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Macrolactam formation catalyzed by the thioesterase domain of vicenistatin polyketide synthase

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Abstract—The excited thioesterase (TE) domain from the vicenistatin polyketide synthase (PKS) efficiently catalyzed the macrolactam formation of the *N*-acetylcysteamine thioester of the *seco*-amino acid of the aglycon vicenilactam. This result indicates that the vicenistatin PKS TE domain cyclizes the extended polyketide chain on the ACP domain in the PKS. Furthermore, the simple ethyl ester of the *seco*-amino acid was also found to be used as a substrate of the TE domain with similar efficiency.

Vicenistatin **1** was isolated from *Streptomyces halstedii* HC34 as an antitumor antibiotic, and consists of a unique 20-membered macrolactam aglycon, vicenilactam **2**, and an aminosugar visenisamine.[1] The characteristic macrolactam **2** was proposed to be biosynthesized by a standard polyketide synthase (PKS), which uses the unique amino acid starter unit, 3-amino-2-methylpropionate, as shown in Figure 1.[2,3] On the aspect of the polyketide macrolactam formation, the post PKS amide bond formation was suggested in the biosynthesis of ansamycin antibiotics including rifamycin and ansatrienin, in which an amide synthase just after the PKS was proposed to be responsible to the macrolactam formation.[4] On the other hand, the vicenistatin PKS obviously contains the thioesterase (TE) domain at the end of polypeptide, so that the TE domain was anticipated to be responsible to the macrolactam formation of the extended polyketide leading to the macrocyclic vicenilactam.[2,3]

In fact, the thioesterase-catalyzed macrolactone formation appears in many PKS system.[5] The isolated PKS TE domains in the epothilone PKS[6] and the pikromycin PKS[7] have recently shown to catalyze the macrolactonization using the suitable *N*-acetylcysteamine (NAC) thioester analogues of the natural polyketide substrate. In addition, some of the isolated nonribosomal peptide synthetase (NRPS) TE domains have been reported to catalyze the macrocyclic peptide formation of linear peptides as well.[8] These reports, therefore, encouraged us to investigate the function of the isolated TE domain of the vicenistatin PKS to confirm the biosynthetic role and to envision the synthetic potential as a macrocycle forming enzyme.

At first, the expected linear amino acyl chain substrate was prepared from vicenilactam **2** (Scheme 1). Activation of vicenilactam **2** as the *tert*-butyl carbamate was necessary to hydrolyze the amide bond giving the desired linear amino acid. Vicenilactam **2** was not hydrolyzed at all or decomposed under the several basic conditions. The protected *seco*-amino acid was then converted to the corresponding NAC thioester and then deprotected under the acidic condition giving the NAC thioester of *seco*-vicenilactam to mimic the S-ACP substrate in the vicenistatin PKS.[9]

The TE domain of the vicenistatin PKS was designed based on the crystal structure of the TE domain of 6-deoxyerythronolide B synthase (DEBS) and the pikromycin PKS.[10] Although the DEBS TE domain has not been shown to have the macrocyclic forming activity, the hydrolytic activity for the specific NAC substrates has been reported.[11] The structural studies clearly showed that the linker region between the TE domain

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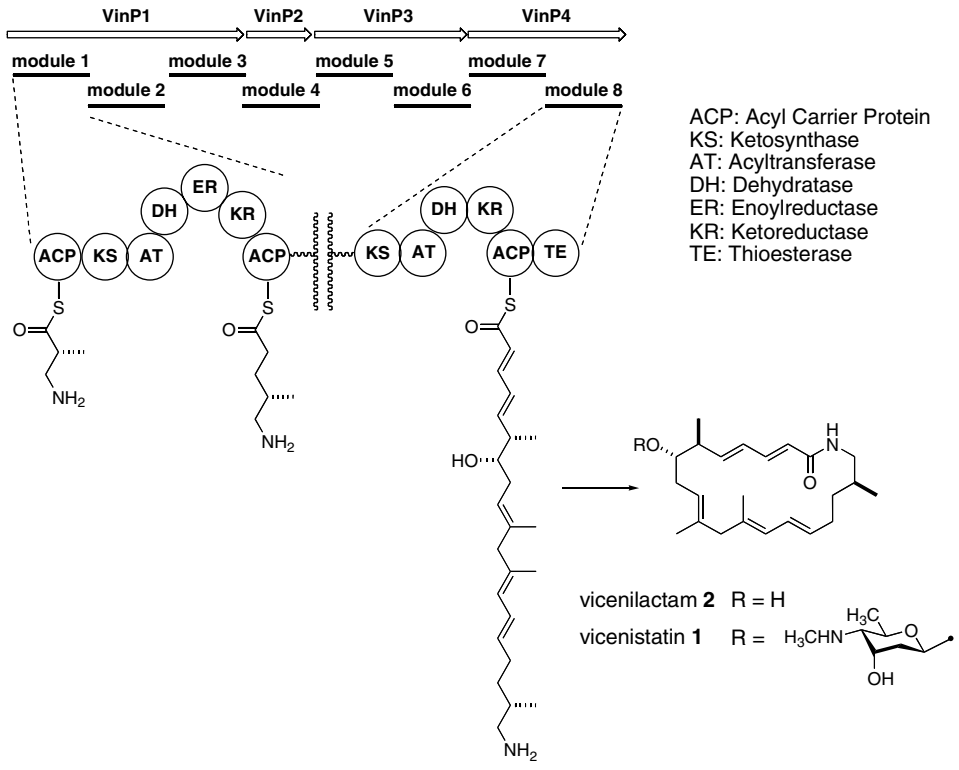
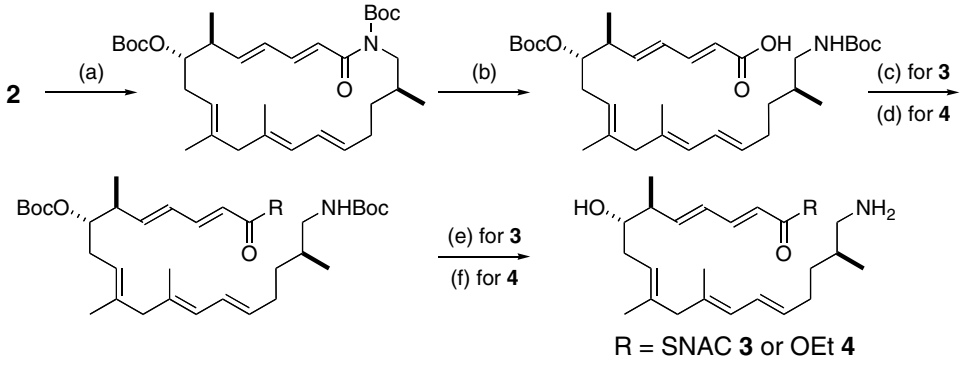


Figure 1. A part of modular organization of the vicenistatin (Vin) PKS in *Streptomyces halstedii*.



Scheme 1. Synthesis of the *seco*-vicenilactam esters. Reagents and conditions: (a) $(Boc)_2O$, pyridine, 98%; (b) LiOH, dioxane, H_2O, 82%; (c) DCC, DMAP, NAC–SH, CH_2Cl_2, 96%; (d) DCC, DMAP, ethanol, 65%; (e) $BF_3{\cdot}OEt_2$, 4 Å—MS, CH_2Cl_2 67%; TsOH, EtOH, reflux; 74%; (f) TsOH, EtOH, reflux; 61%.

and the acyl carrier protein (ACP) domain is important to form the hydrophobic interface of the dimeric TE domain. Thus, the position close to the ACP of module 8 in the vicenistatin PKS was chosen as the N-terminal of the TE domain in the present study.[12] The DNA sequence for the vicenistatin TE domain was amplified by a standard PCR and cloned into the expression vector (pET30, Novagen, USA) after confirmation of the DNA sequence. The TE domain was overexpressed in *Escherichia coli* BL21(DE3) by addition of isopropyl β-D-thiogalactoside and the cells of *E. coli* was homogenized by sonication. The TE domain was purified by the DEAE ion chromatography and the gel filtration chromatography to homogeneity with yield of 20 mg/L of culture.

The TE reaction was carried out with the NAC thioester of *seco*-vicenilactam **3** as shown in Figure 2.[13] The formation of the macrolactam product **2** was confirmed by HPLC equipped with a photodiode array detection system and LC–ESI mass spectrometry. Using 1.25 mM of substrate (apparently saturated) and 0.13 mM of the TE domain, 37% of NAC thioester **3** was converted to vicenilactam **2** for 40 min at 28 °C. It should be noteworthy that the hydrolyzed compound was not detected at all under the present reaction conditions. Thus, it was clearly proved that the TE domain is responsible to the macrolactam formation in the biosynthesis of vicenilactam **2**. This result also clearly suggested that the unique amino acid starter unit, 3-amino-2-methylpropionate, is somehow loaded on

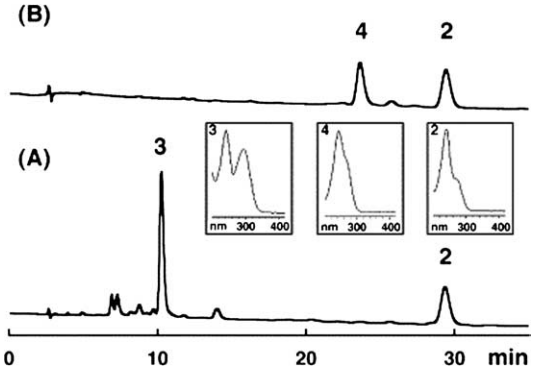


Figure 2. HPLC traces (OD at 238 nm) of (A) macrocyclization by the vicenistatin TE domain (Vin TE) with *seco*-vicenilactam NAC thioester **3** (LC–ESI (positive), $[M+H]^+$: *m*/*z* 477), (B) with ethyl ester **4** (LC–ESI (positive), $[M+H]^+$: *m*/*z* 404) to vicenilactam **2** (LC–ESI (positive), $[M+Na]^+$: *m*/*z* 380). Off sets show the PDA spectrum of each peak. Reaction conditions: 1.25 mM of substrates (apparently saturated), 0.13 mM of the TE domain, 10% DMSO, in a 100 mM phosphate buffer (pH 7.0), 28 °C, 40 min.

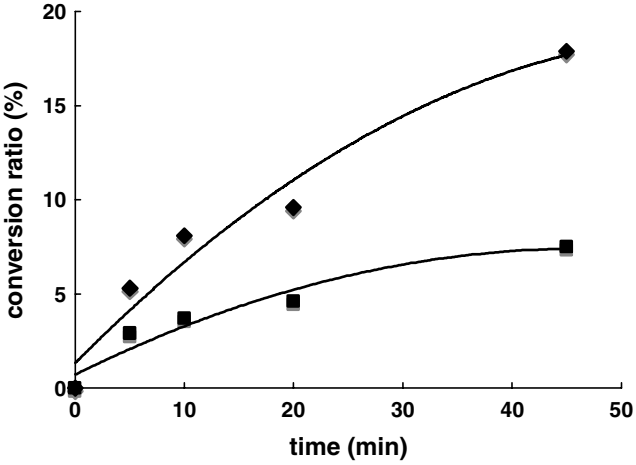


Figure 3. Time course of the TE-catalyzed cyclization with the ethyl ester **4** (♦) and the NAC thioester **3** (■). Reaction conditions: same as conditions in Figure 2 except for the concentration of substrates (0.25 mM). Conversion ratio was calculated as follows: conversion ratio (%) = (vicenilactam production)/[(remaining substrate) + (vicenilactam production)] × 100. The amounts of vicenilactam **1** and substrate **3** and **4** were estimated from OD at 238 nm using the same absorption coefficient for normalization.

the vicenistatin PKS and the polyketide chain containing the quite nucleophilic amino group is extended by the PKS leading to the vicenilactam. Although the biosynthetic genes for the other macrolactam-containing polyketides such as hitatimycin, BE-14106, GT32-B, cremimycin, fluvirucins, etc. have not been identified so far, similar TE-catalyzed macrolactam formations could be proposed in the biosynthesis.

To investigate the substrate specificity of the TE domain, the ethyl ester of *seco*-vicenilactam **4** was also prepared as a simple convenient substrate analogue.[9] To our surprise, 51% of ethyl ester **4** was cyclized to the macrocyclic lactam **2** by the TE domain under the same conditions (Fig. 2B). Although the precise kinetic analysis could not be examined due to the low solubility of the substrate in the buffer, the time dependent production of vicenilactam **2** with a certain concentration (0.25 mM) of the substrates clearly indicated that the ethyl ester **3** appeared to be even better substrate than the NAC thioester **4** (Fig. 3).

In most of the reports about the TE-catalyzed macrocyclization including NRPS TEs and PKS TEs, the NAC thioester has been used as a mimic for the phosphopantetein thioester on the ACP/PCP domain.[5a] In addition to NAC thioester, several thioester including thiophenol, phosphopantetein, and coenzyme A have been also investigated and it was shown that the thioester of thiophenol, which is chemically more reactive but sterically less hindrance than the natural substances, was found to be a superior substrate for the macrocyclization of linear peptides by NRPS TE.[8b] In other words, the modified natural thioester is not always, but the sterically less hindered ester can be favored for the TE-catalyzed reactions. In addition, the *N*-acetylethanolamine ester of the linear peptides instead of the NAC thioester has been used to construct the combinatorial macrocyclic peptides with the modest decrease in catalytic rate.[8c] Accordingly, the chemical reactivity is not so important, once the acyl substrate is incorporated to the active site. Therefore, our result could be rationally explained, because the ethyl ester was sterically less hindrance than the corresponding NAC thioester, although it is chemically less active. In any case, our result clearly established that the simple ethyl ester could be used for the TE-catalyzed macrocyclization as a stable and convenient substrate.

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9. Physico-chemical data; **3**, $[\alpha]_D^{26}$ −6.7 (*c* 0.45, CH_3OH); ^1H NMR (400 MHz, CD_3OD): δ 7.22 (m, 1H), 6.30 (dd, $J = 10.8$, 15.2 Hz, 1H), 6.23 (m, 2H), 6.17 (d, $J = 15.2$ Hz, 1H), 5.80 (d, $J = 10.8$ Hz, 1H), 5.55 (ddd, 1H, $J = 14.3$, 14.3, 6.8 Hz), 5.28 (t, $J = 6.8$ Hz, 1H), 3.50 (dt, $J = 5.8$, 5.8 Hz, 1H), 3.33 (t, $J = 6.7$ Hz, 2H), 3.06 (t, $J = 6.7$ Hz, 2H), 2.90 (dd, $J = 12.7$, 5.8 Hz, 1H), 2.73 (dd, $J = 12.7$, 8.0 Hz, 1H), 2.69 (s, 2H), 2.37 (m, 1H), 2.22 (m, 2H), 2.13 (m, 2H), 1.91 (s, 3H), 1.81 (m, 1H), 1.64 (s, 3H), 1.49 (s, 3H), 1.32 (m, 2H), 1.08 (d, $J = 6.8$ Hz, 3H), 1.01 (d, $J = 6.6$ Hz, 3H); HR FAB-MS: found 477.3128 $(M+H)^+$; calcd for $C_{27}H_{45}N_2O_3S$ 477.3151. Compound **4**, $[\alpha]_D^{26}$ −28 ($c = 0.50$, CH_3OH); ^1H NMR (400 MHz, $CDCl_3$): δ 7.26 (dd, $J = 15.4$, 10.5 Hz, 1H), 6.24 (dd, $J = 14.9$, 10.5 Hz, 1H), 6.22 (dd, $J = 15.4$, 10.5 Hz, 1H), 6.10 (dd, $J = 15.4$, 7.7 Hz, 1H,), 5.82 (d, $J = 15.4$, 1H), 5.80 (d, $J = 10.5$ Hz, 1H), 5.59 (dt, $J = 14.9$, 7.1 Hz, 1H), 5.21 (t, $J = 6.9$ Hz 1H), 4.20 (q, $J = 7.1$ Hz, 2H), 3.54 (ddd, $J = 8.6$, 5.6, 4.2 Hz, 1H), 2.71 (s, 2H), 2.62 (dd, $J = 12.4$, 5.2 Hz, 1H), 2.50 (dd, $J = 12.4$, 6.6 Hz, 1H), 2.42 (m, 1H), 2.15 (m, 4H), 1.64 (s, 3H), 1.6 (m, 1H), 1.54 (s, 3H), 1.3 (m, 2H), 1.29 (t, $J = 7.1$ Hz, 3H), 1.10 (d, $J = 6.8$ Hz, 3H), 0.90 (d, $J = 6.6$ Hz, 3H); HR FAB-MS: found $(M+H)^+$ 404.3183; calcd for $C_{25}H_{42}NO_3$ 404.3165.
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12. Expression of VinTE: The gene for the vicenistatin TE domain (3515–3808 of VinP4, 294 aa, 30.7 kda) was amplified by PCR with primers VinTE-F: 5′-CAG-CCGGCGCatatGTCGGACGACG-3′ and VinTE-R: 5′-TCCGGCGCTCGACaaGCTtATGGGG-3′ using the cosmid K1B10[3] derived from *S. halstediii* HC34 as a template. The amplified PCR product was cloned into the LITMUS28. After confirmation of the DNA sequence, the appropriate DNA fragment was inserted into the pET30b (Novagen) to obtain pET-vinTE. The pET-vinTE was introduced into *E. coli* BL21(DE3) by a standard chemical transformation. The *E. coli* harboring pET-vinTE was grown in a LB medium supplemented with 30 μg/mL kanamycin at 220 rpm, 37 °C. When OD_{600} reached to 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM. After 3 h of further incubation, the cells were harvested and suspended in a 100 mM phosphate buffer (pH 7.0), and then disrupted by sonication. Unbroken cells and debris were removed by centrifugation at 12,000*g* for 30 min. This supernatant was applied onto a DEAE-Sepharose Fast Flow column at a flow rate of 1 mL/min. The protein was eluted with a linear gradient from 0 to 1.0 M NaCl in the same buffer. The fractions containing the vicenistatin TE domain were combined and concentrated by ultracentrifugation. After a gel-filtration using a Hi Load 26/60 Superdex 200 on a FPLC with the same buffer containing 50 mM NaCl, the fractions containing the TE domain were combined and concentrated into ca. 7.0 mL (45 mg/2.4 L culture).
13. A reaction mixture [50 μL of final volume in a 100 mM phosphate buffer (pH 7.0)] containing substrates in DMSO (final concentration of 0.25 or 1.25 mM, 10% DMSO), and the vicenistatin TE (0.13 mM) was incubated at 28 °C. Reaction was terminated by addition of EtOAc. The organic layer was separated, and concentrated. The residue was redissolved in 100 μL of MeOH. The samples were analyzed by HPLC or LCQ-ESI mass spectrometer.