Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

N-terminal dual protein functionalization by strain-promoted alkyne–nitrone cycloaddition

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Supplementary information

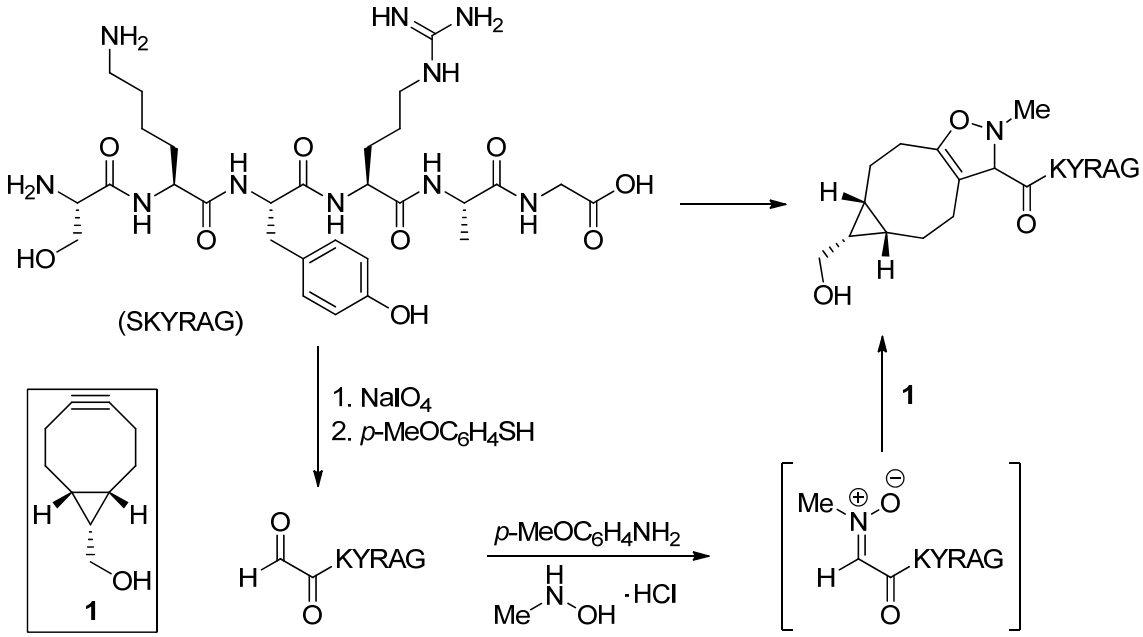


Table S1. SPANC experiments on the model peptide SKYRAG to find optimal conditions for peptide modification.

Entry	MeNHOH·HCl (eq)	BCN-OH (eq)	Conv. after 2h	Conv. after 3d
1	1	1	--	--
2	1	2	--	--
3	2	2	--	-
4	2	4	--	-
5	3	3	+/-	+/-
6	3	6	-	+/-
7	4	4	+/-	+
8	4	8	+/-	+
9	5	5	+	++
10	5	10	+/-	++
11	10	10	++	++
12	10	20	+	++

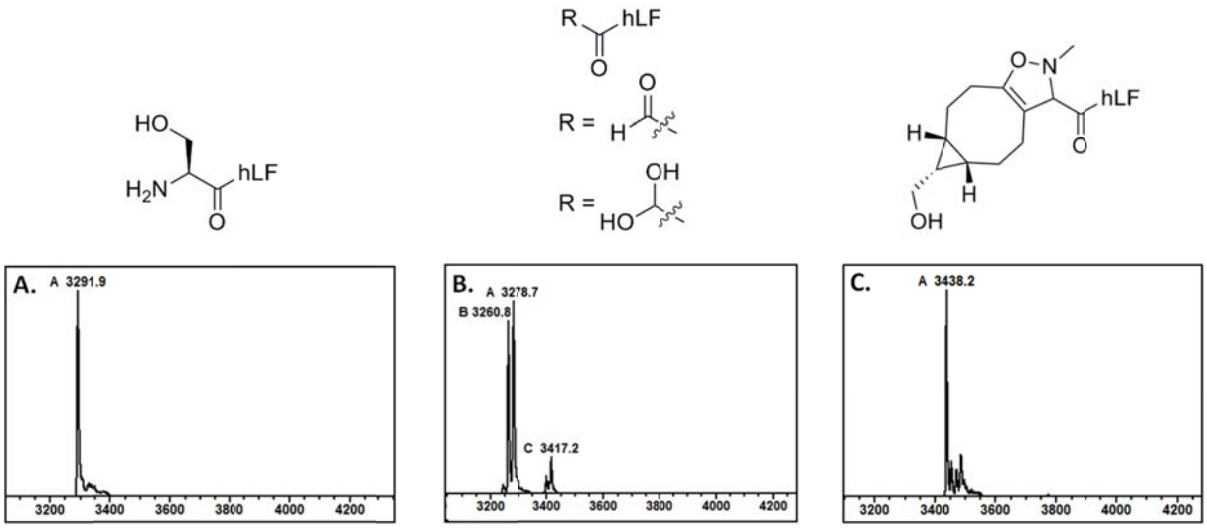


Figure S1. Deconvoluted mass spectra of S-hLF. A. native peptide; B. after oxidation with $NaIO_4$; C. after dual SPANC labeling with BCN-OH and *N*-methylhydroxylamine.

Table S2. SPANC experiments on the model protein HspB2/3 to find optimal conditions for protein modification.

Entry	p-anisidin (eq)	N-propargyl hydroxylamine	BCN-OH	Conv. after 16h
1	10	10	10	--
2	0	10	10	--
3	100 mM	10	10	+
4	10	10	100	--
5	10	100	10	++
6	10	100	100	++
7	100 mM	50	20	++

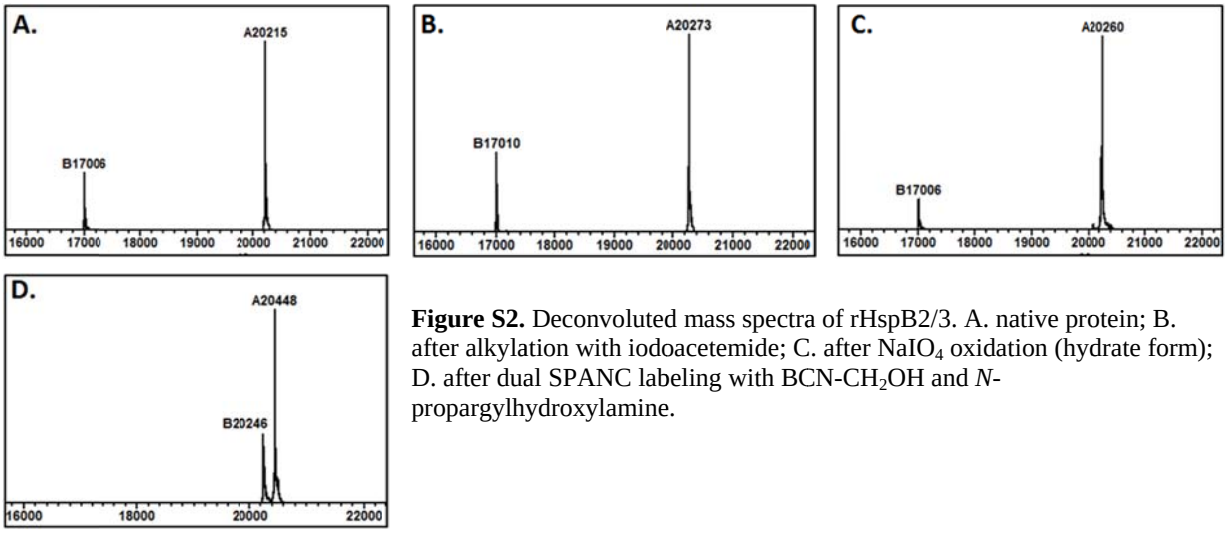


Figure S2. Deconvoluted mass spectra of rHspB2/3. A. native protein; B. after alkylation with iodoacetemide; C. after $NaIO_4$ oxidation (hydrate form); D. after dual SPANC labeling with BCN-CH_2OH and *N*-propargylhydroxylamine.

Experimental procedures for synthesis of SKYRAG and expression of S-eGFP

Synthesis of the peptide SKYRAG

SKYRAG synthesis was done using standard Fmoc solid phase peptide synthesis on a semi-automatic synthesizer.

SKYRAG as a model peptide for optimization of SPANC

SKYRAG (1.02 mg, 1.5 µmol) was dissolved in 1.5 mL 0.1 M NH_4OAc buffer (pH 6.8) and $NaIO_4$ (0.35 mg, 1.8 µmol) was added. After incubation for 20 min at rt, *p*-methoxybenzenethiol (2.1 mg, 1.8 µL, 15 µmol) was added and the mixture was allowed to react for 1 h. *p*-anisdine (1.8 mg, 15 µmol) and the required amounts of *N*-methylhydroxylamine (1-10 equiv in water) and BCN-CH_2-OH **5** (Synaffix B.V, 1-20 equiv in MeCN/water 1:1) were added. The conversion was measured with mass spectrometry after 2 h and 3 d.

Expression and purification of S-eGFP

Cloning of expression vector

All cloning techniques were performed according to standard molecular biology protocols. Restriction enzymes (NdeI and HindIII), ligase (T4 DNA Ligase), Antarctic Phosphatase and DNA polymerase (Phusion® Hot Start DNA Polymerase) were obtained from New England Biolabs.

The eGFP gene (Clontech) was modified by PCR using primers (Biolegio) with overhangs. The forward primer was used to introduce a NdeI restriction site and a serine codon (5'-GACGAGCATATGAGCGGCGTGAGCAAGGGCGAGGAGCTGT-3') and the reverse primer was used to introduce a HindIII restriction site (5'-CTCGTCAAGCTTGTACAGCTCGTCCATGCC -3'). The PCR product and the pET21a(+) expression vector (Novagen) were then digested with NdeI and HindIII. After dephosphorylation of the vector, the digested PCR product was ligated into the vector to yield pET21a(+)-S-eGFP-H_6. This plasmid was transformed into *E. coli* XL1 Blue cells and then the DNA was extracted and the sequence was confirmed by DNA sequencing. The pET21a(+)-S-eGFP-H_6 plasmid was transformed into *E. coli* BLR(DE3)pLysS cells (Novagen), which were used for the expression of S-eGFP.

Expression and purification of S-eGFP

For a typical expression, 100 mL 2xYT medium, supplemented with ampicillin (100 mg/L) and chloroamphenicol (50 mg/L), was inoculated with a single colony of *E. coli* BLR(DE3)pLysS containing pET21a(+)-S-eGFP-H_6 and was incubated at 30 °C overnight. This overnight culture was used to inoculate 900 mL of 2xYT medium supplemented with ampicillin (100 mg/L) and chloroamphenicol (50 mg/L). The culture was grown at 37 °C and protein expression was induced during logarithmic growth (OD_{600} = 0.4-0.6) by addition of IPTG (Sigma-Aldrich) up to

1 mM. After 4 h of expression, the cells were harvested by centrifugation (4000 g at 4 °C for 15 min) and the pellet was stored at -20 °C overnight.
After thawing, the cell pellet was resuspended in 20 mL lysis buffer (50 mM NaH_2PO_4, 10 mM imidazole and 300 mM NaCl, pH 8.0) and incubated with lysozyme (1 mg/mL) for 30 min on ice. The cells were then further lysed by ultrasonic disruption (6 times 10 s, 100% duty cycle and output control 3, Branson Sonifier 250, Marius Instruments Nieuwegein, the Netherlands). The lysate was centrifuged (10000 RPM at 4 °C for 15 min, Sorvall HB-4) to remove the cellular debris. The supernatant was incubated with 2 mL Ni-NTA agarose beads (Qiagen) for 1 h at 4 °C. Subsequently, the suspension was loaded onto a column, the flow-through was collected and the column was washed twice with 10 mL wash buffer (50 mM NaH_2PO_4, 20 mM imidazole and 300 mM NaCl, pH 8.0). Finally, the S-eGFP was eluted from the column using 5 mL elution buffer (50 mM NaH_2PO_4, 250 mM imidazole and 300 mM NaCl, pH 8.0). The volume of the protein solution was reduced to 1 mL using centrifugal filtration (Amicon Ultra-0.5 mL, 10 kDa). The protein was then further purified and the buffer was exchanged to 100 mM ammonium acetate (pH 6.8) via size exclusion chromatography (Superdex 75 HR 10/30). 300 µL fractions were collected and were analyzed by SDS-PAGE (Suppl info, Figure S3) and mass spectrometry. The fractions containing the S-eGFP were combined and 1 mg aliquots were stored at -20 °C. The protein was produced with a yield of 5 mg/L of culture and the purity was verified by SDS-PAGE. ESI-TOF: calculated 28325.9 Da, found 28326.3 Da (Suppl. Info, Figure S3b). The observed mass confirmed efficient processing of the N-terminal methionine residue, resulting in eGFP with an N-terminal serine (Suppl. Info, table S3).

Table S3. Amino acid sequence of S-eGFP-H_6

SGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSR YPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSH NVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEF VTAAGITLGMDELYKLAAALEHHHHHH

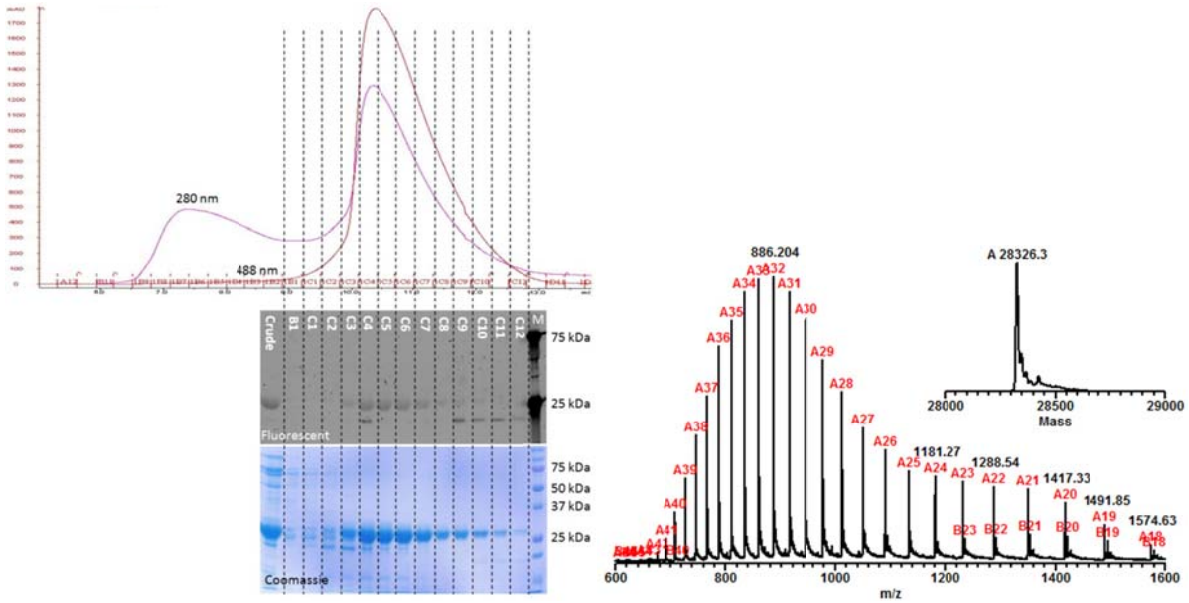
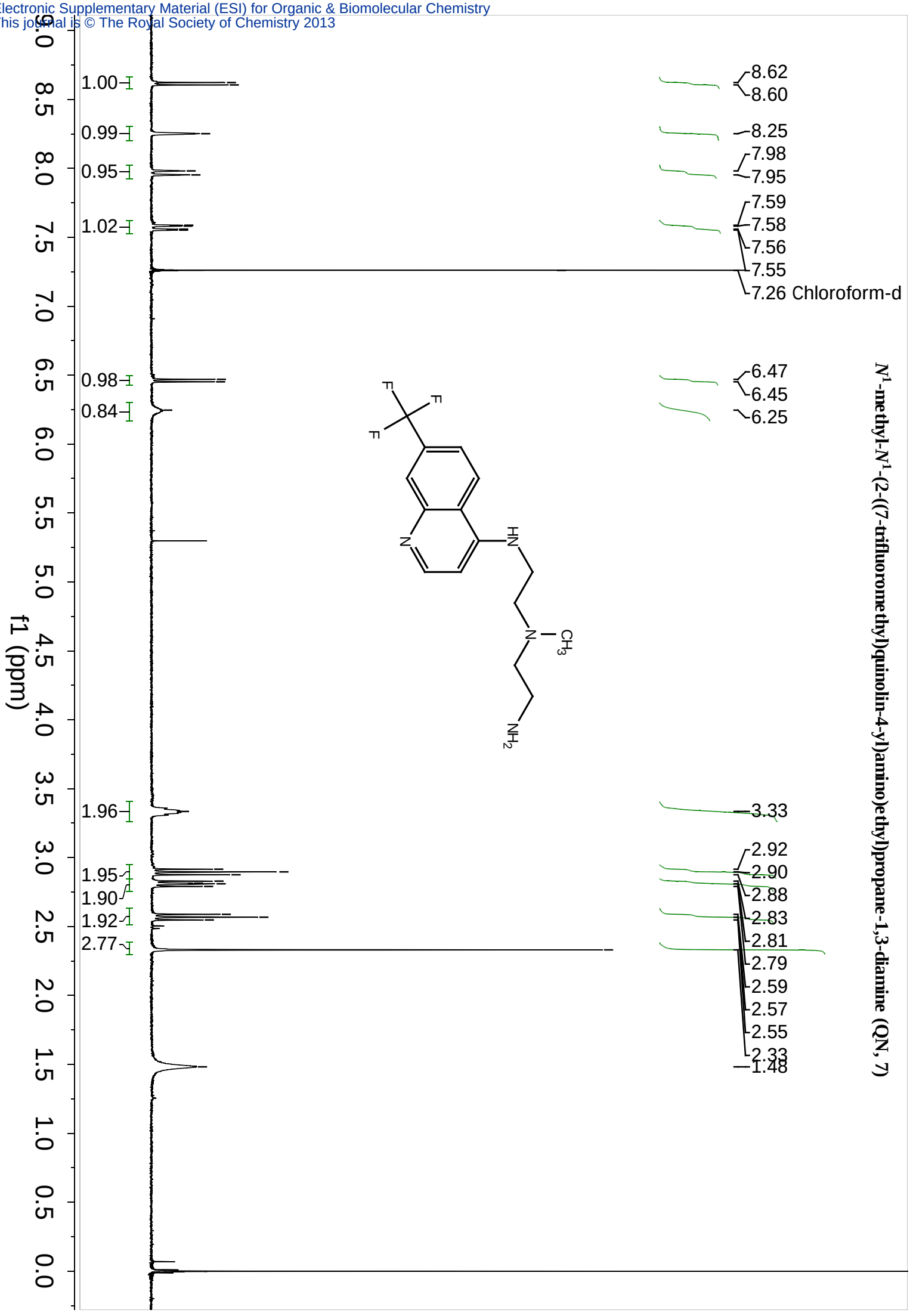
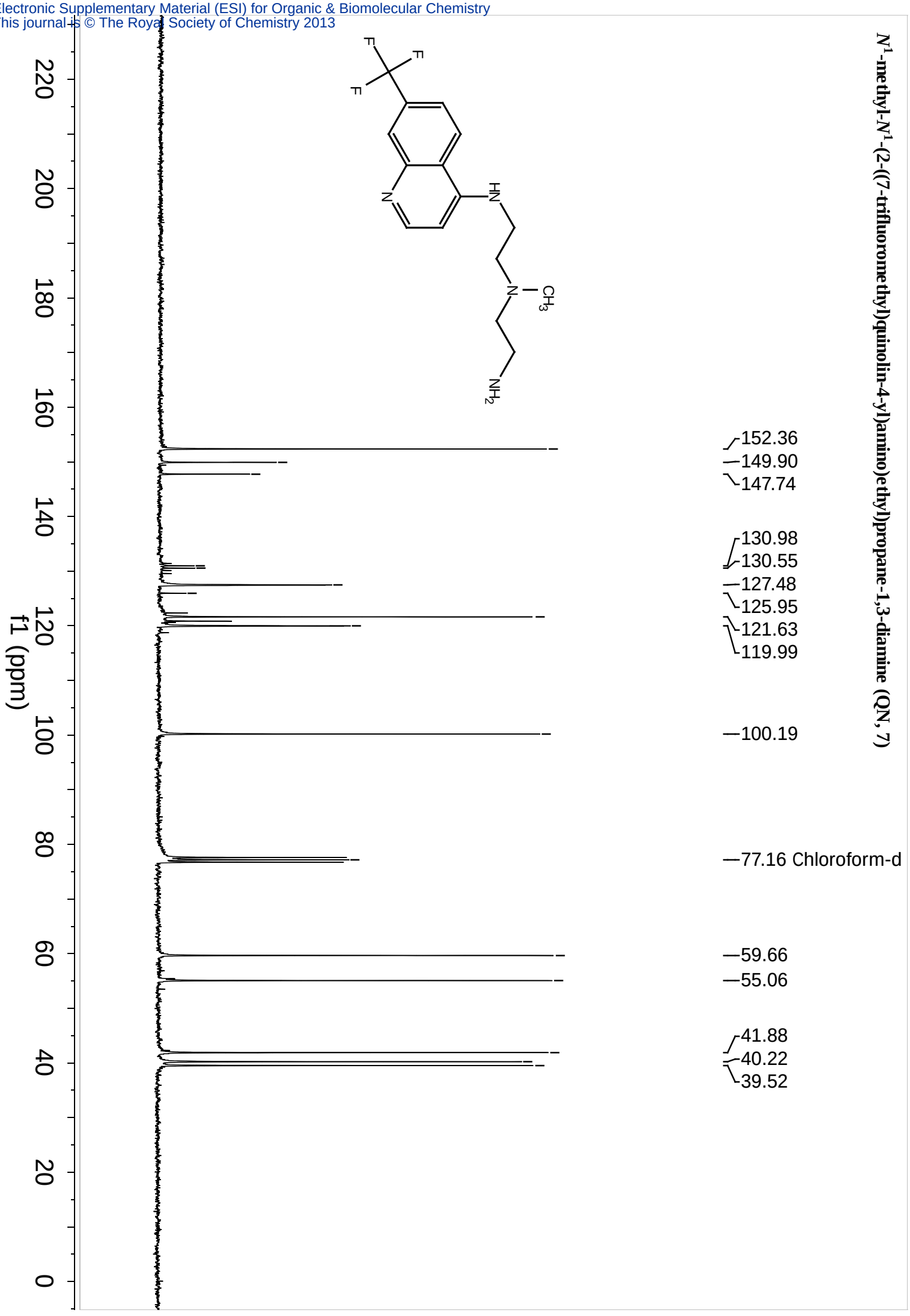


Figure S3. A. Size exclusion chromatography and SDS-PAGE analysis of fractions; B. mass spectrometry spectrum and deconvoluted spectrum of S-eGFP





N^1-methyl-N^1-(2-((7-trifluoromethyl)quinolin-4-yl)amino)ethyl)propane-1,3-diamine (QN, 7)