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Supplemental Material

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The 2'-hydroxy group of flavin mononucleotide influences the catalytic function and promiscuity of the flavoprotein iodotyrosine dehalogenase

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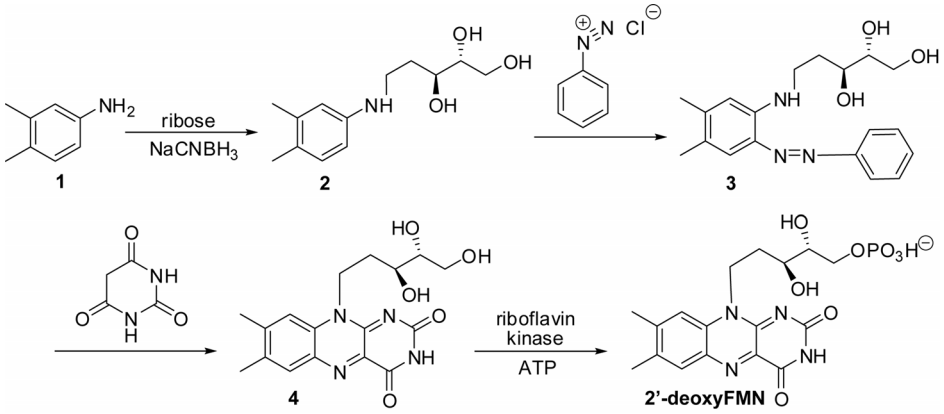


Scheme S1. Synthesis of 2'-deoxyFMN.[1,2]

2-Deoxyribitylated-3,4-dimethylaniline (2). 3,4-Dimethylaniline (394 mg, 3.25 mmol), 2'-deoxy-D-ribose (485 mg, 3.62 mmol) and sodium cyanoborohydride (385 mg, 6.13 mmol) were dissolved in methanol (50 mL) and heated to 65°C for 48 h. Solvent was removed via rotary evaporation and the residue was stirred with 1 N HCl (35 mL) to quench the excess sodium cyanoborohydride until gas evolution ceased. The solution was then neutralized with a saturated sodium bicarbonate solution and extracted with ethyl acetate (2 x 50 mL). The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated to yield the desired product as a light yellow/tan solid (558 mg, 71.7 % yield). ^{1}H NMR (400 MHz, d_4-MeOD) δ 6.90 (d, J = 8.15 Hz, 1H), 6.54 (s, 1H), 6.47 (dd, J = 2.45, 8.15 Hz, 1H), 3.70 (dd, J = 3.84, 11.4 Hz, 1H), 3.65 (m, 1H), 3.60 (dd, J = 6.51, 4.73 Hz, 1H), 3.49 (m, 2H), 3.20 (m, 2H), 2.16 (s, 3H), 2.11 (s, 3H), 1.98 (m, 1H) 1.74 (m, 1H). ESI+ (m/z) [M+H] calcd: 240.1555, found: 240.1464.

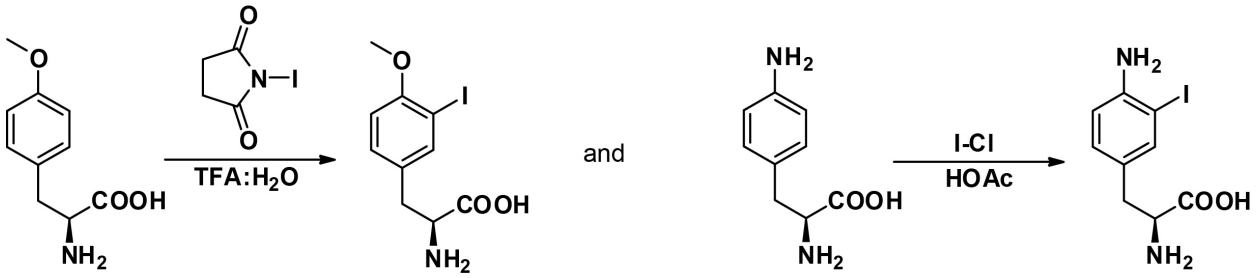
Benzene diazonium chloride. Aniline (189 mg, 2.03 mmol) was dissolved in glacial acetic acid (2 mL) and diluted with water (2 mL). This solution was cooled on ice and concentrated HCl (1 mL) was then added dropwise. Solid sodium nitrite (171 mg, 2.48 mmol) was next added slowly to this solution while maintaining a temperature below 5 °C. This mixture was stirred at 4 °C for 10 min and then used below without further purification.

Diazo-2-deoxyribitylated-3,4-dimethylaniline (3). The ribitylated aniline **2** (538 mg, 2.25 mmol) was suspended in a minimum amount of glacial acetic acid (~2 mL), cooled on ice and stirred at 4 °C for 10 min. The solution containing benzene diazonium chloride was added dropwise to this mixture over ~5 min and stirred at 4 °C for 10 min. A concentrated solution of sodium hydroxide (approx. 400 mg NaOH in 1 mL water) was added with care to maintain a pH below 4 (monitored by pH paper) and the mixture was stirred at 4 °C for 2 h. The reaction mixture was extracted with ether (100 mL), followed by two more extractions of the aqueous layer (2 x 50 mL of ether). The organic layers were combined, neutralized with a saturated sodium bicarbonate solution, washed with water and dried over anhydrous magnesium sulfate. The solvent was removed via rotary evaporation to yield the crude azo product as a red oil (468 mg, 60.6% yield). Chromatography purification on silica using ethyl acetate yielded the desired product as a red solid. ^{1}H NMR (400 MHz, d_6- acetone) δ 7.87 (d, J = 8.19 Hz, 2H), 7.57 (s, 1H), 7.50 (t, J = 7.32, 5.2 Hz, 2H), 7.40 (d, J = 6.75 Hz, 1H), 6.77 (s, 1H), 4.06 (d, J = 6.98 Hz, 1H), 3.85 (m, 1H), 3.75 (m, 1H), 3.67 (m, 1H), 3.56 (m, 1H), 3.52 (m, 2H), 2.32 (s, 3H) 2.26 (s, 3H), 1.85 (m, 2H). ESI+ (m/z) [M+H] calcd: 344.1929, found: 344.2645.

2'-Deoxyriboflavin (4). The diazotizated material **3** (468 mg, 1.36 mmol) and barbituric acid (180 mg, 1.40 mmol) were suspended in *n*-butyl alcohol (10 mL). Glacial acetic acid (2.5 mL) was then added and the mixture was heated to reflux overnight. The solvent was then removed via rotary evaporation to yield a reddish orange solid (438 mg). This solid was purified by HPLC using 0 - 50% acetonitrile in 0.1%

trifluoroacetic acid (TFA) in water over 15 min to provide a yellow solid (34 mg, 70 % yield). ^{1}H NMR (400 MHz, d_4-MeOD): δ 7.98 (s, 1H), 7.90 (s, 1H), 3.62-3.55 (m, 6H), 2.60 (s, 3H), 2.48 (s, 3H), 2.28 (m, 1H), 1.98 (m, 1H) as expected from the literature.[3] ESI+ (m/z) [M+H] calcd: 361.1467, found: 361.1508.[1]

2'-DeoxyFMN. 2'-Deoxyriboflavin (200 μM), $MgCl_2$ (10 mM), and ATP (4 mM) were combined in 100 mM potassium phosphate pH 7.4 (1 mL) and pre-incubated at 37°C for 5 min before addition of 4.42 nM riboflavin kinase.[4,5] The mixture was incubated at 37 °C for 96 h to allow full conversion of 2-deoxyriboflavin to 2'-deoxyFMN prior to its purification by HPLC using an Econosphere C18 reverse-phase semi-preparative column (250 mm×10.0 mm) to yield a yellow solid (21 mg, 90% yield). The solvent system for HPLC comprised of 0.1 M ammonium acetate pH 3.8 (A) and acetonitrile (B). A gradient of 0-25% B for 15 min and a wash (25-95% B for 5 min, 95% B for 5 min) were used at 5 mL/min. ^{1}H NMR (400 MHz, D2O): δ 7.57 (s, 1H), 7.55 (s, 1H), 3.85 (m, 1H), 3.75 (m, 1H), 3.60 (m, 1H), 3.52 (m, 1H), 2.36 (s, 3H), 2.24 (s, 3H), 2.04 (m, 2H), 1.78 (m, 1H). ESI+ (m/z) [M+H] calcd: 441.1131, found: 441.1166.



Scheme S2. Iodination of tyrosine analogs

2-Amino-3-(3-iodo-4-methoxyphenyl)propanoic acid (*O*-Me I-Tyr). L-*O*-Methyl tyrosine (100 mg, 0.5 mmol) was dissolved in a mixture of TFA (5 mL) and water (0.5 mL), cooled to 4 ºC and combined with *N*-Iodosuccinimide (126 mg, 0.52 mmol). The reaction mixture was warmed to 40 ºC for 3 h before the solvent was evaporated under vacuum. The residue was re-dissolved in water, cooled to 4 ºC and quenched with 1 M Na_2SO_3. The resulting solution was filtered and then purified by preparative HPLC using an Econosphere C18 10 μm 250×10 mm column at a flow rate of 5 mL/min. The solvent system comprised of 0.1% TFA in water (A) and acetonitrile (B). A solution of 5% B washed the column for 4 min and then a linear gradient of 5-30 % B (16 min) was used. *O*-Methyl iodotyrosine was obtained as a TFA salt (108 mg, 50%). ^{1}H NMR (400 MHz, MeOD) δ 7.72 (d, 1H, J = 2.3 Hz), 7.27 (dd, 1 H, J = 2.3 Hz, J = 8.0 Hz), 6.93 (d, 1H, J = 8.0 Hz), 4.17 (dd, 1 H, J = 5.3 Hz, J = 7.5 Hz), 3.86 (s, 3H), 3.21 (dd, 1 H, J = 5.3 Hz, J = 14.9 Hz), 3.07 (dd, 1 H, J = 14.9 Hz, J = 7.5 Hz). ^{13}C NMR (100 MHz, D_2O) δ 34.5, 53.9, 90.6, 123.8, 130.9, 133.5, 136.6, 141.1, 171.2. ESI+ (m/z) [M+H] calcd: 321.9929, found: 321.9862.

2-Amino-3-(4-amino-3-iodophenyl)propanoic acid (I-aminoPhe). 4-Amino-L-phenylalanine hydrochloride (30 mg, 0.14 mmol) was dissolved in methanol (1 mL). Glacial acetic acid (0.1 mL) was added to increase the solubility of the starting material. Iodine monochloride (7 μL, 1 eq) was added to the reaction mixture and allowed to stir at room temperature for 3 h before quenching with 1 M $Na_2S_2O_3$. Methanol was removed under reduced pressure and the remaining mixture was redissolved in water. The product was purified by preparative HPLC using an Econosphere C18 10 um 250×10 mm column with a flow rate of 5 mL/min. The solvent system comprised of 0.1% TFA in water (A) and acetonitrile (B) with a gradient of 5-25 % B over 20 min. I-AminoPhe was obtained as a TFA salt (108 mg, 50%). ^{1}H NMR (400 MHz, D_2O) δ 7.86 (m, 1H), 7.33 (m, 2H), 4.20 (dd, 1 H, J = 6.7 Hz, J = 7.0 Hz), 3.24 (dd, 1 H, J = 6.7 Hz, J = 15.3 Hz), 3.13 (dd, 1 H, J = 7.0 Hz, J = 15.3 Hz). ESI+ (m/z) [M+H] calcd: 306. 9865, found: 306.9940.

Figure S1. Substrate affinity for HsIYD. HsIYD (4.5 μM) alternatively containing FMN and 2'-deoxyFMN in sodium phosphate (100 mM, pH 7.4) and potassium chloride (200 mM) was stirred gently (25 °C) while titrating with I-Tyr. Binding of the ligand was monitored by a diagnostic quenching of FMN_{ox} fluorescence (λ_{ex} 450 nm, λ_{em} 516 nm).[6] Data represent the average of 3 independent measurements and the error represents their standard deviation. The values of K_d were calculated by non-linear regression to eq. 1 (Origin 7.0).[7]

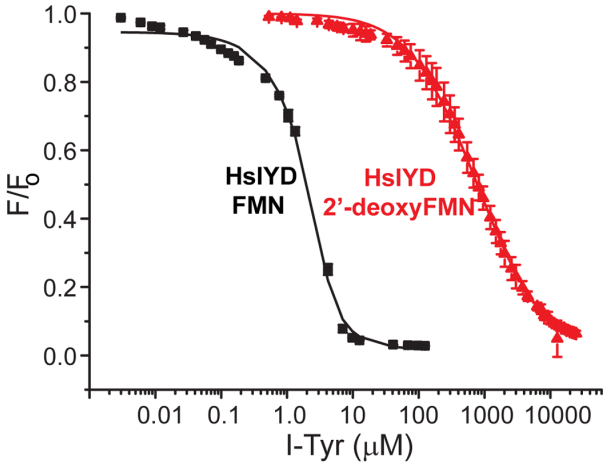
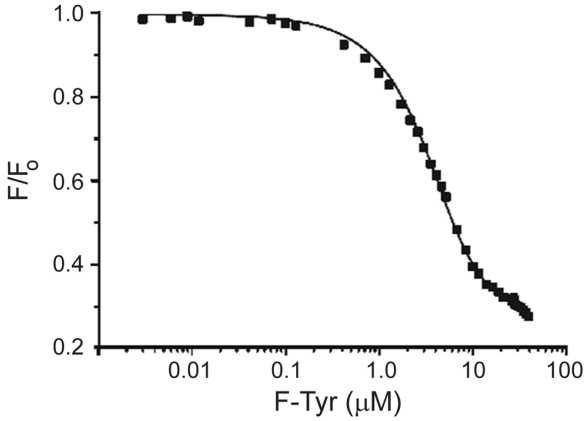


Figure S2. Affinity of an inert substrate analog for HsIYD containing 2'-deoxyFMN. Binding of F-Tyr to 2'-deoxyFMN HsIYD (4.5 μM) in 100 mM postassium phosphate pH 7.4 and 200 mM postassium chloride was monitored by the diagnostic quenching of 2'-deoxyFMN fluorescence at 25 °C using λ_{ex} of 450 nm and λ_{em} of 527 nm. The values of K_d were calculated by a non-linear regression to eq. 1 (Origin 7.0).[7]



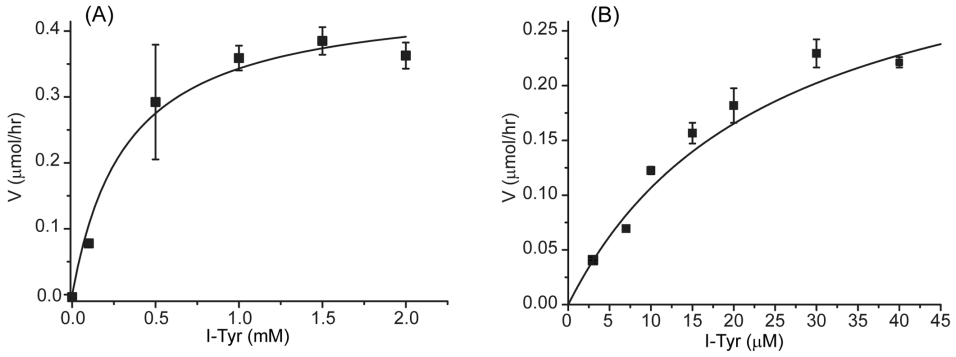


Figure S3. Catalytic deiodination after exchange of FMN for 2'-deoxyFMN. Generation of Tyr from I-Tyr by (A) 2'-deoxyFMN HsIYD and (B) 2'-deoxyFMN TnIYD was monitored by reverse-phase C-18 HPLC under standard assay conditions. Data represent an average of three independent determinations and error bars represent the standard deviation for (A). Data represent the average of two independent determinations and the error bars represent their range for (B). The kinetic parameters were determined from the best fit of data (solid lines) to Michaelis-Menten kinetics using Origin 9.

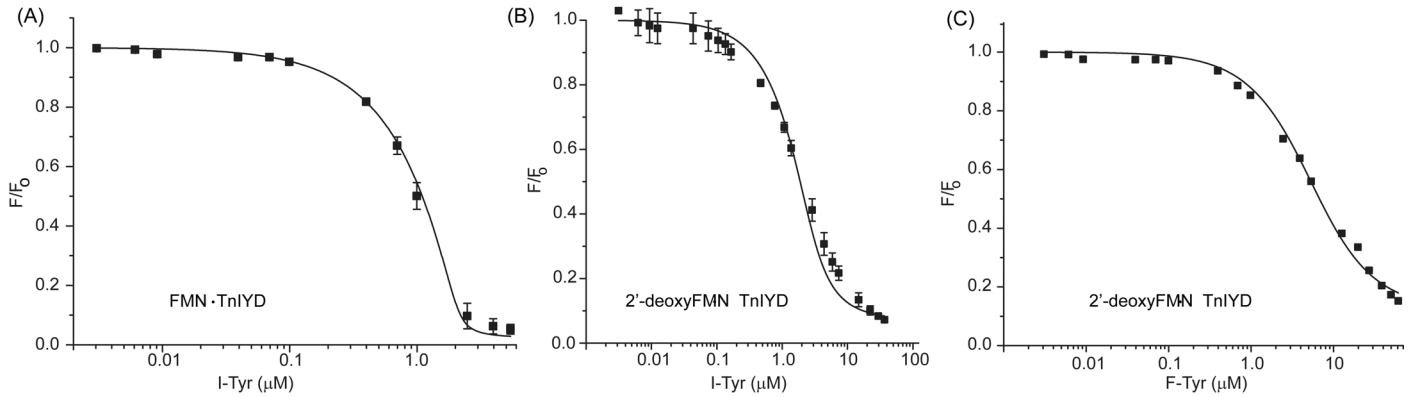


Figure S4. Affinity of a substrate and its analog for TnIYD. TnIYD (2.1 μM) reconstituted alternatively with FMN_{ox} or 2'-deoxyFMN_{ox} was titrated with (A) I-Tyr, (B) I-Tyr and (C) F-Tyr in 100 mM potassium phosphate (pH 7.4) while gently stirring at 25 °C. Binding was monitored by the diagnostic quenching of flavin fluorescence using λ_{ex} of 450 nm and λ_{em} of 516 nm.[6] Data for (A) and (B) represent the average of two independent measurements and the error represents their range. Analysis for (C) was performed once and individual measurements did not deviate from the predicted response. All K_d values were calculated by non-linear regression to eq. 1.

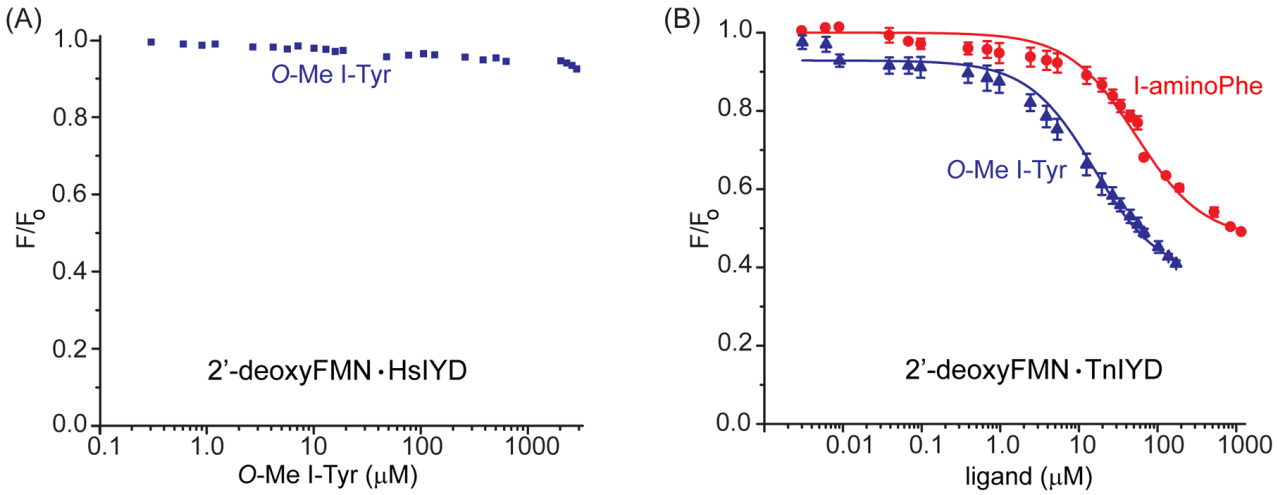


Figure S5. Affinity of substrate analogs for 2'-deoxyFMN IYD from human and *T. neapolitana*. (A) 2'-deoxyFMN HsIYD (4.5 μM) in sodium phosphate (100 mM, pH 7.4) and potassium chloride (200 mM) was titrated with *O*-Me I-Tyr while gently stirring at 25 °C. (B) 2'-deoxyFMN TnIYD (2.1 μM) in sodium phosphate (100 mM, pH 7.4) was titrated with the indicated ligands while gently stirring at 25 °C. Binding of the ligands was monitored by the diagnostic quenching of 2'-deoxyFMN_{ox} fluorescence (λ_{ex} 450 nm, λ_{em} 516 nm).[6] Data of (A) represent one set of observations and data of (B) represent the average of 2 independent measurements and the error was set by their range. The values of K_d were calculated by non-linear regression to fit eq. 1.[7]

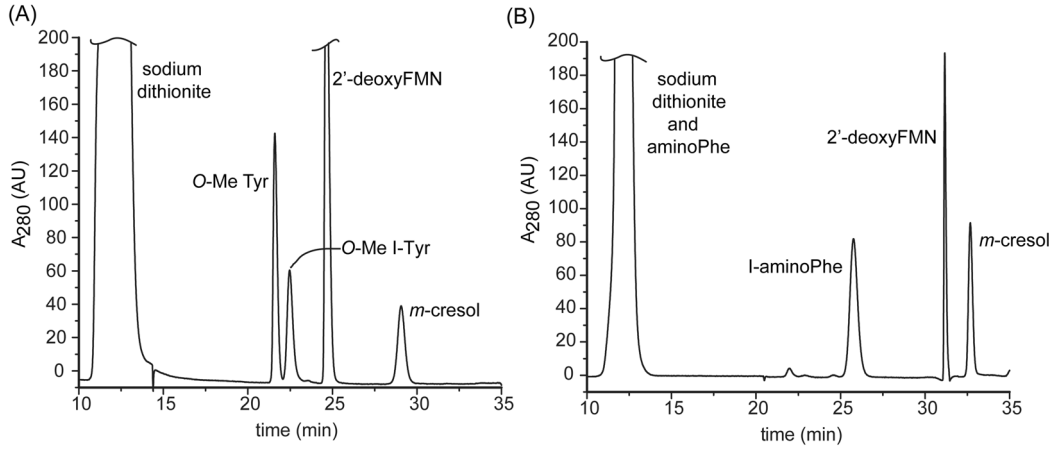


Figure S6. Chromatographic separation and detection of I-Tyr analogs and their deiodinated products. Reverse phase (C18) HPLC separation of reaction components and their detection at 280 nm was used to monitor potential deiodination of substrate analogs. (A) For the possible formation of *O*-Me Tyr, a gradient of buffer B (0.44 % formic acid in acetonitrile) in buffer A (0.44 % aq. formic acid) was used as follows: 0-5% B over 0-10 min, 5-60% B from 10-25 min and a wash of 60-95% acetonitrile from 25-30 min and then 100% acetonitrile for 10 min (1 mL/min). (B) The potential consumption of I-aminoPhe was monitored with a gradient of acetonitrile in buffer A (25 mM ammonium formate pH 6.4) as follows: 0% acetonitrile from 0-10 min, 0-10 % acetonitrile from 10-17 min, a constant 10 % acetonitrile from 17-22 min and a wash of 10-100 % acetonitrile from 22-26 min (1 mL/min).

References

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