PAPER

Peptide bond formation by aminolysin-*A* catalysis: A simple approach to enzymatic synthesis of diverse short oligopeptides and biologically active puromycins†

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A new S9 family aminopeptidase derived from the actinobacterial thermophile *Acidothermus cellulolyticus* was cloned and engineered into a transaminopeptidase by site-directed mutagenesis of catalytic Ser[491] into Cys. The engineered biocatalyst, designated aminolysin-*A*, can catalyze the formation of peptide bonds to give linear homo-oligopeptides, hetero-dipeptides, and cyclic dipeptides using cost-effective substrates in a one-pot reaction. Aminolysin-*A* can recognize several C-terminal-modified amino acids, including the L- and D-forms, as acyl donors as well as free amines, including amino acids and puromycin aminonucleoside, as acyl acceptors. The absence of amino acid esters prevents the formation of peptides; therefore, the reaction mechanism involves aminolysis and not a reverse reaction of hydrolysis. The aminolysin system will be a beneficial tool for the preparation of structurally diverse peptide mimetics by a simple approach.

Introduction

Small molecules composed of natural or unnatural amino acid moieties comprise an attractive group of biologically active compounds. Typical examples are short oligopeptides composed of amino acid residues connected by amide bonds. Tripeptide mimic leupeptin is one of the best characterized serine/cysteine proteinase inhibitors.[1–3] E-64 and its derivatives also comprise peptide-mimicking skeletons and reportedly show selective inhibition toward thiol proteinases.[4] Furthermore, unmodified short oligopeptides have been reported to show diverse biological activities.[5–9] Therefore, incorporation of diverse amino acid moieties into other peptide skeletons by amide bonds is an attractive strategy for the development of novel bioactive small molecules.

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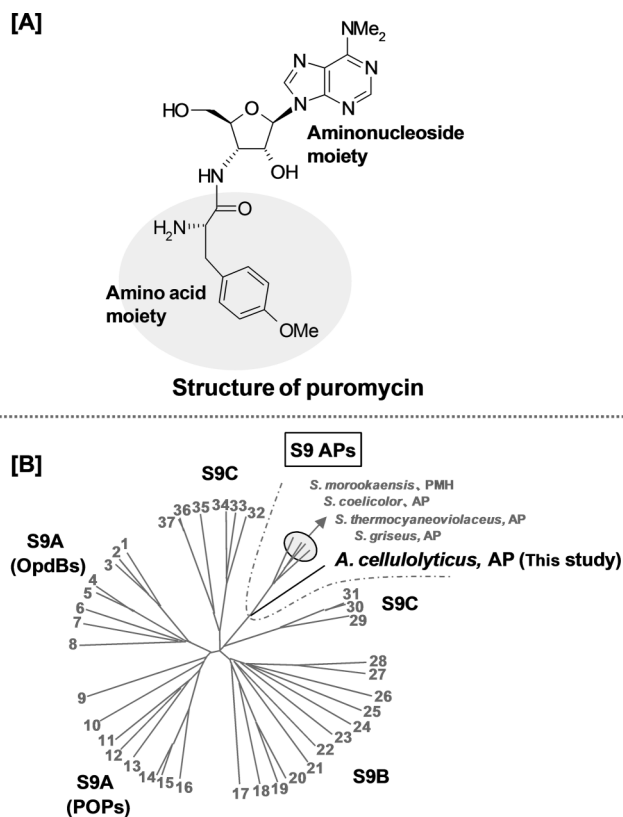
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† Electronic supplementary information (ESI) available: A list of the known S9 family enzymes, enzymatic characteristics of the parent S9-ACAP, and MS and MS/MS data of produced compounds. See DOI: 10.1039/c0ob00403k

Puromycin (PM) is an aminoacyl nucleoside antibiotic produced by *Streptomyces alboniger* (Fig. 1[A]). PM, which is categorized as a mimic of aminoacyl-tRNA (aa-tRNA), acts as an inhibitor of protein synthesis.[10,11] The compound and its analogues are widely used as chemical probes for labelling the C-terminal end of proteins.[12] Furthermore, Starck *et al.* demonstrated that PM derivatives in which the amino acid moiety (the 4-methoxy-L-tyrosine residue of PM) was changed to D and L forms or *β*-amino acid analogues can act as pseudo-substrates in an intact eukaryotic translation system and result in the incorporation of unnatural amino acid residues at the C-terminal end of the full length protein. The authors also demonstrated the inhibitory effect of PM analogues on mRNA translation.[13] Therefore, PM and its analogues are attractive small molecules for the development of new antibiotics or chemical probes for biochemical studies. Herein, the chemical structure of PMs is explained as follows: the amino group of the 3′-amino-3′-deoxynucleoside skeleton is acylated by the carboxyl group of 4-methoxy-L-tyrosine to form an amide bond. Therefore, the compound could also be categorized as a biologically active molecule containing an amino acid moiety linked by an amide bond.

Chemical syntheses of the aforementioned peptidic compounds have already been well characterized. Nevertheless, the chemical methods remain to be developed because of the protection/deprotection procedures required for reacting groups, purification procedures involving excessive amounts of catalysts or byproducts, and the use of excessive amounts of organic solvents. Enzymatic synthesis can be regarded as an alternative method for overcoming such difficulties.[14]

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Org. Biomol. Chem., 2011, **9**, 2327–2335 | 2327

Fig. 1 Search for S9 family aminopeptidase (AP) possessing puromycin hydrolyzing activity. [A] Structure of puromycin. [B] Phylogenetic relationships among S9 family APs and other S9 family peptidases including subfamilies S9A, S9B, and S9C. The 37 types of S9 oligopeptidases, including S9A, S9B, and S9C (gray numbers; origins of each enzyme are omitted for clarity), were collected from the MEROPS database (http://merops.sanger.ac.uk) and a previously published paper.[40] The enzymes are oligopeptidases, not APs. Each subfamily is proposed to be distinct in its substrate specificity. The origins of the enzymes are shown in Table S1, ESI.† The four types of S9 APs (gray characters) have previously been reported.[15,18–20] Each enzyme is an AP and not an oligopeptidase. The full-length amino acid sequences were compared with that of putative S9 AP derived from *A. cellulolyticus* (black character) using the CLUSTAL W program (http://align.genome.jp/) to construct the unrooted dendrogram.

Our group recently reported engineering a novel S9 family aminopeptidase (AP) derived from an actinomycetes into transaminopeptidase aminolysin-*S* by site-directed mutagenesis of catalytic Ser into Cys.[15] The engineered transaminopeptidase catalyzed the formation of peptide bonds to give linear dipeptides, oligopeptides, and cyclic dipeptides *via* a one-pot reaction. In addition, the site-directed mutagenesis also reflected the substrate specificity in the aminolysis reaction[16,17] In fact, the parent S9 AP possessing catalytic Ser can also catalyze the aminolytic peptide bond formation to give peptides such as *β*-Ala and Pro containing dipeptides. However, it cannot produce hydrophobic homopeptides including L-Phe-L-Phe-OEt because of its re-hydrolysis by the used enzyme. By contrast, our engineered transaminopeptidase aminolysin-*S*, in which the catalytic Ser of the parent S9 AP is mutated into Cys, can recognize L-Phe-OEt as a good acyl donor/acceptor to give L-Phe-L-Phe-OEt in good yield because re-hydrolysis of the produced dipeptide

does not occur. This observation suggests that the substrate recognition property of aminolysin-*S* in the aminolysis reaction would correlate with that of the parent S9 AP in the hydrolysis reaction. The amino acid sequence of this parent S9 AP is 69.8% identical to a previously reported AP derived from *Streptomyces morookaensis*, puromycin hydrolase (PMH).[18,19] PMH reportedly catalyzes the hydrolysis of amide bonds between the amino acid moiety and the 3′-amino-3′-deoxynucleoside of PM. In addition, we demonstrated the wide variety of its homologous APs in the field of actinomycetes.[20] The hydrolytic activities of PMH homologues toward PM remain to be clarified. Nevertheless, our continuing database-mining approach resulted in the discovery of a putative S9 family AP in the gram-positive actinobacteria *Acidothermus cellulolyticus*.[21] Accordingly, we expect that S9 family APs, which exhibit hydrolytic activities toward oligopeptides and PM, are widely distributed from Gram-negative mesophilic *Streptomyces* spp. to Gram-positive thermophilic *A. cellulolitics*. If this hypothesis is correct, such an S9 AP would be an ideal "parent enzyme" for generating biocatalysts that catalyze the production of diverse biologically active small molecules, including linear peptides, cyclic dipeptides, peptide mimics, and diverse puromycin analogues. Such a transaminopeptidase has not been developed to date and will be a beneficial tool for the structural modification of amino acid conjugates by simple enzymatic procedures.

First, we report the cloning and characterization of a putative S9 family AP derived from the Gram-positive bacteria *A. cellulolytics* ATCC43068. As expected, the enzyme (S9-ACAP) showed AP activity toward synthetic substrates, linear di- and tri-peptides, and PM. In addition, amidase and esterase activities were observed. Next, the parent S9-ACAP was engineered into the transaminopeptidase aminolysin-*A* by site-directed mutagenesis of catalytic Ser[491] into Cys, after which its peptide bond formation activity was evaluated. The biological activities of the synthesized puromycin analogues were also assessed.

Results and discussion

Database-mining for S9 family aminopeptidases

We recently reported the wide distribution of S9 family APs in the field of actinomycetes.[20] The enzymes are homologues of previously reported PMH derived from *S. morookaensis*.[18,19] S9 family enzymes generally include the catalytic Ser residue and reportedly show oligopeptidase activity with strict substrate specificities toward Pro-containing oligopeptides (for an overview and further information about the peptidases, visit the MEROPS database at http://merops.sanger.ac.uk).[22–24] However, the enzymatic characteristics of PMH and other S9 APs derived from *Streptomyces* spp. are distinct from those of known S9 enzymes, especially with respect to their substrate specificities.[15,20,25] We also reported the engineering of this S9 AP into a transaminopeptidase that catalyzes the formation of peptide bonds to give diverse peptides *via* aminolysis.[15] The strategy of protein engineering involved the site-directed mutagenesis of catalytic Ser into Cys to mimic the catalysis of non-ribosomal peptide synthetase (NRPS),[26] a similar peptidic catalyst,[27] known transpeptidases including sortase A,[28–33] engineered peptide ligases,[34–38] and cyclodipeptide synthase.[39] The above observations indicate that S9 family APs have the potential for use as parent enzymes to generate new

biocatalysts for the formation of peptide bonds. Our continuous database-mining approach resulted in the discovery of a putative S9 family AP gene (Acel_1489) in the genomic DNA of *A. cellulolyticus*.[21] Fig. 1[B] shows the phylogenetic relationships among S9 family APs and other S9 family oligopeptidases, including the subfamilies S9A, S9B, and S9C. For these analyses, we prepared an unrooted dendrogram by comparing the full-length amino acid sequences of the enzymes using the CLUSTAL W program. S9 APs formed a single cluster separated from other subfamilies. Furthermore, our targeted S9 AP derived from *A. cellulolyticus* was classified into the cluster of known S9 APs. The enzyme was then cloned and characterized.
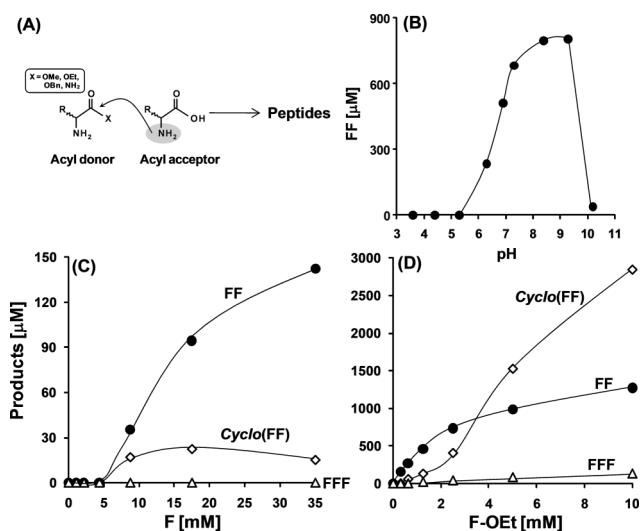
Enzymatic characteristics of ACAP, an S9 family aminopeptidase derived from *A. cellulolyticus*

The S9 AP gene was cloned and over-expressed in *E. coli* to yield the corresponding enzyme, S9-ACAP, as described in the experimental section. The enzymatic characteristics of S9-ACAP are listed in Table S2, ESI.† As expected, the enzyme showed hydrolytic activity toward *p*-nitroanilide derivatives of amino acids, which are typical substrates for APs. Furthermore, we observed hydrolytic activities toward dipeptides, tripeptides, and puromycin. We also observed amidase and esterase activities toward L-phenylalanine amide (L-Phe-NH$_2$) and L-phenylalanine methyl ester (L-Phe-OMe).

Characterization of aminolysin-*A* catalysis

The catalytic Ser[491] of S9-ACAP was mutated into Cys to generate a transaminopeptidase, and its aminolytic activities were then evaluated. In this study, the mutant was designated as aminolysin-*A* on the basis of its catalytic mechanism (aminolysis) and the genus of the microorganism (*Acidothermus*) from which it was derived. As shown in Fig. 2(A), the reaction mechanism of aminolysis that was used to generate peptides requires two distinct substrates, an acyl donor and an acyl acceptor. Fig. 2(B) shows an
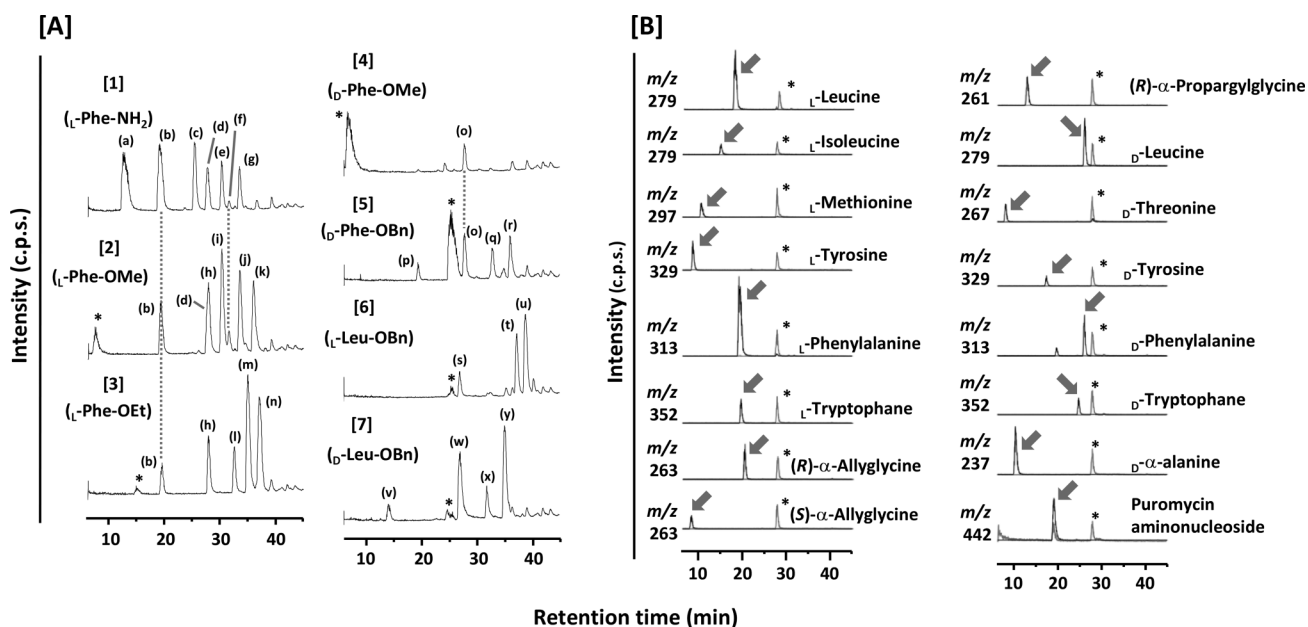
optimal pH profile of aminolysin-*A* catalysis. In these experiments, 2.0 mM L-Phe-OMe as the acyl donor and 25.0 mM L-Phe as the acyl acceptor were subjected to aminolysin-*A* catalysis under several pH conditions. The reaction proceeded efficiently under basic pH to give L-Phe-L-Phe as the major product; however, the yield rapidly decreased at pH 10.0. The reason for this rapid decrease at pH 10.0 was likely the non-enzymatic hydrolysis of the acyl donor (L-Phe-OMe). Therefore, the enzyme reactions of the following experiments were conducted at pH 8.0 as the standard condition to avoid such non-enzymatic hydrolysis of the acyl donor substrates. Next, we evaluated the effect of the concentration of substrates (acyl donor/acyl acceptor) on the reaction efficiency. Fig. 2(C) shows the dose–response curve of the effect of the concentration of an acyl acceptor. For these experiments, 313 µM L-Phe-OEt as the acyl donor and several concentrations of L-Phe as acyl acceptor were utilized in the reaction. In this reaction, two distinct products, dipeptide L-Phe-L-Phe and cyclic dipeptide *cyclo*(L-Phe-L-Phe) were formed. The production efficiency for L-Phe-L-Phe was correlated with the amounts of added acceptor, L-Phe, indicating that reaction efficiency could be controlled by the ratio of acyl donor to acyl acceptor. As shown in Fig. 2(C), under the appropriate conditions (acyl donor 313 µM, acyl acceptor 35 mM) approximately 50% of the L-Phe-OEt (acyl donor) was converted into the corresponding dipeptide. The effect of concentration of an acyl donor on the reaction efficiency is shown in Fig. 2(D). In these experiments, several levels of acyl donor (L-Phe-OEt) and 35 mM L-Phe as acyl acceptor were utilized. As shown in the figure, two major products, linear and cyclic dipeptides, and trace amounts of tripeptides were formed. The dipeptide L-Phe-L-Phe was the major product when there was a low concentration (below 2.5 mM) of acyl donor substrate. However, an increase in the acyl donor substrate (L-Phe-OEt) led to a significant increase in the yield of the cyclic dipeptide *cyclo*(L-Phe-L-Phe). In our previous study,[15] the cyclic dipeptide was believed to primarily be produced *via* the intramolecular aminolysis of intermediate L-Phe-L-Phe-OEt. Herein, ester derivatives of amino acids, including L-Phe-OEt, could be recognized by aminolysin-*A* as both the acyl donor and acyl acceptor because the compound possesses an ester bond and a free amino group. However, free amino acids, including L-Phe, could only be recognized as acyl acceptors because of the absence of an ester bond. Therefore, the results shown in Fig. 2(D) could be reasonably explained as follows: The aminolysin-*A* requires low amounts of acyl donor and excess free amine as acyl acceptor to effectively produce the corresponding dipeptide.

Linear homo-peptide synthesis

C-terminal modified amino acids at a concentration of 10 mM were subjected to catalysis by aminolysin-*A* (Fig. 3). As shown in panels A-1 to A-3, L-Phe derivatives were recognized as good acyl donors and acyl acceptors. In all cases, oligopeptides composed of L-Phe residues with varying degrees of polymerization (DP) up to 2 were produced. The peak (h) in panels A-2 and A-3 was identified as the cyclic dipeptide *cyclo*(L-Phe-L-Phe) on the basis of its MS spectrum (Fig. S6, ESI†) and a comparison of the retention time with that of an authentic standard. As shown in panel A-1 and in Fig. S6-1, ESI,† this cyclic dipeptide was not formed when using L-Phe-NH$_2$ as the substrate. Such cyclic dipeptides

Fig. 2 Enzymatic characteristics of aminolysin-*A*. (A) Mechanism of aminolysis reaction. (B) Optimal pH profile of aminolysin-*A*. (C) The effect of the concentration of acyl acceptor on aminolysin-*A* catalysis. (D) The effect of the concentration of acyl donor on aminolysin-*A* catalysis.

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Org. Biomol. Chem., 2011, **9**, 2327–2335 | 2329

Fig. 3 Peptide synthesis. [A] Homo-peptide synthesis. The represented C-terminal-modified amino acids (10 mM) were subjected to catalysis by aminolysin-*A*. Their total ion chromatograms are shown. Asterisks denote the remaining substrates. The newly produced compounds are denoted by letters; (a), (L-F)$_2$-NH$_2$; (b), (L-F)$_2$; (c), (L-F)$_3$-NH$_2$; (d), (L-F)$_3$; (e), (L-F)$_4$-NH$_2$; (f), (L-F)$_4$; (g), (L-F)$_5$-NH$_2$; (h), *cyclo*(L-F-L-F); (i), (L-F)$_3$-OMe; (j), (L-F)$_4$-OMe; (k), (L-F)$_5$-OMe; (l), (L-F)$_3$-OEt; (m), (L-F)$_4$-OEt; (n), (L-F)$_5$-OEt; (o), *cyclo*(D-F-D-F); (p), (D-F)$_2$; (q), (D-F)$_2$-OBn; (r), (D-F)$_3$-OBn; (s), *cyclo*(L-L-L-L); (t), (L-L)$_4$-OBn; (u), (L-L)$_5$-OBn; (v), (D-L)$_2$; (w), *cyclo*(D-L-D-L); (x), (D-L)$_2$-OBn; (y), (D-L)$_3$-OBn. The MS spectrum of each compound is shown in Fig. S6, ESI.† [B] Hetero-dipeptide synthesis. 2 mM of L-F-OMe and 22.5 mM of the represented amines were subjected to catalysis by aminolysin-*A*. Their extracted ion chromatograms (XICs) are shown. The *m/z* values for monitoring the produced dipeptide (F-X, X corresponding to the used acceptor amines) are shown in each panel. The resulting XICs are shown as black lines, in which the arrowed peaks represent the produced dipeptide containing the N-terminal phenylalanine moiety. The gray lines are the XICs for monitoring the by-product *cyclo*(L-Phe-L-Phe). The *m/z* value for these chromatograms (gray lines) was 295, which was the [M+H]$^+$ ion of *cyclo*(L-Phe-L-Phe).

should originate from esterified dipeptides (L-Phe-L-Phe-OMe or L-Phe-L-Phe-OEt) due to the following observations: First, L-Phe-L-Phe-NH$_2$, the C-terminal modified dipeptide corresponding to the substrates used, only appears in panel A-1. Furthermore, the simple dipeptide L-Phe-L-Phe was observed in all cases.

Panels A-4 and A-5 show that the aminolysin-*A* recognized esterified D-Phe as an acyl donor and acyl acceptor. When using D-Phe-OMe as the substrate, only *cyclo*(D-Phe-D-Phe) was formed (panel A-4, peak o). In contrast, linear oligopeptides (DP = 2–3) and *cyclo*(D-Phe-D-Phe) were produced when D-phenylalanine benzyl ester (D-Phe-OBn) was used as the substrate, indicating that D-Phe-OBn was a better substrate than D-Phe-OMe for producing linear peptides. Therefore, it is suggested that the recognition of the aminolysin-*A* depended on the type of substrate esterification.

In cases using the L- and D-forms of Phe-OMe as substrates (panels A-2 and A-4), the L-form acted as the better substrate, resulting in the production of diverse oligopeptides with different DPs (DP = 2–5). Indeed, the peak area of the residual substrate (asterisked) obtained when using L-Phe-OMe was at least 5.4 times smaller than when using D-Phe-OMe (panel A-4). In contrast, as shown in panels A-6 and A-7, the L- and D- forms of Leu-OBns were almost completely consumed to give linear oligopeptides (peaks t, u, x, and y) and corresponding cyclic dipeptides (peaks s and w). It should be noted that the sensitivity of ESI-MS detection depends on ionization efficiencies. In our observations, high molecular weight peptides (high DPs) were more sensitively detected than lower molecular weights. Therefore, the quantification of

each product was impossible in the experiments conducted herein. Nevertheless, we propose that the substrate recognition properties of aminolysin-*A* depend on the types of amino acids, including their chiralities and types of esterifications, because the sensitivity of the pair of enantiomers identified by the ESI-MS detection is proposed to be the same.

To determine if cyclization of the reaction intermediate dipeptide ester into cyclic dipeptide occurred enzymatically or non-enzymatically, the intermediate dipeptide ester was isolated from the reaction mixture and subjected to heat treatment (50 °C for 6 h) with or without the enzyme. For this experiment, D-Phe-OBn was selected as a model substrate. The isolated D-Phe-D-Phe-OBn was easily cyclized to give *cyclo*(D-Phe-D-Phe) with or without the enzyme (Fig. S7, ESI†). Based on these findings, we concluded that the cyclization (intramolecular aminolysis) of the intermediate dipeptide ester occurred in a non-enzymatic manner.

Subjecting free L-Phe (10 mM) to the same assays did not result in the production of peptide (data not shown). Furthermore, the parent ACAP did not catalyze the formation of peptide bonds when L-Phe-OMe (10 mM) and free L-Phe (10 mM) were used as substrates (data not shown). Based on these observations, we concluded that the catalytic mechanism of the aminolysin-*A* was just an aminolysis and not the reverse reaction of hydrolysis, and that the Cys^{491} residue was responsible for the peptide bond formation. The proposed mechanism is shown in Scheme 1. In addition, aminolysin-*A* recognized C-terminal modified-L-Ile, L-Trp, D-Trp, L-Met, and L-Tyr to give the corresponding linear
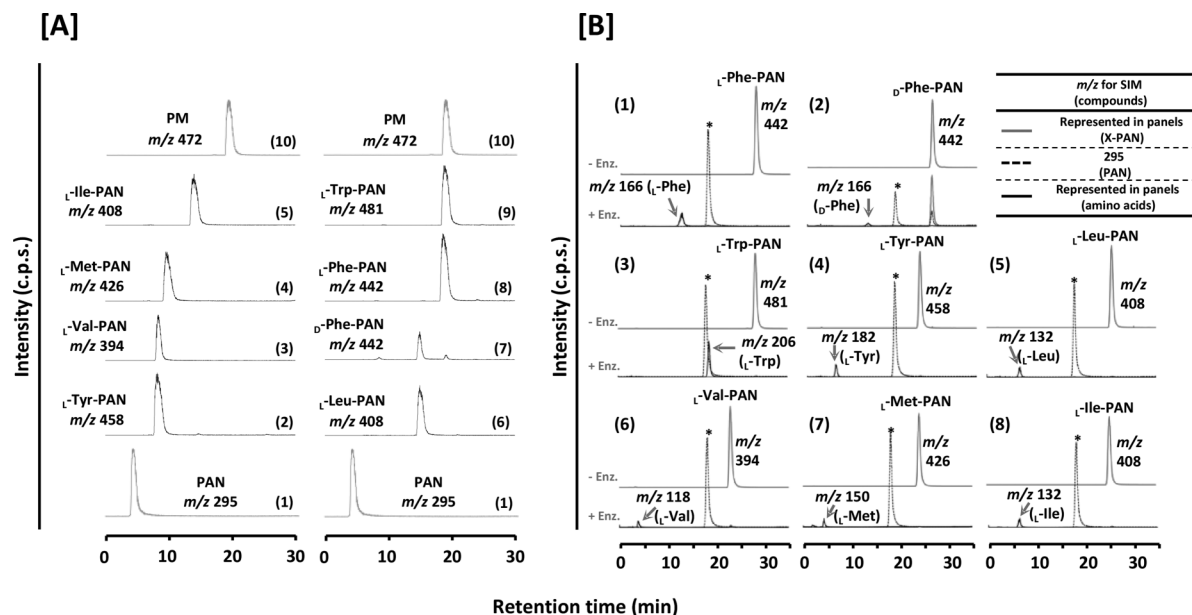
Fig. 4 Puromycin analogue synthesis. [A] Catalysis of aminolysin-*A* to give diverse puromycin analogues. 4 mM C-terminal esterified amino acids and 35 mM of puromycin aminonucleoside (PAN) were subjected to catalysis by aminolysin-*A*. Their extracted ion chromatograms (XICs) are shown. The procedure for the construction of each XIC was similar to that described in the legend of Fig. 3[B]. The XICs of authentic puromycin—PM, panel (10)—and PAN—panel (1)—are shown in both the left and right panels for clarity. The MS/MS spectra of produced X-PAN (X, amino acid residues) and authentic PM are shown in the ESI† (Section 7). [B] Hydrolytic activity of the parent hydrolase S9-ACAP toward puromycin analogues synthesized by aminolysin-*A*. 10 μg of isolated X-PANs were subjected to enzymatic hydrolysis by S9-ACAP. Their selected ion monitoring (SIM) chromatograms are shown. The upper chromatogram (gray line) in each panel was obtained by an enzyme control reaction (enzyme-free treatment). The *m/z* values for the construction of chromatograms are shown in the panels. The lower chromatograms in each panel (solid black and dotted gray lines) denote the enzyme-treated reaction mixtures. The dotted line was plotted to monitor puromycin aminonucleoside (PAN, asterisked peak) released by the enzymatic hydrolysis of the corresponding X-PAN. The solid black lines denote the amino acid residues from the corresponding X-PAN. The released amino acids are highlighted by arrows.
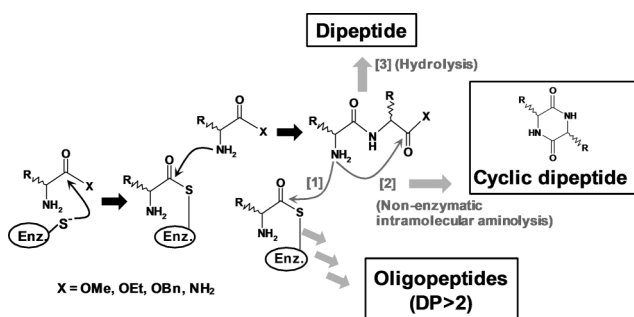
Scheme 1 Reaction of aminolysin-*A* used peptide synthesis.

oligopeptides and cyclic dipeptides (Tables S3 and S4, ESI†), indicating its broad substrate specificity.

Synthesis of hetero-dipeptides and PM analogues by aminolysin-*A*

A set of acyl donors (2.0 mM L-Phe-OMe) and acyl acceptors (22.5 mM of several free amines including amino acids and puromycin aminonucleoside (PAN)—Table S5, ESI†) was subjected to catalysis by aminolysin-*A*. The extracted ion chromatograms (XICs) of positive samples are shown in Fig. 3[B]. In this analysis, the amines shown in the figure were subjected to the reaction and then LC–MS analysis. Their total ion chromatograms were extracted by the *m/z* of the molecular ion corresponding to the produced dipeptides as well as to the by-product *cyclo*(L-Phe-

L-Phe). In this analysis, putative by-products including oligomers of phenylalanine were not detected. The *m/z* values for the monitoring of produced dipeptides are shown in each panel. The produced dipeptides are indicated by arrows, and the asterisks denote the by-products *cyclo*(L-Phe-L-Phe). When L-Phe-OMe was used as the acyl donor, various amino acids including L-forms, D-forms, and α-substituted derivatives were recognized as acceptors. The incorporated free amino acids should be positioned at C-terminal positions of the produced dipeptide given that the catalytic mechanism of aminolysin-*A* is aminolysis. Furthermore, aminonucleoside PAN was also found to give a new puromycin analogue. This result confirmed the broad substrate specificity of aminolysin-*A*. When L-Met-OMe was used as the acyl donor, the represented free amines in Fig. 3[B] also acted as acyl acceptors to give the corresponding L-Met-containing dipeptides (Fig. S8, ESI†), again indicating the broad substrate specificity of aminolysin-*A*.

Synthesis of puromycin analogues

Eight types of amino acids, L-Tyr, L-Val, L-Met, L-Ile, L-Leu, D-Phe, L-Phe, and L-Trp, were incorporated into the PAN skeleton *via* aminolysin-*A* catalysis. In this study, each analogue is mentioned as X-PAN, where X is the amino acid residue. Fig. 4[A] shows the XICs of the reaction mixtures. In these analyses, XICs were created in a manner similar to that described in the section on hetero-dipeptide synthesis. Their MS/MS spectra (Fig. S9,

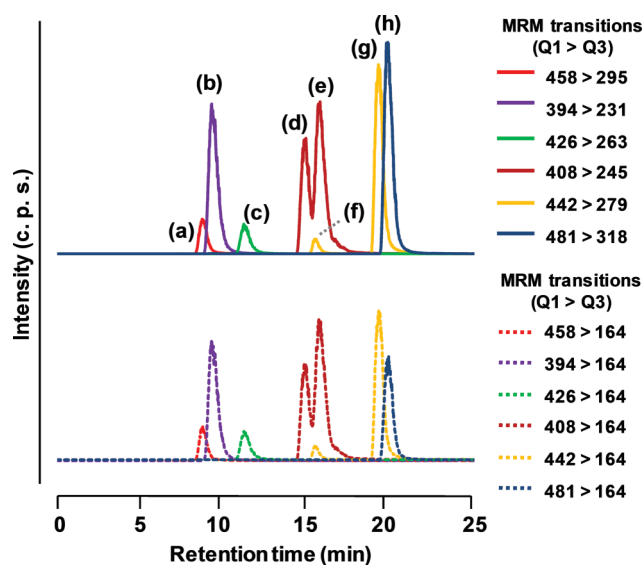**Table 1** Conversion efficiency of C-terminal-esterified amino acid to puromycin analogues

Product	Acyl donor	Conversion efficiency (%)[a]	
		UV270	MRM
L-Phe-PAN	L-Phe-OMe	18[b]	28[b]
			(17)[c]
D-Phe-PAN	D-Phe-OBn	1.8[b]	3.2[b]
			(1.3)[c]
L-Trp-PAN	L-Trp-OBn	26[b]	12[b]
			(12)[c]
L-Tyr-PAN	L-Tyr-O-Bn	24[b]	22[b]
			(16)[c]
L-Leu-PAN	L-Leu-OBn	7.0[b]	15[b]
			(20)[c]
L-Ile-PAN	L-Ile-OBn	13[b]	15[b]
			(10)[c]
L-Val-PAN	L-Val-OBn	8.0[b]	8.5[b]
			(3.6)[c]
L-Met-PAN	L-Met-OMe	13[b]	16[b]
			(3.8)[c]

[a] Each compound was quantified as authentic PM as described in the experimental section. [b] Single donor/single acceptor system. [c] Multi donor/single acceptor system.

Fig. 5 Puromycin analogue synthesis by a multi-donor system. 50 mM puromycin aminonucleoside (PAN) as the acyl acceptor and a mixture of L-Tyr-OBn, L-Val-OBn, L-Met-OMe, L- Ile-OBn, L-Leu-OBn, D-Phe-OBn, L-Phe-OMe, and Trp-OBn as acyl donors (0.85 mM each) were subjected to catalysis by aminolysin-*A*. Multiple-reaction monitoring (MRM) chromatograms are shown. The precursor *m/z* and fragment *m/z* for each MRM transition are shown in the figure (precursor *m/z* > fragment *m/z*). The upper panel (solid lines) confirms the types of incorporated amino acids. The lower panel (dotted lines) shows the puromycin skeleton (Section 7-2, ESI†). (a), L-Tyr-PAN; (b), L-Val-PAN; (c), L-Met-PAN; (d), L-Ile-PAN; (e), L-Leu-PAN; (f), D-Phe-PAN; (g), L-Phe-PAN; (h), L-Trp-PAN.

ESI†) reasonably identified the chemical structures as puromycin analogues, differing only in their amino acid moieties. The HR-ESI-MS analysis of the isolated compounds also supported their structure. Therefore, this experiment confirmed our speculation that an S9 family AP with broad substrate specificity could be a template for generating a transaminopeptidase capable of catalyzing the formation of peptide bonds to give diverse oligopeptides and puromycin analogues. Here, we wondered whether the broad substrate specificity of the transaminopeptidase aminolysin-*A* was inherited from that of the parent hydrolase S9-ACAP. To answer this question, the isolated puromycin analogues were subjected to hydrolysis by its parent enzyme, ACAP. As shown in Fig. 4[B], all of the synthesized puromycin analogues were hydrolyzed by the parent ACAP to give PAN (gray lines, asterisks) and the corresponding amino acids (solid black lines, arrowed). Nevertheless, D-Phe-PAN was not completely hydrolyzed (panel B-2), indicating that the parent ACAP only weakly recognized the compound. Here, the conversion efficiency of D-Phe-OBn to D-Phe-PAN was lower than those of others (Table 1). Therefore, we concluded that the substrate specificity of aminolysin-*A* originated from that of its parent hydrolase, ACAP. In addition, this experiment also confirmed the broad substrate recognition of ACAP.

Synthesis of diverse puromycin analogues by a multi-donor system

We attempted to use an enzymatic multi-donor/single acceptor approach for the development of our aminolysin system to produce diverse puromycin analogues. In this approach, a mixture of eight types of esterified amino acids were used as acyl donors (0.85 mM each) and PAN (50 mM) was used as a single acceptor (Table 1). Fig. 5 shows the multiple-reaction monitoring (MRM) chromatograms of the reaction mixture. The MRM transitions for the lower panel (dotted lines) were the *m/z* of the expected precursor ions > 164 (Q1 > Q3), which were used to confirm the presence of the *N,N*-dimethyl adenine moiety. That for upper panel (solid lines) were the *m/z* of the expected precursor ion >

that of the daughter ion, corresponding to the loss of the *N,N*-dimethyl adenine moiety from the produced puromycin analogues. The above parameters were defined based on characteristic fragmentation ions of the MS/MS spectrum of authentic puromycin (Fig. S9, ESI†). The compounds (a)–(h) were detected in both the upper and lower panels. The retention time of each compound in the lower and upper panels was the same. The *m/z* of the molecular ions of compounds (a), (b), (c), and (h) enabled easy identification of each compound as L-Tyr-PAN, L-Val-PAN, L-Met-PAN, and L-Trp-PAN, respectively. We inferred that compounds (d) and (e), which were detected based on an MRM transition of 408 > 245 (Q1 > Q3), were L-Ile-PAN or L-Leu-PAN as judged from the *m/z* of the precursor ions. Here, isolated L-Ile-PAN was eluted more rapidly than L-Leu-PAN during ODS HPLC analysis (Fig. 4[B]). Moreover, cochromatography experiments between the peak (d) and the isolated L-Ile-PAN in Fig. 4[B] gave a single peak (data not shown), and similar results were obtained for the isolated L-Leu-PAN and compound (e). Based on these observations, compounds (d) and (e) were identified as L-Ile-PAN and L-Leu-PAN, respectively. Compounds (f) and (g) were identified as D-Phe-PAN and L-Phe-PAN, respectively, in a manner similar to that described above. Therefore, eight types of amino acid residues of the used acyl-donors were incorporated into PAN to give puromycin analogues *via* a multi-donor system. Conversion efficiencies for the esterified amino acids are shown in Table 1. A comparison of the data from the two systems revealed that a single-donor/single acceptor system (Table 1, footnote *b*) was

Table 2 Antimicrobial activity of synthesized puromycins and puromycin aminonucleoside

Compound	MIC ($\mu g\ mL^{-1}$)		
	S. aureus	*B. subtilis*	*E. coli*
PAN[a]	I.A.[d]	I.A.[d]	I.A.[d]
PM[b]	25.0	12.5	50.0
L-Phe-PAN[c]	12.5	12.5	25
L-Met-PAN[c]	50.0	50.0	100
L-Leu-PAN[c]	75.0	100	I.A.[d]
L-Ile-PAN[c]	75.0	100	I.A.[d]

[a] Puromycin aminonucleoside. [b] Puromycin dihydrochloride. [c] Counter ion was not identified. [d] Inactive at $100\ \mu g\ mL^{-1}$.

more effective than a multi-donor/single acceptor system (Table 1, footnote *c*) in terms of reaction efficiency.

Antimicrobial activities of puromycin analogues

In preliminary experiments, eight types of partially purified X-PANs (synthesized by the experiments in Fig. 4) were tested in an antimicrobial assay against *Staphylococcus aureus*, *Bacillus subtilis*, and *Esherichia coli*. As a result, L-Phe-PAN, L-Ile-PAN, L-Leu-PAN, and L-Met-PAN showed inhibitory activities (data not shown). Therefore, these four compounds were completely purified and used for further analysis (Table 2). Authentic puromycin showed potent antimicrobial activity against the three microorganisms mentioned above. Similarly, the four types of purified puromycin analogues were also active. Here, PAN was inactive against all organisms at a concentration of $100\ \mu g\ mL^{-1}$. Therefore, the critical role of amino acid moieties in the antimicrobial activity of puromycin analogues was clarified. It should be noted that newly synthesized L-Phe-PAN was more active than puromycin against *S. aureus* and *E. coli*. Therefore, a future challenge will be to prepare diverse puromycin analogues using the aminolysin system to clarify their biological activities. Such studies are currently under way.

Conclusions

The parent hydrolase S9-ACAP showed broad substrate specificity toward peptidic substrates and high stability against heat treatment (Table S2, ESI†). In addition, its unique characteristic, *viz.*, puromycin hydrolyzing activity, was detected. These findings were expected based on the reported activity of its homologous hydrolase PMH [18,19] Therefore, we speculated that the enzyme would be an ideal template for generation of biocatalysts for the synthesis of diverse short oligopeptides and amino-acid-containing small molecules such as puromycin and its analogues. We succeeded in engineering the peptide-bond-hydrolyzing S9-ACAP into a peptide-bond-forming biocatalyst by site-directed mutagenesis of catalytic Ser[491] into Cys. While this strategy has already been described, and is well recognized and widely adapted for serine-oligopeptidases, [14,34–38] it has not been very successful for APs because such "serine-aminopeptidases" have seldom been recognized by enzymologists. [15–20] Therefore, one of the themes emerging from this study, our previous findings [15–17,20] and those of another group [18,19] is that it might be possible to divide the S9 family peptidases into oligopeptidase and AP subfamilies. In this

classification, the already reported subfamilies S9A, S9B, S9C, and S9D are categorized as the former, while the APs are categorized as the latter. However, further investigations are needed to determine if this is indeed the case.

The generated biocatalyst, which was designated aminolysin-A, catalyzed the formation of peptide bonds *via* an aminolytic mechanism to give short oligopeptides. Its high substrate recognition ability enabled the one-pot synthesis of short oligopeptides, hetero-dipeptides, and cyclic dipeptides (Fig. 3). In addition, the substrate scope and part of the reaction mechanism of the aminolysin system was investigated (Scheme 1). Furthermore, diverse puromycin analogues composed of distinct amino acid residues could be synthesized by single-donor and multi-donor systems (Fig. 4 and 5). We used our aminolysin system to modify the puromycin skeleton and succeeded in making the antimicrobial activity of natural puromycin more potent (Table 2). To the best of our knowledge, this is the first report of the enzymatic derivatization of the puromycin skeleton by the aminolytic reaction. Such one-pot synthesis should be difficult if conducted through the use of parent S9 AP because of the re-hydrolysis of product. [16,17] Hence, the strategy of this study, the mutagenesis of the catalytic Ser into Cys to give the aminolysis activity and to decrease the hydrolysis activity for S9 AP, would be interesting for the enzymatic synthesis of diverse biologically active chemicals possessing peptide bonds. Herein, one of the limitations for the clinical use of puromycin was its toxicity. The aminolysin system described in this paper might be a beneficial tool for one-step high-throughput derivatization of the puromycin skeleton to decrease its toxicity.

The major obstacle remaining for our aminolysin system is its reaction efficiency. Indeed, the best conversion efficiency from an acyl donor to puromycin analogue, which was observed for L-Phe-PAN, was 28% (Table 1). One of the reasons for such a moderate reaction efficiency is the non-enzymatic hydrolysis of the ester bonds of the donor substrates. Indeed, L-Phe-OMe, which was a typically good substrate for aminolysin-A catalysis, was almost completely hydrolyzed after 6 h at 50 °C (pH 8.0) under enzyme-free conditions (data not shown). Accordingly, improving the reaction efficiency will be our next step.

Finally, the results of this study and our previous studies [15–17,20] underscore the following points. The S9 family APs (also known as puromycin hydrolase) will be widely distributed in the field of microorganisms, including Gram-positive and Gram-negative bacteria. Such enzymes are promising "templates" for generating biocatalysts for the formation of peptide bonds to give diverse peptide mimetics, including linear dipeptides, cyclic dipeptides, linear oligopeptides, and amino-acid-conjugated small molecules such as puromycins.

Experimental

General

L-Phenylalanine *p*-nitroanilide (Phe-*p*NA), Pro-*p*NA, Leu-*p*NA, Lys-*p*NA, D-Phe-*p*NA, puromycin dihydrochloride, and puromycin aminonucleoside (PAN) were purchased from Sigma-Aldrich. Ala-*p*NA was obtained from the Peptide Institute, Inc. Other *p*NA derivatives of amino acids (a.a.-*p*NAs) were purchased from Bachem AG. L-Leu-L-Leu and L-Phe-L-Phe were obtained from Sigma-Aldrich. L-Phe-L-Phe-NH₂, L-Phe-L-Phe-L-Phe, and

cyclo(L-Phe-L-Phe) were purchased from Bachem. The sources of ester derivatives of amino acids for the aminolysis reaction are listed in Table S3, ESI.† 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. A Waters 2690 Separation Module, a Waters 2487 Dual λ Absorbance Detector, and an API 2000 LC–MS/MS System (Applied Biosystems) were used for LC–UV–MS/MS analysis. The column used for HPLC analysis was a TSKgel ODS-120T, 5 μm, 2.0 × 150 mm (Tosoh Corp.). Other chemicals were commercially available. HRESIMS experiments were conducted using a micrOTOFII-SKA (Bruker Daltonics).

Construction of a phylogenetic tree for the S9 family enzymes

The S9 family enzymes derived from different sources were collected by searching the MEROPS database (http://merops.sanger.ac.uk) and a previously published paper.[40] Using the CLUSTAL W program (http://align.genome.jp/), an unrooted dendrogram was prepared by comparing the full-length amino acid sequences of the collected enzymes. The enzymes used for this experiment are listed in Table S1, ESI.†

Cloning, over-expression, and purification of S9-ACAP, an S9 family aminopeptidase derived from *A. cellulolyticus*

Genomic DNA was prepared from *A. cellulolyticus* ATCC43068 as described in our previous paper.[15,20,25] The gene encoding ACAP (locus tag of *A. cellulolyticus* 11B genome project (http://gib.genes.nig.ac.jp/single/main.php?spid=Acel_11B): Acel_1489) was amplified using PCR with a set of sense primers incorporating the *Nde*I site upstream of a start codon (5′-CAT<u>ATG</u>CCCGAGATTGCTCCGTA -3′; the start codon is underlined) and an anti-sense primer incorporating the *Hin*dIII site downstream of a stop codon (5′-AAGCTT<u>CTA</u>AGGAGCCGGCGGCGCCGG - 3′; the stop codon is underlined) and the genomic DNA of *A. cellolyticus* ATCC43068. In addition, a set of Prime Star GXL and Prime Star GXL buffers (Takara) was used for the PCR reaction. The generation of expression plasmid (pET28-His₆-ACAP), as well as the over-expression and purification of proteins, were conducted as described previously using expression vectors designed for the N-terminal His₆-tag.[15,20,25] The purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions with Coomassie blue staining.

Construction of aminolysin-*A*, an S491C mutant

Site-directed mutagenesis was conducted by inverse PCR using a sense primer (5′- GCGATCCGCGGCGGC(A→T, Ser→Cys)GTGCCGGCGGCTGG-3′), its complementary strand, and the pCR-Blunt II-TOPO:*ACAP* plasmid. Experimental procedures used to generate the expression plasmid (pET-28: S491C) and to over-express and purify the protein were the same as those used in our past studies.[15]

Hydrolytic activity of wild-type ACAP

The hydrolytic activity of wild-type ACAP toward a.a.-*p*NAs was evaluated as described in a previous study.[20] The activity toward phenylalanyl-phenylalanine amide (Phe-Phe-NH₂), Phe-Phe, Phe-Ala, Leu-Phe, and phenylalanine methyl ester (Phe-OMe) was measured as previously described by our lab.[41] The details are described in the ESI.†

Characterization of aminolysin-*A* catalysis

The optimal pH profiles were evaluated by the use of 2.0 mM L-Phe-OMe as the acyl donor and 25.0 mM of L-Phe as the acyl acceptor in 140 mM citrate-phosphate-borate buffer. The volume of the reaction mixture was 100 μl, in which 10% (v/v) DMSO and 5 μg of purified aminolysin-*A* was included. The reaction mixture was incubated at 50 °C for 1 h, after which 100 μl of CH₃CN was added to quench the reaction. The mixture was then centrifuged and the supernatant was subjected to the following LC–UV analysis. The mobile phase was water containing 0.1% (v/v) HCOOH (A) and MeOH containing 0.1% (v/v) HCOOH (B), which was applied at a flow rate of 0.2 ml min⁻¹. The LC condition was 20% B during 0–5 min, after which it increased linearly from 20%–99% B from 5–40 min, and then remained 99% B from 40–45 min. UV monitoring at 210 nm was conducted to detect and quantify the produced L-Phe-L-Phe. For this experiment, commercially available L-Phe-L-Phe was used as an authentic standard. The effects of the concentration of the acyl donor and acyl acceptor were evaluated under the following assay conditions. The reaction mixture (100 μl) was composed of 0.1 M phosphate buffer (pH 8.0), several amounts of L-Phe-OEt and L-Phe, 5 μg purified aminolysin-*A*, and 10% (v/v) DMSO. The enzyme reaction was conducted at 50 °C for 3 h. The identification and quantification of the product by LC–UV analysis was similar to that described above. Commercially available L-Phe-L-Phe, L-Phe-L-Phe-L-Phe, and *cyclo*(L-Phe-L-Phe) were used as authentic standards.

Homo-oligopeptide synthesis by aminolysin-*A*

The reaction mixture (100 μl) was composed of 0.1 M phosphate buffer (pH 8.0), 10 mM ester derivatives of amino acids, 10 μg purified aminolysin-*A*, and 10% (v/v) DMSO. The substrates used are listed in Table S3, ESI.† The reaction was conducted at 50 °C for 6 h, after which 300 μl of 10% (v/v) HCOOH–MeOH–DMSO mixture (1 : 1 : 1, v/v) was added to quench the reaction. After centrifugation, 20 μL of the supernatant was subjected to LC–MS analysis as described above. Identification of the produced L-Phe-L-Phe, L-Phe-L-Phe-NH₂, L-Phe-L-Phe-L-Phe, *cyclo*(L-Phe-L-Phe), and L-Leu-L-Leu was conducted on the basis of their retention time during LC–UV analysis and their *m/z* values upon LC–MS analysis. To accomplish this, commercially available compounds were used as authentic standards. Identification of the other products was conducted based on the *m/z* values of the molecular ions observed upon LC–MS analysis.

Hetero-dipeptide synthesis by aminolysin-*A*

The reaction mixture (100 μl) was composed of 0.1 M phosphate buffer (pH 8.0), 2.0 mM L-phenylalanine methyl ester (Phe-OMe) as an acyl donor, 22.5 mM free amino acids (Table S5, ESI†), PAN as an acyl acceptor, 10 μg purified aminolysin-*A*, and 10% (v/v) DMSO. The enzyme reaction was conducted at 50 °C for 6 h, after which 200 μl of 5% (v/v) HCOOH was added to quench the

reaction. Following centrifugation, 20 µl of the supernatant was subjected to LC–MS analysis as described above.

Puromycin analogue synthesis by aminolysin-*A*

In the case of the single donor/single acceptor system (Fig. 4A), the reaction mixture (2.5 mL) was composed of 0.1 M phosphate buffer (pH 8.0), 4.0 mM ester derivatives of amino acids as acyl donors, 35 mM PAN as an acyl acceptor, 1.0 mg aminolysin-*A*, and 10% (v/v) DMSO. Enzyme reaction, quenching of the reaction, and LC–UV–MS and LC–MS/MS analysis were conducted in a similar manner as described above. In this experiment, UV monitoring at 270 nm and MS/MS monitoring with the MRM method were adopted for the detection and quantification of the produced X-PANs as a substitute for authentic PM. In this analysis, the calibration curve for authentic PM was used for the quantification of each product (Section 7-2, ESI†). HRESIMS data describing the isolated puromycin analogues were as follows. L-Phe-PAN, calcd for $C_{21}H_{28}N_7O_4$ [M+H]$^+$: 442.2197, found: 442.2190. D-Phe-PAN, calcd for $C_{21}H_{28}N_7O_4$ [M+H]$^+$: 442.2197, found: 442.2193. L-Trp-PAN, calcd for $C_{23}H_{29}N_8O_4$ [M+H]$^+$: 481.2306, found: 481.2305. L-Tyr-PAN, calcd for $C_{21}H_{28}N_7O_5$ [M+H]$^+$: 458.2146, found: 458.2139. L-Leu-PAN, calcd for $C_{18}H_{30}N_7O_4$ [M+H]$^+$: 408.2354, found: 408.2350. L-Ile-PAN, calcd for $C_{18}H_{30}N_7O_4$ [M+H]$^+$: 408.2354, found: 408.2348. L-Val-PAN, calcd for $C_{17}H_{28}N_7O_4$ [M+H]$^+$: 394.2197, found: 394.2201. L-Met-PAN, calcd for $C_{17}H_{28}N_7O_4S$ [M+H]$^+$: 426.1918, found: 429.1910. In the case of the multi-donor/single acceptor system (Fig.5), the reaction mixture (1.2 mL) was composed of 0.1 M phosphate buffer (pH 8.0), a mixture of L-Phe-OMe/D-Phe-OBn/L-Leu-OBn/L-Ile-OBn/L-Trp-OBn/L-Tyr-OBn/L-Met-OMe/Val-OBn (0.85 mM each), 50 mM PAN, 100 µg of aminolysin-*A*, and 10% (v/v) DMSO. The enzyme reaction was conducted at 50 °C for 6 h, followed by the addition of 300 µl of 10% (v/v) HCOOH for quenching. After centrifugation, 10 µL of the supernatant was subjected to LC–MS/MS analysis. The procedures for the quantification of each product by the MRM method are described above.

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