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The Iterative Gramicidin S Thioesterase Catalyzes Peptide Ligation and Cyclization

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SUMMARY

Here, we present a comprehensive in vitro characterization of the excised iterative, bimodular PCP-TE of the gramicidin S synthetase GrsB, which is able to act both as a ligation and a cyclization catalyst. Using the native pentapeptidyl-thioester substrates, GrsB PCP-TE catalyzes the dimerization and subsequent formation of the decapeptide lactam gramicidin S. Interestingly, the detection of linear decapeptidyl-SNAC as an enzyme-dependent intermediate supports the iterative mechanism in vivo, in which two pentapeptides, one bound as an ester to the active site serine of the TE domain and the second bound as a thioester to the adjacent pan-PCP, are ligated to a decapeptidyl-pan-PCP that subsequently transferred to the adjacent TE domain and cyclized. Moreover, GrsB PCP-TE can handle different substrates length, leading not only to dimerization, but also to trimerization and the formation of different ring sizes.

INTRODUCTION

Many bioactive peptides of diverse structures are produced via a template-directed manner by multimodular enzymes called nonribosomal peptide synthetases (NRPSs) [1]. A common feature of such nonribosomally synthesized bioactive peptides is their macrocyclic structure, which causes reduction in structural flexibility and may therefore constrain them into the biologically active conformation [2]. Macrocyclic products are released from NRPS assembly lines usually by the action of C-terminal thioesterase domains (TE domains, cyclases). These accomplish product release by diverse mechanisms, mainly including a head-to-tail or branched side chain cyclization resulting in the formation of the final cyclic product [3, 4]. Excised TE domains, separated from their biosynthetic machinery, are active enzymes in vitro when provided with substrate mimics. Due to the fact that stereoselective peptide cyclization is a difficult task in organic synthesis, chemoenzymatic methods have emerged for the production of natural products and their derivatives [5]. These approaches combine substrate generation by solid-phase peptide synthesis and stereoselective cyclization utilizing excised TE domains.

Based on the mechanism of peptide assembly and product release, NRPSs can be subdivided into three different classes [6]. Best characterized are the linear NRPSs (Type A), which are exemplified by the tyrocidine and the surfactin synthetases [1]. In these systems, the sequence of the assembled peptide chain is entirely determined by the number and order of modules within the corresponding peptide synthetase. Several TE domains of linear NRPSs are biochemically and structurally well characterized and are frequently used in chemoenzymatic approaches to generate macrocyclic peptide derivatives [7–13]. In contrast, iterative (Type B) and nonlinear (Type C) NRPS assembly line templates are more complex. For example, iterative synthetases use their modules and domains more than once to assemble their final products from repetitive building blocks. Prominent candidates for the iterative mechanism are the antibiotic gramicidin S, the siderophore enterobactin, the antitumor agent thiocoraline, and the potent anti-SARS-CoV valinomycin, as well as the anticancer drug bleomycin and the siderophore yersiniabactin for nonlinear NRPS-PKS hybrids [14–19]. To clarify the mechanistic aspects and to explore the biocombinatorial potential of the iterative reaction, we investigated the TE domain of the gramicidin S synthetase for its substrate specificity, ligation capacity, and cyclization potential.

Gramicidin S is a potent and well-characterized antibiotic synthesized by *Bacillus brevis* [14]. It is a cyclic decapeptide with the primary structure *cyclo*(-D-Phe$_1$-L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$ -D-Phe$_6$- L-Pro$_7$- L-Val$_8$- L-Orn$_9$- L-Leu$_{10}$-), which is assembled on the GrsA and GrsB peptide synthetases that comprise one and four modules, respectively (Figure 1). The modular organization of the peptide synthetases suggests a mechanism in which the gramicidin TE (GrsB TE) serves as a holding bay for the gramicidin linear pentapeptide intermediate. After formation of the first pentapeptide, the TE domain awaits the assembly of the second pentapeptide and catalyzes the dimerization and the release of the cyclic decapeptide lactam. This type of mechanism was first proposed by Stoll et al. back in 1970 and was previously supported by mass spectrometric studies on the TE domain of the iterative enterobactin (Ent) synthetase [15, 20]. Early studies also discussed enzyme dimerization, and they determined that the utilization of two TE domains to catalyze this two-step mechanism would lead to the

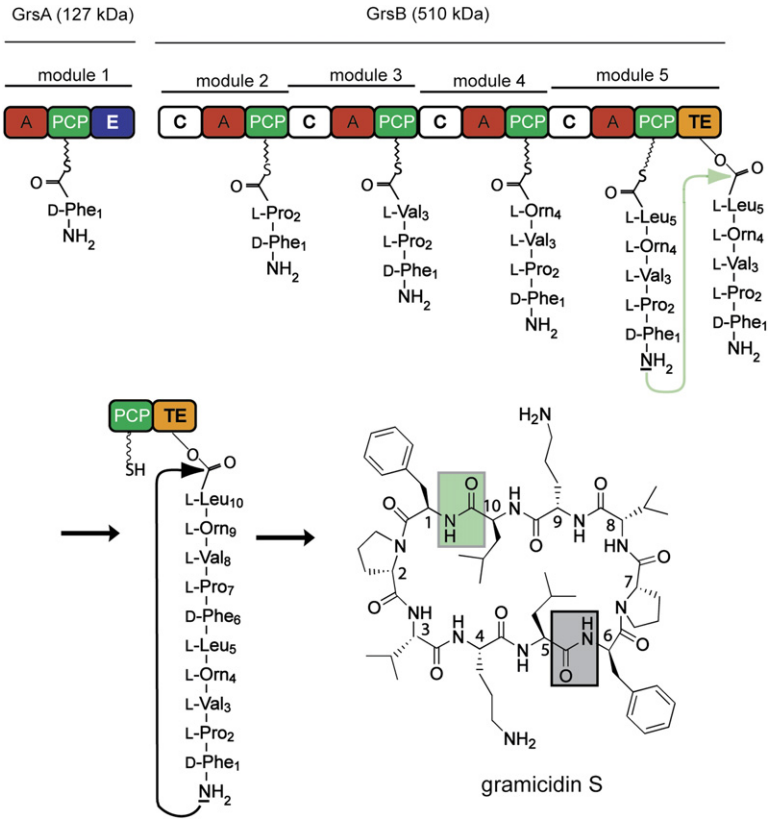

Figure 1. The Gramicidin S Biosynthetic Machinery

The enzymatic assembly line consists of two NRPSs (GrsA and GrsB), which are composed of one and four modules, respectively. Each module is responsible for the incorporation of one monomeric amino acid. The thioesterase domain (TE domain) catalyzes the dimerization of two assembled pentapeptides and subsequent cyclization, resulting in gramicidin S. A, adenylation; PCP, peptidyl carrier protein; E, epimerization; C, condensation; TE, thioesterase.

same product. However, this seems rather unlikely, as it has been shown for GrsA and the iterative enterobactin synthetase EntF, harboring the TE domain, that NRPSs work as monomers [21].

Here, we report in vitro studies on the characterization of an iterative TE domain. We demonstrate that the excised GrsB TE can act in vitro both as a ligation and a cyclization catalyst. Results obtained with peptidyl thioester substrates of different lengths enable a new biocombinatorial approach and a suggestion for a reaction mechanism.

RESULTS

Definition of the GrsB TE N-Terminal Border

In order to explore the potential of the excised GrsB TE domain to catalyze the cyclization of artificial, linear gramicidin thioester substrates, we generated a recombinant protein comprising the TE domain together with its adjacent peptidyl-carrier protein (GrsB PCP-TE, 59 kDa, 365 residues) [22]. Heterologous expression in *Escherichia coli* BL21 cells at 25°C resulted in the production of the recombinant *apo* protein in sufficient yields (4 mg/l, Figure S1; see the Supplemental Data available with this article online).

In order to exclude the possibility that the PCP domain is indispensable for the iterative reaction of the PCP-TE construct, we additionally investigated the activity of the single TE domain. To define the borders of the TE domain, we performed sequence alignments, which revealed that the published borders of gramicidin thioesterase [7] comprise about 25 amino acid residues as an N-terminal extension compared to most characterized linear TE domains [7–13]. Sequence alignments of GrsB TE with the iterative TE domains of EntF, DhbF, and TioS revealed that this N-terminal extension seems to be a unique feature of GrsB TE (Figure 2). Based on these sequence alignments, it was possible to define two putative N-terminal borders of the GrsB TE domain. Based on this finding, a long version (TE_{Long}, 33 kDa, 275 residues) and a short version (TE_{Short}, 30 kDa, 250 residues) were cloned and expressed in the soluble and active forms (Figure S1). Comparison of the reaction time courses of GrsB PCP-TE and GrsB TE_{Long} incubated with the pentapeptidyl-thioester and the decapeptidyl-thioester revealed similar rates of product formation. In contrast, GrsB TE_{Short} catalyzes a 5-fold slower product formation. Due to good solubility and the best yields of the GrsB PCP-TE, all further experiments were conducted with this construct.

Characterization of GrsB PCP-TE Activity with Native Linear Substrates

Based on previous results [7, 23], in which the decapeptidyl-SNAC (**GS10**) was used as the substrate, we synthesized **GS10** (Table 1) for initial activity studies. Incubation of the excised GrsB PCP-TE with **GS10** resulted in the formation of the expected cyclic decapeptide gramicidin S (t_R = 43.0, Figure 3A). The kinetic parameters for the

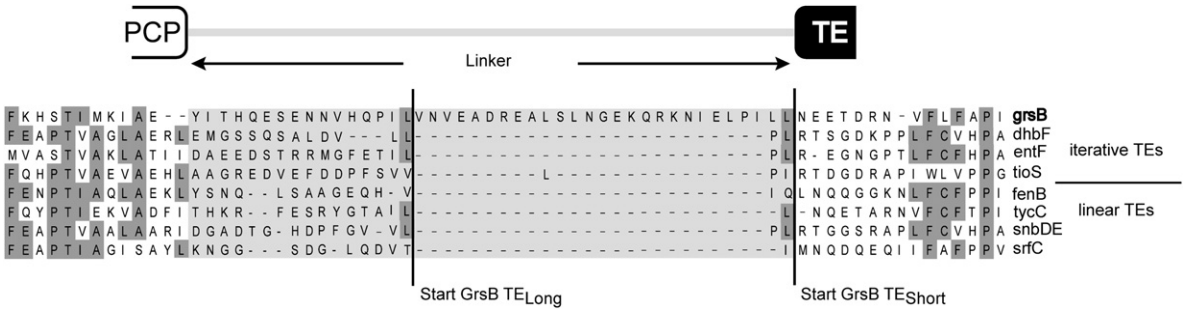

Figure 2. Sequence Alignment of the Linker Region between PCP and Thioesterase of Different NRPSs

The vertical bars mark the start of the short and long versions of the GrsB thioesterase (GrsB TE$_{Long}$, GrsB TE$_{Short}$). Conserved residues are marked in dark gray. The putative linker region of all synthetases is marked in bright gray. grsB, gramicidin S synthetase B; dhbF, bacillibactin synthetase F; entF, enterobactin synthetase F; tioS, thiocoraline synthetase S; fenB, fengycin synthetase B; tycC, tyrocidine synthetase C; snbDE, pristinamycin synthetase DE; srfC, surfactin synthetase C.

cyclization reaction revealed a catalytic efficiency of k_{cat}/K_M = 5.5 min^{-1} mM^{-1} (K_M = 0.24 mM, k_{cat} = 1.3 min^{-1}).

A common mechanistic feature in iterative NRPS systems is the ability to combine two or more identical monomers. Therefore, the major question for excised GrsB PCP-TE was whether the recombinant protein is able to catalyze an iterative reaction in vitro. For this purpose, we synthesized the native pentapeptidyl-SNAC and -thiophenol substrates, **GS5$_{SNAC}$**, **GS5$_{SPh}$** (Table 1), respectively, and incubated them with the GrsB PCP-TE. In both cases, the HPLC trace revealed that GrsB PCP-TE catalyzes the dimerization of the pentapeptides to a decapeptide and its subsequent cyclization, resulting in the formation of gramicidin S (t_R = 43.1) (Figures 3B and 3C). The formation of gramicidin S in this reaction occurred with a k_{cat} value of 0.4 min^{-1} for **GS5$_{SNAC}$** and a k_{cat} value of 2.4 min^{-1} for **GS5$_{SPh}$**. Taking into account that the linear gramicidin substrate possesses two nucleophiles, L-Orn$_4$ and the N terminus of D-Phe$_1$, MS-MS sequencing of the cyclic decapeptide was conducted. The results obtained excluded the possibility that L-Orn$_4$ is involved in ring formation. Cyclization is exclusively mediated through a nucleophilic attack of the α amino group of D-Phe$_1$; fragment ions proving the predicted linkage of D-Phe$_1$ to L-Leu$_5$ were unambiguously identified (Figure S2). In addition to the main product, gramicidin S, and hydrolysis of the pentamer, linear decapeptidyl-SNAC (5+5) and a 15-membered ring (5+5+5) (at low concentrations) consisting of three pentapeptide monomers were detected as side products (ratio hydrolysis/gramicidin S: 1/4). In the case of **GS5$_{SPh}$**, such a linear decepeptidyl intermediate was not observed (ratio hydrolysis/gramicidin S: 1/3). Interestingly, **GS5$_{SPh}$** forms a 5-membered ring in an enzyme-independent reaction, which is not observed with the SNAC-activated substrate. Comparison of the assay conducted with and without enzyme showed that the enzyme-catalyzed reaction is faster than the uncatalyzed formation of the 5-membered ring. For stability reasons, we used peptidyl-SNACs instead of thiophenols for all further studies.

Substrate Specificity of GrsB PCP-TE

Encouraged by the obtained results, we wanted to gain insight into the substrate tolerance of the thioesterase. For this purpose, single amino acid residues in the native pentapeptide substrate were exchanged stepwise for alanine and were screened for the formation of the corresponding decapeptides. Substrates substituted in positions 2 (L-Pro$_2$), 3 (L-Val$_3$), and 4 (L-Orn$_4$) revealed the formation of a 10-membered ring with the corresponding linear SNAC intermediate and small amounts of the 15-membered ring, indicating the tolerance of GrsB PCP-TE for those positions within the pentapeptide (Tables 1 and 2, the ratio of hydrolysis/linear and cyclic products is: **GS5$_{SNAC}$**, 1/4; **GS5$_{Ala2}$**, 1/2.5; **GS5$_{Ala3}$**, 6/1; **GS5$_{Ala4}$**, 1/1.5). The velocity of the reaction was about the same for the tested substrates (conversion of substrate after 10 min: **GS5$_{SNAC}$**, 11%; **GS5$_{Ala2}$**, 6%; **GS5$_{Ala3}$**, 9%; **GS5$_{Ala4}$**, 9%). Interestingly, in the assays with the alanine pentapeptide derivatives, the ratio of the 15-membered ring to the 10-membered ring is different (ratio 10-membered ring/15-membered ring: **GS5$_{SNAC}$**, 6/1; **GS5$_{Ala2}$**, 5/1; **GS5$_{Ala3}$**, 2/1; **GS5$_{Ala4}$**, n.d.). To corroborate these results, we determined the kinetic parameters for the cyclization reaction of the corresponding SNAC decapeptides **GS10$_{Ala2,7}$**, **GS10$_{Ala3,8}$**, and **GS10$_{Ala4,9}$** (Tables 1 and 2; **GS10$_{Ala2,7}$**: K_M, 357 μM, k_{cat}, 4 min^{-1}; **GS10$_{Ala3,8}$**: K_M, 485 μM, k_{cat}, 3.4 min^{-1}). Unfortunatley, we had solubility problems with **GS10$_{Ala4,9}$**; thus, we were not able to determine kinetic parameters. Additionally, we altered the configuration of the amino acids at positions 1 and 5. Substrates carrying D-Leu at the C terminus (**GS5$_{D\text{-}Leu5}$** and **GS10$_{D\text{-}Leu10}$**) showed low hydrolysis rates, whereas substrates carrying L-Phe at the N terminus (**GS5$_{L\text{-}Phe1}$** and **GS10$_{L\text{-}Phe1}$**) were mainly hydrolyzed. Cyclization and dimerization were not observed.

Variations of Substrate Length

Focusing on the impact of different substrate lengths for the oligomerization and cyclization reaction, we created a library based on the structure of gramicidin S that

Table 1. ESI-Mass Spectrometric Characterization of Synthesized Linear Substrates

Compound	Sequence	Species (m/z)	Observed Mass (Calculated Mass)
GS2	D-Phe$_1$- L-Leu$_2$- SNAC	$[M + H]^+$	380.2 (380.2)
GS3	D-Phe$_1$- L-Pro$_2$- L-Leu$_3$- SNAC	$[M + H]^+$	477.2 (477.3)
GS4	D-Phe$_1$- L-Pro$_2$- L-Orn$_3$- L-Leu$_4$- SNAC	$[M + H]^+$	591.2 (591.3)
GS5$_{SNAC}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- SNAC	$[M + H]^+$	690.5 (690.4)
GS5$_{SPh}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- SPh	$[M + H]^+$	681.5 (681.4)
GS5$_{Ala2}$	D-Phe$_1$- L-Ala$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- SNAC	$[M + H]^+$	664.5 (664.4)
GS5$_{Ala3}$	D-Phe$_1$ -L-Pro$_2$ -L-Ala$_3$ -L-Orn$_4$ -L-Leu$_5$ -SNAC	$[M + H]^+$	662.5 (662.4)
GS5$_{Ala4}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Ala$_4$- L-Leu$_5$- SNAC	$[M + H]^+$	647.4 (647.4)
GS5$_{L\text{-}Phe1}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- SNAC	$[M + H]^+$	690.5 (690.4)
GS5$_{D\text{-}Leu5}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- D-Leu$_5$- SNAC	$[M + H]^+$	690.5 (690.4)
GS5$_{NAc}$	NAc-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- SNAC	$[M + H]^+$	732.4 (732.4)
GS5$_{OH}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$	$[M + H]^+$	589.4 (589.4)
GS6	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Val$_4$- L-Orn$_5$- L-Leu$_6$- SNAC	$[M + H]^+$	789.5 (789.5)
GS7	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Pro$_4$- L-Val$_5$- L-Orn$_6$- L-Leu$_7$- SNAC	$[M + H]^+$	886.5 (886.5)
GS8	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Pro$_5$- L-Val$_6$- L-Orn$_7$- L-Leu$_8$- SNAC	$[M + H]^+$	1000.6 (1000.6)
GS9	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- L-Pro$_6$- L-Val$_7$- L-Orn$_8$- L-Leu$_9$- SNAC	$[M + H]^+$	1113.8 (1113.7)
GS10	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- D-Phe$_6$- L-Pro$_7$- L-Val$_8$- L-Orn$_9$- L-Leu$_{10}$- SNAC	$[M + H]^+$	1260.7 (1260.7)
GS10$_{L\text{-}Phe1}$	L-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- D-Phe$_6$- L-Pro$_7$- L-Val$_8$- L-Orn$_9$- L-Leu$_{10}$- SNAC	$[M + H]^+$	1260.7 (1260.7)
GS10$_{D\text{-}Leu10}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- D-Phe$_6$- L-Pro$_7$- L-Val$_8$- L-Orn$_9$- D-Leu$_{10}$- SNAC	$[M + H]^+$	1260.7 (1260.7)
GS10$_{Ala2,7}$	D-Phe$_1$- L-Ala$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- D-Phe$_6$- L-Ala$_7$- L-Val$_8$- L-Orn$_9$- L-Leu$_{10}$- SNAC	$[M + H]^+$	1208.7 (1208.7)
GS10$_{Ala3,8}$	D-Phe$_1$- L-Pro$_2$- L-Ala$_3$- L-Orn$_4$- L-Leu$_5$- D-Phe$_6$- L-Pro$_7$- L-Ala$_8$- L-Orn$_9$- L-Leu$_{10}$- SNAC	$[M + H]^+$	1204.7 (1204.7)
GS10$_{Ala4,9}$	D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Ala$_4$- L-Leu$_5$- D-Phe$_6$- L-Pro$_7$- L-Val$_8$- L-Ala$_9$- L-Leu$_{10}$- SNAC	$[M + H]^+$	1174.7 (1174.7)

contained peptides that varied in length. These substrates were synthesized by excluding stepwise internal amino acids of the linear gramicidin S decapeptide. Starting by assaying the smallest dipeptide, **GS2** (Table 1), only hydrolysis could be detected. In addition, the slow formation of diketopiperazin as a prominent nonenzymatic reaction was visible. These results indicate that the dipeptide **GS2** seems to be too short for the conversion catalyzed by the thioesterase. By using additional substrates of varying lengths it was possible to detect, in addition to hydrolysis, the formation of different ring sizes. The tripeptide **GS3** (Tables 1 and 2) was dimerized by the thioesterase and was subsequently cyclized into a 6-membered ring (3+3) as the single product. Consecutively, we incubated a tetrapeptide, **GS4** (Table 1), with GrsB PCP-TE. After 90 min of incubation, one-third of this tetrapeptide was converted into two products, an 8-membered (48%, 4+4) and a 12-membered (21%, 4+4+4) ring (Table 2). Side product formation could be assigned to the hydrolyzed monomer (10%), the hydrolyzed octapeptide (4+4, 5%), and the activated octapeptidyl-SNAC (4+4, 16%).

With further increases of the peptide length, the ability of GrsB PCP-TE to dimerize or even trimerize the incubated substrate is reduced. Assaying the reaction of a hexapeptide, **GS6** (Table 1), exclusively led to a 6-membered ring. The heptapeptide **GS7** (Tables 1 and 2) was dimerized and was subsequently cyclized into a 14-membered ring (7+7). The octapeptide **GS8** (Tables 1 and 2) was converted into an 8-membered ring and a linear dimer (8+8) of the substrate. For the nonapeptide **GS9** (Tables 1 and 2), cyclization was exclusively observed, as described for the decapeptide **GS10**.

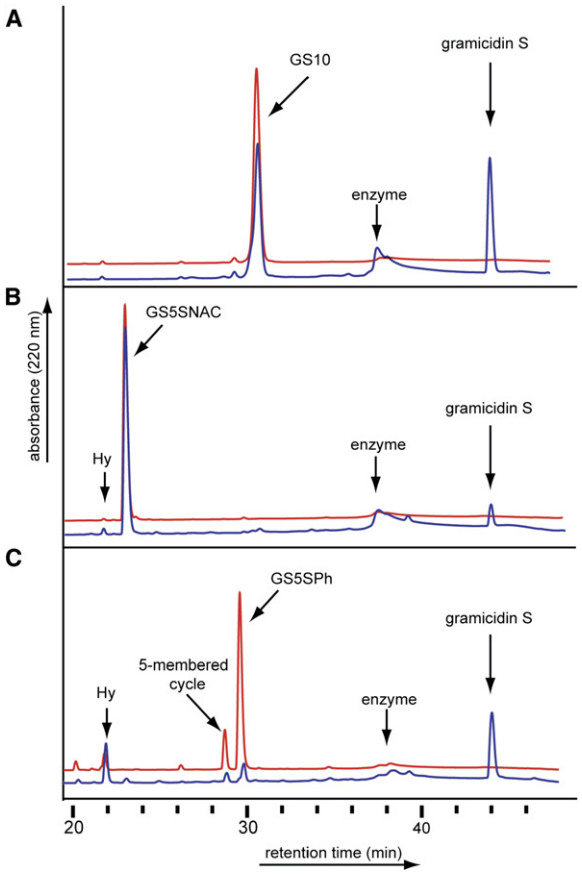

Figure 3. Dimerization and Cyclization of Pentapeptidyl- and Decapeptidyl-Thioesters Mediated by the GrsB PCP-TE

(A) Incubation of **GS10** (300 μM) with PCP-TE (10 μM) for 60 min.
(B) Incubation of **$GS5_{SNAC}$** (300 μM) with PCP-TE (10 μM) for 60 min.
(C) Incubation of **$GS5_{SPh}$** (300 μM) with PCP-TE (10 μM) for 60 min.
The blue HPLC trace corresponds to the assay with enzyme, and the red trace shows incubation of the substrate in the absence of the enzyme. Analysis was performed on a C_{18} Nucleodur column (Macherey and Nagel; 250/2; pore diameter, 100 Å; particle size, 3 μM) with the following gradient: 0–40 min, 5%–60% MeCN/0.1% TFA into H_2O/0.1% TFA, 40–45 min, 60%–95% MeCN/0.1% TFA into H_2O/0.1% TFA, 0.3 ml/min, 45°C.

These results indicate that GrsB PCP-TE catalyzes the ring formation of 6- to 15-membered rings achieved by ligation and cyclization reactions.

Construction of a Cyclic Peptide Library

In order to get an impression of the ligation and cyclization potential of GrsB PCP-TE, we performed assays in which substrates differing in composition and length were combined and screened for the assembly of novel products. Mixing the tetrapeptide **GS4** and the pentapeptide **$GS5_{SNAC}$** in the presence of GrsB PCP-TE led to an 8-membered (4+4), a 10-membered (5+5), and a 12-membered (4+4+4) ring (Figure 4). In addition, formation of a novel 13-membered ring (4+4+5) consisting of two tetra- and one pentapeptide monomer could be detected.

These results were exploited in two additional experiments, in which the substrates **GS3** and **GS4** were combined and led to the formation of an 8-membered (4+4), a 12-membered (4+4+4), and an 11-membered (4+4+3) ring (Table 2). Likewise, the incubation of GrsB PCP-TE with the two substrates, **GS4** and **GS6**, resulted in the formation of an additional 10-membered ring (6+4) (Table 2).

Probing the Ligase Activity of GrsB PCP-TE

Generally, NRPS condensation (C) domains catalyze the peptide bond ligation of activated amino acids along the nascent peptide chain [24]. As iterative TE domains mediate the linkage of smaller monomers before cyclization, the question of whether the excised GrsB PCP-TE can potentially act as a ligase arises. To test this ability, we had to impede the GrsB PCP-TE from acting as a cyclase. This was achieved by blocking either the N or C terminus of the suitable linear monomers. In a first set of experiments, we incubated GrsB PCP-TE with a pentapeptide, **$GS5_{OH}$**, that was not activated as a thioester. As expected, no conversion was detectable. Additionally, an activated pentapeptidyl-SNAC, **$GS5_{NAc}$**, that was N-terminally acylated, was constructed. Assaying this substrate with GrsB PCP-TE resulted exclusively in hydrolysis, whereas coincubation of both substrates, **$GS5_{OH}$** and **$GS5_{NAc}$**, led to the ligation of these pentapeptides, which, in turn, resulted in a linear decapeptide (ratio ligation/hydrolysis: 1/2, using 300 μM of each substrate). This reaction clearly indicated the ability of GrsB PCP-TE to catalyze a ligation reaction. Following Michaelis-Menten kinetics, this ligation reaction was determined to a k_{cat} of 4.9 min^{-1} and a K_M of 0.62 mM for **$GS5_{OH}$** and a k_{cat} of 7.3 min^{-1} and a K_M of 1.1 mM for **$GS5_{NAc}$**.

DISCUSSION

Recent studies on several TE domains dissected from various NRPS systems opened new approaches for the in vitro generation of macrocyclic bioactive compounds and allowed the characterization of their dedicated regio- and stereoselective cyclization mechanisms. With the biochemical characterization of the iterative TE domain of gramicidin S we present here a class of TE domains with high potential for biocombinatorial synthesis. In contrast to linear TE domains, iterative GrsB TE not only catalyzes the regio- and stereoselective cyclization of linear peptide precursors, but additionally catalyzes peptide bond ligation.

The GrsB PCP-TE was tested by using the native linear decapeptide **GS10** and pentapeptides **$GS5_{SNAC}$** and **$GS5_{SPh}$**, which leads to the formation of the desired cyclic decapeptide lactam gramicidin S (Figure 3). This clearly indicated that the excised TE domain catalyzes both elongation and cyclization in vitro. Former studies with the same pentapeptidyl-SNAC indicated a barely detectable dimerization activity catalyzed by GrsB TE [7]. In contrast, our results prove not only dimerization, but also subsequent cyclization, resulting in gramicidin S in sufficient yields for thiophenol- and SNAC-activated substrates.

Table 2. ESI-Mass Spectrometric Characterization of Cyclic Products

Ring Size	Substrate	Cyclic Species	Species (m/z)	Observed Mass (Calculated Mass)
6-membered	GS6	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Val$_4$- L-Orn$_5$- L-Leu$_6$-)	$[M + H]^+$	670.5 (670.4)
6-membered	GS3	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Leu$_3$-)$_2$	$[M + H]^+$	715.5 (715.4)
8-membered	GS4	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Orn$_3$- L-Leu$_4$-)$_2$	$[M + H]^+$	943.5 (943.6)
8-membered	GS8	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Pro$_5$- L-Val$_6$- L-Orn$_7$- L-Leu$_8$-)	$[M + H]^+$	881.5 (881.6)
9-membered	GS9	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$- L-Pro$_6$- L-Val$_7$- L-Orn$_8$- L-Leu$_9$-)	$[M + H]^+$	994.5 (994.6)
10-membered	GS5$_{SNAC}$, GS5$_{SPh}$ or GS10	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$-)$_2$	$[M + H]^+$	1141.7 (1141.7)
10-membered	GS5$_{Ala2}$	*Cyclo*(-D-Phe$_1$- L-Ala$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$-)$_2$	$[M + H]^+$	1089.8 (1089.7)
10-membered	GS5$_{Ala3}$	*Cyclo*(-D-Phe$_1$ -L-Pro$_2$ -L-Ala$_3$ -L-Orn$_4$ -L-Leu -)$_2$	$[M + H]^+$	1085.5 (1085.6)
10-membered	GS5$_{Ala4}$	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Ala$_4$- L-Leu$_5$-)$_2$	$[M + H]^+$	1055.6 (1055.6)
10-membered	GS10$_{Ala2,7}$	*Cyclo*(-D-Phe$_1$- L-Ala$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$-)$_2$	$[M + H]^+$	1089.7 (1089.7)
10-membered	GS10$_{Ala2,8}$	*Cyclo*(-D-Phe$_1$ -L-Pro$_2$ -L-Ala$_3$ -L-Orn$_4$ -L-Leu -)$_2$	$[M + H]^+$	1085.7 (1085.6)
10-membered	GS10$_{Ala2,9}$	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Ala$_4$- L-Leu$_5$-)$_2$	$[M + H]^+$	1055.7 (1055.6)
10-membered	GS6 and GS4	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Val$_4$- L-Orn$_5$- L-Leu$_6$-D-Phe$_7$- L-Pro$_8$- L-Orn$_9$- L-Leu$_{10}$-)	$[M + H]^+$	1141.7 (1141.7)
11-membered	GS4 and GS3	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Orn$_3$- L-Leu$_4$- D-Phe$_5$- L-Pro$_6$-L-Orn$_7$- L-Leu$_8$- D-Phe$_9$- L-Pro$_{10}$- L-Leu$_{11}$-)	$[M + H]^+$	1300.7 (1300.8)
12-membered	GS4	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Orn$_3$- L-Leu$_4$-)$_3$	$[M + H]^+$	1414.8 (1414.9)
13-membered	GS4 and GS5$_{SNAC}$	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Orn$_3$- L-Leu$_4$- D-Phe$_5$- L-Pro$_6$-L-Orn$_7$- L-Leu$_8$- D-Phe$_9$- L-Pro$_{10}$- L-Val$_{11}$- L-Orn$_{12}$- L-Leu$_{13}$-)	$[M + H]^+$	1513.8 (1513.9)
14-membered	GS7	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Pro$_4$- L-Val$_5$- L-Orn$_6$- L-Leu$_7$-)$_2$	$[M + H]^+$	1533.8 (1533.9)
15-membered	GS5$_{SNAC}$	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$-)$_3$	$[M + H]^+$	1712 (1712.1)
15-membered	GS5$_{Ala2,7}$	*Cyclo*(-D-Phe$_1$- L-Ala$_2$- L-Val$_3$- L-Orn$_4$- L-Leu$_5$-)$_3$	$[M + H]^+$	1633.9 (1634)
15-membered	GS5$_{Ala3,8}$	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Ala$_3$- L-Orn$_4$- L-Leu$_5$-)$_3$	$[M + H]^+$	1627.9 (1628)
15-membered	GS5$_{Ala4,9}$	*Cyclo*(-D-Phe$_1$- L-Pro$_2$- L-Val$_3$- L-Ala$_4$- L-Leu$_5$-)$_3$	$[M + H]^+$	1582.9 (1582.9)

Dimerization activity by TE domains of linear NRPSs has been described only once thus far [13]. Here, the gramicidin S pentapeptidyl-SNAC was used in combination with the excised tyrocidine TE, leading to hydrolysis as the major product and small amounts of dimerized and cyclized products. In comparison, dimerization and cyclization catalyzed by GrsB PCP-TE is indeed slower, but they are only accompanied by low rates of hydrolysis (ratio cycle(5+5)/hydrolysis 4/1 for **GS5$_{SNAC}$**). Kinetic investigations show that the conversion of the pentapeptide into gramicidin S is slower than the conversion of the decapeptide, possibly due to the fact that the ligation reaction of the pentapeptide is the rate-determining step. Using the pentapeptidyl-SNAC as the substrate, a decapeptidyl-SNAC was generated in an enzyme-dependent reaction. The detection of this decapeptidyl-SNAC gave rise to speculations about the mechanism of iterative TE domains. The SNAC intermediate can be formed by the nucleophilic attack of the amino group of a free SNAC pentapeptide on the peptidylester of the TE-bound pentapeptide. If the free amino group of the TE-bound substrate would attack the thioester of the SNAC-activated peptide, only hydrolyzed linear substrates could be detected as side products. These observations can also aid in the understanding of the reaction catalyzed by iterative thioesterases in vivo. For the enterobactin synthetase, Shaw-Reid et al. [15] postulated that the first monomer is built up by the synthetase and is subsequently transferred to the active site serine of the TE domain. The second monomer is presented by the pan-arm of the PCP domain adjacent to Ent-TE. The ligation reaction results in a dimer bound as an ester to the active site serine of the TE domain. Two possible mechanisms for this elongation reaction can be postulated (Figure 5). Either the amino group of the first monomer bound to the TE domain nucleophilicaly attacks the second monomer presented by the

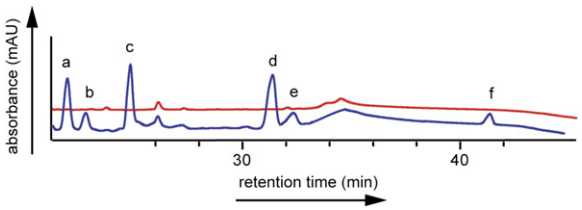

Figure 4. Coincubation of GS4 and $GS5_{SNAC}$ with GrsB PCP-TE, Resulting in the Formation of a Mixture of Cyclic Products
Blue trace: incubation of **GS4** (D-Phe- L-Pro- L-Orn- L-Leu-SNAC, 300 μM) and $\mathbf{GS5_{SNAC}}$ (D-Phe- L-Pro- L-Val- L-Orn- L-Leu-SNAC, 300 μM) with GrsB PCP-TE (10 μM) for 1 hr at 25°C. Red trace: control reaction without enzyme. Due to high retention time differences between substrates **GS4** and $\mathbf{GS5_{SNAC}}$ and products, only product composition is shown. a, (4+4)-SNAC; b, (5+5); c, *cyclo*(4+4); d, *cyclo*(4+4+4); e, *cyclo*(5+4+4); f, *cyclo*(5+5).

pan-PCP, resulting in a dimer bound to the active site serine of the TE domain (forward reaction), or the amino group of the PCP-bound substrate attacks the TE-bound monomer, resulting in a dimer bound to the PCP domain, that is subsequently transferred to the active site serine of the TE domain for cyclization (backward reaction). According to our results, there is strong evidence that the GrsB TE utilizes the backward reaction, as we were able to detect the formation of the decapeptidyl-SNAC. To date, it cannot be verified whether the decapeptidyl-SNAC is a side product or an intermediate of the reaction. In the forward reaction, a second catalytic center would be required to catalyze the ligation reaction because the serine of the catalytic triad is blocked by the first monomer. Comprehensive sequence alignments of several TE domains revealed that the gramicidin TE domain has an extension of about 25 amino acids (Figure 2). Expression of this longer version of the GrsB TE domain (GrsB TE_{Long}) published by Kohli et al. [7], and a short version (GrsB TE_{Short}) missing the 25 amino acids resulted in comparable dimerization and cyclization activity. Although the obtained products were found in equal parts, the catalytic efficiency of GrsB TE_{Short} is 5-fold lower, indicating an important role of the N-terminal extension for protein stability. Also, the iterative gramicidin TE domain has a longer linker region between the PCP and the TE domain that was not observed in other PCP-TE junctions. This extended linker region may be important for flexibility of the thioesterase needed to catalyze two different reactions: the ligation of two substrates and intramolecular cyclization. Structural analysis of this iterative TE might provide insights into these mechanistic issues.

To investigate the cyclization and ligation ability of GrsB PCP-TE for biocombinatorial approaches, we determined its substrate tolerance. The alanine screen of the linear pentapeptide clearly indicates that residues at positions 2–4 in the substrate are tolerant of substitutions, which is shown by the time course of the reaction and the given product ratios (ratio of hydrolysis/linear and cyclic products: $\mathbf{GS5_{SNAC}}$, 1/4; $\mathbf{GS5_{Ala2}}$, 1/2.5; $\mathbf{GS5_{Ala3}}$, 6/1; $\mathbf{GS5_{Ala4}}$, 1/1.5, and conversion of substrate after 10 min: $\mathbf{GS5_{SNAC}}$, 11%; $\mathbf{GS5_{Ala2}}$, 6%; $\mathbf{GS5_{Ala3}}$, 9%; $\mathbf{GS5_{Ala4}}$, 9%). To corroborate these results, we synthesized the corresponding decapeptides and were able to show, for two of these substrates, that the cyclization reaction is characterized by higher K_M and k_{cat} values (**GS10**: K_M, 241 μM, k_{cat}, 1.3 min^{-1}; $\mathbf{GS10_{Ala2,7}}$: K_M, 357 μM, k_{cat}, 4 min^{-1}; $\mathbf{GS10_{Ala3,8}}$: K_M, 485 μM, k_{cat}, 3.4 min^{-1}). However, positions 1 and 5, which are directly involved in the elongation and/or cyclization reaction, are not accessible to alteration of the stereogenic configuration. The tolerance for substitutions of L-Val_3, L-Orn_4, and D-Phe_6 of the decapeptide in the cyclization reaction mediated by GrsB TE was shown by Guo [23]. Similar substrate tolerance

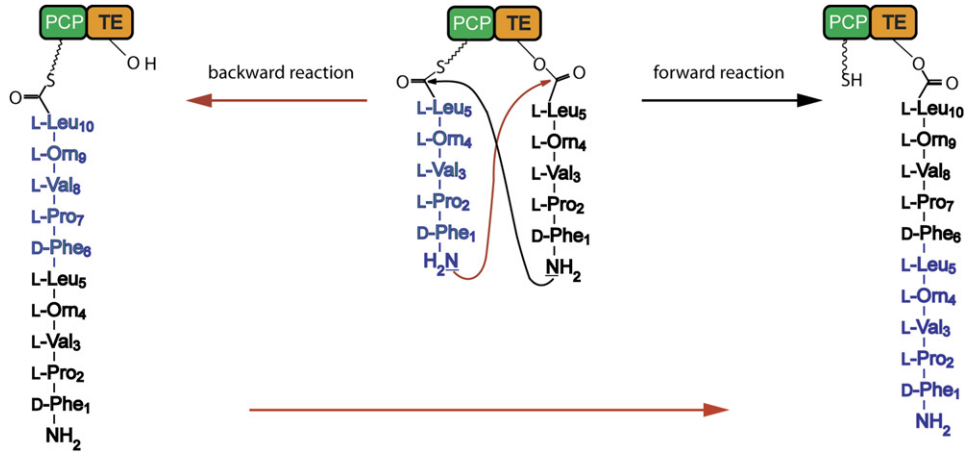

Figure 5. Postulated Mechanisms of the Dimerization Reaction Catalyzed by GrsB PCP-TE
In the forward reaction (indicated by black arrows), the pentapeptide attached to the TE nucleophilically attacks the thioester of pentapeptidyl-pan-PCP, resulting in the formation of a linear decapeptide directly bound to the active site serine of the TE domain. In the backward reaction (indicated by red arrows), the amino group of pentapeptidyl-pan-PCP attacks the ester of the pentapeptide attached to the thioesterase. This leads to the decapeptide bound to the PCP domain, which is subsequently transferred to the TE domain for cyclization.

for cyclization reactions was observed for Type A TE domains [7–13].

How iterative thioesterases can control the number of ligation steps of the repetative basic units remains unknown. The results obtained from assays with substrates of different lengths and substrate mixtures revealed that whether an elongation or a cyclization reaction occurs is determined by two different aspects: the preorganization of the substrate and the size of the obtained ring. This mechanism was suggested recently based on the comparison of the two known crystal structures of TE domains of linear NRPSs [12].

Previously, Wadhwani and Bu investigated the pre-fold of the linear native gramicidin S decapeptide substrate [25, 26]. They were able to show that even the linear substrate adopts a similar β sheet structure to gramicidin S itself. Encouraged by these results, we propose that the pre-fold of the substrate might be important for the efficiency of the catalytic reaction. The impact of the substrate pre-fold was also shown for the thioesterase excised from the tyrocydine synthetase, whose cyclization potential depends on the pre-fold of the linear substrate that is used [27]. Taking into consideration the 2(2n+1) rule [28] and the reported structures of various ring sizes of gramicidin S [29], we propose that some linear substrates possess a higher β sheet content than others.

The smallest ring formed by the GrsB PCP-TE contains six residues and a stabilized β sheet structure, obtained by the cyclization of the hexapeptide **GS6** and by the dimerization prior to cyclization of the smallest accepted tripeptide, **GS3**. The heptapeptide **GS7** is elongated once, and subsequent cyclization led to a 14-membered ring. The 14-membered ring likewise has a stabilized β sheet structure that is preferred to an unstabilized 7-membered ring. This indicates that the elongation and subsequent cyclization to an energetically favored ring size is preferred by GrsB PCP-TE. Larger peptides containing more than seven amino acids were exclusively cyclized. Although the larger 18-membered ring is believed to have a β sheet structure, this cyclization was not observed when the TE domain was incubated with the nonapeptide **GS9**; instead, a 9-membered ring was exclusively formed. This can be explained by the maximum capacity of the catalytic pocket of the thioesterase. GrsB PCP-TE can sufficiently cyclize linear peptides consisting of 6–15 amino acids, reflecting the high variability, flexibility, and limitation of the binding pocket. For the tetrapeptide **GS4**, an 8-membered (4+4) and 12-membered (4+4+4) ring were observed. These products are not in accordance with the β sheet rule. It becomes clear that β sheet structures are impossible to assemble by monomers of this size. In these cases, size exclusion mediated by the thioesterase led to the formation of the dedicated ring sizes.

Reactions including different sizes of peptide substrates revealed that not only dimerization, but also ring compositions of both incubated substrates, of the dedicated monomers could be detected. These findings open the gate for generating peptide libraries derived from mixtures of small peptides that are randomly assembled to cyclic compounds.

Interestingly, the GrsB PCP-TE can also function as a ligase. This can be achieved by blocking the cyclization reaction with suitable substrate monomers, whose cyclization nucleophile is blocked. Such ligation products are detected by a coincubation of two substrates, one of which is not activated at the C terminus, whereas the other is blocked at the N terminus. Results obtained so far indicated a high degree of substrate tolerance. Therefore, it remains an interesting task to determine the maximum length that can be utilized for this ligation reaction.

In summary, we show that the excised thioesterase of gramicidin S has relaxed substrate specificity and is able to convert substrates of different lengths into the dedicated cycles. Because GrsB TE can catalyze two different reactions, cyclization and ligation, it is possible to obtain cyclic peptide libraries built by ligation and cyclization of linear peptides differing in length and amino acid composition. We believe that GrsB TE, or iterative operating TE domains in general, will become a powerful tool for biocombinatorial engineering of novel cyclic and linear peptidic compounds.

SIGNIFICANCE

Many bioactive compounds, like valinomycin, thiocoraline, and enterobactin, are synthesized on iterative types of NRPSs. In contrast to linear NRPS systems, iterative synthetases use their modules more than once. In these systems, thioesterases were suggested to catalyze a ligation of repetitive peptidyl intermediates prior to a cyclization reaction. Here, we present a comprehensive in vitro characterization of the excised iterative, bimodular PCP-TE of the gramicidin S synthetase GrsB. We show that the TE domain catalyzes the ligation and subsequent cyclization of peptidyl-SNAC and -thiophenols. Detection of linear thioester intermediates supports a mechanism in vivo, in which the ligation product of the two pentapeptides is transferred backward to the PCP domain to be cyclized by the TE domain afterwards (backward reaction). Using different substrates, we were able to define the substrate specificity of this particular iterative thioesterase. Most importantly, GrsB PCP-TE is able to handle peptides of different lengths, leading to direct cyclization or dimerization or even trimerization reactions and the formation of various ring sizes. Encouraged by these observations, we were able to show that this class of thioesterases can be applied to the generation of libraries of cyclic compounds by randomly triggered assembly of monomers out of a pool of small peptides. Additionally, GrsB PCP-TE can be used as a ligation catalyst. Together, our investigations characterize this iterative thioesterase as a useful tool for biocombinatorial approaches that may lead to the synthesis of new bioactive products.

EXPERIMENTAL PROCEDURES

Strains, Culture, Media, and General Methods

Escherichia coli strains were grown in LB medium supplemented, if applicable, to the final concentration of 100 μg/ml ampicillin, 25–50 μg/ml kanamycin. Oligonucleotides were purchased from Qiagen Operon. Standard protocols were applied for all DNA manipulations [30]. Amino acids were purchased from Novabiochem, Bachem Biosciences, and IRIS biotech. All other compounds, except HBTU and HOBt·H_2O (IRIS Biotech), were purchased from Sigma-Aldrich.

Construction of Expression Plasmids

Gene fragments encoding for GrsB PCP-TE (12,262–13,356 bp) and TE (GrsB TE_{Short}, 12,608–13,351 bp; GrsB TE_{Long}, 12,527–13,351 bp) were amplified by PCR from chromosomal DNA of *Bacillus brevis* ATCC 9999 by using Pfu Turbo DNA polymerase (Stratagene) with the following oligonucleotides: GrsB PCP-TE, 5′- CACCGAGTATG TTGCACCAAGGAA-3′ and 5′-TTTTACTACAAATGTCCCTTGTAG-3′; GrsB TE_{Long}, 5′-TTTCCATGGTAAACGAAGCAGATAG-3′ and 5′-ATT GGATCCTACAAATGTCCCTTGTAG-3′; GrsB TE_{Short}, 5′-TTTCCATGG TAAACGAAGAAACAGATCG-3′ and 5′- ATTGGATCCTACAAATGT CCCTTGTAG-3′. The PCR product of GrsB PCP-TE was inserted into the pBAD202/D-TOPO vector (Invitrogen). The PCR product of GrsB TE_{Long} was cloned into the NcoI/BamHI site of pQE60 (Qiagen), and the PCR product of GrsB TE_{Short} was inserted into the NcoI and BamHI sites of pCL11, a pET16b derivative (Novagen) containing the BamHI/HindIII fragment of pQE60 encoding a hexahistidine tag [31]. All inserted fragments were confirmed by sequencing (GATC Biotech).

Expression and Purification of Recombinant Enzymes

E. coli BL21 (Amersham Biosciences) or M15/pREP4 (Qiagen) was transformed with the desired plasmids. Cells were grown to OD 0.6 (600 nm), induced with 0.01% arabinose or 0.1 mM IPTG, and cultivated for an additional 2.5 hr at 25°C/30°C. The expressed proteins were purified by Ni-NTA affinity chromatography (GE Healthcare). Fractions containing the recombinant proteins, identified by SDS-PAGE (12.5%, Supplemental Data), were pooled and dialyzed against assay buffer (25 mM HEPES, 50 mM NaCl [pH 7.5]).

The concentration of the purified proteins was determined spectrophotometrically by using the estimated extinction coefficient at 280 nm. After being flash frozen in liquid nitrogen, the proteins were stored at −80°C.

Synthesis of Linear Thioester Substrates

Peptide synthesis was carried out on an Advanced ChemTech APEX 396 synthesizer (0.1 mmol scale) with 2-chlorotrityl resin (IRIS Biotech) as described previously [32]. Preparation and purification of *N*-acetylcysteamine- and thiophenol-thioester peptides were performed as described earlier [32]. The identity of the thioester substrates was determined by reverse-phase liquid chromatography-mass spectrometry (RP-LCMS, Agilent 1100 MSD) (Table 1). Peptides were solubilized in standard assay buffer to a final concentration of 15 mM and variable amounts of DMSO (<20% v/v).

Enzyme Assays

Standard reactions were conducted in a total volume of 50 μl, at 25°C, in assay buffer (25 mM HEPES, 50 mM NaCl [pH 7.5]) containing a substrate concentration of 300 μM. Reactions were initiated by the addition of 10 μM enzyme and were quenched after 90 min by the addition of 10 μl 4% TFA/H_2O. In order to verify the role of the thioesterase in these reactions, a control without enzyme in which the formation of products was completely abolished was always performed. Assays were analyzed by RP-LCMS (Agilent 1100 MSD) on a Nucleodur 125/3 C_{18} (ec) column (Macherey and Nagel; pore diameter, 100 Å; particle size, 3 μm) utilizing the following gradient: 0–40 min, 5%–60% MeCN/0.1% TFA into H_2O/0.1% TFA, 40–45 min, 60%–95% MeCN/0.1% TFA into H_2O/0.1% TFA, 0.5 ml/min, 40°C; the identities of the products were verified by ESI-MS.

Kinetic characterizations of the dimerization and cyclization reactions were performed by determining initial rates at 5–8 substrate concentrations by using 2 time points at each concentration within the linear region of the reaction. Kinetic parameters of the ligation reaction were determined by using one substrate in excess and by varying the other substrate concentration. The concentrations of all thioester substrates were calculated by using experimentally determined extinction coefficients at a wavelength of 215 nm. Concentrations of products were calculated by comparison with synthesized linear peptides with identical sequences. Assays were analyzed by RP-LCMS (Agilent 1100 MSD) on a Nucleodur 125/2 C_{18} (ec) column (Macherey and Nagel; pore diameter, 100 Å; particle size, 3 μm) with the following gradients: native penta- and decapeptide substrates activated as thioester: 0–35 min, 5%–90% MeCN/0.1% TFA into H_2O/0.1% TFA, 0.4 ml/min, 45°C; ligation of $\mathbf{GS5_{OH}}$ and $\mathbf{GS5_{SNAC}}$: 0–45 min, 5%–90% MeOH/0.1% TFA into H_2O/0.1% TFA, 0.4 ml/min, 45°C.

Supplemental Data

Supplemental Data include SDS-PAGE of GrsB PCP-TE, GrsB TE_{Long}, and GrsB TE_{Short} and MS-MS sequencing of the product derived from $\mathbf{GS5_{SNAC}}$ and are available at http://www.chembiol.com/cgi/content/full/14/1/13/DC1/.

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