**Table of contents:**

**Figure S1**. Kinetic isotope effect (KIE) experiments. a) GC-MS spectrum of the mixture of benzyl azide (**1**; *m/z* 133.2) and deuterated benzyl azide (***d*$_2$-1**; *m/z* 135.2) prior to the reaction with P450 FL#62 (**1** : ***d*$_2$-1** ratio of 0.9 : 1). b) GC-MS spectrum of the benzaldehyde (**2a**) and deuterated benzaldehyde (***d*-2a**) products after the reaction of P450 FL#62 with the **1** / ***d*$_2$-1** mixture. The KIE value was calculated from the integrated MS signals corresponding to the molecular ions of **2a** (*m/z* 106.1) and ***d*-2a** (*m/z* 107.1). Reaction conditions: 400 µL-scale reaction containing 5 µM P450 FL#62, 10 mM **1** + ***d*$_2$-1**, 10 mM $Na_2S_2O_4$ in phosphate buffer (pH 7.0) at room temperature for 6 hours.

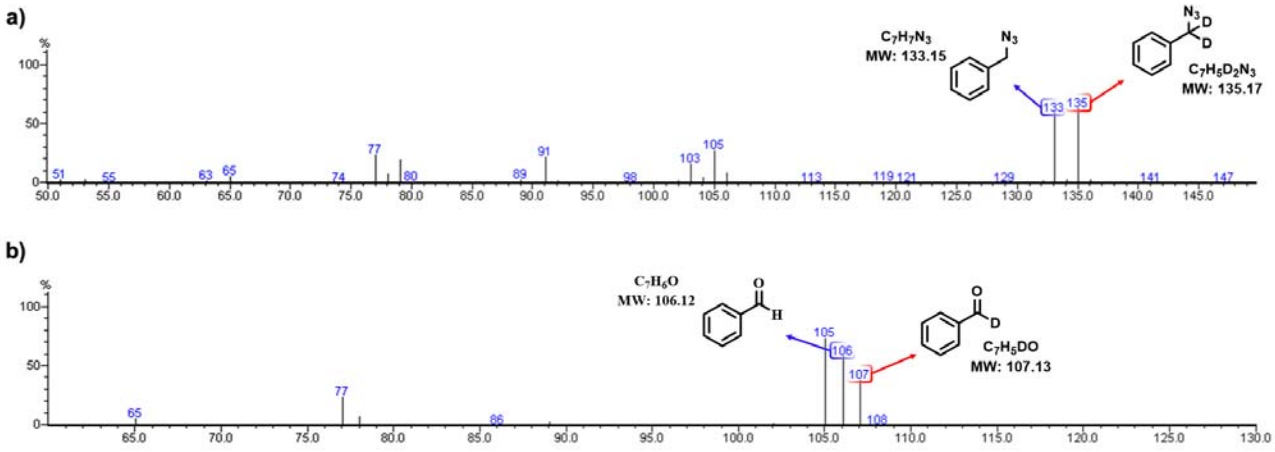


**Figure S2**. $^{18}O$ labeling experiments. GC-MS spectrum of benzaldehyde (**2a**) and $^{18}O$-containing benzaldehyde (**2a($^{18}O$)**) formed upon the reaction of P450 FL#62 with benzyl azide (**1**) in the presence of 50% $H_2{}^{18}O$ under standard reaction conditions (1 μM protein, 10 mM benzyl azide, 10 mM sodium dithionite in KPi (50% $H_2{}^{18}O$) at pH 7.0 (not adjusted), room temperature, 24 hours). The isotopic distribution (62 % $^{16}O$, 38% $^{18}O$) was calculated from integration of the MS signals corresponding to the molecular ions of **2a** (*m/z* 106.1) and **2a($^{18}O$)** (*m/z* 108.1).

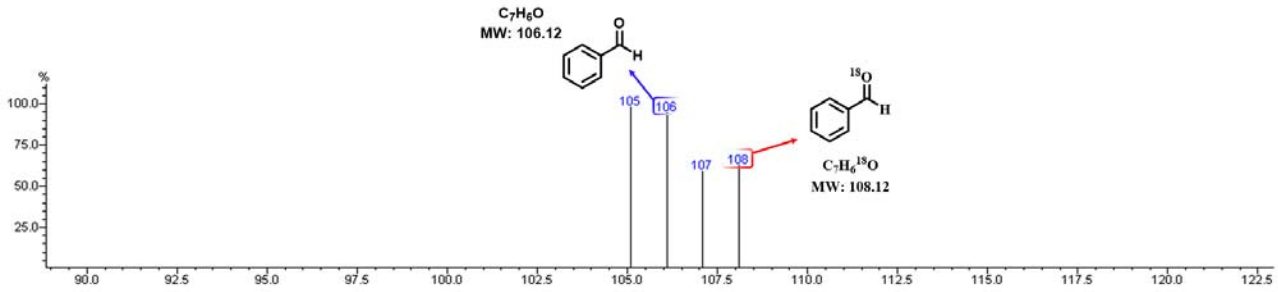


**Figure S3**. Isotopic labeling studies. a) Measurement of kinetic isotope effect (KIE) for H/D substitution of α protons in FL#62-catalyzed oxidation of benzyl azide. b) $^{18}O$ labelling experiment in FL#62 reaction with benzyl azide in the presence of $H_2{}^{18}O$.

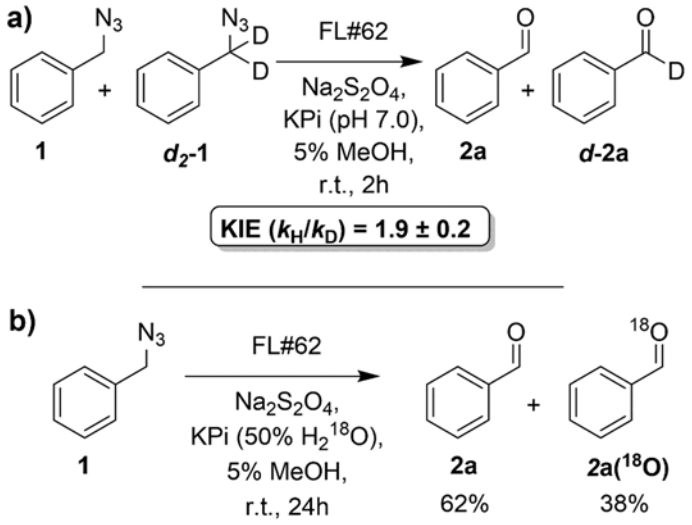
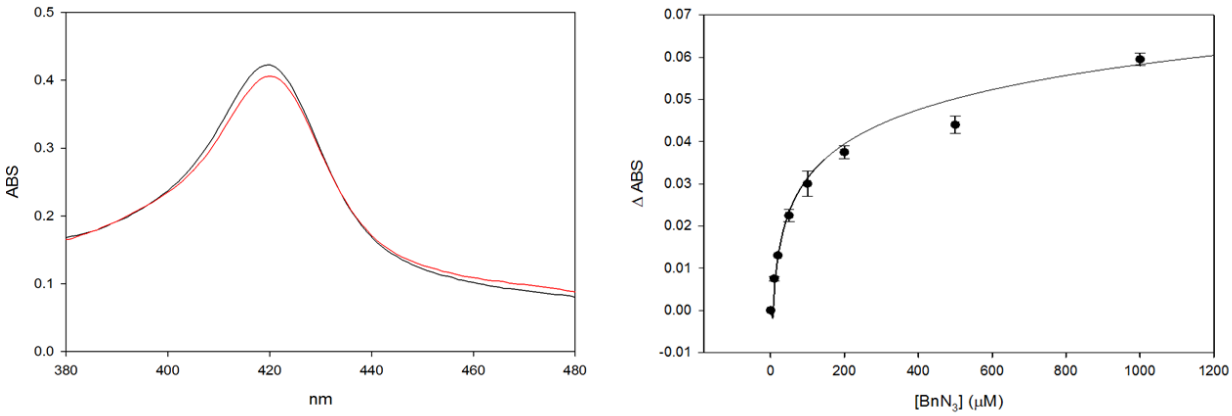
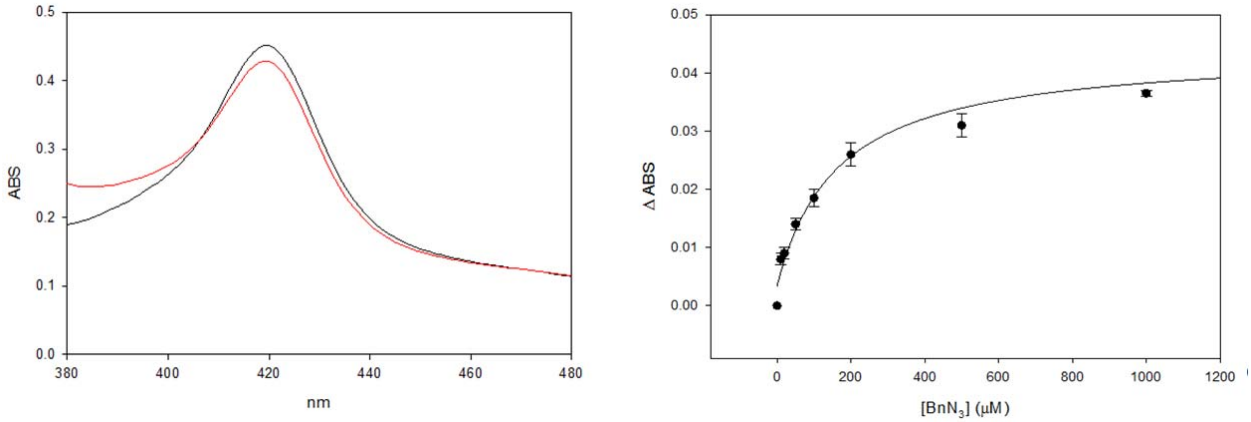


**Figure S4**. Substrate binding studies with selected P450 enzymes and azide substrates. *Left panel*: overlay of the visible absorbance spectrum before (gray line) and after (red line) addition of the indicated substrate (0.5 mM), illustrating the substrate-induced shift of the heme spin state equilibrium. *Right panel*: plot of substrate-induced heme spin shift versus substrate concentration. The equilibrium dissociation constant ($K_D$) for the enzyme − substrate complex was calculated via nonlinear fitting of the experimental data (dots) to a noncooperative 1:1 binding model equation (solid line).

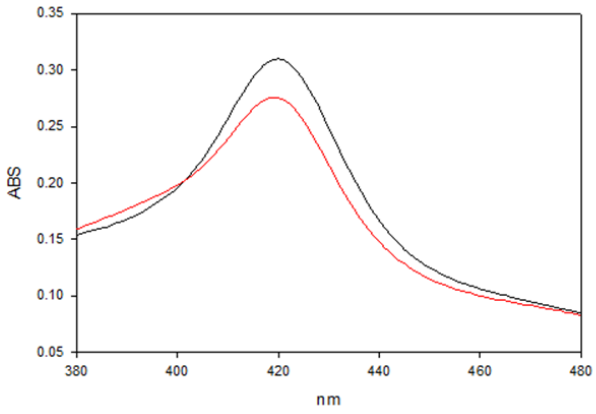
A) CYP102A1 and benzyl azide (**1**)

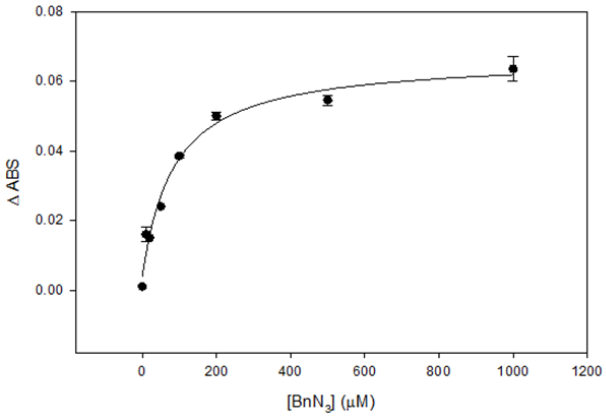



B) CYP102A1(F87A) and benzyl azide (**1**)

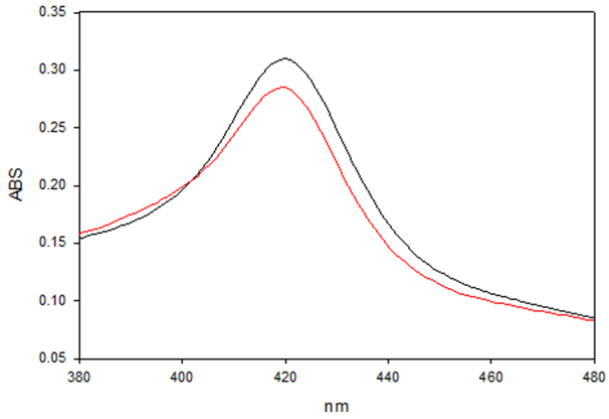


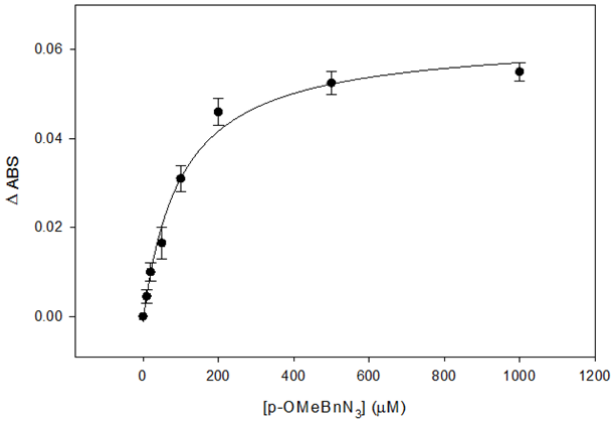
C) FL#62 and benzyl azide (**1**)



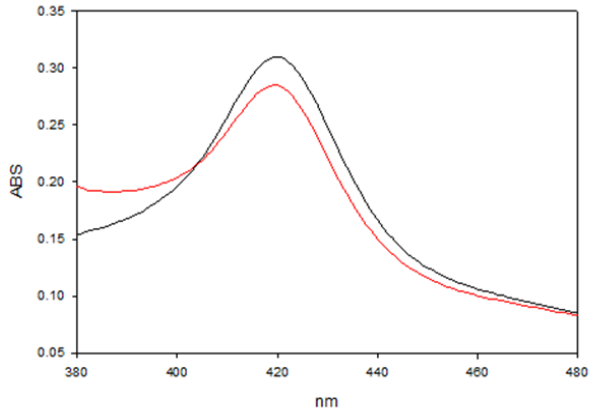




D) FL#62 and p-methoxy-benzyl azide (**4a**)




E) FL#62 and p-nitro-benzyl azide (**5a**)



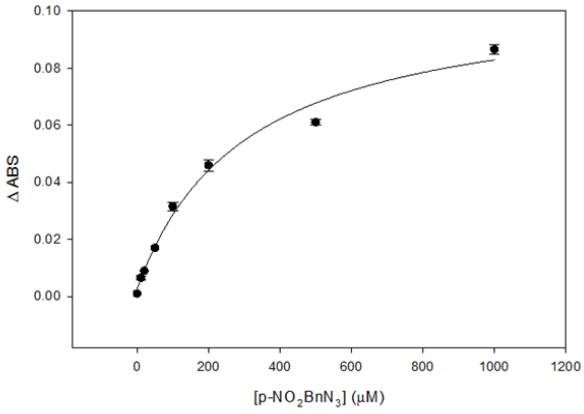
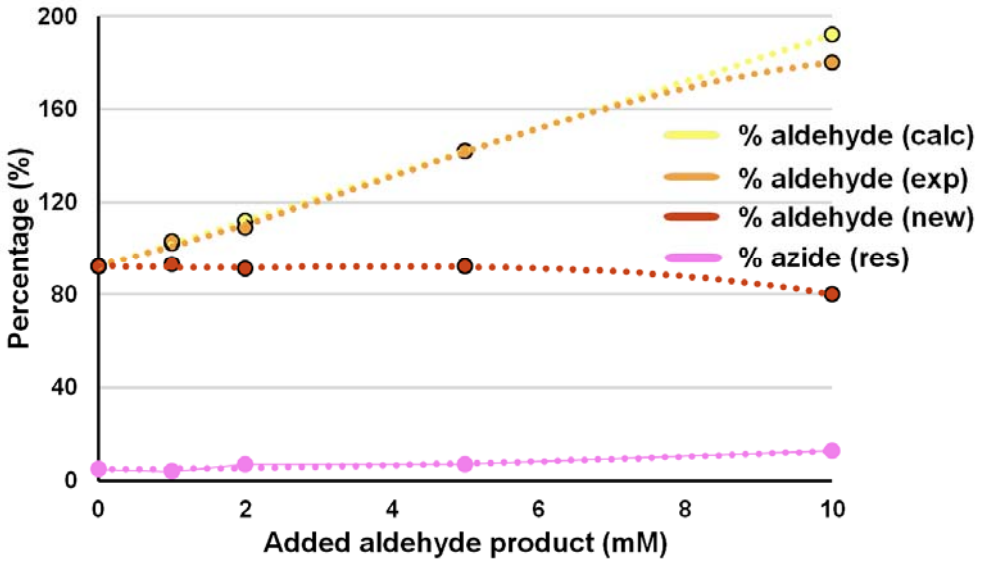



**Figure S5**. Product inhibition experiment for the FL#62-catalyzed conversion of benzyl azide (**1**) to benzaldehyde (**2a**). The amount of benzaldehyde product (**2a**) formed in the presence of exogenously added benzaldehyde (1, 2, 5, and 10 mM) is compared to that of a reference reaction containing no exogenous benzaldehyde. The amounts of the aldehyde or azide species in the graph are reported as relative to the % conversion and % residual substrate, respectively, in the reference reaction. "% aldehyde (calc)" refers to the relative amount of total aldehyde product expected in the absence of product inhibition (= aldehyde formed in the reference reaction + added aldehyde). "% aldehyde (exp)" refers to the experimentally observed total aldehyde product in the reaction. "% aldehyde (new)" refers to the relative amount of newly formed aldehyde product. "% azide (res)" refers to the relative amount of residual azide substrate at the end of the reaction.



**Experimental Procedures:**

**Reagents and Analytical Methods.** All the chemicals and reagents were purchased from commercial suppliers (Sigma-Aldrich, ACS Scientific, Acros, VWR, Alfa Aesar) and used without any further purification, unless otherwise stated. Bovine catalase, human hemoglobin, and horseradish peroxidase were purchased from Sigma Aldrich. $^{18}$O-Water (Normalized, 97.4 atom %) for isotopic labeling experiments was purchased from Icon Isotopes. All dry reactions were carried out under argon atmosphere in oven-dried glassware with magnetic stirring using standard gas-light syringes, cannulae and septa. Gas chromatography (GC) analyses were carried out using a Shimadzu GC-2010 gas chromatograph equipped with a FID detector and an Agilent J&W GC Chiral Cyclosil-B Column (30 m x 0.25 mm x 0.25 μm film). Separation method: 1 μL injection, injector temp.: 200 ºC, detector temp: 300 ºC. Gradient: column temperature set at 60 ºC for 0.10 min, then to 100 ºC at 60 ºC/min, to 200 ºC at 8 ºC/min, to 230 °C at 30 °C/min and then at 230 °C for 1.0 min. Total run time was 15.27 min. UV-Vis spectra were recorded on a Shimadzu UV-2401PC UV-VIS spectrophotometer, Wavelength Range (nm) from 700 to 300, Scan Speed: Fast, Sampling Interval (nm): 1.0, Scan Mode: Single.

**Substrates and reaction products.** The azide substrates and aldehyde/ketone products were prepared as described in our previous report[1], which also provides full characterization data ($^{1}$H/$^{13}$C(/$^{19}$F) NMR, MS) for these compounds.

**Protein expression and purification**. Wild-type CYP102A1 and its engineered variants were expressed from pCWori-based plasmids containing the P450 gene under the control of an IPTG-inducible promoter (*Bam*HI/*Eco*RI cassette) according to procedures described previously.[2] FL#62[3] contains the following mutations: <u>V78A</u>, <u>F81S</u>, <u>A82V</u>, <u>F87A</u>, P142S, T175I, <u>A180T</u>, <u>A184V</u>, A197V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V, where the active site mutations are underlined. Typically, cultures of recombinant DH5α cells were grown at 37 °C (200 rpm) in Terrific Broth (TB) medium supplemented with ampicillin (100 mg/L) until $OD_{600}$ reached 1.0 and then induced with 0.25 mM β-D-1-thiogalactopyranoside (IPTG) and 0.3 mM δ-aminolevulinic acid (ALA). After induction, cultures were shaken at 150 rpm and 27 °C and harvested after 20 h by centrifugation at 4000 rpm at 4 °C. Cell lysates were prepared by sonication and loaded on Q Sepharose resin. P450s were eluted using 20 mM Tris, 340 mM NaCl, pH 8.0. After buffer exchange (50 mM potassium phosphate buffer, pH 8.0), the enzymes were stored at

−80 °C. P450 concentration was determined from CO-binding difference spectra ($\varepsilon_{450–490}$ = 91 000 $M^{-1}$ $cm^{-1}$).

**Enzymatic reactions**. The enzymatic reactions were typically carried out at a 400 µL scale in KPi buffer (50 mM) using 1 µM catalyst (standard concentration for all the enzymes) and 10 mM sodium dithionite. A solution containing sodium dithionite (100 mM stock solution) in KPi buffer (50 mM, pH 7.0) was degassed by bubbling argon into the mixture for 3 min in a sealed vial. A separate vial containing the catalyst was carefully degassed in a similar manner. The solution was transferred to the catalyst-containing vial via cannulation. Reaction was initiated by addition of a 10 mM solution of the appropriate azide (200 mM stock solution in methanol, final percentage of methanol 5% v/v), with a syringe, and the reaction mixture was stirred for 24 h at 25 °C, under positive argon pressure.

**Product analysis.** The reactions were analyzed by adding 20 µL of internal standard (benzodioxole, 5 mM in methanol) to the reaction mixture, followed by extraction with 400 µL of dichloromethane and analyzed by GC-FID as described previously.[1] TON = nmol aldehyde / nmol catalyst. Product yield is based on conversion of the appropriate azide to the corresponding aldehyde as determined by gas chromatography. Calibration curves for quantification of the different products were constructed using authentic standards produced synthetically as described previously.[1] All measurements were performed at least in duplicate.

**$^{18}$O labeling experiments.** For the $^{18}$O incorporation experiments, reactions were carried out at a 400 µL scale in KPi buffer (pH 7.0) containing 50% $H_2{}^{18}O$ using 1 µM P450 FL#62, 10 mM sodium dithionite, and 10 mM benzyl azide in methanol (5% v/v) under an argon atmosphere. The reactions were gently stirred 24 hours at 25 °C, then added of 20 µL of internal standard (benzodioxole, 5 mM in methanol). The products were extracted with 400 µL of dichloromethane and analyzed by GC-MS as described above.

**Kinetic analyses.** Reactions were carried out on a 400 µL scale using the enzyme at a fixed concentration of 1 µM, 10 mM sodium dithionite, and substrate **1**, **4a**, or **5a** at varying concentration (1, 2, 5, 10, 15, 20, 30 mM) in KPi buffer (50 mM, pH 7.0). Initial velocity ($V_0$) was measured based on the amount of aldehyde product (**2a**, **4b**, or **5b**, respectively) formed after 90 minutes. The kinetic parameters $V_{max}$, $k_{cat}$ , and $K_M$ were obtained by fitting the resulting plot of

initial velocity ($V_0$) vs. substrate concentrations ([S]) to the Michaelis-Menten equation using software GraphPad Prism (version 6.01).

**Heme spin shift experiments.** Substrate binding experiments were performed using 3 μM purified P450 in potassium phosphate buffer (50 mM, pH 8.0) by titrating increasing amounts of alkyl azide (10 μM to 1 mM) from an ethanol stock solution (50 mM). At each concentration, a difference spectrum from 350 to 500 nm was recorded and binding curves were generated by plotting the change in absorbance at 390 and 420 nm corresponding to the high-spin and low-spin state of the enzyme, respectively, against the substrate concentration. $K_D$ values were calculated using Graph Pad Prism via nonlinear fitting of the experimental binding curves to an equation describing a standard 1:1 binding interaction. Reported mean and standard deviation values were calculated from experiments performed in duplicate.

**Product inhibition experiments.** Product inhibition experiments were carried out using 10 mM substrate (**1**, **4a**, or **5a**), 1 μM FL#62, and 10 mM $Na_2S_2O_4$ in KPi buffer (50 mM, pH 7.0). Each reaction mixture was added with increasing amounts (1, 2, 5, 10 mM) of the corresponding aldehyde product (**2a**, **4b**, and **5b**, respectively). A reference reaction was carried out under identical conditions but without the addition of the product. The reactions were gently stirred 24 hours at 25 °C, then added of 20 μL of internal standard (benzodioxole, 5 mM in methanol). The products were extracted with 400 μL of dichloromethane and analyzed by GC-MS as described above. The occurrence of product inhibition was determined by comparing the theoretical maximal amount of aldehyde product (given by the amount of aldehyde produced in the reference reaction *plus* the amount of exogenous aldehyde product added to the reaction) with the actual amount of aldehyde produced in the reaction.

## References


[1] S. Giovani, R. Singh, R. Fasan, *Chem. Sci.* **2016**, *7*, 234-239.

[2] K. Zhang, S. El Damaty, R. Fasan, *J. Am. Chem. Soc.* **2011**, *133*, 3242-3245.

[3] K. D. Zhang, B. M. Shafer, M. D. Demars, H. A. Stern, R. Fasan, *J. Am. Chem. Soc.* **2012**, *134*, 18695-18704.