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Synthesis, characterization and reactivity of 3-mercaptopyruvic acid.

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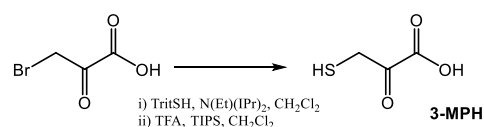
Supporting information for this article is given at the end of the document.

Abstract: A synthesis of the sulfur metabolic compound 3-mercaptopyruvic acid is reported. It allowed its isolation and thorough spectroscopic characterization for the first time. The compound is isolated without tedious work-up, with a purity of 97%, as indicated by chemical and biochemical analyses. Detailed kinetic, and thermodynamic studies of its complex behaviour in solution are discussed. 3-MPH is stable under its enol form in non-polar solvent, and in polar solvent a fast equilibrium between the α -ketoacid and a cyclic dimer dithiane is observed. The formation of the dimer confers increased stability towards hydrogen peroxide to 3-3-mercaptopyruvic acid when compared to cysteine.

3-mercaptopyruvic acid is a sulfur metabolic compound formed during the catabolism of cysteine.^[1] It has regained considerable interest in the last decade, because it is the substrate of the enzyme 3-mercaptopyruvatesulfurtransferase (3-MST). As such, it is involved in the production of reactive sulfur species taking part in redox signalling^[2] (hydrogen sulfide,^[3-4] persulfides,^[5] polysulfides)^[4,6], as well as in cyanide detoxification.^[7-8] So far, the main synthetic route to 3-mercaptopyruvic acid proposed in the literature involves the reaction of sodium hydrosulfide with bromopyruvic acid^[9-11] However, a recent study proposed that the product is not 3-mercaptopyruvate, but the cyclic dimer 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylate.^[8] Although the use of the dimer is under most circumstances not problematic *per se* for chemical, biochemical or biological studies (see below), several major drawbacks are associated with this synthesis: i) a tedious and harmful purification of sodium hydrogen sulfide is required prior to the reaction to avoid erratic yield and purity ii) a second purification step is necessary prior to certain biochemical experiments to remove the traces of hydrosulfide which could affect the data.^[12] Last year, an alternative synthesis was proposed,^[13] but the resulting product was barely characterized.^{††} Here, we present a synthetic route unambiguously leading to 3-mercaptopyruvic acid (from now on in the manuscript, we will use 3-MPH to refer to 3-mercaptopyruvic acid synthesized in this work, and 3-MP for the commercially available "sodium 3-mercaptopyruvate" or for the product derived from the synthesis using sodium hydrosulfide and

bromopyruvic acid, regardless of the protonation state of the compounds in solution) with purity greater than 97%, after simple purification steps. The isolation of 3-MPH allowed its first characterization by standard spectroscopic techniques, and permitted to study its reactivity in solution. In non-polar solvents, 3-MPH is stable and in its enol form. In polar solvents, 3-MPH is in rapid equilibrium with the dimer 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylic acid. At physiological pH, the equilibrium is strongly shifted towards the dimeric moiety at high, non-physiological concentrations. However, at more relevant concentration, the amount of 3-MPH becomes more significant.

Based on previous work on 3-MP,^[1,14] we anticipated that acidic conditions and non-polar solvents would facilitate the isolation of monomeric 3-MPH. We therefore designed the synthesis presented in Scheme 1, in which the sulfur atom is first incorporated in its trityl-protected form. Deprotection in the presence of trifluoroacetic acid and the trityl cation quencher triisopropylsilane leads, after simple washing with pentane, to 3-MPH with an overall yield of 54%.



Scheme 1. Synthetic route towards 3-MPH..

Since the spectroscopic data reported for 3-MP in the literature are probably ascribable to the dimeric compound 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylate (Scheme 2),^[1,8] we undertook the characterization of 3-MPH. The product isolated from the reaction described in Scheme 1 is the enol tautomer, as indicated by ¹H and ¹³C NMR spectra recorded in CDCl₃. Thus, the ¹H-¹³C HSQC spectrum shows a resonance of a single proton at $\delta = 6.49$ ppm, attached to a carbon atom with a chemical shift at $\delta = 108.4$ ppm (Figure S1). The enolic form of 3-MPH is further supported by FT-IR (Figure S2) and UV-Vis spectroscopy (Figure S3). The FT-IR spectrum of 3-MPH strongly differs from that recorded with 3-MP^[15]. Hence, the spectrum of 3-MP (Figure S2B) exhibits a single strong band in the carbonyl region at 1714 cm⁻¹, while the spectrum of 3-MPH (Figure S2A) displays two strong bands at 1703 and 1652 cm⁻¹, which are attributed to the acid and enol moieties, respectively. In addition, 3-MPH also displays an absorbance around 2550 cm⁻¹ (S-H stretching) and at 3091 cm⁻¹ (C-H alkene stretching). The UV-visible spectrum recorded in chloroform also confirms the enolic form, with a strong absorption ($\epsilon = 9500 \text{ L.M}^{-1}.\text{cm}^{-1}$) at 274 nm (Figure S3) which is absent in the spectrum of 3-MP.

To assess the purity of 3-MPH, we used physicochemical and biochemical techniques. When commercial 3-MP is essentially sold with a purity of >90%, elemental analyses for 3-MPH indicate a purity of over 99% for 3-MPH, with the remaining 1% being triphenylmethane.

To confirm this result, we explored the stoichiometry of the reaction between *E. Coli* 3-MST and 3-MPH in the presence of the recycling system thioredoxin (Trx) / NADPH-dependant Trx reductase (TrxR) (see the Supplementary Information). Under these conditions, the variation in NADPH concentration monitored by UV-vis spectroscopy at 340 nm allows a biochemical determination of the purity of the 3-MPH. The curve displayed in Figure 1 confirms that 3-MPH is at least 97% pure (vs 89% for the commercial sample). Additional experiments carried out with 3-MST under either

single turnover or steady-state conditions give identical enzymatic parameters for both the commercial 3-MP and the synthetic 3-MPH (Figure S4 and S6).

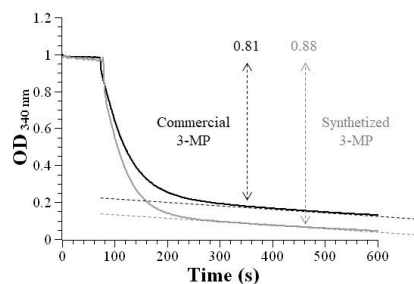
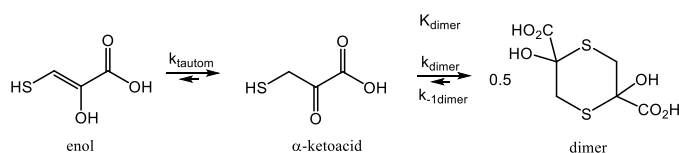


Figure 1. Determination of the purity of 3-MPH by monitoring its reaction with 3-MST / Trx-regenerating system. Reaction conditions: Tris⁺ buffer, *E. coli* 3-MST: 10 μ M, Trx: 250 μ M, TrxR: 1.25 μ M, NADPH: 0.3 mM, 3-MP (Santacruz biotechnology, > 90%, as a reference (—)) or 3-MPH (---): 145 μ M. The theoretical variation in absorbance is 0.91.



Scheme 2. Simplified equilibria taking place upon dissolution of 3-MPH, and thermodynamic and kinetic constants experimentally determined in this work.

3-mercaptopyruvic acid has not been unambiguously isolated before, due to its conversion to the corresponding cyclic dithiane (Scheme 2) in polar solvents.^[1] Indeed, while 3-MPH is stable for hours in chloroform, its typical ¹H NMR signature ($\delta = 6.59$ ppm) is slowly converted to new peaks over time upon dissolution in D₂O (pH approximately 2.5). These new peaks (Figure 2) are found at similar chemical shifts, and display the same features as those reported for the diastereoisomers of the cyclic dimer 2,5-dihydroxy-1,4-dithiane-2,5-dicarboxylic acid.^[14] During the course of the reaction, we did not detect any intermediate. In addition, the comparative integration of the ¹H NMR signals of the species in solution clearly indicates that there is no incorporation of deuterium into the enol over the course of the reaction. This suggests that no equilibrium takes place between the α -ketoacid and the enol forms of 3-MPH under these conditions, and that the former is rapidly converted into the dimer. The reaction rate constant obtained from the NMR data presented in Figure 2 ($k_{\text{autom}} = (1.71 \pm 0.02) \times 10^{-3} \text{ s}^{-1}$) is similar to that obtained by monitoring the decrease of the enol absorbance at 262 nm by UV-vis (Figure S7) ($k_{\text{autom}} = (1.12 \pm 0.04) \times 10^{-3} \text{ s}^{-1}$).

The conversion of 3-MPH to the cyclic dithiane is pH dependent, and at physiological pH (7.4) the reaction is too fast to be monitored by ¹H NMR spectroscopy. Indeed, only the isomers of the dithiane are present in solution after the preparation of a 10 mM solution of 3-MPH (Figure S8).

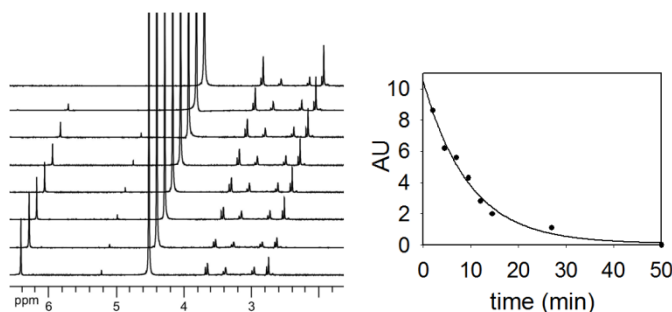


Figure 2. Evolution of the ^1H NMR spectrum of 3-MPH (10 mM) in D_2O (pH~ 2.5, left, $t=0$ at the bottom, $t= 50$ min at the top, X-axis units are chemical shifts (ppm)) and of the integration of the signal at 6.59 ppm over time (arbitrary reference: solvent residual peak) (right) with the resulting curve fit obtained using a single exponential equation.

However, monitoring the absorbance at 262 nm by stopped-flow experiments (Figure S9) allows the determination of rate constants of $k_{\text{tautom}} = 5.02 \times 10^{-2} \cdot \text{s}^{-1}$ and $4.82 \times 10^{-2} \cdot \text{s}^{-1}$ for solutions at 25 and 12.5 μM , respectively. Together, these data clearly indicate that the conversion of the enol to the corresponding α -ketoacid is the limiting step of the reaction leading to the formation of 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylic acid from 3-MPH.

Although neither we nor others have evidence for the presence of monomeric 3-MPH at NMR concentrations,^[1,14] the full conversion of a 0.5 μM solution of reduced 3-MST to its persulfide form within 0.1s in the presence of a 5 μM solution of 3-MPH (Figure S4A) seems to indicate that either a substantial amount of 3-MPH is present in solution at this concentration, or that the conversion of the dimer to 3-MPH is very fast on the experimental time-scale. However, monitoring the incorporation of deuterium at the α -carbon position of the dithiane by ^1H NMR previously gave $t_{1/2}$ values of 2 hours for the ring opening.¹⁴ To further investigate the equilibrium K_{dimer} and the related kinetics, we studied the reactivity of solutions containing various initial concentrations of 3-MPH, at pH 7.4, with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) by absorbance stopped-flow experiments. Only the open forms of the 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylic acid and 3-MPH ("free thiols" in Scheme S1) can react with the thiophilic reagent, allowing the determination of their concentration in solution. A typical kinetic experimental trace is shown in Figure 3 (inset). The fitting of the data at each concentration with the biphasic first order equation (1) (Experimental Section) gives observed rate constants $k_{\text{obs1}} = 3.79 \pm 0.04 \text{ s}^{-1}$ and $k_{\text{obs2}} = 0.16 \pm 0.01 \text{ s}^{-1}$ (Figures S5). The total variation in absorbance for each experiment corresponds to the theoretical value expected for a full conversion of $[\text{3-MPH}]_0$ to the mixed disulfide 3-MPH-TNB. The fast phase can be unambiguously attributed to the reaction of "free thiols" with DTNB (a similar rate of $3.24 \pm 0.01 \text{ s}^{-1}$ is obtained with cysteine under identical conditions, data not shown). The slower one most likely represents the rate limiting conversion of the dithiane into 3-MPH (along with the conversion of the open dimer-TNB adduct to 3MPH-TNB and 3-MPH), that is $k_{-1\text{dimer}}$. The rate constant thus obtained is 3 orders of magnitude higher than the one determined by ^1H NMR ($k = 9.6 \times 10^{-5} \cdot \text{s}^{-1}$),^[14] probably reflecting the strongly unfavourable formation of the enol tautomer^[1] required for deuterium incorporation (Scheme S1).

When assuming that only 3-MPH and 5-dihydroxy-1-4-dithiane-2,5-dicarboxylic acid are present in solution, and that the variation in the absorbance during the first phase is directly related to the concentration of 3-MPH, a K_{dimer} value of $(3.64 \pm 0.19) \times 10^4 \text{ M}^{-1}$ and a corresponding k_{dimer} value of $(6.3 \pm 2.0) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ are obtained by fitting the data of Figure 3.

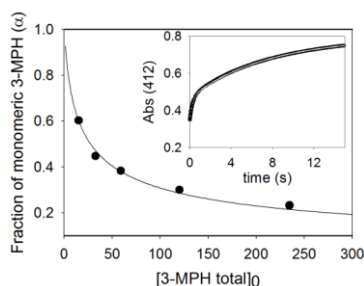


Figure 3. Plot of the fraction of monomeric 3-MPH (α , calculated as described in the Experimental Section) vs the initial 3-MPH concentration. The data were fitted with equation (2) (Experimental Section). Inset: Representative kinetic trace (black line) obtained when reacting 30 μM 3-MPH in KPi with 1.6 mM DTNB in KPi, and the fit curve (white line over the black line) obtained with equation (1) (Experimental Section).

The determination of this equilibrium constant allowed the refinement of the $K_{3\text{-MP}}$ value calculated for the sulfur transfer step from 3-MPH to 3-MST ($70 \pm 4 \mu\text{M}$ with 3-MPH and $73 \pm 3 \mu\text{M}$ with commercial 3-MP, previously $160 \pm 30 \mu\text{M}$)¹² (Figure S4). The parameters obtained under steady state conditions remain unchanged, since the conversion of the dithiane to 3-MPH ($t_{1/2} = 4\text{s}$) is fast on the experiment time scale. Similarly, 3-MPH or 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylate can indiscriminately be used for biological experiments such as the study of 3-mercaptopyruvate analogues as antidote for cyanide poisoning.^[8,14]

A full investigation of the potential physiological significance of the formation of the dimer is beyond the scope of this study. However, in this context, it is interesting to note that 3-MPH is less sensitive to oxidation by hydrogen peroxide than the related amino-acid cysteine (Figure 4).

In conclusion, we report a simple synthesis of 3-mercaptopyruvic acid, allowing detailed spectroscopic characterization of this biologically important derivative, and the study of its behaviour in solution. Our data indicate that commercially available 3-MP is not sodium 3-mercaptopyruvate (CAS 10255-67-1), but its cyclic dimer disodium 2,5-dihydroxy-1-4-dithiane-2,5-dicarboxylate (CAS 1309654-46-3 / 1001081-70-4). The dimer formation may be relevant in biological systems.

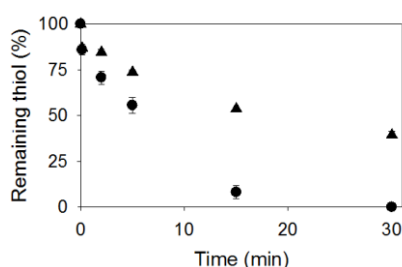


Figure 4. Percentage of remaining thiol (determined by DTNB titration after quenching the reaction with 500U of catalase) over time upon reacting 200 μM of Cysteine (●) or 3-MPH (▲) with 1 mM of H_2O_2 in KPi at 25°C.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

E.G. is grateful to Dr D. Over and to Dr D. Padovani for useful discussions and for critically *reading the manuscript*.

Keywords: mercaptopyruvate • Hydrogen sulfide • H₂S Donor

‡ We use the following abbreviations in the text: KPi: potassium phosphate buffer (50 mM, pH 7.4, containing 1 mM diethylenetriaminepentaacetic acid as chelating agent), Tris: trishydroxyméthylaminomethane buffer (Tris-HCl 50 mM, ethylenediaminetetraacetic acid 2 mM, pH 8.0).

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Experimental Section

Physical measurements. The ^1H and ^{13}C NMR spectra were recorded at 293K on a Bruker AVANCE II-500 or a Bruker Advance-500 equipped with a cryogenically cooled probe. FT-IR spectra were recorded on a Perkin-Spectrum One equipped with an ATR MIRacle (ZnSe). UV-vis were recorded on a Cary 300Scan at 293K or a SAFAS UVmc2 at 303K. All kinetics and thermodynamic parameters were obtained by fitting the experimental data with the appropriate equation using SigmaPlot. Stopped-flow kinetic experiments were performed on a Biologic SFM-300 at 293K or a SX19MV-R (Applied PhotoPhysics) at 303 K.

Materials. Chemicals for the synthesis were purchased from Aldrich, and used as-is. 3-MP was purchased from Santa Cruz Biotechnology (>90%), NADPH from Roche. *E. coli* 3-MST, thioredoxin1 (Trx) and NADPH-dependent thioredoxine reductase (TrxR) were prepared following reported procedures. $^{1-3}$ KPi refers to potassium phosphate buffer (50 mM, pH 7.4, containing 1 mM diethylenetriaminepentaacetic acid as chelating agent) and Tris to trishydroxyméthylaminomethane buffer (Tris-HCl 50 mM, ethylenediaminetetraacetic acid 2 mM, pH 8.0).

Synthesis of 3-MPH.

Synthesis of 3-MPH-Trit: To a solution of 9.46 g of bromopyruvic acid (56 mmol) dissolved in 200 mL of dichloromethane was slowly (2 minutes) added 15.70 g of triphenylmethanethiol (56 mmol) (greenish color) and at 0°C 19.34 mL of N,N-diisopropylethylamine (114 mmol) were slowly added. After 30 min in ice and 3h at RT, the yellow solution was washed 5 times with 1.2N HCl (decantation is getting easier with each wash). After drying over sodium sulfate, the solvent was removed under vacuum to yield a pale yellow residue. Check ^1H NMR to see if tritylthiol (3.1) or bromo (4.5 ppm) remains. Pdt : 6.16 + 3.64

Purif: Either 1) si tritylthiol reste: extraction with hot diethylether (5x100 mL) and evaporation gave a beige powder or 2) si couleur un peu jaune et pas de tritylthiol: addition of ether, heating to boil, and fridge overnight. NMR should look like 500cryoerwa0707 53 (2020).

^1H NMR (500 MHz, CDCl_3 , δ , ppm): 7.33-7.23 (m, 15H), 6.17 (s, 1H), 5.68 (br, 1H). ^{13}C NMR (125 MHz, CDCl_3 , δ , ppm): 166.1, 144.1, 135.4, 129.8, 128.1, 127.9, 127.3, 68.9.

Synthesis of 3-MPH: To 16.20 g of 3-MPH-Trit (44 mmol) in 0.6 L of dichloromethane were added triisopropylsilane (27.77 mL, 135 mmol) and trifluoroacetic acid (34.63 mL, 452 mmol), and the resulting yellow solution was stirred at rt for 2 h. After removal of all volatiles under vacuum, the residue was washed with pentane (5x300 mL) to give a beige solid (3.62 g, 68%). Verifier : plus de silane (1.4-,1.0), plus de tritylH aromatique + 5.6), plus de TFA (-76 en 19F)

^1H NMR (500 MHz, CDCl_3 , δ , ppm): 6.49 (d, $J^2=12.2\text{Hz}$), 5.81 (br, 1H), 3.62 (d, $J^2=12.2\text{Hz}$). ^{13}C NMR (125 MHz, CDCl_3 , δ , ppm): 165.1, 136.7, 108.1. ^1H NMR (500 MHz, D_2O , δ , ppm): 6.59 (s, 1H). ^{13}C NMR (125 MHz, D_2O , δ , ppm): 164.8, 138.5, 108.5. ESI-MS (ESI $^-$, m/z = 118.98 ([M-H] $^-$, 100%). Elemental Analysis: calculated (found) for 99.3% $\text{C}_3\text{H}_4\text{O}_3\text{S}$ + 0.07% $\text{C}_{19}\text{H}_{16}$: C, 30.89

(30.92) H, 3.40 (3.26); S, 26.31 (26.31). UV-vis (CHCl₃): 274 nm ($\epsilon=9500 \text{ L.M}^{-1}.\text{cm}^{-1}$). FT-IR (cm⁻¹, neat): 3091, 2570, 2549, 1703, 1652, 1445, 1237, 1128.

Kinetic and thermodynamic studies.

Stopped flow studies for the dissolution of 3-MPH in KPi.

Three syringes S1-S3 were used, with the following contents: S2: H₂O, S1 3-MPH in H₂O ([3-MPH]₀ = 100 or 50 μM), S3 : KPi. Each run consisted of mixing 0.5/0.5/1 parts of S1/S2/S3, resulting in a 4-times dilution of [3-MPH]₀. An average of three runs was recorded and used to extract the rate constant.

Stopped flow studies for the reactivity of 3-MPH with DTNB.

Three syringes S1-S3 were used, with the following contents: S2: KPi, S1: 3-MPH in KPi ([3-MPH]₀), S3 : 3.2 mM solution of DTNB in KPi. Each run consisted of mixing 0.5/0.5/1 parts of S1/S2/S3, resulting in a 4-times dilution of [3-MPH]₀. An average of at least three runs was recorded for each substrate concentration.

Equation (1) was used to fit the collected data $\text{Abs}(412) = f(t)$.

$$\text{Abs}(412) = y_0 + \Delta 1(1 - \exp(-k_{\text{obs}1}t)) + \Delta 2(1 - \exp(-k_{\text{obs}2}t)) + (at)$$

(equation (1))

, where y_0 represents the starting absorbance, ($\Delta 1$ and $\Delta 2$) and ($k_{\text{obs}1}$ and $k_{\text{obs}2}$) the variation in absorbance and the observed rate constant of the two single order phases, respectively. The linear function accounts for the hydrolysis of DTNB.

The k_{obs} values are as expected almost independent of [3-MPH]₀ (Figure S5), and therefore we assigned the $k_{\text{obs}1}$ and $k_{\text{obs}2}$ values by averaging the values of ($k_{\text{obs}1}$, $k_{\text{obs}1} + \text{SE}$, $k_{\text{obs}1} - \text{SE}$) and ($k_{\text{obs}2}$, $k_{\text{obs}2} + \text{SE}$, $k_{\text{obs}2} - \text{SE}$) obtained for each concentration. The error corresponds to the standard error calculated from the 15 values used for each k_{obs} .

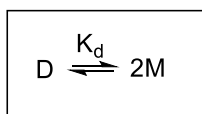
The fraction of monomeric 3-MPH (α) in the syringe S1 was calculated as follows: $\alpha = ((4 * \Delta 1 / (14000) / [3\text{-MPH}]_0))$, where 14000 is the molar extinction coefficient calculated under these conditions for TNB using cysteine, and the factor 4 accounts for the dilution of [3-MPH]₀ during the run.

The plot of $\alpha = f([3\text{-MPH}]_0)$ was fitted with the following equation (derivation given below):

$$\alpha = [-K_d + (K_d^2 + 8[3\text{-MPH}]_0 K_d)^{0.5}] / [4[3\text{-MPH}]_0]$$

(equation (2))

, with $K_d = 1/K_{\text{dimer}}$.



D : dimeric 3-MPH

M : monomeric 3-MPH

α : fraction of monomeric 3-MPH in solution

M_0 : initial concentration of 3-MPH (monomeric + dimeric forms)

$$\left(\alpha = \frac{M}{M_0} \right) \quad \text{and} \quad \left(1-\alpha = \frac{2D}{M_0} \right)$$

$$K_d = \frac{M^2}{D} = \frac{2\alpha^2 M_0^2}{(1-\alpha)M_0} = \frac{2\alpha^2 M_0}{(1-\alpha)}$$

$$\text{so} \quad 2\alpha^2 M_0 + \alpha K_d - K_d = 0$$

yielding the unique solution $\alpha = \frac{-K_d + (K_d^2 + 8M_0K_d)^{1/2}}{4M_0}$

3-MP analysis under single turn-over conditions.

These studies were carried out on a SX19MV-R stopped-flow apparatus (Applied PhotoPhysics) as described in the literature.¹ Experiments were recorded at 30°C, excitation wavelength was set at 295 nm and emitted light was detected using a 320 nm cut-off filter. The first syringe contained *E. coli* 3-MST at 0.5 μM in TE buffer (Tris-HCl 50 mM, EDTA 2 mM, pH 8.0) the other contained variable concentrations of commercial 3-MP or synthesized 3-MPH in the same buffer.

3-MP analysis under steady-state conditions.

Steady-state kinetic parameters were determined at 30 °C in TE buffer with saturated concentration of Trx1 (250 μM) and variable concentrations of 3-MP in the presence of a Trx-regenerating system (1.25 μM *E. coli* Trx reductase (TrxR) and 0.3 mM NADPH). A concentration of 10 nM of *E. coli* 3-MST was added and initial rate measurements were carried out on a SAFAS UVmc² spectrophotometer.

Figure S1. ^1H - ^{13}C HSQC spectrum of 3-MPH recorded on a Bruker Advance-500 in CDCl_3 . X- and Y-axis units are chemical shift (ppm).

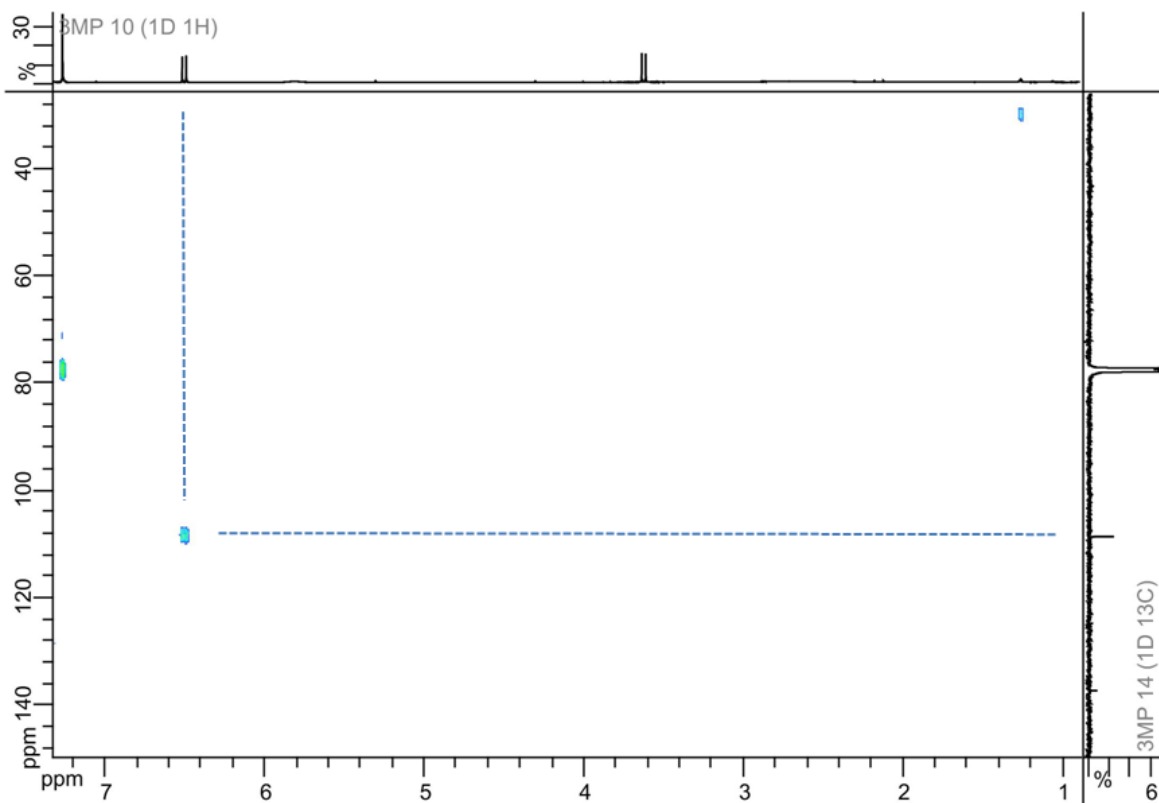


Figure S2. FT-IR spectrum of 3-MPH (A) and 3-MP (B) recorded on a Perkin-Elmer Spectrum One (ATR Miracle ZnSe).

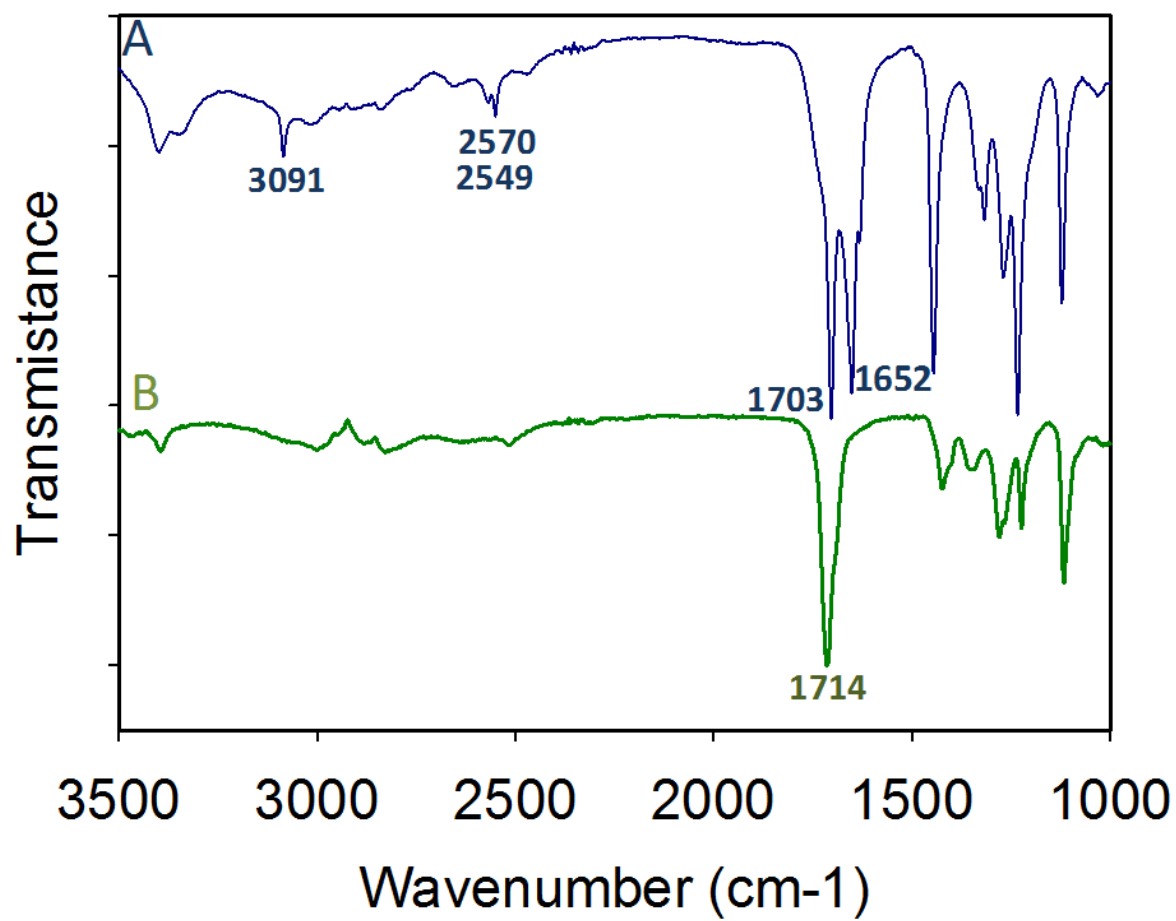


Figure S3. UV-vis spectrum of 3-MPH recorded in CHCl_3 (140 μM).

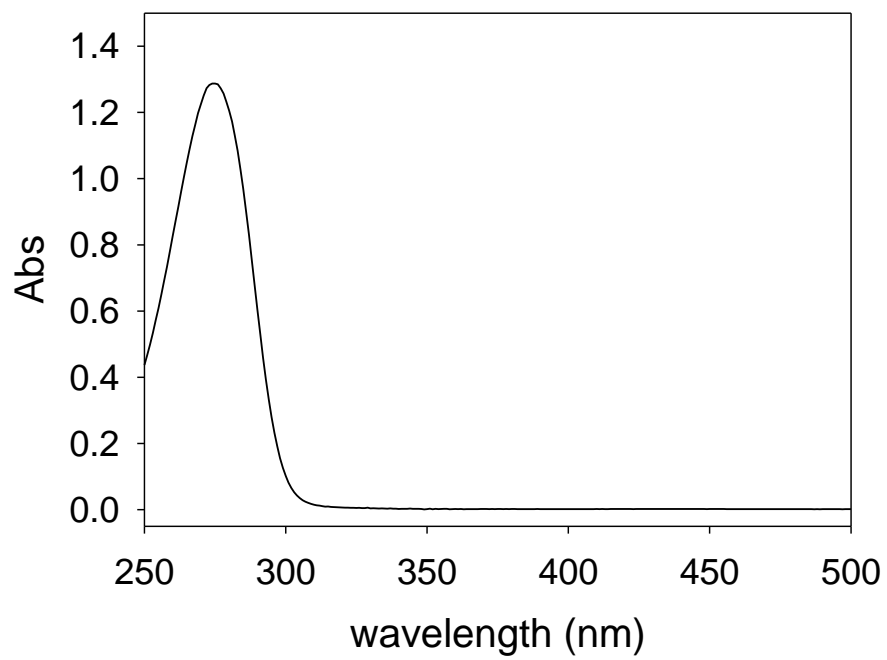
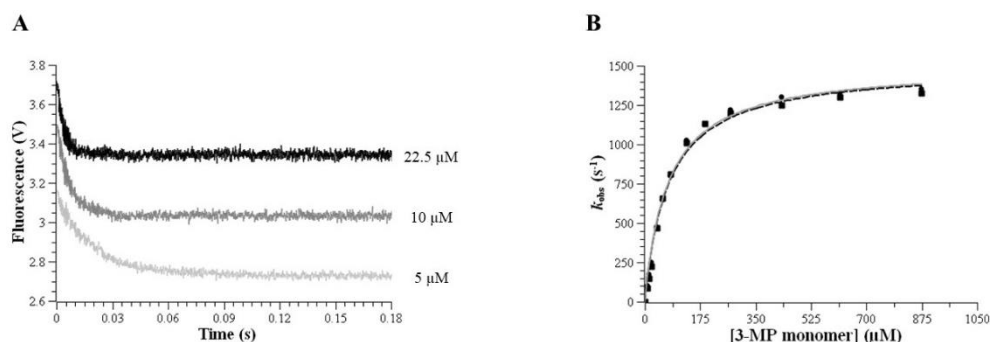


Figure S4. Determination of the enzymatic parameters for the sulfur transfer step.

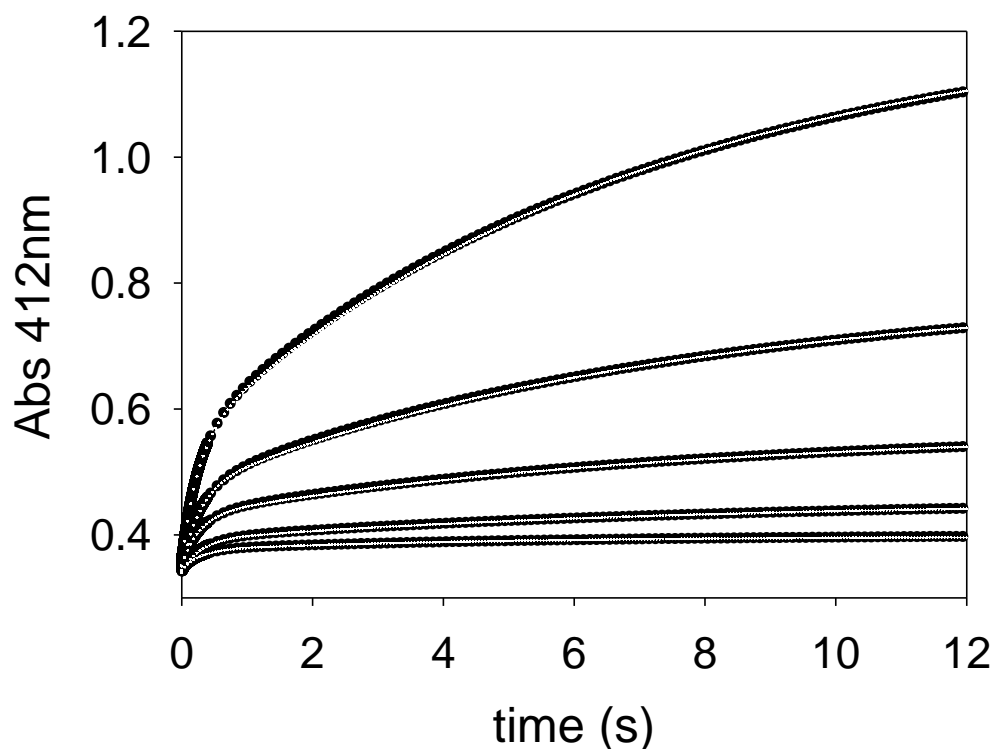
A: Fluorescence experimental traces obtained in the presence of 0.5 μM of *E. coli* 3-MST and 5, 10 and 22.5 μM of synthesized 3-MPH for the black, dark grey and grey traces, respectively. These fluorescence traces were adjusted to an exponential equation allowed to obtain k_{obs} values.

B: Superposition of first step k_{obs} values with 3-MP concentrations (corrected using the value of $K_{\text{dimerization}}$ reported in the main text) for commercial 3-MP (■) and synthesized 3-MPH (●). Experimental data were adjusted to hyperbolic equation corresponding to the commercial 3-MP (---) and synthesized 3-MPH (—).



Kinetic parameters thus obtained are $k_{\text{max}1} = 1450 \pm 9 \text{ s}^{-1}$ and $K_{3\text{-MP}} = 70 \pm 4 \mu\text{M}$ for the synthesized 3-MPH, vs. $k_{\text{max}1} = 1500 \pm 42 \text{ s}^{-1}$ and $K_{3\text{-MP}} = 73 \pm 3 \mu\text{M}$ for the commercial 3-MP.

Figures S5. Determination of the observed rate constants. Kinetic trace (black line) obtained when reacting various concentrations of 3-MPH in KPi with 1.6 mM DTNB in KPi, and best-fit curves (white line over the black line) obtained with non-linear regression (equation (1)). The concentrations and the resulting values are below (underline values are those used to determine kobs1 and kobs2).



Nonlinear Regression

Equation: Exponential Rise to Maximum; Double, 5 Parameter + slope

$$f = y_0 + a * (1 - \exp(-b * x)) + c * (1 - \exp(-d * x)) + e * x$$

[3-MPH] in cuvette = 60 μ M

R	Rsqr	Adj Rsqr	Standard Error of Estimate		
1.0000	1.0000	1.0000	0.0008		
Coefficient		Std. Error	t	P	VIF
y0	0.3517	4.8954E-005	7183.3018	<0.0001	17.0098<
a	0.1912	0.0001	1377.5053	<0.0001	52.3706<
b	4.0876	0.0061	669.6498	<0.0001	52.0540<
c	0.6309	0.0003	2124.7087	<0.0001	47.6283<
d	0.1554	0.0002	985.4730	<0.0001	13.9974<
e	0.0024	9.3956E-006	260.1409	<0.0001	36.4576<

[3-MPH] in cuvette = 30 μ M

R	Rsqr	Adj Rsqr	Standard Error of Estimate		
1.0000	1.0000	1.0000	0.0006		
Coefficient		Std. Error	t	P	VIF
y0	0.3420	3.5714E-005	9577.0219	<0.0001	16.0347<
a	0.1265	0.0001	1108.5778	<0.0001	59.4964<
b	3.8358	0.0068	565.8970	<0.0001	56.2155<
c	0.2986	0.0002	1219.2445	<0.0001	56.2175<
d	0.1458	0.0003	582.2119	<0.0001	16.3123<
e	0.0012	7.6668E-006	151.0597	<0.0001	42.9978<

[3-MPH] in cuvette = 15 μ M

	R	Rsqr	Adj Rsqr	Standard Error of Estimate		
	1.0000	1.0000	1.0000	0.0003		
	Coefficient	Std. Error	t	P	VIF	
y0	0.3500	1.9674E-005	17788.8493	<0.0001	15.9483<	
a	0.0787	6.3556E-005	1238.1490	<0.0001	60.1732<	
b	3.8141	0.0060	634.8590	<0.0001	56.5758<	
c	0.1276	0.0001	935.5850	<0.0001	56.9868<	
d	0.1451	0.0003	447.6620	<0.0001	16.5332<	
e	0.0005	4.2640E-006	115.4268	<0.0001	43.5896<	

[3-MPH] in cuvette = 7.5 μ M

	R	Rsqr	Adj Rsqr	Standard Error of Estimate		
	0.9997	0.9995	0.9995	0.0006		
	Coefficient	Std. Error	t	P	VIF	
y0	0.3400	3.5086E-005	9689.6283	<0.0001	14.9125<	
a	0.0512	0.0001	404.5264	<0.0001	66.4819<	
b	3.5741	0.0165	217.0718	<0.0001	59.5716<	
c	0.0631	0.0003	218.7876	<0.0001	72.6484<	
d	0.1329	0.0012	109.8515	<0.0001	20.6090<	
e		4.9386E-013	8.8428E-006	5.5848E-008	1.0000	55.1220<

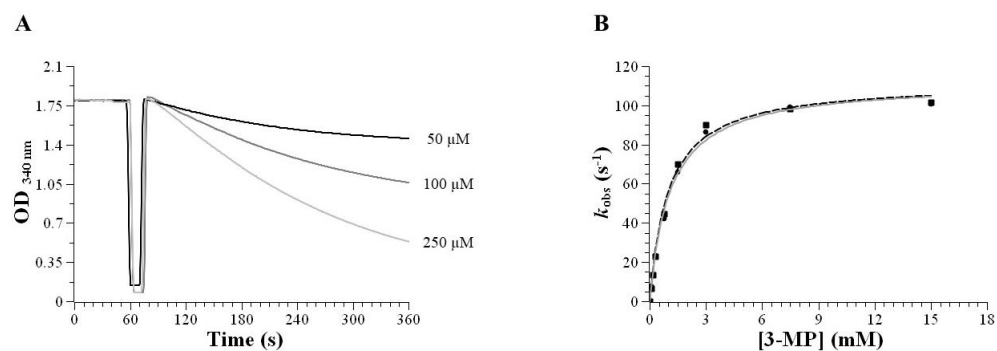
[3-MPH] in cuvette = 3.75 μ M

	R	Rsqr	Adj Rsqr	Standard Error of Estimate		
	0.9984	0.9968	0.9968	0.0007		
	Coefficient	Std. Error	t	P	VIF	
y0	0.3436	4.3341E-005	7928.4113	<0.0001	16.1219<	
a	0.0318	0.0002	173.9496	<0.0001	100.0572<	
b	3.6548	0.0357	102.4331	<0.0001	73.4019<	
c	0.0221	0.0002	102.2390	<0.0001	33.5823<	
d	0.2235	0.0054	41.1514	<0.0001	11.7133<	
e		9.5584E-013	6.0146E-006	1.5892E-007	1.0000	18.0648<

Figure S6. Comparison of 3-MP samples under steady-state conditions.

A: Experimental absorbance traces obtained in presence of 10 nM of *E. coli* 3-MST and 50, 100 and 250 μM of synthesized 3-MPH for the black, dark grey and grey traces, respectively. Initial velocity rate determination allowed to obtain k_{obs} values for each substrate concentrations.

B: Superposition of steady-state k_{obs} values with different 3-MP concentrations for commercial 3-MP (■) and synthesized 3-MPH (●). Experimental data were adjusted to Michaelis-Menten equation corresponding to the commercial 3-MP (---) and synthesized 3-MPH (—).



Steady-state kinetic parameters thus obtained are $k_{\text{cat}} = 112 \pm 2 \text{ s}^{-1}$ and $K_{\text{M}} = 1087 \pm 86 \mu\text{M}$ for the synthesized 3-MPH vs. $k_{\text{cat}} = 112 \pm 3 \text{ s}^{-1}$ and $K_{\text{M}} = 1001 \pm 101 \mu\text{M}$ for the commercial 3-MP.

Figure S7. Evolution of the UV-vis spectrum of 3-MPH recorded in H₂O (initial concentration : 180 μ M) and curve fit obtained by fitting the absorbance at 262 nm over time by an exponential equation.

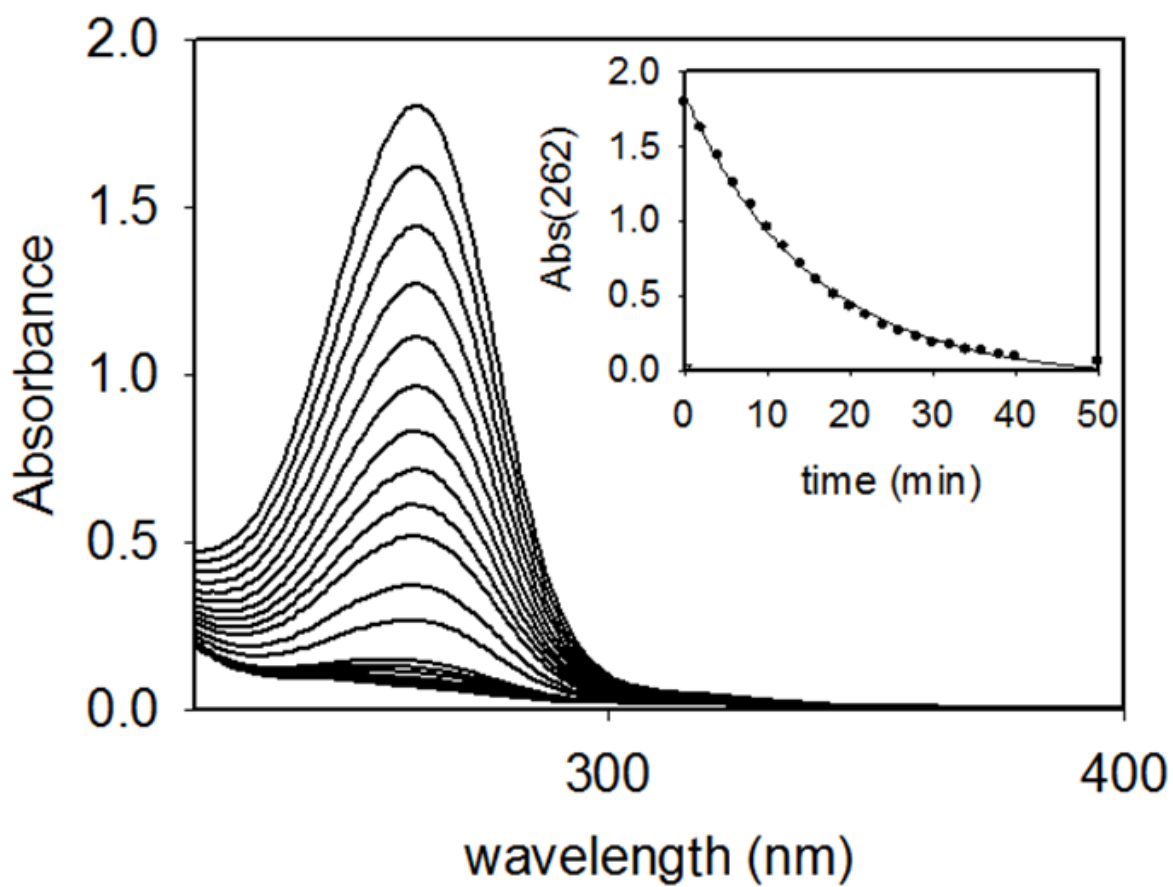


Figure S8. ^1H NMR spectrum of 3-MPH (10mM) recorded on a Bruker Advance-500 in KPi containing 10% D_2O . X-axis units are chemical shift (ppm).

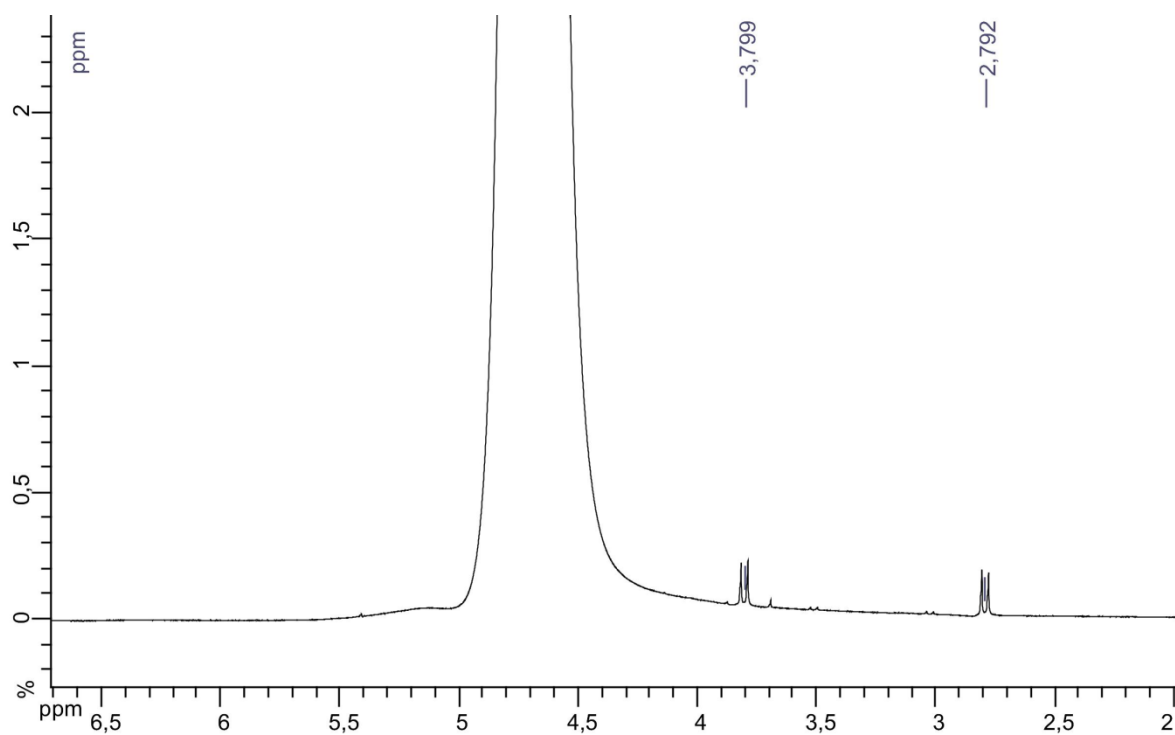
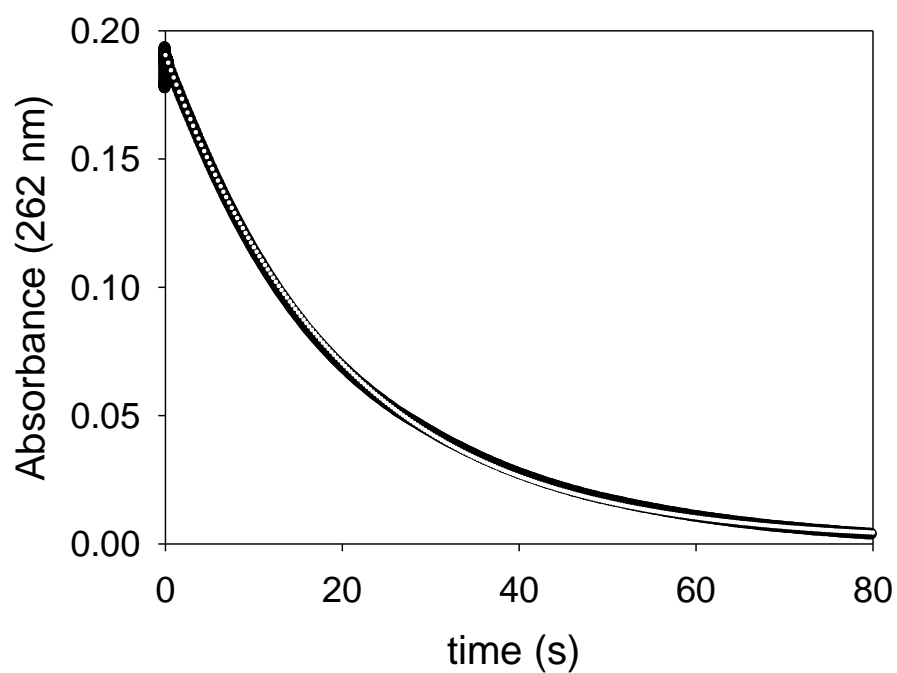
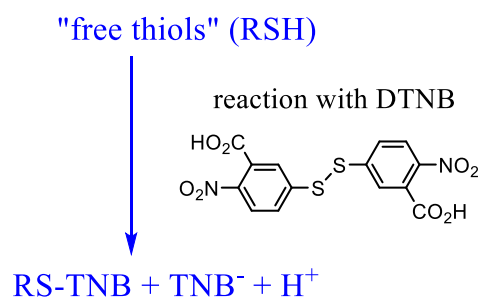
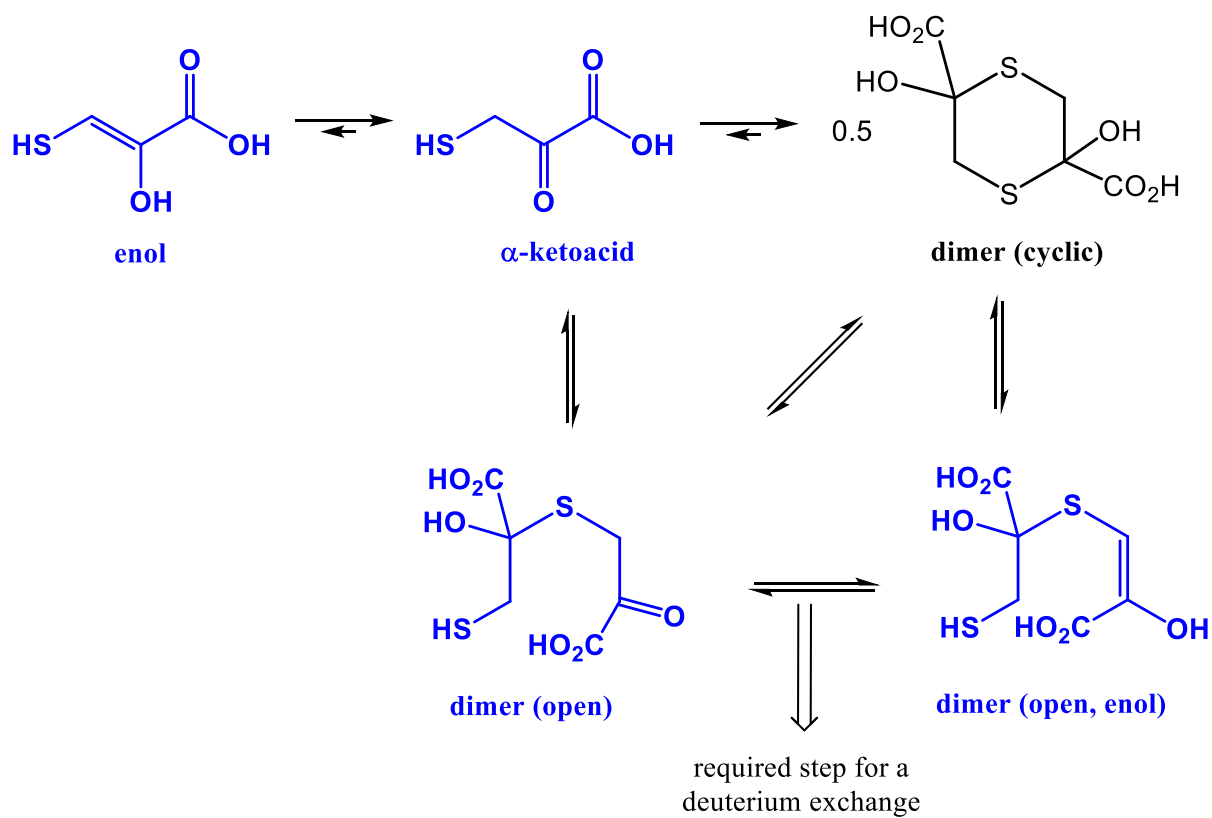


Figure S9. Stopped-flow experimental trace (black line) obtained at 262 nm when mixing a freshly prepared solution of 3-MPH in H₂O (final concentration: 25 μ M) with Kpi, and the curve fit obtained by fitting the data with an exponential equation (white line).



Scheme S1. Various equilibria taking place in solution upon dissolution of 3-MPH.



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- 2 E. Mossner, M. Huber-Wunderlich, R. Glockshuber, *Protein Sci.*, 1998, **7** 1233.
- 3 S. B. Mulrooney, C. H. Williams, *Protein Sci.*, 1997, **6** 2188.