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Autocatalytic backbone N-methylation in a family of ribosomal peptide natural products

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Peptide backbone N-methylations as in cyclosporin A have been an exclusive characteristic of non-ribosomal peptides. We have identified the first posttranslationally modified peptide or protein harboring internal α -N-methylations through discovery of the genetic locus for the omphalotins, cyclic N-methylated peptides produced by the fungus *Omphalotus olearius*. We show iterative autocatalytic activity of an N-methyltransferase fused to its peptide substrate is the signature of a new family of ribosomally encoded metabolites.

The fidelity and complexity of protein- and peptide-mediated interactions is predominantly achieved through the vast chemical space provided by the 20 canonical proteinogenic amino acids. This space is extended by posttranslational modifications (PTMs) such as phosphorylation, glycosylation, acetylation, amidation, and many others¹. An expanded repertoire of PTMs can be found in ribosomally synthesized and posttranslationally modified peptides (RiPPs), a class of natural products that is found in all kingdoms of life and invokes an extensive array of biological activities². RiPPs are produced by translation of protein/peptide precursors followed by posttranslational modification of these precursors by additional enzymes. Many of these accessory proteins are presumably recruited by the N-terminal leader sequence of the precursor to amend the C-terminal peptide core². Proteolytic release and export of the core affords the active metabolite.

In contrast to RiPP biosynthesis, nonribosomal peptide synthetases (NRPSs) create structurally diverse peptides through direct incorporation of non-proteinogenic amino acids³. This feat is achieved in an assembly line-like fashion where individual modules within giant multimodular NRPS proteins are responsible for the activation, incorporation, and optional further modification of amino acids.

Recent insights into RiPP enzymes have begun to challenge the view that certain residues and peptide modifications are only accessible to NRPS pathways⁴. Nonetheless, while the methylation of peptide N-termini and side chains have been observed in RiPPs², backbone N-methylation has only been found in NRPS-derived peptides and shown to occur prior to amide bond formation⁵. These modifications have been demonstrated to improve therapeutic peptide metabolic stability, membrane permeability, target selectivity, affinity and oral bioavailability⁵. Despite these favorable properties, backbone α -N-methylation has to our knowledge not been reported as a PTM in any naturally occurring ribosomal peptide or protein⁵.

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Cyclic peptides such as the nematotoxic omphalotins from the basidiomycete *O. olearius*, in which 9 of the 12 backbone nitrogens are methylated, were therefore assumed to be products of an NRPS⁶ (**Fig. 1a**). However, a basic local alignment search tool (BLAST) search of the circularly permuted omphalotin peptide sequence (WVIVVGIVGVIG) against the *O. olearius* genome revealed a protein-encoding gene (*ophA*) containing a stretch of codons consistent with the omphalotin peptide scaffold (core peptide) as part of a gene cluster (**Fig. 1b**). Surprisingly, the OphA sequence N-terminal of the core peptide (leader sequence) comprises 399 amino acid residues and contains a domain with sequence homology to SAM-dependent methyltransferases (**Fig. 1c**). Such a protein domain architecture has, to our knowledge, never been observed before in RiPP precursors. Recognition motifs present in other RiPP family leader sequences, namely the RiPP precursor peptide recognition element (RRE) or the α -helical sequence conserved in microviridins⁷, were not detected in OphA.

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To test whether OphA was both the genetic source of omphalotins and the methyltransferase responsible for α -N-methylation, *ophA* cDNA was cloned and the gene encoding OphA with an N-terminal histidine affinity tag was expressed in *E. coli* and purified via metal chelate chromatography (**Supplementary Results, Supplementary Fig. 1**). After one day of expression, electrospray ionization time of flight mass spectrometry (ESI-TOF-MS) analysis of recombinant full-length OphA showed a mass shift corresponding to ten methylations (**Supplementary Note**). In contrast, omission of the C-terminal omphalotin-encoding region of OphA (OphA-NP) resulted in the expected mass of the unmodified recombinant protein (**Supplementary Note**). Detailed peptide fingerprinting of trypsin-digested OphA was performed using high performance liquid chromatography coupled tandem mass spectrometry with high-energy collision dissociation (HPLC-MS/MS HCD). OphA expressions from one to five days revealed a suite of differentially methylated species with up to 11 methylations sequentially extending from the N- to C-terminus of the precursor core peptide (**Fig. 2a, Supplementary Note**). The pattern of methylation was the same as in the natural products, with two additional methylations in the sequence C-terminal of the core peptide (C-terminal recognition sequence). Electron-transfer dissociation (ETD) fragmentation unambiguously localized the methylation sites to the backbone nitrogens (**Fig. 2b**).

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Based on homology of OphA with homodimeric SAM-dependent methyltransferases, we hypothesized that residues Y98 and S129 might be located within the OphA SAM-binding pocket (**Supplementary Fig. 2**). In agreement with this hypothesis, alanine substitutions for Y98 and S129 indeed abolished methylation (**Supplementary Fig. 3a-c**). These results suggest that the methyltransferase domain of OphA is required for the observed methylation of the omphalotin peptide sequence and that this activity is likely SAM-dependent. Gel filtration experiments suggested OphA associates into homodimers (**Supplementary Fig. 1**), incurring the possibility that the apparent automethylation occurred either intra- or intermolecularly. In order to distinguish between these two possibilities, the inactive mutants OphA S129A and OphA Y98A were coexpressed in trans-complementation experiments with the catalytically active core peptide mutant OphA V5T. After 36-hour expression in *E. coli*, the otherwise inactive OphA mutants harbored up to ten α -N-methylations, revealing the capability of OphA to act in trans (**Supplementary Fig. 3d-f**). Attempts to further address this issue, through observing and characterizing in vitro activity of purified OphA, have thus far been unsuccessful despite observing activity in cell-free extracts with or without supplementation of S-adenosylmethionine (**Supplementary Fig. 4**); this has been seen in

other RiPP systems⁸. Future experiments will be necessary to further validate that OphA catalyses intermolecular α -N-methylation and to test whether the enzyme accepts peptide substrates unbound to the leader sequence.

To determine OphA sequence requirements for methylation, we introduced several mutations into the flanking regions of the OphA core peptide. Removal of the C-terminal recognition sequence (OphA-NF) resulted in incomplete methylation near the C-terminus (**Supplementary Note**). Sequences following RiPP core peptides have been postulated to be able to replace leader sequences in the case of bottromycins⁹, and have been shown to be involved in other PTMs such as dehydration/decarboxylation¹⁰ and macrocyclization¹¹⁻¹³. On the other hand, mutation of residues 392 through 396 to incorporate a TEV cleavage site (OphA-TEV) preceding the core peptide resulted in a methylation-competent precursor (**Supplementary Note**). Thus, there appears to be a moderate sequence flexibility for the flanking residues of the core peptide sequence, opening the possibility of engineering cleavable N-methylated linear peptides without the necessity of additional biosynthetic enzymes.

We exchanged the omphalotin core peptide in OphA with sequences closely resembling two naturally occurring, backbone N-methylated therapeutic peptides: the NRPS-derived cyclosporin A (CycA), used as an immunosuppressant drug in organ transplantations¹⁴, and dictyonamide A (DicA), shown to be a cyclin-dependent kinase 4 inhibitor¹⁵. HPLC-MS/MS HCD analysis of the trypsinized fragments comprising the CycA- and DicA-like cores revealed up to five methylations for the CycA-like core (**Fig. 2c**) and up to nine for the DicA-like peptide fragment (**Fig. 2d**) upon expression for five days in *E. coli*. Whole-protein ESI-TOF-MS of these samples revealed the most abundant species were the two- and five-times methylated CycA-like peptide and the three- and eight-times methylated DicA-like peptides (**Supplementary Note**). Of note, the core methylation patterns of these hybrids did not fully correspond to the patterns of the natural products (**Fig. 2**). Nonetheless, these data are exciting proof-of-principle findings since synthetic production of custom backbone N-methylated peptides in large quantities is still troublesome due to low coupling yields and side reactions¹⁶. While these types of peptides can be produced by in vitro translation using custom-loaded aminoacyl-tRNAs¹⁷, the yields from this process are limited. Thus, a scalable in vivo method for the production of backbone methylated peptides, such as with this system capable of producing differentially methylated species on alternative OphA core sequences, would be an attractive strategy.

All of the above results strongly suggest that omphalotins are not NRPS products but the founding member of a novel family of RiPPs. Analysis of the *ophA* locus in the *O. olearius* genome revealed that the gene is part of a gene cluster with enzymes possibly involved in tailoring and maturation of the core peptide (**Fig. 1b**). Two putative P450 monooxygenases and an O-acyltransferase are likely involved in posttranslational modification of the precursor to create the peptide scaffolds found in omphalotins B-I¹⁸ (**Supplementary Fig. 5**). Macrocyclization of omphalotins is likely mediated by a putative prolyl oligopeptidase; endopeptidases have been shown to cyclise peptides in bacteria, fungi, and plants as seen in the biosynthesis of patellamides¹¹, amatoxins¹² and cyclotides¹³. Experiments to confirm the function of the other enzymes flanking *ophA* in the *O. olearius* genome are ongoing.

BLAST searches of other sequenced fungal genomes using the proteins encoded in the omphalotin gene cluster as queries revealed a homologous gene cluster in the basidiomycete *Dendrothele bispora* CBS 962.96 (**Supplementary Fig. 6a**). As an initial proof of concept, the putative precursor gene found in *D. bispora* (*dbophA*) was synthesized (**Supplementary Fig. 6b**). DbOphA was functionally competent in that it methylated its core peptide, whose sequence is highly homologous to the omphalotins, in a similar pattern to OphA (**Supplementary Note**).

Examples of autocatalysis are widespread in nature and range from autoproteolysis, autophosphorylation, and automethylation to isopeptide bond formation and pseudo-cofactor/chromophore maturation¹⁹. Intermolecular autocatalysis has been observed less frequently but has been described in the maturation of proteases like thrombin²⁰ and capsid assembly of bacteriophage HK97²¹. However, nearly all of these processes have one of two functions: either they modulate the catalytic activity or stabilize the structure of the respective enzyme. By contrast, OphA is an enzyme that contains the structural information of a metabolite and the catalytic domain to modify its structure.

In summary, we demonstrate that the omphalotins are the founding members of a fourth family of fungal RiPPs besides the amatoxins²², dikaritins (comprising ustiloxins and phomopsins)^{23,24} and epichloëcyclins²⁵. The hallmark of this newly described family is N-methylation of the peptide backbone accomplished by a methyltransferase domain encoded within the leader of the peptide precursor protein. The methyltransferase domain in OphA appears to act iteratively, in an N-to-C-terminal consecutive manner, on the core peptide sequence that is located at the C-terminus of the precursor protein and destined to be cleaved off for the generation of the mature natural product. Although we are unaware of examples where a RiPP modifying enzyme was directly fused to a core peptide to circumvent the need for a leader, several research groups have successfully engineered artificial fusions of RiPP modifying enzymes to leader sequences in order to observe in trans modification of core peptides^{26,27}. To our knowledge, such a self-modifying reaction as part of a natural product biosynthetic pathway is without precedent.

For this newly discovered family of RiPPs, we propose the name ‘Borosins’ after the ancient mythological symbol Ouroboros, a serpent biting into its own tail. Based on our findings that the identified methyltransferase is functional in the *E. coli* cytoplasm and appears to be promiscuous with regard to acceptor peptide sequence specificity, this RiPP family opens the possibility to produce backbone N-methylated peptide libraries in *E. coli* for the identification of new bioactive peptides.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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Author contributions

M.K. initiated and supervised the project. M.F.F. and N.S.V. designed the experiments. N.S.V. and N.K. conducted the experiments. N.S.V., M.F.F. and M.J.H. analysed the data. All authors discussed the results. M.F.F., N.S.V. and M.K. wrote the manuscript. J.P. edited the manuscript and provided experimental expertise.

Competing financial interests

M.K., M.F.F., N.S.V., N.K. and J.P., are inventors on European Patent Application no. EP16164245.9 filed by ETH Zürich titled “Novel multiply Backbone N-methyl transferase and use thereof”.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. Correspondence and requests for materials should be addressed to M.K. and M.F.F.

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288

289 Figure legends

290

291 **Figure 1 | Omphalotin A: Natural product and encoding genetic locus.** (a) Structure of
 292 omphalotin A, with the α -nitrogen methyl groups indicated in green. (b) The putative
 293 biosynthetic gene cluster for omphalotin A (further details in **Supplementary Table 2**). (c)
 294 The core sequence (italicized residues 1 to 12) of the precursor protein OphA, encoding the
 295 omphalotins, is flanked by a leader (residues 1 to 399) and C-terminal recognition sequence
 296 (CRS; residues +1 to +6). Due to methyltransferase domain homology (PF00590) within the
 297 leader, residue numbering is modified as suggested for RiPPs².

298

299 **Figure 2 | Observed methylation states of wild-type and hybrid omphalotin precursors.**
 300 Each peptide sequence represents MS/MS spectra for the C-terminal tryptic fragment of
 301 OphA purified from various *E. coli* expressions. Solid green circles indicate MS/MS
 302 confirmation of N-methylated residues; unfilled circles represent detected but undefined N-
 303 methylation positions. Schematics of relevant natural products are depicted at the top of the
 304 panels. ESI-TOF-MS, HPLC-MS/MS and estimated relative amounts of methylated OphA
 305 tryptic fragments are in the **Supplementary Note**. (a) Methylation of OphA. Zero through 10
 306 methylations (with 10 being most abundant) were observed after one-day expressions, while
 307 11 methylations could only be observed after five days. (b) Confirmation of methylations
 308 localized to backbone α -nitrogens by HPLC-MS/MS ETD. Observed b-, c- and y-ions of the
 309 three-times methylated OphA fragment after four hours of expression are denoted on the
 310 omphalotin-encoding peptide structure; the observed methylations are in perfect agreement
 311 with the natural product. (c,d) Methylation states of hybrid omphalotin precursors OphA-
 312 CycA and OphA-DicA after five-day expressions, where the omphalotin-encoding sequence
 313 is exchanged with a peptide resembling cyclosporin A (c) or dictyonamide A (d). * = D-
 314 alanine; R1 = (2S, 3R, 4R, 6E)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid; R2 = L- α -
 315 aminobutyric acid; Abz = 2-aminobenzoic acid.

316

317 Online Methods

318 **cDNA synthesis and cloning of the *ophA* precursor.** *O. olearius* strain DSM3398 (DSMZ)
 319 was used for RNA extraction and cDNA synthesis. *O. olearius* mycelium was grown on
 320 cellophane disks (Celloclair) on 1.5% (w/v) agar plates containing yeast extract maltose agar
 321 (0.4% (w/v) yeast extract (Oxoid AG), 1% (w/v) malt extract (Oxoid AG), 0.4% (w/v) glucose)
 322 for 5 days at 28 °C in the dark. To extract RNA, 8.2 mg of lyophilized mycelium was lysed
 323 with 8 mg of 0.5 mm glass beads in three FastPrep (Thermo Savant) steps of 45 s at 4.5, 5.5
 324 and 6.5, cooling the sample for 5 min on ice between each step. RNA was extracted with 1
 325 ml Qiazol (Qiagen) and 0.2 ml chloroform. After centrifugation at 12000 g at 4 °C for 15 min,
 326 RNA was recovered in the aqueous phase, loaded and washed on-column using the RNeasy
 327 Lipid Tissue Mini Kit (Qiagen) and eluted with RNase-free water. cDNA was synthesized
 328 from 2 μ g of extracted RNA using the Transcriptor-first strand cDNA synthesis kit (Roche
 329 Applied Science) following the manufacturer's instructions. The putative coding sequence of
 330 the omphalotin precursor gene was amplified from cDNA by PCR using Phusion high-fidelity
 331 DNA polymerase (ThermoFisher) with primers Fw-OMP and Rv-OMP (**Supplementary Table**
 332 **1**). The PCR product was subjected to an A-tailing reaction before it was cloned into the
 333 pGEM-T-easy vector (Promega) and transformed to *E. coli* DH5 α . For construction of an *E.*
 334 *coli* expression plasmid, a PCR reaction was performed using the pGEM-T-easy-cDNA
 335 plasmid as template and pET24-Fw-OMP or pET24-Fw-HisOMP and pET24-Rv-OMP

(**Supplementary Table 1**) as primers. The resulting PCR product encoding an N-terminal His tag was cloned into pET24 (Merck Millipore) using the restriction sites *NdeI* and *NotI* (Thermo Scientific) and the resulting plasmids were transformed into *E. coli* BL21(DE3) (Promega). All DNA constructs were verified by DNA-sequencing (Microsynth AG).

Synthesis and cloning of *dbophA*. *dbophA* was synthesized based on publicly available RNA sequencing data that can be found on the JGI website (http://genome.jgi.doe.gov/vista_embed/?organism=Denbi1) and codon-optimized for expression in *E. coli* by Genscript. The codon optimized sequence containing an N-terminal His tag and *NdeI* and *HindIII* restriction sites was as follows: 5'-CATATGGAACATCATCATCATCATCATCATATTCTTCTACTCAAACCAAACCCGGTTTCGCTCATCGTCGTCGGTACAGGCATCGAGAGCATCGGTCAAATGACGCTCCAAGCCTTATCGTACATTGAAGCTGCTTCCAAAGTCTTCTATTGTGTGCATCGACCCTGCGACAGAGGCTTTTATCCTCACCAAGAACAAGAATTGTGTGCGACTTGTATCAGTACTACGACAATGGCAAGTCTAGAATGGATACTTACACCCAAATGGCTGAGCTCATGCTCAAGGAAGTCCGCAATGGCCTCGACGTTGTGCGGGTATTCTATGGCCATCCGGGCGTGTTTCGTGAACCCTTCTCACAGGGCATTGGCTATCGCCAGAAGCGAGGGATACCAAGCTCGTATGCTTCCAGGAGTATCTGCAGAGGACTGCCTCTTTGCCGACTTATGCATCGACCCCTCGAACCCCGGCTGCTTGACCTACGAAGCATCCGATTTCTCATCAGAGAGAGACCAGTGAACGTTACAGTCACCTCATTCTTTTCCAAGTTGGATGCGTCGGTATCGCAGACTTCAATTCAGCGGATTTCGACAATTCGAAATTTACCATTCTGGTTGACCGTCTCGAGCAAGAATACGGCCCAGACCATACCGTCGTGCATTATATCGCAGCTATGATGCCTACCAAGATCCCGTCAACGACAAGTTCACGATCGGACAACCTCCGTGAACCCGAAATCGCCAAACGAGTAGGTGGTGTTCGACTTTTTACATCCCTCCAAAGGCCAGAAAGGATATCAACACCGATATCATCCGACTCTTGAATTCCTACCTGCTGGTAAAGTTCCCGACAAACACACCCAAATCTACCCACCTAATCAATGGGAACCCGACGTGCCTACTCTACCTCCCTATGGACAAAACGAACAGGCTGCTATCACTAGATTGGAAGCTCACGCTCCCCCGAAGAGTATCAGCCTCTGGCCACTTCAAAGCTATGACTGACGTCATGACCAAAGTACTTTGGATCCCAAGGCGCTCGCCGAGTATAAGGCTGATCATCGAGCCTTCGCTCAGTCTGTTCTGACTTGACGCCTCAAGAGAGAGCAGCTTTGGAGCTAGGAGATTCATGGGCTATTCGGTGCGCCATGAAGAACATGCCGTCCTCTCTCTTGAAGCCGCTAGCCAGTCCGTGCAAGAGGCATCCATGAACGGTTTCCCATGGGTCATCGTCACGGGTATCGTTGGGGTCATCGGATCGGTTGTGAGCAGTGCTTGAAGCTT-3'. The codon-optimized ORF was recloned into pET24 for expression in *E. coli* BL21(DE3) using the restriction sites *NdeI* and *HindIII*.

Cloning of *ophA-NF*, *-NP*, *-TEV*, *-CycA* and *-DicA*. In order to remove the sequence encoding the omphalotin core peptide (*ophA-NP*) a stop codon was introduced after the proline followed by a *BsaI* restriction site using ligation of a synthetic adapter consisting of oligonucleotides Fw-OMP-*BsaI* and Rv-OMP-*BsaI* (**Supplementary Table 1**). Primers were annealed by combining 10 µl of each oligonucleotide (100 µM) and mixing it with 80 µl of 10 mM Tris pH 7.5. The mixture was subjected to the following program: 5 min at 98 °C with a cooling rate of 4 °C/s, 30 seconds at 80 °C with a cooling rate of 0.01 °C/s and 30 seconds at 40 °C with a cooling rate of 0.02 °C/s. The annealed primers were phosphorylated and ligated into pET24 containing *ophA* and digested with restriction endonucleases *NcoI* and *NotI* (Thermo Scientific) at a vector to insert mole ratio of 1:7. The inserted *BsaI* site was then used for removal of the C-terminal recognition sequence or to insert sequences coding for CycA and DicA using primers 8 to 13 in **Supplementary Table 1**. Primer annealing was performed as described above and the synthetic DNA fragments were ligated into pET24-*ophA* containing the introduced *BsaI* site using restriction sites *BsaI* and *NotI*. For the introduction of the TEV cleavage site in the *ophA* gene the following DNA fragment was

synthesized by Genscript: 5'-
GGATCCCAAGGCACTCGCCGACTACAAAGCTGATCACCGCGCCTTTGCTCAATCTGTCC
CCGACTTGACGCCTCAGGAGCGTGCGGCTTTGGAGCTCGGTGATTCGTGGGCTATTCCG
TTGCGCGATGAAGAATATGCCCTCGTCGCTCTTGGACGCTGCTCGTGAATCCGGCGAA
AACTTGTACTTCCAAGGTTTCCCATGGGTCATCGTCGTTGGTGTATCGGTGTCATCGG
ATAAGAATTC-3'. The fragment was ligated into pET24 containing the *ophA* ORF using the
restriction sites *Bam*HI and *Eco*RI.

Generation of constructs encoding OphA P399A, V5T, Y98A, and S129A. To generate the *ophA* mutants, site directed mutagenesis was performed with the Phusion Site-directed Mutagenesis Kit from Thermo Scientific according to the instructions of the manufacturer using primers 14 to 21 (**Supplementary Table 1**) using pET24-*ophA* as a template. To clone non-tagged *ophA* V5T into pCDFDUET-1 (Merck Millipore), a PCR reaction was performed using the pET24-*ophA* V5T plasmid as template and the primers pCDF-Fw-OphA V5T and pCDF-Rv-OphA V5T (**Supplementary Table 1**). The resulting PCR product was digested with *Bsa*I and *Hind*III and cloned into pCDFDUET-1 using the restriction sites *Nco*I and *Hind*III.

Purification of His-tagged OphA, OphA variants and DbOphA from *E. coli* BL21(DE3). *E. coli* BL21(DE3) transformants containing the various *ophA* and *dbophA* expression vectors were pre-cultured overnight in LB medium containing 50 µg/ml kanamycin at 37 °C. 1 ml of pre-culture was diluted in 1 L of TB medium (2.4% (w/v) yeast extract (Oxoid AG), 1.2% (w/v) tryptone (Oxoid AG), 0.4% (w/v) glycerol, 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄) containing 50 µg/ml kanamycin. Cultures were grown at 37 °C to an OD₆₀₀ between 1.5-2 after which they were chilled on ice for 20 min. 0.2 mM IPTG was added and further incubation was performed at 16 °C for the indicated expression times. Bacterial cells were spun down at 8000 g for 5 min at 4 °C, pellets were resuspended in ice-cold buffer (50 mM HEPES pH 8, 0.1% (v/v) triton X-100, 10% (v/v) glycerol) containing 20 mM imidazole and lysed using a French press. Cell debris was removed by centrifugation at 16000 g for 30 min. The supernatant was incubated with Ni-NTA beads (Thermo Scientific) at 4 °C for 1 h, washed with 10 column volumes of ice-cold buffer and the protein was finally eluted in buffer containing 400 mM imidazole. Purified protein was concentrated on an Amicon Ultra-4 centrifugal filter device (Millipore) with a molecular weight cutoff of 30 kDa, desalted using a PD-10 column (Amersham Biosciences) in buffer without imidazole and flash-frozen in liquid nitrogen before being stored at -20 °C. Protein concentrations were measured using the Pierce BCA protein assay (Thermo scientific) using a standard curve derived from bovine serum albumin.

Size exclusion chromatography. Size exclusion chromatography was performed using a Superdex-200 Increase column (GE healthcare). The column was calibrated using high and low molecular weight gel filtration standards (GE healthcare) in 50 mM HEPES pH 8, 0.1% (v/v) triton X-100, 10% (v/v) glycerol. The column was pre-equilibrated in the same buffer and OphA (5 mg/mL) was applied to the column in a total volume of 100 µl. Flow-through fractions were collected and analyzed by 12% (w/v) SDS-PAGE.

HPLC-MS/MS HCD. An Amicon Ultra column (30 kDa cutoff) was washed two times with ddH₂O. Protein (100 µg) diluted with ddH₂O to a final volume of 400 µL was added. The sample was centrifuged (11000 g for 5 min) and washed with 500 µl of ddH₂O. A buffer

containing 50 mM NH_4HCO_3 pH 8 was added and the sample was concentrated to 4 $\mu\text{g}/\mu\text{l}$. 25 μl was transferred to a glass vial, trypsin (Promega) was added in a molar ratio of 1:70 and the sample incubated for 3 h at 37 °C. 3 μl of each sample was used for mass spectrometry analysis. HPLC-MS/MS data was recorded on a Thermo Scientific Q Exactive mass spectrometer equipped with a Dionex Ultimate 3000 UHPLC system using a Phenomenex Kinetex 2.6 μm C18 100 Å (150 × 4.6 mm) column heated at 50 °C. Elution was performed with a linear gradient using water with 0.1% (v/v) formic acid (FA) (solvent A) and acetonitrile (ACN) with 0.1 % (v/v) FA (solvent B) at a flow rate of 0.8 mL/min. The column was equilibrated with 5 % solvent B for 2 min followed by a linear increase of solvent B to 85 % over 15 min and a final elution step with 98% solvent B for 5 min. Mass spectra were acquired in positive ion mode with the following settings; spray voltage 3500 V, capillary temperature at 268.75 °C, probe heater at 437.5 °C and an S-lens level at 50. Full MS was done at a resolution of 35000 (AGC target 1e6, maximum IT 100 ms, range between 500-1800 m/z) and data-dependent MS/MS was performed at a resolution of 17500 (AGC target at 2e5, maximum IT of 300 ms isolation window 1.2) using a stepped NCE of 16, 20 and 24. The inclusion list contained the mass of the trypsinized fragment encoding the omphalotin peptide (or one of the variants) and its different methylation states. Data were processed using ThermoFisher Xcalibur software.

HPLC-MS/MS ETD. Sample preparation was performed as stated above (see: HPLC-MS/MS HCD). 50 μl of trypsinized sample was desalted and purified using C18 ZipTips (Merck Millipore). ZipTips were washed 5 times with 10 μl of 90% ACN with 0.1% (v/v) FA followed by ten times equilibration with 10 μl of 0.1% (v/v) FA. After equilibration, peptides were bound to the column by pipetting 10 μl of sample up and down 10 times and washed 10 times with 10 μl of 0.1% (v/v) FA. Peptides were eluted in 10 μl 70% ACN with 0.1% (v/v) FA by pipetting up and down ten times followed by an additional elution with 90% ACN with 0.1% (v/v) FA. Eluates were pooled and dried using a speedvac and dissolved in 20 μl of 2.5% ACN with 0.1% (v/v) FA. 3 μl of each sample was used for MS analysis. HPLC-MS/MS data was recorded on a LTQ-Orbitrap Fusion mass spectrometer (Thermo Fischer Scientific) coupled to an Easy-nLC 1000 UHPLC system (Thermo Fischer Scientific) using a self-made fritted column (75 μm × 150 mm) packed with reverse-phase C18 material (ReproSil-Pur 120 C18-AQ, 1.9 μm , Dr. Maisch GmbH) heated at 50 °C. Elution was performed with a linear gradient using water with 0.1% (v/v) FA (solvent A) and ACN with 0.1% (v/v) FA (solvent B) at a flow rate of 0.3 $\mu\text{L}/\text{min}$. The column was equilibrated with 5% solvent B for 2 min followed by a linear increase of solvent B to 85% over 15 min and a final elution step with 98% solvent B for 5 min. For MS analysis, one scan cycle comprised of a full scan MS survey spectrum, followed by electron-transfer/higher-energy collision dissociation (ETHcD) MS/MS scans with a maximum duty cycle of 3 seconds using a target list. Full-scan MS spectra (600–2000 m/z) were acquired in the FT-Orbitrap at a resolution of 120,000 at 400 m/z , while ETHcD MS/MS spectra were recorded in the FT-Orbitrap at a resolution of 30,000 at 400 m/z . ETD was performed with a target value of 2e5 with supplementary activation energy set at 30. For all experiments, dynamic exclusion was used with 1 repeat count, 30 s repeat duration, and 10 s exclusion duration. Data were processed using ThermoFisher Xcalibur software.

ESI-TOF-MS. Determination of total mass by ESI-TOF-MS was performed by the Functional Genomics Center Zürich (FGCZ). In order to get rid of detergent, proteins were precipitated with 20% (w/v) trichloroacetic acid (TCA), reconstituted in hexafluorisopropanol (HFIP) prior to the analysis. ESI MS data was recorded on a Waters Q-TOF SYNAPT G2 HDMS in a

mass range between 50 and 5000 Da; the sampling cone energy was set at 35-50V. The m/z data were then deconvoluted into MS data using the MaxEnt1 software (Waters).

Trans-complementation assays. *E. coli* BL21(DE3) containing pCDF-*ophA* V5T and either the pET24-*ophA* Y98A or S129A expression vector was grown in LB medium containing a final concentration of 50 µg/ml kanamycin or streptomycin, respectively. Protein expression was induced for 36 h after which His-tagged OphA-Y98A or OphA-S129A was purified via metal chelate chromatography. Methylation was assessed via HPLC-MS/MS HCD as described above.

Cell-free assays. An *E. coli* BL21(DE3) culture was grown overnight at 37 °C. Bacterial cells were spun down at 8000 g for 5 min at 4 °C, pellets were resuspended in ice-cold buffer (50 mM HEPES pH 8, 0.1% (v/v) triton X-100, 10% glycerol) and lysed using a French press. Cell debris was removed by centrifugation at 16000 g for 30 min. Previously purified OphA expressed for four hours in *E. coli* was added to 300 µl of cell lysate to a final concentration of 5 µM. After three days of incubation at room temperature, 25 µl of the cell lysate was transferred to a glass vial, trypsin (Promega) was added in a mole ratio of 1:35 and the sample incubated for three hours at 37 °C. 15 µl of the sample was used for mass spectrometry analysis as described previously.

Data Availability. The data that support the findings of this study are available from the corresponding authors upon request.

a **b** **c**

a

Omphalotin A:

ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
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ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E
ESGEEASQNGFPW V I V V G V I G V I G S V M S T E

b

c

Cyclosporin A:

ESGEEASQNGFPL V L A A L L V I V G S V M S T E
ESGEEASQNGFPL V L A A L L V I V G S V M S T E
ESGEEASQNGFPL V L A A L L V I V G S V M S T E
ESGEEASQNGFPL V L A A L L V I V G S V M S T E
ESGEEASQNGFPL V L A A L L V I V G S V M S T E
ESGEEASQNGFPL V L A A L L V I V G S V M S T E

d

Dictyonamide A: A T T V V V V V I V G -Abz

ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
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ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G
ESGEEASQNGFPA T T V V V V V I V G

Position of N-Me confirmed by MS/MS MS/MS detected but undefined N-Me position