Supporting Information:

Changes in regioselectivity of H atom abstraction during the hydroxylation and cyclization reactions catalyzed by hyoscyamine 6β-hydroxylase

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Contents

S1 General

S1.1 Reagents

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were used without further purification unless otherwise specified. Tetrahydrofuran (THF) was distilled from sodium/benzophenone, and dichloromethane (DCM) was distilled from calcium hydride under an argon atmosphere. Silica gel column chromatography was carried out using SiliaFlash P60 (230–400 mesh, Silicycle Inc., Quebec, Canada).

S1.2 Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Beckman System Gold 125 Solvent Module with a 166 UV-vis detector equipped with a C18 reversed-phase column (Microsorb 100-5 C18 250 $\times$ 4.6 mm, Agilent Technologies, Santa Clara, CA). LC-ESI-ToF-MS analysis was performed using an Agilent Technologies HPLC system equipped with a pump (G1311C), an auto sampler (G1329B) and a ToF mass spectrometer (G6230B) with an electrospray ionization (ESI) source. LC-MS separations were performed using a Poroshell 120 EC-C18 column (2.7 μm, 4.6 $\times$ 100 mm) with an Eclipse plus C18 guard column (1.8 μm, 2.1 $\times$ 5 mm) at a flow rate of 0.5 mL/min using 0.1% formic acid in H_2O (solvent A) and acetonitrile (solvent B). The obtained LC-MS data were analyzed using MassHunter software (Agilent Technologies). NMR spectra were recorded using a Varian DirectDrive 400 MHz NMR spectrometer at the Nuclear Magnetic Resonance Facility at the University of Texas at Austin. Deuterated solvents were used as the internal reference during acquisition of NMR spectra unless stated otherwise. Chemical shifts are reported as parts per million (ppm) relative to those of $CDCl_3$, 7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C NMR, respectively. Anaerobic work was performed in an anaerobic glovebox (Coy, Grass Lake, MI) under an atmosphere of $>$ 98% N_2 and ca. 2% H_2.

S2 Enzyme assays using H6H

S2.1 Evidence for C7 hydroxylation

H6H (68 μM) was incubated with 1.0 mM substrate (i.e., **1**, **11** or **12**) in a 200 μL reaction volume containing 0.4 mM $FeSO_4$, 5.0 mM αKG (α-ketoglutarate), 4.0 mM sodium ascorbate and 50 mM Tris buffer (pH 7.4) for 30 min at room temprature. A 20 μL aliquot of the resulting solution was then quenched with 40 μL of acetonitrile, centrifuged, and 10 μL of the supertant was diluted into 1 mL water and filtered using a 0.2 mm PTFE syringe filter (VWR International, Rad-

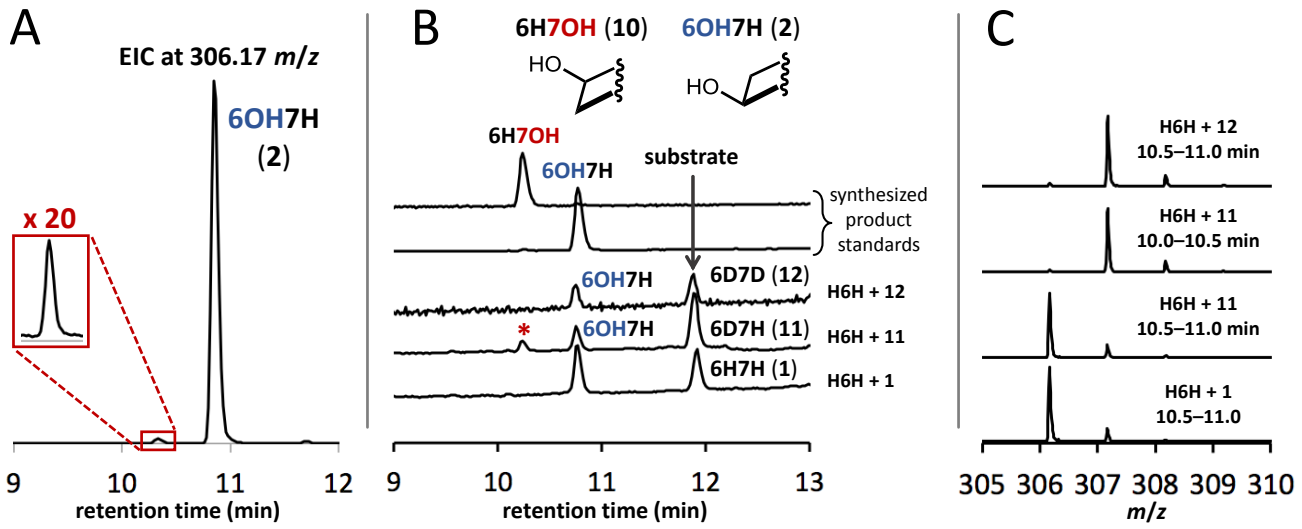


Figure S1: LC-MS evidence for low-level hydroxylation at C7 of **1** catalyzed by H6H. (A) Prolonged incubation of H6H with the hydroxylation substrate **1** results in the formation of a minor peak observable in the EIC for **2** (306.17 m/z). (B) Total-ion-chromatograms of H6H reactions with substrate and its isotopologs demonstrate that the C7-hydroxylated species **10** has the same retention time as the minor hydroxylation product, which increases significantly (asterisk) versus **2** upon substrate deuteration at C6 (i.e., **11**). Standard chromatograms are reduced by 80%. (C) Normalized MS signals in the 305–310 m/z interval integrated over the 10.0–10.5 (minor product), 10.5–11.0 min (major product) LC-retention times. The neutral, unlabeled hydroxylation product **2** has molecular weight 305 Da.

nor, PA) prior to LC-MS analysis. The filtrate (1 μL) was analyzed by LC-MS using a Poroshell 120 EC-C18 column (2.7 μm, 4.6 $\times$ 100 mm) at a flow rate of 0.5 mL/min using 0.1% formic acid in H_2O (solvent A) and acetonitrile (solvent B) with the gradient program: 0–10 min: 0–30% B, 10–12 min: 30–100% B, 12–13 min: 100–0% B and 13–18 min: 0% B.

A representative extracted ion chromatogram (EIC) is shown in Fig. S1A demonstrating a major and minor peak both characterized by an m/z value of 306.17 consistent with the $[M+H]^+$ ion of hydroxyhyoscyamine. The minor peak coelutes with a synthetic standard of **10**. When the reaction was run with the 6-deutero isotopolog **11**, there was a +1 increase in the m/z value of the minor product by MS as well as a significant increase in its overall signal intensity versus the major product (see Fig. S1B & C). Finally, when the reaction was run with the dideuterated substrate isotopolog **12**, the minor peak intensity was again reduced to levels comparable to the **1** case and both signals showed +1 increases in their respective m/z values (see Fig. S1B & C). Based on these observations, the minor peak was assigned as the C7-hydroxylation product **10**.

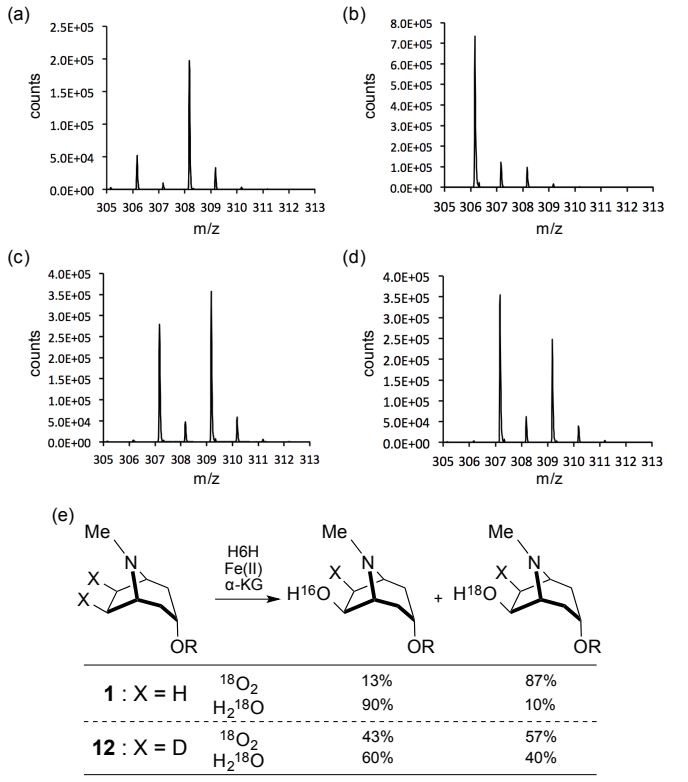
S2.2 Quantitative partitioning analysis

Partitioning between C6- versus C7-hydroxylation during the H6H-catalyzed reaction was determined by incubating 16 μM H6H with 0.5 mM substrate (e.g., **1** or **11**) in a 100 μL reaction volume containing 0.4 mM $FeSO_4$, 10 mM αKG, 4 mM sodium ascorbate and 50 mM HEPES buffer (pH 6.9). Reactions were run in 2 mL glass vials at room temperature, open to air and stirred at 100 rpm with a small stirbar. A 10 μL aliquot of the reaction solution was transferred to 20 μL of acetonitrile after 5, 10 and 20 min of incubation to quench the reaction. The quenched reaction solution was centrifuged, and the supernatant (20 μL) was diluted into 480 μL water and filtered using a 0.2 mm PTFE syringe filter (VWR International, Radnor, PA) prior to LC-MS analysis. The filtrate (1 μL) was analyzed by LC-MS using a Poroshell 120 EC-C18 column (2.7 μm, 4.6 $\times$ 100 mm) at a flow rate of 0.5 mL/min using 0.1% formic acid in H_2O (solvent A) and acetonitrile (solvent B) with the following gradient program: 0–10 min: 0–30% B, 10–12 min: 30–100% B, 12–13 min: 100–0% B and 13–18 min: 0% B.

The hydroxylated products **10** (or **13**) and **2** were observed exclusively as the $[M+H]^+$ ion adducts in the ESI spectra. Compounds **10** (or **13**) and **2** are separable on the Poroshell 120 EC-C10 column (see Fig. S1B). Therefore, the MS signal intensities were determined by integrating the corresponding peaks from the extracted ion chromatogram (EIC). The product concentration ratio was then obtained from the ratio

$$\frac{[\mathbf{10}]}{[\mathbf{2}]} = \frac{I_{\mathbf{10}} + \beta}{I_{\mathbf{2}} + \beta}, \tag{S1}$$

where $I_{\mathbf{10}}$ and $I_{\mathbf{2}}$ are the observed signal intensities from the integrated EIC peaks for **10** (or **13**) and **2**, respectively, and β is a correction equal to 1.5×10^4 count·min. The correc-



1 : X = H	$^{18}O_2$	13%	87%
	$H_2{}^{18}O$	90%	10%
12 : X = D	$^{18}O_2$	43%	57%
	$H_2{}^{18}O$	60%	40%

Figure S2: ^{18}O incorporation experiments. ESI-MS spectra of hydroxyhyocyamine formed in the H6H-catalyzed reaction of (a) **1** under $^{18}O_2$, (b) **1** in $H_2{}^{18}O$, (c) **12** under $^{18}O_2$, (d) **12** in $H_2{}^{18}O$. (e) Summary of the ^{18}O incorporation experiments.

tion term compensates for loss of sensitivity in the MS detector, and its use is justified in Sec. S3.3. No corrections were made for scopolamine formation, because the ratio of scopolamine to total hydroxylated product was negligible with respect to **2** from which it primarily forms (interquartile ranges: $(0.02, 0.06)$ & $(0.0, 0.001)$ for assays with **1** and **11**, respectively). For each reaction, the ratios (S1) at each of the three time points were averaged arithmetically to obtain the ratio for the experiment. The entire experiment was repeated five times and the results of each experiment averaged arithmetically to provide the reported estimate, sample standard deviation and standard error of the mean.

S2.3 Initial rate measurements

Reactions were initiated by adding H6H to solutions containing 100 μM substrate (i.e., **1**, **12**, **2** or **10**), 0.4 mM $FeSO_4$, 10 mM αKG, 4 mM sodium ascorbate and 50 mM HEPES buffer (pH 6.9) at room temperature in a 2 mL glass vial. The final enzyme concentration was 1.6 μM. The total initial reaction volume was 100 μL, open to air and stirred at ca. 100 rpm using a small stirbar. These conditions were chosen to match those for the partitioning experiments and to ensure that the αKG and O_2 concentrations remained roughly constant throughout the reaction time courses. Controls demonstrated that H6H was saturated in the case of each substrate under these conditions, and there was no evidence of substrate inhibition. In particular, the Michaelis constant for each substrate was found to be less than 10 μM in the presence of 10 mM αKG and 0.21 atm O_2.

Reaction aliquots were quenched with acetonitrile at three time points such that the conversion of the substrate was less than 20%. After centrifugation, the supernatant (20 μL) was added to 280 μL water and a 10 μL aliquot was analyzed by LC-MS as described in Sec. S2.2 to obtain signal intensities for the product formed and residual substrate. A correction of 1.5×10^4 count·min was added to each observed signal as described in Sec. S2.2 and justified in Sec. S3.3. Since **1**, **2** and **10** all have similar ionization efficiencies in the MS source (see Sec. S3.1) no additional corrections were applied to the hydroxylation reaction. In the case of the cyclization reaction, however, scopolamine was found to ionize roughly 50% as efficiently as the hydroxylated substrates (i.e., **2** and **10**, see Sec. S3.1). Therefore, scopolamine MS signal intensities were multiplied by 2. Fractions of reaction were obtained from the corrected signal intensities and used to determine the extent of reaction given the inital substrate concentration. Linear regression of the residual substrate concentration versus time then provided the initial rate as a measure of V-max. Three independent measurements were made of V-max in the above manner for each substrate.

S2.4 H6H reaction under $^{18}O_2$

The H6H reaction was carried out under $^{18}O_2$ as previously described.[1] In an anaerobic chamber under an atmosphere of $>$ 98% N_2 and $<$ 2% H_2, a 50 μL solution containing 1 mM substrate **1**, 0.4 mM $FeSO_4$, 5 mM αKG, 4 mM ascorbate and 68 μM H6H in 50 mM Tris (pH 7.4) was prepared in a 1.5 mL tube. The tube was then placed in a 20 mL vial that was subsequently capped with a rubber septum. The vial was then removed from the anaerobic chamber, and the headspace was evacuated using a needle connected to a vacuum pump. The reaction was initiated by introducing $^{18}O_2$ (99 atom %) using a balloon. After 1 h, the reaction mixture was quenched by the addition of 100 μL acetonitrile and centrifuged under an aerobic atmosphere. The supernatant was analyzed by LC-MS as described above to obtain the M and $M + 2$ signal intensities for the protonated adduct of the hydroxylated product. The same experiment and analysis was also performed aerobically (i.e., with natural abundance O_2) and buffer containing 97 atom % $H_2{}^{18}O$. In this case, the reaction was incubated for 5 h prior to quenching and ESI-MS analysis. ESI-MS spectra and summary data for all experiments are shown in Fig. S2. Reaction conditions for substrate **12** were the same as those for **1**.

S3 Controls

S3.1 Relative ionization efficiencies

The relative ionization efficiencies of **1** vs. **2** as well as **2** vs. scopolamine (**3**) were determined by running the H6H-catalyzed hydroxylation of **1** or cyclization of **2** in the presence of the dihydroxylated species 6β,7β-dihydroxyhyoscyamine, which is inert to the activity of H6H. The substrate (0.5 mM **1** or **2**) and standard 6β,7β-dihydroxyhyoscyamine (0.5 mM) were incubated with H6H (35 μM), $FeSO_4$ (0.4 mM), αKG (5 mM), sodium ascorbate (4 mM) and tris buffer (pH 7.4, 50 mM) in a 1.5 mL tube (0.1 mL total volume, under air, cap closed) at room temperature. A 20 μL aliquot was added to 40 μL of acetonitrile after 30 min, 1 h, 4 h, and 16 h. After centrifugation, the supernatant (5 μL) was diluted into 1 mL water. The sample was analyzed by LC-MS (1 μL injection) as described above to obtain the EIC peak integrations for **1**, **2**, 6β,7β-dihydroxyhyoscyamine and **3**. No significant scopolamine formation was observed during the hydroxylation of **1**.

Assuming signal intensities (I) are directly proportional to ionization efficiency, we have the following relationships

$$I_s^i = \gamma_i \varepsilon_s s_i, \qquad I_p^i = \gamma_i \varepsilon_p p_i, \qquad I_r^i = \gamma_i \varepsilon_r r_i, \tag{S2}$$

where s_i, p_i and r_i are the concentrations of substrate (i.e., **1** or **2**), product (i.e., **2** or **3**) and standard (i.e., 6β,7β-dihydroxyhyoscyamine) in the i-th reaction aliquot, respectively; ε_s, ε_p and ε_r are the corresponding ionization efficiencies and γ_i is a constant specific to the i-th sample injection. Conservation of mass requires that

$$p_i - p_j = s_j - s_i, \tag{S3}$$

for any i,j-pair of reaction aliquots. Furthermore, $r_i = r_j$, because 6β,7β-dihydroxyhyoscyamine is inert to H6H activity, such that

$$p_i/r_i - p_j/r_j = s_j/r_j - s_i/r_i. \tag{S4}$$

Substitution of Eqns. (S2) into (S4), and rearranging gives

$$\frac{\varepsilon_s}{\varepsilon_p} = \frac{I_s^i/I_r^i - I_s^j/I_r^j}{I_p^j/I_r^j - I_p^i/I_r^i}, \quad i \neq j. \tag{S5}$$

We thus obtain n-choose-2 dependent estimates of the ionization efficiency ratio given n reaction aliquots. Taking the median as the best overall estimator and the interquartile range as an estimate of the uncertainty yields

$$\frac{\varepsilon_{\mathbf{1}}}{\varepsilon_{\mathbf{2}}} = 1.1 \pm 0.2 \qquad \text{and} \qquad \frac{\varepsilon_{\mathbf{2}}}{\varepsilon_{\mathbf{3}}} = 2.0 \pm 0.5.$$

Therefore, for all practical purposes, the ionization efficiencies of **1** and **2** may be regarded as equal while that of **2** is approximately twice the ionization efficiency of **3**.

S3.2 Linear dynamic range

The MS signals associated with **2** and **10** produced via the H6H-catalyzed hydroxylation reaction differ by nearly two orders of magnitude. Therefore, it is important to ensure that both signals can be measured simultaneously within the linear dynamic range of the MS. To check for this, the 5 min sample from one of the experimental trials was analyzed over a series of different injection volumes ranging from 0.2 to 10 μL (the standard assay volume was 1 μL). Plots of the observed signal intensities $I_{\mathbf{10}}$ and $I_{\mathbf{2}}$ are shown in Fig. S3.

The $I_{\mathbf{10}}$ signal shows a linear response to injection volume at intensities above ca. 2.5×10^4 count·min doubling with a doubling of the injection volume. However, loss of detector sensitivity becomes apparent below ca. 2.0×10^4 count·min where a nonlinear dependence is observed (discussed further below). With respect to the high intensity $I_{\mathbf{2}}$ signals, there is evidence for the onset of saturation as the signal intensity exceeds ca. 1×10^7 count·min. Below this cutoff, however, the response is linear with a doubling of intensity following a doubling of the injection volume. These observations suggest a linear dynamic range for sample concentrations yielding responses in the approximate range of 2.0×10^4 to 1.0×10^7 count·min. The ranges of observed intensities among all samples in the experimental trials were 3.4×10^6 to 1.35×10^7 for $I_{\mathbf{2}}$, and 3.1×10^4 to 1.6×10^5 for $I_{\mathbf{10}}$. Thus, all measurements appear to have been made within the linear dynamic range of the instrument.

S3.3 Sensitivity

As shown in Fig. S3 (right-most plot), the MS begins to show a nonlinear response as the peak intensity drops below approximately 2.0×10^4 count·min. This is likely due to loss of sensitivity as the ionized fraction of the sample drops below a certain detection limit. As a consequence, linear regressions applied to the linear range of the responses undershoot zero by approximately 1.5×10^4 count·min. This undershoot may be approximated as signal lost due to a discrete cutoff when only signals within the linear range are considered. Therefore, these signals should be corrected by adding 1.5×10^4 count·min to the observed values. While such a correction amounts to only 1% of signals exceeding ca. 1.0×10^6 count·min (i.e., those from **2**), this was not true for the **10** signals, which were typically in the 3.1×10^4 to 1.6×10^5 count·min range. Therefore, the correction was applied to correct for any potential bias due to instrument sensitivity as shown in Eqn. S1.

S4 Analysis of V-max ratios

Let X be the undeuterated hydroxylation substrate **1** and Y denote any of the four substrates **1**, **12**, **2** or **10**. We are in-

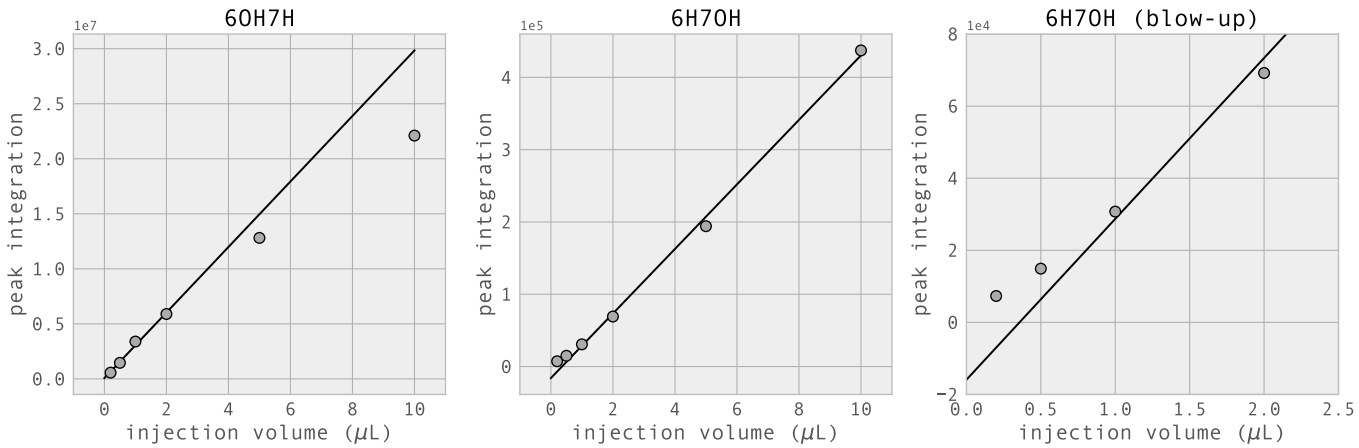


Figure S3: Controls for the linear dynamic range in signal response measured as integrated EIC peaks. A C6- versus C7-hydroxylation partitioning experimental sample was injected at a range of injection volumes and the integrated EIC peaks were plotted versus injection volume. Circles denote the observed signal response at each injection volume for the **2** product (6OH7H, left-most plot) or the **10** product (6H7OH, two right-most plots). Solid lines denote linear regressions to a subset of each data series: first four data points (**2**), last five data points (**10**). The ordinates are scaled by (left-to-right) 1×10^{-7}, 1×10^{-5} and 1×10^{-4}. See text for further detail.

terested in evaluating the V-max ratio V_X/V_Y given the experimental design and reported results. The H6H catalytic cycles for all four substrates may be reasonably modeled as serial (see Fig. S4), because there is little to no branching of reaction flux in each case (including **1** and **12**) and product dissociation is effectively irreversible under the experimental conditions. Our approach will be equivalent to that of Northrop[2,3] but with an additional emphasis on net rate constant abstractions as a means of further generalization.

The enzyme intermediates in the H6H catalytic cycle for each Y can be divided into four mutually exclusive and exhaustive groups as shown in Fig. S4. The first group (A) consists of all enzyme species after an irreversible step of product dissociation up to but not including intermediate $E_{bnd,Y}$, which binds the variable substrate Y. The second group (B) includes all enzyme species following $E_{bnd,Y}$ up to and including the iron(IV)-oxo complex $E_{abs,Y}$ that decomposes via H atom abstraction. The third group (C) is all remaining enzyme species other than $E_{bnd,Y}$, and the fourth and final group is just $E_{bnd,Y}$ alone.

With the enzyme intermediates thus grouped, the reciprocal of V-max for saturating substrate Y can be expressed as the sum of the reciprocal net rate constants for each intermediate in each group,[4]

$$\frac{e_0}{V_Y} = \sum_A \frac{1}{k'_{iY}} + \sum_B \frac{1}{k'_{iY}} + \sum_C \frac{1}{k'_{iY}}, \tag{S6}$$

where e_0 is the total enzyme concentration and k'_{iY} is the net rate constant associated with the i-th intermediate E_{iY}. Note that the net rate constant associated with $E_{bnd,Y}$ is not represented in this sum, because the substrate Y is saturating.[4]

We can now include the net rate constants for the H6H catalytic cycle specifically with $X = \mathbf{1}$ by multiplying each term in (S6) by unity to obtain

$$\frac{e_0}{V_Y} = \sum_A \frac{1}{k'_{iX}} \cdot \frac{k'_{iX}}{k'_{iY}} + \sum_B \frac{1}{k'_{iX}} \cdot \frac{k'_{iX}}{k'_{iY}} + \sum_C \frac{1}{k'_{iX}} \cdot \frac{k'_{iX}}{k'_{iY}} + \sum_{C'} \frac{0}{k'_{iX}}.$$

In this expression we have also included a dummy term, which is equal to 0, involving the sum over enzyme intermediates in a fourth group C'. This group accounts for any enzyme species in group C of the H6H catalytic cycle for X that do not have a counterpart in group C of the catalytic cycle for Y. For example, hydroxylation of $X = \mathbf{1}$ may involve a discreet substrate radical intermediate, whereas it has been suggested that cyclization of **2** and **10** may be concerted with H atom abstraction.[1]

If the only microscopic rate constants to differ significantly (i.e., by a factor of 10 or greater) between the H6H catalytic cycles of X and Y are substrate binding and H atom abstraction, then it is easy to show that the net rate constant ratios for enzyme intermediates in groups A and C are all approximately 1.[5] We can thus combine the A- and C-sums to get

$$\frac{e_0}{V_Y} = \sum_{A,C} \frac{1}{k'_{iX}} + \sum_B \frac{1}{k'_{iX}} \cdot \frac{k'_{iX}}{k'_{iY}} + \sum_{C'} \frac{0}{k'_{iX}}. \tag{S7}$$

Since by definition $V_X = e_{iX} k'_{iX}$, where e_{iX} is the steady-state concentration of the i-th intermediate in the hydroxylation of X, we can rewrite Eqn. (S7) in terms of fractional intermediate concentrations (i.e., $f_{iX} = e_{iX}/e_0$) as[5]

$$\frac{V_X}{V_Y} = \sum_{A,C} f_{iX} + \sum_B f_{iX} \frac{k'_{iX}}{k'_{iY}} + 0 \cdot \sum_{C'} f_{iX}. \tag{S8}$$

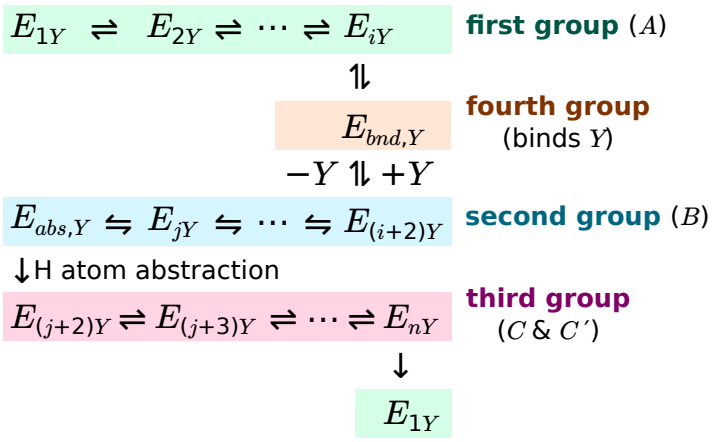


Figure S4: Generalized kinetic model for the H6H catalyzed hydroxylation of $Y = \mathbf{1}, \mathbf{12}$ and cyclization of $Y = \mathbf{2}, \mathbf{10}$ with the intermediates grouped as discussed in the text. Intermediate $E_{bnd,Y}$ binds the variable substrate Y, and intermediate $E_{abs,Y}$ represents the iron(IV)-oxo complex poised for H atom abstraction from the bound substrate. The first, second and fourth groups are treated as essentially the same for all four substrates. Group C' allows for the possibility that additional intermediates may be present in the third group for $X = \mathbf{1}$ compared to $Y = \mathbf{2}, \mathbf{10}$ (see text for details).

Based on the experimental results of the present work and prior computational studies by other investigators,[6] the elementary reaction involving H atom abstraction may be reasonably modeled as irreversible for all Y (as depicted in Fig. S4). Generalized formulations of net rate constant ratios arising in serial kinetic models have been previously studied in considerable theoretical detail,[5,7] and according to these results the net rate constant ratios in the B-sum under the stated hypotheses take the form,

$$k'_{iX}/k'_{iY} = (1 - \phi_{iX}) + \phi_{iX} k_X/k_Y. \tag{S9}$$

In this expression, ϕ_{iX} is a weighting term associated with E_{iX} that depends only on the rate constants in the catalytic cycle for X such that $0 \leq \phi_{iX} \leq 1$. The ratio k_X/k_Y is the ratio of microscopic rate constants for H atom abstraction from X versus Y. Combining the previous two results, rearranging to consolidate the sums of f_{iX} terms and setting $\alpha_X = \sum_B f_{iX}\phi_{iX}$, we have the result

$$\frac{V_X}{V_Y} = \sum_{A,B,C} f_{iX} + 0 \cdot \sum_{C'} f_{iX} - \alpha_X + \alpha_X \frac{k_X}{k_Y}. \tag{S10}$$

Now, if there are no enzyme species in group C',[a] then $\sum_{A,B,C} f_{iX} = 1$, and we obtain Eqn. (5),

$$\frac{V_X}{V_Y} = 1 - \alpha_X + \alpha_X \frac{k_X}{k_Y}. \tag{5}$$

[a]This is necessarily the case when $Y = X$, almost certainly to be the case under deuteration alone (i.e., $Y = \mathbf{12}$) and at least possible for $Y = \mathbf{2}, \mathbf{10}$.

This expression is identical to the result obtained by Northrop under equivalent assumptions for V-max isotope effects.[2,3] In contrast, if there are enzyme species in group C', which would be the case if hydroxylation is stepwise and cyclization is concerted with H atom abstraction, then $\sum_{A,B,C,C'} f_{iX} = 1$ such that

$$\frac{V_X}{V_Y} \lesssim 1 - \alpha_X + \alpha_X \frac{k_X}{k_Y}. \tag{S11}$$

Here, the approximation becomes better as the fractional concentrations of the intermediate species in the catalytic cycle for X but missing from Y (e.g., a discreet radical intermediate) get smaller.

S5 Preparation of compounds

S5.1 Syntheses

S5.1.1 Synthesis of [6β,7β-$^2H_2$]hyoscyamine

Methyl 3-((*tert*-butyldimethylsilyl)oxy)[6β,7β-$^2H_2$]-8-azabicyclo[3.2.1]octane-8-carboxylate (S2). Compound **S1** (synthesized as previously reported,[8] 0.793 g, 2.69 mmol) was dissolved in MeOH (30 mL) and 100 mg 10% Pd/C was added. The reaction mixture was stirred under a deuterium atmosphere (99.8 atom % D, 1 atm) for 4 h. The reaction solution was filtered through a pad of Celite, and the filtrate was concentrated to yield **S2** (0.780 mg, 96%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.25 (1H, H-1 or H-5), 4.16 (1H, H-1 or H-5), 4.00 (1H, m, H-3), 3.67 (3H, s, NCO_2Me), 2.20 (2H, s, H-6, H-7), 2.05 (1H, d, J = 10.6 Hz, H-2 or H-4), 1.95 (1H, d, J = 10.6 Hz, H-2 or H-4), 1.64 (2H, m, H-2, H-4), 0.88 (9H, s, Si-*t*Bu), −0.02 (6H, s, Si-Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.1, 65.5, 53.0 (m), 52.1, 39.4 (m), 38.6 (m), 27.7 (m), 25.7, 17.8, −5.2. ESI-HRMS Calcd. for $C_{15}H_{28}D_2NO_3Si^+$ $[M+H]^+$ 302.2115, found 302.2097.

Methyl 3-*endo*-hydroxy[6β,7β-$^2H_2$]-8-azabicyclo[3.2.1]octane-8-carboxylate (S3). To a solution of **S2** (780 mg, 2.59 mmol) in THF (25 mL), tetrabutylammonium fluoride (1 M solution in THF, 10.4 mL, 10.4 mmol) was added at 0 °C, and the mixture was warmed to room temperture. After 48 h, the mixture was diluted with DCM and water, and the resulting solution was extracted with DCM (3 × 20 mL). The combined organic phase was washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified using flash chromatography on silica gel (ethyl acetate/hexanes = 2/1) to yield **S3** (351 mg, 72%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.25 (1H, H-1 or H-5), 4.18 (1H, H-1 or H-5), 4.11 (1H, t, J = 4.9 Hz, H-3), 3.67 (3H, s, NCO_2Me), 2.14 (2H, s, H-6, H-7), 2.11 (1H, d, J = 13.8 Hz, H-2 or H-4), 2.01 (1H, d, J = 13.8 Hz, H-2 or H-4), 1.79 (1H, s, OH), 1.71 (2H, m, H-2, H-4). ^{13}C NMR

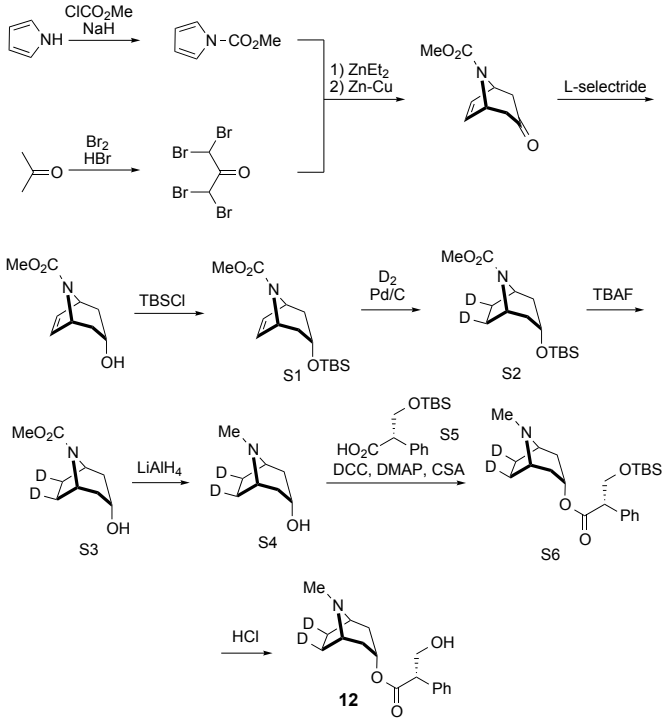


Figure S5: Synthesis of [6β,7β-$^2H_2$]hyoscyamine (**12**).

(101 MHz, $CDCl_3$) δ 154.1, 65.1, 52.7 (m), 52.2, 38.8 (m), 38.3 (m), 28.1 (m), 27.4 (m). ESI-HRMS Calcd. for $C_9H_{14}D_2NO^+_3$ $[M+H]^+$ 188.1250, found 188.1258.

8-Methyl[6β,7β-^{2}H$_2$]-8-azabicyclo[3.2.1]octan-3-ol (S4). To a suspension of lithium aluminum hydride (0.284 g, 7.5 mmol) in ether (40 mL), a solution of **S3** (0.351 g, 1.87 mmol) in ether (10 mL) was added at 0 °C, and the mixture was warmed to room temperture. After 4 h, the reaction was quenched with water (0.15 mL) at 0 °C. The resulting solution was filtered through Celite. The filtrate was evaporated and the obtained residue was purified using flash chromatography on silica gel ($CHCl_3$/MeOH/30% NH_3 = 80/20/1) to yield **S4** (0.175 g, 65%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.03 (1H, t, J = 4.9 Hz, H-3), 3.11 (2H, m, H-1, H-5), 2.79 (3H, s, NMe), 2.11 (2H, ddd, J = 4.0 Hz, J = 5.0 Hz, J = 15.0 Hz, H-2, H-4), 2.06 (2H, s, H-6, H-7), 1.68 (2H, m, H-2, H-4), ^{13}C NMR (101 MHz, $CDCl_3$) δ 64.5, 60.1, 40.6, 39.2, 25.1 (m). ESI-HRMS Calcd. for $C_8H_{14}D_2NO^+$ $[M+H]^+$ 144.1352, found 144.1360.

[6β,7β-^{2}H$_2$]Hyoscyamine (12). A mixture of **S4** (170 mg, 1.20 mmol), **S5** (synthesized as previously reported,[1] 483 mg, 1.82 mmol), N,N'-dicyclohexylcarbodiimide (DCC, 1.84 mg, 1.94 mmol), N,N-dimethyl-4-aminopyridine (DMAP, 15 mg, 0.12 mmol), and 10-camphorsulfonic acid (CSA, 84 mg, 0.36 mmol) in DCM (15 mL) was stirred at room temperature for 3 d. The resulting solution was filtered through Celite to remove the white precipitate. The filtrate was loaded on a silica gel column pre-equilibrated with chloroform. The product was eluted with $CHCl_3$/MeOH = 20/1. The fractions containing the ester product **S6** (monitored by ^{1}H NMR) were combined and concentrated. The obtained residue was used in the next step without further purification. The residue was dissolved in MeOH (40 mL) and 1 N HCl (2.5 mL) was added. The mixture was stirred overnight at room temperature before evaporation to a small volume. The resulting acidic solution (pH 1) was diluted with water (15 mL) and washed with $CHCl_3$ (3 $\times$ 10 mL). The aqueous solution was neutralized to pH 7–8 with freshly prepared, saturated $NaHCO_3$ and subsequently extracted with $CHCl_3$ (3 $\times$ 10 mL). The combined chloroform phase was dried over Na_2SO_4, filtered, and concentrated to give **12** (328 mg, 94% in 2 steps) with a trace amount of the unwanted 2′R isomer. Further purification will be described in Sec. S5.2. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.28–7.17 (5H, m, Ph), 4.91 (1H, dd, J = 5.2 Hz, J = 5.4 Hz, H-3), 4.60 (1H, s, br, OH), 4.09 (1H, m, H-3), 3.70 (1H, m, H-3′), 3.70 (1H, m, H-2′), 2.94 (1H, m, H-1 or H-5), 2.83 (1H, m, H-1 or H-5), 2.10 (3H, s, NMe), 2.02 (1H, ddd, J = 3.7 Hz, J = 5.4 Hz, J = 15.2 Hz, H-2 or H-4), 1.95 (1H, ddd, J = 3.9 Hz, J = 5.2 Hz, J = 15.2 Hz, H-2 or H-4), 1.70 (1H, s, H-6 or H-7), 1.61 (1H, d, J = 15.2 Hz, H-2 or H-4), 1.61 (1H, d, J = 15.2 Hz, H-2 or H-4), 1.29 (1H, s, H-6 or H-7). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 135.9, 128.7, 128.1, 127.5, 67.7, 63.8, 59.5, 59.4, 54.7, 40.1, 36.0, 35.9, 24.9 (m), 24.4 (m). ESI-HRMS Calcd. for $C_{17}H_{22}D_2NO_3^+$ $[M+H]^+$ 292.1876, found 292.1889.

S5.1.2 Synthesis of [6β-^{2}H]hyoscyamine

Reagents. (+)-Ipc$_2$BD was prepared from $BD_3\cdot SMe_2$ (98 atom % D, Cambridge Isotope laboratories, Inc. (Tewksbury, MA)) and (−)-α-pinene as previously reported.[9]

Methyl (1*S*,3*R*,5*S*,6*R*)-3-((*tert*-butyldimethylsilyl)oxy)-6-hydroxy[7β-^{2}H]-8-azabicyclo[3.2.1]octane-8-carboxylate (S7). A solution of **S1** (1.76 g, 5.92 mmol) in THF (120 mL) was added dropwise at −30 °C to crystals of (+)-Ipc$_2$BD (4.10 g, 14.2 mmol). After 4 h, MeOH (10 mL), NaOH (3 N, 10 mL), and H_2O_2 (30%, 10 mL) were added to the reaction mixture sequentially. The resulting mixture was warmed to room temperature and stirred for 14 h. The resulting mixture was diluted with ethyl acetate (200 mL) and H_2O (100 mL). The aqueous solution was extracted with ethyl acetate (3 $\times$ 150 mL), and the combined organic phase was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified using flash chromatography on silica gel (hexanes/ethyl acetate = 2/3) to yield **S7** (1.69 g, 90%). The optical purity was determined to be > 99% ee by Mosher's method as described previously.[1]

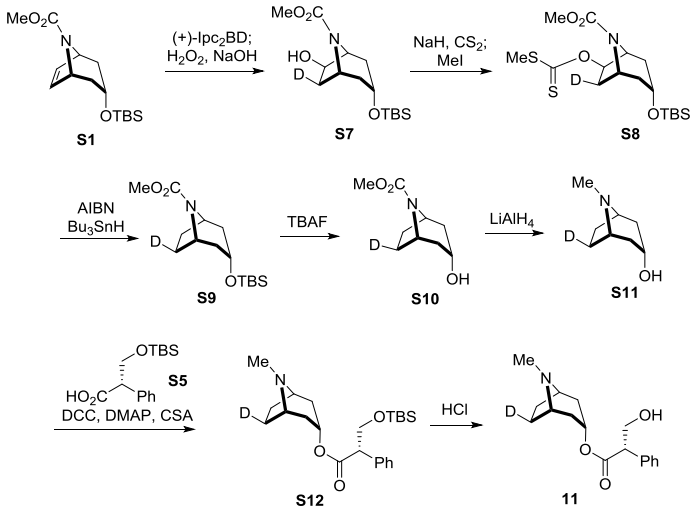


Figure S6: Synthesis of [6β-^{2}H]hyoscyamine (**11**).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.72 (1H, m, H-6), 4.33 (1H, m, H-1), 4.02 (1H, m, H-5), 3.96 (1H, m, H-3), 3.69 (3H, s, NCO_2Me), 2.82 (1H, d, J = 6.8 Hz, H-7), 1.96 (2H, m, H-2 and H-4), 1.72 (1H, br, OH), 1.72 (1H, d, J = 14.5 Hz, H-4), 1.56 (1H, d, J = 14.2 Hz, H-2), 0.88 (9H, s, Si-*t*Bu), 0.01 (6H, s, Si-Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.9, 74.8 (m), 65.1, 62.7, 53.4, 52.3, 40.5 (m), 37.8 (m), 36.6 (m), 25.7, 17.8, −5.1. ESI-HRMS Calcd. for $C_{15}H_{29}DNO_4Si^+$ $[M+H]^+$ 317.2001, found 317.2031.

Methyl (1*S*,3*R*,5*S*,6*R*)-3-((*tert*-butyldimethylsilyl)oxy)-6-(((methylthio)carbonothioyl)oxy)[7β-^{2}H]-8-azabicyclo[3.2.1]octane-8-carboxylate (S8). To a solution of **S7** (1.0 g, 3.16 mmol) in THF (20 mL), NaH (60% in mineral oil, 252 mg, 6.32 mmol) was added portionwise at 0 °C, and then CS_2 (0.62 mL, 12.6 mmol) was added dropwise to the mixture at 0 °C. The mixture was warmed to room temperature and MeI (1.57 mL, 25.3 mmol) was added. After 3 h, the reaction was quenched with H_2O (50 mL) and diluted with ethyl acetate (50 mL). The organic phase was separated and the aqueous solution was extracted with ethyl acetate (3 × 50 mL). The combined organic phase was washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified using flash chromatography on silica gel (ethyl acetate/hexanes = 1/8) to yield **S8** (1.26 g, 98%). This compound was observed as a mixture of two conformers (56 : 44 ratio) by NMR analysis. NMR assignments for both conformers are shown. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.21 (1H, d, J = 7.2 Hz, H-6), 4.44 (1H, s, H-1, major), 4.37 (1H, s, H-5, minor), 4.32 (1H, s, H-1, minor), 4.29 (1H, s, H-5, major), 4.02 (1H, m, H-3), 3.69 (3H, s, NCO_2Me, minor), 3.68 (3H, s, NCO_2Me, major), 2.86 (1H, d, J = 7.3 Hz, H-7, minor), 2.83 (1H, d, J = 7.3 Hz, H-7, major), 2.51 (3H, s, SMe), 2.19–1.86 (3H, m, H-2, H-4, H-4), 1.62 (1H, d, J = 14.4, H-2), 0.89 (9H, s, Si-*t*Bu), 0.04 (3H, s, Si-Me), 0.02 (3H, s, Si-Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 215.0, 215.0, 154.5, 154.2, 87.4, 86.4, 65.0, 59.4, 59.2, 53.3, 53.2, 52.4, 38.2, 37.5, 37.5 (m), 36.8, 36.7 (m), 35.9, 25.8, 18.9, 17.8, −5.1, −5.2. ESI-HRMS Calcd. for $C_{17}H_{31}DNO_4S_2Si^+$ $[M+H]^+$ 407.1599, found 407.1636.

Methyl (1*R*,3*S*,5*S*)-3-((*tert*-butyldimethylsilyl)oxy)[6β-^{2}H]-8-azabicyclo[3.2.1]octane-8-carboxylate (S9). A mixture of **S8** (1.2 g, 2.96 mmol), 2,2′-azobis(2-methylpropionitrile) (128 mg, 0.78 mmol), tributyltin hydride (1.52 mL, 5.63 mmol) in toluene (20 mL) was stirred at 95 °C. After 20 min, the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The crude product was purified using flash chromatography on silica gel (ethyl acetate/hexanes = 1/20 then 1/10) to yield **S9** (0.834 g, 94%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.25 (1H, s, H-1 or H-5), 4.17 (1H, s, H-1 or H-5), 4.00 (1H, m, H-3), 3.67 (3H, s, NCO_2Me), 2.20 (2H, m, H-6, H-7), 2.10–1.91 (2H, m, H-2, H-4), 1.86 (1H, dd, J = 6.4, J = 7.4, H-7), 1.64 (2H, m, H-2, H-4), 0.88 (9H, s, Si-*t*Bu), 0.02 (6H, s, Si-Me). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.1, 65.5, 53.0, 53.0, 52.1, 39.4, 38.6, 28.3 (m), 27.6 (m), 25.7, 17.8, −5.2. ESI-HRMS Calcd. for $C_{15}H_{29}DNO_3Si^+$ $[M+H]^+$ 301.2052, found 301.2068.

Methyl (1*R*,3*S*,5*S*)-3-hydroxy[6β-^{2}H]-8-azabicyclo[3.2.1]octane-8-carboxylate (S10). Compound **S10** was synthesized in 80% yield based on the same procedure as described for **S3**. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.25 (1H, s, H-1 or H-5), 4.18 (1H, s, H-1 or H-5), 4.11 (1H, m, H-3), 3.66 (3H, s, NCO_2Me), 2.20–1.82 (5H, m, H-2, H-4, H-6, H-7, H-7), 1.71 (2H, d, J = 14.9 Hz, H-2, H-4), 1.47 (1H, OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.1, 65.0, 52.7, 52.7, 52.2, 38.8, 38.2, 28.4 (m), 27.7 (m). ESI-HRMS Calcd. for $C_9H_{15}DNO_3^+$ $[M+H]^+$ 187.1187, found 187.1197.

(1*R*,3*S*,5*S*)-8-Methyl[6β-^{2}H]-8-azabicyclo[3.2.1]octan-3-ol (S11). Compound **S11** was synthesized in 96% yield based on the same procedure as described for **S4**. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.99 (1H, t, J = 5.0 Hz), 3.05 (2H, s, H-1, H-5), 2.23 (3H, s, NMe), 2.18 (1H, br, OH), 2.12–1.91 (5H, m, H-2, H-4, H-6, H-6, H-7), 1.65 (2H, d, J = 14.4 Hz, H-2, H-4). ^{13}C NMR (101 MHz, $CDCl_3$) δ 63.4, 59.9, 59.8, 40.1, 39.7, 25.6, 25.6 (m), 25.3 (m). ESI-HRMS Calcd. for $C_8H_{15}DNO^+$ $[M+H]^+$ 143.1289, found 143.1309.

[6β-^{2}H]Hyoscyamine 11. Based on the same procedure as described for **12**, compound **11** was synthesized in 92% yield with a trace amount of the unwanted 2′*R* isomer. Further purification will be described in Sec. S5.2. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.32–7.21 (5H, m, Ph), 4.97 (1H, t, J = 5.3 Hz, H-3), 4.13 (1H, m, H-3′), 3.76 (1H, m, J = 5.3 Hz, H-3′), 3.74

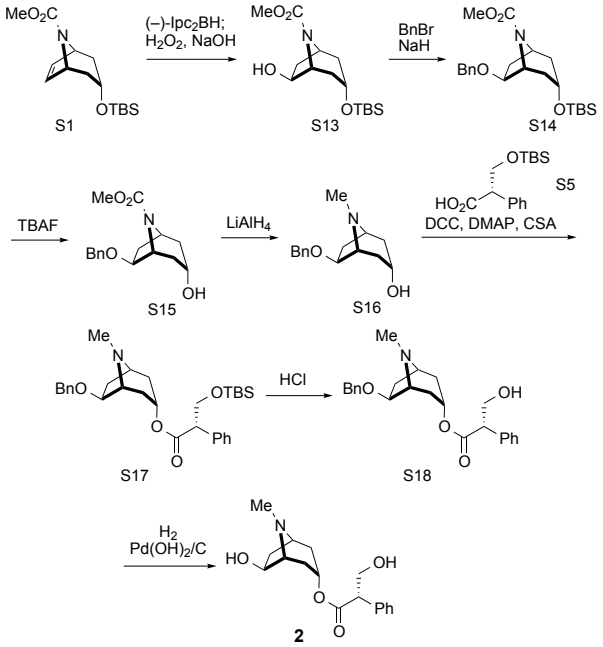


Figure S7: Synthesis of 6β-hydroxyhyoscyamine (**2**).

(1H, m, H-2′), 3.72 (1H, br, OH), 2.98 (1H, m, H-5), 2.87 (1H, m, H-1), 2.14 (3H, s, NMe), 2.05 (1H, ddd, J = 3.8 Hz, J = 5.5 Hz, J = 15.1 Hz, H-4), 1.98 (1H, m, H-2), 1.75–1.63 (2H, m, H-6, H-7), 1.64 (1H, d, J = 14.7 Hz, H-4), 1.43 (1H, d, J = 15.0 Hz, H-2), 1.18 (1H, dd, J = 9.4 Hz, J = 12.8, H-7). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 135.8, 128.8, 128.1, 127.6, 68.0, 64.0, 59.5, 59.4, 54.6, 40.2, 36.3, 36.1, 25.2 (m), 24.5. ESI-HRMS Calcd. for $C_{17}H_{23}DNO_3^+$ $[M+H]^+$ 291.1813, found 291.1847.

S5.1.3 Synthesis of 6β-hydroxyhyoscyamine

6β-hydroxyhyoscyamine (2). 6β-hydroxyhyoscyamine (**2**) was synthesized based on the same procedure previously described for 7β-hydroxyhyoscyamine (**10**),[1] except that $(-)$-Ipc_2BH was used for enantioselective hydroboration of **S1** (Fig. S7). The obtained NMR and MS data were consistent with the natural product isolated from plants.[10] ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37–7.23 (5H, m, Ph), 5.02 (1H, dd, J = 5.3 Hz, J = 5.5 Hz, H-3), 4.16 (1H, m, H-3′), 3.81 (1H, m, H-3′), 3.78 (1H, m, H-2′), 3.07 (1H, m, H-1), 3.02 (2H, br, OH), 2.94 (1H, m, H-5), 2.46 (3H, s, NMe), 2.15 (1H, dd, J = 5.5 Hz, J = 15.6 Hz, H-4), 2.05 (1H, dd, J = 5.3 Hz, J = 15.6 Hz, H-2), 1.60 (1H, d, J = 15.6 Hz, H-4), 1.22 (1H, d, J = 15.6 Hz, H-2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 135.4, 128.9, 128.1, 127.8, 75.6, 67.7, 66.8, 64.1, 58.1, 54.4, 39.7, 36.4, 29.8, 28.7. ESI-HRMS Calcd. for $C_{17}H_{24}NO_4^+$ $[M+H]^+$ 306.1700, found 306.1710.

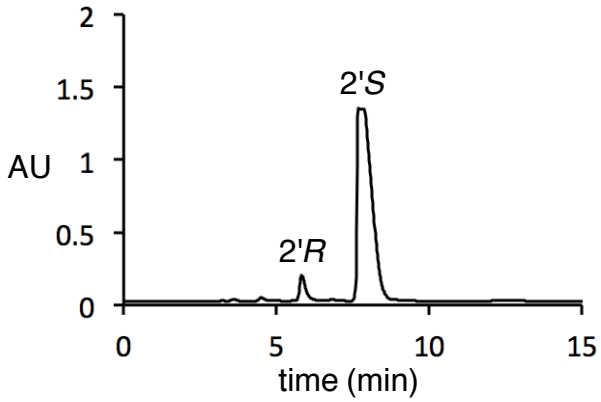


Figure S8: Separation of (2′*S*)-hyoscyamine. A representative HPLC trace for **1** is shown.

S5.2 Purification of hyoscyamine analogues

(2′*S*)-hyoscyamines (e.g. **1**, **11**, **12**) were separated from mixtures with minor (2′*R*) isomer using a chiral cellulose column (CHIRAL ART Cellulose-C, 5 μm, 250 × 4.6 mm, YMC America, Inc. (Allentown, PA)) under the following conditions: 10% ethanol in hexanes with 0.1% diethylamine (isocratic), flow rate 1 mL/min, detection at 230 nm (Fig. S8). The 2′*S* isomers were eluted at approximately 8 min. The desired compounds were collected and concentrated under reduced pressure. The resulting residue was dissolved in methanol and acidified with 1 N HCl. The mixture was dried and redissolved in water to prepare the stock solution.

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