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Two Prenyltransferases Govern a Consecutive Prenylation Cascade in the Biosynthesis of Echinulin and Neoechinulin

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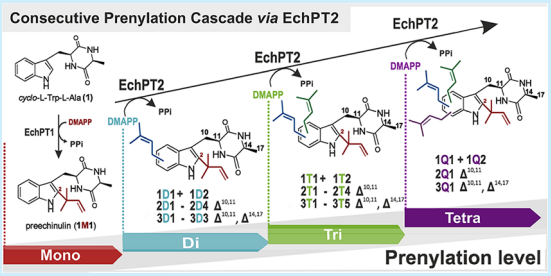
Supporting Information

ABSTRACT: Two prenyltransferases from *Aspergillus ruber* control the echinulin biosynthesis via exceptional sequential prenylations. EchPT1 catalyzes the first prenylation step, leading to preechinulin. The unique EchPT2 attaches, in a consecutive prenylation cascade, up to three dimethylallyl moieties to preechinulin and its dehydro forms neoechinulins A and B, resulting in the formation of at least 23 2- to 4-fold prenylated derivatives. Confirming these products in fungal extracts unravels the unprecedented catalytic relevance of EchPT2 for structural diversity.



Indole diketopiperazine (DKP) alkaloids have been established as a steadily growing and reliable source for compounds of significant biological activity.[1,2] Their indole nucleus backbone, as a privileged structure, has become a focus of fragment-based drug discovery.[1,3] This core ring system is commonly assembled via one L-tryptophan and a second amino acid, usually catalyzed by a nonribosomal peptide synthetase (NRPS),[4] providing an initial biosynthetic starting point in the form of an indole DKP skeleton.[5] Subsequent modification reactions including prenylations increase not only structural complexity but also biological and pharmacological activities.[4−8]

Echinulins and neoechinulins derived from L-tryptophan and L-alanine exemplify such DKPs and are highly decorated with dimethylallyl (DMA) moieties.[1,2,5] Their eponymous group member echinulin (Figure 1) was first isolated from *Aspergillus*

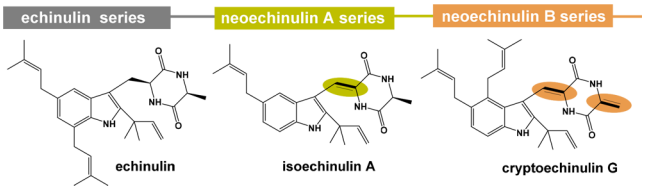


Figure 1. Representatives of echinulin and neoechinulins.

amstelodami[5] and later, together with congeners, from different terrestrial and marine-derived fungi,[5] e.g. *Aspergillus cristatus*[9] and *Aspergillus glaucus*.[10] These prenylated *cyclo*-L-Trp-L-Ala derivatives can be classified into the echinulin, neoechinulin A, and neoechinulin B series (Figures 1 and S1 in Supporting Information (SI)).[11] They share a reverse *C2*-prenyl moiety and differ from each other in *exo* double bounds at the DKP ring. In turn, the members of each series differ from each other in the number and position of additional DMA moieties. C-5 of the indole ring seems to be the second preferred prenylation position in echinulins and neoechinulins. A third DMA moiety is mainly found at C-7 or at C-4.[11,12] Various intriguing pharmacological activities have been identified for echinulin and congeners,[5] such as protection against neuronal cell death[13] and antiviral[14] and antitumor activities.[15] In spite of over 40 years of studies on structures, as well as biological and pharmacological activities of this intriguing substance group, the enzymes involved in the biosynthesis, especially those for the transfer of the different prenyl moieties, have not been reported prior to this study.[2,5]

In nature, prenyl transfer reactions are catalyzed by prenyltransferases (PTs). These enzymes employ isoprenic precursors of various number of C_5-units such as DMAPP (C_5).[8] Known indole PTs from fungi belong to the dimethylallyltryptophan synthase (DMATS) superfamily and usually catalyze regio- and stereoselective regular or reverse prenylations. In most cases, one PT only catalyzes one specific transfer reaction.[8] For example, fumitremorgin A from *Neosartorya fischeri* contains three prenyl moieties, which are transferred from DMAPP by three different PTs.[16] Contrary examples of one PT involved in more than one prenylation step are rare. Two membrane-bound PTs, from *Humulus lupulus*, have been reported to be responsible for three sequential prenylation steps in the biosynthesis of *β*-bitter acid,[17] and in the biosynthesis of the fungal metabolite shearinine D, JanD from the DMATS superfamily catalyzes a tandem diprenylation.[18]

A large number of echinulin congeners with different numbers of prenyl moieties have been identified in and isolated from

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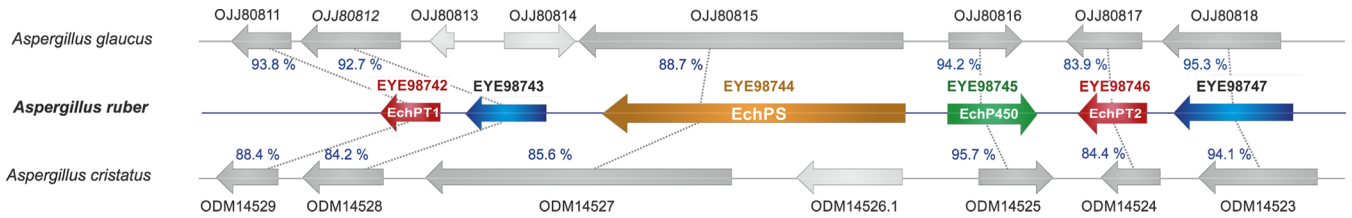

Figure 2. Putative echinulin gene clusters in *Aspergillus* strains. Genes with high sequence identities at the amino acid level are indicated by dotted lines. Details of alignment and the putative gene functions can be found in Table S1.

Aspergillus ruber.[12,14] In analogy to most biosynthetic pathways, it could be speculated that three or even four PTs are necessary for the attachment of the prenyl moieties in echinulin and its congeners. Unexpectedly, mining the *A. ruber* CBS 135680 genome[19] reveals just three DMATS PT genes. Two of them, coding for EYE98742 (termed EchPT1 in this study) and EYE98746 (EchPT2), build a cluster with an NRPS (EYE98744) gene. The third one (EYE95342) is located in a separate cluster with a polyketide synthase gene. Homologues of the putative NRPS-containing cluster harboring only two PT genes are also identified in the genomes of *Aspergillus cristatus* YKY807 and *Aspergillus glaucus* CBS 516.65 (Figure 2, Table S1). This unusual genetic organization prompted us to investigate the functions of the three PTs from *A. ruber*.

Sequence analysis revealed the incorrect annotation of the putative NRPS EYE98744 in the database by lacking 789 amino acids at the N-terminus, which was corrected in this study (SI). The revised sequence of EYE98744 comprises 2113 amino acid residues (Table S1) and shares a sequence identity of 23.5% with the known *cyclo*-L-Trp-L-Pro synthetase FtmPS.[20] It can be speculated that this enzyme, tentatively named EchPS, might be responsible for the assembly of *cyclo*-L-Trp-L-Ala. The two putative echinulin prenyltransferases EchPT1 and EchPT2 are polypeptides of 417 and 408 amino acids, respectively. They share a clear sequence similarity with the members of the DMATS superfamily (Figure S2).[8] It seems unbelievable that these two PTs should catalyze three or four prenylations deduced from the number and positions of DMA moieties in echinulins and congeners.

To investigate the possible involvement of the three PTs in the biosynthesis of echinulin, the coding sequences of EchPT1, EchPT2, and EYE95342 were PCR amplified from cDNA of the endophytic fungus *A. ruber* QEN-0407-G2[12] and cloned into pQE9 and pQE70 for heterologous expression in *E. coli* (SI, Figures S3–S5). All three purified proteins were incubated separately with the putative product of EchPS, *cyclo*-L-Trp-L-Ala (**1**), in the presence of DMAPP. LC-MS analysis revealed product formation in the reaction mixture with EchPT1, but with neither EchPT2 nor EYE95342 (Figure 3i–iii). The $[M + H]^+$ ion of the single EchPT1 product **1M1** indicates a monoprenylation of **1**. For a better understanding, we use **M**, **D**, **T**, and **Q** after the substrate number for mono-, di-, tri-, and tetraprenylation, respectively. The number after these letters refers to the order of the identified products. Formation of **1M1** is strictly dependent on the presence of **1**, DMAPP, and active EchPT1. NMR and MS analyses of the isolated product confirmed **1M1** to be preechinulin. This proved unequivocally that EchPT1 catalyzes the first prenylation in the biosynthesis of echinulin in *A. ruber* (for structure elucidation and kinetic parameters see SI, Tables S2 and S3, Figures S9–11, S16, and S18).

To verify the roles of the two other PTs in the biosynthesis of echinulins, we carried out incubations containing either two or all

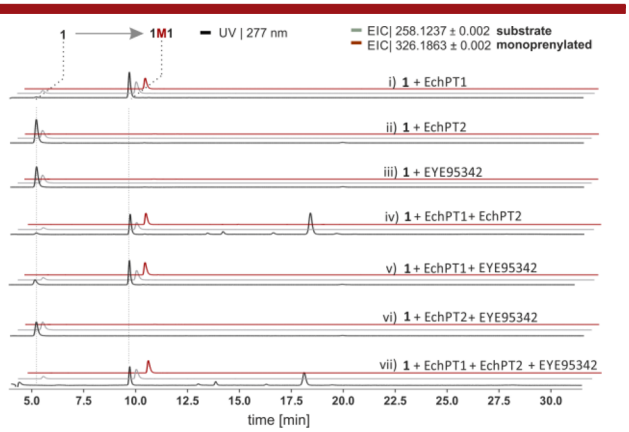

Figure 3. LC-MS analysis of the incubation mixtures of **1** with EchPT1, EchPT2, and EYE95342 alone or in combinations.

three PTs (Figure 3iv–vii). Assaying **1** with EchPT1 and EchPT2 resulted in the formation of **1M1** and at least four additional products (iv). The same peaks were also detected in the assay with all three enzymes (vii). In contrast, the combination of EchPT1 and EYE95342 (v) yields just the EchPT1 product **1M1**, while EchPT2 and EYE95342 catalyze no conversion of **1** at all (vi). These results prove that EYE95342 is likely not involved in the biosynthesis of echinulins and EchPT2 catalyzes the further metabolism of **1M1.**

To gain detailed insights into the reaction mechanism of **1** with EchPT1 and EchPT2 (Figure 3iv), we assayed **1M1** with EchPT2 in the presence of DMAPP. LC-MS analysis reveals six product peaks on three consecutive prenylation levels (Figures 4a and S6a), i.e. two di-, tri-, and tetraprenylated products each, which was confirmed by detection of their exact $[M + H]^+$ ions (Table S3). The $[M + H]^+$ ions of the first two products in ascending order of retention times (Figure S6a) are 68 Da larger than that of **1M1**, proving the presence of an additional prenyl moiety in their structures. These putative diprenylated products are termed **1D1** and **1D2**. The $[M + H]^+$ ions of the following two triprenylated products, **1T1** and **1T2**, indicate the attachment of two prenyl residues to **1M1**, and the $[M + H]^+$ ions of the last two products **1Q1** and **1Q2** indicate even three additional prenyl units attached to the already monoprenylated substrate **1M1**. Notably, while a large number of echinulin-related structures were described in the literature,[5,12] no derivative with four prenyl moieties has been reported to date. From Figure 4a, it is obvious that **1T2** is the main product of the EchPT2 reaction. To the best of our knowledge, six products across three consecutive prenylation levels from an incubation mixture with only one enzyme have not been reported prior to this study. These fascinating results prompted us to investigate the relationships between these products and their dependence on reaction time, DMAPP and protein concentrations (Table S2, Figures S12–15 and S17). HPLC analysis of the reaction mixtures revealed **1T2** as the

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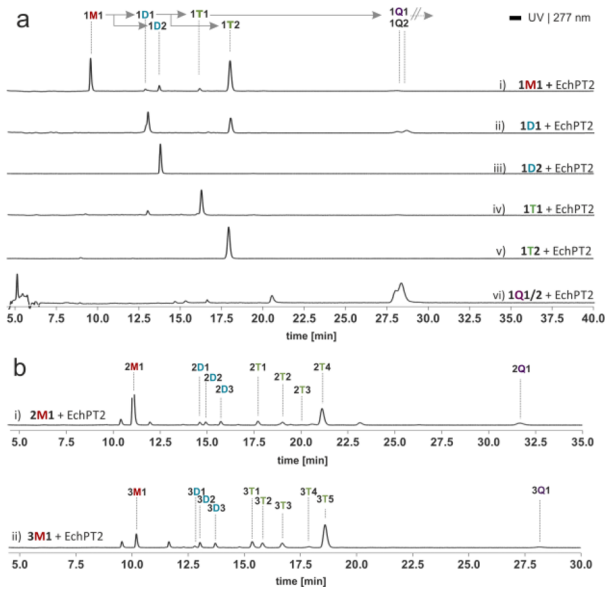


Figure 4. LC-MS analysis of EchPT2 assays with **1M1**, **1D1**, **1D2**, **1T1**, **1T2**, and **1Q1**/**1Q2** (a), neoechinulins A (**2M1**) and B (**3M1**) (b).

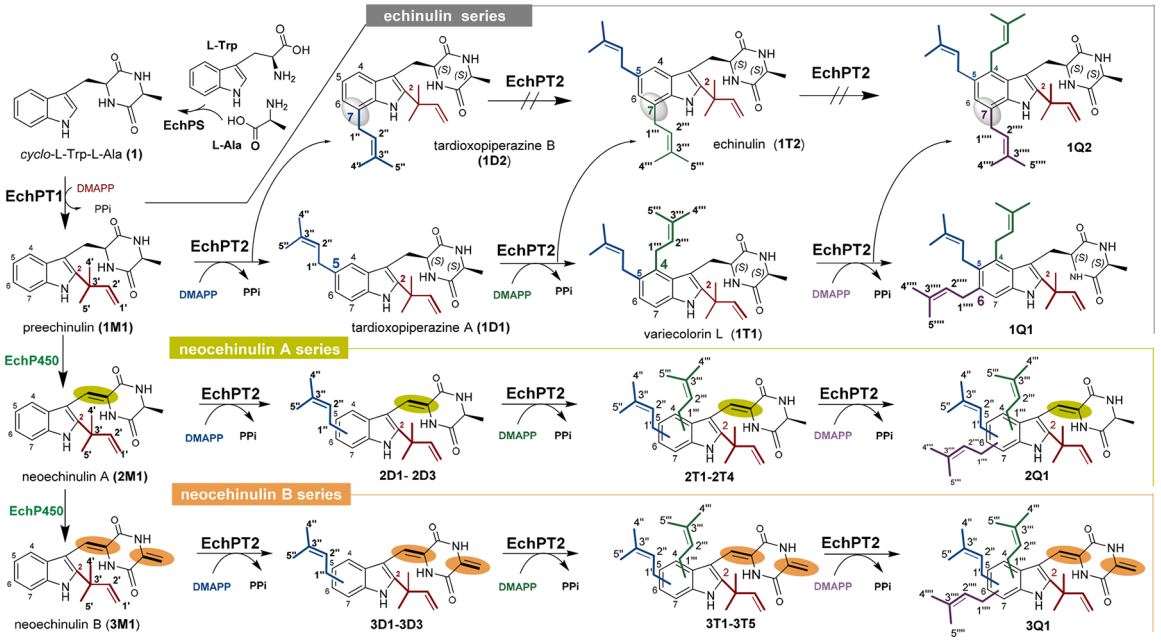
predominant product in all enzyme assays. Most interestingly, there are two divergent fate patterns for the enzyme products. In all three dependency assays, the formation of **1D1** and **1T1** showed an initially rapid, yet short increase, followed by a continuous decrease. In contrast, the yields of **1D2**, **1T2**, **1Q1**, and **1Q2** increased steadily in all assays (Figure S13). This indicates that **1D1** and **1T1** could serve as intermediates in a consecutive prenylation cascade, whereas **1D2** and **1T2** as well as **1Q2** and **1Q2** may represent end products of the (branch) pathways.

To confirm our hypothesis, we isolated EchPT2 products for structure elucidation. Interpretation of the spectra and literature search confirmed **1D1** and **1D2** to be tardioxopiperazines A and B with an additional regular prenyl moiety at C-5 and C-7, respectively, compared to that of **1M1**. The two triprenylated products **1T1** and **1T2** bear prenyl moieties at C-2 and C-5 and differ from each other in the position of the third prenyl residue, which is located at C-4 in **1T1** and C-7 in the case of **1T2**. **1T1** and **1T2** can therefore be unequivocally identified as variecolorin L and echinulin. Due to the low amount, the structure of **1Q1** cannot be determined in this study. **1Q2** was isolated from fungal extracts (Figure S8) and identified as a reversely *C2*-, regularly *C4*-, *C5*-, and *C7*-tetraprenylated derivative. Judging by incubation results described below, **1Q1** must be a reversely *C2*-, regularly *C4*-, *C5*-, and *C6*-tetraprenylated derivative (Scheme 1; Tables S3−S7; Figures S19−29).

The single EchPT2 products were then incubated independently with EchPT2 (Figures 4a and S6a). LC-MS analysis showed further conversion of **1D1** by EchPT2 to **1T1**, **1T2**, **1Q1**, and **1Q2** (ii). The last two products were also detected in the assay of **1T1** with EchPT2 (iv). Conversely, **1D2**, **1T2**, **1Q1**, and **1Q2** underwent no further conversion by EchPT2 (iii, v, and vi). These confirmed the hypothesis that **1D2** and **1T2** are end products of different branches of the EchPT2 reaction and *C7*-prenylation serves as a termination step in a consecutive prenylation cascade as shown in Scheme 1. To reach the tetraprenylated products, the putative NRPS product **1** will be reversely prenylated at C-2 by EchPT1. The resulting product **1M1** undergoes regular prenylation at C-5, then at C-4, and finally at C-6 or C-7. No pentaprenylated derivative was detected in the reaction mixtures of **1M1**, **1D1**, **1T1**, **1Q1**, or **1Q2** with EchPT2 (Figure S6a), indicating that the tetraprenylated derivatives **1Q1** and **1Q2** are the final products of the cascade. **1M1**, **1D1**, **1D2**, **1T1**, **1T2**, and a mixture of **1Q1** and **1Q2** were also incubated with EYE95342. LC-MS analysis revealed no conversion of these substrates (Figure S7).

Having explored the outstanding catalytic skill of EchPT2, we wondered if the products of such a prenylation cascade, especially the previously undescribed tetraprenylated derivatives **1Q1** and **1Q2**, coexist in the fungal cultures. Based on previous results

Scheme 1. Proposed Biosynthetic Pathway of Echinulin and Neoechinulin Series via Consecutive Multiprenylations by EchPT1 and EchPT2 in *A. ruber*



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reporting a strong influence of salt concentration on metabolism,[19] we cultivated *A. ruber* QEN-0407-G2 under different conditions (SI). The fungal cultures were extracted and analyzed by LC-MS (Figure S1). The enzyme products described above, i.e. **1M1** of the EchPT1 reaction, as well as **1D1**, **1D2**, **1T1**, **1T2**, and **1Q2** of the EchPT2 reaction, were detected by LC-MS analysis, corresponding to the enzyme reactions with EchPT1 and EchPT2 (Figures S1, S6a, and S8).

As previously mentioned, neoechinulins with one (A series) or two *exo* double bonds (B series) at the DKP ring are also frequently identified in echinulin producers.[14] LC-MS analysis of the obtained fungal extract indeed revealed the presence of members of both series with up to three prenylation levels (Figure S1). In total, one mono- (neoechinulin A, **2M1**), two di-, and two triprenylated derivatives from the A series were detected. In the case of the B series, one mono- (neoechinulin B, **3M1**), two di-, and four triprenylated derivatives were observed. In contrast to the triprenylated **1T2** as the main metabolite of the echinulin series (Figure S1), the monoprenylated derivatives **2M1** and **3M1** are found to be the major products of the neoechinulin series A and B. Furthermore, *cyclo*-L-Trp-L-Ala (**1**), but not its di- or tetradehydrogenated derivatives, i.e. the unprenylated precursors of **2M1** and **3M1**, was detected in the fungal cultures. This could indicate that the first prenylation catalyzed by EchPT1 takes place before dehydrogenation, probably catalyzed by the cytochrome P450 enzyme EchP450 (Scheme 1). We speculated that both series of neoechinulins are also EchPT2 products of sequential prenylations and, thus, isolated the monoprenylated **2M1** and **3M1** from the extracts (SI for structure elucidation, Tables S3 and S8, Figures S30 and S31). LC-MS analysis of the reaction mixture of **2M1** and **3M1** with EchPT2 indeed demonstrated the clear acceptance of both substrates (Figures 4b, S6b) and the formation of eight and nine products with two to four prenyl residues, respectively. In comparison to the fungal extract, tetraprenylated products **2Q1** and **3Q1** were also clearly detected in the EchPT2 assays with **2M1** and **3M1**. This proves that EchPT2 also catalyzes a prenylation cascade with **1M1** analogs bearing exo double bonds and that the biosynthetic pathway illustrated in Scheme 1 can be expanded by neoechinulins. That is, conversion of **1M1** to neoechinulins A (**2M1**) and B (**3M1**) by a putative cytochrome P450 enzyme (EchP450)[21] marks the starting point of the neoechinulin formation. In analogy to **1M1**, **2M1** and **3M1** undergo a prenylation cascade catalyzed by EchPT2, resulting in the formation of products with different prenylation grades.

In conclusion, this study provides the first example of a prenyltransferase catalyzing an exceptional consecutive prenylation cascade. The unique feature of EchPT2 is its ability to accept its own mono-, di-, and triprenylated derivatives as substrates and to catalyze prenylations at different positions, leading to the formation of echinulin and congeners. It is of the utmost interest to solve such intriguing enzymatic structures and to comprehend the ability of EchPT2 to bind different substrates and catalyze diverse prenylations. This knowledge would also provide a basis for controlling the prenylation cascade by site-directed mutagenesis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b02926.

Detailed experimental procedures including structural elucidation, kinetic parameters, MS and NMR data, and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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