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PenA, a penicillin-binding protein-type thioesterase specialized for small peptide cyclization

Kenichi Matsuda [1], Kei Fujita[1], Toshiyuki Wakimoto [1,2]

[1]Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, Japan
[2]Global Station for Biosurfaces and Drug Discovery, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan
Correspondence should be addressed to: Toshiyuki Wakimoto at wakimoto@pharm.hokudai.ac.jp

Abstract: Penicillin-binding protein-type thioesterases (PBP-type TEs) are a recently identified group of peptide cyclases that catalyze head-to-tail macrolactamization of nonribosomal peptides. PenA, a new member of this group, is involved in the biosyntheses of cyclic pentapeptides. In this study, we demonstrated the enzymatic activity of PenA *in vitro*, and analyzed its substrate scope with a series of synthetic substrates. A comparison of the reaction profiles between PenA and SurE, a representative PBP-type TE, showed that PenA is more specialized for small peptide cyclization. A computational model provided a possible structural rationale for the altered specificity for substrate chain lengths.

Keywords: Cyclopeptide, Biocatalyst, Cyclase

Introduction

Cyclic peptides exhibit improved membrane permeability and resistance against proteolytic enzymes, as compared to their linear counterparts (Tsomaia, 2015). Cyclization confers structural rigidity and constrains the peptide shape, similar to the binding state to biological targets, thus enhancing affinity and specificity. Although macrocyclization is a general strategy of peptide modification when considering pharmaceutical applications, the control of undesired epimerization and oligomerization during the ring-closure remains challenging (White & Yudin, 2011). Cyclization generally becomes more problematic when the chain length is decreased, as the preferable *E*-geometry of peptide bonds prevents cyclization points from being in proximal space. Several strategies have been invented to tackle this obstacle, such as incorporation of turn-inducing protecting groups (Skropeta et al., 2004), cyclization on solid support (Alcaro et al., 2004) and contraction of the ring size of a larger intermediate (Meutermans et al., 2003). On the other hand, small cyclic peptides such as cyclic tetrapeptides and cyclic pentapeptides are frequently found in nature (Abdalla, 2016; Jing & Jin, 2020), and the chemo- and regio-specific ring-closure during their biosyntheses is achieved by a group of enzymes referred to as peptide cyclases, which could be exploited as alternative catalytic tools for the cyclization of small peptides.

Penicillin-binding protein (PBP)-type TEs are a recently discovered family of peptide cyclases involved in the biosynthesis of a series of nonribosomal macrolactams (Kuranaga et al., 2018; Matsuda, Kuranaga, et al., 2019). In contrast to canonical peptide cyclase domains, such as the type-I thioesterase (TE) and the terminal condensation domain (C_T), the PBP-type TEs are physically discrete from an assembly line and thus act *in trans*. The PBP-type TE group members examined thus far exclusively catalyze macrolactamization. PBP-type TEs are encoded in the biosynthetic gene clusters of several nonribosomal macrolactams ranging from hexapeptide to decapeptide, including surugamides (Kuranaga et al., 2018) and desotamides (Li et al., 2015; Ding et al., 2020), ulleungmycins (Son et al., 2017), noursamycins (Mudalung et al., 2019), and mannopeptimycins (Magarvey et al., 2006). Among them, SurE, a PBP-type TE for surugamide biosynthesis, is of particular interest because it acts *in trans* to two distinct nonribosomal peptide synthetases (NRPSs) to cyclize a structurally unrelated set of linear intermediates (i.e., octapeptide for surugamides A–E and decapeptide for cyclosurugamide F) (Fig. 1) (Matsuda, Kobayashi, et al., 2019). SurE exhibits broad substrate tolerance *in vivo* and *in vitro*, indicating its potential use as a biocatalyst for peptide cyclization and a genetic tool for synthetic biology (Matsuda et al., 2020). A promiscuous peptide cyclase with multiple physiological substrates is a promising candidate for the further development of tools for producing cyclic peptides. However, SurE is currently the only example of a biochemically characterized PBP-type TE that acts on more than one NRP assembly line.

Pentaminomycins are a growing family of cyclic pentapeptides isolated from several *Streptomyces* species (Carretero-Molina et al., 2020; Hwang et al., 2020; Jang et al., 2018; Kaweewan et al., 2020). Interestingly, the BE-18257s, a structurally distinct group of cyclic pentapeptides, are concomitantly produced along with pentaminomycins (Carretero-Molina et al., 2020; Hwang et al., 2020; Kaweewan et al., 2020), suggesting their biosynthetic relationship. The biosynthetic gene cluster of pentaminomycins (*pen*) was proposed (Hwang et al., 2020; Kaweewan et al., 2020). and experimentally validated quite recently by heterologous expression (Fig. 1) (Román-Hurtado et al., 2021). We herein refer to the genes according to the report by Hwang et al. (Hwang et al., 2020). In the *pen* cluster, the NRPS gene *penN2*, responsible for pentaminomycin biosyntheses, is encoded in the neighboring region of the NRPS gene *penN1*, responsible for BE-18257s. Notably, both assembly lines lack canonical termination domains at their C-termini, suggesting the presence of unusual cyclization mechanisms. Analysis of the *pen* cluster identified the single PBP-like enzyme PenA, encoded in the upstream region of *penN1*. As no other candidate

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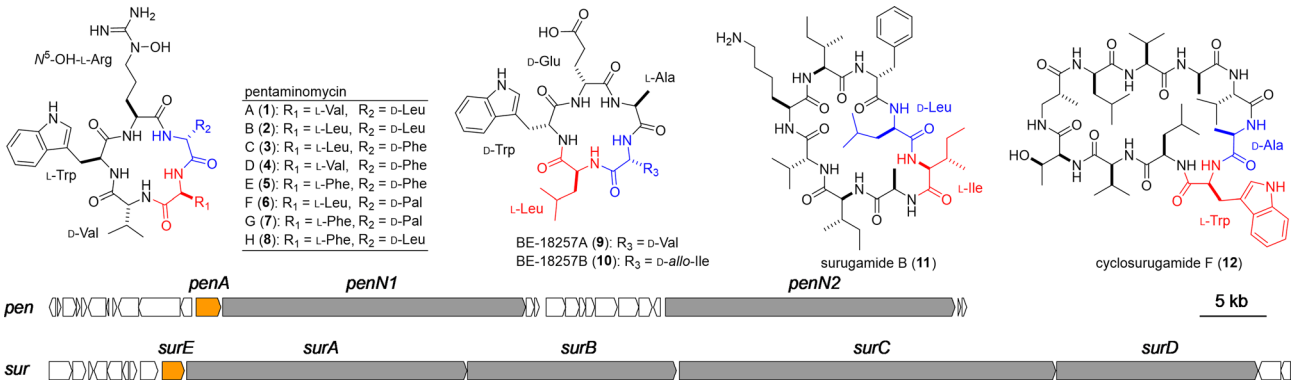


Fig. 1. Structures of pentaminomycins, BE-18257s, surugamides, and their biosynthetic gene clusters. The residues at N- and C-termini of linear precursors were colored in red and blue, respectively. Genes encoding PBP-type TEs and NRPSs are colored orange and gray, respectively.

gene for chain-termination and cyclization is encoded in *pen*, PenA is hypothesized to act on both the *penN1* and *penN2* assembly lines to catalyze the macrolactamization of two families of cyclic pentapeptides (Hwang et al., 2020; Kaweewan et al., 2020).

In this study, we demonstrated the macrolactamization activity of PenA *in vitro*, validating it as a new member of the PBP-type TEs. A series of synthetic substrates revealed that the scope of PenA is similar to that of SurE, in terms of the strict requirement for heterochiral residues at both termini and the broad tolerance of residues in the middle. On the other hand, PenA and SurE exhibit distinct preferences for the substrate chain length. The structural model of PenA provides a possible rationale for the difference in the enzymatic properties.

Material and methods

Synthesis of substrate mimics

The detailed procedure for solid phase peptide synthesis (SPPS) and synthesis of substrate mimics are provided in supplementary materials.

Preparation of recombinant PBP-type TEs

The recombinant PenA (GenBank protein ID: WP_158102277) in *Streptomyces cacaoi* NBRC12748 was prepared by following procedure. DNA fragment coding for PenA was amplified by KOD One (Toyobo) using a set of primer PenA_Fw/PenA_Rv (PenA_Fw: CCGgaattccatatgtccgaatccgctg, PenA_Rv: CCCaagctttcagccgagccgg, restriction enzyme sites are underlined). The genomic DNA of *Streptomyces cacaoi* NBRC12748 was used as template. Amplified fragments were digested by EcoRI and HindIII and inserted into the multicloning site of pUC19 (Takara). After sequence confirmation, the fragment was transferred to the NdeI/HindIII site in multicloning site of expression vector pColdII (Takara) to generate PenA-pColdII. PenA-pColdII was introduced into *E. coli* BL21(DE3) and the transformant was cultivated for overnight at 16°C, with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Following Ni-NTA affinity column chromatography yielded recombinant PenA with the His-tag fused at its *N*-terminus. Imidazole in the eluted fraction was removed by an Amicon Ultra 0.5ml filter (Merck Millipore). The concentrations of proteins were measured using a Bio-Rad protein assay kit. Preparation of recombinant SurE was described in previous reports (Matsuda et al., 2020).

In vitro assay

In vitro reaction was performed with following condition; 50 μL volume of reaction mixture containing 20 mM Tri-HCl (pH8.0), 400 μM substrate, 1 μM enzyme. Reaction mixture was incubated at 30°C for 2 hours, then reaction was quenched by adding equal volume of 0.1% TFA. Resultant mixture was analyzed by HPLC equipped with reversed-phase column COSMOSIL-5C_{18}-MS-II 4.6 × 250 mm (nacalai tesque). Sample was eluted with gradient method (30-70% in 20 min for mobile phase B) using H_2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively, unless otherwise noted. Reaction yields were calculated based on area value of reaction products. ε (220 nm) values were assumed to be equal for SNAC substrate and corresponding enzymatic product. ε (220 nm) values for dimerized products **37** and **38** were assumed to be double of that for monomer.

Kinetic assay

50 μL of reaction mixture containing 20 mM Tri-HCl (pH8.0), 12.5–400 μM substrate were pre-incubated at 30°C for 10 min. Reaction was initiated by adding 1 μM PenA or 50 nM SurE then mixture was incubated for 2 min. Reaction was quenched by adding equal volume of 0.1% TFA, then 20 μL of resultant mixture was loaded onto COSMOSIL 3C_{18}-MS-II 2.0 × 150 mm (nacalai tesque) then eluted with gradient method (30-70% in 5 min for mobile phase B) using H_2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively. Column elutes were monitored with UV absorption at 210 nm. Initial velocity was calculated assuming that ε (210 nm) values are equal for SNAC substrate and corresponding enzymatic product. Kinetic parameters were estimated using the Michaelis–Menten equation and the curve-fitting program Kaleidagraph.

Computational modeling of PenA

Structural model of PenA was generated by web tool SWISS-MODEL (https://swissmodel.expasy.org/) (Waterhouse et al., 2018), using crystal structure of SurE (PDB ID: 6KSU) as template.

Results and discussion

A series of linear substrates resembling pentaminomycin C (**3**) was synthesized by conventional solid-phase peptide synthesis with DIC/Oxyma-mediated amide coupling and piperidine-promoted Nα-deprotection. Elongated peptides were cleaved from the resin and coupled with *N*-acetyl cysteamine (SNAC), to afford thioester substrates as the mimics

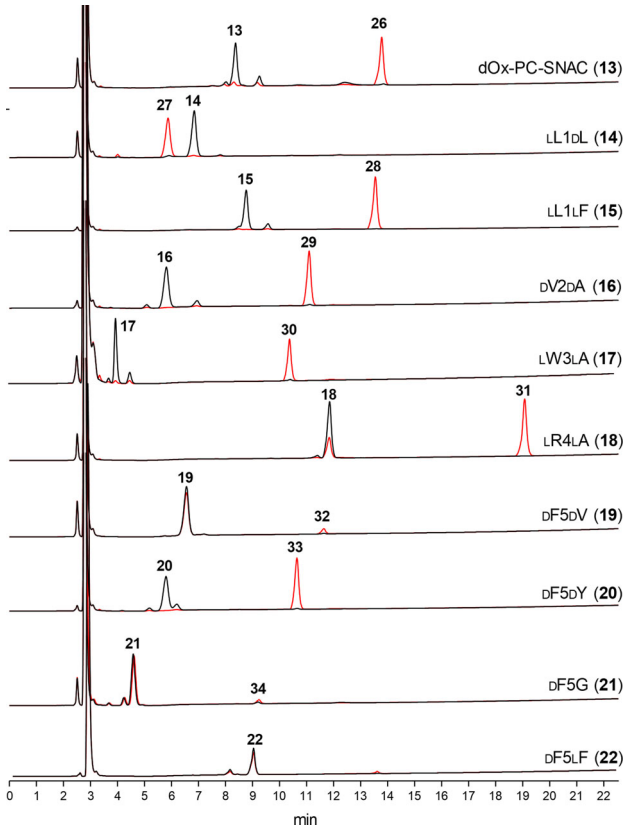


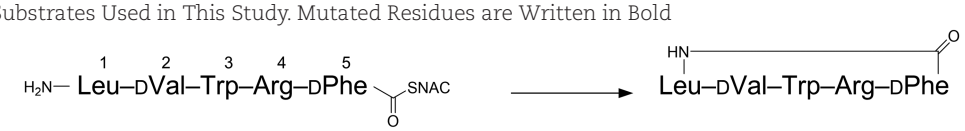
Fig. 2. *In vitro* assays of PenA, using **13**–**22** as substrates. The reaction substrates were described on each chromatograph. Chromatographs of samples with active enzyme (red) and boiled enzyme (black) are overlaid. Samples were eluted with a gradient method (30–70% in 20 min for mobile phase B) with H_2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively. Column eluates were monitored with UV absorption at 220 nm.

of peptidyl-carrier protein (PCP)-bound linear peptides. The nonproteogenic residue, N^5-OH-L-Arg, was substituted with L-Arg for simplification in these synthetic peptides. PenA from *Streptomyces cacaoi* NBRC12748 was expressed with a His-tag fused at its N-terminus in *E. coli* BL21 (DE3), and then was incubated with 400 μM of **13** for 2 hr at 30°C. As a result, PenA cyclized **13** to afford the head-to-tail cyclic peptide without the detectable accumulation of a hydrolytic product (Fig. 2). This validates that PenA is indeed a peptide cyclase, and that the N^5-OH moiety at position 4 is dispensable for PenA-mediated cyclization.

With the established reaction conditions in hand, we next evaluated the substrate scope of PenA with the series of synthetic peptides listed in Table 1. First, we investigated its stereospecificity against the residues at both termini (positions 1 and 5 in Table 1). PenA was incapable of cyclizing **14** with the substitution of L-Leu1 with D-Leu, but yielded the linear carboxylic acid **27**, indicating that the N-terminal L-configured residue is indispensable for the cyclization step. PenA also failed to consume **22** with the substitution of D-Phe5 with L-Phe, indicating that the D-configured residue at the C-terminus is required for the formation of the peptide-*O*-PenA complex. Therefore, PenA specifically couples a L-configured residue at the N-terminus and an D-configured residue at the C-terminus, as in the case of SurE. The specificity of the heterochiral coupling is likely to be a common trait of PBP-mediated macrolactamization.

The diversity of the residues at the cyclization site among the pentaminomycin derivatives suggests that PenA possesses somewhat relaxed specificity for the terminal residues. Indeed, PenA cyclized **15** and **20**, with terminal residues mutated to aromatic residues, in quantitative manners. On the other hand, PenA failed to efficiently cyclize **21**, a derivative with a Gly residue at the C-terminus. PenA also showed decreased cyclization activity against **19**, a derivative with D-Val at the C-terminus. Next, to investigate PenA's specificity toward the side chains in the

Table 1. Synthetic Substrates Used in This Study. Mutated Residues are Written in Bold



13 **26**

Substrate	Sequence 1	2	3	4	5	6	7	8	Reaction product[a]	Cyclic or linear[b]
deOx-PC (**13**)	Leu	D-Val	Trp	Arg	D-Phe	–	–	–	**26**	Cyclic
LL1DL (**14**)	**D-Leu**	D-Val	Trp	Arg	D-Phe	–	–	–	**27**	Linear
LL1LF (**15**)	**Phe**	D-Val	Trp	Arg	D-Phe	–	–	–	**28**	Cyclic
DV2DA (**16**)	Leu	**D-Ala**	Trp	Arg	D-Phe	–	–	–	**29**	Cyclic
LW3LA (**17**)	Leu	D-Val	**Ala**	Arg	D-Phe	–	–	–	**30**	Cyclic
LR4LA (**18**)	Leu	D-Val	Trp	**Ala**	D-Phe	–	–	–	**31** (73%)	Cyclic
DF5DV (**19**)	Leu	D-Val	Trp	Arg	**D-Val**	–	–	–	**32** (9%)	Cyclic
DF5DY (**20**)	Leu	D-Val	Trp	Arg	**D-Tyr**	–	–	–	**33**	Cyclic
DF5G (**21**)	Leu	D-Val	Trp	Arg	**Gly**	–	–	–	**34** (8%)	Cyclic
DF5LF (**22**)	Leu	D-Val	Trp	Arg	**Phe**	–	–	–	N.D.	–
tetrapeptide (**23**)	Leu	Trp	Arg	D-Phe	–	–	–	–	**35**–**38**[c]	Cyclic/linear
tripeptide (**24**)	Leu	Arg	D-Phe	–	–	–	–	–	**39**, **40**	Linear
SB-SNAC (**25**)	Ile	D-Ala	Ile	D-Val	Lys	Ile	D-Phe	D-Leu	**11**	Cyclic

[a]Chemical structures of reaction products are depicted in Supplementary Fig. S2. Reaction yields are described when a substrate was not completely consumed within 2 hr of incubation. N.D.: not detected.
[b]This column indicates whether the reaction products of PBP-type TEs are cyclic or linear.
[c]Yields are described in Fig. 4.

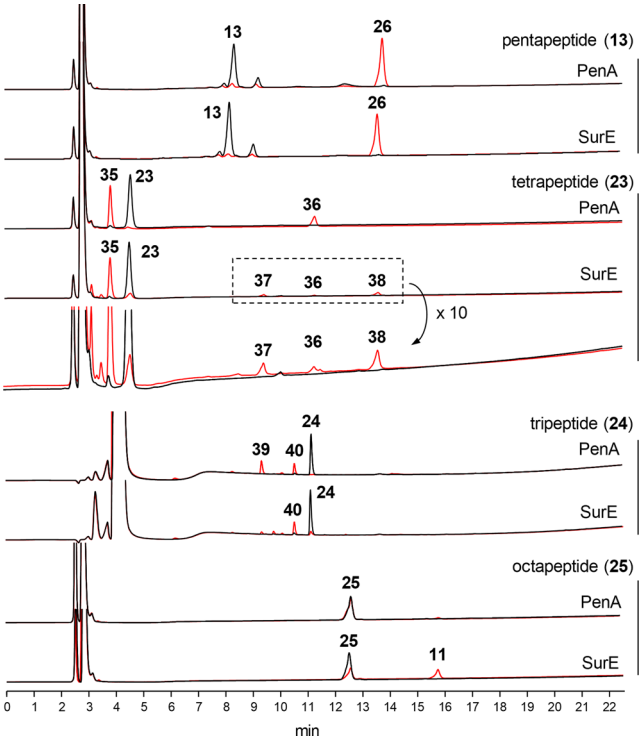


Fig. 3. *In vitro* assays of PenA and SurE, using **13** and **23**–**25** as substrates. Chromatographs for samples with active enzyme (red) and boiled enzyme (black) are overlaid. Samples were eluted with a gradient method (30–70% in 20 min for mobile phase B) with H_2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively, except for samples with **24**, which were eluted with a different gradient method (2–98% in 20 min for mobile phase B). Column eluates were monitored with UV absorption at 220 nm.

middle of the sequence, each internal residue was individually substituted with Ala with the retained Cα configuration, to afford **16**–**18**. These substitutions were generally accepted by PenA. Notably, the substitution of L-Arg4 with L-Ala severely affected the cyclization yield, suggesting the importance of ionic interactions between substrates and the PenA active site.

Thus far, PenA exhibited specificity similar to that of SurE, in terms of the strict requirement for heterochirality at the cyclization site and relaxed specificity for the residues in the middle. As the physiological substrates of PenA are pentapeptides, whereas those of SurE are octa- and decapeptides, these PBP-type TEs could have distinct preferences for the substrate chain length. To clarify this point, the cyclization activities of PenA and SurE were compared by using the pentapeptide **13**, tetrapeptide **23**, tripeptide **24**, and octapeptide **25** (Fig. 3). The octapeptide **25** is a thioester mimic of the linear surugamide B that was used in the previous study as a model substrate to investigate the scope of SurE. These analyses revealed that the tripeptide **24** was not cyclized by either PenA or SurE, whereas the pentapeptide **13** was efficiently cyclized by both enzymes with the following kinetic parameters: PenA: $K_m = 64.4 \pm 7.5\ \mu M$, $k_{cat} = 6.1 \pm 0.3\ min^{-1}$, SurE: $K_m = 297.7 \pm 53.0\ \mu M$, $k_{cat} = 96.4 \pm 17.1\ min^{-1}$. Stark differences between PenA- and SurE-mediated cyclizations were observed when the tetrapeptide **23** and octapeptide **25** were used as substrates. In the case of the octapeptide **25**, while SurE efficiently converted it into the cyclic peptide surugamide B (**11**), PenA gave only a trace amount of **11**. In the tetrapeptide **23** reactions, while PenA generated the monomeric cyclopeptide **36** and the hydrolytic linear peptide **35**, SurE generated not only the monomeric cyclopeptide **36** but also the dimeric linear peptide **37** and dimeric cyclopeptide **38**. Perhaps the tetrapeptide **23** tethered on the catalytic serine residue does not fully occupy the active site of SurE, allowing a second peptide molecule to approach the active site for intermolecular ligation to generate the linear dimeric thioester (Fig. 4, path c), which would successively be hydrolyzed or cyclized to give the linear or cyclic dimer, respectively. In the reactions with the tripeptidyl substrate **24**, the concomitant accumulation of the dipeptide **39**, possibly generated from the peptidase activity of PBP-type TEs, was observed. As the peptidase activity of PBP-type TEs is not known, this phenomenon requires further investigation in future work.

PBP-type TEs generally consist of an N-terminal PBP domain with a highly conserved catalytic tetrad and a C-terminal lipocalin domain that participates in substrate binding (Matsuda et al., 2020). The catalytic tetrad is located at the bottom of a large cleft between the PBP and lipocalin domains. To gain insight into the structural features that differentiate the chain length specificities between PenA and SurE, we first compared their primary sequences. Although they share high sequence identity (39.32%), a notable difference was observed in the region between β-strands 5 and 6 in the lipocalin domain (Matsuda et al., 2020). These strands are connected by 7 amino acid residues in SurE, while this region is elongated up to 17 residues in PenA (Fig. 5a). This extended loop is located at the side of a β-barrel that forms the substrate binding cleft (Fig. 5b). In the model structure of PenA that was generated using structure of SurE (PDB ID: 6KSU) as a template, this extended loop protrudes toward the PBP domain and substantially narrows the binding cleft (Fig. 5c), which may explain why PenA is incapable of accommodating a large octapeptidyl substrate. The extended loop may also play a role in shielding the tetrapeptide-*O*-PenA intermediate from a second substrate molecule, thus hampering the intermolecular ligation of peptides, in contrast to the case of SurE.

Enzyme-mediated peptide cyclization has been an area of intense research for many years, because of its high chemo- and regio-selectivities and mild aqueous reaction conditions (Schmidt et al., 2017). However, the use of enzymes to cyclize small peptides, such as tetra- and pentapeptides, is considerably less characterized (Mandalapu et al., 2018; Sardar et al., 2015) as compared to the enzymatic cyclizations of medium and large sized peptides. This is probably because small peptides are beyond the scope of the currently available, well-investigated peptide cyclases, such as sortase A, butelase, and subtilisin derivatives, which all accept substrates with ten or more residues (Schmidt et al., 2017). As both naturally occurring and synthetic small peptides are a fruitful class of compounds with a wide range of biological activities (Abdalla, 2016; Sarojini et al., 2019), keen interest is being focused on the development of versatile biocatalysts that enable rapid access to this chemical space.

PenA exhibits a strict requirement for the heterochirality of terminal residues as well as broad tolerance for the side chains in the middle of the sequence. PenA converted most of the tested substrates into their cyclic counterparts, in a nearly quantitative manner. As compared with SurE, PenA is more specialized to small cyclic peptides, as it accepts tetra- and pentapeptides, but not octapeptides. Notably, PenA generated a cyclic tetrapeptide without a detectable amount of the dimerized product. Taken together, this study highlights the potential use of PBP-type TEs for the cyclization of small peptides, which are usually challenging to cyclize by conventional chemical approaches. The data presented

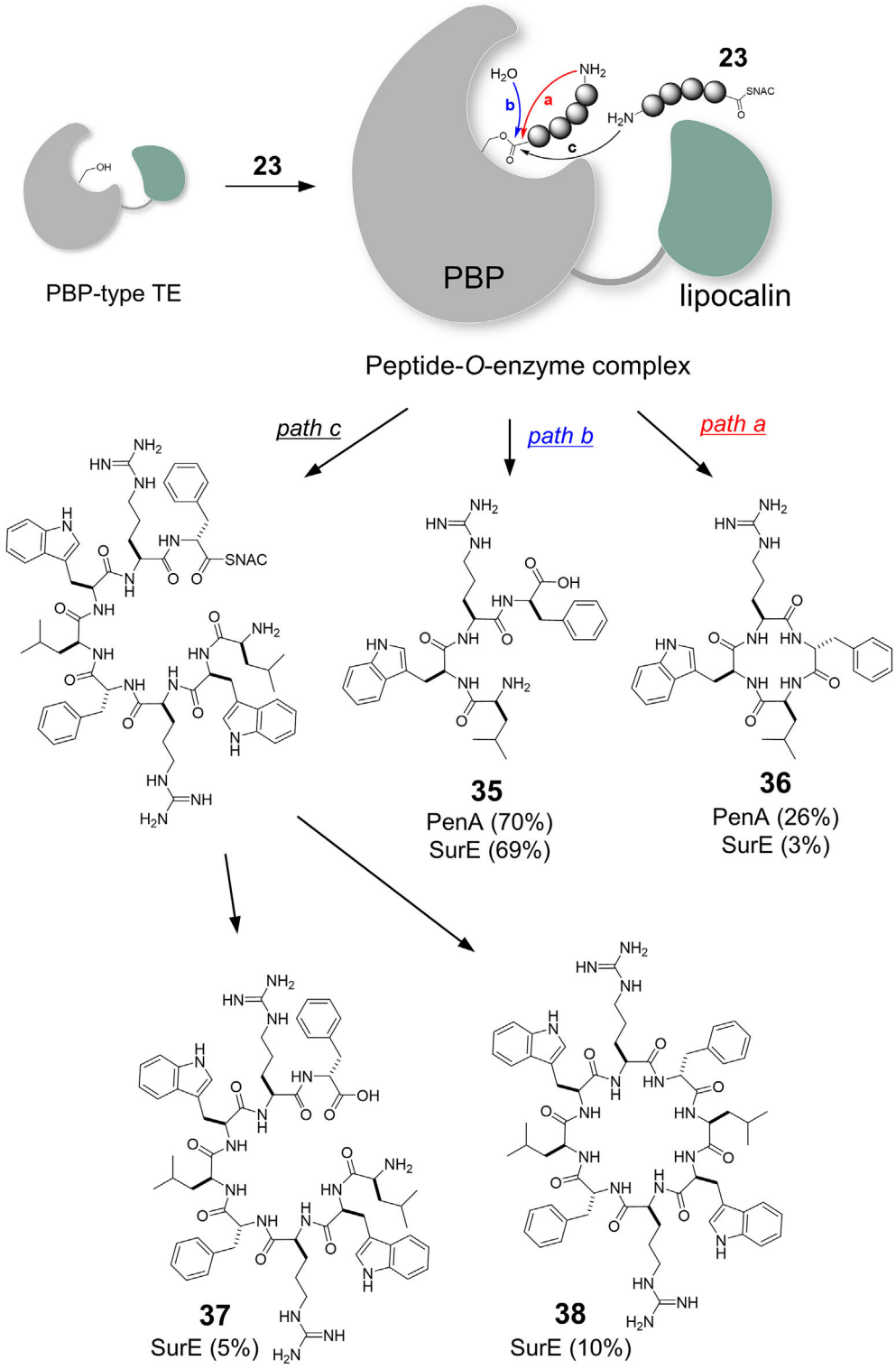


Fig. 4. Proposed reaction scheme of PBP-type TEs with the tetrapeptidyl substrate **23**. Yields of each enzyme are described.

here provide an important basis for the future development of engineered biocatalysts with broadened substrate scopes.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

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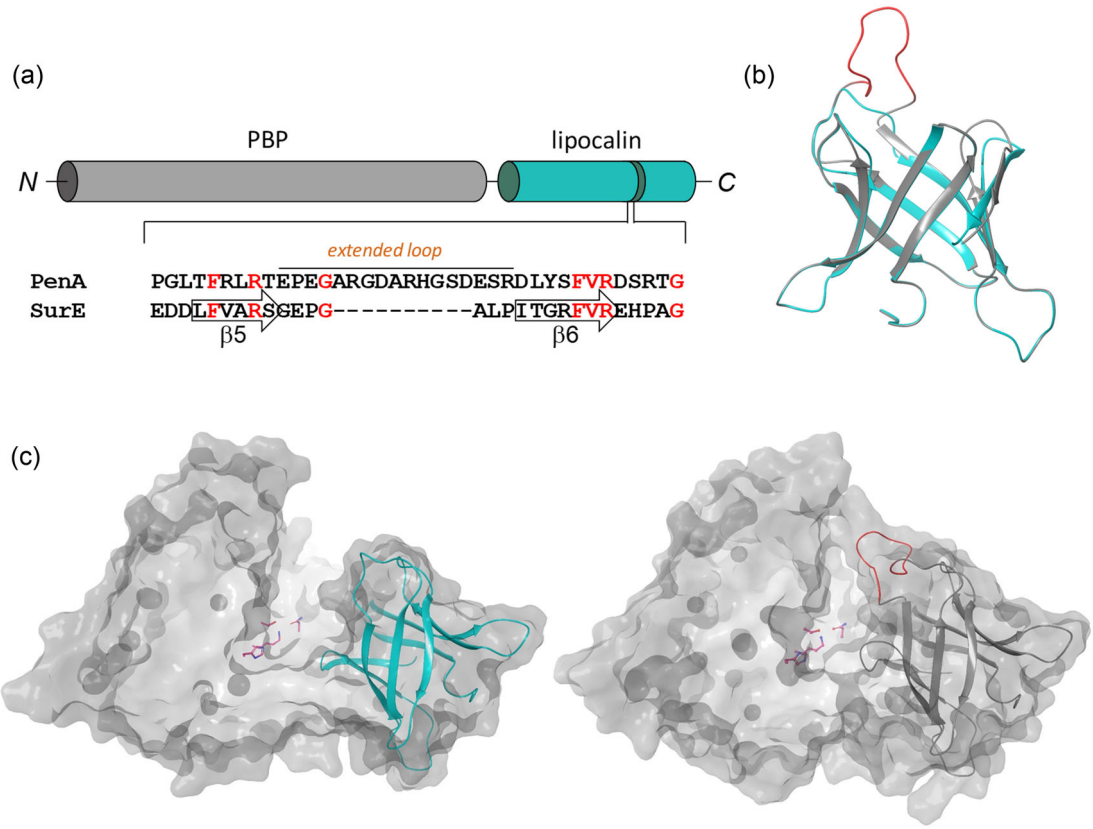

Fig. 5. Structural comparison between PenA and SurE. The model of PenA was generate by SWISS-MODEL (Waterhouse et al., 2018) using crystal structure of SurE (PDB ID: 6KSU) as template. (a) Domain architecture of PBP-type TEs and primary sequence alignment of the lipocalin domains. (b) Comparison between the lipocalin domain of SurE (6KSU, cyan) and that of the PenA model (gray). The extended loop region in PenA is colored red. (c) Overall structure of SurE (6KSU, left) and that of the PenA model (right). The catalytic tetrad is shown in magenta. The color scheme for the lipocalin domains is the same as in (b).

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Conflict of Interest

The authors declare no conflict of interest.

References

Abdalla, M. A. (2016). Medicinal significance of naturally occurring cyclotetrapeptides. *Journal of Natural Medicines*, 70, 708–720.

Alcaro, M. C., Sabatino, G., Uziel, J., Chelli, M., Ginanneschi, M., Rovero, P., & Papini, A. M. (2004). On-resin head-to-tail cyclization of cyclotetrapeptides: Optimization of crucial parameters. *Journal of Peptide Science*, 10, 218–228.

Carretero-Molina, D., Ortiz-Lopez, F. J., Martín, J., González, I., Sánchez-Hidalgo, M., Román-Hurtado, F., Díaz, C., de la Cruz, M., Genilloud, O., & Reyes, F. (2020). Pentaminomycins F and G, first non-ribosomal peptides containing 2-pyridylalanine. ChemRxiv. Preprint. https://doi.org/10.26434/chemrxiv.13142786.v1.

Ding, W., Dong, Y., Ju, J., & Li, Q. (2020). The roles of genes associated with regulation, transportation, and macrocyclization in desotamide biosynthesis in *Streptomyces scopuliridis* SCSIO ZJ46. *Applied Microbiology and Biotechnology*, 104, 2603–2610.

Hwang, S., Le, L., Jo, S.-I., Shin, J., Lee, M. J., & Oh, D.-C. (2020). Pentaminomycins C-E: Cyclic pentapeptides as autophagy inducers from a Mealworm Beetle gut bacterium. *Microorganisms*, 8, 1390.

Jang, J. P., Hwang, G. J., Kwon, M. C., Ryoo, I. J., Jang, M., Takahashi, S., Ko, S. K., Osada, H., Jang, J. H., & Ahn, J. S. (2018). Pentaminomycins A and B, hydroxyarginine-containing cyclic pentapeptides from *Streptomyces* sp. RK88-1441. *Journal of Natural Products*, 81, 806–810.

Jing, X. & Jin, K. (2020). A gold mine for drug discovery: Strategies to develop cyclic peptides into therapies. *Medicinal Research Reviews*, 40, 753–810.

Kaweewan, I., Hemmi, H., Komaki, H., & Kodani, S. (2020). Isolation and structure determination of a new antibacterial peptide pentaminomycin C from *Streptomyces cacaoi* subsp. cacaoi. *Journal of Antibiotics*, 73, 224–229.

Kuranaga, T., Matsuda, K., Sano, A., Kobayashi, M., Ninomiya, A., Takada, K., Matsunaga, S., & Wakimoto, T. (2018). Total synthesis of the nonribosomal peptide surugamide B and identifcation of a new offloading cyclase family. *Angewandte Chemie, International Edition*, 57, 9447–9451.

Li, Q., Song, Y., Qin, X., Zhang, X., Sun, A., & Ju, J. (2015). Identification of the biosynthetic gene cluster for the anti-infective desotamides and production of a new analogue in a heterologous host. *Journal of Natural Products*, 78, 944–948.

Magarvey, N. A., Haltli, B., He, M., Greenstein, M., & Hucul, J. A. (2006). Biosynthetic pathway for mannopeptimycins, lipoglycopeptide antibiotics active against drug-resistant gram-positive pathogens. *Antimicrobial Agents and Chemotherapy*, 50, 2167–2177.

Mandalapu, D., Ji, X., Chen, J., Guo, C., Liu, W. Q., Ding, W., Zhou, J., & Zhang, Q. (2018). Thioesterase-mediated synthesis of teixobactin

analogues: Mechanism and substrate specificity. *Journal of Organic Chemistry*, 83, 7271–7275.

Matsuda, K., Kobayashi, M., Kuranaga, T., Takada, K., Ikeda, H., Matsunaga, S., & Wakimoto, T. (2019). SurE is a *trans*-acting thioesterase cyclizing two distinct non-ribosomal peptides. *Organic & Biomolecular Chemistry*, 17, 1058–1061.

Matsuda, K., Kuranaga, T., & Wakimoto, T. (2019). A new cyclase family catalyzing head-to-tail macrolactamization of non-ribosomal peptides. *Journal of Synthetic Organic Chemistry, Japan*, 77, 1106–1115.

Matsuda, K., Zhai, R., Mori, T., Kobayashi, M., Sano, A., Abe, I., & Wakimoto, T. (2020). Heterochiral coupling in nonribosomal peptide macrolactamization. *Nature Catalysis*, 3, 507–515.

Meutermans, W. D. F., Bourne, G. T., Golding, S. W., Horton, D. A., Campitelli, M. R., Craik, D., Scanlon, M., & Smythe, M. L. (2003). Difficult macrocyclizations: New strategies for synthesizing highly strained cyclic tetrapeptides. *Organic Letters*, 5, 2711–2714.

Mudalungu, C. M., von Törne, W. J., Voigt, K., Rückert, C., Schmitz, S., Sekurova, O. N., Zotchev, S. B., & Süssmuth, R. D. (2019). Noursamycins, chlorinated cyclohexapeptides identified from molecular networking of *Streptomyces noursei* NTR-SR4. *Journal of Natural Products*, 82, 1478–1486.

Román-Hurtado, F., Sánchez-Hidalgo, M., Martín, J., Ortiz-López, F. J., Carretero-Molina, D., Reyes, F., & Genilloud, O. (2021). One pathway, two cyclic non-ribosomal pentapeptides: Heterologous expression of BE-18257 antibiotics and pentaminomycins from *Streptomyces cacaoi* CA-170360. *Microorganisms*, 9, 135.

Sardar, D., Lin, Z., & Schmidt, E. W. (2015). Modularity of RiPP enzymes enables designed synthesis of decorated peptides. *Chemistry & Biology*, 22, 907–916.

Sarojini, V., Cameron, A. J., Varnava, K. G., Denny, W. A., & Sanjayan, G. (2019). Cyclic tetrapeptides from nature and design: A review of synthetic methodologies, structure, and function. *Chemical Reviews*, 119, 10318–10359.

Schmidt, M., Toplak, A., Quaedflieg, P., van Maarseveen, J. H., & Nuijens, T. (2017). Enzyme-catalyzed peptide cyclization. *Drug Discovery Today: Technologies*, 26, 11–16.

Skropeta, D., Jolliffe, K. A., & Turner, P. (2004). Pseudoprolines as removable turn inducers: Tools for the cyclization of small peptides. *Journal of Organic Chemistry*, 69, 8804–8809.

Son, S., Hong, Y. S., Jang, M., Heo, K. T., Lee, B., Jang, J. P., Kim, J. W., Ryoo, I. J., Kim, W. G., Ko, S. K., Kim, B. Y., Jang, J. H., & Ahn, J. S. (2017). Genomics-driven discovery of chlorinated cyclic hexapeptides ulleungmycins A and B from a *Streptomyces* species. *Journal of Natural Products*, 80, 3025–3031.

Tsomaia, N. (2015). Peptide therapeutics: Targeting the undruggable space. *European Journal of Medicinal Chemistry*, 94, 459–470.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., de Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., & Schwede, T. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Research*, 46(W1), W296–W303.

White, C. J. & Yudin, A. K. (2011). Contemporary strategies for peptide macrocyclization. *Nature Chemistry*, 3, 509–524.