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Mutasynthesis of Physostigmines in *Myxococcus xanthus*

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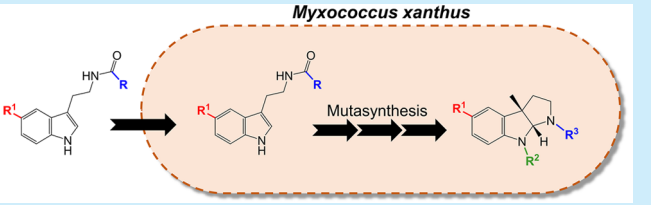
ABSTRACT: The alkaloid physostigmine is an approved anticholinergic drug and an important lead structure for the development of novel therapeutics. Using a complementary approach that merged chemical synthesis with pathway refactoring, we produced a series of physostigmine analogues with altered specificity and toxicity profiles in the heterologous host *Myxococcus xanthus*. The compounds that were generated by applying a simple feeding strategy include the promising drug candidate phenserine, which was previously accessible only by total synthesis.



Physostigmine is a potent parasympathomimetic alkaloid that is medically used for the treatment of glaucoma and cholinergic poisoning.[1] The narrow therapeutic window of this drug as well as its short duration of action have stimulated the search for derivatives with better toxicity and pharmacokinetic profiles that could be administered systemically, for example, for the treatment of Alzheimer's dementia (AD) or other diseases.[2] Therefore, the generation of physostigmine analogues has attracted considerable attention over the years.[2] From a chemical perspective, the asymmetric synthesis of the pyrroloindoline moiety of physostigmine is of particular interest, and it has been achieved, among others, from various oxindole derivatives featuring a C3-quaternary stereogenic center.[3] The preparation of these chiral oxindole precursors typically involves multiple steps and requires precious metal catalysts or toxic reagents.[4] Nature generates the pyrroloindoline scaffold in a much more sustainable way, although the assembly strategy is remarkably similar to chemical synthesis.

The biosynthesis of physostigmine starts from L-tryptophan, which is initially oxidized by a housekeeping enzyme to 5-hydroxy-L-tryptophan and then further processed by the pathway-specific decarboxylase PsmH to give the intermediate serotonin (**1**).[5] A series of concerted enzymatic reactions, of which many are catalyzed by transferases, ultimately give rise to physostigmine (**7**) (Figure 1).[5] Particularly noteworthy in this context is the PsmD-mediated stereospecific methylation of the indole backbone at C3. This enzymatic conversion is assumed to lead to a highly reactive iminium species, which is prone to an intramolecular nucleophilic attack by a side-chain amine, thereby forming the pyrroloindoline skeleton.[5,6]

The total synthesis of natural product derivatives is, in general, laborious due to the repeated use of identical linear transformations and the need to individually optimize reaction conditions. To produce physostigmine analogues, we thus planned to redirect the biosynthetic pathway and to generate the desired compounds by fermentation. For this, a mutasynthetic strategy was pursued. Mutasynthesis involves the genetic disruption of the targeted biosynthesis at an early step. Afterward, the disrupted pathway can be reactivated by feeding synthetic analogues of the missing intermediate.[7] In the present case, we decided to use serotonin (**1**) as an entry point to reroute the biosynthesis, as derivatives of this achiral intermediate are easily synthetically prepared.[8] However, because a genome sequence of the native physostigmine producer, *Streptomyces griseofuscus* NRRL 5324, has not been published, it remained unclear whether the serotonin-forming decarboxylase PsmH could be complemented by a housekeeping enzyme upon its genetic inactivation. To avoid this scenario, we decided to reconstitute the subsequent biosynthetic reactions in a heterologous host, which is not capable of producing **1**. We selected the myxobacterium *Myxococcus xanthus* as a production chassis due to its abundant cellular pool of *S*-adenosyl methionine (SAM),[9] which was expected to promote the biosynthesis.

After the required biosynthesis genes *psmA-F* had been obtained through gene synthesis, they were cloned into a plasmid derived from the *E. coli-Myxococcus* shuttle vector pZJY156.[10,11] For expression, the genes were placed under the control of a σ^{54}-dependent, constitutive myxobacterial promoter.[12] In comparison with σ^{70}, σ^{54} allows a very tight control of gene transcription because the corresponding RNA polymerase-promoter DNA complex is not prone to spontaneous isomerization to form the transcriptionally

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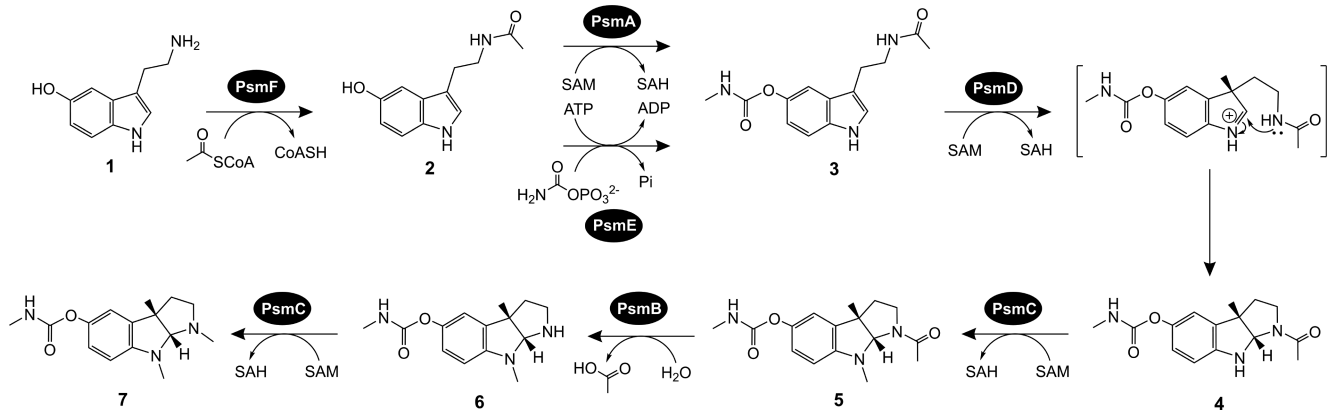

Figure 1. Proposed biosynthesis of physostigmine (7) from serotonin (**1**).[5]

competent open complex.[13] After the transformation of the *M. xanthus* type strain FB with the generated expression plasmid pMEX04, the bacterium was grown in a medium supplemented with **1**. The analysis of this culture by LC-MS did not provide any evidence of the production of physostigmine (7), whereas the mass of the fed substrate was found in high intensity. To test whether this failure was due to cellular uptake issues of **1**, we repeated the feeding study with the synthetically prepared intermediates *N*-acetylserotonin (**2**) and 5-*O*-methylcarbamoyl-*N*-acetylserotonin (**3**). Although both **2** and **3** are more lipophilic than **1**, evidence of a biosynthetic conversion was obtained only with **3** (Figure 2).

Despite the successful reconstitution of physostigmine biosynthesis in *M. xanthus*, only a very low titer had been achieved (<1 mg/L; Figure 2B). The expression plasmid was redesigned to improve the productivity. Because our analyses had corroborated the necessity to feed the carbamoylated intermediate **3**, *psmA*, *psmE*, and *psmF* became dispensable. The removal of these genes reduced the size of the expression plasmid and thereby also its metabolic burden. The remaining biosynthesis genes were fused to the first 45 bp of the endogenous *pilA* gene to improve their expression.[14] (See the Supporting Information.) Furthermore, they were assembled in a multimonocistronic fashion, in which every gene was placed under the control of a discrete promoter. Using the modified expression plasmid, pMEX10, the titer of **7** increased to 4.7 mg/L after the feeding of **3** (Figure 2C). This value could be further raised when *M. xanthus* NM was used as the host. The latter strain is closely related to the original host *M. xanthus* FB but lacks swarming capabilities due to mutations in its motility systems.[15] In *M. xanthus* NM:pMEX10, the average production titer of **7** was determined to be 28.1 mg/L (Figure 2D). Because the substrate **3** had been completely consumed in the initial feeding study of *M. xanthus* NM:pMEX10, we assumed that even higher titers of **7** would be possible, contingent upon the availability of a sufficient precursor. Effectively, a maximal physostigmine titer of 72.0 mg/L could be achieved by increasing the concentration of **3** in the fermentation broth from 40 to 200 mg/L.

With an *M. xanthus* strain producing sufficient amounts of **7** for further functionalization at our disposal, the structural boundaries for the *in vivo* production of analogues could be explored. We first probed the replacement of the methylcarbamoyl moiety in **7** by more lipophilic carbamoyl residues, which is a proven strategy to improve both the duration of

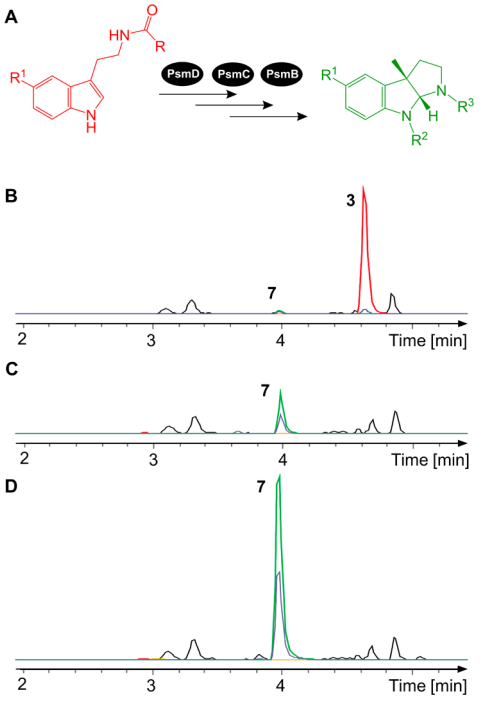

Figure 2. (A) Biosynthesis of physostigmine (**7**: R^1 = $OCONHCH_3$, R^2, R^3 = CH_3) or physostigmine analogues in the generated *M. xanthus* strains, depending on the feeding of synthetically prepared precursor molecules (marked in red, e.g., **3**: R^1 = $OCONHCH_3$, R = CH_3). (B–D) Total ion chromatograms of raw extracts from *M. xanthus* FB:pMEX04 (B), *M. xanthus* FB:pMEX10 (C), and *M. xanthus* NM:pMEX10 (D) after the feeding of **3**. Red: Extracted ion chromatogram (EIC) of **3** (*m*/*z* 276.13). Green: EIC of physostigmine (**7**) (*m*/*z* 276.17). The identities of **3** and **7** were confirmed by comparison with external standards.

action and the toxicity profile of this cholinesterase inhibitor.[16] The synthetic substrate 5-*O*-ethylcarbamoyl-*N*-acetylserotonin (**3a**), which is closely related to **3**, was readily converted by the reconstituted biosynthetic machinery and gave the derivative **7a** with a good titer (9.5 mg/L). Moreover, we isolated the analogue 8-desmethyl-ethyl-physostigmine (**8a**) with a titer of 19.0 mg/L. We assume that **8a** is a biosynthetic precursor of **7a** and that the sequence of biosynthetic reactions in *M. xanthus* deviates from the original proposal displayed in Figure 1.[5] However, the possibility that **7a** is metabolized to **8a** in the heterologous host cannot be completely ruled out, even though a feeding experiment with **7a** did not support this scenario.

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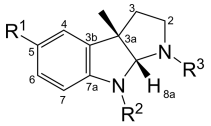
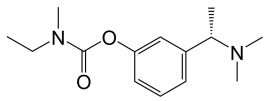
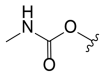
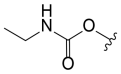
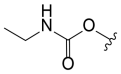
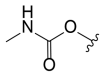
Table 1. IC_{50} Values of Physostigmines for the Inhibitory Effects on AChE, BChE, and L6 Cells





Compound	R^1	R^2	R^3	AChE IC_{50} [nM]	BChE IC_{50} [nM]	Selectivity ratio[a]	Cytotoxicity IC_{50} [μM]
rivastigmine				36059.2	1031.0	35.0	n.d.[b]
physostigmine (7)		CH_3	CH_3	127.9	122.5	1.0	139.8
7a		CH_3	CH_3	1559.2	158.1	9.9	163.5
8a		H	CH_3	1095.2	126.1	8.7	n.d.
7c	F	CH_3	CH_3	> 10^{10}	34435.0	n.d.	> 300
7d	Cl	CH_3	CH_3	> 10^{10}	> 10^5	n.d.	> 300
7e	Br	CH_3	CH_3	> 10^{10}	> 10^5	n.d.	> 300
7f		H		116.8	5.7	20.5	> 300
podophyllotoxin				n.d.	n.d.	n.d.	0.01

[a]Selectivity was calculated from the ratio AChE IC_{50}/BChE IC_{50}. [b]Not determined.

Next, we evaluated the production of the phenylcarbamate derivative of physostigmine, which is known under the name phenserine (**7b**) and represents a promising drug candidate for the treatment of AD.[16b,17] Upon the feeding of 5-*O*-phenylcarbamoyl-*N*-acetylserotonin (**3b**) to a culture of *M. xanthus* NM:pMEX10, a new metabolite with a mass corresponding to **7b** was detected. The identity of this compound was verified by chromatography with a commercial standard of **7b** and LC-MS/MS analysis (Figure S1). Although the titer of the fermentation-derived **7b** (<1 mg/L) was low in comparison with **7** or **7a**, this result was still very encouraging regarding the plasticity of the physostigmine pathway.

In the following studies, we thus attempted to entirely replace the carbamate moiety of **7** with halogen atoms. The substitution of this pharmacophore seemed worthwhile despite the anticipated loss of anticholinergic properties because it would provide analogues that would be potentially useful as analgesics or antiplasmodial agents.[18] Furthermore, the introduction of chlorine or bromine atoms into natural products facilitates subsequent chemical derivatizations, such as Pd-catalyzed Suzuki−Miyaura and related cross-coupling reactions.[19] For the halogen incorporation, *M. xanthus* NM:pMEX10 was fed with *N*-acetyltryptamines featuring a fluoro, chloro, or bromo substituent at C5. To our delight, all three tested substrates were successfully converted into correspondingly halogenated physostigmine analogues. The titer of the fluorinated analogue **7c** (2 mg/L) was lower than the chlorinated **7d** (4.9 mg/L) or brominated physostigmine **7e** (5.6 mg/L). This might indicate a preference for at least one Psm enzyme for large substituents at C5 but could also be due to different cellular uptake rates of the substrates. According to high-performance liquid chromatography (HPLC) analysis, the lipophilicity increases from *N*-acetyl-5-fluorotryptamine via *N*-acetyl-5-chlorotryptamine to *N*-acetyl-5-bromotryptamine.

Except for C5, the methyl group at N1 of **7** was identified as another possible target for mutasynthetic modifications. The *N*-butanoylated analogue of 5-*O*-methylcarbamoyl-serotonin (**3f**), which had been added to cultures of *M. xanthus* NM:pMEX10, was converted to the pyrroloindoline **7f** (4.7 mg/L). An inspection of the ion chromatogram from the corresponding culture extract suggested the presence of a putatively methylated derivative of **7f** (*m*/*z* 332.19; Figure S40), but the identity of this compound could not be clarified due to its low titer. Because no further physostigmine-related compounds were observed after the feeding of **3f**, we assume that the PsmD product **7f** is not efficiently processed by PsmB or PsmC.

The produced derivatives **7a**, **8a**, and **7c**−**7f** were tested together with **7** for their inhibitory activities against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Although AChE is the major enzyme for regulating the acetylcholine level in healthy humans, BChE can compensate its function.[20] In patients suffering from AD, the activity ratio of both enzymes is strongly shifted to BChE, which is why the latter represents an important pharmacological target in the treatment of this disease.[21] Many AD drugs actually show a preference for BChE over AChE inhibition. This is illustrated by the approved drug rivastigmine,[22] which served as a positive control in our study (Table 1).

A comparison of rivastigmine with physostigmine (**7**), **7a**, **8a**, and **7f** revealed that the physostigmines are more potent

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inhibitors of AChE and BChE, as documented by their lower IC_{50} values. On the contrary, the tested physostigmines were less selective regarding the inhibition of the two cholinesterases. Whereas the natural product **7** did not discriminate between AChE and BChE, the selectivity ratio could be improved in the mutasynthetic analogues **7a**, **8a**, and **7f**. In the case of **7a** and **8a**, the gain of selectivity was at the expense of the activity. In stark contrast, the new derivative **7f** not only showed a significantly improved selectivity ratio but also improved bioactivity. Notably, this compound also exhibited reduced toxicity against the myoblast cell line L6 in comparison with **7**.

The halogenated physostigmine analogues **7c**−**7e**, which lack a carbamoyl moiety, were found to be inactive toward AChE and BChE, as expected. Because previous studies had suggested that **7** and its degradation products eseroline and rubreserine might inhibit *Plasmodium falciparum*,[23] we also investigated the antiprotozoal activities of the generated compounds (Table S1). Although the halogenated derivatives showed only moderate activity in the respective assays, it became obvious, once more, that the activity profile of **7** can be tailored by mutasynthetic modification.

In summary, we refactored and reconstituted the late steps of the physostigmine pathway in the host bacterium *Myxococcus xanthus*. This approach, in combination with the feeding of precursor analogues, enabled the mutasynthetic production of various physostigmine derivatives, including the drug candidate phenserine which, until then, had been accessible only by total synthesis. Of particular note is the generation of a previously not described physostigmine analogue featuring a butanoyl moiety at N1. The corresponding compound possesses an improved selectivity and toxicity profile in comparison with the parental natural product, which underlines the usefulness of mutasynthesis in drug development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c02374.

Details of the cloning strategy, expression and mutasynthesis experiments, preparation of the substrates, chemical characterization of the generated physostigmine derivatives, and bioactivity testing (PDF)

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Notes

The authors declare no competing financial interest.

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