Supporting Information for:

Structure and Mechanism for Iterative Amide N-Methylation in the Biosynthesis of Channel-forming Peptide Cytotoxins

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Materials and Methods

Materials
Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT) and enzymes for PCR, plasmid digestion, proteolysis, and Gibson Assembly were purchased from New England Biolabs. Bacterial growth media and other reagents were purchased from Thermo Fisher Scientific, Millipore Sigma, Bachem, and Cayman Chemical.

General Methods
Protein affinity and size-exclusion chromatography (SEC) were carried out using an ÄKTApel prime plus purifier (GE Healthcare). High-performance liquid chromatography (HPLC) was performed with a Shimadzu Prominence Liquid Chromatography system. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) data were collected on UltraflexXtreme and Autoflex Speed MS instruments (Bruker Daltonics). Unless otherwise stated, reactions for the synthesis of 6 were carried out under a stream of N2 gas in flame-dried round-bottom flasks. Protein concentrations were determined using the Bradford assay with bovine serum albumin standards1 by measuring absorbance at 595 nm in 1 cm cuvettes using a NanoDrop One (Thermo Fisher Scientific).

Cloning and heterologous expression of AerA, AerD, and AerE (wild-type and site-directed variants)

AerA and AerD: The aerA and aerD genes from M. aerodenitrificans DSM 15089 were cloned as previously described into expression vectors encoding for His8-AerA in pACYCDuet-1 and tag-free AerD in a pCDFDuet-1 derivative plasmid (pBADcdf) containing the arabinose-inducible promoter (pBAD).2 Following co-transformation of E. coli BL21 (DE3) using the above plasmids, transformants were grown in 4 L of terrific broth (TB) medium containing appropriate antibiotics until an optical density at 600 nm (OD600) of 1.6–2.0 was achieved. Cells were cooled on ice for 30 min and His8-AerA expression was induced by addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) with growth at 18 °C for 1 h (220 rpm) followed by the addition of 0.2% (w/v) L-arabinose for induction of AerD and continued growth for 20 h at 18 °C (220 rpm).

AerE: All aerE expression plasmids were constructed using the pET hexahis (His8) small ubiquitin-like modifier (SUMO) tobacco etch virus (TEV) protease ligation-independent cloning (PLIC) vector (2S-T) (Table S1). This vector was a gift from Scott Gradia (Addgene plasmid # 29711). Primers designed to include the LICv1 tags were used for PCR amplification of aerE from M. aerodenitrificans DSM 15089 gDNA. Amplicons were used in a Gibson Assembly reaction with SspI-linearized 2S-T following the manufacturer (NEB) protocol and products were used to transform E. coli DH5α. Site-directed mutations were incorporated by three-component Gibson Assemblies containing two aerE gene-fragments containing the PCR-derived mutations (Table S1). Transformant plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced by services from ACGT, Inc. (Wheeling, IL). Sequence-verified His8-SUMO-AerE plasmids were used to transform E. coli Rosetta (DE3) competent cells for protein overexpression. Protein was overexpressed by growth at 37 °C in Luria-Broth (LB) containing appropriate antibiotics to an OD600 of 0.4–0.6. Protein expression was then induced by addition of 0.5 mM.
IPTG and growth for 18–20 h at 18 °C. 2–4 L of LB media was used for the overexpression of wild-type and site-directed variants of His$_6$-SUMO-AerE.

**Protein purification**

**AerE (tag-free)**

Cells from a 4 L culture were harvested by centrifugation at 3,000 x g for 30 min at 4 °C and resuspended in buffer A (0.5 M NaCl, 20 mM tris-HCl, and 10% glycerol (v/v), pH 8.0) before lysing via sonication. The lysate was cleared by centrifugation at 15,400 x g for 1 h at 4 °C and the supernatant was injected onto a 5 mL HisTrap nickel-nitrioltriacetic acid (Ni-NTA) affinity column (GE Healthcare) equilibrated with buffer B (1 M NaCl, 30 mM imidazole, 20 mM tris-HCl, pH 8.0). The column was washed with 70 mL of buffer B prior to eluting the bound protein with a linear gradient of increasing buffer C (1 M NaCl, 500 mM imidazole, 20 mM tris-HCl, pH 8.0). Fractions containing His$_6$-SUMO-AerE were pooled and treated with 30 µg/mL of tobacco etch virus (TEV) protease while dialyzing against 0.2 M NaCl, 2.5 mM β-mercaptoethanol (BME), and 20 mM tris-HCl, pH 8.1 for 12 h at 4 °C. Dialysis was continued for another 4 h against a similar buffer lacking BME before injecting the sample onto a 5 mL Ni-NTA affinity column (GE Healthcare) equilibrated with buffer A for subtractive Ni-affinity purification. The column was subsequently washed with buffer A (50 mL) and eluted using the following step-wise gradient of increasing buffer B: 10%, 20%, 30%, 40%, 50%, and 100% in 5–10 mL increments. Tag-free AerE primarily eluted at 30% B. Pooled AerE fractions were concentrated to ≤5 mL and purified by size-exclusion chromatography (SEC) by injecting the sample onto a 120 mL Superdex 200 10/300 GL column (GE Healthcare) with 300 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES, pH 7.5 used for equilibration and isocratic elution. AerE eluted as a monomer and appeared ≥95% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). AerE variants used in the mutational analysis displayed in Figure 6A were purified in the same manner.

**SUMO-AerE (wild-type and SDMs used for mutational analysis in Fig. S32)**

Cells from a 2 L culture were harvested, lysed, and centrifuged as described for tag-free AerE. The clarified lysate was added to 3 mL of Ni-NTA resin (Thermo Fisher Scientific) and allowed to bind by gravity elution. The loaded resin was washed with 70 mL of buffer B and eluted with 2 mL fractions of 10% C (2x), 50% C (2x), and 100% C (5x). Pooled fractions were concentrated and used directly in $N$-methylation assays (Fig. S32).

**AerA$_{DL}$**

Cells from a 4 L culture were harvested, lysed, and centrifuged as above. The clarified lysate was purified by Ni-NTA chromatography in the same manner as for tag-free AerE (above). The purified fractions of AerA$_{DL}$/AerD were pooled and denatured by dialysis into denaturing buffer (8 M urea, 150 mM NaCl, 10 mM tris, 2 mM dithiothreitol [DTT], pH 7.8) with vigorous stirring at room temperature for 16 h. The solution was purified again by Ni-NTA chromatography by injecting the dialyzed contents onto a 5 mL Ni-NTA affinity column (GE Healthcare) followed by washing the column with 150 mL of denaturing buffer sans DTT. Denaturing buffer sans DTT and containing 500 mM imidazole was used to elute the bound AerA$_{DL}$ in a single 50 mL fraction. Purified AerA$_{DL}$ was dialyzed against 10 mM HEPES pH 7.5 and concentrated by lyophilization prior to usage in enzyme reactions.

**Preparative proteolysis of AerA$_{DL}$ for isolation of minimal substrates**

AerA$_{DL}$ (250 mM) was treated with either trypsin (0.4 µg/µL) or proteinase K (0.2 µg/µL; New England Biolabs, cat. # P8107) in reaction buffer (final reaction volume = 500 µL) and incubated for 12 h at ambient temperature (20–22 °C). The reactions were terminated by heat inactivating/precipitating the enzymes at 95 °C for 5 min, followed by centrifugation removal of the precipitate and HPLC purification of the proteolyzed fragments in the supernatant (AerA$_{DL,21L0}$ and AerA$_{DL,31-35}$; see HPLC purification).

s3
In vitro N-methylation of AerADL, AerADL21LP, AerADL31-35, and AerADL34 (SPPS-derived)

AerADL: N-methylation reactions (20 µL) with substrate AerADL were carried out at 22 °C and contained 25 µM AerE (tag-free; following SEC), 100 µM AerADL, and 1 mM SAM in reaction buffer (50 mM KCl, 50 mM K2HPO4 pH 8, 200 µM MgCl2, 10% glycerol). Reactions were terminated by heat-precipitating the enzyme at 95 °C for 5 min. The precipitate was removed by centrifugation, and the peptide products in the supernatant were trypsinized (0.4 µg/µL) for 30 min at 22 °C before desalting with a C4 ZipTip (Millipore Sigma) following the manufacturer instructions. Full extent of methylation (+70 Da) was observed by MALDI-TOF MS after ~16 h (Fig. 2B and 3A).

AerADL21LP, AerADL31-35, and AerADL34: N-methylation of HPLC-purified AerADL21LP (tryptic fragment), AerADL31-35 (proteinase K fragments), and AerADL34 (SPPS-derived) by AerE was carried out and analyzed by MALDI-TOF MS as for the AerADL substrate, sans the trypsinization. Full extent of methylation (+70/70.2 Da, depending on the mass resolution) was observed by MALDI-TOF MS for all species (Figs. 2C, 6A, and S1).

Processivity analysis of AerE

Reactions were carried out and terminated as described (see In vitro N-methylation of AerADL), except 20 µM enzyme was used and a larger reaction volume of 300 µL. At each time point, 20 µL were extracted for heat-quenching, trypsinization, desalting, and MALDI-TOF MS analysis. The peak intensities were calculated using Bruker Analysis software and normalized before plotting in OriginPro 2016 (OriginLab) software (Fig. 3A).

Tandem MS analysis (ESI MS/MS) of methylated AerADL intermediates

Four 100 µL reactions were setup as described (see In vitro N-methylation of AerADL) and quenched at four different time points (i.e., 5, 30, 75, and 200 min) to isolate mono-, di-, tri-, and tetra-N-methylated (1Me – 4Me) AerADL intermediates. After heat-denaturing and trypsinization, the reaction products were isolated by HPLC using a GRACE Denali C18 (120 Å; 5 µm; Part No. 238DE54; length 250 mm; ID 4.6 mm), flow-rate of 1 mL/min, and a detection wavelength of 280 nm. Elution consisted of a mobile phase of H2O/ACN (A/B) + 0.1% (v/v) formic acid and a linear gradient of 2–70% B over 35 min followed by a linear step to 90% B held for 10 min. 1Me – 4Me AerADL21 peptides eluted between 35–42 min (determined by MALDI-TOF MS). HPLC-isolated peptides were directly infused into a ThermoFisher Scientific Orbitrap Fusion electrospray-ionization mass-spectrometer (ESI-MS) using an Advion TriVersa Nanomate 100. The following parameters were used: resolution = 120,000; isolation width (MS/MS) = 2 m/z; normalized collision energy (MS/MS) = 35; activation q value (MS/MS) = 0.4; and activation time (MS/MS) = 30 ms. Collision-induced dissociation (CID) and high-energy collisional dissociation (HCD) were used to fragment the parent ion at 35%. Data analysis was performed in the Qualbrowser application of Xcalibur software (Thermo Fisher Scientific). Only peaks with less than 5 ppm error were tabulated and included in the analysis (Tables S2-S6).

Synthesis of N2-((9H-fluoren-9-yl)methoxy)carbonyl)-N4-methyl-N4-(2,4,6-trimethoxybenzyl)-D-asparagine (6)
Scheme S1: Synthesis of precursor (6) for the SPPS of 5Me-AerA<sub>DL,34</sub>. Tmb = 2,4,6-trimethoxybenzyl.

**N-methyl-1-(2,4,6-trimethoxyphenyl)methanamine (1)**
The procedure reported by Itoh et al. was performed with a slight adaptation. Specifically, to a solution of 2,4,6-trimethoxybenzaldehyde (3 g, 15.3 mmol) stirring in 80 mL of THF was added aqueous methylamine (2.64 mL, 34.0 mmol) from a 40% (w/w) stock solution. The reaction was stirred for 2 h at 22 °C before removing THF by rotary evaporation and adding 100 mL of dichloromethane (DCM). The organic layer was washed twice with 50 mL of H<sub>2</sub>O, dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated in vacuo to afford the crude imine as a yellow crystalline solid. To the imine dissolved in 50 mL of EtOH was added NaBH₄ (1.74 g, 46.0 mmol) at 0 °C. The reaction was stirred at 0 °C for 20 min followed by stirring at 22 °C for 14 h. The solution was filtered through Whatman filter paper and the filtrate quenched by the addition of H<sub>2</sub>O (50 mL) at 0 °C. The EtOH was removed by rotary evaporation, and the aqueous solution was extracted twice with DCM (150 mL). The basic aqueous layer was basified to pH 9 with K₂CO₃. The basic aqueous layer was extracted with DCM (3 x 150 mL), and the organic layers were dried with Na₂SO₄ and concentrated in vacuo to afford 1 as a yellowish oil (2.28 g, 70%). <sup>1</sup>H NMR (500 MHz, CDCl₃) δ 2.34 (3H, s, NMe), 3.70 (2H, s, Tmb CH₂), 3.77 (6H, s, OMe), 3.78 (3H, s, OMe), 6.09 (2H, s, Ar) (Fig. S6).
Benzyl N2-(tert-butoxycarbonyl)-N4-methyl-N4-(2,4,6-trimethoxybenzyl)-D-asparaginate (3)
The procedure reported by Itoh et al. was performed with a slight adaptation. To a solution of N-Boc-D-aspartate O-benzyl ester (2) (2.96 g, 9.15 mmol) in 60 mL THF was added 1-hydroxybenzotriazole monohydrate (3.3 g, 21.5 mmol), N,N'-disopropylcarbodiimide (2.17 mL, 14.0 mmol), and 1 (2.28 g, 10.8 mmol). The solution was allowed to stir for 10 min before adding N-ethylmorpholine (1.36 mL, 10.8 mmol) dropwise over 3 min. The reaction mixture was stirred for 20 h at room temperature before quenching with saturated aqueous NH₄Cl (30 mL) at 0 °C. The organic layer was diluted with EtOAc (100 mL) and washed with brine (30 mL). The aqueous layer was extracted with additional EtOAc (2 x 100 mL) before pooling the organic layers, drying with Na₂SO₄, and concentrating in vacuo. The crude product was purified using flash column chromatography with a hexane:EtOAc (5:1 to 2:1) mobile phase to provide 3 (4.20 g, 76%) as an eggshell-white foam. ¹H NMR (500 MHz, CDCl₃, signals were derived from two different rotamers: major [A] and minor [B]) δ 1.37 (9H, s, Boc CH, [B]), 1.38 (9H, s, Boc CH, [A]), 2.59 (3H, s, NMe, [B]), 2.63 (3H, s, NMe, [A]), 3.06 (1H, dd, J = 17.0, 4.1 Hz, Cβ-H, [A]), 3.43 (1H, dd, J = 16.9, 4.0 Hz, Cβ–H, [A]), 3.67 (6H, s, OMe, [A]), 3.69 (6H, s, OMe, [B]), 3.73 (3H, s, OMe, [A/B]), 4.30 (2H, s, Tmb CH₂, [A]), 4.46–4.70 (1H, m, Cα–H, [A/B]), 5.05–5.23 (2H, m, Bn CH₂, [A/B]), 5.69 (1H, d, J = 9.1 Hz, NH, [B]), 5.92 (1H, d, J = 9.4 Hz, NH, [A]), 6.04 (2H, s, Tmb Ar, [A]), 6.06 (2H, s, Tmb Ar, [B]), 7.20–7.31 (5H, m, Bn Ar, [A/B]) (Fig. S7).

Benzyl N4-methyl-N4-(2,4,6-trimethoxybenzyl)-D-asparaginate (4)
The procedure reported by Itoh et al. was performed with a slight adaptation. To a solution of 3 (100 mg, 0.19 mmol) dissolved in DCM (5 mL) was added ZnBr₂ (218 mg, 0.97 mmol). The suspension was stirred vigorously for 2 h before concentrating by rotary evaporation and quenching with acidified H₂O (7 mL, pH 4) at 0 °C. The DCW was removed by rotary evaporation and the aqueous solution was washed once with EtOAc (50 mL). The aqueous layer was basified to pH 9 with solid K₂CO₃ and extracted with EtOAc (3 x 50 mL). The pooled organic layers were dried with Na₂SO₄ and concentrated in vacuo to afford the amine 4 (78 mg, 98%). It is noted that an increased scale of this reaction (i.e., 1.2 g of 3) resulted in product retention in the organic phase following the H₂O (70 mL, pH 4) quench and addition of EtOAc (100 mL). The crude product 4 was then purified by flash column chromatography using an initial mobile phase of CHCl₃ followed by CHCl₃:MeOH (95:5) to yield 4 (660 mg, 68%) as a light-yellow foam. ¹H NMR (500 MHz, CDCl₃, signals were derived from two different rotamers: major [A] and minor [B]) δ 2.67 (3H, s, NMe, [A/B]), 3.27–3.51 (2H, m, Cβ-H, [B]), 3.65 (6H, s, OMe, [A/B]), 3.80 (3H, s, OMe, [A/B]), 3.70–3.78 (2H, m, Cβ-H, [A]), 4.32 (2H, s, Tmb CH₂, [A/B]), 4.68–4.75 (1H, m, Cα–H, [B]), 4.75–4.82 (1H, m, Cα–H, [A]), 5.18 (1H, d, J = 12.2 Hz, Bn CH₂, [A]), 5.37 (1H, d, J = 12.2 Hz, Bn CH₂, [A]), 6.03 (2H, s, Tmb Ar, [A]), 6.09 (2H, s, Tmb Ar, [B]), 7.21–7.38 (5H, m, Bn Ar, [A/B]), 7.9–9.0 (2H, br. s, NH₂ [A/B]) (Fig. S8).

benzyl N2-((9H-fluoren-9-yl)methoxy)carbonyl)-N4-methyl-N4-(2,4,6-trimethoxybenzyl)-D-asparaginate (5)
Similar to reported procedures³, the amine 4 (78 mg, 0.19 mmol) was dissolved in 1,4-dioxane/H₂O (10:9) (2 mL) before adding NaHCO₃ (90 mg, 1.07 mmol) and 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) (73 mg, 0.28 mmol) at 0 °C while stirring. The reaction was stirred at 0 °C for 1 h then at 22 °C for 30 min before quenching by addition of saturated NH₄Cl (2 mL) at 0 °C. The solution was diluted with H₂O (2 mL) and extracted with EtOAc (3 x 50 mL). The pooled organic layers were washed once with brine (5 mL), dried with Na₂SO₄, and concentrated by rotary evaporation. The crude product 5 was purified by flash column chromatography using a hexanes:EtOAc (5:1 to 2:1) mobile phase; affording 5 as a white foam (87 mg, 73%). ¹H NMR (500 MHz, CDCl₃, signals were derived from two different rotamers: major [A] and minor [B]) δ 2.67 (3H, s, NMe, [B]), 2.72 (3H, s, NMe, [A]), 3.17 (1H, dd, J = 16.7, 4.0 Hz, Cβ–H, [A]), 3.56 (1H, dd, J = 16.9, 3.9 Hz, Cβ–H, [A]), 3.72 (6H, s, OMe, [A]), 3.73 (6H, s, OMe, [B]), 3.81 (3H, s, OMe, [A/B]), 4.10–4.57 (5H, m, Fmoc CH₂/CH[3°], Tmb CH₂, [A/B]), 4.67–4.78 (1H, m, Cα–H, [A/B]),
N2-([(9H-fluoren-9-yl)methoxy]carbonyl)-N4-methyl-N4-(2,4,6-trimethoxybenzyl)-D-asparagine (6)

Similar to Itoh et al., compound 5 (82 mg, 0.130 mmol) was stirred in EtOAc:EtOH (1:1) (1.5 mL) before adding 5% Pd/C catalyst (30 mg, 0.013 mmol). The reaction flask was purged with H₂ from a balloon thrice for 30 sec and allowed to stir under H₂ at 22 °C for 2 h. The reaction was terminated by filtering the Pd/C through celite while eluting the product from the solid layer with EtOAc:MeOH (1:1) (25 mL). The filtrate was concentrated by rotary evaporation and the crude product was purified by flash column chromatography using a mobile phase of CHCl₃ followed by CHCl₃:MeOH (95:5) to yield 6 (63 mg, 89%) as a white foam.

1H NMR (500 MHz, CDCl₃, signals were derived from two different rotamers: major [A] and minor [B]) δ 2.72 (3H, s, NMe [B]), 2.77 (3H, s, NMe [A]), 3.10 (1H, dd, J = 17.1, 6.9 Hz, Cβ–H, [A]), 3.22 (1H, dd, J = 16.9, 3.2 Hz, Cβ–H, [B]), 3.59 (1H, dd, J = 17.2, 3.6 Hz, Cβ–H, [A]), 3.75 (6H, s, OMe, [A/B]), 3.79 (3H, s, OMe, [A/B]), 4.22 (1H, t, J = 7.0 Hz, Fmoc CH[3°], [A/B]), 4.30–4.47 (4H, m, Fmoc/Tmb CH₂, [A]), 4.60 (1H, d, J = 13.6 Hz, Tmb CH₂, [B]), 4.65–4.80 (1H, m, Cα–H, [A/B]), 6.09 (2H, s, Tmb Ar, [A]), 6.11 (2H, s, Tmb Ar, [B]), 6.36 (1H, d, J = 7.6 Hz, NH, [A]), 6.40 (1H, d, J = 7.3 Hz, NH, [B]), 7.27–7.77 (8H, m, Fmoc Ar, [A/B]) (Fig. S10).

Solid-phase peptide synthesis (SPPS) of AerADL₃₄ and 5Me-AerADL₃₄

Automated SPPS was carried out using a Rainin PS3 synthesizer on a 0.1 mM scale with Fmoc-Ala Wang resin. Coupling reactions contained 4 eq of the appropriate D- or L-configured Fmoc-amino acid or 6 and 4 eq of HCTU dissolved in DMF + 0.4 M N,N,N'-dimethylformamide. Each coupling step was performed twice prior to deprotection with 20% (v/v) piperidine in DMF. Following all rounds of double-coupling/deprotection, the protected resin-bound products corresponded to (D-configured amino acids in lower-case and side-chain protecting groups in superscript; Fig. S11):

AerADL₃₄: Fmoc-A-A-A-A-V-V-\text{Trt}^{Bu}-Y^{Bu}-L-G-A-A-N^{Trt}-V-V-G-A-A-N^{Trt}-G-T^{Bu}-V-T^{Bu}-A-N^{Trt}-A-V-A-A-N^{Trt}-T^{Bu}-N^{Trt} \text{A}-V-A-A -- Wang resin
AerADL₃₄: Fmoc-a-a-a-a-v-v-\text{Trt}^{Bu}-Y^{Bu}-l-g-a-n^{Trt}-v-v-g-a-a-n^{Trt}-g-t^{Bu}-v-t^{Bu}-a-n^{Trt}-a-v-a-n^{Trt}-t^{Bu}-n^{Trt} \text{a}-v-a-a -- Wang resin
5Me-AerADL₃₄: Fmoc-a-a-a-a-v-v-t^{Bu}-Y^{Bu}-l-g-a-a-(Me-n^{Tmb})-v-v-g-a-a-(Me-n^{Tmb})-g-t^{Bu}-V-t^{Bu}-(Me-n^{Tmb})-a-v-a-(Me-n^{Tmb})-T^{Bu}-(Me-n^{Tmb})-a-v-a -- Wang resin

The product was washed with DCM and dried with a N₂ stream. Resin-bound, crude peptide was globally deprotected by incubation with 4 mL of 95% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) triisopropylsilane (TIPS), and 2.5% (v/v) H₂O for 1 h at 20 °C. The resin was removed by filtration through glass wool and the peptide was precipitated by collecting the filtrate in cold ether (-20 °C). The precipitate was spun down (3,000 × g for 10 min at 4 °C), supernatant discarded, and the pellet resuspended in ice-cold ether. This process was iterated two more times before the crude-peptide pellet was dried under a stream of N₂.

HPLC purification of AerADL₃₄/5Me-AerADL₃₄ from SPPS, AerADL₂₁LP (trypsin fragment), and AerADL₂₁₃⁻₅₋ (proteasine K fragments; 31-35mer)

AerADL₃₄/5Me-AerADL₃₄ from SPPS

The deprotected, crude peptides were purified by reversed phase HPLC using a Macherey Nagel VP 250/10 NUCLEODUR C8 Gravity (5 µm) column with a mobile phase consisting of H₂O/ACN (A/B) + 0.1% FA and a detection wavelength of 280 nm. A linear gradient of 2–50% B over 35 min eluted the peptides at 26 min (AerADL₃₄) or 30 min (5Me-AerADL₃₄) as detected by MALDI-TOF MS (Fig. S11).

HPLC purification of AerADL₂₁LP (trypsin fragment)

Following trypsinization of AerADL (see In vitro N-methylation of AerADL), the AerADL₂₁LP product was purified by HPLC using a GRACE Denali C18 (120 Å; 5 µm; Part No. 238DE54; length 250 mm; ID 4.6
mm), a detection wavelength of 220 nm, and a mobile phase consisting of H$_2$O/ACN (A/B) + 0.1% FA with the following linear gradient: 2–70% B over 40 min. AerADL.21LP eluted at 22.5 min as detected by MALDI-MS TOF (Fig. S1).

HPLC purification of AerADL.31-35 (proteinase K fragments; 31-35mer)
Following overnight proteinase K treatment (see Proteinase K digestion of AerADL) a mixture of proteinase K fragments (Fig. 2) were purified by HPLC as described for AerADL.21LP and co-eluted at 30 min as detected by MALDI-TOF MS. The mixture was used directly for in vitro N-methylation assays (Figs. 2C and S2).

NMR analysis of AerADL.34 and 5Me-AerADL.34
The AerADL.34 and 5Me-AerADL.34 peptides purified by HPLC were dissolved in a mixture of CD$_3$OH (~93%) and H$_2$O (~7%) at a concentration of 1.5 mM in 5 mm Wilmad 528-PP-7 NMR tubes. A 750 MHz Varian NMR spectrometer (University of Illinois at Urbana-Champaign, NMR Laboratory) with a 5mm Indirect-detection HCN probe equipped with the Z-gradient capability was used to collect all proton, TOCSY, and NOESY spectra at 298 K (Figs. S12-S25). Mixing times of 30 ms and 70 ms for TOCSY and 300 ms for NOESY spectra were used for both peptides. All spectra were processed using the software Mnova 14.1.0 of Mestrelab. The proton shifts were assigned based on spin system identification using TOCSY spectra and sequential connectivities observed in the NOESY spectra, referenced to the solvent CD$_3$OH peak at 3.30 ppm.

Crystallization of AerE
Native AerE, SeMet-AerE, and site-directed AerE variants (Y137F, D141A, and N231A) bound to SAH Purified AerE was concentrated to 30 mg/mL prior to crystallization via centrifugation in Amicon Ultra Centrifugal Filters (10 kDa MWCO, Millipore Sigma). In all crystallization trials, AerE was first incubated with 3-5 molar equivalents of S-adenosyl homocysteine (SAH) on ice for 30 min. Initial sparse matrix screening of AerE was conducted in 96-well sitting-drop vapor diffusion trays using the Crystal Gryphon with each 0.4 µL drop consisting of a 1:1 volume ratio of protein to reservoir solution. Tetragonal bipyramid-like crystals with rounded edges appeared using reservoir solutions (i) 40% (v/v) PEG 300, 0.1 M sodium cacodylate pH 6.5, 0.2 M magnesium chloride and (ii) 40% (v/v) PEG 300, 0.1 M sodium cacodylate pH 6.5, 0.2 M calcium acetate after 24 h at 9 °C. Optimization of these crystallization conditions was conducted using 2 µL hanging-drop vapor diffusion with a 1:1 volume ratio of protein to reservoir solution. Selenomethionine (SeMet) derivatized AerE, prepared as previously$^4$, and site-directed variants were crystallized using similar conditions and methods.

AerE bound to SAH and peptide AerADL.34
Co-crystallization of AerE with purified AerADL.34 was conducted using similar methods with the addition of a prior incubation of AerE with 3 molar equivalents of AerADL.34 on ice for 30 min. Co-crystallization of the peptide-bound enzyme was conducted using hanging-drop vapor diffusion with a reservoir solution consisting of 18% (v/v) PEG 3350, 0.1 M sodium citrate pH 4.0, and 0.2 M sodium citrate. Each 2 µL drop consists of a 1:1 volume ratio of protein to reservoir solution and similar shaped crystals grown at 9 °C appeared within 24 h.

Crystallographic data collection, structure solution, and model refinement
X-ray diffraction data were collected at the Advanced Photon Source (Argonne National Lab) synchrotron beamlines 21-ID-D, 21-ID-F, and 21-ID-G operated by the Life Sciences Collaborative Access Team (LS-CAT). For detector and wavelength details, see the Protein Data Bank (PDB) entries 7RC2, 7RC3, 7RC4, 7RC5, and 7RC6. The raw reflection data were initially processed using autoPROC.$^5$ Phases were determined by single-wavelength anomalous dispersion (SAD) methods taking diffraction data from SeMet derivatized AerE crystals, prepared as previously.$^4$ SAD phasing was implemented in Phenix AutoSol$^6$, and
initial models of AerE were built using Buccaneer\(^7\) and Phenix AutoBuild.\(^8\) Model refinement was implemented in phenix.refine\(^9\) and Refmac5\(^10\) in combination with manual building and refinement in Coot.\(^11\)

**In vitro N-methylation of AerA\(_{DL,34}\) in the presence of excess EDTA or EGTA**

AerE from SEC purification (~80 nmol) was dialyzed against a 1 L solution of 100 mM NaCl, 20 mM HEPES, (± 5 mM EDTA or EGTA; ~60,000 molar excess), pH 7.5 for 12 h at 4 °C with vigorous stirring. Note, the protein solution contained KCl from SEC purification (~240 µmol). The dialyzed AerE solution was used to prepare N-methylation reactions containing 20 µM AerE, 100 µM AerA\(_{DL,34}\), and 1.5 mM SAM in 100 mM NaCl, 20 mM HEPES, pH 7.5 reaction buffer. Control reactions lacking AerE or featuring AerE dialyzed against a solution lacking EDTA/EGTA were setup in parallel. The reactions were heat-quenched and analyzed by MALDI-TOF MS as above (Fig. S27).

**Zn-inhibition of AerE and effect of additional metal ions on Zn-inhibition**

Reactions were setup in the same manner as for AerA\(_{DL,34}\) (above) with the addition of ZnCl\(_2\) (60 µM – 1 mM). Control reactions lacking ZnCl\(_2\) or lacking AerE were setup in parallel, and all reactions were analyzed by MALDI-TOF MS. To test the ability of Mg\(^{2+}\), Ca\(^{2+}\), or Mn\(^{2+}\) to rescue the Zn-inhibition, MgCl\(_2\), CaCl\(_2\), or MnCl\(_2\) were added to similar reactions at 25 mM and analyzed in the same manner (Fig. S29).

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**Protein Sequences**

**His\(_6\)-AerA** (bold = leader peptide; red = core peptide)

\[
\text{His}^6-\text{AerA} (\text{bold} = \text{leader peptide}; \text{red} = \text{core peptide}) \\
\text{MGSSHSHHSHHSSQDPMTTTTPASTQVFQTRDLETHIITKAWKDEYKAQLKPDPAALQDALKSIDPSLSLPDSLQVQHEENANLHVLPRNPEISLAEVGDNEAVAPQTIAVVLAVVGAAAAAVTVYLGANVGAANGTVTANAVANTANA}
\]

**AerD**

\[
\text{AerD} \\
\text{MPTQGTSYAEQHIFAADATPDPWPPITIGCQAKRVLWSSSSAVFRELVATDPERAGRDYKLKFSPELIRPLWDDRYHLDAAKDRQHPFIVAYEYHYHTQWDREVKECPRDEPLRLKWRTTRQIARANAMENGLYDNIIHSLAEISDSGC}
\]

**His\(_6\)-SUMO-AerE**

\[
\text{His}^6-\text{SUMO-AerE} (\text{bold} = \text{leader peptide}; \text{red} = \text{core peptide}) \\
\text{MKSSHHHSHHSSMASHDSEVQEAKEPVKEPVTEHINLKVDGSSEIIFFKIKKTTPRRLMLEFAFKRGKEMD}
\]

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s9
Supporting Figures

**Figure S1:** $N$-methylation of a tryptic fragment (AerA$_{DL,21LP}$) of AerA$_{DL}$ by AerE. Treatment of AerA$_{DL,21LP}$ with AerE and SAM resulted in a +70 Da mass shift, detected by MALDI-TOF MS, that is consistent with five-fold methylation.
**Figure S2:** *N*-methylation of proteinase K fragments (AerADL,32-35) of AerADL by AerE. AerADL,32-35 correspond to 32 – 35mer products afforded by proteinase K treatment. Their ability to serve as AerE substrates was probed by MALDI-TOF MS analysis. Green traces contain reactions with AerADL,32-35, AerE, and SAM; whereas, black traces have the same components but lack AerE. For 31mer (AerADL,31) results, see Figure 2C. Note, the +71.1 Da shifts in the -AerE reactions (AerADL,32-34) correspond to desmethylated proteinase K fragments (AerADL,33-35), which differ from the [M+H]+ species by one Ala residue.
Figure S3: N-methylation of epimerized (AerA_{DL,34}) versus non-epimerized (AerA_{L,34}) AerA. Both AerA_{DL,34} and AerA_{L,34} were synthesized by SPPS. Reactions were quenched by heat-denaturation at various time points following the addition of AerE to reactions containing peptides and SAM then analyzed by MALDI-TOF MS.
Figure S4: Fragmentation mass spectrometry (ESI-MS/MS) analysis of AerA<sub>DL</sub>. Observed y-ions are mapped onto the structure and a corresponding collision-induced dissociation (CID) mass spectrum is shown below with a representative set of high-abundance y-ions labeled. Data were collected using a ThermoFisher Scientific Orbitrap Fusion electrospray-ionization mass-spectrometer.
Figure S5: ESI-MS/MS analysis of N-methylated 1Me – 4Me AerA_{DL}. The observed y-ions containing (green) or lacking (black) N-methylations are mapped onto each of the structures.
(Tables S2-S6). Data were collected using a ThermoFisher Scientific Orbitrap Fusion electrospray-ionization mass-spectrometer.

Figure S6: $^1$H NMR spectrum of 1 (Scheme 1) in CDCl$_3$ collected on a Varian Unity Inova 500 MHz NMR spectrometer.
Figure S7: $^1$H NMR spectrum of 3 (Scheme 1) in CDCl$_3$ collected on a Varian Unity Inova 500 MHz NMR spectrometer.
Figure S8: $^1$H NMR spectrum of 4 (Scheme 1) in CDCl$_3$ collected on a Varian Unity Inova 500 MHz NMR spectrometer.
**Figure S9:** $^1$H NMR spectrum of 5 (Scheme 1) in CDCl$_3$ collected on a Varian Unity Inova 500 MHz NMR spectrometer.
Figure S10: $^1$H NMR spectrum of 6 (Scheme 1) in CDCl$_3$ collected on a Varian Unity Inova 500 MHz NMR spectrometer.
Figure S11: Solid-phase peptide synthesis of (A) AerADL,34 (0Me-AerADL,34) and (B) 5Me-AerADL,34. Shown on the left are the products following SPPS with side chain protecting groups (tert-butyl = tBu, trityl = Trt, and 2,4,6-trimethoxybenzyl = Tmb). L-Ala Wang resin is represented as a blue sphere. Deprotection was achieved by addition of a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and H₂O in a ratio of 95:2.5:2.5, respectively (see Methods). Expected exact masses and observed masses (by MALDI-TOF MS) are shown.
**Figure S12:** $^1$H NMR spectrum of 0Me-AerADL$_{34}$ (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer.
Figure S13: $^1$H NMR spectrum of 5Me-AerADL,34 (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer.
Figure S14: 2D TOCSY NMR spectrum of 0Me-AerADL_{34} (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer.
Figure S15: 2D TOCSY NMR spectrum of 5Me-AerADL,34 (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer.
Figure S16: 2D NOESY NMR spectrum of 0Me-AerADL\textsubscript{34} (Fig. S11) in CD\textsubscript{3}OH:H\textsubscript{2}O (93:7) collected on a 750 MHz Varian NMR spectrometer.
Figure S17: 2D NOESY NMR spectrum of 5Me-AerA\textsubscript{DL,34} (Fig. S11) in CD\textsubscript{3}OH:H\textsubscript{2}O (93:7) collected on a 750 MHz Varian NMR spectrometer.
Figure S18: Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 0Me-AerADL\textsubscript{34} (Fig. S11) in CD\textsubscript{3}OH:H\textsubscript{2}O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential \(d_{\gamma\beta}(i, i-1)\) peaks – are labeled with smaller text.
**Figure S19**: Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 0Me-AerADL,34 (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential $d_{N\beta}(i, i-1)$ peaks – are labeled with smaller text.
**Figure S20:** Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 0Me-AerA$_{DL,34}$ (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential or intraresidue $d_{N\beta}(i, i-1)$ peaks – are labeled with smaller text.
**Figure S21**: Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 0Me-AerADL34 (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential $d_{N\alpha}(i, i-1)$ peaks – are labeled with smaller text.
Figure S22: Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 5Me-AerADL,34 (Fig. S11) in CD$_2$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential $d_{N\gamma\beta}(i, i-1)$ peaks – are labeled with smaller text.
Figure S23: Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 5Me-AerADL,34 (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential $d_{N\beta}(i, i-1)$ peaks – are labeled with smaller text.
**Figure S24:** Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 5Me-AerADL\textsubscript{34} (Fig. S11) in CD\textsubscript{3}OH:H\textsubscript{2}O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential or intraresidue $d_{\text{NH}}(i, i-1)$ peaks – are labeled with smaller text.
Figure S25: Overlay of 2D TOCSY (gray) and NOESY (red) spectra of 5Me-AerA_{DL,34} (Fig. S11) in CD$_3$OH:H$_2$O (93:7) collected on a 750 MHz Varian NMR spectrometer. Residue assignments are labeled with large text, whereas NOE cross-peaks – corresponding to sequential $d_N (\alpha/\beta) (i, i-1)$ peaks – are labeled with smaller text.
**Figure S26:** Structural alignment of AerE with N-methyltransferases GenN (RMSD of 2.8 Å over 274 aligned Cα atoms) and PrmC (RMSD of 3.9 Å over 216 aligned Cα atoms). The active site region containing SAH and the putative Na⁺ ion (magenta) is circled in dashed red. Whereas the α/β-Rossmann catalytic subdomains are well aligned, the N-terminal α-helical subdomains are not.
Figure S27: Effect of EDTA and EGTA on AerE N-methylation of AerA_{DL,34}. Following prolonged dialysis (12 h) with excess EDTA or EGTA, AerE remained catalytically competent (see Methods).
Figure S28: Testing metal ion occupancy in the AerADL,34 bound and unbound AerE crystals structures. (A) Metal-ligand distance distributions after Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\) were fitted into the octahedrally-coordinated electron density in the active site of the AerADL,34 bound AerE crystal structure. Due to poor occupancy of the D-Asn25 ligand to the putative structural ion, the side chain was substituted for a water prior to refinement. The refined coordinate .pdb files were then analyzed using the CheckMyMetal (CMM) server.\(^{12}\) The graphs in panel A are directly taken from the CMM generated results which draw ideal bond lengths from the Cambridge Structural Database (CSD).\(^{13}\) (B) Given the ambiguity in assigning Na\(^+\) or Ca\(^{2+}\) on the basis of metal-ligand distances alone, which are comparably acceptable, we looked at their corresponding difference Fourier maps (\(F_o - F_c\)) following crystallographic refinement in phenix.refine. The \(F_o - F_c\) maps are shown in red (negative) and green (positive) and contoured at 3 \(\sigma\), and the \(2F_o - F_c\) maps are shown in blue and contoured at 2.5 \(\sigma\). Together, our results are most consistent with an octahedrally bound Na\(^+\) ion. Of note, the F234 ligand was not recognized in the CMM analysis, but the Na\(^+\)-Phe separation of \(~3.0\) Å is typical of cation-\(\pi\) interactions.\(^{14,15}\) (C) An octahedrally bound Na\(^+\) ion is modelled in the active site of the AerADL,34 unbound AerE crystal structure with the \(2F_o - F_c\) electron density map contoured at 1.7 \(\sigma\).
**Figure S29:** Inhibition of AerE by Zn\(^{2+}\) and lack of rescue by Mn\(^{2+}\), Ca\(^{2+}\), or Mg\(^{2+}\), or Na\(^{+}\). (A) Dose-dependent inhibition of AerE-catalyzed Aer\(_{DL,34}\) N-methylation by ZnCl\(_2\). Full inhibition is observed between 60–250 µM, as measured by MALDI-TOF MS. (B) To probe whether additional ions might ameliorate Zn-inhibition by competitive binding to the same site, excess MgCl\(_2\), CaCl\(_2\), or MnCl\(_2\) were added to the reactions at 25 mM. The MALDI-TOF MS data suggest that Mg\(^{2+}\), Ca\(^{2+}\), or Mn\(^{2+}\) do not rescue Zn-inhibition of AerE. Note: Na\(^{+}\), present at 100 mM in the reaction buffer, also does not rescue Zn-inhibition. The inhibitory effect is most likely due to Zn-induced aggregation of AerE, as protein precipitation was observed upon addition of ZnCl\(_2\).
Figure S30: (A) Multiple sequence alignment between AerE and eight of its homologs including four orthologs (i.e., PoyE, WP_095985947.1, RYY26915.1, and KJS38421.1), two homologs with unknown function (i.e., WP_092072996.1 and WP_013652859.1), and the two structural homologs PrmC and GenN (Fig. S26). AerE and its orthologs, which are either confirmed or putative proteusin N-methyltransferases (NMTs), are indicated by red circles. The seven residues targeted for mutagenesis are boxed in red and those that are catalytically essential are demarcated with red stars (Figs. 6A and S30). (B) Multiple sequence alignment between the AerA core peptide and five additional proteusin core peptide orthologs associated with the NMTs in panel A. Five of the most conserved Asn residues are marked with arrows and their numbers correspond to the AerA core peptide numbering. Five N-terminal residues of the leader peptide used in the alignment are colored gray. (C) A sequence consensus WebLogo corresponding to the -2 through +2 residues to the five most conserved Asn residues (panel B). A nearly identical incidence of Asn is observed in the -2 (7 occurrences) and +2 (9 occurrences) positions.
Figure S31: Superposition of the AerE wild-type and site-directed variants D141A, N231A, and Y137F all bound to SAH. The AerAD_{L,34} peptide is made partially transparent for clarity and its residues are labeled light gray or gold with outlines (for L- and D-configured residues, respectively); whereas all AerE residues are labeled in gray.
Figure S32: Mutational analysis of AerE. Additional structure-based AerE variants were assessed for their N-methylation activity towards AerADL,34 by end-point analysis (16 h) and MALDI-TOF MS (Fig. 6A and Methods). The top eight spectra correspond to reactions featuring AerE, SAM, and AerADL,34, and the bottom spectrum is derived from a control reaction lacking AerE. All proteins used in this analysis contain N-terminal SUMO tags and were purified by Ni-NTA affinity chromatography, except for “WT(SEC)”, which features a tag-free AerE sample whose final purification step terminated in SEC (see Methods). Note, the mutational analysis displayed in Figure 6A features tag-free AerE samples that were all purified by SEC.
### Supporting Tables

**Table S1:** Oligonucleotides used in this study.

| Oligonucleotide | Sequence |
|-----------------|----------|
| AerE_F_LICv1    | TACTTCCAATCCAATGCAATGACCCGACACTGCCACACCAC |
| AerE_R_LICv1    | TTATCCAATCCAATGTAATCTACGTCCGCCGCACCCGGGGAG |
| AerE-H134A_F    | TTTCCGCCGAGCTCGCCGAGGCTATATCGGTCCCG |
| AerE-H134A_R    | GGACCCATATAACACCTCGGCCACCCGGCAGAA |
| AerE-Y137F_F    | GAAGTCCACGAGGTGGCTATCGGTACCCGGACAGCT |
| AerE-Y137F_R    | CTGTCCGAGCAGTTATCGGTACCCGGACTTCC |
| AerE-D141A_F    | GTGTATATCGGTCCCGAGCTACCTGGATGCTG |
| AerE-D141A_R    | AGCATCCAGTAGCTGGCCGCCGACACTACCC |
| AerE-D141N_F    | GTGTATATCGGTCCCGAGCTACCTGGATGCT |
| AerE-D141N_R    | CAGATCCAGTAGCTGGCCGCCGACACTACCC |
| AerE-S142A_F    | TATATCGGTCCCGAGCTACCTGGATGCT|
| AerE-S142A_R    | ACTACAGCTCCAGTAGCTGGCCGCCGACACTAC |
| AerE-N231F_F    | GACCTGCTACCTCGGCCGCCCTTTCGTCG |
| AerE-N231A_R    | GCCACGAGGAGGCGGCGGCGGACGGTGACGAGGTCG |
| AerE-F234A_F    | CACCTGCAACCCGACCTCGGCTACCCGGCTC |
| AerE-F234A_R    | GCCCGGAGGAGGCGGCGGCGGACGGTGACGAGGTCG |
| AerE-V235A_F    | CCTGCAACCCGCTTTCGCGGCCTTCCCTCCCG |
| AerE-V235A_R    | AGCCGGAGGAGGCGGCGGCGGACGGTGACGAGGTCG |
Table S2: Calculated and observed ions from ESI-MS/MS analysis of 0Me-AerADL.

| Fragment Ion | Observed Mass (Da) | Calculated Mass (Da) | Mass Error (ppm) |
|--------------|--------------------|----------------------|------------------|
| y22          | 1999.0224          | 1999.0203            | 1.05             |
| y22-NH₃      | 1981.9982          | 1981.9938            | 2.22             |
| y21          | 1884.9727          | 1884.9774            | 2.49             |
| y20          | 1785.9069          | 1785.9090            | 1.18             |
| y19          | 1686.8386          | 1686.8406            | 1.19             |
| y19-NH₃      | 1669.8132          | 1669.8140            | 0.48             |
| y18          | 1629.8134          | 1629.8191            | 3.50             |
| y17          | 1558.7762          | 1558.7820            | 3.72             |
| y16          | 1487.7435          | 1487.7449            | 0.94             |
| y16-H₂O      | 1469.7329          | 1469.7343            | 0.95             |
| y15          | 1373.7005          | 1373.7019            | 1.02             |
| y14          | 1316.6792          | 1316.6805            | 0.99             |
| y13          | 1215.6319          | 1215.6328            | 0.74             |
| y12          | 1116.5631          | 1116.5644            | 1.16             |
| y11          | 1015.5153          | 1015.5167            | 1.38             |
| y10          | 944.4782           | 944.4796             | 1.48             |
| y9           | 830.4355           | 830.4367             | 1.45             |
| y8           | 759.3985           | 759.3995             | 1.32             |
| y7           | 660.3303           | 660.3311             | 1.21             |
| y6           | 589.2933           | 589.2940             | 1.19             |
| y5           | 475.2506           | 475.2511             | 1.05             |
| y4           | 374.2031           | 374.2034             | 0.80             |
| y22          | 1999.0224          | 1999.0203            | 1.05             |
| y22-NH₃      | 1981.9982          | 1981.9938            | 2.22             |
| y21          | 1884.9727          | 1884.9774            | 2.49             |
| y20          | 1785.9069          | 1785.9090            | 1.18             |
| y19          | 1686.8386          | 1686.8406            | 1.19             |
| y19-NH₃      | 1669.8132          | 1669.8140            | 0.48             |
| y18          | 1629.8134          | 1629.8191            | 3.50             |
| y17          | 1558.7762          | 1558.7820            | 3.72             |
| y16          | 1487.7435          | 1487.7449            | 0.94             |
| y16-H₂O      | 1469.7329          | 1469.7343            | 0.95             |
| y15          | 1373.7005          | 1373.7019            | 1.02             |
Table S3: Calculated and observed ions from ESI-MS/MS analysis of 1Me-AerADL.

| Fragment Ion | Observed Mass (Da) | Calculated Mass (Da) | Mass Error (ppm) |
|--------------|--------------------|----------------------|------------------|
| y22-NH$_3$+Me | 1996.0134          | 1996.0095            | 1.98             |
| y21          | 1884.9749          | 1884.9774            | 1.33             |
| y20          | 1785.9079          | 1785.9090            | 0.62             |
| y19+Me       | 1700.8596          | 1700.8563            | 1.97             |
| y19-NH$_3$+Me| 1683.8297          | 1683.8297            | 0.03             |
| y19-NH$_3$   | 1669.8149          | 1669.8140            | 0.54             |
| y18          | 1629.8183          | 1629.8191            | 0.49             |
| y17          | 1558.7809          | 1558.7820            | 0.71             |
| y16          | 1487.7441          | 1487.7449            | 0.54             |
| y16-H$_2$O   | 1469.7344          | 1469.7343            | 0.07             |
| y15          | 1373.7006          | 1373.7019            | 0.95             |
| y14          | 1316.6761          | 1316.6805            | 3.34             |
| y13          | 1215.6321          | 1215.6328            | 0.58             |
| y12          | 1116.5636          | 1116.5644            | 0.72             |
| y11          | 1015.5154          | 1015.5167            | 1.28             |
| y10          | 944.4787           | 944.4796             | 0.95             |
| y9           | 830.4358           | 830.4367             | 1.08             |
| y8           | 759.3989           | 759.3995             | 0.79             |
| y7           | 660.3305           | 660.3311             | 0.91             |
| y6           | 589.2936           | 589.2940             | 0.68             |
| y5           | 475.2508           | 475.2511             | 0.63             |
Table S4: Calculated and observed ions from ESI-MS/MS analysis of 2Me-AerADL.

| Fragment Ion   | Observed Mass (Da) | Calculated Mass (Da) | Mass Error (ppm) |
|----------------|--------------------|----------------------|------------------|
| y20+Me         | 1799.9239          | 1799.9247            | 0.42             |
| y19+Me         | 1700.8540          | 1700.8563            | 1.32             |
| y19-NH₃+Me     | 1683.8295          | 1683.8297            | 0.09             |
| y18+Me         | 1643.8392          | 1643.8348            | 2.71             |
| y16+Me         | 1501.7642          | 1501.7606            | 2.43             |
| y16-H₂O+Me     | 1483.7473          | 1483.7500            | 1.79             |
| y15+Me         | 1387.7167          | 1387.7176            | 0.61             |
| y15            | 1373.7005          | 1373.7019            | 1.02             |
| y14+Me         | 1330.7025          | 1330.6962            | 4.77             |
| y14            | 1316.6759          | 1316.6805            | 3.49             |
| y13+Me         | 1229.6484          | 1229.6485            | 0.04             |
| y13            | 1215.6321          | 1215.6328            | 0.58             |
| y12+Me         | 1130.5794          | 1130.5801            | 0.57             |
| y12            | 1116.5634          | 1116.5644            | 0.90             |
| y11+Me         | 1029.5297          | 1029.5324            | 2.57             |
| y11            | 1015.5156          | 1015.5167            | 1.08             |
| y10+Me         | 958.4939           | 958.4953             | 1.41             |
| y10            | 944.4791           | 944.4796             | 0.53             |
| y9+Me          | 844.4516           | 844.4524             | 0.89             |
| y9             | 830.4357           | 830.4367             | 1.20             |
| y8+Me          | 773.4163           | 773.4152             | 1.49             |
| y8             | 759.3988           | 759.3995             | 0.92             |
| y7+Me          | 674.3463           | 674.3468             | 0.67             |
| y7             | 660.3304           | 660.3311             | 1.06             |
| y6+Me          | 603.3097           | 603.3097             | 0.08             |
| y6             | 589.2936           | 589.2940             | 0.68             |
| y5             | 475.2507           | 475.2511             | 0.84             |
### Table S5: Calculated and observed ions from ESI-MS/MS analysis of 3Me-AerADL.

| Fragment Ion | Observed Mass (Da) | Calculated Mass (Da) | Mass Error (ppm) |
|--------------|--------------------|----------------------|------------------|
| y21+2Me     | 1913.0083          | 1913.0087            | 0.21             |
| y20+2Me     | 1813.9381          | 1813.9403            | 1.21             |
| y19+2Me     | 1714.8698          | 1714.8719            | 1.22             |
| y19-NH$_3$+2Me | 1697.8450          | 1697.8453            | 0.18             |
| y18+2Me     | 1657.8503          | 1657.8504            | 0.06             |
| y17+2Me     | 1586.8105          | 1586.8133            | 1.76             |
| y16+2Me     | 1515.7758          | 1515.7762            | 0.26             |
| y16-H$_2$O+3Me | 1511.7815          | 1511.7813            | 0.17             |
| y16-H$_2$O+2Me | 1497.7635          | 1497.7656            | 1.40             |
| y15+2Me     | 1401.7303          | 1401.7323            | 2.07             |
| y15+Me      | 1387.7162          | 1387.7176            | 0.97             |
| y14+2Me     | 1344.7112          | 1344.7118            | 0.45             |
| y14+Me      | 1330.6955          | 1330.6962            | 0.49             |
| y13+2Me     | 1243.6616          | 1243.6641            | 2.01             |
| y13+Me      | 1229.6468          | 1229.6485            | 1.34             |
| y12+2Me     | 1144.5947          | 1144.5957            | 0.87             |
| y12+Me      | 1130.5788          | 1130.5801            | 1.11             |
| y11+2Me     | 1043.5459          | 1043.5480            | 2.01             |
| y11+Me      | 1029.5308          | 1029.5324            | 1.51             |
| y10+2Me     | 972.5093           | 972.5109             | 1.65             |
| y10+Me      | 958.4943           | 958.4953             | 0.99             |
| y9+Me       | 844.4509           | 844.4524             | 1.72             |
| y9          | 830.4355           | 830.4367             | 1.45             |
| y8+Me       | 773.4144           | 773.4152             | 0.97             |
| y8          | 759.3984           | 759.3995             | 1.45             |
| y7+Me       | 674.3462           | 674.3468             | 0.82             |
| y7          | 660.3302           | 660.3311             | 1.36             |
| y6+Me       | 603.3086           | 603.3097             | 1.74             |
| y6          | 589.2935           | 589.2940             | 0.85             |
| y5+Me       | 489.2660           | 489.2668             | 1.53             |
| y5          | 475.2501           | 475.2511             | 2.10             |
### Table S6: Calculated and observed ions from ESI-MS/MS analysis of 4Me-AerA<sub>DL</sub>.

| Fragment Ion | Observed Mass (Da) | Calculated Mass (Da) | Mass Error (ppm) |
|--------------|--------------------|----------------------|------------------|
| y22+4Me     | 2055.0849          | 2055.0829            | 0.97             |
| y21+3Me     | 1927.0275          | 1927.0244            | 1.63             |
| y20+3Me     | 1827.9588          | 1827.9560            | 1.56             |
| y19+3Me     | 1728.8901          | 1728.8876            | 1.47             |
| y18+3Me     | 1671.8643          | 1671.8661            | 1.05             |
| y17+3Me     | 1600.8310          | 1600.8290            | 1.28             |
| y16+3Me     | 1529.7939          | 1529.7919            | 1.34             |
| y16-H<sub>2</sub>O+3Me | 1511.7823 | 1511.7813 | 0.69 |
| y15+3Me     | 1415.7473          | 1415.7489            | 1.09             |
| y15+2Me     | 1401.7354          | 1401.7332            | 1.57             |
| y14+3Me     | 1358.7284          | 1358.7275            | 0.70             |
| y14+2Me     | 1344.7153          | 1344.7118            | 2.60             |
| y13+3Me     | 1257.6808          | 1257.6798            | 0.83             |
| y13+2Me     | 1243.6656          | 1243.6641            | 1.21             |
| y12+3Me     | 1158.6132          | 1158.6114            | 1.60             |
| y12+2Me     | 1144.5971          | 1144.5957            | 1.22             |
| y11+3Me     | 1057.5655          | 1057.5637            | 1.75             |
| y11+2Me     | 1043.5505          | 1043.5480            | 2.40             |
| y10+3Me     | 986.5282           | 986.5266             | 1.67             |
| y10+2Me     | 972.5118           | 972.5109             | 0.93             |
| y9+2Me      | 858.4694           | 858.4680             | 1.63             |
| y9+1Me      | 844.4539           | 844.4524             | 1.84             |
| y8+2Me      | 787.4325           | 787.4308             | 2.16             |
| y8+1Me      | 773.4169           | 773.4152             | 2.26             |
| y7+2Me      | 688.3637           | 688.3624             | 1.89             |
| y7+1Me      | 674.3480           | 674.3468             | 1.85             |
| y6+2Me      | 617.3264           | 617.3253             | 1.78             |
| y6+1Me      | 603.3109           | 603.3097             | 2.07             |
| y5+1Me      | 489.2679           | 489.2668             | 2.35             |
| y5          | 475.2530           | 475.2511             | 4.00             |
**Table S7:** Table of NMR peak assignments for 0Me-AerADL,34 and 5Me-AerADL,34. The total number of residues are in parentheses in the “Residue” column. Asm = Nγ-γ-Me-D-Asn.

### 0Me-AerADL,34

| Residue | NH          | Hα          | Hβ          |
|---------|-------------|-------------|-------------|
| Ala(13) | 7.88-8.47, 8.71 | 4.21-4.40   | 1.32-1.41   |
| Asn(5)  | 8.25, 8.33, 8.33, 8.38, 8.57 | 4.67-4.73   | 2.65-2.84   |
| Gly(3)  | 8.41        | 3.77, 3.84, 3.90, 3.94, 3.96, 4.02 | –           |
| Leu(1)  | 8.26 (L21)  | 4.17 (L21)  | 1.50 (L21)  |
| Thr(4)  | 7.93 (T33), 8.13 (T42), 8.12 (T19), 8.29 (T35) | 4.27 (T19), 4.28 (T42/T35), 4.30 (T33) | 4.19 (T19), 4.20 (T42/T35), 4.22 (T33) |
| Tyr(1)  | 8.23 (Y20)  | 4.45 (Y20)  | 2.94-2.98 (Y20) |
| Val(7)  | 7.90, 8.05, 8.08, 8.13 (V34), 8.24 (V27) | 4.16-4.25   | 2.12-2.18   |

### 5Me-AerADL,34

| Residue | NH          | Hα          | Hβ          |
|---------|-------------|-------------|-------------|
| Ala(13) | 7.95-8.38, 8.67 | 4.15-4.37   | 1.32-1.37   |
| Asm(5)  | 8.22, 8.23, 8.36, 8.41, 8.50 | 4.66-4.75   | 2.58-2.80   |
| Gly(3)  | 8.39        | 3.79, 3.87, 3.93, 3.96, 4.00 | –           |
| Leu(1)  | 8.29 (L21)  | 4.12 (L21)  | 1.51 (L21)  |
| Thr(4)  | 8.03 (T33), 8.07 (T42), 8.18 (T19), 8.28 (T35) | 4.26 (T19), 4.29 (T42), 4.29 (T35), 4.37 (T33) | 4.19 (T19), 4.22 (T42), 4.21 (T35), 4.29 (T33) |
| Tyr(1)  | 8.23 (Y20)  | 4.44 (Y20)  | 2.96 (Y20)  |
| Val(7)  | 7.93, 7.95, 8.02, 8.04, 8.15 (V34), 8.21 (V27) | 4.12-4.19   | 2.11-2.13   |
Table 7 (continued): Table of NMR peak assignments for 0Me-AerADL,34 and 5Me-AerADL,34. The total number of residues are in parentheses in the “Residue” column. Asm = $N_\gamma$-Me-D-Asn.

| Residue  | $H_N$ | other                        |
|----------|-------|------------------------------|
| **0Me-AerADL,34** |       |                              |
| Ala(13)  | –     | –                            |
| Asn(5)   | –     | –                            |
| Gly(3)   | –     | –                            |
| Leu(1)   | 1.42 (L21) | H$\delta$: 0.72 (L21), 0.79 (L21) |
| Thr(4)   | 1.07 (T19), 1.17-1.20 (T42/T33/T35) | – |
| Tyr(1)   | –     | Ar: 7.01 (Y20), 6.71 (Y20)   |
| Val(7)   | 0.88, 0.90-0.99 | – |
Table S8: Summary of NOE cross peaks for 0Me-AerADL,34 (n.d. = not detected). Dashes denote positions for which NOE cross peaks are irrelevant or could not be measured due to peak overlap.

| Residue | $i, i-1$ | $i, i-3$ | $i, i+6$ | $i, i-6$ |
|---------|----------|----------|----------|----------|
| Ala13   | –        | –        | –        | –        |
| Ala14   | –        | –        | –        | –        |
| Ala15   | –        | –        | –        | –        |
| Ala16   | –        | –        | –        | –        |
| Val17   | –        | –        | –        | –        |
| Val18   | –        | –        | –        | –        |
| Thr19   | –        | –        | n.d. NH/β| –        |
| Tyr20   | NH/γ     | –        | n.d. α/NH, n.d. β/NH| –  |
| Leu21   | NH/α, NH/β| –        | –        | –        |
| Gly22   | NH/α, NH/γ| n.d. NH/γ| n.d. α/NH| –        |
| Ala23   | NH/α     | –        | –        | –        |
| Ala24   | –        | n.d. NH/α, n.d. NH/β| –        | –        |
| Asm25   | –        | –        | n.d. NH/β| n.d. β/NH|
| Val26   | NH/β     | –        | n.d. β/NH| n.d. NH/α, n.d. NH/β|
| Val27   | NH/α, NH/β| –        | n.d. NH/α, n.d. NH/γ| –  |
| Gly28   | NH/α, NH/β, NH/γ| n.d. NH/β| –        | n.d. NH/α|
| Ala29   | NH/α     | n.d. NH/α| –        | –        |
| Ala30   | –        | –        | –        | –        |
| Asm31   | –        | –        | n.d. NH/β| n.d. β/NH|
| Gly32   | NH/β     | –        | n.d. α/NH| n.d. NH/β|
| Thr33   | NH/α     | –        | –        | n.d. α/NH, n.d. γ/NH|
| Val34   | NH/α, NH/γ| n.d. NH/β| –        | –        |
| Thr35   | NH/α, NH/β| n.d. NH/α| n.d. NH/β| –        |
| Ala36   | NH/γ     | n.d. NH/γ| –        | –        |
| Asm37   | –        | –        | n.d. NH/β| n.d. β/NH|
| Ala38   | NH/β     | –        | –        | n.d. NH/α|
| Val39   | –        | –        | –        | –        |
| Ala40   | –        | –        | –        | –        |
| Asm41   | –        | –        | –        | n.d. β/NH|
| Thr42   | NH/β     | –        | –        | –        |
| Asm43   | NH/α, NH/γ| –        | –        | n.d. β/NH|
| Ala44   | –        | –        | –        | –        |
| Val45   | –        | –        | –        | –        |
| Ala46   | –        | –        | –        | –        |
Table S9: Summary of NOE cross peaks for 5Me-AerADL,34 (n.d. = not detected). Dashes denote positions for which NOE cross peaks are irrelevant or could not be measured due to peak overlap.

| Residue | i, i-1 | i, i-3 | i, i+6 | i, i-6 |
|---------|--------|--------|--------|--------|
| Ala13   | –      | –      | –      | –      |
| Ala14   | –      | –      | –      | –      |
| Ala15   | –      | –      | –      | –      |
| Ala16   | –      | –      | –      | –      |
| Val17   | –      | –      | –      | –      |
| Val18   | –      | –      | –      | –      |
| Thr19   | NH/γ   | –      | n.d. NH/β | –      |
| Tyr20   | NH/α, NH/γ | – | n.d. α/β, n.d. β/β | – | |
| Leu21   | NH/α, NH/β | – | n.d. NH/α, n.d. NH/β | – | |
| Gly22   | – | n.d. NH/β, n.d. NH/γ | n.d. α/β | – | |
| Ala23   | NH/α | n.d. NH/α, n.d. NH/β | – | – | |
| Ala24   | – | – | – | – | |
| Asm25   | – | – | n.d. NH/β | n.d. β/β | |
| Val26   | NH/β | – | n.d. α/β | n.d. NH/α, n.d. NH/β | |
| Val27   | NH/β | – | n.d. NH/α, n.d. NH/γ | n.d. α/β, n.d. β/β | |
| Gly28   | NH/α, NH/β | – | n.d. NH/β | n.d. α/β | |
| Ala29   | NH/α | – | n.d. NH/γ | – | |
| Ala30   | – | – | – | – | |
| Asm31   | – | – | n.d. NH/β | n.d. β/β | |
| Gly32   | NH/β | – | – | n.d. NH/α | |
| Thr33   | NH/α | – | – | n.d. α/β, n.d. γ/γ | |
| Val34   | NH/α, NH/γ | – | n.d. NH/β | – | n.d. NH/α | |
| Thr35   | NH/α, NH/β, NH/γ | – | n.d. NH/α | n.d. NH/β | n.d. γ/γ | |
| Ala36   | NH/α, NH/γ | – | n.d. α/β | – | |
| Asm37   | NH/β | – | n.d. NH/β | n.d. NH/β | n.d. β/β | |
| Ala38   | NH/β | – | – | – | |
| Val39   | – | – | – | – | |
| Ala40   | – | – | – | – | |
| Asm41   | – | – | – | n.d. β/β | |
| Thr42   | NH/β | – | – | n.d. NH/α | |
| Asm43   | NH/β, NH/γ | – | – | n.d. β/β | |
| Ala44   | – | – | – | – | |
| Val45   | – | n.d. NH/γ | – | – | |
| Ala46   | NH/α, NH/β | n.d. NH/β | – | – | |
**Table S10**: Crystallographic data collection and refinement statistics.

| Data collection | AerE | AerE-AerADL,34 | AerE-D141A | AerE-N231A | AerE-Y137F |
|-----------------|------|----------------|------------|------------|------------|
| Space group     | P3\_21 | P3\_21 | P3\_21 | P3\_21 | P3\_21 |
| Cell dimensions |                  |                  |            |            |            |
| a, b, c (Å)     | 77.566, 77.566, 152.314 | 78.029, 78.029, 153.399 | 77.595, 77.595, 151.734 | 77.518, 77.518, 151.794 | 77.697, 77.697, 152.239 |
| α, β, γ (°)     | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| Resolution (Å)² | 1.508 – 40.500 | 1.715 – 67.575 | 1.659 – 67.199 | 1.876 – 67.133 | 1.533 – 19.519 |
| Rmerge¹         | 0.077 (1.151) | 0.084 (1.098) | 0.104 (1.186) | 0.104 (1.550) | 0.092 (1.193) |
| R_pim¹         | 0.018 (0.280) | 0.023 (0.327) | 0.025 (0.331) | 0.025 (0.464) | 0.022 (0.309) |
| | 23.2 (2.2) | 20.0 (2.2) | 15.5 (2.2) | 18.4 (1.8) | 18.7 (2.1) |
| CC_{1/2}       | 1.000 (0.854) | 1.000 (0.805) | 0.999 (0.876) | 0.999 (0.639) | 1.000 (0.776) |
| Completeness (%)¹ | 99.98 (99.99) | 100.0 (100.0) | 97.2 (99.9) | 81.0 (74.1) | 99.9 (80.9) |
| Redundancy¹ | 20.2 (17.6) | 14.8 (11.1) | 17.5 (13.6) | 17.5 (11.6) | 17.8 (15.8) |

| Refinement | | | | | |
| Resolution (Å) | 1.508 – 40.500 | 1.715 – 40.78 | 1.659 – 32.80 | 1.876 – 40.41 | 1.530 – 19.52 |
| No. reflections | 83,933 (8,283) | 58,722 (5,831) | 61,470 (6,238) | 35,333 (1,298) | 80,525 (7,969) |
| R_work / R_free² (%) | 16.1 (21.9) / 17.5 (23.4) | 16.5 (25.2) / 18.8 (28.9) | 17.0 (22.7) / 18.8 (28.1) | 18.8 (28.1) / 22.1 (38.8) | 16.6 (21.3) / 18.7 (23.2) |
| No. atoms | 3,525 | 3,571 | 3,361 | 3,173 | 3,456 |
| Protein | 2,929 | 2,977 | 2,919 | 2,923 | 2,956 |
| Ligand/ion | 41 | 27 | 61 | 37 | 45 |
| Water | 555 | 547 | 381 | 213 | 455 |
| B-factors (Å²) | | | | | |
| Average | 21.31 | 23.99 | 23.93 | 31.59 | 20.62 |
| Protein | 18.73 | 21.17 | 22.29 | 30.96 | 18.50 |
| Ligand/ion | 25.87 | 48.20 | 28.67 | 46.25 | 29.30 |
| Water | 34.95 | 37.77 | 36.16 | 39.62 | 33.55 |
| R.m.s. deviations | | | | | |
| Bond lengths (Å) | 0.006 | 0.008 | 0.007 | 0.008 | 0.006 |
| Bond angles (°) | 0.96 | 0.96 | 0.99 | 0.95 | 1.03 |

1. Highest resolution shell is shown in parentheses.
2. R-work = Σ(|F_{o}-kF_{w}|)/Σ|F_{o}| and R-free is the R value for a test set of reflections consisting of a random 5% of the diffraction data not used in refinement.
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