Supporting Information

Phase-Separated Multienzyme Condensates for Efficient Synthesis of Imines from Carboxylic Acids with Enhanced Du-al-Cofactor Recycling

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1. Experimental procedures

1.1. General information

ASR, BID and UTX strain were retained from the laboratory, and PopZ, *Ni*CAR, PPK12, *Ec*PPase, and *Bp*GDH were synthesized by Beijing Tsingke Biotech Co., Ltd. (Beijing, China). DNA primers were also synthesized from Beijing Tsingke Biotech Co., Ltd. (Beijing, China). All plasmid vectors, restriction enzymes, and ligases were purchased from Takara Bio (Shanghai, China). DNA gel extraction kits, plasmid extraction kits were purchased from Yeasen Biotech (Shanghai, China). Reagents for bacteria culture were purchased from Oxide and ThermoFisher (Shanghai, China). All chemicals were purchased from Titan Chemical (Shanghai, China) and used without further treatment unless otherwise indicated. NADPH and $NADP^+$ were purchased from Bontac Bioengineering (Shenzhen, China). HPLC analysis was performed on a Shimadzu LC-2010A HT apparatus equipped with an Agilent XDB-C18 column (5.0 μm, 4.6 × 250 mm). GC-FID analysis was performed on a Shimadzu GC-2014 Pro gas chromatography with a flame ionization detector (FID) equipped with a 25 m CP-Chirasil-DEX CB column with 0.25 mm inner diameter and 0.25 μm film thickness. Fluorescence images of condensate formation and cofactor recycling were captured using a in a microreactor were taken using a Nikon $A1R^+$ laser scanning confocal microscope (Japan). All experiments were performed in triplicate unless otherwise stated. Data are presented as mean ± standard deviation (SD).

1.2. Enzyme-catalyzed reactions

1.2.1. Investigation of the in vitro carboxylate reduction

The concentrations in the reactions were as follows: benzoic acid **1a** (15 mM), ATP (1.0 mM), NADPH (0.2 mM), MOPS buffer (100 mM, pH 7.5), $MgCl_2$ (25 mM), β-$_D$-glucose (100 mM), sodium hexametaphosphate (8 mM), *Ni*CAR/ BID-*Ni*CAR (0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL) and *Bp*GDH/BID-*Bp*GDH (0.2 U/mL). All reactions were shaken at 30°C at 750 rpm in an Eppendorf thermomixer. Acetonitrile/formic acid 19:1 was added to aliquots to stop the reaction over due time and these solutions were analyzed by HPLC after prior centrifugation.

1. Enzyme stock solution used for the investigation of the in vitro carboxylate reduction.

Entry	Volume
*Ni*CAR/BID-*Ni*CAR	1.25 U/mL
PPK12/BID-PPK12	5 U/mL
*Ec*PPase/BID-*Ec*PPase	5 U/mL
*Bp*GDH/BID-*Bp*GDH	1.25 U/mL
Water	Up to 400 µL

2. Reaction mixture used for the investigation of the *in vitro* carboxylate reduction.

Entry	Volume
$MgCl_2$/ β-$_D$-glucose/ MOPS buffer[1)]	250 µL
ATP/ NADPH stock solution[2)]	40 µL
Enzyme stock[3)]	80 µL
100 mM $PolyP_6$ pH 7.5[4)]	40 µL
250 mM carboxylic acid stock[5)]	30 µL
Water	60 µL
Total reaction volume	500 µL

1) $MgCl_2$ (47.6 mg, 50 mM in stock solution) + β-$_D$-glucose (360.3 mg, 200 mM in stock solution) + 200 mM MOPS buffer (10 mL); 2) ATP (12.5 mg, 12.5 mM in stock solution) + NADPH (8.33 mg, 6.25 mM in stock solution) + water (1600 µL); 3) see **1**; 4) prepared by dissolving sodium hexametaphosphate (183.5 mg, 100 mM) in water (3.0 mL total volume); 5) carboxylic acids dissolved in 250 mM NaOH.

*1.2.2. Impact of substrate concentration on the conversion of benzoic acid **1a***

The standard reaction conditions for *in vitro* reduction of carboxylic acid **1a** were applied at increased substrate concentration. The concentrations in the reactions were as follows: benzoic acid **1a** (5, 10, 20, 50 mM), ATP (1 mM), NADPH (0.2 mM), MOPS buffer (100 mM, pH 7.5), $MgCl_2$ (25 mM), β-$_D$-glucose (100 mM), sodium hexametaphosphate (8 mM), *Ni*CAR/BID-*Ni*CAR (0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL) and *Bp*GDH/BID-*Bp*GDH (0.2 U/mL). All reactions were shaken at 30°C at 750 rpm in an Eppendorf thermomixer. Acetonitrile/formic acid 19:1 was added to aliquots to stop the reaction over due time and these solutions were analyzed by HPLC after prior centrifugation.

*1.2.3. Impact of various buffers on the conversion of benzoic acid **1a***

Three distinct kinds of buffer were selected to investigate whether they have an effect on the multienzyme reaction. The concentrations in the reactions were as follows: benzoic acid **1a** (15 mM), ATP (1 mM), NADPH (0.2 mM), HEPES/Tris-HCl/MOPS buffer (100 mM, pH 7.5), $MgCl_2$ (25 mM), β-$_D$-glucose (100 mM), sodium hexametaphosphate (8 mM), *Ni*CAR/BID-*Ni*CAR (0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL) and *Bp*GDH/BID-*Bp*GDH (0.2 U/mL). All reactions were shaken at 30°C at 750 rpm in an Eppendorf thermomixer. Acetonitrile/formic acid 19:1 was added to aliquots to stop the reaction over due time and these solutions were analyzed by HPLC after prior centrifugation.

*1.2.4. Impact of sodium hexametaphosphate on the conversion of benzoic acid **1a***

To estimate the limits for ATP regeneration under the general reaction conditions, further investigations with varying $PolyP_6$ concentrations were made. The concentrations in the reactions were as follows: benzoic acid **1a** (15 mM), ATP (1 mM), NADPH (0.2 mM), MOPS buffer (100 mM, pH 7.5), $MgCl_2$ (25 mM), β-$_D$-glucose (100 mM), sodium hexametaphosphate (2, 4, 8, 20 mM), *Ni*CAR/BID-*Ni*CAR (0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL) and *Bp*GDH/BID-*Bp*GDH (0.2 U/mL). All reactions were shaken at 30°C at 750 rpm in an Eppendorf thermomixer. Acetonitrile/formic acid 19:1 was added to aliquots to stop the reaction over due time and these solutions were analyzed by HPLC after prior centrifugation.

*1.2.5. Impact of various ATP and NADPH concentrations on the conversion of benzoic acid **1a***

The concentrations in the reactions were as follows: benzoic acid **1a** (15 mM), ATP (100 or 1000 μM), NADPH (2, 20 or 200 μM), MOPS buffer (100 mM, pH 7.5), $MgCl_2$ (25 mM), β-$_D$-glucose (100 mM), sodium hexametaphosphate (8 mM), *Ni*CAR/BID-*Ni*CAR(0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL) and *Bp*GDH/BID-*Bp*GDH (0.2 U/mL) All reactions were shaken at 30°C at 750 rpm in an Eppendorf thermomixer. Acetonitrile/formic acid 19:1 was added to aliquots to stop the reaction over due time and these solutions were analyzed by HPLC after prior centrifugation.

3. Reaction mixtures used for the investigation of the *in vitro* carboxylate reduction of **1a** under varied ATP and NADPH concentrations.

Entry	ATP (mM)	NADPH (mM)	ATP stock conc. used[1)] (mM)	NADPH stock conc. used[2)] (mM)	A/N stock used in reactions			
					ATP (μL)	NADPH (μL)	Main stock (μL)	Water (μL)
1	1	0.2	10	1	40	80	400	280
2	1	0.02	10	1	40	8	400	352
3	1	0.002	10	1	40	0.8	400	359.2
4	0.1	0.2	10	1	4	80	400	316
5	0.1	0.02	10	1	4	8	400	388
6	0.1	0.002	10	1	4	0.8	400	395.2

1) 10 mM ATP: ATP (6 mg, 10 mM) dissolved in water (1000 μL); 2) 10 mM NADPH: NADPH (8.3 mg, 10 mM) dissolved in water (1000 μL).

1.3. Procedures for biocatalytic N-alkylation of amines with carboxylic acids

N-propargylhexylamine

To a 20 mL centrifuge tube were added hexanoic acid (10 mM, 5.81 mg), NADPH (0.04 mM, 10 mM in ddH_2O), ATP (0.2 mM, 10 mM in ddH_2O), glucose (20 mM, 1M in ddH_2O), $MgCl_2$ (25 mM, 1M in ddH_2O), $PolyP_6$ (8 mM, 500 mM in 100 mM pH 7.5 MOPS buffer), BID-*Asp*RedAm (1 U/mL), BID-*Bp*GDH (1 U/mL), BID-*Ni*CAR (1 U/mL), BID-PPK12(5 U/mL), BID-*Ec*PPase (5 U/mL) and a pH balanced stock solution of propargylamine (20 mM, 1M in 100 mM pH 7.5 MOPS buffer). The reaction volume was adjusted to 5 mL with MOPS buffer (100 mM, pH 7.5) and the tube was sealed before incubation at 30℃ in a thermomixer at 250 rpm. The reaction was decanted into falcon tubes and basified to a pH of 12 with 5M NaOH solution before extraction into MTBE (2 × 10 mL) with intermediate centrifugation to improve the separation of phases (4℃, 12000 rpm, 5 min). The combined organic extracts were dried over $MgSO_4$ before concentration under reduced pressure. The crude product was

purified by column chromatography (silica, 95:5 petroleum ether: ethyl acetate) to yield the title compound as a orange oil (6.96 mg, 7.9 mM, 79%).



N-cyclopropylhexylamine

To a 20 mL centrifuge tube were added hexanoic acid (10 mM), NADPH (0,04 mM), ATP (0.2 mM), glucose (20 mM), $MgCl_2$ (25 mM), $PolyP_6$ (8 mM), BID-*Asp*RedAm (1 U/mL), BID-*Bp*GDH (1 U/mL), BID-*Ni*CAR (1 U/mL), BID-PPK12(5 U/mL), BID-*Ec*PPase (5 U/mL) and a pH balanced stock solution of cyclopropylamine (20 mM, 1M in 100 mM pH 7.5 MOPS buffer). The reaction volume was adjusted to 5 mL with MOPS buffer (100 mM, pH 7.5) and the tube was sealed before incubation at 30℃ in a thermomixer at 250 rpm. The reaction was decanted into falcon tubes and basified to a pH of 12 with 5M NaOH solution before extraction into MTBE (2 × 10 mL) with intermediate centrifugation to improve the separation of phases (4℃, 12000 rpm, 5 min). The combined organic extracts were dried over $MgSO_4$ before concentration under reduced pressure. The crude product was purified by column chromatography (silica, 95:5 petroleum ether: ethyl acetate) to yield the title compound as a yellow oil (6.36 mg, 6.4 mM, 64%).



2. Nucleotide sequence information

(1) PopZ-*Ni*CAR

ATGTCTGACCAGTCTCAAGAACCGACTATGGAAGAGATTCTGGCGTCTATCCGTCGTATCAT
CTCTGAAGACGACGCTCCGGCAGAACCGGCTGCAGAAGCTGCACCGCCGCCACCACCGGA
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(2) ASR-*Ni*CAR

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(3) UTX-*Ni*CAR

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(6) BID-*Bp*GDH

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(9) EYFP-BID-*Bp*GDH

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(10) mCherry-BID-PPK12

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(11) BID-*Asp*RedAm

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3. Figures and tables

Table S1. Primers used for PCR amplification.

Primer	Sequence
pRSF-MCS1-F	GAATTCGGATCCTGGCTGTG
pRSF-MCS1-R	AAGCTTGCGGCCGCATAATGC
BID-F	CACAGCCAGGATCCGAATTCCATGGATTGTGAAGTTAACAAC
BID-*Ni*CAR-R	GAATCAACAGCCATATCCATACCATTACGAGCCAGG
PopZ-F	AGCAAATGGGTCGCGATGTCTGACCAGTCTCA
PopZ-*Ni*CAR-R	AGGGGAATCAACAGCCATAGCACCACGACCACGAGA
ASR-F	CAGGATCCGAATTCCATGAAAAAAGTATTAGCTCTGG
ASR-*Ni*CAR-R	GGAATCAACAGCCATCGCTGCGGGTTGTGCAGC
UTX-F	TGGGTCGCGGATCCGAATTCGTGCCTAGCGTCTCTCAGCC
UTX-*Ni*CAR-R	GGAATCAACAGCCATGTGAGGGCTGTTAAGGCTGG
*Ni*CAR-BID-F	CCGGAATTCATGGCAAGTAATGATTATAC
*Ni*CAR-PopZ-F	AGGGGAATCAACAGCCATAGCACCACGACCACGAGA
*Ni*CAR-ASR-F	CTGCTGCACAACCCGCAGCGATGGCTGTTGATTCCC
*Ni*CAR-UTX-F	CTTAACAGCCCTCACATGGCTGTTGATTCCCCTGA
*Ni*CAR-R	TGGTGGTGGTGCTCGAGTTACAGCAGCTGGAGCA
pRSF-MCS2-F	AAGGAGATATACATATG
pRSF-MCS2-R	TTTCTTTACCAGACTCGAG
PPTase-F	AAGGAGATATACATATGATGGTTGACATGAAGACT
PPTase-R	TCTTTACCAGACTCGAGGTCGTGCTGACACAGAGT
pET28a-*EcoR*I-F	GAATTCGGATCCGCGACC
pET28a-*Hind*III-R	AAGCTTGCGGCCGCACT
BID-PPK12-R	TTGTAGATGTTGATCATATCCATACCATTACGAGC
BID-*Ec*PPase-R	AACGTTCAGCAGGCTCATATCCATACCATTACGAG
BID-*Bp*GDH-R	TTCCAGATCACTGTACATATCCATACCATTACGAG
PPK-BID-F	GCTCGTAATGGTATGGATATGATCAACATCTACAA
PPK-BID-R	TCCGTCCGATCGAGAAGAAGCTTGCGGCCGCACT
*Ec*PPase-BID-F	CTCGTAATGGTATGGATATGAGCCTGCTGAACGTT
*Ec*PPase-BID-R	AACGTGCTAAGAACAAGAAGCTTGCGGCCGCACTC
*Bp*GDH-BID-F	CTCGTAATGGTATGGATATGTACAGTGATCTGGAA
*Bp*GDH -BID-R	AGGCCGGTCGCGGTTAAAAGCTTGCGGCCGCACTC
BFP-F	GGTCGCGGATCCGAATTCCATATGAGCGAACTGATTA
BFP-R	TTGTTAACTTCACAATCCATCTCGAGATTCAGTTTA
sfGFP-F	AGCCAGGATCCGAATTCCATGCGTAAAGGCGAAGAGC
sfGFP-R	GTTAACTTCACAATCCATTTTGTACAGTTCATCCAT
EYFP-F	CGCGGATCCGAATTCATGGTGAGCAAAGGCGAAG
EYFP-R	AACTTCACAATCCATTTTATACAGTTCATCCATGCCCAGG
mCherry-F	GTCGCGGATCCGAATTCATGGCAAGCATGACGGGTGG
mCherry-R	ATGGATGAACTGTATAAAATGGATTGTGAAGTTAAC
*Asp*RedAm-BID-F	CGTAATGGTATGGATATGAGCAAACACATCGGCATC
*Asp*RedAm-BID-R	TGCGGCCGCAAGCTTGTCAACGTTCTTGCCAACTTTG

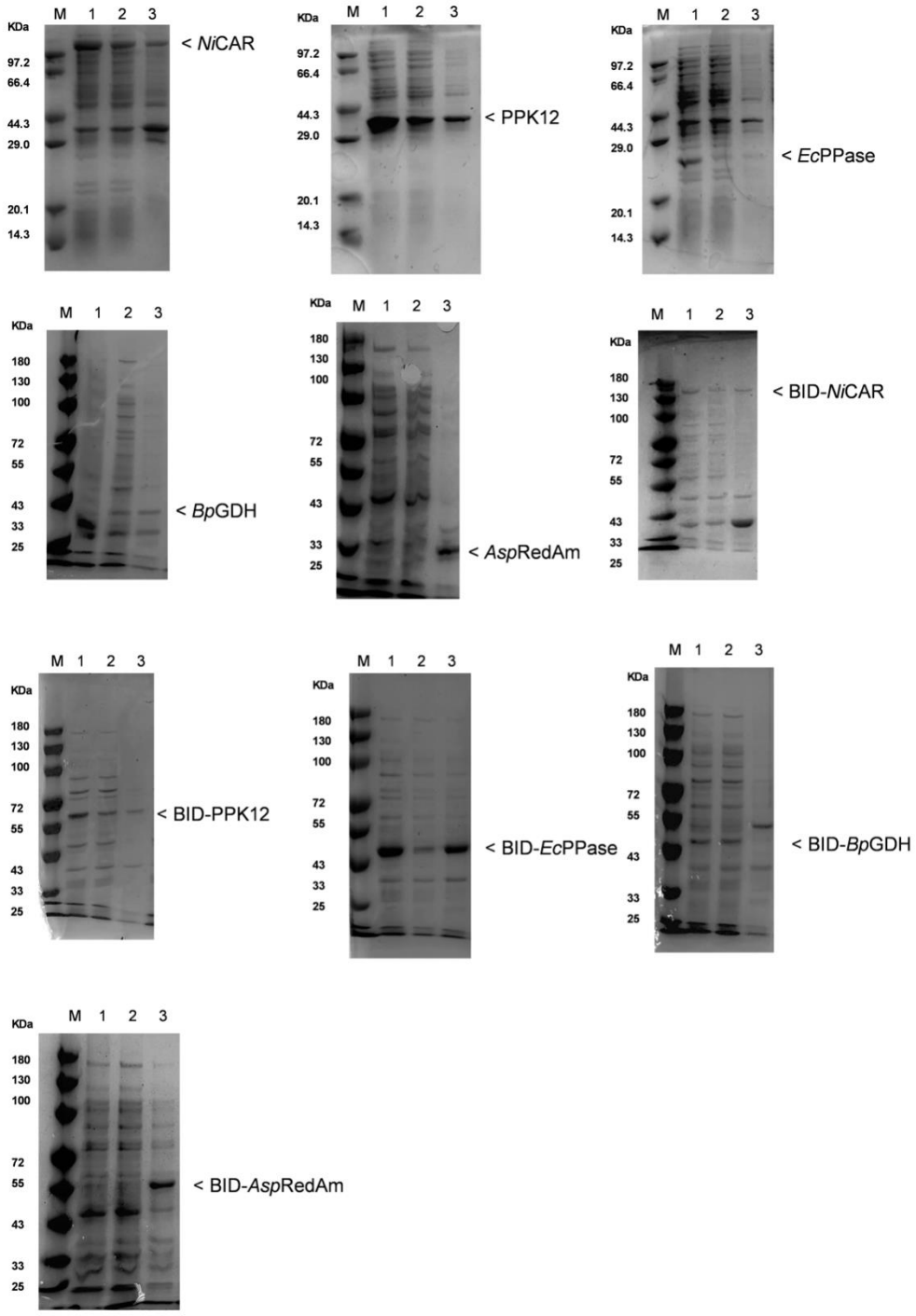


Figure S1. SDS-PAGE of free enzyme and BID-linked fusion enzymes. Lane M: protein marker; lane 1: supernatant of cell lysates; lane 2: Ni-NTA column flow through; lane 3: insoluble protein precipitate.

Table S2. Kinetic parameters of fusion proteins and free enzymes

Entry	Enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
1	*Ni*CAR[a]	0.45 ± 0.03	235.36 ± 2.27	523.02 ± 35.23
2	BID-*Ni*CAR[a]	1.06 ± 0.03	243.37 ± 2.65	229.59 ± 6.96
3	*Ec*PPase[b]	7.47 ± 1.98	295.15 ± 44.96	39.53 ± 12.13
4	BID-*Ec*PPase[b]	5.77 ± 0.76	102.89 ± 6.41	17.83 ± 2.60
5	*Bp*GDH[c]	0.97 ± 0.05	1472.02 ± 16.53	1512.25 ± 77.51
6	BID-*Bp*GDH[c]	1.51 ± 0.10	269.27 ± 2.52	178.35 ± 11.61
7	PPK12[d]	21.22±2.67	1027.22±81.75	48.41±7.21
8	BID-PPK12[d]	26.55±7.17	1810.20±310.62	68.18±21.82
9	*Asp*RedAm[e]	5.24 ± 0.50	76.64 ± 1.25	14.62 ± 1.43
10	BID-*Asp*RedAm[e]	5.53 ± 0.41	90.86 ± 2.77	16.41 ± 1.30
11	*Ni*CAR+ *Ec*PPase+ *Bp*GDH+ PPK12 (toward substrate **1a**)[f]	3.41 ± 0.12	965.64 ± 31.41	283.18 ± 14.57
12	BID-*Ni*CAR+ BID-*Ec*PPase+ BID-*Bp*GDH+ BID-PPK12+ BID protein (toward substrate **1a**)[g]	0.48 ± 0.03	286.49 ± 0.89	594.69 ± 38.62

a: Benzoic acid **1a** (0.5-20 mM), $MgCl_2$ (10 mM), DTT (1 mM), ATP (1 mM), NADPH (0.2 mM), 0.5 μM enzyme. Reaction was performed in 100 mM MOPS, pH 7.5 at 30℃; b: Activities of pyrophosphatase preparations were assessed using an assay described by Sigma-Aldrich for the characterization of inorganic phosphatase; c: Glucose (1-20 mM), $NADP^+$ (0.2 mM), 0.1 μM enzyme. Reaction was performed in 100 mM MOPS, pH 7.5 at 30℃; d: Activities of PPK preparations were assessed using an assay described in the reference[1]; e: Hexanol (0.5-20 mM), propargylamine (20 mM), NADPH (0.2 mM), 0.5 μM enzyme. Reaction was performed in 100 mM Tris-HCl, pH 9.0 at 30℃; f: Benzoic acid **1a** (1-25 mM), ATP (1 mM), NADPH (0.2 mM), *Ni*CAR (8 μM), PPK12 (5 U/mL), *Ec*PPase (5 U/mL), *Bp*GDH (8 μM), β-$_D$-glucose (100 mM), $MgCl_2$ (25 mM), $PolyP_6$ (8 mM), 100 mM MOPS buffer pH 7.5, at 30 ℃, 750 rpm. g: Benzoic acid **1a** (1-25 mM), ATP (1 mM), NADPH (0.2 mM), BID-*Ni*CAR (8 μM), BID-PPK12 (5 U/mL), BID-*Ec*PPase (5 U/mL), BID-*Bp*GDH (8 μM), BID protein (2 μM), β-$_D$-glucose (100 mM), $MgCl_2$ (25 mM), $PolyP_6$ (8 mM), 100 mM MOPS buffer pH 7.5, at 30 ℃, 750 rpm.

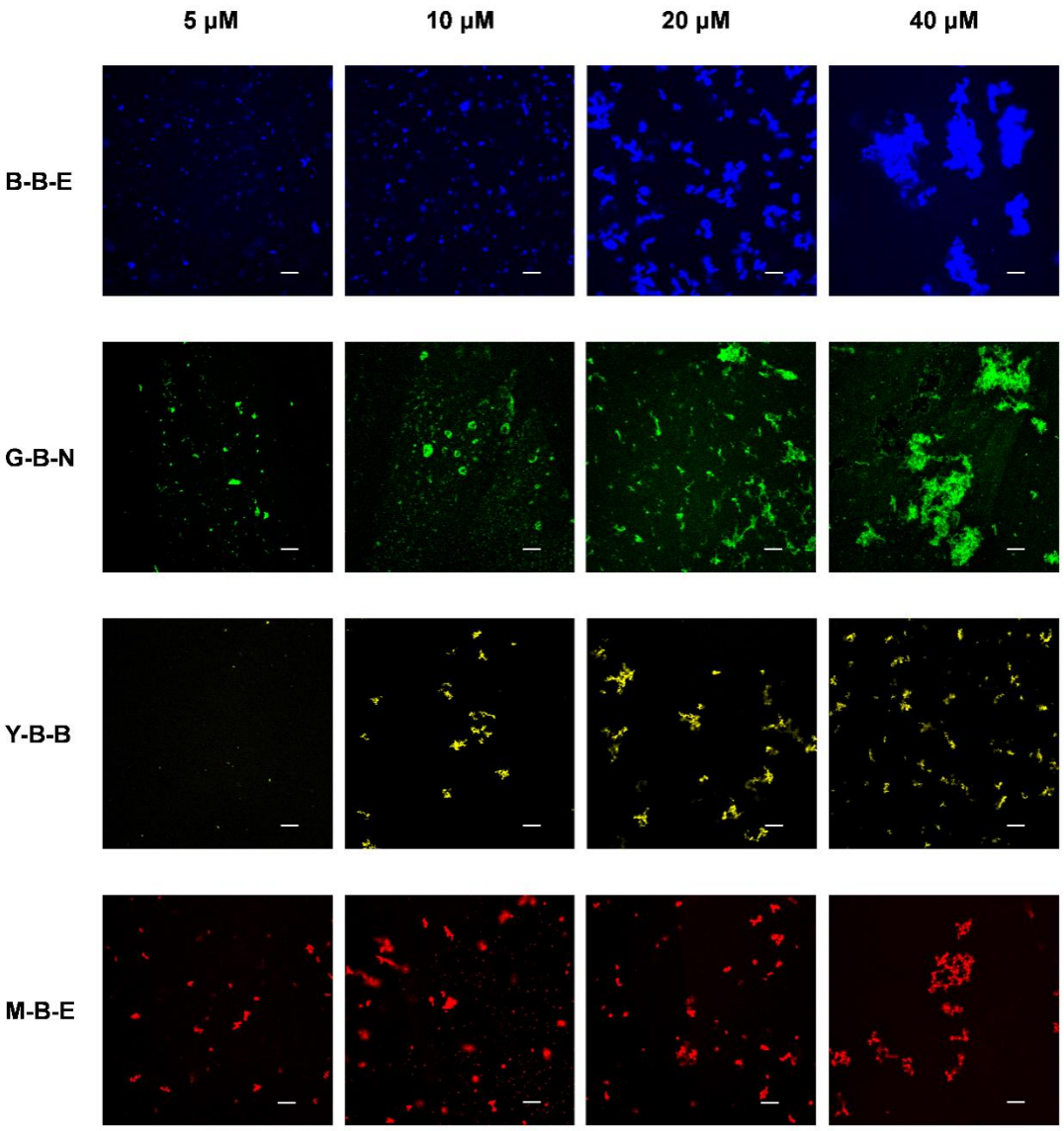


Figure S2. Confocal fluorescence images of four fusion proteins at different concentrations (5, 10, 20, 40 μM). Scale bar, 10 μm.

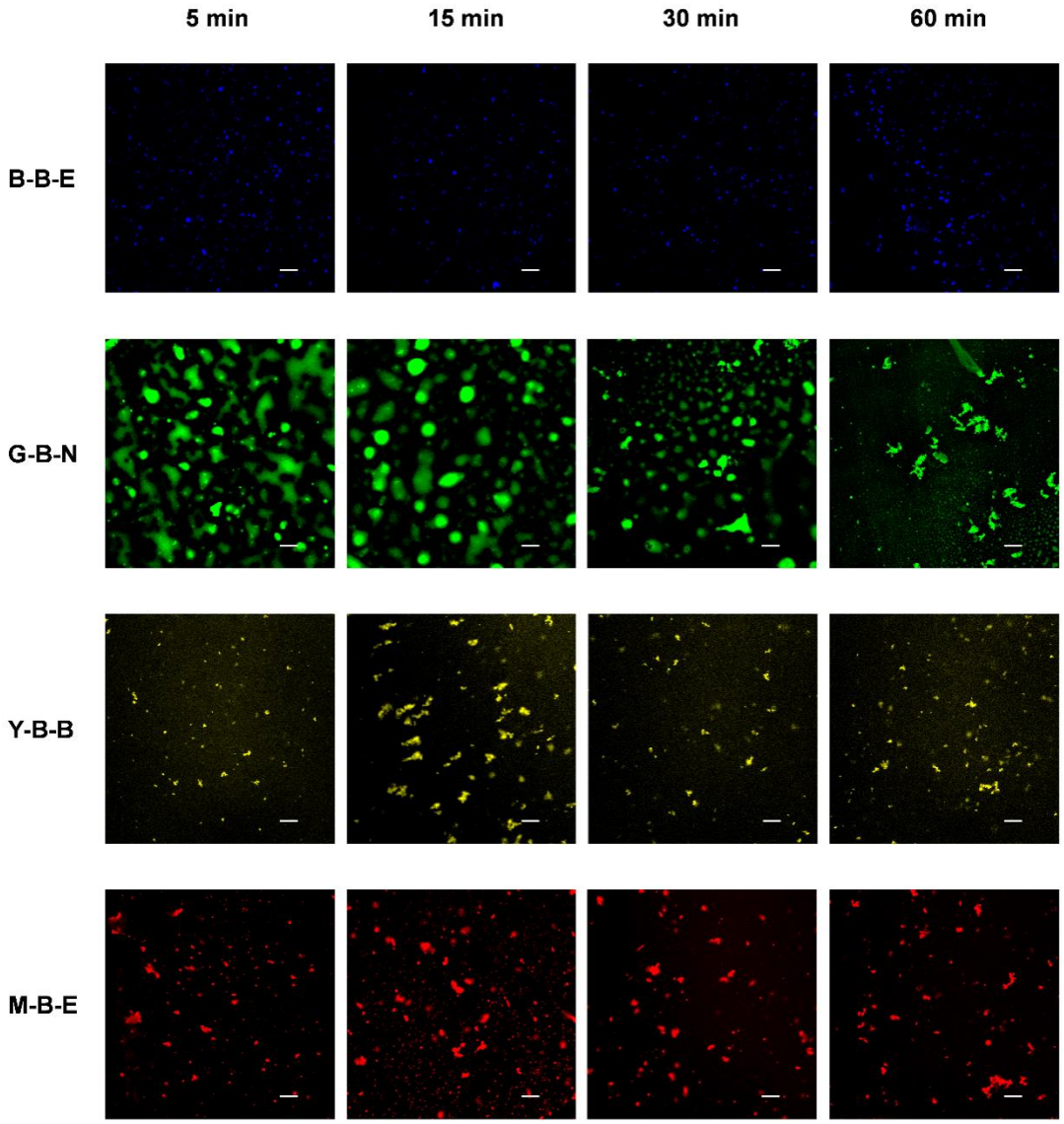


Figure S3. Confocal fluorescence images of four fusion proteins under different incubation time (5, 15, 30, 60 min). Scale bar, 10 μm.

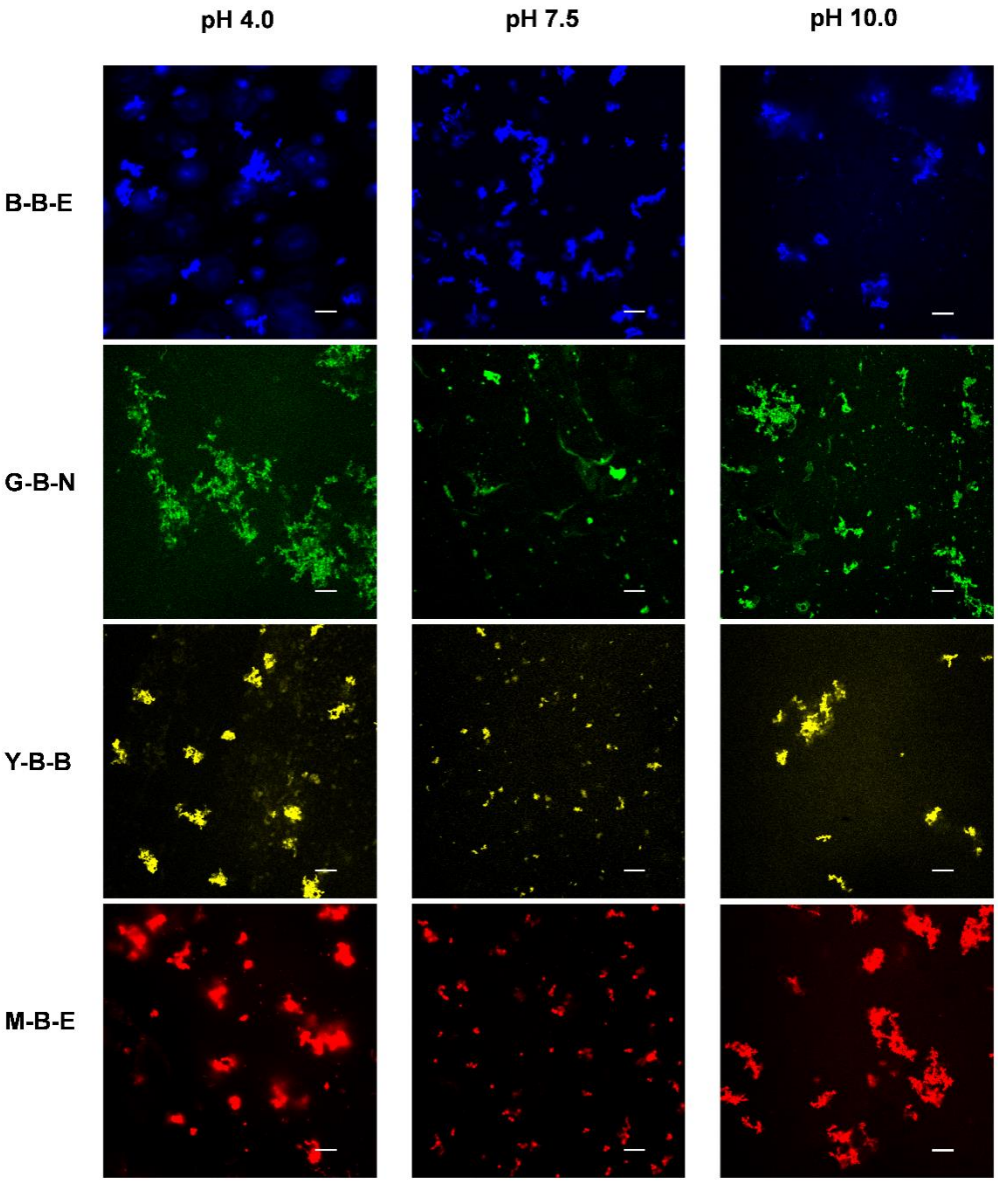


Figure S4. Confocal fluorescence images of four fusion proteins under different pH (4.0, 7.5, 10.0). Scale bar, 10 μm.

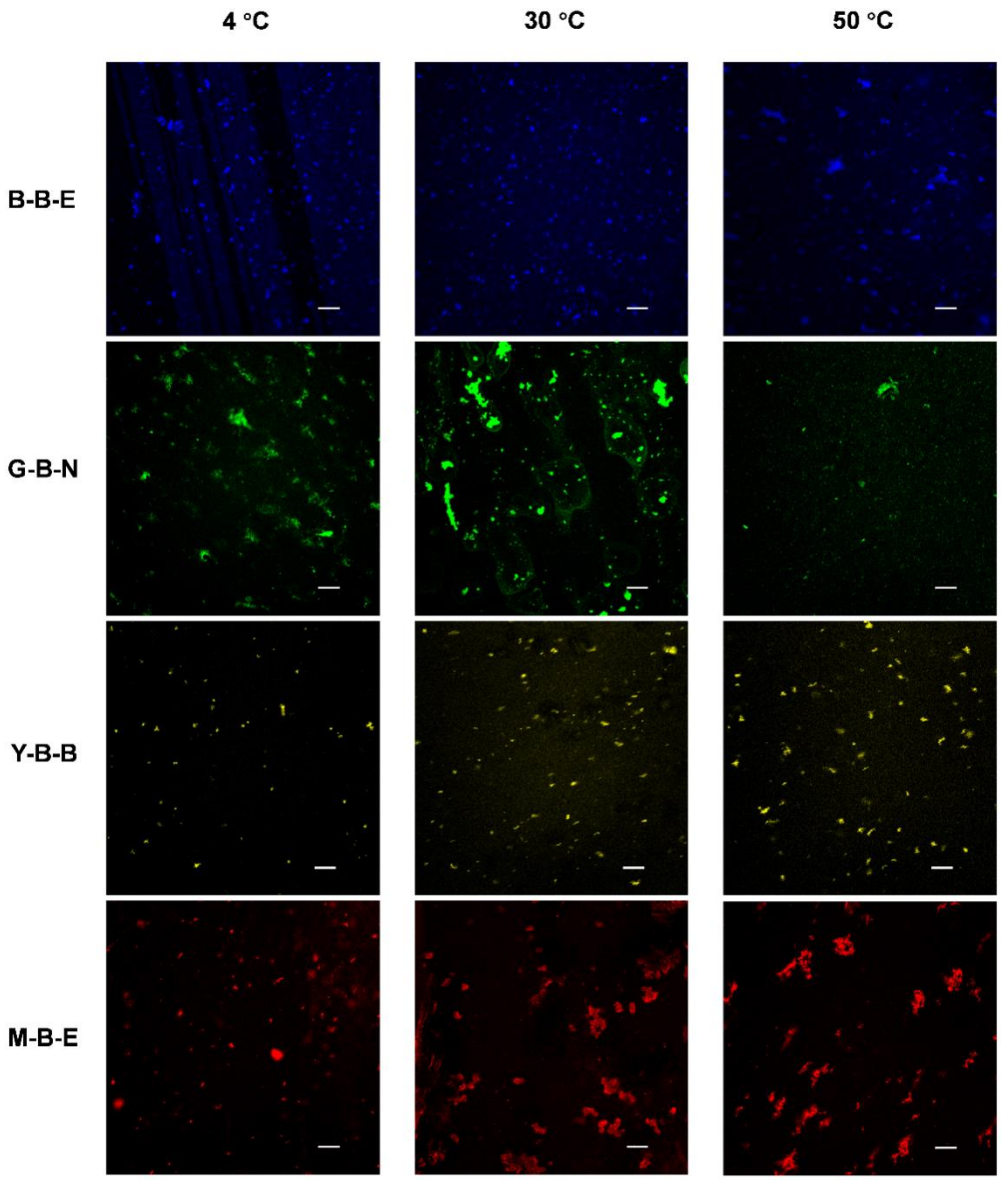


Figure S5. Confocal fluorescence images of four fusion proteins under different incubate temperatures (4, 30, 50 °C). Scale bar, 10 μm.

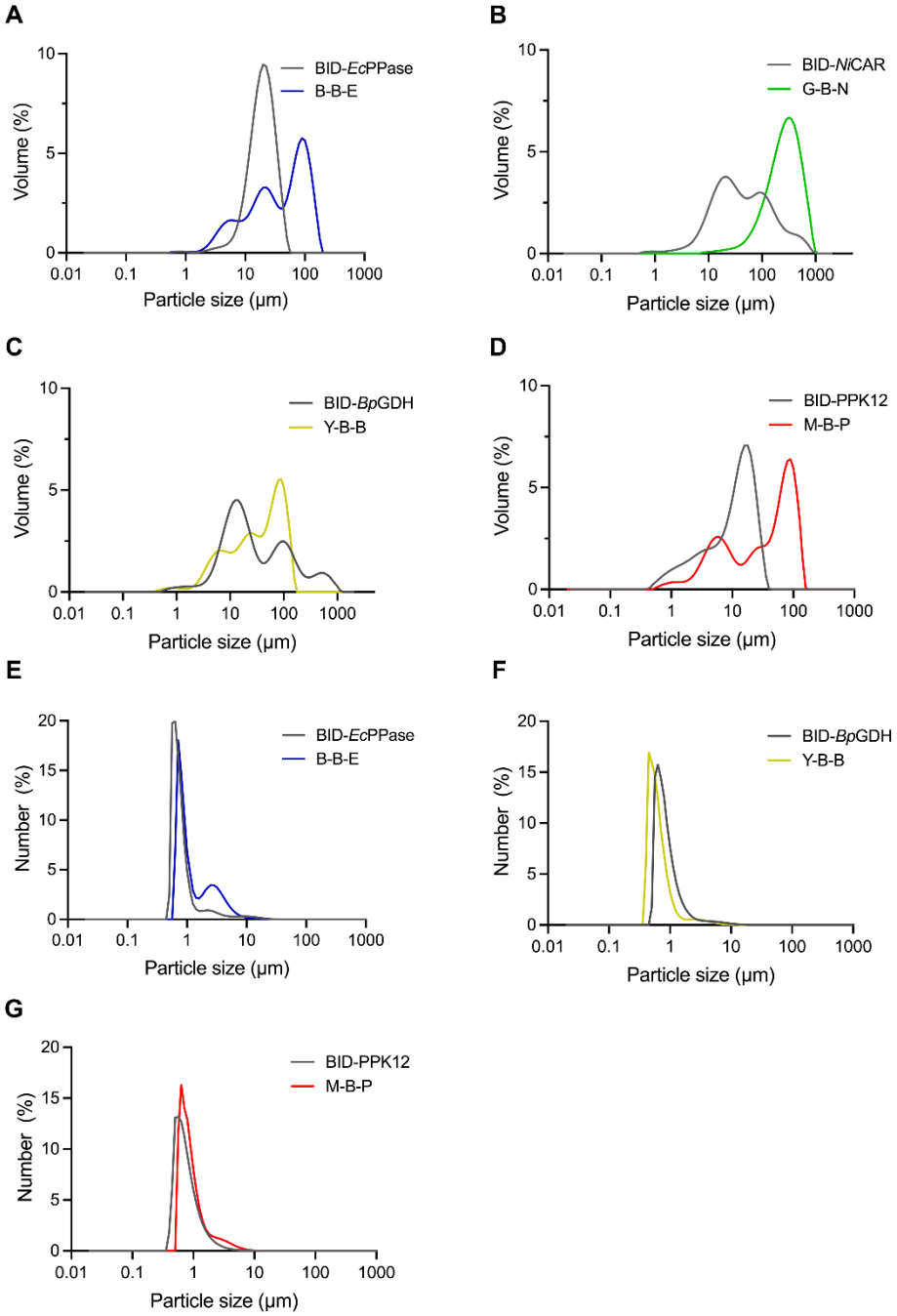


Figure S6. Particle size and size distribution analysis of free enzyme and phase-separated condensates. (A) Analysis of the particle size of BID-*Ec*PPase and BFP-BID-*Ec*PPase with the same concentration of 1 mg/mL. (B) Analysis of the particle size of BID-*Ni*CAR and sfGFP-BID-*Ni*CAR with the same concentration of 1 mg/mL. (C) Analysis of the particle size of BID-*Bp*GDH and EYFP-BID-*Bp*GDH with the same concentration of 1 mg/mL. (D) Analysis of the particle size of BID-PPK12 and mCherry-BID-PPK12 with the same concentration of 1 mg/mL. (E) Size distribution of the particle size of BID-*Ec*PPase and BFP-BID-*Ec*PPase with the same concentration of 1 mg/mL. (F) Size distribution of the particle size of BID-*Bp*GDH and EYFP-BID-*Bp*GDH with the same concentration of 1 mg/mL. (G) Size distribution of condensates generated by 1 mg/mL BID-PPK12 and 1 mg/mL mCherry-BID-PPK12.

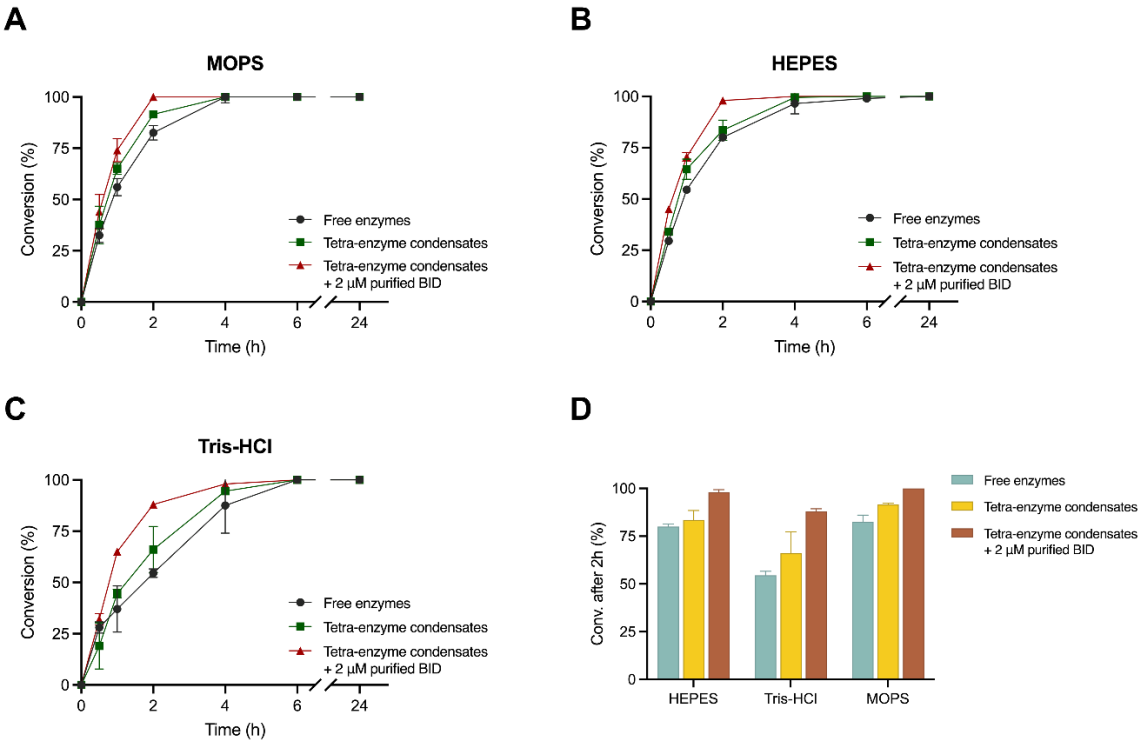


Figure S7. *In vitro* reduction of **1a** under the free enzyme system and the tetra-enzyme systems in the presence of different buffers. (A) MOPS. (B) HEPES. (C) Tris-HCl**.** (D) Conversions of substrate **1a** under free enzyme system and tetra-enzyme condensate systems after 2 h.

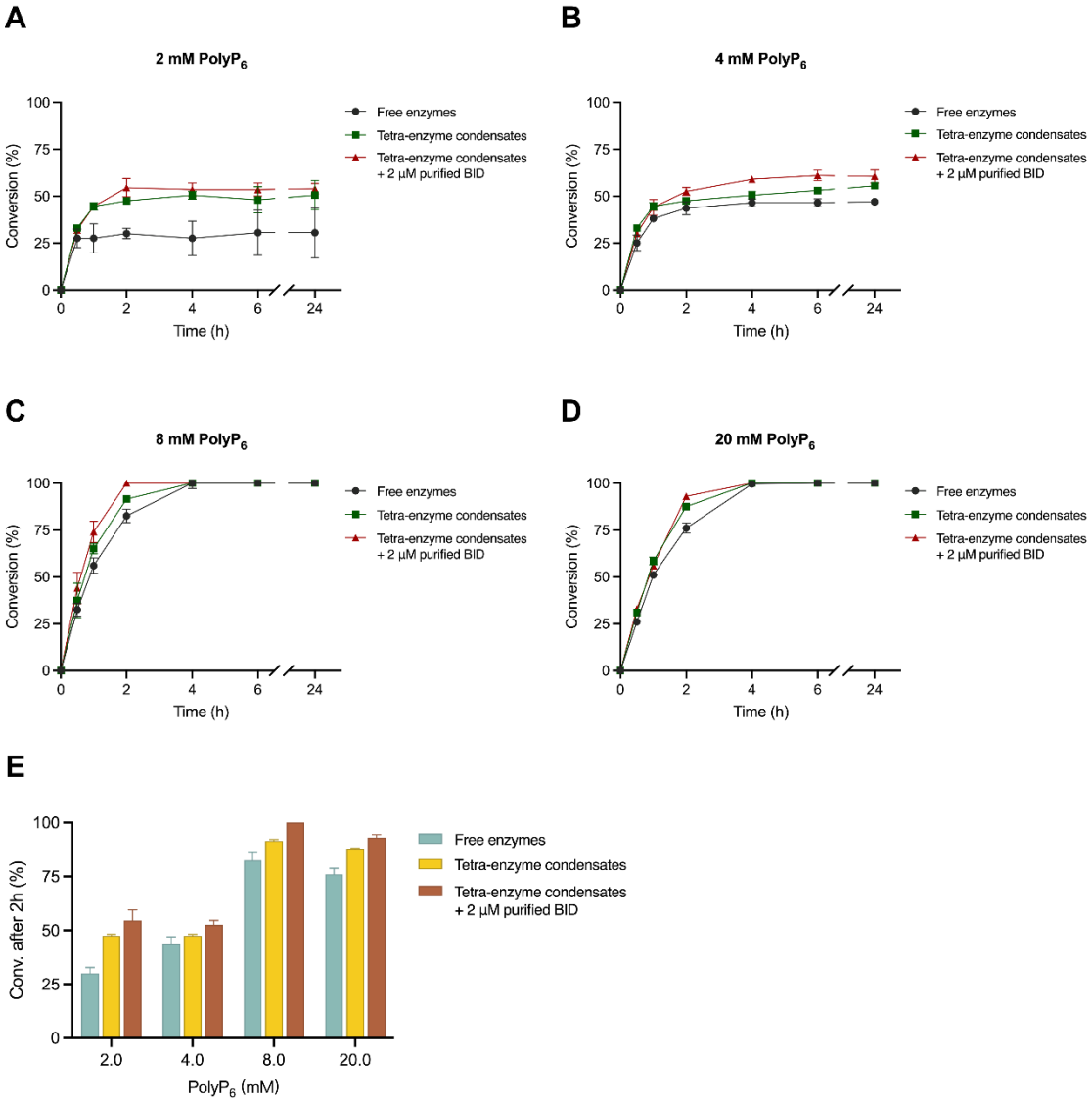


Figure S8. *In vitro* reduction of **1a** under the free enzyme system and the tetra-enzyme systems in the presence of different concentration of $PolyP_6$. (A) 2 mM $PolyP_6$. (B) 4 mM $PolyP_6$. (C) 8 mM $PolyP_6$. (D) 20 mM $PolyP_6$. (E) Conversions of substrate **1a** under free enzyme system and tetra-enzyme condensate systems after 2 h.

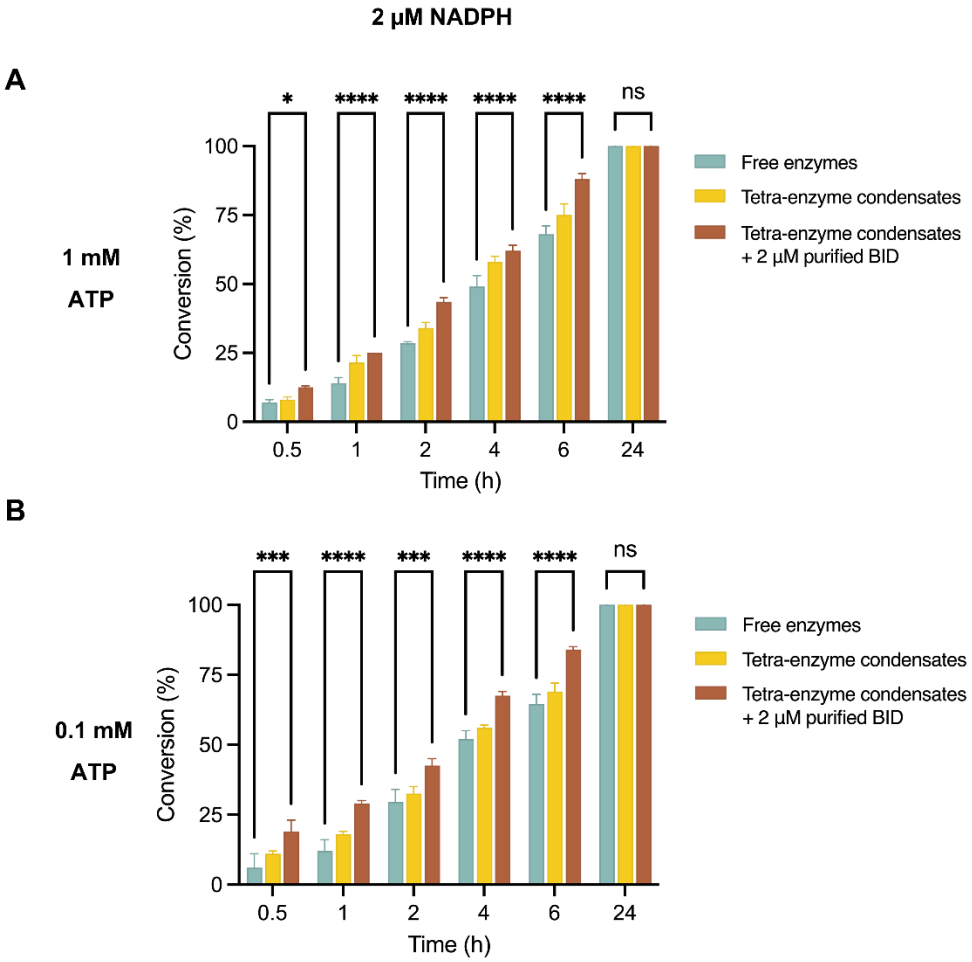


Figure S9. The conversion of 1a over time in the free enzyme system and the tetra-enzyme condensate systems. (A) Reaction conditions: substrate 1a (15 mM), ATP (1 mM), $NADP^+$ (2 μM), $MgCl_2$ (25 mM), $PolyP_6$ (8 mM), β-D-glucose (100 mM), *Ni*CAR/BID-*Ni*CAR (0.2 U/mL), *Bp*GDH/BID-*Bp*GDH (0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL), 2 μM BID protein, MOPS Buffer (100 mM, pH 7.5), at 30°C. (B) Reaction conditions: substrate 1a (15 mM), ATP (0.1 mM), $NADP^+$ (2 μM), $MgCl_2$ (25 mM), $PolyP_6$ (8 mM), β-D-glucose (100 mM), *Ni*CAR/BID-*Ni*CAR (0.2 U/mL), *Bp*GDH/BID-*Bp*GDH (0.2 U/mL), PPK12/BID-PPK12 (5 U/mL), *Ec*PPase/BID-*Ec*PPase (5 U/mL), 2 μM BID protein, MOPS Buffer (100 mM, pH 7.5), at 30°C. Data in panel (A) and (B) are presented as mean ± SD from three independent experiments (n=3). Statistical significance was determined by two-way ANOVA followed by Sidak's multiple comparisons test. Asterisks indicate significant differences compared to the free enzyme system at the corresponding time point (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

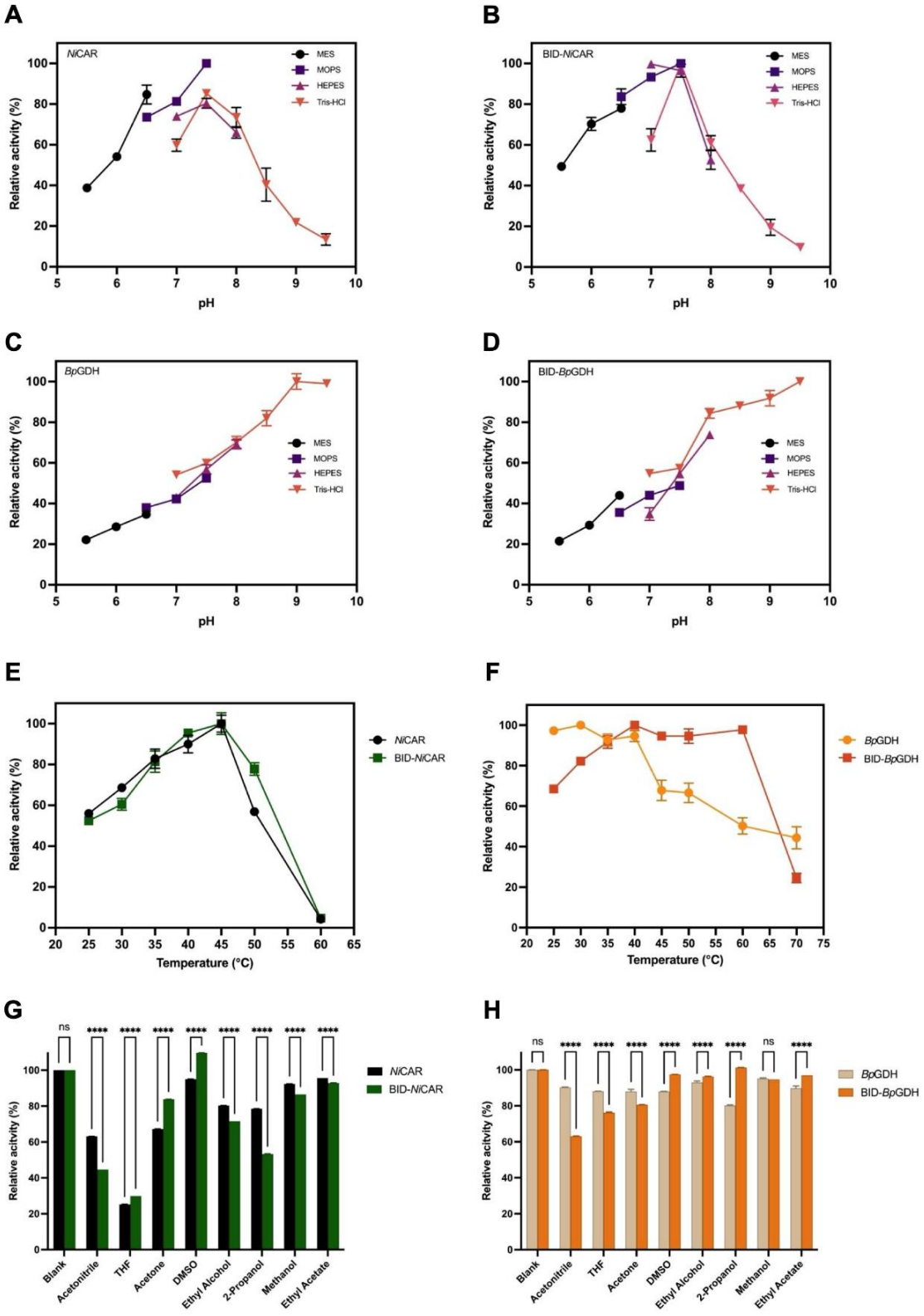
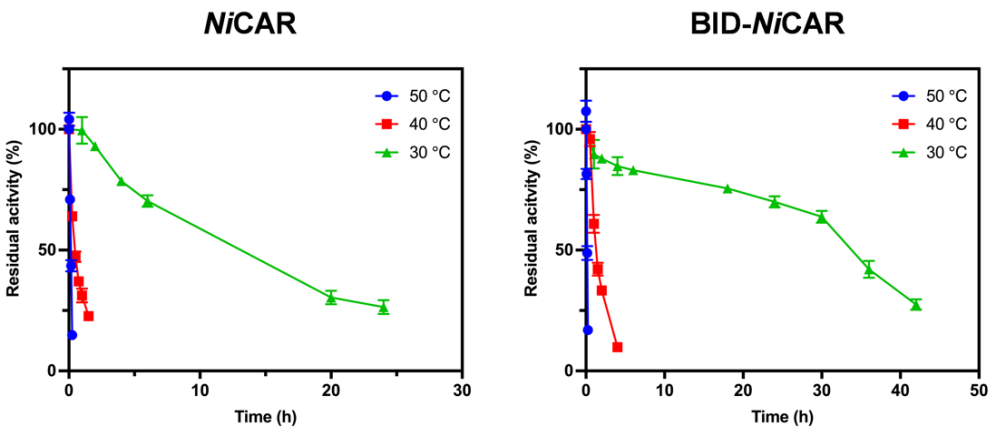


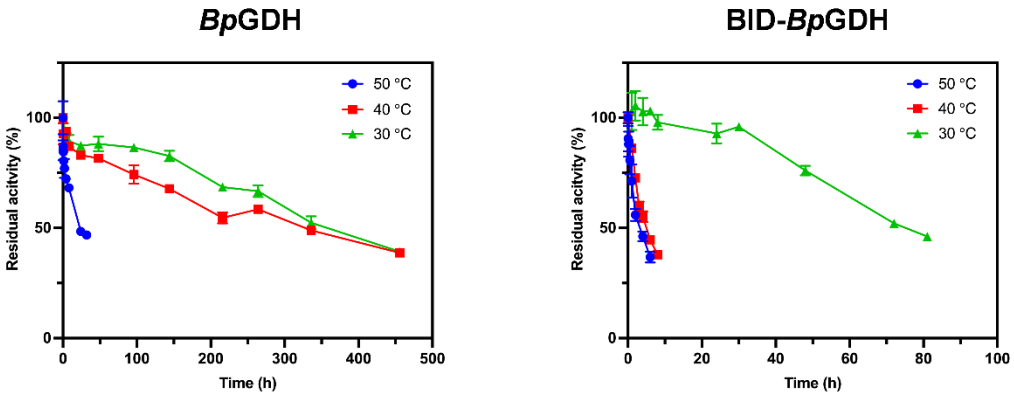
Figure S10. Characterization of CARs and GDHs. (A) Optimal pH of *Ni*CAR. Buffers with various pH were used: MES pH 5.5-6.5, MOPS pH 6.5-7.5, HEPES pH 7.0-8.0, Tris-HCl pH 7.0-9.5. (B) Optimal pH of BID-*Ni*CAR. Buffers with various pH were used as mentioned before. (C) Optimal pH of *Bp*GDH. Buffers with various pH were used as mentioned before. (D) Optimal pH of BID-

*Bp*GDH. Buffers with various pH were used as mentioned before. (E) Optimum reaction temperature of *Ni*CAR and BID-*Ni*CAR. (F) Optimum reaction temperature of *Bp*GDH and BID-*Bp*GDH. (G) Tolerance of *Ni*CAR and BID-*Ni*CAR to common organic solvents. (H) Tolerance of *Bp*GDH and BID-*Bp*GDH to common organic solvents.



Enzyme	30 ℃		40 ℃		50 ℃	
	k_d/h^{-1}	$t_{1/2}$/h	k_d/h^{-1}	$t_{1/2}$/h	k_d/min^{-1}	$t_{1/2}$/min
*Ni*CAR	0.058	11.95	0.9576	0.72	0.1243	5.58
BID-*Ni*CAR	0.0231	30	0.6053	1.14	0.1164	5.95

Figure S11. Thermostability of *Ni*CAR and BID-*Ni*CAR at 30°C, 40°C, 50°C.



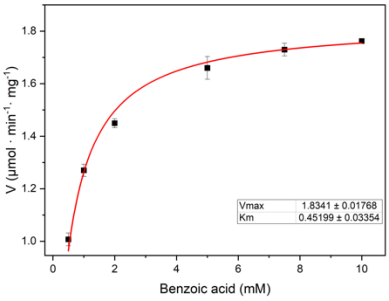
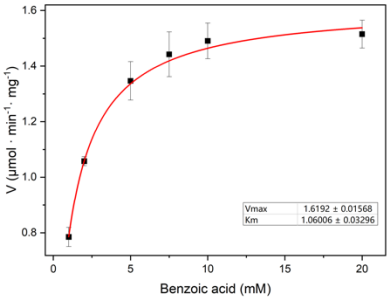
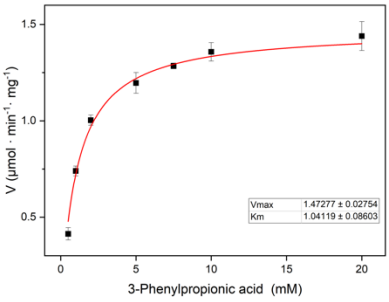
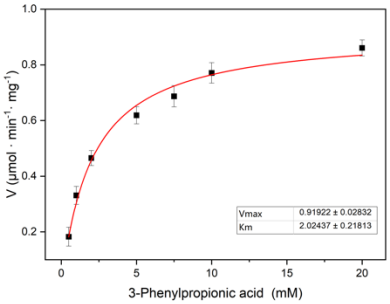
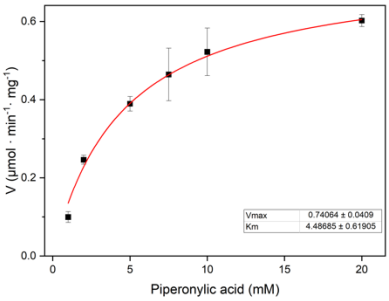
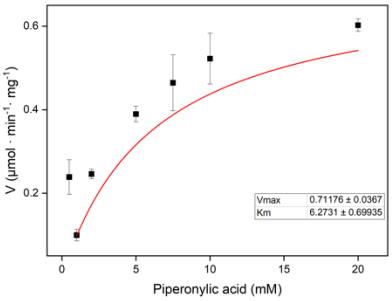
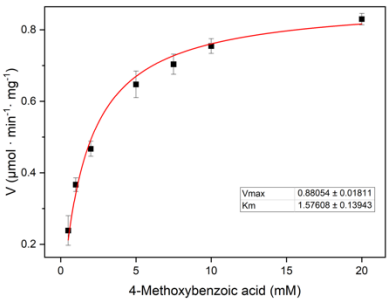
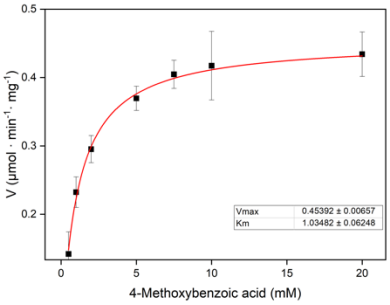
Enzyme	30°C		40°C		50°C	
	k_d/h^{-1}	$t_{1/2}$/h	k_d/h^{-1}	$t_{1/2}$/h	k_d/h^{-1}	$t_{1/2}$/h
*Bp*GDH	0.0018	385	0.0019	364.74	0.0208	33.32
BID-*Bp*GDH	0.0095	72.95	0.1219	5.68	0.1604	4.32

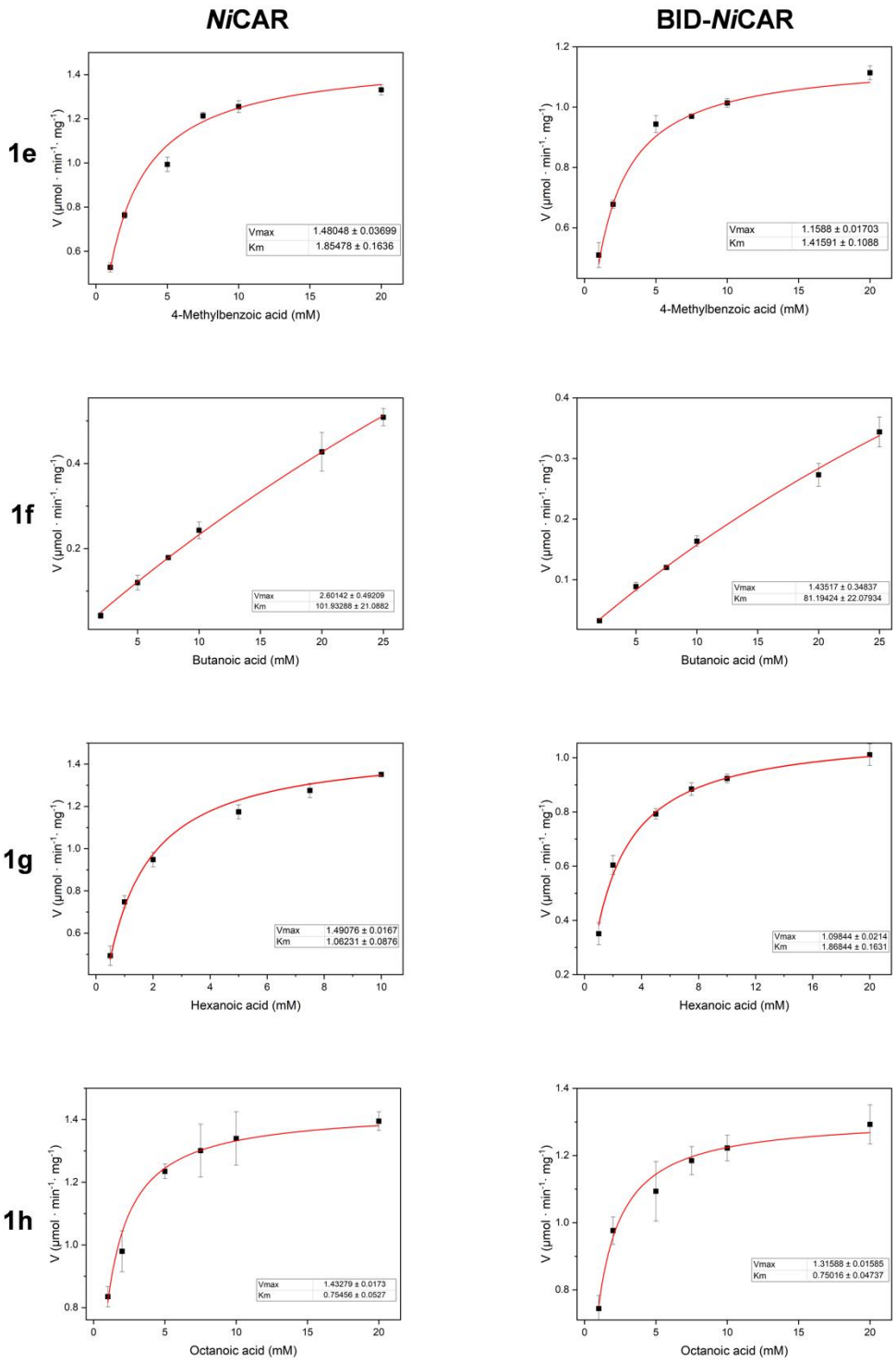
Figure S12. Thermostability of *Bp*GDH and BID-*Bp*GDH at 30°C, 40°C and 50°C.

Table S3. Substrate scope and catalytic activity of *Ni*CAR and BID-*Ni*CAR.

		1a	**1b**	**1c**	**1d**	**1e**
*Ni*CAR	K_m (mM)	0.45±0.03	1.04±0.09	4.49±0.62	1.58±0.14	1.85±0.16
	k_{cat} (min^{-1})	235.36±2.27	189.00±3.53	95.04±5.25	113.62±2.32	189.98±4.75
	k_{cat}/K_m (min^{-1} mM^{-1})	520.72±38.96	181.52±15.38	21.18±3.14	72.09±6.55	102.43±9.39
BID-*Ni*CAR	K_m (mM)	1.06±0.03	2.02±0.22	6.27±0.70	1.03±0.06	1.41±0.11
	k_{cat} (min^{-1})	243.37±2.36	138.16±4.26	106.98±5.52	68.23±0.99	174.17±2.56
	k_{cat}/K_m (min^{-1} mM^{-1})	229.58±7.48	68.25±7.65	17.05±2.09	65.93±4.09	123.01±9.62
		1f	**1g**	**1h**	**1i**	
*Ni*CAR	K_m (mM)	101.93±21.09	1.06±0.09	0.75±0.05	1.42±0.05	
	k_{cat} (min^{-1})	333.83±63.15	191.30±2.15	183.86±2.22	86.02±1.35	
	k_{cat}/K_m (min^{-1} mM^{-1})	3.27±0.92	180.08±14.99	243.67±17.27	60.75±2.48	
BID-*Ni*CAR	K_m (mM)	81.19±22.07	1.87±0.16	0.75±0.05	1.21±0.08	
	k_{cat} (min^{-1})	215.71±52.36	165.10±3.22	197.78±2.38	94.33±075	
	k_{cat}/K_m (min^{-1} mM^{-1})	2.66±0.97	88.36±7.91	263.65±16.95	77.96±4.88	

Reaction conditions: Carboxylic acid **1a-1i** (10 mM), $MgCl_2$ (10 mM), DTT (1 mM), ATP (1 mM), NADPH (0.2 mM), 0.5 μM enzyme. Reaction was performed in 100 mM MOPS, pH 7.5 at 30°C.

	***Ni*CAR**	**BID-*Ni*CAR**
1a		
1b		
1c		
1d		


NiCAR
BID-NiCAR
1e
1f
1g
1h
V (μmol · min-1 · mg-1)
4-Methylbenzoic acid (mM)
Vmax 1.48048 ± 0.03699
Km 1.85478 ± 0.1636
Vmax 1.1588 ± 0.01703
Km 1.41591 ± 0.1088
Butanoic acid (mM)
Vmax 2.60142 ± 0.49209
Km 101.93288 ± 21.0882
Vmax 1.43517 ± 0.34837
Km 81.19424 ± 22.07934
Hexanoic acid (mM)
Vmax 1.49076 ± 0.0167
Km 1.06231 ± 0.0876
Vmax 1.09844 ± 0.0214
Km 1.86844 ± 0.1631
Octanoic acid (mM)
Vmax 1.43279 ± 0.0173
Km 0.75456 ± 0.0527
Vmax 1.31588 ± 0.01585
Km 0.75016 ± 0.04737

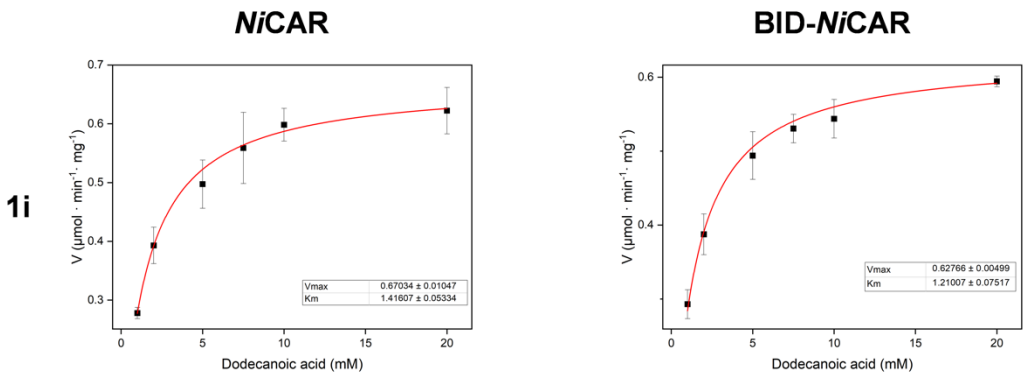
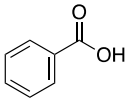
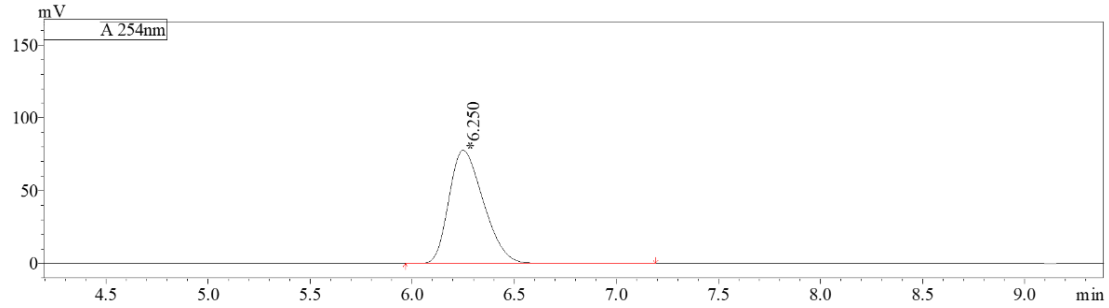


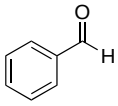
Figure S13. The kinetic constants of *Ni*CAR and BID-*Ni*CAR toward different acid substrates. The enzyme activities were assayed in 100mM MOPS pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.2 mM NADPH, 0.5-20 mM substrate, 0.5 μM enzyme, at 30℃.

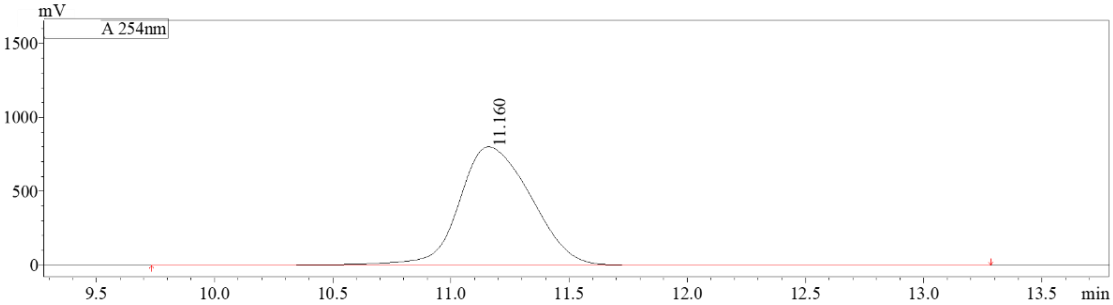
4. HPLC and GC chromatograms

(1) HPLC

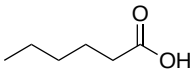
Benzoic acid **1a** 

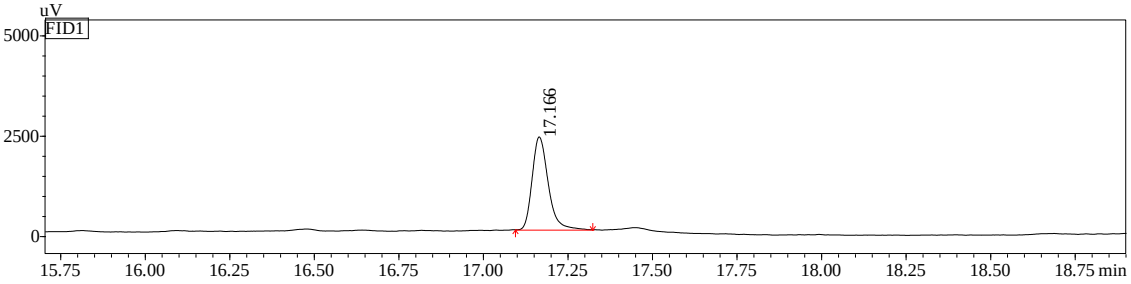


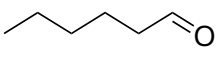
Benzaldehyde **2a** 

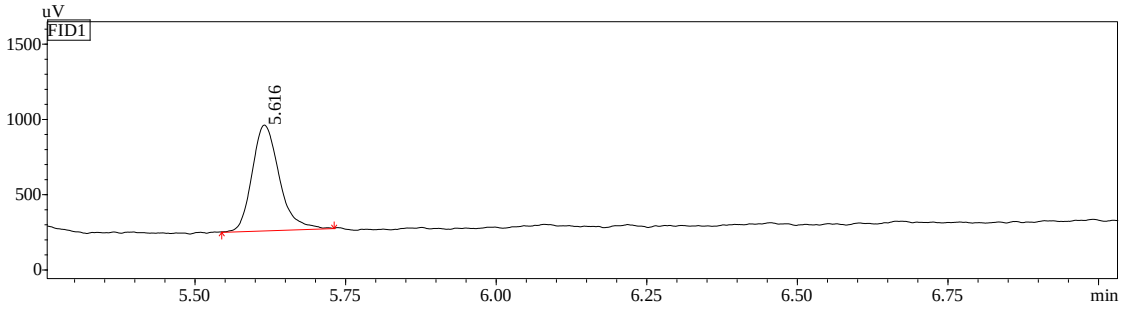


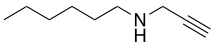
(2) GC-FID

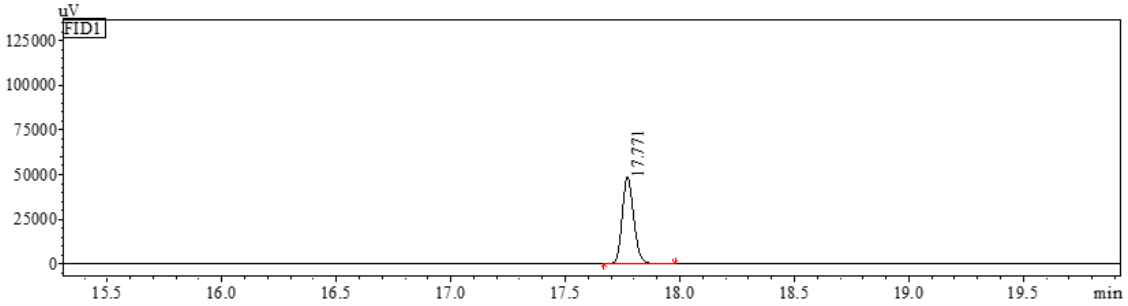
Hexanoic acid **1g** 

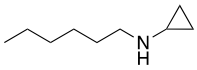


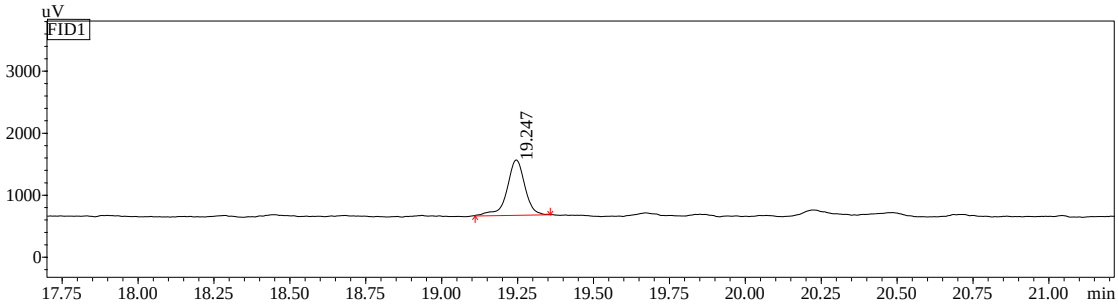
Hexanal **2g** 



N-propargylhexylamine 



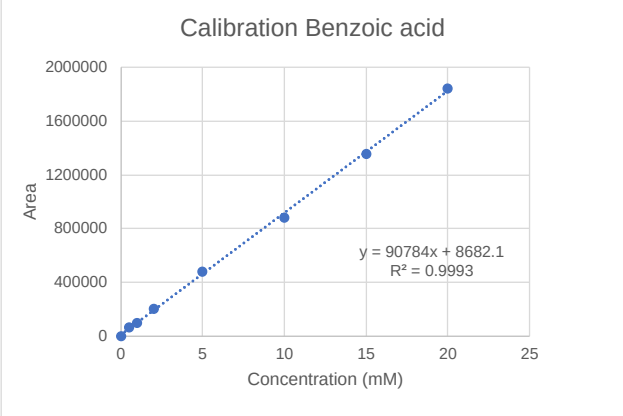
N-cyclopropylhexylamine 



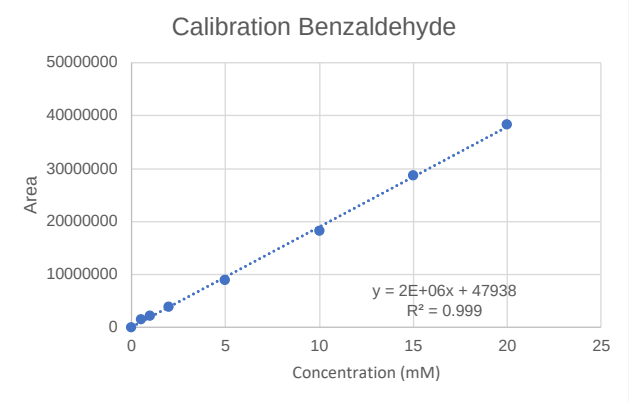
5. Calibration curves and representative HPLC traces

Calibration Curves

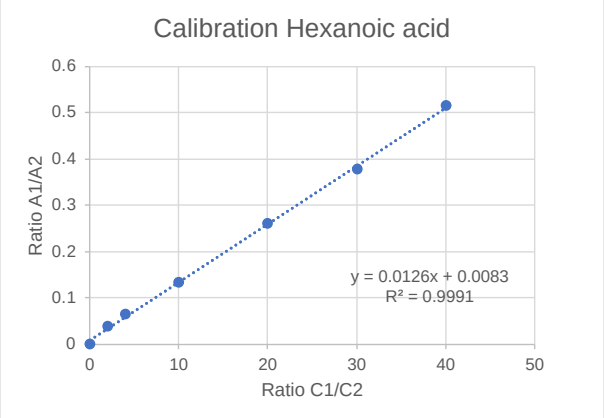
(1) Benzoic acid **1a**



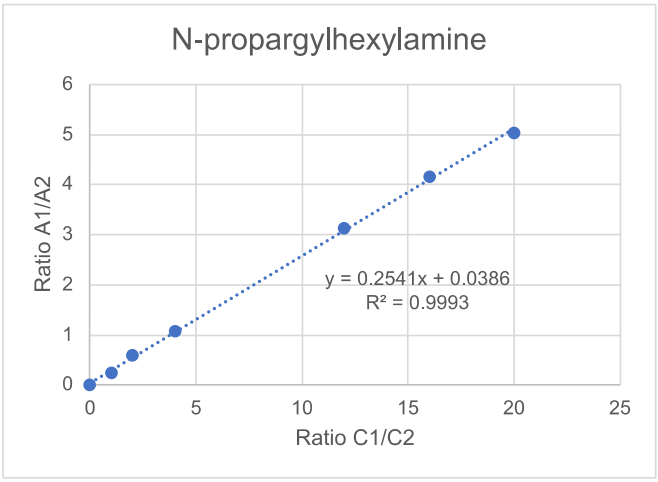
(2) Benzaldehyde **2a**



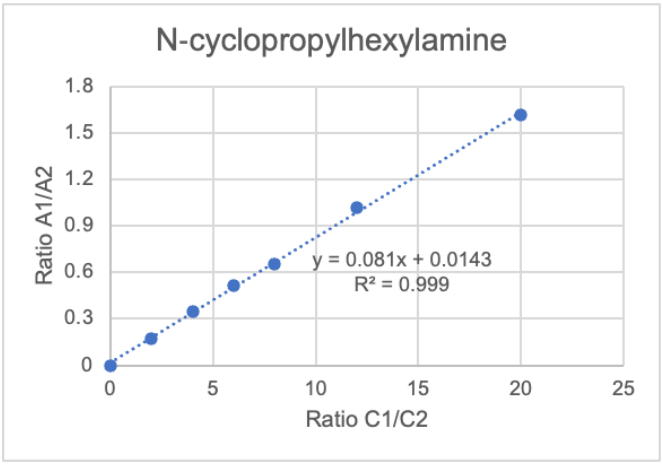
(3) Hexanoic acid **1g**



(4) N-propargylhexylamine

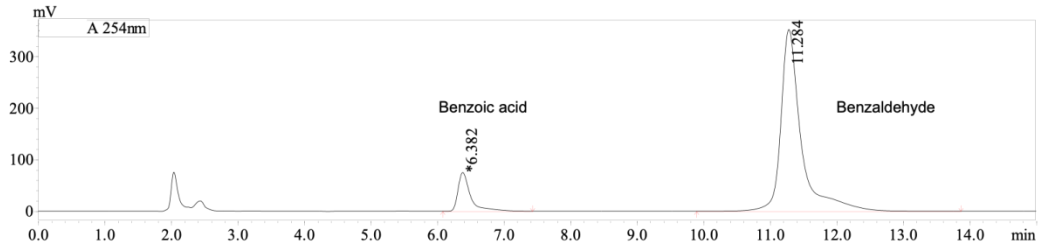


(5) N-cyclopropylhexylamine

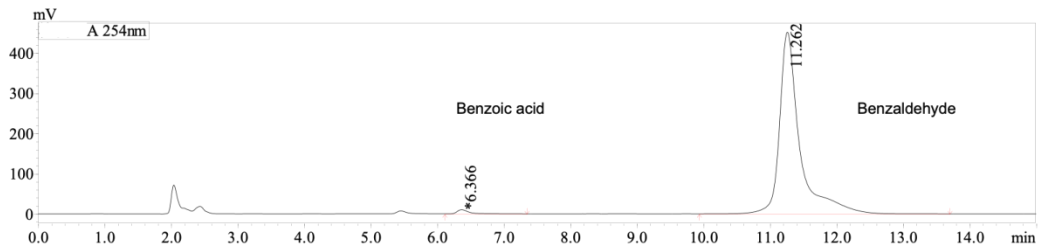


HPLC traces

(1) Tetra-enzyme condensates, 0.5 h



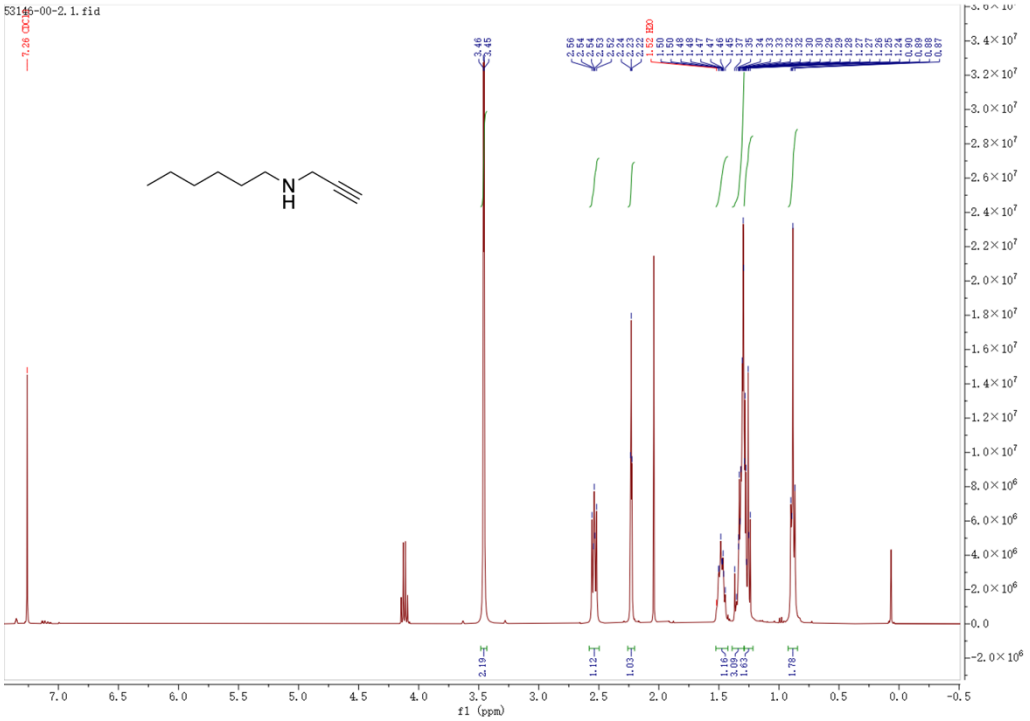
(2) Tetra-enzyme condensates, 4 h



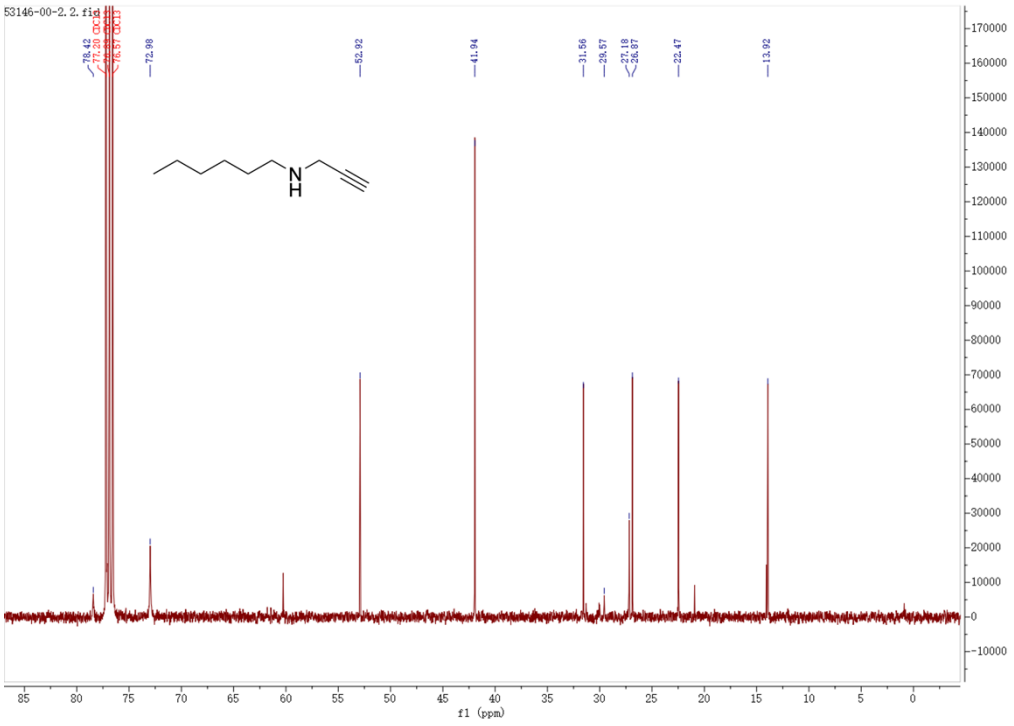
6. ^{1}H-NMR and ^{13}C-NMR

N-propargylhexylamine

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.46 (d, J = 2.5 Hz, 2H), 2.58 – 2.50 (m, 1H), 2.23 (t, J = 2.4 Hz, 1H), 1.53 – 1.42 (m, 1H), 1.39 – 1.29 (m, 3H), 1.29 – 1.22 (m, 2H), 0.92 – 0.84 (m, 2H).

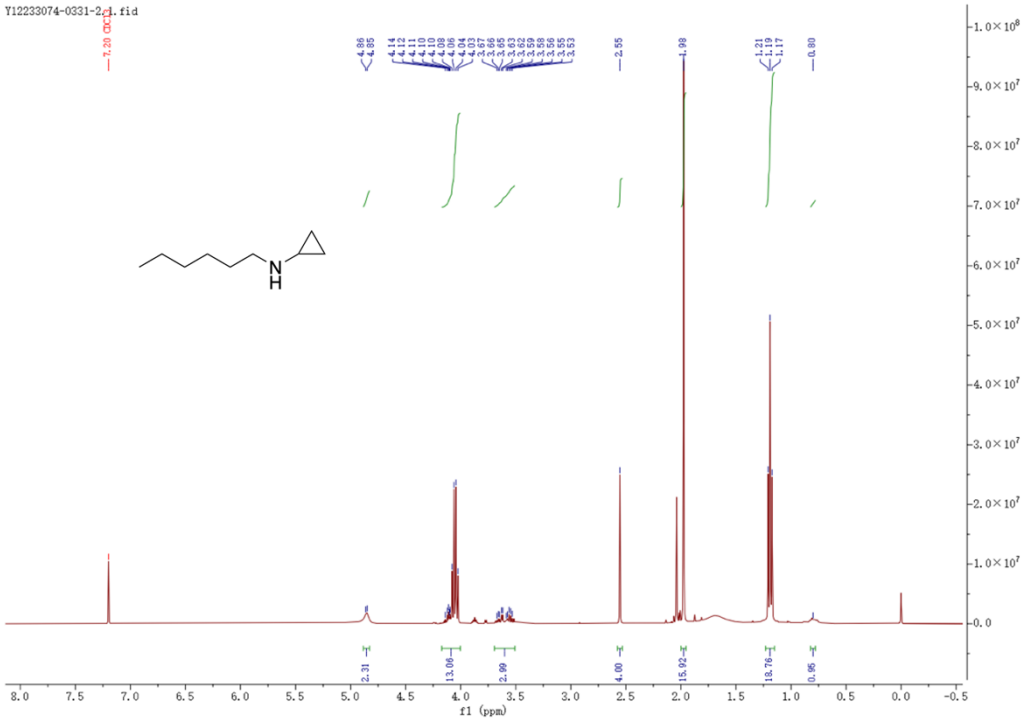


^{13}C NMR (101 MHz, $CDCl_3$) δ 78.42, 72.98, 60.27, 52.92, 41.94, 31.56, 29.57, 27.18, 26.87, 22.47, 13.92.

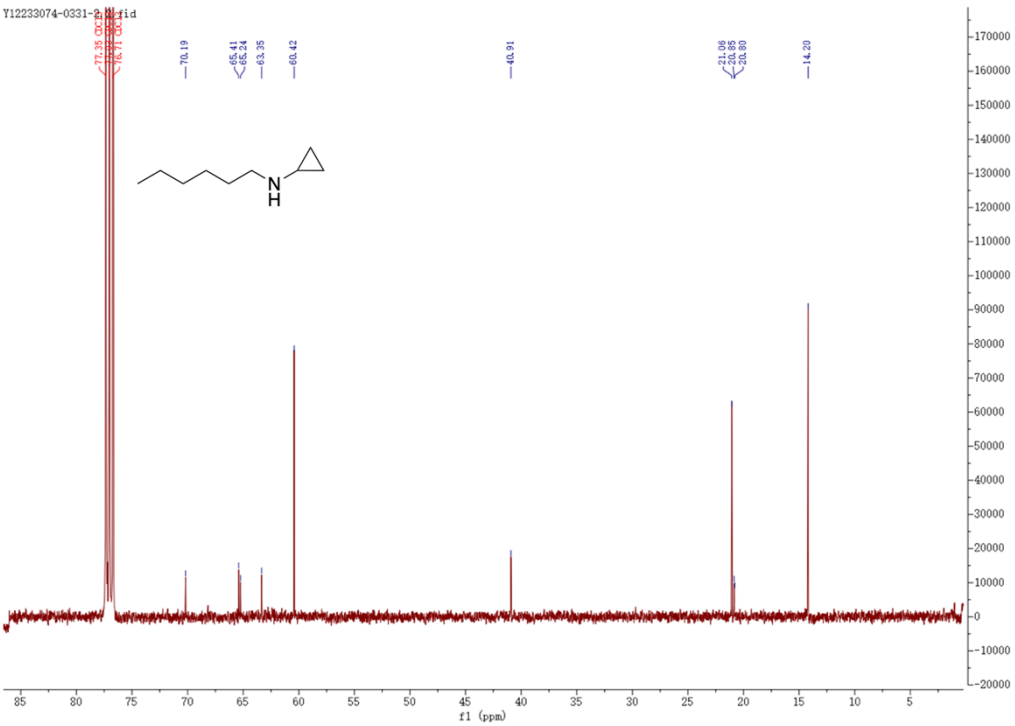


N-cyclopropylhexylamine

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.88 – 4.83 (m, 2H), 4.17 – 4.00 (m, 13H), 3.60 (ddt, J = 34.8, 11.5, 5.5 Hz, 3H), 2.55 (s, 4H), 1.98 (s, 16H), 1.19 (t, J = 7.1 Hz, 19H), 0.80 (s, 1H).



^{13}C NMR (101 MHz, $CDCl_3$) δ 70.19, 65.41, 65.24, 63.35, 60.42, 40.91, 21.06, 20.85, 20.80, 14.20.



7. References

1. Nocek, B.; Kochinyan, S.; Proudfoot, M.; Brown, G.; Evdokimova, E.; Osipiuk, J.; Edwards, A.M.; Savchenko, A.; Joachimiak, A.; Yakunin, A.F. Polyphosphate-Dependent Synthesis of ATP and ADP by the Family-2 Polyphosphate Kinases in Bacteria. *Proc. Natl. Acad. Sci.* **2008**, *105*, 17730–17735, doi:10.1073/pnas.0807563105.