Amino Acid-Accepting Ketosynthase Domain from a *trans*-AT Polyketide Synthase Exhibits High Selectivity for Predicted Intermediate

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Experimental

Cloning of the BaeJ KS1 Domain

The amplification of the BaeJ KS1 gene region of *Bacillus amyloliquefaciens* FZB42 was performed with Phusion (NEB). The primers BaeKS1_for (5'-AAACCATGGAACAGAACAAAAACCGAAAGAA-3') and BaeKS1_rev (5'-AAAAAAAGCGGCCGCTCAATTTTGCTGCACGAGCGGGTG-3') were used. The PCR product was digested with *Bam*HI and *Not*I and then purified on a TAE-agarose gel. The bands were excised and purified with a gene jet gel extraction kit (Fermentas). The insert was ligated into the pHis8[1] vector using T4 ligase (NEB) at 16 °C (overnight). The ligated vector was transformed into *E. coli* BL21(DE3) cells and plated on LB agar containing kanamycin for resistance selection. Colonies were picked, grown overnight in LB media, miniprepped, and sequenced to verify insertion of vector. An appropriate colony was used for protein expression.

Construction of BaeJ KS1(N206A) Mutant

The mutant KS1 gene was constructed using the Phusion (NEB) mutagenesis procedure. The entire expression plasmid, pHis8+KS1, was amplified as a linear product using the primers KS1NA_For (5'-TGCTCATCTTCGTTAATCGGCCTGC-3') and KS1NA_Rev (5'- TGCGGCGTGGACAAAATAGCTCGG-3') which had been previously phosphorylated using polynucleotide kinase (NEB). KS1_Rev introduced an AAC to GCA mutation (Asn206Ala). The PCR product was digested with DpnI to remove the template DNA, gel purified from a TAE-Agarose gel using a Nucleospin® gel purification kit (Macherey-Nagel), ligated overnight using T4 Ligase (NEB) and transformed into *E. coli* strain XL1-Blue. The presence of the correct mutation was confirmed by sequencing.

KS Domain Expression & Purification

Ketosynthase (KS) domains were overexpressed as N-terminally His-tagged proteins in E. coli BL21(DE3) cells. Typically, LB media (1 L) containing kanamycin (30µg/mL) was inoculated with 0.01 volumes (10 mL) overnight culture of cells carrying the relevant plasmid and incubated for 3-4 hours at 37 °C until an O.D. of 0.7 was reached. Expression of KS domains was induced by the addition of IPTG (1 mM) and cultures were incubated for a further 14 hours at 16°C. Cells were harvested by centrifugation, resuspended in resuspension buffer (25 mM Tris-HCl, 0.5M NaCl, pH 7.6), lysed by sonication and clarified by centrifugation.

The supernatant was collected and loaded onto a 5ml Hitrap Chelating column (GE Healthcare) which had been charged with 0.1 M $NiSO_4$ and equilibrated in resuspension buffer. The column was washed in resuspension buffer containing 2.5 mM imadazole, and protein was eluted using a linear gradient of 2.5-500 mM imidazole. Fractions containing KS domain protein were identified by SDS-PAGE, pooled, and concentrated using

vivaspin spin concentrators (Vivascience). Glycerol was added to 10% (v/v) and aliquots were snap frozen in liquid nitrogen before storage at -80˚C.

SDS-PAGE Gel and ESI-MS Spectra of KS Domains

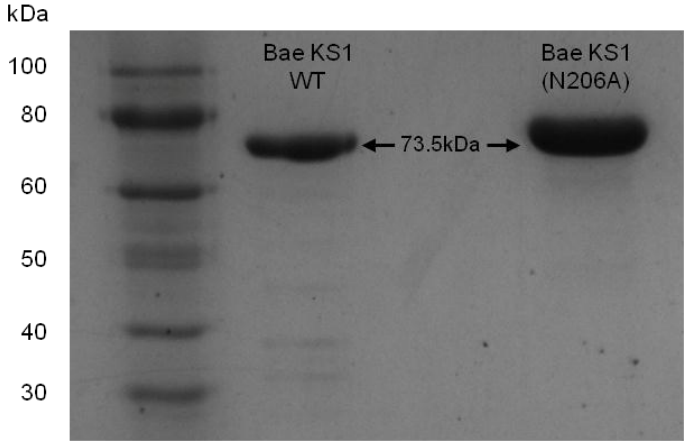


Figure S1: 12% SDS-PAGE gel of WT BaeJ KS1 and BaeJ KS1(N206A) post His-tag purification.

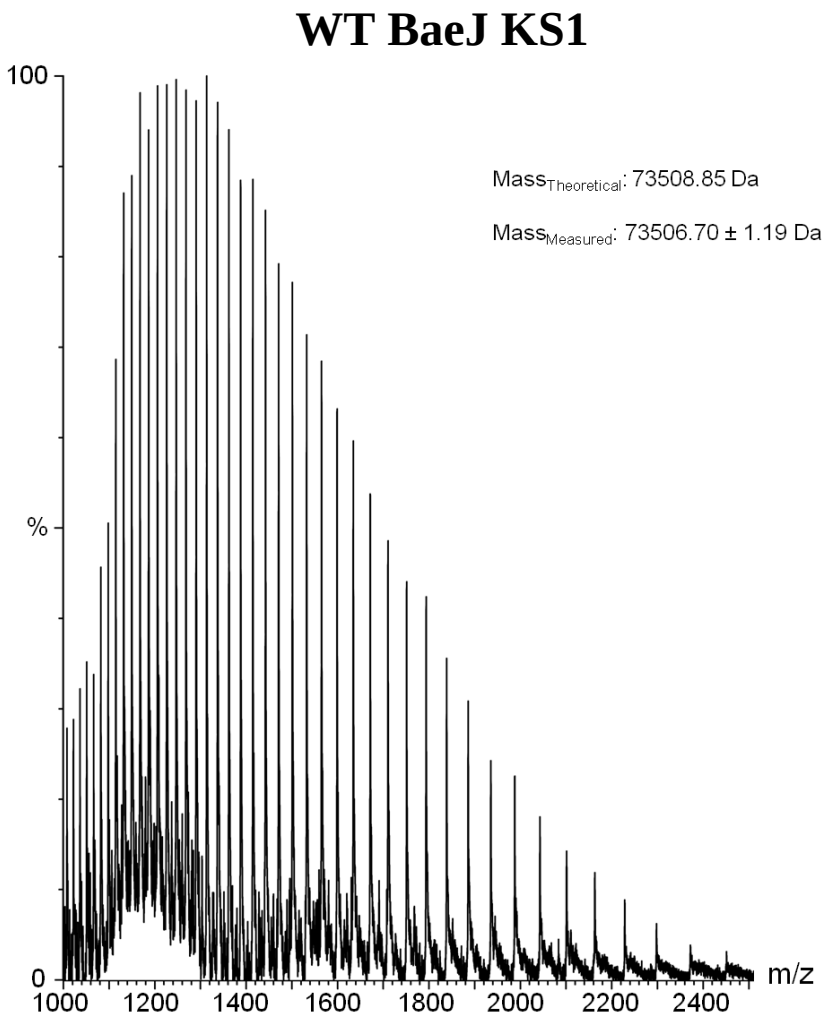


Figure S2: nESI mass spectrum of WT BaeJ KS1 sprayed from 80:20 MeCN:H_2O 0.1% TFA, resulting in a measured mass of 73,506 Da.

BaeJ KS1 (N206A)

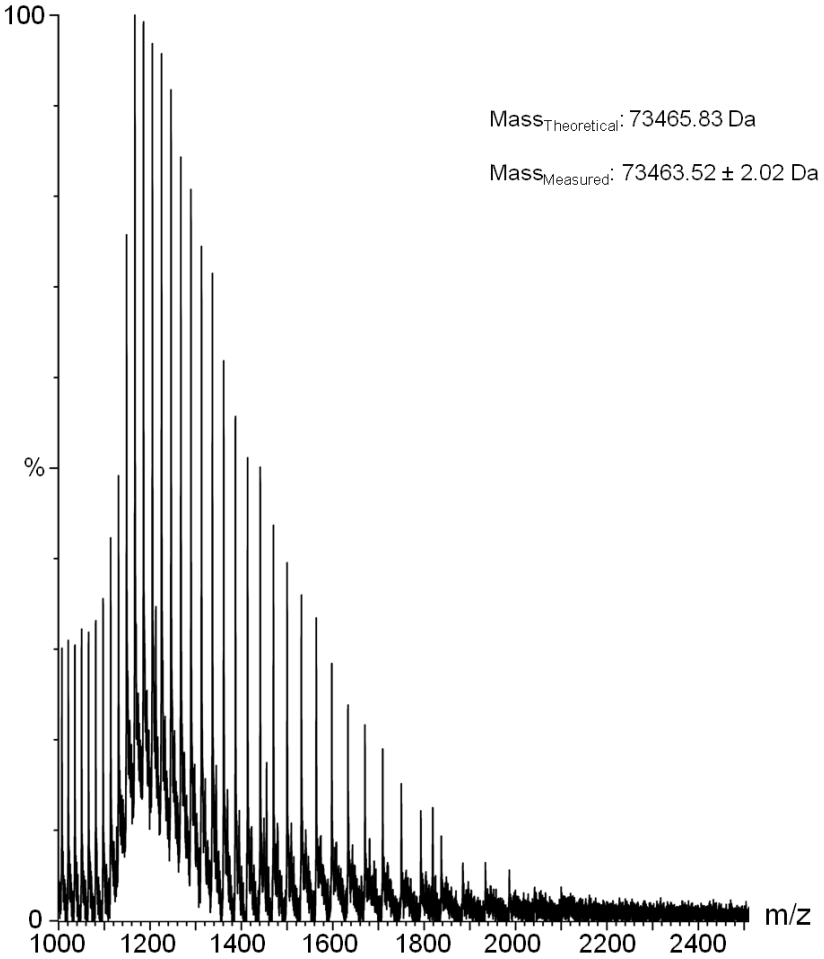


Figure S3: nESI mass spectrum of BaeJ KS1(N206A) sprayed from 80:20 MeCN:H_2O 0.1% TFA, resulting in a measured mass of 73,463 Da.

SNAC Thioester Synthesis

General information

All chemicals were purchased from commercial sources and were used without further purification. Solvents were dried as described in the literature.[2] All reactions were carried out under an atmosphere of argon unless otherwise noted. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker dpx 400 (400 MHz/ 100 MHz) or Bruker dpx 300 (300 MHz/ 75 MHz) spectrometer at room temperature. Chemical shifts were reported in parts per million (ppm) and the solvent residual peak was used as internal reference: ^{1}H-NMR ($CHCl_3$: 7.26 ppm, DMSO: 2.50 ppm), ^{13}C-NMR ($CDCl_3$: 77.16 ppm, DMSO-d_6: 39.52). Multiplicities were indicated as the following: s (singlet), d (doublet), t (triplet), q (quartet), h (heptet), m (multiplet) or br (broad). High resolution mass spectra (HRMS) were recorded with a Bruker micrOTOF-Q mass-spectrometer. IR was recorded on a Thermo Smart Orbit Nicolet 380 FT-IR spectrometer. TLC was performed on silica gel plates (Merck Silica gel

60 F_{254}) and visualized with Seebach reagent (12.5 g phosphomolybdic acid, 5.0 g cerium(IV)sulphate tetrahydrate, 16.0 mL water, 450.0 mL conc. sulfuric acid). Flash column chromatography was performed on silica gel (Merck-Kieselgel 60, 40 – 60 mesh). Abbreviations used: Boc – *tert*-butyloxycarbonyl, CDI – Carbonyldiimidazole, DCC – dicyclohexylcarbodiimid, EDC-HCl – *N*-(3-Dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride, HOBt - 1-Hydroxybenzotriazole hydrate, NMM – *N*-Methylmorpholine.

Benzyl 2-(4-methyl-2-oxopentanamido)acetate (14a)[3]

EDC-HCl (1.77 g, 9.23 mmol, 1.2 eq.) and HOBt (1.25 g, 9.23 mmol, 1.2 eq.) were added to a stirred solution of 4-methyl-2-oxovaleric acid (**12**) (1.00 g, 7.69 mmol) in dry THF (45 mL) at 0 °C. After stirring for 30 min, NMM (2.5 mL) was added, followed by glycine benzylester hydrochloride (**13a**) (1.86 g, 9.23 mmol, 1.2 eq). The reaction mixture was warmed to room temperature, stirred for 24 h, then diluted with EtOAc (100 mL). The organic phase was washed with saturated aqueous NH_4Cl-solution, (3 x 20 mL), saturated aqueous $NaHCO_3$-solution (3 x 20 mL) and brine (2 x 20 ml). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by column chromatography (SiO_2, EtOAc : c-hexane, 1:3, v/v, R_f = 0.50) gave the desired amide **14a** (1.67 g, 78%) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (d, J = 6.8 Hz, 6H), 2.18 (th, J = 6.8, 6.8 Hz, 1H), 2.89 (d, J = 6.8 Hz, 2H), 4.11 (d, J = 5.8 Hz, 2H), 5.20 (s, 2H), 7.33-7.40 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.6 (CH_3), 24.5 (CH), 41.2 (CH_2), 45.4 (CH_2), 67.6 (CH_2), 128.6 (CH), 128.79 (CH), 128.83 (CH), 136.1 (C), 160.5 (C), 168.9 (C), 197.9 (C); IR (neat) ν_{max} 6970, 1151, 1191, 1680, 1721, 1747, 2356, 2958, 3367 cm^{-1}; HRESIMS *m/z* 300.1201 (calcd for $C_{15}H_{19}NO_4Na$, 300.1206).

Benzyl 2-(4-methyl-2-oxopentanamido)propanoate (14b)

Prepared from alanine benzyl ester *p*-toluenesulfonate (**13b**) (2.97 g, 9.23 mmol, 1.2 eq.) according to the procedure described for the amide **14a**. Purification by column chromatography (SiO_2, EtOAc : *c*-hexane, 1:2.5, *v*/*v*, R_f = 0.72) gave the alanine amide **14b** (1.19 g, 53%) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (d, J = 6.6 Hz, 6H), 1.46 (d, J = 7.2 Hz, 3H), 2.17 (th, J = 6.6, 6.6 Hz, 1H), 2.78 (d, J = 6.6 Hz, 2H), 4.58 (dq, J = 7.2, 7.7 Hz, 1H), 5.10 (d, J = 12.5 Hz, 1H), 5.14 (d, J = 12.5 Hz, 1H), 7.30-7.40(m, 5H), 7.43 (brd, J = 7.70, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) *δ* 18.2 (CH_3), 22.6 (CH_3), 22.7 (CH_3), 24.5 (CH), 45.3 (CH_2), 48.3 (CH_2), 67.5 (CH_2), 128.3 (CH), 128.7 (CH), 128.8 (CH), 135.3 (C), 159.8 (C), 171.9 (C), 198.2 (C); IR (neat) ν_{max} 668., 1116, 1683, 1742, 2341, 2956 cm^{-1}; HRESIMS *m/z* 314.1353 (calcd for $C_{16}H_{21}NO_4Na$ 314.1363).

Benzyl 3-methyl-2-(4-methyl-2-oxopentanamido)butanoate (14c)

Prepared from valine benzyl ester *p*-toluenesulfonate (**13c**) (3.50 g, 9.23 mmol, 1.2 eq.) according the procedure described for the amide **14a**. Purification by column chromatography (SiO_2, EtOAc : *c*-hexane, 1:3, *v*/*v*, R_f = 0.59) gave the valine amide **14c** (1.43 g, 58%) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (d, J = 6.9 Hz, 3H), 0.92 (d, J = 6.9 Hz, 3H), 0.94 (d, J = 6.6 Hz, 3H), 0.95 (d, J = 6.6 Hz, 3H), 2.17 (th, J = 6.6, 6.6 Hz, 1H), 2.24 (dh, J = 6.9, J = 4.9 Hz, 1H), 2.78 (d, J = 6.6 Hz, 2H), 4.51 (dd, J = 9.9, 4.9 Hz, 1H), 5.14 (d, J = 12.2 Hz, 1H), 5.21 (d, J = 12.2 Hz, 1H), 7.30-7.40 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.7 (CH_3), 19.1 (CH_3), 22.62 (CH_3), 22.63 (CH_3), 24.5 (CH), 31.5 (CH), 45.4 (CH_2), 57.3 (CH_2), 67.3 (CH_2), 128.5 (CH), 128.6 (CH), 128.7 (CH), 135.3 (C), 160.2 (C), 170.9 (C), 198.2 (C); IR (neat) ν_{max} 697, 1148, 1513, 1683, 1739, 2357, 2961 cm^{-1}; HRESIMS *m/z* 314.1667 (calcd for $C_{18}H_{25}NO_4Na$ 342.1676).

2-(4-Methyl-2-oxopentanamido)acetic acid (15a)[4]

A suspension of amide **14a** (0.40 g, 1.44 mmol) and Pd/C (0.04 g, 10 mol%) in anhydrous methanol (4 mL) was stirred under hydrogen atmosphere (1 bar) at room temperature for 16 h. The suspension was filtered through a plug of Celite ®545 and rinsed with EtOAc (20 mL). The solvents of the combined organic fractions were removed under reduced pressure, yielding the desired acid **15a** (0.27 g, quant.) as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.94 (d, J = 6.70 Hz, 6H), 2.16 (th, J = 6.70, 6.70 Hz, 1H), 2.78 (d, J = 6.70 Hz, 2H), 4.09 (s, 2H), 7.55 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 22.6 (CH_3), 24.4 (CH), 41.0 (CH_2), 45.4 (CH_2), 160.7 (C), 172.9 (C), 197.9 (C); IR (neat) ν_{max} 669, 1212, 1680, 1747, 2339, 2356, 2957, 3350 cm^{-1}; HRESIMS *m/z* 186.0763 (Calcd for $C_8H_{12}NO_4$ 186.0761)$^{-}$.

2-(4-Methyl-2-oxopentanamido)propanoic acid (15b)

Prepared from amide **14b** (1.19 g, 4.08 mmol) according to the procedure described for acid **15a**. Hydrogenation was performed in anhydrous methanol (12 mL), yielding in the desired acid **15b** (0.74 g, 90%) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (d, J = 6.76, 6H), 1.51 (d, J = 7.22, 3H), 2.17 (th, J = 6.76, 6.76 Hz, 1H), 2.79 (d, J = 6.76 Hz, 2H), 4.57 (dq, J = 7.45, 7.22 Hz, 1H), 7.43 (brd, J = 7.45 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.9 (CH_3), 22.6 (CH_3), 24.5 (CH), 45.3 (CH_2), 48.1 (CH), 160.0 (C), 176.5 (C), 198.1 (C); IR (neat) ν_{max} 669, 1158, 1522, 1675, 1723, 2341, 2357, 2959 cm^{-1}; HRESIMS *m/z* 200.0927 (calcd for $C_9H_{14}NO_4$ 200.0928).

3-Methyl-2-(4-methyl-2-oxopentanamido)butanoic acid (15c)

Prepared from amide **14c** (1.43 g, 4.48 mmol) according to the procedure described for acid **15a**. Hydrogenation was performed in anhydrous methanol (15 mL), yielding in the desired acid **15c** (0.94 g, 92%) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84-0.98 (m, 12H), 2.13 (th, J = 6.75, 6.75 Hz, 1H), 2.25 (dh, J = 6.66, 4.95 Hz, 1H), 2.75 (d, J = 6.75 Hz, 2H), 4.44 (dd, J = 9.13, 4.95 Hz, 1H), 7.43 (brd, J = 9.13 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.6 (CH_3), 19.1 (CH_3), 22.6 (CH_3), 24.4 (CH), 31.1 (CH), 45.4 (CH_2), 57.3 (CH), 160.4 (C), 174.7 (C), 198.1 (C); IR (neat) ν_{max} 668, 1150, 1370, 1525, 1673, 1718, 2341, 2358, 2962 cm^{-1}; HRESIMS*m/z* 228.1228 (calcd for $C_{11}H_{18}NO_4$ 228.1241).

***S*-2-Acetamidoethyl 2-(4-methyl-2-oxopentanamido)ethanethioate (2)**[5]

Carbonyldiimidazole (CDI) (1.47 g, 9.07 mmol, 1.2 eq.) was added to a stirred solution of acid **15a** (1.41 g, 7.54 mmol) in anhydrous THF (30 mL) at room temperature. After 10 min *N*-acetylcysteamine (0.96 mL, 1.08 g, 9.05 mmol, 1.2 eq.) was added and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (20 mL). The organic phase was washed with water (3 x 5 mL) and brine (2 x 5 mL). The organic phase was dried (Na_2SO_4) and the solvent was removed under reduced pressure. Purification by column chromatography (SiO_2, EtOAc, R_f = 0.28) gave the thioester **2** (1.30 g, 60%) as a white waxy solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (d, J = 6.50 Hz, 6H), 1.95 (s, 6H), 2.16 (th, J = 6.50, 6.50 Hz, 1H), 2.77 (d, J = 6.50 Hz, 2H), 3.06 (t, J = 6.54 Hz, 2H), 3.36-3.44 (m, 2H), 4.21 (d, J = 6.43 Hz, 2H), 6.09 (brs, 1H) 7.65 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 22.6 (CH_3), 23.2 (CH_3), 24.5 (CH), 28.6 (CH_2), 39.2 (CH_2), 45.4 (CH_2), 49.0 (CH_2), 160.7 (C), 170.6 (C), 196.1 (C), 197.9 (C); IR (neat) ν_{max} 592, 1367, 1466, 1521, 1675, 2362, 2958, 3305 cm^{-1}; HRESIMS *m/z* 311.1023 (calcd for $C_{12}H_{20}N_2O_4SNa$ 311.1036).

S-2-Acetamidoethyl 2-(4-methyl-2-oxopentanamido)propanethioate (3)

Prepared from acid **15b** (0.74 g, 3.68 mmol) according to the procedure described for thioester **2**. Anhydrous CH_2Cl_2 (25 mL) was used instead of THF. Purification by column chromatography (SiO_2, EtOAc, R_f = 0.40) gave the thioester **3** (0.69 g, 62%) as a white waxy solid; ^{1}H MR (400 MHz, $CDCl_3$) δ 0.89 (d, J = 6.66 Hz, 3H), 0.90 (d, J = 6.70 Hz, 3H), 1.47 (d, J = 7.37 Hz, 3H), 1.96 (s, 3H), 2.11-2.44 (m, 1H), 2.79 (dd, J = 6.91, 2.96 Hz, 2H), 3.03-308 (m, 2H), 3.40-3.47 (m, 2H), 4.62 (dq, J = 7.57, 7.37 Hz, 1H), 5.85 (brs, 1H), 7.35 (brd, J = 7.57 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.5 (CH_3), 22.65 (CH_3), 22.67 (CH_3), 23.3 (CH_3), 24.6 (CH), 28.7 (CH_2), 39.3 (CH_2), 45.4 (CH_2), 55.3 (CH), 160.0 (C), 170.5 (C), 198.0 (C), 200.0 (C); IR (neat) ν_{max} 669, 1683, 2340, 2358, 2958, 3301 cm^{-1}; HRESIMS *m/z* 325.1179 (calcd for $C_{13}H_{22}N_2O_4SNa$ 325.1192).

S-2-Acetamidoethyl 3-methyl-2-(4-methyl-2-oxopentanamido)butanethioate (4)

Prepared from acid **15c** (0.59 g, 2.56 mmol) according to the procedure described for thioester **2**. Anhydrous CH_2Cl_2 (10 mL) was used instead of THF. Purification by column chromatography (SiO_2, EtOAc, R_f = 0.48) gave the thioester **4** (0.57 g, 49%) as a white waxy solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85-0.98 (m, 12H), 1.92 (s, 3H), 2.15 (th, J = 6.56, 6.25 Hz, 1H), 2.25 (dh, J = 6.72, 6.66 Hz, 1H), 2.76 (d, J = 6.81 Hz, 2H), 3.02 (t, J = 6.49 Hz, 2H), 3.33-3.42 (m, 2H), 4.48 (dd, J = 9.25, 5.00 Hz, 1H), 6.10 (brs, 1H), 7.35 (brd, J = 9.25 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.1 (CH_3), 19.4 (CH_3), 22.57 (CH_3), 22.59 (CH_3), 23.2 (CH_3), 24.6 (CH), 28.6 (CH_2), 31.2 (CH), 39.3 (CH_2), 45.3 (CH_2), 64.2 (CH), 160.4 (C), 170.5 (C), 198.0 (C), 199.1 (C); IR (neat) ν_{max} 594, 1287, 1369, 1511, 1661, 2360, 2873, 2960, 3292 cm^{-1}; HRESIMS *m/z* 353.1508 (calcd for $C_{15}H_{26}N_2O_4SNa$ 353.1505).

S-2-Acetamidoethyl 5-oxohexanethioate (8)

Prepared from 4-Acylbutyric acid (1.00 g, 7.68 mmol) according the procedure described for thioester **2**. Anhydrous CH_2Cl_2 (30 mL) was used instead of THF. Purification by column chromatography (SiO_2, EtOAc, R_f = 0.22) gave the thioester **8** (1.74 g, quant.) as a white waxy solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.92 (tt, J = 7.08, 7.08 Hz, 2H), 1.96 (s, 3H), 2.13 (s, 3H), 2.49 (t, J = 7.08 Hz, 2H), 2.59 (t, J = 7.08 Hz, 2H), 3.01 (t, J = 6.20 Hz, 2H), 3.38-3.45 (m, 2H), 5.97 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.5 (CH_3), 23.3 (CH_2), 28.7 (CH_3), 30.1 (CH_2), 39.6 (CH_2), 42.2 (CH_2), 43.0 (CH_2), 170.4 (C), 199.52 (C), 207.90 (C); IR (neat) ν_{max} 669, 1371, 1540, 1653, 1683, 1712, 2340, 2358, 3294 cm^{-1}; HRESIMS *m/z* 254.0822 (calcd for $C_{10}H_{17}NO_3SNa$ 254.0821).

S-2-Acetamidoethyl 2-(tert-butoxycarbonyl(ethyl)amino)ethanethioate (17)[6]

N-acetylcysteamine (0.32 mL, 0.36 g, 2.98 mmol) was added to stirred solution of acid **16** [7](0.55 g, 2.71 mmol) and HOBt (0.62 g, 4.10 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0 °C. A solution of DCC (0.76 g, 3.68 mmol) in anhydrous CH_2Cl_2 (5 ml) was then added dropwise at 0 °C, and the mixture was stirred at 0 °C for 2 h and 16 h at room temperature. The precipitate was removed by filtration and the solvent was removed under reduced pressure. Purification of the residue by column chromatography (SiO_2, EtOAc, R_f = 0.31) gave the thioester **17** (0.70 g, 85%) as a colorless oil in two rotamers; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.11 (t, J = 7.21 Hz, 3H), 1.41 and 1.47 (s, 9H), 1.94 (s, 3H), 3.03 (t, J = 6.61 Hz, 2H), 3.29 (q, J = 6.11 Hz, 2H), 3.36-3.45 (m, 2H), 3.98 and 4.04 (s, 2H), 6.05 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.4 and 14.0 (CH_3), 23.3 (CH_3), 28.1 and 28.2 (CH_2), 28.4 and 28.5 (CH_3), 39.5 and 39.7 (CH_2), 43.3 and 44.0 (CH_2), 56.6 and 56.7 (CH_2), 80.7 (C), 154.6 and

155.8 (C), 170.4 and 170.5 (C), 199.3 and 199.5 (C); IR (neat) ν_{max} 584, 1147, 1167, 1654, 2341, 2359, 2975, 3293 cm^{-1}; HRESIMS *m/z* 227.0823 (calcd for $C_8H_{16}N_2O_4SNa$ 227.0825).

S-2-Acetamidoethyl 2-(ethylamino)ethanethioate hydrochloride (5)

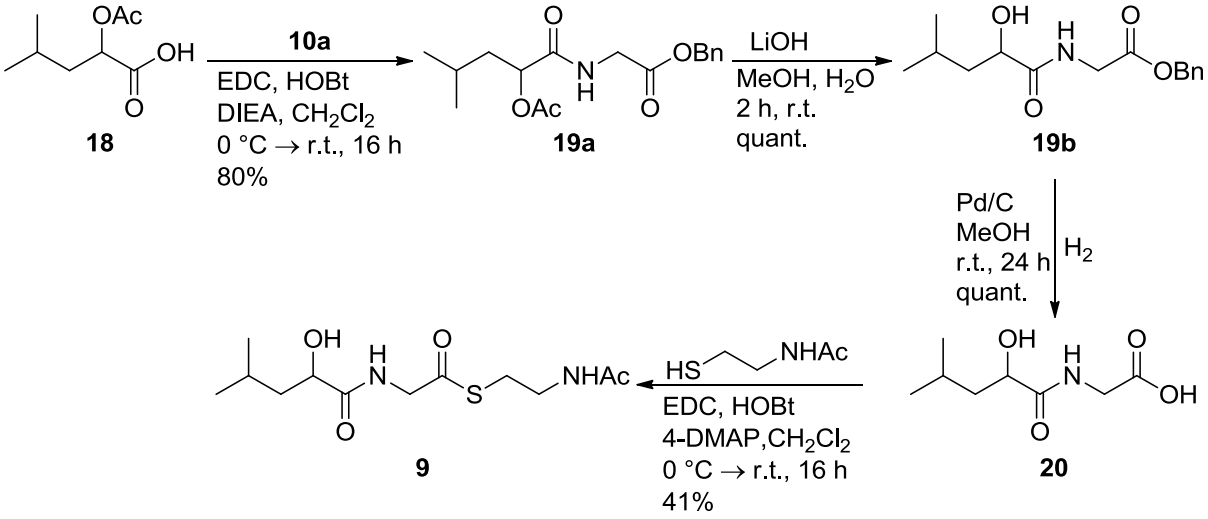
A solution of the thioester **17** (0.70 g, 2.30 mmol) in 4N HCl/dioxane (10.0 mL) was stirred for 1 h at room temperature. The solvent was removed under reduced pressure to give the desired hydrochloride **5** (0.54 g, quant.) as a white salt; ^{1}H NMR (400 MHz, d_6-DMSO) δ 1.19 (t, J = 7.31 Hz, 3H), 1.80 (s, 3H), 2.88-2.98 (m, 2H), 3.04 (t, J = 6.85 Hz, 2H), 3.19-3.26 (m, 2H), 4.21 (d, J = 5.76 Hz, 1H), 4.22 (d, J = 5.76 Hz, 1H), 8.21 (brt, J = 5.24 Hz, 1H), 9.54 (brs, 2H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 10.7 (CH_3), 22.5 (CH_3), 28.2 (CH_2), 37.9 (CH_2), 42.0 (CH_2), 53.4 (CH_2), 169.4 (C), 192.7 (C); IR (neat) ν_{max} 591, 976, 1644, 1692, 2430, 2677, 2930, 3221 cm^{-1}; HRESIMS *m/z* 353.1508 (calcd for $C_{15}H_{26}N_2O_4SNa$ 353.1505).

S-2-Acetamidoethyl 4-acetamidobutanethioate (7)

EDC (1.58 g, 8.24 mmol, 1.2 eq.) and HOBt (1.27 g, 8.24 mmol, 1.2 eq.) were added to a stirred solution of 4-acetamidobutyric acid (1.00 g, 6.89 mmol) in anhydrous CH_2Cl_2 (25 ml) at 0 °C. After stirring for 30 min at 0 °C, *N*-acetylcysteamine (0.88 mL, 0.99 g, 8.24 mmol, 1.2 eq.) and a catalytic amount of 4-DMAP were added. The reaction mixture was allowed to warm to room temperature and stirred for 16 h. Saturated aqueous NH_4Cl-solution (10 mL) was added to the mixture and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (10 mL). The combined organic fractions were extracted with water (2 x 10 mL) and dried over Na_2SO_4. After removing of the solvents under reduced pressure the thioester **7** (1.20 g, 71%) was purified by column chromatography (SiO_2, EtOAc, R_f = 0.1) as white waxy solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.85 (tt, J = 7.08, 7.08 Hz, 2H), 1.96 (2 x s, 6H), 2.60 (t, J = 7.08 Hz, 2H), 3.01 (t, J = 6.31 Hz, 2H), 3.22-3.29 (m, 2H), 3.37-3.45 (m, 2H), 6.10 (brs, 1H), 6.50 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.2 (CH_3), 23.4 (CH_3), 25.4 (CH_2), 29.0 (CH_2), 38.4 (CH_2), 39.3 (CH_2), 41.1 (CH_2), 170.7 (C), 170.8 (C), 199.3 (C); IR (neat) ν_{max} 649, 1289, 1366, 1544, 1634, 2359, 2930, 3080, 3273 cm^{-1}; HRESIMS *m/z* 269.0930 (calcd for $C_{10}H_{18}N_2O_3SNa$ 269.0930).

S-2-Acetamidoethyl 4-oxopentanethioate (6)

Prepared from levulinic acid (0.50 g, 4.30 mmol) according to the procedure described for thioester **7** in anhydrous CH_2Cl_2 (50 mL). Purification by column chromatography (SiO_2, EtOAc, R_F= 0.23) gave the thioester **6** (0.68 g, 73%) as a white waxy solid; ^{1}HNMR (400 MHz, $CDCl_3$) δ 1.92 (s, 3H), 2.15 (s, 3H), 2.73-2.86 (m, 4H), 3.00 (t, J = 6.28 Hz, 2H), 3.34-3.44 (m, 2H), 6.11 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.2 (CH_3), 28.6 (CH_3), 29.8 (CH_2), 37.6 (CH_2), 38.2 (CH_2), 39.5 (CH_2), 170.6 (C), 198.7 (C), 206.34 (C); IR (neat) ν_{max} 989, 1286, 1370, 1540, 1652, 1714, 2357, 3291 cm^{-1}; HRESIMS *m/z* 240.0669 (calcd for $C_9H_{15}NO_3SNa$ 240.0665).



Scheme S1: Synthesis of *N*-acetylcysteamine (SNAC) thioester **9**.

Benzyl 2-(2-acetoxy-4-methylpentanamido)acetate (**19a**)

Prepared from 2-acetoxy-4-methylpentanoic acid[8] (**18**) (2.00 g, 11.48 mmol, 1.0 eq.) and glycine benzyl ester hydrochloride (**13a**) (2.78 g, 13.78 mmol, 1.2 eq.) according to the procedure described for the amide **14a**. Purification by column chromatography (SiO_2, EtOAc : *c*-hexane, 1:1, *v*/*v*, R_f = 0.64) gave the amide **19a** (2.96 g, 80%) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (d, J = 5.9 Hz, 3H), 0.93 (d, J = 6.2 Hz, 3H), 1.61 – 1.80 (m, 3H), 2.14 (s, 3H), 4.08 (d, J = 3.9 Hz, 1H) 4.09 (d, J = 3.9 Hz, 1H), 5.19 (s, 2H), 5.21 – 5.26 (m, 1H), 5.53 (brs, 1H), 7.30 – 7.41 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.0 (CH_3), 21.9 (CH_3), 23.2 (CH_3), 24.7 (CH), 40.9 (CH_2), 42.2 (CH_2), 67.5 (CH_2), 72.8 (CH), 128.6 (CH), 128.76 (CH), 128.81 (CH), 135.1 (C), 169.6 (C), 169.9 (C), 170.7 (C); IR (neat) ν_{max} 697, 736, 1185, 1217, 1665, 1741, 2957, 3325 cm^{-1}; HRESIMS *m/z* 344.1456 (calcd for $C_{17}H_{23}NO_5Na$ 344.1468).

Benzyl 2-(2-hydroxy-4-methylpentanamido)acetate (**19b**)[8]

LiOH (0.22 g, 9.33 mmol) was added to a cooled solution of benzyl ester **19a** (1.00 g, 3.11 mmol) in methanol (9 mL) and water (3 mL). After stirring the solution for 2 h at room temperature, the solvents were removed under reduced pressure. The residue was dissolved in EtOAc (30 mL) and washed with saturated, aqueous NH_4Cl solution (2 x 10 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The ester **19b** (0.87 g, quant.) was obtained as a colorless oil and was used without further purification; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (d, J = 6.6 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 1.42 – 1.63 (m, 2H), 1.73 – 1.91 (m, 1H), 3.93 (dd, J = 18.2, 5.3 Hz, 1H), 4.04 – 4.25 (m, 2H), 4.70 (s, 2H), 7.25 – 7.42 (m, 5H), 7.53 (brt, J = 5.3 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.4 (CH_3), 23.5 (CH_3), 24.6 (CH), 41.0 (CH_2), 43.3 (CH_2), 65.5 (CH_2), 70.9 (CH), 127.2 (CH), 127.9 (CH), 128.7 (CH), 140.8 (C), 173.0 (C), 176.9 (C); IR (neat) ν_{max} 697, 835, 1209, 1533, 1651, 1724, 2955, 3352 cm^{-1}; HRESIMS *m/z* 302.1358 (calcd for $C_{15}H_{21}NO_4Na$ 302.1363).

2-(2-hydroxy-4-methylpentanamido)acetic acid (**20**)

Prepared from benzyl ester **19b** (0.87 g, 3.11 mmol) according to the procedure described for acid **15a**. Hydrogenation was performed in anhydrous methanol (10 mL), yielding in the desired acid **20** (0.58 g, 88%) as

a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (d, J = 6.0 Hz, 3H), 0.93 (d, J = 6.2 Hz, 3H), 1.42 – 1.72 (m, 2H), 1.74 – 1.92 (m, 1H), 3.92 (dd, J = 18.0, 4.6 Hz, 1H), 4.11 (dd, J = 18.0, 5.8 Hz, 1H), 4.19 (dd, J = 9.3, 3.5 Hz, 1H), 7.61 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.4 (CH_3), 23.5 (CH_3), 24.6 (CH), 41.1 (CH_2), 43.3 (CH_2), 70.9 (CH), 172.9 (C), 177.1 (C); ν_{max} 1073, 1140, 1203, 1537, 1623, 2871, 2956, 3376 cm^{-1}; HRESIMS *m/z* 188.0926 (calcd for $C_8H_{14}NO_4$ 188.0928).

S-(2-acetamidoethyl) 2-(2-hydroxy-4-methylpentanamido)ethanethioate (**9**)

EDC-HCl (0.64 g, 3.34 mmol) was added to a stirred solution of acid **20** (0.53 g, 2.78 mmol), *N*-acetylcysteamine (0.44 mL, 0.50 g, 4.17 mmol), HOBt (0.57 g, 3.34 mmol) and catalytic amounts of 4-DMAP in anhydrous CH_2Cl_2 (10 mL) at 0 °C. After stirring over night at room temperature the solution was diluted with EtOAc (50 mL) and washed with saturated, aqueous NH_4Cl-solution (10 mL), saturated, aqueous $NaHCO_3$-solution (10 mL), water (10 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$, filtered and the solvents were removed under reduced pressure. Purification of the residue by column chromatography (SiO_2, EtOAc, R_f = 0.15) gave the thioester **9** (0.33 g, 41%) as a white waxy solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (d, J = 6.6 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.49 – 1.65 (m, 2H), 1.79 – 1.90 (m, 1H), 1.93 (s, 3H), 3.01 (t, J = 6.5 Hz, 2H), 3.31 – 3.40 (m, 2H), 4.12 – 4.23 (m, 3H), 6.68 (brt, J = 5.7 Hz, 1H), 7.68 (brt, J = 6.1 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.4 (CH_3), 23.1 (CH_3), 23.6 (CH_3), 24.5 (CH), 28.4 (CH_2), 39.2 (CH_2), 43.5 (CH_2), 49.0 (CH_2), 70.7 (CH), 171.2 (C), 176.2 (C), 197.7 (C); ν_{max} 1077, 1142, 1235, 1290, 1360, 1547, 1626, 1650, 2957, 3303 cm^{-1}; HRESIMS *m/z* 313.1188 (calcd for $C_{12}H_{22}NO_4SNa$ 313.1192).

SNAC Acylation Assays

Acylation reactions were conducted in storage buffer (25mM Tris, 0.5M NaCl, 10% (v/v) glycerol, pH 7.6). SNAC-thioesters were incubated with KS domains at a final concentration of 0.5mM for comparative substrate studies. The Michalis-Menton treatment of KS1 with (**2**) required a range of SNAC-thioester concentrations. Both WT BaeJ KS1 and BaeJ KS1(N206A) were maintained at 80μM for acylation assays. To ensure the SNAC-thioesters remained in solution, the concentration of DMSO was adjusted to 5% (v/v). Acylation reactions were allowed to run for various periods of time between 0-10mins at 25°C, before quenching with 0.1% TFA.

Sample Preparation for MS Analysis

C_4 ZipTips™ (Millipore) were used to prepare each sample; the C_4 ZipTip was washed with two 10μL aspirations of 50% MeCN, followed by five 10uL aspirations of H_2O/0.1% TFA solution. The protein sample was then loaded onto the ZipTip column by 15-20 10μL aspirations of the sample, each time injecting back into the sample vial. The loaded protein sample was then desalted by x15 10μL aspirations of H_2O/5% MeOH/0.1% TFA, followed by elution of the sample into 5μL H_2O/80% MeCN/0.1% TFA.

Data Analysis

Denatured KS spectra obtained from acylation experiments (see section **4**) were subjected to minimal smoothing and noise reduction. The spectra were deconvoluted using the transform function of MassLynx, taking an average of all charge states. Relevant peak intensities were recorded and converted into the concentration of [KS-SH] and [KS-acyl] respectively. Data was recorded in triplicate, and an average taken for kinetic plots.

Instrumentation

All experiments described within this study were analysed using a Waters SYNAPT™HDMS™, which is a hybrid quadrupole-ion mobility-orthogonal acceleration TOF instrument (oa-TOF), produced by Waters™. This instrument has multiple ionisation capabilities, of which nanoESI was utilised during this study. Mass spectrometer parameters used: cone voltage: 20V, capillary voltage: 1.5kV, trap CE: 10V, transfer CE: 5V, backing pressure: ~4 mbar, trap pressure: 2.3 x 10^{-3}, TOF pressure: 1.6 x 10^{-6} mbar. A quadrupole profile was applied which gave optimal transmission between 1000-3000 m/z. Spectra were acquired between 1000-4000m/z

MassLynx™ 4.1 software was used to control the parameters of the SYNAPT, and allows data obtained to be manipulated in real-time. Spectra were generated from the raw data acquired and often smoothed, centered and noise reduced for analysis.

Construction and Modelling of C207A, M268A and L450A Mutants

Site directed mutagenesis for the KS1 C207A mutant was performed using a Stratagene Quikchange II kit following standard protocol. Oligonucleotides used are shown below.

KS1C207A_For (5'- CTATTTTGTCCACGCCAAC**GC**CTCATCTTCGTTAATCGGC-3')

KS1C207A_Rev (5'- GCCGATTAACGAAGATGAG**GC**GTTGGCGTGGACAAAATAG-3')

For the M268A and L450A mutants, the entire expression plasmid, pHis8+KS1, was amplified as a linear product using the primers:

KS1M268A_For (5'-GCCATCGGCGGTGAAGGGG-3')

KS1M268A_Rev (5'-GCCGTCAGCATCCGCGTCAAACG-3')

KS1L450A_For (5'-GCCGGCGGAACAAACACACACG-3')

KS1L450A_Rev (5'-GCCGAAAGAGCTTAAAGCCATGCGG-3')

KS1M268A_Rev introduced an ATG to GCC mutation (Met268Ala), and KS1L450A_Rev introduced an CTT to GCC mutation (Leu450Ala).

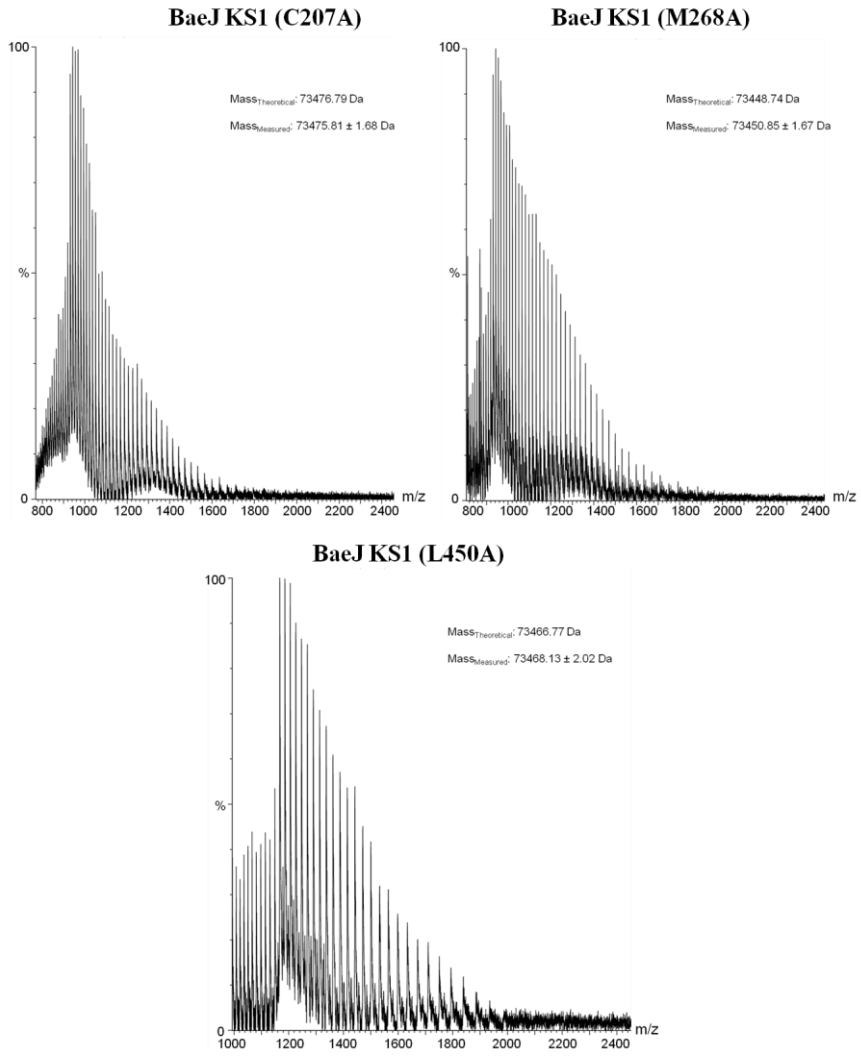


Figure S4: nESI mass spectra of BaeJ KS1(C207A), BaeJ KS1(M268A) and BaeJ KS1(L450A) sprayed from 80:20 MeCN:H_2O 0.1% TFA. Measured and theoretical masses are shown in the inset.

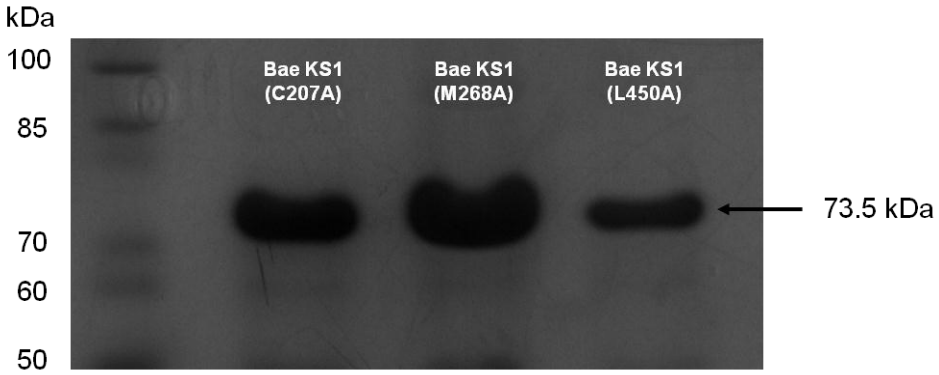


Figure S5: 12% SDS-PAGE gel of BaeJ KS1(C207A), BaeJ KS1(M268A) and BaeJ KS1L450A) post His-tag purification.

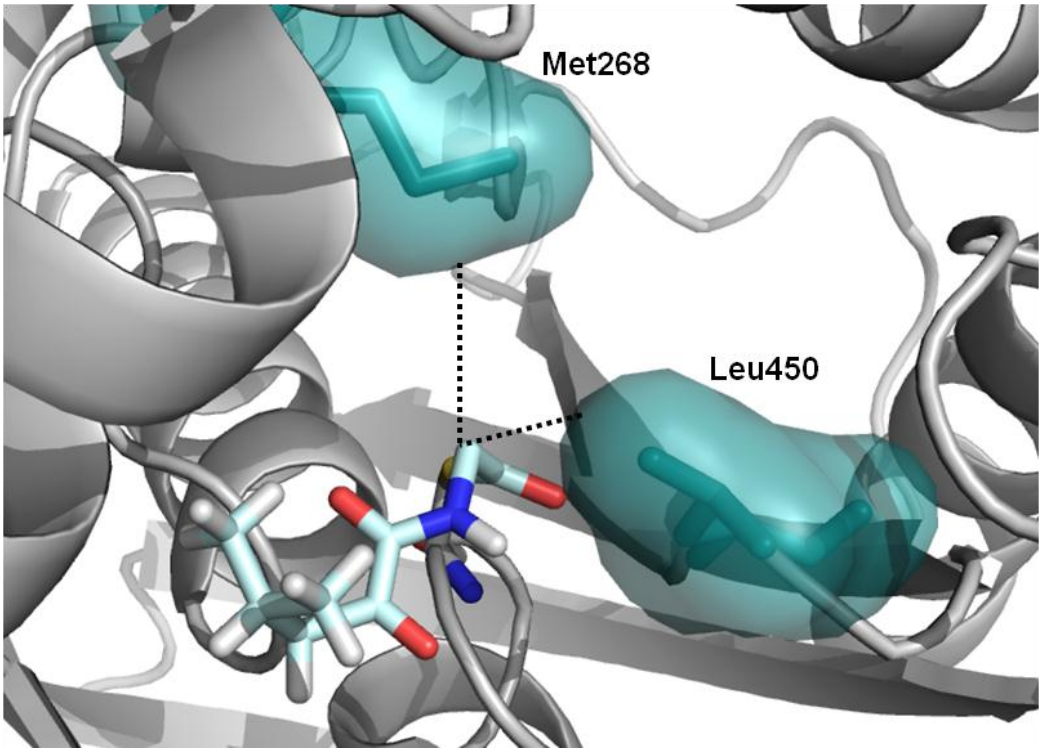


Figure S6: Model of SNAC **2** covalently bound to active site Cys of WT BaeJ KS1. Residues Met268 and Leu450 are shown in teal, and highlight the steric hindrance they apply to the α-position of the substrate.

Sequence Analysis of KS Domains

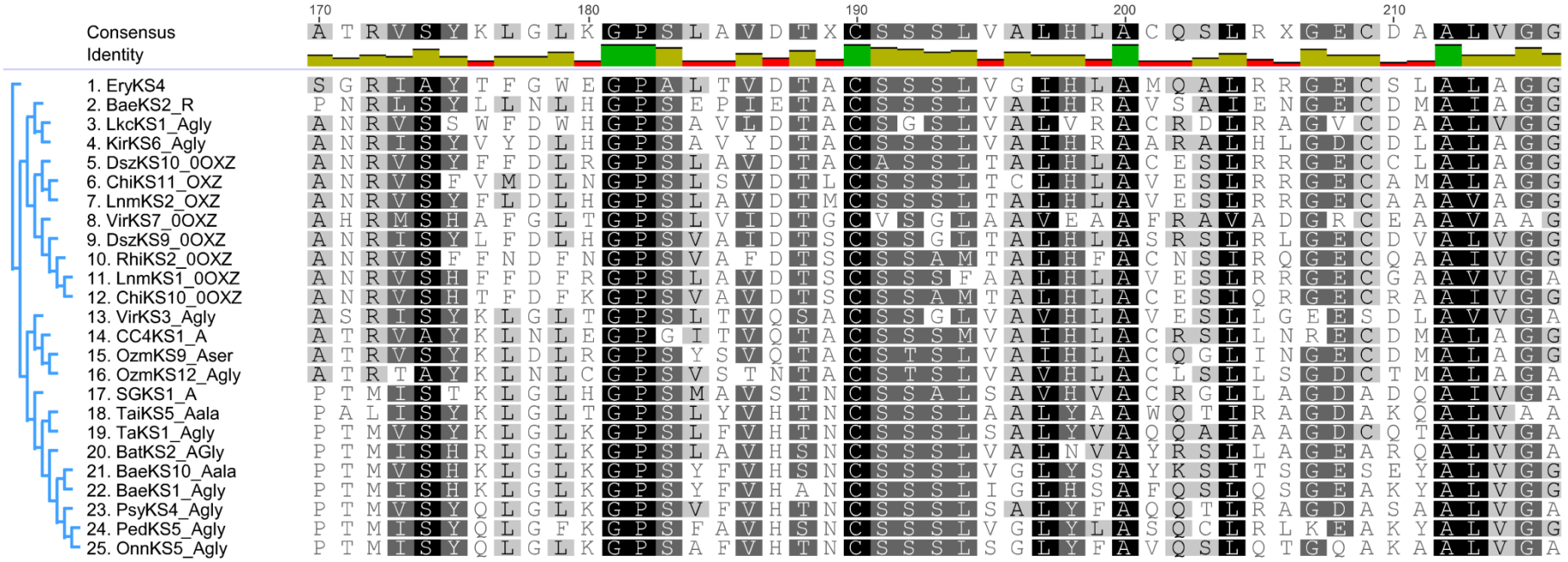


Figure S7: Multiple sequence alignment of KS domains predicted to accept amino acid-derived intermediates. The residue preceding the active-site Cys (X-Cys position) is highlighted in cyan (Asn) or green (Ala). In a high proportion of cases X=Asn, indicating potential importance for substrate selectivity. *N.B.* BaeKS1 and BaeKS10 also represent PksKS1 and PksKS10.

Abbreviations: R, fully reduced, unbranched intermediate; Agly, glycine-dervived intermediate; Aala, alanine-dervived intermediate; Aser, serine-dervived intermediate; OXZ, oxazole/thiazole intermediate; 0OXZ, oxazole/thiazole intermediate & non-elongating.

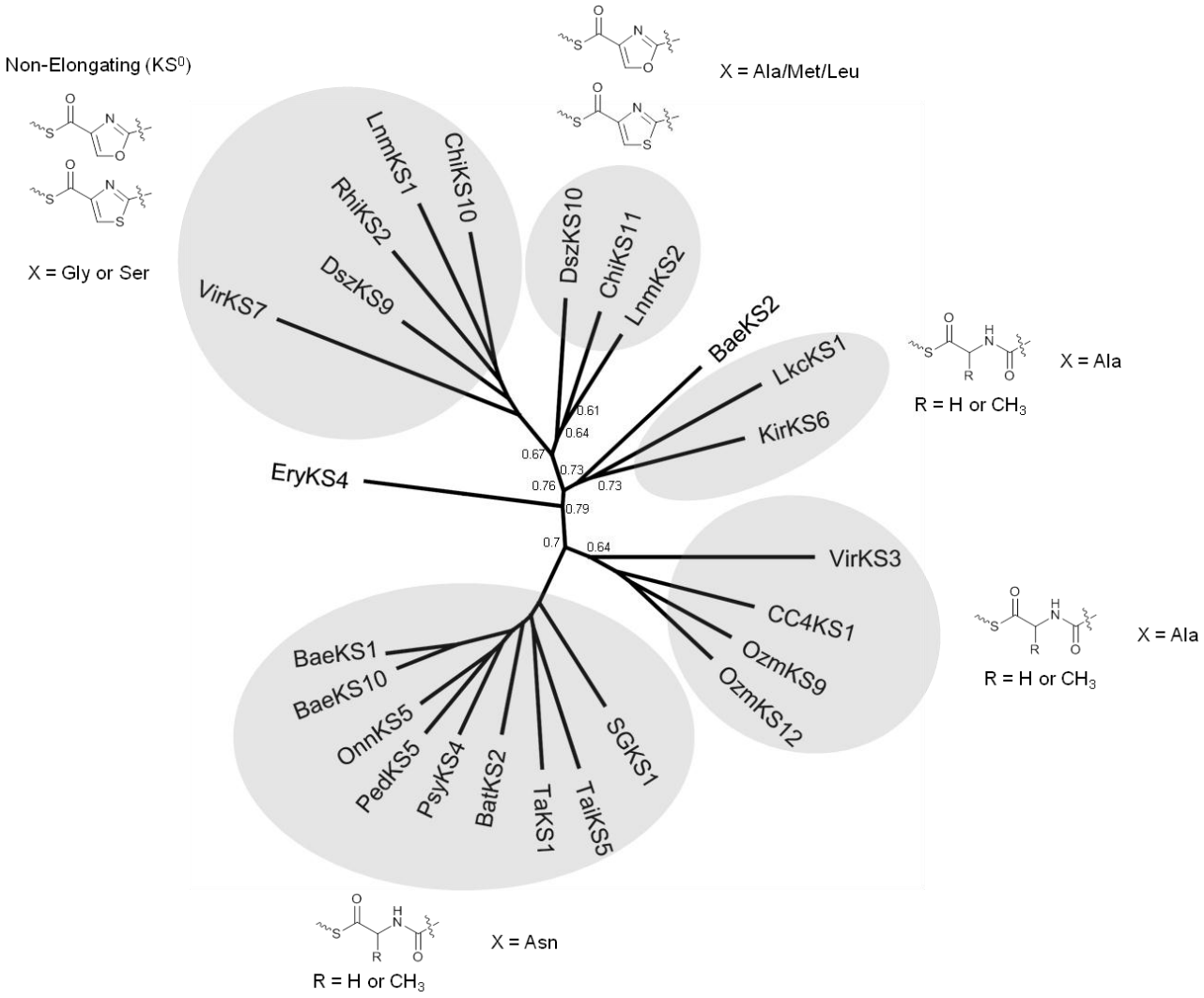


Figure S8: Phylogram of KS domains predicted to accept amino-acid derived substrates. Tip labels consist of KS number and cluster, with an outgroup of the erythromycin KS4 domain. X indicates the identity of the amino acid preceeding the active site Cys residue. The phylogram shows a distinct clade for KS domains which harbor an Asn residue at the X-Cys position. However, amino acid-accepting KSs where X = Ala fall into separate clades. *N.B.* BaeKS1 and BaeKS10 from *B. amyloliquefaciens* FZB42 are orthologous to PksKS1 and PksKS11, respectively, from the bacillaene PKS of *Bacillus subtilis* 168. Bootstrap values ≥0.60 are highlighted.

Abbreviations: Bat, batumin; Bae, bacillaene; CC4, uncharacterized PKSs from *Clostridium cellulolyticum*; Chi, chivosazol; Dsz, disorazol; Ery, erythromycin; Kir, kirromycin; Lkc, lankacidin; Lnm, leinamycin; Onn, onnamide; Ozm, oxazolomycin; Ped, pederin; Psy, psymberin; Rhi, rhizoxin; SG, uncharacterized PKSs from *Streptomyces griseus*; Ta, myxovirescin; Tai, thailandamide; Vir, virginiamycin.

Modelling of BaeJ KS1 Binding Pocket

The homology model of BaeJ KS1 was constructed using the CPHmodel server[9], using published KS structure PDB:2QO3[10] as a template. The SNAC substrate **2** was produced using the bound dodecanoic acid from PDB:1EK4[11] as a template.

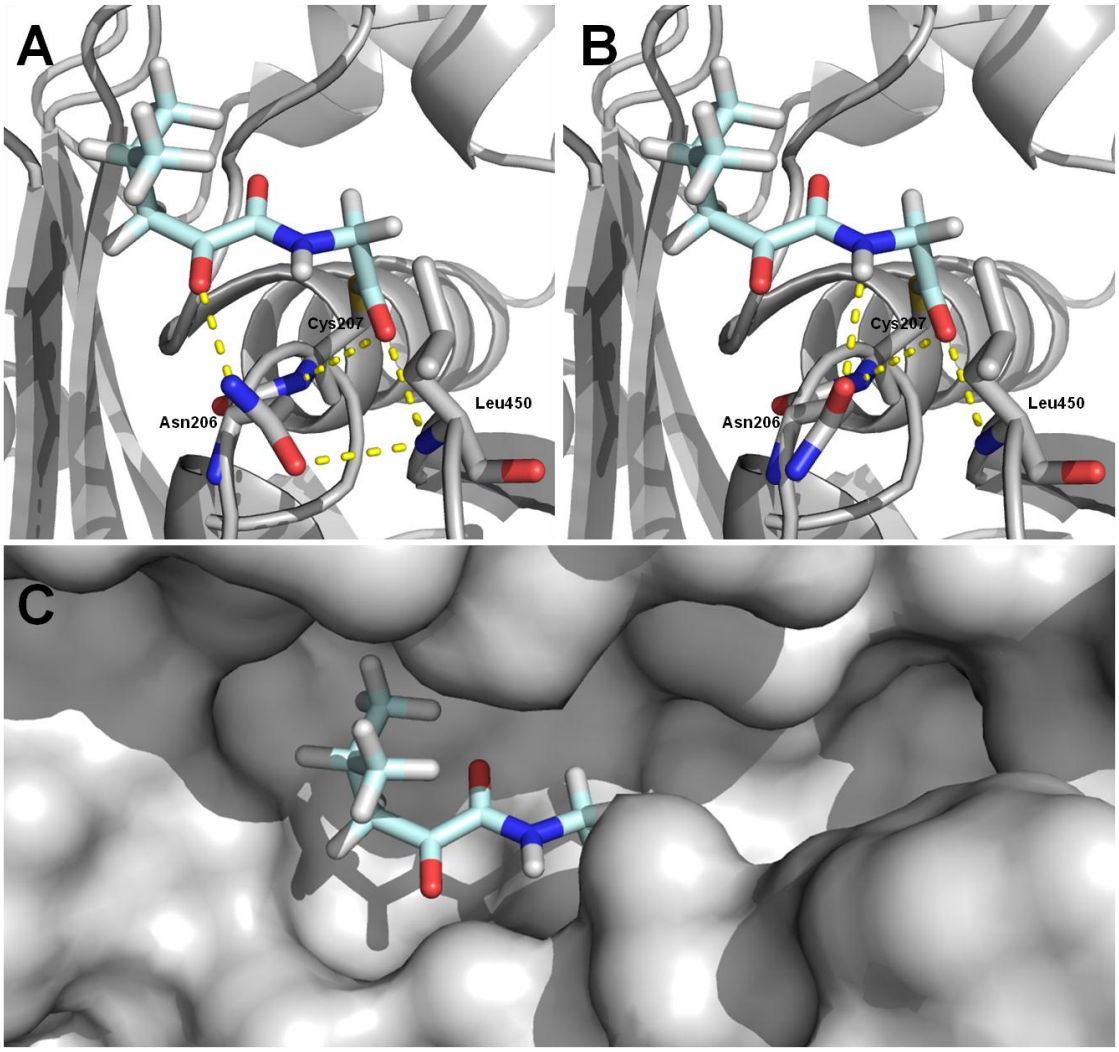
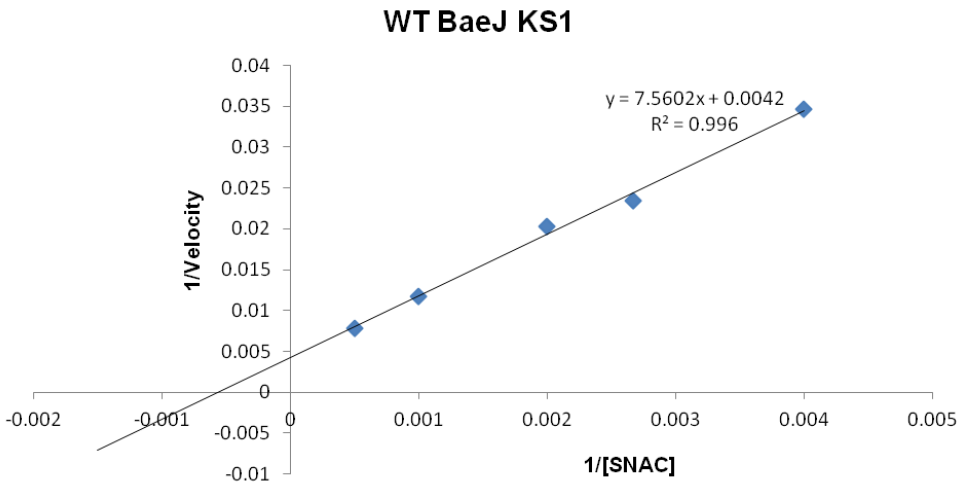
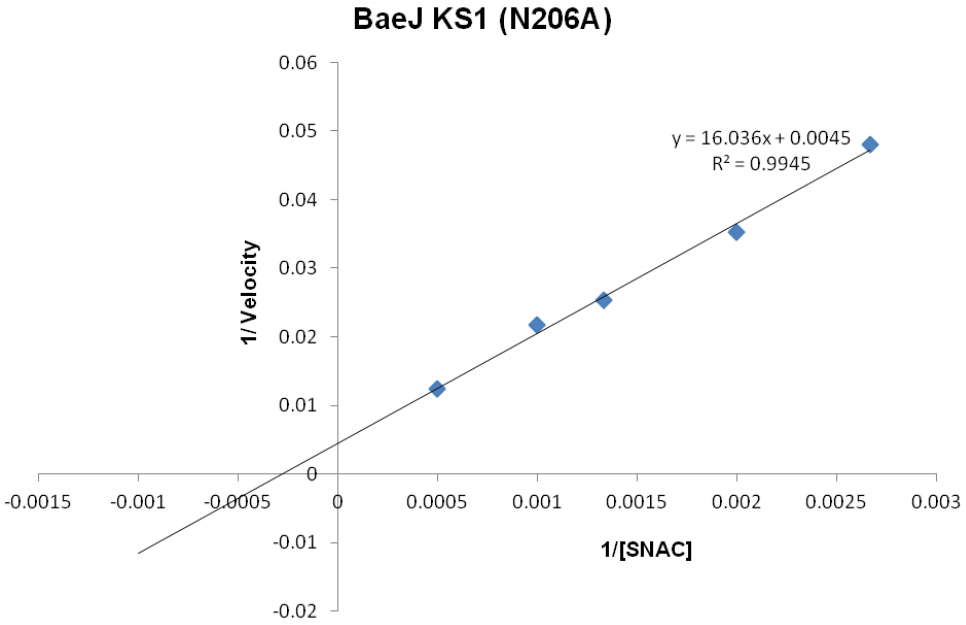


Figure S9: Models of SNAC thioester **2** bound to BaeJ KS1. **A).** BaeJ KS1 with Asn206 able to form a hydrogen bond to the δ-carbonyl of SNAC **2** and to the backbone amide nitrogen of Leu450. **B).** In contrast, BaeJ KS1 with the carboxamide of the Asn206 residue rotated ~90° anticlockwise, which is now able to form a hydrogen bond with the β-amide nitrogen of SNAC **2**. In both cases the thioester carboxyl is stabilized by an oxyanion hole formed by the backbone amide of Leu450, in addition to a potential stabilizing interaction with the backbone amide of Cys207. Dashed yellow lines designate distances between 2.4 - 3.3Å. **C).** Surface representation of BaeJ KS1 binding pocket with SNAC **2**. The distal region of the binding cleft provides space to accommodate the rest of the acyl chain.

Michalis-Menten Treatment of WT KS1 and KS1(N206A)



V_{max} = 0.24 mM min^{-1}
K_m = 1.8 mM
k_{cat} = 3.0 min^{-1}
k_{cat}/K_m = 1.7 x 10^{-6} mM^{-1} min^{-1}



V_{max} = 0.22 mM min^{-1}
K_m = 3.7 mM
k_{cat} = 2.8 min^{-1}
k_{cat}/K_m = 7.8 x 10^{-7} mM^{-1} min^{-1}

Figure S10: Michaelis-Menten analysis of WT KS1 and KS1(N206A). Lineweaver-Burk plots for BaeJ KS1 WT and N206A are shown. Kinetic parameters were calculated from the plot using the equations shown, and errors were determined using a σ confidence interval.

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