Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Iron-Sensitive Protein Conjugates Formed with a Wittig Reaction Precursor in Ionic Liquid

Zeinab M. Nizam, Ashton M. Stowe, Jada K. Mckinney, and Jun Ohata*

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States.

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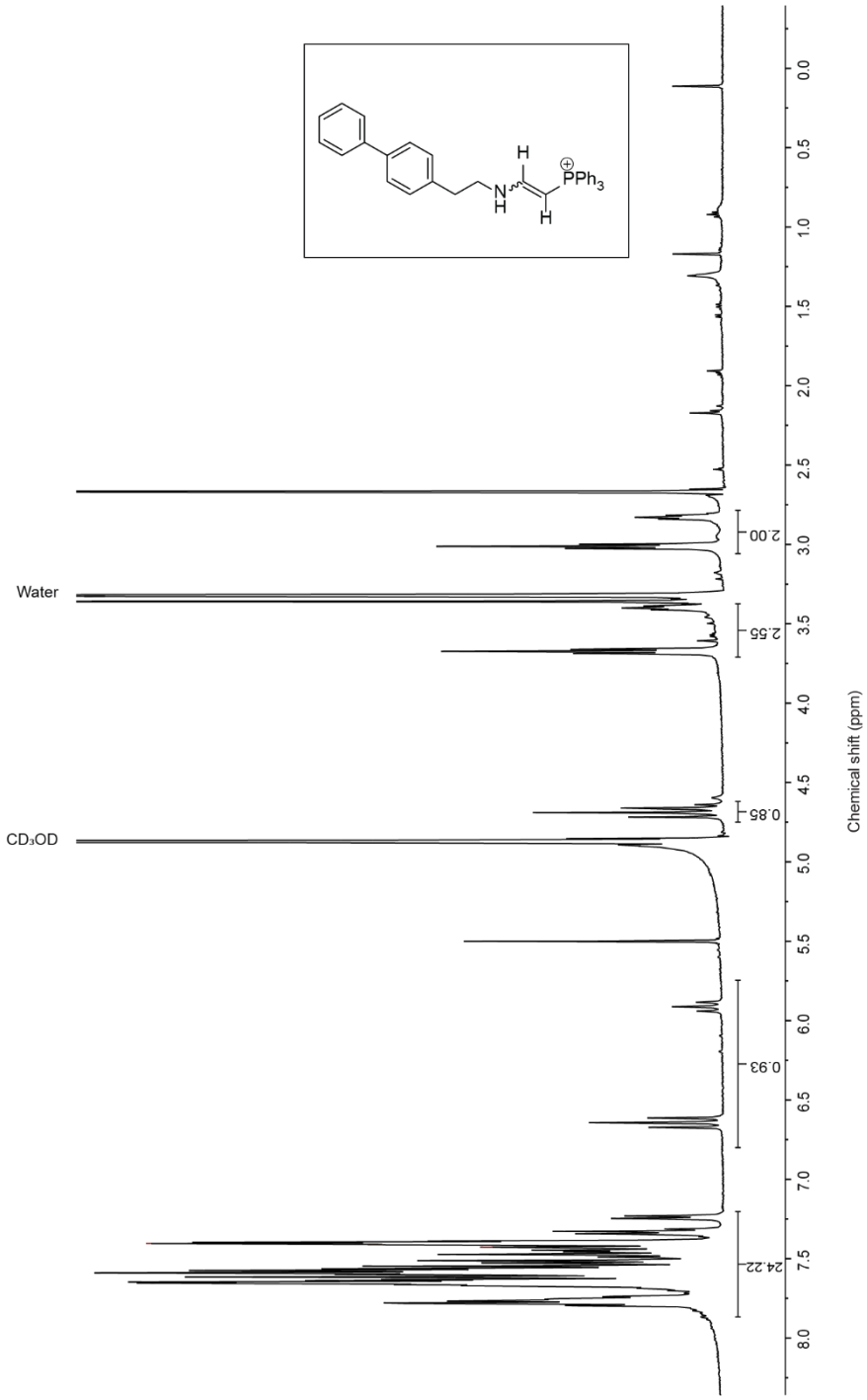


Fig. S1. ^{1}H NMR spectrum of the product of a reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

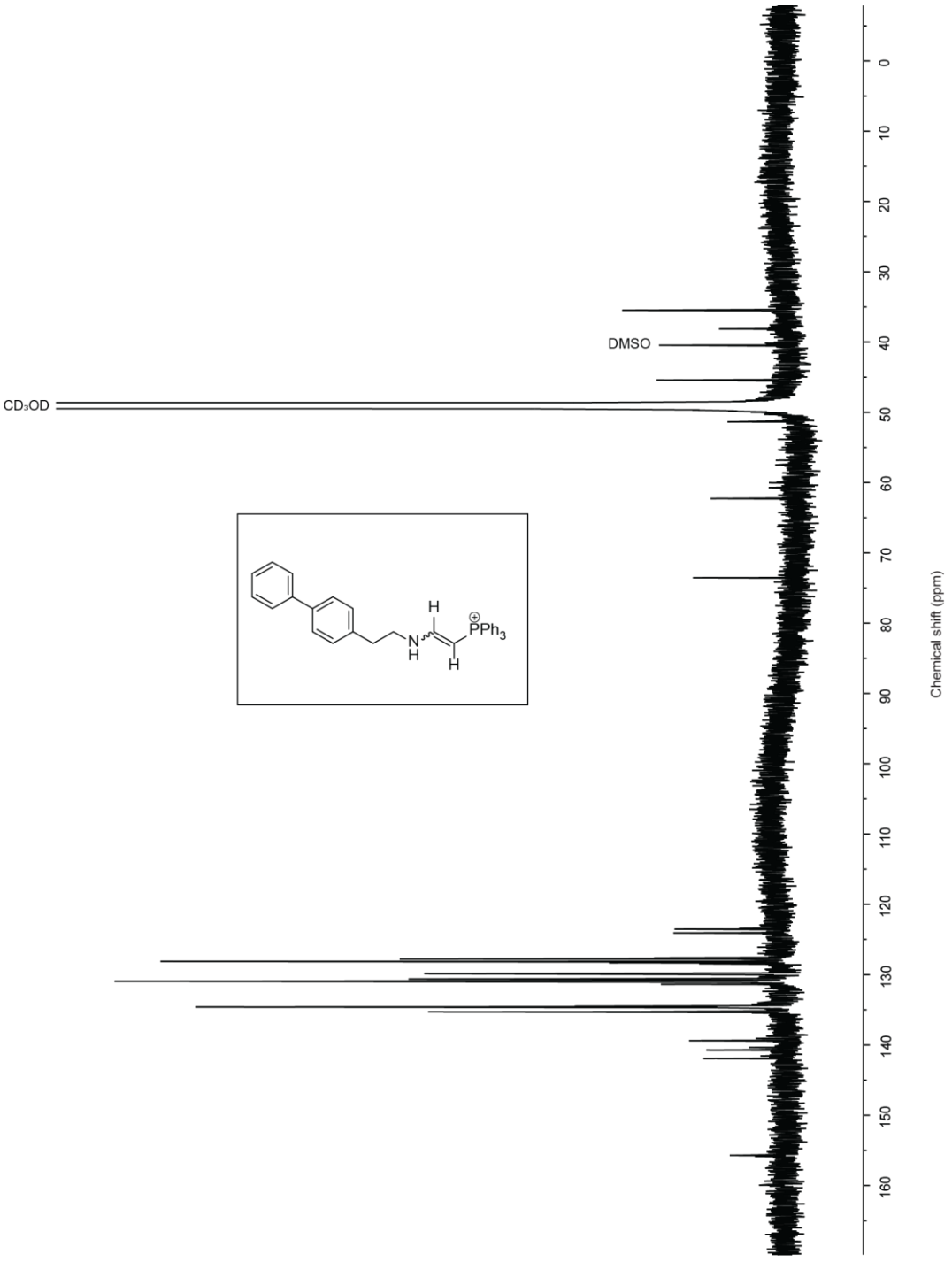


Fig. S2. ^{13}C NMR spectrum of the product of reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

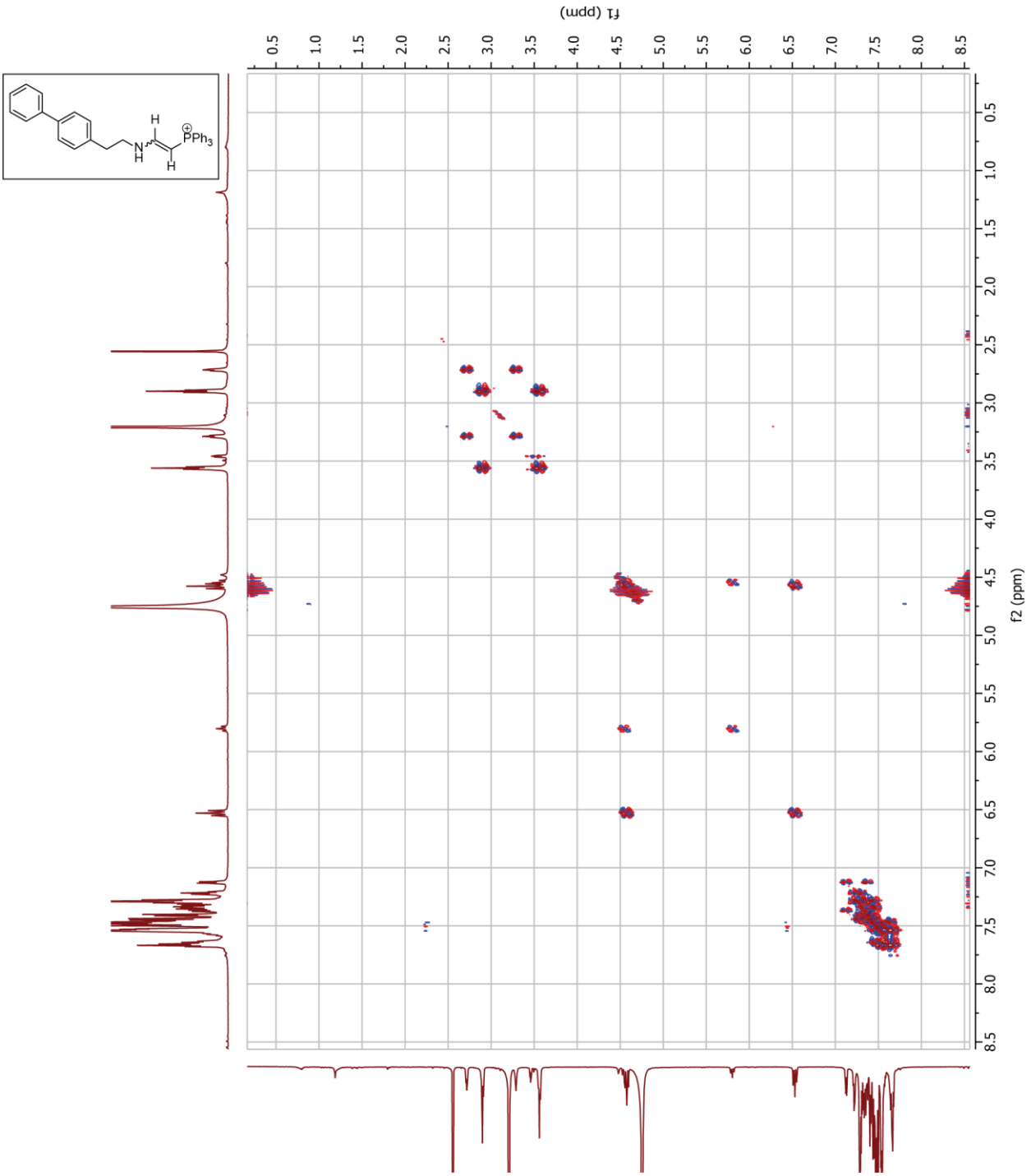


Fig. S3. ^{1}H-^{1}H COSY NMR spectrum of the product of a reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

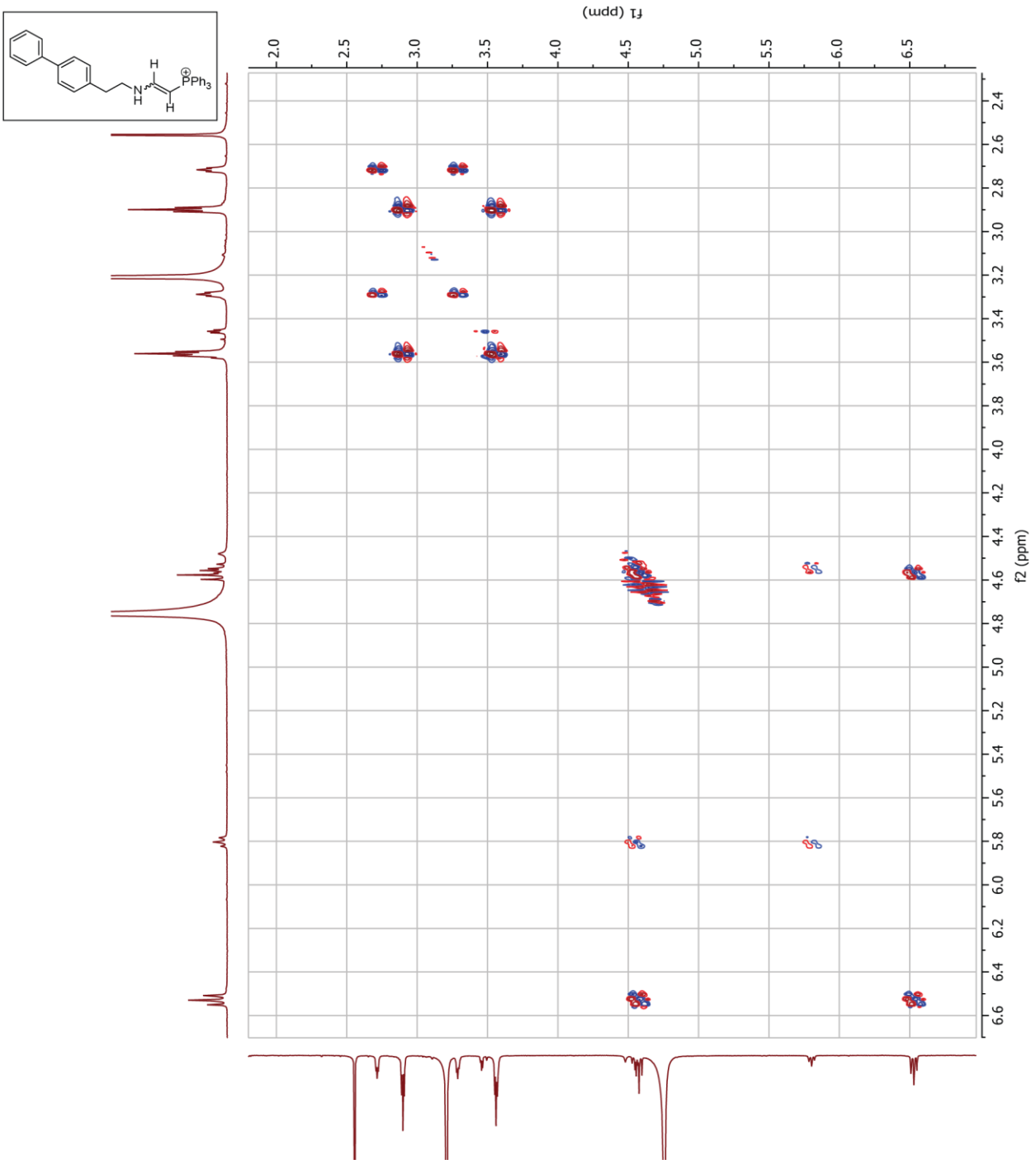


Fig. S4. Zoomed ^{1}H-^{1}H COSY NMR spectrum the product of a reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

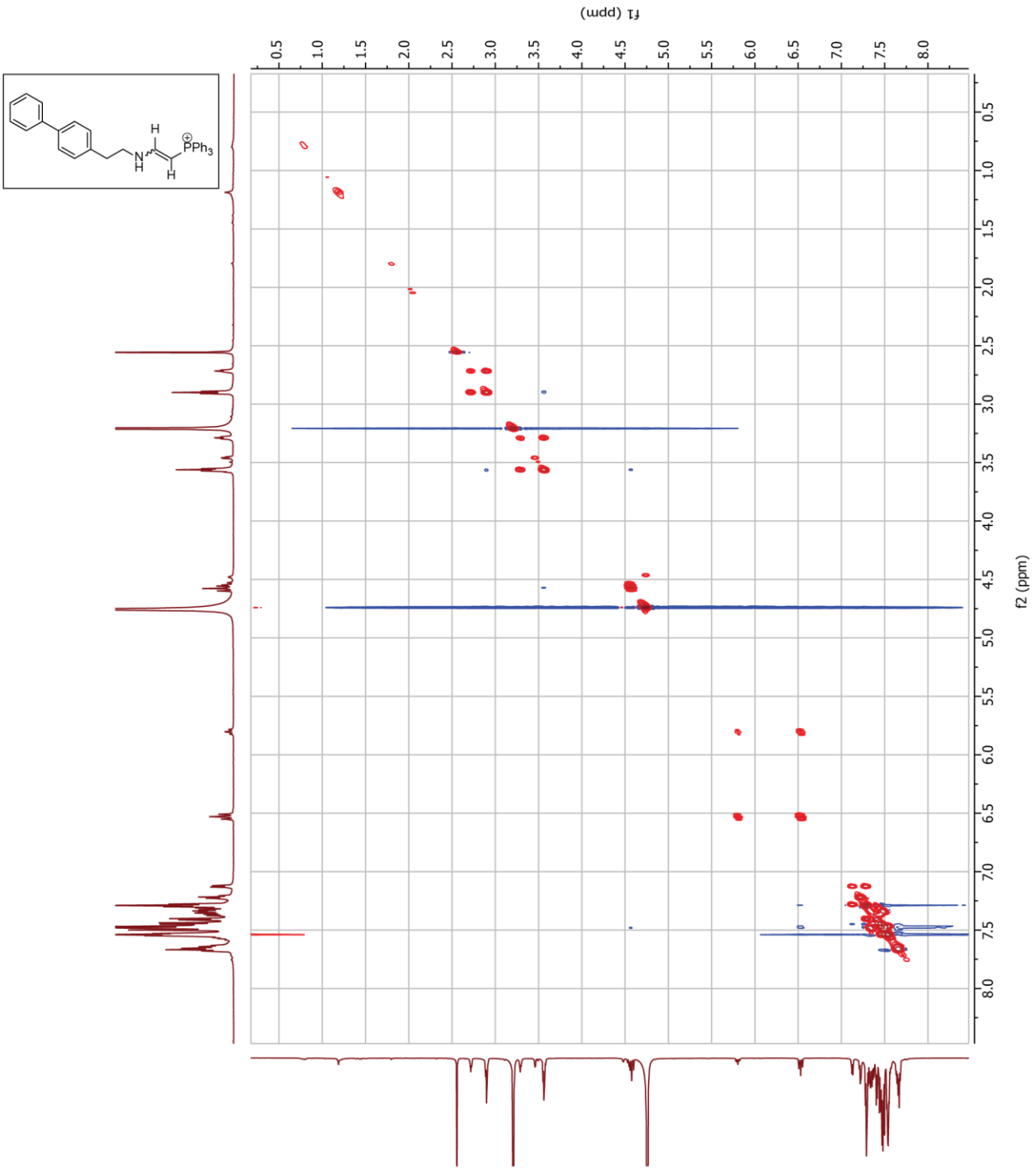


Fig. S5. ^{1}H-^{1}H NOESY NMR spectrum of the product of a reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

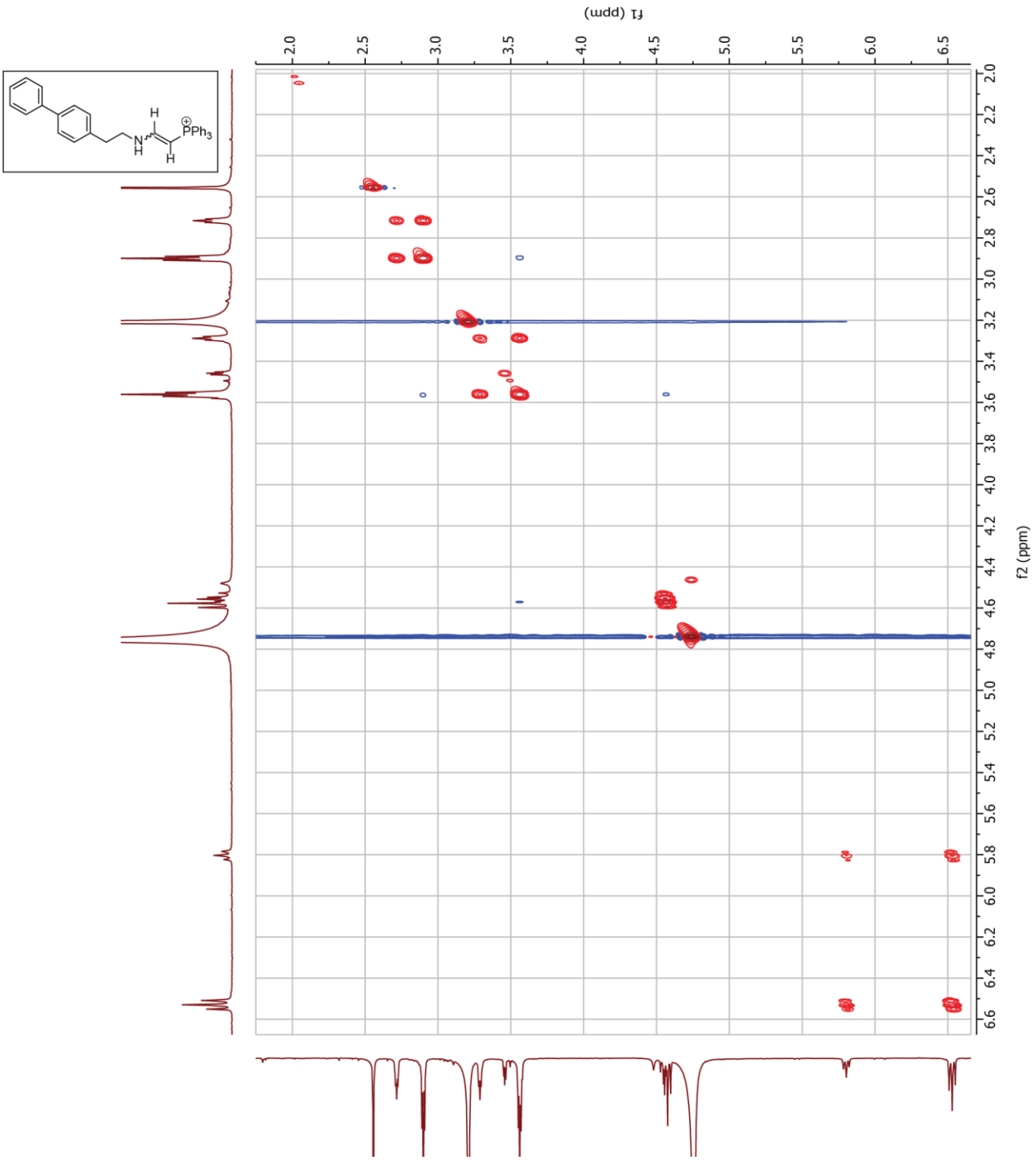


Fig.S6 Zoomed ^{1}H-^{1}H NOESY NMR spectrum of the product of a reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

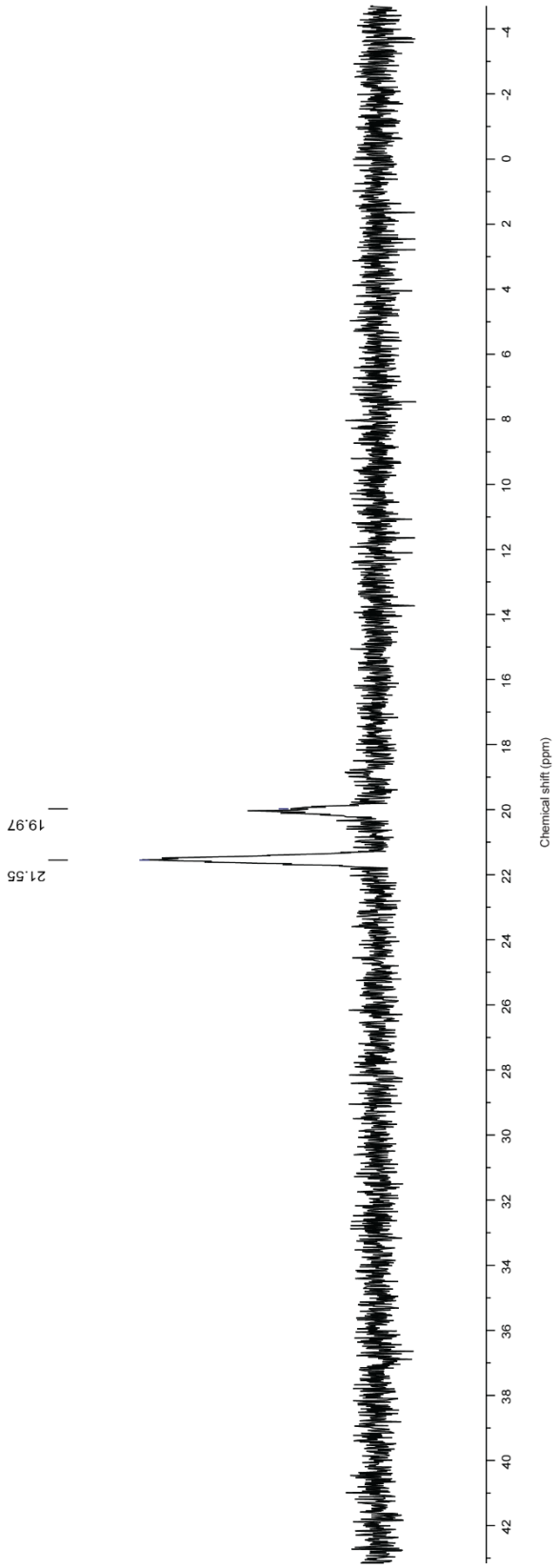


Fig.S7. ^{31}P NMR spectrum of the product of reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

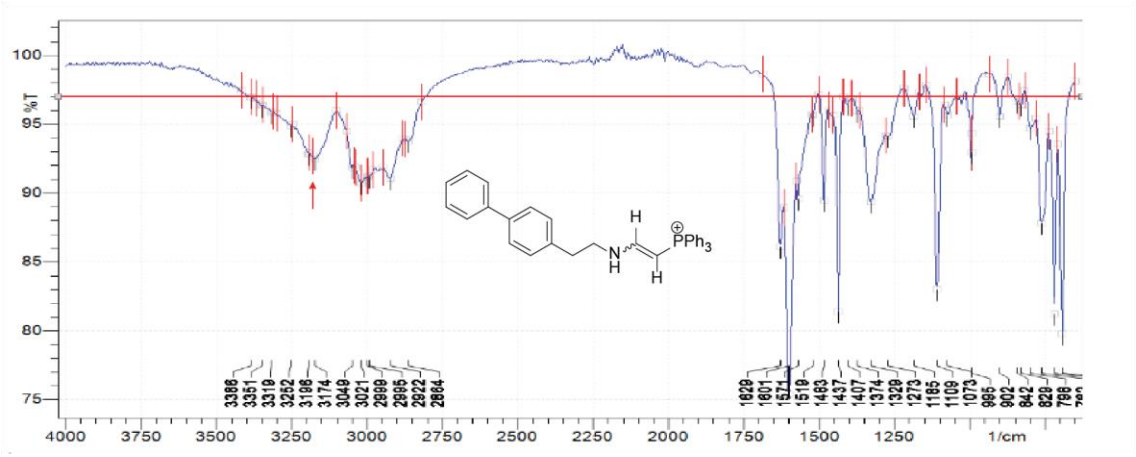


Fig. S8. Infrared (IR) spectrum of the product of a reaction between 2-(4-biphenyl)ethylamine and (formylmethyl)triphenylphosphonium chloride in CD_3OD.

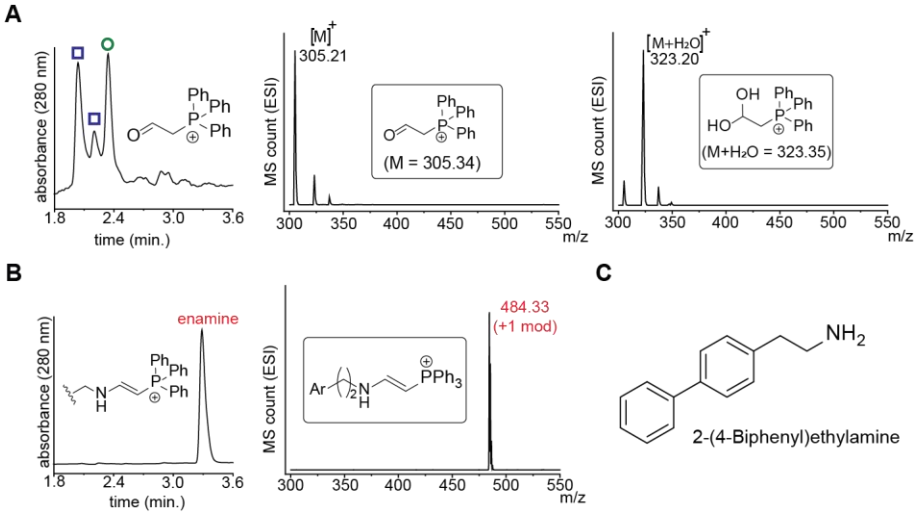


Fig. S9. LC-MS analysis of the reaction mixture of 2-(4-biphenyl)ethylamine modification with (formylmethyl)triphenylphosphonium chloride. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the phosphonium reagent, non-hydrated form (middle), and hydrated form (right). (B) Liquid-chromatography mass spectrometry (LC-MS) analysis of the formed enamine product from the condensation reaction with an alkylamine-containing substrate. (C) Chemical structure of the model substrate. Phosphonium reagent in parent form or non-hydrated (green circles) or phosphonium reagent in hydrated form (blue rectangles).

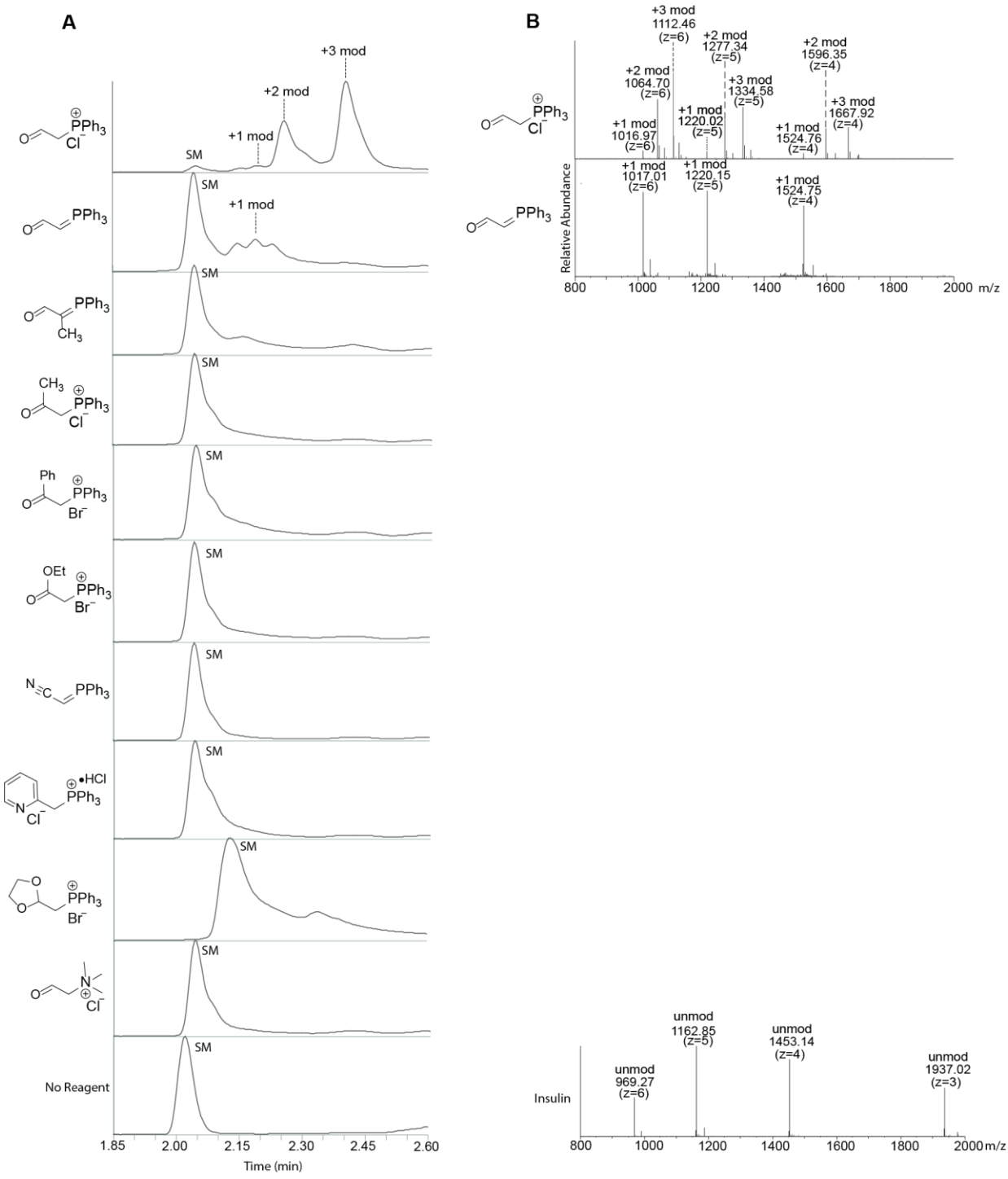


Fig. S10. Liquid chromatography-mass spectrometry (LC-MS) analysis of modification of insulin containing a single lysine residue and N-terminus amines with a variety of phosphonium derivatives possessing different moieties. (A) UV chromatograms of the reaction mixtures. (B) MS spectra of the modification (1 mod, 2 mod and 3 mod) or starting material peptide (SM).

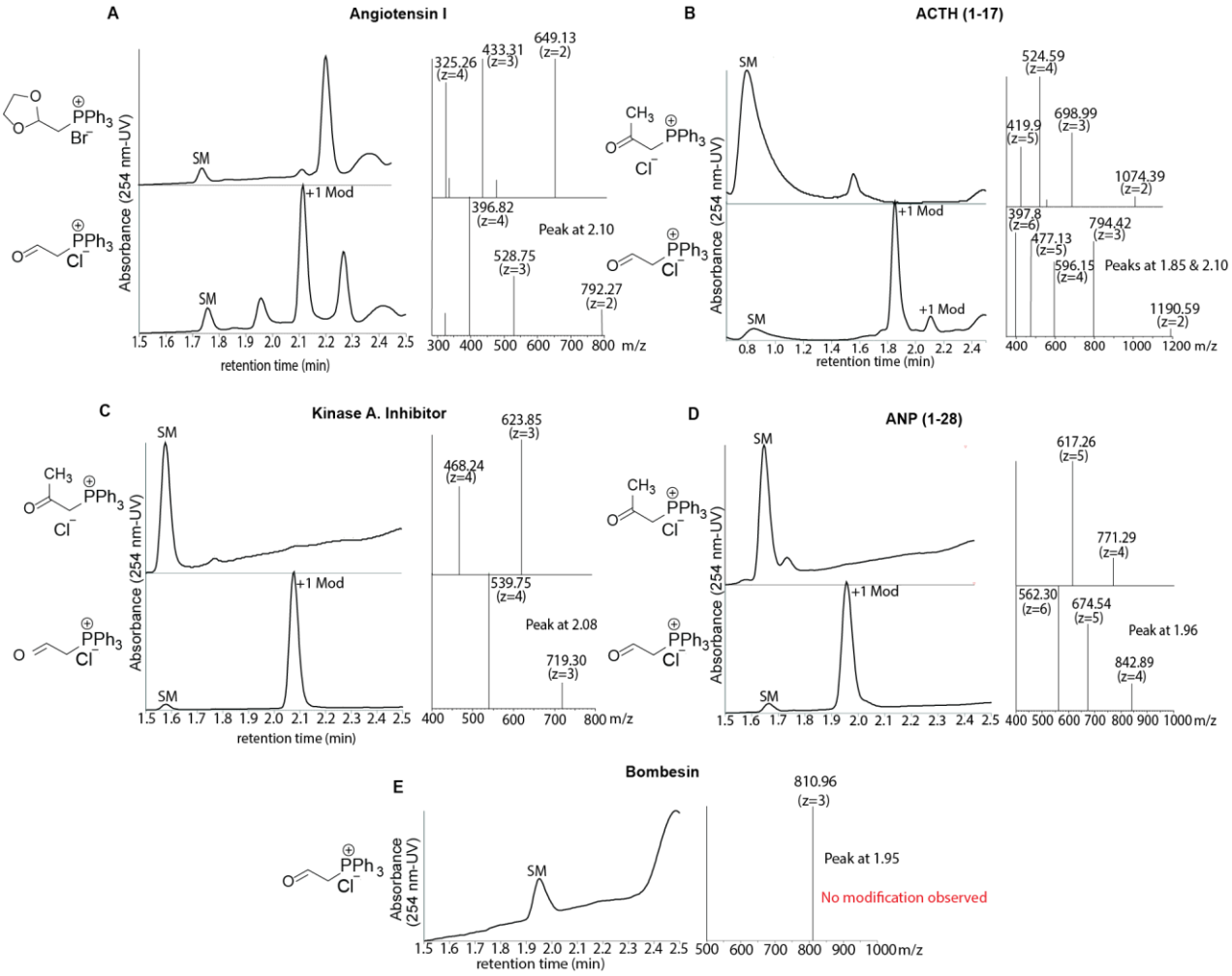


Fig. S11. Liquid-chromatography mass spectrometry (LC-MS) analysis of reaction mixtures of modification of peptide substrates. 254-nm chromatograms were used to obtain the conversions shown in Fig. 2 in the main manuscript. (A) Angiotensin I.(B) Adrenocorticotropic hormone (ACTH) 1-17. (C) Kinase A inhibitor 6-22. (D) Atrial natriuretic peptide (ANP) 1-28. (E) Bombesin, a peptide with capped N-terminus and lacks lysine residues. (1,3-Dioxolan-2-ylmethyl)triphenylphosphonium bromide and acetonyltriphenylphosphonium chloride as a negative controls.

Angiotensin I modified with Formyl-Phosphonium

y ion series		y10	y9	y8	y7	y6	y5	y4	y3	y2	y1	
mass (theoretical)		1583.685	1181.658	1025.557	926.4883	763.425	650.341	513.282	416.2293	269.1609	132.102	
	N-term											C-term
Sequence	H-	D	R	V	Y	I	H	P	F	H	L	— OH
mass (theoretical)		403.0343	559.1354	658.2038	821.2671	934.3512	1071.41	1168.463	1315.531	1452.59	1565.674	
b ion series		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	

Red: Amino acid residue modified
Blue: y ions found in the MS/MS spectrum
Green: b ions found in the MS/MS spectrum

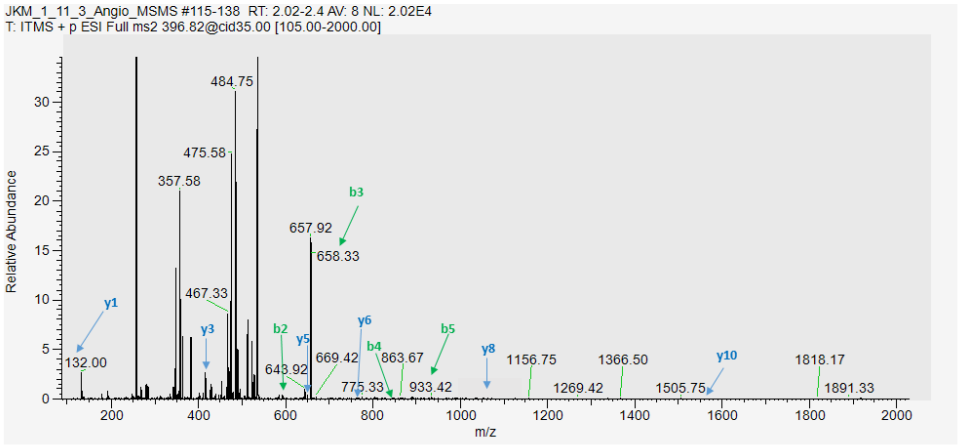


Fig. S12. Tandem mass spectrum (MS/MS) analysis of angiotensin I modified with (formylmethyl)triphenylphosphonium chloride.

y ion series		y17	y16	y15	y14	y13	y12	y11	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1	
mass (theoretical)		2155.9	1767.9	1604.8	1533.8	1418.7	1271.7	1158.5	1087.6	1000.5	943.5	787.4	686.4	629.3	473.2	317.1	203.1	132.1	
	N-term																		C-term
Sequence	H-	T	Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	— OH
mass (theoretical)		389.0	552.1	623.1	738.1	885.2	998.3	1069.3	1156.4	1213.4	1369.5	1470.5	1527.5	1683.6	1839.7	1953.8	2024.8	2137.9	
b ion series		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	

Kinase A. Inhibitor modified with Formyl-Phosphonium

Red: Amino acid residue modified
Blue: y ions found in the MS/MS spectrum
Green: b ions found in the MS/MS spectrum

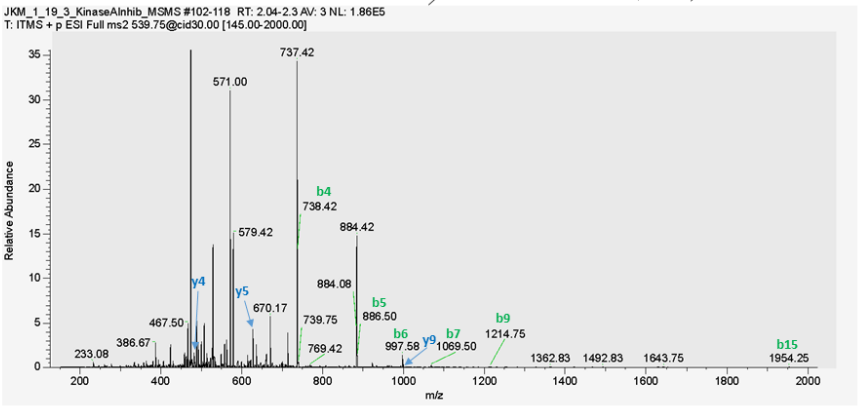


Fig. S13. Tandem mass spectrum (MS/MS) analysis of kinase Inhibitor modified with (formylmethyl)triphenylphosphonium chloride.

y ion series		y17	y16	y15	y14	y13	y12	y11	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1	
mass (theoretical)		2380.086	2006.054	1842.991	1755.959	1624.918	1495.876	1358.817	1211.748	1055.647	869.568	812.5465	684.4516	587.3988	488.3304	431.3089	303.214	175.119	
	N-term																		C-term
Sequence	H-	S	Y	S	M	E	H	F	R	W	G	K	P	V	G	K	K	R	— OH
mass (theoretical)		375.0394	538.1027	625.1347	756.1752	885.2178	1022.277	1169.345	1325.446	1511.526	1568.547	1696.642	1793.695	1892.763	1949.785	2077.88	2205.975	2362.076	
b ion series		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	

ACTH (1-17) modified with Formyl-Phosphonium

Red: Amino acid residue modified
Blue: y ions found in the MS/MS spectrum
Green: b ions found in the MS/MS spectrum

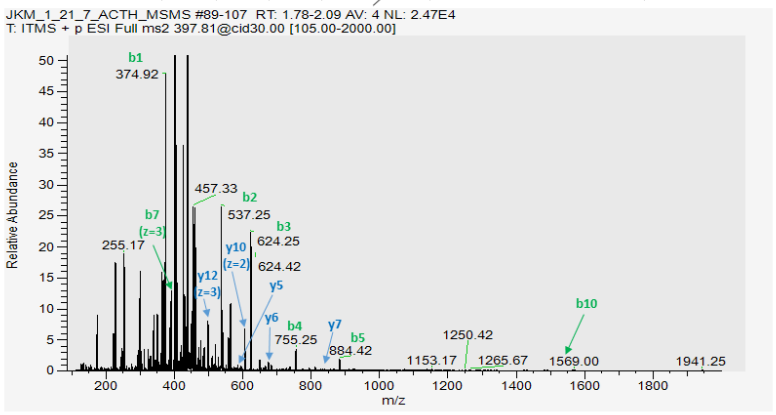


Fig. S14. Tandem mass spectrum (MS/MS) analysis of ACTH (1-17) modified with (formylmethyl)triphenylphosphonium chloride.

y ion series		y28	y27	y26	y25	y24	y23	y22	y21	y20	y19	y18	y17	y16	y15	y14	y13	y12	y11	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1	
mass (theoretical)		3368.468	2994.436	2881.352	2725.25	2569.149	2482.117	2395.085	2292.076	2145.008	2087.986	2030.965	1874.864	1743.823	1628.796	1472.695	1359.611	1302.59	1231.552	1103.494	1016.462	959.404	846.3563	789.3349	686.3257	572.2828	485.2507	338.1823	182.0812	
	N-term																													N-term
Sequence	H—	S	L	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y	— OH
mass (theoretical)		375.0394	488.1234	644.2245	800.3256	887.3577	974.3897	1077.399	1224.467	1281.489	1338.51	1494.611	1625.652	1740.679	1896.78	2009.864	2066.885	2137.923	2265.981	2353.013	2410.035	2523.119	2580.14	2683.149	2797.192	2884.224	3031.293	3187.394	3350.457	
b ion series		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25	B26	B27	B28	

ANP(1-28) modified with Formyl-Phosphonium

Red: Amino acid residue modified
Blue: y ions found in the MS/MS spectrum
Green: b ions found in the MS/MS spectrum

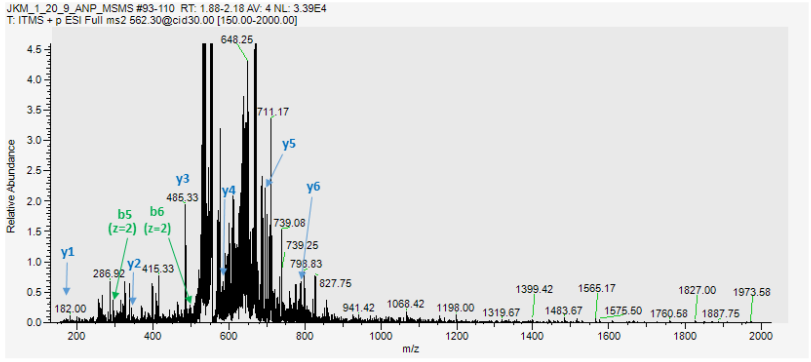


Fig. S15. Tandem mass spectrum (MS/MS) analysis of ANP (1-28) modified with (formylmethyl)triphenylphosphonium chloride.

y ion series			y21	y20	y19	y18	y17	y16	y15	y14	y13	y12	y11	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1	
mass (theoretical)			2670	2325.9	2212.9	2113.8	1984.7	1856.7	1753.7	1650.7	1549.6	1462.6	1349.5	1246.5	1159.5	1046.4	883.3	755.3	642.2	513.1	399.1	236.07	133.06	
	N-term																						N-term
Sequence	H —		G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N	— OH
mass (theoretical)			345.02	458.1	557.1	686.2	814.2	917.2	1020.3	1121.3	1208.3	1321.4	1424.4	1511.5	1624.5	1787.6	1915.7	2028.7	2157.8	2271.8	2434.9	2537.9	2651.9	
b ion series			B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	

Insulin Chain A modified with Formyl-Phosphonium

Red: Amino acid residue modified
Blue: y ions found in the MS/MS spectrum
Green: b ions found in the MS/MS spectrum

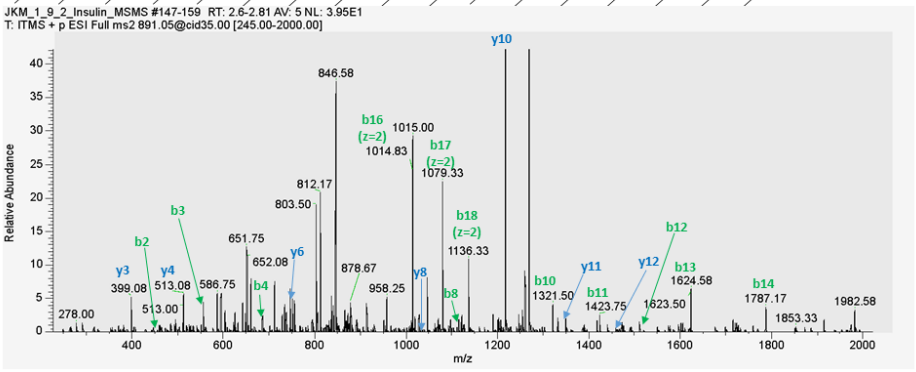


Fig.S16. Tandem mass spectrum (MS/MS) analysis of insulin (Chain A) modified with (formylmethyl)triphenylphosphonium chloride.

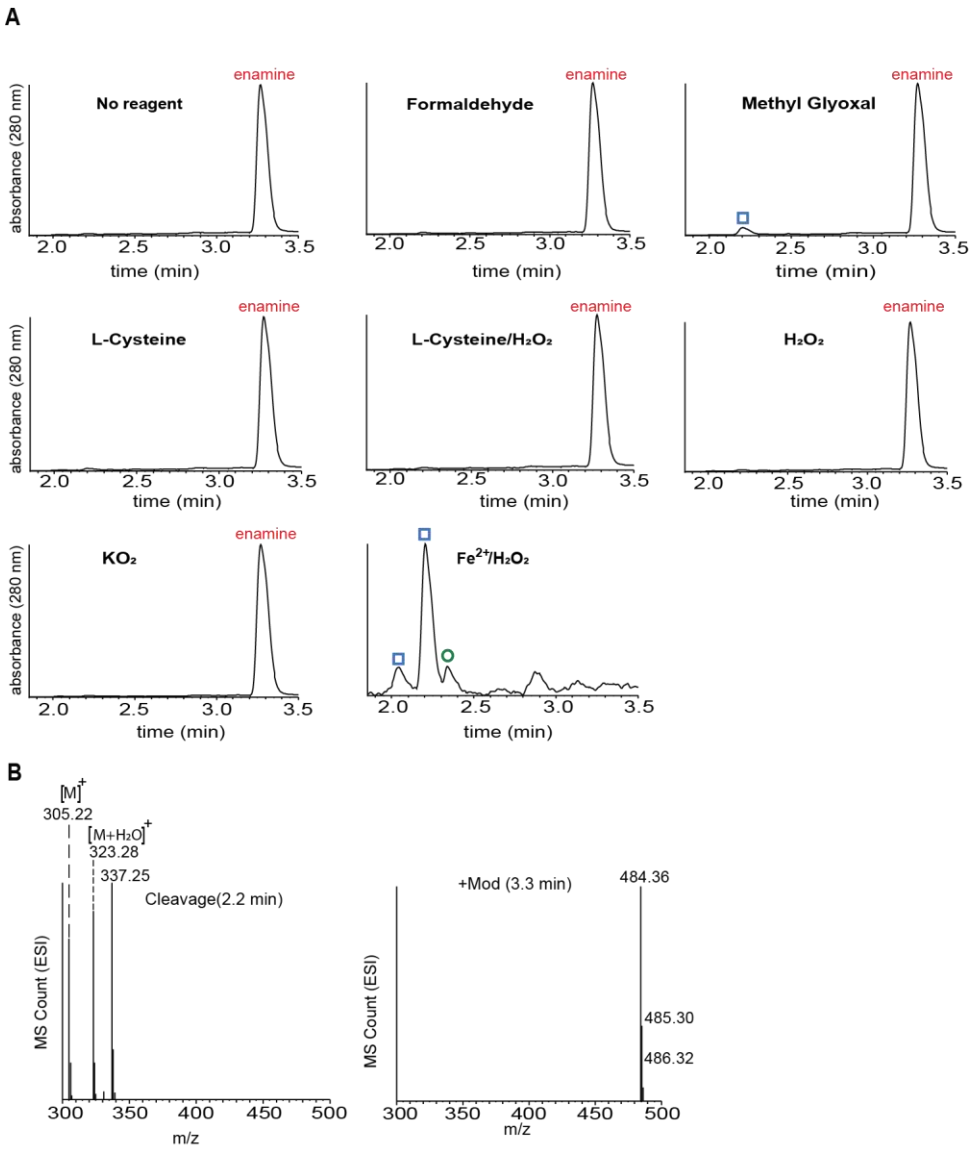


Fig. S17. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the biphenyl-enamine-phosphonium incubated in H_2O: MeOH (8:2) with reagents: paraformaldehyde (PFA), methylglyoxal (MGO), L-cysteine (Cys), oxidized cysteine (Cys/H_2O_2), hydrogen peroxide (H_2O_2), potassium superoxide (KO_2), and hydroxyl radical (Fe^{2+}/H_2O_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). Phosphonium reagent in parent form or non-hydrated (green circles) or phosphonium reagent in hydrated form (blue rectangles).

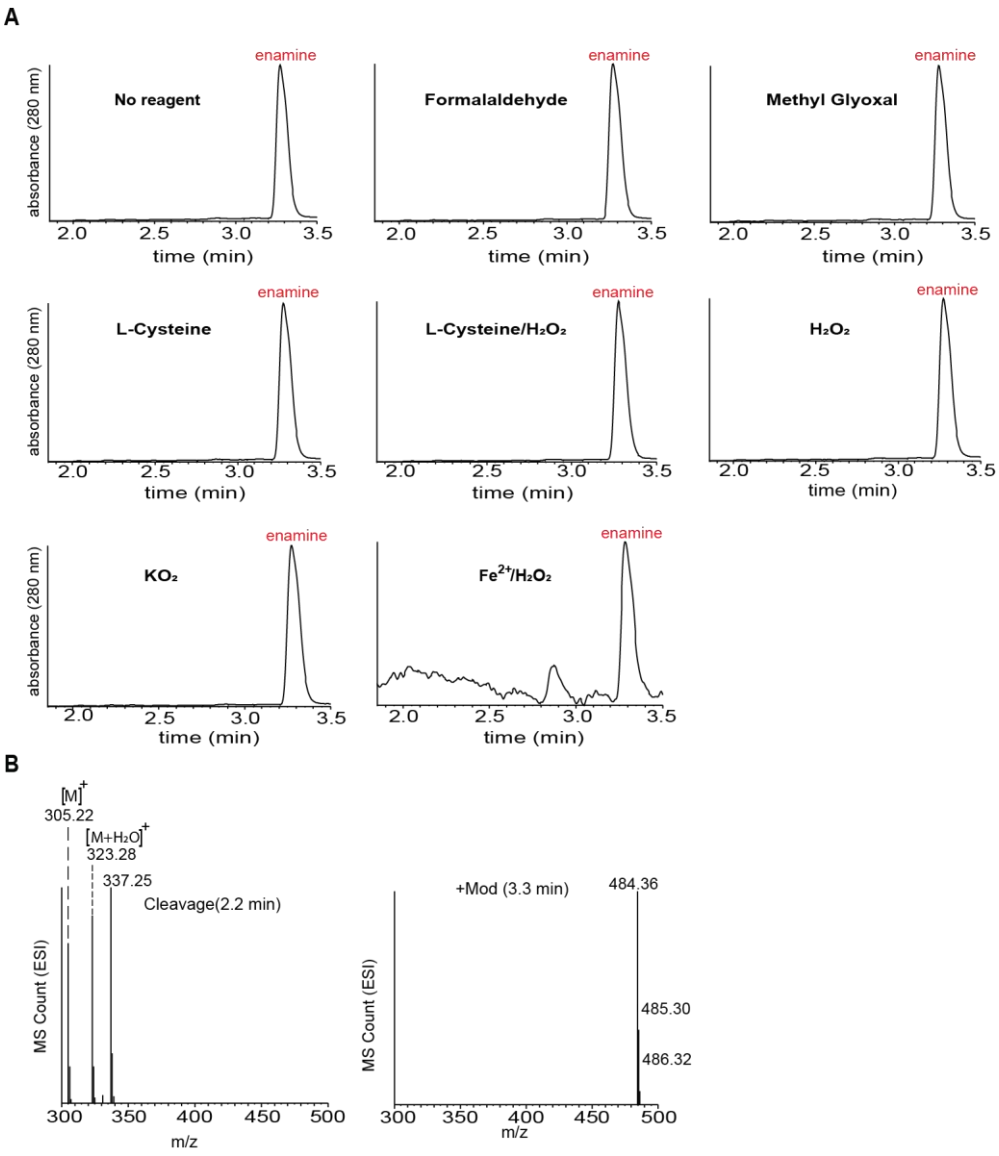


Fig. S18. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the biphenyl-enamine-phosphonium incubated in $(NH_4)_2CO_3$ buffer: MeOH (8:2) with reagents: paraformaldehyde (PFA), methylglyoxal (MGO), L-cysteine (Cys), oxidized cysteine (Cys/H_2O_2), hydrogen peroxide (H_2O_2), potassium superoxide (KO_2), and hydroxyl radical (Fe^{2+}/H_2O_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).

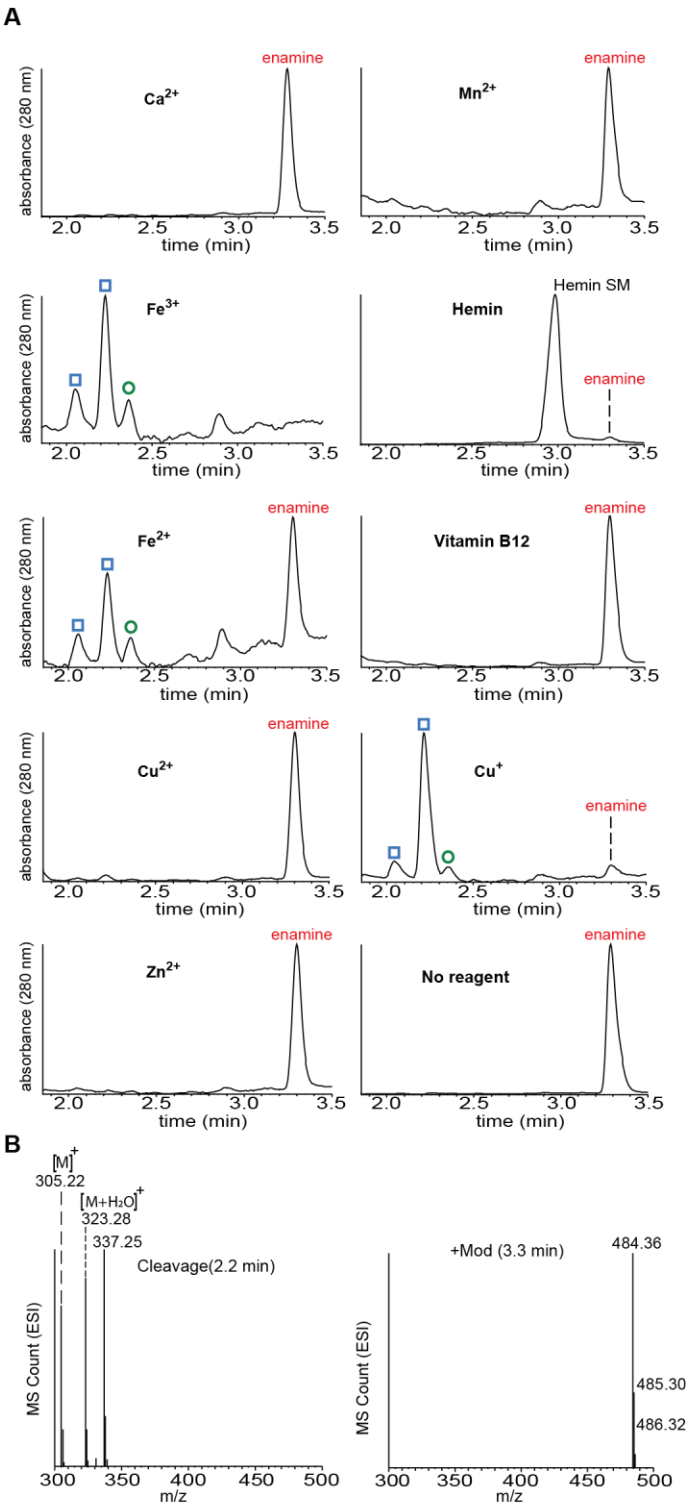


Fig. S19. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the biphenyl-enamine-phosphonium incubated with different metal ions in H_2O: MeOH (8:2). (B) Mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium showing cleavage after incubation with different metals (left), and 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).

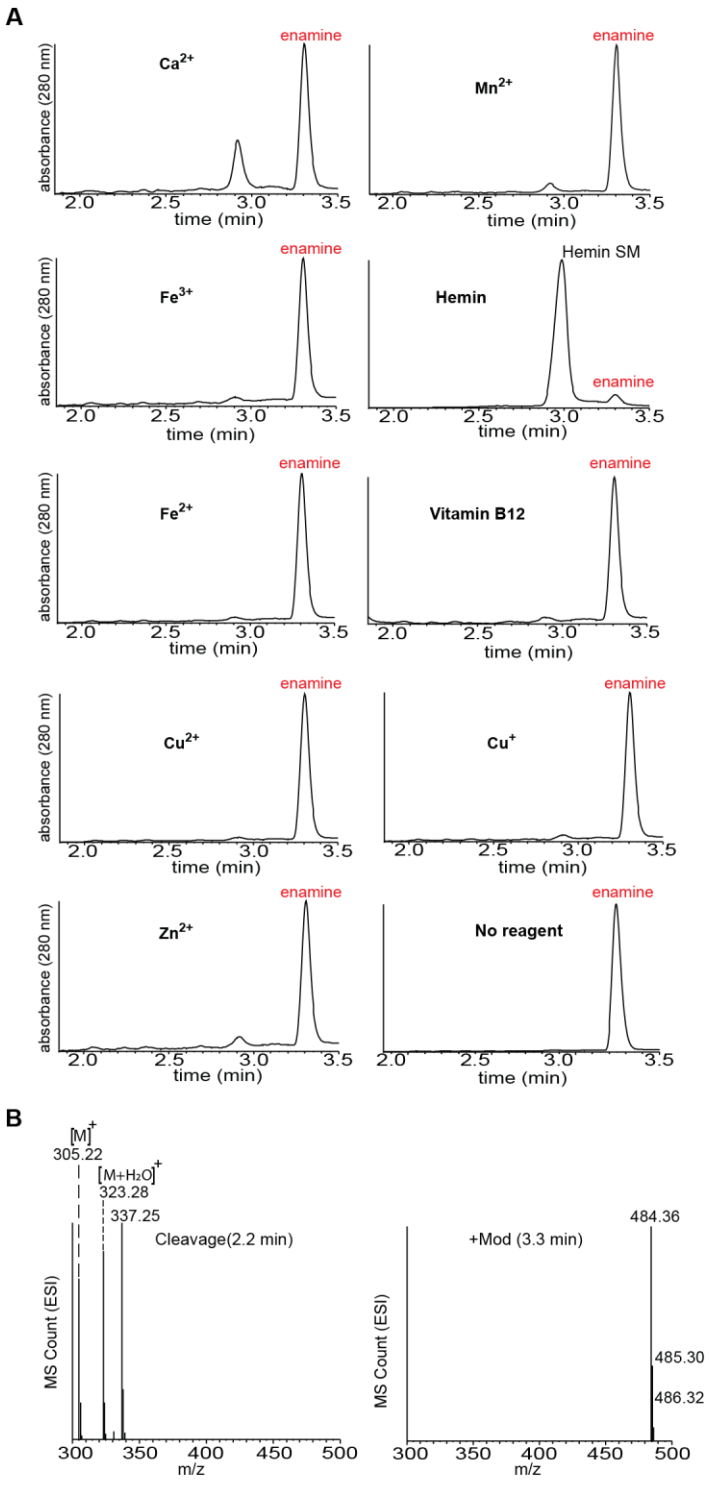


Fig. S20. (A) Liquid-chromatography mass spectrometry (LC-MS) analysis of the biphenyl-enamine-phosphonium incubated with different metal ions in $(NH_4)_2CO_3$ buffer: MeOH (8:2). (B) Mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium showing cleavage after incubation with different metals (left), and the mass spectrometry (MS) analysis of the biphenyl-enamine-phosphonium (right).

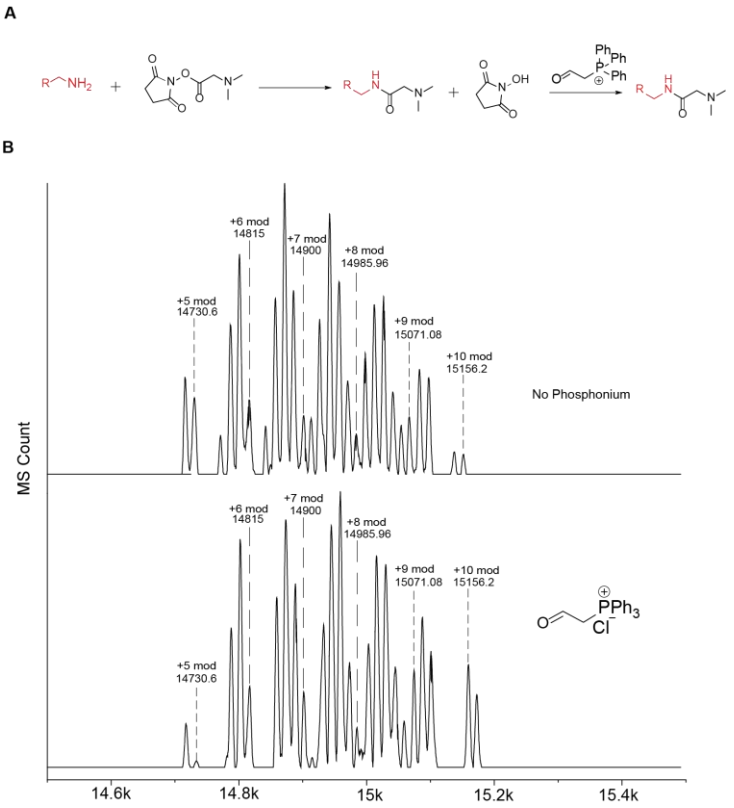


Fig. S21. ESI-MS analysis of a reaction mixture of modification of amino-capped lysozyme with (formylmethyl)triphenylphosphonium chloride. (A) General reaction scheme showing the addition of NHS ester to the amine moiety of lysozyme (first step), followed by the amino-capped lysozyme subjected to conditions 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.

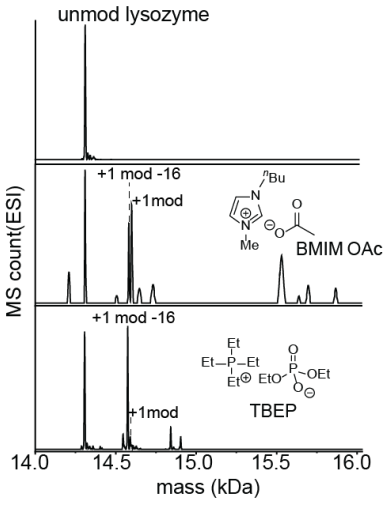


Fig. S22. Mass spectrometry (MS) analysis of reaction mixtures of modification of lysozyme with (formylmethyl)triphenylphosphonium chloride in 1-butyl-3-methylimidazolium acetate (BMIM OAc, middle) and in tributyl(ethyl)phosphonium diethyl phosphate (TBEP, bottom).

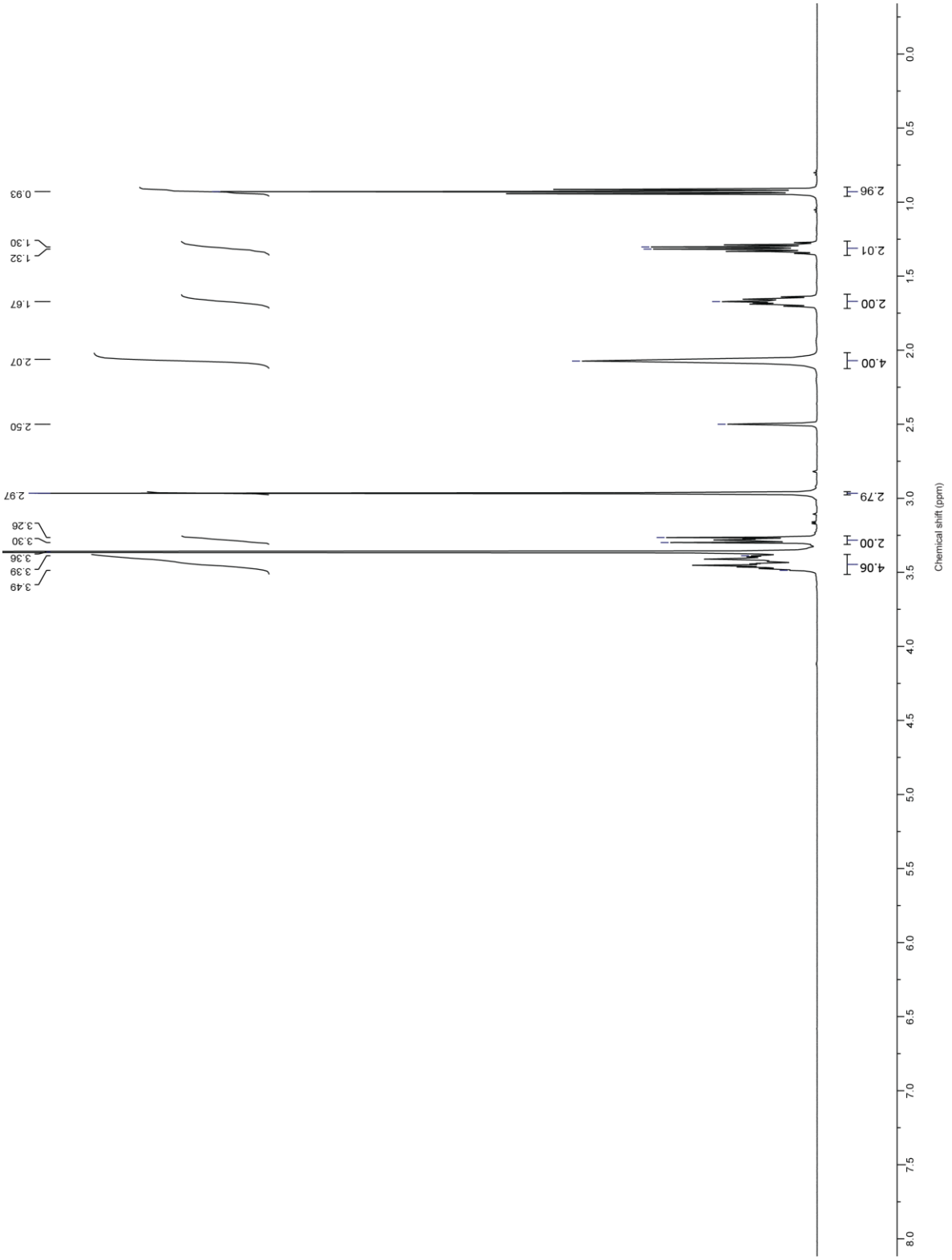


Fig. S23. [1]H NMR spectrum of the commercially 1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate (BMPy OTf) in DMSO-d_6. The spectrum is virtually identical to the previous reported one, and no major impurity peaks were observed.[1]

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 conc from 2-5 mM stock solution in H_2O), and (formylmethyl)triphenylphosphonium chloride (10 mM final conc from 250 mM stock solution in DMSO) were added. The final concn of H_2O 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 H_2O 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 conc from 0.5-2 mM pH 7.4 MES buffer), and (formylmethyl)triphenylphosphonium chloride (10 mM final conc from 250 mM stock solution in DMSO) were added. The final concn of H_2O 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% H_2SO_4 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_2O or $(NH_4)_2CO_3$ (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_2O/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_2O 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.

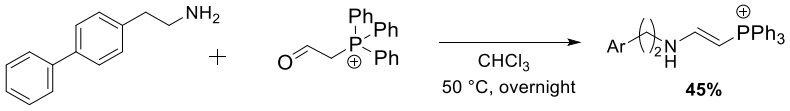
Procedure to prepare Cuprisorb and removal of metals by Cuprisorb

Preparation of Cuprisorb (Seachem, FM-SC120-1). Cuprisorb (200 mg) was washed with H_2O (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_2O (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 $CHCl_3$ (6.4 mL), (formylmethyl)triphenylphosphonium chloride (10 mM final concn from 250mM stock solution in DMSO), and 2-(4-biphenyl) ethylamine (30 mM final concn from 500mM 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 CH_2Cl_2/MeOH). The reaction mixture was purified by Yamazen Smart Flash W-Prep dual channel chromatography with CH_2Cl_2 / 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.[4] For NMR purpose, the purification process was repeated for the recovered product to get a pure compound with CH_2Cl_2 / MeOH (94:6) as the eluent**.** NMR spectra of the product are available in the Supporting figures section. ^{1}H NMR (700 MHz, CD_3OD, 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).^{13}C-NMR (700 MHz, CD_3OD, 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 $C_{34}H_{31}NP$, 484.21886; found 484.21805.

High resolution mass spectrometry (HRMS) spectra of the synthesized compound.

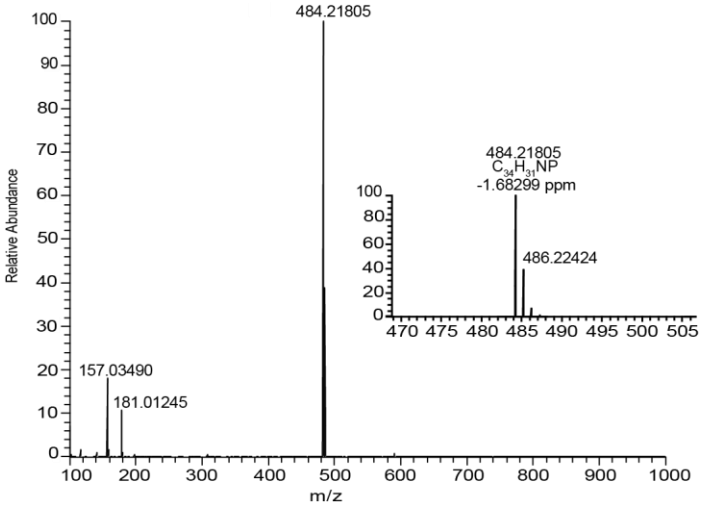


Fig. S22. HRMS-ESI spectra of the biphenyl-enamine-phosphonium.

References for Supplementary Information

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