Supporting Information

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1. NMR spectra

Figure 1. ^{1}H NMR spectrum of 6-bromo-4-hydroxy-3-nitrocoumarin, **10**, in DMSO.

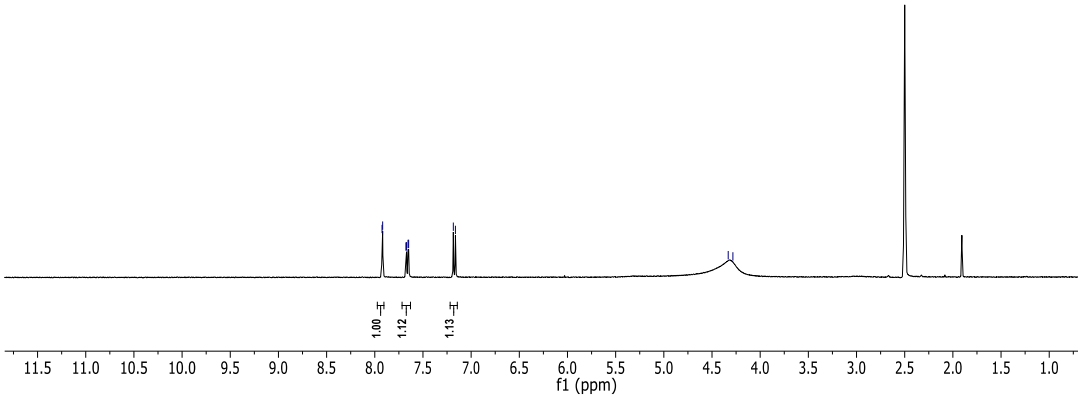


Figure 2. ^{13}C NMR spectrum of 6-bromo-4-hydroxy-3-nitrocoumarin, **10**, in DMSO.

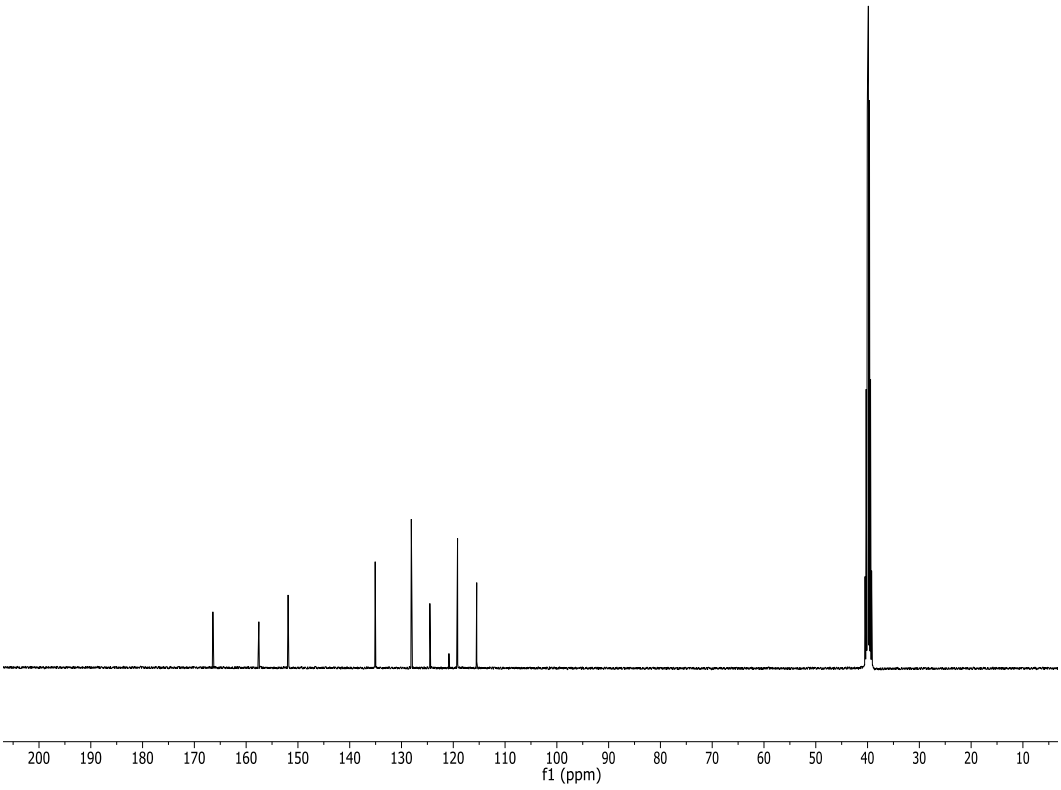


Figure 3. ^{1}H NMR spectrum of 4-hydroxy-6-(4-methoxyphenyl)-3-nitrocoumarin, **11**, in $CDCl_3$.

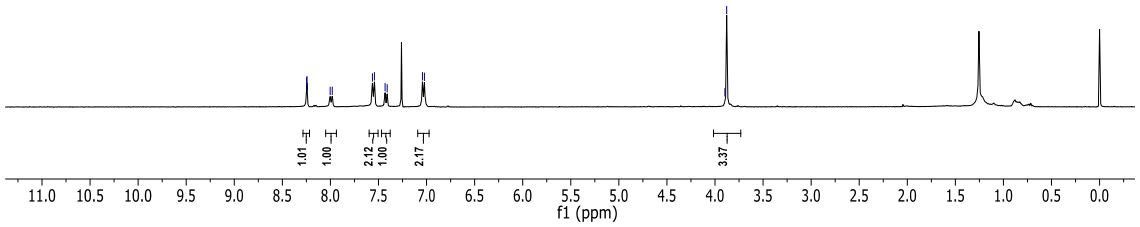


Figure 4. ^{13}C NMR spectrum of 4-hydroxy-6-(4-methoxyphenyl)-3-nitrocoumarin, **11**, in $CDCl_3$.

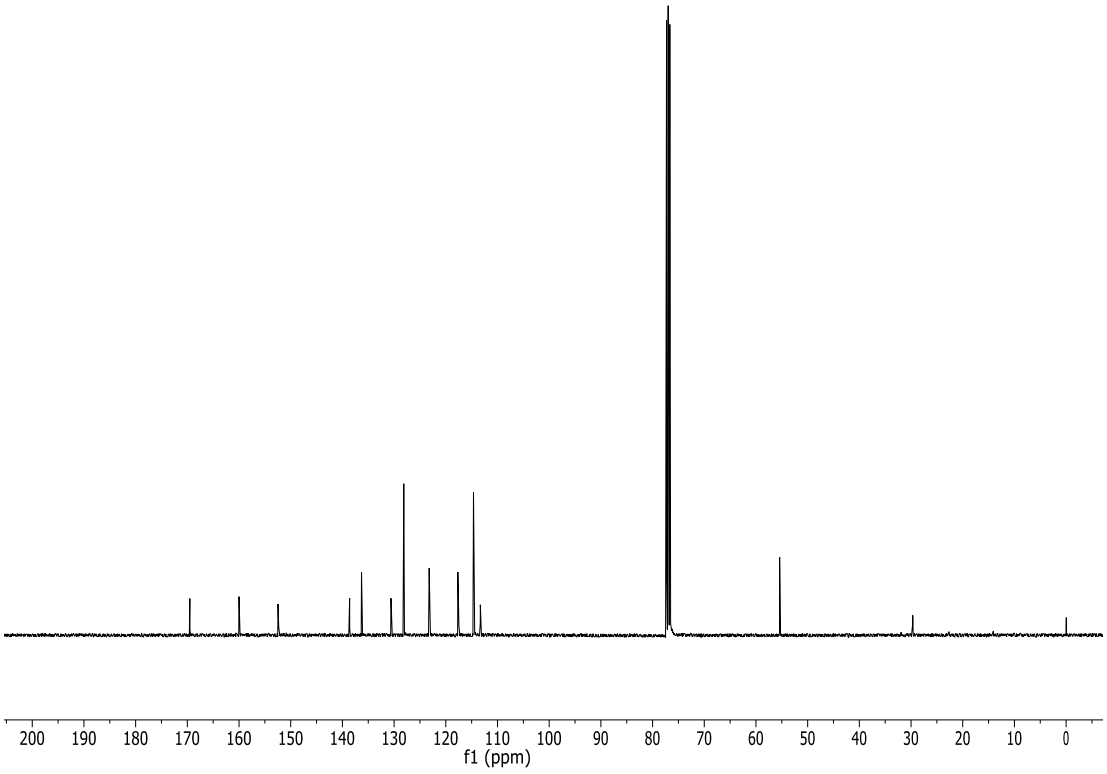


Figure 5. ^{1}H NMR spectrum of 4-bromo-6-(4-methoxyphenyl)-3-nitrocoumarin, **4**, in $CDCl_3$.

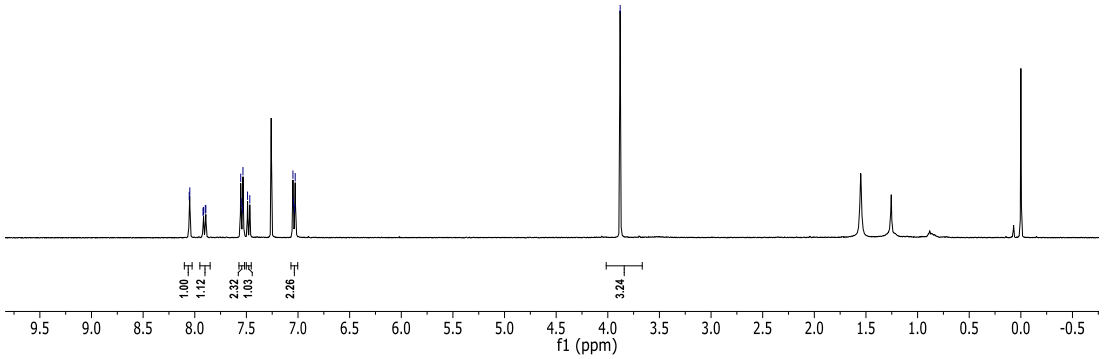
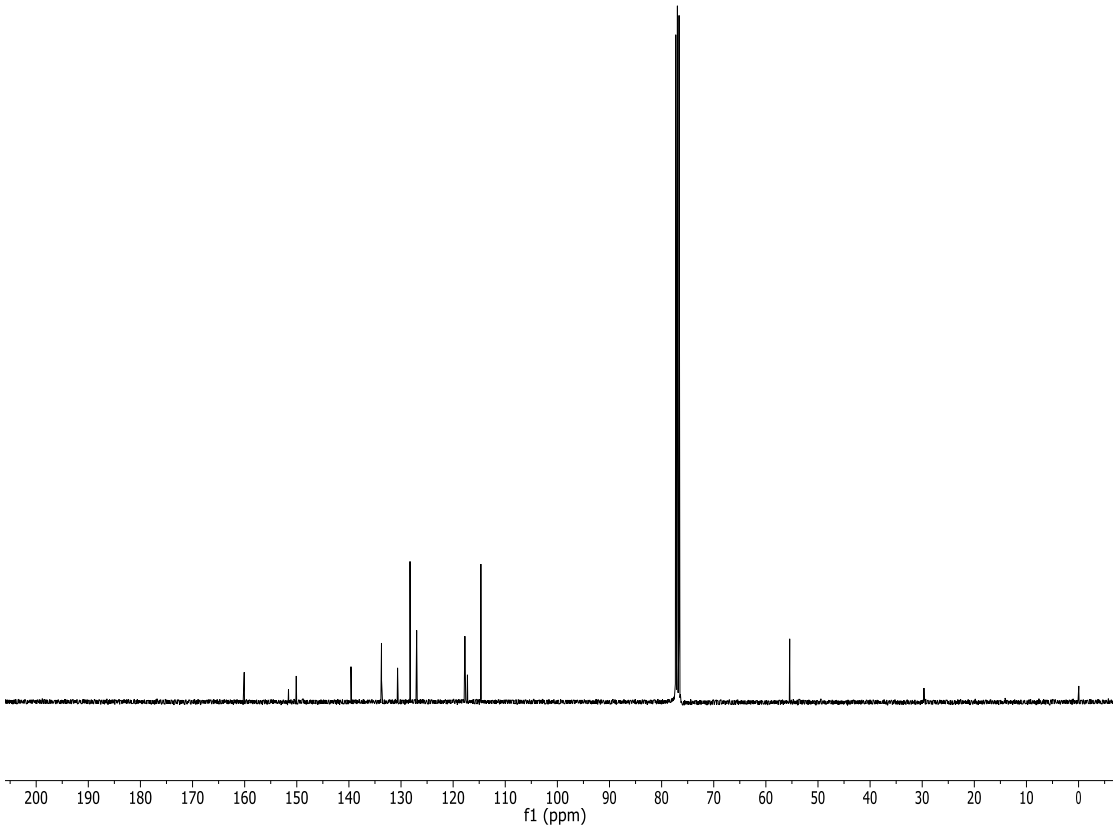


Figure 6. ^{13}C NMR spectrum of 4-bromo-6-(4-methoxyphenyl)-3-nitrocoumarin, **4**, in $CDCl_3$.

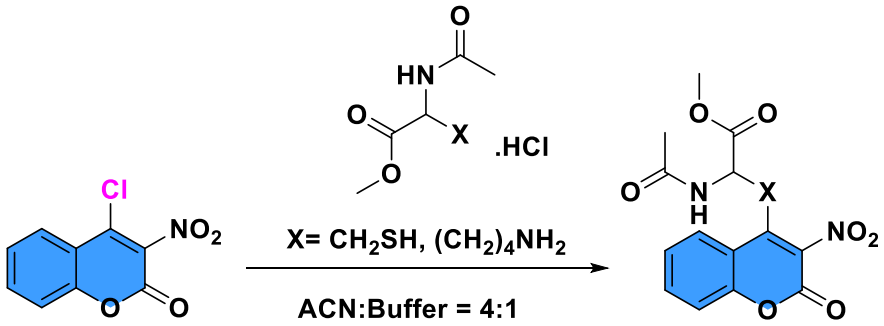


2. Development of selective thiol ligation

2.1. Initial NMR studies with protected amino acids

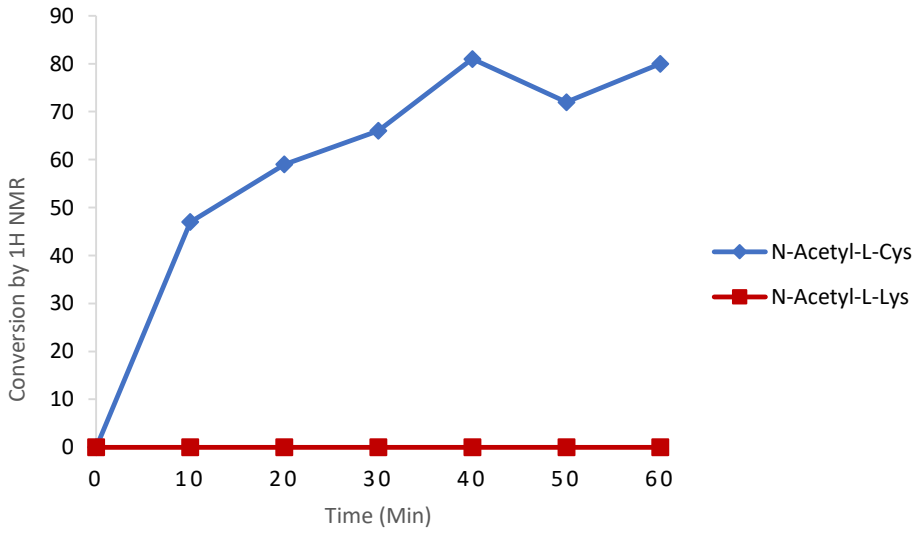
NMR analysis of the reaction between *N*-protected and *C*-protected amino acids with probe **1** at pH 5.0 buffered solutions.

A solution of *N*-acetyl-L-Cys methyl ester (10.0 mM in pH 5.0 citrate-phosphate buffer (100.0 mM, 200.0 µL) was diluted to a total volume of 0.8 mL using d_3-acetonitrile (600.0 µL). A solution of **1** (10.0 mM in d_3-acetonitrile, 200.0 µL) was added to the above mixture and allowed to stir. The reaction conversion was monitored using ^{1}H-NMR spectroscopy. The same procedure was repeated with *N*-acetyl-L-Lys methyl ester.



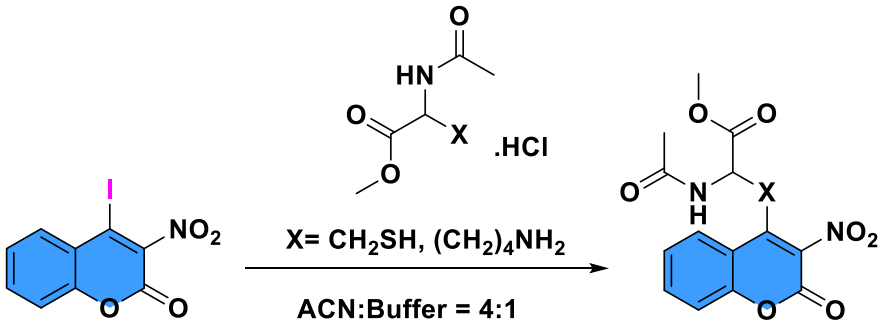
Scheme 1. Probe **1** reaction with *N*-protected and *C*-protected amino acids

Figure 7. Conversion versus time: Probe **1** reaction with *N*-protected and *C*-protected amino acids in pH 5.0 citrate-phosphate buffer



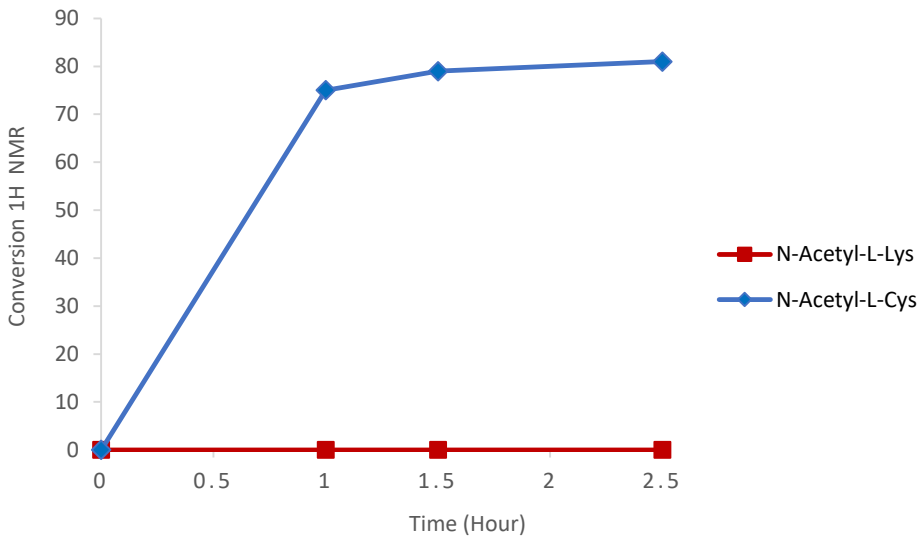
NMR analysis of the reaction between *N*-protected and *C*-protected amino acids with probe **2** under pH 5.0 buffered solutions

A solution of *N*-acetyl-L-Cys methyl ester (10.0 mM in pH 5.0 citrate-phosphate buffer (100.0 mM, 200.0 µL) was diluted to a total volume of 0.8 mL using d_3-acetonitrile (600.0 µL). A solution of **2** (10.0 mM in d_3-acetonitrile, 200.0 µL) was added to the mixture and allowed to stir. The reaction conversion was monitored using ^{1}H-NMR spectroscopy. The same procedure was repeated with *N*-acetyl-L-Lys methyl ester.

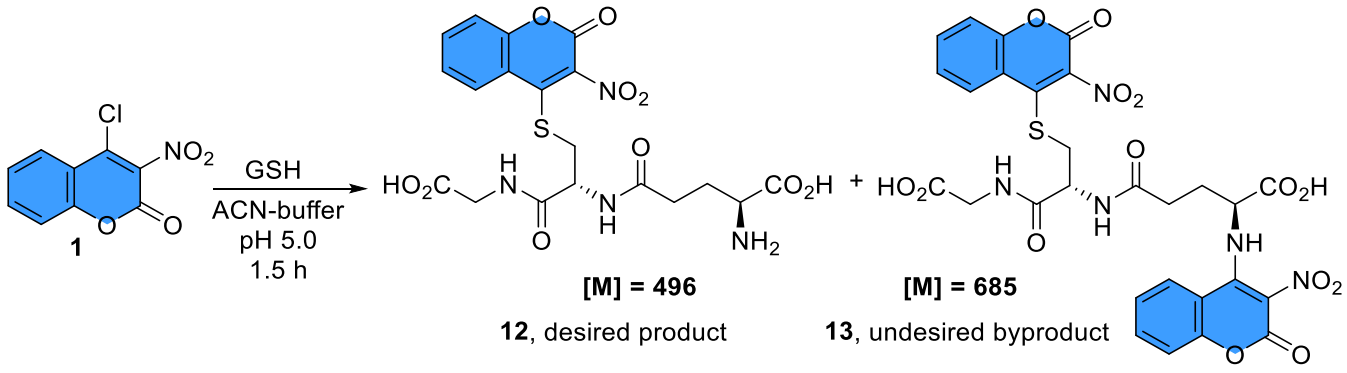


Scheme 2. Probe **2** reaction with *N*-protected and *C*-protected amino acids.

Figure 8. Conversion versus time: Probe **2** reaction with *N*-protected and *C*-protected amino acids in pH 5.0 citrate-phosphate buffer



2.2. GSH bioconjugation with 1



Scheme 3. Reaction of **1** with L-GSH.

To a solution of **1** (50.0 mM in acetonitrile, 200.0 µL) was added L-GSH (50.0 mM in pH 5.0 citrate phosphate buffer, 300.0 mM, 200.0 µL) and the mixture was diluted with 600.0 µL of acetonitrile. After 1.5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 10.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with 0.35 M, 0.40 M, 0.45 M and 0.50 M buffer solutions.

Representative ESI-MS spectra are shown below. The undesired formation of **13** decreased at higher buffer concentrations.

Figure 9. ESI-MS spectrum of the reaction mixture prepared with 0.3 M citrate buffer.

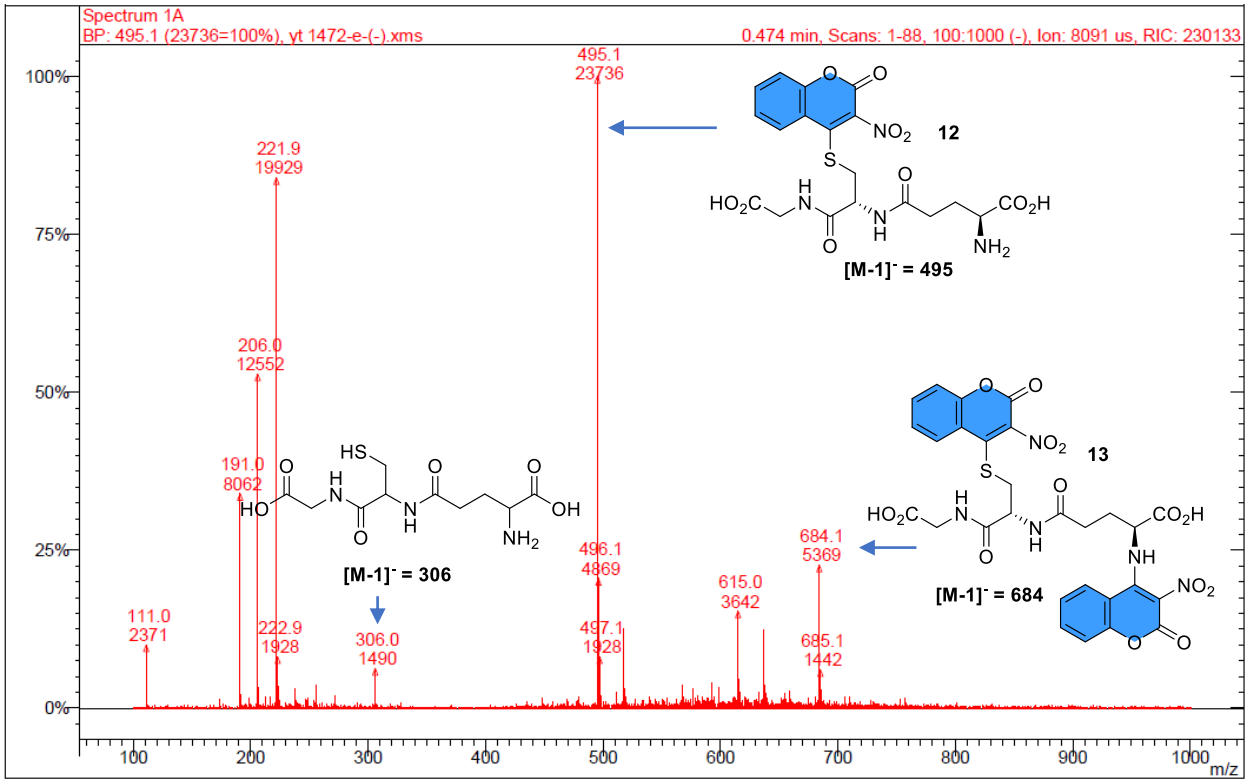


Figure 10. ESI-MS spectrum of the reaction mixture prepared with 0.5 M citrate buffer.

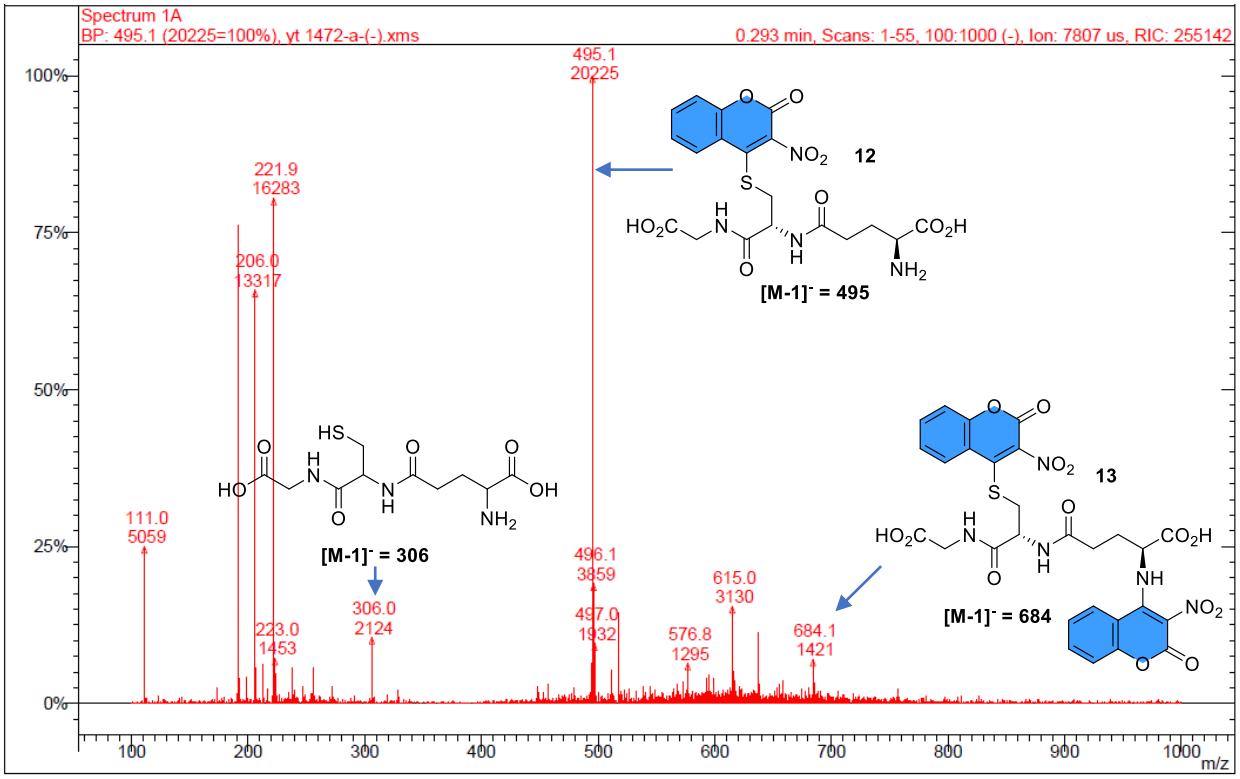
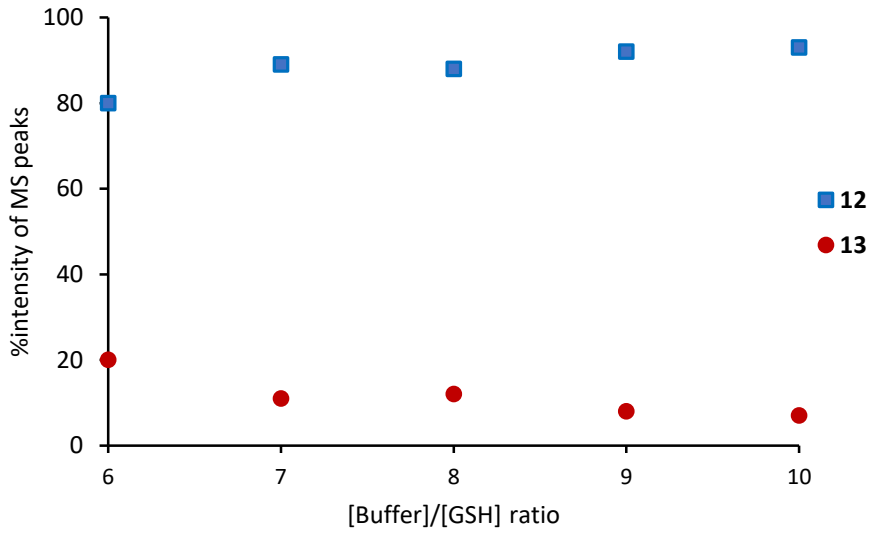
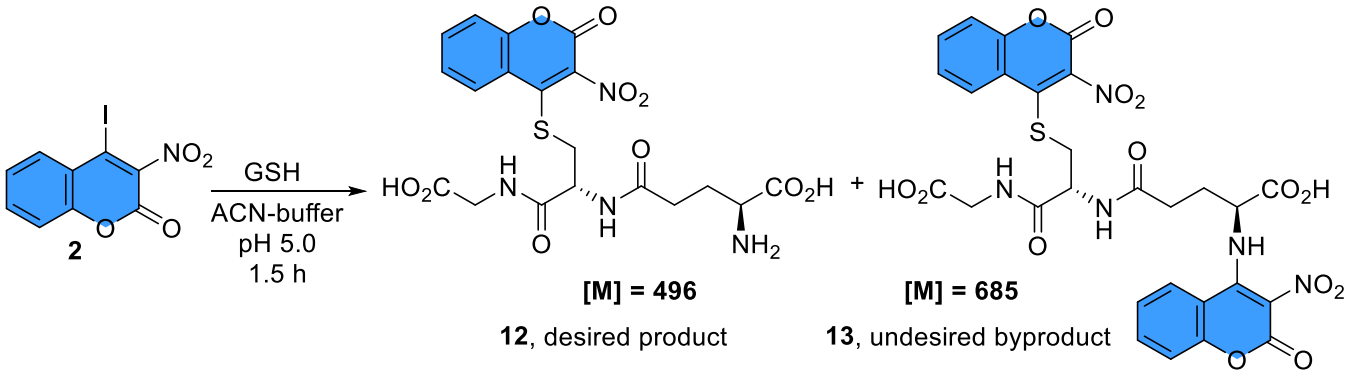


Figure 11. Relative %intensity of MS peaks corresponding to **12** and **13** obtained with different [Buffer]/[GSH] ratios.



2.3 GSH bioconjugation with 2

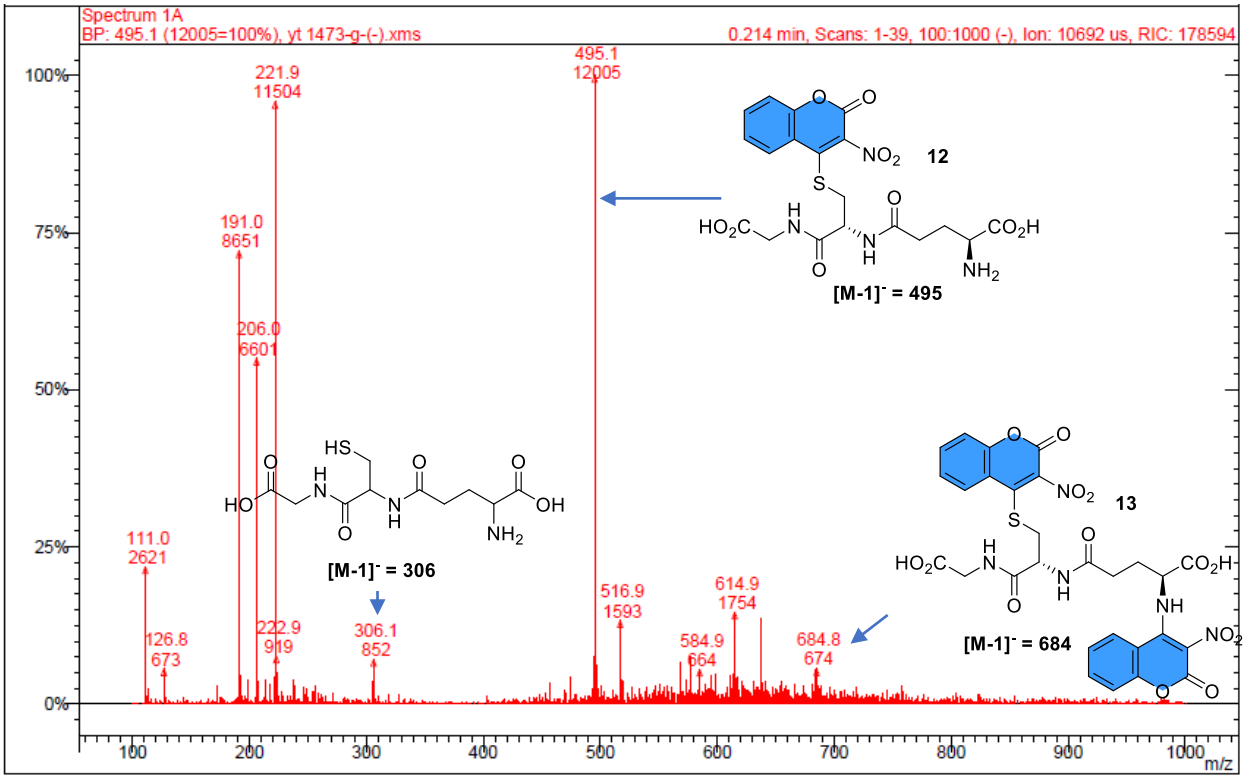


Scheme 4. Reaction of **2** with L-GSH.

To a solution of **2** (50.0 mM in acetonitrile, 200.0 µL) was added L-GSH (50.0 mM in pH 5.0 citrate phosphate buffer, 500.0 mM, 200.0 µL) and the mixture was diluted with 600.0 µL of acetonitrile. Under these reaction conditions the ratio of GSH:buffer was 1:10. After 1.5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 10.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Changing the bioconjugation agent from **1** to **2** did not completely stop formation of the undesired byproduct **13**.

Figure 12. ESI-MS spectrum of the reaction mixture with **2**.



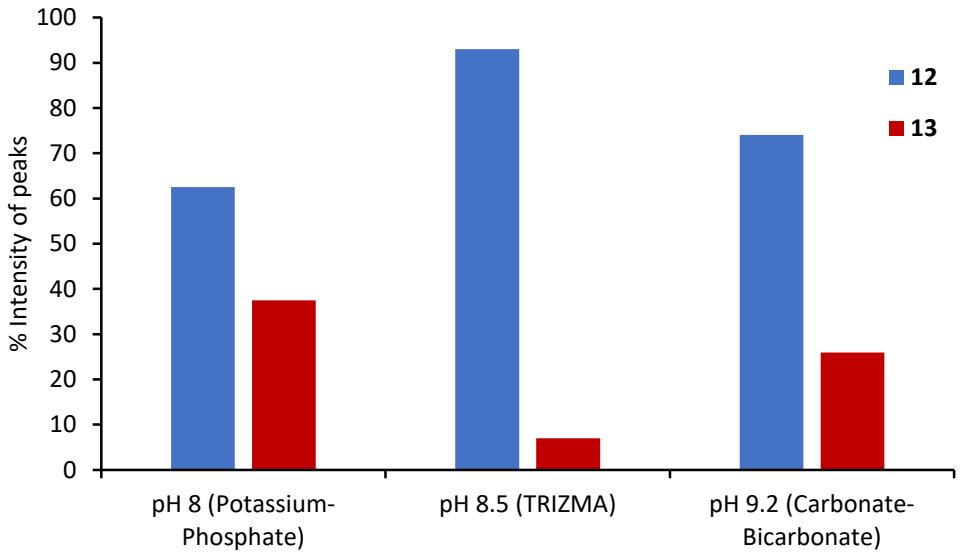
3. Optimization of buffer type and pH for selective bioconjugation

3.1 GSH ligation using 1 in different buffers

To a solution of **1** (50.0 mM in acetonitrile, 200.0 µL) was added GSH (50.0 mM in pH 8.0 potassium phosphate buffer, 500.0 mM, 200.0 µL) and the mixture was diluted with 600.0 µL of acetonitrile. After 1.5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 10.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with pH 8.5 TRIZMA buffer and pH 9.2 sodium carbonate-bicarbonate buffer.

In the presence of TRIZMA buffer, formation of the undesired byproduct **13** is largely reduced. At the same time, the peak corresponding to remaining GSH is no longer observed in the ESI-MS spectrum, which shows that the conversion is almost quantitative.

Figure 13. The relative %intensity of peaks corresponding to of **12** and **13** products formed in different buffers.



3.2 GSH conjugation with 1 in different pH TRIZMA buffers

To a solution of **1** (50.0 mM in acetonitrile, 200.0 µL) was added L-GSH (50.0 mM in pH 8.0 TRIZMA buffer, 500.0 mM, 200.0 µL) and the mixture was diluted with 600.0 µL of acetonitrile. After 1.5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 10.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with pH 9.0 TRIZMA buffer.

Representative ESI-MS spectra are shown below. In the presence of pH 8.5 and 9.0 TRIZMA buffers, the conversion of GSH is quantitative. At pH 9.0 the formation of the undesired byproduct **13** is less than 2%.

Figure 14. The relative %intensity of peaks corresponding to **12** and **13** formed in reaction mixtures prepared in different pH TRIZMA buffers.

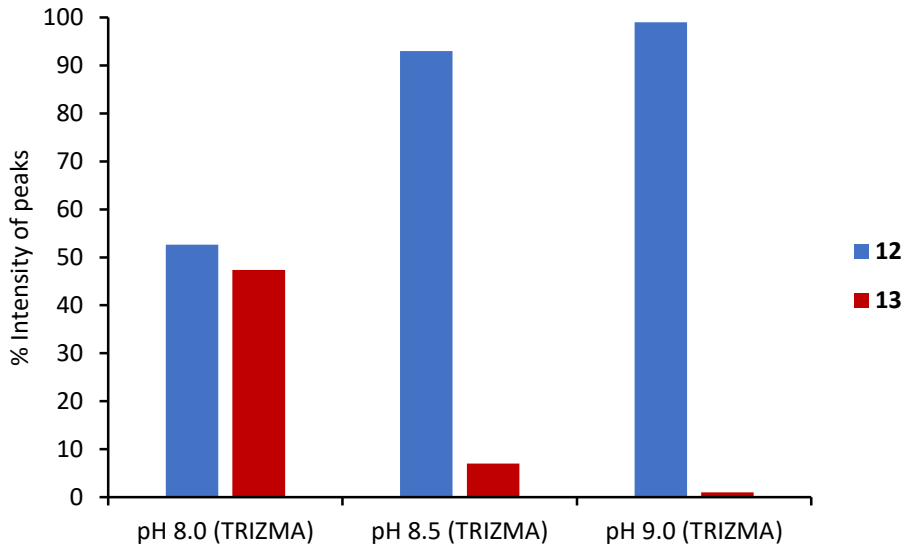


Figure 15. ESI-MS spectrum of the reaction mixture of GSH and **1** in pH 8.5 TRIZMA buffer.

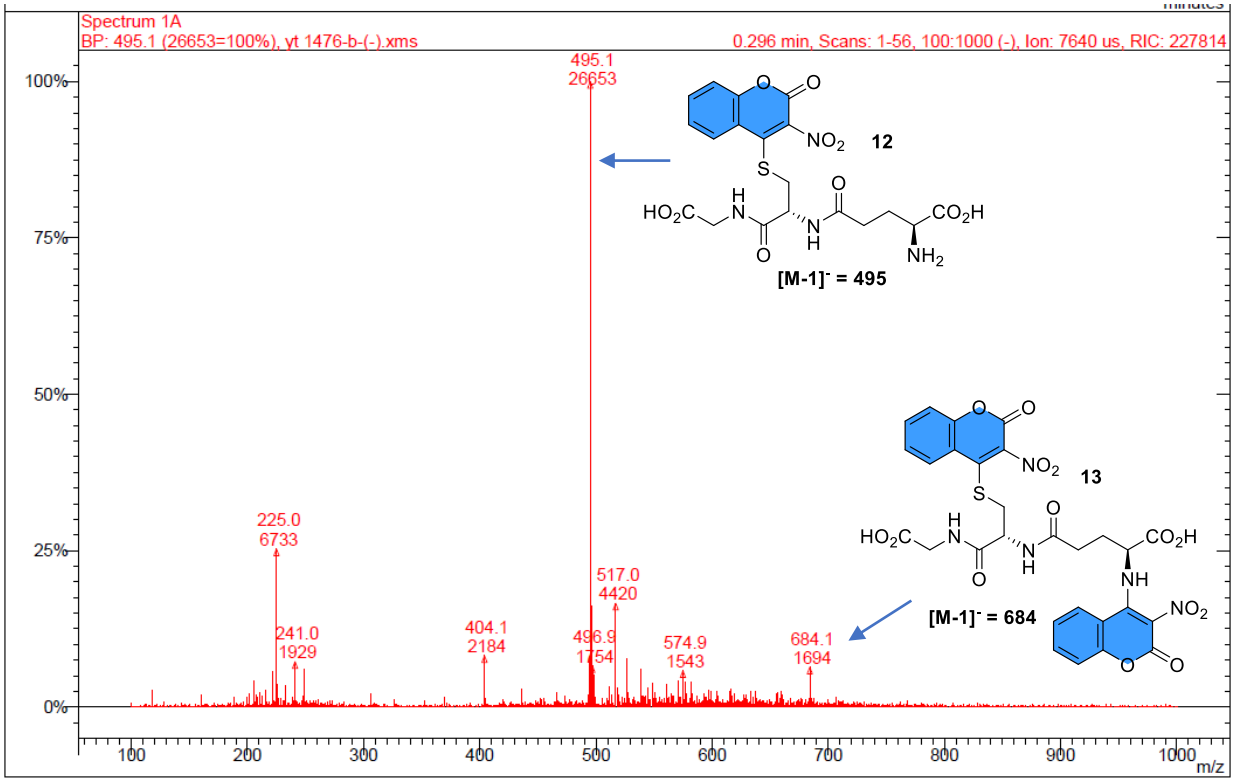
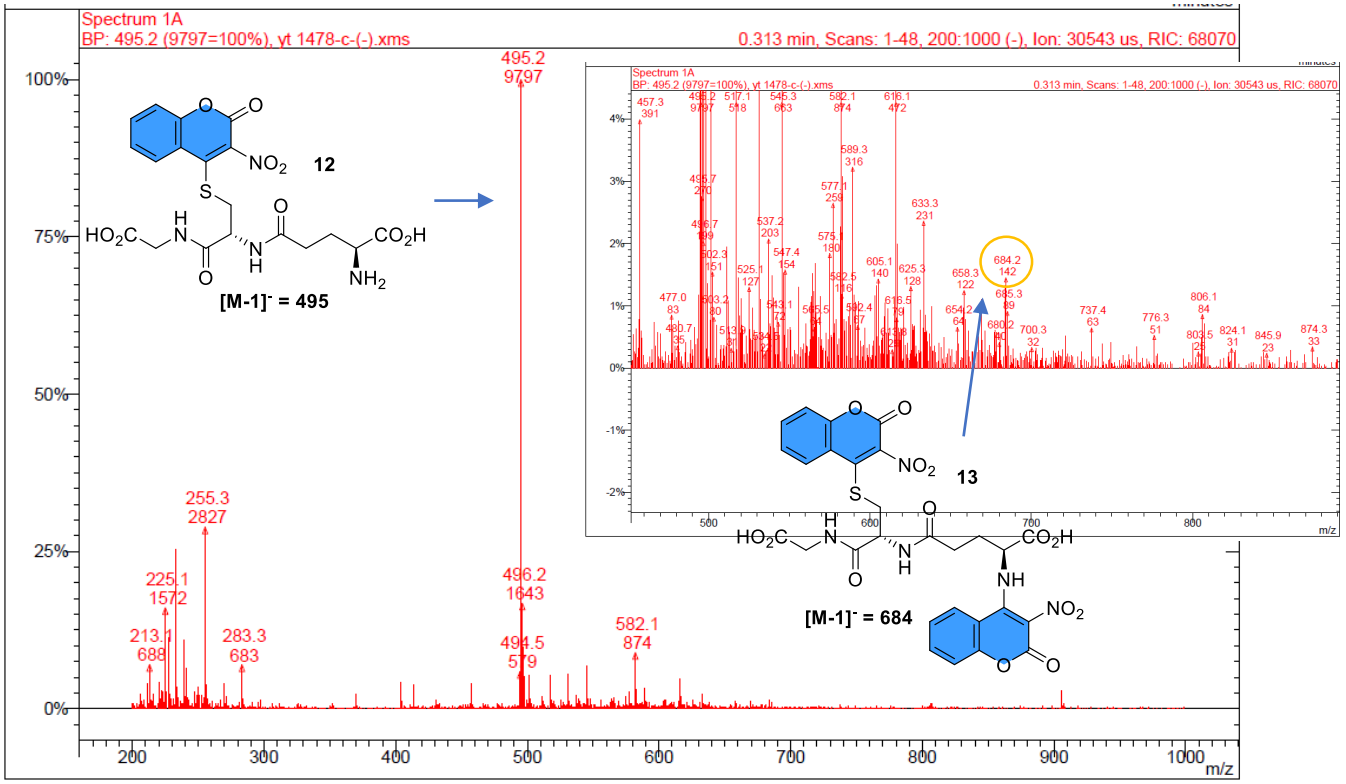
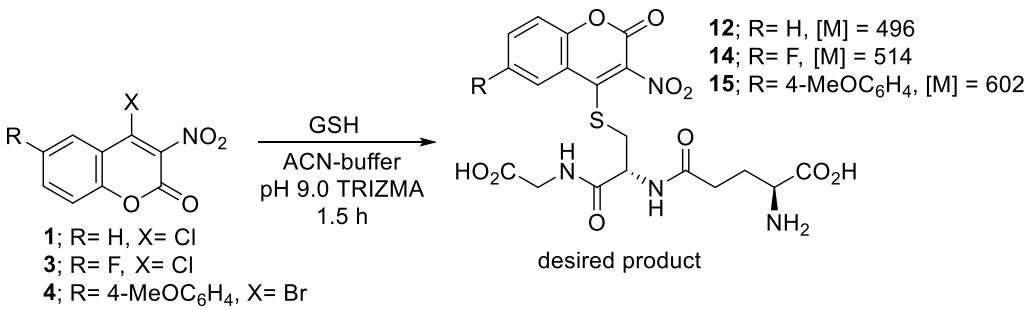


Figure 16. ESI-MS spectrum of the reaction mixture of GSH and **1** in pH 9.0 TRIZMA buffer.



4. Selective GSH ligation with different agents



Scheme 5. Reaction of **1**, **3** and **4** with L-GSH.

To a solution of **4** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). A 4.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with the bioconjugation agents **3** and **1**. Exclusive monoconjugation of GSH can be achieved by bioconjugation agents **1**, **3** and **4** at pH 9.0 TRIZMA buffer.

Figure 17. ESI-MS spectrum of the reaction mixture of GSH and **4** in pH 9.0 TRIZMA buffer.

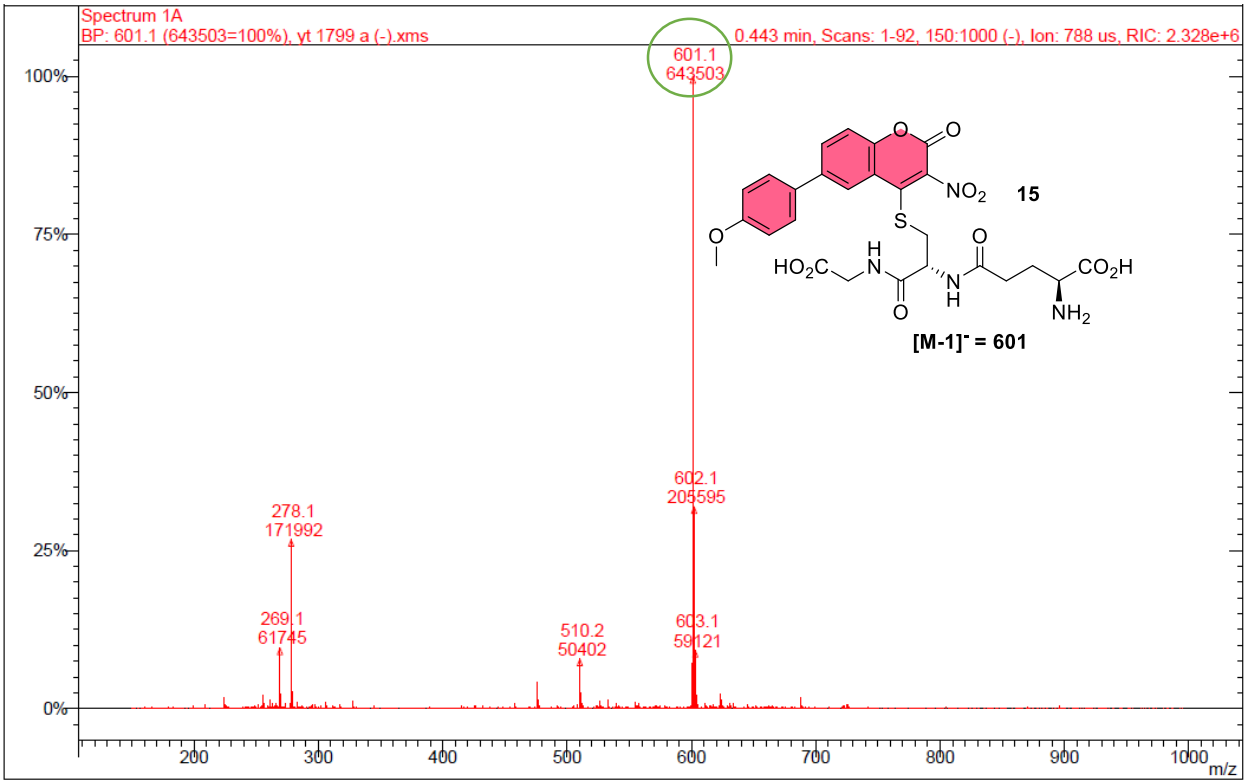


Figure 18. ESI-MS spectrum of the reaction mixture of GSH and **3** in pH 9.0 TRIZMA buffer.

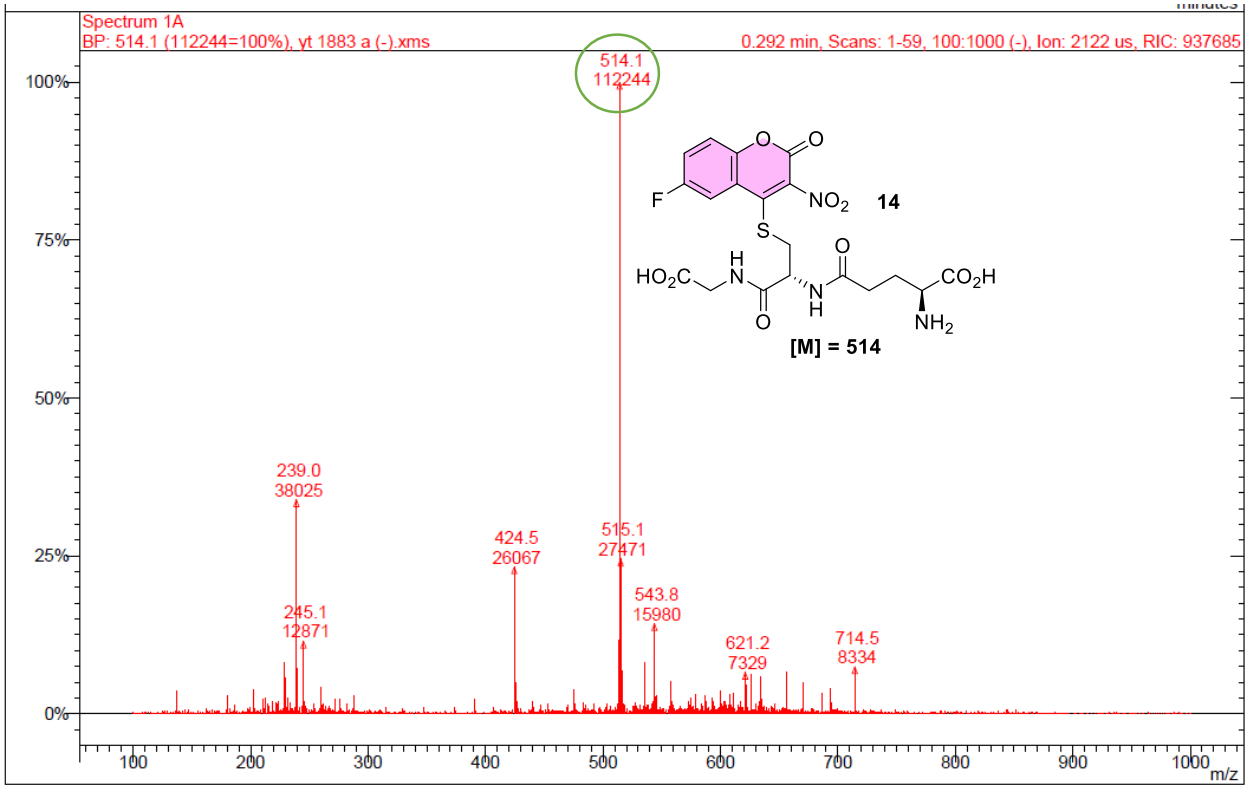
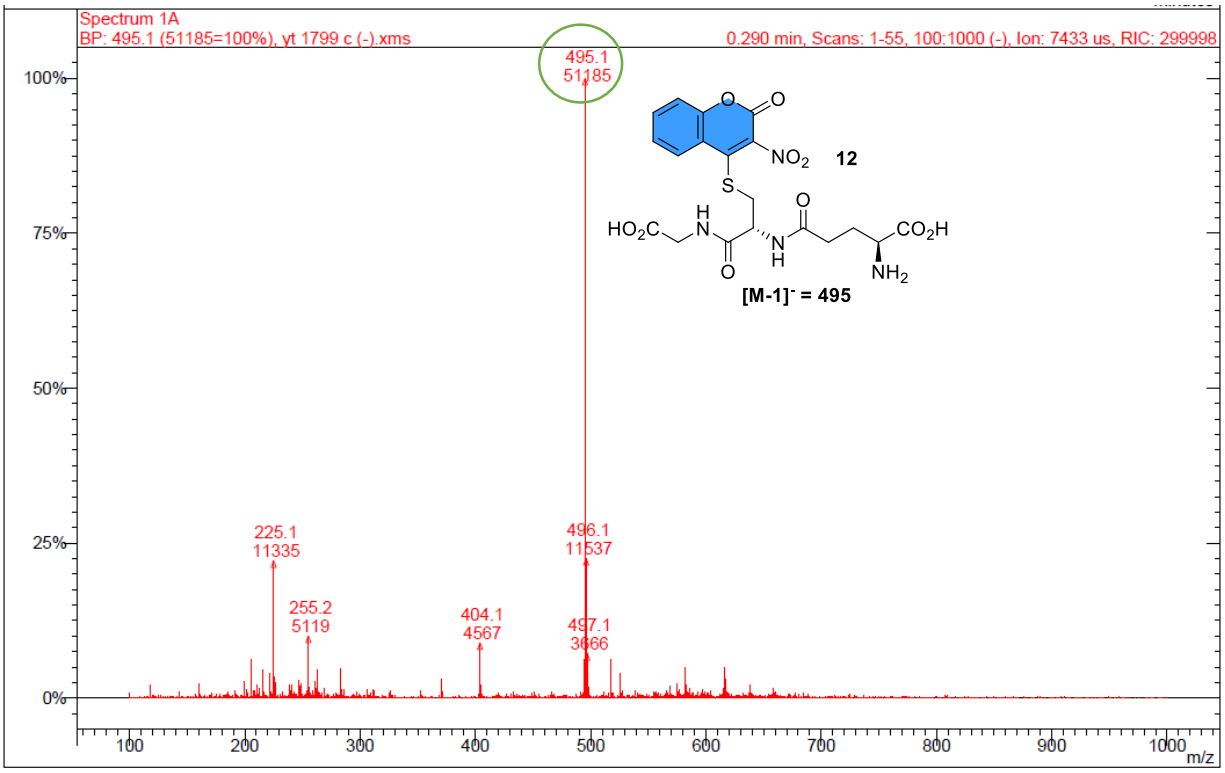
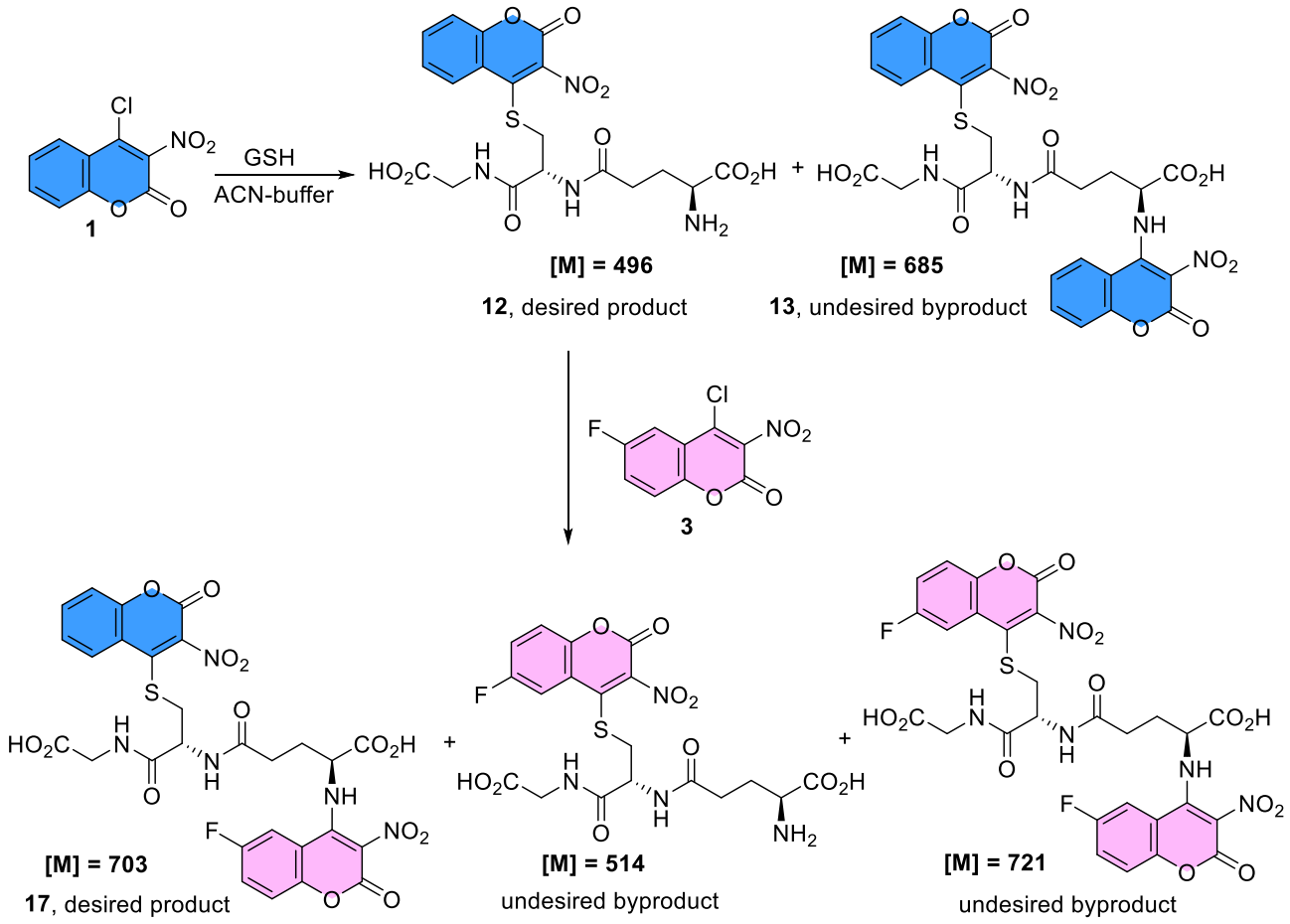


Figure 19. ESI-MS spectrum of the reaction mixture of GSH and **1** in pH 9.0 TRIZMA buffer (control).



5. Selective bioconjugation of thiol and amino groups in GSH using two different coumarin agents



Scheme 6. Selective bioconjugation of GSH with two coumarin agents.

5.1. Optimization of buffer pH

To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 8.5 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 30 minutes, bioconjugation agent **3** (25.0 mM in acetonitrile, 400.0 µL) was added. After 1.5 hours, the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis in negative ion mode. The above procedure was repeated with pH 9.0 TRIZMA buffer.

The conversion of **12** to **17** was higher in pH 8.5 TRIZMA buffer than at pH 9.0.

Figure 20. ESI-MS spectrum of the reaction mixture at pH 8.5 TRIZMA buffer.

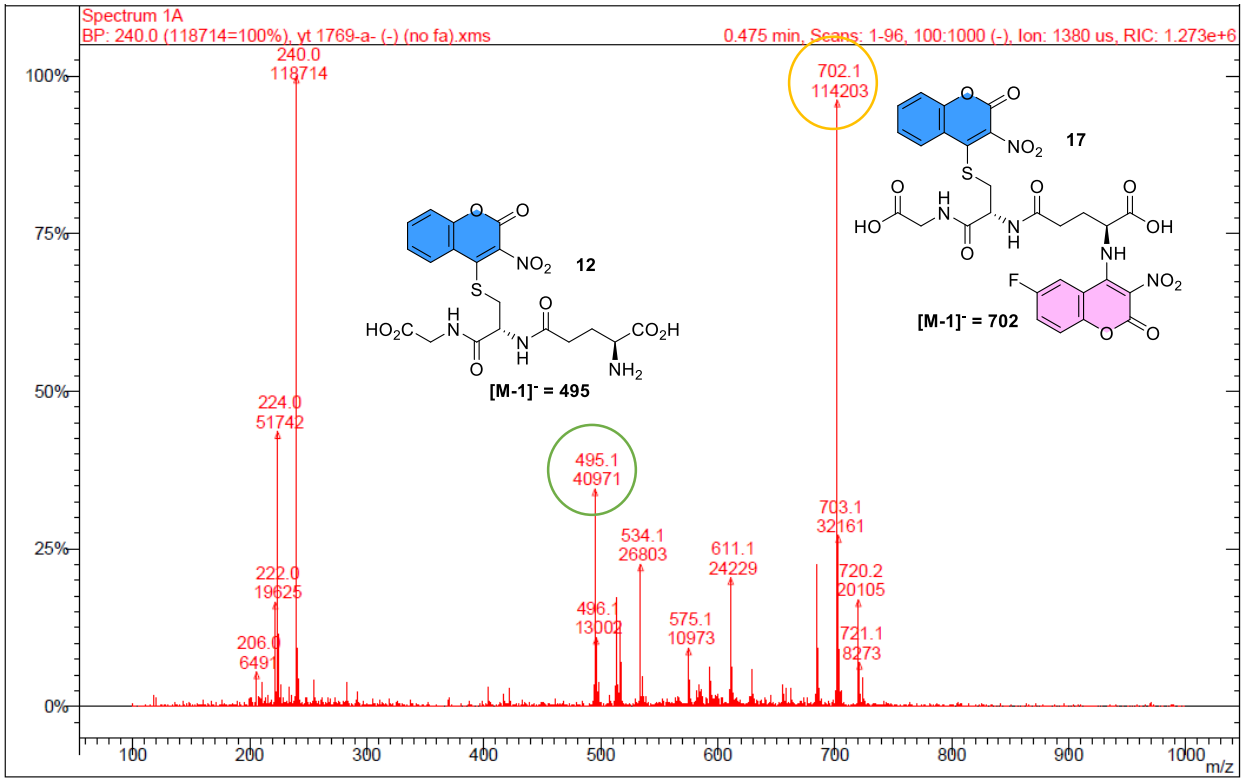
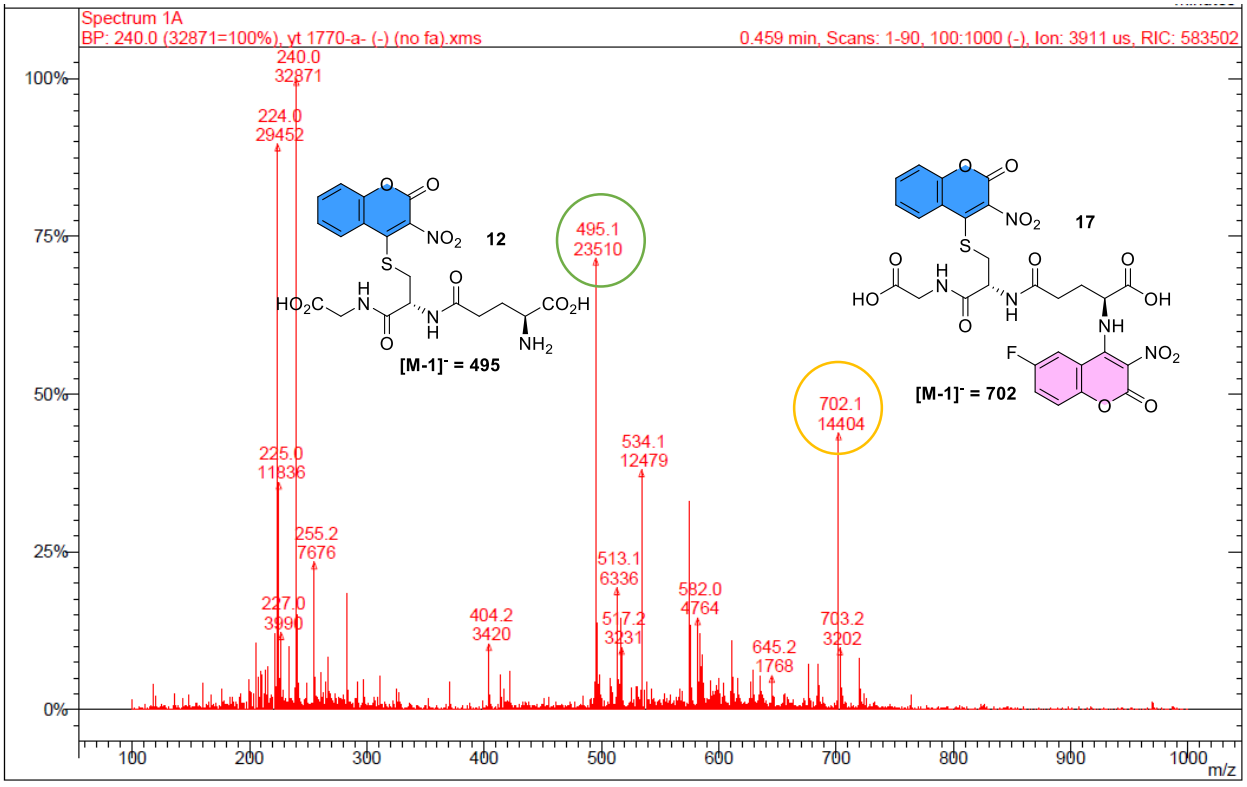


Figure 21. ESI-MS spectrum of the reaction mixture at pH 9.0 TRIZMA buffer.

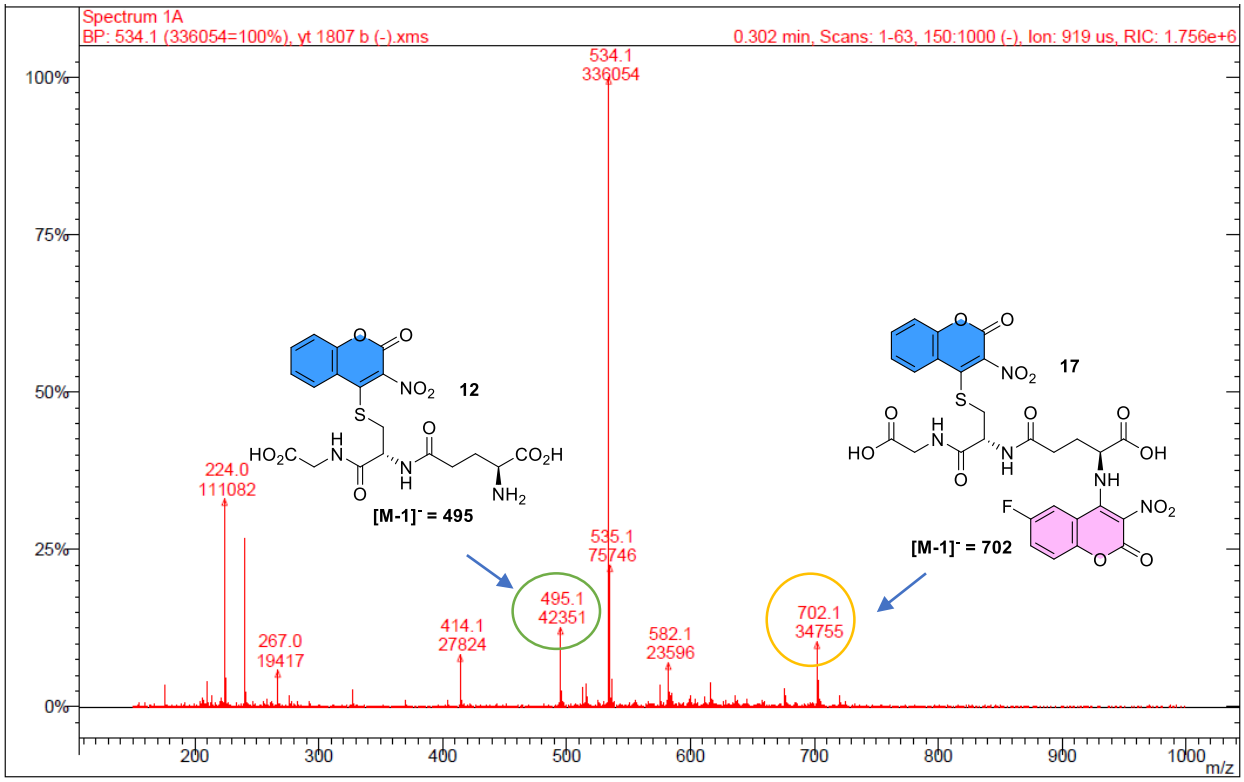


5.2. Increasing the equivalents of 3 to favor quantitative conversion of 12 to 17

To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, bioconjugation agent **3** (0.025 M in acetonitrile, 800.0 µL) was added. After stirring for 1.5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis in negative ion mode.

The conversion from **12** to **17** was low under this condition.

Figure 22. ESI-MS spectrum of the reaction mixture of [**1**+GSH] with two equivalents of **3** at pH 9.0 TRIZMA buffer.

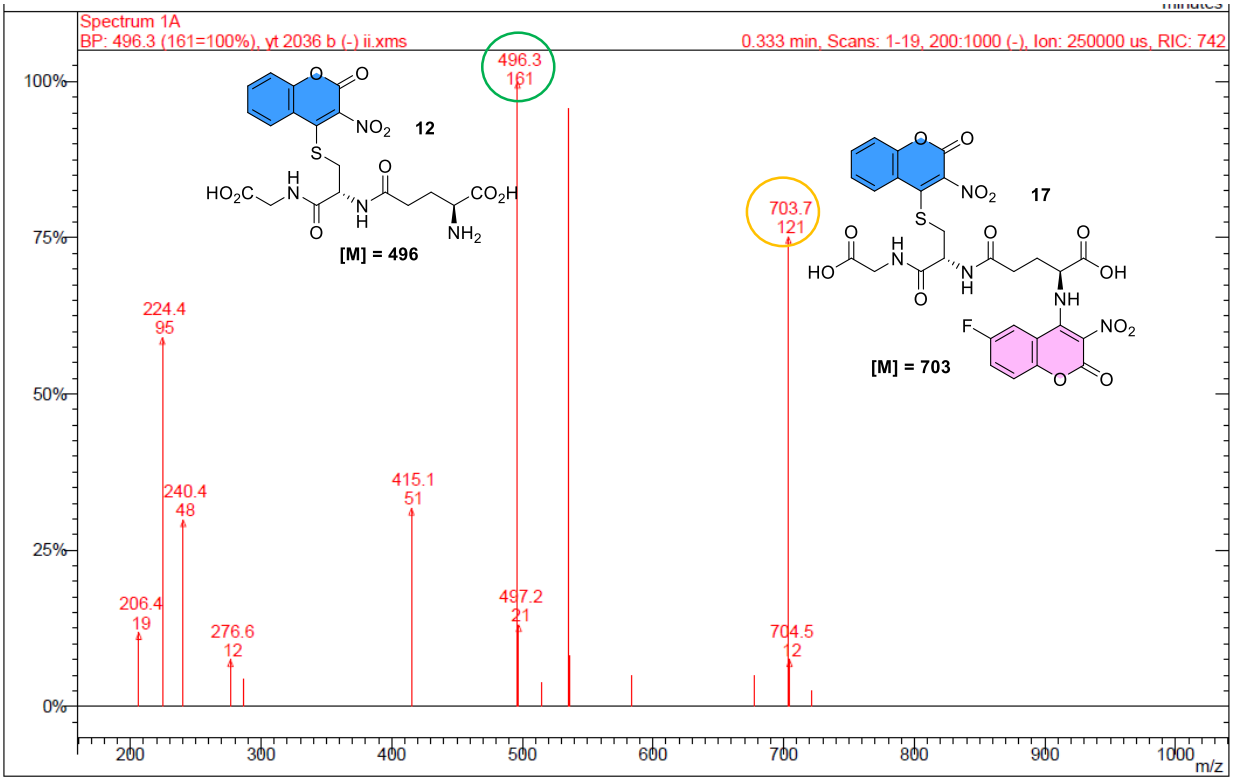


5.3. Increasing the time for the second bioconjugation to favor quantitative conversion of 12 to 17

To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, bioconjugation agent **3** (25.0 mM in acetonitrile, 800.0 µL) was added. After stirring 5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

The conversion from **12** to **17** improved but was not complete under this condition.

Figure 23. ESI-MS spectrum of the reaction mixture of [**1**+GSH] at pH 9.0 TRIZMA buffer with one equivalent of **3**, after 5 hours.

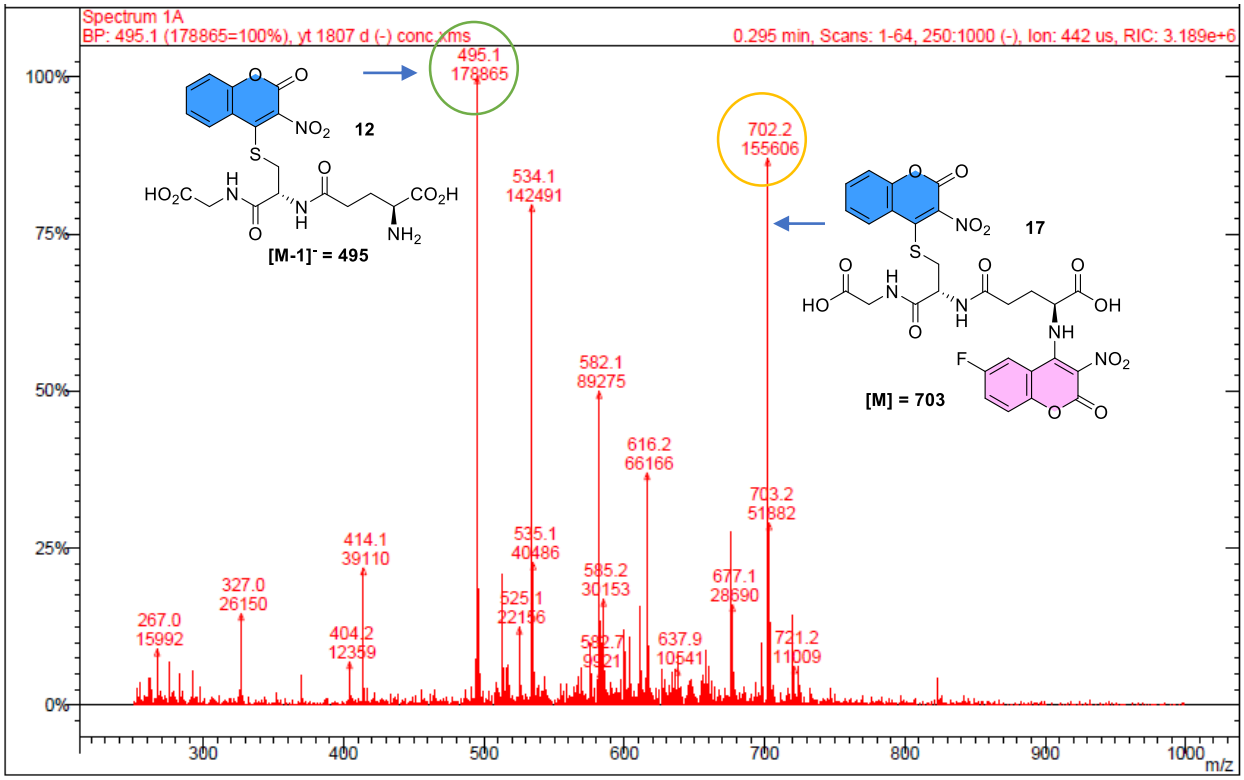


5.4. Second bioconjugation in the presence of additional base

NaOH

To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 2.5 equivalents of NaOH were added (5.0 µL, 5.0 M) and stirred for 5 minutes. Next, the bioconjugation agent **3** (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring for 2 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

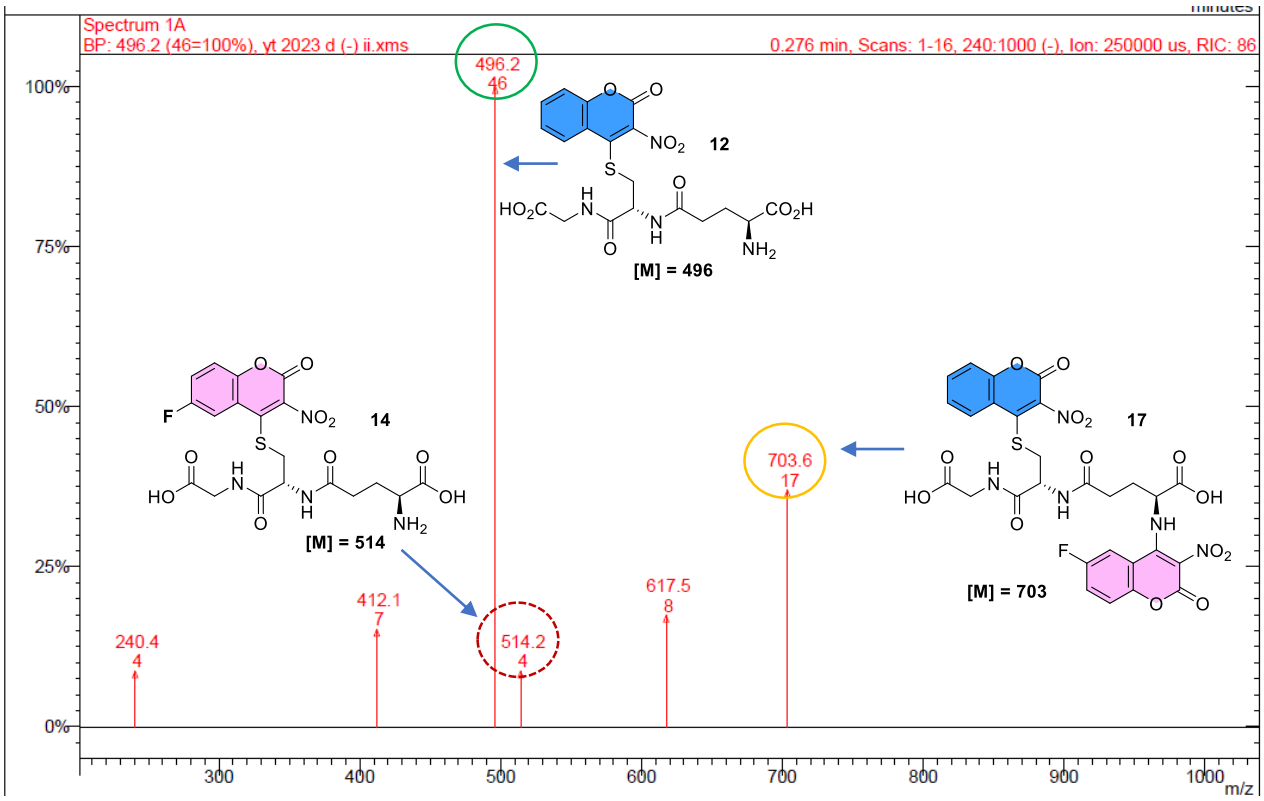
Figure 24. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with one equivalent of **3** followed by addition of 2.5 equivalents of NaOH, after 2 hours.



$\underline{K_2CO_3}$

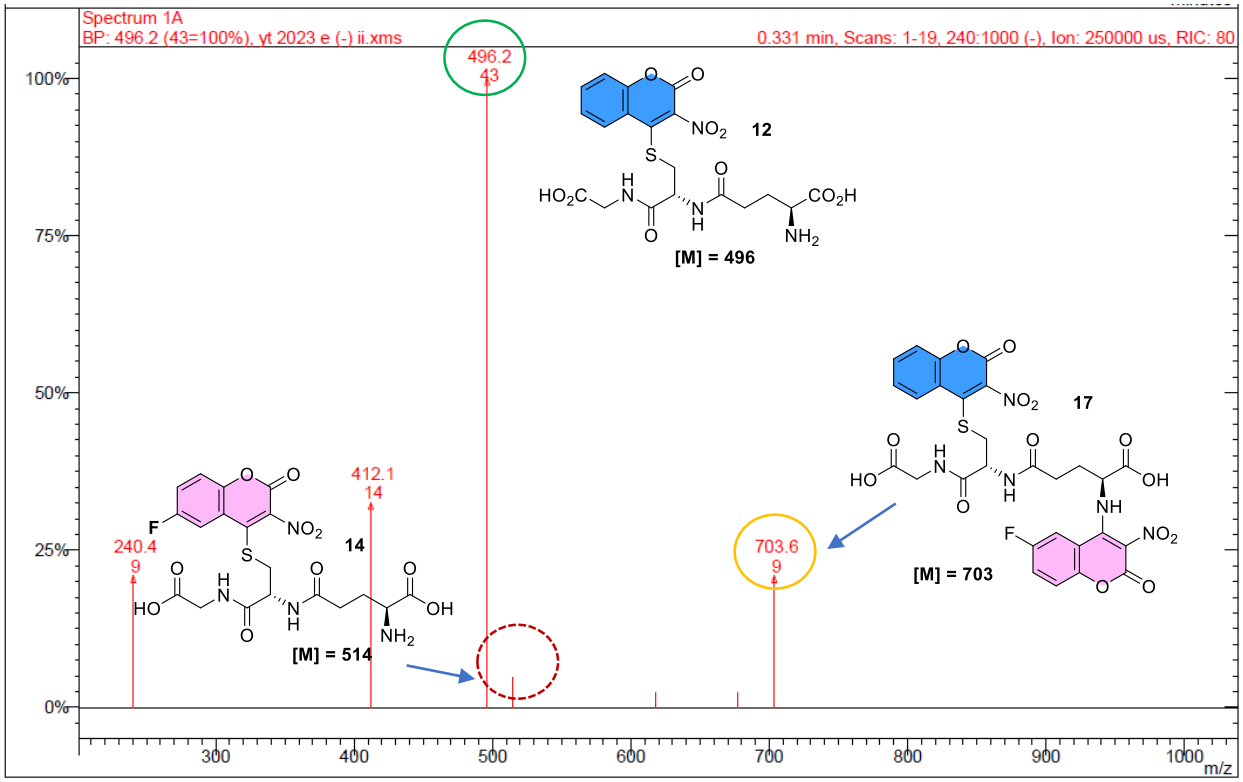
To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 10 equivalents of K_2CO_3 were added (100.0 µL, 1.0 M) and stirred for 5 minutes. Next, the bioconjugation agent **3** (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring for 2 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 25. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with one equivalent of **3** followed by addition of 10 equivalents of K_2CO_3, after 2 hours.



To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 20 equivalents of K_2CO_3 were added (200.0 µL, 1.0 M) and stirred for 5 minutes. Next, the bioconjugation agent **3** (250.0 mM in acetonitrile, 400.0 µL) was added. After stirring for 2 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 26. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with one equivalent of **3** followed by addition of 20 equivalents of K_2CO_3, after 2 hours.



To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 5 equivalents of K_2CO_3 were added (50.0 µL, 1.0 M) and stirred for 5 minutes. Next, the bioconjugation agent **3** (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring for 5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 27. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with one equivalent of **3** followed by addition of 5 equivalents of K_2CO_3, after 5 hours.

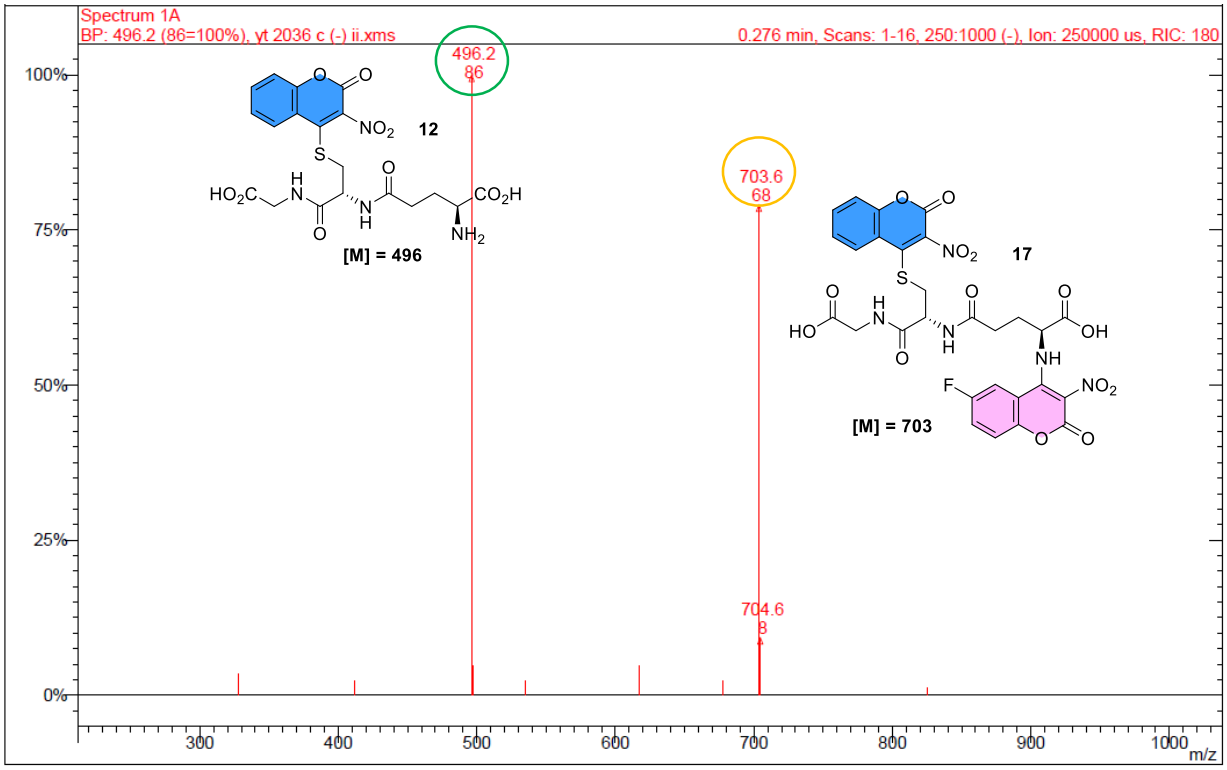
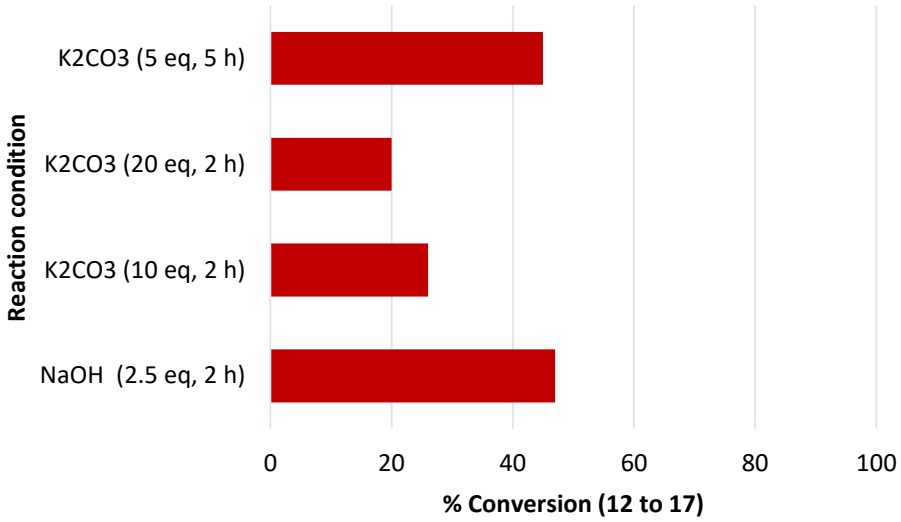


Figure 28: Conversion from **12** to **17** with different bases, equivalents and reaction times.



Adding base (NaOH and K_2CO_3) did not improve the conversion from **12** to **17**. In addition, formation of the undesired product **14** was observed with 10-20 equivalents of K_2CO_3.

5.5. Increasing time and equivalents of the probe 3 to favor conversion of 12 to 17

To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of bioconjugation agent **3** (25.0 mM in acetonitrile, 400.0 µL) was added. After 12 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with 2 and 3 equivalents of bioconjugation agent **3**.

The conversion from **12** to **17** was complete with two equivalents of bioconjugation agent **3**, after 12 hours. The peak at 535.5 is from the reaction of two equivalents of the bioconjugation agent with TRIZMA.

Figure 29. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with one equivalent of **3** after 12 hours.

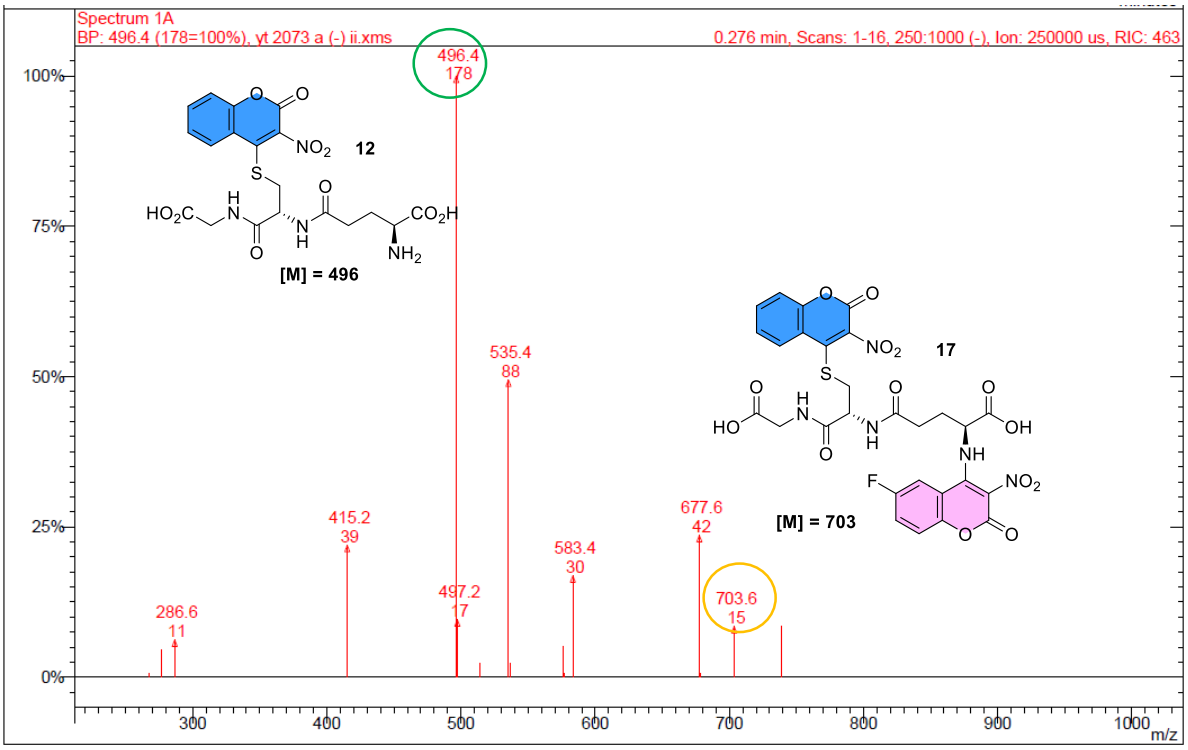


Figure 30. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with two equivalents of **3** after 12 hours.

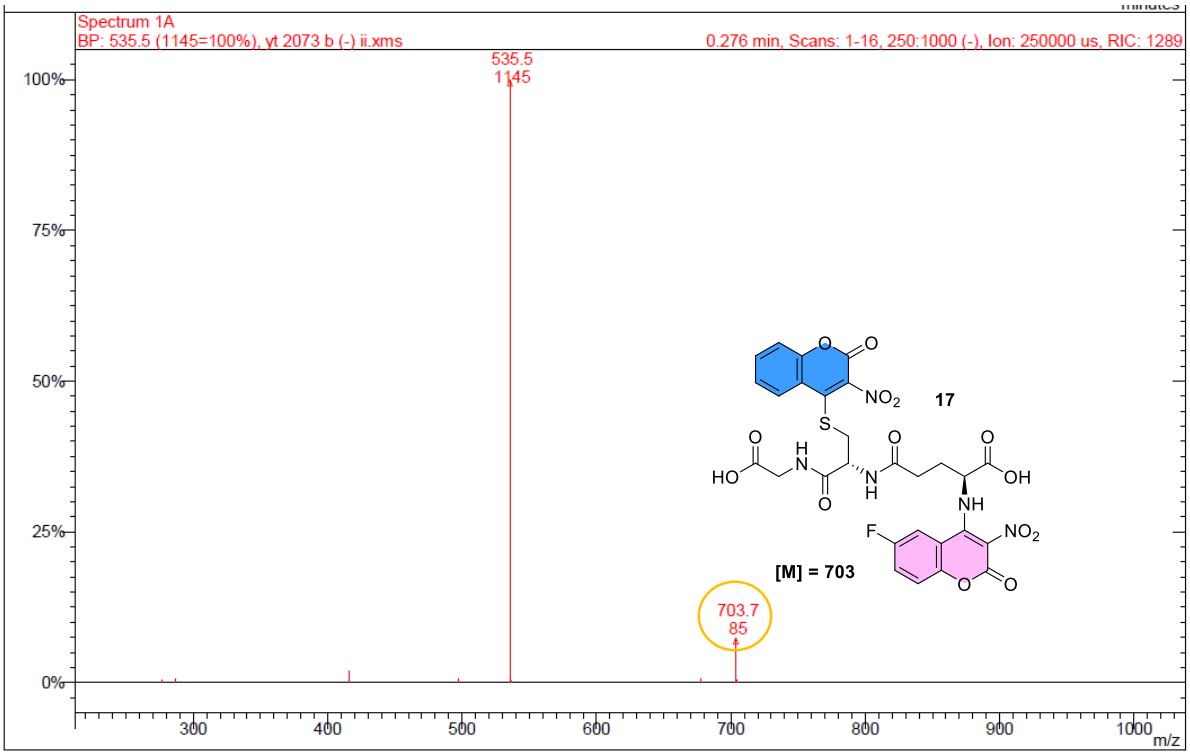
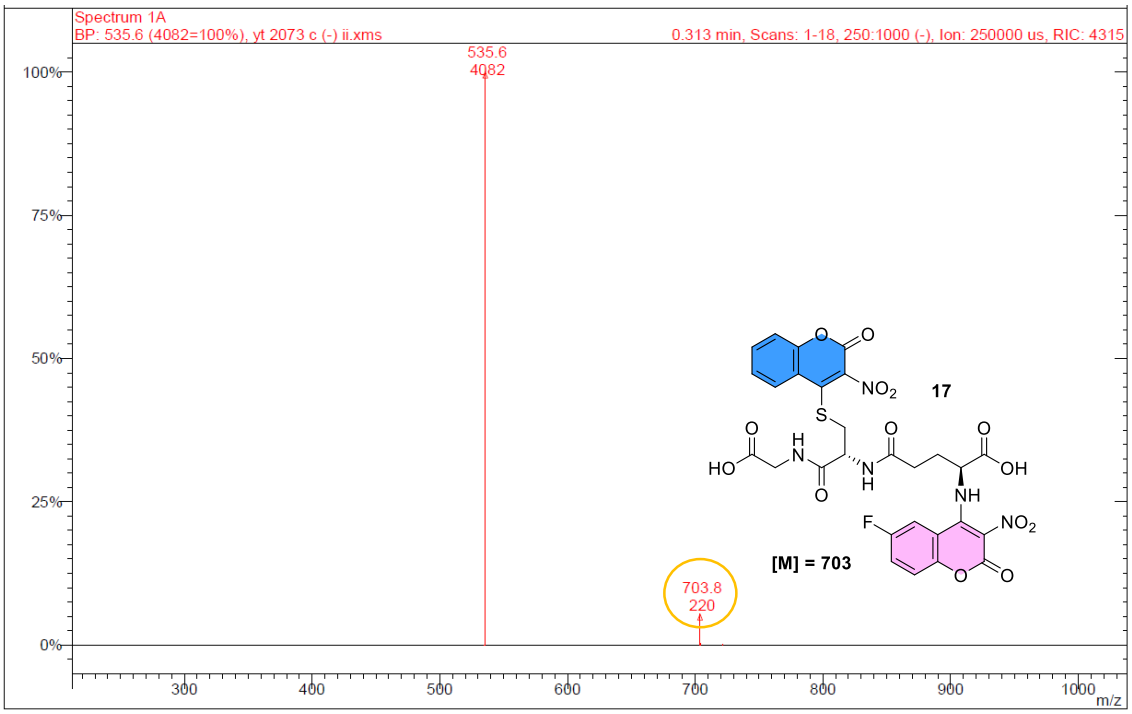


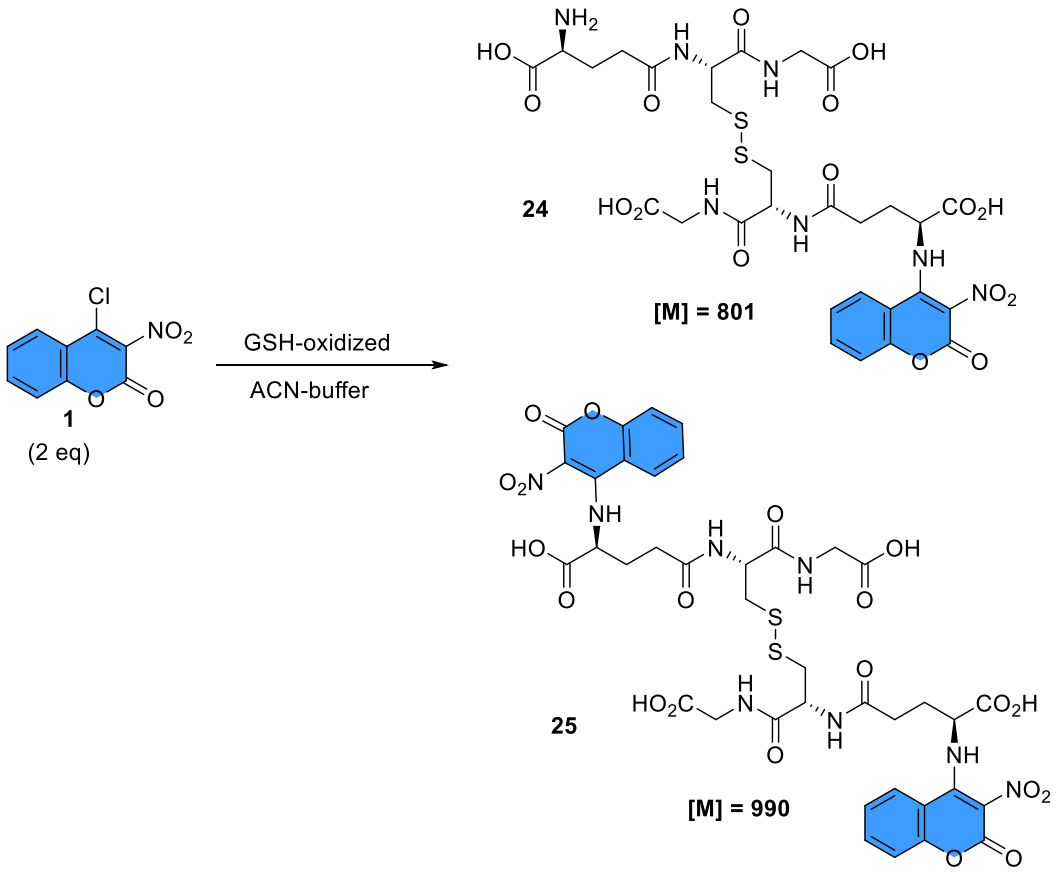
Figure 31. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with three equivalents of **3** after 12 hours.



6. Mechanistic study

6.1. Evidence for thiol ligation

In order to confirm the monoconjugation takes place at the thiol functional group of GSH, bioconjugation agent **1** was reacted with GSH-oxidized (GSSG).



Scheme 7. Reaction of **1** with GSSG to confirm thiol ligation.

To a solution of **1** (25.0 mM in acetonitrile, 800.0 µL) was added GSSG (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 0.8 mL of acetonitrile. After 2 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL using deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with pH 8.5 TRIZMA buffer. Neither the monoconjugated product **24** nor byproduct **25** was observed. This confirms that under these conditions, the first conjugation occurs at the free thiol in GSH.

Figure 32. ESI-MS spectrum of GSSG in the negative ion mode (control).

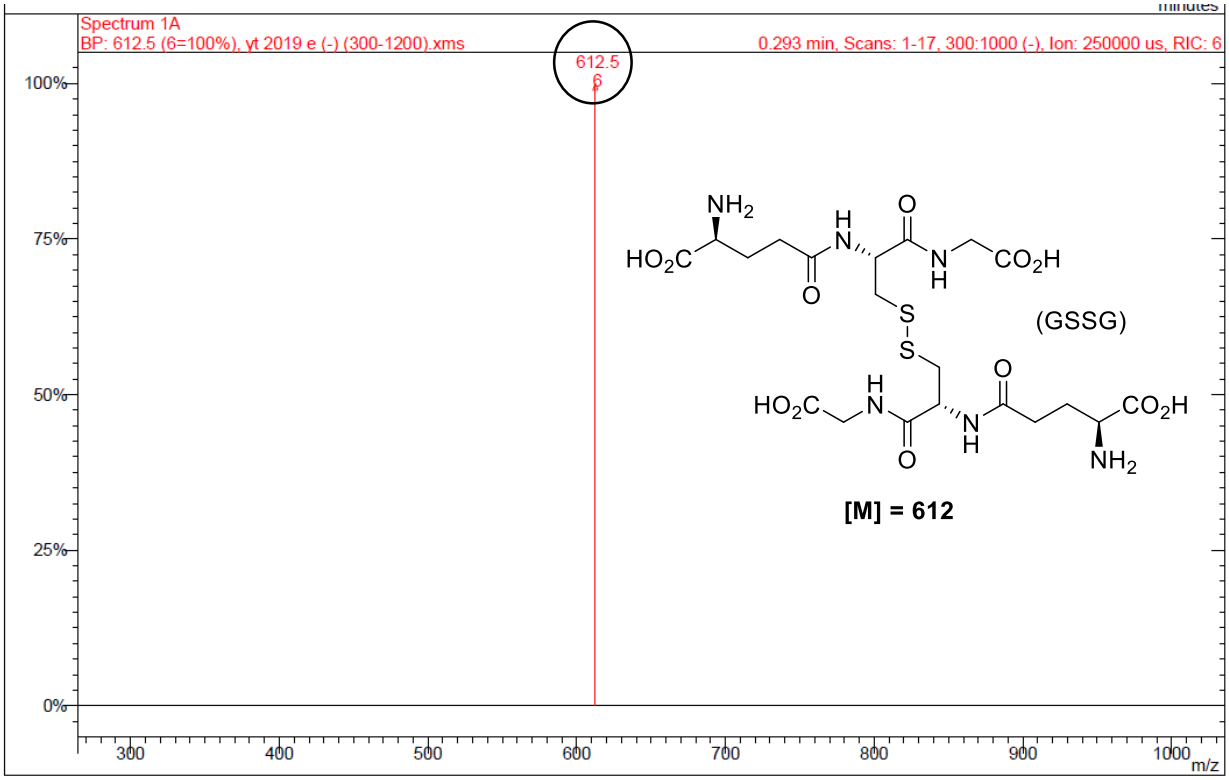


Figure 33. ESI-MS spectrum of the reaction mixture of [**1** + GSSG] in pH 8.5 TRIZMA buffer.

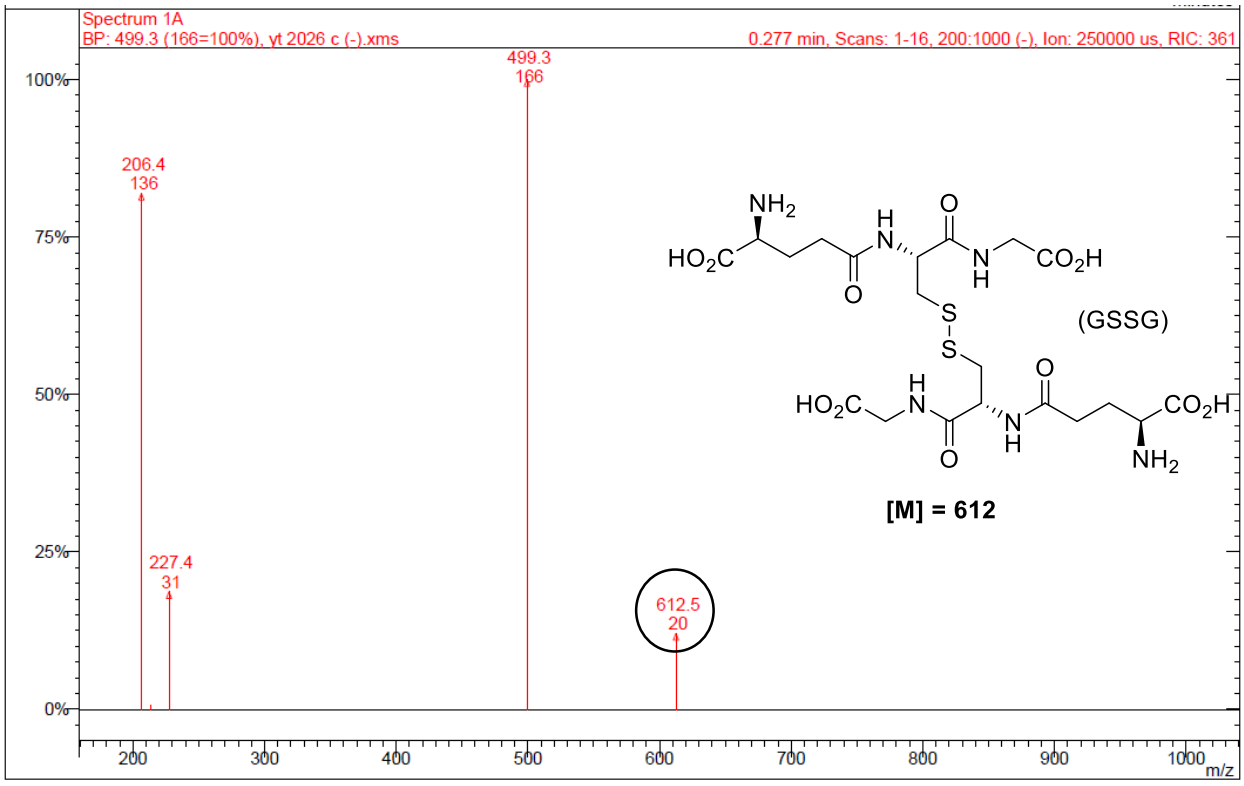
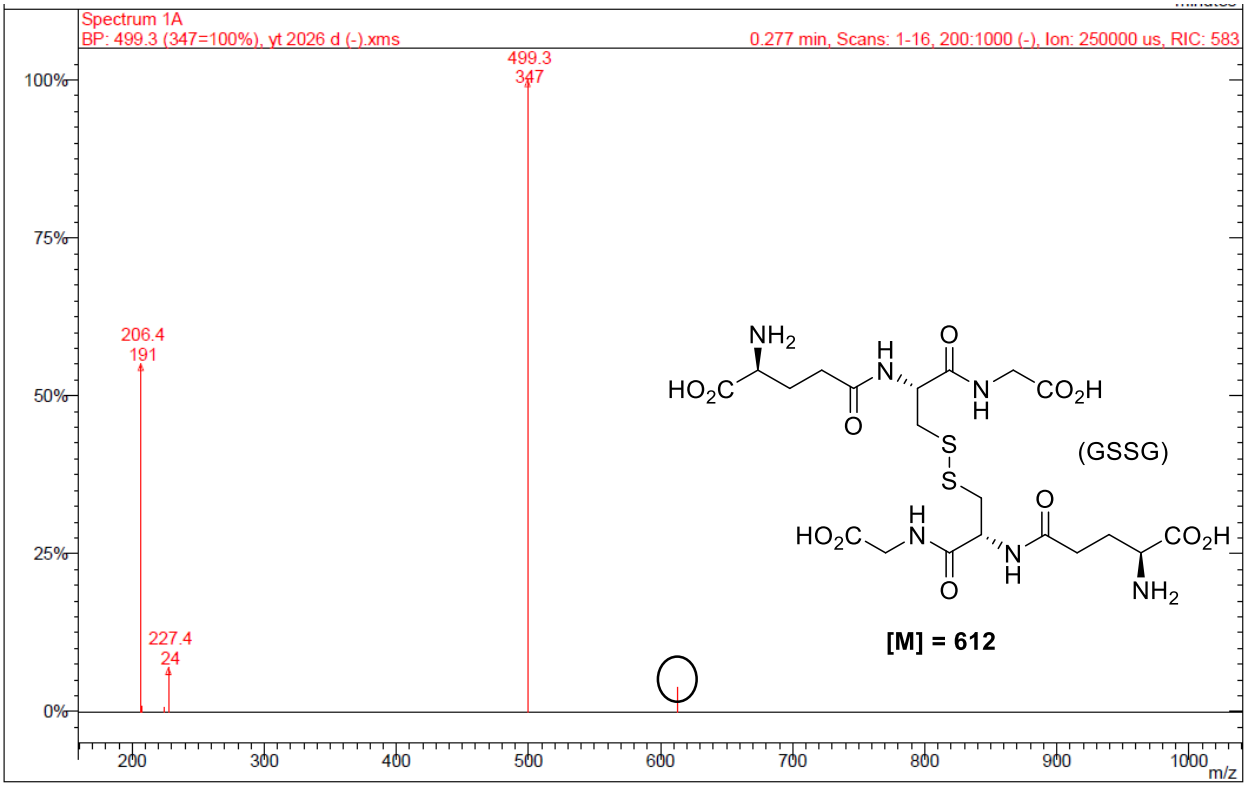
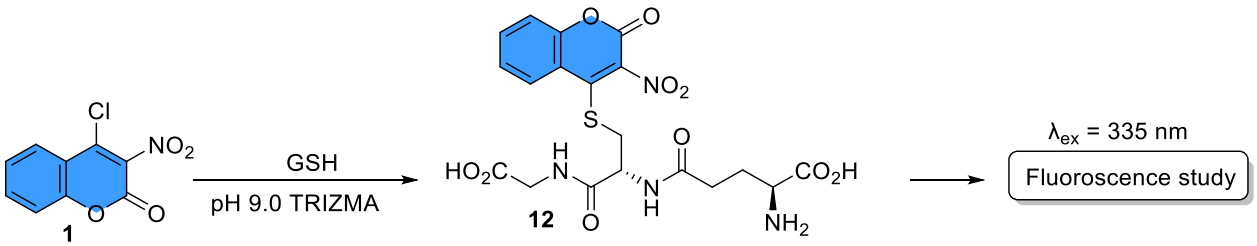


Figure 34. ESI-MS spectrum of the reaction mixture of [**1** + GSSG] in pH 9.0 TRIZMA buffer.



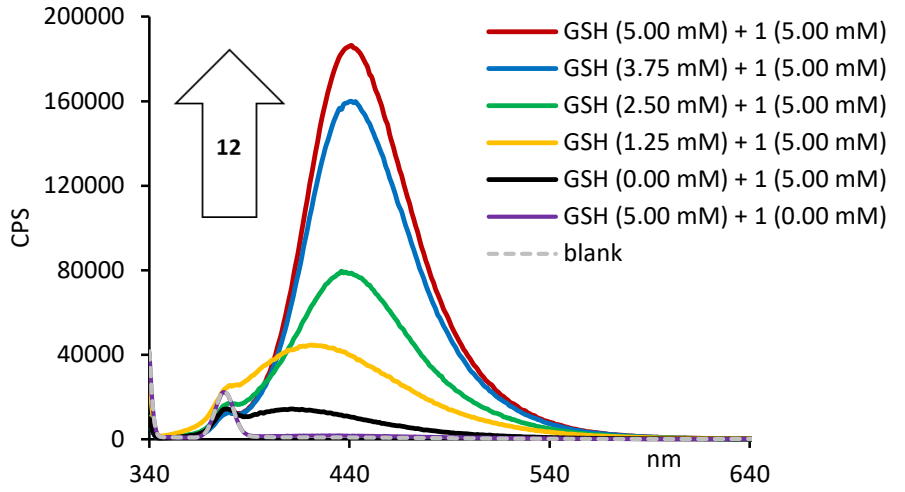
6.2. Fluorescence study with coumarin GSH conjugates



Scheme 8. Fluorescence study of coumarin GSH conjugates.

The change in the fluorescence of probe **1** reactions with varying GSH concentrations was studied. To a solution of probe **1** (25.0 mM in acetonitrile, 200.0 µL, 5.0 mM) was added L-GSH (25.0 mM in pH 9.0 TRIZA buffer) in varying amounts (0.0, 50.0, 100.0, 150.0 and 200.0 µL) and the mixture was diluted to 1.0 mL with acetonitrile: deionized water (4:1). The reactions were stirred overnight, and fluorescence measurements were taken by diluting 40.0 µL aliquots with 2.0 mL of degassed deionized water. Fluorescence spectra were collected using an excitation wavelength of 335 nm with 5 nm slit widths for both excitation and emission, and a quartz cuvette (1 cm path length). The fluorescence intensity at 441.0 nm increased as the concentration of **12** increased from 0.0 to 5.0 mM.

Figure 35. Emission spectra of [GSH+**1**] with increasing equivalents of GSH (λ_{ex} = 335 nm)



Fluorescence measurements were taken at 98.0 µM.

Figure 36. Fluorescence intensity at 441.0 nm with increasing concentration of **12** (λ_{ex} = 335 nm)**.**

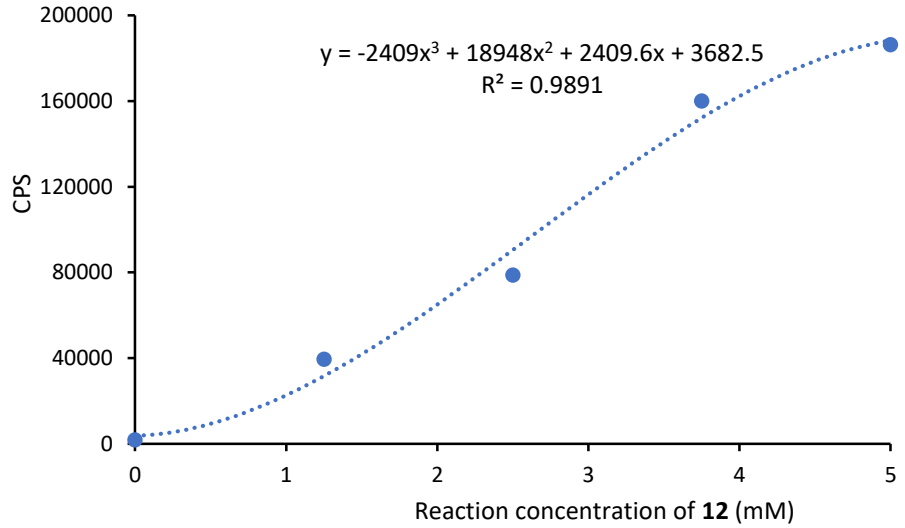
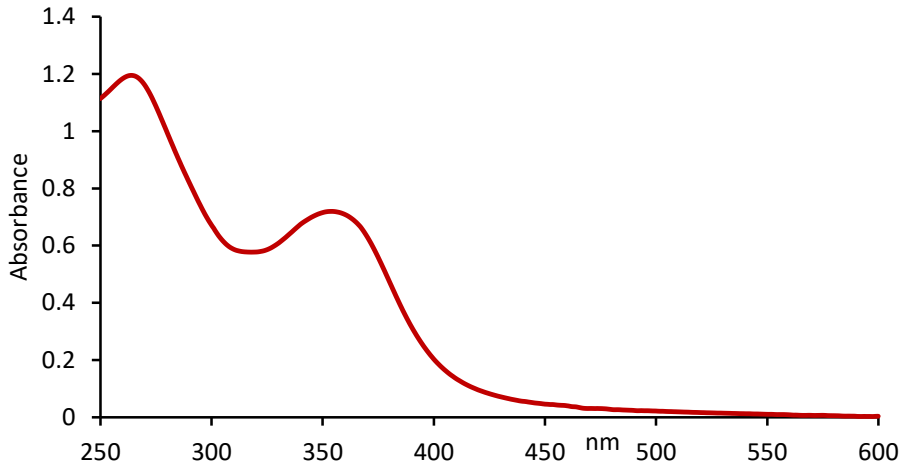
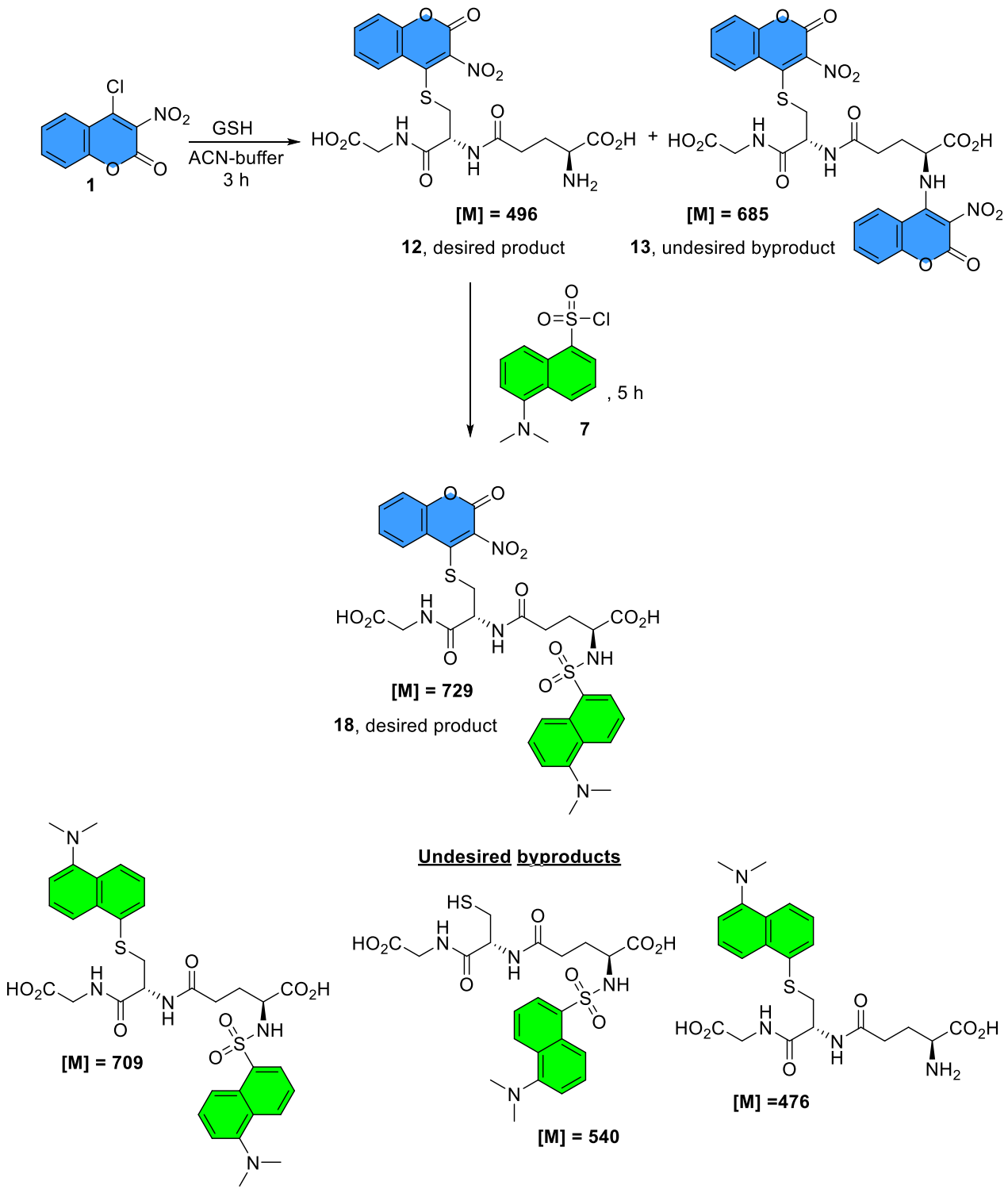


Figure 37. UV Absorption spectra of **12** under basic conditions (2 equiv K_2CO_3).



UV measurement was taken at 100.0 µM.

7. Selective bioconjugation of thiol and amino groups of GSH using a coumarin agent and dansyl chloride



Scheme 9. Selective bioconjugation of GSH with a coumarin agent **1**, followed by dansyl chloride, **7**.

To a solution of **1** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of dansyl chloride, **7** (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring for 5 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with 1.5 equivalents of **16**. MS analysis showed 71% conversion to **18** with 1 equivalent of **7** and >98% conversion with 1.5 equivalents of **7**.

Figure 38. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with 1 equivalent of **7** after 5 hours.

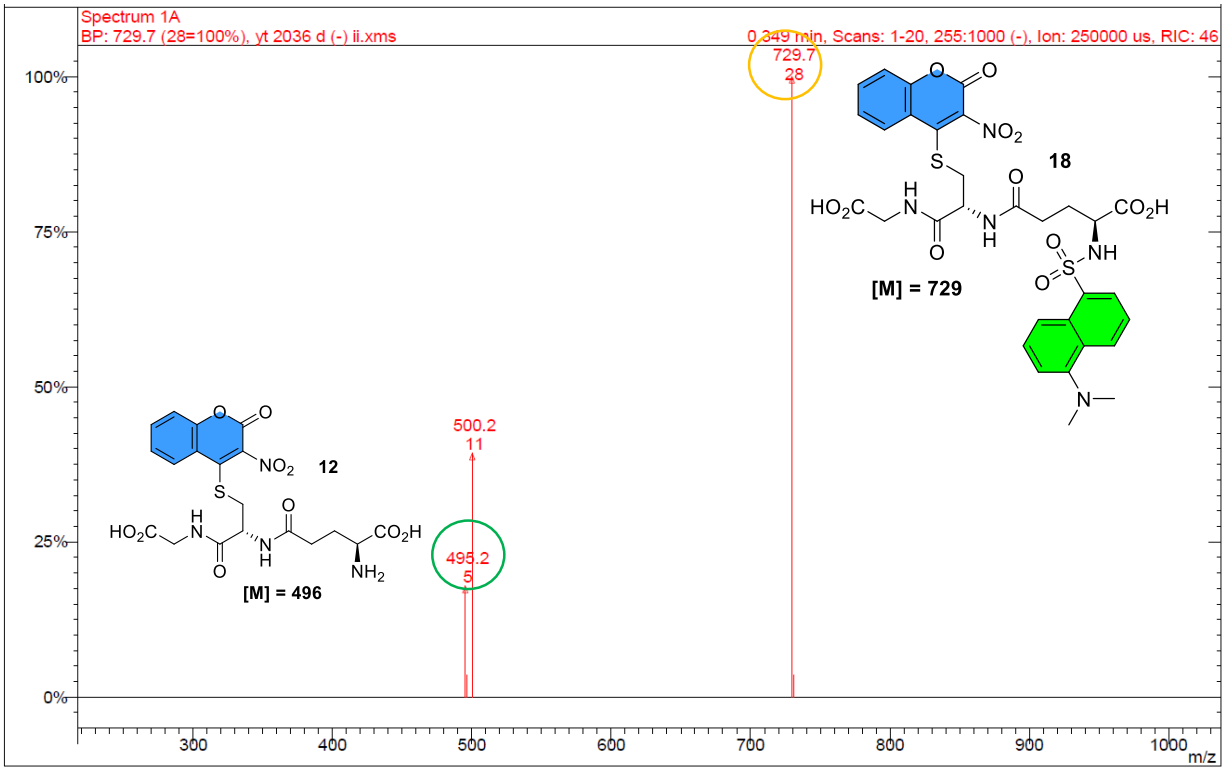
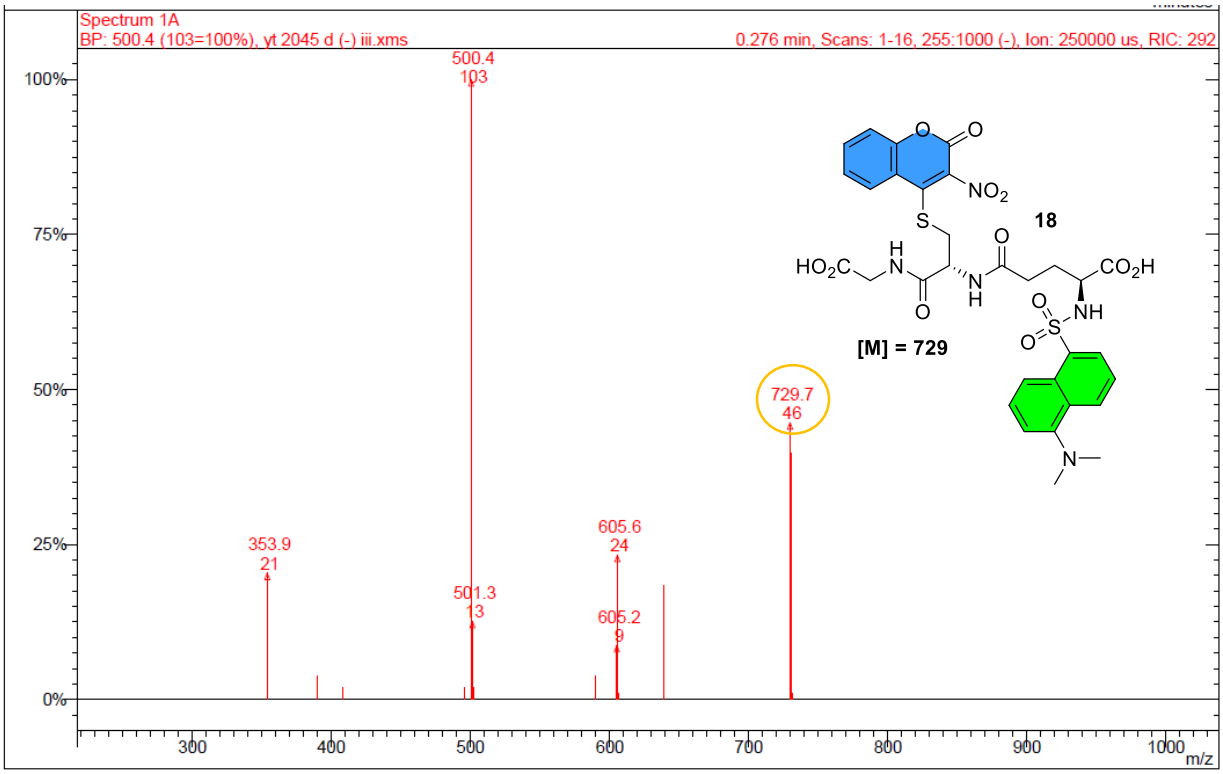
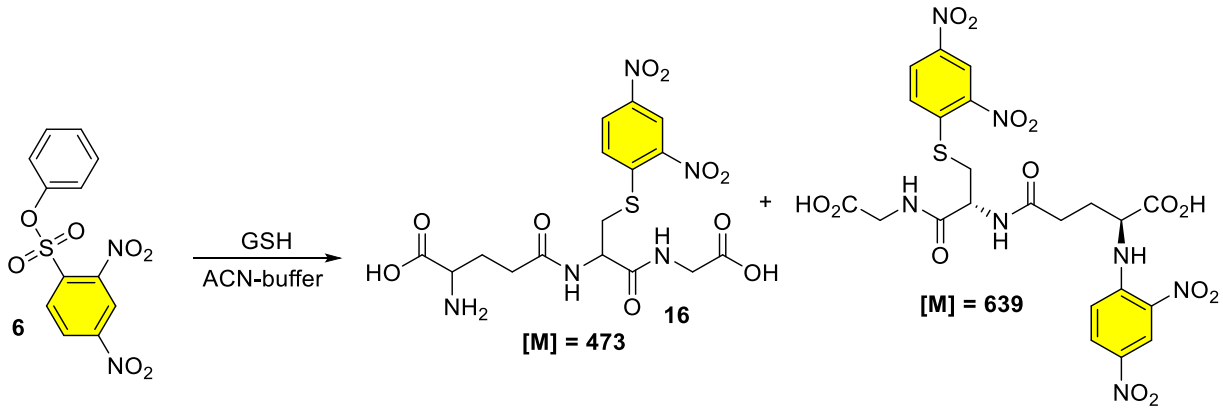


Figure 39. ESI-MS spectrum of the reaction mixture of [**1** + GSH] with 1.5 equivalents of **7** after 5 hours.



8. Selective bioconjugation of thiol and amino groups in GSH using phenyl 2,4-dinitrobenzenesulfonate, 6, and coumarin derivatives

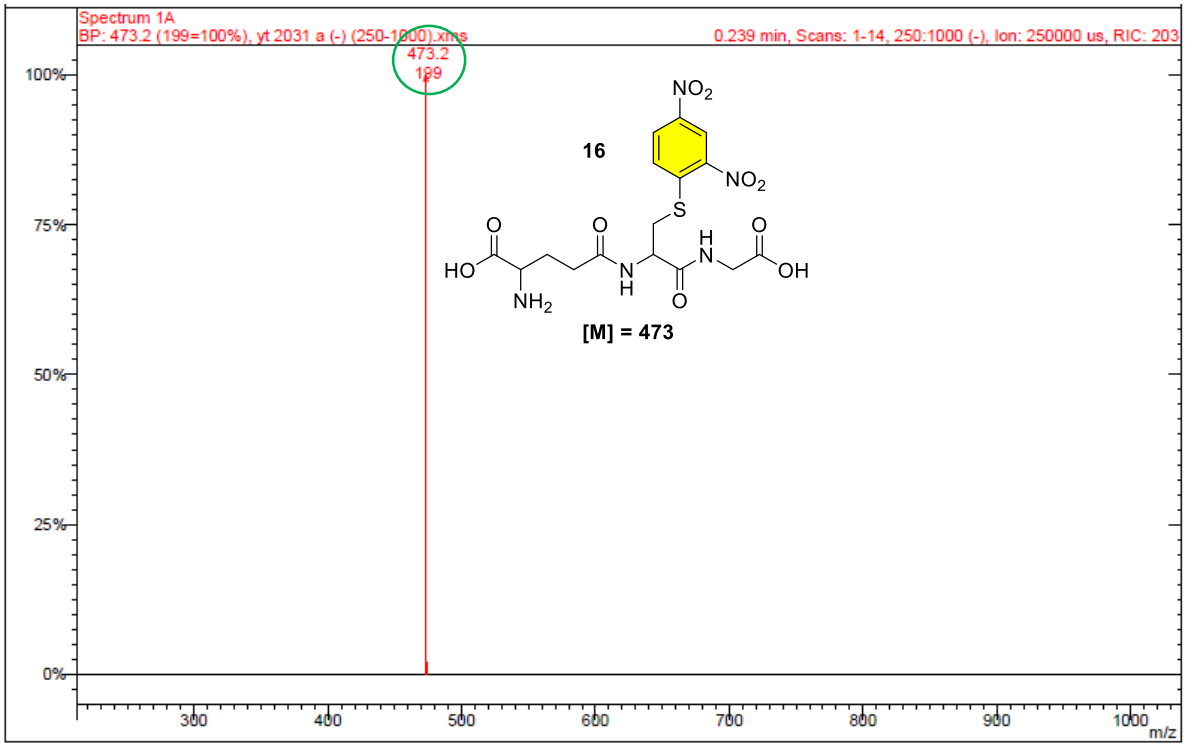
8.1. GSH selective thiol ligation with 6



Scheme 10. Monoconjugation of GSH with bioconjugation agent **6**.

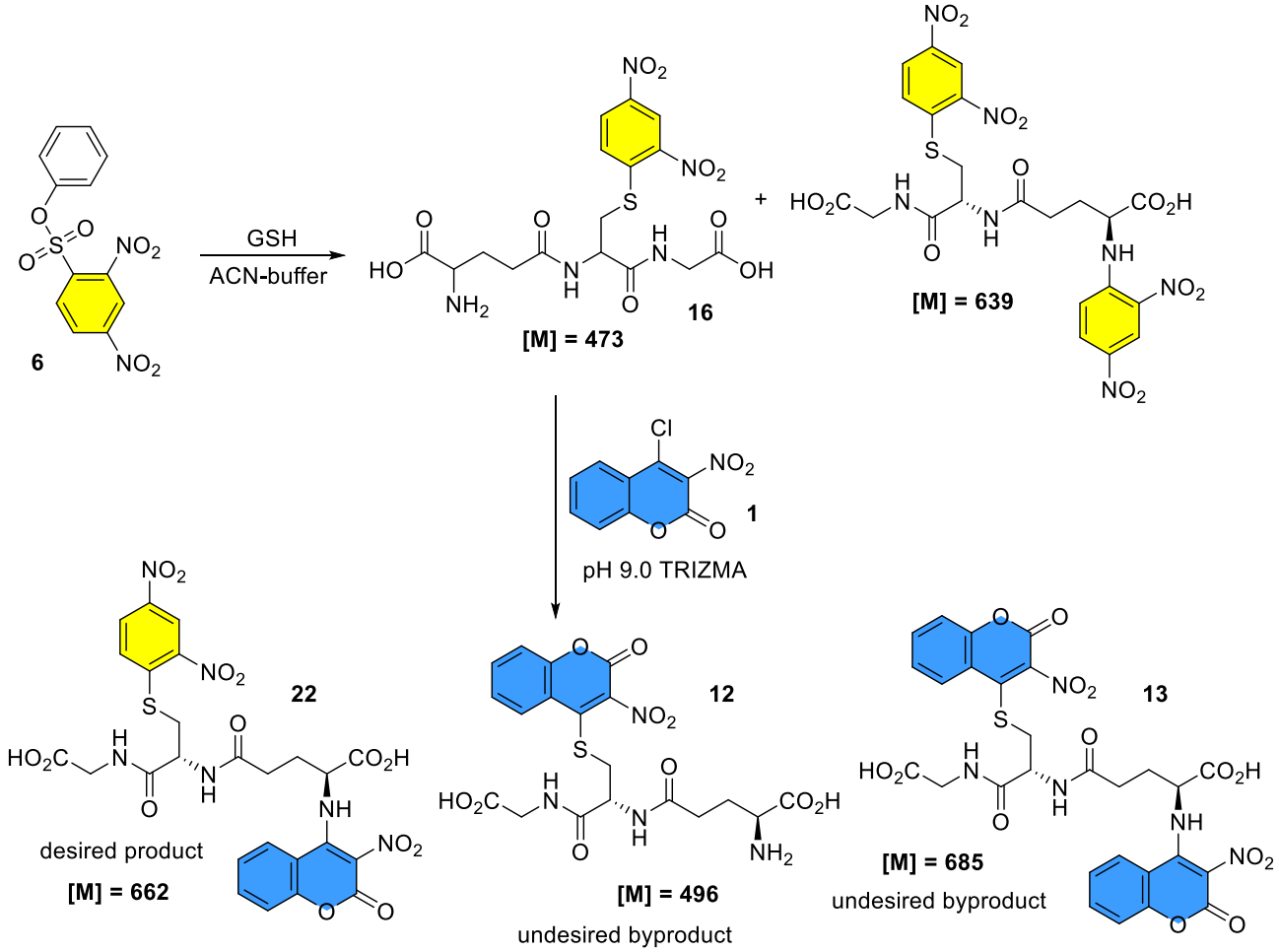
To a solution of **6** (25.0 mM in acetonitrile, 800.0 µL) was added L-GSH (25.0 mM in pH 8.5 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). Exclusive monoconjugation of GSH can be achieved with **6** using pH 9.0 TRIZMA buffer.

Figure 40. ESI-MS spectrum of the reaction mixture of GSH and **6** in pH 9.0 TRIZMA buffer.



8.2 Selective bioconjugation of thiol and amino groups in GSH using 6 and 1

8.2.1. Initial study: Thiol ligation of GSH with one equivalent of 6, followed by amine ligation with 1



Scheme 11. Selective bioconjugation of GSH with **18**, followed by coumarin **1**.

To a solution of **6** (25.0 mM in acetonitrile, 400.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of bioconjugation agent 4-chloro-3-nitrocoumarin, **1** (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring overnight, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with pH 8.5 TRIZMA buffer. Representative ESI-MS spectra are shown below. The undesired formation of **12** was observed at both pHs.

Figure 41. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of **1**, at pH 9.0 TRIZMA buffer.

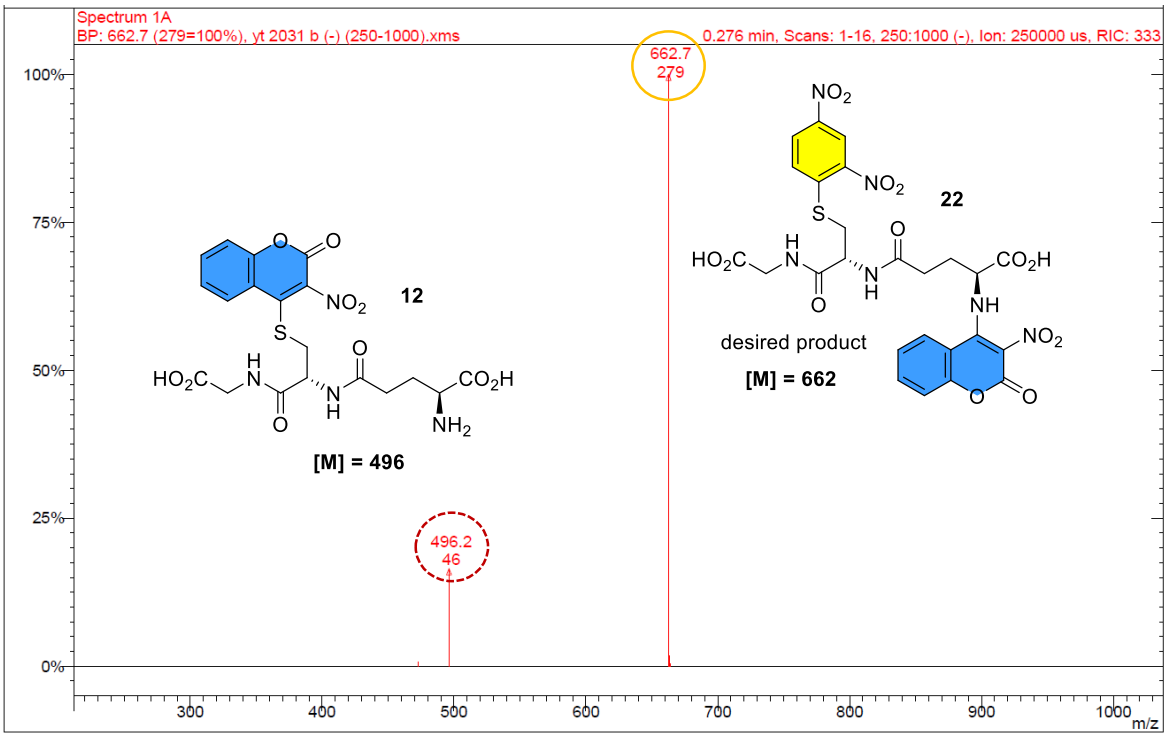
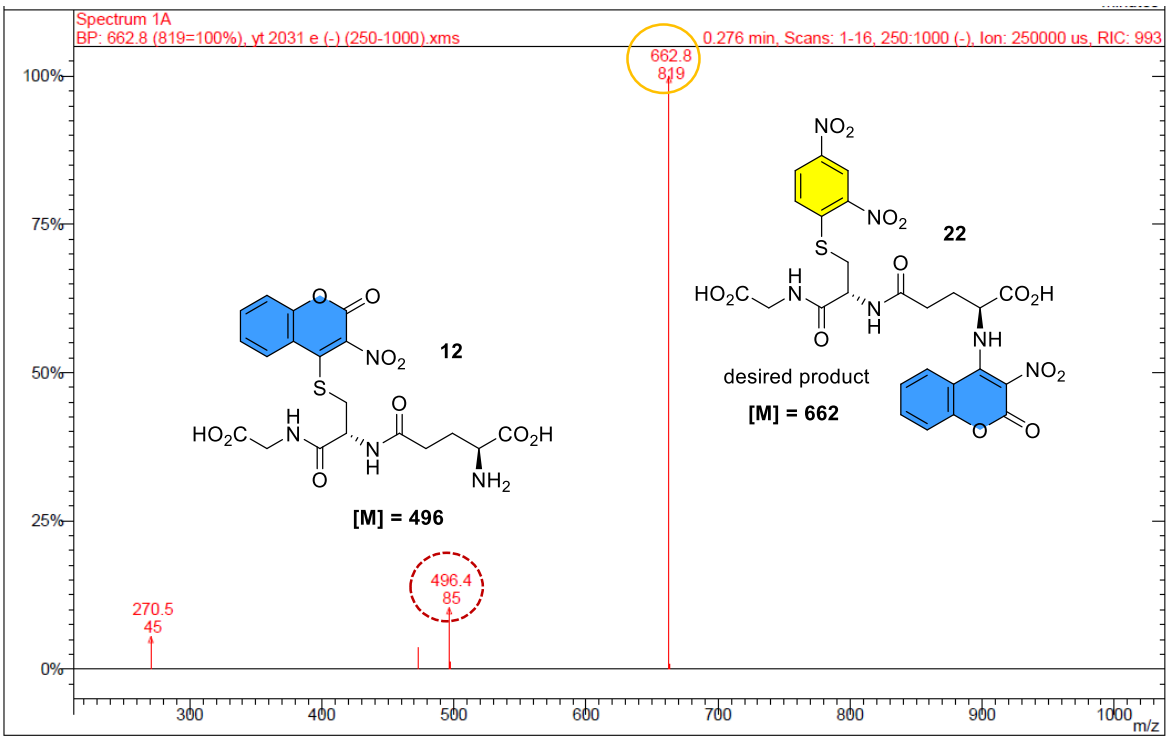


Figure 42. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of **1**, at pH 8.5 TRIZMA buffer.



8.2.2. Thiol ligation of GSH with 1.5 equivalents of 6 followed by amine ligation with 1

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of bioconjugation agent 4-chloro-3-nitrocoumarin, **1**, (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring overnight, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with pH 8.5 TRIZMA buffer. Representative ESI-MS spectra are shown below.

In the presence of 1.5 equivalents of **6**, 96% conversion of **16** to **22** in the presence of pH 8.5 TRIZMA buffer and >99% conversion to **22** at pH 9.0 TRIZMA buffer conditions were observed.

Figure 43. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of **1**, at pH 9.0 TRIZMA buffer.

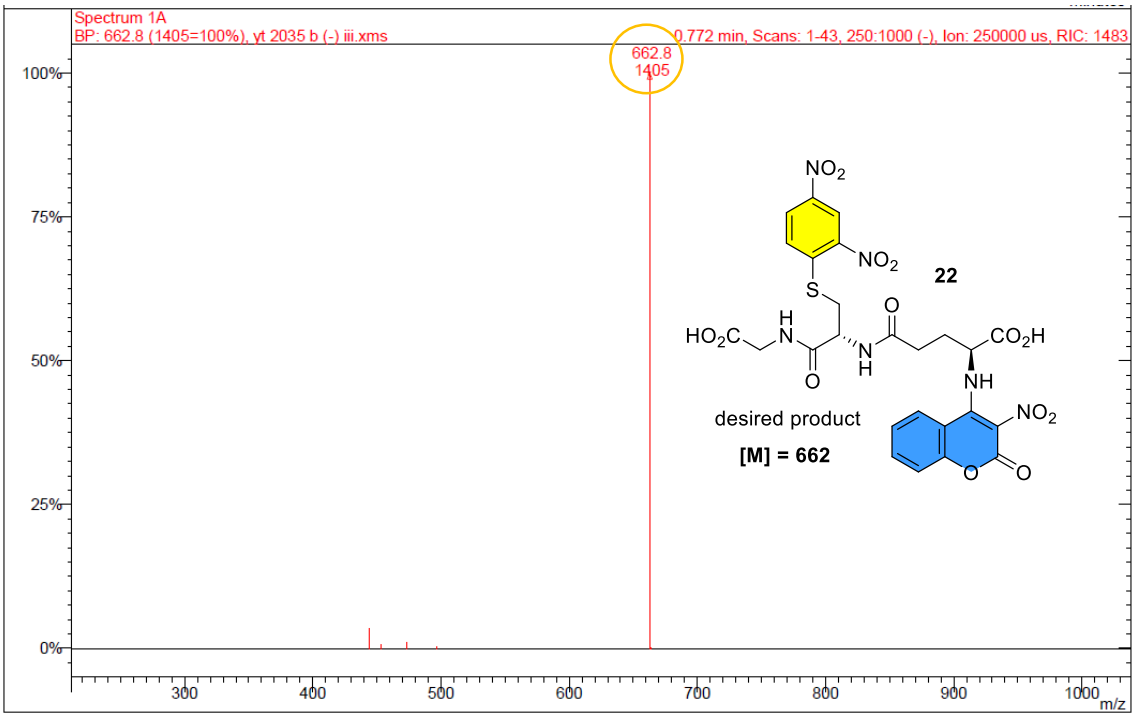
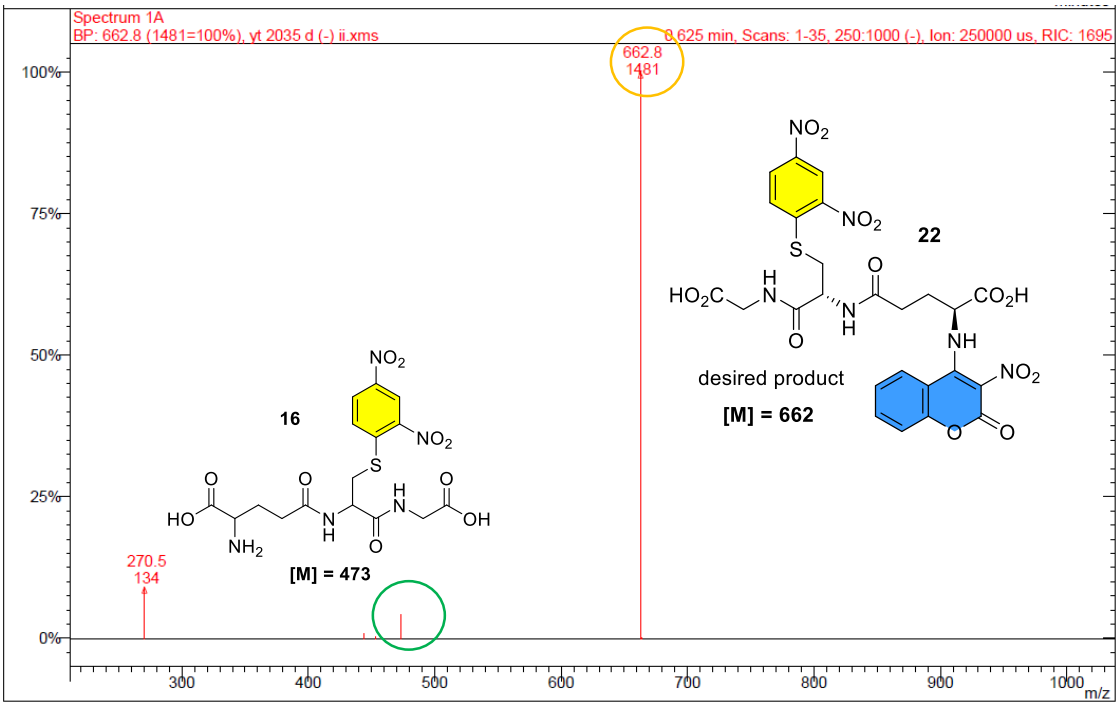
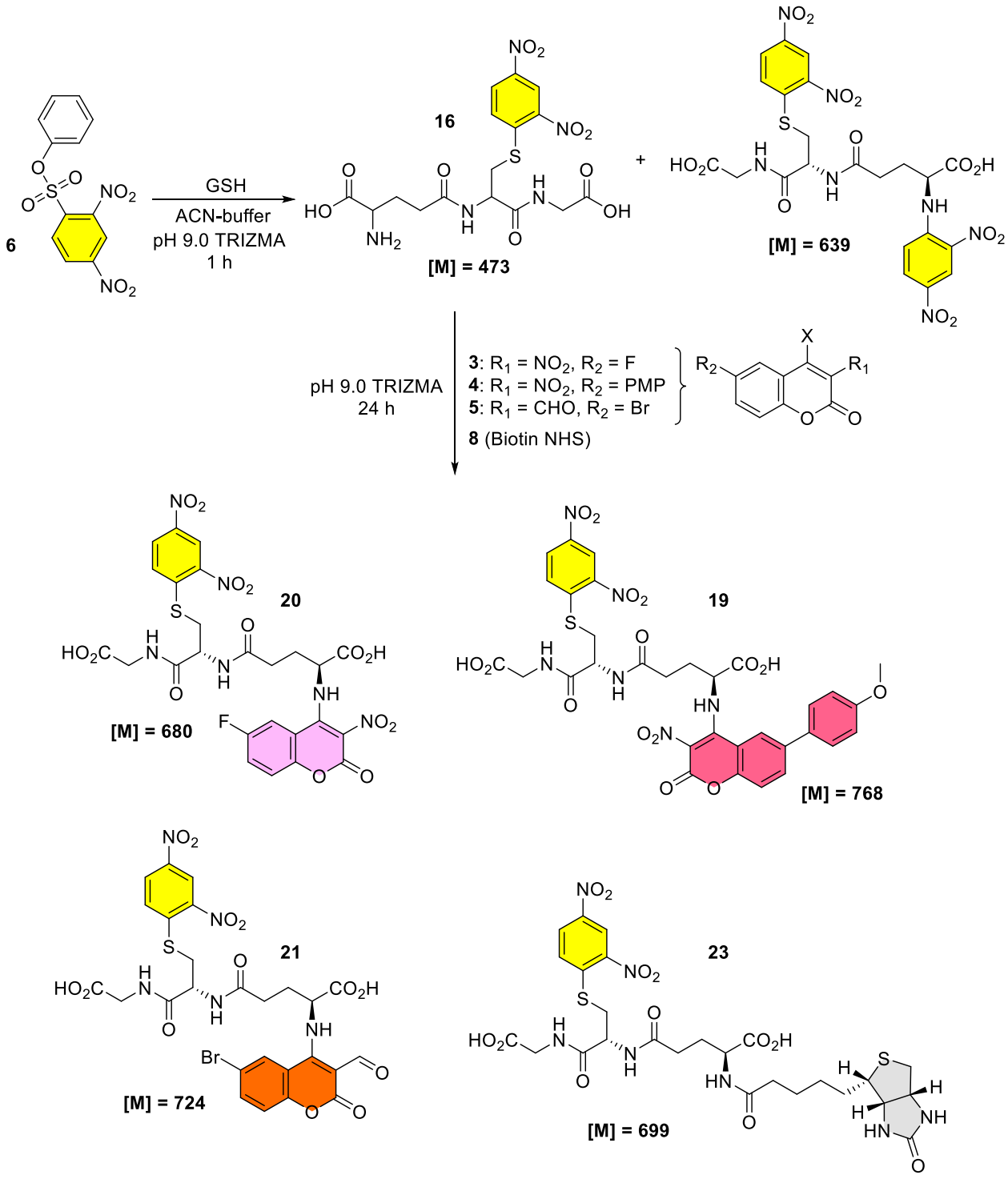


Figure 44. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of **1**, at pH 8.5 TRIZMA buffer.



8.3. Selective bioconjugation of thiol and amino groups in GSH using 6 and other bioconjugation agents



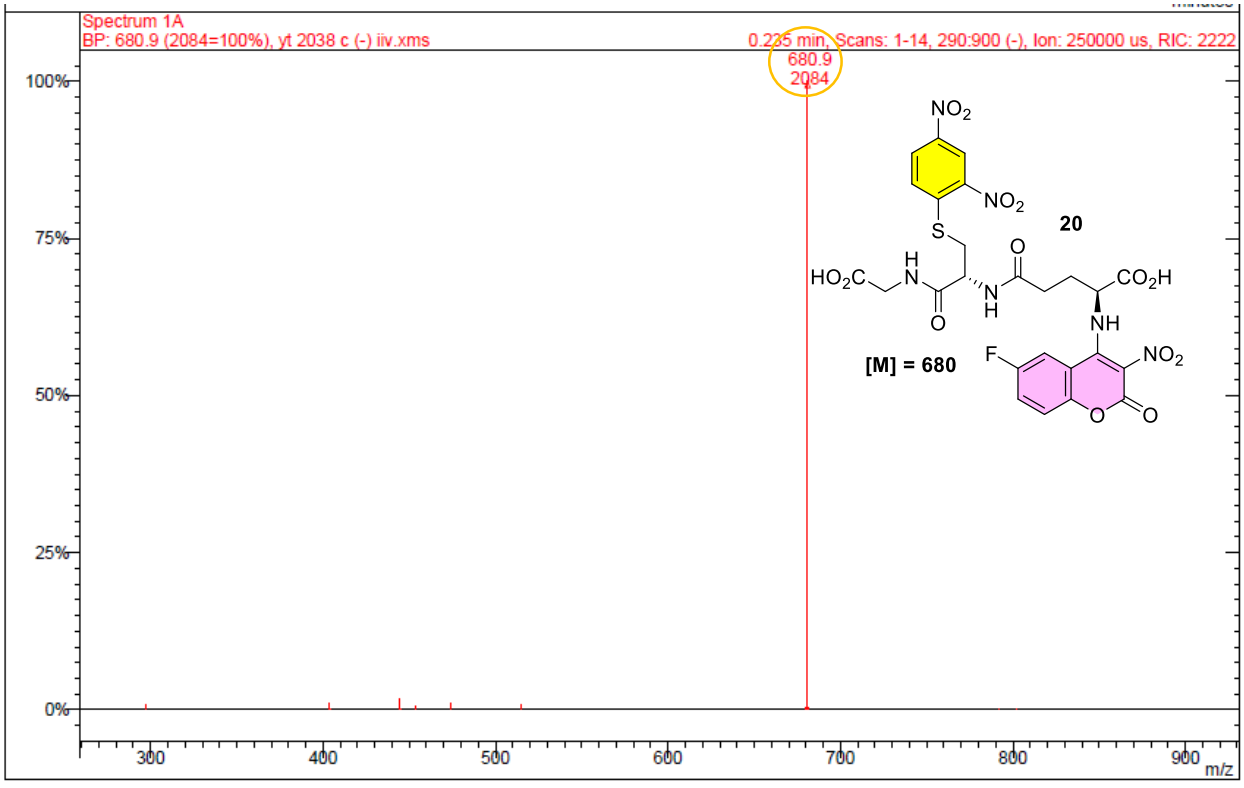
Scheme 12. Selective bioconjugation of GSH with **6** followed by reaction with other bioconjugation agents.

Bioconjugation agent **3**

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of bioconjugation agent 6-fluoro-4-chloro-3-nitrocoumarin, **3**, (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring overnight, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). Representative ESI-MS spectra are shown below.

Selective diconjugation of GSH to **20** can be achieved with **6** and **3** in pH 9.0 TRIZMA buffer.

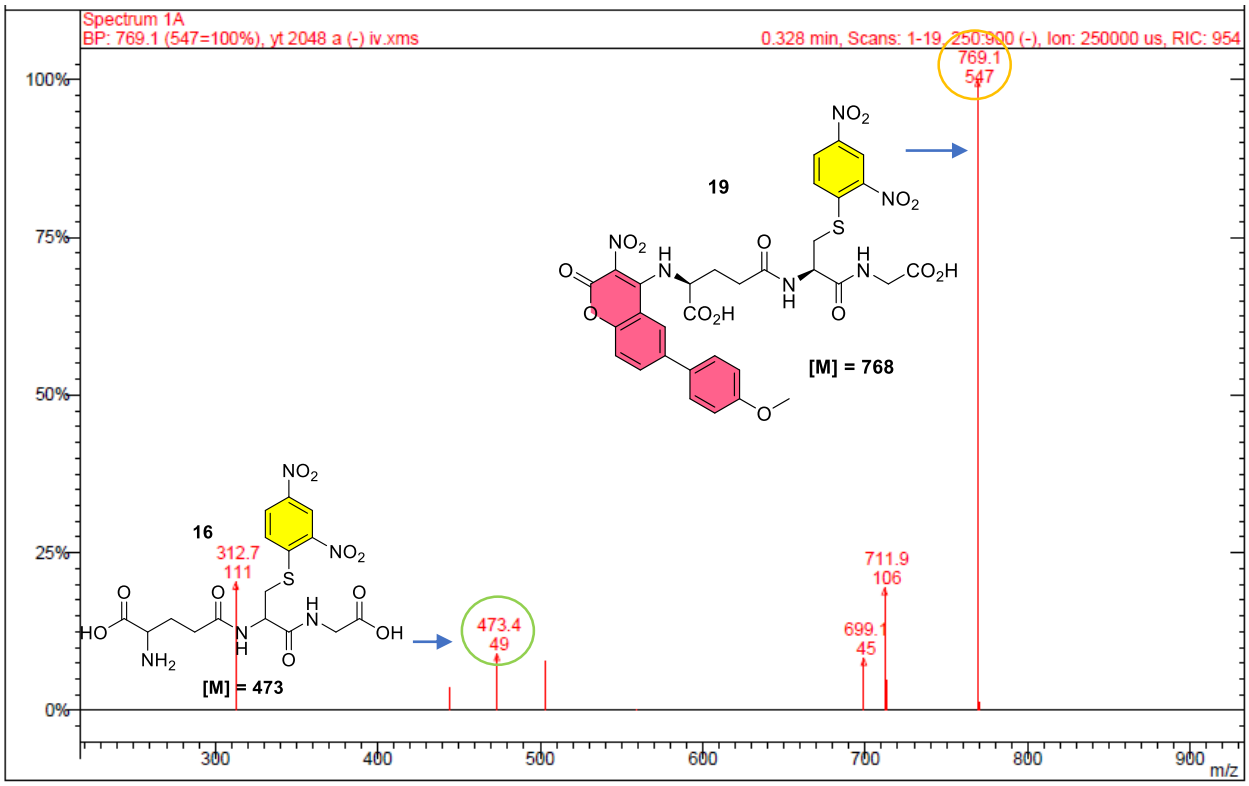
Figure 45. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of **3**, in pH 9.0 TRIZMA buffer



Bioconjugation agent **4**

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1.5 equivalent of bioconjugation agent 6-PMP-4-chloro-3-nitrocoumarin, **4**, (25.0 mM in acetonitrile, 600.0 µL) was added. After stirring overnight, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The ESI-MS spectrum shows 93% conversion from **16** to **19** after 24 hours.

Figure 46. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of **4**, in pH 9.0 TRIZMA buffer.



Bioconjugation agent **5**

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of 6-bromo-4-chloro-3-formylcoumarin, **5**, (25.0 mM in acetonitrile, 400.0 µL) was added. After stirring overnight, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with 1 equivalent of **5** for 48 hours and 2 equivalents of **5** for 24 hours.

Complete conversion to the diconjugation product **21** was observed with 2 equivalents of **5** after 24 hours.

Figure 47. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of 1 equivalent of **5**, in pH 9.0 TRIZMA buffer after 24 hours.

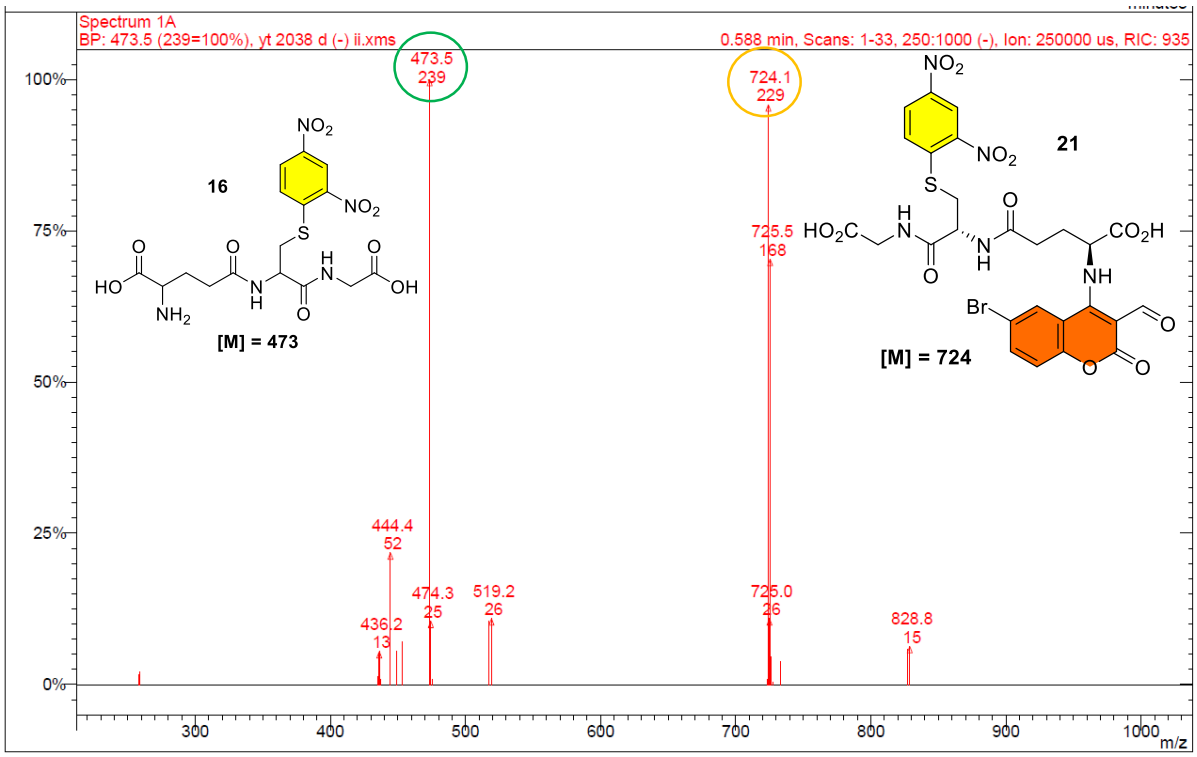


Figure 48. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of 1 equivalent of **5**, in pH 9.0 TRIZMA buffer after 48 hours.

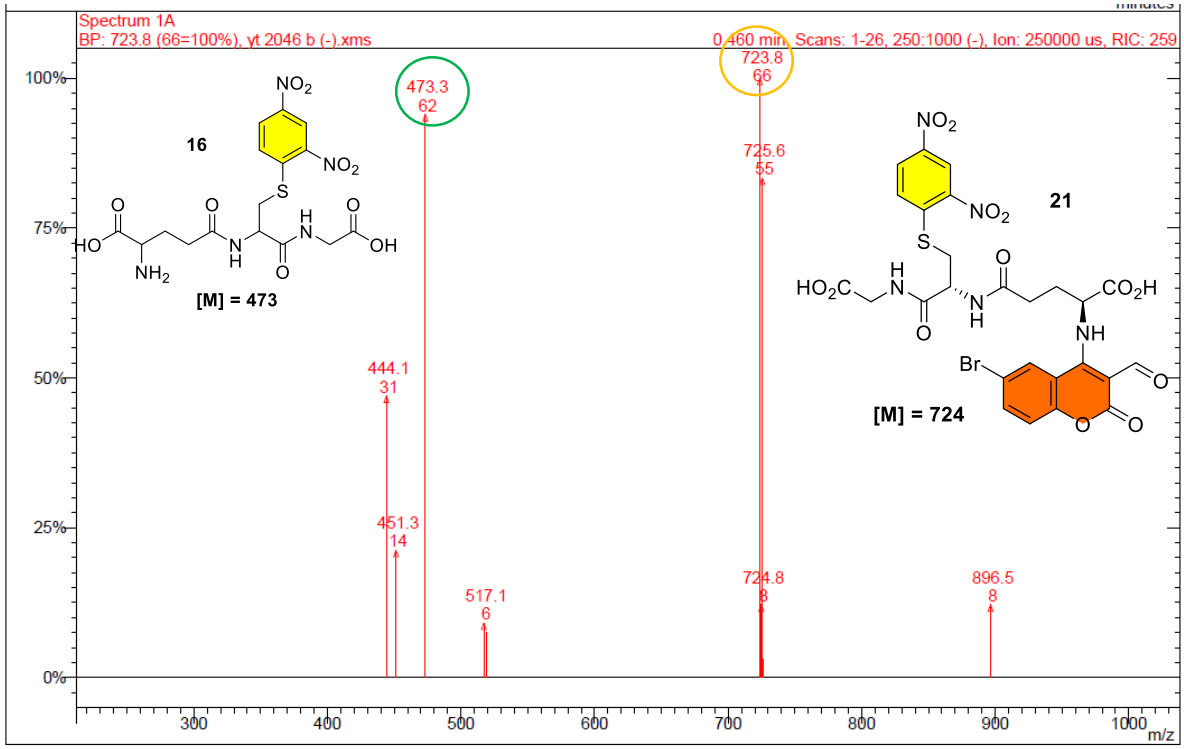
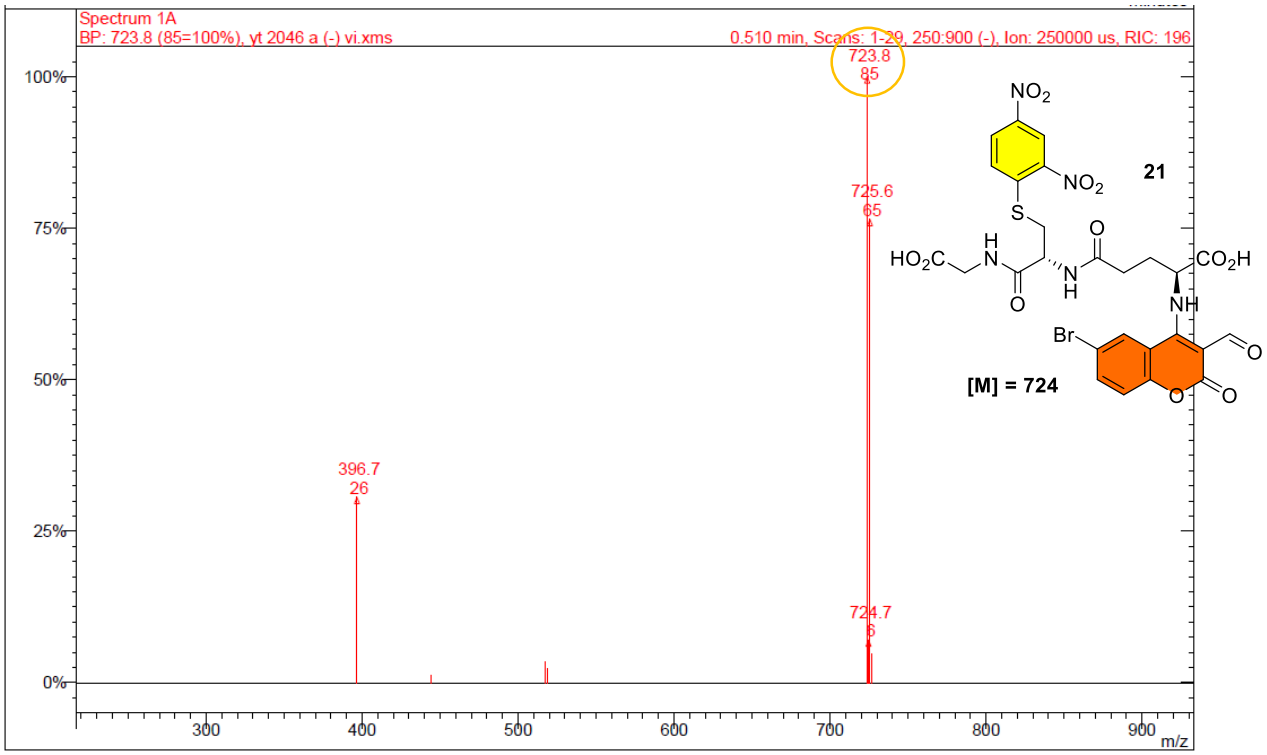


Figure 49. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of 2 equivalents of **5**, in pH 9.0 TRIZMA buffer after 24 hours.

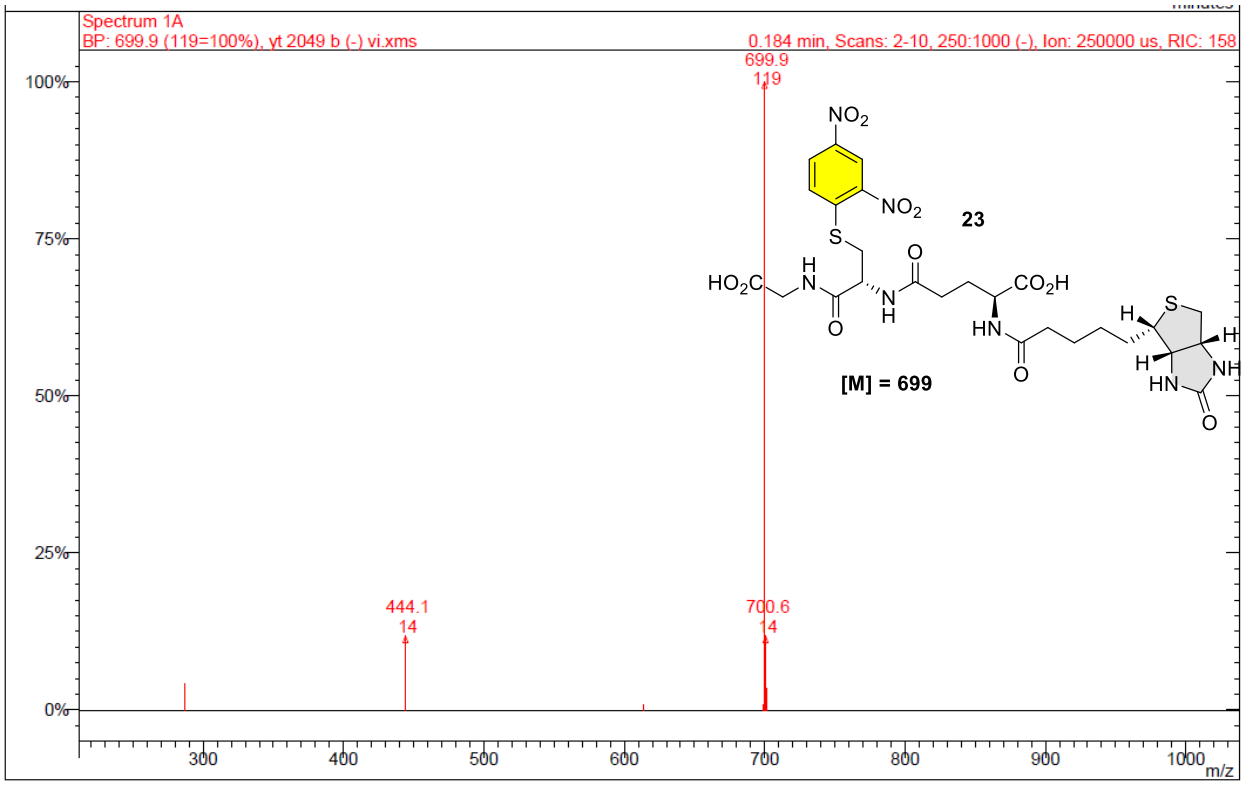


Bioconjugation agent **8 (**Biotin-NHS)

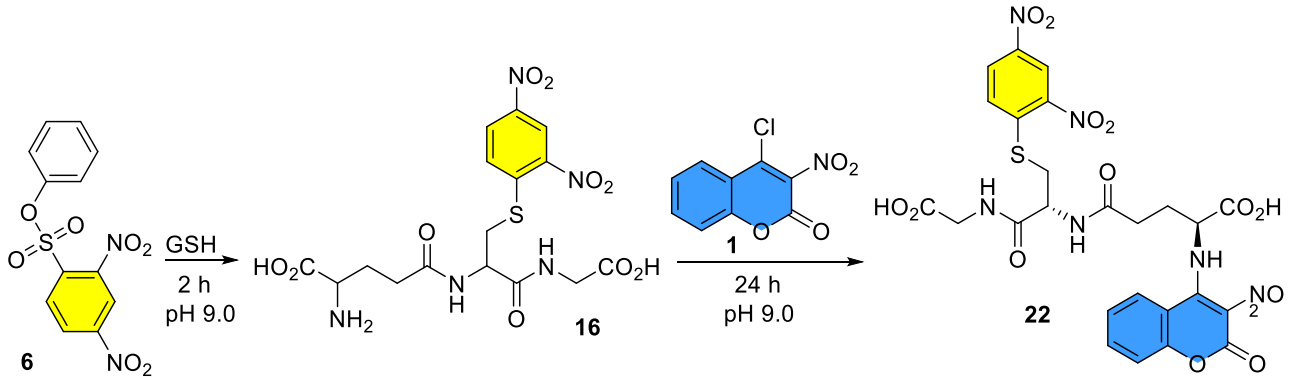
To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of Biotin-NHS, **8**, (12.5 mM in acetonitrile, 1000.0 µL in deionized water and acetonitrile (1:1)) was added. After stirring overnight, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Complete conversion to the diconjugation product **23** was observed with 1.2 equivalents of **8** after 24 hours.

Figure 50. ESI-MS spectrum of the reaction mixture of [**6**+GSH], followed by addition of 1.2 equivalents of **8**, in pH 9.0 TRIZMA buffer after 24 hours.



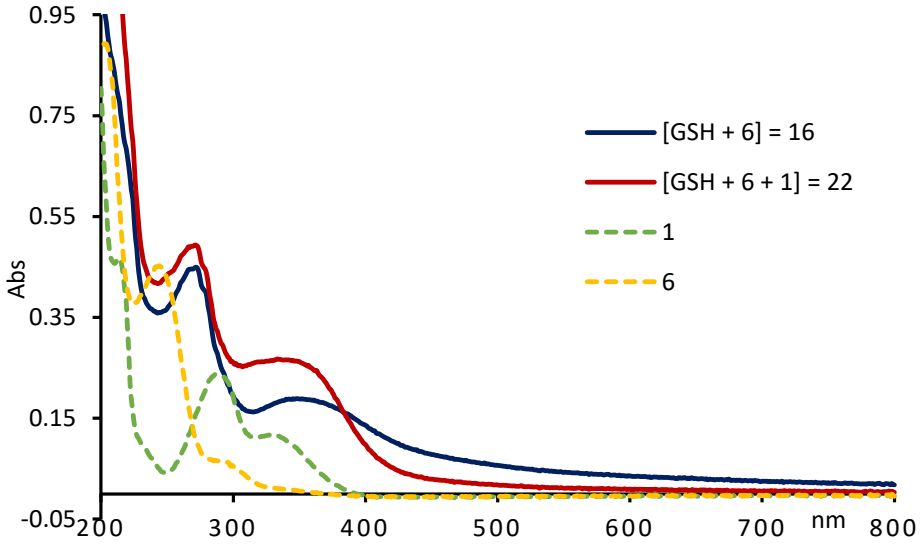
8.4. UV and fluorescence study



Scheme 13. Selective thiol and amine conjugation with **6** and coumarin **1**.

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added L-GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 1.2 mL of acetonitrile. After 2 hours, 1 equivalent of 4-chloro-3-nitrocoumarin, **1**, (25.0 mM in acetonitrile, 400.0 µL in acetonitrile) was added and the solution was allowed to stir overnight. UV measurements were taken by diluting 10.0 µL of the reaction solution with 2.0 mL of acetonitrile. UV spectra were collected with an average scanning time of 0.0125 s, a data interval of 5.00 nm and a scan rate of 400 nm/s.

Figure 51. UV change of mono- and diconjugated GSH with **1** and **6**.



Fluorescence measurements were taken by diluting 30.0 µL aliquots with 2.0 mL of deionized degassed water, with an excitation wavelength of 275 nm and 5 nm slit widths, using a quartz cuvette (1 cm path length).

Figure 52. Emission spectra from 285 nm – 525 nm GSH (λ_{ex} = 275 nm).

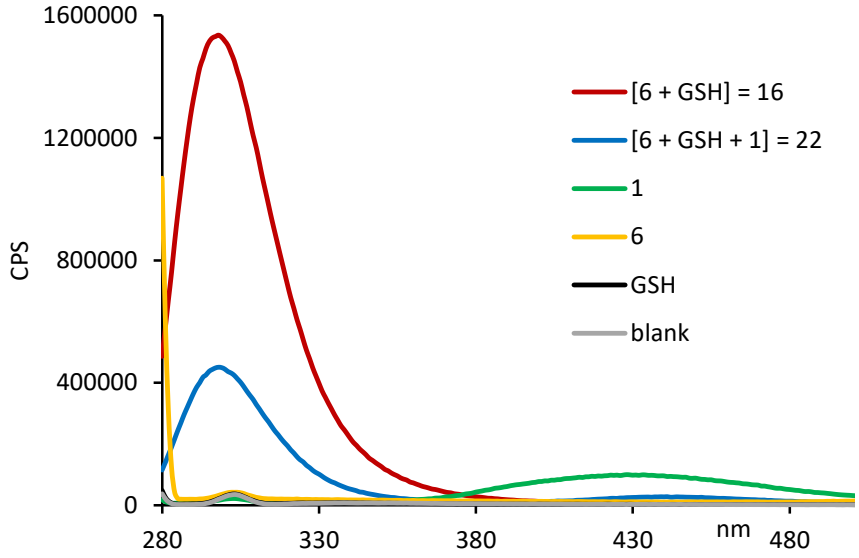
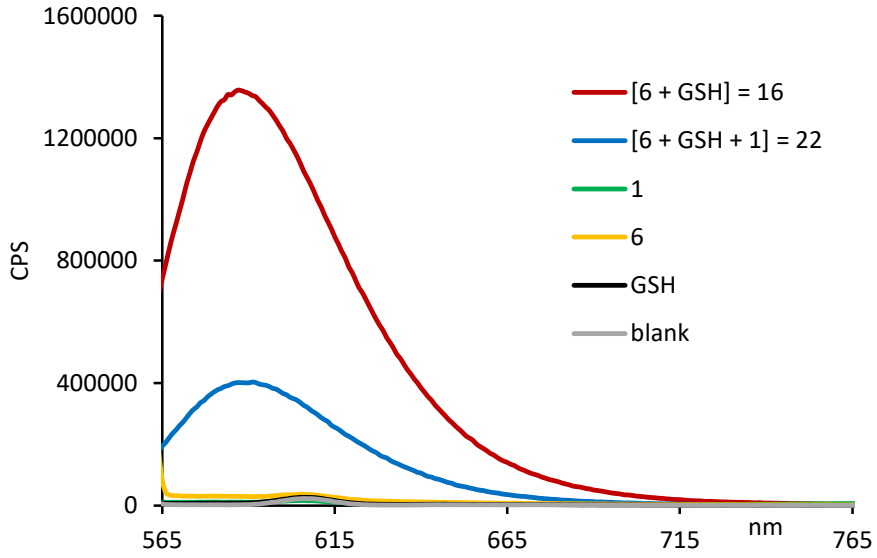
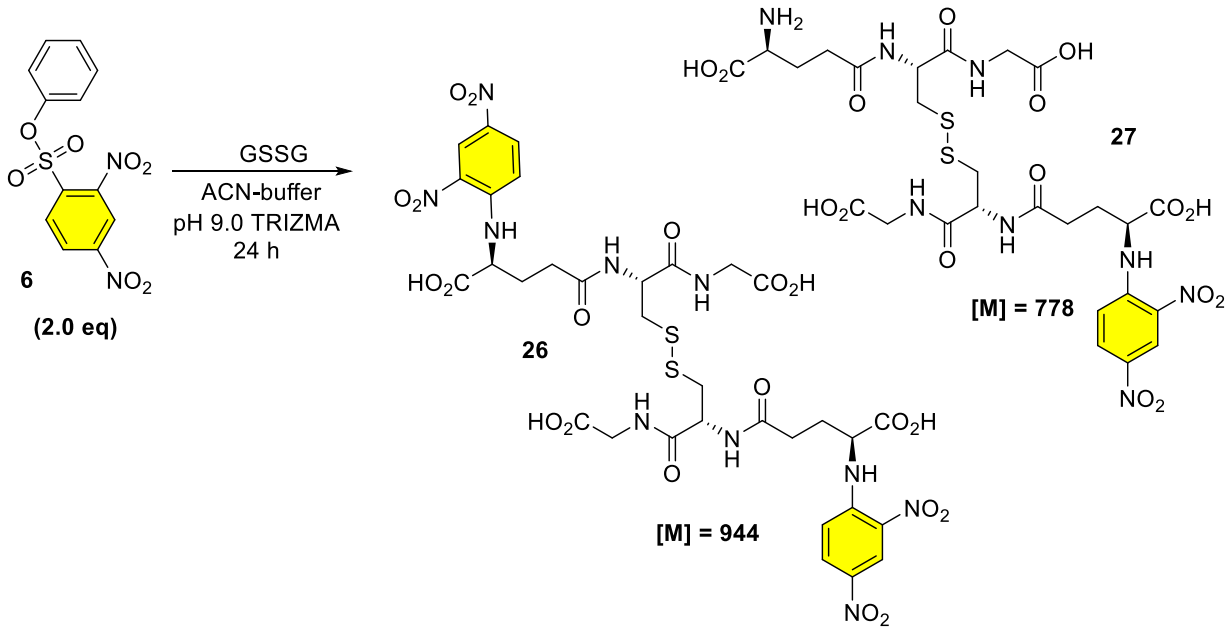


Figure 53. Emission spectra from 565 nm – 765 nm GSH (λ_{ex} = 275 nm).



8.5. Mechanistic studies

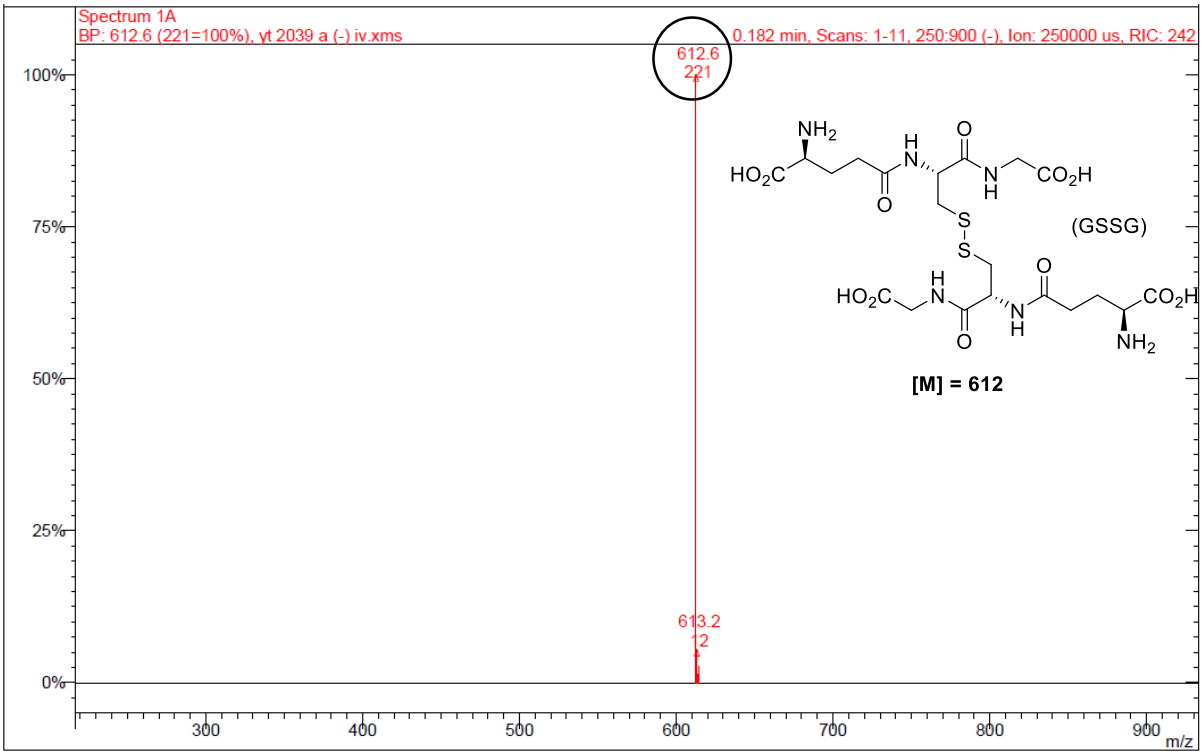
8.5.1. Evidence for thiol ligation: GSSG conjugation with 6



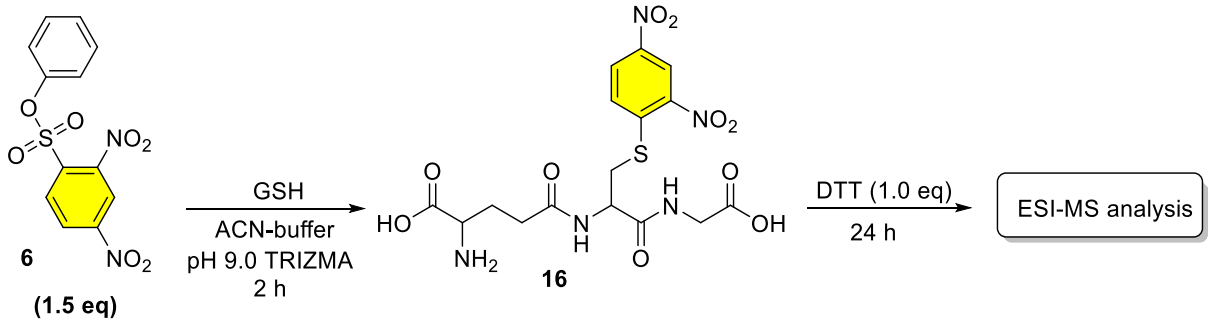
Scheme 14. Evidence for thiol ligation: Reaction of GSSG with bioconjugation agent **6**.

To a solution of **6** (25.0 mM in acetonitrile, 800.0 µL) was added GSSG (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and the mixture was diluted with 0.8 mL of acetonitrile. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL using deionized deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted with 2.0 mL of deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The peaks corresponding to amine ligation products **26** and **27** were not observed. This confirms that under these conditions, the first conjugation occurs at the free thiol group in the peptide.

Figure 54. ESI MS spectrum of GSSG in the presence of **6**.



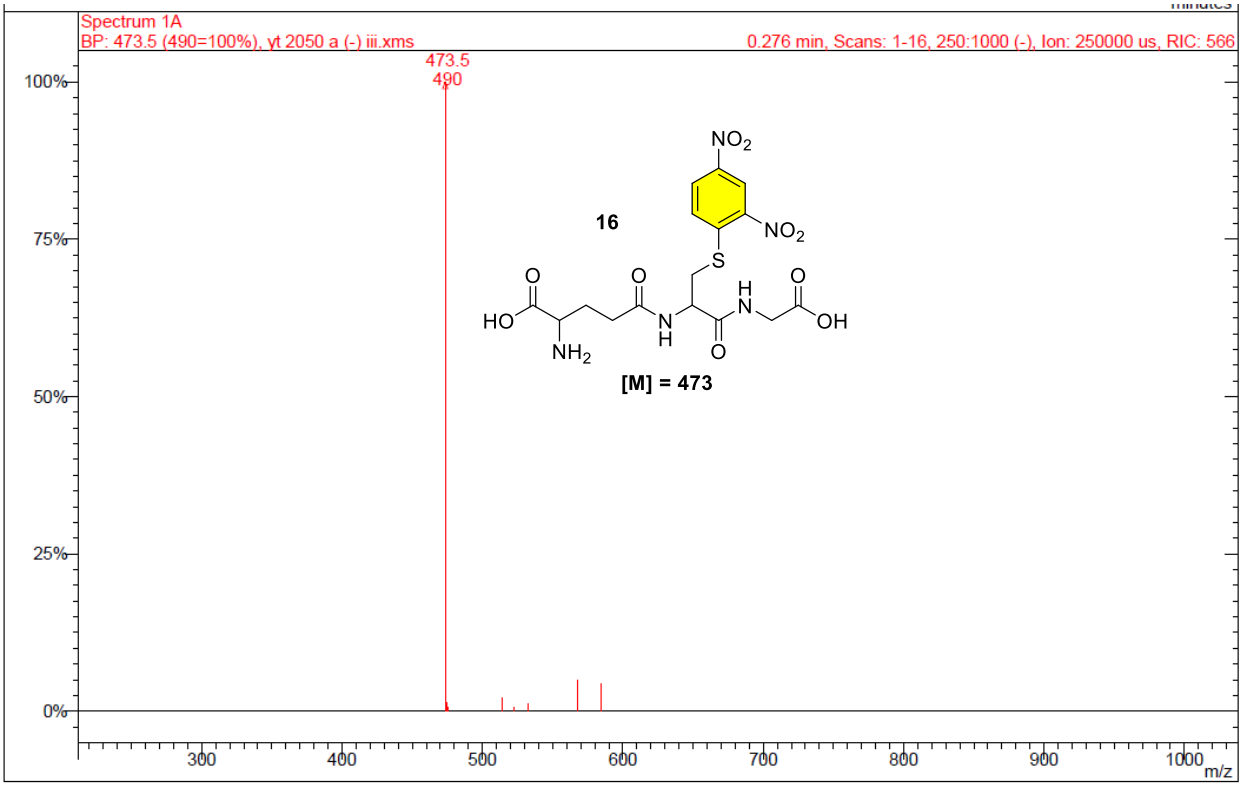
8.5.2. Stability of DNP-GSH conjugate in the presence of DTT (dithiothreitol)



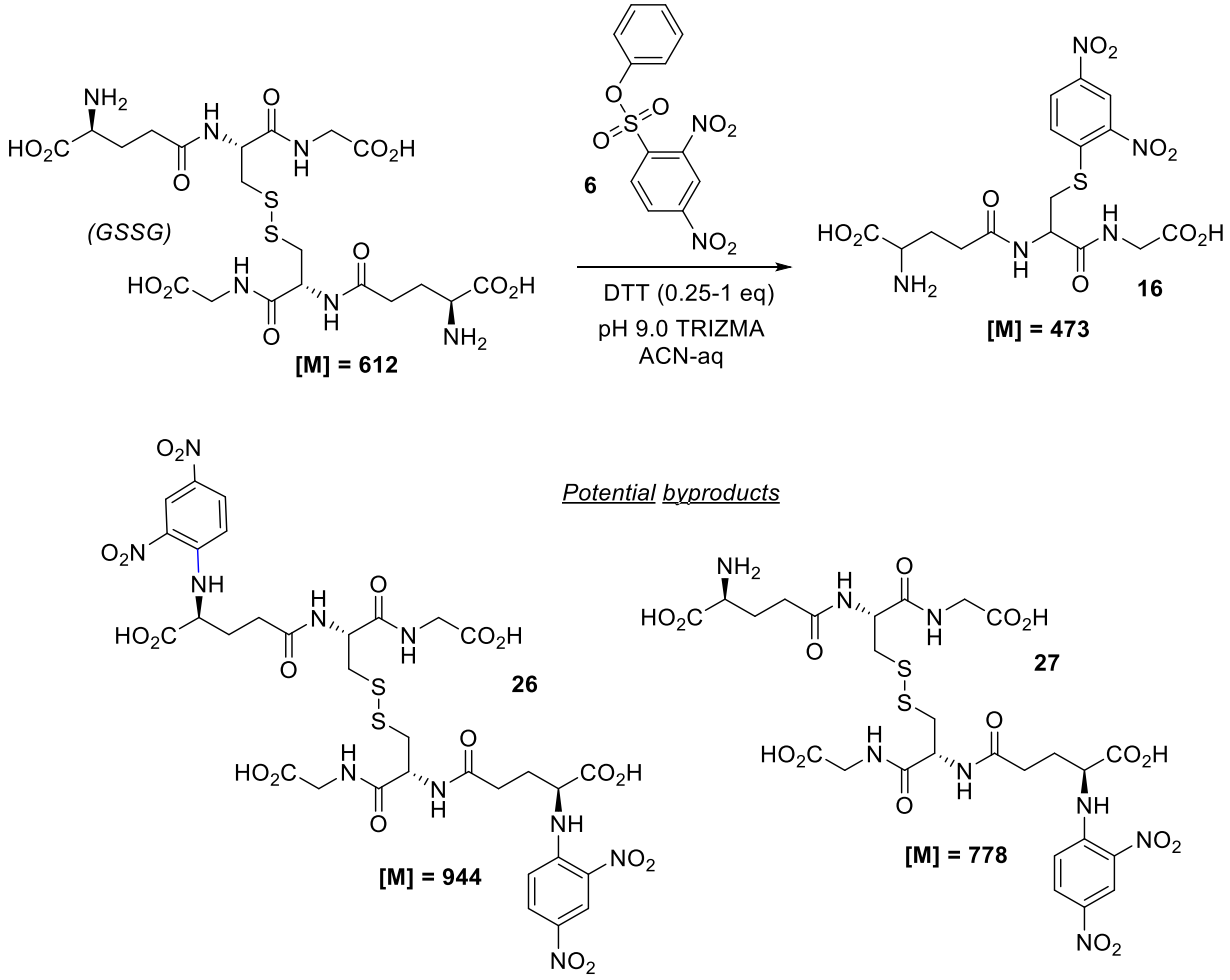
Scheme 15. Stability check of monoconjugated **16** in the presence of DTT.

To a solution of **6** (25.0 mM in acetonitrile, 800.0 µL) was added GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 M, 400.0 µL) and the mixture was diluted with 0.8 mL of acetonitrile. After 2 hours, DTT (25.0 mM in deionized water, 400.0 µL) was added. After stirring for 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The peak corresponding to monoconjugated product, **16** was observed with no other major peaks. This confirms that in the presence of DTT, the monoconjugated product remains stable.

Figure 55. ESI MS spectrum of the reaction between **6** and GSH in the presence of DTT.



8.5.3. Monoconjugation of GSSG in the presence of DTT (dithiothreitol)



Scheme 16. Monoconjugation of GSSG with agent **6** in the presence of DTT.

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) were added GSSG (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and DTT (25.0 mM in deionized water, 400.0 µL) and the mixture was diluted with 0.8 mL of acetonitrile. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The above procedure was repeated with 0.25 equivalent of DTT (25.0 mM in deionized water, 100.0 µL).

Figure 56. ESI-MS spectrum of the reaction mixture of [**6**+GSSG] followed by the addition of 1 equivalent of DTT after 24 hours.

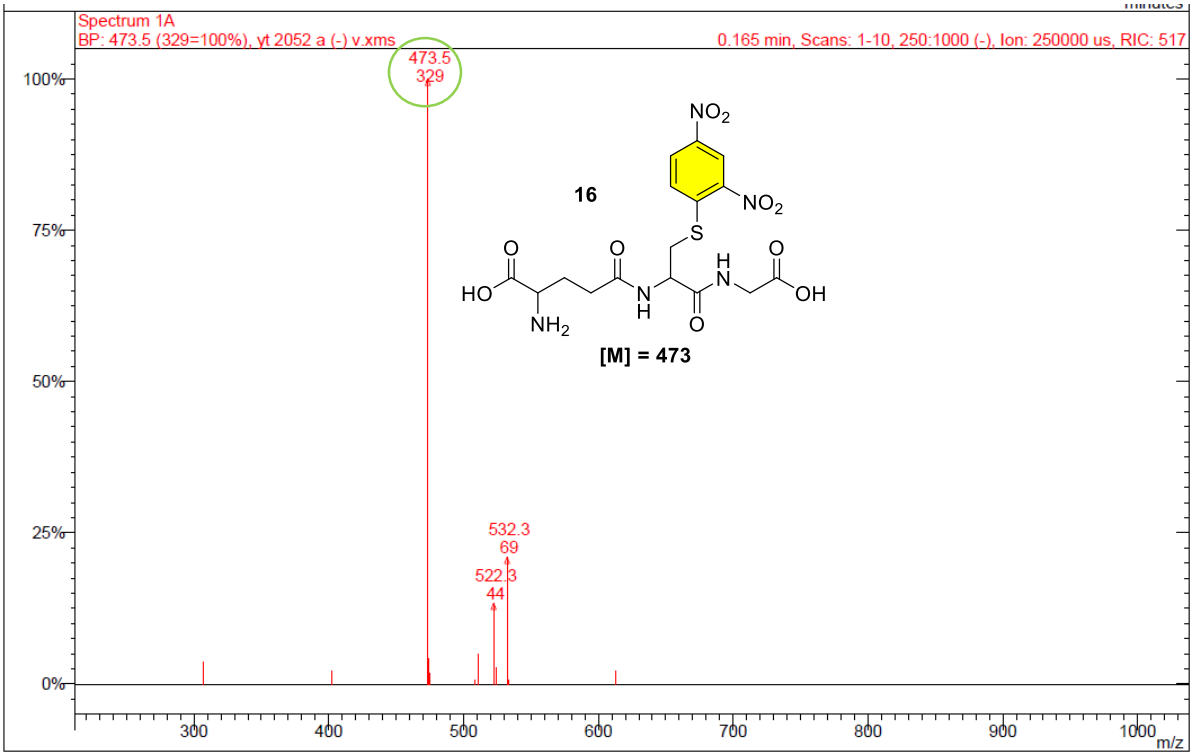
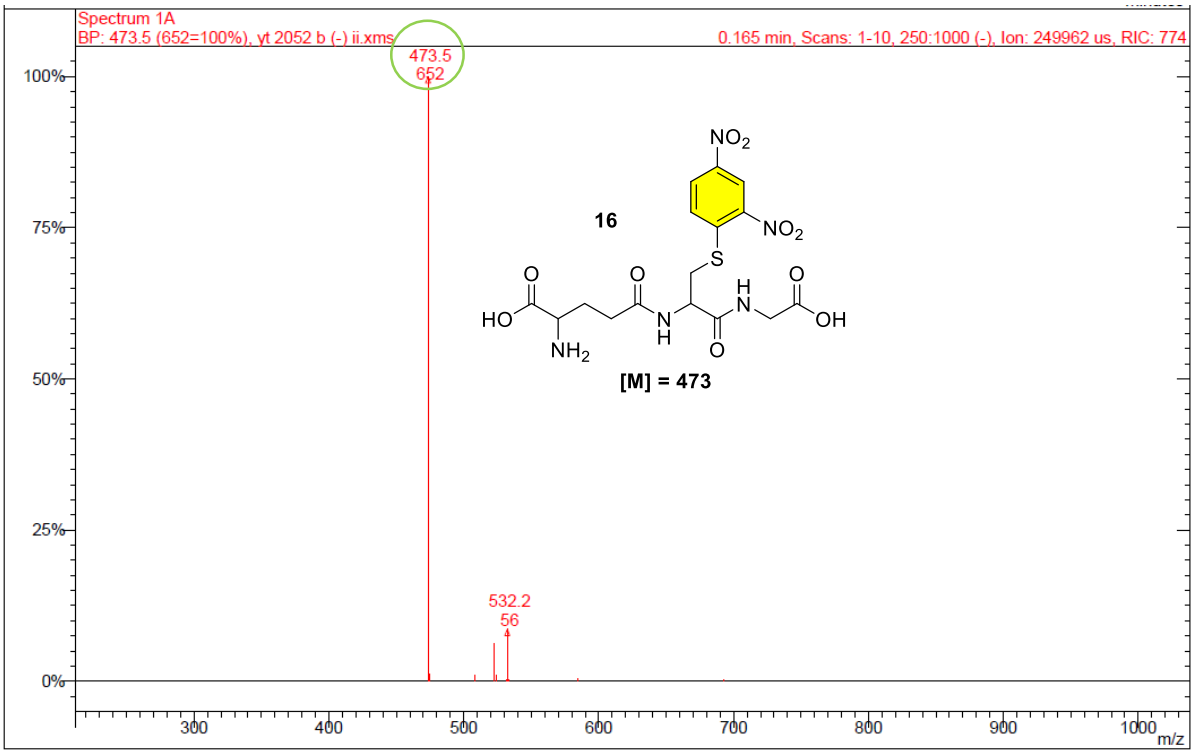
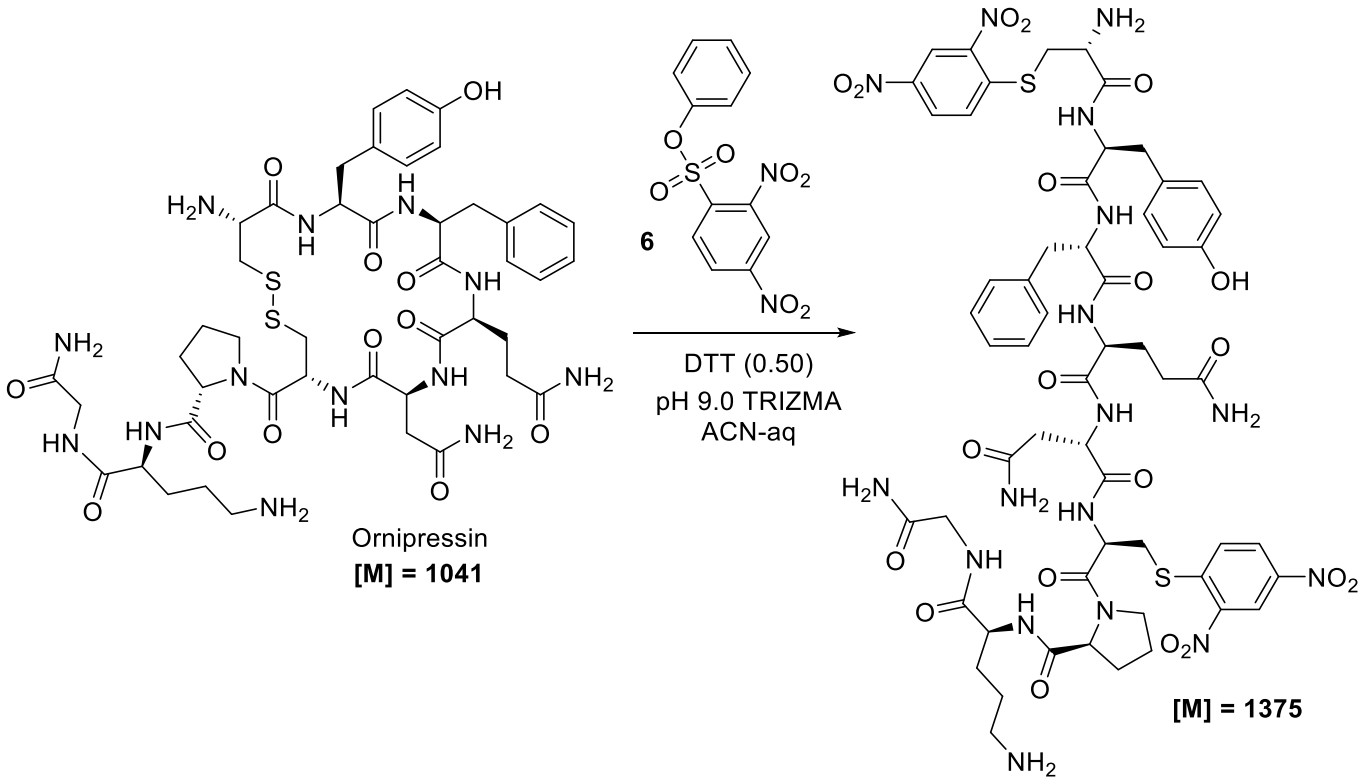


Figure 56. ESI-MS spectrum of the reaction mixture of [**6**+GSSG] followed by the addition of 0.25 equivalent of DTT after 24 hours.



8.5.4. Monoconjugation of Ornipressin in the presence of DTT



Scheme 17. Monoconjugation of Ornipressin with agent **6** in the presence of DTT.

To a solution of **6** (25.0 mM in acetonitrile, 200.0 µL) were added Ornipressin (12.5 mM in pH 9.0 TRIZMA buffer, 125.0 mM, 100.0 µL) and DTT (25.0 mM in water, 25.0 µL) and the mixture was diluted with 45.0 µL deionized water and 360.0 µL acetonitrile. After stirring for 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). A 25.0 µL aliquot of this mixture was diluted to 1.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (positive ion mode).

Figure 58. ESI MS spectrum of the reaction between **6** and Ornipressin.

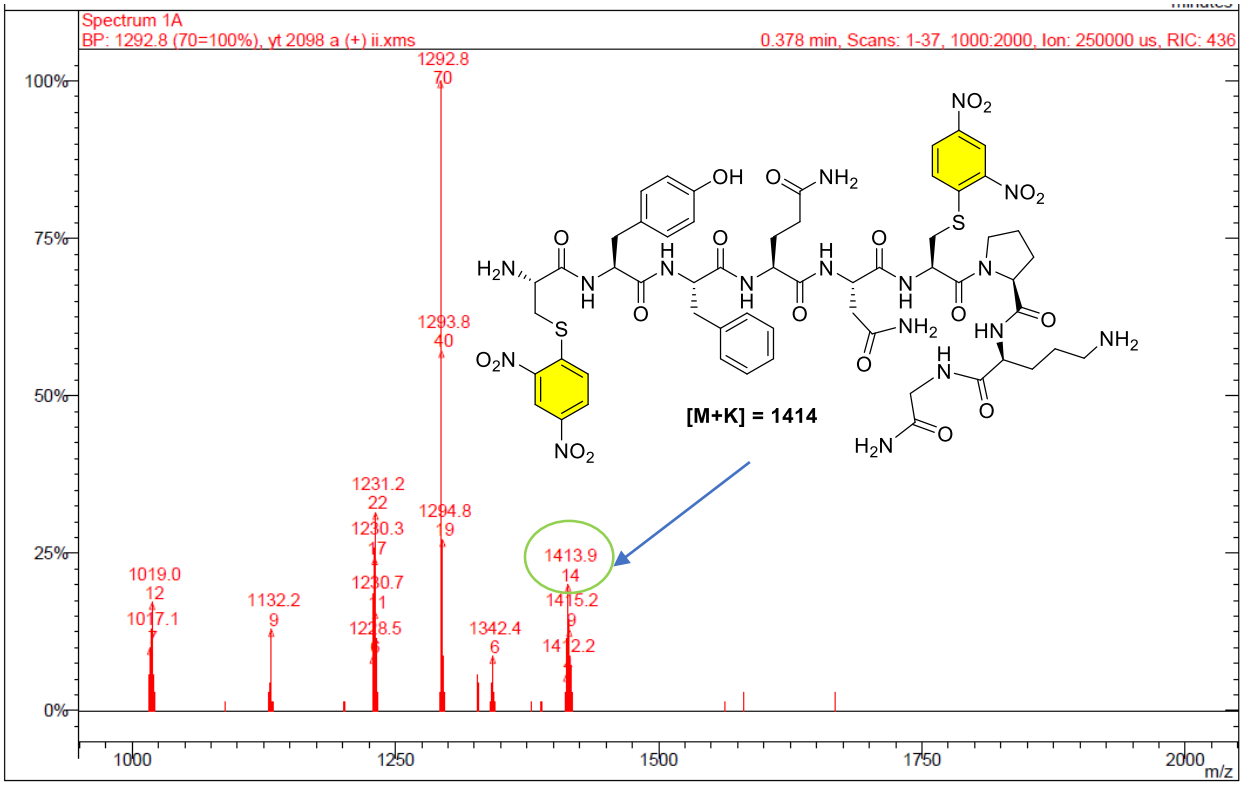
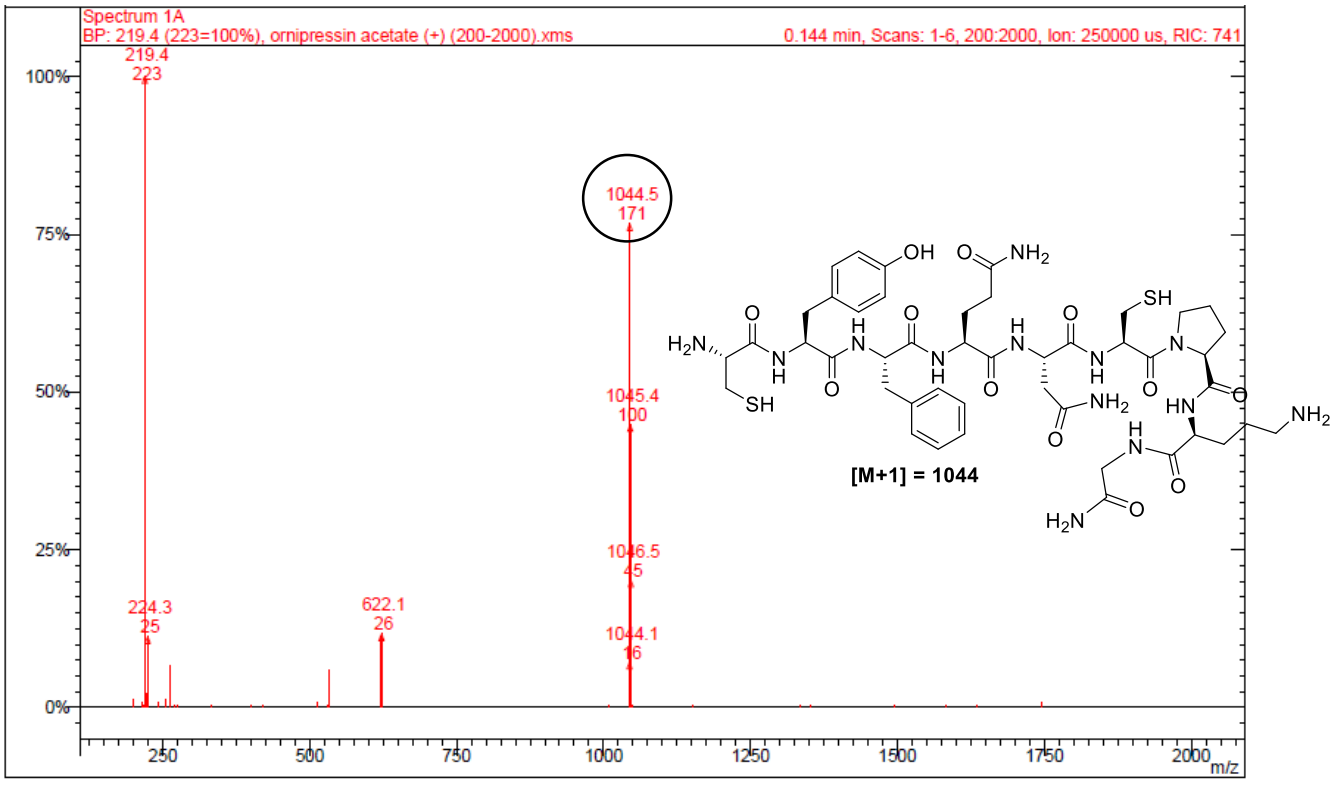
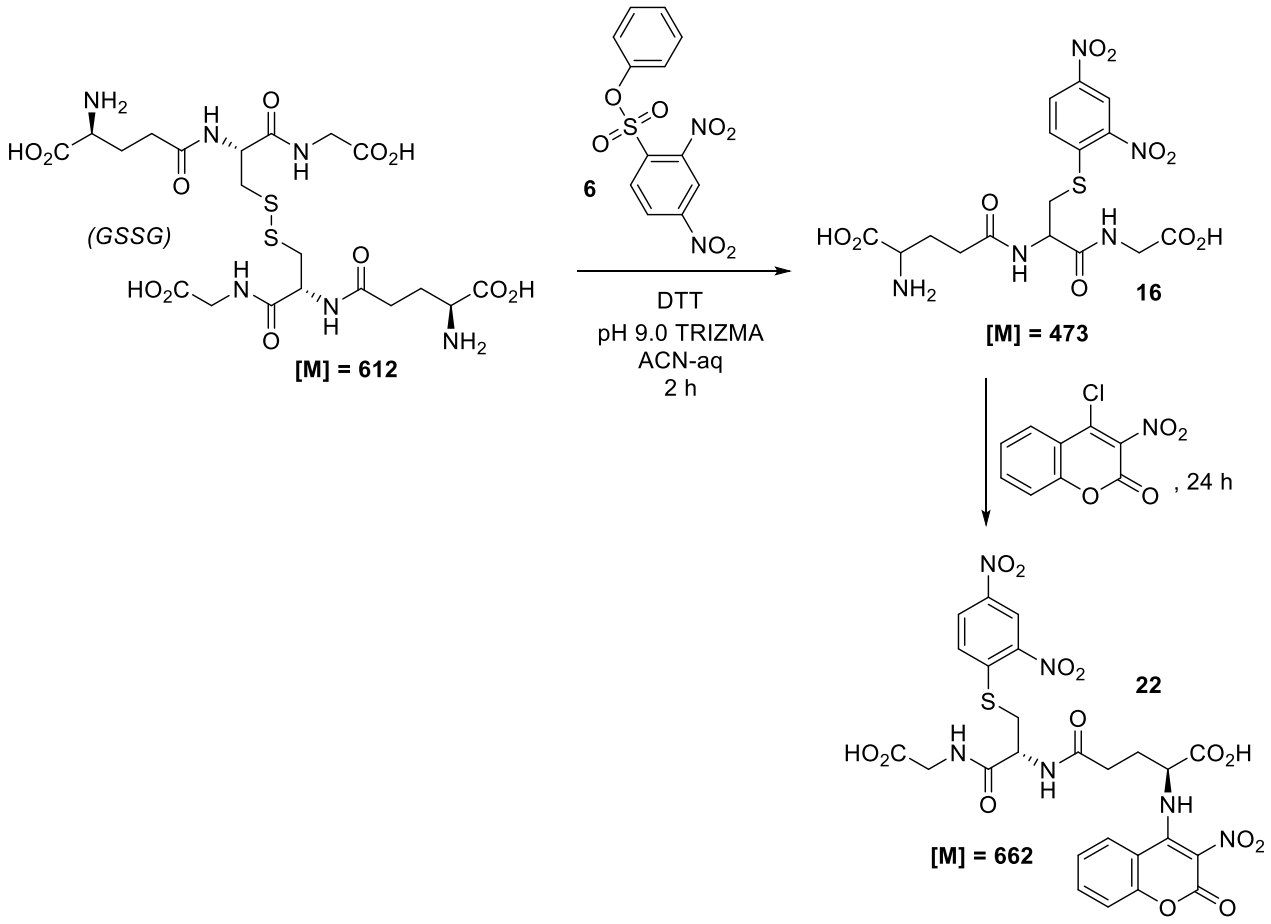


Figure 59. ESI MS spectrum of Ornipressin.



8.5.5. Diconjugation of GSSG in the presence of DTT

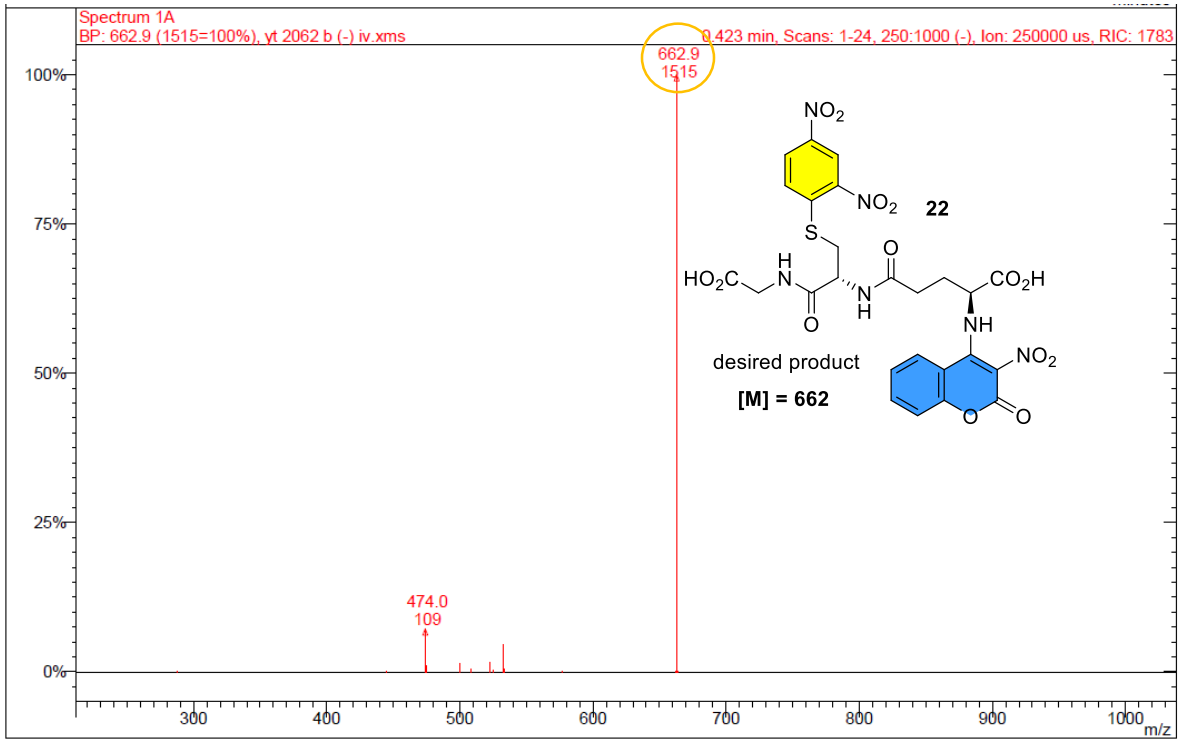
GSSG + DTT + **6** + **1**



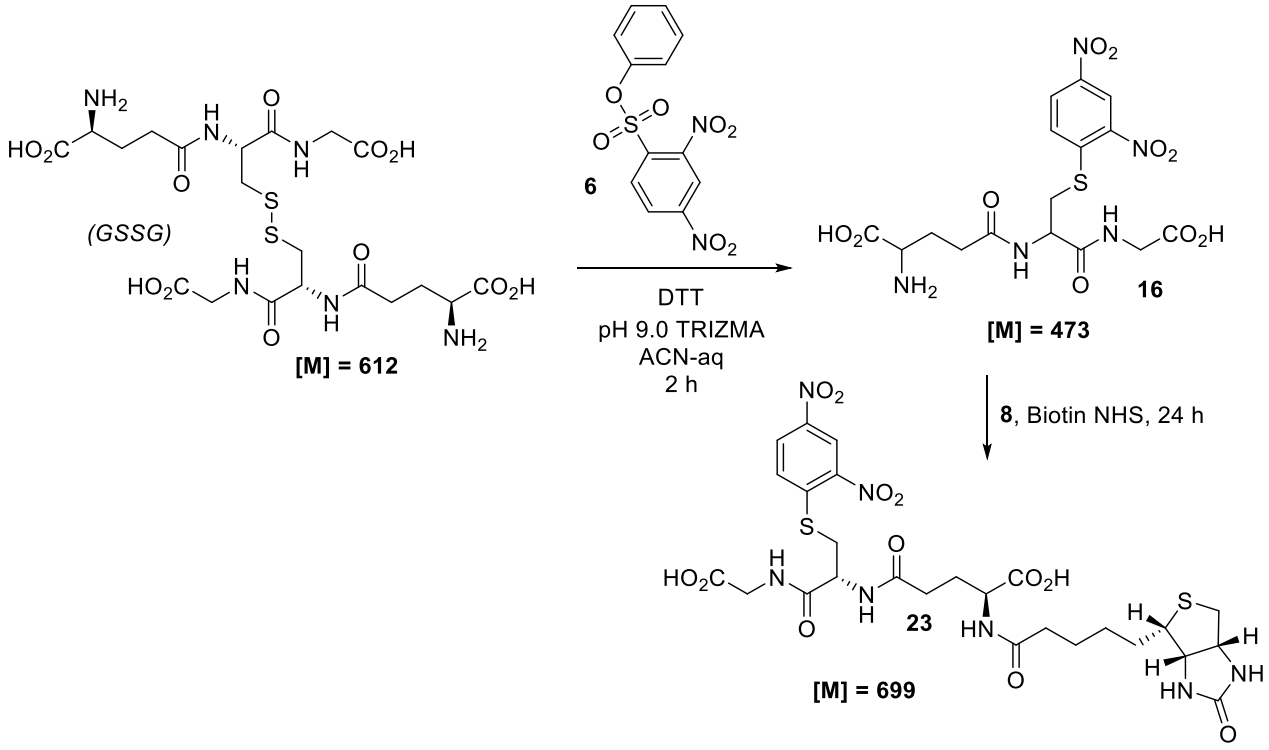
Scheme 18. Diconjugation of GSSG with agent **6** and **1** in the presence of DTT.

To a solution of **6** (25.0 mM in acetonitrile, 600.0 µL) were added GSSG (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 400.0 µL) and DTT (25.0 mM in deionized water, 100.0 µL) and the mixture was diluted with 0.8 mL of acetonitrile. After 2 hours, 1 equivalent of 4-chloro-3-nitrocoumarin, **1**, (25.0 mM in acetonitrile, 400.0 µL in ACN) was added. After stirring for 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted with 2.0 mL of deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 60. ESI-MS spectrum of the reaction mixture of [**6**+GSSG+DTT] followed by **1**.



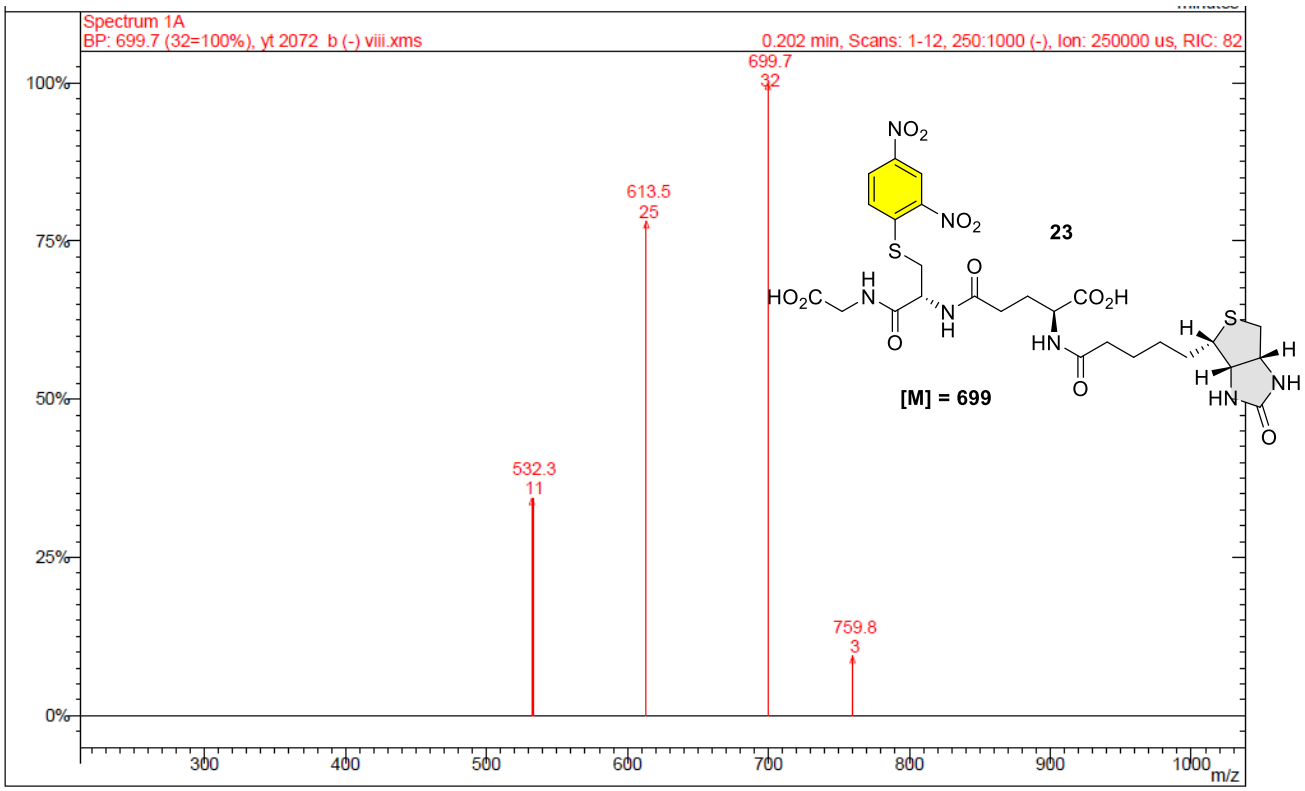
GSSG + DTT + DNP + **8** (Biotin NHS)



Scheme 19. Diconjugation of GSSG with agent **6** and **8** in the presence of DTT.

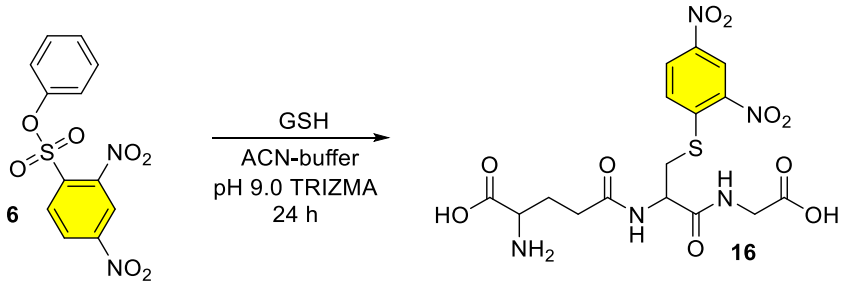
To a solution of **6** (25.0 mM in acetonitrile, 120.0 µL) were added GSSG (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 50.0 µL) and DTT (25.0 mM in water, 50.0 µL) and the mixture was diluted with 45.0 µL deionized water and 360.0 µL acetonitrile. After 2 hours, 1 equivalent of Biotin NHS, **8**, (12.5 mM in deionized water and acetonitrile (1:1), 120.0 µL) was added. After stirring for 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and the solution was diluted to 5.0 mL with deionized water and acetonitrile (1:1). An 8.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode). The MS analysis shows successful bioconjugation although GSSG was not completely reductively cleaved.

Figure 61. ESI-MS spectrum of the reaction mixture of [**6**+GSSG+DTT] followed by **8**.



9. Peptide conjugation at low concentrations

9.1. Monoconjugation at low concentrations

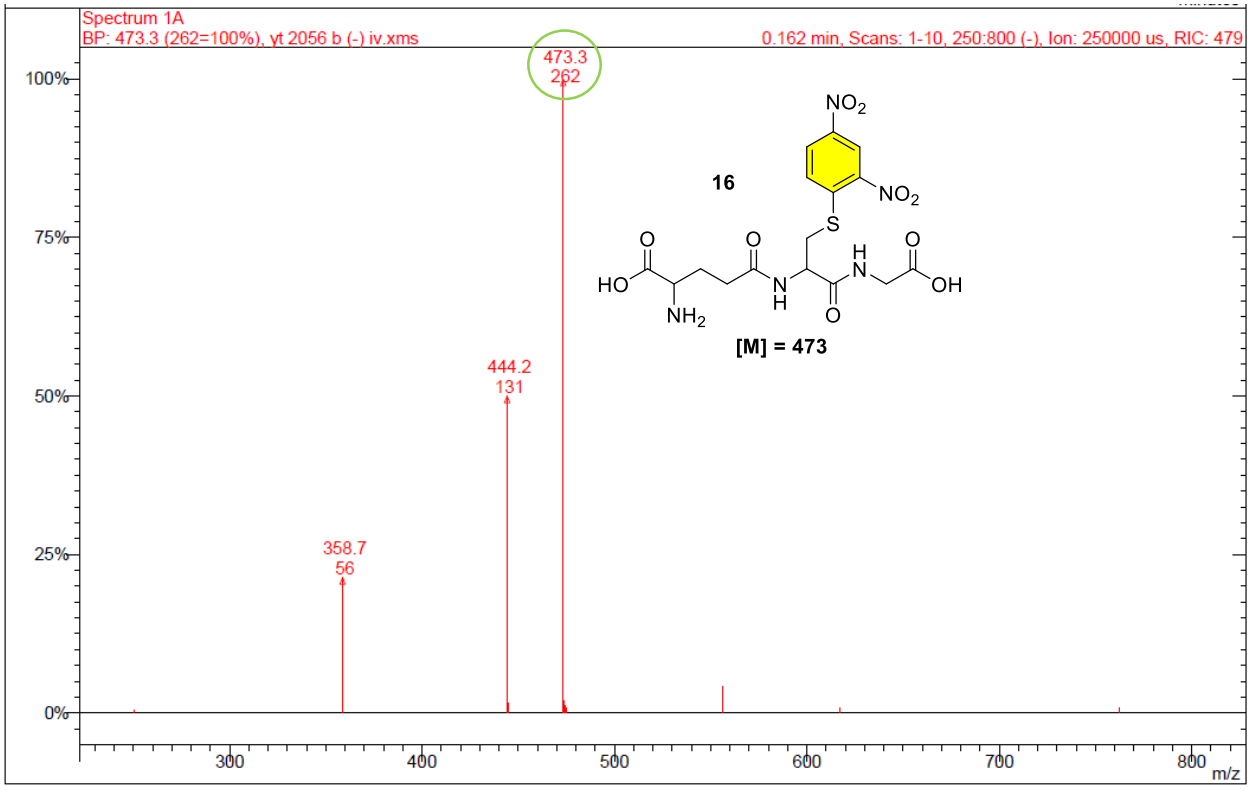


Scheme 20. Monoconjugation with **6** at low concentrations.

[GSH] = 500.0 µM

A solution of GSH (50.0 mM in pH 9.0 TRIZMA buffer, 500.0 mM, 20.0 µL) was diluted to a total volume of 1.40 mL using deionized water (180.0 µL), pH 9.0 TRIZMA buffer (500.0 mM, 200.0 µL) and acetonitrile (1000.0 µL). A solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added to the above mixture and allowed to stir. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and a 32.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

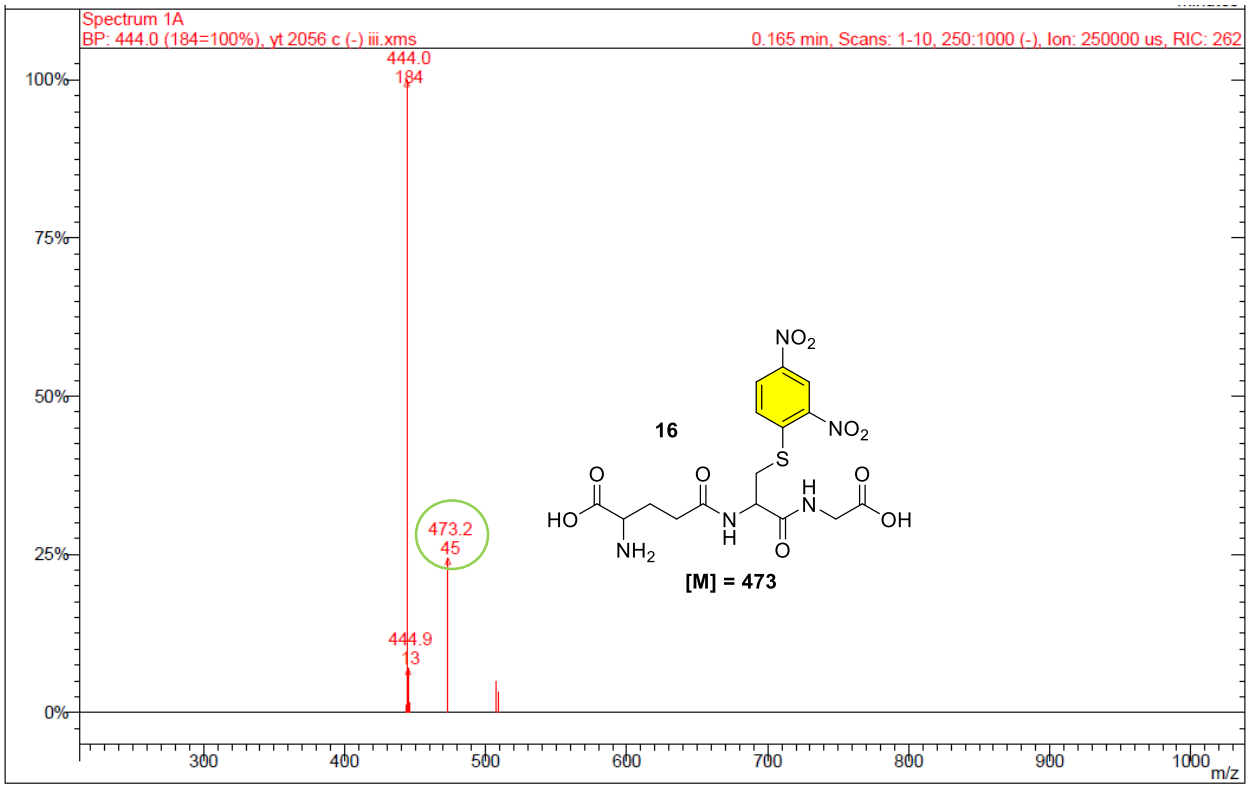
Figure 62. ESI-MS spectrum of the reaction mixture of [**6**+GSH] at 500.0 µM.



[GSH] = 50.0 µM

A solution of GSH (50.0 mM in pH 9.0 TRIZMA buffer, 500.0 mM, 2.0 µL) was diluted to a total volume of 1.40 mL using deionized water (198.0 µL), pH 9.0 TRIZMA buffer (500.0 mM, 200.0 µL) and acetonitrile (1000.0 µL). A solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added to the above mixture and allowed to stir. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and a 320.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

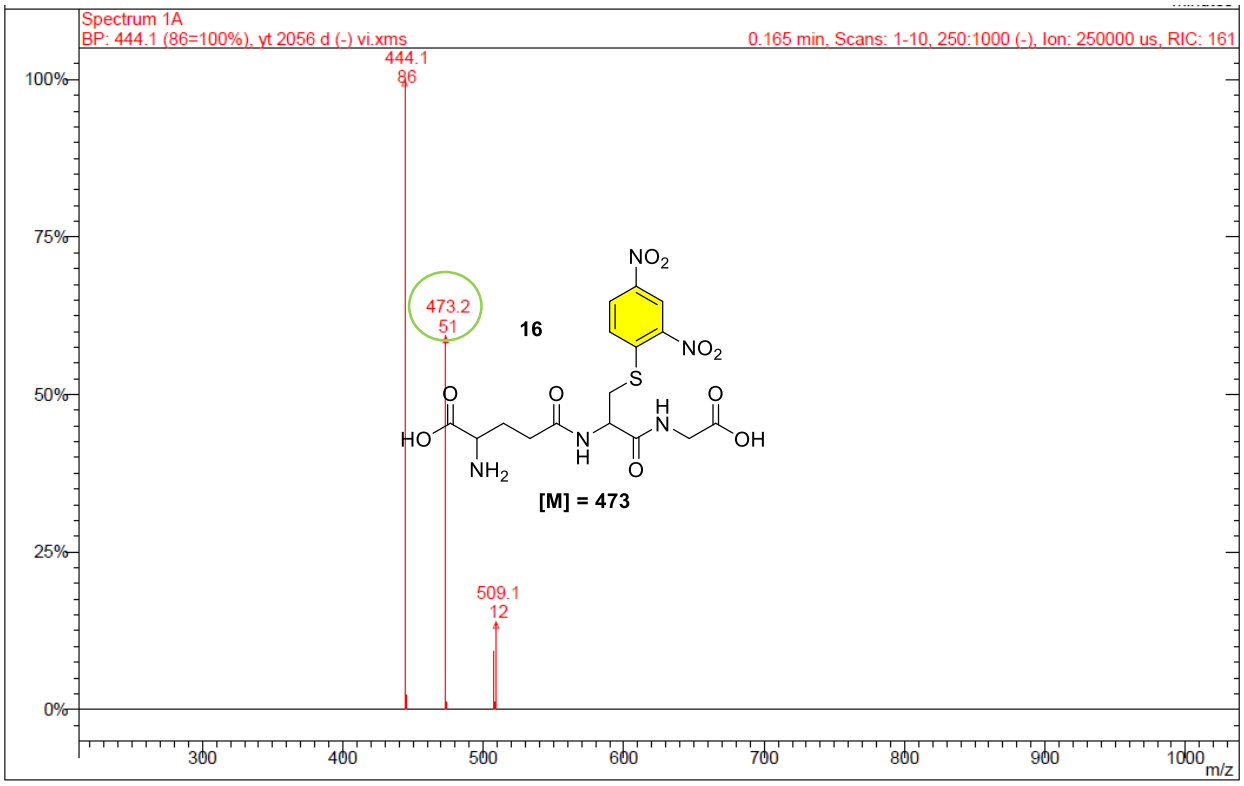
Figure 63. ESI-MS spectrum of the reaction mixture of [**6**+GSH] at 50.0 µM.



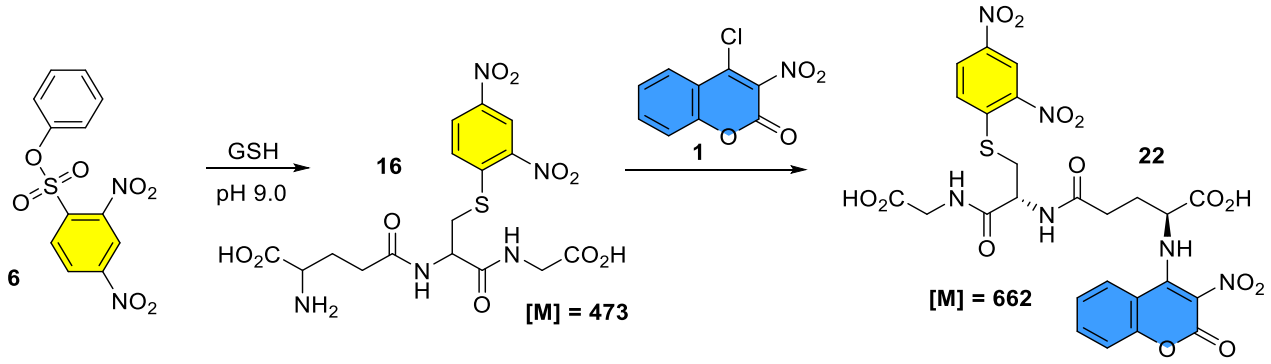
[GSH] = 25.0 µM

A solution of GSH (50.0 mM in pH 9.0 TRIZMA buffer, 500.0 mM, 2.0 µL) was diluted to a total volume of 3.40 mL using deionized water (598.0 µL), pH 9.0 TRIZMA buffer (500.0 mM, 200.0 µL) and acetonitrile (2600.0 µL). A solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added to the above mixture and allowed to stir. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and a 300.0 µL aliquot of this mixture was diluted to 0.9 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 64. ESI-MS spectrum of the reaction mixture of [**6**+GSH] at 25.0 µM.



9.2. Diconjugation at low concentrations

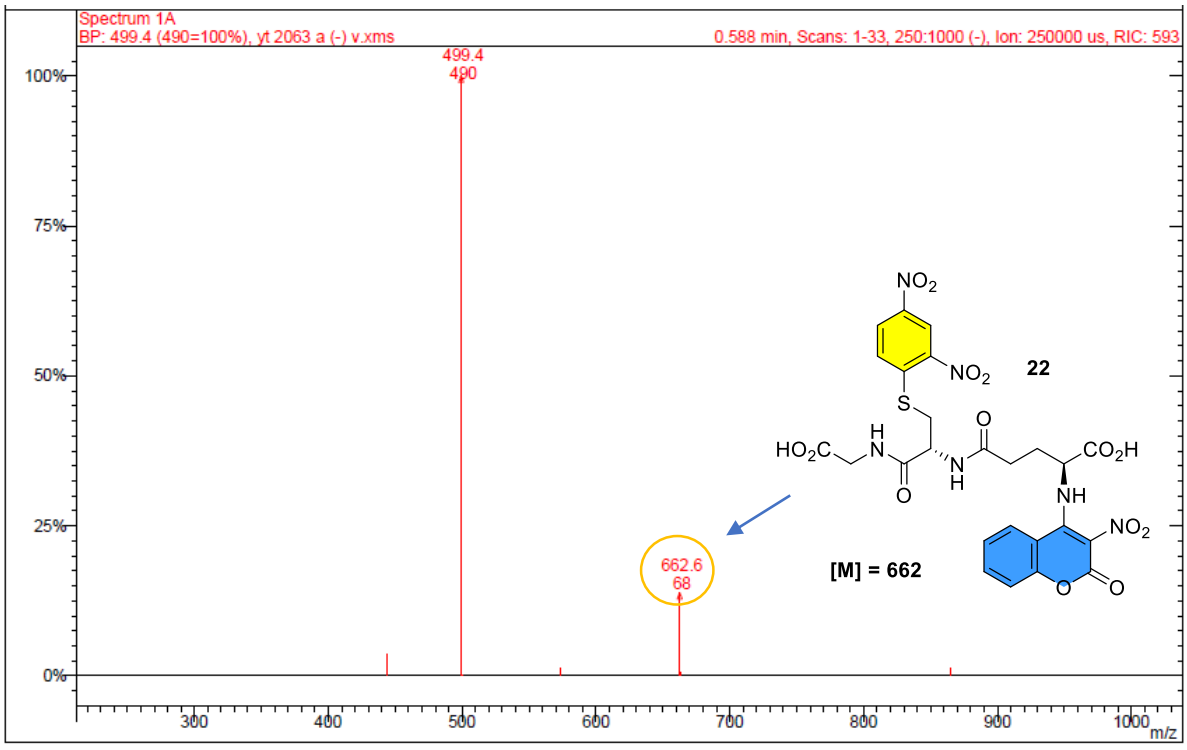


Scheme 21. Diconjugation with **6** and **1** at low concentrations.

[GSH] = 500.0 µM

A solution of GSH (50.0 mM in pH 9.0 TRIZMA buffer, 500.0 mM, 10.0 µL) was diluted to a toal volume of 0.70 mL using deionized water (90.0 µL), pH 9.0 TRIZMA buffer (500.0 mM, 100.0 µL) and acetonitrile (500.0 µL). A solution of **6** (25.0 mM in acetonitrile, 300.0 µL) was added to the above mixture and allowed to stir for 24 hours. Then, bioconjugation agent 4-chloro-3-nitrocoumarin, **1**, (25.0 mM in acetonitrile, 300.0 µL) was added. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and a 32.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

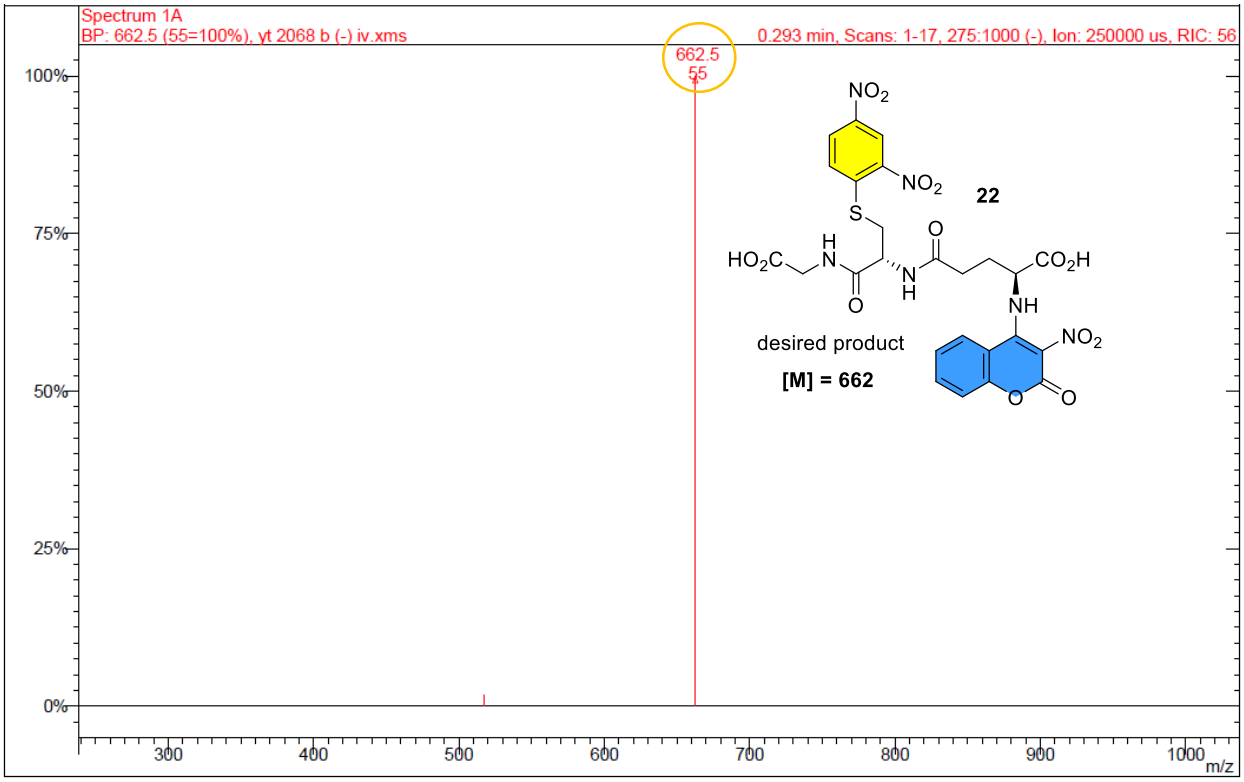
Figure 65. ESI-MS spectrum of the reaction mixture of [**6**+GSH+**1**] at 500.0 µM.



[GSH] = 250.0 µM

A solution of GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 20.0 µL) was diluted to a total volume of 1.40 mL using deionized water (380.0 µL) and acetonitrile (1000.0 µL). A solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added to the above mixture and allowed to stir for 24 hours. Then, bioconjugation agent 4-chloro-3-nitrocoumarin, **1**, (25.0 mM in acetonitrile, 400.0 µL) was added. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and a 64.0 µL aliquot of this mixture was diluted to 2.0 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 66. ESI-MS spectrum of the reaction mixture of [**6**+GSH+**1**] at 250.0 µM.



[GSH] = 25.0 µM

A solution of GSH (25.0 mM in pH 9.0 TRIZMA buffer, 250.0 mM, 20.0 µL) was diluted to a total volume of 1.40 mL using deionized water (398.0 µL) and acetonitrile (1000.0 µL). A solution of **6** (25.0 mM in acetonitrile, 600.0 µL) was added to the above mixture and allowed to stir for 24 hours. Then, bioconjugation agent 4-chloro-3-nitrocoumarin, **1**, (25.0 mM in acetonitrile, 400.0 µL) was added. After 24 hours, 1.0 M formic acid (20.0 µL in deionized water) was added and a 300.0 µL aliquot of this mixture was diluted to 0.9 mL with deionized water and acetonitrile (1:1) for ESI-MS analysis (negative ion mode).

Figure 67. ESI-MS spectrum of the reaction mixture of [**6**+GSH+**1**] at 25.0 µM.