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学位の種類	博士（薬科学）
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学位論文題目	Enzymatic formation of prenyl *β*-carbolines by a fungal indole prenyltransferase （真菌由来インドールプレニル基転移酵素を用いたプレニルβ-カルボリンの酵素生成）

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様式３

論　文　要　旨

論文題目　Enzymatic formation of prenyl *β*-carbolines by a fungal indole prenyltransferase

課程･専攻名：博士後期課程･薬科学専攻

氏　名：Sherif Ahmed Muhammed Ahmed Hamdy

The prenylation of natural products is often an important factor to gain and/or diversify the activities of non-prenylated products. Accordingly, prenylated natural products have attracted keen attention as promising drug leads with versatile and promising pharmacological properties and health benefits for multitarget tissues. Due to the importance of the prenylation of the compounds, various prenylation methods for natural products have been developed. During these developments, some fungal indole prenyltransferase (IPT) have been recognized as promising biocatalysts to produce diverse prenyl compounds, due to their remarkable substrate promiscuity toward various prenyl acceptors and donors. In particular, CdpNPT identified in *Aspergillus fumigatus* and *Neosartorya fischeri* has been recognized as the most potent IPT to generate various prenylated alkaloids.

β-Carboline is a pharmaceutically important indole alkaloid produced by various organisms, including plants and marine creatures. It is also present in food products, alcoholic beverages, and tobacco smoke, as well as in human tissues and body fluids. Previous studies revealed the wide range of biological properties of *β*-carbolines including antimicrobial activities. In this study, the catalytic potential of CdpNPT with naturally occurring *β*-carbolines, harmol (**1**), harmine (**2**), and harman (**3**) (**Fig. 1**) as well as the effects of the prenylation of **1**–**3** on their antibacterial activities against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* were investigated.

1. Prenylated compounds produced by CdpNPT from 1–3 [1,2]

CdpNPT was heterologously expressed in *E. coli* BL21(DE3)pLysS as a C-terminal His_6-tag fused protein. The recombinant CdpNPT was purified by Ni-chelate affinity and size-exclusion chromatography and used for the enzyme reaction with **1**–**3** as the prenyl acceptor and dimethylallyl diphosphate (DMAPP) as the prenyl donor. The LC-ESI-MS analysis revealed that CdpNPT accepted **1** and **2** as the prenyl acceptor and afforded the prenylated compound **4** with a yield of 4.5% and **5** with a yield of 10.0%, respectively (**Figs. 1, 2a, and 2b**). The analyses of the NMR spectra, including ^{1}H and ^{13}C NMR, HMQC, HMBC, and NOESY experiments and HR-ESI-MS of **4** and **5** unambiguously revealed that CdpNPT possessed the ability to generate 6-(3',3'-dimethylallyl)harmol (**4**) from **1** and 6-(3',3'-dimethylallyl)harmine (**5**) from **2** in the presence of DMAPP as prenyl donor. In contrast, the LC-ESI-MS analysis revealed that CdpNPT afforded the two prenylated compounds **6** and **7** as major products, with yields of 10.1% and 14.4%, respectively, together with a trace amount of compound **8** (**Figs. 1 and 2c**). Both compounds **6** and **7** gave a cationized molecular ion peak at *m/z* 251 $[M+H]^+$, which is one dimethylallyl unit higher than that of the prenyl acceptor substrate **3**, whereas **8** had a cationized molecular ion peak at *m/z* 319 $[M+H]^+$, which is two dimethylallyl units higher

than that of the prenyl acceptor substrate **3**. Furthermore, the 1D- and 2D-NMR spectra analysis indicated that CdpNPT possessed the ability to produce two prenylated compounds, 6-(3',3'-dimethylallyl)harman (**6**) and 9-(3',3'-dimethylallyl)harman (**7**) from **3** as the major products. Considering the prenylated positions of **6** and **7**, compound **8** is most likely to be 6,9-(3',3'-dimethlallyl)harman, although the structure was not determined due to the low yield.

2. Catalytic mechanism of CdpNPT for the synthesis of prenyl *β*-carbolines [1,2]

Two mechanistically distinct catalytic pathways of the regular-type dimethylallylation have been proposed for IPTs. One is the one-step regular-type prenylation that occurs by a direct attack from the prenylation site of the activated indole ring to the primary center of the dimethylallyl carbocation *via* the abstraction of the indole amine proton by the catalytic residue Glu116 (numbering according to CdpNPT). The other is the two-step prenylation consisting of the reverse-type prenylation by the attack of the activated indole ring to the tertiary center of the dimethylallyl carbocation, followed by subsequent rearrangement and rearomatization after the indole amine proton is abstracted by Glu116.

To further clarify the catalytic mechanism, crystal structure analyses of CdpNPT complexed with **1** or **3** [CdpNPT (38-440)-**1** and CdpNPT (38-440)-**3**] in conjugation with docking simulation of the prenyl donor, DMAPP, were performed, using the N-terminal truncated mutant, CdpNPT (38-440). The analyses predicted that C-4b of **1** is located at a 3.5 Å distance from C-3 of DMAPP, whereas C-6 of **1** is located 1.1 Å further away from C-3 of DMAPP as compared to C-4b of **1**, suggesting that the initial prenylation first occurred at C-4b (**Fig. 3a**). A similar case was also found in the model structure of CdpNPT (38-440)-**3** with the DMAPP model, where the distance between C-4b in **3** and C-3 in DMAPP was around 1.0 Å shorter than that between C-6 in **3** and C-1 in DMAPP (**Fig. 3b**). These observations suggested that rather than the one-step regular-type prenylation at C-6 of the prenyl acceptors, compounds **4**–**6** were produced by the two-step prenylation by CdpNPT *via* the proton abstraction of the indole amine by Glu116, a nucleophilic attack from C-4b to the tertiary center of the dimethylallyl carbocation, the rearrangement of the terminal olefinic carbon of the intermediate to C-6, and the final rearomatization (**Fig. 4a**). Considering the high structural similarity between **2** and **1**, which only differ by the presence of the methoxy group in **2** and the hydroxy group at C-7 in **1**, CdpNPT could produce **5** by using the two step prenylation catalytic pathway, in a similar manner to that of **4** (**Fig. 4a**). Hence, the N-9 regular-type prenylated product **7** is also expected to be converted from the C-4b reverse-type prenylated intermediate for **6** *via* a subsequent prenyl rearrangement reaction (**Fig. 4b**). However, the docking study also predicted that C-3 and C-1 of DMAPP are located at a 3.9 Å distance from both C-4b and N-9 of **3** (**Fig. 3b**), which is inconsistent with those between C-4b in **1** and C-3 in DMAPP (3.5 Å distance) and between N-9 in **1** and C-1 in DMAPP (4.3 Å distance) in the model structure of CdpNPT complexed with **1**. Thus, a one-step prenylation reaction may also be triggered in **3** to produce **7**, after CdpNPT has abstracted the hydrogen atom of the indole amine *via* the Glu116 residue (**Fig. 4c**).

3. Effects of prenylations of 1–3 and 4 on their antibacterial activities [2]

The non-prenylated *β*-carbolines **1**–**3** reportedly possess antibacterial activities against Gram-positive bacteria, *S. aureus* and *B. subtilis*, and a Gram-negative bacterium, *E. coli*, using the disc diffusion method. To investigate the effects of the prenylations of **1**–**3**, the antibacterial activities of **4**–**7** were assessed against the three bacteria using the MTT assay method, and were compared with those of **1**–**3** (**Table 1**). In contrast to the previous report, **1**–**3** did not show any antibacterial activities against the tested bacteria. The use of different assay systems in this study and the previously reported study may cause this different result. Furthermore, the assay in this study indicated no effect of the regular-type dimethylallylation of **3** at the N-9 position on the antibacterial activities against the three bacteria. However, the assay revealed that the 6-(3',3'-dimethylallyl)-*β*-carbolines **4**–**6** showed weak antibacterial activities against the Gram-positive bacteria with MIC values of 100, 100, and 200 μM, respectively, suggesting that the regular-type dimethylallylations of **1**–**3** at C-6 were effective to enhance their antibacterial activities against *S. aureus* and *B. subtilis*.

Conclusion

This study demonstrated that CdpNPT has the ability to dimethylallylate harmol (**1**), harmine (**2**), and harman (**3**) and produce regular-type 6-(3',3'-dimethylallyl)harmol (**4**) from **1**, 6-(3',3'-dimethylallyl)harmine (**5**) from **2**, and 6-(3',3'-dimethylallyl)harman (**6**) and 9-(3',3'-dimethylallyl)harman (**7**) from **3**. The antibacterial assay also revealed that the regular-type dimethylallylations of **1**–**3** enhanced their antibacterial activities against *S. aureus* and *B. subtilis*. This is the first demonstration of the prenylation of *β*-carbolines by IPT enzymes. The findings suggest that further investigations of the substrate promiscuity and catalytic potential of CdpNPT with other *β*-carbolines could lead to the production of various prenyl *β*-carbolines.

Reference

1. Hamdy SA, Kodama T, Nakashima Y, Han X, Matsui T, Morita H (2022) Enzymatic formation of a prenyl *β*-carboline by a fungal indole prenyltransferase. *J Nat Med*, *76*, 873-879.
2. Hamdy SA, Kodama T, Nakashima Y, Han X, Morita H (2022) Catalytic potential of a fungal indole prenyltransferase toward *β*-carbolines, harmine and harman, and their prenylation effects on antibacterial activity. *J Biosci Bioeng*, in press. 10.1016/j.jbiosc.2022.07.004.

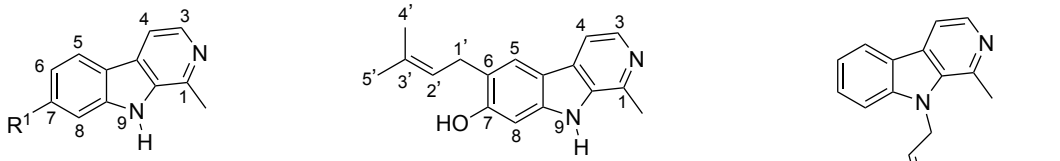

Figure 1. Structures of compounds **1**–**7**.

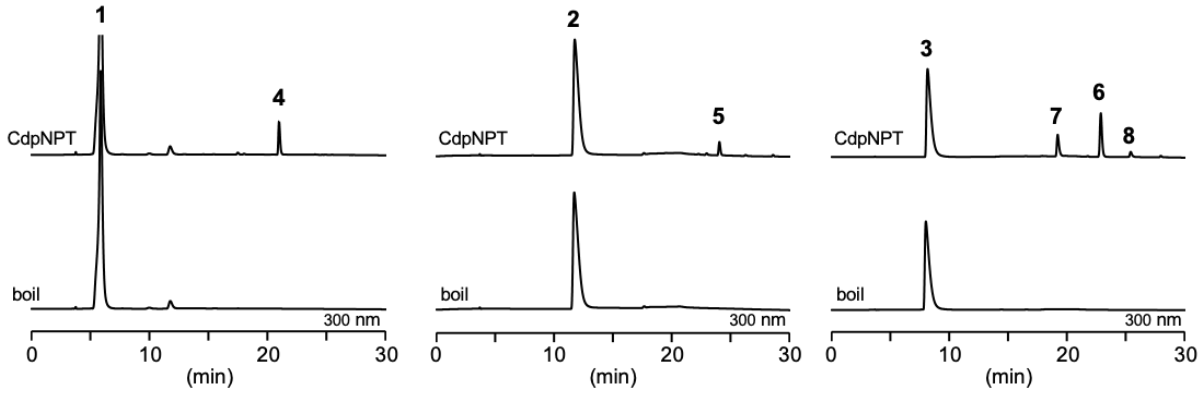

Figure 2. HPLC elution profiles of the enzyme reaction products (a) from **1** and DMAPP, (b) from **2** and DMAPP, and (c) from **3** and DMAPP by CdpNPT and its boiled enzyme.

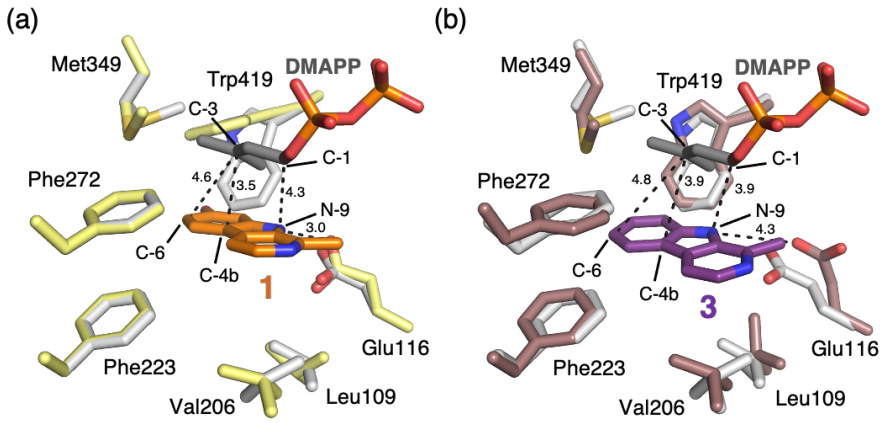

Figure 3. Close-up view of the active-site of docking models of (a) CdpNPT (38-440) with **1** and DMAPP and (b) **3** and DMAPP.

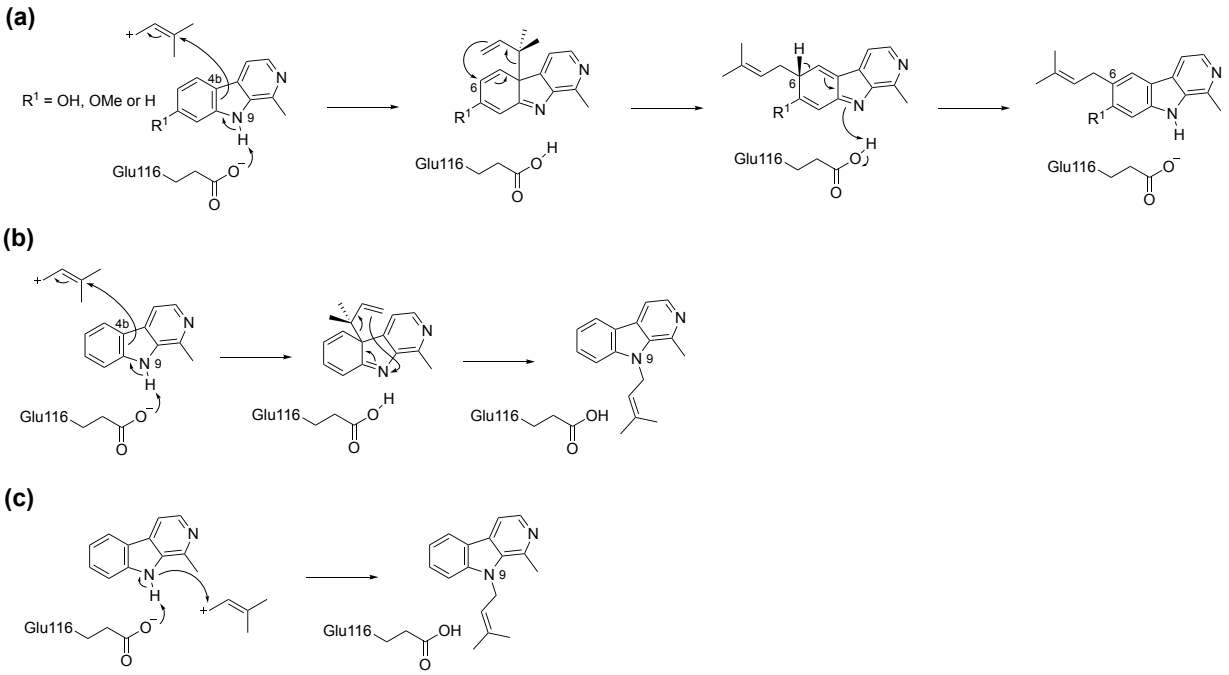

Figure 4. Proposed mechanism for the formation of **4**–**7** by CdpNPT.

Table 1. Antibacterial activities of compounds **1**–**7**.

Sample	MIC (μM)		
	S. aureus	*B. subtilis*	*E. coli*
1	>200	>200	>200
2	100	100	>200
3	>200	>200	>200
4	>200	>200	>200
5	100	100	>200
6	>200	>200	>200
7	200	200	>200
ampicillin	3.13	1.56	-
kanamycin	-	-	1.56

様式７

学位論文審査の要旨

報告番号	富医薬博甲第　　号 富医薬博乙第　　号	氏　　名	Sherif Ahmed Muhammed Ahmed Hamdy

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（論文題目）（英語の場合は和訳，日本語の場合は英訳を付記すること。英語（訳）は，最初の文字のみを大文字で表記し，他は小文字で表記すること（ただし，学名等を除く）。）

Enzymatic formation of prenyl *β*-carbolines by a fungal indole prenyltransferase
（真菌由来インドールプレニル基転移酵素を用いた
プレニル*β*-カルボリンの酵素生成）

（判定）

合格

（論文審査の要旨）（２頁以内）

人類の健康や産業に重要な天然有機化合物には抗生物質や抗ガン剤等の医薬品となる化合物や食品添加物等があるが，これらは天然資源から数多く見出されてきた。これらの二次代謝産物は，生物が持つ生合成酵素の組合せにより母格が構築された後，種々の修飾を経て多様かつ複雑な構造を持つ化合物へと変換されて得られたものである。そのような修飾の一つとして挙げられるのがプレニル化である。天然物のプレニル化修飾は，プレニル化天然物の抗腫瘍，抗菌，抗ウイルス，抗炎症活性等，多岐に渡る生物活性の発現に大きく寄与することが報告されている。これまでにプレニル化された天然物は多数報告されている一方で，多様かつ強力な生物活性を示す*β*-カルボリン類へのプレニル化が生物活性に及ぼす影響については殆ど検討されていない。さらに，真菌由来インド−ルプレニル基転移酵素（IPT）については，これまでにインド−ル基を持つ様々な化合物のプレニル化を行うことができる有用な触媒として報告されているが，*β*-カルボリン類への適用についての検証事例はない。申請者は，生物活性の向上における*β*-カルボリン類へのプレニル修飾の有用性とIPTのプレニル化触媒としての可能性を検証することを目的に，IPTの中でも特に曖昧な基質特異性を示すことが報告されている*Aspergillus fumigatus*由来サイクリックジペプチド-*N*-プレニル基転移酵素(CdpNPT)に，ハマビシ科ハルマラ*Peganum harmala*の主要成分である3種の*β*-カルボリン，ハルモール(**1**)，ハルミン(**2**)，及びハルマン(**3**)をジメチルアリル二リン酸(DMAPP)と共に基質として作用させることで，CdpNPTを用いて*β*-カルボリンをプレニル化できることを明らかにした。さらに，**1**−**3**の6位をプレニル化すると抗菌活性が向上することを見出すに至った。本研究に関する内容の骨子と審査結果は，下記に示すとおりである。

1. CdpNPTの1−3に対する触媒能力の検証

CdpNPTの全長をC末His_6-tagとの融合タンパク質として大腸菌において異種発現させ，Ni-キレートアフィニティーカラム及びゲル濾過を用いて精製したCdpNPTに**1**−**3**をDMAPPとともに作用させ，LC-ESI-MS，HR-ESI-MS，及びNMRを用いて生成物を精査した。その結果，CdpNPTは**1**と**2**から6位が3',3'-dimethylallyl化された新規プレニル化ハルモール(**4**，収率4.5%)とハルミン(**5**，収率10.0%)を各々生成できることを明らかにした。さらに，**3**をプレニル基受容体と

して用いた場合では，CdpNPTは6位が3',3'-dimethylallyl化された新規プレニル化ハルマン(**6**，収率10.1%)と9位が3',3'-dimethylallyl化されたプレニル化ハルマン(**7**，収率14.4%)を生産できることを明らかにした。構造決定には至っていないものの，CdpNPTが**3**からジプレニル化されたハルマン(**8**)を極少量生産できることも明らかにした。IPTが*β*-カルボリンをもプレニル化できることを示した事例は，本研究が最初である。

2. CdpNPTの1–3に対する触媒機構の解析

これまでに，N末端の37番目アミノ酸残基を欠損させたCdpNPTについては，良質な結晶を与えるとの報告がある。そこで，CdpNPTの37番目までのアミノ酸を欠損させた変異体をC末His_6-tagとの融合タンパク質として大腸菌に異種発現させ，Ni-キレートアフィニティーカラムとゲル濾過を用いてCdpNPT(38-440)を精製した。次に，CdpNPT(38-440)に**1**及び**3**を結晶化の際に加えることで，CdpNPT(38-440)と**1**及び**3**の複合体結晶構造を2.40 Åと2.43 Åの分解能で取得し，これらの構造にDMAPPをドッキングさせることで，触媒機構の解明を目指した。その結果，**1**と**3**の4b位は，CdpNPTの活性中心キャビティー内で，6位よりもDMAPPの3位に近いところに配置されること，及び**3**の4b位とDMAPPの3位は，**3**の9位とDMAPPの1位と同じ距離に配置されることを見出した。**1**と**2**の構造的相違は7位の官能基のみであり，このことから**2**は**1**と同様にCdpNPTに結合すると強く推定できる。これらの結果に基づき，**4**–**6**の生成については，プレニル基受容体基質**1**–**3**各々の4b位が3',3'-dimethylallyl化された後，6位へのCope転移が生じたことで生成する可能性が高いこと，**7**の生成については，4bから9位への転移反応を伴うことは無く，9位が直接3',3'-dimethylallyl化されることで生成した可能性が高いと提唱するに至った。

3. 化合物1–7の抗菌活性の評価

1–**3**については，ペーパーディスク法を用いた検証により，*Staphylococcus aureus*，*Bacillus subtilis*，及び*Escherichia coli*に対する抗菌活性が報告されている。**1**–**7**について，ペーパーディスク法よりも化合物の最小発育阻止濃度(MIC)をより詳細に評価可能なMTT法を用いて，*S. aureus*，*B. subtilis*，及び*E. coli*に対する抗菌活性を評価した。その結果，**1**–**3**はこれらの3種の菌体に対して抗菌活性は示さないものの，6位がプレニル化された**4**–**6**は*S. aureus*と*B. subtilis*に対して100 μMのMIC値で，9位がプレニル化された**7**は，*S. aureus*と*B. subtilis*に対して200 μMのMIC値で抗菌活性を示すことが判明した。これらの結果から，**1**–**3**への6位のプレニル化は抗菌活性を高める効果があることを明らかにした。

以上のように，Sherif Ahmed Muhammed Ahmed Hamdyは，IPTが*β*-カルボリンをプレニル化できることを初めて明らかにした。さらに，**1**–**3**の6位をプレニル化すると抗菌活性が劇的に上昇することを明らかにした。CdpNPTや他のIPTに他の*β*-カルボリンや炭素数の異なるプレニル供与体を基質として作用させることで，新たな医薬品シードを生み出すことが期待される。本研究結果は，IPTのプレニル化触媒としての可能性と*β*-カルボリンへのプレニル化修飾の有用性について新たな科学的知見を与えたと言える。

主査及び副査は，論文内容と面接試験を通して，申請者 Sherif Ahmed Mohamed Ahmed Hamdyに，博士（薬科学）の学位を授けるに値すると判定した。

(学位論文のもとになる論文　著者名, 論文題目, 掲載誌名, 巻, 最初の頁と最後の頁, 年を記載)

1. Hamdy SA, Kodama T, Nakashima Y, Han X, Matsui T, Morita H (2022) Enzymatic formation of a prenyl *β*-carboline by a fungal indole prenyltransferase. *J Nat Med*, *76*, 873-879.
2. Hamdy SA, Kodama T, Nakashima Y, Han X, Morita H (2022) Catalytic potential of a fungal indole prenyltransferase toward *β*-carbolines, harmine and harman, and their prenylation effects on antibacterial activity. *J Biosci Bioeng*, in press. 10.1016/j.jbiosc.2022.07.004.