Supporting Information

Iron-Sensitive Protein Conjugates Formed with a Wittig Reaction Precursor in Ionic Liquid
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Fig. S18. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the biphenyl-enamine-phosphonium incubated in (NH$_4$)$_2$CO$_3$ buffer: MeOH (8:2) with reagents: paraformaldehyde (PFA), methylglyoxal (MGO), L-cysteine (Cys), oxidized cysteine (Cys/H$_2$O$_2$), hydrogen peroxide (H$_2$O$_2$), potassium superoxide (KO$_2$), and hydroxyl radical (Fe$^{2+}$/H$_2$O$_2$). (B) Mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium showing cleavage after being subjected to a stimulus (left), and the mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium (right).
Fig. S19. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the biphenyl-enamine-phosphonium incubated with different metal ions in H₂O: MeOH (8:2). (B) Mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium showing cleavage after incubation with different metals (left), and the mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium (right). Phosphonium reagent in parent form or non-hydrated (green circles) or phosphonium reagent in hydrated form (blue rectangles).
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Fig. S21. ESI-MS analysis of a reaction mixture of modification of amino-capped lysozyme with (formylmethyl)triphenylphosphonium chloride. (A) General reaction scheme showing the addition of NHS ester to the amine moiety of lysozyme (first step), followed by the amino-capped lysozyme subjected to conditions with or without formyl-phosphonium. (B) MS spectra of analysis of the reactions of the amino-capped lysozyme modification in the absence (top) or presence (bottom) of the phosphonium reagent. A number of oxidized species (+16) were observed in the mass spectra after the NHS ester labeling process, but not after the phosphonium labeling process.

Fig. S22. Mass spectrometry (MS) analysis of reaction mixtures of modification of lysozyme with (formylmethyl)triphenylphosphonium chloride in 1-butyl-3-methylimidazolium acetate (BMIM OAc, middle) and in tributyl(ethyl)phosphonium diethyl phosphate (TBEP, bottom).
Fig. S23. $^1$H NMR spectrum of the commercially 1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate (BMPy OTf) in DMSO-$d_6$. The spectrum is virtually identical to the previous reported one, and no major impurity peaks were observed.\(^1\)
General Information

Material and reagents
All chemicals including peptides and proteins were purchased from commercial suppliers unless otherwise noted. All the chemical synthesis were performed under air unless otherwise noted. Ionic liquid, 1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate (BMPy OTf) was purchased from Synthonix (B52266) of which purity was confirmed by $^1$H NMR (Fig. S23). (formylmethyl)triphenylphosphonium chloride was purchased from Tokyo Chemical Industry (F0331). Acetophenone-phosphonium (compound 5, Fig 1D) was synthesized according to a previous report.$^2$
Instrumentation

NMR

NMR was performed on Bruker AVANCE NEO 500 and 700.

LC-MS

LC-MS analysis was performed on Thermo Vanquish LC system and LTQ-XL linear ion trap MS system. A C18 reverse-phase column (Hypersil Gold 25003-032130, particle size 3 µm, diameter: 2.1 mm, length 30 mm) was used for analysis of small molecules and peptides by using 280-nm UV detection unless otherwise noted. The flow rate was 0.4 mL/min with the gradient of acetonitrile (10–90% for 3.5 min, and then 90% for 1.5 min) in the presence of 0.1% formic acid. A phenyl reverse-phase column (MAbPac 088648, particle size 4 µm, diameter 2.1 mm, length 50 mm) was used for analysis of proteins by using positive MS ion detection. The flow rate was 0.2 mL/min with the gradient of acetonitrile (10–90% for 3.5 min, and then 90% for 1.5 min) in presence of 0.1% formic acid. Deconvolution of the protein mass spectra was performed by Promass.

The conversion of the LC-MS-based experiments shown in the manuscript was calculated by dividing the product peak area by the sum of the product peak area and starting material peak area.

Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) for peptide substrates were performed on Thermo Vanquish LC system and LTQ-XL linear ion trap MS system with the same setup described in LC-MS.

Circular Dichroism

Spectral data was measured from 200-300 nm using a JASCO J-1500 spectrometer at ambient temperature under the following parameters: data pitch = 0.2 nm; CD scale = 200 mdeg/0.1 dOD; DIT = 2 sec; bandwidth = 1.00 nm; scanning speed = 100 nm/min; accumulations = 3.
Experimental procedures

Typical peptide modification procedure in ionic liquid.

To BMPy OTf (typically 30–40 µL scale) in a 1.7-mL Eppendorf tube, potassium carbonate aqueous solution (20 mM final concn from 2 M stock solution), aqueous solution of peptide (0.05-0.2 mM final concn from 2-5 mM stock solution in H2O), and (formylmethyl)triphenylphosphonium chloride (10 mM final concn from 250 mM stock solution in DMSO) were added. The final concn of H2O was kept lower than 6% v/v. The reaction mixture was incubated at 50 °C for 1 h and subjected to Post-reaction cleanup process for peptide modification before LCMS analysis.

Post-reaction cleanup process for peptide modification.

To the reaction mixture (40 µL) in a 1.7-mL Eppendorf tube, a mixture of 1:1 cold acetone/toluene (600 µL) was added in one portion. The mixture was mixed by upside-down shaking and set at -80 °C overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/toluene was removed. The pellet was air-dried on the bench at rt for 15 min. The samples were washed by an additional cycle of acetone addition and centrifugation. The pellet was air-dried on the bench again at rt for 15 min after removing the final acetone solution and then reconstituted with 60 µL H2O and analyzed by LCMS.

General procedure for protein modification in ionic liquid

To BMPy OTf (typically 30–40 µL scale) in a 1.7-mL Eppendorf tube, potassium carbonate aqueous solution (20 mM final concn from 2 M stock solution), aqueous solution of protein (0.05-0.15 mM final concn from 0.5-2 mM pH 7.4 MES buffer), and (formylmethyl)triphenylphosphonium chloride (10 mM final concn from 250 mM stock solution in DMSO) were added. The final concn of H2O was kept lower than 6% v/v. The reaction mixture was incubated at 37 °C for 3 h and subjected to Post-reaction cleanup process for protein modification before analysis.

Post-reaction cleanup process for protein modification.

To the reaction mixture of lysozyme (40 µL) in a 1.7-mL Eppendorf tube, a mixture of 5:1 cold acetone/methanol (600 µL) was added in one portion. The mixture was mixed by upside-down shaking and set at -80 °C for 1-2 h. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/methanol was removed. The sample was washed by an additional cycle of methanol addition and centrifugation. The pellet was air-dried on the bench again at rt for 15 min after removing the final methanol solution and then reconstituted with 40 µL NMM buffer (50 mM, pH 7.4) and analyzed by LCMS.

To the reaction mixture of streptavidin (30 µL), 9 µL of 5% H2SO4 aqueous solution was added before the addition of cold acetone (600 µL). The mixture was mixed by upside-down shaking and set at -80 °C for 1-2 h. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone was removed. The sample was washed by an additional cycle of acetone addition and centrifugation. The pellet was air-dried on the bench at rt for 15 min after removing the final acetone solution and then reconstituted with 150 µL NMM buffer (5 mM, pH 7.0) and analyzed by LCMS.

Synthesis of NHS ester and capping amine of lysozyme procedure.

The NHS ester was prepared following a reported protocol.³ To DMF (1212 µL), N,N-Dimethylglycine (12.5 mg, 100 mM) and N,N,N'N'-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU-36.5 mg, 100 mM) were added and sonicated for 5 min, followed by the addition of triethylamine (25.3 µL). The reaction mixture was stirred at rt for 30 min, and the generated NHS ester was used without further purification.
To NMM buffer (5mM-100 µL scale) in a 1.7-mL Eppendorf tube, aqueous solution of lysozyme (0.1 mM final conc from 2 mM stock NMM buffer), and NHS ester (5 mM final concn from 100 mM stock solution in DMF) were added. The reaction mixture was incubated at rt for 1 h. and subjected to a post-reaction cleanup process, where a mixture of 5:1 cold acetone/methanol (600 µL) was added in one portion. The mixture was mixed by upside-down shaking and set at −80 °C for overnight. The precipitates were collected by centrifugation (15,000 rcf, 15 min, 4 °C), and acetone/methanol was removed. The pellet was air-dried on the bench at rt for 15 min and then reconstituted with 5 µL NMM buffer (5 mM, pH 7.0) and analyzed by LCMS.

**Linkage Stability experiment procedure.**

To an 8:2 mixture of either H$_2$O or (NH$_4$)$_2$CO$_3$ (5 mM) buffer (31 uL) and MeOH (8 uL), biphenyl-enamine-phosphonium (0.05 mM final concn from 5 mM stock solution in 1:1:1 H$_2$O/MeOH/DMSO), reagent (such as ROS, cellular aldehydes, oxidized sulfur, and metal ions 0.5 mM final concn from 25 mM stock solution in either H$_2$O or DMSO) were added (40 uL total volume). The reaction mixture was analyzed by LCMS after incubation of the enamine compound with the reagent at rt for 1 h. For the non-metal reagents, the samples were injected to the LCMS system at the 1-h time point. For the metal reagents, the metal ions in the samples were removed by Cuprisorb (see below) at the 1-h time point, and then, the samples were analyzed by LCMS.

**Procedure to prepare Cuprisorb and removal of metals by Cuprisorb**

**Preparation of Cuprisorb (Seachem, FM-SC120-1).** Cuprisorb (200 mg) was washed with H$_2$O (3 × 1 mL) in a 1.7-mL Eppendorf tube, and the supernatant was discarded after each wash. The washed Cuprisorb was suspended in H$_2$O (200 uL).

**Removal of metals by Cuprisorb.** The washed Cuprisorb suspended in water (10 uL) was transferred to another Eppendorf tube. The supernatant in the other tube was discarded, and the reaction mixture containing metal samples was added. After incubation of the reaction mixture and with Cuprisorb for a couple of minutes, the supernatant was used for the LCMS analysis.
Preparative synthesis of small molecules

Organic synthesis procedure

Biphenyl-enamine-phosphonium: To CHCl₃ (6.4 mL), (formylmethyl)triphenylphosphonium chloride (10 mM final concn from 250 mM stock solution in DMSO), and 2-(4-biphenyl) ethylamine (30 mM final concn from 500 mM stock solution in acetone), were added to a 20-mL vial equipped with a magnetic stir bar. The reaction mixtures were heated at 50 °C overnight. After the reaction mixture was heated at 50 °C overnight, the formation of the product was confirmed by TLC (9:1 CH₂Cl₂/MeOH). The reaction mixture was purified by Yamazen Smart Flash W-Prep dual channel chromatography with CH₂Cl₂/MeOH (96:4) as the eluent to afford a brown-orange solid (14.0 mg, 45%) as a mixture of rotamers, confirmed by NOESY NMR. For NMR purpose, the purification process was repeated for the recovered product to get a pure compound with CH₂Cl₂/MeOH (94:6) as the eluent. NMR spectra of the product are available in the Supporting figures section. ¹H NMR (700 MHz, CD₃OD, mixture of rotamers): δ 7.83-7.19 (m, 24H), 6.77-5.71 (m, 1H), 4.74-4.57 (m, 1H), 3.71-3.37 (m, 2H), 3.05-2.78 (m, 2H). ¹³C-NMR (700 MHz, CD₃OD, mixture of rotamers): δ 155.72, 141.9, 140.7, 139.4, 135.34, 135.32, 135.27, 135.26, 134.7, 134.6, 134.58, 134.52, 134.45, 131.4, 131.02, 130.95, 130.9, 130.74, 130.7, 129.96, 129.9, 128.5, 128.3, 128.1, 127.8, 127.6, 124.06, 123.96, 123.54, 123.44, 73.5, 62.3, 51.3, 45.4, 38.1, 35.5. IR: 3174. HRMS-ESI (m/z) [M⁺] calcd for C₃₄H₂₁NP, 484.21886; found 484.21805.
High resolution mass spectrometry (HRMS) spectra of the synthesized compound.

Fig. S2. HRMS-ESI spectra of the biphenyl-enamine-phosphonium.
References for Supplementary Information