Formylbenzene diazonium hexafluorophosphate reagent for tyrosine-selective modification of proteins and the introduction of a bioorthogonal aldehyde

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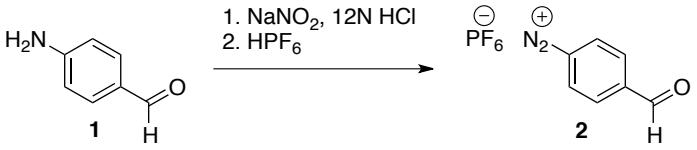
1. General Experimental

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DRX-600 (600 MHz), Varian Inova-400 (400 MHz), or Varian MER-300 (300 MHz) spectrometers in the stated solvents using tetramethylsilane as an internal standard. Chemical shifts were reported in parts per million (ppm) on the δ scale from an internal standard (NMR descriptions: s, singlet; d, doublet; t, triplet; q, quartet; h, heptet; m, multiplet; br, broad). Coupling constants, *J*, are reported in Hertz.

Mass spectroscopy was performed by the Scripps Research Institute Mass Spectrometer Center. Analytical thin-layer chromatography and flash column chromatography were performed on Merck Kieselgel 60 F254 silica gel plates and Silica Gel ZEOprep 60 ECO 40-63 Micron, respectively. Visualization was accomplished with anisaldehyde or KMnO4.

Unless otherwise noted, all the materials were obtained from commercial suppliers, and were used without further purification. All solvents were commercially available grade.

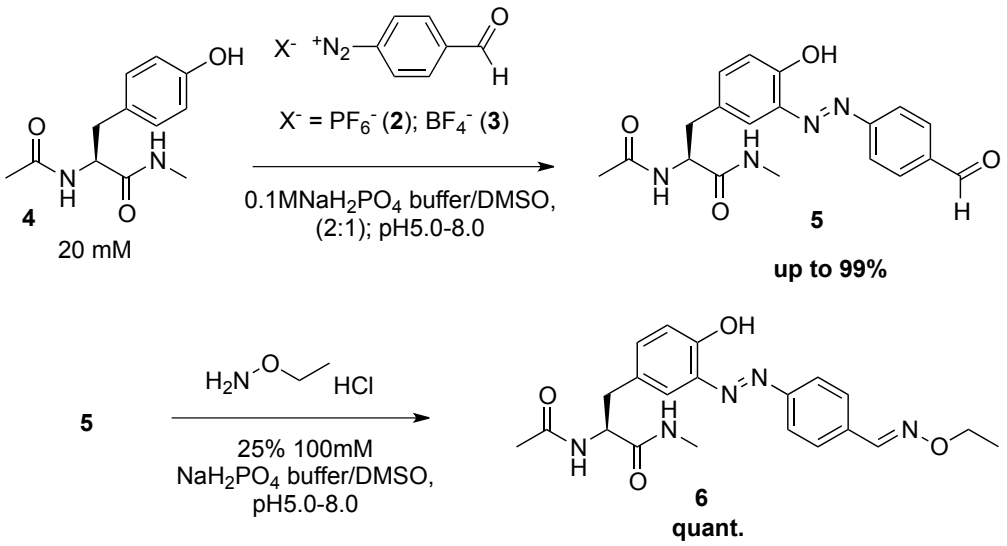
2. Synthesis of diazonium reagent



4-Formyl benzene diazonium hexafluorophosphate (2). To a suspension of 4-aminobenzaldehyde polymer (5.00 g, 41.3 mmol) in 12N HCl (85 mL) was added the solution of $NaNO_2$ (3.42 g, 49.5 mmol) in water (67 mL) at -10 ^{0}C. The resulting solution was stirred at -10 ^{0}C. After 1.5 h, 60% HPF_6 in water (10.3 mL, 70.2 mmol) was added at -10 ^{0}C and stirred for 30 min. Then the reaction mixture was stirred at room temperature for 30 min. The resulting solids were collected by filtration, and washed with water and AcOEt to give **2 (FBDP)** (4.77 g, 42%) as an off-white solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 10.2 (s, 1H), 8.85 (d, *J* = 5.2 Hz, 2H), 8.39 (d, *J* = 5.0 Hz, 2H). ^{13}C NMR (75 MHz, acetone-d6): δ 190.32, 143.69, 133.39, 131.02, 119.41. ^{31}P NMR (162 MHz, acetone-d6): δ -143.89 (h, J_{P-F} = 707 MHz), ^{19}F NMR (376 MHz, acetone-d6): δ -72.57 (d, J_{P-F} = 707 MHz).HRMS: calcd for $C_7H_5N_2O^+$ (M^+) 133.0402, found 133.0401.

3. General procedure for the diazonium coupling reaction



(*S*,*E*)-2-acetamido-3-(3-((4-formylphenyl)diazenyl)-4-hydroxyphenyl)-*N*-methylpropanamide (5). To a solution of *N*-acyl tyrosine methylamide **4** (20 mg, 0.0846 mmol) in 100 mM pH 7.0 NaH_2PO_4/Na_2HPO_4 buffer (2.83 mL) - DMSO (1.41 mL) was added the diazonium reagent **2** (25.9 mg, 0.0931 mmol) at room temperature. The resulting solution was stirred at room temperature for 45 min. After the reaction, water (2.82 mL) was added. The generated solid was filtered then washed with water and AcOEt to give **5** (31.0 mg, 99%) as a yellow solid.

^{1}H NMR (300 MHz, DMSO-d6): δ 10.9 (s, 1H), 10.1 (s, 1H), 8.16 (d, J = 8.6 Hz, 2H), 8.11 (d, J = 8.7 Hz, 2H), 7.93 (q, J = 4.7 Hz, 1H), 7.63 (d, J = 2.1 Hz, 1H), 7.32 (dd, J = 8.5, 2.2 Hz, 1H), 7.00 (d, J = 8.5 Hz, 1H), 4.45-4.37 (m, 1H), 2.94 (dd, J = 13.3, 5.0 Hz, 1H), 2.70 (dd, J = 13.5, 9.5 Hz, 1H), 2.56 (d, J = 4.6, 3H), 1.77 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ 192.59, 171.36, 168.93, 154.82, 154.14, 138.40, 137.05, 135.61, 130.65, 129.53, 123.08, 121.33, 117.94, 54.03, 36.82, 25.40, 22.40.

HRMS: calcd for $C_{19}H_{21}N_4O_4$ (MH^+) 369.1557, found 369.1556.

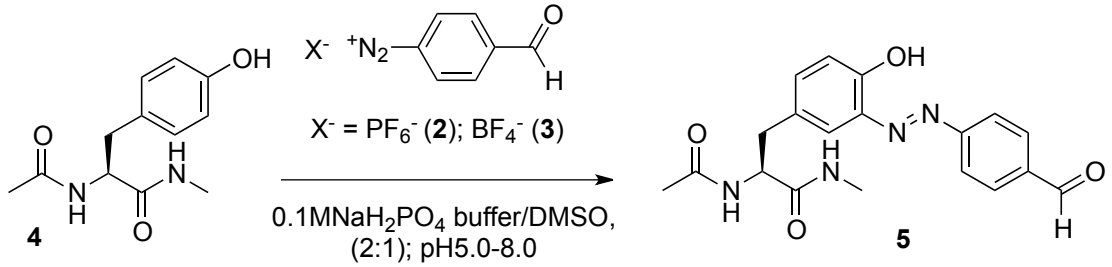


Table S1

Entry	Buffer	X^-	time	Isolated yield (%)
1	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH5-DMSO	PF_6^-	12 h	9
2	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH6-DMSO	PF_6^-	12 h	33
3	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH7-DMSO	PF_6^-	45 min	99
4a	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH7-DMSO	PF_6^-	45 min	93
5b	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH7-DMSO	PF_6^-	45 min	91
6	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH8-DMSO	PF_6^-	5 min	96
7	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH7-DMSO	BF_4^- c	45 min	84
8b	67 % 100 mM NaH_2PO_4-Na_2HPO_4, pH7-DMSO	BF_4^- c	45 min	63

a. the reagent was used after stocking at 4 ^oC for 3 months under air. b. the reagent was used after stocking at room temperature for a week under air. c. *Helv. Chim. Acta* **2002**, *85*, 108-114.

4. General procedure for oxime formation

(*S*)-2-acetamido-3-(3-((*E*)-(4-((*E*)-(ethoxyimino)methyl)phenyl)diazenyl)-4-hydroxyphenyl)-*N*-methylpropanamide (6). To a suspension of compound **3** (15 mg, 0.041 mmol) in 100 mM pH 7.0 NaH_2PO_4/Na_2HPO_4 buffer (0.531 mL) - DMSO (1.54 mL) was added ethoxyamine hydrochloride **4** (4.37 mg, 0.045 mmol) at room temperature. The resulting suspension was stirred at room temperature for 2 h. After the reaction, water (4.00 mL) was added. The generated solid was filtered then washed with water and AcOEt to give **5** (17.2 mg, quant.) as a yellow solid.

^1H NMR (300 MHz, DMSO-d6) (E:Z isomer ratio = 9 : 1): δ 10.9 (s, 1H), 8.33 (s, H), 8.13 (d, *J* = 8.5 Hz, 1H), 8.01 (d, *J* = 8.6 Hz, 2H), 7.93 (q, *J* = 4.5 Hz, 1H), 7.82 (d, *J* = 8.4 Hz, 2H), 7.62 (d, *J* = 2.0 Hz, 1H), 7.28 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 4.44-4.37 (m, 1H), 4.21 (q, *J* = 7.0 Hz, 2H), 2.94 (dd, *J* = 13.7, 5.1 Hz, 1H), 2.69 (dd, *J* = 13.6, 9.6 Hz, 1H), 2.56 (d, *J* = 4.6, 3H), 1.77 (s, 3H), 1.28 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ 171.39, 168.93, 153.30, 151.92, 147.74, 138.05, 134.38, 129.48, 127.71, 122.96, 122.57, 117.72, 69.30, 54.07, 36.84, 25.40, 22.40, 14.41. HRMS: calcd for $C_{21}H_{26}N_5O_4$ (MH^+) 412.1979, found 412.1980.

Table S2

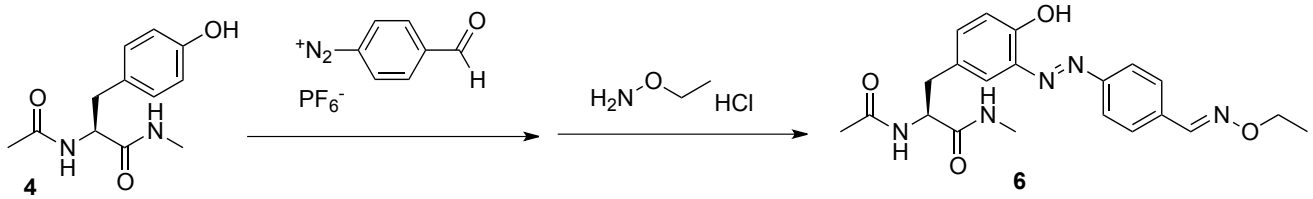
Entry	Buffer	time	Isolated yield

			(%)
1	25 % 100 mM NaH_2PO_4-Na_2HPO_4, pH5-DMSO	30 min	Quant. [a]
2	25 % 100 mM NaH_2PO_4-Na_2HPO_4, pH6- DMSO	1 h	Quant.
3	25 % 100 mM NaH_2PO_4-Na_2HPO_4, pH7- DMSO	2 h	Quant.
4	25 % 100 mM NaH_2PO_4-Na_2HPO_4, pH8- DMSO	2 h	Quant.

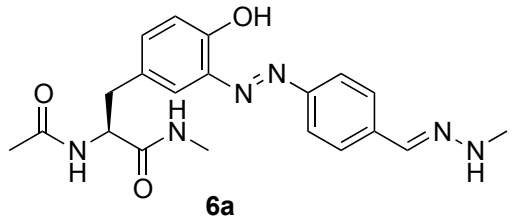
a. quantitative yield.

5. General procedure for the three component reaction

Method 1



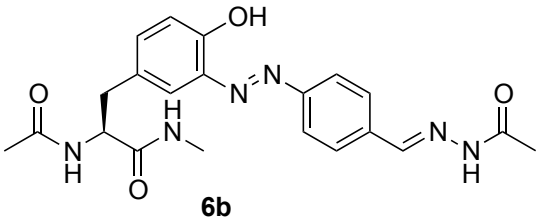
Compound (6). To a solution of *N*-acyl tyrosine methylamide **4** (20 mg, 0.0846 mmol) in 100 mM pH 7.0 NaH_2PO_4/Na_2HPO_4 buffer (2.83 mL) - DMSO (1.41 mL) was added the diazonium reagent **2** (25.9 mg, 0.0931 mmol) at room temperature. The resulting solution was stirred at room temperature for 1 h. After adding DMSO (1.41 mL), ethoxyamine hydrochloride (8.25 mg, 0.0846 mmol) was added at room temperature. The resulting suspension was stirred at room temperature for 2 h. The generated solid was filtered then washed with water and AcOEt to give **6** (32.5 mg, 93%) as a yellow solid.



Compound (6a). The compound **6a** was prepared from methylhydrazine (4.50 μL, 0.0846 mmol), and was obtained as a brown solid (30.7 mg, 92%). The mixed suspension was stirred at room temperature for 12 h.

^{1}H NMR (300 MHz, DMSO-d6): δ 11.1 (s, 1H), 8.13 (d, *J* = 8.4 Hz, 1H), 7.92-7.91 (m, 3H), 7.82 (q, *J* = 4.4 Hz, 1H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.62 (m, 1H), 7.46 (s, 1H), 7.26-7.23 (m, 1H), 6.94 (d, *J* = 8.4 Hz, 1H), 4.45-4.37 (m, 1H), 2.94 (dd, *J* = 14.3, 5.4 Hz, 1H), 2.87 (d, *J* = 4.1 Hz, 3H), 2.72 (dd, *J* =

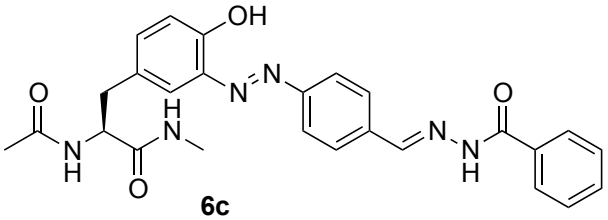
13.5, 9.4 Hz, 1H), 2.57 (d, *J* = 4.4, 3H), 1.77 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ 171.43, 168.94, 152.62, 149.66, 140.13, 137.83, 133.99, 129.42, 129.13, 125.37, 123.73, 122.93, 117.51, 54.09, 36.85, 33.13, 25.40, 22.40. HRMS: calcd for $C_{20}H_{25}N_6O_3$ (MH^+) 397.1983, found 397.1982.



6b

Compound (6b). To a solution of *N*-acyl tyrosine methylamide **4** (9.69 mg, 0.041 mmol) in 100 mM pH 7.0 NaH_2PO_4/Na_2HPO_4 buffer (1.37 mL) - DMSO (0.686 mL) was added the diazonium reagent **2** (12.5 mg, 0.045 mmol) at room temperature. The resulting solution was stirred at room temperature for 1 h. After adding DMSO (2.05 mL), acetyl hydrazine (3.33 mg, 0.045 mmol) was added at room temperature. The resulting suspension was stirred at room temperature for 12 h. AcOEt and brine were added. The organic layer was separated and the aqueous layer was extracted once with AcOEt. The combined organic layer was concentrated *in vacuo*. The residue was washed with water and AcOEt to give **7** (14.8 mg, 85%) as a brown solid.

^{1}H NMR (300 MHz, DMSO-d6) (E:Z isomer ratio = 5 : 2): δ 11.4 (s, 1H), 11.0 (br, 1H), 8.14(d, *J* = 8.5 Hz, 1H), 8.07 (s, 1H), 8.02 (d, *J* = 8.5 Hz, 2H), 7.93 (q, *J* = 4.7 Hz, 1H), 7.86 (d, *J* = 8.5 Hz, 2H), 7.62 (d, *J* = 2.1 Hz, 1H), 7.28 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 4.44-4.37 (m, 1H), 2.94 (dd, *J* = 13.7, 4.7 Hz, 1H), 2.71 (dd, *J* = 13.2, 9.1 Hz, 1H), 2.56 (d, *J* = 4.5 Hz, 3H), 2.24 (s, 1H), 1.77 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ 172.04, 171.42, 168.95, 153.26, 151.78, 141.42, 138.08, 136.63, 134.74, 129.49, 127.54, 122.99, 122.67, 117.71, 54.10, 36.85, 25.42, 22.41, 20.19. HRMS: calcd for $C_{21}H_{25}N_6O_4$ (MH^+) 425.1932, found 425.1925.



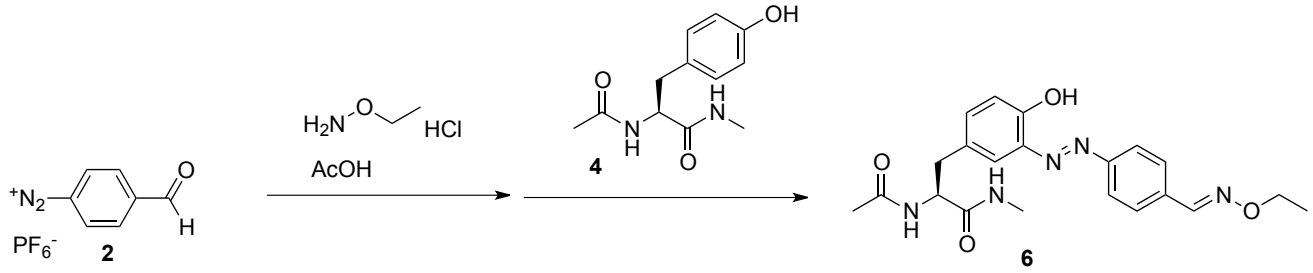
6c

Compound (6c). The compound **6c** was prepared from benzohydrazide (6.13 mg, 0.045 mmol), and was obtained as a brown solid (17.4 mg, 87%).

^{1}H NMR (300 MHz, DMSO-d6): δ 12.1 (br, 1H), 11.0 (br, 1H), 8.85 (br, 1H), 8.15(d, *J* = 8.4 Hz, 1H), 8.07 (d, *J* = 8.5 Hz, 2H), 7.96-7.93 (m, 5H), 7.64-7.52 (m, 4H), 7.31-7.28 (m, 1H), 6.98 (d, *J* = 8.4 Hz, 1H), 4.45-4.38 (m, 1H), 2.95 (dd, *J* = 13.7, 4.7 Hz, 1H), 2.72 (dd, *J* = 13.2, 9.9 Hz, 1H), 2.57 (d, *J* =

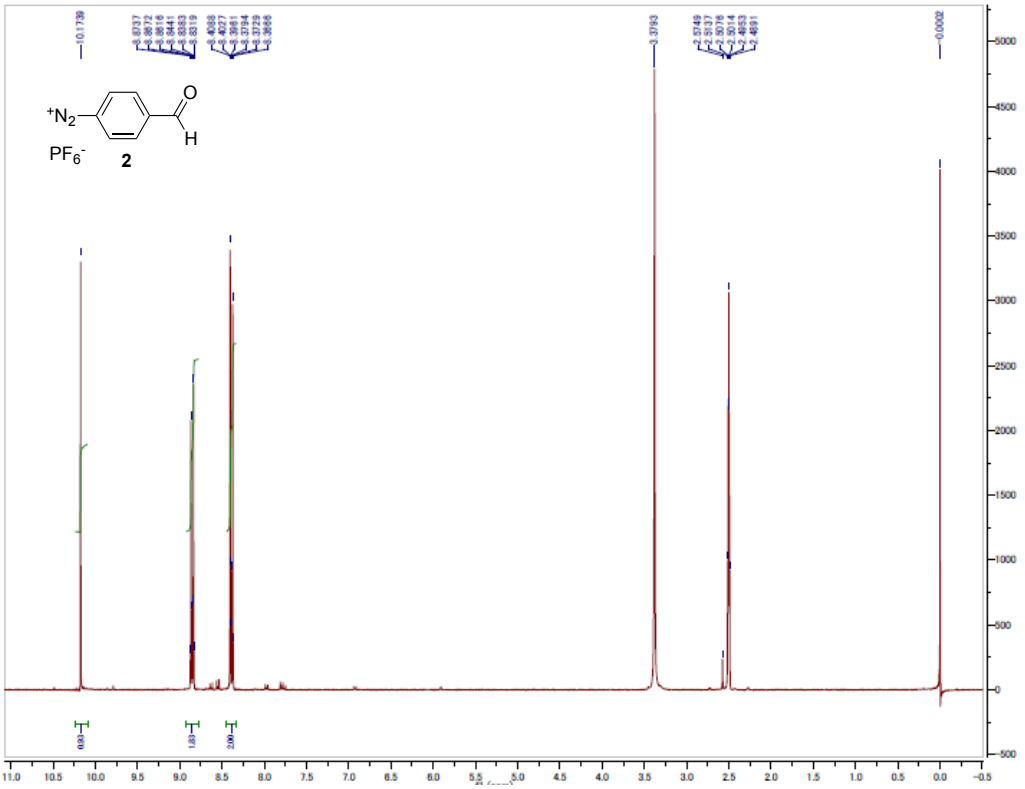
4.5 Hz, 3H), (s, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ 171.44, 168.97, 163.15, 153.35, 152.09, 146.59, 138.14, 136.69, 134.81, 133.19, 131.82, 129.51, 128.44, 128.03, 127.63, 123.06, 122.68, 117.75, 54.12, 36.86, 25.43, 22.43. HRMS: calcd for $C_{26}H_{27}N_6O_4$ (MH^+) 487.2088, found 487.2086.

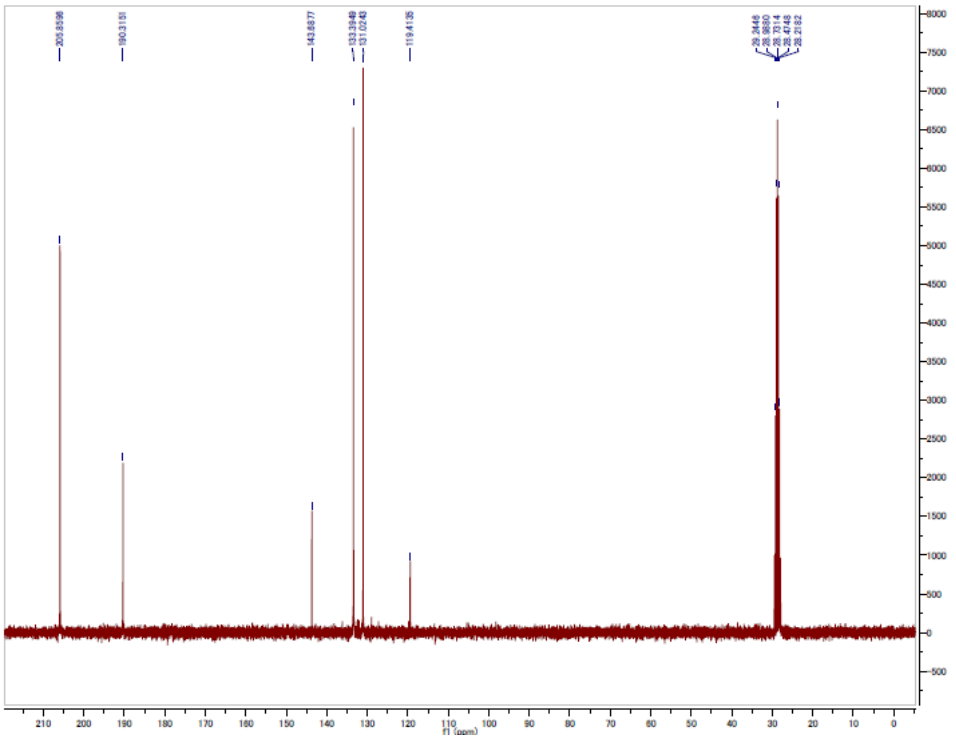
Method 2

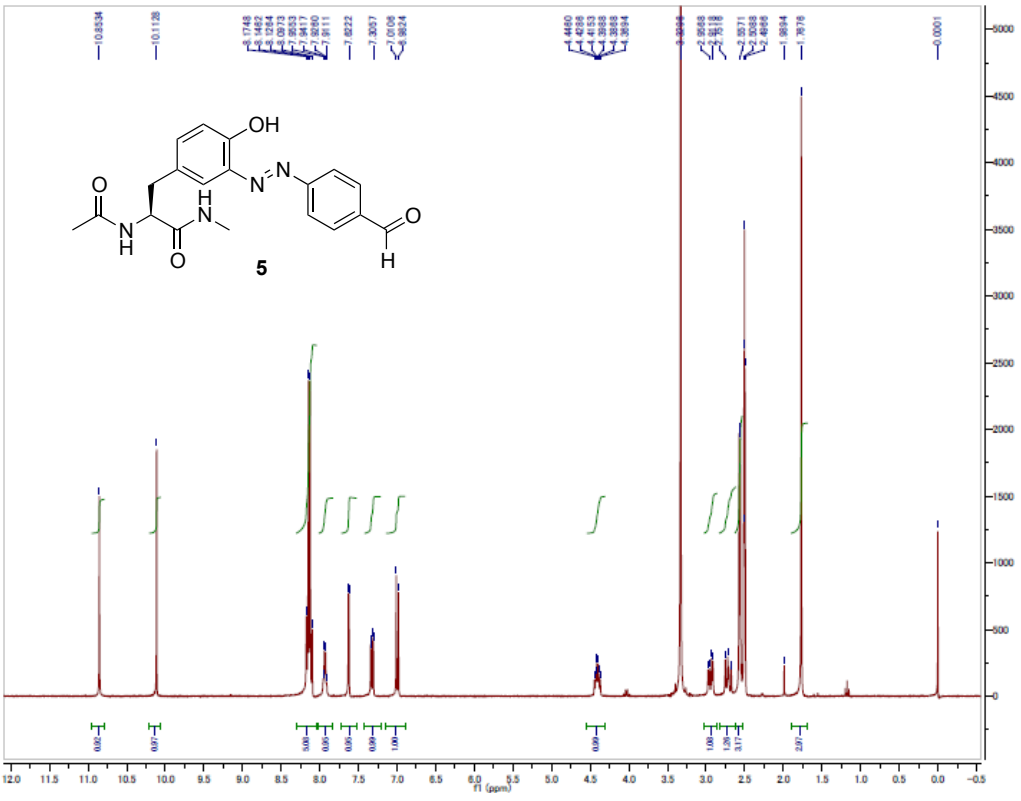


Compound (6). To a solution of diazonium salt 2 (25.9 mg, 0.0931 mmol) in DMSO (1.41 mL) were added the ethoxyamine hydrochloride (9.95 mg, 0.102 mmol) and AcOH (5.32 µL, 0.0931 mmol) at room temperature. The resulting solution was stirred at room temperature for 2 h, and was added to the solution of *N*-acyl tyrosine methylamide **4** (20.0 mg, 0.0847 mmol) in 138 mM pH 8.0 NaH_2PO_4/Na_2HPO_4 buffer (4.25 mL) - DMSO (0.686 mL). After the reaction, water (4.00 mL) was added. The generated solid was filtered and then washed with water and AcOEt to give **6** (15.8 mg, 45%) as a yellow solid.

6. NMR spectra of compounds



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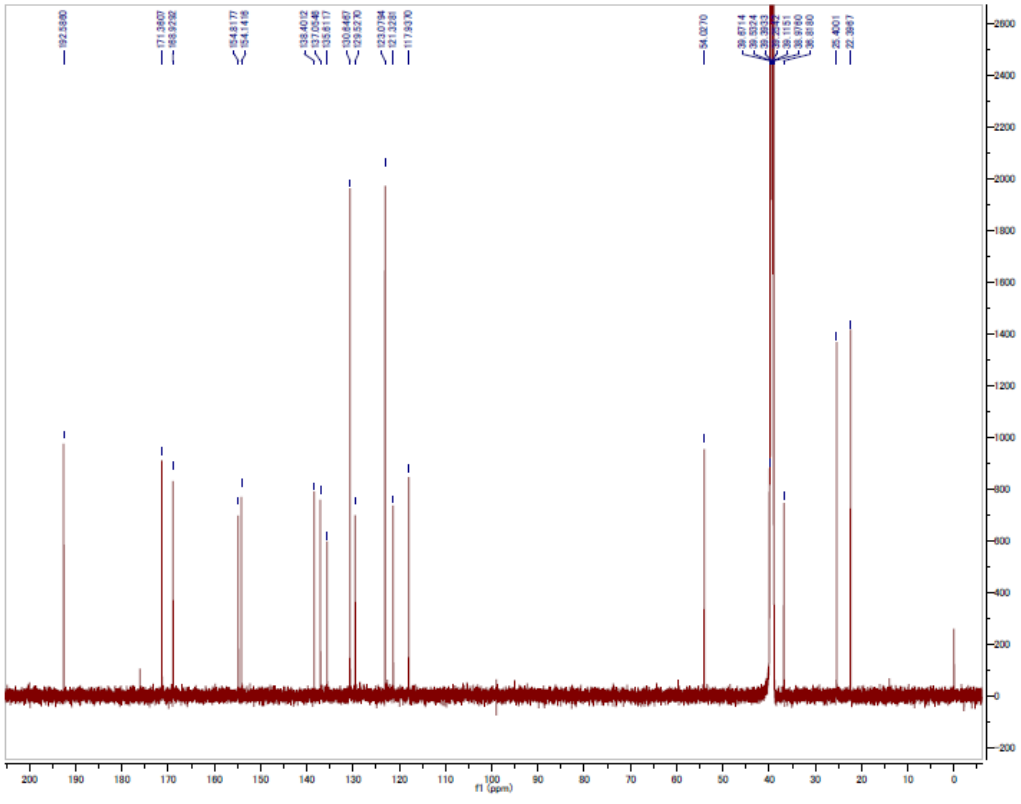

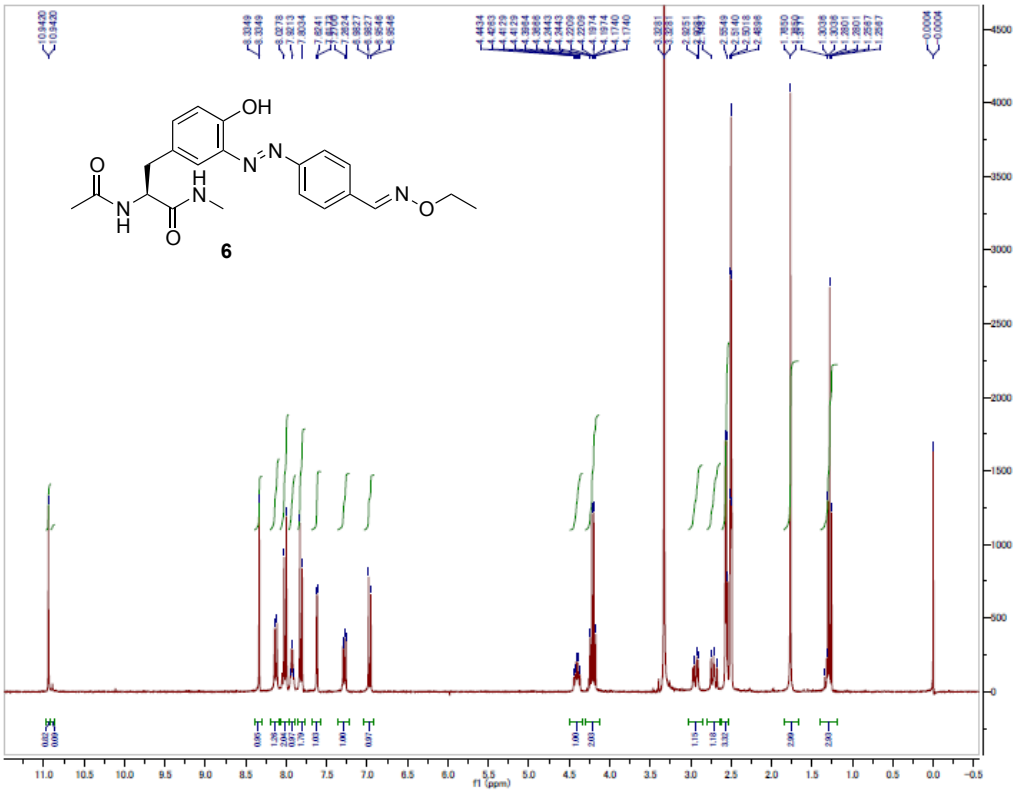
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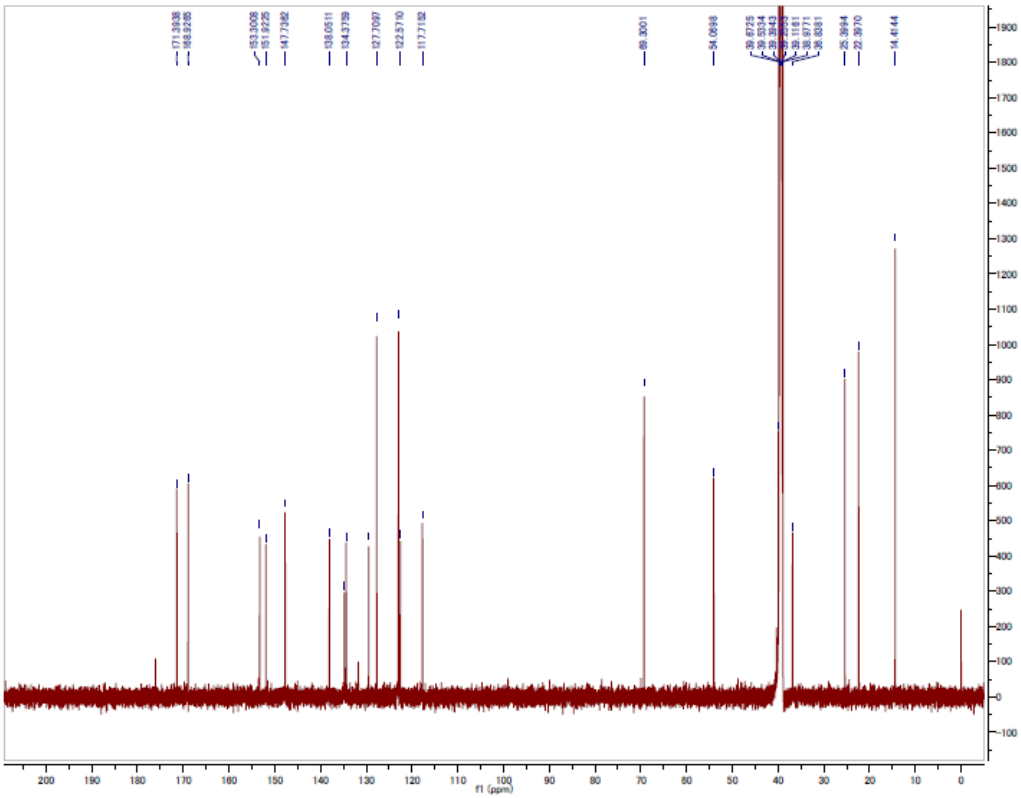

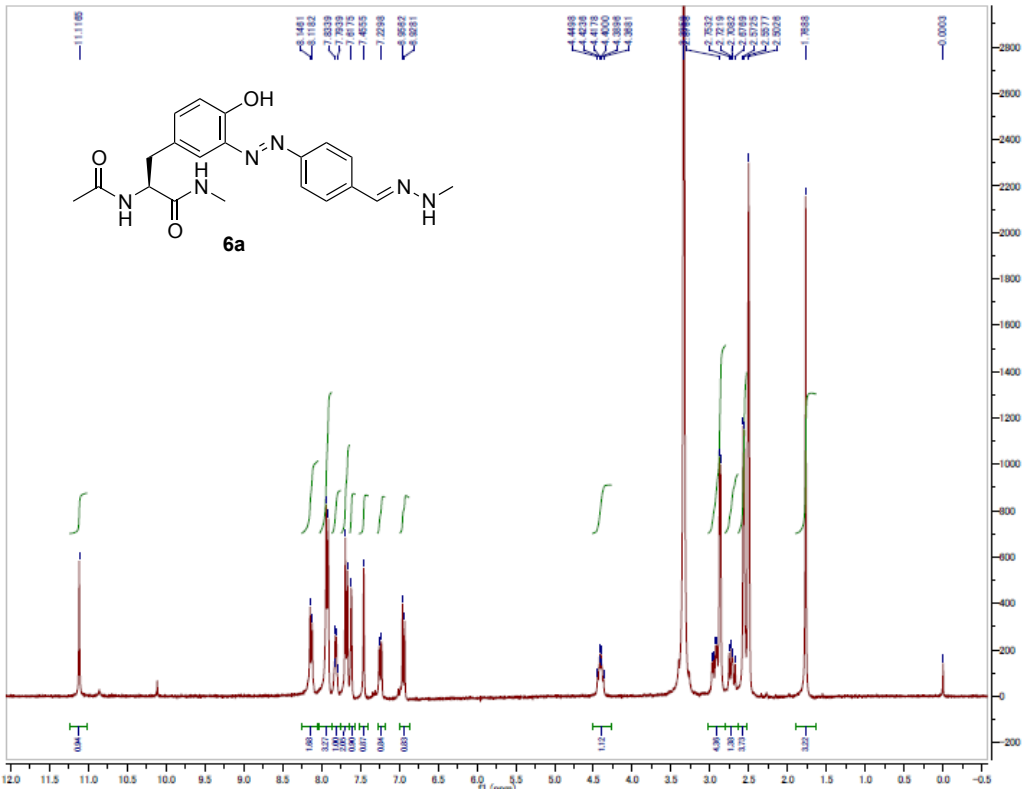
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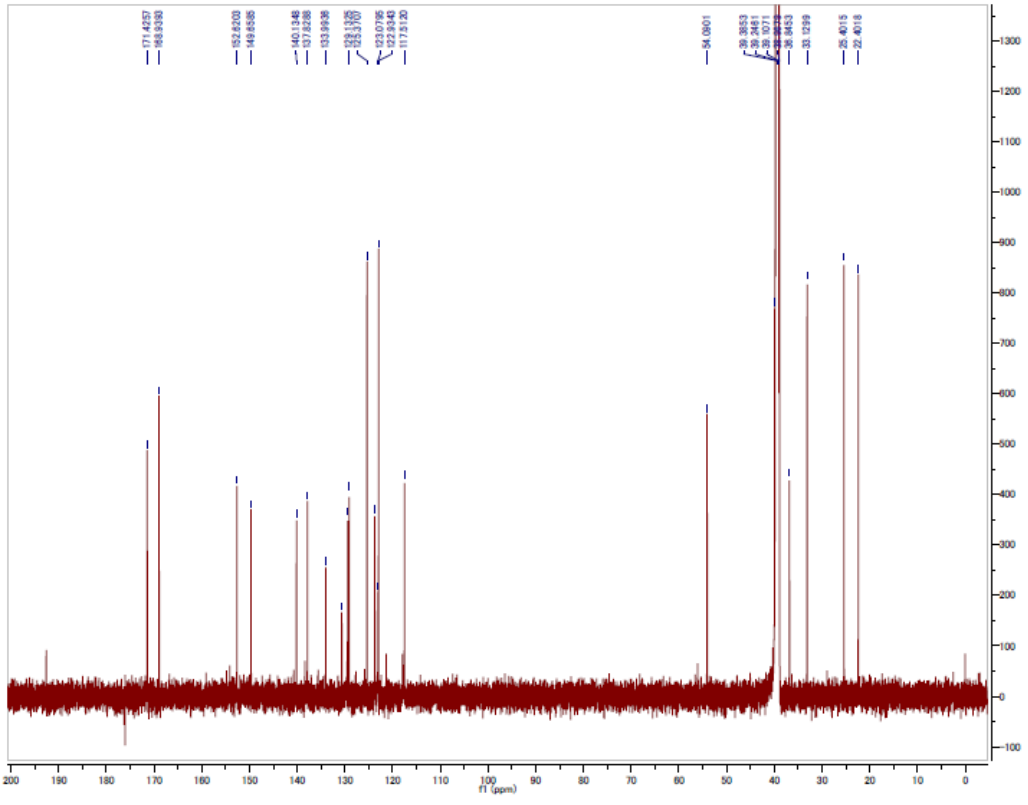

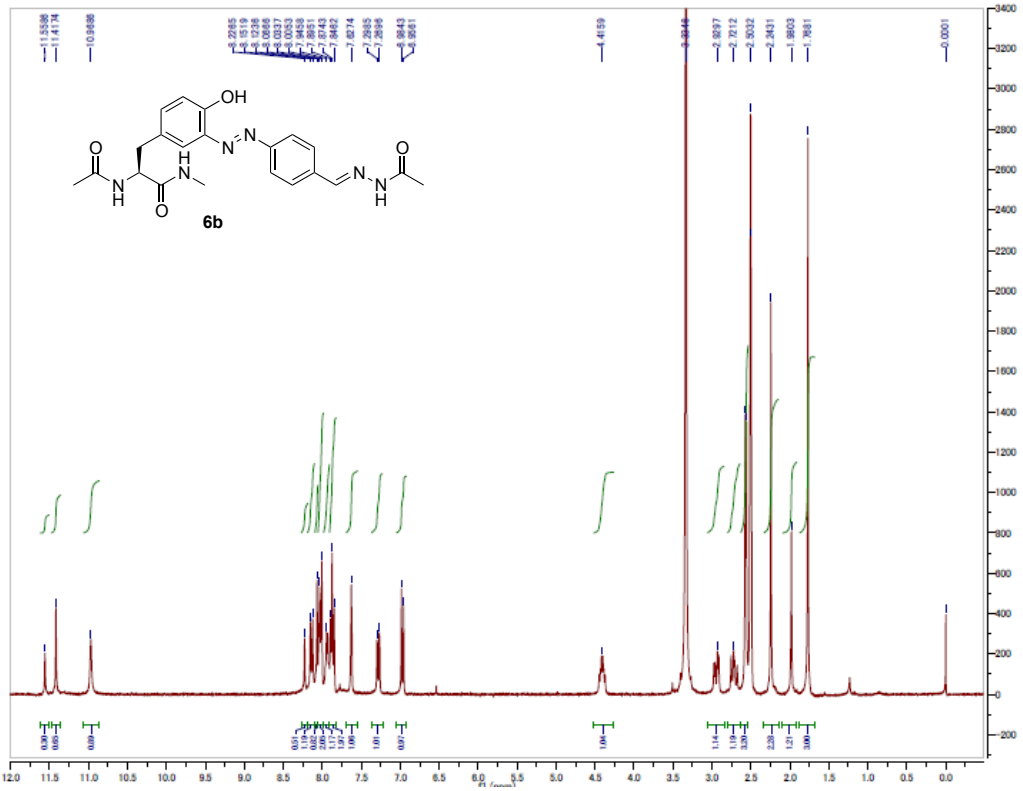
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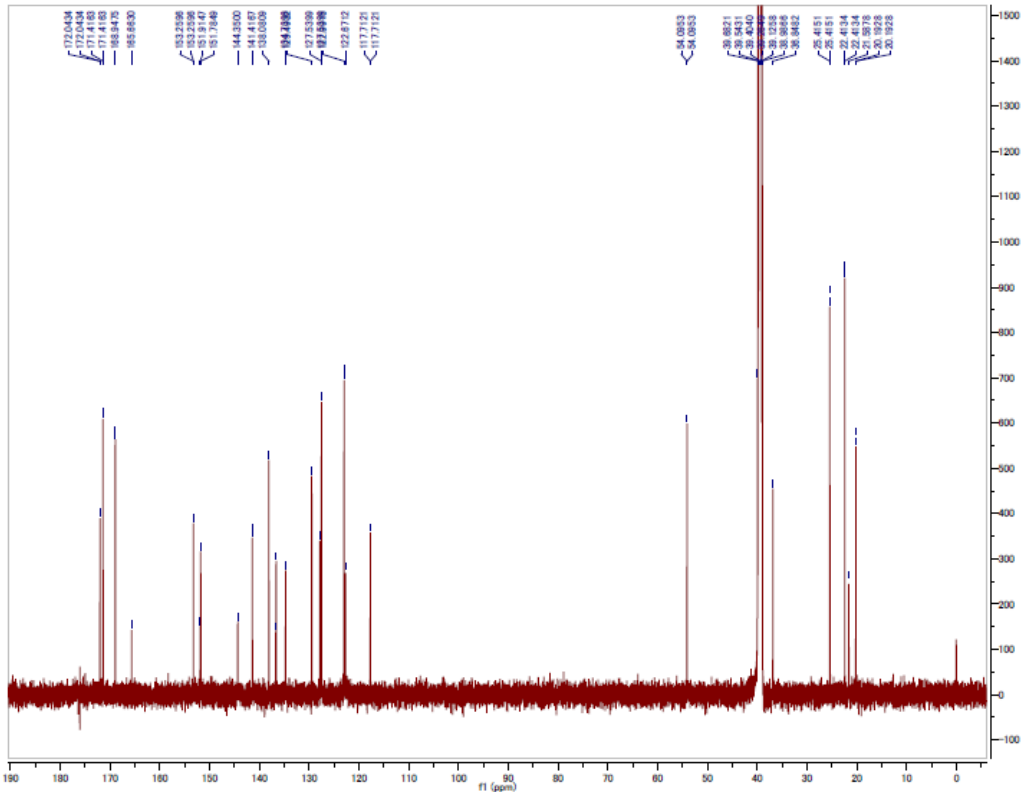

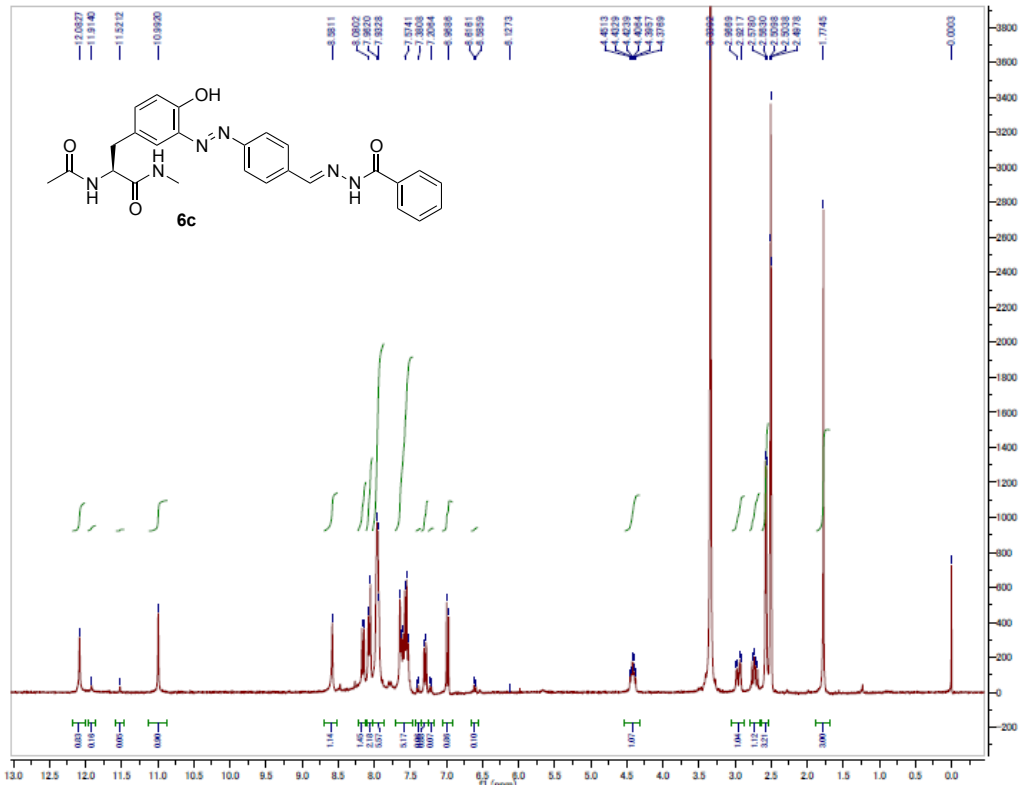
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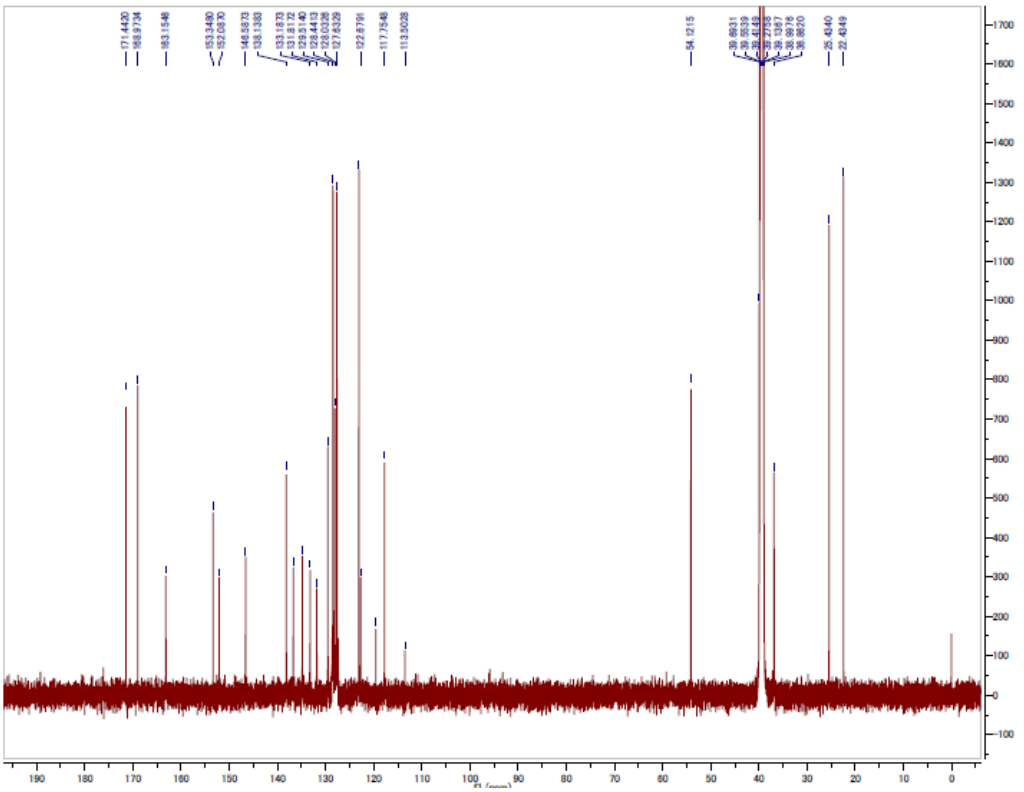


7. Amino Acid Selectivity Study

General procedure.

N-acyl methyl amides of amino acid were obtained as previously described.[3] Equimolar solution of six amino acids *N*-acyl methyl amides (Tyr, His, Cys, Ser, Lys, Trp) was prepared in 0.1M Na_2HPO_4 buffer (pH 7, 7.4, 8). Final concentration of each amino acid was 100 μM (1 eq). 1 mL of the amino acid mixture solution was transferred into LCMS sample vial and 5 μL of FBDP solution in acetonitrile (20 mM solution, 1 eq) was added. Reaction mixture was vortexed gently and allowed to react at room temperature. Each reaction was performed independently at least 3 times. Aliquot of reaction mixture (5 μL) was analyzed by LCMS after 30 min of reaction time using Agilent LC/MSD SL mass spectrometer with Zorbax 300SP-C8, 5 μm, 4.6x50 mm column (Agilent). HPLC parameters were as follows: flow rate 0.5 ml/min, 5% mobile phase B over 2 min followed by a gradient to 5 to 95% B from 2 to 8 min, post time 4 min. Mobile phase A was 0.1% formic acid (v/v) in HPLC-grade H_2O, and mobile phase B was 0.1% formic acid (v/v) in acetonitrile (v/v). API-ESMS data was collected for 115-1000 m/z positive and negative polarity. Data was analyzed using Agilent analysis software. MS TIC positive polarity trace was used to evaluate reaction selectivity. For clarity and quantification purposes traces corresponding to expected product ions were extracted from the MS TIC spectra (Figures S1-S6). Area under the peak was used to calculate content of each product (Tables S3, S4, S5).

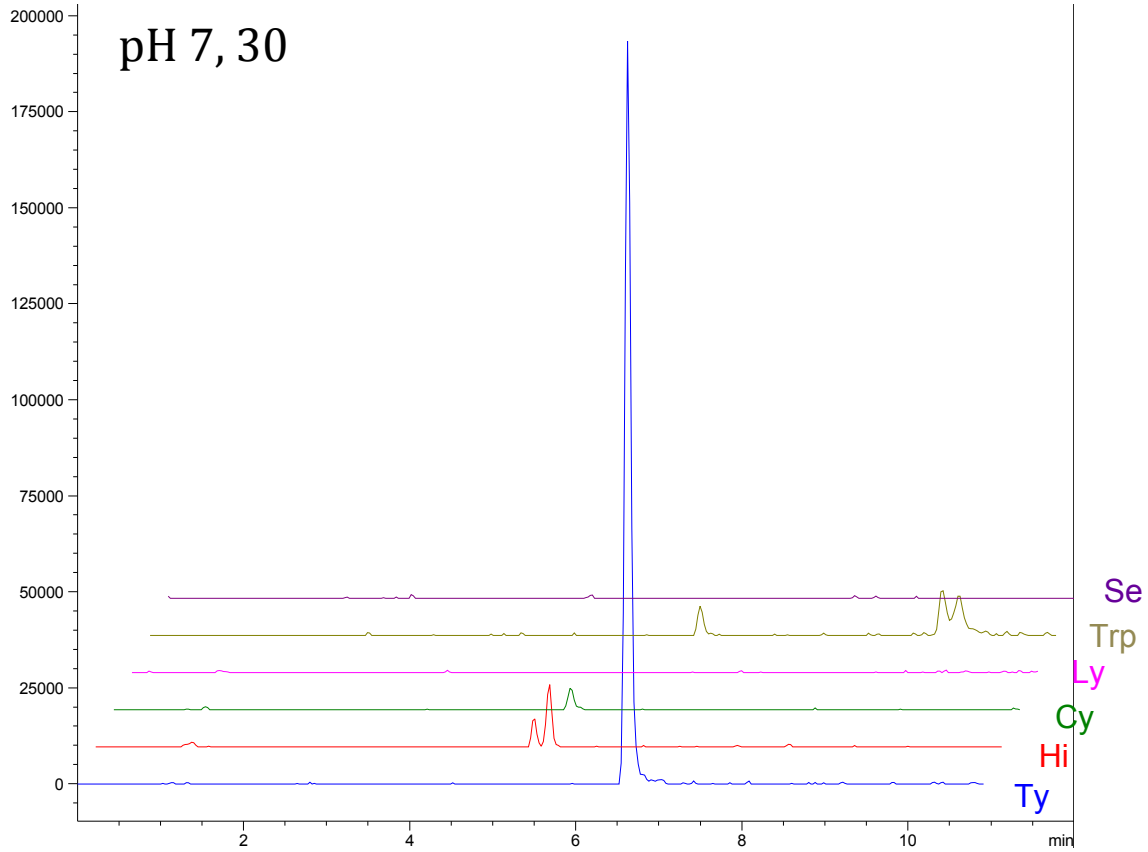


Figure S1. Example of 3D overlay of the extracted product ion traces for amino acids in the reaction mixture at pH 7.

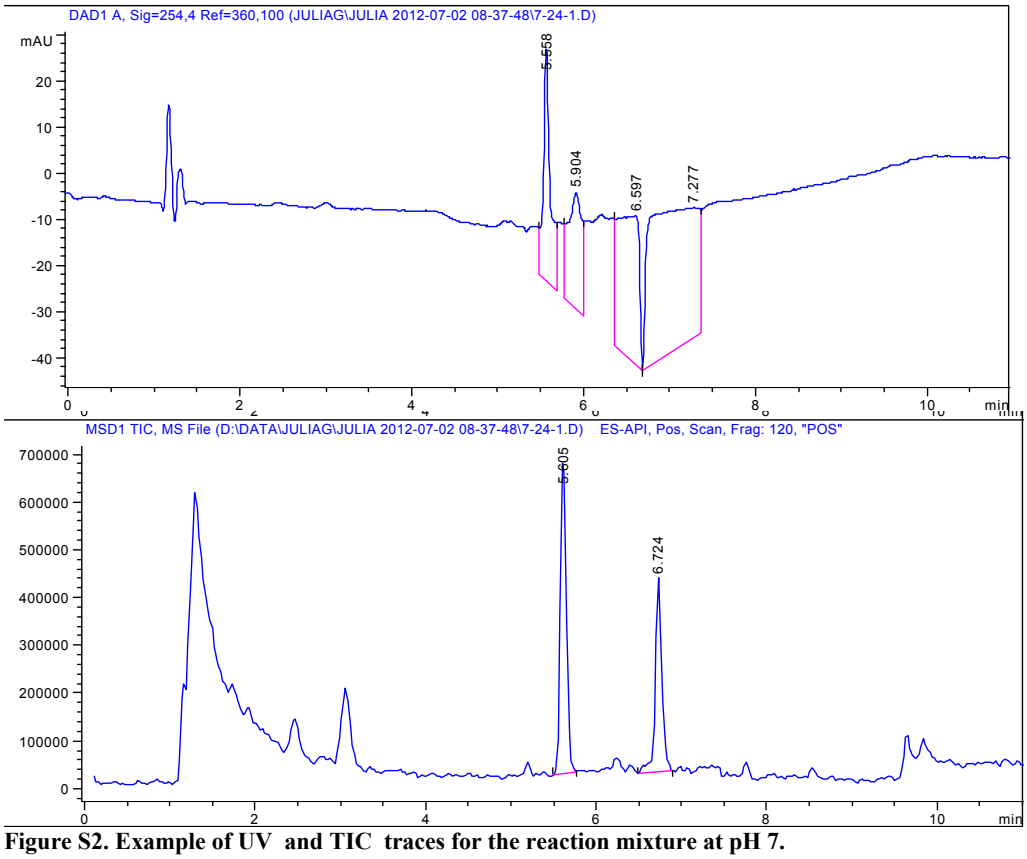


Figure S2. Example of UV and TIC traces for the reaction mixture at pH 7.

Table S3. Summary of the amino acid modification in the equimolar mixture at pH 7.

pH 7	% conversion				
Amino Acid Adduct	**1**	**2**	**3**	**Average**	**STD**
Tyr	89.2	84.7	87.7	87.2	2.305
His	7.1	11.0	2.8	7.0	4.091
Cys	2.0	1.9	2.7	2.2	0.429
Lys	0.0	0.0	0.0	0.0	0
Trp	1.0	2.4	6.8	3.4	3.021
Ser	0.6	0.0	0.0	0.2	0.361

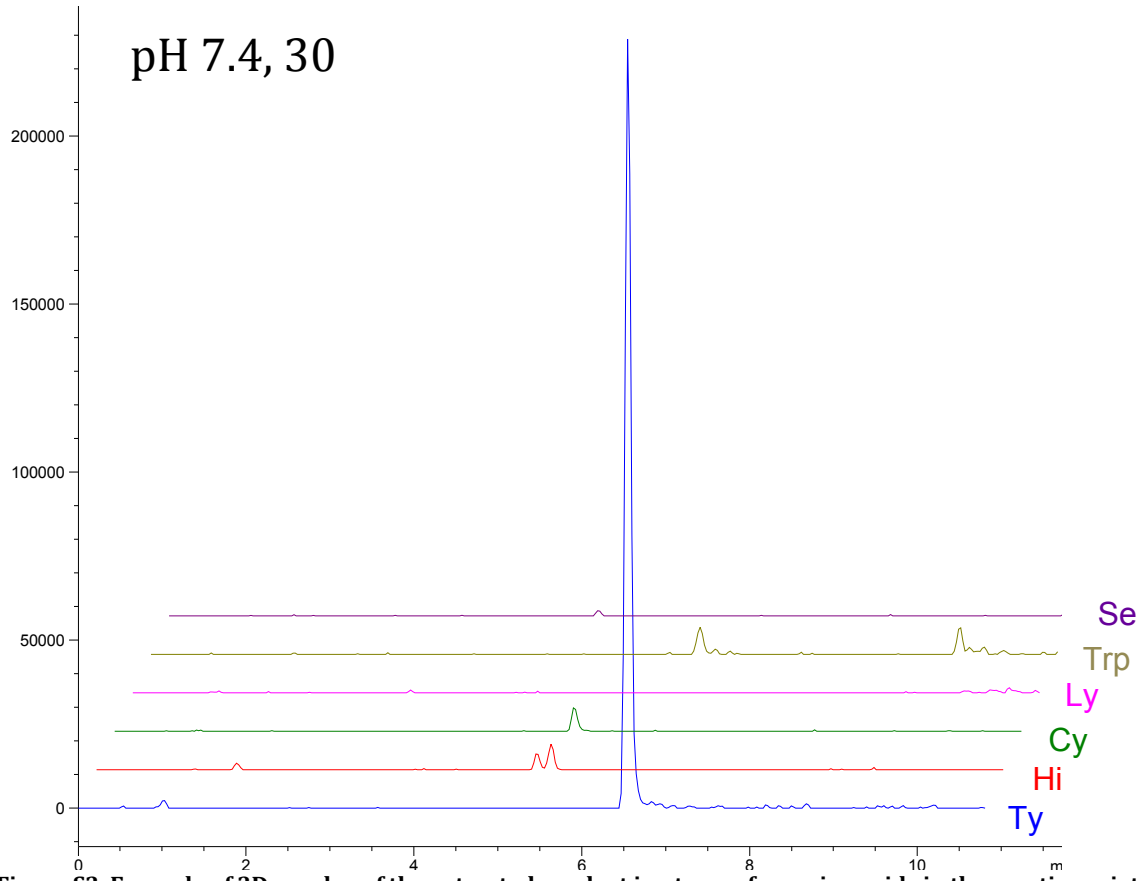


Figure S3. Example of 3D overlay of the extracted product ion traces for amino acids in the reaction mixture at pH 7.4.

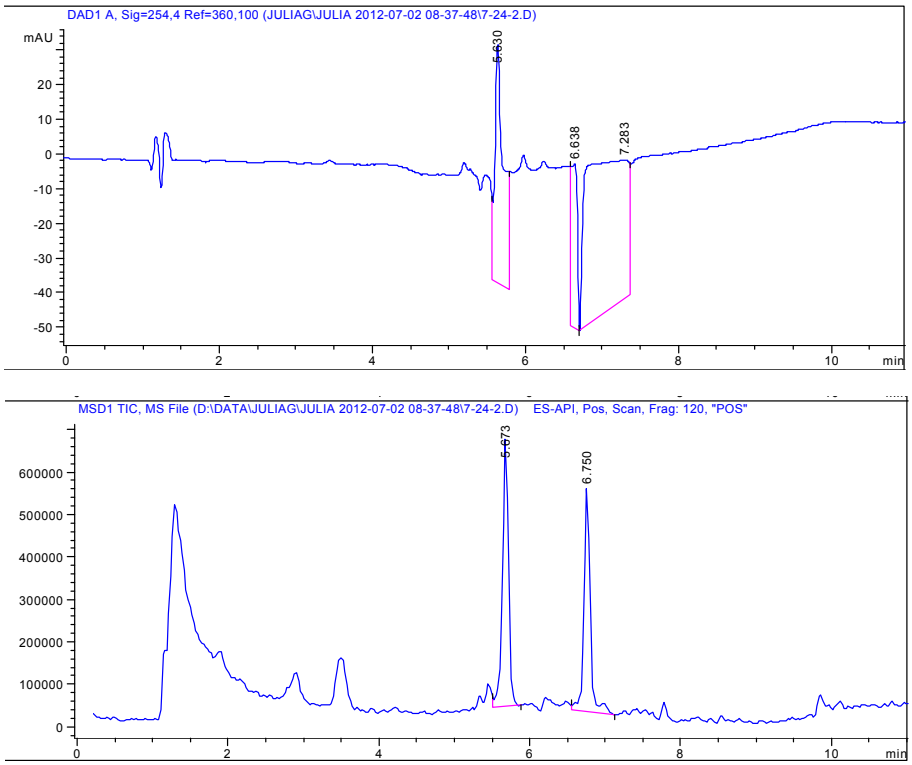


Figure S4. Example of UV and TIC traces for the reaction mixture at pH 7.4.

Table S4. Summary of the amino acid modification in the equimolar mixture at pH 7.4.

pH 7.4	% conversion				
Amino Acid Adduct	**1**	**2**	**3**	**Average**	**STD**
Tyr	92.8	90.4	84.0	89.1	4.537
His	0.6	5.3	10.6	5.5	5.005
Cys	2.6	1.7	2.4	2.2	0.463
Lys	0.0	0.0	0.0	0.0	0
Trp	4.0	2.7	3.0	3.2	0.697
Ser	0.0	0.0	0.0	0.0	0

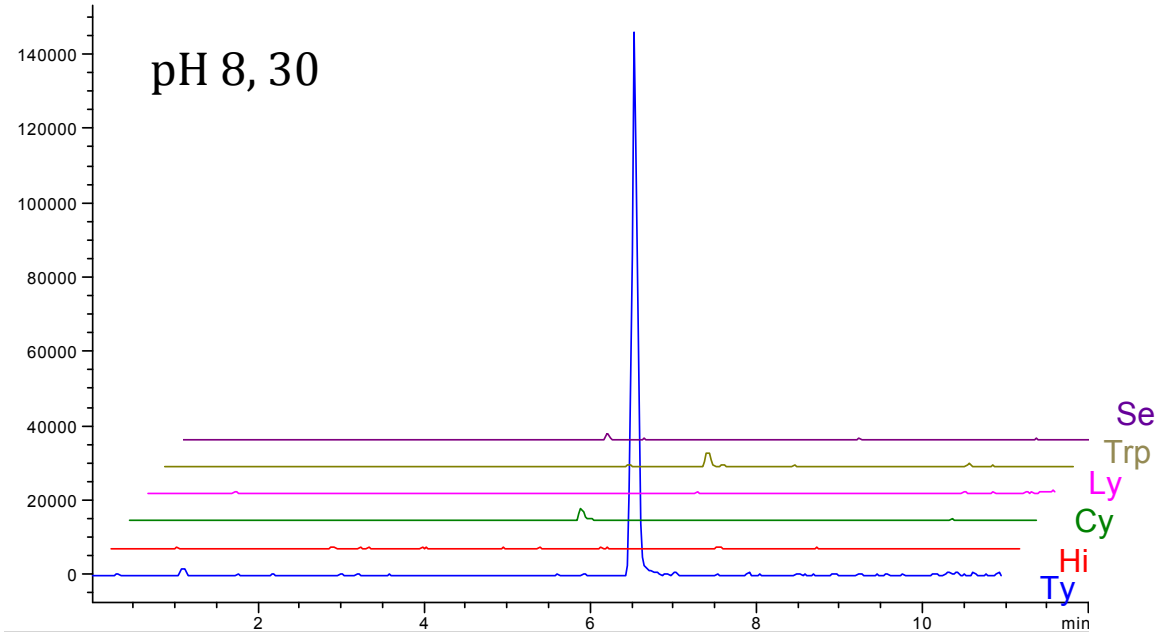


Figure S5. Example of 3D overlay of diazocoupling products MS traces for reaction at pH 8.

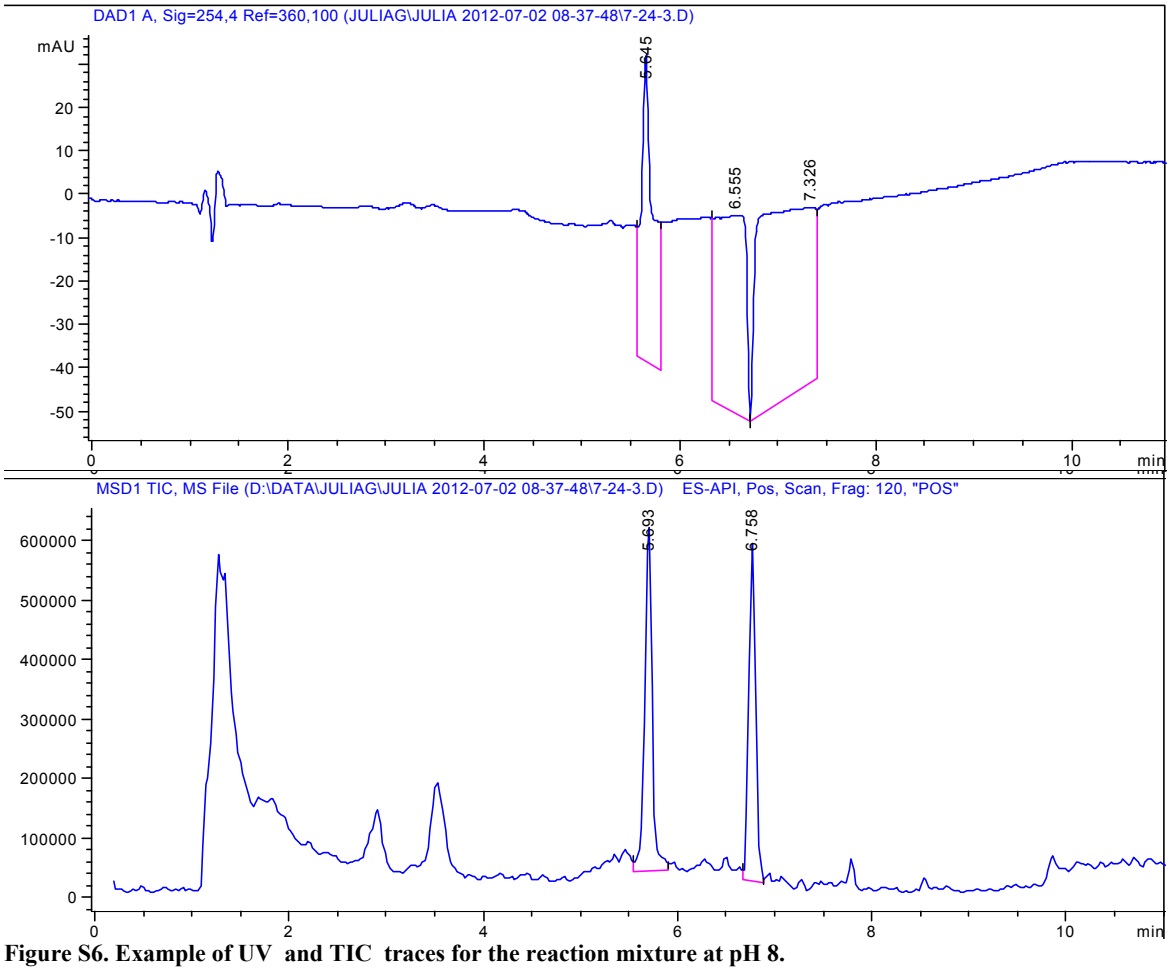


Figure S6. Example of UV and TIC traces for the reaction mixture at pH 8.

Table S5. Summary of the amino acid modification in the equimolar mixture at pH 8.

pH 8	% Conversion				
Amino Acid Adduct	**1**	**2**	**3**	**Average**	**STD**
Tyr	97.0	94.8	94.0	95.3	1.559
His	0.0	1.2	1.7	1.0	0.874
Cys	2.1	2.0	1.2	1.8	0.454
Lys	0.0	0.0	0.0	0.0	0
Trp	0.9	2.0	3.1	2.0	1.076
Ser	0.0	0.0	0.0	0.0	0

8. Tocinoic adic modification with FBDP

To the 1 ml of 100 μM solution of tocinoic acid (Sigma-Aldrich) in 0.1 M Na_2HPO_4 buffer pH 7 or pH 8 was added 10 μL of 20 mM solution of FBDP in acetonitrile. Reaction was vortexed gently and allowed to react at room temperature. Aliquot of reaction mixture (5 μL) was analyzed by LCMS after 30 min of reaction time using Agilent LC/MSD SL mass spectrometer with Zorbax 300SP-C8, 5 μm, 4.6x50 mm column (Agilent). HPLC parameters were as follows: flow rate 0.5 ml/min, 5% mobile phase B over 2 min followed by a gradient to 5 to 95% B from 2 to 8 min, post time 4 min. Mobile phase A was 0.1% formic acid (v/v) in HPLC-grade H_2O, and mobile phase B was 0.1% formic acid (v/v) in acetonitrile (v/v). API-ESMS data was collected for 115-1000 m/z positive and negative polarity. Data was analyzed using Agilent analysis software. MS TIC positive polarity trace was used to evaluate reaction selectivity. For clarity and quantification purposes traces corresponding to starting material and expected product ions were extracted from the MS TIC spectra (Figure S7). Area under the peak was used to calculate content of remaining starting material and product (Table S6). No *N*-terminal modification product has been found in the reaction mixture.

Table S6. Tocinoic acid modification summary.

pH	Tocinoic acid	Expected [MH+]	Found [MH+]	Peak area	%
7	Starting material	741.2	741.2	4304.12646	11.5
	Product	873.2	873.2	3.32795e4	88.5
8	Starting material	741.2	741.2	8853.909	8.6
	Product	873.2	873.2	9.4148e4	91.4

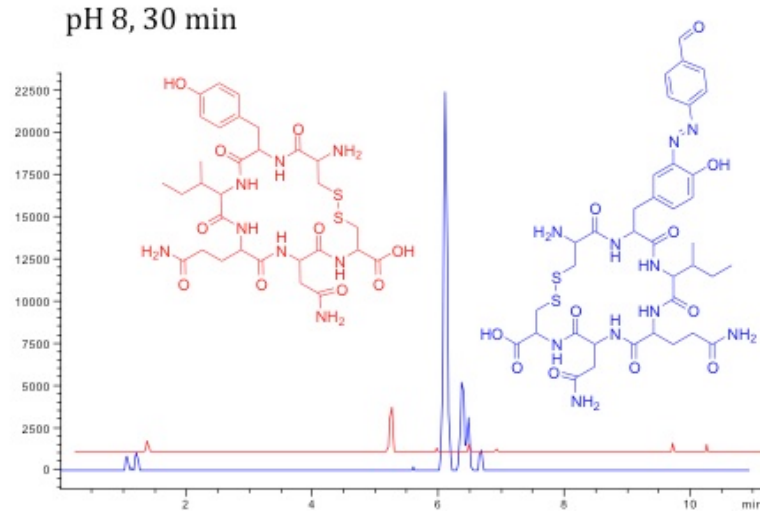


Figure S7. Example of the 3D overlay of extracted starting material and product ions, reaction at pH 8.

9. BSA modification with FBDP

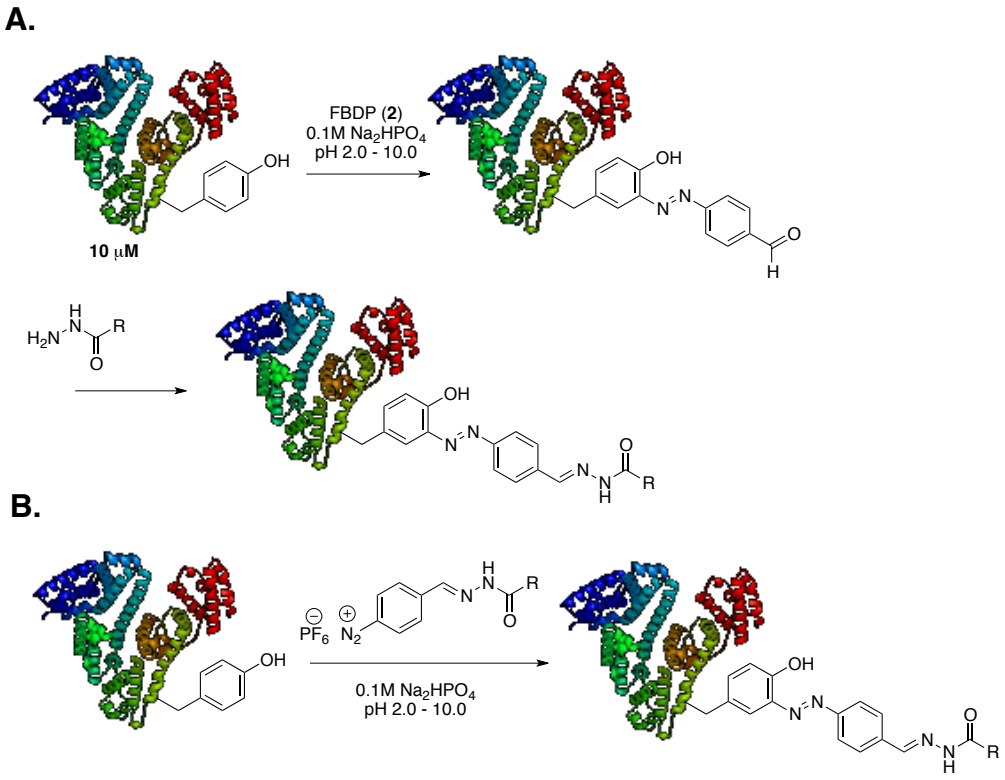


Figure S8. Schematic representation of the BSA modification methods.

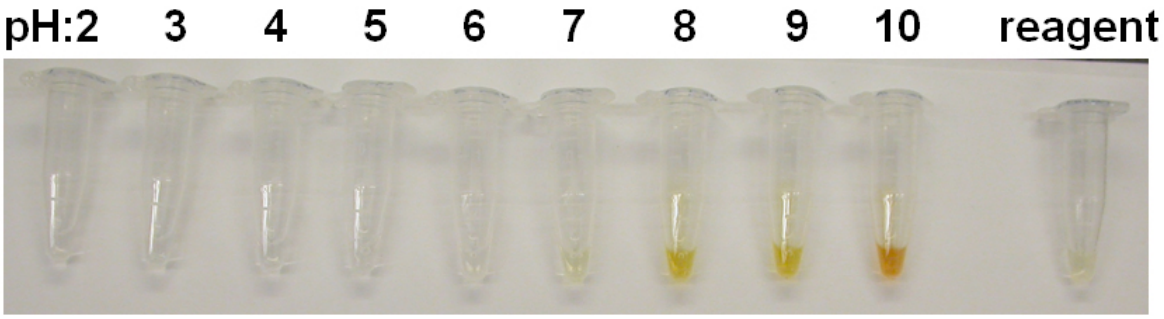


Figure S9. Visual follow up of the reaction of 100μM FBDP reagent 2 with 30 μM solution of BSA at pH 2-10.

General modification procedure.

Method A (Figure S8).

To the 0.5 ml Eppendorf tube containing 99 μL of BSA solution (30 μM solution in 0.1M phosphate buffer pH 2-10) was added 1 μL or reagent solution (10 mM solution in CH_3CN) and reaction mixture was vortexed briefly. Reaction was kept at room temperature for 30 minutes and the conversion was followed by visual observation (Figure S9) and UV-vis measurement at 340 nm that corresponds to the absorbance of diazene functionality (data not shown). The reaction products were purified using Zeba Spin desalting column (7,000Da MWC, Pierce) and the buffer was exchanged to PBS pH 5. In the 1.5 mL Eppendorf tube 99 mL of 30 μM solution of BSA-aldehyde in 0.1 M PBS pH 5 was treated with biotin hydrazide (1 μL of 100 mM solution in DMSO), over night 4 ^{o}C, followed by removal of the excess of small molecule by Zeba Spin Desaltin column. The reaction products were tested in streptavidin ELISA (Figure S11) and characterized by MALDI-TOF (Figure S13).

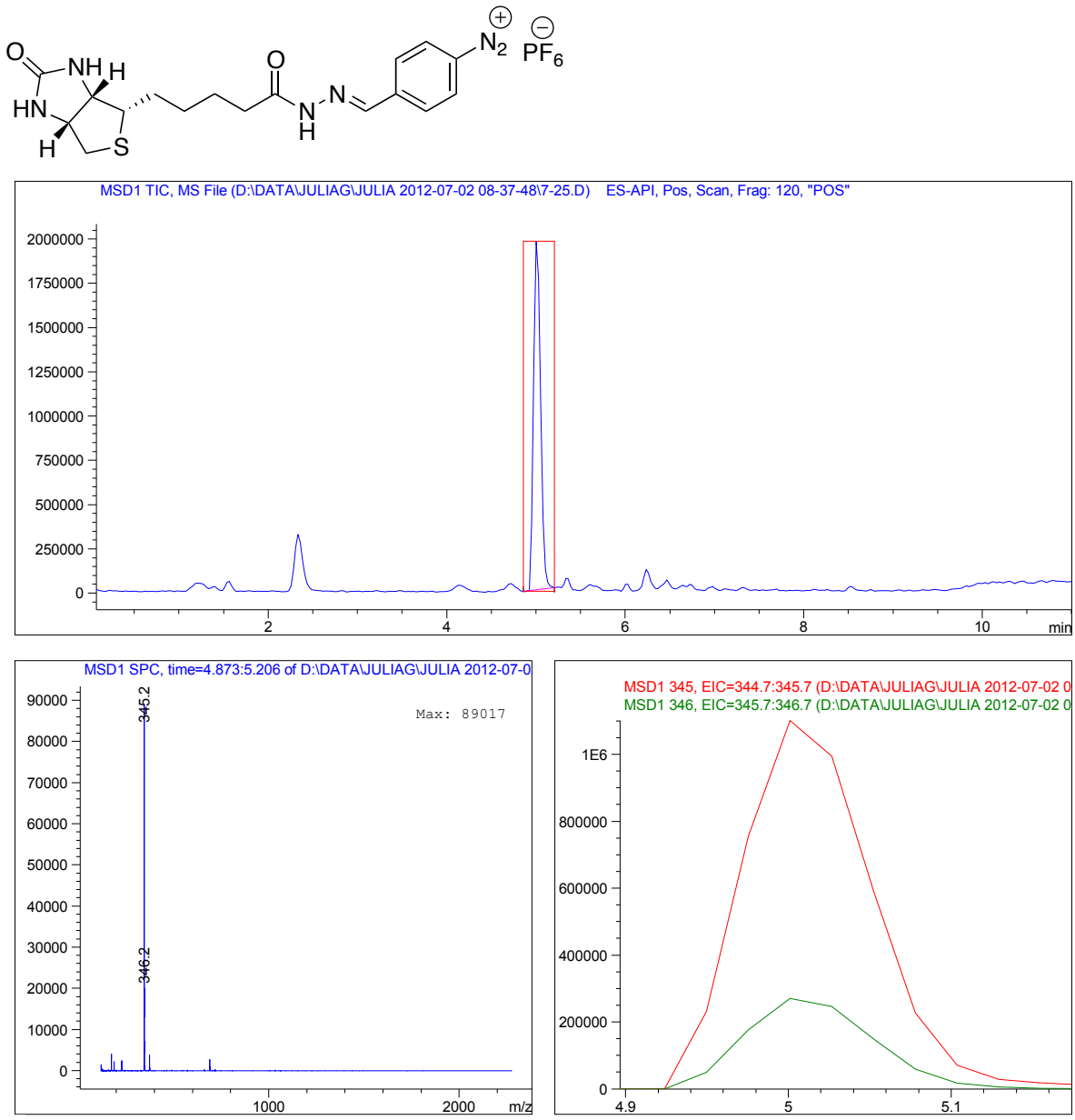
Method B (Figure S8).

In the 0.5 ml Eppendorf tube were combined biotin hydrazide (10 μL of 100 mM solution in CH_3CN; 1 eq), trifluoroacetic acid (10 μL of 200 mM solution in CH_3CN; 2 eq) and FBDP (10 μL of 100 mM solution in CH_3CN; 1 eq). The reaction mixture was mixed and allowed to react for 1 hr. Aliquot of the reagent was diluted and analyzed by LCMS (Figure S10) confirming formation of the hydrazide.

To the 0.5 ml Eppendorf tube containing 99 μL of BSA solution (30 μM solution in 0.1M phosphate buffer pH 2-10) was added 1 μL or reagent solution (10 mM solution in CH_3CN) and reaction mixture was vortexed briefly. Reaction was kept at room temperature for 30 minutes and reaction products were purified using Zeba Spin desalting column (7,000Da MWC, Pierce). The reaction products were tested in streptavidin ELISA (Figure S12) and characterized by MALDI-TOF.

Streptavidin ELISA.

Costar 96-well ELISA plate (Corning, Acton, MA) was coated with 100 ng of protein in 100 μL of PBS pH 7.4 and incubated overnight at 4 ^{o}C. The plate was washed with PBS containing 0.01% tween-20 (100 μL x 3). After blocking with 100 μL of 3% BSA/PBS, 0.01% tween-20 for 1 h at 37 ^{o}C and washing (100 μL of PBS containing 0.01% tween-20 x 5), 100 μL of streptavidin-HRP (Zymed, 1:3000 dilution in PBS) was added to each well and the plate was incubated for 1 hr at 37 ^{o}C. The plate was thoroughly washed with PBS/0.01% tween (100 μL x 5). Upon addition of the ABTS developing solution and incubation at room temperature for 10 min the absorbance was read at 405 nm. All measurements were done in triplicate and an average normalized result was graphed.




```
Peak #1 at  5.008 min ( 4.862 to  5.209 min)
-> The analysis found only one component, indicating a pure peak. <-

Component 1:  Peak at Scan 193.3. Top ions are  345 346 344
```

Figure S10. **LCMS analysis of the *in situ* formed biotin-hydrazine-diazonium. Found [M+]=345.2 corresponds to –N_2 adduct formed under MS ionization conditions**

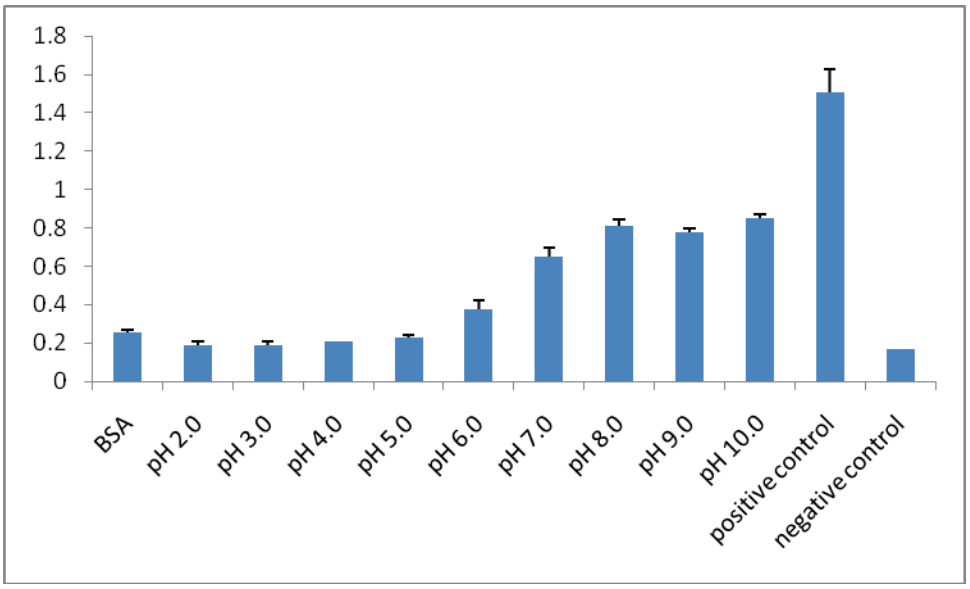


Figure S11. Streptavidin ELISA for the products of Method A. Biotinylated human IgG was used as positive control. Negative control reaction: 99 mL of 30 mM solution of BSA in 0.1 m PBS pH 6 was treated with biotin hydrazide(1 mL of 100 mM solution in DMSO), over night 4 ^{o}C, followed by removal of the excess of small molecule by Zeba Spin Desalting column.

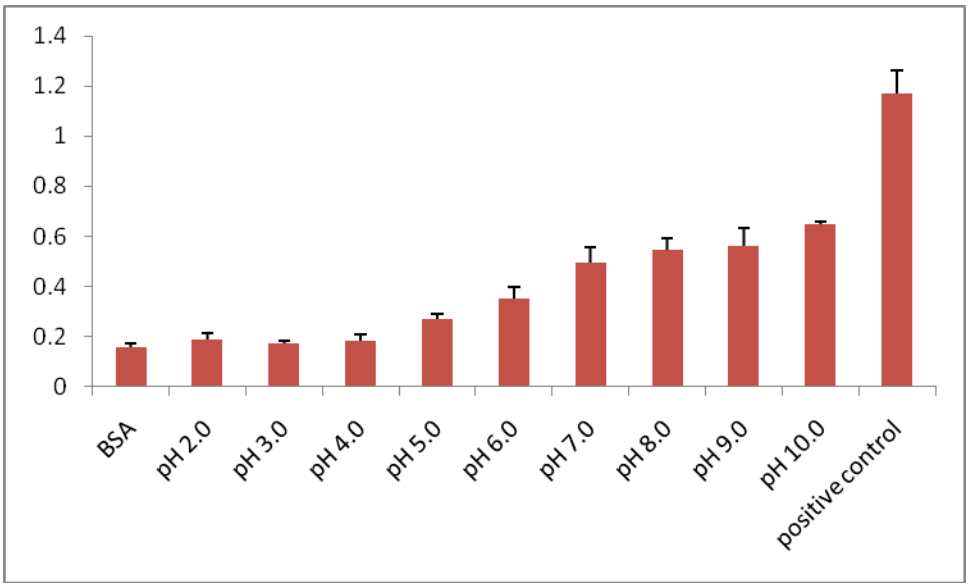
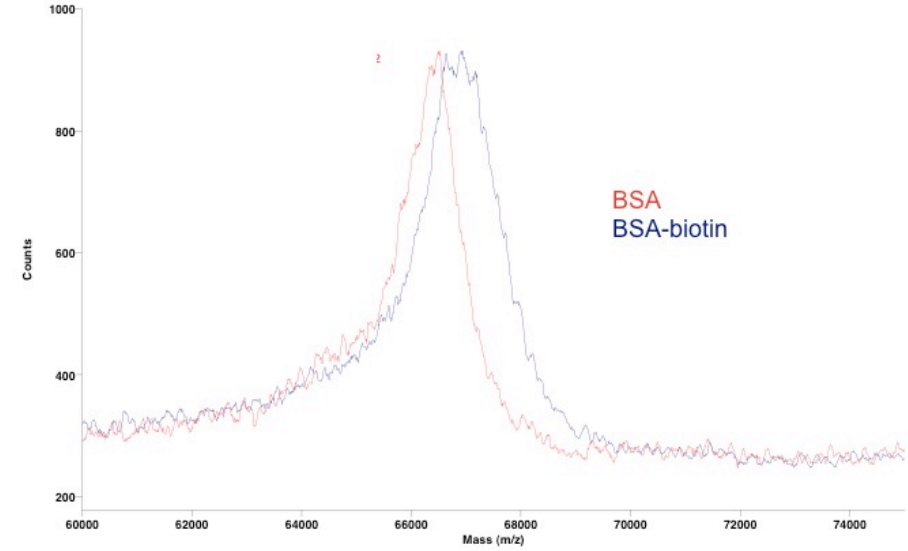


Figure S11. Streptavidin binding ELISA results for the products of Method B. Biotinylated human IgG was used as a positive control.



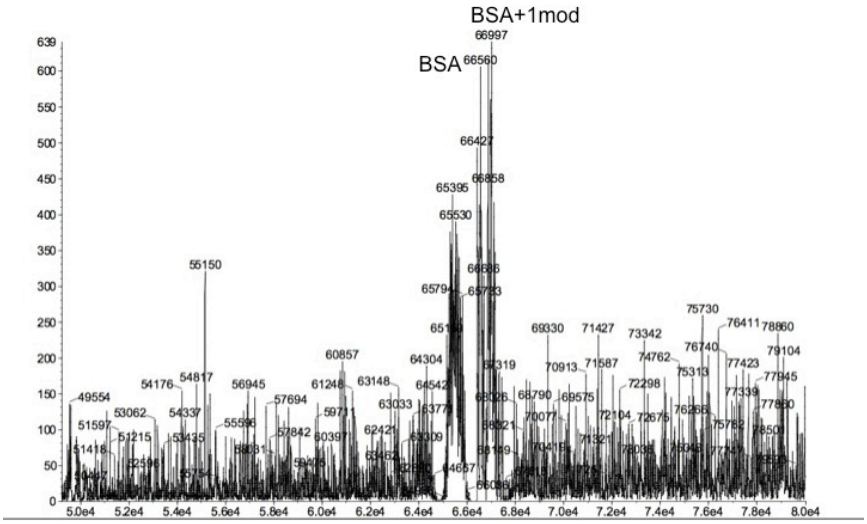


Figure S12. (A)Example of MALDI-TOF characterization of FBDP-based biotin modification of BSA. (BSA, red, MWav= 66417; BSA-biotin, blue, MWav=66892). (B) ESI-MS characterization of biotin modified BSA (MW of modification 446Da).

10. Chymotrypsinogen A modification and activity assay

Chymotrypsinogen was modified with 3.3 equivalents of FBDP at pH 8.0 following BSA modification procedure described above. Activity assay was performed essentially as described by Francis et al.[1] In brief, an 114 µL aliquot of a reaction mixture for the modification of chymotrypsinogen (1.0 mg/mL, 38 µM total protein content) was treated with 2.6 µL of a solution of sequencing grade modified trypsin (Promega, 20 µg reconstituted with 200 µL of 50 mM acid). The

activation of the zymogen was allowed to proceed for 10 minutes at room temperature before being purified and performing buffer exchange into 0.1 M Tris buffer (pH 7.6) using Zeba spin desalting column (Pierce). 40 µL aliquots of the activated protease were then added to 160 µL of 0.5 mM chymotrypsin substrate I, colorimetric (Suc- GGF-pNA, Calbiochem 230912) in 50 mM CaCl2, 20 mM Tris buffer, pH 7.6 (light green triangles). An analogous procedure was followed for reagent modified chymotrypsinogen A samples (light purple squares). In a negative control experiment, the enzyme was not activated with trypsin before addition of the tripeptide substrate (red squares), or the substrate was monitored with addition of only Tris buffer (blue diamonds). The progress of the reaction was monitored by UV-Vis spectrophotometry at 379 nm every 30 sec for 10 min (Figure S14). Each measurement was done in duplicate and the average value was plotted.

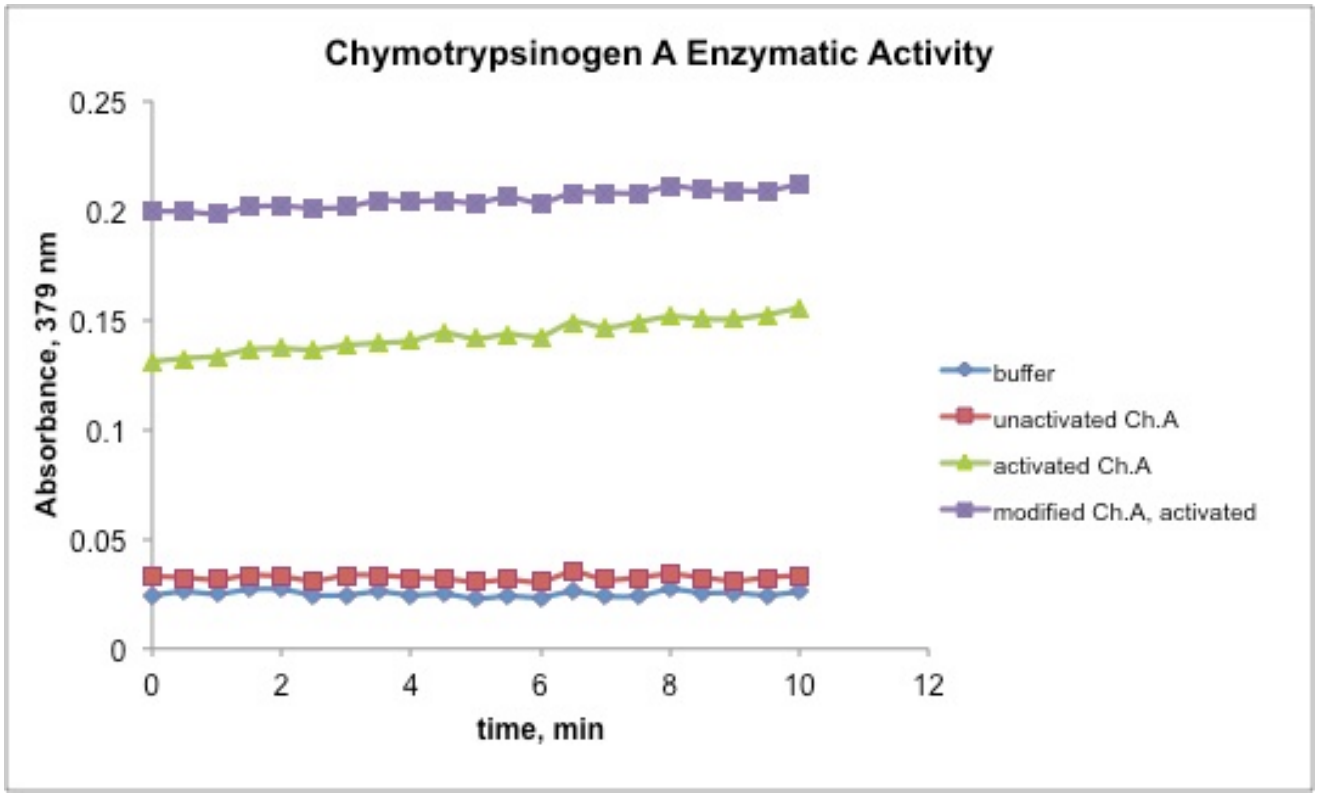


Figure S13. Chymotrypsinogen A activity assay.

11. FBDP-based PEGylation of Human Serum Albumin

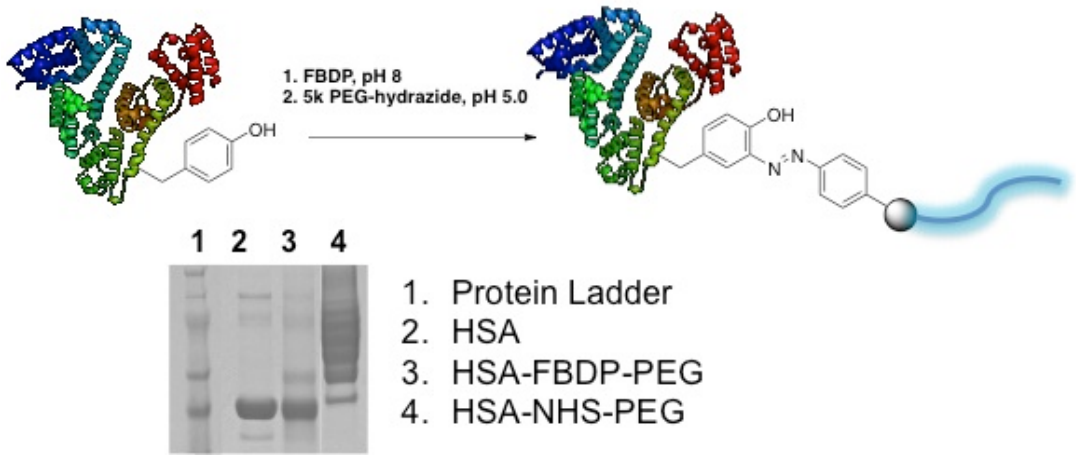


Figure S14. PEGylation of Human Serum Albumin.

5K linear PEG-hydrazide was prepared from commercially available 5k linear PEG-NHS (NOF corporation) by reacting it with 10 equivalents of anhydrous hydrazine in MeOH for 24 h, rt, followed by capping of any unreacted activated ester groups with excess methylamine. Resulting PEG-hydrazide was precipitated out with cold diethyl ether and washed extensively with ice-cold ether to provide desired hydrazide polymer as fine white powder. Hydrazide formation was confirmed by MALDI-TOF characterization (data not shown, MW_{av} (PEG-hydrazide)=5312, MW_{av} (PEG-NHS)=5533).

To the 100 μL of human serum albumin solution (2.5 mg/ml in 0.1M phosphate buffer pH 8.0, 1 eq) was added 1 μL of FBDP (37.5 μM in acetonitrile, 10 eq). Reaction mixture was vortexed gently and left at room temperature for 30 min. Reaction mixture turned yellow. Excess FBDP was removed using Zeba Spin desalting column (7K MWCO, Pierce) and the buffer was exchanged to 0.1M phosphate buffer pH 5. To the solution of HSA-aldehyde was added 5k PEG-hydrazide (1 μL of 37.5 μM solution in DMF, 10 eq). Reaction mixture was vortexed gently and allowed to react for 18h at rt. For a comparison, reaction of HSA (100 μL, 2.5 mg/ml, PBS pH 7.4) with 5k PEG-NHS (NOF corporation; 1 μL of 37.5 μM solution in DMF, 10 eq)) was set in parallel. Crude reaction products were characterized by SDS-PAGE gel electrophoresis using 4-13% Bis-Tris gel (Invitrogen)(Figure S15).

12. Optimization study for FBDP modification of HSA

Reaction procedure to determine pH effect.

Each reaction performed in duplicate. To the100 μL of HSA solution (2.5 mg/ml) in 0.1M phosphate buffer (pH 7; 7.4; 7.6; 7.8; 8; 8.4) was added 1 μL of FBDP solution in acetonitrile (37.7 μM). Reaction mixture was vortexed gently and allowed to react for 30 min at rt followed by removal of excess FBDP using Zeba Spin desalting column. Protein concentration was measured by two independent methods (Bradford assay and absorption at 280nm (Nanodrop, ThermoFisher Scientific), correction factor for diazene adduct = 0.436). Diazene content was estimated based on the absorption at 340 nm and by streptavidin ELISA of HSA-biotin conjugate derived from HSA-aldehyde reaction with biotin-oxyamine (20 eq). The values in all assays were found to correlate well and number of modifications/protein derived from measurement of absorption at 280nm and 340nm were used thereafter. HSA-biotin conjugate was also characterized by MALDI-TOF and LSMS-ESI (Figure S17)

Number of modifications/HSA was plotted as a function of pH (Figure 16).

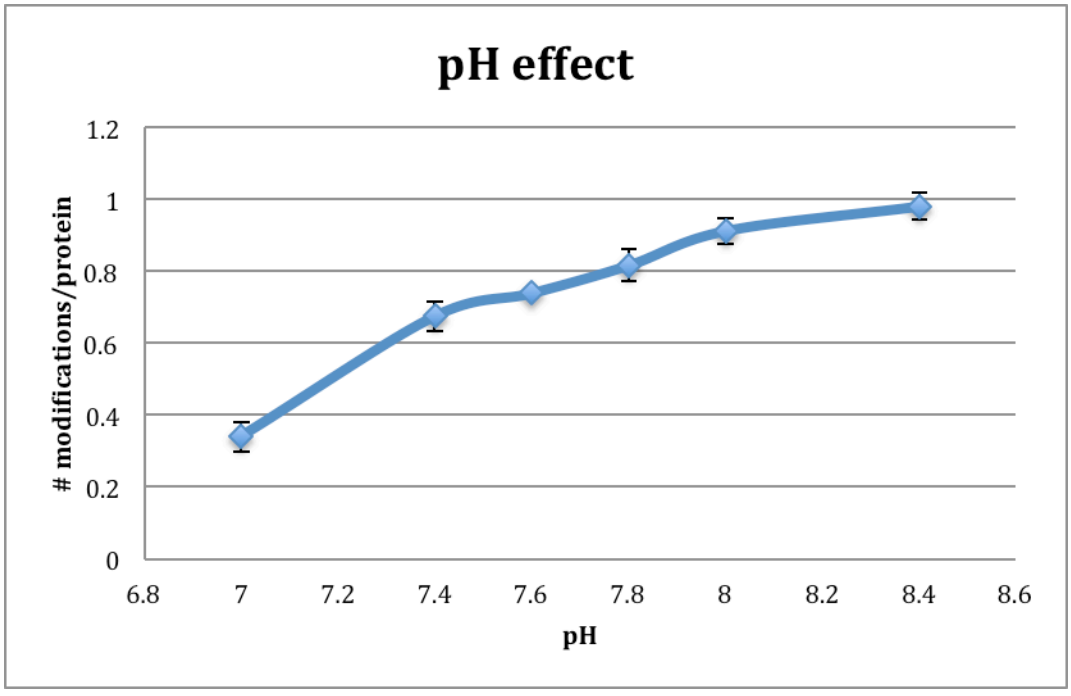
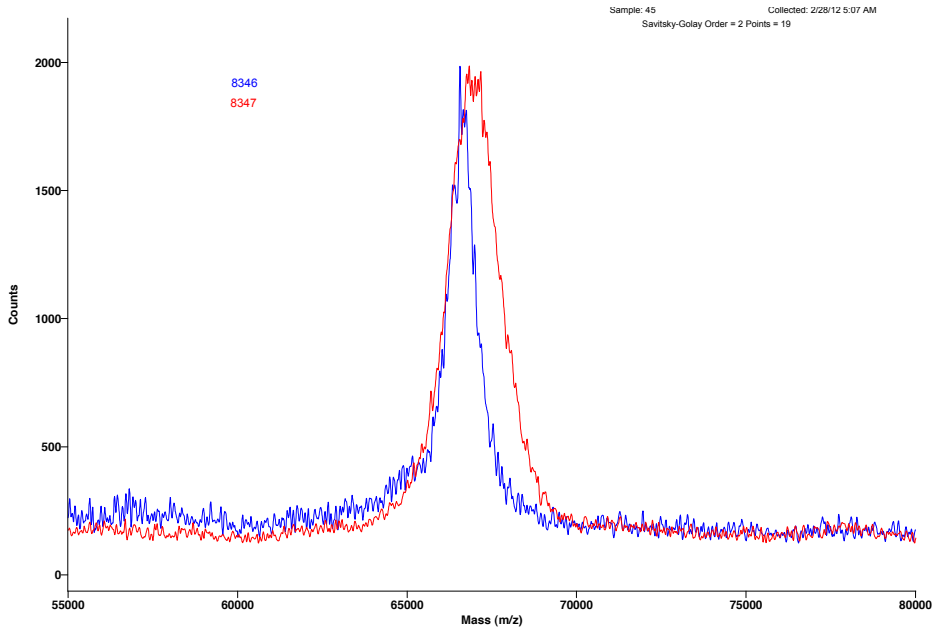


Figure S15.

A.



B.

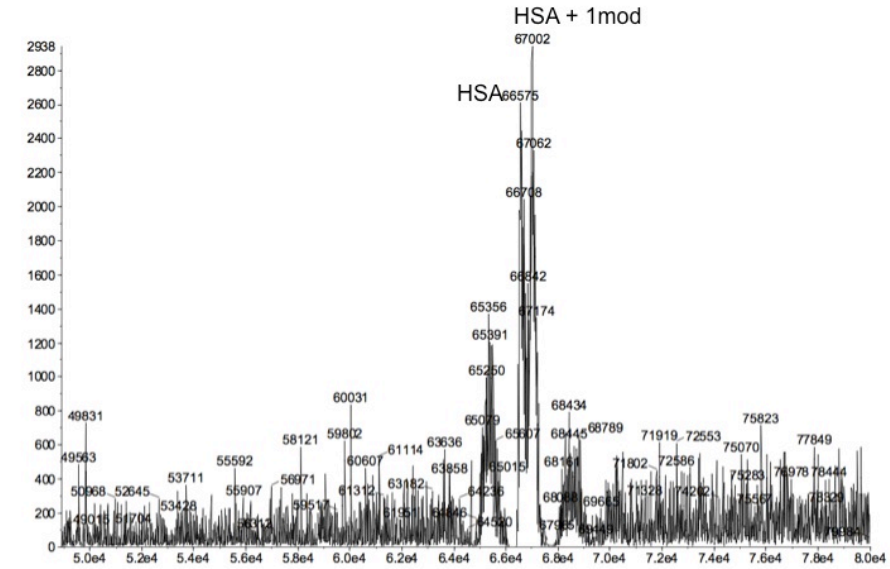


Figure S16. (A) Overlay of MALDI-TOF spectra of HSA (blue, MW_{av}=66525) and HSA-biotin (red, MW_{av}=66931). (B) ESI-MS characterization of HSA-FBDP-biotin conjugate (MW of modification 446 Da)

Reaction procedure to determine time effect.

Each reaction performed in duplicate. To the100 μL of HSA solution (2.5 mg/ml) in 0.1M phosphate buffer (pH 8) was added 1 μL of FBDP solution in acetonitrile (37.7 μM). Reaction mixture was vortexed gently and allowed to react for set amount of time (0.5hr, 1hr, 2hr, 3hr, 4hr) at rt followed by removal of excess FBDP using Zeba Spin desalting column. Protein concentration was measured by two independent methods (Bradford assay and absorption at 280nm (Nanodrop, ThermoFisher Scientific), correction factor for diazene adduct = 0.436). Diazene content was estimated based on the absorption at 340 nm and by streptavidin ELISA of HSA-biotin conjugate derived from HSA-aldehyde reaction with biotin-oxyamine (20 eq).

Number of modifications/HSA was plotted as a function of time (Figure S18).

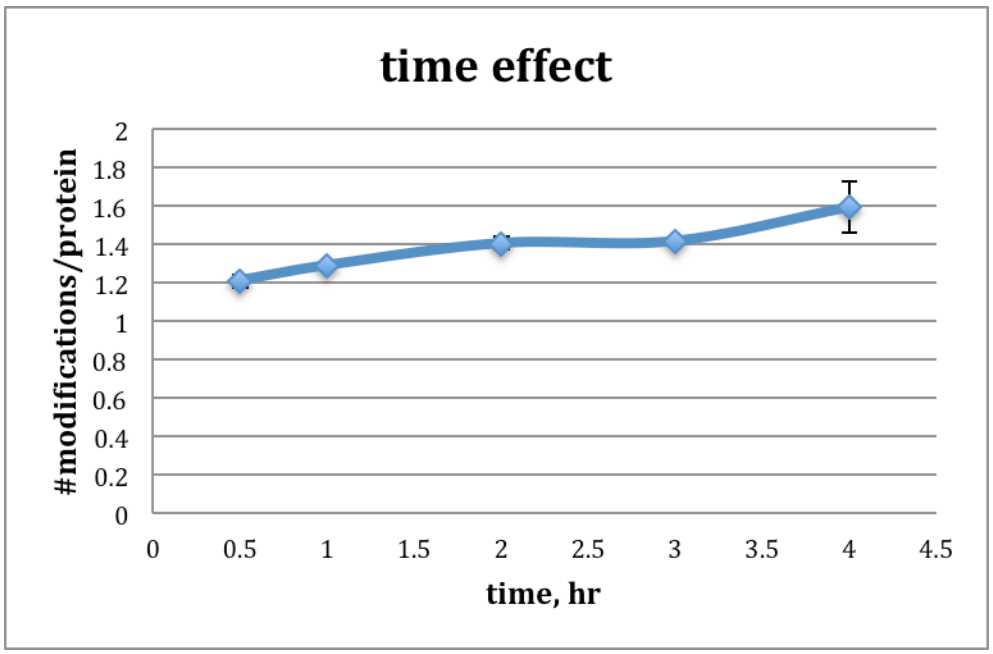


Figure S17

Reaction procedure to determine temperature effect.

Each reaction performed in duplicate. To the100 μL of HSA solution (2.5 mg/ml, 1 eq) in 0.1M phosphate buffer (pH 8) was added 1 μL of FBDP solution in acetonitrile (37.7 μM, 10 eq). Reaction mixture was vortexed gently and allowed to react for 30 min at either 4 ^{o}C or rt or 37 ^{o}C followed by removal of excess FBDP using Zeba Spin desalting column. Protein concentration was measured by two independent methods (Bradford assay and absorption at 280nm (Nanodrop, ThermoFisher Scientific), correction factor for diazene adduct = 0.436). Diazene content was estimated based on the absorption at 340 nm and by streptavidin ELISA of HSA-biotin conjugate derived from HSA-aldehyde reaction with biotin-oxyamine (20 eq).

Number of modifications/HSA was plotted as a function of temperature (Figure S19).

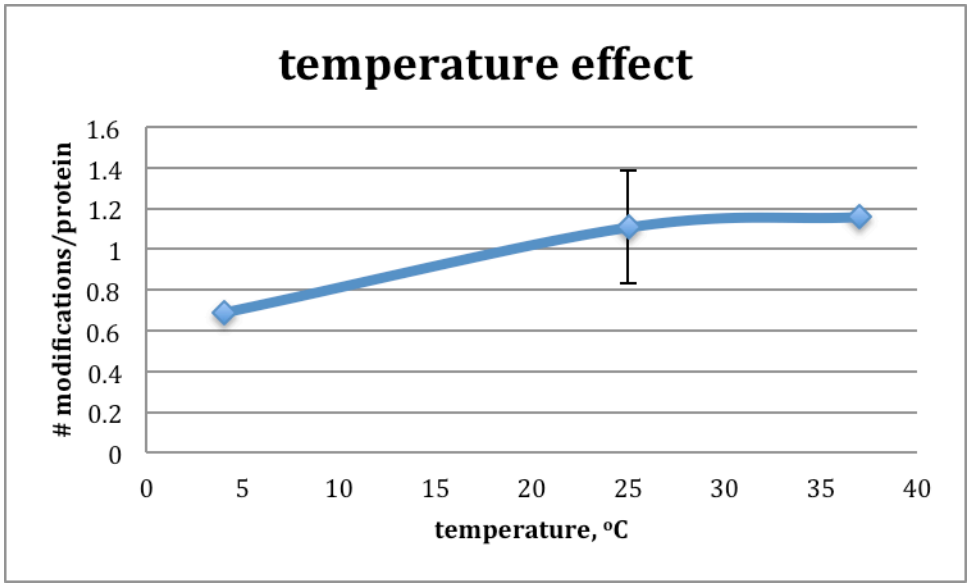


Figure S18

Reaction procedure to determine effect of the excess of reagent.

Each reaction performed in duplicate. To the100 μL of HSA solution (2.5 mg/ml, 1 eq) in 0.1M phosphate buffer (pH 8) was added 1 μL of FBDP solution in acetonitrile at concentrations that would result in the following excess of the reaget: 1 eq, 5 eq, 10 eq, 20 eq, 50 eq. Reaction mixture was vortexed gently and allowed to react for 30 min at rt followed by removal of excess FBDP using Zeba Spin desalting column. Protein concentration was measured by two independent methods (Bradford assay and absorption at 280nm (Nanodrop, ThermoFisher Scientific), correction factor for diazene adduct = 0.436). Diazene content was estimated based on the absorption at 340 nm and by streptavidin ELISA of HSA-biotin conjugate derived from HSA-aldehyde reaction with biotin-oxyamine (20 eq).

Number of modifications/HSA was plotted as a function of reagent excess (Figure S20).

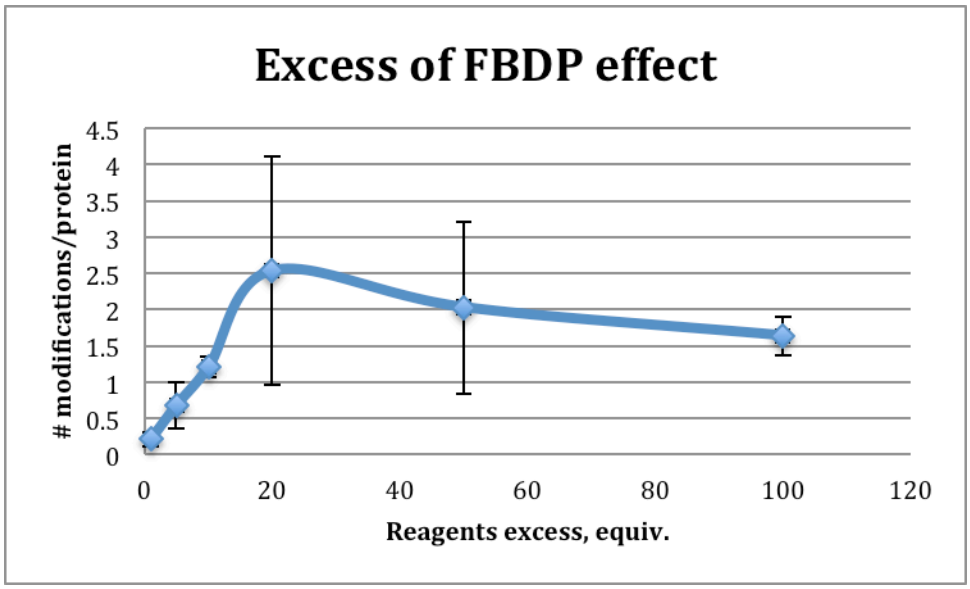


Figure S19

Reaction procedure to determine effect of HSA concentration.

Each reaction performed in duplicate. To the100 μL of HSA solution at the following concentrations: 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml in 0.1M phosphate buffer (pH 8) was added 1 μL of FBDP solution in acetonitrile at concentrations that would result in the 10 fold excess of the reaget. Reaction mixture was vortexed gently and allowed to react for 30 min at rt followed by removal of excess FBDP using Zeba Spin desalting column. Protein concentration was measured by two independent methods (Bradford assay and absorption at 280nm (Nanodrop, ThermoFisher Scientific), correction factor for diazene adduct = 0.436). Diazene content was estimated based on the absorption at 340 nm and by streptavidin ELISA of HSA-biotin conjugate derived from HSA-aldehyde reaction with biotin-oxyamine (20 eq).

Number of modifications/HSA was plotted as a function of protein concentration (Figure S21).

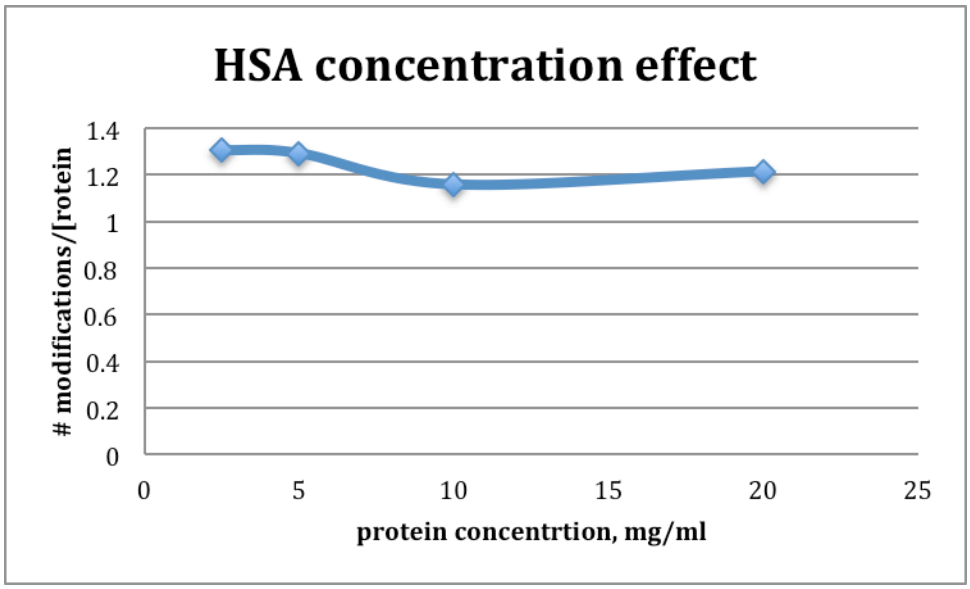
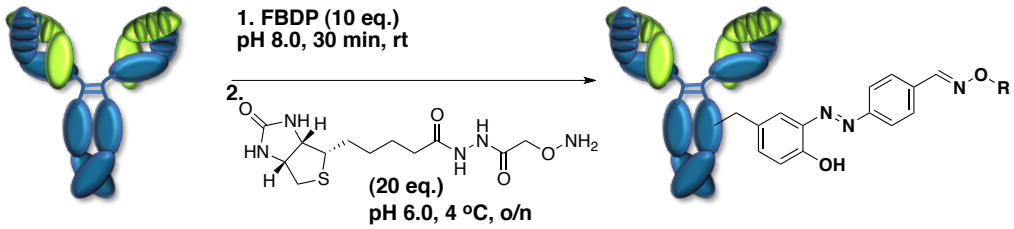


Figure S20

13. FBDP modification of antibody trastuzumab



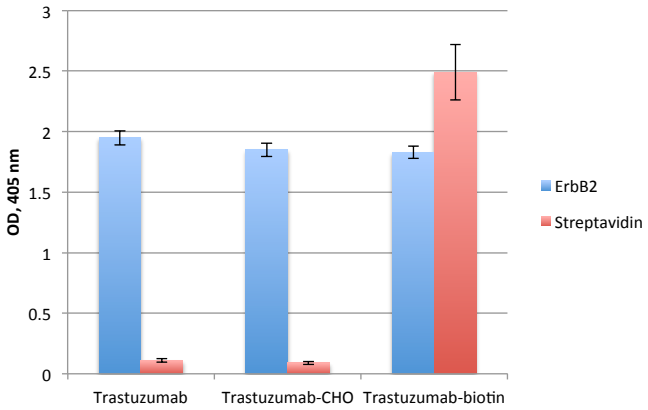


Figure S21.

Modification procedure.

To the 200 μL of trastuzumab solution in 0.1M phosphate buffer pH 8 (1.5 mg/ml) was added FBDP solution in CH_3CN (2 μL of 20 μM solution). Reaction mixture was vortexed gently and allowed to react for 30 min. Reaction mixture turned yellow. The reaction was run through the Zeba spin desalting column (7K MWCO, Pierce) to remove excess FBDP and exchange into phosphate buffer pH 6.0. Then, 4 μL of 20 μM solution of biotin-oxyamine (Cayman Chemical, Cat.# 10009350) was added, reaction was vortexed and allowed to react at 4 ^{o}C for 18h. Excess of unconjugated biotin was removed using Zeba desalting column. Obtained conjugate was characterized by MALDI-TOF (Figure S22) and ELISA assays (Figure S21).

ErbB2 ELISA.

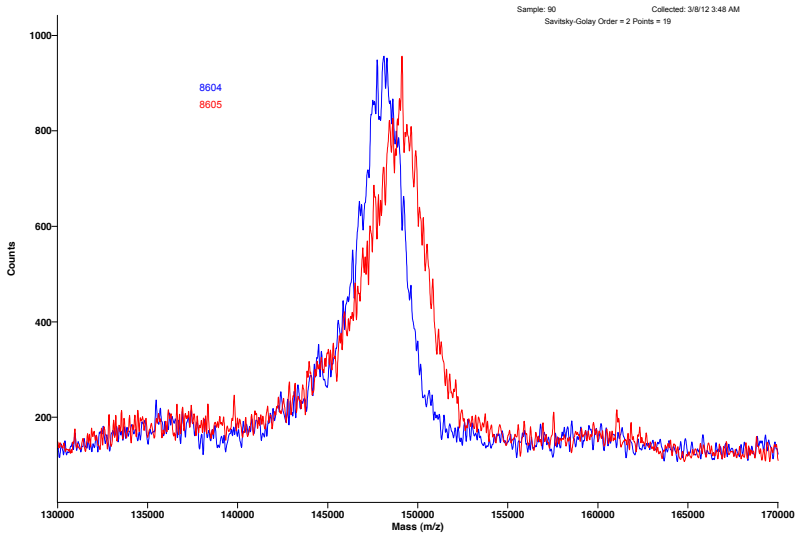
ErbB2 binding ELISA was performed as described.[2] In brief; Costar 96-well ELISA plates (Corning, Acton, MA) were coated with 25 ng of antigen (human ErbB2 or BSA) in 25 μL of PBS pH 7.4 and incubated overnight at 4 ^{o}C. The plate was washed with PBS containing 0.01% tween-20 (100 μL x 3). After blocking with 100 μL of 3% BSA/PBS, 0.01% tween-20 for 1 h at 37 $_{o}$C and washing (100 μL of PBS containing 0.01% tween-20 x 5), 100ng/50 μL /well of trastuzumab, trastuzumab-CHO (trastuzumab modified only with FBDP, but not ligated with biotin-oxyamine) or trastuzumab-biotin(trastuzumab modified with FBDP followed by oxime ligation with biotin-oxyamine) construct or rituximab (negative control) solution was added and the plates were incubated for 1 hr at 37 ^{o}C. The plate was thoroughly washed with PBS/0.01% tween (100 μL x 5) followed by addition of 100 μL /well of secondary antibody solution (donkey anti-Human HRP, diluted 1:1000). The plate was incubated for 1h at 37 ^{o}C and washed with PBS/0.01% tween (100 μL x 5). Upon addition of the ABTS developing solution and incubation at room temperature for 20 min the absorbance was read at 405 nm. All measurements were done in triplicate and an average normalized result was graphed.

Streptavidin ELISA.

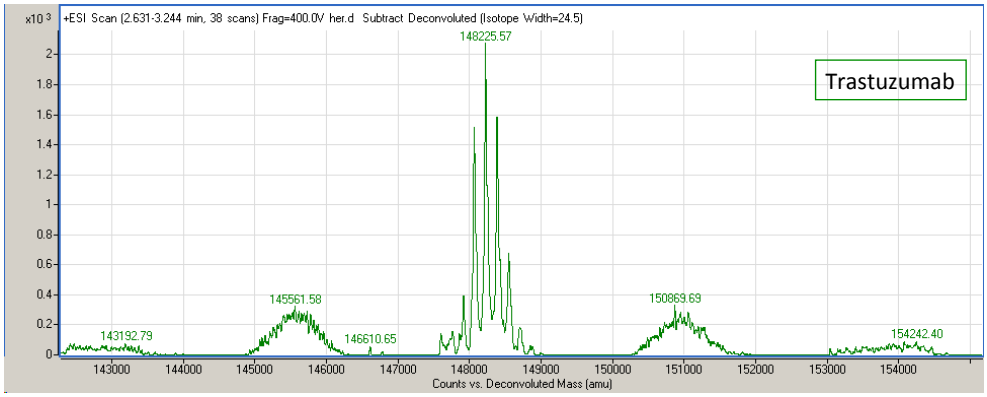
Streptavidin ELISA was performed as previously described. In brief, Costar 96-well ELISA plates (Corning, Acton, MA) were coated with 200 ng of antigen (streptavidin or BSA) in 100 μL of PBS pH 7.4 and incubated overnight at 4 ^{o}C. The plate was washed with PBS containing 0.01% tween-20 (100 μL x 3). After blocking with 100 μL of 3% BSA/PBS, 0.01% tween-20 for 1 h at 37 ^{o}C and washing (100 μL of PBS containing 0.01% tween-20 x 5), 100ng/100 μL /well of trastuzumab, trastuzumab-CHO (trastuzumab modified only with FBDP, but not ligated with biotin-oxyamine) or trastuzumab-biotin (trastuzumab modified with FBDP followed by oxime ligation with biotin-oxyamine) construct, rituximab (negative control), biotinylated goat anti-rabbit antibody (positive

control; Vector, Cat. # F1207) solution was added and the plates were incubated for 1 hr at 37 ^{o}C. The plate was thoroughly washed with PBS/0.01% tween (100 µL x 5) followed by addition of 100 µL /well of appropriate secondary antibody solution (AP goat anti-Human IgG F(ab)$_2$ from Pierce, diluted 1:3000; or AP rabbit anti-goat IgG from Pierce, diluted 1:3000). The plate was incubated for 1h at 37 ^{o}C and washed with PBS/0.01% tween (100 µL x 5). Upon addition of the AP developing solution and incubation at room temperature for 20 min the absorbance was read at 405 nm. All measurements were done in triplicate and an average normalized result was graphed.

(A)



(B)



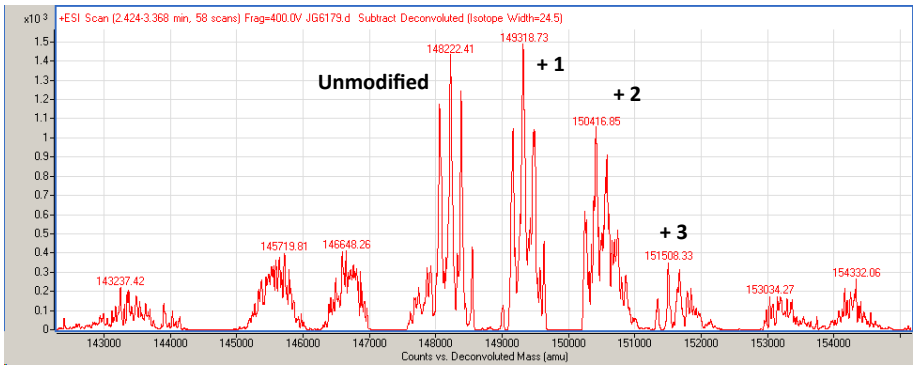
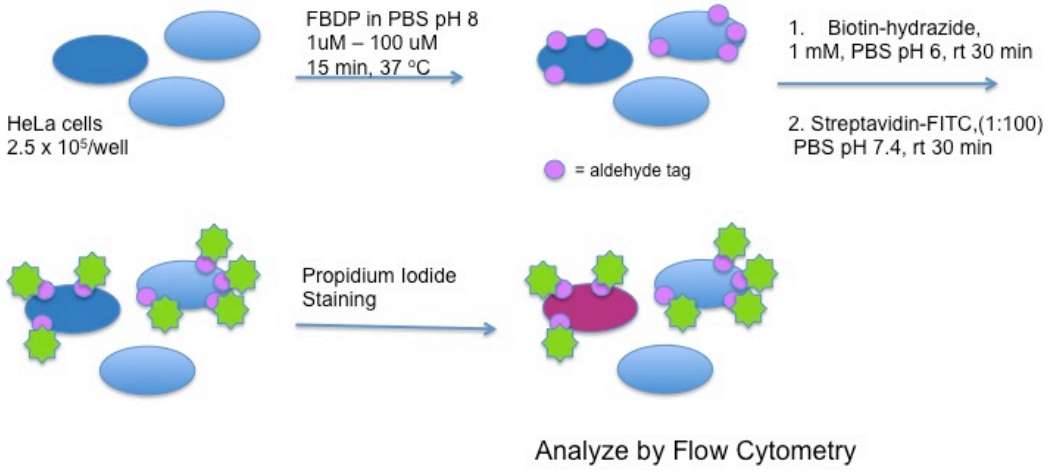
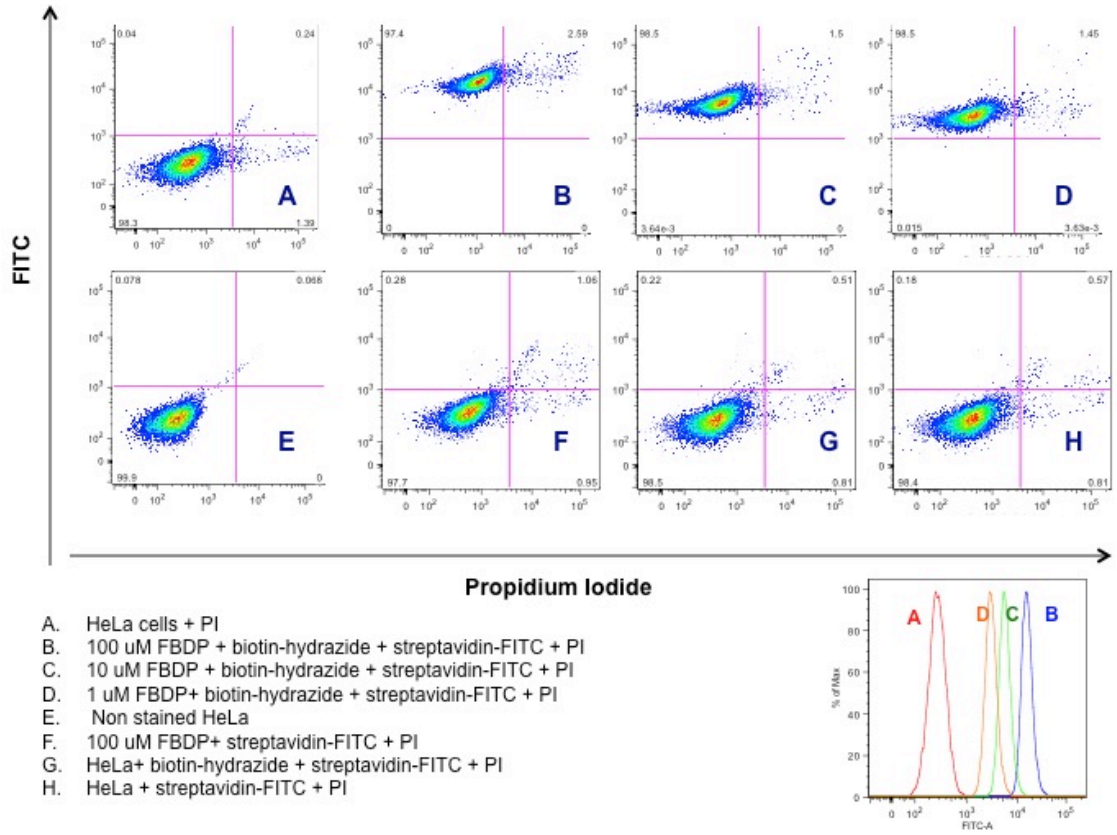


Figure S22. (A) Overlay of MALDI-TOF spectra for trastuzumab (blue, MWav=148302) and trastuzumab-biotin (red, MW_{av}=149053); MWmodification =446Da (B) Deconvoluted ESI spectra of trastuzumab (green) and trastuzumab conjugate (red); MWmodification = 1096Da.

14. Cell Surface Labeling with FBDP



HeLa cells were harvested and washed twice with PBS pH 8.0. Washed cells were resuspended in PBS pH 8.0 to a final concentration of $5x10^6$ cells/ml and 50 μL aliquots were transferred to the V-bottom non-TC treated plate followed by addition of FBDP (50 μL aliquots in PBS pH 8.0, 0.01% acetonitrile; final concentrations of FBDP were 100 μM, 10 μM and 1 μM). Cells were incubated with the reagent for 15 min at 37 °C. Cells were washed with PBS pH 6.0 (3 x 150 μL/well) followed by addition of 100 μL/well of 1 mM biotin-hydrazide (Sigma-Aldrich) in PBS pH 6.0 and incubation at room temperature for 30 min. Cells were washed with FACS buffer (3 x 150 μL/well; PBS pH 7.4, 1% BSA, 0.01% NaN_3). Streptavidin-FITC (Jackson Laboratories) solution in FACS buffer was added to the cells (100 μL/well; 1:100 dilution) followed by incubation for 30 min at room temperature. The cells were washed with FACS buffer (5 x 150 μL/well) followed by Propidium Iodide staining for 15 min at room temperature (1 μg/ml; 100 μL/well). Cells were washed and transferred to the filter-top FACS tubes. Cells were analyzed on LSRII flow cytometer, 30000 events were collected. Data was analyzed usingFlowJo 8.7.1 and Microsoft Excel. All samples were tested in triplicate. Representative graphs from two independent experiments are shown (Figure S23).



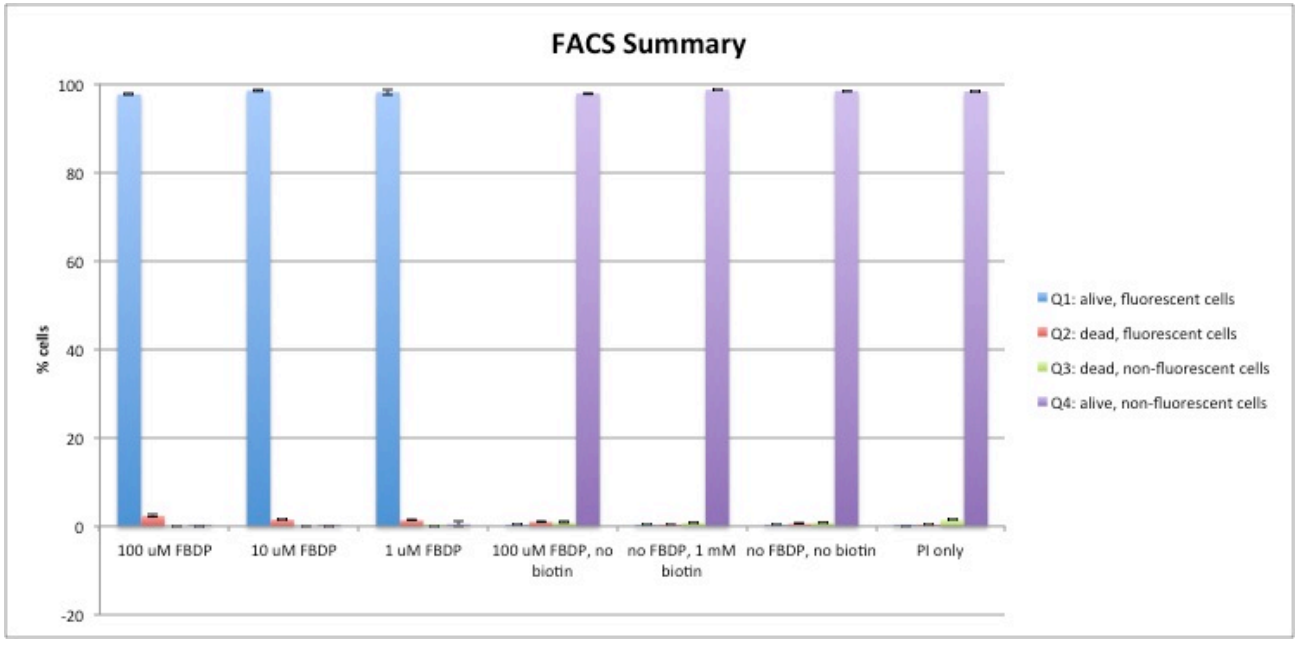


Figure S23. Summary of the HeLa cells surface labeling with FBDP.

15. References

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