A remarkable oxidative cascade that replaces the riboflavin C8 methyl with an amino group during roseoflavin biosynthesis

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Supporting Information

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Materials:

All chemicals were purchased from Sigma-Aldrich. A dehydrated form of LB broth was purchased from EMD Millipore. Kanamycin and IPTG were obtained from Lab Scientific Inc. Amicon Ultra centrifugal filter devices (10,000 MWCO) were obtained from Millipore. Histrap Ni NTA columns were obtained from GE Healthcare. Econo-Pack 10DG desalting columns were purchased from Bio-Rad. The baffled ultra yield flasks (2.5 L) for cell growth were obtained from Thomson Instrument Company. The ZORBAX Eclipse XDBC18 column (15 cm x 4.6 mm, 5 μm particles) was purchased from Agilent Technologies.

Over-expression and purification of the orf7989 gene product:

Orf7989 (codon optimized, Figure S1) was cloned in the pTHT vector (a derivative of pET28b vector with a TEV protease cleavage site after the N-terminal His-tag). The resulting plasmid (pIJ-1) was transformed into *Escherichia coli* BL21 (DE3). A starter culture was grown in 60 ml of LB medium containing 40 μg/ml of Kanamycin at 37 °C with overnight shaking at 220 rpm. 6 flasks each containing 1.5 L LB medium (20 g/L) and 40 μg/ml of Kanamycin, were inoculated with 10 ml of starter culture. The cells were grown at 37 °C with shaking (220 rpm) until the culture reached an OD_{600} of 0.6. The flasks were then kept at 4 °C for ~1h without shaking. IPTG was added to a final concentration of 0.5 mM to induce protein overexpression. The flasks were incubated at 15 °C with shaking (180 rpm) for another 15 hours. After that the cells were harvested by centrifugation at 10,000g for 10 min at 4 °C. Cell pellet was re-suspended in 30 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0) and allowed to thaw on ice. Subsequently, the cell pellet was sonicated for 30 second in a Misonix sonicator 3000 (pulse 'on' time 1.0 sec, pulse 'off' time 1.0 sec, output level 0.8). The process was repeated 6 times with an interval of 10 min before each cycle. The cell debris was separated by centrifugation at 15,000 rpm for 40 minutes at 4 °C. The filtered cell lysate was loaded onto a 5 mL Ni-NTA-affinity column pre-equilibrated with lysis buffer at 4 ^{o}C. The column was then washed with 50 ml wash buffer (50 mM Tris-HCl, 150 mM NaCl, 25 mM imidazole, pH 8.0). The protein was eluted from the column with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 250 mM imidazole, pH 8.0) at 4 °C. The fractions containing yellow protein were collected and concentrated using YM-10 Amicon ultracentrifugal filters at 5000 g to a final

volume of 3 mL. The concentrated sample was desalted by passing through an Econo-Pac 10DG desalting column (Bio-rad), into 50 mM Tris-HCl buffer, 100 mM NaCl and 10% glycerol, pH 7.5. Protein aliquots were frozen in liquid nitrogen and stored at -80 ^{o}C. Protein concentration was determined using the Bradford assay. A typical yield was 45 mg/ml.

ATGGCGCTGAAGGCGCTGATTCTGAACACCACCCTGCGTCGTAGCCCGAGCCGTAG
CCAAACCCAAGGTCTGATTGACAAGGCGGTTCCGCTGTACGAGAAGGAAGGCATTG
AAACCGAGGTGGTTCGTGTGATCGACCACGATATTGAGCAGGAATACTGGGACGAT
TATGACGATTGGAACGCGGGCGAGAAGGCGCGTCGTGAGGACGAATGGCCGTGGCT
GCTGGAGAAAATCCGTGAAGCGGACATCCTGGTGATTGCGACCCCGATTACCCTGA
ACATGTGCACCAGCGCGGCGCACGTTATCCTGGAGAAACTGAACCTGATGGACGAA
CTGAACGGCGATACCAAGCAATTCCCGCTGTATAACAAAGTGGCGGGTCTGCTGAT
GTGCGGCAACGAGGATGGTGCGCACCATGTGGCGGGCACCGTTCTGAACAACCTGG
GCCGTCTGGGTTACAGCGTTCCGCCGAACGCTGCGGCGTATTGGCTGGGTCCGGCGG
GCACCGGTCCGGGTTACATTGAAGGCAAGGGTGACCGTCACTTCCACACCAACAAA
CTGATCCGTTTTATGGTGGCGAACACCAGCCACCTGGCGCGTATGCTGCAGGAAACC
CCGTATACCACCGATCTGGAAGCGTGCGCGCAAGCGGCGCGTGAGGAAAGCGACGA
TGTTTTTGCGATCCGTGTGAACGTTAACACCCCGGCGATTCGCTACAAACGCTTTCA
AAAACTGGGCGAAGTGAAGGTGGAGGAGAGCCAACTGGGTTAA

Figure S1: Sequence for the codon optimized Orf7989.

Over-expression and purification of FAD synthetase:

The FAD synthetase gene, cloned in the pET24b vector with a C-terminal His-tag, was transformed into *Escherichia coli* BL21 (DE3) cell line. A 10 mL starter culture was grown at 37 ^{o}C containing 40 μg/mL kanamycin for 6 hrs. 1.5 litres of LB medium was inoculated with this starter culture. The cells were grown at 37 ^{o}C with shaking till the OD_{600} reached 0.6. The protein expression was then induced by adding IPTG (final concentration 1mM) and the culture was incubated at 15 ^{o}C for 15 hrs. The cells were then harvested by centrifugation and resuspended in 30 mL of binding buffer (50mM KPi buffer containing 150 mM NaCl, 10mM imidazole, pH 7.8). The cells were lysed by sonication followed by centrifugation at 15000 rpm for 45 mins. The supernatant containing the soluble protein was loaded on a Ni-NTA affinity column (Histrap-GE Healthcare) and was then washed with 50 mL of wash buffer (50 mM KPi

buffer containing 150 mM NaCl, 20mM imidazole, pH 7.8). The protein was eluted with the elution buffer (100 mM KPi buffer containing 100 mM NaCl, 250 mM imidazole, pH 7.8). The eluted protein fractions were pooled and concentrated using 10kDa Amicon ultracentrifugal filters to a final volume of 3 mL. The concentrated protein solution was buffer exchanged into 100 mM KPi buffer containing 150 mM NaCl and 15% glycerol, pH 7.8 using an Econo-Pac 10DG desalting column.

HPLC parameters:

An Agilent 1260 HPLC equipped with a quaternary pump was used. The system included a diode array UV-Vis detector and products were detected using absorbance at 260 nm, and 450 nm. Analysis was performed on a ZORBAX Eclipse XDB-C18 column (15 cm x 4.6 mm, 5 μm particles, Agilent Technologies).

HPLC conditions (for C18 column):

A-Water

B-10 mM ammonium acetate buffer, pH 6.6

C-Methanol

HPLC method:

0 min- 0% A 100% B, 4 min- 10% A 90% B, 10 min-25% A 60% B 15% C, 25 min-20% A 30% B 50% C, 28 min-18% A 20% B, 62% C, 30 min-15% A 10% B 75% C, 32 min- 25% A, 75% B, 35 min- 0% A 100% B, 40 min- 0% A 100% B.

LC-MS parameters:

LC-ESI-TOF-MS was performed using an Agilent 1260 HPLC system equipped with a binary pump and a 1200 series diode array detector followed by a MicroToF-Q II mass spectrometer (Bruker Daltonics) using an ESI source either in negative or positive mode. Analysis was performed on an LC-18-T column (15 cm x 3 mm, 3 μm particles, Supelco).

LC conditions:

A-5 mM Ammonium acetate buffer, pH 6.6

B-75% Methanol and 25 % Water.

LC method: (for positive and negative mode on MS)

0 min-100% A, 4 min-100% A, 10 min- 80% A 20% B, 29 min-35% A 65% B, 31 min-20% A, 80% B, 33 min-100% B, 35 min- 100% A, 40min- 100% A.

Enzymatic assay conditions for AFMN synthase using FMN as the substrate:

The enzymatic assay was performed in 100 mM phosphate buffer, pH 7.5 containing AFMN synthase (150 μM) and FMN (500 μM). The enzymatic reaction mixture was incubated at 30 ^{o}C for 6 hr. The protein was heat-denatured and the remaining solution was filtered (10kda cut-off). Then the filtered solution was treated with 25 mM of pentafluorobenzyl hydroxylamine (PFBHA) either before or after the treatment with Calf Intestinal Phosphatase (CIP). The derivatization reaction was carried out at 60 ^{o}C for 3 hrs. Finally the samples were analyzed by HPLC (C-18 column) and LC-MS for the formyl-FMN/riboflavin PFBHA oxime (Figure S2).

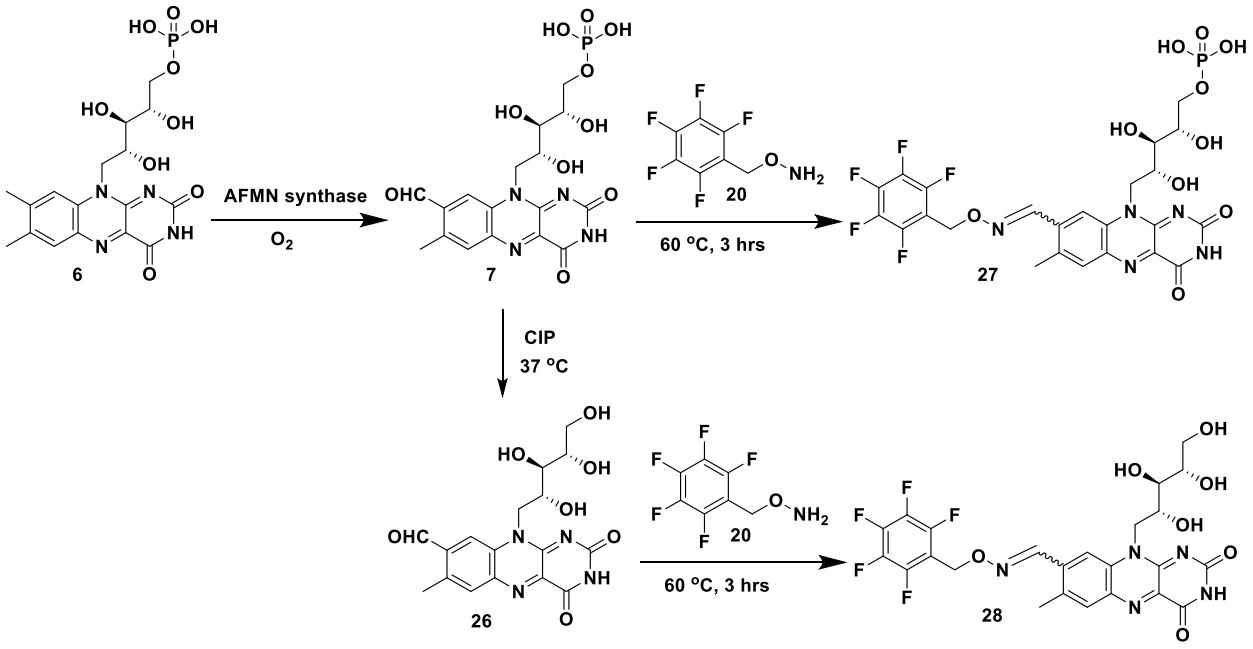


Figure S2: Enzymatic formation of formyl-FMN and formyl-riboflavin after CIP treatment and derivatization of the products as PFBHA oximes.

Enzymatic assay conditions for AFMN synthase using FMN and glutamic acid as the substrates:

The full enzymatic assay was performed in 100 mM phosphate buffer, pH 7.5 containing AFMN synthase (150 μM), FMN (500 μM) and glutamic acid (100 mM). The enzymatic reaction mixture was incubated at 30 ^{o}C for 6 hr. The protein was heat-denatured and the remaining solution was filtered (10kda cut-off) and reacted with pentafluorobenzyl hydroxylamine (25 mM) at 60 ^{o}C for 3 hrs. This derivatization was essential to achieve separation of unreacted formyl-FMN from amino-FMN under our HPLC conditions. Finally the samples were analyzed by HPLC (C-18 column) for the formation amino-FMN (Figure S3).

Another aliquot of the reaction mixture was analyzed by LC-MS before and after the treatment with CIP for the formation of amino-FMN and amino-riboflavin.

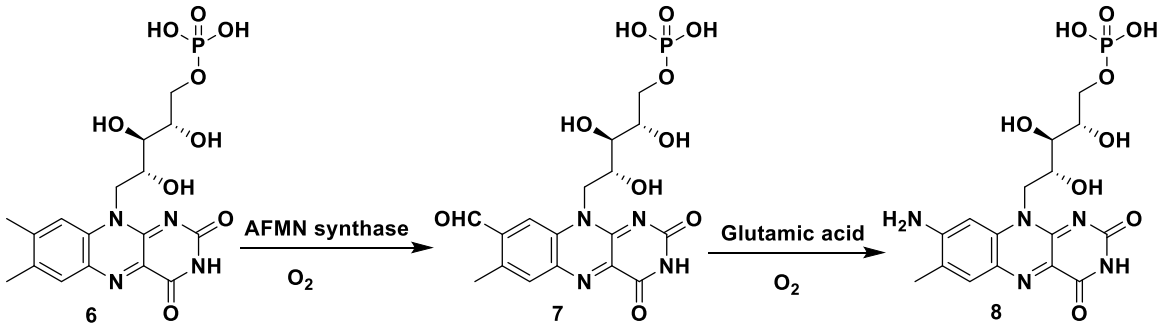


Figure S3: AFMN synthase catalyzed formation of amino-FMN from FMN and glutamate.

Enzymatic assay conditions for AFMN synthase using FMN and ^{15}N-glutamic acid as the substrates:

The full enzymatic assay was performed in 100 mM phosphate buffer, pH 7.5 containing AFMN synthase (150 μM), FMN (500 μM) and ^{15}N-glutamic acid (100 mM). The enzymatic reaction mixture was incubated at 30 ^{o}C for 6 hr. The protein was heat-denatured and the remaining solution was filtered (10kda cut-off) and reaction mixture was analyzed by LCMS (Figure S4).

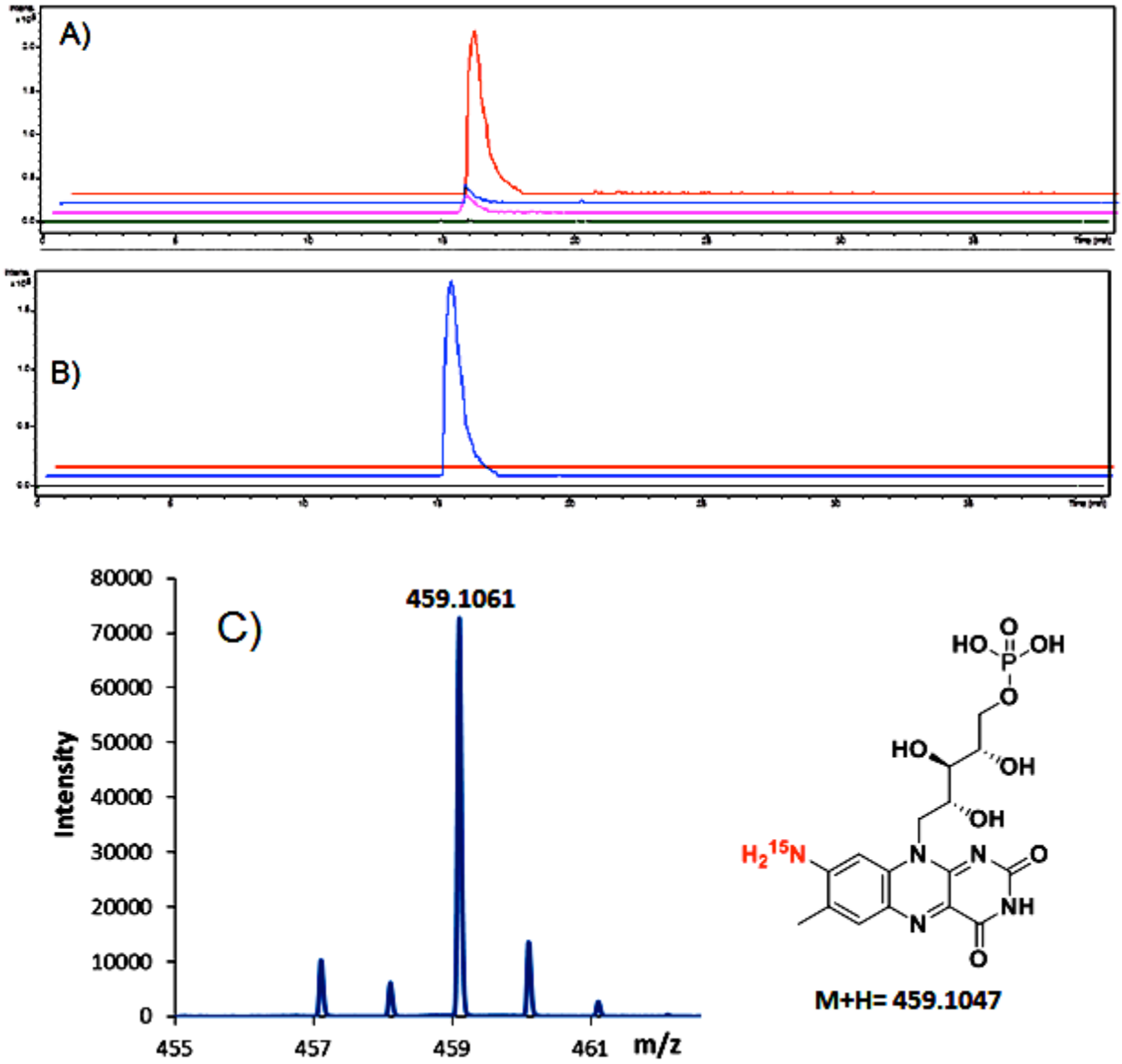


Figure S4: . LC-MS analysis of the AFMN synthase-catalyzed reaction in presence of ^{15}N-glutamate. A) EIC at m/z 458.1077 for the reaction mixture having unlabeled glutamate (red trace), having ^{15}N- glutamate (blue trace), lacking FMN (pink trace) and lacking AFMN synthase (green trace). B) EIC at m/z 459.1047 for the reaction mixture containing ^{15}N-glutamate (blue trace), unlabeled glutamate (red trace) and lacking AFMN synthase (green trace) C) ESI-MS of the 459.1047 species indicating the presence of ^{15}N-Amino-FMN as the product when ^{15}N-labeled glutamate is used.

Oxygen consumption during formyl-FMN formation:

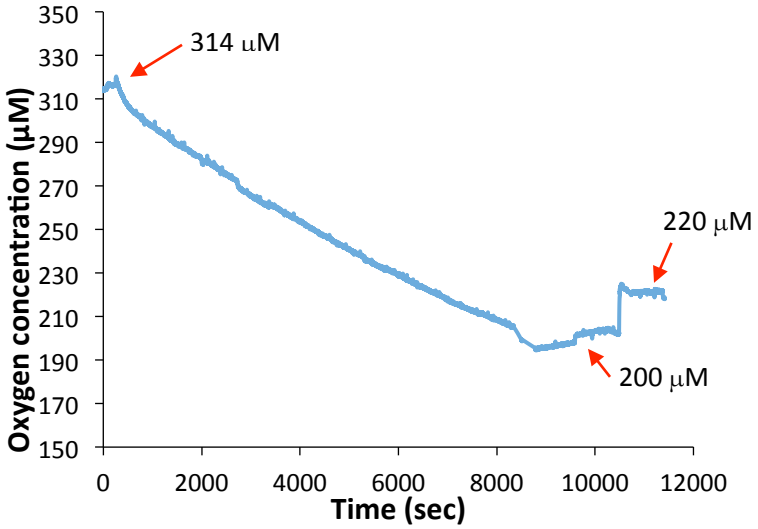


Figure S5: Measurement of variation in oxygen concentration for 8-formyl-FMN formation in the AFMN synthase reaction. 114 μM oxygen was consumed for 100 μM FMN under single turn-over condition indicating only 1 equivalent of oxygen is required to convert FMN to 8-formyl-FMN.

Hansatech Oxygen Electrode Instrument was used to measure the oxygen consumption during the AFMN synthase catalyzed reaction. The electrode consisted of Platinum as cathode and Silver as anode with saturated KCl as the electrolyte. The initial solution contained 100 μM FMN in a total volume of 450 μL. At this point the system was sealed. The starting concentration of oxygen was 314 μM. Now, aerobic enzyme (final concentration is 200 μM) was added to this solution using a syringe to have a final volume of 550 μL and then the change in oxygen concentration was monitored. The result showed a steady decrease in oxygen concentration indicating consumption of oxygen in the reaction of AFMN synthase with FMN. The signal stabilized at 200 μM indicating completion of the reaction. At this point 50 μL catalase was added to check if any H_2O_2 was produced in the reaction. The graph after adding catalase showed only a stoichiometric increase in oxygen concentration due to dilution with aerobic buffer but, no increase due to H_2O_2 production. For the experiment shown in figure S6 the catalase was added initially in the system and similar results to those shown in figure S5 were

obtained.. The reaction mixture was analyzed and confirmed for 8-formyl-FMN formation by LCMS.

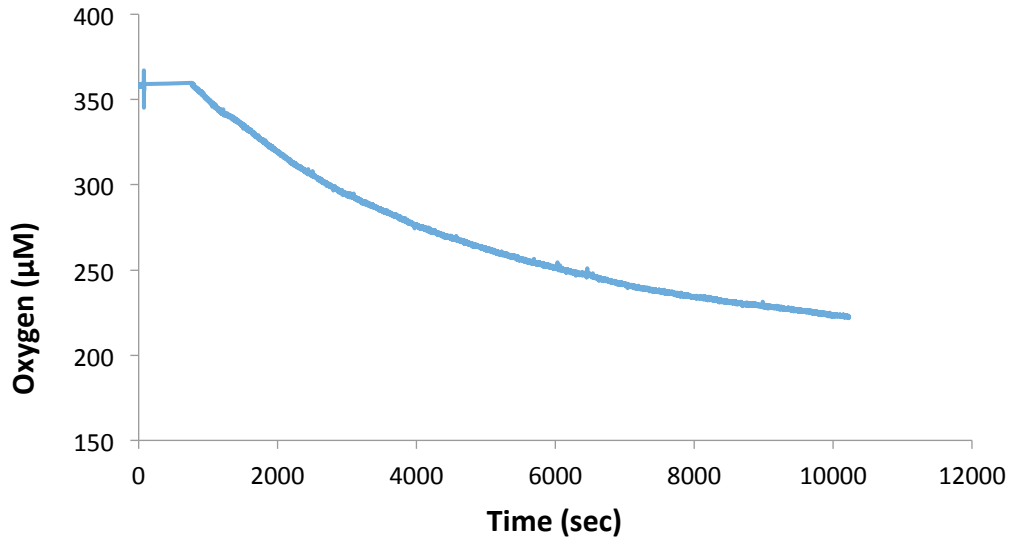


Figure S6: Measurement of oxygen consumption during the AFMN synthase catalyzed formation of 8-formyl-FMN in presence of catalase. 136 µM oxygen was consumed in the oxidation of 100 µM FMN under single turn-over conditions. This result is consistent with a 4-electron reduction of oxygen to water rather than a 2-electron reduction to hydrogen peroxide.

H/D Exchange Experiment:

100 µL aliquot of the enzyme (~2 mM) was buffer exchanged in D_2O buffer using a *Bio-Rad* Spin Column inside the glove box. All the substrate solutions were prepared in D_2O buffer. Enzyme (final concentration 100 uM) and substrates (500 uM FMN and 100 mM glutamic acid) were incubated inside the glove box for 2 hr and the reaction mixture was then analyzed by LCMS (Figure S7).

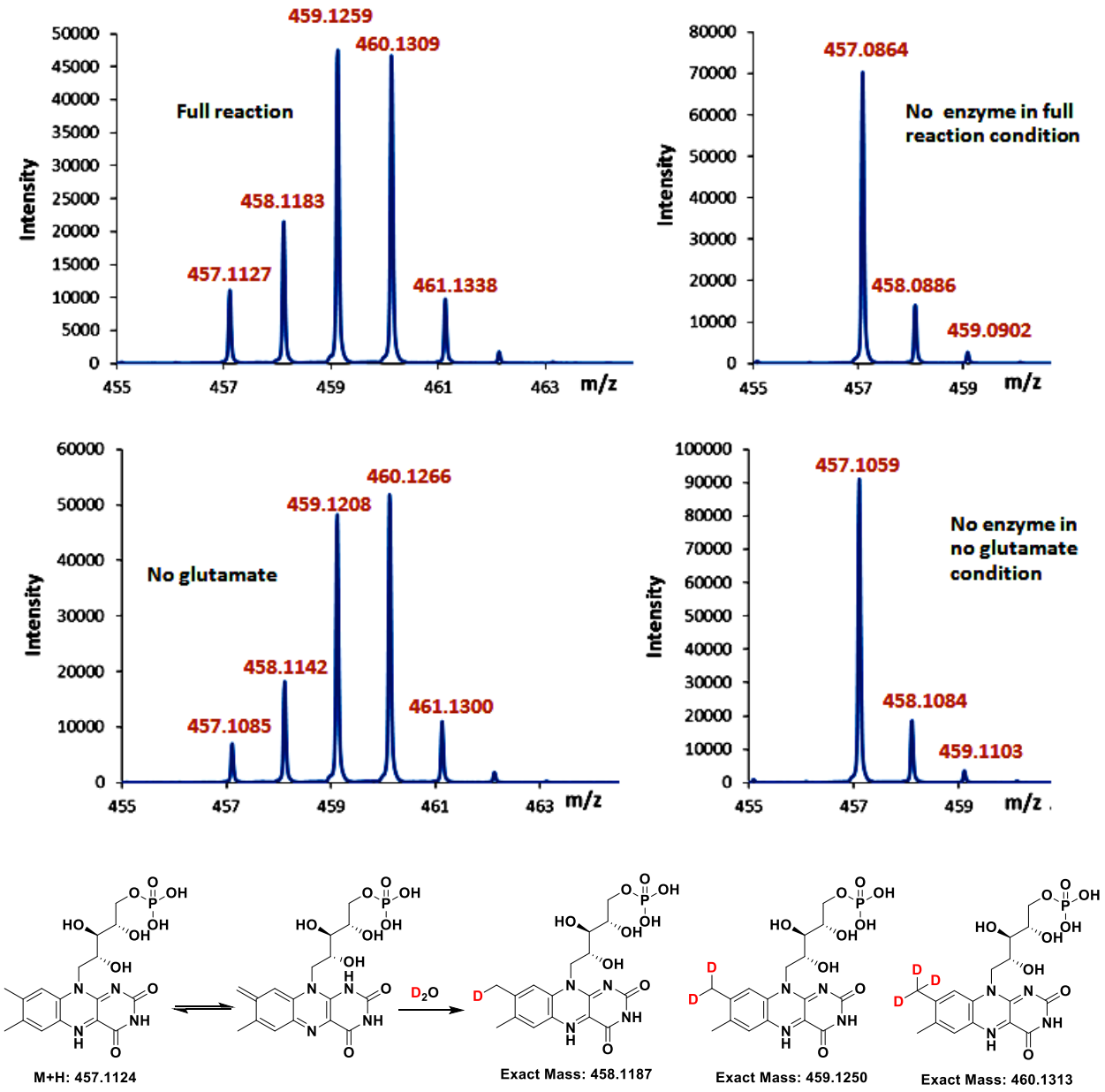


Figure S7: ESI-MS of the unreacted FMN substrate indicating partial exchange of the 8-methyl protons with deuterium.

Synthesis of model compounds:

1) Lumiflavin synthesis:

Lumiflavin was synthesized via following route (Figure S8).

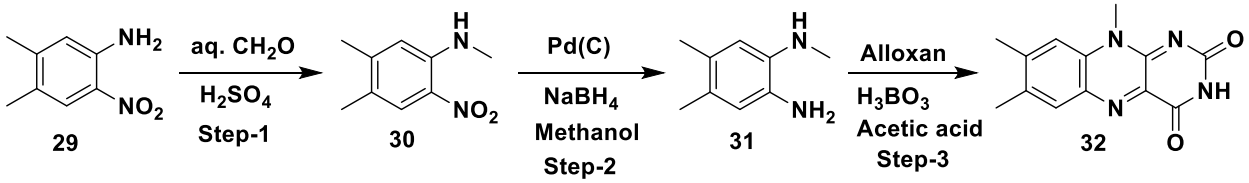


Figure S8: Scheme for synthesis of lumiflavin.

Procedure:

Step-1: Aqueous formaldehyde (37%; 20 ml) was added dropwise over 3 h to a stirred mixture of 4,5-dimethyl-2-nitroaniline (Compound **29**) (2 g, 0.01 mol) and H_2SO_4 (conc.; 20 mL). The reaction mixture was heated at 65 ^oC for 3 h, then cooled to room temperature and poured into ice water (400 mL). The solid was collected by filtration and dissolved in ethyl acetate. The solution was washed with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, filtered, and evaporated to give compound **30** (yield 90%) as a red-orange solid. This compound was used in the next step without further purification.[2] ^1H NMR (400 MHz, $CDCl_3$): δ 7.925 (s, 1H), 6.611 (s, 1H), 3.00 (d, 3H), 2.283 (s, 3H), 2.182(s, 3H).

Step-2: Catalytic reduction of compound **30** was carried out with Pd/C (10% Pd/C, 4% Pd w/w) and $NaBH_4$ (3 eqv.) in MeOH at r.t. under argon. After 40 mins the reaction mixture was filtered through celite using methanol as eluent. The solvent was evaporated to give the product (Yield >90%) which is used for the next step without further purification.[3]

Step-3: Compound **31** (1.3mmol), alloxan monohydrate (3 eqv.) and H_3BO_3 (2 eqv.) in 20 ml acetic acid were stirred under argon for 12 hrs. The fluorescent yellow precipitate obtained was collected by filtration and washed with acetic acid (30 ml) followed by ether to get the final product lumiflavin (Yield ~40%). [2] ^1H NMR (400 MHz, CD_3OD): δ 7.146 (s, 1H), 6.6952 (s, 1H), 3.317 (s, 3H), 1.769 (s, 3H), 1.656(s, 3H).

2) Formyl lumiflavin Synthesis:

Lumiflavin (Compound **32**) was converted to formyl-lumiflavin as shown in Figure S9. Lumiflavin (2 g, 7.8 mmol) was dissolved in 8 ml conc. sulfuric acid and 1.2 ml (23.4 mmol) of bromine was added. The reaction mixture was stirred at 50 ^{o}C for 4-5 days; the course of the reaction was followed by thin-layer chromatography (an aliquot was taken, diluted with water. TLC was developed using 5% MeOH in $CHCl_3$). Tetrahydrofuran (8 ml) was added and the resulting deep-red solution was added dropwise, with stirring, to 200 ml of ice-water. The suspension was allowed to stand at 60 ^{o}C overnight to hydrolyze the dibromo intermediate (Compound **33**). The resulting precipitate was filtered off, washed with water, ethanol and ether and dried in vacuo to give pure product (50% yield). [4] ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.42 (s, 1H), 10.38 (s, 1H), 8.27 (s, 1H), 8.04 (s, 1H), 3.98(s, 3H), 2.72(s, 3H). ^{13}C NMR (100 MHz, CD3OD): δ 192.95, 159.52, 155.52, 150.95, 141.04, 137.63, 136.57, 136.39, 133.68, 131.59, 119.98, 31.97, 18.39.

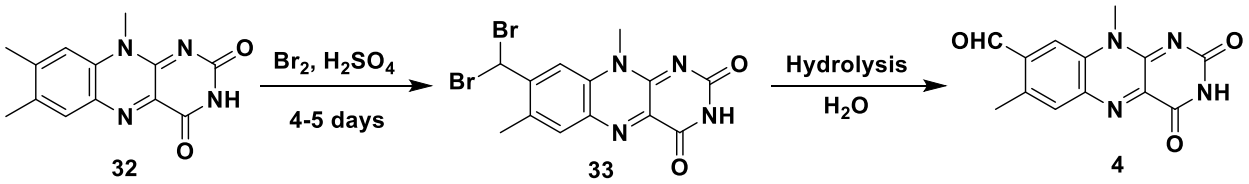


Figure S9: Synthesis of formyl-lumiflavin from lumiflavin.

Treatment of formyl-lumiflavin with ammonia:

Formyl-lumiflavin was treated with methanolic ammonia (7M) in a sealed tube at 40 ^{o}C for 12 hr (Figure S10). The solvent was then removed and the resulting crude product was acetylated by reacting with acetic anhydride and acetic acid to facilitate chromatographic purification (10% methanol in chloroform). The resulting product (30 % yield) was characterized by LCMS and ^{1}H NMR. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.62 (s, 1H), 8.38 (s, 1H), 7.95 (s, 1H), 3.88 (s, 3H), 2.42(s, 3H), 2.22(s, 3H). ^{13}C NMR (100 MHz, CD3OD): δ 169.91, 160.14, 155.68, 143.53, 136.23, 132.60, 132.49, 131.82, 128.92, 107.44, 31.83, 24.22, 17.47.

An analytical sample of amino-lumiflavin was purified by HPLC (C-18 column) from the crude reaction product.

HPLC condition for purification of amino-lumiflavin:

A-Water

B-100 potassium phosphate buffer, pH 6.6

C-Methanol

HPLC method:

0 min- 0% A 100% B, 4 min- 10% A 90% B, 7 min-25% A 60% B 15% C, 16 min-15% A 10% B 75% C, 19 min- 25% A, 75% B, 25 min- 0% A 100% B, 30 min- 0% A 100% B.

Fraction collection method:

Peak eluted at 14.4 to 14.65 min was collected for 20 runs. All the fractions were lyophilized and re-dissolved in methanol thus removing the phosphate salts. The resulting solution in methanol was analyzed by LC-MS using the same conditions as mentioned above for amino-lumiflavin.

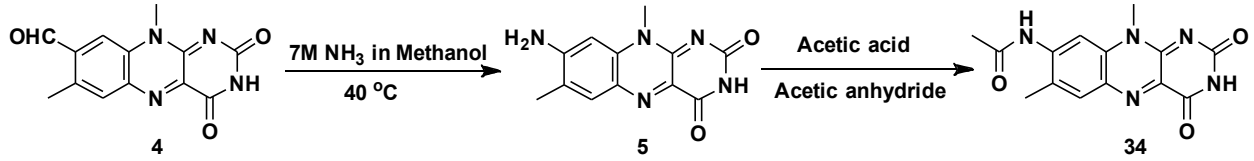


Figure S10: Formation of amino-lumiflavin from formyl-lumiflavin by treatment of methanolic ammonia and protection of the amino group by acetylation.

Synthesis of Formyl-riboflavin and formyl-riboflavin PFBHA oxime:

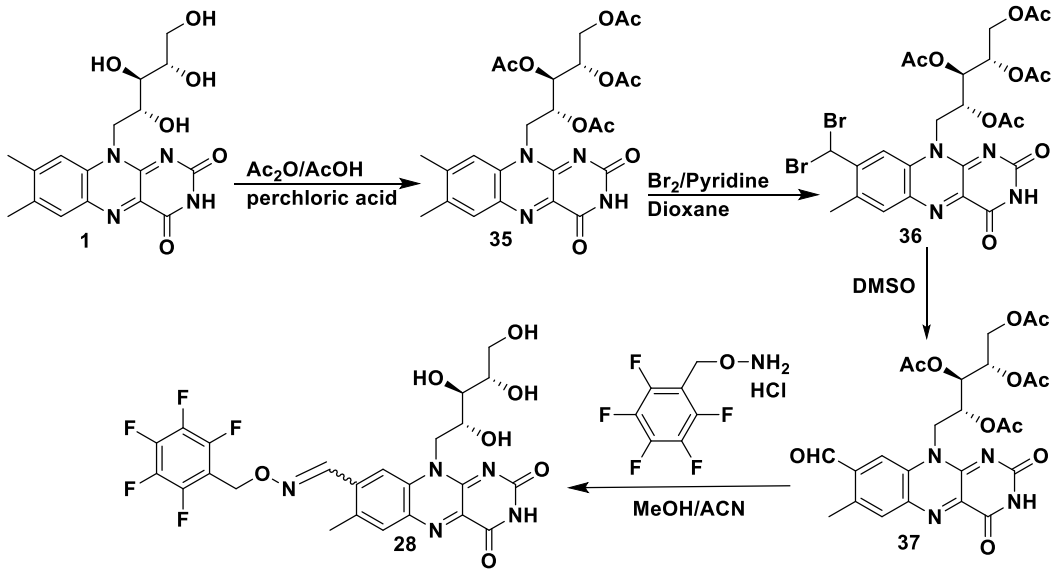


Figure S11: Scheme for the synthesis of 8-Formylriboflavin & derivatization of the product as an oxime.[5,6]

Procedure

2',3',4',5'-Tetraacetylriboflavin (Compound 35):

Riboflavin (0.5 g) was suspended in 40 mL of glacial acetic acid-acetic anhydride (1:1). 0.1 mL of 70 % perchloric acid was added dropwise and the reaction mixture was stirred for 1 h at 40 °C under an argon atmosphere. After cooling in an ice bath, and diluting with an equal volume of cold water, the product was extracted twice with 40 mL of dichloromethane and the combined organic phase was washed twice with 50 mL portions of water and sodium bicarbonate solution, followed by drying over anhydrous magnesium sulfate. After filtration, the solvent was removed under reduced pressure to produce 0.7 g (Yield ~95%) of the tetraacetylriboflavin.[5,6] 1H NMR (400 MHz, CDCl3): δ 8.48 (s, 1H) 8.03 (s, 1H), 7.58 (s, 1H), 5.66 (m, 1H), 5.44 (m, 2H), 5.12 (bm, 2H), 4.29 (dd, 2H), 2.57(s, 3H), 2.44(s, 3H), 2.28 (s, 3H), 2.22 (s, 3H), 2.08 (s, 3H), 1.67 (s, 3H).

2',3',4',5'-Tetraacetyl-8-ω-dibromoriboflavin (Compound 36):

Tetraacetylriboflavin (0.7 g) was dissolved in warm dioxane (10 mL) and dry pyridine (0.5 mL) was added. Bromine (0.5 mL) was added to this mixture, followed by 5 h of refluxing. The mixture was concentrated to dryness in vacuo. The oily residue was re-dissolved in dichloromethane (50 mL), shaken briefly with an equal volume of 0.5 N hydrochloric acid, and the organic phase was washed twice with 50 mL portions of water. The dichloromethane solution was dried over anhydrous magnesium sulfate, the volume was reduced to about 10 mL by evaporation under reduced pressure to give crude product which was further purified by silica-gel column chromatography using ethyl acetate to produce 0.5 g (Yield~50%) of the tetraacetyldibromoriboflavin. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 1H) 8.30 (s, 1H), 8.07 (s, 1H), 7.00 (s, 1H), 5.8.0 (m, 1H), 5.64 (m, 1H), 5.44 (m, 1H), 5.12 (bm, 2H), 4.4.3 (dd, 2H), 2.62 (s, 3H), 2.32 (s, 3H), 2.12 (s, 3H), 2.08 (s, 3H), 1.56 (s, 3H).

2',3',4',5'-Tetraacetyl-8-formylriboflavin (Compound 37):

Tetraacetyl-8-ω-dibromoriboflavin (0.2 g) was dissolve in Dimethyl sulfoxide (5 mL) and the mixture was stirred overnight at room temperature in the dark. DMSO was removed

under reduced pressure to give 100 mg of almost pure product which is further purified by silica-gel column chromatography using ethyl acetate as eluent (Yield ~63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.55 (s, 1H), 8.90 (s, 1H) 8.25 (s, 1H), 8.19 (s, 1H), 5.70 (m, 1H), 5.54 (m, 1H), 5.40 (m, 1H), 5.12 (bm, 2H), 4.40 (dd, 2H), 2.85 (s, 3H), 2.35 (s, 3H), 2.33 (s, 3H), 2.08 (s, 3H), 1.71 (s, 3H).

Derivatization of 8-Formyl riboflavin with Pentafluorobenzyl hydroxyl amine (Compound 28):

Tetraacetyl-8-Formylriboflavin (50 mg, 0.09 mmol) was dissolve in 5 mL of acetonitrile-methanol (1:1). O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (35 mg, 0.15 mmol) was added and the mixture was stirred overnight at 80 ^{o}C in the dark. The oxime precipitated and was removed from the reaction mixture by filtration, followed by washing with methanol and drying to give pure product (Yield ~63%). ^{1}H NMR (400 MHz, DMSO-*d6*): δ 11.40 (s, 1H), 8.60 (s, 1H), 8.19 (s, 1H), 7.98 (s, 1H), 5.38 (bs, 2H), 4.66 (m, 1H), 4.24 (m, 1H), 3.73 (m, 1H), 3.65 (m, 2H), 3.52 (m, 2H), 2. 25 (s, 3H) ppm. ^{13}C NMR (400 MHz, DMSO-*d6*): δ 160.10, 155.87, 151.47, 150.76, 150.65,146.94, 144.47, 142.65, 139.32, 138.65, 136.19, 136.01, 135.67, 134.96, 133.14, 132.48, 118.59, 111.41, 79.40, 74.03, 73.20, 69.15, 63.82, 63.38, 49.05, 47.85, 21.24, 20.53, 20.53 ppm.

Synthesis of 8-formyl flavin mononucleotide (Formyl-FMN, Compound 7):

2',3',4',5'-Tetraacetyl-8-formyl-riboflavin was first converted to 8-formylriboflavin by hydrolysis of the acetyl groups by heating at 100 ^{o}C in 1 N HCl for ~15 mins. The reaction mixture was then neutralized with an equivalent amount of 10 N NaOH solution. This neutral mixture was immediately buffered with 20 mM NaOAc (pH~ 5.5) so that the pH of the final solution becomes ~5.5.[7] This solution containing 8-formyl-riboflavin was phosphorylated to produce 8-formyl-FMN using riboflavin kinase (RibK) from *Microbacterium maritypicum*. 8-formyl riboflavin was incubated overnight at 37 ^{o}C with 10mM ATP, 10 mM $MgCl_2$ and RibK in 100 mM potassium phosphate buffer, pH=7.5. The enzyme was heat denatured and the reaction mixture was passed through a 10 kDa cut-off filter and purified by reverse phase high performance liquid chromatography. The peak eluting at ~8-9 min was collected and lyophilized to give a synthesized standard of 8-formyl-FMN which was used for all the co-elution experiment.

HPLC conditions for the purification of 8-Formyl-FMN:

Analysis was performed on a ZORBAX Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 µm particles, Agilent Technologies). The following gradient, at a flow rate of 1 mL/min, was used. Solvent A is water; solvent B is 10 mM ammonium acetate pH 6.6; solvent C is methanol. 0 min: 90% A, 10% B; 2 min: 90% A, 10% B; 17 min: 15% A, 10% B, 75% C; 23 min: 15% A, 10% B, 75% C; 25 min: 90% A, 10% B; 30 min: 90% A, 10% B.

Synthesis of 8-Amino-riboflavin and 8-Amino-FMN:

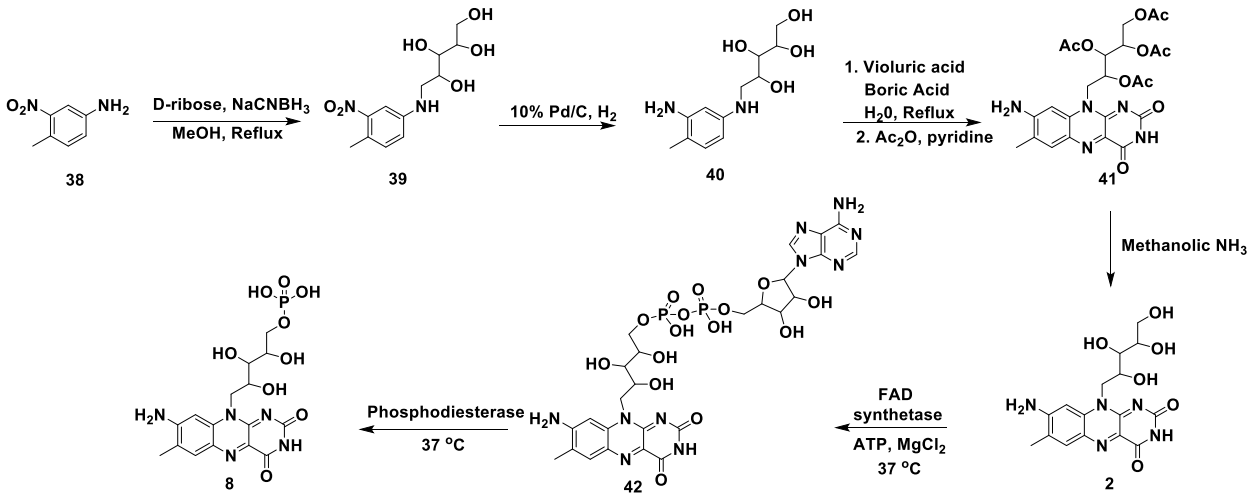


Figure S12: Scheme for synthesis of 8-Amino-FMN.[8]

Synthesis of 5-((4-Methyl-3-nitrophenyl)amino)pentane-1,2,3,4-tetraol (Compound 39):

Methyl-3-nitrophenylamine (Compound **38**) (152 mg, 1.0 equiv.), D-ribose (450 mg, 3.0 equiv.) and sodium cyanoborohydride (126 mg, 3.0 equiv.) were dissolved in anhydrous MeOH (15 mL). The mixture was refluxed at 80 ^{o}C for 2 days under an argon atmosphere. Then the solvent was removed under reduced pressure and excess $NaCNBH_3$ was quenched using 1M HCl. The resulting mixture was neutralized using saturated $NaHCO_3$ solution and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using chloroform/methanol (98:2 to 80:20). Yield: 50%, LC-MS *m/z* 285.1 (M-H). ^{1}H NMR (400 MHz, CD_3OD): □ 2.36 (s, 3H), 3.15-3.21 (m, 1H), 3.45 (dd, 1H), 3.61-3.67 (m, 2H), 3.72-3.80 (m, 2H), 3.89-3.94 (m, 1H), 6.87 (dd, 1H), 7.08 (d, 1H), 7.21 (d, 1H) ppm. ^{13}C NMR (100 MHz, CD_3OD): □ 19.2, 47.1, 64.4, 72.1, 74.1, 74.4, 108.3, 119.0, 121.2, 133.9, 149.3, 151.0 ppm.

Synthesis of 5-((3-Amino-4-methylphenyl)amino)pentane-1,2,3,4-tetraol (Compound 40):

Compound **39** (100 mg) was dissolved in 50 mL MeOH and passed through a Thalesnano H-cube hydrogenator with 10 % Pd/C catalyst at 40 ^{o}C and 1 bar hydrogen gas pressure at a flow rate of 1.0 mL/min. The solution was then concentrated under reduced pressure and the resulting compound **40** was used for the next coupling step without further purification. Yield: 90%, LC-MS *m/z* 255.1 (M-H). ^{1}H NMR (400 MHz, CD_3OD): □ 2.02 (s, 3H), 3.39 (dd, 1H), 3.44-3.51 (m, 1H), 3.61-3.68 (m, 2H), 3.74-3.80 (m, 2H), 3.88-3.92 (m, 1H), 6.09 (dd, 1H), 6.16 (d, 1H), 6.75 (d, 1H) ppm. ^{13}C NMR (100 MHz, CD_3OD): □ 16.5, 48.1, 64.4, 72.1, 74.1, 74.8, 102.7, 106.2, 114.1, 131.7, 146.6, 148.9 ppm.

Synthesis of 8-Amino-riboflavin tetraacetate (Compound 41):

To a solution of **40** in 15 mL of water, violuric acid monohydrate (175 mg) and boric acid (62 mg) was added. The mixture was refluxed at 105 ^{o}C for 12 hours and was then concentrated under reduced pressure. Crude product was dissolved in 8 mL of pyridine. 1 mL of acetic anhydride was added and the mixture and was stirred at RT for 6 hrs. The solvent was then removed under reduced pressure with a toluene azeotrope. The residue was purified by silica gel column chromatography using chloroform/methanol (95:5 to 80:20). LC-MS *m/z* 544.2 (M-H). ^{1}H NMR (400 MHz, CD_3OD): □ 1.98 (s, 3H), 2.01 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.28 (s, 3H), 4.24 (dd, 2H), 4.48 (dd, 1H), 5.37-5.41 (m, 2H), 5.48 (t, 1H), 5.63-5.67 (m, 1H), 6.81 (s, 1H), 7.61 (s, 1H) ppm. ^{13}C NMR (100 MHz, CD_3OD): □ 17.1, 20.4, 20.6, 20.7, 20.9, 45.9, 62.9, 70.4, 71.0, 72.0, 96.0, 127.8, 127.9, 132.9, 134.8, 137.4, 152.4, 158.3, 158.7, 163.4, 171.4, 171.6, 171.9, 172.4 ppm.

Synthesis of 8-Amino-riboflavin (Compound 2):

Compound **41** was converted to compound **2** by overnight stirring in ammonia (7N in methanol). LC-MS m/z 376.1 (M-H). 1H NMR (400 MHz, DMSO-*d6*): □ 2.23 (s, 3H), 3.53-3.56 (m, 1H), 3.64-3.66 (m, 2H), 4.24 (m,1H), 4.45-4.51 (m, 2H), 4.75 (t, 1H), 6.97 (s, 1H), 7.15 (s, 2H), 7.64 (s, 1H), 10.88 (s, 1H) ppm.

Synthesis of 8-Amino-flavin adenine dinucleotide (8-amino-FAD, Compound 42):

Compound **2** was then converted to 8-amino-FAD, compound **42**, enzymatically using FAD synthetase from *Corynebacterium ammoniagenes*. 8-NH_2 riboflavin was incubated overnight at 37 ^oC with 5 mM ATP, 20 mM $MgCl_2$ and FAD synthetase in 100 mM potassium phosphate buffer, pH=7.5[9]. The reaction mixture was passed through a 10kDa cut-off filter and purified by reverse phase high performance liquid chromatography. LC-MS *m/z* 785.1 (M-H).

Synthesis of 8-Amino-flavin mononucleotide (8-amino-FMN, Compound 8):

8-amino-FAD **42** purified by HPLC was incubated overnight at 37 ^oC with 8 mg of phosphodiesterase I from *Crotalus atrox* (Western Diamondback Rattlesnake) in 10 mL of 100 mM potassium phosphate buffer, pH=7.5. The reaction mixture was passed through a 10kDa cut-off filter and then purified by reverse phase high performance liquid chromatography to yield 8-amino-FMN **8.** LC-MS *m/z* 456.1 (M-H). ^1H NMR (400 MHz, D_2O): □ 2.18 (s, 3H), 3.91-3.94 (m, 1H), 3.99-4.03 (m, 2H), 4.05-4.10 (m, 1H), 4.29-4.31 (m, 1H), 4.40 (d, 1H), 4.75-4.82 (m, 1H), 6.66 (s, 1H), 7.34 (s, 1H). ^{13}C NMR (100 MHz, D_2O): □ 16.4, 47.2, 66.1, 69.1, 71.2, 71.3, 72.8, 95.1, 124.3, 128.9, 132.6, 132.7, 136.2, 150.4, 157.3, 158.1, 162.5 ppm.

HPLC conditions for purification of 8-amino-FAD and 8-amino-FMN:

Analysis was performed on a ZORBAX Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 μm particles, Agilent Technologies). The following gradient, at a flow rate of 1 mL/min, was used. Solvent A is water; solvent B is 10 mM ammonium acetate pH 6.6; solvent C is methanol. 0 min: 90% A, 10% B; 2 min: 90% A, 10% B; 17 min: 15% A, 10% B, 75% C; 23 min: 15% A, 10% B, 75% C; 25 min: 90% A, 10% B; 30 min: 90% A, 10% B.

NMR and LCMS data:

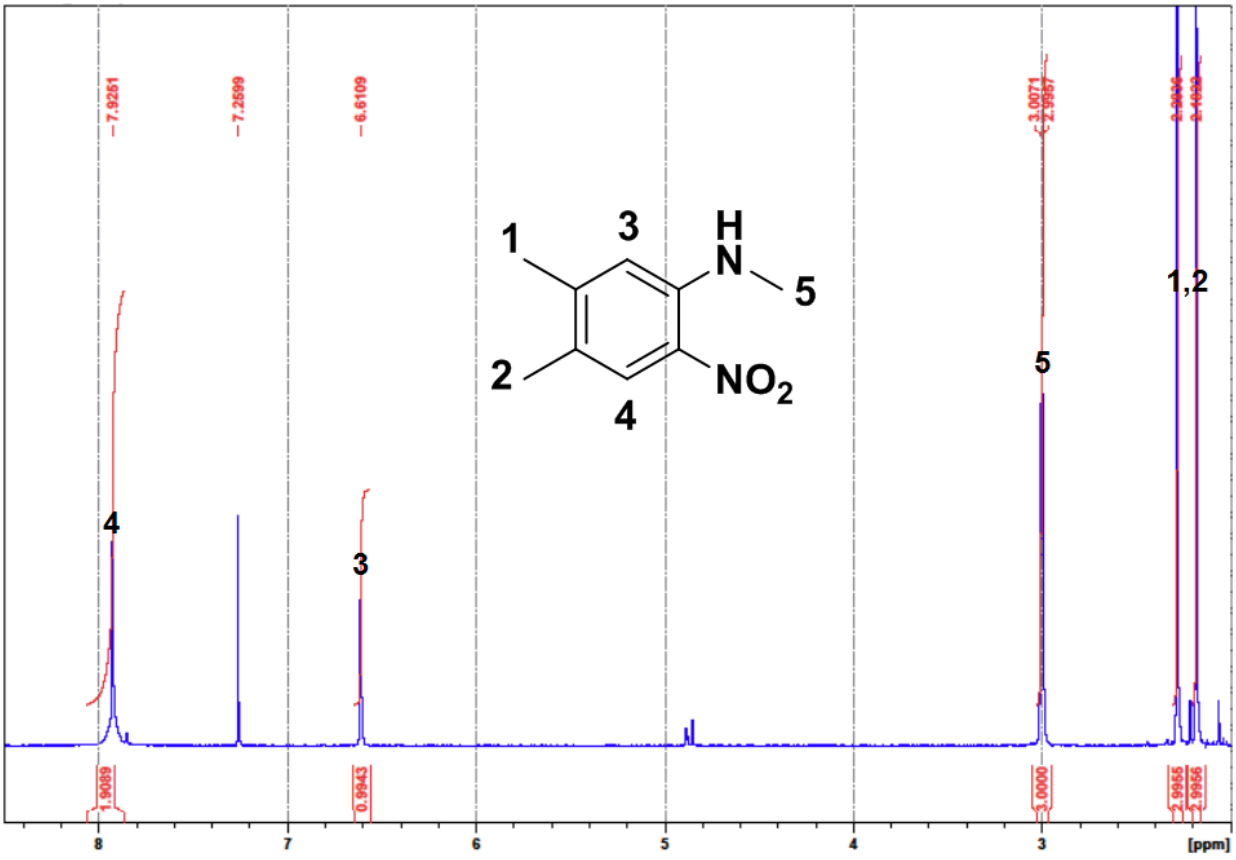


Figure S13: ^{1}H NMR of compound **30**.

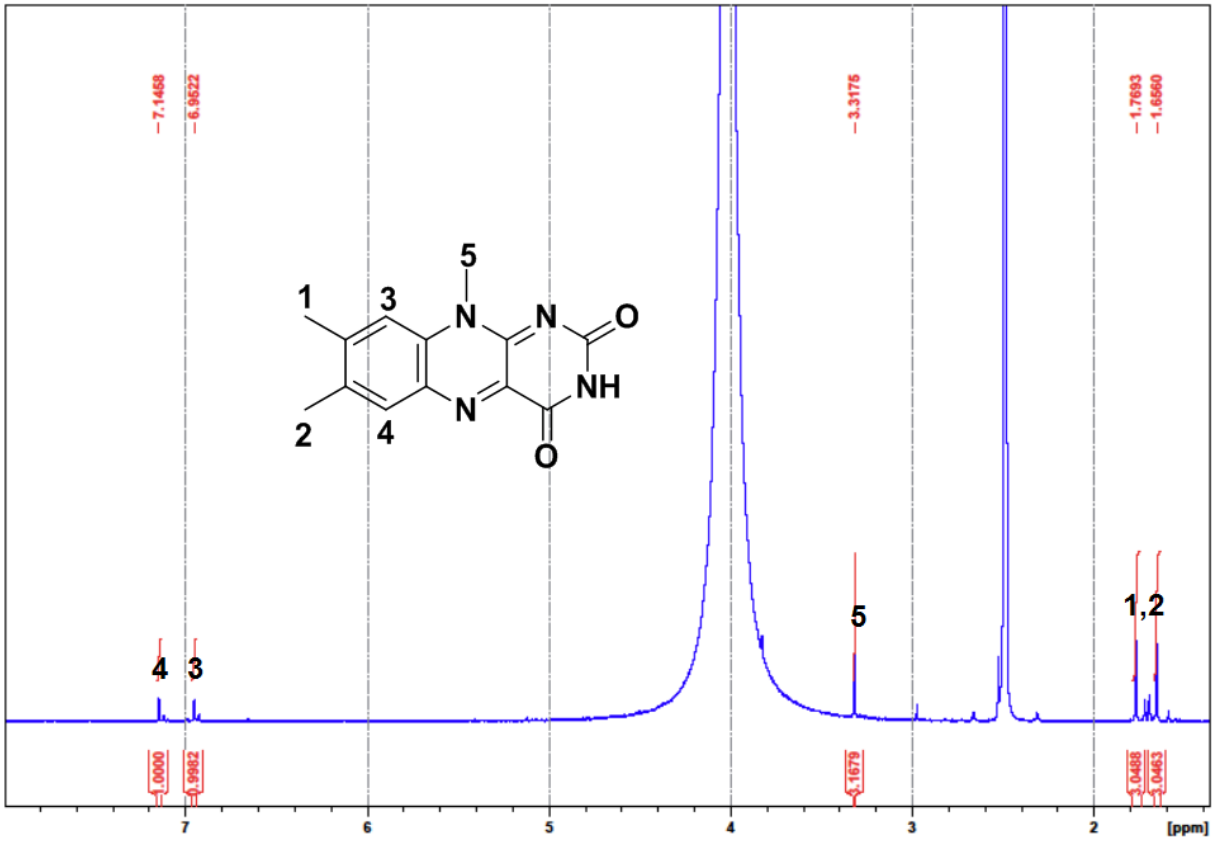


Figure S14: ^{1}H NMR of compound **32.**

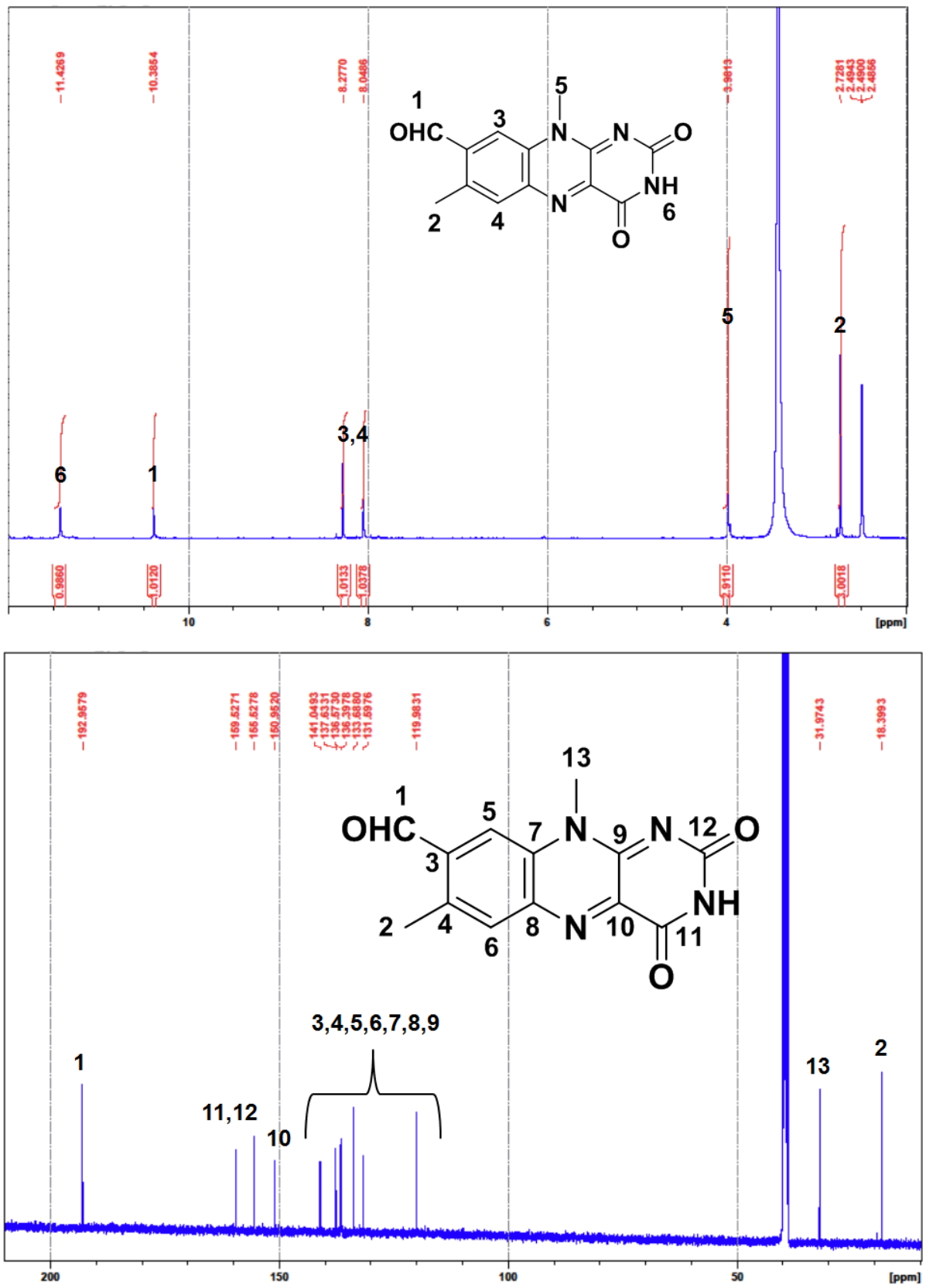


Figure S15: ^{1}H NMR and ^{13}C NMR of formyl-lumiflavin (Compound **4**).

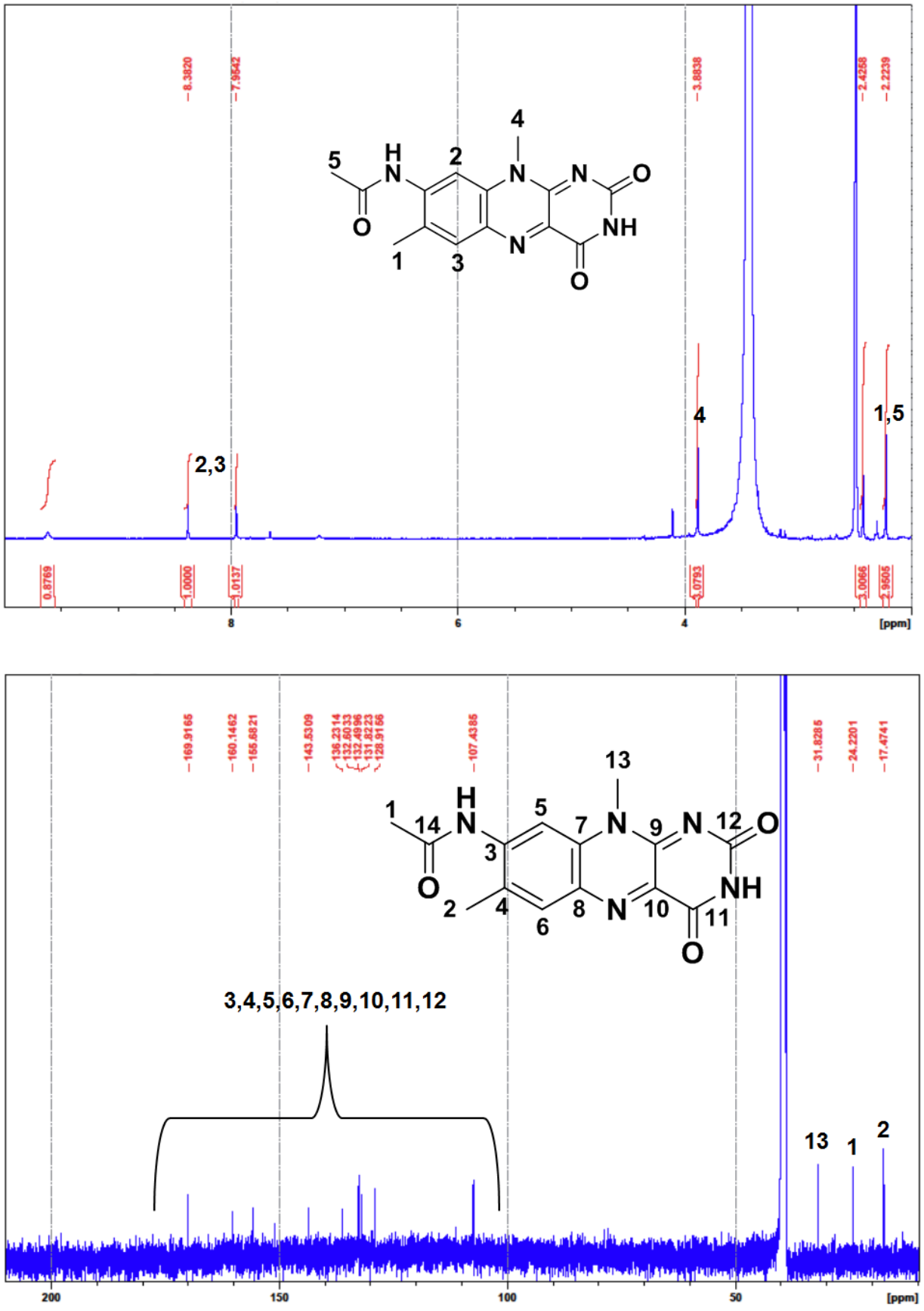


Figure S16: ^{1}H NMR and ^{13}C NMR of acetylamino-lumiflavin (Compound **34**).

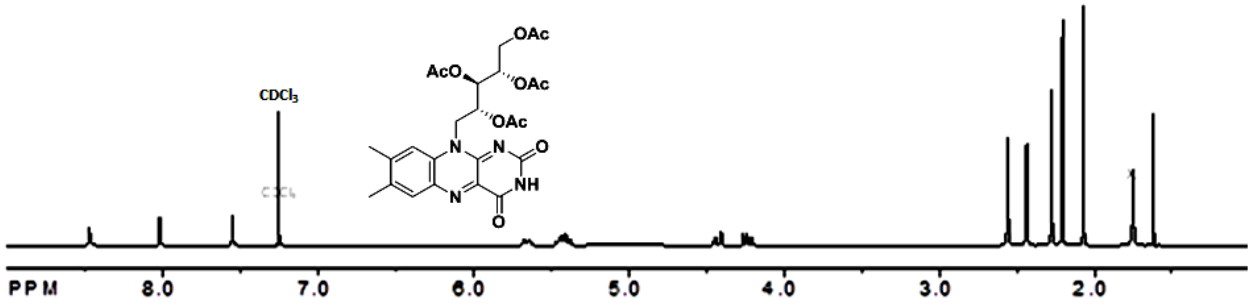


Figure S17: [1]H NMR of 2',3',4',5'-Tetraacetyl-riboflavin (Compound **35**)

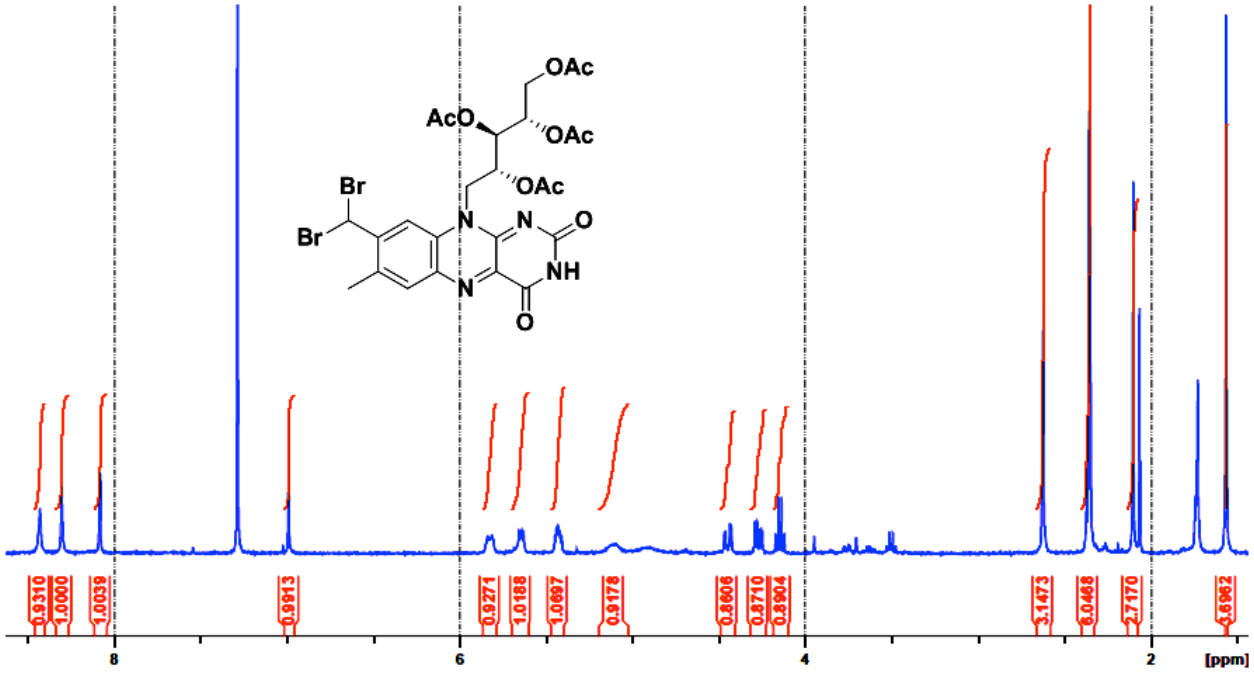


Figure S18: [1]H NMR of 2',3',4',5'-Tetraacetyl-8-ω-dibromoriboflavin (Compound **36**).

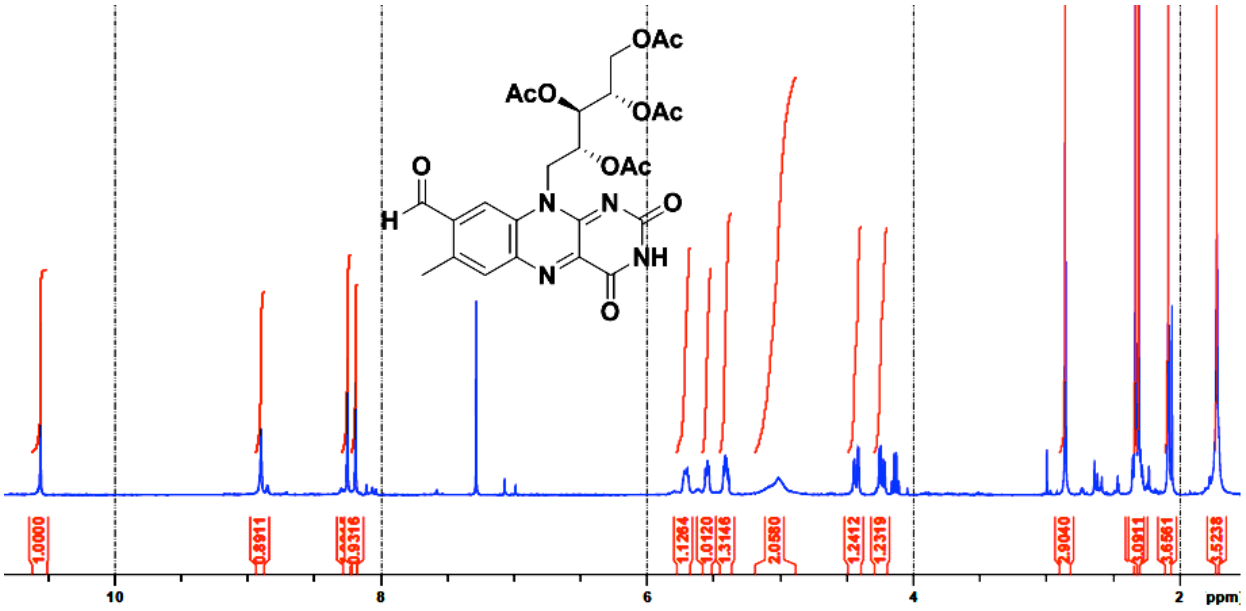
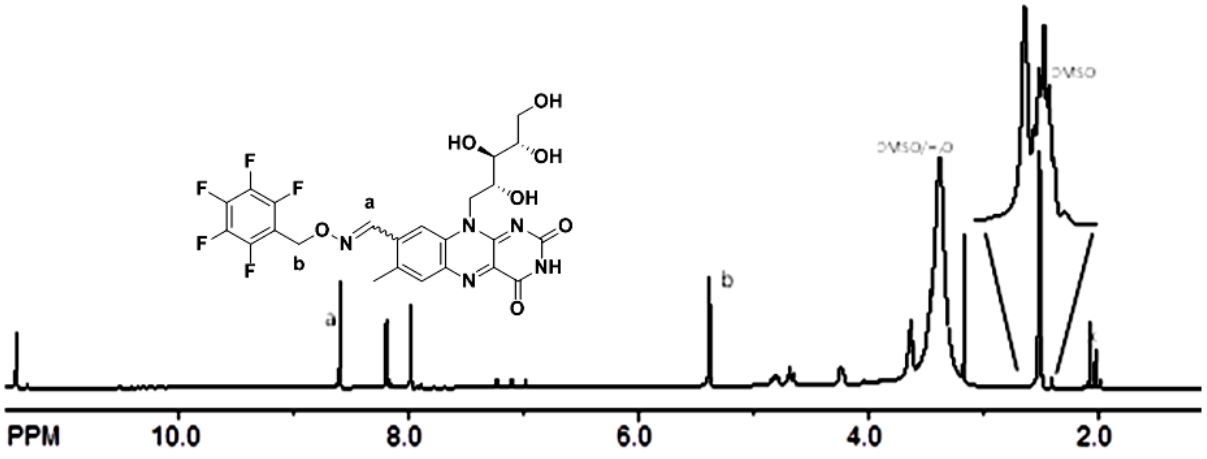


Figure S19: ^{1}H NMR of 2',3',4',5'-Tetraacetyl-8-Formyl-riboflavin (Compound **37**).



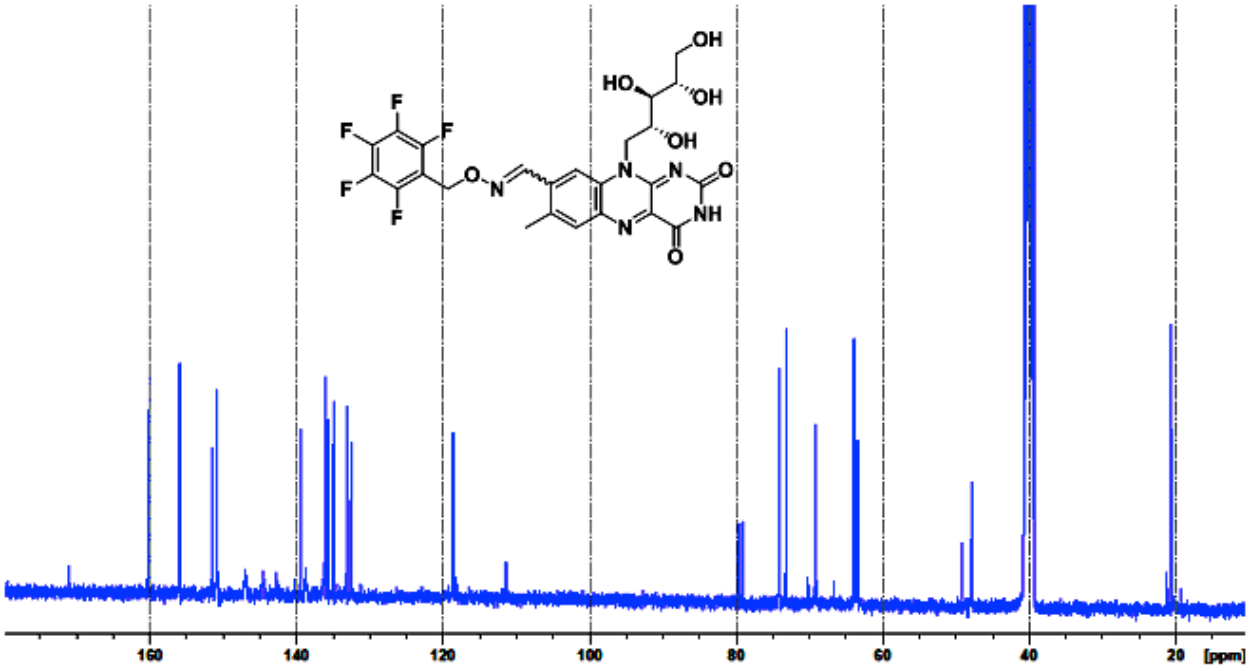
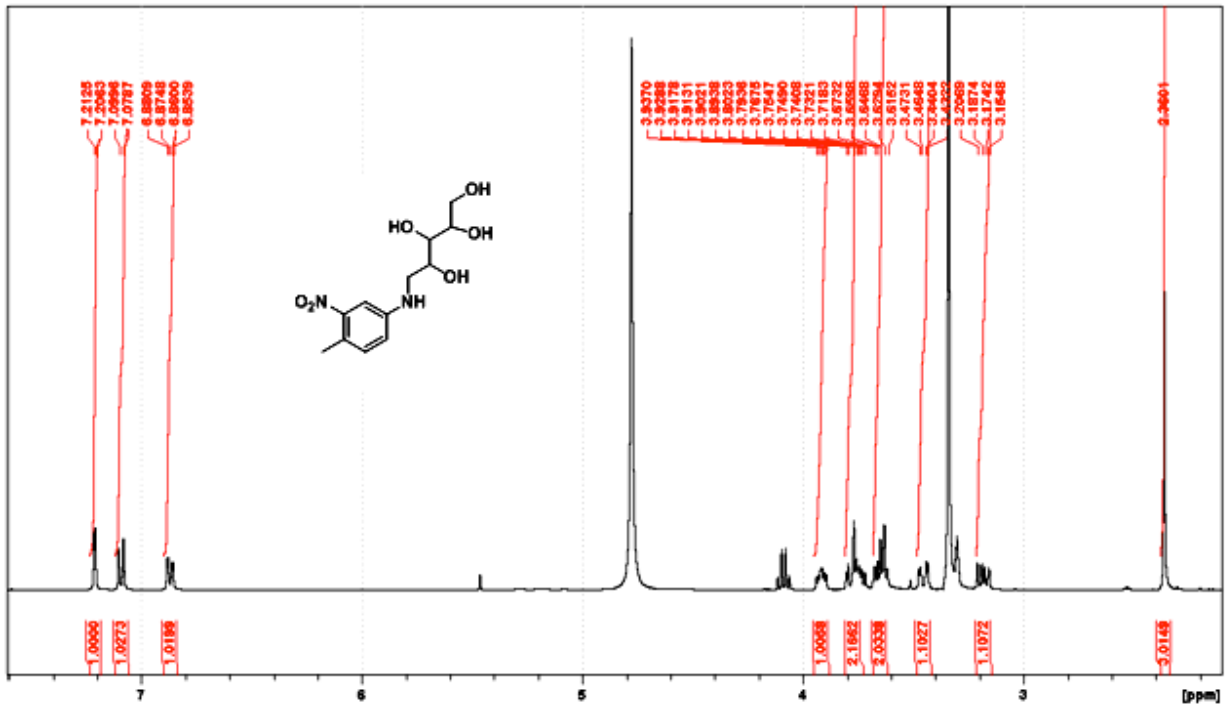


Figure S20: ^{1}H and ^{13}C NMR of PFBHA derivatized-8-Formyl-riboflavin (Compound **28**).



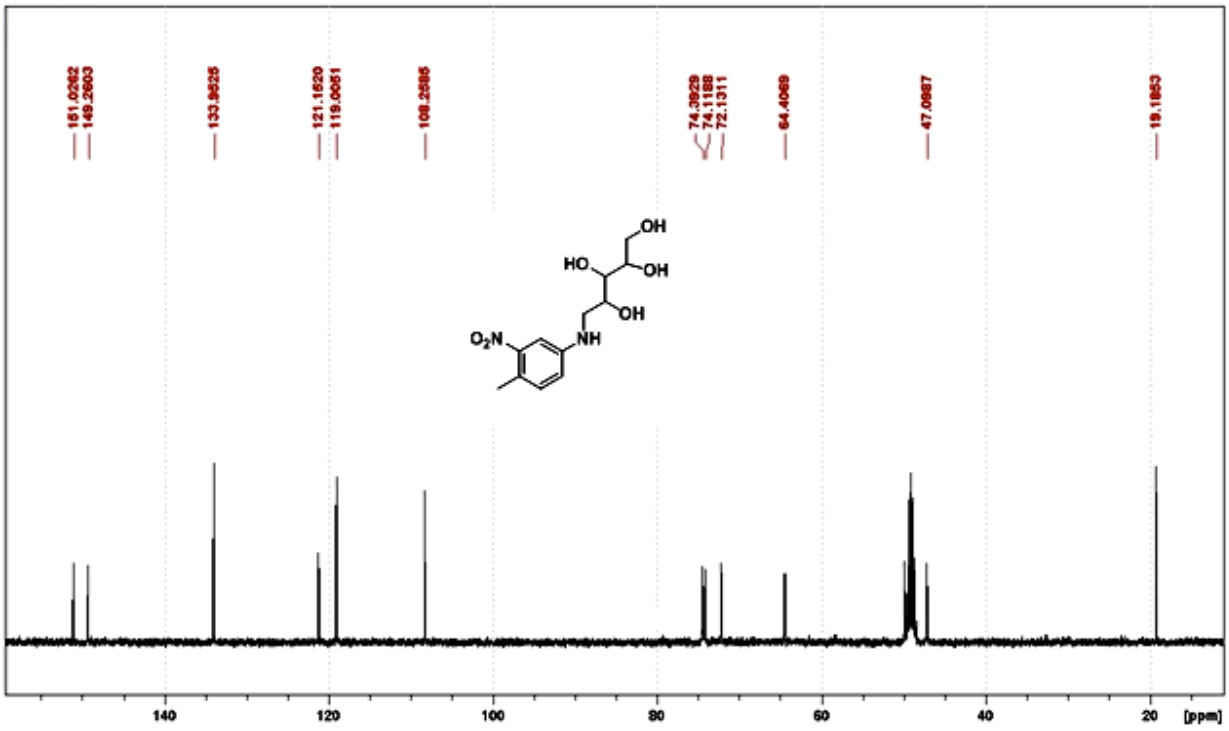
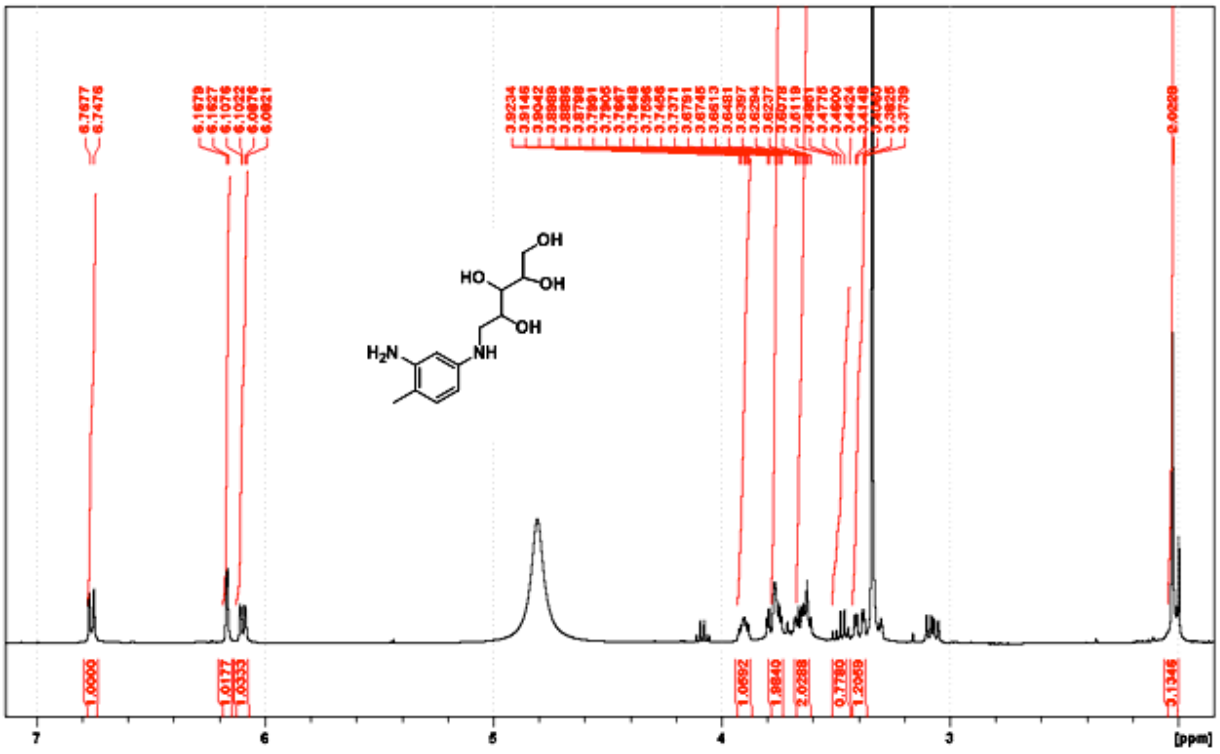


Figure S21: ^{1}H NMR and ^{13}C NMR spectrum of compound **39.**



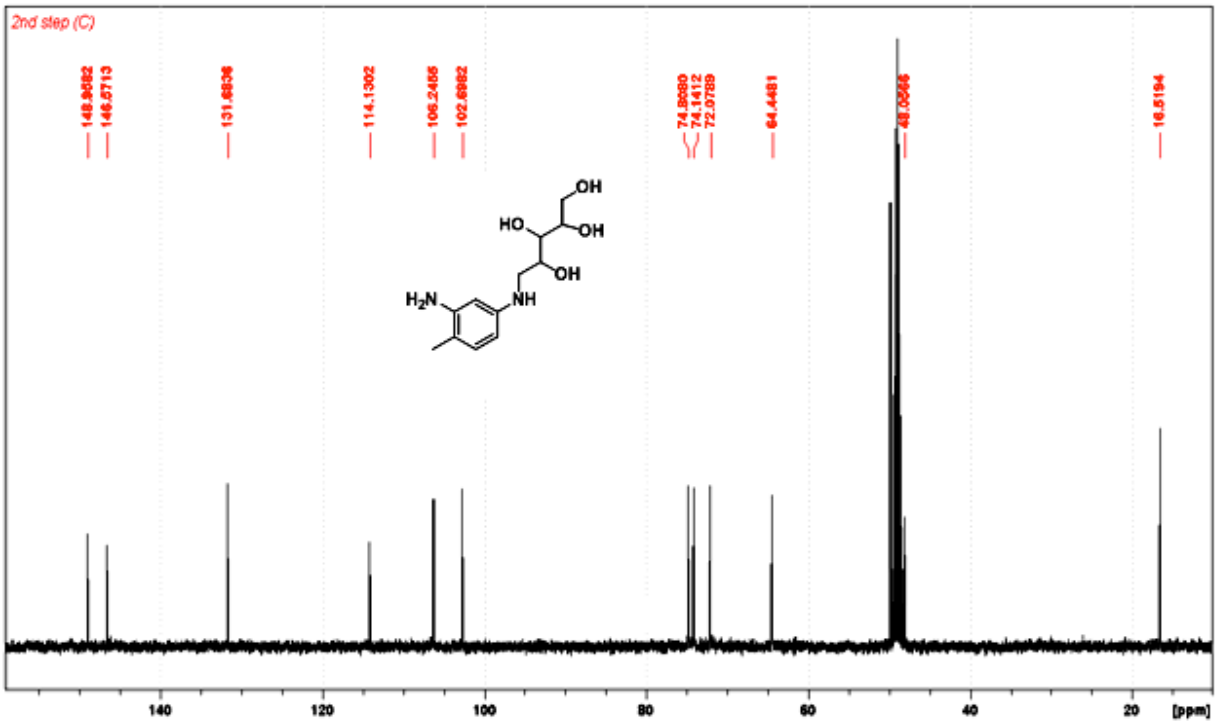
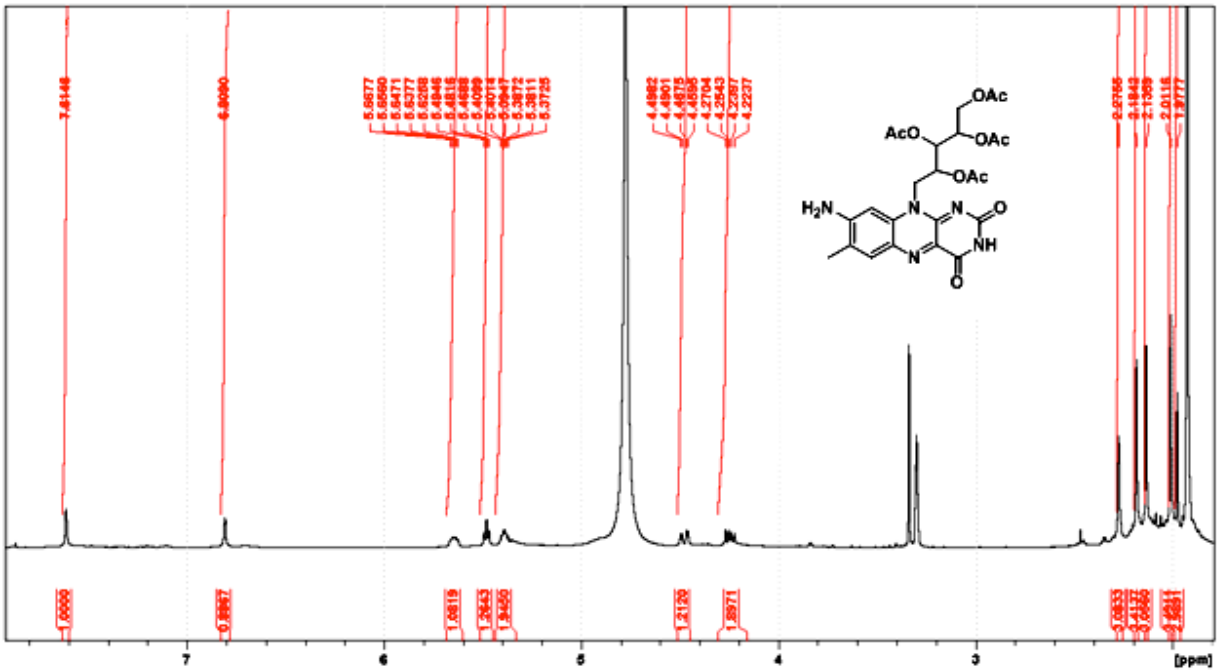


Figure S22: ^{1}H NMR and ^{13}C NMR spectrum of compound **40.**



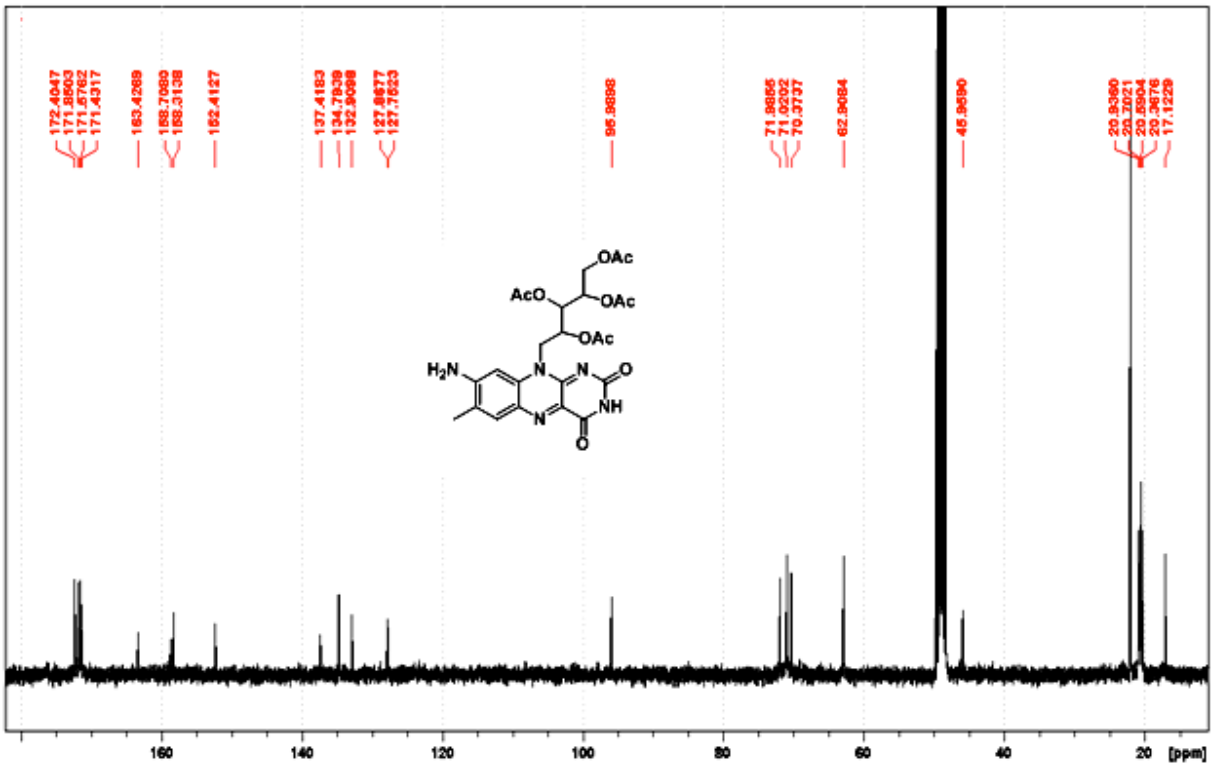


Figure S23: ^{1}H NMR and ^{13}C NMR spectrum of compound **41.**

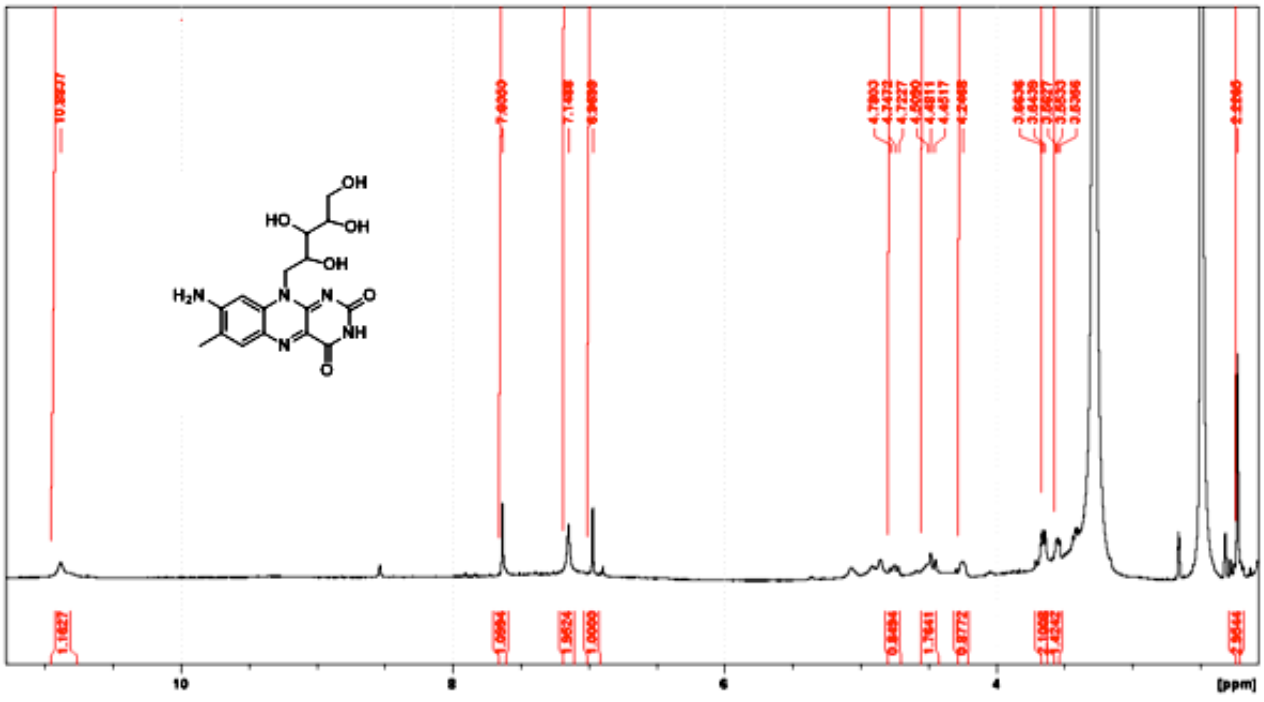


Figure S24: ^{1}H NMR spectrum of compound **2.**

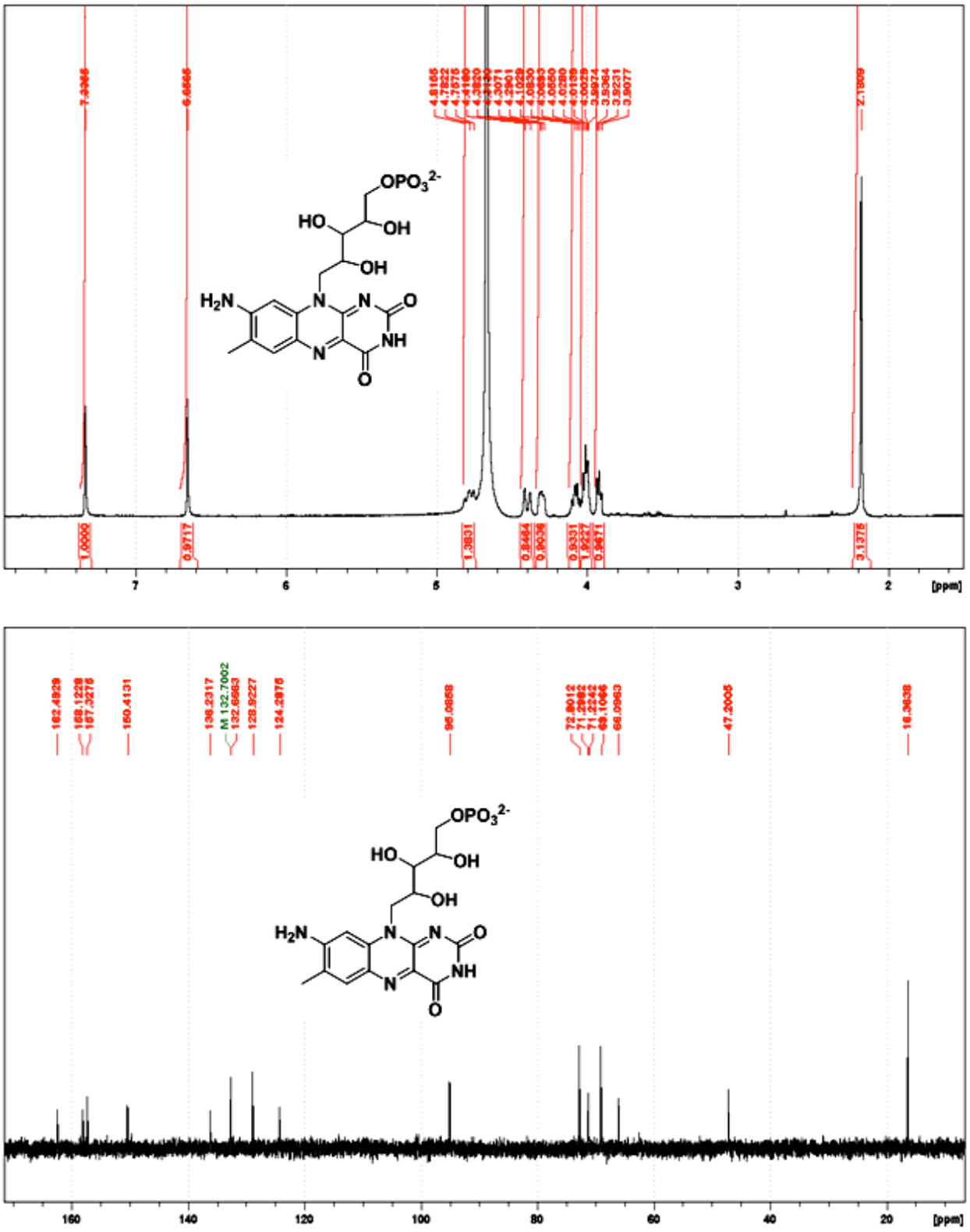


Figure S25: ^{1}H NMR and ^{13}C NMR spectrum of compound **8.**

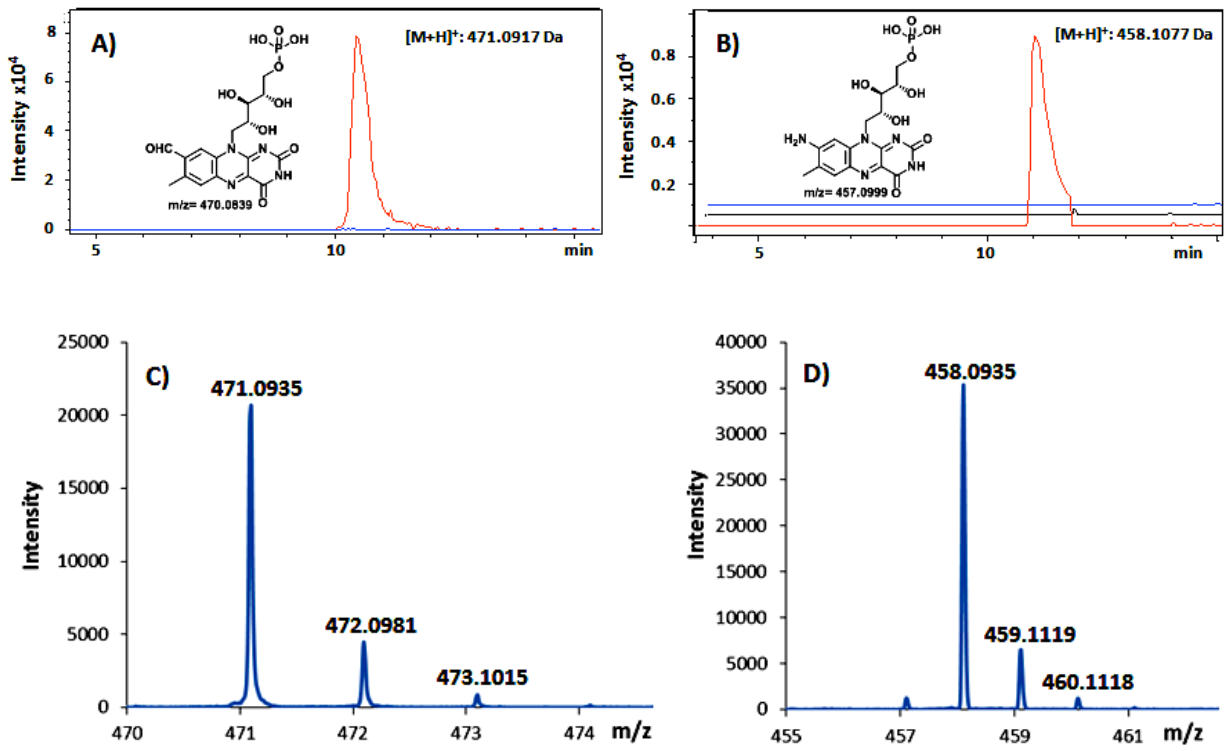


Figure S26: LC-MS analysis of the AFMN synthase-catalyzed reaction in the absence and presence of glutamate. A) EIC at m/z 471.0917 for the reaction mixture lacking glutamate (red trace) and for the reaction mixture lacking glutamate and AFMN synthase (blue trace). B) EIC at m/z 458.1077 for the complete reaction (red trace) and for the reaction mixture lacking AFMN synthase (blue trace) and the reaction mixture lacking glutamate (green trace). C) ESI-MS of the 471.0917 Da species in Panel A consistent with formyl-FMN. D) ESI-MS of the 458.1077 Da species in Panel B consistent with amino-FMN. All spectra were obtained in positive ion mode.

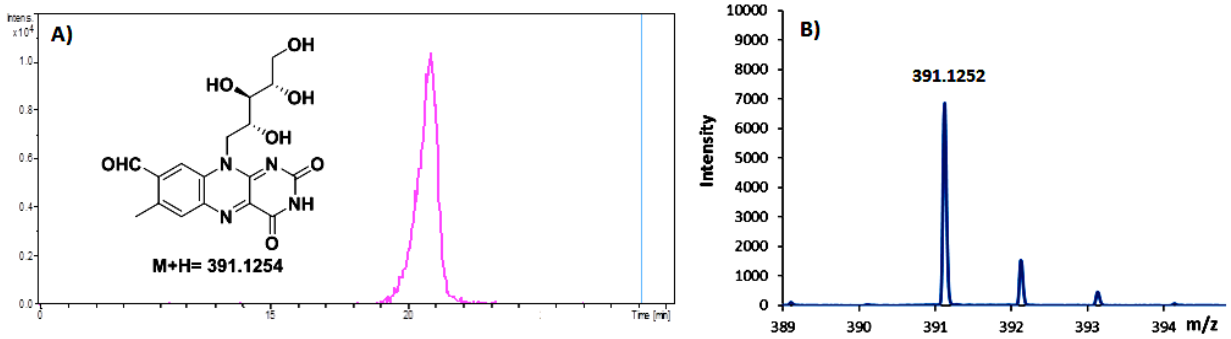


Figure S27: LC-MS analysis of the AFMN synthase catalyzed reaction in absence of glutamate and after CIP treatment to cleave the phosphate group of the product. A) EIC at m/z 391.1254 (positive ion mode). B) ESI-MS of the 391.1254 Da species consistent with formyl-riboflavin.

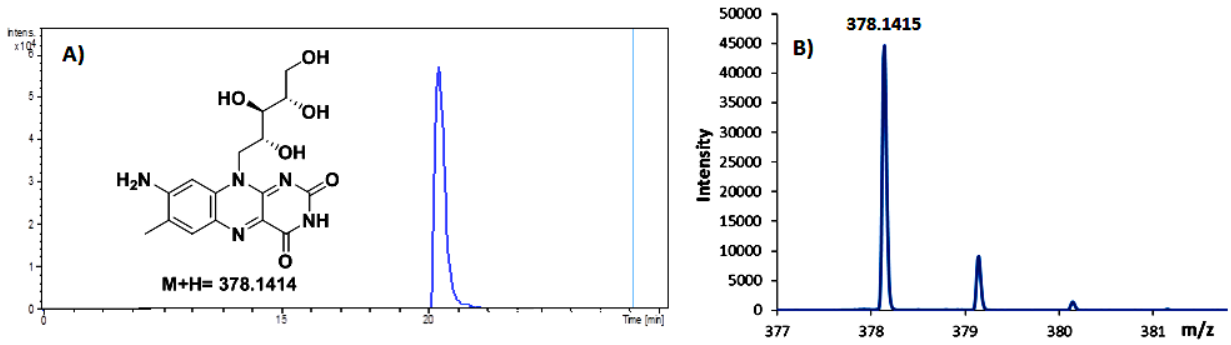


Figure S28: LC-MS analysis of the AFMN synthase catalyzed reaction after CIP treatment to cleave the phosphate group of the product. A) EIC at m/z 378.1414 (positive ion mode). B) ESI-MS of the 378.1414 Da species consistent with amino-riboflavin.

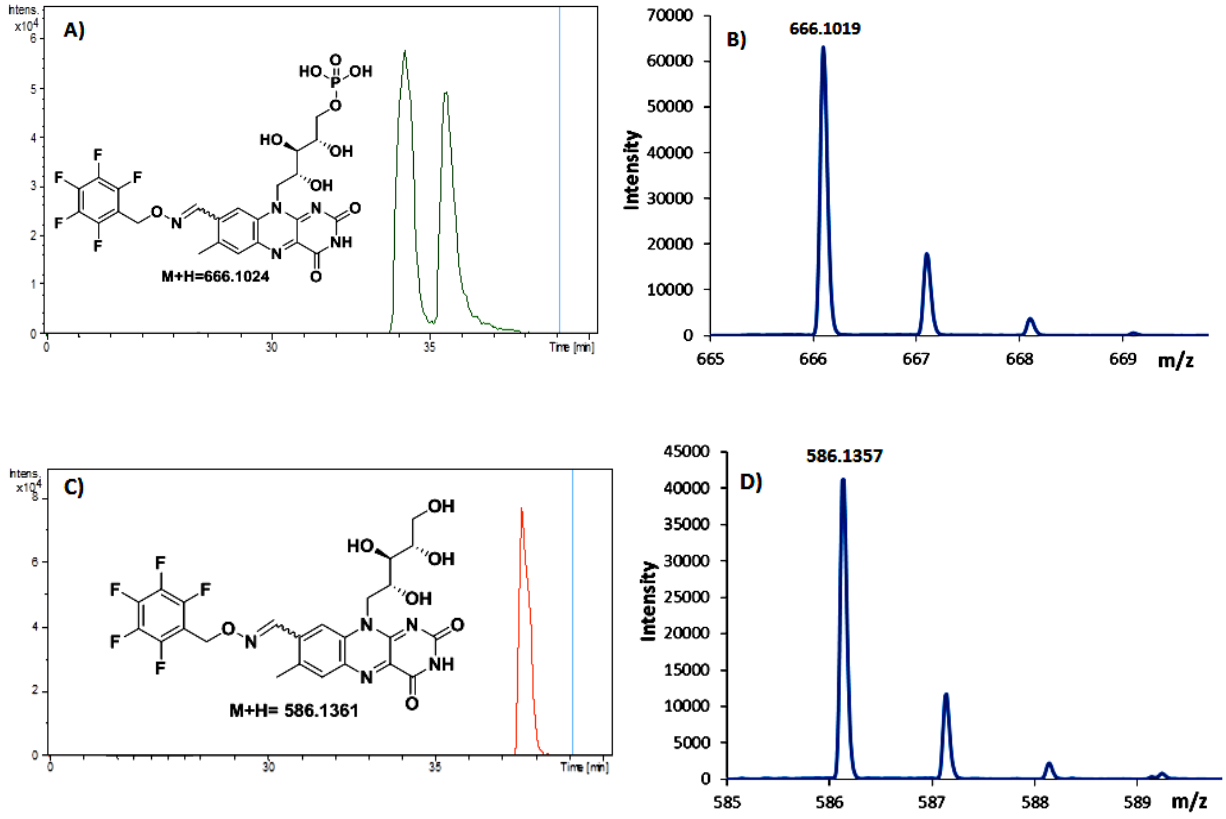


Figure S29: LC-MS analysis of the PFBHA treated AFMN synthase catalyzed reaction before and after CIP treatment in the absence of glutamate. A) EIC at m/z 666.1024 (positive ion mode). B) ESI-MS of the 666.1024 Da species consistent with the formyl-FMN PFBHA oxime. C) EIC at m/z 586.1361 (positive ion mode). D) ESI-MS of the 586.1361 Da species consistent with formyl-riboflavin PFBHA oxime.

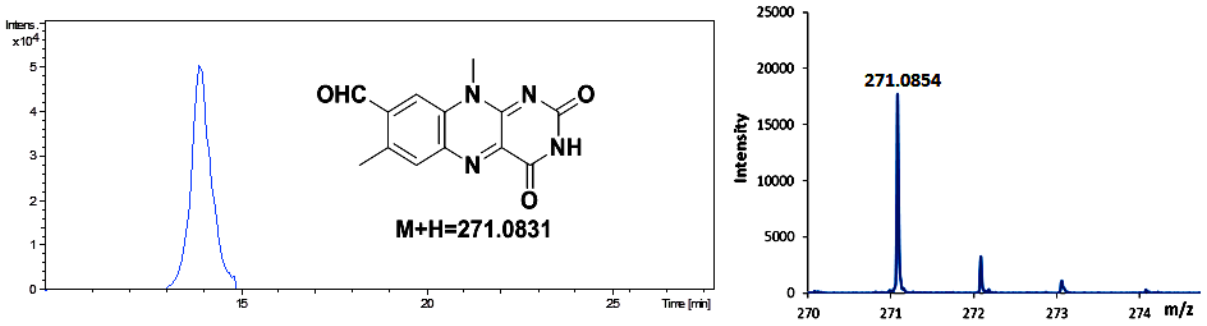


Figure S30: LC-MS analysis of the synthesized formyl-lumiflavin. Left: EIC at m/z 271.0831 (positive ion mode). Right: ESI-MS of the 271.0831 Da species consistent with formyl-lumiflavin.

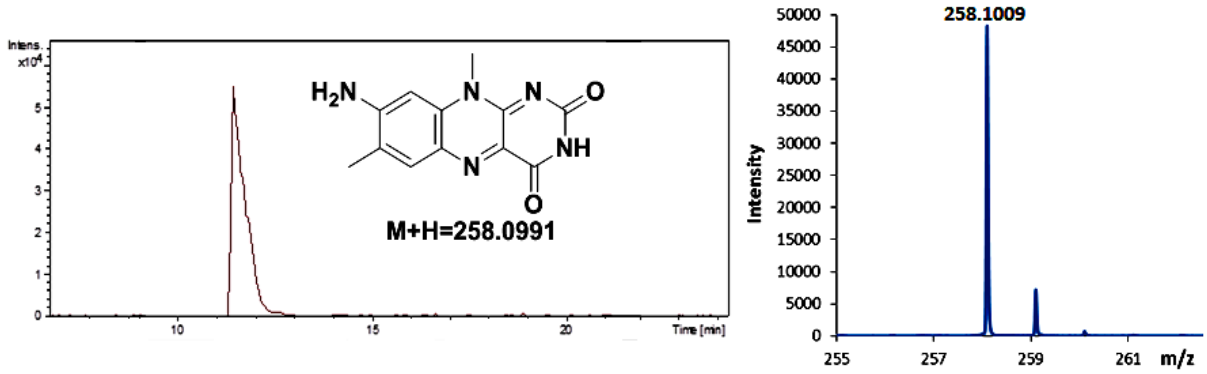


Figure S31: LC-MS analysis of amino-lumiflavin. Left: EIC at m/z 258.0991 (positive ion mode). Right: ESI-MS of the 258.0991 Da species consistent with amino-lumiflavin.

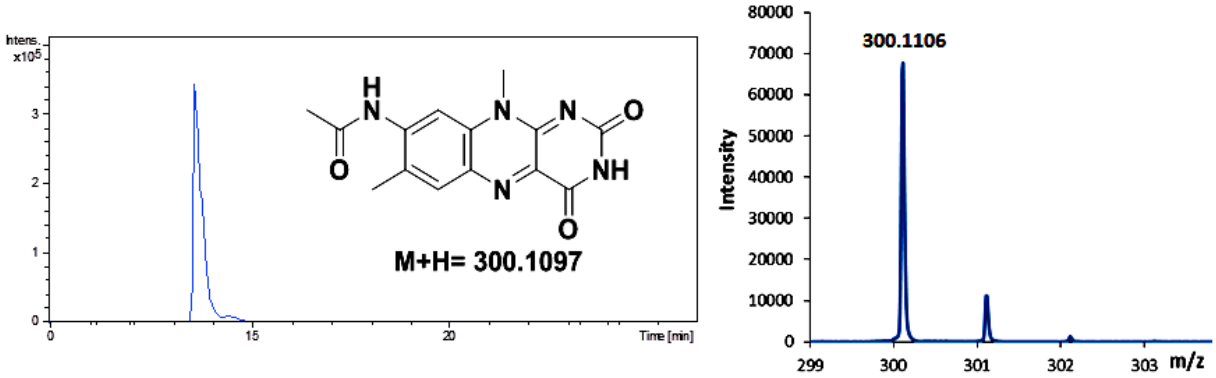


Figure S32: LC-MS analysis of acetylamino-lumiflavin. Left: EIC at m/z 300.1097 (positive ion mode). Right: ESI-MS of the 300.1097 Da species consistent with acetylamino-lumiflavin.

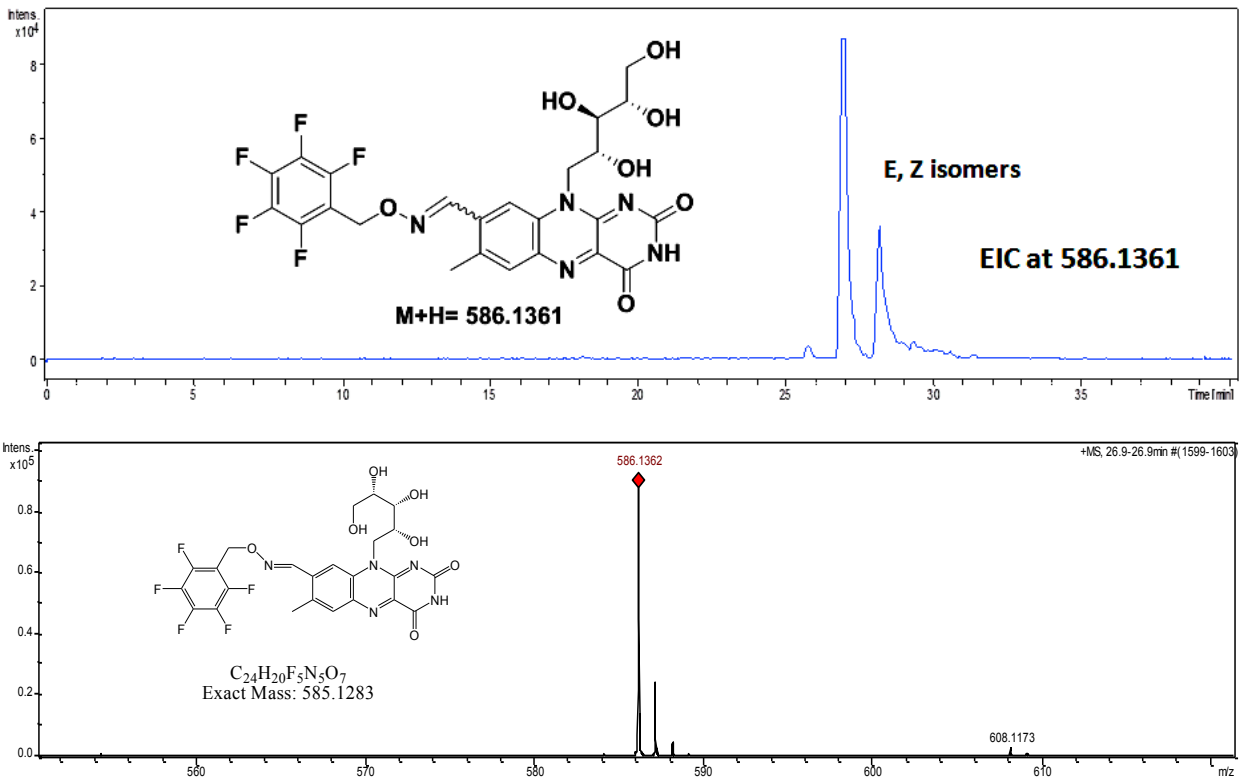


Figure S33: LCMS analysis of synthesized 8-formyl-riboflavin PFBHA oxime. Top: EIC at m/z 586.1361 (positive ion mode). Bottom: ESI-MS of the 586.1361 Da species consistent with the oxime.

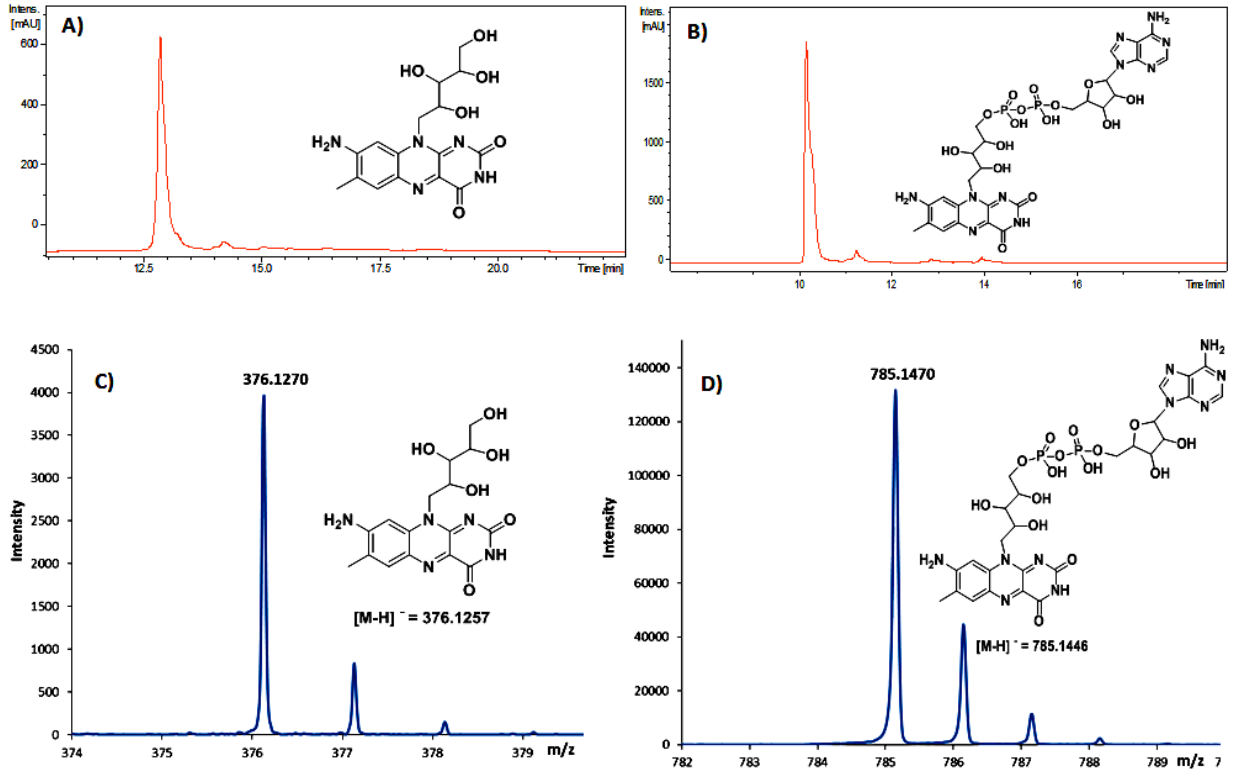


Figure S34: LC-MS analysis of synthesized 8-Amino-riboflavin (Compound **2**) and 8-amino-FAD (Compound **42**). A) UV chromatogram of compound **2** at 450 nm. B) UV chromatogram of compound **42** at 450 nm**. C)** ESI-MS of compound **2** having an exact mass of [M-H]- = 376.1257 Da. D) ESI-MS of compound **42** having an exact mass of $[M-H]^-$ = 785.1446 Da.

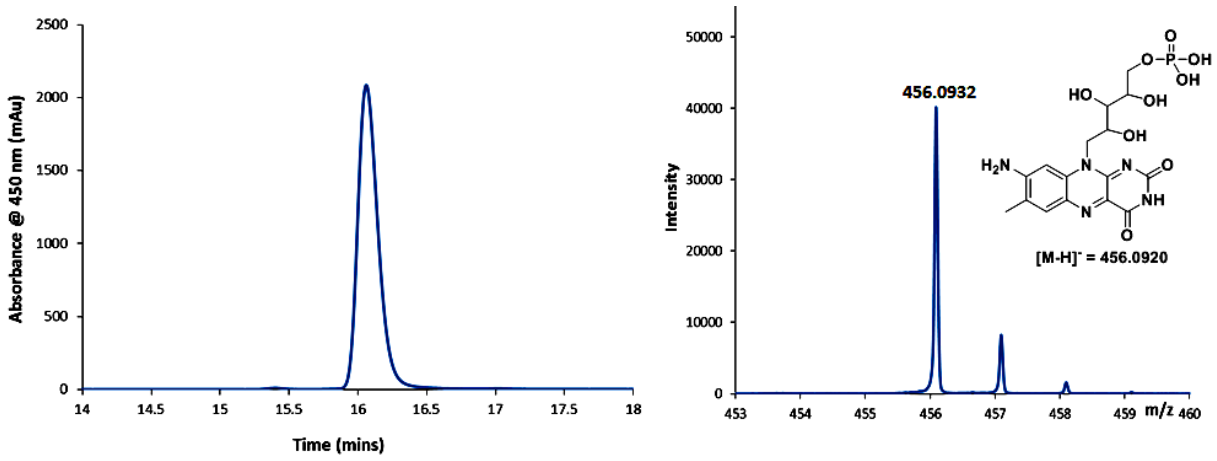


Figure S35: Analysis of synthesized 8-amino-FMN (Compound **8**). A) HPLC analysis of compound **5** at 450nm. B) ESI-MS of **8** having an exact mass of $[M-H]^-$ = 456.0920 Da.

Co-elution experiment with synthesized formyl-riboflavin PFBHA oxime:

Enzymatic reaction was carried out in the absence of glutamate as described above. The protein was heat-denatured and the remaining solution was filtered (10kda cut-off). Then the filtered solution was derivatized with 25 mM of pentafluorobenzyl hydroxylamine (PFBHA) after cleaving the phosphate group of formyl-FMN by treatment with CIP. Finally the samples were analyzed by HPLC (C-18 column) and the co-elution experiment was done with the synthesized sample of formyl-riboflavin PFBHA oxime (Figure S36).

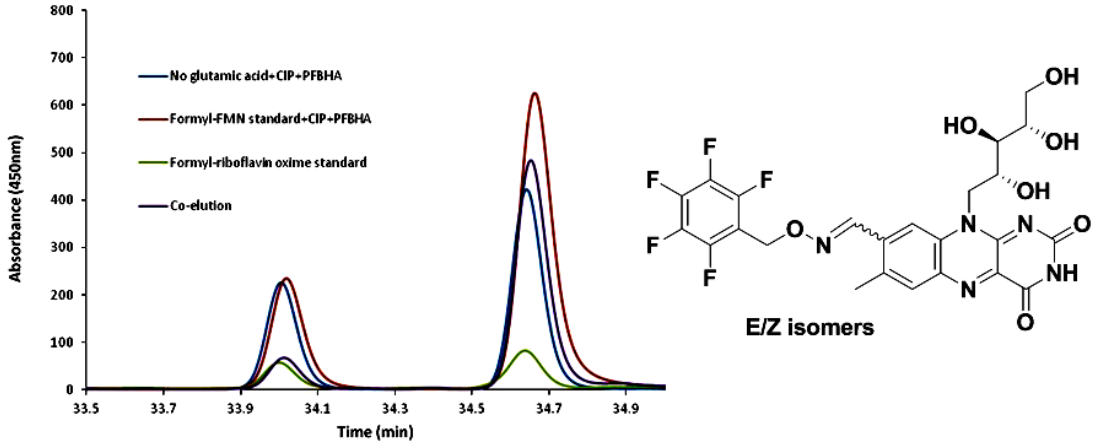


Figure S36: Co-migration of enzymatically formed formyl-FMN after CIP treatment followed by derivatization with PFBHA and a synthesized standard of formyl-ribofavin PFBHA oxime.

Detection of formate formed in the AFMN synthase catalyzed reaction:

The enzymatic assay for amino-FMN formation was performed as described above. Upon completion, the reaction mixture was acidified with 1 N HCl. This acidic mixture was evaporated to dryness and the vapor generated was condensed on the outer wall of a cold thin glass tube containing dry ice and acetone. The condensed liquid was colorless as all the chromophoric compounds remained in the dry residue. This liquid was assayed for formate using formate dehydrogenase (FDH) from *Candida boidinii*. The result (Figure S37) shows an increase in the absorbance at 340 nm with time corresponding to the reduction of NAD^+ due to formate oxidation to CO_2. The unoptimized formate yield was 25% (180 μmols of amino-FMN, 46 μmols of formate).

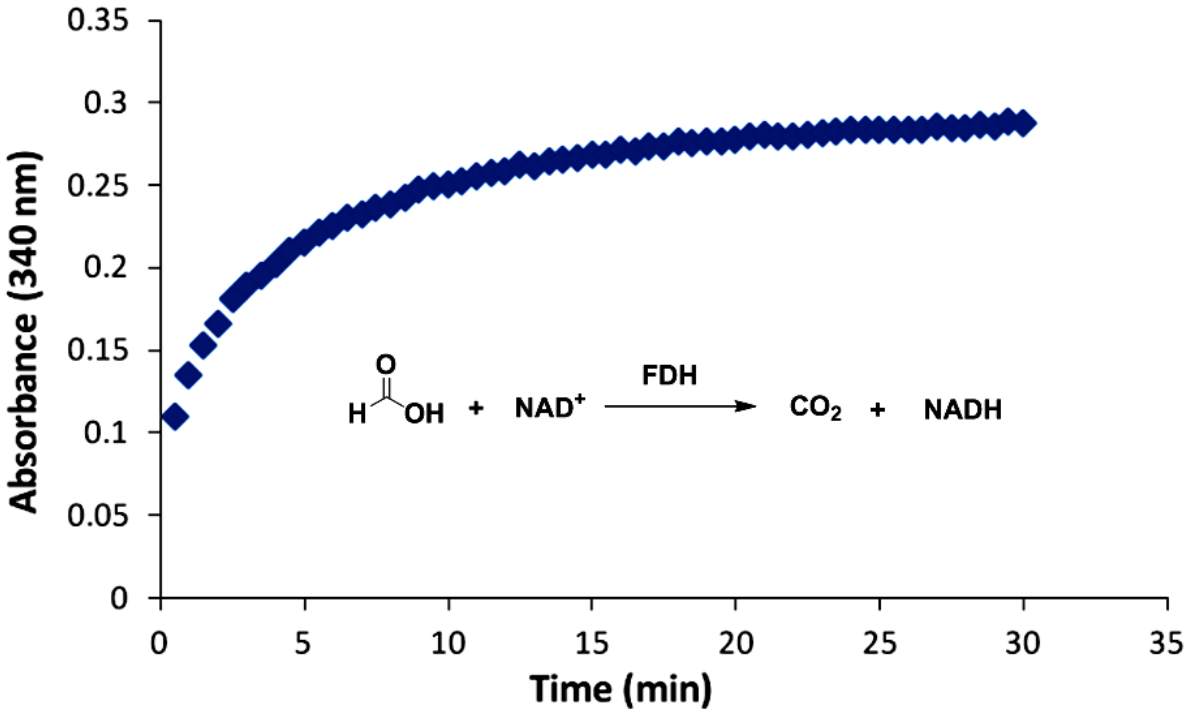


Figure S37: Change in absorbance at 340 nm (λ_{max} of NADH) with time in the FDH assay for formate production in the AFMN synthase catalyzed reaction. No formate was detected in controls lacking AFMN synthase, glutamate or FDH.

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