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SurE is a *trans*-acting thioesterase cyclizing two distinct non-ribosomal peptides†

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SurE is a new, stand-alone thioesterase (TE) offloading the non-ribosomal peptide (NRP) assembly line found in surugamide biosynthesis. It is homologous to penicillin binding protein (PBP) and capable of cyclizing two structurally unrelated substrates derived from two different NRP assembly lines, highlighting the broad substrate tolerance of the SurE offloading cyclase.

Macrocyclization confers peptide resistance to proteolytic degradation, and improves membrane permeability and target specificity.[1] Many of the naturally occurring cyclic peptides are biosynthesized by the non-ribosomal peptide synthetase (NRPS) pathway. In general, the thioesterase (TE) domain located at the C terminus of an NRPS catalyzes the chain termination and macrocyclization of a grown peptide, tethered on the peptidyl carrier protein (PCP) domain in the last module.[2] The efficient peptide cyclization process is catalyzed by the conventional TE domain, which has attracted considerable interest as a potential biocatalyst.[2,3] However, the *cis*-acting TEs usually have a one-to-one relationship with a single assembly line so they can discriminately accept the cognate PCP-bound substrate, and this has hampered the efforts to develop the useful engineering tools to date.[4,5]

Surugamide A (**1**), a cathepsin B inhibitor, and its derivatives surugamides B–E (**2–5**) are cyclic octapeptides isolated from marine *Streptomyces* (Fig. 1).[6] Its biosynthetic gene cluster (*sur* cluster, Fig. 2) consists of four NRPS genes, *surA–surD*. The genes encoding SurA/SurD, which are responsible for the syntheses of **1–5**, are unexpectedly separated by two additional NRPS genes, *surB*/*surC*.[7–9] Successive analysis led to the discovery of a new linear decapeptide, surugamide F (**6a**), derived from these two genes (Fig. 1 and 2).[7] Therefore, the *sur* cluster uniquely encodes biosynthetic routes for two structurally unrelated peptides.[7] An additional notable feature of the *sur* cluster is the lack of the canonical thioesterase (TE) domain that normally plays roles in chain termination (*i.e.*, hydrolysis or macrocyclization) of the grown peptide chain in the NRPS system. In a previous study, we discovered that SurE, a penicillin-binding protein (PBP) encoded just upstream of the NRPS genes, is responsible for the macrocyclization step in the biosynthesis of surugamides A–E.[10] To the best of our knowledge, SurE is the first offloading peptide cyclase that is physically discrete from a mega-synthetase (*trans*-acting TE).[11] The genes encoding for the SurE homologs are widely distributed among actinomycetes, and are frequently encoded in the neighboring regions of NRPSs that lack the canonical TE domain, including those responsible for the biosyntheses of mannopeptimycin,[12] desotamide[13] and ulleungmycin.[14] We proposed the name "PBP-type TE" for this newly found group of *trans*-acting TEs, since they exhibit amino acid sequence homology to PBP.[15–17]

The remaining question in the surugamide pathway is the offloading mechanism in the biosynthesis of **6a**, the linear decapeptide synthesized by SurB/SurC, which also lack the TE domain at their C termini (Fig. 2). In a previous study, we demonstrated that the recombinant SurE efficiently cyclized the SNAC substrate mimicking the PCP-bound octapeptide intermediate *in vitro* and exhibited preference for a substrate with a C-terminal D-amino acid (Fig. 2).[10] Since the C-terminal residue of **6a** is also D-Ala as in the case of D-Leu in **2**, we envisioned that SurE is capable of offloading both the SurA/SurD and SurB/SurC assembly lines (Fig. 2), even though this is an unprecedented *trans*-acting mechanism of a TE, except for the type II TE.[18]

To confirm the function of SurE *in vivo*, we constructed the *surE*-knockout mutant (Δ*surE*) of *Streptomyces albidoflavus* NBRC 12854 and monitored the production of **1–5**. The in-frame deletion of the *surE* gene was accomplished by a double-cross over method, and the culture broth of the Δ*surE* mutant was analyzed

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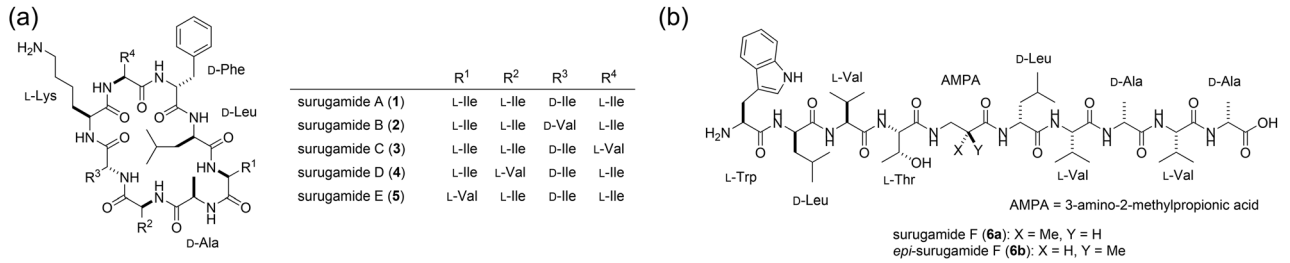


	R^1	R^2	R^3	R^4
surugamide A (**1**)	L-Ile	L-Ile	D-Ile	L-Ile
surugamide B (**2**)	L-Ile	L-Ile	D-Val	L-Ile
surugamide C (**3**)	L-Ile	L-Ile	D-Ile	L-Val
surugamide D (**4**)	L-Ile	L-Val	D-Ile	L-Ile
surugamide E (**5**)	L-Val	L-Ile	D-Ile	L-Ile

Fig. 1 (a) Structures of cyclic octapeptide surugamide A (**1**) and its derivatives surugamides B–E (**2–5**). (b) Structures of linear decapeptide surugamide F (**6a**) and its stereoisomer *epi*-surugamide F (**6b**).

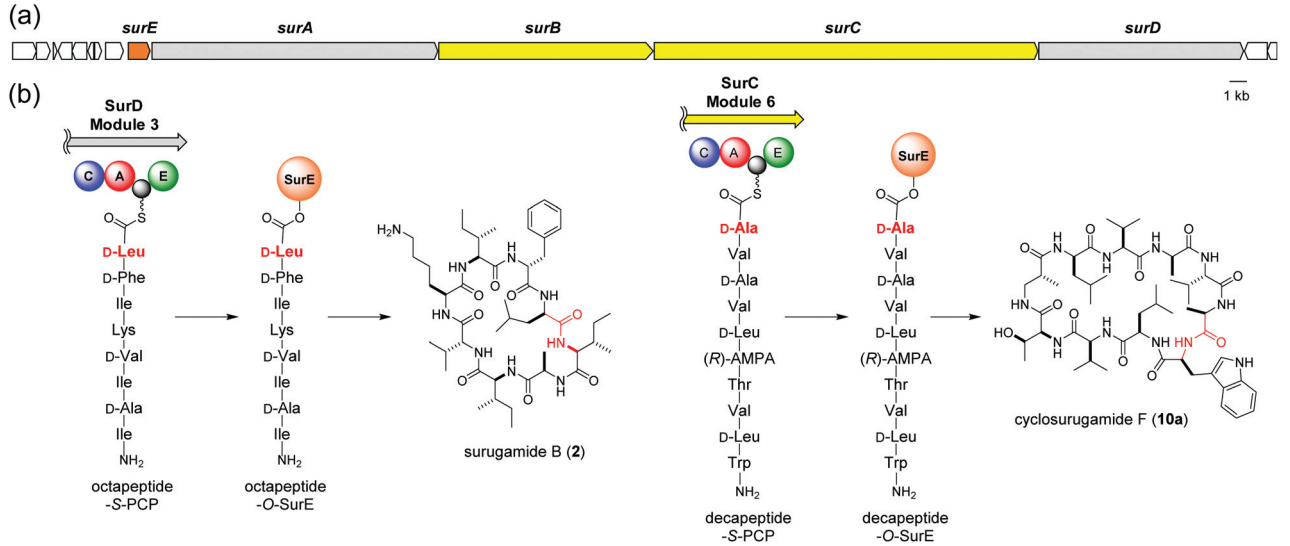


Fig. 2 Biosynthetic gene cluster of surugamides and the proposed SurE-dependent cyclization mechanism. (a) Biosynthetic gene cluster of surugamides. The NRPS genes *surA*/*surD* for the octapeptide biosynthesis, *surB*/*surC* for the decapeptide biosynthesis and *trans*-acting thioesterase gene, *surE*, are colored in gray, yellow and orange, respectively. (b) Proposed SurE-dependent cyclization mechanism in both octapeptidic (left) and decapeptidic (right) surugamide biosyntheses. The D-amino acid at the C-terminus of the PCP-bound intermediates and the peptide bonds formed *via* the cyclization are highlighted in red. Each colored ball represents a functional domain: blue, condensation; red, adenylation; gray, peptidyl carrier protein (PCP); green, epimerization, orange; *trans*-acting TE (SurE).

by LC-MS. As a result, the Δ*surE* mutant no longer produced **1–5** (Fig. 3), showing that SurE is indeed indispensable for the biosynthesis of the cyclic peptides. In addition, we also found that the Δ*surE* strain no longer produced **6a** (Fig. 3), strongly suggesting that SurE is involved in the chain termination of the linear peptide **6a**, as well as the cyclic octapeptides **1–5**.

Prompted by this observation, we performed an *in vitro* reaction with SurE using a synthetic mimic (surugamide F-SNAC, **7a**) of the biosynthetic intermediate as the substrate. Solid phase peptide synthesis followed by conjugation with SNAC and the removal of the Boc and *t*-butyl groups yielded **7a** (Scheme 1).[19] The LC-MS analysis of the reaction mixture demonstrated the efficient consumption of **7a**, whereas only a small amount of **6a** was generated (Fig. 4). However, closer inspection of the LC-MS data revealed the accumulation of a new compound with an ion peak at *m*/*z* 1038.7. HRMS analysis of this compound showed an ion peak at *m*/*z* 1060.6184 (calcd

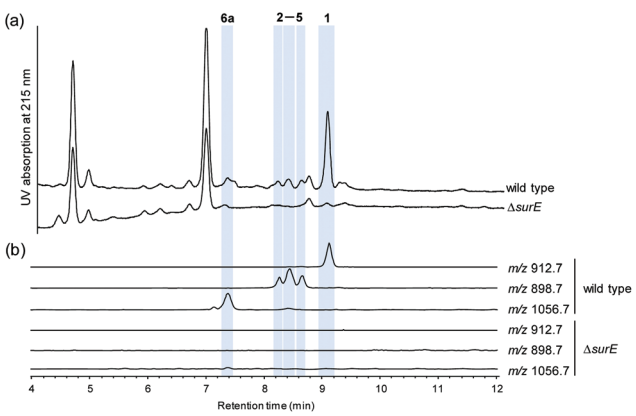
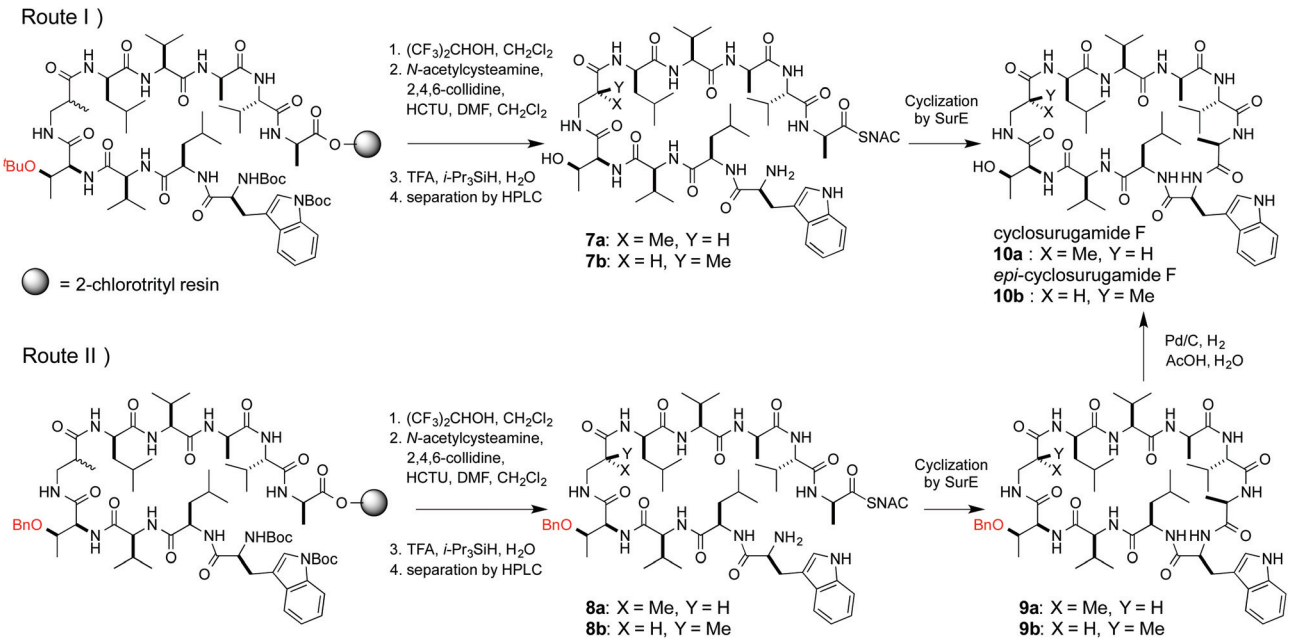


Fig. 3 Metabolic profile of the Δ*surE* strain. (a) LC analysis monitored by UV absorption at 215 nm. (b) LC-MS analysis of wild type and Δ*surE* mutant. Extracted ion chromatographs (EICs) at *m*/*z* 912.7 for **1**, *m*/*z* 898.7 for **2–5** and *m*/*z* 1056.7 for **6a** are presented.



Scheme 1 Synthetic scheme of cyclosurugamide F (**10a**) for structural confirmation.

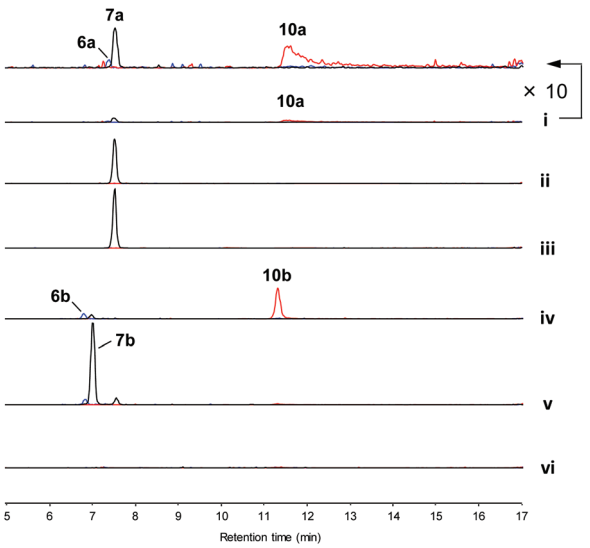


Fig. 4 *In vitro* SurE reaction with surugamide F-SNAC (**7a**) and its epimer **7b**. EIC for **7a** and its epimer **7b** (*m*/*z* 1157.8, black), EIC for **6a** and its epimer **6b** (*m*/*z* 1056.8, blue), and EIC for **10a** and its epimer **10b** (*m*/*z* 1038.7, red) are depicted. i: reaction mixture containing SurE and **7a**; ii: reaction mixture containing SurE and **7a** incubated for 0 min; iii: reaction mixture with **7a** only; iv: reaction mixture containing SurE and **7b**; v: reaction mixture with **7b** only; vi: reaction mixture with SurE only.

for $C_{52}H_{83}O_{11}N_{11}Na$ $[M + Na]^+$ 1060.6166), indicating that this corresponds to the dehydrated **6a**. Based on this result, we postulated that this is a cyclized form of **6a**; namely, cyclosurugamide F (**10a**). However, **6a** has two possible cyclizing modes: head-to-tail macrolactamization and head-to-side-chain macrolactonization through an ester bond with the β-hydroxyl group of a threonine in the middle of the sequence, which must be elucidated. We attempted to isolate this compound, but it was only soluble in DMSO or AcOH and broadly eluted in both normal and reverse phase column chromatography. The shape of the peak corresponding to the target compound was not improved dramatically under any conditions, and thus we sought an alternative way to establish its structure (Scheme 1).

A substrate analog, *O*-benzyl surugamide F-SNAC (**8a**) with the Thr-OH group protected by a benzyl group, was synthesized and subjected to the SurE reaction. We observed the efficient consumption of **8a** and the generation of a new compound with *m*/*z* 1128.7, corresponding to the *O*-benzyl cyclosurugamide F (**9a**) (Scheme 1 and Fig. S1†). Removal of the benzyl group by hydrogenation successfully yielded the compound identical to **10a** (Fig. 5 and Fig. S2†), confirming that **10a** is a head-to-tail cyclopeptide. It is noteworthy that SurE is capable of cyclizing **8a**, a substrate derivative with a bulky protecting group. Moreover, SurE efficiently cyclized the epimeric

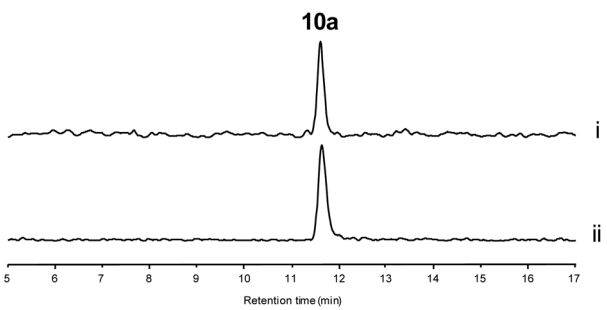


Fig. 5 LC-MS comparison of **10a** synthesized *via* routes I and II. EICs for **10a** (*m*/*z* 1038.8) are depicted. i: **10a** synthesized *via* route I; ii: **10a** synthesized *via* route II.

substrate **7b**, a derivative with the (*S*) configuration at the AMPA residue, to afford **10b** with an ion peak at *m*/*z* 1060.6184 (calcd for $C_{52}H_{83}O_{11}N_{11}Na$ $[M + Na]^+$ 1060.6166 (Fig. 4)). SurE also cyclized **8b** (Fig. S1†). The tolerance of SurE against non-natural substrates highlights its potential use as a biocatalyst for peptide cyclization.

Based on this result, we postulated that the genuine product of the SurB/SurC assembly line is **10a**, rather than the linear surugamide F (**6a**). Indeed, further exploration revealed the presence of **10a** as a metabolite of *S. albidoflavus* NBRC 12854 at the early stage of fermentation (Fig. S3†). While the titer of **6a** increased with longer cultivation, that of **10a** was retained, even though both are derived from the same assembly line, SurB/SurC (Fig. S4†). Based on this observation, we speculate that **6a** is generated *via* the hydrolysis of **10a** by an unidentified peptidase. Taken together, these results show that SurE is responsible for the offloading and cyclization of two distinct types of peptides that differ in terms of both amino acid sequence and chain length, highlighting its wide substrate tolerance.

Conclusions

In the present study, our gene knock-out experiment and characterization of recombinant SurE using synthetic substrates showed that SurE acts as an offloading cyclase not only in the biosynthesis of cyclic octapeptides **1–5**, but also in the biosynthesis of structurally unrelated decapeptide **6a**. Furthermore, during the course of *in vitro* experiments, we identified a new cyclic peptide, cyclosurugamide F (**10a**), generated by the SurE-catalyzed offloading process of the SurB/SurC assembly line. Two distinct TE-less NRPS assembly lines prominently feature the biosynthetic pathway of surugamides, in which the *trans*-acting TE, SurE, remarkably cyclizes two structurally unrelated substrates originating from the respective NRPS assembly lines. We demonstrated that the epimeric and/or benzyl derivatives of **7a** were also cyclized by SurE, suggesting its good tolerance for a range of non-natural substrates. Our results highlight the substrate promiscuity of SurE and its potential use as a biocatalyst for the macrolactamization of linear peptides. Examinations of the scope, limitations and *in trans* interactions between SurE and the last PCPs at the ends of two assembly lines[20] will be the topics of further investigations.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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