Palladium–Protein Oxidative Addition Complexes by Amine-Selective Acylation

Heemal H. Dhanjee[†], Ivan Buslov[†], Ian W. Windsor, Ronald T. Raines, Bradley L. Pentelute,* Stephen L. Buchwald*

Correspondence to: sbuchwal@mit.edu (S.L.B.), blp@mit.edu (B.L.P)

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

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1. General Experimental Details

1.1. Materials

N,*N*′-Disuccinimidyl carbonate and 3-(4-bromophenyl)propanoic acid were purchased from Combi-Blocks and used as received. For purification of protein-palladium oxidative addition complexes, HPLC grade acetonitrile was purchased from Sigma-Aldrich (HPLC, gradient grade, ≥99.9%, catalog no. 34851-4L) or Fisher Scientific (HPLC grade acetonitrile, ≥99.9% pure, catalog no. A998-4). 3-mercaptopropionic acid was purchased from MilliporeSigma and used without additional purification. All deuterated solvents were purchased from Cambridge Isotopes and used without further purification. All other reagents were purchased from Sigma-Aldrich and used as received.

Reactions monitored by analytical thin-layer chromatography (TLC) utilized glass-backed plates pre-coated with silica gel (250 µm, 60-Å por size, Extra Hard Layer, *SiliaPlate*) containing a fluorescent indicator (254 nm). SiliCycle SiliaFlash® F60 silica gel was used for silica flash chromatography (40–63 µm, 230–400 mesh, 60 Å pore diameter). Tetrahydrofuran (THF), diethyl ether (Et_2O), and dichloromethane (CH_2Cl_2) were unstabilized and obtained from J. T. Baker. These solvents were degassed by purging with Ar and successively filtered through packed columns of neutral alumina and CuO under Ar pressure. Solvents for extraction and flash-column chromatography were purchased as A.C.S. reagent grade from Millipore-Sigma.

Trypsin Gold, mass spectrometry grade was purchased from Promega and used as received. Trypsin/Lys-C Mix, Mass Spec Grade was purchased from Promega (Ca. No. V5071) and used as received. Hen egg white lysozyme and ribonuclease A were purchased from Amresco or VWR Life Sciences and used as received.

Dimethylsulfoxide (DMSO), pentane, and cyclohexane were purchased from Millipore-Sigma in SureSeal™ bottles and were purged with argon before use.

The ligand SPhos was a generous gift from Millipore-Sigma. sSPhos was prepared as per literature protocol.[1]

TISCH brand SPEC1798 filters were used for filtrations (Nylon 0.22 µm syringe filters).

Gels for SDS-PAGE were Invitrogen by Thermo Fisher Scientific, Bolt™ 4-12% Bis-Tris Plus either 1.0 mm × 15 well or 1.0 mm × 10 well plates. The molecular weight standard used was Invitrogen SeeBlue® Plus2 standard, using Bolt™ LDS Sample Buffer (4×) or, in the case of antibodies, Amresco Laemmli Loading Buffer (4×).

Zeba™ Spin Desalting Columns (7K MWCO, 0.5 mL) were obtained from ThermoFisher Scientific and used as received (Cat. No. 89882).

1.2. NMR Spectroscopy

NMR spectra were recorded on Bruker AVIII HD 400 MHz or Bruker Neo 500 MHz. All ^{1}H NMR chemical shifts are reported in parts per million (ppm, δ scale) and are referenced to the residual protonated NMR solvent ($CDCl_3$: δ 7.26, DMF-d7: 8.03). All ^{13}C spectra recorded are proton decoupled with chemical shifts reported in parts per million (ppm, δ scale) and are referenced to the carbon resonance of the NMR solvent ($CDCl_3$: 77.16, DMF-d7: 163.15). All ^{31}P chemical shifts are reported in parts per million (ppm, δ scale) and referenced to PPh_3 (-6.00 ppm) using the internal/external reference method. ^{1}H NMR spectroscopic data are reported as follows: chemical shift in ppm (multiplicity, coupling constants *J* (Hz), integration intensity, assigned number of protons in molecule). The multiplicities are abbreviated with s (singlet), br. s (broad

singlet), d (doublet), t (triplet), q (quartet), hept (heptet), and m (multiplet). In the case of combined multiplicities, the multiplicity with the larger coupling constant is stated first. The chemical shift of all signals is reported as the center of the resonance range, except in the case of multiplets, which are reported as ranges in chemical shift. All raw fid files were processed and the spectra analyzed using the program MestReNOVA 12.0 from Mestrelab Research S. L. Copies of the ^{1}H, ^{13}C, and ^{31}P NMR spectra can be found at the end of the Supporting Information (unless otherwise noted).

1.3. Mass Spectrometry and Liquid-Chromatography Mass Spectrometry (LCMS)

High resolution mass spectra for small molecules prepared in this study were obtained on an Agilent Technologies 6545 Q-TOF LC/MS system.

LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6520 ESI-Q-TOF mass spectrometer. Solvent compositions used in the LC-MS are 0.1% formic acid in H2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LC-MS methods were used:

Method A LC conditions: Zorbax 300SB C3 column: 2.1 × 150 mm, 5 µm, column temperature: 40 °C, gradient: 0-2 min 1% B, 2-11 min 1-61% B, 11-12 min 61-95% B, flow rate: 0.8 mL/min. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300–3000 *m*/*z*, temperature of drying gas = 350 °C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octapole rf voltages were set at 4000, 175, and 750, respectively.

Method B LC conditions: Zorbax 300SB C3 column: 2.1 × 150 mm, 5 µm, column temperature: 40 °C, gradient: 0-2 min 1% B, 2-11 min 1-91% B, 11-12 min 91-95% B. flow rate: 0.8 mL/min. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300–3000 *m*/*z*, temperature of drying gas = 350 °C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octapole rf voltages were set at 4000, 175, and 750, respectively.

Data were processed using Agilent MassHunter Workstation Qualitative Analysis Version B.06.00 with BioConfirm Software. Deconvoluted masses of proteins were obtained using a maximum entropy algorithm. Unless otherwise specified, the following parameters were used for deconvolution:

A range 3,000 less than the starting mass rounded down to the nearest 10,000 was used as a lower limit (not lower than 4,000). For the higher limit, a mass to the nearest 10,000 rounding up was used. A limited *m*/*z* range from 400-3,000 was used with a baseline subtraction factor of 3 and a mass step of 1.

Y-axis in all chromatograms shown in supplementary figures represents total ion current (TIC); mass spectrum insets correspond to the maxima point of the TIC peak.

1.4. Determination of Bioconjugation Yields

Yield determination by SDS-PAGE was carried as previously reported and is reproduced here for clarity (Sections 1.4.1 and 1.4.2).[2]

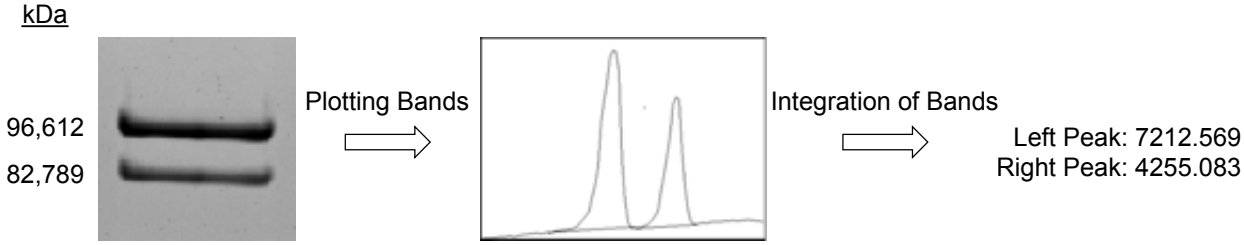
1.4.1. Determination by LCMS

Reported yields based on LC-MS spectra were determined by extracting the total ion current (TIC) spectra of all protein containing species in the chromatogram utilizing Agilent MassHunter 6.0 software with BioConfirm package. These extracted chromatograms were deconvoluted utilizing a maximum entropy algorithm and abundance of each species determined using total ion count.

NOTE: Dissolving Pd-protein-OAC complexes in acetonitrile was found to produce nitrile adducts (+26 Da). Thus, exposure of all OAC compounds to acetonitrile was avoided, except during LC-MS runs with flowing solvent.

1.4.2. Determination by SDS-PAGE

Band densitometry was calculated using ImageJ software (https://imagej.nih.gov/ij/). Yields based on densitometry were calculated based on the ratio of product : starting material protein within a lane and standardized based on molecular weight. For clarity, an example of SDS-PAGE yield determination follows:



After plotting bands and integration using ImageJ software:
Ligation Product: Left Peak Standardization (MW 96,612) | 7,212.569/96,612 = 0.074655
Starting material: Right Peak Standardization (MW 82,789) | 4,255.083/82,789 = 0.051397
Thus, the determined yield would be Standardized Product/(Standardized Total):

0.074655/(0.074655 + 0.051397) *100 = 59% in the example above.

1.5. Circular Dichroism Analysis of Proteins

Far-UV circular dichroism (CD) spectra for Ribonuclease A, Lysozyme C, and derivatives were acquired on an Aviv Model 420 Circular Dichroism Spectrometer at 25 °C in a 1.00 mm quartz cuvette from 260 to 190 nm, a 0.5 mm step, and with five second averaging times at each wavelength measured. Proteins were prepared at the specified concentrations (0.05 mg to 0.17 mg/mL) by dissolving solid samples in water followed by dilution with an equal volume of sodium phosphate buffer (20 mM, pH 7.4). The final concentration of buffer for all CD samples was: 10 mM sodium phosphate, pH 7.4. Data processing included solvent background correction (subtraction) and adjustment for pathlength and concentration (molar ellipticity, [θ], in deg cm^2 $dmol^{-1}$ = (millidegrees × mean residue weight)/(pathlength in millimeters × concentration in mg mL^{-1}). The resulting data was plotted as [θ], in deg cm^2 $dmol^{-1}$ vs wavelength (nm). The data were smoothed using 2nd order smoothing (5 neighbors) in GraphPad Prism 9.0.0.

2. Protein Sequences, Expression, and Purification

2.1. General Procedures for Protein Expression and Purification

pD444 PAC-CO.[3] Cysteine mutations were introduced by site-directed mutagenesis using QuickChange Lightning Single Site-directed Mutagenesis Kit (Agilent) following the manufacturer's instructions. Sequences of the generated protein constructs are summarized in Tables S1-S5.

PA[K653C] (mPA) was expressed in the periplasm of *E. coli* BL21(DE3) cells. Cells were grown in LB medium containing ampicillin (100 µg/mL) at 37 °C to an A_{600} of 0.8. Expression was induced by addition of IPTG to final concentration of 0.4 mM, and the culture was grown overnight at 18 °C. The protein was purified by anion exchange chromatography followed by size exclusion chromatography. The protein was analyzed by LC-MS confirming sample purity and molecular weight. Protein aliquots were flash frozen and stored in –80 °C freezer.

2.2. Protein Sequences and Calculated Masses

Table S1. Protective Antigen [K563C] (mPA)

Sequence[a]	Calculated mass (average)
MEVKQENRLLNESESSSQGLLGYYFSDLNFQAPMVVTS STTGDLSIPSSELENIPSENQYFQSAIWSGFIKVKKSDEYT FATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQI KIQYQRENPTEKGLDFKLYWTDSQNKKEVISSDNLQLP ELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEVEGYT VDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDP YSDFEKVTGRIDKNVSPEARHPLVAAYPIVHVDMENIIL SKNEDQSTQNTDSETRTISKNTSTSRTHTSEVHGNAEVH ASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAET MGLNTADTARLNANIRYVNTGTAPIYNVLPTTSLVLGK NQTLATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDD FSSTPITMNYNQFLELEKTKQLRLDTDQVYGNIATYNFE NGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNLVERR IAAVNPSDPLETTKPDMTLKEALKIAFGFNEPNGNLQYQ GKDITEFDFNFDQQTSQNICNQLAELNATNIYTVLDKIK LNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVI NSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYD MLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVY AVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG	82781 Da (Observed: 82785 Da)

[a] Cysteine residue highlighted in red.

Table S2. Wild Type Protective Antigen (PA)

Sequence	Calculated mass(average)
MEVKQENRLLNESESSSQGLLGYYFSDLNFQAPMVVTS STTGDLSIPSSELENIPSENQYFQSAIWSGFIKVKKSDEYT FATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQI KIQYQRENPTEKGLDFKLYWTDSQNKKEVISSDNLQLP ELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEVEGYT VDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDP YSDFEKVTGRIDKNVSPEARHPLVAAYPIVHVDMENIIL SKNEDQSTQNTDSETRTISKNTSTSRTHTSEVHGNAEVH ASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAET MGLNTADTARLNANIRYVNTGTAPIYNVLPTTSLVLGK NQTLATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDD FSSTPITMNYNQFLELEKTKQLRLDTDQVYGNIATYNFE NGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNLVERR IAAVNPSDPLETTKPDMTLKEALKIAFGFNEPNGNLQYQ GKDITEFDFNFDQQTSQNIKNQLAELNATNIYTVLDKIK LNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVI NSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYD MLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVY AVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG	82806 Da (Observed: 82677[b] or 82800)

[b] The difference in calculated and observed mass is suspected to be an *N*-terminal processed variant of the desired protein, resulting in removal of the *N*-terminal methionine.

Table S3. Ribonuclease A

Sequence	Calculated mass(average)
KETAAAKFERQHMDSSTSAASSSNYCNQMMKSRNLTK DRCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQ SYSTMSITDCRETGSSKYPNCAYKTTQANKHIIVACEGN PYVPVHFDASV	13682 Da (Observed: 13683 Da)

Table S4. Hen Egg White Lysozyme C

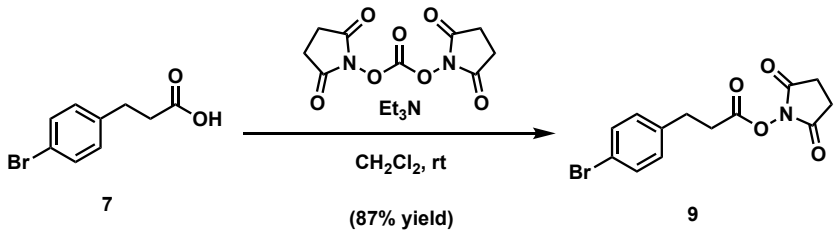
Sequence	Calculated mass(average)
KVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFES NFNTQATNRNTDGSTDYGILQINSRWWCNDGRTPGSRN LCNIPCSALLSSDITASVNCAKKIVSDGNGMNAWVAWR NRCKGTDVQAWIRGCRL	14305 Da (Observed: 14305 Da)

3. Synthesis of Pd Transfer Reagents

3.1. Synthesis of Palladium Transfer Reagents 10a and 10b

Synthesis of [(cod)Pd($CH_2TMS)_2$] (cod = 1,5 cyclooctadiene). This compound was prepared according to literature procedure.[4] The ^{1}H and ^{13}C NMR spectra of the obtained material are identical to those reported in the literature. The title compound was stored in a freezer at –20 °C.

Preparation of Reagent 9



A 100 mL round-bottom flask was sequentially charged with a Teflon-coated stir-bar, 3-(4-bromophenyl)propanoic acid (**7**) (1.152 g, 5.029 mmol, 1.000 equiv), and disuccinimidyl carbonate (1.547 g, 6.039 mmol, 1.201 equiv). The solid mixture was suspended in CH_2Cl_2 (20 mL) with stirring followed by the addition of triethylamine (0.70 mL, 5.0 mmol, 1.0 equiv). The round-bottom flask was sealed with a rubber septum and this septum pierced with a needle attached to an empty balloon. The reaction exhibited moderate effervescence over the course of the first *ca*. 10 min. The initially white suspension was stirred for 1.5 h after which time the reaction mixture had turned into a clear solution, faint yellow in color. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and poured into a separatory funnel containing 1 N aqueous HCl (20 mL). The reaction flask was rinsed with an additional portion of CH_2Cl_2 (10 mL) and added to the separatory funnel. The organic layer was separated and washed again with 1 N aqueous HCl (20 mL), dried over $MgSO_4$ and filtered. The filtrate was concentrated with the aid of a rotary evaporator to afford a white solid that was purified by flash column chromatography using SiO_2 (26.9 g). The SiO_2 was dry packed into a 26 mm internal diameter column prepacked with 8 mm of sand and subsequently saturated with CH_2Cl_2. The crude reaction mixture was dissolved in a minimal amount of CH_2Cl_2, loaded on top of the column and a layer of sand (8 mm) placed on top of the SiO_2 bed. CH_2Cl_2 (500 mL) was passed through the column and collected in 50 mL fractions that were monitored by TLC (UV, 254 nm, 100% CH_2Cl_2). Fractions 3-8 were collected and concentrated to a white solid with the aid of a rotary evaporator. To remove residual CH_2Cl_2, the resulting white solid was suspended and sonicated in diethyl ether and concentrated to dryness. This procedure repeated once more with hexanes and dried under high vacuum overnight to give the desired product **9** (1.43 g, 87 % yield) as a white amorphous solid.

R_f: 0.35 in 100% CH_2Cl_2.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, J = 8.3 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 3.01 (t, J = 7.8 Hz, 2H), 2.92–2.87 (m, 2H), 2.83 (s, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.1, 167.8, 138.1, 131.9, 131.9, 130.2, 120.7, 32.5, 30.0, 25.7.

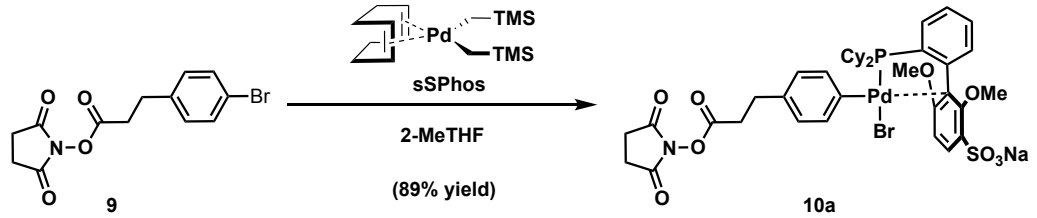
IR (Diamond-ATR, neat) $\tilde{\upsilon}_{max}$ 2998, 2934, 1818, 1777, 1729, 1489, 1371, 1287, 1067, 1014, 902, 833, 825, 809, 766, 654.

Melting Point 134.4–135.9 ºC.

HRMS (ESI) calc. $C_{13}H_{12}BrNNaO_4^+$ $[M+Na]^+$: 347.9842. Found: 347.9844.

Elemental Analysis: calc. $C_{13}H_{12}BrNO_4$: C, 47.88; H, 3.71. Found: C, 47.94; H, 3.63

Preparation of Reagent 10a



A tared 20 mL vial (Kimble Chase, catalog no. FS74504-20 with polypropylene cap and cork-backed foil liner) was sequentially charged with a stir-bar, **9** (109 mg, 334 µmol, 1.11 equiv), and sSPhos (169 mg, 330 µmol, 1.10 equiv). The reaction vial was brought into a nitrogen-filled glovebox and then 2-MeTHF (2.0 mL) was added while stirring for *ca*. 2 min to give a clear, colorless solution. To this was added (cod)Pd(CH_2TMS)$_2$ (117 mg, 300 µmol, 1.00 equiv), the vial was sealed with the polypropylene cap, and the reaction mixture was stirred for *ca*. 3 min (in the glovebox) to give a beige suspension. At this time the reaction vial was then brought out of the glovebox, sonicated for 30 s, and its content allowed to stir for 5 h. After this time, the vial containing the beige suspension was placed in a centrifuge and subjected to 3220 rcf (relative centrifugal force) for 2 min, the cap was removed and the supernatant decanted. The stir-bar was removed and the solid was resuspended in 2-MeTHF (3.0 mL), the vial was capped and then sonicated for 1 min to give a homogeneous suspension. The vial was then again centrifuged at 3220 rcf for 2 min. The cap was then removed and the supernatant decanted. This sonication/centrifugation/decanting procedure was repeated with 2-MeTHF (3.0 mL) and pentane (3 × 7 mL). The resulting beige solid was then dried under high vacuum for 48 h to give the crude product **10a** (253 mg, 89% yield).

Note 1: To minimize hydrolysis of the NHS ester, the compound was stored under an atmosphere of nitrogen in a desiccator. After *ca*. 6 months of use, LC analysis indicated ~10% hydrolysis of the NHS ester group. An LC trace of this material is provided in section 12 with the associated spectra.

Note 2: Unless otherwise specified, all studies disclosed herein were carried out utilizing the crude NHS ester reagent **10a**. If desired, we found that the NHS ester reagent could be purified by reverse-phase chromatography. In one instance, a ~20% hydrolyzed sample of **10a** (hydrolysis of the NHS ester, as assessed by peak area in the LCMS trace of reagent **10a**, see section 9 for methods of analysis), was purified by reverse phase chromatography utilizing a 12 g Biotage® SNAP cartridge (KP-C18-HS) on a CombiFlash NextGen 300 automated chromatography system. Solvent A was 0.1% TFA in H_2O, and solvent B was acetonitrile. The column was equilibrated in 20% B, followed by loading of 50.7 mg of crude **10a** (containing ~20% hydrolyzed NHS ester) in DMSO (1.0 mL). 1 CV of 20% B was passed through the column, followed by a gradient elution to 40% B over 3 CV, then a gradient over 9 CV to 50% B, and last 8 CV of 50% B was passed

through the column. The desired product eluted during the 50% B isochratic elution. Fractions containing **10a** were flash frozen and lyophilized overnight to give an 18.6 mg recovery of **10a** as a pale-yellow solid. An LC trace of this purified material is provided in section 9 (page S44).

^{1}H NMR Complex spectrum, see section 9.

^{31}P NMR (162, MHz, $CDCl_3$) δ 37.7 (br. s), 32.5 (br. s), 26.7 (br. s).

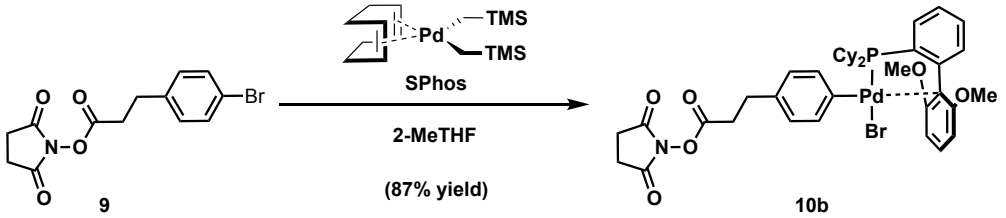
IR (Diamond-ATR, neat) $\tilde{\upsilon}_{max}$ 3435, 2927, 2851, 1813, 1784, 1739, 1575, 1450, 1397, 1200, 1098, 1085, 1051, 1010, 916, 807, 759, 693, 655.

Melting Point decomposition, 204 ºC (black solid)

HRMS (ESI) calc. $C_{39}H_{46}NNaO_9PPdS^+$ $[M-Br]^+$: 864.1558. Found: 864.1558.

Elemental Analysis: calc. $C_{39}H_{46}BrNNaO_9PPdS$: C, 49.56; H, 4.91. Found: C, 49.65; H, 4.98.

Preparation of Reagent 10b



A tared 20 mL vial (Kimble Chase, catalog no. FS74504-20 with polypropylene cap and cork-backed foil liner) was sequentially charged with a stir bar, **9** (36.8 mg, 113 µmol, 1.00 equiv), and SPhos (49.7 mg, 121 µmol, 1.07 equiv). The reaction vial was brought into a nitrogen-filled glovebox. To it was added 2-MeTHF (2.0 mL) while stirring for *ca*. 2 min to give a clear, colorless solution. To this was added (cod)Pd(CH_2TMS)$_2$ (46.1 mg, 118 µmol, 1.05 equiv) was added to give an orange-colored solution. The vial containing the reaction mixture was sealed with the polypropylene cap, stirred for *ca*. 3 min, and brought out of the glovebox and stirring was continued for 5 h. After this time, the cap was removed, the vial was opened to air and the mixture diluted with 1:1 pentane:2-MeTHF (10 mL). The vial was again capped, and the mixture was placed in a centrifuge and subjected to 3220 rcf (relative centrifugal force) for 2 min after which the cap and stir bar were removed and the supernatant decanted. The resulting white precipitate was resuspended in 1:1 pentane:2-MeTHF (1.5 mL), the vial was recapped and was sonicated for 1 min. After sonicating, the vial containing the milky white suspension was centrifuged for 2 min at 3220 rcf and the vial was uncapped and the resulting supernatant decanted. This sonication/centrifugation/decanting procedure was repeated with 1:1 pentane:2-MeTHF (3.0 mL) and pentane (2 × 10 mL). The resulting white solid was dried under high vacuum for 48 h to give **10b** (83.1 mg, 87% yield) as a grey amorphous powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (t, J = 8.4 Hz, 1H), 7.63 (t, J = 7.1 Hz, 1H), 7.50–7.38 (m, 2H), 7.03 (dd, J = 7.8, 1.3 Hz, 2H), 6.88–6.80 (m, 3H), 6.64 (d, J = 8.4 Hz), 3.78 (s, 6H), 2.97–

2.89 (m, 2H), 2.88–2.75 (m, 9H), 2.22–2.10 (m, 2H), 1.98–1.51 (m, 17H), 1.29–1.03 (m, 7H), 0.62–0.49 (m, 2H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 169.3, 169.2, 168.3, 168.2, 160.6, 157.9, 144.3, 144.1, 137.4, 137.3, 135.9, 135.7, 135.4, 134.4, 133.8, 132.9, 132.5, 131.9, 131.8, 131.5, 131.4, 131.4, 129.4, 128.8, 127.2, 127.2, 126.9, 126.9, 126.2, 125.8, 108.2, 108.2, 106.3, 56.0, 34.3, 34.1, 33.3, 33.3, 30.6, 30.1, 30.0, 27.7, 27.6, 27.5, 27.5, 27.2, 27.2, 26.8, 26.0, 25.7.

^{31}P NMR (203, MHz, $CDCl_3$) δ 33.2

IR (Diamond-ATR, neat) $\tilde{\upsilon}_{max}$ 2933, 2850, 1816, 1784, 1739, 1587, 1471, 1451, 1429, 1360, 1288, 1249, 1198, 1112, 1065, 1011, 848, 809, 782, 759, 727, 641.

Melting Point 163 ºC (decomposition, black tar)

HRMS (ESI) calc. $C_{39}H_{47}NO_6PPd^+$ $[M\text{-}Br]^+$: 762.2170. Found: 762.2185.

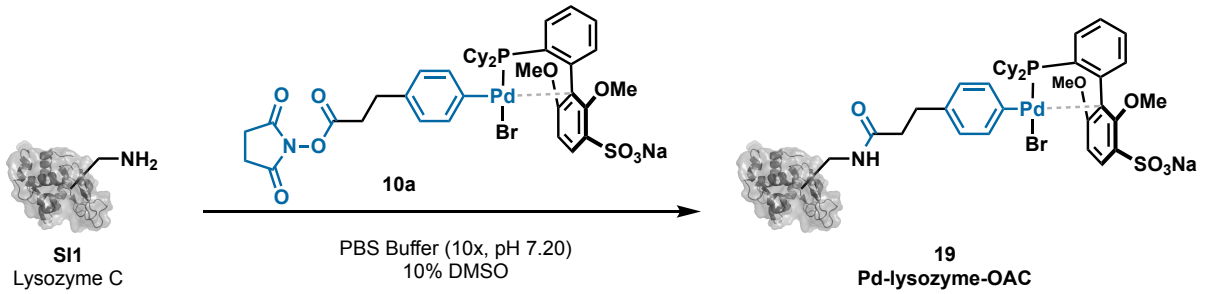
Elemental Analysis: calc. $C_{39}H_{47}BrNO_6PPd$: C, 55.56; H, 5.62. Found: C, 55.49; H, 5.46.

4. Synthesis of Palladium-Protein Oxidative Addition Complexes from Native Proteins

4.1. Synthesis of Pd-Protein-OACs using Pd-Transfer Reagent 10a

Preparation of 10× PBS Buffer: A solution of 10× PBS buffer was prepared by dissolving NaCl (80.0 g), KCl (2.0 g), $Na_2HPO_4 \cdot 2H_2O$ (14.4 g), and KH_2PO_4 (2.4 g) in 750 mL of water. The solution was adjusted to a pH of 7.2 by the addition of aqueous NaOH (5 N solution) and subsequently diluted to a total volume of 1.00 L.

4.1.1. Pd-lysozyme-OAC



To a 250 mL round-bottom flask was added lysozyme C **SI1** (20 mg of a crystallized powder, 1.0 equiv) and dissolved in PBS buffer (20 mL, 10×, pH 7.2). While stirring at 400 rpm, palladium transfer reagent **10a** (1.98 mg, 2.1 µmol, 1.5 equiv) dissolved in DMSO (2.22 mL, stored over 4 Å MS) was added over a period of 2 min, and the reaction mixture stirred for 1 h. An aliquot of the mixture was taken and analyzed by LC-MS (~ 30% conversion to the mono-modified species was observed, LCMS method B). The reaction mixture was centrifuged at 3220 rcf for 7 min and the supernatant directly purified by mass-directed RP-HPLC (Agilent Zorbax SB C3 column: 9.4 × 250 mm, 5 µm, solvent **A** was water with 0.1% TFA additive and solvent **B** was acetonitrile with 0.1% of TFA additive. A linear gradient from 1-65% **B** changed at a rate of 0.5% **B**/min was used). Fractions containing the desired product were lyophilized to yield Pd-lysozyme-OAC (**19**) as a white solid (4.67 mg). Total ion current chromatogram and CD spectra are shown below (Fig. S1).

Preparation of samples for CD spectroscopy:

<u>Solution A:</u> A solution of Pd-lysozyme C-OAC **19** in water was prepared by adding solid **19** (0.2 mg) to a 1.5 mL Eppendorf tube following by the addition of water (200 µL). This was subsequently diluted with sodium phosphate buffer (200 µL, 20 mM sodium phosphate, pH 7.4).

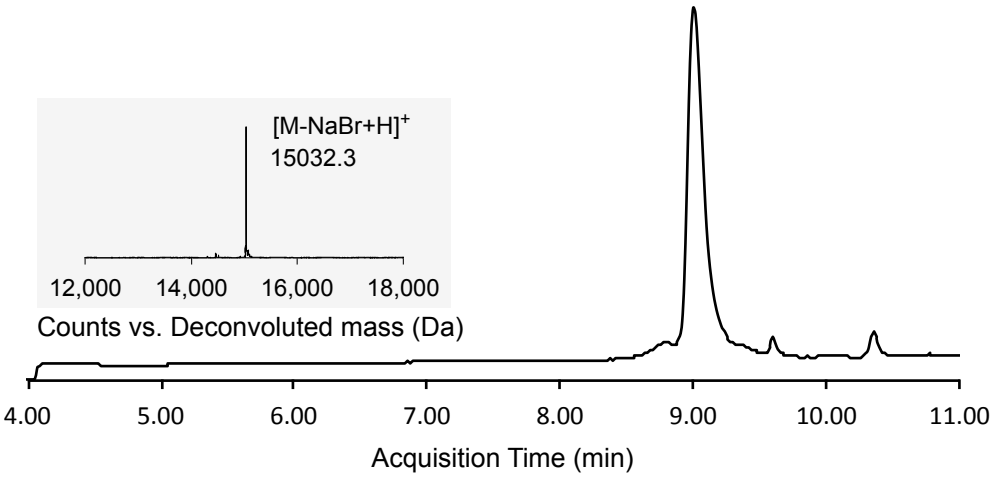
<u>Solution B:</u> In a separate Eppendorf tube, 200 µL of Solution A was diluted with sodium phosphate buffer (400 µL, 10 mM sodium phosphate, pH 7.4) to give a 0.17 mg/mL solution of Pd-lysozyme C-OAC **19**. 400 µL of this sample was transferred to a 1.00 mm quartz cuvette and the CD spectrum obtained according to the general experimental (Section 1.5) and the results depicted below in Fig. S1B.

<u>Lysozyme C-3MPA:</u> Solution A (100 µL) was added to a 0.6 mL Eppendorf tube followed by the addition of a solution of 3-mercaptopropionic acid (3MPA) (5 µL, itself prepared by the dilution of 2 µL of neat 3-MPA into 998 µL of sodium phosphate buffer (20 mM sodium phosphate, pH 7.4). The mixture was incubated for 30 minutes and subsequently buffer exchanged into sodium phosphate buffer (10 mM sodium phosphate, pH 7.4) by the use of a Zeba™ Spin Desalting

Column (7K MWCO, 0.5 mL) according to the manufacturers protocol. The concentration of the solution was estimated by A280 (0.15 mg/mL using the extinction coefficient of unmodified Lysozyme C, 26.4 L g^{-1}). 100 µL of the filtered solution was diluted with 300 µL of sodium phosphate buffer (10 mM sodium phosphate, pH 7.4) to give a calculated concentration of 0.05 mg/mL and the entirety of this solution transferred to a 1.00 mm quartz cuvette. The CD spectrum recorded according to the general experimental (Section 1.5). The results are depicted below in Fig. S1B.

Lysozyme C: A solution of lysozyme C was prepared by dissolving lysozyme C (0.7 mg) in water (700 µL) in a 1.5 mL Eppendorf tube followed by the addition of sodium phosphate buffer (700 µL, 20 mM sodium phosphate, pH 7.4). This gave a 0.5 mg/mL of lysozyme C. 200 µL of this 0.5 mg/mL solution of lysozyme C was then diluted with 400 µL of sodium phosphate buffer (400 µL, 10 mM sodium phosphate, pH 7.4). 400 µL of the resulting solution of lysozyme C (0.17 mg/mL in 10 mM sodium phosphate buffer) was transferred to a 1.00 mm quartz cuvette and the CD spectrum recorded according to the general experimental (Section 1.5). The results are depicted below in Fig. S1B.

A. Total ion count chromatogram and deconvoluted mass spectra of Pd-lysozyme-OAC



B. Far UV circular dichroism spectra of lysozyme C and lysozyme C derivatives

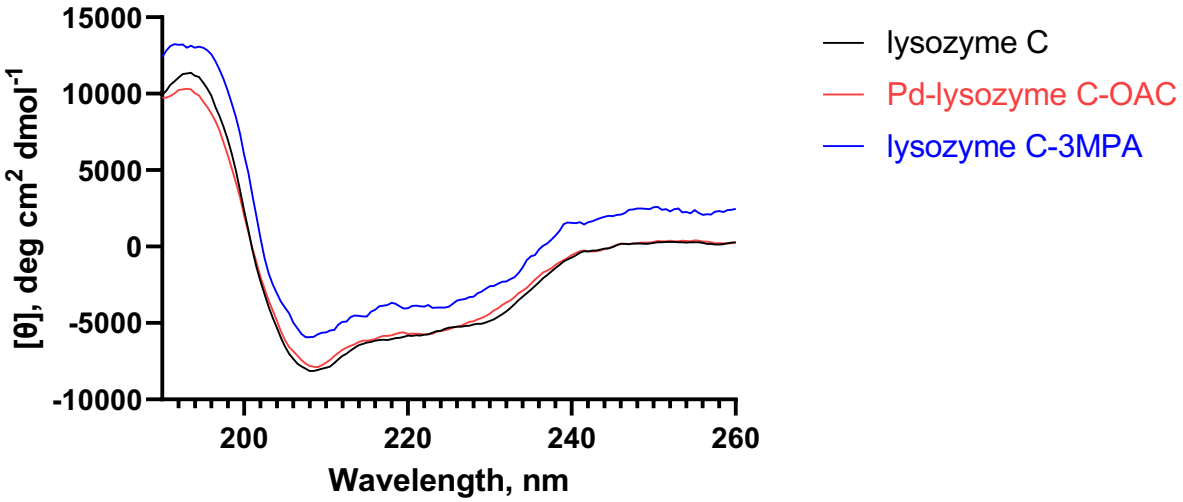
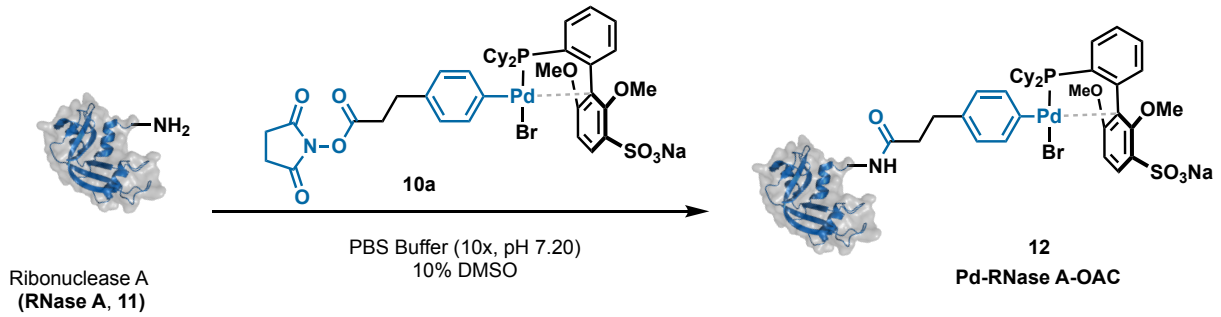


Fig. S1. (A) Total ion current chromatogram and deconvoluted mass spectra (inset) of Pd-lysozyme-OAC after RP-HPLC. Deconvoluted mass, calc.$[M\text{-}NaBr+H]^+$: 15032 (Calculated as the observed mass of lysozyme C + the depicted modification = 14305 + 728 = 15033 Da) , observed: 15032.3. (B) CD spectra of lysozyme C (0.17 mg/mL), Pd-lysozyme C-OAC (0.17 mg/mL), and lysozyme C-3MPA (0.05 mg/mL). The final concentration of buffer for each protein solution was 10 mM sodium phosphate, pH 7.4. Data were smoothed using GraphPad Prism 9.0.0 using a 2nd order smoothing polynomial with 5 neighbors.

4.1.2. Ribonuclease A-OAC



To a 50 mL falcon tube containing ribonuclease A **11** (20.7 mg, 1.51 µmol, 1.00 equiv) was added PBS (21.6 mL, 10×, pH 7.2). A stir-bar was added and the mixture was stirred for 15 min at 400 rpm. After all the RNase A had dissolved, a solution of transfer reagent **10a** (2.4 mL, 0.89 mg/mL in DMSO, 1.5 equiv) was added in three 800 µL portions down the wall of the Falcon tube over the course of ~30 s. The reaction was stirred for 1 h after which the reaction had turned into a cloudy suspension. An aliquot of the reaction mixture was taken and analyzed by LC-MS (method B, ~30% conversion to a mono-modified species). The reaction was centrifuged, and the supernatant transferred into another 50 mL falcon tube and purified by mass-directed RP-HPLC (Agilent Zorbax SB C3 column: 9.4 × 250 mm, 5 µm, solvent **A** was water with 0.1% TFA additive and solvent **B** was acetonitrile with 0.1% of TFA additive. A linear gradient from 0-65% **B** changed at a rate of 0.76% **B**/min was used). Fractions were collected in 1 min time intervals. Fractions 44, 45, 46, and 47 were individually collected in 15 mL falcon tubes and separately lyophilized. After lyophilization, the falcon tubes were centrifuged to pellet the resulting white foam to give Pd-RNase A-OAC fraction 44 (2.22 mg), fraction 45 (1.47 mg), fraction 46 (1.39 mg), and fraction 47 (0.34 mg). Total isolated Pd-RNase A-OAC **12**: 5.42 mg.

<u>96 mg scale Reaction</u>

A procedure identical to that described above was used with ribonuclease A (96 mg, 7.0 µmol, 1.0 equiv), PBS buffer (90 mL, 10×, pH 7.2) with palladium transfer reagent **10a** (9.9 mg, 10.5 µmol, 1.5 equiv) dissolved in DMSO (10 mL, stored over 4 Å MS). The palladium reagent was added over a period of 5 min, and the reaction mixture stirred for 1 h. An aliquot of the mixture was taken and analyzed by LC-MS (~30% conversion to the mono-modified species was observed, LCMS method B). The reaction mixture was split into two equal volumes, and one portion flash frozen and stored in a freezer at -80 ºC. The remaining aliquot was filtered through a 0.22 µm nylon filter and purified by mass-directed RP-HPLC (Agilent Zorbax SB C3 column: 9.4 × 250 mm, 5 µm, solvent **A** was water with 0.1% TFA additive and solvent **B** was acetonitrile with 0.1% of TFA additive. A linear gradient from 20-61% **B** changed at a rate of 0.28% **B**/min was used). Fractions containing the desired product were lyophilized to yield Pd-RNase A-OAC **12** (14.6 mg) as a white solid. Total ion current chromatogram and CD spectra are shown on the next page below.

Preparation of samples for CD spectroscopy:

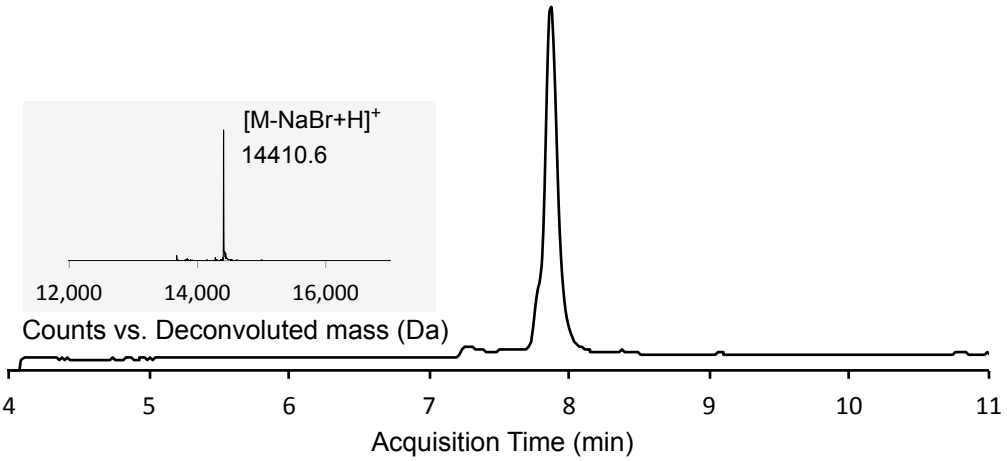
<u>Solution A:</u> A solution of Pd-RNase A-OAC **12** in water was prepared by adding solid **12** (0.2 mg) to a 1.5 mL Eppendorf tube following by the addition of water (200 µL). This was subsequently diluted with sodium phosphate buffer (200 µL, 20 mM sodium phosphate, pH 7.4).

Solution B: In a separate Eppendorf tube, 200 µL of Solution A was diluted with sodium phosphate buffer (400 µL, 10 mM sodium phosphate, pH 7.4) to give a 0.17 mg/mL solution of Pd-RNase A-OAC **12**. 400 µL of this sample was transferred to a 1.00 mm quartz cuvette and the CD spectrum obtained according to the general experimental (Section 1.5) and the results depicted below in Fig. S2B.

RNase A-3MPA: Solution A (100 µL) was added to a 0.6 mL Eppendorf tube followed by the addition of a solution of 3-mercaptopropionic acid (3-MPA) (5 µL, itself prepared by the dilution of 2 µL of neat 3-MPA into 998 µL of sodium phosphate buffer (20 mM sodium phosphate, pH 7.4). The mixture was incubated for 30 minutes and subsequently buffer exchanged into sodium phosphate buffer (10 mM sodium phosphate, pH 7.4) by the use of a Zeba™ Spin Desalting Column (7K MWCO, 0.5 mL) according to the manufacturers protocol. The concentration of the solution was estimated by A280 (0.39 mg/mL using the extinction coefficient and MW parameters of unmodified RNase A, $\varepsilon = 9440$ L mol^{-1} cm^{-1}, MW: 13683). 100 µL of the filtered solution was diluted with 300 µL of sodium phosphate buffer (10 mM sodium phosphate, pH 7.4) to give a calculated concentration of 0.13 mg/mL and the entirety of this solution transferred to a 1.00 mm quartz cuvette and the CD spectrum recorded according to the general experimental (Section 1.5). The results are depicted below in Fig. S2B.

RNase A: A solution of RNase A was prepared by dissolving RNase A (0.4 mg) in water (400 µL) in a 1.5 mL Eppendorf tube followed by the addition of sodium phosphate buffer (20 mM sodium phosphate, pH 7.4). This gave a 0.5 mg/mL of RNase A. 200 µL of this 0.5 mg/mL solution of RNase A was then diluted with 400 µL of sodium phosphate buffer (400 µL, 10 mM sodium phosphate, pH 7.4). 400 µL of the resulting solution of RNase A (0.17 mg/mL in 10 mM sodium phosphate buffer) was transferred to a 1.00 mm quartz cuvette and the CD spectrum recorded according to the general experimental (Section 1.5). The results are depicted below in Fig. S2B.

A. Total ion count chromatogram and deconvoluted mass spectra Pd-RNase A-OAC



C. Far UV circular dichroism spectra of RNase A and RNase A derivatives

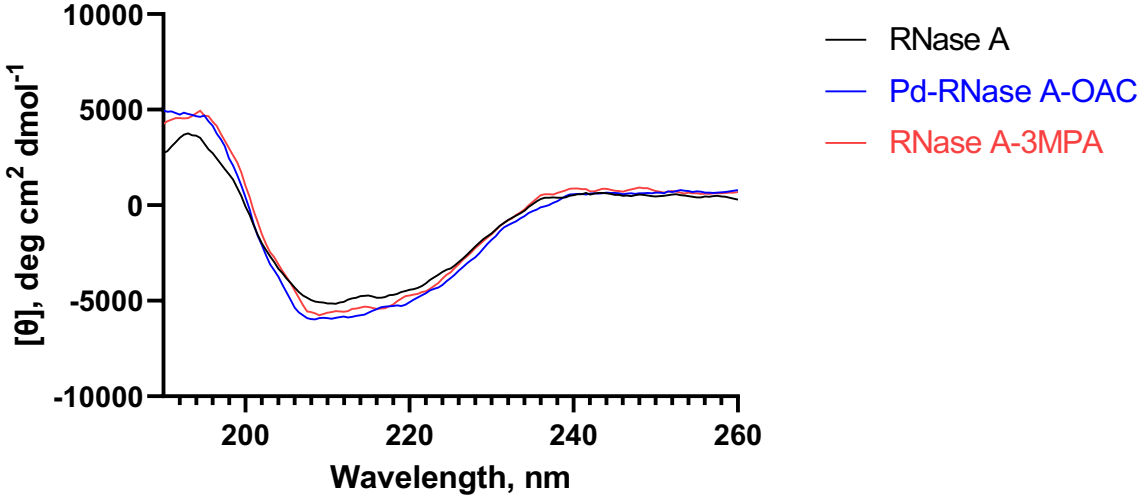


Fig. S2. (A) Total ion current chromatogram and deconvoluted mass spectra of Pd-RNase A-OAC after RP-HPLC. Deconvoluted mass, calc. $[M\text{-}NaBr+H]^+$ 14409 Da (Calculated as the observed mass of RNase A + the depicted modification, 13682 + 728.2 = 14409 Da), observed: 14410.6 Da. (B) CD spectra of RNase A (0.17 mg/mL), Pd-RNase A-OAC (0.17 mg/mL), and RNase A-3MPA (0.13 mg/mL). The final concentration of buffer for each protein solution was 10 mM sodium phosphate, pH 7.4. Data were smoothed using GraphPad Prism 9.0.0 using a 2nd order smoothing polynomial with 5 neighbors.

4.2. Supporting Evidence for Modification Sites by MS/MS Analysis

Identification of Modification Sites by Trypsin Digestion

To obtain information about the sites of modification of the overall NHS-ester acylation reaction, the reaction conjugation reactions outlined in section 4.1.1 and 4.1.2 were carried out on a 1 mg scale. Briefly:

Preparation of crude samples for MS/MS analysis (Protocol A): To a 1.5 mL Eppendorf tube containing ribonuclease A **11** (1.0 mL, 0.88 mg/mL in 10× PBS buffer at pH 7.2) or lysozyme C **SI1** (1.0 mL, 1.0 mg/mL in 10× PBS buffer at pH 7.2). A solution of transfer reagent **10a** (111 µL, purified reagent, 0.89 mg/mL in DMSO, 1.5 equiv) was added and the reaction mixed by pipetting up and down 10× with a 111 µL volume. The Eppendorf tube was capped with the attached cap, and the reaction was placed on a nutating mixer at room temperature. After a 1 h incubation time, any excess NHS ester reagent was quenched by uncapping the Eppendorf tube followed by the addition of Glycine (50 µL, prepared as a 2 mg/mL in PBS 10× buffer, pH 7.2). The reaction was again capped and placed on a nutating mixer for 10 min. After these 10 min, the Eppendorf tube was uncapped followed by the addition of a solution containing 3-mercaptopropionic acid (3-MPA) (90 µL, prepared by the addition of 2 µL of neat 3-MPA to 998 µL of 10× PBS buffer at pH 7.2. The final pH of this solution was 6.5). The Eppendorf tube was again capped and placed on a nutating mixer for 30 minutes. After this time, the reaction was filtered/buffer exchanged into Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.5) with the aid of a disposable PD-10 column (Cytiva [formerly GE Healthcare Life Sciences], 8.3 mL capacity, Supplier No. 17-0851-01) according to the manufacturer's gravity filtration protocol. The recovery of the conjugates RNase A-3MPA and lysozyme C-3MPA were 0.35 mg/mL and 0.24 mg/mL respectively as estimated by A280 with an extinction coefficient of lysozyme C (26.4 L g^{-1} cm^{-1}) and RNase A (9440 L mol^{-1} cm^{-1} and MW:13683).

Digestion and MS/MS protocol:

For the analysis of protein derivatives prepared by Protocol A above, the PD-10 filtered material was placed in a PCR tube followed by the addition of 1,4-dithiothreitol (2 µL, 85 mM solution in water) and mixed by pipetting up and down 20×.

For the analysis of purified, lyophilized products: Lyophilized solids were dissolved to a concentration of 2 mg/mL in water. Protein solution (16 µL) was added to a PCR tube after which each fraction was quenched by the addition of 3-mercaptopropionic acid solution (2 µL, 11.5 mM solution in water) and mixed by pipetting up and down 20× and incubated for 15 min.

A solution of 1,4-dithiothreitol (2 µL, 85 mM solution in water) was added to each protein solution to be analyzed by MS/MS and the reaction mixed by pipetting up and down 20×. After a 1.5 h incubation period, iodoacetamide (6 µL, 100 mM solution in water) was added, mixed by pipetting up and down 20×, and let stand in the dark for 30 min. Lastly, trypsin gold (0.75 µL, 0.5 mg/mL solution) or Trypsin/Lys-C Mix (0.5 µL, 0.5 mg/mL) was added, and the reaction incubated overnight in a preheated 37 ºC bath. 1 µL, 3 µL, and 10 µL were directly injected into a 6550 Q-ToF instrument with method (2) below. The resulting data was compiled and fragments with an ion intensity greater than or equal to 5% are included in section 10 of the Supporting Information.

The resulting digests were analyzed by Q-TOF LC-MS on a 6550 Q-ToF instrument with either (1) a Phenomenex Jupiter C4 column with a gradient from 1% and ramping to 61% CH_3CN over 28 min, flowrate 100 µL/min with MS/MS analysis from 4 min to 18 min or (2) a Aeris C4

column with a gradient from 1% to 91% CH_3CN over 50 min, flowrate 100 µL/min with MS/MS analysis from 5 min to 45 min.
The resulting MS/MS spectrum analyzed with PEAKS Studio v8.5. The results are summarized in Section 10.

5. Bioconjugation Procedures

5.1. Protein-Protein Cross-Coupling

5.1.1. Determination of protein concentrations

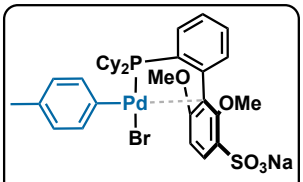
Protein concentrations for proteins comprised of the canonical 20 amino acids were calculated using ExPASy SIB Bioinformatics Resource Portal. For clarity, the molar extinction coefficient is estimated with the following formula:

ϵ_{280} = [(No. of Trp × 5500) + (No. of Tyr × 1490) + (No. of Cys-Cys × 125)] M^{-1} cm^{-1}

In the case of protein-OACs prepared with reagent **10a**, two methods were interchangeably used to estimate concentration. Protein concentrations were determined by Bradford assay using bovine serum albumin as standard or by the use of Beer's law and the absorbance of the protein at 280 nm.

In the case of absorbance, a molar extinction coefficient of 2207 M^{-1} cm^{-1} was used to represent the non-canonical Pd-complex. Thus the molar extinction coefficient for a protein bearing the non-canonical functional group derived from reagent **10a** is calculated as follows:

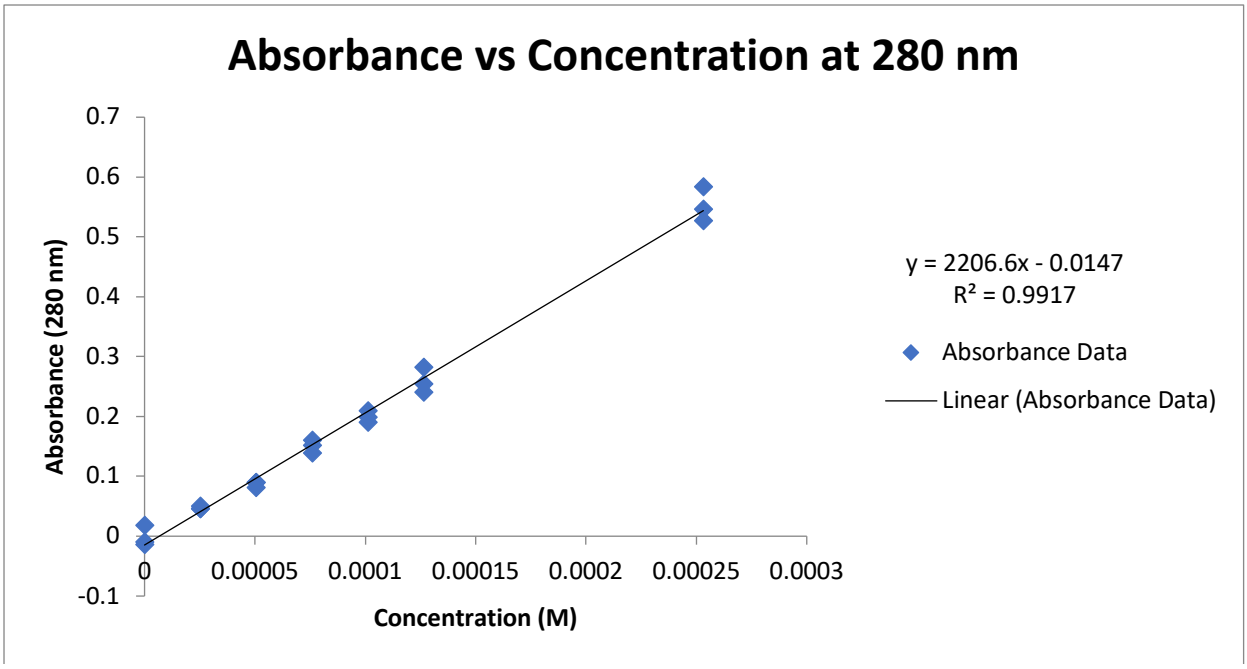
ϵ_{280} = [(No. of Trp × 5500) + (No. of Tyr × 1490) + (No. of Cys-Cys × 125) +2207] M^{-1} cm^{-1}



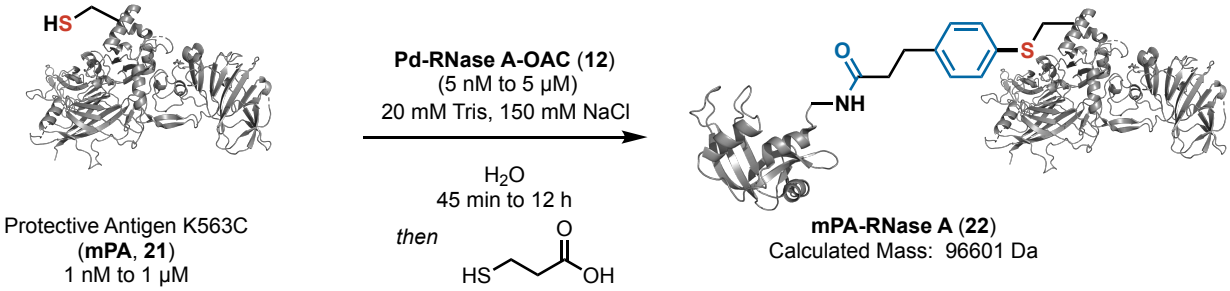
SI2
(ϵ_{280} = 2207 M^{-1} cm^{-1})

The value 2207 M^{-1} cm^{-1} was experimentally determined from the model reagent **SI2** as follows:

3.50 mg, 3.84 mg, and 4.07 mg of compound **SI2** were independently weighed out into three separate vials. These were diluted to a concentration of 1.00 mg/mL in 10% DMF in water. Subsequently, each of these stock 1.00 mg/mL solutions were aliquoted/diluted to 0.2, 0.1, 0.08, 0.06, 0.04, and 0.02 mg/mL via serial dilution to give a total of 18 solutions, three at each concentration. The absorbance of each solution was measured by averaging two independent readings of the same sample on a BioTek Synergy HT plate-reader outfitted with a BioTek Take 3 micro-volume plate. These were then plotted as Absorbance vs concentration (M) a least-squared analysis performed. Utilizing Beer's law, the slope of the line was used to calculate the corresponding extinction coefficient 2207 ± 47 M^{-1} cm^{-1}.



5.1.2. Pd-RNase A-OAC cross-coupling with mutant Protective Antigen[K563C] (mPA)



<u>Cross-Coupling from μM to nM Concentrations</u>

To a PCR tube was added **mPA** (3.83 μL, 145 μM, 12 mg/mL in buffer P) followed by the addition of water (5 μL) and TCEP•HCl (2 μL, 560 μM in water). After an incubation time of 30 min, this gave a solution of mPA (51 μM) free of disulfides as judged by LC-MS (method B). The reactions were then set up as follows:

Note: This additional TCEP reduction step was later found to be unnecessary, and only utilized when the starting material was deemed to have significant (>5%) dimerized protein as determined by LCMS deconvolution.

Note: A solution of Pd-RNase-A OAC was prepared in water whose concentration was determined by Bradford assay (0.388 mg/mL). This solution was used for the following reactions:

<u>1 μM</u>: In a PCR tube was added Tris buffer (39 μL, 100 mM, pH 7.5), and Pd-RNase A-OAC (10 μL, 26.7 μM). To this was added the above TCEP-reduced mPA solution (1 μL, 51 μM), and the resulting solution was mixed by pipetting up and down 20×.

100 nM: In a 0.6 mL Eppendorf tube was added Tris buffer (489 µL, 100 mM, pH 7.5), and Pd-RNase A-OAC (10 µL, 26.7 µM). To this was added the above TCEP-reduced mPA solution (1 µL), and the resulting solution was mixed by pipetting up and down 20×.

10 nM: In a 1.6 mL Eppendorf tube was added Tris buffer (1496 µL, 100 mM, pH 7.5), and Pd-RNase A-OAC (3 µL, 26.7 µM). To this was added the above TCEP-reduced mPA solution (0.3 µL), and the resulting solution was mixed by pipetting up and down 20×.

1 nM: In a 15 mL falcon tube was added Tris buffer (15 mL, 100 mM, pH 7.5), and Pd-RNase A-OAC (3 µL, 26.7 µM). To this was added the above TCEP-reduced mPA solution (0.3 µL), and the resulting solution was mixed by pipetting up and down 20×.

The reactions were incubated for 45 min, after which an aliquot was taken and quenched by the addition of 3-mercaptopropionic acid (5 µL, 11.5 mM solution in water). The quenched aliquots were let incubate for 15 min then analyzed by LCMS (method B).

After 12 h, the reactions were quenched by the addition of 3-mercaptopropionic acid (5 µL, 11.5 mM solution in water). The 2 nM reaction was concentrated using a 10 K spin-mass filter (EMD-Millipore) to a volume of ~200 µL and analyzed by LC-MS (method B). Results were described in the main text (Fig. 4).

Cross-Coupling on 1.0 mg scale mPA to determine Pd content before and after cross-coupling

To a 1.5 mL Eppendorf tube was added Pd-RNase A-OAC (0.92 mg of lyophilized solid), dissolved in buffer P (900 µL), and mixed by pipetting up and down. This was let stand for 5 min followed by the addition of **mPA** (100 µL, 121 µM, 10 mg/mL in buffer P). The reaction was subsequently quenched by the addition of 3-mercaptopropionic acid (50 µL, 1.2 mg / mL solution in buffer P). On this scale, the conversion as determined by densitometry analysis with SDS-PAGE, showed a 68% conversion of the starting material. The mixture was incubated for 15 min and subsequently purified on a HiLoad 26/600 Superdex 200 pg preparative size-exclusion column with buffer P as eluent. Fractions were analyzed by SDS-PAGE and fractions containing the product and starting mPA protein were pooled and concentrated using an Amicon 10 K MWCO centrifugal filter.

Pd content analysis:

ICP-MS was used to measure the palladium content of the crude reaction mixture and the purified product after size-exclusion chromatography. The crude reaction mixture (20 µL) was diluted five-fold to a total volume of 100 µL. 100 µL of the purified conjugate was additionally prepared and both samples were sent to Merck for Pd concentration determination by ICP-MS analysis. The five-fold diluted sample of the crude reaction mixture had a measured palladium concentration of 202 ppb, and the purified conjugate after SEC purification measured 81.4 ppb.

Note: A density of 1.00 mg/µL was used for the aqueous protein solutions.

Mass of Pd in the crude reaction mixture = Measured Pd content * 5 * total volume of the reaction mixture * density = (202 ppb) * 5 * 1050 µL * 1.00 mg/µL = 0.00106 mg Pd present in the crude reaction mixture

Mass of Pd in the purified material after SEC chromatography = Measured Pd content * total volume of the purified material * density of protein solution = (81.4 ppb) * 459 µL * 1.00 mg/µL = 0.0000374 mg Pd

Percent Pd removed = [1 - (mass Pd in crude reaction mixture / mass Pd in the purified material) * 100] = 96.3% of the Pd was removed in the purification process.

SDS-PAGE Analysis of Reaction

The Effect of Varying Concentration of mPA with Constant Pd-RNase A-OAC

To a PCR tube was added Tris buffer (pH 7.5, 100 mM Tris, 150 mM NaCl). To this was added mPA (1 µL, 1.0 mg/mL in buffer P) and last the addition of Pd-RNase A-OAC (0.65 mg/mL as determined by A280). After the addition of Pd-RNase A-OAC, the reactions were mixed by pipetting up and down 30×. The reactions were incubated at room temperature for 45 min after which 3 µL of each reaction was diluted with 15 µL of buffer P followed by the addition of a solution of 3-mercaptopropionic acid (5 µL, 11.5 mM solution in buffer P, pH ~6.3) to quench the reaction. These solutions were analyzed by LC-MS using method A.

The unquenched reaction mixtures were let incubate for another 15 minutes (1 hr total) after which these were each quenched by the addition of 3-mercaptopropionic acid (5 µL). The reactions were let incubate for 15 min after which 16 µL of each reaction mixture was diluted with 4× LDS loading dye (6 µL) and the entirety of this mixture loaded for SDS-PAGE analysis (see Fig. S12 for SDS-PAGE analysis and Fig. S13 for LCMS analysis).

The reactions were set up as per the general procedure, except utilizing the following concentrations and volumes, without additional TCEP reduction of the starting mPA.

mPA (1.0 mg/mL), Pd-RNase A-OAC (0.65 mg/mL determined by A280).

Reaction 1: Tris buffer (20 µL), mPA (10 µL) and Pd-RNase A-OAC (0 µL).
Final Concentrations: 4 µM in mPA, 3 µM in Pd-RNase A-OAC

Reaction 2: Tris buffer (23 µL), mPA (5 µL) and Pd-RNase A-OAC (2 µL).
Final Concentrations: 2 µM in mPA, 3 µM in Pd-RNase A-OAC

Reaction 3: Tris buffer (18 µL), mPA (10 µL) and Pd-RNase A-OAC (2 µL).
Final Concentrations: 4 µM in mPA, 3 µM in Pd-RNase A-OAC

Reaction 4: Tris buffer (13 µL), mPA (15 µL) and Pd-RNase A-OAC (2 µL).
Final Concentrations: 6 µM in mPA, 3 µM in Pd-RNase A-OAC

Reaction 5: Tris buffer (8 µL), mPA (20 µL) and Pd-RNase A-OAC (2 µL).
Final Concentrations: 8 µM in mPA, 3 µM in Pd-RNase A-OAC

Reaction 6: Tris buffer (3 µL), mPA (25 µL) and Pd-RNase A-OAC (2 µL).
Final Concentrations: 10 µM in mPA, 3 µM in Pd-RNase A-OAC

The reactions were incubated for 1 h and subsequently quenched by the addition of 3-mercaptopropionic acid (11.5 mM, pH ~6.3 in buffer P). Results are summarized in Fig. S3 and Fig. S4.

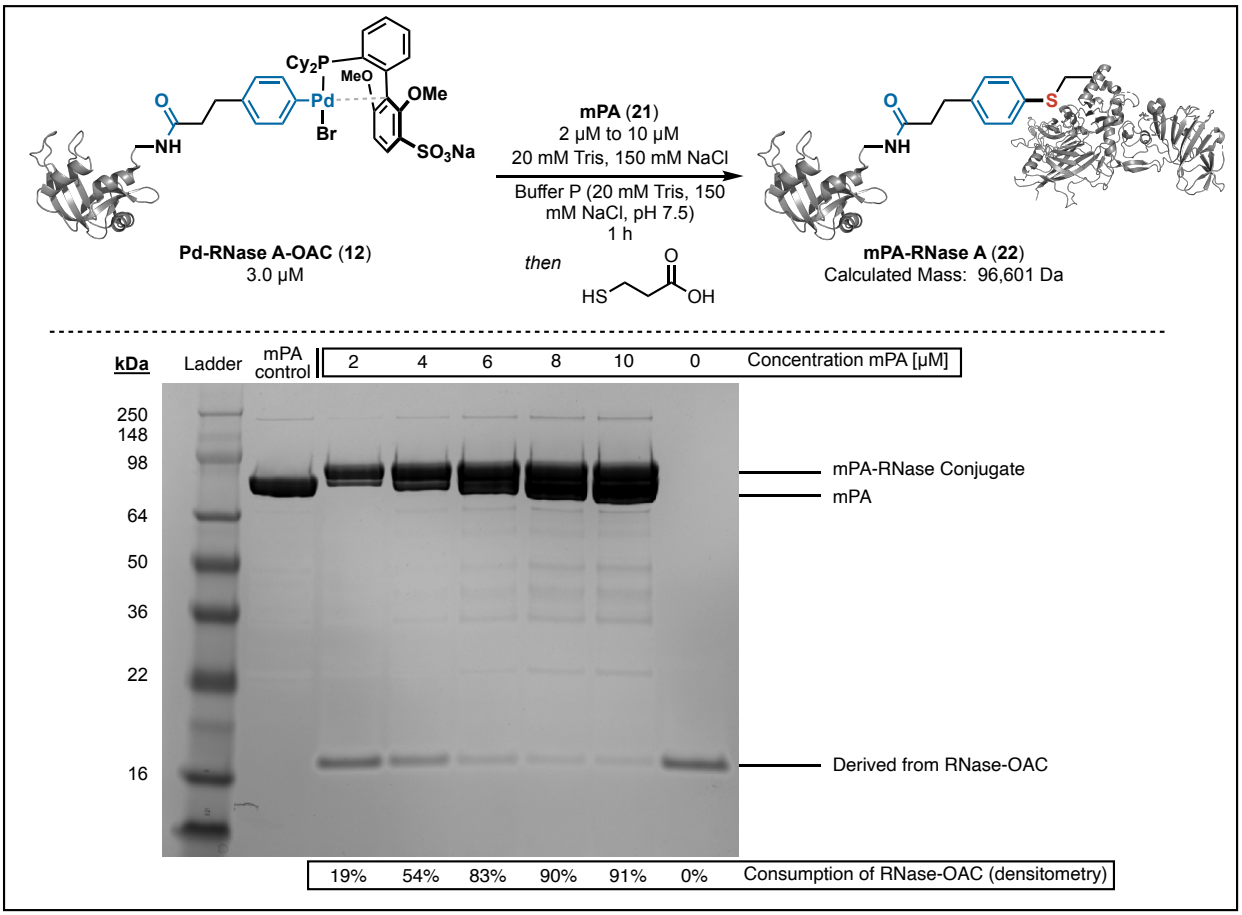


Fig. S3. Reaction with Pd-RNase A-OAC with varying concentration of mPA and analysis by SDS-PAGE and densitometry. Conversion of starting material was estimated using a loading standard (right-most lane) and the ratio of starting material present : initial loading used.

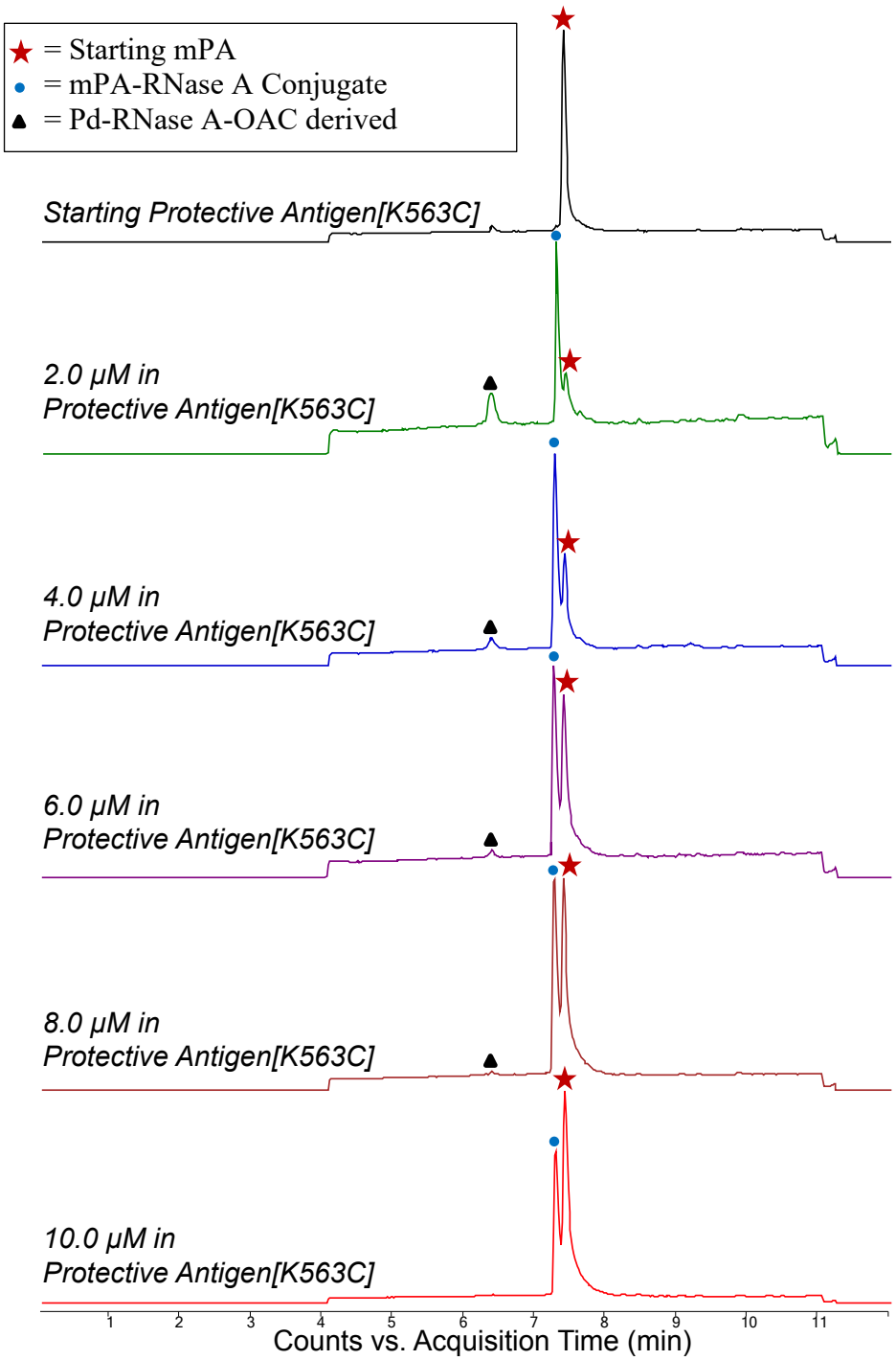
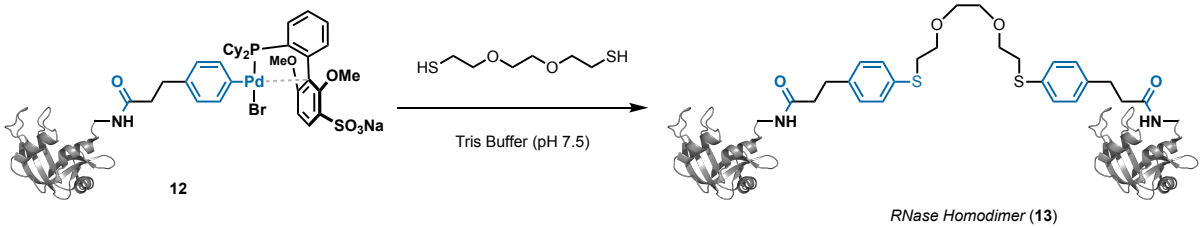


Fig. S4. Reaction with Pd-RNase A-OAC with varying concentrations of Protective Antigen [K563C] (mPA) and analysis by LC-MS [total ion count chromatogram shown]. In the reaction mixture at 10 μM in mPA, trace residual starting material was deconvoluted to give unmodified RNase A (MW: 13682 Da) as the major protein-containing species, proto-demetalation of Pd-RNase A-OAC (MW: 13815 Da), and Pd-RNase A-OAC quenched with 3-mercaptopropionic acid (MW: 13919 Da). Of note, starting Pd-RNase A-OAC contains approximately 5% unmodified RNase A as determined by TIC. Peaks are labelled above the maximum height of the peak with

the following symbols: red star, starting mPA; blue circle, conjugate mPA-RNase A; black triangle, products derived from RNase A.

5.1.3. Protein Homodimerization via Cross-Coupling



To a 2 mL centrifuge tube was added Pd-RNase A-OAC **12** (1.85 mg of a lyophilized solid) dissolved in Tris buffer (pH 7.5, 100 mM Tris, 150 mM NaCl). To this was added a solution of 2,2'-(ethylenedioxy)diethanethiol (40 µL, 139 µM solution in DMSO). The reaction was mixed by pipetting up and down 20× followed by a 5 min incubation time. This addition-incubation procedure was repeated 5×. The reaction was then filtered through a 0.22 µm nylon filter and purified by size-exclusion chromatography utilizing a HiLoad 26/600 Superdex 200 pg column with buffer P as eluent with 3-thiopropionic acid (pH 7.5, 20 mM Tris, 150 mM NaCl, 10 mM 3-mercaptopropionic acid). Fractions were analyzed by SDS-PAGE and fractions containing the desired dimer were pooled and buffer exchanged with 1× PBS Buffer and concentrated to a total volume of 2.79 mL and concentration determined by UV absorbance at 280 nm (0.24 mg/mL, 0.67 mg total, solution measured as 24.4 ppb Pd). The molar extinction coefficient was estimated by doubling the sequence of RNase A and using ExPASy SIB Bioinformatics Resource Portal (18880 M^{-1} cm^{-1}). The pooled fractions were additionally analyzed by LC-MS (method B, Fig. S15).

<u>Pd concentration as measured by ICP-MS Analysis</u>: 100 µL of the purified RNase A homodimer solution was sent to Merck for ICP-MS analysis and the measured concentration of the solution was determined to be 24.4 ppb.

Thus, the percent of Palladium in Protein is calculated as follows:

<u>*Note*</u>: A density of 1.00 mg/µL was used for the aqueous protein solutions.

Mass of Pd in the purified material after = Measured Pd content * total volume of the purified material * density of protein solution = (24.4 ppb) * 2790 µL * 1.00 mg/µL = 0.0000681 mg Pd

Mass Percent of Pd in Protein = (mass of palladium in solution/mass of protein in solution) *100 = (0.0000681 mg Pd)/(0.67 mg Protein) * 100 = 0.01 % Pd/Protein.

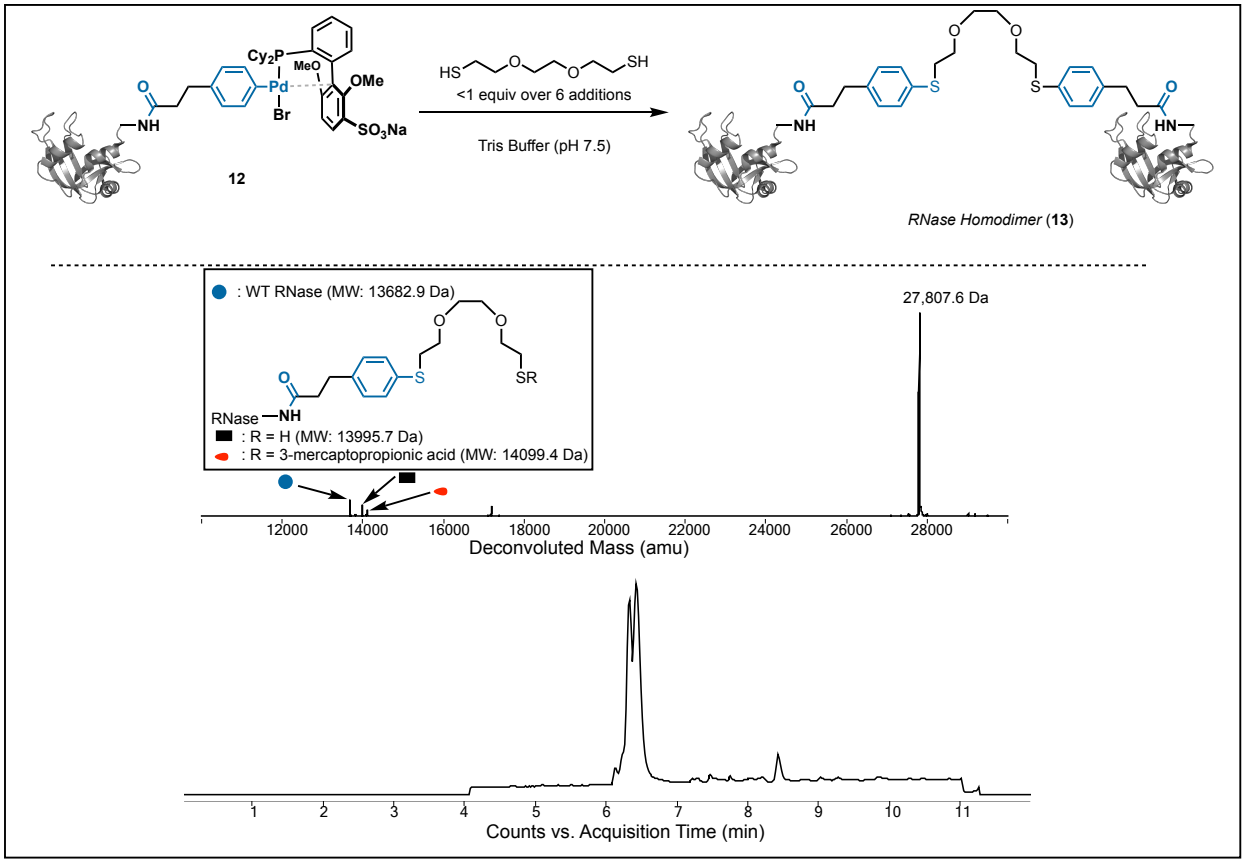


Fig. S5. Homodimerization of Pd-RNase A-OAC utilizing sub-stoichiometric additions of a dithiol. Top: reaction scheme. Conditions: Pd-RNase A-OAC [70 µM], Tris buffer (pH 7.5, 100 mM Tris, 150 mM NaCl). Bottom: LC-MS of RNase A homodimer purified by size-exclusion chromatography (SEC) and deconvolution of all proteinogenic peaks. Product after SEC contains unmodified RNase A (MW: 13682.9), and products derived from single thiol conjugation (R = H [M: 13995.7 Da] or 3-mercaptopropionic acid [M: 14099.4 Da]).

6. Antibody Labeling Experiments

6.1. Antibody Sequence and Source

Trastuzumab was purchased from Sydlabs (52 mg/mL in PBS 1× Buffer, pH 7.2) and stored at 4 ºC.

Table S5. Light Chain of Trastuzumab

Sequence[a]	Calculated mass (average)
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQ KPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQ PEDFATYYCQQHYTTPPTFGQGTKVEIKRTRTVAAPSVF IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY ACEVTHQGLSSPVTKSFNRGEC	23700.3 Da

[a] Cysteine residue highlighted in red.

Table S6. Heavy Chain of Trastuzumab

Sequence[a]	Calculated mass (average)
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWG QGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG	49124.3 Da

[a] Cysteine residue highlighted in red.

6.2. Synthesis of Trastuzumab-RNase A Conjugate

In an Eppendorf tube, to a solution of Trastuzumab **14** (66 µL, 57 µM) in 1× PBS, TCEP•HCl (1.5 µL, 25 mM solution in water neutralized to pH 6.7) and Tris buffer (7.5 µL, 1 M, pH 8.0) were added. The reaction mixture was pipetted up and down 10× and incubated in a water bath at 37 ºC for 2 h. The final concentrations for the reduction step were: 50 µM antibody, 500 µM TCEP, and 0.1 M Tris.

Tris buffer (30 µL, 1.0 M, pH 8.0) was added to the aqueous solution of Pd-RNase A-OAC (645 µL, 116 µM in water). The partially reduced antibody (75 µL, 50 µM solution) was added to a solution of Pd-Rnase A-OAC **12** (300 µL, 250 µM in water) and the reaction mixture was mixed by pipetting up and down 10×. The reaction mixture was allowed to incubate for 12 h. The final reaction conditions are: 5 µM antibody **14**, 100 µM Pd-RNase A-OAC **12**, 0.05 M Tris pH 8.0.

After the 12 h incubation time, the reaction mixture was quenched by the addition of 3-mercaptopropionic acid (25 µL, 30 mM in buffer P, 7 equiv relative to the amount of palladium reagent used), and the resulting solution was incubated at room temperature for 15 min. A 20 µL aliquot of the reaction mixture was used for analysis and the remainder was flash frozen and stored in a -80 ºC freezer. *N*-linked glycans were removed by the addition of 0.5 µL of PNGase F (New England BioLabs) to this 20 µL aliquot of quenched antibody solution, the resulting mixture incubated for 1 h at 45 ºC. Excess RNase A was removed by buffer exchange to Tris (0.1 M, pH 8.0) using a 50K spin concentrator (EMD Millipore). The resulting antibody solution was reduced with 5 µL of 200 mM TCEP solution (pH 8.0) and incubated for 30 min at 45 ºC. LC-MS analysis of this reduced antibody was conducted using method B. SDS-PAGE analysis was performed using Invitrogen Bolt 4-12% Bis-Tris Plus Gels (10-wells), utilizing pre-stained SeeBlue™ Plus2 pre-stained molecular weight standard with Laemmli's buffer as a loading dye.

6.3. Trastuzumab labeling with EGP peptide

In an Eppendorf tube, to a solution of Trastuzumab **14** (350 μM, 8.7 μL in 1× PBS), MOPS buffer was added (441.3 μL, 0.1 M, pH 7.5), followed by the addition of DMF (22 μL). Lastly, to this was added a solution of palladium transfer reagent **10b** (28 μL, 1.19 mM in DMF) and the reaction mixed by pipetting up and down 10×. The reaction was subsequently placed on a nutating mixer for 1 h. After this time, the excess of Pd reagent was removed by exchanging buffer into MOPS buffer (0.1 M, pH 7.5) : DMF (9 : 1) using a 50K spin concentrator (EMD Millipore).

To 40 μL of the resulting solution, EGP peptide **AVGALEGPRNQDWLGVPRQLC 17** (with *C*-terminal Cys for conjugation) (60 μL, 80 μM in MOPS buffer, 0.1 M, pH 7.5) was added. The reaction was allowed to incubate at room temperature for 12 h and subsequently quenched by the addition of 3-mercaptopropionic acid (2.5 μL, 10 mM in Buffer P, 5 equiv relative to the amount of Pd reagent used), and the resulting solution was incubated at room temperature for 15 min. *N*-linked glycans were removed by the addition of 0.5 μL of PNGase F (New England BioLabs) to the antibody solution and the resulting mixture was incubated for 1 h at 45 ºC. The antibody solution was reduced with 5 μL of 200 mM TCEP solution (pH 8.0) and incubated for 30 min at 45 ºC. LC-MS was performed using method A. SDS-PAGE analysis was performed using Bolt 4-12% Bis-Tris Plus Gels (10-wells), utilizing pre-stained SeeBlue Plus2 molecular weight standard with Laemmli's buffer as a loading dye.

7. Chemical Stability and Biological Activity of Oxidative Addition Complexes

7.2. Protein-Palladium Oxidative Addition Complex Stability in Solution

The stability of Pd-RNase A-OAC in aqueous solution was evaluated by the analysis at final concentrations of 1.0 mg/mL (69 µM) in water (no buffer added, final pH ~3.5), in 25 mM acetate buffer (pH 5.0), in 25 mM sodium phosphate buffer (pH 7.0), and in 25 mM Tris buffer (pH 8.0). To prepare these solutions, Pd-RNase A-OAC was dissolved in water to a concentration of 1.33 mg/mL (91 µM). To 30 µL aliquots of this solution, 10 µL of water or 10 µL of a corresponding buffer solution was added. The solutions were analyzed by LC-MS using method A immediately after preparation, and again for 1, 2, and 3 days. A small peak at ~7.3 min corresponds to unmodified RNase A. For the unbuffered solution, the peak at ~7.7 min has a mass of 14303.8 Da (marked with a blue circle), corresponding to the loss of the Pd ([M-PdNaBr+H]). For the samples at pH 5.0 and pH 7.0, the peak at ~7.5 min has a mass of 13815.9 Da (marked above the peak with a black square), corresponding to proto-demetallation.

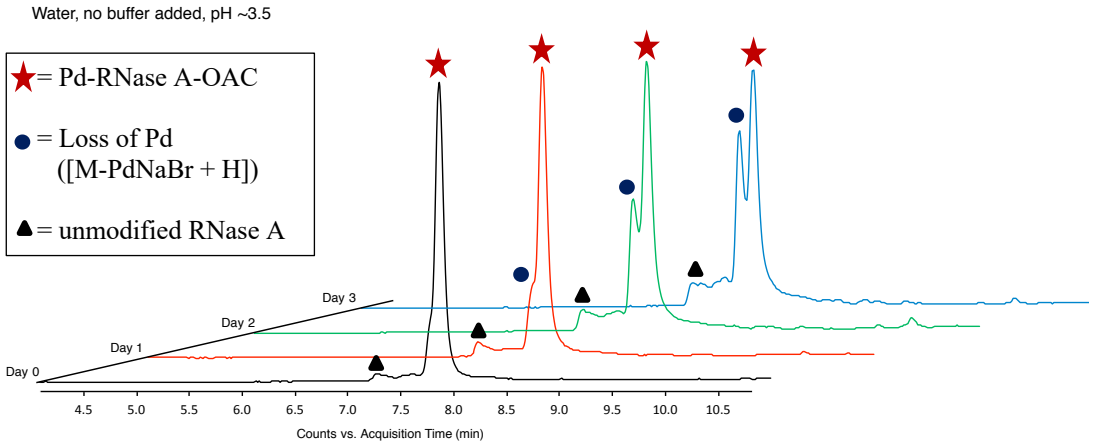


Fig. S6. Stability of Pd-RNase A-OAC in unbuffered water at pH reaching approximately 3.5.

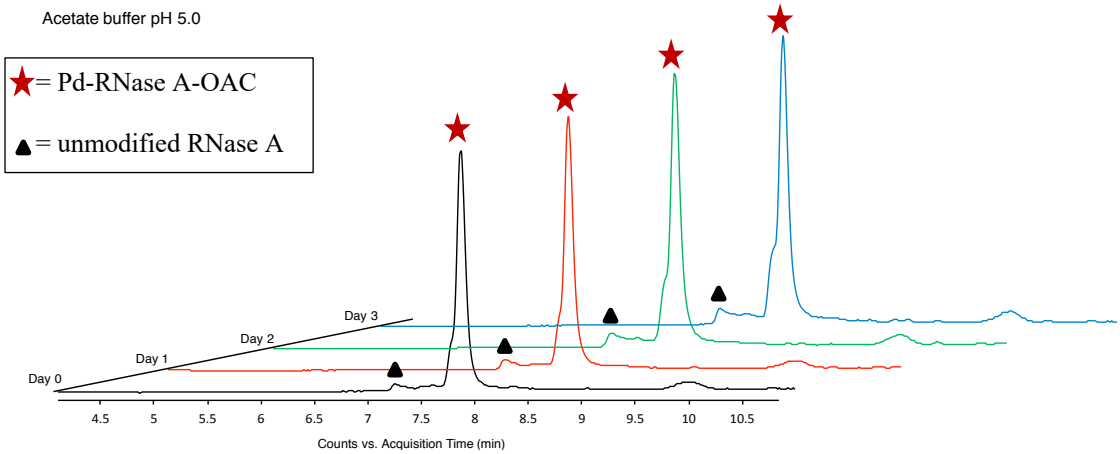


Fig. S7. Stability of Pd-RNase A-OAC in 25 mM acetate buffer at pH 5.0.

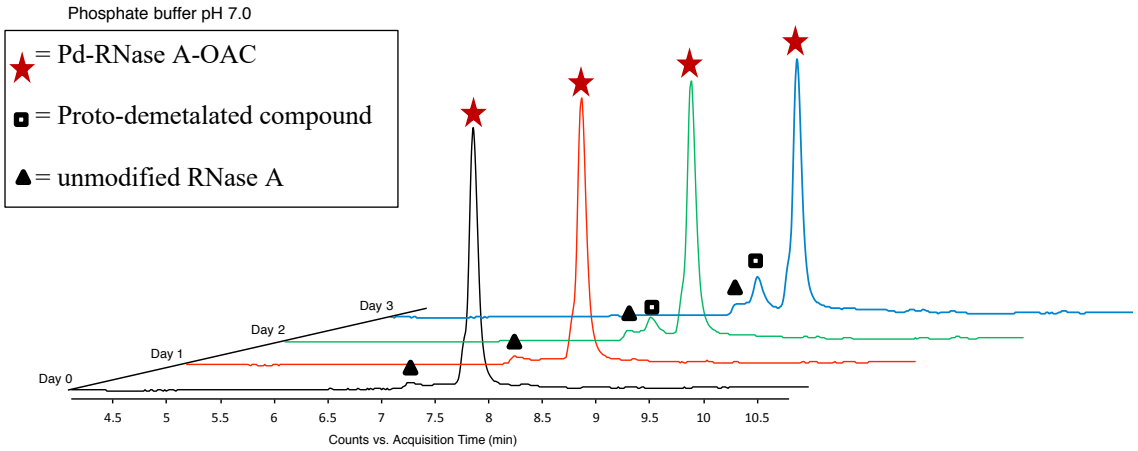


Fig. S8. Stability of Pd-RNase A-OAC in sodium phosphate buffer at pH 7.0.

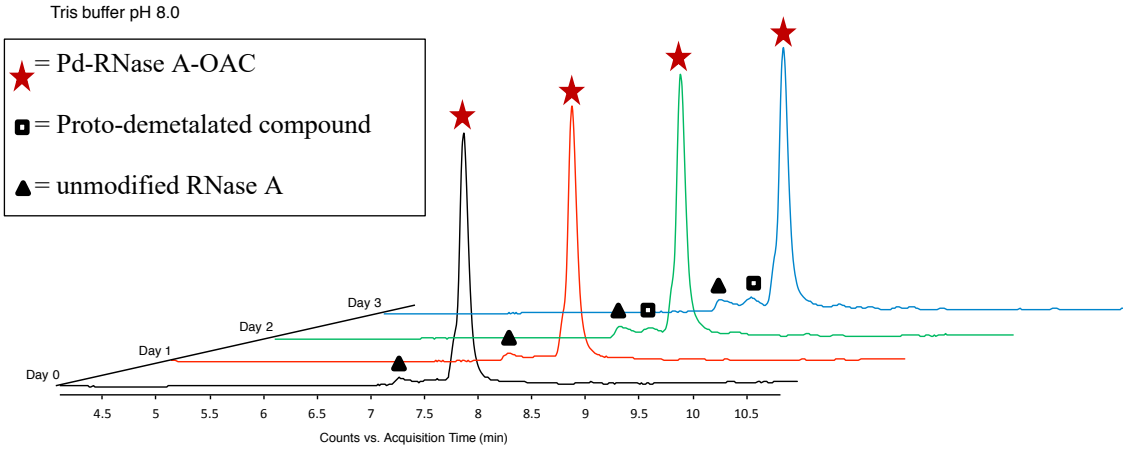


Fig. S9. Stability of Pd-RNase A-OAC in Tris buffer at pH 8.0.

7.3. Enzymatic Assay of Pd-lysozyme-OAC

The measurement of lysozyme activity was performed as described previously.[5]

In a 50 mL falcon tube, lyophilized *Micrococcus lysodeikticus* cells (ATCC 4698) (9 mg, Sigma, M3770) were suspended in potassium phosphate buffer (30 mL, 0.1 M, pH 6.2). 200 µL of cell suspension was placed in the wells of a 96-well plate, and 50 µL of buffered solution containing lysozyme was added to each well and quickly mixed by pipetting up and down, and the measurement of A_{450nm} was started immediately. Reactions were monitored for 7 min, with no change in A_{450nm} of blank sample (cells in buffer without lysozyme) observed. A calibration curve was obtained using egg white lysozyme (Amresco, ultra pure grade, 0663). The reactions were run in triplicate. The activity of lysozyme and lysozyme conjugates was calculated from the slopes of A_{450nm} regression by converting the decrease of absorbance in 0.001/min to 1 unit of enzyme under our conditions (potassium phosphate buffer, 0.1 M, pH 6.2, 25 °C). The specific activity of a batch of egg white lysozyme was 71,000 units/mg determined by this method. Pd-lysozyme-OAC demonstrated a specific activity of 27,000 units/mg. When a buffered solution of 3-mercaptopropionic acid (20 equiv) was added to the same aliquot to remove the Pd-complex from enzyme prior measurements, the specific activity of the enzyme was 63,000 units/mg.

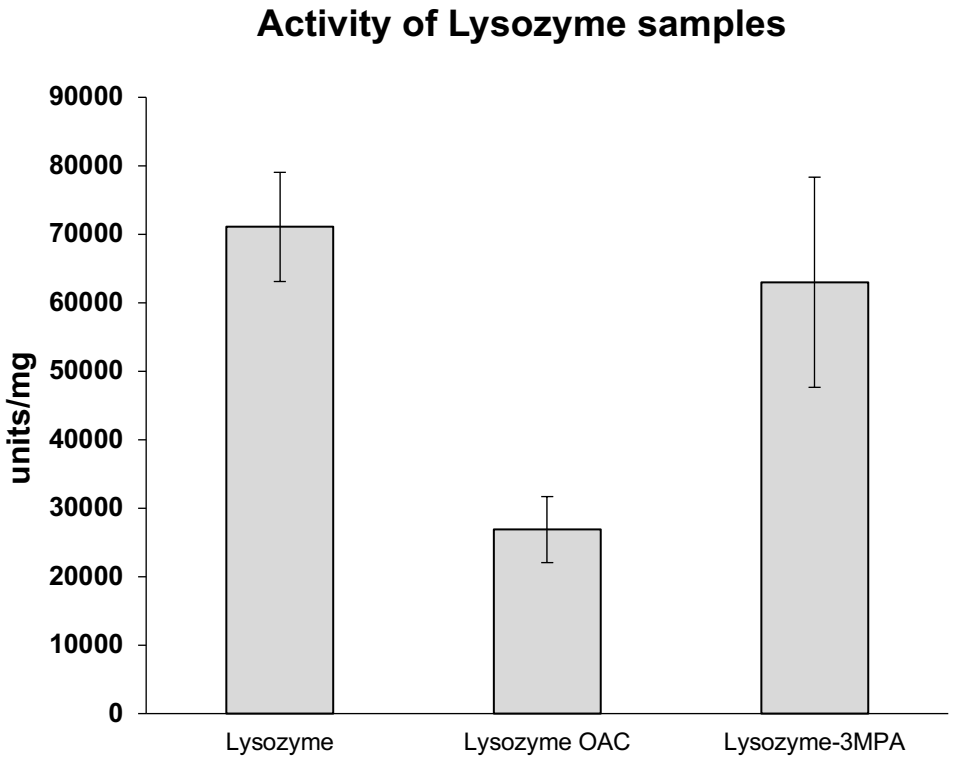


Fig. S10. Specific activity of native lysozyme (71,000 ± 8,000 units/mg), Pd-lysozyme-OAC (27,000 ± 4,800 units/mg), and lysozyme conjugated with 3-mercaptopropionic acid (63,000 ± 15,000 units/mg). Assays were carried out in triplicate. (3MPA, 3-mercaptopropionic acid).

7.4. Enzymatic Assay of Pd-RNase A-OAC

The measurement of ribonucleolytic activities was performed as described previously.[6]

The substrate was tetranucleotide 6-FAM–dArU(dA)$_2$–6-TAMRA (6-FAM: 5′-6-carboxyfluorescein group; 6-TAMRA, 3′-6-carboxytetramethylrhodamine group), was purchased from Integrated DNA Technologies (Coralville, IA).

The enzymatic activities of RNase A and Pd-RNase A-OAC were determined by measuring the initial velocity of cleavage of 6-FAM-dArU(dA)$_2$–6-TAMRA (20 nM). The fluorescence measurements were made with a Tecan M1000 fluorescence plate reader (Maennedorf, Switzerland) by excitation at 493 nm and observation of emission at 515 nm. Assays were performed in 96-well plates (Corning) at 25 °C in a ribonuclease-free buffer (20 mM MES, 0.10 M NaCl, pH 6.0). The final concentration of enzymes in wells was 5 pM. All assays were carried out in quadruplicates. Values of k_{cat}/K_M were determined from initial velocity data, as previously described.[6]

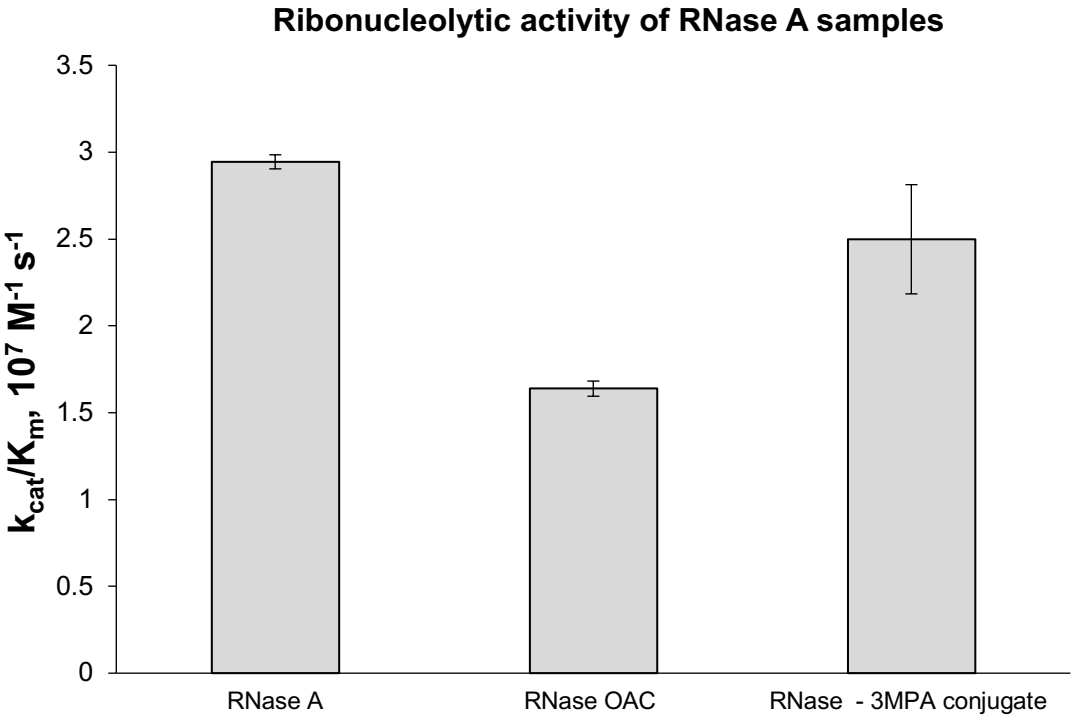
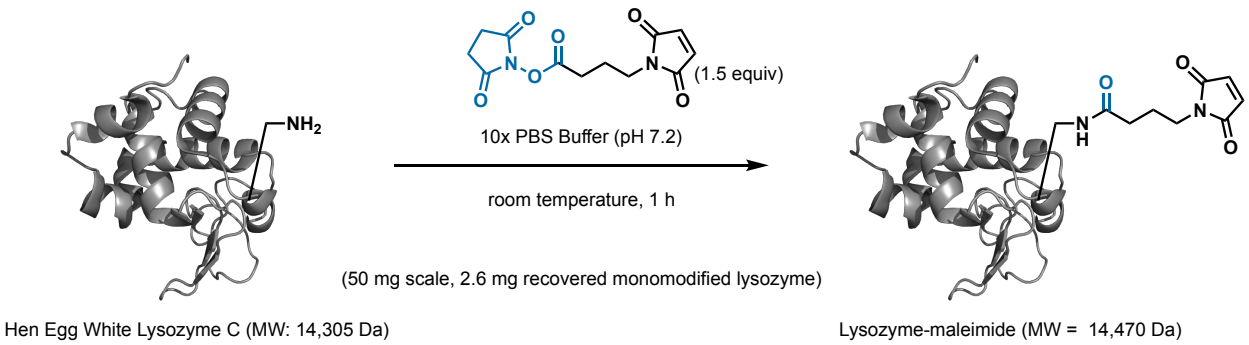


Fig. S11. Ribonucleolytic activity of native RNase A (k_{cat}/K_M = 2.94 ± 0.04 × 10^7 M^{-1} s^{-1}), Pd-RNase A-OAC (1.64 ± 0.04 × 10^7 M^{-1} s^{-1}), and RNase A-3-mercaptopropionic acid (2.50 ± 0.31 × 10^7 M^{-1} s^{-1}). All assays were carried out in quadruplicate. (3MPA, 3-mercaptopropionic acid).

8. Lysozyme-Maleimide Preparation and Competition Experiments with Pd-lysozyme-OAC

8.1. Preparation of Lysozyme-Maleimide



A procedure identical to that for the preparation of Pd-lysozyme-OAC **19** was used except utilizing Hen egg white lysozyme C (45.7 mg, 1.00 equiv, 3.19 µmol) and bifunctional reagent *N*-succinimidyl 3-maleimidoprionate (1.34 mg, 1.50 equiv, 4.79 µmol) dissolved in DMSO (5.0 mL). (See section 5.1.1 for preparation of Pd-lysozyme-OAC **19**). Analysis of fractions after RP-HPLC identified a single fraction that contained predominantly mono-modified lysozyme (**20**) (2.6 mg), which was dissolved to a concentration of 1.0 mg/mL in water. See Fig. S12.

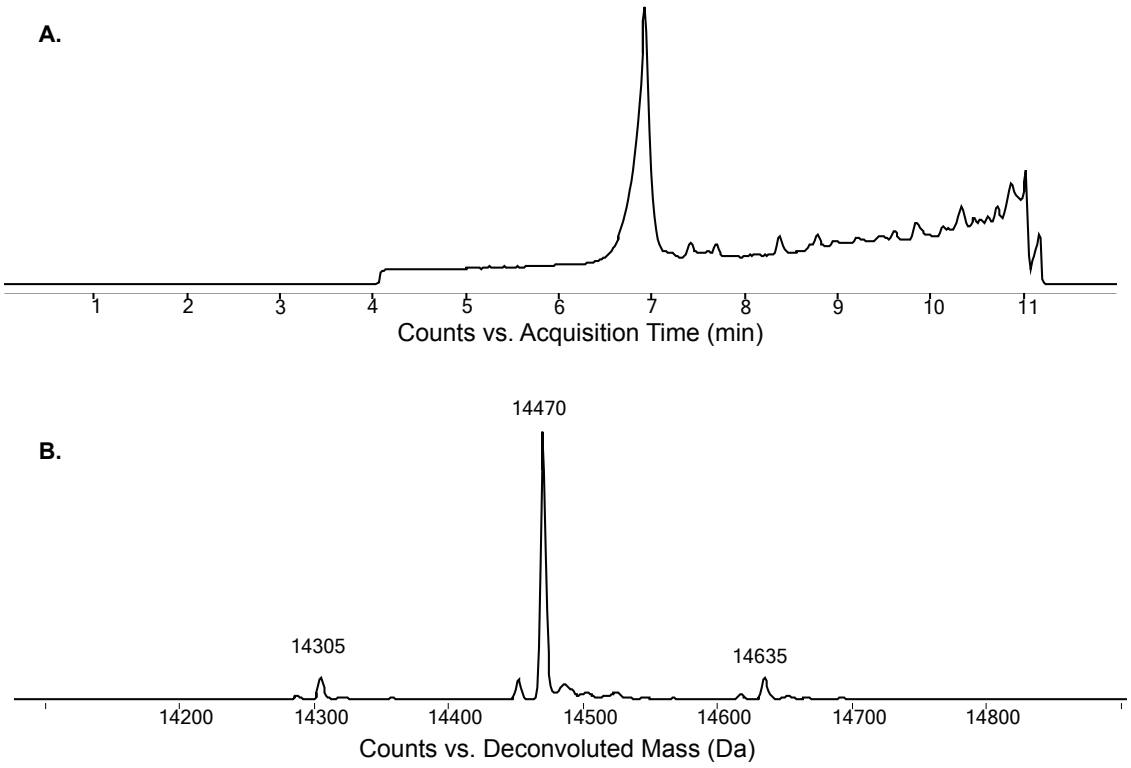
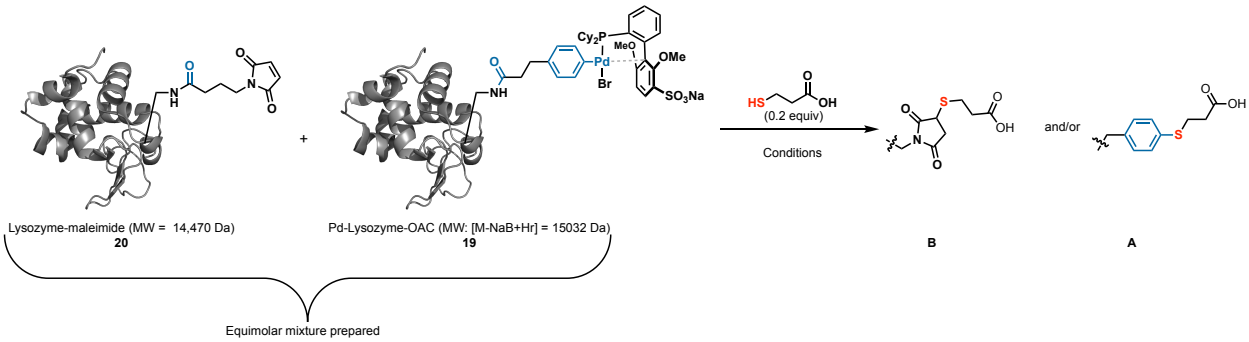


Fig. S12. Total ion count chromatogram (A) and deconvoluted mass spectrum (B) of lysozyme-maleimide. Lysozyme-maleimide purified by RP-HPLC with acetonitrile and water containing 0.1% TFA as eluent. Lysozyme, MW = 14305 Da; mono-modified lysozyme, MW = 14470; bis-modified lysozyme, MW = 14635 Da.

8.2. Competition Experiments



To a PCR tube was added Pd-lysozyme-OAC **19** (1 mg/mL of a lyophilized solid in water) and lysozyme-maleimide **20** (1 mg/mL of lyophilized protein in water), and the mixture was evaluated by LCMS (Method B). The mixture was adjusted accordingly and evaluated by LCMS until a ~1:1 mixture resulted as monitored both by total ion current and UV absorbance at 214 nm. The final concentration of both species in solution was ~0.5 mg/mL. A solution of 3-mercaptopropionic acid was prepared by the serial dilution of neat 3-mercaptopropionic acid (1.0 µL) diluted with water (999 µL) (solution A). To a separate Eppendorf tube was prepared a new solution (solution B) by adding solution A (50 µL) to water (950 µL) and mixed by pipetting up and down. A last solution was prepared (solution C) by the dilution of 10 µL of solution B with 990 µL of water. Final concentration, solution C: 5.74 µM solution of 3-mercaptopropionic acid in water.

To a set of PCR tubes was added the lysozyme-maleimide/lysozyme-OAC mixture (3.0 µL, ~0.5 mg/mL each) followed by the addition of the appropriate buffer (17 µL either of: pH 3.0, 100 mM citric acid buffer; pH 5.0, 100 mM citric acid buffer; pH 7.0, 100 mM sodium phosphate buffer; pH 7.5, buffer P [20 mM Tris, 150 mM NaCl]; pH 8.0, 100 mM Tris buffer) and mixed by pipetting up and down 20×. Last, to each reaction tube was added 3.0 µL of solution C (5.74 µM 3-mercaptopropionic acid in water). The reactions were mixed by pipetting up and down 30× and allowed to incubate for 1 h. After 1 h, the reactions were analyzed by LCMS (method B).

See Fig. S13 for results.

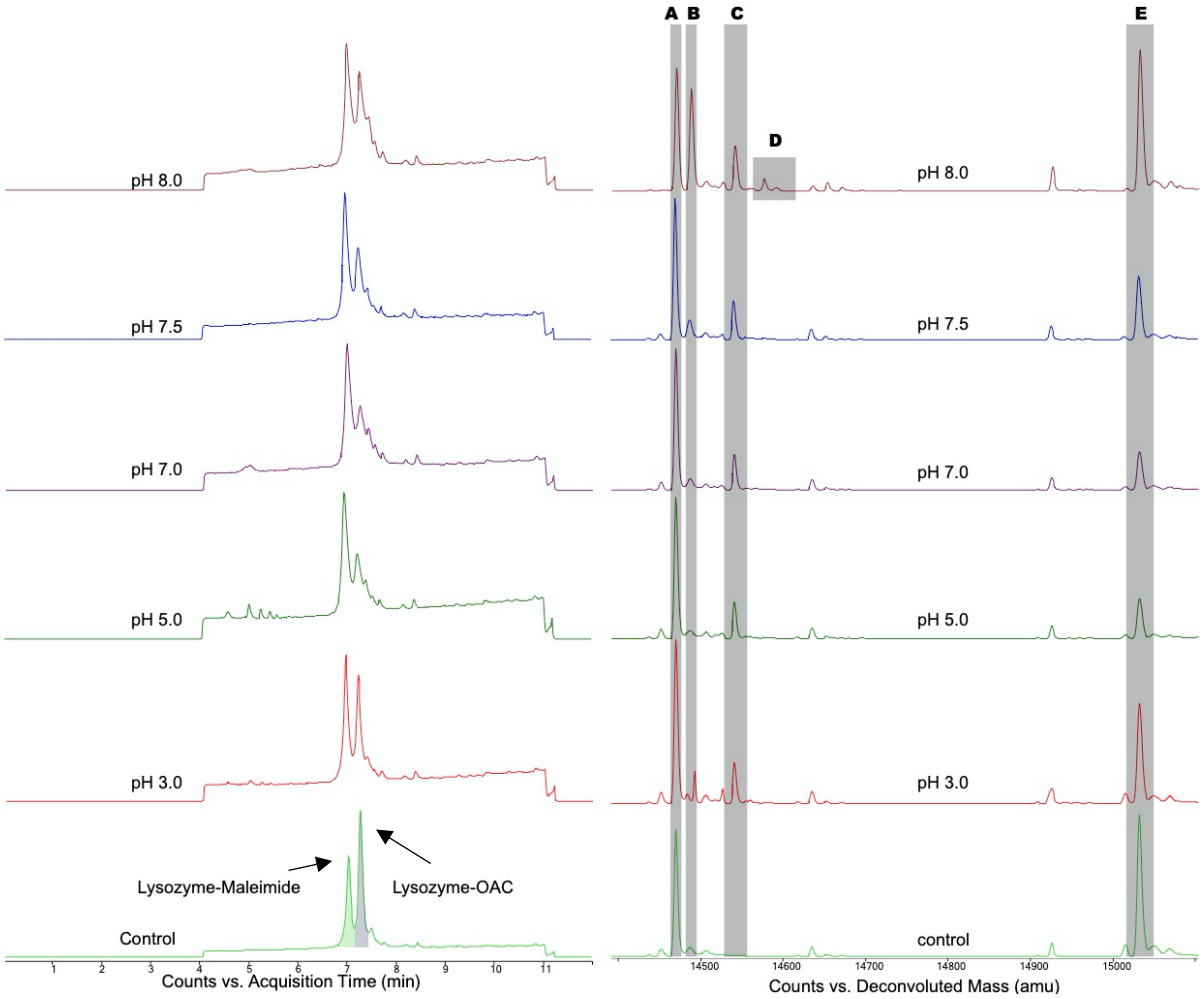


Fig. S13. Pd-Lysozyme-OAC outcompetes lysozyme-maleimide in competition experiments from pH 3.0-8.0. LC-MS Total ion current spectrum (left) and deconvolution data of all proteinogenic peaks (right) for experiments evaluating relative rates of 3-mercaptopropionic acid bioconjugation with Pd-lysozyme-OAC **19** and lysozyme-maleimide **20** at pH 3.0-8.0. A, MW = 14471 Da, lysozyme-maleimide **20**; B, MW = 14488 Da, hydrolyzed lysozyme-maleimide; C, MW = 14542 Da, Pd-lysozyme-OAC **19** conjugation with 3-mercaptopropionic acid; D, MW = 14577 Da and 14591 Da, lysozyme-maleimide conjugation with 3-mercaptopropionic acid and it's hydrolyzed form; E, MW [M-NaBr+H] = 15032 Da, Pd-lysozyme-OAC **19**. Only the case of pH 8.0 is the 3-mercaptopropionic acid conjugate observed with lysozyme-maleimide **20** at a B : C ratio of 3 : 1.

8.3. Protein-Protein Cross-Coupling of Lysozyme-Maleimide or Pd-lysozyme-OAC with mPA

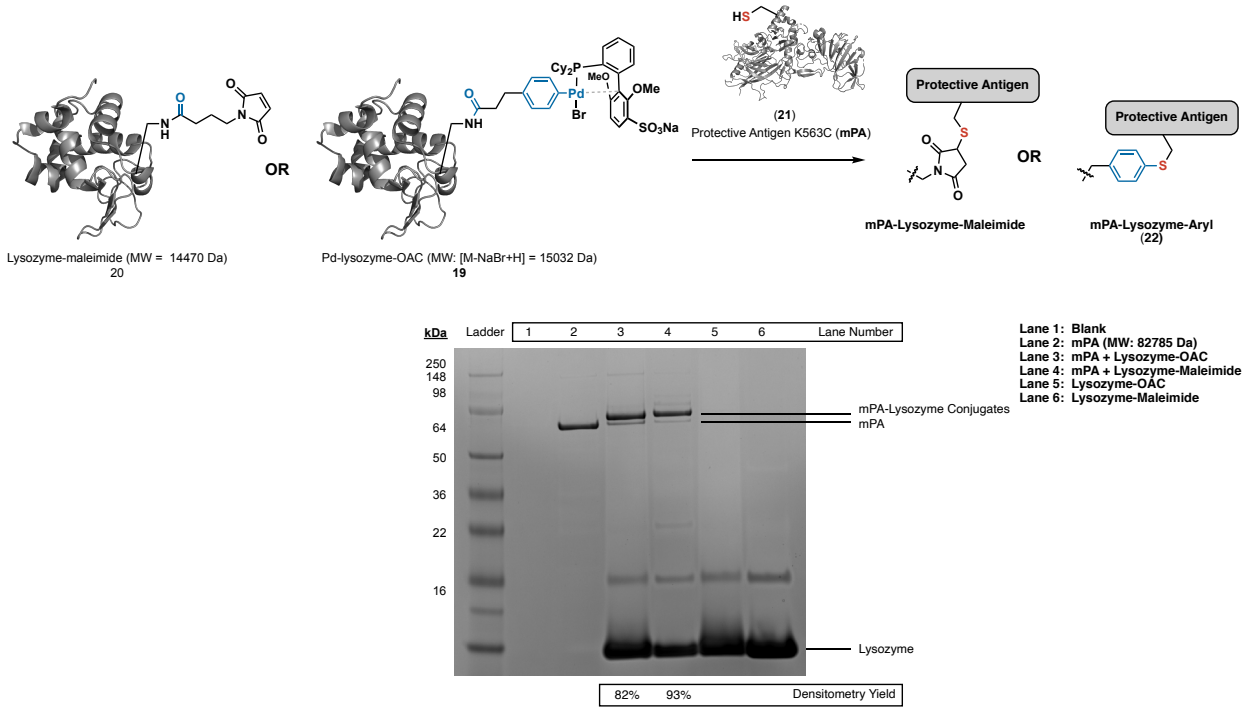
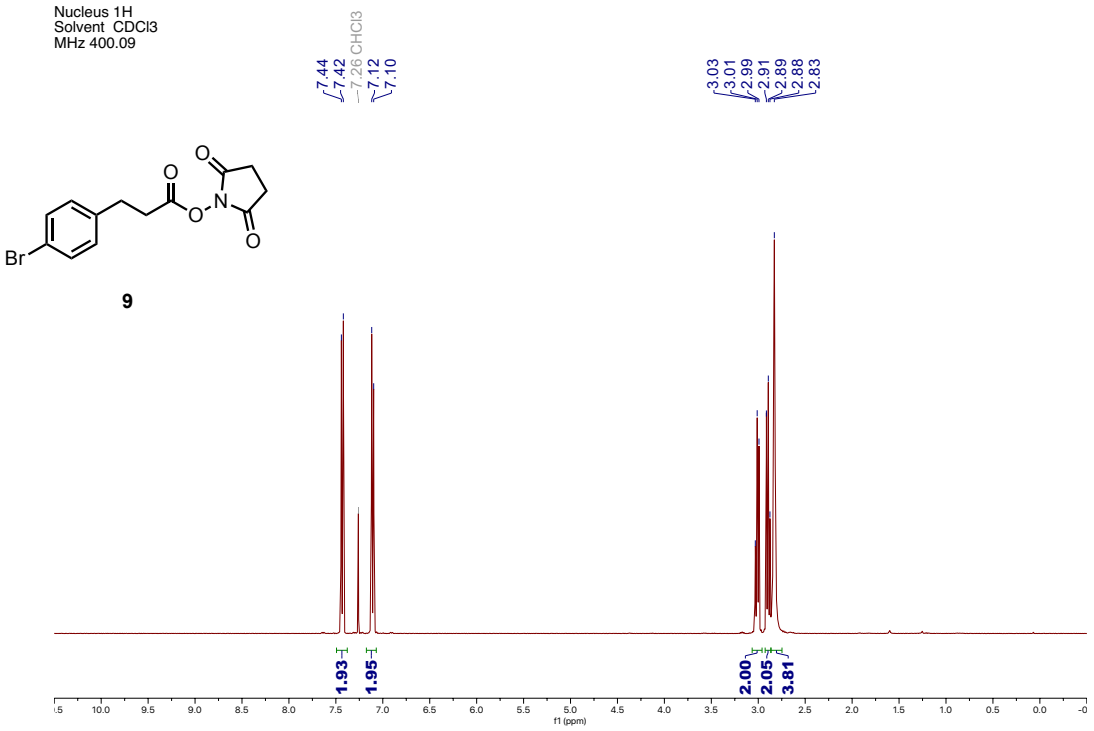


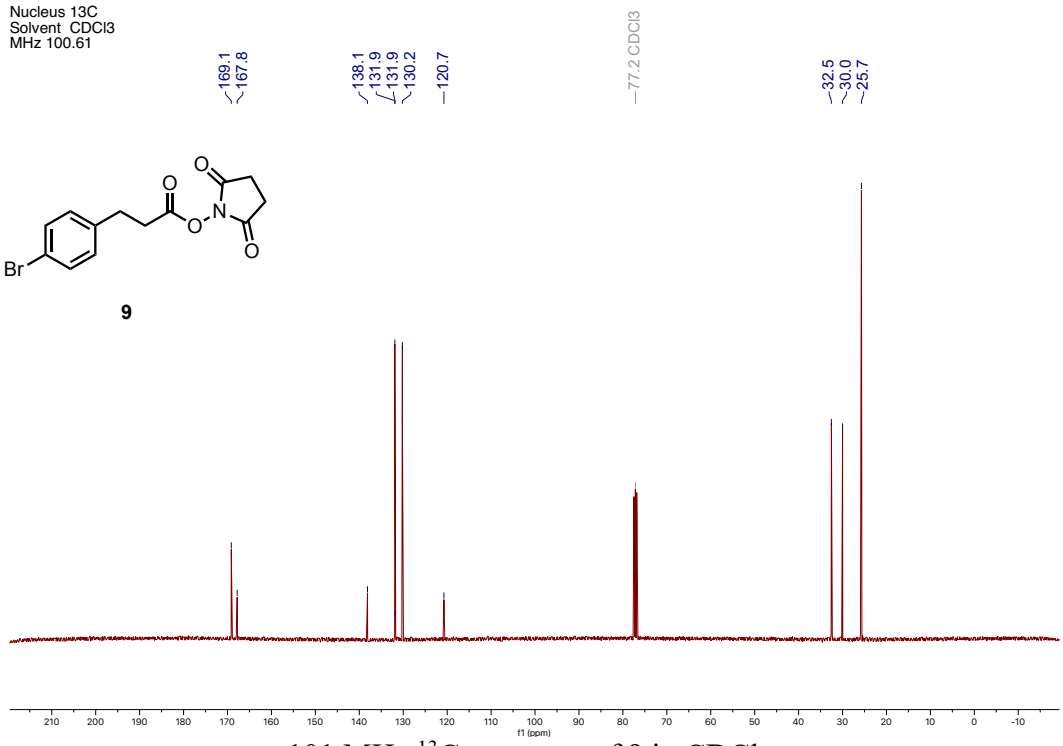
Fig. S14. Comparison of conjugation efficiencies with lysozyme-maleimide or Pd-lysozyme-OAC with protective antigen [K563C] (mPA).

9. NMR Spectra

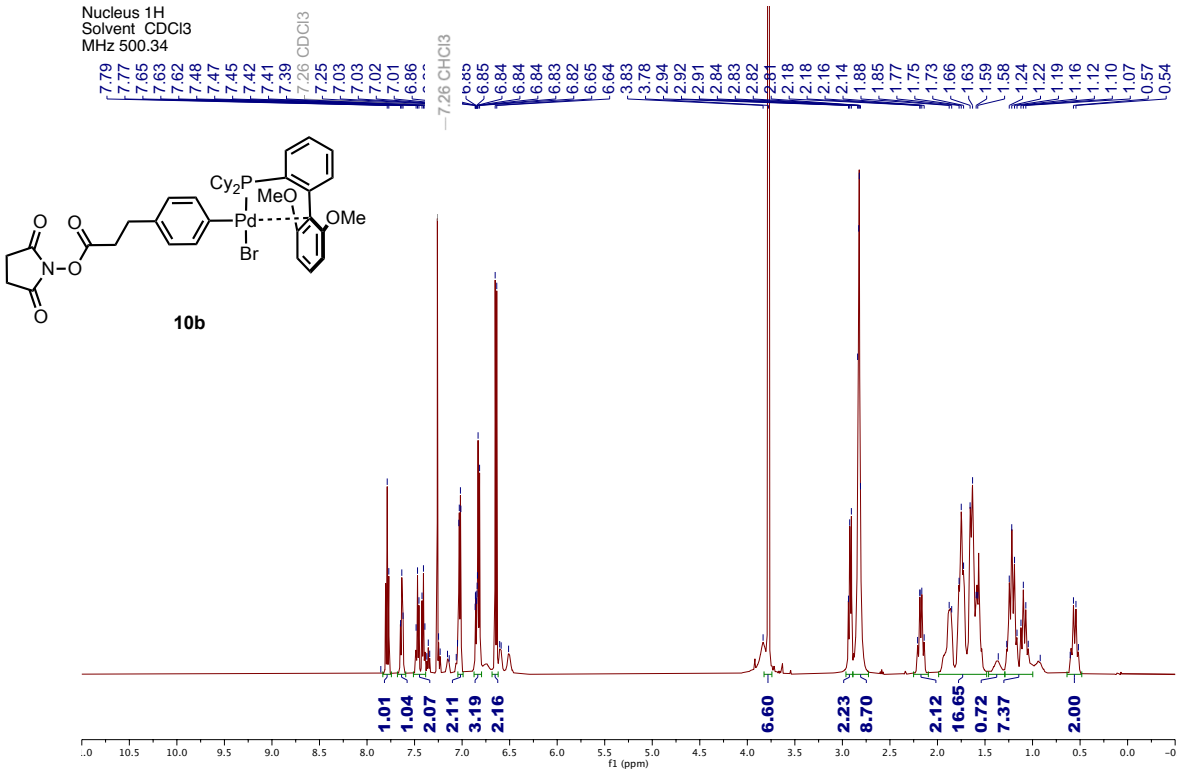
9.1. NMR Spectra of Small-Molecules



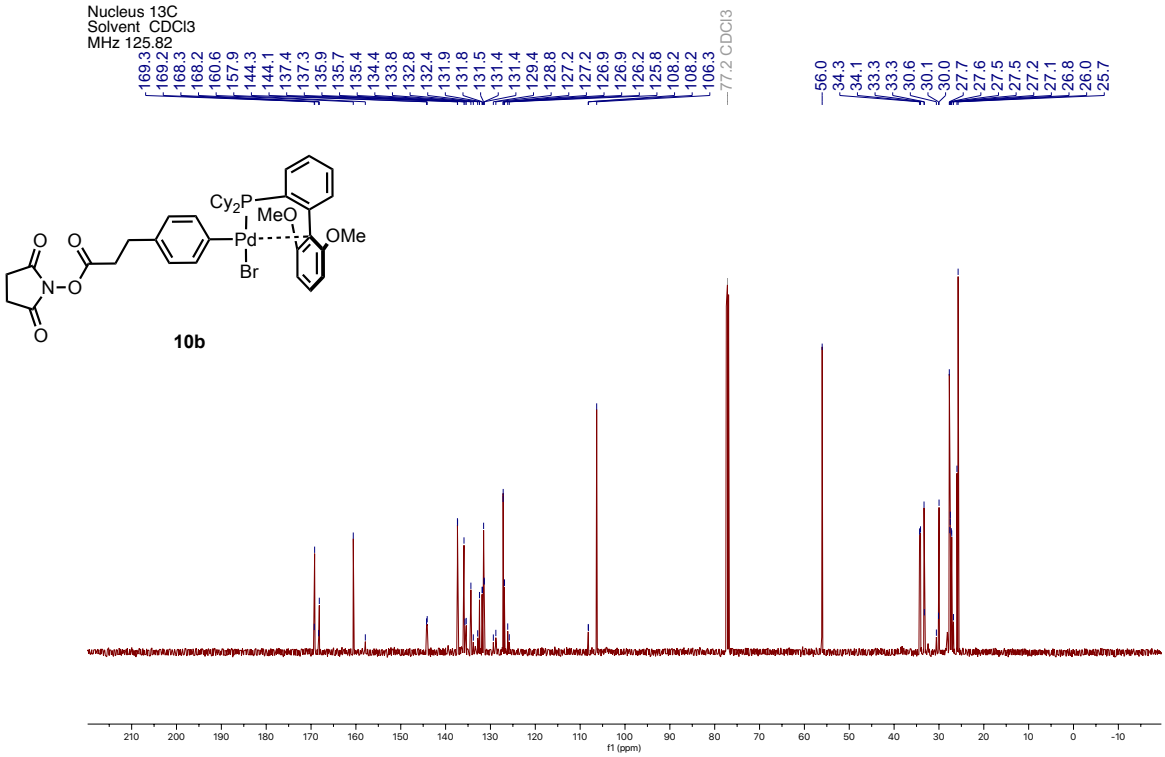
400 MHz ^{1}H spectrum of **9** in $CDCl_3$.



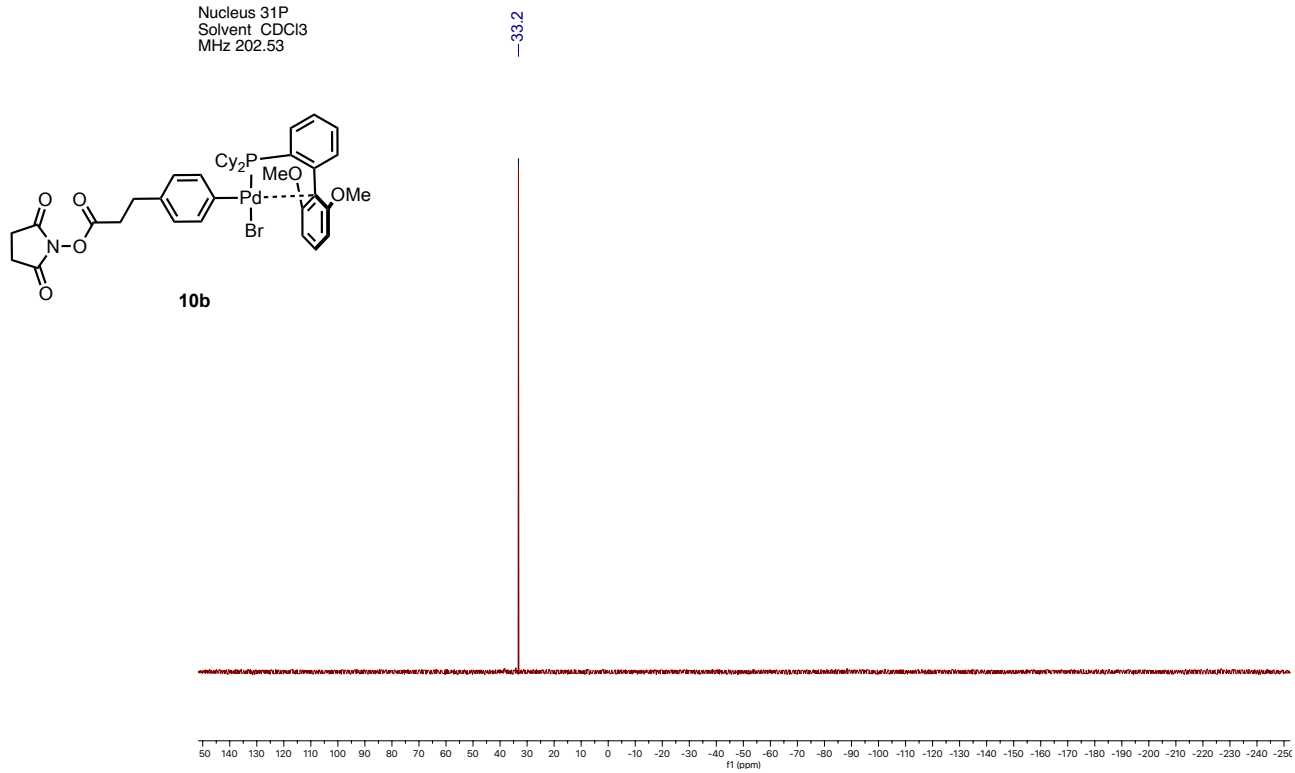
101 MHz ^{13}C spectrum of **9** in $CDCl_3$.



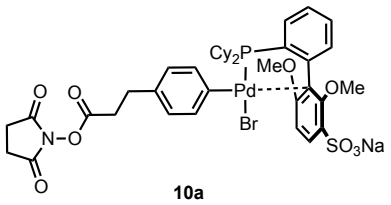
500 MHz ^{1}H spectrum of **10b** in $CDCl_3$.



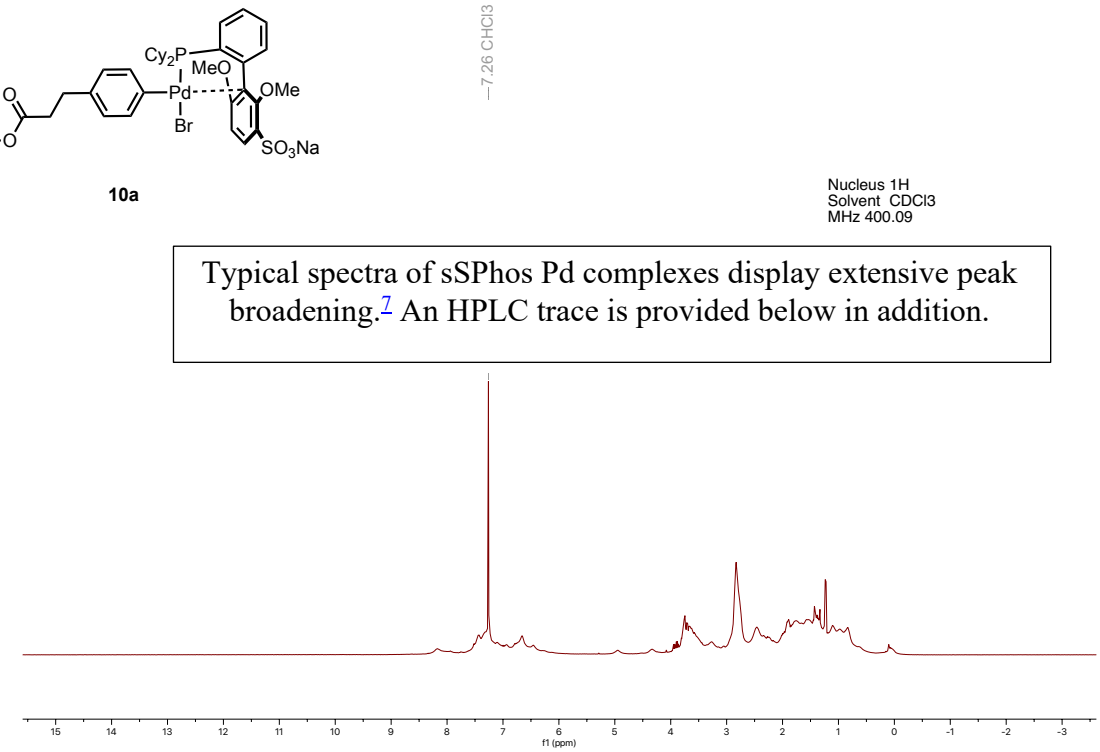
126 MHz ^{13}C spectrum of **10b** in $CDCl_3$.



203 MHz ^{31}P spectrum of **10b** in $CDCl_3$.

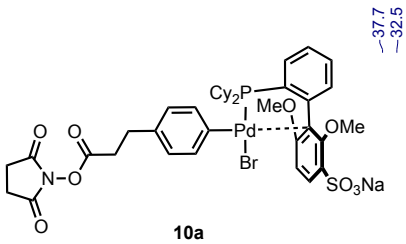


10a

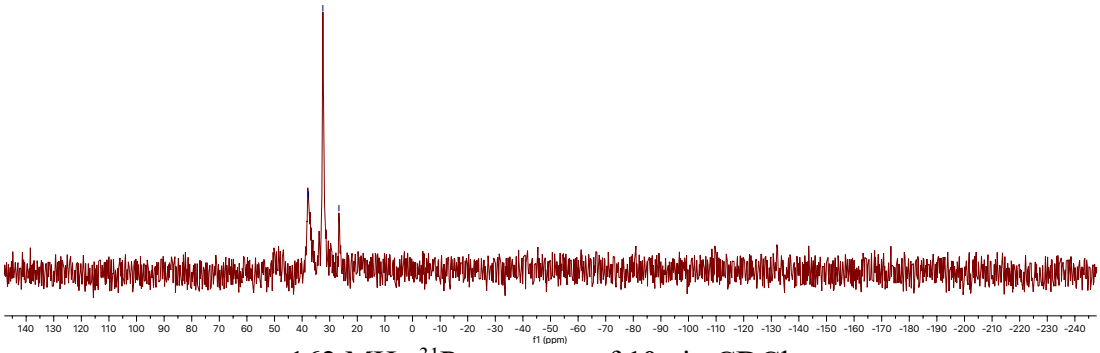


Typical spectra of sSPhos Pd complexes display extensive peak broadening.[7] An HPLC trace is provided below in addition.

400 MHz ^{1}H spectrum of **10a** in $CDCl_3$. sSPhos-Pd complexes display extensive peak broadening, thus, in addition to this spectrum, an HPLC trace of the material is provided below.

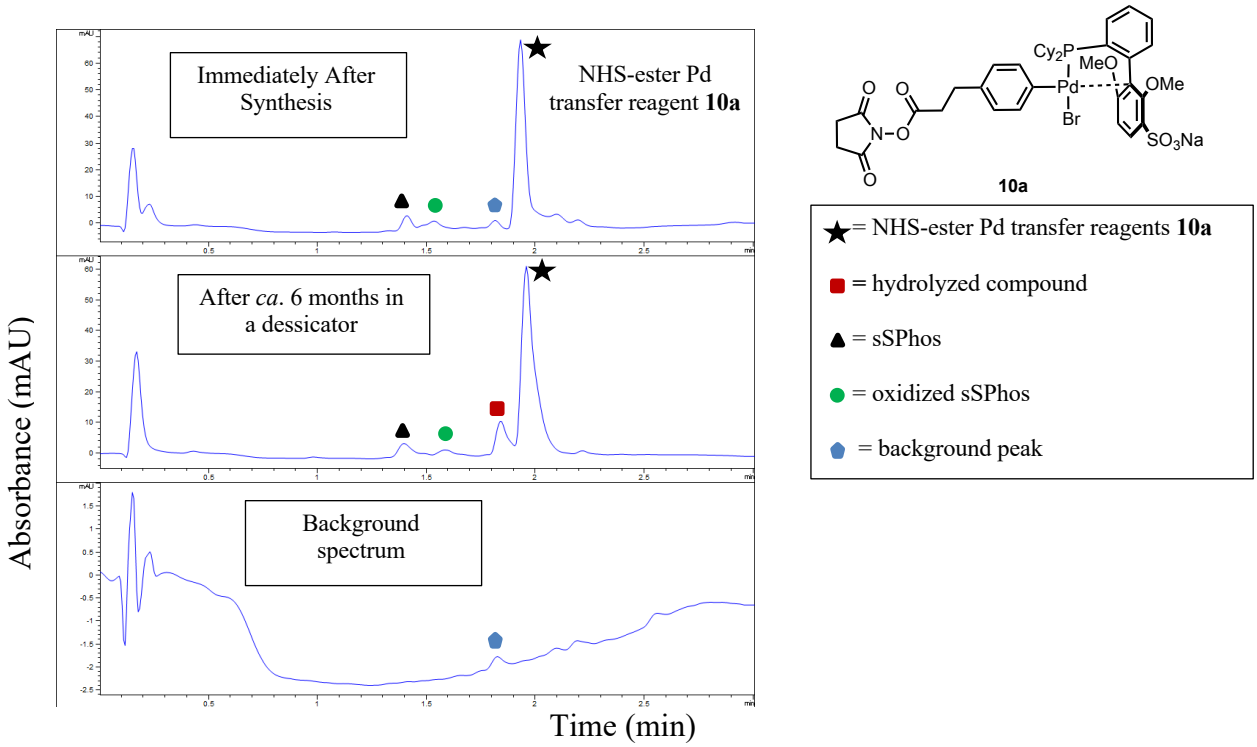


10a

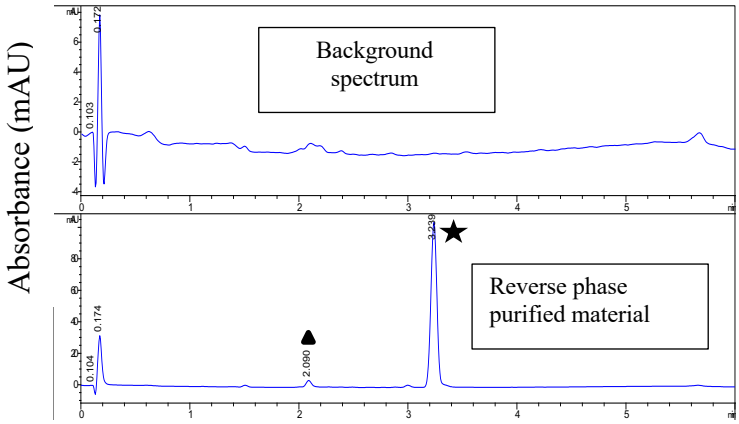


162 MHz ^{31}P spectrum of **10a** in $CDCl_3$.

A. HPLC chromatogram of crude **10a**



B. HPLC chromatogram of purified **10a**



(A) HPLC chromatogram of reagent **10a** within hours after synthesis and after *ca*. 6 months. After *ca*. 6 months of storage, the compound had shown ~10% hydrolysis as determined by integration of the peaks after HPLC. The analysis was performed on an Agilent 6120 LC/MS instrument with an Agilent 1260 binary pump on an Accucore C18 LC column and monitored at a wavelength of 254 nm. A gradient from 7% **B** to 95% **B** was run over 2.5 min, then held isocratic at 95% **B** for 0.5 min (Solvent **A**: 0.1% TFA in water, Solvent **B**: 0.1% TFA in CH_3CN). The nominal mass of reagent **10a** (retention time t_R = 1.95 min) was observed to be 842 Da and the peak highlighted in red the hydrolyzed compound (t_R = 1.84 min) with observed mass 745 Da. Peaks at 1.4 min and 1.55 min correspond to free sSPhos and sSPhos-oxide ligand respectively. *Note:* Near baseline peaks were identified via tandem mass spectrometry and are labelled above

the peak and assigned as depicted. The amount of residual sSPhos and its oxidized form were minimized via trituration as described in the synthetic protocols.

(B) HPLC chromatogram of reverse-phase purified **10a** after lyophilization. The analysis was performed as described in (A) except with a gradient from 7% **B** to 95% **B** over 5.5 min, then held isocratic at 95% **B** for 0.5 min.

10. Evidence Supporting Sites of Modification via MS/MS

As noted in footnote 21 of the main text, in typical proteins, the N-terminal amino group has a p*K*a value that is 2.6 ± 1.2 units lower than those in lysine side-chains[8] and, if sterically accessible, is likely to be the most readily acylated amino group. We expect this to be the major product during transfer of the palladium reagent onto the protein via acylation. However, we note that the isolated, singly-modified products **12**, **19**, and **20** are produced as regioisomeric mixtures of proteins. That is, a regioisomeric mixture of proteins containing a single modification at different sites. Of note, both RNase A (**11**) and lysozyme C contain N-terminal lysine residues and differentiation between possible N-terminal amine or N-terminal lysine side-chain modification was not attempted.

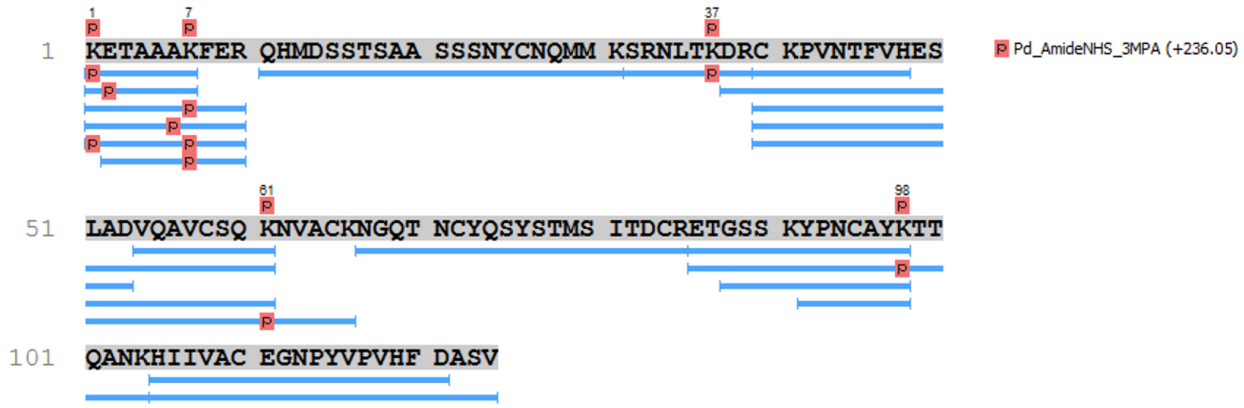
To provide evidence for sites of modification, MS/MS data were obtained according to the protocols in Section 4.2 and the data is presented below. Data were analyzed with Peaks v8.5 software. Sites of modification are highlighted in red (Tables S7 and S8). Only signals with an ion intensity greater than or equal to 5% are reported.

We also note that similar data were obtained when analyzing individual fractions of purified mono-modified material as well as those samples prepared for analysis by Protocol A outlined in Section 4.2.

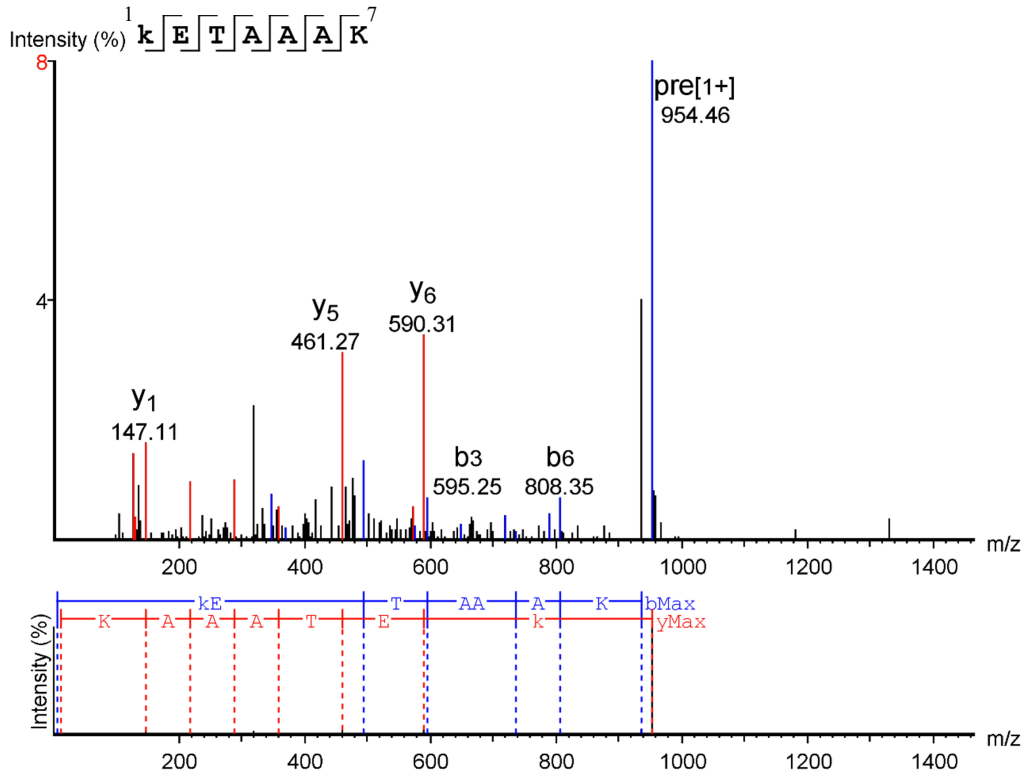
10.1. Assigned Modification Sites of Ribonuclease A

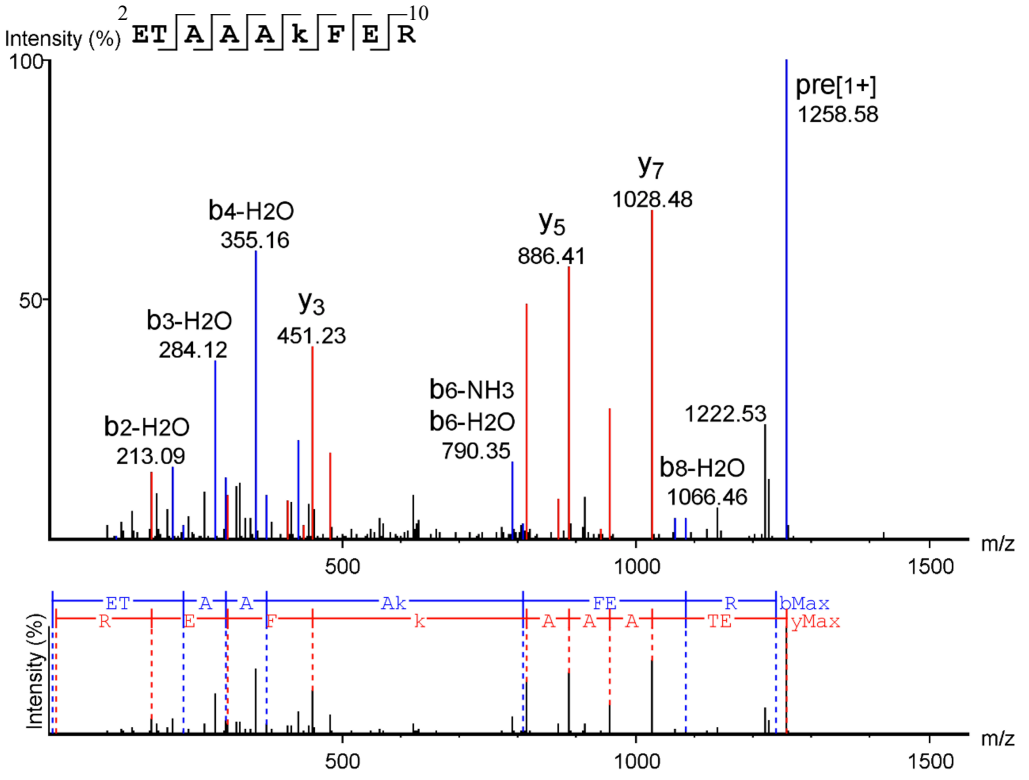
Table S7. Ribonuclease A Sites of Modification

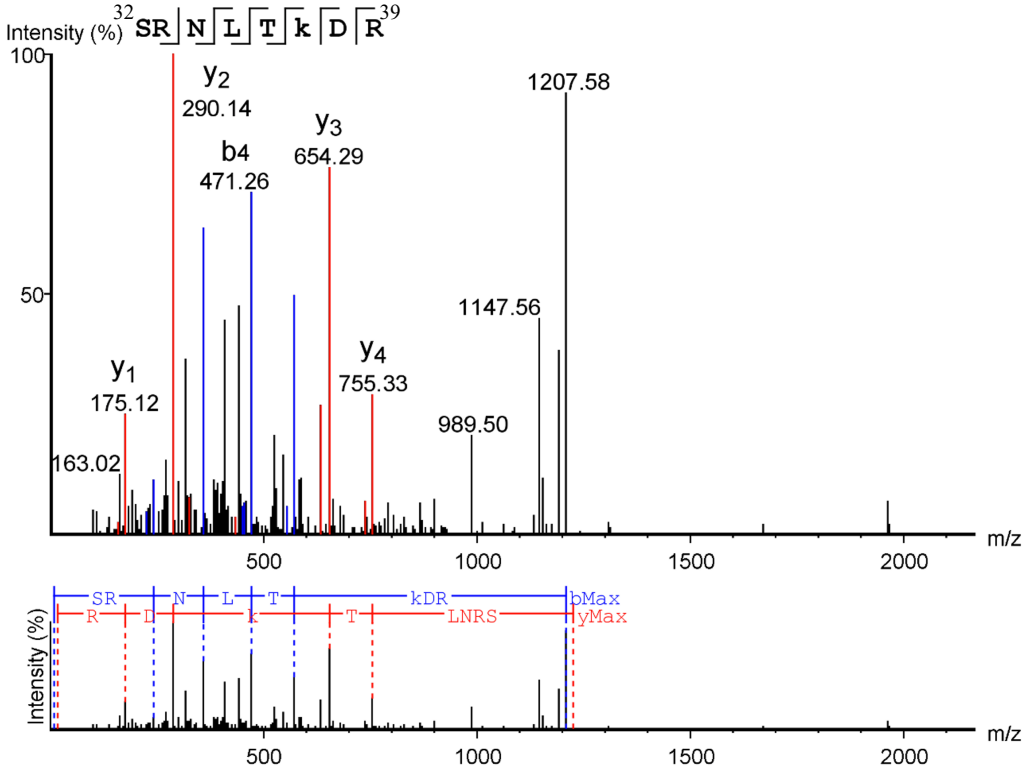
Sequence	Calculated mass(average)
^{1}KETAAAKFER^{10} ^{11}QHMDSSTSAA^{20} ^{21}SSSNYCNQMM^{30} ^{31}KSRNLTKDRC^{40} ^{41}KPVNTFVHES^{50} ^{51}LADVQAVCSQ^{60} ^{61}KNVACKNGQT^{70} ^{71}NCYQSYSTMS^{80} ^{81}ITDCRETGSS^{90} ^{91}KYPNCAYKTT^{100} ^{101}QANKHIIVAC^{110} ^{111}EGNPYVPVHF^{120} ^{121}DASV^{124}	13682 Da (Observed: 13683 Da)

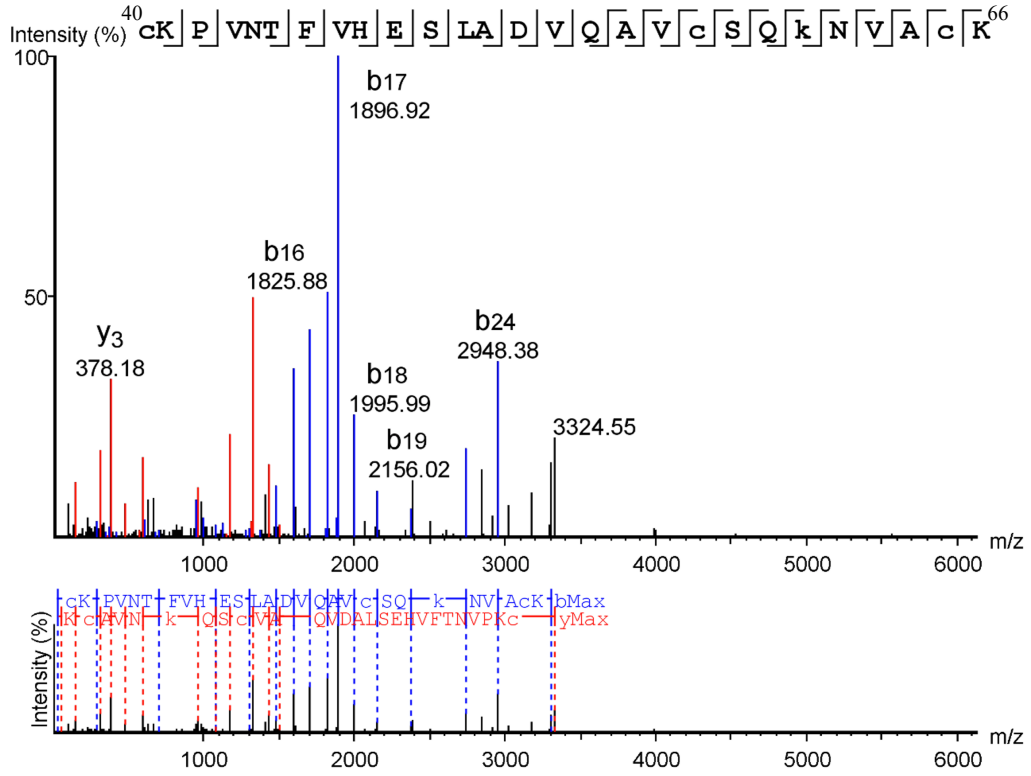


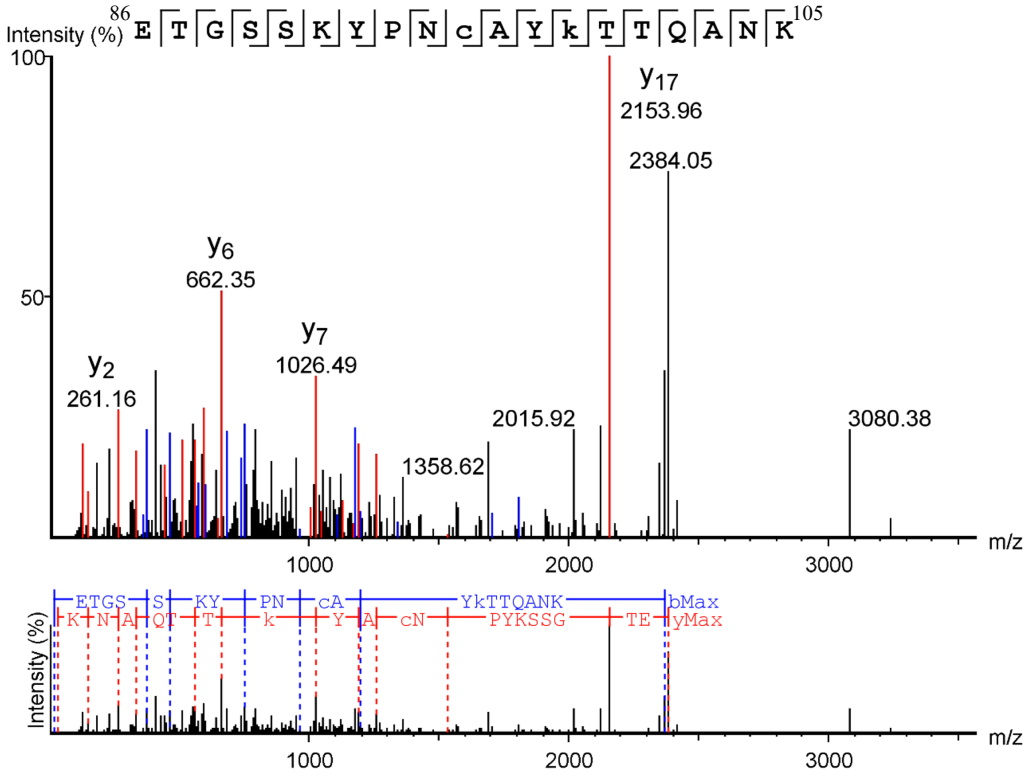
Above the sequence are highlighted modification sites identified from fragments with an ion intensity greater than or equal to 5% from all MS/MS experiments. Fragments found are highlighted in blue. The figure was generated by and taken directly from PEAKS v8.5.


Intensity (%)
1 kETAAAK 7
8
4
pre[1+]
954.46
y5
461.27
y6
590.31
y1
147.11
b3
595.25
b6
808.35
200
400
600
800
1000
1200
1400
m/z
kE
T
AA
A
K
bMax
K
A
A
A
T
E
k
yMax
Intensity (%)
200
400
600
800
1000
1200
1400
m/z


Intensity (%)
2 ETAAAkFER 10
100
50
pre[1+]
1258.58
y7
1028.48
b4-H2O
355.16
y5
886.41
y3
451.23
b3-H2O
284.12
b6-NH3
b6-H2O
790.35
1222.53
b2-H2O
213.09
b8-H2O
1066.46
500
1000
1500
m/z
ET
A
A
Ak
FE
R
bMax
R
E
F
k
A
A
A
TE
yMax
Intensity (%)
500
1000
1500
m/z


Intensity (%)
32 SRNLTkDR 39
100
50
y2
290.14
b4
471.26
y3
654.29
1207.58
1147.56
y4
755.33
y1
175.12
989.50
163.02
500
1000
1500
2000
m/z
SR N L T kDR bMax
R D k T LNRS yMax
Intensity (%)
500
1000
1500
2000
m/z


Intensity (%)
40 cKPVNTFVHESLADVQAVcSQkNVAcK 66
100
50
b17
1896.92
b16
1825.88
y3
378.18
b24
2948.38
b18
1995.99
b19
2156.02
3324.55
1000
2000
3000
4000
5000
6000
m/z
cK PVNT FVH ES LADVQAVcSQ k NV AcK bMax
kcAVN k QScVA QVDALSEHVFTNVPKc yMax
Intensity (%)
1000
2000
3000
4000
5000
6000
m/z

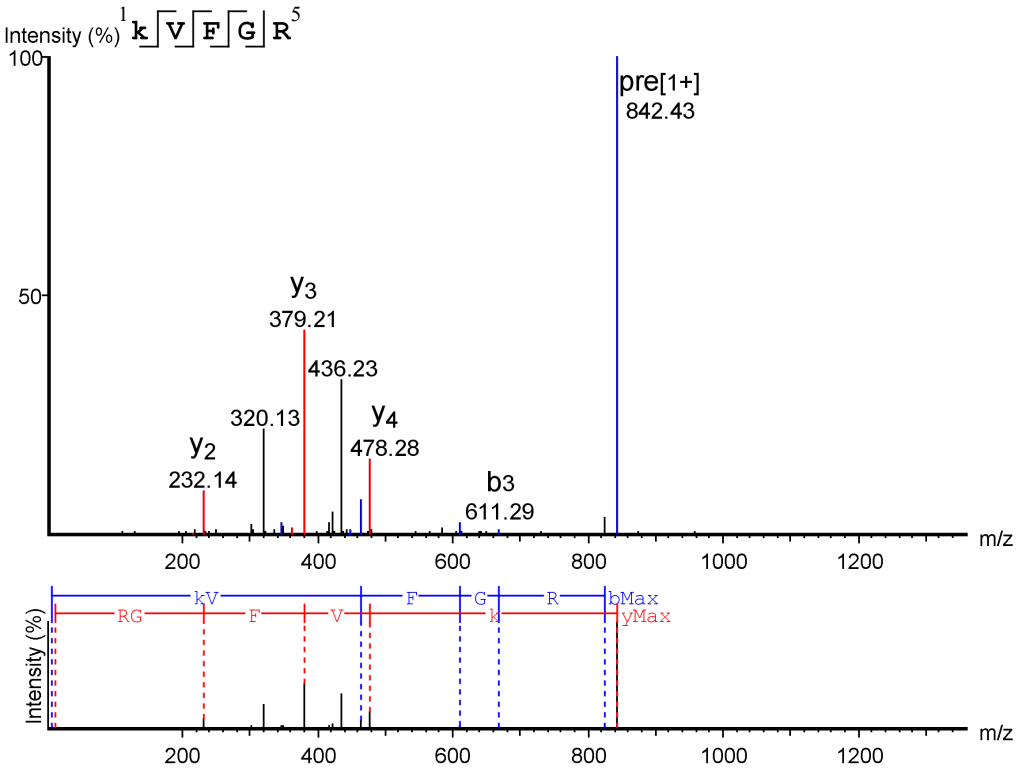

86 E T G S S K Y P N c A Y k T T Q A N K 105
Intensity (%)
100
50
y17
2153.96
2384.05
y6
662.35
y7
1026.49
y2
261.16
2015.92
3080.38
1358.62
m/z
1000
2000
3000
ETGS S KY PN cA YkTTQANK bMax
K N A QT T k Y A cN PYKSSG TE yMax
Intensity (%)
m/z
1000
2000
3000

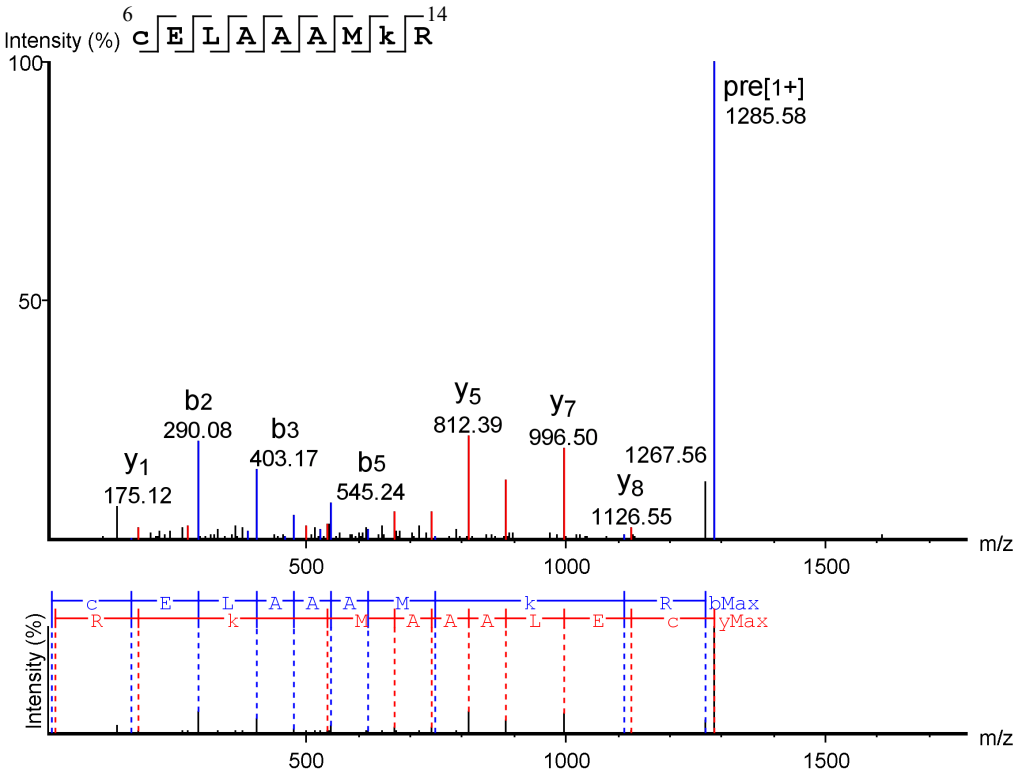
10.2. Assigned Modification Sites of Lysozyme C

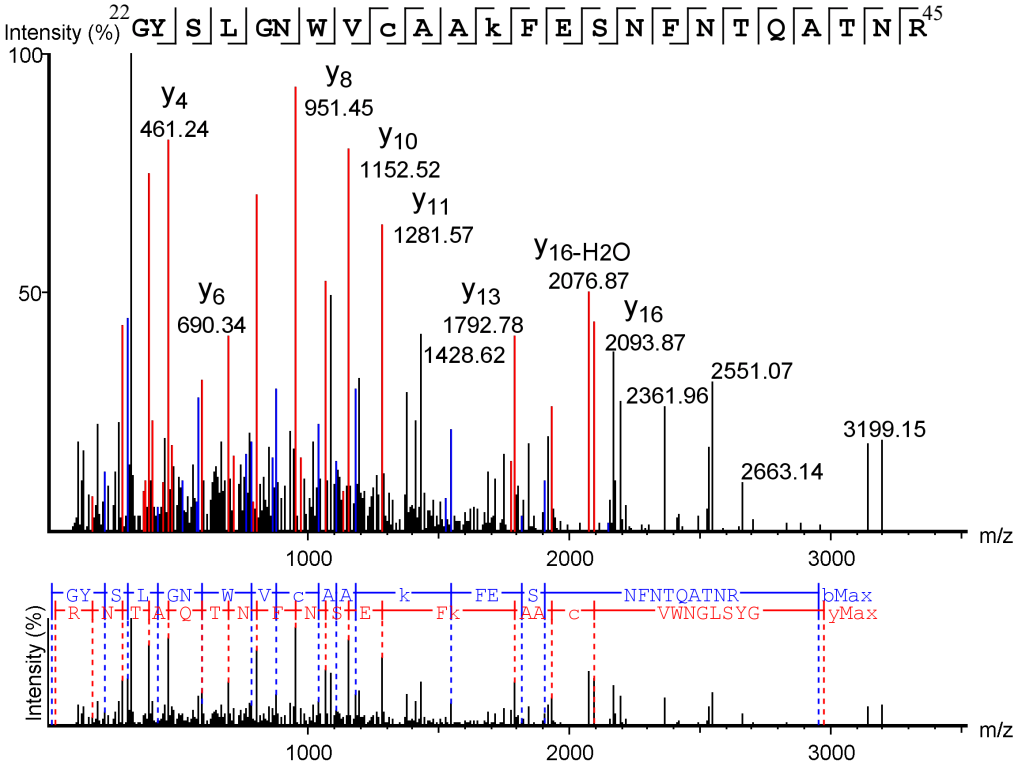
Table S8. Hen Egg White Lysozyme C

Sequence			Calculated mass(average)
^{1}KVFGRCELAA^{10}	^{11}AMKRHGLDNY^{20}	^{21}RGYSLGNWVC^{30}	14305 Da (Observed: 14305 Da)
^{31}AAKFESNFNT^{40}	^{41}QATNRNTDGS^{50}	^{51}TDYGILQINS^{60}	
^{61}RWWCNDGRTP^{70}	^{71}GSRNLCNIPC^{80}	^{81}SALLSSDITA^{90}	
^{91}SVNCAKKIVS^{100}	^{101}DGNGMNAWVA^{110}	^{111}WRNRCKGTDV^{120}	
^{121}QAWIRGCRL^{129}			

Above the sequence are highlighted modification sites identified from fragments with an ion intensity greater than or equal to 5% from all MS/MS experiments. Fragments found are highlighted in blue. The figure was generated by and taken directly from PEAKS v8.5.


Intensity (%)
1 k V F G R 5
100
50
pre[1+]
842.43
y3
379.21
436.23
320.13
y4
478.28
y2
232.14
b3
611.29
m/z
200
400
600
800
1000
1200
kV
F
G
R
bMax
RG
F
V
k
yMax
Intensity (%)
m/z
200
400
600
800
1000
1200


6 cELAAAMkR 14
Intensity (%)
100
50
pre[1+]
1285.58
b2
290.08
b3
403.17
y1
175.12
b5
545.24
y5
812.39
y7
996.50
1267.56
y8
1126.55
500
1000
1500
m/z
Intensity (%)
c E L A A A M k R bMax
R k M A A A L E c yMax
500
1000
1500
m/z


22 GYSLGNWVcAAkFESNFNTQATNR 45
Intensity (%)
100
50
y4
461.24
y8
951.45
y10
1152.52
y11
1281.57
y6
690.34
y13
1792.78
1428.62
y16-H2O
2076.87
y16
2093.87
2551.07
2361.96
3199.15
2663.14
1000
2000
3000
m/z
Intensity (%)
GY S L GN W V c AA k FE S NFNTQATNR bMax
R N T A Q T N F N S E Fk AA c VWNGLSYG yMax
1000
2000
3000
m/z

References

(1) Anderson, K. W.; Buchwald, S. L. General catalysts for the Suzuki-Miyaura and Sonogashira coupling reaction of aryl chloride and for the coupling of challenging substrate combinations in water. *Angew. Chem. Int. Ed.* **2005**, *44*, 6173-6177; *Angew. Chem.* **2005**, *117*, 6329-6333.

(2) Dhanjee, H. H.; Saebi, A.; Buslov, I.; Loftis, A. R.; Pentelute, B. L.; Buchwald, S. L. Protein–protein cross-coupling via palladium–protein oxidative addition complexes from cysteine residues. *J. Am. Chem. Soc.* **2020**, doi: 10.1021/jacs.0c03143.

(3) Su, L.; Pan, Y.; Huang, Y.; Cho, C.; Chen, C.; Huang, S. A novel E2F-like mprotein involved in transcriptional activation of cyst wall protein genes in Giarda lamblia. *J. Biol. Chem.,* **286**, 34101–34120 (2011).

(4) Vinogradova, E. V.; Zhang, C.; Spokoyny, A. M.; Pentelute, B. L.; Buchwald, S. L. Organometallic palladium reagents for cysteine bioconjugation. *Nature* **526**, 687–691 (2015).

(5) Lee, C. Y.; Yang, D. Determination of lysozyme activities in a microplate format. *Anal. Biochem.* **310**, 223-224 (2002).

(6) Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. Hypersensitive substrate for ribonucleases. Nucleic Acids Res. **27**, 3696-3701 (1999).

(7) Rojas, A. J.; Pentelute, B. L.; Buchwald, S. L. Water-soluble palladium reagents for cysteine *S*-arylation under ambient aqueous conditions. *Org. Lett.* **2017**, *19*, 4263-4266.

(8) Grimsley, G. R.; Schotlz, J. M.; Pace, C. N. A summary of the measured p*K* values of the ionizable groups in folded proteins. *Protein Sci.* **2009**, *18*, 247-251.