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Functional characterization of recombinant hyoscyamine 6β-hydroxylase from *Atropa belladonna*

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ABSTRACT

(−)-Hyoscyamine, the enantiomerically pure form of atropine, and its derivative scopolamine are tropane alkaloids that are extensively used in medicine. Hyoscyamine 6β-hydroxylase (H6H, EC 1.14.11.11), a monomeric α-ketoglutarate dependent dioxygenase, converts (−)-hyoscyamine to its 6,7-epoxy derivative, scopolamine, in two sequential steps. In this study, H6H of *Atropa belladonna* (AbH6H) was cloned, heterologously expressed in *Escherichia coli*, purified and characterized. The catalytic efficiency of AbH6H, especially for the second oxidation, was found to be low, and this may be one of the reasons why *Atropa belladonna* produces less scopolamine than other species in the same family. 6,7-Dehydrohyoscyamine, a potential precursor for the last step of epoxidation, was shown not to be an obligatory intermediate in the biosynthesis of scopolamine using purified AbH6H with an in vitro ^{18}O labeling experiment. Moreover, the nitrogen atom in the tropane ring of (−)-hyoscyamine was found to play an important role in substrate recognition.

1. Introduction

Tropane alkaloids comprise a large group of plant secondary metabolites that are considered to be involved in self-defense systems against pathogens, pests and herbivores.[1] Many tropane alkaloids exhibit interesting biological effects and some are used in medicinal applications.[2] (−)-Hyoscyamine and scopolamine ((−)-hyoscine) are two of the most medicinally valuable tropane alkaloids and are produced by members of the family Solanaceae such as *Atropa*, *Anisodus*, *Duboisia*, and *Hyoscyamus*.[3] These two biologically active tropane alkaloids compete with acetylcholine for binding at the muscarinic receptors of the parasympathetic nervous system preventing the transmission of certain nerve impulses, which causes sedative effects.[4] Due to their strong effect on the parasympathetic nervous system, plants from Solanaceae have been employed as traditional medicines to relieve pain dating back to ancient times.[2,5] Atropine (the racemic form of hyoscyamine) and the (+)-enantiomers of these two tropane alkaloids have lower biological activity than the (−)-enantiomers.[4] In addition, scopolamine shows greater pharmacological effects and fewer side effects than (−)-hyoscyamine. Accordingly, scopolamine has been widely used as an anesthetic, mydriatic, antispasmoidic, gastrointestinal sedative, and in the treatment of motion sickness and Parkinson's disease.[2,6]

Biosynthesis of scopolamine begins with methylation of putrescine by putrescine *N*-methyltransferase to afford *N*-methylputrescine, which is incorporated into the pyrrolidine ring of tropinone (Fig. 1). Tropinone is then reduced by tropinone reductase I, followed by condensation with phenylalanine-derived phenyl-lactic acid. Thereafter, rearrangement catalyzed by a mutase yields (−)-hyoscyamine.[1,4] The last two steps in the biosynthesis of scopolamine are catalyzed by hyoscyamine 6β-hydroxylase (H6H, EC 1.14.11.11). H6H first hydroxylates hyoscyamine to form 6β-hydroxyhyoscyamine, followed by the epoxidation of 6β-hydroxyhyoscyamine to produce scopolamine (Fig. 1). Both steps require 2-oxoglutarate, Fe(II), catalase, and ascorbate as cofactors for maximal activity.[7] This bifunctional H6H belongs to the family of 2-oxoglutarate and ferrous iron (2OG/Fe(II)) dependent dioxygenases.[8] Chemical synthesis of scopolamine is difficult and costly, thus the commercial supply of this alkaloid relies on the extraction from plant sources.[6,9] To meet the ever-growing international market for scopolamine, plant metabolic engineering and use of hairy root culture systems are two popular strategies that have been investigated to enhance its production.[10–14]

Some species of Solanaceae have been found to produce more scopolamine than others, potentially due to variable activities of H6H. For example, a perennial herb *Atropa belladonna,* commonly known as 'deadly nightshade', has been reported to contain hyoscyamine as the principal tropane alkaloid, with only small amounts of scopolamine being present (<0.1% dry weight).[15] It has been speculated that the low production of scopolamine in *Atropa belladonna* results from low expression levels of the H6H

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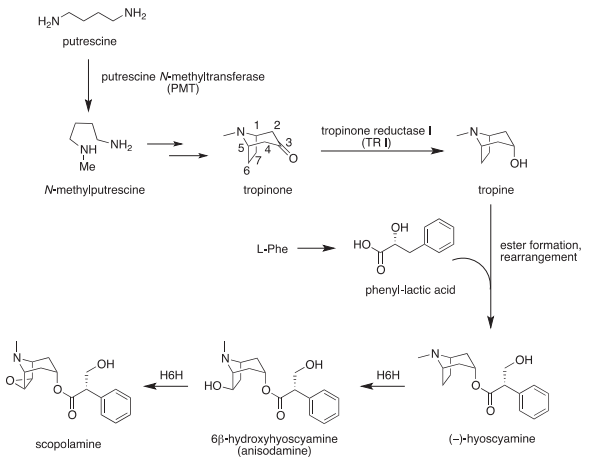


Figure 1. Biosynthetic pathway of (−)-hyoscyamine and scopolamine.

gene, or a less efficient H6H enzyme, or both.[16] However, *Hyoscyamus niger*, a close relative of *Atropa belladonna*, is a scopolamine-rich plant.[17] Scopolamine content was found to be increased fivefold in *A. belladonna* hairy roots transformed with the H6H gene of *Hyoscyamus niger* (*Hn*H6H).[18] Moreover, the transcript level of H6H in *Atropa belladonna* and the production of scopolamine can be elevated by exogenous chromium in the culture.[19] However, the catalytic efficiency of H6H from *Atropa belladonna* has not been reported. In this study, we describe the cloning, heterologous expression and purification of H6H of *Atropa belladonna* (*Ab*H6H). The activity of the recombinant protein was examined and analogues were synthesized and tested for substrate conversion and enzyme inhibition. Additionally, an ^{18}O-labeled 6β-hydroxyhyoscyamine was fed to purified *Ab*H6H to demonstrate that dehydration is not an obligatory biosynthetic step for the biosynthesis of scopolamine.

2. Results and discussion

2.1. Expression and purification of AbH6H

Expression plasmid pQE60 was used to overexpress *Ab*H6H (GenBank accession number AB017153) with a C-terminal hexahistidine tag in *Escherichia coli* strain JM109 cells. Cell lysate from bacterial cultures was subjected to purification by nickel-chelate affinity chromatography. Protein fractions isolated from cell lysate were examined by SDS–PAGE (Fig. 2). The fractions (lane 7 and 8) containing pure *Ab*H6H were combined, dialyzed against phosphate buffer (20 mM, pH 7.5), concentrated and stored at −20 °C. The mass of the purified *Ab*H6H detected by high resolution ESI mass spectrometry is 39238 Da. This is 131 Da less than the calculated molecular weight of 39369 Da, which implies loss of the first amino acid methionine (fMet) of *Ab*H6H. The fMet residue is often cleaved by Met aminopeptidase when the second residue is a non-bulky amino acid.[20]

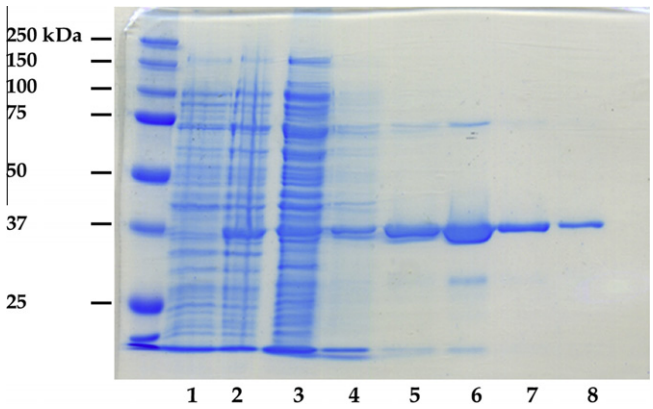


Figure 2. Purification of *Ab*H6H as analyzed by coomassie blue stained SDS–PAGE. Lane 1: pre-induction cell lysate; lane 2: post-induction lysate; lane 3: flow through; lane 4: lysis buffer wash; lane 5: elution with lysis buffer containing 40 mM imidazole; lane 6, 7: elution with lysis buffer containing 60 mM imidazole; lane 8: elution with lysis buffer containing 80 mM imidazole.

2.2. Biochemical characterization of AbH6H

To study the kinetics of *Ab*H6H, a HPLC method was developed to detect the formation of products from the enzymatic reaction. After incubation of (−)-hyoscyamine with *Ab*H6H (0.61 μM) and other cofactors for 2 h, followed by EtOAc extraction and concentration in vacuo, the resulting residue was analyzed by HPLC. Retention times of 6β-hydroxyhyoscyamine and (−)-hyoscyamine are 14.2 and 17.4 min, respectively (Fig. 3). The HPLC fractions with retention time of 14.2 min were pooled and extracted with EtOAc to give 6β-hydroxyhyoscyamine, which was characterized by ^{1}H NMR, ESI-HRMS and optical rotation. When the biosynthetic 6β-hydroxyhyoscyamine served as substrate, scopolamine was produced with retention time of 19.1 min. As long as the reaction mixture contained hyoscyamine, only 6β-hydroxyhyoscyamine was detected. No product was detected in the control mixture without *Ab*H6H. The recombinant *Ab*H6H is stable in 20 mM phosphate buffer at −20 °C, and it loses approximately 20% of activity after 2 months. However, after 4 days at 4 °C, more than 50% of activity was lost. The optimal temperature and pH for the enzymatic activity of this recombinant *Ab*H6H are 34 °C and 7.6, respectively.

During the first hour of the hydroxylation reaction, 6β-hydroxyhyoscyamine was produced nearly linearly with respect to time. Therefore, the first hour time period was chosen to study the enzyme kinetics. The K_m and V_{max} values for hyoscyamine under optimal conditions were 52.1 ± 11.5 μM and 1.17 ± 0.08 nmol s^{-1} mg^{-1}, respectively. The K_m value of *Ab*H6H is higher than that of the enzyme from *Hyoscyamus niger Hn*H6H (35 μM) and from *Anisodus tanguticus At*H6H(15.1 ± 0.3 μM); this implies that *Ab*H6H has lower affinity for the substrate than *Hn*H6H and *At*H6H (Table 1). The V_{max} value of *Ab*H6H is considerably smaller than the one of *Hn*H6H (3.28 nmol s^{-1} mg^{-1}) and *At*H6H (11.1 ± 0.5 nmol s^{-1} mg^{-1}).[5,7]

Additionally, the K_m and V_{max} values for 6β-hydroxyhyoscyamine were 84.4 ± 15.8 μM and (3.36 ± 0.14) × 10^{-3} nmol s^{-1} mg^{-1}, respectively. The V_{max} value for 6β-hydroxyhyoscyamine is approximately 0.1% of the V_{max} value for hyoscyamine, which suggests that the epoxidation step is much slower than the hydroxylation step. Comparison of the K_m and V_{max} values between *Ab*H6H and *At*H6H indicates that *Ab*H6H possesses much lower affinity to 6β-hydroxyhyoscyamine in addition to much lower catalytic efficiency.

To investigate whether the position of the His_6-tag affects the activity of H6H, the same assay was used to determine kinetic constants for *Ab*H6H with N-terminal hexahistidine tag. We observed that the enzyme with His_6-tag at either the N- or C-terminus has approximately the same activity.

2.3. ^{18}O-Labeling experiment

Previous studies have shown that the 7β-hydrogen atom of 6β-hydroxyhyoscyamine is lost during the epoxidation catalyzed by H6H.[21,22] Furthermore, 6,7-dehydrohyoscyamine was found to be converted to scopolamine. Based on this, 6,7-dehydrohyoscyamine was proposed to be an intermediate in the epoxidation step in the

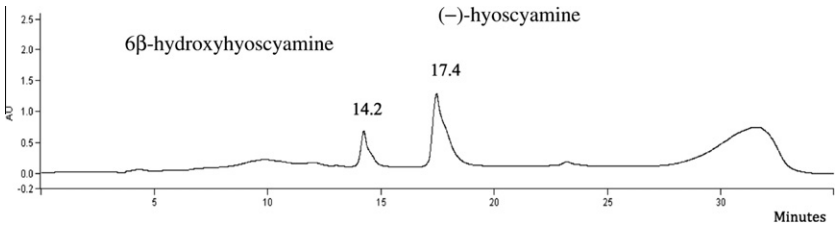


Figure 3. HPLC analysis of *Ab*H6H assay mixture. 6β-Hydroxyhyoscyamine and (−)-hyoscyamine have retention times of 14.2 and 17.4 min, respectively.

Table 1
K_m and V_{max} values of H6H from different species

Species	*Atropa belladonna*	*Hyoscyamus niger*[7]	*Anisodus tanguticus*[5]
K_m (μM) for hyoscyamine	52.1 ± 11.5	35	15.1 ± 0.3
K_m (μM) for 6β-hydroxyhyoscyamine	84.4 ± 15.8	N/A[a]	17.0 ± 0.2
V_{max} (nmol s^{-1} mg^{-1}) for hyoscyamine	1.17 ± 0.08	3.28	11.1 ± 0.5
V_{max} (nmol s^{-1} mg^{-1}) for 6β-hydroxyhyoscyamine	(3.36 ± 0.14) × 10^{-3}	N/A[a]	4.2 ± 0.5

[a] N/A means the value is not reported.

biosynthesis of scopolamine.[23] Subsequently, ^{18}O-labeling experiments using *Duboisia* shoot cultures and partially purified H6H were reported in which ^{18}O from 6-hydroxyhyoscyamine was unexpectedly found to be retained in the epoxide functionality of scopolamine.[21,24] However, this in vivo approach and partially purified enzyme preparations are subject to many uncontrollable variables. Hence we decided to reexamine the ^{18}O-labeling experiment using the pure recombinant *Ab*H6H enzyme. The ^{18}O-labeling experiment illustrated in Figure 4 can be used to determine whether intermediacy of 6,7-dehydrohyoscyamine is obligatory.

First, [6-^{18}O]β-hydroxyhyoscyamine was obtained from the incubation of *Ab*H6H and hyoscyamine under an atmosphere of $^{18}O_2$ and fully purified. Subsequently, the [6-^{18}O]β-hydroxyhyoscyamine was added to the *Ab*H6H enzyme and incubated for 4 h under a natural atmosphere having only $^{16}O_2$. This was then extracted with EtOAc. The FTICR-ESI-MS analysis of the EtOAc extract shows that ^{18}O-labeled scopolamine and [6-^{16}O]β-hydroxyhyoscyamine were baseline separated with *m/z* values of 306.1585 and 306.1699, respectively (Fig. 5). The ratio of mass/charge peaks between [6-^{16}O]β-hydroxyhyoscyamine and [6-^{18}O]β-hydroxyhyoscyamine is 0.205, and the ratio of mass/charge peaks between scopolamine and ^{18}O-labeled scopolamine is 0.264. These two ratios are close enough to indicate ^{18}O in the 6β-hydroxyl group of 6β-hydroxyhyoscyamine was retained in the epoxide functionality of scopolamine after biotransformation by pure *Ab*H6H.[24] Furthermore, ^{18}O-labeled scopolamine was not detected in a control experiment containing 6β-hydroxyhyoscyamine performed under $^{18}O_2$. These results suggest that the dehydration step, if it occurs at all, is not the predominant route to scopolamine.

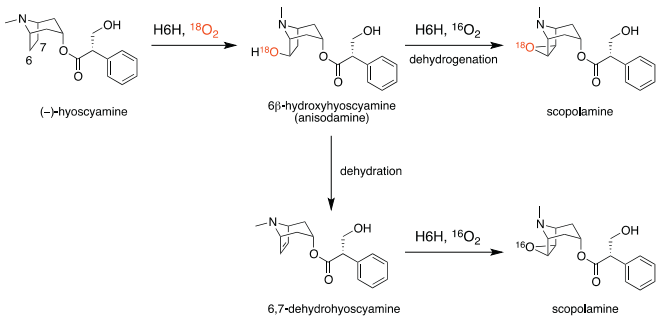


Figure 4. Two possible pathways for the epoxidation in the biosynthesis of scopolamine.

It also demonstrates that the last step of scopolamine biosynthesis is likely to involve direct oxidative closure of the 6-hydroxyl group on to the 7-position catalyzed by the same dioxygenase, *Ab*H6H.[22,24] However, in intact plants, the dehydration step could potentially be catalyzed by other enzymes.

Although the detailed mechanism of epoxidation of 6β-hydroxyhyoscyamine still remains undetermined, a general hydroxylation mechanism for 2-oxoglutarate (2OG)/Fe(II) dependent oxygenases has previously been proposed.[25] Several crystallographic studies indicate that the active site of this type of enzyme binds to Fe(II) by coordinating with two His residues, an Asp/Glu residue, and 2OG chelates Fe(II) in a bidentate manner (Fig. 6).[25,26] Presumably, molecular oxygen first binds to the Fe(II) and then attacks on the 2-oxoglutarate to form a very active bicyclic intermediate. A potent Fe(IV)═O species is formed after the collapse of the bicyclic moiety with the release of CO_2. The reaction between Fe(II) and O_2 triggered by substrate binding has been shown from studies of the chlorinating enzyme SyrB2.[27] This substrate triggering is thought to protect the high-valent iron species from side reactions.[27] Nevertheless, in some cases, oxygen binds Fe(II) prior to the target substrate, which indicates some variations during the early stage of the catalysis.[26] The existence of a reactive ferryl intermediate has been supported by spectroscopic studies on taurine dioxygenase (TauD), prolyl-4-hydroxylase, and SyrB2.[28,29] However, whether the potent oxidizing moiety is always a high-valent ferryl iron still remains uncertain.[26]

In the case of H6H, the hydroxylated product, 6β-hydroxyhyoscyamine, results from rebound of the substrate radical and the hydroxyl group on the ferryl moiety. Clearly, this product can be released from the enzyme, and this is predominantly what is observed with *Ab*H6H. The enzyme can then reload for another catalytic cycle. However, in the absence of the preferred substrate, (−)-hyoscyamine, 6β-hydroxyhyoscyamine can bind and be transformed as a substrate. We postulate that a carbon-based radical may be formed on C-7 of 6β-hydroxyhyoscyamine by abstraction of the β-hydrogen from that position by the Fe(IV)═O species. Epoxidation could potentially be achieved by interaction of the hydroxyl oxygen and C-7 radical with concomitant oxidation (Fig. 6).

2.4. Substrate specificity test

Based on previous research on *Hn*H6H, it has been revealed that H6H has low specificity in terms of the alkaloid substrate. The *N*-methyl group of the tropane ring and the hydroxymethylene group

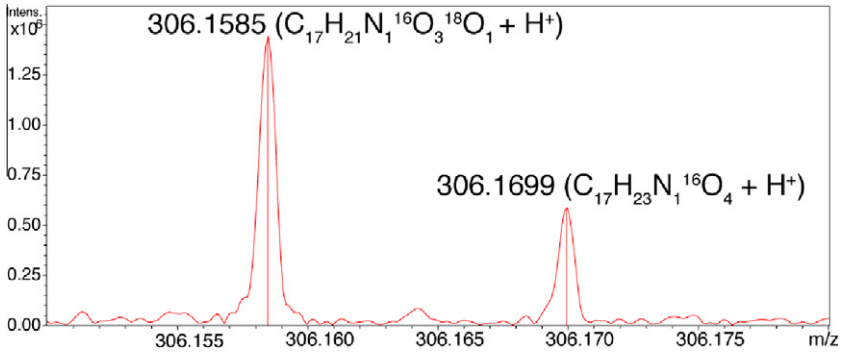


Figure 5. FTICR-ESI-MS spectrum of ^{18}O-labeled scopolamine and [6-^{16}O]β-hydroxyhyoscyamine.

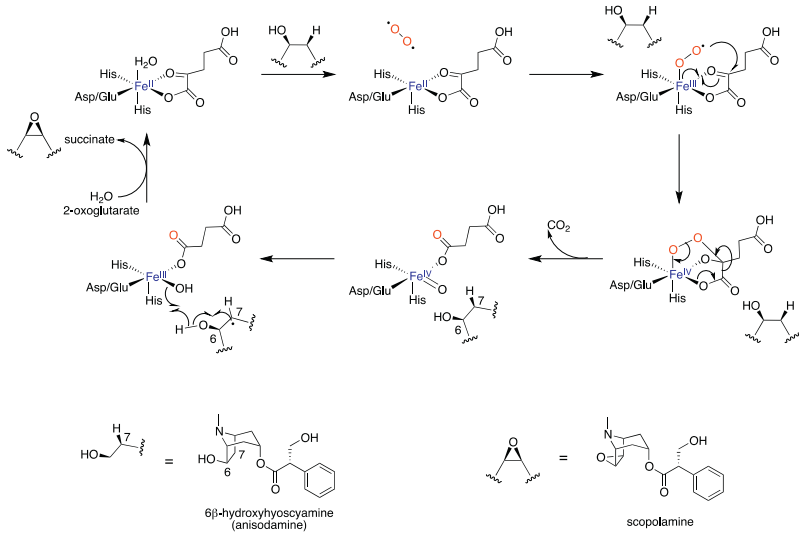


Figure 6. Proposed mechanism of epoxidation catalyzed by H6H.

of the tropic acid are not essential for the hydroxylation step.[7] Still, the enantiomer (+)-hyoscyamine is not a substrate nor do (+)-hyoscyamine and scopolamine inhibit enzyme activity.[7] We examined a series of analogues (Fig. 7) to determine whether they may be converted as substrates by *Ab*H6H or whether they inhibit enzyme activity.

To synthesize analogues **1**, **2**, **5** and **6** (Fig. 7), a general DCC/DMAP coupling method was utilized to couple the corresponding carboxylic acid and alcohol.[30] The route to analogue **3** is shown in scheme 1. Analogue **7** was synthesized from scopolamine.[31] Analogue **4** is commercially available.

Testing results of analogues are summarized in Table 2, and will be discussed below. After addition of analogues **1**, **2**, **3**, **4** to *Ab*H6H, no *m/z* values corresponding to hydroxylated products were found by ESI-MS. Additionally, analogues **1**, **2**, and **3** did not show an inhibitory effect on enzyme activity, but methylated scopolamine (**4**) showed very weak inhibition (approximately 7%) at 0.1 mM. Although an earlier study revealed that the *N*-methyl group of tropane ring is not important for the hydroxylation reaction,[7] when

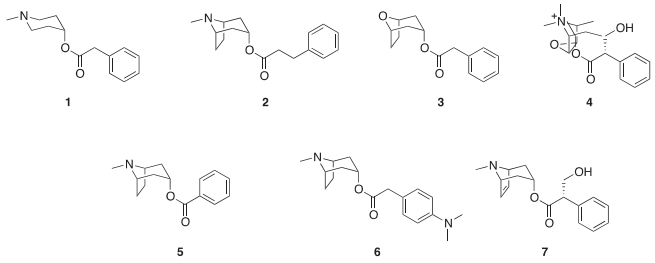
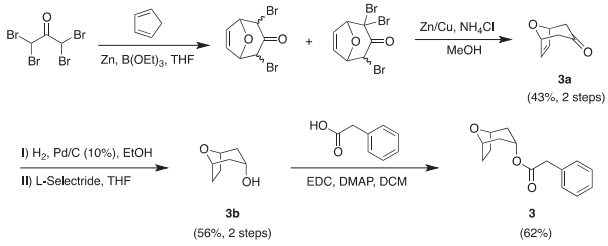


Figure 7. Analogues of hyoscyamine



Scheme 1. Synthesis of analogue 3.

Table 2
Summary of results from testing of analogues **1**–**7**

Analogues	1	2	3	4	5	6	7
Substrate	–[a]	–	–	–	+[b]	+	+
Inhibition (0.1 mM)	–	–	–	7	N/A[c]	N/A	N/A

[a] A (–) sign means that there was no inhibition/activity observed.
[b] A (+) sign means that analogues were converted to products.
[c] N/A: not determined.

nitrogen is replaced by oxygen as shown in **3**, neither hydroxylation nor inhibition were observed. This indicates that **3** does not bind to the active site. In the physiological environment of plants, the nitrogen atom in the tertiary amine functionality of the tropane ring will be protonated. The positively charged nitrogen atom may interact with some residues in the active site to aid in enzyme–substrate binding. However, replacing nitrogen by oxygen in analogue **3** loses the benefit from the positive charge, which may lead to no binding between the enzyme and substrate. Interestingly, analogue **1** has no inhibition effect on enzyme activity. This indicates that the bicyclic skeleton of the tropane ring is essential for substrate binding. Compound **2** has one additional carbon between the ester and the phenyl ring compared to hyoscyamine. The length of this analogue may prevent its fit into the active site of *Ab*H6H.

Compound **7** was found to be converted to scopolamine by the enzyme with a conversion rate approximately 20% higher than the hydroxylation of (−)-hyoscyamine. This result is expected as epoxidation of an olefin is known to be faster than the hydroxylation of an unactivated carbon.[7] The expected *m/z* values of hydroxylated products converted from **5** and **6** by the enzyme were observed by ESI-MS. However, the conversion rates of **5** and **6** were so low that quantification could not be achieved by HPLC. These results indicate that the shorter tether between the phenyl and ester group can be tolerated, albeit with low conversion rates. Furthermore, the presence of a dimethyl amino group at the para position of phenyl ring limits the extent of hydroxylation to a miniscule amount.

3. Conclusions

*Ab*H6H has been cloned, expressed in *E. coli* and fully purified using a modified assay. The kinetic parameters of this enzyme have been characterized, and it was found that the catalytic efficiency, especially for the second step, is much lower than both *Hn*H6H and *At*H6H. This might be one of the reasons why *Atropa belladonna* produces less scopolamine than *Hyoscyamus niger* and *Anisodus tanguticus*. At physiological pH, the amine moiety in the tropane ring of hyoscyamine will be protonated, and the positive charge on the nitrogen atom appears to play an important role in substrate recognition. This idea is supported by failure to detect conversion or inhibition by the oxygen analogue **3**. However, benzoyl tropine (**5**) and 4-(dimethylamino)phenylacetyl tropine (**6**) can be hydroxylated by *Ab*H6H with a low conversion rate. Hydroxylation of 3-phenylpropanoyl tropine (**2**) was not detected. The ^{18}O labeling experiments with purified *Ab*H6H show that dehydration of 6β-hydroxyhyoscyamine is at best a very minor route to scopolamine, and is probably not involved at all in the biosynthetic pathway. Instead, it seems likely that the epoxidation is be achieved by iron-oxo oxidation at C-7 of β-hydroxyhyoscyamine followed by interaction of the hydroxyl at C-6.

4. Experimental protocols

4.1. Materials and general methods

All results were obtained using C-terminal His_6-tagged *Ab*H6H. $^{18}O_2$ (>98.1 atom%) was purchased from Matheson, USA. All other reagents were purchased from Sigma–Aldrich, Alfa-Aesar or TCI and were used as received, unless otherwise noted. Reactions requiring anhydrous conditions were carried out under an atmosphere of argon, using flame-dried glassware. Anhydrous tetrahydrofuran was distilled under argon over sodium metal, with benzophenone added as an indicator. All other solvents were used as received. Infrared spectra were recorded with an FT-IR Nicolet Magna 750 spectrophotometer and Nic-Plan FT-IR Microscope. ^1H and ^{13}C NMR spectra were recorded with Varian Inova 500 and 600 MHz spectrometers. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) are recorded in Hertz (Hz). High-resolution mass spectra were obtained with an Applied BioSystems Mariner BioSpectrometry Workstation (orthogonal acceleration time-of-flight (TOF) detector, positive and negative ion ESI and APCI) and Kratos MS50G (positive ion EI). The mass of *Ab*H6H was recorded by an Agilent 6220 oaTOF. The mass analysis of ^{18}O labeling experiment was performed on a Bruker 9.4T Apex-Qe FTICR with electrospray ionization. Analytical high performance liquid chromatography (HPLC) were carried out with a Varian ProStar 210, equipped with a dual wavelength detector, a 1 mL Rheodyne manual injector and a Phenomenex Luna C18(2) column (5 μm, 4.6 × 250 mm). All HPLC solvents were filtered with a Millipore filtration system before use.

4.2. Cloning and heterologous expression of AbH6H in *E. coli*

The DNA encoding *Ab*H6H with a C-terminal hexahistidine tag (His_6) was chemically synthesized and purchased from BioBasic Inc. (Ontario, Canada). The codon usage of this gene had been optimized for expression in *E. coli*. The DNA fragment containing *Ab*H6H was designed with *Nco*I and *Hin*dIII restriction sides adjacent to the gene and cloned into *Nco*I and *Hin*dIII restriction sites of the expression vector pQE60 (Qiagen). The resulting plasmid was transformed into *E. coli* JM109 to overexpress *Ab*H6H.

A fresh overnight culture (40 mL) of *E. coli* containing the recombinant plasmid was added to 500 mL of Luria-Bertani media containing ampicillin (150 μg/mL). The culture was grown at 37 °C to an A_{600} of 0.6 with shaking. The culture was then put in an ice bath for 10 min to chill the cells, after which isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM, and culture growth was continued for additional 8 h at 20 °C. Cells were harvested by centrifugation at 8,000×*g* for 20 min at 4 °C.

4.3. Cloning of AbH6H with an N-terminal hexahistidine tag

The *Ab*H6H gene in pQE60 was amplified using forward primer (5′-GCC CCATATGGCTACC CTGGTTTCTAACTGG-3′), reverse primer (5′-CCGATCTGTCGACCTAAGCGTTGATTTTGTACGGTTTAACACC-3′) and *pfuUltra* II fusion HS DNA polymerase (Stratagene) according to manufacturer instructions. The PCR product was purified using a QIAquick PCR purification kit (Qiagen). The resulting DNA was ligated into *Nde*I/*Sal*I sites of the pET28a expression vector (Novagen), creating a fusion with an N-terminal His-tag followed by a thrombin cleavage site. The resulting plasmid was transformed into *E. coli* BL21 (DE3) to overexpress *Ab*H6H

4.4. Purification of recombinant AbH6H protein from *E coli*

All purification steps were performed at 0–4 °C. Approximately 5 g of cell pellets was suspended in 40 mL of buffer A (50 mM sodium phosphate, pH 7.5, 300 mM NaCl, 15 mM imidazole) with lysozyme (1 mg/mL) and DNase I (5 μg/mL). After incubation for 15 min at room temperature followed by 20 min on ice, this cell suspension was lysed by sonication. The cellular debris was removed by centrifugation at 20,000×*g* for 30 min at 4 °C, and the supernatant was loaded onto 1 mL of Ni-NTA resin (Qiagen). 30 mL of buffer A were used to wash out the weakly bound and unbound contaminant proteins. The target protein was eluted by gravity by 7 column volumes using a stepwise increase in imidazole concentration of buffer A (buffer A containing 25, 40, 60, or 80 mM imidazole). Fractions were analyzed by SDS–PAGE. Fractions containing pure *Ab*H6H were collected, dialyzed against phosphate buffer (20 mM sodium phosphate, pH 7.5), concentrated

using Amicon centrifugal filter units with a MWCO of 30 kDa and stored at −20 °C. Protein concentration was determined using absorbance at 280 nm.

4.5. General procedure of AbH6H assay

The hydroxylase activity and epoxidase activity were assayed by measuring the formation of 6β-hydroxyhyoscyamine and scopolamine respectively. The reaction mixture contained: 50 mM Tris/HCl buffer (pH 7.6), 0.4 mM $FeSO_4$, 4 mM sodium ascorbate, 1 mM 2-oxoglutaric acid, 0.2 mM (−)-hyoscyamine hydrobromide or 6β-hydroxyhyoscyamine, 2 mg/mL of catalase (Sigma–Aldrich, cat. no. C9322) and *Ab*H6H.[5,7] In a final volume of 20 mL, the enzyme assay was incubated at 34 °C for 2 h, and then quenched with 300 μL of saturated Na_2CO_3 solution to raise the pH to ~9.5, followed by extraction with 50 mL of EtOAc and drying over Na_2SO_4. After removing EtOAc under reduced pressure, the residue was dissolved in 0.5 mL of diluted HCl (20 mM). The quantification of the alkaloids was performed by HPLC analysis.[32]

Using the previously described *Ab*H6H assay, the K_m and V_{max} of the hydroxylation reaction catalyzed by *Ab*H6H were determined by varying the concentration of (−)-hyoscyamine (0.01, 0.02, 0.04, 0.08, 0.15, 0.25 mM) while keeping the amount of *Ab*H6H constant (0.61 μM) with 1 h incubation time. To test the conversion of analogues, each analogue was added to the assay at a final concentration of 0.2 mM. For analogues that were not recognized as substrates by *Ab*H6H, their inhibitory properties were tested by the same *Ab*H6H assay (containing 0.2 mM (−)-hyoscyamine) with 0.1 mM of each analogue, and HPLC quantification was performed to observe a potential reduction of the production of 6β-hydroxyhyoscyamine.

4.6. HPLC methods

The separation of alkaloids was monitored by UV absorbance at 210 nm. The mobile phase contained solvent A, water with 0.3% phosphoric acid (adjusted pH to 7.4 by addition of TEA), and solvent B, MeOH. The baseline separation was achieved using the following gradient: 0–5 min, 5–38% B; 5–18 min, 38–46% B; 18–22 min, 46% B; 22–25 min, 46–90% B; 25–27 min, 90% B; 27–28 min, 90–5% B; 28–35 min, 5% B. The flow rate was 1 mL/min.

4.7. Isolation of biosynthetic 6β-hydroxyhyoscyamine

A flask of 600 mL of *Ab*H6H assay was prepared according to the general procedure of *Ab*H6H assay, and it was incubated overnight at 34 °C, followed by quench with saturated Na_2CO_3 solution and EtOAc extraction. The organic solvent was dried on Na_2SO_4 and concentrated on a rotary evaporator. The residue was separated by silica gel flash column (DCM/EtOH/NH_4OH, 80:12:3) to afford 3 mg of 6β-hydroxyhyoscyamine. The ^1H NMR (600 MHz, $CDCl_3$) spectrum is consistent with a previous report;[33] δ 7.35 (m, 5H), 5.04 (t, J = 5.4 Hz, 1H), 4.36 (d,, J = 6.3 Hz, 1H), 4.18 (dd, J = 11.1, 8.9 Hz, 1H), 3.82 (ddd,, J = 29.6, 11.1, 5.3 Hz, 2H), 3.13 (s, 1H), 3.05 (s, 1H), 2.52 (s, 3H), 2.35 (m, 2H),1.79 (dd,, J = 13.8, 7.8 Hz, 1H), 1.63 (m, 2H), 1.25 (m, 1H). The optical rotation value ($[\alpha]_D^{23}$) is −9.7° (c = 0.50, $CHCl_3$) and the ESI-HRMS m/z value of calcd for $C_{15}H_{19}NO_2$ is 306.17 [M+H$^+$], found 306.1699.

5. ^{18}O-Labeling experiment

200 mL of the enzyme assay with hyoscyamine was prepared and the reaction system was purged with argon for 30 min. The reaction mixture was kept in a warm water bath (~30 °C). The reaction mixture was flashed with $^{18}O_2$ for 30 min, thereafter, pure *Ab*H6H was added to the system and $^{18}O_2$ was flushed for another 2 h. Then the enzymatic reaction was quenched with saturated Na_2CO_3 solution (3 mL), followed by EtOAc extraction. After removal of the solvent in vacuo, the resulting residue was dissolved in 1.5 mL of HCl (20 mM) and subjected to HPLC separation. [6-^{18}O]β-hydroxyhyoscyamine was isolated from the extraction of HPLC fraction with EtOAc. After feeding [6-^{18}O]β-hydroxyhyoscyamine to the pure *Ab*H6H under a natural atmosphere ($^{16}O_2$) and incubating for 4 h, the enzymatic reaction was quenched. The reaction mixture was extracted by EtOAc, dried over Na_2SO_4 and solvent was removed in vacuo. The resulting residue was dissolved in diluted HCl (20 mM) and further analyzed on a Bruker 9.4T Apex-Qe FTICR with electrospray ionization.

5.1. Chemical synthesis of hyoscyamine analogues

5.1.1. 1-Methylpiperidin-4-yl 2-phenylacetate (1)

N-methyl-4-piperidinol (0.27 g, 2.33 mmol), hydrocinnamic acid (0.39 g, 2.59 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.55 g, 2.86 mmol) and 4-dimethylaminopyridine (0.029 g, 0.26 mmol) were dissolved in DCM (12 mL) and stirred overnight at ambient temperature. The reaction solution was washed with $NaHCO_3$ (2 × 15 mL), H_2O (2 × 15 mL), brine (15 mL), dried over Na_2SO_4, and concentrated in vacuo. The product was purified by silica gel flash column (DCM/MeOH/NH_4OH, 80:5:0.5) to afford **1** as a pale white solid (0.50 g, 88%). FTIR (neat, cm^{-1}) ν 3031, 2941, 2783, 1732, 1467; ^1H NMR (500 MHz, $CDCl_3$) δ 7.25 (m, 5H), 4.78 (m, 1H), 3.60 (s, 2H), 2.55 (s, 2H), 2.20 (m, 5H), 1.87 (m, 2H), 1.70 (m, 2H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.0, 134.2, 129.2, 128.5, 127.0, 52.7, 46.1, 41.8, 30.7; ESI-HRMS m/z calcd for $C_{14}H_{19}NO_2$: 234.1489 [M+H$^+$], found: 234.1489.

5.1.2. 8-Methyl-8-azabicyclo[3.2.1]octan-3-yl 3-phenylpropanoate (2)

Tropine (0.5 g, 3.47 mmol), 3-phenylpropanoic acid (0.57 g, 3.81 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.75, 3.89 mmol) were dissolved in DCM (35 mL) and stirred for 24 h at ambient temperature. The reaction mixture was washed with $NaHCO_3$ (2 × 30 mL), H_2O (2 × 30 mL), brine (30 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification via silica gel flash column chromatography (DCM/MeOH/TEA, 5:1:0.01) afforded product **2** as a light yellow solid (0.50 g, 51%). FTIR (neat, cm^{-1}) ν 3062, 3028, 2934, 2875, 1729, 1471; ^1H NMR (500 MHz, $CDCl_3$) δ 7.20 (m, 5H), 4.96 (t, J = 5.3 Hz, 1H), 3.04 (s, 2H), 2.94 (t, J = 8.0 Hz 2H), 2.59 (t, J = 8.1 Hz, 2H), 2.23 (s, 3H), 2.07 (dt, J = 14.3, 5.1 Hz, 2H), 1.94 (m, 2H), 1.80 (m, 2H), 1.60 (d, J = 15.4, 2H); ^{13}C NMR (100.7 MHz, $CDCl_3$) δ 172.0, 140.7, 128.7, 128.5, 126.5, 67.7, 60.0, 40.6, 36.8, 36.7, 31.2, 25.8; ESI-HRMS m/z calcd for $C_{17}H_{23}NO_2$: 274.1802 [M+H$^+$], found 274.1803.

5.1.3. 8-Oxabicyclo[3.2.1]oct-6-en-3-one (3a)

This compound was prepared using an adapted protocol.[34] Activated Zn is obtained by brief treatment of Zn dust with 2% HCl (2 × 10 mL), then washing with water (2 × 10 mL), acetone (2 × 10 mL), ether (3 × 10 mL) and drying in an oven (120 °C). Furan (0.44 mL, 6.0 mmol) in dry THF (1 mL) was added to a 50 mL three-neck round-bottom flask charged with activated Zn (0.36 g, 5.5 mmol). Triethyl borate (1.1 mL, 65 mmol) was added to a solution of 1,1,3,3-tetrabromopropanone (1.80 g, 5.0 mmol) in dry THF (1 mL), in a separate dried 10 mL round-bottom flask. The tetrabromopropanone mixture is added at room temperature to the Zn suspension dropwise over 30 min via a syringe. After addition of 80% of the tetrabromopropanone mixture, approximately 25 μL of bromine was added to activate the reaction. After complete addition, the reaction mixture was heated to reflux for 1.5 h. The mixture was removed from heat and stirred at room temperature

for 1 h, then cooled to 0 °C. Ice-cold water (3 mL) was added and the reaction mixture was stirred for 20 min at room temperature. The reaction mixture was filtered through a pad of Celite and rinsed with ether (4 × 10 mL). The filtrate is washed with water (2 × 5 mL), then brine (20 mL). The product was re-extracted with from the combined aqueous layers with ether (4 × 10 mL). The organic phases were combined and dried over $MgSO_4$, and the solvent was removed under reduced pressure (max. 30 °C), to yield the brominated cycloaddition product as a dark brown oil (1.7 g). This product was directly used in the next step.

The brominated cycloaddition product (1.5 g) was dissolved in MeOH (1.5 mL), and 10% of this solution was added to a suspension of zinc-copper couple (1.2 g) and NH_4Cl (1.26 g, 2.35 mmol) in MeOH (5 mL) at −78 °C. After stirring for 15 min, the mixture was warmed to 0 °C and the remaining cycloaddition product was added dropwise, followed by stirring for 1.5 h at room temperature. The reaction mixture was cooled to 0 °C and then filtered. The residue was rinsed with ether (4 × 10 mL). The combined organic phases were washed with brine (20 mL), and the aqueous phase was extracted with $CHCl_3$ (5 × 20 mL). The combined organic phases were dried over $MgSO_4$, concentrated in vacuo, filtered through a pad of K_2CO_3, and washed with $CHCl_3$ (3 × 20 mL). The filtrate was concentrated under reduced pressure yielding a brown oil. The crude product was purified by flash column chromatography on silica gel (hexane/ether, 10:1) to give final bicyclic ketone (**3a**) as a pale solid (0.18 g, 43% over two steps). FTIR (neat, cm^{-1}) ν 2962, 2916, 1714, 1246, 1043; ^1H NMR (500 MHz, $CDCl_3$) δ 6.27 (s, 2H), 5.06 (d, J = 5.1 Hz, 2H), 2.76 (dd, J = 17.0, 5.2 Hz, 2H), 2.34 (dd, J = 16.3, 0.6 Hz, 2H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 205.3, 133.3, 77.2, 46.7; EI-HRMS m/z calcd for $C_7H_8O_2$: 124.05243 [M^+], found: 124.05256.

5.1.4. 8-Oxabicyclo[3.2.1]octan-3-ol (3b)

8-Oxabicyclo[3.2.1]oct-6-en-3-one **3a** (0.12 g, 0.97 mmol) was dissolved in anhydrous EtOH (10 mL), followed by addition of 10% Pd/C (12 mg). The mixture was stirred under under an atmosphere of hydrogen for 8 h at the room temperature. The reaction mixture was filtered through Celite and the solvent was removed under reduced pressure to generate crude product as a pale oil. The oil was diluted in THF (5 mL) and cooled to −78 °C, followed by addition of L-Selectride (0.16 mL, 0.75 mmol) dropwise. After stirring for 1 h at −78 °C, the reaction mixture was stirred in an ice-water bath for 2 h. The reaction was quenched by adding EtOH (5 mL). The mixture was concentrated under reduced pressure and re-dissolved in DCM (20 mL). The organic layer was washed with water (1 × 10 mL) and brine (10 mL). The combined aqueous phases were re-extracted with DCM (3 × 15 mL). Combined organic phases were dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 7:1) to yield the final product **3b** as a pale white solid (70 mg, 56%). FTIR (neat, cm^{-1}) ν 3420, 2946, 2921, 1243; ^1H NMR (500 MHz, $CDCl_3$) δ 4.38 (s, 2H), 4.34 (t, J = 4.8 Hz, 1H), 2.26 (m, 2H), 2.14 (ddd, J = 14.9, 9.1, 14.9, Hz, 2H), 1.95 (m, 2H), 1.66 (dd, J = 14.9, 1.3 Hz); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 73.5, 64.6, 39.0, 28.8; EI-HRMS m/z calcd for $C_7H_{12}O_2$: 128.08372 [M^+], found: 128.08379.

5.1.5. 8-Oxabicyclo[3.2.1]octan-3-yl 2-phenylacetate (3)

8-Oxabicyclo[3.2.1]octan-3-ol **3b** (0.04 g, 0.31 mmol), phenyl acetic acid (0.048 g, 0.04 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.066 g, 0.34 mmol), and 4-dimethylaminopyridine (10 mol %, 0.004 g) were dissolved in DCM (5 mL). The reaction mixture was stirred for 36 h at room temperature. The reaction solution was washed with $NaHCO_3$ (2 × 10 mL), H_2O (2 × 10 mL), brine (10 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification via silica gel flash column chromatography (hexane/ether, 4:1) generated product **3** as a white solid (0.047 g, 62%). FTIR (neat, cm^{-1}) ν 3059, 3031, 2985, 2960, 2924, 2877, 1729, 1492, 1465; ^1H NMR (500 MHz, $CDCl_3$) δ 7.35 (m, 2H), 7.27 (m, 3H), 5.05 (t, J = 4.9 Hz, 1H), 4.27 (s, 1H), 2.09 (dt, J = 15.2, 4.6 Hz, 2H), 1.76 (m, 4H), 1.59 (dd, J = 15.4, 1.0 Hz, 2H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.7, 134.0, 129.2, 128,6, 127.2, 73.1, 67.8, 42.3, 35.8, 28.3; ESI-HRMS m/z calcd for $C_{15}H_{18}O_3$: 269.1148 [M+Na^+], found 269.1150.

5.1.6. 8-Methyl-8-azabicyclo[3.2.1]octan-3-yl benzoate (5)

Tropine (0.13 g, 0.92 mmol), benzoic acid (0.13 g, 1.06 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.194 g, 0.101 mmol), and 4-dimethylaminopyridine (0.092 mmol, 0.011 g) were dissolved in DCM (15 mL). The reaction mixture was stirred for 48 h at room temperature. The reaction solution was washed with $NaHCO_3$ (2 × 10 mL), H_2O (2 × 10 mL), brine (10 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification *via* silica gel flash column chromatography (DCM/MeOH/NH_4OH, 50:5:1) affords product **5** as a light yellow solid (0.14 g, 64%). FTIR (neat, cm^{-1}) ν 2931, 1714, 1601, 1450; ^1H NMR (500 MHz, $CDCl_3$) δ 8.02 (m, 2H), 7.55 (m, 1H), 7.45 (m, 2H), 5.26 (t, J = 5.4 Hz, 1H), 3.26 (s, 2H), 2.32 (s, 3H), 2.24 (dt, J = 14.4, 4.0 Hz, 2H), 2.10 (m, 4H), 1.84 (d, J = 14.6 Hz 2H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 165.9, 132.8, 130.9, 129.4, 128.4, 68.1, 59.9, 40.5, 36.7, 25.8; ESI-HRMS m/z calcd for $C_{15}H_{19}NO_2$: 246.1489 [M+H^+], found 246.1489.

5.1.7. 8-Methyl-8-azabicyclo[3.2.1]octan-3-yl 2-(4-(dimethylamino)phenyl)acetate (6)

Tropine (0.30 g, 2.12 mmol), 4-(dimethylamino)phenylacetic acid (0.31 g, 1.70 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.49 g, 2.54 mmol) and 4-dimethylaminopyridine (0.21 mmol, 0.026 g) were dissolved in DCM (12 mL) and stirred for 20 h at room temperature. The reaction mixture was washed with $NaHCO_3$ (2 × 15 mL), H_2O (2 × 15 mL), brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The product was purified by silica gel flash column (DCM/MeOH/NH_4OH, 4:1:0.1) to yield **6** as a yellow oil (0.37 g, 71%). FTIR (neat, cm^{-1}) ν 2933, 2873, 2848, 1726, 1567; ^1H NMR (500 MHz, $CDCl_3$) δ 7.19 (m, 2H), 6.66 (m, 2H), 4.97 (t, J = 5.4, 1H), 3.50 (s, 2H), 3.05 (s, 2H), 2.94 (s, 6H), 2.26 (s, 3H), 2.09 (dt, J = 14.7, 4.8 Hz, 2H), 1.92 (m, 2H), 1.78 (m, 2H), 1.66 (d, J = 14.4 Hz); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.4, 149.8, 129.9, 122.0, 112.9, 67.6, 59.8, 41.3, 40.8, 40.4, 36.5, 25.5; ESI-HRMS m/z calcd for $C_{18}H_{26}N_2O_2$: 303.2067 [M+H^+], found 303.2067.

5.1.8. 6,7-Dehydrohyoscyamine (7)

Scopolamine (0.1 g, 0.33 mmol) and zinc-copper couple (3 g) in anhydrous EtOH were heated to reflux for 8 h. The reaction mixture was filtered through a pad of Celite and concentrated under reduced pressure. Purification was via silica gel flash chromatography (DCM/MeOH/NH_4OH, 20:1:0.05) to afford **7** a white solid (0.08 g, 80%). FTIR (neat, cm^{-1}) ν 3063.3, 2936.2, 2855.3, 1724.3, 1493.7; ^1H NMR (500 MHz, $CDCl_3$) δ 7.30 (m, 5H), 5.80 (dd, J = 5.6, 1.9 Hz, 1H), 5.42 (dd, J = 5.5, 1.9 Hz, 1H), 5.01 (t, J = 6.1 Hz), 4.12 (dd, J = 11.3, 8.8 Hz, 1H), 3.80 (dd, J = 11.3, 5.2 Hz, 1H), 3.72 (dd, J = 8.8, 5.2 Hz, 1H), 3.34 (m, 1H), 3.24 (m, 1H), 2.20 (m, 4H), 2.14 (ddd, J = 15.0, 6.0, 3.6 Hz, 1H), 1.68 (d, 14.9 Hz, 1H), 1.52 (d, 14.9 Hz, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.9, 135.9, 131.6, 131.6, 128.7, 128.3, 127.6, 67.9, 65.4, 65.3, 64.2, 54.3, 41.5, 33.7, 33.5; ESI-HRMS m/z calcd for $C_{17}H_{21}NO_3$: 288.1594 [M+H^+], found 288.1594. $[\alpha]_D^{24}$ −9.7° (c = 0.50, $CHCl_3$)

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