Identification of Highly Reactive Sequences For PLP-Mediated Bioconjugation Using a Combinatorial Peptide Library

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Supporting Information

General Procedures. Unless otherwise noted, the chemicals and solvents used were of analytical grade and were used as received from commercial sources. Water (dd-H_2O) used as reaction solvent and in biological procedures was deionized using a Barnstead NANOpure purification system (ThermoFisher, Waltham, MA). Pyridoxal 5'-phosphate monohydrate was obtained from Aldrich (St. Louis, MO). All Fmoc-protected amino acids were obtained from Novabiochem (EMD, Germany). Tentagel S OH resin was obtained from Advanced ChemTech (Louisville, KY). Centrifugations were conducted with a Sorvall RC 5C plus (Sorvall, USA) for samples greater than 50 mL, a Sorvall LEGEND mach 1.6R for samples between 1 and 50 mL, and an Eppendorf Mini Spin Plus for samples less than 1 mL (Eppendorf, Hauppauge, NY).

Solid-Phase Peptide Synthesis. Peptides were synthesized using standard conditions for Fmoc-based chemistry. The side chain protecting groups used were: Asn(Trt), Asp(tBU), Arg(Pbf), Cys(Trt), Gln(Trt), Glu(tBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu). The C-terminal amino acid (10 equiv) was preactivated with 5 equivalents of diisopropylcarbodiimide (DIC) then coupled to the Tentagel S OH resin with 0.1 equivalents of *N*,*N*-dimethylaminopyridine (DMAP) as an additive. Deprotection of Fmoc groups was accomplished by incubation with a 20% v/v piperidine in *N*,*N*-dimethylformamide (DMF) solution for 20 minutes. Coupling reactions were carried out using 10 equivalents of amino acid with 10 equivalents of 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU)[1] and 20 equivalents of *N*,*N*-diisopropylethylamine (DIPEA) in DMF for 10 minutes. Side-chain deprotection was achieved using a 1-2 h incubation with a 95:2.5:2.5 ratio of trifluoroacetic acid (TFA) to H_2O to triisopropylsilane (TIPS), followed by equilibration in 50 mM phosphate buffer (pH 6.5) using three 5 min exposures.

Split-Pool Library Synthesis with Partial Truncation. Construction of the combinatorial library began with the synthesis of the 5-residue base sequence (WSNAG) on 500 mg of resin.[2] Capping was subsequently performed using 0.15 equivalents of bromobenzoic acid, 0.1 equivalents of HCTU and 1 equivalent of DIPEA. The resin was then split into 20 reaction vials, and each vial was exposed to coupling conditions using one of the twenty amino acids. After coupling and rinsing, the resins were then recombined, with the exception of the vials corresponding to Gln or Leu, which were kept separate. After Fmoc deprotection, the next capping step was performed. Bromobenzoic acid was attached to the mixed resin sample and methylbromobenzoic acid was used to cap the Gln and Leu samples to distinguish them from isobaric residues during sequencing. The Gln and Leu samples were then mixed with the rest of the resin before splitting into separate vials for the next coupling reaction.

General Procedure for Library Screening. Portions of resin-bound peptides (appx. 25 mg each) were treated with 1 mL of PLP solutions of varying concentration in 50 mM phosphate buffer, pH 6.5 with 10% DMF. Prior to use, the pH of each PLP solution was adjusted to 6.5 with NaOH following the addition of

the PLP. After 18 h of reaction time, the resin samples were washed with three portions of deionized water, followed by three portions of DMF to remove residual PLP. The peptides were then incubated with 1 mL of a 10 mM solution of disperse red alkoxyamine in DMF for 3 h at rt. The excess disperse red alkoxyamine was removed by rinsing with three portions of dichloromethane (DCM), followed by three portions of DMF. The beads were then rinsed with ethanol and transferred to a Petri dish for visual inspection. The beads were examined using a Leica S6D Microscope and L2 Light Source (Leica, Germany) equipped with a Moticam 2300 3.0 MP camera using Motic Image Plus 2.0 ML Software for capturing images. Individual red beads were manually removed using a Pipet-Lite LTS L-20 pipet (Rainin, Oakland, CA) and transferred to PCR tubes for sequencing. The residual ethanol in the tubes was removed by pipetting.

General Procedure for Library Sequencing. Individual beads identified in the library were incubated with 100 mM NaOH to cleave the peptide from the resin.[2] The solution was desalted using Ziptips with a 0.2 µL C18 resin (Millipore, Billerica, MA). The peptide was eluted from the Ziptip with 2 µL of matrix solution (described below) directly onto a MALDI sample plate (Applied Biosystems, Foster City, CA). MALDI-TOF analysis was performed on a Voyager-DE instrument (Applied Biosystems), and all spectra were analyzed using Data Explorer software. The matrix solution was a saturated α-Cyano-4-hydroxycinnamic acid solution in 50% acetonitrile, 50% water, 0.1 % TFA.

General Procedure for the Modification of Resin-Bound Peptide Substrates. Portions of resin-bound peptides (appx. 10 mg each) were treated with 1 mL of PLP solutions of varying concentration in 50 mM phosphate buffer, pH 6.5 with 10% DMF. Prior to use, the pH of each PLP solution was adjusted to 6.5 with NaOH following the addition of the PLP. After 18 h of reaction time, the resin samples were washed with three portions of deionized water, followed by three portions of DMF to remove residual PLP. The peptides were then incubated with 1 mL of a 250 mM *O*-benzylhydroxylamine hydrochloride ($BnONH_2$) solution in water for 3 h at rt. Excess alkoxyamine was removed by rinsing with three portions of deionized water, followed by three portions of DMF. The peptides were then cleaved from the resin through incubation with 300 µL of a 100 mM sodium hydroxide solution for 30 min. The resulting peptide solution was added to 700 µL of 50 mM phosphate buffer (pH 6.5). The resulting solution was diluted 20-fold into dd-H_2O for mass spectrometry analysis.

Liquid Chromatography and Mass Spectrometry Materials. Acetonitrile (Fisher Optima grade, 99.9%) and formic acid (1 mL ampules, 99+%) were used to prepare mobile phase solvents for liquid chromatography-mass spectrometry (LC-MS). Water used in these experiments was purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA).

LC-MS analysis of peptide bioconjugates. Peptide bioconjugates were analyzed using a nanoAcquity ultraperformance liquid chromatograph (UPLC; Waters, Milford, MA) that was connected in-line with a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters).

The UPLC was equipped with C18 trapping (5 µm, 20 mm × 180 µm) and analytical (1.7 µm, 100 mm × 100 µm) columns and a 10 µL sample loop. Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). Sample solutions contained in 0.3 mL polypropylene snap-top vials sealed with septa caps were loaded into the nanoAcquity autosampler compartment prior to analysis. Following sample injection (1.5 µL, partial loop), trapping was performed for 5 min with 100% A at a flow rate of 3 µL/min. The injection needle was washed with 500 µL each of solvents A and B after injection to avoid cross-contamination between samples. The elution program consisted of a linear gradient from 25% to 50% B over 30 min, a linear gradient to 95% B over 0.33 min, isocratic conditions at 95% B for 6.67 min, a linear gradient to 1% B over 0.33 min, and isocratic conditions at 1% B for 11.67 min, at a flow rate of 500 nL/min. The analytical column and sample compartment were maintained at 35

ºC and 8 ºC, respectively.

The column exit was connected to a NanoEase nanoelectrospray ionization (nanoESI) emitter mounted in the nanoflow ion source of the Q-Tof. The nanoESI source parameters were as follows: nanoESI capillary voltage 2.3 kV, nebulizing gas (nitrogen) pressure 0.15 mbar, sample cone voltage 30 V, extraction cone and ion guide voltages 4 V, and source block temperature 80 ºC. No cone gas was used. The collision cell contained argon gas at a pressure of 8×10^{-3} mbar. The Tof analyzer was operated in "V" mode. Under these conditions, a mass resolving power of 1.0×10^{4} (measured at m/z = 771) was routinely achieved, which was sufficient to resolve the isotopic distributions of the singly and multiply charged peptide bioconjugate ions measured in this study. Thus, an ion's mass and charge could be determined independently, i.e., the ion charge was determined from the reciprocal of the spacing between adjacent isotope peaks in the m/z spectrum. External mass calibration was performed immediately prior to analysis using solutions of sodium formate. Mass spectra were acquired in the positive ion mode over the range m/z = 100-2000, in continuum data format, using a 0.95 s scan integration and a 0.05 s interscan delay. Mass chromatograms and spectra were processed using MassLynx software (version 4.1, Waters).

LC-MS analysis of protein bioconjugates. Protein bioconjugates were analyzed using an Agilent 1200 series liquid chromatograph (LC; Santa Clara, CA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA).

The LC was equipped with C8 guard (Poroshell 300SB-C8, 5 µm, 12.5 × 2.1 mm, Agilent) and analytical (75 × 0.5 mm) columns and a 100 µL sample loop. Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). Sample solutions contained in 0.3 mL polypropylene snap-top vials sealed with rubber septa caps were loaded into the Agilent 1200 autosampler compartment prior to analysis. For each sample, approximately 100 to 200 picomoles of protein analyte was injected onto the column. Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90 µL/min. The elution program consisted of a linear gradient from 30% to 95% B over 19.5 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 0.5 min, and then isocratic conditions at 0.5% B for 9.5 min, at a flow rate of 90 µL/min. The column and sample compartments were maintained at 35 ºC and 10 ºC, respectively. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection, to avoid cross-contamination between samples.

The connections between the LC column exit and the mass spectrometer ion source were made using PEEK tubing (0.005" i.d. × 1/16" o.d., Western Analytical, Lake Elsinore, CA). External mass calibration was performed prior to analysis using the standard LTQ calibration mixture containing caffeine, the peptide MRFA, and Ultramark 1621 dissolved in 51% acetonitrile/25% methanol/23% water/1% acetic acid solution (v/v). The ESI source parameters were as follows: ion transfer capillary temperature 275 ºC, normalized sheath gas (nitrogen) flow rate 25%, ESI voltage 2.5 kV, ion transfer capillary voltage 33 V, and tube lens voltage 125 V. Mass spectra were recorded in the positive ion mode over the range m/z = 500 to 2000 using the Orbitrap mass analyzer, in profile format, with a full MS automatic gain control target setting of 5×10^{5} charges and a resolution setting of 6×10^{4} (at m/z = 400, FWHM). Raw mass chromatograms and spectra were processed using Xcalibur software (version 4.1, Thermo) and measured charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ), using default "small protein" parameters and a background subtraction factor of 1.5.

Constuction of GFP and ISP Expression Plasmids. The construction of the ISP-pTXB1 and EGFP-pET28b plasmids has been previously reported.[3,4] The Quick Change II System Kit (Stratagene, La Jolla, CA) was used to mutate the N-termini of each plasmid to AKT. The plasmids used had previous N-terminal mutations, therefore site-directed mutagenesis was also used to make mutants containing the

wild type N-terminal sequence. The ISP wild-type sequence was obtained by removing two amino acids that had been added to the N-terminus during the original construction of the plasmid using the primers:

Forward: 5'-gaaggagatatacatatgggtaaccaggcgagcgt-3'
Reverse: 5'-acgctcgcctggttacccatatgtatatctccttc-3'

The AKT-terminal ISP was obtained on the extended N-terminus using the primers:
Forward: 5'-ggagatatacatatggcgaaaaccaaccaggcgagcgt-3'
Reverse: 5'-acgctcgcctggttggttttcgccatatgtatatctcc-3'

The wild-type GFP plasmid was constructed using the primers:
Forward: 5'-atacatatggtgagcaagggcgaggagctgttcaccg-3'
Reverse: 5'-gaaattcttcctctatatgtataccactcgttcccgc- 3'

The GFP AKT mutant was constructed using the primers:
Forward: 5' -catatggccaaaacgggcgaggagctgttcaccggggtg- 3'
Reverse: 5' -cctcgcccgttttggccatatgtatatctccttcttaaag- 3'

Incorporation of the point mutations was verified by sequencing.

General Procedure for Expression and Purification of GFP and ISP. The plasmids were transformed into T7 Express LysY/Iq Competent *E. coli* (NEB, Ipswich, MA) and plated on LB Agar Plates (ampicillin at 100 μg/mL). Cells were grown in 1 L of Luria Broth (LB) containing ampicillin at 100 μg/ml at 37 °C until an optical density (OD) of 0.7 was observed at 600 nm. Protein expression was induced by the addition of 1 mL of 0.3 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). Cultures were grown for an additional 16 h at 16 °C. The cells were then centrifuged for 10 min at 8,000 rcf at 4 °C. The collected cells were re-suspended in 10 mL of Lysis/Wash Buffer (0.02 M Tris, 0.5 M NaCl, 1 mM EDTA, pH 7.5) by vortexing. The cells were lysed by sonication using a Branson Digital Sonifier (VWR Scientific, West Chester, PA) for 20 min with a blunt ended tip. Debris was removed by centrifugation. The protein-containing lysate was incubated with 5-6 mL of chitin resin (NEB) in for 1 h. The resulting resin-bound protein was washed with 100 mL of Wash Buffer that was cooled to 4 °C. A 30 mL solution of 50 mM sodium 2-sulfanylethanesulfonate (MESNA) in Wash Buffer was flowed over the resin-bound protein using suction in order to cleave it from the intein-chitin binding domain. The column bed was then allowed to stand in a minimal amount of this solution at 4 °C for 15 h. The protein was eluted from the column with the addition of 15 mL of Wash Buffer. Purified protein was then buffer exchanged 3 times with Wash Buffer using Amicon Ultra 15 mL 3,000 MWCO (Millipore, Billerica, MA) and incubated with an additional 5 mL of chitin resin for 1 h. The chitin resin was removed by filtration through a Steriflip (Millipore). Purified protein was then buffer exchanged into 25 mM sodium phosphate (pH 6.5) using Amicon Ultra 15 mL 3,000 MWCO (Millipore) centrifugal ultrafiltration membranes. The entire procedure was performed in a cold room maintained at 4 °C. Purity was evaluated by SDS-PAGE with Coomassie staining.

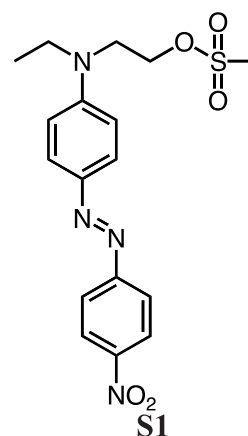
General Procedure for Modification of Protein Substrates. Protein and PLP stock solutions were prepared at twice the desired final concentration and mixed in equal volumes in a 1.5 mL Eppendorf tube. The final volume of each reaction was 100 μL. The 2x protein stock solutions were prepared at 40-600 μM using 25 mM phosphate buffer at pH 6.5. The 2x PLP stock solutions were prepared in 25 mM phosphate buffer, pH 6.5. Following addition of the PLP, the pH was re-adjusted to pH 6.5 using NaOH. The reaction mixture was briefly agitated to ensure mixing and then incubated without further agitation at 37 °C for 1-18 h. Following the reaction, the PLP was removed using Milipore 0.5 mL spin concentrators

(MWCO 3 kDa). The buffer exchange first involved the dilution of each sample to 500 µL with 25 mM phosphate buffer (pH 6.5). Each sample was then concentrated to 100 µL, and the process was repeated 2-4 times, depending on the initial concentration of PLP that was used. The resulting 100 µL of reacted protein was then treated with 25 µL of 250 mM $BnONH_2$ (pH adjusted to 5.5) in a 1.5 mL Eppendorf tube and incubated at rt for 18 h. Buffer exchange steps were again repeated to stop the reaction and remove the excess alkoxyamine.

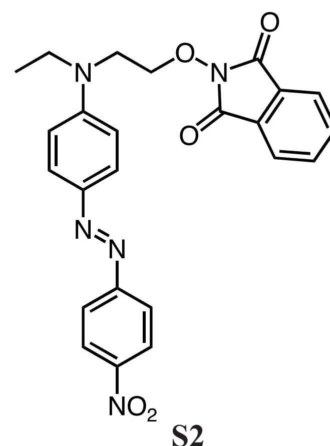
Synthesis of Disperse Red Alkoxyamine.

NMR. ^{1}H and ^{13}C spectra were measured with a Bruker AVQ-400 (400 MHz) or AV-300 (300 MHz) spectrometer. ^{1}H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-*d* (δ 7.26, s). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (n) for a given resonance is indicated nH, and is based on spectral integration values. ^{13}C NMR spectra are reported as δ in units of part per million (ppm) relative to chloroform-*d* (77.23, t).

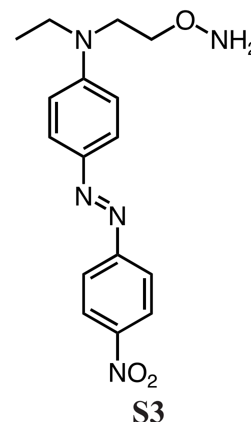
Disperse Red mesylate (S1). To an oven-dried 500 mL round bottom flask was added Disperse Red 1 (2.0 g, 6.4 mmol, 1.0 equiv) and distilled methylene chloride (70 mL). The mixture was stirred in an ice bath under an atmosphere of nitrogen until the Disperse Red had dissolved (<5 min). *N*,*N*-Diisopropylethylamine (DIPEA) (2.5 mL, 14.6 mmol, 2.3 equiv) was added dropwise, followed by the dropwise addition of mesyl chloride (590 µL, 7.6 mmol, 1.2 equiv). The reaction was allowed to warm to room temperature and was stirred for an additional 2 h. The mixture was washed with saturated ammonium chloride and brine, and the organic layer was dried over anhydrous sodium sulfate, filtered through glass wool and concentrated under reduced pressure. The resulting red solid was used in subsequent experiments without further purification (2.5 g, quant. yield). High resolution FAB-MS M^{+}: calculated 392.1154, found 392.1149. ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 8.31 (d, 2H, *J*=9.2) 7.90 (m, 4H) 6.77 (d, 2H, *J*=9.3) 4.39 (t, 2H, *J*=6.0) 3.79 (t, 2H, *J*=6.0) 3.54 (q, 2H, *J*=7.2) 3.06 (s, 3H) 1.25 (t, 3H, *J*=7.2) ^{13}C-NMR (400 MHz, $CDCl_3$) *δ* 156.6, 150.7, 147.5, 144.1, 126.2, 124.7, 122.7, 111.5, 66.0, 53.4, 49.3, 46.0, 37.6, 12.3.



Disperse Red phthalimide (S2). To a 200 mL round bottom flask was added **S1** (2.5 g, 6.4 mmol, 1.0 equiv), *N*,*N*-dimethylformamide (DMF) (20 mL) and *N*-hydroxyphthalimide (1.25 g, 7.6 mmol, 1.2 equiv). To this solution was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (1.14 mL, 7.6 mmol, 1.2 equiv), and the mixture was stirred under nitrogen and heated (50 °C) for 3 h. Following the reaction, the DMF was evaporated under reduced pressure. The mixture was dissolved in a 2:1 mixture of dicholoromethane and isopropyl alcohol and eluted through a silica gel plug. The resulting solution was transferred to a separatory funnel and washed with sodium bicarbonate solution, water, and brine. The organic layer was then dried over sodium sulfate, filtered through glass wool, and concentrated under reduced pressure (1.9 g, 66% yield). High resolution FAB-MS M^{+}: calculated 459.1543, found 459.1540. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.31 (d, 2H, *J*=9.2) 7.90 (m, 4H) 7.83 (q, 2H, *J*=2.8) 7.75 (q, 2H, *J*=2.8) 6.78 (d, 2H, *J*=9.2) 4.41 (t, 2H, *J*=6.0) 3.87 (t, 2H, *J*=6.0) 3.64 (q, 2H, *J*=7.2) 1.29 (t, 3H, *J*=7.2) ^{13}C-NMR (400 MHz, $CDCl_3$) δ 163.4, 156.7, 151.0, 147.4, 143.9, 134.7, 128.7, 126.3, 124.7, 123.7, 122.6, 111.5, 75.9, 48.7, 46.1, 12.3.



Disperse Red alkoxyamine (S3). To an oven-dried 500 mL round bottom flask was added **S2** (1.9 g, 4.2 mmol, 1.0 equiv), methylene chloride (50 mL), and hydrazine hydrate (1.02 mL, 21.1 mmol, 5.0 equiv). The reaction was stirred for 1 h at rt, during which time an off-white precipitate formed. The white precipitate was removed by filtration and the red filtrate was concentrated under reduced pressure and used without further purification (0.89 g, 68% yield). High resolution FAB-MS M^+: calculated 329.1488, found 329.1494. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.31 (d, 2H, *J*=9.2) 7.89 (t, 4H, *J*=9.6) 6.78 (d, 2H, *J*=9.2) 3.86 (t, 2H, *J*=6.0) 3.65 (t, 2H, *J*=6.0) 3.52 (q, 2H, *J*=7.2) 1.23 (t, 3H, *J*=7.3). ^{13}C-NMR (400 MHz, $CDCl_3$) δ 157.1, 151.7, 147.5, 143.8, 126.5, 124.9 122.8, 111.5, 72.8, 49.1, 46.0, 12.4.



Supporting Figures

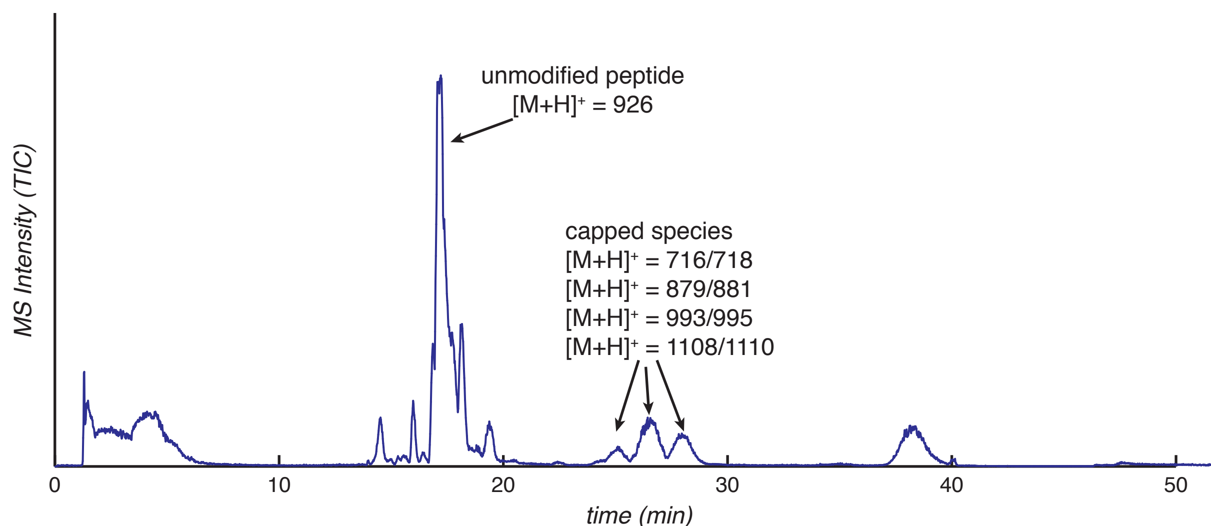


Figure S1. ESI-QTOF MS analysis of the peptide mixture released from a single bead from the library prior to exposure to PLP. The main species identified was the unmodified peptide. The capped species (identified by their bromine isotope patterns) and other impurities comprised a small portion of the total material on the bead. The mass differences observed between the capped species corresponded to the amino acids DNY, and the sequence assignment was confirmed when the calculated mass of the peptide (DNYWSNAG $[M+H]^+$ = 926.31 *m*/*z*) matched the observed $[M+H]^+$ = 926.25 *m*/*z*.

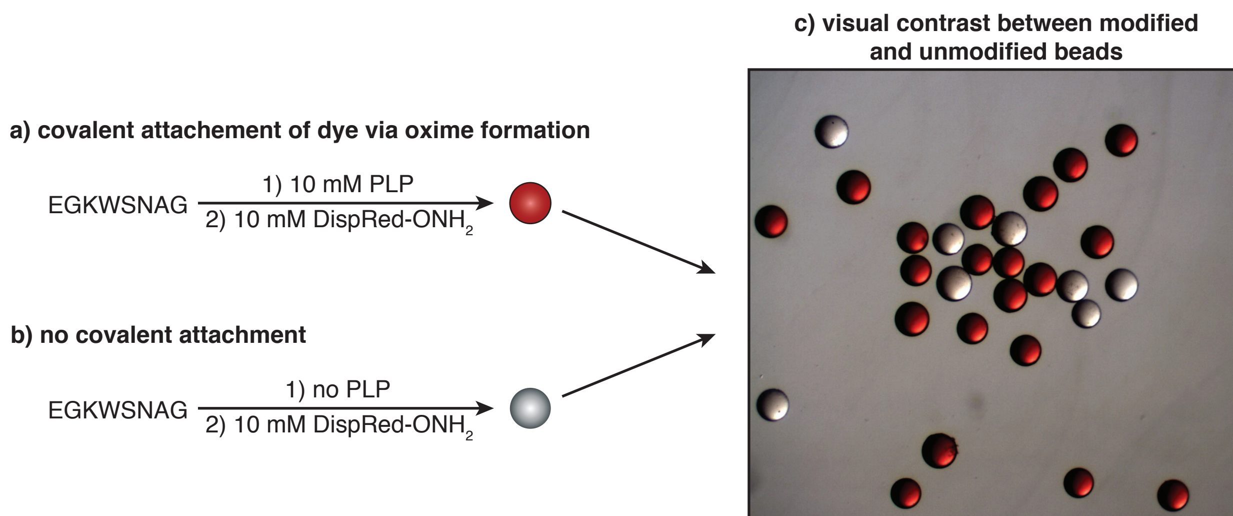


Figure S2. Testing contrast for modified and unmodified samples. (a) A peptide known to give high yields under standard transamination conditions, EGKWSNAG, was subjected to standard PLP transamination conditions to install a keto group. The beads were then incubated with Disperse Red alkoxyamine to form an oxime with the dye, resulting in a visually red bead. (b) The same peptide was subjected to Disperse Red alkoxyamine without prior transamination to check for non-specific binding of the dye to the bead. The resulting colorless beads confirmed that there was no association of the dye in the absence of a colvalent linkage. (c) The beads with and without covalent attachment of Disperse Red were mixed and viewed under a light microscope. The contrast was sufficient to distinguish beads bearing modified and unmodified peptides easily.

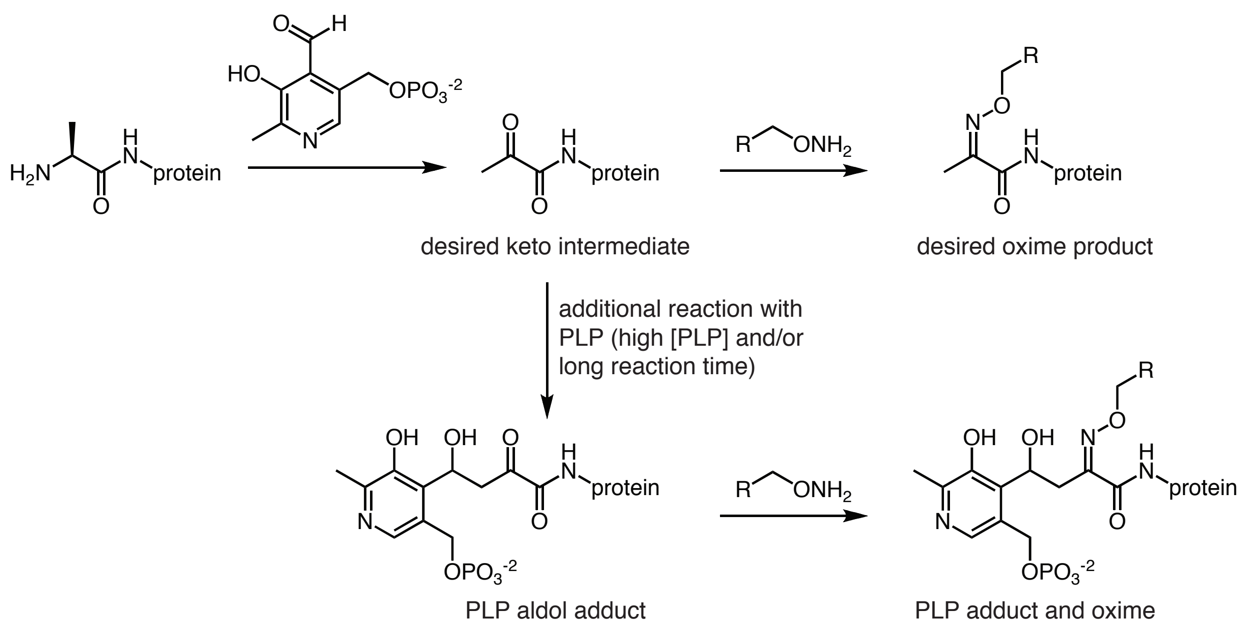


Figure S3. Proposed pathway for the formation of PLP adducts. In addition to the desired pathway, the enol form of the keto-protein intermediate reacts with the aldehyde of PLP to form an aldol adduct. The PLP adduct structure still contains a ketone and is able to undergo oxime formation in some cases. Because the aldol reaction is bimolecular, reaction conditions found to result in higher purity oxime product and minimal PLP adduct formation were (1) the use of lower concentrations of PLP for longer reaction times, or (2) the use of higher concentrations of PLP for shorter times.

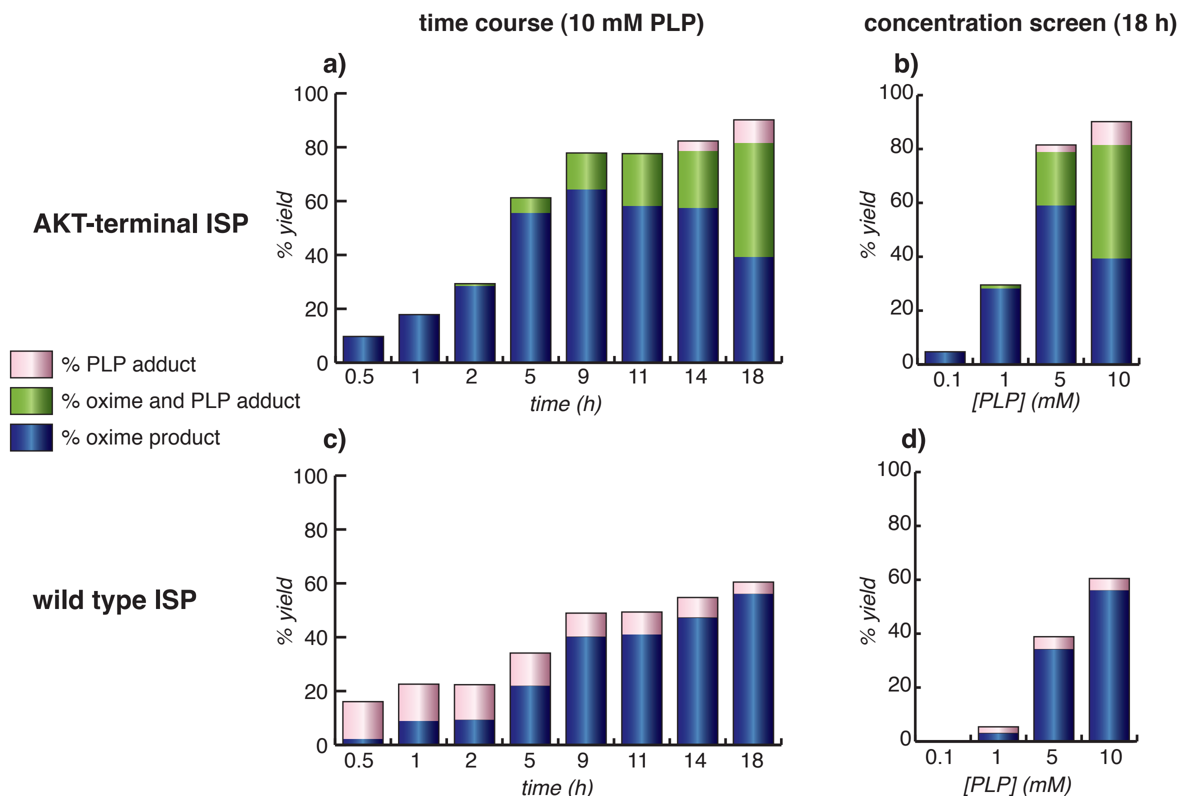


Figure S4. Ice Structuring Protein (ISP) product distributions after modification with PLP and benzylalkoxyamine. (a) AKT-terminated ISP reached high oxime yields after 9 hours. Longer reaction times led to increased byproduct formation. (b) The AKT-mutant product distribution showed similar behavior at different concentrations of PLP, with a high yield of oxime product being formed with 5 mM PLP, and higher concentrations resulting in high amounts of byproduct. The wild type ISP (with a GNQ terminus) was less reactive than the AKT mutant, in terms of both oxime product and byproduct formation. The product distributions are shown for a time course (c) and concentration screen (d).

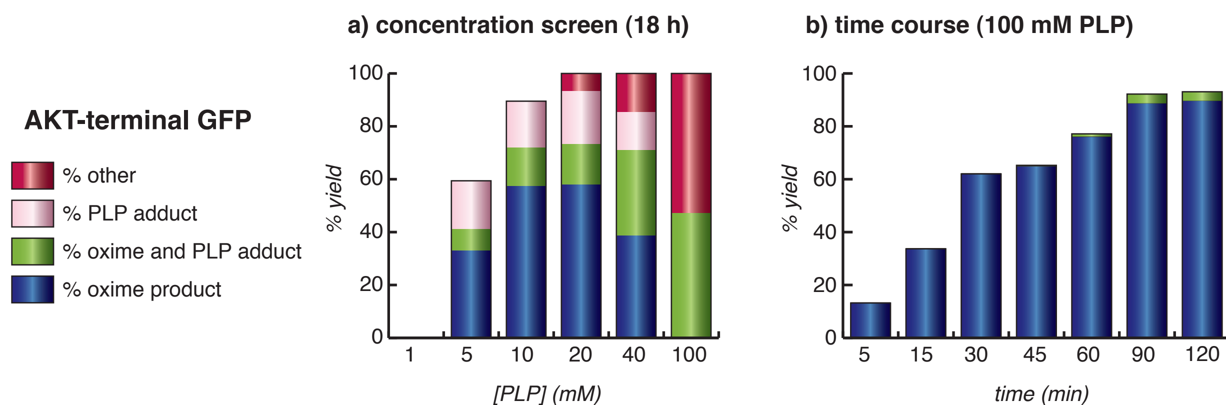


Figure S5. Product distributions for the modification of GFP with PLP. (a) AKT-terminal GFP was particularly reactive towards byproduct formation, especially at high concentrations of PLP. The bar labeled 'other' included unknown modification products. (b) When strategies to minimize byproduct formation were applied, we found that using 100 mM PLP for short reaction times (1-2 h) led to high yields of oxime product with little-to-no adduct formation. Wild type GFP (MVS terminal sequence, data not shown) provided only trace amounts of product using the conditions screened for the AKT mutant.

Supporting Information References:

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4. Esser-Kahn, A. P.; Trang, V.; Francis, M. *J. Am. Chem. Soc.* **2010***, 132,* 13264–13269