Supporting Appendix for

Entropy drives selective fluorine recognition in the fluoroacetyl-CoA thioesterase from Streptomyces cattleya

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Materials and Methods

Commercial Materials. Acetyl coenzyme A sodium salt, trifluoroacetic acid (TFA), 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), magnesium chloride hexahydrate, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), streptomycin sulfate, calcium pantothenate, 2,2-dimethoxypropane, anhydrous N, N-dimethylformamide (DMF), ethyl chloroformate, ethanolamine, diisopropylethylamine, palladium on carbon (10 wt% loading), acetic anhydride, iodine, sodium thiosulfate, anhydrous magnesium sulfate, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride, 2,2-dimethyl-1,3-dioxane-4,6-dione, 4-dimethylaminopyridine, sodium fluoroacetate, Selectfluor, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Monosodium phosphate monohydrate, disodium phosphate heptahydrate, hexanes, ethyl acetate, methanol, anhydrous methanol, acetonitrile, toluene, triethylamine, adenosine-5’-triphosphate trisodium salt, isopropyl-β-D-thiogalactopyranoside (IPTG), sodium chloride, anhydrous sodium acetate, sodium bicarbonate, potassium carbonate, imidazole, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Fisher Scientific (Pittsburg, PA). 2-Fluoropropionic acid was purchased from Oakwood Products (West Columbia, SC). Benzyl-N-(2-aminoethyl) carbamate hydrochloride and t-butyI-γ-aminobutanoate hydrochloride were purchased from Bachem (Torrance, CA). 1-Hydroxybenzotriazole hydrate and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) were purchased from Advanced ChemTech (Louisville, KY). Hydrochloric acid, potassium chloride, and KOD DNA polymerase were purchased from EMD (Darmstadt, Germany). Platinum Taq HF was purchased from Invitrogen (Carlsbad, CA). Complete Mini EDTA-free protease inhibitor was purchased from Roche Applied Science (Penzberg, Germany). [1-13C]-glucose and L-phenylalanine (ring, 13C6) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA).

Expression and purification of FIK and variants. Wt and F36A FIK were expressed and purified as described previously (1).

Synthesis of substrates. Fluoroacetyl-CoA was synthesized as described previously (1).

Pre-steady state kinetic analysis of wild-type and F36A FIK. Pre-steady-state kinetic experiments were performed using rapid chemical quench followed by HPLC separation of coenzyme A from unhydrolyzed acyl-CoA. FIK (50 µM in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl) was mixed with substrate (750 µM for fluoroacetyl-CoA or 6 mM for acetyl-CoA, in water) using a Chemical Quench Flow Model RQF-3 (KinTek). The reaction was stopped at various times by mixing with 50% TFA to achieve a final concentration of 17% TFA. Quenched samples were analyzed by HPLC on an Agilent Eclipse XDB-C18 (3.5 µm, 3.0 × 150 mm) using a linear gradient from 0 to 100% acetonitrile over 15 min (0.6 mL/min) with 50 mM sodium phosphate, pH 4.5 containing 0.1% TFA as the aqueous mobile phase. Conversion percentages were calculated based
on the peak areas for substrate and product based on the CoA absorbance at 260 nm. Plots of coenzyme A released versus time were fit to the equation $[\text{CoA}] = A^* (1 - \exp(-k^2_*t)) + V^*t$, where $A$ is the burst amplitude, $k_2$ is the burst-phase rate constant, and $V$ is the steady-state rate. In cases where data could not be fit with a burst phase, they were fit as pseudo first-order reactions according to the equation $[\text{CoA}] = k\text{[FIK]}t + [\text{CoA}]_0$, where the y-intercept, $[\text{CoA}]_0$, is the concentration of CoA released before the first data point, $k$ is the rate constant, and $t$ is time.

**Construction of vectors for protein expression.** The genes encoding pantothenate kinase (coaA), phosphopantheine adenylyltransferase (coaD), and dephospho-CoA kinase (coaE) were amplified from a saturated overnight culture of *E. coli* BL21(de3)-T1R using primers coaA F1 and R1, coaD F1 and R1, or coaE F1 and R1 below using either Platinum Taq HF (coaA and coaD) or KOD polymerase (coaE) according to manufacturer protocol. The PCR products were digested with SfoI and XhoI and ligated into the SfoI-XhoI sites of a modified pET23a vector containing an N-terminal His$^{10}$ tag and a Tobacco Etch Virus (TEV) protease cleavage site. Wild-type and F36A FIK plasmids were constructed as previously described (1). The expression plasmid for the F40L FIK mutant was constructed using the QuikChange protocol on pET23a-His-TEV-FIK plasmid with primers FIK F40L F1 and R1. The resulting plasmids were verified by Sanger sequencing (Quintara Biosciences).

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**Expression and purification of CoA biosynthetic enzymes.** LB containing carbenicillin (50 µg/mL) was inoculated to OD$_{600}$ nm = 0.05 using an overnight culture of freshly transformed *E. coli* BL21(de3)-T1R containing the appropriate overexpression plasmid. The cultures were grown at 37°C at 200 rpm to OD$_{600}$ nm = 0.6. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and cells were incubated at 30 °C at 200 rpm for 4 h. Cells were harvested by centrifugation at 2,060 × g at 4°C for 15 min. The
cell pellet was resuspended in 10 mL/L cell culture of lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 100 µM phenylmethanesulfonyl fluoride) with one Complete Mini EDTA-free protease inhibitor tablet per 50 mL buffer. Cells were lysed by passing through a French pressure cell at 14,000 psi. Insoluble material was removed by centrifugation at 18,500 × g at 4°C for 20 min. Streptomycin sulfate (10% w/v solution) was added to the supernatant to a final concentration of 1% and precipitated DNA was removed by centrifugation at 18,500 × g at 4°C for 20 min. The supernatant was incubated for 1 h at 4°C on a nutating mixer with Ni-NTA resin (1 mL/L of cell culture) that had been equilibrated with wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). The resin was then pelleted by centrifugation at 500 × g for 3 min and the supernatant was carefully decanted. The resin was resuspended in wash buffer, packed into a column, and washed wash buffer (10 column vol) followed by wash buffer containing 8% elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole; 10 column vol). Proteins were then eluted with elution buffer (10 column vol) in 5 mL fractions. Protein containing fractions were pooled, dialyzed into storage buffer (50 mM HEPES, pH 8.0, 250 mM NaCl, 2 mM MgCl2), and stored at -80°C until further use. Final protein concentrations before storage were estimated using the ε280 nm calculated by ExPASY ProtParam as follows: His10-PanK: 16 mg/mL (ε280 nm = 45,380 M⁻¹cm⁻¹), His10-PPAT: 10 mg/mL (ε280 nm = 8,480 M⁻¹cm⁻¹); His10-DPCK: 6 mg/mL (ε280 nm = 16,960 M⁻¹cm⁻¹)

**Synthesis of substrate analogs.** Dichloromethane and tetrahydrofuran used in chemical synthesis were dried using a VAC Solvent Purifier System (Vacuum Atmospheres Company, Amesbury, MA). HPLC purifications were performed using Agilent Eclipse XDB-C18 or XDBC8 columns (9.4 × 250 mm, 5 µm) connected to an Agilent 1200 binary pump and Agilent G1315D diode-array detector. Fractions were assayed for the desired compounds by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1290 binary pump coupled to an Agilent 6130 single-quadrupole electrospray ionization mass spectrometer. Fractions containing the desired compounds were pooled, organic solvents were removed on a rotary evaporator, and the pooled fractions were lyophilized. Substrate analogs were dissolved in water, quantified by absorbance at 260 nm (ε280 nm = 13,100 M⁻¹cm⁻¹), and stored at -80°C until further use. High resolution mass spectra were collected at the QB3/Chemistry Mass Spectrometry Facility at the University of California, Berkeley. NMR spectra were recorded at the College of Chemistry NMR Facility at the University of California, Berkeley. One-dimensional spectra were collected on Bruker AV-600 or AVQ-400 NMR spectrometers at 298 K. Chemical shifts are expressed in parts per million (δ, ppm) downfield from tetramethylsilane (¹H and ¹³C) or trichlorofluoromethane (¹⁹F) and are referenced to the solvent signal.
**O,O’-isopropylidene-D-pantothenic acid (2)**

Calcium pantothenate (2 g, 4.2 mmol) and p-toluenesulfonic acid (1.6 g, 4.2 mmol) were suspended in DMF (40 mL). 2,2-Dimethoxypropane (62 mL) was added and the reaction was stirred at room temperature for 12 h. Toluene was added to form an azeotrope with DMF and the solvent was removed under vacuum on a rotary evaporator. The resultant tan residue was suspended in dichloromethane (40 mL) and precipitate was removed by vacuum filtration. The filtrate was evaporated to dryness to yield \( \text{O,O’-isopropylidene-D-pantothenic acid} \) as a tan solid (2.17 g, 98%).

**1H NMR** (400 MHz, CDCl3, 25°C) \( \delta \) (ppm): 4.05 (s, 1H, H3´´), 3.62 (d, \( J = 11.6 \) Hz, 1H, H1´´), 3.45 (m, 2H, H5´´), 3.21 (d, \( J = 11.6 \) Hz, 1H, H1´´), 2.50 (t, \( J = 6 \) Hz, 2H, H6´´), 1.39, 1.36 (each s, 3H, OC(CH3)2), 0.96, 0.90 (each s, 3H, H10´´ and H11´´); **13C NMR** (100.6 MHz, CDCl3, 25°C) \( \delta \) (ppm): 174.9, 170.2 (C4´´ and C7´´), 99.1 (OC(CH3)2), 77.1 (C3´´), 71.4 (C1´´), 34.3 (C5´´), 33.0 (C4´´), 29.4 (C2´´), 22.1 (OC(CH3)2), 18.9, 18.7 (C10´´ and C11´´).

**HR-ESI-MS** calcd (M-H+) m/z 258.1347, found (M-H+) m/z 258.1344.

**O,O’-isopropylidene-oxa(dethia)-pantetheine (3)**

\( \text{O,O’-isopropylidene-D-pantothenic acid} \) (836 mg, 3.2 mmol) and triethylamine (0.56 mL, 4 mmol) were dissolved in dry dichloromethane (10 mL). The reaction mixture was cooled to 0°C and ethyl chloroformate (0.32 mL, 3.36 mmol) was added dropwise by syringe. After stirring for 30 min, ethanolamine (0.28 mL, 4.64 mmol) was added. The reaction mixture was stirred for 30 min at 0°C and for 2 h at room temperature. The solvent was removed under vacuum and the resultant residue was suspended in ethyl acetate and purified by silica chromatography using a stepwise gradient from ethyl acetate to 60 methanol: 40 ethyl acetate to yield \( \text{O,O’-isopropylidene-oxa(dethia)-pantetheine} \) as a white solid (540 mg, 55%).

**1H NMR** (600 MHz, MeOD, 25°C) \( \delta \) (ppm): 4.05 (s, 1H, H3´´), 3.67 (d, \( J = 12 \) Hz, 1H, H1´´), 3.52 (t, \( J = 6 \) Hz, 2H, H9´´), 3.45 (m, 2H, H5´´), 3.42 (m, 2H, H8´´), 3.19 (d, \( J = 12 \) Hz, 1H, H1´´), 2.37 (t, \( J = 6.6 \) Hz, 2H, H6´´), 1.38 (s, 3H, OC(CH3)2), 1.37 (s, 3H, OC(CH3)2), 0.92, 0.90 (each s, 3H, H10´´ and H11´´); **13C NMR** (150.9 MHz, MeOD, 25°C) \( \delta \) (ppm): 174.2, 174.1 (C4´´ and C7´´), 100.5 (OC(CH3)2), 78.5 (C3´´), 72.4 (C1´´), 61.7 (C9´´), 43.18 (C8´´), 36.5 (C5´´), 34.1 (C4´´), 29.9 (C2´´), 22.6 (OC(CH3)2), 19.5, 19.2 (C10´´ and C11´´).

**HR-ESI-MS** calcd (M+H+) m/z 303.1914, found (M+H+) m/z 303.1915.

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Crosspeak numbering for HMBC spectra

| 1  | H4-C2’ | 10 | H16-C11’ | 19 | H9-C8’ | 28 | H2-C4’ |
| 2  | H3-C2’ | 11 | H15-C12’ | 20 | H10-C8’ | 29 | H2-C4’ |
| 3  | H2-C11’ | 12 | H16-C11’ | 21 | H17-C7’ | 30 | H2-C4’ |
| 4  | H13-C10’ | 13 | H17-C7’ | 22 | H10-C8’ | 31 | H2-C4’ |
| 5  | H2-C11’ | 14 | H15-C10’ | 23 | H16-C10’ | 32 | H2-C4’ |
| 6  | H2-C10’ | 15 | H15-C10’ | 24 | H14-C9’ | 33 | H2-C4’ |
| 7  | H2-C1’ | 16 | H14-C9’ | 25 | H13-C8’ | 34 | H2-C4’ |
| 8  | H3-C1’ | 17 | H13-C8’ | 26 | H12-C7’ | 35 | H2-C4’ |
| 9  | H2-C4’ | 18 | H12-C7’ | 27 | H11-C6’ | 36 | H2-C4’ |
**O,O'-isopropylidene-acetyl-oxa(dethia)-pantetheine (4a)** (3). Sodium acetate (45 mg, 0.55 mmol) and HATU (228 mg, 0.60 mmol) were dissolved in dry THF (5 mL). Diisopropylethylamine (0.26 mL, 1.5 mmol) was added, followed by **O,O'-isopropylidene-oxa(dethia)-pantetheine** 3 (150 mg, 0.5 mmol). The reaction was stirred for 16 h under N₂ at room temperature. Solvent was removed under vacuum and the residue was purified on an Agilent Eclipse XDB-C18 column using a linear gradient from 0 to 100% acetonitrile over 30 min (3 mL/min) with 0.1% (v/v) formic acid as the aqueous mobile phase. Fractions were screened for the desired compound by LC-MS, pooled, and lyophilized to yield **O,O'-isopropylidene-acetyl-oxa(dethia)-pantetheine** as a clear oil (49 mg, 28%).

\[\delta (ppm): 4.09 (s, 1H, H₃⁻), 4.01 (t, J = 5.4 Hz, 2H, H₉⁻), 3.70 (d, J = 12 Hz, 1H, H₁⁻), 3.31 (m, 4H, H₅⁻ and H₈⁻), 3.20 (d, J = 12 Hz, 1H, H₁⁻), 2.33 (t, J = 6.6 Hz, 2H, H₆⁻), 1.94 (s, 3H, COCH₃), 1.329 (s, 3H, OC(CH₃)₂), 1.326 (s, 3H, OC(CH₃)₂), 0.79 (each s, 3H, H₁₀⁻ and H₁₁⁻); \]

\[\delta (ppm): 175.1, 174.1 (C₄⁻ and C₇⁻), 171.7 (COCH₃), 99.8 (OC(CH₃)₂), 77.0 (C₃⁻), 70.6 (C₁⁻), 38.5 (C₅⁻), 38.2 (C₈⁻), 35.4 (C₅⁻), 32.3 (C₄⁻), 30.2 (COCH₃), 28.3 (C₂⁻), 20.9, 20.4 (OC(CH₃)₂), 19.0, 18.2 (C₁₀⁻ and C₁₁⁻). \]

**O,O'-isopropylidene-fluoroacetyl-oxa(dethia)-pantetheine (4b)** (3, 4). Sodium fluoroacetate (55 mg, 0.55 mmol) and HATU (228 mg, 0.60 mmol) were dissolved in dry THF (5 mL). Diisopropylethylamine (0.26 mL, 1.5 mmol) was added, followed by **O,O'-isopropylidene-oxa(dethia)-pantetheine** 3 (150 mg, 0.5 mmol). The reaction mixture was stirred for 16 h under N₂ at room temperature. Solvent was removed under vacuum and the residue was purified on an Agilent Eclipse XDB-C18 column using a linear gradient from 0 to 100% acetonitrile over 30 min (3 mL/min) with 0.1% (v/v) formic acid as the aqueous mobile phase. Fractions were screened for the desired compound by LC-MS, pooled, and lyophilized to yield **O,O'-isopropylidene-fluoroacetyl-oxa(dethia)-pantetheine** as a clear oil (38 mg, 21%).

\[\delta (ppm): 4.99 (d, J = 46.2 Hz, 2H, COCH₂F), 4.29 (t, J = 6 Hz, 2H, H₉⁻), 4.23 (s, 1H, H₁⁻), 3.83 (d, J = 12 Hz, 1H, H₁⁻), 3.48 (m, 4H, H₅⁻ and H₈⁻), 3.36 (d, J = 12 Hz, 1H, H₁⁻), 2.46 (t, J = 6 Hz, 2H, H₆⁻), 1.463 (s, 3H, OC(CH₃)₂), 1.460 (s, 3H, OC(CH₃)₂), 0.94, 0.93 (each s, 3H, H₁₀⁻ and H₁₁⁻); \]

\[\delta (ppm): 174.1, 171.7 (C₄⁻ and C₇⁻), 170.4 (COCH₂F), 99.8 (OC(CH₃)₂), 77.0 (C₃⁻), 70.6 (C₁⁻), 38.5 (C₅⁻), 38.2 (C₈⁻), 35.4 (C₅⁻), 32.3 (C₄⁻), 30.2 (COCH₃), 28.3 (C₂⁻), 20.9, 20.4 (OC(CH₃)₂), 19.0, 18.2 (C₁₀⁻ and C₁₁⁻). \]

**O,O'-isopropylidene-N-Cbz-aza(dethia)-pantetheine (6)**. **O,O'-isopropylidene-N-(2-aminoethyl)carbamate hydrochloride** (822 mg, 4 mmol) was added. The reaction mixture was cooled to 0°C and ethyl chloroformate (0.32 mL, 3.36 mmol) was added dropwise by syringe. After stirring the reaction mixture on ice for 30 min, benzyl-N-(2-aminoethyl)carbamate hydrochloride (822 mg, 4 mmol) was added. The reaction mixture was stirred at 0°C for 30 min and at room temperature for 2 h. The solvent was removed on a rotary evaporator and the residue was purified by silica chromatography using 75 ethyl
acetate: 25 methanol as the mobile phase. The resultant oil was further purified by silica chromatography using a stepwise gradient from ethyl acetate to 10 methanol: 90 ethyl acetate to yield the desired compound as a white solid (600 mg, 43%). $^1$H NMR (600 MHz, MeOD, 25°C) δ (ppm): 7.32 (m, 5H, C$_6$H$_5$), 5.12 (s, 2H, OCH$_2$Ph), 4.11 (s, 1H, H$_{3'}$), 3.74 (d, J = 12.6 Hz, 1H, H$_{1'}$), 3.41 (m, 2H, H$_{5'}$), 3.26 (m, 2H, H$_{8'}$), 3.23 (t, J = 6 Hz, 2H, H$_{9'}$), 2.38 (t, J = 6.6 Hz, 2H, H$_6'$), 1.54 (s, 3H, OC(CH$_3$)$_2$), 1.52 (s, 3H, OC(CH$_3$)$_2$), 0.98, 0.96 (each s, 3H, H$_{10'}$ and H$_{11'}$); $^{13}$C NMR (150.9 MHz, MeOD, 25°C) δ (ppm): 174.3, 172.4 (C$_{4'}$ and C$_{7'}$), 138.5, 129.6, 129.1, 129.03 (C$_6$H$_5$), 100.5 (OC(CH$_3$)$_2$), 78.5 (C$_3'$), 72.4 (C$_{1'}$), 67.6 (OCH$_2$Ph), 40.5 (C$_{9'}$), 36.5 (C$_{8'}$), 34.1 (C$_{5'}$), 30.8 (C$_{4'}$), 29.9 (C$_{2'}$), 19.5, 19.2 (C$_{10'}$ and C$_{11'}$). HR-ESI-MS calcd (M+H$^+$) m/z 436.2442, found (M+H$^+$) m/z 436.2441.

$O,O'$-isopropylidene-aza(dethia)-pantetheine (7). $O,O'$-isopropylidene-$N$-Cbz-aza(dethia)-pantetheine 6 (446 mg, 1 mmol) was dissolved in methanol (10 mL) in the presence of palladium (10 wt% on activated carbon, 50 mg). The flask was purged with N$_2$ and the reaction mixture was saturated with H$_2$ and stirred under H$_2$ atmosphere for 10 min. The reaction mixture was then filtered through a bed of Celite, which was washed extensively with methanol. Removal of the solvent in vacuo yielded $O,O'$-isopropylidene-aza(dethia)-pantetheine as a clear oil (260 mg, 86%). $^1$H NMR (600 MHz, MeOD, 25°C) δ (ppm): 4.13 (s, 1H, H$_{3'}$), 3.74 (d, J = 12 Hz, 1H, H$_{1'}$), 3.46 (m, 2H, H$_{5'}$), 3.44 (m, 2H, H$_{8'}$), 3.20 (d, J = 12 Hz, 1H, H$_{1'}$), 2.73 (t, J = 6.6 Hz, 2H, H$_{9'}$), 2.45 (t, J = 6.6 Hz, 2H, H$_{6'}$), 1.45 (s, 3H, OC(CH$_3$)$_2$), 1.44 (s, 3H, OC(CH$_3$)$_2$), 0.99, 0.97 (each s, 3H, H$_{10'}$ and H$_{11'}$); $^{13}$C NMR (150.9 MHz, MeOD, 25°C) δ (ppm): 174.3, 172.3 (C$_{4'}$ and C$_{7'}$), 99.8 (OC(CH$_3$)$_2$), 77.0 (C$_{3'}$), 70.6 (C$_{1'}$), 38.7 (C$_{9'}$), 38.6 (C$_{8'}$), 35.3 (C$_{5'}$), 32.3 (C$_{4'}$), 28.3 (C$_{2'}$), 18.2, 18.1 (C$_{10'}$ and C$_{11'}$). HR-ESI-MS calcd (M+H$^+$) m/z 302.2074, found (M+H$^+$) m/z 302.2074.

$O,O'$-isopropylidene-acetyl-aza(dethia)-pantetheine (8a). Sodium acetate (47 mg, 0.57 mmol) and HATU (238 mg, 0.63 mmol) were dissolved in dry DMF (6 mL). Diisopropylethylamine (0.3 mL, 1.82 mmol) was added, followed by $O,O'$-isopropylidene-aza(dethia)-pantetheine 7 (155 mg, 0.51 mmol). The reaction mixture was stirred for 16 h under N$_2$ at room temperature. Solvent was removed under vacuum and the residue was purified directly on an Agilent Eclipse XDB-C18 column using a linear gradient from 0 to 100% acetonitrile over 30 min (3 mL/min) with 0.1% formic acid as the aqueous mobile phase. Fractions were screened for the desired compound by LC-MS, pooled, and lyophilized to yield $O,O'$-isopropylidene-acetyl-aza(dethia)-pantetheine as a clear oil (45 mg, 24%). $^1$H NMR (600 MHz, D$_2$O, 25°C) δ (ppm): 4.23 (s, 1H, H$_{3'}$), 3.83 (d, J = 12 Hz, 1H, H$_{1'}$), 3.47 (m, 2H, H$_{5'}$), 3.44 (m, 2H, H$_{8'}$), 3.36 (d, J = 11 Hz, 1H, H$_{1'}$), 3.27 (t, J = 6 Hz, 2H, H$_{9'}$), 2.45 (t, J = 6.6 Hz, 2H, H$_{6'}$), 1.96 (s, 3H, COCH$_3$), 1.462 (s, 3H, OC(CH$_3$)$_2$), 1.459 (s, 3H, OC(CH$_3$)$_2$), 0.94, 0.93 (each s, 3H, H$_{10'}$ and H$_{11'}$); $^{13}$C NMR (150.9 MHz, D$_2$O, 25°C) δ (ppm): 174.3, 174.1 (C$_{4'}$ and C$_{7'}$), 171.7 (COCH$_3$), 99.8 (OC(CH$_3$)$_2$), 77.0 (C$_{3'}$), 70.6 (C$_{1'}$), 38.7 (C$_{9'}$), 38.6 (C$_{8'}$), 35.3 (C$_{5'}$), 32.3 (C$_{4'}$), 28.3 (C$_{2'}$), 21.9, 20.9 (OC(CH$_3$)$_2$), 18.2, 18.1 (C$_{10'}$ and C$_{11'}$). HR-ESI-MS calcd (M+H$^+$) m/z 344.2180, found (M+H$^+$) m/z 344.2179.

S7
O,O'-isopropylidene-fluoroacetyl-aza(dethia)-pantetheine (8b). Sodium fluoroacetate (36 mg, 0.36 mmol) and HATU (152 mg, 0.40 mmol) were dissolved in dry DMF (4 mL). Diisopropylethylamine (0.2 mL, 1.16 mmol) was added, followed by O,O'-isopropylidene-aza(dethia)-pantetheine 7 (100 mg, 0.33 mmol). The reaction mixture was stirred for 12 h at room temperature under N₂ atmosphere. Toluene was added to form an azeotrope with DMF and solvents were removed on a rotary evaporator. The residue was dissolved in water and purified on an Agilent Eclipse XDB-C8 column using a linear gradient from 0 to 100% methanol over 30 min (3 mL/min) with 0.1% formic acid as the aqueous mobile phase. Fractions were screened for the desired compound by LC-MS, pooled, and lyophilized to yield O,O'-isopropylidene-fluoroacetyl-aza(dethia)-pantetheine as a clear oil (16.4 mg, 14%). ¹H NMR (600 MHz, D₂O, 25°C) δ (ppm): 4.99 (d, J = 46.2 Hz, 2H, COCH₂F), 4.29 (t, J = 6 Hz, 2H, H₉⁻⁻), 4.23 (s, 1H, H₃⁻⁻), 3.83 (d, J = 12 Hz, 1H, H₁⁻⁻), 3.48 (m, 4H, H₅⁻⁻ and H₈⁻⁻), 3.36 (d, J = 12 Hz, 1H, H₁⁻⁻), 2.46 (t, J = 6.6 Hz, 2H, H₆⁻⁻), 1.463 (s, 3H, OC(CH₃)₂), 1.460 (s, 3H, OC(CH₃)₂), 0.94, 0.93 (each s, 3H, H₁₀⁻⁻ and H₁₁⁻⁻); ¹³C NMR (150.9 MHz, D₂O, 25°C) δ (ppm): 174.1, 171.7 (C₄⁻⁻ and C₇⁻⁻), 170.4, 170.3 (COCH₂F), 99.8 (OC(CH₃)₂), 78.5, 77.4 (COCH₂F), 77.0 (C₃⁻⁻), 70.6 (C₁⁻⁻), 64.2 (C₀⁻⁻), 38.0 (C₀⁻⁻), 35.2 (C₈⁻⁻), 32.3 (C₄⁻⁻), 28.3 (C₂⁻⁻), 20.9 (OC(CH₃)₂), 18.2, 18.1 (C₁₀⁻⁻ and C₁₁⁻⁻). HR-ESI-MS calcd (M+H⁺) m/z 363.1926, found (M+H⁺) m/z 363.1926.

O,O'-diacetyl-D-pantothenic acid (10) (3). Calcium pantothenate (2 g) and I₂ (140 mg) were dissolved in acetic anhydride (40 mL). The reaction mixture was stirred at 0°C for 2 h and at room temperature for 16 h. Toluene was added to the reaction mixture to form an azeotrope with acetic anhydride and the solvents were removed on a rotary evaporator. The resultant red oil was dissolved in dichloromethane (60 mL) and washed with 1 M sodium thiosulfate (60 mL). The organic layer was dried over MgSO₄ and the solvent was removed under vacuum to yield O,O'-diacetyl-D-pantothenic acid as a clear oil in quantitative yield. ¹H NMR (600 MHz, CDC₁₃, 25°C) δ (ppm): 4.88 (s, 1H, H₃⁻⁻), 4.01 (d, J = 11 Hz, 1H, H₁⁻⁻), 3.80 (d, J = 11 Hz, 1H, H₁⁻⁻), 3.55 (m, 2H, H₅⁻⁻), 2.55 (t, J = 6.6 Hz, 2H, H₆⁻⁻), 2.12 (s, 3H, COCH₃), 2.01 (s, 3H, COCH₃), 1.04, 0.98 (each s, 3H, H₁₀⁻⁻ and H₁₁⁻⁻); ¹³C NMR (150.9 MHz, CDC₁₃, 25°C) δ (ppm): 175.7 (COOH), 171.4, 170.2 (C₄⁻⁻ and C₇⁻⁻), 168.5 (COCH₂F), 77.0 (C₃⁻⁻), 69.5 (C₁⁻⁻), 37.3 (C₅⁻⁻), 34.8 (C₄⁻⁻), 29.7 (C₂⁻⁻), 21.5, 21.0 (COCH₃), 20.9, 20.7 (C₁₀⁻⁻ and C₁₁⁻⁻). HR-ESI-MS calcd (M+H⁺) m/z 304.1396, found (M+H⁺) m/z 304.1391.

N-(O,O'-diacetyl-D-pantothenoyl)-g-aminobutyric acid (11) (3). O,O'-diacetyl-D-pantothenic acid 10 (480 mg, 1.6 mmol), t-butyl-γ-aminobutanoate hydrochloride (353 mg, 1.8 mmol), 1-hydroxybenzotriazole hydrate (243 mg, 1.8 mmol), and triethylamine (0.45 mL, 3.3 mmol) were dissolved in dry dichloromethane (10 mL) under N₂. The reaction mixture was cooled to 0°C and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (353 mg, 1.8 mmol) was added, and the mixture was stirred at 0°C for 30 min and at room temperature for 16 h. The reaction mixture was then diluted with dichloromethane, washed with 0.1 M HCl (20 mL) and saturated sodium bicarbonate (20 mL), and the organic layer was dried over MgSO₄. Evaporation of the
solvent yielded \(N-(O,O'-\text{diacetyl-d-pantothenoyl})-\text{g-aminobutyric acid \(t\)-butyl ester 12}\) as a yellow oil (650 mg). This material was dissolved in dry dichloromethane and TFA and the reaction mixture was stirred at room temperature for 3 h. The solvent was removed by forming an azeotrope with toluene and evaporating under vacuum followed by lyophilization. \(N-(O,O'-\text{diacetyl-d-pantothenoyl})-\text{g-aminobutyric acid was obtained as a yellow oil (620 mg, 85%). 1H NMR (600 MHz, CDCl\textsubscript{3}, 25°C) \(\delta\) (ppm): 4.91 (s, 1H, H\textsubscript{3}´´), 4.01 (d, \(J = 11\) Hz, 1H, H\textsubscript{1}´´), 3.80 (d, \(J = 11\) Hz, 1H, H\textsubscript{1}´´), 3.55 (m, 2H, H\textsubscript{5}´´), 3.47 (m, 2H, H\textsubscript{8}´´), 2.55 (t, \(J = 6.6\) Hz, 2H, H\textsubscript{6}´´), 2.41 (t, \(J = 6.6\) Hz, 2H, CH\textsubscript{2}COOH), 2.28 (quintet, \(J = 8\) Hz, 2H, H\textsubscript{9}´´), 2.20 (s, 3H, COCH\textsubscript{3}), 2.10 (s, 3H, COCH\textsubscript{3}), 1.04, 1.03 (each s, 3H, H\textsubscript{10}´´ and H\textsubscript{11}´´); 13C NMR (150.9 MHz, CDCl\textsubscript{3}, 25°C) \(\delta\) (ppm): 178.5 (COOH), 173.4, 171.7 (C\textsubscript{4}´´ and C\textsubscript{7}´´), 159.9, 159.7 (COCH\textsubscript{3}), 76.5 (C\textsubscript{3}´´), 75.3 (C\textsubscript{1}´´), 39.6 (C\textsubscript{8}´´), 37.4 (CH\textsubscript{2}COOH), 36.1 (C\textsubscript{5}´´), 35.3 (C\textsubscript{4}´´), 31.5 (C\textsubscript{2}´´), 20.9, 20.8 (C\textsubscript{10}´´ and C\textsubscript{11}´´). HR-ESI-MS calcd (M+H\textsuperscript{+}) \(m/z\) 389.1932, found (M+H\textsuperscript{+}) \(m/z\) 389.1927.

\(O,O'-\text{diacetyl-malonyl-carba(dethia)-pantetheine methyl ester (13a) (3)}. \(N-(O,O'-\text{diacetyl-d-pantothenoyl})-\text{g-aminobutyric acid 11}\) (620 mg, 1.6 mmol), 2,2-dimethyl-1,3-dioxane-4,6-dione (225 mg, 1.6 mmol), and 4-dimethylaminopyridine (225 mg, 1.8 mmol) were dissolved in dry dichloromethane (10 mL). The reaction mixture was stirred under N\textsubscript{2} atmosphere at room temperature for 10 min, and \(N-(3\text{-dimethylaminopropyl})-N'\text{-ethylcarbodiimide hydrochloride}\) (335 mg, 1.8 mmol) was added. The reaction mixture was stirred at room temperature for 4 h. The solution was diluted with dichloromethane (10 mL) and washed with 0.1 M HCl (20 mL). The organic layer was dried over MgSO\textsubscript{4} and the solvent removed under vacuum to yield the desired compound 12 as a yellow oil (590 mg, 70%). This material (590 mg, 1.1 mmol) was dissolved in dry methanol (20 mL) and heated to reflux for 18 h under N\textsubscript{2} atmosphere. The reaction mixture was cooled and methanol was removed on a rotary evaporator to yield \(O,O'-\text{diacetyl-malonyl-carba(dethia)-pantetheine methyl ester (13a) (3)}. \(O,O'-\text{diacetyl-malonyl-carba(dethia)-pantetheine methyl ester (13a) (3)}\) (50 mg, 0.1 mmol) and Selectfluor (40 mg, 0.1 mmol) were suspended in acetonitrile (500 mL). The mixture was heated to 82°C under 100 W microwave irradiation for 10 min in a Discover Labmate microwave reactor (CEM). The reaction mixture was then diluted to 5 mL with water and purified on an Agilent Eclipse-XDB C8 column using a linear gradient from 0 to 100% methanol over 30 min (3 mL/min) with 0.1% (v/v)
formic acid as the aqueous mobile phase. Fractions containing the desired product as determined by LC-MS analysis were then pooled and lyophilized (12 mg, 25%). $^1$H NMR (600 MHz, D$_2$O, 25°C) $\delta$ (ppm): 5.68 (d, $J = 47$ Hz, 1H, CHF), 4.80 (s, 1H, H$_{1''}$), 4.07 (d, $J = 11$ Hz, 1H, H$_{1''}$), 3.86 (s, 3H, Me), 3.85 (d, $J = 11$ Hz, 1H, H$_{1''}$), 3.50-3.40 (m, 2H, H$_{5''}$), 3.20 (m, 2H, H$_{8''}$), 2.85 (m, 2H, H$_{6''}$), 2.44 (m, 2H, CH$_2$), 2.16 (s, 3H, COCH$_3$), 2.10 (s, 3H, COCH$_3$), 1.77 (quintet, $J = 7.2$ Hz, 2H, H$_{9''}$), 1.03, 1.02 (each s, 3H, H10$^{''}$ and H11$^{''}$); $^{13}$C NMR (150.9 MHz, D$_2$O, 25°C) $\delta$ (ppm): 211.0 (CH$_2$COCHF), 174.4, 173.6 (C$_4''$ and C$_7''$), 173.1 (COCH$_3$), 170.6 (COOMe), 92.0 (CHF), 77.4 (C$_3''$), 69.5 (C$_1''$), 53.9 (Me), 38.4 (C$_8''$), 36.6 (C$_5''$), 36.6 (C$_4''$), 35.8 (C$_2''$), 21.7, 20.6 (COCH$_3$), 20.1, 19.9 (C$_{10''}$ and C$_{11''}$). HR-ESI-MS calcd (M+H$^+$) m/z 463.2086, found (M+H$^+$) m/z 463.2089.

General protocol for enzymatic synthesis of coenzyme A analogs from pantetheine analogs. Acetonide-protected oxa(dethia) (4a, 4b) and aza(dethia) (8a, 8b) pantetheine analogs were deprotected and used directly in enzymatic reactions to generate the CoA analog. In brief, 4a, 4b, 8a, and 8b were resuspended in water at a concentration of approximately 40 mg/mL. Dowex 50WX8-400 (hydrogen form, 200-400 mesh, 25 mg/mL) was then added and stirred at room temperature (3)Reactions were monitored by LC-MS and found to achieve completion at after 2-3 h. Dowex resin was removed by filtration with a 0.2 µM syringe filter. The solution was then neutralized and compounds 5a, 5b, 9a, and 9b were used without further purification.

$O,O'$-diacetyl-malonyl-carba(dethia)-pantetheine methyl esters (13a, 13b) (50 mg) and potassium carbonate (50 mg) were dissolved in water (1 mL). The mixture was heated to 82°C under 100 W microwave irradiation for 10 min in a CEM Discover Labmate microwave reactor to yield fluoroacetyl-carba(dethia)-pantetheine. After cooling, the mixture was neutralized with 1 M HCl and compounds 14a and 14b were used without further purification.

Deprotected pantetheine analogs were mixed with CoA synthesis buffer (final: 50 mM Tris-HCl, pH 7.5, 20 mM KCl, 10 mM MgCl$_2$, 15 mM ATP) to a final concentration of 5 mM based on literature protocol (2). The reaction was then initiated by addition of PanK, PPAT, and DPCK (2 mg each). For acid-labile oxa(dethia)-CoA analogs, the reaction mixture was lyophilized and the lyophilizate was dissolved in 5 mL water and 0.2 µm-filtered. For aza(dethia)-CoA analogs, the reaction mixture was lyophilized, the lyophilizate was dissolved in 5 mL water with 70% (v/v) perchloric acid (250 µL, 0.05 volumes) was added to precipitate protein. Precipitated protein was removed by centrifugation and the solution was neutralized and filtered (0.2 µm). The filtered solutions of crude CoA analogs were injected onto an Agilent Eclipse XDB-C18 equipped with an Agilent Eclipse XDB-C18 analytical guard column (4.6 × 12.5 mm, 5 µm). The column was washed with 0.2% TFA (v/v) in water until the absorbance at 260 nm returned to baseline. A linear gradient was then applied from 0 to 100% methanol over 90 min (3 mL/min) with 0.2% TFA as the aqueous mobile phase. Fractions were screened by LC-MS, pooled, and lyophilized to yield pure CoA analogs as a white powder.
**Fluoroacetyl-oxa(dethia)-CoA.** \(^1\text{H NMR (600 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 8.55 (s, 1H, H\(_8\)), 8.35 (s, 1H, H\(_2\)), 6.12 (d, \(J = 6\) Hz, 1H, H\(_1\)), 4.90 (d, \(J = 46\) Hz, 2H, CH\(_2\)F), 4.24 (m, 2H, H\(_2\)' and H\(_3\)'), 4.21 (t, \(J = 6\) Hz, 2H, H\(_5\)'), 4.54 (s, 1H, H\(_4\)'), 4.21 (d, \(J = 13.8\) Hz, 2H, H\(_3\)'), 3.93 (s, 1H, H\(_3\)'), 3.83 (m, 1H, H\(_1\)'), 3.60 (m, 1H, H\(_1\)'), 3.39 (m, 4H, H\(_8\) and H\(_9\)'), 2.39 (t, \(J = 6\) Hz, 2H, H\(_6\)'), 0.87 (s, 3H, H\(_10\)'), 0.76 (s, 3H, H\(_11\)'), \(^1\text{C NMR (150.9 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 174.63 (C\(_7\)'), 173.14 (C\(_4\)'), 170.4, 170.2 (FCH\(_2\)CO), 149.75 (C\(_6\)), 148.39 (C\(_2\)), 144.67 (C\(_4\)), 142.41 (C\(_8\)), 118.45 (C\(_5\)), 87.49 (C\(_1\)'), 83.06 (C\(_4\)'), 78.53, 77.37 (FCH\(_2\)CO), 74.43 (C\(_2\)'), 74.14 (C\(_3\)'), 73.62 (C\(_3\)'), 72.24 (C\(_1\)'), 64.10 (C\(_5\)'), 64.10 (C\(_9\)'), 38.29 (C\(_8\)'), 38.02 (C\(_2\)''), 35.33 (C\(_5\)'), 35.18 (C\(_6\)'), 20.61 (C\(_10\)'), 18.27 (C\(_11\)').

**Acetyl-oxa(dethia)-CoA.** \(^1\text{H NMR (600 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 8.47 (s, 1H, H\(_8\)), 8.32 (s, 1H, H\(_2\)), 5.97 (d, \(J = 6\) Hz, 1H, H\(_1\)'), 4.75 (m, 2H, H\(_2\)' and H\(_3\)'), 4.40 (s, 1H, H\(_4\)'), 4.10 (d, \(J = 14.4\) Hz, 2H, H\(_5\)'), 3.90 (s, 1H, H\(_1\)'), 3.79 (m, 1H, H\(_1\)'), 3.41 (m, 4H, H\(_8\) and H\(_9\)'), 2.26 (t, \(J = 6\) Hz, 2H, H\(_6\)'), 1.85 (s, 3H, CH\(_3\)CO), 0.75 (s, 3H, H\(_10\)'), 0.63 (s, 3H, H\(_11\)').

**Fluoroacetyl-aza(dethia)-CoA.** \(^1\text{H NMR (600 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 8.50 (s, 1H, H\(_8\)), 8.32 (s, 1H, H\(_2\)), 6.08 (d, \(J = 6\) Hz, 1H, H\(_1\)'), 4.30 (m, 2H, H\(_2\)' and H\(_3\)'), 3.90 (s, 1H, H\(_1\)'), 3.79 (m, 1H, H\(_1\)'), 3.41 (m, 4H, H\(_8\) and H\(_9\)'), 2.35 (t, \(J = 6\) Hz, 2H, H\(_6\)'), 0.85 (s, 3H, H\(_10\)'), 0.73 (s, 3H, H\(_11\)''); \(^1\text{C NMR (150.9 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 174.60 (C\(_7\)'), 174.16 (C\(_4\)'), 170.85, 170.74 (FCH\(_2\)CO), 149.70 (C\(_6\)), 148.32 (C\(_2\)), 144.66 (C\(_4\)), 142.36 (C\(_8\)), 118.25 (C\(_5\)), 87.44 (C\(_1\)'), 82.83 (C\(_4\)), 74.49 (C\(_2\)'), 74.06 (C\(_3\)'), 72.28 (C\(_1\)'), 65.13 (C\(_5\)'), 63.22 (C\(_9\)'), 38.25 (C\(_8\)'), 38.20 (C\(_2\)'), 35.28 (C\(_3\)'), 35.08 (C\(_9\)'), 20.51 (C\(_10\)'), 20.18 (CH\(_3\)CO), 18.22 (C\(_11\)'').

**Acetyl-aza(dethia)-CoA.** Acetyl-oxa(dethia)-CoA. \(^1\text{H NMR (600 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 8.47 (s, 1H, H\(_8\)), 8.32 (s, 1H, H\(_2\)), 6.08 (d, \(J = 6\) Hz, 1H, H\(_1\)'), 4.30 (m, 2H, H\(_2\)' and H\(_3\)'), 3.90 (s, 1H, H\(_1\)'), 3.79 (m, 1H, H\(_1\)'), 3.41 (m, 4H, H\(_8\) and H\(_9\)'), 2.35 (t, \(J = 6\) Hz, 2H, H\(_6\)'), 0.85 (s, 3H, H\(_10\)'), 0.73 (s, 3H, H\(_11\)''); \(^1\text{C NMR (150.9 MHz, D}_2\text{O, 25°C)}\) \(\delta\) (ppm): 174.60 (C\(_7\)'), 174.16 (C\(_4\)'), 170.85, 170.74 (FCH\(_2\)CO), 149.70 (C\(_6\)), 148.32 (C\(_2\)), 144.66 (C\(_4\)), 142.36 (C\(_8\)), 118.25 (C\(_5\)), 87.44 (C\(_1\)'), 82.83 (C\(_4\)), 74.49 (C\(_2\)'), 74.06 (C\(_3\)'), 72.28 (C\(_1\)'), 65.13 (C\(_5\)'), 63.22 (C\(_9\)'), 38.25 (C\(_8\)'), 38.20 (C\(_2\)'), 35.28 (C\(_3\)'), 35.08 (C\(_9\)'), 20.51 (C\(_10\)'), 20.18 (CH\(_3\)CO), 18.22 (C\(_11\)'').
38.25 (Cₘ), 38.20 (C₂'), 35.28 (Cₙ), 35.08 (C₀'), 20.51 (C₁₀'), 20.18 (CH₃CO), 18.22 (C₁₁'). HR-ESI-MS calcd (M-H⁺) m/z 791.1573, found (M-H⁺) m/z 791.1552.

Fluoroacetyl-carba(dethia)-CoA. ¹H NMR (600 MHz, D₂O, 25°C) δ (ppm): 8.53 (s, 1H, H₈), 8.33 (s, 1H, H₂), 6.10 (d, J = 6 Hz, 1H, H₁''), 4.95 (d, J = 47 Hz, 2H, CH₂F), 4.85 (m, 2H, H₂' and H₃'), 4.52 (s, 1H, H₄''), 4.20 (d, J = 13.8 Hz, 2H, H₅''), 3.91 (s, 1H, H₆''), 3.80 (m, 1H, H₁'''), 3.59 (m, 1H, H₂'''), 3.37 (t, J = 7.2 Hz, 2H, H₃''), 3.05 (t, J = 7.2 Hz, 2H, H₄''), 2.44 (t, J = 7.2 Hz, 2H, H₅''), 2.37 (t, J = 7.8 Hz, 2H, CH₂CO), 1.65 (quintet, J = 6.6 Hz, 2H, H₆''), 0.85 (s, 3H, H₁₀''), 0.74 (s, 3H, H₁₁''); ¹³C NMR (150.9 MHz, D₂O, 25°C) δ (ppm): 210.0, 209.9 (CH₂FCO), 174.6 (C₇''), 173.8 (C₄''), 149.7 (C₆), 148.4 (C₂), 144.7 (C₄), 142.4 (C₈), 118.5 (C₅), 87.5 (C₁'), 84.3 (CH₂F), 83.0 (C₄), 74.4 (C₅'), 74.1 (C₃'), 73.6 (C₃'), 72.2 (C₄'), 65.1 (C₅'), 38.5 (C₇'), 38.4 (C₆'), 38.3 (CH₂COCH₂F), 35.4 (C₃'), 35.2 (C₆'), 22.3 (C₉'), 21.8 (C₁₀'), 18.3 (C₁₁'). ¹⁹F NMR (564.7 MHz, D₂O, 25°C) δ (ppm): -229.5 (t, J = 46.3 Hz). HR-ESI-MS calcd (M-H⁺) m/z 808.1527, found (M-H⁺) m/z 808.1505.

Acetyl-carba(dethia)-CoA. ¹H NMR (600 MHz, D₂O, 25°C) δ (ppm): 8.39 (s, 1H, H₈), 8.22 (s, 1H, H₂), 5.97 (d, J = 6 Hz, 1H, H₁''), 4.75 (m, 2H, H₂' and H₃'), 4.40 (s, 1H, H₄''), 4.10 (d, J = 12.6 Hz, 2H, H₅''), 3.80 (s, 1H, H₆''), 3.72 (m, 1H, H₁'''), 3.49 (m, 1H, H₂'''), 2.92 (t, J = 6 Hz, 2H, H₅''), 2.35 (t, J = 6 Hz, 2H, H₆''), 2.25 (t, J = 6 Hz, 2H, H₇'), 1.96 (s, 3H, CH₃CO), 1.49 (t, J = 6 Hz, 2H, H₉'), 0.74 (s, 3H, H₁₀''), 0.64 (s, 3H, H₁₁'); ¹³C NMR (150.9 MHz, D₂O, 25°C) δ (ppm): 215.4 (CH₃CO), 174.50 (C₇''), 173.6 (C₄''), 149.60 (C₆), 148.24 (C₂), 144.64 (C₄), 142.28 (C₈), 87.44 (C₁'), 82.85 (C₄), 74.46 (C₂), 74.07 (C₃'), 73.50 (C₅'), 72.26 (C₆'), 65.14 (C₅'), 40.17 (CH₂CH₂COCH₃), 38.48 (C₇'), 38.26 (C₈') 35.33 (C₉'), 35.13 (C₁₀'), 29.14 (CH₃CO), 22.52 (C₉'), 20.52 (C₁₀'), 18.26 (C₁₁'). HR-ESI-MS calcd (M-H⁺) m/z 790.1621, found (M-H⁺) m/z 790.1598.

Preparation of proteins for NMR spectroscopy. LB (10 mL) containing carbenicillin (50 µg/mL) was inoculated with a single colony of freshly transformed E. coli BL21(de3)-T1R containing the appropriate overexpression plasmid for wt or F36A FIK. The cultures were grown at 37°C at 200 rpm to for 4-6 h.

For preparation of ¹⁵N-Phe-labeled wild-type or F36A FIK, the culture was diluted into M9 media prepared with unlabeled ammonium chloride and supplemented with 100 mg/L of ¹⁵N-Phe and 200 mg/L of each of the other 19 proteinogenic amino acids (per L: 12.8 g NaHPO₄ · 7H₂O, 3.0 g KH₂PO₄, 0.5 g NaCl, 1.0 g NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4% (w/v) glucose, 40 µg/mL thiamine, 5 mg FeCl₃, 0.5 mg ZnCl₂, 0.1 mg CuCl₂, 0.1 mg CoCl₂ · 6H₂O, 0.1 mg HBO₃, 16 mg MnCl₂, and 0.1 mg each of D-biotin, folic acid, nicotinamide, calcium D-pantothenate, pyridoxal-HCl, riboflavin, lipoic acid, pyridoxine, and 4-aminobenzoic acid).

For preparation of wild-type FIK with uniformly ¹³C-labeled phenylalanine, the culture was diluted into M9 media as described above but the amino acid mixture used for supplementation was prepared with 100 mg/L of L-Phe (ring,¹³C₆) rather than ¹⁵N-Phe.
For preparation of $^{13}$C-labeled wild-type or F36A FlK, $[1^{-13}$C]-glucose and unlabeled NH$_4$Cl was used for preparation of the M9 media.

These cultures were grown at 37°C at 200 rpm to an OD$_{600 \text{ nm}}$ = 0.7 - 0.8. The cultures were then cooled on ice for 20 min. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and cells were incubated at 16°C at 200 rpm for 12-16 h. Wild-type or F36A FlK with various isotope labels was then purified as described previously and flash frozen in NMR buffer (50 mM sodium phosphate pH 7.6, 100 mM NaCl) then stored at -80°C (1).

**Calculations for percent of ligand or protein in bound form.** Using the equation:

$$K_D = \frac{[P][L]}{[PL]}$$

where $[P]$ is the concentration of protein, $[L]$ the concentration of ligand and $[PL]$ the concentration of the bound protein-ligand complex, one can transform this into a quadratic equation solving for $[PL]$ as follows:

$$[PL] = \frac{1}{2} (K_D + [P]_o + [L]_o) - \sqrt{\frac{1}{4} (K_D + [P]_o + [L]_o)^2 - [L]_o[P]_o}$$

At a given concentration of $[P]_o$ and $[L]_o$ and given the $K_D$ value obtained from the ITC experiments, one is able to calculate the fraction of ligand bound ($f_{LB}$) or protein bound ($f_{PB}$) with the following equations:

$$f_{LB} = \frac{[PL]}{[L]_o}; \quad f_{PB} = \frac{[PL]}{[P]_o}$$

**$^1$H-NMR experiments for ligand binding.** The carba(dethia) ligands (FAcCCoA and AcCCoA) used for substrate analog NMR experiments were synthesized as previously described in the methods section. Purified ligands were lyophilized to dryness, then resuspended in D$_2$O and stored at -80°C with a stock concentration ranging from 10-40 mM. The concentration of these ligands were quantified by A$_{260 \text{ nm}}$ ($\varepsilon_{280 \text{ nm}} = 13,100 \text{ M}^{-1}\text{cm}^{-1}$). For NMR experiments, both FAcCCoA and AcCCoA were diluted with a buffer of 50 mM sodium phosphate pH 7.6, 100 mM NaCl in 100% D$_2$O to a final concentration of 500 µM. Wild-type or F36A FlK in NMR buffer (50 mM sodium phosphate pH 7.6, 100 mM NaCl in 100% H$_2$O) starting with stock concentrations of 700-1000 µM was then added to a final concentration of 100 µM. The 1D $^1$H-NMR experiments were performed on a Bruker Avance II 900 MHz spectrometer equipped with a TCI cryoprobe at 298K and the resulting spectra were processed using Topspin version 3.2. 500 µM of FAcCCoA was calculated to be 12.5% bound with 100 µM wild-type FlK and 3% bound with 100 µM F36A FlK.
500 uM of AcCoA was calculated to be 1.2% bound with 100 uM wild-type FlK. Similar experiments were carried out where the protein concentration was varied so that in all three combinations the ligand was all 5% bound, the observed trend in line broadening of the $^1$H-NMR peaks were still similar to that of Figure S11. Results with uniform protein concentrations were ultimately used for ease of comparison using the equation $k_{ex} = k_{on}[P] + k_{off}$. After NMR experiments, the carba(dethia) substrate analogs could be re-purified with a yield of >85%. This suggests that the substrate analogs were relatively stable throughout the course of the NMR experiment.

$^1$H/$^{15}$N HSQC and NOE NMR experiments. The samples used for NMR experiments consisted of 250-300 μM of $^{15}$N-Phe-labeled wild-type or F36A FlK in 50 mM sodium phosphate pH 7.6, 100 mM NaCl containing 10% (v/v) D$_2$O. The 2D $^1$H/$^{15}$N HSQC and NOE experiments were performed on a Bruker Avance II 900 MHz spectrometer equipped with a TCI cryoprobe at 298K and the resulting spectra were processed using Topspin version 3.2. The NOE mixing time was set to 70 ms.
Supplementary Results

Figure S1. Sequence alignment of FlK and homologs. The homologs are the top ten hits from NCBI BLAST drawn from sequences that were available as of May 12, 2017. Sequences were aligned using MUSCLE. Phe 36 is shown in red, and the equivalent position in homologs lacking Phe 36 is shown in blue.

Streptomyces cattleya  MRDGRUGGERFTDHDFVPPHPHRHLVESPEFAEFPEVFSATGFMVGLMNACVRAMAPY
Streptomyces fradiae  MRDGLVGEVFHVHYTPFTKVPDRTVHLLYESPEFAGFPEVFSATGFMVGLNMETTVRALAPY
Streptomyces sp. MA37  MREDLTVKTHGHRPHTYPFFPEVFSATGFMVGLNMETTVRALQFPF
Streptomyces xinghaiensis  MREGLLTGEKTHHYRVPKTHYRLYESPEFSTFFPEVFSATGFMVGLNMETTVRALMYPH
Syderoxydans sp. RIFOXYD2_FULL_59_7  MKDTLKPG1RYEHRFVPIPTKVPALEVSPESQMFPEVFSATGFMVGLWACLMMPH
Gallionella capsiferriformans  MKDTLKPG1RFSEHKYLVPKPALEVSPESQMFPEVFSATGFMVGLWACLMMPH
Deltaproteobacteria bacterium  MKETLKAGLEHETYRFVTPKRVPEAKVFPMKVFATGFMVGLWCEAMAPH
Rhodovulum sp. PH10  MKFLSVGLPTHRFSTYFPTKVPFVCAPEAEMSFMKVFATGFMVGLCTVMAPRH
Gallionellas bacterium  MKDTL1IPG1RFSEHYRPVKPALEVSPESQMFPEVFSATGFMVGLWACLMMPH
Solemya velesiana gill symbiont  MNDGLKVGLYHQRHTVPKPALEVSPESAEVFMPEVFSATGFLVWALILMKPH
Gallionellas bacterium  MKDTL1IPG1RFSEHYRPVKPALEVSPESQMFPEVFSATGFMVGLWACLMMPH

Streptomyces cattleya  LE-PGEGLST1AICYTHTAATTPGALTVTAELRSVEGGLLSWRVSASAHGDVEIGSGT
Streptomyces fradiae  LE-PGEGLST1GTYVHTAATTPGALTVAELLSVEKRRISWRVTAHGDVDSSGT
Streptomyces sp.MA37  LA-PGEGLST1AICYVHTAATTPGALTVAELLARLGRSTWQTAHGDDEIGSGT
Streptomyces xinghaiensis  LE-AEGESL1GCVAHTAATTPGALTVAELLOGRKLRKWVTADHGVEIGSGT
Syderoxydans sp. RIFOXYD2_FULL_59_7  LDWAFQSLTH1MVHSEAATTPGLEVTAETVSWGRERR1FASASAHGDVLDIASGT
Gallionella capsiferriformans  LDWAFQSLTH1MVHSEAATTPGLEVTAETVSWGRERR1FASASAHGDVLDIASGT
Deltaproteobacteria bacterium  ME-PGEGLST1GTYVHTAATTPGALTVAELLSVEKRRISWRVTAHGDVDSSGT
Rhodovulum sp. PH10  LD-AEGESL1GTVHDVHTAATTPGALTVAELLSVEKAEMVEIGSGT
Gallionellas bacterium  LDWAFQSLTH1MVHSEAATTPGLEVTAETVSWGRERR1FASASAHGDVLDIASGT
Solemya velesiana gill symbiont  LDWAFQSLTH1MVHSEAATTPGLEVTAETVSWGRER1FASASAHGDVLDIASGT
Gallionellas bacterium  LDWAFQSLTH1MVHSEAATTPGLEVTAETVSWGRERR1FASASAHGDVLDIASGT

Streptomyces cattleya  RAV1HELEFNKVRQKTPAG--------
Streptomyces fradiae  RAV1TDLRFTAGMREKLARGTAAGPAAT
Streptomyces sp.MA37  RAVVVDLRFTQGREEKLRATAVPE----
Streptomyces xinghaiensis  RAVIVERTPTSSLKGRASVETGES--
Syderoxydans sp. RIFOXYD2_FULL_59_7  RFV1DRARFDKLSKQRSKQT---------
Gallionella capsiferriformans  RYVINKEKFDNKLDRKREKINQ--------
Deltaproteobacteria bacterium  RPAFVPAKFNPARMAKRTA---------
Rhodovulum sp. PH10  RYVVSWDKNPARTGKEAGAAGLAASEEG
Gallionellas bacterium  RYVINKEKFDNKLDRKDKPTLIG-------
Solemya velesiana gill symbiont  RFIINREKFDAGVASKTSK--------
Gallionellas bacterium  RFIVIDKARFDKSKQINSN--------
**Figure S2.** Pre-steady-state kinetics of F36A FlK. (A) Pre-steady-state time course for F36A FlK hydrolysis of acetyl-CoA. (B) Pre-steady-state time course for F36A FlK hydrolysis of fluoroacetyl-CoA.

![Graphs showing pre-steady-state kinetics](image)

- **Figure S2.** Pre-steady-state kinetics of F36A FlK. (A) Pre-steady-state time course for F36A FlK hydrolysis of acetyl-CoA. (B) Pre-steady-state time course for F36A FlK hydrolysis of fluoroacetyl-CoA.

**Figure S3.** Chemoenzymatic synthesis of non-hydrolyzable substrate analogs. (A) SDS-PAGE gel showing purified pantothenate kinase (PanK), phosphopantetheine adenylyltransferase (PPAT), and dephospho-CoA kinase (DPCK) from *E. coli*. (B) Enzymatic conversion of pantetheine analogs to the corresponding CoA analogs by PanK, PPAT, and DPCK.

![SDS-PAGE gel and enzymatic conversion](image)

- **Figure S3.** Chemoenzymatic synthesis of non-hydrolyzable substrate analogs. (A) SDS-PAGE gel showing purified pantothenate kinase (PanK), phosphopantetheine adenylyltransferase (PPAT), and dephospho-CoA kinase (DPCK) from *E. coli*. (B) Enzymatic conversion of pantetheine analogs to the corresponding CoA analogs by PanK, PPAT, and DPCK.
Figure S4. $^1$H/$^{13}$C HMBC spectrum of fluoroacetyl-oxa(dethia)-CoA. The carbonyl carbon and $\alpha$-carbon chemical shifts are consistent with an ester.
Figure S5. $^1$H/$^{13}$C HMBC spectra of amide acyl-CoA analogs. (A) Acetyl-aza(dethia)-CoA. (B) Fluoroacetyl-aza(dethia)-CoA. The carbonyl carbon and $\alpha$-carbon chemical shifts are consistent with an amide.
Figure S6. $^1$H/$^{13}$C HMBC spectrum of ketone acyl-CoA analogs. (A) Acetyl-carba(dethia)-CoA. (B) Fluoroacetyl-carba(dethia)-CoA. The carbonyl carbon and $\alpha$-carbon chemical shifts are consistent with a ketone.
Figure S7. Stability of non-hydrolyzable analogs of fluoroacetyl-CoA. (A) HPLC trace of a fluoroacetyl-oxa(dethia)-CoA standard (black), fluoroacetyl-oxa(dethia)-CoA after incubation in 100 mM Tris-HCl, pH 7.6 for 18 h (red), and after incubation in 100 mM Tris-HCl, pH 7.6 in the presence of 3 µM FlK for 18 h (grey). (B) HPLC trace of a fluoroacetyl-aza(dethia)-CoA standard (black), fluoroacetyl-aza(dethia)-CoA after incubation in 100 mM Tris-HCl, pH 7.6 for 18 h (red), and fluoroacetyl-aza(dethia)-CoA after incubation in 100 mM Tris-HCl, pH 7.6 in the presence of 100 µM FlK for 18 h. (C) HPLC trace of a fluoroacetyl-carba(dethia)-CoA standard (black), 125 µM fluoroacetyl-carba(dethia)-CoA after incubation in 100 mM Tris-HCl, pH 7.6 for 18 h (red) and after incubation in 100 mM Tris-HCl, pH 7.6 in the presence of 62.5 µM FlK for 18 h (grey). (Panels A and B: Buffer A, 10 mM ammonium acetate; Panel C: Buffer A 10 mM ammonium acetate with 10% acetonitrile).
Figure S8. ITC traces for binding of substrate analogs to FlK. (A) FAcOCoA, fluoroacetyl-oxa(dethia)-CoA. (B) AcOCoA, acetyloxa(dethia)-CoA. (C) FAcNCoA, fluoroacetyl-aza(dethia)-CoA. (D) AcNCoA, acetyl-aza(dethia)-CoA. (E) FAcCCoA, fluoroacetylcarba(dethia)-CoA. (F) AcCCoA, acetyl-carba(dethia)-CoA.
Figure S9. ITC traces for substrate analog binding to F36A Flk. (A) Fluoroacetyl-ox(dethia)-CoA. (B) Fluoroacetyl-carba(dethia)-CoA. (C) Fluoroacetyl-aza(dethia)-CoA. (D) Acetyl-aza(dethia)-CoA.
**Table S1.** Inhibition constants for competition of substrate analogs with fluoroacetyl-CoA and acetyl-CoA. Errors are derived from propagation of errors from nonlinear fitting of Michaelis-Menten curves in the presence and absence of inhibitor. Data are mean ± s.e. (n = 3).

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>substrate</th>
<th>wild-type FIK</th>
<th>F36A FIK</th>
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<tbody>
<tr>
<td>AcOCoA</td>
<td>FAcCoA</td>
<td>2500 ± 1100</td>
<td>1500 ± 300</td>
</tr>
<tr>
<td>AcOCoA</td>
<td>AcCoA</td>
<td>510 ± 40</td>
<td>300 ± 30</td>
</tr>
<tr>
<td>FAcOCoA</td>
<td>FAcCoA</td>
<td>320 ± 50</td>
<td>260 ± 60</td>
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<tr>
<td>FAcOCoA</td>
<td>AcCoA</td>
<td>130 ± 20</td>
<td>330 ± 60</td>
</tr>
<tr>
<td>AcNCoA</td>
<td>FAcCoA</td>
<td>33000 ± 7000</td>
<td>2800 ± 700</td>
</tr>
<tr>
<td>AcNCoA</td>
<td>AcCoA</td>
<td>8400 ± 2100</td>
<td>4700 ± 500</td>
</tr>
<tr>
<td>FAcNCoA</td>
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<td>FAcNCoA</td>
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<td>1200 ± 400</td>
<td>7500 ± 700</td>
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<td>FAcCoA</td>
<td>11000 ± 2000</td>
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<td>3600 ± 500</td>
</tr>
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<td>FAcCCoA</td>
<td>FAcCoA</td>
<td>900 ± 150</td>
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</tr>
<tr>
<td>FAcCCoA</td>
<td>AcCoA</td>
<td>460 ± 60</td>
<td>4500 ± 800</td>
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</table>
Figure S10. Fitting plots of inhibition ($K_i$) vs dissociation ($K_D$) constants. The following kinetic scheme was to derive equations for curve fitting:

\[ E + R_O\text{S-CoA} \xrightarrow{k_1} E \cdot R_O\text{S-CoA} \quad \text{acyl-enzyme intermediate} \]

\[ \text{acylation} \quad R_O\text{S-CoA} \xrightarrow{k_2} R_O\text{S-CoA} \quad \text{deacylation} \]

\[ k_r \quad \text{no reaction} \]

The inhibition constant $K_i$ is defined as:

\[ K_i = \frac{[E][I]}{[EI]} \quad (1) \]

where $[E]$ is unbound enzyme concentration, $[I]$ is the inhibitor concentration, and $[EI]$ is the concentration of enzyme inhibitor complex. Using the partition analysis and net rate constant method developed by Cleland (5):

\[ [E] = \frac{k_1 k_2 [S]}{k_{-1} + k_2} [E]_{\text{total}} \quad (2) \quad \text{and} \quad [EI] = \frac{k_{-1'} k_1 [S]}{k_1'[I]} [E]_{\text{total}}, \quad (3) \]

where $[S]$ is the substrate concentration and $[E]_{\text{total}}$ is the enzyme concentration. Substituting (2) and (3) into (1),

\[ K_i = \frac{k_1 k_2 [S]}{k_{-1} + k_2} \times \frac{k_{-1'} k_1 [S]}{k_{-1'} k_1 [S]} \times [I] = \frac{k_2}{k_{-1} + k_2} \times \frac{k_{-1'}}{k_{-1'}} \times [I] = \left(\frac{k_2}{k_{-1}} + 1\right) \times K_D \times [I]. \quad (4) \]

Therefore, the slope of the plot of $K_i$ versus $K_D$ is

\[ \frac{K_i}{K_D} = \left(\frac{k_2}{k_{-1}} + 1\right). \quad (5) \]

When $k_{-1} \gg k_2$, corresponding to rapid equilibrium of substrate, $\frac{k_2}{k_{-1}} \to 0$, and $\frac{K_i}{K_D} = 0$.

On the other hand, if the rapid equilibrium assumption is not met, (i.e., $k_2 > k_{-1}$, and the slope of the plot is given by (5).
**Figure S11.** $^1$H-NMR spectra of carba(dethia) substrate analogs FAcCCoA and AcCoA upon binding to FlK. (A) FAcCCoA in the presence and absence of wild-type FlK. (B) FAcCCoA in the presence and absence of F36A FlK. (C) AcCCoA in the presence and absence of wild-type FlK. Upon addition of protein, the ligand will exist in both the free state and the protein-bound state. If the exchange rate between these two states is within a fast exchange regime, then the resulting NMR peak representing the combination of both states will shift but the line width of that peak will remain similar. However, if the exchange rate is within an intermediate exchange regime, the line width of the resulting peak will broaden significantly. We see significant line broadening of the representative methyl peak of the ligand in panel A but not in panel B and C. One must note that the intensity decrease of the protein-bound peak in panel B is due to a decrease in substrate analog concentration upon addition of protein, and at the same time the line width of this peak is not significantly changed. This suggests that the exchange rate of FAcCCoA ↔ FAcCCoA · wild-type FlK is within the intermediate exchange regime. In contrary, the exchange rate of FAcCCoA ↔ FAcCCoA · F36A FlK and AcCCoA ↔ AcCCoA · wild-type FlK are within the fast exchange regime.
**Figure S12.** $^1$H/$^{13}$C HSQC spectra of the aromatic region of wt FlK with $^{13}$C-labeled phenylalanines. Phenylalanines were labeled either uniformly by feeding $^{13}$C-Phe (grey) or at the $\delta$ position by feeding [1-$^{13}$C] glucose (black). Uniformly $^{13}$C-labeled Phe will appear as three distinct $^1$H/$^{13}$C cross peaks for each Phe in the aromatic region, whereas [1-$^{13}$C] glucose will be incorporated into the $\delta$ position of either Phe or Tyr. Therefore, black peaks that overlay with grey peaks can be assigned to Phe residues and black peaks without corresponding grey peaks can be assigned to Tyr residues by comparison of the spectra of protein labeled by uniformly $^{13}$C-labeled Phe (grey) and [1-$^{13}$C] glucose incorporation (black).
Figure S13. $^{1}H^{13}C$ TROSY-HSQC of $^{13}C$ labeled wild-type FIK (black) overlaid with $^{1}H^{13}C$ TROSY-HSQC of $^{13}C$ labeled (A) F36A FIK (red), and (B) F40L FIK (red). The mutants are missing peaks corresponding to F36 or F40, this facilitated assignment of the residues as indicated on the spectra. However the overlay spectra in panel A do not unambiguously suggest the labeled peak as F36, as there is an extra peak appearing in the top right corner annotated with a “?” This extra peak is possibly from a different residue whose peak has shifted due to the overall change in protein folding upon point mutation, but an additional experiment (Fig. S14) is needed to confirm the assignment of F36.
**Figure S14.** $^1$H/$^{15}$N HSQC spectra of $^{15}$N Phe-labeled wild-type and F36A FlK. (A) Overlay of wt and F36A FlK enabled assignment of the $^1$H/$^{15}$N cross peak for F36 by its disappearance in the spectrum for the mutant. Other Phe peaks shift as several are in close proximity to position 36 and could be perturbed by the F36A mutation. (B) $^1$H/$^{15}$N HSQC-NOESY spectrum of $^{15}$N Phe-labeled FlK. The Nuclear Overhauser effect (NOE) is the transfer of nuclear spin polarization from one spin bath to another spin bath via cross-relaxation. Protons that are within proximity to the labeled nucleus through-space will appear as NOE peaks. In this specific experiment, the protons connected to the δ-carbon of Phe are close enough in space to the $^{15}$N-labeled backbone nitrogen of the Phe residue to produce an NOE peak. These protons are the same ones contributing to the $^1$H/$^{13}$C cross peaks in the $^1$H/$^{13}$C TROSY-HSQC spectra (Figure S13). Once $H_δ$ of F36 is identified in the NOESY spectrum, a cross peak with the same $^1$H peak shift in the former $^1$H/$^{13}$C TROSY-HSQC can also be confirmed to be attributed by F36. In panel B, the circled peaks are NOE cross peaks corresponding to the $^1$H/$^{15}$N HSQC peaks in squares. One such set is the NOE peaks of F36 as labeled. The $^1$H chemical shift of the up-field cross peak corresponds to the $^1H_δ$ resonance of F36 observed in the $^1$H/$^{13}$C TROSY-HSQC (Figure S13), further confirming the assignment of F36 in the $^1$H/$^{13}$C TROSY-HSQC spectra.
References


