SUPPLEMENTARY INFORMATION

Seven-enzyme *in vitro* cascade to (3*R*)-3-hydroxybutyryl-CoA

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Characterization

Analysis of compounds was performed with the following instruments and parameters:

NMR: MR 400 MHz Agilent

HPLC (C18): Varian Microsorb-MV 300-5 C18 250 x 4.6 mm, flow rate 1.0 mL/min, gradient 0-15 min of 2-50% B, detection at 256 nm [A: water with 0.1% TFA, B: acetonitrile with 0.1% TFA, v/v].

HPLC (chiral): Daicel Chiralcel OC-H column, 4.6 mm x 25 cm, 5 μm, flow rate 0.75 mL/min, 93% hexane/7% ethanol (v/v), detection at 235 nm, 25 °C.

LC/MS: Agilent 1260 HPLC with an Agilent 6120 Quadrupole ESI instrument, in positive mode for analysis of acyl-*S*-*N*-acetylcysteamine (SNAC) compounds and negative mode for analysis of acyl-CoA compounds; ZORBAX Eclipse Plus 95 Å C18 column, 2.1 mm × 50 mm, 5 μm; column temperature of 40 °C; flow rate of 0.7 mL/min; gradient 0-12 min of 5-100% B and 12-15 min of 100% B (A: water, B: methanol, v/v).

High resolution MS: For (3*R*)-3-hydroxybutyryl-CoA, Agilent Technologies 6530 Accurate-Mass Q-TOF, Direct Inject, Jet Stream Ion Source ESI, in negative mode. For (*R*)-3-hydroxy-SNAC and (*S*)-3-hydroxy-SNAC, Agilent 1260 Infinity II HPLC with an Agilent 6230 TOF ESI instrument, in positive mode; Agilent Eclipse Plus C18 RRHD column, 2.1 mm × 50 mm, 1.8 μm; column temperature of 30 °C; flow rate of 0.1 mL/min; gradient 0-10 min of 10-60% B, 10-19 min of 60-90% B (A: water, B: acetonitrile, v/v).

All pH adjustments were made with aqueous HCl and NaOH.

Expression vectors

The CoaA (*S. aureus*), CoaD (*E. coli*), and CoaE (*E. coli*) vectors were gifts from E. Strauss[1]. The BktB (*C. necator*) vector was a gift from K.L.J. Prather[2]. Generation of the GDH (*B. subtilis*) vector has been described[3]. The CDS of ACS (*S. coelicolor*) was amplified from genomic DNA (NCBI Gene ID: 1098999). The amplicon was cloned into a pET28b backbone with an N-terminal His-tag through ligation independent cloning. The CDS of PhaB (*B. pseudomallei*) was obtained from NCBI (Gene ID: 3693808). This sequence was optimized using DNA Works for expression in *E. coli* BL21(DE3) cells. The optimized sequence was converted into 18 overlapping oligonucleotide primers each ~60 nucleotides long and assembled using overlapping PCR to yield the full CDS. The CDS was cloned into a pNIC-Bsa4 expression vector through ligation independent cloning.

Enzyme precipitates

E. coli BL21(DE3) cells were transformed with expression plasmids and grown at 37 °C in LB media (6 flasks, each with 1 L) and either 50 mg/L kanamycin or 100 mg/L ampicillin. When OD_{600} = 0.6 the temperature was lowered to 15 °C, and the cells were induced with 0.8 mM IPTG. After shaking overnight cells were centrifuged (4000 x g, 20 min, 4 °C), resuspended in 100 mL lysis buffer (500 mM NaCl, 30 mM HEPES, pH 7.7), and sonicated (3 cycles of 45 s with 1 s on on 1 s off, 100 W, in an ice bath). After centrifugation (30,000 x g, 30 min, 4 °C), 40 g ammonium sulfate was added for every 100 mL of supernatant, and the mixture was centrifuged (30,000 x g, 30 min, 4 °C) to yield a pellet (~5 g) that was stored at -80 °C. Precipitates were analyzed for enzyme expression through Coomassie-stained SDS-PAGE gels (Figure S1).

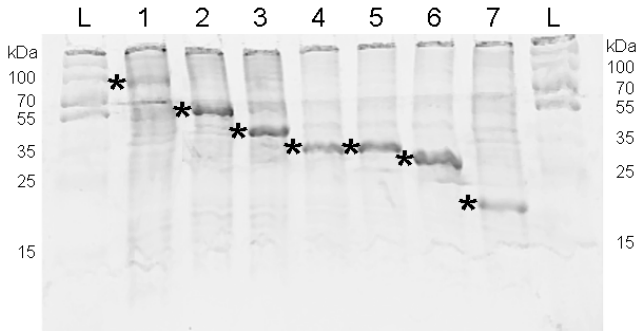


Figure S1. Coomassie-stained SDS-PAGE gel of ammonium sulfate precipitates: 1) ACS, 73 kDa, 2) BktB, 43 kDa, 3) CoaA, 38 kDa, 4) GDH, 30 kDa, 5) PhaB, 28 kDa, 6) CoaE, 25 kDa, 7) CoaD, 20 kDa.

Cascade reactions

Biocatalysis of (3R)-3-hydroxybutyryl-CoA. Production of (3*R*)-3-hydroxybutyryl-CoA was achieved at 25 °C in 100 mL containing 150 mM NaCl, 100 mM Tris-HCl, 10 mM disodium ATP, 20 mM $MgCl_2$, 1 mM pantethine, 2 mM DTT, pH 8.0, as well as 10 mg ammonium sulfate precipitate for each of CoaA, CoaD, and CoaE. The reaction was stirred at room temperature for 24 h, at which time 200 mM D-glucose, 50 µM $NADP^+$, 65 mM sodium acetate, and 5 mM additional disodium ATP were added, the solution was adjusted back to pH 8.0, and 10 mg of ammonium sulfate precipitated ACS, BktB, GDH, and PhaB were supplied. The reaction was stirred at 25 °C for another 24 h.

Reactions yielding (3*R*)-3-hydroxybutyryl thioesters worked when PhaB was employed as the reductase and CoA was chosen as the handle. To explore the promiscuity of the system, cascade reactions were run with *N*-acetylcysteamine (NAC) or pantetheine as the handle. ACS acetylated both handles with high efficiency. 3-Hydroxybutyryl-SNAC products were not observed. 3-Hydroxybutyryl-*S*-pantetheine products were formed, albeit at a lower yield compared to when CoA was employed as the handle (Figures S2-S4).

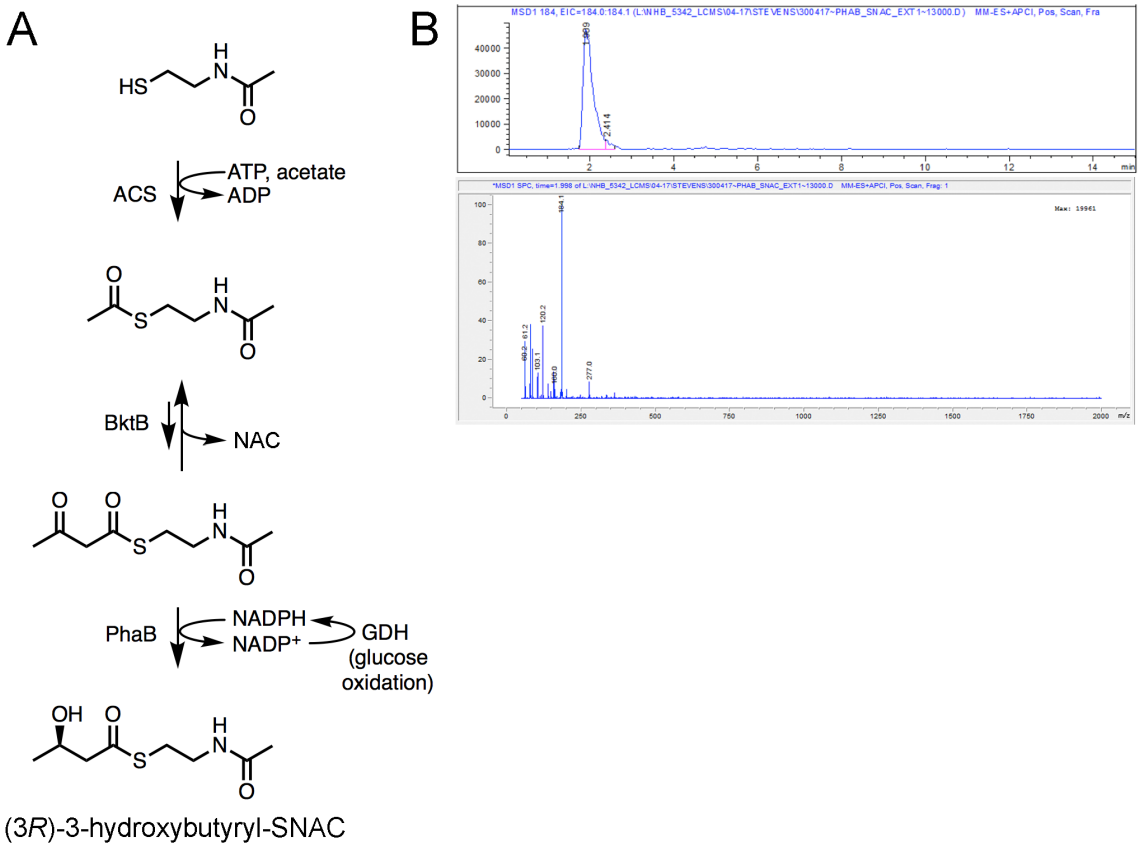


Figure S2. LC/MS data of one-pot reaction in the attempt to generate (*R*)-3-hydroxybutyryl-SNAC. **A)** Reaction scheme. **B)** The extracted ion peak chromatogram for the acetyl-SNAC sodium adduct ($[M+Na]^+$, 184.1 *m*/*z*) and its mass spectrum show acetyl-SNAC was produced.

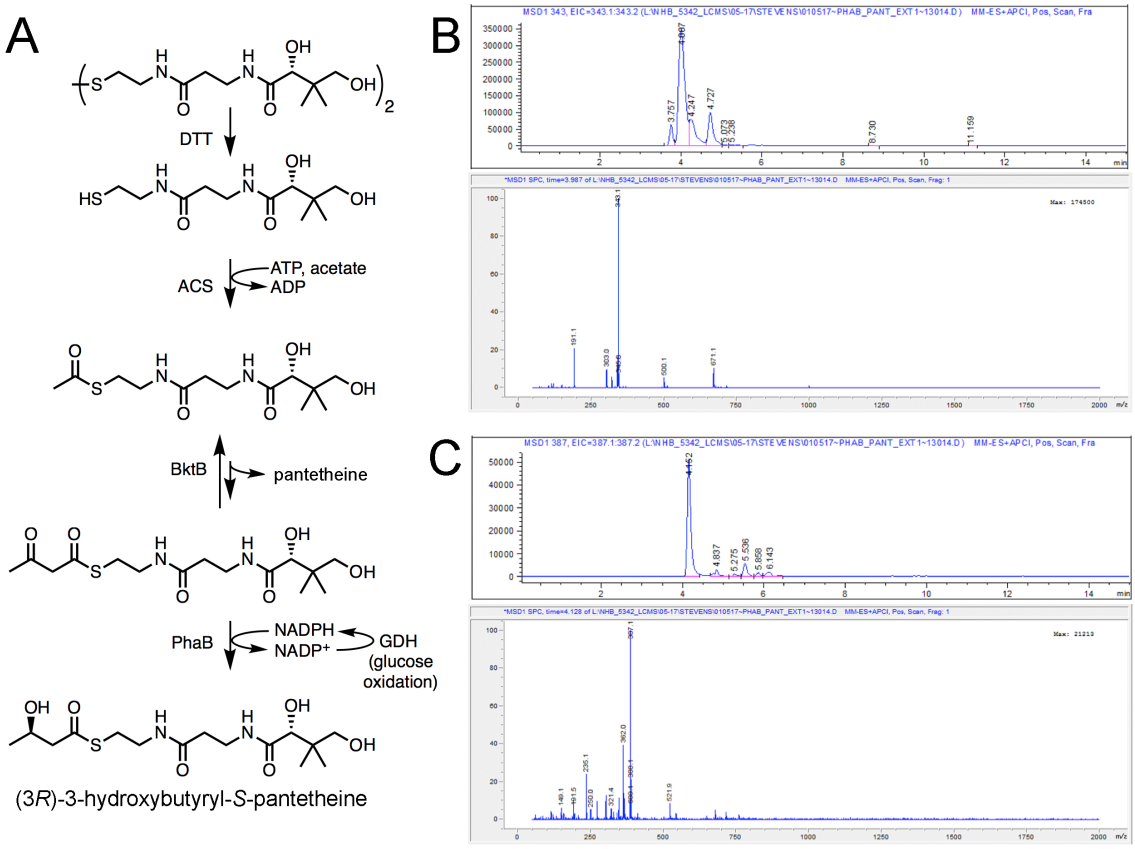


Figure S3. LC/MS data of one-pot reaction to generate (3*R*)-3-hydroxybutyryl-*S*-pantetheine. **A)** Reaction scheme. **B)** The extracted ion peak chromatogram for the acetyl-*S*-pantetheine sodium adduct ($[M+Na]^+$, 343.1 *m*/*z*) and its mass spectrum show acetyl-*S*-pantetheine was produced. **C)** The extracted ion peak chromatogram for the 3-hydroxybutyryl-*S*-pantetheine sodium adduct ($[M+Na]^+$ adduct (387.1 *m*/*z*) shows the target compound was produced, although at ~15% the level of acetyl-*S*-pantetheine.

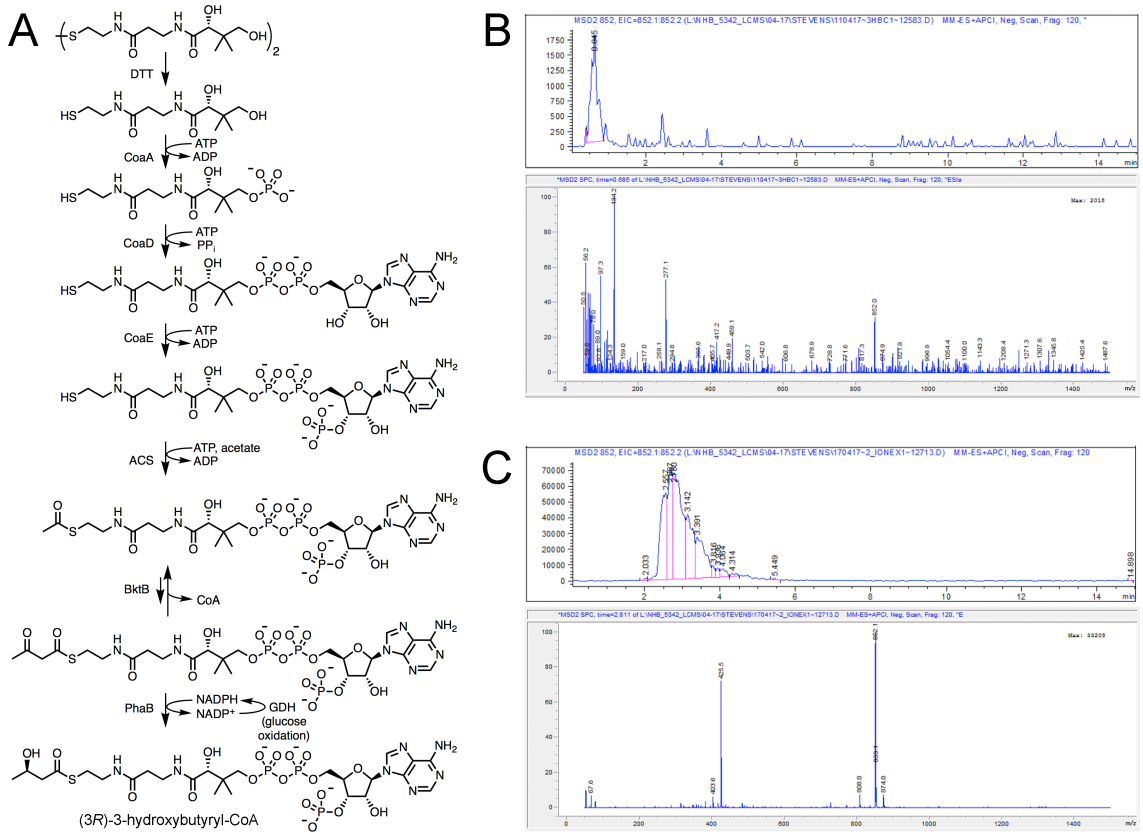


Figure S4. LC/MS data of one-pot reaction to generate (3*R*)-3-hydroxybutyryl-CoA. **A)** Reaction scheme. **B)** The extracted ion peak chromatogram for acetyl-CoA ([M-H]$^-$, 852.1 *m*/*z*) and its mass spectrum show acetyl-CoA was produced. **C)** The extracted ion peak chromatogram for 3-hydroxybutyryl-CoA ([M-H]$^-$ adduct (852.1 *m*/*z*) shows the target compound was produced.

Product analysis

From the completed cascade reaction, (3*R*)-3-hydroxybutyryl-CoA was either purified and analyzed by LC/MS or subjected to thiol/thioester exchange to determine the diastereomeric purity of the 3*R*-hydroxybutyryl group.

Purification of (3R)-3-hydroxybutyryl-CoA. The (3*R*)-3-hydroxybutyryl-CoA species was purified by ion exchange on a Q-sepharose column (100 mL column volume). The 100 mL cascade reaction was heated to 90 °C and centrifuged (4000 x g, 10 min, 25 °C) to remove protein, then diluted 20-fold and poured over Q-sepharose beads prewashed with water. Four column volumes of water were run over the beads to remove loosely bound products. Four column volumes of 50 mM HCl were run over the beads followed by four column volumes of 100 mM HCl to elute (3*R*)-3-hydroxybutyryl-CoA. ~35-mL fractions were collected, with fractions 10-21 containing (3*R*)-3-hydroxybutyryl-CoA (Figure S5). These fractions were combined. Low resolution LC/MS and high-resolution MS analysis were performed (Figure S6). The combined fractions contain >90% pure (3*R*)-3-hydroxybutyryl-CoA as judged by HPLC, ^{1}H NMR, and ^{31}P NMR (Figure S7). After bringing the elution to pH 7.0 with 2 M LiOH, the product (and LiCl) could be precipitated by through the addition of acetone to 95% (v/v) and centrifugation (4000 x g, 10 min, 25 °C).

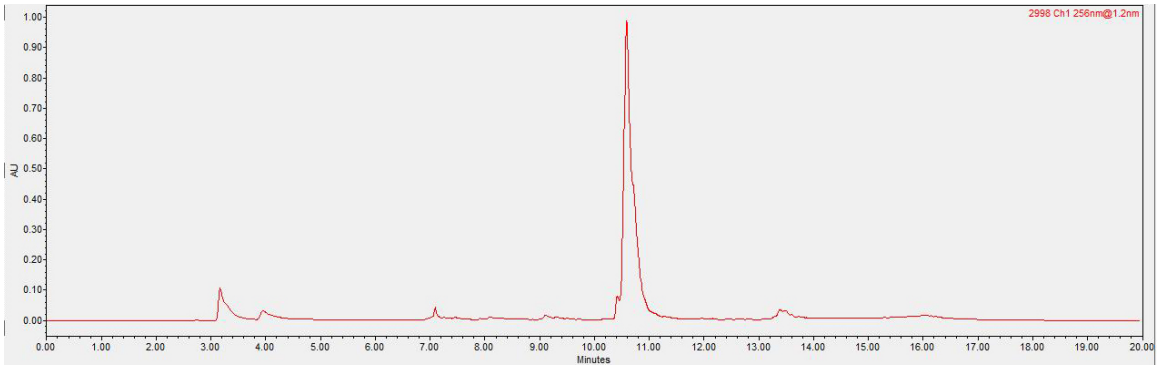
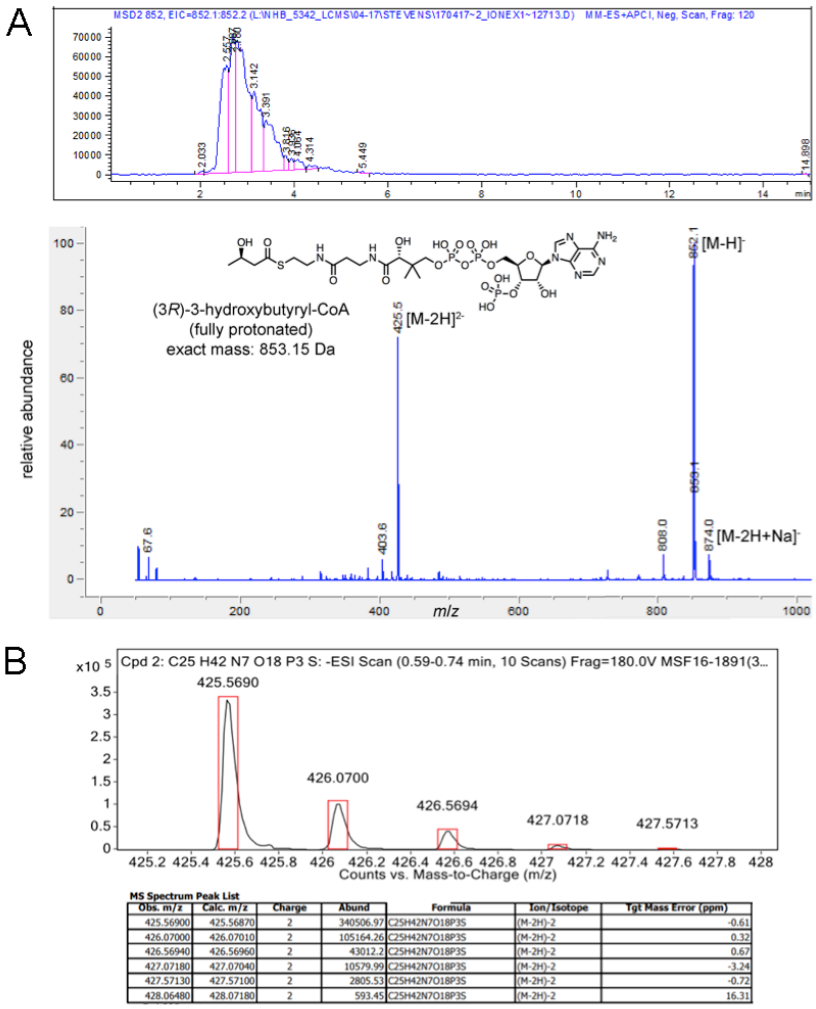


Figure S5. Combined fractions containing (3*R*)-3-hydroxybutyryl-CoA eluted from the Q-sepharose column.



MS Spectrum Peak List

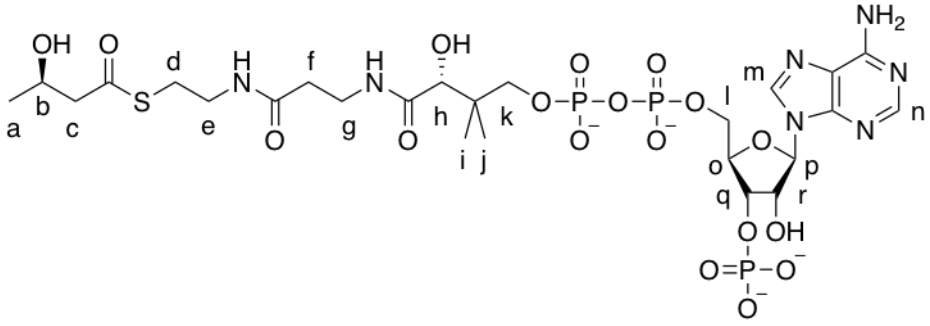
Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
425.56900	425.56870	2	340506.97	C25H42N7O18P3S	(M-2H)-2	-0.61
426.07000	426.07010	2	105164.26	C25H42N7O18P3S	(M-2H)-2	0.32
426.56940	426.56960	2	43012.2	C25H42N7O18P3S	(M-2H)-2	0.67
427.07180	427.07040	2	10579.99	C25H42N7O18P3S	(M-2H)-2	-3.24
427.57130	427.57100	2	2805.53	C25H42N7O18P3S	(M-2H)-2	-0.72
428.06480	428.07180	2	593.45	C25H42N7O18P3S	(M-2H)-2	16.31

Figure S6. Analysis of purified (3*R*)-3-hydroxybutyryl-CoA. A) LC/MS chromatogram and mass spectrum of (3*R*)-3-hydroxybutyryl-CoA. B) High-resolution mass spectral analysis shows the correct molecular formula for (3*R*)-3-hydroxybutyryl-CoA.

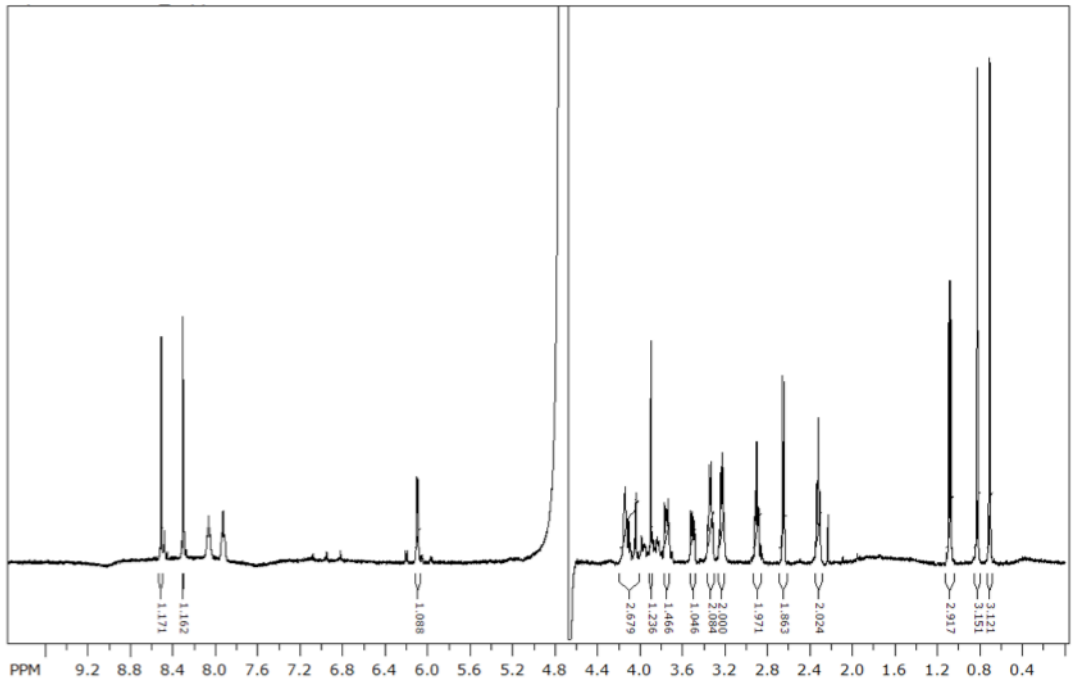
^{1}H NMR [400 MHz, 10% (v/v) D_2O, suppression of HOD and H_2O] δ 8.54 (s, 1H), 8.33 (s, 1H), 6.11 (d, *J* = 4.9 Hz, 1H), 4.18–4.02 (m, 3H), 3.91 (s, 1H), 3.76 (dd, *J* = 9.1, 4.8 Hz, 1H), 3.51 (dd, *J* = 9.6, 4.7 Hz, 1H), 3.35 (d, *J* = 6.1 Hz, 2H), 3.27–3.19 (m, 2H), 2.94–2.87 (m, 2H), 2.66 (d, *J* = 6.4 Hz, 2H), 2.36–2.29 (m, 2H), 1.08 (d, *J* = 6.3 Hz, 3H), 0.82 (s, 3H), 0.71 (s, 3H).

4.7 1H (q) and 4.4 1H (o) are not visible due to the water suppression technique.

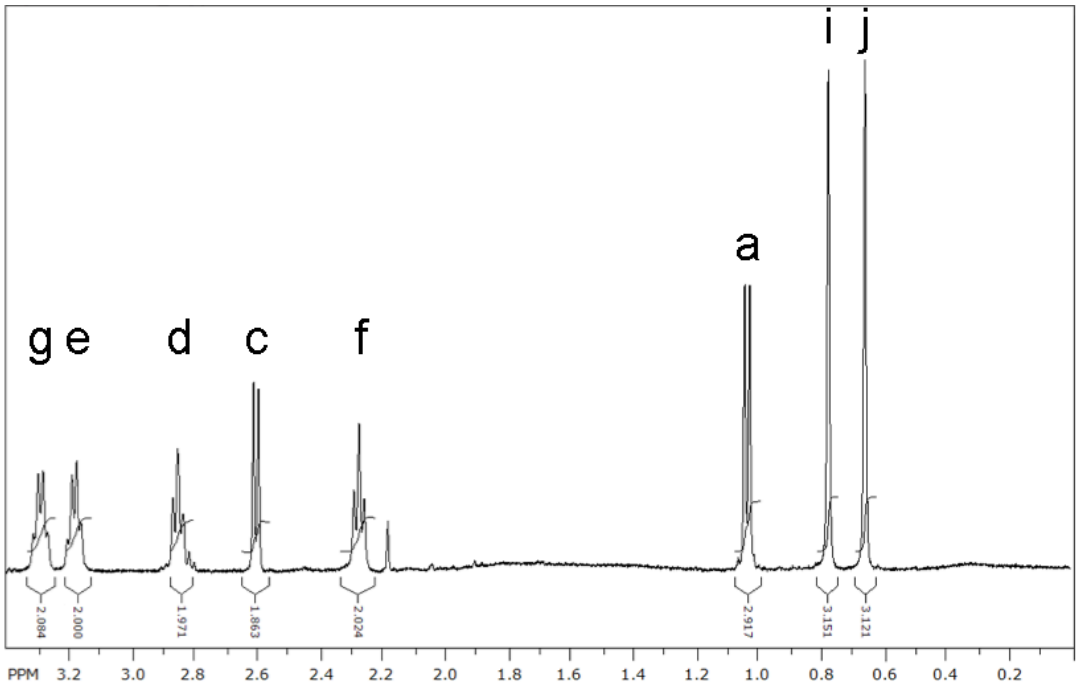
A



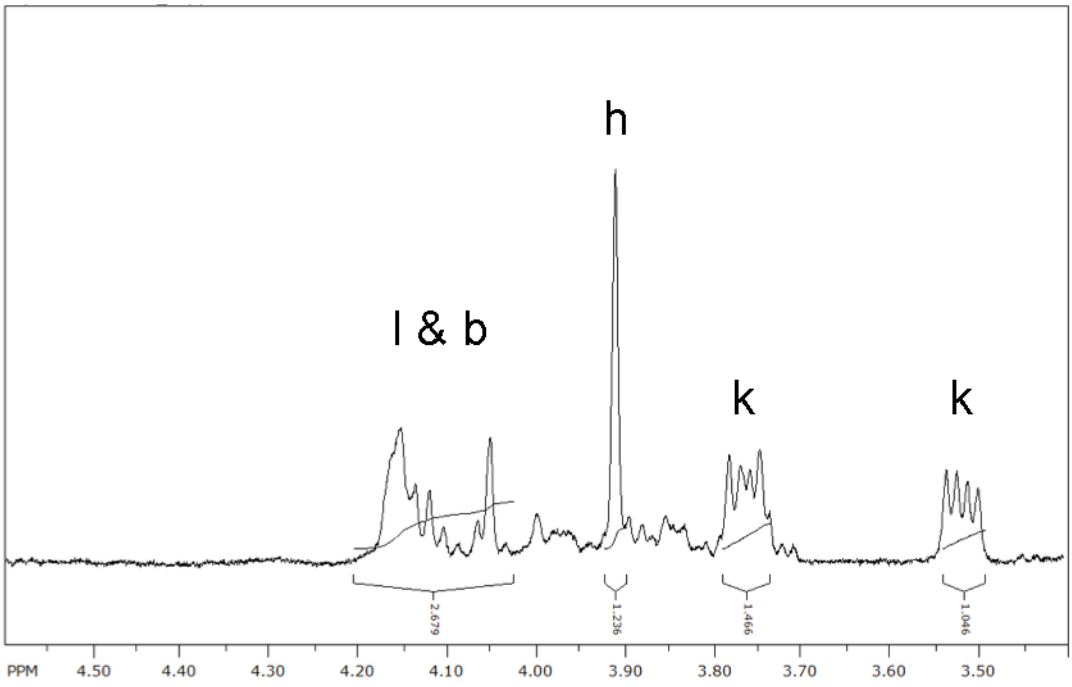
B



c


i
j
a
g e
d
c
f
2.084
2.000
1.971
1.863
2.024
2.917
3.151
3.121
PPM 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2

D


h
l & b
k
k
2.679
1.236
1.466
1.046
PPM 4.50 4.40 4.30 4.20 4.10 4.00 3.90 3.80 3.70 3.60 3.50

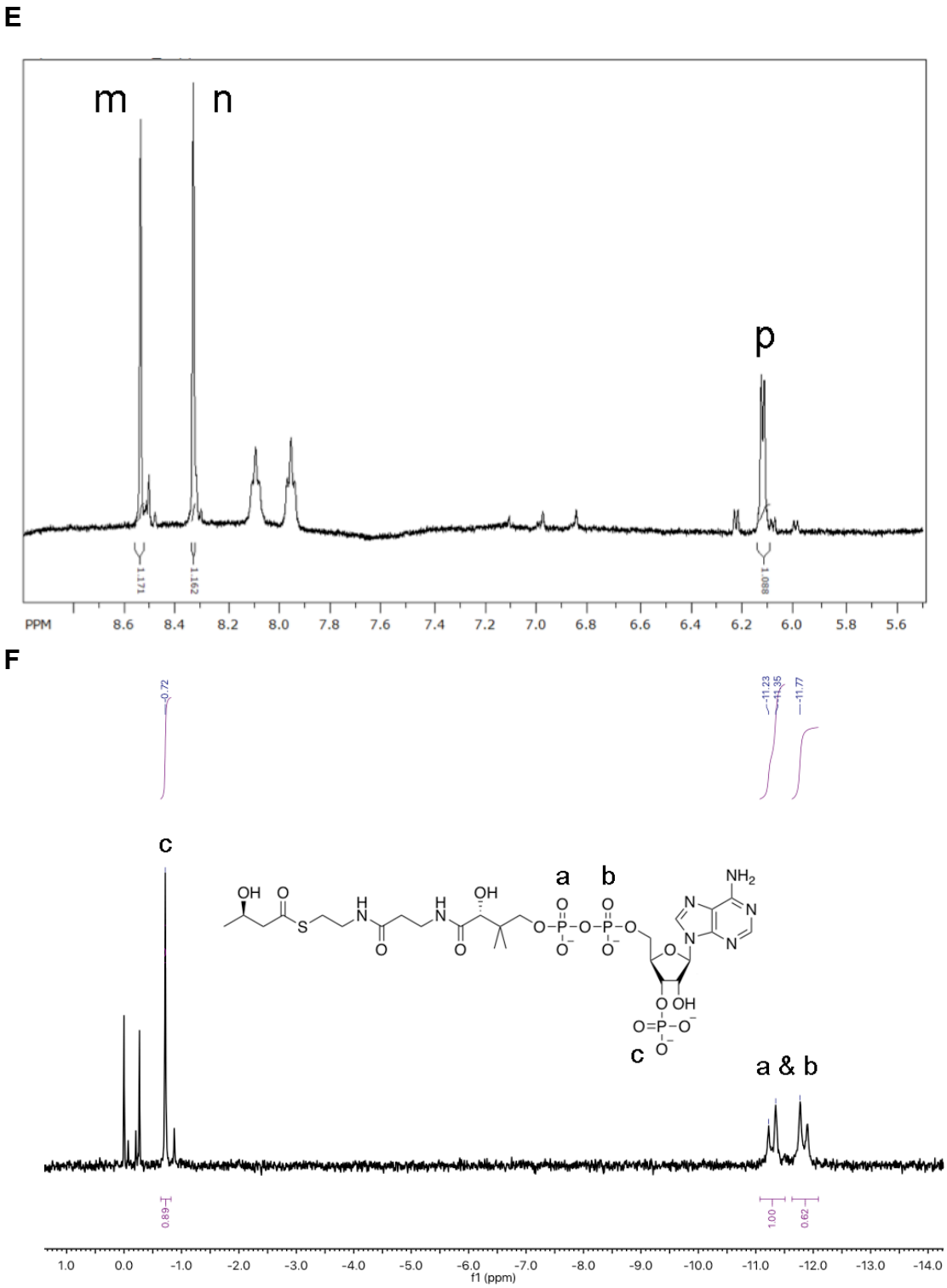


Figure S7. NMR analysis of purified (3*R*)-3-hydroxybutyryl-CoA. A) Structure of (3*R*)-3-hydroxybutyryl-CoA with protons labeled. B) ^{1}H-NMR spectrum of purified (3*R*)-3-hydroxybutyryl-CoA. C) Integration of ^{1}H-NMR peaks present in 0.0–3.4 ppm. D) Integration of ^{1}H-NMR peaks present in 3.4–4.6 ppm. E) Integration of ^{1}H-NMR peaks present in 5.5–9.0 ppm. F) Integration of ^{31}P-NMR spectrum of purified (3*R*)-3-hydroxybutyryl-CoA.

Quantification of purified (3R)-3-hydroxybutyryl-CoA. Absorbance at 259 nm was measured for fractions determined to contain pure (3*R*)-3-hydroxybutyryl-CoA by HPLC and NMR (Fractions 10-21)(Table S1). The absorbance at 259 nm of 2 μL of each fraction was measured on a NanoDrop 1000 (Thermo Scientific). The extinction coefficient for acyl-CoAs (15,000 $M^{-1}cm^{-1}$ at 259 nm)[5] was used to calculate the concentration of (3*R*)-3-hydroxybutyryl-CoA in each fraction. The total yield was calculated to be 119 mg in 451 mL of eluted fractions, resulting in a 69.6% yield.

Fraction	Abs @ 259nm	Volume (mL)	conc (mM)	Mass (mg)
10	0.744	34.6	0.496	14.64
11	0.638	37.0	0.425	13.43
12	0.541	24.0	0.361	7.38
13	0.538	42.0	0.359	12.85
14	0.465	50.0	0.310	13.22
15	0.372	32.5	0.248	6.88
16	0.416	37.0	0.277	8.75
17	0.447	36.5	0.298	9.28
18	0.496	36.0	0.331	10.16
19	0.425	44.0	0.283	10.64
20	0.286	37.0	0.191	6.02
21	0.236	40.5	0.157	5.44

Theoretical Total Yield (mg)	Experimental Total Yield (mg)	Percent Yield
170.62	118.68	69.56

NanoDrop 1000 path length = 1mm

Acyl-CoA Extinction Coefficient = 15000 M^{-1} cm^{-1}

Table S1. Quantification of (3*R*)-3-hydroxybutyryl-CoA in fractions 10-21.

Transfer to NAC and chiral analysis. The completed cascade reaction was adjusted to pH 8.5, NAC (240 mg, 10 equiv.) was added, and the mixture was stirred for 2 h at room temperature. The enzymes were removed through heating (80 °C for 1 h) and centrifugation (4000 x g, 10 min, 25 °C), and the resulting solution was thrice extracted with 50 mL ethyl acetate and dried with anhydrous Na_2SO_4. The solvent was removed through reduced pressure, and the residue was passed through a $CuSO_4$-impregnated silica gel column with ethyl acetate, yielding a sticky, yellowish oil after solvent evaporation. Chiral chromatography showed (*R*)-3-hydroxybutyryl-SNAC and no (*S*)-3-hydroxybutyryl-SNAC (Figures S8-S9).

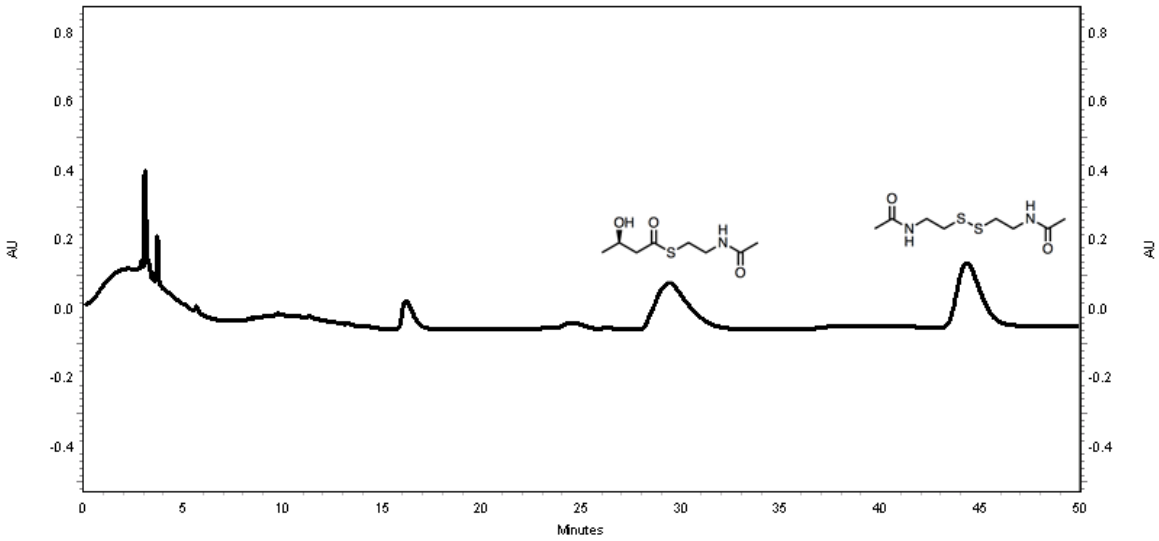


Figure S8. Chiral chromatography of (*R*)-3-hydroxybutyryl-SNAC generated through the cascade reaction. t_r = 29.3 min. The peak at 44.2 min is the NAC dimer, a contaminant that also serves as an internal standard (see Figure S9).

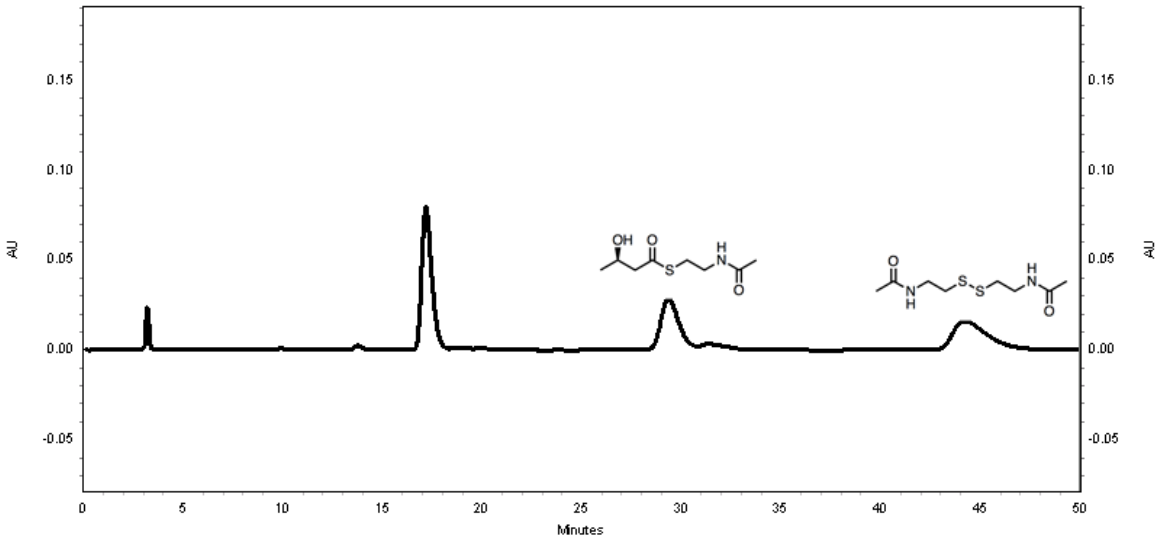


Figure S9. Chiral chromatography of authentic (*R*)-3-hydroxy-SNAC (t_r = 29.2 min) and authentic NAC dimer (t_r = 44.1 min).

Synthesis of chemical standards

Racemic 3-hydroxybutyryl-SNAC. The synthesis was performed following an established protocol.[4] Racemic 3-hydroxybutyric acid (220 mg, 1.06 mmol) was dissolved in 5 mL THF and cooled to 0 °C. DMAP (52 mg, 0.42 mmol) and EDC-HCl (441 mg, 2.30 mmol) were then added. The reaction was stirred for 5 min, and NAC (560 mg, 4.69 mmol) was added. The reaction was gradually warmed up to room temperature and stirred overnight. Next, the reaction was quenched with 1 M HCl (20 mL), extracted with ethyl acetate (40 mL), and dried over anhydrous Na_2SO_4. Solvent was removed by reduced pressure. Purification of the residue by column chromatography gave racemic 3-hydroxybutyryl-SNAC (20 mg, 9.2%) as a sticky, yellowish oil (Figures S10-S12).

Column: 1 x 9 cm using $CuSO_4$-impregnated silica gel, 150 mL (2:1 EtOAc/dichloromethane).
^{1}H NMR (400 MHz, chloroform-*d*) δ 5.97 (b, 1H), 4.25–4.17 (m, 1H), 3.43 (q, *J* = 6.1 Hz, 2H), 3.02 (t, *J* = 6.4 Hz, 2H), 2.60–2.41 (m, 2H), 1.98 (s, 3H), 1.25 (d, *J* = 6.3 Hz, 3H).
^{13}C NMR (400 MHz, chloroform-*d*) δ 196.62, 170.80, 64.18, 39.73, 30.65, 28.68, 23.15, 22.40.
LRESIMS *m*/*z* 228.1 $[M+Na]^+$ (calculated for $C_7H_{10}O_2Na$: 228.08).
HPLC (chiral) t_r = 29.6 min for (*R*)-3-hydroxybutyryl-SNAC, t_r = 31.2 min for (*S*)-3-hydroxybutyryl-SNAC

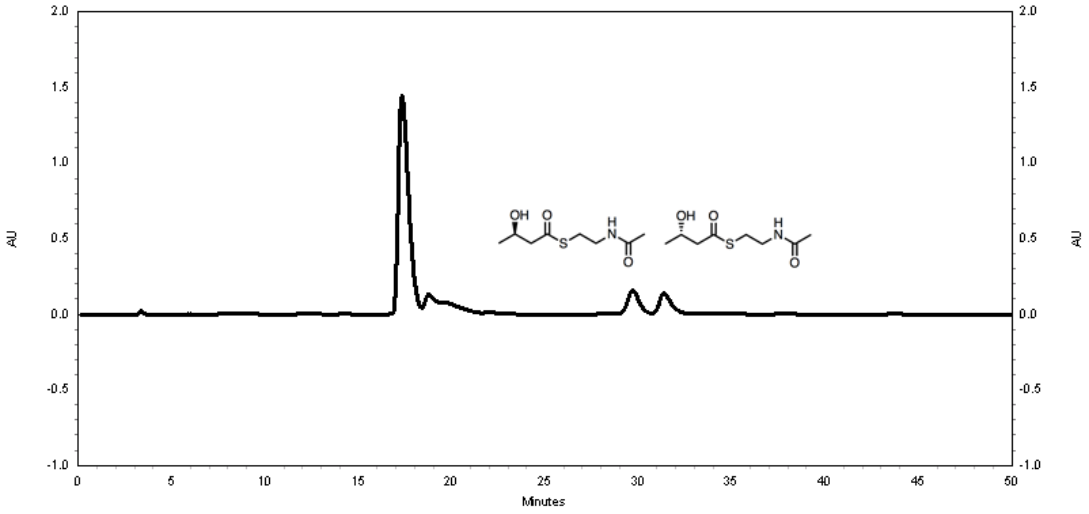


Figure S10. Chiral chromatography of synthetic racemic 3-hydroxybutyryl-SNAC. t_r = 29.6 min for (*R*)-3-hydroxybutyryl-SNAC, t_r = 31.2 min for (*S*)-3-hydroxybutyryl-SNAC.

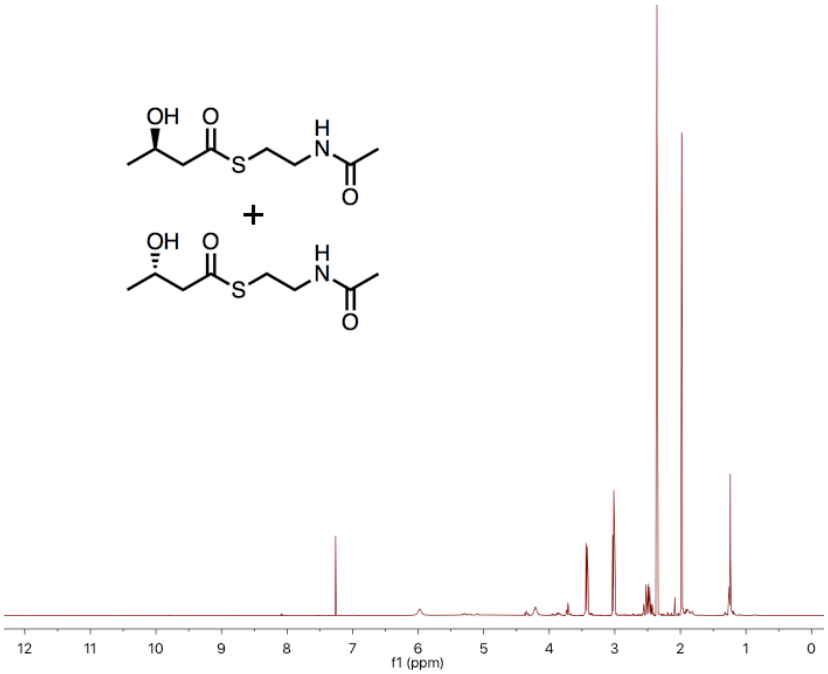


Figure S11. [1]H NMR of racemic 3-hydroxybutyryl-SNAC.

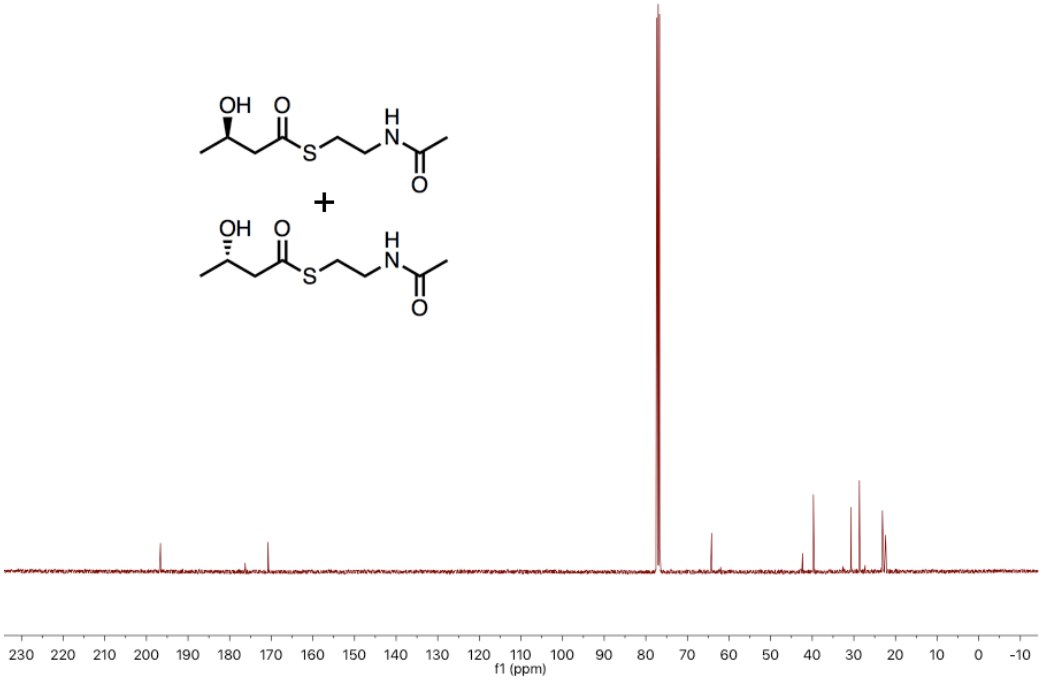


Figure S12. [13]C NMR of racemic 3-hydroxybutyryl-SNAC.

(R)-3-hydroxybutyryl-SNAC. The synthesis for racemic 3-hydroxybutyryl-SNAC was followed except for the use of authentic (*R*)-3-hydroxybutyryic acid rather than racemic 3-hydroxybutyric acid (Figures S13-S14). The ^{1}H-NMR matched that of racemic 3-hydroxybutyryl-SNAC.

Figure S13. HRESIMS of authentic (*R*)-3-hydroxybutyryl-SNAC, *m/z* 228.0666 $[M+Na]^{+}$ (calculated for $C_8H_{15}NO_3SNa$: 228.0670).

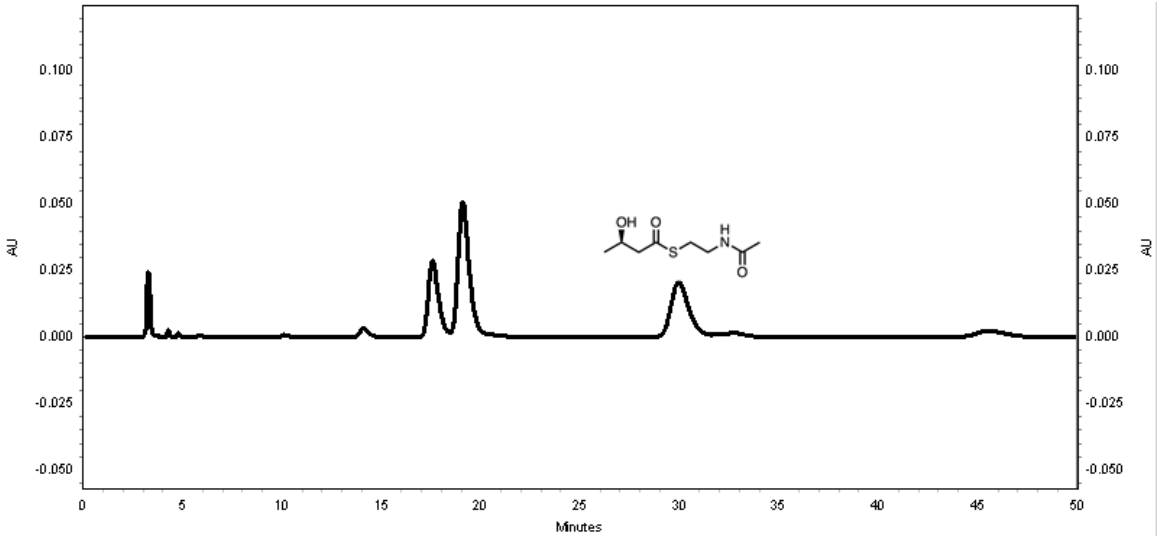


Figure S14. Chiral chromatography of synthetic (*R*)-3-hydroxybutyryl-SNAC (t_r = 29.6 min).

3S-hydroxybutyryl-SNAC. The synthesis for racemic 3-hydroxybutyryl-SNAC was followed except for the use of authentic (*S*)-3-hydroxybutyryic acid rather than racemic 3-hydroxybutyric acid (Figures S15-S16). The ^{1}H-NMR matched that of racemic 3-hydroxybutyryl-SNAC.

Figure S15. HRESIMS of authentic (*S*)-3-hydroxybutyryl-SNAC, *m/z* 228.0665 $[M+Na]^{+}$ (calculated for $C_8H_{15}NO_3SNa$: 228.0670).

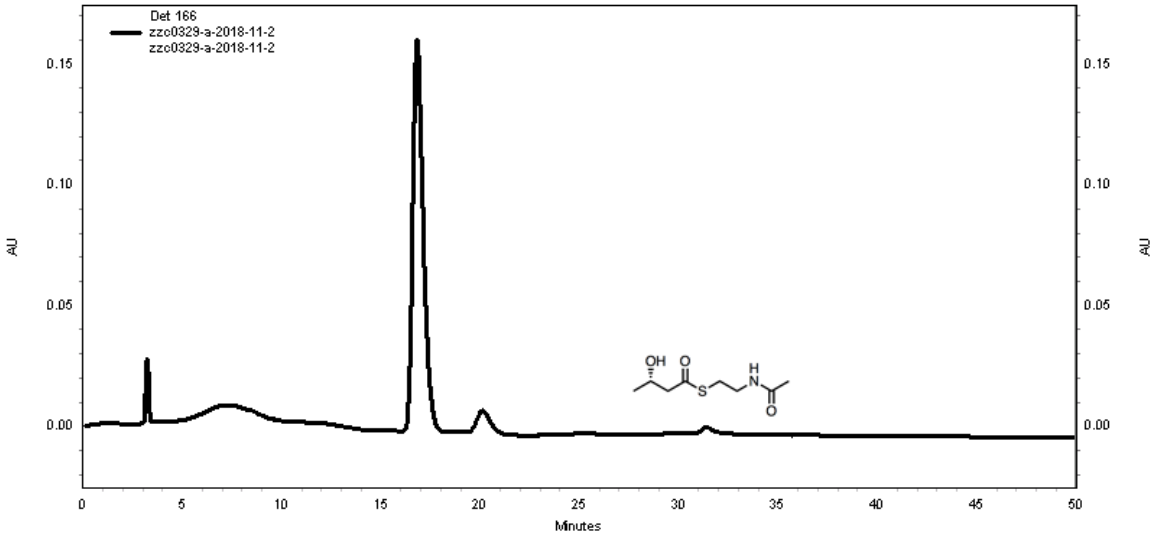


Figure S16. Chiral chromatography of synthetic (*S*)-3-hydroxybutyryl-SNAC (t_r = 31.2 min).

References

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