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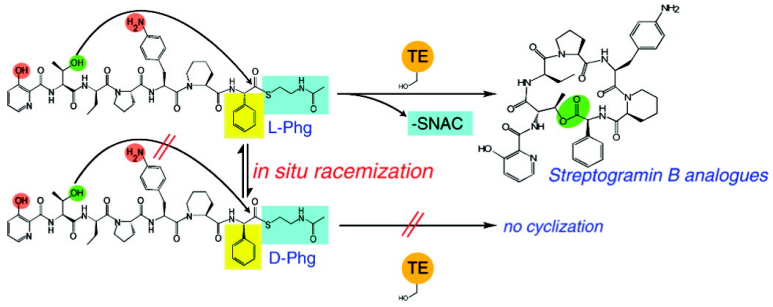

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Chemoenzymatic Approach to Enantiopure Streptogramin B Variants: Characterization of Stereoselective Pristinamycin I Cyclase from *Streptomyces pristinaespiralis*

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Abstract: Streptogramin B antibiotics are cyclic peptide natural products produced by *Streptomyces* species. In combination with the synergistic group A component, they are "last line of defense" antimicrobial agents against multiresistant cocci. The racemization sensitivity of the phenylglycine (Phg_7) ester is a complex challenge in total chemical synthesis of streptogramin B molecules. To provide fast and easy access to novel streptogramin antibiotics, we introduce a novel chemoenzymatic strategy in which diversity is generated by standard solid phase protocols and stereoselectivity by subsequent enzymatic cyclization. For this approach, we cloned, overproduced, and biochemically characterized the recombinant thioesterase domain SnbDE TE of the pristinamycin I nonribosomal peptide synthetase from *Streptomyces pristinaespiralis*. SnbDE TE catalyzes regioselective ring closure of linear peptide thioester analogues of pristinamycin I as well as stereoselective cyclization out of complex in situ racemizing substrate mixtures, enabling synthesis of Streptogramin B variants via a dynamic kinetic resolution assay. A remarkable substrate tolerance was detected for the enzymatic cyclization including all the seven positions of the peptide backbone. Interestingly, SnbDE TE was observed to be the first cyclase from a macrolactone forming NRPS which is additionally able to catalyze macrolactamization of peptide thioester substrates. An *N*-methylated peptide bond between positions 4 and 5 is mandatory for a high substrate turnover. The presented strategy is potent to screen for analogues with improved activity and guides our understanding of structure−activity relationships in the important class of streptogramin antibiotics.

Introduction

A large class of natural products with a remarkable structural diversity is produced by nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), and hybrid NRPS/PKS systems. Prominent examples are the antibiotics daptomycin and erythromycin, or the anticancer agent epothilone.[1] The macrocyclic structure of many of these compounds enhances the rigidity of their ring skeleton leading to stabilization of their bioactive conformation and decreases sensitivity against proteolytic digest.[2]

An important subclass are the cyclic streptogramin antibiotics, which always exist as two structurally unrelated compounds, the streptogramin A (S_A) and the streptogramin B (S_B) group molecules, both composed of macrocyclic lactones[3] (Figure 1).

S_A and S_B synergistically inhibit bacterial protein synthesis by binding to different sites of the 50S subunit of the prokaryotic ribosom.[4] Recently, chemical modification of the streptogramin components Pristinamycin II_A (PII_A) and Pristinamycin I_A (PI_A) lead to the development of Synercid, a 30:70 combination of dalfopristin (S_A) and quinupristin (S_B) (Figure 1).

Synercid in contrast to the natural streptogramins shows a high water solubility and is therefore the first injectable streptogramin antibiotic. It is of great pharmacological and clinical importance due to its efficacy against multiresistant Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) strains.[5] The crystal structure of the 50S ribosomal subunit of *Deinococcus radiodurans* in complex with quinupristin and dalfopristin was very recently solved and gives new insights into their unique inhibitory mechanism.[6]

The biosynthesis of the S_B antibiotic PI_A is catalyzed by an NRPS system composed of three proteins, SnbA (61.4kDa), SnbB (276.1 kDa), and SnbDE (522.1 kDa).[7−9] These three

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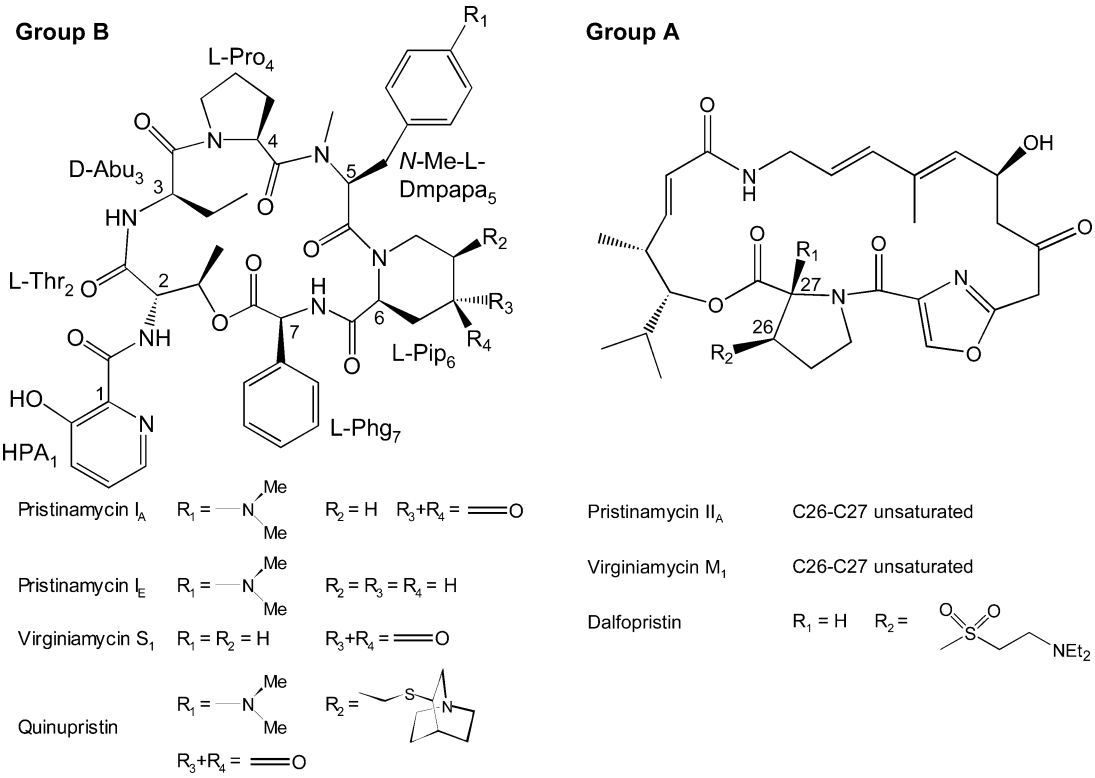

Figure 1. Structures of important streptogramin antibiotics. Group A and group B molecules act synergistically. The group B antibiotic Pristinamycin I_E is the molecule produced by the herein investigated NRPS system. PI_E is the precursor of PI_A, the main streptogramin B component produced by *Streptomyces pristinaespiralis*.

subunits are further subdivided into seven modules, which are responsible for the incorporation of seven building blocks into the PI peptide backbone. The final linear peptide precursor is cyclized by a regioselective nucleophilic attack of L-Thr_2 onto the C-terminal carboxyl group of L-phenylglycine$_7$ (Phg) under release from the synthetase. The action of the three synthetases yields the PI_A precursor PI_E (Figure 1). The tailoring oxidation reaction of L-pipecolic acid (L-Pip) (PI_E) to 4-oxo-L-Pip (PI_A) is performed postsynthetically putatively under participation of the protein SnbF.[7] An interesting feature is the presence of an *N*-methylated peptide bond between L-Pro_4 and 4-(dimethylamino)-phenylalanine$_5$ (L-$Dmpapa_5$). This *N*-methylation is carried out by an *N*-methyl transferase domain of the L-Dmpapa incorporating module of SnbDE.[9]

Various chemical modifications of natural S_B compounds have been reported and reviewed by Barriere et al.[3] All of these modifications must preserve the lactone function, which is essential for bioactivity. This structural feature complicates the synthesis of novel variants as chemical methods are mostly inefficient for regio- and stereospecific cyclization of peptides.[10] There are several additional problems which have to be addressed in the total chemical synthesis of S_B molecules: Phg esters are highly sensitive to both epimerization and elimination, and peptides containing a homochiral amino acid triad are sensitive to strong acids.[11,12] Recently, the first solid-phase synthesis of the S_B-antibiotic Dihydrovirginiamycin S_1 was published.[13] In this publication, a rather complex strategy circumvents the noted problems by an optimized set of protection groups and deprotection and coupling conditions.

Naturally, the complexity of macrocyclization of natural products produced by NRPS, such as tyrocidine, surfactin, S_B or CDA, is solved by catalysis of thioesterase domains (TE, cyclase). These 28−35 kDa cyclases catalyze the macrocyclization of linear precursors bound as thioesters to the most downstream peptidyl carrier protein (PCP) under release from the synthetase.[14] The mechanism of TE catalysis involves transfer of the peptide chain from the PCP bound 4′-phosphopantetheine (ppant) to the TE active site serine residue, followed by deacylation of the resulting peptidyl-*O*-TE intermediate.[15]

It was shown that TE domains excised from NRPS assembly lines retain autonomous catalytic cyclization activity with artificial linear peptide thioester substrates.[16−19] One described possible leaving group is *N*-acetyl-cysteamin (SNAC), which mimics the last part of the ppant-cofactor. Alterations in the primary sequence of soluble substrates identified TE domains as permissive cyclization catalysts tolerating variations in the identity of most individual side chains. They have been shown to be versatile tools for the discovery of new bioactive compounds.[19,20] Recently, a chemoenzymatic synthesis of chimeric S_B-tyrocidine antibiotics using the recombinant tyro-

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cidine thioesterase (TycC TE) was developed to prepare bioactive compounds that overcome S_B resistance.[21] However, the substrate tolerance of the TycC TE restricts the variability and the S_B-similarity of the compounds produced by this strategy.

Here we report a detailed biochemical characterization of the TE domain of PI synthetase SnbDE of the actinomycete *Streptomyces pristinaespiralis* (SnbDE TE) and its ability to generate variants of PI chemoenzymaticly by cyclization of soluble peptide thioester substrates with remarkable regio- and stereoselectivity. Substrates with the natural Phg at the C-terminal position undergo an in situ racemization under the assay conditions, but only the natural L-Phg diastereomers are accepted by the cyclase for the cyclization reaction. This dynamic kinetic resolution[22] simplifies S_B synthesis to standard peptide chemistry and subsequent enzymatic reaction. Furthermore, the cyclase shows a broad tolerance for variations of the peptide backbone. Therefore, the presented chemoenzymatic approach gives rise to be suitable for the synthesis of novel analogues of this clinically important antibiotic with improved biological activities.

Experimental Section

Cloning and Expression of the SnbDE TE Domain. The *snbDE te* gene fragment was amplified by PCR from chromosomal DNA of *Streptomyces pristinaespiralis* ATCC 25486 using Pfu Turbo DNA polymerase (Stratagene). Amplification of *snbDE te* (containing SnbDE residues 13 729−14 544[9]) was carried out using the oligonucleotides 5′-C ACC GGC GCC GAC ACG GGC and 5′-GTG GCC GGC CTG CGG. The blunt-end PCR product was cloned into pBAD202/D-TOPO vector (Invitrogen) using the pBAD directional TOPO expression kit (Invitrogen). This vector appends a C-terminal His_6 tag and an N-terminal His-patch (HP) thioredoxin leader to the expressed protein. The cloning product was confirmed by GATC Biotech by sequencing with an ABI prism 310 genetic analyzer (Applied Biosystems). For expression, the recombinant plasmid was transformed into *Escherichia coli* BL21 (Amersham Biosciences). The transformed cells were grown to OD = 0.5 (600 nm), induced with 0.01% arabinose, and again grown at 25 °C for 2.5 h. The expressed protein was purified by Ni−NTA affinity chromatography (Amersham Pharmacia Biotech). Dialysis into 25 mM Hepes and 50 mM NaCl, pH 7.0, was carried out using HiTrap Desalting columns (Amersham Pharmacia Biotech). The concentration of the purified protein was determined spectrophotometrically using the estimated extinction coefficient at 280 nm. After being flash frozen in liquid nitrogen, the protein was stored at −80 °C over several months.

Synthesis of Peptide Thioester Substrates. Peptide synthesis was carried out on an Advanced ChemTech APEX 396 synthesizer (0.1 mmol scale) by using 2-chlorotrityl resin (IRIS biotech) as described earlier.[23] All *N*-(9-fluoromethoxycarbonyl)-amino acids and unprotected amino acids were purchased from Novabiochem, Bachem Biosciences and IRIS biotech. All other compounds were purchased from Sigma-Aldrich, except HBTU and HOBt*H_2O (IRIS biotech). The preparation and purification of peptide-SNAC substrates were described previously.[24] Coupling of *N*-methylated amino acids was carried out using a racemization free triphosgene coupling method described elsewhere.[25] Thr_2 was incorporated without a side chain protecting group in peptides bearing an acid sensitive N-alkyl amino acid triade to circumvent the acid deprotection step. Cleavage from the resin of such peptides was performed by treatment of the peptide resin with a 1:5 mixture of 1,1,1,3,3,3-hexafluor-2-propanol/CH_2Cl_2 for 15 min.[25] The identities of peptide-SNAC substrates were determined by reversed-phase liquid chromatography−mass spectroscopy (RP-LCMS) (Table 1).

Table 1. Characterization of Substrates and Products by ESI-MS

		observed mass (calculated mass) (Da)[a]		
compd[b]	species	substrate	cyclized product	hydrolyzed product
1	$[M + H]^+$	930.4 (930.4)	811.3 (811.4)	829.3 (829.4)
2	$[M + H]^+$	944.4 (944.4)	825.4 (825.4)	843.4 (843.4)
3	$[M + H]^+$	898.3 (898.4)		797.3 (797.4)
4	$[M + H]^+$	899.3 (899.4)		798.3 (798.4)
5	$[M + H]^+$	944.4 (944.4)	825.4 (825.4)	843.4 (843.4)
6	$[M + H]^+$	944.4 (944.4)	825.4 (825.4)	843.4 (843.4)
7	$[M + H]^+$	944.4 (944.4)	825.4 (825.4)	843.4 (843.4)
8	$[M + H]^+$	936.3 (936.5)		835.3 (835.4)
9	$[M + H]^+$	930.3 (930.4)	811.3 (811.4)	829.3 (829.4)
10	$[M + H]^+$	918.3 (918.4)	799.3 (799.4)	817.3 (817.4)
11	$[M + H]^+$	853.3 (853.4)	734.3 (734.3)	752.3 (752.4)
12	$[M + H]^+$	904.3 (904.4)	785.3 (785.4)	803.3 (803.4)
13	$[M + H]^+$	868.3 (868.4)	749.3 (749.4)	767.3 (767.4)
14	$[M + H]^+$	928.3 (928.4)	809.3 (809.4)	827.3 (827.4)
15	$[M + H]^+$	943.3 (943.4)	824.3 (824.4)	842.3 (842.4)
16	$[M + H]^+$	927.3 (927.4)	808.3 (808.4)	826.3 (826.4)
17	$[M + H]^+$	1015.5 (1015.5)	896.3 (896.4)	914.4 (914.4)
18	$[M + H]^+$	859.3 (859.4)	740.3 (740.3)	758.3 (758.4)
19	$[M + H]^+$	930.3 (930.4)	811.3 (811.4)	829.3 (829.4)
20	$[M + H]^+$	914.3 (914.4)	795.3 (795.4)	813.3 (813.4)
21	$[M + H]^+$	972.5 (972.5)	853.4 (853.4)	871.4 (871.4)
22	$[M + Na]^+$	965.5 (965.4)	846.3 (846.4)	864.3 (864.4)
23	$[M + H]^+$	929.3 (929.4)	810.4 (810.4)	828.4 (828.4)

[a] Ionization method: ESI. [b] For substrate structures see Table 2.

Synthesis of Fmoc-*N*-methyl-4-(dimethylamino)-L-phenylalanine. To a mixture of Fmoc-*N*-methyl-4-(amino)-L-phenylalanine (928.8 mg, 2.30 mmol), 37% aqueous formaldehyde (632 μL, 23.00 mmol), and acetic acid (1.28 mL, 2.3 mmol) in 15 mL acetonitrile was added sodium cyanoborhydride (222.91 mg, 3.69 mmol) at room temperature. After being stirred overnight, the mixture was poured into water and acidified to pH 2 with concentrated hydrochloric acid. The product was extracted with dichloromethane and washed with 0.1 N HCl and water. After drying over anhydrous sodium sulfate, the combined organic phases were evaporated. The product was purified by column chromatography (dichloromethane/methanol 12/1) to give Fmoc-*N*-methyl-4-(dimethylamino)-L-phenylalanine (878,9 mg, 2.02 mmol, 88% yield).

^{1}H NMR: δ_H (300 MHz, $CDCl_3$) 2.96−3.25 [m, 2 H, Ph-CH_2], 3.08 [s, 6 H, NMe_2], 4.16−4.70 [4 H, m, C**H**C**H$_2$**O, C**H**CH_2Ph], 5.40 [d, 1 H, J = 7.3 Hz, NH], 6.90−7.60 [m, 10 H, H aryl], 7.77 [d, 2 H, J = 7.6 Hz, H aryl (Fmoc)]. ESI-MS: m/z = 431.1 $[M + H]^+$ (431.2 calc.)

Assays of Peptide-SNAC Substrates. Enzymatic reactions were carried out in 25 mM Hepes and 50 mM NaCl, pH 7.0, at 25 °C in a total volume of 50 μL. Dissolution of **21**, **22**, and **23** was facilitated by the addition of 5% DMSO (v/v). In standard reactions the substrate concentration was 250 μM and the enzyme concentration 5 μM. For kinetic investigations the substrate concentration was varied in the range 25 μM−3 mM. Reactions were initiated by addition of enzyme to a final concentration of 10 μM in these studies. Reactions were quenched by addition of 35 μL of 4% trifluoroacetic acid (TFA)/H_2O. All assays were analyzed by RP-LCMS on a C_{18} Nucleodur column (Macherey and Nagel, 250/3, pore diameter 100 Å, particle size 3 μM) with the following gradients: **1**−**21**, 0−40 min, 10−60% acetonitrile/0.1% TFA in water/0.1% TFA, 0.3 mL/min, 40 °C; **22**−**23**, 0−40 min, 35−95% acetonitrile/0.1% TFA in water/0.1% TFA, 0.3 mL/min, 40 °C.

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Table 2. Cyclization to Hydrolysis Ratios and Kinetic Parameters of SnbDE Cyclase Mediated Product Formation Compared to the Reference Substrate Phe_7-PLP-SNAC (**2**)[a]

Compound Number	Compound Name	Peptide Sequence	C/H ratio	kinetic parameters for total substrate turnover (sum of cyclization and hydrolysis)	
				k_{cat}/K_M ($mM^{-1}min^{-1}$)	K_M (mM)
1	**Phg_7-PLP-SNAC (most natural substrate)**	HPA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phg_7-SNAC	2.0	2.70 ± 0.17	n.d.
2	**Phe_7-PLP-SNAC (racemization stable reference substrate)**	HPA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	1.0	0.54 ± 0.10	2.70 ± 0.35
3	**PA_1-Ala_2-Phe_7-PLP-SNAC**	PA_1-Ala_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0	n.d.	n.d.
4	**Ala_2-Phe_5-Phe_7-PLP-SNAC**	HPA_1-Ala_2-D-Abu_3-Pro_4-Phe_5-Pip_6-Phe_7-SNAC	0	n.d.	n.d.
5	**D-Thr_2-Phe_7-PLP-SNAC**	HPA_1-D-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0	n.d.	n.d.
6	**L-allo-Thr_2-Phe_7-PLP-SNAC**	HPA_1-L-allo-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0	n.d.	n.d.
7	**D-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-D-Phe_7-SNAC	0	n.d.	n.d.
8	**Ac-Ala_1-Phe_7-PLP-SNAC**	Ac-Ala_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0	n.d.	n.d.
9	**D-Ala_3-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Ala_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0.6	0.40 ± 0.02	3.31 ± 0.31
10	**Ala_4-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Ala_4-$Papa_5$-Pip_6-Phe_7-SNAC	1.7	0.51 ± 0.05	1.68 ± 0.30
11	**Ala_5-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-Ala_5-Pip_6-Phe_7-SNAC	0.9	1.15 ± 0.10	1.25 ± 0.22
12	**Ala_6-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Ala_6-Phe_7-SNAC	1.2	0.16 ± 0.01	4.10 ± 0.45
13	**Ala_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Ala_7-SNAC	1.7	0.15 ± 0.01	3.63 ± 0.33
14	**PA_1-Phe_7-PLP-SNAC**	PA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0.5	0.58 ± 0.06	2.57 ± 0.45
15	**HBA_1-Phe_7-PLP-SNAC**	HBA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0.4	n.d.	n.d.
16	**BA_1-Phe_7-PLP-SNAC**	BA_1-Thr_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	0.4	n.d.	n.d.
17	**In-Ala_4-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Ala_4-Pro_5-$Papa_6$-Pip_7-Phe_8-SNAC	0.4	n.d.	n.d.
18	**Del-D-Abu_3-Phe_7-PLP-SNAC**	HPA_1-Thr_2-Pro_3-$Papa_4$-Pip_5-Phe_6-SNAC	0.5	n.d.	n.d.
19	**L-Ser_2-Phe_7-PLP-SNAC**	HPA_1-Ser_2-D-Abu_3-Pro_4-$Papa_5$-Pip_6-Phe_7-SNAC	5.8	0.75 ± 0.06	2.66 ± 0.35
20	**L-Dap_2-Phe_5-Phe_7-PLP-SNAC**	HPA_1-Dap_2-D-Abu_3-Pro_4-Phe_5-Pip_6-Phe_7-SNAC	1.5	n.d.	n.d.
21	**L-$Dmpapa_5$-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-$Dmpapa_5$-Pip_6-Phe_7-SNAC	0.7	0.88 ± 0.04	n.d.
22	***N*-Me-Phe_5-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-*N*-Me-Phe_5-Pip_6-Phe_7-SNAC	0.9	3.83 ± 0.25	n.d.
23	**Phe_5-Phe_7-PLP-SNAC**	HPA_1-Thr_2-D-Abu_3-Pro_4-Phe_5-Pip_6-Phe_7-SNAC	2.0	0.92 ± 0.05	n.d.

BA, benzoic acid; Dap, 2,3-diaminopropionic acid; Del, deletion; Dmpapa, 4-(dimethylamino)-phenylalanine; HBA, hydroxybenzoic acid; HPA, 3-hydroxypicolinic acid; In, insertion; PA, picolinic acid; Papa, 4-amino-phenylalanine

[a] The reference sequence and deviations from this sequence in all other substrates are highlighted by shading.

The identities of the products were verified by ESI-MS (Table 2). Concentrations of various peptide thioesters were calculated using experimentally determined extinction coefficients at a wavelength of 215 nm. The extinction coefficients of peptide SNAC substrates were assumed to be identical to the corresponding cyclized and hydrolyzed products. Kinetic characterization of the cyclization and hydrolysis reactions was performed by determining initial rates at five to eight substrate concentrations using two time points at each concentration within the linear region of the reaction, which was verified by time courses. Substrates **21**−**23** show low water solubility and were dissolved in DMSO after the purification resulting in a DMSO concentration of 5% in standard assays. DMSO inhibits the enzymatic reaction at a concentration higher than 20%. Therefore, a separate determination of k_{cat} and K_M was hindered for theses two substrates.

Results

Construction of the SnbDE TE-Thioredoxin Fusion. Due to the high GC content (75%) of the SnbDE TE DNA template, cloning and expression of SnbDE TE were employed using the pBAD202/D-TOPO vector (Invitrogen). This expression system attaches a His-patch thioredoxin domain (11.7 kDa) to the N-terminus of the expressed protein. With this strategy a soluble recombinant SnbDE TE (46.1 kDa) was obtained.

SnbDE TE is able to catalyze macrolactonization of linear streptogramin B analogues. To examine the ability of SnbDE TE to cyclize streptogramin B thioester substrates, we synthesized the linear peptide thioester analogue of PI_E **Phg_7-PLP-SNAC** (**1**), whose amino acid sequence represents the peptide backbone of unoxidized streptogramin B (L-Pip instead of 4-oxo-L-Pip at position 6) (Table 2). For synthetic reasons, *N*-methyl-$Dmpapa_5$ was substituted by 4-amino-phenylalanine$_5$ ($Papa_5$). During the synthesis of the **Phg_7-PLP** peptide, problems with the integrity of the stereocenter of the C-terminal L-Phg occurred, caused by a high base sensitivity of this amino acid. HPLC-MS analysis of the peptides after cleavage from the resin showed two different epimers in a ratio of 1:1. In contrast, analogues containing L-Phe at position 7 instead of Phg showed only one substrate peak clearly demonstrating that only the Phg-residue shows loss of chiral integrity during the synthesis. Nevertheless, a 1:1 mixture containing both epimers was used for the synthesis of **Phg_7-PLP-SNAC** (**1**). All attempts to quantitatively separate the resulting two diastereomers of **1** by HPLC failed. Only an enrichment of each particular epimer could be obtained due to a very high racemization sensitivity of **Phg_7-PLP-SNAC** even under neutral buffer conditions. The stereocenter of Phg_7 racemizes completely within a few hours at room temperature (data not shown). Therefore, in all enzymatic assays mixtures of both L- and D-**Phg_7-PLP-SNAC** diastereomers in various ratios were used as substrates. Assaying for the SnbDE TE-mediated cyclization of the substrate mixture and analysis by HPLC-MS showed two hydrolyzed linear peptide products as expected but only one cyclization product (Figure 2A).

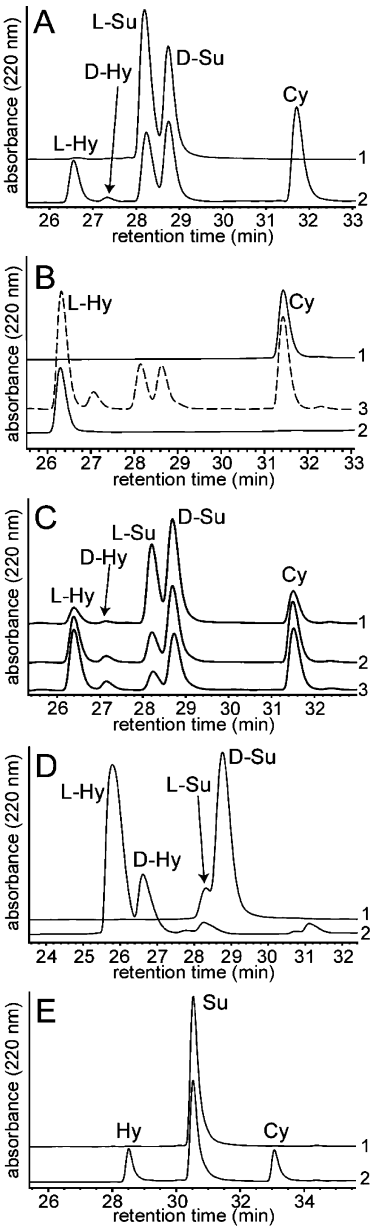

Figure 2. Reaction profile of SnbDE cyclase mediated substrate turnover of racemization sensitive **Phg$_7$-PLP-SNAC** (**1**) and the racemization resistant reference substrate **Phe$_7$-PLP-SNAC** (**2**). (A) HPLC trace of SnbDE cyclase incubated with a diastereomer mixture of **1** for 3 h at 25 °C (trace 2). Trace 1 shows substrate incubation in the absence of the enzyme. (B) Enzymatic reopening of isolated cycle. Trace 1: Isolated cyclic product of **1** before enzymatic incubation. Trace 2: Analysis of isolated cycle after 24 h incubation with SnbDE cyclase at 25 °C. Fresh enzyme was added at regular intervals after 4 h. Trace 3 (dashed line): HPLC trace of a diastereomer mixture of **1** incubated with SnbDE cyclase for comparison of retention times. (C) Time-resolved HPLC trace of SnbDE cyclase incubated with equal amounts of each diastereomer of **1** at beginning of the reaction at 25 °C. After 2 h fresh enzyme was added to the mixture to a final concentration of 500 μM. Trace 1: 34 min. Trace 2: 175 min. Trace 3: 265 min. (D) HPLC analysis of an assay containing mainly the unnatural D-**Phg$_7$-PLP-SNAC** at the beginning of the reaction. Trace 1: substrate mixture at the begin of the reaction. Trace 2: analysis after 24 h incubation with SnbDE cyclase at 25 °C. Fresh enzyme was added every 4 h. (E) HPLC trace of SnbDE cyclase incubated with **2** for 3 h at 25 °C (trace 2). Trace 1 shows incubation of **2** without enzyme. Su = substrate, Cy = cyclized product, Hy = hydrolyzed product.

In contrast to the substrates, these products showed no racemization sensitivity under assay conditions. To investigate this result in more detail, we tested the ability of SnbDE TE to cyclize substrates with racemization insensitive L- and D-amino acids at position 7. Therefore, Phe was used instead of Phg. The results gained with these well-defined substrates L-**Phe$_7$-PLP-SNAC** (**2**) and D-**Phe$_7$-PLP-SNAC** (**7**) demonstrated that SnbDE cyclase exhibits a high stereoselectivity for the C-terminal amino acid: only the natural L-form is accepted in this position for the cyclization reaction, whereas the D-form is hydrolyzed very slowly (see below). Obviously, because of this high stereoselectivity of the cyclase, the one cyclization peak (t_R = 31.6 min) detected in enzymatic assays with substrate **1** represents the natural L-Phg containing macrolactone, whereas the D-form shows only hydrolysis. To confirm that this cyclic product exclusively consists of one diastereomer, the cyclic product was isolated by HPLC, lyophilized, and incubated again with SnbDE TE in assay buffer. If the cyclase is able to reopen the macrolactone as reported previously for other TEs,[18,26] the resulting linear products could provide information about the identity of the macrocycle. Incubation for 24 h resulted in quantitative hydrolysis of the cycle. Only the left hydrolysis peak (t_R = 26.43 min) was detected in the HPLC analysis (Figure 2B), clearly demonstrating the optical purity of the enzymatically built macrocycle. Furthermore, this result gives strong evidence that this hydrolysis product is the natural L-Phg containing hydrolysate. This observation also corresponds to the faster increase of this hydrolysis peak (t_R = 26.43 min) compared to the right one (t_R = 27.18 min) when a substrate diastereomer mixture of equal initial concentrations was used (Figure 2C). Obviously, also in the case of hydrolysis, the L-Phg containing substrate is favored. Moreover, time-resolved assays with **1** showed that the left substrate diastereomer peak (t_R = 28.26 min) is transformed into the products faster than the right substrate diastereomer peak (t_R = 28.74 min) (Figure 2C). Therefore, most likely the left substrate peak corresponds to the natural L-Phg-diastereomer, which is either cyclized to the macrolactone or hydrolyzed, whereas the right one represents the unnatural substrate containing D-Phg, which is slowly hydrolyzed exclusively. The total product formation with the natural L-Phg substrate (sum of cyclization and hydrolysis) was 30-fold faster than product formation (hydrolysis) with the unnatural D-Phg-substrate at equal substrate concentrations. The cyclic as well as the L- and D-hydrolyis products were formed in a ratio of 2:1:0.1.

Most interestingly, the decrease of the unnatural substrate in enzymatic assays is more significant than the increase of the unnatural hydrolysis product. Furthermore, the increase of the cycle and the natural hydrolysis product exceeds the decrease of the L-Phg substrate (Figure 2C). This observation demonstrates a preparative useful reaction pathway. The D-Phg substrate is converted via in situ racemization to the L-Phg substrate and vice versa. Selective subsequent enzymatic transformation of L-**Phg$_7$-PLP-SNAC** continuously shifts the equilibrium of the racemization. This dynamic kinetic resolution enables the yield of natural configured products higher than 50% out of a 1:1 mixture of substrate diastereomers. This reaction pathway was further proven by an assay with a substrate mixture containing mainly the unnatural D-**Phg$_7$-PLP-SNAC**: Complete enzymatic reaction including reopening of the formed cycle

(26) Tseng, C. C.; Bruner, S. D.; Kohli, R. M.; Marahiel, M. A.; Walsh, C. T.; Sieber, S. A. *Biochemistry* **2002**, *41*, 13350−13359.

converted this substrate mixture into the two hydrolysis products with 77% L-Phg and 23% D-Phg configuration (Figure 2D).

Phe$_7$-PLP-SNAC - A Simplified Substrate for Substrate Specificity Scans. The D-**Phg$_7$-PLP-SNAC**, which is present in all substrate mixtures of **1**, presumably inhibits the enzymatic catalyzed transformation of the L-Phg diastereomer. Therefore, the investigations of the substrate specificity of the SnbDE cyclase and the separate determination of the kinetic parameters k_{cat} and K_M of the enzymatic reaction are hindered for all substrates with C-terminal Phg. For this reason, the racemization stable **Phe$_7$-PLP-SNAC** (**2**) was utilized as a template for substrate specificity scans and as a reference for kinetic investigations (Table 2). **2** was successfully cyclized to a streptogramin B analogue by the cyclase with a cyclization-to-hydrolysis ratio of 1:1 (Figure 2E). To enable a comparison of the kinetic properties of the natural substrate **1** and the simplified reference substrate **2** in the enzymatic reaction, the initial formation rates of the cyclic and hydrolysis products starting from **2** and an enriched diastereomer mixture of **1** (L-**1**/D-**1** ≈ 95/1 by analytical HPLC), respectively, were determined. The k_{cat}/K_M values of both substrates were extrapolated from initial gradients of Michaelis−Menten plots. A 5-fold higher k_{cat}/K_M was observed for the substrate **1** with the natural C-terminus (Table 2). Nevertheless, for the mentioned experimental reasons, all the following investigations were carried out with the sequence of the simplified racemization insensitive substrate **Phe$_7$-PLP-SNAC** (**2**) as reference.

Regioselectivity of SnbDE Cyclase. The reference substrate **Phe$_7$-PLP-SNAC** (**2**) contains three competing nucleophiles: the hydroxyl functions of 3-hydroxypicolinic acid$_1$ (HPA$_1$) and L-Thr$_2$ as well as the amino group of L-Papa$_5$, which is methylated in the natural peptide sequence (Table 2). To determine the regioselectivity of the enzymatic cyclization, we synthesized the two additional SNAC substrates **picolinic acid$_1$-(PA$_1$)-Ala$_2$-Phe$_7$-PLP-SNAC** (**3**) and **Ala$_2$-Phe$_5$-Phe$_7$-PLP-SNAC** (**4**). In both compounds, L-Thr$_2$, the cyclization nucleophile in natural pristinamycin,[27] was replaced by L-Ala to prevent this favored cyclization. Compound **3** contains only the amino group of L-Papa$_5$ and compound **4** only the hydroxyl group of HPA$_1$ as nucleophile. Enzymatic assays of both substrates showed only hydrolysis but no detectable cyclization demonstrating high selectivity of the cyclase for the natural nucleophile.

Stereoselectivity of SnbDE Cyclase. Two additional substrates were synthesized in order to investigate the stereoselectivity of SnbDE cyclase for the cyclization nucleophile L-Thr$_2$. In substrate D-**Thr$_2$-Phe$_7$-PLP-SNAC** (**5**), the natural L-Thr$_2$ was replaced by D-Thr$_2$. Enzymatic assays of this substrate revealed no cyclization product. Only hydrolysis could be observed, indicating the importance of L-configured threonine in position 2 for cyclization (Figure 3A). To investigate the role of the relation between the two stereocenters of L-Thr for cyclization, we utilized **6**, which contains L-allo-Thr at position 2. We detected no cyclization at all in this assay but a large amount of hydrolysis (Figure 3B). These results emphasize a high stereoselectivity of SnbDE TE for both stereocenters of L-Thr$_2$. To determine the stereoselectivity for the C-terminal amino acid, we synthesized the substrate **7** in which L-Phe$_7$ was replaced by D-Phe$_7$. No remarkable enzymatic substrate turnover

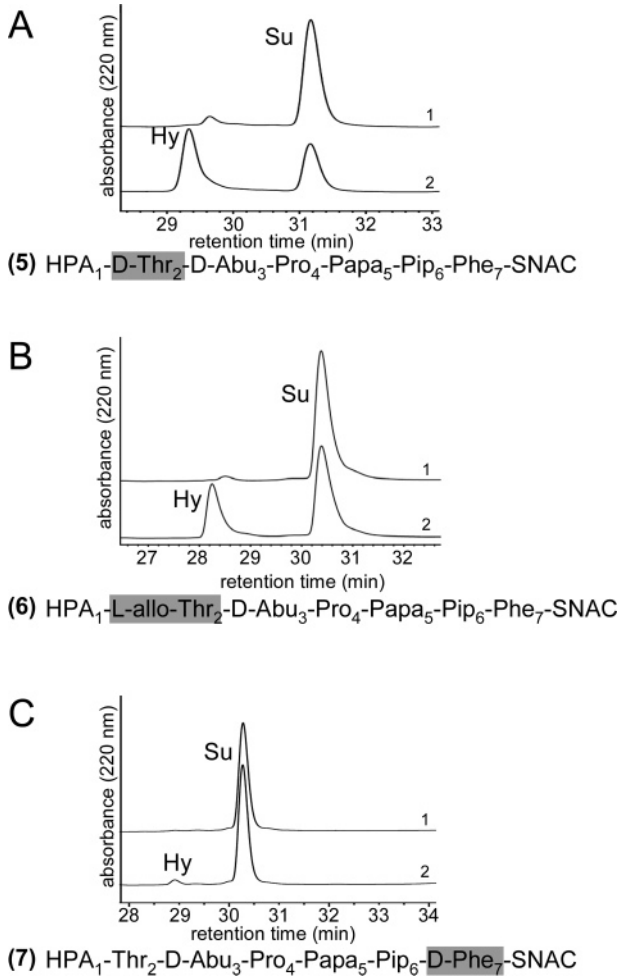

Figure 3. Probing the stereoselectivity of SnbDE cyclase mediated macrolactonization. HPLC traces of SnbDE cyclase incubated with peptide-SNAC substrates **5**, **6**, and **7** (A−C) (traces 2) for 3 h at 25 °C. Traces 1 show incubations in absence of the enzyme. Changed stereochemistry compared to the structure of the reference substrate **2** is highlighted by shading.

was detected; only a very small amount of hydrolysis was observed (Figure 3C). In summary SnbDE TE is shown to be a cyclase with a distinct stereoselectivity for the cyclization nucleophile L-Thr$_2$ and the C-terminal L-Phg$_7$ or L-Phe$_7$, respectively.

Substrate Specificity of SnbDE Cyclase. To further investigate the substrate tolerance of SnbDE cyclase, we synthesized a series of peptide SNAC variants that differ from the reference substrate **Phe$_7$-PLP-SNAC** (**2**) sequence by single or double amino acid substitutions. Specifically, we substituted residue 1 by Ac-L-Ala and the residues 3−7 by Ala without a change of α-C-atom stereochemistry, generating substrates **8**−**13**. Cyclization-to-hydrolysis ratios were determined in enzymatic assays (Table 2). Substrate **8 Ac-Ala$_1$-Phe$_7$-PLP-SNAC** yielded no cyclization, indicating that position 1 is relevant for cyclization efficiency. A substitution of D-Abu$_3$ (Abu, aminobutyric acid) by D-Ala$_3$ resulting in substrate **9** lead to a decrease of cyclization-to-hydrolysis ratio from 1 (reference substrate **2**) to 0.6. Separate exchange of positions 4, 5, 6, and 7 was shown to be well tolerated by SnbDE TE resulting in comparable or improved cyclization-to-hydrolysis ratios (substrates **10**−**13**). In summary, the presented Ala-Scan identifies the residue HPA$_1$ to be invariant and D-Abu$_3$ to be important for the cyclization-

(27) Preud'homme, J.; Tarridec, P.; Belloc, A. *Bull. Soc. Chim. Fr.* **1968**, *2*, 585−591.

to-hydrolysis ratio. Both amino acids enclose the nucleophile L-Thr_2 and therefore seem to be important for the positioning of the nucleophile. The only invariant position is position 1. Exchanges of all other residues are tolerated by the enzyme.

To investigate the observed invariability of position 1 in more detail, we assayed the substrates **14**−**16**, in which HPA_1 of the reference substrate **2** was replaced by picolinic acid (PA), 2-hydroxybenzoic acid (HBA), or benzoic acid (BA), respectively. These three substrates allow us to evaluate the influence of the hydroxyl function and the N-atom of HPA_1 on the cyclization reaction. With these three substrates, the cyclization-to-hydrolysis ratio dropped from 1:1 (reference substrate **2**) to 1:2 indicating the relevance of all functional groups of HPA for cyclization efficiency (Table 2). However, neither of these functionalities seems to be essential for the cyclization reaction. Therefore, SnbDE shows a certain tolerance in the details of the structure of position 1 but no tolerance for replacements by building blocks with deviant structures.

To test the substrate tolerance of SnbDE TE with regard to the formation of macrolactones of various ring sizes, substrates **17** and **18** were synthesized and assayed with the cyclase. In substrate **17** an additional L-Ala was inserted between the positions 3 and 4 in comparison to the reference substrate **2** sequence, and in substrate **18** D-Abu_3 was deleted, respectively. With both substrates the formation of cyclic products was observed with a cyclization-to-hydrolysis ratio of 1:2 showing a certain tolerance for variations in the distance between the cyclization nucleophile and the C-terminal amino acid (Table 2).

Further investigations concerning the cyclization nucleophile were undertaken to determine the tolerance of SnbDE TE for variations in this important position. In substrate **19**, L-Thr_2 was replaced by the smaller L-Ser, which lacks the β-methyl group. Interestingly, a 6-fold increase of the cyclization-to-hydrolysis ratio in comparison to the reference substrate **2** was observed with this substrate, presumably caused by the lower steric hindrance of serine in comparison to threonine. Substrate **20** contains an L-diamino propionic acid (Dap) instead of Thr at position 2 and a Phe in position 5 instead of Papa. Dap differs from L-Ser by replacement of the hydroxyl function with an amino group. The additional replacement of $Papa_5$ by Phe_5 in this sequence was undertaken after the observation that substrates containing Phe_5 are cyclized more efficiently than peptides containg $Papa_5$ (Table 2, substrate 23). Cyclization and hydrolysis were observed with this substrate in a ratio of 3:2 with a comparable kinetic efficiency to reference substrate **2**, clearly demonstrating that SnbDE TE is able to catalyze also macrolactamization of peptide thioester substrates.

To qualify the substrate tolerance of SnbDE in more detail, the kinetic parameters of the enzymatic reactions of the reference substrate **2**, the Ala-Scan (substrates **9**−**13**), PA_1-Phe_7-PLP-SNAC (**14**) and Ser_2-Phe_7-PLP-SNAC (**19**) were determined (Table 2). Obtained K_M values range from 1.25 to 4.1 mM. Substrates **12** and **13**, in which residues 6 and 7 are individually replaced by L-Ala, respectively, are processed 3.6-fold more slowly than the reference substrate **2**. This indicates that both C-terminal residues have an influence onto peptidyl-*O*-TE intermediate formation. All other tested substrates with deviations from the reference sequence show kinetic efficiencies comparable or higher than the parameters of substrate **2**.

Influence of the *N*-Methylations at Position 5. Interestingly, the substrate **Ala_5-Phe_7-PLP-SNAC** shows a 2-fold increased k_{cat}/K_M value of total product formation and a similar cyclization-to-hydrolysis ratio in comparison to the reference substrate **2**. The reference substrate **2** differs at this position from the natural peptide sequence of PI in the methylation grade of the para amino group of $Papa_5$ and the peptide bond between positions 4 and 5 for synthetic reasons. Possibly, these deviations from the natural substrate structure negatively influence the substrate turnover. Ala_5 seems to be a better replacement for *N*-methyl-$Dmpapa_5$ at this position than $Papa_5$, which is present in the reference substrate **2**.

To test the influence of the dimethylation of the para amino group of $Papa_5$, we synthesized and assayed the substrate **21**, which contains L-$Dmpapa_5$ instead of $Papa_5$. Due to the low water solubility of this substrate, reaction rates at substrate concentrations higher than 500 μM could not be obtained. A separate determination of k_{cat} and K_M values was therefore not possible, but k_{cat}/K_M values could be extrapolated from a Michaelis−Menten-plot. The *para*-dimethylated substrate **21** shows a cyclization-to-hydrolysis ratio and kinetic parameters comparable to the reference substrate **2** (Table 2). Therefore, the missing dimethylation of the para amino group of $Papa_5$ in the herein used substrates has no negative influence on cyclization efficiency.

To investigate the influence of *N*-methylation of the peptide bond between residues 4 and 5, we synthesized the substrates ***N*-Me-Phe_5-Phe_7-PLP-SNAC** (**22**) and **Phe_5-Phe_7-PLP-SNAC** (**23**). **22** and **23** differ only in the presence of the peptide *N*-methylation. Therefore, **23** is an excellent reference substrate for determining the influence of this *N*-methylation. To determine the methylation influence, we compared the reaction courses of two enzymatic assays containing equal amounts **22** and **23**, respectively. Furthermore, we investigated the cyclization-to-hydrolysis ratios and k_{cat}/K_M values of the two substrates (Figure 4). A 4.2-fold higher kcat/K_M value is detectable for the *N*-methylated substrate **22** compared to the nonmethylated substrate **23**. In comparison to the reference substrate **$Papa_5$-Phe_7-PLP-SNAC** (**2**), the k_{cat}/K_M value of **22** for total product formation increases 7-fold with a comparable cyclization-to-hydrolysis ratio. Interestingly, the nonmethylated substrate **23** shows an unusually high cyclization-to-hydrolysis ratio of 2:1 compared to the reference substrate **2.** In summary, the *N*-methylation of the peptide bond has a fundamental influence on the reaction rate and is an important feature of the enzyme-catalyzed reaction, whereas the dimethylation of the para amino group plays a minor role for cyclization efficiency.

Discussion

Modification of natural products is a promising strategy in drug discovery. The herein investigated S_B molecule exhibits some remarkable structural features: First, S_B antibiotics contain an *N*-methylated peptide bond within the iminoacid triad Pro_4-*N*-Me-$Dmpapa_5$-Pip_6. Second, five of the seven building blocks are nonproteinogenic (HPA_1, D-Abu_3, $Dmpapa_5$, 4-oxo-Pip_6, and Phg_7). These two features are a peculiar property of peptides from the secondary metabolism of actinomycetes and fungi. Impressive examples are the cyclosporins with seven or the enniatins with three *N*-methylations.[28] Nonproteinogenic amino

(28) Velkov, T.; Lawen, A. *J. Biol. Chem.* **2003**, *278*, 1137−1148.

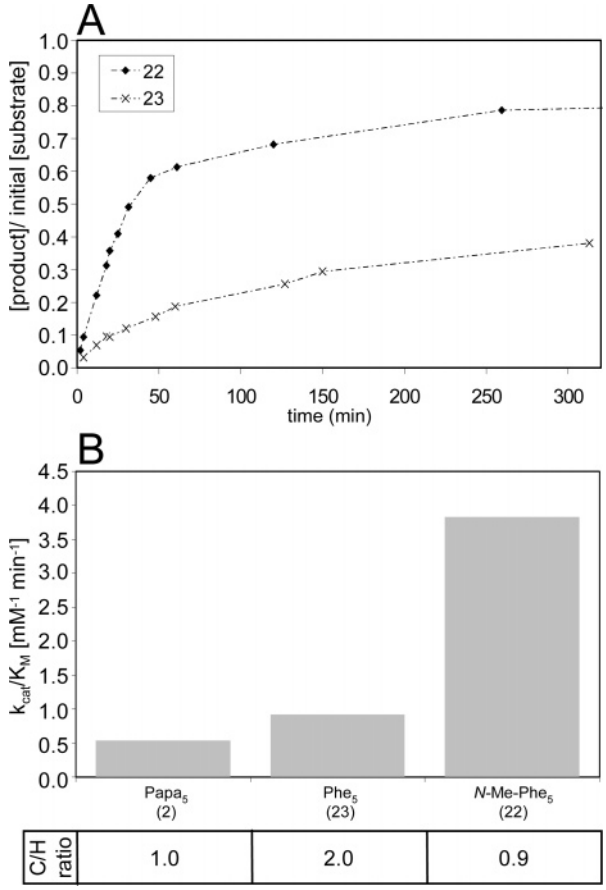

Figure 4. Influence of the *N*-methylated peptide bond between positions 4 and 5 onto the SnbDE cyclase mediated reaction. (A) Time course of total product formation by incubation of the substrates ***N*-Me-Phe$_5$-Phe$_7$-PLP-SNAC** (**22**) and **Phe$_5$-Phe$_7$-PLP-SNAC** (**23**) with SnbDE cyclase at 25 °C. The initial substrate concentration of **22** and **23** was 350 μM. (B) Comparison of kinetic properties and cyclization-to-hydrolysis ratios of substrates **2**, **22**, and **23**. Given are the amino acid residues at position 5. *C*/*H* ratio = cyclization-to-hydrolysis ratio.

acids often play an important role for the bioactivity of natural products, as it was shown recently for the L-3-methylglutamate in the clinically approved antibiotic daptomycin.[29]

Artificial modifications of such complex molecules are constrained by the high content of diverse functional groups, which require selective protection during chemical transformations.[30] A general problem of cyclic peptide synthesis is the regio- and stereospecific closure of the macrocycle without racemization of the activated C-terminal amino acid.[10,31] Furthermore, all total synthesis methods of streptogramin B molecules described so far revealed the challenge of epimerization sensitivity of the phenylglycine ester.[13] This sensitivity restricts choice of coupling and protection/deprotection conditions in chemical syntheses.

Herein we report the characterization of the recombinant TE domain of SnbDE from *Streptomyces pristinaespiralis* and present the combination of solid-phase peptide synthesis and enzymatic cyclization by this enzyme as a simple solution of the aforementioned difficulties in streptogramin B synthesis and as a versatile tool for the design of large peptide libraries of S_B variants.

The SnbDE cyclase was probed with 23 different peptide SNAC substrates based on the simplified **Phe$_7$-PLP-SNAC** (**2**)-sequence to determine its substrate tolerance and the characteristics of the cyclization reaction.

The enzymatic cyclization is characterized by high stereoselectivity for the cyclization nucleophile and the C-terminal position (Figure 3). The stereoselectivity for the nucleophile corresponds to results obtained for CDA and syringomycin cyclases.[18,24]

A change in the stereocenter of the C-terminal amino acid effects cylization and hydrolysis reaction simultaneously. This emphasizes the importance of C-terminal amino acids stereochemistry for the peptidyl-*O*-TE intermediate formation. To overcome difficulties and shortcomings of chemical S_B synthesis predominantly due to racemization sensitivity of the Phg ester, we tested the possibility to selectively cyclize the natural **Phg$_7$-PLP-SNAC** (**1**) from the diastereomer mixture. The peptide thioester **1** is highly sensitive to epimerization of the C-terminal Phg due to an electronic stabilization of the enol form of Phg and a simultaneous activation of Phg by the thioester.[32] During the course of our studies, we observed that the combination of **Phg$_7$-PLP-SNAC** and SnbDE TE fulfils the conditions of a dynamic kinetic resolution (DKR) (Figure 5).[22] DKR means the combination of a stereoselective transformation with an in situ racemization process. In principle, both diastereomers of the starting material can be converted to the desired product in high yield and optical purity by such a process. Therefore, there is no inherent limitation of maximum 50% yield from the substrate mixture with this method. This DKR in the system **Phg$_7$-PLP-SNAC**−SnbDE TE improves the synthetic relevance of this cyclase and allows the use of acid and basic conditions in coupling and deprotection protocols without paying attention to epimerization sensitivity of phenylglycine.

SnbDE TE exploits a distinct regioselectivity important for its synthetic potential. Out of three individual nucleophilic amino acids in the **Phe$_7$-PLP-SNAC** (**2**) reference sequence, only the natural nucleophile L-Thr$_2$ is used for the cyclization reaction. This high regioselectivity allows cyclization to proceed without protection of the alternative nucleophiles HPA_1 and $Papa_5$. Insertion and deletion variants **17** and **18** of **Phe$_7$-PLP-SNAC** revealed that SnbDE is able to catalyze the formation of cycles with various ring sizes albeit with a lower cyclization-to-hydrolysis ratio (Table 2). This observation is in consistence with results obtained from tyrocidine synthetase TE domain (Tyc TE): This cyclase is able to cyclize peptides from 6, 8, 10, 12, and 14 residues in length with comparable kinetic efficiency.[23] The surfactin TE (Srf TE) shows only hydrolysis for shorter or longer substrates.[26]

The structural similarity between Ser and Thr enables the SnbDE cyclase to accept also L-Ser instead of the natural L-Thr as cyclization nucleophile. Interestingly, the cyclization-to-hydrolysis ratio increases 6-fold with L-Ser at position 2. This seems to be due to a lower steric hindrance during the nucleophilic attack onto the C-terminal amino acid. This observation also corresponds to an increased cyclization-to-hydrolysis ratio after exchange of the native C-terminal amino

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(31) Benoiton, N. L.; Lee, Y. C.; Steinaur, R.; Chen, F. M. *Int. J. Pept. Protein Res.* **1992**, *40*, 559−566.
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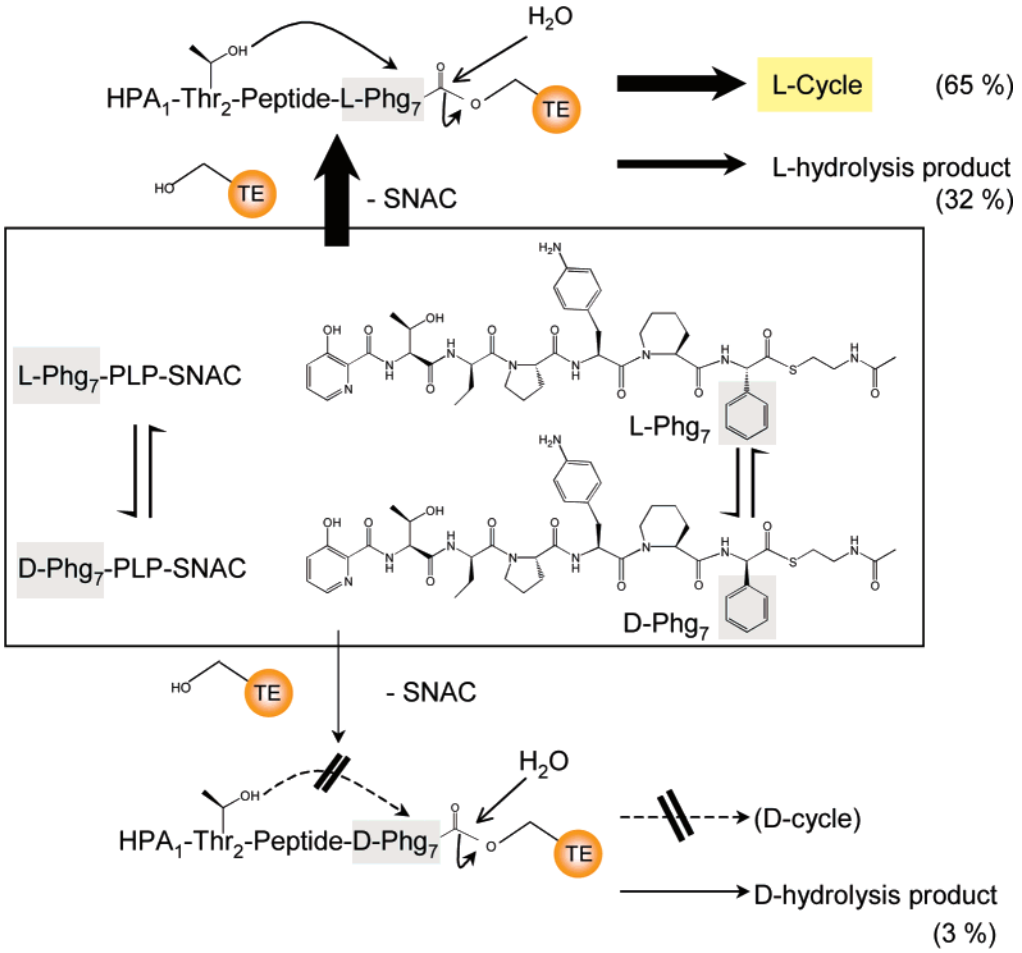

Figure 5. Dynamic kinetic resolution of Phg_7-PLP-SNAC diastereomers during incubation with SnbDE cyclase. The two substrate diastereomers equilibrate in situ. Both diastereomers are able to acylate the active Ser residue of SnbDE-TE resulting in two different peptidyl-*O*-TE intermediates. Only the peptidyl-*O*-TE intermediate with the L-Phg-configuration is able to undergoe a macrolactonization by an intramolecular attack of the hydroxyl function of Thr_2. A side reaction by an extramolecular attack of water results in the L-hydrolysis product. This side reaction is the only reaction pathway for the peptidyl-*O*-TE intermediate with D-Phg configuration: It is unable to cyclize and is transformed into the D-hydrolysis product exclusively. The cyclization-to-hydrolysis ratio of the L-Phg products is 2:1. The reaction rate of product formation of the natural products with L-Phg configuration is 30-fold higher than the D-Phg product formation rate at equal substrate concentrations. This difference in reaction rates affects the racemization equilibrium and results in a rebuilding of the transformed L-Phg substrate by racemization of the D-Phg substrate. The presented relative yields are values for equal substrate concentrations.

acid Phg by Ala. However, the k_{cat}/K_M value decreases by an exchange of the C-terminal position. Therefore, both the steric hindrance between the reactive residues and the recognition of the C-terminal amino acid are of special importance for the cyclization efficiency.

Interestingly, SnbDE TE is able to catalyze macrolactamization shown by the replacement of the Ser_2 hydroxyl group by an amino group. So far only the TE domain from tyrocidine was shown to generate macrolactams and macrolactones by catalyzing a head-to-tail cyclization of linear precursors.[33] The macrolactonization catalyst Srf TE in contrast cannot be used for macrolactamization of substrates with structures similar to the native surfactin structure.[26] Therefore, SnbDE TE is the first cyclase from a macrolactonization NRPS system which can additionally be used as macrolactamization catalyst and the first cyclase which is able to form branched lactams in vitro.

In the course of our studies, the ability of SnbDE TE to cyclize substrates deviating from the natural substrate structure was tested (substrates **8**−**13**) (Figures 3 and 4, Table 2). **Phe$_7$-PLP-SNAC** (**2**) was used as a reference sequence in this systematic Ala-Scan. The results of the scan demonstrated no restrictions for replacements but the need of an aromatic residue at position 1 (Table 2, substrates 8, 14−16). Variations of this aromatic residue and that at position 3, both flanking the cyclization nucleophil, reduce the cyclization-to-hydrolysis ratio. These results are comparable to the tolerance described for the surfactin (Srf TE) and the tyrocidine (TycC TE) TE domains.[26,33]

A characteristic feature of S_B antibiotics is the presence of the imino acid triad Pro_4-*N*-Me-$Dmpapa_5$-Pip_6. This triad is disrupted for synthetic reasons in the here presented substrates by replacement of *N*-Me-$Dmpapa_5$ by unmethylated residues. Assaying the substrate **22** which contains *N*-Me-Phe at position 5 showed a strong increase of the total substrate turnover (Figure 4). Therefore, the *N*-methylation has a fundamental influence onto peptidyl-*O*-TE intermediate formation and enzyme−substrate recognition. There are two possible effects on how *N*-methylation of peptide bonds can influence the substrate conformation: First, the presence of an *N*-methylation in the peptide backbone abolishes the formation of hydrogen bonds. Second, imino acids are known to induce turns into peptide chains because of their higher tendency for cis peptide bond

(33) Trauger, J. W.; Kohli, R. M.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7092−7098.

formation.[34] Cyclization of peptides shorter than seven amino acids without turn-inducing elements such as imino acids is very difficult.[34] Possibly, the missing *N*-methylation of the peptide bond between positions 4 and 5 facilitates the formation of secondary structures of the substrates which are poorly recognized by the cyclase or show a preorganization unfavorable for the cyclization reaction.

In conclusion, SnbDE cyclase is a versatile tool for the synthesis of streptogramin B antibiotics allowing in principle the generation of variations at all seven positions with various efficiencies. Substitutions at positions 1 and 3 affect the cyclization-to-hydrolysis ratio, whereas replacements at positions 6 and 7 reduce the reaction rate. The presence of the unnatural amino acid L-Phg and the *N*-methylation of the peptide bond between positions 4 and 5 are relevant for substrate turnover. Synthesis of S_B molecules can now be approached by standard solid-phase peptide synthesis of the peptide chain followed by stereoselective enzymatic cyclization using the SnbDE cyclase. However, the described chemoenzymatic approach will enable the generation of Streptogramin B analogues, which can be screened for improved therapeutic activity and could give new insights into structure activity relationships in this important class of antibiotics.

(34) Sewald, N.; Jakubke, H. *Peptides: Chemistry and Biology*; Wiley-VCH: Weinheim, 2002; p 313.

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