Fermentative production of tetrahydropapaverine and its derivatives using *Escherichia coli*.

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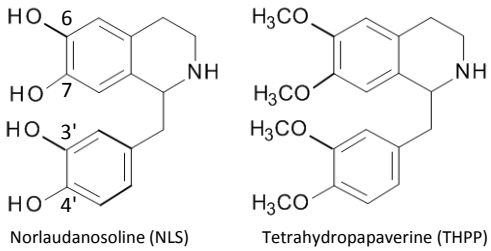
Research aims

Benzylisoquinoline alkaloids (BIAs) have powerful physiological effects and are widely used as pharmaceuticals. They include drugs such as morphine (analgesics), berberine (stegnotic), and magnoflorine (anticancer). BIAs have complex structures with asymmetric carbons, making chemical synthesis of BIAs particularly difficult. Recently, the potential for microbial production of BIAs has attracted attention.

The antispasmodic drug atracurium is listed in the World Health Organization's (WHO) Model List of Essential Medicines, although it is relatively expensive compared to other listed drugs. Therefore, it is important to develop a cost-effective method for atracurium production. Atracurium is manufactured from tetrahydropapaverine (THPP), itself a type of BIA. To potentially develop a new supply of inexpensive atracurium, we attempted to construct a microbial production system for THPP. Intermediates of THPP production, such as methylated norlaudanosolines (NLSs), are also important compounds that can be used or converted into pharmaceuticals. An additional aim of the project is to catalogue a methylated NLS library, in concert with developing THPP production. This research will not only contribute to the supply of inexpensive atracurium, but also affect drug discovery using BIAs.



Norlaudanosoline (NLS) Tetrahydropapaverine (THPP)

Fig.1 Chemical formulae of NLS and THPP

Methods

We have previously shown BIA production from simple carbon sources such as glucose and glycerol, using *Escherichia coli* (*E. coli*). Tools developed by us during this research will be applied to THPP production. THPP can be obtained from NLS by methylation of four hydroxyl groups (Fig. 1). Four enzymes were used to perform the methylation; coclaurine 6-*O*-methyltransferase (6OMT) from *Coptis japonica* (*C. japonica*) for 6-*O* methylation, reticuline 7-*O*-methyltransferase (7OMT) from *Eschscholzia californica* for 7-*O* methylation, human catechol *O*-methyltransferase (COMT) for 3′-*O* methylation, and 3′-hydroxy-*N*-methylcoclaurine methyltransferase (4'OMT) from *C. japonica* for 4′-*O* methylation.

After confirming expression of these enzymes in *E. coli*, the properties of each enzyme were analyzed *in vitro* with crude extracts. Subsequently, the possible reaction orders suitable for THPP production were investigated. Finally, THPP production was attempted using multiple strains and a stepwise culture method to control the order of reactions.

Results

1. Synthesis of various methylated NLSs using different enzyme combinations

In total, there are fifteen possible combinations to mix the four enzymes; this creates fifteen different methylated NLSs. Initially, products were analyzed in the reaction mixtures containing combinations of enzymes. Twelve of the fifteen NLSs could be identified in these mixtures (Table 1). 3′, 4′-*O*-dimethyl NLS was not detected in any reactions. It was not possible to discriminate between 6,3′,4′- and 7,3′,4′-*O*-trimethylated NLSs because of the fact that a suitable LC-MS protocol to separate these NLS derivatives could not be determined. Surprisingly, THPP could be detected in the reaction mixture without 6OMT. Subsequently, it was shown that 4′OMT possesses some 6OMT activity and THPP can be

Table 1 Products when NLS was reacted with various combinations of methyltransferases

Methylated positions of products	Combination of methyltransferases															
	6	4'	C	7	6+4'	6+C	6+7	4'+C	4'+7	C+7	6+4'+C	6+4'+7	6+C+7	4'+C+7	6+4'+C+7
6	○	○	○		○	○	○	○	○	○	○	○	○	○	○
7			○					○		○				○	
3'			○					○		○				○	
4'		○							○					○	
67						○	○		○		○	○	○		○
63'			○			○		○		○	○		○	○	○
64'		○	○		○	○		○	○	○	○	○	○	○	○
73'			○					○		○				○	
74'			○					○		○				○	
3'4'															
673'						○		○		○	○		○	○	○
674'						○		○	○	○	○	○	○	○	○
63'4'								○			○			○	○
73'4'															
673'4'														○	○

produced even without 6OMT. As the amount of THPP in the reaction with 6OMT was higher than that without 6OMT, 6OMT was used for THPP production in further experiments. Additionally, the 6OMT-like activity of 4′OMT can produce the R-form of the NLS derivative (*R*)-reticuline, which is a very important intermediate chemical in the production of opiates[1)]. Although this is not directly related to this study, it is an important discovery for BIA production in general.

2. Optimization of reaction order for THPP production

Although THPP could be produced by simultaneous reactions using all four enzymes, productivity was low. The substrate recognition of some methyltransferases is affected by positions of the methoxy group in methylated NLSs. Therefore, optimizing the reaction order will be important for THPP production. When COMT was reacted with NLS as the first reaction, productivities were relatively high (Fig. 2A). Furthermore, after COMT reaction, subsequent reactions still occurred efficiently using the three remaining enzymes mixed at the same time (Fig. 2B). These data suggest that efficient THPP production could be developed using a 2-step culture method, with an initial step using a strain that expresses COMT, and a second strain that simultaneously expresses 4′OMT-6OMT-7OMT .

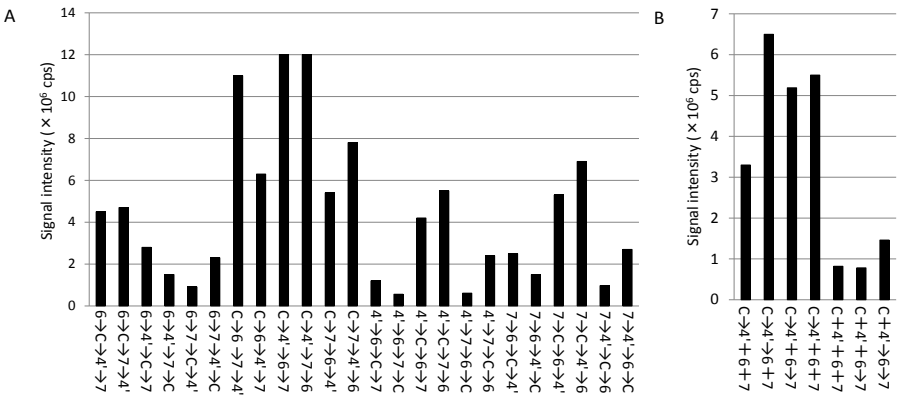


Fig.2 Suitable reaction order for THPP synthesis in in vitro
A. THPP synthesis by four-step reaction. B. Detail investigation of C→4'→7→6. →and＋ indicate stepwise and simultaneous, respectively.

3. THPP production by 2-step culture

The COMT gene was introduced into an NLS production strain. The strain was cultured in a glucose-containing medium. After incubation, methylated NLSs could be detected. THPP production was conducted by adding these NLSs in solution to the culture of the 4'OMT-6OMT-7OMT expressing strain. Unfortunately, THPP was not detected in the second culture. However, trimethylated-NLSs were detected, suggesting that part of the pathway is functional in the second strain.

Conclusions

In this study, reaction order was shown to be important for THPP production. How and which enzymes determine the importance of the reaction order requires further investigation. Although THPP was successfully synthesized using *in vitro* experiments, it was not replicated *in vivo*. This study suggests that COMT reacts with dopamine prior to NLS synthesis, reducing NLS productivity. Furthermore, dimethyl-NLSs could be detected in the second culture step, indicating that activities of 4′OMT, 6OMT, and/or 7OMT may be reduced in comparison to the *in vitro* experiments. Fully understanding why this reduced activity is observed will be important for further development of THPP production systems.

References

1) Nakagawa A, Matsumura E, Koyanagi T, Katayama T, Kawano N, Yoshimatsu K, Yamamoto K, Kumagai H, Sato F, and Minami H (2016) Total biosynthesis of opiates by stepwise fermentation using engineered *Escherichia coli* *Nat Commun* **7**:10390