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_2O (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 *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.[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_2PO_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_2PO_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_2PO_4 buffer

NaH_2PO_4 buffer (20 mL, 20 mM; pH 7.3) was lyophilized. Subsequently, the residue was dissolved in D_2O (2 mL), and stirred for 60 min. This solution was again lyophilized, dissolved in D_2O (2 mL), and stirred for 60 min. The resulting mixture was lyophilized once more, after which the residue was dissolved in D_2O (20 mL) yielding a stock solution of NaD_2PO_4 (20 mM; pD 7.6).

Redissolving 4-OT in NaD_2PO_4 buffer

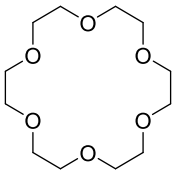
A Vivaspin 2 concentrator (from Sartorius Stedim Goettingen, Germany) with a cut-off filter of 5000 Da was washed four times with H_2O by centrifugation (4000 rpm, 20 min). Subsequently, the concentrator was charged with a solution of 4-OT (either wild-type or mutant; 300 μL with concentration of ~10 mg/mL in 20 mM NaH_2PO_4 buffer, pH 7.3) and centrifuged (4000 rpm, 30 min). The enzyme was

retained on the filter and redissolved in NaD_2PO_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_2PO_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)

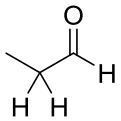
In separate experiments, WT 4-OT, 4-OT F50A and 4-OT P1A (290 µM) were incubated with **3** (30 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 µL in an NMR tube). A control sample was prepared containing all components 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_2PO_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)



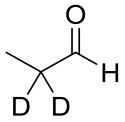
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 3.68 (s, 24H)

Propanal (3)



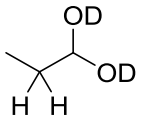
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.69 (t, *J* = 1.3 Hz, 1H), 2.57 (dq, *J* = 7.3, 1.3 Hz, 2H), 1.06 (t, *J* = 7.3 Hz, 3H)

Propanal-2,2-d_2 (3-d_2)



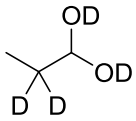
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.69 (s, 1H), 1.03 (s, 3H)

Propane-1,1-diol-d_2 (3', hydrate of 3)



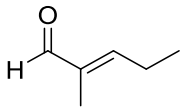
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 4.96 (t, *J* = 5.5 Hz, 1H), 1.59 (dq, *J* = 7.5, 5.5 Hz, 2H), 0.92 (t, *J* = 7.5 Hz, 3H)

Propane-2,2-d_2-1,1-diol-d_2 (3'-d_2)



^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 4.95 (s, 1H), 0.88 (s, 3H)

2-Methyl-2-pentenal (5)



^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 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)

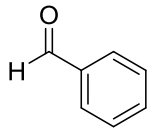
UV spectroscopic assay for cross-coupling of propanal (3) and benzaldehyde (6)

The cross-coupling of **3** and **6** was monitored by following the decrease in absorbance at 250 nm ($\lambda_{max,\mathbf{6}}$ = 250 nm) indicating depletion of **6** (Figure S2). Simultaneously, the increase in absorbance at 288 nm, corresponding to the formation of **8** ($\lambda_{max,\mathbf{8}}$ = 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_2PO_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)

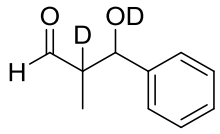
In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (290 μM) were incubated with **3** (30 mM), **6** (15 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 μL in an NMR tube) (Figure S3). A control sample 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_2PO_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)



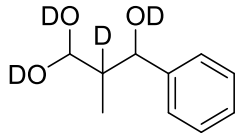
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.94 (s, 1H), 7.97 (d, *J* = 7.9 Hz, 2H), 7.76 (d, *J* = 7.5 Hz, 1H), 7.63 (dd, *J* = 7.9, 7.5 Hz, 2H)

3-(Hydroxy-*d*)-2-methyl-3-phenylpropanal-2-*d* (7)



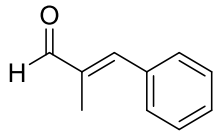
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.74 (s, 1H), 7.48 – 7.36 (m, 5H), 5.23 (s, 1H), 1.03 (s, 3H)

2-Methyl-3-phenylpropane-2-*d*-1,1,3-triol-d_3 (7')



^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 7.48 – 7.36 (m, 5H), 5.21 (s, 1H), 4.94 (s, 1H), 0.94 (s, 3H)

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



^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.49 (s, 1H), 7.67 (d, J = 7.5 Hz, 2H), 7.55 – 7.50 (m, 4H), 2.03 (s, 3H)

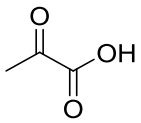
^{1}H NMR spectroscopic assay for cross-coupling of propanal (3) and pyruvate (9)

In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (290 µM) 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_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 µL 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).

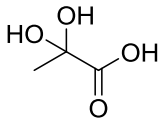
The experiment with WT 4-OT was repeated in NaH_2PO_4 buffer (20 mM; pH 7.3) with D_2O (10% v/v) to avoid H-D exchange and enable the detection of all proton signals of **10** by ^{1}H NMR spectroscopy (Figures S5 and S7). Propanal (**3**, 50 mM) and pyruvate (**9**, 50 mM) were incubated with WT 4-OT (90 µM) in NaH_2PO_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_2PO_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)



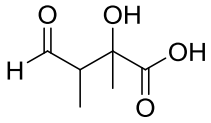
^{1}H NMR (500 MHz, 20 mM NaH_2PO_4; pH 7.3): δ 2.39 (s, 3H)

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



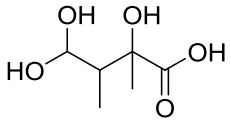
^{1}H NMR (500 MHz, 20 mM NaH_2PO_4; pH 7.3): δ 1.50 (s, 3H)

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



^{1}H NMR (500 MHz, 20 mM NaH_2PO_4; 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')

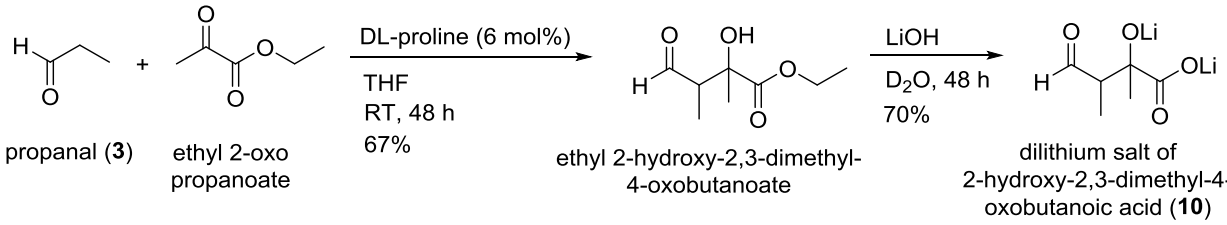


The ^{1}H NMR spectroscopic data for **10'** are listed below (see chemically prepared **10**).

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_2O 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**.

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: δ 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: δ 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: δ 202.72, 175.86, 74.15, 62.14, 53.13, 24.08, 13.96, 7.96; minor diastereomer: δ 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 $C_8H_{15}O_4$).

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_2O (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_2O, 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_2O layer was concentrated to ~1.0 mL *in vacuo* to remove residual EtOAc. The remaining D_2O 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_2O, 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_2O, 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 $C_6H_9O_4Li_2$).

^{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_2PO_4, pH 7.3) were incubated with **10** (300 μL from a 150 mM stock solution in D_2O, pH 4.5; final concentration of 30 mM), and the total volume was set at 1.5 mL using 20 mM NaH_2PO_4, pH 7.3 (Figure S8). The reactions were performed in NaH_2PO_4, instead of NaD_2PO_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_2PO_4 and 80 μL D_2O

(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 heptanedial (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).[4] General procedure: a 100 mM solution of diol and a 100 mM solution of sodium periodate were prepared in D_2O. 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**[4] and **14**[12] are in accordance with data reported in the literature.

UV spectroscopic assay for cyclization of 11 and 14

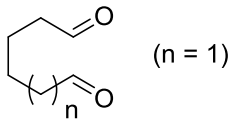
In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (150 µM) were incubated with dial (**11** or **14**, 16.7 mM) in a 1 mm cuvette at room temperature in NaH_2PO_4 buffer (20 mM, pH 7.3; 0.3 mL final volume) (Figures S9 and S13). UV spectra were recorded from 200 to 400 nm at t = 6 and 12 h ($\lambda_{max,\mathbf{13}}$ = 245 nm (Lit : 236 nm in 99.5% EtOH)[13], $\lambda_{max,\mathbf{16}}$ = 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_2PO_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-

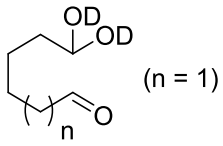
keto tautomerization of phenylpyruvate[15] (5 mM phenylpyruvate, 1 µg/mL WT 4-OT or 0.1 µg/mL 4-OT F50A in NaH_2PO_4 buffer, pH 7.3). ^{1}H NMR spectroscopic data of **13**[16] and **16**[17] are consistent with data in the literature. Hydrated forms of **13** and **16** were not observed.

Hexanedial (11)



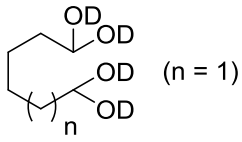
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.71 (t, *J* = 2.1 Hz, 2H), 2.62 – 2.56 (m, 4H), 1.72 – 1.61 (m, 4H)

6,6-Di(hydroxy-*d*)hexanal (11')



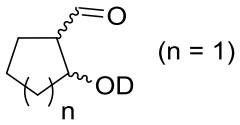
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.71 (t, *J* = 2.1 Hz, 1H), 5.06 (t, *J* = 5.6 Hz, 1H), 2.62 – 2.56 (m, 2H), 1.72 – 1.61 (m, 4H), 1.47 – 1.38 (m, 2H)

Hexane-1,1,6,6-tetraol-d_4 (11'')



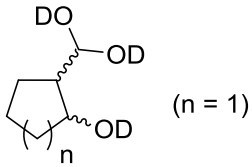
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 5.06 (t, *J* = 5.6 Hz, 2H), 1.72 – 1.61 (m, 4H), 1.47 – 1.38 (m, 4H)

2-(Hydroxy-*d*)cyclopentane-1-carbaldehyde (12)



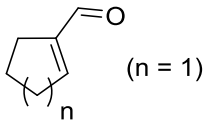
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): (major diastereomer) δ 9.67 (s, 1H), 4.30 (m, 1H), 4.14 (m, 1H, overlapping with signal of compound **12'** (hydrate)), 1.96 – 1.46 (m, 6H); characteristic signal of minor diastereomer: δ 9.81 (s, 1H)

(2-(Hydroxy-*d*)cyclopentyl)methanediol-d_2 (12')



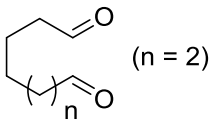
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): (major diastereomer) δ 4.97 (s, 1H), 4.52 (m, 1H), 4.14 (m, 1H, overlapping with signal of compound **12**), 1.96 – 1.46 (m, 6H); characteristic signal of minor diastereomer: δ 5.03 (s, 1H)

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



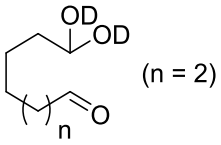
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.68 (s, 1H), 7.25 (s, 1H), 2.66 (t, J = 7.6 Hz, 2H), 2.49 (t, J = 7.0 Hz, 2H), 2.05 – 1.99 (m, 2H)

Heptanedial (14)



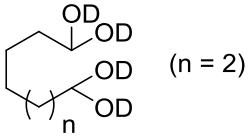
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.71 (t, *J* = 1.9 Hz, 2H), 2.59 – 2.54 (m, 4H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 2H)

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



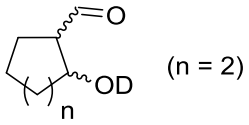
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.71 (t, *J* = 1.9 Hz, 1H), 5.05 (t, *J* = 5.6 Hz, 1H), 2.59 – 2.54 (m, 2H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 4H)

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



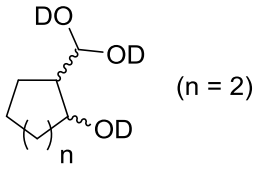
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 5.05 (t, *J* = 5.6 Hz, 2H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 6H)

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



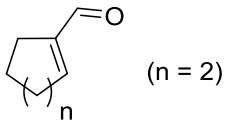
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): (major diastereomer) δ 9.67 (s, 1H), 3.95 – 3.89 (m, 1H), 2.06 – 1.13 (m, 8H), signal of 1 proton overlaps with water signal at 4.80 ppm; characteristic signal of minor diastereomer: δ 9.72 (s, 1H)

(2-(hydroxy-*d*)cyclohexyl)methanediol-d_2 (15')

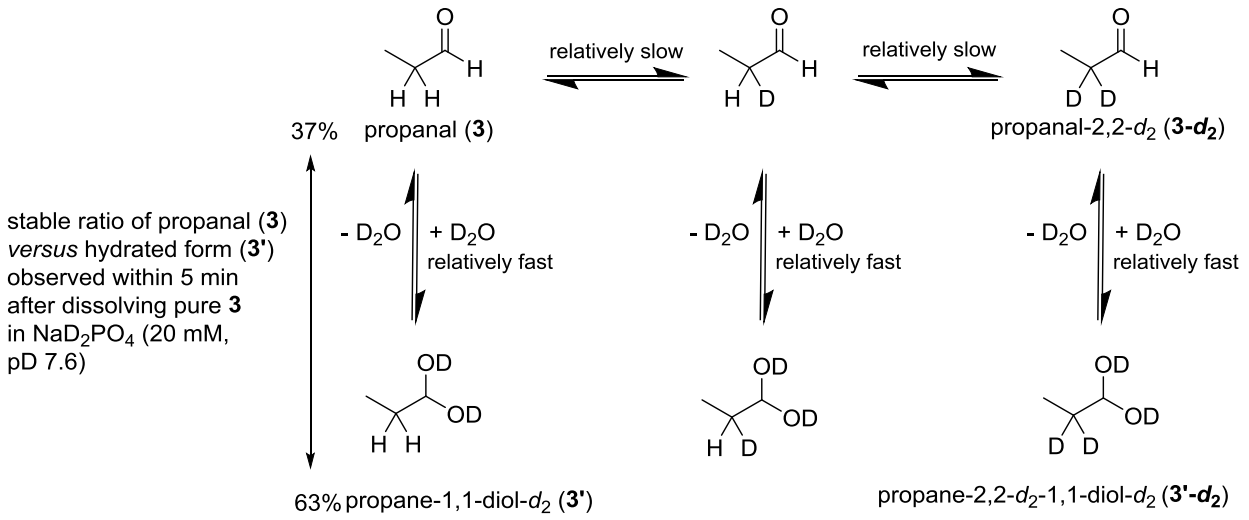


^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): (major diastereomer) δ 5.22 (s, 1H), 4.52 – 4.48 (m, 1H), 3.63 – 3.57 (m, 1H), 2.06 – 1.13 (m, 8H); minor diastereomer was not observed.

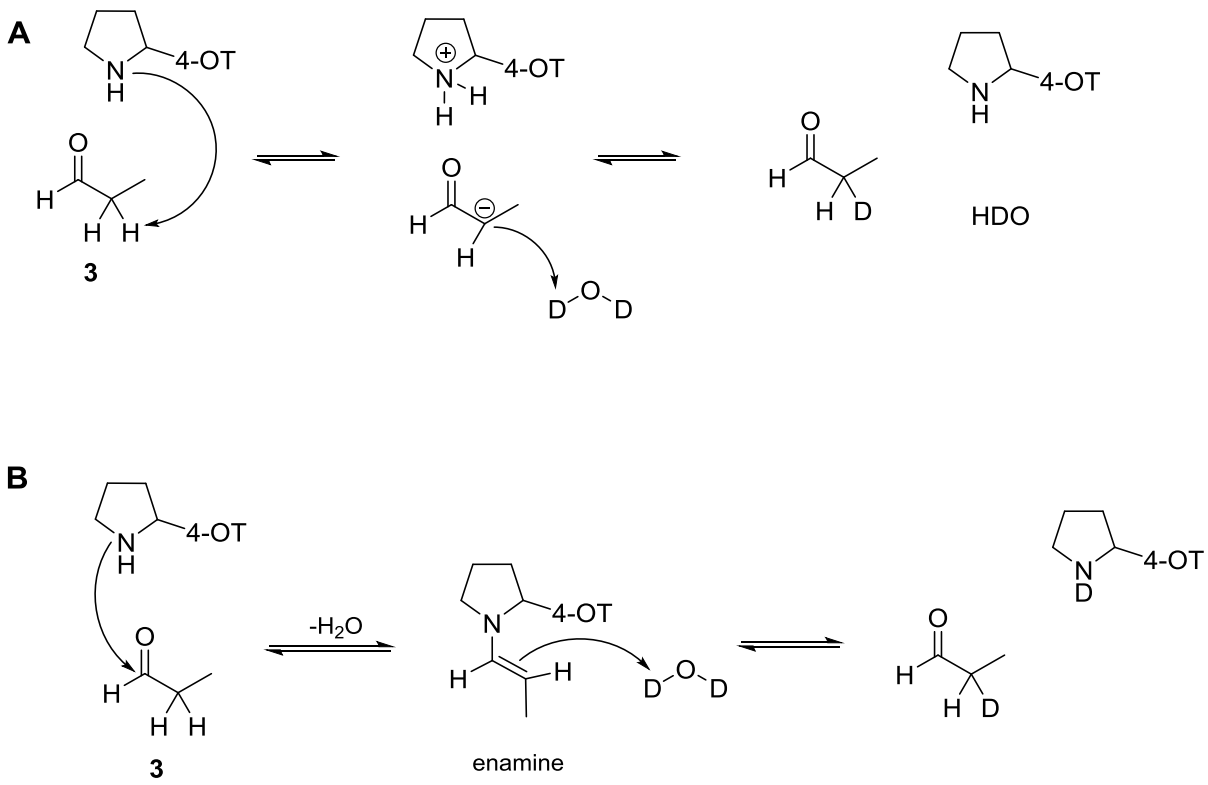
Cyclohex-1-ene-1-carbaldehyde (16)



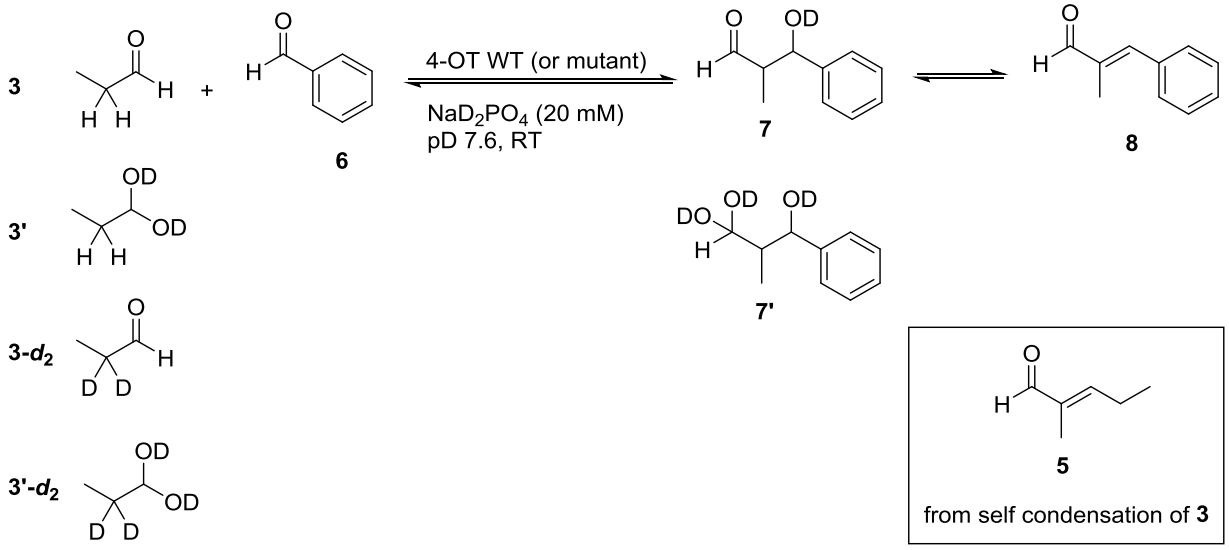
^{1}H NMR (500 MHz, 20 mM NaD_2PO_4; pD 7.6): δ 9.29 (s, 1H), 7.13 (s, 1H), 2.46 – 2.33 (m, 2H), 2.21 – 2.09 (m, 2H), 1.72 – 1.61 (m, 4H)



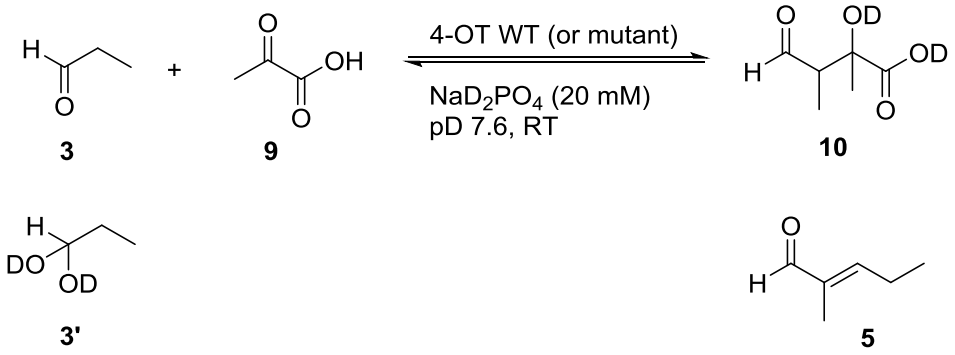
Scheme S2. Stable equilibrium between the hydrated (63%) and unhydrated (37%) form of **3** witnessed immediately after preparing the sample (≤ 5 min, ^{1}H NMR spectroscopy) in the absence as well as presence of enzyme (4-OT WT, F50A, P1A). Hydrogen-deuterium exchange of the acidic protons of substrate **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.



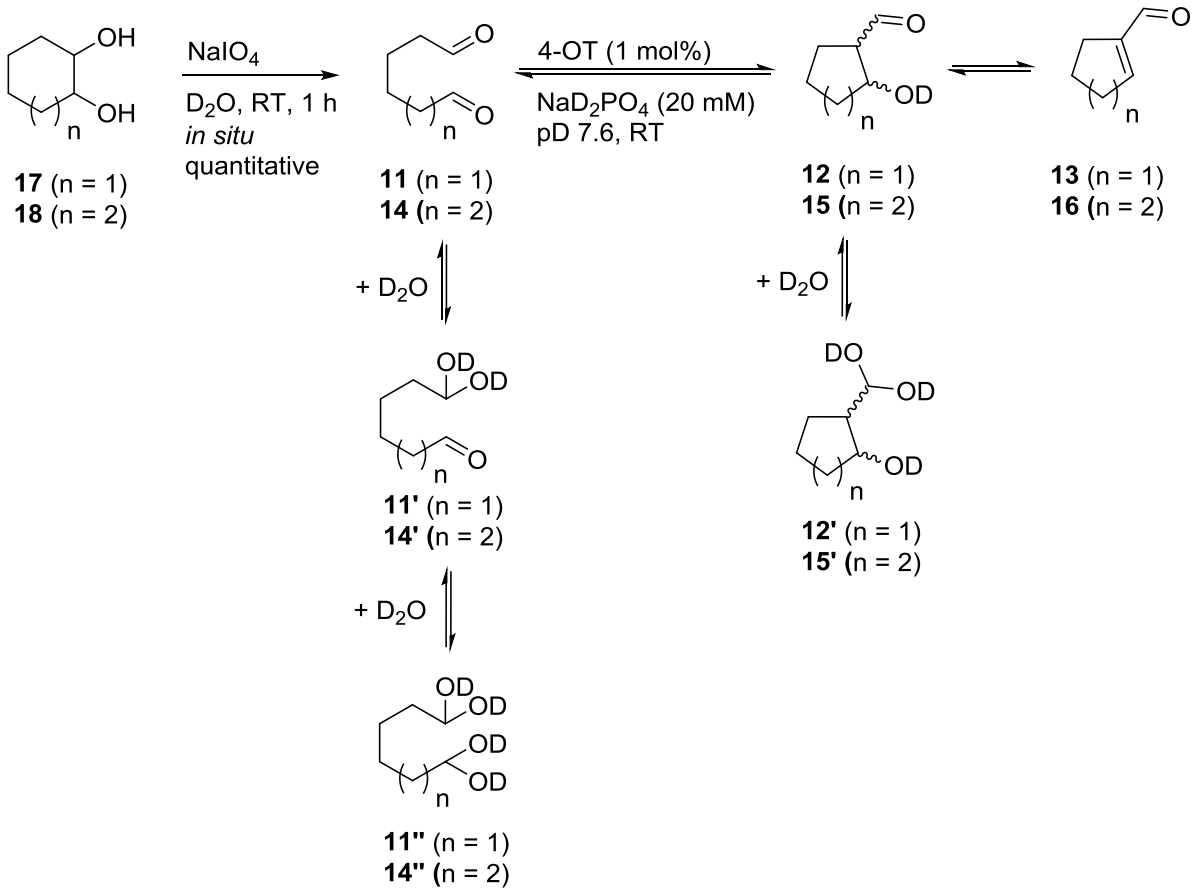
Scheme S3. Proposed mechanisms for the 4-OT-catalyzed hydrogen-deuterium exchange within propanal (**3**) and the role of Pro-1 as base (A) or as nucleophile (B).



Scheme S4. Incubation of **3** and **6** in 20 mM NaD_2PO_4 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 NaD_2PO_4 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 hexanedial (**11**) and heptanedial (**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.

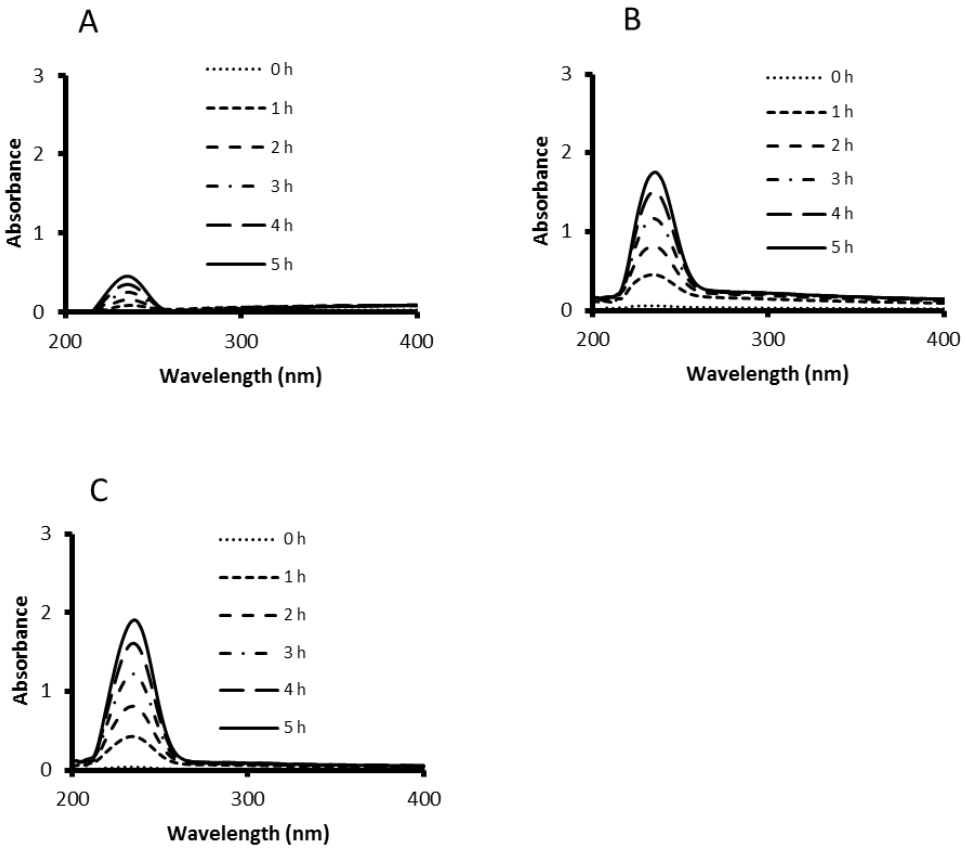


Figure S1. UV spectra showing the conversion of 3-hydroxy-2-methylpentanal (**4**) into 2-methyl-2-pentenal (**5**, λ_{max} = 234 nm). Aldol compound **4** (20 mM in 20 mM NaH_2PO_4 buffer (pH 7.3) was incubated with A) no enzyme (control sample), B) WT 4-OT, or C) 4-OT F50A mutant.

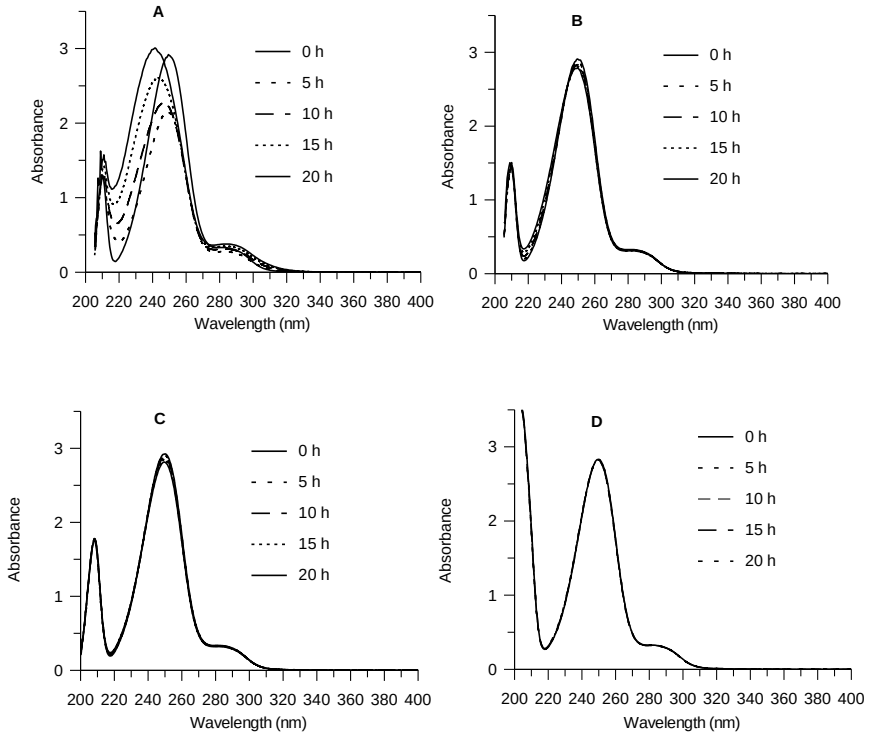


Figure S2. UV spectra recorded after incubation of **3** (50 mM) and **6** (2 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) 4-OT F50A mutant, B) WT 4-OT, C) 4-OT P1A mutant, or D) no enzyme (control sample).

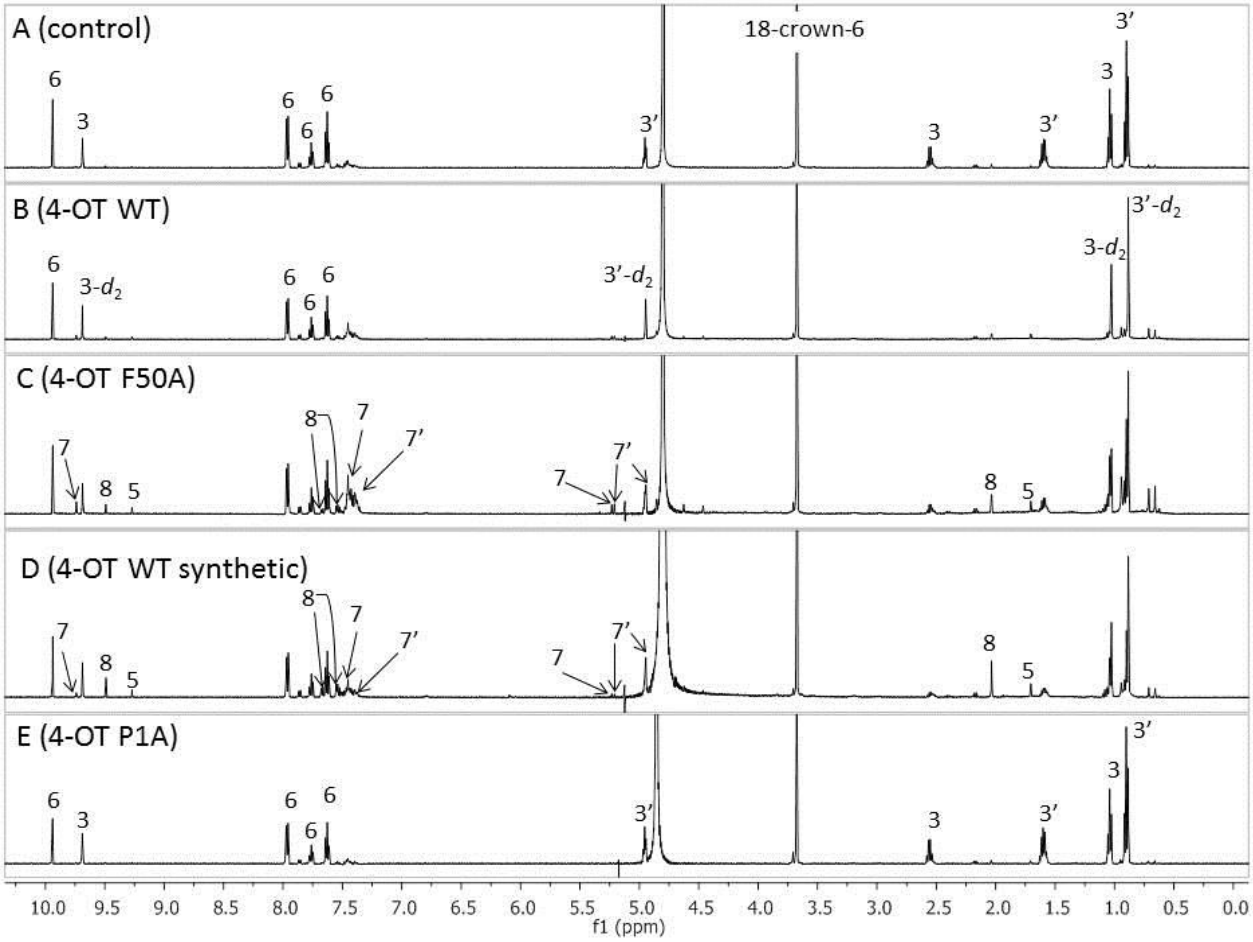


Figure S3. Stack plot of ^{1}H NMR spectra recorded after 4 d of incubation of **3** and **6** in 20 mM NaD_2PO_4 buffer (pD 7.6) with A) no enzyme; B) 4-OT WT; C) 4-OT F50A; D) 4-OT WT synthetic; E) 4-OT P1A (See Scheme S4 for reaction scheme). Spectrum A shows equilibrium was reached between unhydrated 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**.

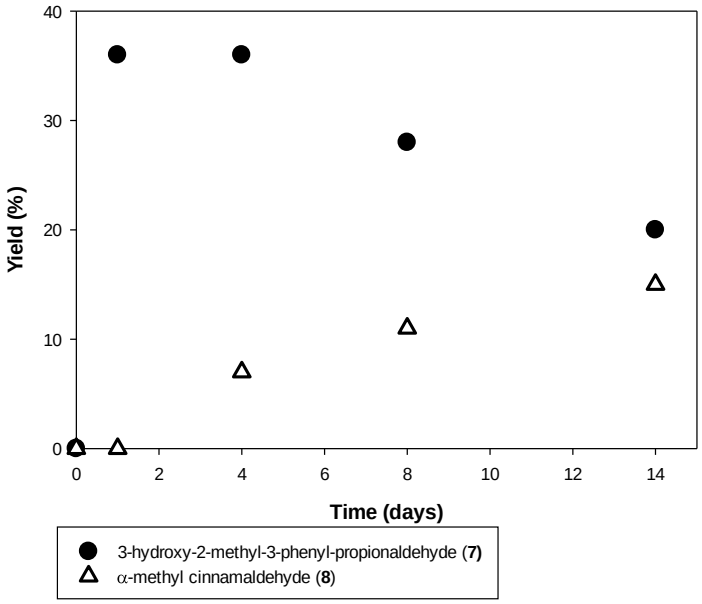


Figure S4. Yields (%) of **7** (●) and **8** (Δ) (based on **6**) in course of time in the reaction mixture (**3** + **6**) incubated with 4-OT F50A.

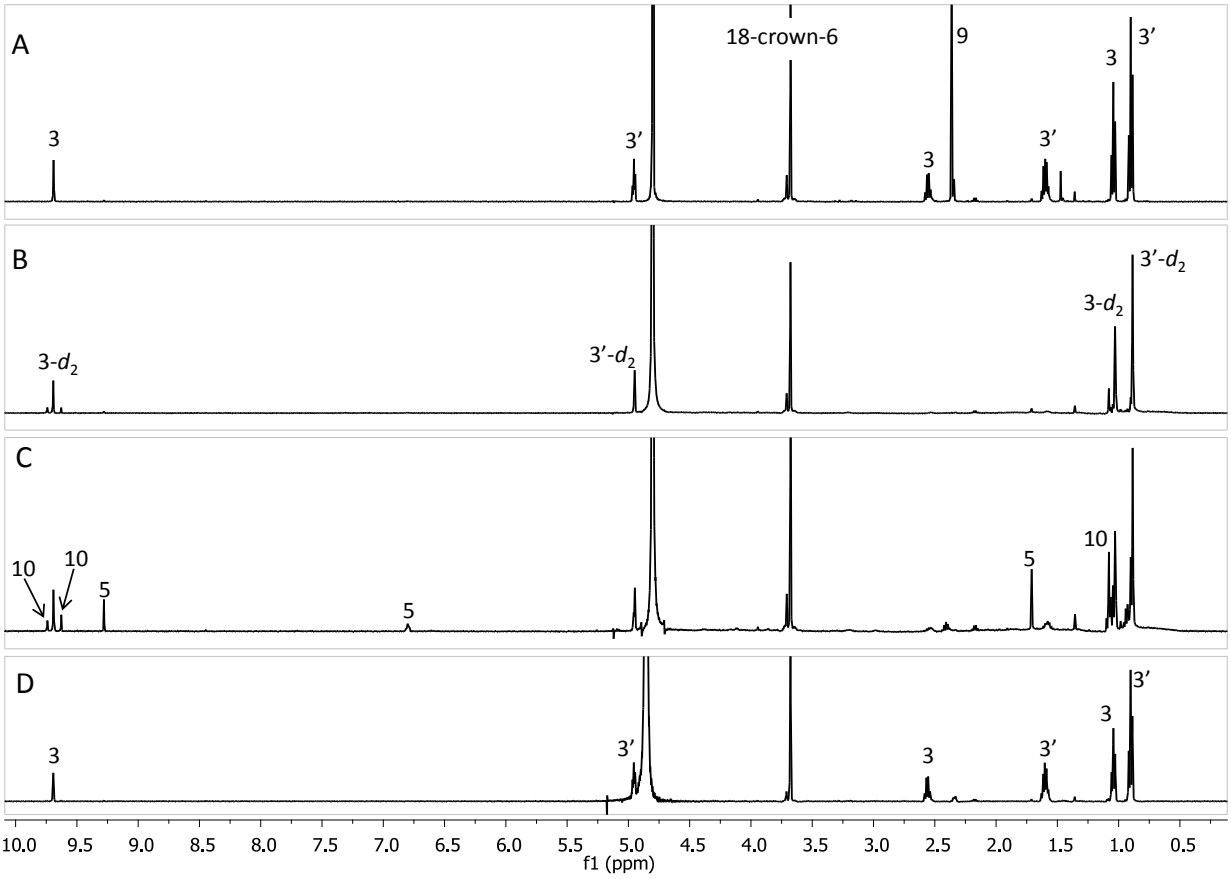


Figure S5. Stack plot of ^{1}H NMR spectra recorded after 4 d of incubation of **3** and **9** in 20 mM NaD_2PO_4 buffer (pD 7.6) with A) no enzyme; B) 4-OT WT; C) 4-OT F50A; D) 4-OT P1A (see Scheme S5 for reaction scheme). Spectrum A shows equilibrium was reached between unhydrated and hydrated (i.e. **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**.

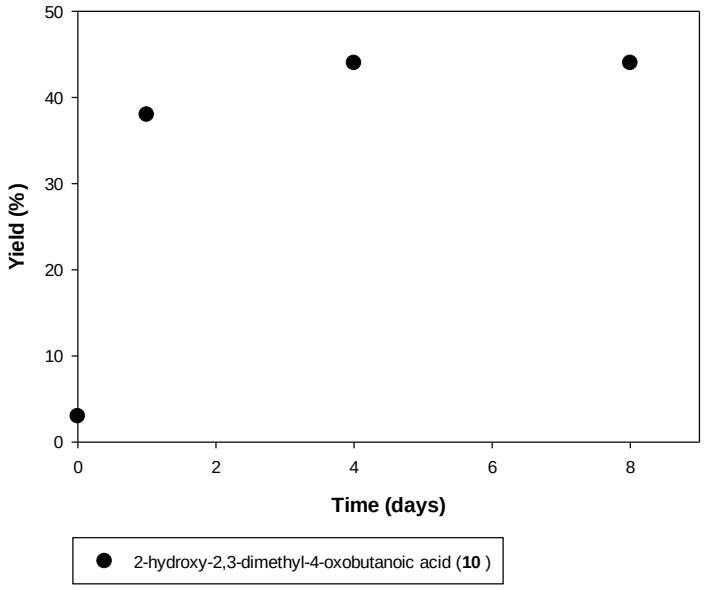


Figure S6. Yields (%) of **10** (based on **9**) in course of time in the reaction mixture (**3** + **9**) incubated with the 4-OT F50A mutant.

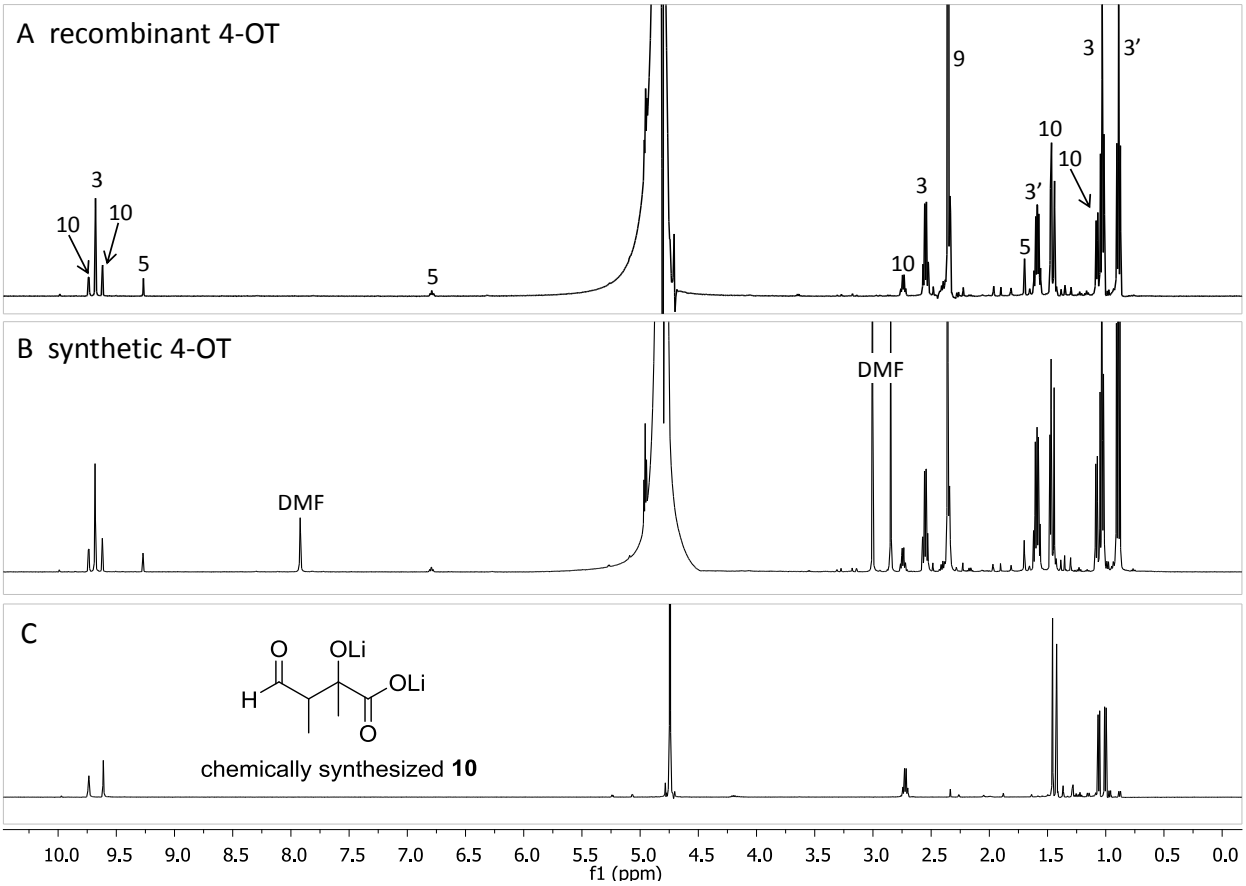


Figure S7. Stack plot of ^{1}H NMR spectra recorded after 4 d of incubation of **3** and **9** in 20 mM NaH_2PO_4 buffer **(**pH 7.3**)** with A) recombinant 4-OT WT and B) synthetic 4-OT WT (DMF used as internal standard). Spectrum C) shows the signals for chemically synthesized **10**. See Scheme S5 for reaction scheme for enzymatically obtained **10** and Scheme S1 for synthetically obtained **10**.

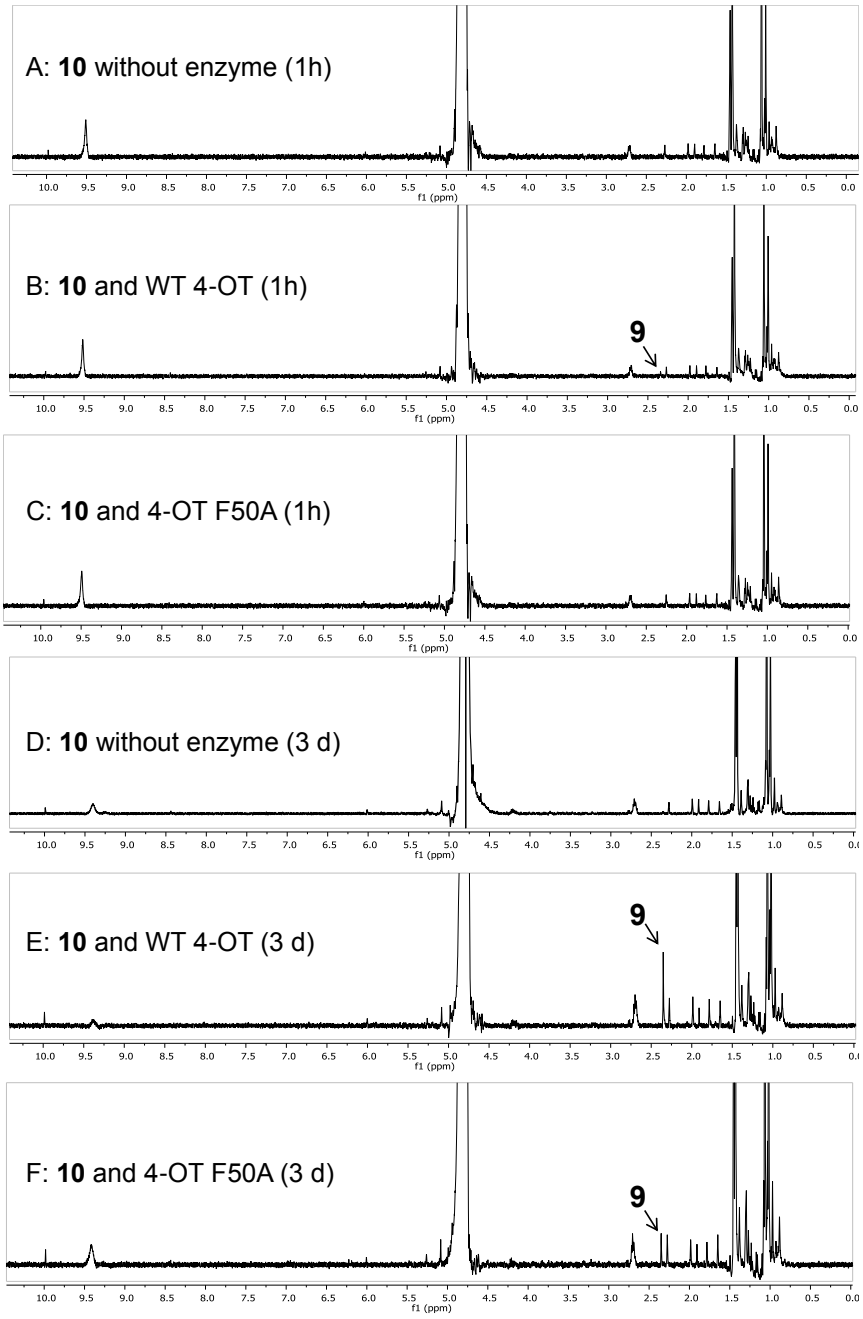


Figure S8. Stack plot of ^{1}H NMR spectra recorded after the incubation of **10** in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) no enzyme for 1 h; B) WT 4-OT for 1 h; C) 4-OT F50A for 1 h; D) no enzyme for 3 d; E) WT 4-OT for 3 d; and F) 4-OT F50A for 3d.

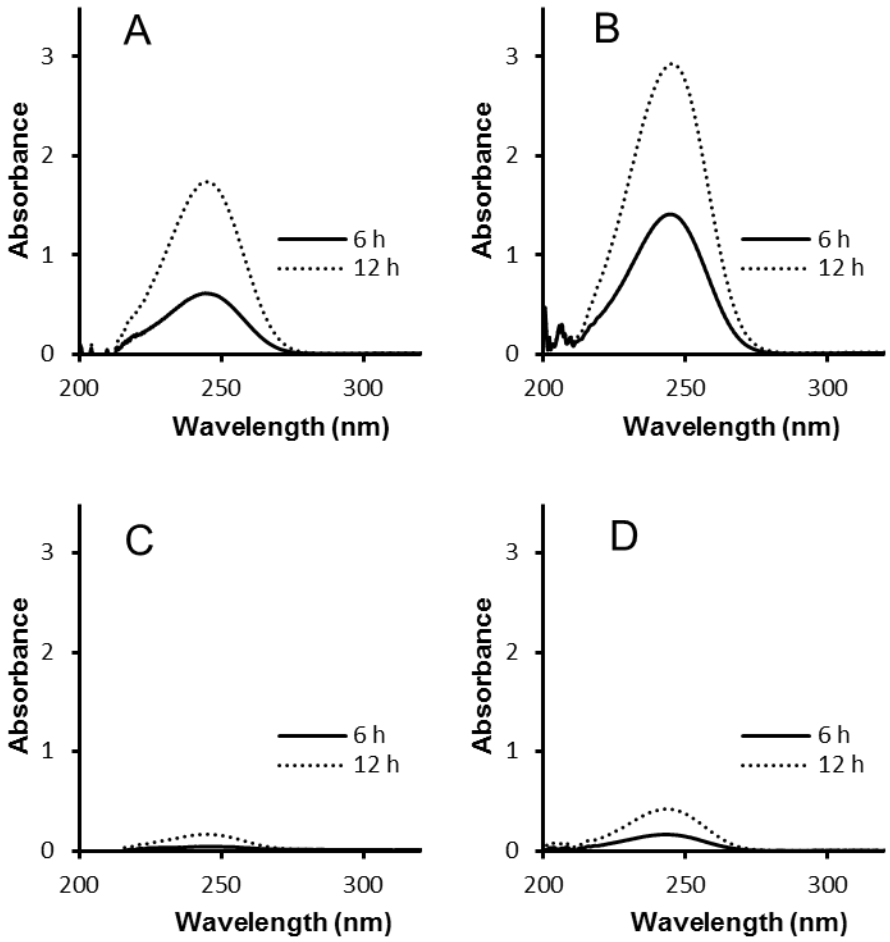


Figure S9. UV spectra recorded after incubation of **11** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) WT 4-OT, B) 4-OT F50A mutant, C) no enzyme (control sample) and D) 4-OT P1A mutant (t = 6 and 12 h). Increase of absorbance at 245 nm indicates formation of **13**.

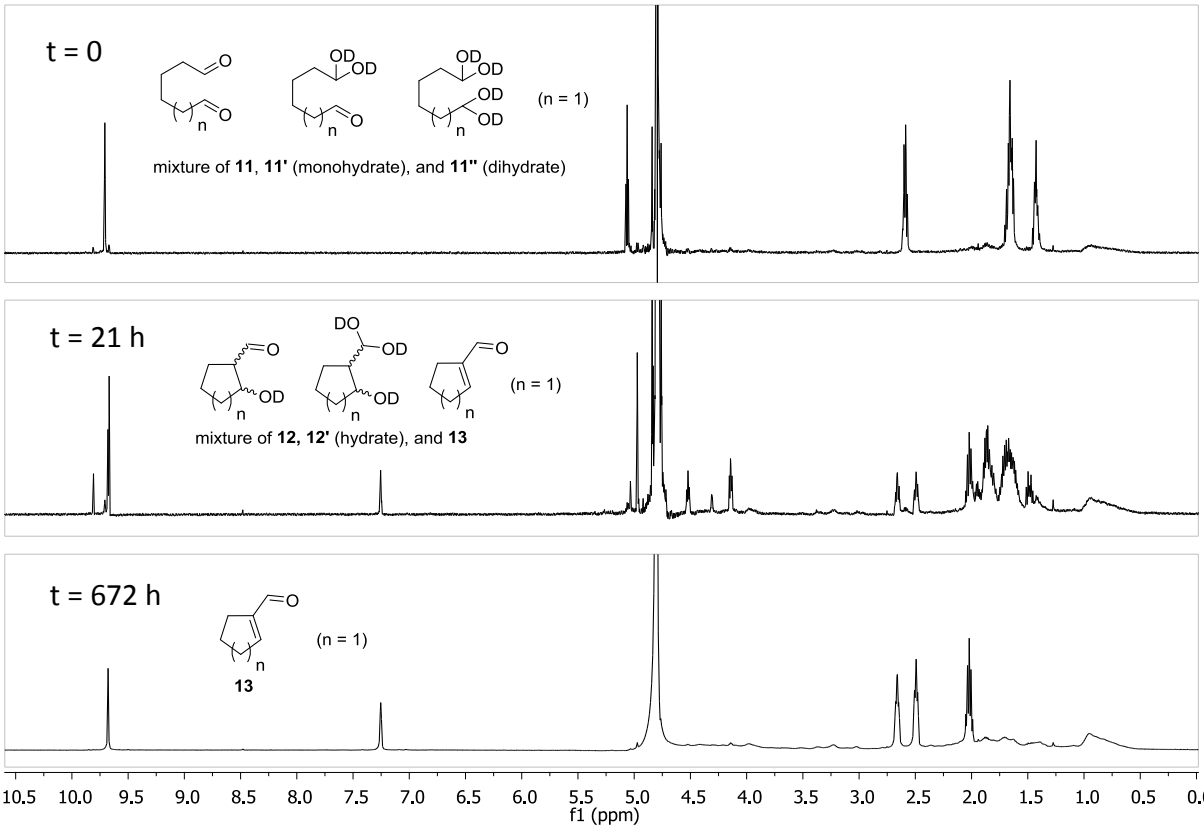


Figure S10. Cyclization of hexanedial **11** (15.4 mM) into **12** in NaD_2PO_4 (20 mM, pD = 7.6), catalyzed by 4-OT F50A (148 µM), and subsequent dehydration into **13** monitored by ^{1}H NMR spectroscopy (t = 0, 21, 672 h) (See Scheme S6 for reaction scheme). The enzyme 4-OT is responsible for broad absorptions between ~2 and 0.5 ppm, especially well-visible in the bottom spectrum.

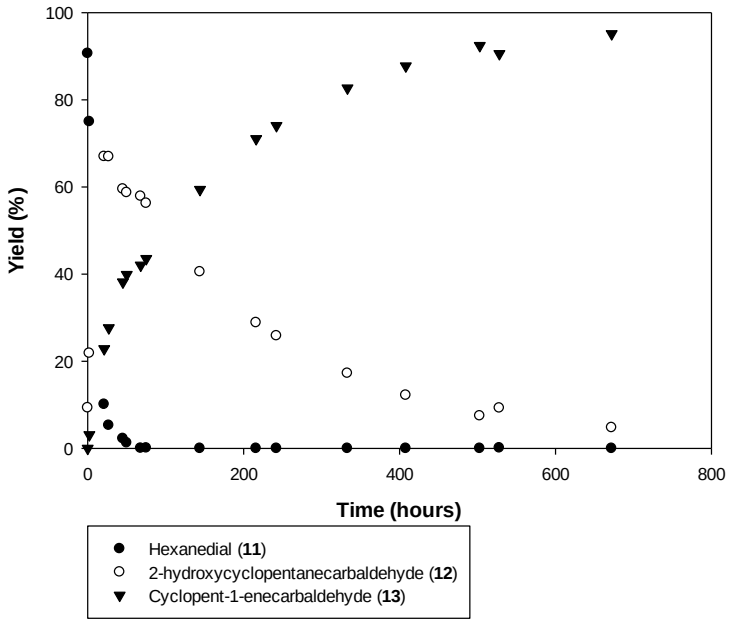


Figure S11. Plot of the presence (%) of **11** and yields (%) of **12** and **13** in course of time in the reaction mixture incubated with the 4-OT F50A mutant (in 20 mM NaD_2PO_4 buffer at pD 7.6).

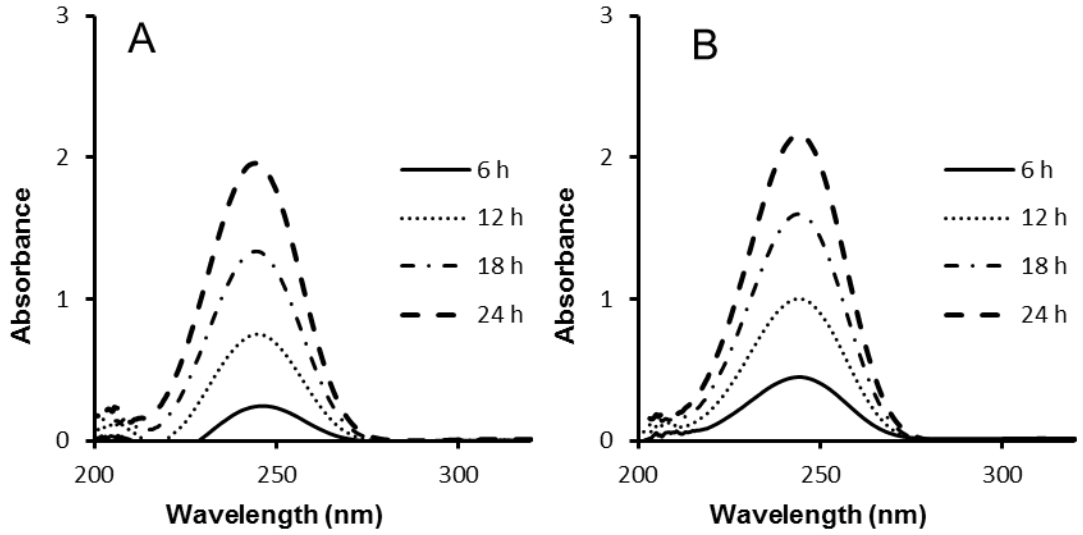


Figure S12. UV spectra recorded after incubation of **11** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with 0.075 mM A) recombinant WT 4-OT and B) synthetic WT 4-OT (t = 6, 12, 18 and 24 h).

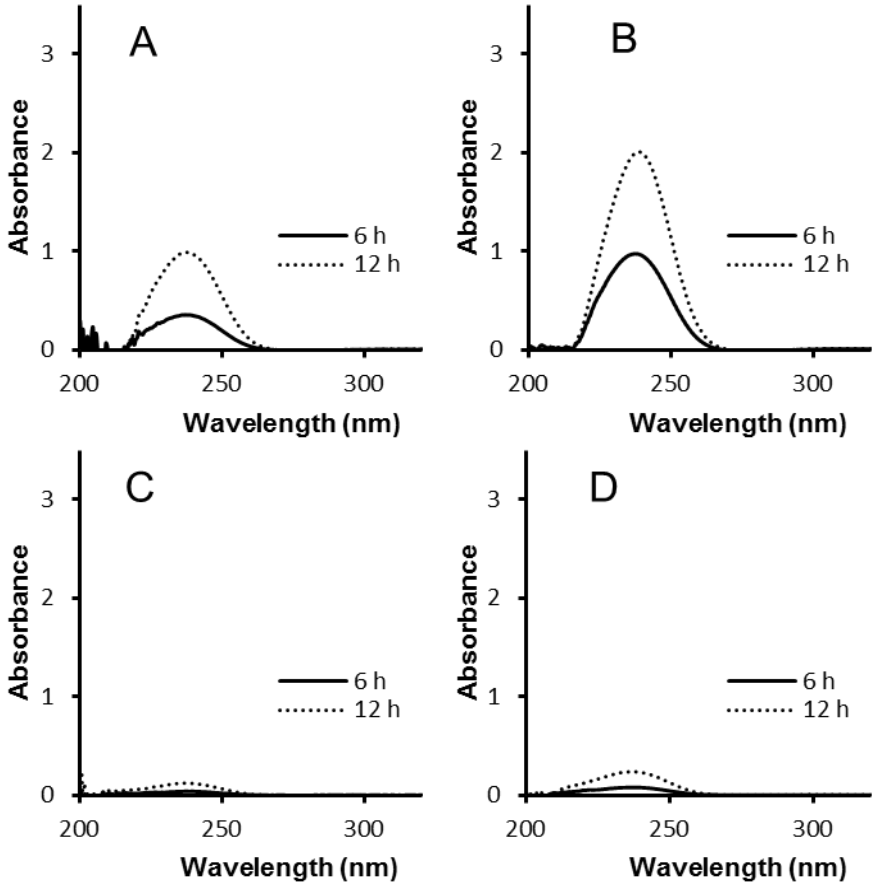


Figure S13. UV spectra recorded after incubation of **14** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) WT 4-OT, B) 4-OT F50A mutant, C) no enzyme (control sample) and D) 4-OT P1A mutant (t = 6 and 12 h). Increase of absorbance at 236 nm indicates formation of **16**.

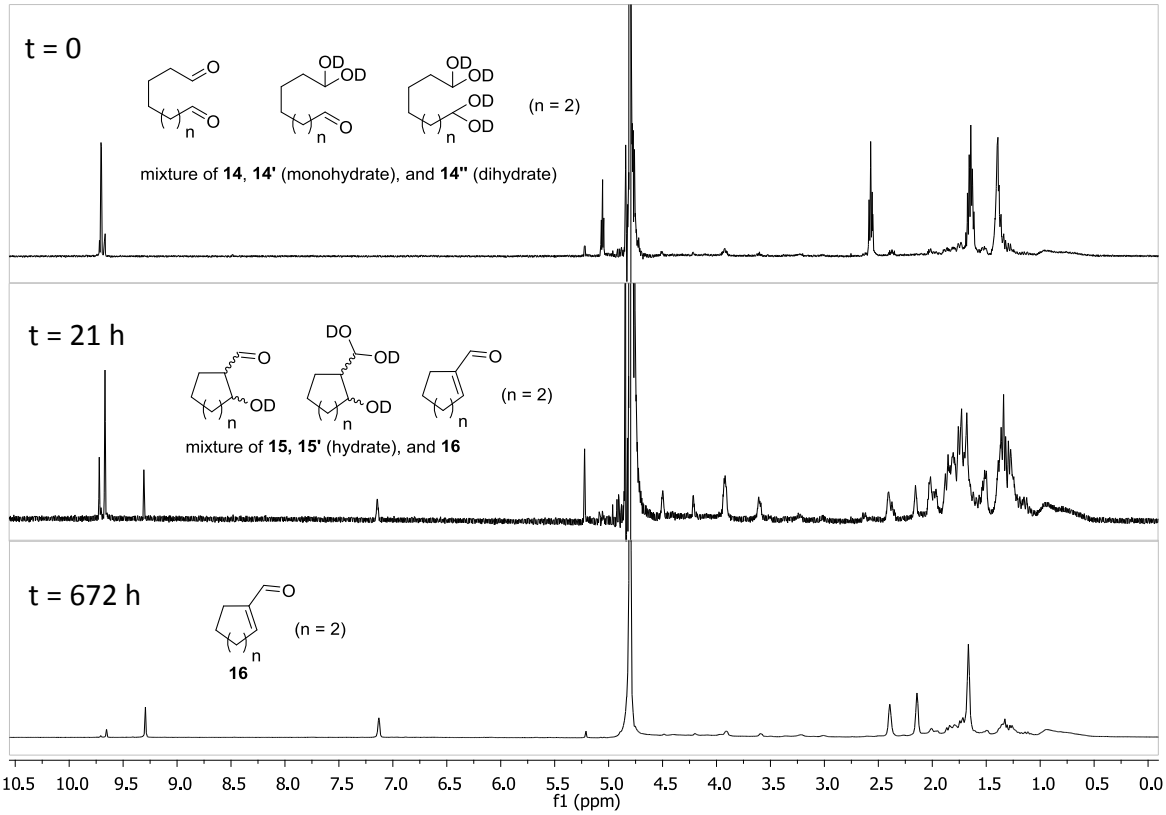


Figure S14. Cyclization of heptanedial **14** (15.4 mM) into **15** in NaD_2PO_4 (20 mM, pD = 7.6), catalyzed by 4-OT F50A (148 µM), and subsequent dehydration into **16** monitored by ^{1}H NMR spectroscopy (t = 0, 21, 672 h) (See Scheme S6 for reaction scheme). The enzyme 4-OT is responsible for broad absorptions between ~2 and 0.5 ppm, especially well-visible in the bottom spectrum.

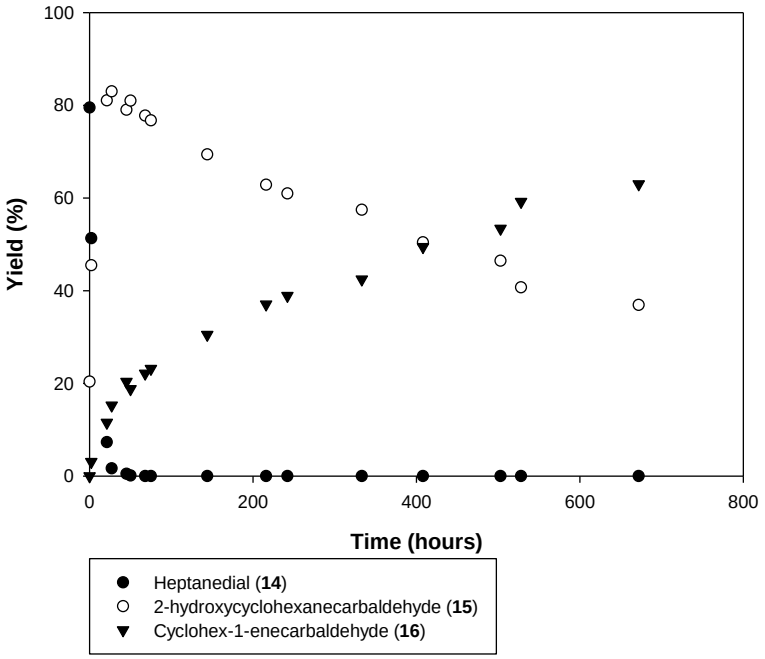


Figure S15. Plot of the presence (%) of **14** and yields (%) of **15** and **16** in course of time in the reaction mixture incubated with the 4-OT F50A mutant (in 20 mM NaD_2PO_4 buffer at pD 7.6).

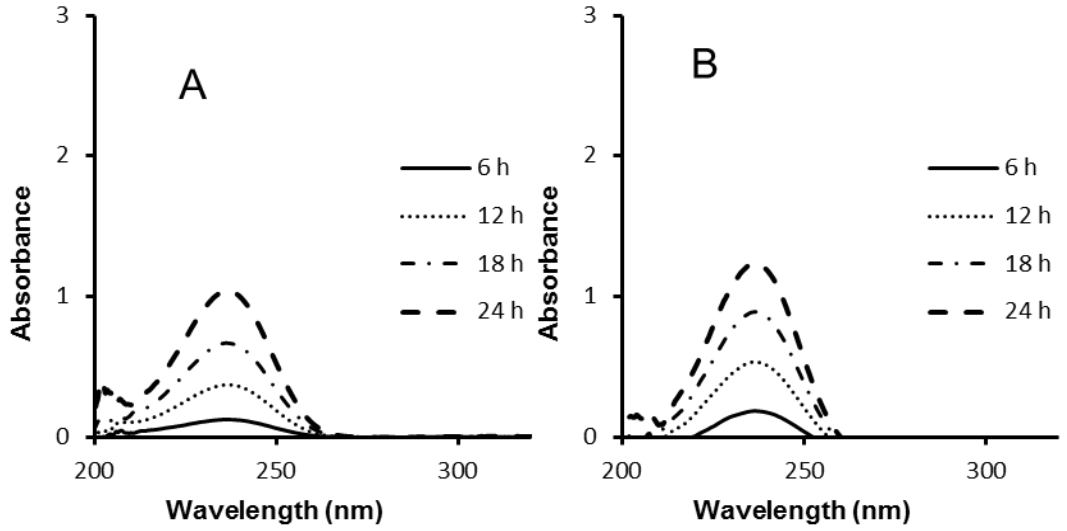


Figure S16. UV spectra recorded after incubation of **14** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with 0.045 mM A) recombinant WT 4-OT and B) synthetic WT 4-OT (t = 6, 12, 18 and 24 h).

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