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Complete Decoration of the Indolyl Residue in *cyclo*-L-Trp-L-Trp with Geranyl Moieties by Using Engineered Dimethylallyl Transferases

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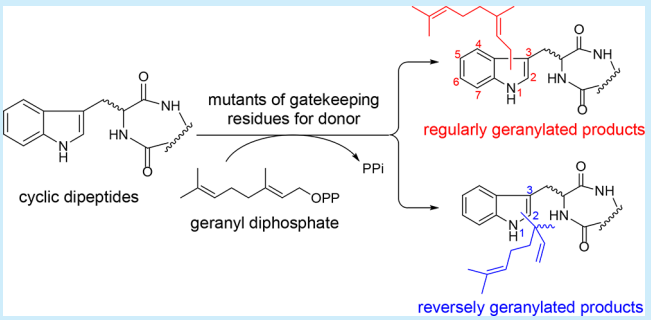
Supporting Information

ABSTRACT: Mutation of the gatekeeping residues for prenyl donor selectivity in six dimethylallyl transferases significantly increased their activities toward geranyl diphosphate. Forty-two geranylated derivatives were obtained from 15 cyclic dipeptides by using the engineered enzymes. Taking *cyclo*-L-Trp-L-Trp as an example, the geranyl moiety can be attached to all seven possible positions of the indole nucleus. This study demonstrates a convenient way to increase the structural diversity of geranylated products by structure-based engineering of the available dimethylallyl transferases.



Prenylated natural products are hybrid structures containing prenyl moieties ($n \times C_5$) or derivatives thereof and are widely distributed in plants and microorganisms.[1−3] Among them, prenylated indole alkaloids including prenylated tryptophan-containing cyclic dipeptides (CDPs) usually exhibit a wide range of biological and pharmacological activities and, therefore, are important sources for drug discovery and development.[2,4,5] Representative structures such as notoamides,[6,7] roquefortines,[8] fumitremorgins,[9] and fellutanines[10−12] are commonly derived from CDPs and often decorated with one or more dimethylallyl (C_5) moieties (Figure 1).

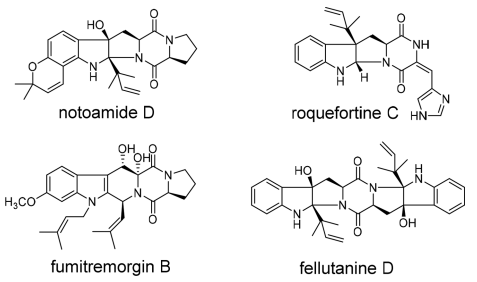
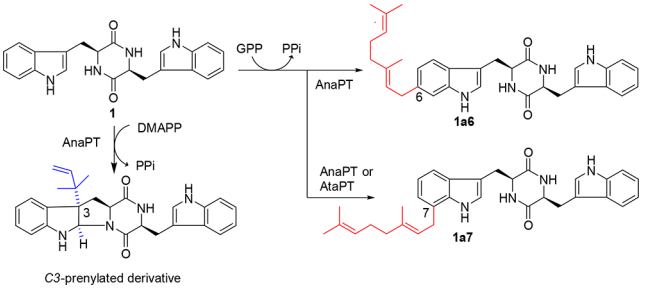


Figure 1. Representative examples of prenylated indole alkaloids derived from cyclic dipeptides.

The key reactions in the biosynthesis of the prenylated natural products are connections of the prenyl moiety with its acceptor catalyzed by prenyltransferases (PTs). PTs belonging to the dimethylallyltryptophan synthase (DMATS) superfamily catalyze the regiospecific Friedel−Crafts alkylation of aromatic substrates, mainly of indole derivatives.[13,14] The attachment of the C_5 unit to *N1* and the six nonbridgehead carbons at the indole ring can be achieved by the available indole PTs.[13−15] However, the transfer of the C_{10} unit (geranyl) onto the indole nucleus was rarely reported. Taking CDPs as examples, AnaPT from *Neosartorya fischeri* catalyzes in the presence of dimethylallyl diphosphate (DMAPP) reverse *C3*-prenylation and also uses geranyl diphosphate (GPP) for their *C6*- and *C7*-geranylation, as exemplified by cWW (**1**; for simplicity, one letter codes are used for proteinogenic amino acids) in Scheme 1.[16,17] AtaPT from *Aspergillus terreus* was reported to catalyze the *C4*-geranylation of **1**.[18] Our recent reinvestigation on this

Scheme 1. Reverse Dimethylallyl *C3*-PT AnaPT from *N. fischeri* Can Also Use GPP for *C6*- and *C7*-Geranylation[a]



[a]AtaPT from *A. terreus* attaches a geranyl moiety to *C7* of **1**. To facilitate the reading flow, geranylated products are named by using substrate number, prenylation pattern (**a** for regular or normal and **b** for reverse prenylation), and prenylation position at the indole ring (**1** to **7** for *N1*- to *C7*-alkylated products), e.g., **1a6** for *C6*-regularly geranylated cWW.

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reaction revealed a *C7*- instead of a *C4*-geranylation (data not shown). Other CDPs with geranyl residues have not yet been reported. Furthermore, only a few members of PTs are able to accept GPP as a prenyl donor and act as geranyl transferases, *e.g.* TleC,[19] VrtC,[20] and AmbP1.[21] Therefore, it is necessary to find new geranyl transferases or to manipulate available dimethylallyl transferases for GPP acceptance to enrich the biocatalysis toolboxes.

Inspired by the activities of AtaPT and AnaPT toward GPP for prenylation of CDPs, we first evaluated the GPP acceptance of five dimethylallyl transferases, FtmPT1,[22] CdpNPT,[23] BrePT,[24] CdpC2PT,[25] and CdpC3PT,[26] in the presence of CDPs as acceptors. These enzymes catalyze regular (normal) or reverse prenylations at C-2 or C-3 of the indole ring. The tested CDPs include **1**, cWP (**2**) and its stereoisomers (**3**–**5**), cWA (**6**) and its stereoisomers (**7**–**9**), cWL (**10**), cWY (**11**), cWF (**12**), cWG (**13**), (*S*)-(**14**), and (*R*)-benzodiazepinedinone (**15**). HPLC analysis of the incubation mixtures showed that the five PTs well accepted their natural or best aromatic substrates in the presence of DMAPP (Figure S1 in the Supporting Information (SI)), confirming the presence of active proteins. In the presence of GPP, CdpC2PT showed clear activity toward **1**, **2**, **14**, and **15** (Figure S2). The other four enzymes exhibited very low (CdpC3PT toward **1**, **2**, **5**, and **14**, Figure 2, Figure S3) or no activity (CdpNPT, FtmPT1, and BrePT) (Figure 2, Figures S4–S6), when GPP was used as a prenyl donor.

AtaPT was previously reported to have broad tolerance for prenyl donors. The residue Gly326 was proven to contribute to the enlarged pocket for donor binding.[18] Mutation of Gly326 to methionine dramatically reduced the activity of G326M toward GPP and FPP, but substantially enhanced the catalytic activity with DMAPP. This proved that the size of the residue at 326 controls the prenyl donor selectivity in AtaPT. Mutation of Met328 in the tryptophan prenyltransferase FgaPT2 to small amino acids such as cysteine, threonine, serine, glycine, or alanine significantly increased the acceptance of GPP.[27] Encouraged by these findings, structure-based protein sequence alignments of AtaPT and FgaPT2 with the five PTs mentioned above led to the identification of the corresponding residues Met364 in FtmPT1, IIe337 in BrePT, Thr351 in CdpC2PT, Met349 in CdpNPT, and Phe335 in CdpC3PT (Figure 3, Figure S7).

Molecular modeling with the ternary crystal structures of FtmPT1 and CdpNPT[23,28] also uncovered Met364 in FtmPT1 and Met349 in CdpNPT as key residues for DMAPP selectivity (Figure S8). The side chain of this residue seals the active site to form a pocket for DMAPP. In the active site of both enzymes, GPP would clash with the side chain of the gatekeeping residue methionine. Therefore, reducing the steric pressure in the DMAPP binding site by replacement of this bulky residue with a small amino acid like glycine would increase the enzymatic activity toward GPP.

To switch the prenyl donor specificity from DMAPP to GPP for FtmPT1, BrePT, CdpNPT, and CdpC3PT and to expand the GPP acceptance of CdpC2PT, we replaced the mentioned amino acids of the five dimethylallyl transferases by glycine, resulting in the mutants FtmPT1_M364G, BrePT_I337G, CdpNPT_M349G, CdpC2PT_T351G, and CdpC3PT_F335G, respectively (see Table S1 for constructs and SI for description). As expected, replacing bulky amino acids by glycine significantly improved in most cases the CDP consumption in the presence of GPP (Figure 2, Figures S2–S6, S9). In comparison, the acceptance of DMAPP by the mutants was reduced to different levels (Figure S1). In sharp contrast to no activity in the presence of GPP by wild type CdpNPT, FtmPT1, and BrePT, all the tested CDPs (**1**–**15**) were accepted by CdpNPT_M349G with total product yields from 13.7% to 95.8% (Figure 2, Figures S4 and S9). **1**–**13** with a diketopiperazine ring were consumed by FtmPT1_M349G with total product yields from 9.6% to 75.4%. The two benzodiazepinediones **14** and **15** were not accepted (Figure 2, Figures S5 and S9).

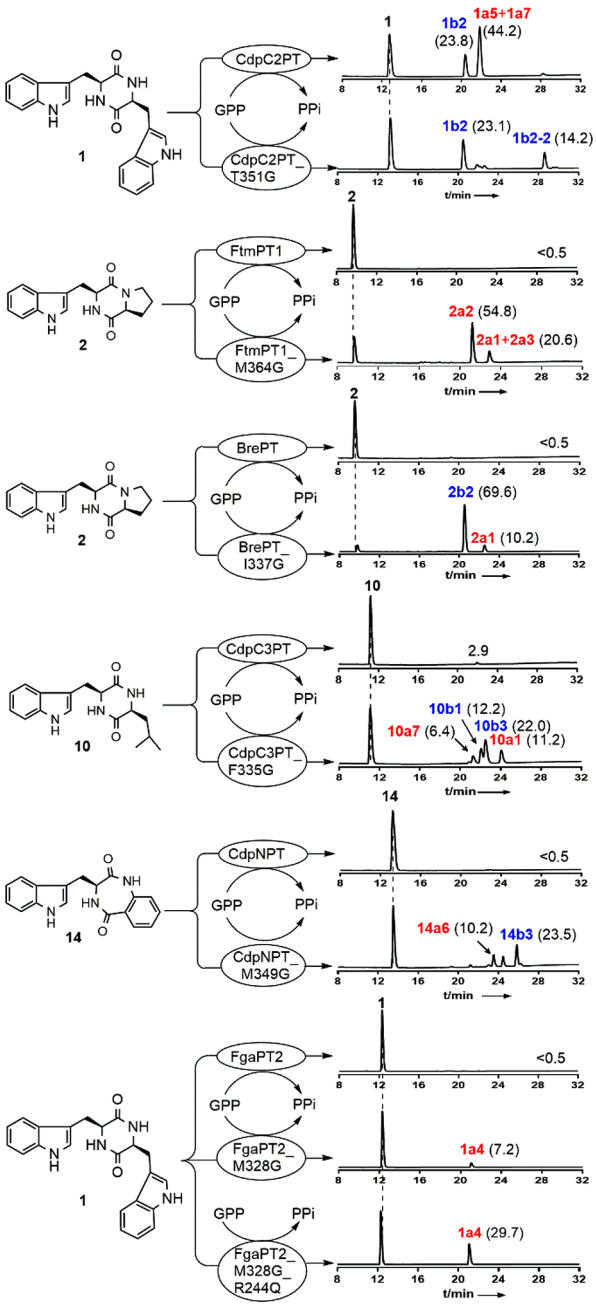

Figure 2. HPLC chromatograms of the incubation mixtures of six dimethylallyl transferases and their corresponding mutants with their natural or best accepted substrates in the presence of GPP. Regular geranylated products are labeled in red, and reverse, in blue. The product yields are given in parentheses after the product number. See please Figure S1 for their behavior toward DMAPP.

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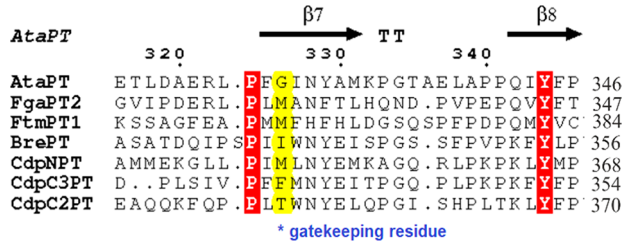


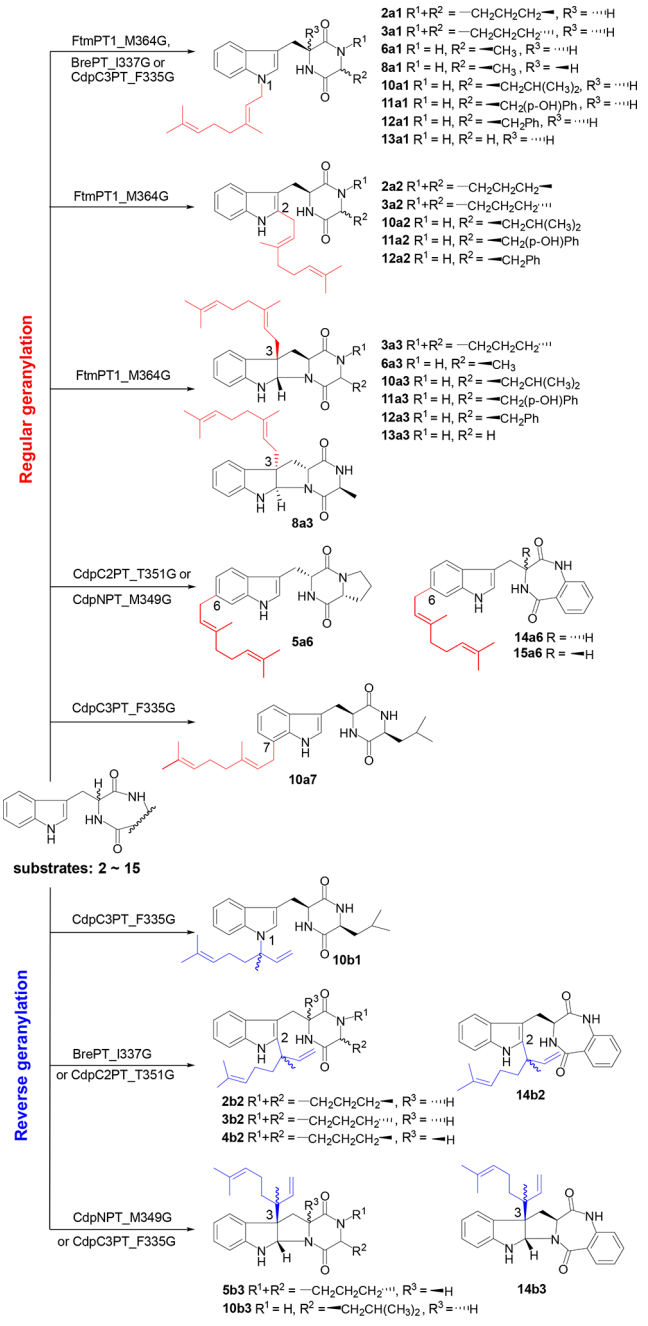
Figure 3. Selected region of structure-based alignments of PTs used in this study with AtaPT and FgaPT2. See Figure S7 for complete alignments.

The activity of BrePT toward GPP can be strongly increased by mutation at the gatekeeping residue Ile337, at least for its natural substrate **2** (79.8%) and the two stereoisomers **3** (71.4%) and **4** (28.4%) (Figure 2, Figure S6). In the case of CdpC3PT_F335G, conversion of almost all substrates was significantly increased, especially for **10**. Its conversion was improved from 2.9% by the wild type enzyme to 51.8% by this mutant (Figure 2, Figure S3). CdpC2PT_T351G exhibited different behaviors toward **1**−**15** from CdpC2PT, with distinct products or different ratios of the same products (Figure 2, Figures S2 and S9). Detailed inspection of the HPLC chromatograms of **1**−**15** with FtmPT1_M364G, BrePT_I337G, CdpNPT_M349G, CdpC2PT_T351G, and CdpC3PT_F335G (Figures S2−S6, S9) revealed the presence of one to four product peaks with different ratios. Most reactions have two or three products. This shows the relaxed regiospecific geranylation by the mutants in comparison to the wild type enzymes in the presence of DMAPP.

For structure elucidation, 41 representative products of selected substrates were isolated by preparative HPLC from different incubation mixtures (Table S2) and subjected to HR-ESI-MS and ^{1}H NMR analyses (Tables S2−S18, Figures S10−S54). Structure elucidation (see the SI) confirmed reverse or regular geranylation at different positions of the indole ring (Scheme 2).

Three of the tested PTs catalyze regular (FtmPT1) or reverse (BrePT and CdpC2PT) *C2*-prenylations of CDPs in the presence of DMAPP.[22,24,25] Structure elucidation proved that BrePT_I337G catalyzes solely or predominantly still reverse *C2*-geranylation of **2**− **4**. In the reaction mixtures of FtmPT1_M364G with six substrates, regularly *C2*-prenylated derivatives were obtained as major products, i.e. **1a2**, **2a2**, **3a2**, **10a2**, **11a2**, and **12a2**. Regularly *C3*-prenylated derivatives such as **1a3**, **3a3**, **6a3**, **8a3**, **10a3**, **11a3**, **12a3**, and **13a3** were identified in at least eight incubation mixtures. This is not surprising, because regularly *C3*-prenylated CDPs were also reported for FtmPT1 in the presence of DMAPP.[29] In that study, however, regularly *C2*-prenylated derivatives have been identified as the major products. Interestingly, in the assays of FtmPT1_M364G with GPP and **6**, **8**, and **13**, no *C2*-, but regularly *N1*- and *C3*-geranylated derivatives were obtained (Scheme 2, Figure S5). The behavior of CdpC2PT_T351G toward GPP also differs significantly from that of the wild type enzyme. For example, **1** was converted by CdpC2PT to **1b2**, **1a5**, and **1a7**, while CdpC2PT_T351G mainly catalyzes *C2*-geranylation, resulting in the formation of mono- (**1b2**) and digeranylated (**1b2-2**) derivatives (Figure 2, Figure S9). CdpC3PT and CdpNPT both catalyze reverse *C3*-prenylation of CDPs in the presence of DMAPP.[23,26] Reverse *C3*-geranylation was also detected for CdpNPT_M349G with

Scheme 2. Structures of Enzyme Products with Different Prenylation Patterns and Positions (Products of 1 Are Presented in Scheme 3)



the selected substrates **1**, **5**, and **14**. In addition, regularly *C6*-geranylated products **1a6**, **5a6**, and **14a6** were also identified. In the incubation mixture of CdpNPT_M349G with **15**, the product **15a6** was even the predominant peak (Figure S4). CdpC3PT_F335G converted **10** to a mixture of at least four products **10a1**, **10b1**, **10b3**, and **10a7** with **10b3** as the major one (Figure S3). These results indicate the trends of *C6*- and *C7*-geranylation of CDPs by dimethylallyl transferases, which catalyze *C3*-prenylation in the presence of DMAPP, as observed for AnaPT (Scheme 1).[16,17]

In summary, our results presented in this study showed that replacing the gatekeeping residue not only changes the tolerance of PTs toward prenyl donor with longer chain but

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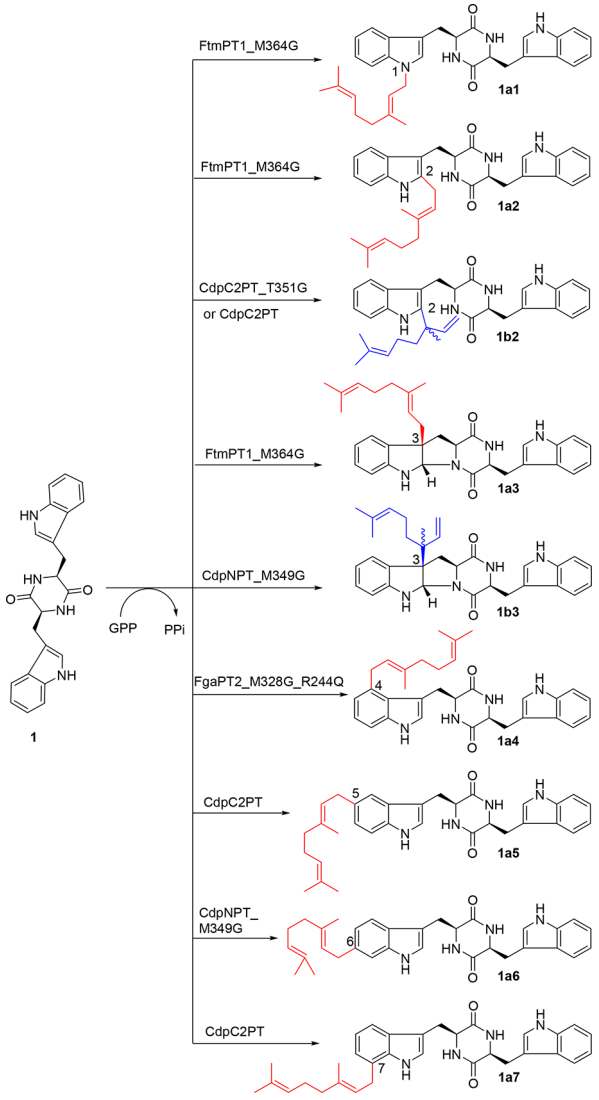
also breaks their regiospecificity, leading to several prenylated products in one incubation. To some extent, this change broadens their application in creation of structure diversity of geranylated indole derivatives. The possible reason for the relaxed regioselective prenylation by the mutants could be that the enlarged pocket allows the binding of aromatic substrates in different orientations. However, the reduced regioselectivity requires more efforts for product isolation. Therefore, further mutagenesis experiments are necessary in the future to increase the desired regio- and stereoselectivity. This would require more structural information, *e.g.* of the available single mutants of the gatekeeping residues. Nevertheless, the single mutants constructed in this study clearly showed different preferences toward the 15 tested CDPs and converted them to geranylated derivatives at various positions. As shown in Scheme 2, products obtained in this study also carry the geranyl moiety at the *N1*-, *C2*-, and *C3*-position of the pyrrole ring in a regular or reverse manner.

To the best of our knowledge, known geranylations of diverse substrates usually occur at the benzene ring of the indole nucleus, *e.g.* *C7*-geranylation of indolactam V by TleC, *C6*-geranylation of tryptophan derivatives by 6-DMATS$_{Sa}$, and *C6*- and *C7*-geranylation of CDPs by AnaPT.[16,19,30] As mentioned above, the reported *C4*-geranylation of **1** by AtaPT[18] should be at C-7. Attachment of the geranyl moiety has been reported at neither C-4 or C-5 nor at N-1, C-2, or C-3 prior to this study.

The achieved relaxed regioselective geranylation by the mutants encouraged us to decorate all nucleophilic reactive positions (N-1 and the six nonbridgehead carbons) at the indole ring of one CDP with the geranyl moiety. For this purpose, **1** has been selected as the favorable CDP. HPLC analysis of the incubation mixture of **1** and GPP with FtmPT1_M364G, CdpNPT_M349G, CdpC2PT, and CdpC2PT_T351G revealed the presence of two to four product peaks each with product yields between 37.4 and 68.0% (Figure S9). Isolation and structure elucidation confirmed geranylations at N-1 (**1a1**), C-2 (**1a2** and **1b2**), C-3 (**1a3** and **1b3**), C-5 (**1a5**), C-6 (**1a6**), and C-7 (**1a7**) of the indole ring (Scheme 3). To get the remaining *C4*-position filled by geranylation, we applied the mutant FgaPT2_M328G, which uses GPP for *C4*-geranylation of tryptophan.[27] HPLC analysis of the incubation mixture of **1** and GPP with FgaPT2_M328G indicated product formation with a low yield (7.2%, Figure 2). In another previous study, we demonstrated that mutation on Arg244 of FgaPT2 significantly increased the catalytic activity toward CDPs in the presence of DMAPP.[31] To enhance the geranylation of **1**, we combined mutations at Met328 and Arg244 and created the double mutant FgaPT2_M328G_R244Q. As shown in Figure 2, one peak with a product yield of 29.7% was detected. Structure elucidation confirmed the desired geranylated product **1a4** (Scheme 3). In this way, nine products with a geranyl moiety at all nucleophilic reactive positions of the indole ring can be obtained from **1** by one-step chemoenzymatic reactions (Scheme 3, Figure S9).

With the tremendously increasing availability of protein structures, structure-based enzyme engineering becomes more important for modification of drug-like molecules. Indolactam geranyl transferase (TleC) can be changed to dimethylallyl transferase by small-to-large mutation.[19] MpnD and PagF were changed from selective dimethylallyl to geranyl transferases by large-to-small substitution.[19,32] In analogy, our study provides

Scheme 3. Attachment of Geranyl Moiety to Different Positions of 1 via One-Step Chemoenzymatic Reactions



an excellent example for rational engineering of desirable enzymes. Mutation of the gatekeeping residues in six PTs turned on or improved the acceptance of GPP for geranylation of diverse CDPs. Forty-two geranylated products were obtained from *in vitro* enzymatic reactions of CDPs and GPP. Using **1** as an acceptor and GPP as a donor, N-1 and the six nonbridgehead carbons of the indole ring were decorated with geranyl moieties. Derivatives of other CDPs could also be obtained by the mutants described in this study or by mutation of additional available dimethylallyl transferase at the corresponding positions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b03124.

Experimental procedures, structural elucidation, and NMR spectra of geranylated products (PDF)

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Notes

The authors declare no competing financial interest.

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