

# **Catalysis of a flavoenzyme-mediated amide hydrolysis**

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## **Supporting information:**

### **S.1. Materials.**

A dehydrated form of LB broth was purchased from EMB Chemicals, (Gibbstown, NJ). Ampicillin and IPTG were obtained from Lab Scientific Inc. (Livingston, N.J.). Uracil, Thymine, FMN, NADH, NADPH, 3,3-diethoxypropanoic acid, were purchased from Sigma (St. Louis, MO.). Triethylamine was obtained from Fisher (Fairlawn, NJ). Trifluoroacetic acid, methanol (HPLC grade), ethanol, chloroform, potassium hydroxide, sodium chloride, imidazole, 2-mercaptoethanol, sodium hydroxide, ether and ethylacetate were purchased from Acros Organics (Morris Plains, NJ). Sodium dihydrogen phosphate monohydrate, concentrated sulfuric acid and anhydrous potassium carbonate were from Mallinckrodt Baker Inc. (Phillipsburg, NJ).  $^{13}\text{C}^{15}\text{N}$  labeled uracil, Deuterium oxide ( $\text{D}_2\text{O}$ ) and Methanol- $\text{d}_4$  were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Microcon YM-10 centrifugal filter devices (10,000 MWCO) and the Amicon Ultra centrifugal filter device (10,000 MWCO) were obtained from Millipore (Billerica, MA). The Supelcosil LC-18-T column for HPLC analysis was from Supelco, (Bellefonte, PA). *E. coli* strain MachI and the Gateway system were from Invitrogen (Carlsbad, CA). Nucleospin Purification kit, Phusion DNA polymerase, *E. coli* BL21(DE3), chaperone plasmid and the Ni-NTA superflow resin were obtained from Macherey-Nagel (Easton, PA), New England Biolabs (Ipswich, MA), Novagen (San Diego, CA), TaKaRa Bio Inc. (Otsu, Japan) and Qiagen (Valencia, CA) respectively.

### **S.2. Cloning, over-expression and purification.**

The over expression plasmid (pCA24N), containing genes encoding for the Rut A (b1012) and Rut F (b1007) proteins, was transformed into *Escherichia coli* BL21(DE3). A starter culture was prepared by growing a single colony of transformed cells in 10 ml of LB media containing 20  $\mu\text{g}/\text{ml}$  of chloramphenicol at 37 °C with overnight agitation. 1 liter LB medium (20 g/L), containing 20  $\mu\text{g}/\text{ml}$  of chloramphenicol, was inoculated with this starter culture. The cells were grown at 37 °C with shaking until the culture reached an  $\text{OD}_{600}$  of 0.6 at which point they were induced by adding IPTG to a final concentration of 0.5 mM, the

temperature was lowered to 15 °C and the cells were allowed to grow for a further 12 hours. The cells were then harvested by centrifugation at 10,000g for 8 min at 4 °C.

Cells from 1 liter of culture were re-suspended in 20 ml of binding buffer (50 mM  $\text{KH}_2\text{PO}_4$ , 150 mM NaCl, 10 mM imidazole, pH 7.7) and approximately 2 mg of lysozyme was added. The cells were then lysed by sonication (Misonix Sonicator 3000, pulse 'on' time 1.0 sec, pulse 'off' time 1.0 sec, output level 0.8, 30 cycles) on ice 5 times. The cell debris was removed by centrifugation at 39,000g for 40 minutes at 4 °C. The clarified supernatant was loaded onto a 2 mL Ni-NTA-affinity column pre-equilibrated with binding buffer kept at 4°C. The Ni-NTA-affinity column was then washed with 100 ml wash buffer (50 mM  $\text{KH}_2\text{PO}_4$ , 150 mM NaCl, 20 mM imidazole, pH 7.7). The protein was eluted from the column with elution buffer (50 mM  $\text{KH}_2\text{PO}_4$ , 150 mM NaCl, 200 mM imidazole, pH 7.7) at 4°C. The fractions containing protein were pooled and concentrated using YM-10 Amicon ultracentrifugal filters at 5000g to a final volume of 500  $\mu\text{l}$ . The concentrated sample was desalted into 100 mM phosphate buffer at pH 8.0 containing 100 mM NaCl and glycerol to a final concentration of 30% using an Econo-Pac 10DG disposable chromatography column. RutA, over expressed well (25mg/L) and soluble protein was obtained upon purification by Ni-affinity chromatography. The protein concentration was measured by the Bradford assay<sup>1</sup>.

RutF had good over expression but very little soluble protein was obtained upon purification. RutF was subcloned into pET28b and was then co-expressed with the chaperone plasmid pG-Tf2 (encoding for chaperones groES-groEL-tig), in order to get soluble protein. Kanamycin (40 mg/L) was used in the later case as the antibiotic.

The overexpression plasmid (pET30LIC)<sup>2</sup> for the Fre protein did not code a 6xHis tag. The *fre* gene was therefore excised from pET30LIC by digestion with *Nde*I and *Eco*RI and ligated into similarly digested pTHT (a pET-28 derived vector which allows attachment of a modified 6xHisTag and a TEV protease cleavage site onto the N-terminus of the expressed protein) to give pFRETHT. The Fre protein was then overexpressed (60mg/L) and purified by Ni-affinity chromatography according to the procedure mentioned above.

### S.3. HPLC analysis.

HPLC analysis of the enzymatic reaction mixture was performed on a Hewlett-Packard 1100 instrument using a Supelcosil LC 18 T (15 cm X 4.6 mm, 3.0  $\mu\text{m}$ ) column.

Solution A contained water, solution B contained 100 mM potassium phosphate buffer at pH 6.6 and solution C contained methanol. The following linear gradient was used. 0% to 10% solution A and 100% to 90% solution B for 0-5 min, 10% to 48% solution A, 90% to 40% of solution B and 0% to 12% of solution C from 5-12 min, 48% to 50% solution A, 40% to 30% of solution B and 12% to 20% of solution C in 12-14 min, 50% to 30% solution A, 30% to 10% of solution B and 20% to 60% of solution C in 14-18 min, 30% to 0% solution A, 10% to 100% of solution B and 60% to 0% of solution C in 18-20 min and 0% of solution A, 100% of solution B and 0% of solution C in 20-25 minutes. The flow rate was 1 ml/min and the absorbance was measured at 254 nm. Under these conditions the following compounds were readily separated (retention time in parenthesis): 3-ureidoacrylate (**3**) (2.3 min), uracil (**1**) (3.7 min), NAD (12.9 min) and NADH (14.3 min).

## S.4. Synthesis.

### S.4.1. 3-ureidoacrylate (**3**).

The synthesis of 3-ureidoacrylate (**3**) began with the known protocols for making 3-oxauracil (**17**) from maleic anhydride (**16**)<sup>3</sup> *supplementary figure.1(a)*, which was converted to 3-ureidoacrylate (**3**) with minor modification of literature procedure<sup>4</sup>. 3-Oxauracil (**17**) (1.13 g, 0.01 mol; well ground) was added to a solution of ammonia in water (20 mL) at 5 °C with constant stirring over 10 min till all of it dissolved, after which the reaction mixture was allowed to stand for another 12 hrs at 4 °C instead of 25 °C as reported in the literature. There was no change in the workup procedure and quantitative yields of 3-ureidoacrylate (**3**) were obtained. This is a considerable improvement on the literature procedure where the reported yield is 12%. <sup>1</sup>H NMR (DMSO, 300MHz)  $\delta$  4.73 (d, 1H), 6.80 (br s, 2H), 7.32 (dd, 1H), 9.75 (br d, 1H).

### S.4.2. (Z)-4-nitrophenyl-3-ureidoacrylate (**19**).

The synthesis of (Z)-4-nitrophenyl 3-ureidoacrylate (**19**) was adapted from known literature procedures of similar compounds<sup>5-6</sup>. To a solution of (Z)-3-ureidoacrylic acid (**3**) (100 mg, 0.77 mmol) and 4-dimethylaminopyridine (DMAP) (10 mg, 0.08 mmol) in anhydrous dimethylformamide (DMF) (2 mL), 4-nitrophenol (**18**) (130 mg, 0.92 mmol) was added, followed by dicyclohexylcarbodiimide (DCC) (190 mg, 0.93 mmol) *supplementary figure 1(b)*. The reaction mixture was stirred at room temperature overnight. N,N-dicyclohexylurea (DCU) was removed by filtration, and DMF was evaporated from the filtrate under reduced

pressure. The residue was suspended in ethyl acetate (30 mL), the insoluble DCU was removed by filtration and the solvent was removed under reduced pressure. The product was washed with water, filtered under vacuum, and dried to give yellow colored 4-nitrophenyl 3-ureidoacrylate (55 mg, 30%).  $^1\text{H}$  NMR (300 MHz, DMSO):  $\delta$  5.14 (d, 1H), 6.85 (br s, 1H), 7.31 (br s, 1H), 7.47 (d, 2H), 7.67 (dd, 1H), 8.32 (d, 2H), 9.80 (d, 1H). GC-MS:  $m/z = 251.1$  ( $\text{M}^+$ ) calculated for  $\text{C}_{10}\text{H}_9\text{N}_3\text{O}_5$ .

#### S.4.3. (Z)-3-ureidoacrylic peracid (**15**).

The synthesis of (Z)-3-ureidoacrylic peracid (**15**) was adapted from known literature procedures with significant modifications *supplementary figure 1(c)*. To a suspension of 4-nitrophenyl 3-ureidoacrylate (**19**) (30 mg, 0.12 mmol) in  $\text{D}_2\text{O}$  (0.5 mL)  $\text{Na}_2\text{O}_2$  (16 mg, 0.14 mmol) was added at 4° C and the reaction mixture was stirred for 5 min. (Z)-3-ureidoacrylic peracid (**15**) was identified by NMR without any further purification which was not possible due to its instability.  $^1\text{H}$  NMR (300 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  5.03 (d, 1H), 7.23 (d, 1H).

### S.5. Enzymatic Assays

#### S.5.1. RutA and RutF catalyzed reaction of uracil (**1**).

A 500  $\mu\text{L}$  of reaction mixture containing 200  $\mu\text{M}$  of uracil (**1**), 5  $\mu\text{M}$  of RutA, 5  $\mu\text{M}$  of RutF, 40  $\mu\text{M}$  of FMN and 2 mM of NADH was incubated at room temperature for 2 hours. All solutions were made in 100 mM potassium phosphate buffer at pH 8.0. The reaction mixture was filtered to remove protein using a Microcon YM10 and analyzed by HPLC (method A), *supplementary figure 2*. Product formation was seen only when all of the components of the reaction mixture were present. No product was detected on substituting RutF with DTT and/or dithionite as alternative chemical reductants. The same product was seen when RutF was substituted with Fre, as shown in *supplementary figure 3*.

#### S.5.2. Oxidation of NADH by RutF

The RutF preparation was found to selectively use NADH instead of NADPH to reduce FMN. To a solution of 100  $\mu\text{M}$  of NADH in 100 mM phosphate buffer pH 8.0 at room temperature, 10  $\mu\text{M}$  of the impure preparation of RutF was added and the oxidation of the NADH was followed by monitoring the absorbance at 340 nm. A decrease in absorbance was seen for NADH but no such decrease was observed for NADPH of equal concentration. No such decrease was observed at 340 nm when the enzyme was omitted

from the reaction mixture. An increase in the rate of NADH oxidation was observed with increase in concentration of the enzyme for the same amount of NADH, *supplementary figure 4*.

### S.6. Product isolation and characterization

All attempts to isolate and characterize the product by NMR and ESI-MS were unsuccessful because the product was unstable to acid and degraded on lyophilization. In order to characterize the product without isolation the enzymatic reaction was set up with  $^{13}\text{C}^{15}\text{N}$  labeled uracil (**1**). 5 mM  $^{13}\text{C}^{15}\text{N}$  labeled uracil (**1**) was incubated overnight with 50 mM NADH, 200  $\mu\text{M}$  FMN and 120  $\mu\text{M}$  of RutA and RutF mixture, at 25 °C. The final volume was 700  $\mu\text{L}$ . A control reaction was set up in an identical manner in which the enzymes were substituted with equal volume of desalting buffer. All solutions were made in 25 mM  $\text{D}_2\text{O}$  phosphate buffer pH 8.0. The enzymes were desalted into 100 mM potassium phosphate buffer at pH 8.0 containing 100 mM NaCl and 2 mM DTT and glycerol was added to a final concentration of 50 %. The complete conversion of the substrate to the product was determined by HPLC analysis (data not shown). The control reaction lacking RutAF shows unreacted  $^{13}\text{C}^{15}\text{N}$  labeled uracil (**1**, top panel, *supplementary figure 6*,  $J_{\text{C4-C5}} = 65.5 \text{ Hz}$ ,  $J_{\text{C5-C6}} = 66.5 \text{ Hz}$ ,  $J_{\text{C6-N1}} = 11.6 \text{ Hz}$ ,  $J_{\text{N1-C2}} = 24.5/20.5 \text{ Hz}$ ,  $J_{\text{C2-N3}} = 20.5/24.5 \text{ Hz}$  and  $J_{\text{N3-C4}} = 10.6 \text{ Hz}$ ). The bottom panel in *supplementary figure 6* shows the product of the RutA/F catalyzed reaction ( $J_{\text{C4-C5}} = 66.6 \text{ Hz}$ ,  $J_{\text{C5-C6}} = 2.3 \text{ Hz}$ ,  $J_{\text{C6-N1}} = 15.5 \text{ Hz}$ ,  $J_{\text{N1-C2}} = 20.3/17.7 \text{ Hz}$ ,  $J_{\text{C2-N3}} = 17.7/20.3 \text{ Hz}$ ,  $J_{\text{N3-C4}} = 0 \text{ Hz}$ ). The  $^{13}\text{C}$  NMR of the product, showed no coupling between N3 and C4, demonstrating that the bond between these atoms is broken during the enzymatic transformation.

The product of the enzymatic reaction was further characterized by HMBC (*supplementary figure 6*), a broad band  $^{15}\text{N}$  decoupled  $^{13}\text{C}$  NMR spectrum (*supplementary figure 7*), and an arrayed narrow band  $^{15}\text{N}$  decoupled  $^{13}\text{C}$  NMR spectrum (*supplementary figure 8*).

### S.7. RutA reaction in the presence of $\text{H}_2\text{O}^{18}$ and $^{18}\text{O}_2$ .

Two identical solutions were made, each (300  $\mu\text{L}$ ) containing 180  $\mu\text{L}$  of  $\text{D}_2\text{O}$ , 10  $\mu\text{L}$  of 1M  $\text{K}_2\text{HPO}_4$  pH 8.0, 10  $\mu\text{L}$  of 20 mM FMN and 100  $\mu\text{L}$  of 1 mM RutA/F. To one of them, 300  $\mu\text{L}$  of 9.4 mM  $\text{C}^{13}\text{N}^{15}$  labeled uracil (**1**) in  $\text{H}_2\text{O}^{18}$  was added. This was the ‘reaction

mixture' which had a final concentration of 50%  $\text{H}_2\text{O}^{18}$ . The other sample was used as a 'control' and contained an equal volume of 9.4 mM  $\text{C}^{13}\text{N}^{15}$  labeled uracil (**1**) in  $\text{H}_2\text{O}^{16}$ . The caps of the eppendorf tubes were left open for atmospheric oxygen to dissolve. The reaction mixture was allowed to incubate at room temperature for 3 hours after which both the samples were analyzed by  $\text{C}^{13}$  NMR. Reaction carried out with  $^{13}\text{C}^{15}\text{N}$  labeled uracil (**1**) in 50%  $\text{H}_2\text{O}^{18}$  showed no isotopic shift at C4 of the product, *supplementary figure 9(a)*.

Two 15 mL Nalgene tube containing 500  $\mu\text{L}$  of  $\text{C}^{13}\text{N}^{15}$  labeled uracil (**1**) dissolved in 25 mM phosphate buffer at pH 8.0 in  $\text{D}_2\text{O}$  were set up and  $^{18}\text{O}_2$  was passed through one (reaction mixture) while  $^{16}\text{O}_2$  was passed through the other (control) using a long needle attached to the respective gas cylinders. The rate of the gas flow was about 1 small bubble per second. The solutions were purged with oxygen for 30 minutes, after which 100  $\mu\text{L}$  of 1 mM RutA/F, 50  $\mu\text{L}$  of 20 mM FMN and 75 mg of NADH were added in quick succession in the order mentioned. The tubes were capped tightly and vortexed briefly to dissolve the NADH. The reaction mixture and the control were incubated at room temperature for 2 hours after which they were analyzed by  $\text{C}^{13}$  NMR. The enzymatic reaction carried out in the presence of  $^{18}\text{O}_2$  shows a clear isotopic shift at C4 for the product *supplementary figure 9(b)*.

The enzymatic reaction product was then treated with 10% trifluoroacetic acid, with the hope that the structure of the acid degraded product could lead to the structure of the parent compound. A triplet was observed at 162.7 ppm which corresponded to the chemical shift of urea, *supplementary figure 10*. The triplet clearly originated from C2 of uracil (**1**) (flanked by 2  $^{15}\text{N}$  nitrogens) and this observation suggested that acid hydrolysis of the product of the RutA/F catalyzed reaction of uracil (**1**) produces urea. The product is likely to be 3-ureidoacrylate (**3**) as it is susceptible to addition elimination reactions under acidic conditions to give urea. Such a reaction can also occur for compound (**5**) but in that case it would have produced hydroxyurea, which has a different chemical shift (164.5 ppm).

### **S.9. Co-injection with 3-ureidoacrylate (**3**).**

The RutA/F product was prepared by incubation of 200  $\mu\text{M}$  of uracil (**1**), 1 mM NADH, 20  $\mu\text{M}$  of FMN and 10  $\mu\text{M}$  of RutA/F at 25°C for 2 hours. All solutions were prepared in 100 mM potassium phosphate buffer at pH 8.0. The synthesized 3-ureidoacrylate (**3**) was dissolved in 100 mM potassium phosphate buffer at pH 8.0. Co-

injection of synthesized sample of 3-ureidoacrylate (**3**) with the product of RutA/F catalyzed reaction of uracil (**1**) showed an increase in intensity of the product peak, *supplementary figure 11*, thereby ascertaining that 3-ureidoacrylate (**3**) was indeed the product of the RutA/F catalyzed reaction of uracil (**1**).

#### S.10. Protein Crystallization and structure determination.

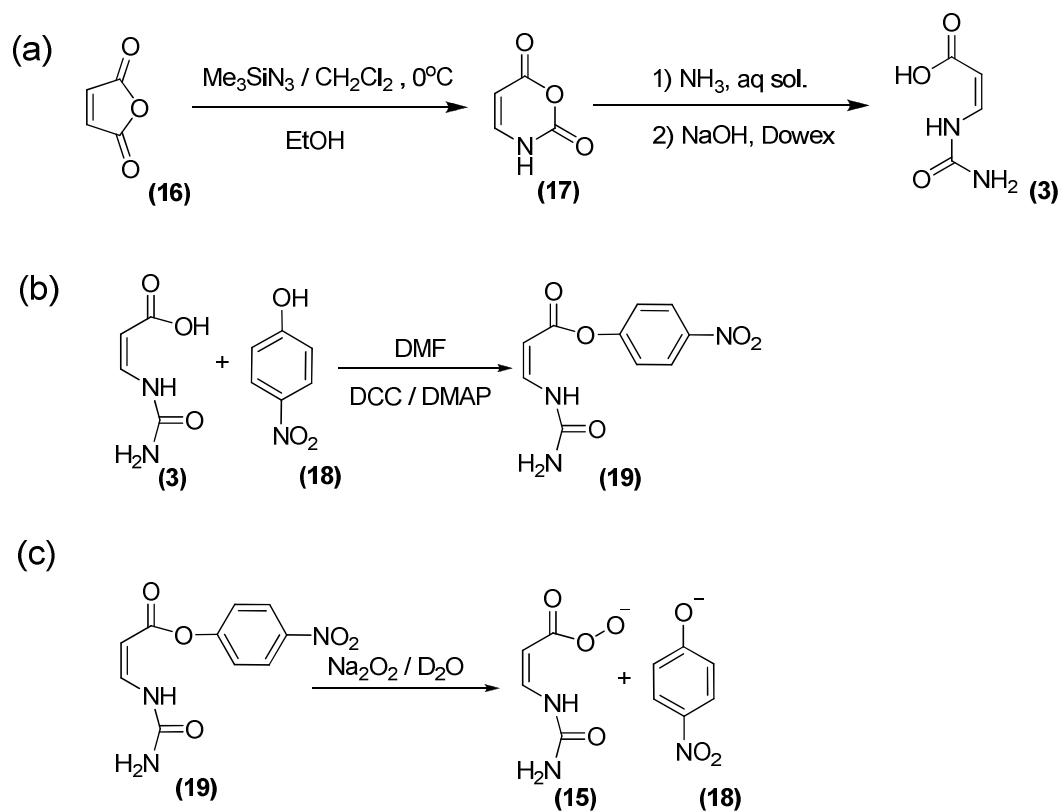
Crystals of RutA were grown from 2 mM FMN, 1.5 M ammonium sulfate, and 100 mM MES pH 6.0 using the hanging drop vapor diffusion method at 18 °C. Yellow crystals appeared after 1 month with a maximum size of approximately 50  $\mu\text{m}$   $\times$  50  $\mu\text{m}$   $\times$  50  $\mu\text{m}$ . Prior to flash freezing in liquid nitrogen, these crystals were cryo-protected by a quick soak in a solution containing 11% glycerol, 10% ethylene glycol, 2.0 M ammonium sulfate, and 100 mM MES pH 6.0. X-ray diffraction data of RuA at 1.8 Å resolution were collected at the Advanced Photon Source NE-CAT beamline ID24-E using an ADSC Quantum 315 detector. The data were integrated and scaled using HKL2000<sup>8</sup>. Crystals of RutA belong to the space group P3<sub>1</sub>21 with unit cell parameters  $a = 87.85$  Å and  $c = 96.37$  Å. The unit cell contains one protomer per asymmetric unit with a solvent content of 49% and Matthews coefficient of 2.41 Å<sup>3</sup>/Da. The structure of RutA was determined using the automated molecular replacement program MrBUMP<sup>9</sup>. The top solution was obtained using one protomer of protein Yntj (1YW1) as the search model, which has approximately 21% sequence identity to RutA. The initial model had an R factor of 50% and R<sub>free</sub> of 55%. The phases were gradually improved by iterative cycles of manually rebuilding in COOT<sup>10</sup> and restrained refinement in REFMAC5<sup>11</sup> and PHENIX<sup>12</sup>. Water molecules and ligand FMN were included after the refinement of the protein molecule converged. The final structure has an R factor of 16.2% and R<sub>free</sub> of 19.0%.

#### S.11. Stability of the 3-ureidoacrylic peracid (**15**).

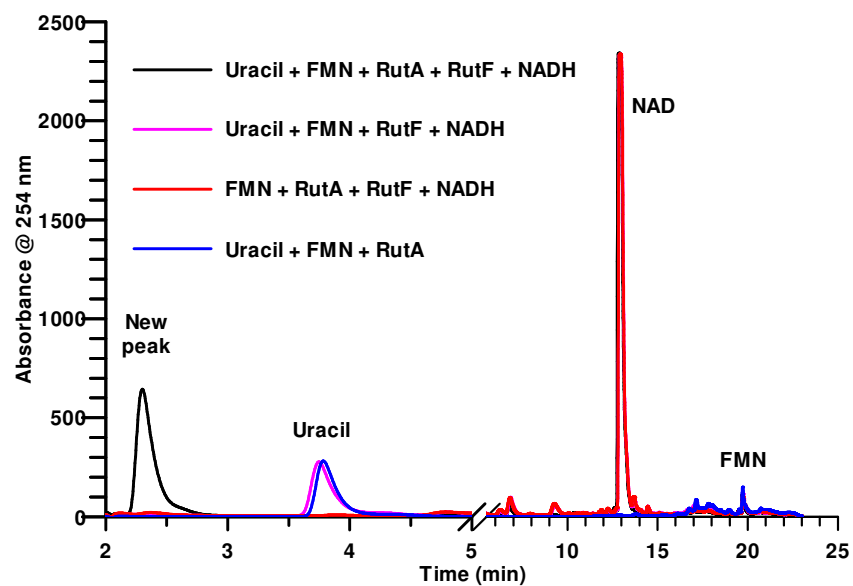
There are many procedures that describe the preparation of peracids from the corresponding acid using hydrogen peroxide in an acidic medium<sup>13-14</sup>. The (Z)-3-ureidoacrylic acid (**3**) was found to be susceptible to addition elimination reactions under acidic condition to produce urea. A similar reaction is likely to occur for (Z)-3-ureidoacrylic peracid (**15**) if it is prepared under acidic conditions, hence basic conditions were employed for its synthesis. Unfortunately, the (Z)-3-ureidoacrylic peracid (**10**) is not stable for



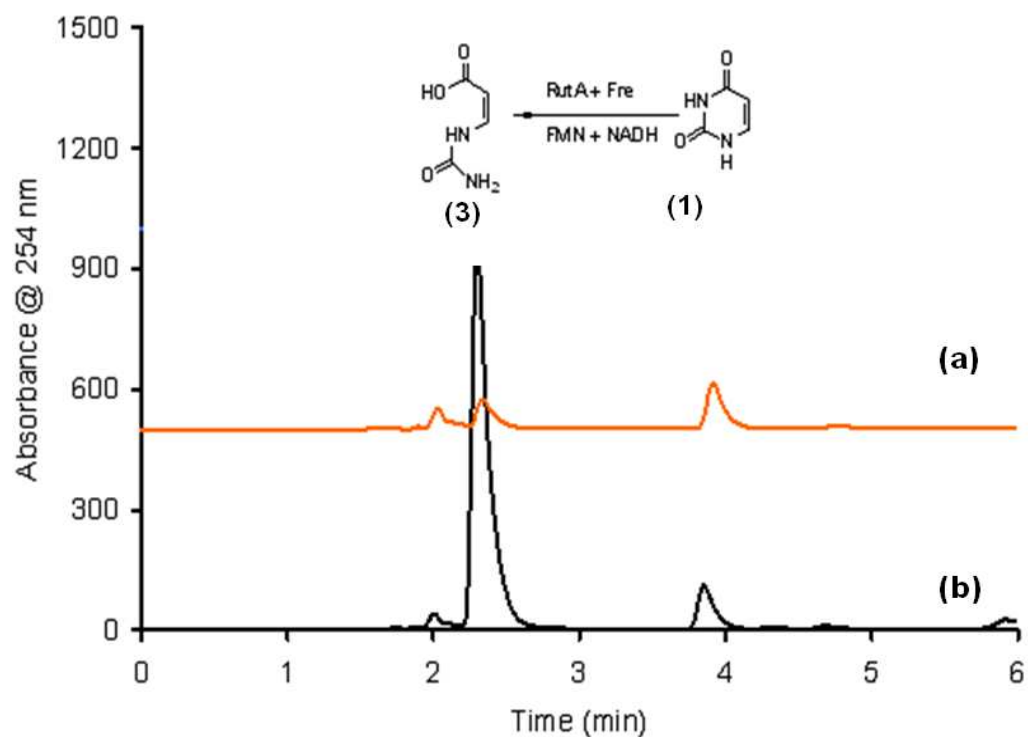
purification as it decomposed over time to form (Z)-3-ureidoacrylic acid (**3**) and uracil (**1**) along with some unidentified species, *supplementary figure 12*. The half life of the (Z)-3-ureidoacrylic peracid (**10**) was calculated to be 3 hrs at 25 °C, pH 10.0. The degradation of (Z)-3-ureidoacrylic peracid (**10**) to (Z)-3-ureidoacrylic acid (**3**) and uracil was confirmed by HPLC analysis of the degraded sample, *supplementary figure 13* as well as spiking the degraded sample with (Z)-3-ureidoacrylic acid (**3**) and uracil (data not shown).



**Supplementary Figure 1:** Synthetic schemes for the preparation of substrates and reference compounds (a) 3-ureidoacrylate (**3**) (b) (*Z*)-4-nitrophenyl 3-ureidoacrylate (**19**) and (c) (*Z*)-3-ureidoacrylic peracid (**15**).

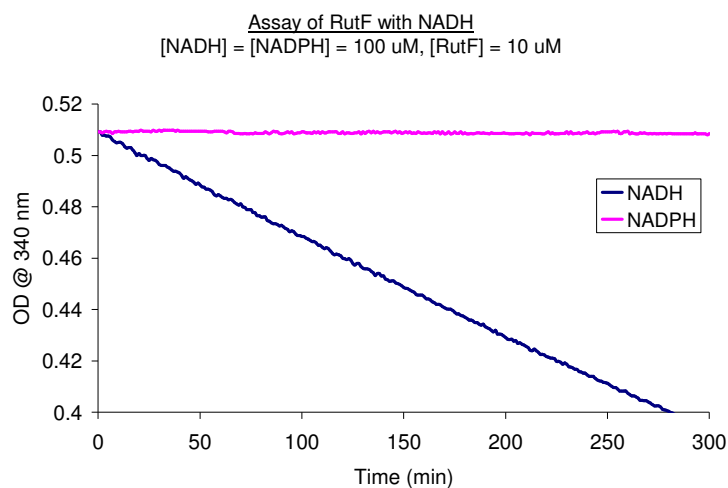


**Supplementary Figure 2:** HPLC analysis showing the formation of a new product as a result of enzymatic reaction of RutA and RutF with uracil (1).

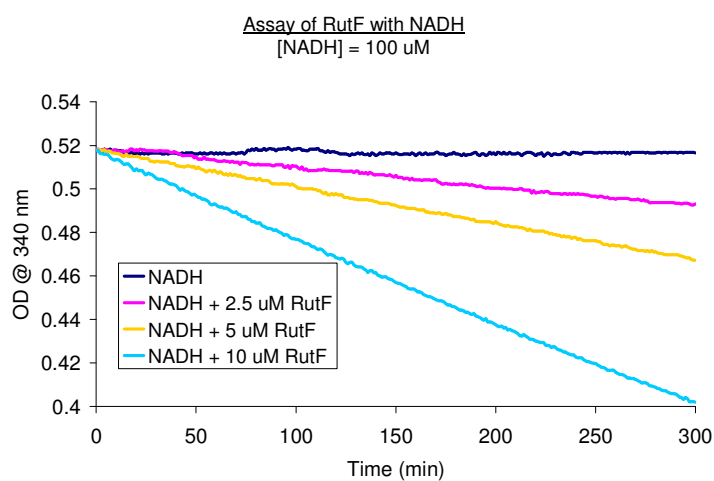


**Supplementary Figure 3:** HPLC chromatogram of (a) the reaction mixture for the RutA-Fre catalyzed reaction of uracil (**1**) (3.7 min) and (b) Co-injection of (a) with chemically synthesized 3-ureidoacrylate (**3**) (2.3 min). Unreacted uracil (**1**) is seen in trace (a) as the reaction mixture was analyzed prior to completion of the reaction.

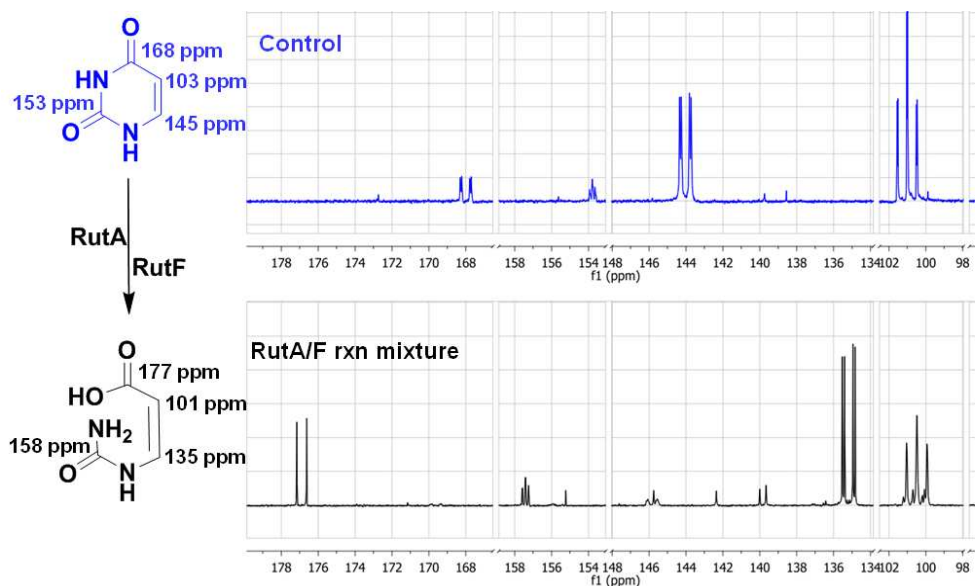
(a)



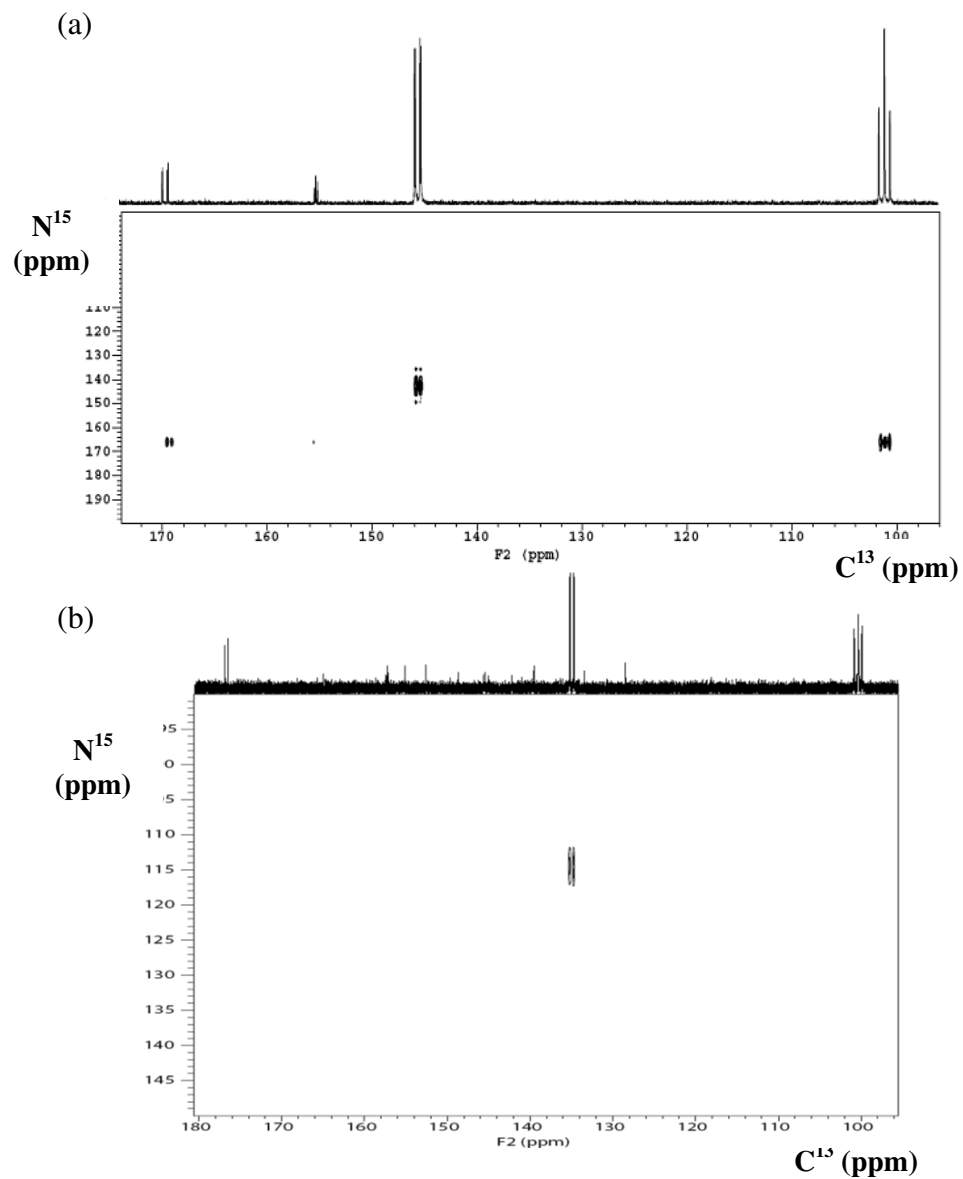
(b)



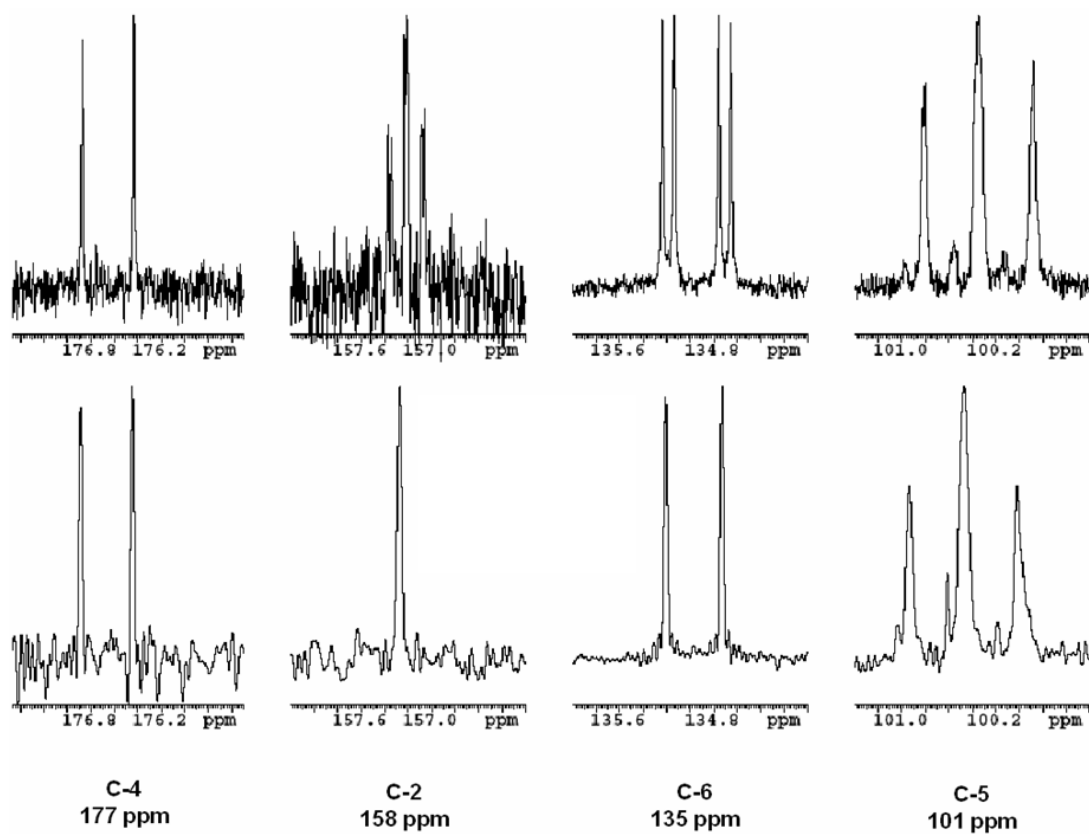
**Supplementary figure 4:** (a) Oxidation of NADH and NADPH by RutF. (b) Oxidation of NADH with varying concentrations of RutF. Assay mixture (500  $\mu$ L) consisted of 100  $\mu$ M NADH and 10  $\mu$ M protein in 100 mM potassium phosphate buffer at pH 8.0.



**Supplementary Figure 5:** NMR (125 MHz, D<sub>2</sub>O, C<sup>13</sup>) of the RutA/F control (top panel) and reaction mixture (bottom panel). The reaction mixture (700  $\mu$ L) consisted of 5 mM <sup>13</sup>C<sup>15</sup>N labeled uracil (**1**), 50 mM NADH, 200  $\mu$ M FMN and 120  $\mu$ M of RutA and RutF incubated overnight at 25 °C. The control sample was identically prepared except the enzymes were substituted with an equal volume of desalting buffer (100 mM potassium phosphate buffer at pH 8.0 containing 100 mM NaCl and 2 mM DTT and glycerol).

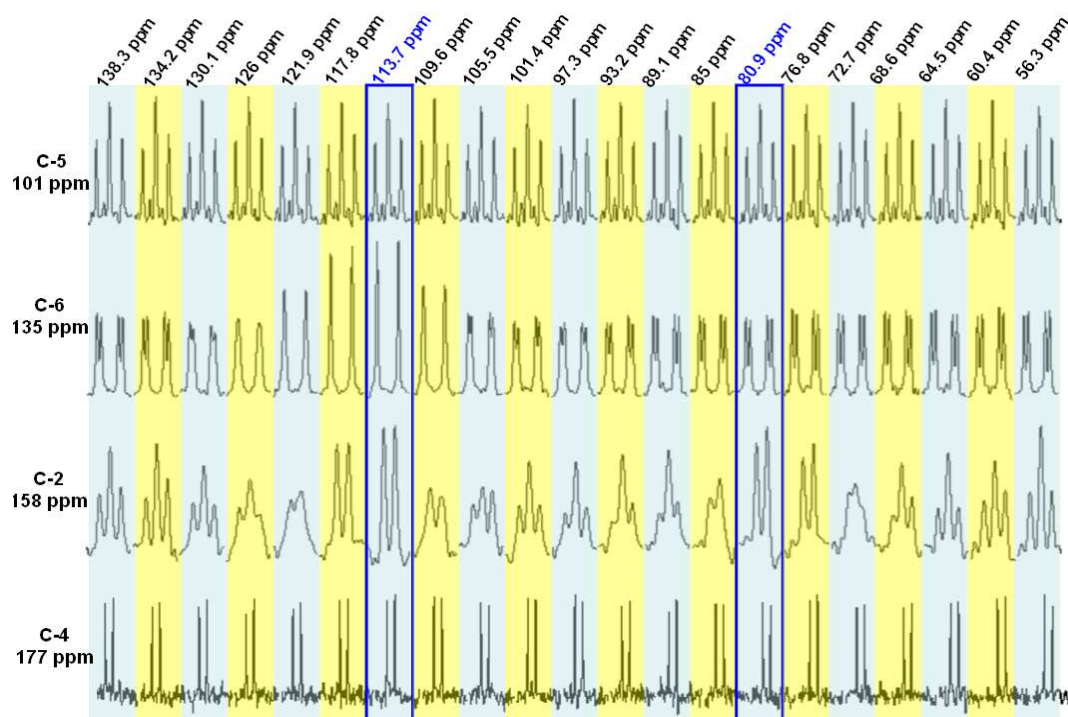


**Supplementary Figure 6:** NMR (150 MHz,  $D_2O$ ,  $N^{15}$ ,  $C^{13}$ ) analysis of the RutA/F reaction product. (a) HMBC of  $[N^{15}, C^{13}]$ -uracil (**1**). (b) HMBC of the reaction product

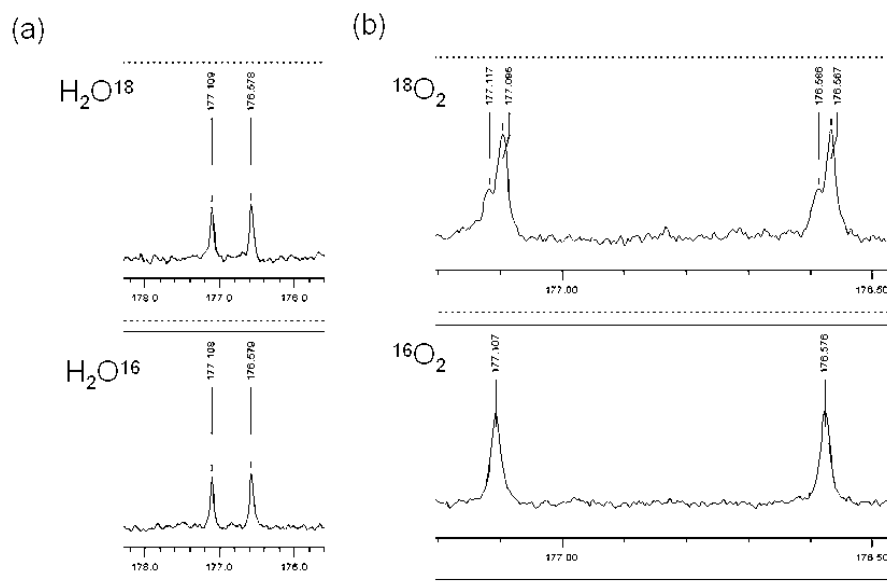


**Supplementary Figure 7:** Broad band  $^{15}\text{N}$  decoupled  $^{13}\text{C}$  NMR analysis of the RutA/F reaction product (150 MHz,  $\text{D}_2\text{O}$ ,  $\text{C}^{13}$ ). Top spectra show the  $^{13}\text{C}$  NMR of the RutA/F reaction mixture with  $^{15}\text{N}$  coupling, while the bottom spectra show the corresponding  $^{15}\text{N}$  decoupled spectra.

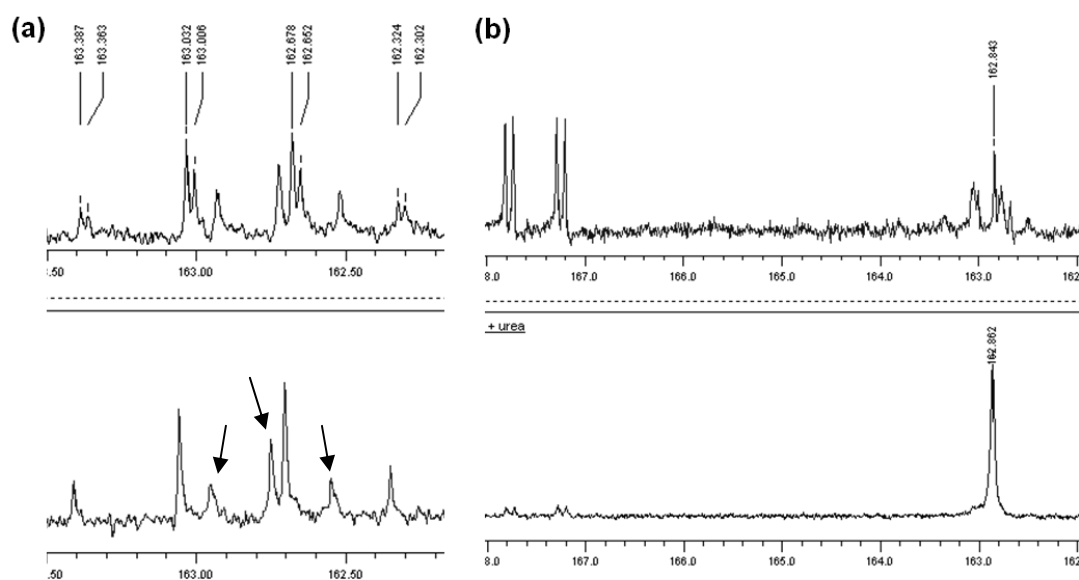




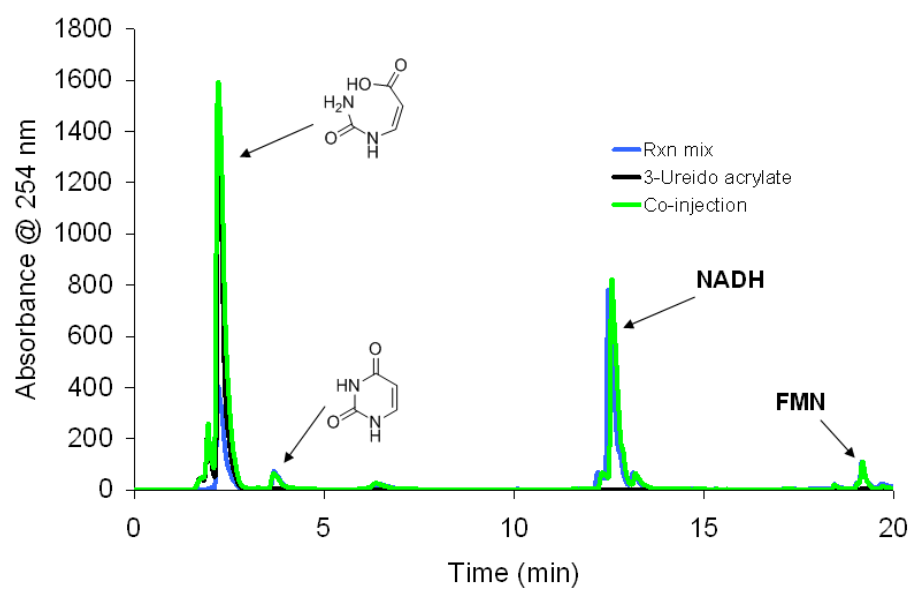
**Supplementary Figure 8:** Arrayed narrow band  $^{15}\text{N}$  decoupled  $^{13}\text{C}$  NMR spectrum of the product of the RutA/F catalyzed reaction (150 MHz,  $\text{D}_2\text{O}$ ,  $\text{C}^{13}$ ). The  $^{15}\text{N}$  decoupling frequency is given on the top of each column. A  $90^\circ$  decoupler pulse was provided for 4.8 msec with the decoupling band width of 15 ppm. The fate of each of the  $^{13}\text{C}$  nuclei at various potential decoupling frequencies of the  $^{15}\text{N}$  is shown in the array.



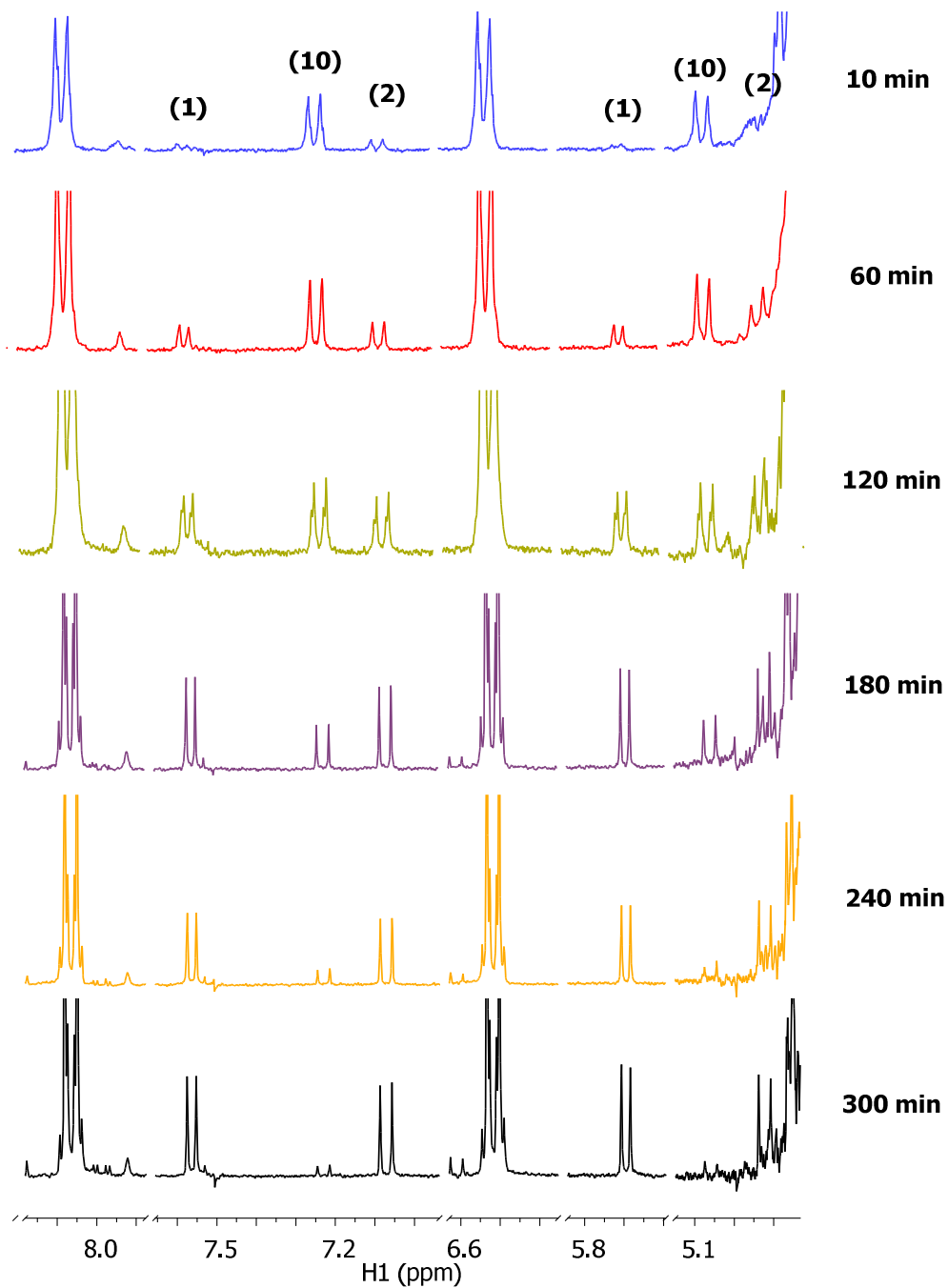
**Supplementary Figure 9:** NMR (125 MHz,  $\text{D}_2\text{O}$ ,  $\text{C}^{13}$ ) analysis of the product of the RutA/F catalyzed reaction run in  $\text{H}_2\text{O}^{18}$  or  $^{18}\text{O}_2$ . (a) Top panel:  $^{13}\text{C}$  NMR spectrum of the enzymatic reaction carried out in 1:1  $\text{H}_2\text{O}^{18}/\text{H}_2\text{O}^{16}$ , Bottom panel:  $^{13}\text{C}$  NMR spectrum of an identical reaction carried out in 100%  $\text{H}_2\text{O}^{16}$ . (b) Top panel:  $^{13}\text{C}$  NMR spectrum of the enzymatic reaction run under  $^{18}\text{O}_2$  gas. Bottom panel:  $^{13}\text{C}$  NMR spectrum of an identical reaction mixture run under  $^{16}\text{O}_2$  gas.



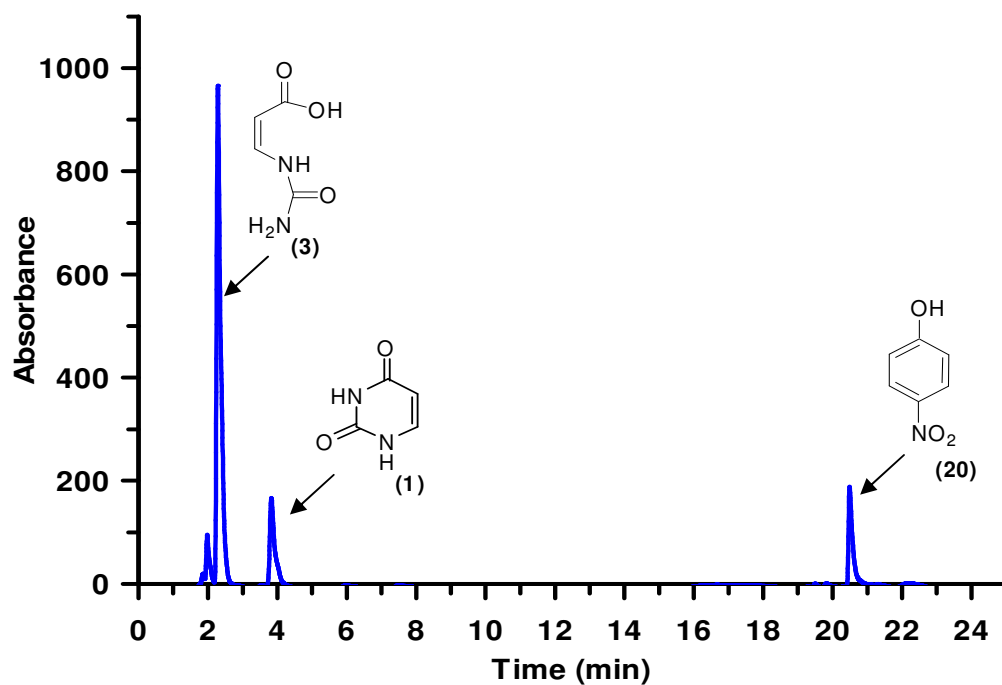
**Supplementary Figure 10:**  $^{13}\text{C}$ -NMR (125 MHz,  $\text{D}_2\text{O}$ ) analysis of the trifluoroacetic acid catalyzed decomposition product of the RutA/F catalyzed reaction. (a). Top panel:  $^{13}\text{C}$  NMR spectrum (163.2–163.5ppm) of the reaction carried out in 50%  $\text{H}_2\text{O}^{18}$  and then treated with 10% trifluoroacetic acid. The carbonyl carbon of trifluoroacetic acid is a quartet of doublets because of the  $^{18}\text{O}$  isotopic shift. Bottom panel: spectrum of an identical reaction mixture carried out in 100%  $\text{H}_2\text{O}^{16}$ . The arrows show the triplet present in both samples arising from the decomposition product. (b) Top panel:  $^{13}\text{C}$  NMR of the sample shown in the top panel of (a) showing the 162.0-168ppm region. Bottom panel shows the same sample after spiking with urea.



**Supplementary Figure 11:** HPLC analysis showing the co-migration of 3-ureidoacrylate (**3**) with the product of the RutA/F catalyzed reaction of uracil (**1**).



**Supplementary Figure 12:**  $^1\text{H}$  NMR analysis of the decomposition of (*Z*)-3-ureidoacrylic peracid (**15**,  $^1\text{H}$  NMR (300 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  5.03 (d,  $J=9$  Hz, 1H), 7.23 (d,  $J=9$  Hz, 1H)), to give (*Z*)-3-ureidoacrylic acid ( $\delta$  4.92 (d,  $J=9$  Hz, 1H), 7.07 (d,  $J=9$  Hz, 1H)) and uracil: ( $\delta$  5.70 (d,  $J=7$  Hz, 1H), 7.50 (d,  $J=7$  Hz, 1H)).



**Supplementary Figure 13:** HPLC analysis of decomposed (*Z*)-3-ureidoacrylic peracid (**15**) after storage at 25 °C for 5 hours

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