A Secondary Kinetic Isotope Effect Study of the 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase-catalyzed Reaction: Evidence for a Retroaldol-Aldol Rearrangement

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Supporting Information

General Methods. The pET24(+) vector was purchased from Novagen (Madison, WI) and DNA polymerase *Pfu* was obtained from Stratagene (La Jolla, CA). Primers used in PCR amplification were prepared by Integrated DNA Technologies (Coralville, IA) and used without further purification. All electrophoretic reagents were acquired from Bio-Rad (Hercules, CA). Culture media were products of Difco (Detroit, MI), and the *Ni*-NTA agarose resin was purchased from Qiagen (Valencia, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). DXP and MEP were prepared by enzymatic synthesis as published,[1,2] except the purification was achieved by cellulose chromatography using a water/THF solvent system.

Anhydrous THF and DMC were purified by the *PURESOLV*^{TM} solvent purification system. NMR spectra (^{1}H at 300 MHz, ^{13}C at 75 MHz, and ^{31}P at 121 MHz) were recorded with a Varian U-300 spectrometer in $CDCl_3$, unless otherwise specified. Chemical shifts (δ in ppm) are given relative to those for $CDCl_3$ (^{1}H, ^{13}C) or external aqueous 85% H_3PO_4 (^{31}P). High resolution mass spectroscopy was performed on a Finnigan Mat 95 instrument. Flash chromatography was conducted on Sorbent Technologies silica gel (230-400 mesh). TLC was carried out on UV_{254}

plates (0.25mm). TLC spots were developed by heating the plate previously stained with a solution of phosphomolybdic acid (3% in EtOH).

Overexpression and Purification of DXR. In our previous studies,[3,4] a C-terminal His-tagged DXR was used. The C-terminal His_6-tag was removed in the current study by inserting a stop codon after the *dxr* sequence and before the coding sequence for the His-tag. This was performed by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The oligonucleotides used for mutagenesis were *dxrn1* (5'-GGTG-ATGCGTCTCGCAAGCTGACTCGAGCACC-3') and *dxrn2* (5'-GGTGGTGGTGGTGCTC-GAGTCAGCTTGCGAGA-3'). The constructed mutant plasmids were amplified in *E. coli* strain DH5α and purified with the QIAprep spin miniprep kit (Qiagen, Valencia, CA). Once the mutation was verified by DNA sequencing (performed by the core facility in the Institute for Cellular and Molecular Biology at the University of Texas at Austin), the mutant plasmids were used to transform *E. coli* BL21 Star™ (DE3) (Invitrogen, Carlsbad, CA) for protein expression.

An overnight culture of the recombinant strain grown in Luria-Bertani (LB) media supplemented with kanamycin (50 μg/mL) at 37 °C was used to inoculate six 1 L cultures of the same medium and antibiotic. These cultures were grown at 37 °C until the OD_{600} reached 0.65, followed by induction with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). The culture was incubated for an additional 3.5 h at 37 °C. The cells were harvested by centrifugation (7000×*g*, 8 min). DXR was purified according to a literature procedure.[5] The purified protein was concentrated, mixed with glycerol to a final concentration of 30%, and divided into aliquots that were flash frozen and stored at –80 °C. The concentration of DXR was determined by the method of fluorescence active-site titration with NADPH as previously described.[3]

Determination of k_{cat} and K_m for DXP, [3-^{2}H]-DXP and [4-^{2}H]-DXP. Enzyme assays were performed at 24 °C in duplicate following a literature procedure.[5] The reaction mixture contained

100 μL of degassed and N_2 saturated 100 mM Tris•HCl buffer (pH 7.6), 2 mM $MgCl_2$, 1 mg/mL bovine serum albumin (BSA), 0.15 mM NADPH, 30 nM DXR, and various (20-2328 μM) concentrations of DXP, [3-^{2}H]-DXP (**11**) or [4-^{2}H]-DXP (**17**). Each reaction was initiated by the addition of DXR and was monitored by the consumption of NADPH at 340 nm. The concentrations of DXP, [3-^{2}H]-DXP, [4-^{2}H]-DXP and MEP were determined according to a literature proceedure.[5] The initial rates were determined by fitting the progress curves to a polynomial and using the constant term as the initial rate. The kinetic parameters, k_{cat} and K_m, were then determined by fitting the initial rates (see Figure **S1**) to the Michaelis-Menten equation using Grafit 5.0.1. Attempts to fit the data to an equation that took into account a constant ratio of [3/4-^{2}H]-DXP to inhibitor, to more accurately determine k_{cat} and K_m, proved unsuccessful due to large errors for the fitted parameters. The k_{cat}, K_m and KIE values are reported in Table **S1**. As stated in the manuscript, the progress curves for the enzymatically synthesized DXP are linear, yet the progress curves for the chemically synthesized [3-^{2}H]-DXP and [4-^{2}H]-DXP are nonlinear. This is depicted in Figure **S2**.

Table S1. Summary of steady-state parameters and K_{eq}s for DXP, [3-^{2}H]-DXP and [4-^{2}H]-DXP.

Substrate	k_{cat} (s^{-1})	K_m (μM)	$^Dk_{cat}$	$^Dk_{cat}/K_m$	K_{eq}
DXP	16.1 ± 0.7	166 ± 20	--	--	22.0 ± 0.3
3[^{2}H]-DXP	13.2 ± 0.7	116 ± 20	1.21 ± 0.08	0.85 ± 0.19	22.7 ± 0.2
4[^{2}H]-DXP	13.7 ± 0.5	154 ± 17	1.17 ± 0.05	1.12 ± 0.17	19.8 ± 0.2

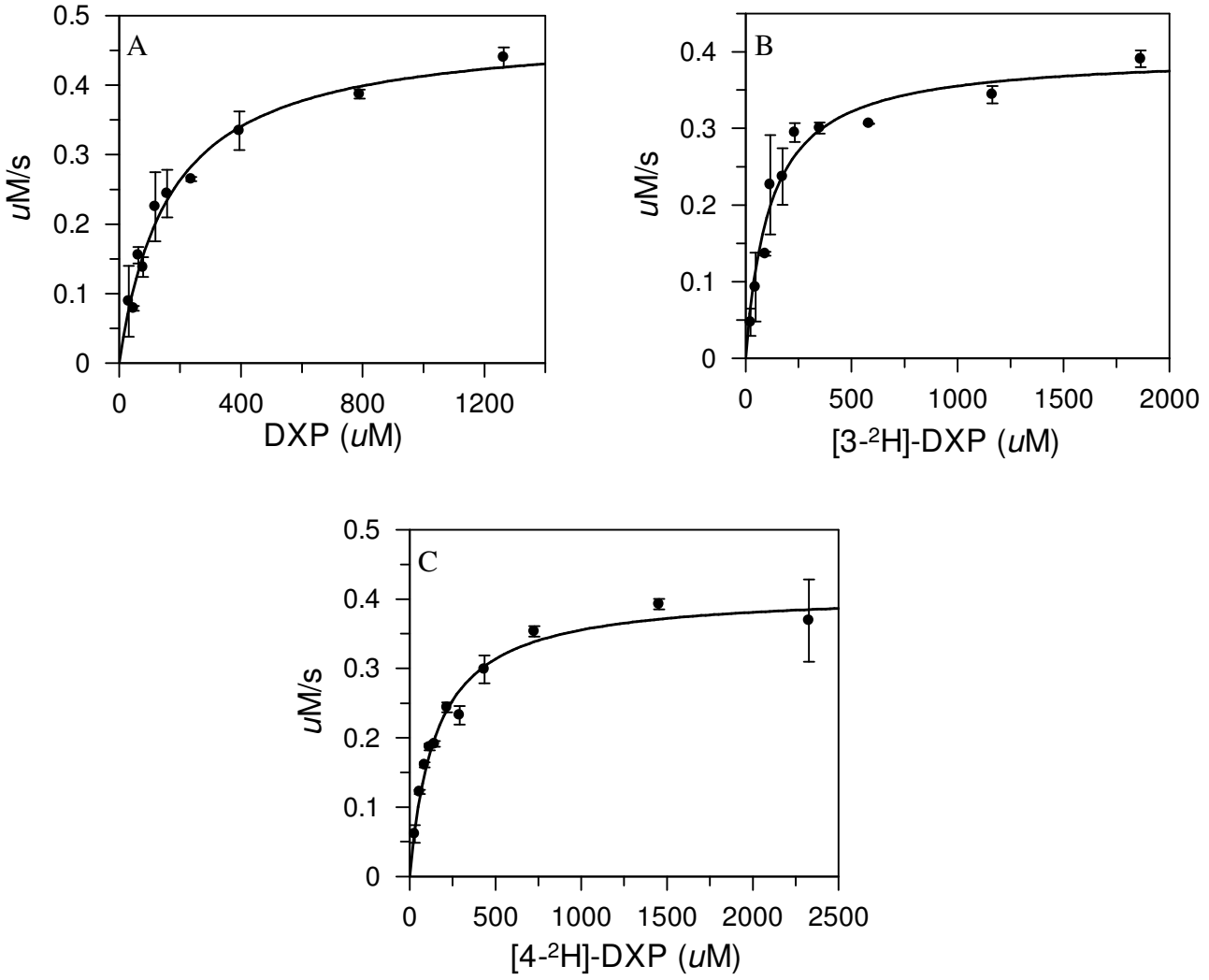


Figure S1. Michaelis-Menten curves for determining k_{cat} and K_m for A) DXP, B) [3-^{2}H]-DXP and C) [4-^{2}H]-DXP.

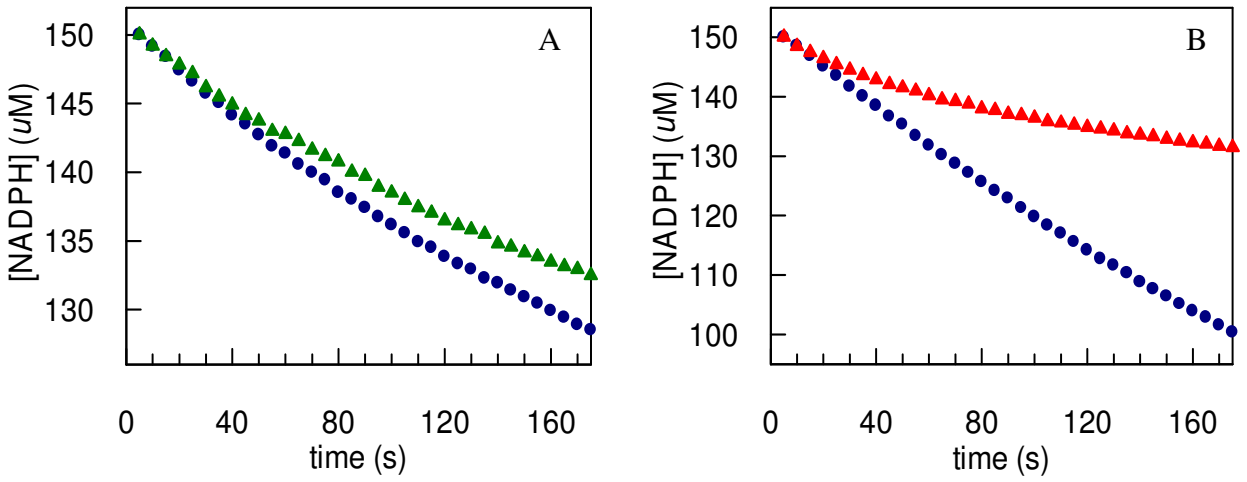


Figure S2. Progress curves in determining ${}^{D}k_{cat}$ and ${}^{D}(k_{cat}/K_M)$ for [3-^{2}H]- and [4-^{2}H]-DXP. Reaction conditions: A) 150 μM NADPH, 118 μM DXP (●) or 116 μM [3-^{2}H]-DXP (▲) (**11**), 2 mM $MgCl_2$, and 30 nM DXR in 100 mM Tris•HCl (pH 7.6); (B) 150 μM NADPH, 2295 μM DXP (●) or 2328 μM [4-^{2}H]-DXP (▲), 2 mM $MgCl_2$, and 30 nM DXR in 100 mM Tris•HCl (pH 7.6).

Determination of K_{eq} for the DXR reaction using DXP, [3-^{2}H]-DXP and [4-^{2}H]-DXP. The equilibrium constants for the reaction with DXP, [3-^{2}H]-DXP, and [4-^{2}H]-DXP were determined by measuring the change in [NADPH] prior to and after the addition of DXR until the reaction mixture reached equilibrium. The assays were carried out at fixed concentrations of $MgCl_2$ (2 mM), NADPH (75 μM), DXP (67 μM) (or [3-^{2}H]-DXP (57 μM), or [4-^{2}H]-DXP (70 μM)), with varying concentrations of $NADP^+$ (0-1.7 mM) in 500 μL of 100 mM Tris buffer (pH 7.6) that was degassed and saturated with N_2. To drive the reaction to equilibrium, DXR was added to a final concentration of 1.7 μM. The assays were carried out at 24 °C in duplicate. To determine the K_{eq} for each reaction, the data were fit to Equation 1 using Grafit 5.0.1.

$$[NADP^+]_o = \frac{K_{eq}[NADPH]_o[DXP]_o - K_{eq}([NADPH]_o + [DXP]_o)[X] + (K_{eq}-1)[X]^2}{[X]} \qquad (1)$$

$[NADPH]_o$, $[DXP]_o$, and $[NADP^+]_o$ are the respective starting concentrations of each component before the addition of DXR. $[X]$ is the difference between $[NADPH]_o$ and the concentration of

NADPH once equilibrium is reached. In the data fitting, K_{eq} was made a parameter, and $[NADPH]_o$ and $[DXP]_o$ were set as constants. The K_{eq}s determined are listed in Table **S2**.

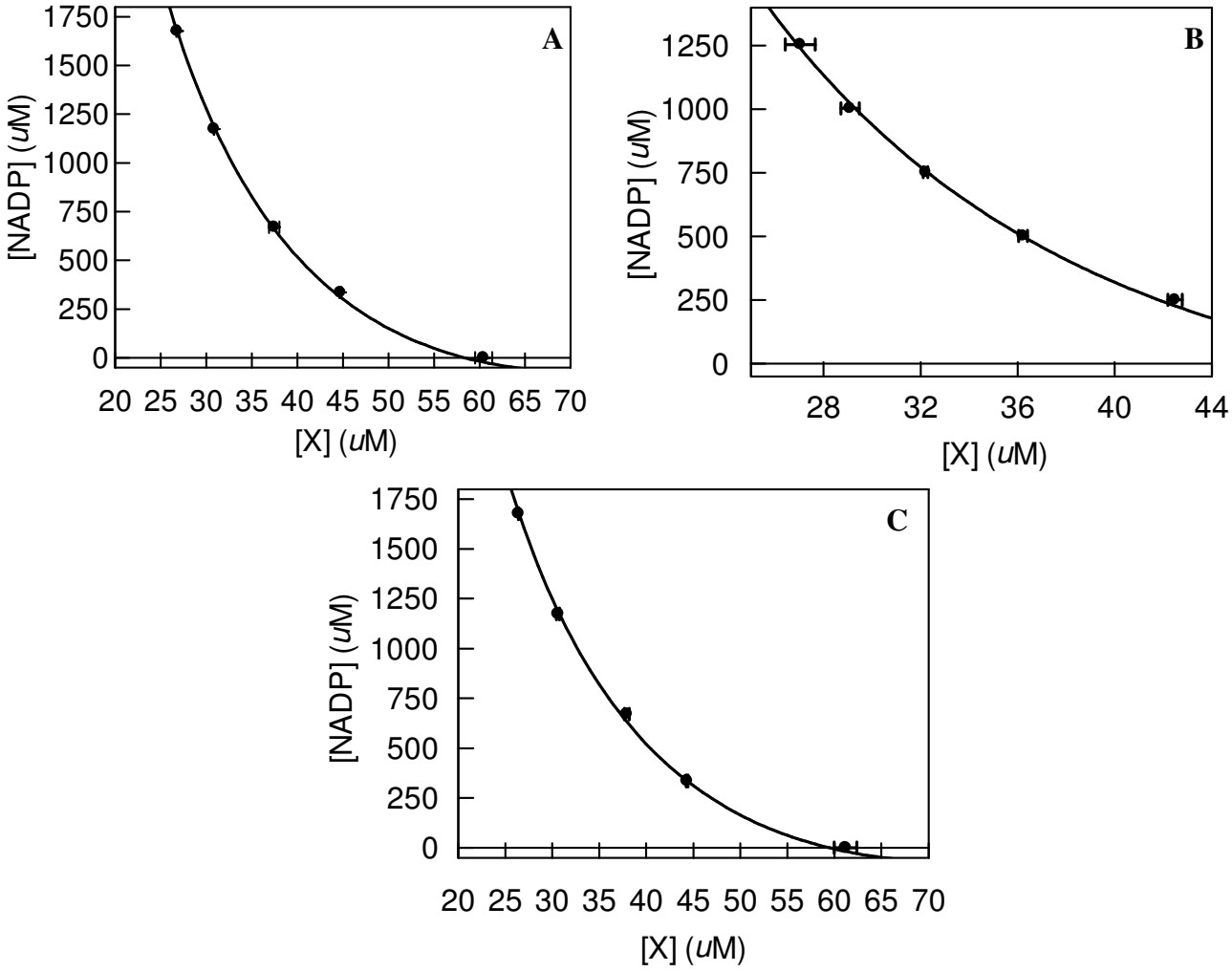


Figure S3. Plots to determine K_{eq} by analyzing the effect [$NADP^+$] has on the amount of NADPH consumed, [X], in reaching equilibrium for reactions with (A) DXP, (B) [3-^{2}H]-DXP, and (C) [4-^{2}H]-DXP. The data were fit to Equation 1.

Equilibrium Perturbation Experiments. The equilibrium perturbation experiments to determine the KIE's with [3/4-^{2}H]-DXP were conducted according to literature procedures.[7-9] The concentrations used in the experiment were based on a similar study with alcohol dehydrogenase.[9] The perturbations were carried out in triplicate at 24 °C. A stock solution containing $MgCl_2$, NADPH, [3- or 4-^{2}H]-DXP, MEP and $NADP^+$ was made, and a 1-mL aliquot

of this stock solution was diluted to 3 mL for each perturbation experiment. The final concentrations for the [3-^{2}H]-DXP perturbation experiments were 165 μM NADPH, 1 mM [3-^{2}H]-DXP, 1.1 mM MEP, 3.1 mM $NADP^+$, and 2 mM $MgCl_2$. For the [4-^{2}H]-DXP perturbation experiments, the final concentrations were 149 μM NADPH, 900 μM [4-^{2}H]-DXP, 990 μM MEP, 2.79 mM $NADP^+$, and 1.8 mM $MgCl_2$. Before the addition of enzyme, the sample in the cuvette was incubated in the UV/Vis cell holder, which was connected to a water circulator, to allow the temperature of the sample to equilibrate and to ensure the sample produced an acceptable baseline reading. The concentration of DXR in each perturbation experiment varied from 300-450 nM.

Kinetic Simulations of Equilibrium Perturbation Experiments. The *E. coli* DXR has been shown to proceed via an ordered substrate binding mechanism, with NADPH binding before DXP.[5] In contrast, studies of *M. tuberculosis* DXR showed that it binds substrate in random order, but with a preference to bind NADPH before DXP.[10] Accordingly, in the computer simulations described here, the ordered substrate binding model is followed. It is also assumed that product release is ordered with MEP dissociating before $NADP^+$. Interestingly, the results were not significantly different when a fully random kinetic mechanism was tested by simulation. Hence, only the ordered mechanism is considered here. The kinetic mechanism used in the simulation is shown in Scheme **S1**.

In the simulations, all the rate constants were allowed to float with limited restrictions. It was assumed that there is no isotope effect on substrate binding or release. Thus, the rate constants for binding of DXP and [^{2}H]-DXP were set equal so that they floated together. This condition was also applied to the binding/dissociation of MEP and [^{2}H]-MEP. In the simulations, the rate constants for NADPH binding and dissociation were allowed to float together so that their ratio equals the K_d of DXR for NADPH, which was previously determined

to be 750 nM in active site titration experiments. Since the K_d's for DXP, MEP and $NADP^+$ are not known, the corresponding rate constants for binding and dissociation were allowed to float independent of each other. To further restrict the simulations, the ratio of the internal equilibrium for the chemistry step of the unlabeled reaction (k_3/k_{-3}) and that for the labeled reaction (k_3^*/k_{-3}^*) were set to the corresponding equilibrium isotope effect (see Table **S2**).

Scheme S1. Kinetic scheme used for computer simulations

$$\text{DXR + NADPH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{DXR•NADPH} \quad \textbf{(1)}$$

$$\text{DXR•NADPH + DXP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{DXR•NADPH•DXP} \quad \textbf{(2)}$$

$$\text{DXR•NADPH•DXP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{DXR•NADP•MEP} \quad \textbf{(3)}$$

$$\text{DXR•NADP•MEP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{DXR•NADP + MEP} \quad \textbf{(4)}$$

$$\text{DXR•NADP} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{DXR + NADP} \quad \textbf{(5)}$$

$$\text{DXR•NADPH + d-DXP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{DXR•NADPH•d-DXP} \quad \textbf{(6)}$$

$$\text{DXR•NADPH•d-DXP} \underset{k_{-3}^*}{\overset{k_3^*}{\rightleftharpoons}} \text{DXR•NADP•d-MEP} \quad \textbf{(7)}$$

$$\text{DXR•NADP•d-MEP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{DXR•NADP + d-MEP} \quad \textbf{(8)}$$

The binding and dissociation rates for DXP, MEP or $NADP^+$ derived from computer simulations are not accurate but relative. The values obtained by simulation depend on the initial starting values. All of the starting binding rates were set less than the diffusion controlled limit to ensure that the simulated values were physically reasonable. When different values for the binding and dissociation rates were used to start the simulation, the program would give different optimal rates and equilibrium binding values. If the K_d for DXP was lowered, then the K_d for either $NADP^+$ or MEP would also decrease so that the reactant concentrations entered into

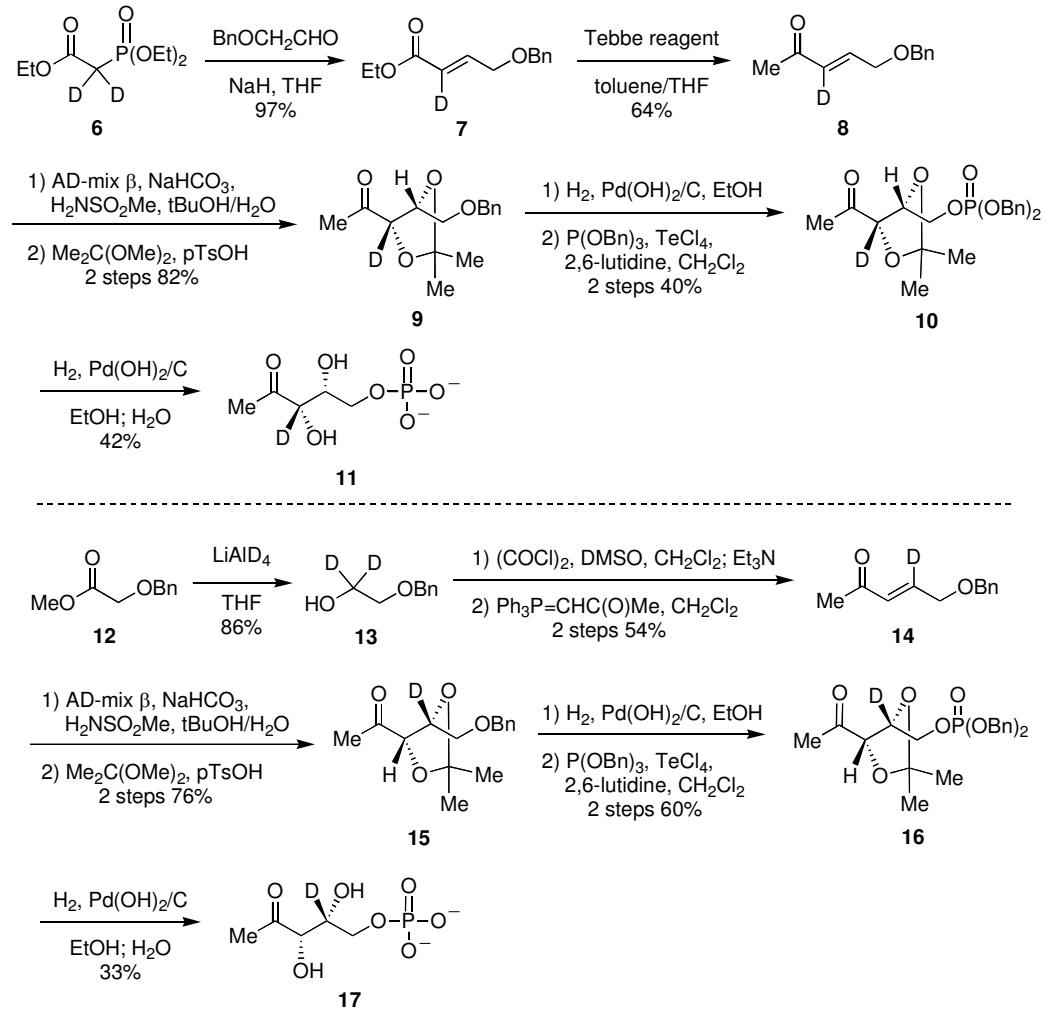
program were at equilibrium. Thus, in the simulation experiments, the exact equilibrium binding value for each reactant is not important, only the relationship among the four reactants is meaningful. The magnitude of the binding and dissociation rates did affect the KIE values, but not in a significant manner. Depending on the initial binding and dissociation rates, the KIE values for [3-^{2}H]-DXP ranged from 1.02 to 1.05, and those for [4-^{2}H]-DXP ranged from 1.09 to 1.14. The rate constants reported in Table **S2** had the smallest standard errors and were used to calculate the KIE's ($^D k_{chem}$'s) reported in Table 1 (see text).

Table S2: Results from Computer Simulations

	[3-^{2}H]-DXP[a]		[4-^{2}H]-DXP[a]	
	Float All	Fix Binding	Float All	Fix Binding
k_1	73 ± 3	73	72 ± 4	72
k_{-1}	55 ± 2	55	54 ± 3	54
k_2	2.15 ± 0.06	2.15	6.9 ± 0.3	6.9
k_{-2}	463 ± 13	463	946 ± 40	946
k_3	19.2 ± 0.5	19.6 ± 0.2	19.8 ± 0.5	19.8 ± 0.3
k_{-3}	9.1 ± 0.2	9.4 ± 0.1	9.4 ± 0.2	9.4 ± 0.1
k_4	2410 ± 130	2410	1660 ± 90	1660
k_{-4}	11.8 ± 0.4	11.8	5.9 ± 0.3	5.9
k_5	127 ± 5	127	91 ± 4	91
k_{-5}	16.5 ± 0.7	16.5	25 ± 1	25
k_3*	18.4 ± 0.4	18.9 ± 0.2	17.8 ± 0.4	17.8 ± 0.2
k_{-3}*	8.4 ± 0.2	8.6 ± 0.1	9.4 ± 0.2	9.4 ± 0.1

[a]The units for second order rate constants are reported as $\mu M^{-1}s^{-1}$, and those for the first order rate constants are reported as s^{-1}.

Scheme S2



Synthesis of [3-^{2}H]-DXP (11)

Ethyl (E)-[2-^{2}H]-4-Benzyloxy-but-2-enoate (**7**). A solution of phosphonate **6**[11] (2.26 g, 10.0 mmol) in THF (5 mL) was slowly added to a suspension of sodium hydride (60% dispersed in mineral oil, 400 mg, 10.0 mmol) in THF (10 mL) at 0 °C. α-Benzyloxyacetaldehyde (2.25 g, 15.0 mmol) was then added to the resulting slurry. The mixture was gradually warmed up to room temperature and stirred overnight. After the complete consumption of the starting material was indicated by TLC analysis, a saturated ammonium chloride solution was added to quench the reaction. The mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The

crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 6:1) to afford compound **7** (2.15 g, 9.72 mmol) in 97% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (t, 3H, *J* = 4.2 Hz), 4.19 (q, *J* = 4.2 Hz, 2H), 4.25 (d, *J* = 6.9 Hz, 2H), 4.59 (s, 2H), 7.01 (m, *J* = 2.1 Hz, 1H), 7.37 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.5, 60.6, 68.9, 73.0, 121.4 (t), 127.9, 128.1, 128.7, 138.0, 144.4, 166.6.

(E)-[3-^{2}H]-5-Benzyloxy-pent-3-en-2-one (**8**). Ester **7** (1.40 g, 6.33 mmol) was dissolved in a mixture of toluene/THF (3/1, 12 mL) and pyridine (0.07 mL). The mixture was cooled to –40 °C and a solution of Tebbe reagent[12] (14.2 mL, 7.10 mmol) was added dropwise. After 30 min, the reaction mixture was gradually warmed up to room temperature, at which time the reaction was quenched with a solution of sodium hydroxide (15%). The aqueous layer was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was re-suspended in a mixture of acetic acid/THF/toluene (1/5/5, 15 mL). After the consumption of the starting material was indicated by TLC analysis, a saturated sodium bicarbonate solution was added to quench the reaction. The aqueous layer was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 6:1) to afford compound **8** (765 mg, 4.00 mmol) in 64% yield. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 2.28 (s, 3H), 4.21 (d, *J* = 4.5 Hz, 2H), 4.59 (s, 2H), 6.80 (m, *J* = 2.1 Hz, 1H), 7.37(m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 27.5, 69.0, 73.2, 127.2, 127.6, 127.9, 128.0, 128.2, 128.7, 128.8, 137.9, 143.4, 198.7. High resolution MS (CI) calcd. for $C_{12}H_{13}DO_2$ 191.1057; found 191.1052.

1-((4′S,5′R)-[4′-^{2}H]-5′-Benzyloxymethyl-2′,2′-dimethyl-1′,3′-dioxolan-4′-yl)-ethanone (**9**). A solution of enone **8** (391 mg, 2.04 mmol) in toluene (1 mL) was added to a solution of AD-mix β

(3.08 g, 1.5 g/mmol), sodium bicarbonate (540 mg, 6.43 mmol), and methanesulfonamide (202 mg, 2.12 mmol) in *t*-BuOH/H_2O (1/1, 20 mL) at 0 °C. The mixture was vigorously stirred at 0 °C until the substrate was consumed. The mixture was then extracted with ethyl acetate (3 × 10 mL), and the combined organic layers were dried over anhydrous sodium sulfate. After concentration under reduced pressure, the crude product was used in the next step without further purification. *p*-Toluenesulfonic acid (38 mg, 0.020 mmol) was added to a solution of the crude diol in 2,2-dimethoxypropane (20 mL) at room temperature. After 10 h, the reaction was quenched by the addition of a saturated sodium bicarbonate solution and the resulting mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 10:1) to afford compound **9** (443 mg, 1.67 mmol) in 82% yield (2 steps). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.47 (s, 3 H), 1.50 (s, 3 H), 2.31 (s, 3 H), 3.68 (dd, *J* = 9.3, 3.3 Hz, 1H), 3.75 (d, *J* = 9.3 Hz, 1H), 4.23 (s, 1H), 4.64 (s, 2H), 7.34 (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 26.5, 26.7, 27.2, 70.4, 73.9, 77.5, 82.2, 111.4, 128.0, 128.7, 138.1, 208.7.

Phosphoric acid (4R,5S)-[5-^{2}H]-5-acetyl-2,2-dimethyl-1,3-dioxolan-4-yl-methyl ester dibenzyl ester (**10**). $Pd(OH)_2$/C (40 mg) was added to a solution of compound **9** (516 mg, 1.94 mmol) in ethanol (10 mL) at room temperature and the mixture was charged with hydrogen gas (1 atm). After stirring overnight, the mixture was filtered over a pad of celite to remove the catalyst. The filtrate was concentrated to afford a crude alcohol, which was re-dissolved in dichloromethane (10 mL). Tribenzyl phosphite (2.72 g, 7.76 mmol), 2,6-lutidine (1.10 g, 10.3 mmol), and tellurium tetrachloride (1.40 g, 5.20 mmol) was added to the resulting mixture at 0 °C. After stirring at 0 °C for 3 h, the reaction mixture was slowly warmed to room temperature. When the substrate was consumed, the reaction was quenched with a saturated ammonium

chloride solution and the resulting mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine solution, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 2:1) to afford compound **10** (338 mg, 0.78 mmol) in 40% yield (2 steps). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 3H), 1.44 (s, 3H), 2.27 (s, 3H), 4.10 (dd, *J* = 10.2, 6.6 Hz, 1H), 4.16-4.26 (m, *J* = 10.2, 6.3 Hz, 2H), 5.08 (d, *J* = 8.1 Hz, 4H), 7.36 (m, 10H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 26.5, 26.8, 27.0, 66.95, 67.02, 69.65, 69.72, 76.3, 76.4, 111.6, 128.2, 128.9, 208.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δ -0.01 (s)

Phosphoric Acid Mono-((2R,3S)-[3-^{2}H]-2,3-dihydroxy-4-oxo-pentyl)ester (**11**). $Pd(OH)_2$/C (20 mg) was added to a solution of compound **10** (50 mg, 0.12 mmol) in ethanol (5 mL) at room temperature. The mixture was charged with hydrogen gas (1 atm) and vigorously stirred overnight. The catalyst was removed by filtration over a pad of celite and the filtrate was concentrated under reduced pressure. The crude residue was dissolved in de-ionized water and stirred at room temperature for 3 days. The resulting free acid was converted to the corresponding sodium salt by adding solid sodium bicarbonate until the pH reached ~7 and then lyophilized to remove water. The yellowish residue was re-dissolved in a minimum amount of water and methanol and subjected to cellulose chromatography (Whatman CF-11, THF:H_2O = 2:1 to 1:1).[3,13] Fractions containing the desired product were pooled, concentrated under reduced pressure to remove THF, and then lyophilized to afford compound **11** (13 mg, 0.05 mmol, 42% yield) as a white foam. ^{1}H-NMR (300 MHz, D_2O) δ 2.13 (s, 3H), 3.83 (*app* q, 2H), 4.21 (t, *J* = 6.2, 1H); ^{13}C-NMR (75 MHz, D_2O) δ 25.9, 66.0, 70.0, 70.1, 212.8. ^{31}P-NMR (121 MHz, D_2O) δ 2.18 (s); High resolution MS (CI) calcd. for $C_5H_{10}DO_7P$ 215.0305; found 215.0330.

Synthesis of [4-^{2}H]-DXP (17)

2-Benzyloxy[1,1-^{2}H]ethanol (**13**). Lithium aluminum deuteride ($LiAlD_4$, 260 mg, 6.1 mmol) was added to a solution of benzyloxyacetic acid methyl ester **12** (1.1 g, 6.1 mmol) in anhydrous THF (30 mL) at 0 °C. The reaction mixture was warmed to room temperature over 30 min and then vigorously stirred for 3 h. After completion of the reaction was indicated by TLC, a saturated ammonium chloride solution was added to quench the reaction. The product was extracted with ethyl acetate (2 × 30 mL) and the combined organic layers were dried over anhydrous sodium sulfate. After concentration under reduced pressure, the crude product was purified by flash column chromatography (hexanes:ethyl acetate = 6:1) to afford compound **13** (806 mg, 5.23 mmol) in 86% yield. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 2.22 (s, 1H), 3.69 (s, 2H), 4.58 (s, 2H), 7.38 (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 61.4, 71.5, 73.6, 128.1, 128.7, 138.2.

(E)-[4-^{2}H]-5-Benzyloxy-pent-3-en-2-one (**14**). Dimethylsulfoxide (0.55 mL, 7.77 mmol) was added to a solution of oxalyl chloride (0.330 mL, 3.90 mmol) in dichloromethane (10 mL) at −78 °C. The mixture was stirred for 15 min followed by the slow addition of alcohol **13** (300 mg, 1.95 mmol) at −78 °C. After stirring for 2 h, the reaction was quenched with anhydrous triethylamine (1.36 mL, 9.75 mmol) and warmed to room temperature over 1 h. The mixture was poured into a saturated ammonium chloride solution and extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 10:1) to give the corresponding aldehyde, which was used in the next step without further purification. 1-(Triphenylphosphoranylidene)-2-propanone (505 mg, 1.59 mmol) was slowly added to a solution of the aldehyde (200 mg, 1.32 mmol) in dichloromethane (10 mL) at 0 °C. The reaction mixture was then warmed to room temperature and stirred for additional 10 h. The reaction was quenched by adding brine solution and the

resulting mixture was extracted with ethyl acetate (2 × 10 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 20:1 to 5:1) to afford compound **14** (200 mg, 1.05 mmol, 54% yield in 2 steps). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 2.28 (s, 3H), 4.21 (s, 2H), 4.59 (s, 2H), 6.36 (m, *J* = 2.1 Hz, 1H), 7.37 (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 27.5, 69.0, 73.2, 128.0, 128.2, 128.8, 130.5, 137.8, 143.0, 198.5. High resolution MS (CI) calcd. for $C_{12}H_{13}DO_2$ 191.1057; found 191.1062.

1-((4′S,5′R)-[5′-^{2}H]-5′-Benzyloxymethyl-2′,2′-dimethyl-1′,3′-dioxolan-4′-yl)-ethanone (**15**). An identical procedure used for the synthesis of **9** was followed to prepare **15**. Briefly, a solution of enone **14** (101 mg, 0.526 mmol) in toluene (1 mL) was added to a solution of AD-mix β (800 mg), sodium bicarbonate (134 mg), and methanesulfonamide (50.4 mg) in *t*-BuOH/H_2O (1/1, 6 mL) at 0 °C. The mixture was vigorously stirred until the substrate was consumed. The mixture was then extracted with ethyl acetate and the combined organic layers were dried and concentrated under reduced pressure. *p*-Toluenesulfonic acid (10.0 mg, 0.0528 mmol) was added to a solution of the crude product in 2,2-dimethoxypropane (10 mL) at room temperature. After 10 h, the reaction was quenched by adding a saturated sodium bicarbonate solution and the reaction mixture was subjected to routine workup. The crude residue was purified by flash column chromatography (hexanes:ethyl acetate = 10:1) to afford compound **15** (106 mg, 0.40 mmol) in 76% yield (2 steps). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 3H), 1.48 (s, 3H), 2.28 (s, 3H), 3.62 (d, *J* = 9.3 Hz, 1H), 3.75 (d, *J* = 9.3 Hz, 1H), 4.21 (s, 1H), 4.61 (s, 2H), 7.34 (m, 5H).

Phosphoric Acid (4R,5S)-[4-^{2}H]-5-Acetyl-2,2-dimethyl-1,3-dioxolan-4-ylmethyl Ester Dibenzyl Ester (**16**). An identical procedure used for the synthesis of **10** was followed to prepare **16**. $Pd(OH)_2$/C (20 mg) was added to a solution of benzyl ether **15** (75 mg, 0.28 mmol) in

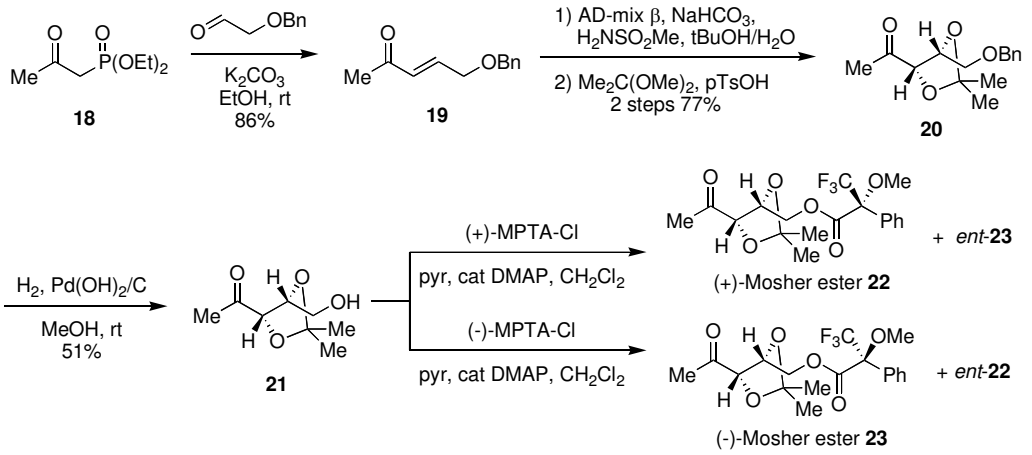
ethanol (10 mL) at room temperature. The mixture was vigorously stirred overnight under a hydrogen atmosphere (1 atm). The catalyst was removed by filtration over a pad of celite and the filtrated was concentrated under reduced pressure. Tribenzyl phosphite (205 mg, 0.57 mmol), 2,6-lutidine (124 mg, 1.14 mmol), and tellurium tetrachloride (141 mg, 0.57 mmol) was added to a solution of the crude alcohol in dichloromethane (3 mL) at 0 °C. The mixtue was stirred at 0 °C for 3 h, and then slowly warmed to room temperature. The reaction was quenched with a saturated ammonium chloride solution and extracted with ethyl acetate. After workup, the crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 2:1) to afford compound **16** (73 mg, 0.17 mmol) in 60% yield (2 steps). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 3H), 1.44 (s, 3H), 2.27 (s, 3H), 4.09 (dd, *J* = 10.2, 6.6 Hz, 2H), 4.16 (s, 1H), 4.26 (dd, *J* = 10.2, 6.3 Hz, 1H), 5.09 (d, *J* = 2.7 Hz, 4H), 7.36 (m, 10H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 26.5, 26.8, 27.0, 66.9, 67.0, 69.67, 69.74, 81.4, 111.6, 128.3, 128.9, 136.0, 208.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δ 1.56 (s). High resolution MS (CI) calcd. for $C_{22}H_{26}DO_7P$ 435.1557; found 435.1554.

Phosphoric Acid Mono-((2R,3S)-[2-^{2}H]-2,3-dihydroxy-4-oxo-pentyl)ester (**17**). Preparation of **17** followed the identical procedure used for the synthesis of **11**. $Pd(OH)_2$/C (10 mg) was added to a solution of phosphonate **16** (50 mg, 0.12 mmol) in ethanol (5 mL) at room temperature. The mixture was charged with hydrogen gas (1 atm) and vigorously stirred overnight. The catalyst was removed and the filtrate was concentrated. The crude residue was dissolved in de-ionized water and stirred at room temperature for 3 days. The resulting free acid was converted to the corresponding sodium salt and then lyophilized. The yellowish residue was purified by cellulose chromatography (Whatman CF-11, THF:H_2O = 2:1 to 1:1)[3,13] to afford compound **17** (10 mg, 0.039 mmol, 33% yield) as a white foam. ^{1}H-NMR (300 MHz, D_2O) δ 2.06 (s, 3 H), 3.79 (q, *J* = 10.5, 8.7 Hz, 2H), 4.23 (s, 1H); ^{13}C-NMR (75 MHz, D_2O) δ 25.8, 65.9,

76.8, 212.8; ^{31}P-NMR (121 MHz, D_2O) δ 5.38 (s). High resolution MS (CI) calcd. for $C_5H_{10}DO_7P$ 215.0305; found 215.0301.

Determination of Stereoselectivity in Sharpless Asymmetric Dihydroxylation

Scheme S3



(E)-5-Benzyloxy-pent-3-en-2-one (**19**). Potassium carbonate (667 mg, 4.83 mmol) was added to a solution of diethyl 2-oxopropylphosphonate (957 mg, 4.83 mmol) in ethanol (10 mL) at room temperature. The mixture was stirred for 2 h, at which time a solution of benzyloxy-acetaldehyde (653 mg, 4.35 mmol) was added at room temperature. After stirring for 10 h, the solid residue was removed by filtration. The filtrate was concentrated under reduced pressure and redissolved in ethyl acetate (100 mL). The solution was washed with a saturated ammonium chloride solution (50 mL) followed by a brine solution (50 mL), dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexane:ethyl acetate = 6:1) to afford enone **19** (711 mg, 3.74 mmol, 86%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.25 (s, 3H), 4.19 (dd, J = 4.3, 2.0 Hz, 2H), 4.55 (s, 2H), 6.33 (dt, J = 16.0, 2.0 Hz, 1H), 6.79 (dt, J = 16.0, 4.5 Hz, 1H), 7.24–7.37 (m, 5H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 27.3, 68.8, 72.9, 127.7, 127.9, 128.5, 130.3, 137.5, 143.0, 198.2.

1-((4′S,5′R)-5′-Benzyloxymethyl-2′,2′-dimethyl-1′,3′-dioxolan-4′-yl)-ethanone (**20**). An identical procedure used for the synthesis of **9** was followed to prepare **15**. Briefly, a solution of enone **19** (711 mg, 3.74 mmol) in toluene (1 mL) was added to a solution of AD-mix β (5.54 g), sodium bicarbonate (972 mg), and methanesulfonamide (364 mg) in *t*-BuOH/H_2O (1/1, 36 mL) at 0 °C. The mixture was vigorously stirred until the substrate was consumed. The mixture was then extracted with ethyl acetate and the combined organic layers were dried and concentrated under reduced pressure. The crude residue was subjected to flash column chromatography to afford a diol (725 mg, 3.23 mmol, 86%). *p*-Toluenesulfonic acid (20.0 mg, 0.105 mmol) was added to a solution of the diol in 2,2-dimethoxypropane (30 mL) at room temperature. After 10 h, the reaction was quenched by adding a saturated sodium bicarbonate solution and the reaction mixture was subjected to routine workup. The crude residue was purified by flash column chromatography (hexanes:ethyl acetate = 10:1) to afford compound **20** (764 mg, 2.89 mmol, 89%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 3H), 1.47 (s, 3H), 2.27 (s, 3H), 3.59–3.65 (m, 1H), 3.71–3.76 (m, 1H), 4.17–4.23 (m, 2H), 4.60 (s, 2H), 7.25–7.38 (m, 5H), ^{13}H-NMR (100 MHz, $CDCl_3$) δ 26.4, 26.6, 27.0, 70.3, 73.7, 77.4, 82.1, 111.2, 127.8, 128.5, 138.0, 208.5. High resolution MS (CI) calcd. for $C_{15}H_{21}O_4$ 265.1440; found 265.1440.

1-((4′S,5′R)-5′-hydroxymethyl-2′,2′-dimethyl-1′,3′-dioxolan-4′-yl)-ethanone (**21**). $Pd(OH)_2$/C (30 mg) was added to a solution of benzyl ether **20** (264 mg, 1.00 mmol) in ethanol (5 mL) at room temperature. The mixture was vigorously stirred overnight under a hydrogen atmosphere (1 atm). The catalyst was removed by filtration over a pad of celite and the filtrate was concentrated under reduced pressure. The crude residue was subjected to flash column chromatography (hexanes:ethyl acetate = 3:2) to afford alcohol **21** (88.5 mg, 0.508 mmol, 51%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.33 (d, *J* = 0.55 Hz, 3H), 1.37 (d, *J* = 0.55 Hz, 3H), 2.20 (s,

3H), 3.47 (ddd, *J* = 11.9, 5.8, 5.1 Hz, 1H), 3.61 (ddd, *J* = 11.9, 5.6, 3.6 Hz, 1H), 4.03 (ddd, *J* = 7.5, 5.1, 3.6 Hz, 1H), 4.21 (d, *J* = 7.5 Hz, 1H), 4.96 (t, *J* = 5.7 Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ 26.1, 26.4, 27.0, 61.5, 78.5, 81.3, 110.0, 208. High resolution MS (CI) calcd. for $C_8H_{15}O_4$ 175.0970; found 175.0974.

Preparation of (+)-Mosher Acid Chloride ((+)-MPTA-Cl). Oxalyl chloride (53 μL, 0.60 mmol) was added to a solution of (+)-Mosher acid ((+)-MPTA, 94.0 mg, 0.402 mmol) in dichloromethane (1 mL) at room temperature. The reaction was initiated by adding one drop of dimethylformate and the evolution of CO_2 gas was observed. After 6 hr, the mixture was concentrated under reduced pressure and half of the resulting residue was used for the next step without further purification.

Preparation of (-)-MPTA-Cl. An identical procedure used for the synthesis of (+)-MPTA-Cl was followed to prepare (-)-MPTA-Cl. Oxalyl chloride (63 μL, 0.72 mmol) was added to a solution of (-)-Mosher acid ((-)-MPTA, 113 mg, 0.483 mmol) in dichloromethane (1 mL) at room temperature. The reaction was initiated by adding one drop of dimethylformate and the evolution of CO_2 gas was observed. After 6 hr, the mixture was concentrated under reduced pressure and half of the resulting residue was used for the next step without further purification.

*Preparation of (+)-Mosher ester of **22**.* Crude (+)-MPTA-Cl in dichloromethane (1 mL) was added to a solution of alcohol **21** (15.4 mg, 0.0884 mmol), pyridine (14.4 μL, 0.177 mmol), and a catalytic amount of *N*,*N*-dimethylaminopyridine in dichloromethane (2 mL) at room temperature. After overnight stirring, the mixture was diluted with dichloromethane (15 mL) and washed with a 0.5 N HCl solution, a saturated sodium bicarbonate solution, and a brine solution sequentially. The solution was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was analyzed using NMR spectroscopy to determine the

enantiomeric purity of the resulting Mosher esters and then purified by column chromatography (hexanes:ethyl acetate = 7:1) to confirm the identity of ester **22**. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 6H), 2.27 (s, 3H), 3.56 (d, *J* = 1.1 Hz, 3H), 4.12 (d, *J* = 7.8 Hz, 1H), 4.24-4.28 (m, 1H), 4.41 (dd, *J* = 12.1, 4.1 Hz, 1H), 4.67 (dd, *J* = 12.1, 2.8 Hz, 1H), 7.36-7.43 (m, 3H), 7.53-7.55 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 26.3, 26.6, 26.8, 55.7, 64.9, 81.1, 111.3, 127.57, 127.58, 128.56, 129.84, 132.17, 166.41, 208.26. ^{19}F-NMR (376 MHz, $CDCl_3$) δ -72.12. High resolution MS (CI) calcd. for $C_{18}H_{22}O_6F_3$ 391.1368; found 391.1370.

*Preparation of (-)-Mosher Ester of **23**.* An identical procedure used for the synthesis of (+)-Mosher ester **22** was followed to prepare (-)-Mosher ester **23**. Crude (+)-MPTA-Cl in dichloromethane (1 mL) was added to a solution of alcohol **21** (19.0 mg, 0.109 mmol), pyridine (27.0 μL, 0.327 mmol), and a atalytic amount of *N*,*N*-dimethylaminopyridine in dichloromethane (2 mL) at room temperature. After overnight stirring, the mixture was diluted with dichloromethane (15 mL) and washed with a 0.5 N HCl solution, a saturated sodium bicarbonate solution, and a brine solution sequentially. The solution was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was analyzed using NMR spectroscopy to determine the enantiomeric purity of the resulting Mosher esters and then purified by column chromatography (hexanes:ethyl acetate = 7:1) to confirm the identity of ester **23**. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H), 1.38 (s, 3H), 2.28 (s, 3H), 3.56 (d, *J* = 1.2 Hz, 3H), 4.15 (d, *J* = 7.7 Hz, 1H), 4.24-4.28 (m, 1H), 4.43 (dd, *J* = 12.0, 4.8 Hz, 1H), 4.64 (dd, *J* = 12.0, 2.9 Hz, 1H), 7.36-7.43 (m, 3H), 7.52-7.57 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 26.3, 26.6, 26.7, 55.7, 65.0, 81.3, 111.3, 127.5, 128.6, 129.8, 132.2, 166.43, 208.31. ^{19}F-NMR (376 MHz, $CDCl_3$) δ -72.04. High resolution MS (CI) calcd. for $C_{18}H_{22}O_6F_3$ 391.1368; found 391.1370.

*Determination of the Ratio Between Enantiomeric Isomers of Alcohol **21***. The enantiomeric purity of alcohol **21** was determined by analyzing ^{19}F-NMR spectra of the crude products of the esterification reactions using (+)-MPTA-Cl and (-)-MPTA-Cl. Mosher ester **22** and **23** show a distinctive singlet peak at δ -72.12 and -72.04, respectively. (Figure **S4-A**). If the Sharpless asymmetric dihydroxylation of enone **19** was not perfectly stereoselective, its enantioisomeric product would be passed down across the course of reactions to finally afford the enantiomer of **21** (*ent*-**21**). Since the reactions of *ent*-**21** with (+)-MPTA-Cl and (-)-MPTA-Cl yield *ent*-**23** and *ent*-**22**, respectively, the ratio between **21** and *ent*-**21** can be determined by comparing the areas of the singlet peaks of the appropriate enantiomers formed in the esterification reactions using either (+)-MPTA-Cl or (-)-MPTA-Cl (Figure **S4-B**), and was found to be >20:1 (the discrepancy in integration values between the two crude products is presumably due to the intrinsic sensitivity of the NMR experiment).

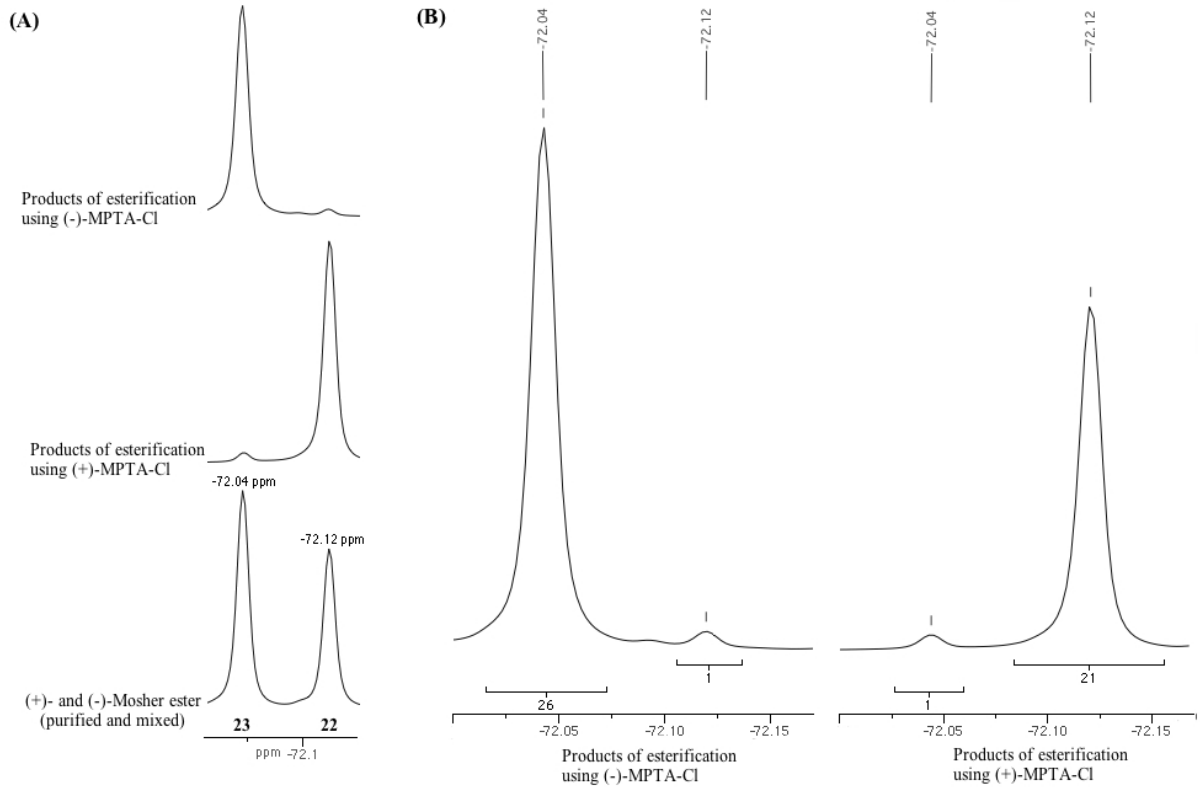


Figure S4. (A) The overlaid ^{19}F-NMR spectra of the crude esterification products. The peaks at δ -72.12 and -72.04 correspond to (+)-Mosher ester **22** and (-)-Mosher ester **23** (or *ent*-**23** and *ent*-**22**), respectively. (B) Integration of each peak to determine the ratio between enantiomeric isomers of alcohol **21**.

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