Installation of the ether bridge of lolines by the iron- and 2-oxoglutarate-dependent oxygenase, LolO: regio- and stereochemistry of sequential hydroxylation and oxacyclization reactions

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Supporting Information

LC–MS analysis of LolO products in the presence of $^{16}$O$_2$ and $^{18}$O$_2$ (p. 2, Figure S1); NMR spectra of isolated 2-OH-AcAP (pp. 3–6, Figures S2–S5); substrate binding and determination of $K_d$ (p. 7, Figure S6); comparison of the kinetics of the Fe$^{IV}$–oxo complex defined by stopped-flow absorption and freeze-quench Mössbauer spectroscopies (p. 8, Figure S7); protein sequence alignment of LolO to T7H and the active site of T7H (p. 9, Figure S8); synthetic procedures and data for intermediates and products (pp. 10–22); NMR spectrum of trans-3-[2H]Pro·HCl showing contamination with cis isomer (p. 23, Figure S9); method of preparation of the LolO expression vector; methods for enzyme purification and fungal culture feeding (pp. 24–26); analysis of the effect of LolC on the fate of C3-2H in Asp (pp. 26–28); references (p. 29); NMR spectra of synthetic compounds (pp. 30–65).
Figure S1. LC–MS chromatograms of the products of the LolO in vitro enzyme assay with $^{16}\text{O}_2$ or $^{18}\text{O}_2$. A reaction mixture consisting of 0.125 mM LolO, 0.1 mM Fe$^{II}$, 0.4 mM AcAP, and varying amounts of 2OG (from less than unity to excess) in 50 mM HEPES buffer, pH 7.5, was prepared in the absence of O$_2$. Reactions were initiated by injection of an equal volume of the same buffer saturated with $^{16}\text{O}_2$ or $^{18}\text{O}_2$ gas, and the reactions were allowed to proceed at ambient temperature for 5 min before the LC–MS analysis.
Figure S2. 600 MHz $^1$H NMR spectrum of the isolated intermediate, 2-OH-AcAP, in $^2$H$_2$O. The sample was contaminated with NH$_4$OAc from the chromatography mobile phase.
Figure S3. 125 MHz $^{13}$C NMR spectrum of the isolated intermediate, 2-OH-AcAP in $^2$H$_2$O. The sample was contaminated with NH$_4$OAc from the chromatography mobile phase.
Figure S4. 400 MHz $^1$H–$^1$H COSY NMR spectrum of the isolated intermediate, 2-OH-AcAP in $^2$H$_2$O.
Figure S5. 600 MHz $^1$H NMR spectrum of 2-OH-AcAP in $^2$H$_2$O (lower, red), and NOE difference spectrum upon irradiation of the resonance at 4.43 ppm (upper, green).
Figure S6. Stopped-flow absorption experiment monitoring the effect of substrate concentration on accumulation of the ferryl intermediate in the LolO hydroxylation reaction. An anoxic solution containing 0.50 mM LolO, 0.40 mM Fe$^{II}$, 2.5 mM 2OG, and 2,2,7,7,8-[²H₅]AcAP in 50 mM HEPES buffer (pH 7.5) was mixed at 5 °C with an equal volume of O₂-saturated (at 20 °C) buffer. A, Change in absorbance at 320 nm as a function of time after mixing. The solid lines are fits of the equation for change in absorbance as a function of time in a sequence of two consecutive first-order reactions with isosbestic reactant and product and more intensely absorbing intermediate. The concentrations indicated in the legend are of the correct enantiomer, (1S)-2,2,7,7,8-[²H₅]AcAP (half the total concentration of the racemic synthetic substrate), in the LolO reactant solution (before mixing). B, Amplitude of the change in absorbance (difference between maximum and minimum values, proportional to the quantity of the intermediate that accumulates) plotted as a function of the concentration of the correct substrate enantiomer. The solid line is a fit of the quadratic equation for binding to the data; it yielded an apparent dissociation constant ($K_d$) of 13 µM.
Figure S7. Comparison of $\Delta A_{320}$ traces from the stopped-flow absorption experiments and the quantities of ferryl intermediate determined by analysis of the Mössbauer spectra of freeze-quenched samples prepared under identical reaction conditions. Black open circles are the $\Delta A_{320}$ data from the stopped-flow experiment with 2,2,8-$[^2\text{H}_3]\text{AcAP}$. Green and blue circles are from simulation of the traces for the reactions with 2,2,8-$[^2\text{H}_3]\text{AcAP}$ and AcAP, respectively, in KinTek Explorer as described in the main text. The simulations for the reaction with unlabeled AcAP employed the kinetic parameters given in Table 1. The red squares and triangles are the concentrations of the ferryl intermediate in the reactions with 2,2,8-$[^2\text{H}_3]\text{AcAP}$ and unlabeled AcAP, respectively, determined from analysis of the 4.2 K and 53 mT Mössbauer spectra. The relative scaling was determined by using $\Delta \varepsilon_{320} = 2000 \text{ M}^{-1} \text{ cm}^{-1}$, the best-fit value for the change in molar absorptivity from the reactant state to the ferryl intermediate state obtained in the regression analysis using KinTek Explorer.
**Figure S8.** Left, Alignment of the (partial) amino acid sequences of LolO and thymine 7-hydroxylase (T7H). The conserved residues are shaded gray, with the ligand residues highlighted in red. Right, model of the T7H active site showing the cofactor coordination sphere, including the two His and one Asp residues that are conserved in the LolO sequence.
Synthetic procedures

Di-tert-butyl (±)-3-hydroxy-3-deuteropyrrolidine-1,2-dicarboxylate (6)

Sodium borodeuteride (1.83 g, 43.9 mmol) was added to a solution of 1⁺ (5.01 g, 17.6 mmol) in dry THF (66 mL) at 0 °C in small portions over a period of 1 h. The reaction mixture was allowed to stir for another 3 h at 0 °C and allowed to warm up to ambient temperature. The solvent was evaporated, and the residue was dissolved in CH₂Cl₂. The pH of the reaction mixture was adjusted to 2 by dropwise addition of 1 N HCl with stirring at 0 °C. The organic layer was washed with brine, dried with MgSO₄, and evaporated to give 6 (4.61 g, 15.9 mmol, 91% yield) as a light-yellow powder that consisted of a mixture of diastereomers. ¹H NMR (400 MHz, CDCl₃) δ 1.36 (s, 9H; major), 1.38 (s, 9H; minor), 1.40 (s, 9H; minor), 1.41 (s, 9H; major), 1.88–2.05 (m, 2H), 3.25–3.64 (m, 2 H), 4.12 (s, 1H; major), 4.17 (s, 1H; minor). ²H NMR (61.5 MHz, CDCl₃): δ 4.44 (broad s, 1H). ¹³C NMR (100 MHz, CDCl₃) δ (major) 28.2, 28.5, 32.1, 44.4, 64.3, 71.9 (t, 22.3 Hz), 80.2, 82.0, 154.2, 169.7; δ (minor) 28.3, 28.4, 32.7, 43.9, 64.0, 70.9 (t, 22.0 Hz), 80.0, 81.9, 154.3, 170.4. IR (ATR): 3428, 2165, 1736, 1678 cm⁻¹. HRMS: m/z calcld for C₁₄H₂₅DNO₅ (M + H): 289.3621; found: 289.1868.

Di-tert-butyl (±)-3,3-dideuteropyrrolidine-1,2-dicarboxylate (7)

Triethylamine (49.1 mL, 48.5 mmol) was added to a mixture of 6⁺ (13.9 g, 48.5 mmol) and
DMAP (5.92 g, 48.5 mmol) in dry CH$_2$Cl$_2$ (320 mL). The reaction mixture was cooled to 0 ºC, and TsCl (10.7 g, 56.2 mmol) was added in portions over 1 h. Stirring was continued for another 54 h at 0 ºC, after which a saturated solution of NH$_4$Cl was added to the reaction mixture at 0 ºC, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic extracts were washed with brine, dried with MgSO$_4$ and evaporated. Silica gel chromatography (30% EtOAc in petroleum ether) afforded crude tosylate (9.83 g, 22.2 mmol, 46% yield), which was taken to the next step without purification. Sodium borodeuteride (5.76 g, 13.8 mmol) was added to a solution of the tosylate (10.5 g, 23.8 mmol) in dry DMSO (310 mL), and the mixture was heated to 95 ºC under nitrogen for 8 h. The reaction mixture was allowed to cool, diluted with brine (effervescence), and extracted with ether. The combined organic layers were dried over MgSO$_4$, and the solvent was evaporated. Flash chromatography (40% EtOAc in petroleum ether) afforded 7 (4.83 g, 17.7 mmol, 79% yield) as a white gum. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.44 (s, 9H; major), 1.45 (s, 9H; minor), 1.47 (s, 9H; major), 1.48 (s, 9H; minor), 1.77–1.97 (m, 2H), 3.47 (m, 2H), 4.10 (s, 1 H; major), 4.18 (s, 1 H; minor). $^2$H NMR (61.5 MHz, CDCl$_3$): $\delta$ 1.81 (broad s, 1H), 2.07 (broad s, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ (major) 23.3, 28.1, 28.4, 30.3 (quintet, 20.3 Hz), 46.4, 59.6, 79.6, 80.8, 154.0, 154.4, 172.4; $\delta$ (minor) 24.1, 28.0, 28.5, 29.3 (quintet, 20.3 Hz), 46.6, 59.6, 79.4, 153.8, 153.8, 172.3. IR (ATR): 2283-2162, 1737, 1697 cm$^{-1}$. GC-MS (EI): 217 (5%), 203 (2%), 187 (3%), 172 (25%), 144(17%), 116 (100%), 72 (97%), 57 (95%).

(±)-3,3-Dideuteroproline hydrochloride (3,3-[2H$_2$]Pro·HCl)
Aqueous HCl (6.0 M, 50 mL) was added to a solution of 7 (9.21 g, 33.7 mmol) and allowed to stir for 2 h at room temperature. The water was evaporated to give (±)-3,3-[\textsuperscript{2}H\textsubscript{2}]Pro·HCl (2.82 g, 18.4 mmol, 55% yield) as a brown gum. \textsuperscript{1}H NMR (400 MHz, \textsuperscript{2}H\textsubscript{2}O) \(\delta\) 2.04 (t, 7.3 Hz, 2H), 3.40 (qt, \(J_\text{t}=7.3\) Hz, \(J_\text{q}=11.81\) Hz, 2H), 4.42 (s, 1H). \textsuperscript{2}H NMR (61.5 MHz, \textsuperscript{2}H\textsubscript{2}O): \(\delta\) 2.14 (broad s, 1H), 2.41 (broad s, 1H), 8.39 (broad s, 1H), 8.92 (broad s, 1H). \textsuperscript{13}C NMR (100 MHz, \textsuperscript{2}H\textsubscript{2}O) \(\delta\) 25.8, 30.4 (quintet, 20.8 Hz), 49.0, 62.1, 174.6. IR (ATR): 3359, 2032–1926, 1727, 1626 cm\textsuperscript{-1}. EI-MS: Positive ion 117.9 amu, negative ion 173 amu.

**Ethyl (±)-3,3-dideuteroprolinate ((±)-3,3-[\textsuperscript{2}H\textsubscript{2}]8)**

![Chemical Structure](image)

Proline derivative (±)-3,3-[\textsuperscript{2}H\textsubscript{2}]Pro·HCl (3.15 g, 20.5 mmol) was suspended in ethanol (28 mL) and heated to 60 °C. Thionyl chloride (12.2 g, 102 mmol) was added dropwise, and the resulting solution was allowed to reflux for 4 h. Excess thionyl chloride and ethanol were evaporated, and the resulting solid was dissolved in CH\textsubscript{2}Cl\textsubscript{2} (18 mL). Water (3 mL) was added to the reaction mixture, and the pH was adjusted by dropwise addition of conc. aq. NH\textsubscript{4}OH (5 mL). The aqueous layer was extracted with CH\textsubscript{2}Cl\textsubscript{2}. The combined organic layers were dried over MgSO\textsubscript{4}, and the solvent was evaporated to afford (±)-3,3-[\textsuperscript{2}H\textsubscript{2}]8 (2.1 g, 14 mmol, 69% yield) as a yellow oil. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 1.28 (t, 7.1 Hz, 3H), 1.65–1.84 (m, 2 H), 2.92 (dt, \(J_\text{d}=10.2\) Hz, \(J_\text{t}=6.7\) Hz, 1H), 3.08 (dt, \(J_\text{d}=10.2\) Hz, \(J_\text{t}=6.8\) Hz, 1H), 3.75 (s, 1H), 4.18 (q, 7.1 Hz, 2H). \textsuperscript{2}H NMR (61.5 MHz, CDCl\textsubscript{3}): \(\delta\) 1.68 (broad s, 1H), 1.96 (broad s, 1H). \textsuperscript{13}C NMR (100 MHz,
CDCl$_3$ δ 14.1, 25.1, 29.4 (quintet, 20.3 Hz), 46.9, 59.5, 60.7, 175.1. IR (ATR): 3347, 2230–
2125, 1727 cm$^{-1}$. GC–MS (EI): 145 (5%), 72 (100%), 55 (2%).

**Ethyl (±)-3,3-dideutero-N-(3-ethoxy-3-oxopropyl)prolinate ((±)-3,3-[2H]$_2$2)**

Proline ester (±)-3,3-[2H]$_2$8 (2.06 g, 14.7 mmol) and ethyl acrylate (7.09 g, 70.8 mmol) were combined and allowed to reflux under nitrogen for 24 h. The reaction mixture was allowed to come to room temperature, and the solvent was evaporated. Silica gel chromatography (40% EtOAc in petroleum ether) afforded (±)-3,3-[2H]$_2$2 (2.97 g, 12 mmol, 85% yield) as a yellow oil.

$^1$H NMR (400 MHz, CDCl$_3$): δ 1.25 (t, 7.1 Hz, 3H), 1.28 (t, 7.1 Hz, 3H), 1.75–1.95 (m, 2H), 2.43 (q, 8.5 Hz, 1H), 2.52 (t, 7.6 Hz, 1H), 2.71-2.81 (m, 1H), 3.04 (dt, $J_d = 12.2$ Hz, $J_t = 7.7$ Hz, 1H), 4.15 (dt, $J_d = 3.4$ Hz, $J_t = 8.0$ Hz, 1H), 3.21 (s, 1H), 4.13 (q, 7.1 Hz, 2H), 4.18 (q, 7.1 Hz, 2H). $^2$H NMR (61.5 MHz, CDCl$_3$): δ 1.71 (broad s, 1H), 1.88 (broad s, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 13.8, 13.9, 22.8, 28.5 (quintet, 20.1 Hz), 33.6, 49.5, 52.9, 60.0, 60.2, 65.3, 171.8, 173.6. IR (ATR): 2233-2131, 1728, 1612 cm$^{-1}$. HRMS: $m/z$ calcd for C$_{12}$H$_{20}$D$_2$O$_4$N (M + H): 246.3227; found: 246.1669.
(±)-7,7-Dideutero-1-oximinopyrrolizidine ((±)-7,7-[2H]3)

To a solution of diisopropylamine (2.18 mL, 21.5 mmol) in dry THF (85 mL) under nitrogen at –78 °C was added n-BuLi (2.4 M in hexane) (7.5 mL). The reaction mixture was allowed to stir for 1 h. A solution of (±)-3,3-[2H]2 (2.21 g, 8.9 mmol) in THF (43 mL) was prepared and added to the reaction mixture dropwise. The reaction mixture was allowed to stir at –78 °C for 18 h and then brought to room temperature. Water (30 mL) was added, and then conc. HCl (30 mL) was added dropwise at 0 °C. After refluxing for 1.5 h, the reaction mixture was adjusted to pH 9 by addition of a saturated solution of K2CO3 in H2O at 0 °C. Next, NH2OH·HCl (0.62 g, 8.9 mmol) was added to the reaction mixture, which was allowed to reflux for 2 h. The mixture was then allowed to stir for 24 h. The solvent was evaporated, and the residue was extracted with CH2Cl2. The solution was concentrated and applied to a silica column, and then eluted with CH2Cl2:CH3OH:NH4OH (8:2:0.2). The solvent was evaporated to give (±)-7,7-[2H]3 (0.6 g, 4.2 mmol, 47% yield) as a light brown powder that consisted of an inseparable mixture of diastereomers. 1H NMR (400 MHz, CDCl3): δ 4.07 (s, 1H), 3.80 (s, 1H), 3.20–2.82 (m, 6H), 2.84–2.39 (m, 6H), 1.94–1.68 (m, 4H). 2H NMR (61.5 MHz, CDCl3): δ 2.29 (broad s, 1H), 2.21 (broad s, 1H), 1.88 (s, 1H), 1.75 (broad s, 1H). 13C NMR (100 MHz, CD3OD): δ (major) 26.0, 30.3 (m, 20 Hz), 30.9, 51.7, 54.4, 64.4, 166.2; δ (minor) 25.6, 30.2 (m, 20 Hz), 51.1, 54.2, 62.9, 165.8. IR (ATR): 3202, 2235-2125, 1842, 1675, 1509 cm⁻¹. HRMS: m/z calcd for C7H11D2N2O (M + H): 143.1148; found: 143.1149.
Sodium borohydride (0.19 g, 5.0 mmol) was added in small portions to a solution of (±)-7,7-
\([\text{\textsuperscript{2}}\text{H}_2]\)3 (0.08 g, 0.5 mmol) and NiCl\(_2\) (0.21 g, 1.6 mmol) in anhydrous MeOH:THF (3:1, 5 mL) at
–60 °C over a period of 2 h (caution: gas evolution!). After the addition was complete, the
resulting black slurry was allowed to warm to –30 °C and was allowed to stir at this temperature
until no starting material could be detected by TLC (18 h). The reaction mixture was allowed to
warm to room temperature, and Ac\(_2\)O (in excess) was added with vigorous stirring. The
heterogeneous mixture was allowed to stir for another 4 h before conc.
NH\(_4\)OH (10 mL) was added while stirring. The mixture was filtered through a short pad of Celite. The organic solvent
was evaporated, the residue was extracted with CH\(_2\)Cl\(_2\) (3 times), and the combined organic layer
was washed with brine (2 times), dried over anhydrous MgSO\(_4\), and concentrated under vacuum.
The crude product was purified by flash chromatography and eluted with
CH\(_2\)Cl\(_2\):CH\(_3\)OH:NH\(_4\)OH (6:4:0.2) to afford (±)-7,7-[\text{\textsuperscript{2}}\text{H}_2]\)AcAP (13.2 mg, 0.078 mmol, 13%
yield) as a gummy yellow solid. \(^1\text{H}\) NMR (400 MHz, CDCl\(_3\)): \(\delta\) 1.77 (m, 2H), 1.86 (dq, \(J_d =
12.1\) Hz, \(J_t = 6.0\) Hz, 1H), 1.99 (s, 3H), 2.21 (d quintet, \(J_d = 13.0\) Hz, \(J_{\text{quintet}} = 6.6\) Hz, 1H), 2.66
(dq, \(J_d = 6.7\) Hz, \(J_q = 9.8\) Hz, 2H), 3.08 (dt, \(J_d = 10.7\) Hz, \(J_t = 6.4\) Hz, 1H), 3.41-3.22 (m, 2H),
4.15 (ddt, \(J_d = 6.7\) Hz, \(J_d = 8.0\) Hz, \(J_t = 5.1\) Hz, 1H), 6.3 (broad s, 1H). \(^2\text{H}\) NMR (61.5 MHz,
CDCl\(_3\)): \(\delta\) 1.98 (broad s, 1H), 1.62 (broad s, 1H). \(^1\text{C}\) NMR (100 MHz, CDCl\(_3\)): \(\delta\) 23.5, 25.3,
30.0 (m, 20 Hz), 32.8, 53.4, 55.2, 55.4, 70.9, 170.2. IR (ATR): 3233, 2233 (weak), 1659, 1541 cm\(^{-1}\). HRMS: \(m/z\) calcld for C\(_9\)H\(_{15}\)D\(_2\)ON\(_2\) (M + H): 171.1461; found: 171.2597.

**(±)-2,2,8-Trideutero-1-oximinopyrrolizidine ((±)-2,2,8-[\(^2\)H\(_3\)]3)**

A solution of \(n\)-BuLi in hexane (2.50 M, 11.1 mL) was added to a solution of diisopropylamine (4.60 mL) in dry THF (100 mL) at \(-78\) °C under nitrogen. After 45 min, a solution of \(2^i\) (3.40 g, 13.8 mmol) in THF was added dropwise. The reaction mixture was allowed to stir at \(-78\) °C for 18 h and then brought to room temperature. Next, D\(_2\)O (30 mL) was added, and then 7.7 M DCl in D\(_2\)O (35 mL) was slowly added to the reaction mixture at 0 °C. The mixture was allowed to reflux for 1.5 h. The pH of the reaction mixture was adjusted to 9 by dropwise addition of saturated K\(_2\)CO\(_3\) in D\(_2\)O at 0 °C. Next, NH\(_2\)OH·HCl (0.96 g, 13.8 mmol) was added, and the mixture was allowed to reflux for 2 h. The mixture was then allowed to stir for 24 h. The solvent was evaporated, and the crude mixture was extracted with CH\(_2\)Cl\(_2\). The solvent was concentrated and applied to a silica column, and then eluted with CH\(_2\)Cl\(_2\)-CH\(_3\)OH-NH\(_4\)OH (8:2:0.2). The solvent was evaporated to give (±)-2,2,8-[\(^2\)H\(_3\)]3 (0.49 g, 3.47 mmol, 25% yield) as a light brown powder and as an inseparable mixture of diastereomers. \(^1\)H NMR (400 MHz, CD\(_3\)OD): \(\delta\) 3.17–2.50 (m, 4H), 2.33–2.18 (m, 1H, major), 2.17–2.05 (m, 1H, minor), 1.99–1.50 (m, 3H); \(^2\)H NMR (61.5 MHz, CDCl\(_3\)): \(\delta\) 4.07 (broad s, minor), 3.82 (broad s, major), 2.88–2.21 (merged broad singlets). \(^13\)C NMR (100 MHz, CD\(_3\)OD): \(\delta\) (major) 26.2, 28.7 (m), 30.8, 50.8, 54.2, 65.9 (t, 21.8
Hz), 166.0; δ (minor) 25.9, 30.2, 51.4, 54.9, 63.9 (t, 21.8 Hz), 165.8. IR (ATR): 3195, 2177, 1844, 1675, 1509 cm\(^{-1}\). HRMS: \(m/z\) calcd for \(C_7H_{10}D_3ON_2\) (M + H): 144.1211; found: 144.1210.

(±)-2,2,8-Trideutero-1-exo-acetamidopyrrolizidine ((±)-2,2,8-\[^2\text{H}_3\]AcAP)

Sodium borohydride (0.24 g, 6.25 mmol), (±)-2,2,8-\[^2\text{H}_3\]3 (96 mg, 0.67 mmol), and NiCl\(_2\) (0.26 g, 2.01 mmol) were combined in the same way as described for the preparation of (±)-7,7-\[^2\text{H}_2\]AcAP to afford (±)-2,2,8-\[^2\text{H}_3\]AcAP (28 mg, 0.16 mmol, 25% yield) as a gummy yellow solid. \(^1\text{H}\) NMR (400 MHz, CDCl\(_3\)): δ 1.60 (dt, \(J_t = 7.4\) Hz, \(J_d = 12.3\) Hz, 1H), 1.74 (dq, \(J_d = 12.4\) Hz, \(J_t = 6.9\) Hz, 1H), 1.83 (ddt, \(J_d = 18.6\) Hz, \(J_d = 7.4\) Hz, \(J_t = 6.2\) Hz, 1H), 1.97 (overlapping s, 4H), 2.61 (dt, \(J_d = 11.9\) Hz, \(J_t = 6.6\) Hz, 2H), 3.00 (dt, \(J_d = 10.5\) Hz, \(J_t = 6.3\) Hz, 1H), 3.18 (d, \(J_d = 10.6\) Hz, 1H), 4.10 (d, \(J_d = 8.0\) Hz, 1H), 5.88 (broad s, 1H). \(^2\text{H}\) NMR (61.5 MHz, CDCl\(_3\)): δ 3.20 (broad s, 1H), 2.18 (broad s, 1H), 1.68 (s, 1H). \(^{13}\text{C}\) NMR (100 MHz, CDCl\(_3\)): δ 23.6, 25.6, 30.7, 53.3, 55.3, 55.5, 170.1. IR (ATR): 3257, 2239 (weak), 1652, 1552 cm\(^{-1}\). HRMS: \(m/z\) calcd for \(C_9H_{14}D_3ON_2\) (M + H): 172.2583; found: 172.1524.
**N-tert-Butoxycarbonyl-trans-3-hydroxy-L-proline (9)**

A 10% aqueous solution of NaOH (2.2 mL) was added to trans-3-hydroxy-L-proline (1.0 g, 7.6 mmol) suspended in a mixture of THF:H₂O (2:1, 10 mL). Di-tert-butyl dicarbonate (1.7 g, 7.6 mmol) was added, and the reaction mixture was stirred for 21 h at room temperature. The organic solvent was evaporated, and the pH of the mixture was adjusted to 2 by addition of 10% aqueous KHSO₄ solution. The mixture was extracted with EtOAc, washed with brine, dried with MgSO₄, and evaporated to give crude 9 (1.7 g, 7.4 mmol, 96% yield) as a yellow viscous oil. ¹H NMR (400 MHz, CDCl₃) δ 1.43 (s, 9H; major), 1.47 (s, 9H; minor), 1.82–1.93 (m, 2H; minor), 1.98–2.10 (m, 2H; major), 3.46–3.62 (m, 4H), 4.10 (br, 1H; major), 4.17 (br, 1H; minor), 4.37–4.41 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ (major) 28.7, 33.0, 45.5, 69.6, 75.8, 81.3, 156.4, 174.3; δ (minor) 28.9, 33.6, 45.9, 69.1, 75.0, 81.5, 156.1, 174.0. IR (ATR): 3283, 1741, 1653 cm⁻¹.

**tert-Butyl N-tert-butoxycarbonyl-trans-3-hydroxy-L-prolinate (4)**

The orthoamide Me₂NCH(O-t-Bu)₂ (5.8 mL) was added to 9³ (1.54 g, 6.67 mmol), and the solution was allowed to stir for 12 h at 80 °C under nitrogen. The solution was cooled to room temperature, and H₂O (5.8 mL) was added to it. The mixture was stirred for 22 h at room
temperature and saturated aqueous NaHCO₃ (15 mL) was added to it, followed by extraction with Et₂O. The organic layer was washed with water and brine and dried with MgSO₄. The crude compound was purified by flash chromatography (5% MeOH in CH₂Cl₂) to give 4 (1.02 g, 3.55 mmol, 53% yield) as a yellow viscous oil.¹H NMR (400 MHz, CDCl₃) δ 1.35 (s, 9H; major), 1.37 (s, 9H; minor), 1.38 (s, 9H; minor), 1.39 (s, 9H; major), 1.78–2.04 (m, 2H), 2.8 (br s, 1H; minor), 2.9 (br s, 1H; major), 3.44–3.60 (m, 2 H), 4.02 (br s, 1H; major), 4.08 (br s, 1H; minor), 4.26–4.35 (m, 1H).¹³C NMR (100 MHz, CDCl₃) δ (major) 27.8, 31.8, 44.2, 68.7, 74.7, 79.7, 81.2, 154.2, 170.3; δ (minor) 28.2, 32.4, 44.5, 68.6, 73.7, 79.5, 81.4, 154.5, 170.2. IR (ATR): 3454, 3357, 1735, 1660 cm⁻¹. HRMS: m/z calcd for C₁₄H₂₆NO₅ (M + H): 288.3635; found: 288.1807.

**tert-Butyl N-tert-butoxycarbonyl-cis-3-hydroxy-L-proline (5)**

To a solution of triphenylphosphine (1.28 g, 4.87 mmol), benzoic acid (0.59 g, 4.9 mmol), and 4² (700 mg, 2.44 mmol) in dry THF (20 mL) at 0 ºC under nitrogen, DEAD (848 mg, 4.87 mmol) in dry THF (8 mL) was added. The reaction mixture was stirred for 5 min at 0 ºC, and then for 22 h at room temperature. The solvent was evaporated, the residue was resuspended in methanolic KOH solution (1.0 M, 43 mL) at 0 ºC, and the mixture was stirred for 30 min. The solvent was evaporated, and the residue was partitioned between EtOAc and water. The organic layer was washed with brine, dried with MgSO₄, and evaporated. Flash chromatography (30% acetone in petroleum ether) afforded 5 (624 mg, 2.17 mmol, 89% over two steps) as a white solid.¹H NMR
(400 MHz, CDCl$_3$) $\delta$ 1.35 (s, 9H; major), 1.37 (s, 9H; minor), 1.39 (s, 9H; minor), 1.40 (s, 9H; major), 1.86–2.06 (m, 2H), 3.23–3.41 (m, 1H), 3.44–3.61 (m, 1H), 4.12 (d, 7.0 Hz, 1H; major), 4.15 (d, 7.0 Hz, 1H; minor), 4.30–4.40 (quintet, 6.6 Hz, 1H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ (major) 28.0, 28.1, 31.7, 43.6, 64.0, 71.9, 79.7, 81.1, 154.0, 169.5; $\delta$ (minor) 27.9, 28.2, 32.2, 44.0, 63.8, 71.0, 79.5, 81.2, 154.2, 169.6. IR (ATR): 3240, 2162, 1740, 1693 cm$^{-1}$. HRMS: m/z calcd for C$_{14}$H$_{26}$NO$_5$ (M + H): 288.3635; found: 288.1807.

**tert-Butyl N-tert-butoxycarbonyl-cis-3-deutero-L-prolinate (10)**

![Chemical Structure](image)

Triethylamine (1.45 mL, 10.4 mmol) was added to a mixture of 4 (0.3 g, 1 mmol) and DMAP (0.13 g, 1.0 mmol) in dry CH$_2$Cl$_2$ (20 mL) TsCl (0.23 g, 1.2 mmol) was added in portions over 1 h at 0 ºC, and the mixture was allowed to stir at 0 ºC for 54 h. A saturated solution of NH$_4$Cl was added at 0 ºC, and aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic extracts were washed with brine, dried with MgSO$_4$ and evaporated. Silica gel chromatography (50% EtOAc in petroleum ether) afforded the tosylate (0.36 g). The tosylate was dissolved in dry DMSO (12 mL), and NaBD$_4$ (0.21 g, 4.9 mmol) was added. The reaction mixture was kept at 95 ºC under nitrogen for 8 h. It was cooled and diluted with brine (effervescence), and the aqueous phase was extracted with Et$_2$O. The combined organic layer was dried over MgSO$_4$, and the solvent was evaporated. Flash chromatography (40% EtOAc in petroleum ether) afforded 10 (0.185 g, 0.68 mmol, 65% yield) as a white gum. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.38 (s, 9H), S20
1.40 (s, 9H), 1.68–1.94 (m, 2H), 2.01–2.18 (m, 1H), 3.12–3.55 (m, 2H), 4.04 (d, 8.6 Hz, 1H; major), 4.12 (d, 8.6 Hz, 1H; minor). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) (major) 23.4, 28.1, 28.5, 30.7 (t, 20.8 Hz), 46.4, 59.8, 79.7, 80.9, 154.1, 172.4; \(\delta\) (minor) 23.2, 28.1, 28.5, 46.6, 59.7, 79.5, 154.4, 172.3. \(^2\)H NMR (61.5 MHz, CDCl\(_3\)): \(\delta\) 1.89 (broad s, 1H).

IR (neat): 1739, 1701 cm\(^{-1}\).

HRMS [M]+: \(m/z\) calcd for C\(_{17}\)H\(_{21}\)D\(_2\)NO\(_4\): 307.1747; found: 307.1744.

**cis-3-Deutero-L-proline-hydrochloride (cis-3-[\(^2\)H]Pro·HCl)**

![Chemical Structure](image)

Compound 10\(^d\) (185 mg, 0.680 mmol) was dissolved in 6 M HCl (7 mL), and the reaction mixture was allowed to stir for 2 h at room temperature. The water was evaporated to give cis-3-[\(^2\)H]Pro·HCl\(^d\) (79 mg, 0.52 mmol, 76% yield) as a brown gum. \(^1\)H NMR (400 MHz, \(\text{\(^2\)H}_2\text{O}\)) \(\delta\) 2.01–2.12 (q, 7.1 Hz, 2H), 2.35–2.49 (m, 1H), 3.42 (qt, \(J_t = 7.3\) Hz, \(J_q = 11.7\) Hz, 2H), 4.43 (d, 8.8 Hz, 1H). \(^{13}\)C NMR (100 MHz, \(\text{\(^2\)H}_2\text{O}\)) \(\delta\) 25.8, 30.5 (t, 20.4 Hz), 48.8, 62.0, 174.4. \(^2\)H NMR (61.5 MHz, \(\text{\(^2\)H}_2\text{O}\)); \(\delta\) 2.14 (broad s, 1H), 8.38 (broad s, 1H), 8.91 (broad s, 1H). IR (ATR): 3398, 2464, 1915, 1725 cm\(^{-1}\). EI-MS: Positive ion 116.9 amu ([M + H]), negative ion 35.1 amu ([Cl]).

**tert-Butyl N-tert-butoxycarbonyl-trans-3-deutero-L-prolinate (11)**

![Chemical Structure](image)

S21
Alcohol 5 (624 mg, 2.17 mmol), DMAP (0.265 g, 2.17 mmol), triethylamine (3.0 mL, 22 mmol), TsCl (0.50 g, 2.6 mmol), and NaBD₄ (260 mg, 6.22 mmol) were combined in the way described for the preparation of 10 to give 11 (256 mg, 0.941 mmol 43% yield), contaminated with a small amount of 10, as a white gum. ¹H NMR (400 MHz, CDCl₃) δ 1.36 (s, 9H), 1.39 (s, 9H), 1.68–1.92 (m, 3H), 2.01–2.18 (m, 1H), 3.19-3.55 (m, 2H), 4.03 (s, 1H; major), 4.11 (s, 1H; minor). ¹³C NMR (100 MHz, CDCl₃) δ (major) 23.4, 28.1, 28.5, 30.7 (t, 20.3 Hz), 46.4, 59.7, 79.7, 80.9, 154.1, 172.3; δ (minor) 24.2, 28.1, 28.5, 29.7 (t, 20.7 Hz), 46.6, 59.7, 79.5, 154.4, 172.4. ²H NMR (61.5 MHz, CDCl₃): δ 2.14 (broad s, 1H). IR (neat): 1738, 1689 cm⁻¹. HRMS [M⁺]: m/z calcd for C₁₇H₂₁D₂NO₄: 307.1747; found: 307.1744.

**trans-3-Deutero-L-proline hydrochloride (trans-3-[²H]Pro·HCl)**

![trans-3-[²H]Pro·HCl](image)

Compound 11⁺ (256 mg, 0.340 mmol) was dissolved in 6 M HCl (7.5 mL) and the reaction mixture was stirred for 2 h at room temperature. The water was evaporated to give trans-3-[²H]Pro·HCl⁺ (107 mg, 0.704 mol, 75% yield), contaminated with a small amount of cis-3-[²H]Pro·HCl, as a brown gum. (See spectra below.) ¹H NMR (400 MHz, D₂O) δ 2.00–2.25 (m, 3H), 3.36–3.53 (m, 2 H), 4.44 (d, 7.0 Hz, 1H). ¹³C NMR (100 MHz, D₂O) δ 25.8, 30.6 (t, 22 Hz), 48.7, 62.1, 174.5. ²H NMR (61.5 MHz, D₂O): δ 2.39 (broad s, 1H), 8.35 (broad s, 1H), 8.88 (broad s, 1H). IR (ATR): 3355, 2165–2066, 1736 cm⁻¹. EI-MS: Positive ion 116.9 amu ([M + H]), negative ion 35.1 amu ([Cl]).
Figure S9. 400 MHz $^1$H NMR spectra showing contamination of $trans$-3-$[^2]$H]Pro·HCl with $cis$-3-$[^2]$H]Pro·HCl. (A) Pro; (B) $cis$-3-$[^2]$H]Pro·HCl; (C) $trans$-3-$[^2]$H]Pro·HCl.
Plasmid construct for expression of lolO in E. coli

The DNA sequence of lolO2 of Epichloë uncinata e167 was codon-optimized for over-expression in E. coli, synthesized, and inserted into the Ndel and BamHI restriction sites of expression vector pET28a by GeneArt (Regensburg, Germany). The construct puts the gene under a T7 promoter and expresses LolO with an N-terminal hexahistidine tag. The codon-optimized gene sequence is shown below.

ATGACCGTTACCAATAAACCGGTTGAACCGGCAAATGTTCCGGTTATGGATTTTGAA
GCAATTTCATGCAAGCGTGGGTAATGAACGTAATAATCTGCGTCAGCTGGATGA
AGCATGTTCCCATCATGTTGCAGTTTTATGTTATTAATCATAGCATTGGCACCAGAAAC
CCTGGAAGAGGCATTTTTGTGTTGCAAAAAATTTCTTTGATCTGCCGCTGGCAGTGA
AAATTTCAGTTCATATTCGCCTGATGTGAAGCAACACATTTTCAAGGTTTGACCCGAC
CGGTGAAGCCATTAGCAGCAGGTTTGGGACCCCGGATGAAATGAACGTCTGC
GTAAAGAAAATGCGGACCGAATGAAAGCAATGGAATGCAAGCAGACCGGTGGC
ACCTATCCGCCTGGTAATCCGGATCTGAATCTGGTTGAACACGATCTGCCTGCTGTAT
CTGGATTTTTCTGAAAAATGGTTCGAGCCTGCTATAACAGAGCTGCAAGAAATATG
CGTCTGGTTTTGGAATTTCTGGGATGGAAGATCTGGATTATTACGGAACACCGGTGGT
AGTCAGCTGGCAAGCGGTAGGAGCAGATCGTGTCAACCTGGAACGTATTATGCGTAGG
TTACCATGCTGTTTTCAAGGATAGGTTGGTGGTCTGGAAATGCAAGTATGATTAAGAA
GATATTTATCGCTCCGGTGCTCCGATATAAGGTGCAGTATTGTCAGGTGGGAC
CTGCTGGAAACACAGGACCATGCTGGTGCTGGGCTACGTCGACCAGTGTTACCGCA
CCGAGCCGGTTATATGTATGGGTGATGCTCAGGTGGGATGATGAAATGATGATTAGG
TATAGCTGGTTTTTTTGTGTCATCTGAACCTGGATGAAATGATTTAACGCGTGGCA
Preparation of anoxic enzyme and buffers

The solution of purified LolO was placed into a vacuum flask and purged with Ar on a vacuum/gas manifold to remove $O_2$. After ~50 cycles of gentle evacuation and refilling with argon over a period of ~1 h while gently stirring on ice, the flask was sealed and kept on ice overnight to allow for equilibration of the solution and gas phase. The protein solution was then divided into aliquots in an anoxic chamber with $N_2$ atmosphere and stored in liquid $N_2$ until being used. The deoxygenated LolO solution was used for all *in vitro* experiments.

The $O_2$-free buffers were prepared by placing the buffers in round-bottom flasks and evacuating for 30 min, then filling the head space with Ar for 10 min while stirring. The buffers were kept under vacuum for another 2 h to remove residual $O_2$ before being refilled with Ar, sealed, and transferred to an anoxic chamber until use.

The air-saturated buffers were prepared by placing the solution in a round-bottom flask and stirring overnight on ice while exposed to air until use. The $O_2$-saturated buffer was prepared in a similar manner. The buffers were stirred on ice on a vacuum/gas manifold and kept under vacuum for 30 min, and then the head space gas was exchanged with $O_2$ by evacuating and refilling with $O_2$ for ~10 cycles. The solution was kept under $O_2$ for another 2 h before use. $^{18}O_2$-equilibrated buffer was prepared by equilibrating the deoxygenated buffer under $^{18}O_2$ (400 torr) while stirring for 30 min.

**Fungal culture feeding experiments**
The procedures for fungal (Ep. uncinata e167) isolation, preparation of fungal culture media, and feeding with labeled substrates have been described. Solutions of substrates were prepared in deionized water and sterilized by filtration through a 0.2 µm membrane before feeding to loline-producing cultures (4 mM final) in triplicate on the 6th or 9th day of fungal growth. The cultures (1.7 mL) were harvested on the 15th or 16th day and lyophilized. Analysis of Variance was used to analyze the Asp feeding data and was carried out in PROC GLM in SAS 9.3 (SAS Institute, Cary, NC). Proline feeding data was analyzed by the standard t-test.

**Analysis of LolC on C3-D fate of labeled Asp**

Chemically synthesized (3S)-3-[2H]Asp and (3R)-3-[2H]Asp were used in this study to probe the stereochemistry of the LolO-mediated abstraction of hydrogen from C2 of AcAP. It was important to consider whether the biosynthetic steps leading from the labeled Asp compounds to the LolO substrate, AcAP, could change the C3 configuration and thus confound the analysis. We previously showed that the first step to the lolines is most likely a γ-substitution of the O-acetyl group of O-acetylhomoserine (O-Ac-Hse) by Pro catalyzed by LolC, which has high similarity (43% identity) to O-acetylhomoserine (thiol) lyase. The anticipated mechanism (Scheme S1) involves removal and restoration of one hydrogen from C3 of O-Ac-Hse, which originates from C3 of Asp, the position that we stereoselectively labeled with D. The action of LolC thus has the potential to affect the extent of D labeling and possibly even the stereochemistry at C3 in feeding the (3R)- and (3S)-3-[2H]Asp compounds to the fungal cultures. However, the structure of a closely related γ-type PLP enzyme, cystathionine γ-synthase, implies that the amine group of Lys261 removes and restores the C3 hydron with retention of stereochemistry, and LolC is expected to behave similarly. The implication is that action of LolC could lead to loss of deuterium from the stereospecifically labeled AcAP precursors but not
Scheme S1. Likely mechanism by which O-acetylhomoserine (thiol)lyase (Nu–H = H$_2$S) and LolC (Nu–H = Pro) catalyze γ-substitution reactions. The highlighted steps could lead to exchange of the involved hydron with solvent, resulting in loss of deuterium in the feeding studies.

For completeness, the outcomes of six different scenarios on the extent of deuteration of the NFL are compiled in Table S1. As noted, it is assumed in these predictions that LolC neither inverts nor racemizes the position originating from C3 of Asp. The fact that deuterium appears in a change in the configuration of the labeled carbon. If it had happened that LolE and LolO removed opposite hydrogens from this carbon, the experiment would almost certainly have failed, because each enzyme would have exchanged out one of the two positions, resulting in retention of deuterium for neither of the two labeled Asp compounds. By contrast, for the actual case that the two enzymes remove the same hydrogen, substitution of the other position allowed the deuterium to remain in place, through the action of both enzymes, to appear in the NFL product.
NFL for one of the isotopologs and is completely solvent-exchanged in the other validates our analysis.

Scheme S2. How LolC could alter D content and configuration of $O$-Ac-Hse isotopologs deuterated at C3. Panel A: LolC removes pro-$R$ H/D, restores a different H to same face. Panel B: LolC removes pro-$S$ H/D, restores a different H to same face. The H/D removed is highlighted in blue.

Table S1. Possible outcomes of feeding experiments of $(3R)$- and $(3S)$-$3$-[2H]Asp after the LolC and LolO reactions. The cells shaded in blue match the observed results.

| 3-[2H]Asp configuration at C3 | H/D that LolC removes from C3 | LolC restores on C3 | Asp C3 D after LolC | NFL from endo C2 H abstraction | NFL from exo C2 H abstraction |
|--------------------------------|-------------------------------|--------------------|---------------------|-------------------------------|-------------------------------|
| $R$                            | pro-$R$ or pro-$S$            | same atom          | $R$                 | $d_1$                         | $d_0$                         |
| $S$                            | pro-$R$ or pro-$S$            | same atom          | $S$                 | $d_0$                         | $d_1$                         |
| $R$                            | pro-$R$                       | different H$^+$    | none                | $d_0$                         | $d_0$                         |
| $S$                            | pro-$R$                       | different H$^+$    | $S$                 | $d_0$                         | $d_1$                         |
| $R$                            | pro-$S$                       | different H$^+$    | $R$                 | $d_1$                         | $d_0$                         |
| $S$                            | pro-$S$                       | different H$^+$    | none                | $d_0$                         | $d_0$                         |
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Figure S10. 400 MHz $^1$H NMR spectrum of 6 in CDCl$_3$. 
Figure S11. 100 MHz $^{13}$C NMR spectrum of 6 in CDCl$_3$. 
Figure S12. 400 MHz $^1$H NMR spectrum of 7 in CDCl$_3$. 
Figure S13. 100 MHz $^{13}$C NMR spectrum of 7 in CDCl$_3$. 
Figure S14. 400 MHz $^1$H NMR spectrum of (±)-3,3-[$^{2}$H$_2$]Pro·HCl in $^2$H$_2$O.
Figure S15. 100 MHz $^{13}$C NMR spectrum of (±)-3,3-$^{2}$H$_{2}$Pro·HCl in $^{2}$H$_{2}$O.
Figure S16. 400 MHz $^1$H NMR spectrum of (±)-3,3-$^2$H$_2$8 in CDCl$_3$. 
Figure S17. 100 MHz $^{13}$C NMR spectrum of (±)-3,3-$[^2]$H$_2$8 in CDCl$_3$. 

S37
Figure S18. 400 MHz $^1$H NMR spectrum of (±)-3,3-[^2H]2 in CDCl₃.
Figure S19. 100 MHz $^{13}$C NMR spectrum of (±)-3,3-[2H$_2$]2 in CDCl$_3$. 

S39
Figure S20. 400 MHz $^1$H NMR spectrum of (±)-7,7-[2H$_2$]3 in CD$_3$OD.
Figure S21. 100 MHz $^{13}$C NMR spectrum of (±)-7,7-$[^2]$H$_2$3 in CD$_3$OD.
Figure S22. 400 MHz $^1$H NMR spectrum of (±)-7,7-$[^2$H$_2$]AcAP in CDCl$_3$. 
Figure S23. 100 MHz $^{13}$C NMR spectrum of (±)-7,7-[2H$_2$]AcAP in CDCl$_3$. 
Figure S24. 400 MHz $^1$H NMR spectrum of (±)-2,2,8-[2H$_3$]3 in CD$_3$OD.
Figure S25. 100 MHz $^{13}$C NMR spectrum of (±)-2,2,8-$[^2]$H$_3$]3 in CD$_3$OD.
Figure S26. 400 MHz $^1$H NMR spectrum of (±)-2,2,8-[$^2$H$_3$]AcAP in CDCl$_3$. 

S46
Figure S27. 100 MHz $^{13}$C NMR spectrum of (±)-2,2,8-[$^2$H$_3$]AcAP in CDCl$_3$. 
Figure S28. 400 MHz $^1$H NMR spectrum of 9 in CD$_3$OD.
Figure S29. 100 MHz $^{13}$C NMR spectrum of 9 in CD$_3$OD.
Figure S30. 400 MHz $^1$H NMR spectrum of 4 in CDCl$_3$. 

S50
Figure S31. 100 MHz $^{13}$C NMR spectrum of 4 in CDCl$_3$. 

S51
Figure S32. 400 MHz $^1$H NMR spectrum of 10 in CDCl$_3$. 

S52
Figure S33. 100 MHz $^{13}$C NMR spectrum of 10 in CDCl$_3$. 
Figure S34. 400 MHz $^1$H NMR spectrum of cis-3-$[^2]$H]Pro·HCl in $^2$H$_2$O.
Figure S35. 100 MHz $^{13}$C NMR spectrum of cis-3-[2H]Pro·HCl in $^2$H$_2$O.
Figure S36. 400 MHz $^1$H NMR spectrum of 5 in CDCl$_3$. 
Figure S37. 100 MHz $^{13}$C NMR spectrum of 5 in CDCl$_3$. 

S57
Figure S38. 400 MHz $^1$H NMR spectrum of 11 in CDCl$_3$. 
Figure S39. 100 MHz $^{13}$C NMR spectrum of 11 in CDCl$_3$. 

S59
Figure S40. 400 MHz $^1$H NMR spectrum of trans-$[^{2}\text{H}]\text{Pro}\cdot\text{HCl}$ in $^{2}\text{H}_2\text{O}$.
Figure S41. 100 MHz $^{13}$C NMR spectrum of trans-3-$^{[2}\text{H}]$Pro·HCl in $^2\text{H}_2\text{O}$. 

S61
Figure S42. 400 MHz $^1$H NMR spectrum of (3S)-2,3-[2H$_2$]Asp in $^2$H$_2$O.
Figure S43. 100 MHz $^{13}$C NMR spectrum of (3$S$)-2,3-[$^2$H$_2$]Asp in $^2$H$_2$O.
Figure S44. 400 MHz $^1$H NMR spectrum of (3$R$)-3-$[^2]$HAsp in $^2$H$_2$O.
Figure S45. 100 MHz $^1$H NMR spectrum of (3R)-3-[2H]Asp in $^2$H$_2$O.