Supporting Information

for

Chemoproteomic identification of phosphohistidine acceptors: Posttranslational activity regulation of a key glycolytic enzyme

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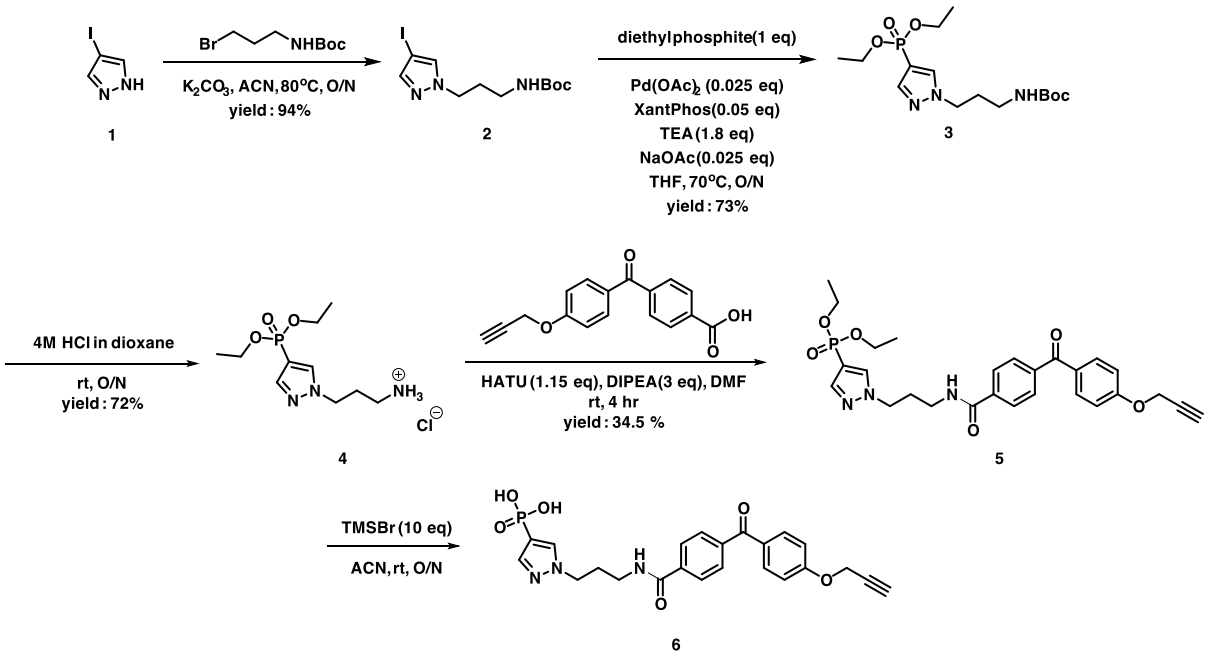
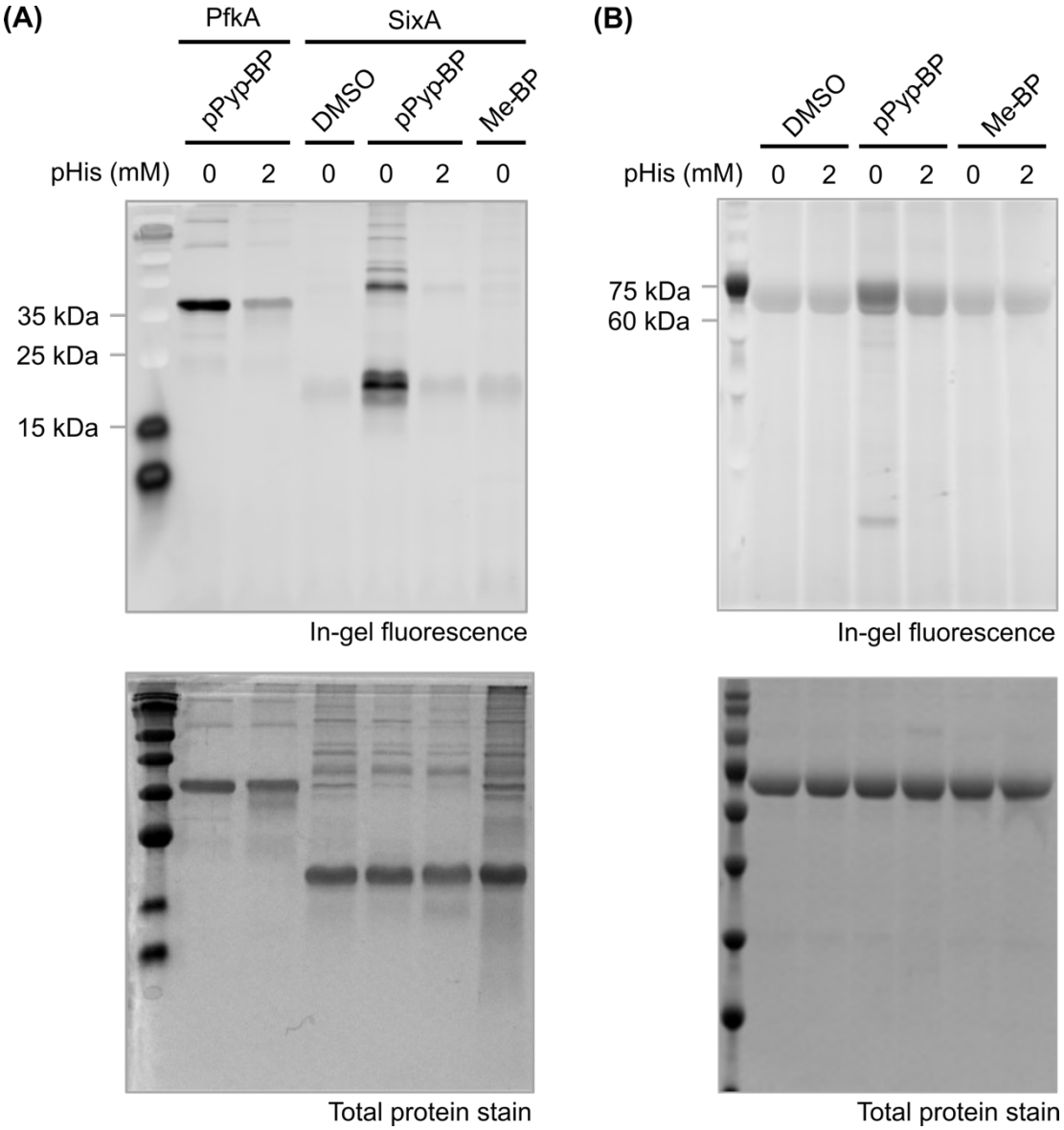
Supplementary Figures and Tables

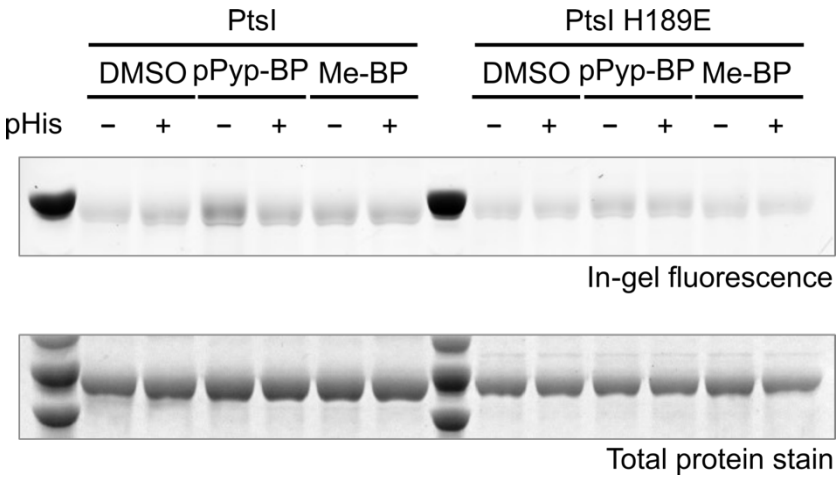
Supplementary Figures



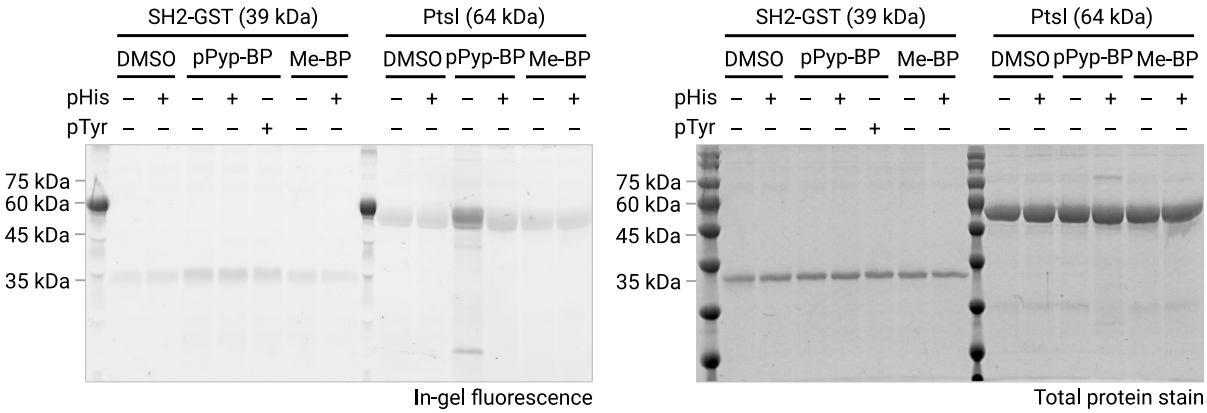
Supplementary Figure S1. Synthetic scheme for the preparation of pPyp-BP. A full description of the synthetic procedure is given in the Experimental Procedure section (see below).



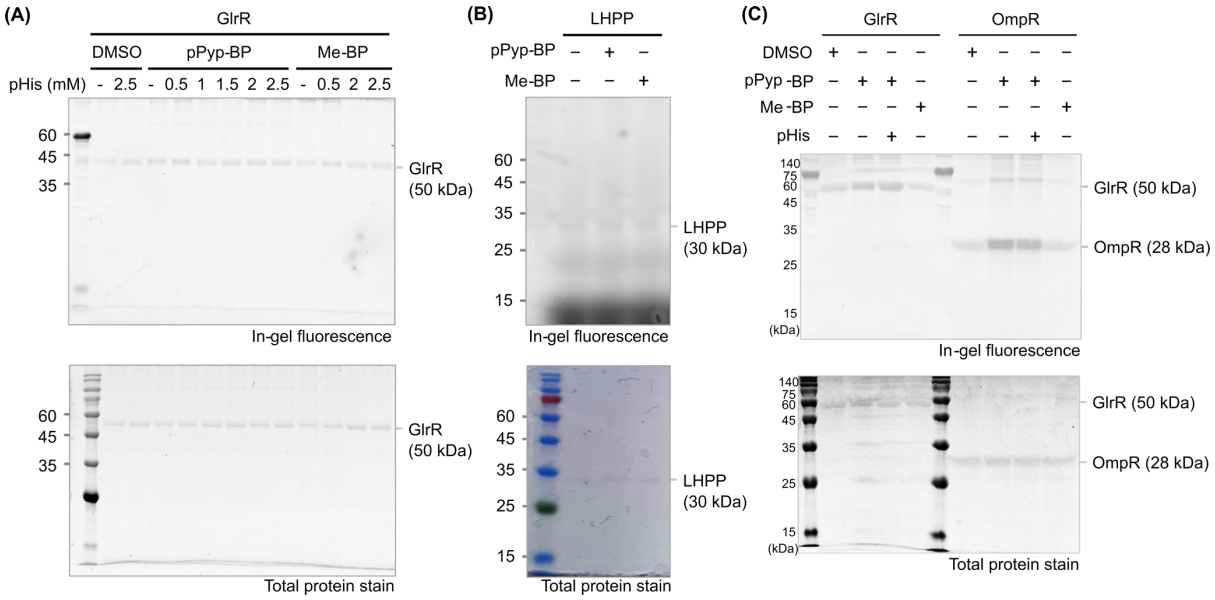
Supplementary Figure S2. Full gel images of Figure 2B. Photolabeling of model *E. coli* SixA and PtsI with pPyp-BP. The photolabeling was visualized by a Cu-catalyzed click reaction with TAMRA-azide (top panel), and the loading was shown with Coomassie stain (bottom panel).



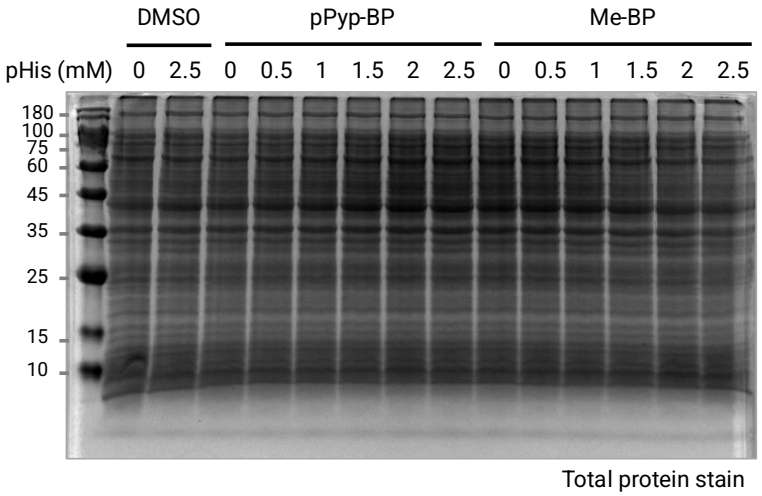
Supplementary Figure S3. Photolabeling of a model pHis acceptor (PtsI) and its pHis-binding site mutant (PtsI H189E). PtsI and its mutant H189E were labeled with pPyp-BP or Me-BP in the presence (+) or absence (-) of pHis. While robust labeling of PtsI by pPyp-BP was observed, labeling was substantially reduced for the H189E mutant, confirming the specificity of pPyp-BP for the pHis-binding site. Importantly, residual labeling of PtsI H189E by pPyp-BP was not diminished in the presence of excess pHis, indicating that this labeling is nonspecific.



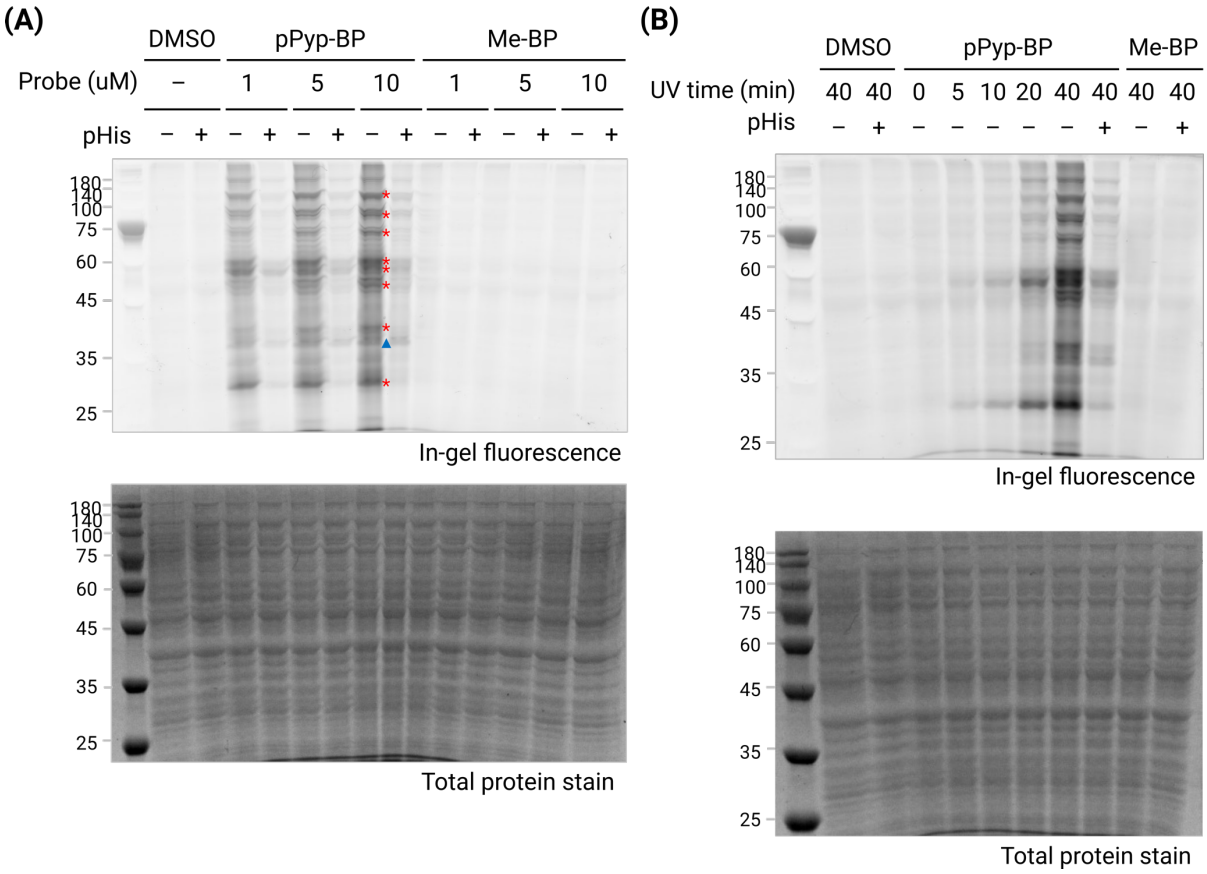
Supplementary Figure S4. Photolabeling of SH2-GST with pPyp-BP. SH2 (from human SH2B), a well-established pTyr-binding domain, was not selectively labeled by pPyp-BP. The weak labeling observed for SH2-GST was not affected by the presence of free pHis or free pTyr, indicating nonspecific labeling (left). In contrast, labeling of the pHis acceptor PtsI (right) was reduced in the presence of excess pHis, confirming specificity. These findings demonstrate that pPyp-BP does not exhibit cross-reactivity to the pTyr reader.



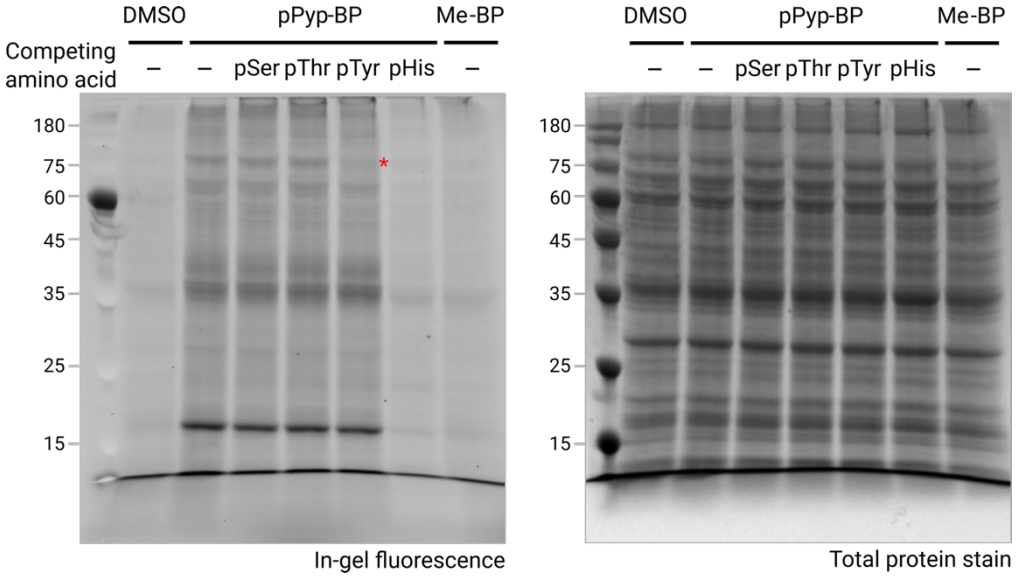
Supplementary Figure S5. Photolabeling of additional known pHis-recognizing proteins with pPyp-BP. LHPP showed no detectable labeling, while GlrR and OmpR exhibited weak labeling by pPyp-BP. However, this labeling was not competed by excess pHis, suggesting these interactions are nonspecific.



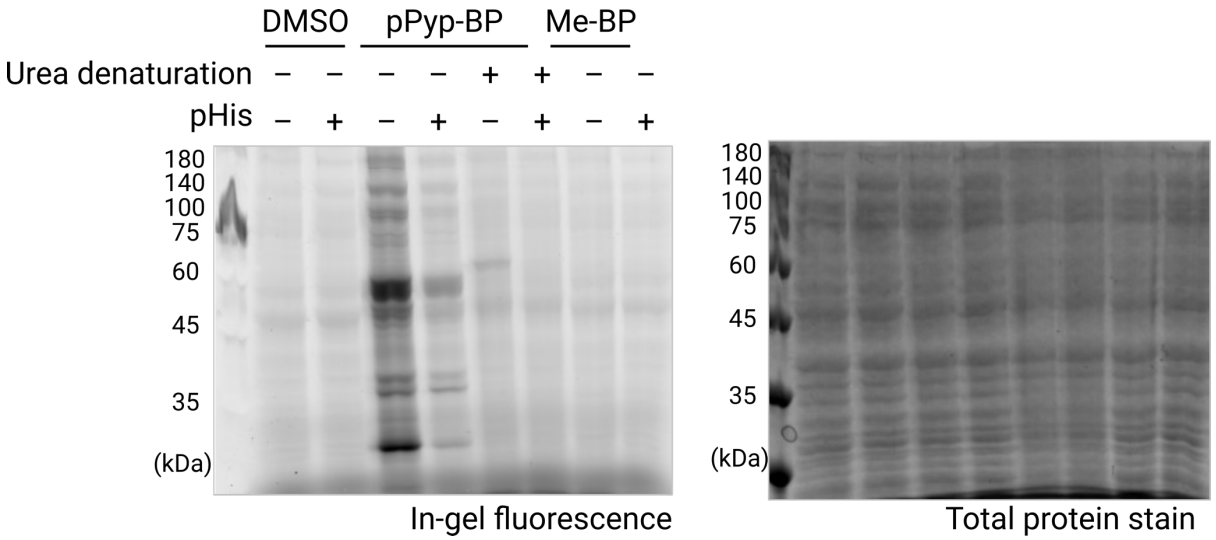
Supplementary Figure S6. Full gel images of Figure 2C in the main text. The total protein loading was shown with the Coomassie stain.



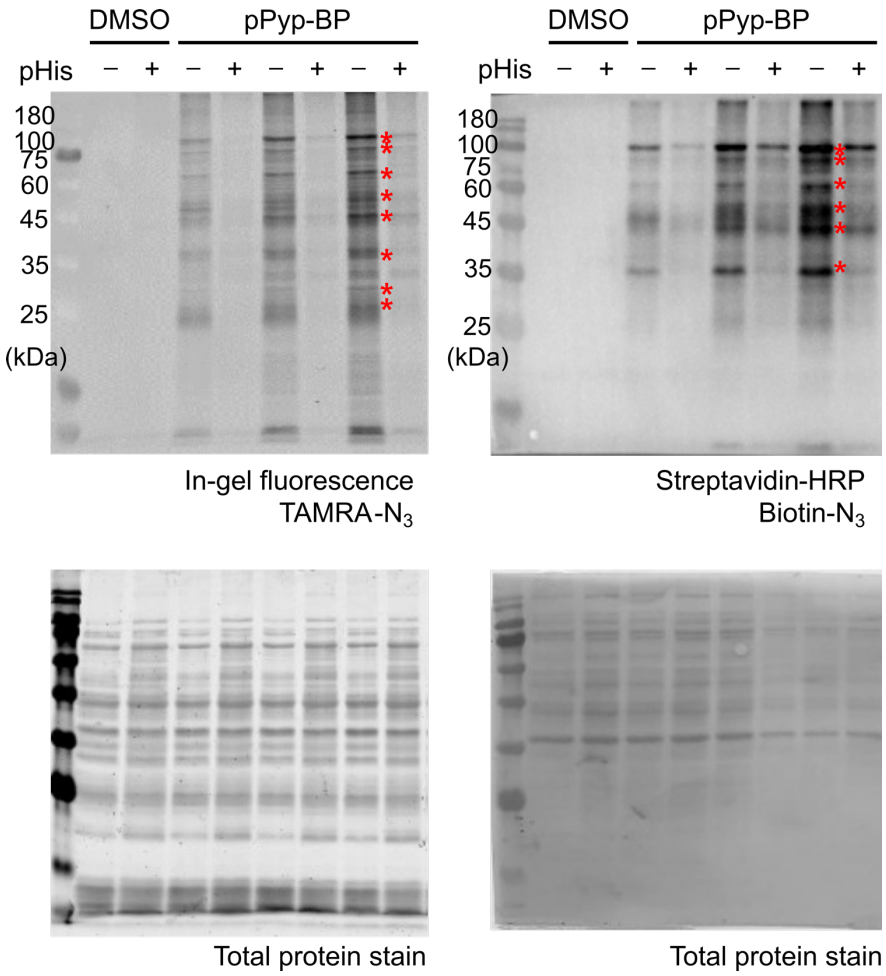
Supplementary Figure S7. Photolabeling of *E. coli* lysates. Labeling intensity with pPyp-BP increased with increasing probe concentration (A) or UV exposure time (B). Weaker photolabeling in the presence of pHis indicated that the probe labeled pHis-binding proteins. Labeling of several bands (red stars) was markedly reduced by competition with excess pHis, whereas the labeling of a nonspecific band (blue triangle) remained unchanged. Negligible labeling by Me-BP (negative control) was observed.



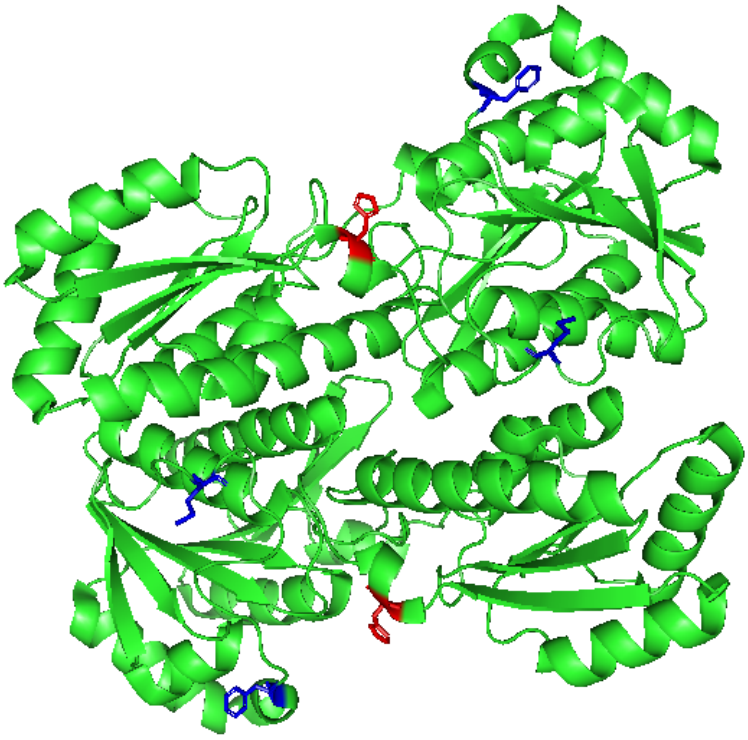
Supplementary Figure S8. Photolabeling of *E. coli* lysate with pPyp-BP in the presence of competing phosphoamino acids. The labeling is competed with pHis, but not with pSer, or pThr. The one protein band at approximately 75 kDa (red star) is reduced in the presence of pTyr, whereas other labeled proteins remain unaffected. In contrast, in the presence of pHis, the labeling intensity of nearly all proteins is reduced. The photolabeling was visualized by a Cu-catalyzed click reaction with TAMRA-azide (top panel), and the loading was shown with Coomassie stain (bottom panel).



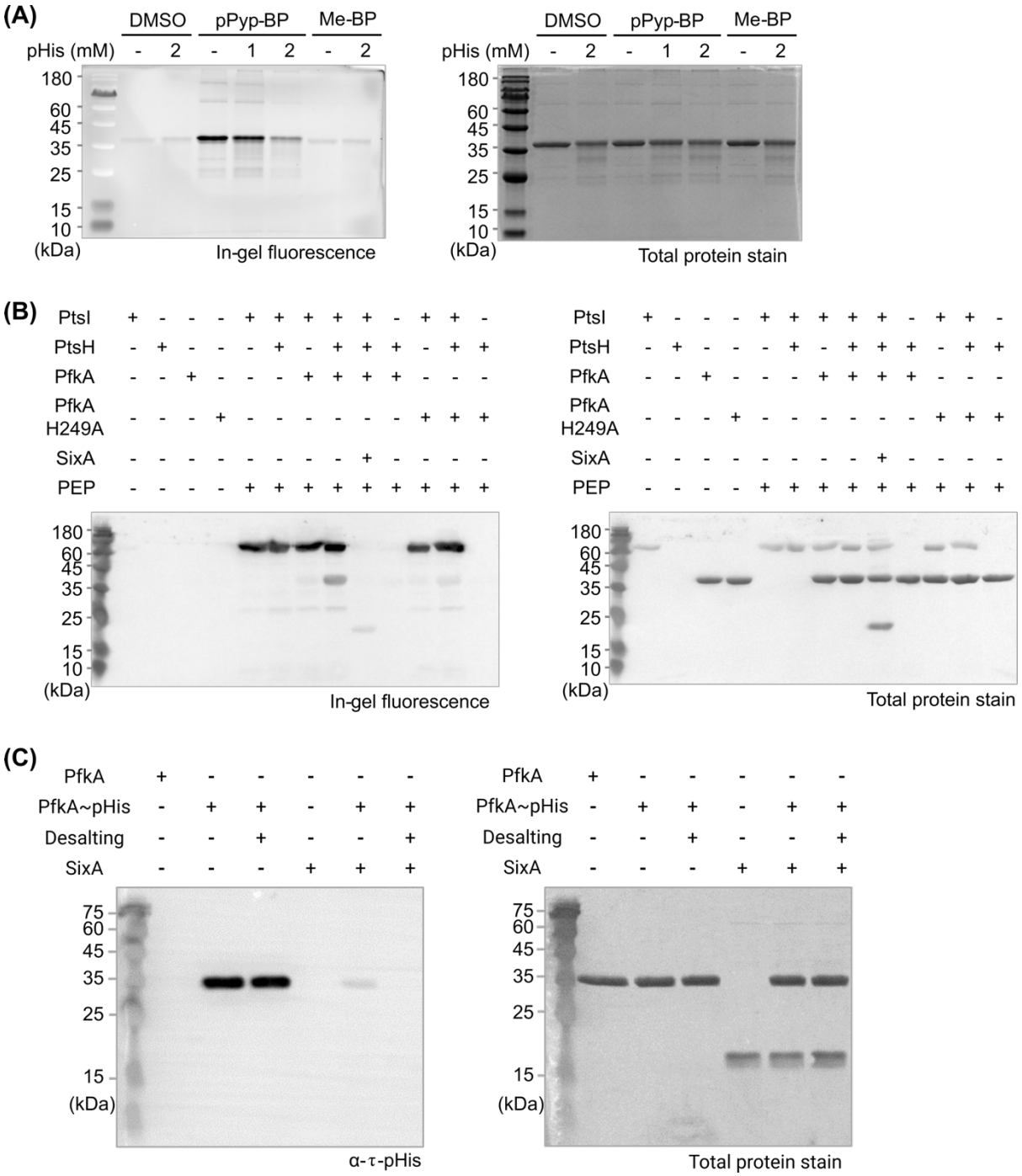
Supplementary Figure S9. pPyp-BP labeling requires native protein structures. *E. coli* lysates were subjected to denaturation with 8 M urea (+) or left untreated (-) before photocrosslinking with pPyp-BP or Me-BP, followed by TAMRA-azide conjugation and in-gel fluorescence detection. Left: In-gel fluorescence image clearly showing reduced pPyp-BP labeling in denatured lysates. Right: Total protein stain confirming equal protein loading. These results indicate that pPyp-BP preferentially labels properly folded proteins.



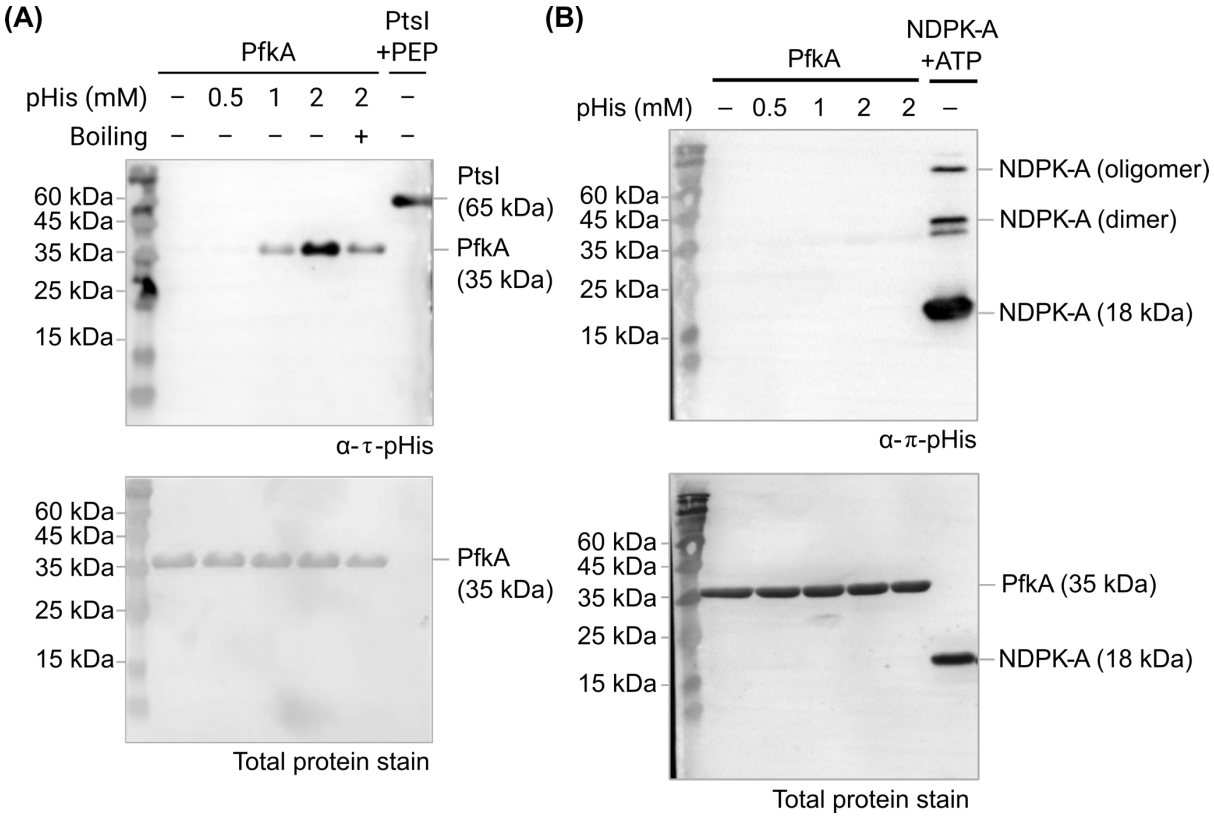
Supplementary Figure S10. E. coli lysates labeled with pPyp-BP were subjected to CuAAC using either TAMRA-N_3 or Biotin-N_3. Both detection methods exhibited similar labeling patterns, indicating consistent and reproducible labeling (red stars). Note that some low molecular weight bands appear faint in the streptavidin blot due to imperfect transfer to the membrane.



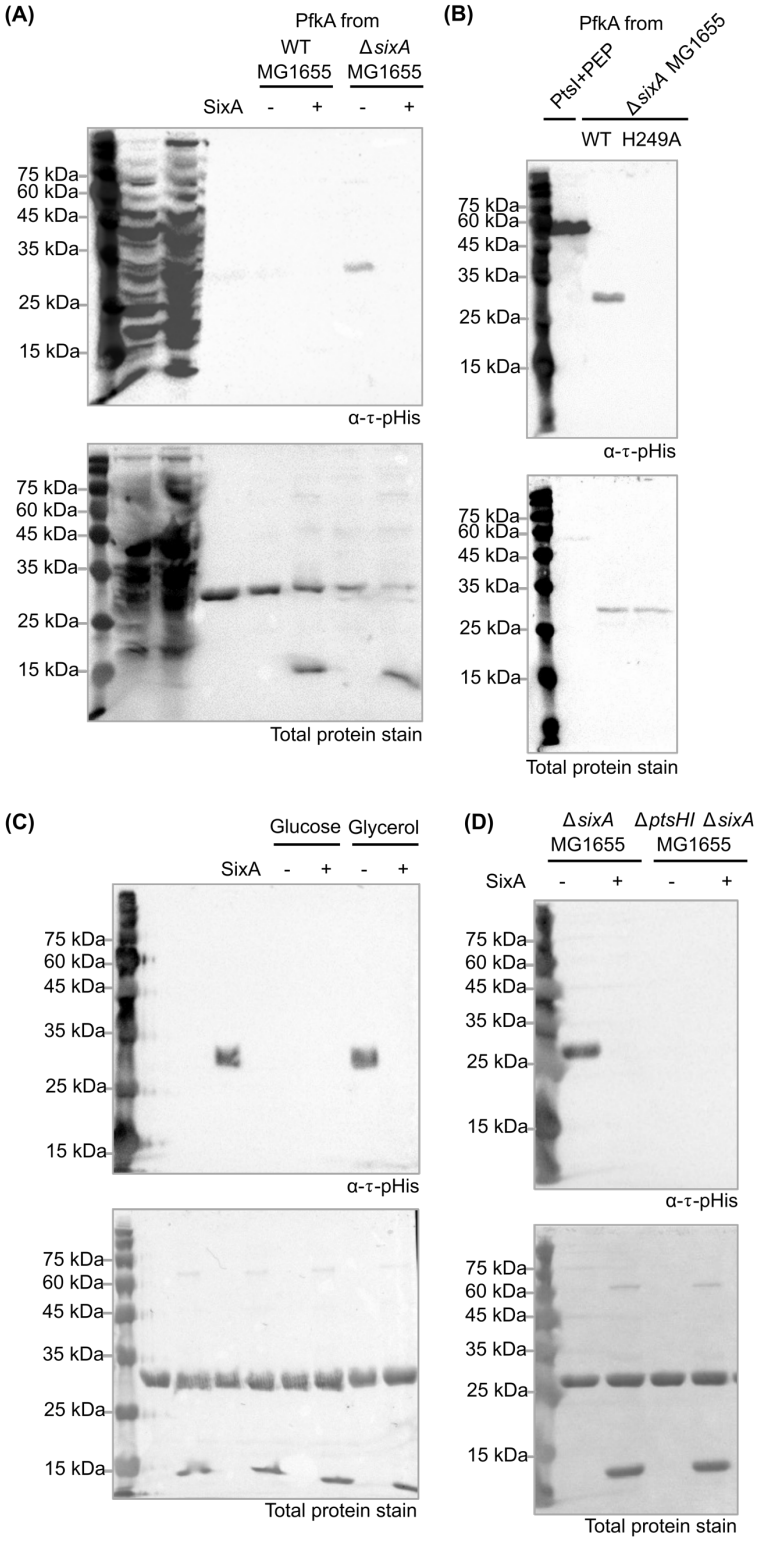
Supplementary Figure S11. Crystal structure PfkA (PDB: 1PFK) highlighting the pPyp-BP labeled residues Met60 and Phe73 (blue) as well as the pHis site His249 (red). The distance between the Met60 and His249 is about 20 Å, while the Phe73 and His249 are about 17 Å apart.



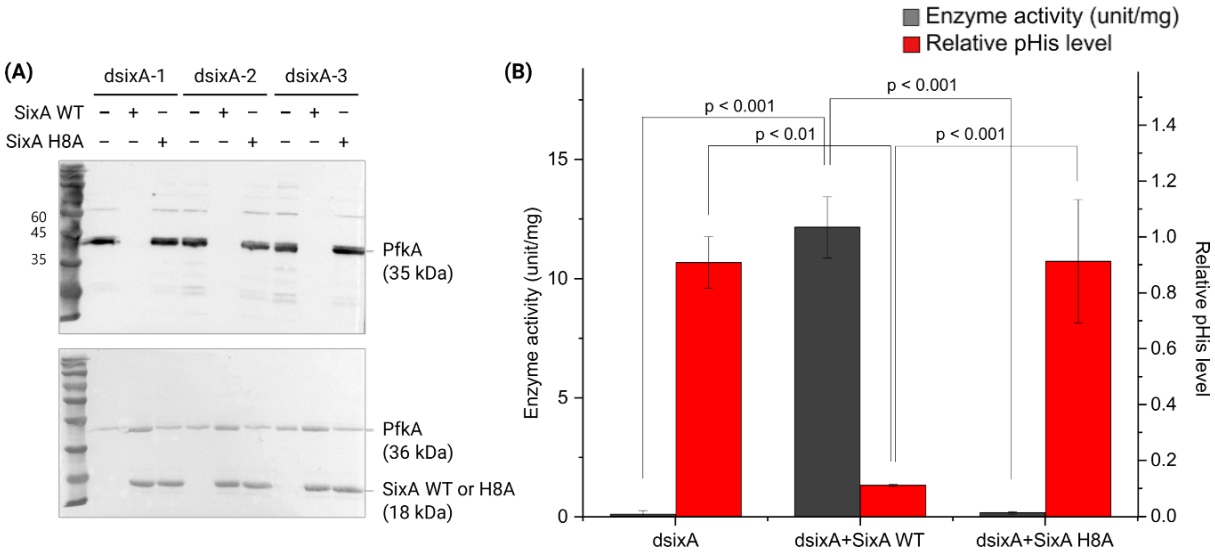
Supplementary Figure S12. Full gel images of Figure 4A (panel A), 4E (panel B), and 4F (panel C) in the main text.



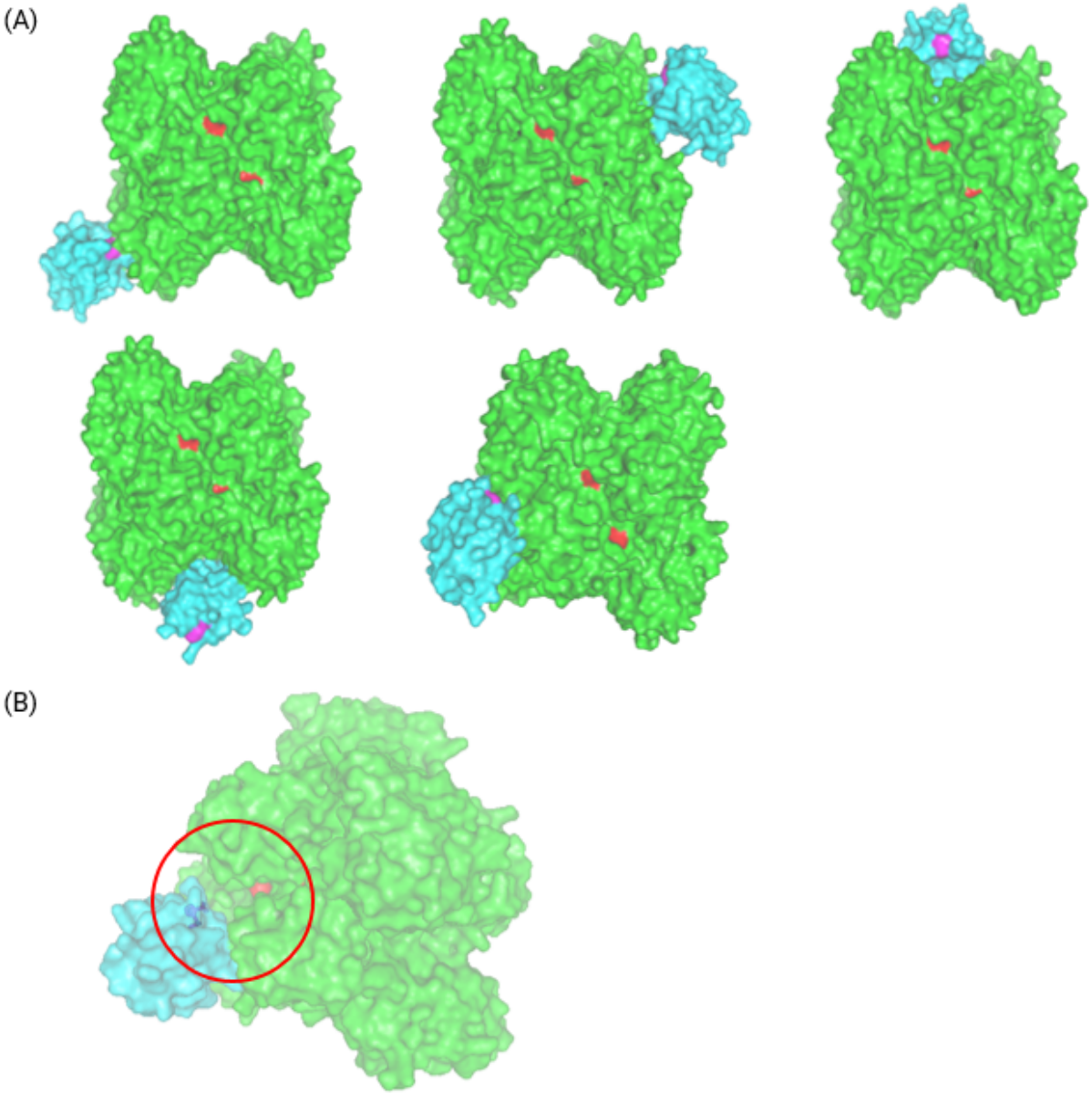
Supplementary Figure S13. Anti-τ-pHis (panel A) and anti-π-pHis (panel B) western blots of phosphorylated PfkA show that PfkA~pHis has a τ-pHis. PtsI phosphorylated with PEP and NDPK-A autophosphorylated with ATP were positive controls for the blots.



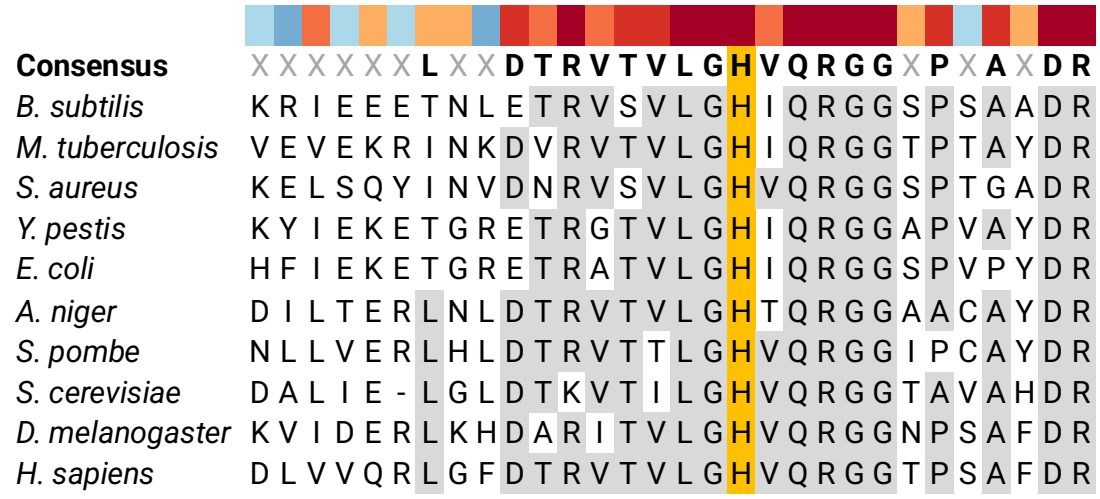
Supplementary Figure S14. Full membrane images of Figure 5A (panel A), Figure 5B (panel B), Figure 5E (panel C), and Figure 5F (panel D) in the main text.



Supplementary Figure S15. Restoration of PfkA activity by SixA is dependent on its phosphatase activity. (A) Anti-pHis western blots (triplicates) of PfkA~pHis treated with WT Six or the catalytically inactive H8A mutant. (B) Comparison of the kinase activity and pHis levels in SixA-treated PfkA~pHis. PfkA~pHis exhibited reduced enzyme activity and elevated histidine phosphorylation. The addition of recombinant WT SixA restored the kinase activity and reduced pHis levels, whereas the catalytically inactive SixA H8A mutant failed to dephosphorylate PfkA~pHis or restore its enzymatic function.



Supplementary Figure S16. PfkA-PtsH complex structures predicted with AlphaFold 3. All structures featured interface predicted template modeling (ipTM) scores higher than 0.80, representing confident, high-quality predictions. (A) Five predicted complexes between PfkA tetramer (cyan) and PtsH (green) are presented. PfkA H249 (red) and PtsH H15 (magenta) are far away in all structures. (B) In one of the predicted complexes between PfkA tetramer and PtsH~pHis, PfkA H249 and PtsH pHis15 are close to each other.



Supplementary Figure S17. Sequence alignment of PfkA in diverse species. Histidine residues corresponding to H249 of *E. coli* are highlighted in yellow.

Supplementary Tables

Supplementary Table S1. Proteomic analysis of *E. coli* proteins labeled by pPyp-BP vs. Me-BP (Excel file)

Supplementary Table S2. Proteomic analysis of pPyp-BP labeling in the presence/absence of excess pHis (Excel file)

Supplementary Table S3. LC-MS data for PfkA labeled with pPyp-BP (Excel file)

Supplementary Table S4. LC-MS data for phosphorylated PfkA (Excel file)

Supplementary Table S5. Raw data for PfkA activity assays (Excel file)

Supplementary Table S6. Sequences of the recombinant proteins used in this study. The polyhistidine tag (orange), the mutation site (green), and the TEV cleavage site (blue) are highlighted.

Protein	Amino acid sequence (N' → C')
PfkA WT	MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYEDRMVQLDRYS VSDMINRGGTFLGSARFPEFRDENIRAVAIENLKKRGIDALVVIGGDGSYMGAMRLTE MGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAIDRLRDTSSSHQRISVVEVMGRYC GDLTLAAAIAGGCEFVVVPEVEFSREDLVNEIKAGIAKGKKHAIVAITEHMCDVDELA HFIEKETGRETRATVLGHIQRGGSPVPYDRILASRMGAYAIDLLLAGYGGRCVGIQNE QLVHHDIIDAIENMKRPFKGDWLDCAKKLYHHHHHH*
PfkA H249A	MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYEDRMVQLDRYS VSDMINRGGTFLGSARFPEFRDENIRAVAIENLKKRGIDALVVIGGDGSYMGAMRLTE MGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAIDRLRDTSSSHQRISVVEVMGRYC GDLTLAAAIAGGCEFVVVPEVEFSREDLVNEIKAGIAKGKKHAIVAITEHMCDVDELA HFIEKETGRETRATVLG**A**IQRGGSPVPYDRILASRMGAYAIDLLLAGYGGRCVGIQNE QLVHHDIIDAIENMKRPFKGDWLDCAKKLYHHHHHH*
PtsI	MGSSHHHHHHGENLYFQSMISGILASPGIAFGKALLLKEDEIVIDRKKISADQVDQEV ERFLSGRAKASAQLETIKTKAGETFGEEKEAIFEGHIMLLEDEELEQEIIALIKDKHMTA DAAAHEVIEGQASALEELDDEYLKERAADVRDIGKRLLRNILGLKIIDLSAIQDEVILVAA DLTPSETAQLNLKKVLGFITDAGGRTSHTSIMARSLELPAIVGTGSVTSQVKNDDYLIL DAVNNQVYVNPTNEVIDKMRAVQEQVASEKAELAKLKDLPAITLDGHQVEVCANIGT VRDVEGAERNGAEGVGLYRTEFLFMDRDALPTEEEQFAAYKAVAEACGSQAVIVRT MDIGGDKELPYMNFPKEENPFLGWRAIRIAMDRREILRDQLRAILRASAFGKLRIMFP MIISVEEVRALRKEIEIYKQELRDEGKAFDESIEIGVMVETPAAATIARHLAKEVDFFSIG TNDLTQYTLAVDRGNDMISHLYQPMSPSVLNLIKQVIDASHAEGKWTGMCGELAGD ERATLLLLGMGLDEFSMSAISIPRIKKIIRNTNFEDAKVLAEQALAQPTTDELMTLVNK FIEEKTIC*
PtsH	MFQQEVTITAPNGLHTRPAAQFVKEAKGFTSEITVTSNGKSASAKSLFKLQTLGLTQ GTVVTISAEGEDEQKAVEHLVKLMAELE*
PtsH H15A	MFQQEVTITAPNGL**A**TRPAAQFVKEAKGFTSEITVTSNGKSASAKSLFKLQTLGLTQ GTVVTISAEGEDEQKAVEHLVKLMAELE*

Supplementary Table S7. Primers used for the site-directed mutagenesis of recombinant proteins. The mutation sites (green) are highlighted.

Gene	Primer sequence (5' to 3')	
PfkA H249A	FWD	CTGGGCGCGATCCAGCGCGGTGGTTC
	REV	GCTGGATCGCGCCCAGCACAGTTG
PtsH H15A	FWD	GAACGGTCTGGCCACCCGCCCTGCTG
	REV	GGTGGCCAGACCGTTCGGAGCGGTAATG

Supplementary Table S8. Strains and plasmids used for the gene knockout.

Strains/plasmids	Genotype and description	Source
Strains		
MG1655	*E. coli* K-12 F^- λ^- *ilvG*$^-$ *rfb-50 rph-1*	Lab stock
MG1655 Δ*sixA* Δ*ptsHI*	MG1655 Δ*sixA*::FRT Δ*ptsHI*::FRT	This study
MG1655 Δ*sixA*	MG1655 Δ*sixA*::FRT	This study
Plasmids		
pCP20	SC101-ts *ori*, carrying constitutively expressed yeast FLP recombinase, Am^R, Cm^R	[66]
pKD13	Template for kanamycin cassette flanked by FRT sites, Km^R	[67]
pSIM5	SC101-ts *ori*, carrying heat-shock inducible λ-RED recombinase system, Cm^R	[68]

Supplementary Table S9. Primers used for the gene knockout.

Primer	Sequence (5'-3')
sixA-del-*FP*	GATGAGCAATTATGCCCAATGAATCTACCTCATTGGGCATAATTTGGAACGT GTAGGCTGGAGCTGCTTCG
sixA-del-*RP*	CTGGTTTCAGATGAAAGAAAACGGGCGAATCTGGTTAACAAAAGCGGTGCAT TCCGGGGATCCGTCGACC
ptsHI-del-FP	CTAGACTTTAGTTCCACAACACTAAACCTATAAGTTGGGGAAATACAATGGT GTAGGCTGGAGCTGCTTC
ptsHI-del-RP	AAGCAGTAAATTGGGCCGCATCTCGTGGATTAGCAGATTGTTTTTTCTTCAT TCCGGGGATCCGTCGACC
sixA-seq-FP	ATAATGCGATTGGCATTAACCCGC
sixA-seq-RP	GTTCCGCTATATCGATTCTCTCGG
ptsHI-seq-FP	GTAATGCCAGCTTGTTAAAA
ptsHI -seq-RP	AAAAACGACATCCGGCACGT

Experimental Sections

General Materials and Equipment

Reagents and Solvents

- 1H-pyrazole, hydrogen peroxide (H_2O_2), 3-bromopropylamine hydrobromide, 98%, palladium (II) acetate, 4-pentynoic acid, 95%, 4-(dimethylamino)pyridine, 99% (DMAP), aluminum chloride, 98+%, extra pure, anhydrous powder ($AlCl_3$), and terephthaloyl chloride, 99% were purchased from Alfa Aesar. 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene (Xantphos), sodium acetate (NaOAc), propargyl bromide (stabilized with MgO), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC*HCl) were purchased from TCI. Diethyl phosphite was purchased from Acros Organics. Di-tert-butyl decarbonate (Boc_2O) and iodine were purchased from DAEJUNG. Triethylamine (TEA), 99.0%, phenol, 99.0%, and trifluoroacetic acid, 98+% (TFA) were purchased from SAMCHUN.
- One Shot™ TOP10 Chemically Competent *E. coli* and BL21 (DE3) pLysS were purchased from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively.
- LB broth and LB agar were purchased from Affymetrix (Santa Clara, CA). Ampicillin sodium salt was purchased from MP Biomedicals (Santa Ana, CA) and Thermo Scientific (Waltham, MA).
- HisPur Ni-NTA Resin and Imperial™ Protein Stain were purchased from Thermo Scientific. 96-well transparent plates were purchased from SPL Life Science (Pocheon, Republic of Korea).
- τ/π-pHis antibodies were purchased from Sigma-Aldrich. τ-pHis antibody, clone SC56-2 and π-pHis antibody, clone SC50-3 were used.

Equipment

High-performance liquid chromatography (HPLC)

For reverse-phase HPLC, Agilent Technologies 1260 Infinity was used. For eluents, solvent A is H_2O with 0.1% TFA, and solvent B is 10% H_2O in acetonitrile with 0.1% TFA. The analytical column is C_{18} 5 µm 4.6 x 150 mm, GL sciences, and the semi-prep column is C_{18} 5 µm 10 x 250 mm, GL sciences.

Photolabeling

BIO-LINK® BLX-LMS, 65 W power supply, 365 nm wavelength, 8W *5 (T-8L lamp) was used for photolabeling.

Spectroscopy

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded at 400 MHz. The chemical shifts are reported on the δ scale (ppm) with tetramethylsilane as the internal standard. Coupling constants (J) are reported in Hertz (Hz). Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 100 MHz and are reported (ppm) relative to the center line from chloroform-d (77.23 ppm) or dimethyl sulfoxide-d_6 (39.54 ppm). Phosphorous nuclear magnetic resonance spectra (^{31}P NMR) were recorded at 160 MHz.

High-resolution mass spectrometry (HRMS) data were recorded with a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific) in electrospray ionization (ESI) mode or using an AccuTOF 4G+ DART (JEOL) in direct analysis in real-time (DART) mode.

Fourier-transform infrared spectroscopy (FT-IR) spectra were recorded on a Spectrum Two FT-IR spectrometer, UATR Two (PerkinElmer), with ν_{max} reported in cm^{-1}.

Safety Considerations

- Ultraviolet light is damaging to biological tissues. Caution is required when working with the lamp, and protective eyewear must be used at all times.
- The GSH category 1 eye and skin damage chemicals 1H-pyrazole, hydrogen peroxide, 4-pentynoic acid, 4-(dimethylamino)pyridine, aluminum chloride, terephthaloyl chloride, propargyl bromide solution, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, diethyl phosphite, di-tert-butyl decarbonate, triethylamine, phenol, and trifluoroacetic acid constitute significant safety hazards and must be handled with extreme care.
- The GSH category 1 corrosive chemical propargyl bromide solution constitutes aspiration hazards and must be handled with extreme care.
- The GSH category 1 toxic to aquatic life chemical palladium (II) acetate and iodine constitute significant environmental hazards and must be handled with extreme care.

Experimental Procedures

DNA constructs

The gene encoding each wild-type protein was amplified from *E. coli* genomic DNA via PCR using synthetic primers (Supplementary Table S6). The resulting PCR product was inserted into the target vector using a Gibson assembly kit. Plasmid extraction was performed in accordance with the manufacturer's protocol. Site-directed mutagenesis using synthetic primers (Supplementary Table S7) was employed to generate the mutant variants of each protein.

Bacterial strains

A single mutant strain of *E. coli* MG1655 lacking the *sixA* gene and a double mutant strain lacking both the *sixA* and *ptsHI* genes were utilized in this study. Chromosomal gene knockout was performed through homologous recombination using a λ RED recombination system, which employed a kanamycin resistance (KanR) gene flanked by flippase (FLP) recognition target sites, followed by FLP-mediated removal.[66, 67] The KanR gene was PCR-amplified with 50 nucleotide overhangs homologous to the upstream and downstream regions of the target chromosomal gene. The resulting PCR products were introduced into electrocompetent cells induced for the RED system. Transformants were screened on kanamycin-containing LB plates, followed by colony PCR and DNA sequencing to verify successful gene knockout. The plasmids and primers used for gene deletion are listed in Supplementary Tables S7 and S8. Primer synthesis and DNA sequencing were conducted by Macrogen Inc. (Seoul, Korea).

Expression and purification of recombinant proteins

Except for PfkA, expression constructs were based on pET21a vectors. These proteins were overexpressed as C-terminal His_6-tagged proteins (except PtsI) in *E. coli* BL21(DE3) pLysS cells. PfkA

(WT, H249A) expression constructs were based on pBbE6k vectors. These proteins were derived from *E. coli* and overexpressed as C-terminal His_6-tagged proteins in Δ*pfkA E.coli* MG1655 cells. The bacterial cultures transformed with the expression vectors were grown at 37 °C in Luria-Bertani (LB) broth media with kanamycin (50 μg/mL) or ampicillin (100 μg/mL) until the OD_{600} reached 0.6, and the expression was induced with 1 mM IPTG for 3 hr at 37 °C. Following induction, the bacterial cells were harvested by centrifugation (4000 rpm, 30 min, 4 °C), lysed using a tip sonicator (1 sec on, 5 sec off, total on time 3 min, 0 °C), and centrifuged again (4000 rpm, 30 min, 4 °C). The resulting supernatant was purified using Ni-NTA affinity column chromatography according to the manufacturer's protocol. PtsH and its mutant were expressed and purified according to established literature procedures.[69]

Lysate preparation for in-gel fluorescence and proteomics

E. coli MG1655 was cultured in 5 mL LB until the OD_{600} reached 0.6~0.7. The culture was then centrifuged (4000 rpm, 30 min, 4 °C), and the resulting cell pellet was resuspended in lysis buffer (20 mM HEPES, 100 mM NaCl, pH 7.5) and lysed using a tip sonicator (1 sec on, 5 sec off, total on time 5 min, 0 °C). Following centrifugation (4000 rpm, 30 min, 4 °C), the supernatant was collected and used for the subsequent labeling experiments.

Recombinant expression and purification of PfkA from *E. coli* MG1655 strains (WT, Δ*sixA*, and Δ*ptsHI* Δ*sixA*) grown in glucose or glycerol

E. coli MG1655 cells (WT, Δ*sixA*, and Δ*ptsHI* Δ*sixA*) were transformed with PfkA-His_6 expression vectors based on the pBbE6k system. The transformed bacteria were cultured at 37 °C for 16 hours in minimal media, composed of salts (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl) supplemented with tryptone and 1 mM $MgSO_4$. The seed culture solution was diluted with minimal media, consisting of the same salts with additional 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.5% glycerol or glucose, and then grown at 37 °C. Cells were cultured until the OD_{600} reached 0.2, and the expression was induced with 0.1 mM IPTG for 1 hr at 37 °C. Following induction, cells were centrifuged (4000 rpm, 20 min, 4 °C) to remove

IPTG. The cell pellet was resuspended in fresh growth media and centrifuged again (4000 rpm, 20 min, 4 °C), and this washing step was repeated twice. The cells were then further cultured until the OD_{600} reached 0.6 in glucose media or 0.5 in glycerol media. The culture was then centrifuged (4000 rpm, 30 min, 4 °C), and the resulting cell pellet was resuspended in lysis buffer (20 mM HEPES, 100 mM NaCl, pH 7.5 with additional phosSTOP) and lysed using a tip sonicator (1 sec on, 5 sec off, total on time 2 min, 0 °C). The lysate was purified using Ni-NTA affinity column chromatography according to the manufacturer's protocol.

Synthesis of free pHis

The free pHis used in these experiments was synthesized according to established protocols, [70] and its structure was confirmed by NMR and MS analyses.

In vitro phosphorylation of PfkA (by pHis or PtsH)

PfkA WT and mutant proteins were phosphorylated using either free pHis or the PtsI-PtsH system. 4 μM of PfkA was incubated with 2 mM free pHis for 4 hr or 0.5 μM of PfkA was incubated with 1 μM PtsI, 20 μM PtsH, and 0.56 mM PEP at 37 °C for 2 hr in 20 mM HEPES, 100 mM NaCl pH 7.5 buffer.

PfkA activity assay

The activity assay was adapted from the procedure by Kotlarz et al. to measure the production of F1,6BP by a series of coupled enzymatic reactions.[49] In brief, F1,6BP was converted by aldolase into glyceraldehyde 3-phosphate, which was subsequently oxidized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). 1 mM F6P as the substrate, 3 μg aldolase, 0.3 μg TpiA, and 0.3 μg GAPDH were used in coupled enzymatic reactions for 12 ng PfkA. The resulting decrease in absorbance at 340 nm, corresponding to NADH consumption by GAPDH, was quantified. Enzyme activity was reported as units per milligram of PfkA. One unit is defined as the amount of PfkA converting one micromole of

fructose-6-phosphate per minute.

Photolabeling of pPyp-BP binding proteins

In the *E. coli* lysate experiment, 1 mg of lysate in the lysis buffer (1 mg/mL, 20 mM HEPES, 100 mM NaCl, pH 7.5) was mixed with pPyp-BP (final concentration 5 μM) and $MgCl_2$ (final concentration 5 mM). As negative controls, DMSO or Me-BP was used in place of pPyp-BP. Following a 1-hour incubation at room temperature in the dark, the mixture was irradiated at 365 nm (5 * 8 W) at 0 °C for 20 minutes using a UV irradiation apparatus (BLX365, Vilber Lourmat).

For single protein labeling, the protein of interest (final concentration 4 μM) was mixed with pPyp-BP (final concentration 5 μM) and $MgCl_2$ (final concentration 5 mM) in the lysis buffer. All other conditions were identical to those used in the lysate experiment.

CuAAC labeling and purification

The photolabeled protein solution was supplemented with 8 M urea in lysis buffer to reach a final concentration of 2 M. Subsequently, TAMRA-azide or biotin-azide (in DMSO, final concentration 1 mM), a pre-mixed solution of $CuSO_4$ and THPTA (final concentration 0.2 mM $CuSO_4$ and 1 mM THPTA), and sodium ascorbate (final concentration 5 mM) were added sequentially. The mixture was then incubated for 2 hours at room temperature and used directly for in-gel fluorescence or proteomic analysis. For proteomic analysis, proteins were isolated by MeOH/$CHCl_3$ precipitation according to a previously reported procedure.[15]

Streptavidin enrichment and on-bead digestion with biotin

The protein pellet was re-dissolved in 200 μL of denaturation buffer (1.6% SDS in DPBS) and quantified using the Pierce BCA protein assay kit (Thermo Scientific). Subsequently, 650 μg of the lysate was diluted 8-fold (final SDS concentration of 0.2%) with DPBS. This mixture was loaded onto pre-

equilibrated Pierce streptavidin agarose beads (100 µL, Thermo Scientific) and incubated for 2 hours on an end-over-end rotator. The beads were washed sequentially with 200 µL of 0.2% SDS in DPBS, followed by 2 times with 200 µL DPBS and 2 times with 200 µL DI water. The beads were resuspended in 200 µL of the trypsin digestion buffer (1 M urea, 50 mM Tris, 1 mM $CaCl_2$, pH 8.0), and 5 µg of trypsin/Lys-C Mix (Promega) was added. The mixture was incubated overnight using a thermomixer (37 °C, 400 rpm). The digested solution was collected, and the beads were washed with an additional 100 µL water. The combined solution was acidified by adding formic acid (final concentration 0.4%). It was then desalted using a homemade C18 stage-tip[71] and dried in a vacuum concentrator for subsequent LC-MS/MS analyses.

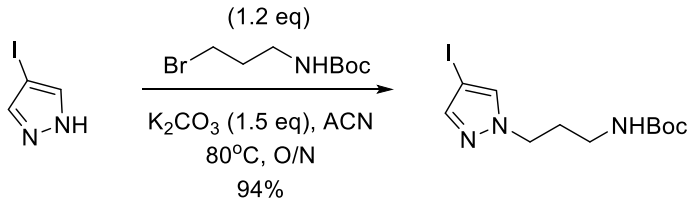
LC-MS/MS analysis of protein samples

All proteomic samples were analyzed using a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific) in combination with a nanoelectrospray ion source (EASY-Spray). After drying, samples were resuspended in an aqueous solution containing 2% acetonitrile and 0.05% TFA and subsequently loaded onto the LC-MS/MS. Digested peptides were separated using a C18 column (PepMap RSLC C18, 2 µm, 100 Å, 75 µm * 50 cm) with a gradient of acetonitrile (from 4% to 32.5% over 120 minutes) in 0.1% aqueous formic acid at a flow rate of 300 nL/min. MS^2 analysis was performed with precursor ion scanning of MS spectra (m/z 400 ~ 2000) in the Orbitrap at a resolution of 70,000 at m/z 400, with an internal lock mass. The top 15 ions were isolated and fragmented using high-energy collision-induced dissociation (HCD). All the experiments were carried out in triplicate.

For individual protein analysis, 400 ng to 1 µg of trypsin-digested proteins were analyzed. Digestion was performed using Pierce Trypsin Protease (MS grade, Thermo Scientific) according to the manufacturer's in-solution digestion protocol, with a minor modification. Peptide separations were carried out using an LC gradient of acetonitrile (from 4% to 23% in 0.1% aqueous formic acid over 50 minutes) was used. All other conditions were consistent with those used for the proteomic sample analysis.

Chemical synthesis and NMR spectra

1) Synthesis of **2**



In a round-bottom flask, 4-iodo-1H-pyrzole[19] (600 mg, 3.1 mmol) was dissolved in dry acetonitrile (7.5 mL), and K_2CO_3 (641 mg) was added. To this suspension, N-Boc-3-bromopropylamine[72] (884 mg, 3.72 mmol, 1.2 equiv) was added at rt, and the reaction mixture was stirred for 24 hr at 80 °C. After evaporation, the reaction mixture was diluted with H_2O (15 mL) and extracted with ethyl acetate (2 x 15 mL). The combined organic phase was dried with Na_2SO_4, evaporated under reduced pressure, and purified by silica gel flash column chromatography (ethyl acetate:hexane = 1:1) to give a yellow oil (1.045 g, 94% yield).

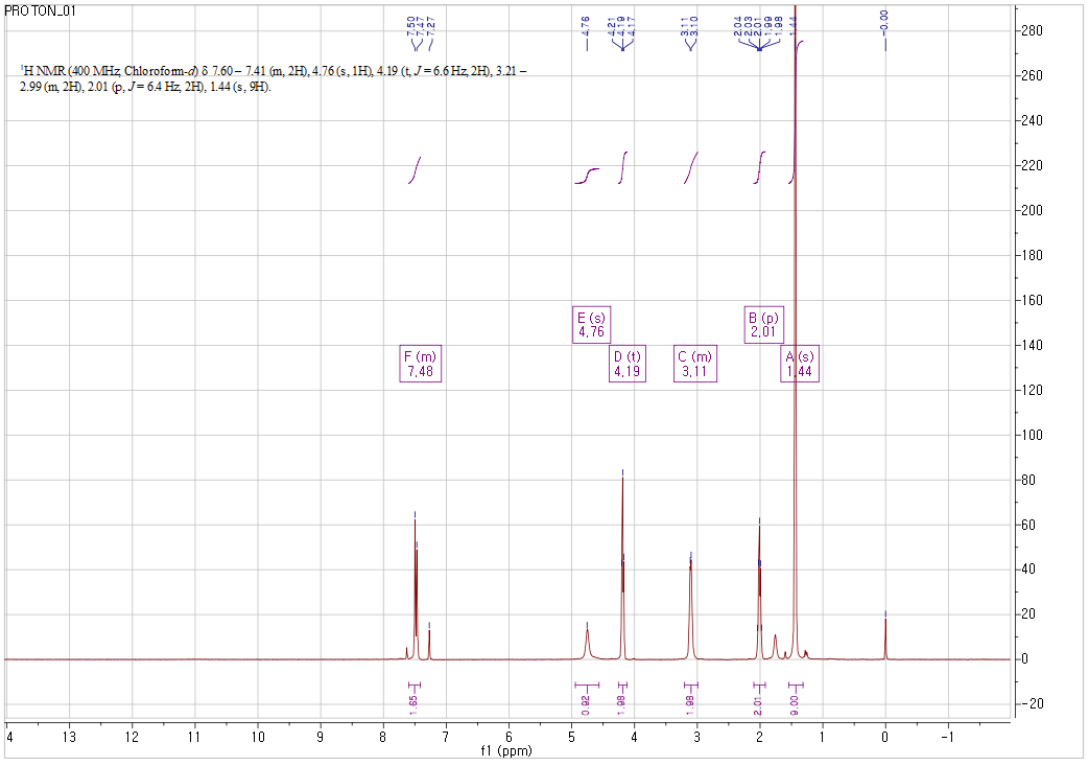
TLC (50% ethyl acetate in hexane): R_f = 0.6, UV-active, brown spot (stained with ninhydrin).

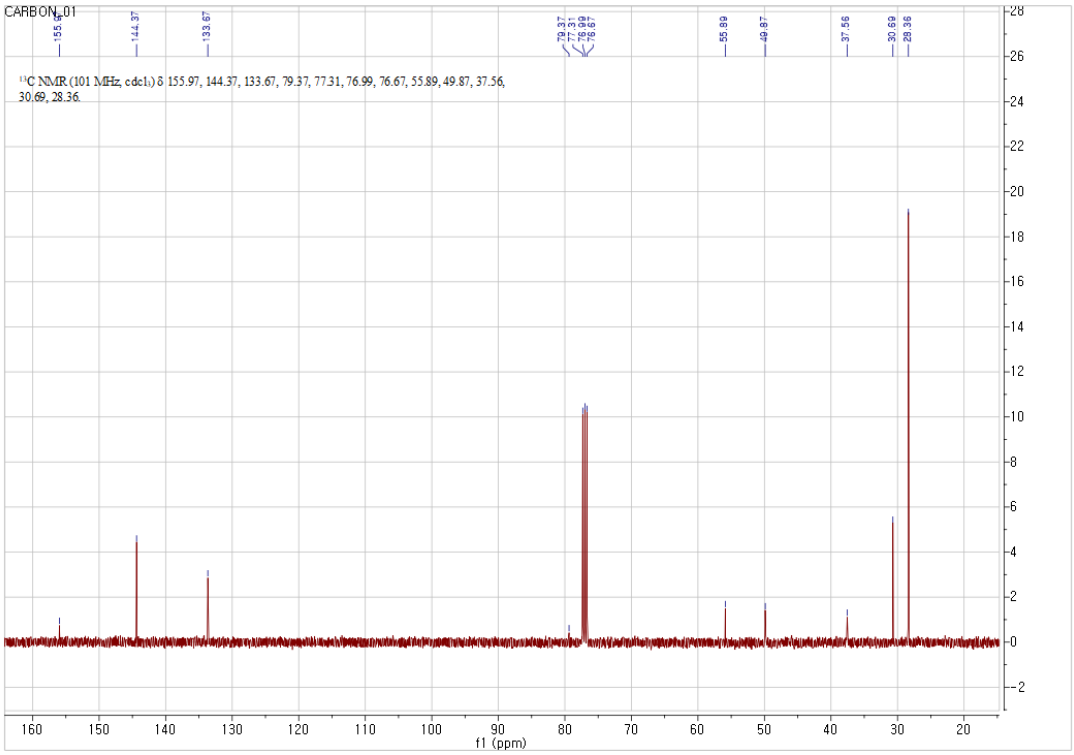
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.60 – 7.41 (m, 2H), 4.76 (s, 1H), 4.19 (t, J = 6.6 Hz, 2H), 3.21 – 2.99 (m, 2H), 2.01 (p, J = 6.4 Hz, 2H), 1.44 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ = 155.97, 144.37, 133.67, 79.37, 55.89, 49.87, 37.56, 30.69, 28.36.

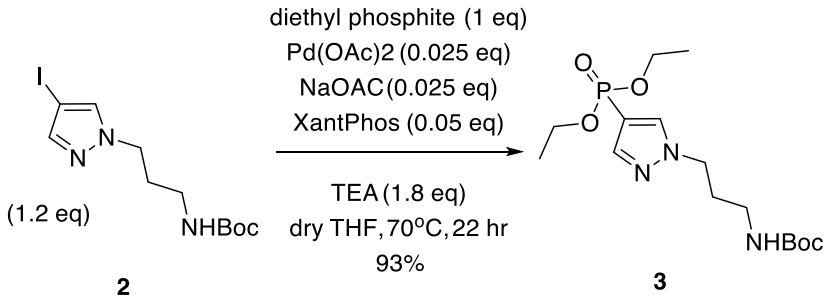
FT-IR: 3346, 2977, 2926, 1698, 1515 cm^{-1}

HRMS (*m/z*): Calculated (for $C_{11}H_{18}IN_3NaO_2^+$): 374.0336; observed 374.0335 (error: 0.2674 ppm)


PROTON_01
^{1}H NMR (400 MHz, Chloroform-d) δ 7.60 – 7.41 (m, 2H), 4.76 (s, 1H), 4.19 (t, J = 6.6 Hz, 2H), 3.21 – 2.99 (m, 2H), 2.01 (p, J = 6.4 Hz, 2H), 1.44 (s, 9H).
F (m) 7,48
E (s) 4,76
D (t) 4,19
C (m) 3,11
B (p) 2,01
A (s) 1,44
f1 (ppm)


CARBON_01
^{13}C NMR (101 MHz, cdcl$_3$) δ 155.97, 144.37, 133.67, 79.37, 77.31, 76.99, 76.67, 55.89, 49.87, 37.56, 30.69, 28.36.
f1 (ppm)

2) Synthesis of **3**



2 **3**

The synthesis of **3** was adapted from a procedure by Tran et al.[73] $Pd(OAc)_2$ (8.5 mg, 0.038 mmol, 0.025 equiv), XantPhos (43.8 mg, 0.076 mmol, 0.05 equiv), and NaOAc (3.2 mg, 0.038 mmol, 0.025 equiv) were mixed in a round-bottom two-neck flask. The flask was sealed with a rubber septum and flushed with Ar. THF (23 mL) and TEA (380 µL, 2.74 mmol, 1.8 equiv) were added with a syringe. The reaction mixture was stirred for 20 min at 70 °C, during which the reaction mixture turned red-brown from yellow. To this mixture, a solution of **2** (800 mg, 1.82 mmol, 1.2 equiv) in THF (5 mL) and diethyl phosphite (196 µL, 1.52 mmol, 1 equiv) were added with a syringe, respectively. The reaction mixture was stirred overnight at 70 °C, during which the reaction mixture turned yellow-orange from red-brown. The reaction mixture was diluted with ethyl acetate (80 mL), washed with saturated aqueous $NaHCO_3$ solution (80 mL), and extracted with ethyl acetate (3 x 80 mL). The combined organic phase was dried with Na_2SO_4, evaporated under reduced pressure, and purified by flash column chromatography (ethyl acetate:methanol = 9:1) to give a yellow-orange oil (510.3 mg, 93% yield).

TLC (10% methanol in ethyl acetate): R_f = 0.6, UV-inactive, brown spot (stained with ninhydrin).

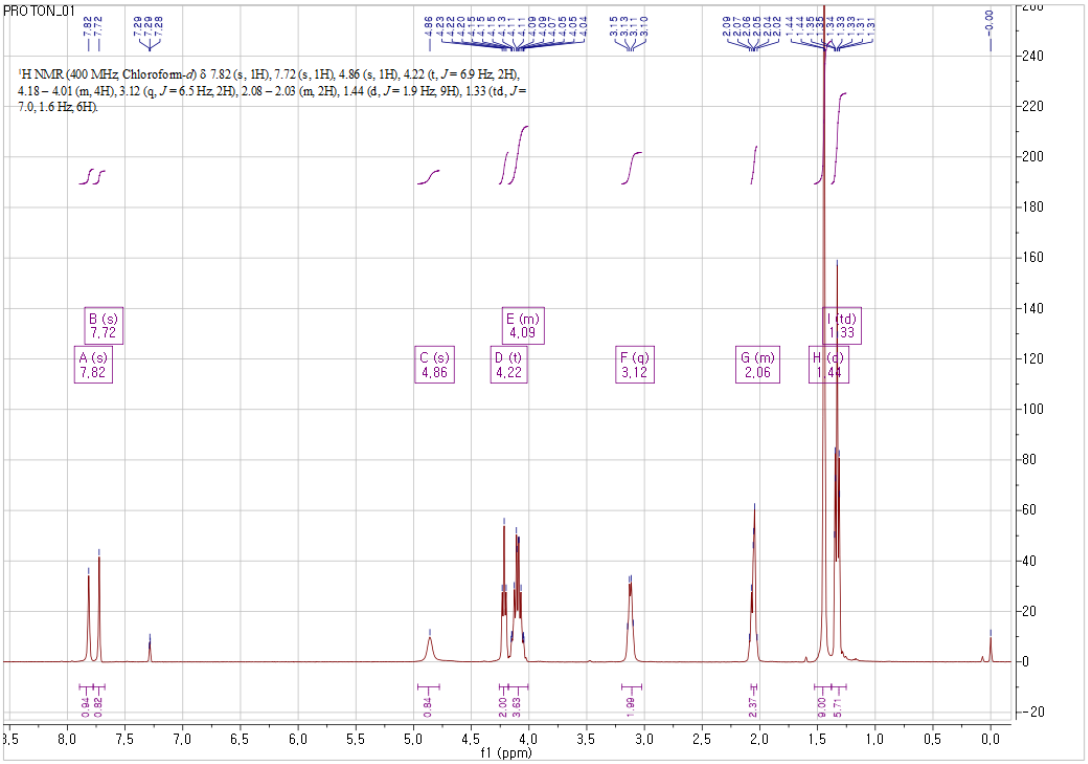
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.82 (s, 1H), 7.72 (s, 1H), 4.86 (s, 1H), 4.22 (t, J = 6.9 Hz, 2H), 4.18 – 4.01 (m, 4H), 3.12 (q, J = 6.5 Hz, 2H), 2.08 – 2.03 (m, 2H), 1.44 (d, J = 1.9 Hz, 9H), 1.33 (td, J = 7.0, 1.6 Hz, 6H).

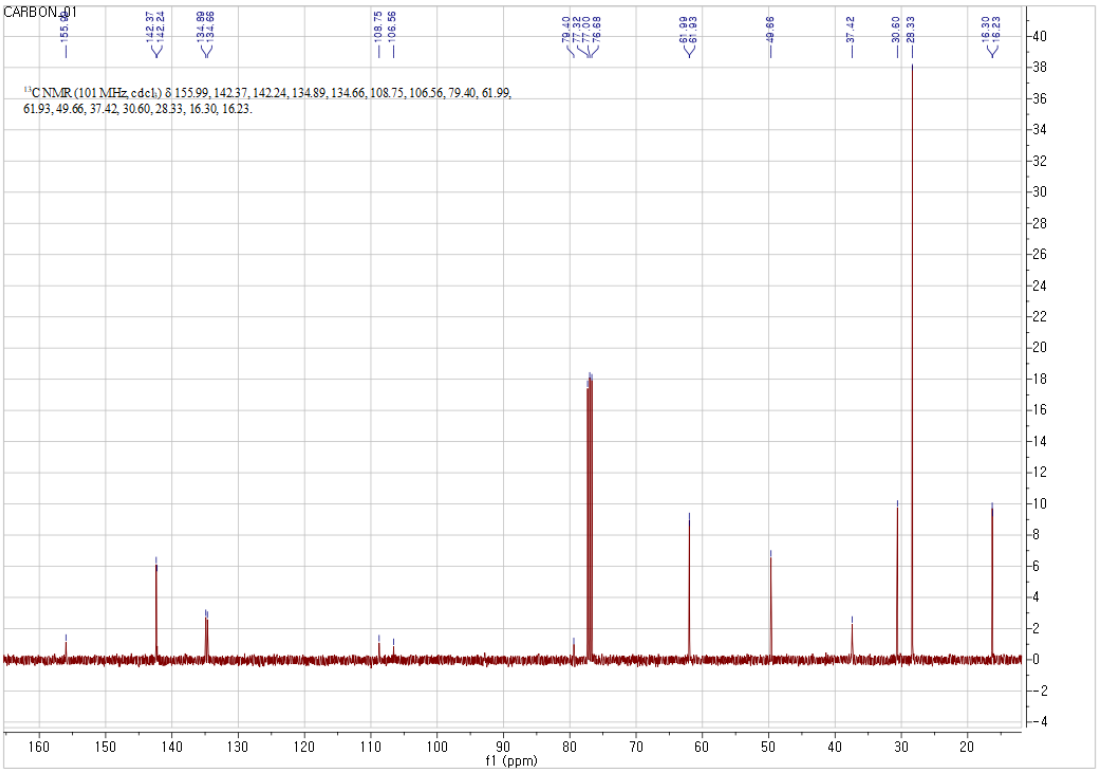
^{13}C NMR (101 MHz, $CDCl_3$): δ = 155.99, 142.37, 142.24, 134.89, 134.66, 108.75, 106.56, 79.40, 61.99, 61.93, 49.66, 37.42, 30.60, 28.33, 16.30, 16.23.

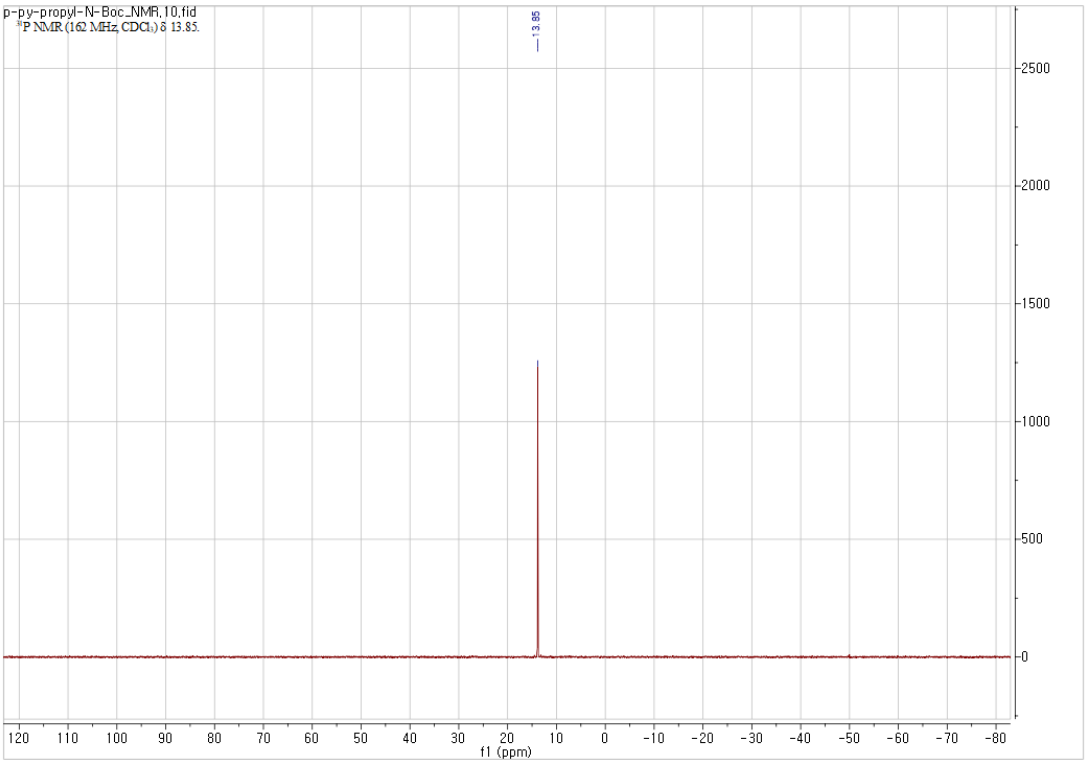
^{31}P NMR (162 MHz, $CDCl_3$): δ = 13.85.

FT-IR: 3309, 2978, 1707, 1528 cm^{-1}

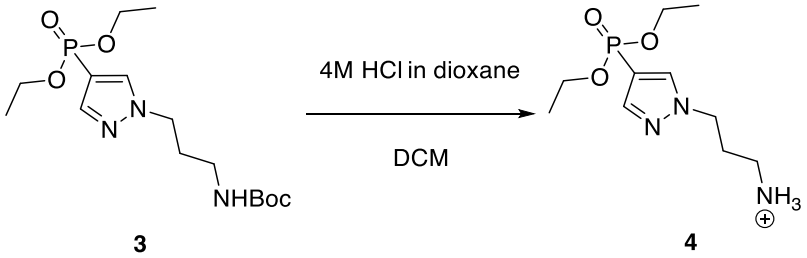
HRMS (*m/z*): Calculated (for $C_{15}H_{29}N_3O_5P^+$): 362.1840; observed 362.1837 (error: 0.8283 ppm)




CARBON_01
155.99
142.37
142.24
134.89
134.66
108.75
106.56
79.40
77.32
77.00
76.68
61.99
61.93
49.66
37.42
30.60
28.33
16.30
16.23
13C NMR (101 MHz, cdcl3) δ 155.99, 142.37, 142.24, 134.89, 134.66, 108.75, 106.56, 79.40, 61.99, 61.93, 49.66, 37.42, 30.60, 28.33, 16.30, 16.23.
f1 (ppm)


p-py-propyl-N-Boc_NMR,10.fid
31P NMR (162 MHz, CDCl3) δ 13.85.
13.85
f1 (ppm)

3) Synthesis of **4**



3 **4**

Compound **3** (400 mg, 1.43 mmol, 1 equiv) was dissolved in dichloromethane (14 mL) in a septum-sealed round bottom flask. A solution of HCl in dioxane (4 M, 1.15 mL) was added with a syringe under an argon atmosphere. The reaction mixture was stirred for 5 hr at rt, and the solution was evaporated under reduced pressure to give a yellow oil (270 mg, 72% yield). This material was used without further purification.

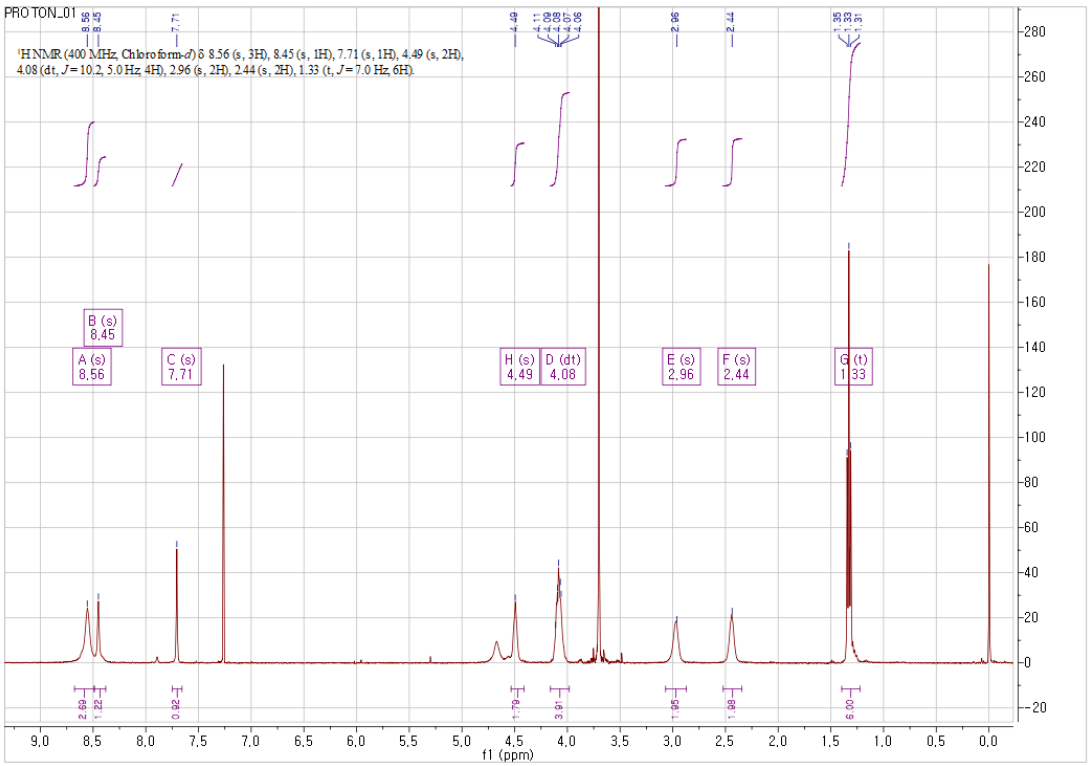
TLC (10% methanol in ethyl acetate): R_f = 0, UV-inactive, brown spot (stained with ninhydrin).

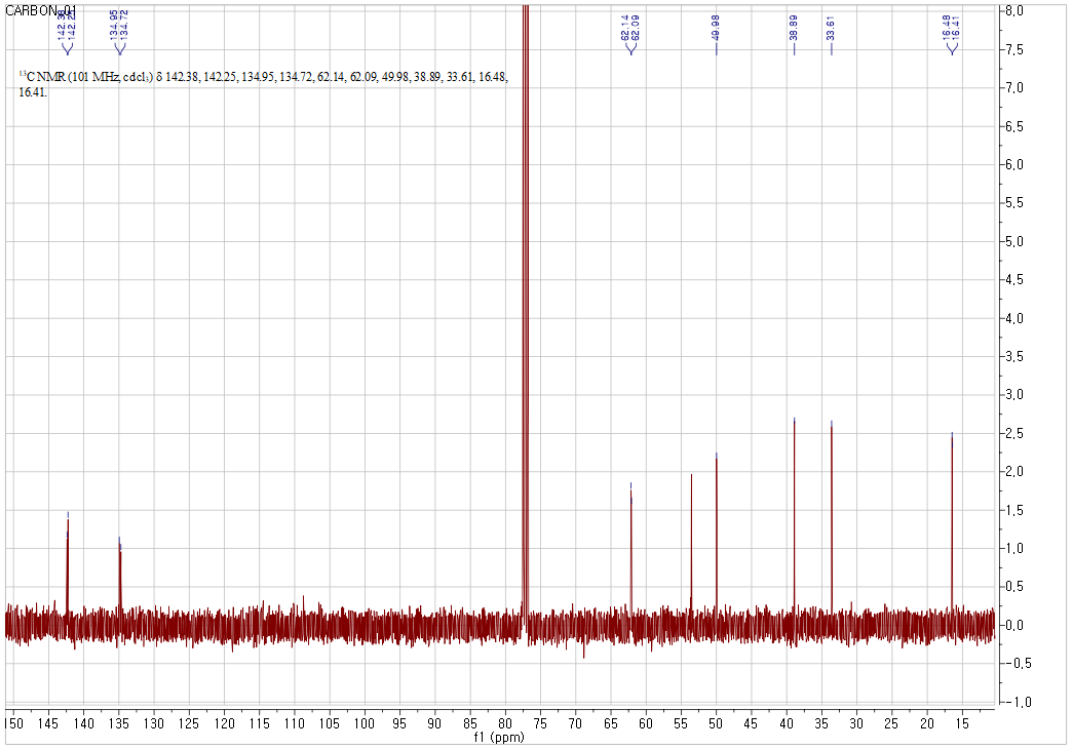
^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.56 (s, 3H), 8.45 (s, 1H), 7.71 (s, 1H), 4.49 (s, 2H), 4.08 (dt, J = 10.2, 5.0 Hz, 4H), 2.96 (s, 2H), 2.44 (s, 2H), 1.33 (t, J = 7.0 Hz, 6H).

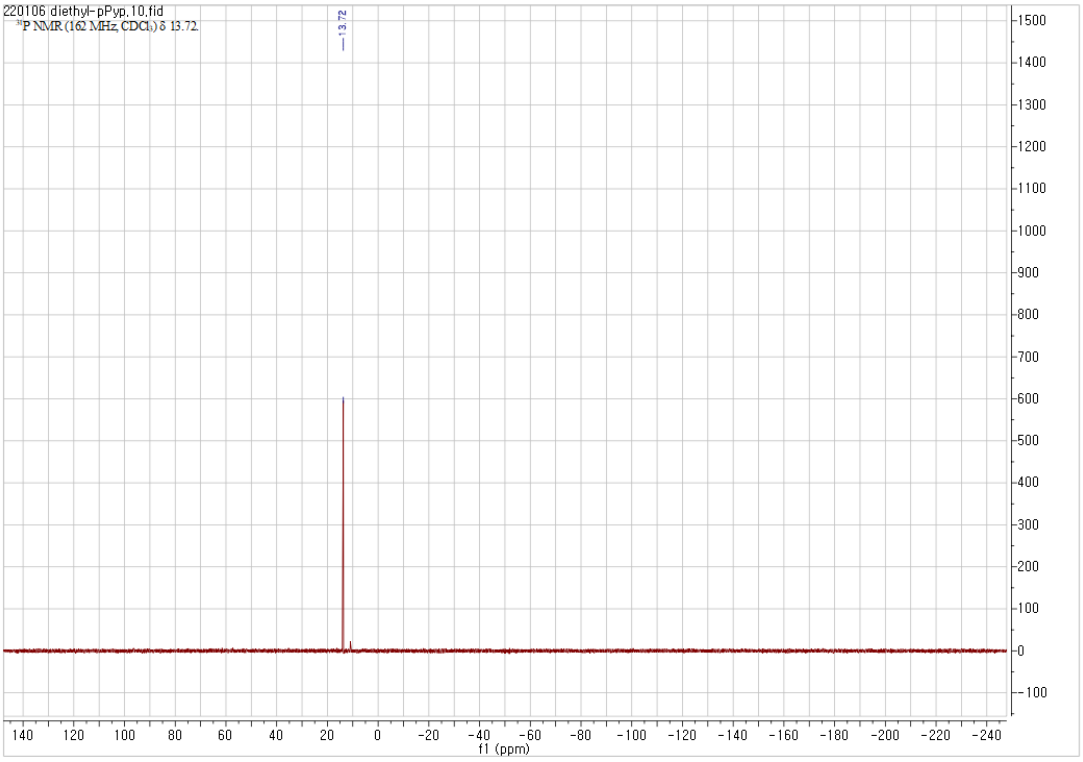
^{13}C NMR (101 MHz, $CDCl_3$): δ = 142.38, 142.25, 134.95, 134.72, 62.14, 62.09, 53.56, 49.98, 38.89, 33.61, 16.48, 16.41.

^{31}P NMR (162 MHz, $CDCl_3$): δ = 13.72.

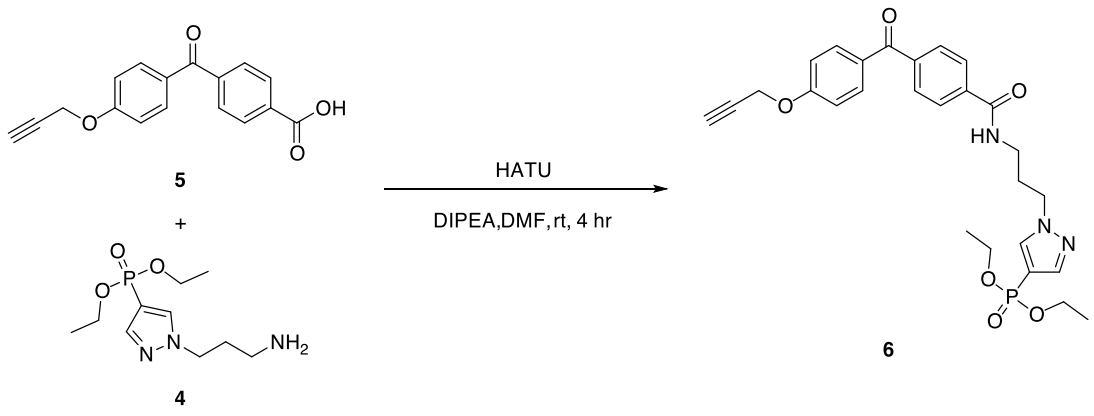
HRMS (*m/z*): Calculated (for $C_{10}H_{21}N_3O_3P_1$+): 262.1315; observed 262.1315 (error: 0 ppm)


PROTON_01
^{1}H NMR (400 MHz, Chloroform-d) δ 8.56 (s, 3H), 8.45 (s, 1H), 7.71 (s, 1H), 4.49 (s, 2H), 4.08 (dt, J = 10.2, 5.0 Hz, 4H), 2.96 (s, 2H), 2.44 (s, 2H), 1.33 (t, J = 7.0 Hz, 6H).
B (s) 8.45
A (s) 8.56
C (s) 7.71
H (s) 4.49
D (dt) 4.08
E (s) 2.96
F (s) 2.44
G (t) 1.33
f1 (ppm)


CARBON_01
^{13}C NMR (101 MHz, cdcl$_3$) δ 142.38, 142.25, 134.95, 134.72, 62.14, 62.09, 49.98, 38.89, 33.61, 16.48, 16.41.
f1 (ppm)



4) Synthesis of **6**



This material was synthesized following a procedure for a similar material.[74] 4-benzoic acid-4'-(prop-2-ynylophenomeone **5**[75] (154 mg, 0.55 mmol, 1.2 equiv) was dissolved in dry DMF (1 mL) under argon atmosphere. HATU (380.23 mg, 0.53 mmol, 1.15 equiv) and DIPEA (240 µL, 1.38 mmol, 3 equiv) were added. The solution was stirred for 5 min, at rt (solution color was changed from colorless to yellow).

Compound **4** (120 mg, 0.46 mmol, 1 equiv) in dry DMF (500 µL) was added with a syringe. The reaction mixture was stirred for 4 hr at rt and subsequently diluted with ethyl acetate (20 mL). The organic phase was washed with brine (20 mL) three times. The combined yellow organic phase was dried with Na_2SO_4, evaporated under reduced pressure, and purified by silica gel flash column chromatography (ethyl acetate:methanol = 9:1) to give a yellow oil (83 mg, 34.5% yield).

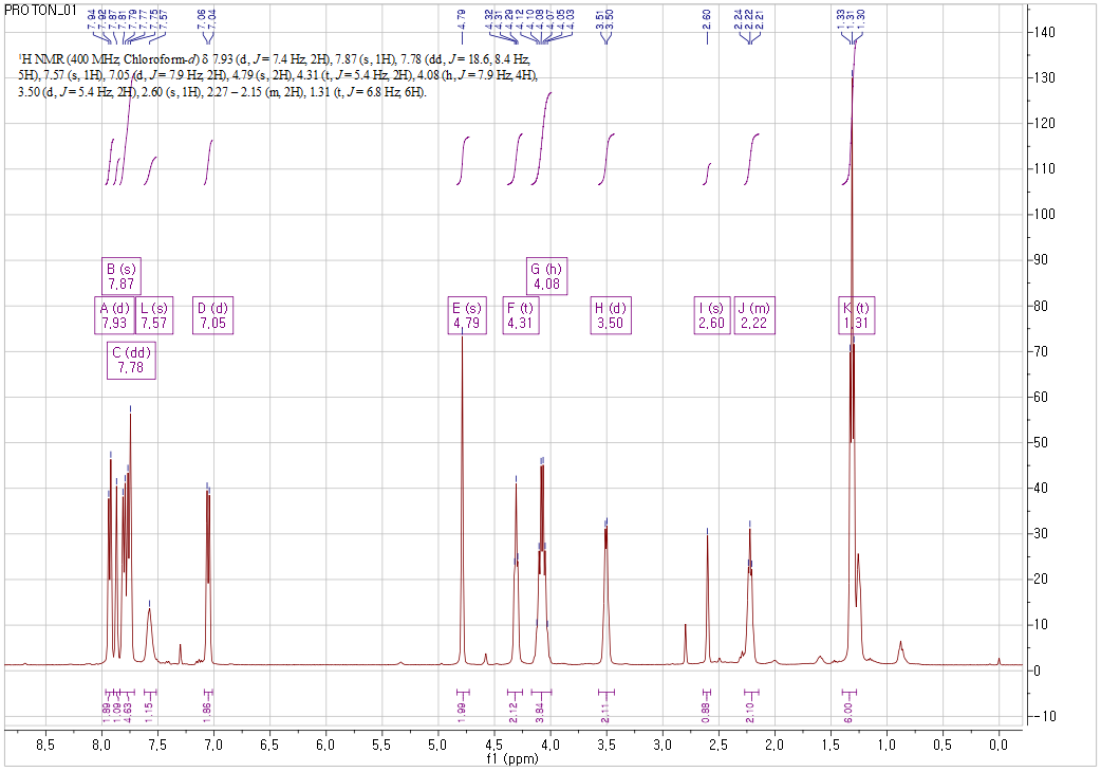
TLC (10% methanol in ethyl acetate): R_f = 0.4, UV-active, yellow spot (stained with $KMnO_4$).

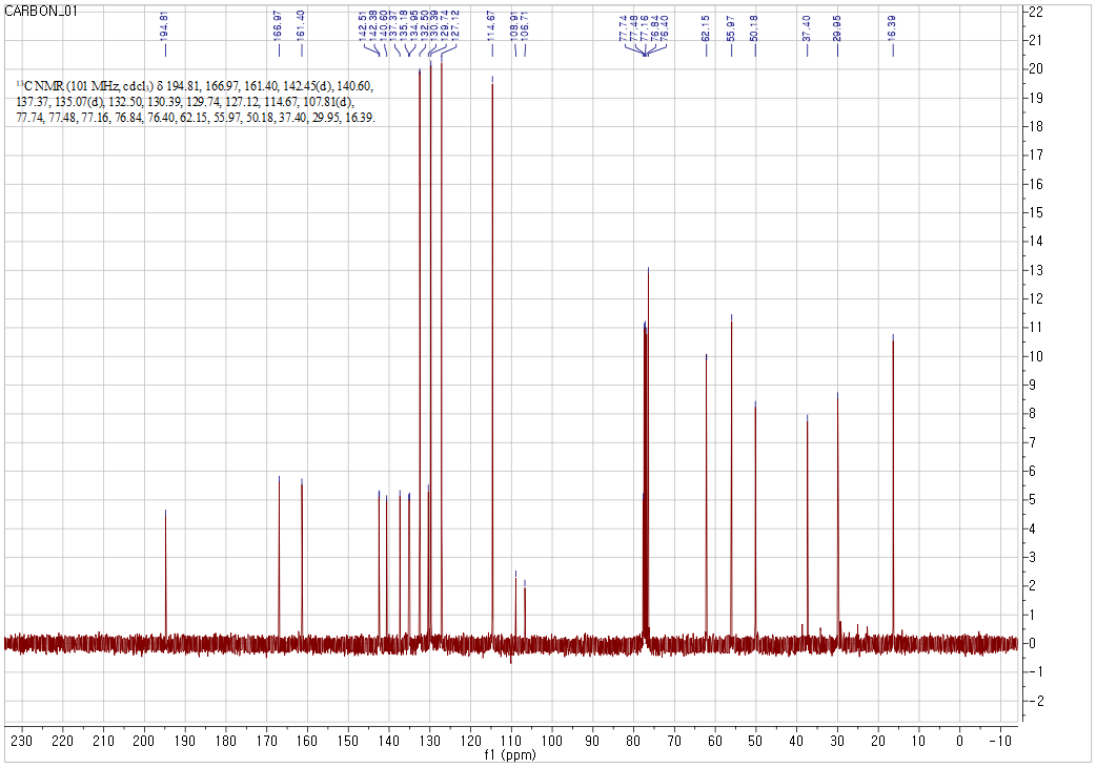
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.93 (d, J = 7.4 Hz, 2H), 7.87 (s, 1H), 7.78 (dd, J = 18.6, 8.4 Hz, 5H), 7.57 (s, 1H), 7.05 (d, J = 7.9 Hz, 2H), 4.79 (s, 2H), 4.31 (t, J = 5.4 Hz, 2H), 4.08 (h, J = 7.9 Hz, 4H), 3.50 (d, J = 5.4 Hz, 2H), 2.60 (s, 1H), 2.27 – 2.15 (m, 2H), 1.31 (t, J = 6.8 Hz, 6H).

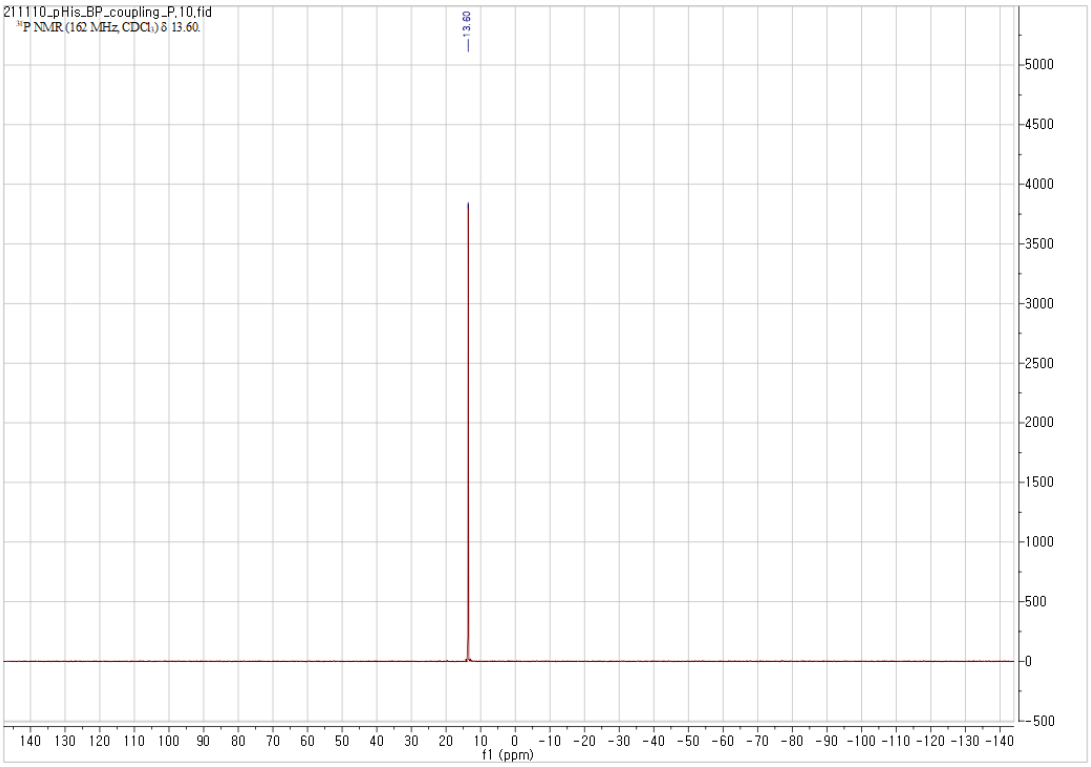
^{13}C NMR (101 MHz, $CDCl_3$): δ = 194.81, 166.97, 161.40, 142.45(d), 140.60, 137.37, 135.07(d), 132.50, 130.39, 129.74, 127.12, 114.67, 107.81(d), 77.74, 76.40, 62.15, 55.97, 50.18, 37.40, 29.95, 16.39.

^{31}P NMR (162 MHz, $CDCl_3$): δ = 13.60.

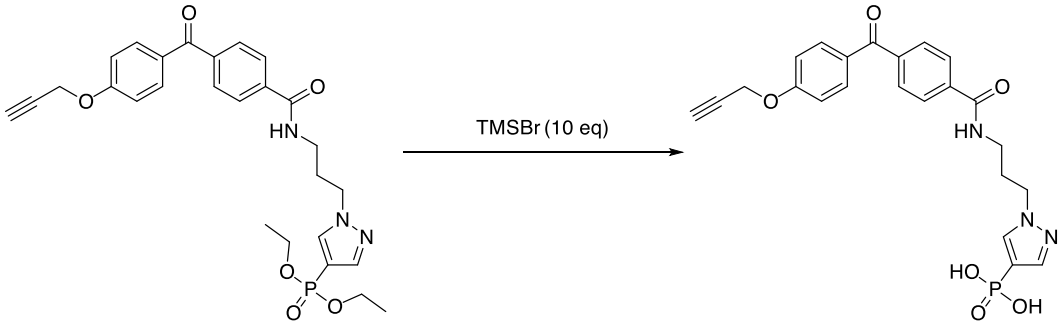
HRMS (*m/z*): Calculated (for $C_{27}H_{31}N_3O_6P_1$+): 524.1945; observed 524.1941 (error: -0.7631 ppm)


PROTON_01
¹H NMR (400 MHz, Chloroform-d) δ 7.93 (d, J = 7.4 Hz, 2H), 7.87 (s, 1H), 7.78 (dd, J = 18.6, 8.4 Hz, 5H), 7.57 (s, 1H), 7.05 (d, J = 7.9 Hz, 2H), 4.79 (s, 2H), 4.31 (t, J = 5.4 Hz, 2H), 4.08 (h, J = 7.9 Hz, 4H), 3.50 (d, J = 5.4 Hz, 2H), 2.60 (s, 1H), 2.27 – 2.15 (m, 2H), 1.31 (t, J = 6.8 Hz, 6H).
B (s) 7,87
A (d) 7,93
L (s) 7,57
D (d) 7,05
C (dd) 7,78
E (s) 4,79
G (h) 4,08
F (t) 4,31
H (d) 3,50
I (s) 2,60
J (m) 2,22
K (t) 1,31
f1 (ppm)


CARBON_01
¹³C NMR (101 MHz, cdcl₃) δ 194.81, 166.97, 161.40, 142.45(d), 140.60, 137.37, 135.07(d), 132.50, 130.39, 129.74, 127.12, 114.67, 107.81(d), 77.74, 77.48, 77.16, 76.84, 76.40, 62.15, 55.97, 50.18, 37.40, 29.95, 16.39.
f1 (ppm)



5) Synthesis of **7** pPyp-BP



Compound **6** (60 mg, 0.11 mmol) was dissolved in dry acetonitrile (6 mL). Bromotrimethylsilane (146 µL, 1.15 mmol) was added, and the mixture was stirred overnight at rt under Ar. The reaction mixture was quenched with MeOH (5 mL) and stirred for 30 min at rt open to air. The solution was concentrated under reduced pressure and purified by semi-preparative reverse-phase HPLC (0-100% solvent B, 20 min, 2.5 mL/min) to give a white oil (9.8 mg, 19.1% yield).

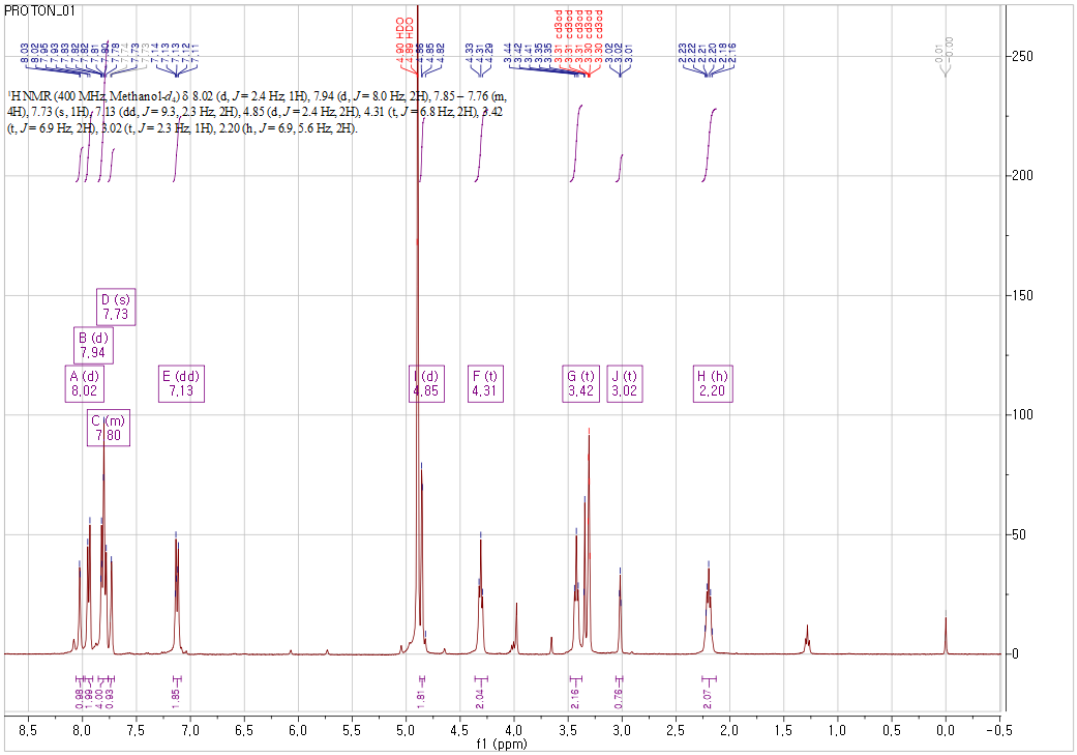
TLC (10% methanol in ethyl acetate): R_f = 0.1, UV-active, yellow spot (stained with $KMnO_4$).

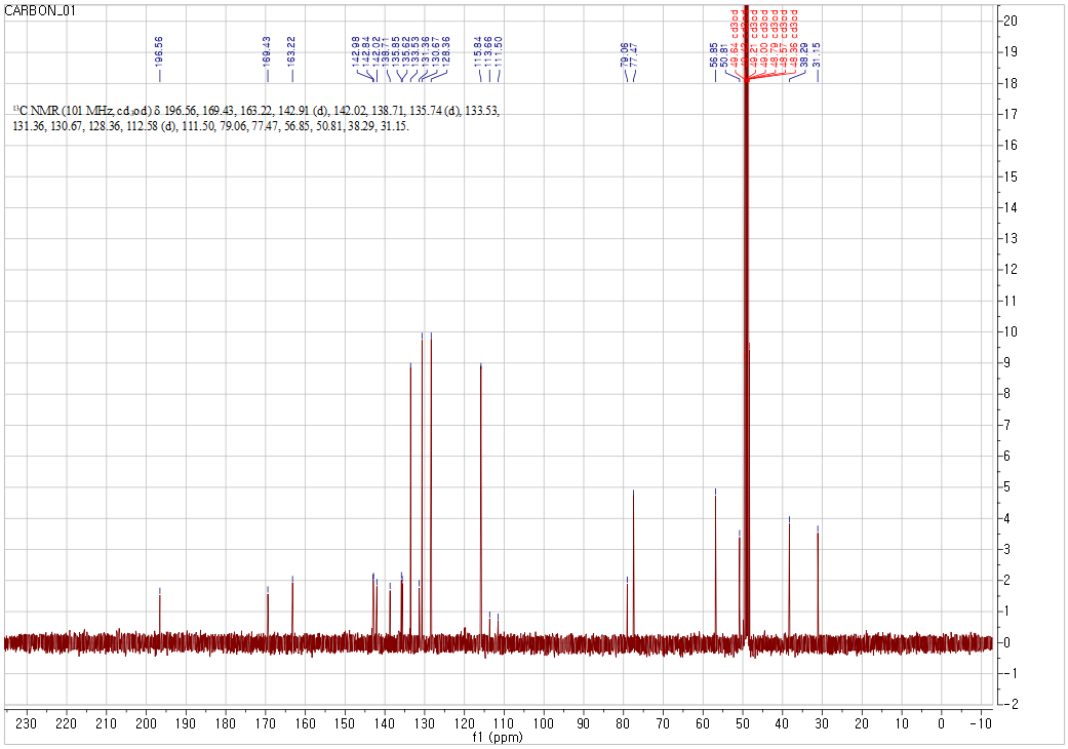
^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.02 (d, J = 2.4 Hz, 1H), 7.94 (d, J = 8.0 Hz, 2H), 7.85 – 7.76 (m, 4H), 7.73 (s, 1H), 7.13 (dd, J = 9.3, 2.3 Hz, 2H), 4.85 (d, J = 2.4 Hz, 2H), 4.31 (t, J = 6.8 Hz, 2H), 3.42 (t, J = 6.9 Hz, 2H), 3.02 (t, J = 2.3 Hz, 1H), 2.20 (h, J = 6.9, 5.6 Hz, 2H).

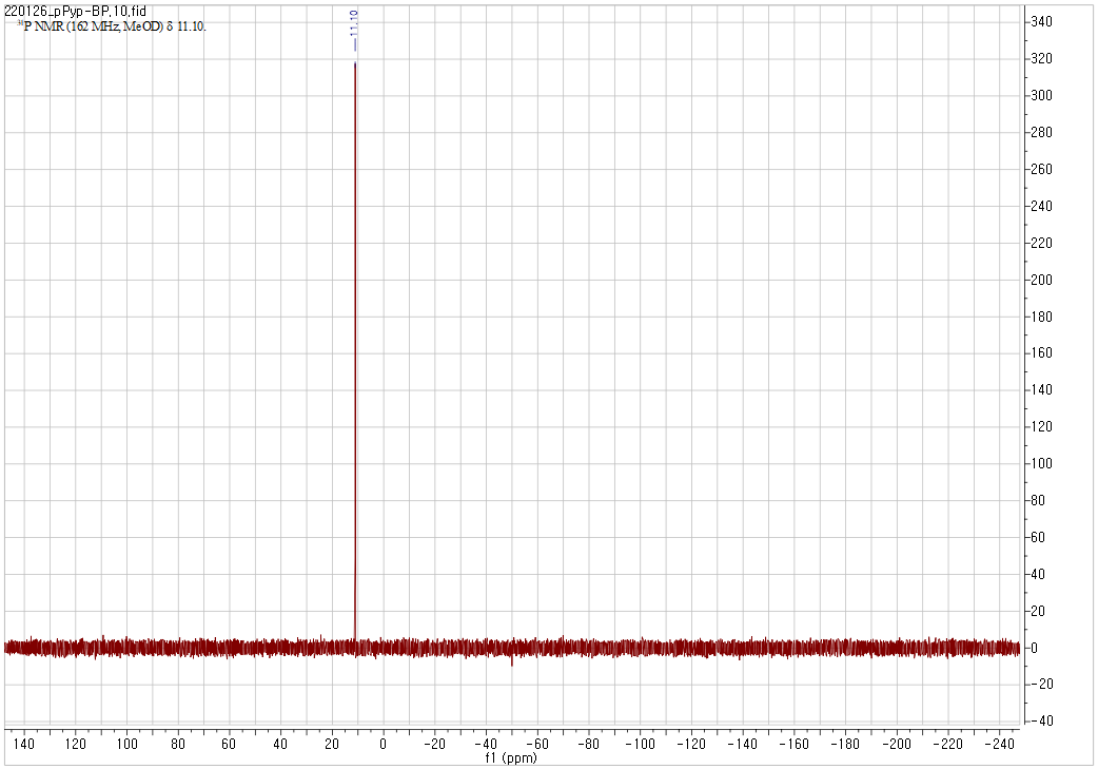
^{13}C NMR (101 MHz, $CDCl_3$): δ = 196.56, 169.43, 163.22, 142.91 (d), 142.02, 138.71, 135.74 (d), 133.53, 131.36, 130.67, 128.36, 112.58 (d), 111.50, 79.06, 77.47, 56.85, 50.81, 38.29, 31.15.

^{31}P NMR (162 MHz, $CDCl_3$): δ = 11.10.

HRMS (*m/z*): Calculated (for $C_{23}H_{21}N_3O_6P^-$): 466.1168; observed 466.1169 (error: 0.2145 ppm)




CARBON_01
196.56
169.43
163.22
142.98
142.84
142.02
138.71
135.85
135.74
133.53
131.36
130.67
128.36
112.58
111.84
111.50
111.50
79.06
77.47
56.85
50.81
49.84
49.63
49.42
49.21
49.00
48.79
48.58
48.37
38.29
31.15
13C NMR (101 MHz, cd3od) δ 196.56, 169.43, 163.22, 142.91 (d), 142.02, 138.71, 135.74 (d), 133.53, 131.36, 130.67, 128.36, 112.58 (d), 111.50, 79.06, 77.47, 56.85, 50.81, 38.29, 31.15.
f1 (ppm)


220126_pPyp-BP.10.fid
31P NMR (162 MHz, MeOD) δ 11.10.
11.10
f1 (ppm)

Proteomics data processing

Raw files were analyzed using Maxquant with the following parameters.

- Trypsin/Lys-C (maximum of 2 missed cleavage)
- *E. coli* K12 database (Uniprot UP000000625, Ver. Apr. 2022)
- Fixed modification: Carbamidomethylation (Cys)
- Variable modification: Oxidation (Met)
- Match-between-run LFQ (label-free quantification) for quantification.
- For pHis site identification, phosphorylation (Ser, The, Tyr, and His) was searched as variable modification, following the reported parameter.[10]
- For pPyp-BP site identification, PTM change of C(23)H(22)O(6)N(3)P was searched as a variable modification for all amino acid residues.

All statistical analyses were conducted using Perseus (Ver. 2.0.10.0)[76] following established literature protocols.[77] VolcaNoseR was employed for the visualization of proteomics results.[78] Additional data processing was carried out using R (Ver. 3.6.3) within the RStudio IDE (Ver. 1.2.5042).

Data availability

The proteomics raw data have been deposited to the ProteomeXchange Consortium (PRIDE, PXD053006 and PXD056526).

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