Biochemistry and Cell Biology
Biochimie et biologie cellulaire

**An enhanced chemo-enzymatic method for loading
 substrates onto carrier protein domains**

Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2017-0275.R1
Manuscript Type:	Article
Date Submitted by the Author:	23-Nov-2017
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Is the invited manuscript for consideration in a Special Issue? :	N/A
Keyword:	non-ribosomal peptide synthetase, post-translational modification, carrier protein, phosphopantetheinyl transferase, coenzyme A

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1 **An enhanced chemo-enzymatic method for loading substrates onto carrier**
2 **protein domains**

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Abstract

Non-ribosomal peptide synthetase (NRPS) machineries produce many medically relevant peptides that cannot be easily accessed by chemical synthesis. Thus, understanding NRPS mechanism is of crucial importance to allow efficient redesign of these machineries in order to produce new compounds. During NRPS-mediated synthesis, substrates are covalently attached to PCPs, and studies of NRPSs are impeded by difficulties in producing PCPs loaded with substrates. Different approaches to load substrates on to PCP domains have been described, but all suffer from difficulties in either the complexity of chemical synthesis or low enzymatic efficiency. Here, we describe an enhanced chemo-enzymatic loading method that combines two approaches into a single, highly efficient one-pot loading reaction. First, D-pantetheine and ATP are converted into dephospho-coenzyme A via the actions of two enzymes from coenzyme A (CoA) biosynthesis. Next, phosphoadenylates are dephosphorylated using alkaline phosphatase to allow linker attachment to PCP domain by Sfp mutant R4-4, which is inhibited by phosphoadenylates. This route does not depend on activity of the commonly problematic dephospho-CoA kinase, and therefore offers an improved method for substrate loading onto PCP domains.

Keywords: carrier protein; post-translational modification; non-ribosomal peptide synthetase; phosphopantetheinyl transferase; coenzyme A.

29 **Introduction**

30 Non-ribosomal peptide synthetases (NRPSs) produce a wide variety of bioactive molecules, often with
31 complex chemical structures. (Al Toma et al. 2015; Süssmuth and Mainz 2017) Due to this
32 complexity, many such compounds of medicinal interest are still produced through fermentation,
33 which makes understanding NRPS function highly important for future efforts in compound
34 reengineering. NRPS machineries typically utilize a modular architecture of repeating domains to
35 synthesise their peptide products, with the core domains for peptide synthesis being adenylation (A)
36 domains, condensation (C) domains and peptidyl carrier protein (PCP) domains (Figure 1) (Süssmuth
37 and Mainz 2017). A domains select and activate amino acids in an ATP-dependant process and load
38 them onto the phosphopantetheine (PPE) arm of a neighbouring PCP domain, where the amino acid
39 remains tethered as a thioester (Kittilä et al. 2016a). This PCP-bound aminoacyl thioester is then
40 accepted by a C domain, where an upstream PCP-bound amino acid or peptide is condensed to form a
41 new peptide bond with concomitant transfer of the upstream molecule onto the downstream
42 aminoacyl-PCP. By reproducing these catalytic steps, and the introduction of additional tailoring
43 domains, multiple modules can in turn generate peptides whose length is governed by the number of
44 modules within the NRPS (Hur et al. 2012; Payne et al. 2017). Once the peptide has been fully
45 synthesised, a thioesterase (TE)-domain typically cleaves the peptide from the NRPS machinery
46 (Horsman et al. 2016).

47 Given the significance of the compounds produced by NRPS systems, these assembly lines are
48 important targets for structural and biochemical characterisation (Süssmuth and Mainz 2017).
49 Studying NRPS systems is challenging however due to the complexity of NRPS machineries and the
50 requirement for intermediates to be covalently loaded onto PCP domains before they are recognized as
51 substrates by any catalytic domains (Kittilä et al. 2016a). A domains can be used to load substrates
52 onto PCP domains, but unfortunately A domains can be very selective for their substrates and
53 therefore it is not possible to load a wide range of compounds *via* this route (Ehmann et al. 2000a;
54 Henderson et al. 2014; Mitchell et al. 2012; Villiers and Hollfelder 2009). In some cases, substrates
55 bound to a small molecule mimic of the PPE-linker (N-acetylcystamine thioesters, SNACs) are

56 accepted (Ehmann et al. 2000b; Luo et al. 2002; Roche and Walsh 2003), but this approach lacks
 57 general utility as it removes crucial protein-protein interactions and not all NRPS systems accept
 58 SNAC-bound substrates (Figure 2). Thus, one of the biggest breakthroughs in NRPS characterization
 59 has been the identification of a promiscuous phosphopantetheinyl transferase (PPTase) that can load
 60 PCP domains with substrates that are coupled to coenzyme A (CoA): the enzyme Sfp from *Bacillus*
 61 *subtilis* (Beld et al. 2014; Belshaw et al. 1999; Lambalot et al. 1996; Quadri et al. 1998). This enzyme
 62 can load unnatural amino acids and peptides onto PCP domains *in vitro*, which has enabled detailed
 63 characterization of the enzymatic steps during NRPS catalysis. As the Sfp enzyme is promiscuous
 64 towards the compounds coupled to CoA, this route has also been used for protein crosslinking, which
 65 has been valuable in crystallographic studies (Haslinger et al. 2014; Liu et al. 2011) as well as for
 66 protein labelling (La Clair et al. 2004; Shute et al. 2005; Yin et al. 2006).

67 The major limitation of this PCP loading strategy is the fact that the essential handle for Sfp function –
 68 CoA – has limited solubility in solvents appropriate for chemical synthesis, which in turn makes the
 69 synthesis of CoA linked substrates challenging – especially when the substrates are hydrophobic in
 70 nature. In addition, CoA coupled substrates are not suitable for *in vivo* studies due to poor membrane
 71 permeability. Thus, modified approaches to overcome these limitations have been developed. A less
 72 commonly used approach is to use a mutant of the Sfp enzyme to allow substrate loading beyond CoA
 73 coupled compounds. The R4-4 Sfp mutant developed by the Yin group was an important development
 74 in the field, as it allows any substrate-linker combinations that are coupled to adenosine 5'-
 75 diphosphate (ADP) to be loaded onto PCP domains (Figure 2) (Zou and Yin 2009). The advantage of
 76 this approach is that ADP coupling removes any restrictions on the linker region between the substrate
 77 and PCP domain. However, a more versatile route for loading carrier proteins is a chemo-enzymatic
 78 route where substrates/probes are synthesized as D-pantetheine derivatives (Figure 2). These can be
 79 converted into CoA derivatives by the use of three promiscuous enzymes from the CoA biosynthesis
 80 pathway: pantothenate kinase (PanK or CoaA), phosphopantetheine adenylyltransferase (PPAT or
 81 CoaD) and dephospho-CoA kinase (DPCK or CoaE) (Nazi et al. 2004). The derivatives thus formed
 82 can then be loaded to PCP domains using Sfp. This route allows a wide range of compounds to be

loaded on to carrier proteins as demonstrated both *in vivo* (Clarke et al. 2005; Meier et al. 2006) and *in vitro* (Hur et al. 2009; Meier et al. 2006; Reimer et al. 2016; van Wyk and Strauss 2007).

Although this enzymatic route to CoA-bound substrates is highly functional, several groups have reported problems with the DPCK enzyme, which may contribute to low functionality of the loading cascade (Francois et al. 2006; Rootman et al. 2010; Strauss et al. 2010). To eliminate this problem, we sought to combine the chemo-enzymatic route to CoA analogues together with the use of the R4-4 mutant of Sfp, as this would eliminate the need for DPCK by virtue of the ability of the Sfp R4-4 mutant to load PCP domains with dephospho-CoA coupled substrates (Figure 2). We have explored this strategy and in doing so have been able to establish a simplified loading route for PCP domains that is fast, simple and removes the problematic DPCK enzyme from the PCP loading process.

Materials and methods

Cloning: The gene encoding *ppat* from *E.coli* was obtained as a synthetic gene (ThermoFisher, Geneart®) and excised from the pEX-K plasmid using NdeI and HindIII restriction enzymes (New England Biolabs). The gene encoding *panK* was amplified from *E. coli* strain DH10β using whole cell PCR including primers GGGAATTCCATATGACCGCCAGAAACATGCTTATGAG (forward, NdeI site underlined) and GCGGTAGAAGAGGTCAGACTACGCAAATAAAAGCTTGGAT (reverse, HindIII site underlined) and GoTaq® Green Premix (Promega). Both genes were then subcloned into pET-28(a) vector (Novagen), with inserts and vector cut using NdeI and HindIII-HF restriction enzymes, ligated with T4 ligase. A synthetic gene codon optimized for expression in *Escherichia coli* encoding *PCP6_{com}* from *Streptomyces lavendulae* (Chiu et al. 2001) was obtained from Eurofins genomics. The *PCP6_{com}* gene was amplified by PCR using Phusion high fidelity polymerase (NEB) and primers TATTACCATGGCAGGCGGTCTGTGATCC (forward, NcoI site underlined) and AATAACTCGAGACCAGTCTCGGGCAGGCTTGCTGCTTCTTCGGC (reverse, XhoI site underlined). *PCP6_{com}* was subcloned into a modified pET vector (Bogomolovas et al. 2009) containing a thioredoxin (Trx) solubility tag followed by a TEV cleavage site and a C-terminal His6-tag under the control of a T7 promoter. All plasmids were used to transform BL21.DE3 Gold cells for protein

expression. Cloning of Trx-PCP2_{tei} was performed as described previously (Kittila et al. 2017). The plasmid encoding the Sfp mutant R4-4 was provided by the Yin group (Sunbul et al. 2009).

Protein expression and purification: The Sfp mutant R4-4 was prepared following a protocol previously published by the Walsh laboratory (Yin et al. 2006); Trx-PCP2_{tei} was also prepared as described previously (Kittila et al. 2017). A single colony was used to inoculate 20-100 mL Luria-Bertani (LB) medium supplemented with kanamycin (50 mg/L) and grown over night at 37°C. 4 L of LB-medium was supplemented with kanamycin (50 mg/L) and inoculated with the overnight culture (1 %). After the OD₆₀₀ reached 0.5-0.8 (37°C, 80 rpm), protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Cultures were then allowed to grow overnight (18°C, 80 rpm), after which the cells were harvested by centrifugation (5500 g, 10 min, 4°C) and the cell pellets resuspended in lysis buffer (50 mM Tris·HCl pH 7.4, 50 mM NaCl, 10 mM imidazole). Resuspended cells were flash frozen and stored at -80°C prior to purification.

Cells and proteins were kept on ice or in a cold room throughout the purification; purification steps performed using an Äkta system were performed at RT. Prior to cell lysis, protein inhibitor cocktail tablets (Sigmafast™, EDTA-free, Sigma-Aldrich) were added to the thawed cells. Cells were lysed by four passes through a microfluidizer (Microfluidics) and the lysate cleared by centrifugation (38800 g, 1 h, 4 °C). The cleared lysate was incubated with Ni-NTA beads (4 mL/construct, 1 h, 4°C, Macherey-Nagel) with gentle shaking. Beads were washed twice with wash buffer (50 mM Tris·HCl, pH 7.4, 300 mM NaCl, 10 mM imidazole) and the bound protein eluted with elution buffer (50 mM Tris·HCl, pH 7.4, 300 mM NaCl, 300 mM imidazole); the elution fraction for PPAT was dialyzed against dialysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, overnight). PanK and PPAT were further purified by size exclusion chromatography using a Superose 12 column connected to an Äkta PURE system (GE Healthcare Life Sciences, 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂). Protein purity was assessed using 15 % SDS-PAGE, with selected fractions combined and concentrated before being aliquoted, flash frozen and stored at -80°C. Protein concentration was determined by A₂₈₀ absorption (Nanodrop 2 000c, ε_(PanK) = 45380 M⁻¹ cm⁻¹, ε_(PPAT) = 8480 M⁻¹ cm⁻¹, ε_(Trx-PCP6com) = 15470 M⁻¹ cm⁻¹). The identity of the purified proteins was confirmed by MALDI-TOF MS peptide mass fingerprinting.

136 **Activity assays:** Activity of PanK was determined using an NADH coupled colorimetric ADP assay
137 (see (Beinker et al. 2005)): 1 μ M PanK was mixed in assay buffer (50 mM Tris, pH 7.4, 20 mM KCl,
138 5 mM $MgCl_2$, 1 mM EDTA, 2 mM TCEP, 0.1 g/L BSA and 0.2 mM NADH, V_{tot} = 500 μ L) with 4 U
139 pyruvate kinase/ 6 U lactic dehydrogenase (Pyruvate kinase/ Lactic dehydrogenase from rabbit muscle
140 (P0294), Sigma-Aldrich) and 0.8 mM phosphoenolpyruvate (Sigma-Aldrich). The reaction was
141 allowed to equilibrate after which 0.5 mM D-pantetheine was added. The reaction was initiated by
142 addition of 1 mM ATP, with the decrease in NADH absorption at 340 nm monitored over time using a
143 Jasco V-650 spectrophotometer (Jasco).

144 The activity of PPAT was determined using an NADH coupled PP_i assay (Kittilä et al. 2016b): 2 μ M
145 PanK, 0.5 mM D-pantetheine, 2 mM ATP and the components for PP_i detection were mixed in 100
146 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EDTA and incubated for 10 min at 25°C to allow the
147 formation of phosphopantetheine. 2 μ M PPAT was subsequently added and the decrease in NADH
148 absorption was observed spectroscopically.

149 Product formation was confirmed by LCMS measurements: briefly, 2.5 μ M PanK, 0.5 mM reduced D-
150 pantetheine and 2.5 mM ATP were mixed in assay buffer (50 mM Tris-HCl, pH 7.4, 20 mM KCl, 10
151 mM $MgCl_2$) and incubated for 30 min (37°C). A sample was taken and 25 μ M of PPAT was added
152 with 1 mM ATP to the remaining sample. The reaction was incubated for 30 min (37°C) and a further
153 sample was taken. From both samples the proteins were denatured at 95°C and the resultant
154 precipitate removed by centrifugation. The clarified supernatant was then analyzed by LCMS on a
155 Shimadzu 2020 system (column: XBridge BEH300 Prep C18 column, 10 μ m, 4.6 x 250 mm, Waters;
156 gradient: 2 % AcN for 4 min, 2-10 % AcN in 11 min, 10-40 % AcN in 10 min; flow rate: 1 mL/min)
157 using positive and negative ionization modes. Product formation was then determined using extracted
158 ion chromatograms.

159 **PCP loading (conventional method):** 120 μ M Trx-PCP_{6_{com}}, 240 μ M CoA or dephospho-CoA and 50
160 nM Sfp mutant R4-4 were mixed in loading buffer (50 mM Tris, pH 7.4, 15 mM $MgCl_2$, 2 mM DTE);
161 the reaction was incubated for 2 h at 30°C and quenched by the addition of 20 mM EDTA. PCP
162 loading efficiency was determined using MALDI-TOF-MS (intact mass determination, Axima

Confidence or Performance, Shimadzu). For examining loading inhibition, 5 mM ATP/ADP/AMP, 4 μ M PanK or 5 μ M PPAT were added to the loading reaction.

PCP loading (one-pot loading reaction): 120 μ M Trx-PCP6_{com} or Trx-PCP2_{tei}, 2.5 μ M PanK and 240 μ M reduced D-pantetheine, panthenol or pantothenic acid were mixed in assay buffer (25 mM Tris·HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM DTE). The reaction was initiated by the addition of 0.5 mM ATP. After 5-15 min incubation (RT), 20 μ M PPAT was added and reaction incubated for a further 10-30 min (RT). Next, 0.4 U/ μ L of alkaline phosphatase (from calf intestine, grade I (Ref 10108146001), Roche) was added and reaction incubated for an additional 10 min (RT). The linker was then enzymatically loaded onto the relevant PCP domain by the addition of 0.5 μ M Sfp R4-4 mutant (2 h, RT). The reaction was quenched by addition of 20 mM EDTA and the loading efficiency analyzed by MALDI-TOF-MS (intact mass determination).

Substrate loading onto PCP domain: 100 μ M Trx-PCP2_{tei} was loaded with 300 μ M (D)-tyrosine-4-hydroxyphenylglycine-pantetheine (for details of the synthesis see Kittilä et al. 2017) as described above, but with the incubation times reduced to protect the thioester bond from hydrolysis (PanK 5 min, PPAT 10 min, CIP 10 min, Sfp R4-4 15 min). Loaded Trx-PCP2_{tei} was washed four times with 50 mM Tris·HCl (pH 7.4) (Amicon® ultra ultracentrifugal filters, MWCO 10 000, 0.5 mL, Merck Millipore) to remove excess peptide. Dipeptide was cleaved from Trx-PCP2_{tei} with excess of methylamine and sample neutralized with formic acid. Precipitate was removed via centrifugation and supernatant analyzed with UPLC-MS (LCMS-8050, Shimadzu) with single ion monitoring (Column: Acquity UPLC Peptide BEH C18, 1.7 μ M, 2.1 x 100 mm, Waters; Gradient: 5 % acetonitrile for 0.5 min, 5-20 % acetonitrile in 20 min; flow rate 0.5 mL/min). A reaction without Trx-PCP2_{tei} (negative control) was handled with the same protocol.

Results and discussion

In our initial experiments, we tested the activity of the PanK and PPAT enzymes prior to optimizing the PCP loading assay. Both enzymes were expressed in *Escherichia coli* and purified in a simple two-step procedure to generate high yields of both purified enzymes (PanK = 0.7 mmol/L, PPAT = 3.8

mmol/L). The enzymatic activities of PanK and PPAT were then assessed using spectroscopic NADH coupled assays (Beinker et al. 2005; Kittilä et al. 2016b). Both enzymes were shown to be active under the assay conditions and product formation was confirmed using LCMS (Figure 3), with the phosphorylated pantetheine needed as a substrate for PPAT produced enzymatically from D-pantetheine by PanK.

After ensuring the production of dephospho-CoA from D-pantetheine, the one-pot PCP-loading reaction was explored. As an acceptor protein, a PCP domain of the NRPS machinery from complestatin biosynthesis (Trx-PCP_{6com} module 6, expressed as a thioredoxin fusion protein to improve yield) was utilized (Chiu et al. 2001). The functionality of the loading reaction was determined by measuring intact mass using MALDI-TOF ($\Delta_{m/z} = 358$ for phosphopantetheine loaded versus the unloaded PCP domain). Surprisingly no loading of the PCP domain was detected in the one-pot loading reaction, although it has been reported that R4-4 mutant of Sfp is able to load the PPE-linker to PCP domains using dephospho-CoA as a substrate (Sunbul et al. 2009). After reviewing the substrate binding pocket of Sfp (Mofid et al. 2004), it appeared plausible that the mutation to allow the wider substrate selectivity of the R4-4 mutant enzyme abolished its ability to distinguish between CoA and phosphoadenylates. If this were the case, then ATP, ADP and AMP could potentially bind to Sfp, preventing phosphopantetheine loading on the PCP domain. To test potential Sfp inhibition, we assessed how the different components of the one-pot loading reaction (ATP, ADP, AMP PanK, PPAT) affect the conventional loading reaction, where the linker is derived from CoA. The components were added individually to the loading reaction although they are not needed for linker loading from CoA. No PPE-loading was detected in the presence of phosphoadenylates, whilst the addition of PanK or PPAT did not influence the loading reaction (Figure 4). Therefore, the hypothesis of phosphoadenylates inhibiting Sfp mutant R4-4 seemed to be the most likely explanation for the lack of loading from our initial one-pot reaction.

To overcome this unwanted inhibition, we introduced alkaline phosphatase from calf-intestinal (CIP) into the loading reaction. CIP is commonly used to dephosphorylate DNA as it cleaves phosphates from the 5' end of DNA. Addition of CIP to the PCP-loading reaction was therefore expected to

dephosphorylate the problematic phosphoadenylates but not the desired dephospho-CoA product. This strategy proved to be successful and loading of PCP domains could now be achieved (Figure 5). As all of the loading enzymes (PanK, PPAT, Sfp) have been reported to be promiscuous, a wide variety of pantetheine analogs should be able to be loaded onto PCP domains. To test this, the one-pot loading reaction was also tested using D-pantothenic acid and D-panthenol to provide the linker regions for PCP loading. Both compounds were converted to dephospho-CoA analogs and loaded successfully on the PCP domain using our established one-pot loading method (Figure 5).

To demonstrate the functionality of this loading strategy towards different PCP domains and that the loading strategy can also be used for substrate loading, loading of a PCP domain derived from the teicoplanin producing NRPS machinery (Trx-PCP_{2tei}, module 2 (Kittila et al. 2017)) was studied. Trx-PCP_{2tei} was first successfully loaded using the one-pot loading strategy with the linker derived from pantetheine (Figure 6A). Next, we successfully loaded linked substrates – pantetheine dipeptide thioesters – onto the PCP domain using our established method (Figure 6B). Furthermore, we have been able to employ these loaded substrates to study catalytic steps in synthesis of glycopeptide antibiotics (Kittila et al. 2017).

In conclusion, the R4-4 mutant of Sfp enables loading of PCP domains in a one-pot loading reaction after dephosphorylation of phosphoadenylylates. The main advantage of this new PCP loading method is that the phosphorylation of dephospho-CoA catalyzed by DPCK is not needed to achieve PCP loading. The reaction catalyzed by DPCK is reversible (Strauss et al. 2010) and removal of the pyrophosphate formed in the step catalyzed by PPAT could well be required for efficient product formation when DPCK is present. In addition, the activity of DPCK has been reported to reduce over time (Rootman et al. 2010) and some groups have reported problems in achieving high activity through protein purification (Francois et al. 2006). Therefore, removing the need for this step is likely to prove advantageous in loading substrates onto PCP domains for future studies on NRPS systems, with the protocol described herein proving to be a simple and robust method to achieve this essential modification of PCP domains.

Acknowledgements

The authors are grateful to G. Stier (BZH-Heidelberg) for the thioredoxin fusion protein vector; to J. Yin (Georgia State University) for the R4-4 Sfp expression plasmid; to Melanie Müller and Marion Gradl (MPIMF-Heidelberg) for mass spectral analysis; to Melanie Schoppet (MPIMF-Heidelberg/Monash University) for dipeptide thioester substrate synthesis; and to Alexa Weinmann and Veronika Ulrich (MPIMF-Heidelberg) for assistance with construct cloning and protein preparation. T.K. is grateful for the support of the Deutsche Akademischer Austausch Dienst (DAAD Graduate School Scholarship Program); M.J.C. is grateful for the support of the Deutsche Forschungsgemeinschaft (Emmy-Noether Program, CR 392/1-1), Monash University and the EMBL Australia program.

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Figure Captions

Figure 1. Non-ribosomal peptide synthesis and examples of important peptide natural products produced by NRPS machineries. NRPS synthesis utilizes a modular architecture that is based upon repeating catalytic domains. Initial amino acid selection and activation is an ATP-dependent process that is performed by adenylation (A) domains. The activated amino acid is then transferred onto a peptidyl carrier protein (PCP) domain, which serve as attachment points for all intermediates during NRPS-mediated biosynthesis. Peptide bond formation is catalyzed by condensation (C) domains, which bind two PCP domains and transfer the upstream substrate onto the amino acid bound on the downstream PCP-domain with concomitant formation of a new peptide bond.

Figure 2. Loading substrates onto PCP domains. Loading substrates onto PCP domains is one of the biggest challenges in NRPS research and therefore several approaches to achieve loaded PCP domains have been established. A domains can be used to load substrates to PCP domains *in vitro*. First, the linker region needs to be attached in a reaction catalyzed by the phosphopantetheine transferase enzyme Sfp before an A domain can attach the substrate to the free thiol group of the linker. Substrates can also be coupled to coenzyme A chemically and Sfp can be used to load them to PCP domains. A mutant Sfp enzyme (R4-4) loads substrates coupled to ADP, which allows loading of varied linker regions in addition to different substrates. The majority of NRPS domains only accept PCP-bound substrates, but in some cases soluble SNAC-substrates can also be accepted. However, acceptance of such derivatives must be tested for each system separately and their use removes important protein-protein interactions between NRPS domains. Substrates can also be coupled to pantetheine, which can in turn be converted to coenzyme A using three enzymatic steps (chemo-enzymatic route). In this work, we explored an improved loading route in which a chemo-enzymatic route is combined with the use of Sfp mutant R4-4. This route is shown with a red arrow, with the steps being bypassed shown in grey. PanK = pantothenate kinase, PPAT = phosphopantetheine adenylyltransferase, DPCK = dephospho-CoA kinase.

384

385 **Figure 3.** PanK and PPAT activity. Both enzymes were initially purified to homogeneity (PanK = 39.3
386 kDa, PPAT = 20.3 kDa), with enzymatic activities analyzed using NADH coupled spectroscopic
387 assays. The PanK reaction was initiated by addition of ATP and subsequent production of ADP was
388 detected. PPAT adenylates phosphopantetheine that is produced by PanK: thus, a PanK reaction was
389 incubated in assay buffer with components to detect PP_i formation. As PanK does not produce PP_i , a
390 steady baseline was detected prior to PPAT addition. Product formation was confirmed by LCMS
391 measurement, with traces showing a combined reaction with PanK and PPAT. No remaining starting
392 material (D-pantetheine) was detected.

393

394 **Figure 4.** Inhibition of the one-pot PCP loading reaction. The Sfp mutant R4-4 can load Trx-PCP_{6com}
395 domain using CoA (positive control) or dephospho-CoA – however, the initial one-pot loading
396 reaction tested did not afford successful PCP loading despite the demonstrated functionality of all
397 enzymes. Inhibition of Sfp R4-4 was studied by addition of phosphoadenylates or other enzymes from
398 the loading cascade into a loading reaction utilizing CoA. No loading was observed in the presence of
399 phosphoadenylates, whilst the additional enzymes did not affect loading. Trx-PCP_{6com} = 23750 Da (-
400 Met); PPE-loaded Trx-PCP_{6com} = 24108 Da (-Met).

401

402 **Figure 5.** One-pot loading reaction including calf intestine alkaline phosphatase (CIP). Addition of
403 CIP to the one-pot loading reaction allowed different linkers to be successfully loaded onto Trx-
404 PCP_{6com}. CIP was stored in 3.2 M ammonium sulfate leading to formation of sulfate adducts (sulfate =
405 96 g/mol); linker loading from CoA by Sfp R4-4 was used as a positive control. Trx-PCP_{6com} = 23750
406 Da (-Met), loading with CoA/pantetheine = 24108 Da (-Met), loading with panthotenic acid = 24049
407 Da (-Met), loading with panthenol = 24035 Da (-Met).

408

409 **Figure 6.** Loading of Trx-PCP2_{tei}. A) A one-pot loading reaction was used to load Trx-PCP2_{tei} with
 410 linker derived from pantetheine to ensure the functionality of the loading cascade towards different
 411 PCP domains. Trx-PCP2_{tei} = 22475 Da (-Met), loaded Trx-PCP2_{tei} = 22833 Da (-Met). B) Trx-PCP2_{tei}
 412 was loaded with dipeptide coupled to a pantetheine linker using the established one-pot loading
 413 cascade. After the loading reaction, the substrate was cleaved from the linker via addition of
 414 methylamine and analyzed via UPLC-MS. Two peaks were observed because of dipeptide
 415 racemization during synthesis. Hydrolyzed dipeptide ((D)-tyrosine-4-hydroxyphenylglycine, 331
 416 g/mol) trace shown in grey, methylamine cleaved dipeptide (344 g/mol) shown in black.

417

Draft

Figure 1. Non-ribosomal peptide synthesis and examples of important peptide natural products produced by NRPS machineries. NRPS synthesis utilizes a modular architecture that is based upon repeating catalytic domains. Initial amino acid selection and activation is an ATP-dependent process that is performed by adenylation (A) domains: the activated amino acid is then transfer onto a peptidyl carrier protein (PCP) domain, which serve as attachment points for all intermediates during NRPS-mediated biosynthesis. Peptide bond formation is catalyzed by condensation (C) domains, which bind two PCP domains and transfer the upstream substrate onto the amino acid bound on the downstream PCP-domain with concomitant formation of a new peptide bond.

83x37mm (300 x 300 DPI)

Figure 2. Loading substrates onto PCP domains. Loading substrates onto PCP domains is one of the biggest challenges in NRPS research and therefore several approaches to achieve loaded PCP domains have been established. A domains can be used to load substrates to PCP domains *in vitro*. First, the linker region needs to be attached in a reaction catalyzed by the phosphopantetheine transferase enzyme Sfp before an A domain can attach the substrate to the free thiol group of the linker. Substrates can also be coupled to coenzyme A chemically and Sfp can be used to load them to PCP domains. A mutant Sfp enzyme (R4-4) loads substrates coupled to ADP, which allows loading of varied linker regions in addition to different substrates. The majority of NRPS domains only accept PCP-bound substrates, but in some cases soluble SNAC-substrates can also be accepted. However, acceptance of such derivatives must be tested for each system separately and their use removes important protein-protein interactions between NRPS domains. Substrates can also be coupled to pantetheine, which can in turn be converted to coenzyme A using three enzymatic steps (chemo-enzymatic route). In this work we explored an improved loading route in which a chemo-enzymatic route is combined with the use of Sfp mutant R4-4. This route is shown with a red arrow, with the steps being bypassed shown in grey. PanK = pantothenate kinase, PPAT = phosphopantetheine adenylyltransferase, DPCK = dephospho-CoA kinase.

112x72mm (300 x 300 DPI)

Figure 3. PanK and PPAT activity: both enzymes were initially purified to homogeneity (PanK = 39.3 kDa, PPAT = 20.3 kDa), with enzymatic activities analyzed using NADH coupled spectroscopic assays. The PanK reaction was initiated by addition of ATP and subsequent production of ADP was detected. PPAT adenylates phosphopantetheine that is produced by PanK: thus, a PanK reaction was incubated in assay buffer with components to detect PPI formation. As PanK does not produce PPI, a steady baseline was detected prior to PPAT addition. Product formation was confirmed by LCMS measurement, with traces showing a combined reaction with PanK and PPAT. No remaining starting material (d-pantetheine) was detected.

79x35mm (300 x 300 DPI)

Figure 4. Inhibition of the one-pot PCP loading reaction. The Sfp mutant R4-4 can load Trx-PCP6_{com} domain using CoA (positive control) or dephospho-CoA – however, the initial one-pot loading reaction tested did not afford successful PCP loading despite the demonstrated functionality of all enzymes. Inhibition of Sfp R4-4 was studied by addition of phosphoadenylates or other enzymes from the loading cascade into a loading reaction utilizing CoA. No loading was observed in the presence of phosphoadenylates, whilst the additional enzymes did not affect loading. Trx-PCP6_{com} = 23750 Da (-Met); PPE-loaded Trx-PCP6_{com} = 24108 Da (-Met).

217x561mm (300 x 300 DPI)

Figure 5. One-pot loading reaction including calf intestine alkaline phosphatase (CIP). Addition of CIP to the one-pot loading reaction allowed different linkers to be successfully loaded onto Trx-PCP6_{com}. CIP was stored in 3.2 M ammonium sulfate leading to formation of sulfate adducts (sulfate = 96 g/mol); linker loading from CoA by Sfp R4-4 was used as a positive control. Trx-PCP6_{com} = 23750 Da (-Met), loading with CoA/pantetheine = 24108 Da (-Met), loading with pantothenic acid = 24049 Da (-Met), loading with panthenol = 24035 Da (-Met).

142x236mm (300 x 300 DPI)

Figure 6. Loading of Trx-PCP2_{tei}. A) A one-pot loading reaction was used to load Trx-PCP2_{tei} with linker derived from pantetheine to ensure the functionality of the loading cascade towards different PCP domains. Trx-PCP2_{tei} = 22475 Da (-Met), loaded Trx-PCP2_{tei} = 22833 Da (-Met). B) Trx-PCP2_{tei} was loaded with dipeptide coupled to a pantetheine linker using the established one-pot loading cascade. After the loading reaction, the substrate was cleaved from the linker via addition of methylamine and analyzed via UPLC-MS. Two peaks were observed because of dipeptide racemization during synthesis. Hydrolyzed dipeptide ((D)-tyrosine-4-hydroxyphenylglycine, 331 g/mol) trace shown in grey, methylamine cleaved dipeptide (344 g/mol) shown in black.

93x49mm (300 x 300 DPI)