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Supporting Information

Broadening the Substrate Range of Serine Palmitoyltransferase by Protein Engineering and Applications to 3-Keto-Dihydrosphingosine Analogs

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GGC

Figure S1. Sequence optimized *Sp*SPTase gene.
*Nde*I restriction site: CATATG
*Xho*I restriction site: CTCGAG

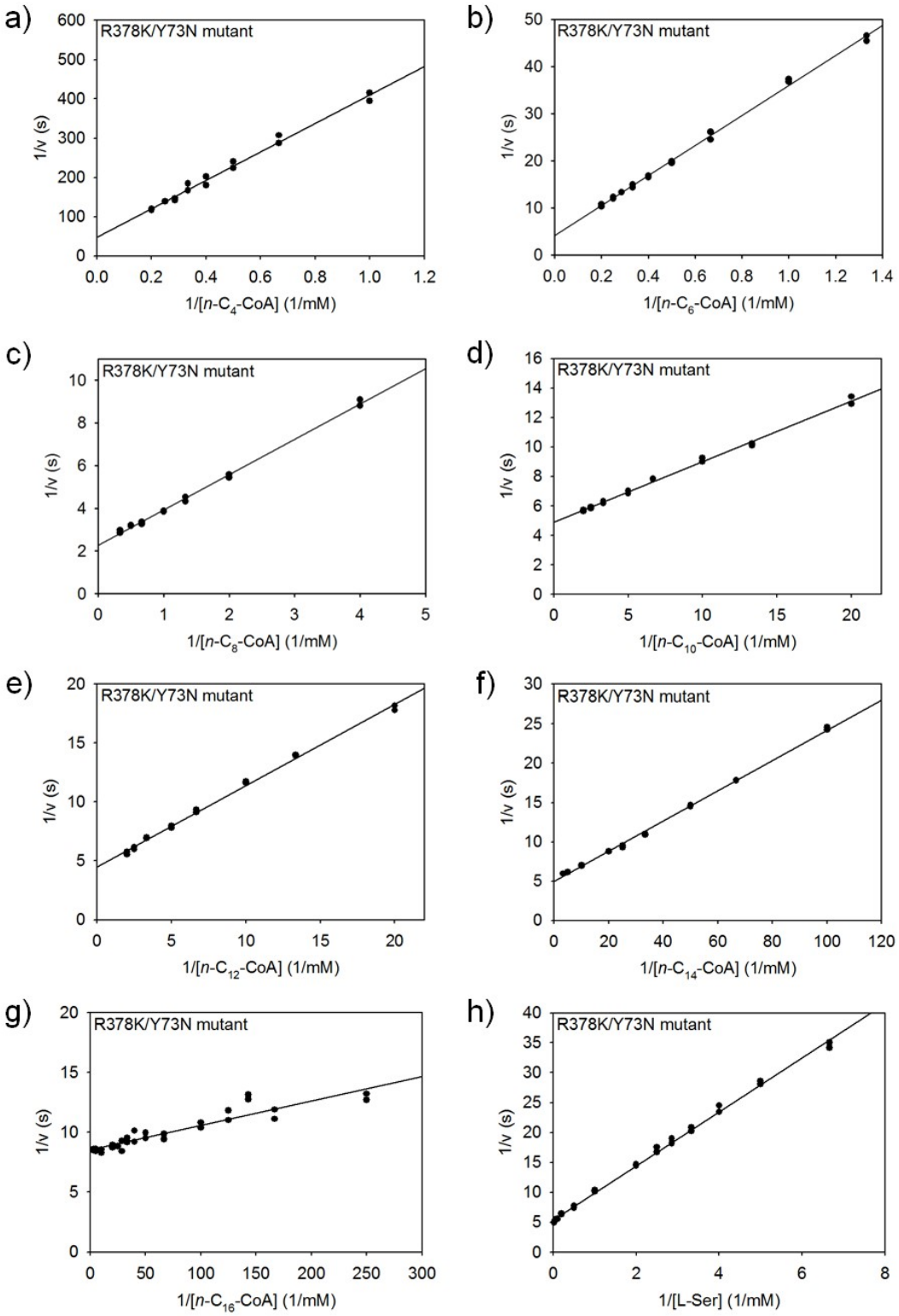


Figure S2. Lineweaver-Burk plots for the production of KDS analogues from L-Ser and Acyl-CoA's (*n*-C_4-CoA to *n*-C_{16}-CoA) by R378K/Y73N *Sp*SPTase. Enzyme reactions with variable substrates *n*-C_4-CoA (a), *n*-C_6-CoA (b), *n*-C_8-CoA (c), *n*-C_{10}-CoA (d), *n*-C_{12}-CoA (e), *n*-C_{14}-CoA (f), *n*-C_{16}-CoA(g), and L-Ser (h).

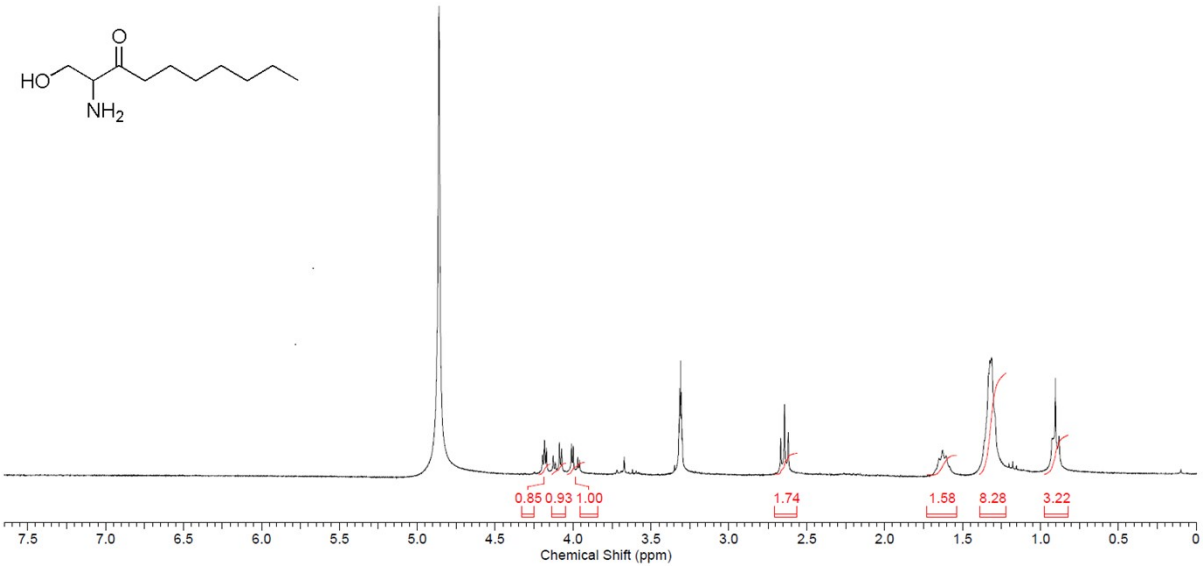


Figure S3. Production of 3-keto-C_{10}-dihydrosphingosine from L-Ser and *n*-C_8-CoA by R378K/Y73N *Sp*SPTase.

^{1}H NMR spectra of 3-keto-C_{10}-dihydrosphingosine were recorded at 300 MHz at 25 °C and calibrated against the residual proton signal of the solvent as internal references (CD3OD: δH = 3.31 ppm).

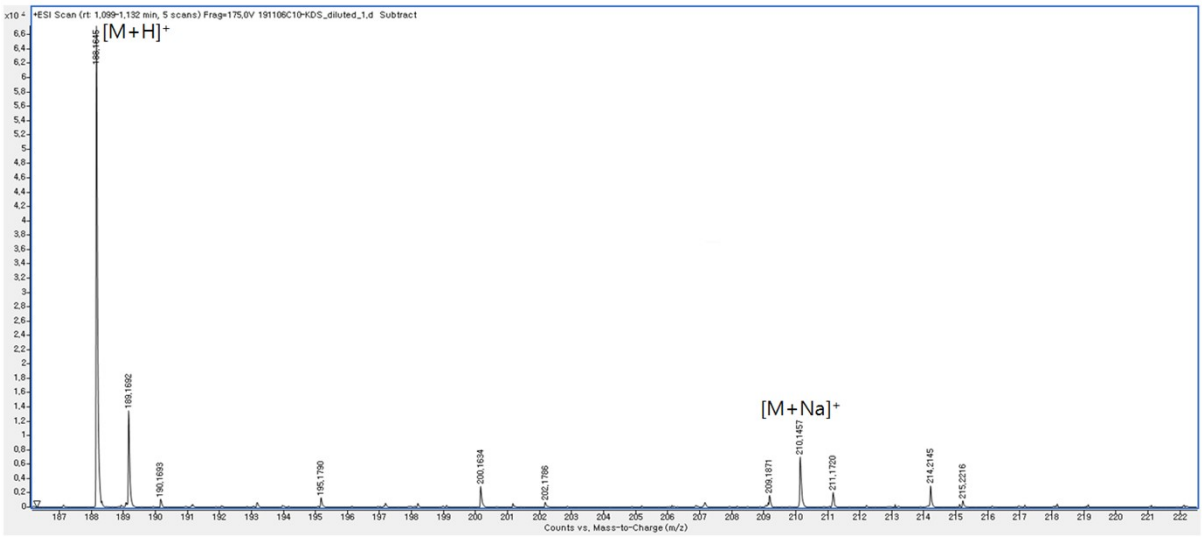


Figure S4. MS analysis of 3-keto-C_{10}-dihydrosphingosine synthesized from L-Ser and *n*-C_8-CoA by R378K/Y73N *Sp*SPTase. The formation of the product was confirmed as previously described.[1] 3-Keto-C_{10}-dihydrosphingosine formation was corroborated ESI MS (positive mode). $C_{10}H_{21}NO_2$ calcd $[M + H]^+$ = 188.1645, $[M + Na]^+$ = 210.1464; obsd: 118.1645 (Δ = 0 ppm), 210.1457 (Δ = 3.3 ppm).

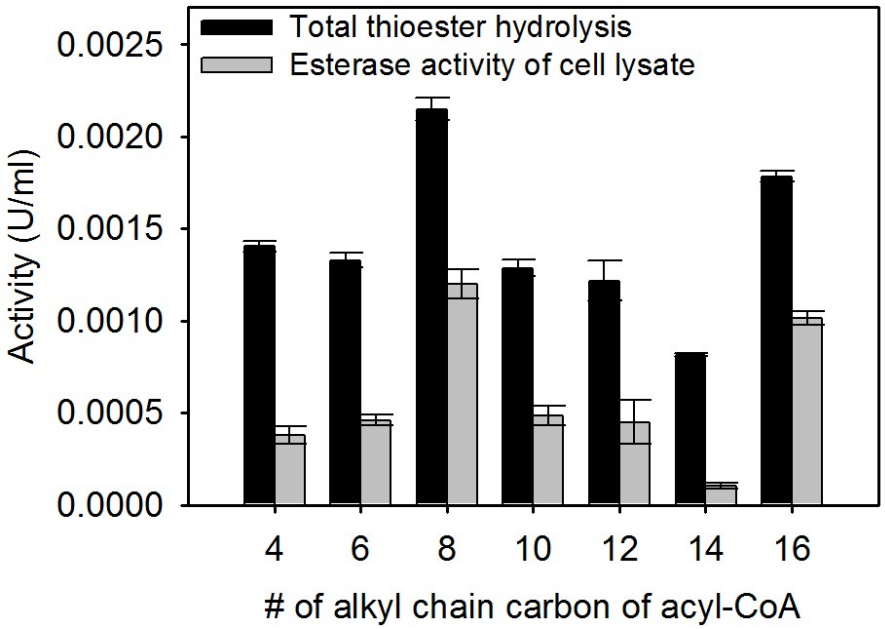
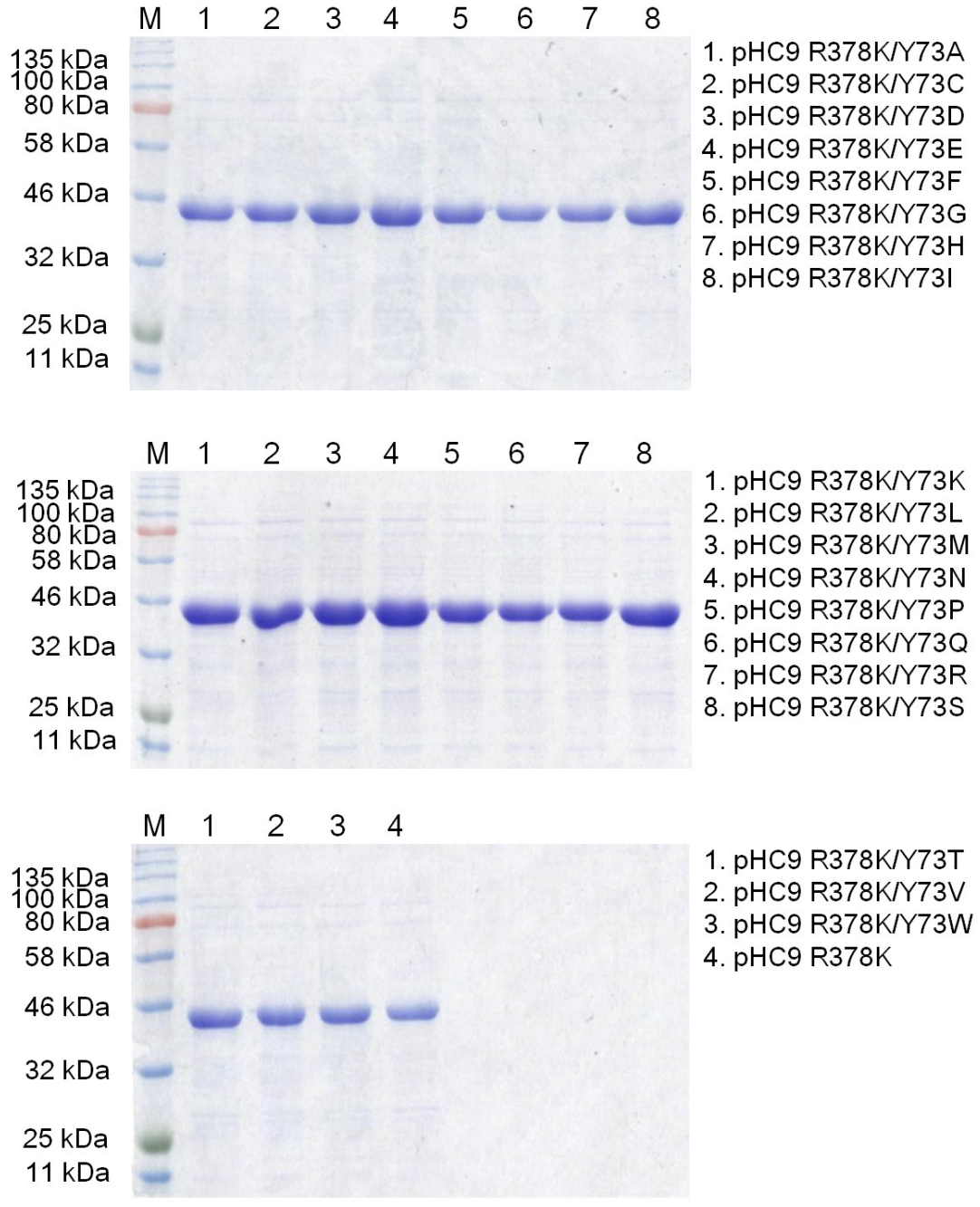


Figure S5. Thioesterase activities of BL21-Gold (DE3) for *n*-acyl-CoA's. The cell lysate (10 µl) was added to 190 µl of a reaction buffer (500 mM KP_i, 150 mM NaCl, 20 mM L-Ser, 10 µM PLP, 0.3 mM Ellman's reagent, 1 mM EDTA, pH 7.5) containing *n*-acyl-CoA's (0.5 mM). The reactions were incubated at 37 °C, and the thioester bond hydrolysis of *n*-acyl-CoA's was determined by monitoring the absorbance at 412 nm for 2 h. The total thioester hydrolysis includes the rates of self-hydrolysis.



M: Color Prestained Protein Standard, broad range (11–245 kDa) (New England Biolabs)

Figure S6. SDS-PAGE analysis of *Sp*SPTase R378K/Y73X mutants expressed under autoinduction conditions.

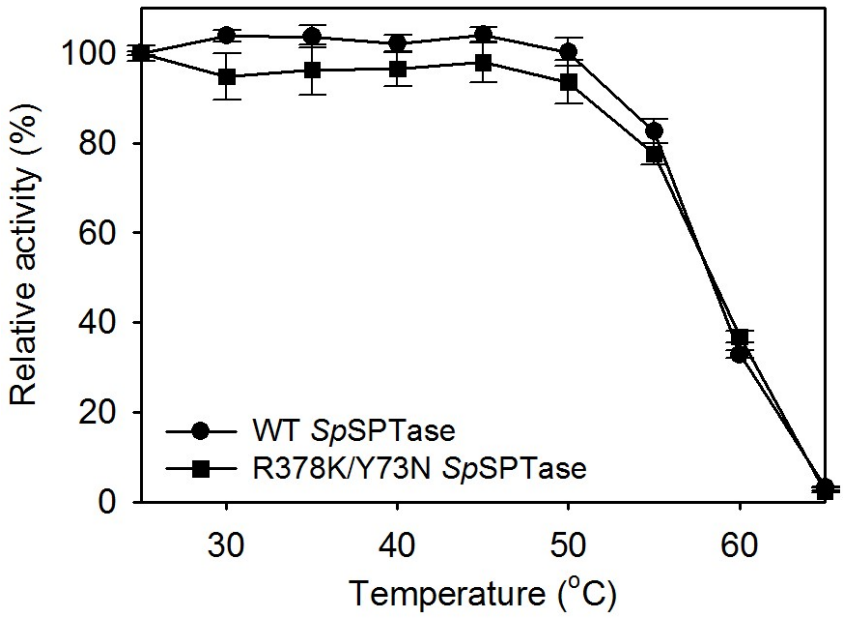


Figure S7. Thermostability of wild-type and R378K/Y73N *Sp*SPTases. *Sp*SPTases (0.2 mg/ml) were incubated at 25-65 °C for 30 min, and then 4 °C for 10 min. The incubated enzyme solution (10 µl or 2 µg) was added to a reaction buffer (500 mM KP_i, pH 7.5, 150 mM NaCl, 20 mM L-Ser, 10 µM PLP, 1 mM EDTA, 0.15 mM *n*-C_{16}-CoA, and 0.3 mM DTNB). Error bars represent standard deviation of triplicate measurement.

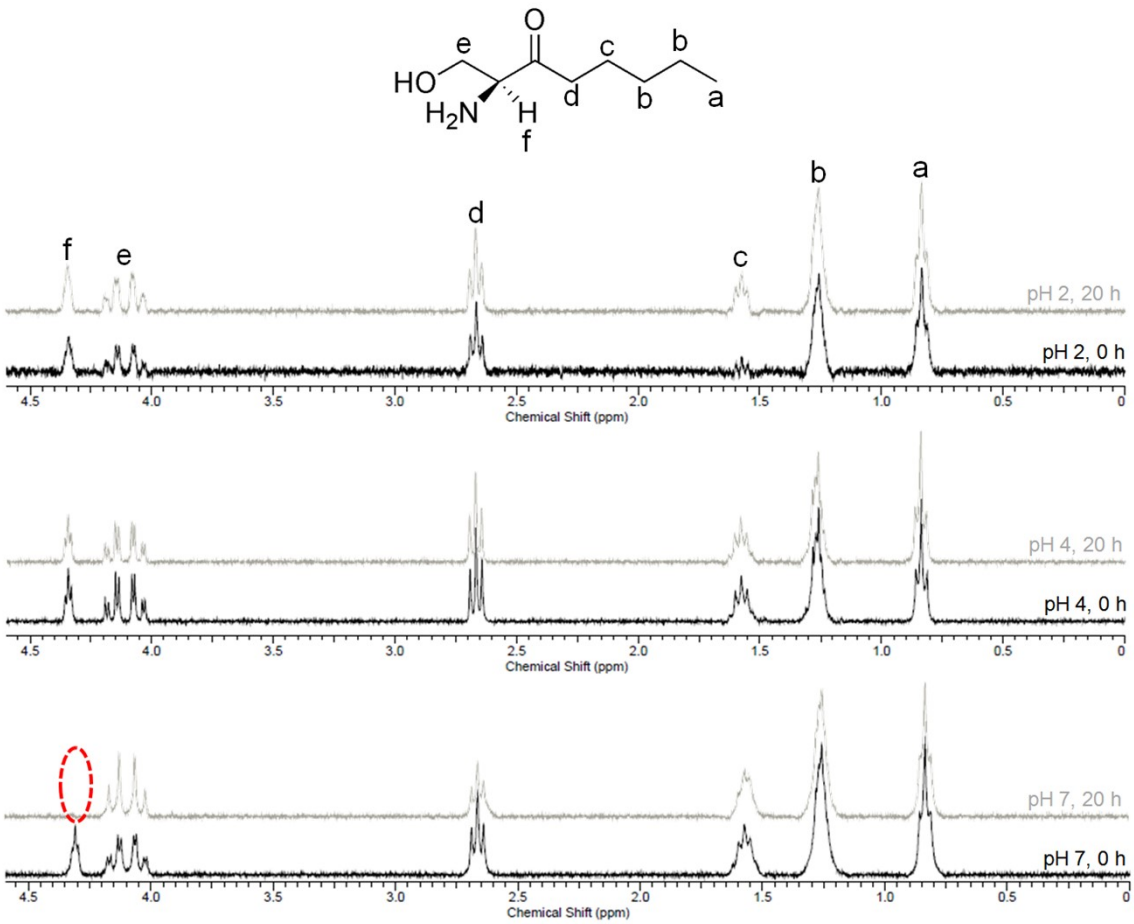


Figure S8. pH-dependent racemization of 3-keto-C_8-dihydrosphingosine. 3-keto-C_8-dihydrosphingosine (C_8-KDS) was synthesized as described previously.[2] The analogue was dissolved in either a deuterated 20 mM KP_i buffer for pH 7 or D_2O for pH2 and 4. The solutions were incubated in the dark at rt. for 20 h, and the exchange of the α-proton was determined by comparing ^1H NMR data.

Table S1. Degenerate primers (NNK and MNN) used for site saturation mutagenesis.

Mutants	Forward primers (NNK)	Reverse primers (MNN)	Q_{pool}
F47X	TCACTGATCCG**NNK**GCTATTGTCATGGAGCAGGTAAAATC	ATGACAATAGC**MNN**CGGATCAGTGACACCGGAATCCAGAA	0.87
Y73X	TGCTTGGCACC**NNK**AATTACATGGGCATGACCTTCGATCC	CCCATGTAATT**MNN**GGTGCCAAGCAGGATGGTATCTTTTC	0.86
G101X	CCGGCACCAAT**NNK**AGCCGTATGTTAAACGGTACTTTTCA	AACATACGGCT**MNN**ATTGGTGCCGGAACCAAATTTCTCCA	0.84
S102X	GCACCAATGGC**NNK**CGTATGTTAAACGGTACTTTTCATGA	TTTAACATACG**MNN**GCCATTGGTGCCGGAACCAAATTTCT	0.93
M104X	ATGGCAGCCGT**NNK**TTAAACGGTACTTTTCATGACCACAT	GTACCGTTTAA**MNN**ACGGCTGCCATTGGTGCCGGAACCAA	0.79
L105X	GCAGCCGTATG**NNK**AACGGTACTTTTCATGACCACATGGA	AAAAGTACCGTT**MNN**CATACGGCTGCCATTGGTGCCGGAAC	0.88
A160X	CAGACAGCCAT**NNK**TCAATCTACGATGGTTGTCAGCAAGG	TCGTAGATTGA**MNN**ATGGCTGTCTGCATCCAGGATTACGT	0.88
S206X	AAGGAGTGTAC**NNK**ATGTTGGGTGACATTGCTCCGCTTAA	TCACCCAACAT**MNN**GTACACTCCTTCAAGGACAACCAGTT	0.82
M207X	GAGTGTACTCT**NNK**TTGGGTGACATTGCTCCGCTTAAGGA	ATGTCACCCAA**MNN**AGAGTACACTCCTTCAAGGACAACCA	0.93
F293X	GTCCGTACATC**NNK**ACGGCATCTCTTCCTCCAAGCGTCGT	AGAGATGCCGT**MNN**GATGTACGGACGGCAAGCAAGACGCA	0.73
T294X	CGTACATCTTC**NNK**GCATCTCTTCCTCCAAGCGTCGTGGC	GGAAGAGATGC**MNN**GAAGATGTACGGACGGCAAGCAAGAC	0.81
S296X	TCTTCACGGCA**NNK**CTTCCTCCAAGCGTCGTGGCCACTGC	CTTGGAGGAAG**MNN**TGCCGTGAAGATGTACGGACGGCAAG	0.86
N375X	GATTATACGTG**NNK**ATGGCCAAACCACCTGCAACTCCTGC	GGTTTGGCCAT**MNN**CACGTATAATCCTCCGTCCAGCAAGG	0.86
M376X	TATACGTGAAT**NNK**GCCAAACCACCTGCAACTCCTGCAGG	GGTGGTTTGGC**MNN**ATTCACGTATAATCCTCCGTCCAGCA	0.89
A377X	ACGTGAATATG**NNK**CGTCCACCTGCAACTCCTGCAGGAAC	GCAGGTGGACG**MNN**CATATTCACGTATAATCCTCCGTCCA	0.86
P379X	ATATGGCCAAA**NNK**CCTGCCACCTGCAACTCCAACTCCTG	GGAGTTGCAGG**MNN**TTTGGCCATATTCACGTATAATCCTC	0.82
P380X	TGGCCAAACCA**NNK**GCAACTCCTGCAGGAACCTTCTTGTT	GCAGGAGTTGC**MNN**TGGTTTGGCCATATTCACGTATAATC	0.91
A381X	CCCGTCCACCT**NNK**ACTCCTGCAGGAACCTTCTTGTTGCG	CCTGCAGGAGT**MNN**AGGTGGACGGGCCATATTCACGTATA	0.89
T382X	AACCACCTGCA**NNK**CCTGCAGGAACCTTCTTGTTGCGTTG	GTTCCTGCAGG**MNN**TGCAGGTGGTTTGGCCATATTCACGT	0.80
R390X	CCTTCTTGTTG**NNK**TGTTCCATTTGTGCGGAACACACCCC	CAAATGGAACA**MNN**CAACAAGAAGGTTCCTGCAGGAGTTG	0.86

Table S2. Primers used for site saturation mutagenesis at Tyr73 position.

Mutants	Forward primers	Reverse primers
Y73A	CTTGGCACC**GCT**AATTACATGGGCATGACCTT	CATGTAATT**AGC**GGTGCCAAGCAGGATGGTAT
Y73C	CTTGGCACC**TGT**AATTACATGGGCATGACCTT	CATGTAATT**ACA**GGTGCCAAGCAGGATGGTAT
Y73D	CTTGGCACC**GAT**AATTACATGGGCATGACCTT	CATGTAATT**ATC**GGTGCCAAGCAGGATGGTAT
Y73E	CTTGGCACC**GAA**AATTACATGGGCATGACCTT	CATGTAATT**TTC**GGTGCCAAGCAGGATGGTAT
Y73F	CTTGGCACC**TTC**AATTACATGGGCATGACCTT	CATGTAATT**GAA**GGTGCCAAGCAGGATGGTAT
Y73G	CTTGGCACC**GGT**AATTACATGGGCATGACCTT	CATGTAATT**ACC**GGTGCCAAGCAGGATGGTAT
Y73H	CTTGGCACC**CAC**AATTACATGGGCATGACCTT	CATGTAATT**GTG**GGTGCCAAGCAGGATGGTAT
Y73I	CTTGGCACC**ATC**AATTACATGGGCATGACCTT	CATGTAATT**GAT**GGTGCCAAGCAGGATGGTAT
Y73K	CTTGGCACC**AAA**AATTACATGGGCATGACCTT	CATGTAATT**TTT**GGTGCCAAGCAGGATGGTAT
Y73L	CTTGGCACC**TTA**AATTACATGGGCATGACCTT	CATGTAATT**TAA**GGTGCCAAGCAGGATGGTAT
Y73M	CTTGGCACC**ATG**AATTACATGGGCATGACCTT	CATGTAATT**CAT**GGTGCCAAGCAGGATGGTAT
Y73N	CTTGGCACC**AAT**AATTACATGGGCATGACCTT	CATGTAATT**ATT**GGTGCCAAGCAGGATGGTAT
Y73P	CTTGGCACC**CCT**AATTACATGGGCATGACCTT	CATGTAATT**AGG**GGTGCCAAGCAGGATGGTAT
Y73Q	CTTGGCACC**CAA**AATTACATGGGCATGACCTT	CATGTAATT**TTG**GGTGCCAAGCAGGATGGTAT
Y73R	CTTGGCACC**CGC**AATTACATGGGCATGACCTT	CATGTAATT**GCG**GGTGCCAAGCAGGATGGTAT
Y73S	CTTGGCACC**AGT**AATTACATGGGCATGACCTT	CATGTAATT**ACT**GGTGCCAAGCAGGATGGTAT
Y73T	CTTGGCACC**ACG**AATTACATGGGCATGACCTT	CATGTAATT**CGT**GGTGCCAAGCAGGATGGTAT
Y73V	CTTGGCACC**GTC**AATTACATGGGCATGACCTT	CATGTAATT**GAC**GGTGCCAAGCAGGATGGTAT
Y73W	CTTGGCACC**TGG**AATTACATGGGCATGACCTT	CATGTAATT**CCA**GGTGCCAAGCAGGATGGTAT

Reference

1. Choe, H.; Cha, M. S.; Stewart, J. D., Expanding the acyl-CoA chain length tolerance of Sphingomonas paucimobilis serine palmitoyltransferase by Mutating Arg 378. *Enzyme Microb. Technol.* **2020,** *137*, 109515.
2. Ishijima, H.; Uchida, R.; Ohtawa, M.; Kondo, A.; Nagai, K.; Shima, K.; Nonaka, K.; Masuma, R.; Iwamoto, S.; Onodera, H.; Nagamitsu, T.; Tomoda, H., Simplifungin and valsafungins, antifungal antibiotics of fungal origin. *J. Org. Chem.* **2016,** *81*, 7373-7383.