Zwitterionic Reagents for Labeling, Cross-Linking and Improving the Performance of Chemiluminescent Immunoassays

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Supplementary Material

Synthetic schemes for compounds 1,2, 4 and 5; HPLC chromatograms of intermediates, final compounds and peptide labeling reactions; MALDI-TOF mass spectra of BSA conjugates and, assay data for theophylline and TSH.

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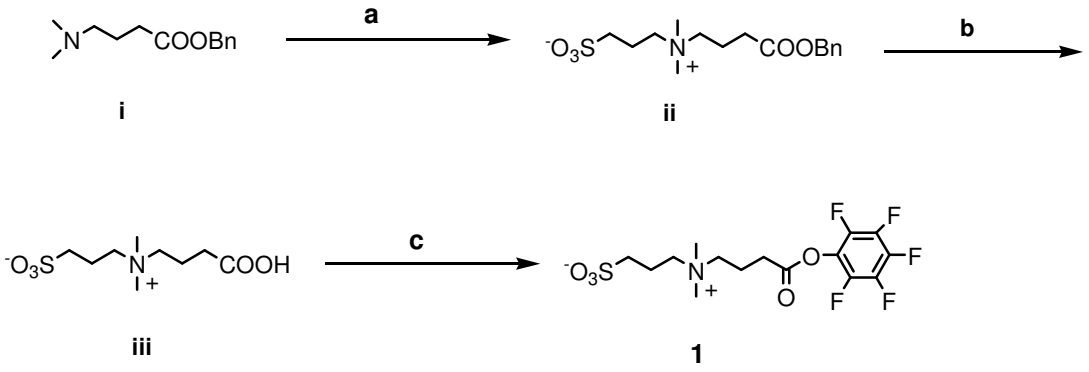


Figure S1. Synthetic scheme for amine-reactive zwitterionic reagent **1**. Reagents: (a) 1,3-propane sultone, 2,6-di-*tert*-butylpyridine, dimethylformamide; (b) hydrogen, 10% palladium on carbon, methanol; (c) pentafluorophenol, 1-ethyl-(dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl), 1:1, 0.1 M HCl/MeCN.

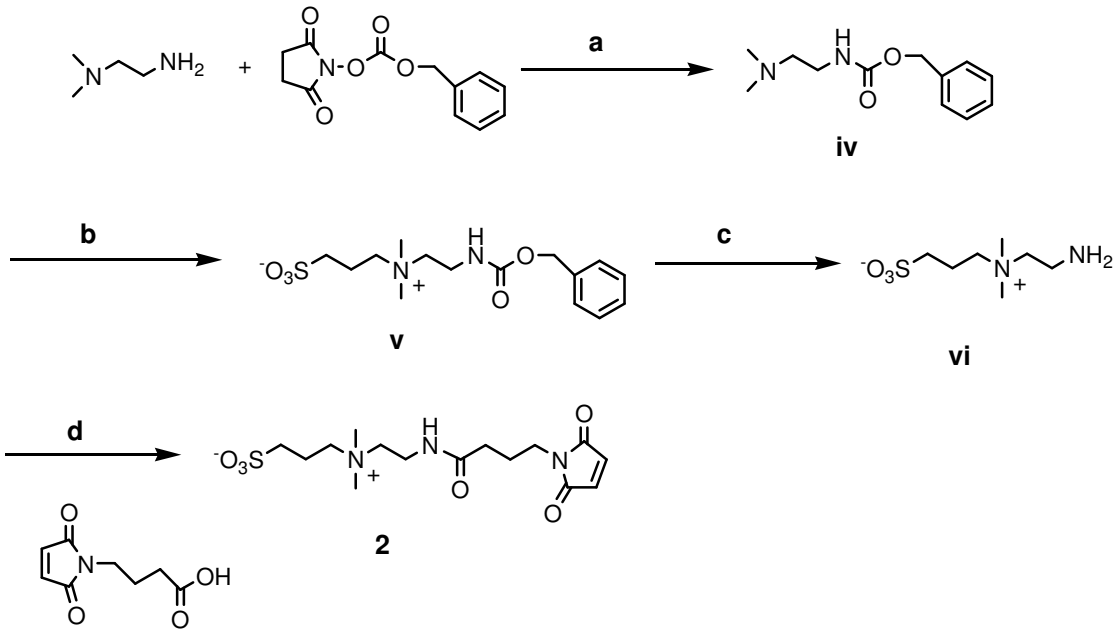


Figure S2. Synthetic scheme for thiol-reactive, zwitterionic reagent **2**.

Reagents: (a) chloroform; (b) 1,3-propane sultone, ethyl acetate; (c) hydrogen, 10% palladium on carbon, methanol/water (d) (BOP) benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate, diisopropylethylamine, dimethylsulfoxide.

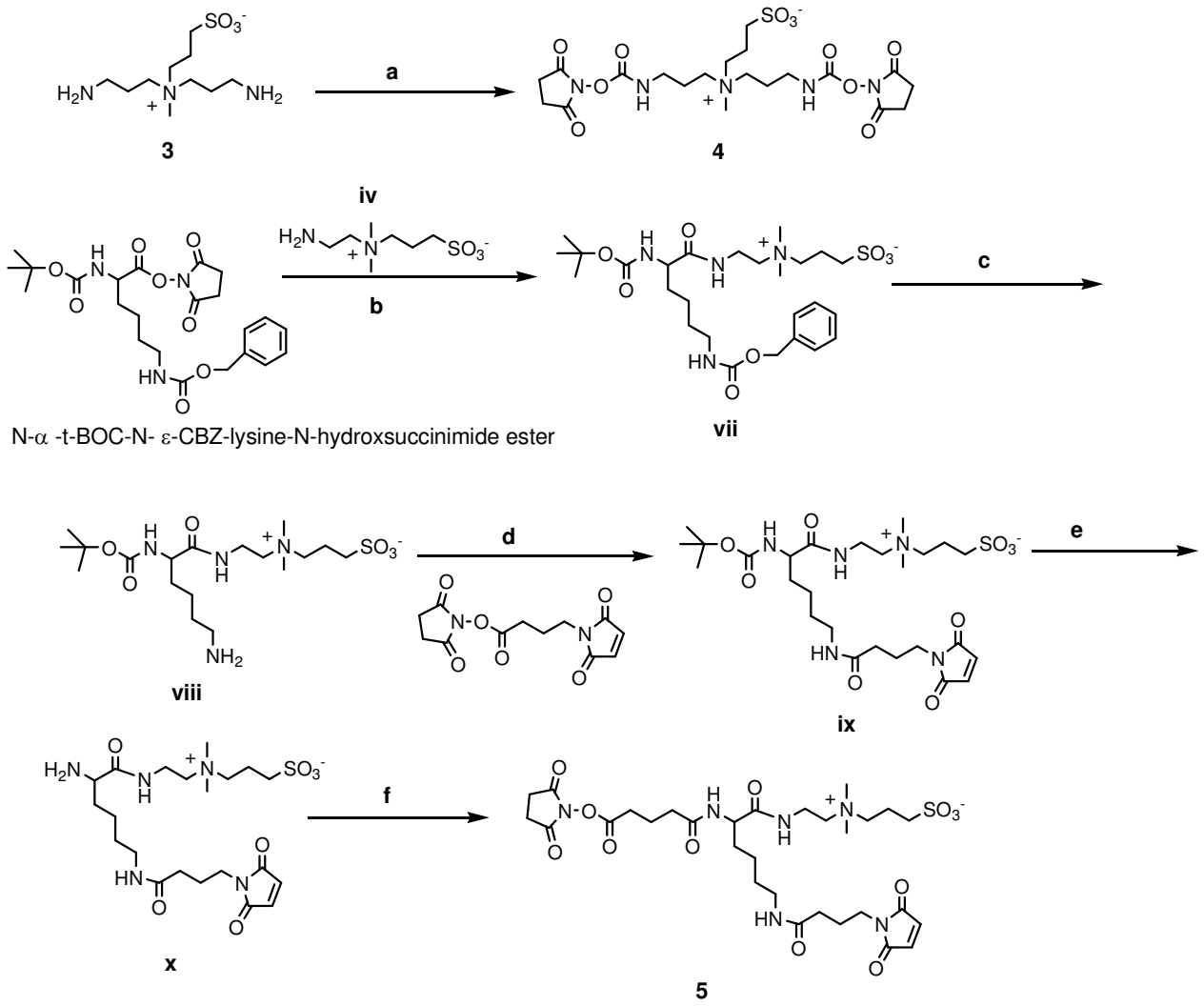


Figure S3. Synthetic schemes for zwitterionic homobifunctional crosslinker **4** and heterobifunctional crosslinker **5**.

Reagents: (a) disuccinimidyl carbonate, diisopropylethylamine, dimethyl sulfoxide; (b) dimethyl sulfoxide; (c) hydrogen, 10% palladium on carbon, methanol/water; (d) dimethyl sulfoxide; (e) trifluoroacetic acid; (f) disuccinimidyl glutarate, dimethyl sulfoxide.

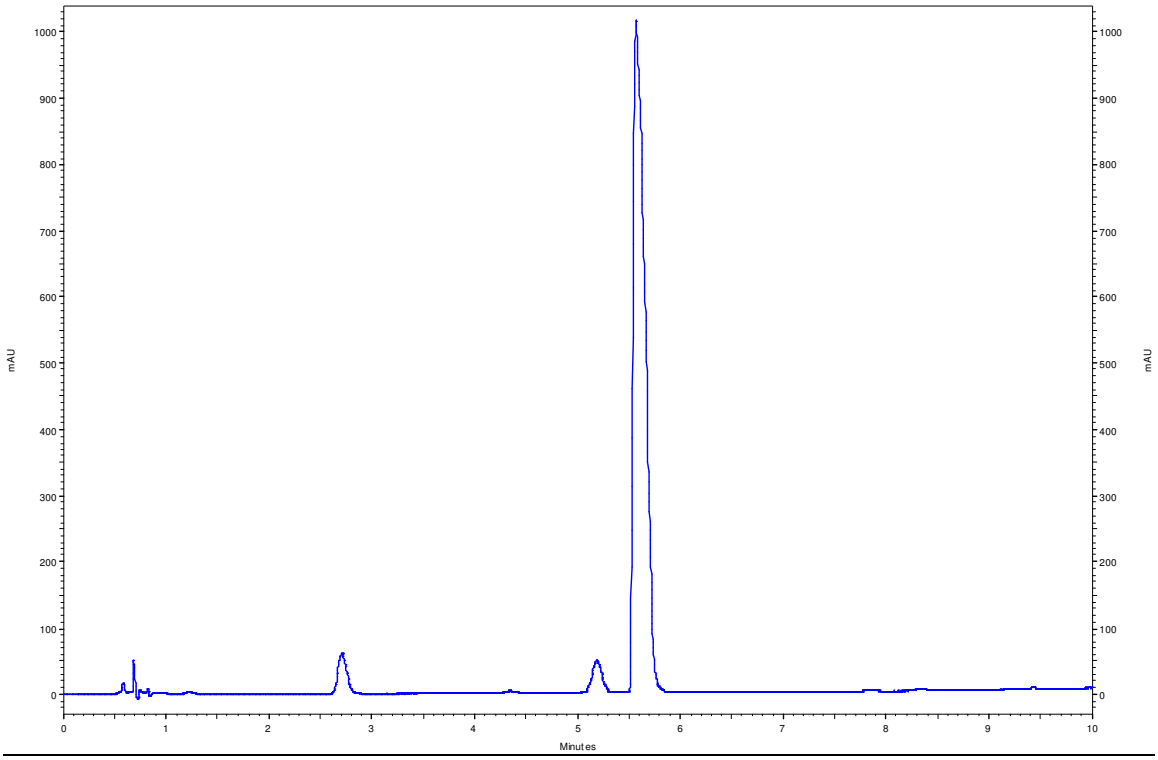
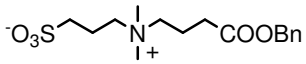


Figure S4. HPLC chromatogram of compound **ii**. HPLC conditions: Phenomenex, Gemini C6-Phenyl, 3 micron, 4.6 x 50 mm column using a 10 minute gradient of 10 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 220 nm.



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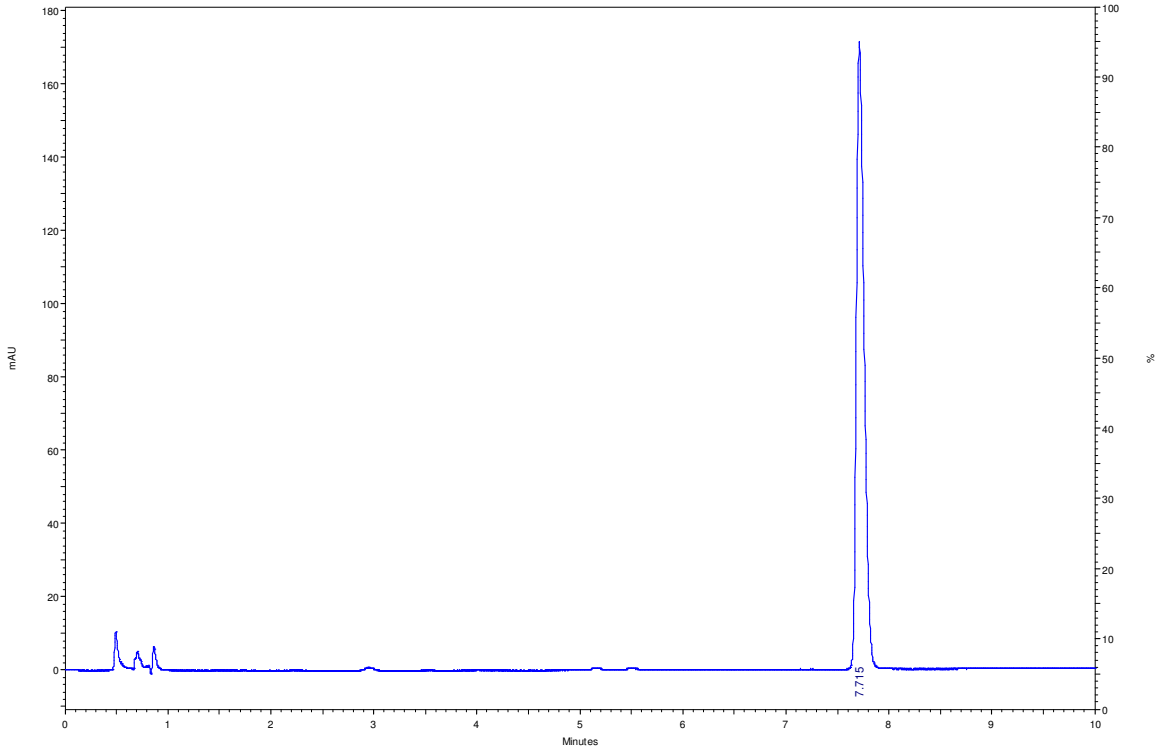
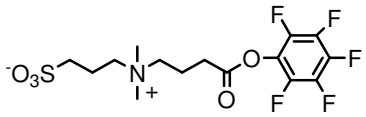


Figure S5. HPLC chromatogram of amine-reactive, zwitterionic reagent **1**. HPLC conditions: Phenomenex, Gemini C6-Phenyl, 3 micron, 4.6 x 50 mm column using a 10 minute gradient of 10 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 260 nm.



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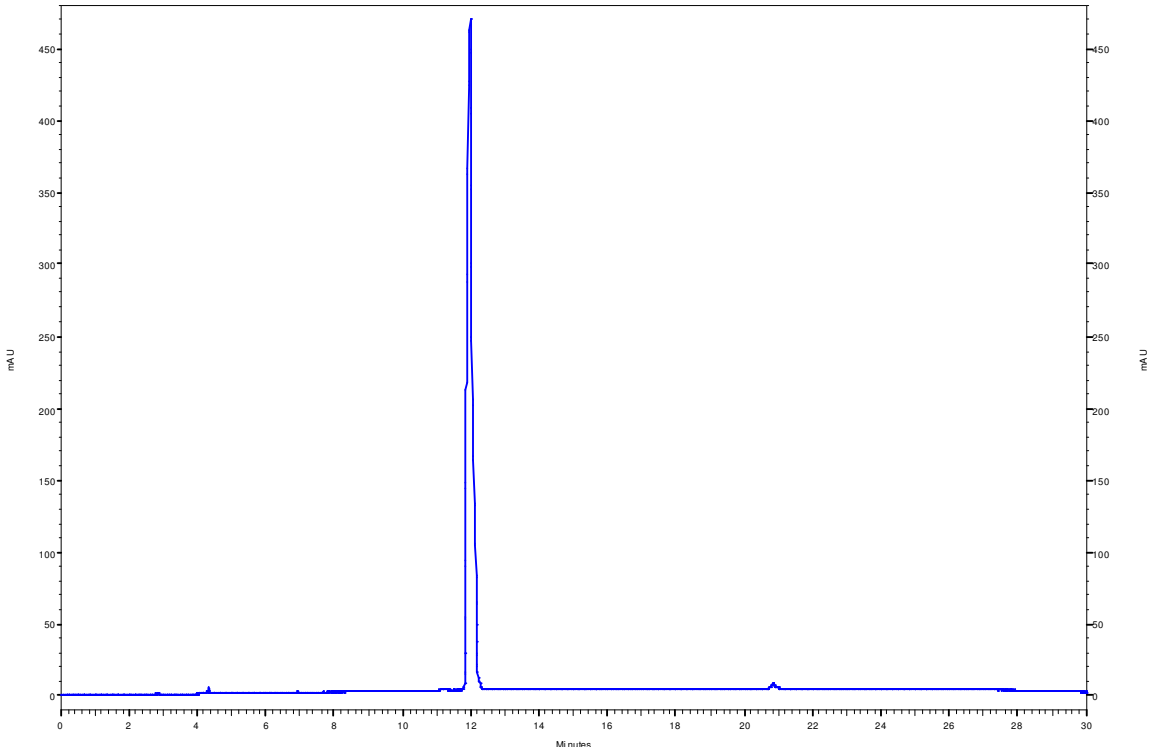
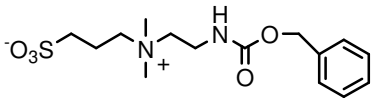


Figure S6. HPLC chromatogram of compound **v**. HPLC conditions: Phenomenex PRODIGY, C_{18}, 10 micron, 10 x 250 mm column using a 30 minute gradient of 10 → 60% MeCN/water (with 0.05% TFA) at a flow rate of 4.0 mL/minute and UV detection at 260 nm.



v

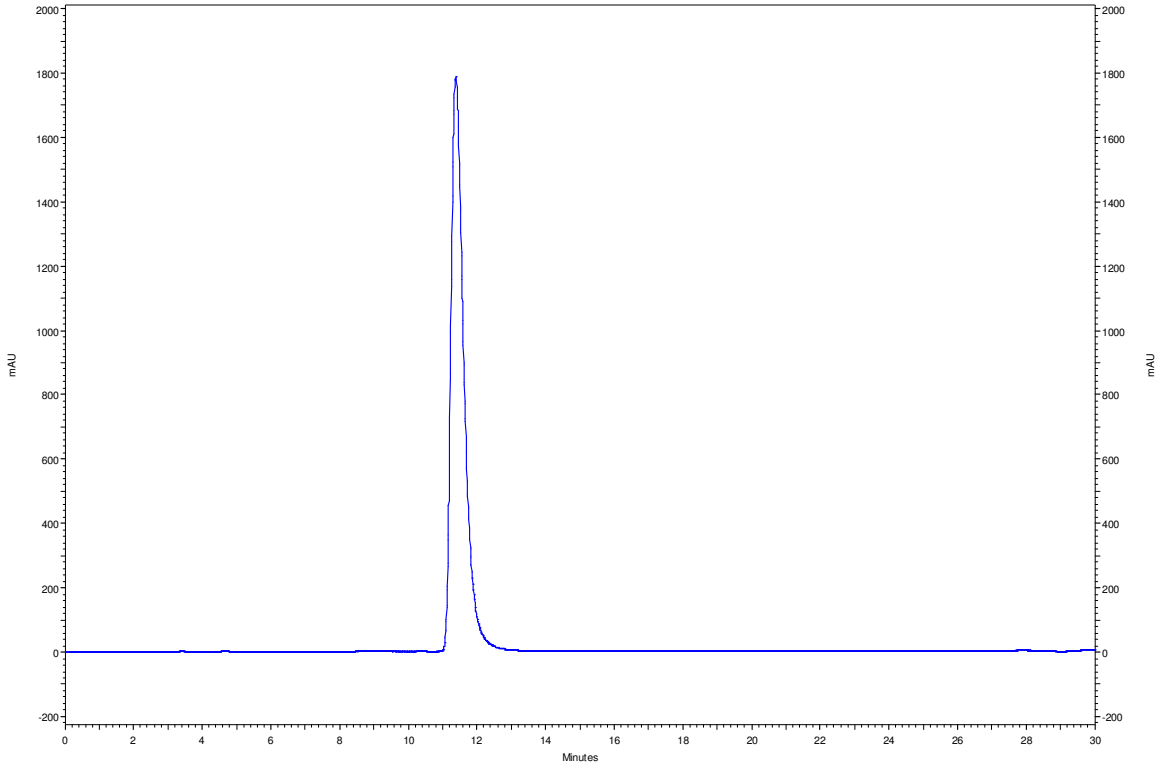
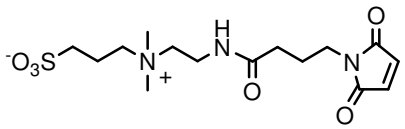


Figure S7. HPLC chromatogram of thiol-reactive, zwitterionic reagent **2**. HPLC conditions: Phenomenex, C_{18}, 10 micron, 3.9 x 300 mm column using a 30 minute gradient of 0 → 20% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 220 nm.



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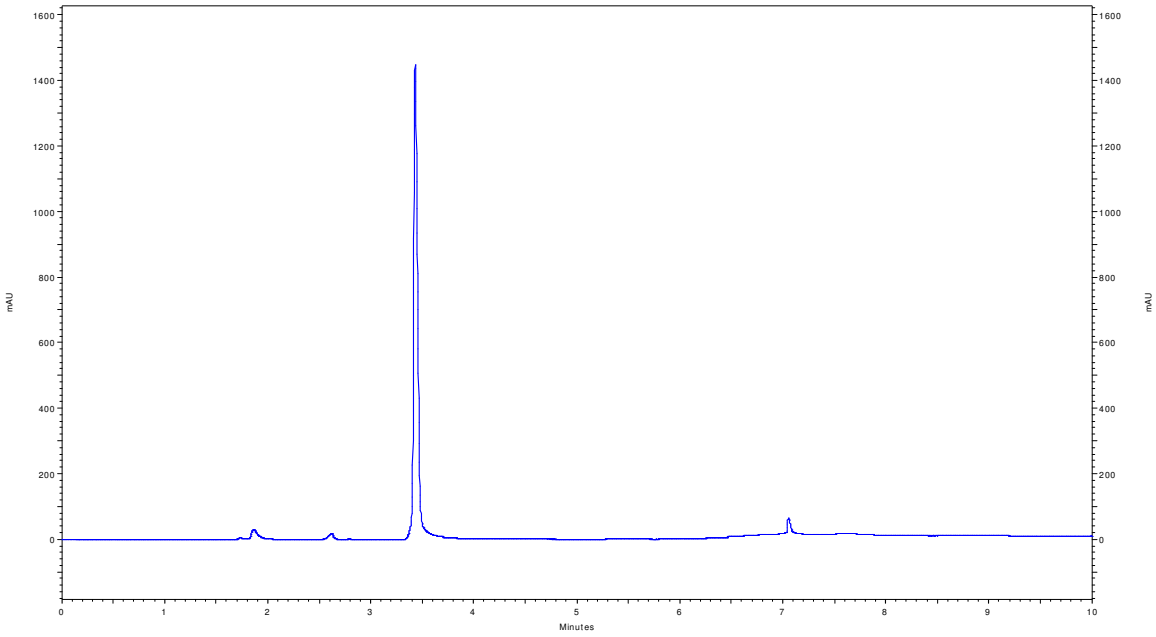
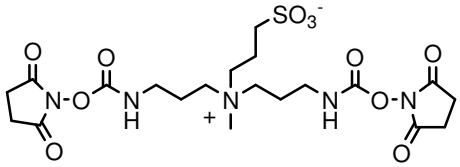


Figure S8. HPLC chromatogram of homobifunctional, zwitterionic, cross-linking reagent **4**. HPLC conditions: Phenomenex, Luna PFP(2), 3 micron, 4.6 x 150 mm column using a 20 minute gradient of 0 → 30% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 220 nm.



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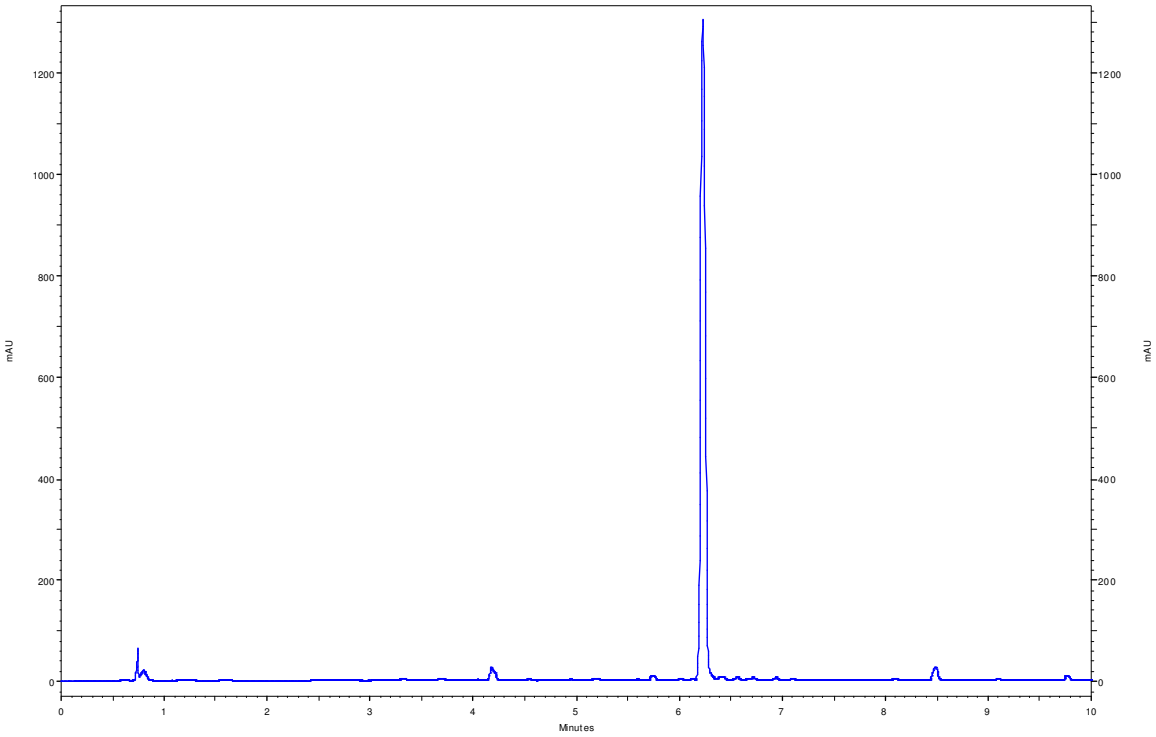
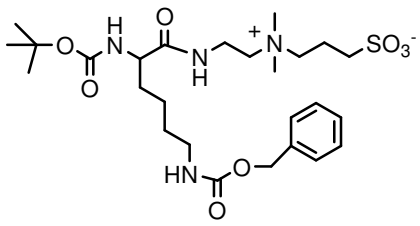


Figure S9. HPLC chromatogram of compound **vii**. HPLC conditions: YMC, C_{18}, 3 micron, 4.0 x 50 mm column using a 10 minute gradient of 10 → 90% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 260 nm.



vii

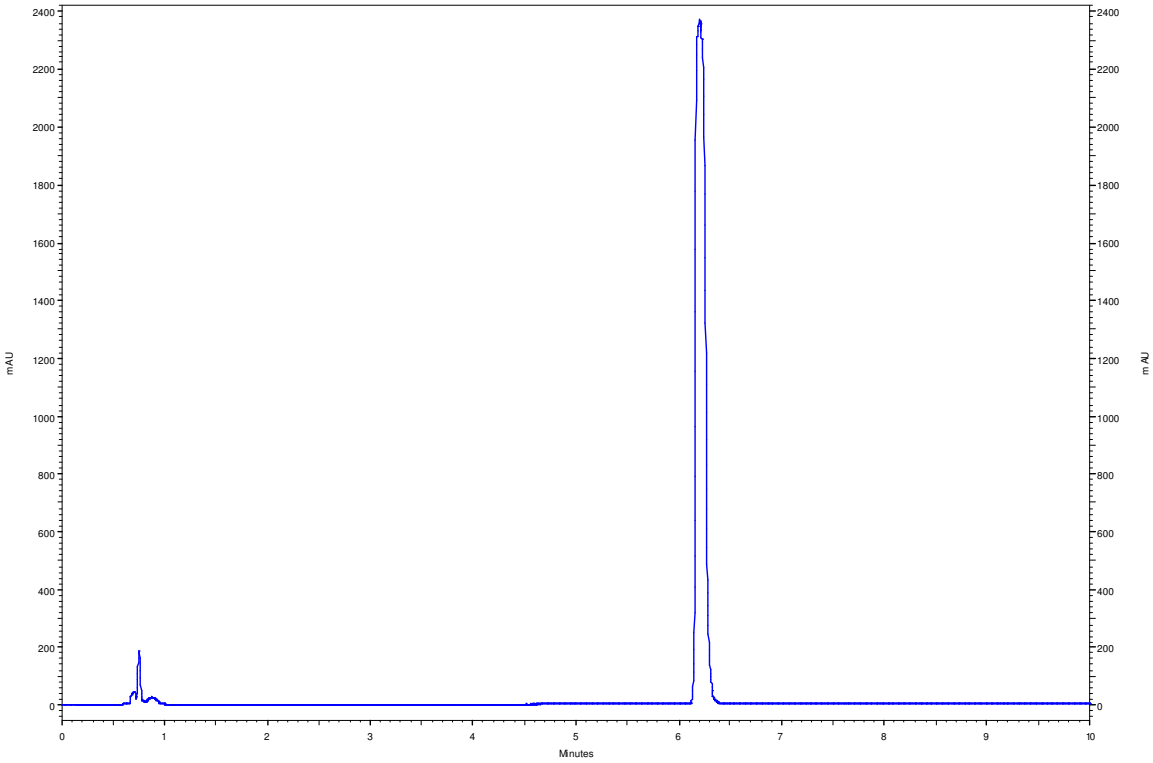
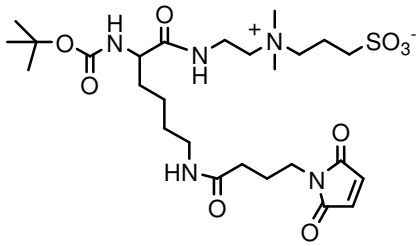


Figure S10. HPLC chromatogram of compound **ix**. HPLC conditions: YMC, C_{18}, 3 micron, 4.0 x 50 mm column using a 10 minute gradient of 10 → 40% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 220 nm.



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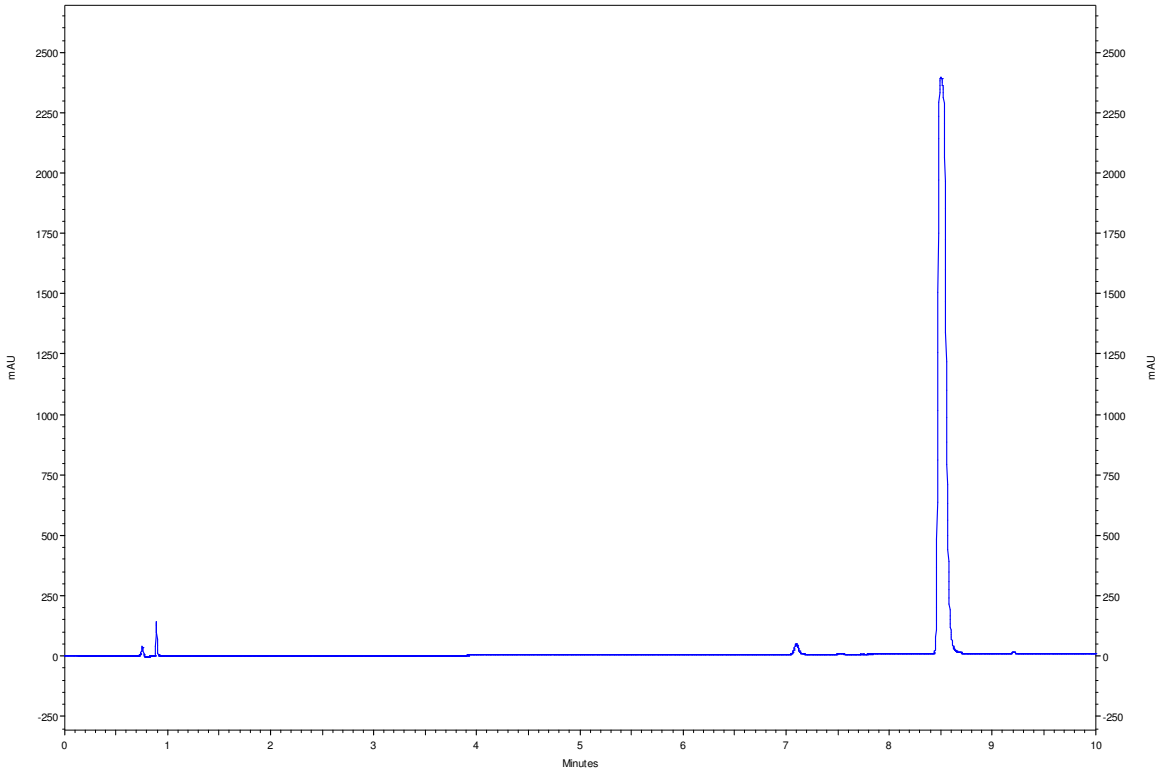
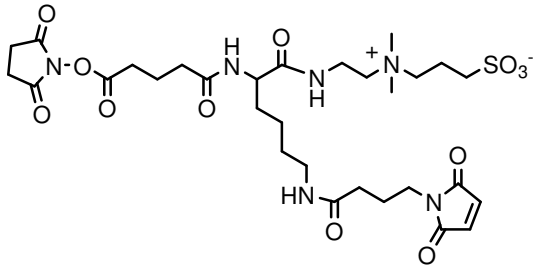


Figure S11. HPLC chromatogram of heterobifunctional, zwitterionic, cross-linking reagent **5**. HPLC conditions: YMC, C_{18}, 3 micron, 4.6 x 50 mm column using a 10 minute gradient of 0 → 30% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 220 nm.



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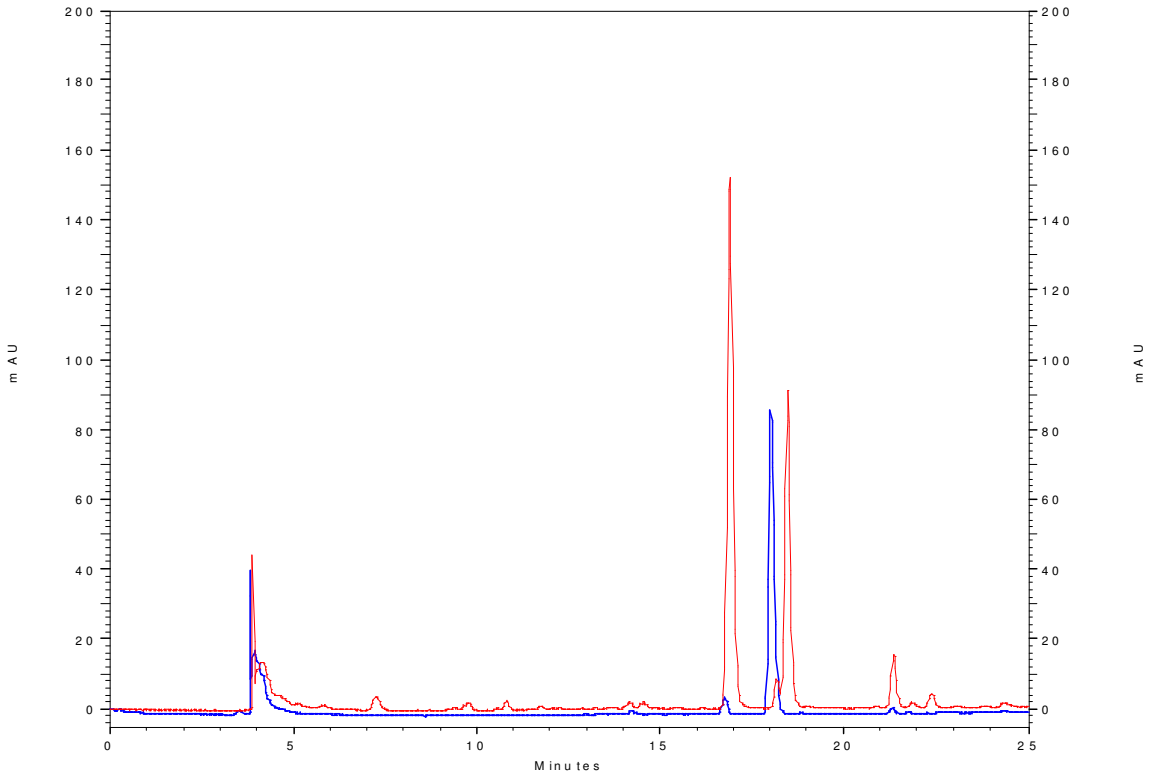
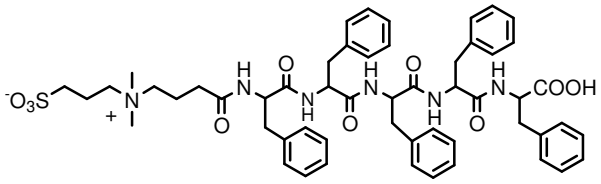


Figure S12. HPLC chromatograms of (a) coupling reaction of zwitterionic reagent **1** with penta(phenylalanine) (red trace) (b) penta(phenylalanine) (blue trace). Compound eluting at 18.5 minutes (red) is the zwitterion-labeled peptide **6**. Compound eluting at 16.5 minutes (red) is pentafluorophenol that is released in the coupling reaction. HPLC conditions: Phenomenex, 10 micron, $C_{18,}$ 3.9 x 300 mm column and a 30 minute gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection 260 nm.



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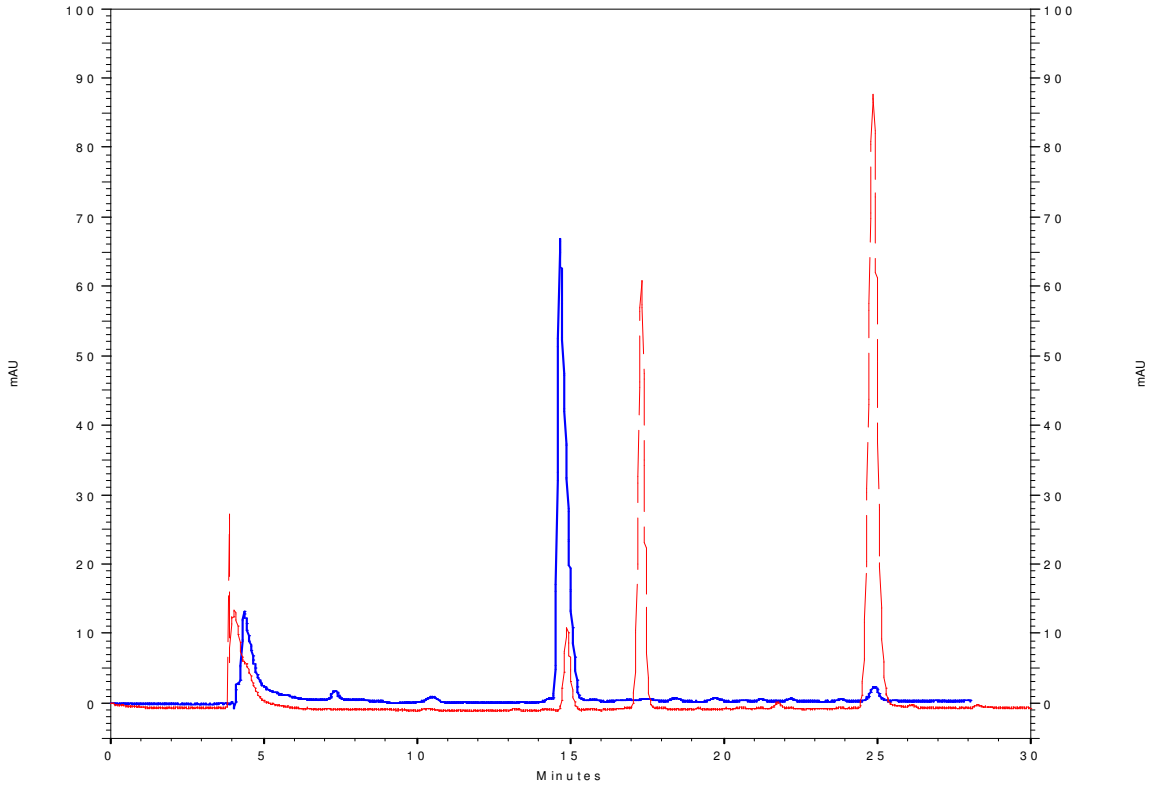
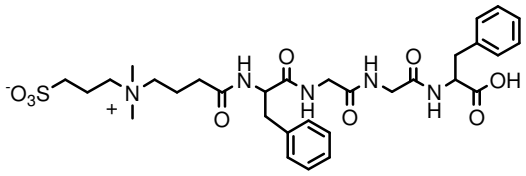


Figure S13. HPLC chromatograms of (a) coupling reaction of zwitterionic reagent **1** with Phe-Gly-Gly-Phe (red trace) (b) Phe-Gly-Gly-Phe (blue trace). Compound eluting at 17.3 minutes (red) is the zwitterion-labeled peptide **7**. Compound eluting at 25 minutes is pentafluorophenol that is released in the coupling reaction. HPLC conditions: Phenomenex, 10 micron, $C_{18,}$ 3.9 x 300 mm column and a 40 minute gradient of 10 → 60% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 260 nm.



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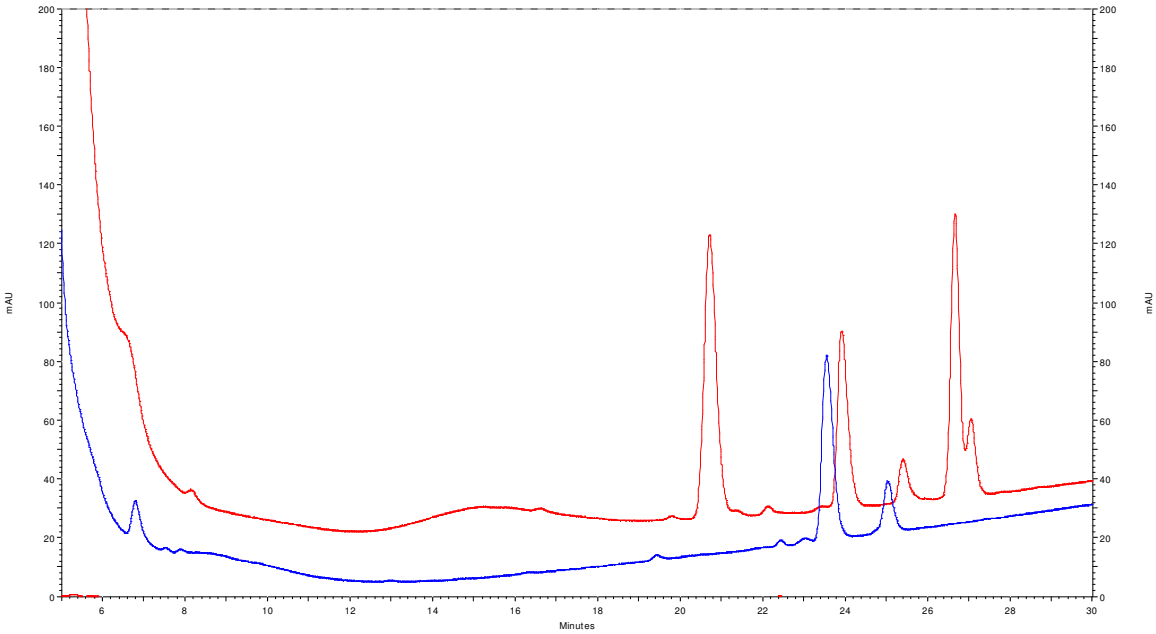
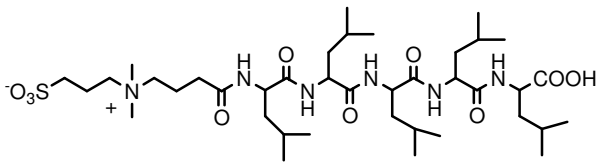


Figure S14. HPLC chromatograms of (a) coupling reaction of zwitterionic reagent **1** with penta(leucine) (red trace) (b) crude penta(leucine) (blue trace) eluting at 23.5 minutes with impurity at 25 minutes. Compound eluting at 27 minutes (red) is the zwitterion-labeled peptide **8**. Compound eluting at 21.5 minutes is pentafluorophenol that is released in the coupling reaction. Unreacted peptide is shifted slightly at 24 minutes. HPLC conditions: Phenomenex, 10 micron, $C_{18,}$ 3.9 x 300 mm column and a 30 minute gradient of 10 → 70% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 220 nm.



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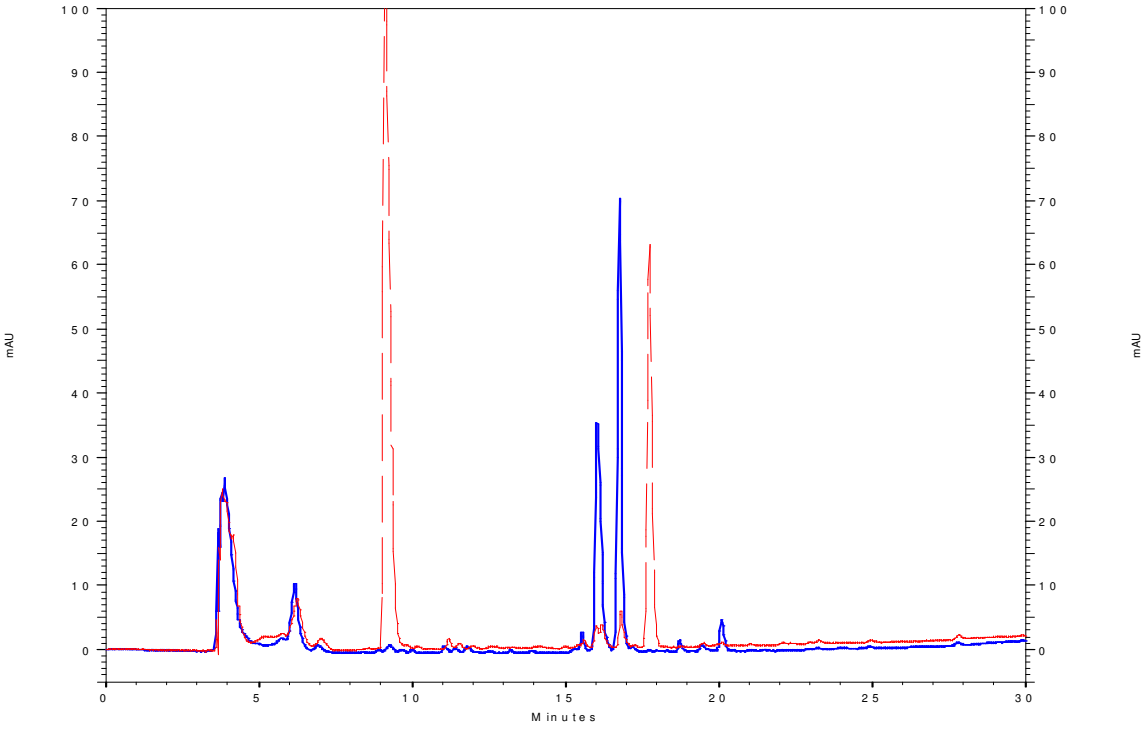
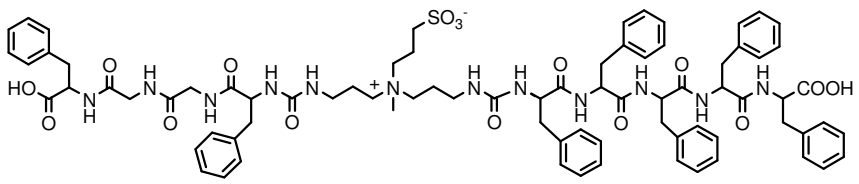


Figure S15. HPLC chromatograms of (a) coupling reaction of zwitterionic cross-linking reagent **4** with penta(phenylalanine) (blue trace) (b) further coupling of zwitterion-modified penta(phenylalanine) intermediate **xi** with Phe-Gly-Gly-Phe (red trace). Compound eluting at 17 minutes (blue) is the zwitterion-labeled penta(phenylalanine) intermediate **xi**. Compound eluting at 16 min (blue) is unreacted penta(phenylalanine). Compound eluting at 18 minutes (red) is the zwitterion cross-linked nonapeptide **9**. Compound eluting at 9 minutes (red) is excess, unreacted Phe-Gly-Gly-Phe. HPLC conditions: Phenomenex, 10 micron, $C_{18,}$ 3.9 x 300 mm column and a 30 minute gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 260 nm.



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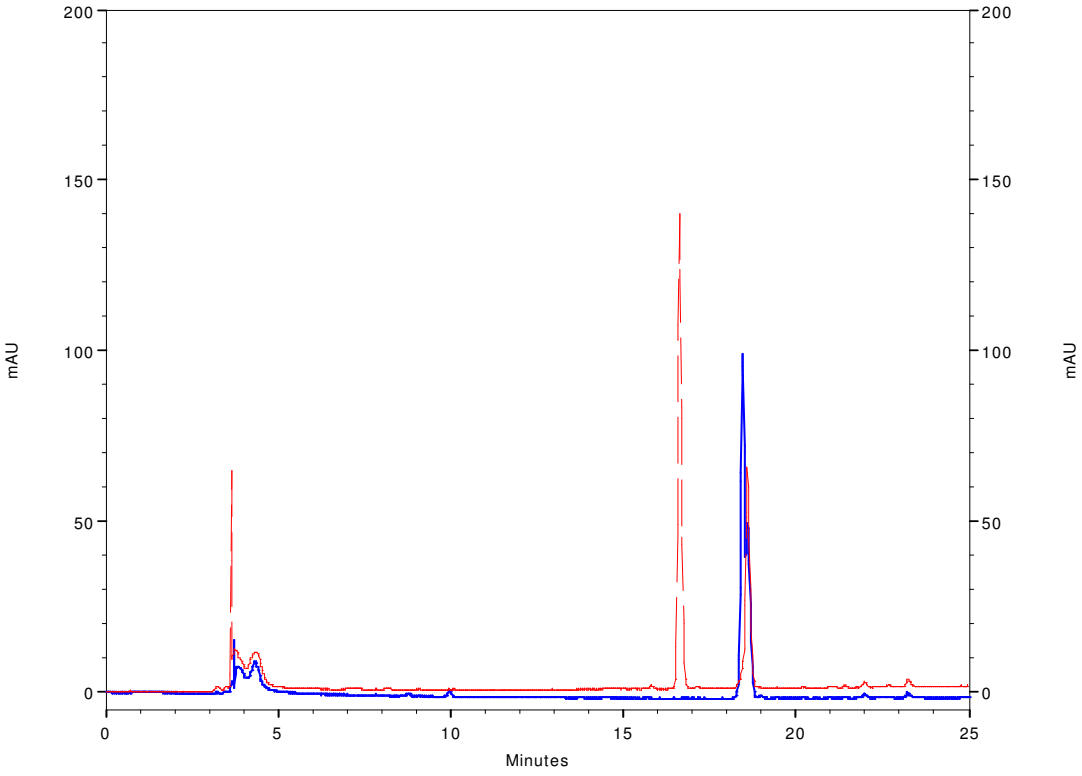
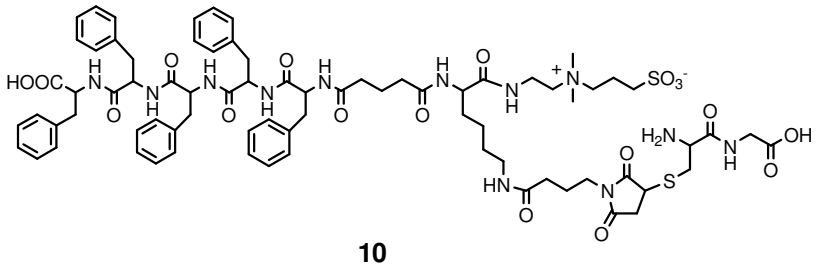


Figure S16. HPLC chromatograms of (a) coupling reaction of heterobifunctional zwitterionic, cross-linking reagent **5** with penta(phenylalanine) (blue trace) (b) further coupling of zwitterion-modified penta(phenylalanine) intermediate **xii** with Cys-Gly (red trace). Compound eluting at 18.5 minutes (blue, earlier eluting) is the zwitterion-labeled penta(phenylalanine) intermediate **xii**. Starting material penta(phenylalanine)elutes as a later eluting shoulder peak. Compound eluting at 16.5 minutes (red) is the zwitterion cross-linked heptapeptide **10**. HPLC conditions: Phenomenex, 10 micron, $C_{18,}$ 3.9 x 300 mm column and a 30 minute gradient of 10 → 100% MeCN/water (with 0.05% TFA) at a flow rate of 1.0 mL/minute and UV detection at 260 nm.



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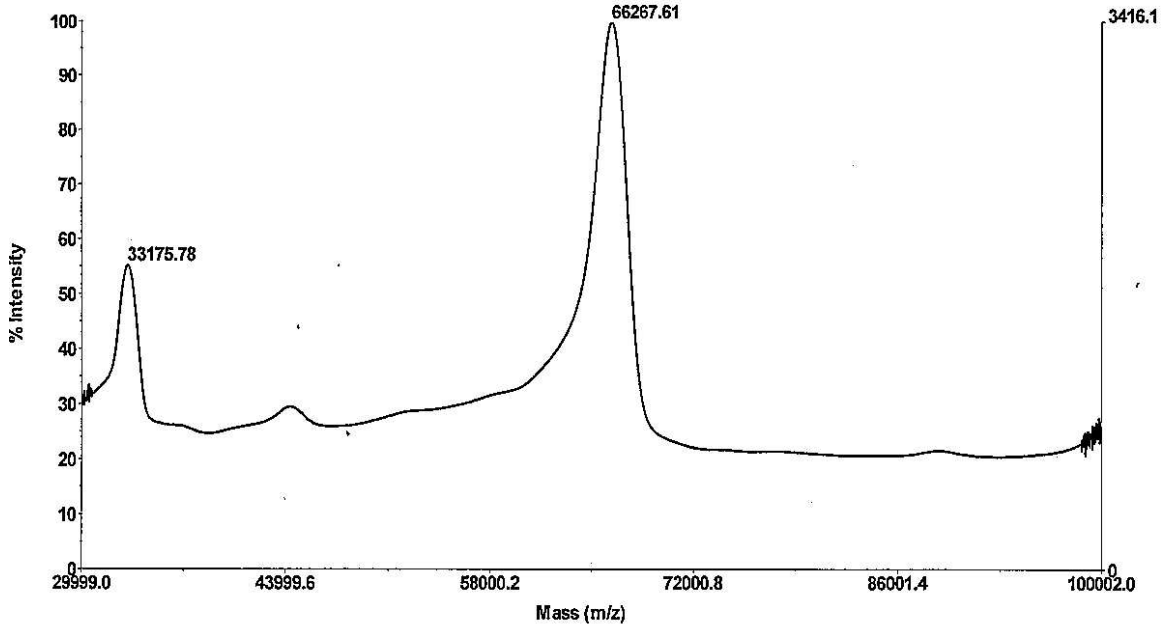
A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Mass (m/z)

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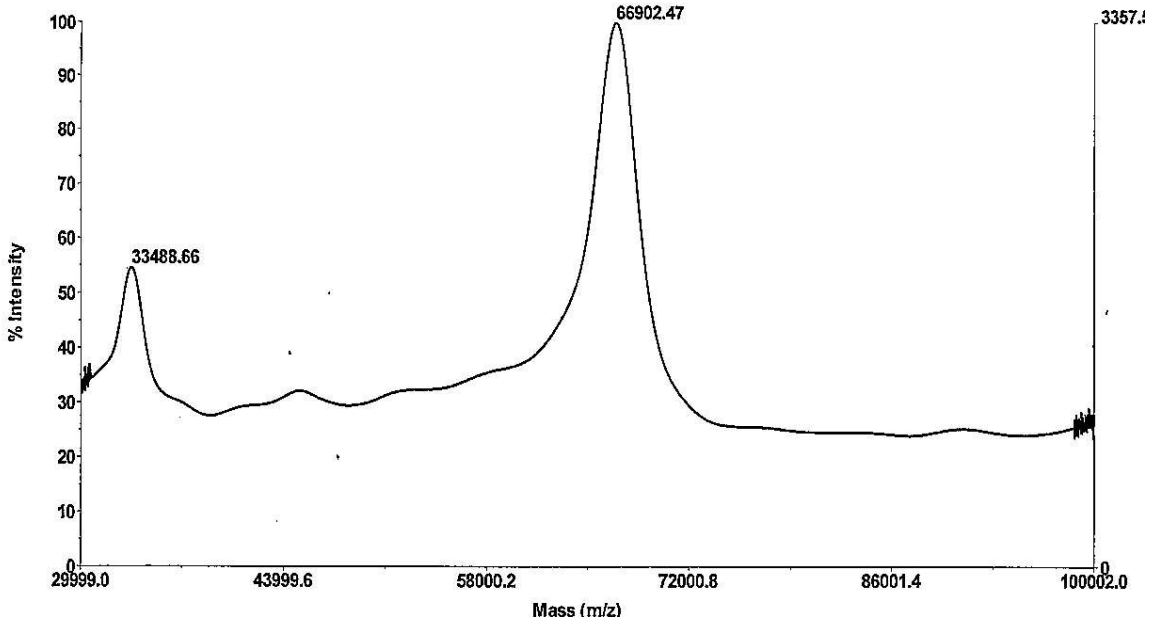
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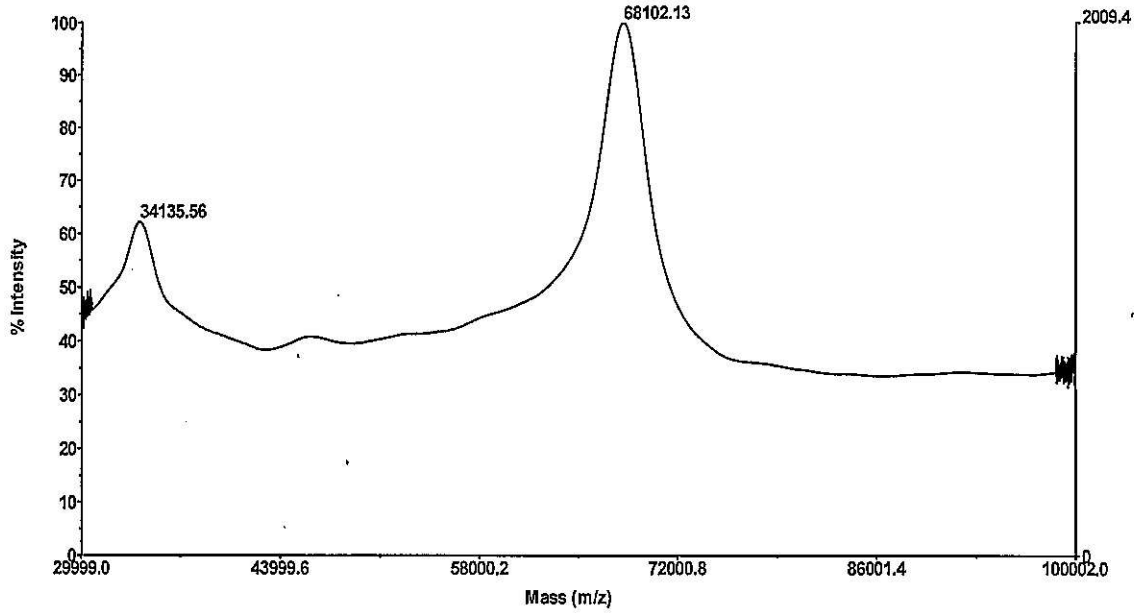
C



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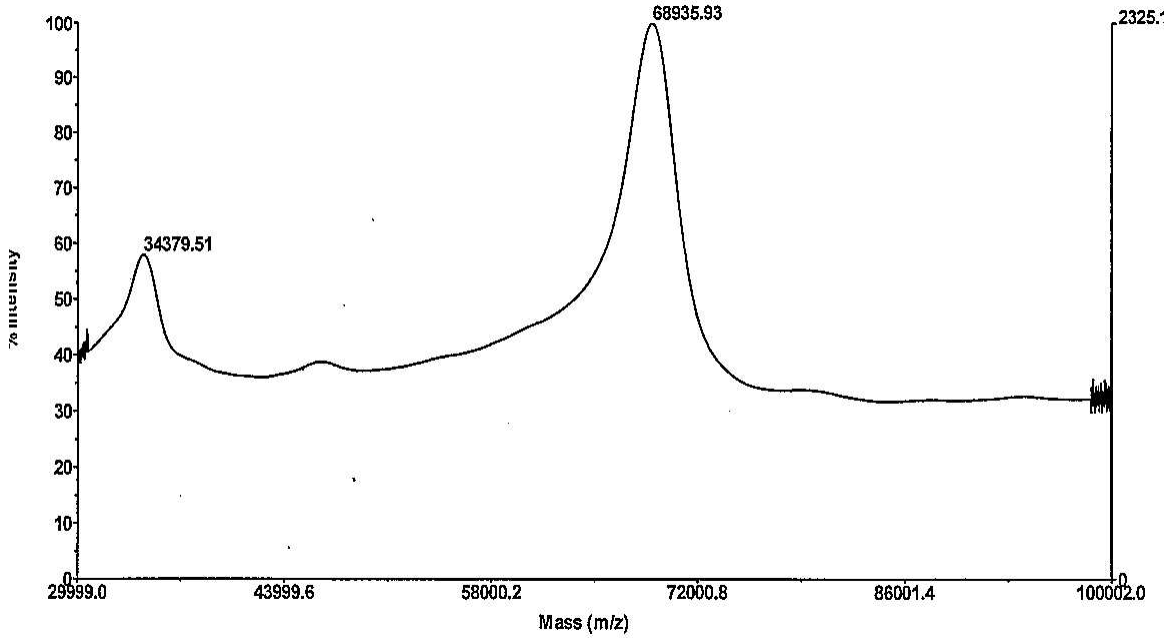
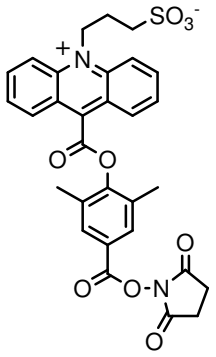
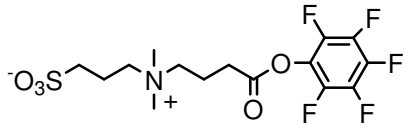


Figure S17. MALDI-TOF mass spectra of (**A**) Unlabeled BSA, (**B**) BSA labeled with NSP-DMAE-NHS indicating a mass increase of 634 units corresponding to ~1.4 acridinium ester labels, (**C**) NSP-DMAE-BSA further labeled with 10 equivalents of zwitterion label **1** indicating a mass increase of 1200 units corresponding to ~5 zwitterion

labels and, (**D**) NSP-DMAE-BSA further labeled with 20 equivalents of zwitterion label **1** indicating a mass increase of 2034 units corresponding to ~8 zwitterion labels.



NSP-DMAE-NHS



1

Table S1. Theophylline assay using conjugates **11** and **12**.

Theophylline Assay						
	Compound 11			Compound 12		
Theophylline [µM]	Mean Chemiluminescence (RLU)	B/B_0 (%)	Relative Precision (c.v.%)	Mean Chemiluminescence (RLU)	B/B0 (%)	Relative Precision (c.v.%)
0	2,138,770	100	6.2	2,321,548	100	5.8
1	2,089,048	98	4.3	2,074,255	89	3.5
2	1,941,391	91	3.3	1,905,715	82	1.5
4	1,675,088	78	3.1	1,651,129	71	4.0
10	1,232,974	58	1.7	1,160,276	50	5.6
20	850,536	40	2.1	849,644	37	3.9
40	511,047	24	0.7	470,761	20	1.8
100	242,815	11	2.4	197,789	8.5	5.0
500	53,563	2.5	0.6	39,429	1.7	0.9
1000	27,932	1.3	1.3	20,006	0.9	1.9

Table S2. TSH assay using anti-TSH conjugate of labels **13** and **14**.

TSH Assay				
	Compound **13**		Compound **14**	
TSH [mIU/L]	Mean Chemiluminescence (RLU)	Relative Precision (c.v.%)	Mean Chemiluminescence (RLU)	Relative Precision (c.v.%)
0	9,186	3.3	10,887	3.6
0.002	9,566	2.0	12,064	2.9
0.004	9,800	2.0	12,652	5.5
0.01	10,697	2.6	12,875	7.4
0.015	11,061	3.0	12,983	4.8
0.02	11,536	4.6	13,295	1.3
0.025	11,871	3.7	13,642	1.4
0.03	12,580	3.3	14,443	1.1
0.1	16,982	3.3	21,818	4.6
1	102,981	3.0	124,400	2.5
10	621,486	1.5	965,677	5.1
100	4,853,307	2.6	6,673,056	3.1