Supplemental Information for

Comparison of *Alicyclobacillus acidocaldarius* OSBS to its promiscuous NSAR/OSBS relatives

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Supplemental Materials and methods

General procedure for synthesis of succinyl amino acids: A solution of amino acid (1.0 equiv) and succinic anhydride (1.0 equiv) in acetic acid (~1 mmol/mL) was heated at 50 °C under N_2 for 5 h. After concentration in vacuo to remove acetic acid, the crude product was purified by flash chromatography using gradient elution (CH_2Cl_2:MeOH from 20:1 to 15:1) with 60 Å silica gel (230-400 mesh) as stationary phase to provide the desired succinylated product. Purity was confirmed by HPLC analysis performed with a Gemini HPLC column (C18, 3 micron, 150 x 4.60 mm). Mobile phase A: 0.2% H_3PO_4 in H_2O; mobile phase B: 0.2% H_3PO_4 in CH_3CN/H_2O (4:1).

N-Succinyl-L-phenylalanine: Following the general procedure, *L*-phenylalanine (1.20 g, 7.26 mmol) and succinic anhydride (730 mg, 7.26 mmol) afforded *N*-succinyl-L-phenylalanine as colorless crystals (1.58 g, 82%). Characterization data matched that previously reported.[1]

N-Succinyl-D-phenylalanine: Following the general procedure, *D*-phenylalanine (1.20 g, 7.26 mmol) and succinic anhydride (730 mg, 7.26 mmol) afforded *N*-succinyl-*D*-phenylalanine as colorless crystals (1.45 g, 75%). Characterization data matched that previously reported.[1]

N-Succinyl-L-valine: Following the general procedure, *L*-valine (937 mg, 8.00 mmol) and succinic anhydride (801 mg, 8.00 mmol) afforded *N*-succinyl-*L*-valine as colorless crystals (1.49 g, 86%). Characterization data matched that previously reported.[1]

N-Succinyl-L-methionine: Following the general procedure, *L*-methionine (1.19 g, 8.00 mmol) and succinic anhydride (801 mg, 8.00 mmol) afforded *N*-succinyl-*L*-methionine as a colorless oil (1.45 g, 73%). Characterization data matched that previously reported.[1]

N-Succinyl-L-tryptophan: Following the general procedure, *L*-tryptophan (1.02 g, 5.00 mmol) and succinic anhydride (501 mg, 5.00 mmol) afforded *N*-succinyl-*L*-tryptophan as a colorless oil (1.22 g, 80%). Characterization data matched that previously reported.[1]

Specific rotation of each substrate was determined by fitting three independent serial dilutions to a straight line. Specific rotations of each substrate at 405 nm are: 6.54 deg M^{-1} cm^{-1} (L- and D-NSPG)[2], 2.2 deg M^{-1} cm^{-1} (*N*-succinyl-L- and D- phenylalanine), 0.9 deg M^{-1} cm^{-1} (*N*-succinyl-L-valine), 1.0 deg M^{-1} cm^{-1} (*N*-succinyl-L-methionine), and 1.9 deg M^{-1} cm^{-1} (*N*-succinyl-L-tryptophan).

References

(1) Sakai, A., Xiang, D. F., Xu, C., Song, L., Yew, W. S., Raushel, F. M., and Gerlt, J. A. (2006) Evolution of enzymatic activities in the enolase superfamily: *N*-succinylamino acid racemase and a new pathway for the irreversible conversion of D- to L-Amino Acids, *Biochemistry 45*, 4455-4462.

(2) McMillan, A. W., Lopez, M. S., Zhu, M., Morse, B. C., Yeo, I. C., Amos, J., Hull, K., Romo, D., and Glasner, M. E. (2014) Role of an active site loop in the promiscuous activities of Amycolatopsis sp. T-1-60 NSAR/OSBS, *Biochemistry 53*, 4434-4444.

Table S1. Primers used for cloning NSAR/OSBS subfamily genes.

Species	**Primer sequence**
AmedNSAR/OSBS	FORWARD: TACTTCCAATCCAATGCCATGAAACTCACCGGGGTGGAACTCC REVERSE: TTATCCACTTCCAATGTTACTAGGCGAGCCAGGACTTCGCGG
LvNSAR/OSBS	FORWARD: TACTTCCAATCCAATGCCATGAAAGTAGAAAAGATTACTTTAAGAC REVERSE: TTATCCACTTCCAATGTTATTATTGATAGACCTCTTTGCTTATTGTCAGC
RcNSAR/OSBS	FORWARD: TACTTCCAATCCAATGCCATGAAGATCGAGTCGATCACATTG REVERSE: TTATCCACTTCCAATGTTATCATCCTTTCCCAATGATCACCTCACG

All DNA primers are shown in the 5' to 3' direction. The red, underlined bases are part of the vector sequence.

Table S2. Primers used for mutagenesis.

Mutation	**Template**	**Primer sequence**
M18F	AaOSBS WT	Forward: GAAATTTCCGttcCGTACGGCGCATG Reverse: AGCGGCAGTGACAGACGA
Y55A	AaOSBS WT	Forward: GGAACCGACCgcgACGGAAGAATGTACCG Reverse: GCCAGAGCCACGCATTCC
Y299I	AaOSBS WT	Forward: CGGTGGCATGatcGAAACCGGTG Reverse: ACCCAAGCTGCCATGCCT
M18F/Y299I	AaOSBS M18F	Forward: CGGTGGCATGatcGAAACCGGTG Reverse: ACCCAAGCTGCCATGCCT

All DNA primers are shown in the 5' to 3' direction. The underlined bases designate the codons where mutations were introduced.