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Late-Stage Chemoenzymatic Installation of Hydroxy-Bearing Allyl Moiety on the Indole Ring of Tryptophan-Containing Peptides

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Abstract: The late-stage functionalization of indole- and tryptophan-containing compounds with reactive moieties facilitates downstream diversification and leads to changes in their biological properties. Here, the synthesis of two hydroxy-bearing allyl pyrophosphates is described. A chemoenzymatic method is demonstrated which uses a promiscuous indole prenyltransferase enzyme to install a dual reactive hydroxy-bearing allyl moiety directly on the indole ring of tryptophan-containing peptides. This is the first report of late-stage indole modifications with this reactive group.

The selective cleavage of a carbon–hydrogen (C–H) bond of final or intermediate compounds to form a C–C or a C-heteroatom (C–X) bond streamlines existing derivatization approaches. This late-stage functionalization, particularly in the context of heterocycle-containing complex compounds, leads to the formation of highly activated systems and provides access to bioactive analogs.[1,2] Yet, this C–H functionalization usually requires transition metals in addition to a high level of stereo-, regio-, and chemoselectivity.[3] An example of C–H activation includes allylic alkylation which has facilitated significant reactions such as the synthesis of indole-derived isatins[4] and oxo-functionalized eburnane alkaloids.[5] Another remarkable form of C–H activation is the introduction of a hydroxy group. Late-stage hydroxylation changes the physical and chemical properties of compounds and enables downstream modifications without additional steps of protection/deprotection.[2,6] Furthermore, hydroxylation of molecules allows for the formation of extended network of hydrogen bonds with biomolecules altering their biological activities.[6]

Hydroxy-bearing allyl (HBA) moiety is one of the most recognized oxyfunctionalized entities as it carries two reactive groups, allyl and alcohol. The presence of HBA enabled the synthesis of complex molecules such as the antibiotic precursor 6-deoxyerythronolide[7] and the polyene pheromone navenone B[8] from late-stage intermediates. Moreover, the presence of HBA facilitated impressive downstream chemical reactions such as Sharpless epoxidation,[9] intramolecular cyclization,[10] and rearranged enone formation,[11] in addition to alkylation[12] and chlorination[13] reactions. The installation of HBA is challenging and has been achieved via hydroxylation of compounds containing allylic groups in multi-step reactions.[14] Yet, no late-stage direct installation of HBA has been reported due to its reactive nature.

Indole- and tryptophan (Trp)-containing compounds are known for their biological activities.[15] Activation of this electron-rich heteroaromatic system has attracted attention either at early[16] or late[17] stage. Approaches of indole prenylation,[18] alkylation,[16] or hydroxylation[19] have been reported (Figure 1a) and used for the synthesis of complex bioactive compounds.[4,5] Trp and indole-containing compounds have also been modified using enzymes such as indole

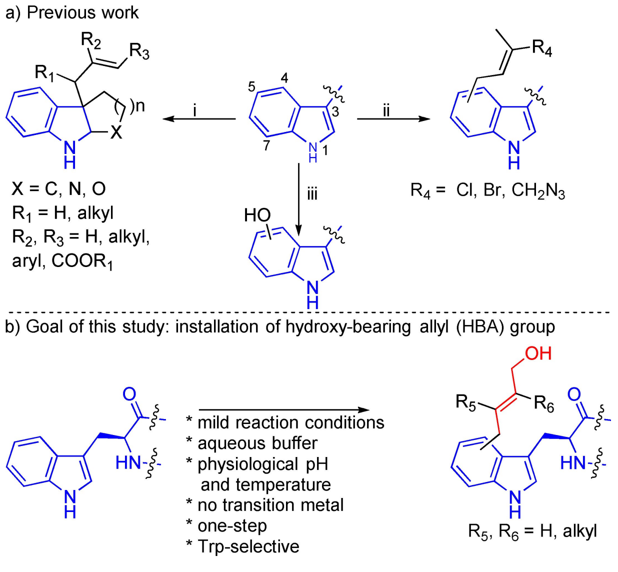

Figure 1. Previous and current introduction of allyl and/or hydroxy groups on indole-derived compounds. a) previously reported late-stage allylic alkylation (i and ii)[16–18] and hydroxylation (iii)[19] catalyzed by transition metals, indole prenyltransferases (IPTs)[21] or other methods. b) Aim of current study. Hydroxy-bearing allyl (HBA) group shown in red.

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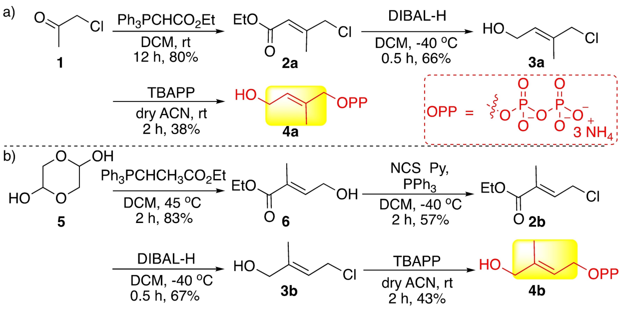
prenyltransferases (IPTs).[20] IPTs catalyze the transfer of a prenyl group, usually from native dimethylallyl, geranyl or farnesyl pyrophosphate donors, to Trp and other indole-derived acceptors. Several IPTs have relaxed substrate specificities allowing them to catalyze C–C bond formation between nonnative acceptors and donors.[21,22] Our lab and others have reported the diversification of Trp-containing simple[21,22] and complex[21,23,24] peptides with alkyl/aryl groups using IPTs. Yet, no reports included an HBA or even a hydroxy-containing moiety (Figure 1b). We hypothesized that we can synthesize HBA pyrophosphate analogs that structurally resemble the IPTs native donor substrate dimethylallylpyrophosphate (DMAPP). We can then exploit a promiscuous IPT to facilitate the late-stage installation of HBA on Trp-containing peptides.

In this report, we strategically synthesize two HBA pyrophosphate isomers, (*E*)-1-hydroxy-3-methyl-2-buten-4-yl diphosphate (**4a**) and (*E*)-1-hydroxy-2-methyl-2-buten-4-yl diphosphate (**4b**) (Scheme 1). We install one of the HBA precursors, **4b** enzymatically on the indole ring of mature peptides using an IPT enzyme. We determine the regiospecificities of the products using HR-ESI-MS and 1- and 2D NMR to be C-5/C-6/C-3 and C-5 selectively on the Trp of simple and complex peptides, respectively.

First, we achieved the synthesis of **4a** and **4b** using key Wittig ylides and corresponding carbonyls (Scheme 1) different than what was previously reported for **4b**.[25–27] Chloroacetone **1** and ethyl(triphenylphosphoranylidene)acetate were used to synthesize **2a** while the glycolaldehyde dimer **5**, ethyl 2-(triphenylphosphoranylidene)-propionate and *N*-chlorosuccinamide were used to synthesize **2b**. Both chloroalkyl esters **2a**/**2b** were reduced with diisobutylaluminum hydride (DIBAL–H) to provide **3a**/**3b** followed by reaction with TBAPP and ion exchange chromatography to provide (*E*)-**4a**/**4b** (Scheme 1). Strong NOE correlations confirmed the *E*-configurations of C-2$''$/C-3$''$ in both compounds.

Next, we had the nucleic acid sequences encoding three promiscuous IPTs, PriB,[21] FgaPT2[28] and CdpNPT[29] synthesized (Figures S1–S3). We evaluated the ability of any of the purified enzymes to couple **4a** and/or **4b** to simple cyclic Trp-containing dipeptides. Cyclo-(L-Trp-L-Trp) **7**, cyclo-(L-Trp-L-Tyr) **8** and cyclo-(L-Trp-Gly) **9** were chosen as representatives (Figure 2a). Analytical scale reactions were carried out after optimization by incubating 0.4 mM of each of **7**–**9** and 1.6 mM of each of **4a** or **4b** with 33.6 μM of one of the above enzymes in 50 mM Tris buffer (pH 8.0) with (FgaPT2 and CdpNPT) and without (PriB) 10 mM $CaCl_2$ at 37 °C for 16 h (Figure 3a). Negative controls lacking enzyme under the same conditions were also included. HPLC and HPLC-MS of the quenched reactions revealed the formation of new products when each of **7**–**9** was incubated with **4b** and CdpNPT but not **4a** or any other tested enzyme. (Figures S5–S7, Table S1). This data is consistent with the higher promiscuity of CdpNPT compared to other IPTs.[22–24] Due to the higher total conversion yield of **4b** with **7** (34%) compared to **8** (12.4%) and **9** (1.7%), we sought to determine the Michaelis constant (K_m) as well as the turnover number k_{cat} of CdpNPT with **7**/**4b** using GraphPad Prism 9.1.2. Subsequent determination of CdpNPT steady-state kinetic parameters of **7**/**4b** showed the total catalytic efficiency to be 0.51 M^{-1} s^{-1} (Figure S8), two-fold lower than when coupling



Scheme 1. Synthesis of **4a** and **4b** HBA pyrophosphates. Dimethylallyl fragment of native acceptor DMAPP is highlighted. Purified yields after column chromatography are stated.

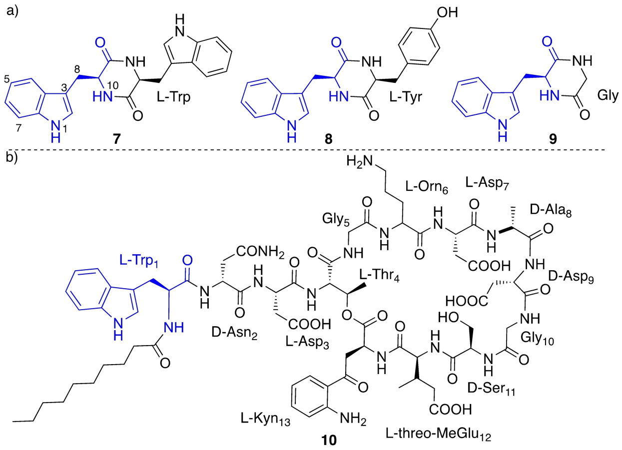


Figure 2. Tryptophan-containing peptides used in this study. Structures of a) cyclic dipeptides **7**–**9**; b) daptomycin **10**.

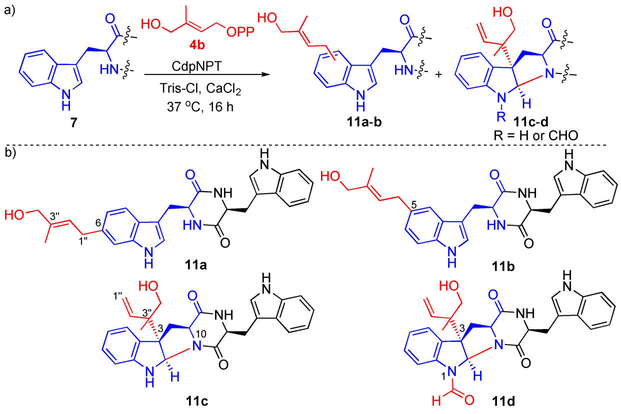


Figure 3. Chemoenzymatic method used and products obtained. a) CdpNPT-catalyzed reaction between **4b** and Trp-containing dipeptides **7**–**9**. Reaction conditions: **7**–**9** (0.4 mM), **4b** (1.6 mM), 33.6 μM CdpNPT, in 50 mM TrisCl/10 mM $CaCl_2$ at 37 °C for 16 h; b) structures of HBA-modified products of cyclo-(L-Trp$_1$-L-Trp$_2$), **11a**–**11d** confirmed by HR-MS and 1- and 2D NMR spectroscopy. **11d** is probably an artifact that arose from HPLC conditions.

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simple dipeptides with alkyl pyrophosphates.[23] The optimized reaction of **7**/**4b** produced **11** [Figure 3; **11a** (10.0%), **11b** (8.1%), **11c** (6.7%) and **11d** (9.4%)].

To identify the structures of the modified dipeptides, we scaled up the reaction between **7** and **4b** using the optimized analytical conditions. Reactions in Tris 50 mM (pH 8.0) supplemented with 10 mM $CaCl_2$ containing 2.3 mM **7**, 1.8 mM **4b** and 46 μM CdpNPT were incubated at 37 °C for 16 h. Preparative RP-HPLC chromatography led to the purification of four modified dipeptides **11a**–**11d** with total isolated yields of 26%. The (+)-HR- ESI-MS of **11a**–**11c** showed *m/z* $[M+H]^+$ 457.2246, 457.2251 and 457.2196, respectively, indicating a molecular formula of $C_{27}H_{28}N_4O_3$ (calculated as 457.2234) and a $\Delta m/z$ of 84 compared to the parent **7** suggesting an extra –C_5H_8O–. The 1- and 2D NMR spectroscopy of **11a** and **11b** reveals a C-1″ hydroxy-prenylated group at C-6 and C-5, respectively (Figure 3b, Figures S9–S10, Table S2, see Experimental Section for detailed structural elucidation). In addition to the HBA-dipeptides **11a** and **11b**, we also obtained **11c** and **11d**. Full NMR spectral data for **11c** showed a C-3″ hydroxy-prenylated group at the indole C-3 with a subsequent formation of C–N bond between C-2 and N-10 generating a hexahydropyrrolo-[2,3-*b*]indole structure (Figure 3b, Figures S11–S12, Table S3, see Experimental Section for detailed structural elucidation) typical of other prenylated analogs.[22] The HRMS of **11d** indicated a molecular formula of $C_{28}H_{28}N_4O_4$ and NMR data were consistent with that of **11c** with an additional formyl group at *N*-1 suggesting **11d** to be an artifact of HPLC conditions of **11c**. This highlights the selectivity of CdpNPT to modify the indole ring of Trp-containing dipeptides yet with moderate regiospecificities. Thus, we synthesized four HBA-modified dipeptides selectively at the Trp moiety (Figure 3b).

We sought to determine if our methodology can be used to modify a larger, more reactive and more diverse Trp-containing peptide such as the cyclic tridecapeptide, daptomycin[30] **10** with HBA (Figure 2b). Thus, analytical assays were carried out after optimization using 0.8 mM of **10**, 1.6 mM of each of **4a** and **4b** and 25.2 μM of one of the three IPTs and incubated in 50 mM Tris (pH 8.0) with and without $CaCl_2$ at 37 °C for 16 h. Similar to reactions with dipeptides **7**–**9**, only reactions with **4b** not **4a** and with only CdpNPT showed a new product. In contrast to **7**–**9**, only one single product **12** was formed (Figure 4a). To identify the regiospecificity of **12**, large-scale reaction containing 0.8 mM **10**, and 2.0 mM of **4b** and 32 μM CdpNPT incubated in 50 mM Tris/10 mM $CaCl_2$ (pH 8.0) at 37 °C for 16 h was carried out. Preparative RP-HPLC purification led to a single modified daptomycin product with isolated yield of 15%. The (+)-HR-ESI-MS indicated a molecular formula of $C_{77}H_{109}\,N_{17}O_{27}$, an additional –$C_5H_8O$– compared to **10**. 1- and 2D NMR data revealed **12** to be 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin (Figures 4b–4c, Figure S13, Table S4; see Experimental Section for detailed structural elucidation).

In this study, we synthesize two HBA-pyrophosphates (Scheme 1) and use one substrate for the direct installation of HBA on the indole ring of Trp-containing peptides. The fact that **4b**, not **4a** was accepted by CdpNPT might be due to the closer structural resemblance of **4b** to the enzyme native donor DMAPP compared to **4a**. Moreover, upon the release of the diphosphate from **4a** or **4b**, an allyl carbocation is formed. The **4b** allyl carbocation formed is stabilized by hyperconjugation with the 3″-methyl C–H and the allyl σ-bonds[31] allowing it to have more resonance structures than the **4a** carbocation. This increases the stability of **4b** and facilitates the reaction between the nucleophilic indole and the electrophilic donor species.[20] To our knowledge, this is the first report for the synthesis of **4a** while **4b** has been previously reported using different approaches and starting materials.[25–27] In addition to their role in late-stage biocatalytic diversification of Trp residues shown here, **4a**/**4b** can act as substrates for other diphosphate-utilizing enzymes such as terpene cyclases and other prenyltransferases. Using different HBA pyrophosphate substrates with variable enzymes has the potential to diversify natural products and other compounds. Moreover, substrate **4b** was shown to be a strong phosphoantigen activator of the Vγ9/Vδ2T of the γδ T-cells involved in immune responses to many diseases and fighting tumor cells.[32] Due to structural resemblance of **4a** with **4b**, we propose similar activity which urges the need for further immunological studies.

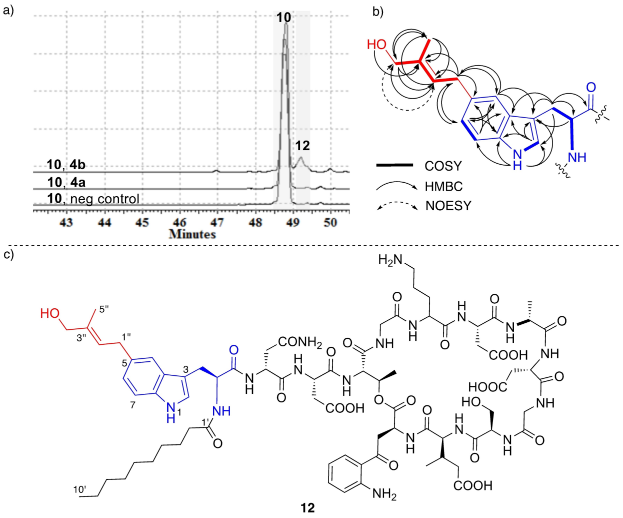

Figure 4. Derivatization of daptomycin **10** with HBA group. a) HPLC analysis of in vitro assays containing **10** (0.8 mM) with **4a** or **4b** (1.6 mM) in Tris 50 mm/$CaCl_2$ 10 mm (pH 8.0) incubated with CdpNPT (32 μM) for 16 h at 37 °C. b) ^{1}H-^{1}H COSY, ^{1}H–^{13}C HMBC and ^{1}H-^{1}H NOESY correlations of the tryptophan of **12** (full NMR correlations are demonstrated in Figure S13). c) Structure of HBA-modified daptomycin **12**. Long-range ^{1}H–^{1}H COSY correlations are shown between H-2″/H-4″, H-5″.

We report a selective late-stage functionalization method for the direct installation of an HBA group onto the Trp indole ring via a chemoenzymatic approach (Figure 3a). This method directly and selectively modifies the Trp indole ring and allows the diversification of Trp-containing peptides with an allyl alcohol group without the need for laborious de novo chemical synthesis. Peptide functionalization have gained great interest as it used as a tool for drug discovery and understanding diseases.[33] Indeed, several groups have functionalized Trp-containing peptides using chemical and photochemical methods.[34,35] However, direct installation of a dual reactive functionality, allyl and alcohol, that enables streamline downstream modifications to target either one or both groups simultaneously is unprecedented. No enzyme has been reported to install an HBA or free hydroxy group on aromatic moieties of complex compounds or peptides. Our one-step method is carried out in aqueous buffer under mild conditions of ambient temperature and pH using a wild-type enzyme and requires no directing groups or tedious enzyme engineering efforts. The Trp-containing peptides tested in this study are chemically diverse and in the case of **10** contains several acidic, basic and polar groups. Yet, only Trp-HBA derivatives were obtained highlighting the selectivity of our method towards the Trp residue in peptides. No diprenylated products were detected with any tested substrates by HPLC or HRMS even on the two Trp residues-containing, **7**. Despite the fact that multiple products were reported with CdpNPT when coupling dipeptides with other alkyl pyrophosphates, C-5 modification was never shown.[22] The increase in conversion yield of **7** compared to either **8** or **9** could be attributed to its higher hydrophobic character and proper size that properly fits into the enzyme active site.[36] Although **11 c** and **11 d** do not contain a typical allyl alcohol group, they contain vinyl and alcohol moieties which are also reactive and prone to further derivatization. In addition to the dual reactive group installed, **11 c** and **11 d** have undergone another remarkable *N-C* bond formation converting the cyclodipeptide to hexahydropyrrolo[2,3-*b*]indole core which is characteristic of several promising biologically active alkaloids.[37] In the case of dipeptides, regiospecificity was moderate giving rise to multiple products (Figure 3b) while with the larger peptide **10**, one single product was obtained (Figure 4a, Figure 4c). This is probably attributed to the steric restraint of **10** in the IPT active site preventing different orientations compared to the simpler **7**–**9** (Figure 4a, 4c). Noteworthy, the Trp C-5 position of **10** was reported to be less accessible to enzymatic functionalization when coupled with any alkyl substrates.[24] However, **4 b** entirely favored accessing the C-5 position with **10**. We provide a proof of concept that HBA can be installed selectively on Trp and suggest that yield and regiospecificity can be improved via screening of other wildtype IPTs and/or engineering efforts as well as using enzyme immobilization techniques. This work highlights the significance of enzymes in catalyzing difficult chemical reactions, in addition to their established role in the biosynthesis of complex natural products[38,39] and resistance against producing hosts.[40]

In short, we synthesized two HBA pyrophosphate isomers with good yield. We developed a method to selectively use these isomers to functionalize the indole ring of the Trp C–H bond of representative peptides with a dual reactive HBA moiety. The method directly modifies the indole ring at multiple sites in the case of small peptides but one specific C-5 modified product was synthesized with a complex tridecapeptide, **10**. The late-stage direct installation of two reactive nucleophilic groups, hydroxyl and allyl, is unprecedented and opens the door for future downstream derivatizations, bioorthogonal chemical reactions, bioconjugation and modulation of the biological activities of Trp-containing peptides and indole-derived compounds.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

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

Keywords: allylation · biocatalysis · C–H activation · functionalization · indole prenyltransferase

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