Supporting Information

Bis-Chlorination of a Hexapeptide-PCP Conjugate by the Halogenase Involved in Vancomycin Biosynthesis

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1. Production of VhaA in *E. coli*

The DNA sequence of the vancomycin gene cluster from *A. orientalis* ATCC19795 (DSM 40040) is available in the GeneBank database (Accession number HE589771). The primary sequence of VhaA produced in *E. coli* is shown below (N-terminal tag underlined):

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        10         20         30         40         50         60
GSSHHHHHHS SGENLYFQGH MSVEDFDVVV AGGGPGGSTV ATLVAMQGHR VLLLEKEVFP

        70         80         90        100        110        120
RYQIGESLLP ATVHGVCRML GVADELAGAG FPIKRGGTFR WGARPEPWTF HFGISAKMAG

       130        140        150        160        170        180
STSHAYQVER ARFDEILLNN AKSKGVVVRE GCSVHDVVED GERVTGAKYT DPDGNEREVS

       190        200        210        220        230        240
ARFVIDASGN KSRLYSKVGG SRNYSEFFRS LALFGYFEGG KRLPEPVSGN ILSVAFDNGW

       250        260        270        280        290        300
FWYIPLSDTL TSVGAVVRRE DADKIQGDRE KALNALIAEC PLISEYLSNA TRVTTGKYGE

       310        320        330        340        350        360
LRVRKDYSYQ QETYWRPGMV LVGDAACFVD PVFSSGVHLA TYSALLAARS INSVLAGDLD

       370        380        390        400        410        420
EKTALNEFEA RYRREYGVFY EFLVSFYQMN VNEESYFWQA KKVTQNQSTD IESFVELIGG

       430        440        450        460        470        480
VSSGETALTA ADRIAARSAE FAAAVDEMAG GDGDNMVPMF KSTVVKQAMQ EAGQVQMKAL

       490        500        510
LGEDAEPELP LFPGGLVTSP DGMKWLPHHP A
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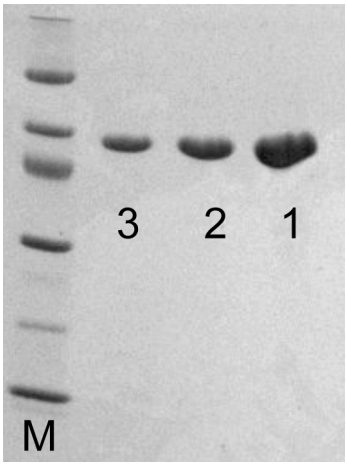
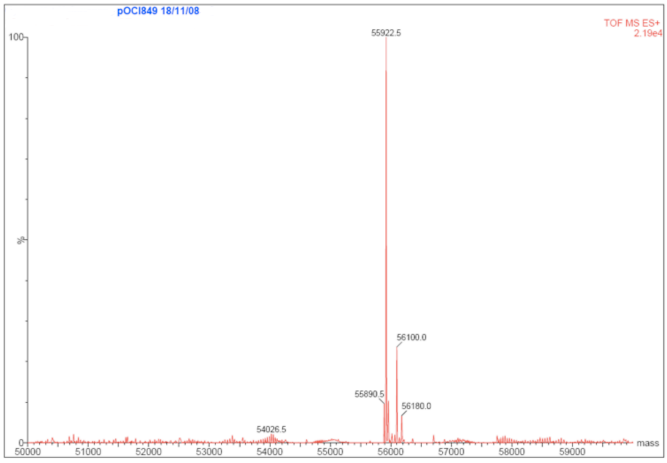
Number of amino acids: 511 **Molecular weight:** 55916.7

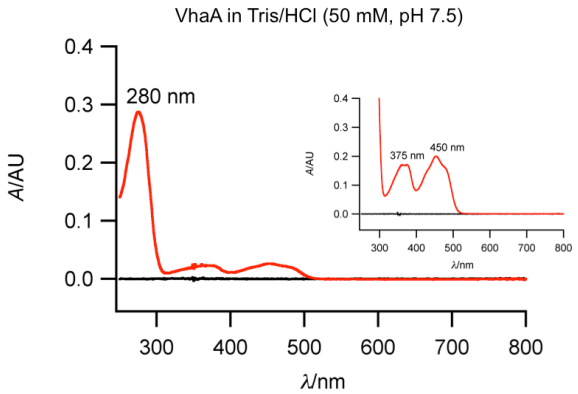
VhaA was purified from cell lysates in three steps: 1) Ni-NTA affinity chromatography; 2) anion exchange chromatography; 3) gel filtration. The protein analyzed in Coomassie-stained SDS-PAGE, at each step of the purification, is shown below (M = molecular weight markers), along with an electrospray time-of-flight MS of the purified protein:

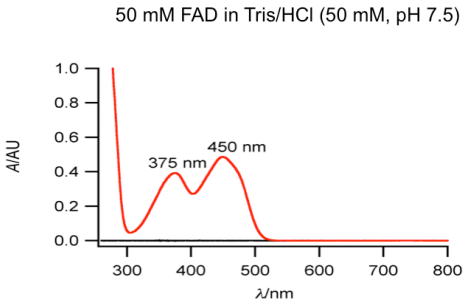




The concentration of VhaA was determined spectrophotometrically at 280 nm using an extinction coefficient $\varepsilon_{280} = 64270\ M^{-1}cm^{-1}$.

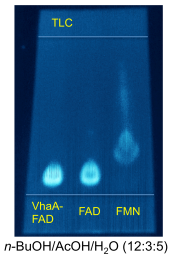
2. UV-Vis spectra of purified VhaA



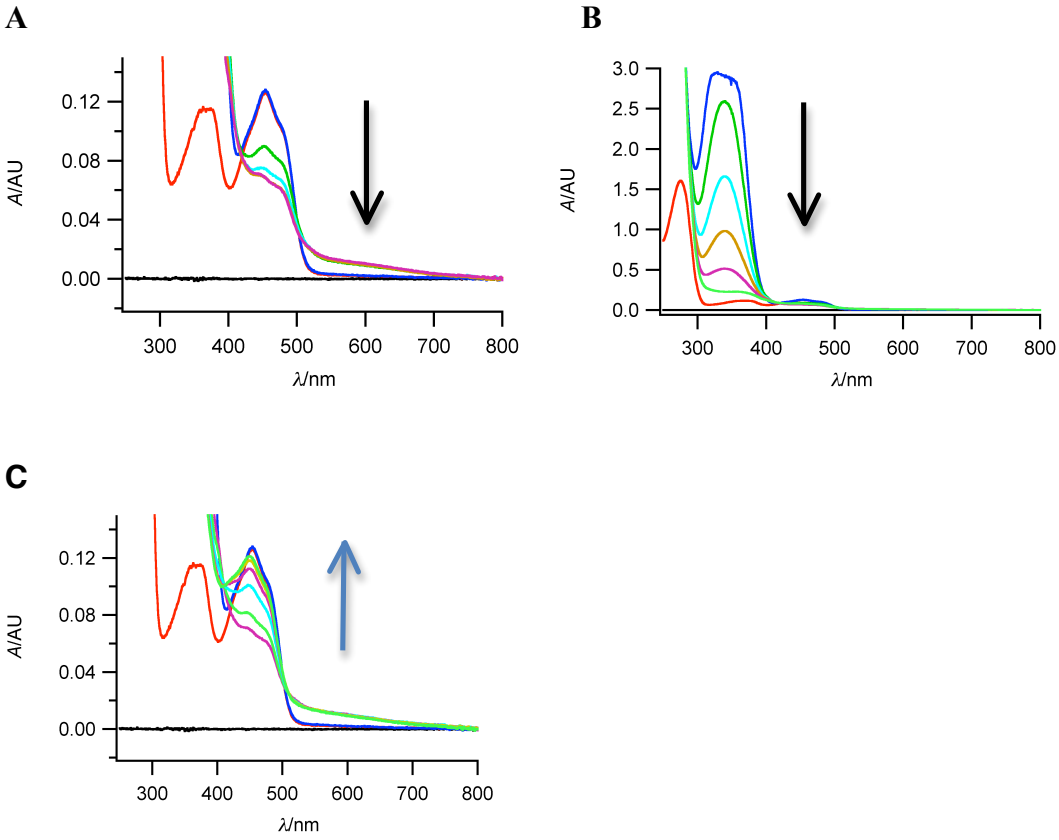


3. VhaA contains bound FAD

Heat denaturation and analysis by TLC (Si plate) of the cofactor isolated from VhaA (*left*), compared to standards (FAD, *middle*; FMN, *right*), with UV detection.



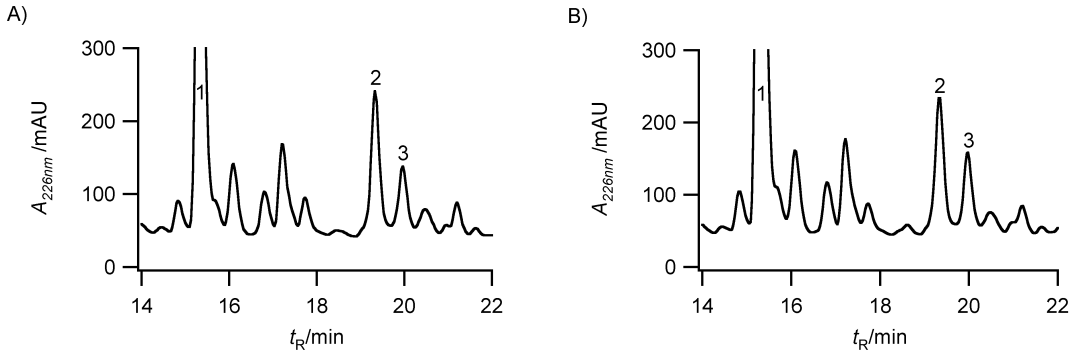
The flavin bound to VhaA could be reduced by NADPH in the presence of the flavin reductase SsuE, as shown below by UV-Vis spectroscopy:



A, Reduction of VhaA with NADPH and SsuE (VhaA 20µM, NADPH 1 mM, SsuE 10 µM; Tris-HCl 50 mM at pH 7.5) over 120 s; **B**, Consumption of NADPH (Δ340 nm) over the same time period; **C**, Re-oxidation of flavin bound to VhaA by air over 120 s.

4. Assays with VhaA

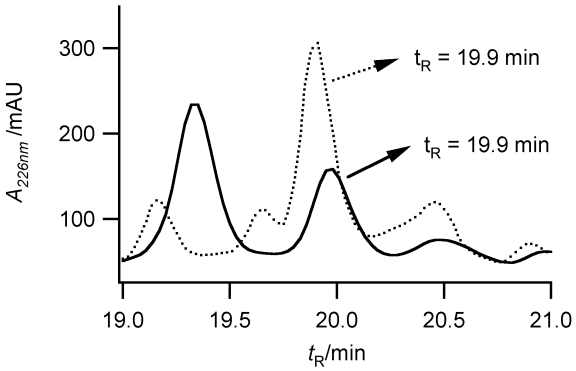
The assay containing the peptide-*S*-PCP substrate (35 μM), VhaA (35 μM), SsuE (10 μM), FAD (100 μM), NaCl (100 mM) in Tris/HCl (50 mM, pH 7.5) was carefully degassed with N_2. This procedure was repeated after addition of NADPH (1 mM) and the mixture was incubated under N_2 at 37 °C for 15 min. The concentration of molecular oxygen within the assay solution was then allowed to increase by opening to air and the solution was incubated another 45 min at 37 °C. During the assay, NADPH (1 mM) was supplemented every *ca*. 3 min in a pulsing mode. Alternatively, an NADPH reduction system comprising glucose-6-phosphate (3 mM) and glucose-6-phosphate dehydrogenase (0.5 U, *Sigma*) could be used to provide NADPH. At the end of the assay, addition of 1/10 volume of aqueous hydrazine (25% v/v) and incubation at 37 °C for 30 min yielded the peptide hydrazides, which were separated from the assay proteins by solid phase extraction (*Waters* OASIS HLB cartridges). Columns were equilibrated with methanol (2 mL) and water (4 mL), washed with 5% methanol (v/v) in water (2 mL) and peptidic fractions were eluted with pure methanol (2 mL). The peptidic fraction was analyzed by analytical RP-HPLC (C_{18} Agilent Zorbax Eclipse XDB, 250 x 4.6 mm, pore diameter 80 Å, particle size 5 μm; 5% MeCN (+0.1% TFA) in H_2O (+0.1% TFA) for 2 min, then 5-40% MeCN (+0.1% TFA) in H_2O (+0.1% TFA) over 33 min at a flow rate of 1 mL/min). Part of a typical HPLC chromatogram is shown below:



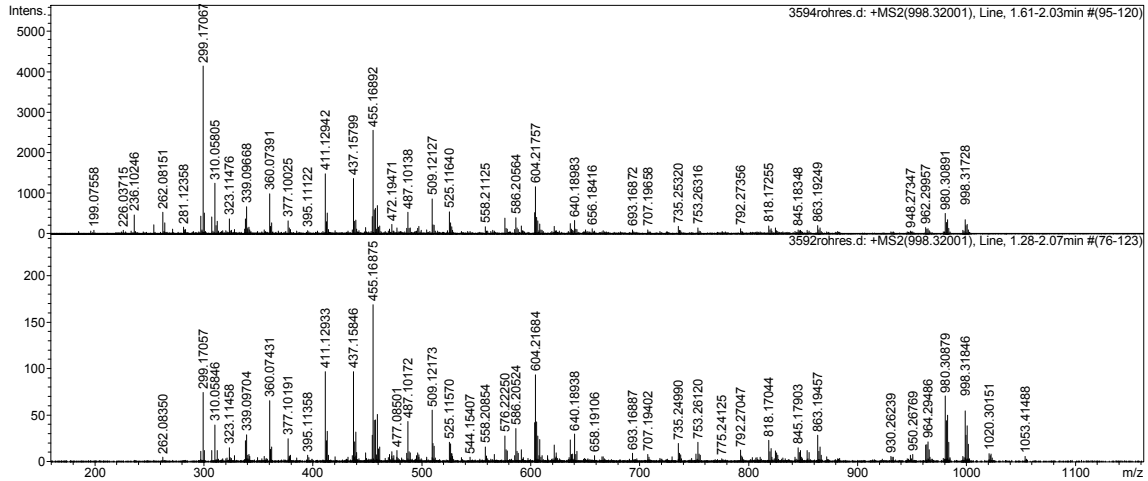
Analytical HPLC chromatograms of assay products isolated from assays with VhaA and β-hydroxylated hexapeptide-PCP7 substrate **2**. **A)** The assay with VhaA was carried out under conditions described above with NADPH-pulses. *Peak 1*, *m/z* 930.4 $[M+H]^+$, residual non-chlorinated hexapeptide hydrazide (**3a**); *peak 2*, *m/z* 942.40014, *m/z* 964.38202 (possible sodium-adduct of *m/z* 942.4). A mass *m/z* 964 corresponds to that of mono-chlorinated product $[M+H]^+$. However, the HR-MS and isotope pattern **do not** correspond to that expected for the molecular formula of mono-chlorinated peptide hydrazide ($[M+H]^+$ *m/z* calc. 964.36024). MS/MS spectra of the *m/z* 942 species

revealed no fragment ions similar to those for **3a** or **3**. The identity of this species is presently unknown; *peak 3*, *m/z* 998.32095 $[M+H]^+$, dichlorinated hexapeptide hydrazide product **3**. **B)** The assay with VhaA was carried out in the presence of an NADPH regeneration system consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. *Peak 1*, *m/z* 930.4 $[M+H]^+$, residual non-chlorinated hexapeptide hydrazide (**3a**); *peak 2*, *m/z* 942.4, *m/z* 964.4; *peak 3*, *m/z* 998.3 $[M+H]^+$, dichlorinated hexapeptide hydrazide product **3**.

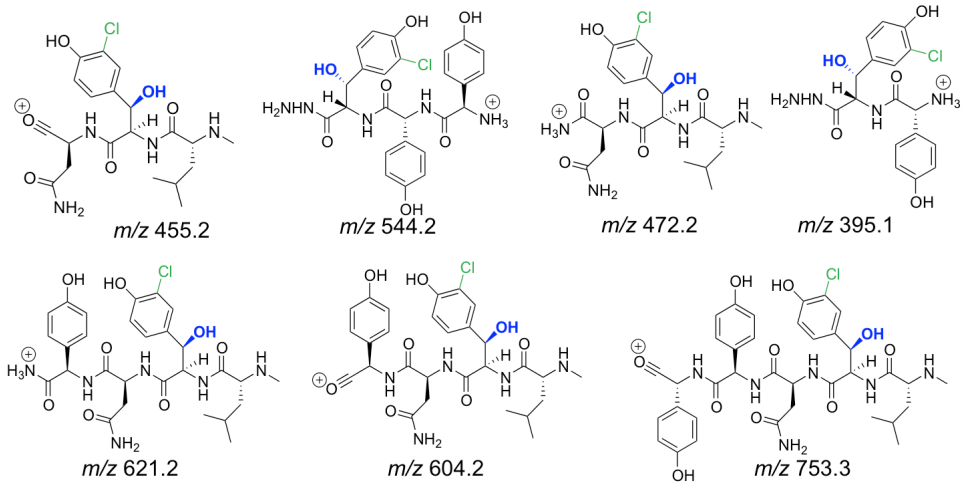
The dichlorinated peptide **3** from the assay was compared by HPLC and MS to material prepared by reacting **4** with hydrazine, as shown below:



Superposition of the analytical HPLC chromatograms of product **3** produced in the VhaA assay (*black line*) and that prepared from **4** (*dotted line*). The peaks corresponding to the linear dichlorinated hexapeptide hydrazides give in each case the same HPLC retention time under identical HPLC conditions.



ESI-MS/MS spectra of the product **3** produced in the VhaA assay (*bottom*) and that prepared synthetically from **4** (*top*).



ESI-MS/MS ion structures assigned to the MS peaks (see above) confirm that chlorination has occurred in residues-2 and -6 in **3**.

Electrospray ionization (ESI) mass spectrometry was performed on a *Bruker Esquire* LC/MS. HPLC/MS measurements were performed on a HPLC system comprising a *Dionex P 580 Pump*, a *Gilson 215* liquid handler, a four channel UV detector *Dionex UVD 170S* and a ESI-MS detector *Thermo AQA*. UV detection was measured at λ = 226, 278 and 254 nm. High-resolution electrospray mass spectra (HR-ESI-MS) were recorded on a Bruker maXis QTOF-MS instrument (*Bruker Daltonics* GmbH, Bremen, Germany). The samples were dissolved in MeOH and analyzed via continuous flow injection at 3 μL/min. The mass spectrometer was calibrated between *m/z* 118 and 2721 using a Fluka electrospray calibration solution (*Sigma-Aldrich*, Buchs, Switzerland) at a resolution of 20'000 and a mass accuracy below 2 ppm. All solvent used were purchased in best LC-MS qualities.