Supporting Information

Protein-Protein Recognition Involved in the Intermodular Transacylation Reaction in Modular Polyketide Synthase in the Biosynthesis of Vicenistatin

Taichi Chisuga, Akimasa Miyanaga,* and Tadashi Eguchi*
Experimental

Table S1. Initial velocity of the transacylation reaction between VinP2 NDD₄KS₄ and acyl-VinP1 ACP₃CDD₃ proteins
Table S2. Initial velocity of the transacylation reaction of VinP2 NDD₄KS₄AT₄ mutant proteins
Table S3. The amount of the complex produced in the crosslinking reaction
Table S4. A list of primers used for preparation of VinP2 NDD₄KS₄AT₄ and the mutants
Table S5. A list of primers used for preparation of VinP1 ACP₃CDD₃, VinP2 ACP₄CDD₄ and VinP3 ACP₅CDD₆ proteins
Table S6. Solvent C concentration of HPLC analysis of the transacylation reaction and the reaction time
Table S7. A list of primers used for preparation of ACPCDD chimeric proteins
Table S8. Summary of the ESI-MS analysis of apo-, holo- and acyl-ACP proteins
Table S9. Summary of the ESI-MS analysis of crypto-ACP proteins

Figure S1. Polyketide synthases in the biosynthesis of vicenistatin
Figure S2. Amino acid sequence alignment of C-terminus of VinP1 and N-terminus of VinP2
Figure S3. Predicted dimer structure of acyl-VinP1 ACP₃CDD₃ and the amino acid sequence of C-terminal VinP1
Figure S4. HPLC analysis of the transacylation reaction between VinP2 NDD₄KS₄ and butyryl-VinP1 ACP₃CDD₃
Figure S5. LC-ESI-MS analysis of VinP1 ACP₃CDD₃ proteins
Figure S6. Quantitative analysis of the transacylation activity of VinP2 NDD₄KS₄ against tiglyl-VinP1 ACP₃CDD₃
Figure S7. The sequence alignment of KS domains
Figure S8. HPLC analysis of the transacylation reaction between VinP2 NDD₄KS₄AT₄ double mutant protein and tiglyl-VinP1 ACP₃CDD₃
Figure S9. Proposed transacylation mechanism between KS domain and acyl-ACP
Figure S10. LC-ESI-MS analysis of VinP2 ACP₄CDD₄ proteins
Figure S11. LC-ESI-MS analysis of VinP3 ACP₅CDD₆ proteins
Figure S12. HPLC analysis of the transacylation reaction between VinP2 NDD₄KS₄ and tiglyl-ACPCDD proteins
Figure S13. LC-ESI-MS analysis of VinP1 ACP₃DE₃ proteins
Figure S14. HPLC analysis of the transacylation reaction between VinP2 NDD₄KS₄ and tiglyl-VinP1 ACP₃DE₃
Figure S15. The amino acid sequence alignment of ACPs.
Figure S16. The strategy for construction of expression plasmids of ACP₅CDD chimeric proteins
Figure S17. The gene fragment and amino acid sequence of ACP₃CDD₃ chimeric protein
Figure S18. The gene fragment and amino acid sequence of ACP₅CDD₅ chimeric protein
Figure S19. The gene fragment and amino acid sequence of ACP₅CDD₇ chimeric protein
Figure S20. LC-ESI-MS analysis of ACP₅CDD₅ proteins
Figure S21. LC-ESI-MS analysis of ACP₆CDD₃ proteins
Figure S22. LC-ESI-MS analysis of ACP₇CDD₃ proteins
Figure S23. HPLC analysis of the transacylation reaction between VinP2 NDD₄KS₄ and tiglyl-ACPCDD chimeric proteins
Figure S24. SDS-PAGE analysis of the crosslinking between VinP2 NDD₄KS₄AT₄ S684G and Br-acetyl pantetheinamide-VinP1 ACP₅CDD₃

References
Experimental

General procedure

All commercial reagents derived from TCI, Kanto Chemical and Sigma Aldrich were used unless otherwise indicated. $^1$H-NMR spectra were recorded with a JEOL ECS-400 spectrometer.

Synthesis of tiglyl-CoA

Tiglic acid (11 mg, 110 μmol) was dissolved in 1.5 mL of THF, and PyBOP (120 mg, 230 μmol) was then added to the solution. After stirring at room temperature for 30 min, CoA lithium salt (85 mg, 109 μmol) dissolved in 0.5 mL of 4% K$_2$CO$_3$ solution was added to the reaction solution. The reaction solution was stirred at room temperature for 1 h, and 0.2 mL of water was then added to the reaction solution. After stirring at room temperature for 30 min, the reaction mixture was washed by AcOEt three times, and the water layer was then lyophilized. The crude residue was dissolved in 1 mL of water and purified by high-performance liquid chromatography (HPLC) with a Hitachi instrument (Chromaster Pump 5110, Diode Array Detector 5430, and Column Oven 5310) equipped with a TSKgel ODS -100V column (5 μm, 250 × 4.6 mm$^2$; TOSOH) in the column oven at 40 °C. Tiglyl-CoA was detected at 260 nm and eluted with a gradient of solvents D (50 mM NaH$_2$PO$_4$ buffer pH 4.5) and E (methanol), flow rate: 1.0 mL/min; 0–10 min 5% E, 10–30 min 5–80% E linear gradient, and 30–35 min 90% E. This fraction was lyophilized to obtain the product with NaH$_2$PO$_4$ salt derived from solvent D. The product was desalted by preparative HPLC with the same Hitachi instrument equipped with an TSKgel ODS-100V column (5 μm, 250 × 4.6 mm$^2$) in the column oven at 40 °C. Desalted tiglyl-CoA was detected at 260 nm and eluted with a gradient of solvents F (200 μM HCl) and G (acetonitrile), flow rate: 1.0 mL/min; 0–5 min 5% G, 5–15 min 5–60% G linear gradient, and 15–25 min 60% G. After lyophilization of this fraction, tiglyl-CoA was obtained (5.5 mg, 6%).

Spectroscopic data: $^1$H NMR (400 MHz, D$_2$O): δ 0.60 (s, 3H), 0.74 (s, 3H), 0.74 (s, 3H), 1.61 (s, 3H), 1.63 (d, J= 6.8 Hz, 3H), 2.26 (t, J= 6.4 Hz, 2H), 2.81 (t, 6.8 Hz, 2H), 3.16 (t, J= 6.4 Hz, 2H), 3.29 (t, J= 6.4 Hz, 2H), 3.41 (dd, J= 4.4, 9.6 Hz, 1H), 3.69 (dd, J= 5.2, 10.0 Hz, 1H), 3.87 (s, 1H), 4.08 (bs, 2H), 4.43 (s, 1H), 5.99 (d, J= 6.0 Hz, 1H), 6.67 (q, J= 6.9 Hz, 1H), 8.08 (s, 1H), 8.38 (s, 1H). ECS-MS (positive mode): m/z 850.0 ([M+H]$^+$)

Preparation of the VinP2 ACP$_4$CDD$_4$ domain and VinP4 ACP$_6$CDD$_6$ domain.

The vinP2 ACP$_4$CDD$_4$ and vinP3 ACP$_6$CDD$_6$ fragments were amplified by PCR using cosmid K1B10$^{[1]}$ as the template DNA with oligonucleotides shown in Table S5. The amplified fragments were cloned into the expression vector pColdW$^{[2]}$ using NdeI and XhoI / NdeI and HindIII restriction sites to form pColdW-vinP2 ACP$_4$CDD$_4$ and pColdW-vinP3 ACP$_6$CDD$_6$, respectively. For the expression of the VinP2 ACP$_4$CDD$_4$ and VinP3 ACP$_6$CDD$_6$, Escherichia coli BL21(DE3) (NIPPON GENE CO., LTD.) cells harboring each gene were grown at 37 °C in Luria-Bertani (LB) broth containing ampicillin (50 μg/mL). When the optical density at 600 nm reached 0.4, protein expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were then cultured for an additional 20 h at 15 °C. The harvested cell pellets were suspended in buffer A [50 mM HEPES-Na (pH 8.0), 100 mM NaCl, and 10% (w/v) glycerol] and lysed by sonication. The supernatant was loaded onto a His60 Ni Superflow affinity column (Clontech).
The column was washed with buffer A containing 20 mM imidazole to remove unbound proteins. The protein was then eluted with buffer A containing 200 mM imidazole. The protein solution was then desalted and concentrated using a PD-10 column (Cytiva) and an Amicon Ultra 10K centrifugal filter (Merck Millipore).

**Preparation of VinP2 NDD\(_4\)KS\(_4\)AT\(_4\) H341A/S684G and H381A/S684G double mutant proteins.**

For preparation of pET30-\(\text{vinP2}\) NDD\(_4\)KS\(_4\)AT\(_4\) H341A/S684G and H381A/S684G, site-directed mutagenesis was performed using pET30-\(\text{vinP2}\) NDD\(_4\)KS\(_4\)AT\(_4\) S684G as the template DNA with the oligonucleotides shown in Table S4.

For the expression of the VinP2 NDD\(_4\)KS\(_4\)AT\(_4\) H341A/S684G and H381A/S684G, E. coli Rosetta\textsuperscript{TM} 2(DE3) (Novagen) cells harboring each gene were grown at 37 °C in LB broth containing kanamycin (50 μg/mL) and chloramphenicol (20 μg/mL). When the optical density at 600 nm reached 0.6, protein expression was induced by the addition of 0.2 mM IPTG, and the cells were then cultured for an additional 20 h at 15 °C. The harvested cell pellets were suspended in buffer A and lysed by sonication. The supernatant was loaded onto a His60 Ni Superflow affinity column. The column was washed with buffer A containing 20 mM imidazole to remove unbound proteins. The protein was then eluted with buffer A containing 200 mM imidazole. The protein solution was then desalted and concentrated using a PD-10 column and an Amicon Ultra 10K centrifugal filter.

**Analysis of transacylation reaction between VinP2 NDD\(_4\)KS\(_4\)AT\(_4\) double mutant proteins and tiglyl-VinP1 ACP\(_3\)CDD\(_3\).**

For analysis of transacylation reaction of VinP2 NDD\(_4\)KS\(_4\)AT\(_4\) double mutants, 50 μM VinP2 NDD\(_4\)KS\(_4\)AT\(_4\) double mutant protein was mixed with 50 μM tiglyl-VinP1 ACP\(_3\)CDD\(_3\) in buffer A. The reaction mixture was incubated at 28 °C for 120 and 240 min, and an equal volume of acetonitrile was then added to the solution. These mixtures were subjected to HPLC analysis with a Hitachi instrument (Chromaster Pump 5110, Diode Array Detector 5430, and Column Oven 5310) equipped with an Aeris C4 WIDEPORE column (3.6 μm, 150×4.6 mm\(^2\); Phenomenex) in the column oven at 50 °C. Holo-VinP1 ACP\(_3\)CDD\(_3\) was detected at 280 nm and eluted with a gradient of solvents B (0.1% TFA in water) and C (0.1% TFA in acetonitrile), flow rate: 1.0 mL/min; 0–5 min 41% C, 5–25 min 41–55% C linear gradient, and 25–30 min 90% C. Initial velocity of the transacylation between VinP2 NDD\(_4\)KS\(_4\) domain and tiglyl-VinP1 ACP\(_3\)CDD\(_3\) was calculated based on the HPLC peak area.

**Preparation of CoaA, CoaD, CoaE and Sfp recombinant proteins.**

CoaA, CoaD and CoaE were prepared as recombinant proteins, each of which is fused to a maltose-binding protein on these N-terminus. The plasmids encoding CoaA, CoaD and CoaE\[3\] were provided by Prof. Michael D. Burkart. These Coa proteins were expressed and purified using a previously described protocol\[3\]. The Sfp recombinant protein was prepared as a His-tagged protein. The Sfp protein was expressed and purified using a previously described protocol.\[2\]

**Preparation of ACPCDD chimeric proteins.**
A expression plasmids of ACP₃CDD₃ chimeric proteins were constructed by Gibson assembly (the strategy is shown in Figure S16). For construction of pColdW-ACP₄CDD₃ and pColdW-ACP₅CDD₃, fragment 1 was amplified by PCR using pColdW-vinP2 ACP₄CDD₄ and pColdW-vinP3 ACP₅CDD₅, respectively, as the template DNA with oligonucleotides shown in Table S7. Fragment 2 was amplified by PCR using pColdW-vinP1 ACP₃CDD₃ as the template DNA with oligonucleotides shown in Table S7. Next, fragment 1 and fragment 2 were combined by Gibson assembly reaction. The reaction mixture (10 μL) containing 0.5 pmol of fragment 1 and 0.5 pmol of fragment 2, and 10 μL of Gibson Assembly® Master Mix (New England Biolabs) was then added to the mixture. The reaction mixture was incubated at 50 °C for 15 min to generate pColdW-ACP₄CDD₃ and pColdW-ACP₅CDD₅.

For construction of pColdW-ACP₇CDD₃, pColdW-vinP4 ACP₇ was firstly prepared as the DNA template. The vinP4 ACP₇ fragment was amplified by PCR using cosmid K1B10[1] as the template DNA with oligonucleotides shown in Table S7. The amplified fragment was cloned into the expression vector pColdW using NdeI and HindIII restriction sites to form pColdW- vinP4 ACP₇. The fragment 1 was then amplified by PCR using pColdW-vinP4 ACP₇ as the template DNA with oligonucleotides shown in Table S7. The fragment 2 was amplified by PCR using pColdW-vinP1 ACP₃CDD₃ as the template DNA with oligonucleotides shown in Table S7. Next, the fragment 1 and fragment 2 were combined by Gibson assembly reaction. The reaction mixture (10 μL) containing 0.5 pmol of fragment 1 and 0.5 pmol of fragment 2, then 10 μL of Gibson Assembly® Master Mix was added to the mixture. The reaction mixture was incubated at 50 °C for 15 min to generate pColdW-ACP₇CDD₃.

**LC-ESI-MS analysis of ACP proteins.**

The conversion of apo-ACP(CDD) proteins to acylated- or crypto-ACP(CDD) proteins was monitored by LC-ESI-MS using LCMS-2020 (Shimadzu) equipped with a Protein-R column (300 Å, 250×2.0 mm; COSMOSIL). The flow rate was 0.2 mL/min, and the detection wavelength was 280 nm. The elution condition was a linear gradient of solvents H (0.1% formic acid in water) and I (0.1% formic acid in acetonitrile): 0–5 min in 20% I and 5–20 min in 20–90% I linear gradient. Observed molecular weights were calculated from multicharged states by deconvolution (LabSolutions LCMS Multi-Charged Ion Analysis Software, Shimadzu). LC-ESI-MS data are summarized in Table S8, S9.
**Table S1.** Initial velocity of the transacylation reaction between VinP2 NDD₄KS₄ and acyl-VinP1 ACP₃CDD₃ proteins.

| ACP protein       | Acyl group | Initial velocity (nM/min)[a] |
|-------------------|------------|-----------------------------|
| VinP1 ACP₃CDD₃    | tiglyl     | 436 ± 34                    |
|                   | butyryl    | 535 ± 37                    |

[a] Initial velocity of 50 μM VinP2 NDD₄KS₄AT₄ S684G and 50 μM acyl-VinP1 ACP₃CDD₃.
**Table S2.** Initial velocity of the transacylation reaction between VinP2 NDD₄KS₄AT₄ mutant proteins and tiglyl-VinP1 ACP₃CDD₃.

| VinP2 NDD₄KS₄AT₄  | Initial velocity (nM/min)\(^{[a]}\) |
|-------------------|--------------------------------------|
| S684G             | 436 ± 34                              |
| C206G/S684G       | trace\(^{[b]}\)                       |
| H341A/S684G       | 31.2 ± 6.7                            |
| H381A/S684G       | 19.8 ± 0.7                            |

\(^{[a]}\) Initial velocity of 50 μM VinP2 NDD₄KS₄AT₄ mutant protein and 50 μM tiglyl-VinP1 ACP₃CDD₃. \(^{[b]}\) Initial velocity < 10 nM/min.
Table S3. The amount of the complex produced in the crosslinking reaction between VinP2 NDD$_4$KS$_4$ and Cl-acetyl pantetheinamide-ACP(CDD) proteins.

| ACP part | CDD part | Production of crosslinked complex (µM)[a] |
|----------|----------|------------------------------------------|
| ACP$_3$  | CDD$_3$  | 31 ± 1                                    |
| ACP$_3$  | none     | 4.0 ± 0.2                                 |
| ACP$_4$  | CDD$_4$  | 3.0 ± 0.1                                 |
| ACP$_6$  | CDD$_6$  | 1.4 ± 0.3                                 |
| ACP$_4$  | CDD$_3$  | 14 ± 2                                    |
| ACP$_6$  | CDD$_3$  | 5.3 ± 0.4                                 |
| ACP$_7$  | CDD$_3$  | 8.7 ± 2.0                                 |

[a] Production of crosslinked complex on the condition of 50 µM VinP2 NDD$_4$KS$_4$AT$_4$ S684G and 200 µM Cl-acetyl pantetheinamide-ACP(CDD).
Table S4. A list of primers used for preparation of VinP2 NDDKSAT and the mutants.

| Primers                  | Nucleic acid sequence of primers                     |
|--------------------------|------------------------------------------------------|
| VinP2 NDDKSAT Fwd        | 5'- AACATATGGAGAACGAAAAGAAACTTCTCGATTACCTCAAGC      |
| VinP2 NDDKSAT Rev        | 5'- TAAGCTTCTGACCCAACCCCCGACACATCCCCCGC            |
| VinP2 NDDKSAT C206G Fwd  | 5'- GACACCGCCCGCTCGTCCTGGCTGGCC                     |
| VinP2 NDDKSAT C206G Rev  | 5'- GGACGACGAGCCGCGGTGAGTGAGTGAGC                  |
| VinP2 NDDKSAT S684G Fwd  | 5'- GGTCATGTCAGGGTGAGATCGCGGGCTG                   |
| VinP2 NDDKSAT S684G Rev  | 5'- ACCCTGACCATGACCCACCCACGCATC                    |
| VinP2 NDDKSAT H341A Fwd  | 5'- GAGCAGCGGACGGGTACCGAAGGTGATCGAGGCTGA          |
| VinP2 NDDKSAT H341A Rev  | 5'- CAGCTGCAGCGGGCTGACCGGCGGCCAGCGGCC          |
| VinP2 NDDKSAT H381A Fwd  | 5'- GTGGGTGCAGCGAGGCTGCCGCCGGG                   |
| VinP2 NDDKSAT H381A Rev  | 5'- CAGCTGACCGGGATCCTCGACTGAGTC                 |
Table S5. A list of primers used for preparation of VinP1 ACP₃CDD₁, VinP2 ACP₄CDD₄ and VinP3 ACP₆CDD₆ proteins.

| Primers       | Nucleic acid sequence of primers                                      |
|---------------|---------------------------------------------------------------------|
| VinP1 ACP₃CDD₁ Fwd | 5’- AACATATGGACCAGGAGGCGCTACGCGG                                    |
| VinP1 ACP₃CDD₁ Rev | 5’- AACTCGAGTCAGTCCGTCGGACAGCTACCGTC                                |
| VinP1 ACP₃DE₂ Fwd | 5’- ACGCCATATCAGCCGAAAGG                                          |
| VinP1 ACP₃DE₂ Rev | 5’- AAAACTCGAGTCACCTGCGACAGCTACCGTC                                 |
| VinP2 ACP₄CDD₁ Fwd | 5’- AAAACATATGAGCGGCGGACTTGAGCAG                                   |
| VinP2 ACP₄CDD₁ Rev | 5’- TTCTCGAGTCAGGACTTGCGGAAGGTGCTGTC                                |
| VinP3 ACP₆CDD₆ Fwd | 5’- TAACATATGGATGACCCCGAGAGGACCCGTG                                 |
| VinP3 ACP₆CDD₆ Rev | 5’- TAATAAGCTTTCAGTTTCAGCCGAGGTCCTTGTTC                              |
| ACP proteins                  | Concentration of solvent C (%) | Reaction time (min) |
|------------------------------|-------------------------------|---------------------|
|                              | 0−5 min                       | 5−25 min           |                      |
| tiglyl-VinP1 ACP1CDD3        | 43                            | 43−53              | 8, 15, 120           |
| butyryl-VinP1 ACP3CDD3       | 43                            | 43−53              | 8, 15, 120           |
| tiglyl-VinP1 ACP3DE3         | 40                            | 40−50              | 240                  |
| tiglyl-VinP2 ACP4CDD4        | 43                            | 43−53              | 240                  |
| tiglyl-VinP3 ACP6CDD6        | 47                            | 47−52              | 240                  |
| tiglyl-ACP3CDD3              | 43                            | 43−53              | 60, 120              |
| tiglyl-ACP5CDD3              | 42                            | 42−52              | 120, 240             |
| tiglyl-ACP7CDD3              | 43                            | 43−60              | 120, 240             |

**Table S6.** Solvent C concentration of HPLC analysis of the transacylation reaction and the reaction time in this study.
Table S7. A list of primers used for preparation of ACP-CDD chimeric proteins.

| Primers                        | Nucleic acid sequence of primers                                      |
|--------------------------------|-----------------------------------------------------------------------|
| ACP₃CDD₃_Fragment1_FWD         | 5’- GCCTTTTTACGGTTCTCTGGCCTTTTGCTGG                                   |
| ACP₃CDD₃_Fragment1_REV         | 5’- CCTCCTCCTCCAAGCCGGTGGCCAGCTC                                      |
| ACP₃CDD₃_Fragment2_FWD         | 5’- ACCCGGCTTTGGAGGAGGAGGACTCGACC                                     |
| ACP₃CDD₃_Fragment2_REV         | 5’- GCCAGGAACCGTAAAAGGCGCGTTGC                                       |
| ACP₆CDD₃_Fragment1_FWD         | 5’- CCTCCTCCTCGTGACAGCCTCCGAC                                         |
| ACP₆CDD₃_Fragment1_REV         | 5’- GCCAGGAACCGTAAAAGGCGCGTTGC                                       |
| ACP₆CDD₃_Fragment2_FWD         | 5’- GCCTTTTTACGGTTCTCTGGCCTTTTGCTG                                   |
| ACP₆CDD₃_Fragment2_REV         | 5’- CCTCCTCCTCGTGACAGCCTCCGAC                                         |
| pColdW-vinP4_ACP₇_FWD          | 5’- AAAAAAATATGGACTCCCGGACGAGCCGAGCACC                                 |
| pColdW-vinP4_ACP₇_REV          | 5’- TAAGCTTCTAGATGGTCGCCGAGGAGGAGTCCGG                                 |
| ACP₇CDD₇_Fragment1_FWD         | 5’- GCCCTTTTTACGGTTCTCTGGCCTTTTGCTG                                   |
| ACP₇CDD₇_Fragment1_REV         | 5’- CCTCCTCCTCGCCAGGACGACCTTGCC                                      |
| ACP₇CDD₇_Fragment2_FWD         | 5’- GCCAGGAACCGTAAAAGGCGCGTTGC                                       |
| ACP₇CDD₇_Fragment2_REV         | 5’- GCCAGGAACCGTAAAAGGCGCGTTGC                                       |
### Table S8. Summary of the ESI-MS analysis of apo-, holo- and acyl-ACP proteins in this study.

| ACP proteins                  | Obs. (Da) | Calc. (Da) |
|-------------------------------|-----------|------------|
| apo-VinP1 ACP$_3$CDD$_3$      | 21,278    | 21,283     |
| holo-VinP1 ACP$_3$CDD$_3$     | 21,618    | 21,624     |
| tiglyl-VinP1 ACP$_3$CDD$_3$   | 21,701    | 21,706     |
| butyryl-VinP1 ACP$_3$CDD$_3$  | 21,688    | 21,694     |
| apo-VinP1 ACP$_3$DE$_3$       | 18,288    | 18,291     |
| holo-VinP1 ACP$_3$DE$_3$      | 18,627    | 18,632     |
| tiglyl-VinP1 ACP$_3$DE$_3$    | 18,709    | 18,714     |
| apo-VinP2 ACP$_4$CDD$_4$      | 22,815    | 22,819     |
| holo-VinP2 ACP$_4$CDD$_4$     | 23,155    | 23,160     |
| tiglyl-VinP2 ACP$_4$CDD$_4$   | 23,237    | 23,242     |
| apo-VinP3 ACP$_6$CDD$_6$      | 22,211    | 22,216     |
| holo-VinP3 ACP$_6$CDD$_6$     | 22,551    | 22,557     |
| tiglyl-VinP3 ACP$_8$CDD$_6$   | 22,634    | 22,639     |
| apo-ACP$_4$CDD$_3$            | 21,244    | 21,248     |
| holo-ACP$_4$CDD$_3$           | 21,584    | 21,589     |
| tiglyl-ACP$_4$CDD$_3$         | 21,666    | 21,671     |
| apo-ACP$_6$CDD$_3$            | 21,743    | 21,748     |
| holo-ACP$_6$CDD$_3$           | 22,083    | 22,089     |
| tiglyl-ACP$_8$CDD$_3$         | 22,165    | 22,171     |
| apo-ACP$_2$CDD$_3$            | 21,495    | 21,501     |
| holo-ACP$_7$CDD$_3$           | 21,838    | 21,842     |
| tiglyl-ACP$_2$CDD$_3$         | 21,918    | 21,924     |
Table S9. Summary of the ESI-MS analysis of *crypto*-ACP proteins in this study.

| ACP proteins                                           | Obs. (Da) | Calc. (Da) |
|--------------------------------------------------------|-----------|------------|
| Br-acetyl pantetheinamide-VinP1 ACP<sub>3</sub>CDD<sub>3</sub> | 21,722    | 21,728     |
| Cl-acetyl pantetheinamide-VinP1 ACP<sub>3</sub>CDD<sub>3</sub> | 21,677    | 21,683     |
| Cl-acetyl pantetheinamide-VinP1 ACP<sub>3</sub>DE<sub>3</sub> | 18,685    | 18,691     |
| Cl-acetyl pantetheinamide-VinP2 ACP<sub>4</sub>CDD<sub>4</sub> | 23,214    | 23,219     |
| Cl-acetyl pantetheinamide-VinP3 ACP<sub>6</sub>CDD<sub>6</sub> | 22,610    | 22,616     |
| Cl-acetyl pantetheinamide-ACP<sub>4</sub>CDD<sub>3</sub> | 21,642    | 21,648     |
| Cl-acetyl pantetheinamide-ACP<sub>6</sub>CDD<sub>3</sub> | 22,142    | 22,148     |
| Cl-acetyl pantetheinamide-ACP<sub>7</sub>CDD<sub>3</sub> | 21,896    | 21,901     |
Figure S1. Polyketide synthases in the biosynthesis of vicenistatin.\textsuperscript{[1,4]} Domains used in this study are colored. CP; carrier protein, ACP; acyl carrier protein, KS; ketosynthase, AT; acyltransferase, DH; dehydratase, KR; ketoreductase, ER; enoylreductase, TE; thioesterase, NDD; N-terminal docking domain, CDD; C-terminal docking domain.
**Figure S2.** A) Amino acid sequence alignment of C-terminus of VinP1 and 6-deoxyerythronolide B synthase 2 (DEBS2). The residues involved in the interaction with NDD are marked with purple dots. B) Amino acid sequence alignment of N-terminus of VinP2 and DEBS3. The residues involved in the interaction with CDD are marked with purple filled circles. These amino acid sequences were aligned by ClustalW\textsuperscript{[5]} and presented by ESPript.\textsuperscript{[6]} C) The solution Nuclear Magnetic Resonance (NMR) structure of DEBS2 NDD\textsubscript{3} (green) and DEBS3 NDD\textsubscript{4} (cyan) complex [Protein Data Bank (PDB) entry 1PZR].\textsuperscript{[7]} The residues involved in the interaction are shown in sticks.
Figure S3. A) Predicted dimer structure of acyl-VinP1 ACP_{3CDD}{\text{a}}. ACP_{3} part, dimerization element (DE) part and C-terminus docking domain (CDD) part of acyl-VinP1 ACP_{3CDD}{\text{a}} are represented by using DEBS1 ACP_{2} [Protein Data Bank (PDB) entry; 2JU1]^{[8]}, DEBS1 DE_{2} [PDB entry; 1PZQ]^{[7]} and DEBS1 CDD_{2} [PDB entry; 1PZR]^{[7]} as the alternative structures, respectively. B) The amino acid sequence of C-terminal region of VinP1.
Figure S4. The transacylation reaction between VinP2 NDD₄KS₄ and butyryl-VinP1 ACP₃CDD₃. A) The substrate of the VinP2 KS₄ domain and butyryl-ACP₃CDD₃, the latter of which is a substrate mimic for the VinP2 KS₄ domain. B) The transacylation reaction with butyryl-VinP1 ACP₃CDD₃.
Figure S5. LC-ESI-MS analysis of VinP1 ACP₃CDD₃ proteins. The mass spectra and deconvoluted mass spectra of VinP1 ACP₃CDD₃ proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the VinP1 ACP₃CDD₃ proteins lacking N-terminal methionine. Non-labeled peaks show the full-length VinP1 ACP₃CDD₃ proteins.
Figure S6. Quantitative analysis of the transacylation activity of VinP2 KS4 domain against tiglyl-VinP1 ACP3CDD3. A) Formation of holo-VinP1 ACP3CDD3. Formation of holo-VinP1 ACP3CDD3 in the reaction of VinP2 NDD4KS4AT4 S684G is shown in blue. Formation of holo-VinP1 ACP3CDD3 in the reaction of VinP2 NDD4KS4AT4 C206G/S684G is shown in orange. B) Initial velocity of the transacylation reaction between VinP2 NDD4KS4AT4 S684G and tiglyl-VinP1 ACP3CDD3. All these data were measured three times each.
**Figure S7.** A) The sequence alignment of KS domains. Conserved KS active site residues related to the transacylation reaction are marked with purple filled circles. The amino acid sequences were aligned by ClustalW\(^{[5]}\) and presented by ESPript.\(^{[6]}\) VinP2 KS\(_4\) (*Streptomyces halstedii*, UniProt; Q76KZ5), DEBS1 KS\(_1\) (*Saccharopolyspora erythraea*, UniProt; Q03131), DEBS2 KS\(_2\) (*Saccharopolyspora erythraea*, UniProt; Q03132), DEBS3 KS\(_3\) (*Saccharopolyspora erythraea*, UniProt; Q03133), CurL KS (*Moorea producens 3L*, UniProt; F4Y424). B) Model structure of the active site of VinP2 KS\(_4\) domain. The model structure was built by SWISS-MODEL\(^{[9]}\) using DEBS3 KS\(_5\) domain [PDB entry; 2HG4]\(^{[10]}\) as a template structure. The active site residues are shown in sticks.
Figure S8. HPLC analysis of the transacylation reaction between VinP2 NDD$_4$KS$_4$AT$_4$ double mutant (H341A/S684G and H381A/S684G, respectively) and tiglyl-VinP1 ACP$_3$CDD$_3$. 
**Figure S9.** Proposed transacylation mechanism between KS domain and acyl-ACP.\textsuperscript{[11–14]} The residue and residue number of VinP2 KS\textsubscript{4} domain are shown in parentheses. In the proposed mechanism of the transacylation reaction of VinP2 KS\textsubscript{4} domain, His\textsubscript{381} promotes deprotonation of Cys\textsubscript{206}, which attacks the acyl group on the terminus of pantetheine arm. The oxyanion hole formed by the main chain amides of Ile\textsubscript{448} and Cys\textsubscript{206} stabilizes the anion generated by the attack of Cys\textsubscript{206}, and His\textsubscript{341} then protonates the thiol of pantetheine.
**Figure S10.** LC-ESI-MS analysis of VinP2 ACP₄CDD₄ proteins. The mass spectra and deconvoluted mass spectra of VinP2 ACP₄CDD₄ proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the VinP2 ACP₄CDD₄ proteins lacking N-terminal methionine. Non-labeled peaks show the full-length VinP2 ACP₄CDD₄ proteins.
Figure S11. LC-ESI-MS analysis of VinP3 ACP₆CDD₆ proteins. The mass spectra and deconvoluted mass spectra of VinP3 ACP₆CDD₆ proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the VinP3 ACP₆CDD₆ proteins lacking N-terminal methionine. Non-labeled peaks show the full-length VinP3 ACP₆CDD₆ proteins.
Figure S12. HPLC analysis of the transacylation reaction between VinP2 NDD4KS4 and tiglyl-ACPcDD4 proteins. A) The transacylation reaction with tiglyl-VinP2 ACPcCDD4. B) The transacylation reaction with tiglyl-VinP3 ACPcCDD6.
Figure S13. LC-ESI-MS analysis of VinP1 ACP3DE3 proteins. The mass spectra and deconvoluted mass spectra of VinP1 ACP3DE3 proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the VinP1 ACP3DE3 proteins lacking N-terminal methionine. Non-labeled peaks show the full-length VinP1 ACP3DE3 proteins.
Figure S14. HPLC analysis of the transacylation reaction between VinP2 NDD\textsubscript{4}KS\textsubscript{4} and tiglyl-VinP1 ACP\textsubscript{3}DE\textsubscript{3}.
**Figure S15.** The amino acid sequence alignment of ACPs. These amino acid sequences were aligned by ClustalW\(^5\) and presented by ESPript.\(^6\) The percentages show the amino acid sequence identity to VinP1 ACP\(_3\). The secondary structural elements of DEBS1 ACP\(_2\) [PDB entry 2JU1]\(^8\) are indicated below the sequence.
Figure S16. The strategy for construction of the expression plasmids of ACP<sub>x</sub>CDD<sub>3</sub> chimeric proteins. The Fragment 1 including gene encoding ACP<sub>x</sub> part and fragment 2 including gene encoding CDD<sub>3</sub> part were combined by Gibson assembly.
The gene encoding ACP₄CDD₃ chimeric protein

5’-CATATGAGCGCGGGGGGACTTTGAGCAGCGGCTCGCGCTCTCACCGCGGAGCAGCGACCGAGACCGCATCGATCCCCGCCGGGCTTTCAAGGAGATCGGGGTTCGACTCGCTCAGGCTGTCGAACTGCGGAACAGGCTGAACGCGGCCACCGGACTGCGGCTGCCCGCCACACCTGGTCTTCGACTATCCGACGCCCACGGTGC
TCTCGCAGTACCTCCTGGCGGAGCTGGCA
CCCGGCTTGGAAGAGAACGGACGCGGGAGACTGATGCGGGAGATCGGGCTGGAGTGCGGCGAAGCTGGCCGACGCGGATGCCCAGCGGGCCGACGAGGACGACCCGGGCCTGGAGTCCGCGACCGCGGACGAACTGTTCGACATCCT
CGGTGAGCTGTCCACCGGACTGACCTCGAG-3’

The amino acid sequence of ACP₄CDD₃ chimeric protein
(Ser2072-Leu2169 of VinP2)-(Glu5747-Asp5826 of VinP1)

SGGGLEQRLAALTAERTETVLELVRERVAAVLGHASADADPARAFKEIGFDSLTAVELRNRLNAATGLRLPAATLVDYPTPTVLQYYLLAEELAPGEEEDSTAELMREIARLEAAVTSASS
AGGAGLAPAVDRLRAMAALKADADAQRAEDDPGLESATADELF DILDGE LSTD

Figure S17. The gene fragment and amino acid sequence of ACP₄CDD₃ chimeric protein. The gene regions encoding ACP₄ and CDD₃ are shown in orange and blue, respectively. The restriction enzyme cleavage sites are underlined. The amino acid sequences of ACP₄ and CDD₃ are shown in orange and blue, respectively.
The gene encoding ACP₆CDD₃ chimeric protein

5’-CATATGGAATCCAGACACCCCCGAAAGGACCATTACCCGCTCACCGCGGCTCGCCGCTCCGCT
CGAGCGTACACCCGCTGCTGAGCTGACACAGGGACAGGCCCCCTTCTCAAGGACCTCGGCTTCGACCTCC
GTCACCCGCGGATGAAGCTCAACAAGCAGGCGCTCCGGCCCTGCGGCCTGCGCCC
CAGGCCCCTCGCTCAGACTACGCAACCCCCGTCGCGCCCTCGCGGCCCATCTGCGCTCGAGAC
TGTTCCAGGAGGAGGAGGAGGAGGACTCGACCACGCGAGGAGCAGGAGATCGCCCGGGCT
GGAGGGCGCCTGACACCTCCCGGCTCACGGCCCTCAGGGCGGCAGCGCCGCGGCGGCTGCGGCTGGACCGGCTGGGCGCTAGGGCTGGCGAAGCTGGCCGACGCGGATGCCCAGCGGGCCGACTG
ACP₆ and CDD₃ are shown in green and blue, respectively. The restriction enzyme cleavage sites are underlined. The amino acid sequences of ACP₆ and CDD₃ are shown in green and blue, respectively.

The amino acid sequence of ACP₆CDD₃ chimeric protein

(Asp3178-Asp3279 of VinP3)-(Glu5747-Asp5826 of VinP1)

DDTPKETVVRLAGLSPAERDHALLELVRTQVAAVRYSMTSDVEQDSKFDLGSVTAV
ELRNKLNAASGLRPLATAVFDYATPVVALAHLRSELFDEEDSTAELEIRLAAVTDASAGGAGLAPAVDLRSLMAAKLADADAQRADEDMPGLESATDLEDFIDGELSTD

Figure S18. The gene fragment and amino acid sequence of ACP₆CDD₃ chimeric protein. The gene regions encoding ACP₆ and CDD₃ are shown in green and blue, respectively. The restriction enzyme cleavage sites are underlined. The amino acid sequences of ACP₆ and CDD₃ are shown in green and blue, respectively.
The gene encoding ACP7CDD3 chimeric protein

5’-
CATATGACTCCGCCGACGACCTCGCGGGCCGCCTCGGCCGGCTGGGCCGAGGGCAGCGCGCCGGCCGTTGGTGCTCGGTGTTCTCGTGCAGAGTGGCTGGGTACGCTCTCCGCTGACGTATCCCTCCTCGACCGACTCTCCTCCGCAAGGTCCGGATGGGAGGAGGACTCGACCGGAGCTGATGCGGGAGATCGCCGGCTGGAGGCCGCGTCACCTCGCGGCCTCATCGGGCAGCGGGCCTGGCACCGCCGGATCTCGTGGAGCCGGAGCGCGTTCCAGGACCTCGGCTTCGATTCGCTGACGACGGTCGAACTGCGCAACGGCCTCACGGCCGTCGCGGGGTCGCCTCCCGGCGACGCTCGTCTTCGACTATCCCTCGACCGACA

The amino acid sequence of ACP7CDD3 chimeric protein
(Asp1643-Ala1744 of VinP4)-(Glu5747-Asp5826 of VinP1)

DSADDLAGRLAGLAEARRPLVLGVVR AQVAQVGLYASADLVEPERAFQDLGFDSLTA VE LRNGLTAVAGVRPALT LVDYPSDLTFLAELS GKVAVAEEEDSTAEMLREARLEAAV TSAASSAGGAGLAPAVDRLRAMAALK ADADAQRADED DPGLESATADEL DILDGELSTD

Figure S19. The gene fragment and amino acid sequence of ACP7CDD3 chimeric protein. The gene regions encoding ACP7 and CDD3 are shown in red and blue, respectively. The restriction enzyme cleavage sites are underlined. The amino acid sequences of ACP7 and CDD3 are shown in red and blue, respectively.
Figure S20. LC-ESI-MS analysis of ACP₄CDD₃ proteins. The mass spectra and deconvoluted mass spectra of ACP₄CDD₃ proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the ACP₄CDD₃ proteins lacking N-terminal methionine. Non-labeled peaks show the full-length ACP₄CDD₃ proteins.
Figure S21. LC-ESI-MS analysis of ACP<sub>6</sub>CDD<sub>3</sub> proteins. The mass spectra and deconvoluted mass spectra of ACP<sub>6</sub>CDD<sub>3</sub> proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the ACP<sub>6</sub>CDD<sub>3</sub> proteins lacking N-terminal methionine. Non-labeled peaks show the full-length ACP<sub>6</sub>CDD<sub>3</sub> proteins.
Figure S22. LC-ESI-MS analysis of ACP\textsubscript{7}CDD\textsubscript{3} proteins. The mass spectra and deconvoluted mass spectra of ACP\textsubscript{7}CDD\textsubscript{3} proteins are shown in left panels and right panels, respectively. Peaks labeled with asterisk show the ACP\textsubscript{7}CDD\textsubscript{3} proteins lacking N-terminal methionine. Non-labeled peaks show the full-length ACP\textsubscript{7}CDD\textsubscript{3} proteins.
**Figure S23.** HPLC analysis of the transacylation reaction between VinP2 NDD₄KS₄ and tiglyl-ACP₄CDD₃ chimeric proteins. A) The transacylation reaction with tiglyl-ACP₄CDD₃. B) The transacylation reaction with tiglyl-ACP₆CDD₃. C) The transacylation reaction with tiglyl-ACP₇CDD₃.
Figure S24. SDS-PAGE analysis of the crosslinking between VinP2 NDD₄KS₄AT₄ S684G and Br-acetyl pantetheinamide-VinP1 ACP₃CDD₃. Lane M) Protein Marker. Lane 1–4) the crosslinking reaction of VinP2 NDD₄KS₄AT₄ S684G, and the reaction times are 0, 15, 120 and 360 min, respectively. Lane 5–8) the crosslinking reaction of VinP2 NDD₄KS₄AT₄ C206G / S684G, and the reaction times are 0, 15, 120 and 360 min, respectively.
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