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Journal Article

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Publication date:
2024-02-01

Permanent link:
https://doi.org/10.3929/ethz-b-000653071

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Originally published in:
ChemBioChem 25(3), https://doi.org/10.1002/cbic.202300626

Funding acknowledgement:
173097 - Molecular dissection of the chemical defense system of multicellular fungi against predation (SNF)

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doi.org/10.1002/cbic.202300626

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Promiscuity of Omphalotin A Biosynthetic Enzymes Allows *de novo* Production of Non-Natural Multiply Backbone N-Methylated Peptide Macrocycles in Yeast

Emmanuel Matabaro,[a] Luca Witte,[a] Fabio Gherlone,[a] Eva Vogt,[a] Hannelore Kaspar,[a] and Markus Künzler*[a]

Multiple backbone N-methylation and macrocyclization improve the proteolytic stability and oral availability of therapeutic peptides. Chemical synthesis of such peptides is challenging, in particular for the generation of peptide libraries for screening purposes. Enzymatic backbone N-methylation and macrocyclization occur as part of both non-ribosomal and ribosomal peptide biosynthesis, exemplified by the fungal natural products cyclosporin A and omphalotin A, respectively. Omphalotin A, a 9fold backbone N-methylated dodecamer isolated from the agaricomycete *Omphalotus olearius*, can be produced in *Pichia pastoris* by coexpression of the *ophMA* and *ophP* genes coding for the peptide precursor protein harbouring an autocatalytic peptide α-N-methyltransferase domain, and a peptide macrocyclase, respectively. Since both OphMA and OphP were previously shown to be relatively promiscuous in terms of peptide substrates, we expressed mutant versions of *ophMA*, encoding OphMA variants with altered core peptide sequences, along with wildtype *ophP* and assessed the production of the respective peptide macrocycles by the platform by high-performance liquid chromatography, coupled with tandem mass spectrometry (HPLC–MS/MS). Our results demonstrate the successful production of fifteen non-natural omphalotin-derived macrocycles, containing polar, aromatic and charged residues, and, thus, suggest that the system may be used as biotechnological platform to generate libraries of non-natural multiply backbone N-methylated peptide macrocycles.

Introduction

Peptide modifications affect the chemical topology of the peptide and can create novel physicochemical properties and functionalities.[1] Particularly, backbone N-methylation and macrocyclization are coveted modifications in drug development, because they provide a rigid architecture that improves proteolytic stability, oral availability, membrane permeability, and selective binding to the target.[2]

Macrocyclization of peptides can be achieved enzymatically or by chemical ligation. Latter method suffers from problems with peptide epimerization, oligomerization, and solubility, which often result in very low yields.[3] Natural, enzyme-mediated peptide macrocyclization strategies, including intramolecular cross-linking (C–O, C–C, or C–S bond formation) and head-to-tail (N-to-C) macrocyclization,[1,4] master such challenges. N-to-C macrocyclization of linear peptides is generally catalyzed by the thioesterase (TE) domains embedded in non-ribosomal peptide synthetases (NRPSs).[5] NRPSs are gigantic multimodular enzymes that act in an assembly-line fashion to form non-ribosomal peptides (NRPs). Alternatively, stand-alone peptide macrocyclases are involved in the synthesis of cyclic ribosomally synthesized and posttranslationally modified peptides (RiPPs).[4d] In both cases, the enzymes utilize a catalytic Ser or Cys to attack the peptide substrate to form an acyl-enzyme intermediate. The structural rearrangement brings the N-terminal end of the peptide substrate close to the C-terminal carbonyl, promoting the transamidation reaction that leads to the formation of a cyclic peptide.[1,5–6] Well-characterized RiPP macrocyclases include PatG from cyanobacteria, POPB from the mushroom *Amanita sp.*, and PCY1 and Butelase 1 found in plants, which are involved in the biosynthesis of cyanobactins, amatoxins, orbitides and cyclotides, respectively.[6–7] Interestingly, these macrocyclases can accept engineered peptide substrates with *D*-, β-, N-methylated, hydroxylated, and other chemically modified amino acids.[7–8] This promiscuity together with high kinetic efficiency make them reliable biotechnological tools for the generation of peptide macrocycle libraries that can be screened for agricultural or pharmaceutical applications.[8a]

Natural peptide macrocycles often carry additional chemical modifications such as oxidation, acylation, C- and N-methylation.[2b,9] Particularly, N-methylation of the amide bond (α- or backbone N-methylation) has been reported in highly bioactive non-ribosomal peptides such as the immunosuppressant cyclosporin A,[10] the antibacterial verrucamides,[11] and the mycotoxin enniatin B.[12] Thus far, the bacterial and fungal peptide natural products of the RiPP family of borosins are the only reported

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202300626

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cases in which backbone N-methylation is introduced as a posttranslational modification (PTM).[13] Borosins are distinctively defined by multiple α-N-methylations (α-N-Me) installed by an S-adenosyl methionine (SAM)-dependent peptide α-N-methyltransferase (α-N-MT). In fungal borosin precursors (types I–III), the α-N-MT domain is fused to the N-terminal substrate (core peptide) via a clasp domain (Figure 1).[6,14] Conversely, for bacterial borosin precursors (split borosins, or type IV borosins), the α-N-MT and the short peptide substrate are encoded by separate genes.[13e,f] After translation, they form a complex (heterodimer) that facilitates intermolecular α-N-methylation of the core peptide.[13d] The omphalotins, 9fold backbone N-methylated macrocyclic dodecapeptides produced by the agaricomycete *Omphalotus olearius*, are the founding members of the borosin RiPP family and exert a strong and selective toxicity towards the plant-parasitic nematode *Meloidogyne incognita*.[15] The omphalotin precursor protein OphMA forms a catenane-like homodimer that efficiently promotes intermolecular *α-N*-methylation of nine residues in the C-terminal 12-residue core peptide region.[13c,14] Since the sequence of the peptide is genetically encoded, it can easily be engineered to extend the chemical space and diversity of this peptide family. Indeed, biochemical studies have demonstrated that OphMA is relatively promiscuous, accepting peptide substrates containing both canonical and non-canonical amino acids.[13a,16]

The *ophMA* gene is located within a gene cluster encoding a serine peptidase of the S9A family, OphP, as well as putative peptide-modifying enzymes of the monooxygenase (OphB) and *O*-acyltransferase (OphD) families and some proteins of unknown function (OphC, OphE).[13b,c] Recent biochemical and structural characterization of OphP shows that the enzyme is able to macrocyclize C-terminal peptides derived from OphMA but not intact OphMA.[17] The study further shows that OphP is promiscuous for the core and follower sequences of the peptide

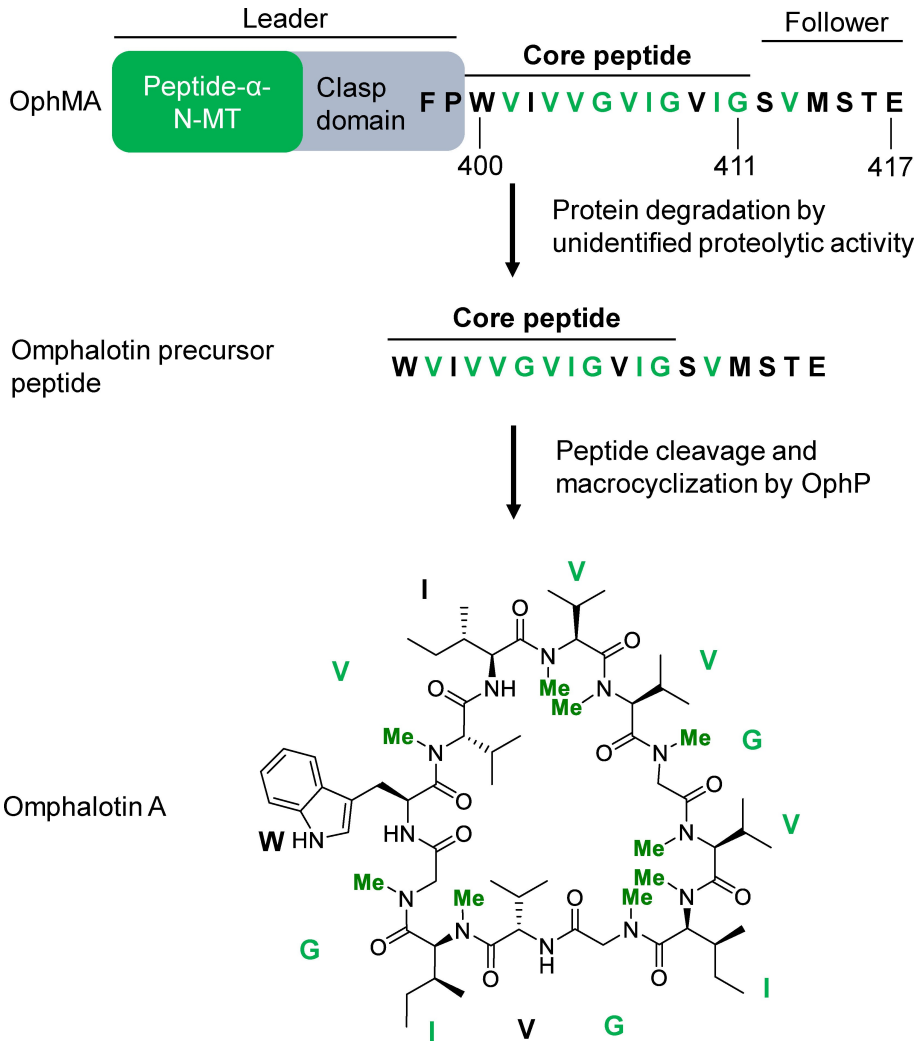

Figure 1. Proposed omphalotin A biosynthetic pathway. Multiple backbone N-methylations are installed in the core peptide (and one residue of the follower sequence) by the peptide α-N-methyltransferase domain (Peptide-α-N-MT) of OphMA. Upon completion of methylation, OphMA is proteolytically degraded but the omphalotin A precursor (core plus follower) peptide withstands degradation due to its multiple backbone N-methylations. This step is still hypothetical as the responsible proteolytic activity has not been identified. The methylated glycine residue of the precursor peptide is then recognized by OphP, the C-terminal (follower) peptide is removed upon formation of a covalent peptide-enzyme intermediate and the intermediate is finally resolved by nucleophilic attack of the free N-terminus of the peptide resulting in the peptide macrocycle. Methylated residues are highlighted in green.

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substrates but prefers hydrophobic and/or multiply backbone N-methylated core residues and is selective for an α-N-methylated glycine residue at the P1 site of macrocyclization. Despite the lack of activity of OphP towards intact OphMA, omphalotin A and homologous peptides from the agaricomycetes *Lentinula edodes* (lentinulin A) and *Dendrothele bispora* (dendrothelin A) can be produced in *P. pastoris* by coexpression of the *ophMA* (*ledMA, dbiMA*) and *ophP* genes[13b,18] suggesting the presence of a yet to be identified proteolytic activity capable of processing OphMA in fungi.[17] Recombinant omphalotin A and its natural variant lentinulin A, produced in this way, demonstrated high potency against the plant parasite *M. incognita* ($LC_{50}<$ 1.0 μM).[18] Since both OphMA and OphP exhibited significant peptide substrate promiscuity,[16a,17] coexpression of *ophMA* and *ophP* in *P. pastoris* may be used as a biotechnological platform for the generation of *de novo* libraries of multiply backbone N-methylated peptide macrocycles. In this study, we assessed the promiscuity of this platform to produce various *de novo* variants of omphalotin A. For this purpose, we expressed *ophMA* mutants with alterations in the core peptide along with *ophP* and analyzed the production of the respective peptide macrocycles by HPLC–MS/MS. Our results demonstrate that the *P. pastoris* OphMA–OphP platform can indeed be exploited for the *de novo* production of non-natural peptide macrocycles with multiple backbone N-methylations.

Results and Discussion

We have previously shown that coexpression of OphP and OphMA variants, where the omphalotin core peptide sequence was replaced by the related sequences of dendrothelin and lentinulin, leads to the production of the respective peptide natural products in the *P. pastoris* omphalotin production platform (Table 1).[18]

In order to further explore the promiscuity of the platform regarding the OphMA core peptide sequence, we set out to produce some non-natural variants of omphalotin A. We first replaced all residues individually by Ala (Table 1). The results of this Ala scan were expected to reveal any critical positions within the core peptide regarding the activities of the unidentified proteolytic activity, OphMA or OphP. Intriguingly, Ala was accepted at every position and the overall methylation pattern of the core peptide was unchanged compared to omphalotin A (Table 1). The produced 9fold α-N-methylated peptide macrocycles were detected using HPLC–MS (Figure 2A; Figure S1A) and their identity was confirmed by HPLC–MS/MS analysis of the respective linear core peptides (Figure S2A). These results suggest that every position of omphalotin A can be substituted, at least by Ala, and showcase the production of twelve non-natural multiply backbone N-methylated peptide macrocycles.

Second, in order to check which types of core peptide residues would be tolerated by the *P. pastoris* peptide production platform, we substituted the OphMA residues at positions 407, 410 and 411 by apolar (Ala, Val, Leu, Ile), polar (Thr), charged (Glu, Arg) and bulky (Phe, Pro, Trp, Tyr) residues (summarized in Table 1). Some of these substitutions were previously analyzed in *E. coli* regarding the promiscuity of the α-N-methyltransferase activity of OphMA.[16a] Substitution of residue Ile407 by Thr, in OphMA(I407T), resulted in the respective 9fold backbone N-methylated peptide macrocycle, including methylation of the introduced Thr residue (Figure 2B and Figures S1B and S2B). This result is in line with previous data demonstrating that Thr is accepted by OphMA peptide α-N-methyltransferase activity at many positions of the core peptide resulting in a methylation pattern similar to the wildtype core peptide.[16a] In contrast, substitution of the same residue with charged residues, in OphMA(I407E) and OphMA(I407R), did not result in any cyclic or linear full-length core peptides. Instead, short linear core peptides with six and five α-N-methylations and terminal Glu and Arg, respectively, were observed (Figures S1C and S2C). In accordance, OphMA variants carrying the I407E and I407R substitutions were previously shown to be significantly impaired in methylation, yielding 5fold and 3fold methylated species, respectively, as the most abundant species.[16a] Surprisingly, the introduction of a bulky hydrophobic (aromatic) Phe residue at the same position, in OphMA(I407F), yielded trace amounts of a mixture of respective 8fold and 9fold α-N-methylated cyclic peptides despite the fact that the core peptide methylation in this OphMA variant is similarly affected (Figure 2B, Figures S1B and S2B).[16a] This result suggests that aromatic residues affect the α-N-methylation but not the macrocyclization of the core peptide. In contrast, OphMA(I407P), which yielded a mixture of 2fold to 7fold methylated OphMA species,[16a] did not lead to any cyclic peptide but a mixture of linear core peptides of up to seven methylations (Figures S1C and S2C). According to previous results, Pro causes steric hindrance by adopting a *trans* conformation in the active site of OphMA, which also precludes methylation of the residue directly upstream (n-1 from Pro).[16a] As a consequence, the most abundant OphMA(I407P) species was 4fold methylated (data not shown). In addition, the reduced conformational flexibility of the core peptide is apparently not compatible with its macrocyclization by OphP.

Since OphP was previously suggested to represent a *bona fide* prolyl oligopeptidase, we substituted the last residue, Gly411, of the core peptide by proline. This construct, OphMA(G411P), did not yield any respective full-length but only short linear core peptides (Figure S1C). These results are in agreement with the *in vitro* analysis of OphP[17] and demonstrate that Oph-mediated macrocyclization cannot be triggered by Pro at the P1 site of the peptide substrate. The crystal structure of OphP reveals that the residue has to be small and α-N-methylated to be recognized.[17] Accordingly, the charged Arg and Glu residues at the same position in OphMA(G411R) and OphMA(G411E), respectively, did also not yield any cyclic full-length core peptides (Figures S1C and S2C). Mutation of Ile410 to Glu in OphMA(I410E), on the other hand, resulted in full-length peptide macrocycles with nine α-N-methylations (Figure 2B, Figures S1B and S2B). Substitution of the same residue by Trp in OphMA(I410W) resulted in respective linear undecameric peptides with eight methylations possibly due to

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Table 1. Chemical diversity of backbone N-methylated peptides produced. Mutations of the core peptide sequence in the respective OphMA variants are indicated in red. Backbone N-methylated residues of the produced peptides are indicated in green. In all OphMA core peptide variants, the leader and follower sequences were unchanged compared to the wildtype protein. Brackets indicate low levels of the respective peptide (see main text for details). Paragraph symbols indicate constructs that were previously analyzed regarding OphMA–OphP promiscuity.[18] Asterisks indicate constructs that were previously analyzed regarding OphMA promiscuity.[13c,16a] CycA: cyclosporin A, DicA: dictyonamide.

OphMA variant	*Core peptide sequence*	*Main peptide product*	*Cyclic*	*Linear*
Omphalotin§	… W V I V V G V I G V I G …	W V I V V G V I G V I G	+	+
Lentinulin§	… W I I V V G V V G V V G …	W I I V V G V V G V V G	+	+
Dendrothelin§	… W V I V T V I V G V I G …	W V I V T V I V G V I G	+	+
W400A	… A V I V V G V I G V I G …	A V I V V G V I G V I G	+	+
V401A	… W A I V V G V I G V I G …	W A I V V G V I G V I G	+	+
I402A	… W V A V V G V I G V I G …	W V A V V G V I G V I G	+	+
V403A	… W V I A V G V I G V I G …	W V I A V G V I G V I G	+	+
V404A	… W V I V A G V I G V I G …	W V I V A G V I G V I G	+	+
G405A	… W V I V V A V I G V I G …	W V I V V A V I G V I G	+	+
V406A	… W V I V V G A I G V I G …	W V I V V G A I G V I G	+	+
I407A	… W V I V V G V A G V I G …	W V I V V G V A G V I G	+	+
G408A	… W V I V V G V I A V I G …	W V I V V G V I A V I G	+	+
V409A	… W V I V V G V I G A I G …	W V I V V G V I G A I G	+	+
I410A	… W V I V V G V I G V A G …	W V I V V G V I G V A G	+	+
G411A	… W V I V V G V I G V I A …	W V I V V G V I G V I A	+	+
I407T*	… W V I V V G V T G V I G …	W V I V V G V T G V I G	+	+
I407R*	… W V I V V G V R G V I G …	W V I V V G V R	-	+
I407E*	… W V I V V G V E G V I G …	W V I V V G V E	-	+
I407F*	… W V I V V G V F G V I G …	W V I V V G V F G V I G	(+)	(+)
I407P*	… W V I V V G V P G V I G …	W V I V V G V P G V I G	-	(+)
I410W	… W V I V V G V I G V W G …	W V I V V G V I G V W	-	+
I410E	… W V I V V G V I G V E G …	W V I V V G V I G V E G	+	+
G411P	… W V I V V G V I G V I P …	W V I V V G V I G	-	+
G411E	… W V I V V G V I G V I E …	W V I V V G V I G V I E	-	+
G411R	… W V I V V G V I G V I R …	W V I V V G V I G	-	+
ΔVIG	… W V I V V G V I G ~~V I G~~ …	W V I V V G V I G	-	+
+VIG	… W V I V V G V I G V I G V I G …	W V I V V G V I G	-	+
CycA*	… L V L A A L L V I V G …	–	-	-
DicA*	… A T T V V V V V I V G …	–	-	-

interference of the bulky residue with the methylation of the subsequent Gly residue (Figures S1C and S2C).

In order to assess the promiscuity of the *P. pastoris* peptide production platform regarding the length of the core peptide, we duplicated or deleted the last three residues of the core

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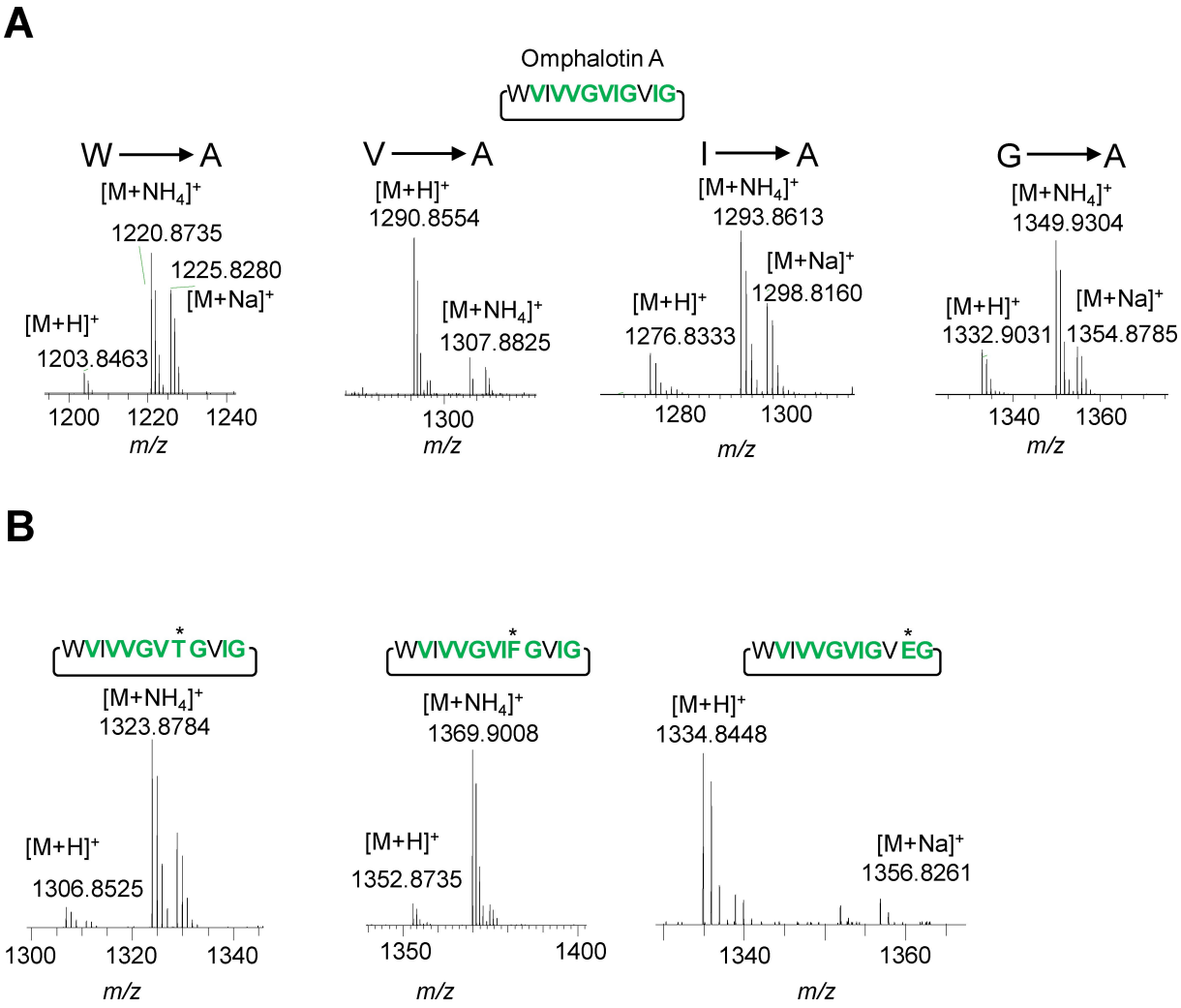

Figure 2. LC–MS analysis of all fifteen non-natural multiply α-N-methylated peptide macrocycles produced in *P. pastoris*. (A) Macrocycles from the Ala scan of the OphMA core peptide. In total, 12 different macrocycles were obtained, by substitution of the only Trp residue, by substitutions of the three Gly residues, by substitutions of the three Ile residues, and by substitutions of the five Val residues. The protonated forms $[M+H]^+$ are shown together with sodium and ammonium adducts $[M+NH_4]^+$ and $[M+Na]^+$, respectively. Blue allows shows that replacement of the same residue at different positions results in the same peptide mass. However, respective cyclic peptides have different hydrophobicity (Figuress S1A). (B) Mass spectra of omphalotin derivatives I417T, I407F and I410E. Asterisks denote the substitution in the core peptide.

peptide (VIG) in OphMA variants OphMA+VIG and OphMAΔ-VIG, respectively. In case of OphMA+VIG, no peptides of full or extended length were observed. Instead, 7fold methylated short linear core peptide was the only detectable peptide (Table 1). We suspect that the extension of the core peptide interferes with the terminal methylation of OphMA similar to OphMA(G411P). This peptide is also produced by OphMAΔVIG demonstrating that methylated Gly408 is recognized by OphP also if it is followed by a Ser rather than a Val residue. Lack of cyclization of this nonameric multiply backbone N-methylated peptide by OphP *in vivo* is consistent with the *in vitro* analysis of OphP[17] and possibly due to steric hindrance. Similarly, the most closely related macrocyclase, GmPOPB (44% identity), shows a significant decrease in macrocyclization efficiency towards peptide substrates shorter than the native octamer core peptide.[8a]

Finally, we swapped the core peptide of OphMA with unrelated sequences resembling the immunosuppressant cyclosporin A in OphMA(CycA)[19] and the cyclin-dependent kinase 4-inhibiting dictyonamide in OphMA(DicA)[20] as done previously for assessing the promiscuity of the α-N-MT activity of OphMA.[13c] In none of these cases, we could detect OphMA-derived peptides even though these proteins were previously shown to carry five and nine α-N-methylations in the core peptide region, respectively.[13c] These results are in accordance with the selectivity of the peptide macrocyclase OphP i.e. the absence of a methylated Gly residue in the respective precursor peptides.[17]

Taken together, these results suggest that there is significant freedom to operate for the production of multiply backbone N-methylated peptide macrocycles using this platform but that composition, conformational flexibility and length of the core peptide are important factors for efficient proteolytic

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processing and macrocyclization as it is the case for other RiPP macrocyclases.[8a,21] Two additional caveats of the current version of the platform are the low yield and the localization of the peptides. Based on the previous production of omphalotin A and its natural variants lentinulin A and dendrothelin A using the same platform, the yields of the non-natural variants are estimated to be in the range of hundreds of μgs per L of *P. pastoris* culture[19] and the peptides have to be extracted from cell lysates as they are not secreted.[23]

Experimental Procedures

Plasmid construction

The *ophMA* gene was obtained from either plasmid PMA1004 (pET24-His8-OphMA_cDNA) or PMA1315 (pPICZA-His8-OphMA) described earlier.[13c,18] For the mutants OphMA(I407E), (I407R), (I407F) and (I407T), sequences were PCR-amplified from our plasmid libraries (PMA), 1483, 1479, 1487 and 1475, respectively, using primers OphMA_Fw and OphMA_Rv.[18] DicA was amplified from PMA140 using primers OphMA_DicA-fl and OphMA_Fw. For OphMA(G411A) and OphMA(CycD), the C-terminal part was obtained by digestion of previously constructed plasmds PMA1518 and PMA1138, respectively, with *Xho*I and *Not*I. For the Ala scanning of the core, OphMA_Fw was used together with primer sequences listed in Table S1. In all cases, genes were inserted into *Not*I and *EcoR*I of the pPICZA vector. The constructs were then used to transform *E. coli* DH5α. The selection was performed on Luria-Bertani (LB) medium zeocin-containing 30 μg/ml zeocin.

Pichia transformation

P. pastoris strain GS115-strepII-SUMO*OphP (SMA2903) was previously used to produce omphalotin A variants. This strain expresses *ophP* from an *AOX1* driven cassette that is inserted into the *HIS4* locus. For transformation and selection, the methods described in[18,22] were followed. Briefly, the SMA 2903 strain was transformed by electroporation with *Pme*I-linearized plasmids for integration into the *AOX1* locus. Transformants were selected on the synthetic complete dextrose medium without histidine (SD-His) containing 400 μl/ml zeocin. Single colonies were verified for gene integration by colony PCR using *AOX*1 standard primers (Table S1). Gene expression was checked by first cultivating selected clones into BMMY medium for three days. Protein production was analyzed by western blot of respective cell lysate supernatants as previously described.[18]

Peptide extraction

For peptide extraction, recommendations from[22] were followed. Briefly, positive clones were cultivated into 25 ml BMMY medium in 250 ml baffled flasks, 200 rpm, 30 °C, for three days, with 0.5% methanol (v/v) supplied every 24 hours. The culture medium and cells were separated by centrifugation at 12000xg for 30 min, 4 °C. Cells were washed twice with cold PBS and then resuspended into 2x PBS containing protease inhibitor cocktail (PIC) (EDTA free, Roche). Cells were lysed with 0.5 mm diameter glass beads using the FastPrep FP120 kit (Thermo Fisher Scientific). Peptides were extracted from cell-lysate supernatant either by phase separation using ethyl acetate or by solid-phase extraction using 1 ml or 3 ml C18 and MCX cartridges (SepPak, Waters). For OphMA(I407F), a mixture of ethyl acetate and n-hexane (1:1, v/v) was used. After extraction, the organic phase was evaporated and the cell extract dissolved in 1 ml MeOH, and kept in a glass vial at −20 °C until use.

Peptide detection and identification by HPLC–ESI-MS/MS

For LC–MS/MS analysis, the method that was previously used for omphalotin and homologs analysis was applied.[18,22] Briefly, 10 μl of the sample was used for analysis via the Dionex Ultimate 3000 UHPLC coupled to mass spectrometer Q Exactive (Thermo Scientific). The Phenomenex Kinetex®μm XB-C18 100 Å (150×2.1 mm) column was first heated at 50 °C. Solvent A (water) and solvent B (acetonitrile) containing 0.1% formic acid (v/v) were used as mobile phases. The HPLC gradient started with equilibrating the column with 30% B for 5 min, a linear gradient to 95% B for 15 min, column wash with 100% B for 7 min, and final equilibration at 30% B for 5 min. HESI and data-dependent MS/MS were not changed. Normalized collision energy (NCE) for cyclic peptides was set at 30%, for linear peptides between 16–25%. Data analysis was performed using the Thermo Fisher Xcalibur 4 software.

Conclusions

Chemical synthesis of multiply backbone N-methylated peptide macrocycles is challenging. Thus, biotechnological production platforms are welcome as environmentally friendly and cost-effective alternatives. The biosynthetic pathway of the ribosomal peptide natural product omphalotin A offers the possibility to produce such peptides in fungal hosts, such as the yeast *P. pastoris*, in a streamlined fashion by employing only two enzymes, OphMA and OphP. Previous studies have shown that OphMA displays considerable substrate promiscuity, accepts almost all canonical amino acids and even tolerates some non-proteinogenic amino acids.[16] Recent biochemical and structural characterization of OphP revealed that (1) the omphalotin A biosynthetic pathway relies on a yet unidentified proteolytic activity for the degradation of OphMA, and that (2) the peptide macrocyclase OphP shows significant promiscuity with regard to the length, sequence and the degree of α-N-methylation of the core peptide.[17]

This study provides insight into the promiscuity of omphalotin A production in *P. pastoris* and led to the successful production of fifteen non-natural multiply backbone N-methylated peptide macrocycles based on the scaffold of omphalotin A (Figure 3). The platform tolerates single substitutions in the core peptide with a preference for small hydrophobic residues, but also accepts bulky, polar or even negatively charged amino acids at some positions. Substitutions with Arg and Pro, however, were not compatible with macrocycle production. We suspect that the positive charge and the reduced conformational flexibility, respectively, of the respective core peptides are not compatible with the substrate channels of OphMA and OphP.[14,17] Similarly, the produced 7fold methylated nonameric peptides were not macrocyclized. Apparently, the reduced length of these core peptides negatively affect the macrocyclization efficiency by OphP as has been shown for the related enzyme GmPOPB.[23] Our results show that, OphP is, similar to PCY1, slightly promiscuous regarding the P1 site of

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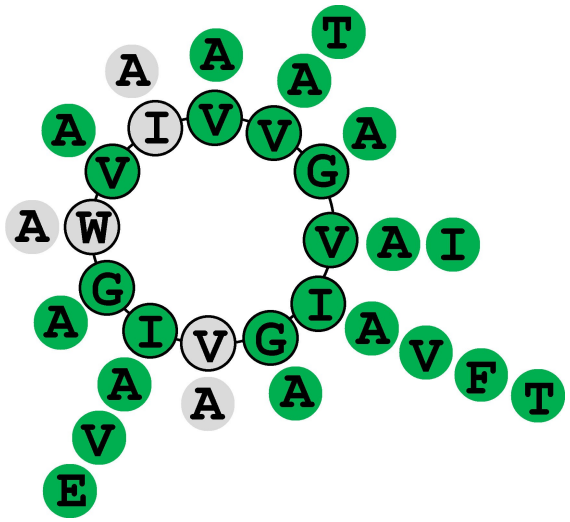

Figure 3. Schematic representation of all omphalotin A-derived multiply α-N-methylated peptide macrocycles produced in *P. pastoris*. The residues in the inner circle represent the scaffold of omphalotin A and the residues in rays indicate substitutions at the individual positions, including those of the natural variants lentinulin A and dendrothelin A as well as the ones of the fifteen non-natural variants presented in this study. Methylated residues are underlied by green, non-methylated residues by grey circles.

macrocyclization in that it accepts both Gly and Ala at position 411. Finally, it is important to note that the system proposed here as a biotechnological platform for the production of multiply backbone N-methylated peptide macrocycles has three mechanisms which ensure that the produced peptide macrocycles are uniform regarding the degree of backbone N-methylation, a feature that will be crucial when it comes to screening of peptide libraries produced *de novo* by the platform. First, methylation of the core peptide occurs processively from the N- to the C-terminus.[13c] Second, only maximally methylated versions of OphMA appear to be processed by the unidentified proteolytic activity.[17] Third, OphP needs a methylated Gly or Ala residue at the P1 site followed by any unmethylated residue (P1′ site) to cleave and does not macrocyclize peptides shorter than 12 residues.[17] The molecular basis of these mechanisms is (except for the P1–P1′ specificity of OphP), however, not understood. For the development of the system into a useful biotechnological platform, future research will focus on the identification of the proteolytic activity responsible for the degradation of OphMA and the maximization of the peptide yield, as well as the development of screening systems for the bioactivity of the peptides given their intracellular localization in yeast.

Supporting Information

Additional references cited within Supporting Information.[13b,18,22]

Author Contributions

MK: Conceptualization, Data Curation, Funding Acquisition, Project Administration, Resources, Supervision and Writing – Review & Editing; EM: Formal Analysis, Investigation, Methodology, Validation, Supervision, Writing – Original Draft; LW, FG and EV: Formal Analysis, Investigation, Validation; HK: Formal Analysis, Methodology, Validation.

Acknowledgements

We would like to thank Prof. Jörn Piel and his staff, in particular Dr. Franziska Hemmerling, Dr. Amy Fraley, Dr. Agneya Bhushan, and Dr. Alexander Oliver Brachmann for the scientific discussions and technical support. This work was funded by the Swiss National Science Foundation (Grant no. 31003A-173097).

Conflict of Interests

MK is an inventor on a patent application filed by ETH Zurich (no. WO2017174760A1, priority date: 7 April 2016). The authors declare no other competing interests.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: α-N-methylation · Borosin · *Komagataella phaffii* · Macrocylization · RiPP

[1] M. Montalban-Lopez, T. A. Scott, S. Ramesh, I. R. Rahman, A. J. van Heel, J. H. Viel, V. Bandarian, E. Dittmann, O. Genilloud, Y. Goto, M. J. Grande Burgos, C. Hill, S. Kim, J. Koehnke, J. A. Latham, A. J. Link, B. Martinez, S. K. Nair, Y. Nicolet, S. Rebuffat, H. G. Sahl, D. Sareen, E. W. Schmidt, L. Schmitt, K. Severinov, R. D. Sussmuth, A. W. Truman, H. Wang, J. K. Weng, G. P. van Wezel, Q. Zhang, J. Zhong, J. Piel, D. A. Mitchell, O. P. Kuipers, W. A. van der Donk, *Nat. Prod. Rep.* **2021**, *38*, 130–239.
[2] a) A. Sharma, A. Kumar, S. A. H. Abdel Monaim, Y. E. Jad, A. El-Faham, B. G. de la Torre, F. Albericio, *Biopolymers* **2018**, *109*, e23110; b) J. Chatterjee, F. Rechenmacher, H. Kessler, *Angew. Chem. Int. Ed. Engl.* **2013**, *52*, 254–269; c) N. Tsomaia, *Eur. J. Med. Chem.* **2015**, *94*, 459–470.
[3] a) H. Y. Chow, Y. Zhang, E. Matheson, X. Li, *Chem. Rev.* **2019**, *119*, 9971–10001; b) X. Jing, K. Jin, *Med. Res. Rev.* **2020**, *40*, 753–810.
[4] a) J. Lu, Y. Li, Z. Bai, H. Lv, H. Wang, *Nat. Prod. Rep.* **2021**, *38*, 981–992; b) K. R. Schramma, L. B. Bushin, M. R. Seyedsayamdost, *Nat. Chem.* **2015**, *7*, 431–437; c) A. W. Truman, *Beilstein J. Org. Chem.* **2016**, *12*, 1250–1268; d) C. Ongpipattanakul, S. K. Nair, *Biochemistry* **2018**, *57*, 3201–3209.
[5] a) X. Gao, S. W. Haynes, B. D. Ames, P. Wang, L. P. Vien, C. T. Walsh, Y. Tang, *Nat. Chem. Biol.* **2012**, *8*, 823–830; b) F. Kopp, M. A. Marahiel, *Nat. Prod. Rep.* **2007**, *24*, 735–749.
[6] C. Ongpipattanakul, S. K. Nair, *ACS Chem. Biol.* **2018**, *13*, 2989–2999.
[7] G. K. Nguyen, S. Wang, Y. Qiu, X. Hemu, Y. Lian, J. P. Tam, *Nat. Chem. Biol.* **2014**, *10*, 732–738.
[8] a) R. M. Sgambelluri, M. O. Smith, J. D. Walton, *ACS Synth. Biol.* **2018**, *7*, 145–152; b) J. R. Chekan, P. Estrada, P. S. Covello, S. K. Nair, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 6551–6556; c) C. N. Alexandru-Crivac, C. Umeobika, N. Leikoski, J. Jokela, K. A. Rickaby, A. M. Grilo, P. Sjo, A. T. Plowright, M. Idress, E. Siebs, A. Nneoyi-Egbe, M. Wahlsten, K. Sivonen,

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M. Jaspars, L. Trembleau, D. P. Fewer, W. E. Houssen, *Chem. Commun. (Camb.)* **2017**, *53*, 10656–10659; d) G. K. Nguyen, X. Hemu, J. P. Quek, J. P. Tam, *Angew. Chem. Int. Ed. Engl.* **2016**, *55*, 12802–12806.
[9] T. R. White, C. M. Renzelman, A. C. Rand, T. Rezai, C. M. McEwen, V. M. Gelev, R. A. Turner, R. G. Linington, S. S. Leung, A. S. Kalgutkar, J. N. Bauman, Y. Zhang, S. Liras, D. A. Price, A. M. Mathiowetz, M. P. Jacobson, R. S. Lokey, *Nat. Chem. Biol.* **2011**, *7*, 810–817.
[10] C. K. Wang, J. E. Swedberg, P. J. Harvey, Q. Kaas, D. J. Craik, *J. Phys. Chem. B* **2018**, *122*, 2261–2276.
[11] X. Zou, S. Niu, J. Ren, E. Li, X. Liu, Y. Che, *J. Nat. Prod.* **2011**, *74*, 1111–1116.
[12] A. Prosperini, H. Berrada, M. J. Ruiz, F. Caloni, T. Coccini, L. J. Spicer, M. C. Perego, A. Lafranconi, *Frontiers in Public Health* **2017**, *5*.
[13] a) M. R. Quijano, C. Zach, F. S. Miller, A. R. Lee, A. S. Imani, M. Kunzler, M. F. Freeman, *J. Am. Chem. Soc.* **2019**, *141*, 9637–9644; b) S. Ramm, B. Krawczyk, A. Muhlenweg, A. Poch, E. Mosker, R. D. Sussmuth, *Angew. Chem. Int. Ed. Engl.* **2017**, *56*, 9994–9997; c) N. S. van der Velden, N. Kalin, M. J. Helf, J. Piel, M. F. Freeman, M. Kunzler, *Nat. Chem. Biol.* **2017**, *13*, 833–835; d) F. S. Miller, K. K. Crone, M. R. Jensen, S. Shaw, W. R. Harcombe, M. H. Elias, M. F. Freeman, *Nat. Commun.* **2021**, *12*, 5355; e) H. Cho, H. Lee, K. Hong, H. Chung, I. Song, J. S. Lee, S. Kim, *Biochemistry* **2022**, *61*, 183–194; f) A. S. Imani, A. R. Lee, N. Vishwanathan, F. de Waal, M. F. Freeman, *ACS Chem. Biol.* **2022**, *17*, 908–917.
[14] H. Song, N. S. van der Velden, S. L. Shiran, P. Bleiziffer, C. Zach, R. Sieber, A. S. Imani, F. Krausbeck, M. Aebi, M. F. Freeman, S. Riniker, M. Kunzler, J. H. Naismith, *Sci. Adv.* **2018**, *4*, eaat2720.
[15] a) A. Mayer, H. Anke, O. Sterner, *Nat. Prod. Lett.* **1997**, *10*, 25–32; b) O. Sterner, W. Etzel, A. Mayer, H. Anke, *Nat. Prod. Lett.* **1997**, *10*, 33–38; c) A. Mayer, M. Kilian, B. Hoster, O. Sterner, H. Anke, *Pestic. Sci.* **1999**, *55*, 27–30.
[16] a) H. Song, J. R. Fahrig-Kamarauskaite, E. Matabaro, H. Kaspar, S. L. Shirran, C. Zach, A. Pace, B. A. Stefanov, J. H. Naismith, M. Kunzler, *ACS Chem. Biol.* **2020**, *15*, 1901–1912; b) H. Song, A. J. Burton, S. L. Shirran, J. Fahrig-Kamarauskaite, H. Kaspar, T. W. Muir, M. Kunzler, J. H. Naismith, *Angew. Chem. Int. Ed. Engl.* **2021**, *60*, 14319–14323.
[17] E. Matabaro, H. Song, L. Sonderegger, F. Gherlone, A. Giltrap, S. Liver, A. Gossert, M. Künzler, J. H. Naismith, *bioRxiv* **2023**, 2022.07.21.500988.
[18] E. Matabaro, H. Kaspar, P. Dahlin, D. L. V. Bader, C. E. Murar, F. Staubli, C. M. Field, J. W. Bode, M. Kunzler, *Sci. Rep.* **2021**, *11*, 3541.
[19] D. Tedesco, L. Haragsim, *Journal of Transplantation* **2012**, *2012*, 230386.
[20] K. Komatsu, H. Shigemori, J. I. Kobayashi, *J. Org. Chem.* **2001**, *66*, 6189–6192.
[21] a) H. Ludewig, C. M. Czekster, E. Oueis, E. S. Munday, M. Arshad, S. A. Synowsky, A. F. Bent, J. H. Naismith, *ACS Chem. Biol.* **2018**, *13*, 801–811; b) S. Sarkar, W. Gu, E. W. Schmidt, *ACS Catal.* **2020**, *10*, 7146–7153; c) X. Mi, E. K. Desormeaux, T. T. Le, W. A. van der Donk, D. Shukla, *Chem. Sci.* **2023**, *14*, 6904–6914.
[22] E. Matabaro, H. Song, C. Chepkirui, H. Kaspar, L. Witte, J. H. Naismith, M. F. Freeman, M. Kunzler, *Methods Enzymol.* **2021**, *656*, 429–458.
[23] R. M. Sgambelluri, M. O. Smith, J. D. Walton, *ACS Synth. Biol.* **2017**, *7*, 145–152.

Manuscript received: September 12, 2023
Revised manuscript received: December 1, 2023
Accepted manuscript online: December 7, 2023
Version of record online: January 4, 2024

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