Supporting Information

Baeyer-Villiger oxidation of ACP-tethered thioester to ACP-linked thiocarbonate catalyzed by a monooxygenase domain in FR901464 biosynthesis

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Table of contents

1. Experimental procedures

1.1 Materials and general methods.

DNA isolation and manipulation in *E.coli* were performed according to standard methods.[1] PCR amplifications were carried out on an authorized thermal cycler (Eppendorf AG 22331, Hamburg, Germany) using PrimeSTAR HS DNA polymerase (TaKaRa). Primer synthesis and DNA sequencing were performed at Shanghai Invitrogen Biotech Co., Ltd. (China). Competent *E.coli* cells were purchased from Invitrogen (Carlsbad, CA) and Novagen (Madison, USA).Restriction enzymes were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). All chemicals and reagents were purchased from Sigma-Aldrich. Analytical HPLC was carried out on a Shimazu Prominence LC-20A HPLC system with DAD detector. LC-MS analysis of small molecular compounds was carried out on an Agilent 1200 HPLC instrument connected to LCQ Fleet electrospray ionization (ESI) mass spectrometer (Thermo Fisher Scientific Inc.). GC-MS analysis was carried out on an Agilent 7890A/5973N GC/MS instrument with a DB-5 MS column (60 m × 250 μm × 0.25 μm). NMR spectra were obtained on a Bruker Avance AV400 NMR spectrometer operating at 400 MHz ^{1}H frequency or 100 MHz ^{13}C frequency. Chemical shifts are referenced to $CDCl_3$ at room temperature.

1.2 Cloning of *fr9H-Ox* and *fr9I-ACP$_1$*.

The genes encoding Ox domain of FR9H and ACP_1 domain of FR9I were amplified by PCR from fosmid pTG1003[2] with primer set FR9H-Ox-For and FR9H-Ox-Rev, and FR9I-ACP_1-For and FR9I-ACP_1-Rev, respectively (Table S1). PCR products were purified, cloned and confirmed by sequencing, then a *Nde* I/*Hin*d III fragment for FR9H-Ox and a *Nde* I/*Xho* I fragment for FR9I-ACP_1 were cloned into the same sites of pET28a and pET37b respectively, to make the expression plasmid pTG1008 (for *fr9H-Ox*) and pTG1009 (for *fr9I-ACP$_1$*). The resulting plasmids were transformed separately into *E. coli* BL21 (DE3) for protein expression.

1.3 Protein Expression and Purification.

For overproduction of proteins, cells harboring the desired plasmid were grown in LB medium supplemented with 50 μg/mL of kanamycin. Cultures (1 L) were grown to an OD_{600} of 0.4-0.6 at 37 °C and then cooled to 16 °C for 30 min, and protein expression was induced by the addition of 100 μM IPTG (final concentration). Cultures were grown for an additional 24 h. Purification of the His-tagged fusion protein with Ni-NTA affinity resin was performed according to manufacturer's

manual (Qiagen, Valencia, CA), and the resultant proteins were dialyzed against 50mM Na_2HPO_4 (pH 9.0), 100mM NaCl, and 10% glycerol for FR9H-Ox or 25 mM HEPES (pH 7.0), 50 mM NaCl, and 10% glycerol for FR9I-ACP_1, and stored at -80 °C. The final concentration of protein was determined by a Bradford assay[3].

1.4 HPLC Analysis of FR9H-Ox Flavin Cofactor.

A 50 μL sample of purified FR9H-Ox (80 μM) was boiled for 10 min, and denatured protein was removed by centrifugation. The flavin present in the supernatant was analyzed by HPLC on an analytic Inertsil ODS-EP column (5 μm, 4.6 × 250 mm, GL Sciences). The LC conditions were as follows. Solvent A was H_2O, and Solvent B was CH_3CN; both solvent contained 0.1% TFA (v/v). The column was equilibrated with 100% solvent A and followed by the following linear gradient program: 100% A/0%B, 0–3 min; ramp to 30% B, 3–18 min; ramp to 80% B, 18–20 min; and return to 100% A/0% B, 20–24 min. The flow rate was 1 mL/min, and elution was monitored at 450 nm. Additionally, the identity of the cofactor was proven by mass spectroscopy (data not shown).

1.5 Biochemical Assays of FR9H-Ox *in vitro*.

Generation of acyl-FR9I-ACP_1. A typical reaction mixture (50 μL) for the generation of acyl-FR9I-ACP_1 was prepared using 100 μM apo-FR9I-ACP_1, 4 μM Sfp (a phosphopantetheinyl transferase from *Bacillus subtilis*[4]), 1 mM TCEP, and 10 mM $MgCl_2$ in 100 mM HEPES buffer (pH 7.0). To the reaction mixtures were added 400 μM acyl-CoA derivatives (**1**-CoA, **2**-CoA, **3**-CoA, or **4**-CoA) individually followed by incubation for 1 h at 30 °C. The reaction mixtures were used directly in the assays of FR9H-Ox or were subjected to HPLC or MS analysis as described bellow.

Assay of the FR9H-Ox reaction. The assay solution (50 μL total volume) consisted of 150 mM HEPES (pH 7.0), 50 μM acyl-FR9I-ACP_1, 10 μM FR9H-Ox, 50 μM FAD and 2 mM NADPH. Reactions were incubated at room temperature and 10 μL aliquots were taken at 30 and 60 min after addition of NADPH and quenched by adding 5μL 10% HCOOH. Control reactions were performed in the absence of NADPH. HPLC analysis of the samples was carried out on a Grace Vydac protein and peptide C8 column. The column was equilibrated with 80% solvent A (H_2O, 0.1% TFA) and 20% solvent B (CH_3CN, 0.1% TFA) and developed with the following program: 0 to 3 min, a linear gradient to 70% A/30% B; 3 to 22 min, a linear gradient to 42% A/58% B; 22 to 25 min, a linear gradient to 5% A/95% B; 25 to 28 min, a linear gradient to 80% A/20% B, and 28 to 30 min, constant 80% A/20% B. It was carried out at a flow rate of 1 mL/min with UV detection at 220 nm,

using a Shimazu Prominence LH-20A HPLC system. MS analysis of protein samples was carried out with an Agilent 6530 Accurate-Mass Quadropole Time-of-Flight (Q-TOF) LC/MS.

To further analysis the product of reactions catalyzed by FR9H-Ox, the reaction (after incubated at room temperature for 2 h in the presence of NADPH) was terminated by the addition of 70% trichloroacetic acid (TCA) to a final concentration of 10% to precipitate all proteins. After incubation on ice for 15 min, the precipitate was separated by centrifugation (4 °C, 12500 rpm for 10 min). The resulting pellet was washed twice with 200 μL of 5% TCA and once with 200 μL of ethanol. After drying by speed-vac, the protein pellet was redissolved in 150 μL of 0.1 M KOH and incubated at 65 °C for 15 min to hydrolyze all FR9I-ACP_1-tethered compounds. Then the released compounds were extracted with ethyl acetate and analyzed by using an Agilent Technologies GC-MS instrument with the following conditions. Helium was used as the carrier gas. The injector temperature was 250 °C. The gradient program used for analysis was constant at 50 °C for 2 min, then 50 to 100 °C at 20 °C/min.

Assay of FR9H-Ox with SNAC substrates. The assay solution (50 μL total volume) consisted of 100 mM HEPES (pH 7.0), 500 μM acyl-SNAC (**1**-SNAC, or **3**-SNAC or **4**-SNAC), 10 μM FR9H-Ox, 50 μM FAD, 2 mM NADPH and 5% DMSO. Reactions were incubated at room temperature for 4 h and quenched by adding 1 μL trichloroacetic acid. And NADPH was not added into the reaction mixtures in the negative controls. Following centrifugation to remove protein, the reactions were analyzed by HPLC or LC-MS using an analytic Inertsil ODS-EP column (5 μm, 4.6 x250 mm, GL Sciences). The LC conditions were as follows. Solvent A was H_2O, and Solvent B was CH_3CN; both solvent contained 0.1% TFA (v/v) in the HPLC analysis or 0.1% HCO_2H (v/v) in the LC-MS analysis. The column was equilibrated with 90% A/10% B and followed by the following linear gradient program: 90% A/10% B, 0 min; ramp to 60% B, 0–15 min; ramp to 95% B, 15–17 min; constant 95% B, 17-22 min; and return to 90% A/10% B, 22–24 min. The flow rate was 1 mL min^{-1}, and elution as monitored at 220 nm.

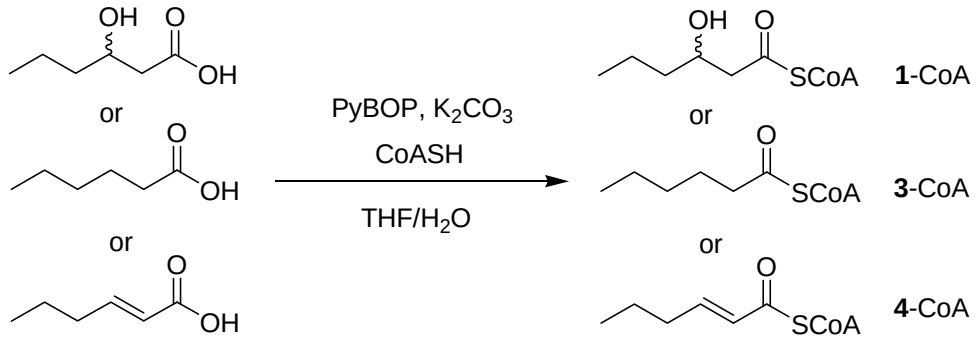
1.6 Whole-cell Biotransformations.

All of the whole-cell biotransformation tests were carried out with growing cells according to the overexpression protocol above. The substrates were dissolved in neat ethanol and added to cell culture (50 mL) 3 h after induction to give a final concentration of 1% (v/v) ethanol in the medium. The final substrate concentrations were 15 mM for each substrate, cyclobutanone, cyclopentanone, and cyclohexanone. Reaction mixtures were extracted from the LB medium with ethyl acetate and

analyzed by using an Agilent Technologies GC-MS instrument[5].

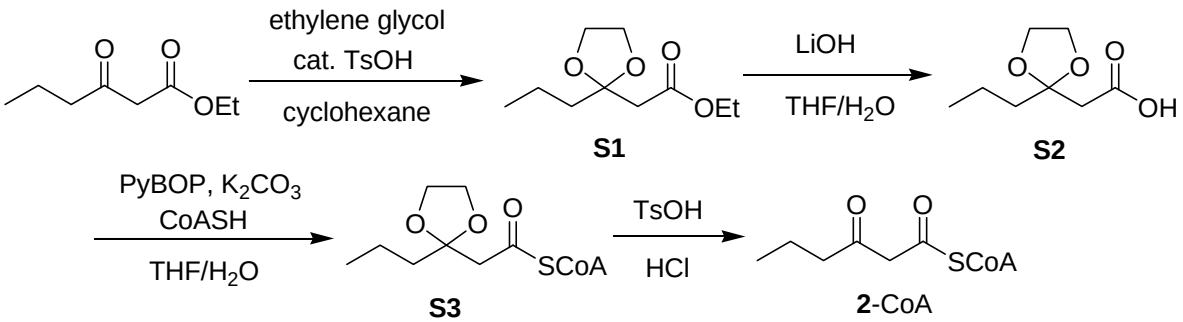
1.7 Chemical synthesis of compounds used in this work.

1.7.1 Synthesis of CoA derivatives (**1**-CoA, **2**-CoA, and **3**-CoA)



Free acid (16 μmol, 1.6 equiv), PyBOP (16 μmol, 1.6 equiv) and K_2CO_3 (40 μmol, 4.0 equiv) were dissolved in 3 mL of freshly distilled THF under argon. Coenzyme A (10 μmol, 1.0 equiv) was dissolved in 1 mL of H_2O (O_2 was removed by sonication), and added dropwise to the THF solution. After stirring at room temperature for 2 h, the reaction mixture was directly subjected to a Venusil XBP-C18 semi-preparative reversed-phase column (10.0 × 250 mm, 5 μm, 100 Å) by HPLC with the following gradient at a flow rate of 3 mL/min at room temperature: 0-20 min, 20%-80% B; 20-25 min, 80%-20% B (buffer A, 10 mM NH_4OAc in H_2O; and buffer B, CH_3CN). UV absorbance was monitored at 260 nm, and fractions containing product were pooled. Acetonitrile was removed by evaporation, and water and NH_4OAc were removed by lyophilization. More than 90% CoA was converted to its derivatives. The mass of the products were confirmed by ESI-MS $[M+H]^+$: **1**-CoA, calcd 892.2, and found 892.3; **3**-CoA, calcd 876.2, and found 876.2; **4**-CoA, calcd 874.2, and found 874.3.

1.7.2 Synthesis of **2**-CoA



To a solution of ethyl 3-oxohexanote (3 mmol, 1.0 equiv) in cyclohexane was added ethylene glycol (6 mmol, 2.0 equiv) followed by TsOH (60 μmol, 0.02 equiv). The mixture was aged for 8 h at reflux. After cooled to room temperature the organic solvent was removed *in vacuo*, and the

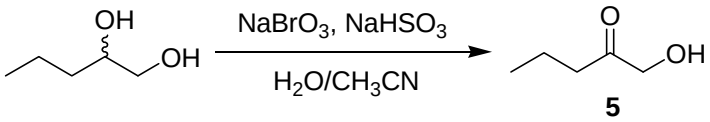
residue was purified by column chromatography (silica gel, hexane: ethyl acetate = 20:1) to give **S1** (489 mg, 81%) as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ 0.92(t, 3H, J=6.0 Hz), 1.25(t, 3H, J=8.0 Hz), 1.41(m, 2H), 1.77(m, 2H), 2.63(s, 2H), 3.92~4.01(m, 4H), 4.14(q, 2H, J=8.0 Hz).

To a solution of **S1** (1 mmol, 1.0 equiv) in THF (3 mL)-H_2O (1 mL) was added lithium hydroxide mono hydrate (4 mmol, 4.0 equiv). The reaction mixture was stirred at room temperature for 8 h, and removed the organic solvent. Then the solution was acidified with 1 M HCl and extracted three times with EtOAc. The combined organic layers were washed with brine, dried over $NaSO_4$, filtered and concentrated *in vacuo* to provide **S2** (164 mg, 95%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.93(t, 3H, J=8.0 Hz), 1.42(m, 2H), 1.79(m, 2H), 2.70(s, 2H), 3.97~4.05(m, 4H), 9.22(brs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.24, 16.95, 39.88, 42.50, 65.23, 65.23, 109.36, 174.84.

The synthesis of **S3** from **S2** (3.6 mg, 1.6 equiv) was carried out as the procedure described above for the synthesis of **1**-CoA. The product **S3** (9 mg) was obtained as a white solid. ESI-MS $[M+H]^+$: calcd 934.2, and found 934.5.

To a solution of **S3** (3 mg) in THF (100 μL)-Acetone (100 μL) was added TsOH (1 mg) followed by 1 M HCl (50 μL). The reaction mixture was static at room temperature for 48 h. Then the desired product was purified by HPLC using the same procedure described for the purification of **1**-CoA. Finally, **2**-CoA (1.5 mg) was obtained. ESI-MS $[M+H]^+$: calcd 890.2, and found 890.4.

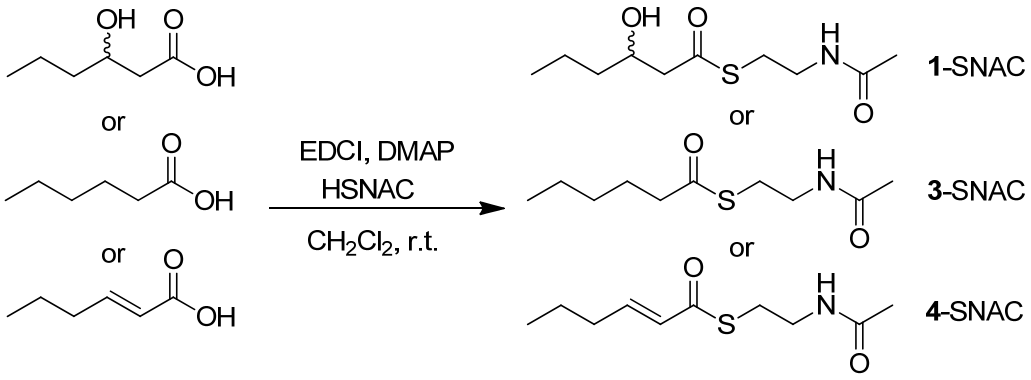
1.7.3 Synthesis of compound **5**[6]



5

To a solution of $NaBrO_3$ (12 mmol) in water (6 mL) was added the diols (5 mmol) in acetonitrile (10 mL) followed by the dropwise addition of a solution of $NaHSO_3$ (12 mmol) in water (12 mL) over a period of about 25 min. The mixture was then stirred at room temperature for 4 h. The reaction mixture was poured into 50 mL of ether. After separation of the organic phase, the aqueous layer was extracted twice with ether. The combined organic layer was washed with anhydrous $Na_2S_2O_3$ solution and dried over Na_2SO_4. After filtration, the ether was removed in vacuum, and the residue was purified by column chromatography (silica gel, hexane: ethyl acetate = 10:1) to give **5** (420 mg, 82%) as a colorless oil. ^{1}H NMR (400MHz, $CDCl_3$): δ 0.92(t, 3H, J=6.0 Hz), 1.59~1.69(m, 2H), 2.36(t, 2H, J=6.0Hz), 3.24(brs, 1H), 4.21(s, 2H).

1.7.4 Synthesis of SNAC derivatives.



4-dimethylaminopyridine (0.3 mmol, 0.3 equiv) was added into the mixture of free acid (1.0 mmol, 1.0 equiv), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (1.2 mmol, 1.2 equiv) and N-acetylcysteamine (1.2 mmol, 1.2 equiv) in CH_2Cl_2 (15 mL), followed by stirring at room temperature for 24 h, washing with 1 N HCl (1 × 10 mL), brine (2 × 10 mL), and dried (Na_2SO_4). After filtered, the solvent was removed at reduced pressure, and the residue was purified by flash column chromatography over silica gel (chloroform/methanol: 80/1) to afford the desired SNAC derivatives. The corresponding products were confirmed by ESI-MS and ^{1}H NMR analysis. **1**-SNAC: ESI-MS $[M+Na]^+$ 256.2; ^{1}H NMR (400MHz, $CDCl_3$): δ 0.89 (t, 3H, J=6.4 Hz), 1.29~1.50 (m, 4H), 1.92 (s, 3H), 2.61~2.72 (m, 2H), 2.94~3.05 (m, 2H), 3.15 (brs, 1H), 3.36~3.41 (m, 2H), 4.03 (m, 1H), 6.36 (brs, 1H). **3**-SNAC: ESI-MS $[M+Na]^+$ 240.2; ^{1}H NMR (400MHz, $CDCl_3$): δ 0.87 (t, 3H, J=6.8 Hz),1.29 (m, 4H), 1.61~1.68 (m, 2H), 1.95 (s, 3H), 2.55 (t, 2H, J=7.6 Hz), 3.00 (t, 2H, J=7.6 Hz), 3.41 (q, 2H, J=6.0 Hz), 5.98 (brs, 1H). **4**-SNAC: ESI-MS $[M+Na]^+$ 238.1; ^{1}H NMR (400MHz, $CDCl_3$): δ 0.90 (t, 3H, J=7.2 Hz), 1.42~1.51 (m, 2H), 1.93 (s, 3H), 2.15 (q, 2H, J=7.2 Hz), 3.05 (t, 2H, J=6.4 Hz), 3.41 (q, 2H, J=6.0 Hz), 6.08 (d, 1H, J=15.2 Hz), 6.22 (brs, 1H), 6.88 (dt, 1H, J_1=15.2 Hz, J_2=7.2 Hz).

1.8 Detailed information of the BVMOs shown in Figure 3 in the text.

ACMO, acetone monooxygenase from *Gordonia* sp. TY-5 (GeneBank: BAF43791.1); AufJ from *Stigmatella aurantiaca* DW4/3-1 (ADO72994.1); BVMO bt from *Burkholderia thailandensis* MSMB43 (ZP_02468377.1); BVMO KT2440 from *Pseudomonas putida* KT2440 (NP_744949.1); BVMO mekA from *Pseudomonas veronii* MEK700 (ABI15711.1); BVMO Pf from *Pseudomonas fluorescens* DSM 50106 (AAC36351.2); BVMO Mtb5 (Rv3049c) and BVMO EtaA (Rv3854c) are from *Mycobacterium tuberculosis* H37Rv genome; Camp from *Pseudomonas putida* (AAR21560.1); CDMO, cyclododecanone monooxygenase from *Rhodococcus ruber* strain SC1 (AAL14233.1); ChlE1 from *Streptomyces antibioticus* (AAZ77692.1); CHMO 9871, cyclohexanone monooxygenase from *Acinetobacter* sp. strain NCIMB 9871 (BAA86293.1); CHMO Arthr from *Arthrobacter* sp.

BP2 (AAN37479.1); CHMO Branch from *Brachymonas petroleovorans* (AAR99068.1); CHMO Brev1 and CHMO Brev2 from *Brevibacterium* sp. HCU (AAG01289.1 and AAG01290.1, respectively); CHMO Rhodo1 from *Rhodococcus* sp. Phi1 (AAN37494.1); CHMO Xantho from *Xanthobacter flavus* (CAD10801.1); CmmOIV from *Streptomyces griseus* subsp. griseus (CAE17536.1); CPMO, cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (BAC22652.1); DKCMO, diketocamphane monooxygenase from *Pseudomonas putida* (AEZ35247.1); GilO1 from *Streptomyces griseoflavus* (AAP69582.1); GrhO6 from *Streptomyces* sp. JP95 (AAM33673.1); HAMPO ACB, 4-hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* (AAK54073.1); MtmOIV from *S. argillaceus* (3FMW_A); PAMO, phenylacetone monooxygenase from *Thermobifida fusca* strain YX (AAZ55526.1); PedG from symbiont bacterium of Paederus fuscipes (AAS47561.1); PenE from *Streptomyces exfoliatus* (ADO85591.1); PntE from *Streptomyces arenae* (ADO85575.1); PtlE from *Streptomyces avermitilis* MA-4680 (BAC70705.1); RubN from *Streptomyces collinus* (AAM97364.1); ScMO, putative monooxygenase from *S. coelicolor* A3(2), (CAB55657.1); SMO, steroid monooxygenase from *R. rhodochrous* strain IFO 3338 (AB010439.1).

2. Supplemental Tables and Figures.

Table S1. PCR primers with restriction sites

Oligonucleotide primers	Sequence (Restriction site in bold)
FR9H-Ox-For with *Xba* I / *Nde* I	5'-ATAT**TCTAGA** **CATATG**GCCGGCGCGCCGCGCCCG-3'
FR9H-Ox-Rev with *Hin*d III	5'-ATAT**AAGCTT**GTCGTCCTGTTTCGTCAAGGCG-3'
FR9I-ACP$_1$-For with *Eco*R I / *Nde* I	5'-ATAT**GAATTC** **CATATG**GAACGTCCCGTCGCTCGCGC-3'
FR9I-ACP$_1$-Rev with *Hin*d III / *Xho* I	5'-ATAT**AAGCTT**A**CTCGAG**TTCGTCCGGTCGCGCGGCTC-3'

Table S2. Q-TOF MS analysis of apo-, holo-, and acyl-*S*-ACPs

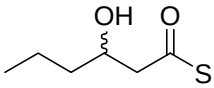
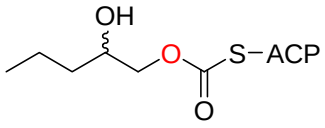
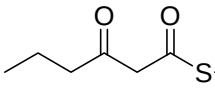
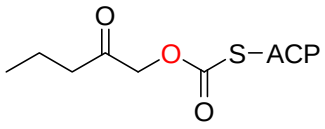
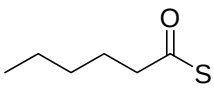
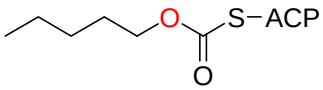
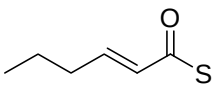
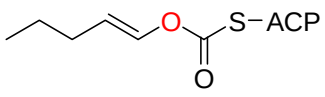
ACPs	MS data	
	calcd (Da)	found (Da)
apo-FR9I-ACP$_1$	12763.2	12763.7
holo-FR9I-ACP$_1$	13103.5	13104.0
 (**1**-FR9I-ACP$_1$)	13217.7	13218.1
 (**1**-FR9I-ACP$_1$-**P**)	13233.7	13234.0
 (**2**-FR9I-ACP$_1$)	13215.7	13216.0
 (**2**-FR9I-ACP$_1$-**P**)	13231.7	13232.3
 (**3**-FR9I-ACP$_1$)	13201.7	13202.3
 (**3**-FR9I-ACP$_1$ -**P**)	13217.7	13218.0
 (**4**-FR9I-ACP$_1$)	13199.7	13200.1
 (**4**-FR9I-ACP$_1$-**P**)	13215.7	not detected

Figure S1. SDS-PAGE analysis of purified FR9H-Ox (A) and FR9I-ACP$_1$ (B). *Lane M*, molecular weight marker; *lane 1*, purified protein. The recombinant *N*-terminal His$_6$-tagged FR9H-Ox consists of 632 amino acids, which is predicted to have the molecular mass of 70.7 kDa. And the recombinant *C*-terminal His$_6$-tagged FR9I-ACP$_1$ consists of 112 amino acids, which is predicted to have the molecular mass of 12.6 kDa.

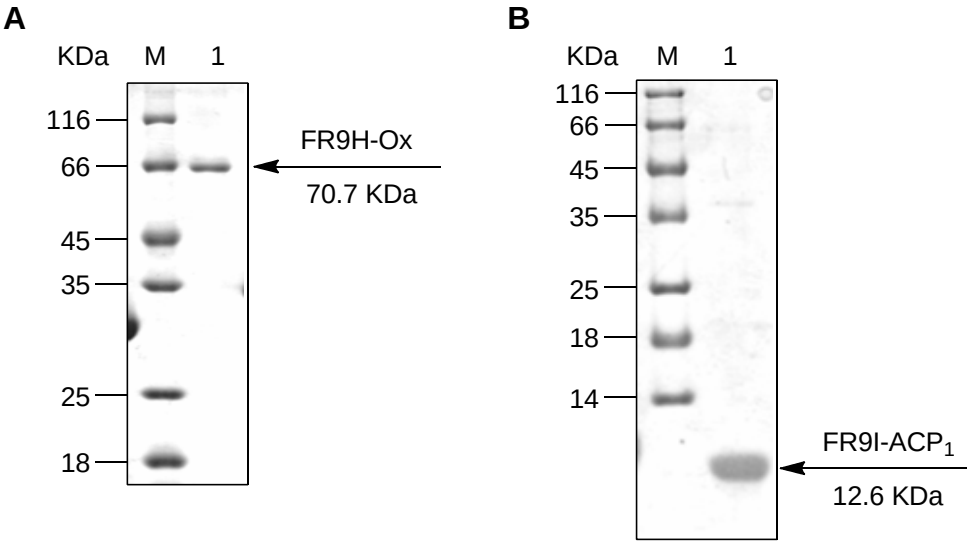


Figure S2. UV-Vis spectroscopy of FR9H-Ox (A, 50 µM) and HPLC analysis of FR9H-Ox containing cofactor with authentic standards (B). FAD (I), FMN (II) and cofactor released from boiled FR9H-Ox (III).

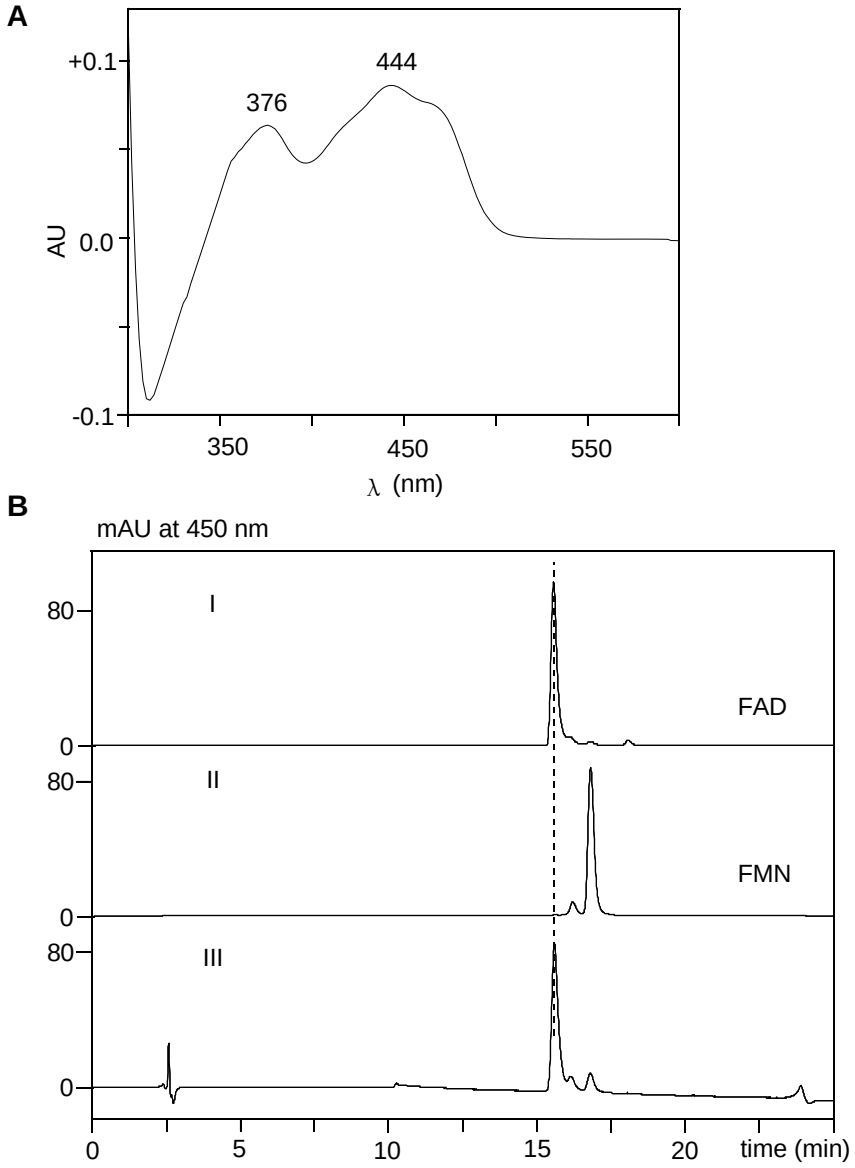


Figure S3. Analysis of FR9H-Ox reactions with 2-FR9I-ACP$_1$. (A) HPLC analysis of **2**-FR9I-ACP$_1$ (I); FR9H-Ox (II); reaction mixtures of FR9H-Ox with **2**-FR9I-ACP$_1$ in the absence of NADPH (III, control), and in the presence of NADPH at different time (IV, 30 min; V, 60 min). (B) Q-TOF-MS data of **2**-FR9I-ACP$_1$ (I) and **2**-FR9I-ACP$_1$-**P** (II). (C) GC-MS data of small molecule compound extracted from the hydrolysis solution of reaction mixtures in the presence of NADPH (II) with authentic standard of 1-hydroxypentan-2-one (compound **5**, I). (D) Proposed reaction catalyzed by FR9H-Ox with model substrate **2**-FR9I-ACP$_1$.

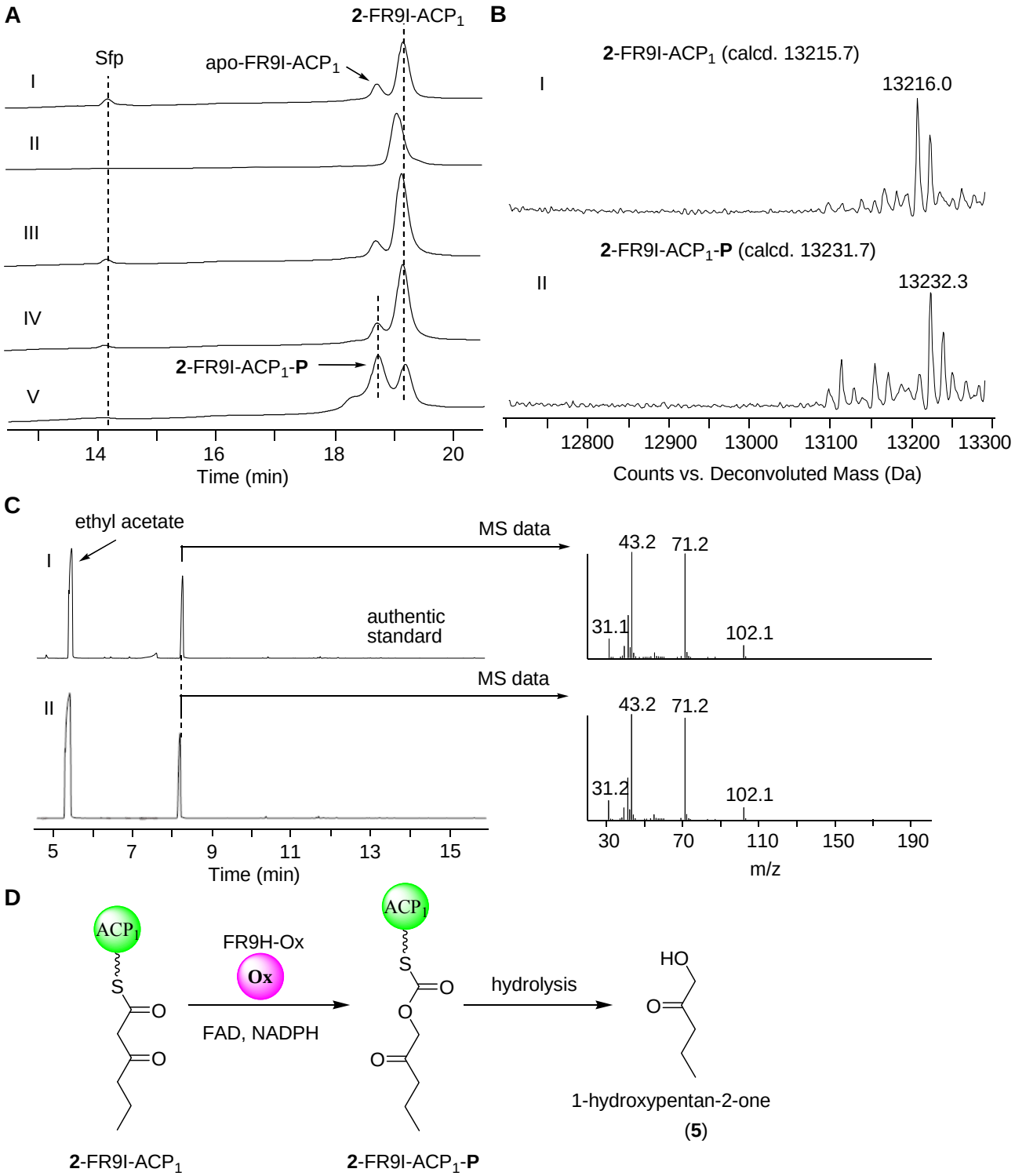


Figure S4. Analysis of FR9H-Ox reactions with 3-FR9I-ACP$_1$. (A) HPLC analysis of **3**-FR9I-ACP$_1$ (I); FR9H-Ox (II); reaction mixtures of FR9H-Ox with **3**-FR9I-ACP$_1$ in the absence of NADPH (III, control), and in the presence of NADPH at different time (IV, 30 min; V, 60 min). (B) Q-TOF-MS data of **3**-FR9I-ACP$_1$ (I) and **3**-FR9I-ACP$_1$-**P** (II). (C) GC-MS data of small molecule compound extracted from the hydrolysis solution of reaction mixtures in the presence of NADPH (II) with authentic standard of pentan-1-ol (I). (D) Proposed reaction catalyzed by FR9H-Ox with model substrate **3**-FR9I-ACP$_1$.

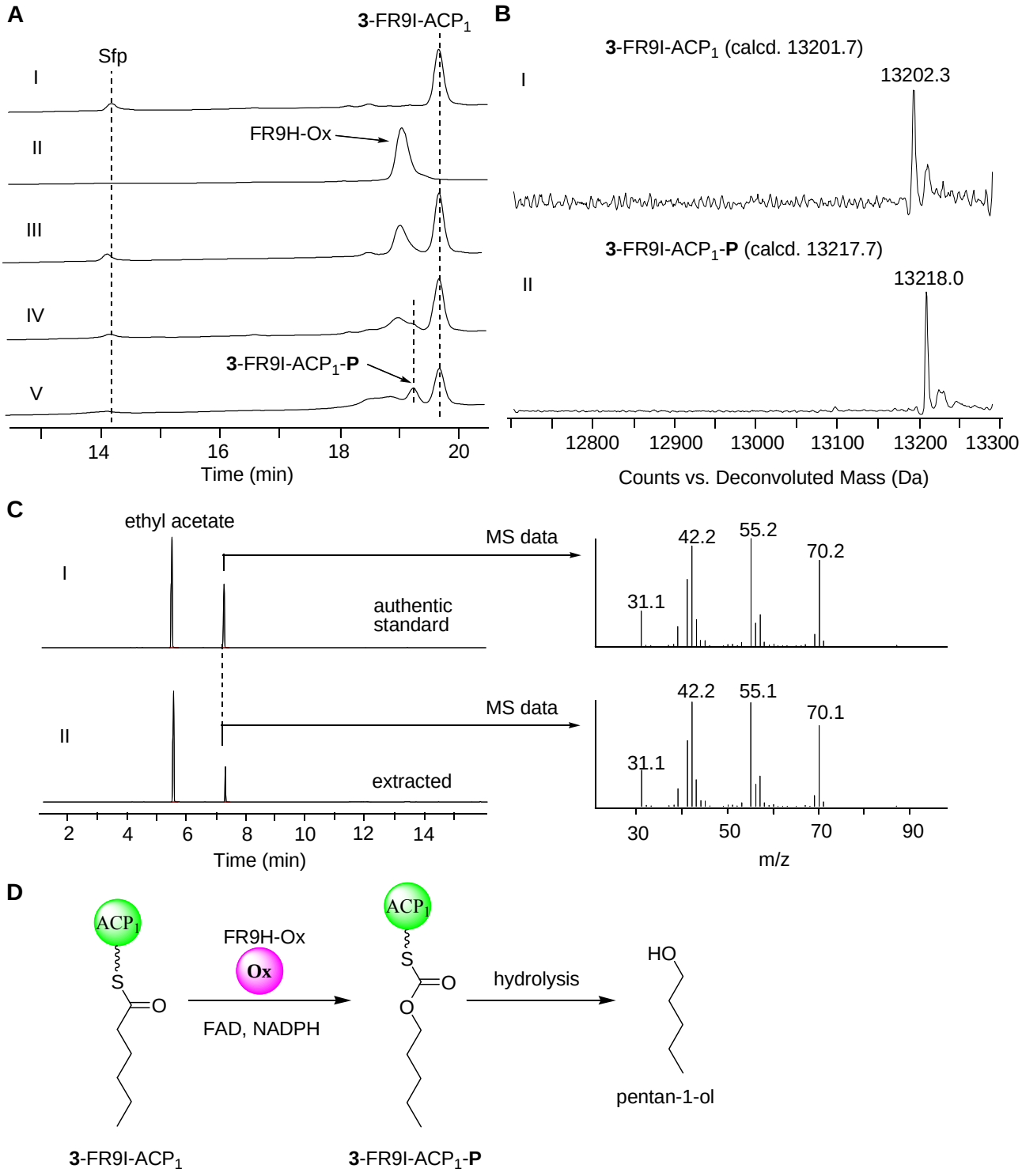


Figure S5. Analysis of FR9H-Ox reactions with 4-FR9I-ACP$_1$. (A) HPLC analysis of **4**-FR9I-ACP$_1$ (I); FR9H-Ox (II); reaction mixtures of FR9H-Ox with **4**-FR9I-ACP$_1$ in the absence of NADPH (III, control), and in the presence of NADPH at different time (IV, 30 min; V, 60 min). (B) Q-TOF-MS data of **4**-FR9I-ACP$_1$ (I) and **4**-FR9I-ACP$_1$-**P** (II, not detected). (C) GC-MS data of small molecule compound extracted from the hydrolysis solution of reaction mixtures in the presence of NADPH (II) with authentic standard of pentanal (I). (D) Proposed reaction catalyzed by FR9H-Ox with model substrate **4**-FR9I-ACP$_1$.

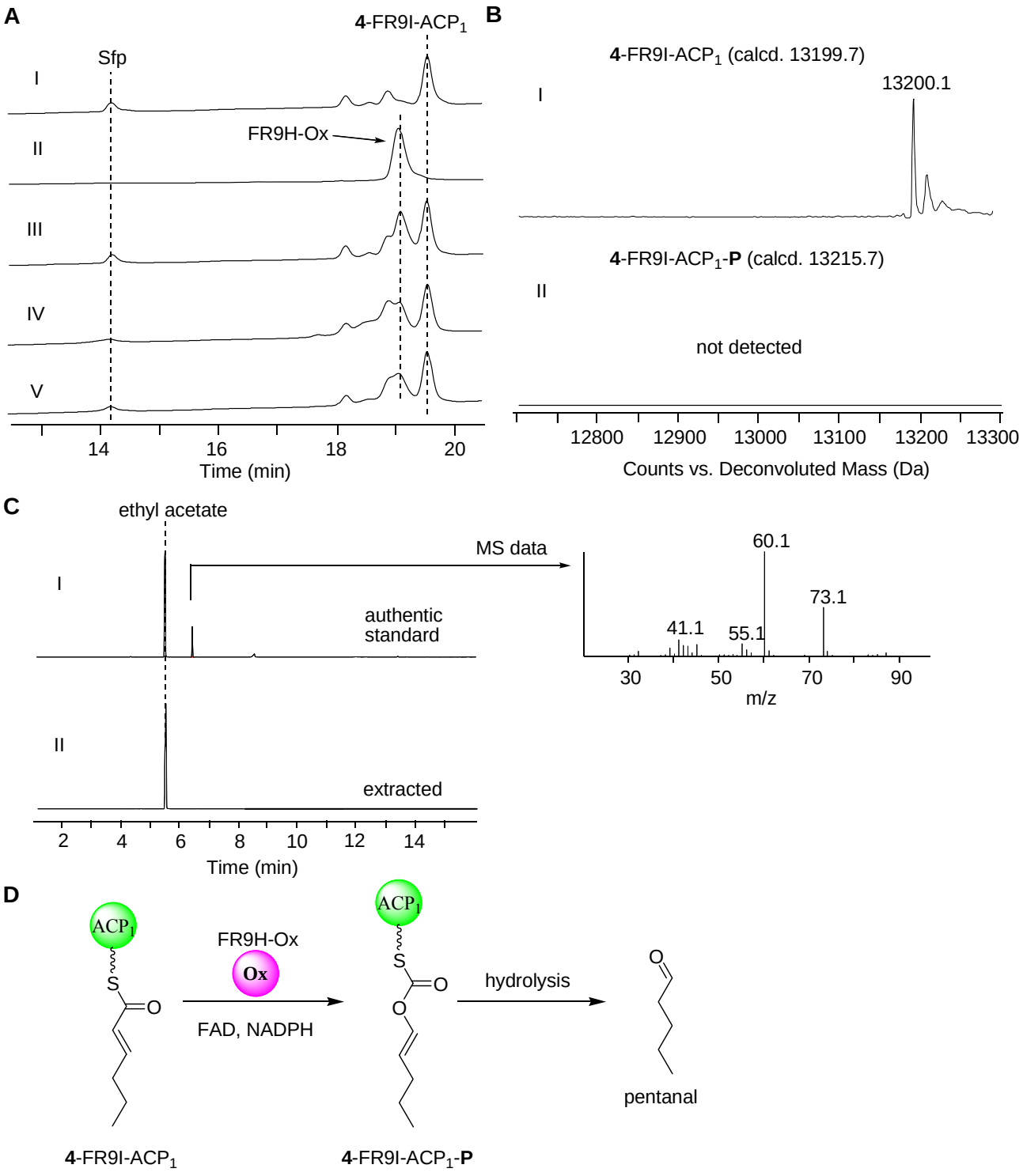


Figure S6. HPLC analysis of FR9H-Ox-catalyzed reactions with different SNAC substrates. (A) **1**-SNAC; (B) **3**-SNAC; (C) **4**-SNAC. (i), reaction mixture; (ii) negative control; (o), substrates. No corresponding BV oxidation products were detected. These results indicated that FR9H-Ox couldn't catalyze the BV oxidation of these SNAC substrates and suggested that the recognition between FR9H-Ox and ACP domain was very important for the catalysis.

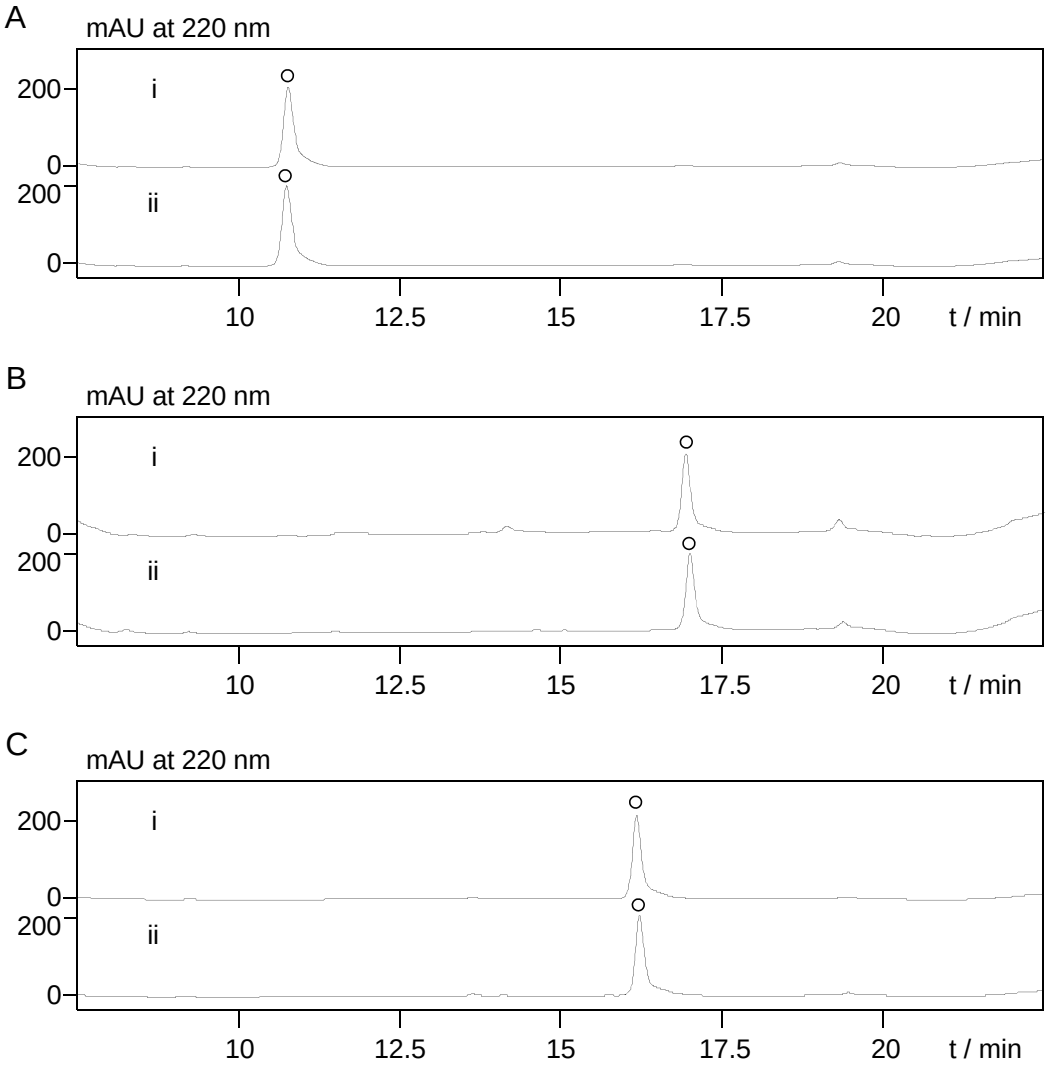
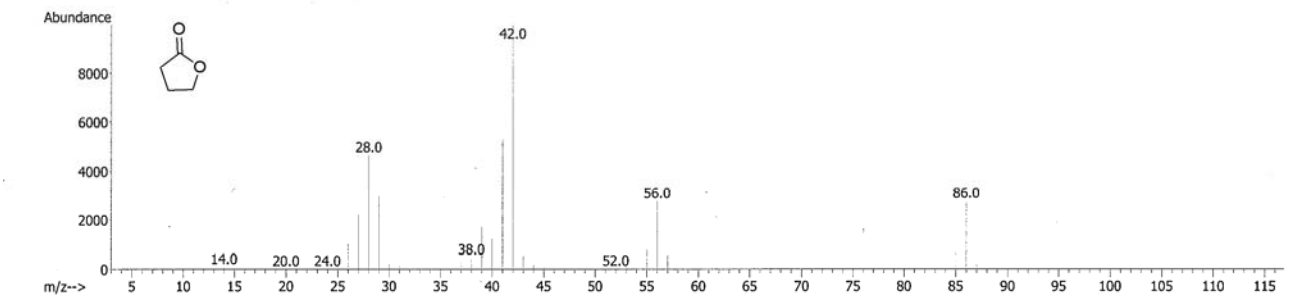


Figure S7. MS data of the products from whole-cell biotransformation assays. (A) MS data of the product from whole-cell biotransformation with cyclobutanone. (B) MS data of the product from whole-cell biotransformation with cyclohexanone.

A



B

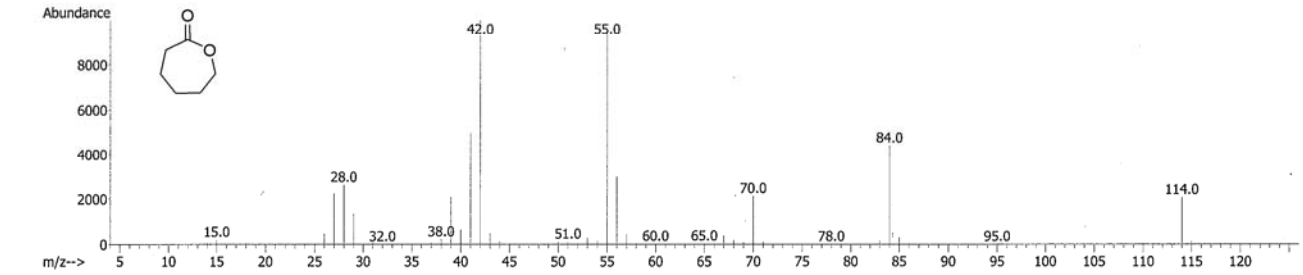


Figure S8. Multiple sequence alignment of FR9H-Ox and its homologs. BVMO_bt, putative Baeyer-Villiger monooxygenase from *Burkholderia thailandensis MSMB43* (GeneBank: ZP_02468377.1); BVMO_EtaA (Rv3854c) from *Mycobacterium tuberculosis* H37Rv genome; BVMO KT2440 from *Pseudomonas putida* KT2440 (NP_744949.1); CHMO_9871, cyclohexanone monooxygenase from *Acinetobacter* sp. strain NCIMB 9871 (BAA86293.1); CHMO_Brev1 and from *Brevibacterium* sp. HCU (AAG01289.1); CHMO_Rhodo1 from *Rhodococcus* sp. Phi1 (AAN37494.1); CPMO, cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (BAC22652.1); PedG from symbiont bacterium of *Paederus fuscipes* (AAS47561.1);

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                                                          ::* *.  *:      *
FR9H-Ox          ---------------AGAPRPAASESQATSIARGSGKRIAVVGAGPAGLVMAKSLLEEG-
BVMO_bt       (65)-HSAASIASPALTASTASAASTASTASTARGLGKRIAVIGAGPAGLVMAKSLLEEG-
PedG             -------------------------------MNQKFRLCIIGGGPLGIGLGRELSEG--
CHMO_9871        ----------------------------MSQKMDFDAIVIGGGFGGLYAVKKLRDEL-
CHMO_Rhodo1      -------------------------MTAQISPTVVDAVVIGAGFGGIYAVHKLHNEQ-
CHMO_Brev1       ----------------------------MPITQQLDHDAIVIGAGFSGLAILHHLR-EI-
CPMO             ---------------MTTMTTMTTEQLGMNNSVNDKLDVLLIGAGFTGLYQLYHLR-KL-
BVMO_EtaA        -------------------------------MTEHLDVVIVGAGISGVSAAWHLQDRCP
BVMO_KT2440      ------------------------MSSHTALPVEPLDVLIMGAGVSGIGAAAYLRRNQP

                       :     .* *              .       :
FR9H-Ox          -HRPDVFERQADLGGVWLLRAENKRAGAYRKTRFQTSKYTSAFSDFDGAPIDAHFHGVAD
BVMO_bt          -HRPDVFERQADLGGVWLLHTENKRAGAYRKTRFQTSKYTSAFSDFDGAPVDGHFHGVAD
PedG             AIDYDLYEAESDLGGVWNREG--KCGRVYPSLHLISPKFNTQVPDYPMPDHYPVYPNHKM
CHMO_9871        ELKVQAFDKATDVAGTWYWNR---YPGALTDTETHLYCYSWDKELLQSLEIKKKYVQGPD
CHMO_Rhodo1      GLTVVGFDKADGPGGTWYWNR---YPGALSDTESHLYRFSFDRDLLQDGTWKTTYITQPE
CHMO_Brev1       GLDTQIVEATDGIGGTWWINR---YPGVRTDSEFHYYSFSFSKEVRDEWTWTQRYPDGEE
CPMO             GYKVHLVDAGADIGGIWHWNC---YPGARVDTHCQIYQYSIP-ELWQEFNWKELFPNWAQ
BVMO_EtaA        TKSYAILEKRESMGGTWDLFR---YPGIRSDSDMYTLGFRFR-----PWTGRQAIADGKP
BVMO_KT2440      NKTFAILESRERMGGTWDLFR---YPGIRSDSDLYTFGFDFK-----PWTKAKSLADAAD

                 :  *.         :          *           * *                 :
FR9H-Ox          IHRYLRDYAERFGVTARIRYRTEVS--RIEPHGDQWHVTTVCD----GASRTDVYDGVAL
BVMO_bt          MHRYLRDYAECFGVTERIRYRTVVR--RVEPHGEQWRVTTERD----GVAHTGIYDGVAL
PedG             MLAYMRSYARDFGVYEHAIFNTSVT--RLEPDGEGWEVELSSG-------ERKRYEVVAV
CHMO_9871        VRKYLQQVAEKHDLKKSYQFNTAVQSAHYNEADALWEVTTEYG-----DKYTARFLITAL
CHMO_Rhodo1      ILEYLESVVDRFDLRRHFRFGTEVTSAIYLEDENLWEVSTDKG-----EVYRAKYVVNAV
CHMO_Brev1       VCAYLNFIADRLDLRKDIQLNSRVNTARWNETEKYWDVIFEDG-----SSKRARFLISAM
CPMO             MREYFHFADKKLDLSKDISFNTRVQSAVFDEGTREWTVRSIGH-----QPIQARFVIANL
BVMO_EtaA        ILEYVKSTAAMYGIDRHIRFHHKVISADWSTAENRWTVHIQS--HGTLSALTCEFLFLCS
BVMO_KT2440      ILEYLSEAIDEHQLAPFIQYQQKVISANWQSDKGLWSVRVEDGRTAQIRTVECRWLFSAG
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                          *       * *   *.   :          *.: ::* * :.   :    :
FR9H-Ox         CQGLYWKPWRPEFAGLDTFRGTILHSAEYVDE-ACLKGKRVLVVGNGISGMDIAEEATQS
BVMO_bt         CQGLYWKPWRPEFAGLDTFRGTILHSAEYVDE-RCLNGKRVLIVGNGISGMDIAEEAARG
PedG            CNGAQRVARFPDPPHPGTFQGKVLHSMDYKSP-DLVRDKRVLVVGAGNSGCDIAVDASHH
CHMO_9871       GLLSAPN--LPNIKGINQFKGELHHTSRWPDD-VSFEGKRVGVIGTGSTGVQVITAVAPL
CHMO_Rhodo1     GLLSAIN--FPDLPGLDTFEGETIHTAAWPEG-KNLAGKRVGVIGTGSTGQQVITALAPE
CHMO_Brev1      GALSQAI--FPAIDGIDEFNGAKYHTAAWPADGVDFTGKKVGVIGVGASGIQIIPELAKL
CPMO            GFGASPS--TPNVDGIETFKGQWYHTALWPQEGVNMAGKRVAIIGTGSSGVQVAQEAALD
BVMO_EtaA       GYYNYDEGYSPRFAGSEDFVGPIIHPQHWPED-LDYDAKNIVVIGSGATAVTLVPALADS
BVMO_KT2440     GYYRYDQGFSPRFEGSEQFKGQIIHPQHWPED-LDYTGKRVVVIGSGATAVTLIPAMADK

                 .   :    *     .
FR9H-Ox         -ASQVFWVMRRPKFIMPRMTGFVPNDFQSPANLLANVDPAQIMERVRHSMPEYFRRYQES
BVMO_bt         -ASQVIWTMRRPKFIMPRMTGFVPNDFQSPASLLANVDPAQMMERVRYSMPEYFRQYQDS
PedG            -AEQVYHSTRRGYHYFPKFIDGKP----------------------------------TP
CHMO_9871       -AKHLTVFQRSAQYSVPIGNDPLS---EEDVKKIKDNYDKIWDGVWNSALAFGLNESTVP
CHMO_Rhodo1     -VEHLTVFVRTPQYSVPVGNRPVT---KEQIDAIKADYDGIWDSVKKSAVAFGFEESTLP
CHMO_Brev1      -AGELFVFQRTPNYVVESNNDKVD---AEWMQYVRDNYDEIFERASKHPFGVDMEYPTDS
CPMO            -AKQVTVYQRTPNLALPMHQKQLS---AEDNLRMKPELPAAFERRGKCFAGFDFDFIAKN
BVMO_EtaA       GAKHVTMLQRSPTYIVSQPDRDGI---AEKLNRWLP--------------------ETM
BVMO_KT2440     -VASITMLQRTPSYIINQPANDGV---AAFLRKVLP--------------------AQT

                        .                                               .
FR9H-Ox         GLLPDADDFRRQPVVQINDGIVALVASGRVEAVIDEIEAFDTHGCRFR-SGRRIEVDAVV
BVMO_bt         GLLPDLDDFRRQPVVQINDGIVSLVASGRVEAVIDEIEAFDAHGCRFRRSGRYVEIDAVV
PedG            QWMLQLGNK----------------------FETKEQTLAYMQQVFKVAGFDGMDYGL
CHMO_9871       AMSVSAEERKAVFEKAWQTGGGFRFMFETFGDIATNMEANIEAQNFIKGKIAEIVKDPAI
CHMO_Rhodo1     AMSVSEEERNRIFQEAWDHGGGFRFMFGTFGDIATDEAANEAAASFIRSKIAEIIEDPET
CHMO_Brev1      AVEVSEEERKRVFESKWEEGG-FHFANECFTDLGTSPEASELASEFIRSKIREVVKDPAT
CPMO            ATELSAAERTEILEELWNAGG-FRYWLANFQDYLFDDKANDYVYEFWRDKVRARIKDPKV
BVMO_EtaA       AYTAVRWKNVLRQAAVYS---------------ACQKWPRRMRKMFLSLIQRQLPEGYDV
BVMO_KT2440     AYSLTRYKNAKITLAFWG---------------FCQRFPKLSKKLLLWLTRKELPKDYPV

                                                                       :
FR9H-Ox         FCTGYQIDERFDYLPGVSMRADFAMGIFHRERPSLVSTSCPLPVAYSGTFFFPEMVARWY
BVMO_bt         FCTGYQIDERLDCLPDVSMRADFAMGIFHRERPSLVSTSCPLPVAYSGTFFFPEMVARWY
PedG            KKPDHPLDGAHPIMNSQILYHIGHGDILPKDN----------------------IEYFE
CHMO_9871       AQKLMPQ----DLYAKRPLCDSGYYNTFNRDNVRLEDVKA-------------NPIVEIT
CHMO_Rhodo1     ARKLMPT----GLYAKRPLCDNGYYEVYNRPNVEAVAIKE-------------NPIREVT
CHMO_Brev1      ADLLCPKSY--SFNGKRVPTGHGYYETFNRTNVHLLDARG-------------TPITRIS
CPMO            AEKLAPMKKPHPYGAKRPSLEQWYYEIFNQNNVTLVDVNE-------------TPVLRIT
BVMO_EtaA       RKHFGPHYN--PWDQRLCLVPNG--DLFRAIRHGKVEVVT-------------DTIERFT
BVMO_KT2440     DVHFNPPYN--PWDQRLCSVPEG--DLFKAISAGNADIVT-------------DHIERFT
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                    :                                                .                    :
FR9H-Ox         ARVMSGRSELAASELDYRLDPRHAAINGPIANVVFGLRLGLLPDPAREFR--AFWQLLNL
BVMO_bt         ARVMSGRADLAGSELDYRLDRRHAAINGPIANVVFGLRLGLLPDPAREFR--EFWRLVNL
PedG            GNTVFFIDGTKADVDLIIYATGYDRDFPFIDHALLEWKDGLPDLFIHIVP--RNLDNIFF
CHMO_9871       ENGVKLENGDFVELDMLICATGFDAVDGNYVRMDIQGKNGLAMKDYWKEG-PSSYMGVTV
CHMO_Rhodo1     AKGVVTEDGVLHELDVLVFATGFDAVDGNYRRIEIRGRNGLHINDHWDGQ-PTSYLGVTT
CHMO_Brev1      SKGIVHGD-TEYELDAIVFATGFDAMTGTLTNIDIVGRDGVILRDKWAQDGLRTNIGLTV
CPMO            EKGIVTAEG-EAEFDLIVFATGFDAVTGGLTSIDFRNNQGQSFKDVWSDG-IRTQLGVAT
BVMO_EtaA       ATGIRLNSGRELPADIIITATGLNLQLFGGATATIDGQQVDITTTMAYKG-------MML
BVMO_KT2440     EHGVLLKSGKMLKADIIVTATGLNVQLFGGITLHKDGKPVVLSETLAYKG-------MML

                          *                                  :
FR9H-Ox         PPFPPIYRLRGEHSDPDAPARLQALNRRNLTSGKADAALDVVRYRLLAGLGDATLRDLLA
BVMO_bt         PPFPPIYRLRGEHADPNAAARLAALNRRNLTSGSAGAALDTVRYRLLAGLGDAALRELLA
PedG            FGFVNAAAGLGDG----------------------LRLQGQFVRSYVRALQQKSKGYFKF
CHMO_9871       NNYPNMFMVLGPNGP-------------FTNLPPSIESQVEWISDTIQYTVENNVESIEA
CHMO_Rhodo1     ANFPNWFMVLGPNGP-------------FTNLPPSIETQVEWISDTVAYAERNEIRAIEP
CHMO_Brev1      NGFPNFLMSLGPQTP-------------YSNLVVPIQLGAQWMQRFLKFIQERGIEVFES
CPMO            AGFPNLLFGYGPQSPA-----------GFCNGPSSAEYQGDLLIQLMNYLRDNNISRIEA
BVMO_EtaA       SGIPNMAYTVGYTN---------------ASWTLKADLVSEFVCRLLNYMDDNGFDTVVV
BVMO_KT2440     SGVPNFAFAVGYTN---------------SSWTLKVCLLCDHFCRLLGLMEREGYNVCEP


FR9H-Ox         RGEIDDNDYRNAGRHAADAIT—LDWRRQYILRDDDVAQARREPAARSERAPHPGSPAWN
BVMO_bt         RGEIDEDDCRHAGRHAADAIT--LDWKRQYILRDDGAEPPGGEPAA--ERAPSPGSPEWD
PedG            IQTKQNDNPD---------------LGQDYFLDSHRHRWEVDFWKFIKCARRYREMLDE
CHMO_9871       TKEAEEQWTQTCANIAEMTLF----PKAQSWIFGANIPGKKNTVYFYLGGLKEYRSALAN
CHMO_Rhodo1     TPEAEEEWTQTCTDIANATLF----TRGDSWIFGANVPGKKPSVLFYLGGLGNYRNVLAG
CHMO_Brev1      SREAEEIWNAETIRGAESTVMSIEGPKAGAWFIGGNIPGKSREYQVYMGGGQVYQDWCRE
CPMO            QSEAQEEWSKLIADFWDSSLF----PRAKSWYQGSNIPGKKVESLNFPLGLPTYISKFNE
BVMO_EtaA       ERPGSDVEERPFMEFTPGYVL-----RSLDELPKQGSRTPWRLNQNYLRDIRLIRRGKID
BVMO_KT2440     KAPEG-VETRPLLDFGAGYVQ-----RALDSMPRQGPREPWVMSMDYFRDVKLLRRGAVT


FR9H-Ox         DYQTLLARLRAGELDMEGVLHALTKQDD
BVMO_bt         DYRTLLARLRAGELDMQGVLHALAKQDD
PedG            I---------------------------
CHMO_9871       CKNHAYEGFDIQLQRSDIKQPANA----
CHMO_Rhodo1     VVADSYRGFELKS-----AVPVTA----
CHMO_Brev1      AEESDYATFLNADSIDGEKVRESAGMK-
CPMO            SAEKGYAGFSLAS---------------
BVMO_EtaA       DEGLRFAKRP-APVGV------------
BVMO_KT2440     DKCLKFTAVPNAPLHADVQLQQQGSRR-
```

3. Supplemental References

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