# Biochemical Investigations of Aromatic Prenyltransferases from the ABBA Superfamily

**Dissertation**

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Tobias Bonitz

aus Altenburg

Tübingen

2013

| | |
|---|---|
| Tag der mündlichen Qualifikation: | 11.06.2013 |
| Dekan: | Prof. Dr. Wolfgang Rosenstiel |
| 1. Berichterstatter: | Prof. Dr. Lutz Heide |
| 2. Berichterstatter: | PD Dr. Bertolt Gust |

## Table of contents

## Summary

The linkage of isoprenoid and aromatic moieties, catalyzed by aromatic prenyltransferases (PTases), leads to an impressive diversity of primary and secondary metabolites, including important pharmaceuticals and toxins (*e.g.* ubiquinone derivatives and ergot alkaloids). In 2005, a hydroxynaphthalene PTase, NphB, featuring a novel ten-stranded β-barrel fold, was identified in *Streptomycetes*. This fold, termed the PT-barrel, remained exclusive to the NphB family until its recent discovery in the DMATS family of indole PTases. Members of both families exist only in fungi and bacteria, and all investigated members catalyze the prenylation of aromatic substrates in the biosynthesis of secondary metabolites. Sequence comparisons using PSI-BLAST do not yield matches between these two families, suggesting that they could have converged upon the same fold independently. However, in the first chapter of this thesis evidence is provided for a common ancestry for the NphB and DMATS families of PTases. In addition sequence repeats were identified that coincide with the structural repeats in proteins belonging to these two families. Therefore it could be speculated that the PT-barrel arose by amplification of an ancestral ααββ module. Because of their homology and their similarities in structure and function, this study proposes to group the NphB and DMATS families together into a single superfamily, the PT-barrel superfamily or ABBA prenyltransferase superfamily.

The second chapter of this thesis reports on a phenazine biosynthetic gene cluster which was discovered in *Streptomyces tendae* Tü1028. In preliminary work the formerly silent phenazine biosynthetic gene cluster was activated by introduction of a constitutive promoter and heterologous expression in *Streptomyces coelicolor* M512. Subsequently the accumulation of the antibiotic phenazine-1-carboxylic acid (PCA) and of a new derivative thereof, *i.e.* a conjugate of PCA and *L*-glutamine (PCA-Gln), was observed. The linkage of PCA to *L*-glutamine was catalyzed by enzymes of the heterologous expression host *Streptomyces coelicolor* M512. In this study I investigated the biological activity of PCA-Gln compared to PCA and could show that PCA showed a strong antibiotic effect, but PCA-Gln did not. It could be speculated that glutamination of PCA represents a resistance mechanism against the antibiotic PCA, which is produced in significant quantities in soil by *Pseudomonas* strains.

The gene cluster from *S. tendae* Tü1028 also contained genes for all enzymes of the mevalonate pathway and for an aromatic PTase, thereby resembling gene clusters for prenylated phenazines. However this study proves by purification and biochemical investigation of the PTase that it does not prenylate phenazines but hydroxynaphthalene substrates, showing very similar properties as NphB of naphterpin biosynthesis.

The third chapter of this thesis deals with a new ABBA PTase which farnesylates a benzodiazepine substrate. The bacterium *Micromonospora* sp. RV115 produces the unusual metabolite diazepinomicin, a prenylated benzodiazepine derivative. Biochemical investigation of the PTase DzmP from this organism showed the farnesylation of the amide nitrogen of dibenzodiazepinone. DzmP is the first member of the ABBA PTase superfamily which utilizes farnesyl diphosphate (C15) as genuine substrate. All previously discovered members utilize either dimethylallyl diphosphate (C5) or geranyl diphosphate (C10). Another putative diazepinomicin biosynthetic gene cluster was identified in the genome of *Streptomyces griseoflavus* Tü4000. The gene cluster contains a gene *ssrg_00986* with 61.4% identity (amino acid level) to *dzmP*. The purified protein showed similar catalytic properties as DzmP.

## Zusammenfassung

Die Verknüpfung von Isoprenketten mit Aromaten, katalysiert von aromatischen Prenyltransferasen (PTasen), erzeugt eine beeindruckende Vielfalt von Primär- und Sekundärmetaboliten, inklusive bedeutender Arzneistoffe und Toxine wie zum Beispiel Ubichinonderivate oder Ergotalkaloide. Im Jahr 2005 wurde eine Hydroxynaphthalin-PTase (NphB) in Streptomyceten gefunden, welche eine neuartige Proteinfaltung mit zehn antiparallelen β-Faltblattsträngen zeigt. Diese Proteinfaltung, genannt das PT-barrel, kam nur in der NphB-Familie vor, bis es vor kurzem ebenfalls in der DMATS Familie von Indol-PTasen gefunden wurde. Mitglieder dieser zwei Familien kommen nur in Pilzen und Bakterien vor und alle bisher untersuchten Vertreter katalysieren die Prenylierung von aromatischen Substraten des Sekundärmetabolismus. Sequenzvergleiche mit PSI-BLAST zeigen keine Ähnlichkeiten zwischen diesen beiden Familien, was zunächst nahe legte, dass sich die Proteinfaltung des PT-barrels in diesen unabhängig entwickelt haben könnte.

Im ersten Kapitel dieser Dissertation werden aber Hinweise aufgezeigt, die für einen gemeinsamen Ursprung der NphB- und DMATS-Familien sprechen. Ebenso wurden Sequenzwiederholungen in Vertretern dieser zwei Familien gefunden, die mit den Sekundärstrukturwiederholungen des PT-barrels übereinstimmen. Man kann also annehmen, dass sich die PT-barrel-Faltung aus einer Vervielfältigung eines vorausgegangenen αααββ Moduls entwickelt hat. Unter Berücksichtigung ihrer Homologie und der Ähnlichkeit der Proteinfaltung, sowie ihrer Funktion, schlägt diese Arbeit vor, die NphB- und DMATS-Familien in einer Superfamilie zu vereinen, der PT-barrel Superfamilie bzw. ABBA PTase Superfamilie.

Das zweite Kapitel dieser Dissertation handelt von einem Phenazinbiosynthese-gencluster, welches in *Streptomyces tendae* Tü1028 gefunden wurde. In einer vorausgegangenen Arbeit konnte das inaktive Phenazinbiosynthesegencluster durch Einfügen eines konstitutiven Promotors und heterologe Expression in *Streptomyces coelicolor* M512 aktiviert werden. Dies führte zu einer Produktion des Antibiotikums Phenazin-1-carbonsäure (PCA) und eines Derivates davon, genauer eines Konjugat von PCA mit *L*-Glutamin (PCA-Gln). Die Verknüpfung von PCA und *L*-Glutamin durch Bildung einer Amidbindung wird durch Enzyme des heterologen Wirtsstammes *Streptomyces coelicolor* M512 katalysiert. In dieser Dissertation wird durch biologische Aktivitätsstudien gezeigt, dass PCA eine deutliche antibiotische

Wirksamkeit zeigt, nicht jedoch PCA-Gln. Somit scheint die Glutaminierung von PCA einen Resistenzmechanismus gegen das Antibiotikum PCA, welches im Boden in signifikanten Mengen von Pseudomonaden gebildet wird, darzustellen.

Das Gencluster aus *S. tendae* Tü1028 beinhaltet des Weiteren Gene für Enzyme der Mevalonatbiosynthese und eine aromatische PTase ($Ptf_{St}$), ähnlich zu Genclustern für prenylierte Phenazine. Im Gegensatz dazu wird in dieser Arbeit durch Reinigung und biochemische Untersuchung der PTase gezeigt, dass von diesem Enzym keine Phenazine prenyliert werden, sondern Hydroxynaphthalinsubstrate. Damit zeigt $Ptf_{St}$ Eigenschaften, die sehr ähnlich zu denen von NphB aus der Naphterpinbiosynthese sind.

Das dritte Kapitel dieser Dissertation beschreibt eine neuartige ABBA PTase, welche ein Benzodiazepin farnesyliert. Das Bakterium *Micromonospora* sp. RV115 produziert den ungewöhnlichen Sekundärmetabolit Diazepinomicin, ein prenyliertes Benzodiazepinderivat. Biochemische Untersuchungen der PTase DzmP aus diesem Organismus zeigt eine Farnesylierung des Amidstickstoffs von Dibenzodiazepinon. DzmP ist das erste Mitglied der ABBA PTase-Superfamilie, welches Farnesyldiphosphat (C15) als natürliches Substrat nutzt. Alle bekannten Mitglieder nutzen entweder Dimethylallyldiphosphat (C5) oder Garanyldiphosphat (C10). Ein weiteres mögliches Diazepinomicingencluster konnte in *Streptomyces griseoflavus* Tü4000 identifiziert werden. Das Gencluster enthält ein Gen (*ssrg_00986*) mit 61,4% Identität (auf Ebene der Aminosäuren) zu *dzmP*. Das gereinigte Protein zeigte vergleichbare katalytische Eigenschaften wie DzmP.

# Publications and presentations

## Publications in international Journals

**Bonitz**, Alva, Saleh, Lupas, Heide (2011) **Evolutionary Relationships of Microbial Aromatic Prenyltransferases.** PLoS ONE 6(11): e27336. DOI:10.1371/journal.pone.0027336

Saleh*, **Bonitz***, Flinspach, Kulik, Burkard, Mühlenweg, Vente, Polnick, Lämmerhofer, Gust, Fiedler, Heide (2012) **Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines.** Med. Chem. Commun., 3, 1009. DOI: 10.1039/c2md20045g
* both authors contributed equally

**Bonitz**, Zubeil, Grond, Heide (2013) **Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily.** submitted

## Conference contributions

| | |
|---|---|
| 01/2013<br>oral presentation | „Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines"<br>ERA IB Workshop in Palma de Mallorca, Spain |
| 10/2012<br>oral presentation | „A new resistance mechanism protecting *Streptomyces* against phenazine-1-carboxylic acid, an antibiotic produced by *Pseudomonas* strains"<br>Symposium „Actinobacteria within soils" in Münster, Germany |
| 03/2012<br>Poster | „Activation of a silent phenazine biosynthetic gene cluster from *Streptomyces tendae* Tü1028 reveals a novel phenazine conjugate"<br>Annual meeting of the VAAM in Tübingen, Germany |

| | |
|---|---|
| 09/2011<br>Poster | „Evolutionary Relationships of Microbial Aromatic Prenyltransferases"<br>Workshop of the VAAM in Bonn, Germany |
| 09/2010<br>Poster | „Cluster analysis of microbial aromatic prenyltransferases"<br>Workshop of the VAAM in Tübingen, Germany |
| 08/2010<br>Poster | „Cluster analysis of microbial aromatic prenyltransferases"<br>Annual meeting of the „Society for Industrial Microbiology" in San Francisco, USA |
| 10/2009<br>Poster | „A new ABBA prenyltransferase from *Streptomyces tendae* Tü1028"<br>Symposium „Biology of Streptomycetes" in Münster, Germany |

## Conference contributions as Co-author

| | |
|---|---|
| 06/2013<br>Poster | „*D*- or *L*-Glutamine? What are bacteria using for making them resistant to their own produced antibiotics? A stereochemical identification. "<br>S. Polnick, O. Saleh, **T. Bonitz**, L. Heide, M. Lämmerhofer<br>HPLC 2013 in Amsterdam, Netherlands |
| 03/2013<br>Poster | „Stereochemical Identification of the *N*-(phenazine-1-yl-carbonyl)glutamine and its 9-Isoprenyl-phenazine analog"<br>Polnick, S., Saleh, O., **Bonitz, T.**, Heide, L., Lämmerhofer, M.<br>Anakon in Essen, Germany |

# Introduction

## Chapter 1 - Evolutionary relationships of aromatic prenyltransferases

from: Evolutionary Relationships of Microbial Aromatic Prenyltransferases.
Tobias Bonitz, Vikram Alva, Orwah Saleh, Andrei N. Lupas, Lutz Heide
PLoS ONE 6(11): e27336. doi:10.1371/journal.pone.0027336

Prenylation of chemical compounds alters their bioactivity compared to their non-prenylated derivatives as revealed by structure–activity relationship studies (Botta *et al.*, 2005). These properties reside in their enhanced permeability of biological membranes and increased affinity for their target proteins (Botta *et al.*, 2005; Cui *et al.*, 2007; Nowicka *et al.*, 2010). Therefore prenylated compounds posses a wide range of biological activities which are applied in medicine, agriculture and food chemistry (Kuzuyama *et al.*, 2005; Yazaki *et al.*, 2009).

Aromatic prenyltransferases (PTases) catalyze the transfer of isoprenyl moieties to aromatic acceptor molecules, forming C-C bonds (Fig. 1). They are key enzymes in the biosynthesis of lipoquinones and of many secondary metabolites in plants, fungi and bacteria (Heide, 2009). Aromatic PTases of lipoquinone biosynthesis are integral membrane proteins. They contain an aspartate-rich motif (*e.g.* NDxxD) for binding of the prenyl diphosphate substrate *via* a $Mg^{2+}$ ion, similar to the corresponding motif of farnesyl diphosphate synthase (Poulter, 2006). A structural model of the membrane bound PTase UbiA involved in the biosynthesis of ubiquinone (Fig. 1) has been proposed (Bräuer *et al.*, 2008).

In contrast to the PTases of lipoquinone biosynthesis, the aromatic PTase CloQ from *Streptomyces roseochromogenes*, involved in the formation of clorobiocin (Fig. 1), was found to be a soluble protein (Pojer *et al.*, 2003; Metzger *et al.*, 2010). CloQ does not contain a NDxxD motif and is active in the absence of $Mg^{2+}$ or other divalent cations. Kuzuyama *et al.* (2005) identified a similar aromatic PTase, NphB, involved in the biosynthesis of the prenylated polyketide naphterpin (Fig. 1) in *Streptomyces* sp. strain CL190. CloQ and NphB were found to display a hitherto unobserved β-barrel fold which was termed the PT-barrel (Fig. 2; PDB 2XLQ and 1ZB6). It consists of five repetitive αααββ elements. The ten β-strands arrange in an

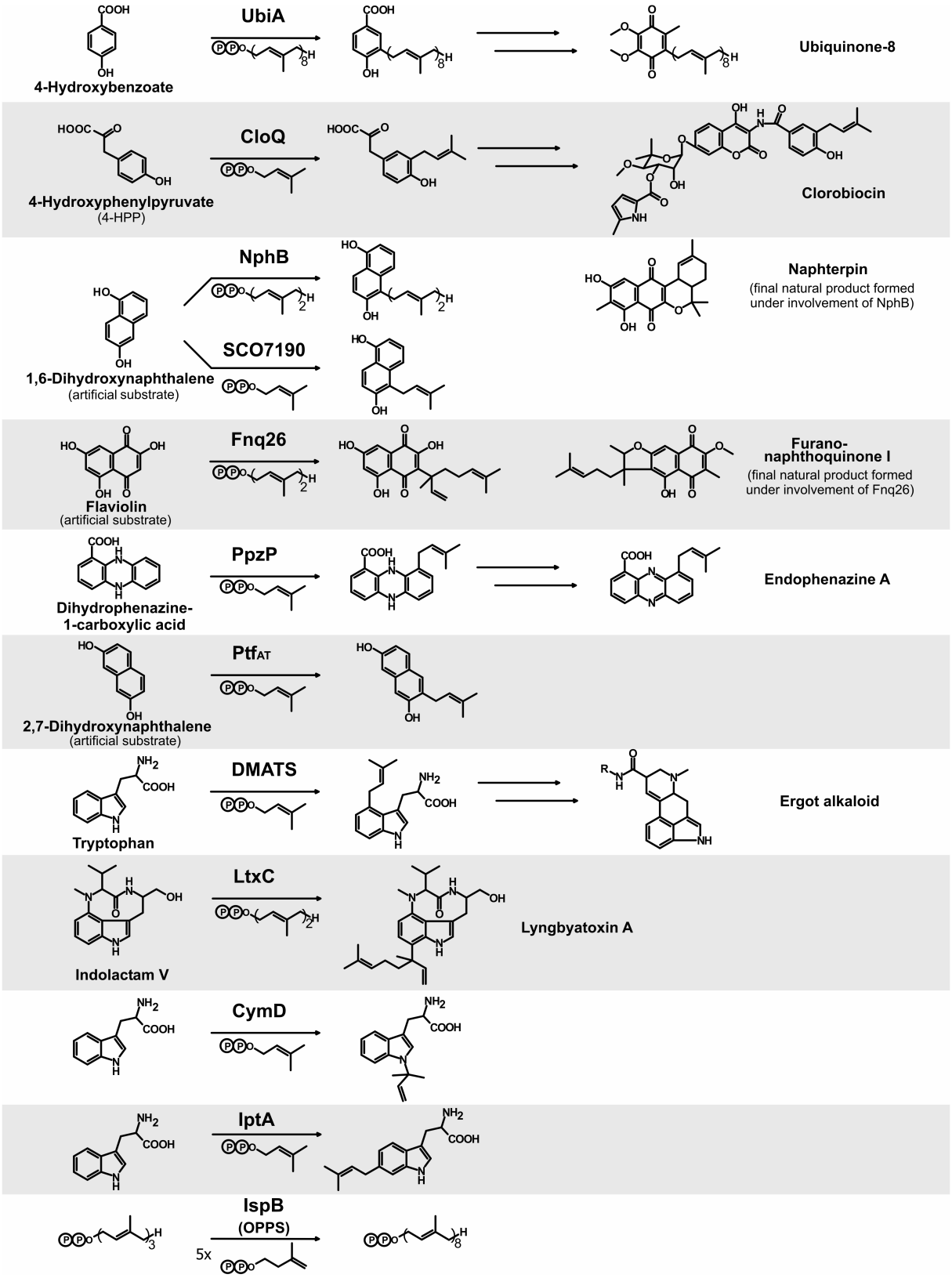



Fig. 1: Reactions catalyzed by aromatic prenyltransferases and by the octaprenyl diphosphate synthase (OPPS) IspB.

antiparallel fashion to form a central β-barrel that contains the active center in its spacious lumen and the α-helices form a solvent-exposed ring around the barrel (Tello *et al.*, 2008). PSI-BLAST searches currently reveal 17 further database entries with sequence similarity to NphB and CloQ, 12 of them in bacteria of the genus *Streptomyces* and five in fungi of the phylum *Ascomycota*. In silico structure predictions suggest that all these proteins adopt the PT-barrel fold. Eleven of these enzymes have been investigated biochemically, and all of them catalyze the *C*-prenylation of aromatic compounds, *i.e.* phenols or phenazines.

The *C*-prenylation of an aromatic compound is also catalyzed by dimethylallyltryptophan synthase (DMATS; Fig. 1), involved in the biosynthesis of the pharmaceutically important ergot alkaloids in different fungi of the phylum *Ascomycota*. DMATS shows no sequence similarity, as evaluated with PSI-BLAST, to the bacterial enzyme NphB or orthologs thereof, and is considerably larger than NphB (459 vs. 307 amino acids). Unexpectedly, however, it was found to adopt the same PT-barrel fold as NphB (Fig. 2; PDB 3I4X) (Metzger *et al.*, 2009). DMATS is the prototype of the fungal indole PTases, involved in the biosynthesis of a large number of complex secondary metabolites in fungi (Steffan *et al.*, 2009). Furthermore, three

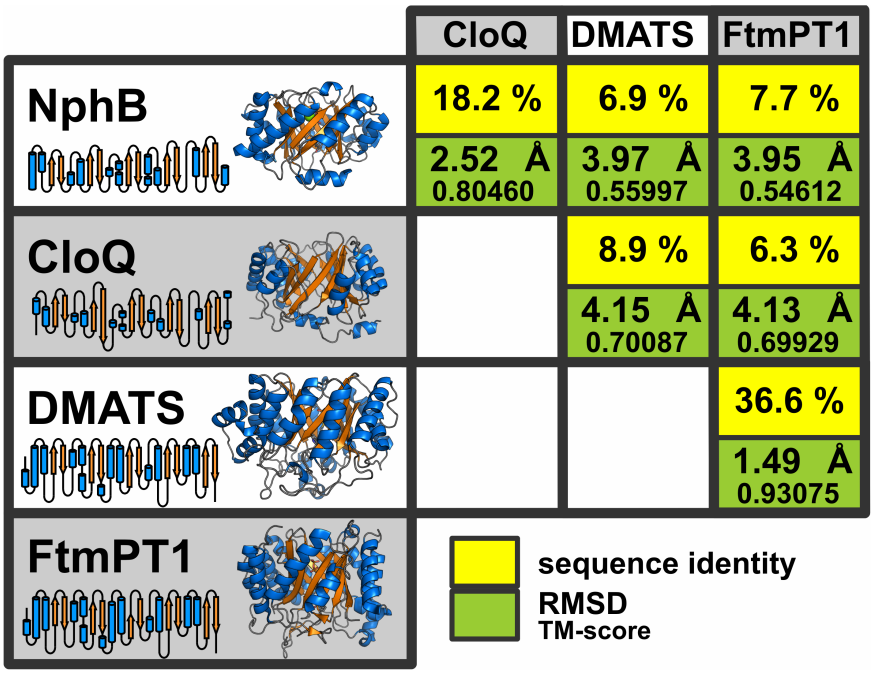



Fig. 2: Structures of PT-barrels. Pairwise sequence identities, RMSDs and TM-scores of the four aromatic prenyltransferases NphB (PDB 1ZB6), CloQ (2XLQ), DMATS (3I4Z) and FtmPT1 (3O2K) are shown. The schemes besides the structures depict the topology of the secondary structural elements.

indole PTases (LtxC, CymD and IptA; Fig. 1) have recently been identified in bacteria (Edwards *et al.*, 2004; Schultz *et al.*, 2010; Takahashi *et al.*, 2010), and GenBank currently contains 16 further entries from bacterial genomes with similarity to these three enzymes. Most of these entries are found in genomes of actinomycetales, but one is from the alphaproteobacterium *Methylobacterium* sp. 4-46, and LtxC is from a cyanobacterium. *In silico* structure prediction suggests that all these bacterial indole PTases adopt the PT-barrel fold.

A similarity in sequence between the phenol/phenazine PTases (NphB/CloQ family) and the indole PTases (DMATS/CymD family) is not detectable using BLAST and PSI-BLAST, despite their similar protein structure (Fig. 2). Only a limited number of structural solutions is available to a polypeptide chain, therefore protein structures are multiply convergent (Cheng *et al.*, 2008). In contrast, sequence space is essentially infinite and many sequences are compatible with a particular fold. For this reason, sequence similarity rather than structure similarity is the primary marker of homology. In the recent years, the enormous growth of protein sequence and structure databases coupled with the development of sensitive sequence comparison methods has shown that proteins may not be as polyphyletic as hitherto assumed (Alva *et al.*, 2010). Indeed, many fold families, for instance families of the TIM $(\beta\alpha)_8$-barrel fold, that were previously considered to be analogous are now thought to be homologous (Copley *et al.*, 2000; Nagano *et al.*, 2002; Söding *et al.*, 2006). Sequence search methods achieve different levels of sensitivity, depending on the amount of information they incorporate. Sequence-to-sequence methods, such as BLAST (Altschul *et al.*, 1990), are the least sensitive as they use only the information from the pairwise comparison of two sequences, scored by a global substitution matrix. Profile-to-sequence methods, such as the iterated version of BLAST, PSI-BLAST (Altschul *et al.*, 1997), are more sensitive, as they include family-specific information for the query sequence in the form of a position-specific scoring matrix derived from homologous sequences. Profile-to-profile comparison methods, such as COMPASS (Sadreyev *et al.*, 2003), provide an additional improvement by using family-specific information for both sequences being compared. Incorporation of position-specific gapping probabilities into the profiles yields profile Hidden Markov Models (HMMs) (Eddy, 1998), which are currently our most sensitive tool for the detection of sequence similarity. HHsearch (Söding, 2005; Söding *et al.*, 2005), an

HMM-to-HMM comparison method, has a sensitivity comparable to that of advanced fold recognition methods, despite using only sequence information.

We used HHsearch to evaluate whether the NphB/CloQ family and the DMATS/CymD family may have originated independently and converged on the PT-barrel fold in response to the biochemical challenge of performing an aromatic prenylation reaction, *i.e.* a reaction corresponding to a Friedel-Crafts alkylation, in an aqueous solution which requires effective shielding of the reactive intermediary allylic cation from reaction with water (Metzger et al., 2009).

## Chapter 2 – A new resistance mechanism against phenazines

From: Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines
Orwah Saleh, Tobias Bonitz, Katrin Flinspach, Andreas Kulik, Nadja Burkard, Agnes Mühlenweg, Andreas Vente, Stefan Polnick, Michael Lämmerhofer, Bertolt Gust, Hans-Peter Fiedler and Lutz Heide
Med. Chem. Commun., 2012, 3, 1009–1019. doi: 10.1039/c2md20045g

In soil diverse, communities of micro- and macro-organism live together in symbiotic or competing relationships. For *Pseudomonas* spp. it was recently demonstrated by Mavrodi *et al.* (2012), that they are able to produce antibiotics in sufficient amounts (0.1 µM in the rhizosphere) to inhibit the growth of sensitive organisms and eventually gaining an advantage on resources and their own survival. This was the first example of effective antibiotic concentration in soil. The investigated antibiotic was phenazine-1-carboxylic acid (PCA). Besides their role as antibiotics, phenazines have a variety of biological functions for the producing bacterium, often related to their capability to shuttle electrons by reversible oxidation and reduction (Pierson *et al.*, 2010).

Phenazine biosynthesis is limited to the actinobacteria (*e.g. Streptomyces*), certain groups of Gram-negative proteobacteria (*e.g. Pseudomonas*) and a few archaea (Mavrodi *et al.*, 2010). *Pseudomonas* strains produce simple phenazines such as PCA and phenazine-1-carboxamide. In contrast, *Streptomyces* strains can form more complex phenazines with very diverse substitution patterns of the phenazine core (Ohlendorf *et al.*, 2012; Zendah *et al.*, 2012). Many phenazine biosynthetic gene clusters have been identified in Gram-negative proteobacteria (Mavrodi *et al.*, 2010), but so far only two have been described in *Streptomyces* (Haagen *et al.*, 2006; Saleh

*et al.*, 2009; Seeger *et al.*, 2011). Both these *Streptomyces* gene clusters show a core of seven biosynthetic genes (*phzBCDEFGA*) responsible for the formation of PCA. Additionally, they show genes involved in the modification of the phenazine core, *e.g.* by prenylation, methylation or oxidation.

In preliminary work for this study the genomic DNA of 190 strains was screened for phenazine biosynthesis genes. Five strains were found to contain a putative phenazine biosynthetic gene cluster (Saleh *et al.*, 2012). In one of these strains, *Streptomyces tendae* Tü1028, additional genes of the mevalonate pathway and for a PTase were found, which suggested the possible formation of a prenylated phenazine (Fig. 3). This strain was selected for further investigation. However, this phenazine biosynthetic gene cluster was silent both in the genuine producer strain and in a heterologous producer strain, despite the use of many different culture media. Eventually, introduction of a constitutive *ermE** promoter upstream of the phenazine biosynthesis genes triggered the formation of two phenazine compounds. One was readily identified as PCA in comparison to an authentic reference substance. The other compound showed an ion mass different from any phenazine derivative described previously. NMR spectroscopy, in comparison to PCA showed that the new compound represented a conjugate of PCA, attached via an amide bond to the amino group of glutamine (α-*N*-(phenazine-1-carbonyl)-*L*-glutamine = PCA-Gln). The stereochemical configuration of the amino acid glutamine in the structure of PCA-Gln was determined by enantioselective HPLC analysis (Lämmerhofer *et al.*, 2008).

Mechanistically, the conjugation of PCA to glutamine requires an activation of the carboxyl group of PCA, *e.g.* in form of a coenzyme A ester or of an acyl adenylate. Surprisingly, no candidate gene for such an amide synthetase was found in the insert of the heterologously expressed cosmid (Fig. 3). In two parallel control experiments, PCA was added to cultures of *S. tendae* Tü1028 or to sterile culture medium (Fig. 4).

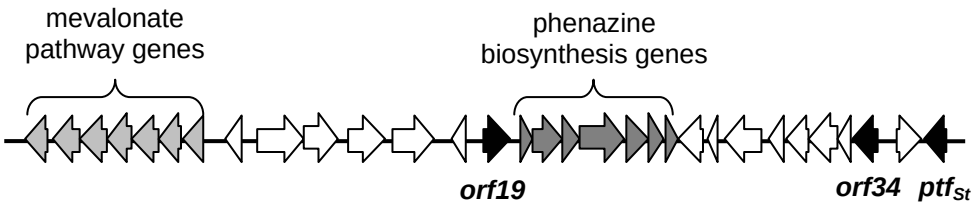



Fig. 3: Insert of the heterologously expressed cosmid containing the secondary metabolic gene cluster from the genome of *S. tendae* Tü1028.

PCA remained unchanged in the sterile medium and in cultures of *S. tendae*, but was nearly quantitatively (97%) converted to PCA-Gln in the cultures of *S. coelicolor* M512. This conversion is therefore carried out by enzymes encoded in the genome of *S. coelicolor* M512.

The antibiotic activity of phenazines results from their ability to reduce molecular oxygen to toxic, highly reactive oxygen species (Pierson *et al.*, 2010). *Pseudomonas aeroginosa*, a producer of phenazines, protects itself from this toxic effect of phenazines by production of superoxide dismutases and catalase (Hassett *et al.*, 1992). *Enterobacter agglomerans* forms a phenazine binding protein which facilitates the export of the toxic molecule (Yu *et al.*, 2011). The resistance mechanisms of phenazine-producing actinobacteria have not been examined. In order to investigate whether the conjugation of PCA to glutamate may offer a mechanism to detoxify PCA, and/or to facilitate its export, in this study the antibiotic activity of PCA and of PCA-Gln against *Escherichia coli*, *Bacillus subtilis* and *Streptomyces coelicolor* M512 were compared in disk diffusion assays.

## Biochemical investigation of the PTase $Ptf_{St}$

Beside the phenazine biosynthesis genes, the cluster of *S. tendae* Tü1028 also contains a complete set of mevalonate pathway genes required for the generation of prenyl moieties and a putative ABBA PTase. Two previously identified gene clusters

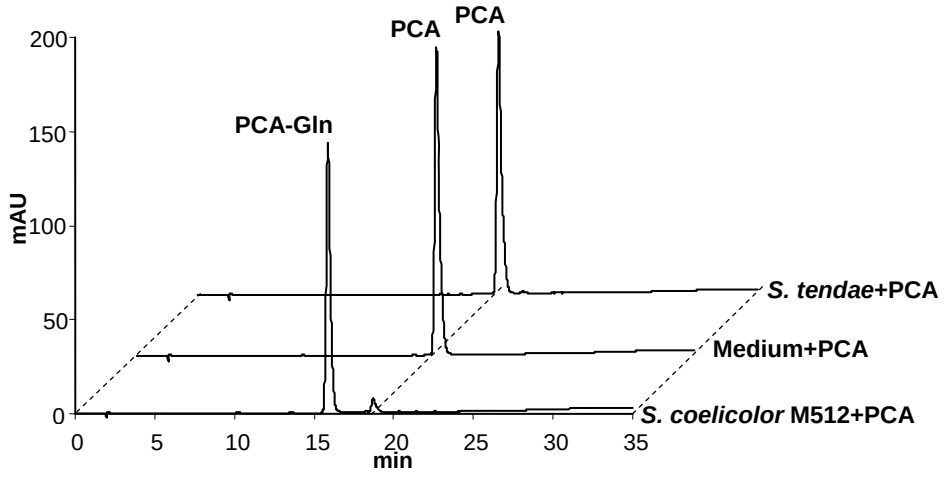



Fig. 4: HPLC analysis of the feeding of phenazine-1-carboxylic acid (PCA) to cultures of *Streptomyces coelicolor* M512, to cultures of *S. tendae*, or to sterile culture medium. Only *S. coelicolor* M512 converted PCA to α-*N*-(phenazine-1-carbonyl)-*L*-glutamine (PCA-Gln).

for prenylated phenazines were found to contain also genes for ABBA PTases, as well as a group of genes of the mevalonate pathway (Haagen *et al.*, 2006; Saleh *et al.*, 2009; Seeger *et al.*, 2011). The left end of the insert of the cosmid as depicted in Fig. 3 contained an operon of seven genes of the mevalonate pathway, encoding all enzymes required for the biosynthesis of the isoprenoid precursor. This operon of mevalonate biosynthesis genes shows very high similarity to the corresponding operons in *S. anulatus* and *S. cinnamonensis*. In all three strains, the genes are organized in the same order, and the entire operons from the three strains show approximately 80% sequence identity to each other on the nucleotide level. At the right end of the insert of the cosmid (Fig. 3), the putative ABBA PTase gene was found and designated *ptf*$_{St}$. Its predicted product (305 aa) showed similarity to the phenazine PTase PpzP from *S. anulatus* and EpzP from *S. cinnamonensis*, and to PTases involved in the biosynthesis of prenylated naphthalene derivatives such as NphB from *Streptomyces sp.* CL190 (Kuzuyama *et al.*, 2005) or Fnq26 from *S. cinnamonensis* (Haagen *et al.*, 2007). Furthermore, the cluster contained a gene coding for a putative polyprenyl diphosphate synthase (*orf19*) and a putative type III polyketide synthase gene (*orf34*). Similar genes have been found in gene clusters for prenylated naphthalenes like naphterpin and furanonaphthoquinone I (Kuzuyama *et al.*, 2005; Haagen *et al.*, 2006). To investigate whether Ptf$_{St}$ is a phenazine or a polyketide PTase its substrate specificity and biochemical properties were determined in this study.

## Chapter 3 – Unusual *N*-prenylation in diazepinomicin biosynthesis

From: Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily
Tobias Bonitz, Florian Zubeil, Stephanie Grond, and Lutz Heide

Typically, ABBA prenyltransferases (PTases) catalyze the *C*-prenylation of aromatic substrates (see chapter 1), *i.e.* a reaction similar to a Friedel-Crafts alkylation, although a few members also catalyze *N*- or *O*-prenylations. Their considerable promiscuity for different aromatic substrates makes them attractive tools for chemoenzymatic synthesis (Tello *et al.*, 2008; Li, 2010). However, their genuine

isoprenoid substrates have so far been strictly limited to either dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP), *i.e.* to substrates with five or ten carbon atoms. In order to expand the substrate range of this group of chemoenzymatic tools, we decided to search for ABBA PTases which might use farnesyl diphosphate (FPP) as genuine isoprenoid substrate. Our attention was drawn to the unusual natural product diazepinomicin (Fig. 5). Its dibenzodiazepinone core is structurally unique in nature. The only related natural products containing benzodiazepine moieties are the pyrrolobenzodiazepines formed by bacteria, and the fungal metabolite cyclopenin (Gerratana, 2010).

Diazepinomicin has been independently isolated from different *Micromonospora* strains from different geographic locations of this world by Bristol-Myers Squibb (Ohkuma *et al.*, 1996; Igarashi *et al.*, 2005), Thallion Pharmaceuticals (formerly Ecopia BioSciences) (Bachmann *et al.*, 2006), Wyeth (Charan *et al.*, 2004) and recently by Abdelmohsen *et al.* (2012). So far this compound has never been described outside of the genus *Micromonospora*. It was named BU-4664L, TLN-4601 (formerly ECO-4601) or diazepinomicin, and the latter name will be used hereafter.

Diazepinomicin binds to peripheral benzodiazepine receptors and has potent antitumor activity (Gourdeau *et al.*, 2008; Boufaied *et al.*, 2010). It has recently been investigated in a phase II clinical trial as anticancer agent (Mason *et al.*, 2012).

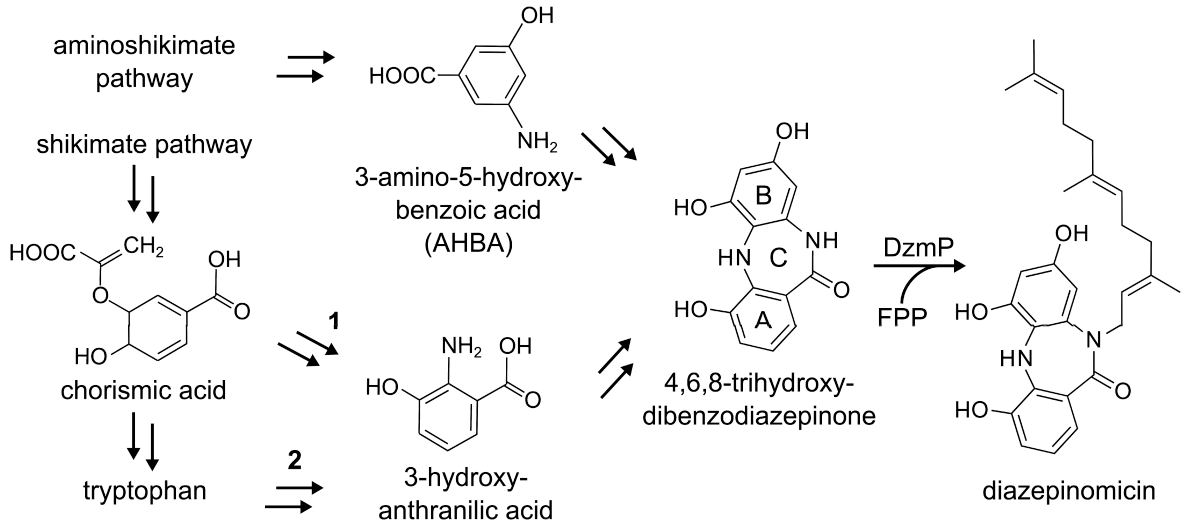



Fig. 5: Diazepinomicin and its hypothetical biosynthetic pathway (modified from McAlpine *et al.* (2008)). 1) Pathway encoded in the gene cluster of diazepinomicin. 2) Degradation of tryptophan via the kynurenine pathway.

Feeding studies have established that ring A of diazepinomicin (Fig. 5) is derived from 3-hydroxy anthranilic acid (McAlpine *et al.*, 2008). This precursor can be formed by the kynurenine pathway which is used in actinobacteria for tryptophan catabolism (Lima *et al.*, 2009; Zummo *et al.*, 2012). Therefore, labeled tryptophan is incorporated into diazepinomicin (McAlpine *et al.*, 2008; Feng *et al.*, 2009; Ratnayake *et al.*, 2009). However, the biosynthetic gene cluster for diazepinomicin contains genes for an even more efficient pathway which is suggested to lead from chorismate via 2-amino-2-deoxyisochorismate to 3-hydroxyanthranilate in three enzymatic steps (McAlpine *et al.*, 2008). The enzymes catalyzing this pathway are similar to PhzE and PhzD of phenazine biosynthesis (Mentel *et al.*, 2009) and to MxcC of myxochelin biosynthesis (Silakowski *et al.*, 2000). Furthermore McAlpine *et al.* (2008) speculated that ring B of diazepinomicin is derived from 3-amino-5-hydroxybenzoic acid (AHBA), a metabolite derived from the aminoshikimate pathway (Floss *et al.*, 2011).

The biosynthetic gene cluster for diazepinomicin has been cloned and sequenced from *Micromonospora* strain 046Eco-11 (McAlpine *et al.*, 2008). Based on a bioinformatic analysis of the DNA sequence, a detailed hypothesis of the biosynthetic pathway leading to diazepinomicin has been formulated (McAlpine *et al.*, 2008), but the function of the individual genes and enzymes has not been investigated experimentally. *orf11* of this gene cluster shows sequence similarity to genes coding for ABBA PTases. It has therefore been speculated that the gene product of *orf11* may catalyze the farnesylation reaction in diazepinomicin biosynthesis (McAlpine *et al.*, 2008).

In this study, we identified a close homolog of *orf11* (96.9% identity on the amino acid level) in the diazepinomicin producer strain *Micromonospora* sp. RV115 (Abdelmohsen *et al.*, 2012). This gene (hereafter called *dzmP*) was expressed in *Escherichia coli* and the protein was purified. In addition, homology searches by BLAST (Altschul *et al.*, 1997) revealed a gene cluster in *Streptomyces griseoflavus* Tü4000 with striking similarity to the diazepinomicin cluster. The *orf11* ortholog from this cluster was also expressed and purified. Both enzymes were characterized biochemically.

# Objective

The main objective of this thesis was to discover and investigate new prenyltransferases which may be used for chemoenzymatic synthesis. Therefore the following tasks were undertaken:

### Chapter 1 - Evolutionary relationships of microbial aromatic PTases

As basis for the exploration of prenyltransferases (PTases), amino acid sequences of different PTases were analyzed using bioinformatic algorithms. Strikingly the families of NphB/CloQ - PTases and DMATS/CymD - PTases showed no homology in PSI-BLAST analysis despite their structural similarity. This thesis therefore investigated the evolutionary relationships between the two families and the origin of their shared protein fold.

### Chapter 2 - A new resistance mechanism of *S. coelicolor* against phenazines and biochemistry of the prenyltransferase $Ptf_{St}$

Expression of the phenazine / polyketide gene cluster from *S. tendae* Tü1028 in the heterologous host *S. coelicolor* M512 by Saleh *et al.* (2012) showed the production of phenazine-1-carboxylic acid (PCA) and a hitherto unknown conjugate of *L*-glutamine to phenazine-1-carboxylic acid (PCA-Gln). This thesis aims to answer the question why *S. coelicolor* M512 produces PCA-Gln.

The mixed phenazine polyketide biosynthetic gene cluster in *S. tendae* Tü1028 contains a hitherto unknown ABBA PTase. This enzyme was characterized during this work, in order to elucidate its function within the biosynthetic gene cluster and its biochemical properties.

### Chapter 3 - Unusual *N*-prenylation in diazepinomicin biosynthesis

Many aromatic PTases of the NphB/CloQ or DMATS/CymD family have been isolated and biochemically characterized. So far none of them was capable to use farnesyl diphosphate as isoprenoid substrate. In order to expand the substrate range of this enzyme group, a search was conducted for aromatic PTases which might use farnesyl diphosphate as genuine isoprenoid substrate.

# Results and discussion

## Chapter 1 - Evolutionary relationships of microbial aromatic prenyltransferases

Evolutionary Relationships of Microbial Aromatic Prenyltransferases.

**Tobias Bonitz**, Vikram Alva, Orwah Saleh, Andrei N. Lupas, Lutz Heide

This manuscript has originally been published in PLoS ONE 6(11): e27336.
doi:10.1371/journal.pone.0027336
**Received:** 9th September 2011; **Accepted:** 14th October 2011; **Published:** 30th November 2011

**Author contributions:**

**Tobias Bonitz**
- involved in initial hypothesis generation
- involved in planning, establishing and accomplishment of experimental setup
- generation of data (BLAST-searches, CLANS-analysis, HHrepID-searches)
- involved in all data discussions and analyses
- writing of the manuscript
- preparation of all figures

Vikram Alva
- involved in planning, establishing and accomplishment of experimental setup
- generation of data (HHpred-analysis)
- involved in data discussion and analyses
- writing of the manuscript
- generation of figure 3

Orwah Saleh
- manuscript preparation

Andrei N. Lupas
- involved in planning of experimental setup
- involved in data discussion and analyses
- manuscript preparation

Lutz Heide
- supervised the project
- involved in initial hypothesis generation
- involved in all data discussions
- manuscript preparation

My personal work for this manuscript involved the generation of the initial hypothesis that the CloQ/NphB and the DMATS/CymD family of ABBA PTases are homologous despite their low sequence similarity. To prove this hypothesis I performed several sequence searches (BLAST, HHsearch and HHrepID) and sequence analysis (CLANS and secondary structure predictions). From this data the hypothesis could be verified in collaboration with V. Alva and A. N. Lupas who supported Prof. L. Heide and me with the data interpretation. The findings revealed in this study established a classification system for ABBA PTases on basis of their homology and their similarities in structure and function. For this manuscript I wrote major text parts, generated all figures and was responsible of the final version.

## HMM-HMM comparisons of PTases featuring the PT-barrel

The PT-barrel is a novel protein fold that was discovered recently and is found exclusively in microbial secondary metabolic PTases with aromatic substrates. For proteins with the PT-barrel fold, the name ABBA PTases has been suggested previously (Tello *et al.*, 2008), owing to the αββα succession of the secondary structure elements in the polypeptide chain which results in the characteristic antiparallel orientation of the β-sheets in the barrel (Fig. 2).

While members of the CloQ/NphB and the DMATS/CymD families display the PT-barrel fold (the structures of NphB and DMATS align at a RMSD of 3.97 Å over 290 aligned residues), they show very little sequence identity (<15%). Nevertheless many instances are known where proteins with such low sequence identity belong to the same superfamily (*e.g.* ubiquitins (Bayer *et al.*, 1998)). We therefore used HHsearch to investigate the evolutionary origins of these two families. Biochemically characterized members of (i) the PTases with similarity to NphB/CloQ, (ii) the fungal indole PTases and (iii) the bacterial indole PTases were selected as representatives for HHsearch analysis. As expected, HHsearch assigns a 100% probability of homology to all pairwise matches within each of these three groups (Fig. 6). We also detected matches between the fungal indole PTases (*e.g.* DMATS) and the bacterial indole PTases (*e.g.* CymD) at a probability of 100%, confirming their evolutionary relatedness. Likewise, we also obtained probability values of 100% for connections between the bacterial phenol PTases NphB and CloQ, and the fungal phenol PTases $Ptf_{At}$, $Ptf_{Bf}$ and $Ptf_{Sc}$. Strikingly we obtained several matches between the CloQ/NphB and the DMATS/CymD families at high probabilities (50%-75%). It has been previously shown that this level of sequence similarity is indicative of common ancestry (Alva *et al.*, 2007; Chaudhuri *et al.*, 2008; Kopec *et al.*, 2010; Remmert *et al.*, 2010). We thus conclude that these two families are homologous.

In the biosynthesis of ubiquinones, menaquinones, plastoquinones and tocopherols, the *C*-prenylation of aromatic substrates is catalyzed by integral membrane proteins with several membrane-spanning helices (Heide, 2009). Similar to the soluble farnesyl diphosphate synthase (FPP synthase) (Poulter, 2006) and the octaprenyl diphosphate synthase IspB (Asai *et al.*, 1994) (Fig. 1), these membrane-bound aromatic PTases show conserved NDxxD motifs for the binding of the isoprenoid substrates in the form of $Mg^{2+}$ complexes.

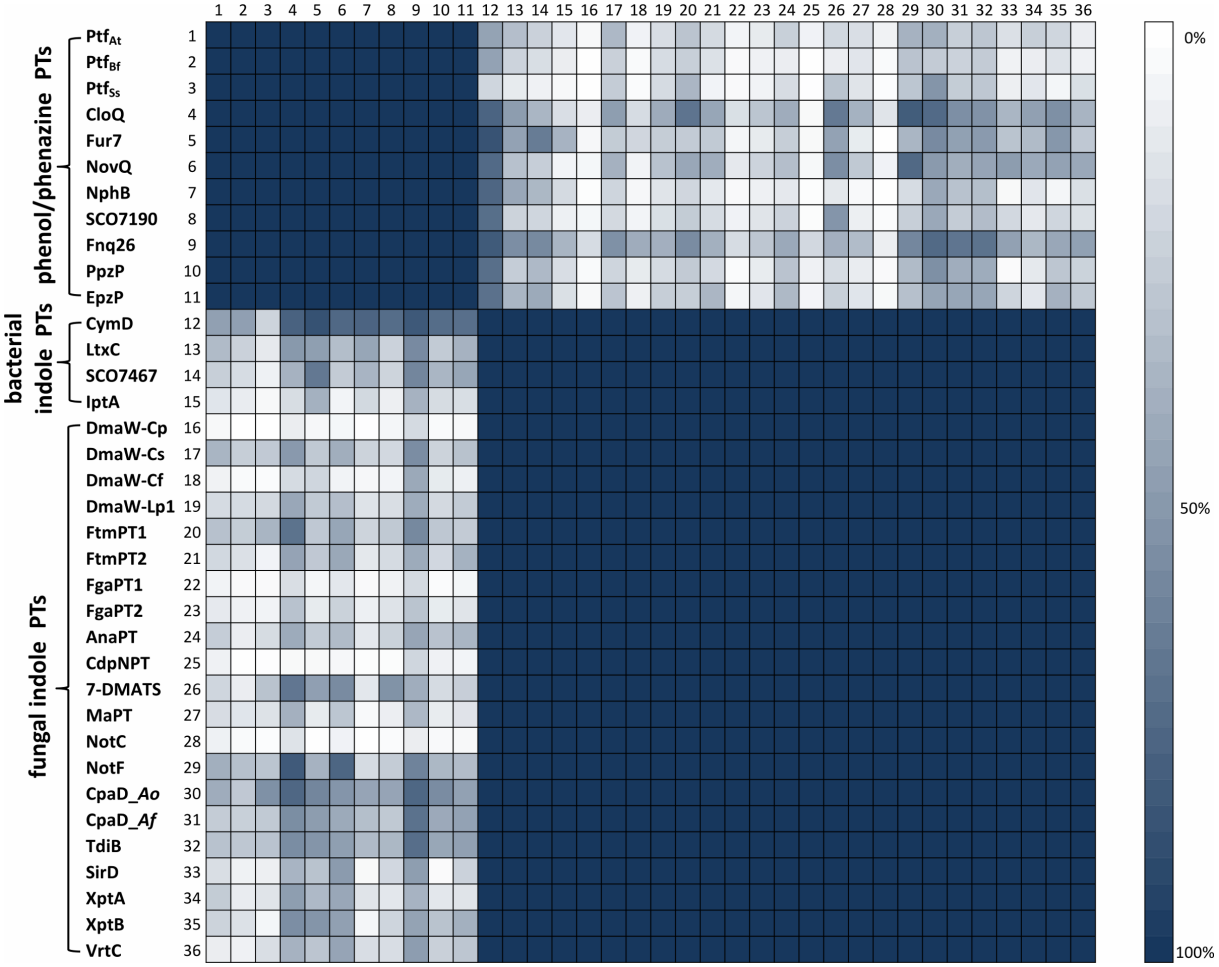



Fig. 6: HHsearch analysis of prenyltransferases with the PT-barrel fold. Pairwise HMM comparisons of 36 biochemically characterized PTases were performed using HHsearch. Group and protein names are shown on the left. Cell color indicates HHsearch probability of the match as depicted in the scale on the right.

In contrast, all aromatic PTases characterized by the PT-barrel fold are soluble enzymes without the NDxxD motifs. As expected, HHsearch detected matches between the membrane-bound aromatic PTases UbiA of ubiquinone biosynthesis, MenA of menaquinone biosynthesis and Str1736 of tocopherol biosynthesis, confirming their homology (data not shown). In contrast, these enzymes did not make any connections to the soluble PTases with the PT-barrel fold.

To check for the existence of possible distant homologs of the aromatic PTases with the PT-barrel fold, we ran HHsearch against a database comprising several complete genomes. The search was seeded with the PTases NphB and DMATS. We did not find matches to proteins outside of the CloQ/NphB and the DMATS/CymD families, indicating that the PT-barrel fold is exclusive to them at this time.

## Detection of sequence repeats in the PT-barrel

The PT-barrel is a toroidal fold, in which five αααββ structural repeats are arranged in a circular fashion to form a closed barrel. While these five repeats are structurally well superimposable with median RMSDs below 2.5 Å, they do not show clear sequence similarity to each other. Therefore, it has remained unclear whether the symmetry displayed by the PT-barrel is a result of five-fold amplification of a single αααββ unit or of structural convergence. If PT-barrels originated by amplification, we might still find residual sequence similarity between their repeats with highly sensitive sequence comparison tools. For this, we used the *de novo* repeat detection method HHrepID, which detects internal sequence symmetries by repeatedly aligning the query HMM with itself. HHrepID has been used successfully to detect highly divergent sequence repeats in several folds including TIM $(\beta\alpha)_8$-barrels (Söding *et al.*, 2006) and outer membrane β-barrels (Remmert *et al.*, 2010). We detected five-fold internal sequence symmetry in both the bacterial and the fungal indole PTases at default settings with a P-value of better than 1E-4. We also found repeats in the phenol/phenazine PTases, albeit at lower detection stringency. In the indole PTases the detected repeats coincide largely with the αααββ structural units, but in the phenol/phenazine PTases the repeats are shorter and coincide only with the ββ hairpins. While we can substantiate a scenario for the origin of indole PTases by amplification based on the presence of residual sequence similarity between their repeats, the repeats of phenol/phenazine PTases are more divergent and a scenario for their origin cannot be established at this time. We note that this range of internal symmetry among members of the same superfamily is not unique to PT-barrels. β-propellers, for instance, display a wide range of internal symmetry, from near-identical to fully diverged, and an origin by amplification has been proposed for them (Chaudhuri *et al.*, 2008).

## Cluster analysis of aromatic PTases

In order to visualize the relationships between the PTases with the PT-barrel fold, we searched the non-redundant protein sequence database at NCBI for homologs of NphB and DMATS and clustered the obtained sequences in CLANS (Frickey *et al.*, 2004). The resulting cluster map (Fig. 7) very clearly shows two distinct clusters that correspond to the phenol/phenazine PTases and the indole PTases.

The two clusters are connected with each other, further confirming the proposed evolutionary relationship between these two enzyme families. No other groups of proteins with similarity to NphB and DMATS were identified by this PSI-BLAST search, showing that the enzymes with PT-barrel fold are not related to other currently known proteins.

The phenol/phenazine PTases (Fig. 7; dark orange) comprise 14 bacterial proteins from the genus *Streptomyces* and 5 fungal proteins from the phylum *Ascomycota.* The cluster analysis did not show a separation of the bacterial and the fungal enzymes within this family, even at higher clustering stringency. In contrast, the indole PTases can be separated into two subclusters, one of which contains all of the 19 bacterial entries, and the other one all of the 186 fungal entries. This separation is already visible in Fig. 7, and becomes very clear at higher clustering stringency.

The family of indole PTases comprises the fungal indole PTases and the bacterial indole PTases, with DMATS and CymD as typical representatives, respectively. It should be noted that the term "indole PTases" is correct for most but not all biochemically investigated members of this family. The exceptions are SirD (NCBI accession AAS92554), which catalyses the *O*-prenylation of the phenolic oxygen of tyrosine in sirodesmin biosynthesis (Zou *et al.*, 2010), VrtC (ADI24928), which *C*- prenylates a phenolic

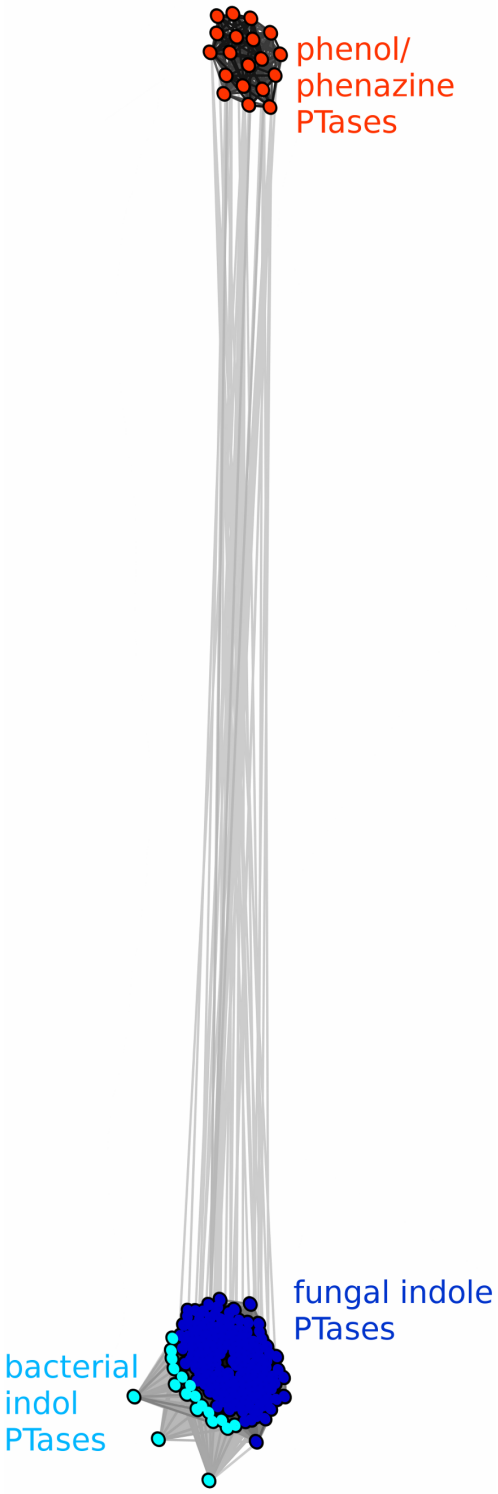



Fig. 7: Cluster map of aromatic prenyltransferases characterized by the PT-barrel fold. Dots represent sequences, line coloring reflects PSI-BLAST P-values; the darker a line, the lower the P-value.

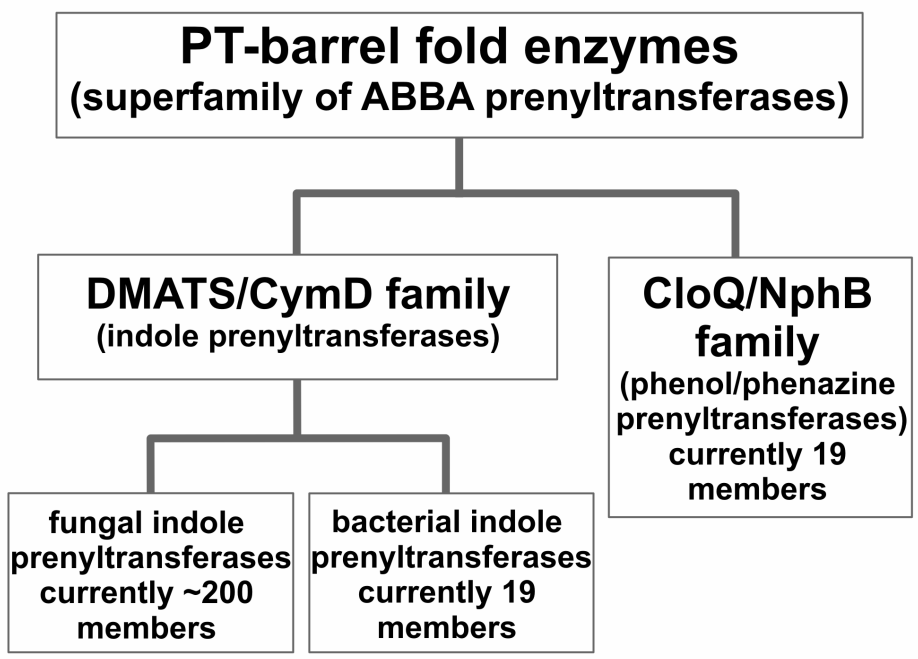



Fig. 8: Classification of prenyltransferases characterized by the PT-barrel fold (= "ABBA prenyltransferases")

substrate related to tetracyclines (Chooi *et al.*, 2010), and TdiB (ABU51603) which catalyses both an indole prenylation and the prenylation of a phenolic moiety during terrequinone biosynthesis A (Balibar *et al.*, 2007).

Our study suggests that all proteins with the PT-barrel fold share a common ancestry and they therefore belong to a single superfamily. As shown in Fig. 8, this superfamily can be divided into two families, *i.e.* the indole PTases and the phenol/phenazine PTases. The state-of-the-art sequence comparison method HHsearch yielded significant matches between these families, indicating a common ancestry.

We also performed a cluster analysis of the membrane-bound aromatic PTases. We searched the non-redundant protein sequence database at NCBI for homologs of the membrane-bound PTases UbiA, MenA and Slr1736 and clustered them in CLANS. As expected, the map (Fig. 9) shows distinct but connected clusters for (i) 4-hydroxybenzoate PTases of ubiquinone biosynthesis, e.g. UbiA of *E. coli* (Melzer *et al.*, 1994), (ii) 1,4-dihydroxy-2-naphthoate-3-prenyltransferases of menaquinone biosynthesis, *e.g.* MenA of *E. coli* (Suvarna *et al.*, 1998), and (iii) homogentisate PTases of plastoquinone and tocopherol biosynthesis (Savidge *et al.*, 2002). In addition, this cluster analysis revealed further enzymes to be related to the aromatic

PTase of lipoquinone biosynthesis. These include the chlorophyll a synthases and protoheme IX farnesyltransferases, both of which attach phytyl or farnesyl moieties to side chains of tetrapyrrole substrates (Saiki *et al.*, 1992; Oster *et al.*, 1997). Another group is formed by the 5-phosphoribose-1-diphosphate:decaprenyl-phosphate 5-phosphoribosyltransferases (DPPRs) which are involved in the biosynthesis of lipids of the bacterial cell wall. The reaction catalyzed by DPPRs is quite different from that catalyzed by aromatic PTases, yet there is obvious sequence similarity between DPPR and UbiA (Huang *et al.*, 2005). A last group of database entries related to membrane-bound aromatic PTases comprises hypothetical proteins, mostly from proteobacteria, which consist of two distinct domains: one similar to hydrolases of the HAD superfamily (Koonin *et al.*, 1994), the other one similar to DPPR or UbiA. The function of these proteins is, to our knowledge, unknown.

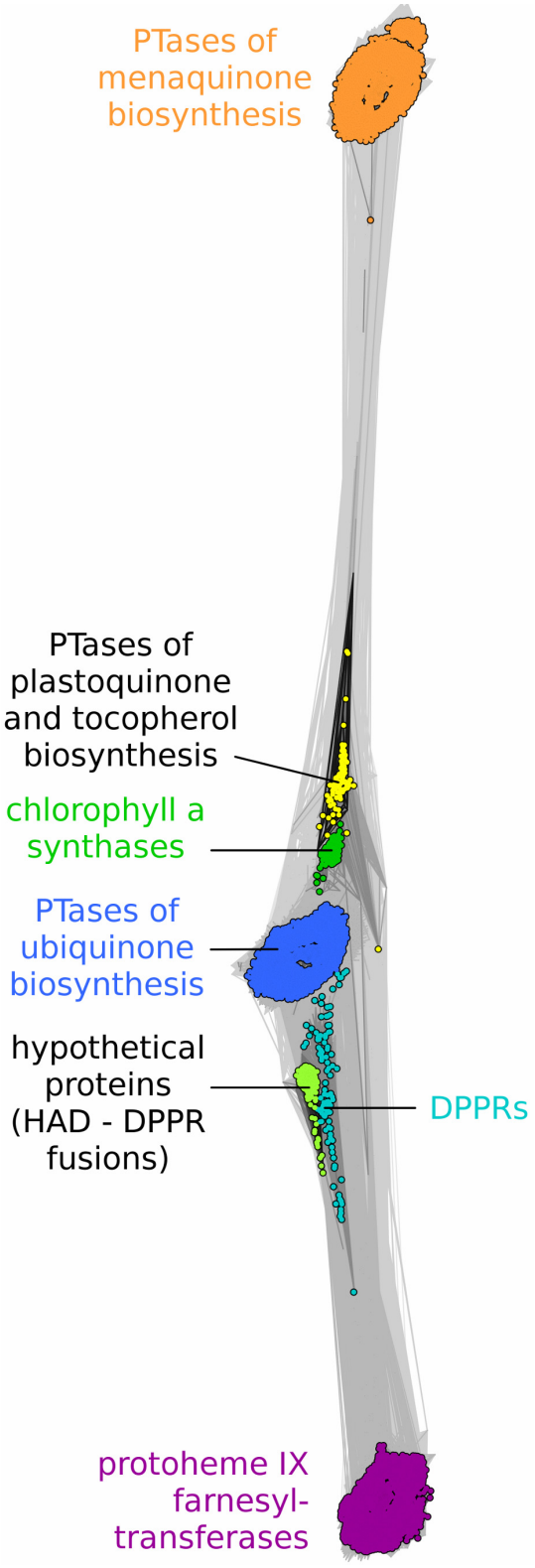



Fig. 9: Cluster map of membrane-bound aromatic prenyltransferases. Dots represent sequences, line coloring reflects PSI-BLAST P-values; the darker a line, the lower the P-value.

As expected, HHsearch did not indicate a relationship between the soluble aromatic PTases with the PT-

barrel fold, such as NphB or DMATS, and the membrane-bound PTases, such as UbiA of ubiquinone biosynthesis. Therefore, two independent solutions have evolved in nature to solve the biochemical problem of catalyzing an aromatic prenylation reaction in an aqueous environment. The indispensable shielding of the reactive allylic cation, generated from the prenyl diphosphate substrate, is achieved by a barrel of antiparallel β-sheets in case of the ABBA PTases, and by a deep lipophilic pocket between the transmembrane helices in case of the membrane-bound aromatic PTases.

All PTases characterized by the PT-barrel fold belong to secondary metabolic pathways; no primary metabolic enzyme with this fold has been discovered yet. In contrast, most of the membrane-bound aromatic PTases are involved in primary metabolism. However, a few enzymes of this group are involved in secondary metabolism. The bacterial PTase AqgD catalyzes the *O*-prenylation of the secondary metabolite alkyl-methoxyhydroquinone (Awakawa *et al.*, 2011), and the fungal PTase XP_751272 is involved in the biosynthesis of pyripyropene A (Itoh *et al.*, 2010); both show similarity to UbiA of ubiquinone biosynthesis. The bacterial putative PTase BAD07390 is likely to be involved in the biosynthesis of the secondary metabolite BE-40644 (Dairi, 2005); it shows similarity to MenA of menaquinone biosynthesis. The recently characterized bacterial PTase AuaA is involved in the biosynthesis of Auracin D (Stec *et al.*, 2011) and is located inbetween the UbiA and protoheme IX farnesyltransferase clusters in the map depicted in Fig. 9.

During our cluster analysis of membrane-bound aromatic PTases, we noticed that many bacterial and fungal genomes contain not one but several genes for (biochemically not yet characterized) proteins annotated as "UbiA prenyltransferase" or similar. For instance, the genome of *Salinispora tropica* contains two genes annotated as "4-hydroxybenzoate polyprenyltransferase" (YP_001160901) and "UbiA PTase" (YP_001161073). The genome of *Catenulispora acidiphila* likewise contains a gene annotated as "4-hydroxybenzoate polyprenyltransferase" (YP_003118736) and in addition three "UbiA prenyltransferase" genes (YP_003112865, YP_003115669 and YP_003116365). Both organisms are Gram-positive bacteria which are believed to not produce ubiquinones (Nowicka *et al.*, 2010). It remains to be shown whether such UbiA-like enzymes may be involved in the biosynthesis of secondary metabolites. In plants several PTases with homology to enzymes of ubiquinone and

plastoquinone biosynthesis have recently been shown to be involved in the biosynthesis of important secondary metabolites (Yazaki *et al.*, 2009).

Both the membrane-bound and the soluble aromatic PTases show remarkable promiscuity for their aromatic substrates and have been used for the chemoenzymatic synthesis of new prenylated aromatic compounds (Kumano *et al.*, 2008; Macone *et al.*, 2009; Ozaki *et al.*, 2009; Xiao *et al.*, 2009; Koeduka *et al.*, 2011; Shindo *et al.*, 2011). Protein engineering has allowed altering the substrate specificity of indole PTases (Li, 2009a; Steffan *et al.*, 2009). Therefore, these PTases may represent promising tools for biotechnological and pharmaceutical research.

# Chapter 2 – A new resistance mechanism of *S. coelicolor* against phenazines and biochemistry of the prenyltransferase $Ptf_{St}$

Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines

Orwah Saleh*, **Tobias Bonitz***, Katrin Flinspach, Andreas Kulik, Nadja Burkard, Agnes Mühlenweg, Andreas Vente, Stefan Polnick, Michael Lämmerhofer, Bertolt Gust, Hans-Peter Fiedler and Lutz Heide

* both authors contributed equally

This manuscript has originally been published in Med. Chem. Commun., 2012, 3, 1009–1019.
doi: 10.1039/c2md20045g

**Received:** 15th February 2012; **Accepted:** 30th March 2012; **Published:** 4th April 2012

**Author contributions:**

Orwah Saleh
- involved in initial hypothesis generation
- involved in experimental setup and all data discussions and analyses
- screening of the cosmid banks
- sequencing of cosmids, generation of heterologous expression strain
- cultivation of strains, analysis of production
- introduction of a new promoter and analysis
- structure elucidation of new phenazine derivative PCA-Gln
- writing of the manuscript
- preparation of figures 1, 2, 3 and Table 1

**Tobias Bonitz**
- planning and accomplishment of experimental setup, data analysis and discussions for biochemical investigation of $Ptf_{St}$ and disc diffusion assays of PCA and PCA-Gln
- cultivation of strains, analysis of production
- submission of sequence data to GenBank
- writing of the manuscript and final submission
- preparation of figures 4 and Table 3 and final submission of all figures

Katrin Flinspach
- screening of the cosmid banks

Andreas Kulik
- isolation of new phenazine derivatives

Nadja Burkard
- structure elucidation of new phenazine derivative

Agnes Mühlenweg
- screening of actinomycetes strains for phenazine biosynthesis genes

Andreas Vente
- screening of actinomycetes strains for phenazine biosynthesis genes

Stefan Polnick
- identification of the stereochemical configuration

Michael Lämmerhofer
- identification of the stereochemical configuration

Bertolt Gust
- introduction of a new promoter and analysis

Hans-Peter Fiedler
- isolation of new phenazine derivatives

Lutz Heide
- supervised the project
- involved in initial hypothesis generation
- involved in all data discussions
- manuscript preparation

My personal part for this manuscript involved the experiments that indicate the resistance mechanism of *S. coelicolor* M512 against PCA and the biochemical investigation of the new ABBA PTase $Ptf_{St}$. The finding that PCA-Gln is likely to be a detoxification product of PCA sheds new light on resistance of *Streptomyces coelicolor* against this antibiotic which is produced in soil by pseudomonads, therefore giving *S. coelicolor* a competitive advantage against Pseudomonads. The ABBA PTase $Ptf_{St}$ has similar biochemical properties as NphB, which indicates that the newly discovered gene cluster may produce a secondary metabolite similar to naphterpin in whose biosynthesis NphB is involved. For this manuscript I was responsible of the final version.

## The conjugation of phenazine-1-carboxylic acid to *L*-glutamine is likely to represent a resistance mechanism

Unexpectedly, we found that the expression host *Streptomyces coelicolor* M512 enzymatically modified the compound formed under direction of the heterologously introduced phenazine biosynthetic gene cluster: phenazine-1-carboxylic acid (PCA) was conjugated to the α-amino group of glutamine in form of an amide (PCA-Gln) (Saleh *et al.*, 2012). This compound is a new, previously undescribed phenazine derivative, and its formation is likely to represent a resistance mechanism against the antibiotic effect of PCA.

To test this we compared the antibiotic activity of PCA and of PCA-Gln against *Escherichia coli*, *Bacillus subtilis* and *Streptomyces coelicolor* M512 in disk diffusion assays. Both compounds showed no effect on *E. coli* in the tested concentrations. However, PCA had a strong antibiotic effect against *B. subtilis*, while PCA-Gln had not (Fig. 10a). *S. coelicolor* M512 rapidly conjugates PCA to *L*-glutamine, and correspondingly this strain was resistant to both PCA and PCA-Gln (Fig. 10b). These results suggest that glutamination of PCA may represent a resistance mechanism. Notably, in soil which is the natural habitat of *Streptomyces* strains PCA is produced by *Pseudomonas* strains and can reach growth-inhibitory concentrations (Mavrodi *et al.*, 2012). Therefore, a resistance mechanism against PCA may offer a competitive advantage to a *Streptomyces* strain in nature.

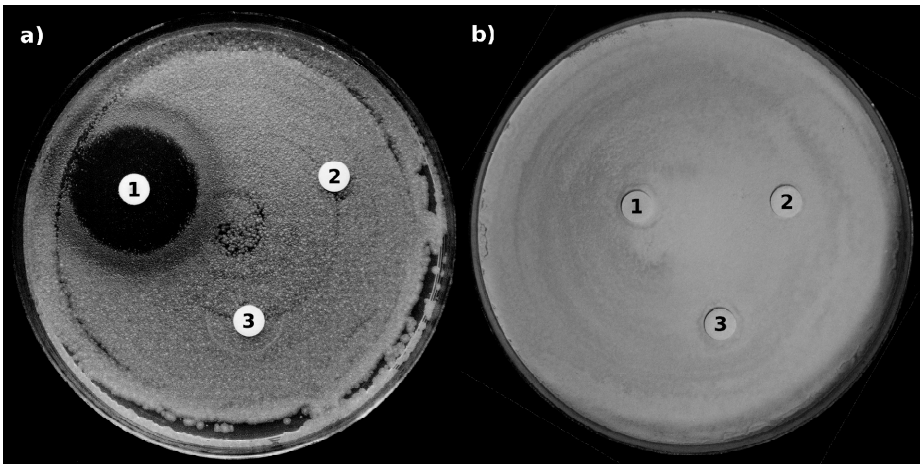
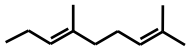
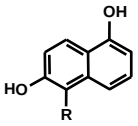
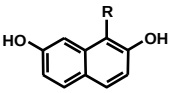
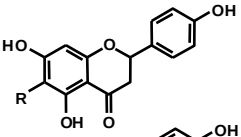
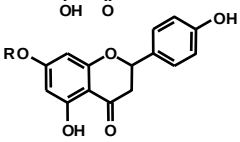
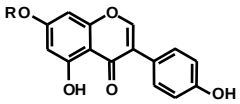
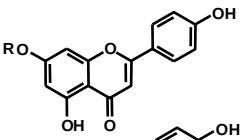
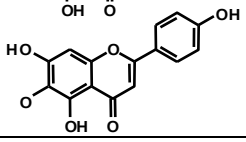



Fig. 10: Antibiotic activity of PCA and of PCA-Gln against (a) *Bacillus subtilis* and (b) *Streptomyces coelicolor* M512. To the paper disks, either, 0.5 µmol PCA (1), or 0.5 µmol PCA-Gln (2) or solvent (3) were applied. In parallel experiments, no growth inhibition on *Streptomyces coelicolor* M512 was exerted by PCA or PCA-Gln in amounts of up to 5 µmol (data not shown).

## Biochemical investigation of the PTase $Ptf_{St}$

The heterologously expressed cosmid from *S. tendae* Tü1028 contained phenazine biosynthesis genes, mevalonate pathway genes and a gene for an aromatic PTase, *$ptf_{St}$*. However no prenylated phenazines could be detected in contrast to a previous study on a gene cluster from *S. anulatus* which contained similar genes (Saleh *et al.*, 2009). We therefore speculated that the PTase gene *$ptf_{St}$* may not code for a phenazine PTase such as PpzP or EpzP (Saleh *et al.*, 2009; Seeger *et al.*, 2011), but for a hydroxynaphthalene PTase such as NphB or Fnq26 (Kuzuyama *et al.*, 2005; Haagen *et al.*, 2007). Phenazine and hydroxynaphthalene PTases are similar in their amino acid sequence, but different in their specificity for the aromatic substrate.

Tab. 1: Prenylation of phenolic compounds under catalysis of $Ptf_{St}$. GPP (1 mM) was used as isoprenoid substrate.
DHN = dihydroxynaphthalene.

| substrate | product | R=  | $k_{cat}$ ($10^{-3}$ $s^{-1}$) |
|---|---|---|---|
| 1,6-DHN | 5-geranyl-1,6-DHN |  | 5.96 |
| 2,7-DHN | 1-geranyl-2,7-DHN |  | 4.96 |
| naringenin | 6-geranyl-naringenin |  | 0.35 |
| | 7-*O*-geranyl-naringenin |  | 0.29 |
| genistein | 7-*O*-geranyl-genistein |  | 0.42 |
| apigenin | 7-*O*-geranyl-apigenin |  | 0.13 |
| | 6-geranyl-apigenin |  | 0.07 |

We therefore expressed $Ptf_{St}$ in *E. coli* as His-tagged protein and purified it by $Ni^{2+}$ affinity chromatography. In contrast to PpzP and EpzP, $Ptf_{St}$ did not show product formation using 5,10-dihydro-PCA and either dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) as substrates. In contrast, prenylated products were readily obtained when $Ptf_{St}$ was incubated with GPP and different phenolic substrates including 1,6-dihydroxynaphthalene (1,6-DHN), 2,7-dihydroxynaphthalene (2,7-DHN) and the flavonoids apigenin, genistein or naringenin (Tab. 1). DMAPP was not accepted as isoprenoid substrate. The $K_m$ values of $Ptf_{St}$ for 1,6-DHN and genistein were determined as 0.36 and 0.16 mM, respectively. Using 1,6-DHN as aromatic substrate, the $K_m$ for GPP was determined as 0.13 mM. In contrast to most other

PTases of the ABBA superfamily (Heide, 2009; Bonitz *et al.*, 2011), $Ptf_{St}$ requires the presence of $Mg^{2+}$ ions for its catalytic activity. These biochemical characteristics (substrate specificity, $K_m$ values, prenylation pattern and magnesium dependency) are very similar to those of NphB, which is involved in the biosynthesis of the prenylated naphthalene derivative naphterpin (Kuzuyama *et al.*, 2005; Kumano *et al.*, 2008). This indicates that $Ptf_{St}$ may be involved in the biosynthesis of a prenylated naphthalene derivative, most likely in the prenylation of a polyketide formed from 1,3,5,8-tetrahydroxynaphthalene (THN), the presumed product of the type III polyketide synthase Orf34 (Fig. 3) (Funa *et al.*, 2002). A mixed biosynthetic gene cluster for prenylated THN derivatives and phenazines was also found in *S. cinnamonensis*, and many of the genes found in the presently described gene cluster of *S. tendae* Tü1028 have orthologs in the cluster of *S. cinnamonensis*. In contrast to the cluster in *S. cinnamonensis*, however, the cluster in *S. tendae* Tü1028 remained silent under many different culture conditions and the structure of the possibly encoded prenylated naphthalene remains unknown.

# Chapter 3 - Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily

Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily.

**Tobias Bonitz**, Florian Zubeil, Stephanie Grond, and Lutz Heide

submitted

**Author contributions:**

**Tobias Bonitz**
- involved in initial hypothesis generation; planning, establishing and accomplishment of experimental setup, and all data discussions and analyses
- writing of the manuscript
- preparation of all figures

Florian Zubeil
- structural elucidation of enzymatic products (MS and NMR analysis)
- writing of the manuscript

Stefanie Grond
- involved in planning of experimental setup
- manuscript preparation

Lutz Heide
- supervised the project
- involved in initial hypothesis generation
- involved in all data discussions
- manuscript preparation

My personal work for this manuscript involved the performance of all the experimental work as well as data analyses (except for the high resolution MS and NMR). DzmP is the first ABBA PTase which accepts farnesyl diphosphate as substrate, and which prenylates an amide nitrogen. DzmP therefore extends the substrate range of ABBA PTases for isoprenoid as well as aromatic substrates. For this manuscript I wrote major text parts, generated all figures and was in charge of the final version.

## Identification and cloning of two putative prenyltransferases from *Micromonospora* sp. RV115 and *Streptomyces griseoflavus* Tü4000

Recently, diazepinomicin was isolated from the marine sponge-associated strain *Micromonospora* sp. RV115. The structure of this compound was unequivocally confirmed by one- and two-dimensional NMR studies (Abdelmohsen *et al.*, 2012). In the diazepinomicin gene cluster of *Micromonospora* strain 046Eco-11 identified by McAlpine *et al.* (2008), the putative PTase gene *orf11* is flanked by the 3-Hydroxy-3-Methylglutaryl-Coenzym-A synthase gene *orf10*, and by *orf12* coding for a putative sensor protein of a two-component regulatory system. We speculated that the gene clusters in both *Micromonospora* strains are very similar, and therefore we designed primers for the 3' terminus of *orf10* and the 5' terminus of *orf12*. Using genomic DNA of *Micromonospora* sp. RV115 as template, PCR readily gave a product of the expected size. Sequencing showed a coding sequence with very high similarity to *orf11* from *Micromonospora* strain 046Eco-11 (96.9% identity on the amino acid level). The *orf11* orthologue from *Micromonospora* sp. RV115 was termed *dzmP* (NCBI accession KC866371).

BLAST searches revealed in the genome of *Streptomyces griseoflavus* Tü4000 a gene termed *ssrg_00986* which is currently annotated as conserved hypothetical protein (NCBI accession ZP_07309813). This gene showed 61.4% identity (amino acid level) to *dzmP*. Inspection of the genes in the vicinity of *ssrg_00986* in the *S. griseoflavus* Tü4000 genome showed a gene cluster with high similarity to the diazepinomicin gene cluster from *Micromonospora* strain 046Eco-11 (Fig. 11). This *S. griseoflavus* Tü4000 cluster contains orthologs of all genes from which an essential function in diazepinomicin biosynthesis has been suggested (McAlpine *et al.*, 2008).

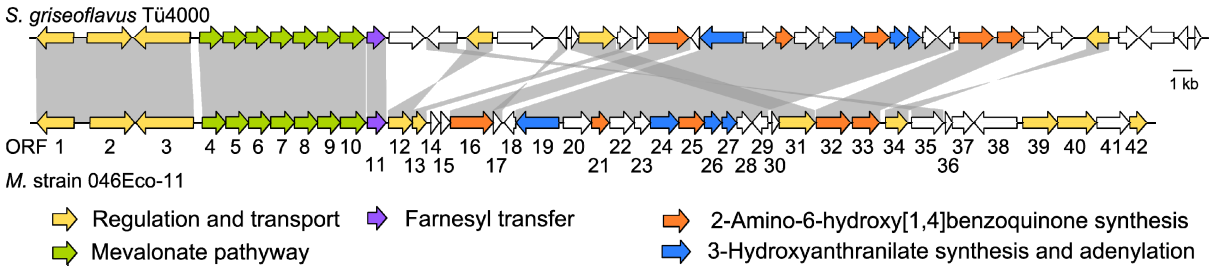



Fig. 11: Comparison of the biosynthetic gene cluster of diazepinomicin in *Micromonospora* strain 046Eco-11 with the newly identified gene cluster in *Streptomyces griseoflavus* Tü4000. Homologous genes are connected by gray areas. The numbering and the suggested function of genes of *Micromonospora* strain 046Eco-11 are adapted from McAlpine *et al.* (2008).

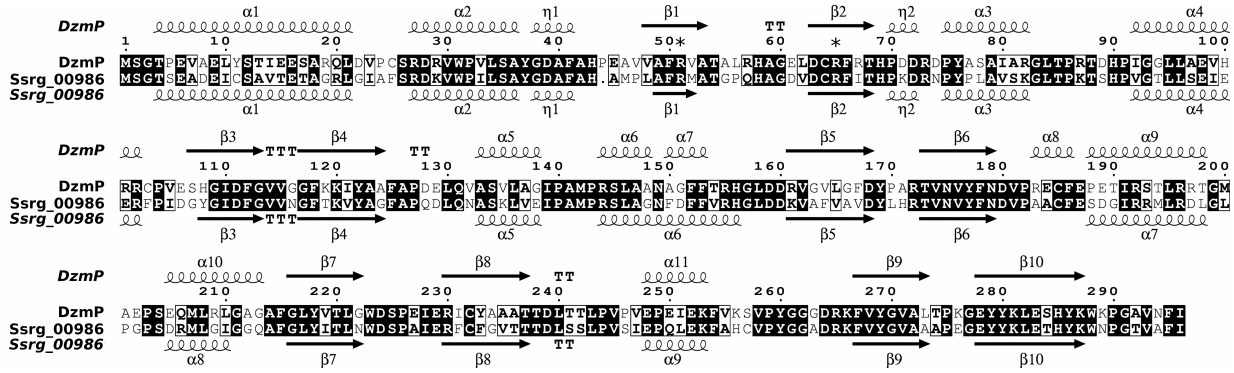



Fig. 12: Amino acid alignment und secondary structure prediction for DzmP and Ssrg_00986 visualized by ESPript (Gouet *et al.*, 1999). Secondary structure elements are: α, α helices; η, $3_{10}$ helices; β, β strands; TT, strict β turns. Strict sequence identity is shown by a *black box* with *white characters*, and similarity is shown by *bold characters* in a *black frame*. The position of the two arginine residues typical for $Mg^{2+}$-independent ABBA prenyltransferases are indicated by asterisks.

However, cultivation of *S. griseoflavus* Tü4000 in HI medium (McAlpine *et al.*, 2008) and Bennett's broth (Abdelmohsen *et al.*, 2012) followed by LC-MS analysis did not show any diazepinomicin production, suggesting that the gene cluster may be silent under the conditions employed. Nevertheless, we amplified the putative PTase gene *ssrg_00986* from genomic DNA of *S. griseoflavus* Tü4000. The correct DNA sequence was confirmed, and both this gene and *dzmP* were cloned into an expression vector for expression as N-terminally His-tagged proteins.

## Bioinformatic sequence analysis of DzmP and Ssrg_00986

The genes *dzmP* and *ssrg_00986* code for proteins of 296 and 295 amino acids, respectively. Secondary structure prediction shows for both gene products the five-fold ααββ repeat which is typical for the ABBA PTases (Fig. 12). A phylogenetic analysis (Fig. 13) places DzmP, Orf11 and Ssrg_00986 into a new, separate branch of the previously described family of phenol / phenazine PTases (Bonitz *et al.*, 2011). DzmP and its orthologs are quite similar to the hydroxynaphthalene PTases NphB (Kumano *et al.*, 2008) and Fnq26 (Haagen *et al.*, 2007), and to the phenazine PTases PpzP (Saleh *et al.*, 2009) and EpzP (Seeger *et al.*, 2011; Zocher *et al.*, 2012) (average sequence identity 43%). While this study was in progress, a further gene cluster with close similarity to the diazepinomicin cluster was deposited in GenBank by the group of Zhongjun Qin from the Shanghai Institutes for Biological Sciences.

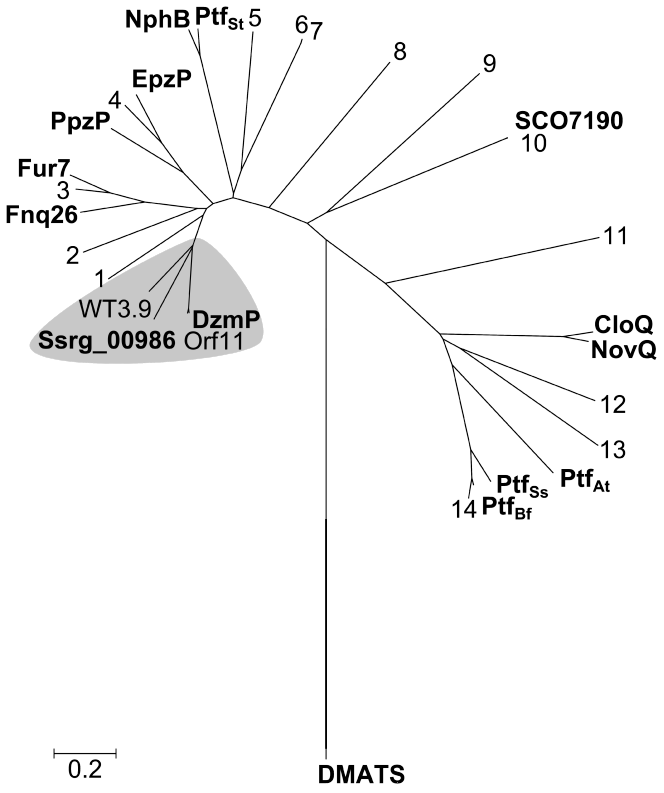



Fig. 13: Evolutionary tree of aromatic prenyltransferases of the CloQ/NphB family (= phenol / phenazine prenyltransferases, (Bonitz *et al.*, 2011; Seeger *et al.*, 2011)). The branch highlighted in grey represents the new prenyltransferases investigated in this study. The evolutionary tree was generated with MEGA4 using the default parameters for pairwise (multiple) alignment. Phylogenetic reconstruction was carried out using the neighbor-joining method. Names of biochemically investigated enzymes (Bonitz *et al.*, 2011) are shown in bold, and further uncharacterized NCBI database entries are ZP_10450727 (1), ABS50461 and ABS50489 (2), CCK32327 (3), AEW22941 (4), CAL34106 (5), ABS50462 (6), ABS50490 (7), YP_005045476 (8), YP_006808349 (9), ZP_06526769 (10), ZP_09171437 (11), XP_002847323 (12), XP_002143864 (13), CCD48995 (14).

This cluster (NCBI accession JQ432566) was obtained from a strain termed *Streptomyces* sp. WT3. It contains a gene named WT3.9 which codes for a 296 aa protein with close similarity to DzmP and Ssrg_00986 (Fig. 13). No further close orthologs of DzmP, and no further gene clusters with close similarity to the diazepinomicin cluster, are currently deposited in GenBank.

The dibenzodiazepinone structure has been described as unique in nature (McAlpine *et al.*, 2008), and has so far only been found in the genus *Micromonospora*. The discovery of gene clusters with similarity to the diazepinomicin cluster in *Streptomyces griseoflavus* Tü4000 (Fig. 11), and in *Streptomyces* sp. WT3

(NCBI accession JQ432566) may indicate that similar compounds can be found also in *Streptomyces* strains.

DzmP and Ssrg_00986 (Fig. 13), as well as Orf11 and Wt3.9, contain arginine residues in position 51 and 64. X-ray crystallographic studies (Metzger *et al.*, 2010) as well as modeling studies (Tello *et al.*, 2008) have suggested that these residues are essential for the cofactor-independent binding of the α-phosphate of the isoprenoid substrate, and therefore are characteristic for ABBA PTases which are independent of $Mg^{2+}$. In contrast, $Mg^{2+}$-dependent ABBA PTases such as NphB (Kuzuyama *et al.*, 2005) and $Ptf_{St}$ (Saleh *et al.*, 2012) contain serine residues in these positions. DzmP and Ssrg_0986 are therefore expected to catalyze a prenyl transfer independent of the presence of magnesium ions.

## Expression and purification of DzmP and Ssrg_00986

DzmP and Ssrg_00986 were expressed as His-tagged proteins in *E. coli*, readily yielding soluble proteins which were purified by $Ni^{2+}$ affinity chromatography. From 1 L of culture 9.5 mg DzmP and 7.5 mg Ssrg_00986 were obtained.

## Prenyltransferase activity of DzmP and Ssrg_00986

In the hypothetical biosynthetic pathway of diazepinomicin suggested by McAlpine *et al.* (2008), the farnesylation of 4,6,8-trihydroxy-dibenzodiazepinone (Fig. 5) is suggested as the final step. Commercially, only non-hydroxylated dibenzodiazepinone is available, and we therefore tested this compound (Fig. 14) as substrate.

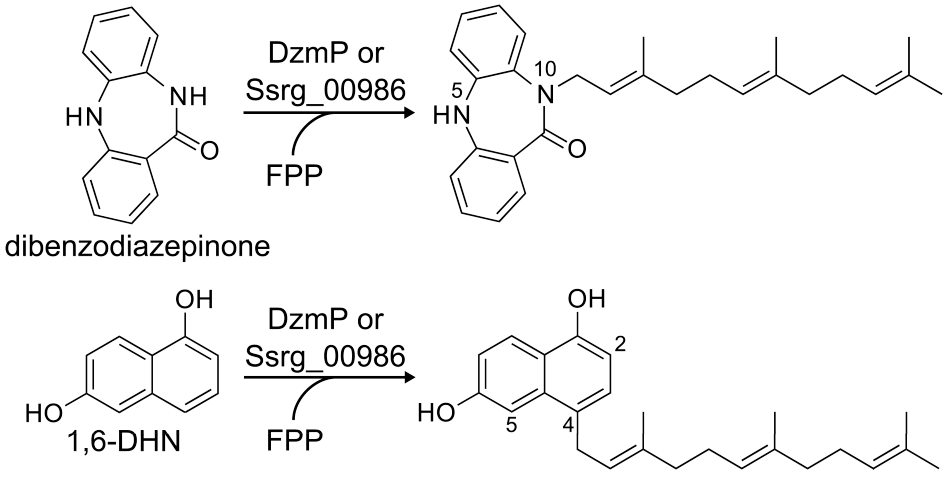



Fig. 14: Reactions catalyzed by DzmP and Ssrg_00986. FPP, farnesyl diphosphate; 1,6-DHN, 1,6-dihydroxynaphthalene.

Furthermore, 1,6-dihydroxynaphthalene (1,6-DHN) was tested, since this compound is known to be accepted by NphB (Kumano *et al.*, 2008), a PTase with similarity to DzmP (Fig. 13). Using farnesyl diphosphate (FPP) as isoprenoid substrate, an enzymatic prenylation of both aromatic compounds by DzmP and, with lower activity, by Ssrg_00986, was readily observed by HPLC-UV and HPLC-MS. Geranyl diphosphate (GPP) was converted with much lower reaction velocity, and therefore the investigations were continued with FPP. Enzymatic product formation showed linear dependence on time for at least 30 min, and linear dependence on protein amount at least up to 12 µM. The addition of 100 mM NaCl increased activity by 10%, whereas the addition of glycerol had no effect. Product formation was readily detectable in the absence of magnesium ions, but the addition of 2 mM $Mg^{2+}$ increased the activity of DzmP approximately 1.5 fold and the activity of Ssrg_00986 approximately 3 fold compared to assays without $Mg^{2+}$. Addition of EDTA (1 mM) did not influence the activity. Maximal product formation for DzmP was observed at pH 8.0, with half-maximal values at pH 5.8 and 10.3. Ssrg_00986 showed maximal activity at pH 7.5, with half-maximal values at pH 6.3 and 8.2.

### Structural identification of the farnesylation product of dibenzodiazepinone

Prenylation of dibenzodiazepinone by an ABBA PTase may occur at many different positions of the molecule. Each of the two aromatic rings (Fig. 14) offers four unsubstituted carbons for *C*-prenylation, in a reaction which would resemble the prenylation of the indole nucleus of *L*-tryptophan by different indole PTases (Li, 2009a; Li, 2009b) or the *C*-prenylation of a dihydrophenazine derivative by PpzP and EpzP (Saleh *et al.*, 2009; Zocher *et al.*, 2012). Notably, PpzP and EpzP show close similarity to DzmP (Fig. 13). However, a few ABBA PTases catalyze *N*-prenylations (Yin *et al.*, 2007; Grundmann *et al.*, 2008; Schultz *et al.*, 2010; Schuller *et al.*, 2012). In the dibenzodiazepinone molecule the nitrogen atom which is chemically most reactive for alkylation reactions is N-5 (Fig. 14). In contrast the amide nitrogen (N-10) is expected to be much less reactive, and enzymatic prenylations of an amide nitrogen are very unusual.

Prenylation of dibenzodiazepinone with FPP under catalysis of DzmP yielded a single product which showed the mass of a mono-farnesylated product. In order to

determine the substitution position, the assay was scaled up to 20 ml and the enzymatic product was purified on preparative scale. By APCI-HRMS, the molecular formula of the reaction product was deduced to be $C_{28}H_{35}N_2O$ ([M+H$^+$], *m/z* calc. 415.27439, found 415.27475, Δ 0.9 ppm). MS-MS showed a fragment $C_{14}H_{11}N_2O$ ([M$^+$], *m/z* calc. 223.08659, found 223.08711, Δ 2.3 ppm) indicating the loss of the farnesyl chain. The position of the farnesylation was unequivocally confirmed by NOESY ($\tau_m$ = 1 s). NOESY signals of H-1' ($\delta$ = 4.48 ppm) and H-9 ($\delta$ = 7.26 ppm), H-5 ($\delta$ = 7.82 ppm) and H-6 ($\delta$ = 7.10 ppm), H-5 and H-4 ($\delta$ = 7.02 ppm) as well as a HMBC signal of H-1' and the carbonyl carbon C-11 ($\delta$ = 167.52 ppm) prove the farnesylation of dibenzodiazepinone at the amide nitrogen in position 10 (Fig. 14).

The farnesylated products generated from dibenzodiazepinone under catalysis of DzmP and Ssrg_00986 showed exactly the same retention time under different HPLC conditions, as well as the same mass and fragmentation in HPLC-MS-MS analysis. This indicates that both enzymes catalyze the same reaction.

The prenylation of an amide nitrogen has not previously been described from an enzyme of the ABBA PTase superfamily. Prenylated amide nitrogens are very rarely found in nature. Among the few exceptions are *N*-prenylated xanthine derivatives which have been described from plants (Auzi *et al.*, 1994), as well as some protozoan secondary metabolites (Kikuchi *et al.*, 2010).

The keto group of the dibenzodiazepinone is subject to a keto-enol tautomerism. Recent X-ray crystallographic investigations suggest that the keto (= amino-oxo) tautomer is the dominant form, compared to the enol (= hydroxyimine) form (Keller *et al.*, 2012). Only the keto form offers a free electron pair at the amide nitrogen, which may be required for the alkylation of this heteroatom. Nevertheless, the reactivity of an amide nitrogen for alkylation reactions is low, making the prenylation catalyzed by DzmP a quite unusual enzymatic reaction.

Benzodiazepines like dibenzodiazepinone form a new class of prenyl acceptor substrates of ABBA PTases. The discovery of DzmP shows that the substrate range of the previously described phenol / phenazine PTase family extends beyond these compound classes, and may be further expanded when other currently uncharacterized GenBank entries with similarity to this family (Fig. 13) are investigated.

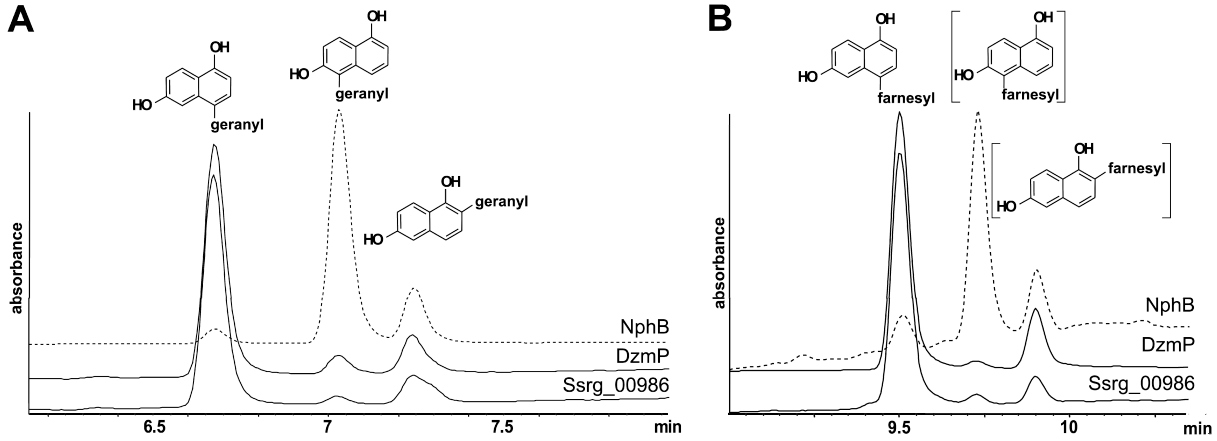



Fig. 15: Geranylation (A) or farnesylation (B) of 1,6-DHN under catalysis of NphB, DzmP and Ssrg_00986. Reaction products were analyzed by HPLC with UV detection at 286 nm. Absorbance was scaled differently for each enzyme in order to improve visibility of the product peaks.

## Structural identification of the major farnesylation product of 1,6-dihydroxynaphthalene

The reaction of 1,6-DHN with FPP under catalysis of DzmP yielded one major product (Fig. 15B; Rt = 9.5 min) and two minor products (Rt = 9.7 min and 9.9 min), each showing the mass of a monofarnesylated 1,6-DHN. A preparative scale assay was carried out and the major product was isolated for structural elucidation.

The molecular formula of the reaction product was deduced to be $C_{25}H_{33}O_2$ ([M+H$^+$], calc. 365.24751, found 365.24717, Δ 0.9 ppm) by HPLC-ESI-HRMS. The fragment $C_{11}H_9O_2$ ([M$^+$], calc. 173.05971, found 173.05969, Δ 0.1 ppm) indicated the loss of the farnesyl chain. The position of the farnesylation of 1,6-DHN was determined using unidimensional ($^1$H NMR, $^{13}$C NMR) and multidimensional ($^1$H-$^1$H COSY, $^1$H HSQC and $^1$H HMBC) NMR spectroscopy, revealing coupling patterns of the aromatic protons in $^1$H NMR and unambiguous COSY-Signals. The coupling pattern indicates H-5 ($\delta$ = 7.20 ppm; d, 2.4 Hz) to be in *meta* position of H-7 ($\delta$ = 7.03 ppm; dd, 2.4 and 9.0 Hz) and H-8 ($\delta$ = 9.02 ppm; d, 9.0 Hz) to be in *ortho* position to H-7 as well as H-2 ($\delta$ = 6.58 ppm; d, 7.6 Hz) to be in *ortho* position of H-3 ($\delta$ = 7.07 ppm; d, 7.6 Hz). Because of the characteristic chemical shifts of H-2 and H-8, and due to the close similarity to the published NMR data of 4-geranyl-1,6-DHN (Kumano *et al.*, 2008), the position of the farnesylation could be deduced to be at C-4 (Fig. 14). The products formed by Ssrg_00986 from the same substrates showed the same retention time as the DzmP products in different HPLC systems, and showed the

same mass and fragmentation in HPLC-MS-MS analysis. This suggested that the products from both enzymes are identical.

### Prenylation of further aromatic substrates

In order to investigate the promiscuity of DzmP for different aromatic substrates, the enzyme was incubated with FPP and either 2,7-dihydroxynaphthalene (2,7-DHN), 5,10-dihydrophenazine-1-carboxylic acid (dihydro-PCA), flaviolin (=2,5,7-trihydroxy-1,4-naphthoquinone) and the isoflavonoid genistein. These compounds have been described to be accepted by ABBA PTases with sequence similarity to DzmP, *e.g.* by NphB (Kumano *et al.*, 2008), PpzP (Saleh *et al.*, 2009) or Fnq26 (Haagen *et al.*, 2007). As determined by HPLC-UV and HPLC-MS-MS, all of these four compounds were converted to mono-farnesylated products, with turn-over rates of approximately 0.57, 0.34, 0.21 and 0.11 $s^{-1} \times 10^{-3}$, respectively. 2,7-DHN and flaviolin yielded a single product, genistein yielded two, and in the prenylation of dihydro-PCA the major product was accompanied by four side products of the same mass, suggesting that farnesylation may occur at different positions of the molecule.

### Comparison of the prenylation reactions of 1,6-DHN catalyzed by NphB, DzmP and Ssrg_00986

NphB catalyzes the geranylation of a (so far unknown) hydroxynaphthalene substrate in the biosynthesis of naphterpin (Kumano *et al.*, 2008). Using the artificial substrate 1,6-DHN, it preferentially prenylates the 5-position. In clear contrast, we found that DzmP preferentially farnesylates the 4-position of 1,6-DHN (see above). This difference prompted us to express and purify NphB following the same procedure as for DzmP and Ssrg_00986, and to compare the reaction products of NphB, DzmP and Ssrg_00986 upon incubation with 1,6-DHN and either GPP or FPP.

As expected, NphB preferentially accepted GPP as substrate; FPP was converted 30 times more slowly. In contrast, DzmP preferentially accepted FPP, and GPP was converted 8 times more slowly. As published previously, NphB forms three geranylated products from 1,6-DHN (Fig. 15A). The major product has been identified as 5-geranyl-1,6-DHN, with 2-geranyl-1,6-DHN as the second most abundant and 4-geranyl-1,6-DHN as the least abundant product (Kumano *et al.*, 2008). As shown in Fig. 15A, also DzmP forms three products from GPP and 1,6-

DHN, and these showed the same HPLC retention time, and the same mass and fragmentation pattern, as the products formed by NphB. In case of DzmP the peak corresponding to 4-geranyl-1,6-DHN is the dominant product, and 5-geranyl-1,6-DHN is the least abundant product. A similar formation of three products is observed when FPP is used as substrate (Fig. 15B). 4-farnesyl-1,6-DHN is the major product formed by DzmP, while this compound is the least abundant product formed by NphB. Therefore, NphB and DzmP show a different regioselectivity in the prenylation of 1,6-DHN. The product spectrum of Ssrg_00986, both with GPP and FPP, was found to be very similar to that of DzmP (Fig. 15A and B), confirming the close similarity of the reaction catalyzed by both enzymes.

**Kinetic investigations of the prenylation of 1,6-DHN and dibenzodiazepinone by DzmP and Ssrg_00986**

The reactions of DzmP and Ssrg_00986 with dibenzodiazepinone and 1,6-DHN as aromatic substrates, and with GPP and FPP as isoprenoid substrates, were investigated. Kinetic constants were calculated using nonlinear regression. Depending on the substrates used, a better fit was obtained by using the equations for sigmoidal curve (indicating cooperativity) or for a substrate inhibition kinetic (Cornish-Bowden, 2004), rather than by using the Michaelis-Menten equation. Using 1,6-DHN, calculations were based on the formation of the 4-prenylated product (*i.e.* the major product). Using 1 mM FPP and different concentrations of 1,6-DHN, both DzmP and Ssrg_0986 gave sigmoidal curves. $k_{0.5}$ values for 1,6-DHN were calculated as 18 ± 1 and 96 ± 11 µM, respectively. For DzmP, $k_{cat}$ was determined as 4.5 ± 0.1 $s^{-1} \times 10^{-3}$, comparable to the value of 4.2 ± 0.2 $s^{-1} \times 10^{-3}$ published for the reaction of GPP and 1,6-DHN catalyzed by NphB (Kumano *et al.*, 2008). For Ssrg_00986, $k_{cat}$ was 0.4 ± 0.01 $s^{-1} \times 10^{-3}$.

Using 1 mM FPP and different concentrations of dibenzodiazepinone, substrate inhibition ($k_i$ = 655 ± 213 µM) was observed. The $K_m$ and $k_{cat}$ values of DzmP for dibenzodiazepinone were determined as 133 ± 30 µM and 0.68 ± 0.09 $s^{-1} \times 10^{-3}$, respectively. For Ssrg_00986, reaction velocity was at least 10 times lower, preventing the reliable measurement of kinetic constants.

Using 1 mM 1,6-DHN and different concentrations of FPP, the plot of reaction velocity over substrate concentration could not be adequately fitted to a Michaelis-

Menten, sigmoidal or substrate inhibition kinetic for DzmP. Half-maximal reaction velocity was observed at approximately 20 µM FPP. With GPP as isoprenoid substrate, a sigmoidal curve was obtained, and $K_m$ and $k_{cat}$ were calculated as 4.9 ± 0.9 µM and 0.5 ± 0.01 $s^{-1} \times 10^{-3}$, respectively.

For Ssrg_00986, a precise comparison of the kinetic constants for FPP and GPP was hampered by the low reaction velocity. However, the enzyme did not show a clear preference for FPP: for both isoprenoid substrates, half-maximal reaction velocities were observed at approximately 45 µM, and maximal turn-over rates were approximately 0.5 $s^{-1} \times 10^{-3}$.

DzmP is the first member of the ABBA PTase superfamily which utilizes farnesyl diphosphate (FPP; $C_{15}$) as genuine substrate. All previously discovered members utilize either DMAPP ($C_5$) or GPP ($C_{10}$). ABBA PTases are useful tools for chemoenzymatic synthesis, due to their nature as soluble, stable biocatalysts which is in contrast to the membrane-bound nature of PTases of lipoquinone biosynthesis. Most ABBA PTases are independent from magnesium as cofactor which is desirable since magnesium ions accelerate the non-enzymatic hydrolysis of prenyl diphosphates (Brems *et al.*, 1977). Finally, ABBA PTases have a remarkable promiscuity for different aromatic substrates (Kuzuyama *et al.*, 2005; Tello *et al.*, 2008; Li, 2010). The discovery of DzmP now provides a welcome extension of the isoprenoid substrate range of this superfamily.

The active center of the ABBA PTases is located within the central barrel which is formed by ten antiparallel β strands. Nearly all of the amino acids interacting with the substrates are part of these β strands, creating a spatially restricted environment (Kuzuyama *et al.*, 2005; Metzger *et al.*, 2009; Metzger *et al.*, 2010). After the discovery of CloQ and NphB which utilize DMAPP ($C_5$) or GPP ($C_{10}$) as isoprenoid substrates, respectively (Pojer *et al.*, 2003; Kuzuyama *et al.*, 2005; Metzger *et al.*, 2010), speculations have been offered which structural features of an ABBA PTase determine the chain length specificity for the isoprenoid substrate (Cui *et al.*, 2007; Tello *et al.*, 2008). *E.g.* it has been suggested that R66 and E281 in CloQ form salt bridges which sterically hinder the accommodation of geranyl diphosphate and thereby restrict the isoprenoid substrate to five carbons, *i.e.* to dimethylallyl diphosphate (Tello *et al.*, 2008). Notably, however, the same residues are now found in DzmP in form of R65 and E283, as evident by modeling of both CloQ and DzmP

using the Phyre 2 (Kelley *et al.*, 2009) and the ASC server (Röttig *et al.*, 2010). This contradicts the above hypothesis that these residues restrict the chain length of the isoprenoid substrate. Further X-ray crystallographic investigations and mutagenesis experiments will be required in order to elucidate how the different chain length specificities of ABBA PTases are determined.

The regiospecific farnesylation of N-10 of dibenzodiazepinone by DzmP supports the hypothesis by McAlpine *et al.* (2008) that this *N*-prenylation reaction may be the last step of diazepinomicin biosynthesis (Fig. 5). The suggested genuine substrate, *i.e.* 4,6,8-trihydroxy-dibenzodiazepinone, is likely to be more reactive towards *N*-alkylation due to the OH-groups which may also contribute to the binding of the substrate in the active center. The absence of these groups in the artificial substrate dibenzodiazepinone (Fig. 14) may explain the relatively low catalytic turnover of that compound.

# References

Abdelmohsen, U. R., M. Szesny, E. M. Othman, T. Schirmeister, S. Grond, H. Stopper and U. Hentschel (2012). "Antioxidant and anti-protease activities of diazepinomicin from the sponge-associated *Micromonospora* strain RV115." Mar. Drugs **10**(10): 2208-2221.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic local alignment search tool." J. Mol. Biol. **215**(3): 403-10.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. **25**(17): 3389-402.

Alva, V., M. Ammelburg, J. Söding and A. N. Lupas (2007). "On the origin of the histone fold." BMC Struct. Biol. **7**: 17.

Alva, V., M. Remmert, A. Biegert, A. N. Lupas and J. Söding (2010). "A galaxy of folds." Protein Sci. **19**(1): 124-30.

Asai, K., S. Fujisaki, Y. Nishimura, T. Nishino, K. Okada, T. Nakagawa, M. Kawamukai and H. Matsuda (1994). "The identification of *Escherichia coli ispB* (cel) gene encoding the octaprenyl diphosphate synthase." Biochem. Biophys. Res. Commun. **202**(1): 340-5.

Auzi, A. A., A. I. Gray, T. G. Hartley, R. D. Waigh and P. G. Waterman (1994). "A xanthine alkaloid from the leaves of *Bosistoa floydii*." Phytochemistry **36**(2): 535-536.

Awakawa, T., N. Fujita, M. Hayakawa, Y. Ohnishi and S. Horinouchi (2011). "Characterization of the biosynthesis gene cluster for alkyl-*O*-dihydrogeranyl-methoxyhydroquinones in *Actinoplanes missouriensis*." Chembiochem **12**(3): 439-48.

Bachmann, B. O., J. B. McAlpine, E. Zazopoulos, C. M. Farnet and M. Piraee (2006). Farnesyl dibenzodiazepinone, and processes for its production. United States, Ecopia BioSciences, Inc. US patent 7101872B2.

Balibar, C. J., A. R. Howard-Jones and C. T. Walsh (2007). "Terrequinone A biosynthesis through *L*-tryptophan oxidation, dimerization and bisprenylation." Nat. Chem. Biol. **3**(9): 584-92.

Bayer, P., A. Arndt, S. Metzger, R. Mahajan, F. Melchior, R. Jaenicke and J. Becker (1998). "Structure determination of the small ubiquitin-related modifier SUMO-1." J. Mol. Biol. **280**(2): 275-86.

Bertrand, J. A., G. Auger, E. Fanchon, L. Martin, D. Blanot, J. van Heijenoort and O. Dideberg (1997). "Crystal structure of UDP-*N*-acetylmuramoyl-*L*-alanine:*D*-glutamate ligase from *Escherichia coli*." EMBO J. **16**(12): 3416-25.

Bonitz, T., V. Alva, O. Saleh, A. N. Lupas and L. Heide (2011). "Evolutionary relationships of microbial aromatic prenyltransferases." PLoS ONE **6**(11): e27336.

Botta, B., G. Delle Monache, P. Menendez and A. Boffi (2005). "Novel prenyltransferase enzymes as a tool for flavonoid prenylation." Trends Pharmacol. Sci. **26**(12): 606-8.

Boufaied, N., M. A. Wioland, P. Falardeau and H. Gourdeau (2010). "TLN-4601, a novel anticancer agent, inhibits Ras signaling post Ras prenylation and before MEK activation." Anticancer Drugs **21**(5): 543-52.

Bräuer, L., W. Brandt, D. Schulze, S. Zakharova and L. Wessjohann (2008). "A structural model of the membrane-bound aromatic prenyltransferase UbiA from *E. coli*." Chembiochem **9**(6): 982-92.

Brems, D. N. and H. C. Rilling (1977). "On the mechanism of the prenyltransferase reaction. Metal ion dependent solvolysis of an allylic pyrophosphate." J. Am. Chem. Soc. **99**(25): 8351-2.

Burger, A., B. Brandt, U. Susstrunk, C. J. Thompson and W. Wohlleben (1998). "Analysis of a *Streptomyces coelicolor* A3(2) locus containing the nucleoside diphosphate kinase (ndk) and folylpolyglutamate synthetase (folC) genes." FEMS Microbiol. Lett. **159**(2): 283-91.

Charan, R. D., G. Schlingmann, J. Janso, V. Bernan, X. Feng and G. T. Carter (2004). "Diazepinomicin, a new antimicrobial alkaloid from a marine *Micromonospora* sp." J. Nat. Prod. **67**(8): 1431-1433.

Chaudhuri, I., J. Söding and A. N. Lupas (2008). "Evolution of the beta-propeller fold." Proteins **71**(2): 795-803.

Cheng, H., B.-H. Kim and N. V. Grishin (2008). "MALISAM: a database of structurally analogous motifs in proteins." Nucleic Acids Res. **36**(suppl 1): D211-D217.

Chooi, Y. H., R. Cacho and Y. Tang (2010). "Identification of the viridicatumtoxin and griseofulvin gene clusters from *Penicillium aethiopicum*." Chem. Biol. **17**(5): 483-94.

Copley, R. R. and P. Bork (2000). "Homology among $(betaalpha)_{(8)}$ barrels: implications for the evolution of metabolic pathways." J. Mol. Biol. **303**(4): 627-41.

Cornish-Bowden, A. (2004). Fundamentals of Enzyme Kinetics, Portland Press Ltd.

Cui, G., X. Li and K. M. Merz, Jr. (2007). "Understanding the substrate selectivity and the product regioselectivity of Orf2-catalyzed aromatic prenylations." Biochemistry **46**(5): 1303-11.

Dairi, T. (2005). "Studies on biosynthetic genes and enzymes of isoprenoids produced by actinomycetes." J. Antibiot. (Tokyo) **58**(4): 227-43.

Eddy, S. R. (1998). "Profile hidden Markov models." Bioinformatics **14**(9): 755-63.

Edwards, D. J. and W. H. Gerwick (2004). "Lyngbyatoxin biosynthesis: sequence of biosynthetic gene cluster and identification of a novel aromatic prenyltransferase." J. Am. Chem. Soc. **126**(37): 11432-3.

Feng, X., A. S. Ratnayake, R. D. Charan, J. E. Janso, V. S. Bernan, G. Schlingmann, H. He, M. Tischler, F. E. Koehn and G. T. Carter (2009). "Probing natural product biosynthetic pathways using Fourier transform ion cyclotron resonance mass spectrometry." Bioorg. Med. Chem. **17**(6): 2154-61.

Frickey, T. and A. Lupas (2004). "CLANS: a Java application for visualizing protein families based on pairwise similarity." Bioinformatics **20**(18): 3702-4.

Funa, N., Y. Ohnishi, Y. Ebizuka and S. Horinouchi (2002). "Properties and substrate specificity of RppA, a chalcone synthase-related polyketide synthase in *Streptomyces griseus*." J. Biol. Chem. **277**(7): 4628-35.

Gerratana, B. (2010). "Biosynthesis, synthesis, and biological activities of pyrrolobenzodiazepines." Med. Res. Rev. **32**(2): 254-93.

Gouet, P., E. Courcelle, D. I. Stuart and F. Métoz (1999). "ESPript: analysis of multiple sequence alignments in PostScript." Bioinformatics **15**(4): 305-308.

Gourdeau, H., J. B. McAlpine, M. Ranger, B. Simard, F. Berger, F. Beaudry, C. M. Farnet and P. Falardeau (2008). "Identification, characterization and potent antitumor activity of ECO-4601, a novel peripheral benzodiazepine receptor ligand." Cancer Chemother. Pharmacol. **61**(6): 911-21.

Grundmann, A., T. Kuznetsova, S. S. Afiyatullov and S.-M. Li (2008). "FtmPT2, an *N*-prenyltransferase from *Aspergillus fumigatus*, catalyses the last step in the biosynthesis of Fumitremorgin B." Chembiochem **9**(13): 2059-2063.

Haagen, Y., K. Glück, K. Fay, B. Kammerer, B. Gust and L. Heide (2006). "A gene cluster for prenylated naphthoquinone and prenylated phenazine biosynthesis in *Streptomyces cinnamonensis* DSM 1042." Chembiochem **7**(12): 2016-2027.

Haagen, Y., I. Unsöld, L. Westrich, B. Gust, S. B. Richard, J. P. Noel and L. Heide (2007). "A soluble, magnesium-independent prenyltransferase catalyzes reverse and regular *C*-prenylations and *O*-prenylations of aromatic substrates." FEBS Lett. **581**(16): 2889-2893.

Hassett, D. J., L. Charniga, K. Bean, D. E. Ohman and M. S. Cohen (1992). "Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase." Infect. Immun. **60**(2): 328-36.

Heide, L. (2009). "Prenyl transfer to aromatic substrates: genetics and enzymology." Curr. Opin. Chem. Biol. **13**(2): 171-9.

Huang, H., M. S. Scherman, W. D'Haeze, D. Vereecke, M. Holsters, D. C. Crick and M. R. McNeil (2005). "Identification and active expression of the *Mycobacterium tuberculosis* gene encoding 5-phospho-a-D-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase, the first enzyme committed to decaprenylphosphoryl-D-arabinose synthesis." J. Biol. Chem. **280**(26): 24539-43.

Igarashi, Y., S. Miyanaga, H. Onaka, M. Takeshita and T. Furumai (2005). "Revision of the structure assigned to the antibiotic BU-4664L from *Micromonopora*." J. Antibiot. **58**(5): 350-352.

Itoh, T., K. Tokunaga, Y. Matsuda, I. Fujii, I. Abe, Y. Ebizuka and T. Kushiro (2010). "Reconstitution of a fungal meroterpenoid biosynthesis reveals the involvement of a novel family of terpene cyclases." Nat. Chem. **2**(10): 858-64.

Keller, M., M. M. Bhadbhade and R. W. Read (2012). "Two dibenzodiazepinone molecules with dissimilar dimeric associations and apparent different tautomeric forms." Acta Cryst. C **68**(6): o240-o246.

Kelley, L. A. and M. J. E. Sternberg (2009). "Protein structure prediction on the Web: a case study using the Phyre server." Nat. Protocols **4**(3): 363-371.

Kikuchi, H., S. Ishiko, K. Nakamura, Y. Kubohara and Y. Oshima (2010). "Novel prenylated and geranylated aromatic compounds isolated from *Polysphondylium* cellular slime molds." Tetrahedron **66**(32): 6000-6007.

Koeduka, T., N. Shitan, T. Kumano, K. Sasaki, A. Sugiyama, P. Linley, T. Kawasaki, H. Ezura, T. Kuzuyama and K. Yazaki (2011). "Production of prenylated flavonoids in tomato fruits expressing a prenyltransferase gene from *Streptomyces coelicolor* A3(2)." Plant Biol. **13**(2): 411-5.

Koonin, E. V. and R. L. Tatusov (1994). "Computer analysis of bacterial haloacid dehalogenases defines a large superfamily of hydrolases with diverse specificity. Application of an iterative approach to database search." J. Mol. Biol. **244**(1): 125-32.

Kopec, K. O., V. Alva and A. N. Lupas (2010). "Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria." Bioinformatics **26**(16): 1927-31.

Kumano, T., S. B. Richard, J. P. Noel, M. Nishiyama and T. Kuzuyama (2008). "Chemoenzymatic syntheses of prenylated aromatic small molecules using *Streptomyces* prenyltransferases with relaxed substrate specificities." Bioorg. Med. Chem. **16**(17): 8117-8126.

Kuzuyama, T., J. P. Noel and S. B. Richard (2005). "Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products." Nature **435**(7044): 983-7.

Lämmerhofer, M. and W. Lindner (2008). "Liquid chromatographic enantiomer separation and chiral recognition by cinchona alkaloid-derived enantioselective separation materials." Adv. Chromatogr. **46**: 1-107.

Laursen, J. B. and J. Nielsen (2004). "Phenazine natural products: biosynthesis, synthetic analogues, and biological activity." Chemical Reviews **104**(3): 1663-1685.

Li, S. M. (2009a). "Applications of dimethylallyltryptophan synthases and other indole prenyltransferases for structural modification of natural products." Appl. Microbiol. Biotechnol. **84**(4): 631-9.

Li, S. M. (2009b). "Evolution of aromatic prenyltransferases in the biosynthesis of indole derivatives." Phytochemistry **70**(15-16): 1746-57.

Li, S. M. (2010). "Prenylated indole derivatives from fungi: structure diversity, biological activities, biosynthesis and chemoenzymatic synthesis." Nat. Prod. Rep. **27**(1): 57-78.

Lima, W. C., A. M. Varani and C. F. Menck (2009). "NAD biosynthesis evolution in bacteria: lateral gene transfer of kynurenine pathway in Xanthomonadales and Flavobacteriales." Mol. Biol. Evol. **26**(2): 399-406.

Macone, A., E. Lendaro, A. Comandini, I. Rovardi, R. M. Matarese, A. Carraturo and A. Bonamore (2009). "Chromane derivatives of small aromatic molecules: Chemoenzymatic synthesis and growth inhibitory activity on human tumor cell line LoVo WT." Bioorg. Med. Chem. **17**(16): 6003-7.

Mason, W. P., K. Belanger, G. Nicholas, I. Vallieres, D. Mathieu, P. Kavan, A. Desjardins, A. Omuro and D. Reymond (2012). "A phase II study of the Ras-MAPK signaling pathway inhibitor TLN-4601 in patients with glioblastoma at first progression." J. Neurooncol. **107**(2): 343-9.

Mavrodi, D. V., R. F. Bonsall, S. M. Delaney, M. J. Soule, G. Phillips and L. S. Thomashow (2001). "Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1." J. Bacteriol. **183**(21): 6454-65.

Mavrodi, D. V., O. V. Mavrodi, J. A. Parejko, R. F. Bonsall, Y. S. Kwak, T. C. Paulitz, L. S. Thomashow and D. M. Weller (2012). "Accumulation of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of dryland cereals." Appl. Environ. Microbiol. **78**(3): 804-12.

Mavrodi, D. V., T. L. Peever, O. V. Mavrodi, J. A. Parejko, J. M. Raaijmakers, P. Lemanceau, S. Mazurier, L. Heide, W. Blankenfeldt, D. M. Weller and L. S. Thomashow (2010). "Diversity and evolution of the phenazine biosynthesis pathway." Appl. Environ. Microbiol. **76**(3): 866-79.

McAlpine, J. B., A. H. Banskota, R. D. Charan, G. Schlingmann, E. Zazopoulos, M. Piraee, J. Janso, V. S. Bernan, M. Aouidate, C. M. Farnet, X. Feng, Z. Zhao and G. T. Carter (2008). "Biosynthesis of diazepinomicin/ECO-4601, a *Micromonospora* secondary metabolite with a novel ring system." J. Nat. Prod. **71**(9): 1585-1590.

Melzer, M. and L. Heide (1994). "Characterization of polyprenyldiphosphate: 4-hydroxybenzoate polyprenyltransferase from *Escherichia coli*." Biochim. Biophys. Acta. **1212**(1): 93-102.

Mentel, M., E. G. Ahuja, D. V. Mavrodi, R. Breinbauer, L. S. Thomashow and W. Blankenfeldt (2009). "Of two make one: the biosynthesis of phenazines." Chembiochem **10**(14): 2295-304.

Metzger, U., S. Keller, C. E. Stevenson, L. Heide and D. M. Lawson (2010). "Structure and mechanism of the magnesium-independent aromatic prenyltransferase CloQ from the clorobiocin biosynthetic pathway." J. Mol. Biol. **404**(4): 611-26.

Metzger, U., C. Schall, G. Zocher, I. Unsöld, E. Stec, S. M. Li, L. Heide and T. Stehle (2009). "The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria." Proc. Natl. Acad. Sci. U. S. A. **106**(34): 14309-14.

Nagano, N., C. A. Orengo and J. M. Thornton (2002). "One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions." J. Mol. Biol. **321**(5): 741-65.

Nowicka, B. and J. Kruk (2010). "Occurrence, biosynthesis and function of isoprenoid quinones." Biochim. Biophys. Acta **1797**(9): 1587-605.

Ohkuma, H. and S. Kobaru (1996). Compound produced by a strain of Micromonospora. United States, Bristol-Myers Squibb Company. US patent 005541181A.

Ohlendorf, B., D. Schulz, A. Erhard, K. Nagel and J. F. Imhoff (2012). "Geranylphenazinediol, an acetylcholinesterase inhibitor produced by a *Streptomyces* species." J. Nat. Prod. **75**(7): 1400-4.

Oster, U., C. E. Bauer and W. Rudiger (1997). "Characterization of chlorophyll a and bacteriochlorophyll a synthases by heterologous expression in *Escherichia coli*." J. Biol. Chem. **272**(15): 9671-6.

Ozaki, T., S. Mishima, M. Nishiyama and T. Kuzuyama (2009). "NovQ is a prenyltransferase capable of catalyzing the addition of a dimethylallyl group to both phenylpropanoids and flavonoids." J. Antibiot. (Tokyo) **62**(7): 385-392.

Pierson, L. S., 3rd and E. A. Pierson (2010). "Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes." Appl. Microbiol. Biotechnol. **86**(6): 1659-70.

Pojer, F., E. Wemakor, B. Kammerer, H. Chen, C. T. Walsh, S. M. Li and L. Heide (2003). "CloQ, a prenyltransferase involved in clorobiocin biosynthesis." Proc. Natl. Acad. Sci. U. S. A. **100**(5): 2316-21.

Poulter, C. (2006). "Farnesyl diphosphate synthase. A paradigm for understanding structure and function relationships in *E*-polyprenyl diphosphate synthases." Phytochem. Rev. **5**(1): 17-26.

Ratnayake, A. S., J. E. Janso, X. Feng, G. Schlingmann, I. Goljer and G. T. Carter (2009). "Evaluating indole-related derivatives as precursors in the directed biosynthesis of diazepinomicin analogues." J. Nat. Prod. **72**(3): 496-9.

Remmert, M., A. Biegert, D. Linke, A. N. Lupas and J. Söding (2010). "Evolution of outer membrane beta-barrels from an ancestral beta beta hairpin." Mol. Biol. Evol. **27**(6): 1348-58.

Röttig, M., C. Rausch and O. Kohlbacher (2010). "Combining structure and sequence information allows automated prediction of substrate specificities within enzyme families." PLoS Comput. Biol. **6**(1): e1000636.

Sadreyev, R. and N. Grishin (2003). "COMPASS: a tool for comparison of multiple protein alignments with assessment of statistical significance." J. Mol. Biol. **326**(1): 317-36.

Saiki, K., T. Mogi and Y. Anraku (1992). "Heme O biosynthesis in *Escherichia coli*: The *cyoE* gene in the cytochrome *bo* operon encodes a protoheme IX farnesyltransferase." Biochem. Biophys. Res. Commun. **189**(3): 1491-1497.

Saleh, O., T. Bonitz, K. Flinspach, A. Kulik, N. Burkard, A. Muhlenweg, A. Vente, S. Polnick, M. Lammerhofer, B. Gust, H.-P. Fiedler and L. Heide (2012). "Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines." Med. Chem. Comm. **3**(8): 1009-1019.

Saleh, O., B. Gust, B. Boll, H. P. Fiedler and L. Heide (2009). "Aromatic prenylation in phenazine biosynthesis: dihydrophenazine-1-carboxylate dimethylallyltransferase from *Streptomyces anulatus*." J. Biol. Chem. **284**(21): 14439-47.

Savidge, B., J. D. Weiss, Y. H. Wong, M. W. Lassner, T. A. Mitsky, C. K. Shewmaker, D. Post-Beittenmiller and H. E. Valentin (2002). "Isolation and characterization of homogentisate phytyltransferase genes from *Synechocystis* sp. PCC 6803 and *Arabidopsis*." Plant Physiol. **129**(1): 321-32.

Schuller, J. M., G. Zocher, M. Liebhold, X. Xie, M. Stahl, S. M. Li and T. Stehle (2012). "Structure and catalytic mechanism of a cyclic dipeptide prenyltransferase with broad substrate promiscuity." J. Mol. Biol. **422**(1): 87-99.

Schultz, A. W., C. A. Lewis, M. R. Luzung, P. S. Baran and B. S. Moore (2010). "Functional characterization of the cyclomarin/cyclomarazine prenyltransferase CymD directs the biosynthesis of unnatural cyclic peptides." J. Nat. Prod. **73**(3): 373-7.

Seeger, K., K. Flinspach, E. Haug-Schifferdecker, A. Kulik, B. Gust, H.-P. Fiedler and L. Heide (2011). "The biosynthetic genes for prenylated phenazines are located at two different chromosomal loci of *Streptomyces cinnamonensis* DSM 1042." Microb. Biotechnol. **4**(2): 252-262.

Shindo, K., A. Tachibana, A. Tanaka, S. Toba, E. Yuki, T. Ozaki, T. Kumano, M. Nishiyama, N. Misawa and T. Kuzuyama (2011). "Production of novel antioxidative prenyl naphthalen-ols by combinational bioconversion with dioxygenase PhnA1A2A3A4 and prenyltransferase NphB or SCO7190." Biosci. Biotechnol. Biochem. **75**(3): 505-10.

Silakowski, B., B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle and R. Müller (2000). "The myxochelin iron transport regulon of the myxobacterium *Stigmatella aurantiaca* Sg a15." Eur. J. Biochem. **267**(21): 6476-85.

Söding, J. (2005). "Protein homology detection by HMM-HMM comparison." Bioinformatics **21**(7): 951-60.

Söding, J., A. Biegert and A. N. Lupas (2005). "The HHpred interactive server for protein homology detection and structure prediction." Nucleic Acids Res. **33**(Web Server issue): W244-8.

Söding, J., M. Remmert and A. Biegert (2006). "HHrep: de novo protein repeat detection and the origin of TIM barrels." Nucleic Acids Res. **34**(Web Server issue): W137-42.

Stec, E., D. Pistorius, R. Muller and S. M. Li (2011). "AuaA, a membrane-bound farnesyltransferase from *Stigmatella aurantiaca*, catalyzes the prenylation of 2-methyl-4-hydroxyquinoline in the biosynthesis of aurachins." Chembiochem **12**(11): 1724-30.

Steffan, N., A. Grundmann, W. B. Yin, A. Kremer and S. M. Li (2009). "Indole prenyltransferases from fungi: a new enzyme group with high potential for the production of prenylated indole derivatives." Curr. Med. Chem. **16**(2): 218-31.

Suvarna, K., D. Stevenson, R. Meganathan and M. E. Hudspeth (1998). "Menaquinone (vitamin K2) biosynthesis: localization and characterization of the *menA* gene from *Escherichia coli*." J. Bacteriol. **180**(10): 2782-7.

Takahashi, S., H. Takagi, A. Toyoda, M. Uramoto, T. Nogawa, M. Ueki, Y. Sakaki and H. Osada (2010). "Biochemical characterization of a novel indole prenyltransferase from *Streptomyces* sp. SN-593." J. Bacteriol. **192**(11): 2839-2851.

Tello, M., T. Kuzuyama, L. Heide, J. P. Noel and S. B. Richard (2008). "The ABBA family of aromatic prenyltransferases: broadening natural product diversity." Cell. Mol. Life Sci. **65**(10): 1459-63.

Xiao, Y., M. Machacek, K. Lee, T. Kuzuyama and P. Liu (2009). "Prenyltransferase substrate binding pocket flexibility and its application in isoprenoid profiling." Mol. Biosyst. **5**(9): 913-7.

Yazaki, K., K. Sasaki and Y. Tsurumaru (2009). "Prenylation of aromatic compounds, a key diversification of plant secondary metabolites." Phytochemistry **70**(15-16): 1739-45.

Yin, W. B., H. L. Ruan, L. Westrich, A. Grundmann and S.-M. Li (2007). "CdpNPT, an *N*-Prenyltransferase from *Aspergillus fumigatus*: Overproduction, Purification and Biochemical Characterisation." Chembiochem **8**(10): 1154-1161.

Yu, S., A. Vit, S. Devenish, H. K. Mahanty, A. Itzen, R. S. Goody and W. Blankenfeldt (2011). "Atomic resolution structure of EhpR: phenazine resistance in *Enterobacter agglomerans* Eh1087 follows principles of bleomycin/mitomycin C resistance in other bacteria." BMC Struct. Biol. **11**: 33.

Zendah, I., N. Riaz, H. Nasr, H. Frauendorf, A. Schuffler, A. Raies and H. Laatsch (2012). "Chromophenazines from the terrestrial *Streptomyces* sp. Ank 315." J. Nat. Prod. **75**(1): 2-8.

Zocher, G., O. Saleh, J. B. Heim, D. A. Herbst, L. Heide and T. Stehle (2012). "Structure-based engineering increased the catalytic turnover rate of a novel phenazine prenyltransferase." PLoS ONE **7**(10): e48427.

Zou, H. X., X. Xie, X. D. Zheng and S. M. Li (2010). "The tyrosine *O*-prenyltransferase SirD catalyzes *O*-, *N*-, and *C*-prenylations." Appl. Microbiol. Biotechnol. **89**(5): 1443-51.

Zummo, F. P., S. Marineo, A. Pace, F. Civiletti, A. Giardina and A. M. Puglia (2012). "Tryptophan catabolism via kynurenine production in *Streptomyces coelicolor*: identification of three genes coding for the enzymes of tryptophan to anthranilate pathway." Appl. Microbiol. Biotechnol. **94**(3): 719-28.

# Abbreviations

| | |
|---|---|
| °C | degree Celsius ($T_K$ – 273,15) |
| µ | micro (metric prefix) |
| Å | Ångström ($1E^{-10}$ m) |
| aa | amino acid |
| 1,6-DHN | 1,6-dihydroxynaphthalene |
| 2,7-DHN | 2,7-dihydroxynaphthalene |
| APCI-HRMS | Atmospheric-Pressure Chemical Ionization - High Resolution MS |
| COSY | Correlation Spectroscopy |
| Da | Dalton ($1{,}6605E^{-24}$ g) |
| dihydro-PCA | 5,10-dihydrophenazine-1-carboxylic acid |
| DMAPP | dimethylallyl diphosphate |
| DNA | deoxyribonucleic acid |
| EDTA | ethylendiamine tetra-acetic acid |
| *et al.* | et alii (and others) |
| Fig. | figure |
| FPP | farnesyl diphosphate |
| g | gram (SI base unit) |
| GPP | geranyl diphosphate |
| h | hour (3600 s) |
| His8 | octahistidine |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HPLC | high performance liquid chromatography |
| HSQC | Heteronuclear Single Quantum Coherence |
| IPTG | isopropyl 1-thio-β-D-galactopyranoside |
| k | kilo (metric prefix) |
| K | Kelvin (SI base unit) |
| $k_{cat}$ | turnover rate |
| $K_m$ | Michaelis-Menten constant |
| L | liter ($1E^{-3}$ $m^3$) |
| M | mol/L |
| m | meter (SI base unit) |

| | |
|---|---|
| m | milli (metric prefix) |
| min | minute (60 s) |
| MS | mass spectrometry |
| MW | molecular weight |
| *m/z* | mass-to-charge ratio |
| n | nano (metric prefix) |
| NOESY | Nuclear Overhauser Effect Spectroscopy |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PTase | prenyltransferase |
| RMSD | root-mean-square deviation |
| RNA | ribonucleic acid |
| rpm | rounds per minute |
| RT | room temperature |
| s | second (SI base unit) |
| SDS | sodium dodecyl sulfate |
| Tris | 2-amino-2-(hydroxymethyl)-1,3-propanediol |
| $v_{max}$ | maximal reaction velocity |
| ×g | ground acceleration |

# Supplementary

Chapter 1

**Evolutionary Relationships of Microbial Aromatic Prenyltransferases**

**Bonitz T**, Alva V, Saleh O, Lupas AN, Heide L

2011, PLoS ONE 6(11): e27336. **doi:10.1371/journal.pone.0027336**

Supplementary Information:

**Table S1.** Proteins included in this study.

**doi:10.1371/journal.pone.0027336.s001**

Chapter 2

**Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines**

Orwah Saleh, **Tobias Bonitz**, Katrin Flinspach, Andreas Kulik, Nadja Burkard, Agnes Mühlenweg, Andreas Vente, Stefan Polnick, Michael Lämmerhofer, Bertolt Gust, Hans-Peter Fiedler and Lutz Heide

2012, Med. Chem. Commun., 2012, 3, 1009–1019. **doi: 10.1039/c2md20045g**

Supplementary Information:

**Figure S1 and Table S1.**

**doi: 10.1039/c2md20045g**

Chapter 3

Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily.

**Tobias Bonitz**, Florian Zubeil, Stephanie Grond, and Lutz Heide

submitted

Supplementary Information:

**Figure S1 and S2, and Table S1 and S2.**

OPEN ACCESS Freely available online

PLoS one

# Evolutionary Relationships of Microbial Aromatic Prenyltransferases

**Tobias Bonitz[1], Vikram Alva[2], Orwah Saleh[1], Andrei N. Lupas[2], Lutz Heide[1]***

**1** Pharmaceutical Institute, Eberhard Karls-Universität Tübingen, Tübingen, Germany, **2** Department of Protein Evolution, Max-Planck-Institute for Developmental Biology, Tübingen, Germany

## Abstract

The linkage of isoprenoid and aromatic moieties, catalyzed by aromatic prenyltransferases (PTases), leads to an impressive diversity of primary and secondary metabolites, including important pharmaceuticals and toxins. A few years ago, a hydroxynaphthalene PTase, NphB, featuring a novel ten-stranded β-barrel fold was identified in *Streptomyces* sp. strain CL190. This fold, termed the PT-barrel, is formed of five tandem ααββ structural repeats and remained exclusive to the NphB family until its recent discovery in the DMATS family of indole PTases. Members of these two families exist only in fungi and bacteria, and all of them appear to catalyze the prenylation of aromatic substrates involved in secondary metabolism. Sequence comparisons using PSI-BLAST do not yield matches between these two families, suggesting that they may have converged upon the same fold independently. However, we now provide evidence for a common ancestry for the NphB and DMATS families of PTases. We also identify sequence repeats that coincide with the structural repeats in proteins belonging to these two families. Therefore we propose that the PT-barrel arose by amplification of an ancestral ααββ module. In view of their homology and their similarities in structure and function, we propose to group the NphB and DMATS families together into a single superfamily, the PT-barrel superfamily.

**Citation:** Bonitz T, Alva V, Saleh O, Lupas AN, Heide L (2011) Evolutionary Relationships of Microbial Aromatic Prenyltransferases. PLoS ONE 6(11): e27336. doi:10.1371/journal.pone.0027336

**Editor:** Vladimir N. Uversky, University of South Florida College of Medicine, United States of America

**Received** September 9, 2011; **Accepted** October 14, 2011; **Published** November 30, 2011



**Funding:** This work was supported by a grant from the German Federal Ministry of Education and Research (BMBF GenBioCom) to LH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: heide@uni-tuebingen.de

## Introduction

Aromatic prenyltransferases (PTases) catalyze the transfer of isoprenyl moieties to aromatic acceptor molecules, forming C-C bonds. They are key enzymes in the biosynthesis of lipoquinones and of many secondary metabolites in plants, fungi and bacteria [1].

Aromatic PTases of lipoquinone biosynthesis are integral membrane proteins. They contain an aspartate-rich motif (e.g. NDxxD) for binding of the prenyl diphosphate substrate *via* a $Mg^{2+}$ ion, similar to the corresponding motif of farnesyl diphosphate synthase [2]. A structural model of the PTase UbiA involved in the biosynthesis of ubiquinone (Fig. 1) has been proposed [3].

In contrast to the PTases of lipoquinone biosynthesis, the aromatic PTase CloQ from *Streptomyces roseochromogenes*, involved in the formation of clorobiocin (Fig. 1), was found to be a soluble, monomeric 35 kDa protein [4]. CloQ does not contain a NDxxD motif and is active in the absence of $Mg^{2+}$ or other divalent cations. Kuzuyama et al. [5] identified a similar aromatic prenyltransferase, NphB, involved in the biosynthesis of the prenylated polyketide naphterpin (Fig. 1) in *Streptomyces* sp. strain CL190. NphB was found to display a hitherto unobserved β-barrel fold which was termed the PT-barrel (Fig. 2; PDB 1ZB6). It consists of five repetitive ααββ elements. The ten β-strands arrange in an antiparallel fashion to form a central β-barrel that contains the active center in its spacious lumen and the α-helices form a solvent-exposed ring around the barrel [6]. The structure of the aforementioned CloQ also displays the PT-barrel fold (Fig. 2; PDB 2XLQ) [7]. PSI-BLAST searches currently reveal 17 further database entries with sequence similarity to NphB and CloQ, 12 of them in bacteria of the genus *Streptomyces* and five in fungi of the phylum *Ascomycota*. In silico structure predictions suggest that all these proteins adopt the PT-barrel fold. Eleven of these enzymes have been investigated biochemically, and all of them catalyze the *C*-prenylation of aromatic compounds, i.e. phenols or phenazines. Fig. 1 shows as examples the reactions catalyzed by the bacterial enzymes CloQ, NphB, SCO7190, Fnq26 and PpzP, and by the fungal enzyme $Ptf_{At}$. Tab. S1 (Supplementary Material) lists the references and accession numbers for all these enzymes and for all other proteins included in this study.

The *C*-prenylation of an aromatic compound is also catalyzed by dimethylallyltryptophan synthase (DMATS; Fig. 1), involved in the biosynthesis of the pharmaceutically important ergot alkaloids in different fungi of the phylum *Ascomycota*. DMATS shows no sequence similarity, as evaluated with PSI-BLAST, to the bacterial enzyme NphB or orthologs thereof, and is considerably larger than NphB (459 vs. 307 amino acids). Unexpectedly, however, it was found to adopt the same PT-barrel fold as NphB (Fig. 2; PDB 3I4X) [8]. DMATS is the prototype of the fungal indole PTases, involved in the biosynthesis of a large number of complex secondary metabolites in fungi [9]. Currently, approximately 200 close orthologs of DMATS are found in different fungal genomes in the database. The structure of a second member of this group, FtmPT1, has recently been published, and it shows the same fold as DMATS (Fig. 2; PDB 3O2K) [10]. Furthermore, three indole

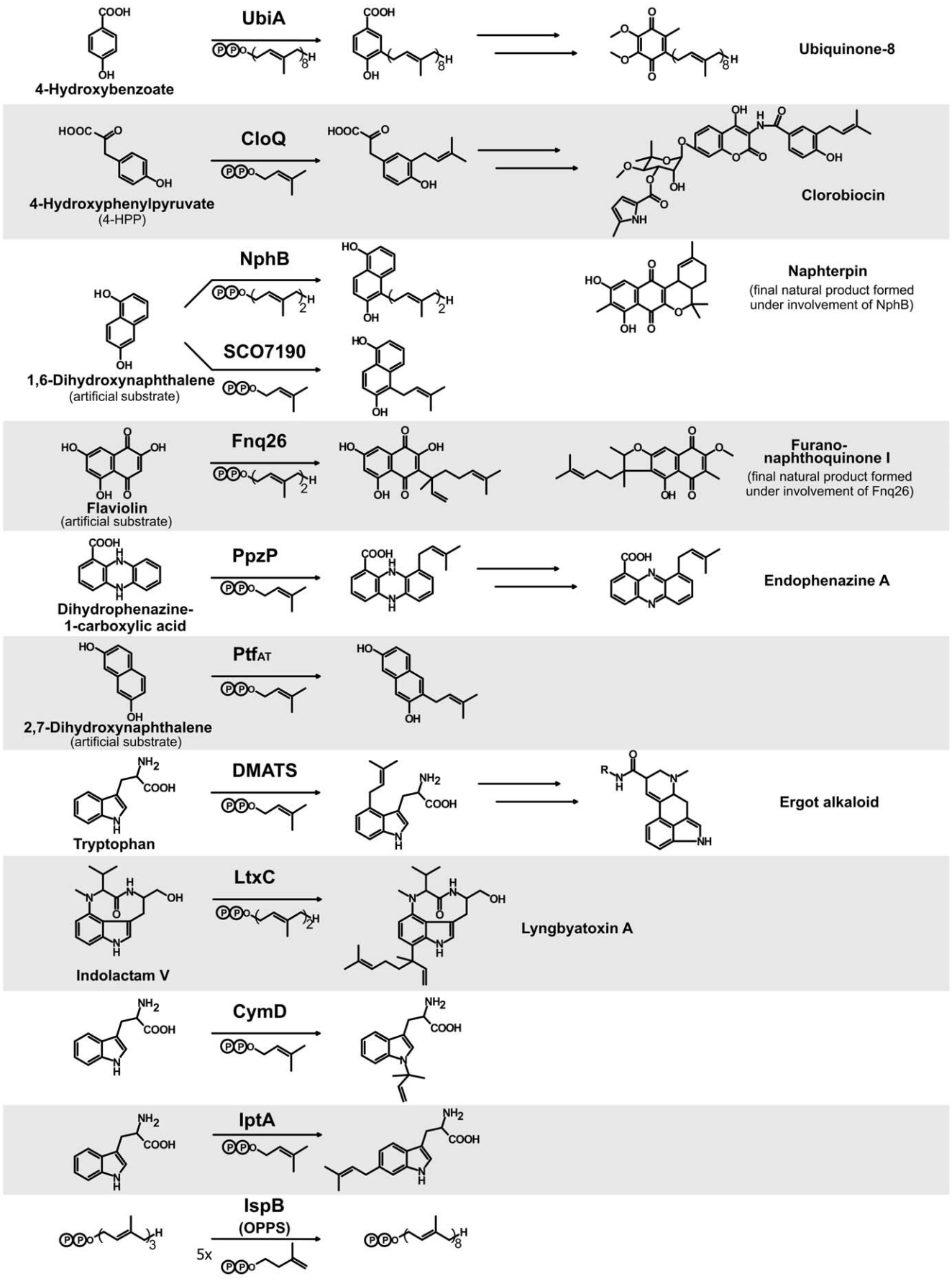



**Figure 1. Reactions catalyzed by aromatic prenyltransferases and by the octaprenyl diphosphate synthase IspB.**
doi:10.1371/journal.pone.0027336.g001

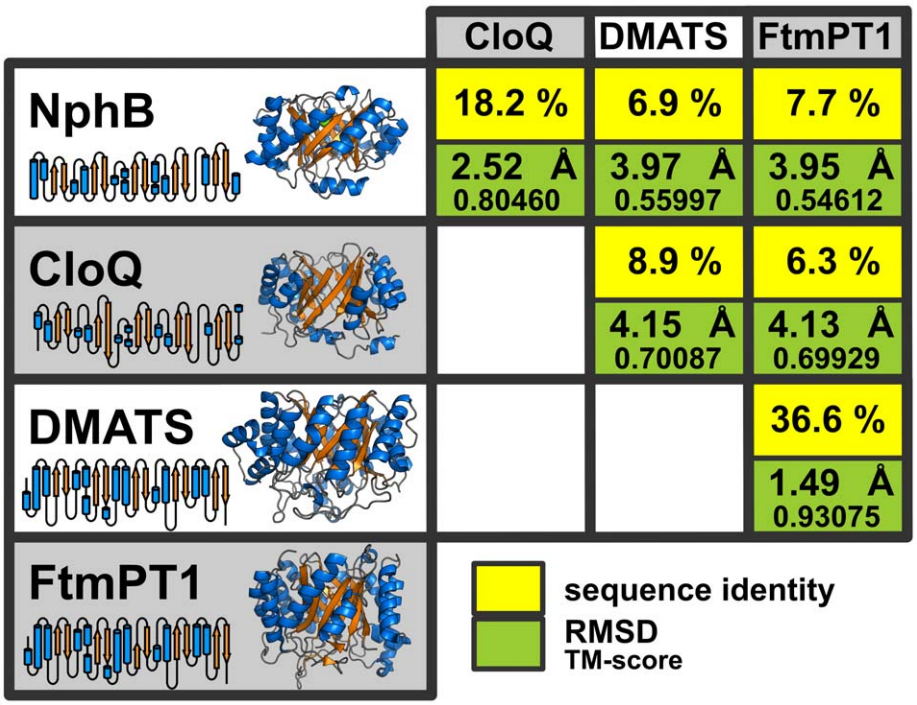



**Figure 2. Structures of PT-barrels.** Pairwise sequence identities, RMSDs and TM-scores of the four aromatic prenyltransferases NphB (PDB 1ZB6), CloQ (2XLQ), DMATS (3I4Z) and FtmPT1 (3O2K) are shown. The schemes besides the structures depict the topology of the secondary structural elements.
doi:10.1371/journal.pone.0027336.g002

PTases (LtxC, CymD and IptA; Fig. 1) have recently been identified in bacteria [11,12,13], and GenBank currently contains 16 further entries from bacterial genomes with similarity to these three enzymes. Most of these entries are found in genomes of actinomycetales, but one is from the alphaproteobacterium *Methylobacterium* sp. 4–46, and LtxC is from a cyanobacterium. In silico structure prediction suggests that all these bacterial indole PTases adopt the PT-barrel fold.

A similarity in sequence between the phenol/phenazine PTases (NphB/CloQ family) and the indole PTases (DMATS/CymD family) is not detectable using BLAST and PSI-BLAST. This raises the question whether the NphB/CloQ family and the DMATS/CymD family may have originated independently and converged on the PT-barrel fold in response to the biochemical challenge of performing an aromatic prenylation reaction, i.e. a reaction corresponding to a Friedel-Crafts alkylation, in an aqueous solution which requires effective shielding of the reactive intermediary allylic cation from reaction with water [8].

Only a limited number of structural solutions is available to a polypeptide chain, therefore protein structures are multiply convergent [14]. In contrast, sequence space is essentially infinite and many sequences are compatible with a particular fold. For this reason, sequence similarity rather than structure similarity is the primary marker of homology. In the recent years, the enormous growth of protein sequence and structure databases coupled with the development of sensitive sequence comparison methods has shown that proteins may not be as polyphyletic as hitherto assumed [15]. Indeed, many fold families, for instance families of the TIM $(\beta\alpha)_8$-barrel fold, that were previously considered to be analogous are now thought to be homologous [16,17,18].

In this study, we used a highly sensitive sequence comparison method, HHsearch [19], based on profile hidden Markov Models (HMMs) to evaluate the evolutionary origins of the CloQ/NphB and the DMATS/CymD families. Our results indicate that they are homologous. We also include an investigation on the membrane-bound aromatic PTases, e.g. of lipoquinone biosynthesis. They show no evolutionary relationship to the CloQ/NphB and the DMATS/CymD families but display evolutionary connections to other PTases such as protoheme IX farnesyltransferases, chlorophyll a synthases and decaprenyl-phosphate 5-phosphoribosyltransferases.

## Materials and Methods

To calculate the root mean square deviation (RMSD) and TM-scores for the proteins included in this study, we used the TM-align server (http://zhanglab.ccmb.med.umich.edu/TM-align/) [20] with default parameters. To evaluate homology between PTases we used HHsearch [21], a sensitive remote homology detection method based on the pairwise comparison of profile hidden Markov models (HMMs). HHsearch was used to perform all-against-all comparison of the 36 biochemically investigated proteins listed in supplementary Table S1. For each of these 36 proteins, we generated multiple sequence alignments using the buildali script from the HHsearch package. The obtained multiple alignments were used to calculate profile HMMs using HHmake, also from the HHsearch package. The profile HMMs were compared with each other using HHsearch and the results were mapped onto a matrix. All tools were run using default settings.

To gather the amino acid sequences of PTases for cluster analysis, we searched the non-redundant protein sequence database (nr) at NCBI for homologs of NphB from *Streptomyces* sp. strain CL190 (PDB identifier 1ZB6) and DMATS from *Aspergillus fumigatus* (3I4X) using the PSI-BLAST algorithm [22] in four iterative steps. The sequences which were shorter than 200 amino acid residues and longer than 600 amino acid residues were

excluded to avoid fragments and multi-domain proteins. The same procedure was applied to search for homologs of UbiA from *Escherichia coli* (AP_004541), MenA from *E. coli* (AAB01207) and Slr1736 from *Synechocystis* sp. PCC 6803 (BAA17774) in four consecutive PSI-BLAST iterations. All identified sequences were pooled together and duplicates were removed using RetrieveSeq tool from the MPI bioinformatic toolkit (http://toolkit.tuebingen.mpg.de) [23]. The sequence XP_003295160 was removed due to the presence of unidentified amino acids in the sequence.

All identified sequences were analyzed and clustered by their pairwise PSI-BLAST P-values [24] with CLANS (http://toolkit.tuebingen.mpg.de/clans; [25]). CLANS treats sequences as point masses in a virtual space which attract or repel each other depending on their pairwise sequence similarities. Clustering was done to equilibrium in 2D at a P-value cutoff of 1E-3 for the cluster map of NphB and DMATS, and 1E-6 for the UbiA, MenA and Slr736 cluster map using default settings.

To detect sequence repeats in PT-barrels, we used the highly sensitive de novo repeat detection method HHrepID [26]. HHrepID takes a multiple sequence alignment as input and converts it into a profile HMM. To detect internal sequence repeats, this profile HMM is repeatedly aligned to itself. We extracted sequences of bacterial and fungal indole PTases, and phenol/phenazine PTases from the aforementioned cluster map and calculated multiple sequence alignments with ClustalW [27]. These alignments were then analyzed for the presence of repeats with HHrepID using default settings.

## Results

*HMM-HMM comparisons of PTases featuring the PT-barrel:* Sequence search methods achieve different levels of sensitivity, depending on the amount of information they incorporate. Sequence-to-sequence methods, such as BLAST [24], are the least sensitive as they use only the information from the pairwise comparison of two sequences, scored by a global substitution matrix. Profile-to-sequence methods, such as the iterated version of BLAST, PSI-BLAST [28], are more sensitive, as they include family-specific information for the query sequence in the form of a position-specific scoring matrix derived from homologous sequences. Profile-to-profile comparison methods, such as COMPASS [29], provide an additional improvement by using family-specific information for both sequences being compared. Incorporation of position-specific gapping probabilities into the profiles yields profile Hidden Markov Models (HMMs) [30], which are currently our most sensitive tool for the detection of sequence similarity. HHsearch [19,21], an HMM-to-HMM comparison method, has a sensitivity comparable to that of advanced fold recognition methods, despite using only sequence information.

While members of the CloQ/NphB and the DMATS/CymD families display the PT-barrel fold (the structures of NphB and DMATS align at a RMSD of 3.97 Å over 290 aligned residues), they show very little sequence identity (<15%). Nevertheless many instances are known where proteins with such low sequence identity belong to the same superfamily (e.g. ubiquitins [31]). We therefore used HHsearch to investigate the evolutionary origins of these two families. Biochemically characterized members of (i) the PTases with similarity to NphB/CloQ, (ii) the fungal indole PTases and (iii) the bacterial indole PTases were selected as representatives for HHsearch analysis. As expected, HHsearch assigns a 100% probability of homology to all pairwise matches within each of these three groups (Fig. 3). We also detected matches between the fungal indole PTases (e.g. DMATS) and the bacterial indole PTases (e.g. CymD) at a probability of 100%, confirming their evolutionary relatedness. Likewise, we also obtained probability values of 100% for connections between the bacterial phenol PTases NphB and CloQ, and the fungal phenol PTases $Ptf_{At}$, $Ptf_{Bf}$ and $Ptf_{Sc}$. Strikingly, we obtained several matches between the CloQ/NphB and the DMATS/CymD families at high probabilities (50%–75%). We have previously shown that this level of sequence similarity is indicative of common ancestry [32,33,34,35]. We thus conclude that these two families are homologous.

In the biosynthesis of ubiquinones, menaquinones, plastoquinones and tocopherols, the *C*-prenylation of aromatic substrates is catalyzed by integral membrane proteins with several membrane-spanning helices [1]. Similar to the soluble farnesyl diphosphate synthase (FPP synthase) [2] and the octaprenyl diphosphate synthase IspB [36] (Fig. 1), these membrane-bound aromatic PTases show conserved NDxxD motifs for the binding of the isoprenoid substrates in the form of $Mg^{2+}$ complexes. In contrast, all aromatic PTases characterized by the PT-barrel fold are soluble enzymes without the NDxxD motifs. As expected, HHsearch detected matches between the membrane-bound aromatic PTases UbiA of ubiquinone biosynthesis, MenA of menaquinone biosynthesis and Str1736 of tocopherol biosynthesis, confirming their homology (data not shown). In contrast, these enzymes did not make any connections to the soluble PTases with the PT-barrel fold.

To check for the existence of possible distant homologs of the aromatic PTases with the PT-barrel fold, we ran HHsearch against a database comprising several complete genomes. The search was seeded with the PTases NphB and DMATS. We did not find matches to proteins outside of the CloQ/NphB and the DMATS/CymD families, indicating that the PT-barrel fold is exclusive to them at this time.

*Detection of sequence repeats in the PT-barrel:* The PT-barrel is a toroidal fold, in which five αααββ structural repeats are arranged in a circular fashion to form a closed barrel. While these five repeats are structurally well superimposable with median RMSDs below 2.5 Å, they do not show clear sequence similarity to each other. Therefore, it has remained unclear whether the symmetry displayed by the PT-barrel is a result of five-fold amplification of a single αααββ unit or of structural convergence. If PT-barrels originated by amplification, we might still find residual sequence similarity between their repeats with highly sensitive sequence comparison tools. For this, we used the de novo repeat detection method HHrepID, which detects internal sequence symmetries by repeatedly aligning the query HMM with itself. HHrepID has been used successfully to detect highly divergent sequence repeats in several folds including TIM $(\beta\alpha)_8$-barrels [18] and outer membrane β-barrels [34]. We detected five-fold internal sequence symmetry in both the bacterial and the fungal indole PTases at default settings with a P-value of better than 1E-4. We also found repeats in the phenol/phenazine PTases, albeit at lower detection stringency. In the indole PTases the detected repeats coincide largely with the αααββ structural units, but in the phenol/phenazine PTases the repeats are shorter and coincide only with the ββ hairpins. While we can substantiate a scenario for the origin of indole PTases by amplification based on the presence of residual sequence similarity between their repeats, the repeats of phenol/phenazine PTases are more divergent and a scenario for their origin cannot be established at this time. We note that this range of internal symmetry among members of the same superfamily is not unique to PT-barrels. β-propellers, for instance, display a wide range of internal symmetry, from near-identical to fully diverged, and an origin by amplification has been proposed for them [35].

*Cluster analysis of aromatic PTases:* In order to visualize the relationships between the PTases with the PT-barrel fold, we

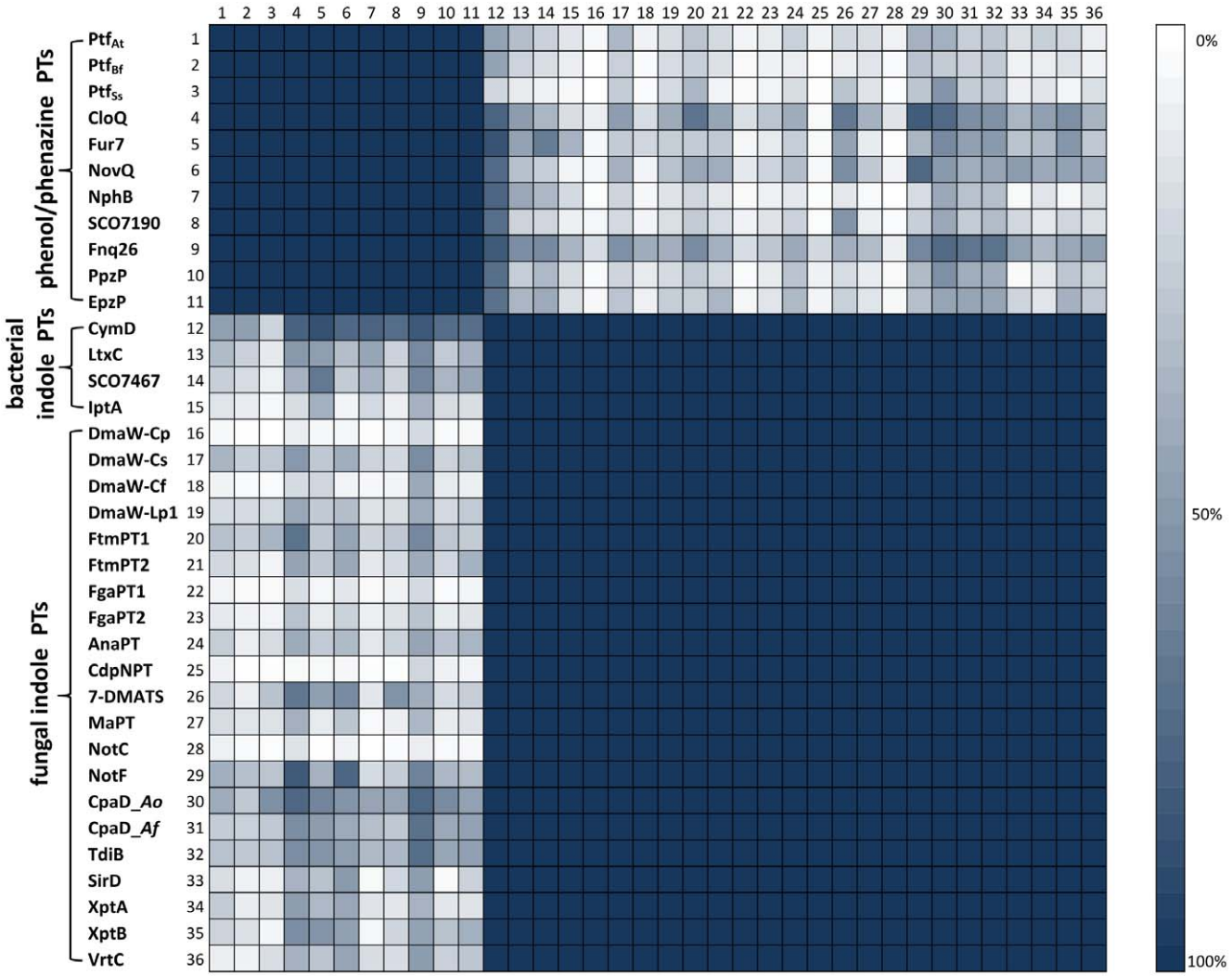



**Figure 3. HHsearch analysis of prenyltransferases with the PT-barrel fold.** Pairwise HMM comparisons of 36 biochemically characterized PTases (see Table S1, Supplementary Material) were performed using HHsearch. Group and protein names are shown on the left. Cell color indicates HHsearch probability of the match as depicted in the scale on the right.
doi:10.1371/journal.pone.0027336.g003

searched the non-redundant protein sequence database at NCBI for homologs of NphB and DMATS and clustered the obtained sequences in CLANS. The resulting cluster map (Fig. 4A) very clearly shows two distinct clusters that correspond to the phenol/phenazine PTases and the indole PTases. The two clusters are connected with each other, further confirming the proposed evolutionary relationship between these two enzyme families. No other groups of proteins with similarity to NphB and DMATS were identified by this PSI-BLAST search, showing that the enzymes with PT-barrel fold are not related to other currently known proteins.

The phenol/phenazine PTases (Fig 4A; dark orange) comprise 14 bacterial proteins from the genus *Streptomyces* and 5 fungal proteins from the phylum *Ascomycota*. The cluster analysis did not show a separation of the bacterial and the fungal enzymes within this family, even at higher clustering stringency. In contrast, the indole PTases can be separated into two subclusters, one of which contains all of the 19 bacterial entries, and the other one all of the 186 fungal entries. This separation is already visible in Fig. 4A, and becomes very clear at higher clustering stringency (data not shown).

We also performed a cluster analysis of the membrane-bound aromatic PTases. We searched the non-redundant protein sequence database at NCBI for homologs of the membrane-bound PTases UbiA, MenA and Slr1736 and clustered them in CLANS. As expected, the map (Fig. 4 B) shows distinct but connected clusters for (i) 4-hydroxybenzoate PTases of ubiquinone biosynthesis, e.g. UbiA of *E. coli* [37], (ii) 1,4-dihydroxy-2-naphthoate 3-prenyltransferases of menaquinone biosynthesis, e.g. MenA of *E. coli* [38], and (iii) homogentisate PTases of plastoquinone and tocopherol biosynthesis [39]. In addition, this cluster analysis revealed further enzymes to be related to the aromatic PTase of lipoquinone biosynthesis. These include the chlorophyll a synthases and protoheme IX farnesyltransferases, both of which attach phytyl or farnesyl moieties to side chains of tetrapyrrole substrates [40,41]. Another group is formed by the 5-phosphoribose-1-diphosphate:decaprenyl-phosphate 5-phosphoribosyltransferases (DPPRs) which are involved in the biosynthesis of lipids of the bacterial cell wall. The reaction catalyzed by DPPRs is quite different from that catalyzed by aromatic PTases, yet there is obvious sequence similarity between DPPR and UbiA [42]. A last

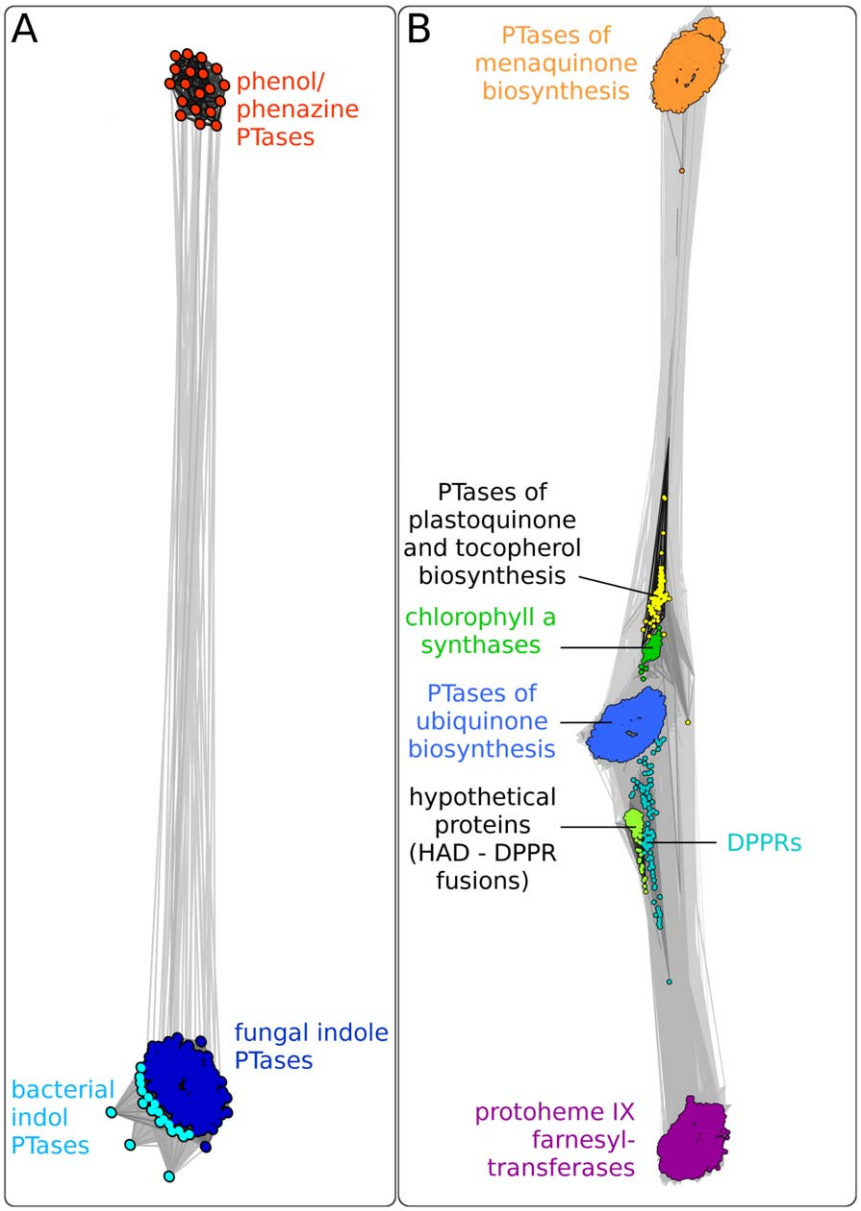



**Figure 4. Cluster map of (A) aromatic prenyltransferases characterized by the PT-barrel fold and (B) membrane-bound aromatic prenyltransferases.** These maps were generated by clustering respective PTases in CLANS based on their all-against-all pairwise similarities as measured by PSI-BLAST P-values. Dots represent sequences, line coloring reflects PSI-BLAST P-values; the darker a line, the lower the P-value.
doi:10.1371/journal.pone.0027336.g004

group of database entries related to membrane-bound aromatic PTases comprises hypothetical proteins, mostly from proteobacteria, which consist of two distinct domains: one similar to hydrolases of the HAD superfamily [43], the other one similar to DPPR or UbiA. The function of these proteins is, to our knowledge, unknown.

## Discussion

The PT-barrel is a novel protein fold that was discovered recently and is found exclusively in microbial secondary metabolic PTases with aromatic substrates. For proteins with the PT-barrel fold, the name ABBA PTases has been suggested previously [6], owing to the αββα succession of the secondary structure elements in the polypeptide chain which results in the characteristic antiparallel orientation of the β-sheets in the barrel. Our study suggests that all proteins with the PT-barrel fold share a common ancestry and they therefore belong to a single superfamily. As shown in Fig. 5, this superfamily can be divided into two families, i.e. the indole PTases and the phenol/phenazine PTases. The state-of-the-art sequence comparison method HHsearch yielded significant matches between these families, indicating a common ancestry. We also found evidence for the origin of the PT-barrel fold by amplification of an ancestral ααββ module.

The family of indole PTases comprises the fungal indole PTases and the bacterial indole PTases, with DMATS and CymD as typical representatives, respectively. It should be noted that the term "indole PTases" is correct for most but not all biochemically investigated members of this family. The exceptions are SirD (NCBI accession AAS92554), which catalyses the *O*-prenylation of

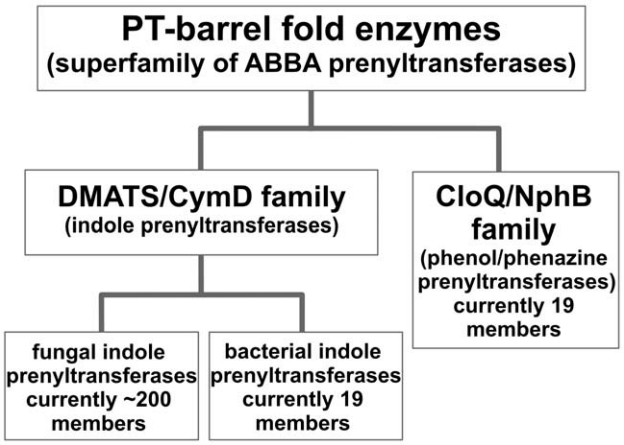



**Figure 5. Classification of prenyltransferases characterized by the PT-barrel fold (="ABBA prenyltransferases").**
doi:10.1371/journal.pone.0027336.g005

the phenolic oxygen of tyrosine in sirodesmin biosynthesis [44], VrtC (ADI24928), which *C*-prenylates a phenolic substrate which is related to tetracyclines [45], and TdiB (ABU51603) which catalyses both an indole prenylation and the prenylation of a phenolic moiety during terrequinone biosynthesis A [46].

As expected, HHsearch did not indicate a relationship between the soluble aromatic PTases with the PT-barrel fold, such as NphB or DMATS, and the membrane-bound PTases, such as UbiA of ubiquinone biosynthesis. Therefore, two independent solutions have evolved in nature to solve the biochemical problem of catalyzing an aromatic prenylation reaction in an aqueous environment. The indispensable shielding of the reactive allylic cation, generated from the prenyl diphosphate substrate, is achieved by a barrel of antiparallel β-sheets in case of the ABBA PTases, and by a deep lipophilic pocket between the transmembrane helices in case of the membrane-bound aromatic PTases.

All PTases characterized by the PT-barrel fold belong to secondary metabolic pathways; no primary metabolic enzyme with this fold has been discovered yet. In contrast, most of the membrane-bound aromatic PTases are involved in primary metabolism. However, a few enzymes of this group are involved in secondary metabolism. The bacterial PTase AqgD catalyzes the *O*-prenylation of the secondary metabolite alkyl-methoxyhydroquinone [47], and the fungal PTase XP_751272 is involved in the biosynthesis of pyripyropene A [48]; both show similarity to UbiA of ubiquinone biosynthesis. The bacterial putative PTase BAD07390 is likely to be involved in the biosynthesis of the secondary metabolite BE-40644 [49]; it shows similarity to MenA of menaquinone biosynthesis. The recently characterized bacterial PTase AuaA is involved in the biosynthesis of Auracin D [50] and is located inbetween the UbiA and protoheme IX farnesyltransferase clusters in the map depicted in Fig. 4B.

During our cluster analysis of membrane-bound aromatic PTases, we noticed that many bacterial and fungal genomes contain not one but several genes for (biochemically not yet characterized) proteins annotated as "UbiA prenyltransferase" or similar. For instance, the genome of *Salinispora tropica* contains two genes annotated as "4-hydroxybenzoate polyprenyltransferase" (YP_001160901) and "UbiA PTase" (YP_001161073). The genome of *Catenulispora acidiphila* likewise contains a gene annotated as "4-hydroxybenzoate polyprenyltransferase" (YP_003118736) and in addition three "UbiA prenyltransferase" genes (YP_003112865, YP_003115669 and YP_003116365). Both organisms are Gram-positive bacteria which are believed to not produce ubiquinones [51]. It remains to be shown whether such UbiA-like enzymes may be involved in the biosynthesis of secondary metabolites. In plants several PTases with homology to enzymes of ubiquinone and plastoquinone biosynthesis have recently been shown to be involved in the biosynthesis of important secondary metabolites [52].

Both the membrane-bound and the soluble aromatic PTases show remarkable promiscuity for their aromatic substrates and have been used for the chemoenzymatic synthesis of new prenylated aromatic compounds [53,54,55,56,57,58]. Protein engineering has allowed altering the substrate specificity of indole PTases [9,59]. Therefore, these PTases may represent promising tools for biotechnological and pharmaceutical research.

## Supporting Information

**Table S1** Proteins included in this study.
(PDF)

## Author Contributions

Conceived and designed the experiments: TB VA ANL LH. Performed the experiments: TB VA. Analyzed the data: TB VA. Contributed reagents/materials/analysis tools: VA ANL. Wrote the paper: TB VA OS ANL LH.

## References


1. Heide L (2009) Prenyl transfer to aromatic substrates: genetics and enzymology. Curr Opin Chem Biol 13: 171–179.
2. Poulter C (2006) Farnesyl diphosphate synthase. A paradigm for understanding structure and function relationships in *E*-polyprenyl diphosphate synthases. Phytochem Rev 5: 17–26.
3. Bräuer L, Brandt W, Schulze D, Zakharova S, Wessjohann L (2008) A structural model of the membrane-bound aromatic prenyltransferase UbiA from *E. coli*. Chembiochem 9: 982–992.
4. Pojer F, Wemakor E, Kammerer B, Chen H, Walsh CT, et al. (2003) CloQ, a prenyltransferase involved in clorobiocin biosynthesis. Proc Natl Acad Sci U S A 100: 2316–2321.
5. Kuzuyama T, Noel JP, Richard SB (2005) Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. Nature 435: 983–987.
6. Tello M, Kuzuyama T, Heide L, Noel JP, Richard SB (2008) The ABBA family of aromatic prenyltransferases: broadening natural product diversity. Cell Mol Life Sci 65: 1459–1463.
7. Metzger U, Keller S, Stevenson CE, Heide L, Lawson DM (2010) Structure and mechanism of the magnesium-independent aromatic prenyltransferase CloQ from the clorobiocin biosynthetic pathway. J Mol Biol 404: 611–626.
8. Metzger U, Schall C, Zocher G, Unsöld I, Stec E, et al. (2009) The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria. Proc Natl Acad Sci U S A 106: 14309–14314.
9. Steffan N, Grundmann A, Yin WB, Kremer A, Li SM (2009) Indole prenyltransferases from fungi: a new enzyme group with high potential for the production of prenylated indole derivatives. Curr Med Chem 16: 218–231.
10. Jost M, Zocher G, Tarcz S, Matuschek M, Xie X, et al. (2010) Structure-function analysis of an enzymatic prenyl transfer reaction identifies a reaction chamber with modifiable specificity. J Am Chem Soc 132: 17849–17858.
11. Edwards DJ, Gerwick WH (2004) Lyngbyatoxin biosynthesis: sequence of biosynthetic gene cluster and identification of a novel aromatic prenyltransferase. J Am Chem Soc 126: 11432–11433.
12. Schultz AW, Lewis CA, Luzung MR, Baran PS, Moore BS (2010) Functional characterization of the cyclomarin/cyclomarazine prenyltransferase CymD directs the biosynthesis of unnatural cyclic peptides. J Nat Prod 73: 373–377.
13. Takahashi S, Takagi H, Toyoda A, Uramoto M, Nogawa T, et al. (2010) Biochemical characterization of a novel indole prenyltransferase from *Streptomyces* sp. SN-593. J Bacteriol 192: 2839–2851.
14. Cheng H, Kim B-H, Grishin NV (2008) MALISAM: a database of structurally analogous motifs in proteins. Nucleic Acids Res 36: D211–D217.
15. Alva V, Remmert M, Biegert A, Lupas AN, Soding J (2010) A galaxy of folds. Protein Sci 19: 124–130.
16. Copley RR, Bork P (2000) Homology among (betaalpha)(8) barrels: implications for the evolution of metabolic pathways. J Mol Biol 303: 627–641.

17. Nagano N, Orengo CA, Thornton JM (2002) One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. J Mol Biol 321: 741–765.
18. Söding J, Remmert M, Biegert A (2006) HHrep: de novo protein repeat detection and the origin of TIM barrels. Nucleic Acids Res 34: W137–142.
19. Söding J (2005) Protein homology detection by HMM-HMM comparison. Bioinformatics 21: 951–960.
20. Zhang Y, Skolnick J (2005) TM-align: a protein structure alignment algorithm based on the TM-score. Nucleic Acids Res 33: 2302–2309.
21. Söding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res 33: W244–248.
22. Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, et al. (2005) Protein database searches using compositionally adjusted substitution matrices. FEBS Journal 272: 5101–5109.
23. Biegert A, Mayer C, Remmert M, Söding J, Lupas AN (2006) The MPI Bioinformatics Toolkit for protein sequence analysis. Nucleic Acids Res 34: W335–339.
24. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
25. Frickey T, Lupas A (2004) CLANS: a Java application for visualizing protein families based on pairwise similarity. Bioinformatics 20: 3702–3704.
26. Biegert A, Söding J (2008) De novo identification of highly diverged protein repeats by probabilistic consistency. Bioinformatics 24: 807–814.
27. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.
28. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.
29. Sadreyev R, Grishin N (2003) COMPASS: a tool for comparison of multiple protein alignments with assessment of statistical significance. J Mol Biol 326: 317–336.
30. Eddy SR (1998) Profile hidden Markov models. Bioinformatics 14: 755–763.
31. Bayer P, Arndt A, Metzger S, Mahajan R, Melchior F, et al. (1998) Structure determination of the small ubiquitin-related modifier SUMO-1. J Mol Biol 280: 275–286.
32. Alva V, Ammelburg M, Söding J, Lupas AN (2007) On the origin of the histone fold. BMC Struct Biol 7: 17.
33. Kopec KO, Alva V, Lupas AN (2010) Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria. Bioinformatics 26: 1927–1931.
34. Remmert M, Biegert A, Linke D, Lupas AN, Söding J (2010) Evolution of outer membrane beta-barrels from an ancestral beta beta hairpin. Mol Biol Evol 27: 1348–1358.
35. Chaudhuri I, Söding J, Lupas AN (2008) Evolution of the beta-propeller fold. Proteins 71: 795–803.
36. Asai K, Fujisaki S, Nishimura Y, Nishino T, Okada K, et al. (1994) The identification of *Escherichia coli ispB* (cel) gene encoding the octaprenyl diphosphate synthase. Biochem Biophys Res Commun 202: 340–345.
37. Melzer M, Heide L (1994) Characterization of polyprenyldiphosphate: 4-hydroxybenzoate polyprenyltransferase from *Escherichia coli*. Biochim Biophys Acta 1212: 93–102.
38. Suvarna K, Stevenson D, Meganathan R, Hudspeth ME (1998) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the *menA* gene from *Escherichia coli*. J Bacteriol 180: 2782–2787.
39. Savidge B, Weiss JD, Wong YH, Lassner MW, Mitsky TA, et al. (2002) Isolation and characterization of homogentisate phytyltransferase genes from *Synechocystis* sp. PCC 6803 and *Arabidopsis*. Plant Physiol 129: 321–332.
40. Saiki K, Mogi T, Anraku Y (1992) Heme O biosynthesis in *Escherichia coli*: The *cyoE* gene in the cytochrome *bo* operon encodes a protoheme IX farnesyltransferase. Biochem Biophys Res Commun 189: 1491–1497.
41. Oster U, Bauer CE, Rudiger W (1997) Characterization of chlorophyll a and bacteriochlorophyll a synthases by heterologous expression in *Escherichia coli*. J Biol Chem 272: 9671–9676.
42. Huang H, Scherman MS, D'Haeze W, Vereecke D, Holsters M, et al. (2005) Identification and active expression of the *Mycobacterium tuberculosis* gene encoding 5-phospho-a-D-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase, the first enzyme committed to decaprenylphosphoryl-D-arabinose synthesis. J Biol Chem 280: 24539–24543.
43. Koonin EV, Tatusov RL (1994) Computer analysis of bacterial haloacid dehalogenases defines a large superfamily of hydrolases with diverse specificity. Application of an iterative approach to database search. J Mol Biol 244: 125–132.
44. Zou HX, Xie X, Zheng XD, Li SM (2010) The tyrosine *O*-prenyltransferase SirD catalyzes *O*-, *N*-, and *C*-prenylations. Appl Microbiol Biotechnol 89: 1443–1451.
45. Chooi YH, Cacho R, Tang Y (2010) Identification of the viridicatumtoxin and griseofulvin gene clusters from *Penicillium aethiopicum*. Chem Biol 17: 483–494.
46. Balibar CJ, Howard-Jones AR, Walsh CT (2007) Terrequinone A biosynthesis through L-tryptophan oxidation, dimerization and bisprenylation. Nat Chem Biol 3: 584–592.
47. Awakawa T, Fujita N, Hayakawa M, Ohnishi Y, Horinouchi S (2011) Characterization of the biosynthesis gene cluster for alkyl-*O*-dihydrogeranylmethoxyhydroquinones in *Actinoplanes missouriensis*. Chembiochem 12: 439–448.
48. Itoh T, Tokunaga K, Matsuda Y, Fujii I, Abe I, et al. (2010) Reconstitution of a fungal meroterpenoid biosynthesis reveals the involvement of a novel family of terpene cyclases. Nat Chem 2: 858–864.
49. Dairi T (2005) Studies on biosynthetic genes and enzymes of isoprenoids produced by actinomycetes. J Antibiot (Tokyo) 58: 227–243.
50. Stec E, Pistorius D, Muller R, Li SM (2011) AuaA, a membrane-bound farnesyltransferase from Stigmatella aurantiaca, catalyzes the prenylation of 2-methyl-4-hydroxyquinoline in the biosynthesis of aurachins. Chembiochem 12: 1724–1730.
51. Nowicka B, Kruk J (2010) Occurrence, biosynthesis and function of isoprenoid quinones. Biochim Biophys Acta 1797: 1587–1605.
52. Yazaki K, Sasaki K, Tsurumaru Y (2009) Prenylation of aromatic compounds, a key diversification of plant secondary metabolites. Phytochemistry 70: 1739–1745.
53. Koeduka T, Shitan N, Kumano T, Sasaki K, Sugiyama A, et al. (2011) Production of prenylated flavonoids in tomato fruits expressing a prenyltransferase gene from *Streptomyces coelicolor* A3(2). Plant Biol 13: 411–415.
54. Shindo K, Tachibana A, Tanaka A, Toba S, Yuki E, et al. (2011) Production of novel antioxidative prenyl naphthalen-ols by combinational bioconversion with dioxygenase PhnA1A2A3A4 and prenyltransferase NphB or SCO7190. Biosci Biotechnol Biochem 75: 505–510.
55. Kumano T, Richard SB, Noel JP, Nishiyama M, Kuzuyama T (2008) Chemoenzymatic syntheses of prenylated aromatic small molecules using *Streptomyces* prenyltransferases with relaxed substrate specificities. Bioorg Med Chem 16: 8117–8126.
56. Macone A, Lendaro E, Comandini A, Rovardi I, Matarese RM, et al. (2009) Chromane derivatives of small aromatic molecules: Chemoenzymatic synthesis and growth inhibitory activity on human tumor cell line LoVo WT. Bioorg Med Chem 17: 6003–6007.
57. Ozaki T, Mishima S, Nishiyama M, Kuzuyama T (2009) NovQ is a prenyltransferase capable of catalyzing the addition of a dimethylallyl group to both phenylpropanoids and flavonoids. J Antibiot (Tokyo) 62: 385–392.
58. Xiao Y, Machacek M, Lee K, Kuzuyama T, Liu P (2009) Prenyltransferase substrate binding pocket flexibility and its application in isoprenoid profiling. Mol Biosyst 5: 913–917.
59. Li SM (2009) Applications of dimethylallyltryptophan synthases and other indole prenyltransferases for structural modification of natural products. Appl Microbiol Biotechnol 84: 631–639.

**SUPPLEMENTAL DATA FOR**

# Evolutionary Relationships of Microbial Aromatic Prenyltransferases

T. Bonitz, V. Alva, O. Saleh, A. N. Lupas, L. Heide

## Table S1: Proteins included in this study

| Name | organism | NCBI Accession | Reference for biochemical data |
|---|---|---|---|
| **NphB/CloQ family (phenol/phenazine prenyltransferases)** | | | |
| CloQ* | *Streptomyces roseochromogenes subsp. oscitans* | AAN65239 | [1] |
| Fnq26* | *Streptomyces cinnamonensis* | CAL34104 | [2] |
| Fur7* | *Streptomyces* sp. KO-3988 | BAE78975 | [3] |
| NphB* | *Streptomyces* sp. Strain CI190 | 1ZB6_A | [4] |
| NovQ* | *Streptomyces niveus* DSM 40088 | AAF67510 | [5] |
| EpzP* | *Streptomyces cinnamonensis* | ADQ43372 | [6] |
| PpzP* | *Streptomyces anulatus* | CAX48655 | [7] |
| $Ptf_{At}$* | *Aspergillus terreus* NIH2624 | EAU39467 | [8] |
| $Ptf_{Bf}$* | *Botryotinia fuckeliana* B05.10 | EDN25735 | |
| $Ptf_{Ss}$* | *Sclerotinia sclerotiorum* 1980 | EDN93598 | |
| SCO7190* | *Streptomyces coelicolor* A3(2) | NP_631248 | [9] |
| **DMATS/CymD family a) fungal indole prenyltransferases** | | | |
| DmaW-Cp* | *Claviceps purpurea* | Q6X2E0 | [10] |
| DmaW-Cs* | *Clavicipitaceae* sp. US2005a | AAZ29613 | [11] |
| DmaW-Cf* | *Claviceps fusiformis* | AAC18893 | [12] |
| DmaW-Lp1* | *Epichloe typhina* x *Neotyphodium lolii* | AAP81206 | |
| FgaPT1* | *Aspergillus fumigatus* Af293 | XP_756136 | [13] |
| FgaPT2* | *Aspergillus fumigatus* | AAX08549/ 3I4Z_A | [14] |
| FtmPT1* | *Aspergillus fumigatus* | AAX56314/ 3O2K_A | [15] |
| FtmPT2* | *Aspergillus fumigatus* | ACF22981 | [16] |
| CdpNPT* | *Aspergillus fumigatus* | ABR14712 | [17] |
| AnaPT* | *Neosartorya fischeri* NRRL 181 | EAW16181 | [18] |
| 7-DMATS* | *Aspergillus fumigatus* | ABS89001 | [19] |
| TdiB* | *Emericella nidulans* | ABU51603 | [20] |
| MaPT* | *Malbranchea aurantiaca* RRC1813 | ABZ80612 | [21] |
| NotC* | *Aspergillus* sp. MF297-2 | ADM34131 | [22] |
| NotF* | *Aspergillus* sp. MF297-2 | ADM34132 | |
| CpaD_*Ao** | *Aspergillus oryzae* RIB40 | XP_001821505 | [23] |
| CpaD_*Af** | *Aspergillus flavus* NRRL3357 | XP_002379958 | |

| | | | |
|---|---|---|---|
| XptA* | *Aspergillus nidulans* FGSC A4 | XP_664388 | [24] |
| XptB* | | see reference | |
| SirD* | *Leptosphaeria maculans* | AAS92554 | [25] |
| VrtC* | *Penicillium aethiopicum* | ADI24928 | [26] |
| **DMATS/CymD family<br>b) bacterial indole prenyltransferases** | | | |
| IptA* = 6-dimethylallyl-tryptophan synthase | *Streptomyces* sp. SN-593 | BAJ07990 | [27] |
| CymD* = Sare_4565 | *Salinispora arenicola* CNS-205 | YP_001539324 | [28] |
| LtxC* | *Lyngbya majuscula* | AAT12285 | [29] |
| SCO7467* | *Streptomyces coelicolor* A3(2) | NP_631515 | -- |
| **Prenyltransferases of lipoquinone biosynthesis** | | | |
| UbiA | *Escherichia coli* str. K-12 substr. W3110 | AP_004541 | [30] |
| MenA | *Escherichia coli* | AAB01207 | [31] |
| Slr1736 | *Synechocystis* sp. PCC 6803 | BAA17774 | [32] |
| UbiA | *Salinispora tropica* CNB-440 | YP_001160901 | -- |
| UbiA | *Salinispora tropica* CNB-440 | YP_001161073 | -- |
| UbiA | *Catenulispora acidiphila* DSM 44928 | YP_003118736 | -- |
| UbiA | *Catenulispora acidiphila* DSM 44928 | YP_003112865 | -- |
| UbiA | *Catenulispora acidiphila* DSM 44928 | YP_003115669 | -- |
| UbiA | *Catenulispora acidiphila* DSM 44928 | YP_003116365 | -- |

* Used in HHpred analysis (Figure 3)

## REFERENCES


1. Pojer F, Wemakor E, Kammerer B, Chen H, Walsh CT, et al. (2003) CloQ, a prenyltransferase involved in clorobiocin biosynthesis. Proc Natl Acad Sci U S A 100: 2316-2321.
2. Haagen Y, Glück K, Fay K, Kammerer B, Gust B, et al. (2006) A gene cluster for prenylated naphthoquinone and prenylated phenazine biosynthesis in *Streptomyces cinnamonensis* DSM 1042. Chembiochem 7: 2016-2027.
3. Kumano T, Tomita T, Nishiyama M, Kuzuyama T (2010) Functional characterization of the promiscuous prenyltransferase responsible for furaquinocin biosynthesis: identification of a physiological polyketide substrate and its prenylated reaction products. J Biol Chem.
4. Kuzuyama T, Noel JP, Richard SB (2005) Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. Nature 435: 983-987.
5. Ozaki T, Mishima S, Nishiyama M, Kuzuyama T (2009) NovQ is a prenyltransferase capable of catalyzing the addition of a dimethylallyl group to both phenylpropanoids and flavonoids. J Antibiot (Tokyo) 62: 385-392.
6. Seeger K, Flinspach K, Haug-Schifferdecker E, Kulik A, Gust B, et al. (2011) The biosynthetic genes for prenylated phenazines are located at two different chromosomal loci of *Streptomyces cinnamonensis* DSM 1042. Microb Biotechnol 4: 252-262.

7. Saleh O, Gust B, Boll B, Fiedler HP, Heide L (2009) Aromatic prenylation in phenazine biosynthesis: dihydrophenazine-1-carboxylate dimethylallyltransferase from *Streptomyces anulatus*. J Biol Chem 284: 14439-14447.
8. Haug-Schifferdecker E, Arican D, Brückner R, Heide L (2010) A new group of aromatic prenyltransferases in fungi, catalyzing a 2,7-dihydroxynaphthalene 3-dimethylallyl-transferase reaction. J Biol Chem 285: 16487-16494.
9. Kumano T, Richard SB, Noel JP, Nishiyama M, Kuzuyama T (2008) Chemoenzymatic syntheses of prenylated aromatic small molecules using *Streptomyces* prenyltransferases with relaxed substrate specificities. Bioorg Med Chem 16: 8117-8126.
10. Steffan N, Grundmann A, Yin WB, Kremer A, Li SM (2009) Indole prenyltransferases from fungi: a new enzyme group with high potential for the production of prenylated indole derivatives. Curr Med Chem 16: 218-231.
11. Markert A, Steffan N, Ploss K, Hellwig S, Steiner U, et al. (2008) Biosynthesis and accumulation of ergoline alkaloids in a mutualistic association between *Ipomoea asarifolia* (Convolvulaceae) and a Clavicipitalean Fungus. Plant Physiol 147: 296-305.
12. Wang J, Machado C, Panaccione DG, Tsai HF, Schardl CL (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genet Biol 41: 189-198.
13. Unsöld IA, Li SM (2006) Reverse prenyltransferase in the biosynthesis of fumigaclavine C in *Aspergillus fumigatus*: gene expression, purification, and characterization of fumigaclavine C synthase FGAPT1. Chembiochem 7: 158-164.
14. Metzger U, Schall C, Zocher G, Unsöld I, Stec E, et al. (2009) The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria. Proc Natl Acad Sci U S A 106: 14309-14314.
15. Jost M, Zocher G, Tarcz S, Matuschek M, Xie X, et al. (2010) Structure-function analysis of an enzymatic prenyl transfer reaction identifies a reaction chamber with modifiable specificity. J Am Chem Soc 132: 17849-17858.
16. Grundmann A, Kuznetsova T, Afiyatullov SS, Li S-M (2008) FtmPT2, an *N*-prenyltransferase from *Aspergillus fumigatus*, catalyses the last step in the biosynthesis of Fumitremorgin B. Chembiochem 9: 2059-2063.
17. Yin WB, Ruan HL, Westrich L, Grundmann A, Li S-M (2007) CdpNPT, an *N*-Prenyltransferase from *Aspergillus fumigatus*: Overproduction, Purification and Biochemical Characterisation. Chembiochem 8: 1154-1161.
18. Yin WB, Grundmann A, Cheng J, Li SM (2009) Acetylaszonalenin biosynthesis in *Neosartorya fischeri*. Identification of the biosynthetic gene cluster by genomic mining and functional proof of the genes by biochemical investigation. J Biol Chem 284: 100-109.
19. Kremer A, Westrich L, Li SM (2007) A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization. Microbiology 153: 3409-3416.
20. Schneider P, Weber M, Hoffmeister D (2008) The *Aspergillus nidulans* enzyme TdiB catalyzes prenyltransfer to the precursor of bioactive asterriquinones. Fungal Genet Biol 45: 302-309.
21. Ding Y, Williams RM, Sherman DH (2008) Molecular analysis of a 4-dimethylallyltryptophan synthase from *Malbranchea aurantiaca*. J Biol Chem 283: 16068-16076.
22. Ding Y, de Wet JR, Cavalcoli J, Li S, Greshock TJ, et al. (2010) Genome-based characterization of two prenylation steps in the assembly of the stephacidin and notoamide anticancer agents in a marine-derived *Aspergillus* sp. J Am Chem Soc 132:

12733-12740.
23. Liu X, Walsh CT (2009) Characterization of cyclo-acetoacetyl-L-tryptophan dimethylallyltransferase in cyclopiazonic acid biosynthesis: substrate promiscuity and site directed mutagenesis studies. Biochemistry 48: 11032-11044.
24. Sanchez JF, Entwistle R, Hung JH, Yaegashi J, Jain S, et al. (2011) Genome-based deletion analysis reveals the prenyl xanthone biosynthesis pathway in *Aspergillus nidulans*. J Am Chem Soc.
25. Zou HX, Xie X, Zheng XD, Li SM (2010) The tyrosine *O*-prenyltransferase SirD catalyzes *O*-, *N*-, and *C*-prenylations. Appl Microbiol Biotechnol 89: 1443-1451.
26. Chooi YH, Cacho R, Tang Y (2010) Identification of the viridicatumtoxin and griseofulvin gene clusters from *Penicillium aethiopicum*. Chem Biol 17: 483-494.
27. Takahashi S, Takagi H, Toyoda A, Uramoto M, Nogawa T, et al. (2010) Biochemical characterization of a novel indole prenyltransferase from *Streptomyces* sp. SN-593. J Bacteriol 192: 2839-2851.
28. Schultz AW, Oh DC, Carney JR, Williamson RT, Udwary DW, et al. (2008) Biosynthesis and structures of cyclomarins and cyclomarazines, prenylated cyclic peptides of marine actinobacterial origin. J Am Chem Soc 130: 4507-4516.
29. Edwards DJ, Gerwick WH (2004) Lyngbyatoxin biosynthesis: sequence of biosynthetic gene cluster and identification of a novel aromatic prenyltransferase. J Am Chem Soc 126: 11432-11433.
30. Melzer M, Heide L (1994) Characterization of polyprenyldiphosphate: 4-hydroxybenzoate polyprenyltransferase from *Escherichia coli*. Biochim Biophys Acta 1212: 93-102.
31. Suvarna K, Stevenson D, Meganathan R, Hudspeth ME (1998) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the *menA* gene from *Escherichia coli*. J Bacteriol 180: 2782-2787.
32. Savidge B, Weiss JD, Wong YH, Lassner MW, Mitsky TA, et al. (2002) Isolation and characterization of homogentisate phytyltransferase genes from *Synechocystis* sp. PCC 6803 and *Arabidopsis*. Plant Physiol 129: 321-332.

MedChemComm

Dynamic Article Links

Cite this: *Med. Chem. Commun.*, 2012, **3**, 1009

www.rsc.org/medchemcomm

CONCISE ARTICLE

# Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines†‡

**Orwah Saleh,§[a] Tobias Bonitz,§[a] Katrin Flinspach,[a] Andreas Kulik,[b] Nadja Burkard,[c] Agnes Mühlenweg,[d] Andreas Vente,[d] Stefan Polnick,[a] Michael Lämmerhofer,[a] Bertolt Gust,[a] Hans-Peter Fiedler[b] and Lutz Heide*[a]**

***Received 15th February 2012, Accepted 30th March 2012***
**DOI: 10.1039/c2md20045g**



The activation of silent biosynthetic gene clusters is a principal challenge for genome mining strategies in drug discovery. In the present study, a phenazine biosynthetic gene cluster was discovered in the Gram-positive bacterium *Streptomyces tendae* Tü1028. This gene cluster remained silent under a multitude of cultivation conditions, both in the genuine producer strain and in a heterologous expression strain. However, introduction of a constitutive promoter upstream of the phenazine biosynthesis genes led to the production of phenazine-1-carboxylic acid (PCA) and of a new derivative thereof, *i.e.* a conjugate of PCA and L-glutamine. The linkage of PCA to L-glutamine by amide bond formation was catalyzed by enzymes of the heterologous expression host *Streptomyces coelicolor* M512. PCA showed a strong antibiotic effect, but PCA-Gln did not. Glutamination of PCA therefore appears to represent a resistance mechanism against the antibiotic PCA, which can be produced in significant quantities in soil by *Pseudomonas* strains. The gene cluster also contained genes for all enzymes of the mevalonate pathway and for an aromatic prenyltransferase, thereby resembling gene clusters for prenylated phenazines. However, purification and biochemical investigation of the prenyltransferase proved that it does not prenylate phenazines but hydroxynaphthalene substrates, showing very similar properties as NphB of naphterpin biosynthesis (Kuzuyma *et al.*, *Nature*, 2005, **435**, 983–987.).

## Introduction

Secondary metabolites of actinomycetes and fungi, and their chemical derivatives, are the primary source of antibacterial agents for medical use.[1] Between 1981 and 2006, 68% of newly introduced antibacterial agents and 54% of anticancer agents were natural products or semisynthetic derivatives thereof.[2,3] The recently initiated large scale microbial genome sequencing projects have triggered a revolution in the genetics and biochemistry of natural product biosynthesis.[4] They have shown that a single strain, *e.g.* of the actinomycetes, has the genetic capacity to produce 10–30 different secondary metabolites, but usually only 2–3 of them are currently known. This indicates that around 90% of secondary metabolic gene clusters of previously investigated strains are still "cryptic", *i.e.* their products are unknown. The investigation of these "cryptic" gene clusters is likely to allow the discovery of many so far unknown natural products and therefore represents a novel avenue to drug discovery.[2] However, many of these clusters are silent, *i.e.* they are not expressed under the currently used culture conditions. The development of methods for the activation of such silent clusters is one of the most important prerequisites for the successful use of genome mining strategies for drug discovery.[5] So far, there are only few examples for the successful activation of silent gene clusters by genetic manipulation. Activation was achieved by either the overexpression of a positive regulator gene[6,7] or the inactivation of a negative regulatory gene.[8–10] However, the regulation of the biosynthesis of secondary metabolites is complex, encompassing different types of transcriptional regulators, environmental factors and an intricate cascade of intracellular signals to achieve the final regulatory effect. In many cases, it may not be possible to achieve the activation of an entire cluster by simple overexpression or deletion of a certain regulatory gene, and also it may be difficult to identify the genes which are most important for the regulation. Therefore, additional strategies which can be used for the activation of silent gene clusters need to be developed.

We encountered the need for such an activation strategy in the present investigation of a silent phenazine biosynthetic gene cluster from a *Streptomyces* strain.

[a]*Pharmaceutical Institute, University of Tübingen, 72076 Tübingen, Germany. E-mail: heide@uni-tuebingen.de; Fax: +497071295250; Tel: +4970712972460*
[b]*Institute of Microbiology and Infection Medicine, University of Tübingen, 72076 Tübingen, Germany*
[c]*Institute of Organic Chemistry, University of Tübingen, 72076 Tübingen, Germany*
[d]*MerLion Pharmaceuticals GmbH, 13125 Berlin, Germany*

† This article is part of the *MedChemComm* natural products themed issue.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c2md20045g

§ These authors contributed equally to this work.

Phenazine natural products show antibacterial, antitumor and antimalarial activity. They are inhibitors of angiotensin-converting enzyme and steroid 5-α-reductase, and they act as virulence factors in pathogenesis.[11] Besides their role as antibiotics, phenazines have a variety of biological functions for the producing bacterium, often related to their capability to shuttle electrons by reversible oxidation and reduction.[12] Phenazine biosynthesis is limited to the actinobacteria (*e.g. Streptomyces*), certain groups of Gram-negative proteobacteria (*e.g. Pseudomonas*) and a few archaea.[13] *Pseudomonas* strains produce simple phenazines such as phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN). In contrast, *Streptomyces* strains can form more complex phenazines with very diverse substitution patterns of the phenazine core.

Many phenazine biosynthetic gene clusters have been identified in Gram-negative proteobacteria,[13] but so far only two have been described in *Streptomyces*.[14–16] Both these *Streptomyces* gene clusters show a core of seven biosynthetic genes (*phzBCDEFGA*) responsible for the formation of PCA. Additionally, they show genes involved in the modification of the phenazine core, *e.g.* by prenylation, methylation or oxidation. In the present study, we screened the genomic DNA of 190 strains for phenazine biosynthesis genes. Five strains were found to contain a putative phenazine biosynthetic gene cluster. In one of these strains, *Streptomyces tendae* Tü1028, we found additional genes which suggested the possible formation of a prenylated phenazine. This strain was selected for further investigation. However, this phenazine biosynthetic gene cluster was silent both in the genuine producer strain and in a heterologous producer strain, despite the use of many different culture media. Eventually, introduction of a constitutive *ermE** promoter upstream of the phenazine biosynthesis genes triggered the formation of two phenazine compounds. One of them is a new, previously undescribed phenazine derivative.

## Results and discussion

### Genomic screening for potential phenazine-producing strains

We screened a collection of 190 *Streptomyces* strains for the presence of phenazine biosynthesis genes. Degenerate primers for the gene *phzB* were developed (see Experimental procedures) and used to screen genomic DNA isolated from all 190 strains. The resulting PCR products were cloned and sequenced. Cosmid libraries of all investigated strains had been established previously using vector pOJ436.[17] From the strains containing a *phzB* ortholog, cosmids containing this gene were identified by Southern hybridization, and the presence of the *phzB* gene was additionally confirmed by PCR. The identified cosmids were re-screened with degenerate primers for the phenazine biosynthesis genes *phzD* and *phzF*.[13] In five of the investigated strains, cosmids were found which contained all three phenazine biosynthesis genes, as also confirmed by sequencing of the PCR products. These five strains were *Streptomyces olivaceus griseolus* Tü2353, *Streptomyces tendae* Tü1028, *Streptomyces* sp. 11412, *Streptomyces* Tü2873 and *Streptomyces* st. 2513/GT. None of these strains had been described previously as a producer of phenazines.

### Screening of cosmids for isoprenoid biosynthesis genes

Our group is involved in the investigation of a recently discovered group of prenyltransferases with aromatic substrates, termed ABBA prenyltransferases.[18–20] Two previously identified gene clusters for prenylated phenazines were found to contain genes for ABBA prenyltransferases, as well as a group of genes of the mevalonate pathway required for the generation of the prenyl moiety.[14–16] Therefore, the cosmids identified in the screening described above were further screened for the genes for 3-hydroxy-3-methylglutaryl-CoA synthase (*hmgs*), 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgr*) and mevalonate diphosphate decarboxylase (*mdpd*), respectively. Only cosmids from *S. tendae* Tü1028 showed PCR products for these mevalonate biosynthesis genes, the cosmids from the other four strains did not. Sequencing of the corresponding PCR products from *S. tendae* Tü1028 confirmed the identity of these genes.

### Screening of cosmids from *S. tendae* Tü1028 for ABBA prenyltransferase genes

17 cosmids from *S. tendae* Tü1028 had been found to contain *phzB*, *phzD* and *phzF*, and eight of these cosmids additionally contained the genes *hmgs*, *hmgr* and *mdpd*. All 17 cosmids were screened with degenerate primers for bacterial ABBA prenyltransferase (see Experimental procedures). Nine cosmids were found to contain the ABBA prenyltransferase gene, but only a single cosmid, termed CB15, showed the presence of all seven genes used in the present screening approach (*phzB*, *phzD*, *phzF*, *hmgs*, *hmgr*, *mdpd* and an ABBA prenyltransferase gene).

### Sequencing of cosmid CB15

Cosmid CB15 from *S. tendae* Tü1028 was subjected to full-length automated sequencing using a shotgun library of DNA fragments. The insert (39 kb) showed an overall G + C content of 70.6% and comprised 31 putative coding sequences (Fig. 1). Table 1 shows the results of database comparisons for these 31 genes. Seven of the putative coding sequences, designated *phzBCDEFGA*, showed obvious similarities to the seven core phenazine biosynthesis genes commonly found in phenazine biosynthesis gene clusters.[13] Of these, *phzC* codes for a DAHP synthase, catalysing the first step of the shikimate pathway. The other six genes code for enzymes which catalyze all steps in the conversion of chorismic acid to 5,10-dihydro-phenazine-1-carboxylic acid, the immediate precursor of phenazine-1-carboxylic acid (PCA).[13] These seven genes in *S. tendae* Tü1028 appear to form a single operon. The intergenic regions between these genes do not exceed 80 bp, and in three instances the adjacent genes show overlapping stop and start codons, suggesting translational coupling. BLAST searches showed very close similarity of *phzBCDEFGA* to the phenazine biosynthetic gene clusters of *Streptomyces anulatus*[14] and *Streptomyces cinnamonensis*.[15,16] The seven core phenazine biosynthesis genes in *S. anulatus* are organized in the same order as in *S. tendae* Tü1028, and the entire operons from the two strains show 82% identity to each other on the nucleotide level, indicating a very close evolutionary relationship.

However, while both *S. anulatus* and *S. cinnamonensis* contain a methyltransferase gene similar to *phzM* of *Pseudomonas* for the

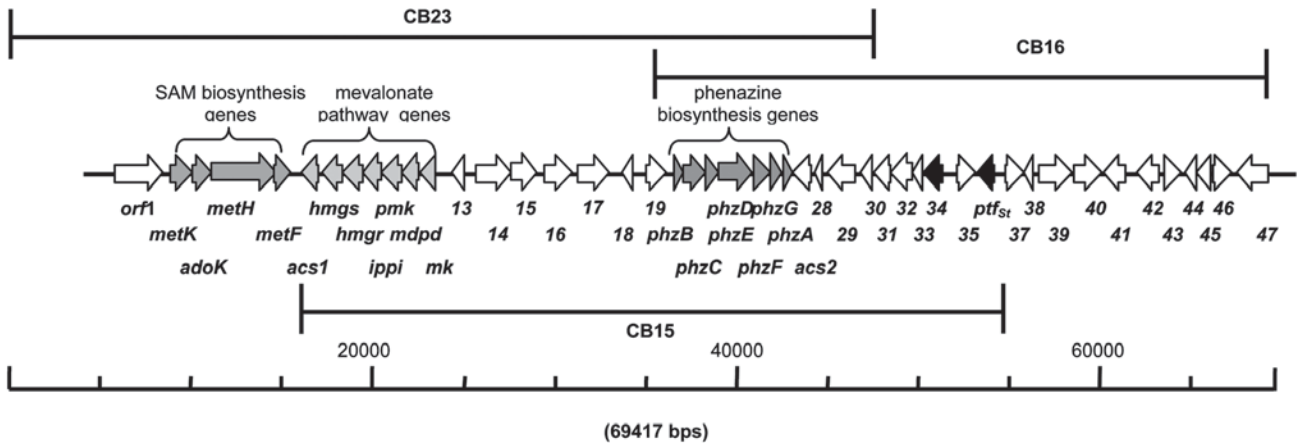



**Fig. 1** Secondary metabolic gene cluster identified in the genome of *S. tendae* Tü1028. The relative positions of the inserts of cosmids CB15, CB16 and CB23 are indicated.



formation of *N*-methylated phenazines,[21] no ortholog of this gene is found in *S. tendae* Tü1028, indicating that this strain cannot synthesize *N*-methylated phenazines.

The left end of the insert of cosmid CB15 as depicted in Fig. 1 contained an operon of seven genes of the mevalonate pathway, encoding all enzymes required for the biosynthesis of the isoprenoid precursor dimethylallyldiphoshate (DMAPP). The first enzyme of this pathway has only recently been characterized in streptomycetes.[22] It catalyzes the conversion of acetyl-CoA and malonyl-CoA to acetoacetyl-CoA, a reaction which is different from the first step of the mevalonate pathway in eukaryotes. Again, this operon of mevalonate biosynthesis genes shows very high similarity to the corresponding operons in *S. anulatus* and *S. cinnamonensis*. In all three strains, the genes are organized in the same order, and the entire operons from the three strains show approximately 80% sequence identity to each other on the nucleotide level.

In contrast to *S. anulatus* and *S. cinnamonensis*, *S. tendae* Tü1028 contained an additional copy of the acetoacetyl-CoA synthase gene, termed *acs2* and located adjacent to the phenazine biosynthesis genes. *acs2* shows 100% identity on the nucleotide level to its paralog *acs1* which is located adjacent to the other mevalonate biosynthesis genes.

At the right end of the insert of cosmid CB15 (Fig. 1), a putative ABBA prenyltransferase gene was found and designated *ptf$_{St}$*. Its predicted product (305 aa) showed similarity to the phenazine prenyltransferase genes *ppzP* from *S. anulatus* and *epzP* from *S. cinnamonensis*, and to prenyltransferases involved in the biosynthesis of prenylated naphthalene derivatives such as NphB from *Streptomyces* sp. CL190 [23] or Fnq26 from *S. cinnamonensis*.[24]

Furthermore, the cluster contained a gene coding for a putative polyprenyl diphosphate synthase (*orf19*) and a putative type III polyketide synthase gene (*orf34*). Similar genes have been found in gene clusters for prenylated naphthalenes like naphterpin and furanonaphthoquinone I.[15,23]

### Cultivation of *S. tendae* Tü1028 and analysis for phenazines and prenylated secondary metabolite

To investigate the production of secondary metabolites, *S. tendae* Tü1028 was cultivated in the medium described for the production of prenylated phenazines and prenylated polyketides in *S. cinnamonensis*[25] for 5, 7 and 9 days. However, HPLC-UV analysis of the culture supernatant and of mycelia extracts did not show any products with the typical UV absorption of phenazines. The strain was then cultivated in the media described for production of the phenazines aestivophoenin,[26] phenazoviridin,[27] saphenamycin[28] and griseolutein.[29] Finally, the strain was cultivated in 9 other media previously used for the production of secondary metabolites by *Streptomyces* strains (see ESI‡), but no phenazines could be detected. Likewise, no product with a similar UV absorption as naphterpin[30] or furanonaphthoquinone I [15] was observed. HPLC-MS was used to search for masses of known phenazines, prenylated phenazines and prenylated polyketides, but without success. Following a suggestion by Seto *et al.*,[56] we cultivated the strain in the presence and absence of the HMG-CoA reductase inhibitor pravastatin (3–14 mM) and compared the chromatograms in order to detect products formed under involvement of the mevalonate pathway, but again without success. The identified gene cluster apparently remained silent under all investigated conditions.

### Heterologous expression of cosmid CB15 from *S. tendae* in *S. coelicolor* M512

In a previous study,[14] the heterologous expression of cosmid 18A9 from *S. anulatus* which comprised similar genes as CB15 from *S. tendae* Tü1028 had resulted in the production of both PCA and its prenylated derivative endophenazine A. Therefore, we decided to heterologously express cosmid CB15.

Cosmid CB15 carries an *oriT* for conjugal transfer into *Streptomyces* and the ΦC31 integration functions for integration into the host genome.[17] CB15 was introduced into *S. coelicolor* M512 by conjugation. Three independent exconjugants were selected and cultivated in the production media used for phenazine production by *S. anulatus*.[14] Extracts of the culture supernatant and of the mycelia were investigated for PCA and for PCA derivatives by HPLC-UV and HPLC-MS. However, we could not detect any phenazine formation. The same heterologous expression experiment was carried out with seven other cosmids from *S. tendae* Tü1028, containing phenazine biosynthesis genes, but again without success. This indicated that the

**Table 1** Genes identified on cosmids CB15, CB16 and CB23 from *S. tendae* Tü1028. The overlap between the three cosmid inserts is depicted Fig. 1. The nucleotide sequence has been deposited in GenBank under accession number JQ659263

| Orf | aa | Proposed function | Ortholog identified by BLAST search | Identity/ similarity% | Accession |
|---|---|---|---|---|---|
| *Cosmid CB23* | | | | | |
| *orf1* | 880 | Putative peptide synthase | *Streptomyces rochei* | 46/56 | NP_851498 |
| *metK* | 402 | Putative *S*-adenosylmethionine synthetase | *Streptomyces pristinaespiralis* ATCC 25486 | 90/95 | ZP_06913987 |
| *adoK* | 327 | Putative adenosine kinase | *Streptomyces pristinaespiralis* ATCC 25486 | 74/81 | ZP_06913988 |
| *metH* | 1167 | Putative methionine synthase | *Streptomyces pristinaespiralis* ATCC 25486 | 86/92 | ZP_06913989 |
| *metF* | 301 | Putative 5,10-methylenetetrahydrofolate reductase | *Streptomyces aureofaciens* | 85/91 | ADM72814 |
| *Cosmid CB15* | | | | | |
| *acs1* | 325 | Acetoacetyl-CoA synthase | PpzT, *Streptomyces anulatus* | 74/82 | CAX48662 |
| *hmgs* | 389 | 3-Hydroxy-3-methylglutaryl CoA synthase | HMGS, *Streptomyces* sp. CL190 | 83/90 | BAB07795 |
| *hmgr* | 353 | 3-Hydroxy-3-methylglutaryl coenzyme A reductase | HMGR, *Streptomyces* sp. CL190 | 91/95 | BAA70975 |
| *ippi* | 364 | Isopentenyl diphosphate isomerase | *Streptomyces* sp. CL190 | 84/93 | Q9KWG2 |
| *pmk* | 365 | Phosphomevalonate kinase | PMEVK, *Kitasatospora griseola* | 71/82 | BAB07819 |
| *mdpd* | 299 | Mevalonate diphosphate decarboxylase | MDPD, *Streptomyces cinnamonensis* | 83/89 | ADQ43374 |
| *mk* | 263 | Mevalonate kinase | NapT6, *Streptomyces* sp. CNQ525 | 77/86 | ABS50475 |
| *orf13* | 226 | Conserved hypothetical protein, Ovm-Z like | NapU1 *Streptomyces* sp. CNQ525 | 65/76 | ABS50476 |
| *orf14* | 666 | Putative nitrogen regulatory protein | *Streptomyces ambofaciens* | 71/79 | CAK50991 |
| *orf15* | 480 | Putative 3-carboxymuconate cycloisomerase | *Streptomyces ambofaciens* | 77/83 | CAK50990 |
| *orf16* | 516 | Putative monooxygenase | Fnq24, *Streptomyces cinnamonensis* | 63/74 | CAL34102 |
| *orf17* | 576 | Putative cytochrome B subunit | Fnq25, *Streptomyces cinnamonensis* | 74/84 | CAL34103 |
| *orf18* | 214 | ECF subfamily RNA polymerase sigma factor | *Geodermatophilus obscurus* DSM 43160 | 43/54 | YP_003409857 |
| *orf19* | 359 | Polyprenyl diphosphate synthase | *Rhodococcus erythropolis* PR4 | 58/71 | BAH34278 |
| *phzB* | 162 | Putative enzyme of phenazine biosynthesis | *Streptomyces anulatus* | 80/91 | CAX48672 |
| *phzC* | 395 | 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate synthase | *Streptomyces anulatus* | 80/90 | CAX48671 |
| *phzD* | 207 | 2,3-Dihydro-3-hydroxy-anthranilate (DHHA) synthase | *Streptomyces cinnamonensis* | 88/92 | CAL34109 |
| *phzE* | 640 | 2-Amino-2-desoxy-isochorismate synthase | *Streptomyces anulatus* | 81/86 | CAX48669 |
| *phzF* | 279 | *trans*-2,3-Dihydro-3-hydroxyanthranilate isomerase | *Streptomyces anulatus* | 87/91 | CAX48668 |
| *phzG* | 233 | FMN-dependent oxidase | *Streptomyces anulatus* | 68/78 | CAX48667 |
| *phzA* | 155 | Putative enzyme of phenazine biosynthesis | *Streptomyces anulatus* | 83/90 | CAX48666 |
| *acs2* | 325 | Acetoacetyl-CoA synthase | PpzT, *Streptomyces anulatus* | 74/82 | CAX48662 |
| *orf28* | 153 | Putative transcriptional regulator | Fnq17, *Streptomyces cinnamonensis* | 52/68 | CAL34095 |
| *orf29* | 527 | Putative fatty acid CoA-ligase | NapB4 *Streptomyces* sp. CNQ525 | 81/88 | ABS50481 |
| *orf30* | 207 | Putative WrbA NAD(P)H:quinone oxidoreductase | Fnq10, *Streptomyces cinnamonensis* | 70/80 | CAL34088 |
| *orf31* | 332 | Putative methyltransferase | *Streptomyces* sp. KO-3988 | 73/85 | BAE78972 |
| *orf32* | 385 | Hypothetical protein | *Streptomyces* sp. KO-3988 | 79/87 | BAE78971 |
| *orf33* | 188 | Putative MomA-like oxygenase | *Streptomyces antibioticus* | 86/90 | BAD89290 |
| *orf34* | 355 | Putative type III polyketide synthase | RppA, *Streptomyces antibioticus* | 94/97 | BAB91443 |
| *orf35* | 332 | Sigma factor, includes region 2 | *Rhodococcus jostii* RHA1 | 51/63 | YP_700910 |
| $ptf_{St}$ | 305 | ABBA prenyltransferase | *Streptomyces* sp. CL190 | 83/90 | BAE00106 |
| *Cosmid CB16* | | | | | |
| *orf37* | 322 | Putative transcriptional regulator | *Thermomonospora curvata* DSM 43183 | 50/65 | YP_003299085 |
| *orf38* | 148 | Hypothetical protein | *Streptomyces hygroscopicus* ATCC 53653 | 88/92 | ZP_07295901 |
| *orf39* | 590 | Putative FAD-binding monooxygenase | *Salinispora tropica* CNB-440 | 60/75 | YP_001159014 |
| *orf40* | 503 | Putative drug resistance transporter | *Catenulispora acidiphila* DSM 44928 | 53/70 | YP_003111195 |
| *orf41* | 424 | Putative bilirubin oxidase | *Streptomyces pristinaespiralis* ATCC 25486 | 61/73 | ZP_06914024 |
| *orf42* | 399 | Hypothetical protein | *Anaeromyxobacter dehalogenans* 2CP-C | 33/49 | YP_463241 |
| *orf43* | 277 | Hypothetical protein | NapU4, *Streptomyces aculeolatus* | 53/67 | ABS50471 |
| *orf44* | 198 | Putative oxidoreductase | *Streptomyces avermitilis* MA-4680 | 90/94 | NP_821545 |
| *orf45* | 272 | Putative myo-inositol 1-monophosphatase | *Streptomyces avermitilis* MA-4680 | 93/96 | YP_003485917 |
| *orf46* | 306 | Putative LysR family transcriptional regulator | *Streptomyces avermitilis* MA-4680 | 93/98 | NP_821547 |
| *orf47* | 594 | Putative ABC transporter permease | *Streptosporangium roseum* DSM 43021 | 51/65 | YP_003338959 |

phenazine biosynthetic gene cluster was not expressed in the heterologous host.

### Introduction of a constitutive promoter upstream of the phenazine biosynthesis genes

Sequence analysis of the phenazine biosynthesis genes in cosmid CB15 gave no indication for any mutation which would impair the catalytic activity of the encoded enzymes. We therefore speculated that these genes were not expressed. The regulatory mechanisms of phenazine biosynthesis in *Pseudomonas* are quite complicated, as *e.g.* shown for the biosynthesis of phenazine-1-carboxamide in *P. chlororaphis* PCL 1391,[31] and the regulatory mechanisms of phenazine biosynthesis in *Streptomyces* have not been investigated at all. This made it difficult to activate the gene cluster by the use of genuine regulatory genes of the pathway. Therefore, we decided to introduce the strong constitutive promoter *ermE** [32] upstream of the operon of phenazine biosynthesis genes into cosmid CB15, using RED/ET-mediated recombination. In previous experiments for gene replacement by this method, we had used gene cassettes containing an *oriT* for conjugal transfer of the resulting constructs.[33,34] Since the cosmid CB15 already contains *oriT*, we constructed a new cassette without *oriT* in order to avoid an undesired recombination with the cosmid backbone (see Experimental procedures). This new cassette contained the *ermE** promoter as well as the hygromycin resistance gene (under the



control of the *lac* promoter) as a selective marker (Fig. 2A). RED/ET-mediated recombination was used to introduce this cassette into cosmid CB15 (Fig. 2A). The recombinants were selected with hygromycin B and verified by restriction analysis. The resulting construct CB15–ermE* was then introduced into the genome of *S. coelicolor* M512 using biparental conjugation. Seven exconjugants were selected with hygromycin B and apramycin, and cultivated in a medium used previously for the heterologous production of phenazines.[14] Extracts of the culture supernatant were analyzed by HPLC. This readily showed the production of two compounds with the typical absorption spectrum of phenazines (Fig. 2B), indicating that the activation of the silent gene cluster by introduction of the *ermE** promoter upstream of the *phzBCDEFGA* operon had been successful.

### Isolation of a new phenazine derivative formed by heterologous expression of the activated phenazine biosynthetic gene cluster

One of the two compounds formed after heterologous expression of the phenazine cluster (Fig. 2B) showed a molecular ion at *m*/*z* 225 ([M + H]$^+$) and was readily identified as phenazine-1-carboxylic acid in comparison to an authentic reference substance. The other compound showed a molecular ion at *m*/*z* 353 ([M + H]$^+$). Positive ion mode high resolution mass spectrometry showed an exact mass of 353.1243310 Dalton, indicating a molecular formula of $C_{18}H_{16}N_4O_4$ (calculated mass 352.1244315 Dalton, Δ 0.28 ppm), different from any phenazine derivative described previously.

To identify the structure of the new product, the heterologous expression strain *S. coelicolor* M512 carrying cosmid CB15–ermE* was cultivated in a 10 litre fermenter. From the culture medium, the compound was isolated using XAD-16 resin and purified by chromatography on Sephadex LH-20 and by preparative reversed phase HPLC. 35 mg of a red solid compound was obtained and 5 mg were investigated by unidimensional ($^1$H and $^{13}$C) and multidimensional ($^1$H-$^1$H COSY, HSQC and HMBC) NMR spectroscopy, in comparison to PCA. This showed that the new compound represented a conjugate of phenazine-1-carboxylic acid, attached *via* an amide bond to the amino group of glutamine (Fig. 2B). The $^1$H and $^{13}$C NMR data of the compound are summarized in Table 2, and the $^1$H-$^1$H-COSY, HSQC and HMBC correlations are depicted in Fig. S1 (ESI‡). It will be termed α-*N*-(phenazine-1-carbonyl)-glutamine (PCA-Gln) hereafter.

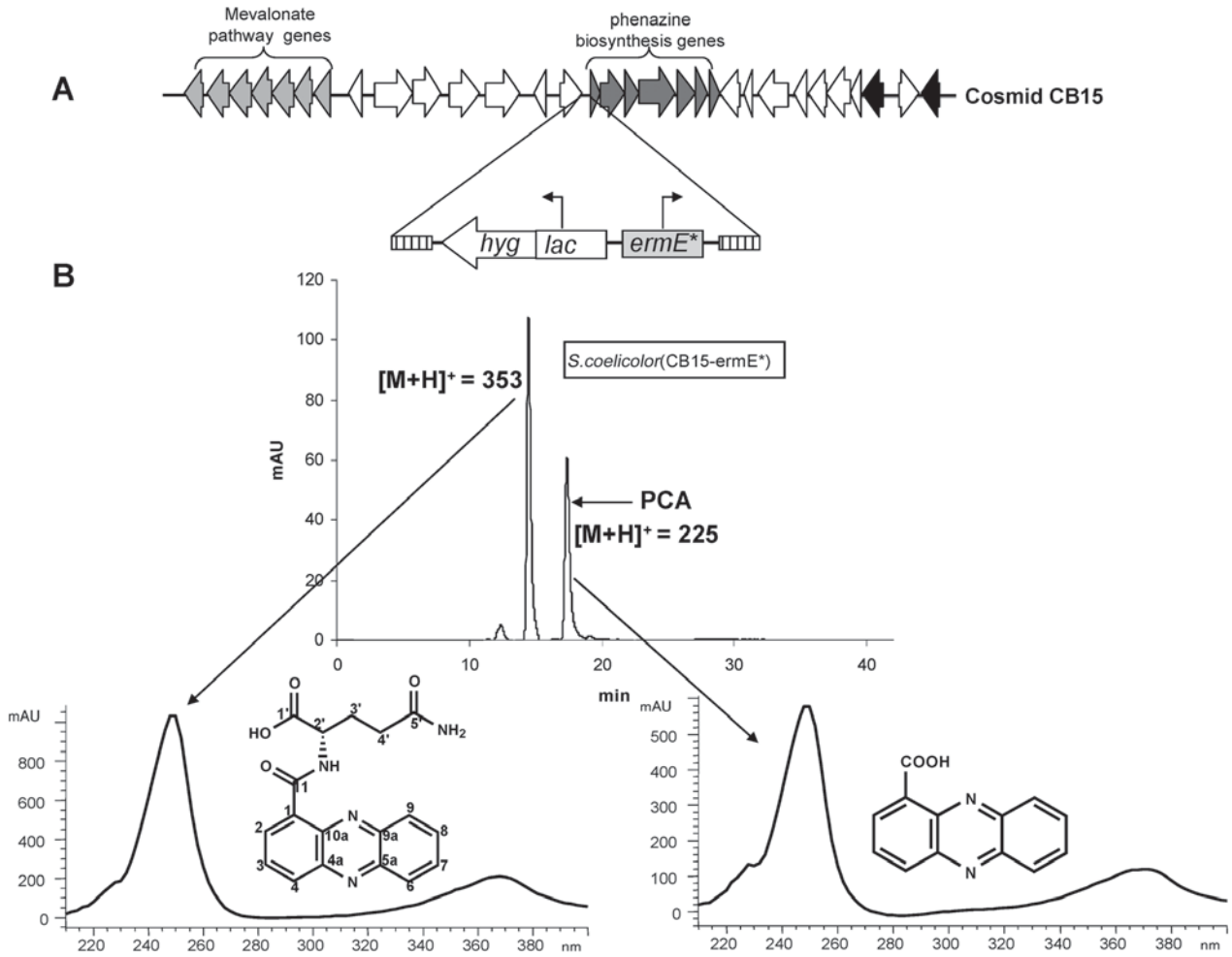



**Fig. 2** (A) Introduction of the constitutive *ermE** promoter into cosmid CB15, resulting in cosmid CB15–ermE*. (B) HPLC analysis of the culture supernatant of the heterologous expression strain *S. coelicolor* (CB15–ermE*); detection: 365 nm. Below the chromatogram, the UV spectra of the two main products are shown.

**Table 2** $^{1}H$ and $^{13}C$ NMR spectroscopic data of α-*N*-(phenazine-1-carbonyl)-L-glutamine. Chemical shifts are expressed in δ values using the solvent as an internal standard (400 MHz, $d_6$-DMSO). Assignments were made using 2D NMR data, shown in Fig. S1.‡ Numbering of the structure is also given in Fig. 2

| Position | $^{13}C$ NMR (100.6 MHz, $d_6$-DMSO) $\delta_C$ [ppm] | $^{1}H$ NMR (400 MHz, $d_6$-DMSO) $\delta_H$ [ppm] |
|---|---|---|
| 1 | 129.0 | |
| 2 | 134.5 | 8.73, 1H, (dd, 7.1, 1.5) |
| 3 | 130.4 | 8.10, 1H, (dd, 8.8, 7.1) |
| 4 | 133.4 | 8.46, 1H, (dd, 8.8, 1.5) |
| 4a | 142.6 | |
| 5a | 142.9 | |
| 6 | 129.4 | 8.36, 1H, (ddd, 7.8, 1.7, 0.6) |
| 7 | 131.8 | 8.06, 1H, (ddd, 7.8, 6.7, 1.6) |
| 8 | 132.4 | 8.10, 1H, (ddd, 8.4, 6.7, 1.7) |
| 9 | 129.3 | 8.32, 1H, (ddd, 8.4, 1.6, 0.6) |
| 9a | 141.0 | |
| 10a | 140.1 | |
| 11 | 163.7 | |
| 1′ | 173.3 | |
| 2′ | 52.6 | 4.69, 1H, (dddd, 7.2, 7.2, 2.2, 2.2) |
| 2′–NH | | 11.18, 1H, (d, 7.2) |
| 3′ | 27.5 | 2.08–2.20, $1H_a$, m<br>2.20–2.39, $1H_b$, m |
| 4′ | 31.2 | 2.20–2.39, 2H, m |
| 5′ | 173.3 | |
| 5′–$NH_2$ | | 6.78, $1H_a$, s<br>7.34, $1H_b$, s |

### Identification of the stereochemical configuration of α-*N*-(phenazine-1-carbonyl)-glutamine

The stereochemical configuration of the amino acid glutamine in the structure of PCA-Gln was determined by enantioselective HPLC analysis.[35] We therefore synthesized α-*N*-(phenazine-1-carbonyl)-L-glutamine and α-*N*-(phenazine-1-carbonyl)-D-glutamine (see Experimental procedures). The isolated PCA-Gln and the two synthesized compounds were analyzed by HPLC using two complementary chiral columns which contained as chiral selectors either quinine (QN) or quinidine (QD) derivatives. As expected, on the QN column the L-Gln derivative was more retained ($R_t = 14.6$ min) in comparison to the D-Gln derivative ($R_t = 6.9$ min). The isolated PCA-Gln showed the same retention time as the synthesized L-Gln derivative. Using the QD column, the D-Gln derivative was more retained in comparison to the L-derivative ($R_t = 14.4$ min and $R_t = 6.3$ min, respectively). Again, the isolated PCA-Gln product showed the same retention time as the L-Gln derivative. These results prove that in the structure of the isolated PCA-Gln, glutamine has the L-configuration.

### The conjugation of phenazine-1-carboxylic acid to L-glutamine is catalyzed by enzymes of the expression host *Streptomyces coelicolor* M512

Except for phenazine-1-carboxamide in *Pseudomonas* and sendomycin A and B from *Streptomyces endus* subsp. *aureus* DO-59,[11] very few amides of PCA have been described in nature. Mechanistically, the conjugation of PCA to glutamine requires an activation of the carboxyl group of PCA, *e.g.* in the form of a coenzyme A ester or of an acyl adenylate, similar to the biosynthesis of phenazine-1-carboxamide under catalysis of PhzH in *Pseudomonas*.[21] Surprisingly, no candidate gene for such an amide synthetase was found in the insert of cosmid CB15. The only adenylate-forming enzyme encoded in CB15 is Orf29, a putative fatty acid CoA ligase. However, this gene shows high similarity to genes found in the biosynthetic gene clusters of the prenylated polyketides napyradiomycin[36] and furaquinocin[37] which are unrelated to phenazines. In order to test whether the conjugation of PCA to glutamine may be carried out by enzymes of the expression host *S. coelicolor* M512 rather than by enzymes encoded in cosmid CB15, we added PCA (final concentration 0.1 mM) to cultures of the expression host *S. coelicolor* M512 (not containing the CB15 cosmid). After 3 days of cultivation, the culture supernatant was extracted and analyzed by HPLC. In two parallel control experiments, PCA was added to cultures of *S. tendae* Tü1028 or to sterile culture medium. The result was strikingly clear (Fig. 3): PCA remained unchanged in the sterile medium and in cultures of *S. tendae*, but was nearly quantitatively (97%) converted to PCA-Gln in the cultures of *S. coelicolor* M512. This conversion is therefore carried out by enzymes encoded in the genome of *S. coelicolor* M512.

Enzymes which can catalyze the transfer of an acyl moiety to the α-amino group of glutamic acid are *e.g.* FolC of folate biosynthesis[38] or MurD of murein biosynthesis.[39] Orthologs of these genes are encoded by the genes *sco2614* and *sco2086* of *S. coelicolor*. These or similar enzymes may be responsible for the observed conjugation of PCA to glutamate.

### The conjugation of phenazine-1-carboxylic acid to L-glutamine is likely to represent a resistance mechanism

Phenazines possess antibiotic activity, owing to the fact that they can reduce molecular oxygen to toxic, highly reactive oxygen species.[12] *Pseudomonas aeroginosa*, a producer of phenazines, protects itself from this toxic effect of phenazines by production of superoxide dismutases and catalase.[40] *Enterobacter agglomerans* forms a phenazine binding protein which facilitates the export of the toxic molecule.[41] The resistance mechanisms of phenazine-producing actinobacteria have not been examined. In order to investigate whether the conjugation of PCA to glutamate may offer a mechanism to detoxify PCA, and/or to facilitate its export, we compared the antibiotic activity of PCA and of PCA-Gln against *E. coli*, *Bacillus subtilis* and *Streptomyces coelicolor* M512 in disk diffusion assays. Both compounds showed no effect on *E. coli* in the tested concentrations.

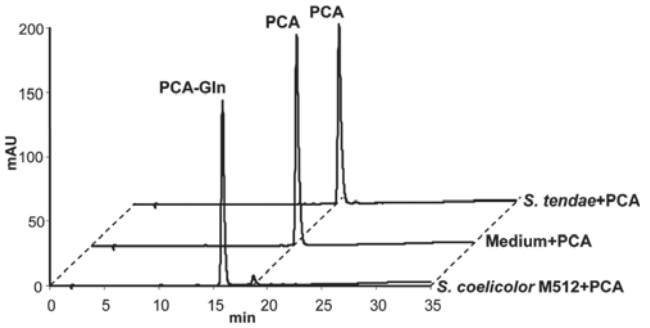



**Fig. 3** HPLC analysis of the feeding of phenazine-1-carboxylic acid (PCA) to cultures of *Streptomyces coelicolor* M512, to cultures of *S. tendae*, or to sterile culture medium. Only *S. coelicolor* M512 converted PCA to α-*N*-(phenazine-1-carbonyl)-L-glutamine (PCA-Gln).

However, PCA had a strong antibiotic effect against *Bacillus subtilis*, while PCA-Gln had not (Fig. 4a). As shown above, *Streptomyces coelicolor* M512 rapidly conjugates PCA to L-glutamine, and correspondingly this strain was resistant to both PCA and PCA-Gln (Fig. 4b). These results suggest that glutamination of PCA may represent a resistance mechanism. Notably, in soil which is the natural habitat of *Streptomyces* strains PCA is produced by *Pseudomonas* strains and can reach growth-inhibitory concentrations.[42] Therefore, a resistance mechanism against PCA may offer a competitive advantage to a *Streptomyces* strain in nature.

To our knowledge, this is the first report of the conjugation of PCA to glutamine or to any other amino acid *via* an amide bond. However, the conjugation of the phenazine–dicarboxylic acid derivative SB 212021 to *N*-acetyl-cysteine has been described.[43] In that case, conjugation occurred *via* the thiol group of cysteine and also resulted in the loss of the (weak) antibacterial activity of the phenazine. A similar *N*-acetyl-cysteine adduct has been described for a polyketide antibiotic, also leading to a loss of biological activity.[44]

### Biochemical investigation of the prenyltransferase $Ptf_{St}$

Cosmid CB15 from *S. tendae* contained phenazine biosynthesis genes, mevalonate pathway genes and a gene for an aromatic prenyltransferase, $ptf_{St}$. However, in the present study we could not detect any prenylated phenazines, in contrast to a previous study on a gene cluster from *S. anulatus* which contained similar genes.[14] We therefore speculated that the prenyltransferase gene $ptf_{St}$ may not code for a phenazine prenyltransferase such as PpzP or EpzP,[14,16] but for a hydroxynaphthalene prenyltransferase such as NphB or Fnq26.[23,24] Phenazine and hydroxynaphthalene prenyltransferases are similar in their amino acid sequence, but different in their specificity for the aromatic substrate.

We therefore expressed $Ptf_{St}$ in *E. coli* as a His-tagged protein and purified it by $Ni^{2+}$ affinity chromatography (see Experimental procedures). In contrast to PpzP and EpzP, $Ptf_{St}$ did not show product formation using 5,10-dihydro-PCA and either DMAPP or GPP as substrates. In contrast, prenylated products were readily obtained when $Ptf_{St}$ was incubated with GPP and different phenolic substrates including 1,6-dihydroxynaphthalene (1,6-DHN), 2,7-dihydroxynaphthalene (2,7-DHN) and the flavonoids apigenin, genistein or naringenin (Table 3). DMAPP was not accepted as an isoprenoid substrate. The $K_m$ values of $Ptf_{St}$ for 1,6-DHN and genistein were determined to be 0.36 and 0.16 mM, respectively. Using 1,6-DHN as an aromatic substrate, the $K_m$ for GPP was determined as 0.13 mM. In contrast to most other prenyltransferases of the ABBA superfamily,[20,45] $Ptf_{St}$ requires the presence of $Mg^{2+}$ ions for its catalytic activity. These biochemical characteristics (substrate and product specificity, $K_m$ values and magnesium dependency) are very similar to those of NphB, which is involved in the biosynthesis of the prenylated naphthalene derivative naphterpin.[23,46] This indicates that $Ptf_{St}$ may be involved in the biosynthesis of a prenylated naphthalene derivative, most likely in the prenylation of a polyketide formed from 1,3,5,8-tetrahydroxynaphthalene (THN), the presumed product of the type III polyketide synthase Orf34 (Table 1).[47] A mixed biosynthetic gene cluster for prenylated THN derivatives and phenazines was also found in *S. cinnamonensis*, and many of the genes found in the

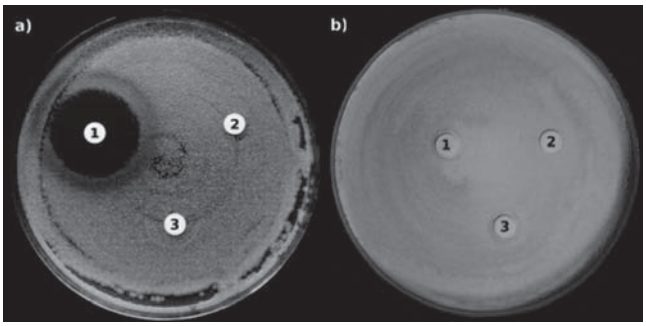
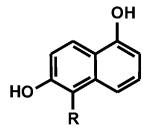
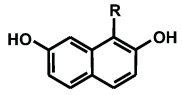
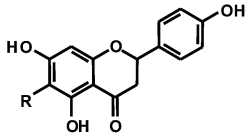
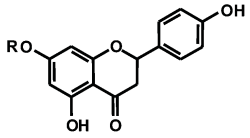
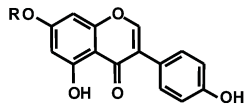
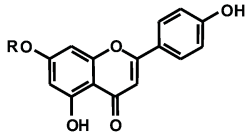
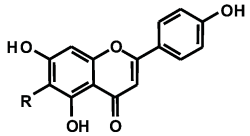



**Fig. 4** Antibiotic activity of PCA and of PCA-Gln against (a) *Bacillus subtilis* and (b) *Streptomyces coelicolor* M512. To the paper disks, either 0.5 μmol PCA (1) or 0.5 μmol PCA-Gln (2) or solvent (3) were applied. In parallel experiments, no growth inhibition on *Streptomyces coelicolor* M512 was exerted by PCA or PCA-Gln in amounts of up to 5 μmol (data not shown).

**Table 3** Prenylation of phenolic compounds under catalysis of $Ptf_{St}$. GPP (1 mM) was used as an isoprenoid substrate. DHN = dihydroxynaphthalene

| Substrate | Product | R = | $k_{cat}$ ($10^{-3}$ $s^{-1}$) |
|---|---|---|---|
| 1,6-DHN | 5-Geranyl-1,6-DHN |  | 5.96 |
| 2,7-DHN | 1-Geranyl-2,7-DHN |  | 4.96 |
| Naringenin | 6-Geranyl-naringenin |  | 0.35 |
| | 7-*O*-Geranyl-naringenin |  | 0.29 |
| Genistein | 7-*O*-Geranyl-genistein |  | 0.42 |
| Apigenin | 7-*O*-Geranyl-apigenin |  | 0.13 |
| | 6-Geranyl-apigenin |  | 0.07 |

presently described gene cluster of *S. tendae* Tü1028 have orthologs in the cluster of *S. cinnamonensis*. In contrast to the cluster in *S. cinnamonensis*, however, the cluster in *S. tendae* Tü1028 remained silent under many different culture conditions and the structure of the possibly encoded prenylated naphthalene remains unknown.

### Sequence analysis of the border regions of the identified gene cluster

In order to check for the presence of further genes for the biosynthesis of prenylated naphthalenes and/or phenazines, we sequenced two cosmids overlapping with the insert of cosmid CB15 (Fig. 1). This revealed on the right end of the cluster (Fig. 1 and Table 1) genes coding for putative regulators and redox enzymes, an ABC transporter gene as well as a gene (*orf43*) with similarity to the hypothetical protein NapU4 from the napyradiomycin cluster.[36] On the left end of the cluster, we found four genes likely to be involved in the recycling of *S*-adenosylhomocysteine (SAH) to *S*-adenosylmethionine (SAM), *i.e. metK*, *adoK*, *metH* and *metF*. The function of these genes may be the supply of SAM for methylation reactions within the biosynthetic pathway, but may also include a role in the regulation of secondary metabolism.[48] Notably, in the silent cluster in *S. tendae* Tü1028, one of the genes required for the recycling of SAH to SAM, *i.e.* the *S*-adenosylhomocysteinase gene *sahH*, was missing, in contrast to the active clusters found in *S. cinnamonensis* and two other organisms.[48] The genes further upstream of *metK* showed similarity to primary metabolic genes, suggesting that this gene may mark the border of the identified secondary metabolic gene cluster.

## Conclusions

The phenazine biosynthetic gene cluster of *S. tendae* Tü1028 was successfully activated by introduction of the constitutive, strong *ermE** promoter upstream of the *phzBCDEFGA* operon. The resulting heterologous expression strain produced 15 mg $l^{-1}$ of phenazine derivatives, showing that the 6.5 kb operon was efficiently transcribed from this promoter. In drug discovery by genome mining, the activation of the silent clusters will present one of the principal challenges. Our study shows that the introduction of a constitutive promoter in front of biosynthetic gene operons may provide a useful tool to meet this challenge. Tetracycline-inducible versions of the *ermE** promoter have been developed,[49] and may allow the controlled expression of secondary metabolic gene clusters.[50]

Heterologous expression of biosynthetic gene clusters in host strains which are completely sequenced, easily cultivatable and amenable for genetic manipulation is another important tool in genomic mining[51] and was also used in the present study. Unexpectedly, we found that the expression host enzymatically modified the compound formed under direction of the heterologously introduced genes: phenazine-1-carboxylic acid, formed under catalysis of *phzBCDEFGA*, was conjugated to the α-amino group of glutamine in the form of an amide. This compound is a new, previously undescribed phenazine derivative, and its formation is likely to represent a resistance mechanism against the antibiotic effect of phenazine-1-carboxylic acid.

## Materials and methods

### Bacterial strains, plasmids, and culture conditions

The 190 *Streptomyces* strains including *S. tendae* Tü1028 were obtained from strain collection of Combinature Biopharm AG (now Merlion Pharmaceuticals GmbH). Strains were grown in liquid YMG medium (4 g of Bacto yeast extract, 10 g of malt extract, 4 g of glucose-monohydrate per l; pH 7.3) or on solid MS medium. For production of phenazine secondary metabolites, the medium described by Sedmera *et al.*[57] was used.

*Escherichia coli* XL1 Blue MRF, *E. coli* SURE (Stratagene, Heidelberg, Germany), *E. coli* BW 25113, and *E. coli* ET12567 (pUB307) were used for cloning and were grown in liquid or on solid (1.5% agar) Luria–Bertani or SOB medium at 37 °C. The REDIRECT technology kit for PCR targeting was obtained from Plant Bioscience Limited (Norwich, UK). Carbenicillin (50–100 μg $ml^{-1}$), apramycin (50 μg $ml^{-1}$), kanamycin (50 μg $ml^{-1}$), chloramphenicol (25 μg $ml^{-1}$), nalidixic acid (20 μg $ml^{-1}$) and hygromycin B (40–100 μg $ml^{-1}$) were used for selection of recombinant strains.

### Chemicals

Dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) were synthesized according to Woodside *et al.*[52] Kanamycin, carbenicillin and hygromycin were purchased from Genaxxon BioSciences GmbH (Biberach, Germany) and phenazine-1-carboxylic acid was from InFarmatik. IPTG, Tris, NaCl, glycerol, dithiothreitol, $MgCl_2$, formic acid, sodium dodecyl sulfate, polyacrylamide, and EDTA were from Carl Roth, Karlsruhe, Germany. Apramycin, nalidixic acid, 1,6-dihydroxynaphthalene (1,6-DHN), methanol, Tween 20, imidazole, *N*-*N*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide were from Sigma Aldrich, Steinheim, Germany. 2,7-Dihydroxynaphthalene (2,7-DHN) was from AcrosOrganics. Merck supplied chloramphenicol, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium carbonate, sodium hydrogen carbonate and β-mercaptoethanol. Lysozyme was from Boehringer Ingelheim, Heidelberg, Germany.

### Genetic procedures

Standard methods for DNA isolation and manipulation were performed as described by Kieser *et al.*[53] and Sambrook and Russell[54] DNA fragments were isolated from agarose gels by using a PCR purification kit (Amersham Biosciences). The genomic DNA of the 190 streptomycetes strains was isolated with the NucleoSpin® 96 Tissue Core Kit from Macherey-Nagel, Düren, Germany.

### Construction and screening of the cosmid library

Cosmid libraries from all investigated strains had been established prior to the present study, using the vector pOJ436.[17] The genomic DNA of the 190 *Streptomyces* strains was initially screened for the gene *phzB* using the following degenerate primers: *phzB_For_1*: 5′-CT(G/C)TGGAC(G/C)AC(G/C)GA(C/T)AC(G/C)GG-3′ and *phzB_R_1*: 5′-GAA(G/C)(G/C)(A/T)(A/G)TG(G/C)AGGAA(A/G)TG(A/G)TT-3′. The resulting PCR



products were verified by sequencing. The amplified PCR product was used as a probe for screening of the cosmid libraries by Southern hybridization. The positive cosmids were screened by PCR screening for the presence of *phzD*, *phzF*, *hmgr* (3-hydroxy-3-methyl-glutaryl-CoA reductase), *hmgs* (3-hydroxy-3-methyl-glutaryl-CoA synthase), *mdpd* (mevalonate diphosphate decarboxylase) and for a putative ABBA prenyltransferase gene.[20] The following primers were used: *PhzD_for* (5′-CGC GCC GTC CTG (A/G)TN CA(C/T) GA(C/T) (A/C/T)T-3′) and *phzD_rev* (5′-CGG TGG TGG TCC CGG (G/C)(A/T)(A/G) AA(A/G) TCN (G/C)-3′); *phzF_for* (5′-CAT CCG GAT CTT GAC CCC NGT NAA (C/T)GA-3′) and *phzF_rev* (5′-GAG GGG CGC CCC AT(C/T) TCN CAN CC-3′); *HMGR_for* (5′-GGG CAT CGC CGC GAC CCT CGT GGA GGA GGG-3′) and *HMGR_rev* (5′-GCG ATG ACG GGG AGG CGC CGG GCG TTC TC-3′); *HMGS_for* (5′-GCC AAG TCC GCC GGN GTN TA(C/T) GT-3′) and *HMGS_rev* (5′-AGC CGG AAG GGG CCN GTN GT(C/T) TG-3′); *MDPD_for* (5′-GAC CCT GGA CGT CTT CCC NAC NAC NAC-3′) *MDPD_rev* (5′-GCG TTC CGC TCG GC(A/G/T) AT(C/T) TCN-3′), *PT_for* 5′-A(G/C)GT(G/C) CCGCT(G/C)GCCAC(G/C)TACGAG-3′ and *PT_rev* 5′-C(G/C)GC(G/C)AG(G/C)CG(G/C)CGGGCGTTCT-3′. Only one cosmid, CB15 from *S. tendae* Tü1028, was found to contain all the investigated phenazine, mevalonate and prenyltransferase genes.

### Heterologous expression of cosmid CB15

Cosmid CB15 was first transformed into the nonmethylating host *E. coli* ET12567 containing plasmid pUZ8002, and the nonmethylated DNA was introduced into *Streptomyces coelicolor* M512 *via* biparental conjugation.[53]

### Construction of the cassette for the activation of the silent phenazine biosynthesis gene cluster

The hygromycin resistance gene together with the *lac* promoter was amplified from plasmid pIJ797 with the following primers: *pLac_kpn_F*: GAC TCA CTA TAG GGC GAA TTG GGTACC GGG CCC CCC CTC GAG GTCGA and *Plac_kpn_R*: GTA ACA TCA AGG CCC GAT CCT T GGTACC CTT GCC CTC CCG CAC GAT GAT CG. The introduced KpnI restriction sites are underlined.

The PCR product was then digested with KpnI and ligated into the KpnI site of pUWL201. The resulting plasmids were transformed into *E. coli* XL1 blue and selected with carbenicillin and hygromycin.

Restriction analysis was used to identify a plasmid which contained the *ermE** and the *lac* promoters in opposite transcriptional directions. This plasmid was used as a template to amplify the cassette with two long primers with homology to the intergenic region upstream of the phenazine biosynthesis gene *phzB: Plac_4_R*: GCC GCG AAA ACC CGT GAC GAC CGT GCG GCC GGG TCC GGA TCG ATA AGC TTG ATT GTA GG and *ErmE_4_R*: CTC TGC GTT CTC GGA AGG CGT GTT CTC GGA AGG CGT GTT GCT TCT AGA ACT AGT GGA TC.

λ-RED recombination was used to introduce the resulting cassette into cosmid CB15 resulting in cosmid CB15–ermE*. Cosmid CB15–ermE* was then transformed into *E. coli* ET12567/pUZ8002 and conjugated with *S. coelicolor* M512. The exconjugants were selected with hygromycin B and apramycin. Seven exconjugants were selected for cultivation.

### Production and analysis of secondary metabolites

The exconjugants as well as wild type *S. tendae* Tü1028 were precultured for 48 h in liquid YMG medium (50 ml). 50 ml of production medium was then inoculated with 2.5 ml of the precultures. The flasks were agitated on a rotary shaker at 30 °C and 200 rpm for 120 h.

For isolation of secondary metabolites, cultures (50 ml) were centrifuged at 3500 × *g* for 10 min, and supernatant and mycelia were analysed separately. Mycelia were extracted with methanol (10 ml) by vortexing. The extract was mixed with sodium acetate buffer (10 ml; 1 M, pH 4.0) and extracted with dichloromethane (5 ml). After separation of the organic phase, the solvent was evaporated, and the residue was redissolved in methanol (0.5 ml).

The supernatant was adjusted to pH 4.0 using 1 M HCl, and extracted with the same volume of ethylacetate. The organic phase was evaporated and the residue was redissolved in methanol.

Extracts were analyzed by HPLC (Agilent 1100 series; Waldbronn, Germany) by using an Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm; Agilent) at a flow rate of 1 ml $min^{-1}$ with a linear gradient from 40 to 100% of solvent B in 20 min (solvent A: water–formic acid (999 : 1); solvent B, methanol) and detection at 252 and 365 nm. Additionally, UV spectra were acquired from 200 to 400 nm by a photodiode array detector. The absorbance at 365 nm was used for quantitative analysis, employing an authentic reference sample of PCA as an external standard.

### Analysis by LC-MS

The extracts were examined with LC-MS and LC-$MS^2$ analyses using a Nucleosil 100-C18 column (2 × 100 mm, 3 μm) coupled to an ESI mass spectrometer (LC/MSD Ultra Trap System XCT 6330; Agilent Technology). Analysis was carried out at a flow rate of 0.4 ml $min^{-1}$ with a linear gradient from 10 to 100% of solvent B in 15 min (solvent A: water–formic acid (999 : 1); solvent B: acetonitrile–formic acid (999.4 : 0.6)). Detection was carried out at 230, 260, 280, 360, and 435 nm. Electrospray ionization (positive and negative ionization) in Ultra Scan mode with a capillary voltage of 3.5 kV and a drying gas temperature of 350 °C was used for LC-MS analysis. For LC-$MS^2$ and LC-$MS^3$, the analysis was carried out in positive ionization mode with a capillary voltage of 3.5 kV at 350 °C.

### Preparative isolation of PCA-Gln

The strain *S. coelicolor* (CB15–ermE*) was precultured in 500 ml of production medium for 48 h at 27 °C. This culture was inoculated into a 10 litre fermenter containing the same production medium and grown at 27 °C for 96 h. The cultures were then filtrated under vacuum using 3% celite. The mycelia were discarded and the culture filtrate was adjusted to pH 7.0 and applied to XDA-16 macro-porous adsorptive resins chromatographic column and eluated with 70% methanol. After concentration, the aqueous solution was extracted with dichloromethane and the separated organic phase was discarded. The aqueous solution

was adjusted to pH 3.0 and extracted three times with ethyl acetate. The ethyl acetate phase was evaporated and the residue was redissolved in methanol and fractioned using a liquid chromatography system with a Sephadex LH20 column (2.5 × 90 cm) and methanol as the mobile phase. The fractions containing PCA-Gln as the main product were pooled and the solvents were evaporated. The residue was redissolved in dimethylsulfoxide (DMSO) and applied to a preparative HPLC system with a Reprosil Basic C18 column (250 × 20 mm). The separation was carried out with a linear gradient from 60 to 70% of solvent B in 15 min (solvent A: water–formic acid (999 : 1); solvent B: methanol) and the fractions containing pure PCA-Gln were pooled and dried using lyophilisation. 35 mg of pure PCA-Gln could be extracted.

### Stereochemical identification of *N*-(phenazine-1-carbonyl)-L-glutamine

For the synthesis of the reference substances, 9.9 mg of PCA were suspended in 400 µl of acetonitrile, added to 49.9 mg of *N*,*N*-dicyclohexylcarbodiimide dissolved in 300 µl of acetonitrile and incubated at 60 °C. After 60 min, 10.5 mg of *N*-hydroxysuccinimide dissolved in 300 µl of acetonitrile were added to the reaction mixture and incubated for further 24 h. The solution was then filled up with acetonitrile to 1 ml and the reaction mixture was divided into three tubes, each containing 300 µl. We added 1 mg of D-glutamine, dissolved in 200 µl of carbonate buffer (0.1 M $NaHCO_3$/0.1 M $Na_2CO_3$; 2 : 1, (v/v)) to the first tube; 1 mg of L-glutamine dissolved in the same buffer to the second tube and the third tube was mixed with 200 µl of the carbonate buffer. The tubes were incubated for 8 days at 25 °C. 20 µl of each reaction solution were diluted with 180 µl of methanol, and 20 µl were injected into an enantioselective HPLC system (LaChrom) with a DAD-detector and Chiralpak QN-AX or QD-AX columns (5 µm, 150 × 4 mm ID, Chiral Technologies Europe, Illkirch, France). We used a mixture of methanol–acetic acid–ammonium acetate (98 : 2 : 0.5; v/v/w) as the mobile phase with an isocratic flow rate of 1 ml $min^{-1}$. The detection was carried out at 210 nm, 250 nm, 280 nm and 340 nm.

### Feeding experiments with PCA

For feeding experiments we used deep-well plates.[55] 2 × 3 ml production medium was inoculated with *S. coelicolor* spores and incubated at 30 °C on a rotary shaker at 250 rpm. After 48 h, PCA was added to the medium to a final concentration of 0.1 mM. As negative control, PCA was added in the same concentration to 2 × 3 ml sterile medium in the same deep-well plate. Another control was done by feeding PCA to two *S. tendae* Tü1028 cultures under the same conditions. 100 µl from each culture were extracted and analyzed by HPLC as described above.

### Antibiotic activity of PCA and of PCA-Gln

Antibacterial activity of PCA and PCA-Gln was tested using *E. coli* K12, *Bacillus subtilis* ATCC 14893 and *Streptomyces coelicolor* M512. For the bioassays, 0.5 µmol of the respective substance (as potassium salt) in 10 µl of 0.1 M Tris HCl pH 8.0 were applied to filter paper disks (6 mm diameter). In tests against *E. coli* K12 or *Bacillus subtilis*, the filter disks were placed on LB nutrient agar inoculated with either *E. coli* or *Bacillus subtilis*. After culturing overnight at 37 °C, the diameter of the growth inhibition zone was determined. In tests against *Streptomyces coelicolor* M512, the filter disks were placed on MS nutrient agar[53] inoculated with a spore suspension of the test strain. After culturing for 48 hours at 30 °C, the diameter of the growth inhibition zone was determined.

### Expression and purification of $Ptf_{St}$

For the construction of the expression plasmid pET28a-OS02, $ptf_{St}$ was amplified with Phusion® DNA polymerase (Finnzymes, Woburn, MA) using the cosmid CB15 as a template. The following primers were used: *F*: GTC AGA ACC AAC GCA TG CATATG TCA ATG TCC GGA GCC GCT GAT G and *R*: GCT CTG CTG CGG CGGCTCGAG TCA GTC CTC CAG CGC GTC G. The underlined letters represent NdeI and XhoI restriction sites, respectively. The resulting PCR fragment was digested with NdeI and XhoI and ligated into plasmid pET28a digested with the same restriction enzymes. The resulting plasmid pET28a-OS02 was verified by restriction analysis and sequencing.

The plasmid pET28a-OS02 was transformed into *E. coli* BL21 (DE3) pLysS (Promega) and a pre-culture of 100 ml of liquid LB medium was cultured overnight at 37 °C and 200 rpm. 35 ml of the pre-culture were inoculated into 1 l of TB medium containing kanamycin (50 µg $ml^{-1}$) and chloramphenicol (25 µg $ml^{-1}$) and grown at 37 °C to an $A_{600}$ of 0.6. The temperature was lowered to 20 °C and isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 6 h of cultivation, the cells were harvested by centrifugation for 10 min at 2700 × *g* at 4 °C. The cells (17 g from 1 l of culture) were resuspended in 43 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 1 M NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 20 mM imidazole, 0.5 mg $ml^{-1}$ lysozyme, 0.5 mM phenylmethylsulfonyl fluoride). After stirring at 4 °C for 30 min, cells were ruptured with a sonifier (Branson W-250 D, Branson, Danbury, CT) and centrifuged for 45 min at 55 000 × *g* at 4 °C. The supernatant was purified by nickel affinity chromatography (5 ml HisTrap™ HP column, GE Healthcare). The protein was eluted using imidazole buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 250 mM imidazole). The buffer was changed using PD-10 desalting columns (GE Healthcare Life Sciences), equilibrated with 50 mM Tris pH 8, 10% (v/v) glycerol, 2 mM DTT. 60 mg of $His_6$–$Ptf_{St}$ could be purified from 1 litre of culture.

### Assay for prenyltransferase activity

The reaction mixture (200 µl) contained 50 mM Tris–HCl pH 9.0, 2.5 mM aromatic substrate, 1 mM DMAPP or GPP, 5 mM magnesium chloride and 0.3 nmol of $Ptf_{St}$. After incubation of the assay for 15 min at 30 °C (for 1,6-DHN and 2,7-DHN) or 45 min (for apigenin, genistein and naringenin), the reaction was stopped by adding 200 µl of ethylacetate : formic acid (39 : 1). After vortexing and centrifugation, 160 µl of the organic phase was evaporated. The residue was dissolved in 50 µl of methanol. 20 µl were analyzed by HPLC-UV and HPLC-MS. HPLC-UV analysis was carried out as described above, with detection at

260 nm for genistein, 290 nm for naringenin, 339 nm for apigenin and 268 nm for 1,3-DHN, 1,6-DHN and 2,7-DHN. LC-MS analysis was carried out as described above, with negative ionization.

For determination of the $K_m$ values for 1,6-DHN and genistein, GPP was kept at a constant concentration of 1 mM. For determination of the $K_m$ value for GPP, 1,6-DHN was kept at 2.5 mM.

## Acknowledgements

This work was supported by a grant from the German Ministry for Education and Research (BMBF/ERA-IB, Project GenoDrug) (to L.H.) and by a scholarship from Tishrin University, Syria (to O.S.).



## References

1 R. H. Baltz, *Nat. Biotechnol.*, 2006, **24**, 1533–1540.
2 C. T. Walsh and M. A. Fischbach, *J. Am. Chem. Soc.*, 2010, **132**, 2469–2493.
3 D. J. Newman and G. M. Cragg, *J. Nat. Prod.*, 2007, **70**, 461–477.
4 C. Corre and G. L. Challis, *Nat. Prod. Rep.*, 2009, **26**, 977–986.
5 A. A. Brakhage, J. Schuemann, S. Bergmann, K. Scherlach, V. Schroeckh and C. Hertweck, *Prog. Drug Res.*, 2008, **66**(1), 3–12.
6 S. Bergmann, A. N. Funk, K. Scherlach, V. Schroeckh, E. Shelest, U. Horn, C. Hertweck and A. A. Brakhage, *Appl. Environ. Microbiol.*, 2010, **76**, 8143–8149.
7 L. Laureti, L. Song, S. Huang, C. Corre, P. Leblond, G. L. Challis and B. Aigle, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 6258–6263.
8 M. Metsä-Ketelä, K. Ylihonko and P. Mantsala, *J. Antibiot.*, 2004, **57**, 502–510.
9 M. Gottelt, S. Kol, J. P. Gomez-Escribano, M. Bibb and E. Takano, *Microbiology*, 2010, **156**, 2343–2353.
10 R. Bunet, L. Song, M. V. Mendes, C. Corre, L. Hotel, N. Rouhier, X. Framboisier, P. Leblond, G. L. Challis and B. Aigle, *J. Bacteriol.*, 2011, **193**, 1142–1153.
11 J. B. Laursen and J. Nielsen, *Chem. Rev.*, 2004, **104**, 1663–1685.
12 L. S. Pierson3rd and E. A. Pierson, *Appl. Microbiol. Biotechnol.*, 2010, **86**, 1659–1670.
13 D. V. Mavrodi, T. L. Peever, O. V. Mavrodi, J. A. Parejko, J. M. Raaijmakers, P. Lemanceau, S. Mazurier, L. Heide, W. Blankenfeldt, D. M. Weller and L. S. Thomashow, *Appl. Environ. Microbiol.*, 2010, **76**, 866–879.
14 O. Saleh, B. Gust, B. Boll, H. P. Fiedler and L. Heide, *J. Biol. Chem.*, 2009, **284**, 14439–14447.
15 Y. Haagen, K. Glück, K. Fay, B. Kammerer, B. Gust and L. Heide, *ChemBioChem*, 2006, **7**, 2016–2027.
16 K. Seeger, K. Flinspach, E. Haug-Schifferdecker, A. Kulik, B. Gust, H. P. Fiedler and L. Heide, *Microb. Biotechnol.*, 2011, **4**, 252–262.
17 M. Bierman, R. Logan, K. O'Brien, E. T. Seno, R. N. Rao and B. E. Schoner, *Gene*, 1992, **116**, 43–49.
18 M. Tello, T. Kuzuyama, L. Heide, J. P. Noel and S. B. Richard, *Cell. Mol. Life Sci.*, 2008, **65**, 1459–1463.
19 U. Metzger, C. Schall, G. Zocher, I. Unsöld, E. Stec, S. M. Li, L. Heide and T. Stehle, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 14309–14314.
20 T. Bonitz, V. Alva, O. Saleh, A. N. Lupas and L. Heide, *PLoSOne*, 2011, **6**, e27336.
21 D. V. Mavrodi, R. F. Bonsall, S. M. Delaney, M. J. Soule, G. Phillips and L. S. Thomashow, *J. Bacteriol.*, 2001, **183**, 6454–6465.
22 E. Okamura, T. Tomita, R. Sawa, M. Nishiyama and T. Kuzuyama, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 11265–11270.
23 T. Kuzuyama, J. P. Noel and S. B. Richard, *Nature*, 2005, **435**, 983–987.
24 Y. Haagen, I. Unsöld, L. Westrich, B. Gust, S. B. Richard, J. P. Noel and L. Heide, *FEBS Lett.*, 2007, **581**, 2889–2893.
25 J. Tax, P. Sedmera, J. Vokoun, J. Urban, J. Karnetová, K. Stajner, Z. Vaněk and V. Krumphanzl, *Collect. Czech. Chem. Commun.*, 1983, **48**, 527–532.
26 K. Shin-ya, S. Shimizu, T. Kunigami, K. Furihata, Y. Hayakawa and H. Seto, *J. Antibiot.*, 1995, **48**, 1378–1381.
27 S. Kato, K. Shindo, Y. Yamagishi, M. Matsuoka, H. Kawai and J. Mochizuki, *J. Antibiot.*, 1993, **46**, 1485–1493.
28 M. Kitahara, H. Nakamura, Y. Matsuda, M. Hamada, H. Naganawa, K. Maeda, H. Umezawa and Y. Iitaka, *J. Antibiot.*, 1982, **35**, 1412–1414.
29 K. Yagishita, *J. Antibiot.*, 1960, **13**, 83–96.
30 K. Shin-Ya, K. Furihata, Y. Hayakawa and H. Seto, *Tetrahedron Lett.*, 1990, **31**, 6025–6026.
31 G. Girard, S. Barends, S. Rigali, E. T. van Rij, B. J. Lugtenberg and G. V. Bloemberg, *J. Bacteriol.*, 2006, **188**, 8283–8293.
32 M. J. Bibb, G. R. Janssen and J. M. Ward, *Gene*, 1985, **38**, 215–226.
33 B. Gust, *Methods Enzymol.*, 2009, **458**, 159–180.
34 B. Gust, G. L. Challis, K. Fowler, T. Kieser and K. F. Chater, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 1541–1546.
35 M. Lämmerhofer and W. Lindner, *Adv. Chromatogr.*, 2008, **46**, 1–107.
36 J. M. Winter, M. C. Moffitt, E. Zazopoulos, J. B. McAlpine, P. C. Dorrestein and B. S. Moore, *J. Biol. Chem.*, 2007, **282**, 16362–16368.
37 T. Kawasaki, Y. Hayashi, T. Kuzuyama, K. Furihata, N. Itoh, H. Seto and T. Dairi, *J. Bacteriol.*, 2006, **188**, 1236–1244.
38 A. Burger, B. Brandt, U. Susstrunk, C. J. Thompson and W. Wohlleben, *FEMS Microbiol. Lett.*, 1998, **159**, 283–291.
39 J. A. Bertrand, G. Auger, E. Fanchon, L. Martin, D. Blanot, J. van Heijenoort and O. Dideberg, *EMBO J.*, 1997, **16**, 3416–3425.
40 D. J. Hassett, L. Charniga, K. Bean, D. E. Ohman and M. S. Cohen, *Infect. Immun.*, 1992, **60**, 328–336.
41 S. Yu, A. Vit, S. Devenish, H. K. Mahanty, A. Itzen, R. S. Goody and W. Blankenfeldt, *BMC Struct. Biol.*, 2011, **11**, 33.
42 D. V. Mavrodi, O. V. Mavrodi, J. A. Parejko, R. F. Bonsall, Y. S. Kwak, T. C. Paulitz, L. S. Thomashow and D. M. Weller, *Appl. Environ. Microbiol.*, 2012, **78**, 804–812.
43 M. L. Gilpin, M. Fulston, D. Payne, R. Cramp and I. Hood, *J. Antibiot.*, 1995, **48**, 1081–1085.
44 D. Schulz, J. Nachtigall, J. Riedlinger, K. Schneider, K. Poralla, J. F. Imhoff, W. Beil, G. Nicholson, H. P. Fiedler and R. D. Sussmuth, *J. Antibiot.*, 2009, **62**, 513–518.
45 L. Heide, *Curr. Opin. Chem. Biol.*, 2009, **13**, 171–179.
46 T. Kumano, S. B. Richard, J. P. Noel, M. Nishiyama and T. Kuzuyama, *Bioorg. Med. Chem.*, 2008, **16**, 8117–8126.
47 N. Funa, Y. Ohnishi, Y. Ebizuka and S. Horinouchi, *J. Biol. Chem.*, 2002, **277**, 4628–4635.
48 X. Q. Zhao, B. Gust and L. Heide, *Arch. Microbiol.*, 2010, **192**, 289–297.
49 A. Rodriguez-Garcia, P. Combes, R. Perez-Redondo and M. C. Smith, *Nucleic Acids Res.*, 2005, **33**, e87.
50 V. Dangel, L. Westrich, M. C. Smith, L. Heide and B. Gust, *Appl. Microbiol. Biotechnol.*, 2010, **87**, 261–269.
51 J. P. Gomez-Escribano and M. J. Bibb, *Microb. Biotechnol.*, 2011, **4**, 207–215.
52 A. B. Woodside, Z. Huang and C. D. Poulter, *Org. Synth.*, 1993, **66**, 211.
53 T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater and D. A. Hopwood, *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, UK, 2000.
54 J. Sambrook and D. W. Russell, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York 2001.
55 C. S. Potten, D. Booth, N. J. Cragg, G. L. Tudor, J. A. O'Shea, C. Booth, F. A. Meineke, D. Barthel and M. Loeffler, *Cell Proliferation*, 2002, **35**(Suppl. 1), 32–47.
56 H. Seto, H. Watanabe and K. Furihata, *Tetrahedron Lett.*, 1996, **37**, 7979–7982.
57 P. Sedmera, S. Pospišil and J. Novák, *J. Nat. Prod.*, 1991, **54**, 870–872.

# Supplemental Data

for

## Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines

Orwah Saleh[1], Tobias Bonitz[1], Andreas Kulik[2], Nadja Burkard[3], Agnes Mühlenweg[4], Andreas Vente[4], Stefan Polnick[1], Michael Lämmerhofer[1], Bertolt Gust[1], Hans-Peter Fiedler[2] and Lutz Heide[1]

[1]Pharmaceutical Institute, University of Tübingen, 72076 Tübingen, Germany
[2]Faculty of Biology, University of Tübingen, 72076 Tübingen, Germany
[3]Institute of Organic Chemistry, University of Tübingen, 72076 Tübingen, Germany
[4]MerLion Pharmaceuticals GmbH, 13125 Berlin, Germany

**Corresponding author**: Lutz Heide, Pharmazeutische Biologie, Pharmazeutisches Institut, Eberhard-Karls-Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany. Fax**:** +49 7071 295250, E-mail**:** **heide@uni-tuebingen.de**

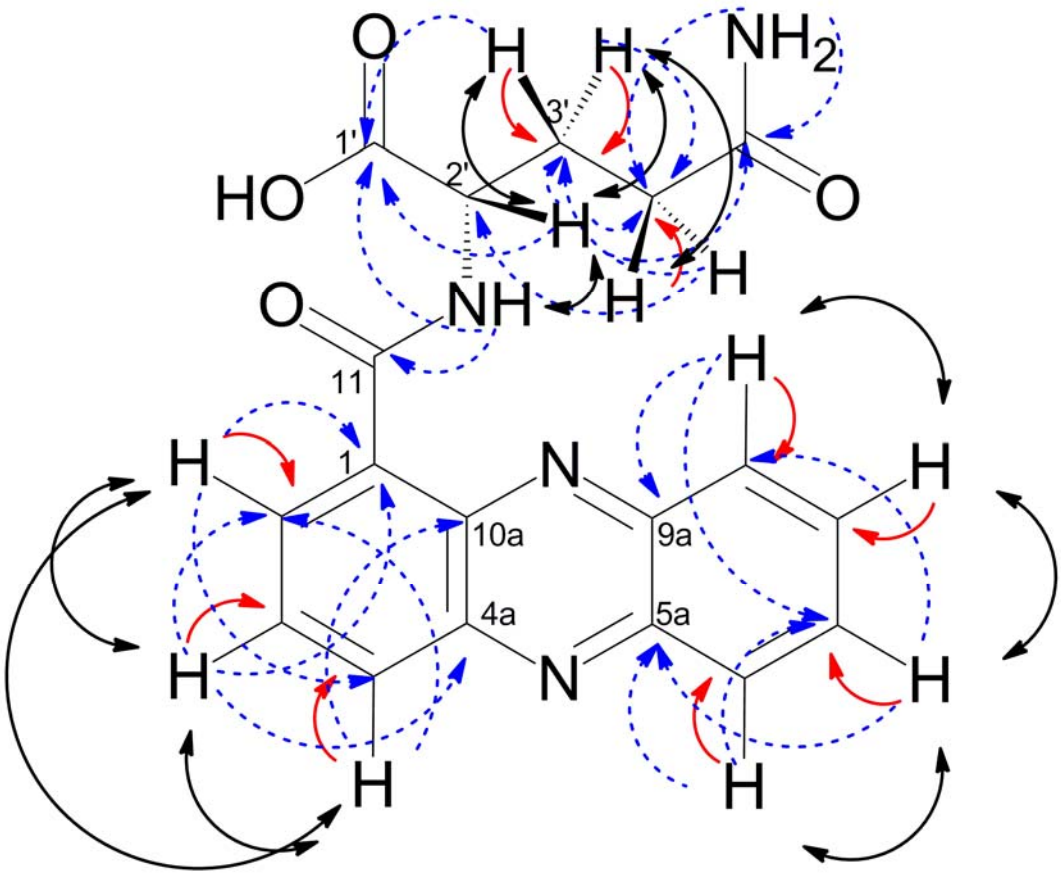



Fig. S1: Selected $^{1}$H-$^{1}$H-COSY ( ↔ ) correlations from 2D NMR experiments for α-N-(phenazine-1-carbonyl)-L-glutamine (400 MHz, $d_6$-DMSO). HSQC ( → ) and HMBC ( ⇢ ) correlations from 2D NMR experiments for α-N-(phenazine-1-carbonyl)-L-glutamine (600 MHz, $d_6$-DMSO).

Tab. S1: Media for cultivation of Streptomyces strains (Flavia Marinelli, University of Insubria, Italy, personal communication)

**Medium I**

| | |
|---|---|
| Glucose | 10 g |
| Glycerol | 10 g |
| Starch | 10 g |
| Corn Steep liquor 0.5% | 5 g |
| Pepton from Casein | 5 g |
| Yeast extract | 2 g |
| Sodium chloride | 1 g |
| Calcium carbonate | 3 g |
| Distilled water | → 1000 ml |

**Medium II**

| | |
|---|---|
| Peptone | 5 g |
| Soluble starch | 20 g |
| Meat extract | 2 g |
| Yeast extract | 3 g |
| Soy-bean meal | 2 g |
| Calcium carbonate | 1 g |
| Distilled water | → 1000 ml |

**Medium III**

| | |
|---|---|
| Soy-bean meal | 15 g |
| Calcium carbonate | 5 g |
| Glycerol | 30 ml |
| Sodium chloride | 2 g |
| Distilled water | → 1000 ml |

**Medium IV**

| | |
|---|---|
| Calcium carbonate | 0.1 g |
| Glycerol | 20 ml |
| Glycine | 2.5 g |
| Sodium chloride | 1 g |
| Potassium dihydrogen phosphate | 1 g |
| Ferrous sulfate | 0.1 g |
| Magnesium sulfate | 0.1 g |
| Distilled water | → 1000 ml |

**Medium V**

| | |
|---|---|
| Peptone | 2 g |
| Yeast extract | 4 g |
| Malt extract | 10 g |
| Glucose | 10 g |
| Glycerol | 5 ml |
| Magnesium chloride | 2 g |
| Distilled water | → 1000 ml |

**Medium VI**

| | |
|---|---|
| Peptone | 5 g |
| Meat extract | 5 g |
| Yeast extract | 5 g |
| Glucose | 20 g |
| Hydrolyzed casein | 3 g |
| Sodium chloride | 1.5 g |
| Distilled water | → 1000 ml |

**Medium VII**

| | |
|---|---|
| Dextrose | 20 g |
| Yeast extract | 2 g |
| Soy-bean meal | 8 g |
| Calcium carbonate | 4 g |
| Sodium chloride | 1 g |
| Distilled water | → 1000 ml |

**Medium VIII**

| | |
|---|---|
| Yeast extract | 4 g |
| Malt extract | 10 g |
| Glucose | 4 g |
| Distilled water | → 1000 ml |

**Medium IX**

| | |
|---|---|
| Soluble starch | 20 g |
| Meat extract | 2 g |
| Yeast extract | 2 g |
| Glucose | 10 g |
| Calcium carbonate | 5 g |
| Hydrolyzed casein | 4 g |
| Distilled water | → 1000 ml |

# Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily*

**Tobias Bonitz[‡], Florian Zubeil[§], Stephanie Grond[§], and Lutz Heide[‡1]**

From the [‡]Pharmaceutical Institute, University of Tübingen, Tübingen, Germany
and the [§]Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

*Running title: *A benzodiazepine farnesyltransferase from Micromonospora sp.*

To whom correspondence should be addressed: Lutz Heide, Pharmaceutical Institute, University of Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany, Tel.: +4970712972460; E-mail: heide@uni-tuebingen.de

**Keywords:** prenyltransferase, *Micromonospora,* farnesyl diphosphate, dibenzodiazepinone

**CAPSULE**
**Background:** ABBA prenyltransferases catalyze the prenylation of aromatic substrates in bacterial and fungal secondary metabolism.
**Results:** DzmP catalyzes the farnesylation of a benzodiazepine substrate in the biosynthesis of diazepinomicin.
**Conclusion:** DzmP is the first ABBA prenyltransferase which accepts farnesyl diphosphate as substrate, and which prenylates an amide nitrogen.
**Significance:** DzmP extends the aromatic and isoprenoid substrate range of ABBA prenyltransferases in chemoenzymatic synthesis.

**SUMMARY**
The bacterium *Micromonospora* sp. RV115, isolated from a marine sponge, produces the unusual metabolite diazepinomicin, a prenylated benzodiazepine derivative. We have cloned the prenyltransferase gene *dzmP* from this organism, expressed it in *Escherichia coli*, and the resulting $His_8$-tagged protein was purified and investigated biochemically. It was found to catalyze the farnesylation of the amide nitrogen of dibenzodiazepinone. DzmP belongs to the ABBA prenyltransferases and is the first member of this superfamily which utilizes farnesyl diphosphate as genuine substrate. All previously discovered members utilize either dimethylallyl diphosphate (C5) or geranyl diphosphate (C10). Another putative diazepinomicin biosynthetic gene cluster was identified in the genome of *Streptomyces griseoflavus* Tü4000, suggesting that the formation of diazepinomicin is not restricted to the genus *Micromonospora*. The gene cluster contains a gene *ssrg_00986* with 61.4% identity (amino acid level) to *dzmP*. The gene was expressed in *E. coli*, and the purified protein showed similar catalytic properties as DzmP. Both enzymes also accepted other phenolic or phenazine substrates.
ABBA prenyltransferases are useful tools for chemoenzymatic synthesis, due to their nature as soluble, stable biocatalysts. The discovery of DzmP and Ssrg_00986 extends the isoprenoid substrate range of this superfamily. The observed prenylation of an amide nitrogen is an unusual biochemical reaction.

**INTRODUCTION**
The isoprenylation of aromatic substrates is important in the biosynthesis of primary metabolites like ubiquinones, menaquinones and plastoquinones, but it also leads to a vast structural diversity of secondary metabolites in bacteria, fungi and plants (1). Within the last ten years, a new superfamily of aromatic prenyltransferases, involved exclusively in bacterial and fungal secondary metabolism, has been discovered and extensively investigated (2-4). Members of this superfamily are characterized by a new protein fold. It consists of a central barrel formed by ten antiparallel β-strands which contains the active center in its lumen and which is surrounded by a ring of solvent exposed α-helices (5). Due to the αββα succession of secondary structure elements, this superfamily has been termed ABBA prenyltransferases (4). It comprises two clearly distinct families (2), *i.e.* the fungal and bacterial indole prenyltransferases involved *e.g.* in ergot alkaloid biosynthesis (3, 6), and the phenol / phenazine prenyltransferases predominantly involved in bacterial secondary metabolism (5, 7, 8). Typically, ABBA prenyltransferases catalyze

the *C*-prenylation of aromatic substrates, *i.e.* a reaction similar to a Friedel-Crafts alkylation, although a few members also catalyze *N*- or *O*-prenylations. The members of the ABBA superfamily are soluble, mostly $Mg^{2+}$-independent catalysts, in contrast to the prenyltransferases of lipoquinone biosynthesis which are integral membrane proteins and require $Mg^{2+}$ as cofactor. Their considerable promiscuity for different aromatic substrates makes them attractive tools for chemoenzymatic synthesis (3, 4). However, their genuine isoprenoid substrates have so far been strictly limited to either dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP), *i.e.* to substrates with five or ten carbon atoms. In order to expand the substrate range of this group of chemoenzymatic tools, we decided to search for ABBA prenyltransferases which might use farnesyl diphosphate (FPP) as genuine isoprenoid substrate. Our attention was drawn to the unusual natural product diazepinomicin (Fig. 1). Its dibenzodiazepinone core is structurally unique in nature. The only related natural products containing benzodiazepine moieties are the pyrrolobenzodiazepines formed by bacteria, and the fungal metabolite cyclopenin (9).

Diazepinomicin has been independently isolated from different *Micromonospora* strains from different geographic locations of this world by Bristol-Myers Squibb (10, 11), Thallion Pharmaceuticals (formerly Ecopia BioSciences) (12), Wyeth (13) and recently by Abdelmohsen *et al.* (14). So far this compound has never been described outside of the genus *Micromonospora*. It was named BU-4664L, TLN-4601 (formerly ECO-4601) or diazepinomicin, and we will use the latter name hereafter.

Diazepinomicin binds to peripheral benzodiazepine receptors and has potent antitumor activity (15, 16). It has recently been investigated in a phase II clinical trial as anticancer agent (17).

Feeding studies have established that ring A of diazepinomicin (Fig. 1) is derived from 3-hydroxy anthranilic acid (18). This precursor can be formed by the kynurenine pathway which is used in actinobacteria for tryptophan catabolism (19, 20). Therefore, labeled tryptophan is incorporated into diazepinomicin (18, 21, 22). However, the biosynthetic gene cluster for diazepinomicin (see below) contains genes for an even more efficient pathway which is suggested to lead from chorismate via 2-amino-2-deoxyisochorismate to 3-hydroxyanthranilate in three enzymatic steps (18). The enzymes catalyzing this pathway are similar to PhzE and PhzD of phenazine biosynthesis (23) and to MxcC of myxochelin biosynthesis (24). Furthermore McAlpine *et al.* (18) speculated that ring B of diazepinomicin is derived from 3-amino-5-hydroxybenzoic acid (AHBA), a metabolite derived from the aminoshikimate pathway (25).

The biosynthetic gene cluster for diazepinomicin has been cloned and sequenced from *Micromonospora* strain 046Eco-11 (18). Based on a bioinformatic analysis of the DNA sequence, a detailed hypothesis of the biosynthetic pathway leading to diazepinomicin has been formulated (18), but the function of the individual genes and enzymes has not been investigated experimentally. *orf11* of this gene cluster shows sequence similarity to genes coding for ABBA prenyltransferases. It has therefore been speculated that the gene product of *orf11* may catalyze the farnesylation reaction in diazepinomicin biosynthesis (18).

In the present study, we identified a close homolog of *orf11* (96.9% identity on the amino acid level) in the diazepinomicin producer strain *Micromonospora* sp. RV115 (14). This gene (hereafter called *dzmP*) was expressed in *Escherichia coli* and the protein was purified. It was found to catalyze the farnesylation of the amide nitrogen of dibenzodiazepinone (Fig. 2). In addition, homology searches by BLAST (26) revealed a gene cluster in *Streptomyces griseoflavus* Tü4000 with striking similarity to the diazepinomicin cluster. The *orf11* ortholog from this cluster was also expressed and purified, and was found to catalyze the same reaction as DzmP. Both enzymes were characterized and found to prenylate various phenolic and phenazine substrates, opening new possibilities for the chemoenzymatic synthesis of prenylated compounds. To our knowledge, DzmP is the first ABBA prenyltransferases which utilizes FPP as its genuine substrate. Both DzmP and its ortholog from *S. griseoflavus* Tü4000 catalyze the prenylation of an amide nitrogen, which is an unusual reaction in nature.

## EXPERIMENTAL PROCEDURES

*Bacterial strains and culture conditions* - *Micromonospora* sp. RV 115 (14) was provided by U. Hentschel, Würzburg, Germany. *Streptomyces griseoflavus* Tü4000 (27) was provided by W. Wohlleben, Tübingen, Germany. They were grown in liquid YMG medium or on solid MS medium. For production of secondary metabolites, the media described by McAlpine *et*

*al.* (18) and Abdelmohsen *et al.* (14) were used. *Escherichia coli* XL1 Blue MRF (Stratagene, Heidelberg, Germany) was used for cloning and was grown in liquid or on solid (1.5% agar) Luria-Bertani medium at 37 °C. Kanamycin (50 μg $ml^{-1}$) was used for selection of recombinant strains.

*Chemicals* - Geranyl diphosphate and farnesyl diphosphate were synthesized as described in (28). Phenazine-1-carboxylic acid (PCA) was purchased from InFarmatic (Budapest, Hungary) and dihydrophenazine-1-carboxylic acid was generated as described by Saleh *et al.* (29). Genistein was from TCI Europe (Eschborn, Germany). 1,6-Dihydroxynaphthalene (1,6-DHN) and 5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one were bought at Sigma Aldrich, Steinheim, Germany. 2,7-Dihydroxynaphthalene (2,7-DHN) was purchased at Acros Organics (Geel, Belgium).

*Genetic procedures* - Standard methods for DNA isolation and manipulation were performed as described by Kieser *et al.* (30) and Sambrook *et al.* (31). DNA fragments were isolated from agarose gels by using a PCR purification kit (Qiagen; Hilden, Germany). Genomic DNA was isolated from *Micromonospora* and *Streptomyces* strains by lysozyme treatment and phenol/chloroform extraction as described by Kieser *et al.* (30). Restriction enzymes were purchased from New England BioLabs, Ipswich, MA.

*Sequence analysis* - Database searches were performed with BLAST (http://www.ncbi.nlm.nih.gov/). Secondary structure predictions were performed with Phyre 2 (32), and sequences were aligned with ClustalX (33) and visualized with ESPript (34) or Jalview (35). Phylogenetic trees were built with MEGA (http://www.megasoftware.net/). Protein structures were modeled with the Schrödinger suite (http://www.schrodinger.com/), examined with Pymol (http://www.pymol.org/) or the ASC server (http://asc.informatik.uni-tuebingen.de/).

*Overexpression and purification of DzmP, Ssrg_00986 and NphB* - *dzmP* and its flanking sequences were amplified from genomic DNA of *Micromonospora* sp. RV115 using the primers orf10_F (5'-CGC GTC TAC GAG CCG CGM TAG-3') and orf12_R (5'-GAH SAK CAG YRC CGC BGC CAC-3'). The PCR product was used for sequencing (NCBI accession KC866371). For the construction of expression plasmids, *dzmP* and *ssrg_00986* were amplified using genomic DNA of *Micromonospora* sp. RV115 and *S. griseoflavus* Tü4000 as templates. The following primers were used for *dzmP*: dzmP_F: (5'-CAT GCC ATG GCA TGT CCG GAA CTC CCG AGG-3') and dzmP_R: (5'-CGG AAT TCA AAT GAA GTT CAC CGC GCC C-3'). The underlined letters represent NcoI and EcoRI restriction sites, respectively. The resulting PCR fragment was cloned into the pGEM-T easy vector (Promega; Mannheim, Germany). After restriction analysis and sequencing the plasmid was digested with NcoI and EcoRI and the insert ligated into plasmid $pHis_8$ (36) digested with the same restriction enzymes. Primers for amplification of *ssrg_00986* were ssrg_00986_$pHis_8$_F (5'-CTG CGC GAA TAC CGG AGC TCT GAT CAG ATG AGG CCA CAG T-3') and ssrg_00986_$pHis_8$_R (5'-GCC TCA ATC GCT TGG GAT CCT ACA TGT CCG GAA CCT CCG AG-3'). The underlined letters represent BamHI and SacI restriction sites, respectively. The resulting PCR fragment was digested with BamHI and SacI and ligated into plasmid $pHis_8$ digested with the same restriction enzymes.

The amino acid sequence of NphB was translated into the nucleotide sequence optimized for expression in *E. coli* with the Gene Designer Tool, provided with a $His_8$ tag similar to $pHis_8$ (36) and synthesized commercially by DNA2.0 (Basel, Switzerland). The resulting *dzmP*, *ssrg_00986* and *nphB* expression plasmids were verified by restriction mapping and sequencing.

*E. coli* Rosetta (DE3) pLysS cells harboring the respective expression plasmid were cultivated in 2 liters of liquid TB medium containing 50 μg $ml^{-1}$ kanamycin and 25 μg $ml^{-1}$ chloramphenicol and grown at 37 °C to an $A_{600}$ of 0.6. The temperature was lowered to 20 °C, and IPTG was added to a final concentration of 0.5 mM. The cells were cultured for a further 15 h at 20 °C. Cells were harvested by centrifugation for 20 min at 2,700 × g at 4 °C and the pellet (40 g) was resuspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 20 mM imidazole, 0.5 mg $ml^{-1}$ lysozyme, 0.5 mM phenylmethylsulfonyl fluoride). After stirring at 4 °C for 30 min, cells were ruptured with a Branson sonifier at 4 °C. Debris and membranes were removed by centrifugation at 38,720 × g for 45 min. The supernatant was applied to a nickel-nitrilotriacetic acid-agarose resin column (GE Healthcare) according to the manufacturer's instructions, using a linear gradient of 20 to 250 mM imidazole (in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM β -

mercaptoethanol) in 60 min for elution. Subsequently, a buffer exchange was carried out by PD10 columns (Amersham Biosciences), which were eluted with 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 10% glycerol and 2 mM 1,4-dithiothreitol. Approximately 19 mg of purified DzmP, 15 mg Ssrg_00986 and 19 mg NphB were obtained from 2 liters of cultures.

*Assay for prenyltransferase activity* - The reaction mixture (100 µl) contained 100 mM Tris-HCl pH 7.5, 0.5 mM aromatic substrate, 0.4 mM isoprenoid substrate, 2 mM $MgCl_2$ and either 0.3 µM DzmP or 0.6 µM Ssrg_00986. After incubation of the assay for 15 min at 30 °C (for DzmP) or 30 min (for Ssrg_00986), the reaction mixture was cooled to 0 °C. 200 µl of ethyl acetate were added, and after vortexing and centrifugation 175 µl of the organic layer was transferred to an Eppendorf tube. The solvent was evaporated, and the residue was dissolved in 50 µl methanol and analyzed by HPLC (Agilent 1100 series; Waldbronn, Germany) using a Kinetex column (2.6 µm, 100 × 4.6 mm; Phenomenex, Aschaffenburg, Germany) at a flow rate of 1 ml $min^{-1}$ with a linear gradient from 40 to 100% of solvent B in 20 min (solvent A: water/formic acid (999:1); solvent B: methanol/formic acid (999:1)) and detection at 230 nm for dibenzodiazepinone, 272 nm for genistein, 286 nm for 1,6-DHN and 2,7-DHN, 307 nm for flaviolin and 365 for PCA. Additionally, a UV spectrum from 200 to 400 nm was logged by a photodiode array detector. The absorbance at 230 and 286 nm were used for quantitative analysis of dibenzodiazepinone and 1,6-DHN. For determination of the $K_m$ values for 1,6-DHN and dibenzodiazepinone, FPP was kept at a constant concentration of 1 mM. For determination of the $K_m$ value for GPP and FPP, 1,6-DHN was kept at 1 mM.

*Analysis by HPLC-MS* - The extracts were examined with HPLC-MS and HPLC-MS-MS analysis using a Nucleosil 100-C18 column (3µm, 100 × 2 mm) coupled to an ESI mass spectrometer (LC/MSD Ultra Trap System XCT 6330; Agilent Technology). Analysis was carried out at a flow rate of 0.4 ml $min^{-1}$ with a linear gradient from 10 to 100% of solvent B in 15 min (solvent A: water/formic acid (999:1); solvent B: acetonitrile/formic acid (999.4:0.6)). Detection was carried out at 230, 260, 280, 360, and 435 nm. Electrospray ionization (positive and negative ionization) in Ultra Scan mode with capillary voltage of 3.5 kV and heated capillary temperature of 350 °C was used for analysis.

*Preparative enzymatic synthesis for structure elucidation* - 50 ml (1,6-DHN) or 20 ml (dibenzodiazepinone) of the reaction mixture for prenyltransferase activity with addition of 10 mM sodium ascorbate was incubated at 20 °C over night and extracted twice with 5 ml dichloromethane. The solvent was evaporated and the products were purified by preparative HPLC using a Multospher 120 RP 18 column (5 µm, 250 × 8 mm, Ziemer Chromatographie, Langerwehe, Germany) at a flow rate of 2.5 ml $min^{-1}$. An isocratic elution with 75% (products of 1,6-DHN) or 80% (product of dibenzodiazepinone) of solvent B was used (solvent A: water; solvent B: methanol).

*Structural elucidation of the products of the DzmP reactions* - The $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-600 spectrometer operating at 600.1 MHz. All spectra were recorded at room temperature. Chemical shifts are expressed in parts per million (ppm, δ) and referenced to tetramethylsilane (TMS, δ=0 ppm). Coupling constants are expressed in Hz. The correct assignment of the chemical shifts was confirmed by application of two-dimensional correlation measurements, including correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond coherence (HMBC) and if required by nuclear Overhauser enhancement spectroscopy (NOESY). High resolution mass spectra were measured using either electron spray ionization or atmospheric-pressure chemical ionization on a Bruker Maxis 4G.

The systematic nomenclature for the enzymatic products are 5,10-dihydro-10-[(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienyl]-dibenzo[*b*,*e*][1,4]diazepin-11-one and 4-[(2*E*,6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienyl]-naphthalene-1,6-diol.

## RESULTS

*Identification and cloning of two putative prenyltransferases from Micromonospora sp. RV115 and Streptomyces griseoflavus Tü4000* - In the diazepinomicin gene cluster of *Micromonospora* strain 046Eco-11 identified by McAlpine *et al.* (18), the putative prenyltransferase gene *orf11* is flanked by the HMG-CoA synthase gene *orf10*, and by *orf12* coding for a putative sensor protein of a two-component regulatory system. Recently, diazepinomicin was isolated from the marine sponge-associated strain *Micromonospora* sp. RV115, in collaboration with one of the authors

of the present study (SG) (14). The structure of this compound was unequivocally confirmed by one- and two-dimensional NMR studies. We speculated that the gene clusters in both *Micromonospora* strains are very similar, and therefore we designed primers for the 3' terminus of *orf10* and the 5' terminus of *orf12*. Using genomic DNA of *Micromonospora* sp. RV115 as template, PCR readily gave a product of the expected size. Sequencing showed a coding sequence with very high similarity to *orf11* from *Micromonospora* strain 046Eco-11 (96.9% identity on the amino acid level). The *orf11* orthologue from *Micromonospora* sp. RV115 was termed *dzmP* (NCBI accession KC866371).

BLAST searches revealed in the genome of *Streptomyces griseoflavus* Tü4000 a gene termed *ssrg_00986* which is currently annotated as conserved hypothetical protein (NCBI accession ZP_07309813). This gene showed 61.4% identity (amino acid level) to *dzmP*. Inspection of the genes in the vicinity of *ssrg_00986* in the *S. griseoflavus* Tü4000 genome showed a gene cluster with high similarity to the diazepinomicin gene cluster from *Micromonospora* strain 046Eco-11 (Fig. 3). This *S. griseoflavus* Tü4000 cluster contains orthologs of all genes from which an essential function in diazepinomicin biosynthesis has been suggested (18). However, cultivation of *S. griseoflavus* Tü4000 in HI medium (18) and Bennett's broth (14) followed by LC-MS analysis did not show any diazepinomicin production, suggesting that the gene cluster may be silent under the conditions employed. Nevertheless, we amplified the putative prenyltransferase gene *ssrg_00986* from genomic DNA of *S. griseoflavus* Tü4000. The correct DNA sequence was confirmed, and both this gene and *dzmP* were cloned into an expression vector for expression as N-terminally His-tagged proteins.

*Bioinformatic sequence analysis of DzmP and Ssrg_00986* - The genes *dzmP* and *ssrg_00986* code for proteins of 296 and 295 amino acids, respectively. Secondary structure prediction shows for both gene products the five-fold αααββ repeat which is typical for the ABBA prenyltransferases (Fig. 4). A phylogenetic analysis (Fig. 5) places DzmP, Orf11 and Ssrg_00986 into a new, separate branch of the previously described family of phenol / phenazine prenyltransferases (2). DzmP and its orthologs are quite similar to the hydroxynaphthalene prenyltransferases NphB (37) and Fnq26 (38), and to the phenazine prenyltransferases PpzP (29) and EpzP (8, 39) (average sequence identity ~ 43%). While this study was in progress, a further gene cluster with close similarity to the diazepinomicin cluster was deposited in GenBank by the group of Zhongjun Qin from the Shanghai Institutes for Biological Sciences. This cluster (NCBI accession JQ432566) was obtained from a strain termed *Streptomyces* sp. WT3. It contains a gene named WT3.9 which codes for a 296 aa protein with close similarity to DzmP and Ssrg_00986 (Fig. 5). No further close orthologs of DzmP, and no further gene clusters with close similarity to the diazepinomicin cluster, are currently deposited in GenBank.

DzmP and Ssrg_00986 (Fig. 5), as well as Orf11 and Wt3.9, contain arginine residues in position 51 and 64. X-ray crystallographic studies (7) as well as modeling studies (4) have suggested that these residues are essential for the cofactor-independent binding of the α-phosphate of the isoprenoid substrate, and therefore are characteristic for ABBA prenyltransferases which are independent of $Mg^{2+}$. In contrast, $Mg^{2+}$-dependent ABBA prenyltransferases such as NphB (5) and $Ptf_{St}$ (40) contain serine residues in these positions. DzmP and Ssrg_0986 are therefore expected to catalyze a prenyl transfer independent of the presence of magnesium ions.

*Expression and purification of DzmP and Ssrg_00986* - DzmP and Ssrg_00986 were expressed as His-tagged proteins in *E. coli*, readily yielding soluble proteins which were purified by $Ni^{2+}$ affinity chromatography (Fig. 6). From 1 L of culture 9.5 mg DzmP and 7.5 mg Ssrg_00986 were obtained.

*Prenyltransferase activity of DzmP and Ssrg_00986* - In the hypothetical biosynthetic pathway of diazepinomicin suggested by McAlpine *et al.* (18), the farnesylation of 4,6,8-trihydroxy-dibenzodiazepinone (Fig. 1) is suggested as the final step. Commercially, only non-hydroxylated dibenzodiazepinone is available, and we therefore tested this compound (Fig. 2) as substrate. Furthermore, 1,6-dihydroxynaphthalene (1,6-DHN) was tested, since this compound is known to be accepted by NphB (37), a prenyltransferase with similarity to DzmP (Fig. 5). Using farnesyl diphosphate (FPP) as isoprenoid substrate, an enzymatic prenylation of both aromatic compounds by DzmP and, with lower activity, by Ssrg_00986, was readily observed by HPLC-UV and HPLC-MS. Geranyl diphosphate (GPP) was converted with much lower reaction velocity, and therefore the

investigations were continued with FPP. Enzymatic product formation showed linear dependence on time for at least 30 min, and linear dependence on protein amount at least up to 12 µM. The addition of 100 mM NaCl increased activity by 10%, whereas the addition of glycerol had no effect. Product formation was readily detectable in the absence of magnesium ions, but the addition of 2 mM $Mg^{2+}$ increased the activity of DzmP approximately 1.5 fold and the activity of Ssrg_00986 approximately 3 fold compared to assays without $Mg^{2+}$. Addition of EDTA (1 mM) did not influence the activity. Maximal product formation for DzmP was observed at pH 8.0, with half-maximal values at pH 5.8 and 10.3. Ssrg_00986 showed maximal activity at pH 7.5, with half-maximal values at pH 6.3 and 8.2.

*Structural identification of the farnesylation product of dibenzodiazepinone* - Prenylation of dibenzodiazepinone by an ABBA prenyltransferase may occur at many different positions of the molecule. Each of the two aromatic rings (Fig. 2) offers four unsubstituted carbons for *C*-prenylation, in a reaction which would resemble the prenylation of the indole nucleus of *L*-tryptophan by different indole prenyltransferases (41, 42) or the *C*-prenylation of a dihydrophenazine derivative by PpzP and EpzP (8, 29). Notably, PpzP and EpzP show close similarity to DzmP (Fig. 5). However, a few ABBA prenyltransferases catalyze *N*-prenylations (43-46). In the dibenzodiazepinone molecule the nitrogen atom which is chemically most reactive for alkylation reactions is N-5 (Fig. 2). In contrast the amide nitrogen (N-10) is expected to be much less reactive, and enzymatic prenylations of an amide nitrogen are very unusual.

Prenylation of dibenzodiazepinone with FPP under catalysis of DzmP yielded a single product which showed the mass of a mono-farnesylated product. In order to determine the substitution position, the assay was scaled up to 20 ml and the enzymatic product was purified on preparative scale. By APCI-HRMS, the molecular formula of the reaction product was deduced to be $C_{28}H_{35}N_2O$ ($[M+H^+]$, *m/z* calc. 415.27439, found 415.27475, Δ 0.9 ppm). MS-MS showed a fragment $C_{14}H_{11}N_2O$ ($[M^+]$, *m/z* calc. 223.08659, found 223.08711, Δ 2.3 ppm) indicating the loss of the farnesyl chain. Full $^1H$ and $^{13}C$ NMR spectroscopic data of the product as well as selected $^1H$-$^1H$ COSY, NOESY and $^1H$ HMBC correlations are given in the Supplementary (Fig. S1 and Table S1). The position of the farnesylation was unequivocally confirmed by NOESY ($\tau_m$ = 1 s). NOESY signals of H-1' ($\delta$ = 4.48 ppm) and H-9 ($\delta$ = 7.26 ppm), H-5 ($\delta$ = 7.82 ppm) and H-6 ($\delta$ = 7.10 ppm), H-5 and H-4 ($\delta$ = 7.02 ppm) as well as a HMBC signal of H-1' and the carbonyl carbon C-11 ($\delta$ = 167.52 ppm) prove the farnesylation of dibenzodiazepinone at the amide nitrogen in position 10 (Fig. 2).

The farnesylated products generated from dibenzodiazepinone under catalysis of DzmP and Ssrg_00986 showed exactly the same retention time under different HPLC conditions, as well as the same mass and fragmentation in HPLC-MS-MS analysis. This indicates that both enzymes catalyze the same reaction.

*Structural identification of the major farnesylation product of 1,6-dihydroxynaphthalene* - The reaction of 1,6-DHN with FPP under catalysis of DzmP yielded one major product (Fig. 7B; Rt = 9.5 min) and two minor products (Rt = 9.7 min and 9.9 min), each showing the mass of a monofarnesylated 1,6-DHN. A preparative scale assay was carried out and the major product was isolated for structural elucidation. The molecular formula of the reaction product was deduced to be $C_{25}H_{33}O_2$ ($[M+H^+]$, calc. 365.24751, found 365.24717, Δ 0.9 ppm) by HPLC-ESI-HRMS. The fragment $C_{11}H_9O_2$ ($[M^+]$, calc. 173.05971, found 173.05969, Δ 0.1 ppm) indicated the loss of the farnesyl chain. The position of the farnesylation of 1,6-DHN was determined using unidimensional ($^1H$ NMR, $^{13}C$ NMR) and multidimensional ($^1H$-$^1H$ COSY, $^1H$ HSQC and $^1H$ HMBC) NMR spectroscopy (Supplementary Fig. S2 and Table S2), revealing coupling patterns of the aromatic protons in $^1H$ NMR and unambiguous COSY-Signals. The coupling pattern indicates H-5 ($\delta$ = 7.20 ppm; d, 2.4 Hz) to be in *meta* position of H-7 ($\delta$ = 7.03 ppm; dd, 2.4 and 9.0 Hz) and H-8 ($\delta$ = 9.02 ppm; d, 9.0 Hz) to be in *ortho* position to H-7 as well as H-2 ($\delta$ = 6.58 ppm; d, 7.6 Hz) to be in *ortho* position of H-3 ($\delta$ = 7.07 ppm; d, 7.6 Hz). Because of the characteristic chemical shifts of H-2 and H-8, and due to the close similarity to the published NMR data of 4-geranyl-1,6-DHN (37), the position of the farnesylation could be deduced to be at C-4 (Fig. 2). The products formed by Ssrg_00986 from the same substrates showed the same retention time as the DzmP products in different HPLC systems, and showed the same mass and fragmentation in HPLC-MS-MS analysis. This suggested that the products from both enzymes are identical.

*Comparison of the prenylation reactions of 1,6-DHN catalyzed by NphB, DzmP and Ssrg_00986* - NphB catalyzes the geranylation of a (so far unknown) hydroxynaphthalene substrate in the biosynthesis of naphterpin (37). Using the artificial substrate 1,6-DHN, it preferentially prenylates the 5-position. In clear contrast, we found that DzmP preferentially farnesylates the 4-position of 1,6-DHN (see above). This difference prompted us to express and purify NphB following the same procedure as for DzmP and Ssrg_00986, and to compare the reaction products of NphB, DzmP and Ssrg_00986 upon incubation with 1,6-DHN and either GPP or FPP.

As expected, NphB preferentially accepted GPP as substrate; FPP was converted 30 times more slowly. In contrast, DzmP preferentially accepted FPP, and GPP was converted 8 times more slowly. As published previously, NphB forms three geranylated products from 1,6-DHN (Fig. 7A). The major product has been identified as 5-geranyl-1,6-DHN, with 2-geranyl-1,6-DHN as the second most abundant and 4-geranyl-1,6-DHN as the least abundant product (37). As shown in Fig. 7A, also DzmP forms three products from GPP and 1,6-DHN, and these showed the same HPLC retention time, and the same mass and fragmentation pattern, as the products formed by NphB. In case of DzmP the peak corresponding to 4-geranyl-1,6-DHN is the dominant product, and 5-geranyl-1,6-DHN is the least abundant product. A similar formation of three products is observed when FPP is used as substrate (Fig. 7B). 4-farnesyl-1,6-DHN is the major product formed by DzmP, while this compound is the least abundant product formed by NphB. Therefore, NphB and DzmP show a different regioselectivity in the prenylation of 1,6-DHN. The product spectrum of Ssrg_00986, both with GPP and FPP, was found to be very similar to that of DzmP (Fig. 7A and B), confirming the close similarity of the reaction catalyzed by both enzymes.

*Kinetic investigations of the prenylation of 1,6-DHN and dibenzodiazepinone by DzmP and Ssrg_00986* - The reactions of DzmP and Ssrg_00986 with dibenzodiazepinone and 1,6-DHN as aromatic substrates, and with GPP and FPP as isoprenoid substrates, were investigated. Kinetic constants were calculated using nonlinear regression. Depending on the substrates used, a better fit was obtained by using the equations for sigmoidal curve (indicating cooperativity) or for a substrate inhibition kinetic (47), rather than by using the Michaelis-Menten equation. Using 1,6-DHN, calculations were based on the formation of the 4-prenylated product (*i.e.* the major product). Using 1 mM FPP and different concentrations of 1,6-DHN, both DzmP and Ssrg_0986 gave sigmoidal curves. $k_{0.5}$ values for 1,6-DHN were calculated as 18 ± 1 and 96 ± 11 µM, respectively. For DzmP, $k_{cat}$ was determined as 4.5 ± 0.1 $s^{-1} \times 10^{-3}$, comparable to the value of 4.2 ± 0.2 $s^{-1} \times 10^{-3}$ published for the reaction of GPP and 1,6-DHN catalyzed by NphB (37). For Ssrg_00986, $k_{cat}$ was 0.4 ± 0.01 $s^{-1} \times 10^{-3}$.

Using 1 mM FPP and different concentrations of dibenzodiazepinone, substrate inhibition ($k_i$ = 655 ± 213 µM) was observed. The $K_m$ and $k_{cat}$ values of DzmP for dibenzodiazepinone were determined as 133 ± 30 µM and 0.68 ± 0.09 $s^{-1} \times 10^{-3}$, respectively. For Ssrg_00986, reaction velocity was at least 10 times lower, preventing the reliable measurement of kinetic constants.

Using 1 mM 1,6-DHN and different concentrations of FPP, the plot of reaction velocity over substrate concentration could not be adequately fitted to a Michaelis-Menten, sigmoidal or substrate inhibition kinetic for DzmP. Half-maximal reaction velocity was observed at approximately 20 µM FPP. With GPP as isoprenoid substrate, a sigmoidal curve was obtained, and $K_m$ and $k_{cat}$ were calculated as 4.9 ± 0.9 µM and 0.5 ± 0.01 $s^{-1} \times 10^{-3}$, respectively.

For Ssrg_00986, a precise comparison of the kinetic constants for FPP and GPP was hampered by the low reaction velocity. However, the enzyme did not show a clear preference for FPP: for both isoprenoid substrates, half-maximal reaction velocities were observed at approximately 45 µM, and maximal turn-over rates were approximately 0.5 $s^{-1} \times 10^{-3}$.

*Prenylation of further aromatic substrates* - In order to investigate the promiscuity of DzmP for different aromatic substrates, the enzyme was incubated with FPP and either 2,7-dihydroxynaphthalene (2,7-DHN), 5,10-dihydrophenazine-1-carboxylic acid (dihydro-PCA), flaviolin (=2,5,7-trihydroxy-1,4-naphthoquinone) and the isoflavonoid genistein. These compounds have been described to be accepted by ABBA prenyltransferases with sequence similarity to DzmP, *e.g.* by NphB (37), PpzP (29) or Fnq26 (38). As determined by HPLC-UV and HPLC-MS-MS, all of these four compounds were converted to mono-farnesylated products, with turn-over rates of approximately 0.57, 0.34, 0.21 and 0.11 $s^{-1} \times 10^{-3}$, respectively. 2,7-DHN and flaviolin yielded a single product,

genistein yielded two, and in the prenylation of dihydro-PCA the major product was accompanied by four side products of the same mass, suggesting that farnesylation may occur at different positions of the molecule.

## DISCUSSION

DzmP is the first member of the ABBA prenyltransferase superfamily which utilizes farnesyl diphosphate (FPP; $C_{15}$) as genuine substrate. All previously discovered members utilize either DMAPP ($C_5$) or GPP ($C_{10}$). ABBA prenyltransferases are useful tools for chemoenzymatic synthesis, due to their nature as soluble, stable biocatalysts which is in contrast to the membrane-bound nature of prenyltransferases of lipoquinone biosynthesis. Most ABBA prenyltransferases are independent from magnesium as cofactor which is desirable since magnesium ions accelerate the non-enzymatic hydrolysis of prenyl diphosphates (48). Finally, ABBA prenyltransferases have a remarkable promiscuity for different aromatic substrates (3-5). The discovery of DzmP now provides a welcome extension of the isoprenoid substrate range of this superfamily.

The active center of the ABBA prenyltransferases is located within the central barrel which is formed by ten antiparallel β strands. Nearly all of the amino acids interacting with the substrates are part of these β strands, creating a spatially restricted environment (5-7). After the discovery of CloQ and NphB which utilize DMAPP ($C_5$) or GPP ($C_{10}$) as isoprenoid substrates, respectively (5, 7, 49), speculations have been offered which structural features of an ABBA prenyltransferase determine the chain length specificity for the isoprenoid substrate (4, 50). *E.g.* it has been suggested that R66 and E281 in CloQ form salt bridges which sterically hinder the accommodation of geranyl diphosphate and thereby restrict the isoprenoid substrate to five carbons, *i.e.* to dimethylallyl diphosphate (4). Notably, however, the same residues are now found in DzmP in form of R65 and E283, as evident by modeling of both CloQ and DzmP using the Phyre 2 (32) and the ASC server (51). This contradicts the above hypothesis that these residues restrict the chain length of the isoprenoid substrate. Further X-ray crystallographic investigations and mutagenesis experiments will be required in order to elucidate how the different chain length specificities of ABBA prenyltransferases are determined.

The prenylation of an amide nitrogen has not previously been described from an enzyme of the ABBA prenyltransferase superfamily. Prenylated amide nitrogens are very rarely found in nature. Among the few exceptions are *N*-prenylated xanthine derivatives which have been described from plants (52), as well as some protozoan secondary metabolites (53).

The keto group of the dibenzodiazepinone is subject to a keto-enol tautomerism. Recent X-ray crystallographic investigations suggest that the keto (= amino-oxo) tautomer is the dominant form, compared to the enol (= hydroxyimine) form (54). Only the keto form offers a free electron pair at the amide nitrogen, which may be required for the alkylation of this heteroatom. Nevertheless, the reactivity of an amide nitrogen for alkylation reactions is low, making the prenylation catalyzed by DzmP a quite unusual enzymatic reaction.

Benzodiazepines like dibenzodiazepinone form a new class of prenyl acceptor substrates of ABBA prenyltransferases. The discovery of DzmP shows that the substrate range of the previously described phenol / phenazine prenyltransferase family (2) extends beyond these compound classes, and may be further expanded when other currently uncharacterized GenBank entries with similarity to this family (Fig. 5) are investigated.

The regiospecific farnesylation of N-10 of dibenzodiazepinone by DzmP supports the hypothesis by McAlpine *et al.* (18) that this *N*-prenylation reaction may be the last step of diazepinomicin biosynthesis (Fig. 1). The suggested genuine substrate, *i.e.* 4,6,8-trihydroxy-dibenzodiazepinone, is likely to be more reactive towards *N*-alkylation due to the OH-groups which may also contribute to the binding of the substrate in the active center. The absence of these groups in the artificial substrate dibenzodiazepinone (Fig. 2) may explain the relatively low catalytic turnover of that compound.

The dibenzodiazepinone structure has been described as unique in nature (18), and has so far only been found in the genus *Micromonospora*. The discovery of gene clusters with similarity to the diazepinomicin cluster in *Streptomyces griseoflavus* Tü4000 (Fig. 3), and in *Streptomyces* sp. WT3 (NCBI accession JQ432566) may indicate that similar compounds can be found also in *Streptomyces* strains. However, the exact structure of such compounds, including the length of their isoprenoid chain, remains to be elucidated.

**REFERENCES**


1. Heide, L. (2009) Prenyl transfer to aromatic substrates: genetics and enzymology. *Curr. Opin. Chem. Biol.* **13**, 171-179
2. Bonitz, T., Alva, V., Saleh, O., Lupas, A. N., and Heide, L. (2011) Evolutionary relationships of microbial aromatic prenyltransferases. *PLoS ONE* **6**, e27336
3. Li, S. M. (2010) Prenylated indole derivatives from fungi: structure diversity, biological activities, biosynthesis and chemoenzymatic synthesis. *Nat. Prod. Rep.* **27**, 57-78
4. Tello, M., Kuzuyama, T., Heide, L., Noel, J. P., and Richard, S. B. (2008) The ABBA family of aromatic prenyltransferases: broadening natural product diversity. *Cell. Mol. Life Sci.* **65**, 1459-1463
5. Kuzuyama, T., Noel, J. P., and Richard, S. B. (2005) Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. *Nature* **435**, 983-987
6. Metzger, U., Schall, C., Zocher, G., Unsöld, I., Stec, E., Li, S. M., Heide, L., and Stehle, T. (2009) The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 14309-14314
7. Metzger, U., Keller, S., Stevenson, C. E., Heide, L., and Lawson, D. M. (2010) Structure and mechanism of the magnesium-independent aromatic prenyltransferase CloQ from the clorobiocin biosynthetic pathway. *J. Mol. Biol.* **404**, 611-626
8. Zocher, G., Saleh, O., Heim, J. B., Herbst, D. A., Heide, L., and Stehle, T. (2012) Structure-based engineering increased the catalytic turnover rate of a novel phenazine prenyltransferase. *PLoS ONE* **7**, e48427
9. Gerratana, B. (2010) Biosynthesis, synthesis, and biological activities of pyrrolobenzodiazepines. *Med. Res. Rev.* **32**, 254-293
10. Igarashi, Y., Miyanaga, S., Onaka, H., Takeshita, M., and Furumai, T. (2005) Revision of the structure assigned to the antibiotic BU-4664L from *Micromonopora*. *J. Antibiot.* **58**, 350-352
11. Ohkuma, H., and Kobaru, S. (1996) Compound produced by a strain of Micromonospora. Bristol-Myers Squibb Company, United States
12. Bachmann, B. O., McAlpine, J. B., Zazopoulos, E., Farnet, C. M., and Piraee, M. (2006) Farnesyl dibenzodiazepinone, and processes for its production. Ecopia BioSciences, Inc., United States
13. Charan, R. D., Schlingmann, G., Janso, J., Bernan, V., Feng, X., and Carter, G. T. (2004) Diazepinomicin, a new antimicrobial alkaloid from a marine *Micromonospora* sp. *J. Nat. Prod.* **67**, 1431-1433
14. Abdelmohsen, U. R., Szesny, M., Othman, E. M., Schirmeister, T., Grond, S., Stopper, H., and Hentschel, U. (2012) Antioxidant and anti-protease activities of diazepinomicin from the sponge-associated *Micromonospora* strain RV115. *Mar. Drugs* **10**, 2208-2221
15. Boufaied, N., Wioland, M. A., Falardeau, P., and Gourdeau, H. (2010) TLN-4601, a novel anticancer agent, inhibits Ras signaling post Ras prenylation and before MEK activation. *Anticancer Drugs* **21**, 543-552
16. Gourdeau, H., McAlpine, J. B., Ranger, M., Simard, B., Berger, F., Beaudry, F., Farnet, C. M., and Falardeau, P. (2008) Identification, characterization and potent antitumor activity of ECO-4601, a novel peripheral benzodiazepine receptor ligand. *Cancer Chemother. Pharmacol.* **61**, 911-921
17. Mason, W. P., Belanger, K., Nicholas, G., Vallieres, I., Mathieu, D., Kavan, P., Desjardins, A., Omuro, A., and Reymond, D. (2012) A phase II study of the Ras-

MAPK signaling pathway inhibitor TLN-4601 in patients with glioblastoma at first progression. *J. Neurooncol.* **107**, 343-349
18. McAlpine, J. B., Banskota, A. H., Charan, R. D., Schlingmann, G., Zazopoulos, E., Piraee, M., Janso, J., Bernan, V. S., Aouidate, M., Farnet, C. M., Feng, X., Zhao, Z., and Carter, G. T. (2008) Biosynthesis of diazepinomicin/ECO-4601, a *Micromonospora* secondary metabolite with a novel ring system. *J. Nat. Prod.* **71**, 1585-1590
19. Lima, W. C., Varani, A. M., and Menck, C. F. (2009) NAD biosynthesis evolution in bacteria: lateral gene transfer of kynurenine pathway in Xanthomonadales and Flavobacteriales. *Mol. Biol. Evol.* **26**, 399-406
20. Zummo, F. P., Marineo, S., Pace, A., Civiletti, F., Giardina, A., and Puglia, A. M. (2012) Tryptophan catabolism via kynurenine production in *Streptomyces coelicolor*: identification of three genes coding for the enzymes of tryptophan to anthranilate pathway. *Appl. Microbiol. Biotechnol.* **94**, 719-728
21. Feng, X., Ratnayake, A. S., Charan, R. D., Janso, J. E., Bernan, V. S., Schlingmann, G., He, H., Tischler, M., Koehn, F. E., and Carter, G. T. (2009) Probing natural product biosynthetic pathways using Fourier transform ion cyclotron resonance mass spectrometry. *Bioorg. Med. Chem.* **17**, 2154-2161
22. Ratnayake, A. S., Janso, J. E., Feng, X., Schlingmann, G., Goljer, I., and Carter, G. T. (2009) Evaluating indole-related derivatives as precursors in the directed biosynthesis of diazepinomicin analogues. *J. Nat. Prod.* **72**, 496-499
23. Mentel, M., Ahuja, E. G., Mavrodi, D. V., Breinbauer, R., Thomashow, L. S., and Blankenfeldt, W. (2009) Of two make one: the biosynthesis of phenazines. *Chembiochem* **10**, 2295-2304
24. Silakowski, B., Kunze, B., Nordsiek, G., Blöcker, H., Höfle, G., and Müller, R. (2000) The myxochelin iron transport regulon of the myxobacterium *Stigmatella aurantiaca* Sg a15. *Eur. J. Biochem.* **267**, 6476-6485
25. Floss, H. G., Yu, T. W., and Arakawa, K. (2011) The biosynthesis of 3-amino-5-hydroxybenzoic acid (AHBA), the precursor of mC7N units in ansamycin and mitomycin antibiotics: a review. *J. Antibiot. (Tokyo)* **64**, 35-44
26. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402
27. Höfer, I., Crüsemann, M., Radzom, M., Geers, B., Flachshaar, D., Cai, X., Zeeck, A., and Piel, J. (2011) Insights into the biosynthesis of hormaomycin, an exceptionally complex bacterial signaling metabolite. *Chem. Biol.* **18**, 381-391
28. Woodside, A. B., Huang, Z., and Poulter, C. D. (1993). *Org. Synth.* **8**, 616
29. Saleh, O., Gust, B., Boll, B., Fiedler, H. P., and Heide, L. (2009) Aromatic prenylation in phenazine biosynthesis: dihydrophenazine-1-carboxylate dimethylallyltransferase from *Streptomyces anulatus*. *J. Biol. Chem.* **284**, 14439-14447
30. Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, UK
31. Sambrook, J. a. R., D. W. (2001) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Kelley, L. A., and Sternberg, M. J. E. (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protocols* **4**, 363-371
33. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948

34. Gouet, P., Courcelle, E., Stuart, D. I., and Métoz, F. (1999) ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* **15**, 305-308
35. Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., and Barton, G. J. (2009) Jalview Version 2 - a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189-1191
36. Jez, J. M., Ferrer, J. L., Bowman, M. E., Dixon, R. A., and Noel, J. P. (2000) Dissection of malonyl-coenzyme A decarboxylation from polyketide formation in the reaction mechanism of a plant polyketide synthase. *Biochemistry* **39**, 890-902
37. Kumano, T., Richard, S. B., Noel, J. P., Nishiyama, M., and Kuzuyama, T. (2008) Chemoenzymatic syntheses of prenylated aromatic small molecules using Streptomyces prenyltransferases with relaxed substrate specificities. *Bioorg. Med. Chem.* **16**, 8117-8126
38. Haagen, Y., Unsöld, I., Westrich, L., Gust, B., Richard, S. B., Noel, J. P., and Heide, L. (2007) A soluble, magnesium-independent prenyltransferase catalyzes reverse and regular *C*-prenylations and *O*-prenylations of aromatic substrates. *FEBS Lett.* **581**, 2889-2893
39. Seeger, K., Flinspach, K., Haug-Schifferdecker, E., Kulik, A., Gust, B., Fiedler, H.-P., and Heide, L. (2011) The biosynthetic genes for prenylated phenazines are located at two different chromosomal loci of *Streptomyces cinnamonensis* DSM 1042. *Microb. Biotechnol.* **4**, 252-262
40. Saleh, O., Bonitz, T., Flinspach, K., Kulik, A., Burkard, N., Mühlenweg, A., Vente, A., Polnick, S., Lämmerhofer, M., Gust, B., Fiedler, H.-P., and Heide, L. (2012) Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines. *Med. Chem. Comm.* **3**, 1009-1019
41. Li, S. M. (2009) Evolution of aromatic prenyltransferases in the biosynthesis of indole derivatives. *Phytochemistry* **70**, 1746-1757
42. Li, S. M. (2009) Applications of dimethylallyltryptophan synthases and other indole prenyltransferases for structural modification of natural products. *Appl. Microbiol. Biotechnol.* **84**, 631-639
43. Grundmann, A., Kuznetsova, T., Afiyatullov, S. S., and Li, S.-M. (2008) FtmPT2, an *N*-prenyltransferase from *Aspergillus fumigatus*, catalyses the last step in the biosynthesis of Fumitremorgin B. *Chembiochem* **9**, 2059-2063
44. Schuller, J. M., Zocher, G., Liebhold, M., Xie, X., Stahl, M., Li, S. M., and Stehle, T. (2012) Structure and catalytic mechanism of a cyclic dipeptide prenyltransferase with broad substrate promiscuity. *J. Mol. Biol.* **422**, 87-99
45. Schultz, A. W., Lewis, C. A., Luzung, M. R., Baran, P. S., and Moore, B. S. (2010) Functional characterization of the cyclomarin/cyclomarazine prenyltransferase CymD directs the biosynthesis of unnatural cyclic peptides. *J. Nat. Prod.* **73**, 373-377
46. Yin, W. B., Ruan, H. L., Westrich, L., Grundmann, A., and Li, S.-M. (2007) CdpNPT, an *N*-Prenyltransferase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterisation. *Chembiochem* **8**, 1154-1161
47. Cornish-Bowden, A. (2004) *Fundamentals of Enzyme Kinetics*, 3 ed., Portland Press Ltd.
48. Brems, D. N., and Rilling, H. C. (1977) On the mechanism of the prenyltransferase reaction. Metal ion dependent solvolysis of an allylic pyrophosphate. *J. Am. Chem. Soc.* **99**, 8351-8352
49. Pojer, F., Wemakor, E., Kammerer, B., Chen, H., Walsh, C. T., Li, S. M., and Heide, L. (2003) CloQ, a prenyltransferase involved in clorobiocin biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 2316-2321

50. Cui, G., Li, X., and Merz, K. M., Jr. (2007) Understanding the substrate selectivity and the product regioselectivity of Orf2-catalyzed aromatic prenylations. *Biochemistry* **46**, 1303-1311
51. Röttig, M., Rausch, C., and Kohlbacher, O. (2010) Combining structure and sequence information allows automated prediction of substrate specificities within enzyme families. *PLoS Comput. Biol.* **6**, e1000636
52. Auzi, A. A., Gray, A. I., Hartley, T. G., Waigh, R. D., and Waterman, P. G. (1994) A xanthine alkaloid from the leaves of *Bosistoa floydii*. *Phytochemistry* **36**, 535-536
53. Kikuchi, H., Ishiko, S., Nakamura, K., Kubohara, Y., and Oshima, Y. (2010) Novel prenylated and geranylated aromatic compounds isolated from *Polysphondylium* cellular slime molds. *Tetrahedron* **66**, 6000-6007
54. Keller, M., Bhadbhade, M. M., and Read, R. W. (2012) Two dibenzodiazepinone molecules with dissimilar dimeric associations and apparent different tautomeric forms. *Acta Cryst. C* **68**, o240-o246

*Acknowledgements* - We thank Prof. W. Wohlleben (University of Tübingen) for providing *Streptomyces griseoflavus* Tü4000, U. Hentschel (University of Würzburg) for providing *Micromonospora* sp. RV115, O. Saleh (University of Tübingen) for advice on the PCR cloning of *ssrg_00986*, J. P. Noel (Salk Institute, La Jolla, CA) for the plasmid pHis$_8$, K. Seeger (University of Tübingen) for the generation of the *nphB* expression plasmid, A. Kulik (University of Tübingen) for MS analyses and M. Kramer (University of Tübingen) for recording the NMR spectra.

**FOOTNOTES**

*This work was supported by a grant from the German Federal Ministry of Education and Research (GenBioCom).
[1]To whom correspondence should be addressed: Lutz Heide, Pharmaceutical Institute, University of Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany, Tel.: +4970712972460; E-mail: heide@uni-tuebingen.de
[2]The abbreviations used are: DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; 1,6-DHN, 1,6-dihydroxynaphthalene; dihydro-PCA, 5,10-dihydrophenazine-1-carboxylic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; aa, amino acid; COSY, Correlation Spectroscopy; HMBC, Heteronuclear Multiple Bond Correlation; HSQC, Heteronuclear Single Quantum Coherence; NOESY, Nuclear Overhauser Effect Spectroscopy; APCI-HRMS, Atmospheric Pressure Chemical Ionization - High Resolution Mass Spectroscopy.

**FIGURE LEGENDS**

**FIGURE 1.** Diazepinomicin and its hypothetical biosynthetic pathway (modified from McAlpine *et al.* (18)). 1) Pathway encoded in the gene cluster of diazepinomicin. 2) Degradation of tryptophan via the kynurenine pathway.

**FIGURE 2.** Reactions catalyzed by DzmP and Ssrg_00986. FPP, farnesyl diphosphate; 1,6-DHN, 1,6-dihydroxynaphthalene.

**FIGURE 3.** Comparison of the biosynthetic gene cluster of diazepinomicin in *Micromonospora* strain 046Eco-11 with the newly identified gene cluster in *Streptomyces griseoflavus* Tü4000. Homologous genes are connected by gray areas. The numbering and the suggested function of genes of *Micromonospora* strain 046Eco-11 are adapted from McAlpine *et al.* (18).

**FIGURE 4.** Amino acid alignment und secondary structure prediction for DzmP and Ssrg_00986 visualized by ESPript (34). Secondary structure elements are: α, α helices; η, $3_{10}$ helices; β, β strands; TT, strict β turns. Strict sequence identity is shown by a *black box* with *white characters*, and similarity is shown by *bold characters* in a *black frame*. The position of the two arginine residues typical for $Mg^{2+}$-independent ABBA prenyltransferases are indicated by asterisks.

**FIGURE 5.** Evolutionary tree of aromatic prenyltransferases of the CloQ/NphB family (= phenol / phenazine prenyltransferases, (2)). The branch highlighted in grey represents the new prenyltransferases investigated in this study. The evolutionary tree was generated with MEGA4 using the default parameters for pairwise (multiple) alignment. Phylogenetic reconstruction was carried out using the neighbor-joining method. Names of biochemically investigated enzymes (2) are shown in bold, and further uncharacterized NCBI database entries are: 1, ZP_10450727; 2, ABS50461 and ABS50489; 3, CCK32327; 4, AEW22941; 5, CAL34106; 6, ABS50462; 7, ABS50490; 8, YP_005045476; 9, YP_006808349; 10, ZP_06526769; 11, ZP_09171437; 12, XP_002847323; 13, XP_002143864 and 14, CCD48995.

**FIGURE 6.** Purification of DzmP (A) and Ssrg_00986 (B) after expression as His$_8$-tagged fusion proteins. *Lane 1*, total protein before IPTG induction; *lane 2*, total protein after IPTG induction; *lane 3*, soluble protein after IPTG induction; *lane 4*, insoluble protein after IPTG induction; *lane 5*, protein after $Ni^{2+}$ affinity chromatography; *lane 6*, protein after buffer exchange over Sephadex G-25; *lane M*,

molecular mass standards. The calculated masses are 34.8 kDa for both enzymes. The 12% polyacrylamide gel was stained with Coomassie Brilliant Blue R-250.

**FIGURE 7.** Geranylation (A) or farnesylation (B) of 1,6-DHN under catalysis of NphB, DzmP and Ssrg_00986. Reaction products were analyzed by HPLC with UV detection at 286 nm. Absorbance was scaled differently for each enzyme in order to improve visibility of the product peaks.

Figure 1

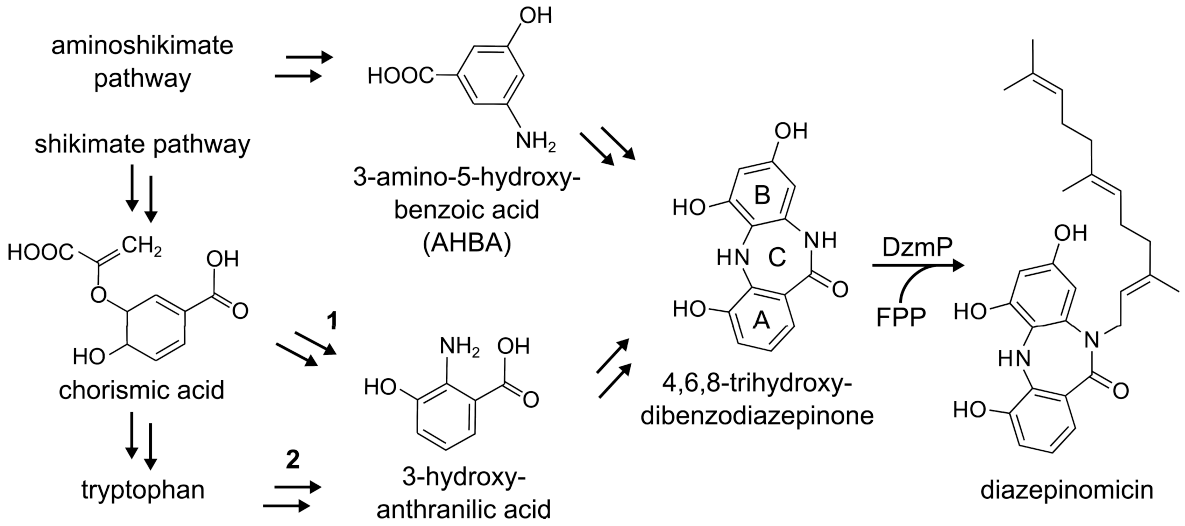

aminoshikimate pathway
shikimate pathway
chorismic acid
tryptophan
1
2
3-amino-5-hydroxy-benzoic acid (AHBA)
3-hydroxy-anthranilic acid
4,6,8-trihydroxy-dibenzodiazepinone
DzmP
FPP
diazepinomicin

Figure 2

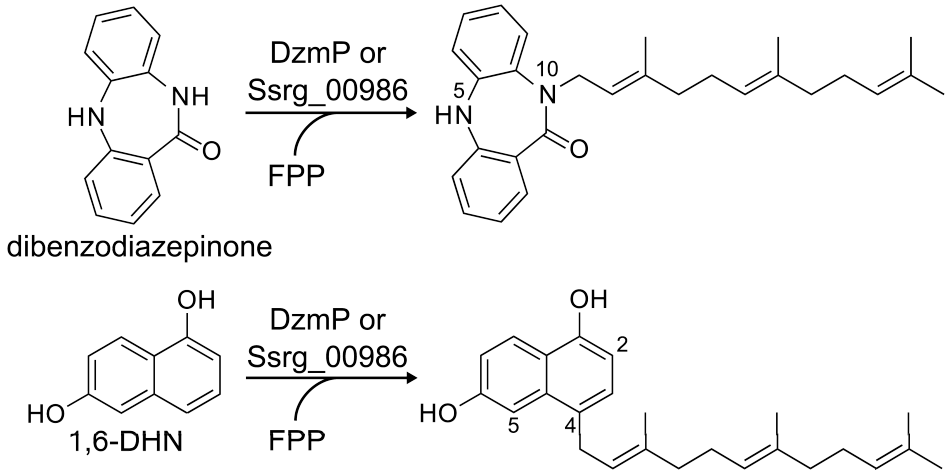

DzmP or
Ssrg_00986
FPP
HN
NH
O
dibenzodiazepinone
10
5
N
HN
O
OH
HO
1,6-DHN
DzmP or
Ssrg_00986
FPP
OH
2
HO
5
4

Figure 3

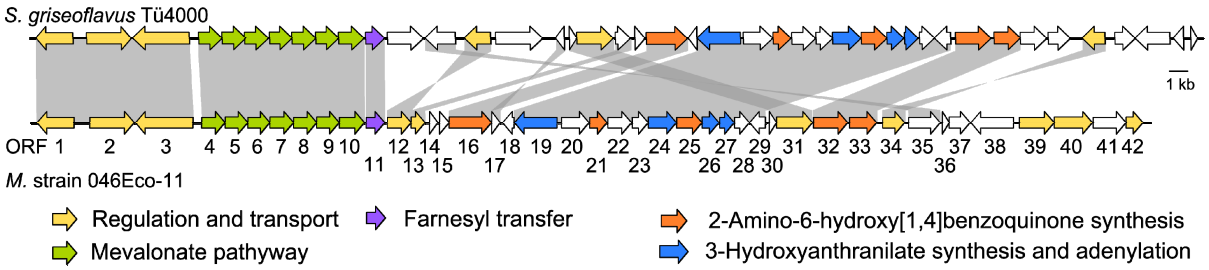

S. griseoflavus Tü4000
1 kb
ORF 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
M. strain 046Eco-11
Regulation and transport
Farnesyl transfer
2-Amino-6-hydroxy[1,4]benzoquinone synthesis
Mevalonate pathyway
3-Hydroxyanthranilate synthesis and adenylation

Figure 4

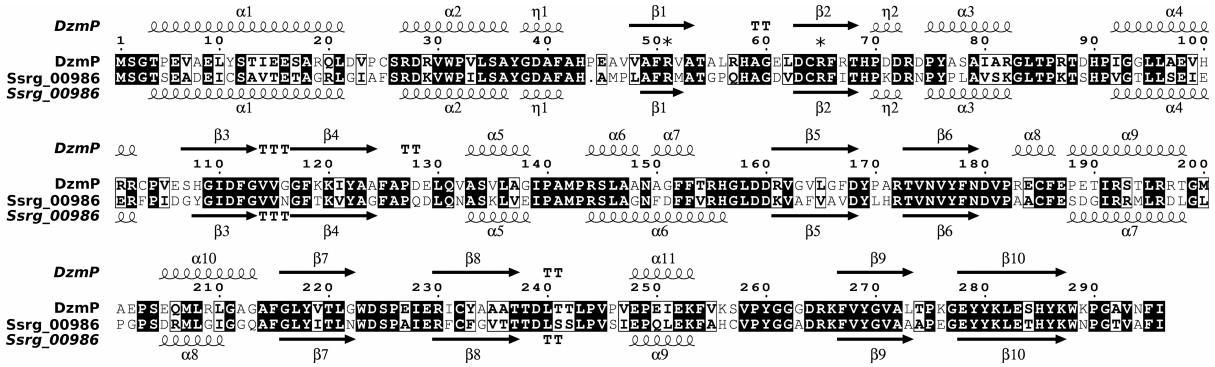


Figure 5

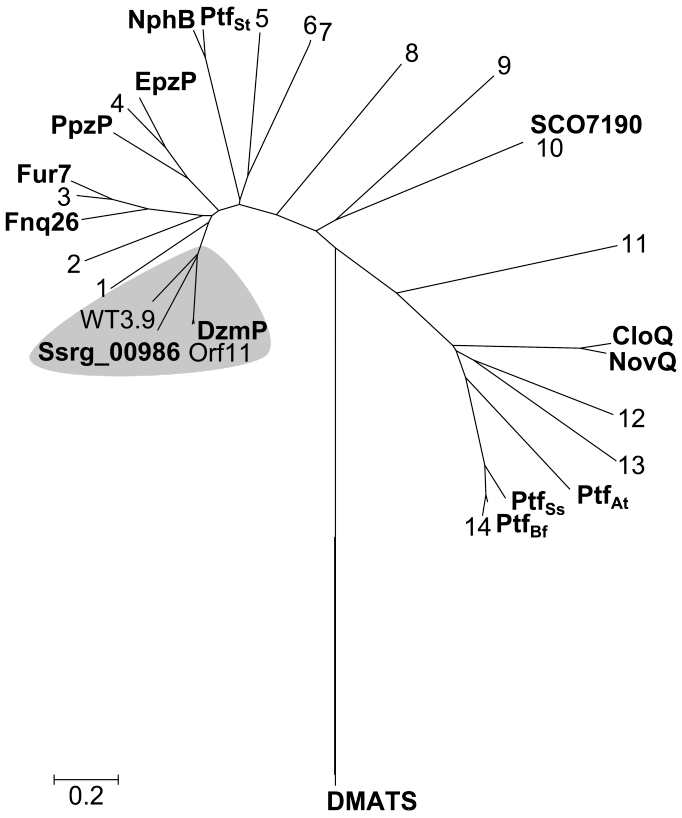

NphB
Ptf$_{St}$
5
6
7
8
9
EpzP
4
PpzP
SCO7190
10
Fur7
3
Fnq26
11
2
1
WT3.9
DzmP
Ssrg_00986
Orf11
CloQ
NovQ
12
13
Ptf$_{Ss}$
Ptf$_{At}$
14
Ptf$_{Bf}$
0.2
DMATS

Figure 6

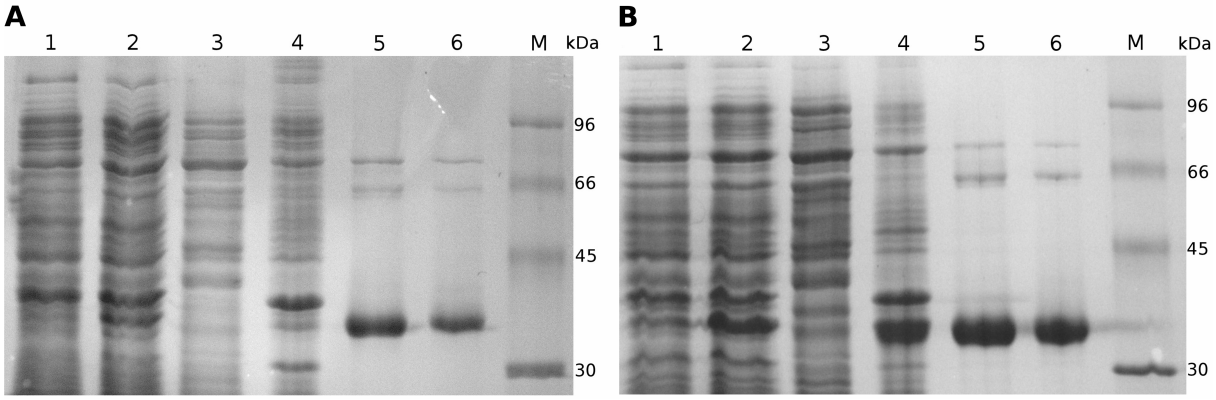
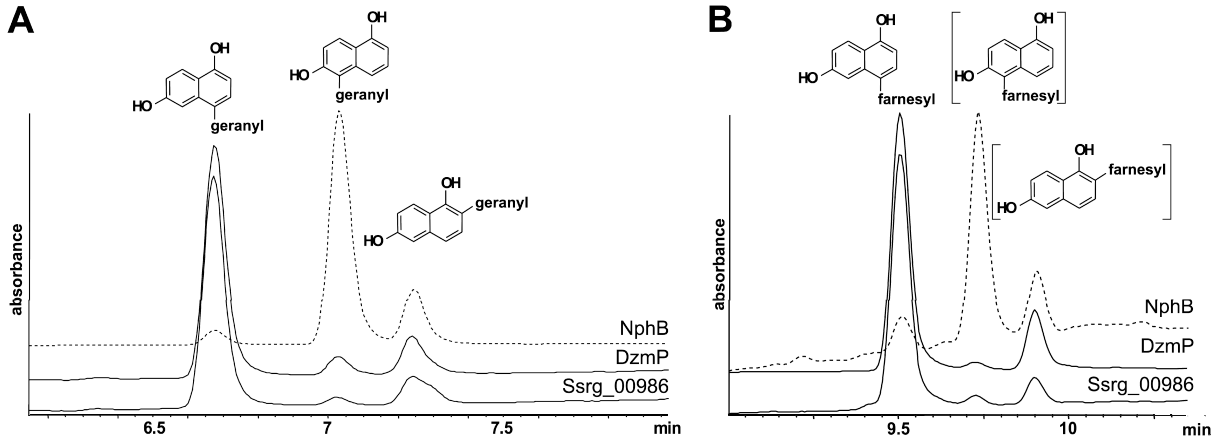


Figure 7


A
OH
HO
geranyl
OH
HO
geranyl
OH
HO
geranyl
absorbance
NphB
DzmP
Ssrg_00986
6.5
7
7.5
min
B
OH
HO
farnesyl
OH
HO
farnesyl
OH
HO
farnesyl
absorbance
NphB
DzmP
Ssrg_00986
9.5
10
min

**Supplemental data**

**for**

# Unusual *N*-prenylation in diazepinomicin biosynthesis: The farnesylation of a benzodiazepine substrate is catalyzed by a new member of the ABBA prenyltransferase superfamily

**Tobias Bonitz[‡], Florian Zubeil[§], Stephanie Grond[§], and Lutz Heide[‡]**

From the [‡]Pharmaceutical Institute, University of Tübingen, Tübingen, Germany and the [§]Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

Corresponding author: Lutz Heide, Pharmaceutical Institute, University of Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany. Tel.: +4970712972460; E-mail: heide@uni-tuebingen.de

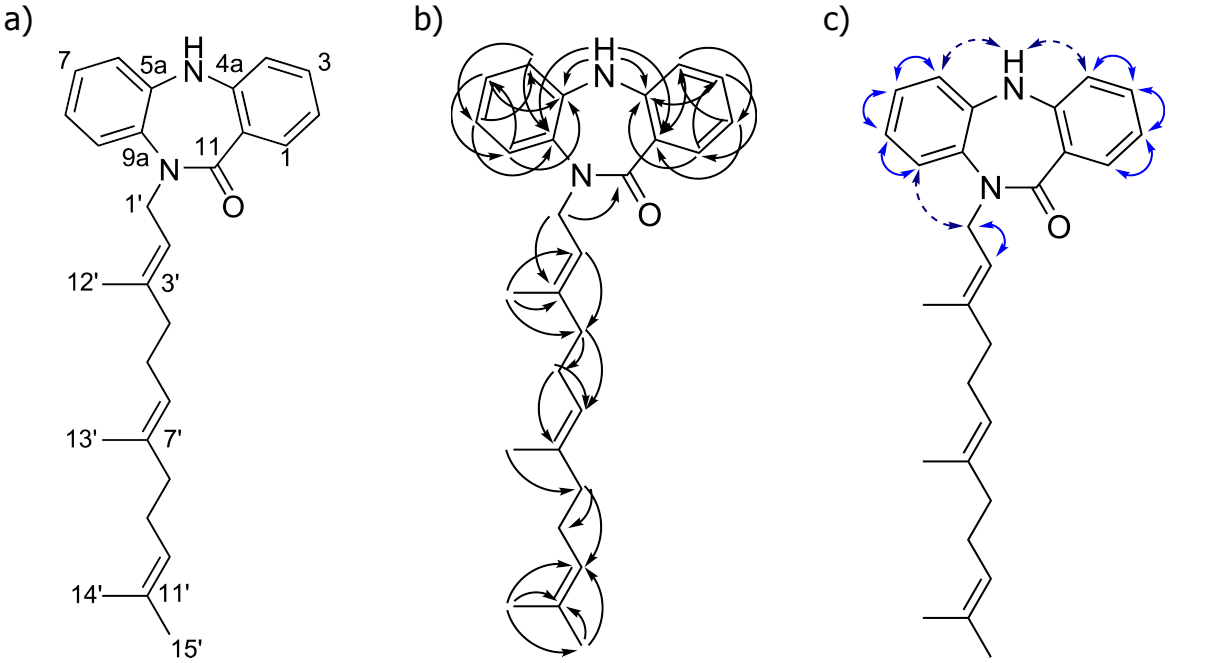



Figure S1. Product formed under catalysis of DzmP from dibenzodiazepinone and FPP (5-hydro-10-farnesyl-11H-dibenzo[b,e][1,4]diazepin-11-one). a) Numbering of atoms; b) selected $^{1}$H HMBC correlations and c) selected NOSEY correlations.

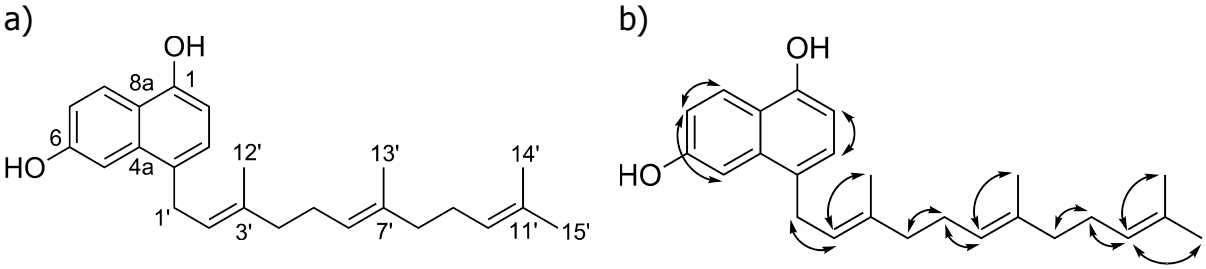



Figure S2. Product formed under catalysis of DzmP from 1,6-DHN and FPP (4-farnesyl-1,6-dihydroxynaphthalene). a) Numbering of atoms and b) selected $^{1}$H-$^{1}$H COSY correlations.

Table S1. $^{1}$H-NMR and $^{13}$C-NMR data for the product formed under catalysis of DzmP from dibenzodiazepinone and FPP (5-hydro-10-farnesyl-11H-dibenzo[b,e][1,4]diazepin-11-one).

| Position | $^{1}$H-NMR data (600.1 MHz, d6-DMSO) $\delta_H$ [ppm] | $^{13}$C-NMR data (150.9 MHz, $d_6$-DMSO) $\delta_C$ [ppm] |
|---|---|---|
| 1 | 7.60, 1H, dd, J = 7.7, 1.6 | 132.0 |
| 2 | 6.93, 1H, ddd, J = 7.7, 7.5, 1.1 | 121.4 |
| 3 | 7.31, 1H, ddd, J = 8.6, 7.5, 1.6 | 132.3 |
| 4 | 7.02, 1H, m | 117.7 |
| 4a | - | 152.0 |
| 5 | 7.82, 1H, bs | - |
| 5a | - | 145.0 |
| 6 | 7.10, 1H, dd, J = 7.9, 1.6 | 120.5 |
| 7 | 7.04, 1H, m | 125.7 |
| 8 | 7.00, 1H, ddd, J = 7.9, 7.6, 1.6 | 123.3 |
| 9 | 7.26, 1H, dd, J = 7.9, 1.5 | 123.5 |
| 9a | - | 134.0 |
| 11 | - | 167.5 |
| 11a | - | 124.7 |
| 1` | 4.48, 2H, d, J = 6.3 | 47.8 |
| 2` | 5.22, 1H, dt, J = 6.2, 3.4 | 121.4 |
| 3` | - | 137.4 |
| 4` | 1.94, 2H, m | 38.9 |
| 5` | 1.97, 4H with 20 | 25.7 |
| 6` | 5.01, 2H with 21, m | 124.1 |
| 7` | - | 134.5 |
| 8` | 1.88, 2H, t, J = 7.0 | 39.2 |
| 9` | 1.97, 4H with 16 | 26.3 |
| 10` | 5.01, 2H with 17, m | 123.6 |
| 11` | - | 130.6 |
| 12` | 1.63, 3H, s | 16.1 |
| 13` | 1.52, 3H, s | 15.8 |
| 14` | 1.49, 3H, s | 17.5 |
| 15` | 1.59, 3H, s | 25.48 |

Table S2. $^{1}$H-NMR and $^{13}$C-NMR data for the product formed under catalysis of DzmP from 1,6-DHN and FPP (4-farnesyl-1,6-dihydroxynaphthalene).

| Position | $^{1}$H-NMR data (600.1 MHz, d3-acetonitrile) $\delta_H$ [ppm] | $^{13}$C-NMR data (150.9 MHz, d3-acetonitrile) $\delta_C$ [ppm] |
|---|---|---|
| 1 | - | 152.3 |
| 1-OH | 7.22, 2H with 6-OH, bs | - |
| 2 | 6.58, 1H, d, *J* = 7.6 | 106.2 |
| 3 | 7.07, 1H, d, *J* = 7.6 | 127.2 |
| 4 | - | 128.3 |
| 4a | - | 135.6 |
| 5 | 7.20, 1H, d, 2.4 | 107.0 |
| 6 | - | 156.1 |
| 6-OH | 7.22, 2H with 1-OH, bs | |
| 7 | 7.03, 1H, dd, *J* = 9.0, 2.4 | 117.2 |
| 8 | 9.02, 1H, d, *J* = 9.0 | 125.1 |
| 8a | - | 120.8 |
| 1` | 3.56, 2H, d, *J* = 7.0 | 31.9 |
| 2` | 5.32, 1H, dq, J = 7.0, 1.1 | 124.5 |
| 3` | - | 136.6 |
| 4` | 2.06, 2H, m | 40.3 |
| 5` | 2.10, 2H, m | 27.14 |
| 6` | 5.11, 1H, dq, J = 7.0, 1.1 | 125.3 |
| 7` | - | 135.9 |
| 8` | 1.91, 2H, m | 40.3 |
| 9` | 1.99, 2H, m | 27.4 |
| 10` | 5.06, 1H, m | 125.3 |
| 11` | - | 132.1 |
| 12` | 1.79, 3H, s | 16.5 |
| 13` | 1.56, 3H, s | 16.3 |
| 14` | 1.55, 3H, s | 17.7 |
| 15` | 1.63, 3H, s | 25.8 |