Supporting Information

Materials

The sources for the buffers, solvents, and components of Luria-Bertani (LB) media are reported elsewhere.\(^1\) High purity synthetic 4-OT was purchased from GenScript USA Inc. (Piscataway, NJ) and folded into the active homohexamer as described before.\(^2\) Chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Compound 4 was synthesized from 3 according to a literature procedure.\(^3\) Compounds 11 and 14 were synthesized from trans-cyclohexane-1,2-diol and trans-cycloheptane-1,2-diol according to a modified literature procedure (Scheme S6).\(^4\)

General methods

Standard molecular biology techniques were performed based on methods described elsewhere.\(^5\) Protein analysis was performed by polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS) gels containing polyacrylamide (10%). Coomassie brilliant blue was used to stain the gels. Protein concentrations were determined based on the Waddell method.\(^6\) Enzymatic assays were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). \(^1\)H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer using a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in parts per million scale and are referenced to H\(_2\)O (4.80 ppm).

Expression and purification of WT 4-OT, 4-OT F50A and 4-OT P1A

The WT 4-OT, 4-OT F50A and 4-OT P1A enzymes were produced in \textit{E. coli} BL21 (DE3) as native proteins (without His-tag) using the pET20b(+) expression system as described before.\(^7\) The construction of the expression vectors and the purification procedure for 4-OT and the mutant enzymes were reported previously.\(^7\) Before applying the 4-OT P1A mutant, which has essentially no aldolase activity, we first
confirmed that purified 4-OT P1A was catalytically active by measuring its promiscuous oxaloacetate decarboxylase activity.\textsuperscript{8}

**UV spectroscopic assay for self-condensation of propanal (3)**

The self-condensation of propanal (3) was monitored by following the increase in absorbance at 234 nm which corresponds to the formation of 2-methyl-2-pentenal (5). The enzyme (150 μM) was incubated in a 1 mm cuvette with 3 (50 mM) in 20 mM NaH\textsubscript{2}PO\textsubscript{4} buffer (pH 7.3; 0.3 mL final volume) and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200 to 400 nm.

**UV spectroscopic assay for dehydration of 3-hydroxy-2-methylpentanal (4)**

The dehydration of 3-hydroxy-2-methylpentanal (4) was monitored by following the increase in absorbance at 234 nm which corresponds to the formation of 2-methyl-2-pentenal (5) (Figure S1). The enzyme (150 μM) was incubated in a 1 mm cuvette with 4 (20 mM) in 20 mM NaH\textsubscript{2}PO\textsubscript{4} buffer (pH 7.3; 0.3 mL final volume) and the reaction was followed for 5 h at room temperature. UV spectra were recorded from 200 to 400 nm.

**Preparation of NaD\textsubscript{2}PO\textsubscript{4} buffer**

NaH\textsubscript{2}PO\textsubscript{4} buffer (20 mL, 20 mM; pH 7.3) was lyophilized. Subsequently, the residue was dissolved in D\textsubscript{2}O (2 mL), and stirred for 60 min. This solution was again lyophilized, dissolved in D\textsubscript{2}O (2 mL), and stirred for 60 min. The resulting mixture was lyophilized once more, after which the residue was dissolved in D\textsubscript{2}O (20 mL) yielding a stock solution of NaD\textsubscript{2}PO\textsubscript{4} (20 mM; pD 7.6).

**Redissolving 4-OT in NaD\textsubscript{2}PO\textsubscript{4} buffer**

A Vivaspin 2 concentrator (from Sartorius Stedim Goettingen, Germany) with a cut-off filter of 5000 Da was washed four times with H\textsubscript{2}O by centrifugation (4000 rpm, 20 min). Subsequently, the concentrator was charged with a solution of 4-OT (either wild-type or mutant; 300 μL with concentration of ~10 mg/mL in 20 mM NaH\textsubscript{2}PO\textsubscript{4} buffer, pH 7.3) and centrifuged (4000 rpm, 30 min). The enzyme was
retained on the filter and redissolved in NaD$_2$PO$_4$ (200 µL, 20 mM; pD 7.6) and centrifuged (4000 rpm, 30 min). Once more, the remaining enzyme on the filter was redissolved in NaD$_2$PO$_4$ (300 µL, 20 mM; pD 7.6), after which the final enzyme concentration was determined.

$^1$H NMR spectroscopic assay for self-condensation of propanal (3)

In separate experiments, WT 4-OT, 4-OT F50A and 4-OT P1A (290 µM) were incubated with 3 (30 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD$_2$PO$_4$ buffer (20 mM; pD 7.6, final volume of 650 µL in an NMR tube). A control sample was prepared containing all components except for the enzyme. $^1$H NMR spectra were recorded ~1 h after the start of the incubation, and subsequently after 1, 4, 8 and 14 d. $^1$H NMR spectroscopic data (in 20 mM NaD$_2$PO$_4$ buffer, pD 7.6) of enzymatically obtained 5 are similar to those of an authentic standard of 5. The hydrated form of 5 was not observed.

Internal standard 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane)

![18-crown-6](image)

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): δ 3.68 (s, 24H)

Propanal (3)

![Propanal](image)

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): δ 9.69 (t, $J$ = 1.3 Hz, 1H), 2.57 (dq, $J$ = 7.3, 1.3 Hz, 2H), 1.06 (t, $J$ = 7.3 Hz, 3H)
Propanal-2,2-d$_2$ (3-$d_2$)

\[
\begin{array}{c}
\text{O} \\
\text{D} \\
\text{D} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 9.69 (s, 1H), 1.03 (s, 3H)

Propane-1,1-diol-d$_2$ (3', hydrate of 3)

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\begin{array}{c}
\text{OD} \\
\text{H} \\
\text{H} \\
\text{OD} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 4.96 (t, $J = 5.5$ Hz, 1H), 1.59 (dq, $J = 7.5$, 5.5 Hz, 2H), 0.92 (t, $J = 7.5$ Hz, 3H)

Propane-2,2-d$_2$-1,1-diol-d$_2$ (3'-$d_2$)

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\text{D} \\
\text{D} \\
\text{OD} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 4.95 (s, 1H), 0.88 (s, 3H)

2-Methyl-2-pentenal (5)

\[
\begin{array}{c}
\text{H} \\
\text{O} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 9.29 (s, 1H), 6.81 (t, $J = 7.6$ Hz, 1H), 2.41 (dq, $J = 7.6$, 7.6 Hz, 2H), 1.71 (s, 3H), 1.09 (t, $J = 7.6$ Hz, 3H)
UV spectroscopic assay for cross-coupling of propanal (3) and benzaldehyde (6)

The cross-coupling of 3 and 6 was monitored by following the decrease in absorbance at 250 nm ($\lambda_{\text{max},6}$ = 250 nm) indicating depletion of 6 (Figure S2). Simultaneously, the increase in absorbance at 288 nm, corresponding to the formation of 8 ($\lambda_{\text{max},8}$ = 288 nm), was monitored. The enzyme (150 µM) was incubated in a 1 mm cuvette with 3 (50 mM) and 6 (2 mM) in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3; 0.3 mL final volume) and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200 to 400 nm.

$^1$H NMR spectroscopic assay for cross-coupling of propanal (3) and benzaldehyde (6)

In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (290 µM) were incubated with 3 (30 mM), 6 (15 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD$_2$PO$_4$ buffer (20 mM; pD 7.6, final volume of 650 µL in an NMR tube) (Figure S3). A control sample was prepared with all the components except for the enzyme. $^1$H NMR spectra were recorded ~2 h after incubation, and then after 1, 4, 8 and 14 d. $^1$H NMR spectroscopic data of $^7$9,10 are in accordance with data in the literature. $^1$H NMR spectroscopic data (in 20 mM NaD$_2$PO$_4$ buffer, pD 7.6) of enzymatically prepared 8 are identical to those of an authentic standard of 8. Hydrated forms of 6 and 8 were not observed.

Benzaldehyde (6)

\[ \text{H} \quad \text{H} \]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 9.94 (s, 1H), 7.97 (d, $J = 7.9$ Hz, 2H), 7.76 (d, $J = 7.5$ Hz, 1H), 7.63 (dd, $J = 7.9, 7.5$ Hz, 2H)
3-(Hydroxy-\textit{d})-2-methyl-3-phenylpropanal-2-\textit{d} (7)

\[
\begin{array}{c}
\text{H} \\
\text{C} \\
\text{O} \\
\text{D} \\
\text{D} \\
\text{OD} \\
\text{H} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 9.74 (s, 1H), 7.48 – 7.36 (m, 5H), 5.23 (s, 1H), 1.03 (s, 3H)

2-Methyl-3-phenylpropane-2-\textit{d}-1,1,3-triol-\textit{d}_3 (7')

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\text{D} \\
\text{D} \\
\text{OD} \\
\text{DO} \\
\text{D} \\
\text{H} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 7.48 – 7.36 (m, 5H), 5.21 (s, 1H), 4.94 (s, 1H), 0.94 (s, 3H)

(E)-2-Methyl-3-phenylacrylaldehyde (8)

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\begin{array}{c}
\text{H} \\
\text{C} \\
\text{O} \\
\text{D} \\
\text{H} \\
\end{array}
\]

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): $\delta$ 9.49 (s, 1H), 7.67 (d, $J$ = 7.5 Hz, 2H), 7.55 – 7.50 (m, 4H), 2.03 (s, 3H)

$^1$H NMR spectroscopic assay for cross-coupling of propanal (3) and pyruvate (9)

In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (290 $\mu$M) were incubated with 3 (30 mM) and 9 (15 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD$_2$PO$_4$ buffer (20 mM; pD 7.6, final volume of 650 $\mu$L in an NMR tube) (Figure S5). The control
sample was prepared with all the components except for the enzyme. $^1$H NMR spectra were recorded ~1 h after incubation, and then after 1, 4, 8 and 14 d. The yield of product 10 was determined on the basis of the sum of the integrations of the two aldehyde signals of the two diastereoisomers of 10. All other proton signals of 10 were either invisible as a result of H-D exchange or overlap with signals of starting materials 3 and 9. To the best of our knowledge, synthesis of 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (10) has not been reported in the literature so far. Therefore, we have chemically synthesized 10 to confirm the identity of enzymatically obtained 10, and for full characterization with $^1$H NMR, $^{13}$C NMR, and exact mass spectroscopy (vide infra).

The experiment with WT 4-OT was repeated in NaH$_2$PO$_4$ buffer (20 mM; pH 7.3) with D$_2$O (10% v/v) to avoid H-D exchange and enable the detection of all proton signals of 10 by $^1$H NMR spectroscopy (Figures S5 and S7). Propanal (3, 50 mM) and pyruvate (9, 50 mM) were incubated with WT 4-OT (90 µM) in NaH$_2$PO$_4$ buffer (20 mM; pH 7.6, final volume of 650 µL in an NMR tube). Reaction progress was monitored with $^1$H NMR spectroscopy which revealed formation of product 10, as a result of cross-coupling of 3 and 9, and of product 5 as a result of self-coupling of 3. In contrast to the experiment in 100% NaD$_2$PO$_4$ buffer (vide supra, Figure S5), the hydrated form of 10 (i.e. 10') was observed in small quantities (<5% compared to 10). This experiment was repeated in the absence of enzyme and in the presence of synthetic 4-OT WT$^2$ instead of recombinant 4-OT. In the absence of enzyme, formation of product 10 was not observed while only trace amounts of 5 were detected. In the presence of synthetic 4-OT WT, formation of products 10 and 5 was observed in equal quantities as in the presence of recombinant 4-OT WT.

**Pyruvate (9)**

![Pyruvate](image)
1H NMR (500 MHz, 20 mM NaH₂PO₄; pH 7.3): δ 2.39 (s, 3H)

2,2-Dihydroxypropanoic acid (9*: hydrated 9)

\[
\text{HO-CH(OH)-COOH}
\]

1H NMR (500 MHz, 20 mM NaH₂PO₄; pH 7.3): δ 1.50 (s, 3H)

2-Hydroxy-2,3-dimethyl-4-oxobutanoic acid (10, enzymatically prepared)

\[
\text{H-C(=O)-CH(OH)-CH(OH)-COOH}
\]

1H NMR (500 MHz, 20 mM NaH₂PO₄; pH 7.3): (diastereomer I) δ 9.74 (d, J = 2.7 Hz, 1H), 2.74 (dq, J = 7.0, 2.7 Hz, 1H), 1.44 (s, 3H), 1.08 (d, J = 7.0 Hz, 3H); (diastereomer II) δ 9.62 (d, J = 0.9 Hz, 1H), 2.74 (dq, J = 7.0, 0.9 Hz, 1H), 1.47 (s, 3H), 1.02 (d, J = 7.0 Hz, 3H)

2,4,4-Trihydroxy-2,3-dimethylbutanoic acid (10’)

\[
\text{HO-CH(OH)-C(=O)-OH}
\]

The 1H NMR spectroscopic data for 10’ are listed below (see chemically prepared 10).

Chemical synthesis of 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (10)

Compound 10 was synthesized by a two-step procedure. During the first step, an aldol coupling of propanal (3) and ethyl 2-oxopropanoate (commercially available), following a modified literature procedure,11 gave ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate in 67% yield after column
chromatography. Subsequently, ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate was hydrolyzed with LiOH in D$_2$O to give 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (10). The $^1$H NMR data of enzymatically obtained 10 matched those of chemically obtained 10 (Figure S7).

![Scheme S1. Chemical synthesis of 10.](image)

**Ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate.**

Ethyl 2-oxopropanoate (2.0 g, 17.2 mmol) and propanal (3, 4.0 g, 68.9 mmol) were dissolved in THF (25 mL). DL-proline (115 mg, 1.0 mmol) was added and the mixture was stirred for 48 h at room temperature. Reaction progress was monitored by thin layer chromatography (silica gel, hexanes/ethyl acetate 3/1). The solvent was evaporated *in vacuo* and the residue was submitted to column chromatography (silica gel, hexanes/ethyl acetate 5/1) to yield two diastereoisomers of ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate (2.0 g, 11.5 mmol, 67%) in a ~55/45 ratio as a colorless oil. $^1$H NMR (500 MHz, CDCl$_3$, 20°C); major diastereomer: $\delta$ 9.63 (s, 1H), 4.31 – 4.20 (m, 2H), 3.25 (b, 1H), 2.62 (q, $J$ = 7.3 Hz, 1H), 1.43 (s, 3H), 1.28 (t, $J$ = 7.2 Hz, 3H), 1.23 (d, $J$ = 7.3 Hz, 3H); minor diastereomer: $\delta$ 9.74 (d, $J$ = 2.9 Hz, 1H), 4.31 – 4.20 (m, 2H), 3.25 (b, 1H), 2.62 (dq, $J$ = 7.1, 2.9 Hz, 1H), 1.50 (s, 3H), 1.30 (t, $J$ = 7.1 Hz, 3H), 1.10 (d, $J$ = 7.1 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$, 20°C); major diastereomer: $\delta$ 202.72, 175.86, 74.15, 62.14, 53.13, 24.08, 13.96, 7.96; minor diastereomer: $\delta$ 203.16, 175.16, 75.03, 62.30, 52.81, 24.53, 14.00, 9.51; HRMS (ESI): $m/z$ = 175.09665 [M+H]$^+$ (calcd. 175.09650 for C$_8$H$_{15}$O$_4$).
2-Hydroxy-2,3-dimethyl-4-oxobutanoic acid (10). A mixture of ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate (112 mg, 0.64 mmol), LiOH (15.3 mg, 0.64 mmol) and D$_2$O (1.5 mL) was stirred for 2 d at room temperature. A $^1$H NMR spectrum of an aliquot of the reaction mixture, diluted with D$_2$O, revealed ~70% conversion of ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate into 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (10). The reaction mixture was washed with EtOAc (7 × 1.5 mL) to remove unhydrolized ester and ethanol. The D$_2$O layer was concentrated to ~1.0 mL in vacuo to remove residual EtOAc. The remaining D$_2$O layer was analyzed by $^1$H NMR spectroscopy revealing the presence of two diastereomers of 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (10) in a ~1/1 ratio. The hydrated forms of both diastereomers (i.e. 2,4,4-trihydroxy-2,3-dimethylbutanoic acid 10') were also observed (~7 mol% relative to aldehyde 10). $^1$H NMR (500 MHz, D$_2$O, 20°C): (diastereomer I) δ 9.80 (d, $J = 2.2$ Hz, 1H), 2.78 (dq, $J = 7.0$, 2.2 Hz, 1H), 1.48 (s, 3H), 1.12 (d, $J = 7.0$ Hz, 3H); (diastereomer II) δ 9.67 (s, 1H), 2.78 (q, $J = 7.0$, 1H), 1.51 (s, 3H), 1.06 (d, $J = 7.0$ Hz, 3H); diastereomers of hydrate: δ 5.30 (d, $J = 4.0$ Hz, 1H) and 5.13 (d, $J = 3.4$ Hz, 1H), 2.12 – 2.08 (m, 1H) and 2.07 – 2.03 (m, 1H), 1.42 (s, 3H) and 1.34 (s, 3H), 1.02 (d, $J = 7.1$ Hz, 3H) and 0.94 (d, $J = 7.1$ Hz, 3H); $^{13}$C NMR (125 MHz, D$_2$O, 20°C): (two diastereomers) δ 207.49 and 207.46, 181.22 and 180.76, 76.88 and 76.53, 53.78 and 52.78, 24.21 and 23.68, 8.72 and 7.47; HRMS (ESI): m/z = 159.08167 [M+H]$^+$ (calcd. 159.08154 for C$_6$H$_9$O$_4$Li$_2$).

$^1$H NMR spectroscopic assay for retro-aldol activity (conversion of 10 into 3 and 9)
In separate experiments, the enzymes WT 4-OT and 4-OT F50A (1 mg/mL, 150 μM in 20 mM NaH$_2$PO$_4$, pH 7.3) were incubated with 10 (300 μL from a 150 mM stock solution in D$_2$O, pH 4.5; final concentration of 30 mM), and the total volume was set at 1.5 mL using 20 mM NaH$_2$PO$_4$, pH 7.3 (Figure S8). The reactions were performed in NaH$_2$PO$_4$, instead of NaD$_2$PO$_4$, to avoid H-D exchange of acidic protons of 9 and to be able to observe the $^1$H NMR signal corresponding to 9. A control experiment without enzyme but under otherwise identical conditions was performed as well. To record $^1$H NMR spectra, 500 μL of each reaction mixture was diluted with 70 μL of 20 mM NaH$_2$PO$_4$ and 80 μL D$_2$O.
(final volume 650 μL), and the spectra were recorded immediately after mixing. The first $^1$H NMR spectrum was recorded after 2 h, and then after 1 and 3 days.

Chemical synthesis of hexanedial (11) and heptanodial (14)

Dials 11 and 14 were synthesized in situ from trans-cyclohexane-1,2-diol (17) and trans-cycloheptane-1,2-diol (18) according to a modified literature procedure (Scheme S6). General procedure: a 100 mM solution of diol and a 100 mM solution of sodium periodate were prepared in D$_2$O. Both solutions (500 μL each) were transferred to a glass vial and the solution was mixed gently. After 1 h, a $^1$H NMR spectrum was recorded which showed quantitative conversion into the dial, which was in equilibrium with its mono- and dihydrates (11’, 11”, 14’, and 14”), without any visible impurities. $^1$H NMR data of 11$^{[8]}$ and 14$^{[12]}$ are in accordance with data reported in the literature.

UV spectroscopic assay for cyclization of 11 and 14

In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (150 μM) were incubated with dial (11 or 14, 16.7 mM) in a 1 mm cuvette at room temperature in NaH$_2$PO$_4$ buffer (20 mM, pH 7.3; 0.3 mL final volume) (Figures S9 and S13). UV spectra were recorded from 200 to 400 nm at t = 6 and 12 h ($\lambda_{max,13} = 245$ nm (Lit : 236 nm in 99.5% EtOH)$^{13}$, $\lambda_{max,16} = 236$ nm (Lit: 230 nm in 100% EtOH)$^{14}$). Control experiments (all components but without enzyme) were performed as well.

$^1$H NMR spectroscopic assay for cyclization of 11 and 14

The enzymes WT 4-OT and 4-OT F50A (148 μM) were incubated separately with dialdehyde (11 or 14, 15.4 mM in 20 mM NaD$_2$PO$_4$, pD = 7.6) (Figures S10 and S14). Total volume of each reaction mixture was 650 μL in an NMR tube. The tubes were stored at room temperature, protected from light. $^1$H NMR spectra were recorded after 0, 2, 21, 27, 45, 50, 68, 75, 144, 216, 242, 333, 408, 503, 528 and 672 h. After 4 weeks, the enzymes were still fully active as determined by an enzymatic assay monitoring the enol-
keto tautomerization of phenylpyruvate\textsuperscript{15} (5 mM phenylpyruvate, 1 µg/mL WT 4-OT or 0.1 µg/mL 4-OT F50A in NaH\textsubscript{2}PO\textsubscript{4} buffer, pH 7.3). \textsuperscript{1}H NMR spectroscopic data of 13\textsuperscript{16} and 16\textsuperscript{17} are consistent with data in the literature. Hydrated forms of 13 and 16 were not observed.

**Hexanediol (11)**

\[
\begin{array}{c}
\text{\Huge O} \\
\text{(n = 1)} \\
\text{\Huge O}
\end{array}
\]

\textsuperscript{1}H NMR (500 MHz, 20 mM NaD\textsubscript{2}PO\textsubscript{4}; pD 7.6): \( \delta \) 9.71 (t, \( J = 2.1 \) Hz, 2H), 2.62 – 2.56 (m, 4H), 1.72 – 1.61 (m, 4H)

**6,6-Di(hydroxy-\textit{d})hexanal (11’)**

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\text{(n = 1)} \\
\text{\Huge O}
\end{array}
\]

\textsuperscript{1}H NMR (500 MHz, 20 mM NaD\textsubscript{2}PO\textsubscript{4}; pD 7.6): \( \delta \) 9.71 (t, \( J = 2.1 \) Hz, 1H), 5.06 (t, \( J = 5.6 \) Hz, 1H), 2.62 – 2.56 (m, 2H), 1.72 – 1.61 (m, 4H), 1.47 – 1.38 (m, 2H)

**Hexane-1,1,6,6-tetraol-\textit{d}_4 (11’’)**

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\text{(n = 1)} \\
\text{\Huge O} \text{\Huge O}
\end{array}
\]

\textsuperscript{1}H NMR (500 MHz, 20 mM NaD\textsubscript{2}PO\textsubscript{4}; pD 7.6): \( \delta \) 5.06 (t, \( J = 5.6 \) Hz, 2H), 1.72 – 1.61 (m, 4H), 1.47 – 1.38 (m, 4H)
2-(Hydroxy-\(d\))cyclopentane-1-carbaldehyde (12)

\[\text{\(n = 1\)}\]

\(^1\)H NMR (500 MHz, 20 mM NaD\(_2\)PO\(_4\); pD 7.6): (major diastereomer) \(\delta\) 9.67 (s, 1H), 4.30 (m, 1H), 4.14 (m, 1H, overlapping with signal of compound 12 (hydrate)), 1.96 – 1.46 (m, 6H); characteristic signal of minor diastereomer: \(\delta\) 9.81 (s, 1H)

(2-(Hydroxy-\(d\))cyclopentyl)methanediol-\(d_2\) (12’)

\[\text{\(n = 1\)}\]

\(^1\)H NMR (500 MHz, 20 mM NaD\(_2\)PO\(_4\); pD 7.6): (major diastereomer) \(\delta\) 4.97 (s, 1H), 4.52 (m, 1H), 4.14 (m, 1H, overlapping with signal of compound 12), 1.96 – 1.46 (m, 6H); characteristic signal of minor diastereomer: \(\delta\) 5.03 (s, 1H)

Cyclopent-1-ene-1-carbaldehyde (13)

\[\text{\(n = 1\)}\]

\(^1\)H NMR (500 MHz, 20 mM NaD\(_2\)PO\(_4\); pD 7.6): \(\delta\) 9.68 (s, 1H), 7.25 (s, 1H), 2.66 (t, \(J = 7.6\) Hz, 2H), 2.49 (t, \(J = 7.0\) Hz, 2H), 2.05 – 1.99 (m, 2H)

Heptanedral (14)
$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): δ 9.71 (t, $J$ = 1.9 Hz, 2H), 2.59 – 2.54 (m, 4H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 2H)

7,7-di(hydroxy-$d$)heptanal (14')

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): δ 9.71 (t, $J$ = 1.9 Hz, 1H), 5.05 (t, $J$ = 5.6 Hz, 1H), 2.59 – 2.54 (m, 2H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 4H)

Heptane-1,1,7,7-tetraol-$d_4$ (14'')

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): δ 5.05 (t, $J$ = 5.6 Hz, 2H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 6H)

2-(Hydroxy-$d$)cyclohexane-1-carbaldehyde (15)

$^1$H NMR (500 MHz, 20 mM NaD$_2$PO$_4$; pD 7.6): (major diastereomer) δ 9.67 (s, 1H), 3.95 – 3.89 (m, 1H), 2.06 – 1.13 (m, 8H), signal of 1 proton overlaps with water signal at 4.80 ppm; characteristic signal of minor diastereomer: δ 9.72 (s, 1H)
(2-(hydroxy-d)cyclohexyl)methanediol-d₂ (15’)

\[
\begin{array}{c}
\text{DO} \quad \text{OD} \\
\text{n} \quad \text{OD}
\end{array}
\]

\(n = 2\)

\(^1\)H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): (major diastereomer) \(\delta 5.22\) (s, 1H), \(4.52 - 4.48\) (m, 1H), \(3.63 - 3.57\) (m, 1H), \(2.06 - 1.13\) (m, 8H); minor diastereomer was not observed.

Cyclohex-1-ene-1-carbaldehyde (16)

\[
\begin{array}{c}
\text{CH}_2 \text{= O} \\
\text{n}
\end{array}
\]

\(n = 2\)

\(^1\)H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): \(\delta 9.29\) (s, 1H), \(7.13\) (s, 1H), \(2.46 - 2.33\) (m, 2H), \(2.21 - 2.09\) (m, 2H), \(1.72 - 1.61\) (m, 4H)
Scheme S2. Stable equilibrium between the hydrated (63%) and unhydrated (37%) form of 3 witnessed immediately after preparing the sample (≤ 5 min, $^1$H NMR spectroscopy) in the absence as well as presence of enzyme (4-OT WT, F50A, P1A). Hydrogen-deuterium exchange of the acidic protons of substrate 3 (monitored by $^1$H NMR spectroscopy. See paragraph ‘$^1$H NMR spectroscopic assay for self-condensation of propanal (3)’). The exchange most likely only takes place at C2 of the unhydrated form of 3 (i.e. propanal) and not at C2 of the hydrated form (3') since protons at C2 of the latter are not acidic.

The rates for reaching equilibrium between unhydrated and hydrated form are relatively high compared to the rates of H-D exchange.
**Scheme S3.** Proposed mechanisms for the 4-OT-catalyzed hydrogen-deuterium exchange within propanal (3) and the role of Pro-1 as base (A) or as nucleophile (B).
Scheme S4. Incubation of 3 and 6 in 20 mM NaD₂PO₄ buffer (pD 7.6) with 4-OT WT, 4-OT F50A, or 4-OT P1A.

Scheme S5. Incubation of 3 and 9 in 20 mM NaD₂PO₄ buffer (pD 7.6) with 4-OT WT, 4-OT F50A, 4-OT P1A, or synthetic 4-OT. Product 10 is not formed in the absence of enzyme.
Scheme S6. *In situ* preparation of hexanediol (11) and heptanediol (14), and their mono- and dihydrates (11’, 11”, 14’, and 14’’), subsequent 4-OT-catalyzed cyclization into products 12 and 15, and final dehydration into α,β-unsaturated adducts 13 and 16. Hydrated forms of 13 and 16 were not observed.
Figure S1. UV spectra showing the conversion of 3-hydroxy-2-methylpentanal (4) into 2-methyl-2-pentenal (5, $\lambda_{\text{max}} = 234$ nm). Aldol compound 4 (20 mM in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3) was incubated with A) no enzyme (control sample), B) WT 4-OT, or C) 4-OT F50A mutant.
**Figure S2.** UV spectra recorded after incubation of 3 (50 mM) and 6 (2 mM) in 20 mM NaH₂PO₄ buffer at pH 7.3 with A) 4-OT F50A mutant, B) WT 4-OT, C) 4-OT P1A mutant, or D) no enzyme (control sample).
Figure S3. Stack plot of $^1$H NMR spectra recorded after 4 d of incubation of 3 and 6 in 20 mM NaD$_2$PO$_4$ buffer (pD 7.6) with A) no enzyme; B) 4-OT WT; C) 4-OT F50A; D) 4-OT WT synthetic; E) 4-OT P1A (See Scheme S4 for reaction scheme). Spectrum A shows equilibrium was reached between unhydrated and hydrated (3’) forms of 3 and shows no formation of products 7, 7’, 8 nor 5 (as a result of self-condensation of 3). Spectrum B shows that protons at C2 of 3 and 3’ have exchanged with deuterium resulting in formation of 3-$d_2$ and 3’-$d_2$ (see Scheme S2 for mechanism). Spectrum B furthermore shows little formation of products 7, 7’, 8 and 5 (indicative signals are specified in spectrum C). Spectra C and D show formation of products 7, 7’, 8 and 5 (only indicative signals are given. Signals of 3 and 6 not marked for the sake of clarity). Hydrated 8 was not observed. Spectrum E shows no formation of products 7, 7’, 8 and 5, nor proton-deuterium exchange of 3.
Figure S4. Yields (%) of 7 (●) and 8 (△) (based on 6) in course of time in the reaction mixture (3 + 6) incubated with 4-OT F50A.
Figure S5. Stack plot of $^1$H NMR spectra recorded after 4 d of incubation of 3 and 9 in 20 mM NaD$_2$PO$_4$ buffer (pD 7.6) with A) no enzyme; B) 4-OT WT; C) 4-OT F50A; D) 4-OT P1A (see Scheme S5 for reaction scheme). Spectrum A shows equilibrium was reached between unhydrated and hydrated (i.e. 3') forms of 3 and shows no formation of product 10 nor 5 (as a result of self-condensation of 3). Spectrum B shows that protons at C2 of 3 and 3' and methyl protons of 9 have exchanged with deuterium resulting in formation of 3-d$_2$, 3'-d$_2$ (see Scheme S2 for mechanism), and 9-d$_3$ (latter not visible in spectrum). Spectrum B furthermore shows little formation of product 10 (two diastereomers) and of 5 (indicative signals are specified in spectrum C). Spectrum C shows formation of 10 (two diastereomers) and 5 (only indicative signals are given. Signals of 3 are not marked for the sake of clarity). Spectrum D shows no formation of products 10 and 5, nor proton-deuterium exchange of 3. It does however indicate complete H-D exchange of acidic methyl protons of 9.
**Figure S6.** Yields (%) of 10 (based on 9) in course of time in the reaction mixture (3 + 9) incubated with the 4-OT F50A mutant.
Figure S7. Stack plot of $^1$H NMR spectra recorded after 4 d of incubation of 3 and 9 in 20 mM NaH$_2$PO$_4$ buffer (pH 7.3) with A) recombinant 4-OT WT and B) synthetic 4-OT WT (DMF used as internal standard). Spectrum C) shows the signals for chemically synthesized 10. See Scheme S5 for reaction scheme for enzymatically obtained 10 and Scheme S1 for synthetically obtained 10.
Figure S8. Stack plot of $^1$H NMR spectra recorded after the incubation of 10 in 20 mM NaH$_2$PO$_4$ buffer at pH 7.3 with A) no enzyme for 1 h; B) WT 4-OT for 1 h; C) 4-OT F50A for 1 h; D) no enzyme for 3 d; E) WT 4-OT for 3 d; and F) 4-OT F50A for 3d.
Figure S9. UV spectra recorded after incubation of 11 (16.7 mM) in 20 mM NaH₂PO₄ buffer at pH 7.3 with A) WT 4-OT, B) 4-OT F50A mutant, C) no enzyme (control sample) and D) 4-OT P1A mutant (t = 6 and 12 h). Increase of absorbance at 245 nm indicates formation of 13.
Figure S10. Cyclization of hexanediol 11 (15.4 mM) into 12 in NaD$_2$PO$_4$ (20 mM, pD = 7.6), catalyzed by 4-OT F50A (148 µM), and subsequent dehydration into 13 monitored by $^1$H NMR spectroscopy (t = 0, 21, 672 h) (See Scheme S6 for reaction scheme). The enzyme 4-OT is responsible for broad absorptions between ~2 and 0.5 ppm, especially well-visible in the bottom spectrum.
Figure S11. Plot of the presence (%) of 11 and yields (%) of 12 and 13 in course of time in the reaction mixture incubated with the 4-OT F50A mutant (in 20 mM NaD₂PO₄ buffer at pD 7.6).
Figure S12. UV spectra recorded after incubation of 11 (16.7 mM) in 20 mM NaH₂PO₄ buffer at pH 7.3 with 0.075 mM A) recombinant WT 4-OT and B) synthetic WT 4-OT (t = 6, 12, 18 and 24 h).
Figure S13. UV spectra recorded after incubation of 14 (16.7 mM) in 20 mM NaH$_2$PO$_4$ buffer at pH 7.3 with A) WT 4-OT, B) 4-OT F50A mutant, C) no enzyme (control sample) and D) 4-OT P1A mutant (t = 6 and 12 h). Increase of absorbance at 236 nm indicates formation of 16.
Figure S14. Cyclization of heptanediol 14 (15.4 mM) into 15 in NaD_2PO_4 (20 mM, pD = 7.6), catalyzed by 4-OT F50A (148 μM), and subsequent dehydration into 16 monitored by ^1H NMR spectroscopy (t = 0, 21, 672 h) (See Scheme S6 for reaction scheme). The enzyme 4-OT is responsible for broad absorptions between ~2 and 0.5 ppm, especially well-visible in the bottom spectrum.
Figure S15. Plot of the presence (%) of 14 and yields (%) of 15 and 16 in course of time in the reaction mixture incubated with the 4-OT F50A mutant (in 20 mM NaD₂PO₄ buffer at pD 7.6).
**Figure S16.** UV spectra recorded after incubation of 14 (16.7 mM) in 20 mM NaH$_2$PO$_4$ buffer at pH 7.3 with 0.045 mM A) recombinant WT 4-OT and B) synthetic WT 4-OT (t = 6, 12, 18 and 24 h).

**References**


13 $\lambda_{\text{max,13}}$ in 99.5% EtOH = 236 nm: G. Magnusson, S. Thoren, *J. Org. Chem.* 1973, **38**, 1380.


