Drug Biosynthesis

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Unusual Acetylation-Dependent Reaction Cascade in the Biosynthesis of the Pyrroloindole Drug Physostigmine**

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Abstract: *Physostigmine is a parasympathomimetic drug used to treat a variety of neurological disorders, including Alzheimer's disease and glaucoma. Because of its potent biological activity and unique pyrroloindole skeleton, physostigmine has been the target of many organic syntheses. However, the biosynthesis of physostigmine has been relatively understudied. In this study, we identified a biosynthetic gene cluster for physostigmine by genome mining. The 8.5 kb gene cluster encodes eight proteins (PsmA–H), seven of which are required for the synthesis of physostigmine from 5-hydroxytryptophan, as shown by in vitro total reconstitution. Further genetic and enzymatic studies enabled us to delineate the biosynthetic pathway for physostigmine. The pathway features an unusual reaction cascade consisting of highly coordinated methylation and acetylation/deacetylation reactions.*

Physostigmine is a tryptophan-derived pyrroloindole alkaloid that reversibly inhibits acetylcholinesterase. It is used clinically as a parasympathomimetic drug to treat a wide variety of disorders, including Alzheimer's disease, glaucoma, delayed gastric emptying, and orthostatic hypertension. Furthermore, physostigmine can cross the blood–brain barrier and is used to counteract the effects on the central nervous system of overdoses of atropine, scopolamine, and other anticholinergic drugs.[1,2] Because of its potent biological activity and unique structure (Scheme 1), physostigmine (**1**) has attracted much interest from organic chemists.[3–5] However, the enzymatic machinery that directs the biosynthesis of physostigmine has not been elucidated until now.

Physostigmine was initially isolated from the seeds of *Physostigma venenosum*.[1] Several structurally related pyrroloindole alkaloids, such as eseramine and physovenine, have also been isolated from this source.[6,7] In addition, many oligomeric pyrroloindole alkaloids, including chimonanthines, hodgkinsines, and psychotridines, have been isolated from other plants, and these compounds are of interest because of their potent analgesic activities (see Figure S1 in the Supporting Information).[8–10] Despite the prevalence of the pyrroloindole ring system in natural products, little is known about the enzymes responsible for the synthesis of the aforementioned alkaloids. Only two types of enzymes have previously been reported to promote the formation of the pyrroloindole ring by exploiting the nucleophilicity of the indole C3 atom. These characterized enzymes include prenyltransferases and monooxygenases, which respectively prenylate and hydroxylate the indole C3 atom in the biosynthesis of pyrroloindole-containing peptide natural products, such as aszonalenin[11] and himastatin.[12] In contrast, physostigmine is methylated at the indole C3 atom, which prompted us to initially hypothesize that its biosynthesis proceeds by the decarboxylation of tryptophan, followed by a rare C3-methylation of the indole ring and cyclization by attack of the primary-amine moiety onto the iminium ion. Further substitutions, including hydroxylation, carbamylation, and three N-methylation steps, then presumably complete the modification of the pyrroloindole skeleton. Herein, we report the characterization of the gene cluster responsible for physostigmine biosynthesis. In addition to the identification of the first indole C3-methyltransferase, we show that the biosynthetic pathway to physostigmine involves an unusual reaction cascade consisting of highly coordinated methylation steps and acetylation/deacetylation reactions.

Because secondary-metabolite biosynthetic genes are typically clustered in microbial hosts, the finding that physostigmine was also produced by the bacterium *Streptomyces griseofuscus* NRRL 5324[13] provided us with the opportunity to quickly identify the genes involved in physostigmine biosynthesis. The genome of *S. griseofuscus* was sequenced and subjected to BLASTP analysis with a carbamoyltransferase as a probe. The bioinformatics search identified one putative gene cluster (GenBank accession no. KF201694), which spans 8.5 kb and encodes eight open reading frames designated *psmA–H* (Scheme 1). The deduced roles of the gene-cluster products do not align well with the originally proposed biosynthetic pathway: The functions of PsmB, F, and G are unknown; four methylation reactions are needed, but only three methyltransferases (PsmA, C, and D) are encoded; and no typical hydroxylase is encoded. To confirm the involvement of this DNA region in physostigmine biosynthesis, we carried out heterologous expression of this cluster in non-physostigmine-producing *Streptomyces* hosts. A fosmid library of *S. griseofuscus* was constructed, and three different fosmids containing the putative gene cluster were identified by PCR screening. The fosmids were then subcloned into an *E. coli–Streptomyces* shuttle vector and introduced into *S. albus* R1 and *S. lividans* K4-114. Analysis of the culture extracts by liquid chromatography–high-resolution mass spectrometry (LC–HRMS) and comparison

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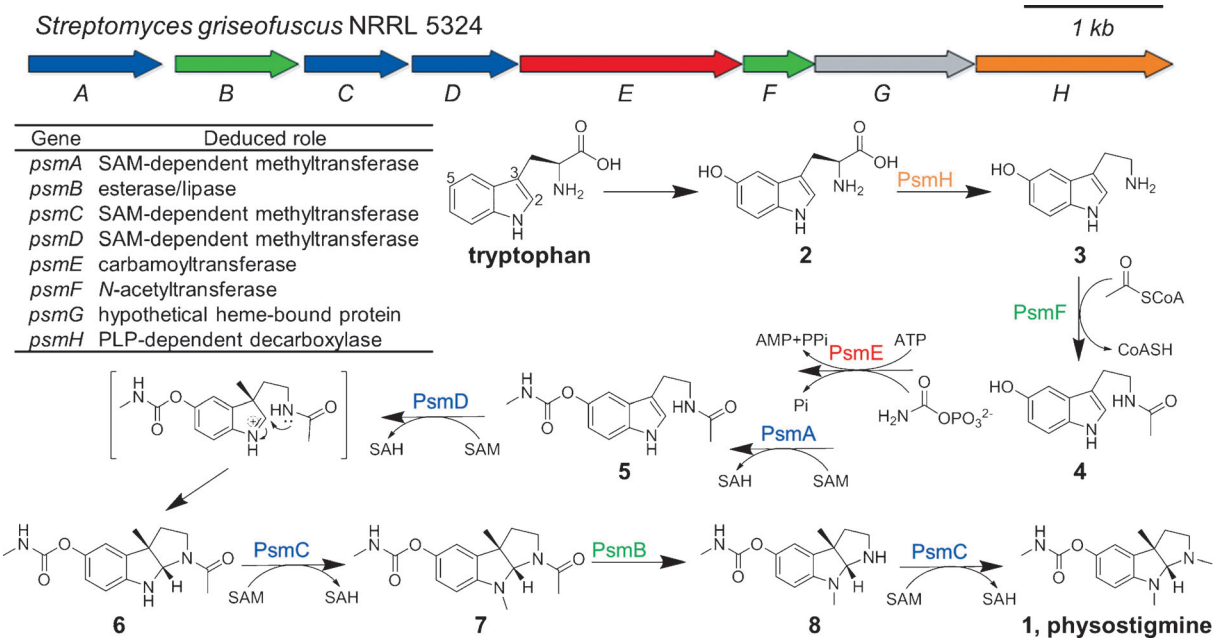

Scheme 1. Map of the physostigmine gene cluster, deduced roles of genes as based on sequence homology, and characterized biosynthetic pathway. SAH = *S*-adenosylhomocysteine.

with the physostigmine standard showed heterologous production of **1** with all three fosmids in both *Streptomyces* hosts, thus verifying that the identified gene cluster is directly involved in the biosynthesis of physostigmine (Figure 1; see also Figure S4). Interestingly, a homologous gene cluster was also found in the published genome of *Streptomyces albulus* CCRC 11814, a known poly-L-lysine producer,[14] although no physostigmine production has been reported from this organism.

The functions of PsmA–H in physostigmine biosynthesis were further demonstrated by in vitro reconstitution. A one-pot assay with all eight of the purified enzymes (see Figure S2) and their possible cosubstrates (*S*-adenosylmethionine (SAM), carbamoyl phosphate, adenosine 5′-triphosphate (ATP), and acetyl coenzyme A (acetyl-CoA)) was performed. We propose that tryptophan is initially hydroxylated by a tryptophan 5-hydroxylase, which was confirmed to be encoded elsewhere on the genome by BLASTP analysis. Indeed, when 5-hydroxytryptophan (**2**) was used as a substrate, **1** was successfully produced in the one-pot assay, as demonstrated by LC–HRMS analysis (see Figures S4 and S5). Individual enzymes were then systematically removed from the assay to determine their necessity for physostigmine biosynthesis. Seven of the enzymes, PsmA–F, H, were determined to be essential for the formation of **1** from **2** (Figure 2).

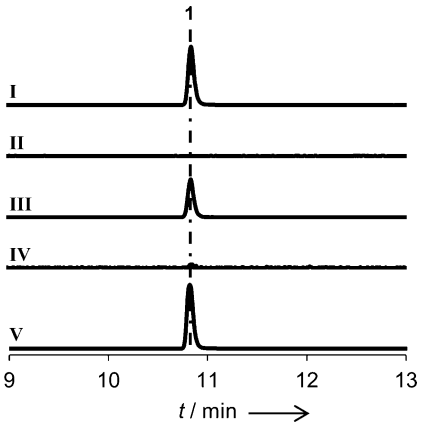

Figure 1. Heterologous production of physostigmine. Extracted ion chromatograms showing I) heterologous production of **1** in *S. lividans*, but not in II) *S. lividans* with an empty vector; III) heterologous production of **1** in *S. albus*, but not in IV) wild-type *S. albus*; V) production of **1** by wild-type *S. griseofuscus*.

The timing of each enzymatic reaction was then delineated by a series of in vitro and in vivo studies. We first set out to determine the substrate (L-tryptophan or **2**) for the pyridoxal 5′-phosphate (PLP)-dependent decarboxylase, PsmH. Incubation of purified PsmH with tryptophan and **2** resulted in the formation of tryptamine and 5-hydroxytryptamine (**3**), respectively (Figure 2). Comparison of the kinetic parameters of PsmH showed that it clearly prefers the hydroxylated substrate: the k_{cat}/K_m value for **2** ($8.6 \pm 0.9\ min^{-1}\,mM^{-1}$) was 650-fold higher than that for tryptophan ($0.013 \pm 0.002\ min^{-1}\,mM^{-1}$). This result is consistent with the one-pot assay in which **2** was efficiently utilized as a substrate. We propose that **3** then undergoes N-acetylation catalyzed by PsmF, an *N*-acetyltransferase homologue. The enzymatic reaction with PsmF, acetyl-CoA, and **3** yielded the expected product **4** (Scheme 1 and Figure 2), thus confirming the function of PsmF as a 5-hydroxytryptamine *N*-acetyltransferase. These early pathway intermediates, **3** and **4**, also known as serotonin and normelatonin, respectively, are common secondary metabolites produced by many other organisms as well.[15,16]

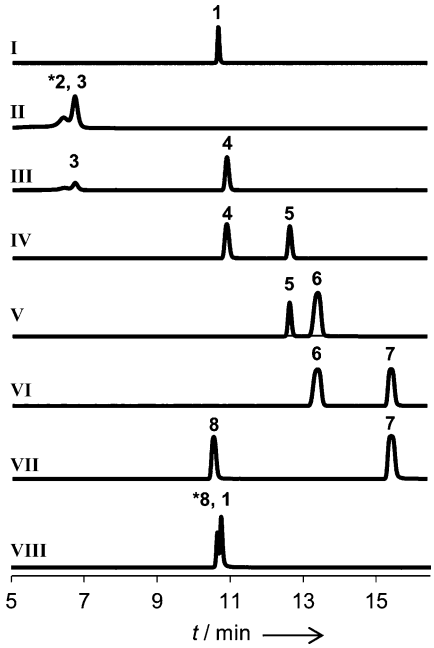

Figure 2. Extracted ion chromatograms for the characterization of PsmA–F, H. The calculated mass with a 10 ppm mass error tolerance was used. I) Reaction of **2** under the catalysis of PsmA–F, H to generate **1**; II) PsmH-catalyzed reaction of **2** to generate **3**; III) PsmF-catalyzed reaction of **3** to generate **4**; IV) PsmE/PsmA-catalyzed reaction of **4** to generate **5**; V) PsmD-catalyzed reaction of **5** to generate **6**; VI) PsmC-catalyzed reaction of **6** to generate **7**; VII) PsmB-catalyzed reaction of **7** to generate **8**; VIII) PsmC-catalyzed reaction of **8** to generate **1**. *Substrate and product have overlapping retention times.

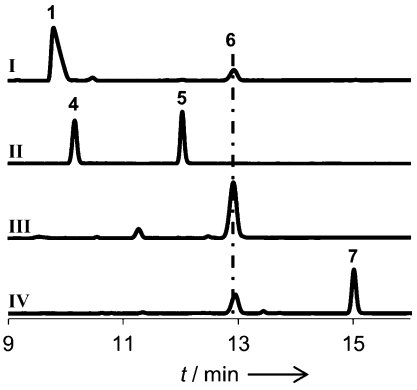

Figure 3. HPLC chromatograms with UV detection of metabolites produced by wild-type and mutant *S. griseofuscus* strains. I) Wild-type *S. griseofuscus* (245 nm); II) Δ*psmD* (280 nm); III) Δ*psmC* (245 nm); IV) Δ*psmB* (245 nm).

The subsequent enzymatic reaction on **4** could be either carbamylation catalyzed by PsmE or C3-methylation catalyzed by one of the methyltransferases. The incubation of PsmE, carbamoyl phosphate, ATP, and **4** resulted in the formation of carbamylated **4**, which could be further methylated by PsmA to yield **5**, which showed a UV absorption spectrum similar to that of **4** (Scheme 1 and Figure 2; see also Figure S5). The production of **5** from **4** confirmed the roles of PsmE as a normelatonin *O*-carbamoyltransferase and PsmA as a carbamoyl *N*-methyltransferase. Compound **5** also accumulated as one of the major metabolites in the culture extracts of the Δ*psmD* mutant of *S. griseofuscus* (Figure 3; see also Figures S7–S11), thus indicating that carbamylation precedes the methylation catalyzed by PsmD. Although PsmE was also capable of carbamylating **2**, **3**, and eseroline (**9**; see Figure S6), further kinetic characterization of the enzyme showed **4** to be the preferred substrate: the k_{cat}/K_m value for **4** ($32.2 \pm 2.1\ min^{-1}\,mM^{-1}$) was higher than those for **2** ($1.55 \pm 0.04\ min^{-1}\,mM^{-1}$), **3** ($13.6 \pm 0.7\ min^{-1}\,mM^{-1}$), and **9** ($0.037 \pm 0.004\ min^{-1}\,mM^{-1}$).

We next probed the putative function of PsmD as a C3-methyltransferase by using purified **5** as a substrate in vitro. C3-methylation would disrupt the conjugated indole ring and lead to a significant change in the UV absorption spectrum of the product. Accordingly, the enzymatic assay consisting of PsmD, SAM, and **5** resulted in the formation of **6**, which has a UV absorption spectrum distinct from that of **5** (Scheme 1 and Figure 2; see also Figure S5). The molecular structure of **6** was confirmed by NMR spectroscopic analysis (Figures S12–S16), and the production of **6** confirmed the role of PsmD as a C3-methyltransferase. It is proposed that the C3-methylated product is highly unstable and undergoes spontaneous cyclization by nucleophilic attack of the amine onto the iminium ion to form the pyrroloindole skeleton (Scheme 1). Similar cyclization mechanisms have been observed in the biosynthesis of aszonalenin and himastatin, whereby C3-prenylation and C3-hydroxylation, respectively, promote ring closure.[11,12,17] Notably, PsmD could also methylate **4**, but it did not exhibit any activity toward **3**, thus indicating that N-acetylation is required for C-methylation.

To complete the biosynthesis of **1** from **6**, one deacetylation and two N-methylation reactions on the pyrroloindole skeleton are necessary. These reactions are presumably catalyzed by PsmB, an esterase/lipase homologue, and PsmC, another methyltransferase homologue, respectively. To examine the timing of these tailoring reactions, *psmB* and *psmC* were individually deleted in frame by double cross-over in *S. griseofuscus* (see Figure S3). Since **6** was observed to accumulate as the major metabolite in the Δ*psmC* culture extracts (Figure 3), we propose that methylation by PsmC directly follows methylation by PsmD. Analysis of the culture extracts of the Δ*psmB* mutant showed accumulation of a new major metabolite, **7**, which was revealed to be an N-methylated derivative of **6** by NMR spectroscopic analysis (Figure 3; see also Figures S17–S21). It is thus proposed that PsmC first catalyzes one N-methylation on **6** to yield **7**, which is subsequently deacetylated by PsmB to yield **8**. Final N-methylation on **8** then completes the biosynthesis of **1** (Scheme 1). To confirm this tailoring-reaction cascade, we biochemically reconstituted each transformation step (Figure 2). An enzymatic assay with PsmC, SAM, and **6** showed the formation of **7** as a major product, thus confirming the function of PsmC as an *N*-methyltransferase. Incubation of purified PsmB and **7** resulted in the formation of **8**, thus indicating that PsmB functions as a deacetylase on **7**. Finally, we incubated the three methyltransferases PsmA, C, and D individually with purified **8** and SAM to identify the enzyme

responsible for the last N-methylation step. Only the assay with PsmC yielded **1**, thus demonstrating that PsmC catalyzes methylation reactions both before and after deacetylation. Although PsmB also exhibited activity toward **6** in vitro, the resulting deacetylated product could not be methylated twice by PsmC to form **1**. Instead, we only observed the formation of a product with the same mass as **8**. This result demonstrates that the N-methylation and deacetylation reactions occur in a very precise order for physostigmine biosynthesis.

In summary, we have identified and dissected the seven enzymes and eight reaction steps involved in the biosynthesis of the pyrroloindole drug physostigmine from 5-hydroxytryptophan. Initial decarboxylation of **2**, followed by acetylation of the amine, results in the well-known intermediate normelatonin, which is modified at the C5 hydroxy group by carbamylation and methylation. C3-methylation of the indole ring yields a highly reactive iminium ion, which is then attacked by the amine nucleophile to form a pyrroloindole skeleton. Further reactions, including tandem N-methylation, N-deacetylation, and N-methylation, complete the biosynthesis of physostigmine (Scheme 1). This biosynthetic logic is rather unusual in that it involves an acetylation and subsequent deacetylation. We then showed that this unexpected acetylation–elimination is due to the highly coordinated nature of the methylation and acetylation/deacetylation reactions involved in physostigmine biosynthesis. Specifically, acetylation is necessary for indole C3-methylation, whereas the two N-methylation reactions of the pyrroloindole ring must occur in concert with deacetylation; no alternative route appears to result in physostigmine formation. Furthermore, this biosynthetic gene cluster also features two unique methyltransferases. To our knowledge, PsmD is the first enzyme that has been characterized to catalyze indole C3-methylation, and PsmA is the first enzyme that has been characterized to methylate a carbamoyl group. Thus, this study not only provides some insight into the biosynthesis of other structurally related pyrroloindole alkaloids, but it also expands the repertoire of enzymes that can functionalize the indole C3 atom and the carbamoyl moiety.

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[1] B. Witkop, *Heterocycles* **1998**, *49*, 9–27.
[2] R. P. Granacher, R. J. Baldessarini, *Clin. Neuropharmacol.* **1976**, *1*, 63–80.
[3] T. Araki, T. Ozawa, H. Yokoe, M. Kanematsu, M. Yoshida, K. Shishido, *Org. Lett.* **2013**, *15*, 200–203.
[4] A. S. ElAzab, T. Taniguchi, K. Ogasawara, *Org. Lett.* **2000**, *2*, 2757–2759.
[5] M. G. Kulkarni, A. P. Dhondge, A. S. Borhade, D. D. Gaikwad, S. W. Chavhan, Y. B. Shaikh, V. B. Ningdale, M. P. Desai, D. R. Birhade, M. P. Shinde, *Tetrahedron Lett.* **2009**, *50*, 2411–2413.
[6] P. M. Dewick, *Medicinal Natural Products: A Biosynthetic Approach*, 3rd ed., Wiley, Chichester, **2009**.
[7] P. Ruiz-Sanchis, S. A. Savina, F. Albericio, M. Álvarez, *Chem. Eur. J.* **2011**, *17*, 1388–1408.
[8] A. Steven, L. E. Overman, *Angew. Chem.* **2007**, *119*, 5584–5605; *Angew. Chem. Int. Ed.* **2007**, *46*, 5488–5508.
[9] T. A. Amador, L. Verotta, D. S. Nunes, E. Elisabetsky, *Planta Med.* **2000**, *66*, 770–772.
[10] T. A. Amador, L. Verotta, D. S. Nunes, E. Elisabetsky, *Phytomedicine* **2001**, *8*, 202–206.
[11] J. M. Schuller, G. Zocher, M. Liebhold, X. Xie, M. Stahl, S. M. Li, T. Stehle, *J. Mol. Biol.* **2012**, *422*, 87–99.
[12] J. Ma, Z. Wang, H. Huang, M. Luo, D. Zuo, B. Wang, A. Sun, Y. Q. Cheng, C. Zhang, J. Ju, *Angew. Chem.* **2011**, *123*, 7943–7948; *Angew. Chem. Int. Ed.* **2011**, *50*, 7797–7802.
[13] J. Zhang, C. Marcin, M. A. Shifflet, P. Salmon, T. Brix, R. Greasham, B. Buckland, M. Chartrain, *Appl. Microbiol. Biotechnol.* **1996**, *44*, 568–575.
[14] T. Yoshida, T. Nagasawa, *Appl. Microbiol. Biotechnol.* **2003**, *62*, 21–26.
[15] M. Berger, J. A. Gray, B. L. Roth, *Annu. Rev. Med.* **2009**, *60*, 355–366.
[16] G. Tosini, K. Ye, P. M. Iuvone, *Neuroscientist* **2012**, *18*, 645–653.
[17] J. D. Rudolf, H. Wang, C. D. Poulter, *J. Am. Chem. Soc.* **2013**, *135*, 1895–1902.

