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A New Generation of Peptide Conjugation Products

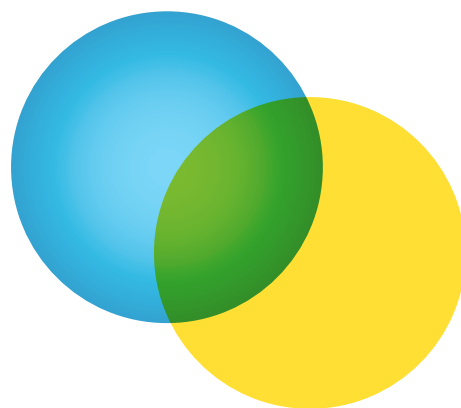


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1.0 Conjugating with HyNic-peptides

Traditionally, two chemistries have been used to conjugate peptides to other biomolecules and surfaces; carbodiimide chemistry (EDC/NHS) and maleimido/thiol chemistry. Both chemistries have significant drawbacks. The EDC/NHS method conjugates peptides through lysine moieties on the peptide and as a consequence, chemically alters the epitope resulting in sub-optimal antibodies. Additionally, (EDC/NHS) chemistry tends to be very inefficient. Maleimido/thiol chemistry is currently the method of choice wherein a cysteine moiety is incorporated on the carboxy-terminus of the peptide and subsequently linked to a maleimido-modified protein carrier. Drawbacks to this method include the requirement to modify the protein carrier just prior to conjugation in order to prevent hydrolysis of the unstable maleimido moiety. Additionally, cysteine-containing peptides readily oxidize to disulfide linked peptides and become un-reactive. While at the same time, precluding the preparation of antibodies against cysteine-containing peptides since these will scramble or change the original epitope.

To overcome these drawbacks, Solulink has developed and introduced HydraLink™ chemistry. HydraLink chemistry is based on the conjugation of HyNic-modified peptides to 4FB-modified carriers. Figure 1 presents the chemistry of the conjugation of a HyNic-peptide (PepLink) to a 4FB-modified protein.

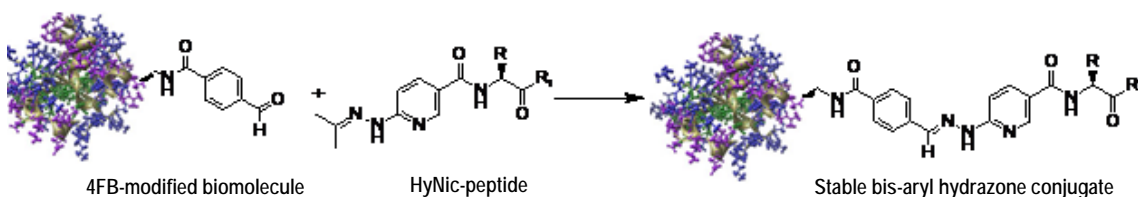
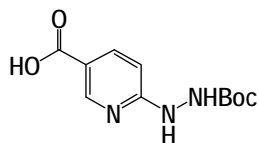


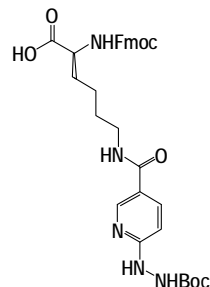
Figure 1: Chemical scheme employed to link HyNic-modified peptides to 4FB-modified biomolecules.

HyNic (**hydrazinonicotinamide**) was originally developed to bind technetium-99M to proteins for *in vivo* imaging.⁶ Subsequently, HyNic was incorporated on chemotactic peptides for imaging of focal sites of inflammation, on oxytocin and octreotide for tumor imaging and for imaging a wide variety of other targets (see PubMed search, keywords: HyNic, peptide).⁷⁻⁹

Incorporation of the HyNic moiety on a peptide is straightforward, high yielding and can be added at either N- or C-terminus using 6-Boc-HyNic (**1**) (Solulink catalog # S-3003) or Fmoc-Lys-(ε-6-BocHyNic)OH (**2**) (Solulink catalog # 3034), respectively.



1. 6-Boc-HyNic



2. Fmoc-Lys-ε-(6-Boc-HyNic)OH

Cleavage from the resin is accomplished using TFA/acetone/water/triisopropylsilane (TIS)/water (92.5/2.5/2.5/2.5) for 2 h. The presence of the acetone forms a hydrazone with the deprotected hydrazine moiety in situ blocking any trifluoroacetamide formation from the reaction of TFA with the strongly nucleophilic hydrazine.^{10,11} Unlike cysteine peptides, HyNic-peptides are stable and do not scramble or dimerize.

The linking of HyNic-peptides (PepLink) to 4FB-modified proteins, oligonucleotides or surfaces is straightforward, stoichiometrically efficient and high yielding. In a standard conjugation, only a 2-5 mole excess of one of the HyNic-peptide (PepLink) is required to give >80% conjugate yield. Hydrazone bond formation can also be catalyzed and reaction kinetics improved 10-100-fold by the presence of aniline, often leading to conjugate yields > 95%. Optimal conjugation kinetics (formation of the hydrazone bond) is between pH 4.5-5.0. However, the reaction also proceeds at higher pH, albeit more slowly. When conjugating to an antibody, it is recommended that reactions be performed at pH 6.0, since antibody immunoreactivity can be deleteriously affected below pH 6.0.¹² A further advantage of using the HyNic/4FB pair is that the degree of conjugation can be conveniently monitored spectrophotometrically (in real-time). The formation of the bis-aryl hydrazone bond can be exploited to both trace and quantify the progress of the conjugation reaction using its known molar extinction coefficient (29,000 @ 354 nm).

2.0 Peptide Conjugation: Some Examples

2.1 HyNic-peptide to a 4FB-Protein

Conjugation of a HyNic-modified peptide to a 4FB-modified protein is a simple, two-step process wherein a 5-fold excess of HyNic-peptide is incubated with a 4FB-modified protein in buffer at pH 6.0 for 2 hours (Figure 2). The final protein-peptide conjugate is readily purified using a diafiltration device (MWCO:1000-3000).

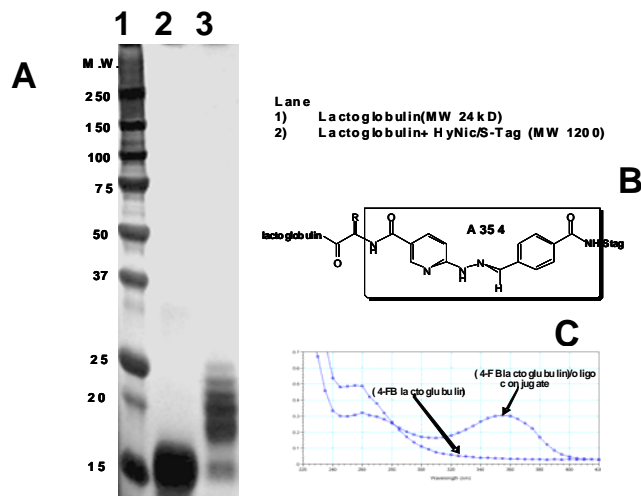


Figure 2: A 15-mer HyNic-peptide (5 equivalents) was added to 4FB-modified lactoglobulin (Lane 2; MW 18300) in 100 mM phosphate, 150 mM NaCl, pH 6.0 and incubated for 2 hours at room temperature. An aliquot of the crude conjugation reaction (Lane 3) was removed and examined using an SDS-PAGE gel (A). The HyNic-peptide efficiently conjugated to the 4FB-modified lactoglobulin with formation of the bis-aryl hydrazone bond structure shown in B. Overlaid spectra of 4FB-modified lactoglobulin and the lactoglobulin-peptide conjugate (C), illustrates the UV-traceable nature of the aromatic hydrazone linkage (λ 354, ϵ 29000).

2.2 HyNic-Peptide to a 4FB-Oligonucleotide

The preparation of peptide/oligonucleotide conjugates with Solulink's PepLink technology is also straightforward. Simple addition of a 3-5 molar excess of a HyNic-peptide to a 4FB-modified oligonucleotide at pH 5.0-6.0 for 2 h @ RT, followed by diafiltration to remove excess peptide, yields the

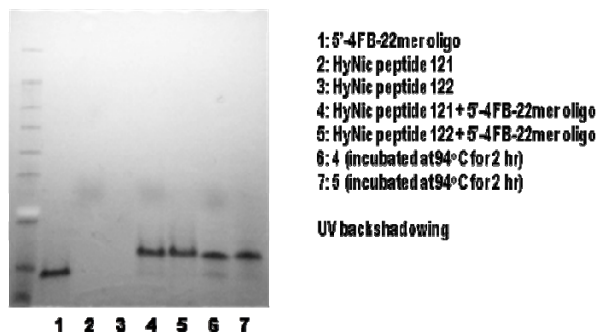


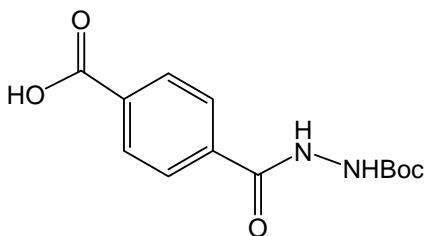
Figure 3: Two N-terminal HyNic-modified PepLink peptides 121 and 122 (lanes 2 and 3) were reacted with a 5'-4FB-modified 18-mer oligo (lane 1) to yield the peptide-oligo conjugate (lanes 4 and 5). Conjugate stability was tested at 94°C in PBS for two hours. Gel results were visualized by UV back-shadowing. As seen in lanes 4 and 5, conjugate formation is efficient and the conjugate bond is stable to 94°C, lanes 6 and 7.

conjugate (see Figure 3). The 4FB-moiety can be incorporated on the oligonucleotide during solid phase synthesis using a 4FB-C6-phosphoramidite (Solulink catalog # S-1005). This is the first example of the preparation of a peptide/oligonucleotide conjugate wherein both moieties had their respective linkers incorporated during solid phase synthesis. To demonstrate the stability of the bis-arylhydrazone bond the

oligo/peptide conjugates were heated at 94°C for 2h and subsequently analyzed by gel electrophoresis (Figure 3, lanes 6 and 7).

2.3 Peptide Conjugates using 4-HTA (4-hydrazidoterephthalamide)

4-BOC-HTA (**3**) (Solulink catalog # S-1014), is an aromatic hydrazide linker which is also available from Solulink for incorporation into peptides. The aromatic acylhydrazine (4-HTA), forms a hydrazide-based hydrazone that is more thermal labile than an aromatic hydrazine-based hydrazone. Conjugates formed with this linker can be cleaved at elevated temperatures in contrast to aromatic hydrazine-based hydrazone (HyNic) conjugates (Figure 4). A more thermal labile bond allows for the cleavage of solution phase conjugates or solid phase immobilized conjugates by simply elevating temperature.



3. 4-Boc-HTA (4-Boc-hydrazidoterephthalic acid)

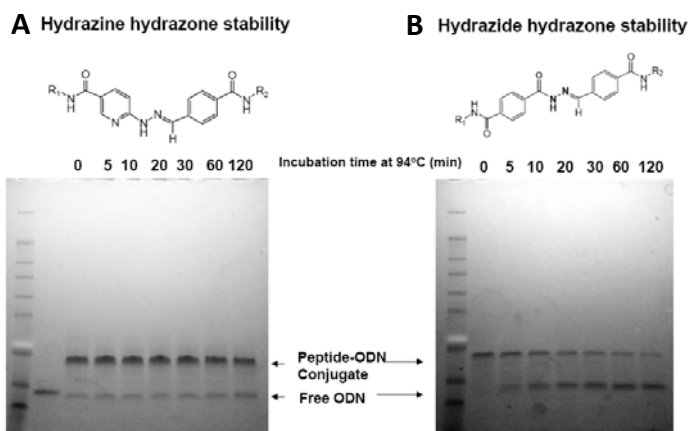
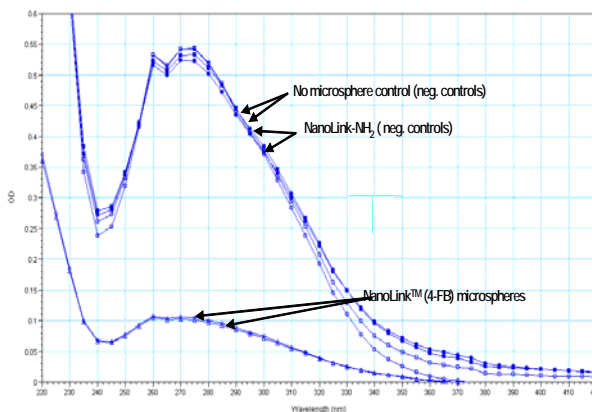


Figure 4: Panel A shows the thermal stability (time course @ 94°C) of a hydrazine-based peptide-oligo (bis-arylhydrazone) conjugate vs. a hydrazide-based hydrazone peptide-oligo conjugate under the same temperature and time conditions.

2.4 HyNic-peptide to a 4FB-Modified Surface (Magnetic Microsphere)

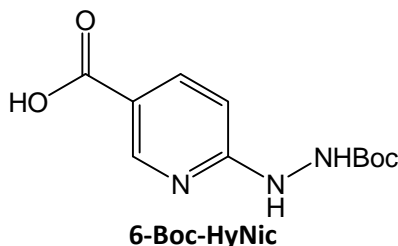
Another application of HyNic-peptides (PepLink) is immobilization of peptides to 4FB-modified solid surfaces. For example, HyNic-peptides are easily immobilized on 4FB-modified magnetic beads (NanoLink; Solulink catalog # M-1001-010). Simple incubation of a HyNic-peptide with 4FB-modified magnetic beads at pH 5.0 for 30-60 minutes, followed by a brief wash, yields the bead immobilized peptide (Figure 5).

Figure 5: A 14mer N-terminus-HyNic-modified peptide was incubated @ 250 uM with Solulink's NanoLink 4FB-magnetic beads in 100 mM MES buffer, pH 5.0 for 1 h. The course of the reaction could be monitored by a reduction in the peptide's A_{280} solution phase absorbance as it became immobilized to the 4FB-beads.



3.0 Solid Phase Synthesis of HyNic-Peptides

3.1 N-terminal HyNic incorporation using 6-Boc-HyNic



Method 1-1: Incorporation of HyNic to the N-terminus of a 10-mer peptide

- 1) Dissolve 6-Boc-HyNic (5 eq), HBTU (5 eq) in a minimum amount of DMF.
- 2) Add DiPEA (15 eq) to mixture and add immediately to Fmoc-deblocked peptide resin.
- 3) Incubate for 1-2 h.
- 4) Check completeness of reaction with Kaiser or TNBS test.
- 5) Wash resin and repeat reaction cycle if required.

Application 1-1: Synthesis of HyNic-10-mer peptide

The peptide was assembled on a Rainin Symphony Peptide Synthesizer using *Rink* amide resin. All acylation reactions were carried out using a 5-fold excess of Fmoc-amino acid activated with HBTU/DiPEA. A coupling time of 1 hour was used for each cycle. The HyNic-peptidyl resin was cleaved using TFA/TIS/acetone/water (92.5/2.5/2.5/2.5) for 2 h. The crude peptide was analyzed by HPLC and ES-MS with an m/e of 1314.6 as expected for the desired peptide (Figure 6). The addition of acetone is critical as the *in situ* formation of the acetone hydrazone of the deprotected peptide blocks the formation of the trifluoroacetamide.^{13, 14} The product was isolated by RP-HPLC using a gradient of 90% A (0.1% TFA/water) to 100% B (0.1% TFA/80% ACN/20% water) in 15 min.

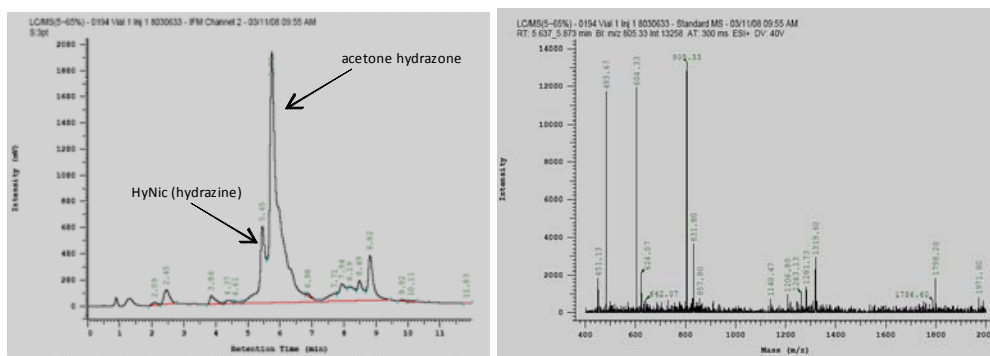
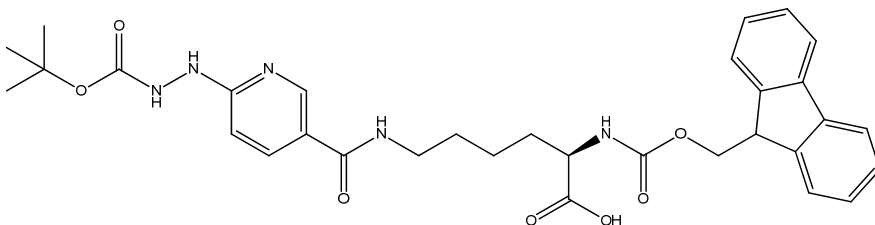


Figure 7: RP-HPLC (left) and mass spectrum (right) of crude C-terminus-HyNic 14mer peptide.

3.2 C-terminal HyNic incorporation using Fmoc-Lysine- ϵ -(6-Boc-HyNic) OH



Fmoc-Lysine- ϵ -(6-Boc-HyNic)OH

Method 2-1: Incorporation of HyNic on the C-terminus of a peptide

- 1) Dissolve Fmoc-Lysine- ϵ -(6-Boc-HyNic)OH (5 eq), HBTU (5 eq) in a minimum amount of DMF.
- 2) Add DiPEA (15 eq) to mixture and add immediately to Fmoc-deblocked peptide resin.
- 3) Incubate for 1-2 h.
- 4) Check completeness of reaction with Kaiser or TNBS test.
- 5) Wash resin and repeat reaction if required.

Application 2-1: Synthesis of C-terminal HyNic-14-mer peptide

The peptide was assembled on a Rainin Symphony Peptide Synthesizer using *Rink* amide resin. All acylation reactions were carried out using a 5-fold excess of Fmoc-amino acid activated with HBTU/DiPEA (5 eq). A coupling time of 1 hour was used for each cycle. The HyNic-peptidyl resin was cleaved using TFA/TIS/acetone/water (92.5/2.5/2.5/2.5) for 2 h. The crude peptide was analyzed by HPLC and ES-MS with an m/e 1612 as expected for the desired peptide (Figure 7). The addition of acetone is critical as the *in situ* formation of the acetone hydrazone of the deprotected peptide blocks the formation of the trifluoroacetamide.^{13, 14} The product was isolated by RP-HPLC using a gradient of 90% A (0.1% TFA/water) to 100% B (0.1% TFA/80% ACN/20% water) in 15 min.

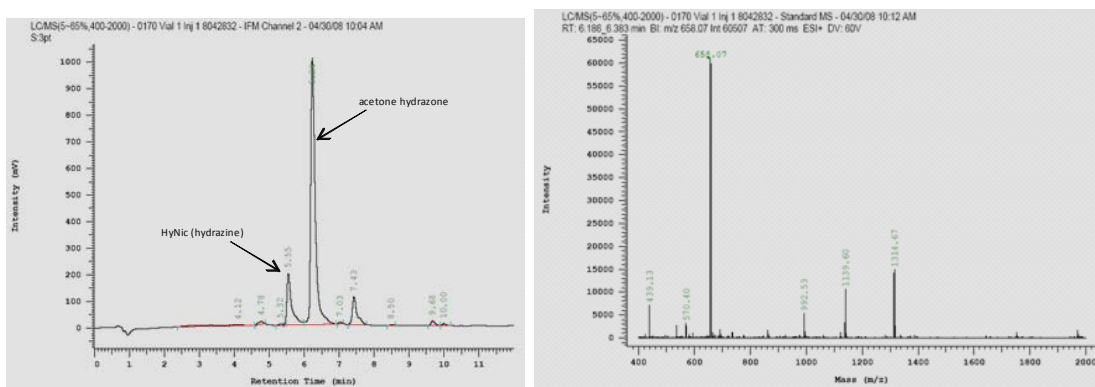
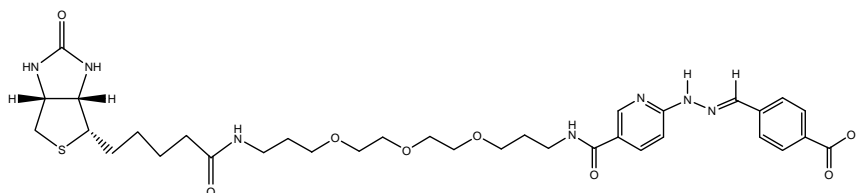


Figure 6: RP-HPLC (left) and mass spectrum (right) of crude N-terminus- HyNic-10mer peptide.

3.3 N-Terminal Biotin incorporation using ChromaLink Biotin



ChromaLink Biotin-COOH

Method 3-1: Incorporation of ChromaLink Biotin-COOH on N-terminus of a peptide

- 1) Dissolve ChromaLink Biotin-COOH (5 eq), HBTU (5 eq) in a minimum amount of DMF.
- 2) Add DiPEA (15 eq) to mixture and add immediately to Fmoc-deblocked peptide resin.
- 3) Incubate for 1-2 h.
- 4) Check completeness of reaction with Kaiser or TNBS test.
- 5) Wash resin and repeat reaction if required.

Application 3-1: Synthesis of N-terminal biotinylated 10-mer peptide

The peptide was assembled on a Rainin Symphony Peptide Synthesizer using *Rink* amide resin. All acylation reactions were carried out using a 5-fold excess of ChromaLink Biotin-COOH with HBTU/DiPEA (15 equiv.). A coupling time of 1 hour was used for each cycle. The ChromaLink Biotin-peptidyl resin was cleaved using TFA/TIS/water (95/2.5/2.5) for 2 h. The crude peptide was analyzed by ES-MS and had m/e 1869 as expected for the desired peptide. The product was isolated by RP-HPLC using a gradient of 90% A (0.1% TFA/water) to 100% B (0.1% TFA/80% ACN/20% water) over 15 min. RP-HPLC of the crude reaction product is illustrated in Figure 8.

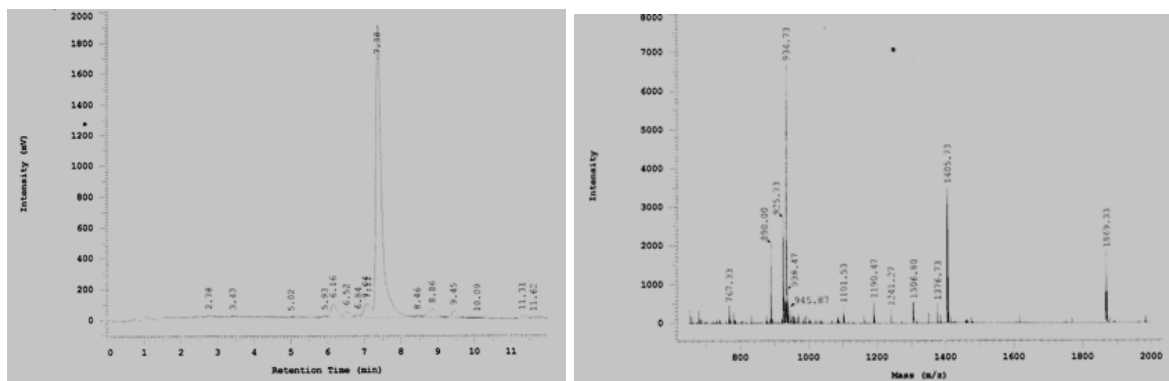


Figure 8: RP-HPLC (left) and mass spectrum (right) of crude 10mer ChromaLink Biotin peptide.

4.0 Recommended HyNic-Peptide Cleavage Conditions

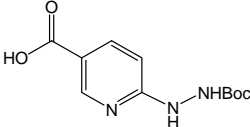
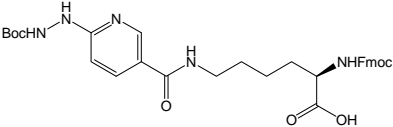
Cleavage

- 1) Suspend resin in 92.5% TFA/2.5% acetone/2.5% triisopropylsilane/2.5% water
- 2) Incubate for 2 hours
- 3) Collect supernatant
- 4) Precipitate with ether then place at -200°C for 15-20 min
- 5) Centrifuge @ 3000 rpm
- 6) Wash pellet with ether (1 eq.)
- 7) Dissolve pellet in 1:1, acetonitrile:water
- 8) Lyophilize
- 9) Purify with HPLC if required; use a C18 column and detect at 214 nm.

Analytical

- 1) The modified peptide will have a longer retention time in HPLC due to the addition of the hydrophobic linker.
- 2) There may be two peaks in the final product, the HyNic acetone hydrazone along with the free hydrazine analog.

5.0 PepLink™ Linker Reagents

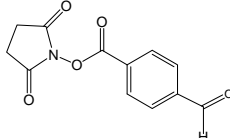
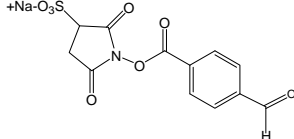
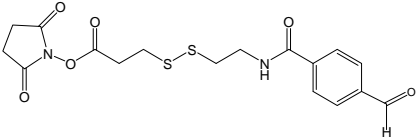
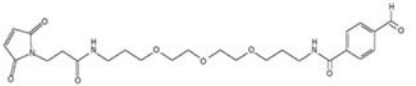
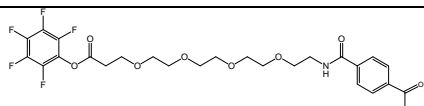
		Catalog #	Quantity	List Price
Peptide Linker				
6-Boc-HNA		S-3003-100	100 mg	\$ 125
		S-3003-500	500 mg	\$ 395
6-FMOC Lysine-ε- (6-BOC-HNA)OH		S-3034-250	100 mg	\$ 375
		S-3034-500	500 mg	\$ 625

6.0 Auxiliary Linker Reagents

Additional 4FB-activated reagents for conjugation to HyNic-peptides

	Catalog #	Quantity	List Price
4FB-Fluorophores and Biofluorescent Proteins			
4FB-fluorescein	S-4006-010	5 X 2.0 mg	\$ 175
4FB-rPE	S-4008-001	1.0 mg	\$ 100
	S-4008-003	1.0 mg	\$ 225
4FB-Nanolink Magnetic Microspheres			
NanoLink 4FB Magnetic Microspheres	M-1001-010	10 mg	\$ 250

4FB Linkers

	Catalog #	Quantity	List Price	
Peptide Linker				
S-4FB (SFB)		S-1004-105	5 x 1.0 mg	\$ 100
		S-1004-011	10 mg	\$ 150
Sulfo-S-4FB (sulfo-SFB)		S-1008-105	5 x 1.0 mg	\$ 150
		S-1008-010	10 mg	\$ 250
SS-S-4FB		S-1037-105	5 x 1.0 mg	\$ 200
		S-1037-010	10 mg	\$ 300
MTFB		S-1035-105	5 x 1.0 mg	\$ 200
		S-1035-105	10 mg	\$ 300
PEG4/PFP		S-1034-010	10 mg	\$ 200
		S-1034-010	5 x 1.0 mg	\$ 250

7.0 References

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