Electronic Supplementary Information

Engineered transaminopeptidase, aminolysin-*S* for catalysis of peptide bond formation to give linear and cyclic dipeptides by one-pot reaction

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CONTENTS

Section-1: Materials

Phe-*p*NA, Pro-*p*NA, Leu-*p*NA, and Lys-*p*NA were purchased from Sigma. Ala-*p*NA was obtained from the Peptide Institute, Inc. Other *p*NA derivatives of amino acids were purchased from Bachem AG. A Zero Blunt II TOPO PCR Cloning kit was purchased from Invitrogen Corp. The plasmids pET28a (+) and *E. coli* Rosetta 2(DE3) pLysS were obtained from Novagen Inc. Other chemicals were commercially available.

Section-2. LC/UV/MS/MS Instrumentation

A Waters 2690 Separation Module, Waters 2487 Dual λ Absorbance Detector, and API 2000 LC/MS/MS System (Applied Biosystems) were used. The column used for this study was a TSKgel ODS-120T, 5 mm, 2.0 × 150 mm (Tosoh Corp.). The mobile phase was water containing 0.1% (v/v) HCOOH (A) and acetonitrile containing 0.1% (v/v) HCOOH (B) at a flow rate of 0.2 ml/ min. The LC condition was 15% B during 0–5 min, a linear increase from 15–50% B during 5–30 min, 50% B during 30–35 min, and a linear increase from 50–80% B during 35–40 min.

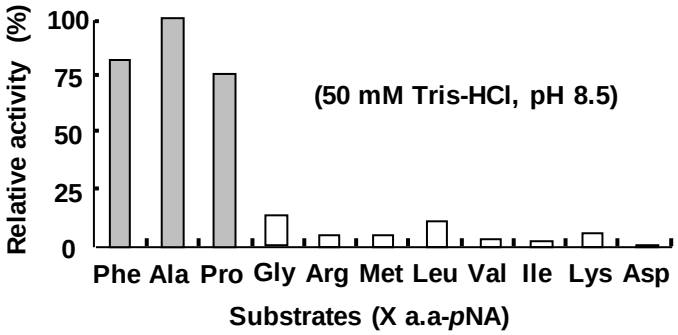
Section-3. Cloning, overexpression, purification, and enzymatic characterization of family S9 aminopeptidase from *Streptomyces thermocyaneoviolaceus* NBRC14271

Genomic DNA was prepared from *Streptomyces thermocyaneoviolaceus* NBRC14271 using the methods described by Hopwood *et al.* (Hopwood *et al.*, 1985). The partial sequence of the targeted gene (*S9AP-ST*) was amplified by PCR using a sense primer (5′-GSGTSATCGAGTACGGAGG-3′), anti-sense primer (5′-CCGTGSCCCTCSCCCTCGAA- 3′), and the genomic DNA. The primer set designated above corresponds to the conserved sequences for SAV 1898 of *S. avermitilis*; SCO 6488 from *S. coelicolor* in those gene products were annotated as putative acyl-peptide hydrolases with high homologies (69.2% and 70.2%, respectively) to the reported S9 aminopeptidase puromycin hydrolase (Nishimura *et al.*, 2006). The PCR product (1.5 kbp) was cloned using a PCR cloning kit (Zero Blunt II TOPO; Invitrogen Corp.) and sequenced. The sequence information for the 5' and 3' regions were obtained using the inverse PCR method, for which *Pvu*II was used for the restriction of the genomic DNA. Finally, the targeted

gene was amplified by PCR using a set that contained a sense primer incorporating the *Nde*I site upstream of a start codon (5′- CATATGTCGGACGTACAGACCC -3′; the start codon is underlined) and an anti-sense primer incorporating the *Hind*III site downstream of a stop codon (5′- AAGCTTCACGTGTGCAGCTCCA- 3′; the stop codon is underlined), along with the genomic DNA. The PCR product (2 kbp) was cloned using a PCR cloning kit (Zero Blunt II TOPO; Invitrogen Corp.) and sequenced, with subsequent digestion using *Nde*I and *Hind*III, to obtain gene fragments encoding the targeted gene. The obtained fragments were introduced into the *Nde*I-*Hind*III gap of pET 28a (+) to generate the plasmids pET28-His_6-S9AP-ST. The expression vectors were designed for the N-terminal His_6-tag. The entire sequence of S9AP-ST has been assigned the accession number AB480284 in the DDBJ database (http://www.ddbj.nig.ac.jp/).

An overnight express system 1 (Novagen Inc.) was used for the overexpression of the protein. Several colonies of Rosetta 2 (DE3) pLysS harboring the expression plasmid described above were inoculated into 50 ml of overnight express Instant TB medium prepared according to the manufacturer's instructions. Cultivation and induction were carried out for 16 h at 30 °C with rotary shaking at 180 rpm; the cells were then collected by centrifugation and disrupted using sonication (Elestin NP035SP; Nepa Gene Co., Ltd.) at 1 min intervals for a total sonication time of 30 min at maximum output. The obtained cell free extract was purified with metal affinity resin (Talon; Clontech) according to the manufacturer's instructions. The purified proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under a reducing condition with Coomassie Blue staining.

The purified S9AP-ST showed aminopeptidase activity with moderately broad substrate specificity, as expected (Fig. S1). Therefore, we tried to change the aminopeptidase S9AP-ST into transaminopeptidase by the site directed mutagenesis of catalytic Ser^{502} to Cys.



• As a substrate, 1 mM of Phe-*p*NA or 2.0 mM of other *p*NA derivatives was used at 37°C in 50 mM Tris-HCl for 1 to 5 min in enzyme assay. The increase in absorbance at 405 nm attributable to release of *p*-nitroaniline was monitored continuously using a spectrometer (U2800; Hitachi Ltd.). The initial rates of the hydrolytic activity were determined from the linear portion of the optical density profile (e_{405nm} of *p*NA=10600 M^{-1}).

Fig. S1 Substrate specificity of S9 aminopeptidase, S9AP-ST

REFERENCES

D. A. Hopwood, M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, H. Schrempf, *A Laboratory Manual*; The John Ines Foundation: Norwich, CT, 1985, pp. 70–84.

M. Nishimura, K. Ikeda, M. Sugiyama, Molecular cloning and characterization of gene encoding novel puromycin-inactivating enzyme from blasticidin S-producing *Streptomyces morookaensis*. J. *Biosci. Bioeng.*, 2006, **101**, 63–69.

Section-4. Construction of S502C mutant, aminolysin-*S*

The pCR-Blunt II-TOPO::*S9AP-ST* plasmid was subjected to PCR using a set containing a sense primer (5'-TGGCCATCCGCGGCGGCT(A→T, Ser→Cys)GCGCCGGCGGCT-3'; corresponding to 1487–1516 of *S9AP-ST*, an internal *Bal*I site is underlined) and an anti-sense primer incorporating the *Hind*III site downstream of a stop codon (5'-AAGCTTCACGTGTGCAGCTCCA-3'; the stop codon is underlined). The PCR product (0.5 kbp) was cloned using a Zero Blunt II TOPO (Invitrogen Corp.) and sequenced, followed by digestion with *Bal*I and *Hind*III to obtain the 0.5 kbp fragment. The fragment was introduced into the *Bal*I and *Hind*III gap of pET28-His_6-S9AP-ST to generate the plasmids pET28-

His_6-*S502C* and sequenced. The *E. coli* Rosetta 2(DE3) pLysS was transformed with the constructed plasmid. The expression vectors were designed for the N-terminal His_6-tag.

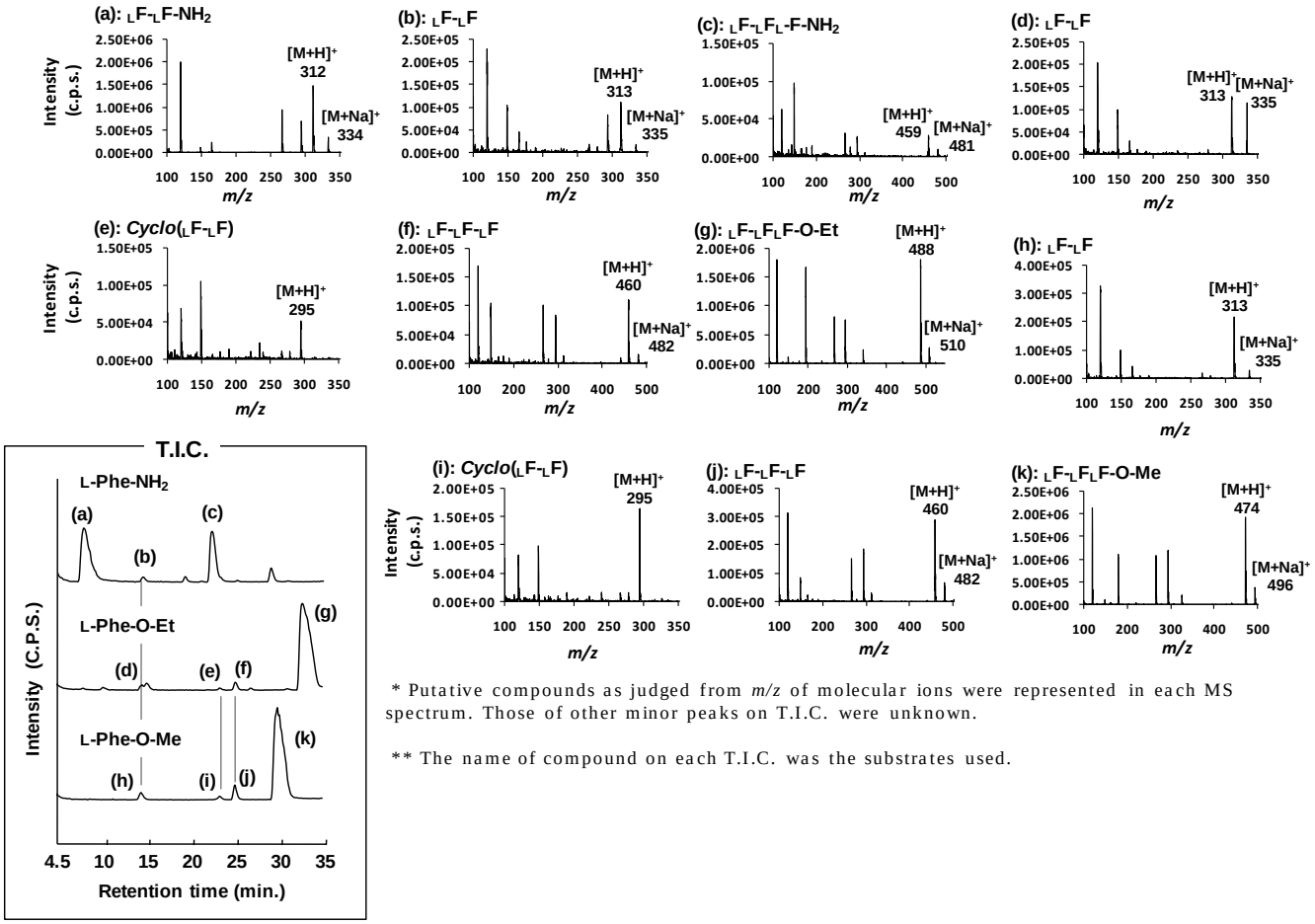
The overexpression and purification of the S502C mutant were done similarly to those of S9AP-ST described above. The enzyme was designated as aminolysin-*S* because of its catalytic mechanism (aminolysis) and the genus of the microorganism (*Streptomyces*).

Section-5. Screening for reactive amino acid derivatives as donors and acceptors

Table S1 lists the amino acid derivatives that were tested. Enzyme reactions were performed as follows. First, 20 mM of each derivative was incubated with 10 μg of aminolysin-*S* in 0.5 M sodium phosphate buffer (pH 7.5) at 50 °C for 23 h. The final volume of each reaction mixture was 100 μl, which contained 5% DMSO (v/v). The reaction was quenched by the addition of 100 μl of 3% (v/v) HCOOH and centrifuged. Then, 20 μl from the upper layer was subjected to ODS-HPLC/UV/MS analysis. The underlined compounds in Table S1 are the reactive amino acid derivatives recognized by aminolysin-*S*. Figs. S2-1–S2-3 show their total ion chromatograms and MS or MS/MS spectra. Those of the non-enzymatic control reaction mixtures were omitted from the figures because of the non-production of peptides.

Table S1. List of tested amino acid derivatives

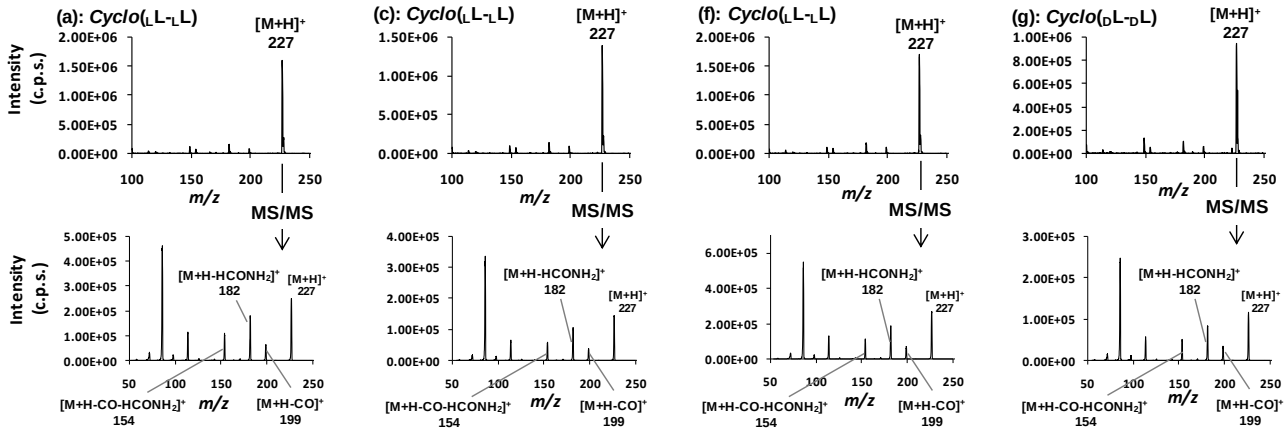
A mino acid moieties	Types of derivatization of carboxyl group	Companies	Amino acid moieties	Types of derivatization of carboxyl group	Companies
L-Lys	O-Me	SIGMA	L-Ala	NH_2	novabiochem
L-Arg	O-Me	BACHEM		O-Me	ALDRICH
L-His	O-Me	ALDRICH	D-Ala	NH2	BACHEM
L-Ile	O-Bzl	BACHEM		O-Me	BACHEM
L-Phe	NH_2	novabiochem		O-Bzl	BACHEM
	O-Me	ALDRICH		O-t-butyl	BACHEM
	O-Et	ALDRICH	Beta-Ala	O-Me	SIGMA
D-Phe	O-Me	ALDRICH	L-Met	O-Me	SIGMA
	O-Bzl	BACHEM	L-Pro	O-Me	ALDRICH
L-Leu	NH2	novabiochem		O-Bzl	BACHEM
	O-Me	ALDRICH	L-Asp	$O-(Me)_2$	ALDRICH
	O-Et	BACHEM	L-Glu	$O-(Et)_2$	ALDRICH
	O-Bzl	PEPTIDE INSTITUE	L-Thr	O-Me	SIGMA
	O-*t*-butyl	SIGMA	L-Gln	NH2	BACHEM
	O-allyl	SIGMA	L-Tyr	O-Me	ALDRICH
D-Leu	O-Bzl	BACHEM	L-Tyr	O-Bzl	BACHEM
L-Trp	O-Me	ALDRICH	Gly	O-Me	ALDRICH
L-Cys	O-Me	ALDRICH	L-Ser	O-Me	ALDRICH
L-Asn	O-Me	BACHEM		O-Bzl	BACHEM
L-Val	O-Me	ALDRICH	D-Ser	O-Bzl	BACHEM
	O-Bzl	BACHEM			



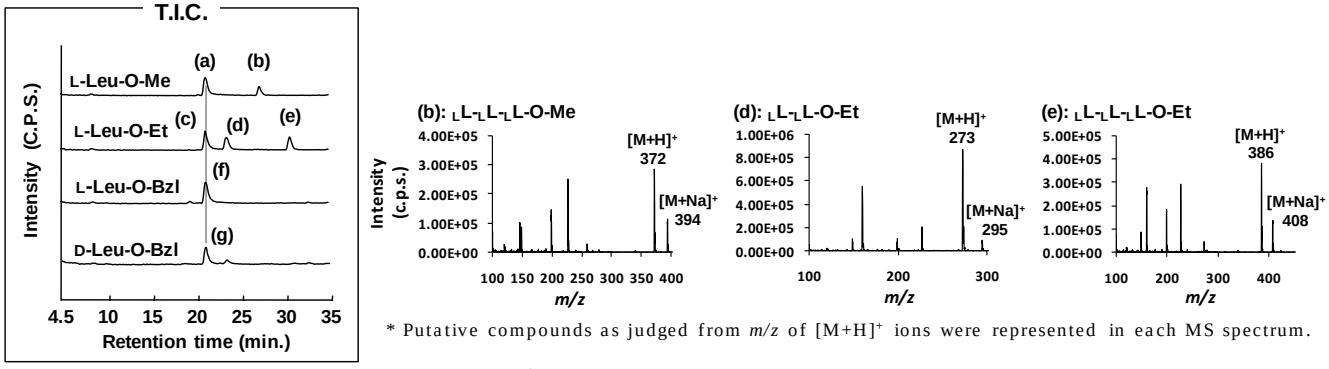
* Putative compounds as judged from *m/z* of molecular ions were represented in each MS spectrum. Those of other minor peaks on T.I.C. were unknown.

** The name of compound on each T.I.C. was the substrates used.

Fig. S2-1 Reactive amino acid derivatives for aminolysin-*S* catalyzed reaction (1)



* Represented fragmentations on each MS/MS spectra were typical for diketopiperazine ling (Xing et al., 2008), and therefore, we judged that the corresponding compound was a cyclic dipeptide, not a simple dipeptide. Putative compounds as judged from *m/z* of molecular ions were represented in each MS spectrum.



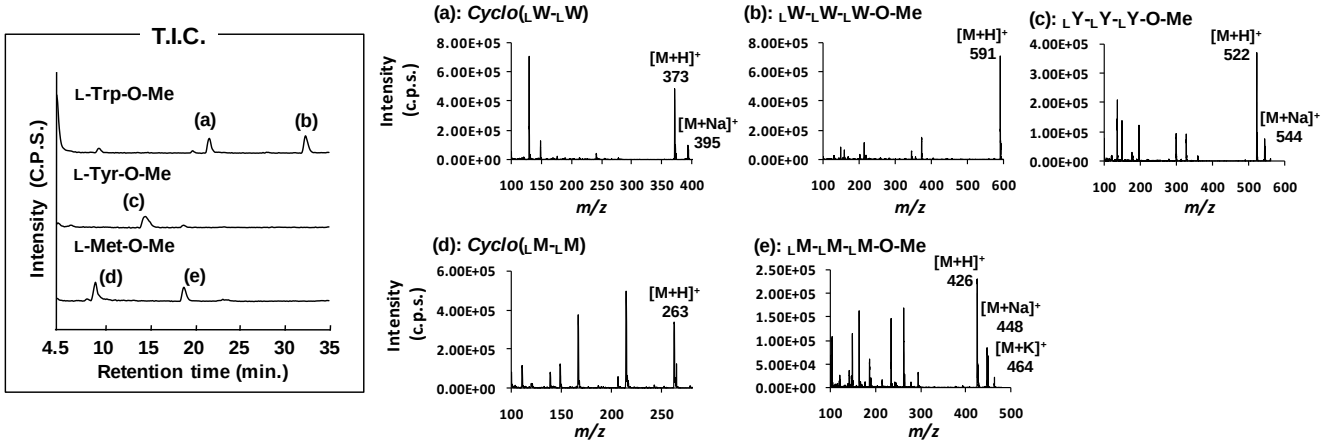
* Putative compounds as judged from *m/z* of $[M+H]^+$ ions were represented in each MS spectrum.

** The name of compound on each T.I.C. was the substrates used.

Fig. S2-2 Reactive amino acid derivatives for aminolysin-*S* catalyzed reaction (2)

REFERENCE

J. Xing, Z. Yang, B. Lv, L. Xiang, Rapid screening for cyclo-dopa and diketopiperazine alkaloids in crude extracts of Portulaca oleracea L. using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.*, 2008, **22**, 1415–1422.



* Putative compounds as judged from *m/z* of molecular ions were represented in each MS spectrum.
** The name of compound on each T.I.C. was the substrates used.

Fig. S2-3 Reactive amino acid derivatives for aminolysin-*S* catalyzed reaction (3)

Section-6. Experimental procedures for characterization of aminolysin-*S* catalyzed peptide bond formation (Fig. 1 of the main text).

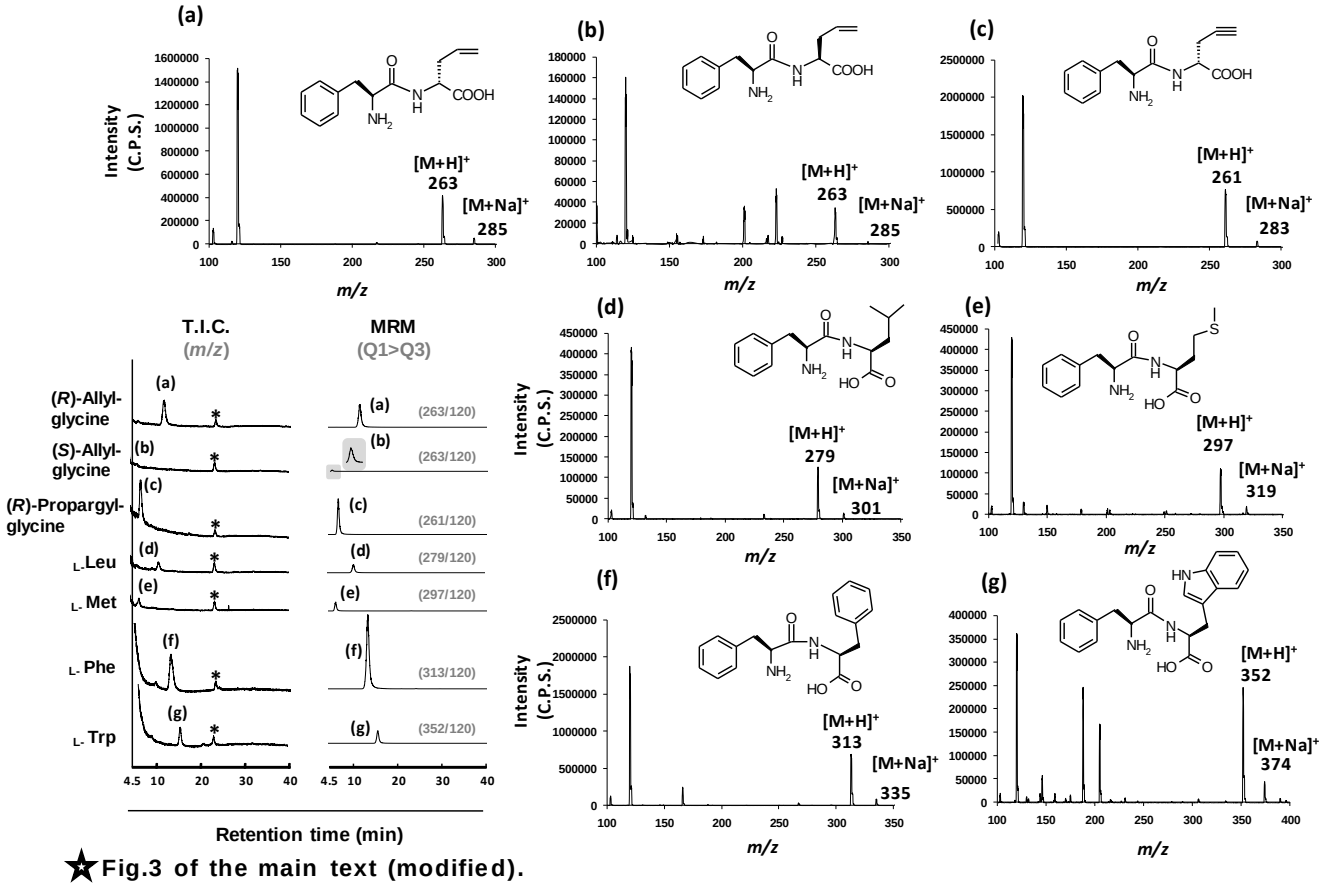
The dependency of the amino acid ligation on the amount of enzyme (Fig. 1[A]) was confirmed as follows. First, 60 mM of $_{L}$F and 0.5 mM of $_{L}$F-O-Et were incubated with 0–30 μg of aminolysin-S in 0.5 M sodium phosphate buffer (pH 7.5) at 50 °C for 1 h. The final volume of each reaction mixture was 100 μl, which contained 5% DMSO (v/v). The reactions were quenched by the addition of 100 μl of 3% (v/v) HCOOH and centrifuged. Then, 20 μl from the upper layer was subjected to ODS-HPLC/UV/MS analysis. Identification of the $_{L}$F-O-Et, $_{L}$F-$_{L}$F, and cyclo($_{L}$F-$_{L}$F) was done by comparing the retention times for the authentic compounds obtained from Sigma. Those of $_{L}$F-$_{L}$F-O-Et were judged from their $[M+H]^+$ ions at *m/z* 341 on the MS spectra. For the time course experiment (Fig. 1[B]), 10 μg of aminolysin-*S* was subjected to the above reaction and incubated for 0–3 h followed by ODS-HPLC/UV/MS analysis, which was performed similarly. Each compound, except for $_{L}$F-$_{L}$F-O-Et, was quantified on the basis of its peak area at 210 nm using a calibration curve obtained from the authentic standard. In the case of $_{L}$F-$_{L}$F-OEt, the calibration curve of $_{L}$F-$_{L}$F was used because of the small effect of ethyl-etherification on its UV adsorption at 210 nm.

Section-7. Detailed characterization of Fig. 3 of the main text for evaluation of diverse free amino acids as acceptor amines

Table S2 lists the free amino acid derivatives that were tested. Enzyme reactions were performed as follows. First, 0.5 mM of the $_{L}$F-O-Et and 60 mM of each amino acid were incubated with 10 μg of aminolysin-*S* in 0.5 M sodium phosphate buffer (pH 7.5) at 50 °C for 15 h. The final volume of each reaction mixture was 100 μl, which contained 5% DMSO (v/v). The reactions were quenched by the addition of 100 μl of 3% (v/v) HCOOH and centrifuged. Then, 20 μl from the upper layer was subjected to an ODS-HPLC/MS analysis.

The underlined compounds shown in Table S2 are the reactive amino acids recognized by aminolysin-*S*. Their MS spectra are presented in Fig. S3. Those of the non-enzymatic control reaction mixtures were omitted because of the non-production of peptides. The MRM experiments were conducted to detect the $_{L}$Phe containing dipeptides. In these experiments, the MRM transition for each dipeptide was the *m/z* of the expected precursor ion > 120 (Q1 > Q3), in which a daughter ion (Q3 value) at *m/z* 120 was an immonium ion of phenylalanine [see Fig. S4, MS/MS spectra of authentic $_{L}$F-$_{L}$F and cyclo($_{L}$F-$_{L}$F)]. Therefore, the compounds detected in this experiment (right panel of Fig. 3 of main text) were dipeptides containing $_{L}$Phe residues. The experimental parameters of the MRM experiment, which were optimized using $_{L}$Phe-$_{L}$Phe, were as follows: DP = 61V, FP = 370 V, EP = 11 V, CEP = 27 V, CE = 51 V, and CXP = 13 V. Those for cyclo ($_{L}$F-$_{L}$F) were DP = 151 V, FP = 370 V, EP = 12 V, CEP = 22 V, CE = 27 V, and CXP = 4 V. Table S3 shows the amounts of the products quantified by these MRM experiments. In this analysis, the calibration curve of $_{L}$F-$_{L}$F was used for the quantification of the linear dipeptides ($_{L}$F-Xs). It should be noted that the product amounts listed in Table S3 are semi-quantitative values, except for those of $_{L}$F-$_{L}$F and cyclo($_{L}$F-$_{L}$F), because of the absence of authentic standards.

Table S2. List of tested free amino acids as acceptors			
Compounds	Companies	Compounds	Company
L-Lys	WAKO	(*R*)-α-Methylphenylalanine	NAGASE and CO., LTD. (Japan)
L-Arg	WAKO	(*S*)-α-Methylphenylalanine	NAGASE and CO., LTD. (Japan)
L-His	WAKO	(*R*)-α-Methyl-3-nitrophenylalanine	NAGASE and CO., LTD. (Japan)
D-His	WAKO	(*S*)-α-Methyl-3-nitrophenylalanine	NAGASE and CO., LTD. (Japan)
L-Ile	nacalai	(*S*)-β-(4-Bisphenyl)-α-methylalanine	NAGASE and CO., LTD. (Japan)
L-Phe	WAKO	(*S*)-4-Bromo-α-methylphenylalanine	NAGASE and CO., LTD. (Japan)
D-Phe	BACHEM	(*S*)-4-Iodo-α-methylphenylalanine	NAGASE and CO., LTD. (Japan)
L-Leu	WAKO	(*R*)-α-Methyltyrosine	NAGASE and CO., LTD. (Japan)
D-Leu	ALDRICH	(*S*)-α-Methyltyrosine	NAGASE and CO., LTD. (Japan)
L-Trp	WAKO	(*R*)-α-Allylglycine	NAGASE and CO., LTD. (Japan)
D-Trp	SIGMA	(*S*)-α-Allylglycine	NAGASE and CO., LTD. (Japan)
L-Ala	WAKO	(*R*)-α-Propargylglycine	NAGASE and CO., LTD. (Japan)
D-Ala	WAKO	(*S*)-α-Propargylglycine	NAGASE and CO., LTD. (Japan)
L-Met	WAKO	(*R*)-α-Methylleucine	NAGASE and CO., LTD. (Japan)
L-Pro	nacalai	(*S*)-α-Methylleucine	NAGASE and CO., LTD. (Japan)
L-Val	WAKO		
L-Asp	WAKO		
L-Glu	WAKO		
L-Thr	nacalai		
D-Thr	SIGMA		
L-Gln	WAKO		
D-Gln	SIGMA		
L-Tyr	WAKO		
D-Tyr	ALDRICH		
Gly	WAKO		
L-Ser	WAKO		
L-Cys	SIGMA		
D-Cys	WAKO		
L-Asn	WAKO		
D-Asn	WAKO		



★Fig.3 of the main text (modified).

Fig. S3 MS spectra of produced peptides of Fig. 3.

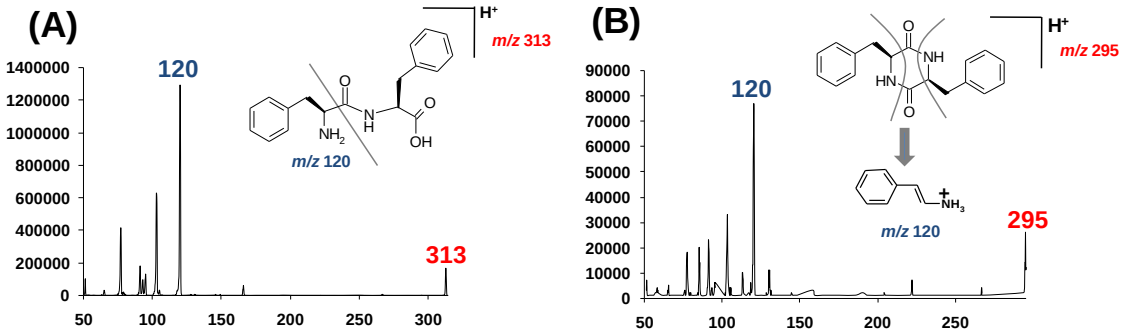


Fig. S4 MS/MS spectra of (A) $_{L}$F-$_{L}$F and (B) cyclo ($_{L}$F-$_{L}$F)

Table S3. Amounts of $_L$phenylalanine-containing dipeptides

Acceptor	Products: $_L$F-X[1)] (μM)	Products: Cyclo($_L$F-$_L$F) (μM)	Ratio of product-amounts [$_L$F-X[1)] to Cyclo($_L$F-$_L$F)]
(R)-a-Allylglycine	75.0	18.6	4.03
(S)-a-Allylglycine	2.76	24.4	0.113
(R)-a-Propargylglycine	80.8	12.7	6.36
$_L$Phenylalanine	246	26.5	9.28
$_L$Tryptophan	32.8	19.9	1.65
$_L$Leucine	23.2	29.5	0.786
$_L$Methionine	29.7	19.6	1.52

1) X: corresponding to acceptor amines. The amount of each compound was quantified as $_L$F-$_L$F.

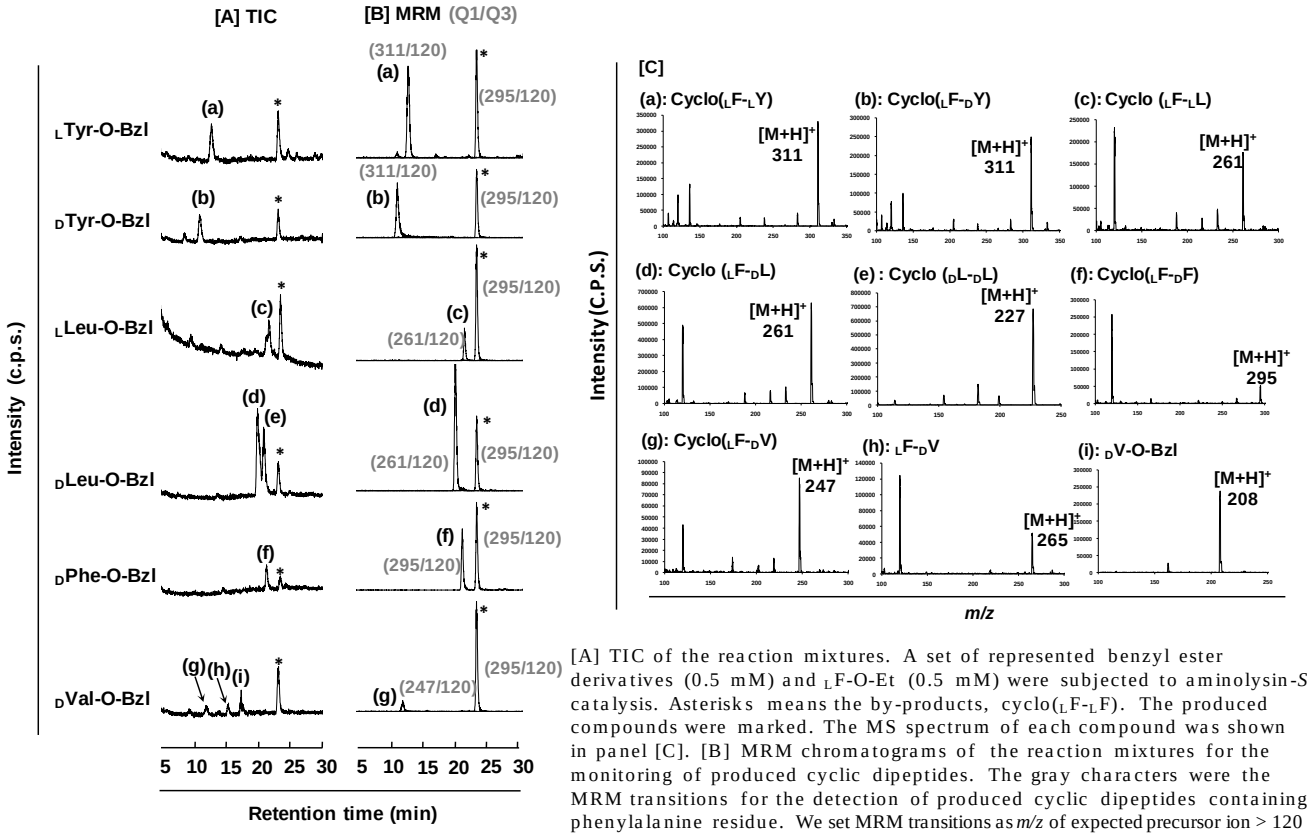
Section-8. Evaluation of benzyl ester derivatives of amino acids for one-pot synthesis of diverse cyclic dipeptides (Fig. 4 of the main text)

Table S4 list the benzyl esters of the amino acids that were tested. Enzyme reactions were performed as follows. First, 0.5 mM of the $_L$F-O-Et and 0.5 mM of each benzyl ester derivative of the amino acids was incubated with 10 μg of aminolysin-*S* in 0.5 M sodium phosphate buffer (pH 7.5) at 50 °C for 12 h. The final volume of each reaction mixture was 100 μl, which contained 1% DMSO (v/v). The reactions were quenched by the addition of 100 μl of 3% HCOOH and centrifuged. Then, 20 μl from the upper layer was subjected to ODS-HPLC/MS or MS/MS analysis. The underlined compounds in Table S4 are the reactive benzyl esters recognized by aminolysin-*S*. Their TIC, MRM chromatograms, and MS spectra are represented in Fig. S5. The MRM transition for each cyclic dipeptide was the *m/z* of the expected precursor ion > 120 (Q1 > Q3), in which the daughter ion (Q3 value) at *m/z* 120 was an immonium ion of phenylalanine [see Fig. S4[B], MS/MS spectrum of cyclo($_L$F-$_L$F)]. Therefore, the detected compounds in Fig.S5[B] were cyclic dipeptides containing $_L$Phe residues. Table S5 shows the amounts of the products quantified by these MRM experiments. In this analysis, the calibration curve of cyclo($_L$F-$_L$F) was used for the quantification of each product. The product amounts listed on Table S5 are semi-quantitative values, except for those of cyclo($_L$F-$_L$F), because of the absence of authentic standards. The most interesting and surprising observation was the aminolysin-*S* catalyzed reaction with a set of $_L$F-O-Et and benzyl ester derivatives of diverse $_D$-amino acids. To confirm such

exciting results, we selected the set of the benzyl esters that were enantiomers, $_{L}$Y-O-Bzl and $_{D}$Y-O-Bzl, for detailed characterization by MS/MS analysis.

Table S4. List of tested benzyl esters of amino acids.

Amino acid moiety	Company
$_{L}$-Leu	Peptide Institute
$_{D}$-Leu	Bachem AG
$_{D}$-Ser	Bachem AG
$_{L}$-Tyr	Bachem AG
$_{D}$-Val	Bachem AG
$_{L}$-Pro	Bachem AG
$_{D}$-Tyr	Bachem AG
$_{D}$-Ala	Bachem AG
$_{D}$-Phe	Bachem AG



[A] TIC of the reaction mixtures. A set of represented benzyl ester derivatives (0.5 mM) and $_{L}$F-O-Et (0.5 mM) were subjected to aminolysin-*S* catalysis. Asterisks means the by-products, cyclo($_{L}$F-$_{L}$F). The produced compounds were marked. The MS spectrum of each compound was shown in panel [C]. [B] MRM chromatograms of the reaction mixtures for the monitoring of produced cyclic dipeptides. The gray characters were the MRM transitions for the detection of produced cyclic dipeptides containing phenylalanine residue. We set MRM transitions as *m/z* of expected precursor ion > 120 (Q1 > Q3), in those, Q 3 values as *m/z* 120 were characteristic as an immonium ion of phenylalanine. Asterisks means the by-products, cyclo($_{L}$F-$_{L}$F).

Fig. 5 Reactive benzyl esters of amino acid for one-pot synthesis of diverse CDPs by aminolysin-*S* catalyzed reaction

Table S5. Amounts of $_{L}$phenylalanine-containing cyclic dipeptides.

Substrates (X-O-Bzl)	Products: Cyclo($_{L}$F-X)[1] (μM)	Products: Cyclo($_{L}$F-$_{L}$F) (μM)	Ratio of product-amounts [Cyclo($_{L}$F-X)[1] to Cyclo($_{L}$F-$_{L}$F)]
$_{L}$Tyr-O-Bzl	25.3	22.0	1.15
$_{D}$Tyr-O-Bzl	15.3	13.6	1.12
$_{L}$Leu-O-Bzl	5.33	22.5	0.237
$_{D}$Leu-O-Bzl	31.1	14.5	2.15
$_{D}$Phe-O-Bzl	16.5	12.5	1.32
$_{D}$Val-O-Bzl	2.49	21.9	1.14

1) X: corresponding to amino acid moiety of the benzyl ester derivative used. The amount of each compound was quantified as cyclo ($_{L}$F-$_{L}$F).

Section-9. Detailed characterization of MS/MS spectra (Fig. 4[B] of the main text) of enzymatically produced cyclo($_{L}$F-$_{L}$Y) and cyclo($_{L}$F-$_{D}$Y)

The enzyme reaction mixtures described in **Section 8** were used for an LC/MS/MS analysis. In the enzyme reaction, 0.5 mM $_{L}$F-O-Et and 0.5 mM $_{L}$Y-O-Bzl or its enantiomer $_{D}$Y-O-Bzl were subjected to the aminolysin-*S* catalyzed reaction. As shown in Fig. 4[A] of the main text and Fig. S5, their TIC chromatograms gave two distinct new products, (a) and (b). Their MS spectra gave $[M+H]^{+}$ ions at *m/z* 311.0, suggesting the presence of a set of diastereomers corresponding to cyclo($_{L}$F-$_{L}$Y) and cyclo($_{L}$F-$_{D}$Y). As depicted in Fig. S6, the MS/MS spectra of the respective ions at *m/z* 311.0 gave identical fragmentations. The ions of (a), (b), and (c) were reported as specific for the diketopiperazine skeleton (Xing *et al.*, 2008). Those of (d) with (f) and (e) with (g), respectively, signaled the presence of Phe and Tyr moieties (Xing *et al.*, 2008). From the observation described above, the produced compounds corresponding to peak (A) and peak (B) were identified as cyclo($_{L}$F-$_{L}$Y) and cyclo($_{L}$F-$_{D}$Y), respectively. Therefore, aminolysin-*S* was demonstrated to recognize the diverse $_{D}$-amino acid derivatives of benzyl esters to synthesize diverse cyclic dipeptides by a one-pot reaction.

REFERENCE

J. Xing, Z.Yang, B. Lv, L. Xiang, Rapid screening for cyclo-dopa and diketopiperazine alkaloids in crude extracts of Portulaca oleracea L. using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.*, 2008, **22**, 1415–1422.

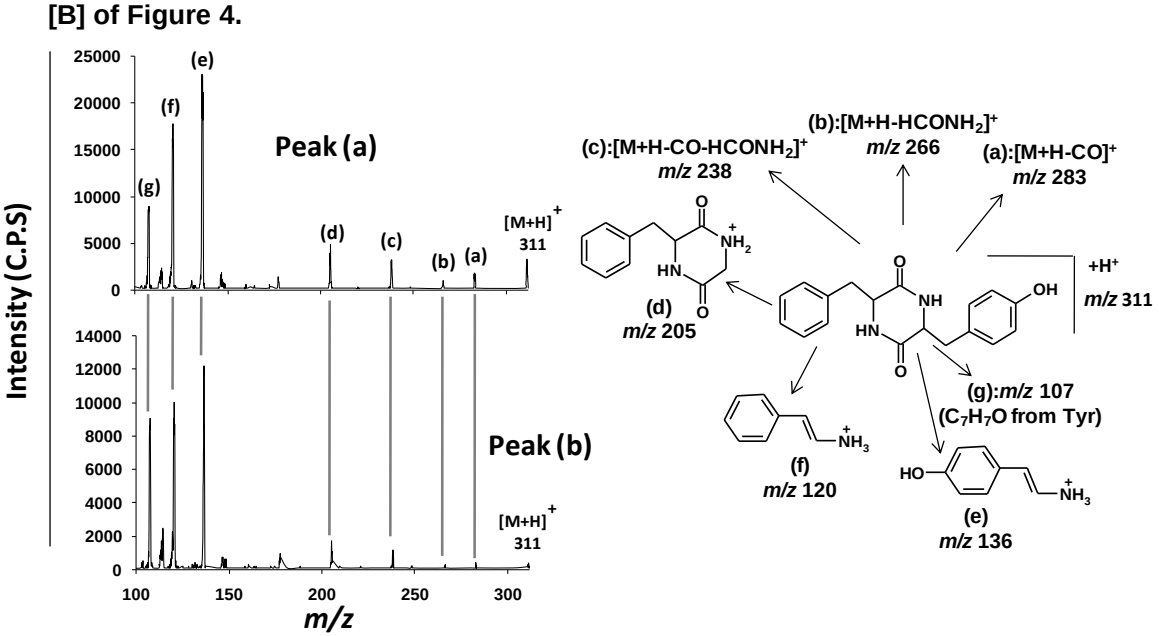


Fig. S6 Detailed characterization of MS/MS spectrums shown in Figure 4 of Main Text.

Section-10. Enzymatic characteristics of aminolysin-*S*

S10-1: Optimum pH

One mM of $_L$F-NH$_2$ as a donor and 80 mM of $_L$-tryptophan as an acceptor were incubated with 10 μg of aminolysin-*S* at several pHs in 0.5 M sodium phosphate or sodium borate buffers at 30 °C for 1 h. The final volume of each reaction mixture was 100 μl, which contained 1% DMSO (v/v). The reaction was quenched by the addition of 100 μl of 20% CH_3CN containing 0.05% TFA (v/v) followed by centrifugation. Then, 20 μl from the upper layer was subjected to LC/UV/MS analysis. The peak on the total ion chromatograms giving the $[M+H]^+$ ions at *m/z* 352 was attributed to dipeptide $_L$F-$_L$W. The quantification of the produced dipeptide was performed using the peak area of the corresponding peak on the UV chromatogram (210 nm). The results are shown in Fig. S7.

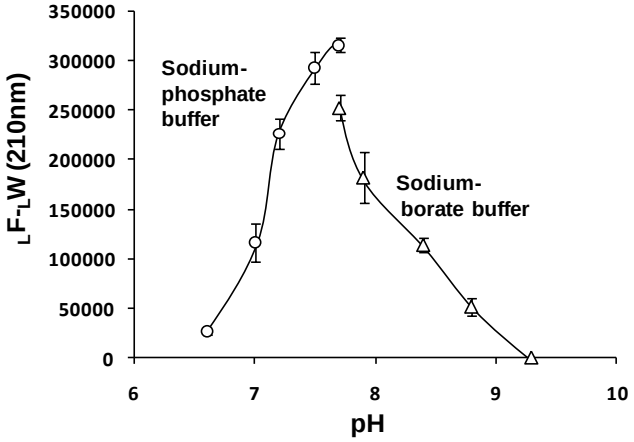


Fig. S7 Optimum pH

S10-2: Effects of DMSO

At 50 °C for 1 h, 0.5 mM of $_{L}$F-O-Et as a donor and 40 mM $_{L}$-phenylalanine as an acceptor were incubated with 10 μg of aminolysin-*S* in 0.5 M sodium phosphate buffer (pH 7.5). The final volume of the reaction mixture was 100 μl, which contained DMSO at several percent. The reaction was quenched by the addition of 100 μl of 3% (v/v) HCOOH, followed by centrifugation. Then, 20 μl from the upper layer was subjected to LC/UV/MS analysis. The identification of the produced compound as $_{L}$F-$_{L}$F was done by comparing the retention time and fragmentation profiles of the MS/MS spectrum of the authentic compound obtained from Sigma. The relative enzymatic activity was calculated as the amount of produced $_{L}$F-$_{L}$F.

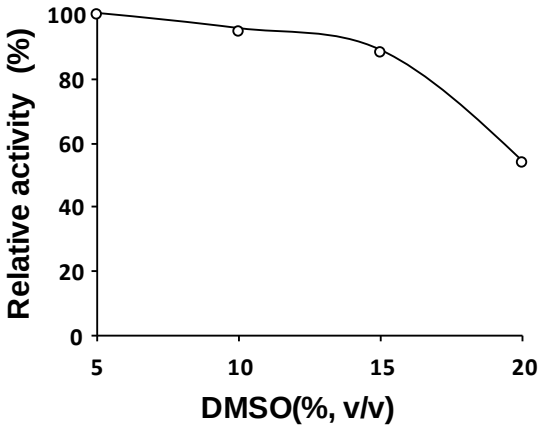


Fig.S8 Effect of DMSO

S10-3: Heat stability

In 50 μl of 0.5 M sodium-phosphate buffer (pH 7.5), 50 μg of aminolysin-*S* was treated at several temperatures for 1 h. Then, 10 μl (10 μg) of each sample solution was subjected to a reaction with 0.5 mM $_{L}$F-O-Et as a donor and 60 mM $_{L}$-phenylalanine as an acceptor in 0.5 M sodium-phosphate buffer (pH 7.5) at 50 °C for 1 h, followed by the addition of 100 μl of 3% (v/v) HCOOH and centrifugation. Then, 10 μl from the upper layer was subjected to LC/MS/MS analysis for the quantification and identification of the produced $_{L}$F- $_{L}$F. The result is shown in Fig. S9[A]. For an indication of the inactivation curve, 50 μg of aminolysin-*S* in 50 μl of 0.5 M sodium-phosphate buffer (pH 7.5) was

incubated for 0–6 h at 50 °C, followed by subjection to the enzyme reaction in the manner described above. The result is shown in Fig. S9 [B].

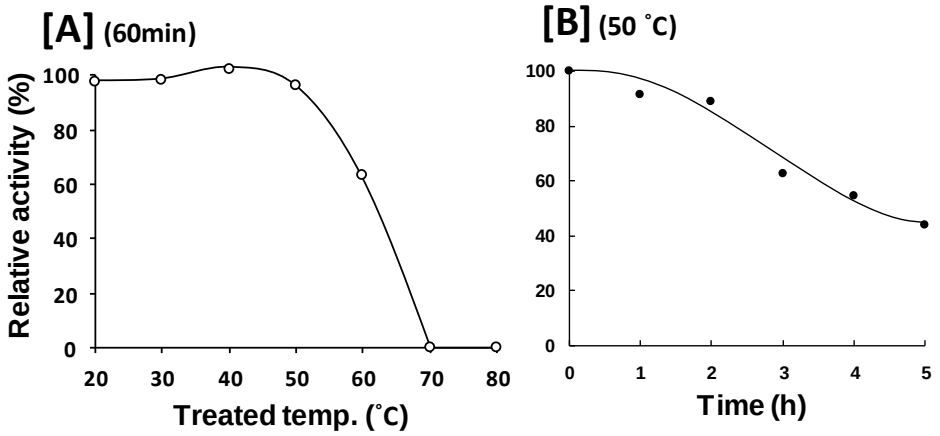


Fig. S9 Heat stability

S10-4: Dose response curve of the acceptor amines

In 0.5 M sodium phosphate buffer (pH 7.5) at 50 °C for 1 h, 0.5 mM of $_{L}$F-O-Et as a donor and several amounts of $_{L}$F or $_{L}$W as an acceptor were incubated with 10 μg of aminolysin-*S*. The procedure for the quenching of the reaction and the quantification of the produced $_{L}$F-$_{L}$F, cyclo($_{L}$F-$_{L}$F), $_{L}$F-O-Et, $_{L}$F-$_{L}$F-O-Et, and $_{L}$F-$_{L}$W were as described above. The results are presented in Fig. S10.

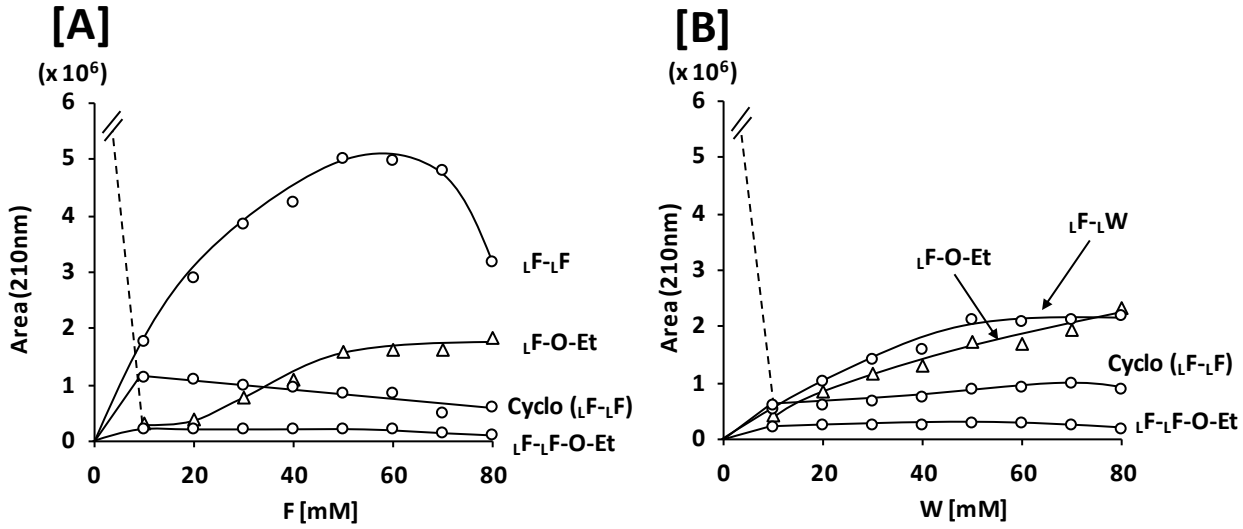


Fig. S10 Dose response curve of acceptor amines