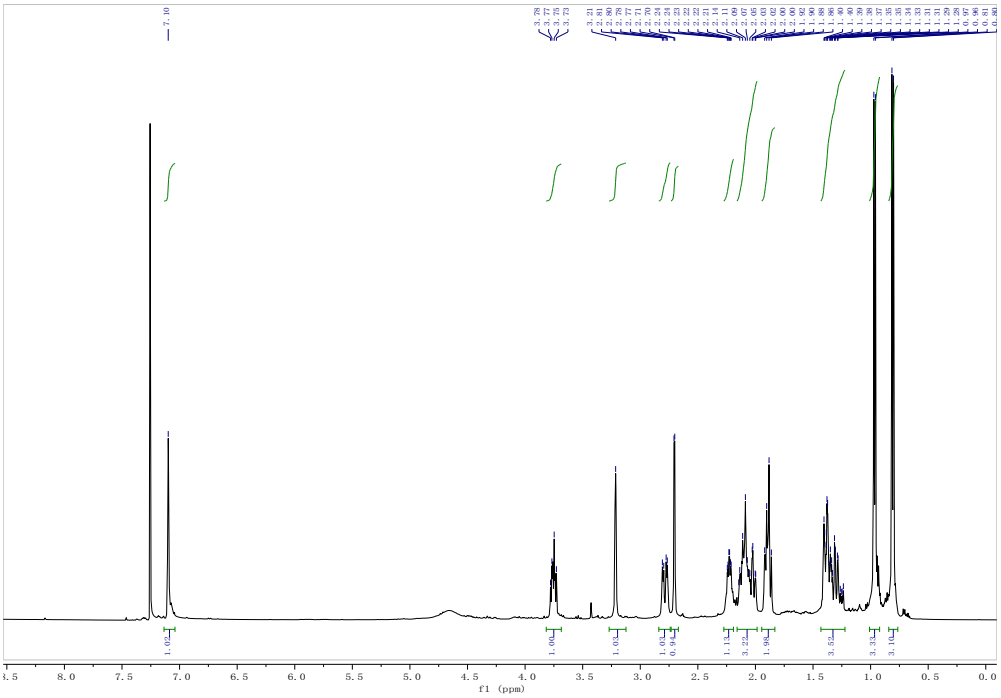
HSQC of compound 3 (500 MHz, $CDCl_3$):



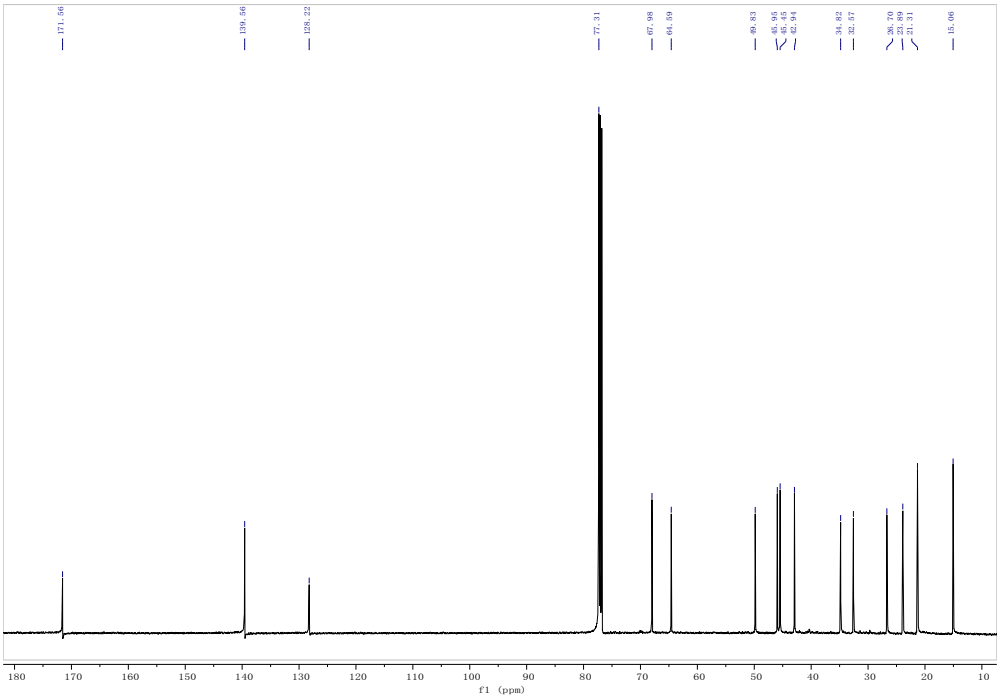
HMBC of compound 3 (500 MHz, $CDCl_3$):



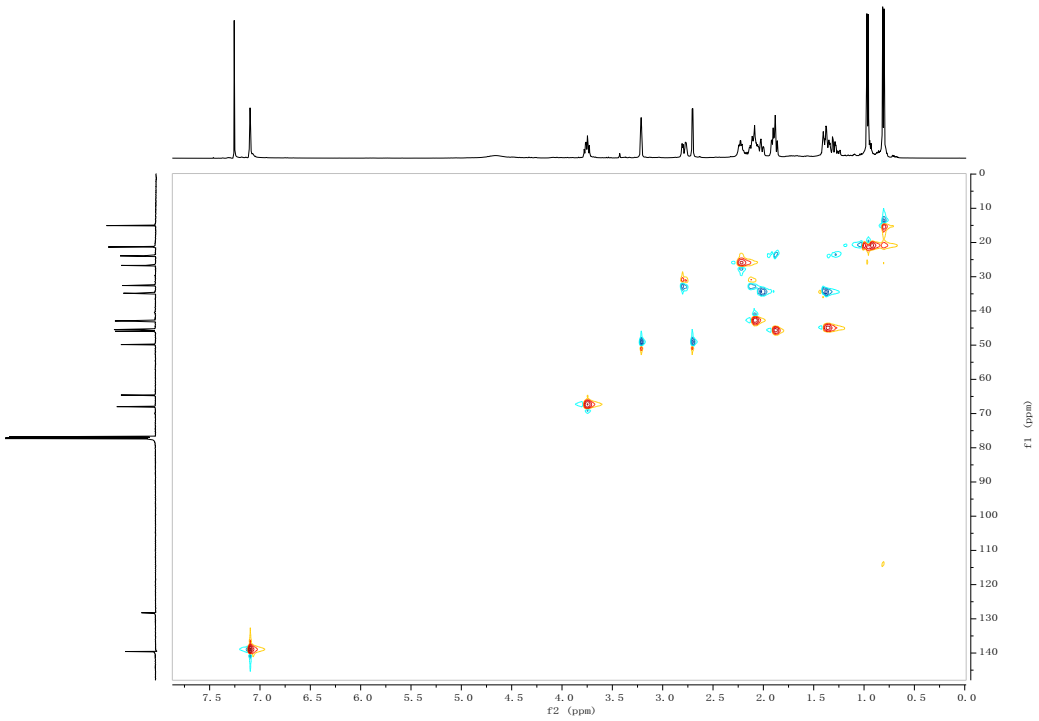
^{1}H NMR of compound 4 (500 MHz, $CDCl_3$):



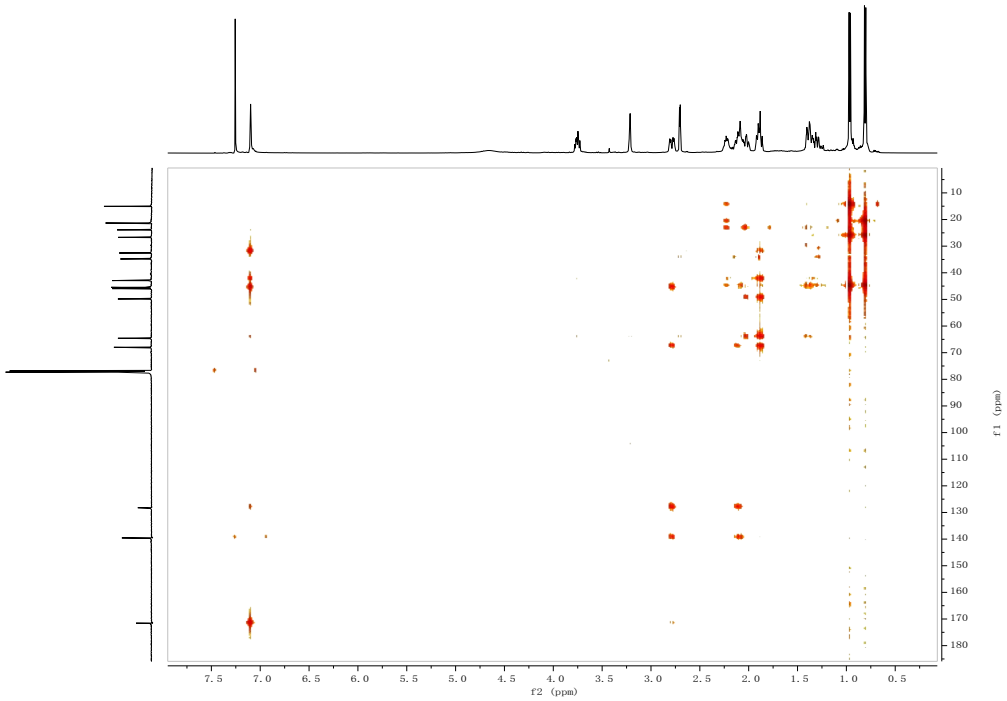
^{13}C NMR of compound 4 (125 MHz, $CDCl_3$):



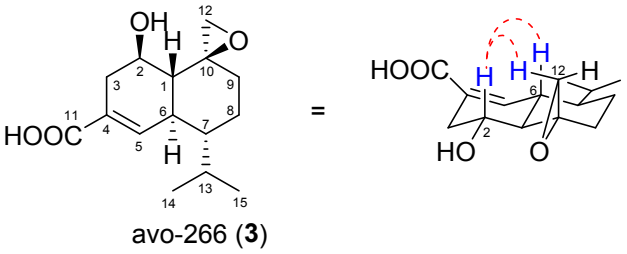
HSQC of compound 4 (500 MHz, $CDCl_3$):



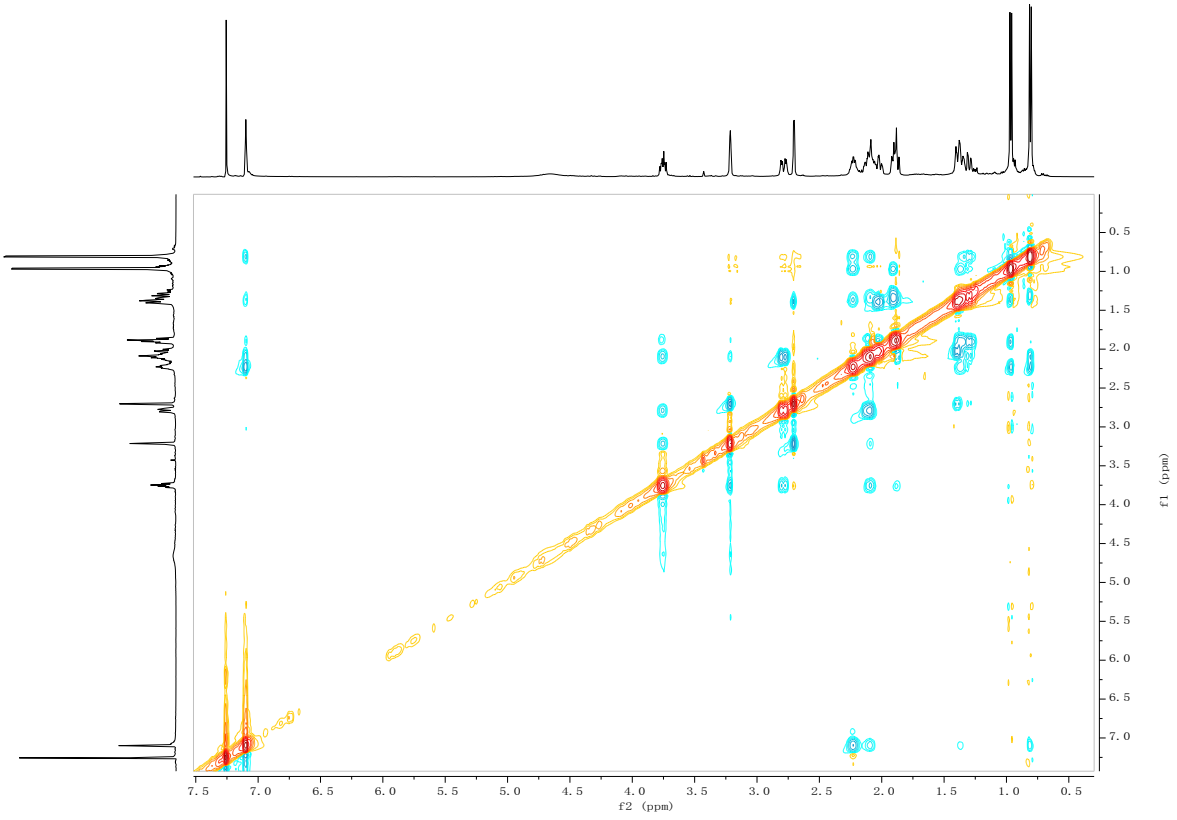
HMBC of compound 4 (500 MHz, $CDCl_3$):



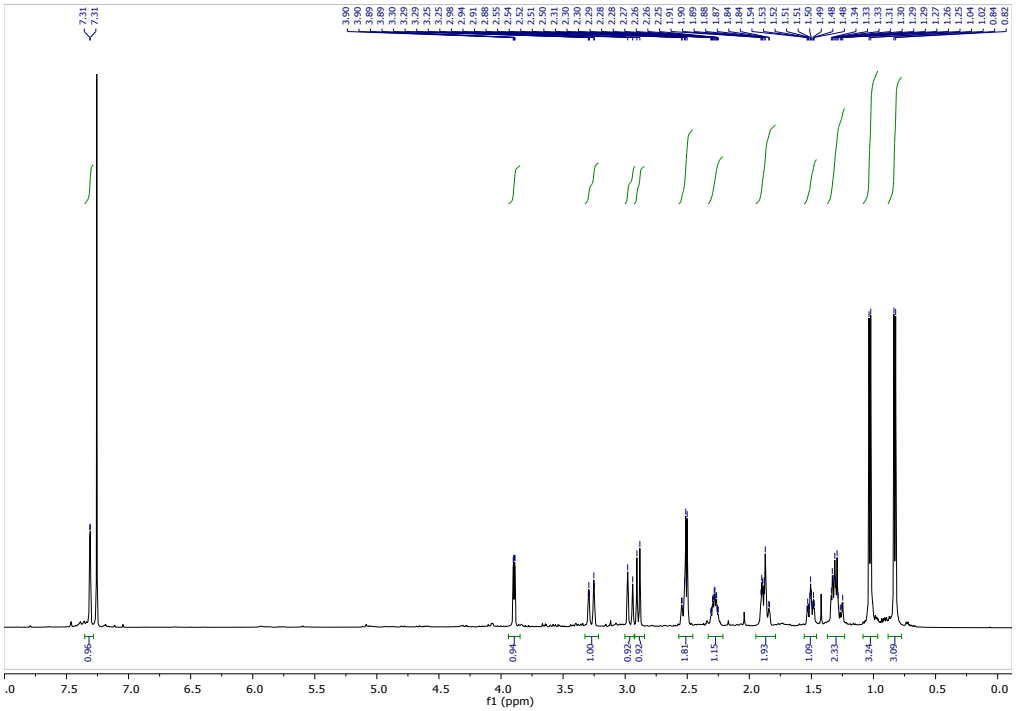
NOESY of compound 4 (500 MHz, $CDCl_3$):



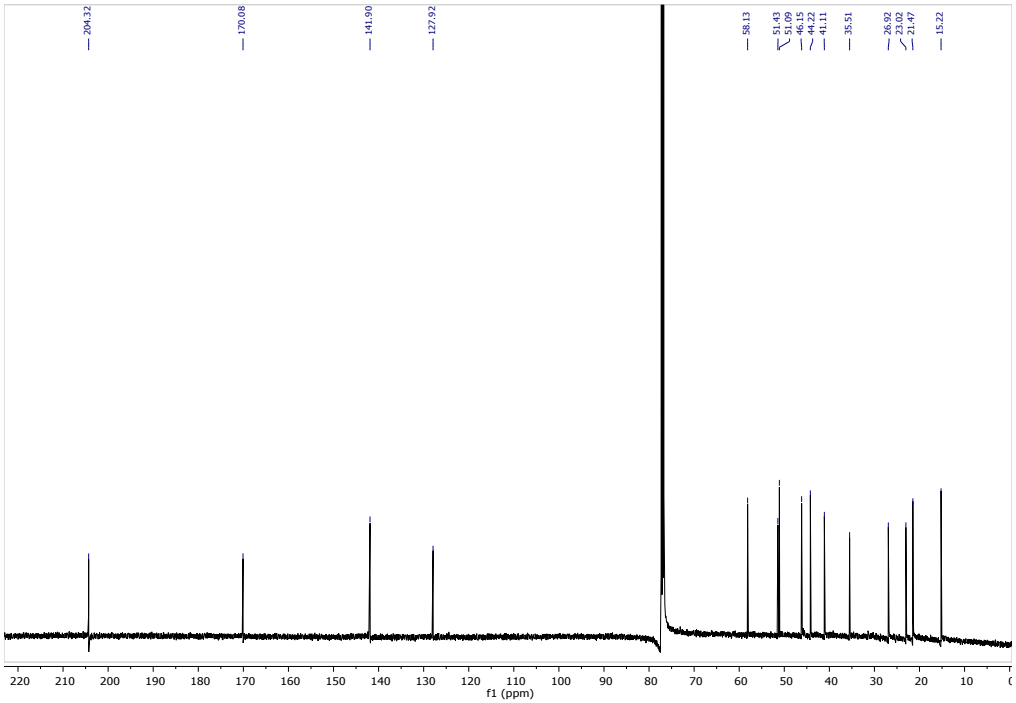
avo-266 (**3**)



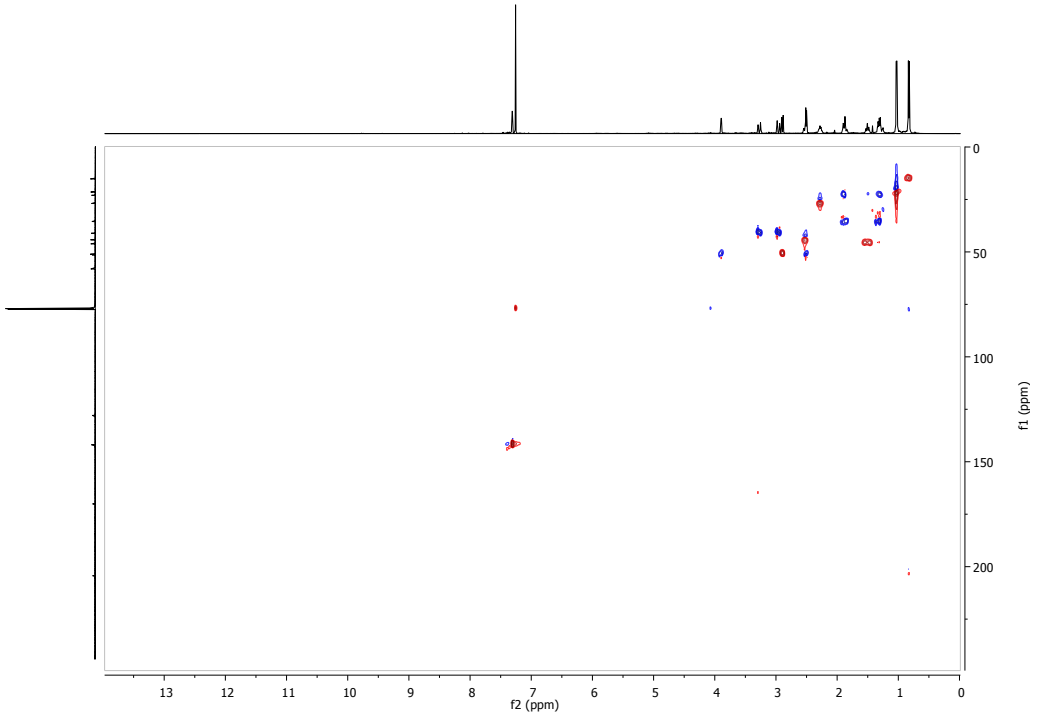
^{1}H NMR of compound 5 (500 MHz, $CDCl_3$):



^{13}C NMR of compound 5 (125 MHz, $CDCl_3$):



HSQC of compound 5 (500 MHz, $CDCl_3$):



HMBC of compound 5 (500 MHz, $CDCl_3$):

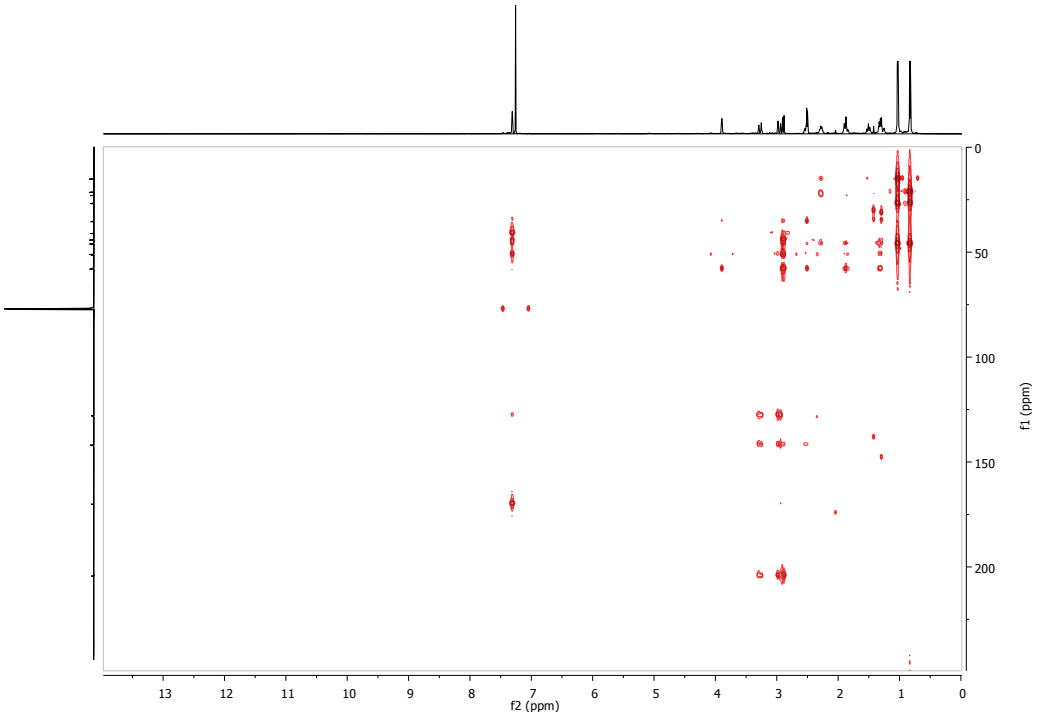


Figure S4. SDS-PAGE analysis of purified proteins. SDS-PAGE analysis of purified *Hs*-GAPDH (**A**), *Tv*-GAPDH (**B**), HepG (**C**), *Hs*-GAPDH-T177A (**D**), *Hs*-GAPDH-S232A-F233V (**E**), *Hs*-GAPDH-I181Y (**F**), *Hs*-GAPDH-L203A (**G**), and HepG-A201L (**H**) from *E. coli* BL21 (DE3).

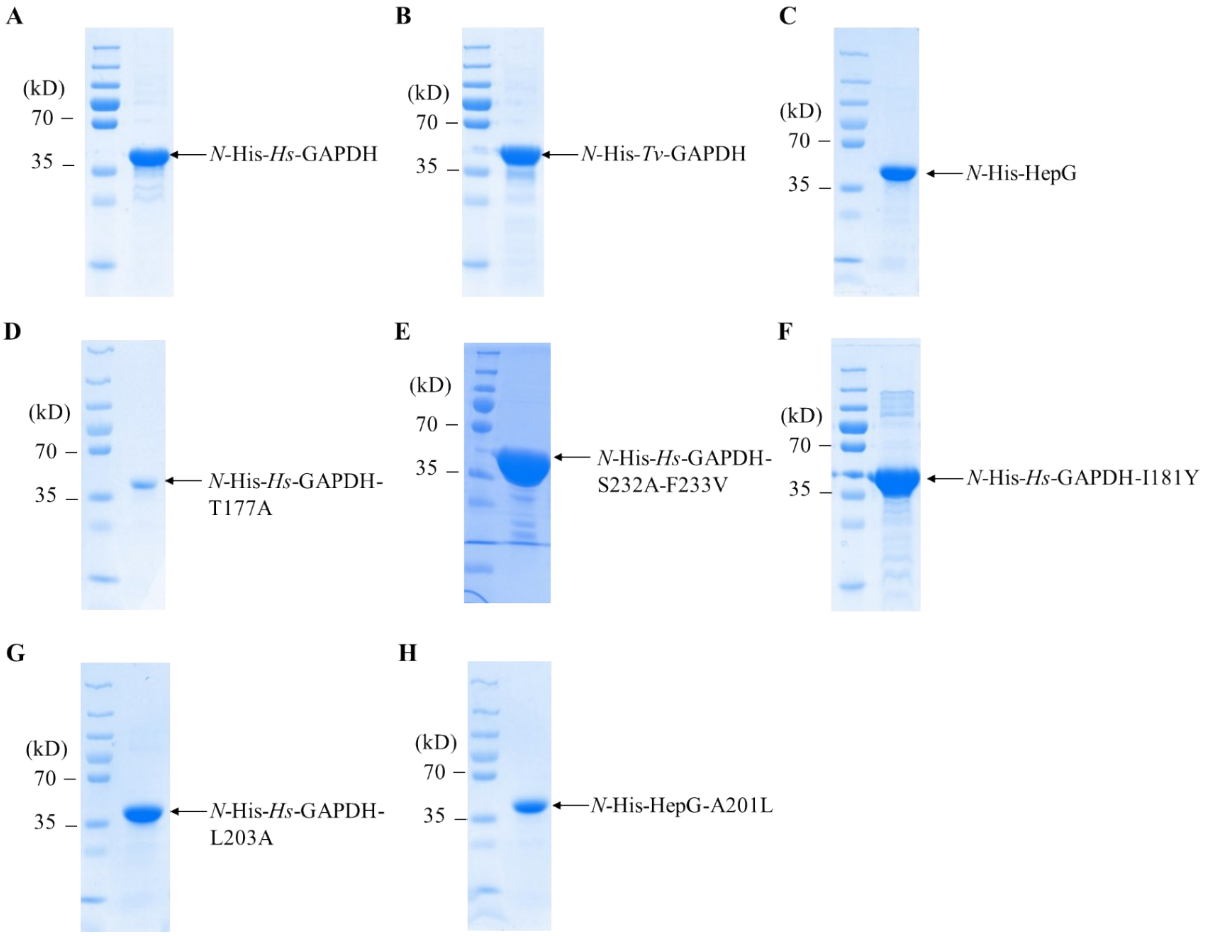
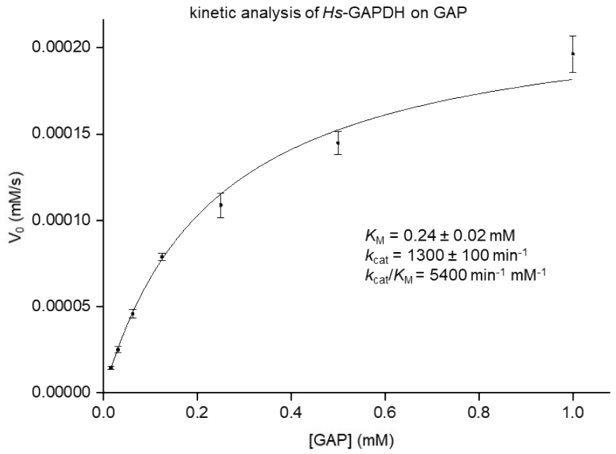
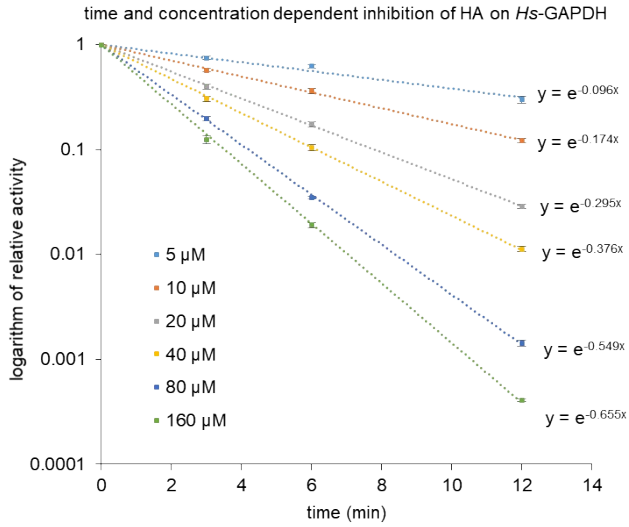


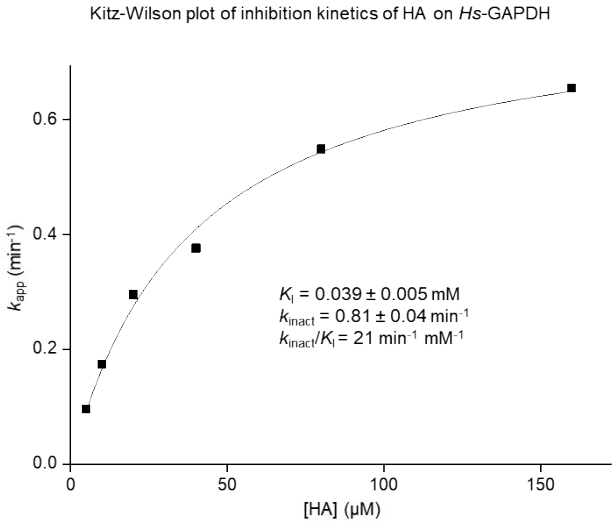
Figure S5. Enzymatic and inhibition kinetic study of *Hs*-GAPDH. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of *Hs*-GAPDH for GAP

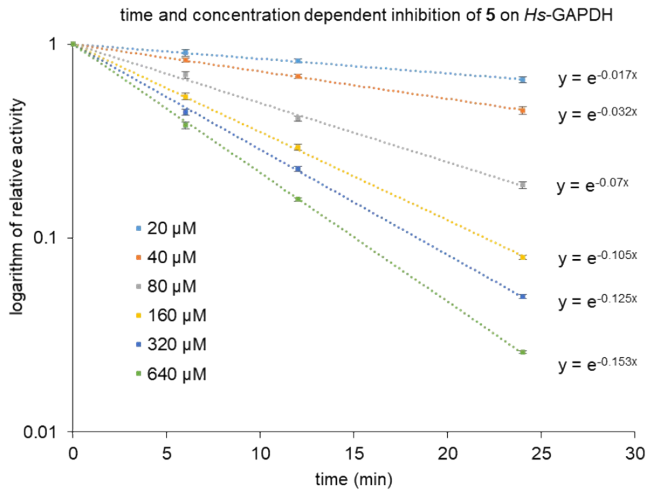


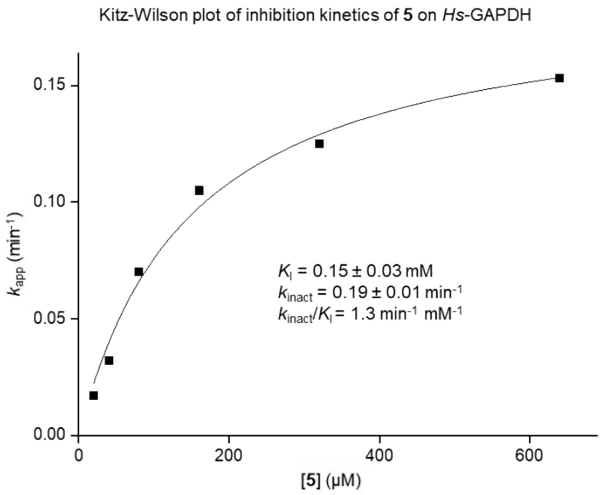
B. Inhibition study of *Hs*-GAPDH using HA



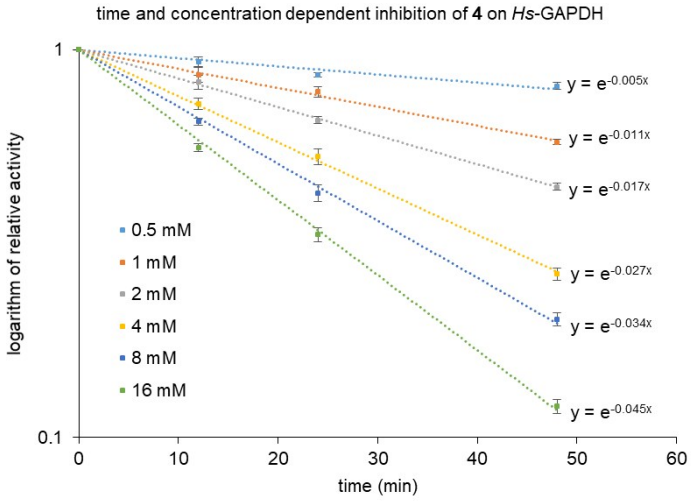


C. Inhibition study of *Hs*-GAPDH using **5**





D. Inhibition study of *Hs*-GAPDH using **4**



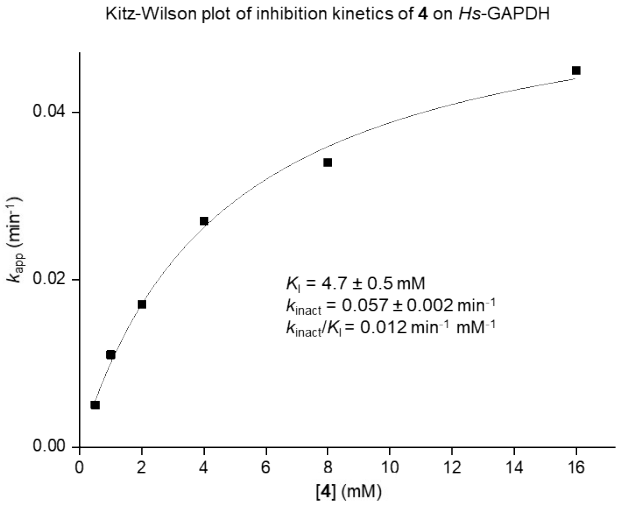
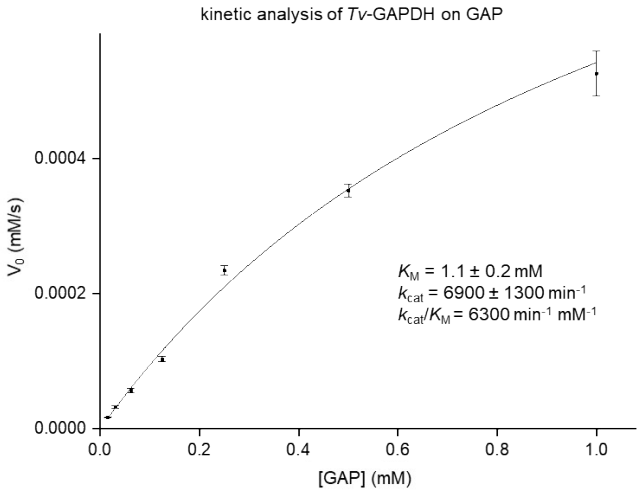
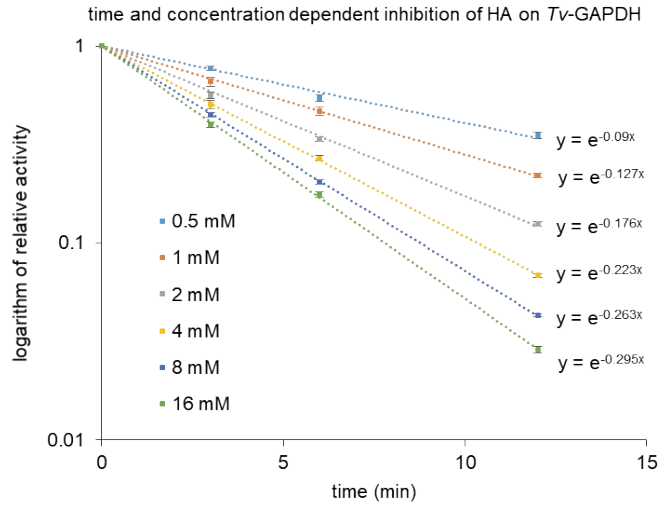


Figure S6. Enzymatic and inhibition kinetic study of *Tv*-GAPDH. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of *Tv*-GAPDH for GAP



B. Inhibition study of *Tv*-GAPDH using HA



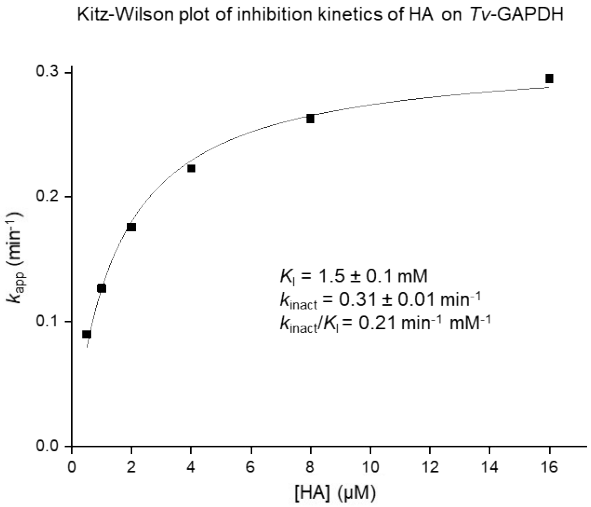
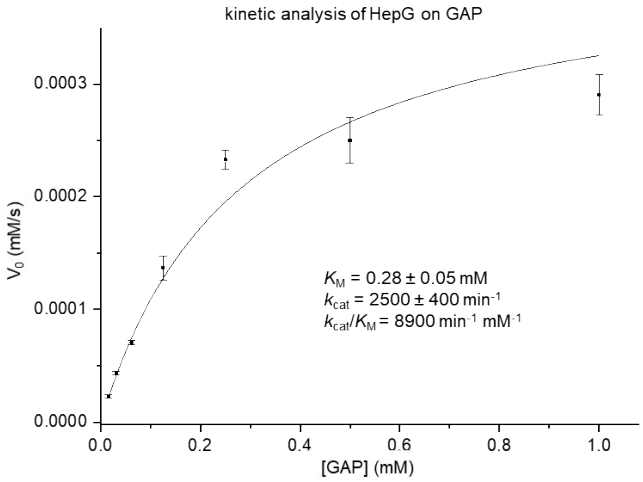
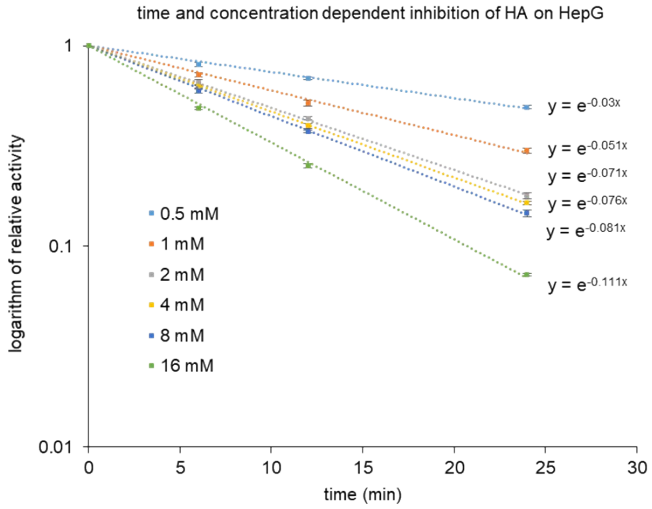


Figure S7. Enzymatic and inhibition kinetic study of HepG. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of HepG for GAP



B. Inhibition study of HepG using HA



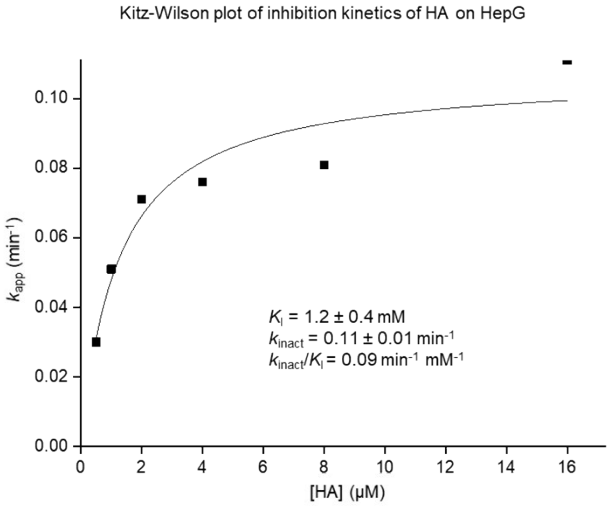
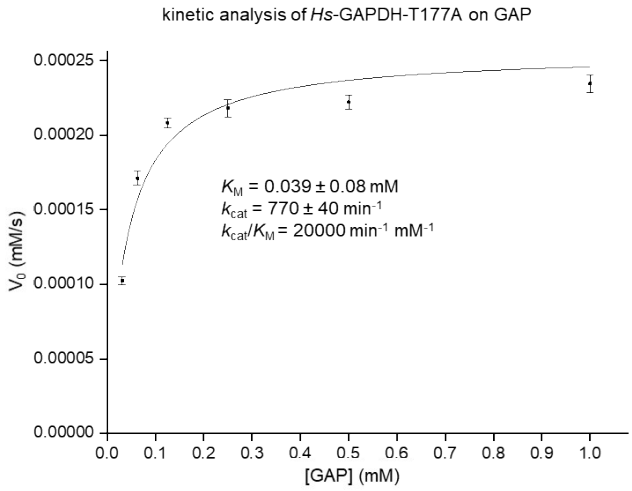
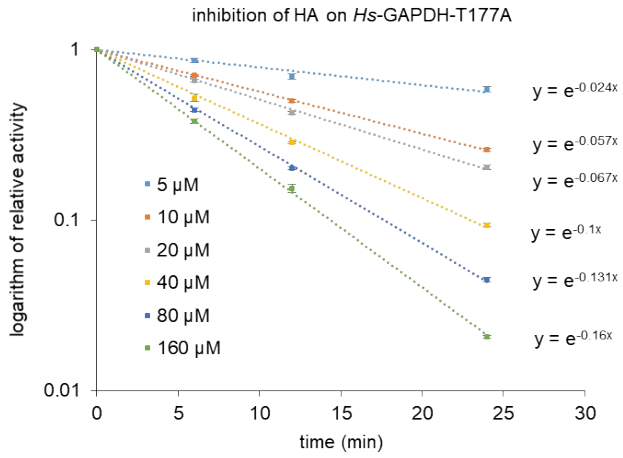


Figure S8. Enzymatic and inhibition kinetic study of *Hs*-GAPDH-T177A. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of *Hs*-GAPDH-T177A for GAP



B. Inhibition study of *Hs*-GAPDH-T177A using HA



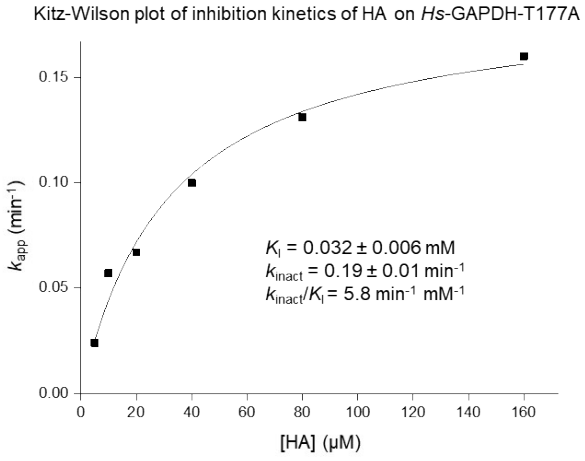
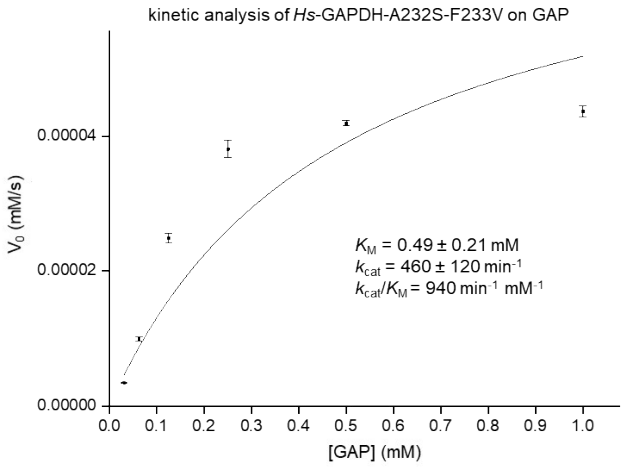
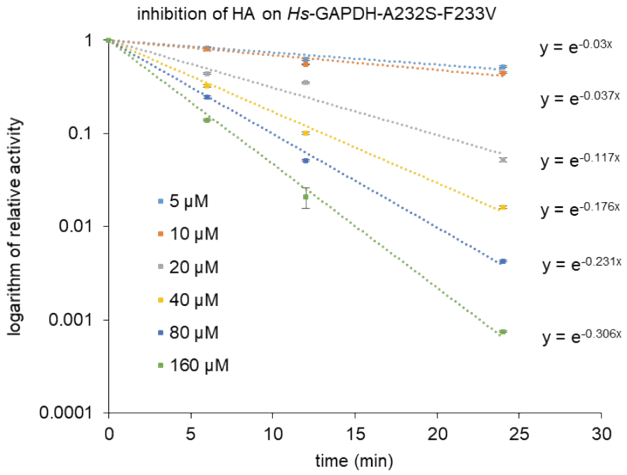


Figure S9. Enzymatic and inhibition kinetic study of *Hs*-GAPDH-A232S-F233V. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of *Hs*-GAPDH-A232S-F233V for GAP



B. Inhibition study of *Hs*-GAPDH-A232S-F233V using HA



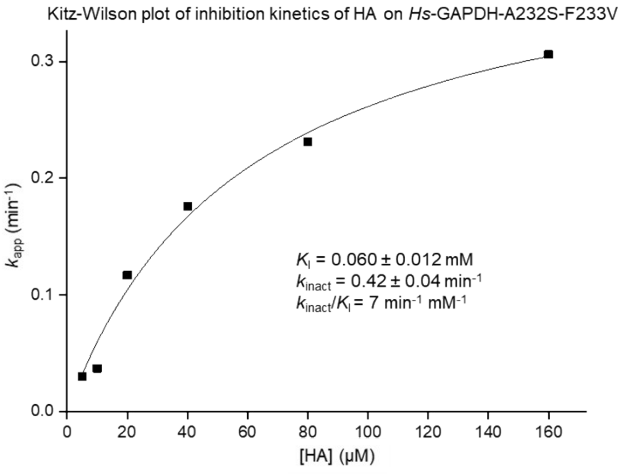
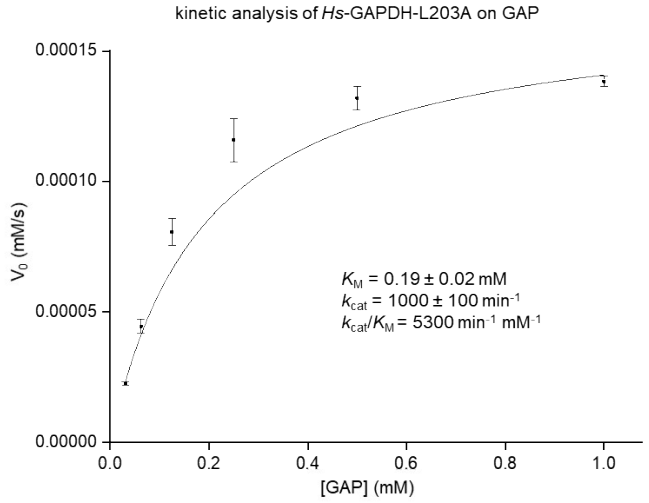


Figure S10. Enzymatic and inhibition kinetic study of *Hs*-GAPDH-L203A. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of *Hs*-GAPDH-L203A for GAP



B. Inhibition study of *Hs*-GAPDH-L203A using HA

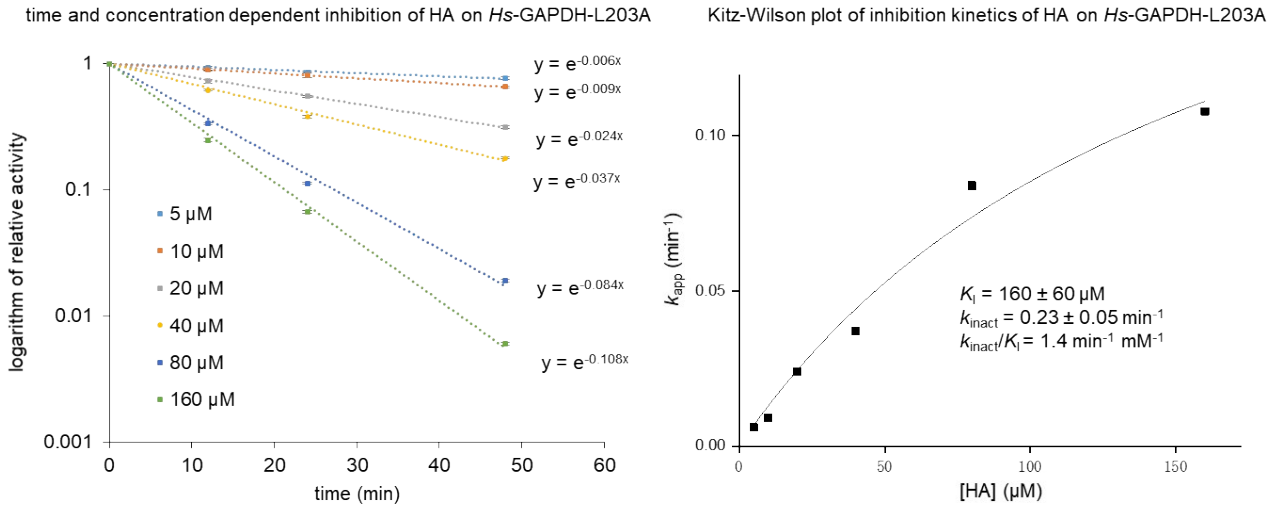
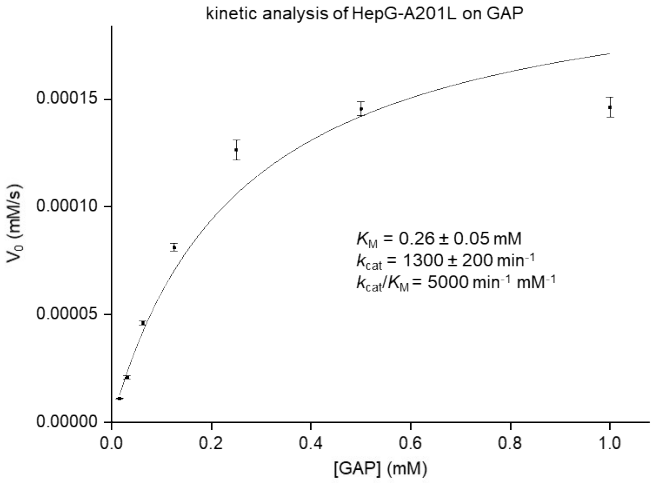
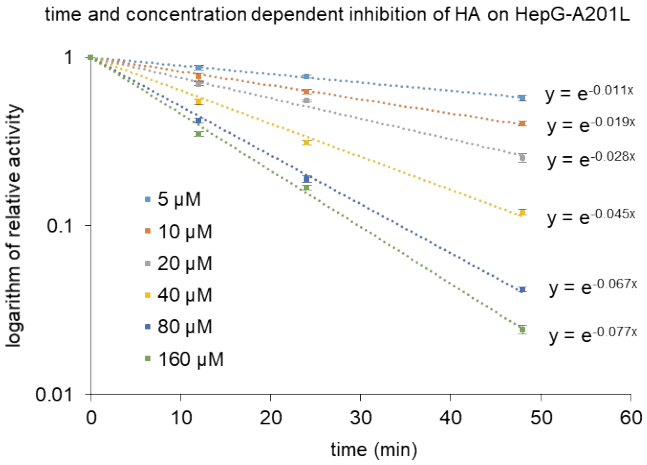


Figure S11. Enzymatic and inhibition kinetic study of HepG-A201L. The standard deviation (SD) is shown in error bars. Data are mean ± SD from three biologically independent experiments.

A. Steady-state kinetics of HepG-A201L for GAP



B. Inhibition study of HepG-A201L using HA



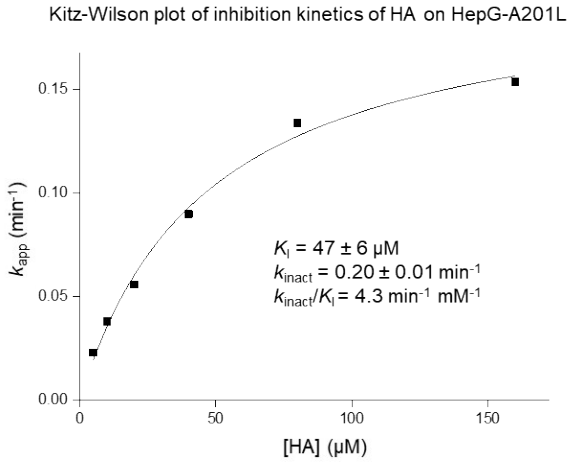


Figure S12. Alignment of the GAPDHs. Secondary structural elements were annotated based on *Hs*-GAPDH (PDB: 1U8F).

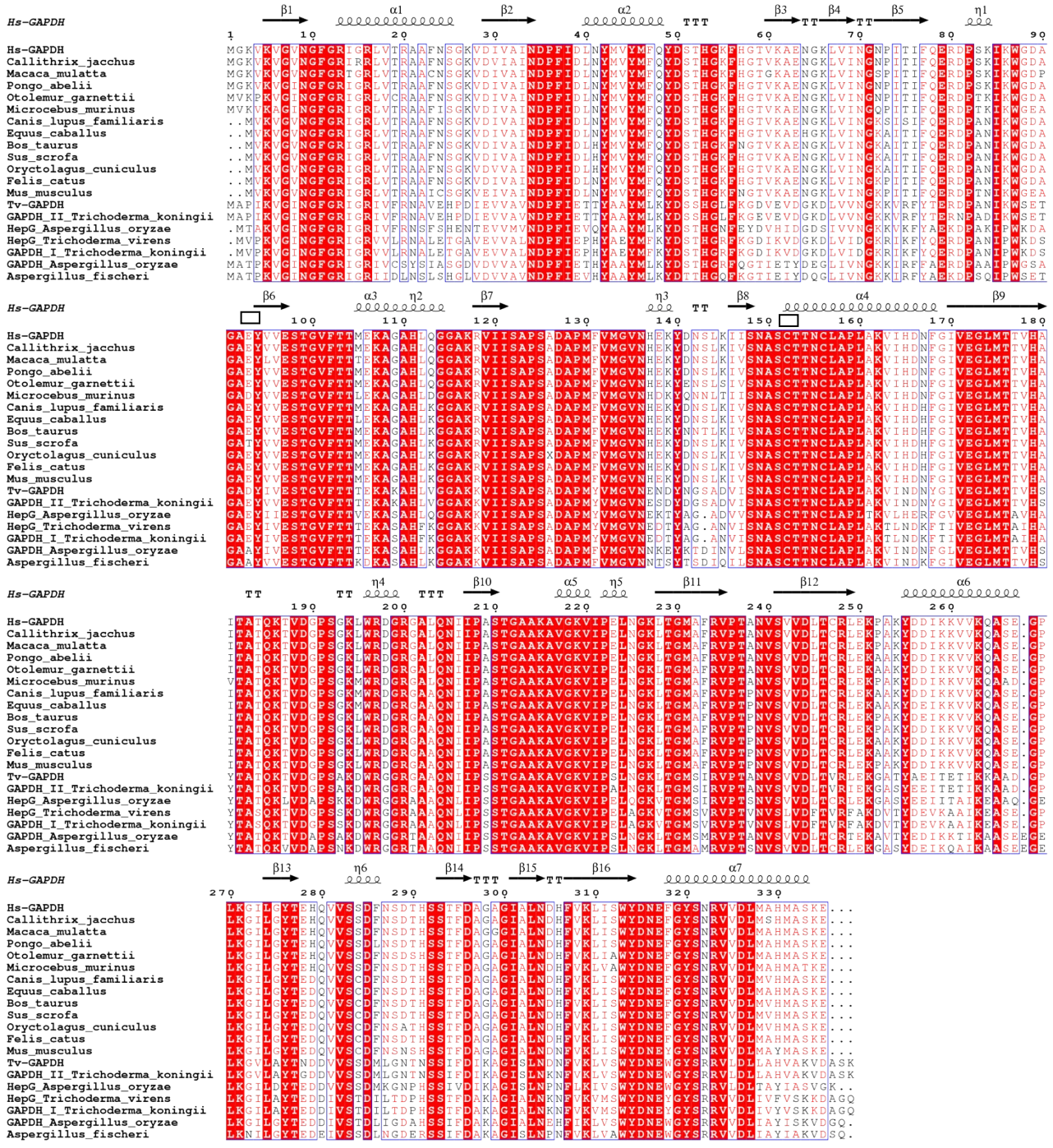
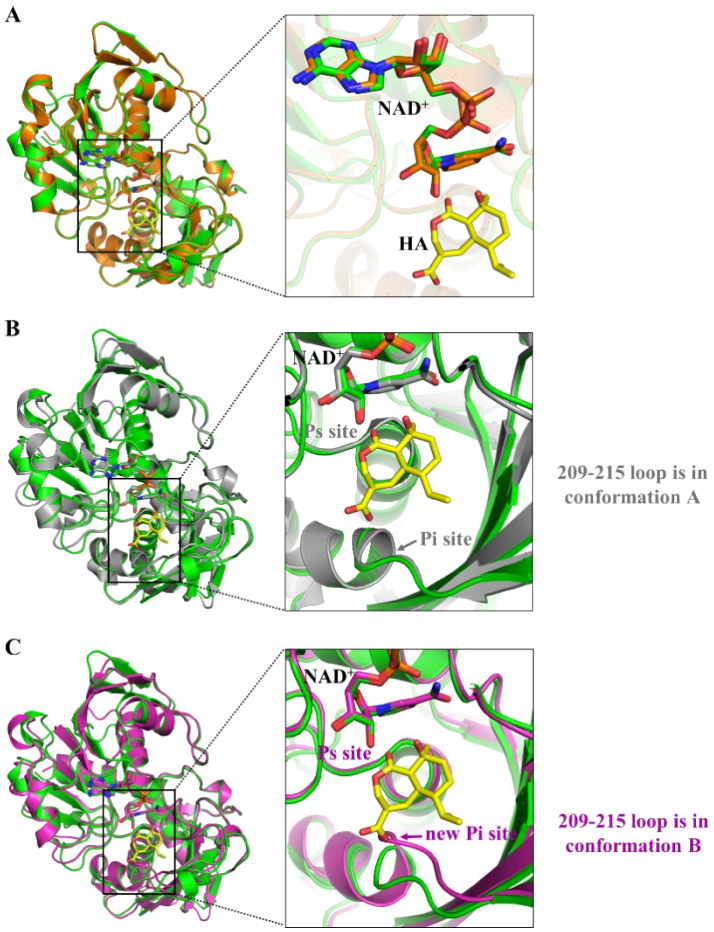
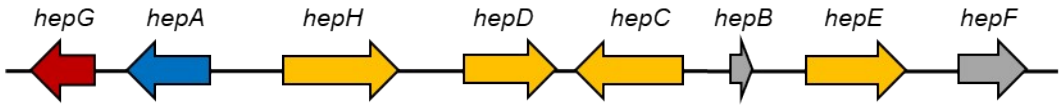


Figure S13. Comparing conformation of the *Hs*-GAPDH-HA complex to three GAPDHs. A. Superimposing chain O of *Hs*-GAPDH-HA complex (green) to human liver GAPDH (PDB: 1ZNQ, orange). The carbon backbone of HA is shown is yellow. The RMSD is 0.304 Å for 332 Cα atoms. Zoom in view of the active site is shown on the right. **B.** Superimposing chain O of *Hs*-GAPDH-HA complex (green) to GAPDH from *Geobacillus stearothermophilus* (PDB: 1NQA, grey), which is in conformation A. RMSD of 209-215 loop pointed out with black arrow is 0.175 Å for for 7 Cα atoms. Zoom in view of the active site is shown on the right. Both Pi site and Ps in *G. stearothermophilus* GAPDH are labeled. **C.** Superimposing chain O of *Hs*-GAPDH-HA complex (green) to GAPDH from *E. coli* (PDB：1DC4，purple), which is in conformation B. RMSD of 209-215 loop pointed out with black arrow is 1.189 Å for for 7 Cα atoms. Zoom in view of the active site is shown on the right. Both new Pi site and Ps in *E. coli* GAPDH are labeled.



Supplementary Tables

Table S1. Heptelidic acid biosynthetic gene cluster of *Trichoderma virens*



Trichoderma virens Gv29-8, scaffold 92 (NW_014013657.1, 1,831,400-1,858,00), 26.6 kb

gene	accession number	Size (gene/protein)	BLASTP homologs	identity/similarity (%)	proposed function
hepA	XP_013949969.1	1424/377	GAP85091.2	59/72	terpene synthase
hepH	XP_013949970.1	1971/534	EIT74228.1	66/80	cytochrome P450
hepD	XP_013949971.1	1730/512	BAJ04474.1	59/75	cytochrome P450
hepC	XP_013949972.1	1759/438	RAQ59865.1	58/74	cytochrome P450
hepE	XP_013949974.1	1847/528	OOO06875.1	70/83	cytochrome P450
hepB	XP_013949973.1	456/115	OOO06872.1	39/58	antibiotic biosynthesis monooxygenase
hepG	XP_013949968.1	1147/336	OAA55922.1	78/88	GAPDH
hepF	XP_013949975.1	1572/503	XP_008081852.1	59/75	MFS multidrug transporters

Table S2. Microbial strains used in this study

strain	genotype	source
Fungi		
Trichoderma virens Gv29-8	wild type	NRRL
Saccharomyces cerevisiae		
RC01	*MATα ura3-52 his3-Δ200 leu2-Δ1 trp1 pep4::HIS3 ura3-52::atCPR prb1 Δ1.6R can1 GAL*	[2]
TY11	RC01 carrying pHepA-xw55	this study
TY12	RC01 carrying pHepH-xw06 & pTvCPR-xw55	this study
TY13	RC01 carrying pHepD-xw02 & pTvCPR-xw55	this study
TY14	RC01 carrying pHepC-xw06 & pTvCPR-xw55	this study
TY15	RC01 carrying pHepE-xw02 & pTvCPR-xw55	this study
Escherichia coli		
DH10β		NEB
BL21 (DE3)		NEB
TY16	BL21 (DE3) carrying HepG-pET	this study
TY17	BL21 (DE3) carrying *Tv*-GAPDH-pET	this study
TY18	BL21 (DE3) carrying *Hs*-GAPDH-pSJ2	this study
TY19	BL21 (DE3) carrying *Hs*-GAPDH-L203A-pSJ2	this study
TY20	BL21 (DE3) carrying HepG-A201L-pET	this study
TY21	BL21 (DE3) carrying *Hs*-GAPDH-T177A-pSJ2	this study
TY22	BL21 (DE3) carrying *Hs*-GAPDH-A232S-F233V-pSJ2	this study
TY23	BL21 (DE3) carrying *Hs*-GAPDH-I182Y-pSJ2	this study

Table S3. Primers for PCR amplification in this study

primer	sequences of primer (5'→3')
HepA-xw55-recomb-F	tatggctagcgattataaggatgatgatgataagactagtatggctcaagtccagcgtgc
HepA-xw55-recomb-R	atttgtcatttaaattagtgatggtgatggtgatgcacgtgagcagcgtgattcaccacg
HepH-xw06-recomb-F	tcaactatcaactattaactatatcgtaataccatatgatggagcaactcaaaactcggc
HepH-xw06-recomb-R	gtggtggtgactcgcgacctcatacacaagcttctaatccgaaacccacctcttattgtc
HepD-xw02-recomb-F	tatcaactattaactatatcgtaataccatatgatgtcttcgtttacatcccctgacatc
HepD-xw02-recomb-R	gataatgaaaactataaatcgtgaaggcatgtttaaacctaattctttcggggaatcatc
HepC-xw06-recomb-F	actatcaactattaactatatcgtaataccatatgatgctcgcatctgtccaatcccttg
HepC-xw06-recomb-R	tggtggtggtgactcgcgacctcatacacaagctttcagtgtgaaacattccaggtggag
HepE-xw02-recomb-F	caactatcaactattaactatatcgtaataccatatgatggacaccttcaatgccactcc
HepE-xw02-recomb-R	tgataatgaaaactataaatcgtgaaggcatgtttaaactcaccactcatccttccgtcg
TvCPR-xw55-recomb-F	tacaatcaactatcaactattaactatatcgtaataccatatggcggaactggacacg
TvCPR-xw55-recomb-R	aaatttgtcatttaaattagtgatggtgatggtgatgcacttatgaccagacatcctcctgg
HepG-pET-F	catcacagcagcggcctggtgccgcgcggcagccatatggttcccaaagttggcatcaac
HepG-pET-R	tctcagtggtggtggtggtggtgctcgagtgcggccgcttactggccggcatccttttttg
tGAPDH-pET-F	tggtgccgcgcggcagccatatgagtcacaaatcagctacaatggctcccatcaaggtcg
tGAPDH-pET-R	gtgctcgagtgcggccgcatctgatcagatcaaacacttatttggaggcatcgaccttgg
Hs-GAPDH-L203A-pSJ2-F	gcggtaaactgtggcgcgatggtcgcggtgccgcacagaatattatcccagcttctaccg
Hs-GAPDH-L203A-pSJ2-R	ctgctttggctgcgccggtagaagctgggataatattctgtgcggcaccgcgaccatcgc
HepG-A201L-pET-F	cgtccaaggattggcgtggcggtcgtgctgccctacaaaacctgattcctagctccactg
HepG-A201L-pET-R	ggagctaggaatcaggttttgtagggcagcacgaccgccacgccaatccttggacgaagg
Hs-GAPDH-T177A-F	aaggcttaatgacggcagtgcacg
Hs-GAPDH-T177A-R	tcgctgtaatagcgtgcactgccgtcattaagc
Hs-GAPDH-A232S-F233V-F	aactgaatggtaaactgacgggcatgtccgttcgcgttcc
Hs-GAPDH-A232S-F233V-R	aaatccacaactgacacattggcggtaggaacgcgaacggacatgc

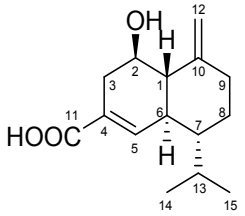
Hs-GAPDH-I182Y-F	ggcatcgtggaaggcttaatgacgacagtgcacgcttatacagcgacccagaaaaccgtg
Hs-GAPDH-I182Y-R	cgctcggaccatccacggttttctgggtcgctgtataagcgtgcactgtcgtcattaagc

Table S4. Plasmids used in this study

plasmids	features	source
pHepA-xw55	pXW55 expressing *hepA*	this study
pHepH-xw06	pXW06 expressing *hepH*	this study
pHepD-xw02	pXW02 expressing *hepD*	this study
pHepC-xw06	pXW06 expressing *hepC*	this study
pHepE-xw02	pXW02 expressing *hepE*	this study
pTvCPR-xw55	pXW55 expressing *Trichoderma virens* cytochrome P450 reductase	this study
HepG-pET	pET28a expressing HepG	this study
Tv-GAPDH-pET	pET28a expressing *Trichoderma virens* GAPDH	this study
Hs-GAPDH-pSJ2	pSJ2 expressing *homo sapiens* GAPDH	this study
Hs-GAPDH-L203A-pSJ2	pSJ2 expressing *homo sapiens* GAPDH L203A mutant	this study
HepG-A201L-pET	pET28a expressing HepG A201L mutant	this study
Hs-GAPDH-T177A-pSJ2	pSJ2 expressing *homo sapiens* GAPDH T177A mutant	this study
Hs-GAPDH-A232S-F233V-pSJ2	pSJ2 expressing *homo sapiens* GAPDH A232S and F233V double mutant	this study
Hs-GAPDH-I182Y-pSJ2	pSJ2 expressing *homo sapiens* GAPDH I182Y mutant	this study
pXW55	protein expression vector in *S. cerevisiae* (*URA3* marker)	[2]
pXW06	protein expression vector in *S. cerevisiae* (*TRP2* marker)	[2]
pXW02	protein expression vector in *S. cerevisiae* (*LEU2* marker)	[2]
pET28a	protein expression vector in *E. coli* BL21 (DE3)	Addgene
pSJ2	protein expression vector in *E. coli* BL21 (DE3)	[3]

Table S5. NMR data and structure

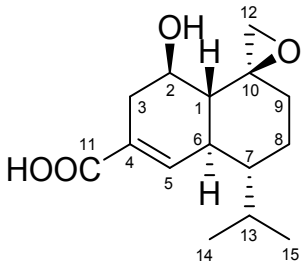
^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) of compound 3:



3

no.	δ_H (mult., *J* in Hz)	δ_C	mult.	HMBC
1	1.97 (1H, m)	51.2	CH	149.5, 141.1, 66.7, 46.4, 45.9, 32.3
2	4.09 (1H, q, 9.3)	66.7	CH	
3	2.92 (1H, dd, 17.4, 5.8)	32.3	CH_2	171.9, 141.1, 127.6, 66.7, 51.2
3’	2.15 (1H, m)			141.1, 127.6, 66.7
4	-	127.6	C	-
5	7.14 (1H, s)	141.1	CH	171.9, 127.6, 51.2, 46.4, 32.3
6	1.97 (1H, m)	45.9	CH	149.5, 141.1, 127.6, 66.7, 51.2, 46.4, 27.3
7	1.44 (1H, m)	46.4	CH	
8	1.87 (1H, d, 12.5)	27.3	CH_2	149.5, 45.9 ,36.9
8’	1.20 (1H, m)			45.9 ,36.9
9	2.41 (1H, d, 12.0)	36.9	CH_2	149.5, 104.8, 51.2, 46.4, 27.3
9’	2.02 (1H, m)			149.5, 104.8, 51.2, 46.4, 27.3
10	-	149.5	C	-
11	-	171.9	C	-
12	4.88 (2H, d, 35.3)	104.8	CH_2	149.5, 51.2, 45.9, 36.9, 27.3
13	2.20 (1H, m)	26.8	CH	45.9, 27.3, 21.3, 15.1
14	0.94 (3H, d, 6.8)	21.3	CH_3	46.4, 26.8, 15.1
15	0.77 (3H, d, 6.9)	15.1	CH_3	46.4, 26.8, 21.3

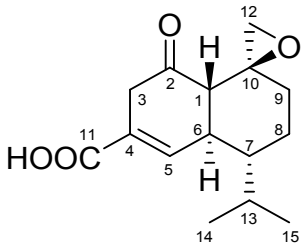
^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) of compound 4:



4

no.	δ_H (mult., *J* in Hz)	δ_C	mult.	HMBC
1	1.88 (1H, m)	45.9	CH	68.0, 64.6, 49.8, 45.5, 42.9, 34.8, 32.6
2	3.75 (1H, m)	68.0	CH	64.6, 42.9
3	2.79 (1H, dd, 15.9, 5.4)	32.6	CH_2	171.6, 139.5, 128.2, 68.0, 45.9
3’	2.12 (1H, m)			139.5, 128.2, 68.0, 45.9
4	-	128.2	C	-
5	7.10 (1H, s)	139.5	CH	171.6, 128.2, 64.6, 45.5, 42.9, 32.6
6	2.09 (1H, m)	42.9	CH	45.9, 45.5, 23.9
7	1.36 (1H, m)	45.5	CH	45.9, 34.8
8	1.91 (1H, m)	23.9	CH_2	64.6, 45.5, 42.9, 34.8
8’	1.29 (1H, m)			64.6, 45.5, 42.9, 26.7
9	2.00 (1H, m)	34.8	CH_2	64.6, 49.8, 45.5
9’	1.38 (1H, m)			64.6, 45.5, 23.9
10	-	64.6	C	-
11	-	171.6	C	-
12	3.21 (1H, s)	49.8	CH_2	64.6
12’	2.70 (1H, d, 3.4)			64.6, 34.8
13	2.23 (1H, td, 6.9, 3.0)	26.7	CH	45.5, 42.9, 23.9, 21.3, 15.1
14	0.97 (3H, d, 6.9)	21.3	CH_3	45.5, 26.7, 15.1
15	0.81 (3H, d, 6.9)	15.1	CH_3	45.5, 26.7, 21.3

^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) of compound 5:



5

no.	δ_H (mult., *J* in Hz)	δ_C	mult.	HMBC
1	2.89 (1H, d, 12.0)	51.1	CH	204.3, 141.9, 58.1, 51.4, 44.2
2	-	204.3	C	-
3	3.27 (1H, dt, 20.2, 2.6)	41.1	CH_2	204.3, 141.9, 127.9
3’	2.96 (1H, dt, 20.1, 2.2)			204.3, 141.9, 127.9
4	-	127.9	C	-
5	7.31 (1H, d, 2.0)	141.9	CH	170.1, 127.9, 51.1, 44.2, 41.1
6	2.57~2.52 (1H, m)	44.2	CH	141.9, 127.9
7	1.51 (1H, tt, 11.7, 3.1)	46.1	CH	
8	1.95~1.80 (1H, m)	23.0	CH_2	58.1, 46.1
8’	1.36~1.23 (1H, m)			58.1, 46.1
9	1.95~1.80 (1H, m)	35.5	CH_2	58.1, 46.1
9’	1.36~1.23 (1H, m)			58.1, 46.1
10	-	58.1	C	-
11	-	170.1	C	-
12	3.89 (1H, dd, 5.5, 1.5)	51.4	CH_2	58.1, 35.5
12’	2.51 (1H, d, 5.7)			58.1, 35.5
13	2.34~2.22 (1H, m)	26.9	CH	46.1, 21.5, 15.2
14	1.03 (3H, d, 6.9)	21.5	CH_3	46.1, 26.9, 15.2
15	0.83 (3H, d, 6.9)	15.2	CH_3	46.1, 26.9, 21.5

Table S6. Data collection and refinement statistics of GAPDHs in this study

Data collection and refinement statistics of *Hs*-GAPDH and HepG (molecular replacement)

	Hs-GAPDH	HepG
PDB ID	6M61	6M5X
Data collection		
Space group	$P2_12_12_1$	$P12_11$
Cell dimensions		
a, *b*, *c* (Å)	81.3, 135.1, 146.2	76.7, 96.6, 96.7
α, β, γ (°)	90, 90, 90	90, 96.9, 90
Resolution (Å)	50-1.83 (1.86-1.83)*	50-2.06 (2.10-2.06)*
R_{sym} or R_{merge}	0.111 (0.866)	0.126 (0.583)
$I/\sigma I$	25.50 (3.00)	13.40 (2.43)
Completeness (%)	99.9 (99.1)	98.6 (90.1)
Redundancy	13.0 (10.2)	5.8 (4.9)
Refinement		
Resolution (Å)	43.03-1.82	39.29-2.06
No. reflections	140724 (11947)	78234 (5635)
R_{work}/R_{free}	0.1916/0.2279	0.1504/0.1933
No. atoms		
Protein	10100	10169
Ligand/ion	190	344
Water	1151	647
B-factors		
Protein	23.8	26.2
Ligand/ion	24.4	32.2
Water	31.1	31.9
R.m.s. deviations		
Bond lengths (Å)	0.006	0.006
Bond angles (°)	0.88	1.00
Ramachandran plot favored (%)	95.10	95.48
Ramachandran plot allowed (%)	4.15	3.84
Ramachandran plot outlier (%)	0.75	0.68

*Number of xtals for each structure should be noted in footnote. *Values in parentheses are for highest-resolution shell.

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