

# A universal and ready-to-use heterotrifunctional cross-linking reagent for facile synthetic access to sophisticated bioconjugates

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## Supporting Information

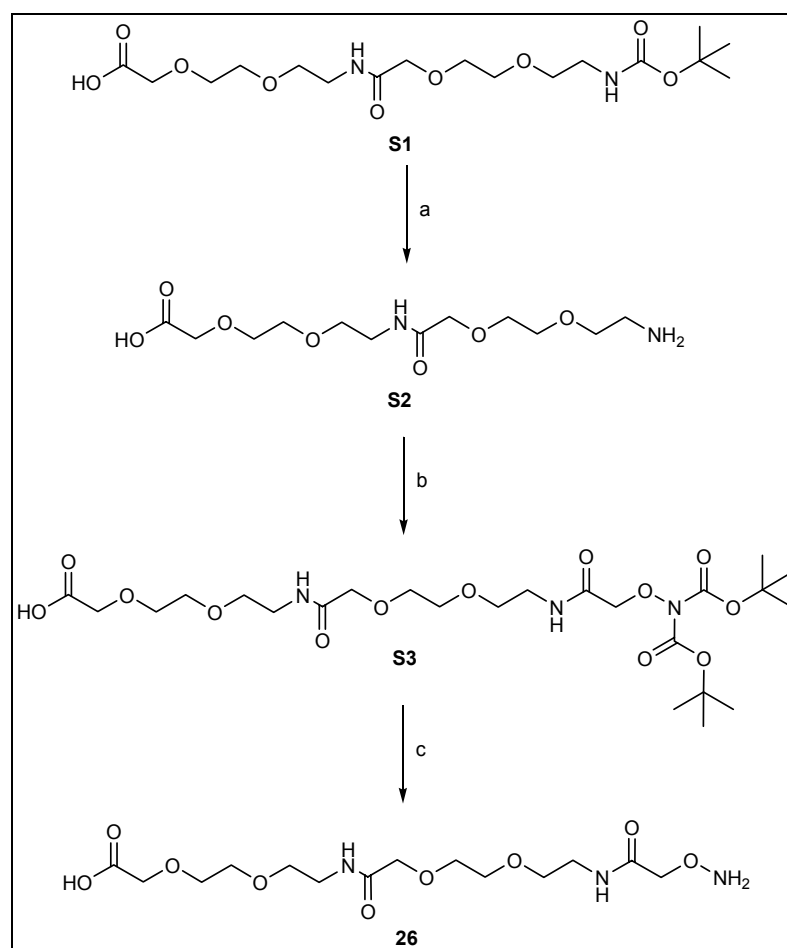
|  |    |
|--|----|
| Experimental: Detailed synthetic procedures for compound 26.....   | 3  |
| <sup>1</sup> H NMR spectrum of 10 recorded in CDCl <sub>3</sub> .....  | 5  |
| <sup>13</sup> C NMR spectrum of 10 recorded in CDCl <sub>3</sub> .....   | 6  |
| ESI-MS spectrum of 10 recorded in the positive mode.....   | 6  |
| ESI-MS spectrum of azido-tripod 3 recorded in the positive mode.....   | 7  |
| (a) 1 <sup>st</sup> step: S <sub>N</sub> 2 reaction with iodoacetyl derivative of R6G-WS: ESI-MS spectrum of 17 (recorded in the positive mode).....   | 8  |
| (b) 2 <sup>nd</sup> step: Oxime ligation with Cy 5.0 aldehyde: ESI-MS spectrum of 19 (recorded in the positive mode).....  | 9  |
| (c) 3 <sup>rd</sup> step: CuAAC reaction with AFB2-alkyne: ESI-MS spectrum of 22 (recorded in the negative mode).....  | 9  |
| (c) 3 <sup>rd</sup> step: CuAAC reaction with ODN-alkyne: ESI-MS spectrum of 23 (recorded in the negative mode with LTQ Orbitrap XL).....  | 10 |
| UV-visible absorption of 23 in deionised water at 25 °C (concentration: 1.30 μM); λ <sub>max</sub> = 267, 534 and 654 nm.....  | 12 |
| Fluorescence emission spectra of 17 (azido-tripod labelled only with donor R6G-WS, (—)) and 23 (—) in deionised water at 25 °C (concentration: 1.30 μM) <sup>a</sup> after excitation at 520 nm..... | 12 |
| (a) 1 <sup>st</sup> step: S <sub>N</sub> 2 reaction with iodoacetyl derivative of Cy 3.0: ESI-MS spectrum of 16 (recorded in the positive mode).....   | 13 |
| (b) 2 <sup>nd</sup> step: Oxime ligation with Cy 5.0 aldehyde: ESI-MS spectrum of 19 (recorded in the positive mode).....  | 14 |
| (c) 3 <sup>rd</sup> step: CuAAC reaction with ODN-alkyne: ESI-MS spectrum of 24 (recorded in the negative mode with LTQ Orbitrap XL).....  | 14 |
| UV-visible absorption of 24 in deionised water at 25 °C (concentration: 0.85 μM); λ <sub>max</sub> = 267, 550 and 652 nm.....  | 15 |
| Fluorescence emission spectra of 16 (azido-tripod labelled only with donor Cy 3.0, (—)) and 24 (—) in deionised water at 25 °C (concentration: 0.85 μM) after excitation at 540 nm.....              | 16 |
| ESI-MS spectrum of 25 recorded in the positive mode.....   | 17 |

Experimental: Detailed synthetic procedures for compound **26**.

### High-performance liquid chromatography separations

Two chromatographic systems were used for the analytical experiments and the purification steps. **System A:** RP-HPLC (Thermo Hypersil GOLD C<sub>18</sub> column, 5  $\mu$ m, 4.6  $\times$  150 mm) with CH<sub>3</sub>CN and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.2) as eluents [0% CH<sub>3</sub>CN (5 min), followed by linear gradient from 0 to 60% (30 min) then from 60 to 90% (10 min) of CH<sub>3</sub>CN] at a flow rate of 1.0 mL min<sup>-1</sup>. Triple UV detection was achieved at 210, 260 and 285 nm. **System B:** semi-preparative RP-HPLC (Thermo Hypersil GOLD C18 column, 5  $\mu$ m, 10  $\times$  250 mm) with CH<sub>3</sub>CN and 0.1% aq. acetic acid (aq. AcOH, 0.1%, v/v, pH 3.3) as eluents [0% CH<sub>3</sub>CN (5 min), followed by linear gradient from 0 to 20% (5 min) and 20 to 70% (50 min) of CH<sub>3</sub>CN] at a flow rate of 4.0 mL min<sup>-1</sup>. Dual UV detection was achieved at 220 and 305 nm.

Aminoxy acid pseudo-PEG linker **26** was prepared from Boc-protected amino-PEG-acid spacer **S1** by using an original 3-step synthetic procedure developed by us and described in Scheme S1 :



**Scheme S1** Reagents and conditions : a) TFA, CH<sub>2</sub>Cl<sub>2</sub>, 4 °C to rt, 3 h, quantitative yield; b) (Boc)<sub>2</sub>-Aoa-OH **9**, DCC, HOBT, NMP, rt, 1 h then DIEA, rt, 1 h, 30% (after RP-HPLC purification); c) TFA, CH<sub>2</sub>Cl<sub>2</sub>, 4 °C to rt, 1 h, quantitative yield.

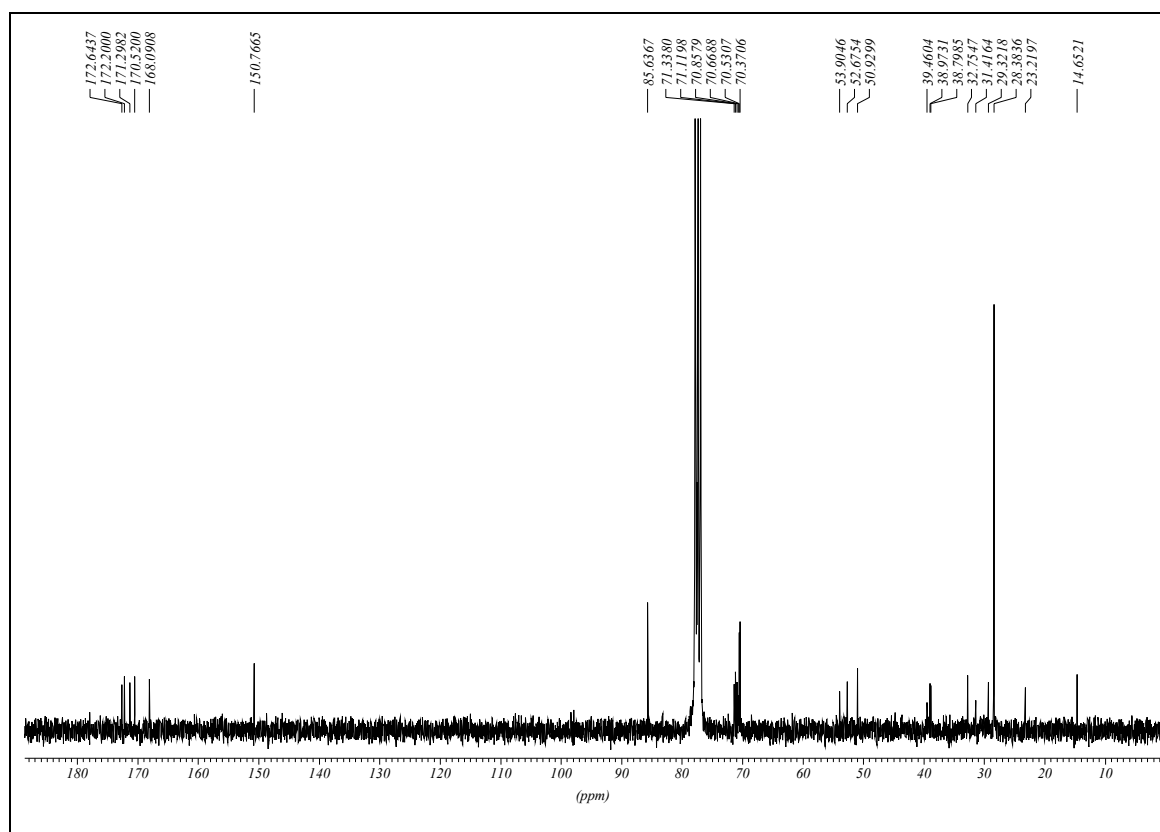
**Amino-PEG-acid (S2).** PEG building block **S1** (68 mg, 0.16 mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4 mL) and the solution was cooled to 4 °C. TFA (956 μL, 12.8 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 3 h. The reaction was checked for completion by TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 9 : 1, v/v) and the mixture was evaporated to dryness. The resulting oily residue was dissolved in deionised water and lyophilised to give free amino acid **S2** (63 mg, 0.15 mmol, quantitative yield) as a yellow oil. This compound was used in the next coupling reaction step without further purification or analysis.

**(Boc)<sub>2</sub>-Aaaa-PEG-OH (S3).** (Boc)<sub>2</sub>-Aaaa-OH **9** (26 mg, 88 μmol) was dissolved in dry NMP (1 mL). HOBt (12.9 mg, 96 μmol) and DCC (18 mg, 88 μmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 1 h. Thereafter the mixture of HOBt active esters was added to the oily amino acid **S2** with dry DIEA (240 μL, 240 μmol) and the mixture was stirred at room temperature for a further 1 h. The mixture was evaporated to dryness, taken up with CH<sub>3</sub>CN (200 μL) and 0.1% aq. AcOH (500 μL) and purified by semi-preparative RP-HPLC (system B, 1 injection). The product-containing fractions were lyophilised to give protected (Boc)<sub>2</sub>-Aaaa-PEG-OH **S3** (14 mg, 24 μmol, yield 30%) as a yellow oil. δ<sub>H</sub>(300 MHz; CDCl<sub>3</sub>) 1.54 (s, 9H, Boc), 3.50-3.75 (m, 16H, 8 × CH<sub>2</sub> PEG), 4.00 (s, 2H, CH<sub>2</sub> PEG), 4.15 (s, 2H, CH<sub>2</sub> PEG), 4.45 (s, 2H, CH<sub>2</sub> Aaaa), 7.93 (bs, 1H, CO<sub>2</sub>H); δ<sub>C</sub>(75.5 MHz; CDCl<sub>3</sub>) 28.2, 38.7, 38.9, 68.9, 69.7, 70.1, 70.4, 70.7, 70.9, 71.2, 76.4, 77.2, 85.4, 150.5, 168.4, 170.2; MS (ESI<sup>-</sup>): *m/z* 580.27 [M - H]<sup>-</sup>, calcd for C<sub>24</sub>H<sub>43</sub>N<sub>3</sub>O<sub>13</sub>: 581.28.

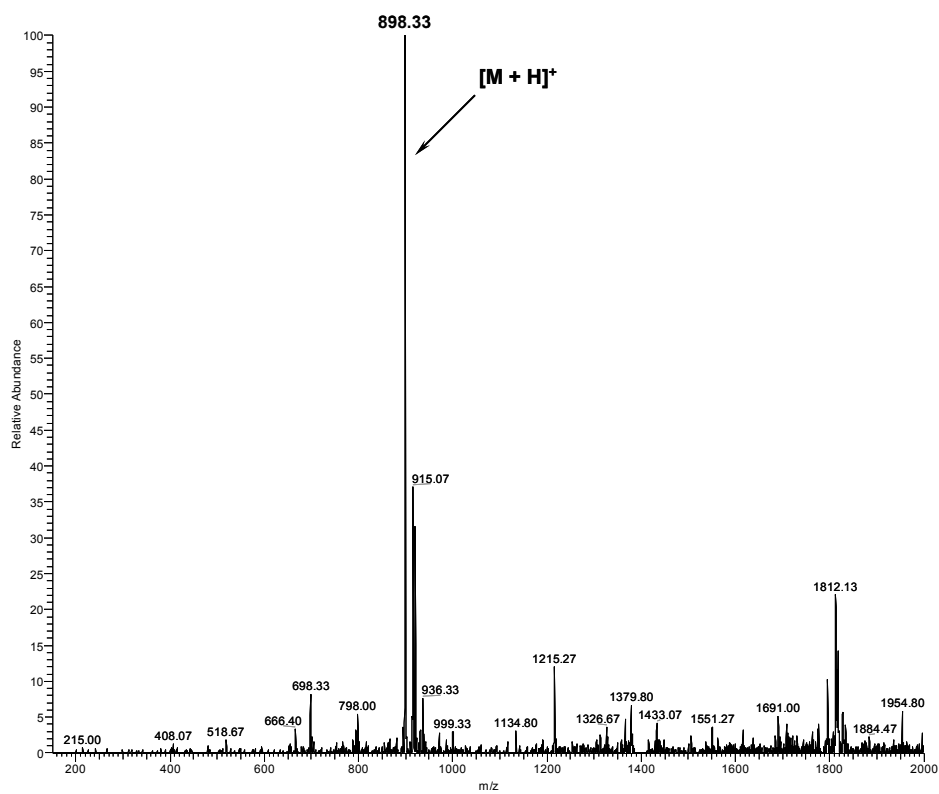
**Compound (26).** (Boc)<sub>2</sub>-Aaaa-PEG-OH **S3** (14 mg, 24 μmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (0.5 mL) and the solution was cooled to 4 °C. TFA (380 μL, 5.1 mmol) was added dropwise and the resulting reaction mixture was stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system A) and the mixture was evaporated to dryness. The resulting oily residue was dissolved in deionised water and lyophilised to give compound **26** (14 mg, 24 μmol, quantitative yield) as a yellow oil. MS (ESI<sup>+</sup>): *m/z* 382.54 [M + H]<sup>+</sup>, calcd for C<sub>14</sub>H<sub>27</sub>N<sub>3</sub>O<sub>9</sub>: 381.17.



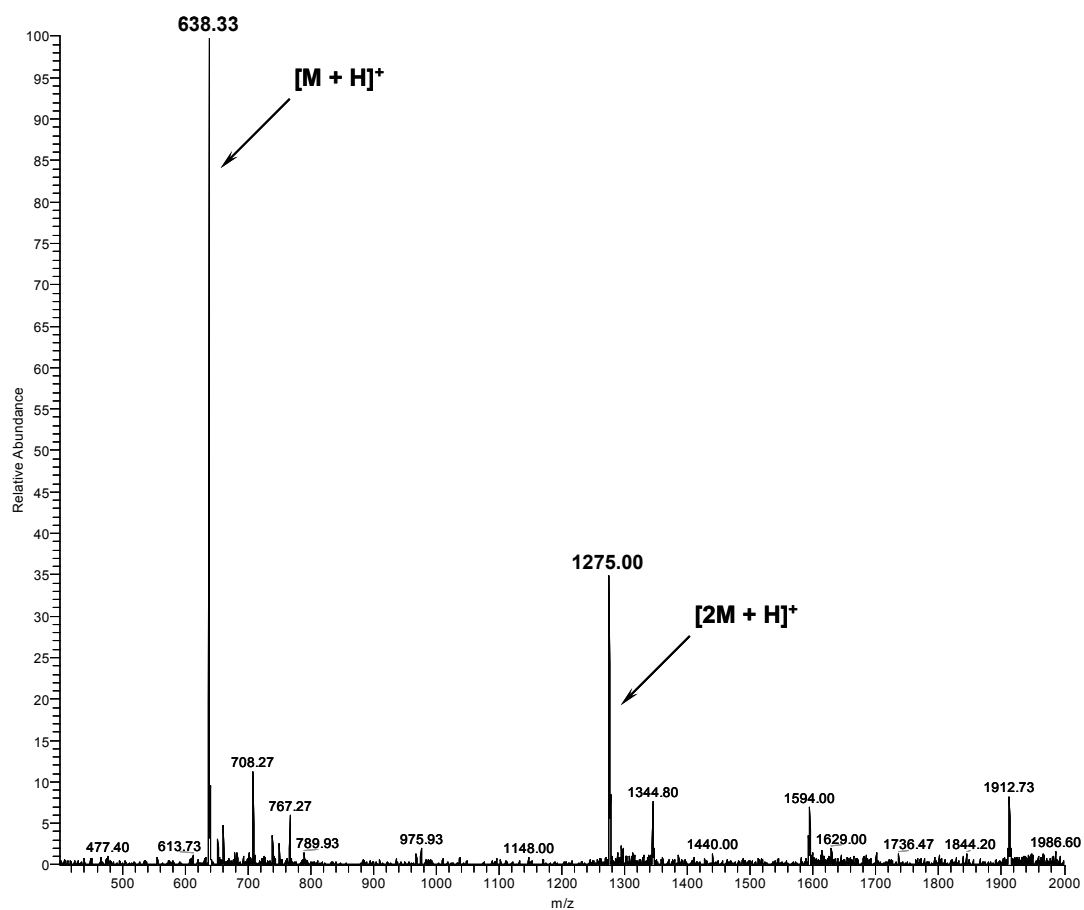
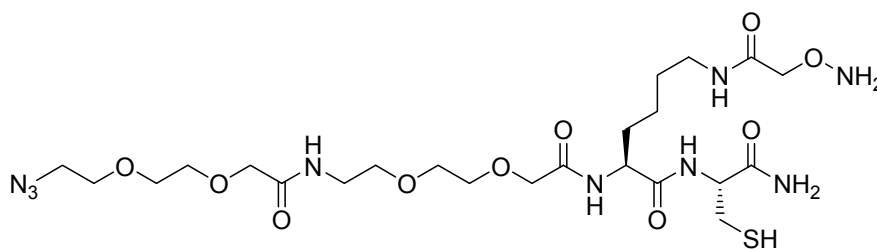
$^{13}\text{C}$  NMR spectrum of **10** recorded in  $\text{CDCl}_3$ .



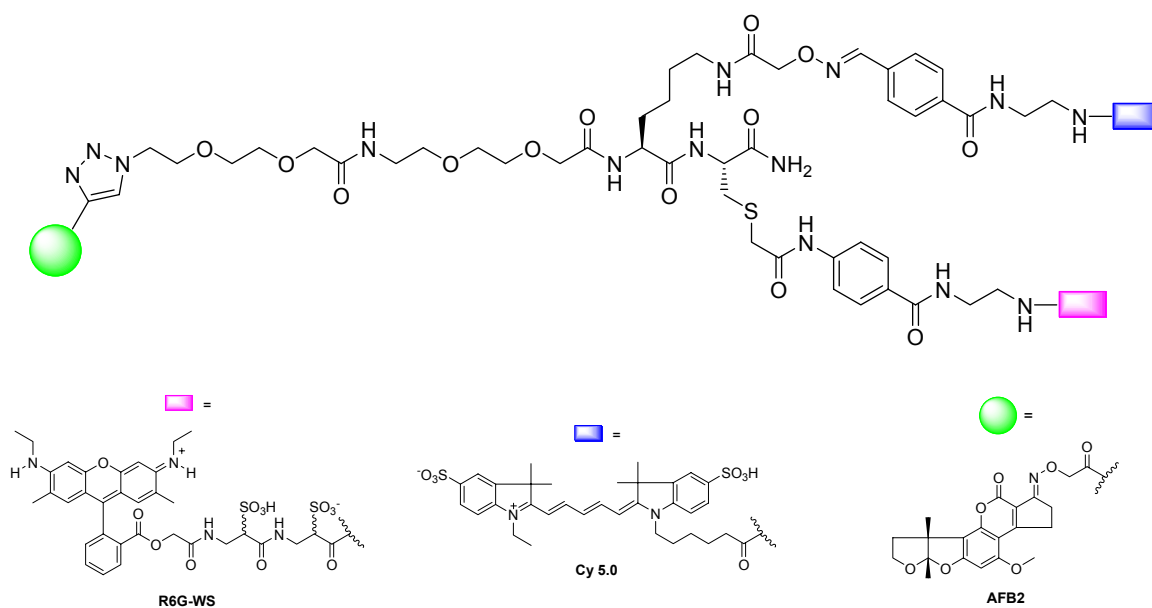
ESI-MS spectrum of **10** recorded in the positive mode.



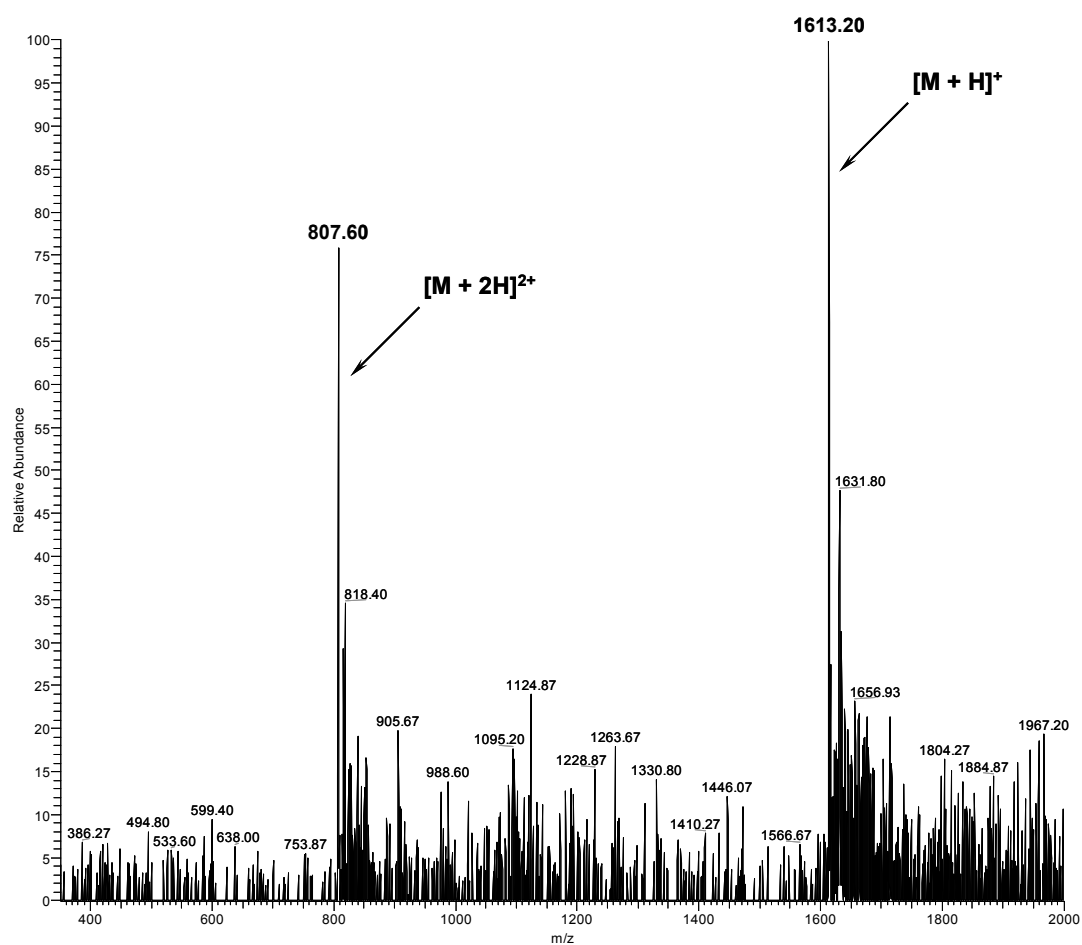
ESI-MS spectrum of azido-tripod **3** recorded in the positive mode.



## FRET cassette (R6G-WS-Cy5.0) labelled AFB2 22

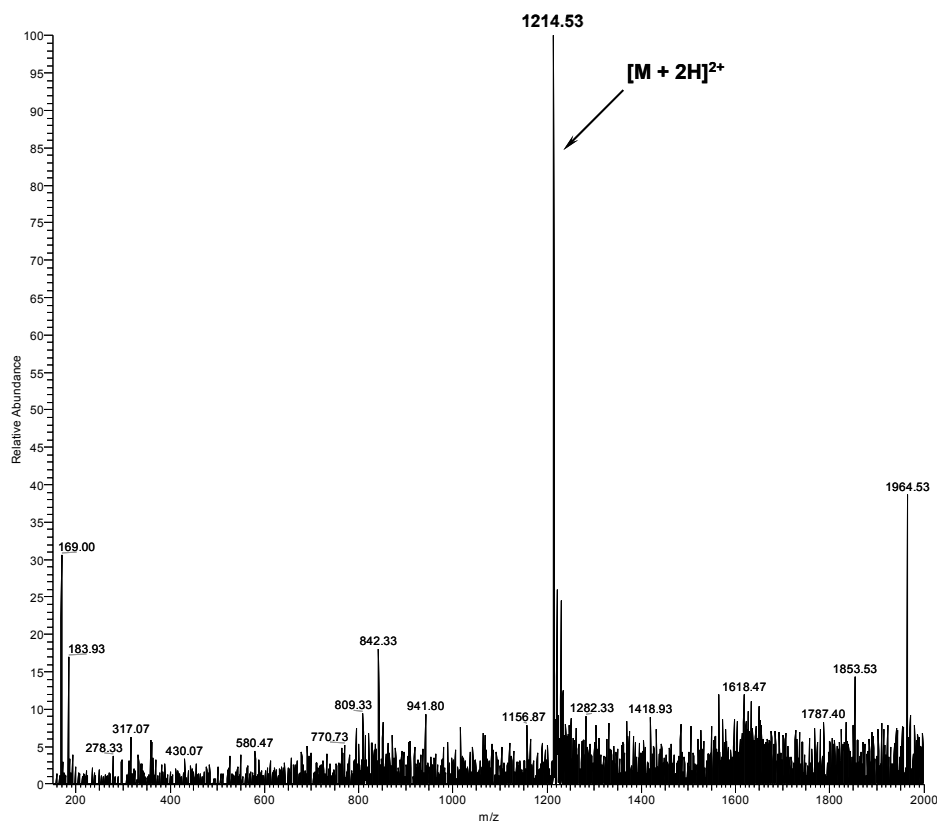


(a) 1<sup>st</sup> step: S<sub>N</sub>2 reaction with iodoacetyl derivative of R6G-WS: ESI-MS spectrum of 17 (recorded in the positive mode).

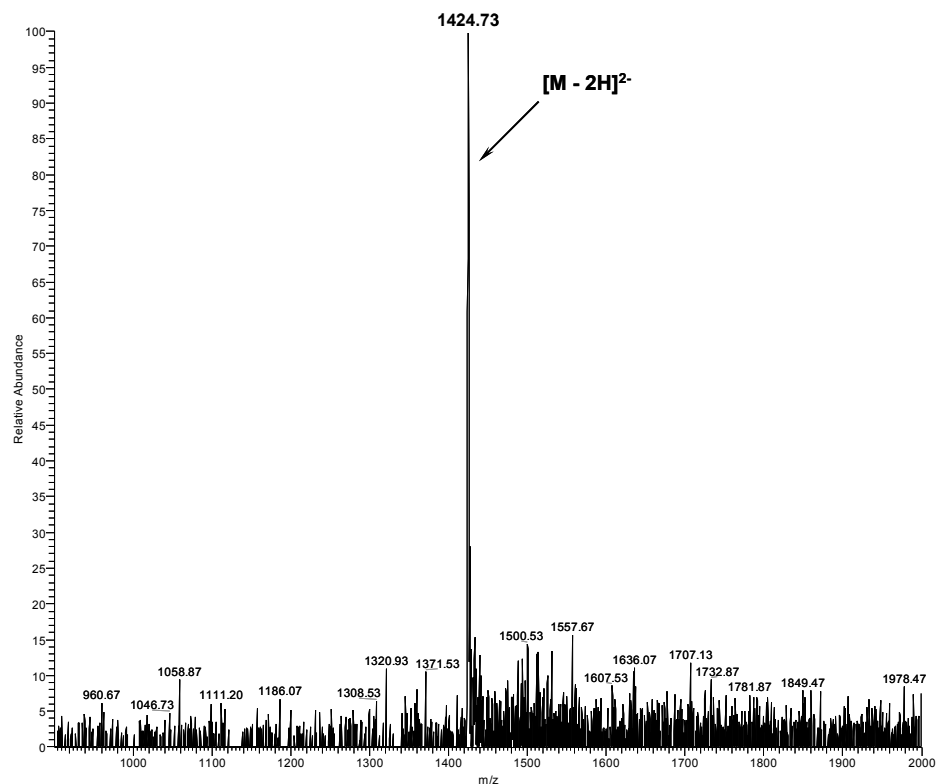




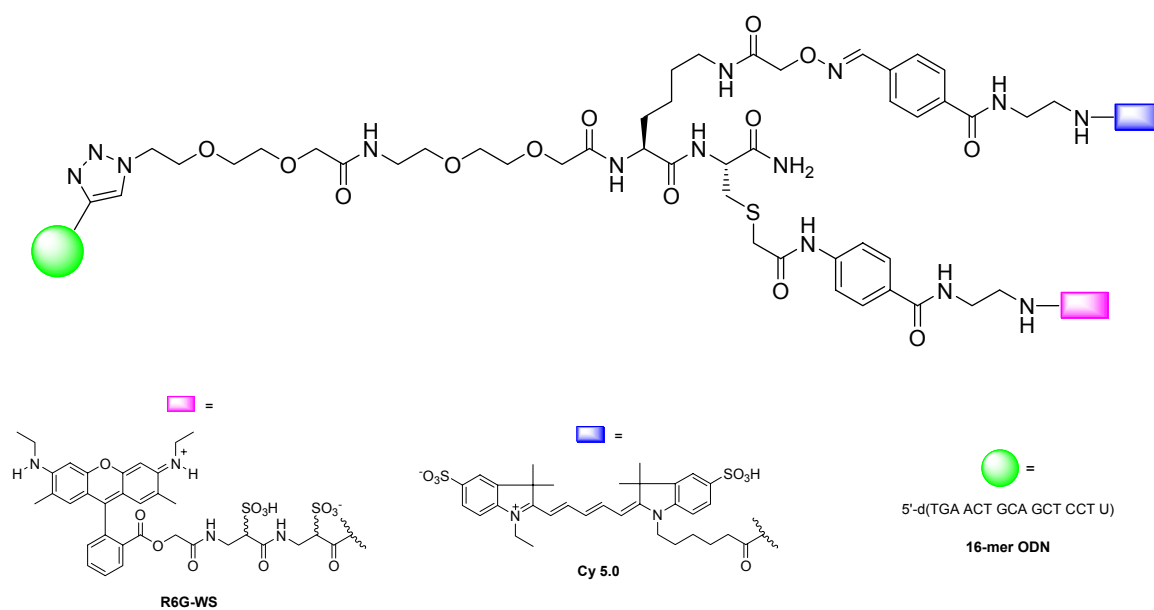
(b) 2<sup>nd</sup> step: Oxime ligation with Cy 5.0 aldehyde: ESI-MS spectrum of **19** (recorded in the positive mode).



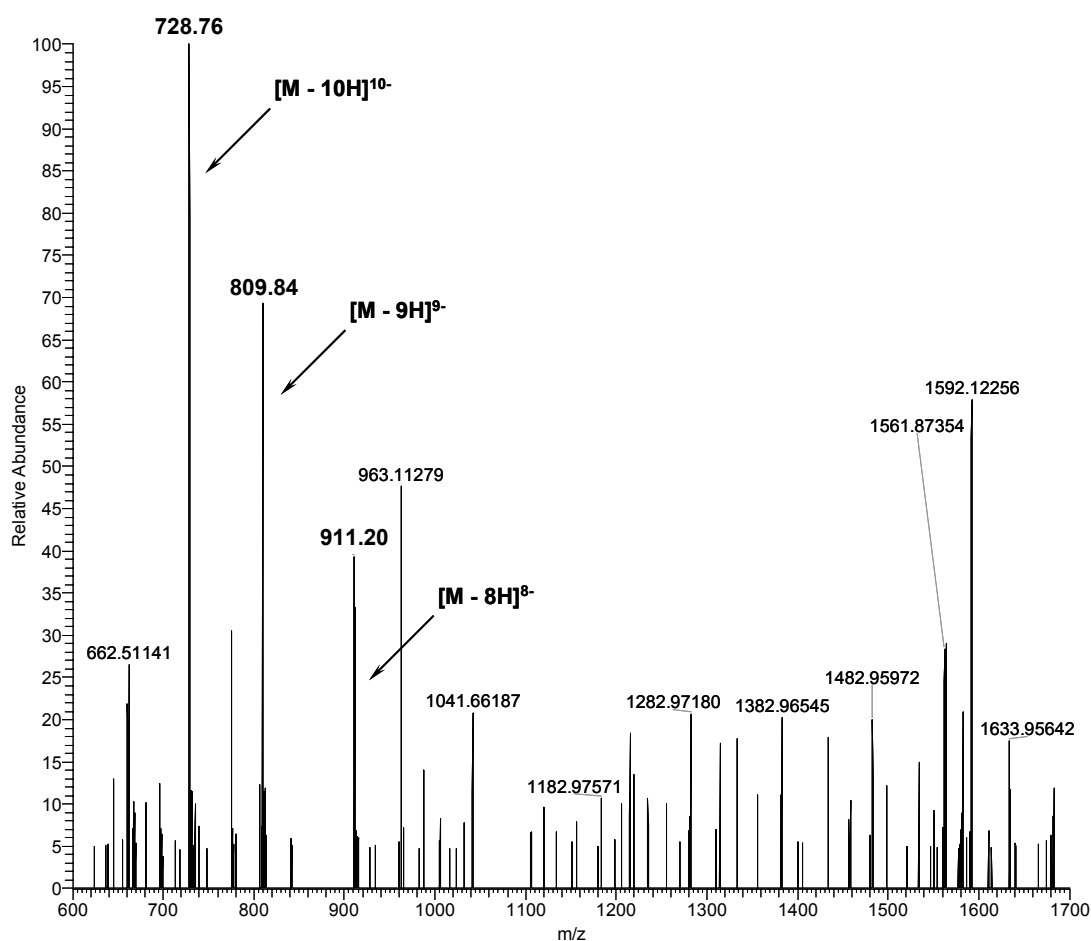
(c) 3<sup>rd</sup> step: CuAAC reaction with AFB2-alkyne: ESI-MS spectrum of **22** (recorded in the negative mode).

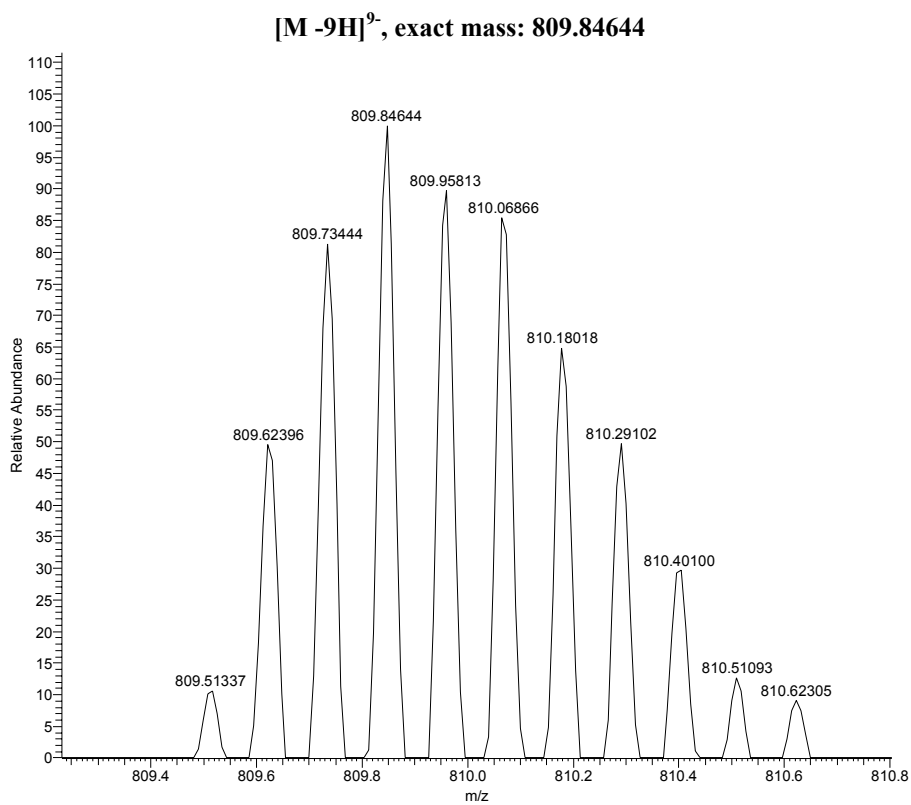
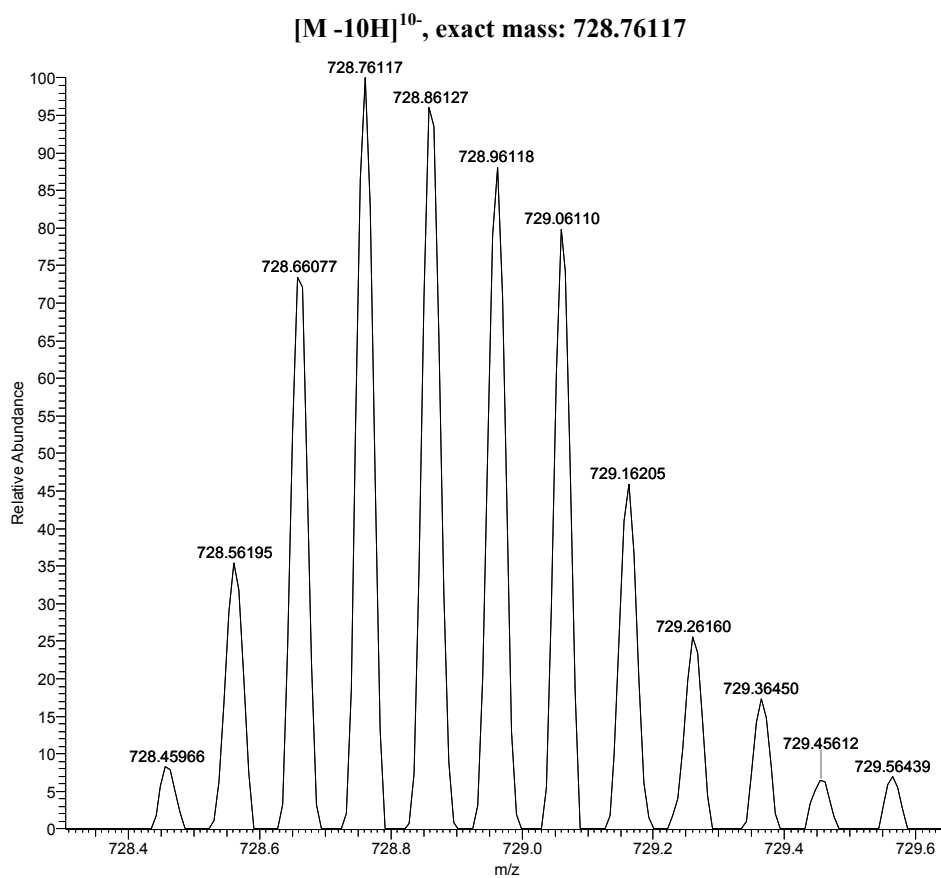


## FRET cassette (R6G-WS-Cy5.0) labelled oligonucleotide **23**

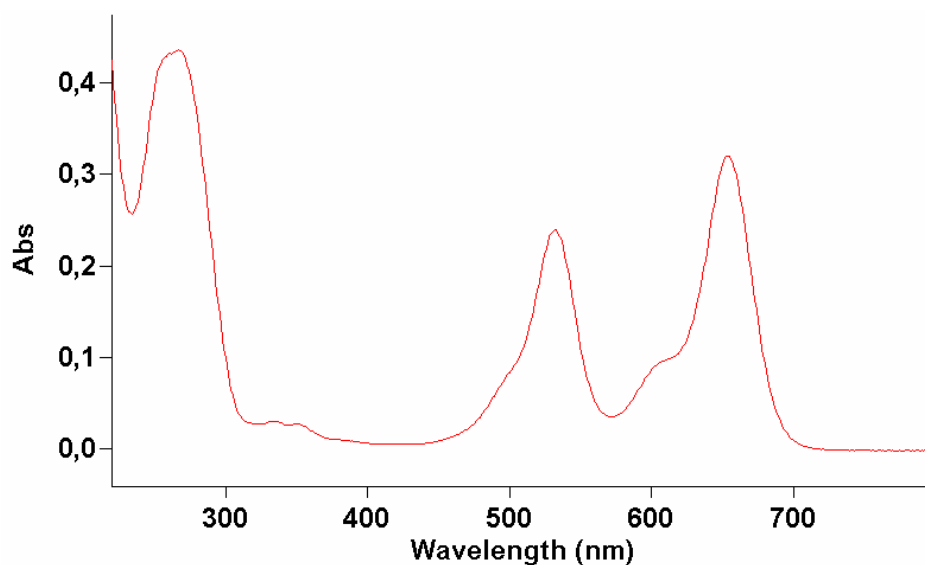


- (a) 1<sup>st</sup> step:  $S_N2$  reaction with iodoacetyl derivative of R6G-WS: see ESI-MS spectrum of **17**.  
(b) 2<sup>nd</sup> step: Oxime ligation with Cy 5.0 aldehyde: see ESI-MS spectrum of **19**.  
(c) 3<sup>rd</sup> step: CuAAC reaction with ODN-alkyne: ESI-MS spectrum of **23** (recorded in the negative mode with LTQ Orbitrap XL)

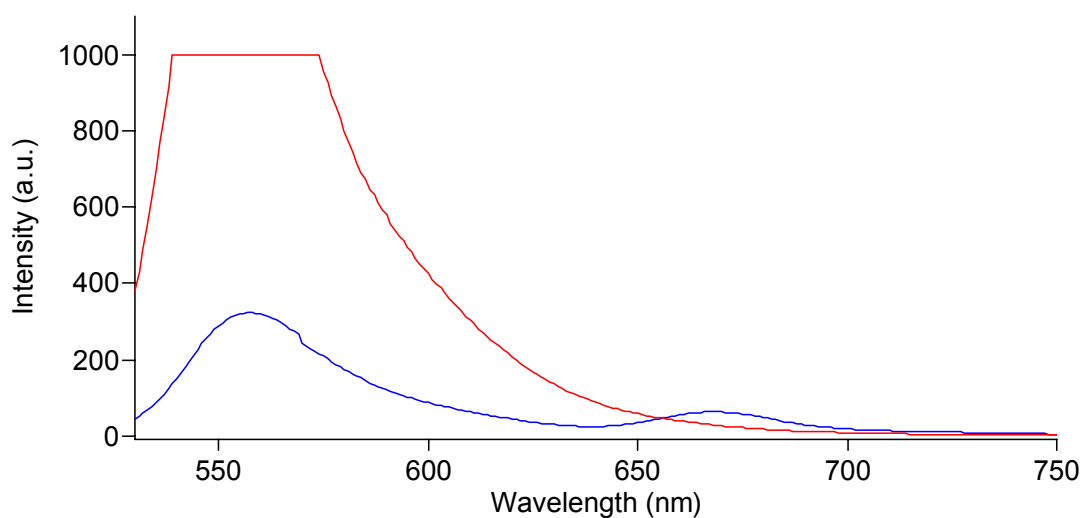




UV-visible absorption of **23** in deionised water at 25 °C (concentration: 1.30  $\mu\text{M}$ );  $\lambda_{\text{max}} = 267$ , 534 and 654 nm.

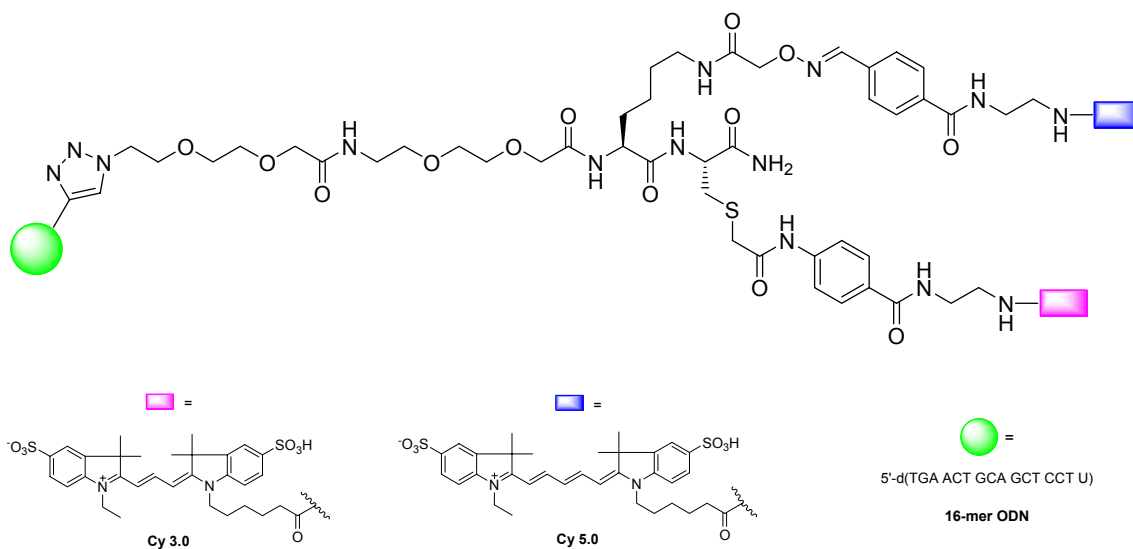


Fluorescence emission spectra of **17** (azido-tripod labelled only with donor R6G-WS, (—)) and **23** (—) in deionised water at 25 °C (concentration: 1.30  $\mu\text{M}$ )<sup>a</sup> after excitation at 520 nm.

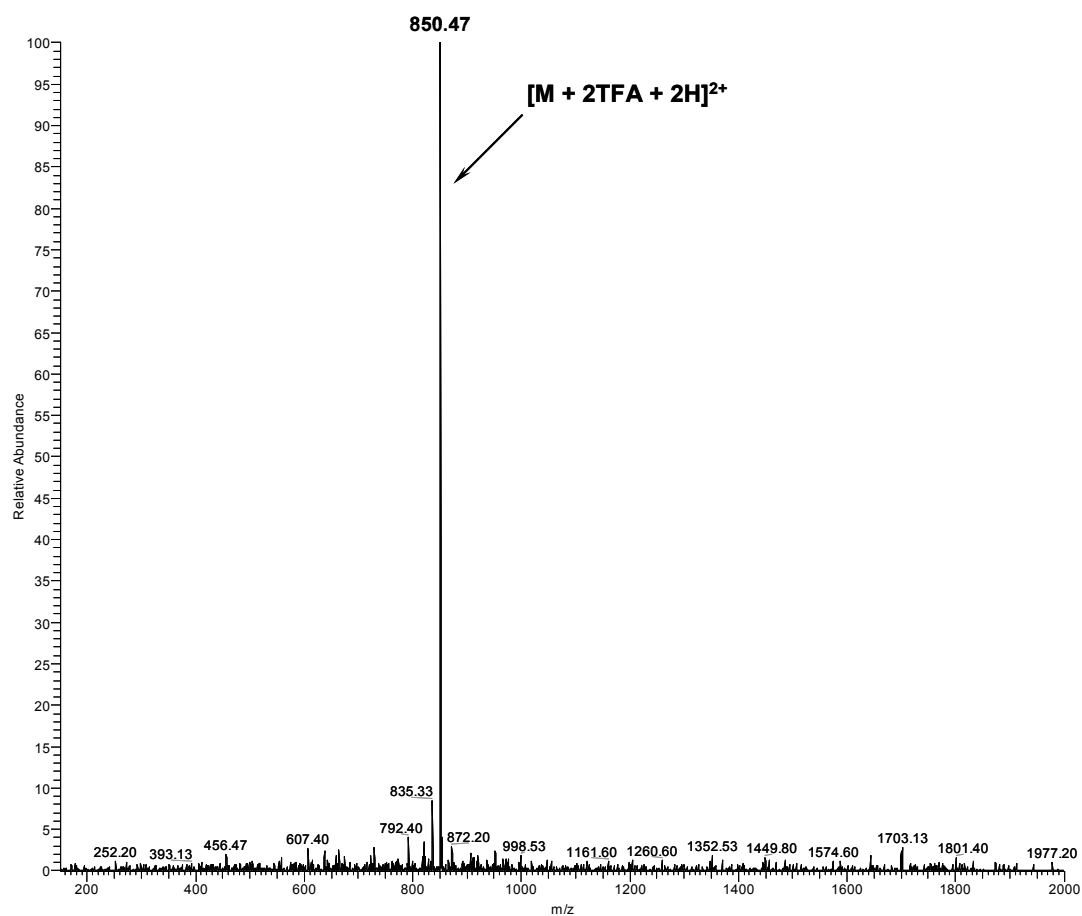


<sup>a</sup>For such FRET cassette concentration, it is not possible to calculate energy transfer efficiency parameter  $E$  because saturation of the relative fluorescence intensity of R6G-WS (in the absence of Cy 5.0) was observed. However, comparison of the fluorescence intensities at  $\lambda = 557$  and  $669$  nm (corrected to account for difference in quantum yields:  $\Phi(\text{R6G-WS})/\Phi(\text{Cy 5.0}) = 0.86/0.20 = 4.3$  in deionised water at 25 °C) enables to calculate an approximate  $E$  parameter equal to 0.40. Furthermore, the distance between the two fluorophores within **23** was not determined because  $R_0$  value for R6G-WS/Cy5.0 pair is not known.

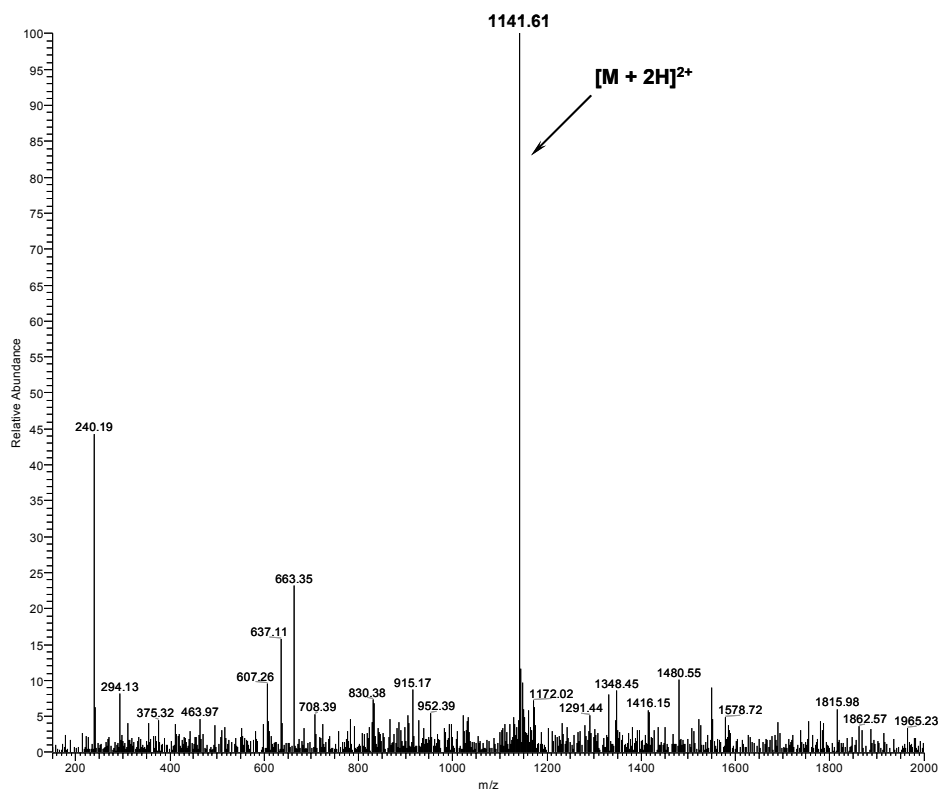
## FRET cassette (Cy3.0-Cy5.0) labelled oligonucleotide 24



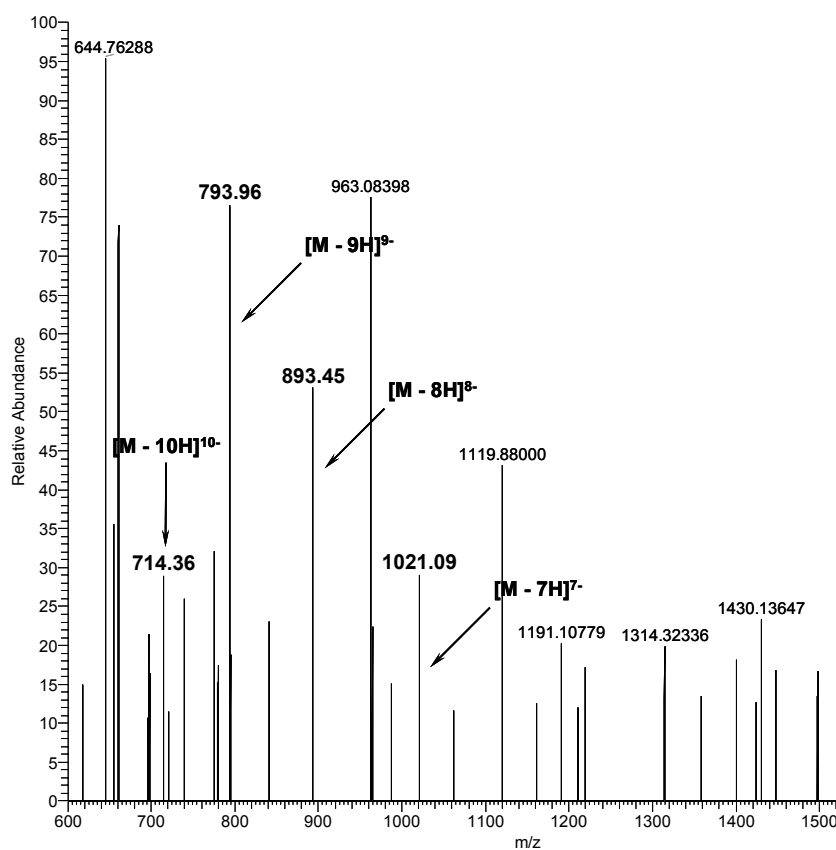
(a) 1<sup>st</sup> step: S<sub>N</sub>2 reaction with iodoacetyl derivative of Cy 3.0: ESI-MS spectrum of 16 (recorded in the positive mode).

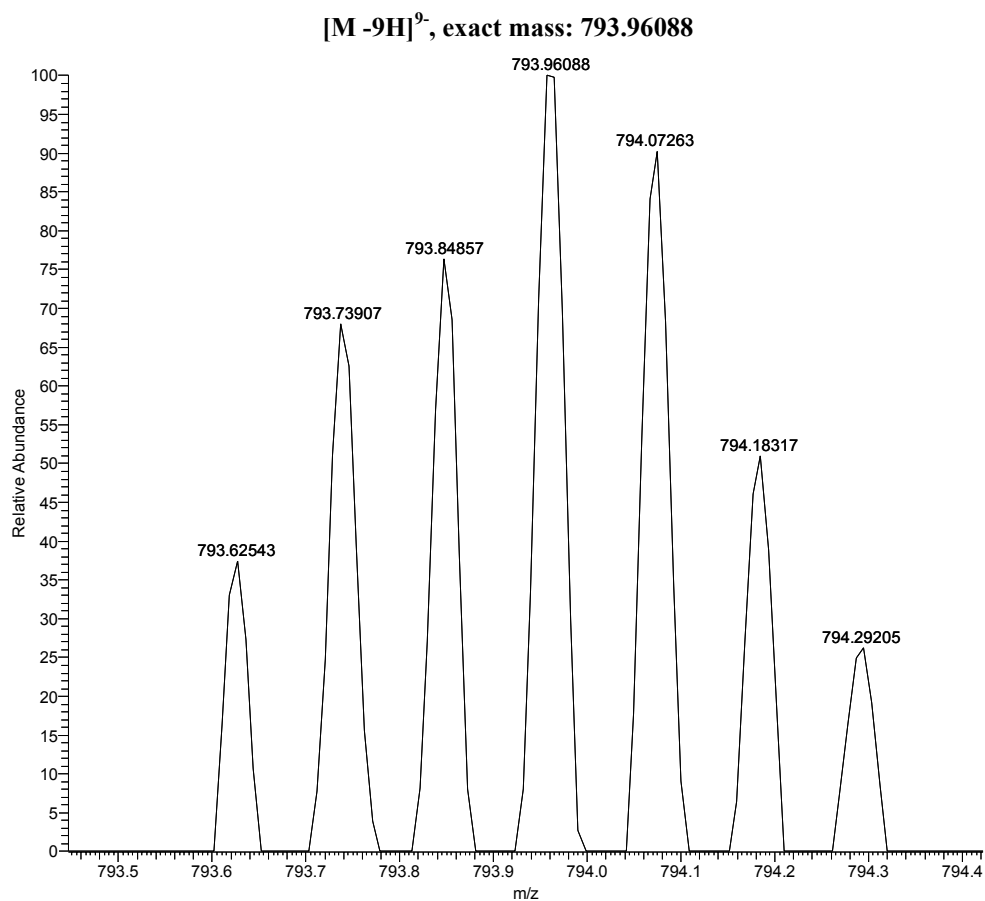


(b) 2<sup>nd</sup> step: Oxime ligation with Cy 5.0 aldehyde: ESI-MS spectrum of **19** (recorded in the positive mode)

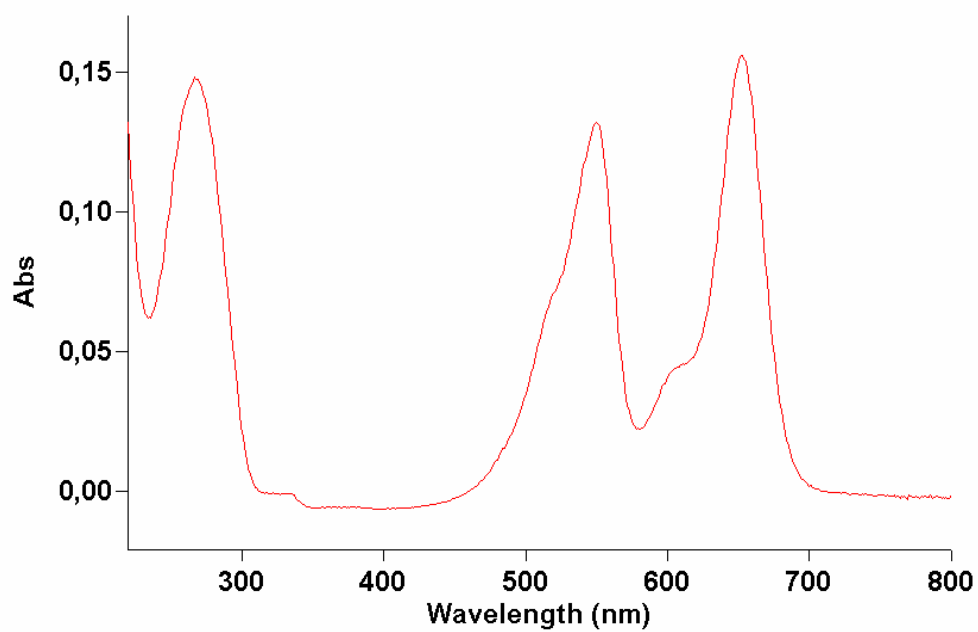


(c) 3<sup>rd</sup> step: CuAAC reaction with ODN-alkyne: ESI-MS spectrum of **24** (recorded in the negative mode with LTQ Orbitrap XL)

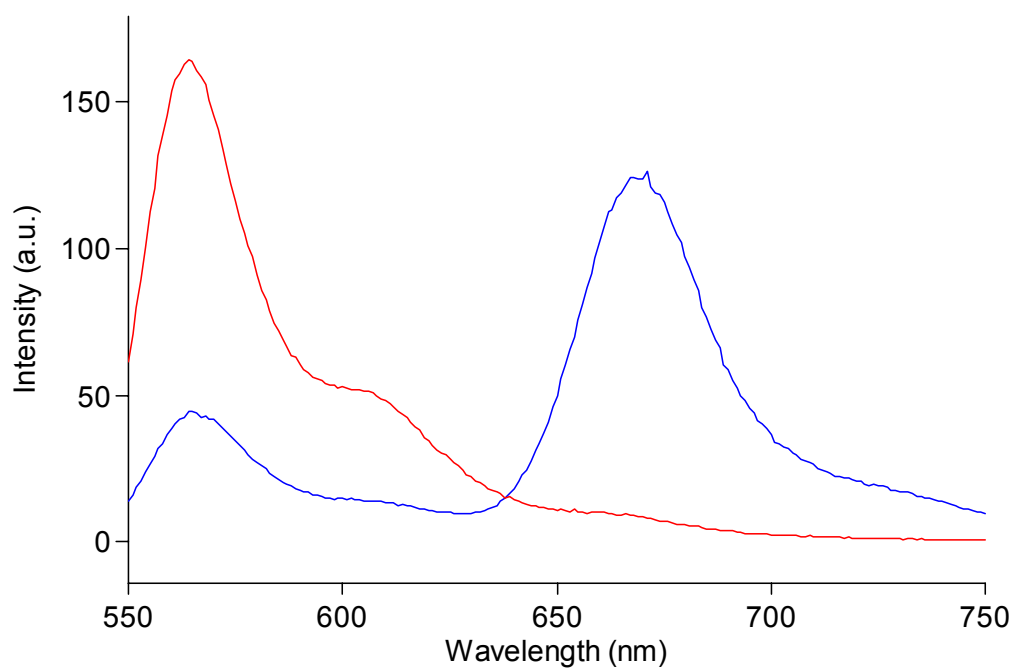




UV-visible absorption of **24** in deionised water at 25 °C (concentration: 0.85  $\mu\text{M}$ );  $\lambda_{\text{max}} = 267$ , 550 and 652 nm.

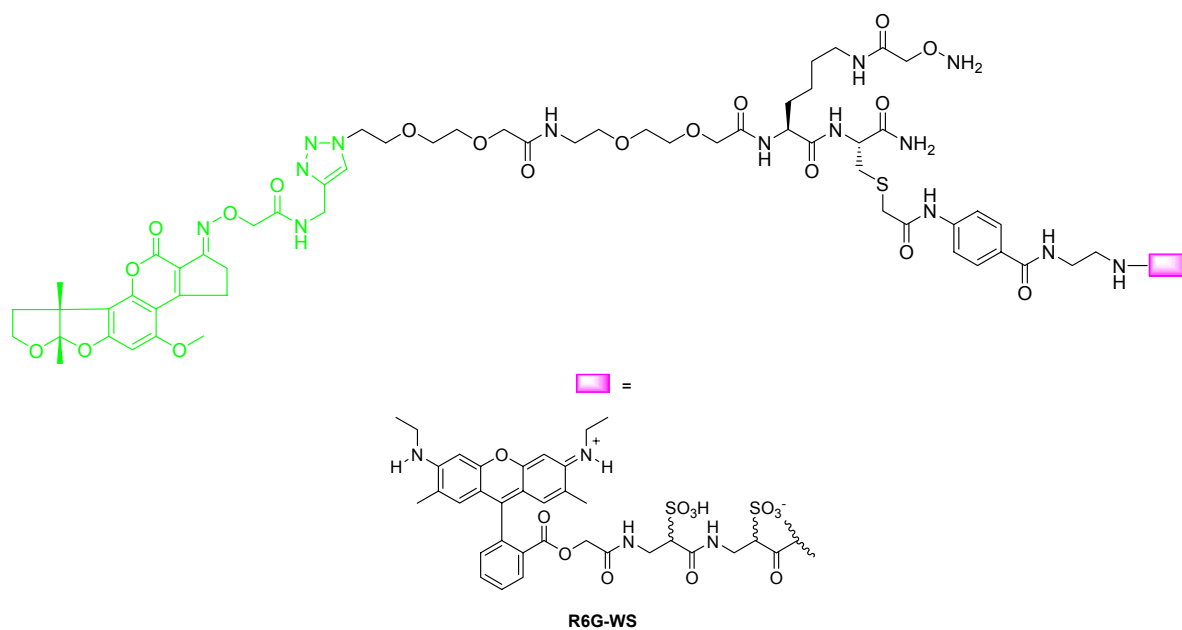


Fluorescence emission spectra of **16** (azido-tripod labelled only with donor Cy 3.0, (—)) and **24** (—) in deionised water at 25 °C (concentration: 0.85  $\mu$ M) after excitation at 540 nm.





## R6G-WS labelled AFB2 aminoxy probe 25



ESI-MS spectrum of **25** recorded in the positive mode.

