Supporting Information for

Metabolic pathway rerouting in *Paraburkholderia rhizoxinica* evolved long-overlooked derivatives of coenzyme F_{420}.

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1 Material and methods

1.1 Chemicals, microorganisms, and synthetic DNA

Chemicals and media components were purchased from VWR, Roth, Sigma-Aldrich, Acros Organics and Alfa Aesar. All chemicals were of the highest purity available and the solvents were of mass spectrometry grade. Endonucleases and DNA polymerases were purchased from New England Biolabs. Synthetic genes (ThermoFisher Scientific, BioCat) were optimized for expression in *E. coli*.

1.2 Microbial strains and cultivation conditions

Rhizopus microsporus van Tieghem ATCC 62417 harboring *Paraburkholderia rhizoxinica* HKI 454 (former name: *Burkholderia rhizoxinica*) and naturally endosymbiont-free *Rhizopus microsporus* var. *chinensis* CBS 344.29 were used in this study. Endobacteria were eliminated from *R. microsporus* ATCC 62417 by antibiotic treatment as described (1). Bacterial symbionts were isolated from the mycelium of *R. microsporus* ATCC 62417 as previously reported (2). For F_{420} extraction, *R. microsporus* strains were cultured in 100 mL of NB medium (Merck Millipore, Darmstadt, Germany) in 500 mL baffled Erlenmeyer flasks at 30 °C, 110 rpm for 7 days. Isolates of *P. rhizoxinica* were cultured in 50 mL MGY M9 medium (0.12% yeast extract, 1% glycerol, 40 mM K_2HPO_4, 14 mM KH_2PO_4, 2.2 mM sodium citrate, 7.5 mM $(NH_4)_2SO_4$, and 0.8 mM Mg_2SO_4) in 300 mL baffled Erlenmeyer flasks at 30 °C, 110 rpm for 7 days. *E. coli* was routinely grown on lysogeny broth (LB, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) at 37 °C and 210 rpm. *E. coli* BL21 (DE3) (New England Biolabs) was used for heterologous gene expression. *Mycolicibacterium* (former name: *Mycobacterium*) *smegmatis* was grown on LB supplemented with 0.05% (v/v) tween 80. Whenever used for the production of coenzyme F_{420}, tween was substituted by sterile glass beads.

1.3 Fluorescence microscopy

To visualize the fluorescence of deazaflavins, *R. microsporus* ATCC 62417, cured *R. microsporus* ATCC 62417, symbiont-free *R. microsporus* CBS 344.29, *M. smegmatis* (3 days old), *E. coli* BL21(DE3)/pETDuet, *E. coli* BL21(DE3)/pDB045, and axenic *P. rhizoxinica* (7 days old) were analyzed using a Zeiss LSM 710 Confocal Laser Scanning Microscope (CLSM, Zeiss, Oberkochen, Germany) with excitation/emission of 405/470 nm. To visualize the localization of *P. rhizoxinica* within the fungal hyphae, a piece of fresh *R. microsporus* ATCC 62417 mycelium was transferred to an Eppendorf tube containing 200 µL physiological saline. The bacterial cells were stained with 5 µM Syto 9 green-fluorescent nucleic acid stain (Invitrogen, USA). The samples were incubated in the dark for 5 min and then analyzed using a Zeiss LSM 710 CLSM at 480/500 nm.

1.4 Plasmid design and cloning

Unless otherwise specified, cloning was based on DNA recombination following the Fast Cloning protocol (3). 50 µL-polymerase chain reactions were composed by 0.2 mM each dNTP, 3 mM $MgCl_2$, 0.2 µM each primer (Table S2), as well as Q5 DNA Polymerase (1 U) and its respective buffer. PCRs started with initial denaturation at 98 °C for 1 minute followed by 18 cycles at 98 °C for 10 s, X °C for 30 s, and 72 °C for X' minutes. A final elongation at 72 °C for two minutes ended the reaction. Annealing temperatures varied with the primers and followed recommendations of the primer manufacturer. Elongation times were calculated as 2 kb per minute. All plasmids created during this study are listed in Table S3. Full plasmid sequences are provided in section 2.6.

The biosynthetic gene cluster for coenzyme F420 from *P. rhizoxinica* HKI 454 (Table S1) was amplified from its genomic DNA and was cloned in two steps. First, ribA and cofE were individually amplified (primers oDB01 and oDB02, oDB05 and oDB06, respectively) and were assembled in pETDuet-1 (Merck) previously linearized with XbaI and BamHI. The resulting construct, pDB044 (6,840 bp), was generated from a tripartite assembly of the two genes and vector backbone using the infusion cloning kit (Takara Clonetech). The gene sequences encoding CofC, the ABC transporter, and CofD were amplified as a single fragment (primers oDB07 and oDB08). pDB044 was PCR-linearized with the primers FC_pDB044_FP and FC_pDB044_RP to receive the latter amplicon. The construct bearing the complete gene cluster for 3PG-F_{420} was termed pDB045 (13,135 bp).

Plasmid pDB045 served as the base to have either *P. rhizoxinica cofD* or both *cofC* and *cofD* swapped by *M. jannaschii* homologous gene sequences. The *cofD* gene (MJ_RS06720) from *M. jannaschii* was obtained in the form of a gene string and amplified with the primers oDB023 and oDB024. pDB045 was amplified with the primers oDB021 and oDB022 in a manner the PCR product would lack *P. rhizoxinica cofD*. The resulting construct (pDB060, 13,045 bp), therefore, bore the BGC for F_{420} with *P. rhizoxinica cofD* replaced by that from *M. jannaschi*. The same strategy was used to exchange *cofC*. This gene from *M. jannaschii* (MJ_0887) was also purchased as a gene string and amplified with the primers oDB128 and oDB069. pDB060 served as template for the amplification/linearization of the plasmid lacking *P. rhizoxinica cofC* using the primers oDB070 and oDB071. The construct obtained (pDB070, 13,136 bp) has, therefore, the BGC for F_{420} from *P. rhizoxinica* with the genes *cofC* and *cofD* replaced by the homologs from *M. jannaschii*. To construct plasmids encoding F_{420}-dependent enzymes, the gene encoding F_{420}-dependent malachite green reductase (MSMEG_5998) was ordered as a gene string. The sequence was amplified (primers DB_FP_MBP_5998 and DB_RP_MBP_5998) and cloned in pMAL-c2x (New England Biolabs) linearized with the primers DB_FP_pMal-c2X and DB_RP_pMal-c2X. The resulting construct was termed pDB061 (7,155 bp). For Fno production (F_{420}:NADPH oxidoreductase from *Archaeoglobus fulgidus)*, the codon-optimized *fno* gene string (Fno, AFULGI_RS04660) was amplified by PCR (primers: FC_fno_ins_fw, FC_fno_ins_rv) and ligated to

the pET28a backbone (primers: FC_fno_vec_fw, FC_fno_vec_rv) by Fast Cloning yielding plasmid pDL008. Plasmids intended to yield reduced 3PG-F_{420} were built with the pCDFDuet-1 backbone (Merck) and bore the codon-optimized sequence of *fno* in the multiple cloning site (MCS) 1. The optimized *fno* sequence was amplified with the primers oDB084 and oDB122. The vector was linearized with the primers oDB081 and oDB123. FastCloning of both fragments yielded pDB065 (4,385 bp), which served as the vector to receive the gene sequence(s) from *P. rhizoxinica* encoding proteins for the production of deazaflavins in its MCS2. pDB065 was linearized with primers oDB105 and oDB106 to receive the trio *cofC*, *fbiC*, and *cofD*, amplified with primers oDB107 and oDB108. The construct was intended to yield reduced 3PG-F_{420}-0 (pDB071, 8,656 bp). For heterologous expression of *cofC* and *cofD*, *cofC* (*P. rhizoxinica*) was amplified by PCR using primers CofC-fw and CofC-rv from genomic DNA. The vector backbone of pACYCDuet was amplified using primers pACYCDuet-fw and pACYCDuet-rv. Both PCR products were joined by FastCloning yielding plasmid pFS03 encoding an N-terminal hexahistidine (His_6) fusion protein of CofC. The coding sequence of *cofD* (*M. jannaschii*) was obtained as synthetic gene string and PCR amplified using primers CofD-fw and CofD-rv. The backbone of pET28a was amplified by primers pET28a-fw and pET28a-rv and ligated with the insert by FastCloning yielding plasmid pFS04 encoding an N-terminal hexahistidine (His6) fusion protein His_6-CofD. For coexpression with *cofE* homologs, a minimal cluster containing cofC, fbiC and cofD from pDB045 was amplified using primers oDB115 and oDB129B. oDB081 and oDB123 primers were used to amplify vector pCDFDuet-1 resulting in plasmid pMH02 (8,085 bp). *CofE* from *Methanocaldococcus jannaschii* was obtained as synthetic gene cloned into pET28a between BamHI and HindIII *sites.* FbiB was obtained as codon-optimized string and amplified by primers fbiB_fw and fbiB_rv, *cofE* from *Paraburkholderia rhizoxinica* was amplified using primers cofE_fw and cofE_rv. Both PCR products were fast-cloned into pET28a (amplified by primers pET28a-fw and pET28a-rv). All *cofE* constructs were designed in frame with the N-terminal His_6-tag.

1.5 Heterologous expression of BGCs in *Escherichia coli*

For the heterologous expression of the biosynthetic gene clusters, *E. coli* BL21 (DE3) transformed, e.g. with plasmid pDB045 was grown to saturation in LB (50 µg mL^{-1} carbenicillin) at 37 °C and 210 rpm. An overnight pre-culture was used (1:100) to inoculate 200 mL of main culture and induced with 200 µM IPTG upon OD_{600} of 0.7. Protein and metabolite production proceeded for additional 20 h in the same culture conditions.

1.6 Extraction of deazaflavins from bacteria and fungi (small scale)

Metabolite extraction for both *E. coli* and *M. smegmatis* followed the same protocol: The biomass was pelleted by centrifugation (4 °C, 8,000 × *g*, 25 min), washed with dH_2O, suspended in -20°C MeOH (MeOH) and left in an ultrasonic homogenizer for 10 minutes. Cell debris was removed by centrifugation. The extract was collected and the solvent was removed by rotary evaporation. The dried extract was dissolved in 500 µl MeOH. If the produced deazaflavin(s) were to be used in an enzymatic assay, the dried extract was dissolved in ddH_2O and subsequently loaded onto a C18 Chromabond cartridge (Macherey-Nagel). Samples were washed and eluted with 20% and 30% (v/v) MeOH:H_2O, respectively. Except for the steps of cell washing and lysis, solvent systems were acidified with 0.1% formic acid (v/v). Mycelium of *R. microsporus* was strained and resuspended in 10 mL ice-cold HPLC-grade MeOH (VWR Chemicals, Darmstadt, Germany), while *P. rhizoxinica* cultures were snap-frozen in liquid nitrogen and freeze-dried. Dry *P. rhizoxinica* cultures were then resuspended in 10 mL ice-cold MeOH. Samples were sonicated for 20 min (Sonorex RK100 Ultrasonic bath, Bandelin) and then shaken (250 rpm) for 1 h followed by centrifugation (10.000 rpm) for 15 min. The supernatant was filtered through a paper filter and the solvent was evaporated. Dry extracts were dissolved in 2 mL H_2O.

1.7 UHPLC-MS analyses of deazaflavins

Ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-HRMS) was performed on a Dionex Ultimate3000 system combined with a Q-Exactive Plus mass spectrometer (Thermo Scientific) with a heated electrospray ion source (HESI). Samples were separated by reverse-phase chromatography in a Luna Omega C18 column (100 x 2.1 mm, 1.6 µm, 100 Å, Phenomenex). H_2O (A) or acetonitrile (B), both acidified with formic acid 0.1% (v/v), served as mobile phases. 5 µl of the bacterial extracts were submitted to a gradient as follows: 0 min, 5% B; 1 min, 15% B; 3 min, 25% B; 6 min, 40% B; 7-9 min, 97% B; 10 min, 5% B at a constant flow rate of 300 µL min^{-1} and at 40 °C. The chromatographic eluent was ionized in positive mode. The MS was carried out within a range of m/z 350 – 1800 followed by a data-dependent MS^2 analysis. MS^1 measurements were set to a resolving power of 70,000, MS^2 experiments to 17,500 at m/z 200, isolation windows m/z 1.0, normalized collision energy (NCE) 30.

1.8 HPLC of F_O

The purification of F_O for subsequent biochemical assays was carried out by analytical HPLC on a Shimadzu system equipped with a Nucleodur 100-5 C8ec column (150 x 4.6 mm, 5 µm, 300 Å, Macherey-Nagel). Solvents were the same as described for LC-HRMS. 20 µl of the SPE-purified samples were submitted to a gradient as follows: 0-2.5 min, 15% B; 5 min, 22% B; 6-11 min, 99% B;

11.5-16.5 min, 15% B. Flow rate and column temperature were held constant at 1 mL min^{-1} and 25 °C, respectively. Compounds were analyzed by a diode array- and a fluorescence detector arranged in tandem. Excitation and emission at 420 and 480 nm, respectively, were set up for the fluorescence-based detection.

1.9 Large-scale production and purification of 3PG-F_{420} and F_{420}

3PG-F_{420} was produced from a total volume of 50 L of *E. coli* BL21 (DE3)/pDB045 grown in LB medium (100 µg/mL carbenicillin) in 2L flasks in several batches. At an OD_{600nm} of 0.6, IPTG was added (0.2 mM) followed by 20 h of further incubation at 37 °C and 160 rpm. *E. coli* cells were harvested in batches by centrifugation (30 min at 3,100 × g), washed with Tris-HCl buffer (25mM, pH 7.4) and stored temporarily at -20°C. Combined *E. coli* pellets (213 g wet weight) were resuspended in Tris-HCl buffer (50 mM, 100 mM NaCl, pH 7.4), to a concentration of about 0.5 g/mL (wet weight). Subsequently, the suspensions were autoclaved for 15 min at 121°C to release 3PG-F_{420}. Extracts were centrifuged for 30 min at 3,100 × g, supernatants were further processed. To generate 3PG-F_{420}-0, the 3PG-F_{420} species were digested by the addition of carboxypeptidase G from *Pseudomonas* sp. (5 units, Sigma-Aldrich) and zinc sulfate (0.2 mM final concentration), and subsequent incubation for 18 hours at 37 °C and 180 rpm. The reaction product was acidified to pH 3.5 with HCl and applied to Chromabond C18 SPE cartridge (10 g, Macherey-Nagel). Step-wise elution was performed with increasing shares of MeOH in H_2O containing 0.25% formic acid. Fluorescence measurements of 1:50 diluted samples in sodium phosphate buffer (50 mM, pH 7.5) with FLUOstar Omega (BMG Labtech) indicated positive fractions. Solvent was evaporated to dryness under reduced pressure in a rotary evaporator. The residues were dissolved in sodium phosphate buffer (25 mM, pH 7.4). Next, anion-exchange chromatography of 3PG-F_{420} was performed on a FPLC system (NGC Quest, Bio-Rad) equipped with a HiPrep QFF column (GE Healthcare) pre-equilibrated with sodium phosphate buffer (25 mM, pH 7.4). After loading, the column was subsequently washed with ten column volumes (CV) of the same buffer. Elution of 3PG-F_{420} was achieved by a NaCl gradient during the elution phase: 0-2 CV: 0-150 mM NaCl, 2-18 CV: 150-700 mM NaCl, 18-20 CV: 700-1000 mM NaCl and monitored by UV/VIS absorption at 420 nm. Retention time depended on the length of the glutamic acid tail, e. g., 5-7 ≥ 450 mM NaCl. Fractions with absorption at 420 nm were applied to C18 SPE cartridges as described above. Finally, the desalted 3PG-F_{420}-0 containing FPLC fractions (A19–A22) were submitted to a semi-preparative HPLC equipped with a Phenomenex Luna C8(2) 250 mm ×10 mm to yield 3PG-F_{420}-0 (golden yellow solid, 2.62 mg, t_R = 13.27 min) using the following gradient: 0–5 min, 20% B; 5–25 min, 20%–100% B; 25–30 min, 100% B (A: ddH_2O + 0.1% formic acid; B: MeCN) with a flow rate of 2.0 mL/min. The desalted 3PG-F_{420}-n containing FPLC fractions (A28–B9) were submitted to semi-

preparative HPLC equipped with a Phenomenex Luna C8(2) 250mm × 10 mm to yield 3PG-F_{420}-n (3.96 mg, t_R = 15.03 min) using the following gradient: 0–5 min, 20% B; 5–20 min, 20%–60% B; 20–25 min, 60%–100% B; 25–30 min, 100% B (A: dd H_2O + 0.1% formic acid; B: MeCN) with a flow rate of 2.0 mL/min.

Classical F_{420} was produced in *M. smegmatis* $mc^2$4517 pYUBDuet-fbiABC as described previously (4). At the end of the cultivations, cells were washed with sodium phosphate buffer (25 mM, pH 7.4). *M. smegmatis* cells (135 g wet weight) were resuspended in sodium phosphate buffer (25 mM, pH 7.4), to about 0.5 g/mL. Subsequently, the suspensions were autoclaved for 15 min at 121°C. Extracts were centrifuged for 30 min at 3,100 x g, the supernatants were further processed.

Anion-exchange purification of F_{420} was performed as described above. Fractions with absorption at 420 nm were applied to C18 SPE cartridges as described above. At this step, for instance, the purity of F_{420}-5-7 eluting at 30% MeOH was already estimated by NMR to be ≥ 95%.

1.10 Extraction of deazaflavins from a biogas–producing microbial community

Samples were kindly provided by the Biogas Jena GmbH & Co. KG (Jena, Germany) two times and with an interval of three months. The substrates of the fermenters are about 30% goat manure and 70% mixed plant residues. Fermenting conditions are anaerobic at temperatures over 42 °C. For extraction of metabolites, 500 mL of MeOH was added to 65 g of the liquid portion of the sludge. The suspension was kept in an ultrasonic homogenizer for 30 minutes and stirred for another three hours followed by filtration through several paper filters and solvent evaporation to dryness under reduced pressure. The remaining solid (300 mg) was resuspended in 50 mL H_2O containing 0.25% formic acid. Subsequently, deazaflavins were SPE-purified as described above on a 10 g Chromabond C18 cartridge (Macherey-Nagel). As high amounts of unknown polymers interfered with LC-MS analyses, these were almost entirely removed by upstream HPLC purification similar to the description of analytical HPLC in section 1.7.

1.11 Acid hydrolysis of 3PG-F_{420} and F_{420} followed by chiral UPLC-MS analysis

3PG-F_{420}-0 (**5**, 1.45 mg yellow solid) was dissolved in 100 µL HCl (1 M) and kept at 100 °C for 12 h. Afterwards, the reaction was neutralized by NaOH (1 M), centrifuged, and submitted to chiral UHPLC-MS on a Shimadzu LCMS-2020 system (single quadrupole) using an Astec CHIROBIOTIC R (10 cm x 4.6 mm, 5 µm, Sigma) column at 40 °C. Scan range of the MS was set to *m*/*z* 70 to 1,000 with a scan speed of 5,000 u/s and event time of 0.25 s under positive and negative mode. DL temperature was set to 250 °C with an interface temperature of 350 °C and a heat block of 400 °C. The nebulizing gas flow was set to 1.5 L/min and dry gas flow to 15 L/min. UPLC-MS analysis was conducted using an isocratic

condition: 0–16 min, 15% A/85% B (A: dd H_2O with 33.3 mM NH_4OAc; B: MeCN) with a flow rate of 0.7 mL/min under negative selected ion monitor (SIM) mode to detect the *m*/*z* 105.3 $(M–H)^-$.

1.12 Nuclear magnetic resonance (NMR) spectroscopy

All measurements were performed on a Bruker AVANCE II 300 MHz and 600 MHz spectrometer equipped with a Bruker Cryoplatform. The chemical shifts are reported in parts per million (ppm) relative to the solvent residual peak of D_2O (^1H: 4.79 ppm, singlet) and DMSO-d_6 (^1H: 2.50 ppm, quintet; ^{13}C: 39.52 ppm, heptet).

1.13 Chemical synthesis of 2-phospho-l-lactate (2-PL)

Synthesis was performed as described in the literature (5). To a solution of benzyl (*S*)-2-hydroxypropanoate (750 mg, 4.16 mmol) in pyridine (5.55 mL) was dropped at 0°C diphenyl phosphoryl chloride (905 µL, 4.37 mmol, 1.05 equiv.) and the suspension was stirred at 25 °C for 24 h. The reaction was stopped by addition of H_2O (1 mL) and the volatiles removed *in vacuo*. The residue was dissolved in toluene (7 mL) and washed with H_2O, 1 M aq. HCl, sat. $NaHCO_3$-solution and brine successively. The organic phase was dried over $MgSO_4$, filtered and the volatiles removed *in vacuo* to yield 1.54 g of colorless oil, which was directly used in the next step. The oil was dissolved in EtOH (41.7 mL) and PtO_2 (82.6 mg, 636 µmol) was added. Hydrogen gas from a balloon was bubbled through the stirred black suspension for 3 h, then the mixture was left stirring under hydrogen atmosphere for additional 22 h with renewal of the atmosphere, when the balloon was depleted. After filtration of the mixture through a pad of Celite the volatiles were removed in vacuo to yield a greenish highly viscous oil. 2-PL was further purified via Chromabond C18 SPE cartridge (Macherey-Nagel). The flow-through was lyophilized and yielded 629 mg of 2-PL as colorless oil (47% yield over two steps). The analytical data was consistent with the literature.

^{1}H NMR (300 MHz, D_2O) δ 4.71 (dq, *J* = 7.5, 6.9 Hz, 1H, 2-H), 1.44 (t, *J* = 6.9 Hz, 3H, 3-H) ppm.
^{13}C NMR (75 MHz, D_2O) δ 175.6 (d, C-1), 70.6 (dd, C-2), 18.8 (dq, C-3) ppm.

1.14 Heterologous production and purification of CofC and CofD

E. coli BL21 (DE3) was individually transformed with pFS03 (encoding His_6-CofC from *P. rhizoxinica*) or pFS04 (His_6-CofD from *M. jannaschii)*. A culture of 100 mL (50 µg/mL kanamycin or 25 µg/mL chloramphenicol) was grown at 37 °C and 180 rpm until OD_{600} = 0.7 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein production followed for 20 h at 17 °C and 180 rpm. Harvesting and purification steps of proteins were conducted at 4 °C. Cells were pelleted for 25 min at 8000 × g and suspended in lysis buffer (NaH_2PO_4 50 mM and NaCl 300 mM, 20 mM imidazole). Pulsed sonication was applied for 60 s with 10 s pause to lyse cells (SONOPLUS Ultrasound-

Homogenizer HD 2070, BANDELIN). Cell debris was separated by centrifugation for 30 min at 11000 × g and the hexa-histidine fusion proteins were purified from the supernatant by metal-affinity chromatography (HisPur™ Ni-NTA Resin column, Thermo Scientific) using lysis buffer with increasing concentration of imidazole (elution by 500 mM imidazole). Re-buffering of proteins was performed in a PD10 column (Sephadex™ G-25 M, GE Healthcare).

1.15 In-vitro CofC/CofD combined enzyme assay

Enzyme assays combining CofC and CofD to assess their substrate specificity were performed analogously to Grochowski *et al.* (6) and product formation of F_{420}-0 derivatives was monitored by LC-MS. Reactions were carried in 50 µl-reaction mixtures of 100 mM HEPES buffer (pH 7.4), 2 mm GTP, 2 mM $MgCl_2$, 50 ng Fo (0.14 nM), 34 µm CofD (WP_010870769), and 0.5 mM of substrates (either 3-phospho-D-glyceric acid, 2-phospho-D-glyceric acid, phosphoenolpyruvic acid or 2-phospho-L-lactate). The reactions were initiated upon addition of 26 µM CofC (WP_013435883). For the competition assay, several substrates were used at the same time. Reactions were stopped after 0, 20, 40 and 60 min to monitor the rate of product formation. All reactions were carried out at 37 °C and 300 rpm. Reactions were quenched by addition of one volume of acetonitrile and 10 µl of formic acid (20 %) and centrifuged at 13000 × g for 30 minutes to pellet precipitates. Supernatants were analyzed by LC-MS. The LC method was the same as stated using a Luna® Omega 1.6 µm C18 100 Å and size of 100 x 2.1 mm (Phenomenex®). ESI-MS analysis was performed with a resolution of 70,000 and restricted to a mass range of 350 to 600 m/z to enhance sensitivity. Individual XICs ($[M+H]^+$) were extracted from raw LC/MS data (3PG-F_{420}-0: m/z 532.09364-532.09896, F_{420}-0: m/z 516.09881-516.10397, DF_{420}-0: m/z 514.08317-514.08831. Quantification was achieved by integration of specific extracted ion chromatograms (XICs). Area under curve (AUC) for each product was then normalized to the area corresponding to F_0 at time point zero. The normalized area was plotted over time and slopes of product formation were determined from the near-linear time range of the assay (20 min to 40 min) to mirror substrate turnover. Relative percentages of substrate turnover were calculated, their mean and standard deviation (SD) were derived from three independent biological replicates (N=3). Error bars of bar charts represent the SD.

16 Heterologous production of Fno and enzymatic reduction of 3PG-F_{420} (kinetics)

For Fno production, *E. coli* BL21 (DE3) / pDL008 was grown in 150 mL of LB added with kanamycin (50 µg/mL) at 37 °C and 180 rpm to an OD_{600} of 0.45. Induction with 1 mM IPTG was followed by 20 h of further incubation at 20 °C and 180 rpm. The pellet was washed with 20 mL potassium phosphate buffer (1.5 M, pH 8.0), resuspended in 5 mL of the same buffer and lysed on ice by 3 cycles

of 1 min sonication (SONOPLUS Ultrasound-Homogenizer HD 2070, BANDELIN) with 30 s between each cycle. After centrifugation (15 min at 3,100 × g), the supernatant was incubated for 30 min at 90 °C to denature most host proteins. This was followed by another centrifugation step (15 min at 3,100 × g) and one-to-four dilution with sodium phosphate buffer (50 mM, pH 7.5, 300 mM NaCl, 20 mM imidazole). Subsequently, metal-affinity chromatography (HisPur^{TM} Ni-NTA Resin column, Thermo Scientific) was performed. After sample loading, the column was washed with two column volumes of the same buffer. Elution of the protein was achieved with buffer containing 500 mM imidazole. Finally, the buffer was exchanged to 1 mL potassium phosphate buffer (50 mM, pH 6) via centrifugal filtration (10 kDa cut-off). Fno-mediated turnover of oxidized 3PG-F_{420} (absorbing at 400 nm, ε_{400}=25.7 mM^{-1} cm^{-1}) to reduced 3PG-$F_{420}H_2$ (non-absorbing) was measured by FLUOstar Omega reader (BMG Labtech). The assay contained 0-14 µM 3PG-F_{420} or 0-9.3 µM F_{420}, 250 µM NADPH and 0.149 µg/mL Fno in potassium phosphate buffer (50 mM, pH 6). Initial reaction rates were obtained by linear regression and plotted against 3PG-F_{420} or F_{420} concentrations. Three biological replicates (N=3) were measured and combined in one analysis. Michaelis-Menten parameters (v_{max} and K_M and corresponding standard errors) were obtained by nonlinear regression using SigmaPlot 12 with Enzyme Kinetics Wizard. Error bars show the standard deviation of the initial reaction speeds of the three replicates.

1.17 In-vivo reduction of malachite green

E. coli BL21 (DE3) transformed with pDB061, pDB071, or a combination of both was cultivated overnight as detailed. At this moment, gene expression was induced with 200 µM IPTG and the cultivation received 50 mg L^{-1} malachite green as described in the literature (7). 1-mL aliquots were centrifuged and UV absorbance at 618 nm of supernatants was measured. The reduction of malachite green to leucomalachite green was indicated by the reduction of absorbance over time. Values were recorded from three independent experiments. All data represent mean ± SD. Statistical comparison was performed in GraphPad Prism version 5.0 for Windows by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The value of $p < 0.05$ was considered statistically significant.

1.18 Fluorescence spectroscopy

Purified 3PG-F_{420} and F_{420} were dissolved in sodium phosphate buffer (50 mM, pH 7.5). Fluorescence spectra were recorded in a CLARIOstar reader (BMG Labtech).

Table S1: The 3PG-F_{420} biosynthetic gene cluster from *P. rhizoxinica* HKI454 (GenBank accession number: NC_014722).

Gene	Locus_tag	Proposed function
ribA	RBRH_RS08950	GTP cyclohydrolase II
	RBRH_RS08955	ABC transporter
cofD	RBRH_RS08960	2-phospho-L-lactate transferase
fbiC	RBRH_RS08965	FO synthase
cofC	RBRH_RS08970	2-phospho-L-lactate guanylyltransferas
cofE	RBRH_RS08970	coenzyme F_{420}-0: γ-L-glutamate ligase

Table S2. Primers used in this study (nucleotide sequences).

Name	5' - 3' sequences
cofC - fw	AGGTCGACAAGCTTGCGATGTCTCCTGTTTCTGTCGC
cofC - re	CTTAAGCATTATGCGGCCTAGATTTGGCATAGCACCCT
cofD - fw	AAATGGGTCGCGGATCCATGGCAAAGTATGTTGCGTTGTG
cofD - re	CGACGGAGCTCGAATTCTTATGCGGTGCTGTGCTGCA
cofE_fw	AAATGGGTCGCGGATCCATGACTGTATCCGCCATC
cofE_rv	CGACGGAGCTCGAATTCTTAGGCCATTTCCACCG
DB_pDB044_FP	AAACCAGCAAGAAGCATGACTGTATCC
DB_pDB044_RP	CCTCTACAATGACAACGGGCAC
DB_FP_MBP_5998	GGATCGAGGGAAGGATTTCAGAA
DB_RP_MBP_5998	AAGCTTGCCTGCAGGTCGAC
DB_FP_pMal-c2X	GTCGACCTGCAGGCAAGCTT
DB_RP_pMal-c2X	TGAAATCCTTCCCTCGATCC
fbiB_fw	AAATGGGTCGCGGATCCATGAGCGCAGCAGCAAATGCA
fbiB_rv	CGACGGAGCTCGAATTCTTATTTACGAACCAGCAGTTCATCGG
oDB08	CATGCTTCTTGCTGGTTTAAACTTATTCAGGCTGCTTGTGTTGCAATG
oDB021	TTTGTGGTAGCCTGTAAGGTTGATGGCTATACAGAAA
oDB022	CTCAGAACGGTAATCACTATCTTCCTGCGTAAAGATC
oDB023	GTGATTACCGTTCTGAGCG
oDB024	TTACAGGCTACCACAAAATTCAATG
oDB069	ATTCCTCAAGGTCGGCATTAACGACGTTTAACAAC
oDB070	ACCATTGCATCTCATATAGGC
oDB071	TGCCGACCTTGAGGAATTC

oDB081	GGATCCGAATTCGAGCTC
oDB084	AGCTCGAATTCGGATCCTCACAGAAATTTGATACCCAGTTCG
oDB085	GGTACCCTCGAGTCTGGT
oDB088	CCAGACTCGAGGGTACCTCATTGTTCCGCTTCACTCAAG
oDB105	CTAATATACTAAGATGGGGAATTG
oDB106	CGAGTCTGGTAAAGAAACC
oDB107	CAATTCCCCATCTTAGTATATTAGGAGATGCAATGGTATGTCTCC
oDB108	GGTTTCTTTACCAGACTCGTTATGCGGTGCTGTGCTGC
oDB115	GAGCTCGAATTCGGATCCTTATGCGGTGCTGTGCTG
oDB122	GTTTAACTTTAATAAGGAGATATACCATGCGTGTGGCTCTGCTG
oDB123	GGTATATCTCCTTATTAAAGTTAAACA
oDB124	ATGTATATCTCCTTCTTATACTTAACT
oDB125	GTTAAGTATAAGAAGGAGATATACATATGATCCAAGAGGTCGAACG
oDB128	GCCTATATGAGATGCAATGGTATGAACTGCGGCATCAAAATGA
oDB129	GAGCTCGAATTCGGATCCTTATGCGGTGCTGTGCTG
oDB129B	TAATAAGGAGATATACCATGTCTCCTGTTTCTGTCGC
pACYCduet-fw	GCCGCATAATGCTTAAGTCG
pACYCduet-rv	CGCAAGCTTGTCGACCTG
pET28a-fw	GAATTCGAGCTCCGTCGACAAG
pET28a-rv	GGATCCGCGACCCATTTGCTGT

Table S3. Plasmids used in the study.

Plasmid	Backbone	Inserted genes	Size (bp)
pDB045	pETDuet-1	*ribA*, *cofC*, *fbiC*, *cofD*, ABC transporter, *cofE*	13,135
pDB060	pETDuet-1	*ribA*, *cofC*, *fbiC*, *cofD**, ABC transporter, *cofE*	13,045
pDB061	pMAL-c2x	MSMEG_5998	6,067
pDB065	pCDFDuet-1	*fno* (MCS1)	4,385
pDB070	pETDuet-1	*ribA*, *cofC**, *fbiC*, *cofD**, ABC transporter, and *cofE*	13,136
pDB071	pCDFDuet-1	*fno* (MCS1) and *cofC*, *fbiC, and cofD* (MCS2)	8,656
pDL008	pET28a	*fno (Archaeoglobus fulgidus)*	5,878
pMH01	pET28a	His6-cofE (*Methanocaldoccoccus jannaschii*)	6,100
pMH02	pCDFDuet	c*ofC*, *fbiC*, *cofD* (*P. rhizoxinica*)	8,085
pFS01	pET28a	His6-*cofE (P.rhizoxinica*)	
pFS03	pACYCDuet	His6-*cofC (P. rhizoxinica)*	4,659
pFS04	pET28a	His6-*cofD (M. jannaschii)*	6,296
pFS06	pER28a	His6-*fbiB (Mycolicibacterium smegmatis)*	6,734

*genes from *Methanocaldococcus jannaschii.*

2 Results

2.1 Fluorescence Microscopy

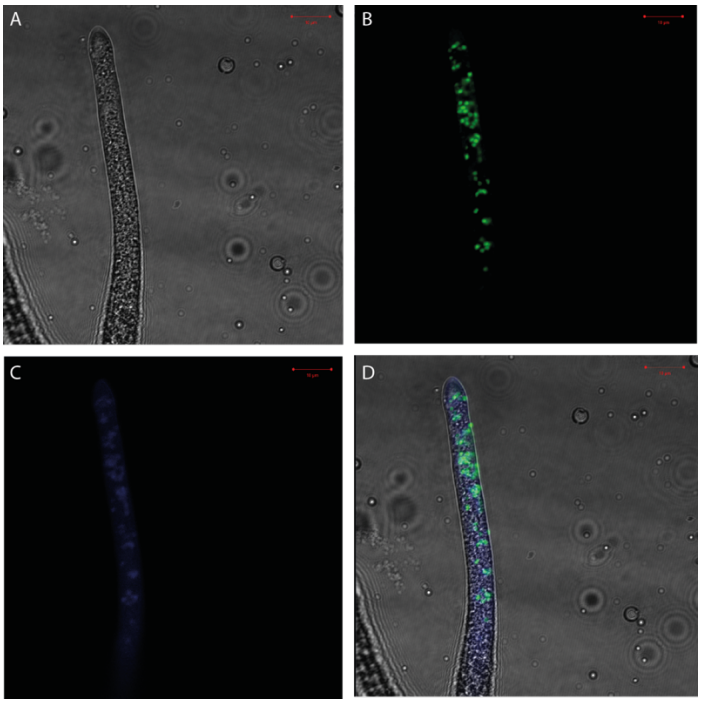


Figure S1. Fluorescence microscopy images of a hyphal tip of *Rhizopus microsporus* ATTC 62417 harbouring *Paraburkholderia rhizoxinica* endosymbionts (green). A) Brightfield B) Syto 9 channel in green C) deazaflavin-channel in blue (excitation: 405 nm, emission: approx. 470 nm; D) Overlay. Symbionts and fluorescence of deazaflavins are present within hyphae. Scale bars represent 10 µm.

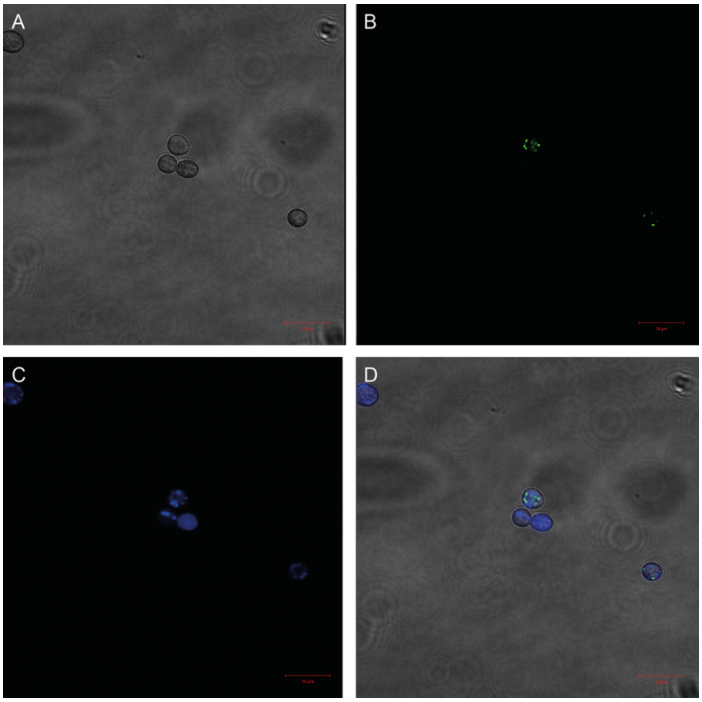


Figure S2. Fluorescence microscopy images of spores of *Rhizopus microsporus* ATTC 62417 harboring *Paraburkholderia rhizoxinica* endosymbionts (stained in green). A) Brightfield B) Syto 9 channel in green C) deazaflavin-channel in blue (excitation: 405 nm, emission: approx. 470 nm; D) Overlay. Symbionts and fluorescence of deazaflavins are present within spores. Scale bars represent 10 µm.

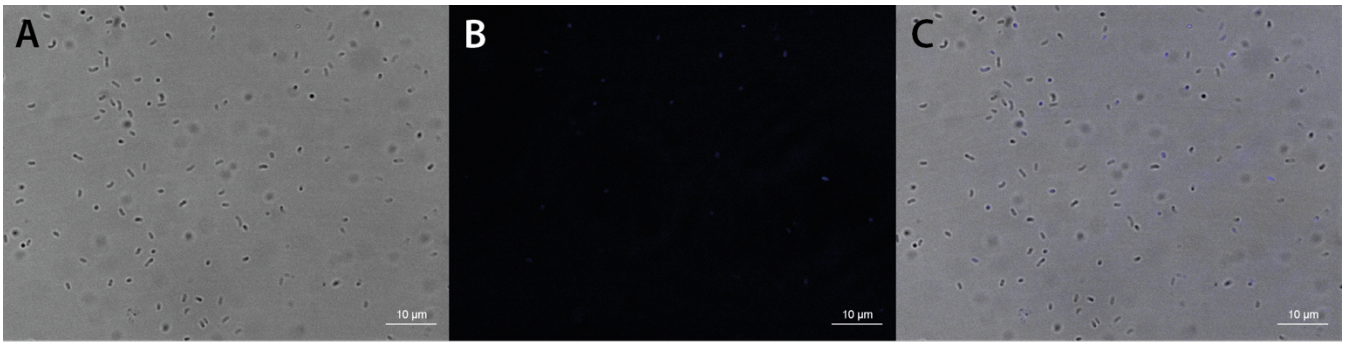


Figure S3. Fluorescence microscopy images of *Paraburkholderia rhizoxinica* in axenic culture. A) Brightfield B) Deazaflavin-channel in blue (excitation: 405 nm, emission: approx. 470 nm; C) Overlay. Bacteria display detectable, but weak, deazaflavin-related fluorescence. Scale bars represent 10 µm.

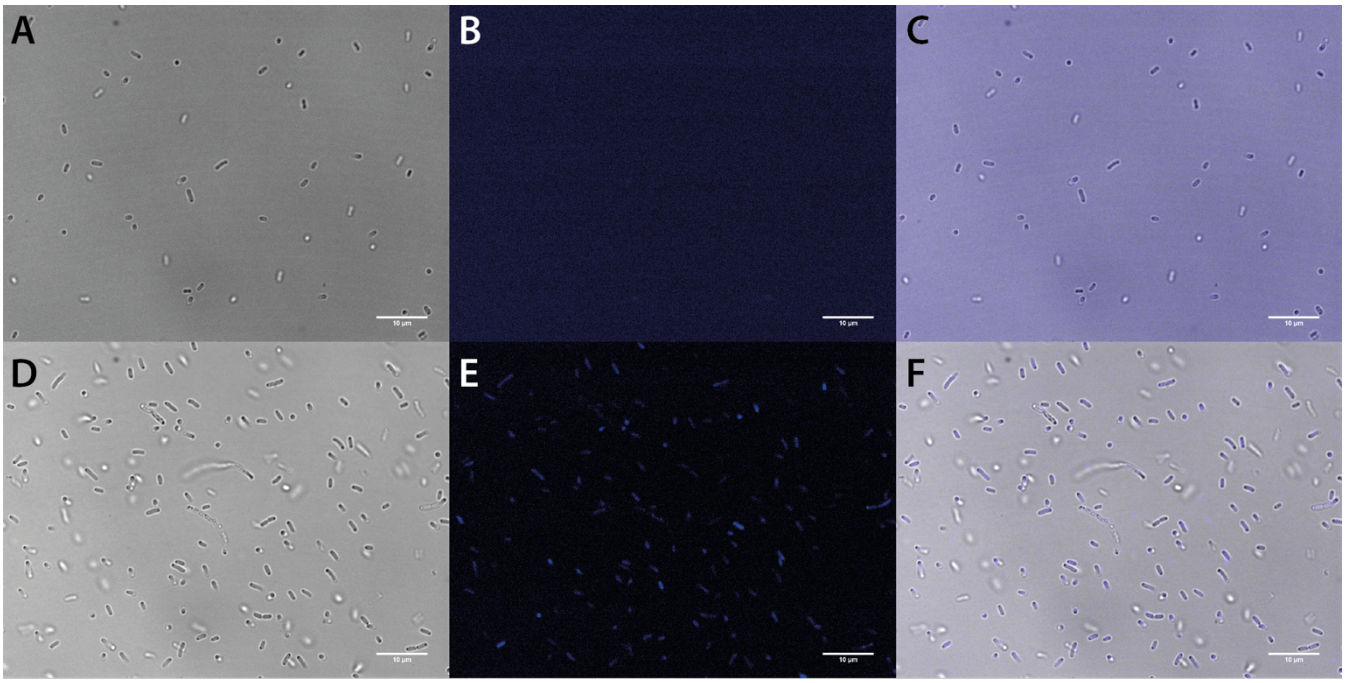


Figure S4. Fluorescence microscopy images of *E. coli*. A – C) Empty vector control (*E. coli* / pETDuet). D – F) 3PG-F_{420}-producing *E. coli* / pDB045. Images shown correlate to brightfield (A, D), blue deazaflavin-channel (excitation: 405 nm, emission: approx. 470 nm; B, E), and brightfield-deazaflavin overlay (C, F). Engineered bacteria *E. coli* / pDB045 display strong deazaflavin-related fluorescence, fluorescence of vector controls *E. coli* / pETDuet is close to noise level.

2.2 Detection of 3PG-F_{420} by LC-MS/MS

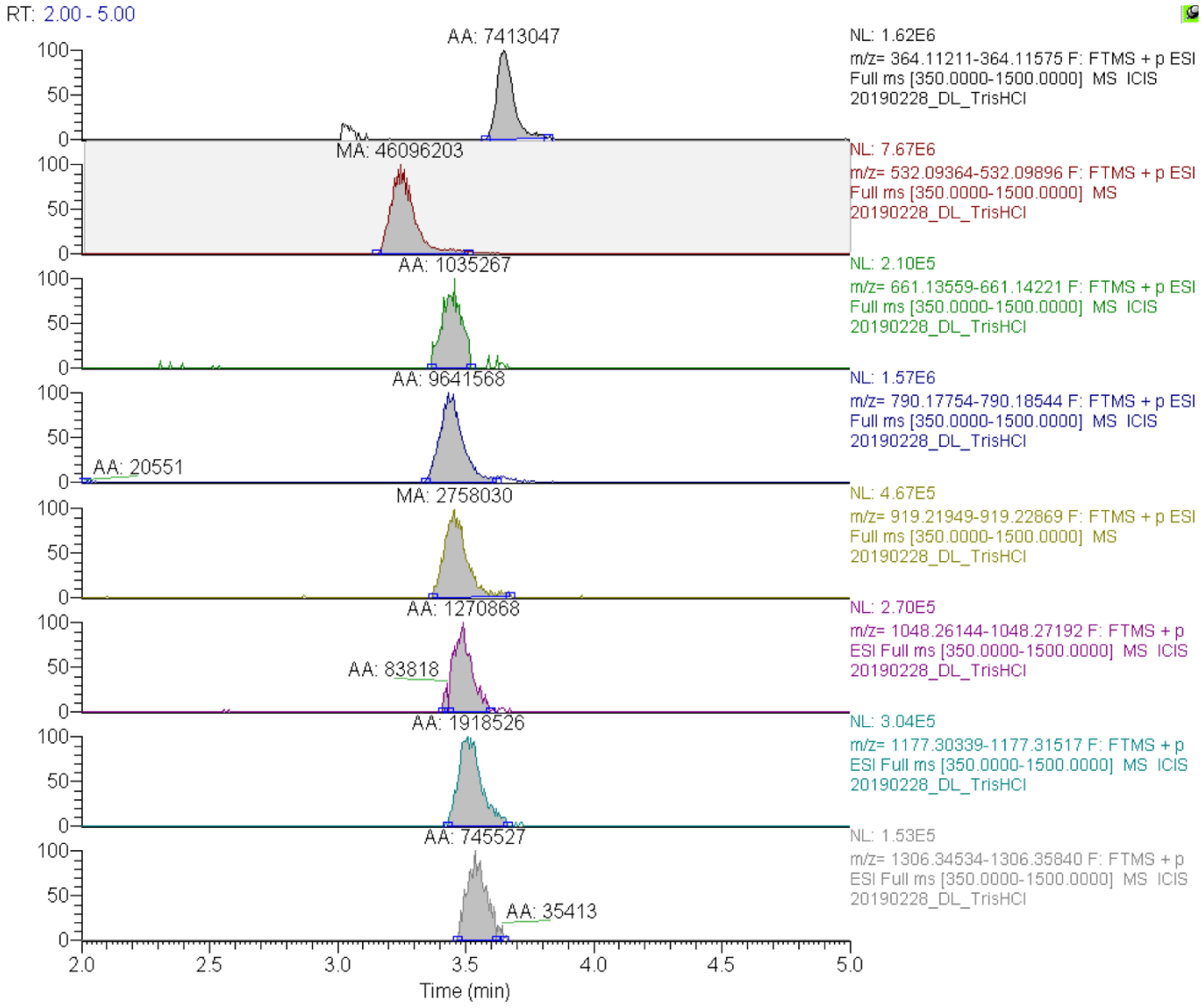


Figure S5. LC-MS analysis of extracts of 3PG-F_{420}-producing *E. coli* BL21(DE3) /pDB045 showing XICs of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 10 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187. Areas under the curve are indicated on top of each peak.

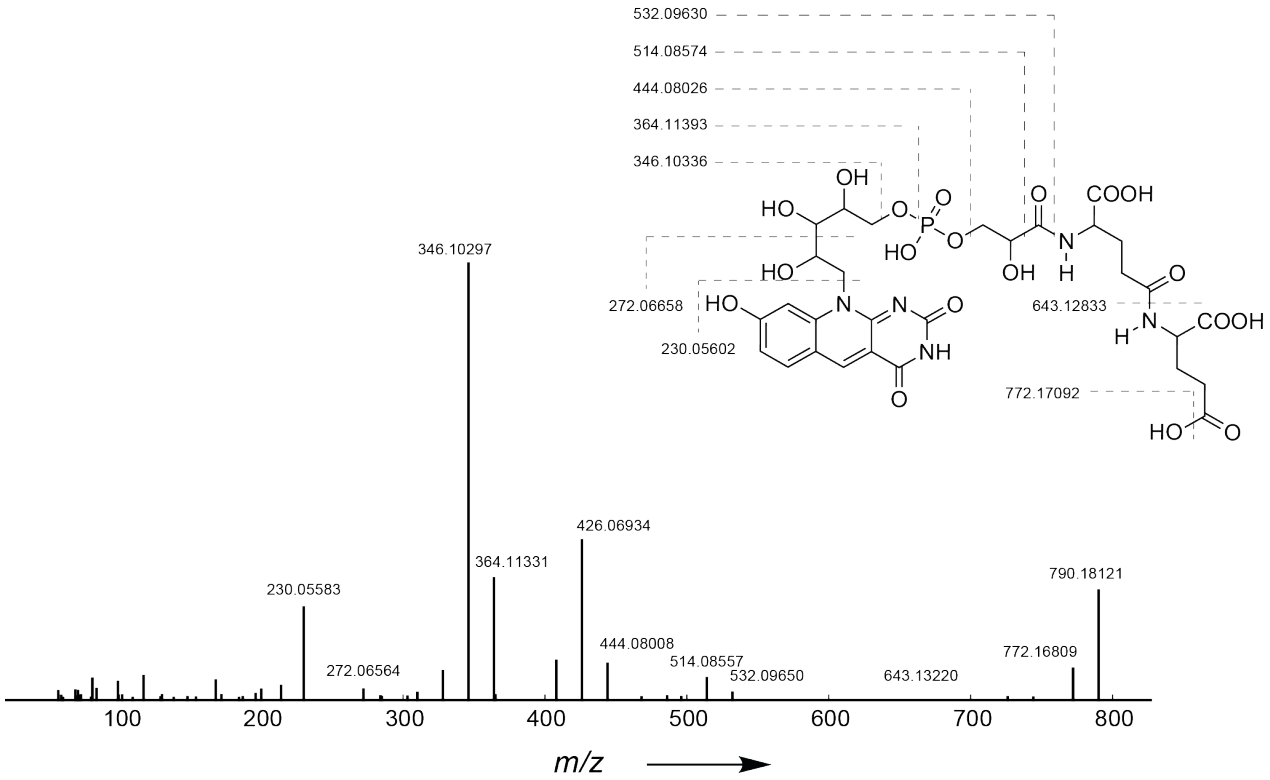


Figure S6 Tandem-mass spectrum (MS^2) of 3PG-F_{420}-2 (precursor mass: $[M+H]^+$ m/z 790.18212, calculated: 790.18149) measured on a Thermo Q Exactive mass spectrometer (NCE 30, resolution: 17,500). Measured masses of characteristic peaks are indicated in the spectrum. The proposed fragmentation pattern with calculated fragment masses is shown (top right).

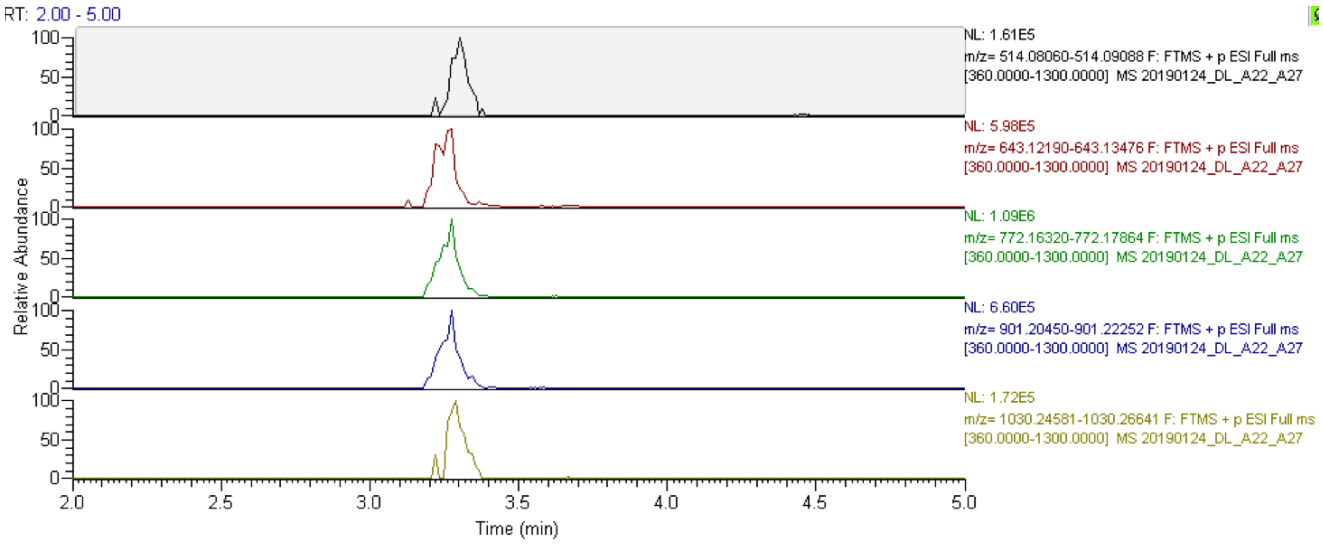


Figure S7. LC-MS analysis of extracts (large-scale cultivation) of *E. coli* BL21(DE3) /pDB45 showing XICs of dehydro-F_{420}-n (DF_{420}-n) species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): DF_{420}-0: 514.08574, DF_{420}-1: 643.12833, DF_{420}-2: 772.17092, DF_{420}-3: 901.21351, DF_{420}-4: 1030.25611.

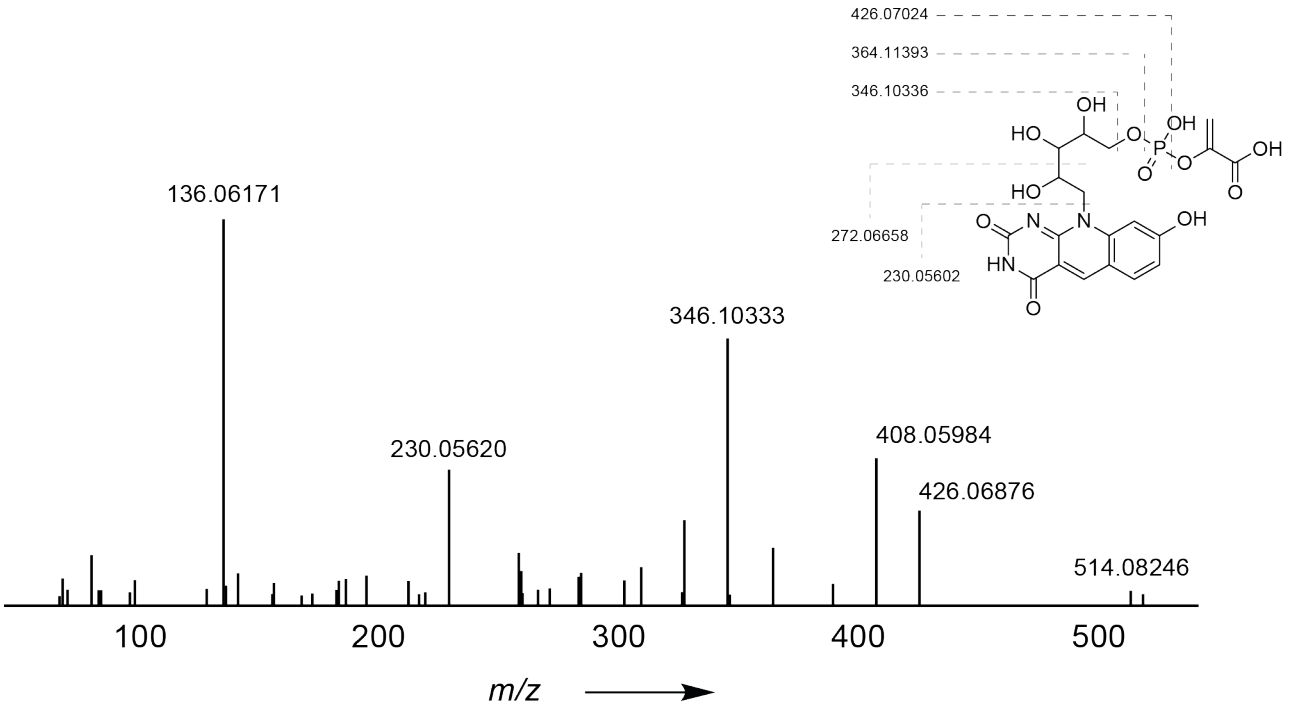


Figure S8 Tandem-mass spectrum (MS^2) of dehydro-F_{420}-0 (DF_{420}-0, precursor mass: $[M+H]^+$:m/z 514.08246, calculated: 514.08574) measured on a Thermo Q Exactive mass spectrometer (NCE 30, resolution: 17,500). Measured masses of characteristic peaks are indicated in the spectrum. The proposed fragmentation pattern with calculated fragment masses is shown (top right). The peak at m/z 408.06 can be explained as fragment ion m/z 426.07 minus H_2O (-18.01).

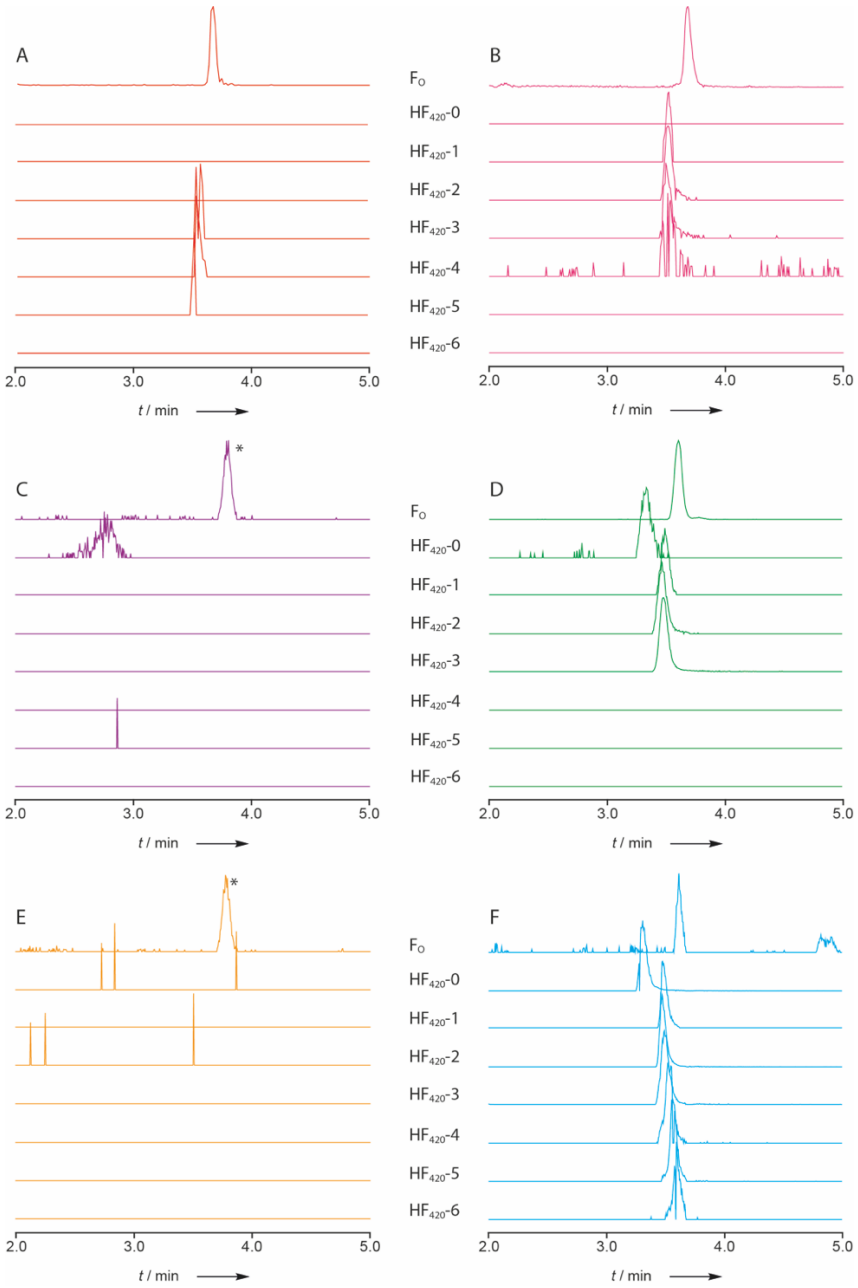


Figure S9. Overview of presence and absence of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues as assessed by LC-MS of microbial extracts (extracted ion chromatograms). Intensities are not drawn to scale, instead, minor peaks are zoomed for better visibility. A) *P. rhizoxinica* axenic culture (red), B) Symbiotic *P. rhizoxinica* isolated from *R. microsporus* ATCC 62417 (magenta), C) cured *R. microsporus* ATCC 62417 without symbionts (purple). D) *R. microsporus* ATCC 62417 with intracellular symbionts (green), E) Naturally symbiont-free *R. microsporus* CBS 344.29 control (yellow), G) 3PG-F_{420} producing *E. coli* BL21(DE3)/pDB045 (blue). Extracted ion chromatograms (10 ppm mass tolerance) were extracted using the following exact masses ($[M+H]^+$): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187. *The asterisks mark a fungal background peak with an m/z similar to the calculated m/z of F_O, but consistently shifted retention time and lower intensity (see Figure S12).

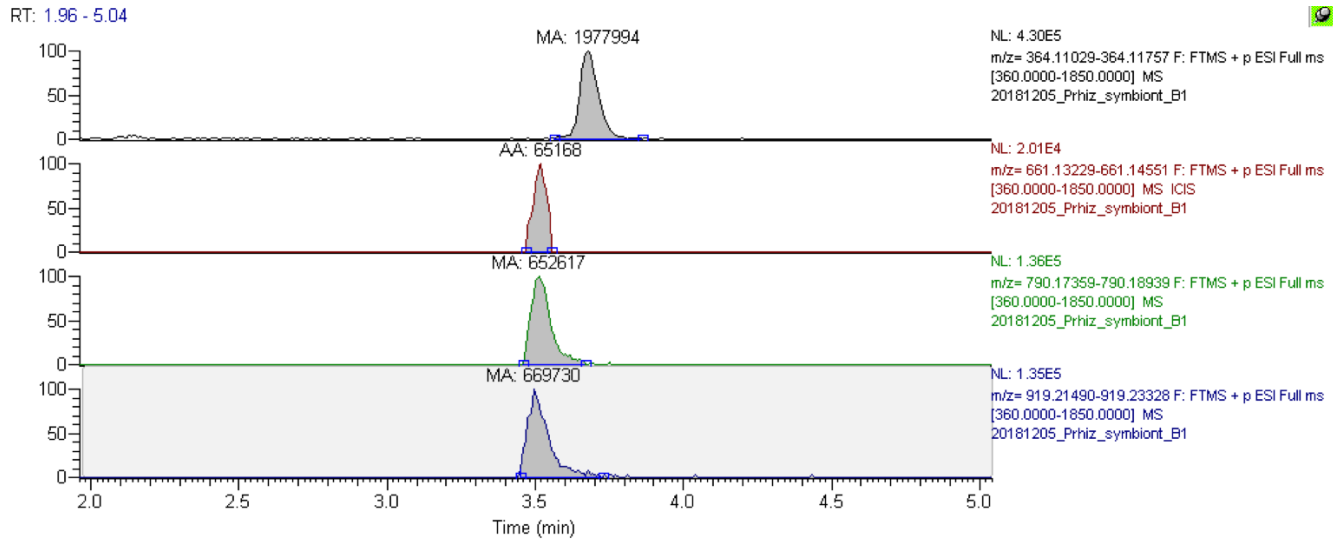


Figure S10. LC-MS analysis of extracts of *P. rhizoxinica* pellets isolated from symbiotic growth conditions showing extracted ion chromatograms (XIC) of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 10 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409. XICs of missing 3PG-F_{420}-n species are not shown. Areas under the curve are indicated on top of each peak.

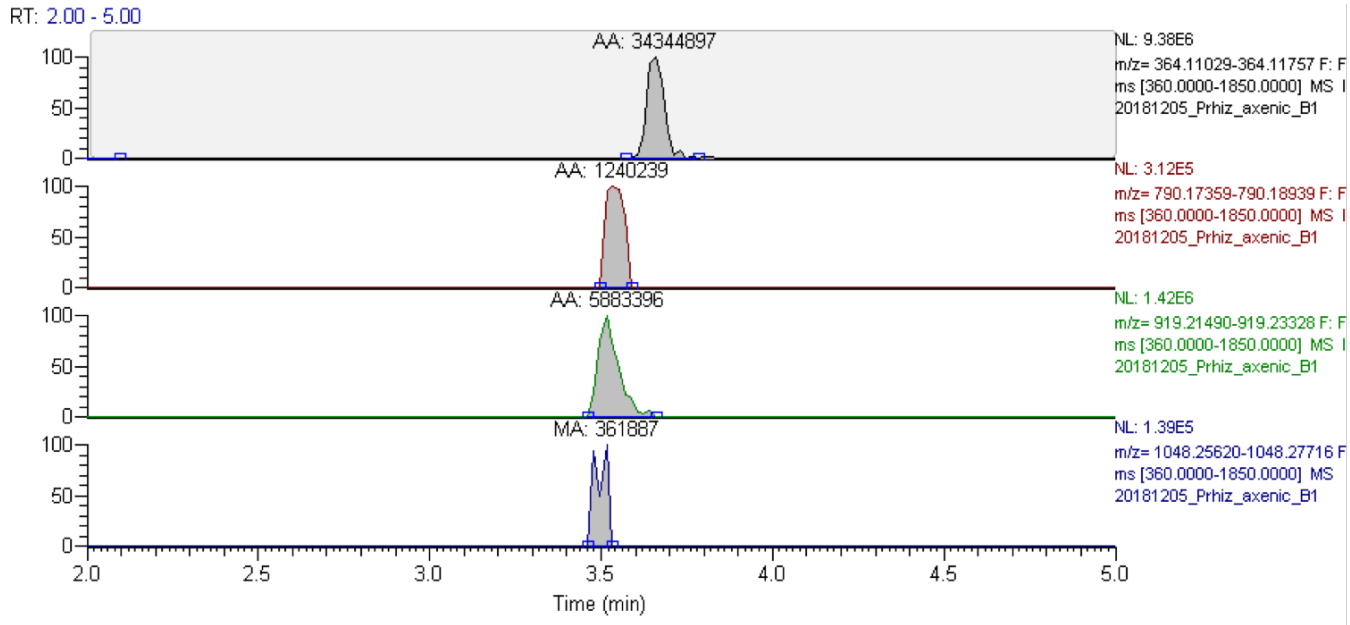


Figure S11. LC-MS analysis of extracts of *P. rhizoxinica* grown in axenic culture showing XICs of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues Expected masses ($[M+H]^+$, 10 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668. XICs of missing 3PG-F_{420}-n species are not shown. Areas under the curve are indicated on top of each peak.

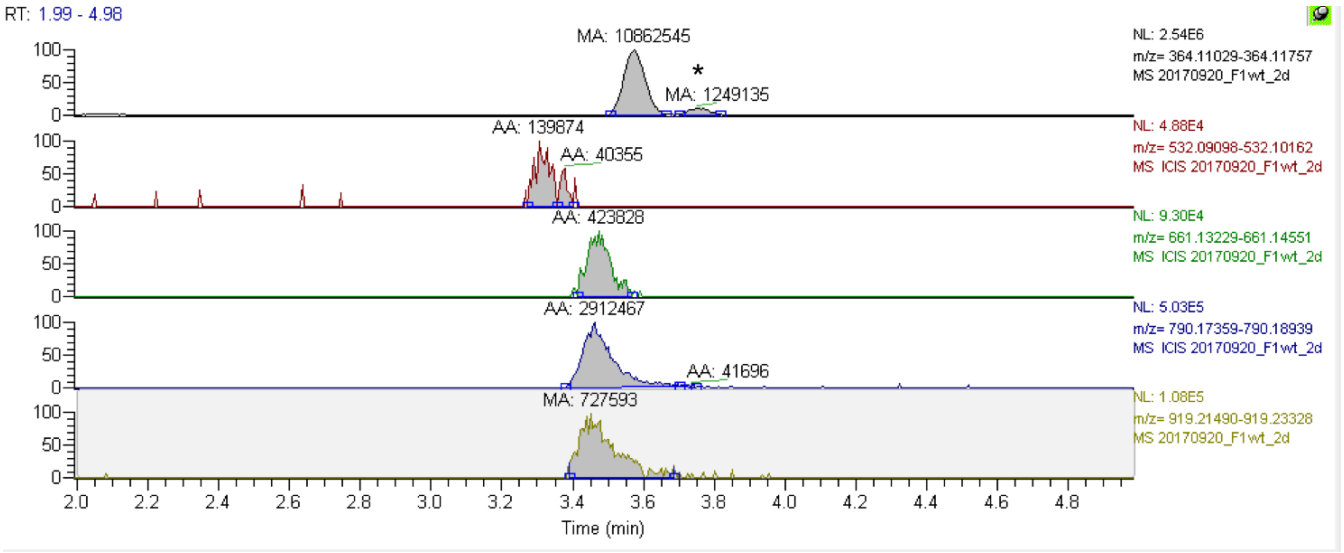


Figure S12. LC-MS analysis of extracts of *R. microsporus* ATCC 62417 with intracellular symbionts (*P. rhizoxinica*) showing XICs of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 10 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409. Areas under the curve are indicated on top of each peak. XICs of missing 3PG-F_{420}-n species are not shown. *A fungal background peak with an m/z close to 364.11393 (F_O) elutes shortly after F_O at 3.8 min. This isobaric compound is not related to deazaflavins.

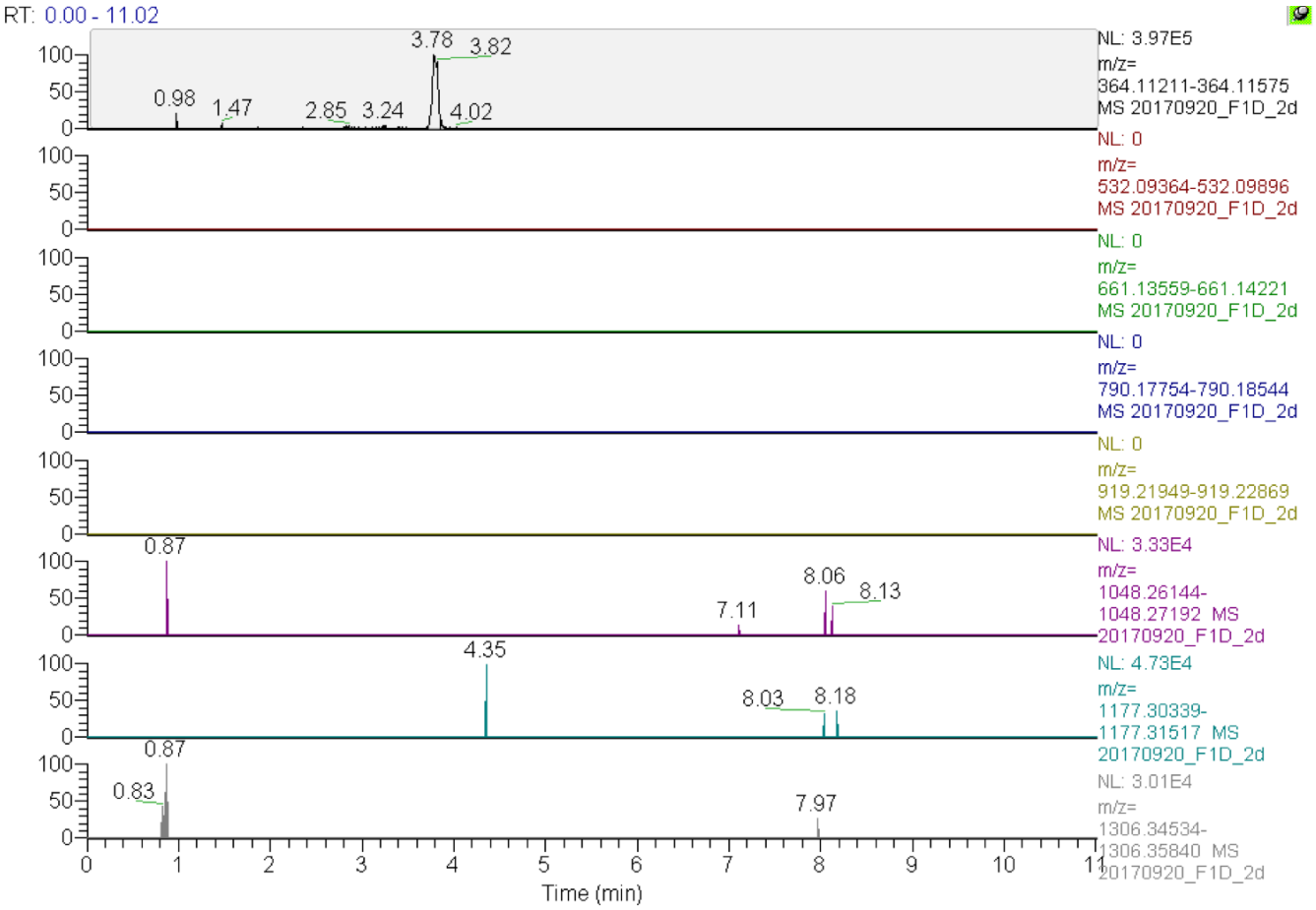


Figure S13. LC-MS analysis of extracts of cured *R. microsporus* ATCC 62417 without symbionts (*P. rhizoxinica*) showing XICs of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 10 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187. The peak eluting at 3.8 is not F_O, but corresponds to the isobaric background compound mentioned above (Figure S12).

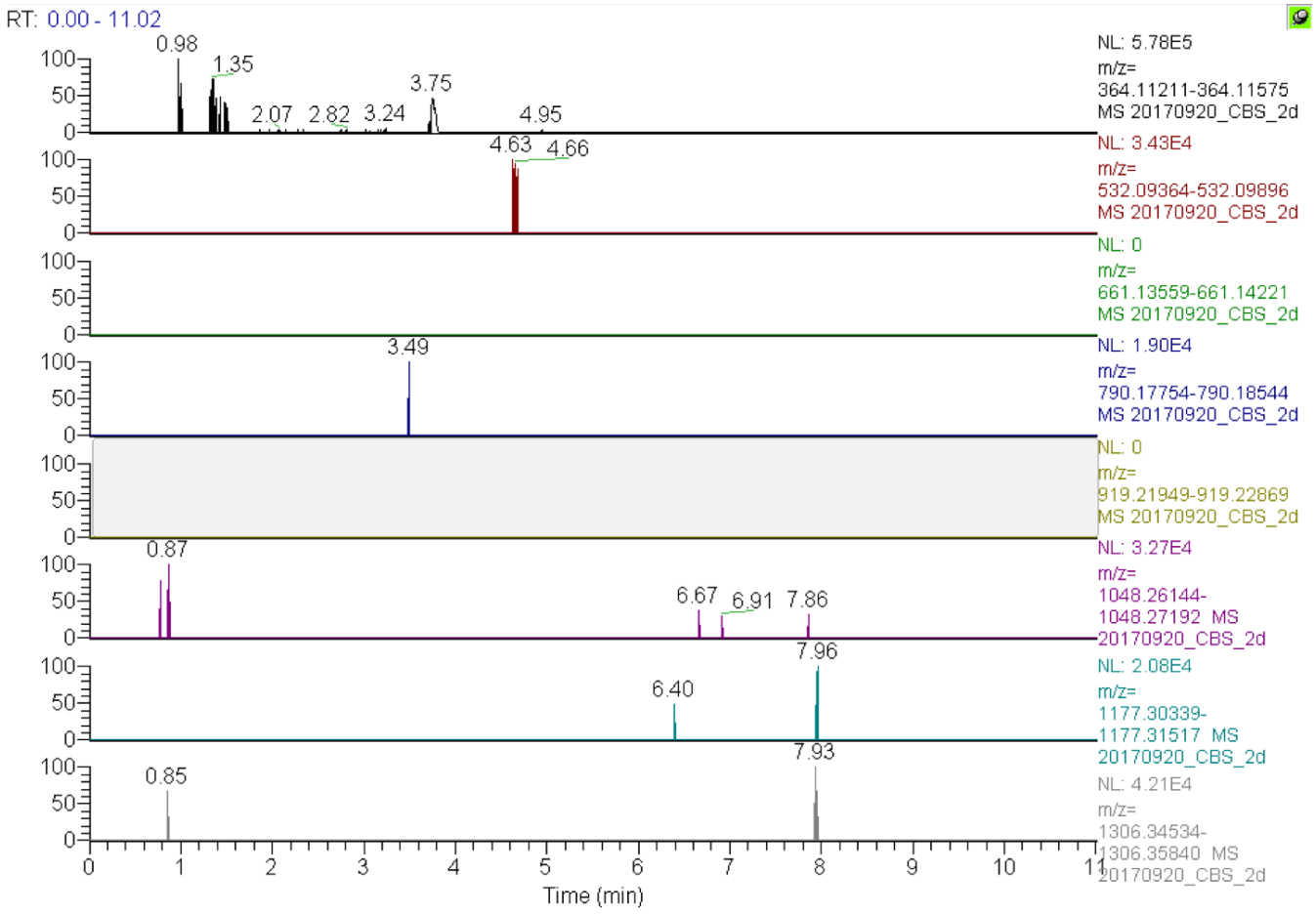


Figure S14. LC-MS analysis of extracts of naturally symbiont-free *R. microsporus* CBS 344.29 showing XICs of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 10 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187. The peak eluting at 3.8 is not F_O, but corresponds to the isobaric background compound mentioned above (Figure S12).

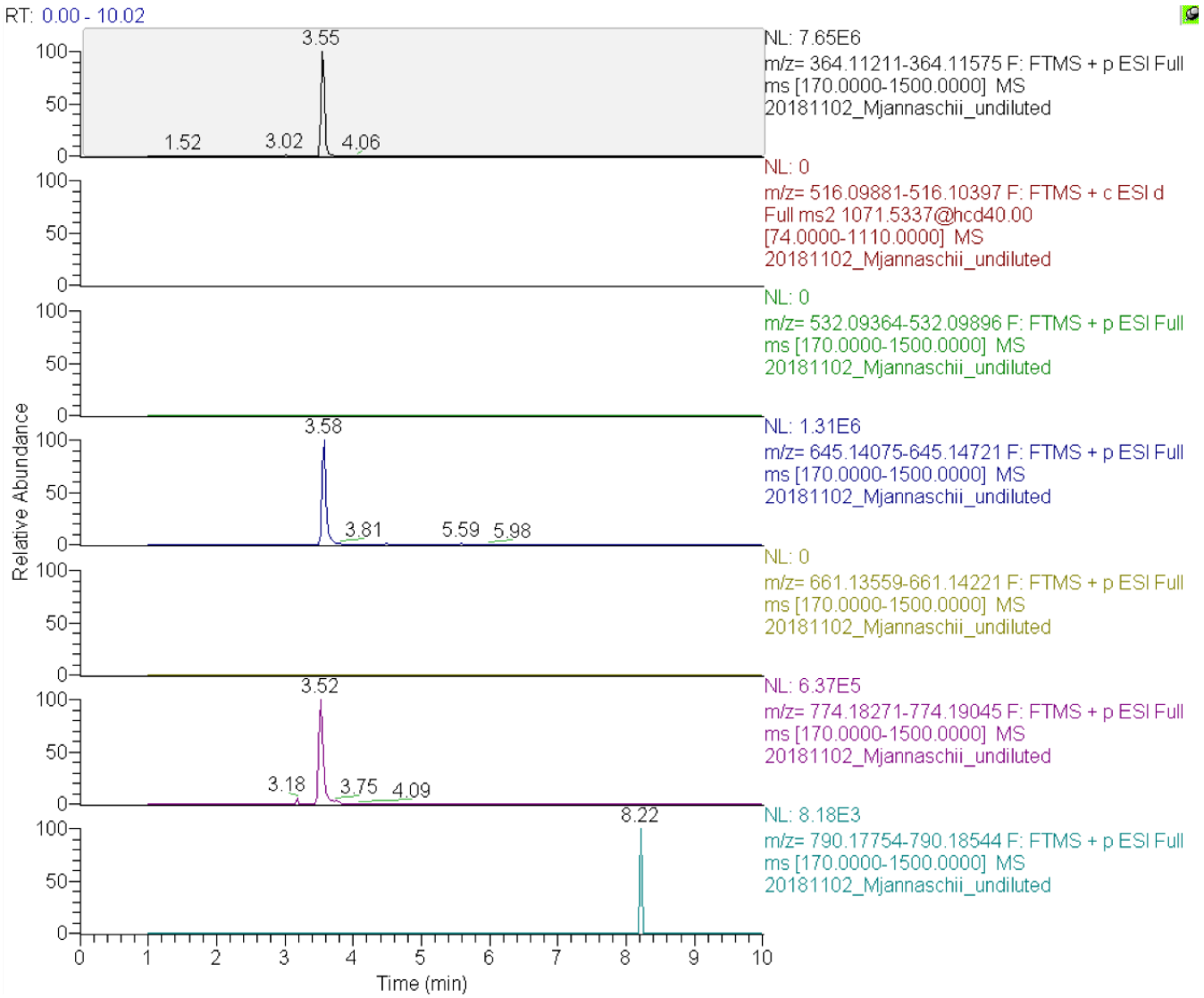


Figure S15. LC-MS analysis of extracts of *M. jannaschii* showing XICs of F_{420}-n and 3PG-F_{420}-n with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, F_{420}-0: 516.10139, 3PG-F_{420}-0: 532.09630, F_{420}-1: 645.14398, 3PG-F_{420}-1: 661.13890, F_{420}-2: 774.18658, 3PG-F_{420}-2: 790.18149. While classical F_{420}-n was produced in good yields, 3PG-F_{420}-0 and F_{420}-0 were not detected.

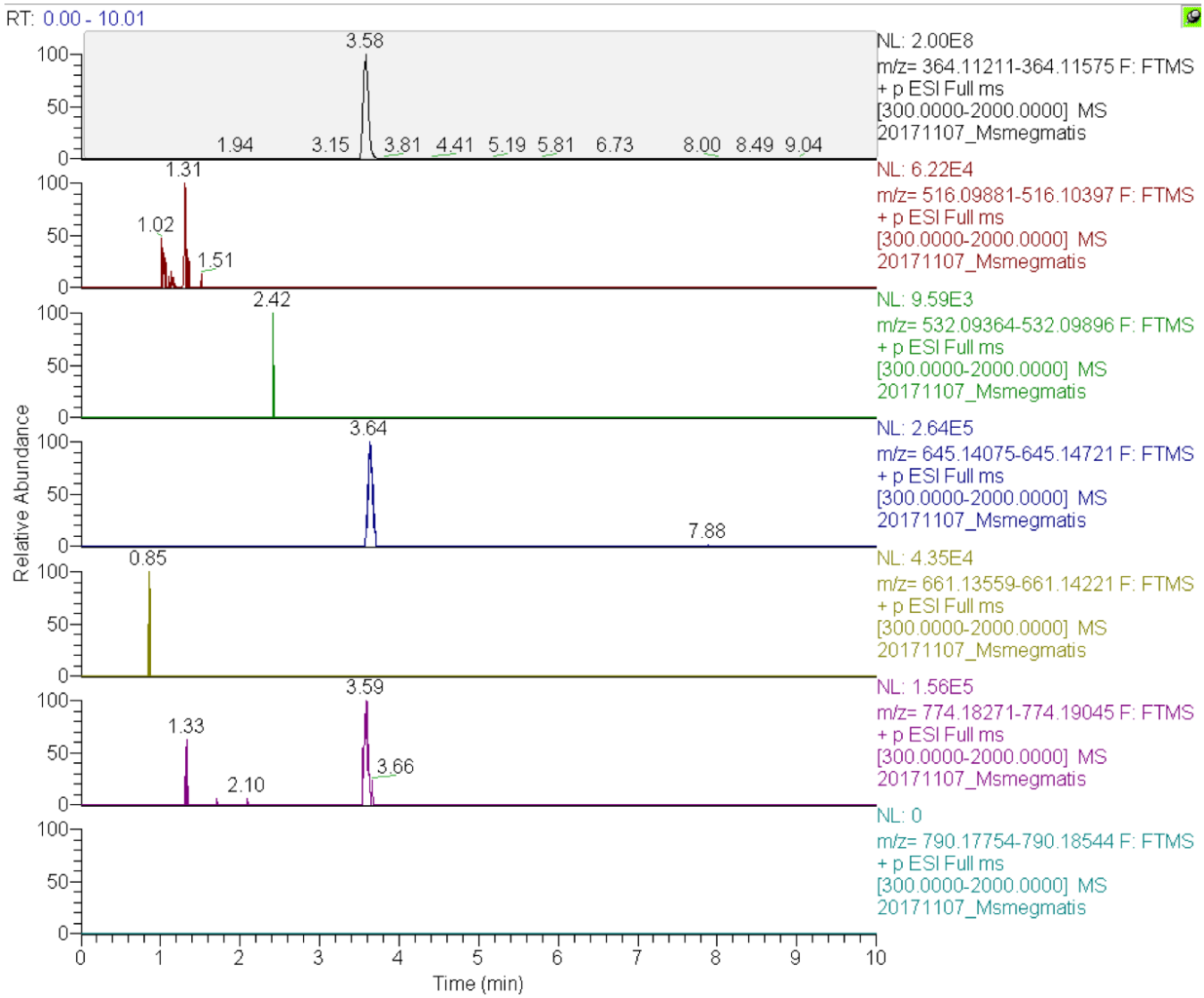
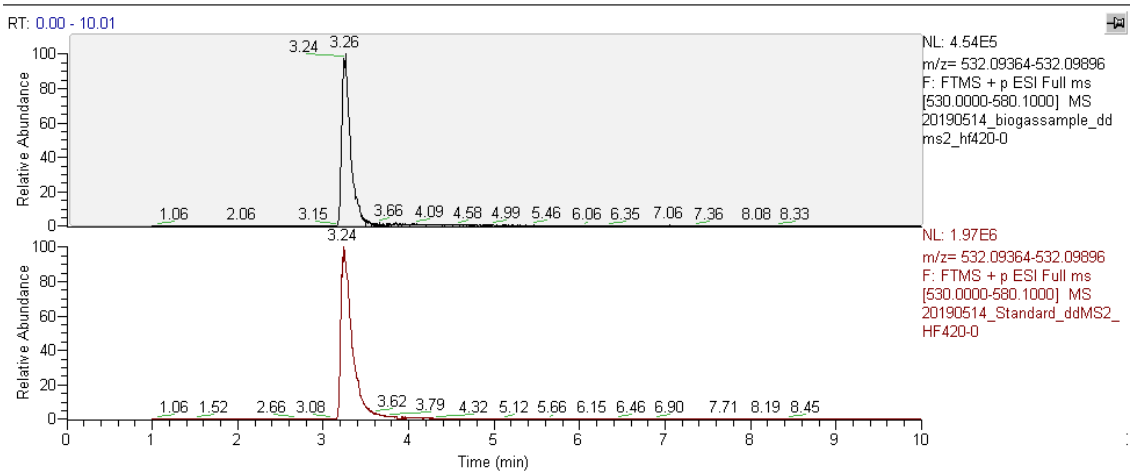


Figure S16. LC-MS analysis of extracts of *M. smegmatis* showing XICs of F_{420}-n and 3PG-F_{420}-n with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, F_{420}-0: 516.10139, 3PG-F_{420}-0: 532.09630, F_{420}-1: 645.14398, 3PG-F_{420}-1: 661.13890, F_{420}-2: 774.18658, 3PG-F_{420}-2: 790.18149. While classical F_{420}-n was produced in good yields, 3PG-F_{420} was not detectable. F_{420}-0 was not found.

A



B

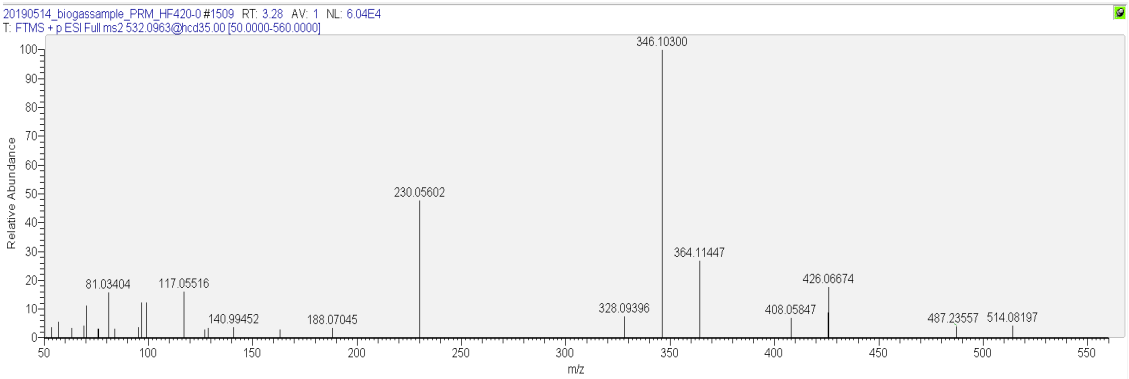


Figure S17. LC-MS analysis of extracts of a biogas-producing microbial community after HPLC purification. A) XIC at m/z 532.09630 (corresponds to 3PG-F_{420}-0 $[M+H]^+$). Upper chromatogram: sample. Lower chromatogram: 3PG-F_{420}-0 standard. B) The MS/MS spectrum of the peak eluting at RT 3.34 min displaying characteristic fragments of F_{420} derivatives (m/z 230.06, 346.10, 364.11, 408.06, 426.07, 514.08).

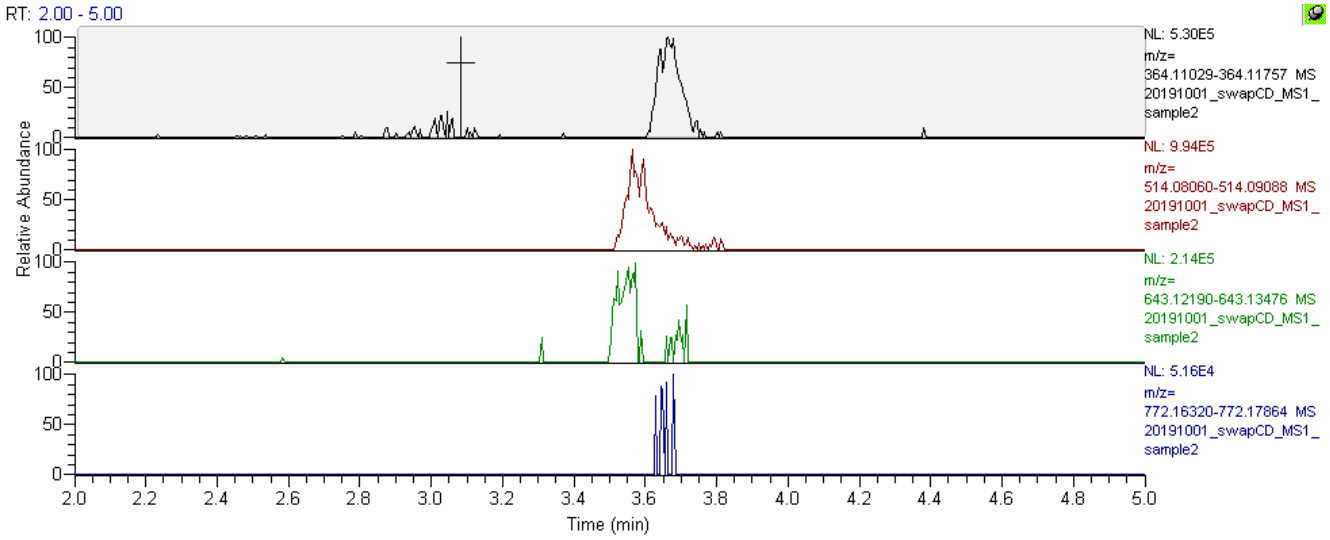


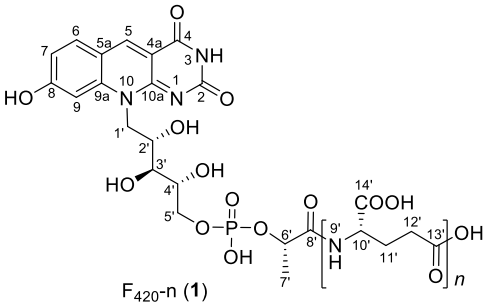
Figure S18. LC-MS analysis of extracts of *E. coli* BL21(DE3) /pDB070 showing XICs of dehydro-F_{420}-n (DF_{420}-n) species with a varying number of (oligo)-γ-glutamate residues. Expected masses ($[M+H]^+$, 10 ppm mass tolerance): DF_{420}-0: 514.08574, DF_{420}-1: 643.12833, DF_{420}-2: 772.17092.

2.3 Structure elucidation of deazaflavins

2.3.1 Structure elucidation of classical F_{420} (control)

Classical F_{420}-n was obtained as described above as yellow solid directly eluted from FPLC and submitted for 1D and 2D NMR analysis. The composition of mixed F_{420}-n was determined as F_{420}-4 with the molecular formula of $C_{39}H_{50}O_{24}N_7P$ based on the ESI-HRMS analysis (*m/z* 1032.26904 ($[M+H]^+$ calcd. 1032.27176 Δ = – 2.63 ppm); F_{420}-5 with the molecular formula of $C_{44}H_{57}O_{27}N_8P$ based on the ESI-HRMS analysis (*m/z* 1161.31250 ($[M+H]^+$ calcd. 1161.31435 Δ = – 1.59 ppm); F_{420}-6 with the molecular formula of $C_{49}H_{64}O_{30}N_9P$ based on the ESI-HRMS analysis (*m/z* 1290.35486 ($[M+H]^+$ calcd. 1290.35694 Δ = – 1.62 ppm); F_{420}-7 with the molecular formula of $C_{54}H_{73}O_{33}N_{10}P$ based on the ESI-HRMS analysis (*m/z* 710.20178 ($[2M+H]^{2+}$ calcd. 710.20341 Δ = – 2.29 ppm). Thorough interpretation of the ^1H NMR and ^{13}C NMR spectra (D_2O) indicated the typical 5-deazaflavin moiety with the observation of $\delta_{H\text{-}5}$ 8.82 ppm, $\delta_{C\text{-}5}$ 144.8 ppm; $\delta_{H\text{-}6}$ 7.91 ppm, $\delta_{C\text{-}6}$ 135.5 ppm; $\delta_{H\text{-}7}$ 7.16 ppm, $\delta_{C\text{-}7}$ 118.1 ppm; $\delta_{H\text{-}9}$ 7.31 ppm, $\delta_{C\text{-}9}$ 102.3 ppm. The ribityl moiety was deduced from the observation of $\delta_{H2\text{-}1'}$ 4.67/4.97 ppm, $\delta_{C\text{-}1'}$ 49.31 ppm; $\delta_{H\text{-}2'}$ 4.37 ppm, $\delta_{C\text{-}2'}$ 70.2 ppm; $\delta_{H\text{-}3'}$ 3.99 ppm, $\delta_{C\text{-}3'}$ 73.3 ppm; $\delta_{H\text{-}4'}$ 4.07 ppm, $\delta_{C\text{-}4'}$ 71.8 ppm; $\delta_{H2\text{-}5'}$ 4.07/4.17 ppm, $\delta_{C\text{-}5'}$ 67.4 ppm. The lactyl moiety was assigned based on the observation of resonance at $\delta_{H\text{-}6'}$ 4.70 ppm, $\delta_{C\text{-}6'}$ 72.6 ppm; $\delta_{H3\text{-}7'}$ 1.49 ppm, $\delta_{C\text{-}7'}$ 19.9 ppm; $\delta_{C\text{-}8'}$ 175.4 ppm. Due to the overlapped signals from the glutamyl chain, the assignment of one unit can be deduced from the observation of $\delta_{H\text{-}10'}$ 4.35 ppm, $\delta_{C\text{-}10'}$ 52.5 ppm; $\delta_{H2\text{-}11'}$ 1.94/2.21 ppm, $\delta_{C\text{-}11'}$ 27.0 ppm; $\delta_{H2\text{-}12'}$ 2.41 ppm, $\delta_{C\text{-}12'}$ 32.0 ppm; $\delta_{C\text{-}13'}$ 175.6 ppm; $\delta_{C\text{-}14'}$ 175.7 ppm. The doublets of C-4', C-5', C-6', C-7' and C-8' indicated the ^{13}C–^{31}P coupling ($^3J_{C\text{-}4'\text{–}P}$ = 7.56 Hz, $^2J_{C\text{-}5'\text{–}P}$ = 5.06 Hz, $^2J_{C\text{-}6'\text{–}P}$ = 4.94 Hz, $^3J_{C\text{-}7'\text{–}P}$ = 2.93 Hz, $^3J_{C\text{-}8'\text{–}P}$ = 6.60 Hz) between the carbon atoms of the ribityl and lactyl moieties assigned to these resonances and the phosphorus of the phosphate group. Finally, the classical F_{420}-n was confirmed based on the match with literature reported NMR data recorded in D_2O.(8) Shift in ^{13}C resonances are presumably a result of the presence of ammonium salts and/or pH differences as indicated in the literature. Detailed assignment is present in Table S4-6.

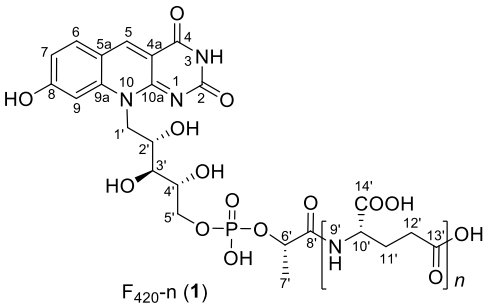
Table S4. NMR Data (DMSO-d_6, at 300 K) for F_{420}-n .[a]



	F_{420}-n				
position	δ_C, mult.[b] (*J* in Hz)[c]	δ_H, mult. (*J* in Hz)	COSY	HMBC	NOESY
N (1)					
2	n.d.				
*N*H (3)		11.04, br s		4a	
4	162.37, qC				
4a	110.65, qC				
5	141.66, CH	8.90, s		4, 5a, 6, 9a, 10a	6
5a	115.64, qC				
6	133.83, CH	8.04, d (*8.87*)	7	5, 5a, 8, 9a	5
7	115.51, CH	7.04, dd (*8.87,*	6, 9	5a, 9	
8	164.53, qC				
9	102.16, CH	7.40, s	7	5a, 8, 9a	1'a, 1'b
9a	144.01, qC				
10					
10a	157.80, qC				
1'a	48.14, CH_2	4.80, m			9
1'b		4.65, m			9
2'	69.28, CH	4.25, m	3'		
3'	73.67, CH	3.62, t (*5.37*)	2', 4'	1', 2', 4'	
4'	70.98, CH_2 (*6.97*)	3.89, m	2', 3'	3', 5'	
5'a	68.25, CH_2	4.13, m	4', 5'b	3'	
5'b		3.94, m	5'a	4'	
6'	71.72, CH (*4.96*)	4.67, q (*7.12*)	8'	7', 8'	*N*H (9')
7'	19.62, CH_3 (*3.19*)	1.39, d (*7.12*)	6'	6', 8'	*N*H (9')
8'	170.77, qC				
*N*H (9')[d]		8.10, d (*8.45*)	10'		6', 7'
10'[d]	51.50, CH	4.15, m	9', 11'b	11', 12', 13', 14'	
11'a[d]	26.97, CH_2	1.96, m	10', 11'a, 12'	10', 12', 13', 14'	
11'b[d]		1.74, m	10', 11'a. 12'	10', 12', 13', 14'	
12'[d]	31.50, CH_2	2.20, m	11'a, 11'b	10', 11', 13'	
13'[d]	171.45, qC				
14'[d]	173.37, qC				

[a] 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR
[b] numbers of attached protons were determined by analysis of 2D spectra.
[c] coupling constant indicated ^{13}C–^{31}P coupling
[d] NMR resonance are overlapped by glutamate chains and only one unit was presented.

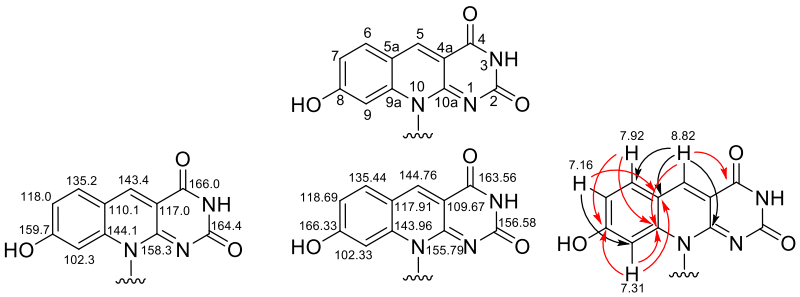
Table S5. NMR Data (D_2O, at 300 K) for F_{420}-n .[a]



	F_{420}-n				
position	δ_C, mult.[b] (*J* in Hz)[c]	δ_H, mult. (*J* in Hz)	COSY	HMBC	NOESY
*N*H (1)					
2	156.58, qC				
*N*H (3)					
4	163.56, qC				
4a	109.67, qC				
5	144.76, CH	8.82, s		4, 5a, 6, 9a, 10a	6
5a	117.91, qC				
6	135.44, CH	7.92, d (*8.80*)	7	5a, 8, 9a	5, 7
7	118.69, CH	7.16, dd (*8.80, 1.70*)	6	5a, 9	5
8	166.33, qC				
9	102.33, CH	7.31, s		5a, 8, 9a	
9a	143.96, qC				
10					
10a	155.79, qC				
1'a	49.30, CH_2	4.96, m	2'		4'
1'b		4.69, m	2'		
2'	70.19, CH	4.37, m	1'a, 1'b, 3'		4'
3'	73.24, CH	3.99, t (*3.65*)	2', 4'	1', 2', 4', 5'	
4'	71.76, CH_2, d, (*7.56*)	4.07, m	3'		1', 2'
5'a	67.42, CH_2, d, (*5.06*)	4.17, m	5'b	3'	
5'b		4.07, m	5'a	3'	
6'	72.53, CH d, (*4.94*)	4.70, q (*7.12*)	7'	7', 8'	
7'	19.93, CH_3, d, (*2.93*)	1.49, d (*6.89*)	6'	6', 8'	
8'	175.29, qC, d, (*6.60*)				
*N*H (9') [d]					
10' [d]	52.31, CH	4.35, m	11'a, 11'b	11', 12', 14'	
11'a [d]	26.98, CH_2	2.21, m	10', 12'	10', 12', 13', 14'	
11'b [d]		1.96, m	10', 12'	10', 12', 13', 14'	
12' [d]	31.98, CH_2	2.41, m	11'a, 11'b	10', 11', 13'	
13' [d]	175.59, qC				
14' [d]	175.54, qC				

[a] 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR
[b] numbers of attached protons were determined by analysis of 2D spectra.
[c] coupling constant indicated ^{13}C–^{31}P coupling
[d] NMR resonance are overlapped by glutamate chains and only one unit was presented.

Table S6. ^{13}C NMR comparison of F_{420}-n with reported for F_{420}-2.(8)



F_{420}-2 (Wolfe, 1978) F_{420}-n (**1**) F_{420}-n (**1**)

	F_{420}-n in D_2O	F_{420}-2 (ammonium salt in D_2O)
position	δ_C, mult.[b] (*J* in Hz)[c]	δ_C, mult. (*J* in Hz)
N (1)		
2	156.58, qC	164.4
*N*H (3)		
4	163.56, qC	166.0
4a	109.67, qC	117.0
5	144.76, CH	143.4
5a	117.91, qC	110.1
6	135.44, CH	135.2
7	118.69, CH	118.0
8	166.33, qC	159.7
9	102.33, CH	102.3
9a	143.96, qC	144.1
10		
10a	155.79, qC	158.3
1'a	49.30, CH_2	48.3
1'b		
2'	70.19, CH	70.5
3'	73.24, CH	73.7
4'	71.76, CH_2, d (*7.56*)	71.9, d (*7.9*)
5'a	67.42, CH_2, d (*5.06*)	67.9, d (*5.1*)
5'b		
6'	72.53, CH, d (*4.94*)	73.0, d (*5.2*)
7'	19.93, CH_3, d (*2.93*)	20.5
8'	175.31, qC, d (*6.60*)	174.9, d (*6.1*)
*N*H (9')		
10'	52.31, CH	55.0
11'a	26.98, CH_2	28.5
11'b		
12'	31.98, CH_2	32.9
13'	175.59, qC	175.8
14'	175.54, qC	178.5

[a]chemical shifts in the table and key HMBC correlations are highlighted in red; shift in ^{13}C resonances are presumably a result of the presence of ammonium salts and/or pH differences as indicated in the literature.

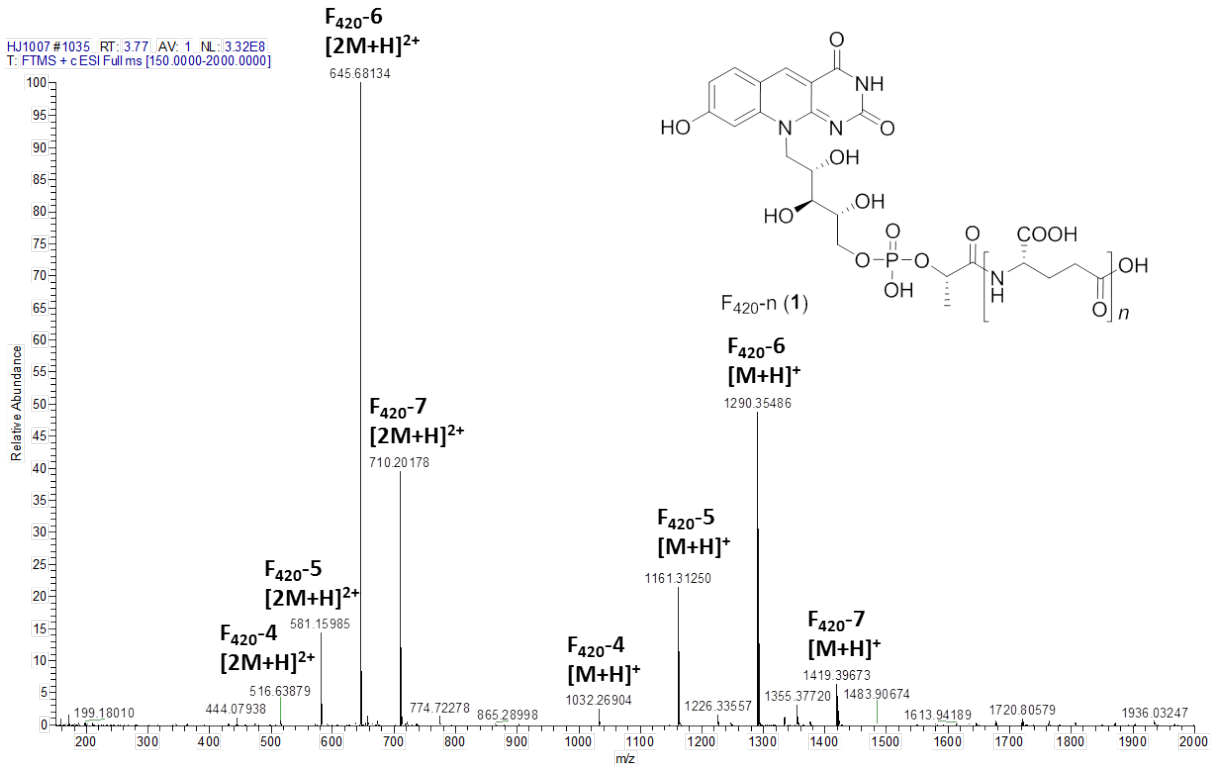


Figure S19. ESI-HRMS spectrum of F_{420}-n.

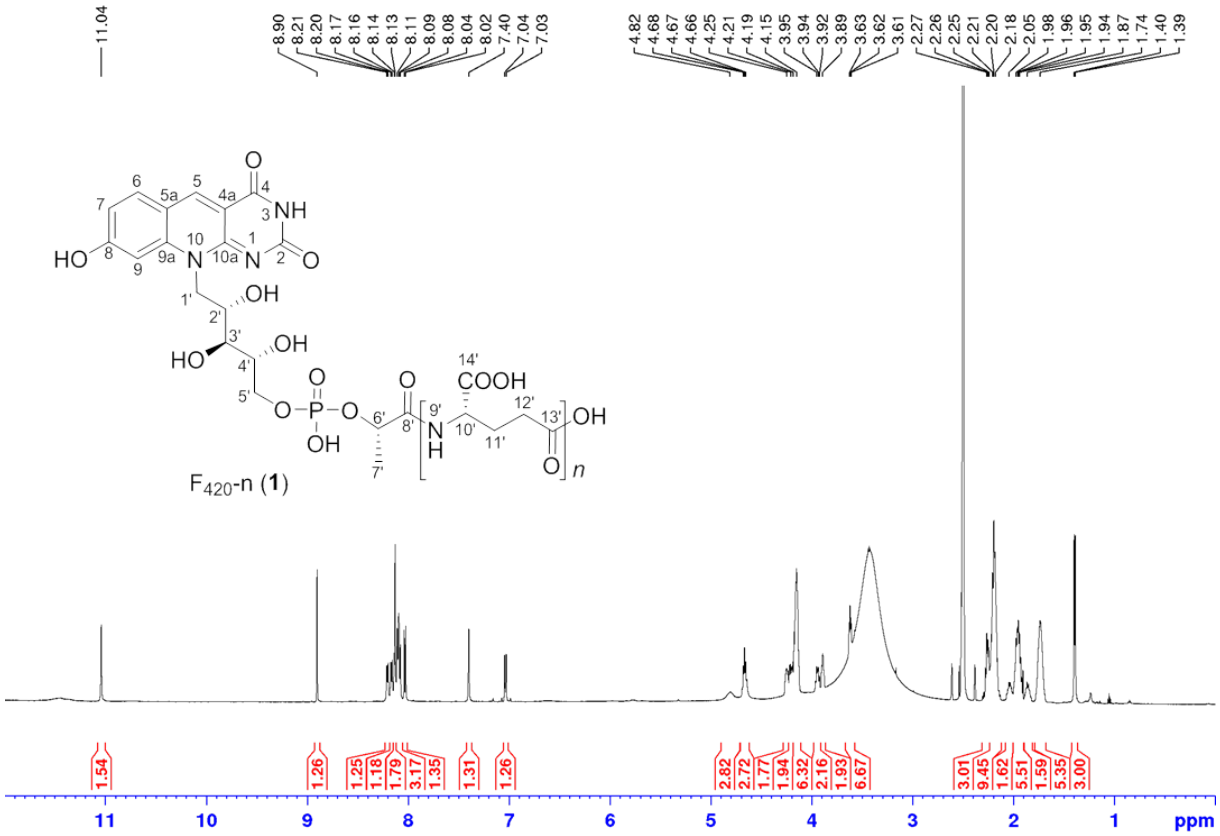


Figure S20. ^{1}H NMR spectrum of F_{420}-n (DMSO-d_6, 600 MHz, 300 K).

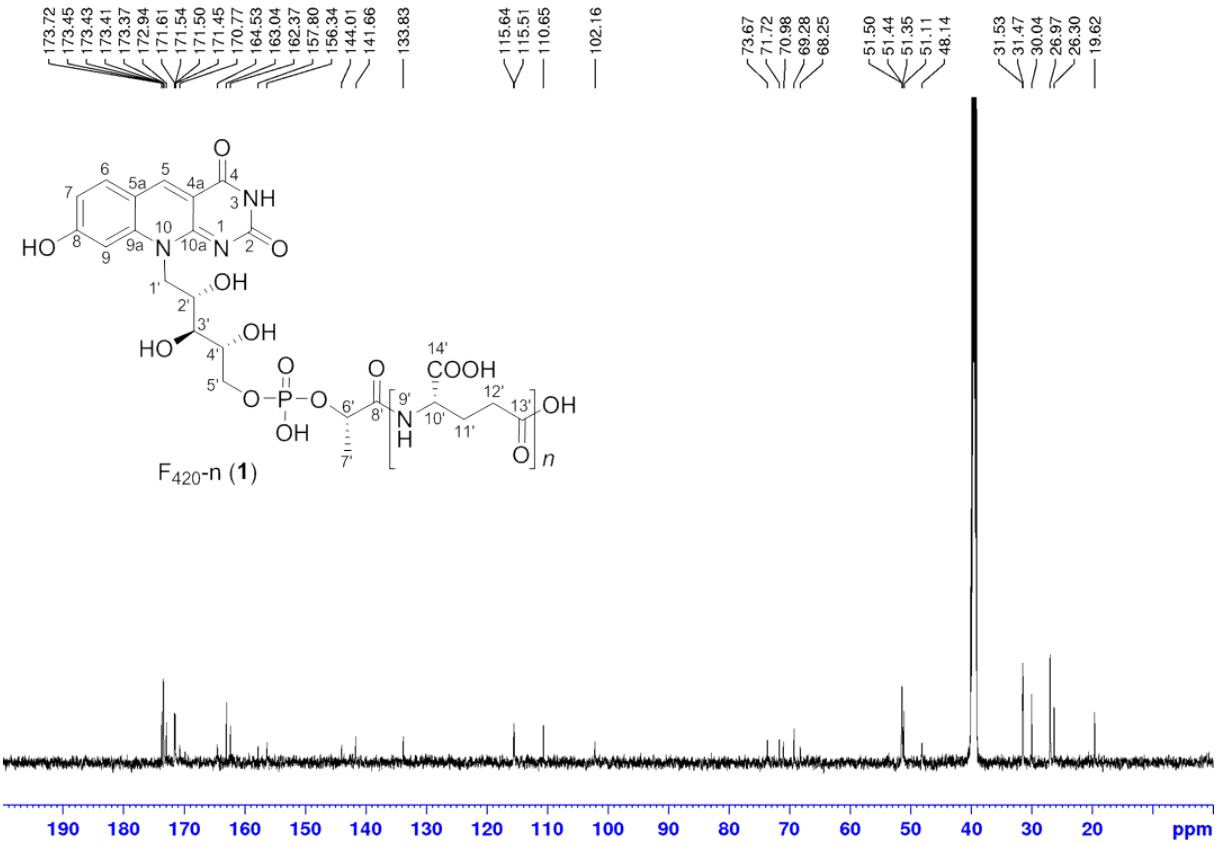


Figure S21. ^{13}C NMR spectrum of F_{420}-n (DMSO-d_6, 150 MHz, 300 K).

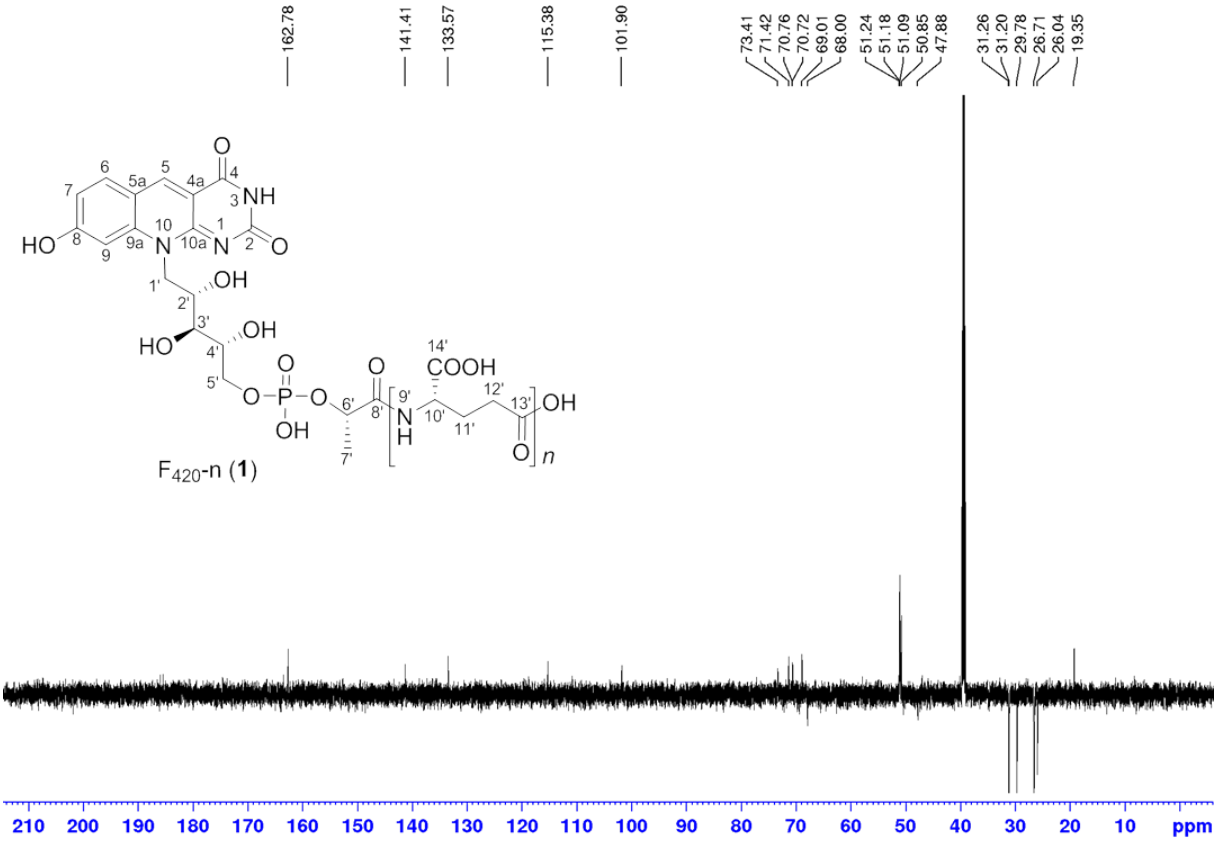


Figure S22. DEPT 135 spectrum of F_{420}-n (DMSO-d_6, 150 MHz, 300 K).

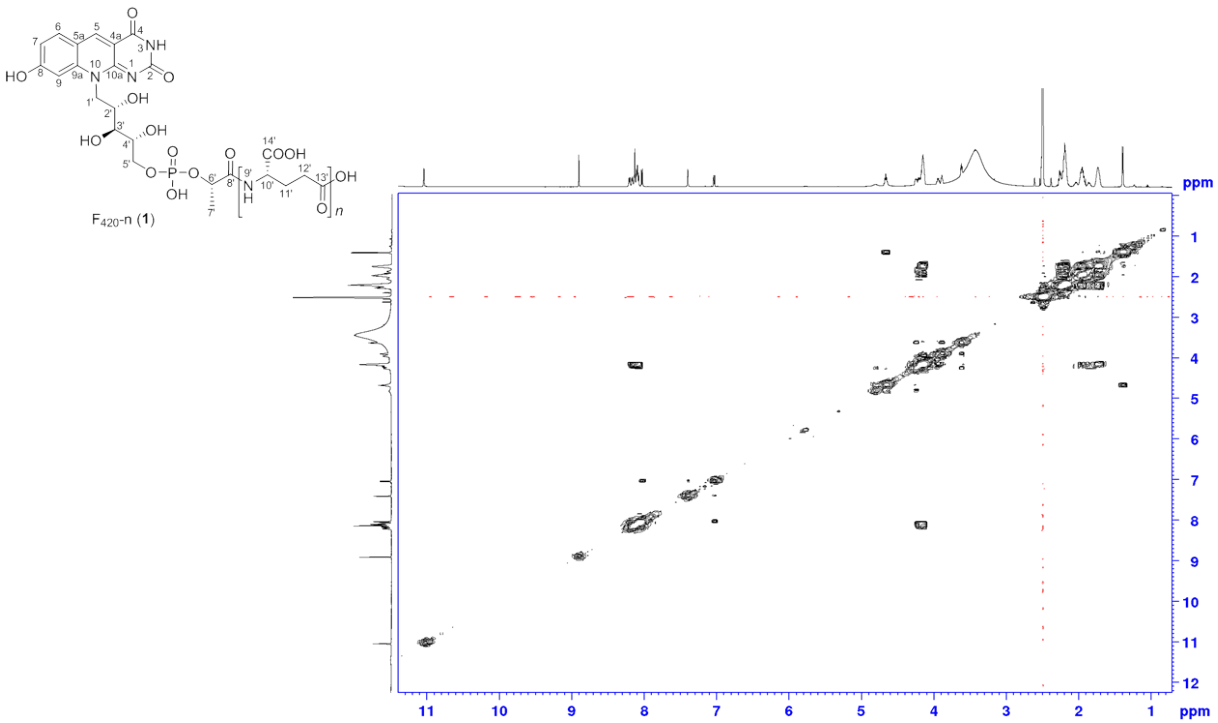


Figure S23. ^{1}H-^{1}H COSY spectrum of F_{420}-n (DMSO-d_6, 600 MHz, 300 K).

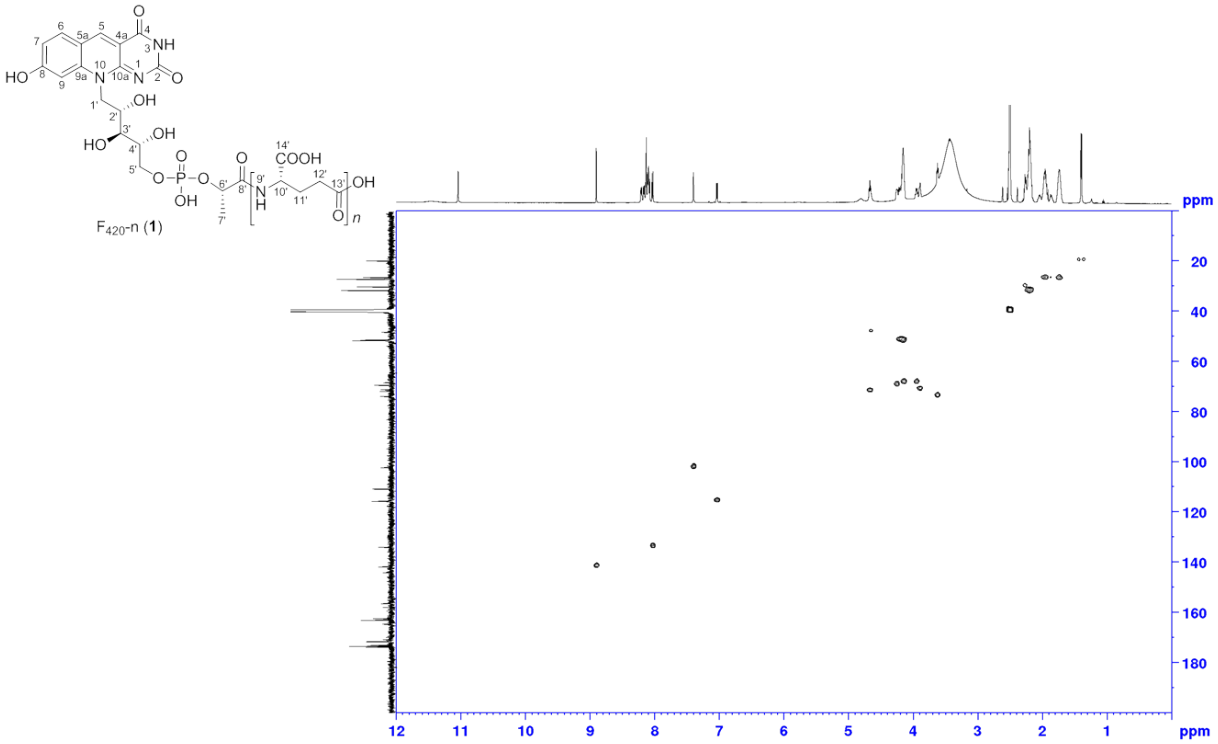


Figure S24. ^{1}H-^{13}C HSQC spectrum of F_{420}-n (DMSO-d_6, 600 MHz, 300 K).

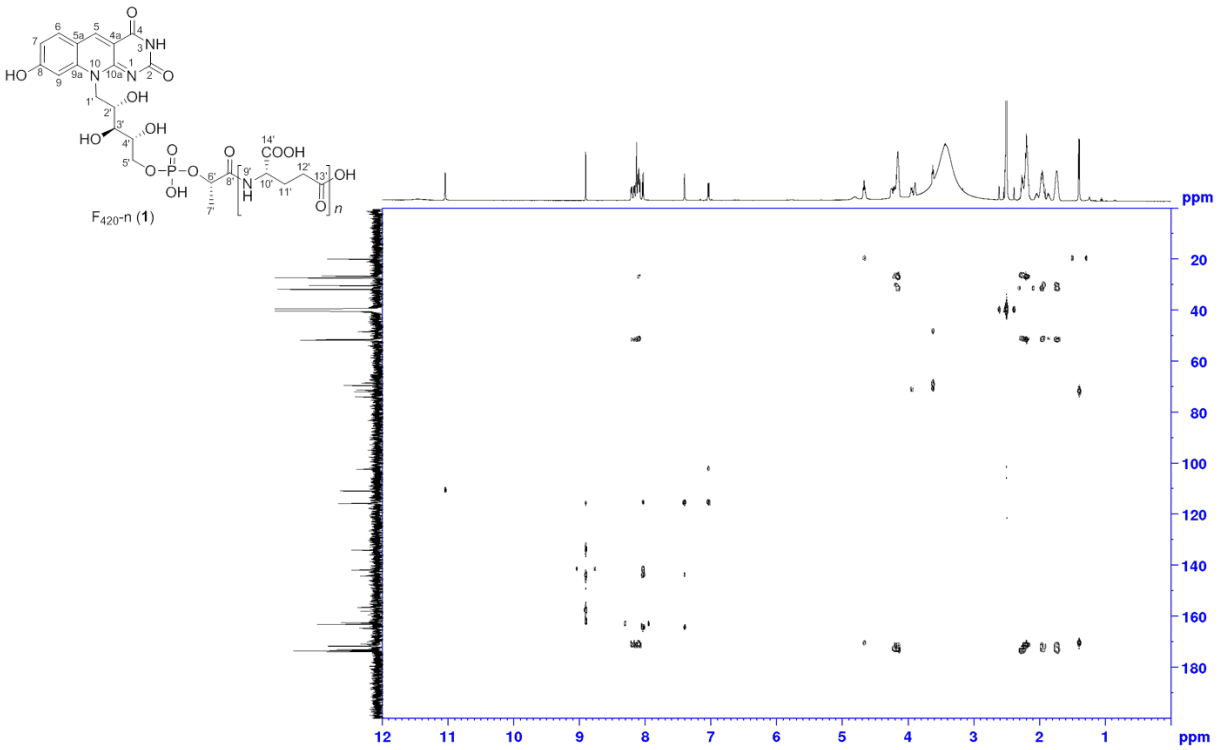


Figure S25. ^{1}H-^{13}C HMBC spectrum of F_{420}-n (DMSO-d_6, 600 MHz, 300 K).

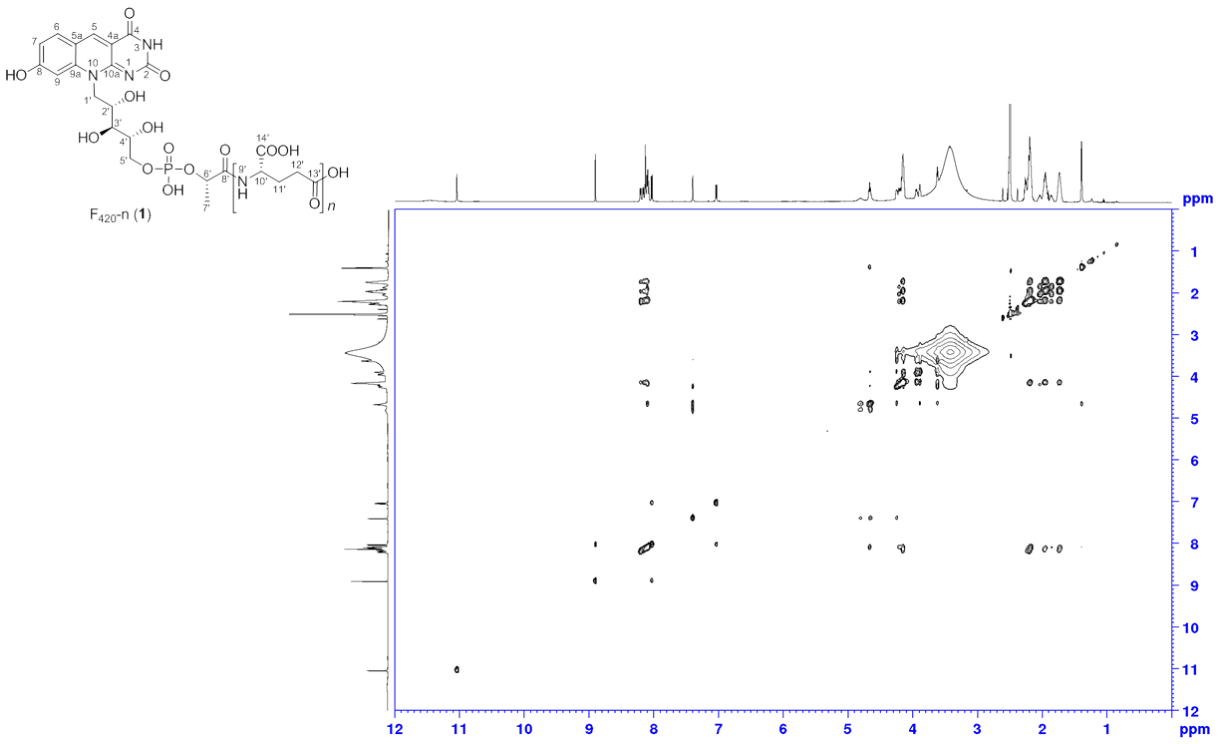


Figure S26. NOESY spectrum of F_{420}-n (DMSO-d_6, 600 MHz, 300 K).

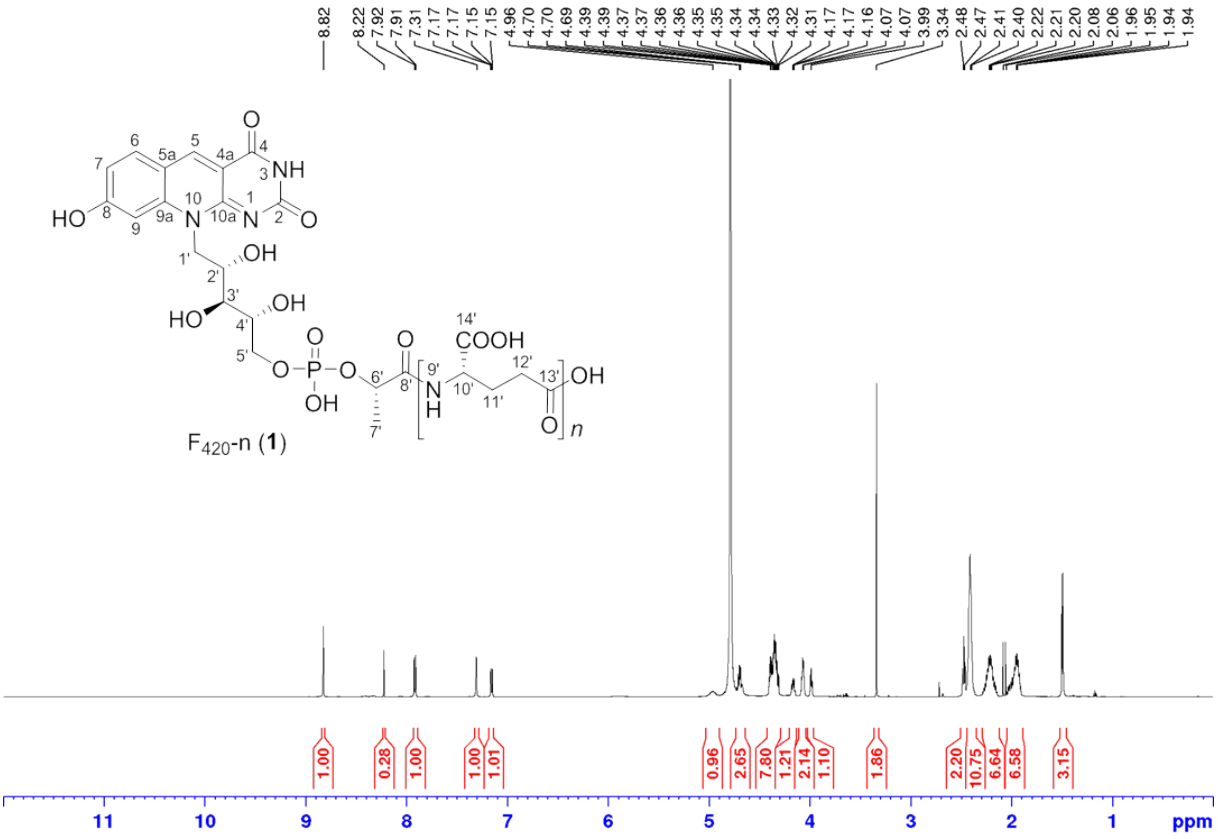


Figure S27. ^{1}H NMR spectrum of F_{420}-n (D_2O, 600 MHz, 300 K).

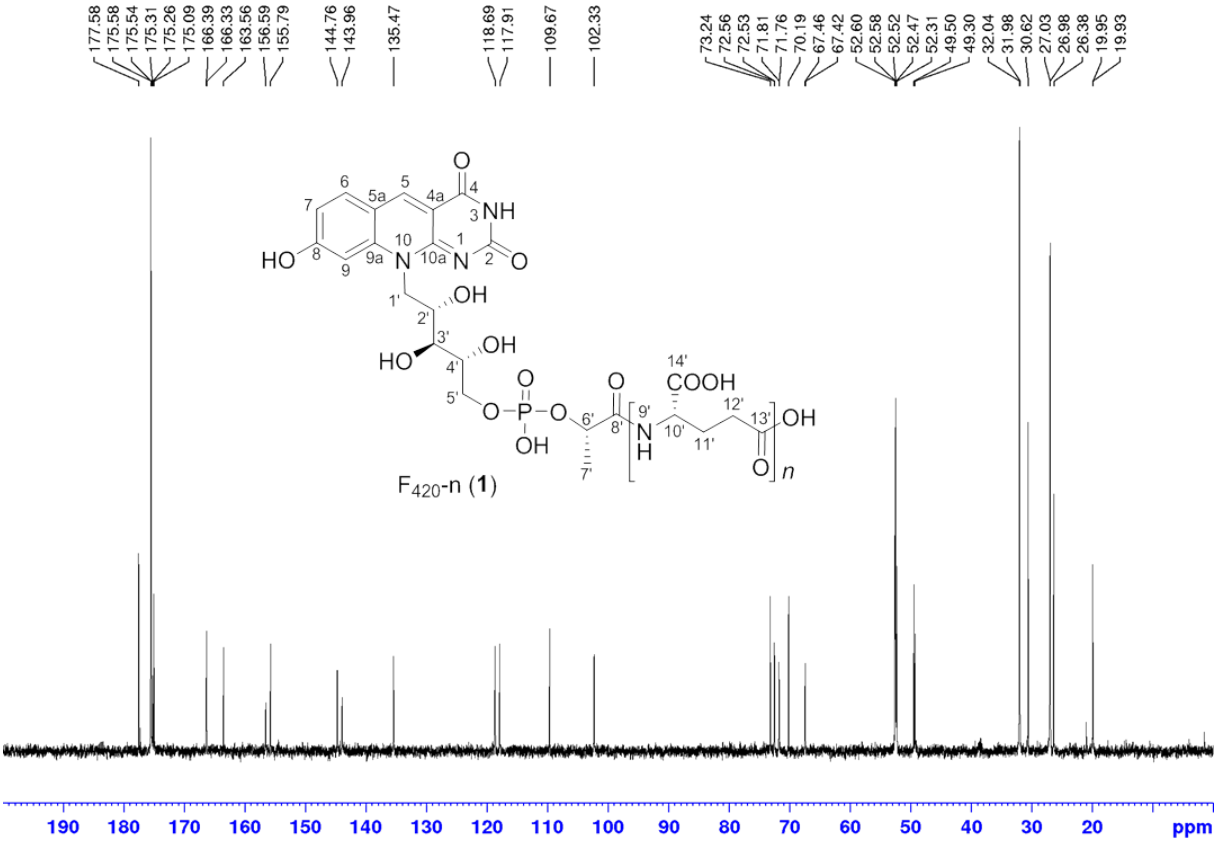


Figure S28. ^{13}C NMR spectrum of F_{420}-n (D_2O, 150 MHz, 300 K).

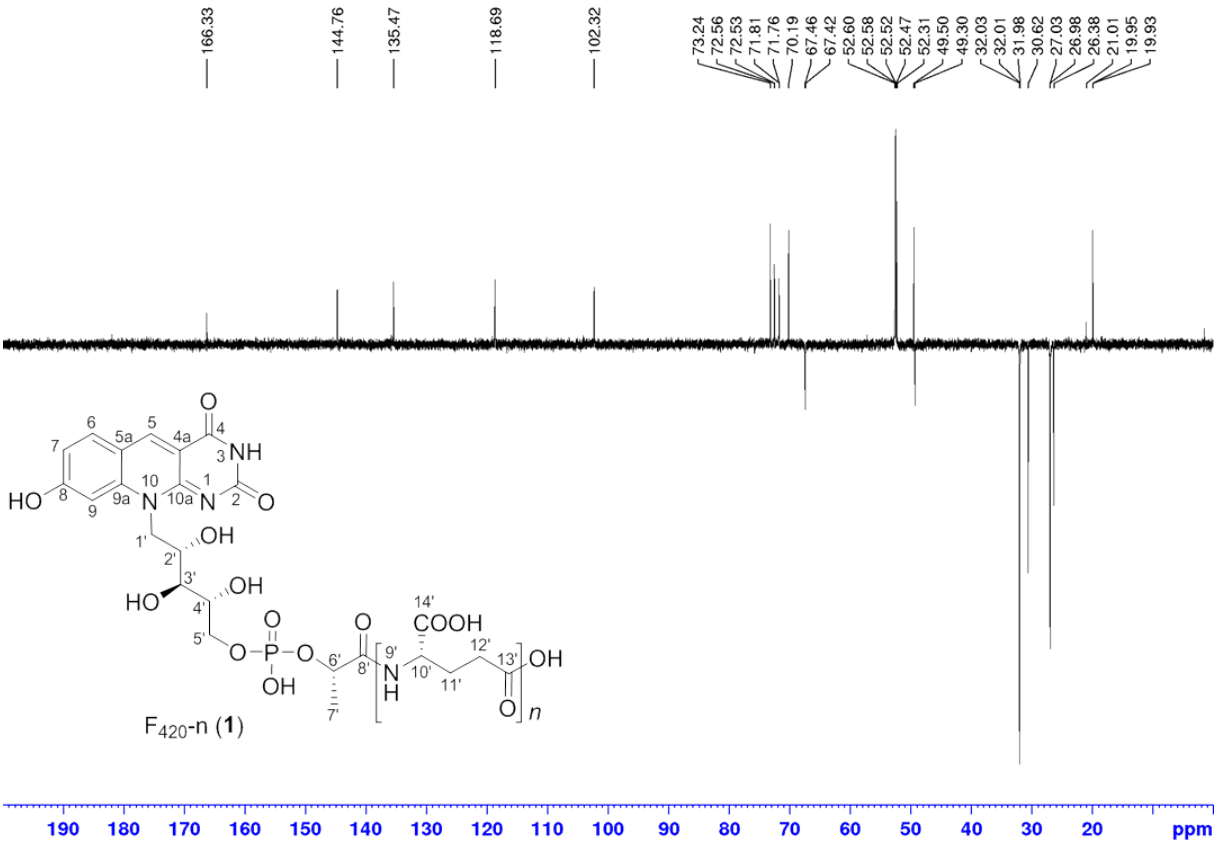


Figure S29. DEPT 135 spectrum of F_{420}-n (D_2O, 150 MHz, 300 K).

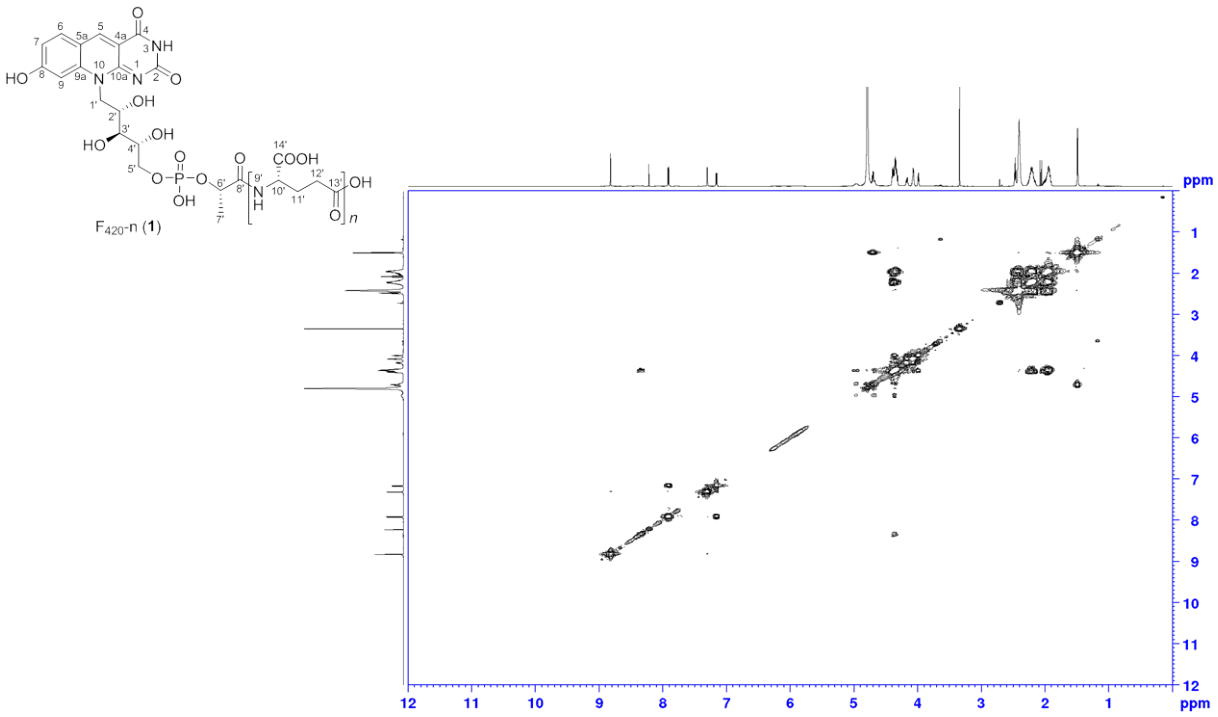


Figure S30. ^{1}H-^{1}H COSY spectrum of F_{420}-n (D_2O, 600 MHz, 300 K).

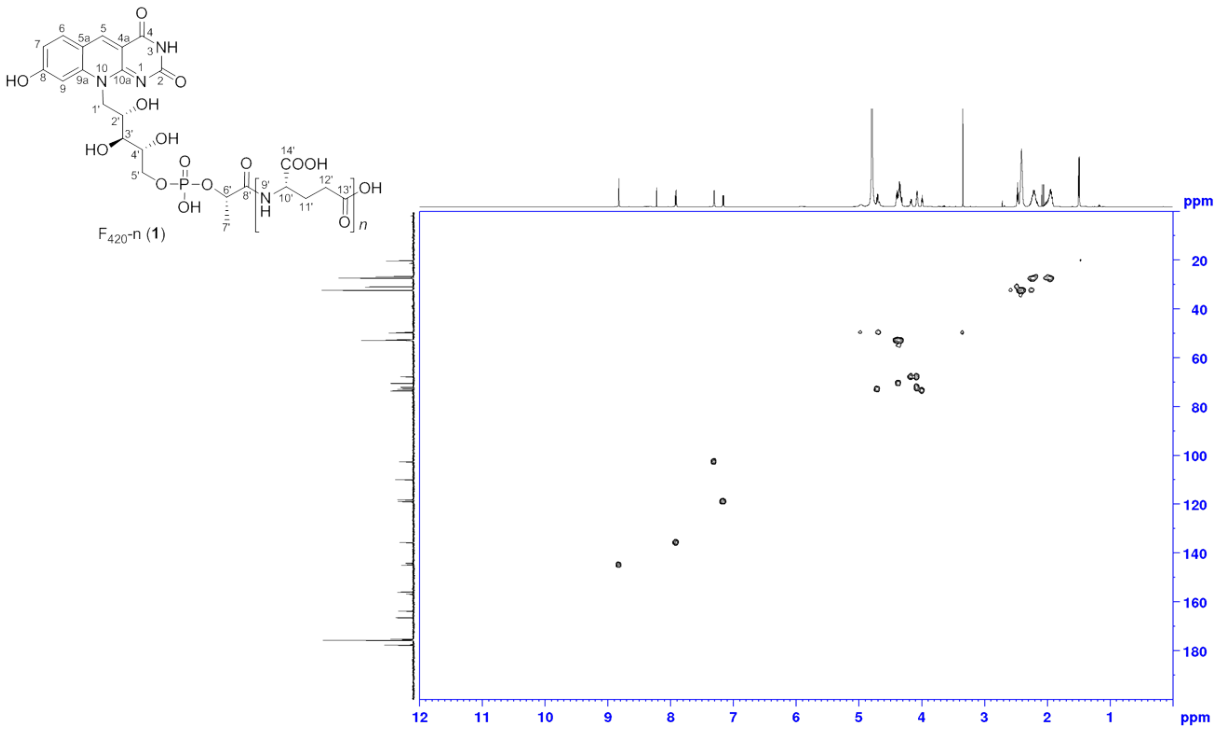


Figure S31. ^{1}H-^{13}C HSQC spectrum of F_{420}-n (D_2O, 600 MHz, 300 K).

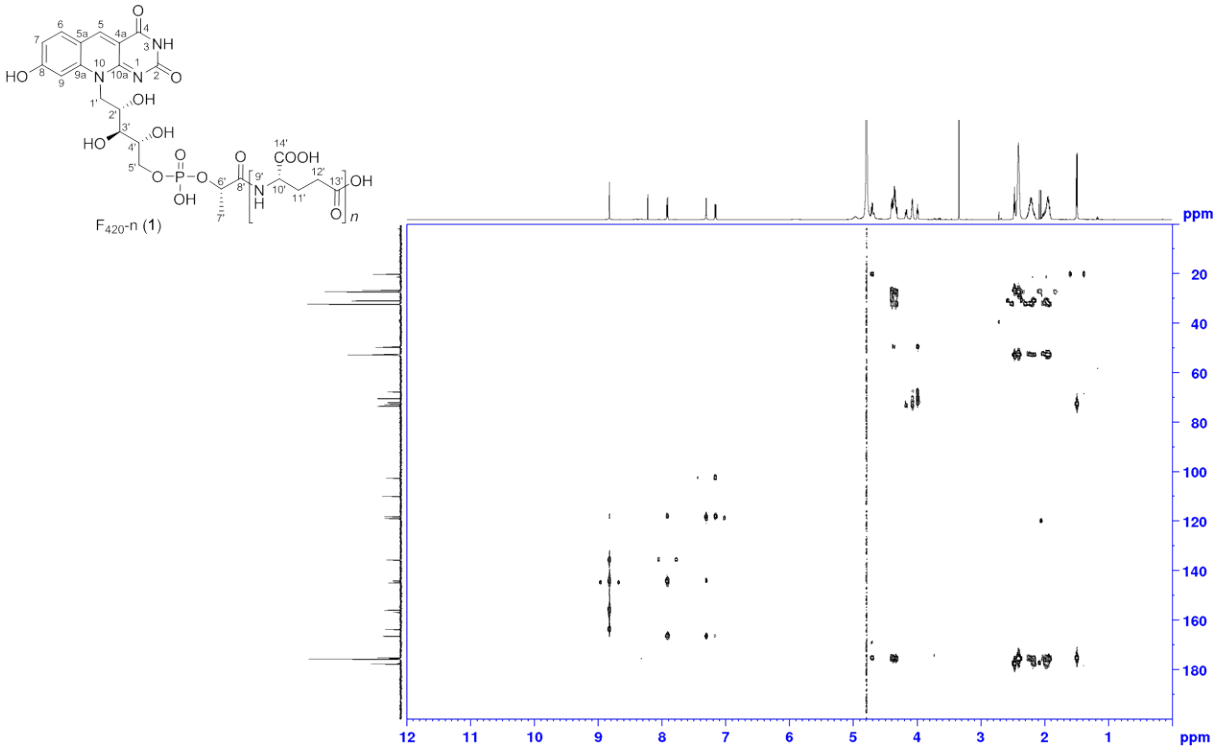


Figure S32. ^{1}H-^{13}C HMBC spectrum of F_{420}-n (D_2O, 600 MHz, 300 K).

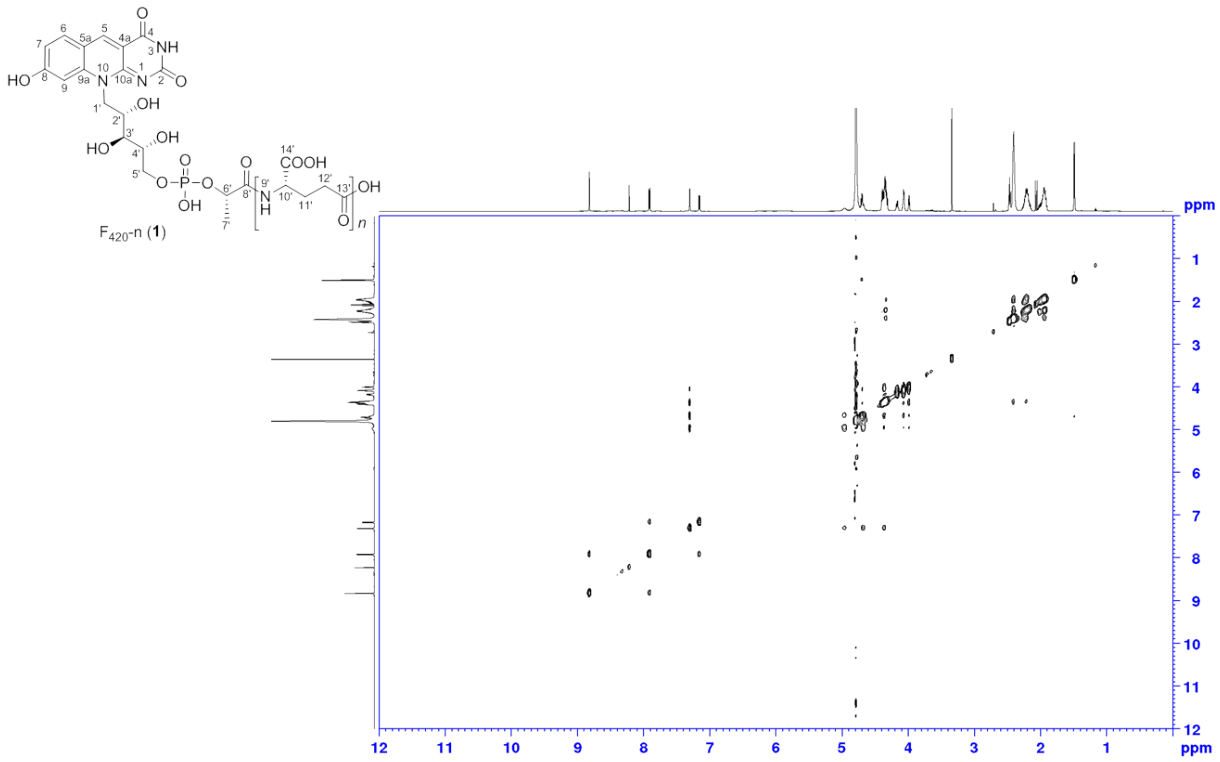


Figure S33. NOESY spectrum of F_{420}-n (D_2O, 600 MHz, 300 K).

2.3.2 Structure elucidation of 3PG-F_{420}-0

3PG-F_{420} species were isolated from large-scale fermentations of *E. coli* / pDB045 as described in Experimental Procedures (section 1.9). The elution from the anion-exchange column is depicted in Figure S34.

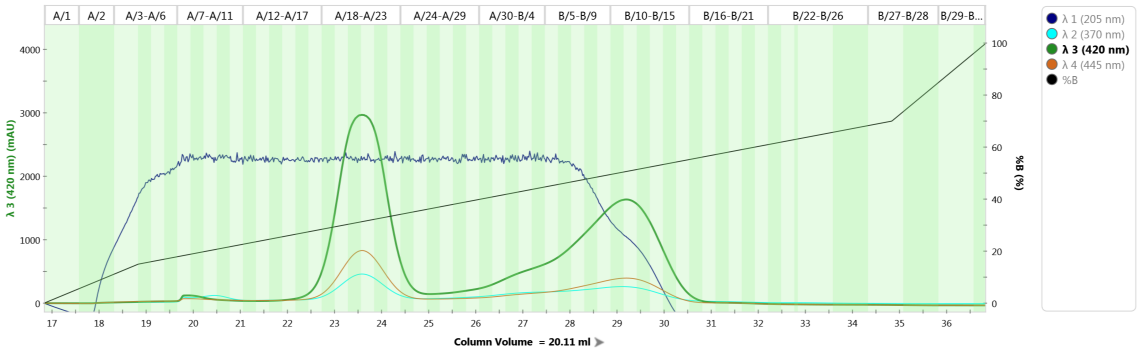


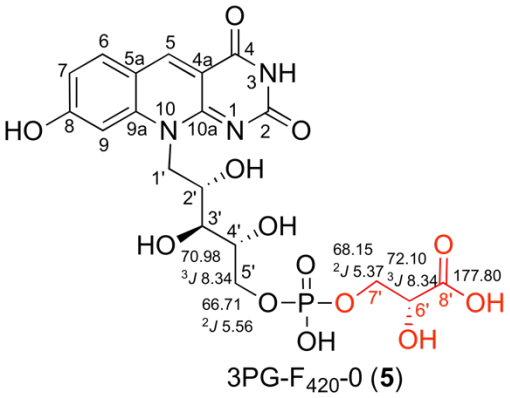
Figure S34 Preparative ion exchange chromatography of *E. coli* / pDB045 lysate on a NGC Quest FPLC (Bio-Rad) equipped with a HiPrep QFF column (GE Healthcare) pre-equilibrated with sodium phosphate buffer (25mM, pH 7.4). A gradient of NaCl was used to elute different 3PG-F_{420} species as peaks monitored by UV/VIS absorption at 420 nm (3PG-F_{420}-0: 22.5 to 25 CV, 3PG-F_{420}-n: 25-31 CV).

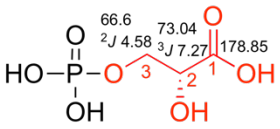
3PG-F_{420}-0 (5) was obtained as golden yellow solid. Due to the solubility, 1D and 2D NMR spectra were recorded in 0.1% ND_3 in D_2O. The molecular formula of 3PG-F_{420}-0 (**5**) was determined as $C_{19}H_{22}O_{13}N_3P$ based on the ESI-HRMS analysis (*m/z* 532.09534 ($[M+H]^+$ calcd. 532.09630 Δ = – 1.81 ppm) and ^1H and ^{13}C NMR spectra. Detailed comparison of the ^1H NMR and ^{13}C NMR spectra with classical F_{420}-n revealed the similar structure feature of 5-deazaflavin moiety and the ribityl moiety, based on comparable ^{13}C resonance, with the slight chemical shift on the 5-deazaflavin moiety due to the ammonium salt and basic condition. The observation of a missing methyl group from the lactyl moiety and additional oxygenated methylene group ($\delta_{H\text{-}7'}$ 4.13/4.03 ppm, $\delta_{C\text{-}7'}$ 68.15 ppm) in 3PG-F_{420}-0 (**5**), and the shift of oxymethine group ($\delta_{H\text{-}6'}$ 4.21 ppm, $\delta_{C\text{-}6'}$ 72.10 ppm) suggested the hydroxy group on the lactyl moiety. HMBC correlations of H-6' and H-7'a to C-8' further suggested the presence of a glyceryl moiety. The doublets of C-4', C-5', C-6' and C-7' indicated the ^{13}C–^{31}P coupling ($^3J_{C\text{-}4'\text{–}P}$ = 8.34 Hz, $^2J_{C\text{-}5'\text{–}P}$ = 5.56 Hz, $^3J_{C\text{-}6'\text{–}P}$ = 8.34 Hz, $^2J_{C\text{-}7'\text{–}P}$ = 5.37 Hz) between the carbon atoms of the ribityl and glyceryl moieties assigned to these resonances and the phosphorus of the phosphate group. ^{13}C–^{31}P coupling also suggested the presence of 3-phosphoglycerate, with the relatively smaller $^2J_{C\text{-}P}$ and larger $^3J_{C\text{-}P}$ value. This hypothesis was confirmed by the measurement of $^2J_{C\text{-}P}$ and $^3J_{C\text{-}P}$ value from 2-phosphoglycerate and 3-phosphoglycerate. The coupling constants of 3PG-F_{420}-0(**5**) exactly matched to 3-phosphoglycerate, ^1H and ^{13}C chemical shift were similar. Therefore, the planar structure of 3PG-

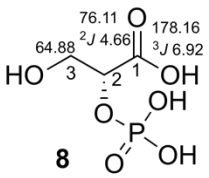
F_{420}-0 (**5**) is assigned as glyceric acid phosphodiester of 7,8-dedemethyl-8-hydroxy-5-deazariboflavin 5'-phosphate. (**Table S7** and **S8**)

Spectral Data: 3PG-F_{420}-0 (**5**): golden yellow solid; UV (H_2O, pH 7.5) λ_{max} 250, 420 nm; IR (ATR) ν_{max} 3233, 2954, 2926, 2867, 2358, 2330, 1587, 1506, 1428, 1354, 1152, 1035, 853, 800 cm^{-1}; NMR spectral data, see Table S7; ESI-HRMS $[M+H]^+$ *m*/*z* 532.09534 (calcd. for $C_{19}H_{23}O_{13}N_3P$, 532.09630).

Table S7. NMR Data (0.1% ND_3 in D_2O, at 300 K) for 3PG-F_{420}-0 (**5**).[a]



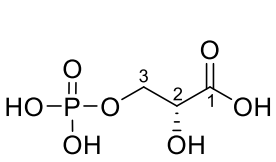




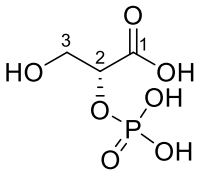
	3PG-F_{420}-0 (**5**)				
position	δ_C, mult.[b] (*J* in Hz)[c]	δ_H, mult. (*J* in Hz)	COSY	HMBC	NOESY
N (1)					
2	159.33, qC				
*N*H (3)					
4	165.45, qC				
4a	104.13, qC				
5	140.80, CH	8.56, s		4, 6, 9a, 10a	
5a	115.41, qC				
6	134.51, CH	7.72, d (*9.06*)	7	5, 5a, 9a, 8	
7	123.52, CH	6.82, d (*8.98*)	6	5a, 9	
8	179.48, qC				
9	102.74, CH	6.77, s		5a, 7, 9a, 8	
9a	145.56, qC				
10					
10a	157.75, qC				
1'a	46.31, CH_2	5.07, br s	1'b, 2'		
1'b		4.56, br d (*13.14*)	1'a		
2'	69.31, CH	4.41, m	1'b, 3'		
3'	72.54, CH	3.96, m	2', 4'	1', 2', 4', 5'	
4'	70.98, CH, d (*8.34*)	4.03, m	3'	2'	
5'a	66.71, CH_2, d (*5.56*)	4.13, m	5'b	3'	
5'b		4.03, m	5'a	3', 4'	
6'	72.10, CH, d (*8.34*)	4.21, m	7'b	8'	
7'	68.15, CH_2, d (*5.37*)	4.13, m	7'b	8'	
		4.03, m	6, 7'a		
8'	177.80, qC				

[a] 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR
[b] numbers of attached protons were determined by analysis of 2D spectra
[c] coupling constant indicated ^{13}C–^{31}P coupling

Table S8. NMR Data (0.1% ND_3 in D_2O, at 300 K) for 3-phospho-D-glyceric acid (**7**) and 2-phospho-D-glyceric acid (**8**).



7



8

	3-phospho-d-glyceric acid (**7**)		2-phospho-D-glyceric acid (**8**)	
position	δ_C, mult.[b] (*J* in Hz)[c]	δ_H, mult. (*J* in Hz)	δ_C, mult.[b] (*J* in Hz)[c]	δ_H, mult. (*J* in Hz)
1	178.85, qC, s		178.16, qC, d (*6.92*)	
2	73.04, CH, d (*7.07*)	4.18, dd (*6.64, 2.70*)	76.11, CH, d (*4.66*)	4.47, dd (*5.81, 2.86*)
3a	66.65, CH_2, d (*4.58*)	4.02, ddd (*11.07 6.14, 2.79*)	64.88, CH_2, s	3.90, dd (*11.62, 5.81*)
3b		3.86, ddd (*11.20, 6.57,*		3.79, dd (*11.62,3.02*)

[a] 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR
[b] numbers of attached protons were determined by analysis of 2D spectra.
[c] coupling constant indicated ^{13}C–^{31}P coupling

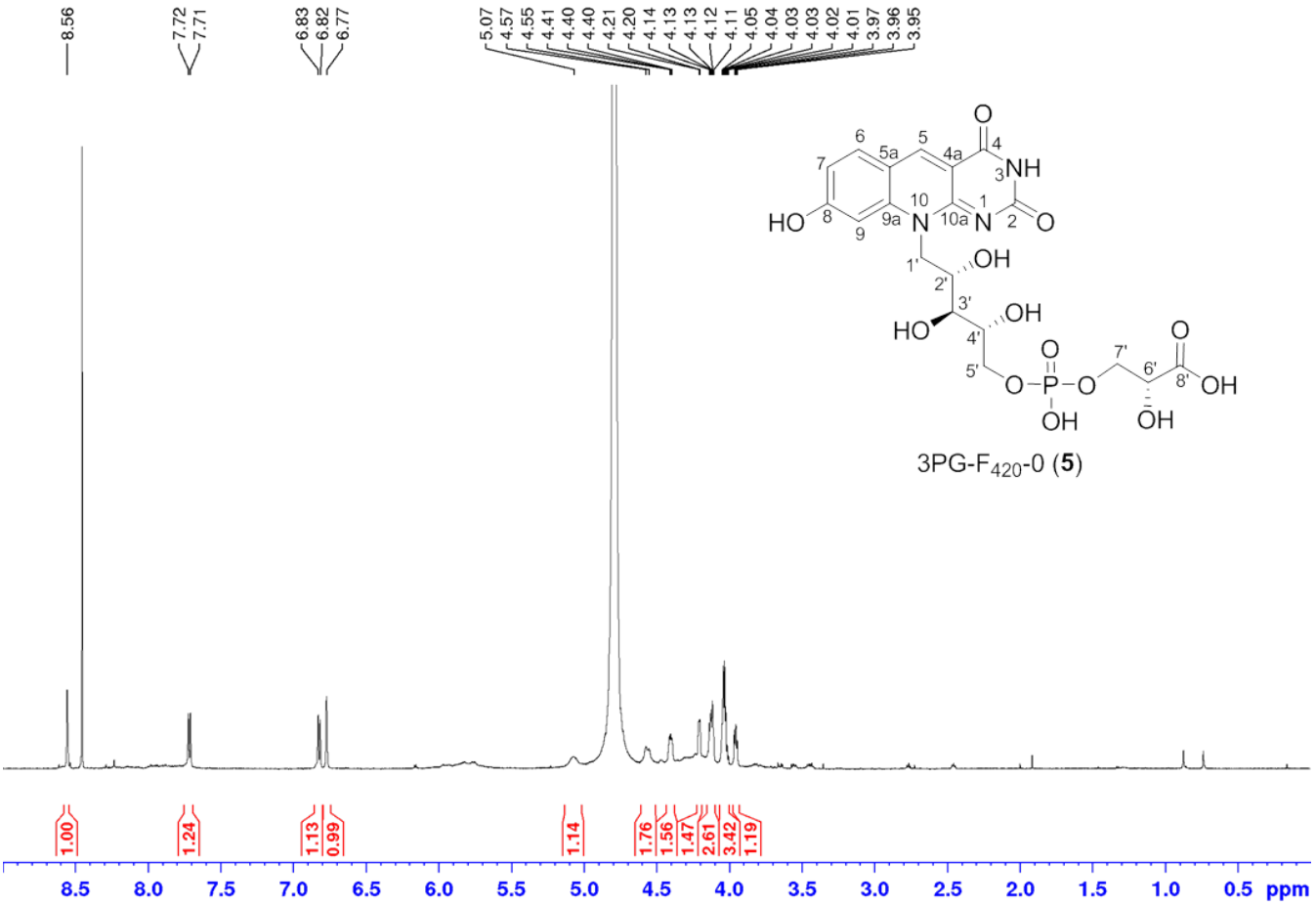


Figure S35. ^1H NMR spectrum of 3PG-F_{420}-0 (**5**) (0.1% ND_3 in D_2O, 600 MHz, 300 K).

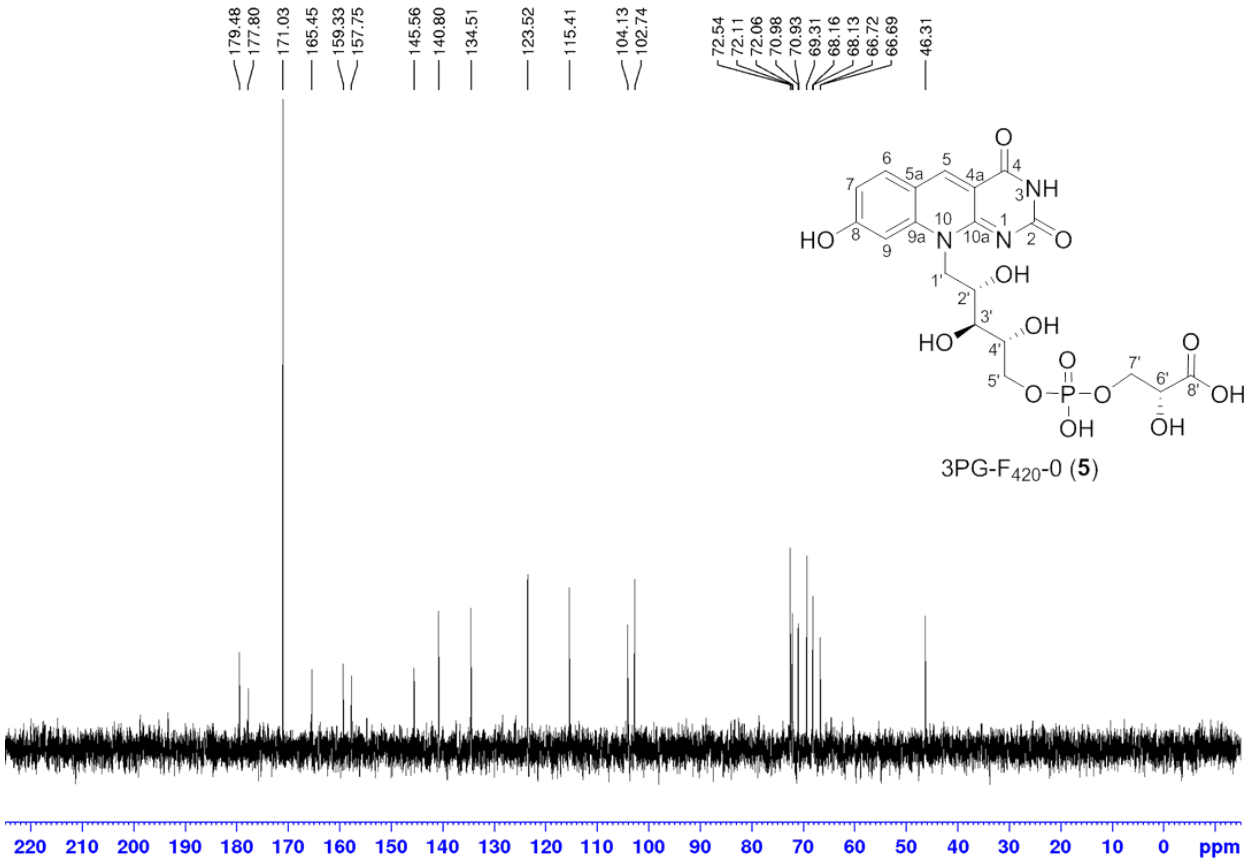


Figure S36. ^{13}C NMR spectrum of 3PG-F_{420}-0 (**5**) (0.1% ND_3 in D_2O, 150 MHz, 300 K).

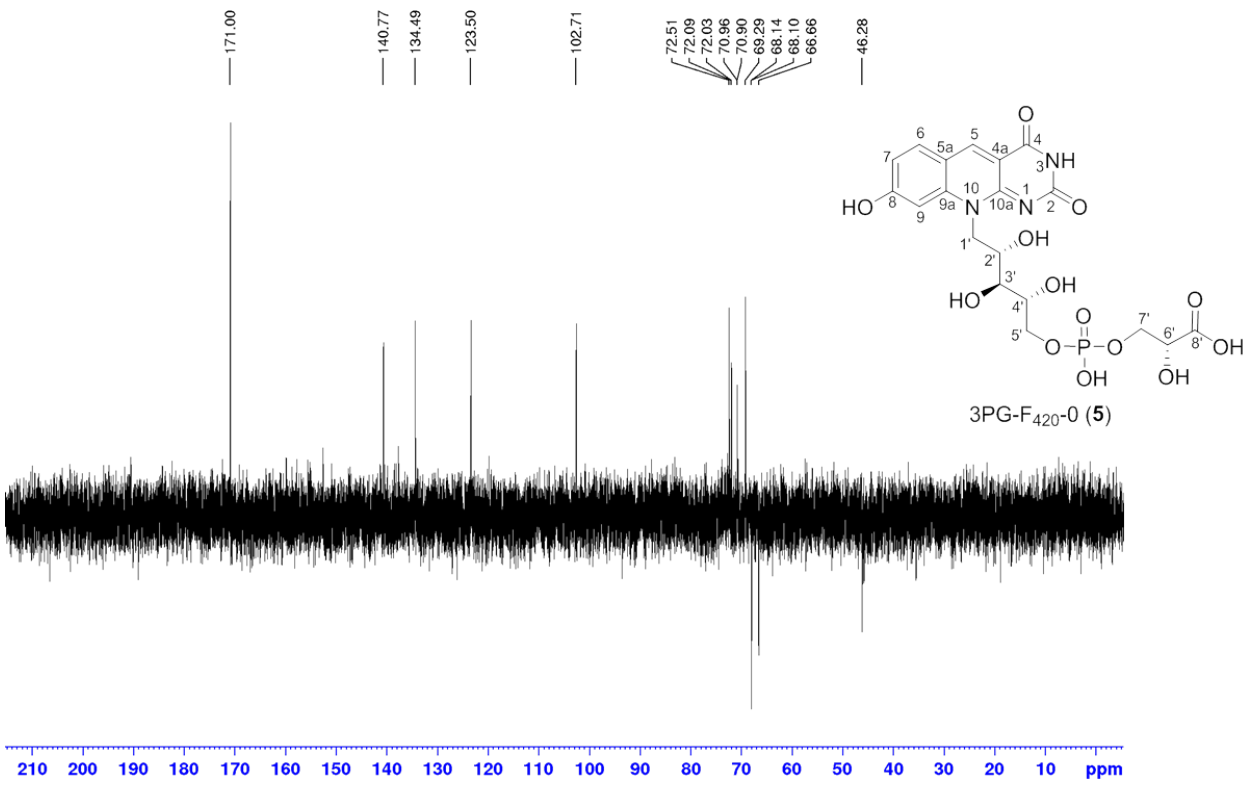


Figure S37. DEPT 135 spectrum of 3PG-F_{420}-0 (**5**) (0.1% ND_3 in D_2O, 150 MHz, 300 K).

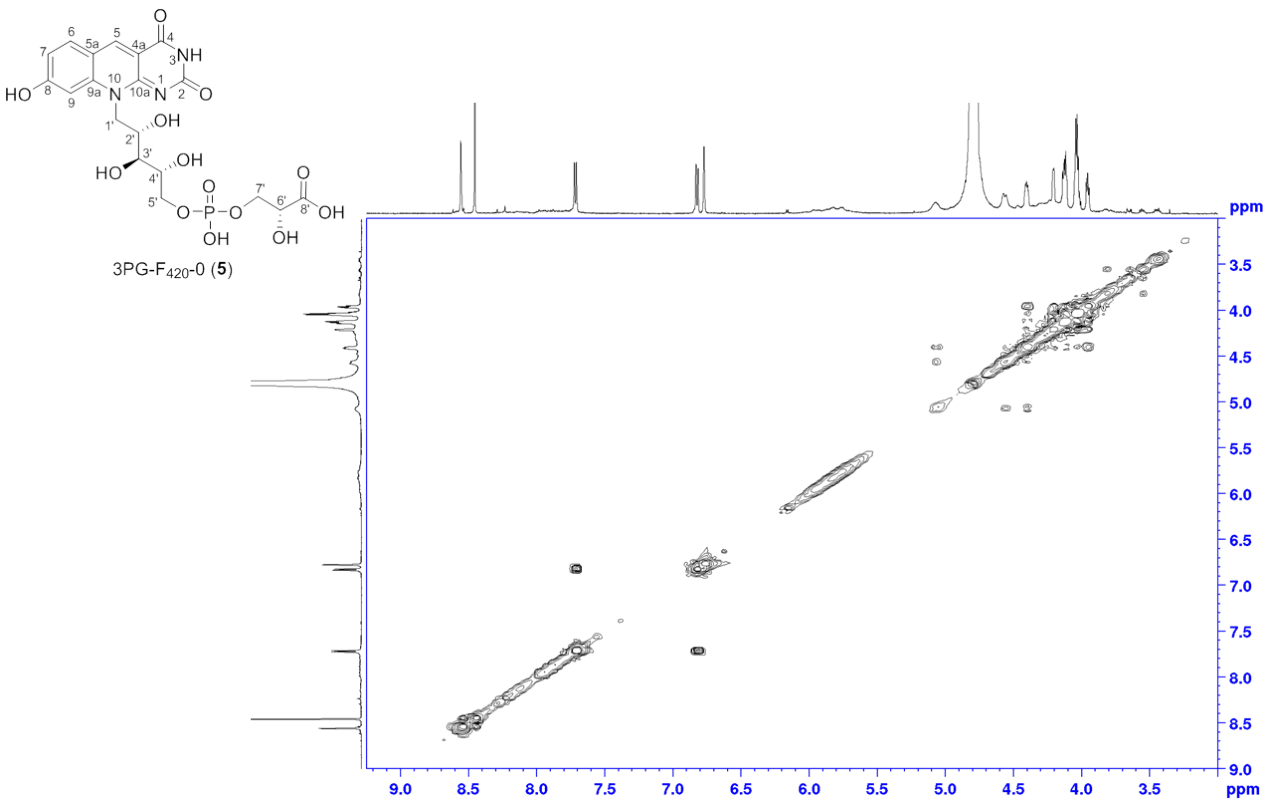


Figure S38. ^{1}H-^{1}H COSY spectrum of 3PG-F$_{420}$-0 (**5**) (0.1% ND_3 in D_2O, 600 MHz, 300 K).

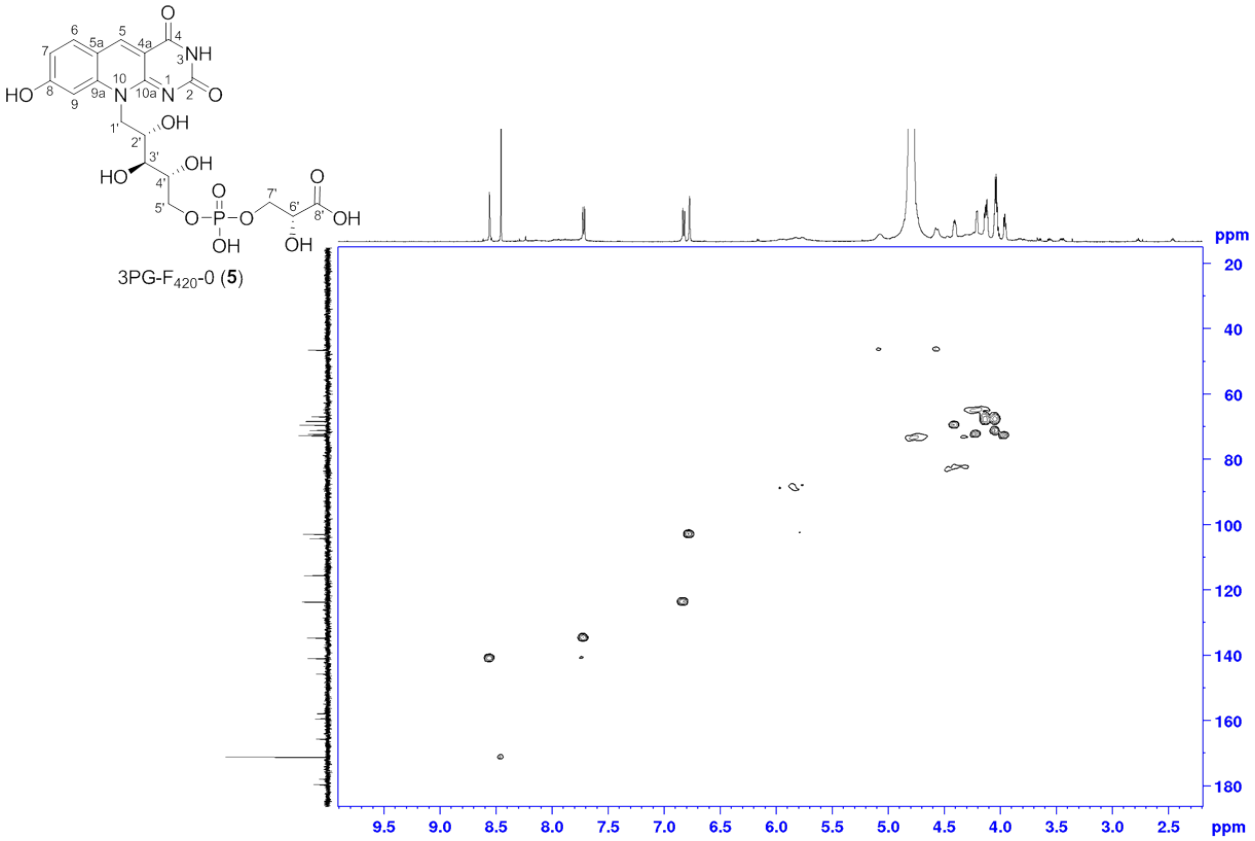


Figure S39. ^{1}H-^{13}C HSQC spectrum of 3PG-F$_{420}$-0 (**5**) (0.1% ND_3 in D_2O, 600 MHz, 300 K).

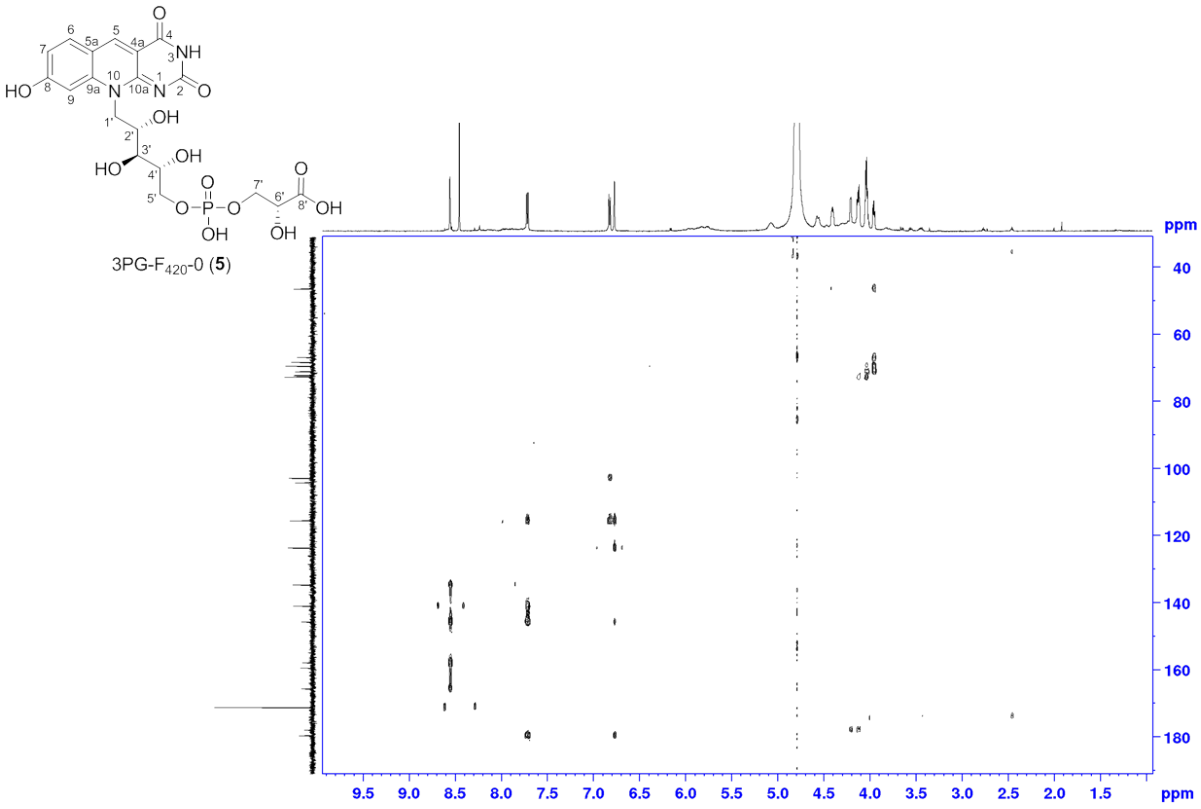


Figure S40. ^{1}H-^{13}C HMBC spectrum of 3PG-F$_{420}$-0 (**5**) (0.1% ND_3 in D_2O, 600 MHz, 300 K).

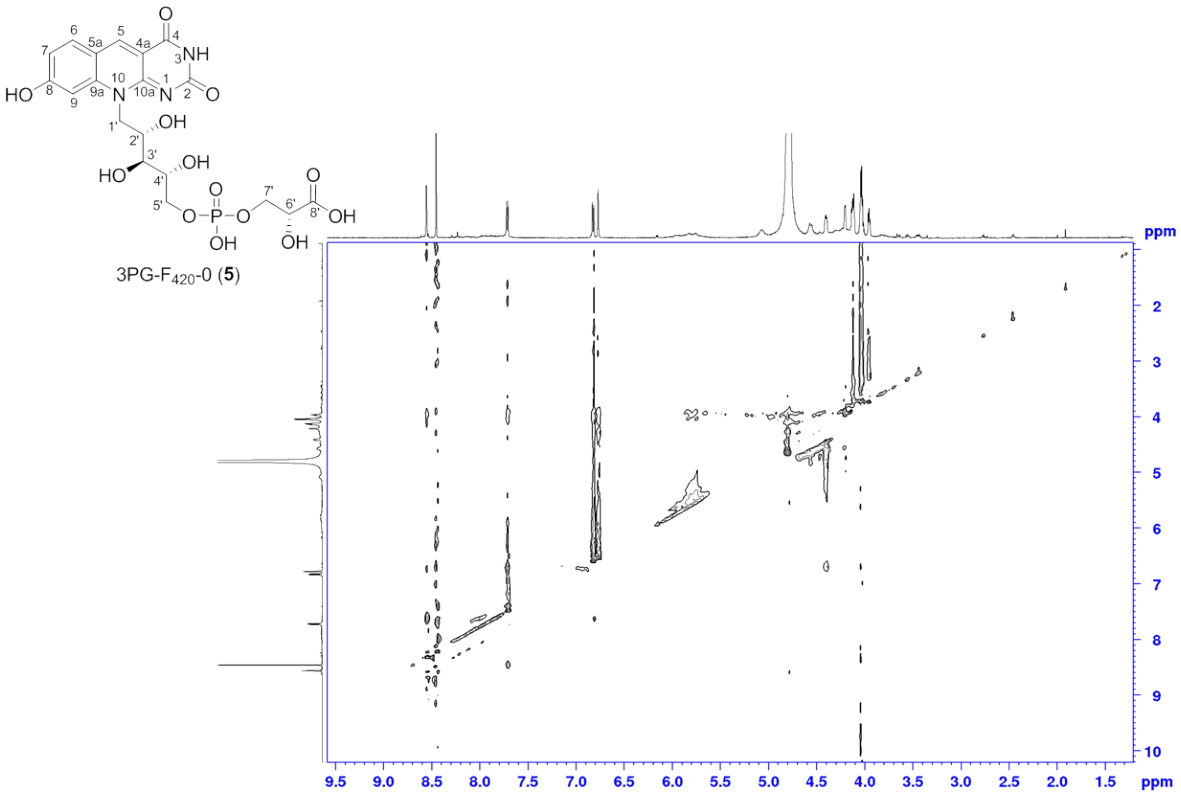


Figure S41. NOESY spectrum of 3PG-F$_{420}$-0 (**5**) (0.1% ND_3 in D_2O, 600 MHz, 300 K).

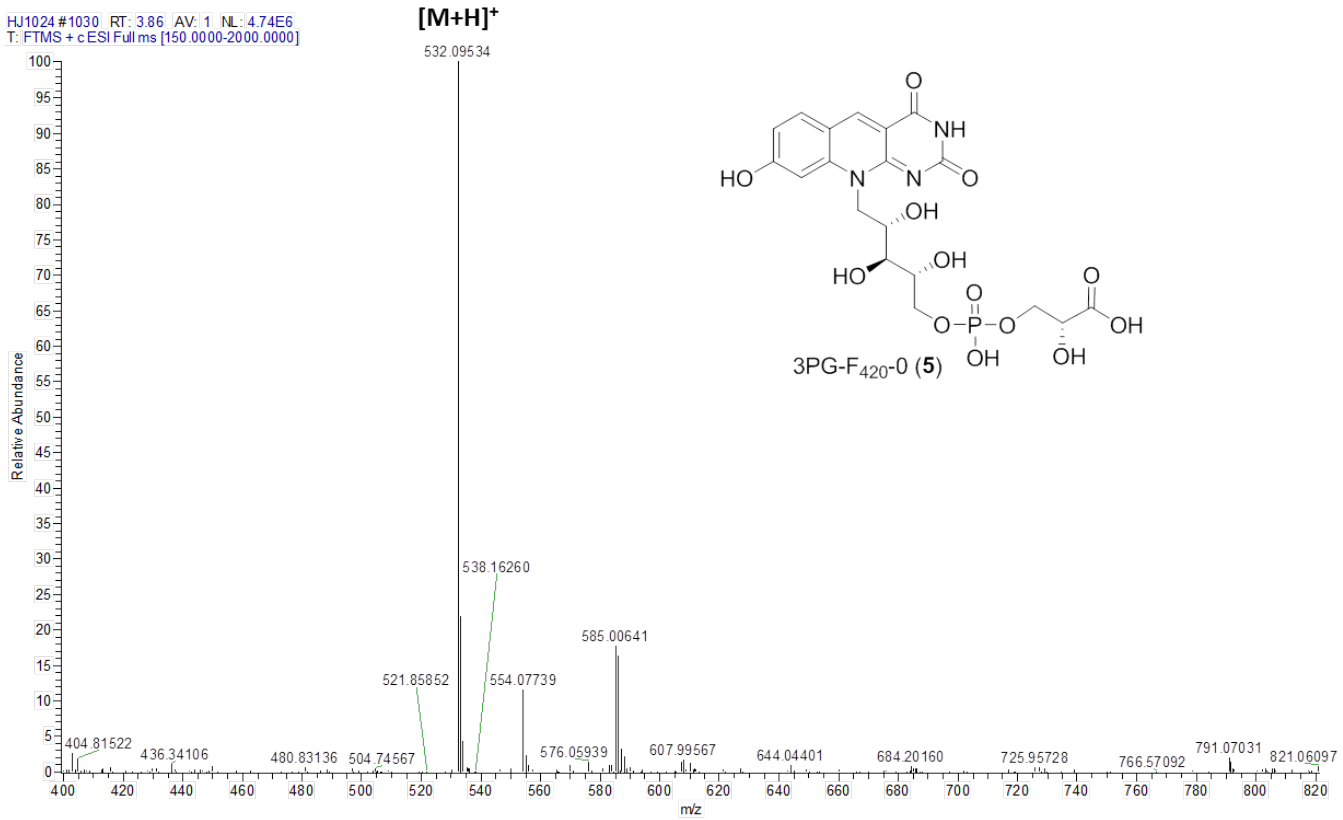


Figure S42. ESI-HRMS spectrum of 3PG-F_{420}-0 (**5**).

2.3.3 Structure elucidation of 3PG-F_{420}-n

3PG-F420-n (4): 3PG-F_{420}-n was obtained as golden yellow solid. 1D and 2D NMR spectra were recorded in 0.1% ND_3 in D_2O as well. The composition of mixed 3PG-F_{420}-n was determined as 3PG-F_{420}-1 with the molecular formula of $C_{24}H_{29}O_{16}N_4P$ based on the ESI-HRMS analysis (*m*/*z* 661.13757 ($[M+H]^+$ calcd. 661.13889 Δ = – 2.00 ppm); 3PG-F_{420}-2 with the molecular formula of $C_{29}H_{36}O_{19}N_5P$ based on the ESI-HRMS analysis (*m*/*z* 790.17981 ($[M+H]^+$ calcd. 790.18149 Δ = – 2.12 ppm); 3PG-F_{420}-3 with the molecular formula of $C_{34}H_{43}O_{22}N_6P$ based on the ESI-HRMS analysis (*m*/*z* 919.22223 ($[M+H]^+$ calcd. 919.22408 Δ = – 2.01 ppm); 3PG-F_{420}-4 with the molecular formula of $C_{39}H_{50}O_{25}N_7P$ based on the ESI-HRMS analysis (*m*/*z* 1048.26489 ($[M+H]^+$ calcd. 1048.26667 Δ = – 1.70 ppm); 3PG-F_{420}-5 with the molecular formula of $C_{44}H_{57}O_{28}N_8P$ based on the ESI-HRMS analysis (*m*/*z* 1177.30750 ($[M+H]^+$ calcd. 1177.30927 Δ = – 1.50 ppm); 3PG-F_{420}-6 with the molecular formula of $C_{49}H_{64}O_{31}N_9P$ based on the ESI-HRMS analysis (*m*/*z* 1306.34961 ($[M+H]^+$ calcd. 1306.35186 Δ = – 1.72 ppm). Detailed comparison of ^1H NMR data of 3PG-F_{420}-n with 3PG-F_{420}-0 (**5**) and $F_{\mathbf{420}}$-n revealed the signals from glutamyl moiety ($\delta_{H'}$ 1.86–2.35 ppm; δ_H 4.25–4.25 ppm).

Spectral Data: 3PG-F_{420}-n**:** golden yellow solid; UV (H_2O, pH 7.5) λ_{max} 250, 420 nm; IR (ATR) ν_{max} 3738, 3339, 2926, 2356, 1678, 1581, 1512, 1365, 1206, 1181, 1138, 1014, 841, 805, 769 722, 664 cm^{-1};

Table S9. NMR Data (0.1% ND_3 in D_2O, at 300 K) for 3PG-F_{420}-n .[a]

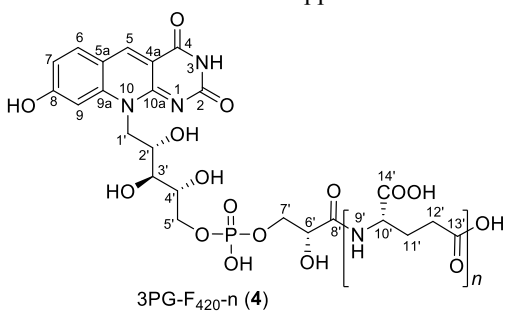
	3PG-F_{420}-n				
position	δ_C, mult.[b] (*J* in Hz)[c]	δ_H, mult. (*J* in Hz)	COSY	HMBC	NOESY
*N*H (1)					
2	159.37, qC				
*N*H (3)					
4	165.59, qC				
4a	104.29, qC				
5	140.93, CH	8.61, s		4, 6, 9a, 10a	6
5a	115.47, qC				
6	134.55, CH	7.75, d (*8.50*)	7	5, 8, 9a	5, 7
7	123.50, CH	6.84, d (*8.60*)	6	5a, 9	5
8	179.45, qC				
9	102.75, CH	6.80, s		5a, 7, 9a	
9a	145.63, qC				
10					
10a	157.86, qC				
1'a	46.37, CH_2	5.09, br s	1'b, 2'		
1'b		4.61, *d* (13.24)	1'a		
2'	69.40, CH	4.41, m	1'a, 3'		
3'	72.60, CH	3.95, t (*5.12*)	2'	1', 2', 4', 5'	
4'	70.94, CH_2, d, (*7.81*)	4.04, m			
5'a	66.95, CH_2, d, (*4.82*)	4.13, m			
5'b		4.01, m			
6'	71.12, CH d, (*8.60*)	4.42, br s	7'a	8'	
7'a	66.85, CH_2, d, (*3.82*)	4.13, m	6'	8'	
7'b		4.01, m			
8'	172.86, qC				
*N*H (9')[d]					
10'[d]	53.64, CH	4.32, m	11'a, 11'b	8', 11', 12'	
11'a[d]	27.86, CH_2	2.04, m	10', 11b'	10', 12', 13', 14'	
11'b[d]		1.91, m	10', 11'a	10', 12', 13', 14'	
12'a[d]	33.57, CH_2	2.28, m		10', 11', 13'	
12'b		2.22, m		10', 11', 13'	
13'[d]	181.56, qC				
14'[d]	173.63, qC				

[a] 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR

[b] numbers of attached protons were determined by analysis of 2D spectra.

[c] coupling constant indicated ^{13}C–^{31}P coupling

[d] NMR resonance are overlapped in the five times glutamate chains and only one unit was presented.



3PG-F_{420}-n (**4**)

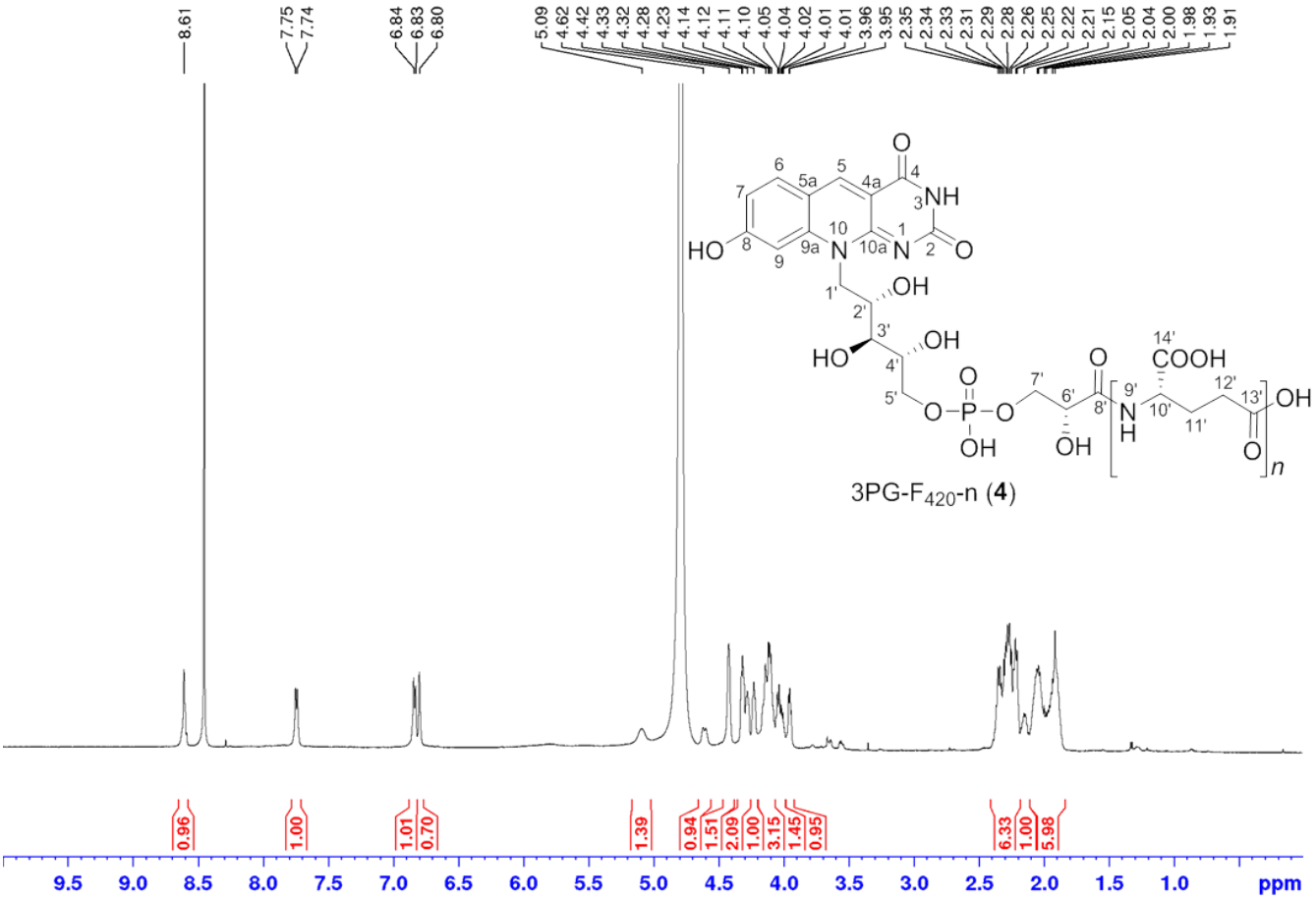


Figure S43. ^{1}H NMR spectrum of 3PG-F_{420}-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

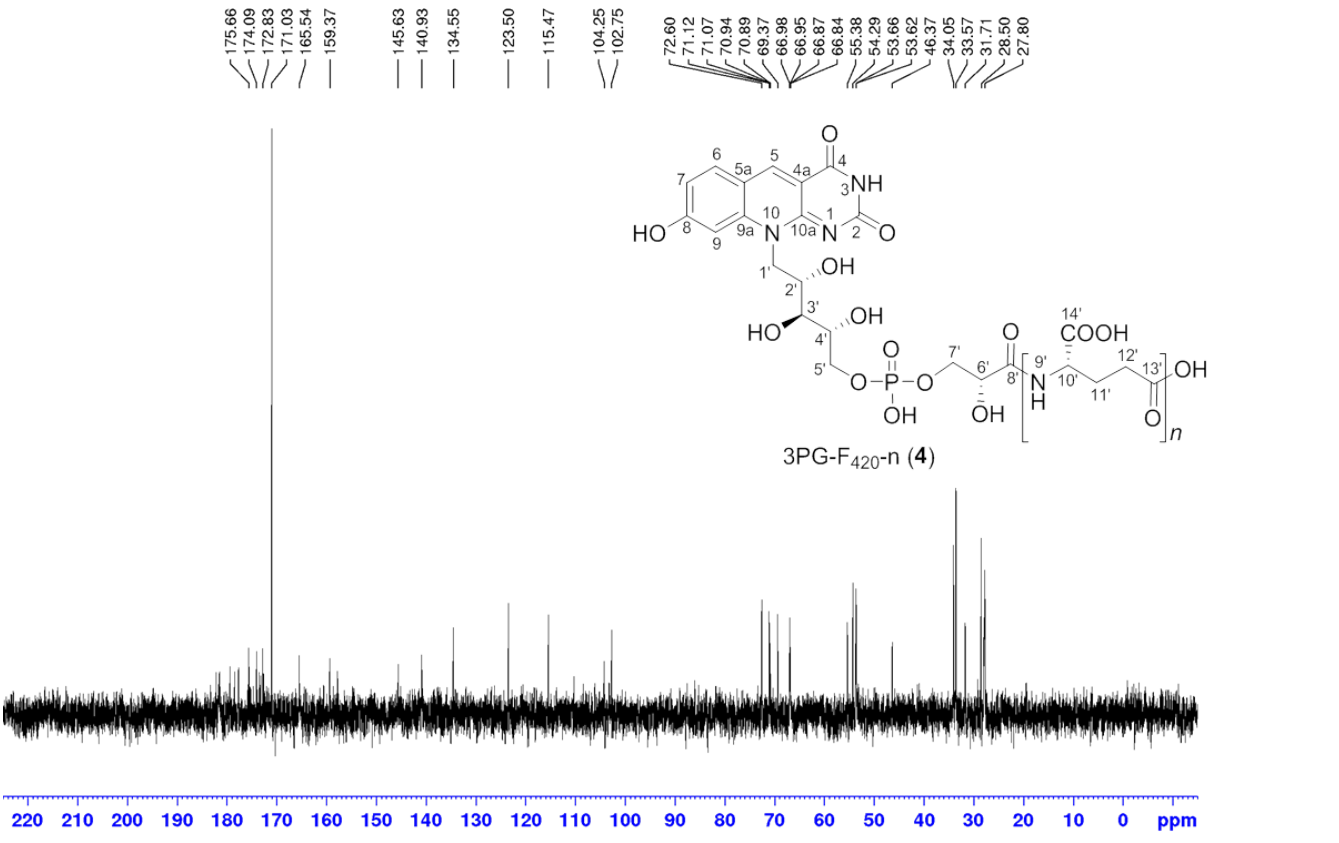


Figure S44. ^{13}C NMR spectrum of 3PG-F_{420}-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

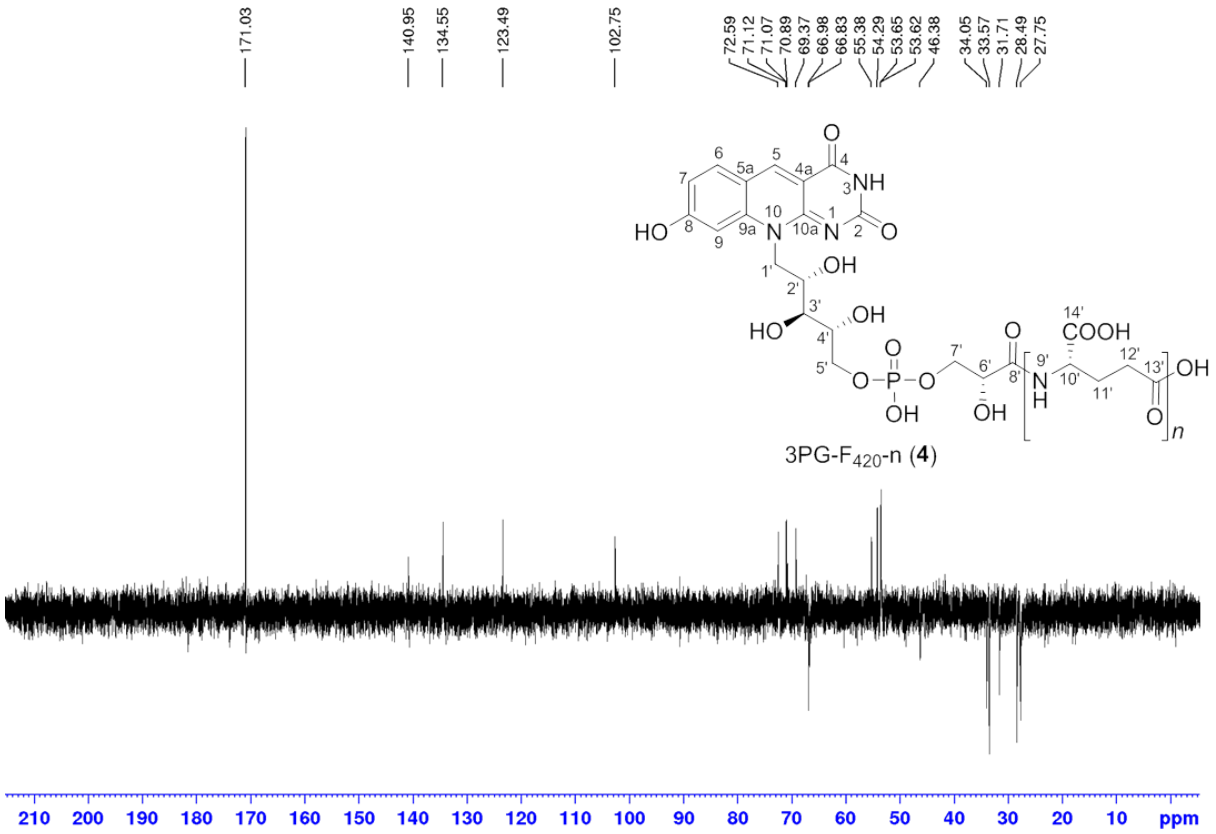


Figure S45. DEPT135 NMR spectrum of 3PG-F_{420}-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

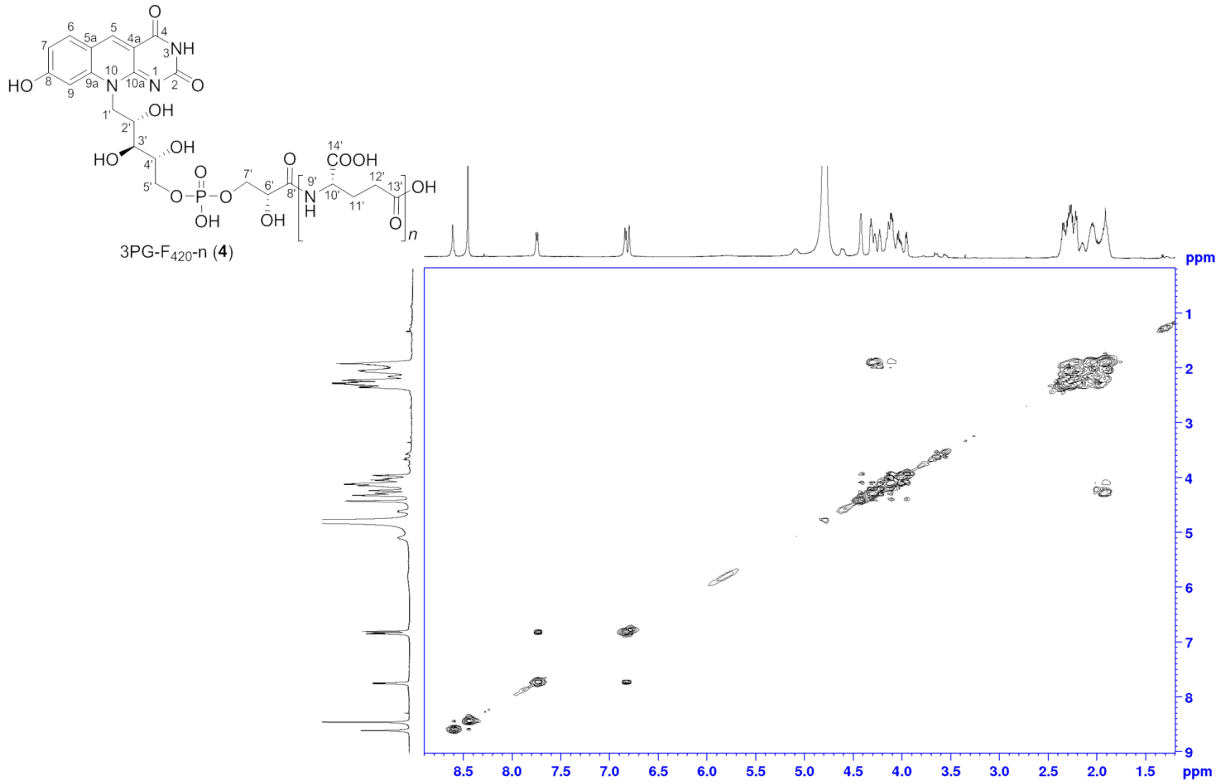


Figure S46. ^{1}H-^{1}H COSY NMR spectrum of 3PG-F_{420}-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

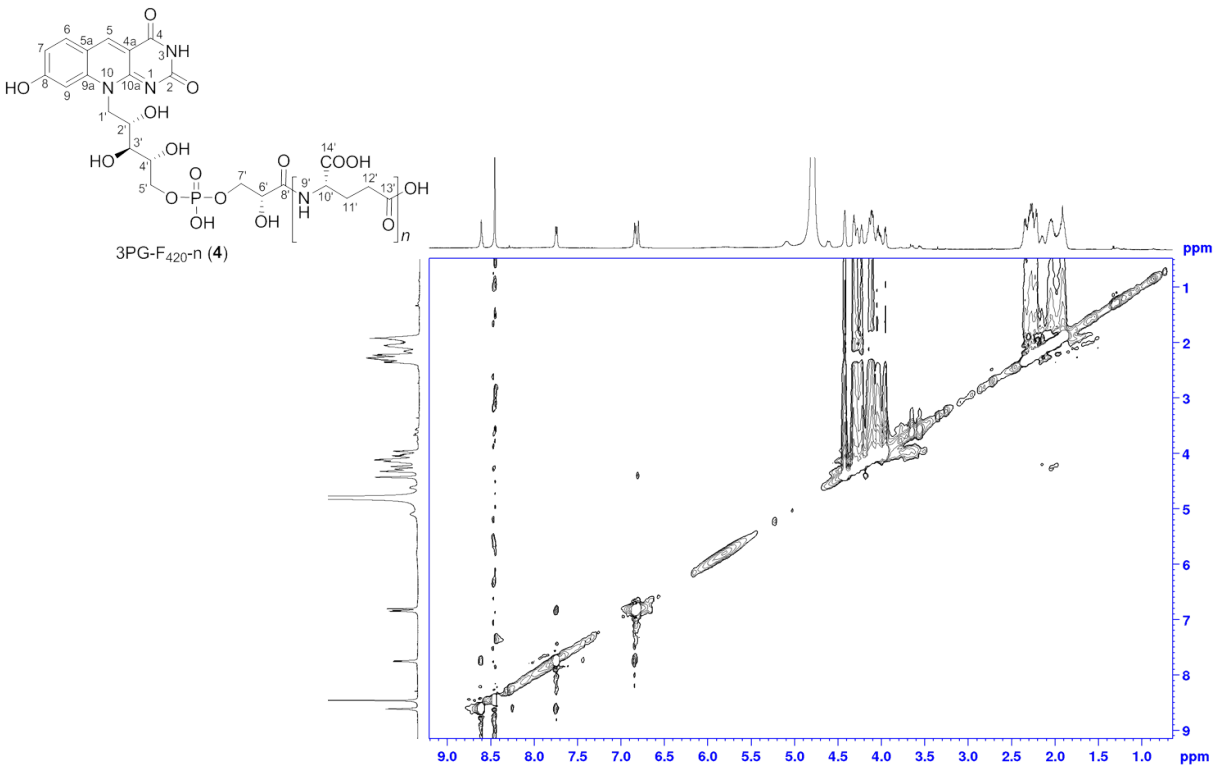


Figure S47. ^{1}H-^{1}H NOESY NMR spectrum of 3PG-F$_{420}$-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

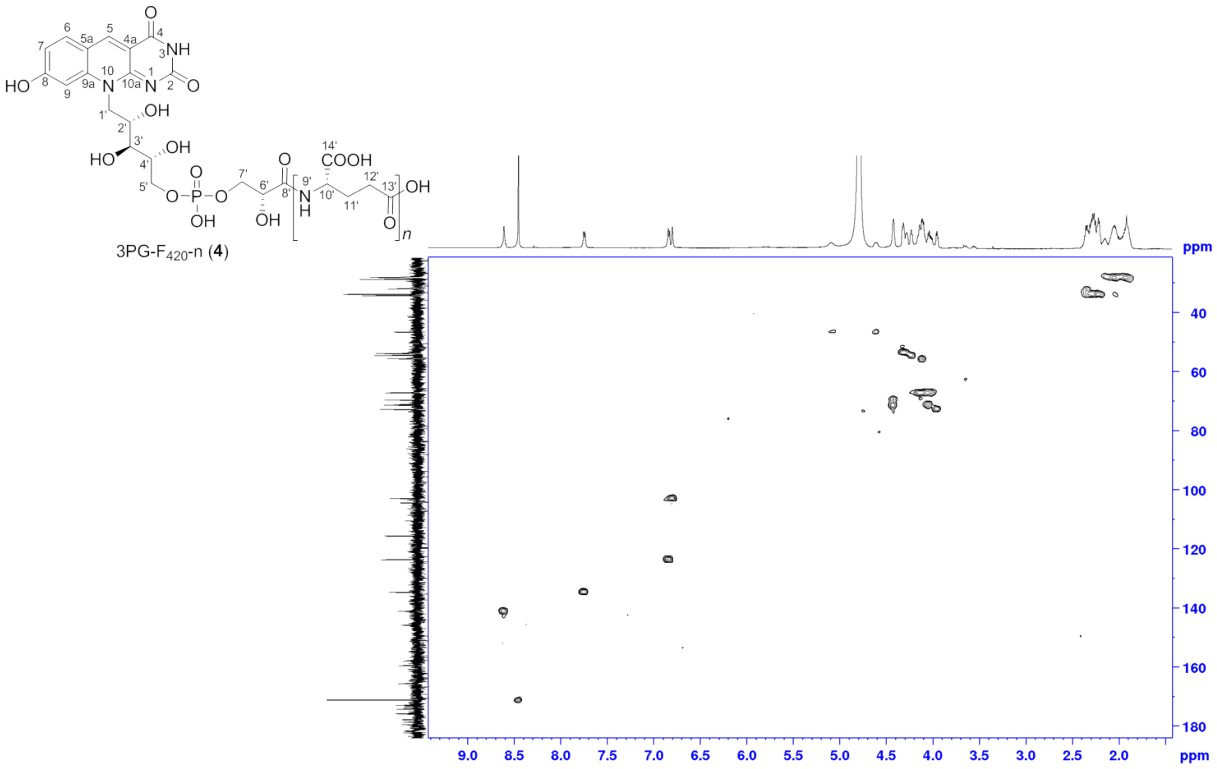


Figure S48. ^{1}H-^{13}C HSQC NMR spectrum of 3PG-F$_{420}$-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

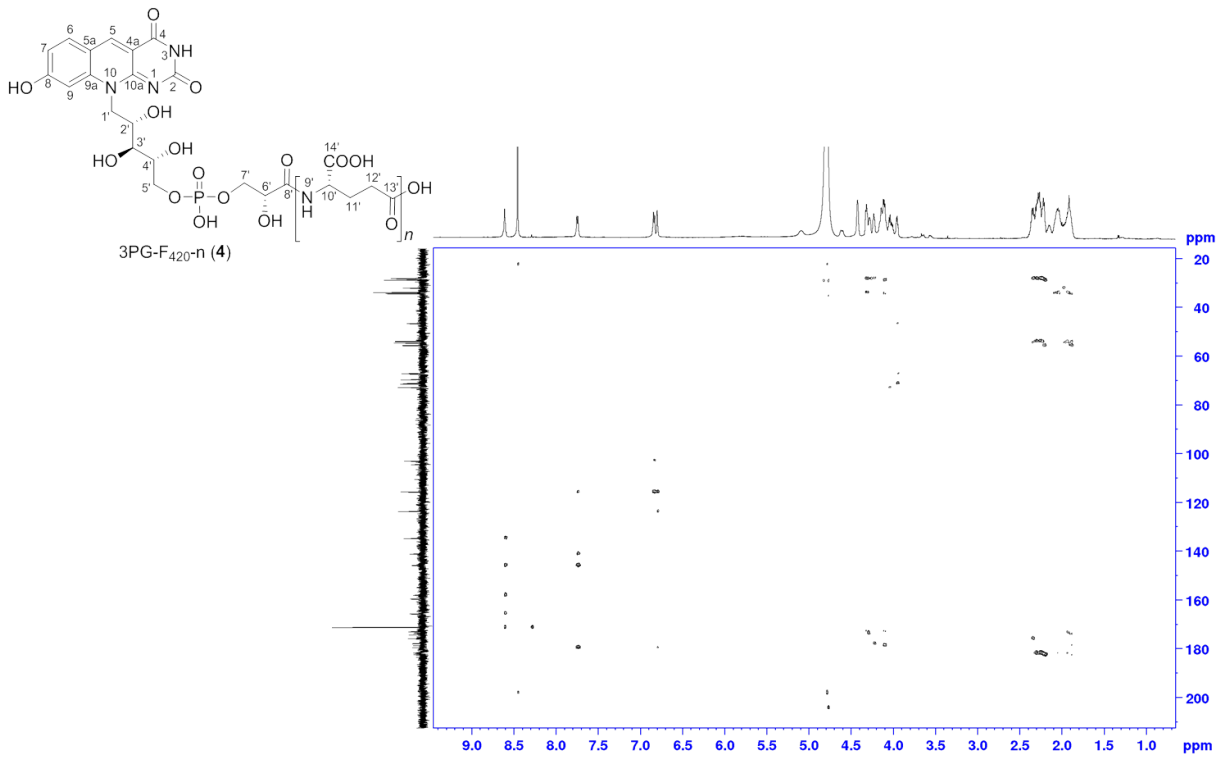


Figure S49. ^{1}H-^{13}C HMBC NMR spectrum of 3PG-F$_{420}$-n (**4**, 0.1% ND_3 in D_2O, 300 MHz, 300 K).

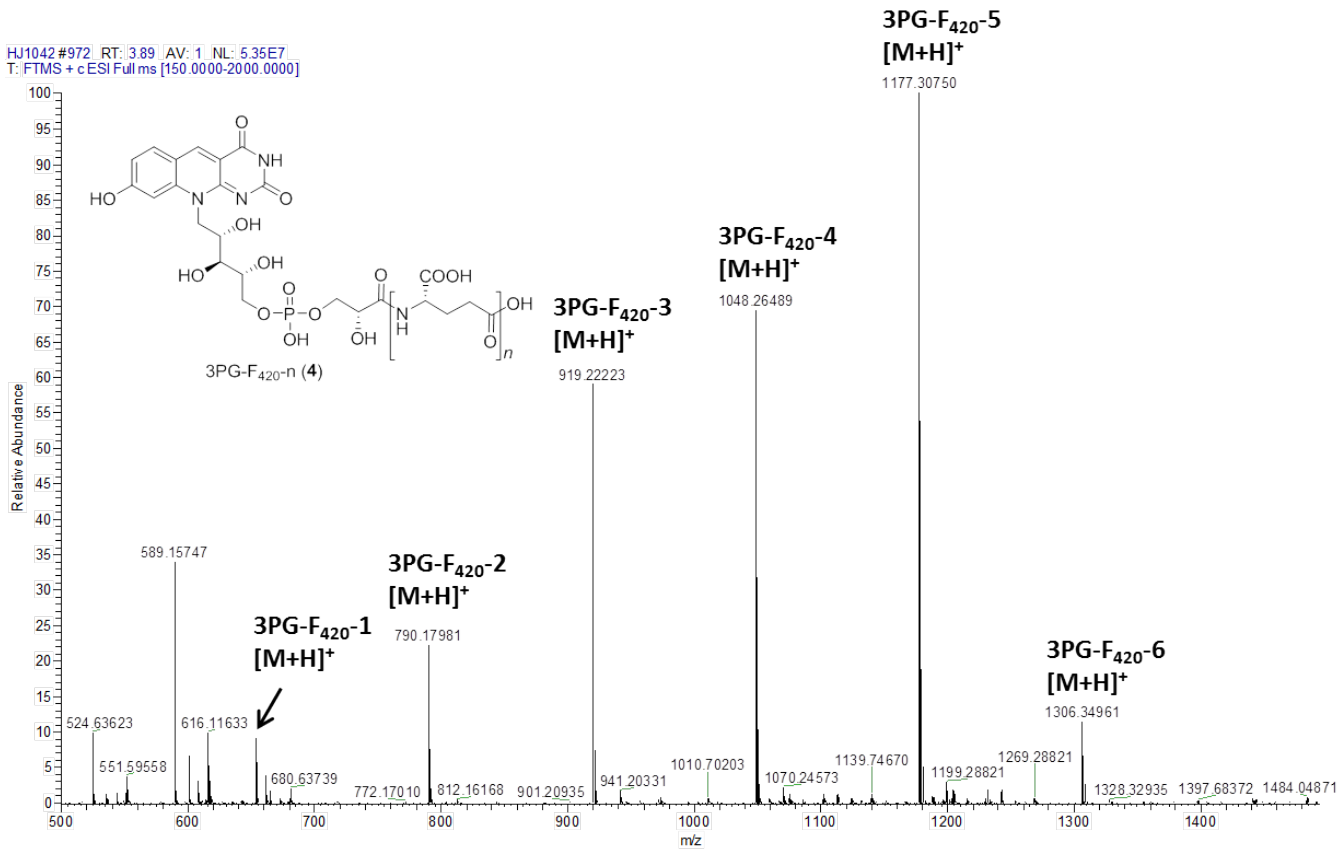


Figure S50. ESI-HRMS spectrum of 3PG-F$_{420}$-n (**4**).

2.3.4 Determination of the absolute stereochemistry of 3PG-F_{420}

In order to determine the stereochemistry of the 3-phosphoglyerate moiety, we performed acid hydrolysis of 3PG-F_{420}-0 (**5**) and analyzed the reaction products by UHPLC-MS as described in Experimental Procedures. Comparison with synthetic standards demonstrated that the retention time of the hydrolyzed product of 3PG-F_{420}-0 (**5**) was identical to the one of D-glyceric acid. Therefore, the stereochemistry of 3-phosphoglycerate moiety in 3PG-F_{420}-0 (**5**) side chain is assigned as the D-configuration (**Figure S51a-c**).

As a control, classical F_{420}-n was submitted to the same analysis. The hydrolyzed products as well as standards of D-lactic acid and L-lactic acid were analyzed under the same analytical condition. The identical retention time of hydrolysis products of classical F_{420}-n with L-lactic acid indicated the stereochemistry of the lactyl moiety as L-configuration as described in the literature(8) (**Figure S51d-f**).

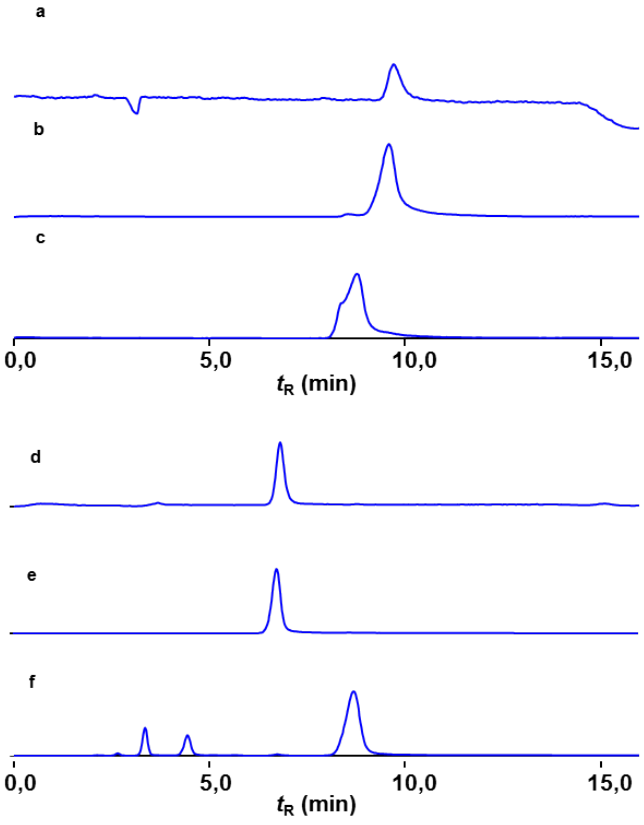


Figure S51. UPLC-MS chromatogram of acid-hydrolyzed product of 3PG-F_{420}-0 and F_{420}-n under SIM mode (–): a) SIM (–) with *m/z* 105.3 of acid hydrolyzed product of 3PG-F_{420}-0, t_R = 9.707 min; b) SIM (–) with *m/z* 105.3 of D-glyceric acid, t_R = 9.607 min; c) SIM (–) with *m/z* 105.3 of L-glyceric acid, t_R = 8.747 min; d) SIM (–) with *m/z* 89.0 of acid-hydrolyzed product of F_{420}-n, t_R = 6.775 min; e) SIM (–) with *m/z* 89.0 of L-lactic acid, t_R = 6.733 min; f) SIM (–) with *m/z* 89.0 of D-lactic acid, t_R = 8.681 min. AnAstec CHIROBIOTIC R (10 cm x 4.6 mm, 5 µm, Sigma) chiral column was used for chromatographic separation of enantiomers.

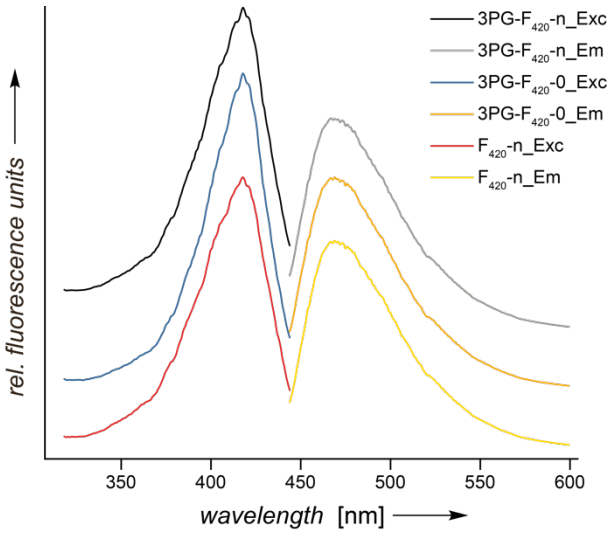


Figure S52: Fluorescence emission and excitation spectra of classical F_{420}-n, 3PG-F_{420}-n and 3PG-F_{420}-0 (50 mM sodium phosphate buffer, pH 7.5). The fluorescence spectra were distributed along the y-axis for better visibility. Em: Emission, Exc: Excitation.

2.4 Combined CofC/D in-vitro enzyme assay

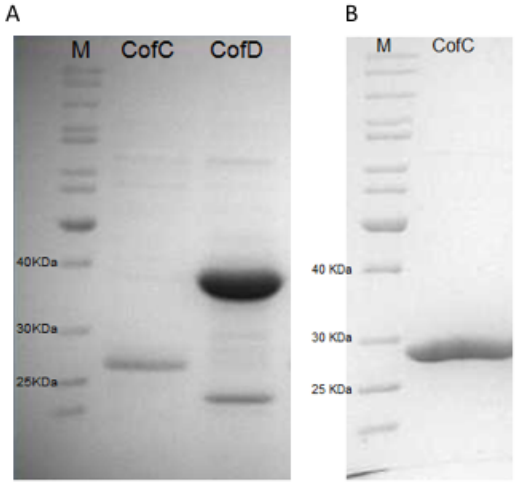


Figure S53. SDS-PAGE analysis of of recombinant proteins used for CofC/D assays. A) His_6-CofC from *P. rhizoxinica* (25.8 kDa) and His_6-CofD from *Methanocaldococcus jannaschii* (37,8 kDa) after metal affinity chromatography (Ni-NTA resin). B) His_6-CofC from *M. jannaschii* (27.65 kDa) after metal affinity chromatography (Ni-NTA resin). All proteins were produced in *E. coli* BL21(DE3). Page Ruler^{TM} (Thermo Scientific) 10-200 KDa protein ladder (M) was used as a marker.

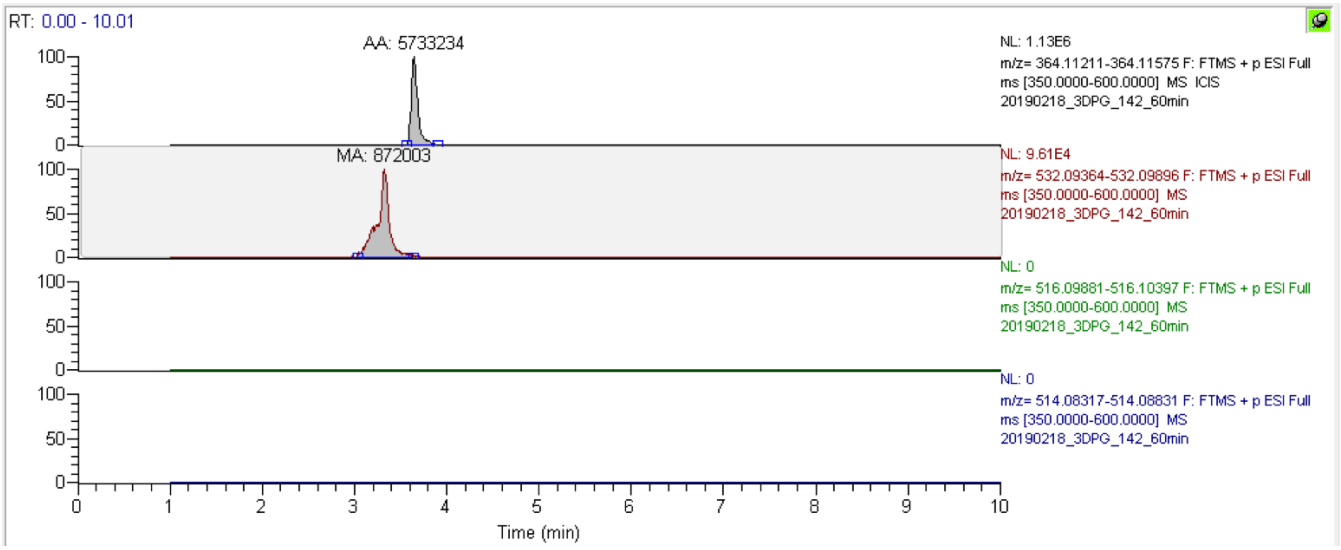


Figure S54. LC-MS analysis of combined CofC/D enzyme (CofC from *P. rhizoxinica*) assays with 3-phospho-D-glycerate showing XICs of reaction products after 60 min of incubation. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, F_{420}-0: F_{420}-0: 516.10139, DF_{420}-0: 514.08246. Areas under the curve are indicated on top of each peak. 3PG-F_{420} is the only product formed.

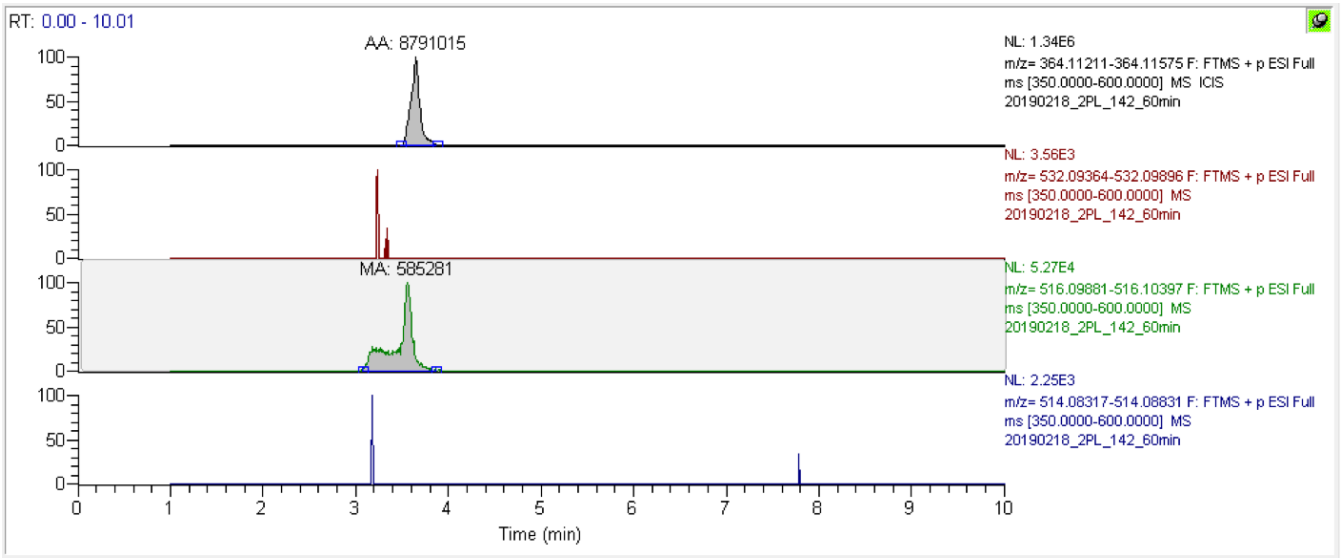


Figure S55. LC-MS analysis of combined CofC/D enzyme assays (CofC from *P. rhizoxinica*) with 2-phospho-L-lactate (2-PL) showing XICs of reaction products after 60 min of incubation. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, F_{420}-0: F_{420}-0: 516.10139, DF_{420}-0: 514.08246. Areas under the curve are indicated on top of each peak. F_{420} is the only product formed. Traces corresponding to the mass of other species are below the noise level.

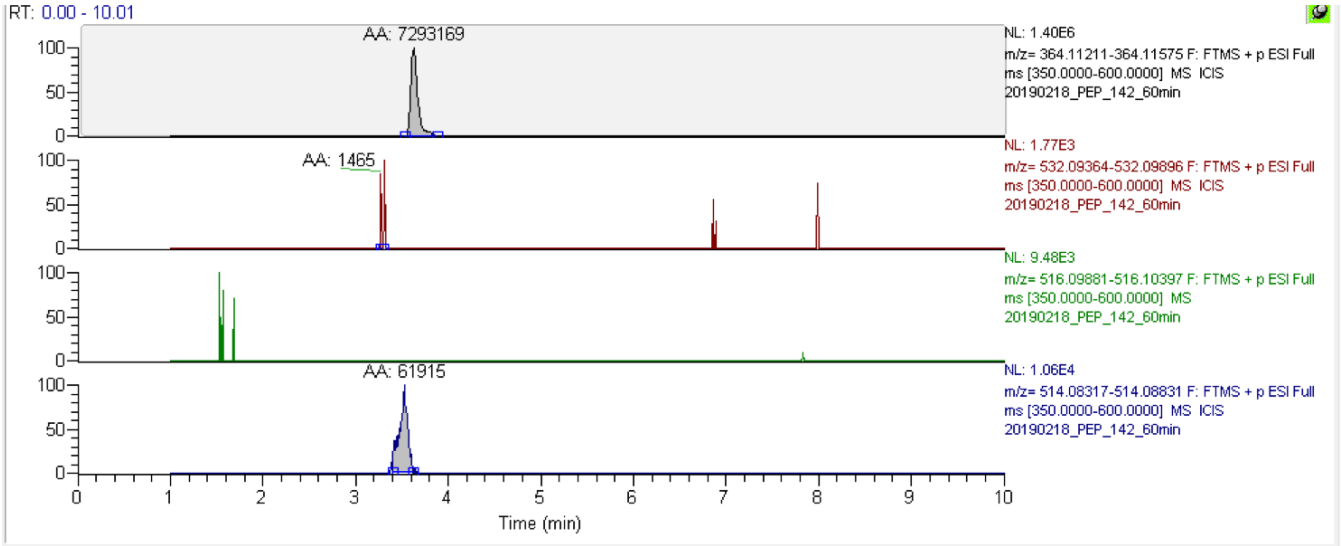


Figure S56. LC-MS analysis of combined CofC/D enzyme assays (CofC from *P. rhizoxinica*) with phosphoenolpyruvate (PEP) showing XICs of reaction products after 60 min of incubation. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, F_{420}-0: F_{420}-0: 516.10139, DF_{420}-0: 514.08246. Areas under the curve are indicated on top of each peak. DF_{420} is the only product formed. Traces corresponding to the mass of other species are below the noise level.

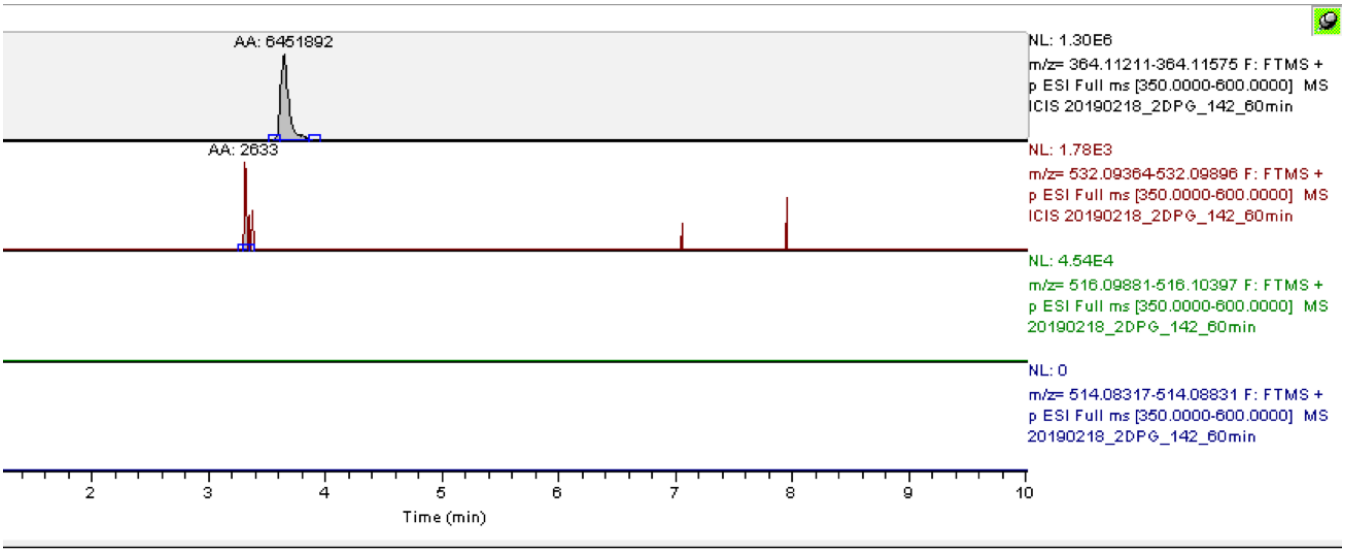


Figure S57. LC-MS analysis of combined CofC/D enzyme assays (CofC from *P. rhizoxinica*) with 2-phospho-D-glycerate (2-D-PG) showing XICs of reaction products after 60 min of incubation. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, F_{420}-0: F_{420}-0: 516.10139, DF_{420}-0: 514.08246. Areas under the curve are indicated on top of each peak. Traces corresponding to the mass of 2PG-F_{420} species are below the noise level.

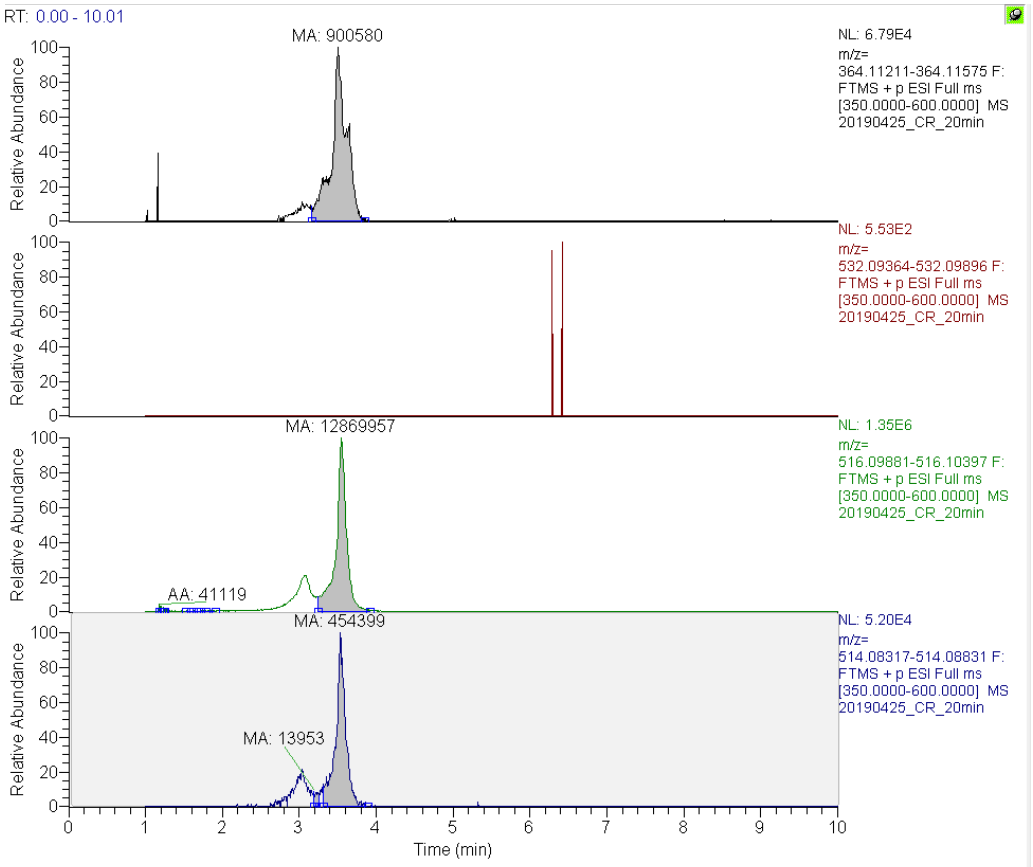


Figure S58. LC-MS analysis of combined CofC/D enzyme assays (CofC from *M. jannaschii*) carried out as substrate competition assay (2-PL, 3-PG, PEP) showing XICs of reaction products after 20 min of incubation. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, F_{420}-0: F_{420}-0: 516.10139, DF_{420}-0: 514.08246. Areas under the curve are indicated on top of each peak. Classical F_{420}-0 and $DF_{420\text{-}0}$ are formed. Traces corresponding to the mass of other species are below the noise level.

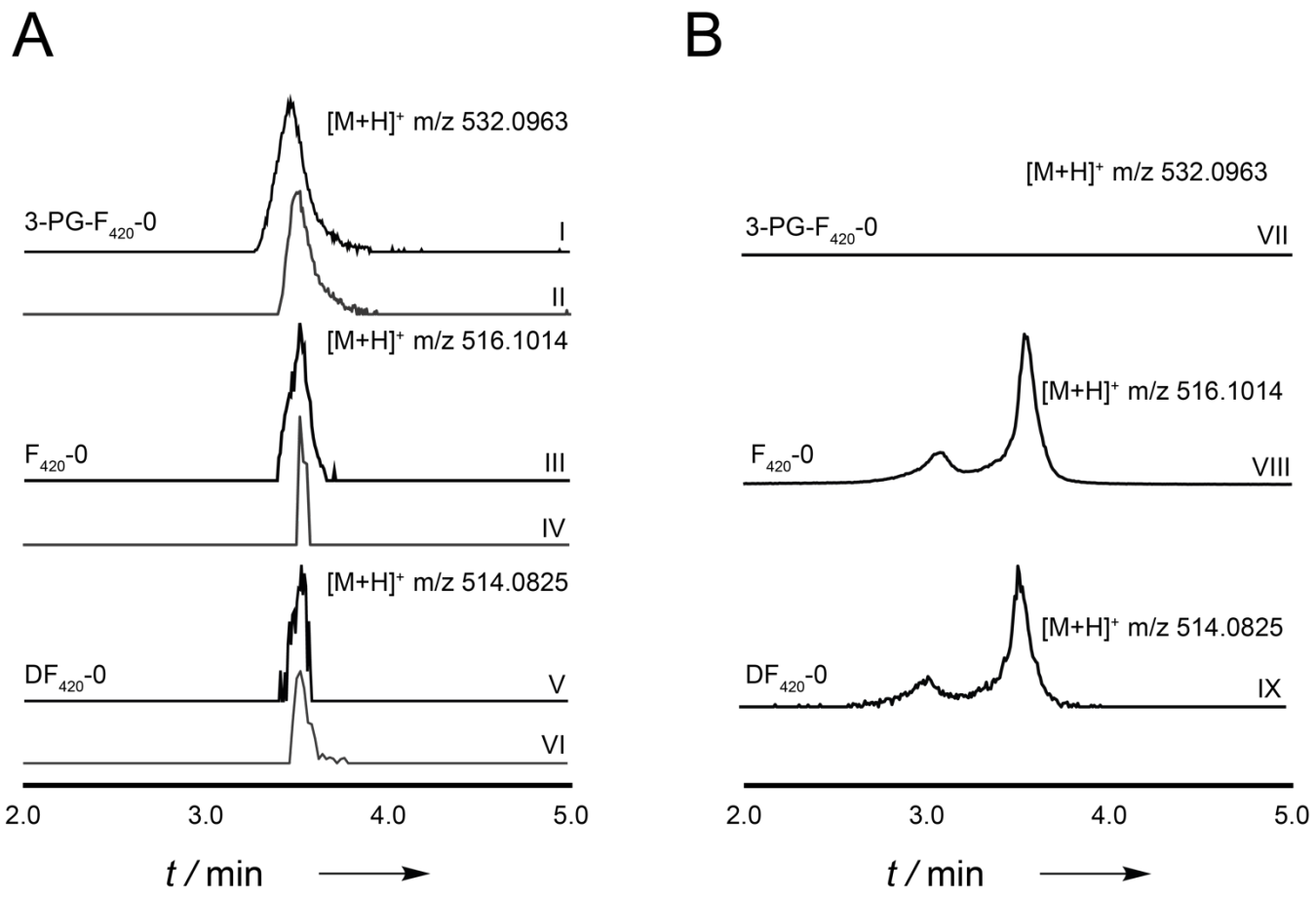


Figure S59. Combined CofC/D enzyme assay carried out as substrate competition assay (3-PG, 2-PL, PEP). LC-MS analyses (XICs) shows reaction products in comparison to corresponding in-vivo products: HF420-0 in vitro, III: F420-0 in vitro, and V: DF420-0 in vitro. II, IV, VI show corresponding in-vivo products A) Reaction with CofC of *P.rhizoxinica*. B) Reaction with CofC from *M. jannaschii*. Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, F_{420}-0: F_{420}-0: 516.10139, DF_{420}-0: 514.08246. Intensities are not drawn to scale.

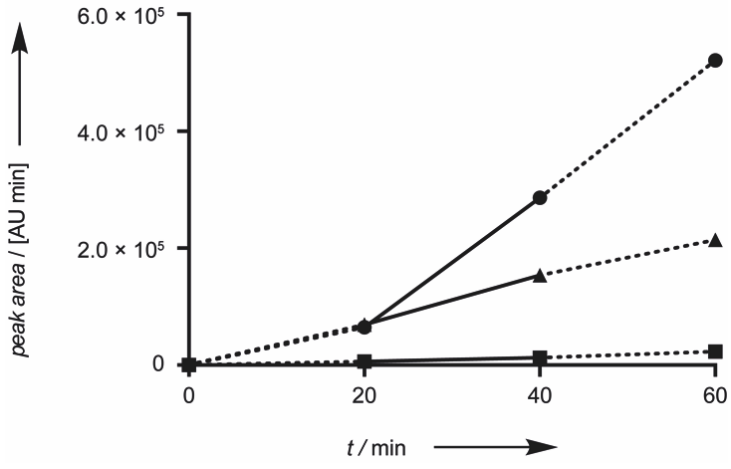


Figure S60. Time course of a combined CofC/D enzyme assay (CofC from *P. rhizoxinica*) with competing substrates (3-phospho-D-glycerate, 2-phospho-L-lactate, phosphoenolpyruvate). Corresponding product formation (circles: 3PG-F_{420}, triangles: DF_{420} squares: F_{420}) was monitored by LC-MS and shown as area under the curve of extracted ion chromatograms (5 ppm mass deviation). Theoretical masses $[M+H]^+$: 3PG-F_{420}: m/z 532.09630, F_{420}-0: m/z 516.10139, and DF_{420}-0: m/z 514.08574. Solid lines indicate the near-linear range of the reaction that was used to determine the rate of product formation.

2.5 Fno and malachite green reduction assays

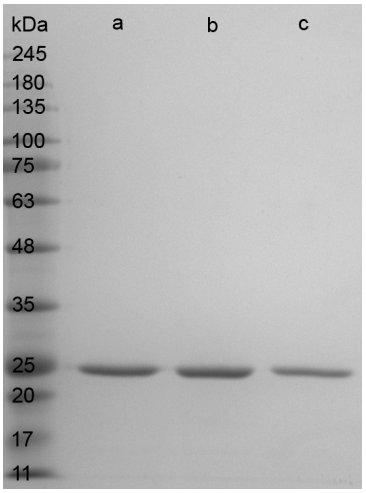


Figure S61 SDS-PAGE gel of recombinant, purified His_6-Fno from *A. fulgidus* (24.1 kDa). Lanes a, b, and c show biological replicates used for kinetic studies. BlueEye Prestained Protein Marker (Jena Bioscience) was used as a marker.

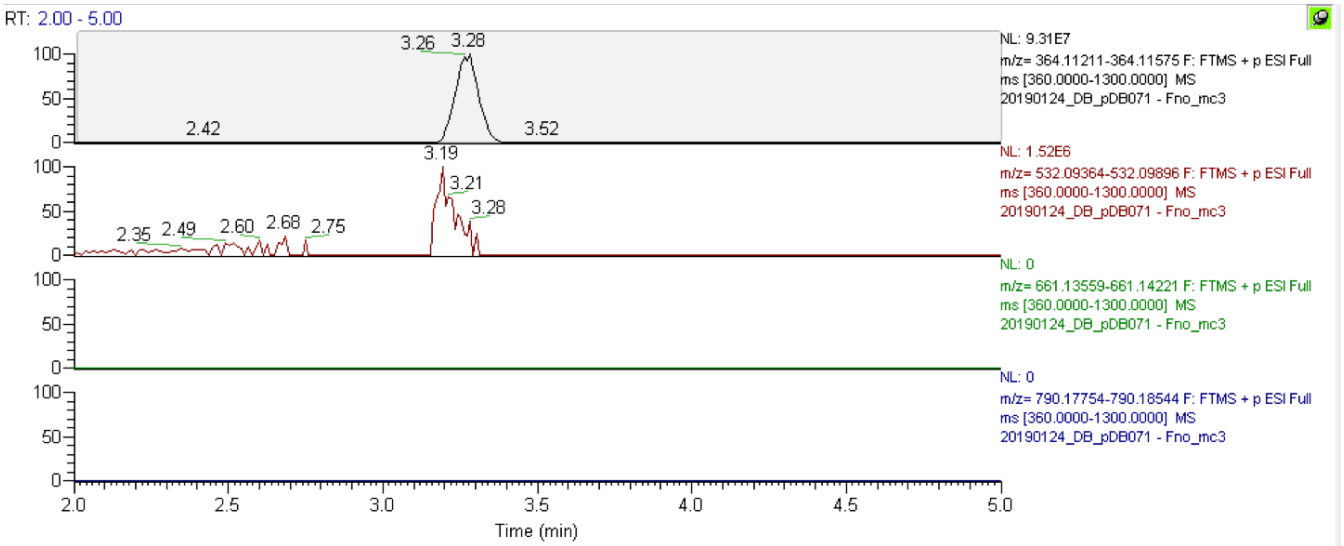


Figure S62. LC-MS analysis of extracts of *E. coli* BL21(DE3) / pDB071 (minimal BGC for 3PG-F_{420}-0 and *fno*) showing XICs of 3PG-F_{420}-n species with a varying number of (oligo)-γ-glutamate residues measured on a Kinetex XB-C18 column (Phenomenex). Expected masses ($[M+H]^+$, 5 ppm mass tolerance): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149. As expected only F_O and 3PG-F_{420}-0 were produced.

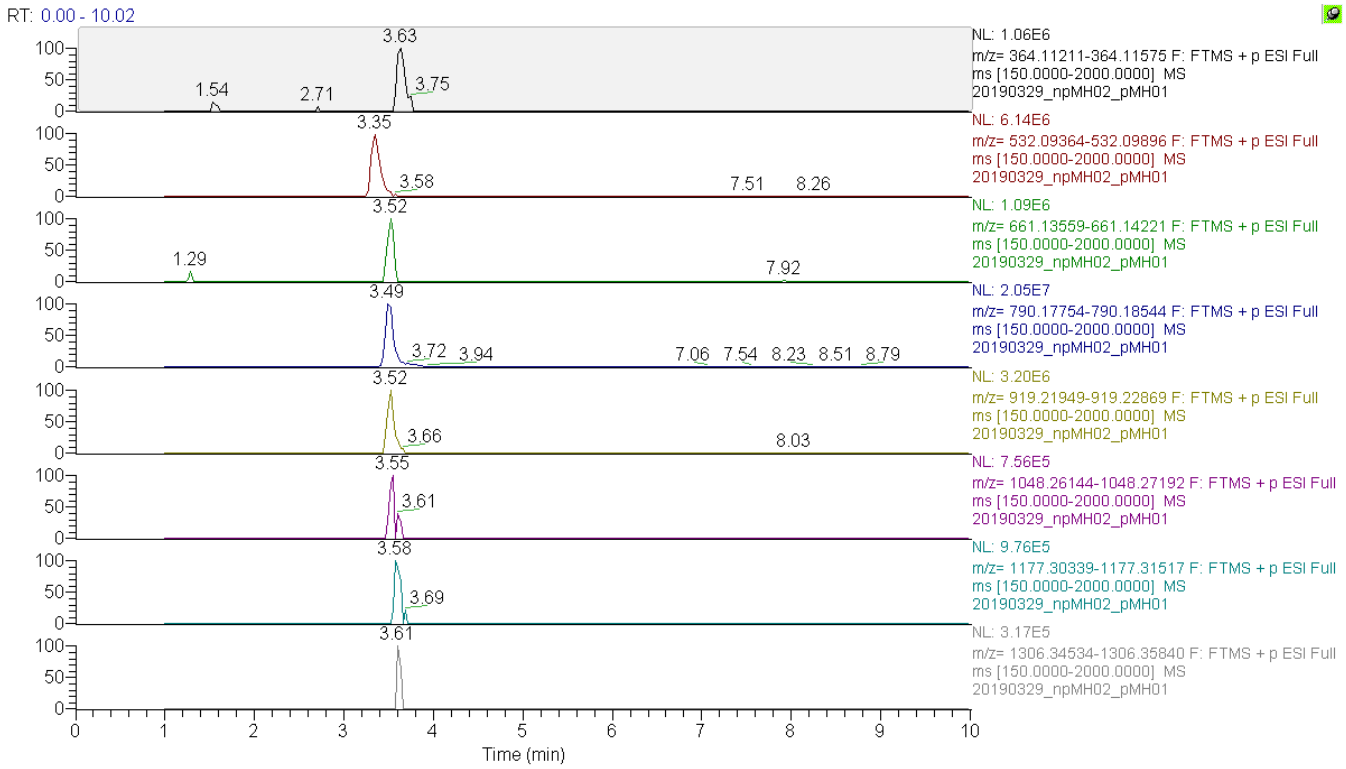


Figure S63: LC-MS analysis of extracts from *E. coli* BL21(DE3) coexpressing *cofE* from *M. jannaschii* (pMH01) and a minimal gene cluster (pMH02) producing 3PG-F420-0. Extracted ion chromatograms (5 ppm mass tolerance) were extracted using the following exact masses ($[M+H]^+$): F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187

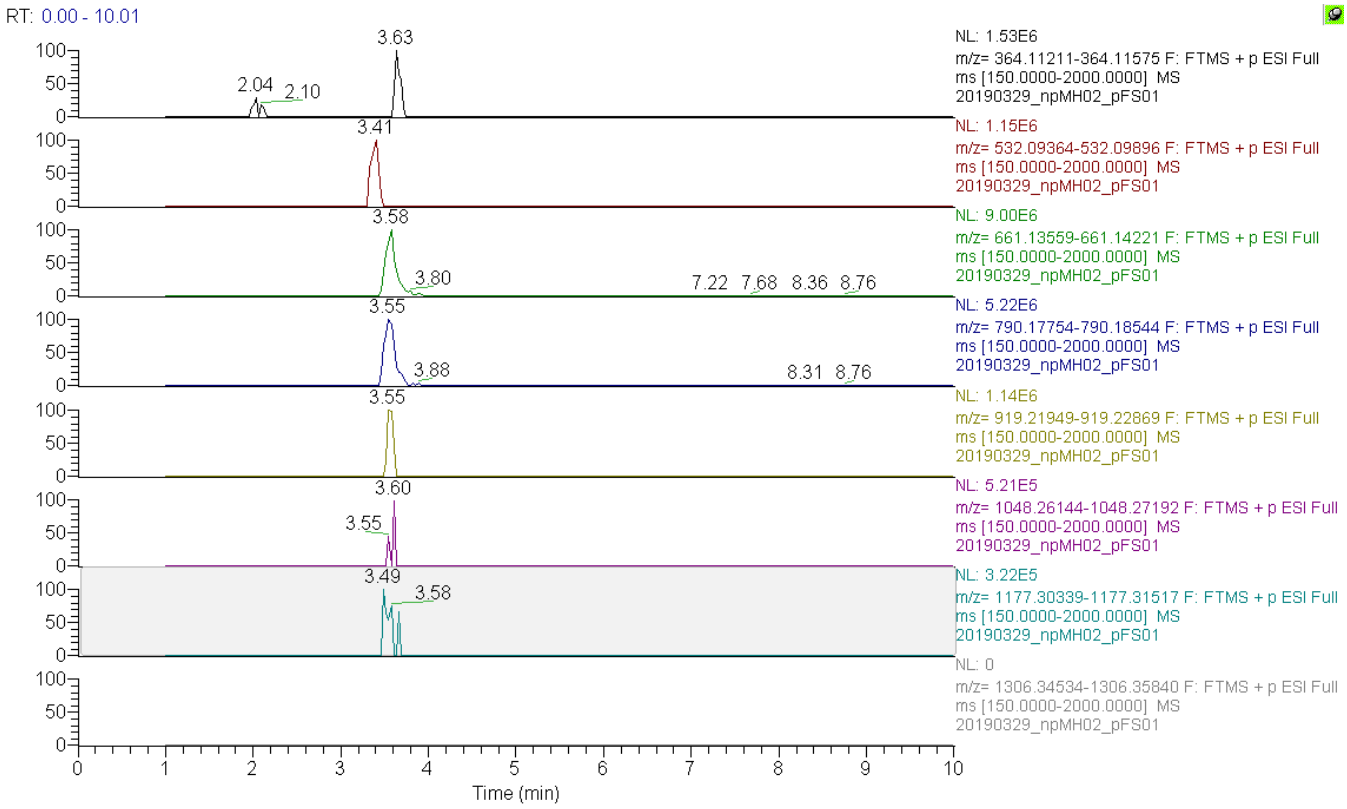


Figure S64: LC-MS analysis of extracts from *E. coli* BL21(DE3) coexpressing *cofE* from *P. rhizoxinica* (pFS01) and a minimal gene cluster (pMH02) producing 3PG-F420-0. F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187

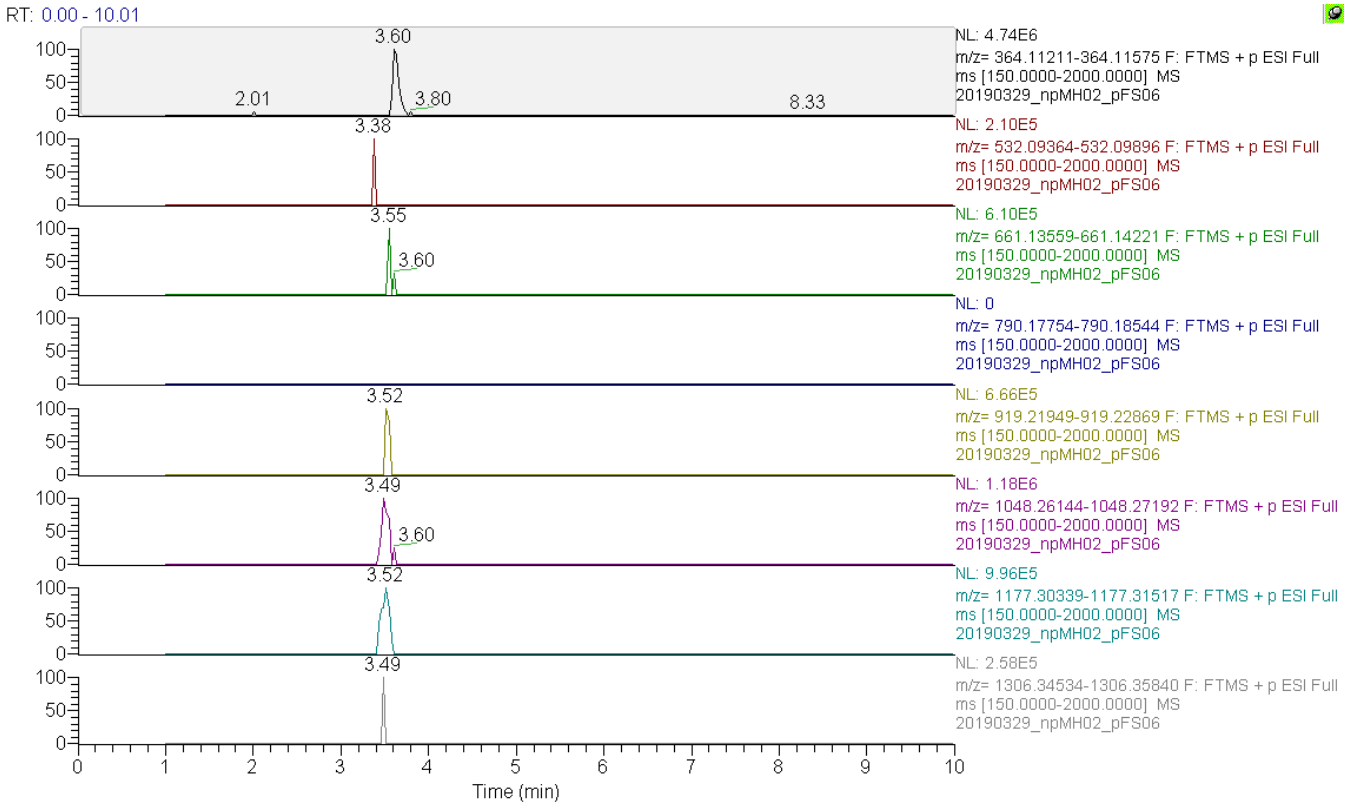


Figure S65: LC-MS analysis of extracts from *E. coli* BL21(DE3) coexpressing *fbiB* from *M. smegmatis* and a minimal gene cluster (pMH02) producing 3PG-F420-0. F_O: 364.11393, 3PG-F_{420}-0: 532.09630, 3PG-F_{420}-1: 661.13890, 3PG-F_{420}-2: 790.18149, 3PG-F_{420}-3: 919.22409, 3PG-F_{420}-4: 1048.26668, 3PG-F_{420}-5: 1177.30928, 3PG-F_{420}-6: 1306.35187

2.6 Plasmids sequences (Fasta format)

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