Supporting Information

A Mechanism-Based Fluorescence Transfer Assay for Examining Ketosynthase Selectivity

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Methods and Materials:

HPLC was performed with a Waters 1525 system. The gradient employed was A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5–95% B over 60 min with a Waters XBridge C18 5u column (4.6 × 100 mm). Mass spectra were acquired with a Waters Micromass ZQ mass detector in EI+mode: Capillary voltage = 3.50 kV, cone voltage = 3 V, RF lens = 0.0 V, source T = 100 °C, desolvation T = 200 °C, desolvation gas = 300 L h⁻¹, esolvation gas = 0.0 L h⁻¹ The system was operated by and spectra were processed using the Waters Empower software suite. Perkin Elmer Spectrum 100 FTIR instrument was used for IR measurements.

General method for preparation of all N-acetylcycteamine (SNAc) thioester derivatives. To a solution of triethylamine (2.80mmol) in dichloromethane (10mL) was added the appropriate acid (1.40mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40mmol), 1-Hydroxybenzotriazole (HOBt) (1.40mmol) and N-acetylcysteamine (SNAc) (1.35mmol) under Argon. The reaction mixture was stirred overnight. The organic layer was washed with saturated NaHCO₃ solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate, concentrated under vacuum, and purified by flash column to provide the final product in pure form.

Propionyl-SNAc (1) Pale yellow oil (74%). ¹H NMR (400 MHz, CDCl₃) δ ppm 0.86(t, J=7.58Hz, 3H) 1.69 (s, 3H) 2.28 (q, J=7.4Hz, 2H) 2.72(t, J=6.8Hz, 2H) 3.07 (q, J=6.6Hz, 2H) 7.39 (br s, 1H). 13C NMR (400 MHz, CDCl₃) δ ppm 199.4, 170.6, 39.0, 36.8, 28.0, 22.6, 9.4. IR: ν 3278, 2979, 1690, 1650, 1546, 1373, 1288, 1090, 935 cm⁻¹ LRMS (ESI+) m/z calcd. for C₇H₁₃NO₂S [M+H]+ 176.073, found 176.1.

Butyroyl-SNAc (2) Pale yellow solid (71%) ¹H NMR (400 MHz, CDCl₃) δ ppm 0.92-0.96(t, J=6.6 Hz, 3H), 1.67-1.69(m, 2H), 1.95(s, 3H), 2.52-2.56(2H), 2.99-3.00(t, 2H), 3.40-3.42(q, 2H), 6.12(s, 1H). 13C NMR (400 MHz, CDCl₃) δ ppm 11.54, 17.17, 21.27, 26.55, 37.64, 44.12, 168.64, 197.97. IR: ν 3291, 2967, 1651, 1547, 1436, 1370, 1284, 1112, 988, 905, 723 cm⁻¹ LRMS (ESI+) m/z calcd. for C₈H₁₅NO₂S [M+H]+ 190.089, found 190.0.

Pentanolyl-SNAc (3) Pale yellow solid (82%). ¹H NMR (400 MHz, CDCl₃) δ ppm 0.87-0.91 (t, J=6.6 Hz, 3H), 1.3-1.36(m, J=6.6 Hz, 2H), 1.58-1.66(m, J=6.6 Hz, 2H), 1.95 (s, 3H), 2.53-2.57 (t, J=6.6 Hz, 2H), 2.98-3.02 (t, J=6.6 Hz, 2H), 3.37-3.42 (q, J=6.5 Hz, 2H), 6.22 (s, 1H). 13C NMR (400 MHz, CDCl₃) δ ppm 11.71, 20.08, 21.10, 25.69, 26.38, 37.58, 41.83, 168.64, 197.92. IR: ν 3288, 3077, 2959, 2933, 2873, 1651, 1546, 1287, 1016, 731 cm⁻¹ LRMS (ESI+) m/z calcd. for C₉H₁₇NO₂S [M+H]+ 204.105, found 204.1.

Hexanoyl-SNAc (4) Colorless solid (73%). ¹H NMR (400 MHz, CDCl₃) δ ppm 0.84-0.87 (t, J=6.3 Hz, 3H), 1.25-1.31 (m, J=6.3 Hz, 4H), 1.58-1.66(m, J=6.3 Hz, 2H), 1.95 (s, 3H), 2.51-2.57 (t, J=6.3 Hz, 2H), 2.97-3.00 (t, J=6.3 Hz, 2H), 3.36-3.40 (q, J=6.3 Hz, 2H), 6.33 (s, 1H). 13C NMR (400 MHz, CDCl₃) δ ppm: 11.93, 20.35, 21.24, 23.41, 26.48, 29.13, 37.77, 42.16, 168.45, 198.24. IR: ν 3293, 3093, 2953, 2930, 2873, 1651, 1546, 817, 1027, 731 cm⁻¹ LRMS (ESI+) m/z calcd. for C₁₀H₁₉NO₂S [M+H]+ 218.120, found 218.0.

Heptanoyl-SNAc (5) Pale yellow solid (60%). ¹H NMR (400 MHz, CDCl₃) δ ppm 0.84-0.87 (t, J=6.3 Hz, 3H), 1.20-1.33 (m, J=6.3 Hz, 6H), 1.58-1.66(m, J=7.5 Hz, 2H), 1.95 (s, 3H), 2.51-2.55 (t, J=6.3 Hz, 2H), 2.97-3.01 (t, J=6.3 Hz, 2H), 3.36-3.40 (q, J=6.3 Hz, 2H), 6.33 (s, 1H). 13C NMR (400 MHz, CDCl₃) δ ppm : 12.12, 20.64, 21.23, 23.41, 26.48, 29.13, 37.77, 42.16, 168.45, 198.24. IR: ν 3291, 3093, 2953, 2930, 2870, 1641, 1551, 1408, 1358, 1292, 970, 745 cm⁻¹ LRMS (ESI+) m/z calcd. for C₁₀H₁₉NO₂S [M+H]+ 218.120, found 218.0.

Octanoyl-SNAc (6) Colorless solid (84%). ¹H NMR (400 MHz, CDCl₃) δ ppm 0.84-0.87 (t, J=6.3 Hz, 3H), 1.20-1.30 (m, J=6.3 Hz, 9H), 1.58-1.66(m, J=7.4 Hz, 2H), 1.94 (s, 3H), 2.52-2.55 (t, J=6.3 Hz, 2H), 2.97-3.01 (t, J=6.3 Hz, 2H), 3.37-3.42 (q, J=6.3 Hz, 2H), 6.27 (s, 1H). 13C NMR (400 MHz, CDCl₃) δ ppm 12.12, 20.64, 21.23, 23.72, 26.46, 26.94, 29.66, 37.76, 42.19, 168.47, 198.21. IR: ν 3291, 3101, 2922, 2848, 1682, 1637, 1555, 1294, 1178, 1045, 967, 728 cm⁻¹ LRMS (ESI+) m/z calcd. for C₁₂H₂₃NO₂S [M+H]+ 246.1522, found 246.1.
3-Methylbutanoyl-SNAc (7) Pale yellow oil (76%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 0.90-0.92 (d, J=6.6 Hz, 6H), 1.92 (s, 3H), 2.07-2.14 (dt, J=13.6, 6.8 Hz, 1H), 2.39-2.41 (d, J=7.1 Hz, 2H), 2.96-3.00 (t, J=6.6 Hz, 2H), 3.55-3.39 (q, J=6.3, 2H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 21.33, 26.51, 37.61, 125.39, 126.81, 131.81, 132.27, 134.78, 168.58, 190.33. IR: ν 3286.82, 2972.41, 2933.13, 1682.23, 1650.32, 1548.77, 1438.83, 1372.86, 1288.66, 1095.01, 1039.33, 893.45, 860.88, 701.62 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_8$H$_{13}$NO$_2$S [M+H]+ 218.1209, found 218.1.

2-Methylpropanoyl-SNAc (9) Pale yellow oil (64%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 1.10-1.14 (dt, J=6.9, 0.7 Hz, 6H), 1.94 (s, 3H), 2.70-2.77 (m, J=6.6 Hz, 1H), 2.97-3.00 (t, J=6.6 Hz, 2H), 3.13-3.16 (q, J=6.4 Hz, 2H), 6.56 (s, 1H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 9.07, 20.73, 21.11, 26.49, 37.66, 168.67, 197.59. IR: ν 3285.27, 3082.62, 2972.41, 2933.13, 1682.23, 1650.32, 1549.77, 1438.83, 1372.86, 1288.66, 1095.01, 1039.33, 995.60, 713.10 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_9$H$_{17}$NO$_2$S [M+H]+ 190.089, found 190.1.

Cyclopropanoyl-SNAc (10) Pale yellow oil (60%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 0.88-0.93 (m, 2H), 1.06-1.10 (m, 2H), 1.90 (s, 3H), 1.94-2.00 (m, 2H), 2.95-2.98 (t, J=6.4 Hz, 2H), 3.07-3.10 (q, J=6.2 Hz, 2H), 6.56 (s, 1H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 9.07, 20.73, 21.11, 26.49, 37.66, 168.67, 197.59. IR: ν 3285.27, 3082.62, 2932.76, 1651.90, 1544.26, 1367.02, 1039.33, 995.60, 713.10 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_8$H$_{13}$NO$_2$S [M+H]+ 188.0739, found 188.1.

Cyclopentanoyl-SNAc (11) Pale yellow oil (73%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 1.56-1.88 (m, J=6.3 Hz, 8H), 1.93 (s, 3H), 2.94-3.00 (t, J=6.6 Hz, 2H), 3.36-3.40 (q, J=6.1 Hz, 2H), 6.27 (s, 1H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 21.22, 23.95, 26.49, 28.7, 37.81, 51.31, 168.51, 201.65. IR: ν 3285.37, 3092.12, 3009.99, 2932.37, 1651.90, 1544.26, 1367.08, 1039.33, 995.60, 713.10 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{10}$H$_{17}$NO$_2$S [M+H]+ 216.105, found 216.1.

Cyclohexanoyl-SNAc (12) Pale yellow oil (78%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 1.15-1.29 (m, 4H), 1.29-1.46 (qd, J=12(3), 2.8 Hz, 2H), 1.61-1.89 (m, 5H), 1.93 (s, 3H), 2.44-2.50 (m, 1H), 2.95-3.00 (t, J=6.6 Hz, 2H), 3.36-3.40 (q, J=6.3 Hz, 2H), 6.33 (s, 1H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 21.32, 23.95, 26.49, 28.7, 37.81, 51.31, 168.51, 201.65. IR: ν 3286.02, 2972.41, 2933.17, 2855.68, 1654.20, 1547.67, 1287.90, 997.65, 730.27 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{11}$H$_{19}$NO$_2$S [M+H]+ 230.120, found 230.1.

Benzoyl-SNAc (13) Yellow oil (75%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 1.93 (s, 3H), 3.22-3.25 (t, J=6.6 Hz, 2H), 3.51-3.57 (q, J=6.6 Hz, 2H), 6.18 (s, 1H), 7.45-7.48 (t, 2H), 7.58-7.62 (t, 1H), 7.95-7.98 (d, 2H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 21.32, 26.67, 37.76, 125.39, 126.81, 131.81, 134.78, 168.58, 190.33. IR: ν 3299.96, 3085.64, 2929.32, 1655.48, 1545.73, 1446.18, 1400.27, 1359.73, 1295.83, 1203.59, 910.55, 768.55, 725.93, 658.99 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{11}$H$_{19}$NO$_2$S [M+H]+ 240.073, found 240.1.

4-Hydroxybenzoyl-SNAc (14) Colorless gelatinous solid (84%). $^1$H NMR (400 MHz, CDCl$_3$) δ ppm 1.81 (s, 3H), 3.05-3.08 (t, J=6.6 Hz, 2H), 3.23-3.27 (q, J=6.6 Hz, 2H), 6.87-6.89-7.48 (d, 2H), 7.80-7.82 (d, 2H), 8.10-8.13 (t, J=5.6 Hz, 1H), 10.55 (s, 1H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ ppm 21.09, 26.51, 36.96, 37.61, 37.81, 38.02, 38.23, 38.65, 114.13, 126.14, 127.94, 161.31, 167.95, 187.45. IR: ν 3436.12, 2251.86, 1655.24, 1215.62, 1023.59, 821.43, 758.88 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{11}$H$_{15}$NO$_2$S [M+H]+ 240.068, found 240.1.
4-Aminobenzoyl-SNAc (15) Pale yellow solid (71%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 1.97 (s, 3H), 3.18-3.21 (t, $J=6.6$ Hz, 2H), 3.52-3.53 (q, $J=6.6$ Hz, 2H), 4.23 (s, 2H), 6.04 (s, 1H), 6.64-6.66 (d, 2H), 7.81-7.83 (d, 2H). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 21.31, 26.27, 38.23, 111.83, 124.76, 127.88, 149.92, 168.58, 188.66. IR: $\nu$ 3435.45, 3337.65, 3214.55, 2926.30, 1734.94, 1627.62, 1566.86, 1512.79, 1166.68, 908.76, 830.93 cm$^{-1}$. LRMS (ESI+) m/z calcd. for C$_{11}$H$_{14}$N$_2$O$_2$S [M+H]$^+$ 239.084, found 239.1.

4-Nitrobenzoyl-SNAc (16) White solid (74%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 2.00 (s, 3H), 3.28-3.32 (t, $J=6.6$ Hz, 2H), 3.54-3.59 (q, $J=6.6$ Hz, 2H), 6.05 (s, 1H), 8.11-8.14 (d, 2H), 8.30-8.34 (d, 2H). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 21.22, 27.25, 37.19, 122.00, 126.37, 139.34, 148.61, 168.79, 188.44. IR: $\nu$ 3286.71, 3079.65, 2931.34, 1648.22, 1547.62, 1518.41, 1348.69, 1194.19, 919.25, 848.83, 718.01 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{11}$H$_{12}$N$_2$O$_4$S [M+H]$^+$ 269.059, found 269.0.

4-Methylbenzoyl-SNAc (17) White solid (67%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 1.96 (s, 3H), 2.39 (s, 3H), 3.18-3.22 (t, $J=6.6$ Hz, 2H), 3.48-3.53 (q, $J=6.6$ Hz, 2H), 6.36 (s, 1H), 7.22-7.24 (d, 2H), 7.83-7.85 (d, 2H). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 19.78, 21.26, 26.54, 37.76, 125.42, 127.43, 132.26, 142.71, 168.64, 189.81. IR: $\nu$ 3305.41, 3076.62, 230.59, 1543.57, 1362.91, 1290.17, 1206.37, 1173.56, 913.35, 815.70 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{12}$H$_{15}$NO$_2$S [M+H]$^+$ 238.089, found 238.1.

4-Methoxybenzoyl-SNAc (18) Pale yellow oil (64%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 1.98 (s, 3H), 3.20-3.23 (t, 2H), 3.51-3.55 (m, $J=6.1$ Hz, 2H), 3.88 (s, 3H), 6.10 (s, 1H), 6.93-6.95 (d, $J=8.6$ Hz, 2H), 7.94-7.96 (d, $J=8.8$ Hz, 2H). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 21.31, 26.50, 37.92, 53.64, 111.95, 127.60, 162.11, 168.54, 188.77. IR: $\nu$ 3303.88, 3075.41, 2836.53, 1650.92, 1600.77, 1575.18, 1542.59, 1506.15, 1363.23, 1223.16, 1028.35, 733.44 cm$^{-1}$ LRMS (ESI+) m/z calcd. for C$_{12}$H$_{15}$NO$_3$S [M+H]$^+$ 254.083, found 254.0.
$^{1}H$ NMR of Poc $\beta$-lactam

$^{13}C$ NMR of Poc $\beta$-lactam
$^1$H NMR 1

$^{13}$C NMR 1
$^1$H NMR

$^{13}$C NMR
$^1$H NMR

$^1$C NMR
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$^1$H NMR of Rhodamine Azide

$^{13}$C NMR of Rhodamine Azide
**Gel Stains:** Full PAGE gels for fluorescence assays.

**Fig. 5:** SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT 6 pre-treated with various concentrations of compound 1

Fluorescence intensities for the above gel were obtained via fluorescence analysis using the ImageJ software. % KS acylation for each concentration of compound 1 assayed was calculated by subtracting the % of maximum fluorescence (determined by comparison to the control lane) for each band from 100%. A plot of % Acylation versus compound 1 concentration is shown below.
**Fig. 6:** SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT 6 pre-treated with compounds 1-6. Repeated runs for compounds 1-6.

**Fig. 8:** SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT 6 pre-treated with compounds 7-12

**Fig. 10:** SDS-PAGE analysis of fluorescence transfer from ACP2 to KSAT 6 pre-treated with compounds 13-18
Fig. 11: SDS-PAGE analysis of fluorescence transfer from ACP2 to apo-module 6 pre-treated with compounds 1-18.

Dimerization of ACP2 is copper dependent and random. It does not affect loading of the KSAT or the full module. The SDS-PAGE gel below demonstrates the copper-dependency of ACP2 dimer formation. Lane 1: ACP2 (25μM). Lane 2: ACP2 (25μM) + N-Poc-β-lactam (2.5mM). Lane 3: ACP2 (25μM) + Copper sulfate (1mM) + sodium ascorbate (1mM). All samples are in 100mM phosphate buffer (pH = 7.2) with 2.5mM TCEP.
HPLC spectra (TIC)

(1)

(2)

(3)