Electronic Supplementary Material (ESI) for Chemical Science.

Luminol Anchors Improve the Electrochemical-Tyrosine-Click Labelling of Proteins

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I. Generalities

Organic synthesis

Reactions requiring anhydrous conditions were performed under positive nitrogen or argon pressure and glassware was dehydrated by three vacuum-argon cycles before each use. Most of the chemical reagents were purchased from Sigma Aldrich®, Carbosynth®, Acros Organics®, Alfa Aesar® or TCI Chemical®. Reagents and solvents such as DCM, THF, Et_2O, TEA, Pyridine, MeOH, Toluene, MeCN or DMF were purchased anhydrous from Sigma Aldrich®. All reagents were stored according to the detailed specifications and used without further purification. Usual reaction monitoring was carried out with thin layer chromatography (TLC) on Merck 60 F_{254} silica gel plates. Revelations were performed under UV light (254 nm) or by dipping in a solution of cerium molybdate, potassium permanganate, sulfuric acid or vanillin and subsequently heated. Purification by silica gel chromatography were carried on Silica 60 M 0.04 – 0.063 mm. Microwaves chemical synthesis were conducted in sealed vials and carried out with a CEM Discover SP-Microwave Synthesizer. ^{1}H and ^{13}C NMR were recorded on Bruker Avance 300 or Bruker Avance 400 spectrometers. NMR spectra were assigned on the basis of the following 1D and 2D experiments: ^{1}H, ^{13}C, DEPT-135, COSY, HSCQ, HMBC and NOESY. All chemical shifts (δ) are shown in ppm on the X-axis using the residual solvent as internal standard (^{1}H NMR: $CDCl_3$ = 7.26 ppm, D_2O = 4.79 ppm, CD_3OD = 3.31 ppm, DMSO-d^6 = 2.50 ppm and ^{13}C NMR: $CDCl_3$ = 77.16 ppm, CD_3OD = 49.00 ppm, DMSO-d^6 = 39.52 ppm). Coupling constants (*J*) are reported in Hz and peak multiplicities are noted according to the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet, br = broad signal. Atom numbering used for NMR attribution is different from the numbers used in nomenclature of compounds. High-resolution mass spectrometry (HRMS) was recorded on a Waters Xevo GL-XS Qtof spectrometer coupled with an Acquity H-class LC apparatus. Ionization sources were performed with

the available methods (ESI^+, ESI^-, $ASAP^+$, $ASAP^-$). A tolerance of 5 ppm was applied between calculated and experimental values.

Electrochemistry

Cyclic and multicyclic voltammetry experiments were conducted using a 3mm diameter glassy carbon electrode (GCE) as working electrode and a flat rod-shaped platinum counter electrode. Reference electrodes used were Saturated Calomel Electrode (SCE) or Ag/AgCl. Before each analysis, electrodes were washed with acetone and distillated water, and GCE was re-polished on high grit sand paper (<1200 grit) to prevent potential passivation. Chronocoulommetry experiments (eY-click) were performed using a conventional three-electrode electrochemical system. Tyrosine free amino acid and polypeptides were tagged using a 50 mL carbon crucible or a 12 cm^2 graphite plate as working electrode, a flat rod-shaped platinum counter electrode, and the reference electrod was SCE. Voltage control was performed using SP-300 potentiostat. Lower scale experiments (proteins, enzymes, antibody) were performed using ElectraSyn 2.0 materials connected to SP-50 potentiostat for voltage control. eY-click were performed in a 5 mL vial, with graphite plate as working electrode, platinum plate as counter electrode, and the reference was Ag/AgCl. Before each experiment, electrodes used were washed with acetone and distillated water, and working electrodes were re-polished on high grit sand paper (<1200 grit). The 50 mL graphite crucible and 12 cm^2 graphite plate were purchased from Mersen®. Electrosynthesis equipment ElectraSyn 2.0 was purchased from IKA® as well as size adapted glass vials and sets of standard size electrodes (4 cm x 0.8 cm x 0.15 cm): graphite, glassy carbon, reticulated vitreous carbon, platinum. The Ag/AgCl reference was a thin silver rod submerged with 1 M aqueous KCl solution and protected from electrolysis mixtures by a porous frit glass. SP-300 and SP-50 potentiostats were purchased from BioLogic. All data were recorded using EC-Lab software.

Bioconjugation

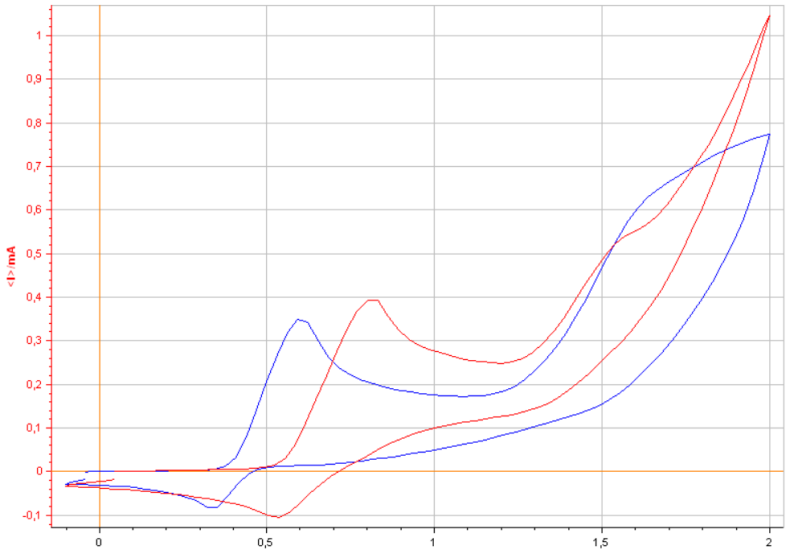
Aqueous buffers were obtained from Sigma Aldrich®. Mutant peptides (TAAQNLYEK, HAWQNLYEK and GWVTDGVSFFLK) were purchased lyophilized from ThermoFischer Scientific® with >95% purity. α-Chymotrypsinogen A, Myoglobin, Bovine Serum Albumin, Jack bean α-Mannosidase and Glucose Oxidase were purchased from Sigma Aldrich®. Trastuzumab was purchased from Carbosynth®. Functionalized cyclooctynes reagents were purchased from specialized suppliers (Jena Biosciences®, Chematech®, Fluorochem®). All biological reagents and octynes were stored according to the detailed specifications and used without further purification. Amino acid sequence and theoretical mass of all proteins investigated were obtained by crossing informations mainly from Uniprot (https://www.uniprot.org) and PDB (https://www.rcsb.org) database. Solvent accessible area of each tyrosines on proteins were obtained from PDBePISA (https://www.ebi.ac.uk/pdbe/prot_int/pistart.html). Conditions for procedure, analysis and characterization of peptides and proteins will be described in the appropriate parts (modification of tyrosine/peptides/proteins).

II. Cyclic Voltammetry

Cyclic voltammetry measurements of **7** and **9** were performed at a graphite carbone electrode as anode (2 mm disc), a platinum plate as cathode and reference was Ag/AgCl. 30 mM of reagent, 1:1 MeCN/PB 50 mM pH 7.4, RT, 100 mV/s.

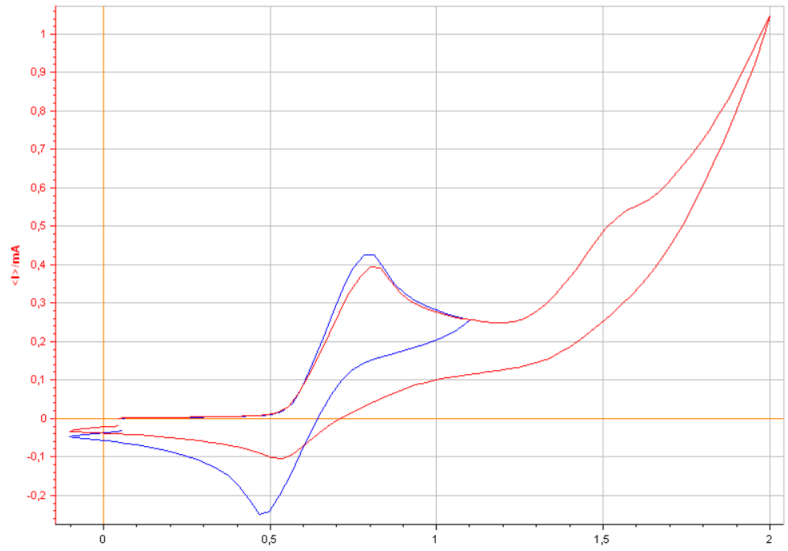
(a)

Blue: Reagent NMePhUr **7**
(Up to 2V)
Red: Reagent NMeLum **9**
(Up to 2V)



(b)

Blue: NMeLum **9**
(Up to 1.1V)
Red: NMeLum **9**
(Up to 2V)



➔ Second small and non-reversible anodic event at 1.5V vs Ag/AgCl (red curve). Partial loss of reversibility of first oxidation event at 0.75V vs Ag/AgCl.

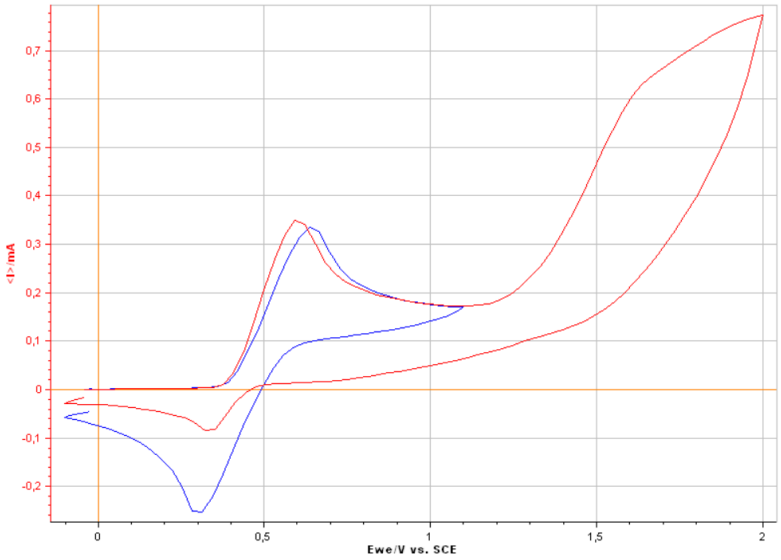
(c)

Blue: NMePhUr **7**

(Up to 1.1V)

Red: NMePhUr **7**

(Up to 2V)



➔ Second small and non-reversible anodic event at 1.6V vs Ag/AgCl (red curve). Loss of reversibility of first oxidation event at 0.6V vs Ag/AgCl.

III. eY-click procedures and characterizations

Modification of tyrosine

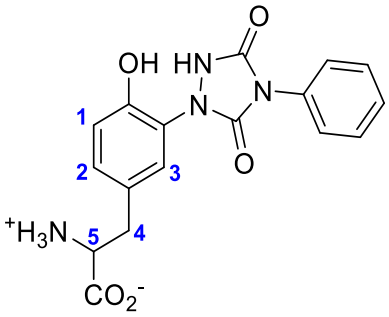
Procedure

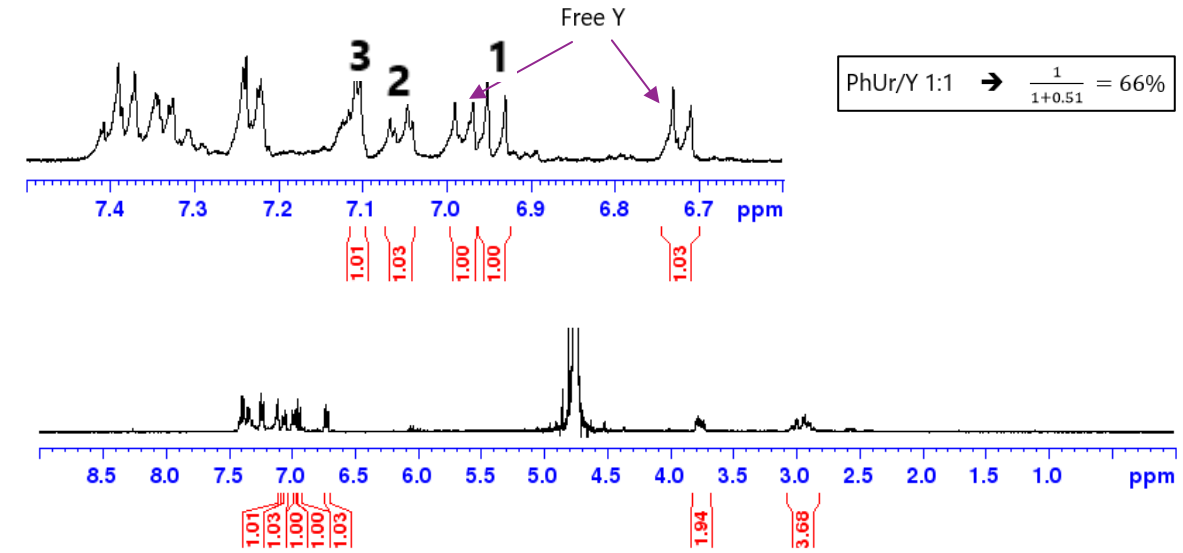
Three electrodes system used was 50 mL carbon crucible as anode, a flat rod-shaped platinum as cathode and the reference electrod was SCE. Appropriate voltage was applied to a mixture of labelling reagent (**1**, **7** or **9,** 10 or 20 mg, 0.05 or 0.10 mmol, 1 or 2 equiv., final concentration 1-2 mM) and L-tyrosine (10 mg, 0.05 mmol, 1 equiv., final concentration 1 mM) in 100 mM phosphate buffer (50 mL). Electrolysis was performed at room temperature (stirring: 700 rpm) until the decay of the current reached more than 90% (2-5h depending on the reagent). After modification, the sample was lyophilized, dissolved in D_2O and directly analysed by ^1H NMR and HRMS to estimate and confirm conversion of tyrosine to the conjugated compound.

^1H NMR analysis of mixtures

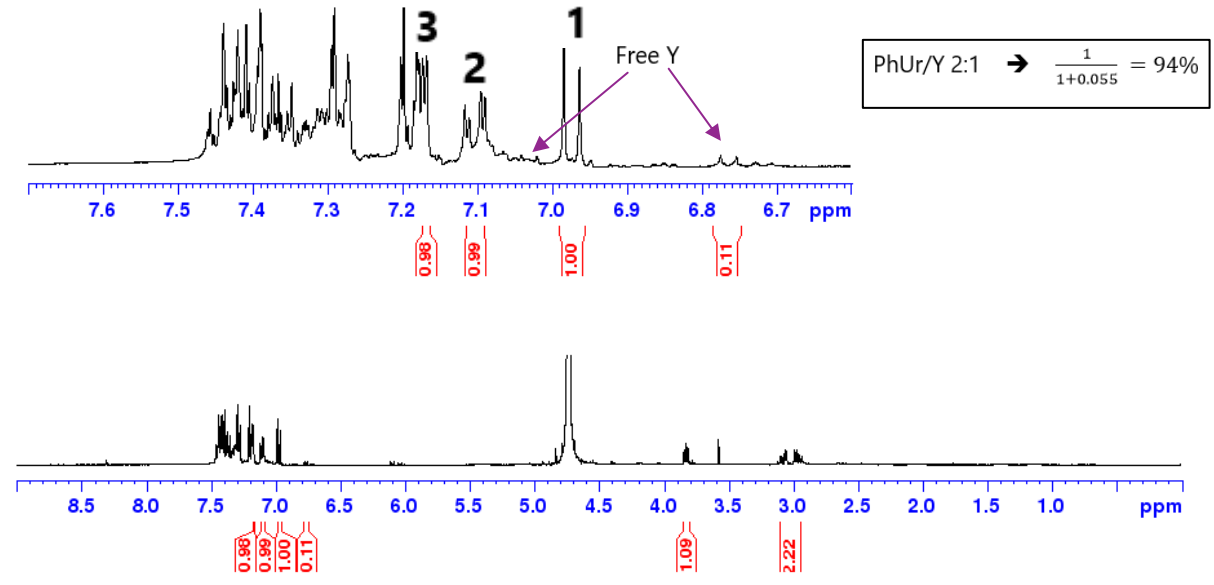
Tyrosine conversion was calculated using the ratio: %Y-clicked / (%Y-clicked + %Y). %Y-clicked was extrapolated by integrating H^1 (of the Y-clicked compounds) which is a clear doublet. Phenol moiety of free Y appears as two AB system multiplets. %Y was obtained from the clear 6.8-6.7 ppm multiplet (supposed to be 2H). When the H^1 doublet and the second Y multiplet overlapped (for NMePhUr and NMeLum), the first Y multiplet was set to 2H, and the value of H_1 was calculated by subtracting 2H to the overall integration.

1-Tyr - ^{1}H NMR (400.16 MHz, D_2O, 298.15 K): δ_H 7.46-7.17 (m, H^{Ar}), 7.17 (d, $^{4}J_{3\text{-}2}$ = 2.13 Hz, 1H, H^3), 7.10 (dd, $^{3}J_{2\text{-}1}$ = 8.45 Hz, $^{4}J_{2\text{-}3}$ = 2.13 Hz, 1H, H^2), 6.97 (d, $^{3}J_{1\text{-}2}$ = 8.45 Hz, 1H, H^1), 3.83 (m, 1H, H^5), 3.10-2.91 (m, 2H, H^4). HRMS (ESI^-): *m/z* calculated for $C_{17}H_{15}N_4O_5$ $[M\text{-}H]^-$ 355.1042 found 355.1050



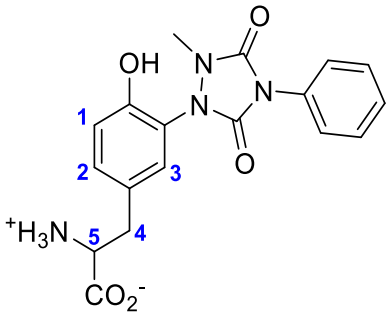


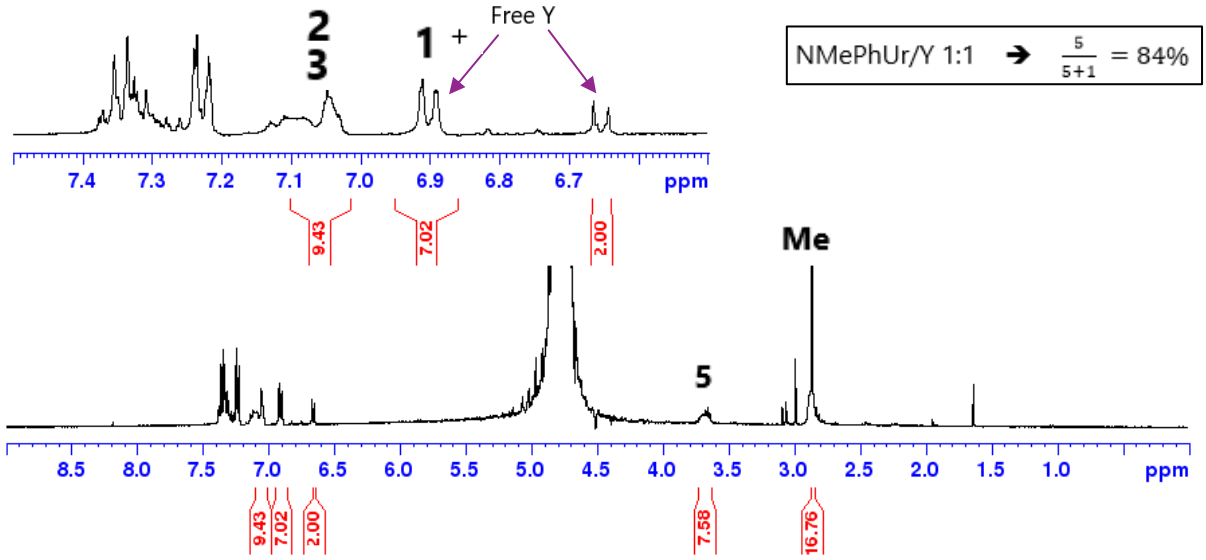
PhUr/Y 1:1 → $\frac{1}{1+0.51}$ = 66%

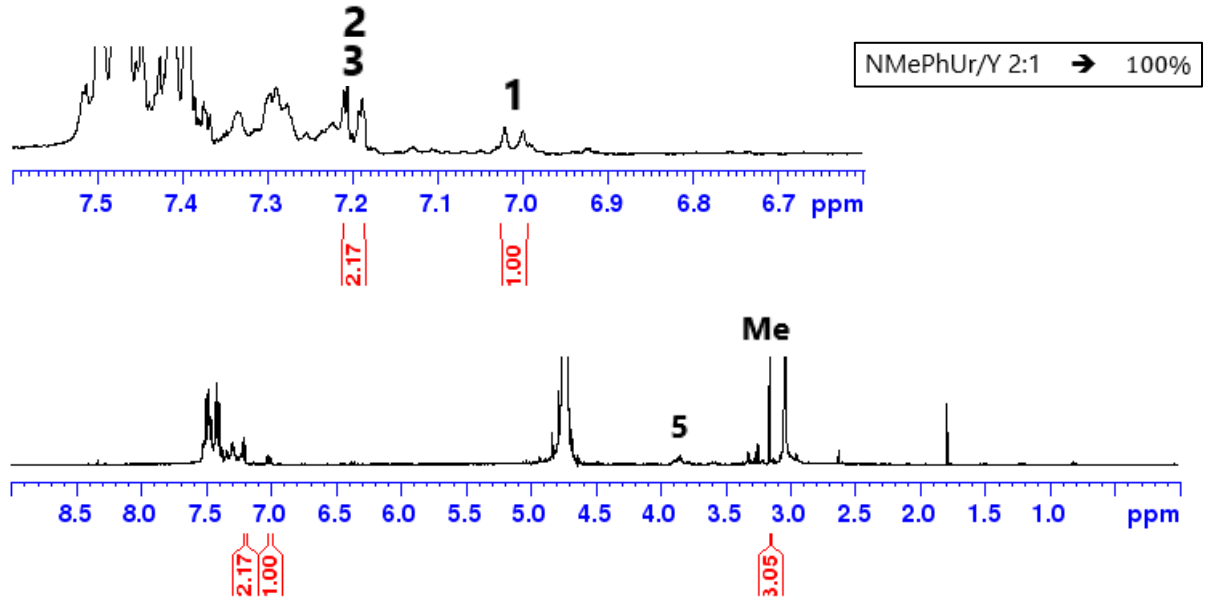


PhUr/Y 2:1 → $\frac{1}{1+0.055}$ = 94%

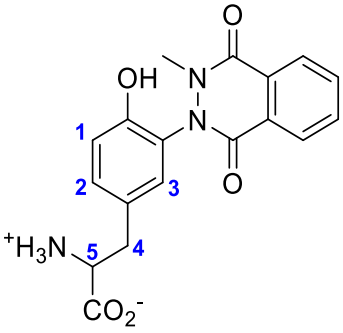
7-Tyr - ^{1}H NMR (400.16 MHz, D_2O, 298.15 K): δ_H 7.52-7.22 (m, H^{Ar}), 7.23-7.18 (m, 2 H, H^2, H^3), 6.97 (d, $^{3}J_{1\text{-}2}$ = 8.34 Hz, 1H, H^1), 3.84 (m, 1H, H^5), 3.19 (s, 3H, Me), 3.08-2.95 (m, 2H, H^4). HRMS (ESI^-): *m/z* calculated for $C_{18}H_{17}N_4O_5$ $[M\text{-}H]^-$ 369.1200 found 369.1199

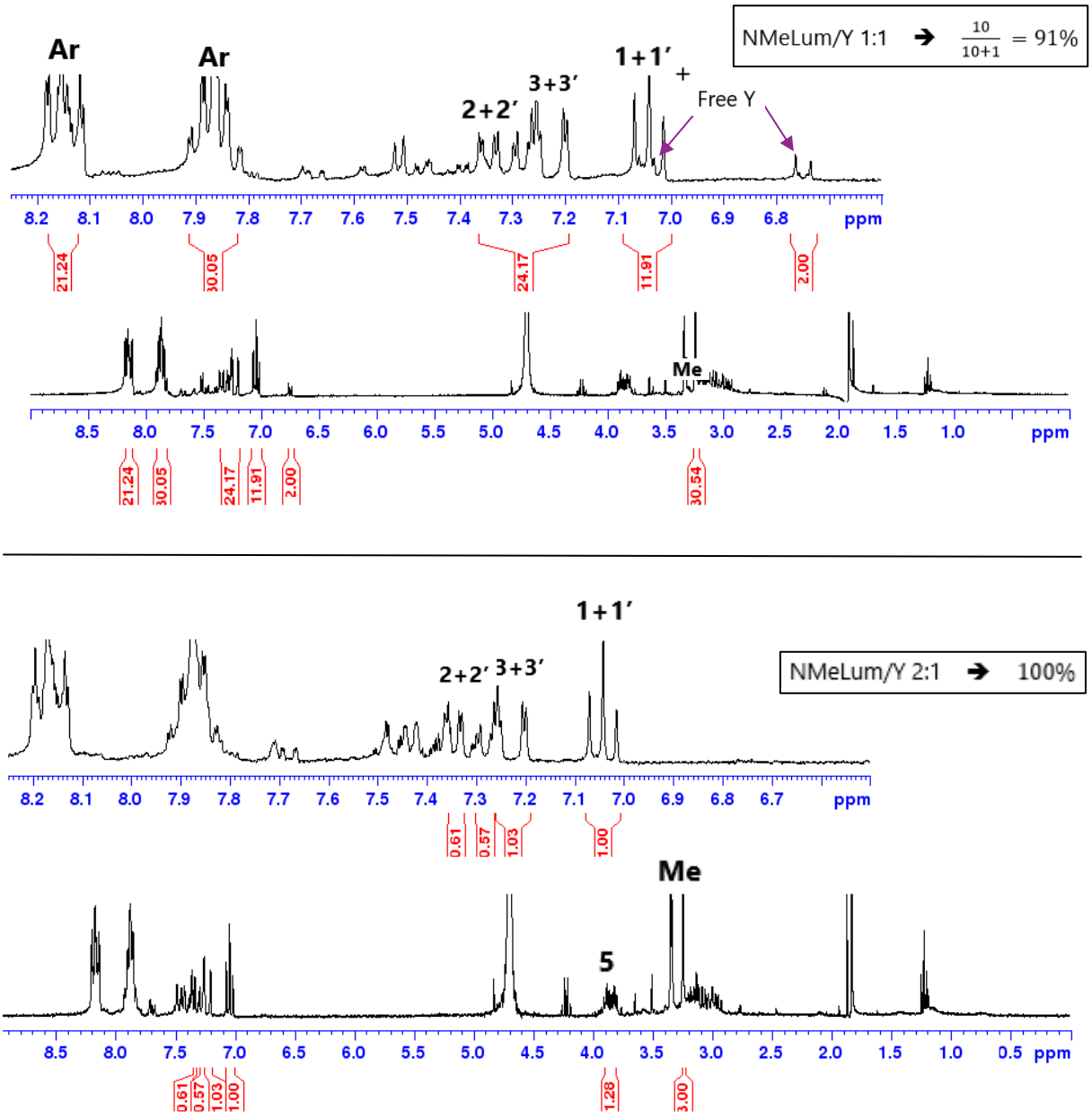






10-Tyr - ^{1}H NMR (400.16 MHz, D_2O, 298.15 K): Mixture of 2 conformers, each H will appear as H^{x} and $H^{x'}$. Integrated both conformers at once (both dd for H^2, both d for H^1 and both d for H^3). δ_H 8.21-8.11 (m, 2H, H^{Ar}), 7.93-7.80 (m, 3H, H^{Ar}), 7.34 (dd, $^{3}J_{2\text{-}1}$ =8.50 Hz, $^{4}J_{2\text{-}3}$ = 2.40 Hz, 1H, H^2), 7.27 (dd, $^{3}J_{2\text{-}1}$ =8.53 Hz, $^{4}J_{2\text{-}3}$ = 2.25 Hz, 1H, $H^{2'}$), 7.25 (d, $^{4}J_{3\text{-}2}$ = 2.40 Hz, 1H, H^3), 7.19 (d, $^{4}J_{3\text{-}2}$ = 2.25 Hz, 1H, $H^{3'}$), 7.05 (d, $^{3}J_{1\text{-}2}$ = 8.50 Hz, 1H, H^1), 7.02 (d, $^{3}J_{1\text{-}2}$ = 8.50 Hz, 1H, $H^{1'}$), 3.86 (m, 1H, H^5), 3.23 (s, 3H, Me), 3.21-2.90 (m, 2H, H^4). HRMS (ESI^-): *m/z* calculated for $C_{18}H_{16}N_3O_5$ $[M\text{-}H]^-$ 354.1090 found 354.1090





Modification of polypeptides

Procedure

In a 15 mL plastic vial, three electrodes system used was 12 cm^2 graphite plate as anode, a flat rod-shaped platinum as cathode and the reference electrod was SCE. Appropriate voltage was applied to a mixture of reagent (**2**, **8** or **10**, 25.0 μmol, 20 equiv., final concentration 2 mM) and peptide (1.25 μmol, 1 equiv., final concentration 0.1 mM) in 100 mM phosphate buffer (12.5 mL). Electrolysis was performed at room temperature (stirring: 700 rpm) during 7h. 100 μL aliquots were taken periodically and were directly analysed by UPLC-HRMS with the system described below.

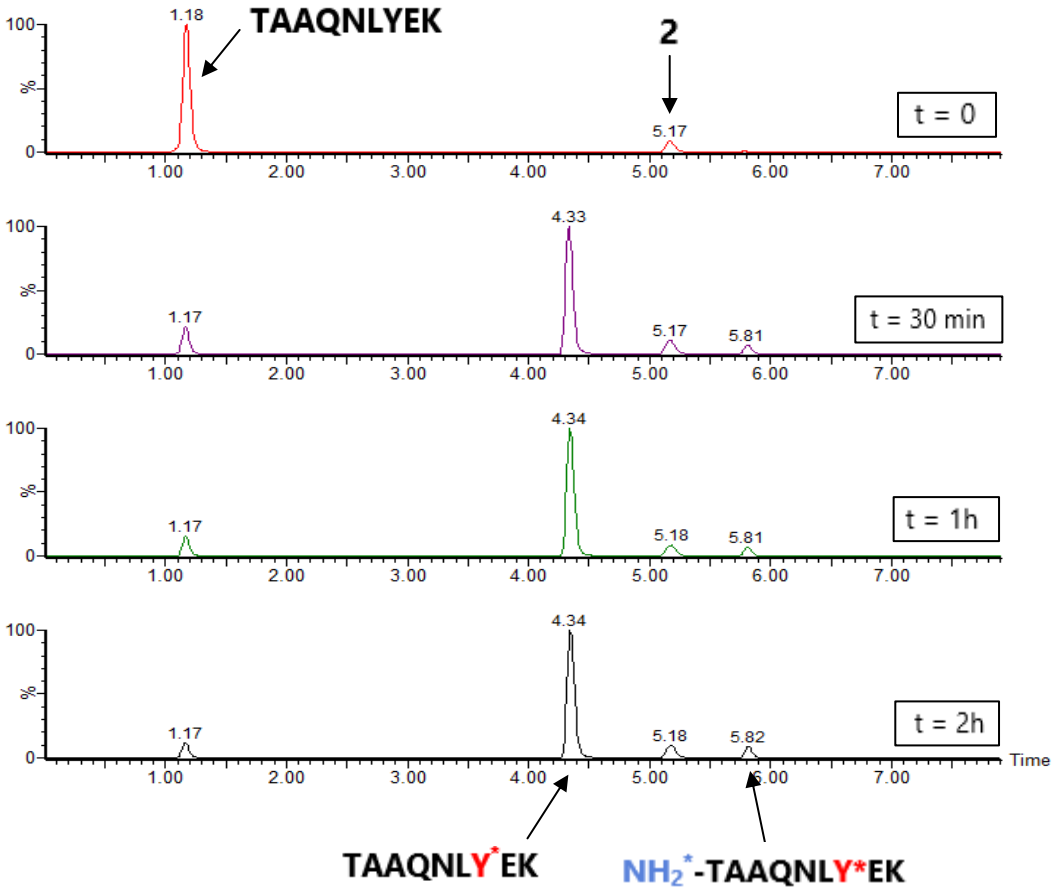
Chromatography and mass spectrometry analysis of peptides

Samples were analyzed by ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS). UPLC-HRMS analyses were performed on a Synapt G2 HRMS Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the positive mode and an Acquity H-Class UPLC device (Waters Corporation, Milford, MA, USA). Samples were injected (10 μL) onto a Acquity CSH C18 Peptide (1.7 μm; 2.1 × 100 mm; 130 Å) reversed-phase LC columns held at 60 °C. Peptides were then eluted over 10 min with a linear gradient of mobile phase B (100% acetonitrile) in mobile phase A (5% acetonitrile), each containing 0.1% formic acid, and at a flow rate of 250 μL/min. Mobile phase B was kept constant at 1% for 0.5 min, then linearly increased from 1% to 80% for 7.5 min, kept constant for 0.5 min, returned to the initial condition over 0.5 min, and kept constant for 0.5 min before the next injection. The full-HRMS mode was applied for peptides and proteins detection (mass-to-charge ratio (*m/z*) range 100-4,000) at a mass resolution of 25,000 full-widths at half maximum. The ionization settings were as follows: capillary voltage, +3 kV; cone voltage, 30 V; desolvation gas (N_2) flow rate, 1000 L/h; desolvation gas/source temperatures, 450/120 °C. Leucine enkephalin solution (2 μg/mL, 50% acetonitrile) was infused at a constant flow rate of 10 μL/min in the lockspray channel, allowing for correction of the measured *m/z* throughout the batch (theoretical *m/z* 556.2771 in

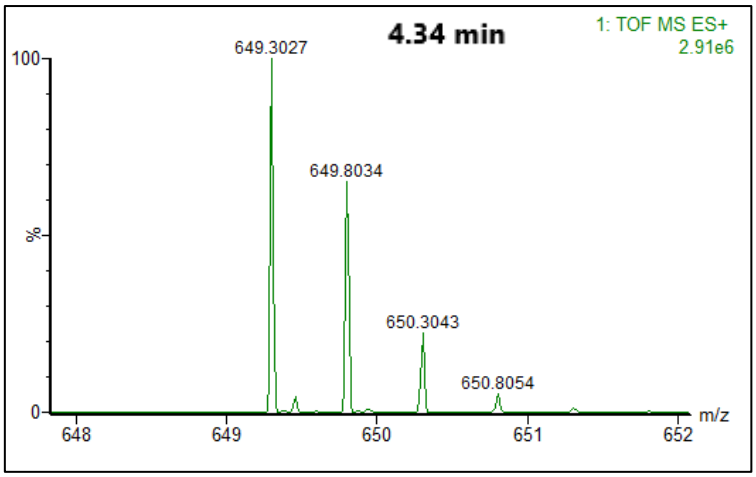
positive mode). Data acquisition and processing were achieved using MassLynx® software (version 4.1, Waters Corporation). MS profiles of the peaks on the chromatogram allowed to identify peptides and confirm conversion to the conjugated compounds. MS/MS fragmentation was then performed on the major ion peak (mono, double or triple charged) to identify the location of conjugation.

TAAQNLYEK + 2 (PhUr)

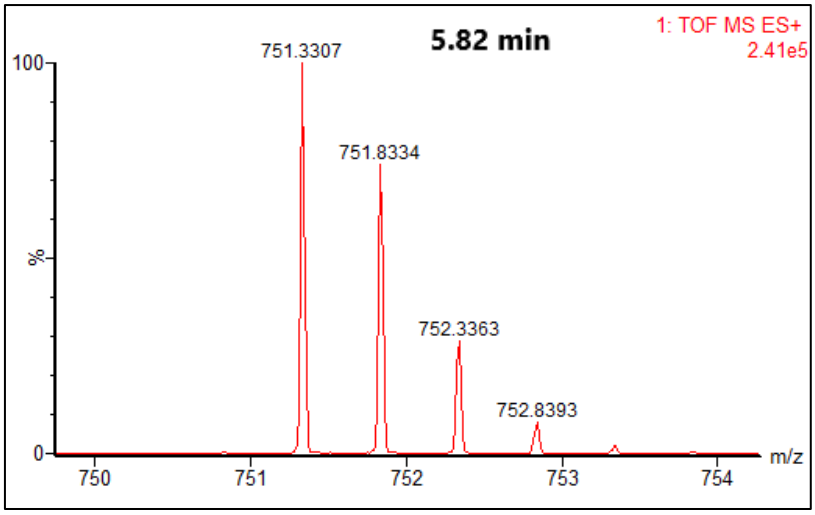
UPLC profile



MS profiles



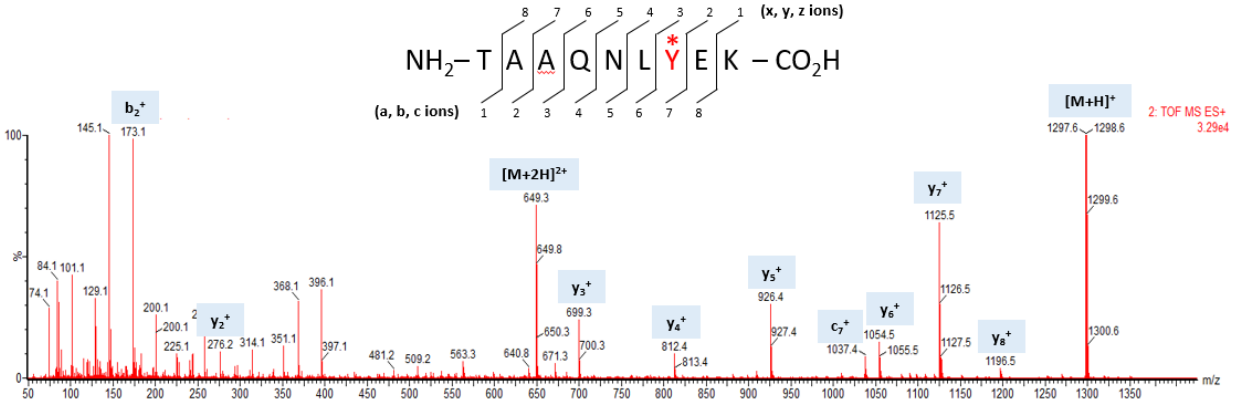
NH_2-TAAQNLY*EK-CO_2H
MW = 1296.5 Da
$[M+2H]^{2+}$ expected = 649.25 found 649.30



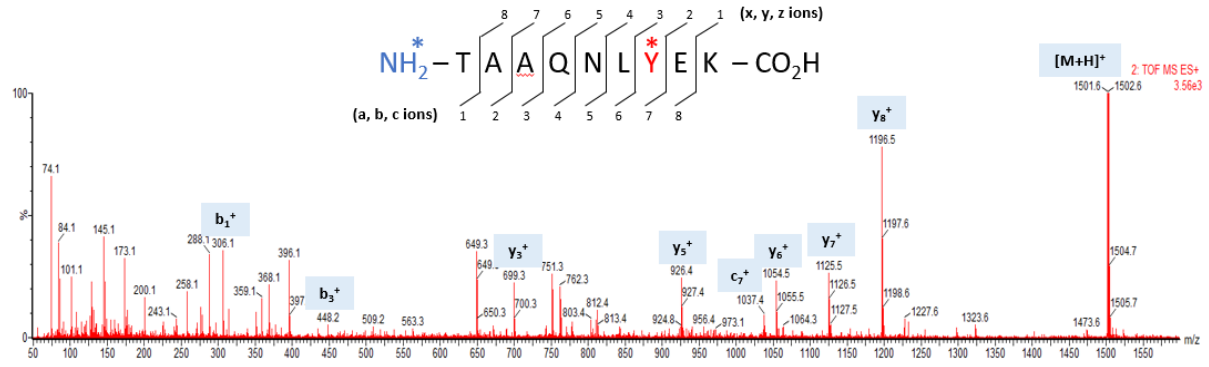
NH_2^*-TAAQNLY*EK-CO_2H
MW = 1500.6 Da
$[M+2H]^{2+}$ expected = 751.3 found 751.33

MS/MS fragmentation

(a) 4.34 min peak (649.3027)

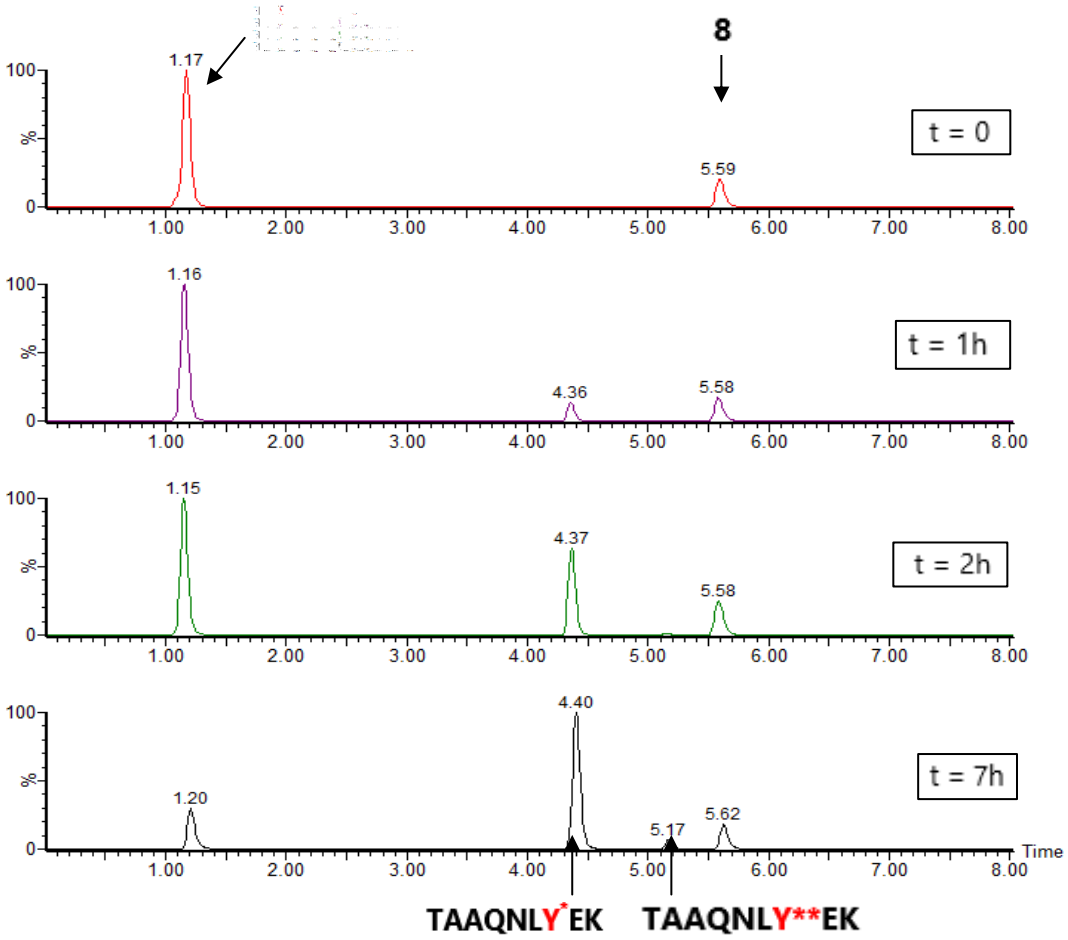


(b) 5.82 min peak (751.3307)

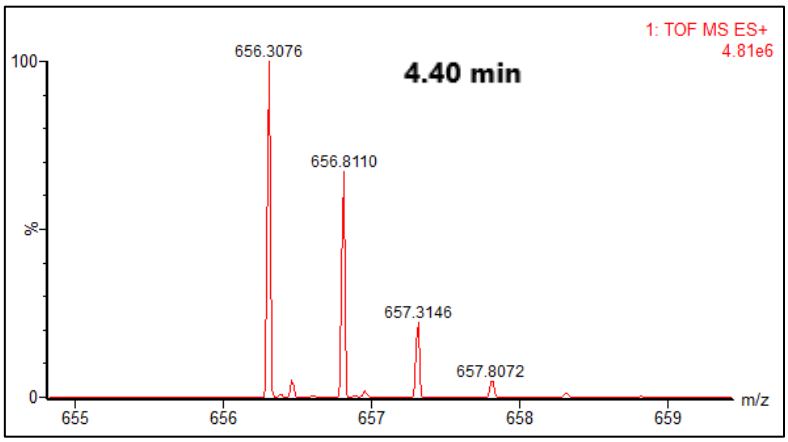


TAAQNLYEK + 8 (NMePhUr)

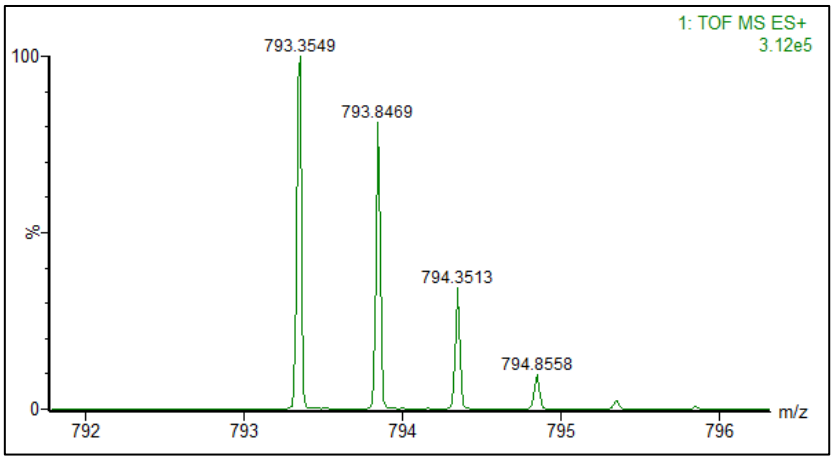
UPLC profile



MS profiles



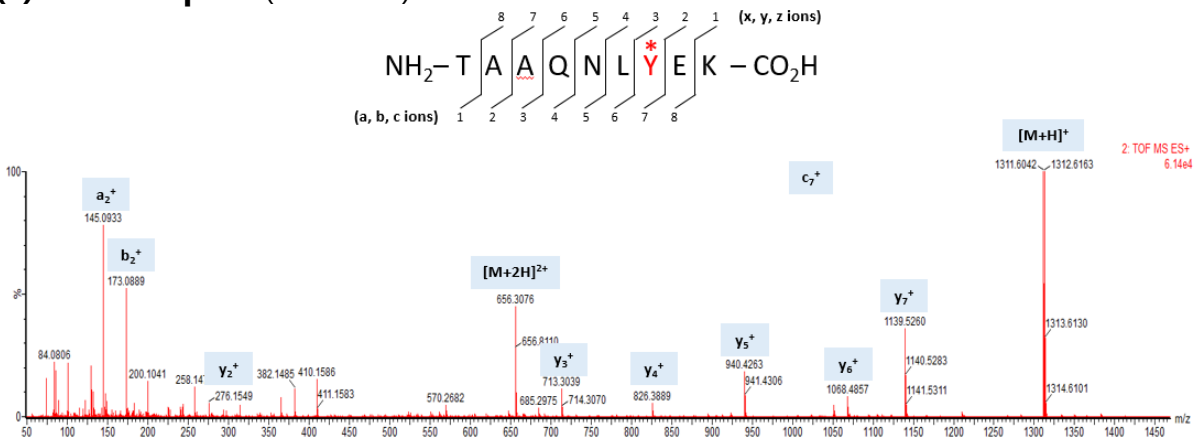
NH_2-TAAQNLY*EK-CO_2H
MW = 1310.5 Da
$[M+2H]^{2+}$ expected = 656.2 **5.17 min** .30



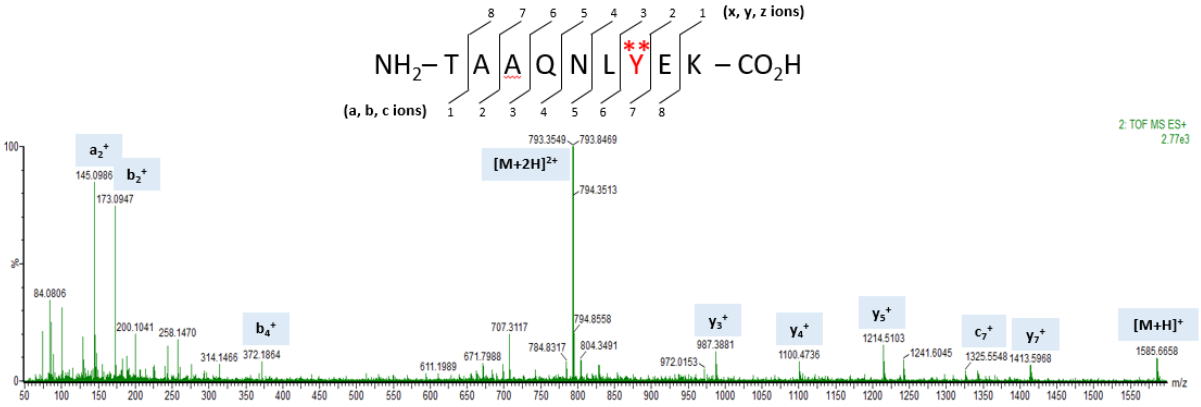
NH_2-TAAQNLYEK-CO_2H**
MW = 1584.5 Da
$[M+2H]^{2+}$ expected = 793.25 found 793.35

MS/MS fragmentation

(a) 4.40 min peak (656.3076)

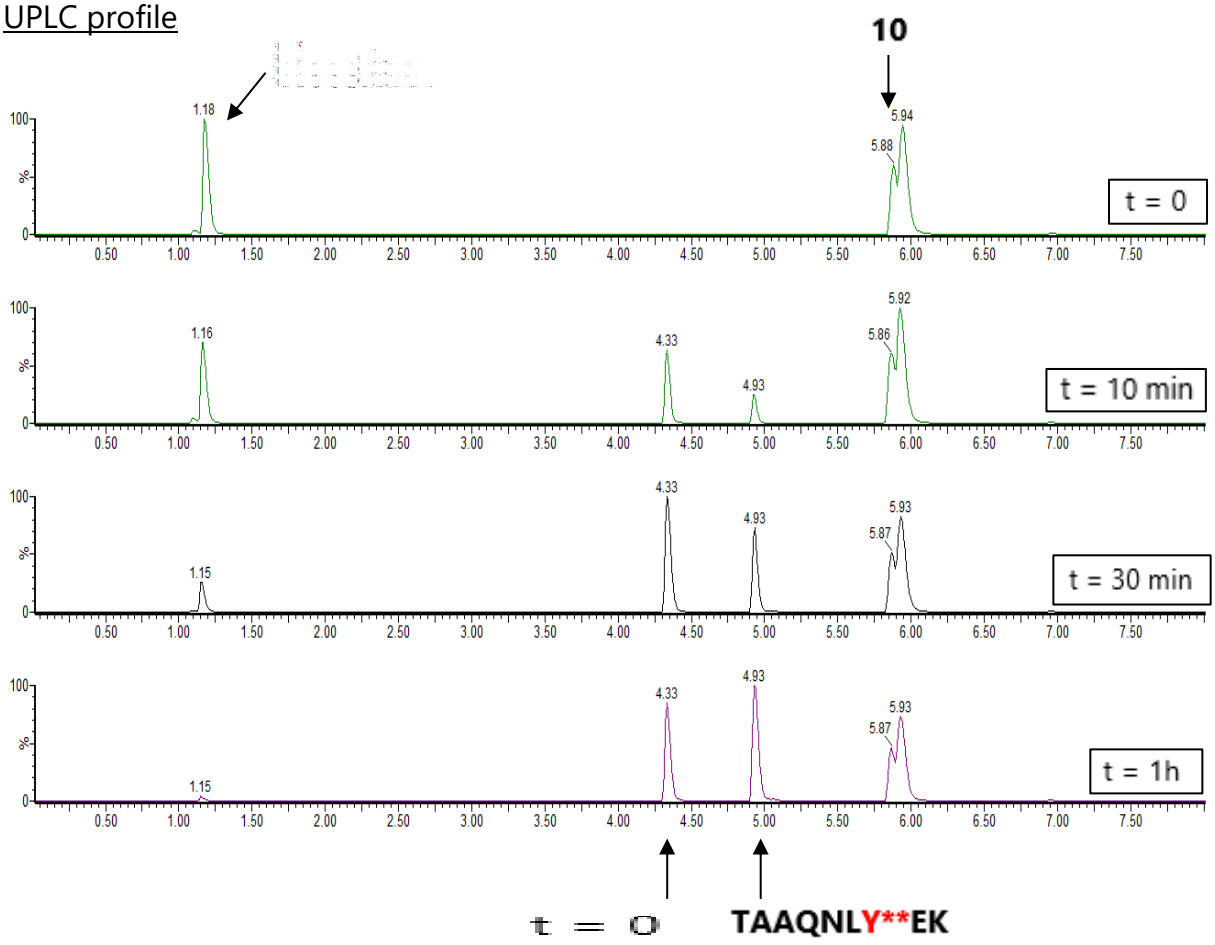


(b) 5.17 min peak (793.3549)

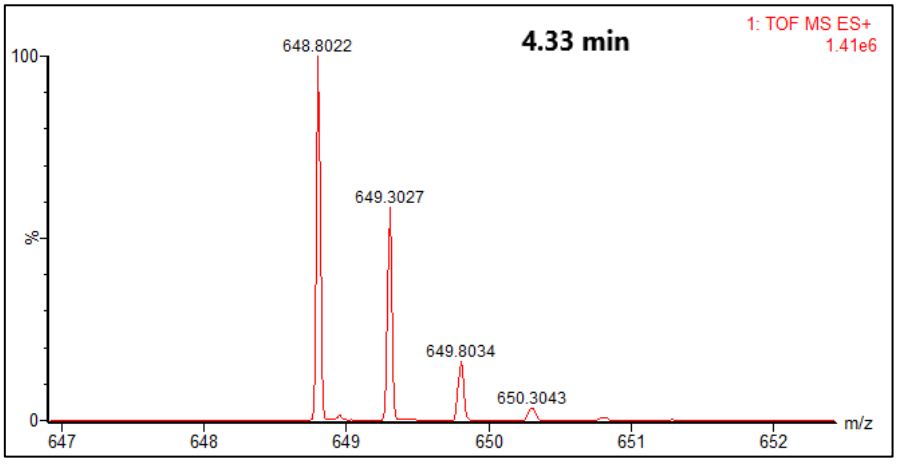


TAAQNLYEK + 10 (NMeLum)

UPLC profile



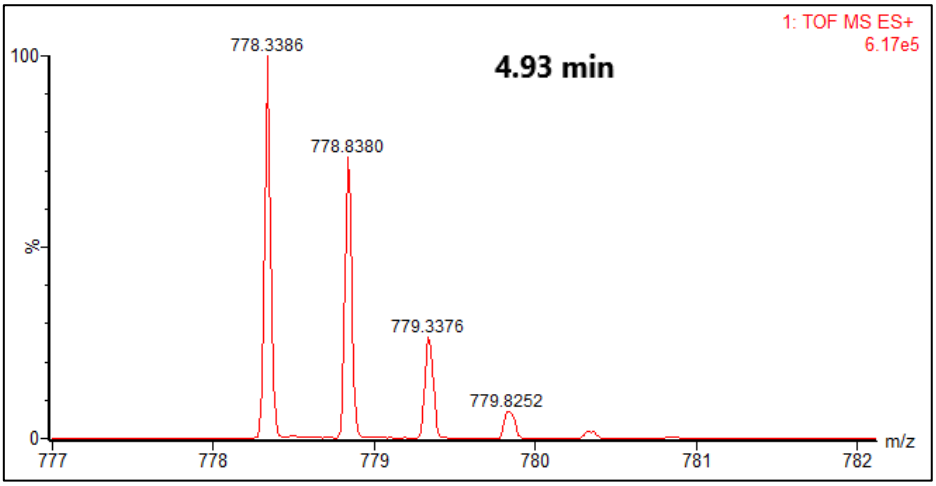
MS profiles



NH_2-TAAQNLY*EK-CO_2H

MW = 1295.5 Da

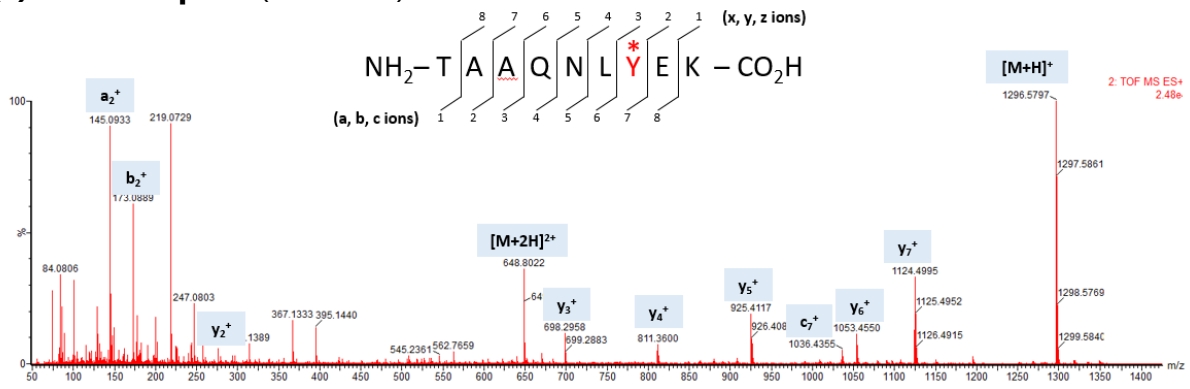
$[M+2H]^{2+}$ expected = 648.75 found 648.80



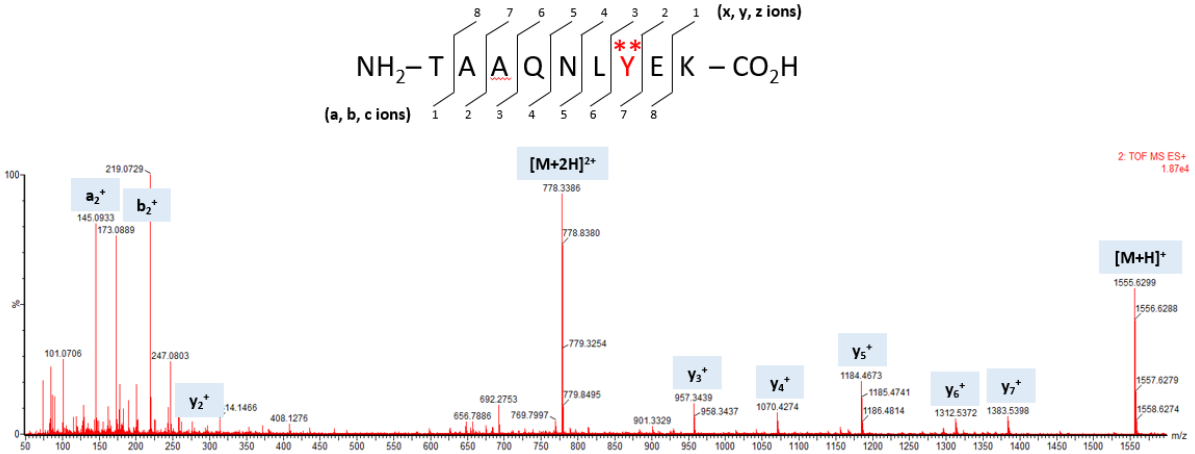
NH_2-TAAQNLYEK-CO_2H**
MW = 1554.5 Da
$[M+2H]^{2+}$ expected = 778.25 found 778.33

MS/MS fragmentation

(a) 4.33 min peak (648.8022)

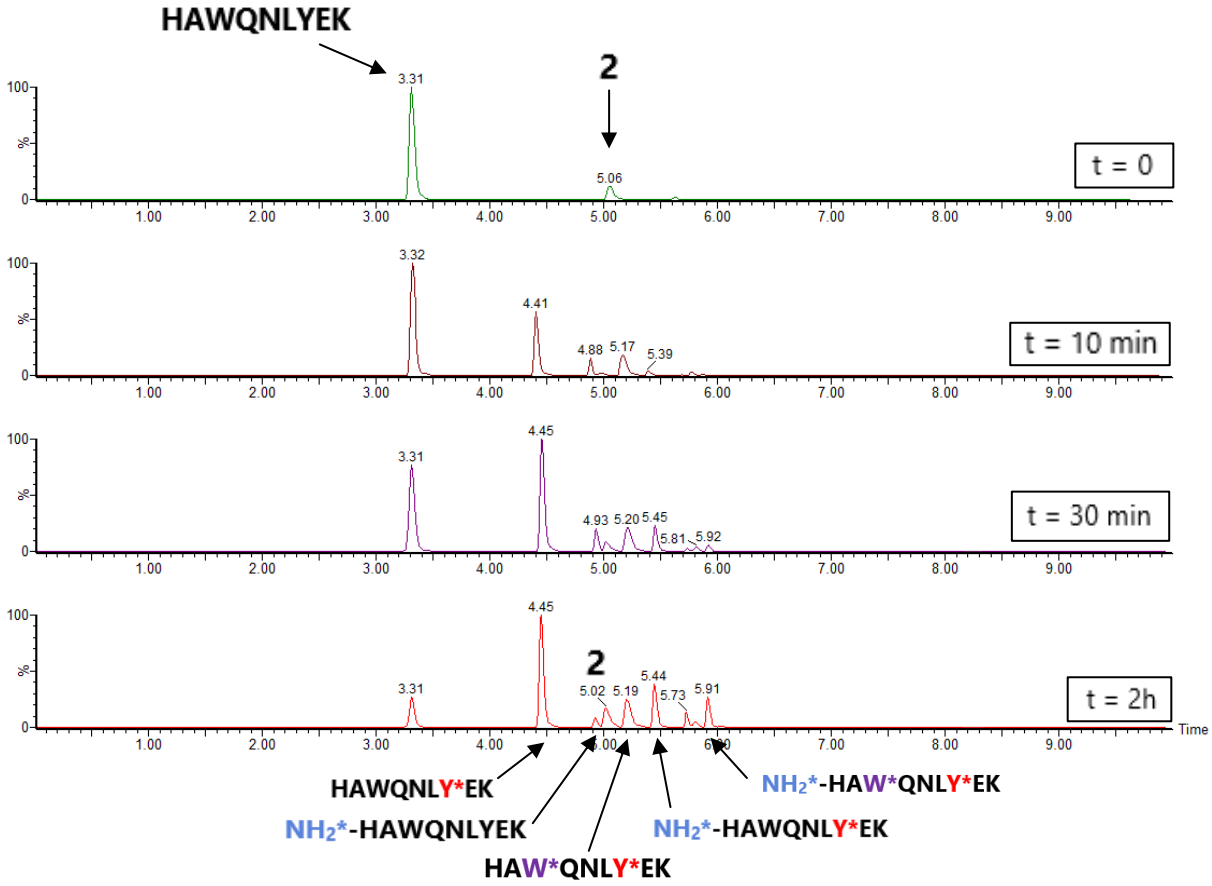


(b) 4.93 min peak (778.3386)

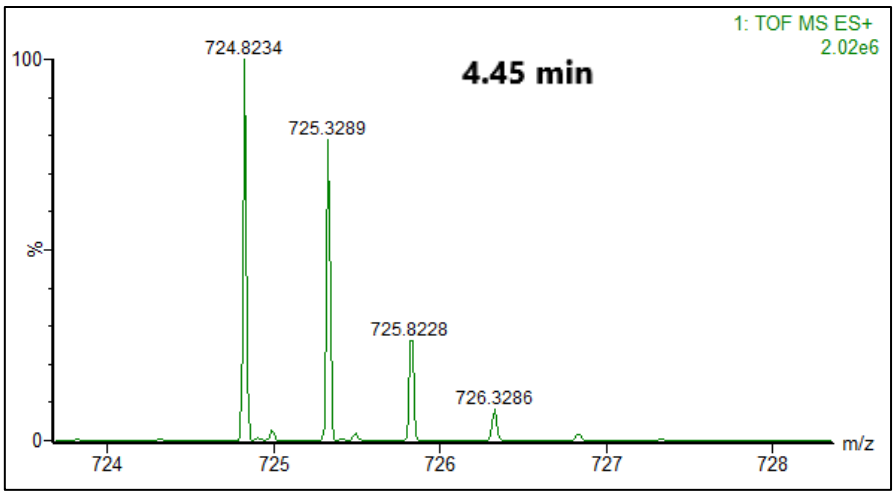


HAWQNLYEK + 2 (PhUr)

UPLC profile



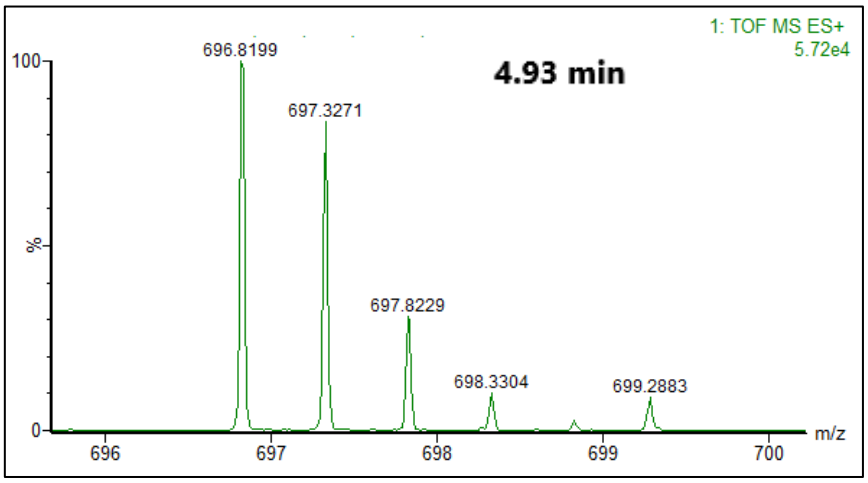
MS profiles



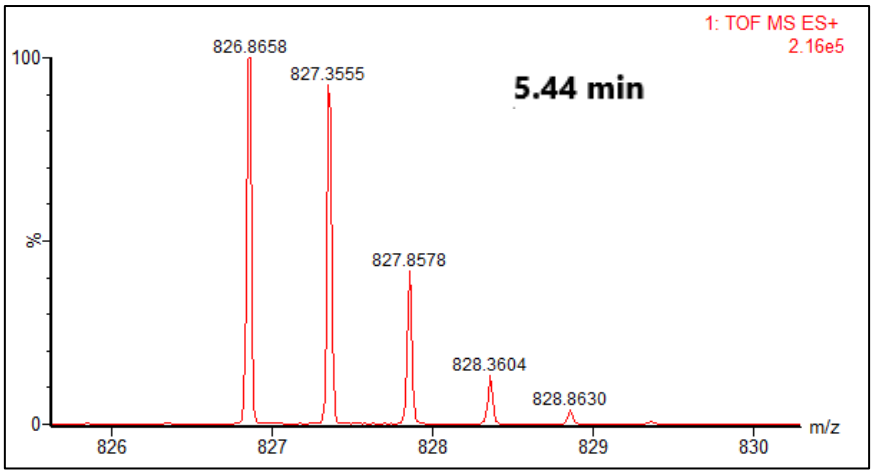
NH_2-HAWQNLY*EK-CO_2H

MW = 1447.6 Da

$[M+2H]^{2+}$ expected = 724.80 found 724.82



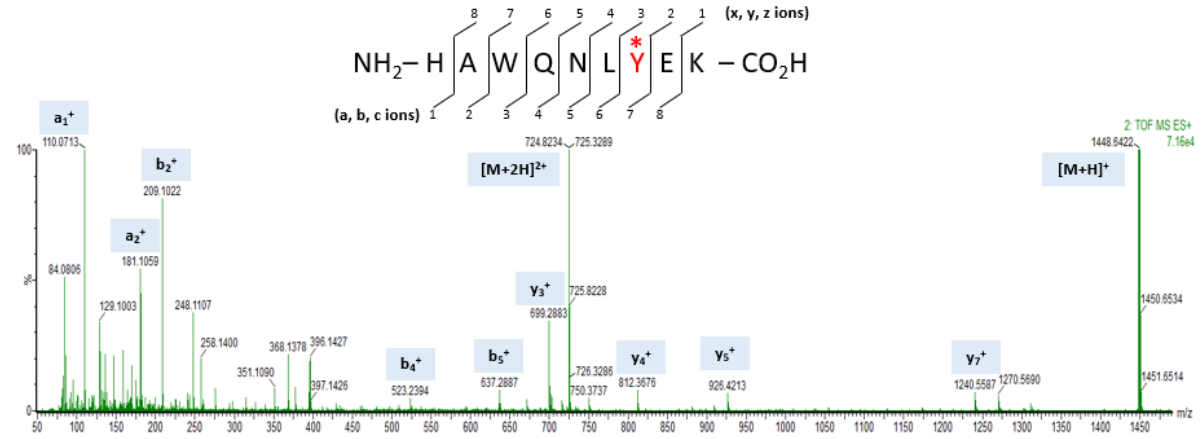
NH_2^*-HAWQNLYEK-CO_2H
MW = 1391.6 Da
$[M+2H]^{2+}$ expected = 696.80 found 696.82



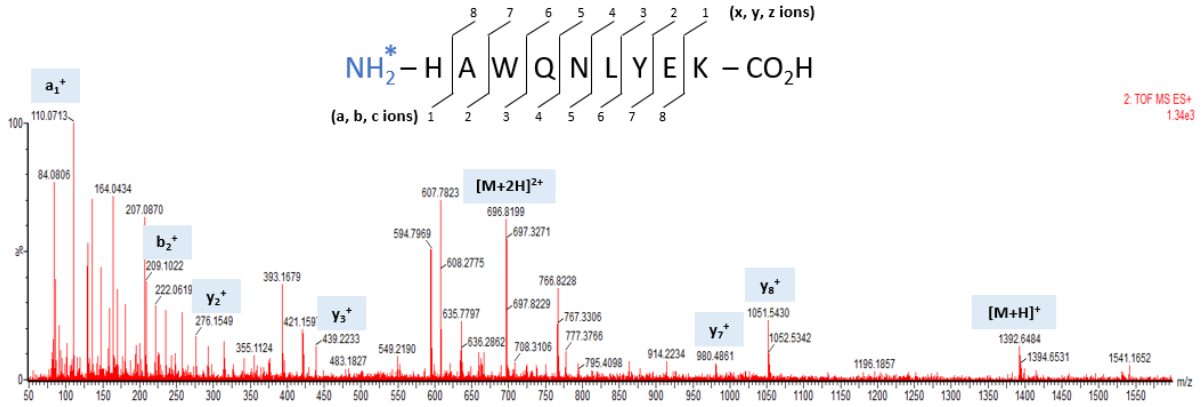
NH_2^*-HAWQNLY*EK-CO_2H
MW = 1651.6 Da
$[M+2H]^{2+}$ expected = 826.80 found 826.87

MS/MS fragmentation

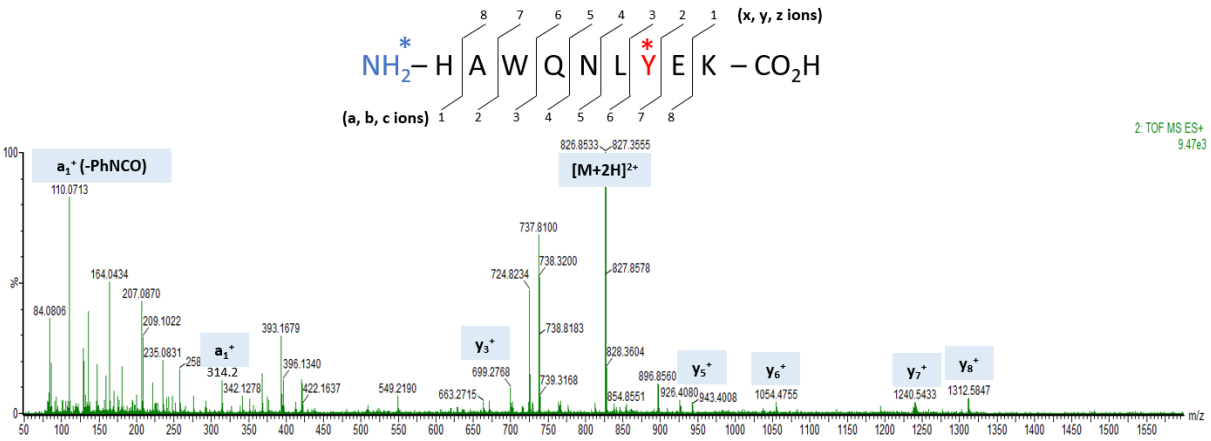
(a) 4.45 min peak (724.8234)



(b) 4.93 min peak (696.8199)

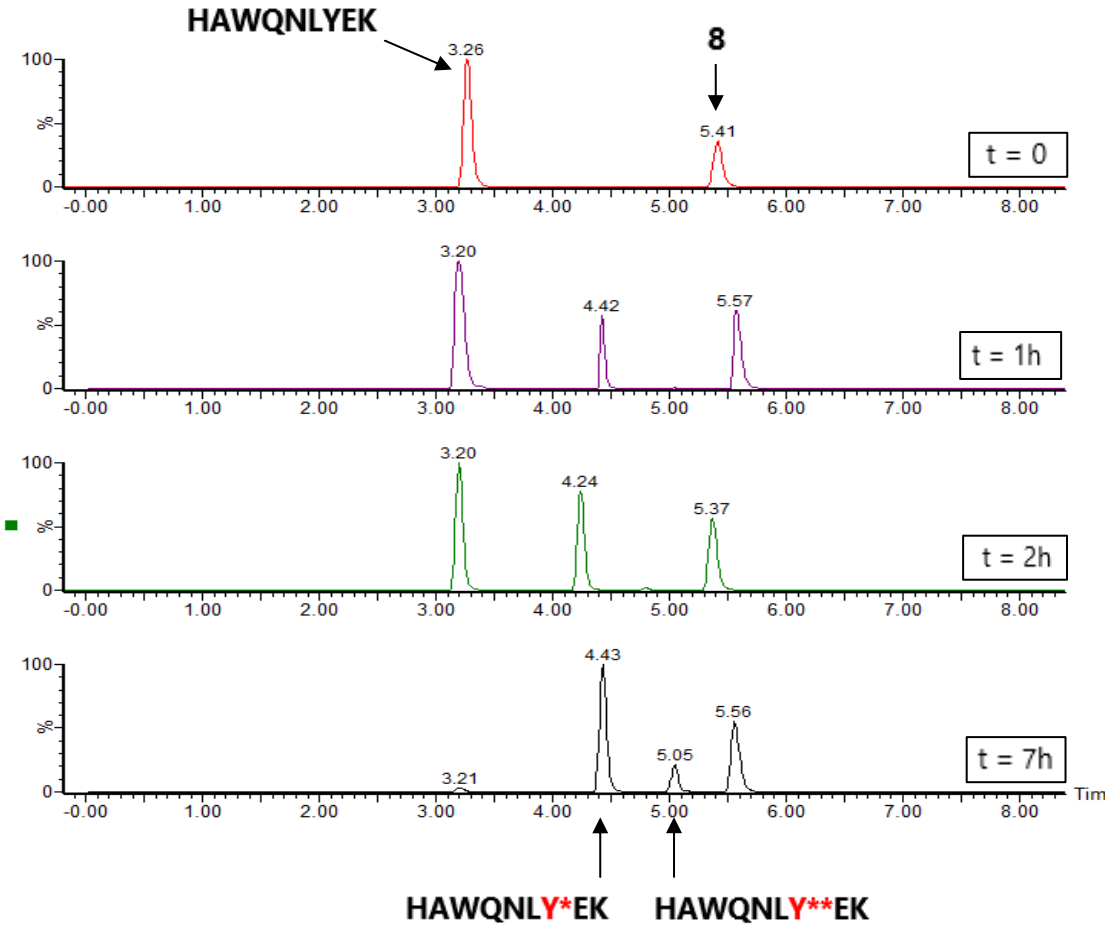


(c) 5.44 min peak (826.8658)

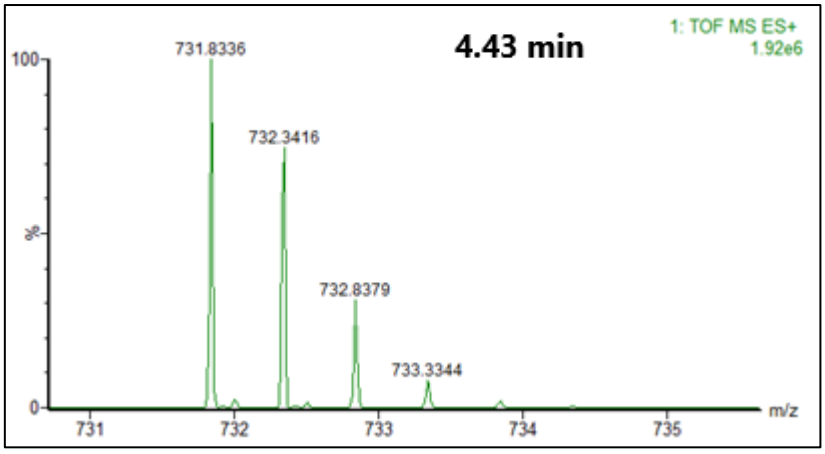


HAWQNLYEK + 8 (NMePhUr)

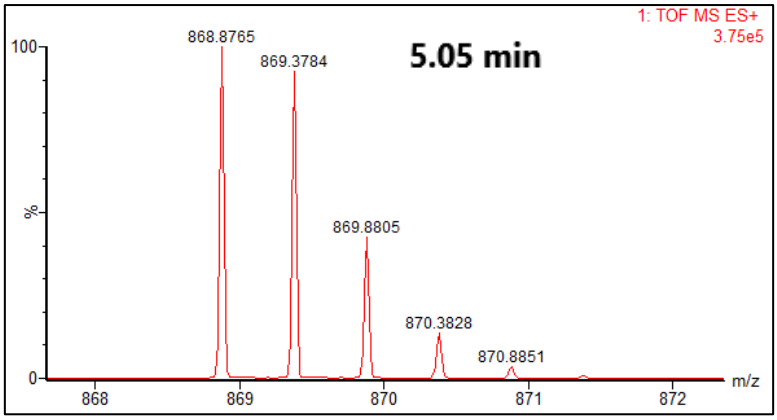
UPLC profile



MS profiles



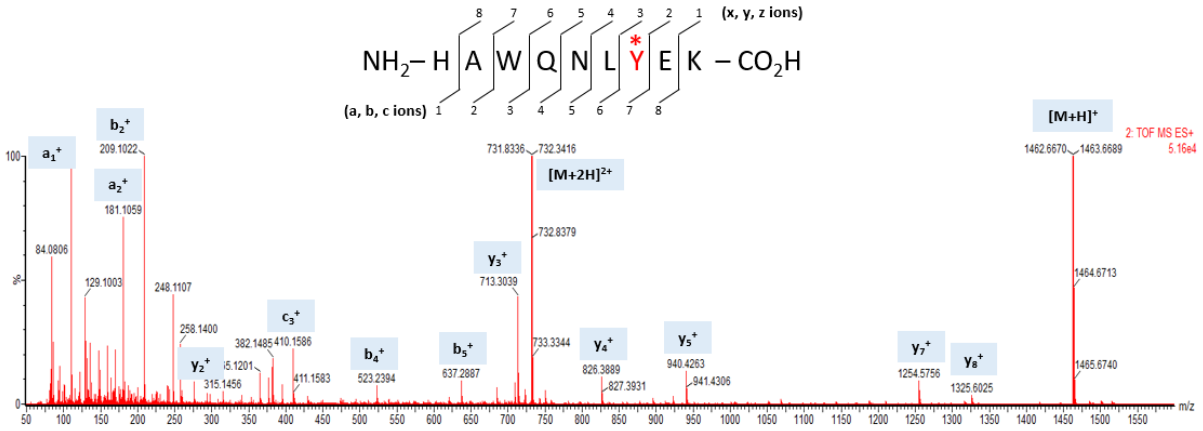
NH_2-HAWQNLY*EK-CO_2H
MW = 1461.6 Da
$[M+2H]^{2+}$ expected = 731.80 found 731.83



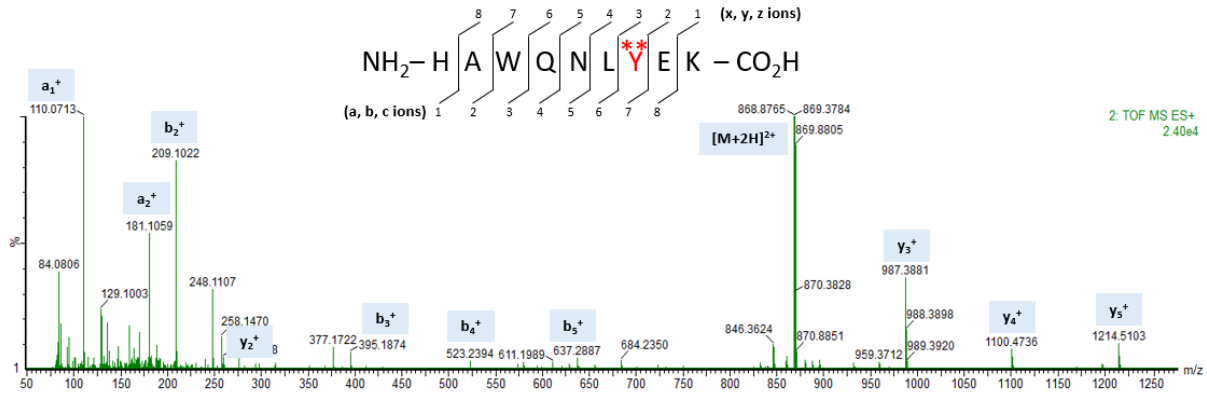
NH_2-HAWQNLYEK-CO_2H**
MW = 1735.6 Da
$[M+2H]^{2+}$ expected = 868.80 found 868.87

MS/MS fragmentation

(a) 4.43 min peak (731.8336)

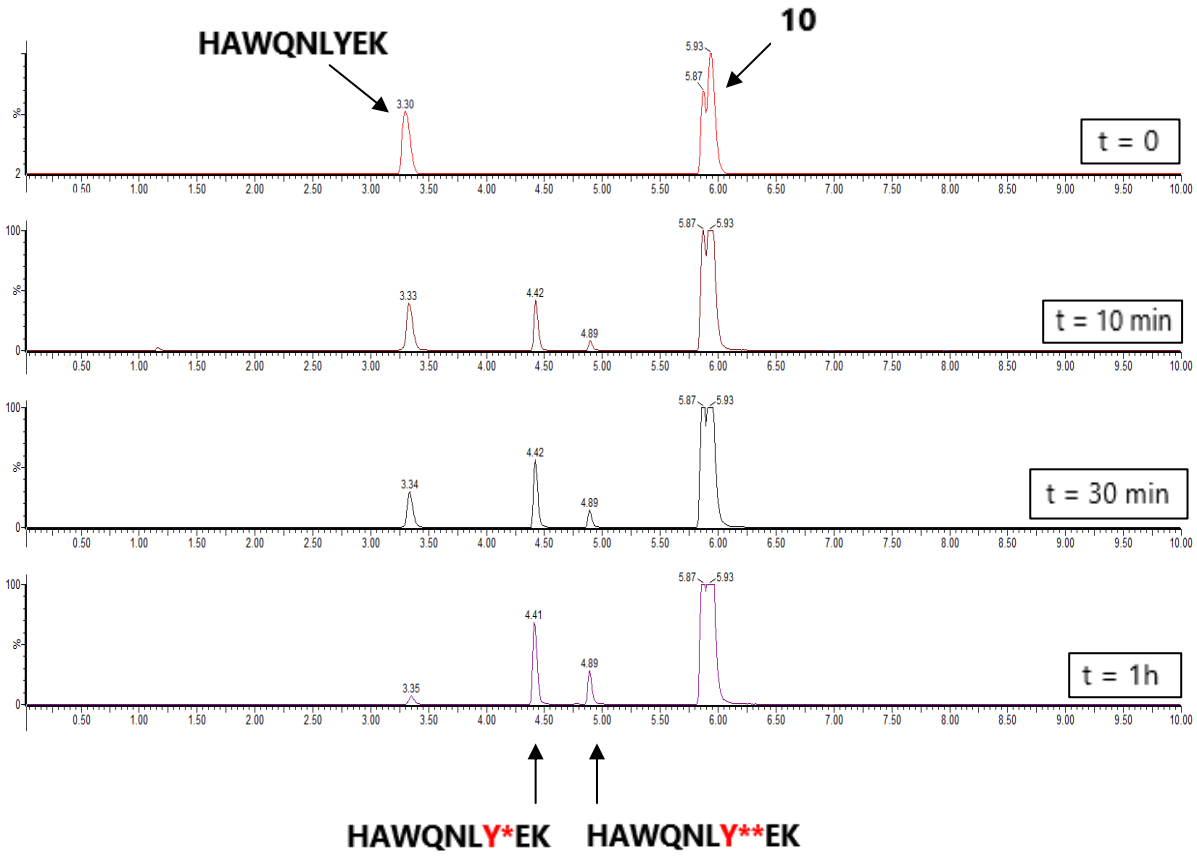


(b) 5.05 min peak (868.8765)

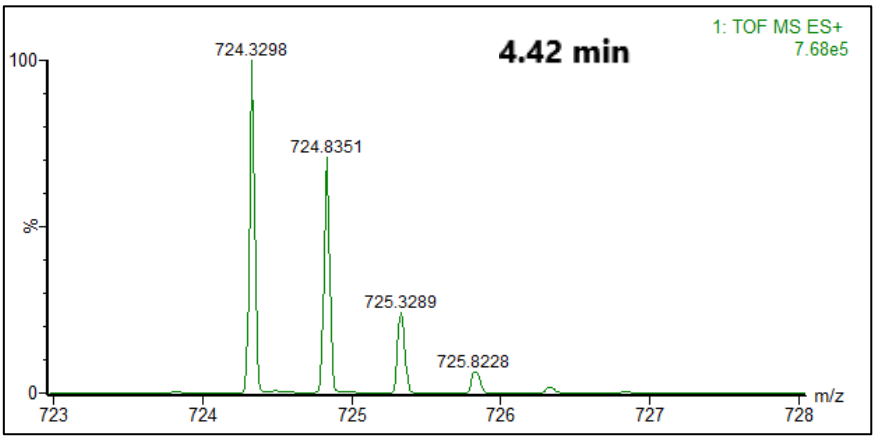


HAWQNLYEK + 10 (NMeLum)

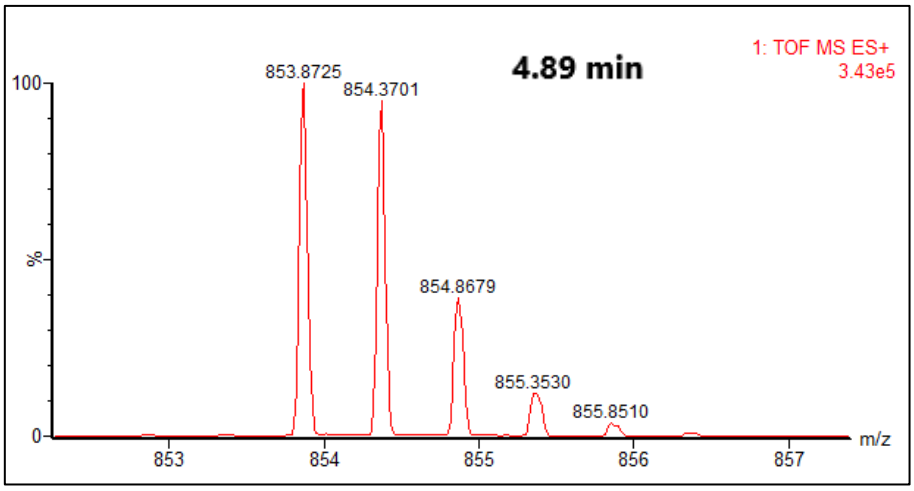
UPLC profile



MS profiles



NH_2-HAWQNLY*EK-CO_2H
MW = 1446.6 Da
$[M+2H]^{2+}$ expected = 724.30 found 724.33



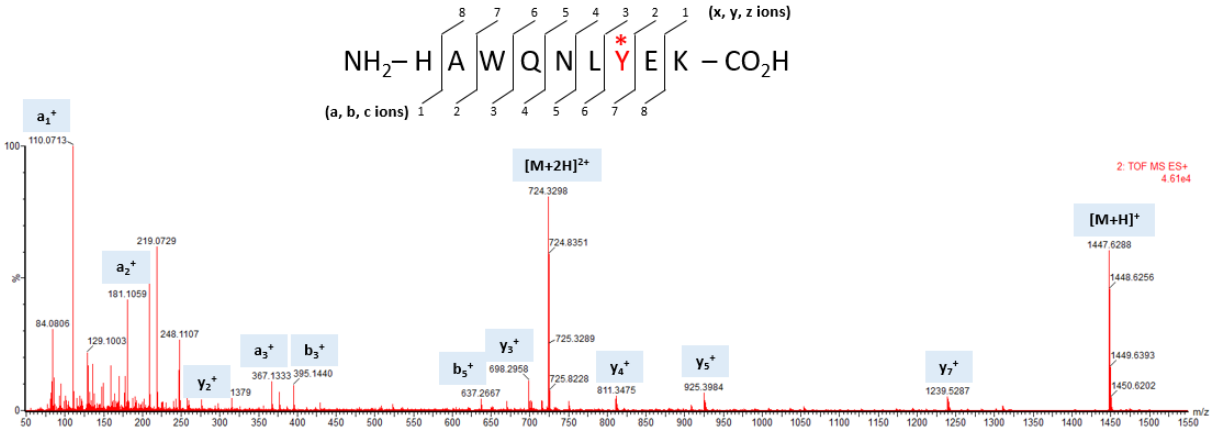
NH_2-HAWQNLYEK-CO_2H**

MW = 1705.6 Da

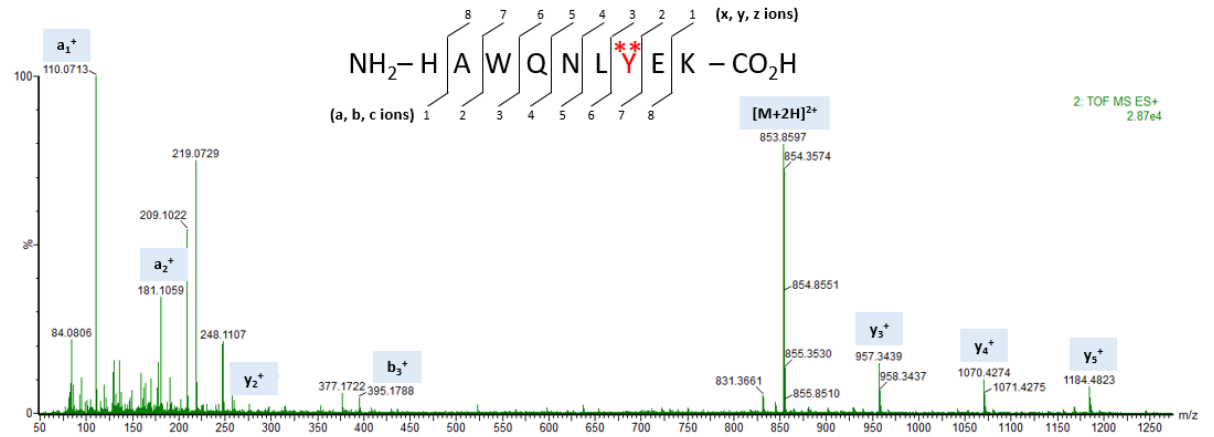
$[M+2H]^{2+}$ expected = 853.80 found 853.88

MS/MS fragmentation

(a) 4.42 min peak (724.3298)

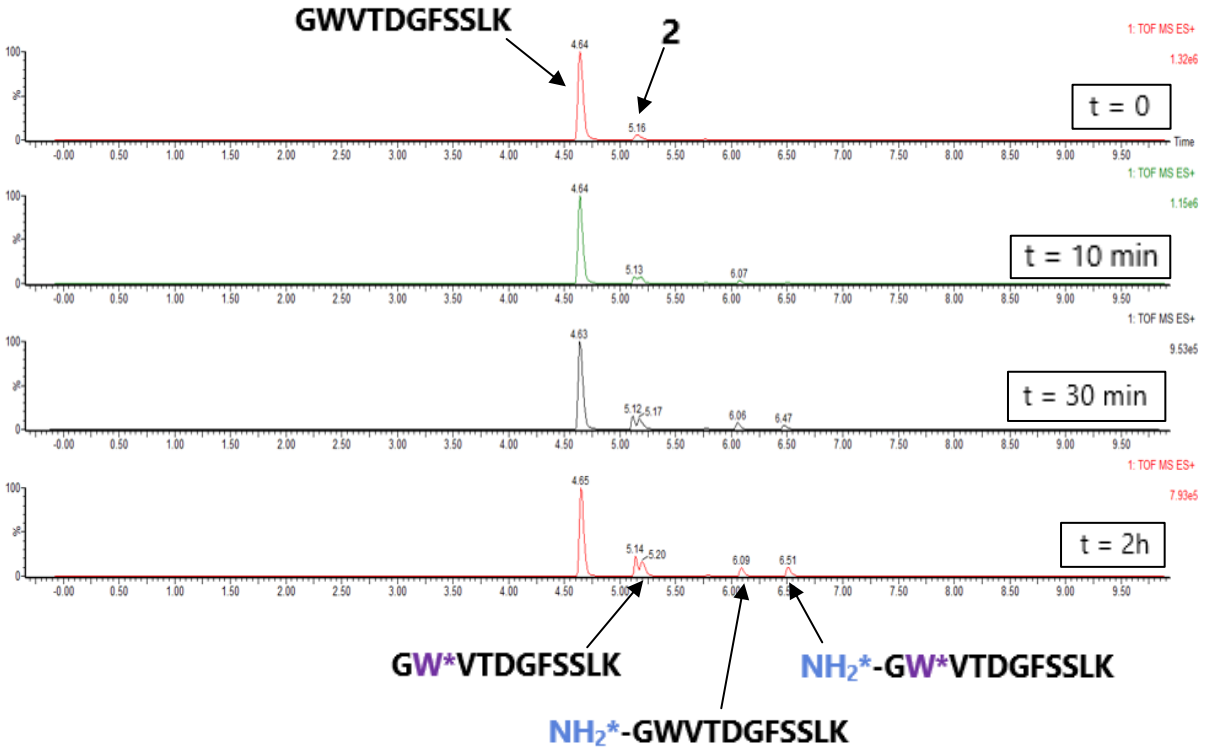


(b) 4.89 min peak (853.8725)

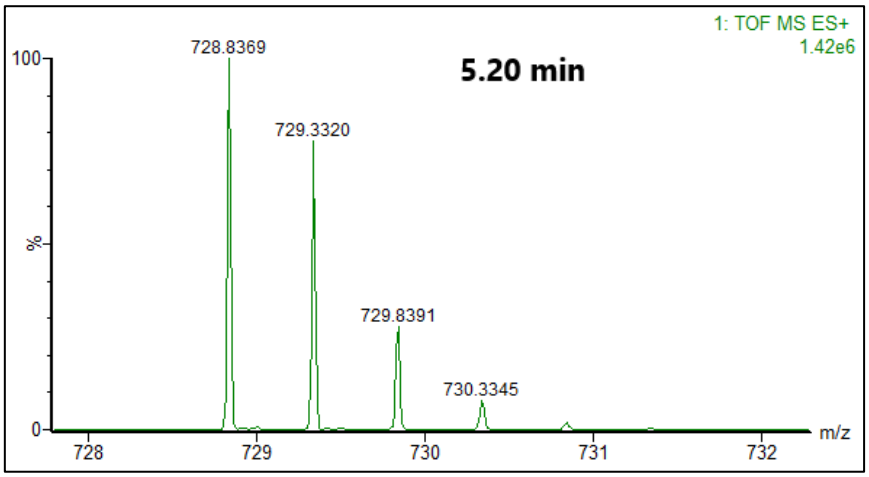


GWVTDGFSSLK + 2 (PhUr)

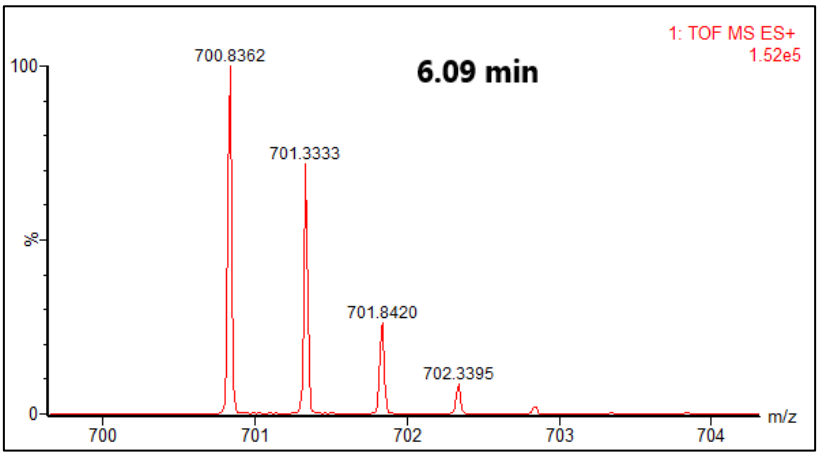
UPLC profile



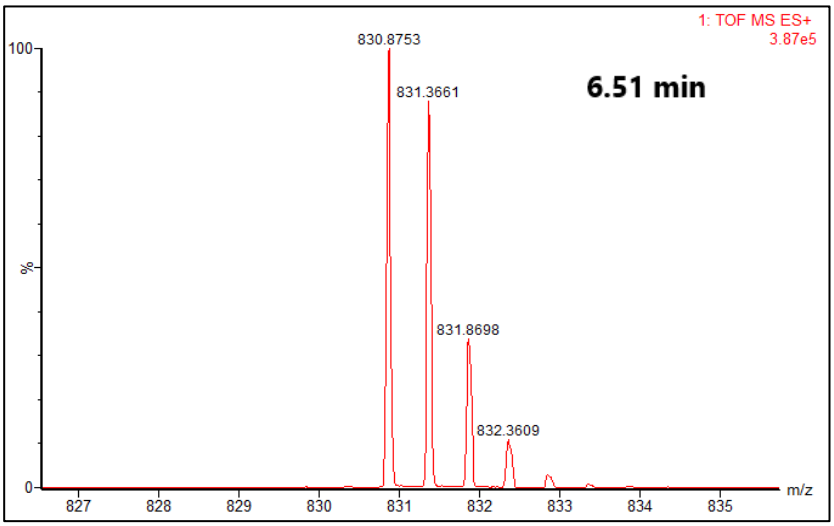
MS profiles



NH_2-GW*VTDGFSSLK-CO_2H
MW = 1455.6 Da
$[M+2H]^{2+}$ expected = 728.80 found 728.84



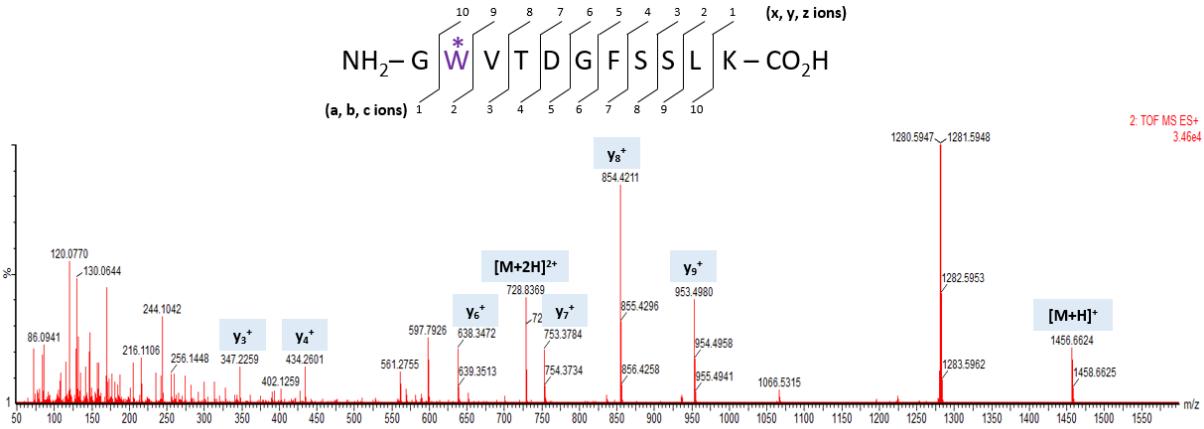
NH_2^*-GWVTDGFSSLK-CO_2H
MW = 1399.6 Da
$[M+2H]^{2+}$ expected = 700.80 found 700.84



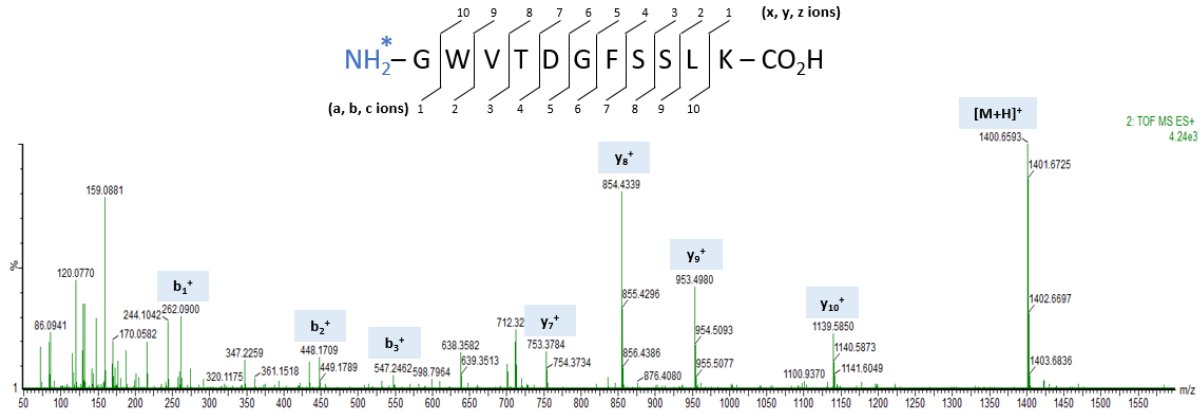
NH_2^*-GW*VTDGFSSLK-CO_2H
MW = 1659.9 Da
$[M+2H]^{2+}$ expected = 830.80 found 830.88

MS/MS fragmentation

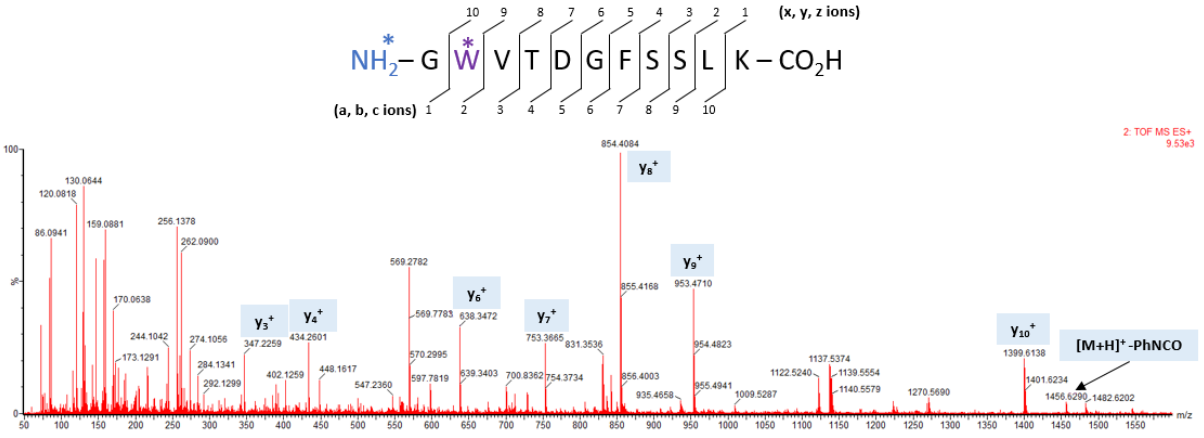
(a) 5.02 min peak (728.8369)



(b) 6.09 min peak (700.8362)

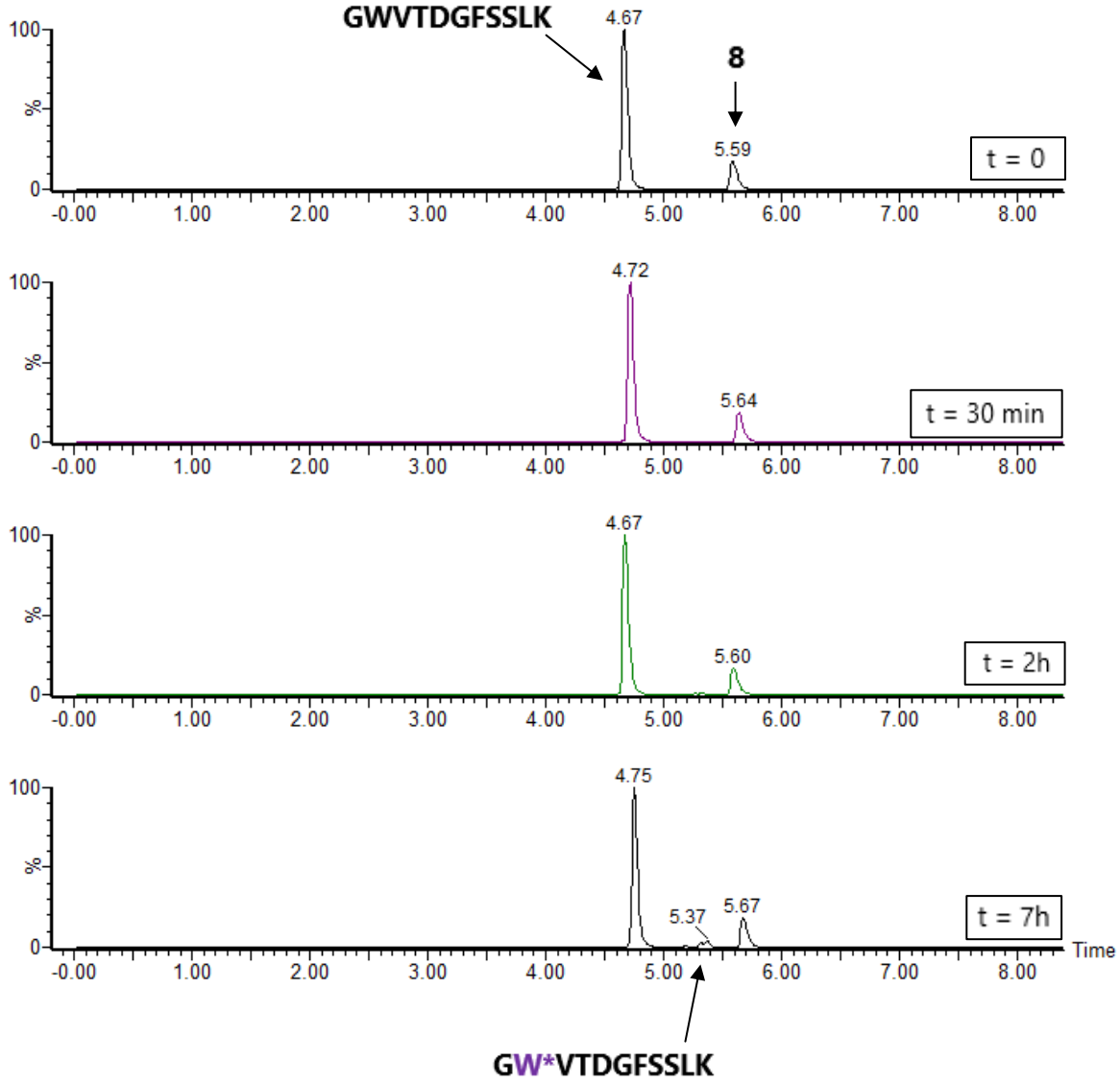


(c) 6.51 min peak (830.8753)

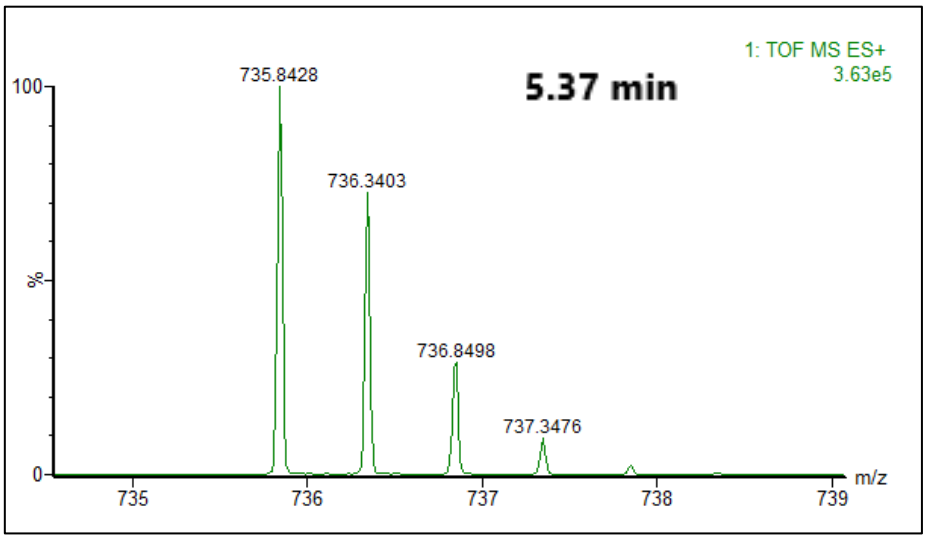


GWVTDGFSSLK + 8 (NMePhUr)

UPLC profile



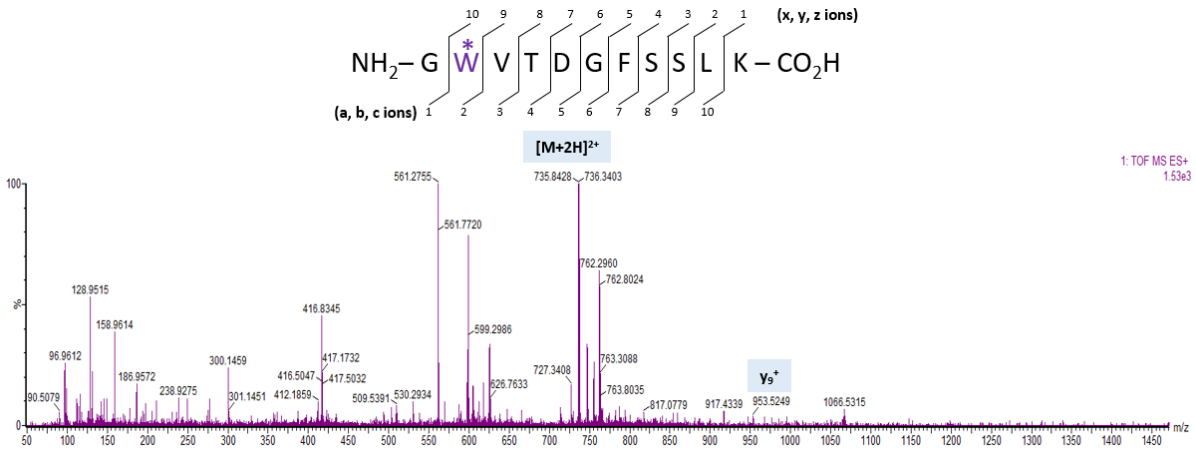
MS profiles



NH_2-GW*VTDGFSSLK-CO_2H
MW = 1469.6 Da
$[M+2H]^{2+}$ expected = 735.80 found 735.84

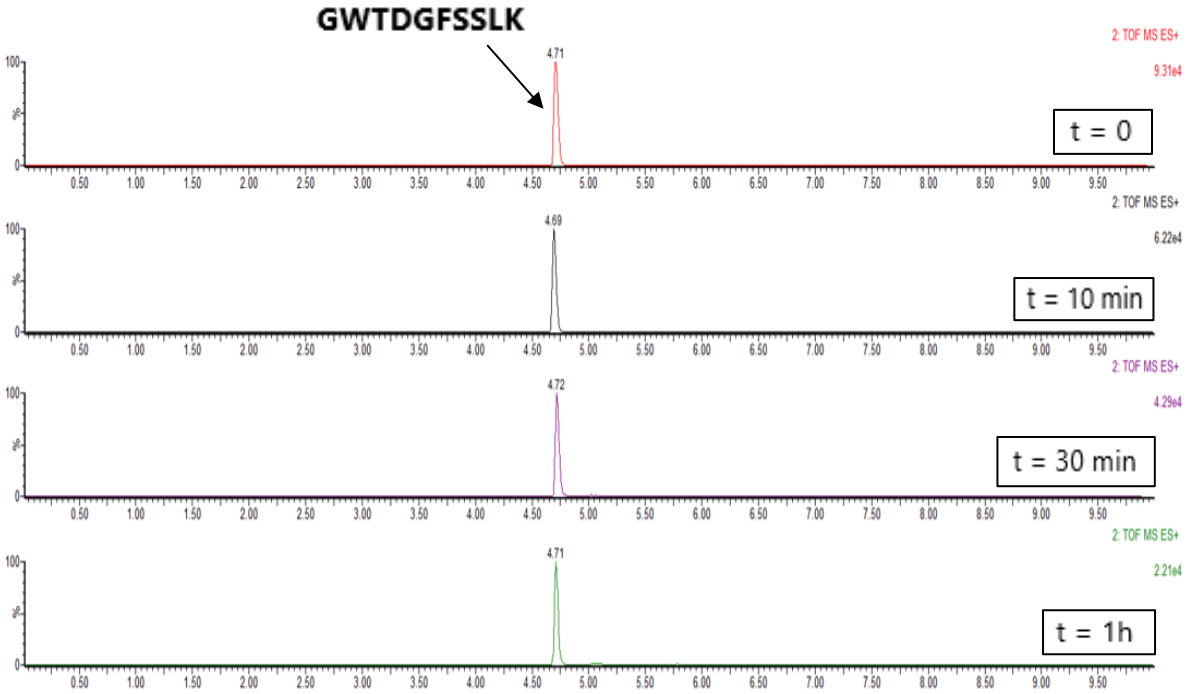
MS/MS fragmentation

(a) 5.37 min peak (735.8428)



GWVTDGFSSLK + 10 (NMeLum)

UPLC profile



Modification of Chymotrypsinogen A

Procedure

In a 5 mL glass vial, three electrodes system used was 7.8 cm^2 graphite plate as anode, 7.8 cm^2 platinum plate as cathode and the reference electrod was Ag/AgCl. Appropriate voltage was applied to a mixture of reagent (**8** or **10**, 5.0 μmol, 1 mM) and Chymo (5 nmol, 1 μM) in 100 mM phosphate buffer (5 mL). Electrolysis was performed at room temperature (stirring: 500 rpm) during 4 h for **8** and during 1 h for **10**. Then, samples were directly analysed by UPLC-HRMS with the system described below.

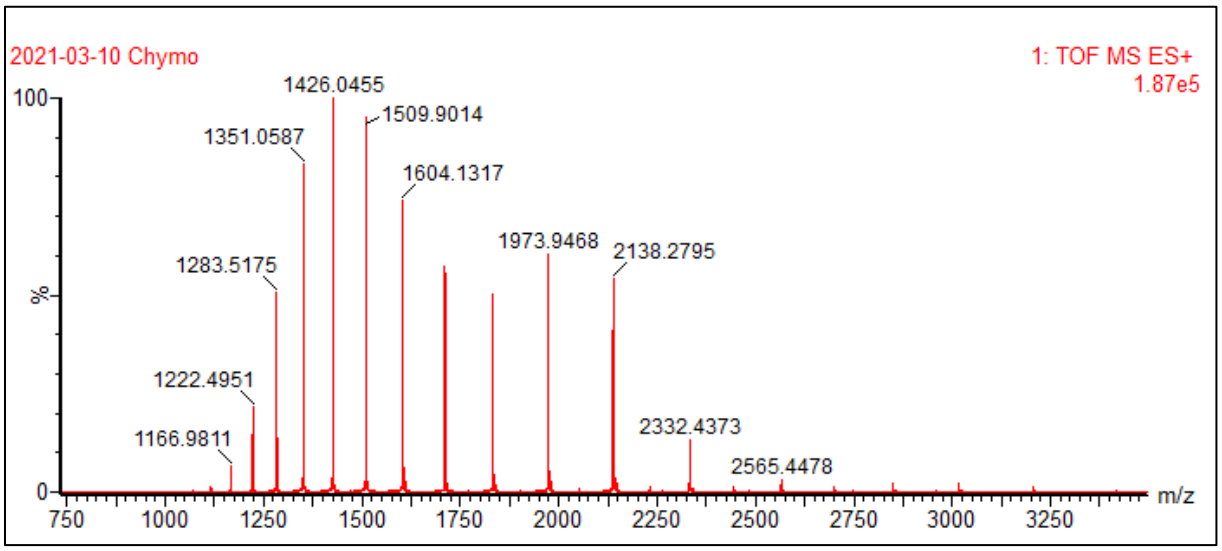
Chromatography and mass spectrometry analysis of proteins

Samples were analyzed by ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS). UPLC-HRMS analyses were performed on a Synapt G2 HRMS Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the positive mode and an Acquity H-Class UPLC device (Waters Corporation, Milford, MA, USA). Samples were injected (10 μL) onto a Acquity CSH C18 Peptide (1.7 μm; 2.1 × 100 mm; 130 Å) reversed-phase LC columns held at 60 °C. Proteins were then eluted over 10 min with a linear gradient of mobile phase B (100% acetonitrile) in mobile phase A (5% acetonitrile), each containing 0.1% formic acid, and at a flow rate of 250 μL/min. Mobile phase B was kept constant at 1% for 0.5 min, then linearly increased from 1% to 80% for 7.5 min, kept constant for 0.5 min, returned to the initial condition over 0.5 min, and kept constant for 0.5 min before the next injection. The full-HRMS mode was applied for protein detection (mass-to-charge ratio (*m/z*) range 100-4,000) at a mass resolution of 25,000 full-widths at half maximum. The ionization settings were as follows: capillary voltage, +3 kV; cone voltage, 30 V; desolvation gas (N_2) flow rate, 1000 L/h; desolvation gas/source temperatures, 450/120 °C. Leucine enkephalin solution (2 μg/mL, 50% acetonitrile) was infused at a constant flow rate of 10 μL/min in the lockspray channel, allowing for correction of the measured *m/z* throughout the batch (theoretical *m/z* 556.2771 in positive mode). Data acquisition and processing were achieved using MassLynx® software (version 4.1, Waters

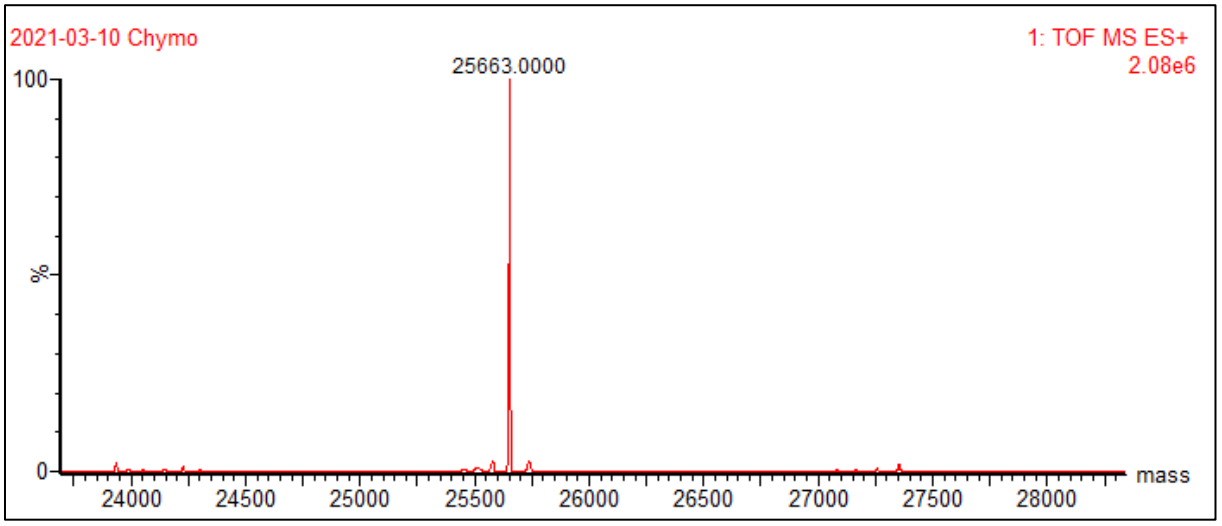
Corporation). The complex mass spectra of proteins under chromatographic peaks were deconvoluted with the MaxEnt1 extension software to get the experimental molecular weights of proteins, which were then compared with those of the unmodified protein (native) to estimate the number of modified tyrosine residues.

Chymo Native

UPLC-MS profile



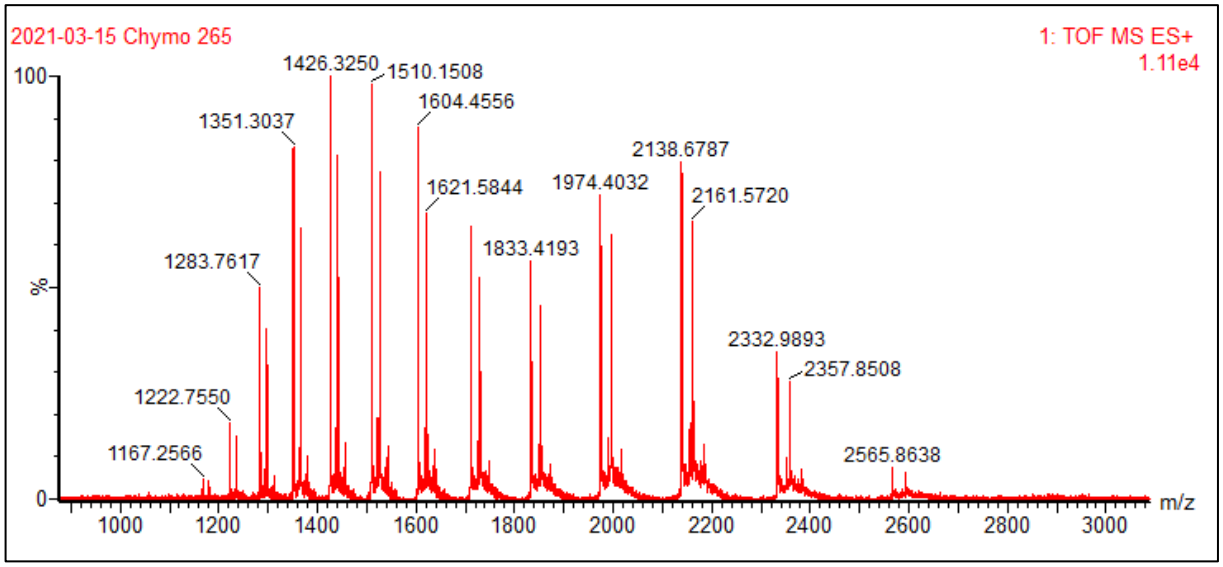
Deconvolution



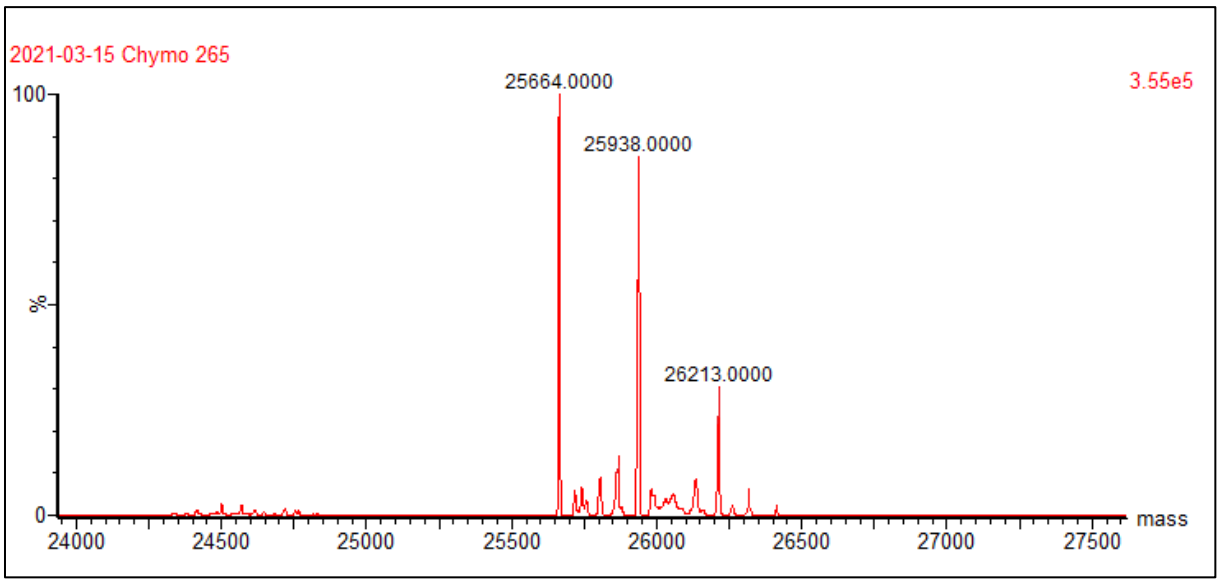
Expected (native): **25666**, found **25663**

Chymo + 8 (NMePhUr)

UPLC-MS profile



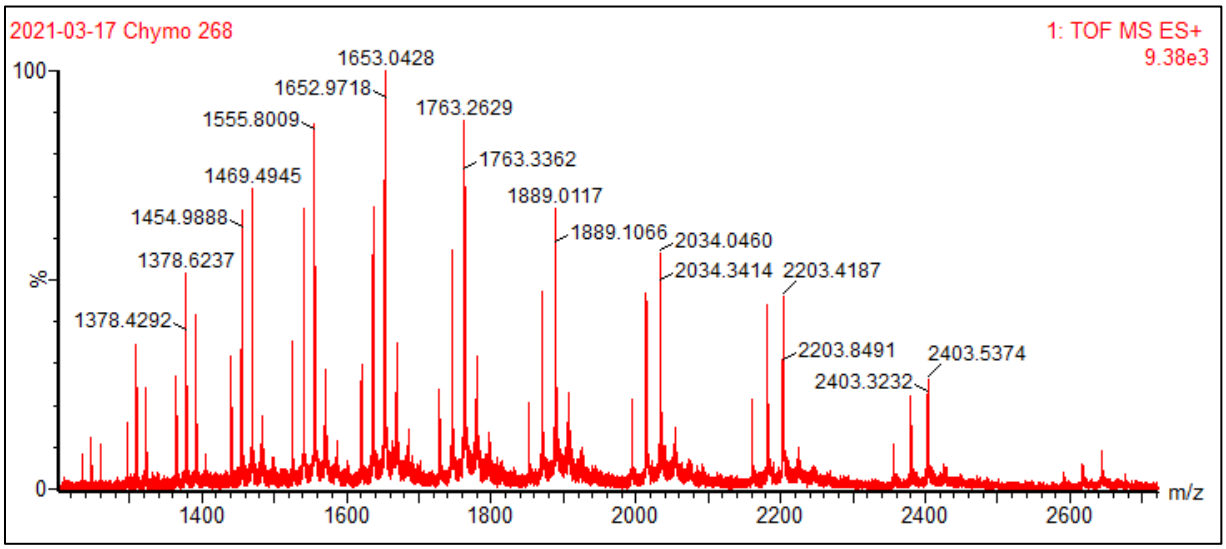
Deconvolution



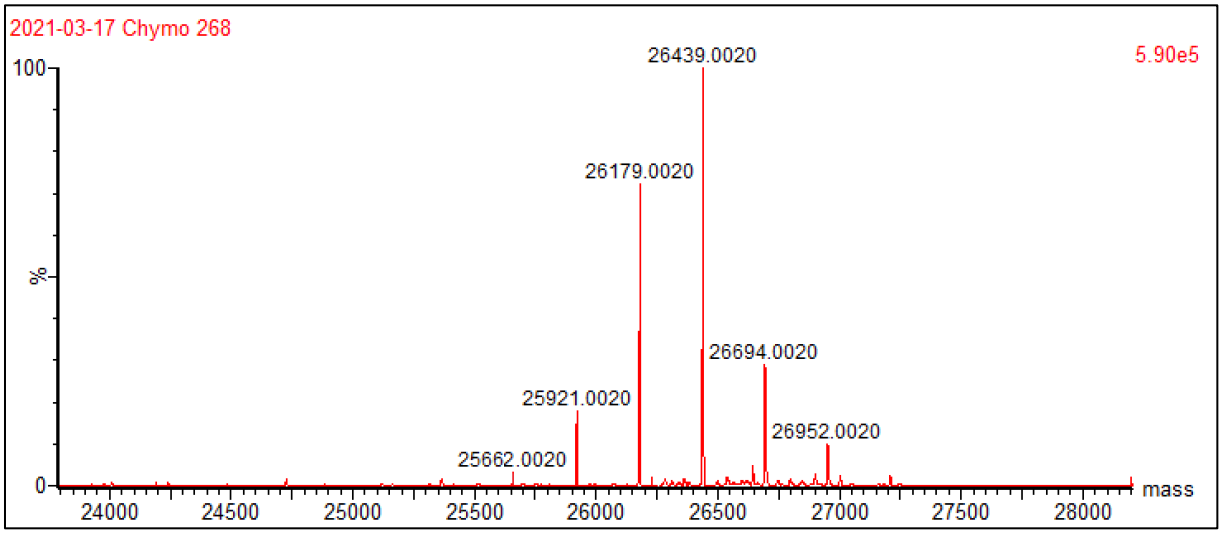
Expected (+1): **25938**, found **25938**
Expected (+2): **26212**, found **26213**

Chymo + 10 (NMeLum)

UPLC-MS profile



Deconvolution

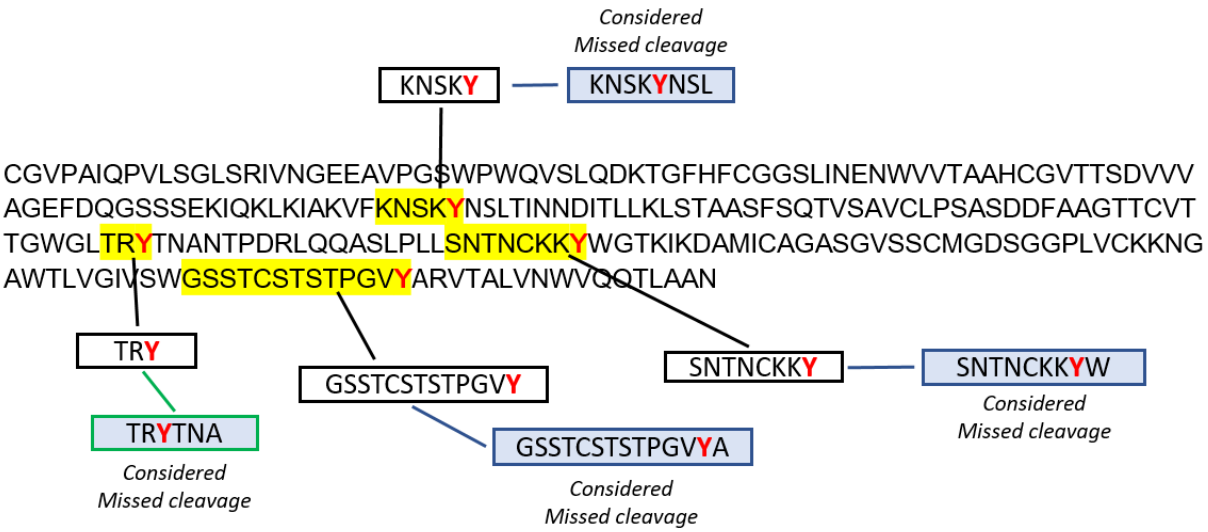


Expected (+1): **25921**, found **25921**
Expected (+2): **26180**, found **26179**
Expected (+3): **26439**, found **26439**
Expected (+4): **26698**, found **26694**
Expected (+5): **29957**, found **26952**

Proteolysis (pepsin) of 10-Chymo and mass spectrometry of peptides

10-Chymo samples were also subjected to pepsin proteolysis in order to form peptides that are more easily detected by mass spectrometry (lower charge states) and suitable for tandem mass spectrometry (MS/MS) analysis. The protein samples were resuspended in phosphate buffer (pH 7.5, 250 µg/mL) and HCl 0.1N was added to adjust the pH to 3. Pepsin solution (Promega, 10 mg/mL in water, 50 µL) was added to 100 µL of protein solutions and then incubated overnight at 37 °C. The reaction was stopped by heating samples at 95 °C for 10 min. Samples were centrifuged (4 °C, 10000 ×g, 10 min) and supernatants were cleaned using 30 mg Oasis HLB 1 cc Cartridges (Waters Corporation). Cartridges were conditioned, equilibrated, loaded, washed and eluted with methanol (1 mL), water (1 mL), samples (~250 µL), 5% methanol (1 mL) and 80% methanol (500 µL), respectively. Eluates were dried under a nitrogen stream, reconstituted with 100 µL of 5% acetonitrile containing 0.1% formic acid, and 10 µL were injected into the analytical system described above (mass spectrometry analysis of peptides). Proteolytic peptides (pepsin proteolysis) were separated and detected by the full-HRMS mode as described above. In parallel, protein sequences were *in silico* digested using the free Expasy software (https://web.expasy.org/peptide_mass). All pepsin peptides (pH > 2) were looked for from their theoretical *m/z* ratios assuming single, double and triple-charged ions. Relevant peptides were then subjected to MS/MS fragmentations to ascertain their amino acid sequences. Then, peptide sequences were compared with each other and between both modified and unmodified protein samples to establish the location of the labelling. Finally, MS/MS fragmentation patterns allowed the identification of the modified tyrosine.

Note: proteolysis with trypsin (pH 8), chymotrypsin (pH 8) and pepsin (pH 1.3) were tried and didn't allow identification of peptides.

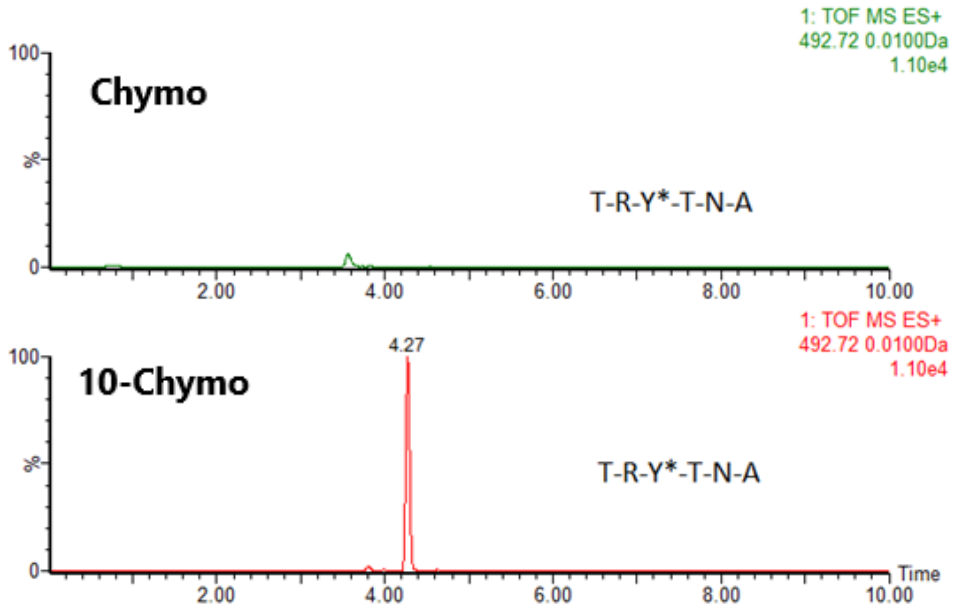


Amino acid sequence of Chymo. Outlined in yellow are the 4 theoretical Y-containing peptides formed *in silico* by pepsin pH >2 proteolysis. Since pepsin cleaves after Y, we also considered that Y-modification could affect proteolysis after Y and investigated each of the 4 missed cleavage peptides (outlined in blue).

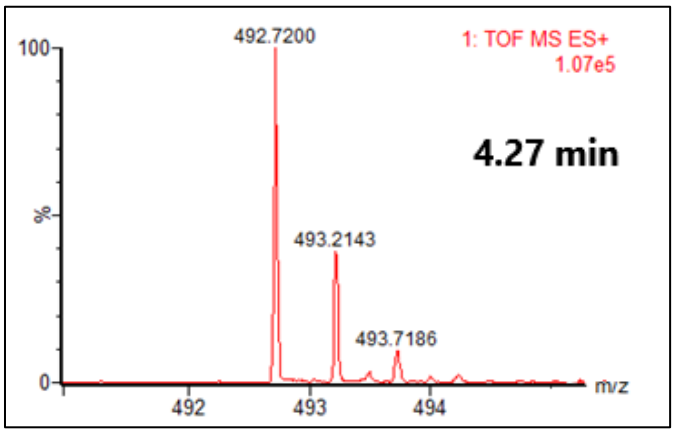
- In the sample of native Chymo, unmodified missed cleavage TRYTNA (*m/z* = 363.18 double charged ion), single-modified missed cleavage (TRYTNA)* (*m/z* = 492.72 double charged ion) and double-modified missed cleavage (TRYTNA)** (*m/z* = 622.26 double charged ion) were not found.
- In the 10-Chymo sample, both Y-modified and double-Y-modified TR**Y**TNA peptides were found (LC, MS and MS/MS below).

10-Chymo ➔ TRYTNA +259

UPLC profiles (TRYTNA + 259)

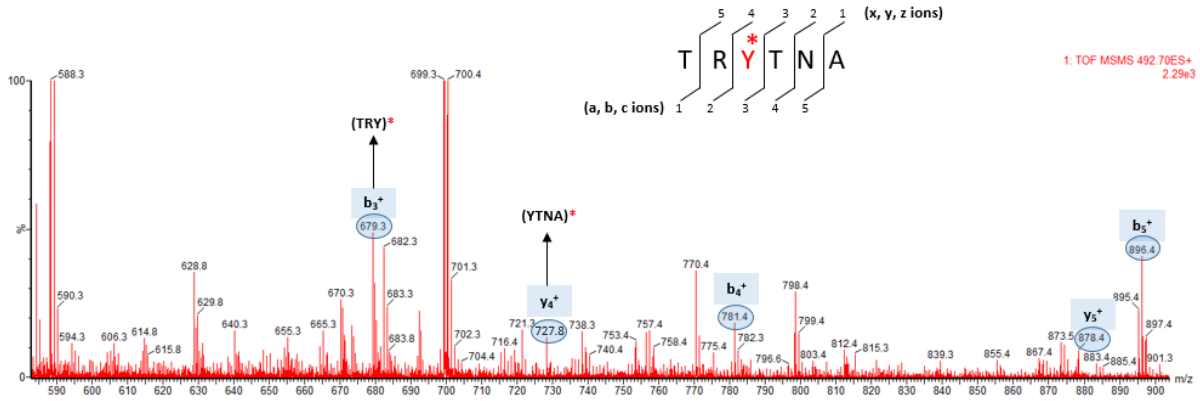


MS profiles (TRYTNA + 259)



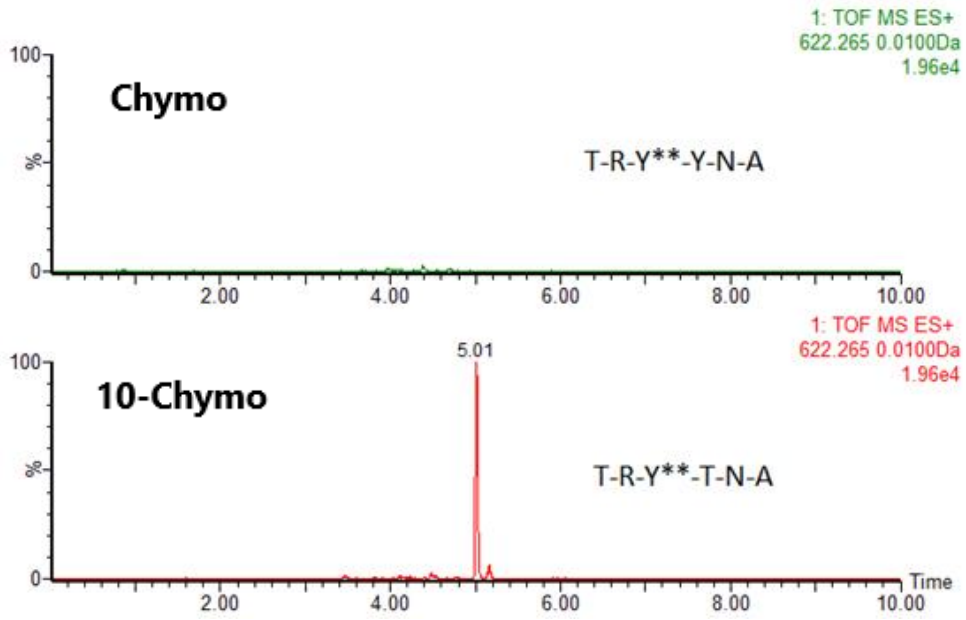
TRY*TNA
MW = 983.4 Da
$[M+2H]^{2+}$ expected = 492.7 found 492.7

MS/MS fragmentation: **4.27 min peak** (492.7200)

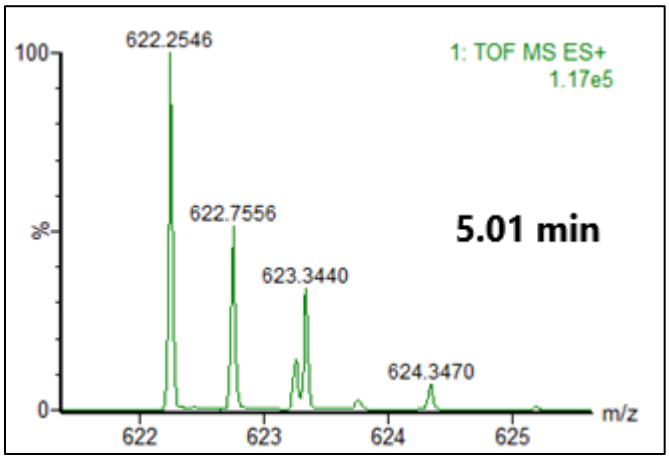


10-Chymo ➔ TRYTNA +2x 259

UPLC profiles (TRYTNA + 2x 259)



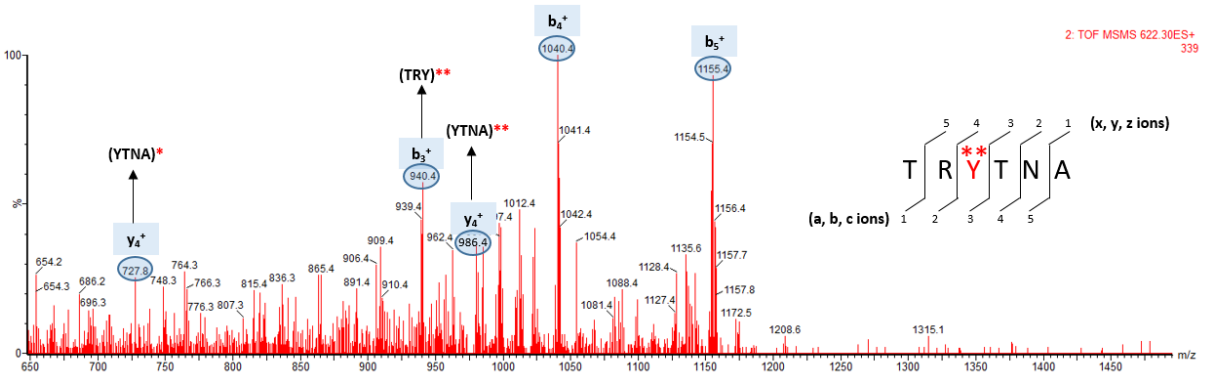
MS profiles (TRYTNA + 2x 259)



TRYTNA**
MW = 1242.4 Da
$[M+2H]^{2+}$ expected = 622.2 found 622.3

Interference with a 623.3 single-charged peptide

MS/MS fragmentation: **5.01 min peak** (622.2546)



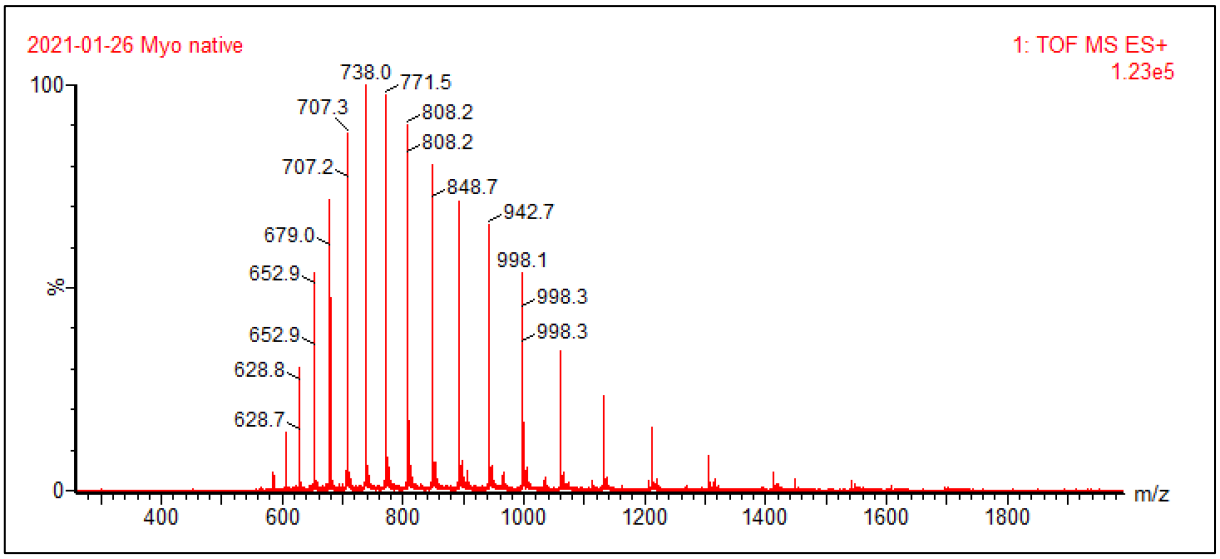
Modification of Myoglobin

Procedure

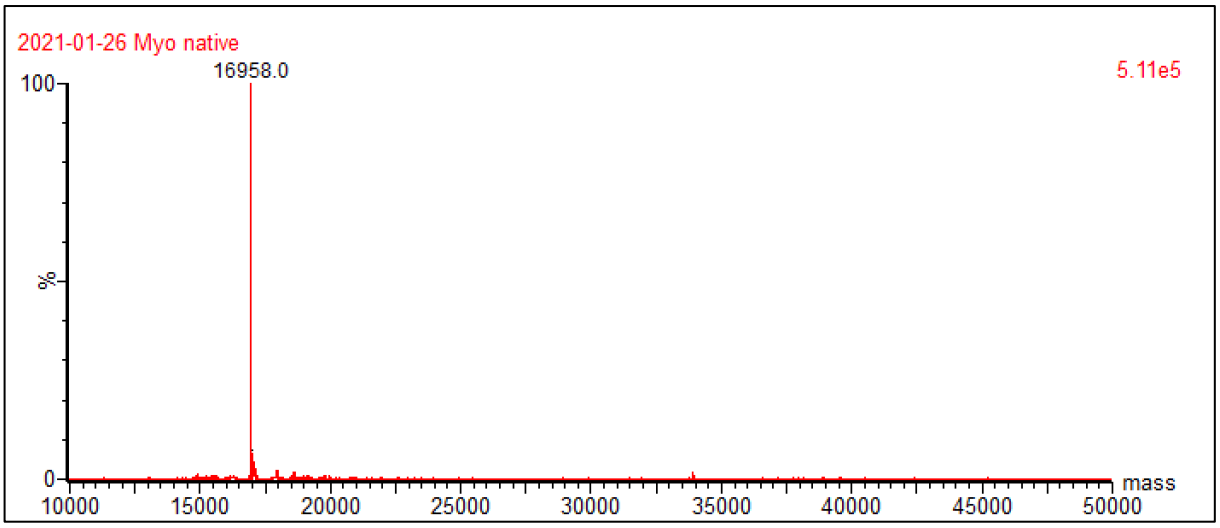
In a 5 mL glass vial, three electrodes system used was 7.8 cm^2 graphite plate as anode, 7.8 cm^2 platinum plate as cathode and the reference electrod was Ag/AgCl. Appropriate voltage was applied to a mixture of **10** (5.0 μmol, 1 mM) and Myo (5 nmol, 1 μM) in 100 mM phosphate buffer (5 mL). Electrolysis was performed at room temperature (stirring: 500 rpm) during 2 h. The excess of reagent was removed using a Sephadex G-50 gel filtration column with distillated water as eluent. The combined fractions were lyophilized and resolubilized in 200 μL of distillated water to be split into two aliquots of 100 μL. First aliquot was analysed by UPLC-HRMS in conditions described for modification of Chymo to estimate the number of modifications. The second aliquot was diluted to 500 μL in a 1 mL plastic Eppendorf and 17 μL of a 5 μg/μL solution of DBCO-PEG_4-4/5-FAM in 8:2 H_2O/DMF (85 μg, 100 nmol, ~50 equiv.) was added. The mixture was incubated at 37°C during 1 h. The excess of DBCO reagent was then removed using Sephadex G-50 gel filtration column with distillated water as eluent. The resulting sample was analysed by UPLC-HRMS in conditions described for modification of Chymo to assess the SPAAC reaction.

Myoglobin Native

UPLC-MS profile

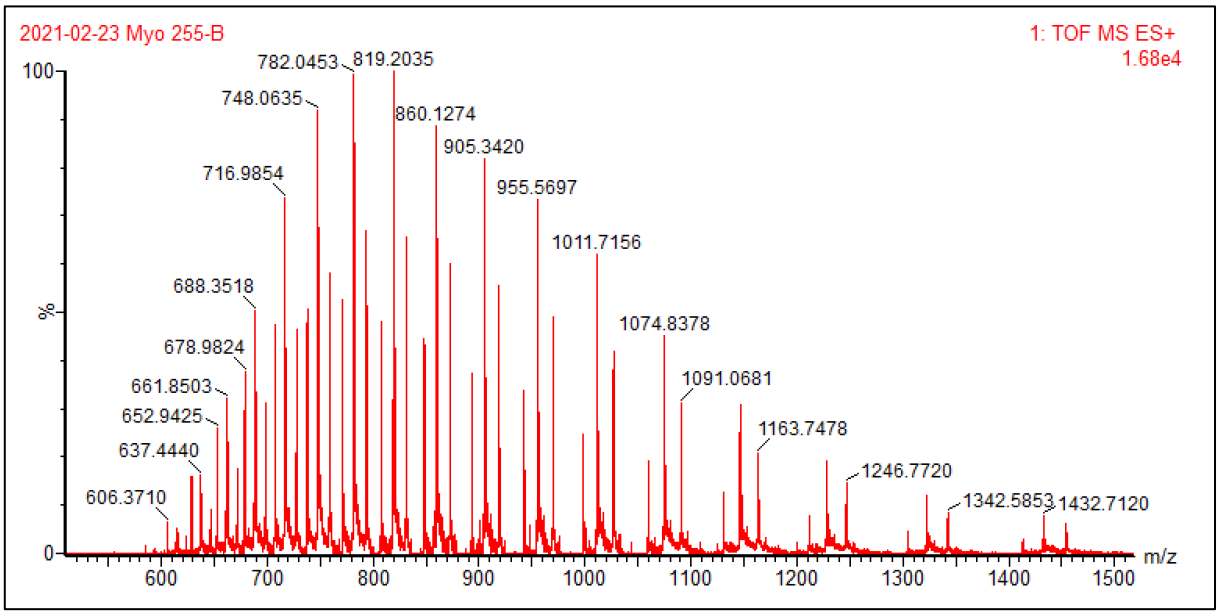


Deconvolution

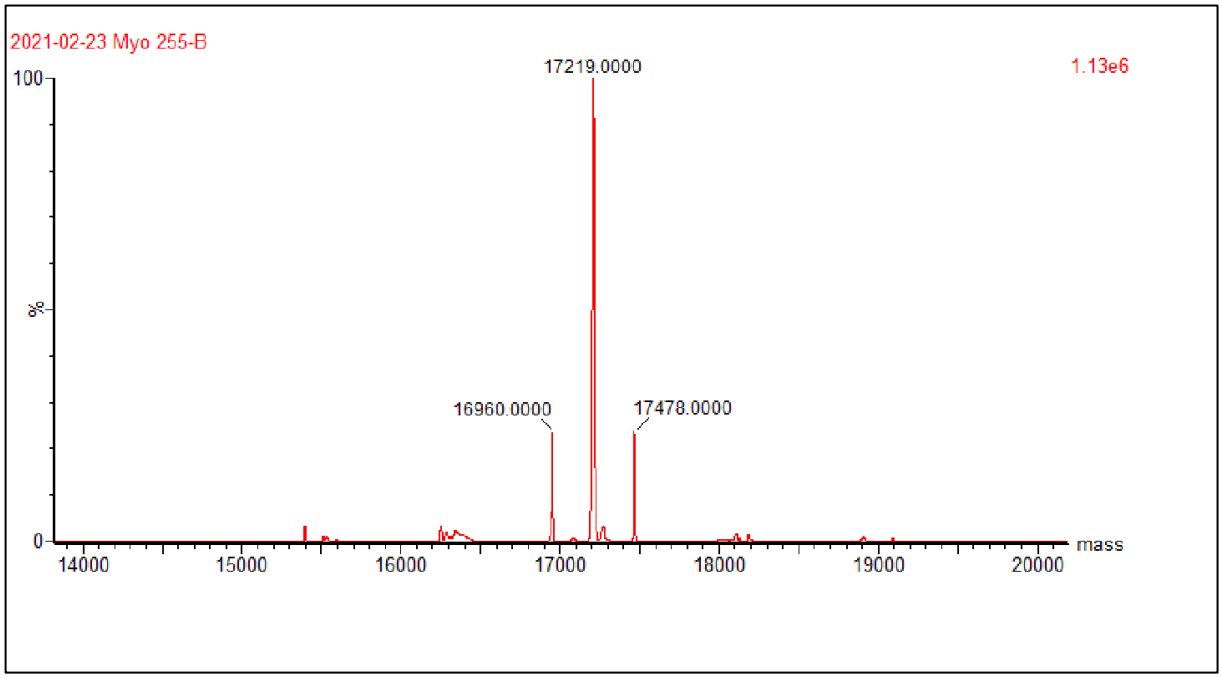


Myoglobin +10 (NMeLum)

UPLC-MS profile



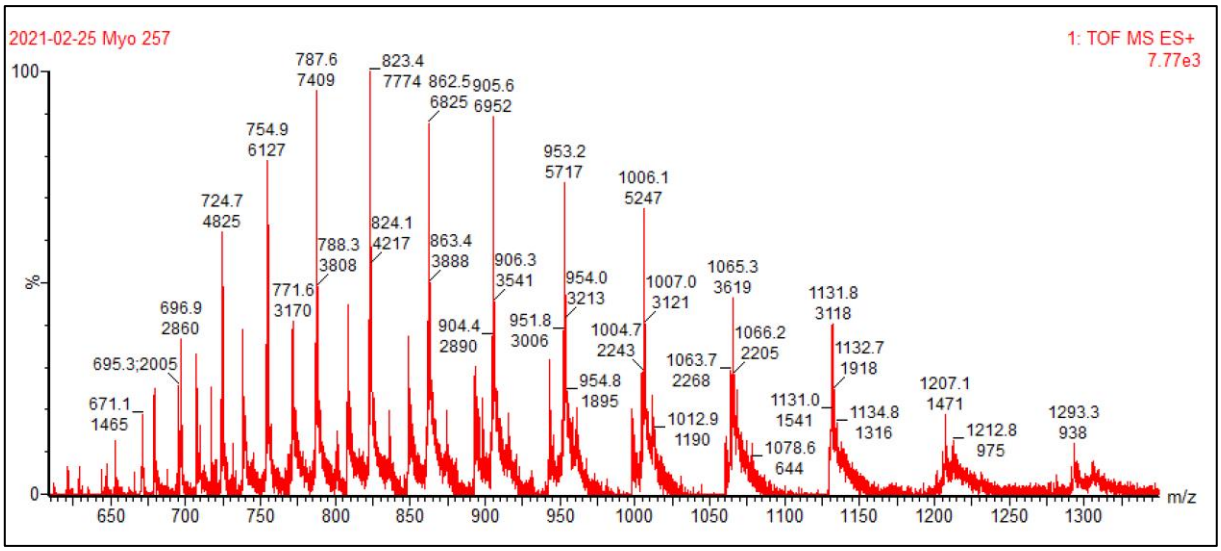
Deconvolution



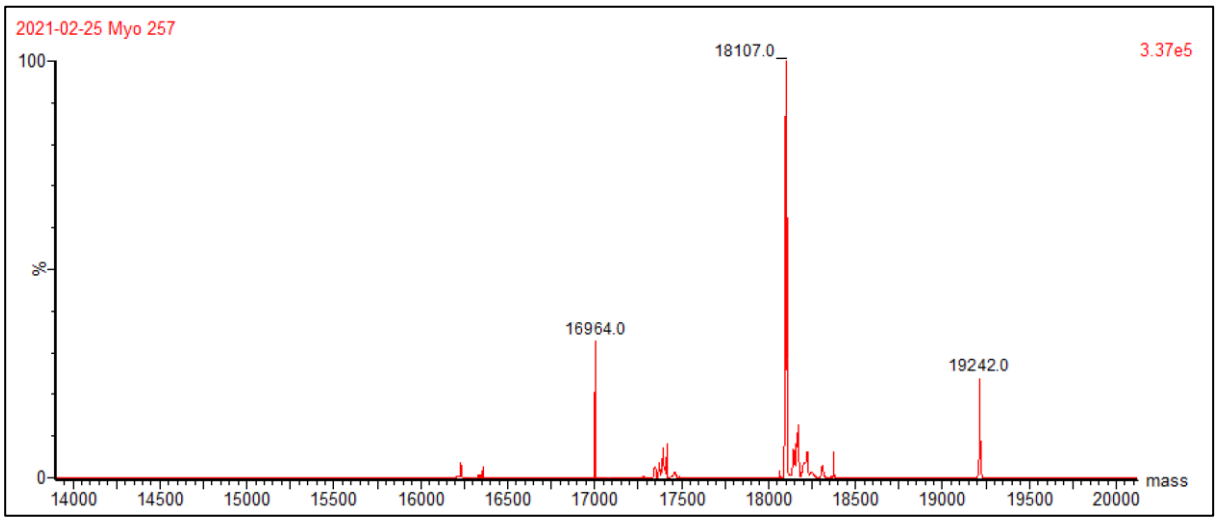
Expected (+1): **17218**, found **17219**
Expected (+2): **17477**, found **17478**

10-Myo + DBCO-PEG-FAM

UPLC-MS profile



Deconvolution



Expected (+1): **18100**, found **18107**
Expected (+2): **19240**, found **19242**

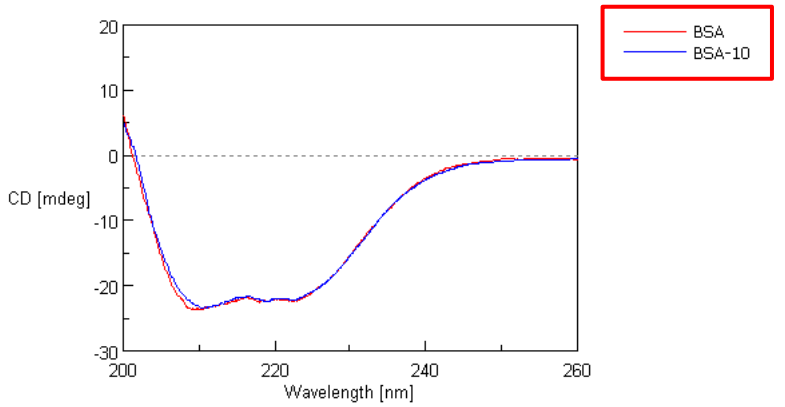
Modification of BSA, ManJB, GOx and Tras

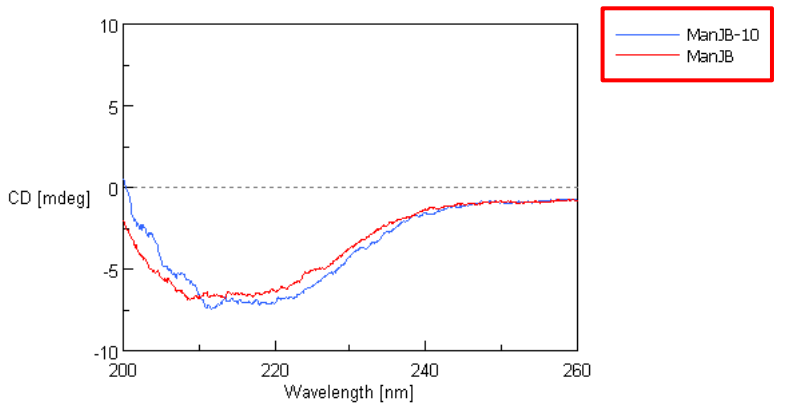
Procedure

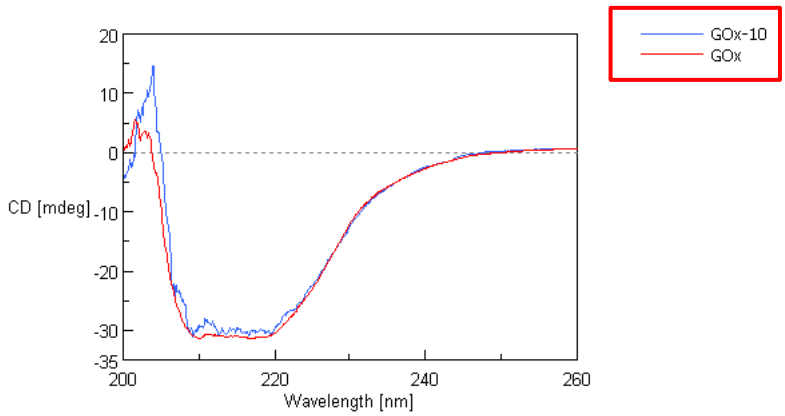
In a 5 mL glass vial, three electrodes system used was 7.8 cm^2 graphite plate as anode, 7.8 cm^2 platinum plate as cathode and the reference electrod was Ag/AgCl. Appropriate voltage vs Ag/AgCl was applied to a mixture of **10** (10 μmol, 2 mM) and protein (BSA: 2 mg or ManJB: 2.4 mg or GOx: 3 mg or Tras: 5 mg) in 100 mM phosphate buffer (5 mL). Electrolysis was performed at room temperature (stirring: 500 rpm) during 1 h. The resulting modified protein sample was then dialyzed using SpectraPor® Dialysis Membrane (MWCO = 1 kDa) to remove the excess of reagent, and was split into two aliquots and lyophilized. First aliquot was analysed by circular dichroism (DC) to confirm structural integrity after the eY-click process. The second aliquot was diluted to 500 μL in a 1 mL plastic Eppendorf and 25-35 μL of a 10 μg/μL solution of DBCO-PEG_4-4/5-FAM in 8:2 H_2O/DMF were added. The mixture was incubated at 37°C during 1 h, lyophilized and analyzed by SDS-PAGE. 10-Tras sample was also subjected to Bio-layer interferometry evaluation.

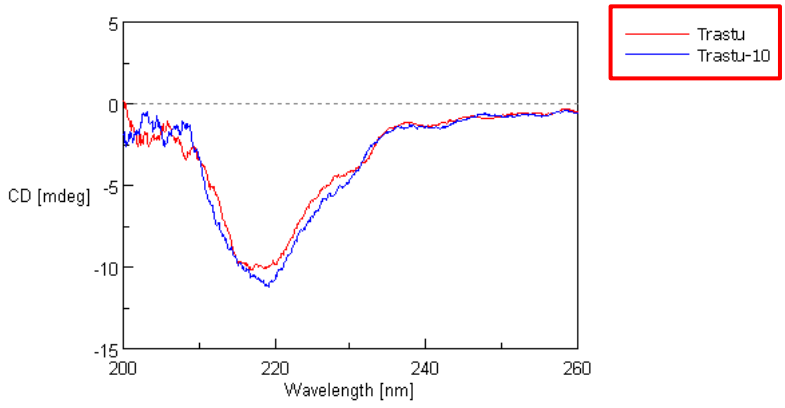
Circular Dichroism measurements

The circular dichroism (CD) spectra of proteins before and after eY-click with **10** were measured using a J-810 spectropolarimeter (Jasco, Japan). The data were acquired in a mini-quartz cell with a path length of 0.02 cm for BSA (6 μM), mannosidase (3 μM), trastuzumab (2 μM) and with a path length of 0.2 cm for glucose oxidase (2 μM). The spectra were recorded in wavelength range from 200 to 260 nm and averaged over three scans (response time 0.125 s; data pitch 0.1 nm, scanning speed 50 nm/min).


BSA
BSA-10
CD [mdeg]
Wavelength [nm]

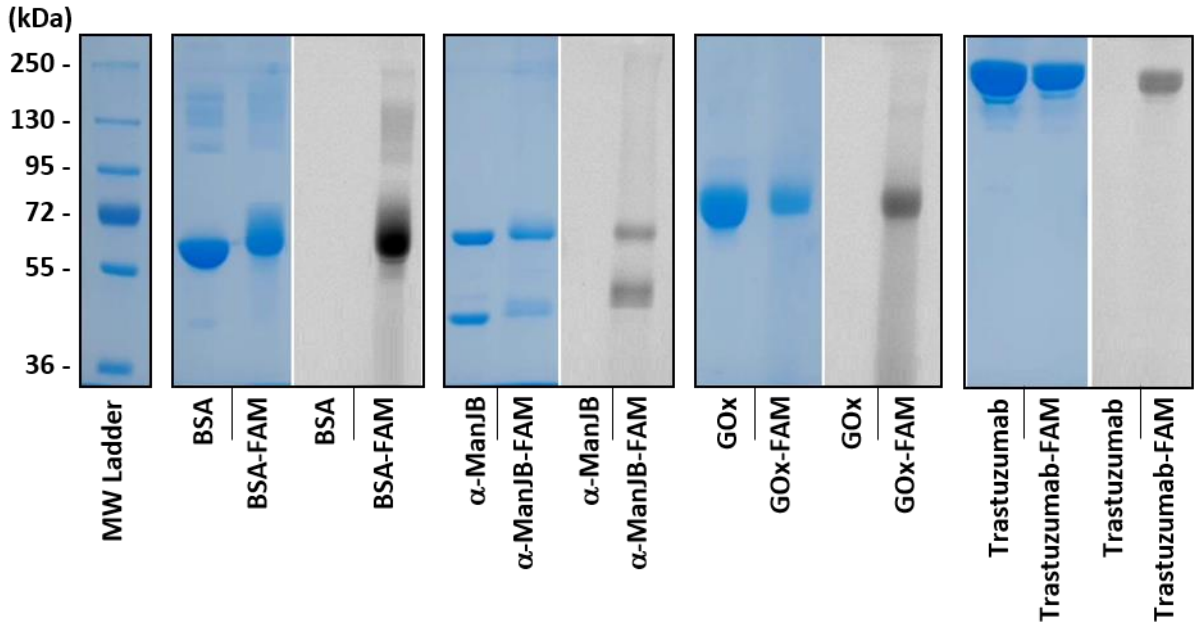

ManJB-10
ManJB
CD [mdeg]
Wavelength [nm]


GOx-10
GOx
CD [mdeg]
Wavelength [nm]


Trastu
Trastu-10
CD [mdeg]
Wavelength [nm]

SDS-PAGE

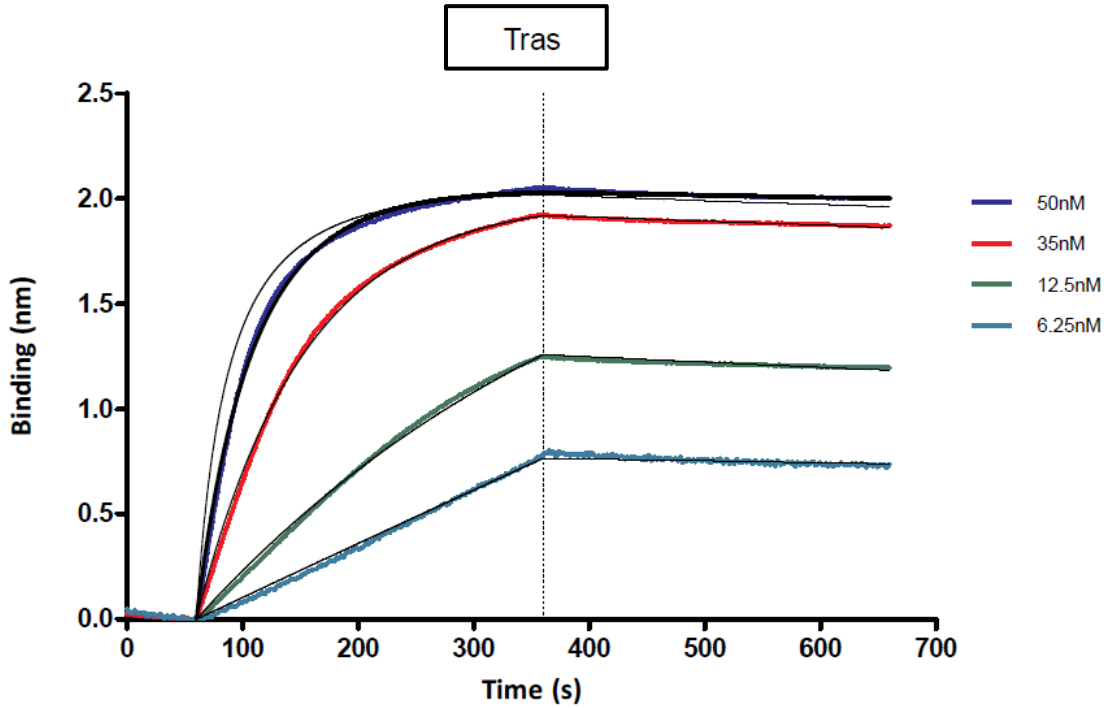
Each protein samples (native and conjugated) were analysed by SDS-PAGE. Samples were diluted with 250 μL distillated water (BSA, α-Man, GOx) or 400 μL (Tras). 2 μL (BSA) or 15 μL (α-Man) or 4 μL (Tras) or 5 μL (GOx) of these solutions were diluted to 20 μL with a loading buffer composed of distillated water (4.8 mL), 0.5M Tris-HCl pH 6.8 (1.2 mL), Glycerol (1 mL), 10% (w/v) SDS (2 mL), 0.1% (w/v) Bromophenol blue (0.5 mL). The resulting proteins solutions were heated at 100 °C for 2 min and separated on a 8 % acrylamide gel (injection volume 20 μL) at 90 V during 30 min then at 120 V until the end. The gel was washed with water and visualized under UV using Molecular Imager Biorad GelDoc XR System for fluorescence detection (right side of each gel/protein). The gel was then visualized with Coomassie brilliant blue (CBB, left side of each gel/protein) stain (1 h colouration and decolouration overnight) to confirm presence of the native/conjugated proteins.

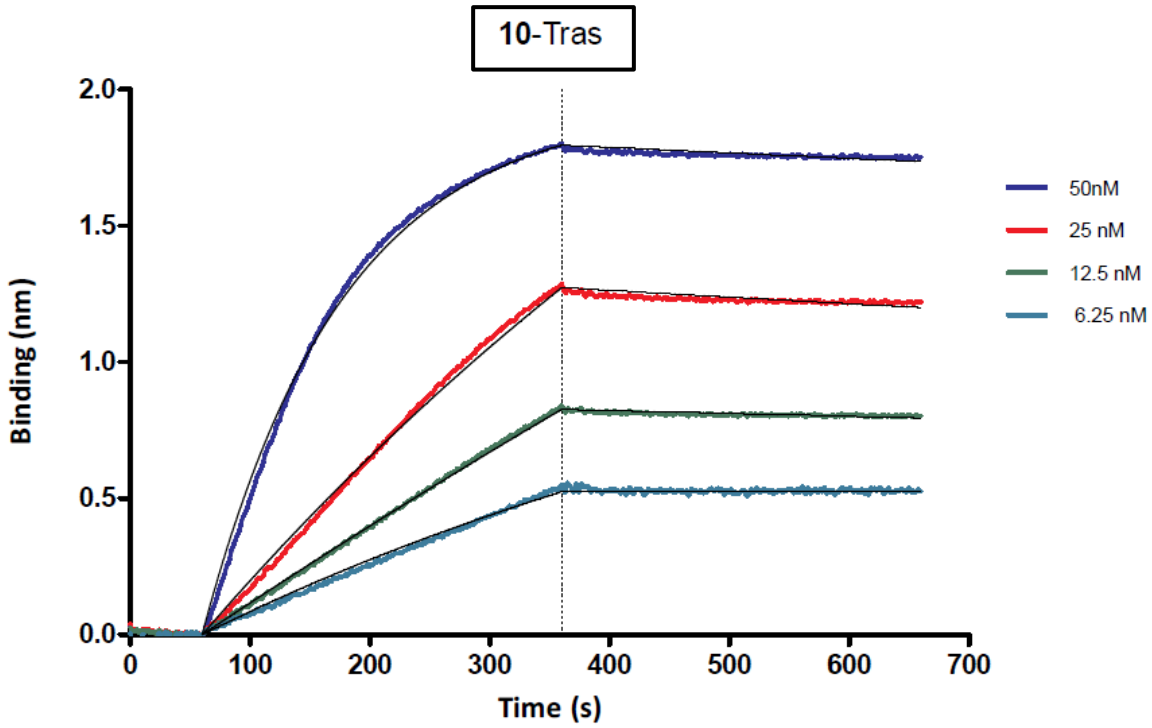


Bio-Layer Interferometry binding assays

Bio-Layer Interferometry (BLI) experiments were performed on a BLItz instrument (Sartorius, Goettingen, Germany) to measure the binding of Human Her2$(His)_6$ receptor (AcroBiosystems, Reference HE2-H5225) to Trastuzumab (Tras) or 10-Tras conjugate. All sample dilutions and baseline steps were carried out using the same reaction buffer (phosphate buffer saline pH 7.4, 0.02% (v/v) Tween-20, 0.1% (w/v) bovine serum albumin). Her2$(His)_6$ (1µM) was first loaded onto Ni-NTA biosensors (ForteBio, reference 18–5101) for 300 s and allowed to equilibrate (60 s) before the binding kinetic steps. Firstly, baseline with the reaction buffer was measured for 60 s. For the association step, each loaded biosensor was dipped into dilutions of Tras or 10-Tras conjugate (from 6.25nM to 50nM) for 300 s with a 2200 rpm shaking speed. Finally, the dissociation step was monitored by dipping the biosensor back into the reaction buffer for 300 s. Control experiments were performed to measure non-specific binding and included binding of reaction buffer with loaded biosensor (subtracted data). Experimental curves were local fitted using a 1:1 binding model with the Blitz pro 1.1 software. Mean values of kinetic constants (k_{on}, k_{off} and K_D) were calculated from the data of four different concentrations of Tras or 10-Tras conjugate. The equilibrium dissociation constant (K_D) was calculated as the ratio of dissociation constant (k_{off})/association constant (k_{on}). Sensorgrams and fitting curves were plotted using Prism 5.0 software (GraphPad Software, La Jolla, CA).

	K_D (M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
Tras	$1.2\ 10^{-9} \pm 9.1\ 10^{-10}$	$2.5\ 10^{5} \pm 5.1\ 10^{3}$	$1.4\ 10^{-4} \pm 4.6\ 10^{-6}$
10-Tras	$3.8\ 10^{-9} \pm 4.6\ 10^{-10}$	$7.7\ 10^{4} \pm 5.0\ 10^{3}$	$1.5\ 10^{-4} \pm 7.7\ 10^{-6}$

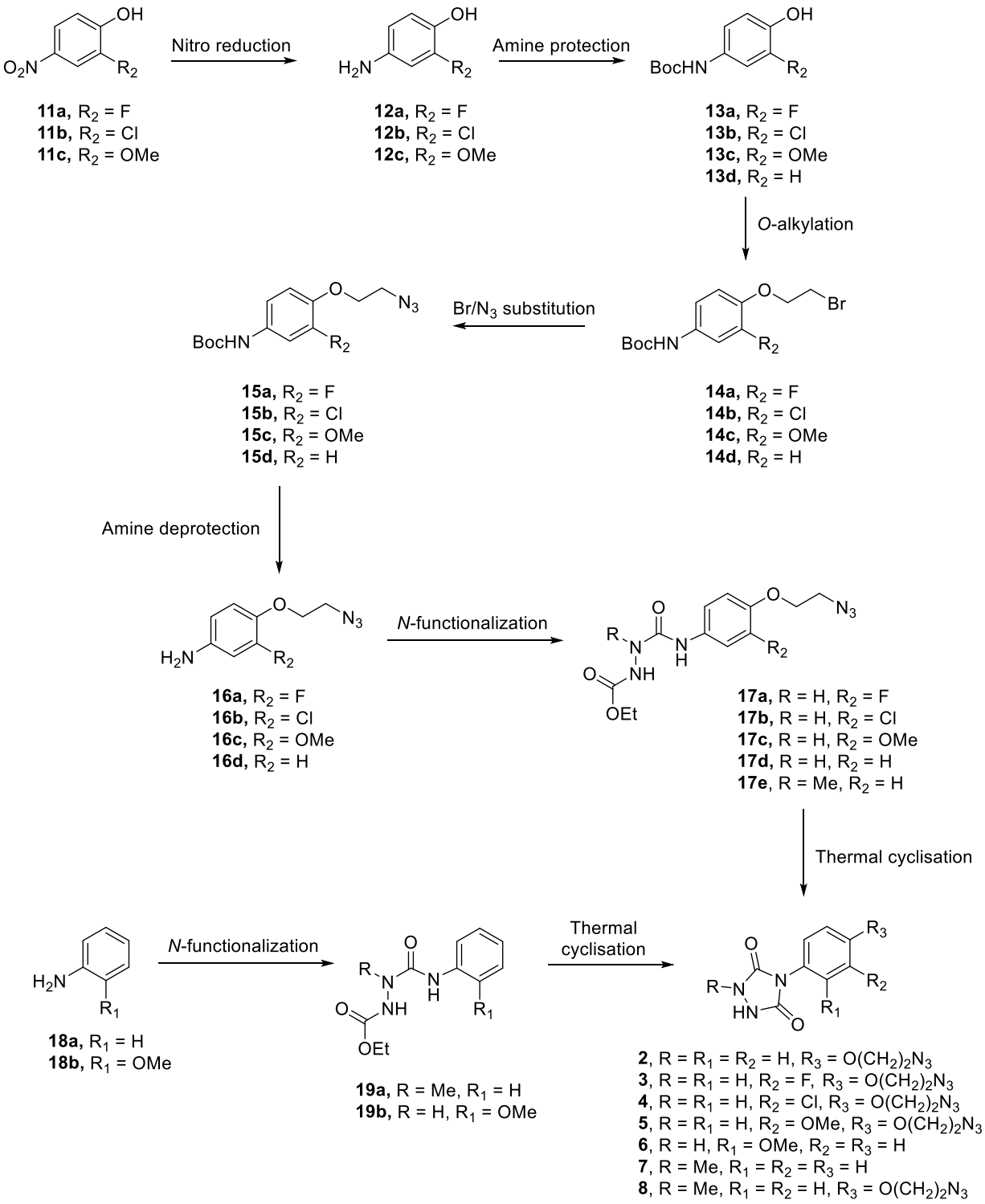

Tras
Binding (nm)
Time (s)
50nM
35nM
12.5nM
6.25nM


10-Tras
Binding (nm)
Time (s)
50nM
25 nM
12.5 nM
6.25 nM

IV. Synthesis of labeling reagents

Urazoles reagents (PhUr 2-6 and NMePhUr 7-8)*

*Urazoles reagents were prepared by adapting the strategy described in the group of Barbas[1]



11a, R_2 = F
11b, R_2 = Cl
11c, R_2 = OMe

12a, R_2 = F
12b, R_2 = Cl
12c, R_2 = OMe

13a, R_2 = F
13b, R_2 = Cl
13c, R_2 = OMe
13d, R_2 = H

14a, R_2 = F
14b, R_2 = Cl
14c, R_2 = OMe
14d, R_2 = H

15a, R_2 = F
15b, R_2 = Cl
15c, R_2 = OMe
15d, R_2 = H

16a, R_2 = F
16b, R_2 = Cl
16c, R_2 = OMe
16d, R_2 = H

17a, R = H, R_2 = F
17b, R = H, R_2 = Cl
17c, R = H, R_2 = OMe
17d, R = H, R_2 = H
17e, R = Me, R_2 = H

18a, R_1 = H
18b, R_1 = OMe

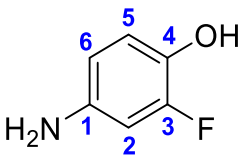
19a, R = Me, R_1 = H
19b, R = H, R_1 = OMe

2, R = R_1 = R_2 = H, R_3 = $O(CH_2)_2N_3$
3, R = R_1 = H, R_2 = F, R_3 = $O(CH_2)_2N_3$
4, R = R_1 = H, R_2 = Cl, R_3 = $O(CH_2)_2N_3$
5, R = R_1 = H, R_2 = OMe, R_3 = $O(CH_2)_2N_3$
6, R = H, R_1 = OMe, R_2 = R_3 = H
7, R = Me, R_1 = R_2 = R_3 = H
8, R = Me, R_1 = R_2 = H, R_3 = $O(CH_2)_2N_3$

General procedure for nitro reduction/hydrogenation (I)

In a dried round-bottom flask fitted with a T-tube glassware, 4-nitrophenol substrate (1 equiv.) was solubilized in anhydrous MeOH (0.15 M) at room temperature. Then, 3 vacuum/azote cycles were performed, Pd/C ($20\%_{w/w}$) was introduced and 3 vacuum/hydrogen cycles were performed before letting mixture stirred at room temperature under positive hydrogen atmosphere. After completion, solids were filtered off on a celite pad and thoroughly washed by MeOH. The obtained solution was then concentrated *in vacuo* to afford crude 4-aminophenol which doesn't require further purification.

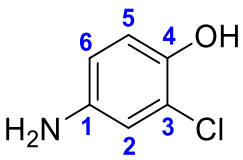
4-Amino-2-fluorophenol (12a)



Commercially available **11a** (1.0 g, 6.37 mmol, 1 equiv.) was hydrogenated following general procedure **(I)** with Pd/C (200 mg) and anhydrous MeOH (40 mL) during 3 h, to afford **12a** (810 mg, crude yield: quant.) as a brown solid. ^{1}H NMR (300.13 MHz, MeOD, 298.15 K): δ_H 6.69 (dd, $^{4}J_{5\text{-}F}$ = 9.71 Hz, $^{3}J_{5\text{-}6}$ = 8.55 Hz, 1H, H^{5}), 6.50 (dd, $^{3}J_{2\text{-}F}$ = 12.78 Hz, $^{4}J_{2\text{-}6}$ = 2.66 Hz, 1H, H^{2}), 6.39 (ddd, $^{3}J_{6\text{-}5}$ = 8.55 Hz, $^{4}J_{6\text{-}2}$ = 2.66 Hz, $^{5}J_{6\text{-}F}$ = 1.24 Hz, 1H, H^{6}). ^{13}C NMR (75.48 MHz, MeOD, 298.15 K): δ_C 153.3 (d, $^{1}J_{3\text{-}F}$ = 238.50 Hz, C^{3}), 141.7 (d, $^{3}J_{1\text{-}F}$ = 8.90 Hz, C^{1}), 138.1 (d, $^{2}J_{4\text{-}F}$ = 13.18 Hz, C^{4}), 119.9 (d, $^{3}J_{5\text{-}F}$ = 3.75 Hz, C^{5}), 112.9 (d, $^{4}J_{6\text{-}F}$ = 3.07 Hz, C^{6}), 105.3 (d, $^{2}J_{2\text{-}F}$ = 21.67 Hz, C^{2}). HRMS (ESI^{+}): *m/z* calculated for C_6H_7FNO $[M+H]^+$ 128.0512 found 128.0509

4-Amino-2-chlorophenol (12b)*

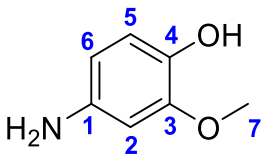
*General procedure (hydrogenation) was followed with **11b** to afford nitro reduction but also removal of Cl.



Commercially available **11b** (1.0 g, 5.76 mmol, 1 equiv.) was solubilized in MeOH (15 mL) and a saturated aqueous solution of NH_4Cl (35 mL). Then, Zn° (1.5 g, 23.04 mmol, 4 equiv.) was added and the mixture was placed on a 80 °C preheated bath. After 5 min stirring, the mixture was cooled down to room temperature. Solids were filtered off on a celite pad and the

resulting solution was diluted with AcOEt (40 mL) and water (20 mL). Aqueous layer was extracted 2x with AcOEt (2x 20 mL), and organic layer was washed 1x with water (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford pure **12b** (830 mg, crude yield: quant.) as a brown solid. ^{1}H NMR (300.13 MHz, MeOD, 298.15 K): δ_H 6.75 (d, $^{4}J_{2\text{-}6}$ = 2.66 Hz, 1H, H^2), 6.72 (d, $^{3}J_{5\text{-}6}$ = 8.55 Hz, 1H, H^5), 6.57 (dd, $^{3}J_{6\text{-}5}$ = 8.55 Hz, $^{4}J_{6\text{-}2}$ = 2.66 Hz, 1H, H^6). ^{13}C NMR (75.48 MHz, MeOD, 298.15 K): δ_C 146.7 (s, C^4), 141.7 (s, C^1), 121.9 (s, C^3), 118.3 (s, C^5), 118.2 (s, C^2), 116.9 (s, C^6). HRMS (ESI^+): *m/z* calculated for $C_6H_7(^{35}Cl)NO$ $[M+H]^+$ 144.0216 found 144.0214

4-Amino-2-methoxyphenol (12c)

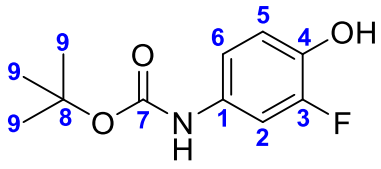


Commercially available **11c** (1.0 g, 5.91 mmol, 1 equiv.) was hydrogenated following general procedure **(I)** with Pd/C (200 mg) and anhydrous MeOH (50 mL) during 3h, to afford **12c** (825 mg, crude yield: quant.) as a brown solid. ^{1}H NMR (400.16 MHz, MeOD, 298.15 K): δ_H 6.67 (d, $^{3}J_{5\text{-}6}$ = 8.31 Hz, 1H, H^5), 6.62 (d, $^{4}J_{2\text{-}6}$ = 2.48 Hz, 1H, H^2), 6.42 (dd, $^{3}J_{6\text{-}5}$ = 8.31 Hz, $^{4}J_{6\text{-}2}$ = 2.48 Hz, 1H, H^6), 3.83 (s, 3H, H^7). ^{13}C NMR (100.62 MHz, MeOD, 298.15 K): δ_C 149.9 (s, C^3), 141.4 (s, C^4), 140.4 (s, C^1), 116.7 (s, C^5), 109.4 (s, C^6), 102.6 (s, C^2), 56.2 (s, C^7). HRMS (ESI^+): *m/z* calculated for $C_7H_{10}NO_2$ $[M+H]^+$ 140.0712 found 140.0708

General procedure for amine protection (II)

Prepared 4-aminophenol (1 equiv.) was dissolved in anhydrous THF (0.15 M). Boc_2O (1 equiv.) and Et_3N (1 equiv.) were then introduced and mixture was left stirred at room temperature. After completion, mixture was concentrated under reduced pressure and purified by silica gel chromatography to afford pure *N*-Boc-4-aminophenol.

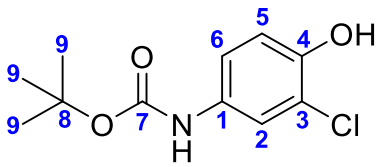
t*-Butyl-(3-fluoro-4-hydroxyphenyl)carbamate (13a)



*General procedure (protection) was performed only once and with 2 equiv. of Boc_2O, affording the pure *N*- and *O*-Boc compound which needed selective *O*-Boc removal.

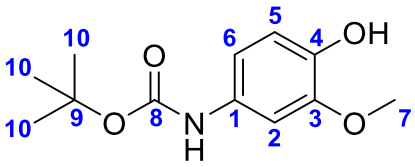
12a (820 mg, 6.45 mmol, 1.0 equiv.) was protected following general procedure **II** with Boc_2O (2.8 g, 12.90 mmol, 2 equiv.), Et_3N (1.7 mL, 12.90 mmol, 2 equiv.) and anhydrous THF (45 mL). After 12h, the obtained crude compound was purified by silica gel chromatography (100:0 to 70:30 CyHex/AcOEt) to obtain 1.3 g (88%) of pure *N*- and *O*-Boc compound and 205 mg (9%) of pure **13a**. First fraction was then redissolved in 1:1 H_2O/Dioxane (0.1 M) and heated at 80°C for 7h30. Mixture was then allowed to cool down to room temperature and the compound was extracted 3x with AcOEt, dried over $MgSO_4$ and concentrated under reduced pressure after filtration. The obtained crude product was purified by silica gel chromatography (100:0 to 70:30 CyHex/AcOEt) to afford **13a** (725 + 205 mg, 64%) as a white solid. ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.37 (d, $^3J_{2\text{-}F}$ = 12.53 Hz, 1H, H^2), 6.90 (dd, $^4J_{5\text{-}F}$ = 8.90 Hz, $^3J_{5\text{-}6}$ = 8.76 Hz, 1H, H^5), 6.81 (ddd, $^3J_{6\text{-}5}$ = 8.76 Hz, $^4J_{6\text{-}2}$ = 2.43 Hz, $^5J_{6\text{-}F}$ = 1.02 Hz, 1H, H^6), 6.35 (*br* s, 1H, *N*H), 4.94 (*br* s, 1H, *O*H), 1.50 (s, 9H, H^9). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 152.8 (s, C^7), 150.7 (d, $^1J_{3\text{-}F}$ = 236.94 Hz, C^3), 139.2 (d, $^2J_{4\text{-}F}$ = 14.45 Hz, C^4), 131.6 (d, $^3J_{1\text{-}F}$ = 9.46 Hz, C^1), 117.1 (s, C^5), 115.1 (s, C^6), 107.4 (d, $^2J_{2\text{-}F}$ = 23.50 Hz, C^2), 80.7 (s, C^8), 28.3 (s, 3x C^9). HRMS (ESI^-): *m/z* calculated for $C_{11}H_{13}FNO_3$ $[M\text{-}H]^-$ 226.0879 found 226.0881

_t_-Butyl-(3-chloro-4-hydroxyphenyl)carbamate (13b)



4-aminophenol **12b** (770 mg, 4.48 mmol, 1.0 equiv.) was protected following general procedure **II** with Boc_2O (1.9 g, 8.96 mmol, 2 equiv.), Et_3N (1.3 mL, 8.96 mmol, 2 equiv.) and anhydrous THF (30 mL). After 12h, the obtained crude compound was purified by silica gel chromatography (70:30 CyHex/AcOEt) to afford pure **13b** (890 mg, 82%) as a white solid. ^1H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.54 (*br* s, 1H, H^2), 7.00 (dd, $^3J_{6\text{-}5}$ = 8.83 Hz, $^4J_{6\text{-}2}$ = 2.53 Hz, 1H, H^6), 6.91 (d, $^3J_{5\text{-}6}$ = 8.83 Hz, 1H, H^5), 6.35 (*br* s, 1H, *N*H), 5.38 (*br* s, 1H, *O*H), 1.51 (s, 9H, H^9). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.0 (s, C^7), 151.1 (s, C^3 or C^1), 147.5 (s, C^3 or C^1), 131.9 (s, C^4), 119.9 (s, C^2), 119.3 (s, C^6), 116.3 (s, C^5), 80.8 (s, C^8), 28.6 (s, 3x C^9). HRMS (ESI^-): *m/z* calculated for $C_{11}H_{13}(^{35}Cl)NO_3$ $[M\text{-}H]^-$ 242.0584 found 242.0582

_t_-Butyl-(3-methoxy-4-hydroxyphenyl)carbamate (13c)

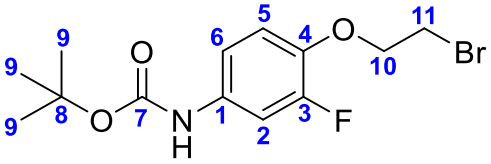


4-aminophenol **12c** (822 mg, 5.91 mmol) was protected following general procedure **II** with Boc_2O (1.4 g, 5.91 mmol) Et_3N (0.9 mL, 5.91 mmol) and anhydrous THF (40 mL). After 12h, the obtained crude compound was purified by silica gel chromatography (70:30 CyHex/AcOEt) to afford pure **13c** (725 mg, 52%) as a white solid. ^1H NMR (400.16 MHz, $CDCl_3$, 298.15 K): δ_H 7.24 (*br* s, 1H, H^2), 6.81 (d, $^3J_{5\text{-}6}$ = 8.42 Hz, 1H, H^5), 6.58 (dd, $^3J_{6\text{-}5}$ = 8.42 Hz, $^4J_{6\text{-}2}$ = 2.41 Hz, 1H, H^6), 6.35 (*br* s, 1H, *N*H), 3.89 (s, 3H, H^7), 1.50 (s, 9H, H^{10}). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.1 (s, C^8), 146.7 (s, C^3), 141.6 (s, C^1), 131.4 (s, C^4), 114.2 (s, C^5), 111.6 (s, C^6), 103.5 (s, C^2), 80.3 (s, C^9), 56.2 (s, C^7), 28.6 (s, 3x C^{10}). HRMS (ESI^+): *m/z* calculated for $C_{12}H_{17}NO_4Na$ $[M+Na]^+$ 262.1055 found 262.1060

General procedure for *O*-alkylation (III)

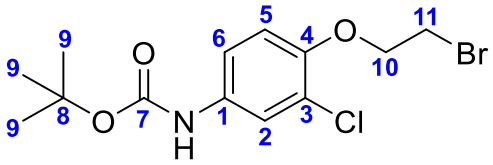
N-Boc-4-aminophenol (1 equiv.) was dissolved in anhydrous MeCN (0.1 M) at room temperature. K_2CO_3 (5 equiv.) and 1,2-dibromoethane (5 equiv.) were then introduced and the suspension was heated to 70 °C. After completion, mixture was cooled to room temperature and mixture was diluted with excess AcOEt and water. Organic compound was extracted with AcOEt (3x), washed with water (1x), dried over $MgSO_4$, filtrated and concentrated under reduced pressure. The crude compound was purified by silica gel chromatography to afford pure *O*-alkylated compound.

***t*-Butyl-(4-(2-bromoethoxy)-3-fluorophenyl)carbamate (14a)**



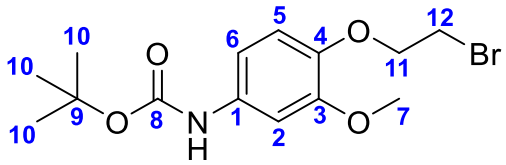
13a (670 mg, 2.95 mmol, 1 equiv.) was dissolved in anhydrous DMF (15 mL, 0.2 M) at room temperature. Then, K_2CO_3 (1.2 g, 8.84 mmol, 3 equiv.) and 1,2-dibromoethane (2.5 mL, 29.5 mmol, 10 equiv.) were added and reaction was heated at 80°C during 8h. DMF was then removed under reduced pressure by co-evaporation with toluene. The obtained crude compound was purified by silica gel chromatography (100:0 to 80:20 CyHex/AcOEt) to afford pure **14a** (585 mg, 59%) as a white solid and recover 193 mg (29%) of pure 13a. ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.33 (d, $^3J_{2\text{-}F}$ = 12.99 Hz, 1H, H^2), 6.92 (m, 2H, H^5, H^6), 6.41 (*br* s, 1H, *N*H), 4.30 (t, $^3J_{10\text{-}11}$ = 6.45 Hz, 2H, H^{10}), 3.61 (t, $^3J_{11\text{-}10}$ = 6.45 Hz, 2H, H^{11}), 1.51 (s, 9H, H^9). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.2 (d, $^1J_{3\text{-}F}$ = 246.10 Hz, C^3), 152.6 (s, C^7), 141.5 (d, $^2J_{4\text{-}F}$ = 11.46 Hz, C^4), 133.5 (d, $^3J_{1\text{-}F}$ = 9.52 Hz, C^1), 117.7 (s, C^5), 114.4 (s, C^6), 108.1 (d, $^2J_{2\text{-}F}$ = 21.88 Hz, C^2), 80.9 (s, C^8), 70.4 (s, C^{10}), 28.9 (s, C^{11}), 28.3 (s, 3x C^9). HRMS (ESI^+): *m/z* calculated for $C_{13}H_{17}(^{79}Br)FNO_3Na$ $[M+Na]^+$ 356.0274 found 356.0267

***t*-Butyl-(4-(2-bromoethoxy)-3-chlorophenyl)carbamate (14b)**



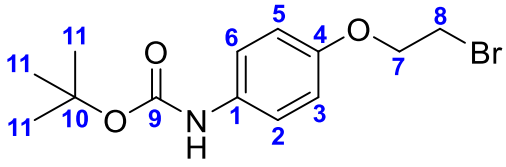
13b (750 mg, 3.08 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (15 mL, 0.2 M) at room temperature. Then, K_2CO_3 (1.2 g, 9.24 mmol, 3 equiv.) and 1,2-dibromoethane (2.7 mL, 30.79 mmol, 10 equiv.) were added and reaction was heated at 80°C during 3h. DMF was then removed under reduced pressure by co-evaporation with toluene. The obtained crude compound was purified by silica gel chromatography (100:0 to 80:20 CyHex/AcOEt) to afford pure **14b** (840 mg, 78%) as a white solid. ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.48 (d, $^4J_{2\text{-}6}$ = 2.61 Hz 1H, H^2), 7.15 (dd, $^3J_{6\text{-}5}$ = 8.70 Hz, $^4J_{6\text{-}2}$ = 2.61 Hz, 1H, H^6), 6.88 (d, $^3J_{5\text{-}6}$ = 8.70 Hz, 1H, H^5), 6.39 (*br* s, 1H, *N*H), 4.29 (t, $^3J_{10\text{-}11}$ = 6.39 Hz, 2H, H^{10}), 3.64 (t, $^3J_{11\text{-}10}$ = 6.39 Hz, 2H, H^{11}), 1.51 (s, 9H, H^9). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.2 (s, C^7), 149.9 (s, C^1), 133.4 (s, C^3), 124.3 (s, C^4), 121.3 (s, C^2), 118.2 (s, C^6), 115.5 (s, C^5), 81.2 (s, C^8), 70.2 (s, C^{10}), 28.7 (s, C^{11}), 28.3 (s, 3x C^9). HRMS (ESI$^+$): *m/z* calculated for $C_{13}H_{17}(^{79}Br)(^{35}Cl)NO_3Na$ $[M+Na]^+$ 371.9978 found 371.9972

***t*-Butyl-(4-(2-bromoethoxy)-3-methoxyphenyl)carbamate (14c)**



General procedure **III** was followed with **13c** (20 mg, 0.08 mmol), anhydrous MeCN (1 mL), K_2CO_3 (57 mg, 0.41 mmol) and 1,2-dibromoethane (40 µL, 0.41 mmol) during 3h. Purification by silica gel chromatography (85:15 CyHex/AcOEt) afforded pure **14c** (24 mg, 85%). ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.15 (*br* s, 1H, H^2), 6.78 (d, $^3J_{5\text{-}6}$ = 8.43 Hz, 1H, H^5), 6.61 (dd, $^3J_{6\text{-}5}$ = 8.43 Hz, $^4J_{6\text{-}2}$ = 2.49 Hz, 1H, H^6), 6.32 (*br* s, 1H, *N*H), 4.21 (t, $^3J_{11\text{-}12}$ = 6.55 Hz, 2H, H^{11}), 3.80 (s, 3H, H^7), 3.55 (t, $^3J_{12\text{-}11}$ = 6.55 Hz, 2H, H^{12}), 1.50 (s, 9H, H^{10}). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.1 (s, C^8), 150.6 (s, C^3), 143.6 (s, C^4), 133.6 (s, C^1), 116.5 (s, C^5), 110.5 (s, C^6), 104.1 (s, C^2), 80.6 (s, C^9), 69.9 (s, C^{11}), 56.2 (s, C^7), 29.2 (s, C^{12}), 28.6 (s, 3x C^{10}). HRMS (ESI$^+$): *m/z* calculated for $C_{14}H_{20}(^{79}Br)NO_4Na$ $[M+Na]^+$ 368.0473 found 368.0459

t-Butyl-(4-(2-bromoethoxy)-phenyl)carbamate (14d)

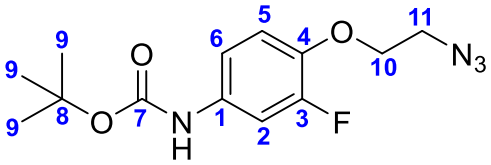


General procedure **III** was followed with commercially available **13d** (5.0 g, 23.90 mmol) anhydrous MeCN (250 mL), K_2CO_3 (6.6 g, 119.50 mmol) and 1,2-dibromoethane (10.5 mL, 119.50 mmol) during 24h. Purification by silica gel chromatography (80:20 CyHex/AcOEt) afforded pure **14d** (3.2 mg, 43%) as a white solid. ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.20 (m, 2H, H^2, H^6), 6.78 (m, 2H, H^3, H^5), 6.29 (*br* s, 1H, *N*H), 4.18 (t, $^3J_{7\text{-}8}$ = 6.32 Hz, 2H, H^7), 3.54 (t, $^3J_{8\text{-}7}$ = 6.32 Hz, 2H, H^8), 1.43 (s, 9H, H^{11}). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 154.2 (s, C^4), 153.3 (s, C^9), 132.4 (s, C^1), 120.5 (s, C^2, C^6), 115.4 (s, C^3, C^5), 80.2 (s, C^{10}), 68.3 (s, C^7), 29.4 (s, C^8), 28.2 (s, 3x C^{11}). HRMS (ESI$^+$): *m/z* calculated for $C_{13}H_{18}(^{79}Br)NO_3Na$ $[M+Na]^+$ 338.0368 found 338.0365

General procedure for bromide/azide substitution (IV)

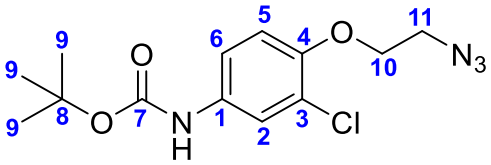
O-alkylated compound (1 equiv.) was dissolved in anhydrous DMF (0.1 M) at room temperature. NaN_3 (3 equiv.) was then added and mixture was heated to 80 °C and left stirred under positive azote atmosphere. After completion monitored by TLC, DMF was removed under reduced pressure by 4 co-evaporations with Toluene. The crude product was purified by silica gel chromatography to afford pure azidoethoxy compound.

***t*-Butyl-(4-(2-azidoethoxy)-3-fluorophenyl)carbamate (15a)**



General procedure IV was followed with **14a** (420 mg, 1.26 mmol), anhydrous DMF (12 mL), and NaN_3 (245 mg, 3.78 mmol) during 30 min at 80 °C, to afford pure **15a** (360 mg, 97%) as a white solid after purification by silica gel chromatography (70:30 CyHex/AcOEt). ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.32 (d, $^3J_{2\text{-}F}$ = 12.86 Hz, 1H, H^2), 6.92 (m, 2H, H^5, H^6), 6.41 (*br* s, 1H, *N*H), 4.16 (t, $^3J_{10\text{-}11}$ = 5.09 Hz, 2H, H^{10}), 3.55 (t, $^3J_{11\text{-}10}$ = 5.09 Hz, 2H, H^{11}), 1.50 (s, 9H, H^9). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.6 (d, $^1J_{3\text{-}F}$ = 244.81 Hz, C^3), 152.7 (s, C^7), 141.8 (d, $^2J_{4\text{-}F}$ = 11.00 Hz, C^4), 133.3 (d, $^3J_{1\text{-}F}$ = 9.63 Hz, C^1), 117.1 (d, $^3J_{5\text{-}F}$ = 2.55 Hz C^5), 114.1 (s, C^6), 108.1 (d, $^2J_{2\text{-}F}$ = 21.88 Hz, C^2), 80.8 (s, C^8), 69.5 (s, C^{10}), 50.3 (s, C^{11}), 28.3 (s, 3x C^9).

***t*-Butyl-(4-(2-azidoethoxy)-3-chlorophenyl)carbamate (15b)**



General procedure **IV** was followed with **14b** (100 mg, 0.29 mmol), anhydrous DMF (3 mL), and NaN_3 (55 mg, 0.86 mmol, 3.0 equiv.) during 30 min at 80 °C, to afford pure **15b** (90 mg, quant.) as a white solid after purification by silica gel chromatography (70:30 CyHex/AcOEt). ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.50 (d, $^4J_{2\text{-}6}$ = 2.61 Hz 1H, H^2), 7.19 (dd, $^3J_{6\text{-}5}$ = 8.66 Hz, $^4J_{6\text{-}2}$ = 2.61 Hz, 1H, H^6), 6.88 (d, $^3J_{5\text{-}6}$ = 8.66 Hz, 1H, H^5), 6.40 (*br* s, 1H, *N*H), 4.16 (t, $^3J_{10\text{-}11}$ = 4.90 Hz, 2H, H^{10}), 3.63 (t, $^3J_{11\text{-}10}$ = 4.90 Hz, 2H, H^{11}), 1.52 (s, 9H, H^9). ^{13}C

NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 152.7 (s, C^7), 149.9 (s, C^1), 133.0 (s, C^3), 123.8 (s, C^4), 122.2 (s, C^2), 118.2 (s, C^6), 114.6 (s, C^5), 80.8 (s, C^8), 68.7 (s, C^{10}), 50.3 (s, C^{11}), 28.3 (s, 3x C^9).

_t_-Butyl-(4-(2-azidoethoxy)-3-methoxyphenyl)carbamate (15c)

General procedure **IV** was followed with **14c** (170 mg, 0.49 mmol), anhydrous DMF (5 mL), and NaN_3 (95 mg, 1.47 mmol) during 12 h at 80 °C, to afford pure **15c** (150 mg, 99%) as a white solid after purification by silica gel chromatography (70:30 CyHex/AcOEt). ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.19 (*br* s, 1H, H^2), 6.84 (d, $^3J_{5\text{-}6}$ = 8.63 Hz, 1H, H^5), 6.69 (dd, $^3J_{6\text{-}5}$ = 8.63 Hz, $^4J_{6\text{-}2}$ = 2.49 Hz, 1H, H^6), 6.39 (*br* s, 1H, *N*H), 4.14 (t, $^3J_{11\text{-}12}$ = 5.51 Hz, 2H, H^{11}), 3.87 (s, 3H, H^7), 3.59 (t, $^3J_{12\text{-}11}$ = 5.51 Hz, 2H, H^{12}), 1.51 (s, 9H, H^{10}). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 153.1 (s, C^8), 150.4 (s, C^3), 143.7 (s, C^4), 133.4 (s, C^1), 116.1 (s, C^5), 110.3 (s, C^6), 104.2 (s, C^2), 80.5 (s, C^9), 68.7 (s, C^{11}), 55.9 (s, C^7), 50.4 (s, C^{12}), 28.6 (s, 3x C^{10}). HRMS (ESI^+): *m/z* calculated for $C_{14}H_{20}N_4O_4Na$ $[M+Na]^+$ 331.1382 found 331.1374

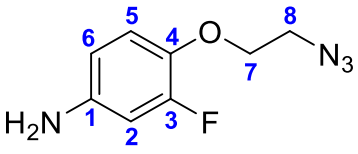
_t_-Butyl-(4-(2-azidoethoxy)-phenyl)carbamate (15d)

General procedure **IV** was followed with **15c** (3.0 g, 9.50 mmol), anhydrous DMF (100 mL), and NaN_3 (1.9 g, 28.46 mmol) during 1 h at 80 °C, to afford pure **15d** (360 mg, 97%) as a white solid after purification by silica gel chromatography (70:30 to 50:50 CyHex/AcOEt). ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.27 (m, 2H, H^2, H^6), 6.86 (m, 2H, H^3, H^5), 6.36 (*br* s, 1H, *N*H), 4.12 (t, $^3J_{7\text{-}8}$ = 5.00 Hz, 2H, H^7), 3.57 (t, $^3J_{8\text{-}7}$ = 5.00 Hz, 2H, H^8), 1.51 (s, 9H, H^{11}). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 154.2 (s, C^4), 153.1 (s, C^9), 132.2 (s, C^1), 120.5 (s, C^2, C^6), 115.2 (s, C^3, C^5), 80.3 (s, C^{10}), 67.3 (s, C^7), 50.2 (s, C^8), 28.2 (s, 3x C^{11}). HRMS ($ASAP^-$): *m/z* calculated for $C_{13}H_{17}N_4O_3$ $[M-H]^-$ 277.1301 found 277.1308

General procedure for amine deprotection (V)

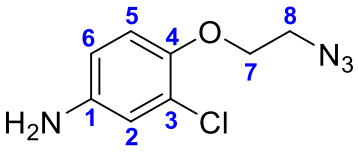
N-Boc compound (1 equiv.) was dissolved in a 1:1 DCM/TFA mixture (0.1 M) and left stirred at room temperature. After completion monitored by TLC, solvents were removed under pressure to obtain the solid ammonium salt. TFA was co-evaporated with DCM and MeOH if necessary. The crude compound was redissolved in MeOH (0.1 M) and an excess of hydroxy ammonium basic resin was introduced before the mixture being left stirred at room temperature. After completion monitored by TLC, resin was filtered off and the resulting solution was concentrated under reduced pressure. The obtained crude product was subsequently purified by silica gel chromatography to afford the pure deprotected aniline.

4-(2-azidoethoxy)-3-fluoroaniline (16a)



General procedure **V** was followed with **15a** (286 mg, 0.966 mmol), DCM (5 mL) and TFA (5 mL) for the formation of the ammonium salt (30 min), and MeOH (10 mL), hydroxy ammonium Amberlite® IRN78 resin (4 spatula, excess) to generate (1 h) the amine **16a** (120 mg) as a yellow oil which has been directly used in next step without further purification. ^{1}H NMR (400.16 MHz, MeOD, 298.15 K): δ_H 6.87 (dd, $^{4}J_{5\text{-}F}$ = 9.18 Hz, $^{3}J_{5\text{-}6}$ = 8.65 Hz, 1H, H^{5}), 6.51 (dd, $^{3}J_{2\text{-}F}$ = 13.08 Hz, $^{4}J_{2\text{-}6}$ = 2.62 Hz, 1H, H^{2}), 6.43 (ddd, $^{3}J_{6\text{-}5}$ = 8.65 Hz, $^{4}J_{6\text{-}2}$ = 2.62 Hz, $^{5}J_{6\text{-}F}$ = 1.30 Hz, 1H, H^{6}), 4.06 (t, $^{3}J_{7\text{-}8}$ = 4.86 Hz, 2H, H^{7}), 3.52 (t, $^{3}J_{8\text{-}7}$ = 4.86 Hz, 2H, H^{8}).

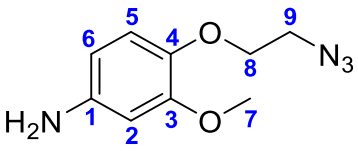
4-(2-azidoethoxy)-3-chloroaniline (16b)



General procedure **V** was followed with **15b** (388 mg, 1.22 mmol), DCM (6 mL) and TFA (6 mL) for the formation of the ammonium salt (30 min), and MeOH (12 mL), hydroxy ammonium Amberlite® IRN78 resin (4 spatula, excess) to generate (1 h) the amine **16b** (170 mg, 66%) as a yellow oil which has been directly used in next step without further purification. ^{1}H NMR (400.16 MHz, MeOD, 298.15 K): δ_H 6.87 (d, $^{3}J_{5\text{-}6}$ = 8.70 Hz, 1H, H^{5}),

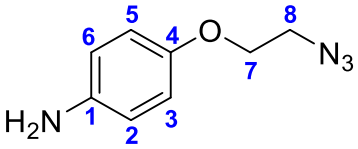
6.78 (d, $^{4}J_{2\text{-}6}$ = 2.68 Hz 1H, H^{2}), 6.61 (dd, $^{3}J_{6\text{-}5}$ = 8.70 Hz, $^{4}J_{6\text{-}2}$ = 2.68 Hz, 1H, H^{6}), 4.08 (t, $^{3}J_{7\text{-}8}$ = 4.94 Hz, 2H, H^{7}), 3.53 (t, $^{3}J_{8\text{-}7}$ = 4.94 Hz, 2H, H^{8}).

4-(2-azidoethoxy)-3-methoxyaniline (16c)



General procedure **V** was followed with **15c** (150 mg, 0.49 mmol), DCM (2.5 mL) and TFA (2.5 mL) for the formation of the ammonium salt (30 min), and MeOH (5 mL), hydroxy ammonium Amberlite® IRN78 resin (2 spatula, excess) to generate (1 h) the amine **16c** (40 mg, 40%) which has been directly used in next step without further purification. ^{1}H NMR (300.13 MHz, DMSO-d^{6}, 298.15 K): δ_H 6.76 (d, $^{3}J_{5\text{-}6}$ = 8.43 Hz, 1H, H^{5}), 6.45 (d, $^{4}J_{2\text{-}6}$ = 2.55 Hz, 1H, H^{2}), 6.26 (dd, $^{3}J_{6\text{-}5}$ = 8.43 Hz, $^{4}J_{6\text{-}2}$ = 2.55 Hz, 1H, H^{6}), 4.74 (*br* s, 2H, *N*H), 4.04 (t, $^{3}J_{8\text{-}9}$ = 5.09 Hz, 2H, H^{8}), 3.78 (s, 3H, H^{7}), 3.49 (t, $^{3}J_{9\text{-}8}$ = 5.09 Hz, 2H, H^{9}).

4-(2-azidoethoxy)aniline (16d)



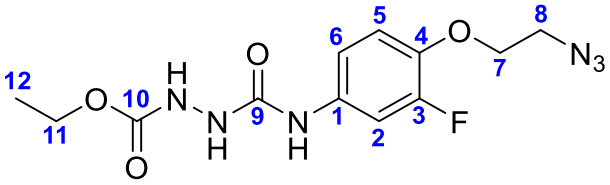
General procedure **V** was followed with **15d** (2.2 g, 7.90 mmol), DCM (40 mL) and TFA (40 mL) for the formation of the ammonium salt (1 h), and MeOH (80 mL), hydroxy ammonium Amberlite® IRN78 resin (6 spatula, excess) to generate (1 h) the amine **16d** (1.4 g, quant.) as a brown oil which has been directly used in next step without further purification. ^{1}H NMR (400.16 MHz, DMSO-d^{6}, 298.15 K): δ_H 6.68 (m, 2H, H^{2}, H^{6}), 6.52 (m, 2H, H^{3}, H^{5}), 4.63 (*br* s, 2H, *N*H), 4.02 (t, $^{3}J_{7\text{-}8}$ = 4.80 Hz, 2H, H^{7}), 3.55 (t, $^{3}J_{8\text{-}7}$ = 4.80 Hz, 2H, H^{8}).

General procedure for aniline functionalization (VI)

Appropriate ethyl carbazate (1 equiv.) and CDI (1 equiv.) were dissolved in anhydrous THF (0.2 M) and the reaction was left stirred at room temperature under positive azote atmosphere for approximatively 4 hours (followed by TLC). Then, a solution of deprotected aniline (1 equiv.) in anhydrous THF (0.2 M, final concentration 0.1 M) was added and mixture was left stirred until completion monitored by TLC. Mixture was

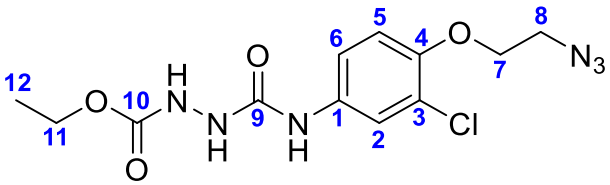
then concentrated under reduced pressure and the obtained crude product was purified by silica gel chromatography (gradient 60:40 to 40:60 to 20:80 CyHex/AcOEt) to afford pure functionalized aniline.

Ethyl 2-((4-(2-azidoethoxy)-3-fluorophenyl)carbamoyl)hydrazine-1-carboxylate (17a)



General procedure **VI** was followed with **16a** (63 mg, 0.32 mmol), anhydrous THF (3.5 mL), CDI (52 mg, 0.32 mmol) and ethyl carbazate (33 mg, 0.32 mmol). Formation of activated carbazide lasted 30 min and reaction with aniline lasted 72h at room temperature to obtain pure **17a** (33 mg, 31%) as a white solid after purification by silica gel chromatography (gradient 100:0 to 40:60 CyHex/AcOEt). ^{1}H NMR (400.16 MHz, MeOD, 298.15 K): δ_H 7.27 (dd, $^{3}J_{2\text{-}F}$ = 13.32 Hz, $^{4}J_{2\text{-}6}$ = 2.32 Hz, 1H, H^{2}), 7.03 (m, 2H, H^{5}, H^{6}), 4.18 (q, $^{3}J_{11\text{-}12}$ = 7.10 Hz, 2H, H^{11}), 4.17 (t, $^{3}J_{7\text{-}8}$ = 4.78 Hz, 2H, H^{7}), 3.58 (t, $^{3}J_{8\text{-}7}$ = 4.78 Hz, 2H, H^{8}), 1.28 (t, $^{3}J_{12\text{-}11}$ = 7.10 Hz, 3H, H^{12}). ^{13}C NMR (100.62 MHz, MeOD, 298.15 K): δ_C 159.5 (s, C^{10}), 158.6 (s, C^{9}), 153.8 (d, $^{1}J_{3\text{-}F}$ = 244.10 Hz, C^{3}), 143.5 (d, $^{2}J_{4\text{-}F}$ = 11.23 Hz, C^{4}), 134.7 (d, $^{3}J_{1\text{-}F}$ = 9.37 Hz, C^{1}), 117.3 (s, C$^{5\text{ or }6}$), 117.1 (s, C$^{5\text{ or }6}$), 110.0 (d, $^{2}J_{2\text{-}F}$ = 23.15 Hz, C^{2}), 70.15 (s, C^{7}), 62.9 (s, C^{11}), 51.5 (s, C^{8}), 14.8 (s, C^{12}), HRMS (ESI^{+}): *m/z* calculated for $C_{12}H_{15}FN_6O_4Na$ [M+Na]$^{+}$ 349.1037 found 349.1039

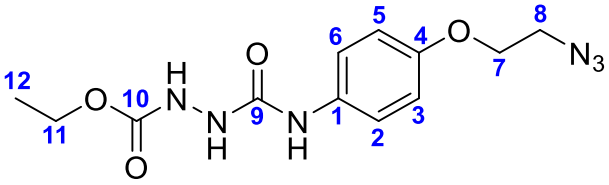
Ethyl 2-((4-(2-azidoethoxy)-3-chlorophenyl)carbamoyl)hydrazine -1-carboxylate (17b)



General procedure **VI** was followed with **16b** (65 mg, 0.31 mmol), anhydrous THF (3 mL), CDI (50 mg, 0.31 mmol) and ethyl carbazate (32 mg, 0.31 mmol). Formation of activated carbazide lasted 20 min and reaction with aniline lasted 72h at room temperature to obtain pure **17b** (34 mg, 31%) as a white solid after purification by silica gel chromatography (gradient 100:0 to 40:60 CyHex/AcOEt). ^{1}H NMR (400.16 MHz, MeOD, 298.15 K): δ_H 7.55 (d, $^{4}J_{2\text{-}6}$ = 2.60 Hz 1H, H^{2}), 7.26 (dd, $^{3}J_{6\text{-}5}$ = 8.88 Hz, $^{4}J_{6\text{-}2}$ = 2.60 Hz, 1H, H^{6}), 7.01 (d, $^{3}J_{5\text{-}6}$ = 8.88 Hz, 1H, H^{5}), 4.17

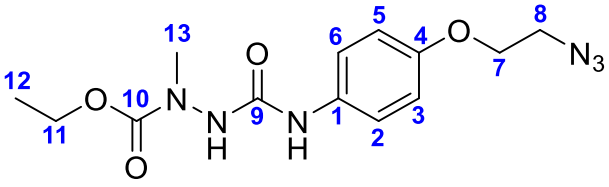
(q, $^{3}J_{11\text{-}12}$ = 7.17 Hz, 2H, H^{11}), 4.17 (t, $^{3}J_{7\text{-}8}$ = 4.87 Hz, 2H, H^{7}), 3.60 (t, $^{3}J_{8\text{-}7}$ = 4.87 Hz, 2H, H^{8}), 1.28 (t, $^{3}J_{12\text{-}11}$ = 7.17 Hz, 3H, H^{12}). ^{13}C NMR (100.62 MHz, MeOD, 298.15 K): δ_C 151.5 (s, C^{9}, C^{10}), 146.8 (s, C^{4}), 134.6 (s, C^{1}), 123.5 (s, C^{2}), 122.2 (s, C^{3}), 120.9 (s, C^{6}), 115.5 (s, C^{5}), 69.7 (s, C^{7}), 63.1 (s, C^{11}), 51.6 (s, C^{8}), 14.9 (s, C^{12}). HRMS (ESI^{+}): *m/z* calculated for $C_{12}H_{15}(^{35}Cl)N_6O_4Na$ $[M+Na]^{+}$ 365.0741 found 365.0730

Ethyl 2-((4-(2-azidoethoxy)phenyl)carbamoyl)hydrazine-1-carboxylate (17d)



General procedure **VI** was followed with **16d** (700 mg, 3.93 mmol), anhydrous THF (40 mL), CDI (637 mg, 3.93 mmol) and ethyl carbazate (409 mg, 3.93 mmol). Formation of activated carbazide lasted 4h and reaction with aniline lasted 96h at room temperature to obtain pure **17d** (850 mg, 70%) as a white solid and pure **16d** (180 mg, 25% recovery) after purification by silica gel chromatography (gradient 60:40 to 20:80 CyHex/AcOEt). ^{1}H NMR (400.16 MHz, DMSO-d^{6}, 298.15 K): δ_H 8.92 (*br* s, 1H, *N*H), 8.59 (s, 1H, *N*H), 7.95 (*br* s, 1H, *N*H), 7.43 (m, 2H, H^{2}, H^{6}), 6.93 (m, 2H, H^{3}, H^{5}), 4.18 (t, $^{3}J_{7\text{-}8}$ = 4.80 Hz, 2H, H^{7}), 4.11 (q, $^{3}J_{11\text{-}12}$ = 7.09 Hz, 2H, H^{11}), 3.68 (t, $^{3}J_{8\text{-}7}$ = 4.80 Hz, 2H, H^{8}), 1.25 (t, $^{3}J_{12\text{-}11}$ = 7.09 Hz, 3H, H^{12}). ^{13}C NMR (100.62 MHz, DMSO-d^{6}, 298.15 K): δ_C 156.9 (s, C^{9} or C^{10}), 155.7 (s, C^{9} or C^{10}), 153.1 (s, C^{4}), 133.2 (s, C^{1}), 120.3 (s, C^{2}, C^{6}), 114.5 (s, C^{3}, C^{5}), 67.1 (s, C^{7}), 60.6 (s, C^{11}), 49.7 (s, C^{8}), 14.5 (s, C^{12}). HRMS (ASAP^{+}): *m/z* calculated for $C_{12}H_{17}N_6O_4$ $[M+H]^{+}$ 309.1311 found 309.1298

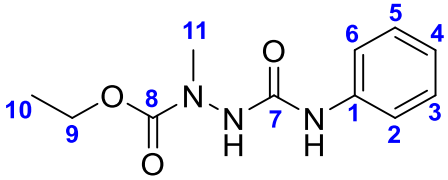
Ethyl 2-((4-(2-azidoethoxy)phenyl)carbamoyl)-1methylhydrazine-1-carboxylate (17e)



Commercially available methylhydrazine (1.9 mL, 36.84 mmol, 2.0 equiv.) was solubilized in anhydrous DCM (70 mL, 0.25 M) at room temperature and under positive argon atmosphere. Mixture was cooled down to -60 °C and ethyl chloroformate (1.8 mL, 18.42 mmol, 1.0 equiv.) was

then added dropwise. After 15 min stirring, the mixture was allowed to warm up to room temperature and a white precipitate formed. After completion (24 h) monitored by TLC, 30 mL of cold water were added and the aqueous layer was extracted once with DCM. The organic layer was washed twice with a saturated solution $NaHCO_3$ and the combined organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford ethyl 1-methylhydrazine-1-carboxylate (1.6 g) as a colourless oil. ^{1}H NMR (300.13 MHz, DMSO-d^6, 298.15 K): δ_H 4.58 (s, 2H, *N*H), 4.02 (q, $^3J_{3\text{-}4}$ = 7.05 Hz, 2H, H^3), 1.17 (t, $^3J_{4\text{-}3}$ = 7.05 Hz, 3H, H^4). HRMS (ESI$^+$): *m/z* calculated for $C_4H_{11}N_2O_2$ $[M+H]^+$ 119.0821 found 119.0822. General procedure **VI** was then followed with **16d** (700 mg, 3.93 mmol), anhydrous THF (40 mL), CDI (637 mg, 3.93 mmol) and freshly prepared ethyl 1-methylhydrazine-1-carboxylate (460.4 mg, 3.93 mmol). Formation of activated carbazide lasted 5h and reaction with aniline lasted 48h at room temperature to obtain pure **17e** (590 mg, 47%) as a white solid after purification by silica gel chromatography (gradient 75:25 to 50:50 CyHex/AcOEt). ^{1}H NMR (400.16 MHz, DMSO-d^6, 298.15 K): δ_H 8.66 (s, 1H, *N*H), 8.26 (s, 1H, *N*H), 7.36 (m, 2H, H^2, H^6), 6.88 (m, 2H, H^3, H^5), 4.12 (t, $^3J_{7\text{-}8}$ = 4.78 Hz, 2H, H^7), 4.07 (q, $^3J_{11\text{-}12}$ = 7.06 Hz, 2H, H^{11}), 3.62 (t, $^3J_{8\text{-}7}$ = 4.78 Hz, 2H, H^8), 3.05 (s, 3H, H^{13}), 1.17 (t, $^3J_{12\text{-}11}$ = 7.06 Hz, 3H, H^{12}). ^{13}C NMR (100.62 MHz, DMSO-d^6, 298.15 K): δ_C 156.6 (s, C^{10}), 154.7 (s, C^9), 153.1 (s, C^4), 132.9 (s, C^1), 120.3 (s, C^2, C^6), 114.6 (s, C^3, C^5), 66.9 (s, C^7), 61.4 (s, C^{11}), 49.5 (s, C^8), 38.7 (s, C^{13}), 14.5 (s, C^{12}). HRMS (ESI$^-$): *m/z* calculated for $C_{13}H_{17}N_6O_4$ $[M-H]^-$ 321.1311 found 321.1308

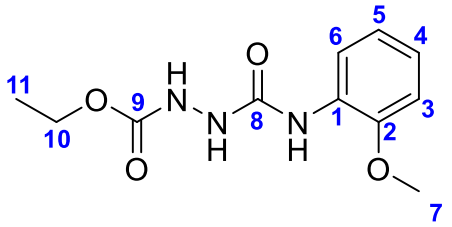
Ethyl 1-methyl-2-(phenylcarbamoyl)hydrazine-1-carboxylate (19a)



General procedure **VI** was followed with commercially available **18a** (80 µL, 0.84 mmol), anhydrous THF (8 mL), CDI (137 mg, 0.84 mmol) and freshly prepared (see synthesis for **17e**) ethyl 1-methylhydrazine-1-carboxylate (100 mg, 0.84 mmol). Formation of activated carbazide lasted 2h and reaction with aniline lasted 24h at 50°C and 72h at room temperature to obtain pure **19a** (46 mg, 24%) as a

white solid after purification by silica gel chromatography (60:40 CyHex/AcOEt). ^{1}H NMR (300.13 MHz, $CDCl_3$, 298.15 K): δ_H 7.59 (*br* s, 2H, 2x *N*H), 7.30 (m, 2H, H^2, H^6), 7.17 (m, 2H, H^3, H^5), 6.97 (m, 1H, H^4), 4.18 (q, $^{3}J_{9\text{-}10}$ = 7.14 Hz, 2H, H^9), 3.21 (s, 3H, H^{11}), 1.26 (t, $^{3}J_{10\text{-}9}$ = 7.14 Hz, 3H, H^{10}). ^{13}C NMR (75.48 MHz, $CDCl_3$, 298.15 K): δ_C 158.4 (s, C^7 or C^8), 155.6 (s, C^7 or C^8), 138.3 (s, C^1), 129.2 (s, C^2, C^6), 123.7 (s, C^4), 120.1 (s, C^3, C^5), 63.2 (s, C^9), 39.2 (s, C^{11}), 14.6 (s, C^{10}). HRMS (ESI^+): *m/z* calculated for $C_{11}H_{15}N_3O_3Na$ $[M+Na]^+$ 260.1011 found 260.1003

Ethyl 2-((2-methoxyphenyl)carbamoyl)hydrazine -1-carboxylate (19b)

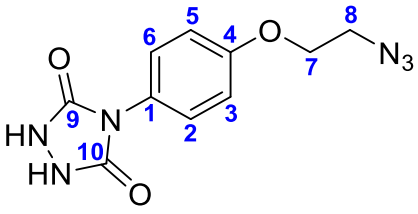


General procedure **VI** was followed with commercially available **18b** (180 µL, 1.62 mmol), anhydrous THF (16 mL), CDI (263 mg, 1.62 mmol) and ethyl carbazate (169 mg, 1.62 mmol). Formation of activated carbazide lasted 5h and reaction with aniline lasted 80h at room temperature to obtain pure **19b** (345 mg, 84%) as a white solid after purification by silica gel chromatography (gradient 100:0 to 40:60 CyHex/AcOEt). ^{1}H NMR (300.13 MHz, DMSO-d^6, 298.15 K): δ_H 8.99 (*br* s, 1H, *N*H), 8.51 (s, 1H, *N*H), 8.02 (m, 2H, *N*H, H^6), 7.02-6.83 (m, 3H, H^3, H^4, H^5), 4.05 (q, $^{3}J_{10\text{-}11}$ = 7.04 Hz, 2H, H^{10}), 3.84 (s, 3H, H^7), 1.18 (t, $^{3}J_{11\text{-}10}$ = 7.04 Hz, 3H, H^{11}). ^{13}C NMR (75.48 MHz, DMSO-d^6, 298.15 K): δ_C 156.8 (s, C^8 or C^9), 155.3 (s, C^8 or C^9), 147.6 (s, C^2), 128.4 (s, C^1), 121.9 (s, C^3 or C^4 or C^5), 118.0 (s, C^3 or C^4 or C^5), 110.7 (s, C^3 or C^4 or C^5), 60.7 (s, C^{10}), 55.8 (s, C^7),14.6 (s, C^{11}). HRMS ($ASAP^+$): *m/z* calculated for $C_{11}H_{16}N_3O_4$ $[M+H]^+$ 254.1141 found 254.1143

General procedure for thermal cyclization (VII)

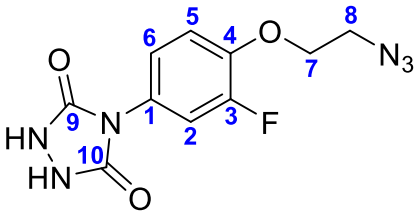
Carbazide functionalized aniline (1 equiv.) was solubilized in MeOH (0.1 M) inside a sealed vial. K_2CO_3 (2 equiv.) was then added and mixture was stirred and heated at 70 °C with the microwave synthesizer for 1 h 30. After completion confirmation by TLC, the mixture was cooled down to room temperature and the vial was removed from microwave. Sulfonic acid resin was added in excess and the neutralization was stirred for approximately 30 min and controlled by pH to reach 4-5 range. Resin was then filtered off and the obtained solution was concentrated under reduced pressure to afford pure urazole.

4-(4-(2-azidoethoxy)phenyl)-1,2,4-triazolidine-3,5-dione (2)



General procedure **VII** was followed with **17d** (350 mg, 1.13 mmol), MeOH (12 mL) and K_2CO_3 (314 mg, 2.27 mmol) to afford pure **2** (600 mg, quant.) as a white-yellow solid. ^{1}H NMR (400.16 MHz, DMSO-d^6, 298.15 K): δ_H 10.36 (s, 2H, *N*H), 7.35 (m, 2H, H^2, H^6), 7.06 (m, 2H, H^3, H^5), 4.22 (t, $^3J_{7\text{-}8}$ = 4.75 Hz, 2H, H^7), 3.67 (t, $^3J_{8\text{-}7}$ = 4.75 Hz, 2H, H^8). ^{13}C NMR (100.62 MHz, DMSO-d^6, 298.15 K): δ_C 157.1 (s, C^1), 153.7 (s, C^9, C^{10}), 127.8 (s, C^4), 125.0 (s, C^2, C^6), 114.8 (s, C^3, C^5), 67.2 (s, C^7), 49.5 (s, C^8). HRMS (ESI$^-$): *m/z* calculated for $C_{10}H_9N_6O_3$ [M-H]$^-$ 261.0736 found 261.0733

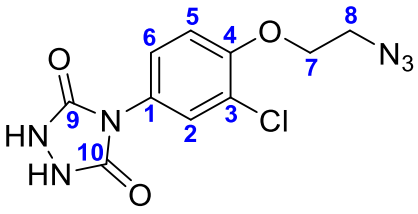
4-(4-(2-azidoethoxy)-3-fluorophenyl)-1,2,4-triazolidine-3,5-dione (3)



General procedure **VII** was followed with **17a** (32 mg, 0.10 mmol), MeOH (1 mL), K_2CO_3 (27 mg, 0.19 mmol) to afford pure **3** (23 mg, 83%) as a white solid. ^{1}H NMR (400.16 MHz, DMSO-d^6, 298.15 K): δ_H 10.45 (s, 2H, 2x *N*H), 7.41 (dd, $^3J_{2\text{-}F}$ = 12.69 Hz, $^4J_{2\text{-}6}$ = 2.29 Hz, 1H, H^2), 7.28 (m, 2H, H^5, H^6), 4.29 (t, $^3J_{7\text{-}8}$ = 4.87 Hz, 2H, H^7), 3.69 (t, $^3J_{8\text{-}7}$ = 4.87 Hz, 2H, H^8). ^{13}C NMR (100.62 MHz, DMSO-d^6, 298.15 K): δ_C 153.2 (s, C^9, C^{10}), 150.9 (d, $^1J_{3\text{-}F}$ = 240.90 Hz, C^3), 148.5 (d, $^2J_{4\text{-}F}$ = 10.45 Hz, C^4), 125.2 (d, $^3J_{1\text{-}F}$ = 9.18 Hz, C^1), 122.6 (d, $^3J_{5\text{-}F}$ = 3.36 Hz, C^5), 114.7 (d, $^4J_{6\text{-}F}$ = 1.74 Hz,

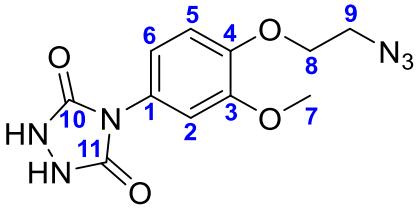
C^6), 114.4 (d, $^2J_{2\text{-}F}$ = 21.08 Hz, C^2), 69.1 (s, C^7), 50.4 (s, C^8). HRMS (ESI^-): *m/z* calculated for $C_{10}H_8FN_6O_3$ $[M\text{-}H]^-$ 279.0642 found 279.0647

4-(4-(2-azidoethoxy)-3-chlorophenyl)-1,2,4-triazolidine-3,5-dione (4)



General procedure **VII** was followed with **17b** (32 mg, 0.09 mmol), MeOH (1 mL) and K_2CO_3 (25 mg, 0.18 mmol) to afford pure **4** (25 mg, 83%) as a white solid. ^1H NMR (400.16 MHz, DMSO-d^6, 298.15 K): δ_H 10.45 (s, 2H, 2x *N*H), 7.41 (dd, $^3J_{2\text{-}F}$ = 12.69 Hz, $^4J_{2\text{-}6}$ = 2.29 Hz, 1H, H^2), 7.28 (m, 2H, H^5, H^6), 4.29 (t, $^3J_{7\text{-}8}$ = 4.87 Hz, 2H, H^7), 3.69 (t, $^3J_{8\text{-}7}$ = 4.87 Hz, 2H, H^8). ^{13}C NMR (100.62 MHz, DMSO-d^6, 298.15 K): δ_C 153.1 (s, C^9, C^{10}), 152.6 (s, C^3), 127.4 (s, C^2), 126.2 (s, C^6), 125.5 (s, C^4 or C^1), 121.3 (s, C^4 or C^1), 114.0 (s, C^5), 67.9 (s, C^7), 49.7 (s, C^8). HRMS (ESI^-): *m/z* calculated for $C_{10}H_8(^{35}Cl)N_6O_3$ $[M\text{-}H]^-$ 295.0346 found 295.0348

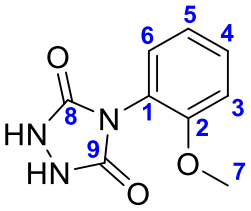
4-(4-(2-azidoethoxy)-3-methoxyphenyl)-1,2,4-triazolidine-3,5-dione (5)



General procedure **VII** was followed with **17c** (40 mg, 0.12 mmol), MeOH (2 mL) and K_2CO_3 (33 mg, 0.24 mmol) to afford pure **5** (33 mg, 99%) as a white solid. ^1H NMR (300.13 MHz, DMSO-d^6, 298.15 K): δ_H 10.37 (s, 2H, 2x *N*H), 7.28 (m, 2H, H^2, H^5), 6.93 (dd, $^3J_{6\text{-}5}$ = 8.52 Hz, $^4J_{6\text{-}2}$ = 2.41 Hz, 1H, H^6), 4.17 (t, $^3J_{8\text{-}9}$ = 5.15 Hz, 2H, H^8), 3.75 (s, 3H, H^7), 3.66 (t, $^3J_{9\text{-}8}$ = 5.15 Hz, 2H, H^9). ^{13}C NMR (75.48 MHz, DMSO-d^6, 298.15 K): δ_C 153.7 (s, C^{10}, C^{11}), 149.1 (s, C^3), 147.1 (s, C^4), 125.4 (s, C^1), 118.9 (s, C^6), 113.4 (s, C^5), 111.1 (s, C^2), 67.8 (s, C^8), 55.9 (s, C^7), 49.7 (s, C^9). HRMS (ESI^-): *m/z* calculated for $C_{10}H_{12}N_6O_4$ $[M\text{-}H]^-$ 291.0842 found 291.0846

4-(2-methoxyphenyl)-1,2,4-triazolidine-3,5-dione (6)

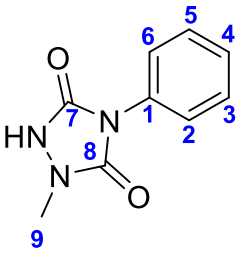
General procedure **VII** was followed with **19b** (300 mg, 1.18 mmol), MeOH (10 mL) and K2CO3 (327 mg, 2.31 mmol) to afford pure **6** (230 mg, 94%) as a white solid.



^{1}H NMR (300.13 MHz, DMSO-d^{6}, 298.15 K): δ_H 7.39 (m, 1H, H^{3} or H^{4} or H^{5} or H^{6}), 7.17 (m, 1H, H^{3} or H^{4} or H^{5} or H^{6}), 7.13 (m, 1H, H^{3} or H^{4} or H^{5} or H^{6}), 6.99 (m, 1H, H^{3} or H^{4} or H^{5} or H^{6}), 3.74 (s, 3H, H^{7}). ^{13}C NMR (75.48 MHz, DMSO-d^{6}, 298.15 K): δ_C 155.6 (s, C^{2}), 154.2 (s, C^{8}, C^{9}), 130.7 (s, C^{3} or C^{4} or C^{5} or C^{6}), 129.9(s, C^{3} or C^{4} or C^{5} or C^{6}), 121.5 (s, C^{1}), 120.1 (s, C^{3} or C^{4} or C^{5} or C^{6}), 112.2 (s, C^{3} or C^{4} or C^{5} or C^{6}), 55.6 (s, C^{7}). HRMS (ASAP^{+}): *m/z* calculated for $C_9H_{10}N_3O_3$ $[M+H]^+$ 208.0722 found 208.0721

1-methyl-4-phenyl-1,2,4-triazolidine-3,5-dione (7)

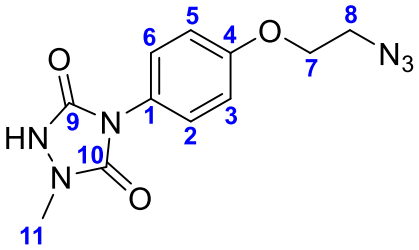
General procedure **VII** was followed with **19a** (40 mg, 0.17 mmol), MeOH (2 mL) and K_2CO_3 (47.4 mg, 0.34 mmol) to afford pure **7** (31 mg, 99%) as a white solid.



^{1}H NMR (400.16 MHz, DMSO-d^{6}, 298.15 K): δ_H 10.79 (*br* s, 1H, *N*H), 7.44 (m, 5H, H^{2}, H^{3}, H^{4}, H^{5}, H^{6}), 3.11 (s, 3H, H^{9}). ^{13}C NMR (100.62 MHz, DMSO-d^{6}, 298.15 K): δ_C 152.7 (s, C^{7} or C^{8}), 152.2 (s, C^{7} or C^{8}), 132.0 (s, C^{Ar}), 128.9 (s, 2x C^{Ar}), 127.8 (s, C^{Ar}), 126.0 (s, 2x C^{Ar}), 32.8 (s, C^{9}). HRMS (ESI^{-}): *m/z* calculated for $C_9H_8N_3O_2$ $[M-H]^-$ 190.0617 found 190.0623

4-(4-(2-azidoethoxy)phenyl)-1-methyl-1,2,4-triazolidine-3,5-dione (8)

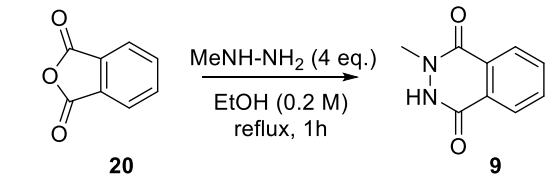
General procedure **VII** was followed with **19b** (590 mg, 1.83 mmol), MeOH (18 mL) and K_2CO_3 (506 mg, 3.66 mmol) to afford pure **8** (470 mg, 93%) as a white solid.

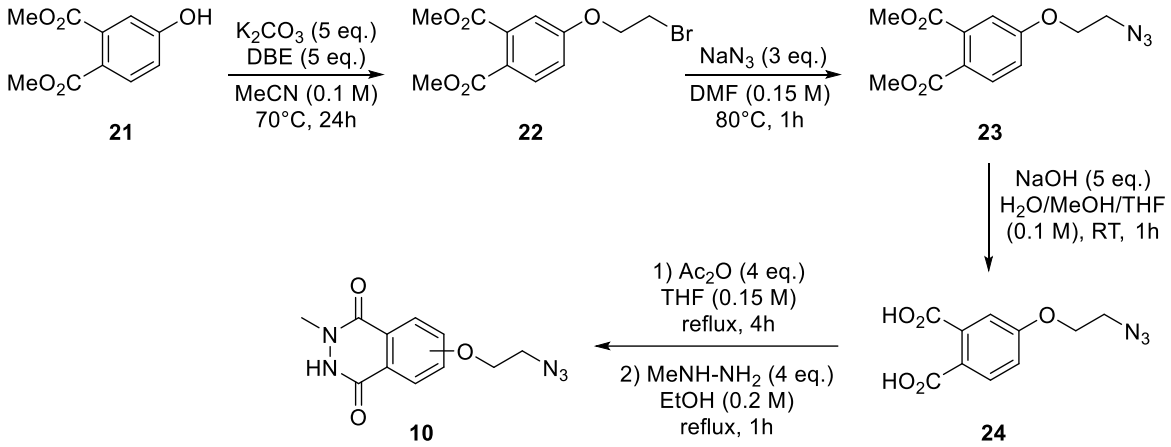


^{1}H NMR (400.16 MHz, DMSO-d^{6}, 298.15 K): δ_H 10.72 (*br* s, 1H, *N*H), 7.35 (m, 2H, H^{2}, H^{6}), 7.07 (m, 2H, H^{3}, H^{5}), 4.23 (t, $^{3}J_{7\text{-}8}$ = 4.75 Hz, 2H, H^{7}), 3.67 (t, $^{3}J_{8\text{-}7}$ = 4.75 Hz, 2H, H^{8}), 3.08 (s, 3H, H^{11}). ^{13}C NMR (100.62 MHz, DMSO-d^{6}, 298.15 K): δ_C 157.1 (s, C^{4}), 153.7 (s, C^{9} or C^{10}), 152.5 (s, C^{9} or C^{10}), 125.0 (s, C^{1}), 114.8 (s, C^{2}, C^{6}), 114.8 (s, C^{3}, C^{5}), 66.9 (s, C^{7}), 49.5 (s, C^{8}), 32.8 (s, C^{11}). HRMS (ESI^{+}): *m/z* calculated for $C_{11}H_{13}N_6O_3Na$ $[M+Na]^+$ 299.0869 found 299.0876

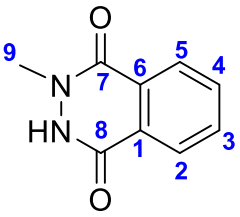
NMeLuminol reagents 9-10*

*10 was prepared with the strategy described by the group of Nakamura[2]





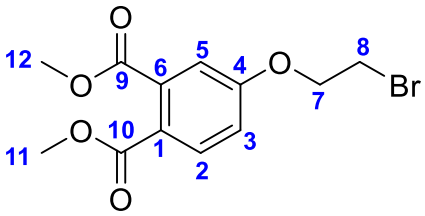
2-Methyl-2,3-dihydrophthalazine-1,4-dione (9)



Commercially available **20** (135 mg, 0.91 mmol) was suspended in anhydrous EtOH (5 mL, 0.2 M), methylhydrazine (160 µL, 3.60 mmol, 4 equiv.) was added and the reaction was refluxed. After 2h, reaction was cooled to room temperature and NH_4Cl (satd.) aqueous solution and AcOEt and H_2O were added. The aqueous layer was extracted 3 times with AcOEt, the organic layer was washed once with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **9** (102 mg, 64%) as a white solid that didn't require further purification. ^1H NMR (400.16 MHz, DMSO-d^6, 298.15 K): δ_H 11.63 (*br* s, 1H, N*H*), 8.22 (m, 1H, H^5), 7.96 (m, 1H, H^2), 7.88 (m, 2H, H^3, H^4), 3.56 (s, 3H, H^9). ^{13}C NMR (100.62 MHz, DMSO-d^6, 298.15 K): δ_C 157.2 (s, C^7), 150.1 (s, C^8), 132.9 (s, C^3 or C^4),

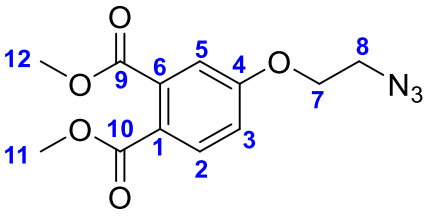
132.2 (s, C^3 or C^4), 128.8 (s, C^1), 126.2 (s, C^5), 124.8 (s, C^6), 124.1 (s, C^2), 37.6 (s, C^9). HRMS (ES^-): *m/z* calculated for $C_9H_7N_2O_2$ $[M-H]^-$ 175.0508 found 175.0508

Dimethyl 4-(2-bromoethoxy)phthalate (22)



To a solution of commercially available **21** (100 mg, 0.48 mmol, 1 equiv.) in anhydrous MeCN (5 mL, 0.1 M) were added K_2CO_3 (329 mg, 2.38 mmol, 5 equiv.) and 1,2-dibromoethane (206 mL, 2.38 µL, 5 equiv.) and reaction was heated to 70°C. After 24h, reaction was cooled to room temperature before adding 10 mL of water. Aqueous layer was extracted 3x with AcOEt, and the combined organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude compound was purified by silica gel chromatography (60:40 CyHex/AcOEt) to afford pure **22** (110 mg, 73%) as an orange oil. ^{1}H NMR (400.16 MHz, $CDCl_3$, 298.15 K): δ_H 7.82 (d, $^3J_{2-3}$ = 8.63 Hz, 1H, H^2), 7.11 (d, $^4J_{5-3}$ = 2.57 Hz, 1H, H^5), 7.03 (dd, $^3J_{3-2}$ = 8.63 Hz, $^4J_{3-5}$ = 2.57 Hz 1H, H^3), 4.37 (t, $^3J_{7-8}$ = 6.22 Hz, 2H, H^7), 3.93 (s, 3H, H^{11} or H^{12}), 3.89 (s, 3H, H^{11} or H^{12}), 3.66 (t, $^3J_{8-7}$ = 6.22 Hz, 2H, H^8). ^{13}C NMR (100.62 MHz, $CDCl_3$, 298.15 K): δ_C 168.7 (s, C^9 or C^{10}), 166.7 (s, C^9 or C^{10}), 160.6 (s, C^4), 135.8 (s, C^1), 131.8 (s, C^2), 123.2 (s, C^6), 116.5 (s, C^3), 114.4 (s, C^5), 68.3 (s, C^7), 52.8 (s, C^{11} or C^{12}), 52.4 (s, C^{11} or C^{12}), 25.8 (s, C^8). HRMS (ESI^+): *m/z* calculated for $C_{12}H_{13}(^{79}Br)O_5Na$ $[M+Na]^+$ 338.9840 found 338.9844

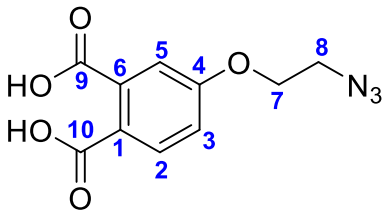
Dimethyl 4-(2-azidoethoxy)phthalate (23)



To a solution of **22** (1.0 g, 3.47 mmol, 1 equiv.) in anhydrous DMF (20 mL, 0.15 M) was added NaN_3 (675 mg, 10.41 mmol, 3 equiv.) and reaction was heated to 80°C. After 1h, reaction was cooled to room temperature and mixture was concentrated under reduced pressure by 3x co-evaporation of DMF with toluene. The crude compound was purified by silica gel chromatography (65:35 CyHex/AcOEt) to afford pure **23** (906 mg, 94%) as an orange oil. ^{1}H NMR (400.16 MHz, $CDCl_3$, 298.15 K): δ_H 7.74 (d, $^3J_{2-3}$ = 8.59 Hz, 1H, H^2), 7.03 (d, $^4J_{5-3}$ = 2.59 Hz, 1H, H^5), 6.95 (dd, $^3J_{3-2}$ = 8.59 Hz, $^4J_{3-5}$ = 2.59 Hz 1H, H^3), 4.13 (t, $^3J_{7-8}$ = 4.92 Hz, 2H, H^7), 3.84 (s, 3H, H^{11} or H^{12}), 3.80 (s, 3H, H^{11}

or H^{12}), 3.55 (t, $^{3}J_{8\text{-}7}$ = 4.92 Hz, 2H, H^{8}). ^{13}C NMR (100.62 MHz, $CDCl_3$, 298.15 K): δ_C 168.6 (s, C^{9} or C^{10}), 166.7 (s, C^{9} or C^{10}), 160.6 (s, C^{4}), 135.8 (s, C^{1}), 131.7 (s, C^{2}), 123.2 (s, C^{6}), 116.5 (s, C^{3}), 114.2 (s, C^{5}), 67.5 (s, C^{7}), 52.9 (s, C^{11} or C^{12}), 52.3 (s, C^{11} or C^{12}), 50.0 (s, C^{8}). HRMS (ESI$^+$): *m/z* calculated for $C_{12}H_{13}N_3O_5Na$ $[M+Na]^+$ 302.0757 found 302.0753

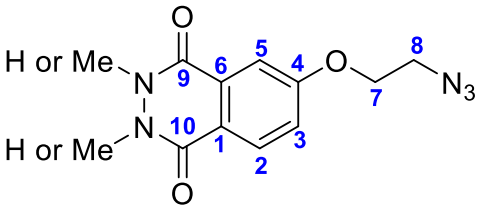
4-(2-azidoethoxy)phthalic acid (24)



To a solution of **23** (900 mg, 3.22 mmol, 1 equiv.) in 1:1 THF (3.5 mL) and MeOH (3.5 mL) was added an aqueous solution (3.5 mL) of NaOH (650 mg, 16.11 mmol, 5 equiv.) and the reaction was stirred at room temperature. After 1h, reaction was quenched with 1M HCl until pH 4-5 (6 mL) before adding AcOEt (15 mL) and H_2O (5 mL). Aqueous layer was extracted 3x with AcOEt and the combined organic layer was washed once with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **24** (725 mg, 89%) as an orange solid that didn't require further purification. ^{1}H NMR (400.16 MHz, MeOD, 298.15 K): δ_H 7.86 (d, $^{3}J_{2\text{-}3}$ = 8.60 Hz, 1H, H^{2}), 7.16 (d, $^{4}J_{5\text{-}3}$ = 2.66 Hz, 1H, H^{5}), 7.12 (dd, $^{3}J_{3\text{-}2}$ = 8.60 Hz, $^{4}J_{3\text{-}5}$ = 2.66 Hz 1H, H^{3}), 4.26 (t, $^{3}J_{7\text{-}8}$ = 4.96 Hz, 2H, H^{7}), 3.65 (t, $^{3}J_{8\text{-}7}$ = 4.96 Hz, 2H, H^{8}). ^{13}C NMR (100.62 MHz, MeOD, 298.15 K): δ_C 171.9 (s, C^{9} or C^{10}), 169.9 (s, C^{9} or C^{10}), 162.3 (s, C^{4}), 138.1 (s, C^{1}), 132.9 (s, C^{2}), 124.6 (s, C^{6}), 116.9 (s, C^{3}), 115.4 (s, C^{5}), 68.9 (s, C^{7}), 51.2 (s, C^{8}). HRMS (ASAP$^-$): *m/z* calculated for $C_{10}H_8N_3O_5$ $[M-H]^-$ 250.0464 found 250.0466

6-(2-azidoethoxy)-2-methyl-2,3-dihydrophthalazine-1,4-dione (10a)

7-(2-azidoethoxy)-2-methyl-2,3-dihydrophthalzine-1,4-dione (10b)

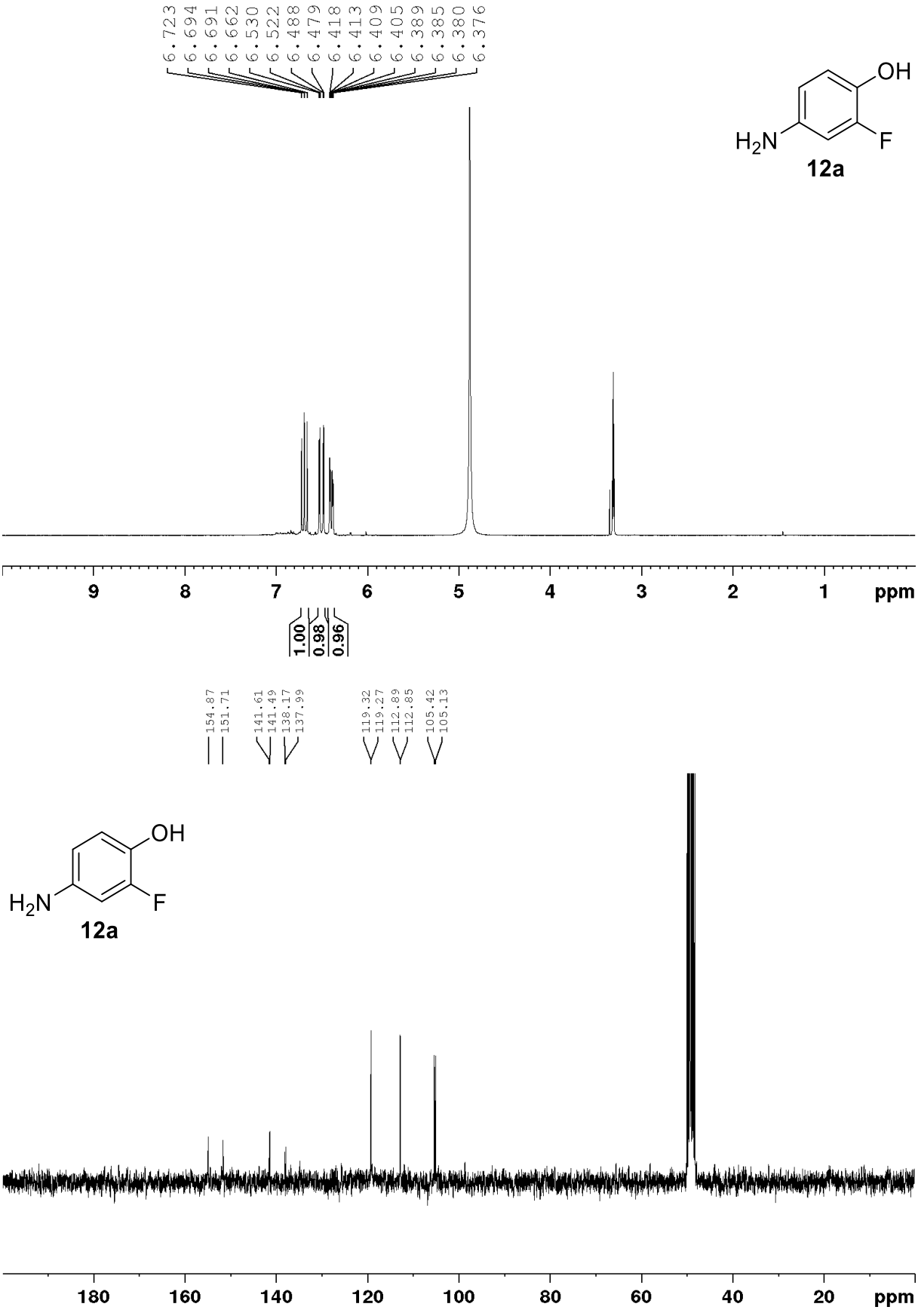


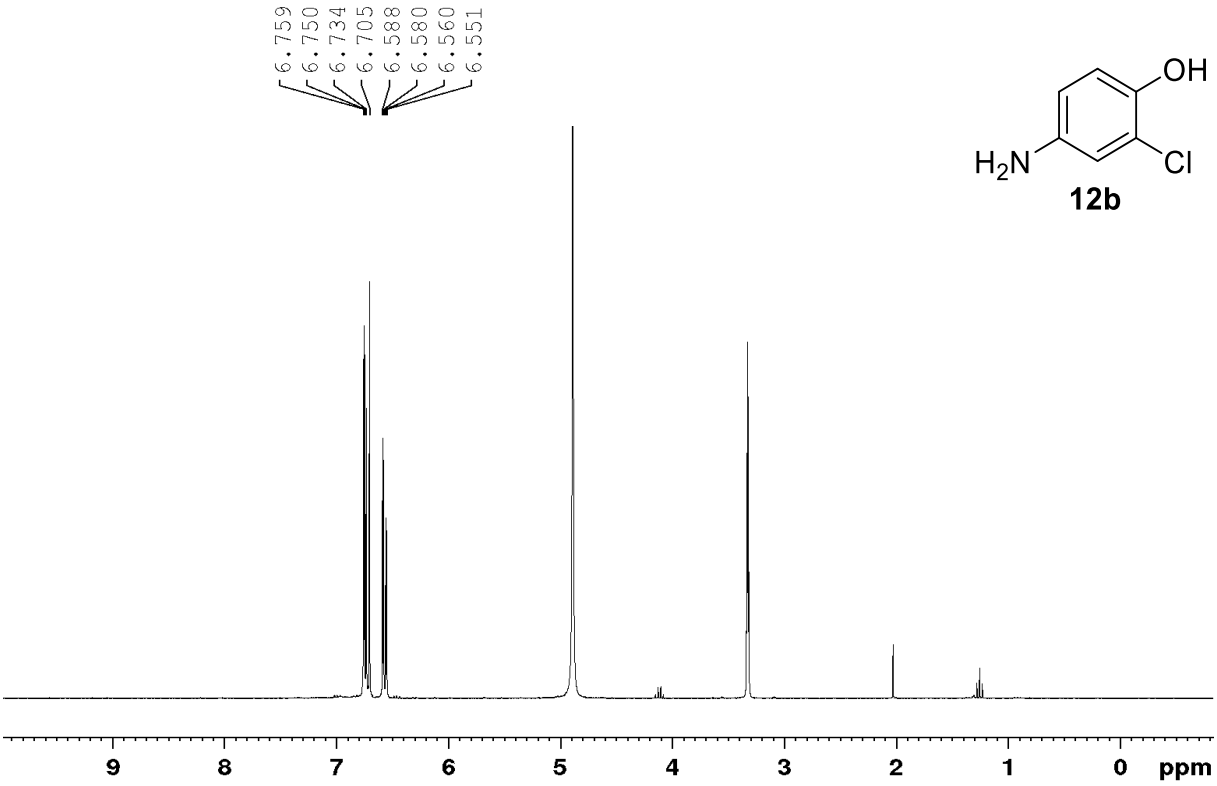
(regioisomers mixture)
ratio 2:1

To a solution of **24** (550 mg, 2.19 mmol, 1 equiv.) in anhydrous THF (15 mL, 0.15 M) was added Ac_2O (830 µL, 8.76 mmol, 4 equiv.) and reaction was refluxed during 8h. Solvents were evaporated under reduced pressure and the crude solid was solubilized in anhydrous EtOH (11 mL, 0.2 M). Methylhydrazine (350 µL, 6.57 mmol, 3 equiv.) was then added and reaction was refluxed. After 1h, reaction was cooled down

to room temperature before being quenched with a saturated solution of NH_4Cl (5 mL). AcOEt (30 mL) and water (20 mL) were added, and aqueous layer was extracted 3x with AcOEt. The combined organic layer was washed with brine, dried over MgSO4, filtered and concentrated under reduced pressure to afford **10a-b** (223 mg, 39% over 2 steps) as a white solid and as a mixture of regioisomers (2:1) that didn't require further purification. ^{1}H NMR (400.16 MHz, DMSO-d^6, 298.15 K): δ_H 8.15 (d, $^3J_{2\text{-}3}$ = 8.79 Hz, 0.5H, mino-H^2), 7.91 (d, $^3J_{2\text{-}3}$ = 8.79 Hz, 1H, majo-H^2), 7.62 (d, $^4J_{5\text{-}3}$ = 2.66 Hz, 1H, majo-H^5), 7.46 (dd, $^3J_{3\text{-}2}$ = 8.79 Hz, $^4J_{3\text{-}5}$ = 2.66 Hz 1H, majo-H^3), 7.43 (dd, $^3J_{3\text{-}2}$ = 8.79 Hz, $^4J_{3\text{-}5}$ = 2.55 Hz, 0.5H, mino-H^3), 7.33 (d, $^4J_{5\text{-}3}$ = 2.55 Hz, 0.5H, mino-H^5), 4.36 (m, 3H, mino-H^7, majo-H^7), 3.72 (m, 3H, mino-H^8, majo-H^8), 3.55 (s, 3H, majo-Me), 3.53 (s, 1.5H, mino-Me). ^{13}C NMR (100.62 MHz, DMSO-d^6, 298.15 K): δ_C 161.2, 160.8, 157.0, 156.9, 150.2, 149.9, 130.8, 128.7, 126.6, 122.7, 121.8, 121.0, 118.7, 108.3, 106.2, 67.4, 49.4, 37.6, 37.3. HRMS (ASAP$^-$): *m/z* calculated for $C_{11}H_{10}N_5O_3$ $[M\text{-}H]^-$ 260.0783 found 260.0784

V. NMR Spectra

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6.705
6.588
6.580
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H2N
Cl
12b
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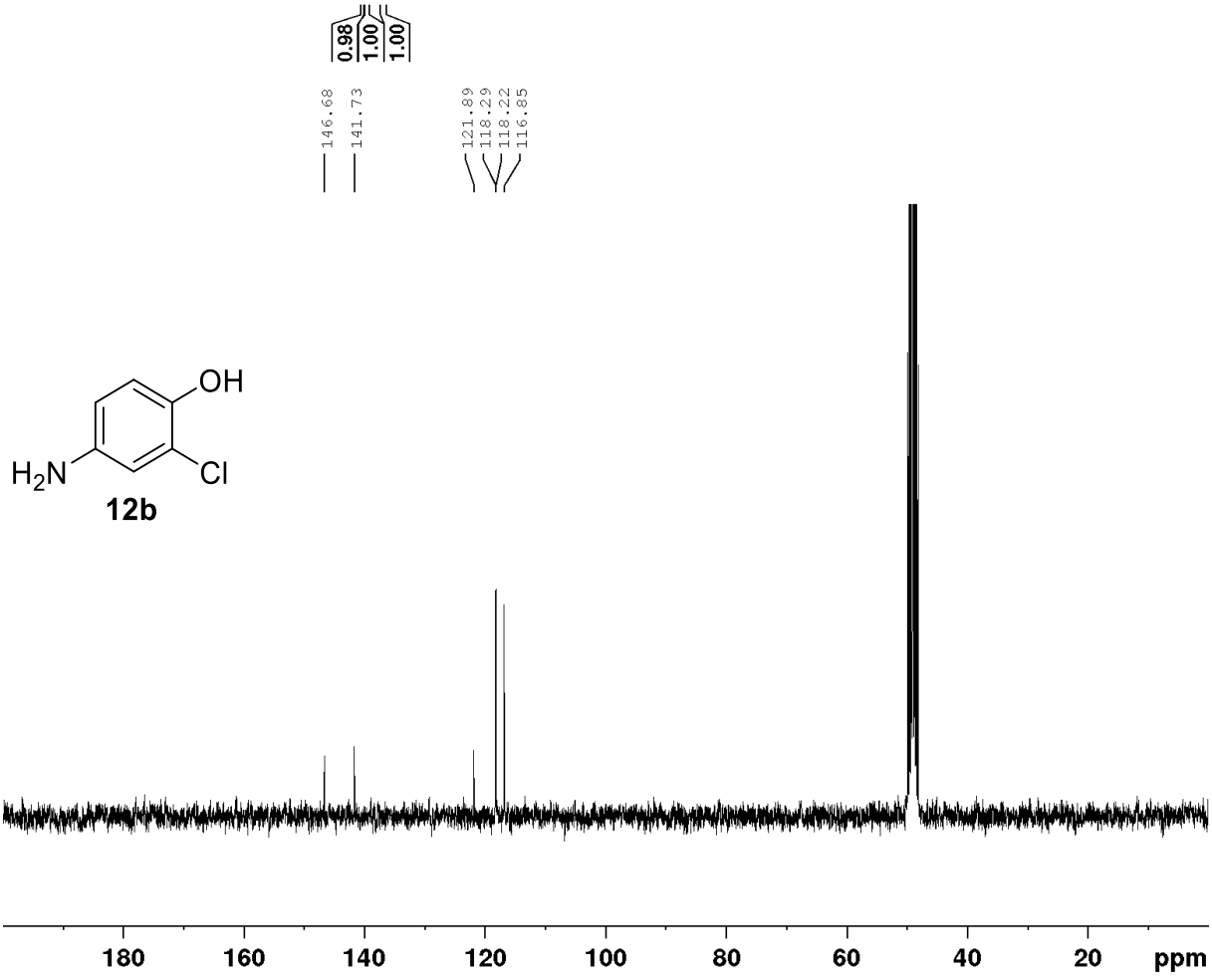
8
7
6
5
4
3
2
1
0
ppm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Cl
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140
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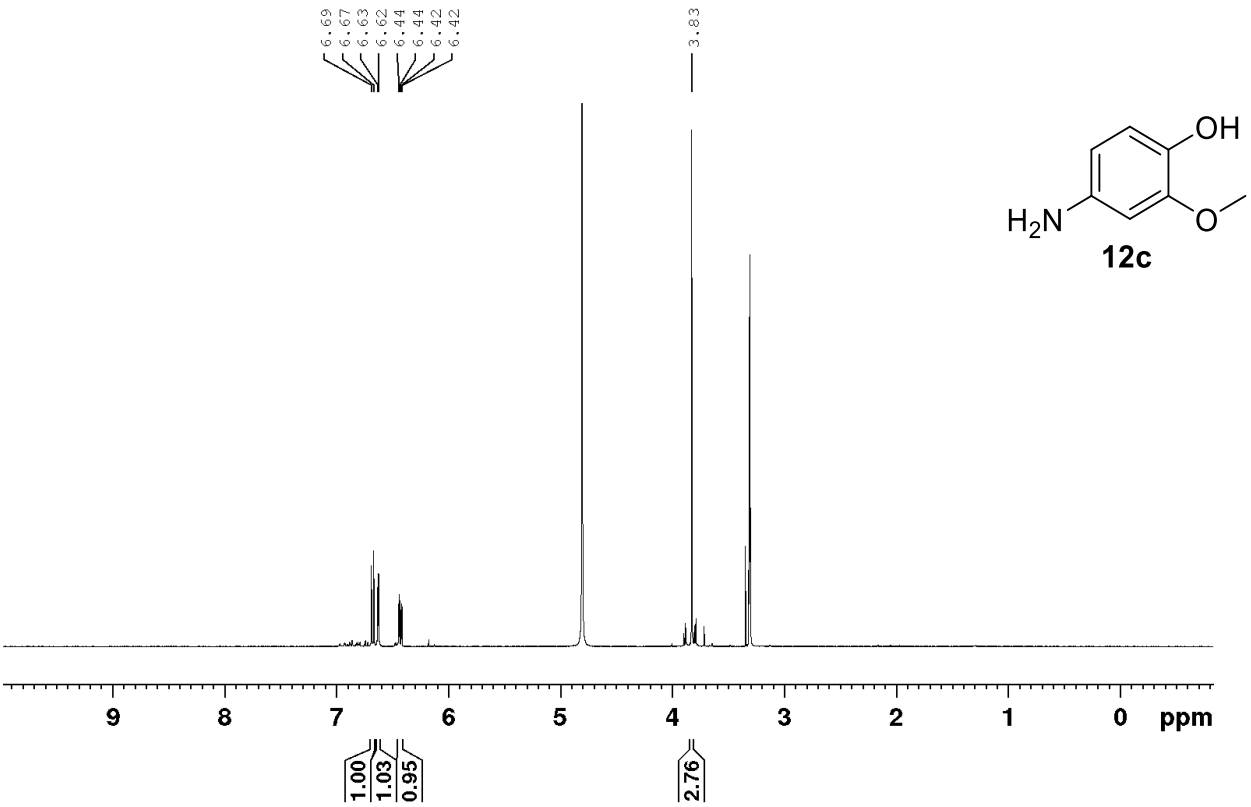
80
60
40
20
ppm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6.42
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H2N
O
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7
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5
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3
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0
ppm
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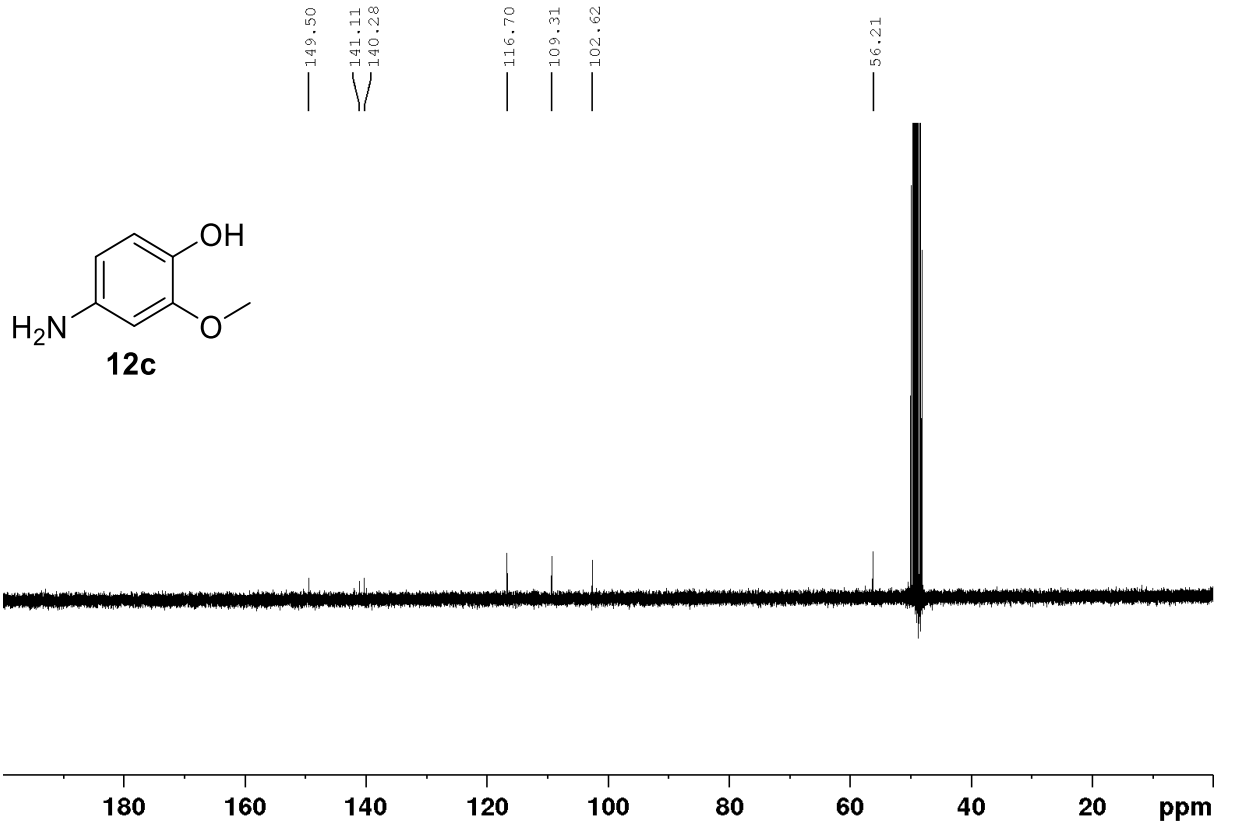
0.95
2.76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H2N
O
12c
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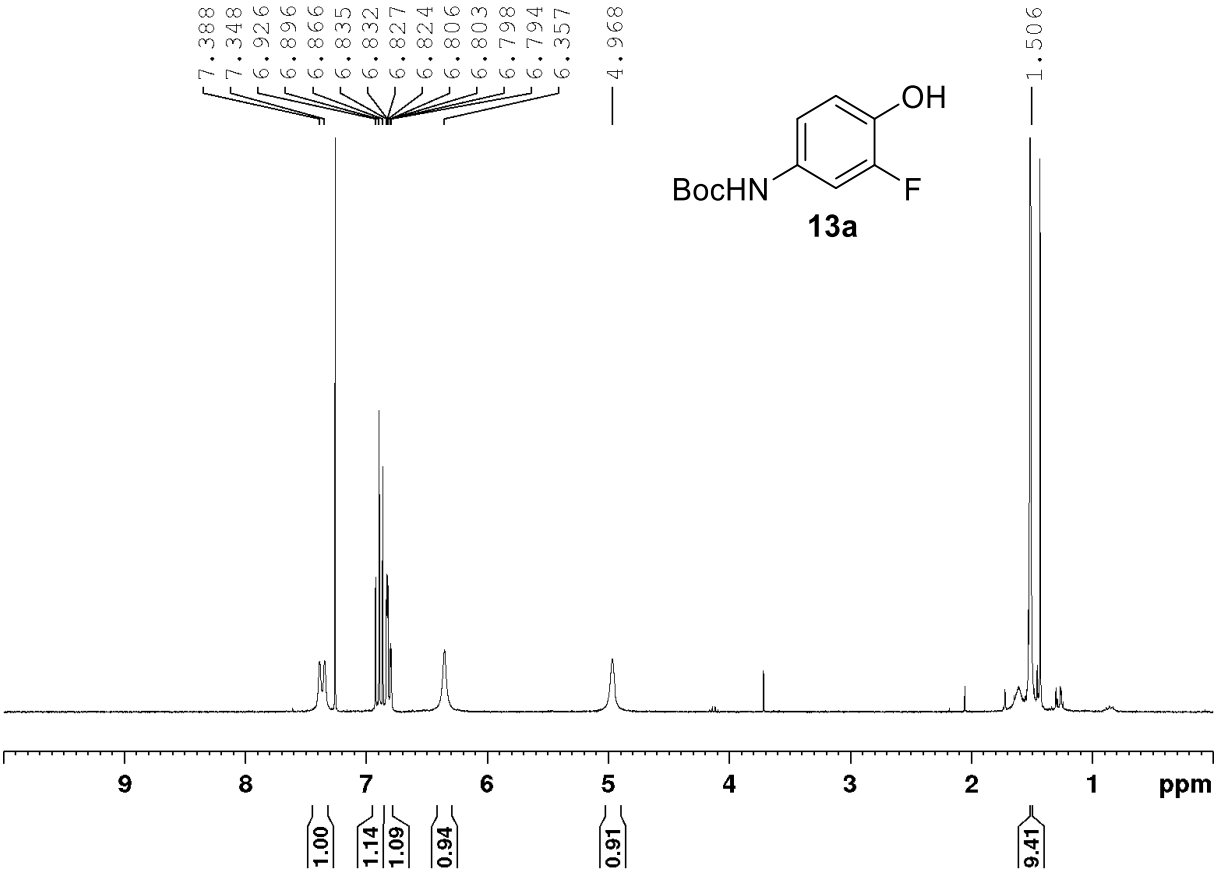
80
60
40
20
ppm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6.827
6.824
6.806
6.803
6.798
6.794
6.357
4.968
1.506
OH
BocHN
F
13a
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8
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6
5
4
3
2
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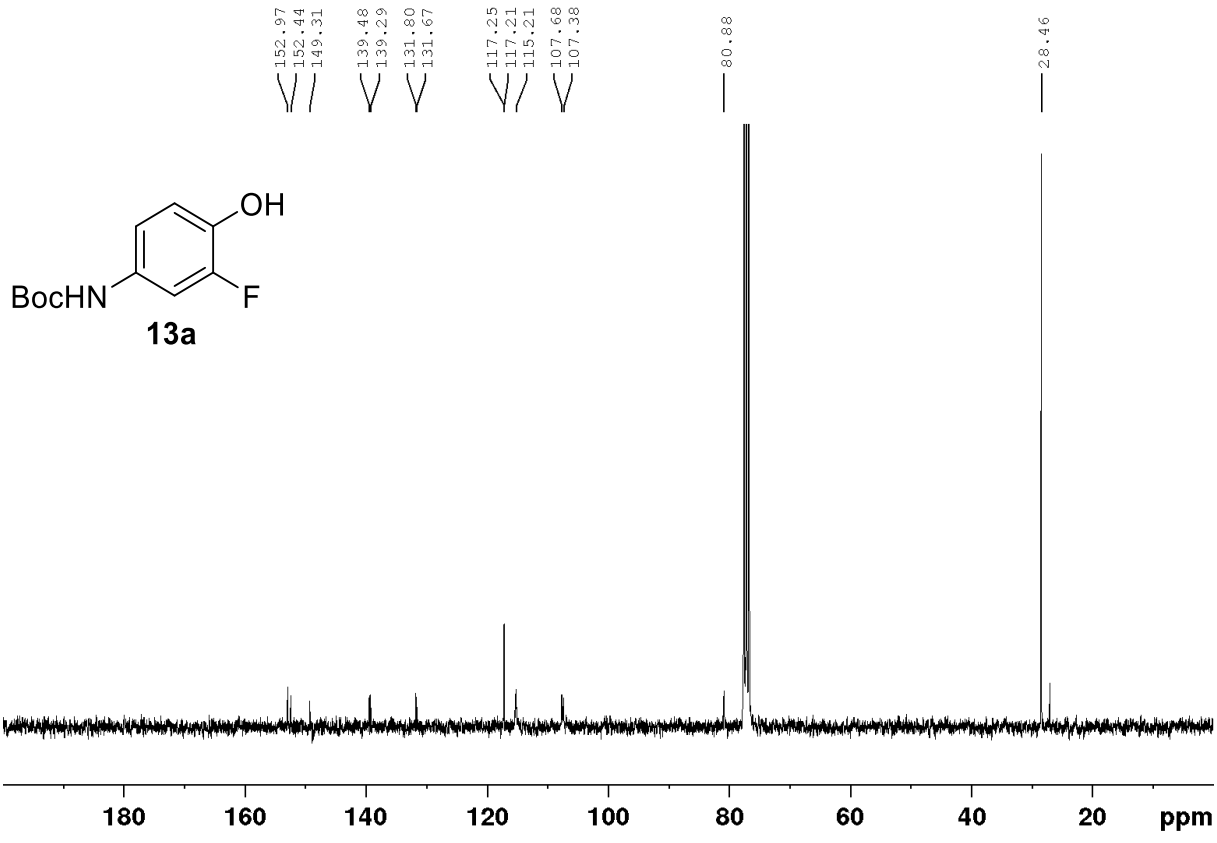
0.91
9.41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152.44
149.31
139.48
139.29
131.80
131.67
117.25
117.21
115.21
107.68
107.38
80.88
28.46
OH
BocHN
F
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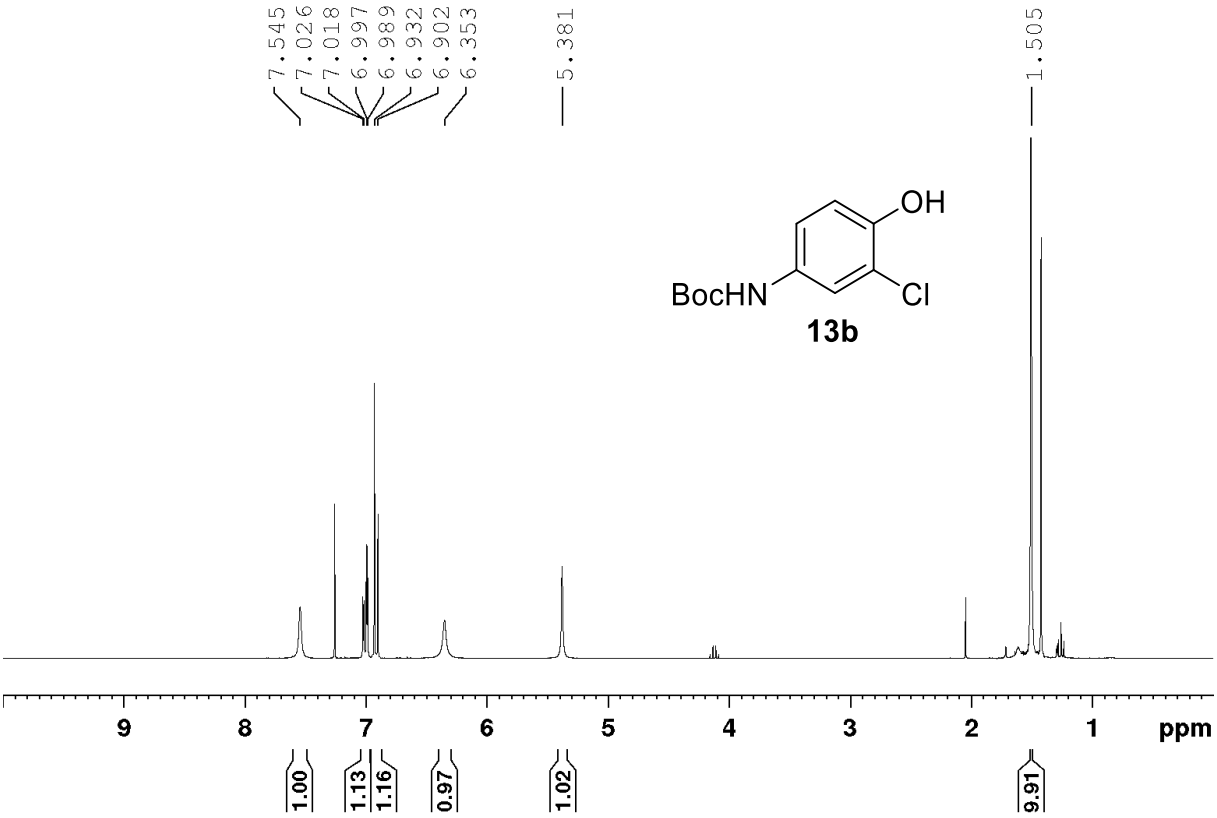
140
120
100
80
60
40
20
ppm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6.932
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6.353
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BocHN
Cl
13b
9
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7
6
5
4
3
2
1
ppm
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1.13
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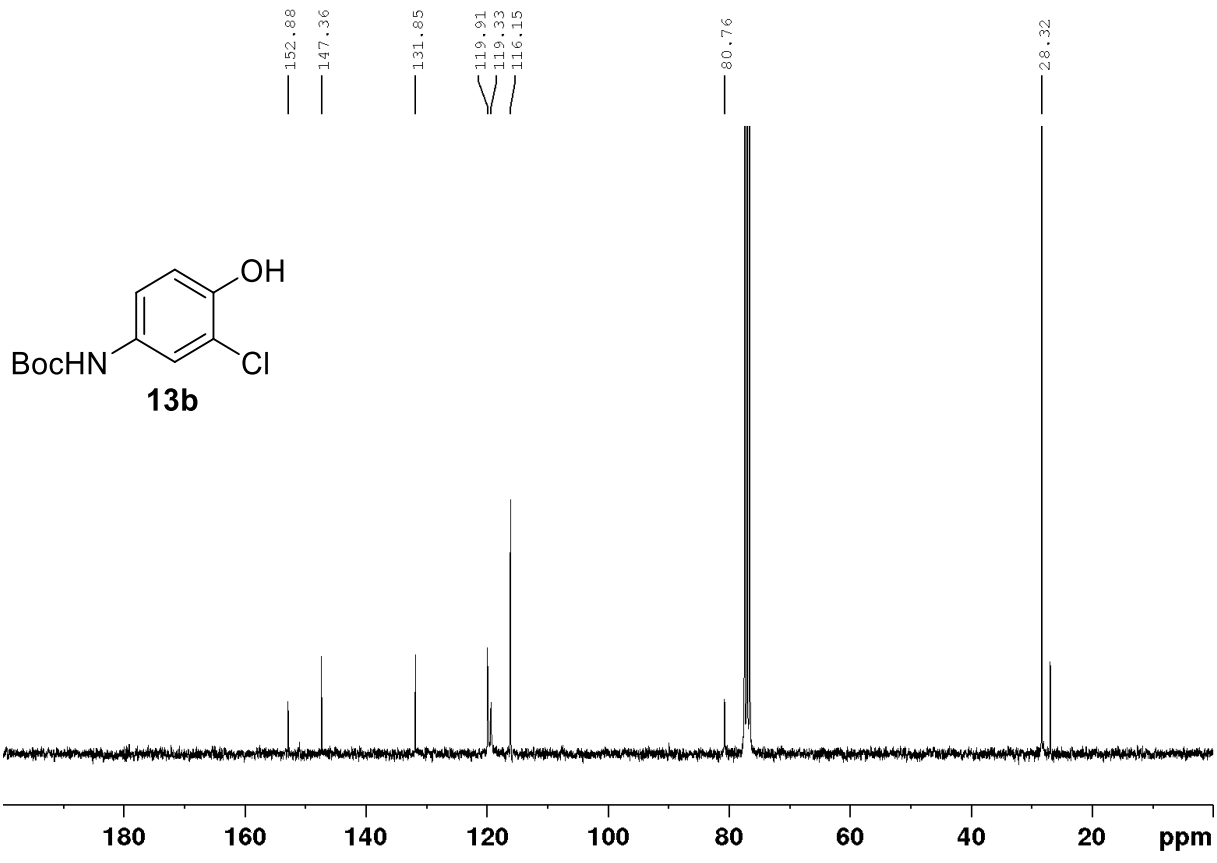
0.97
1.02
9.91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BocHN
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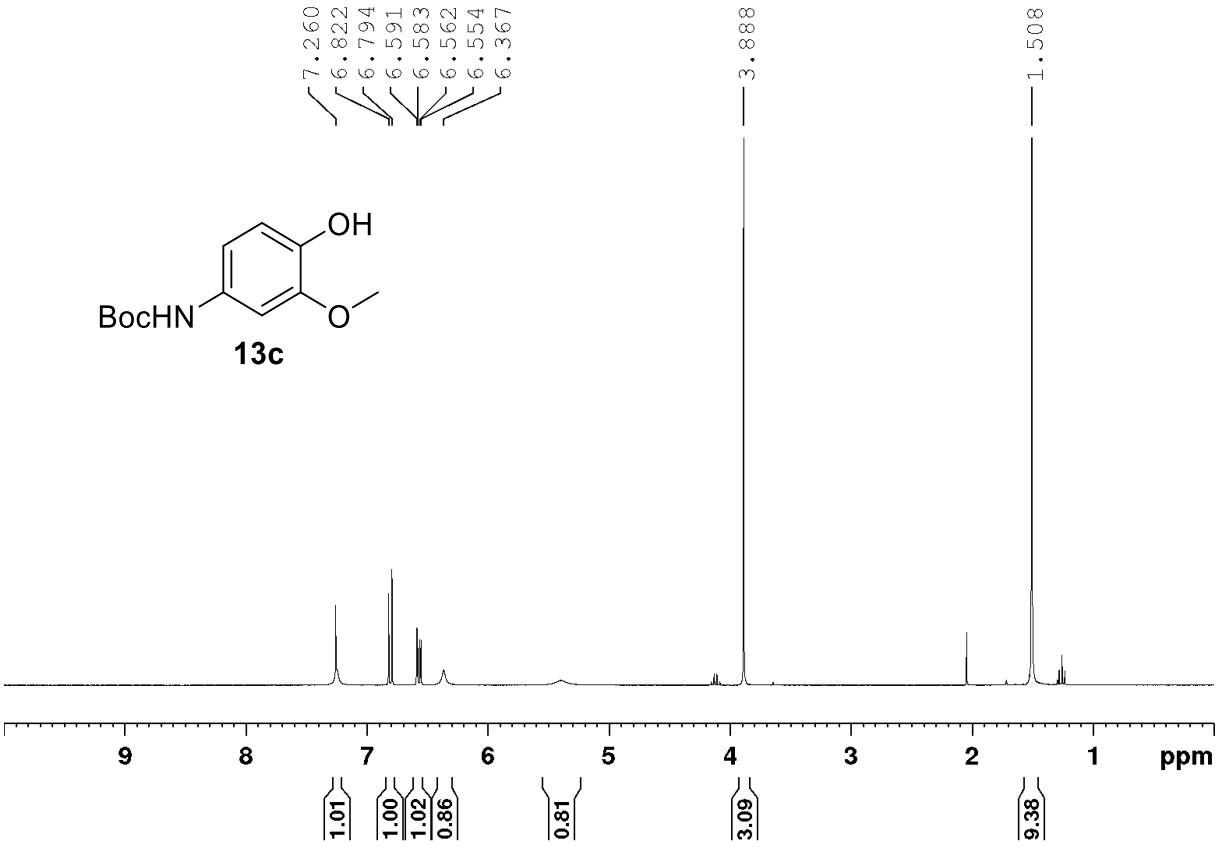
20
ppm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6.583
6.562
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6.367
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1.508
OH
BocHN
O
13c
9
8
7
6
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3
2
1
ppm
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1.00
1.02
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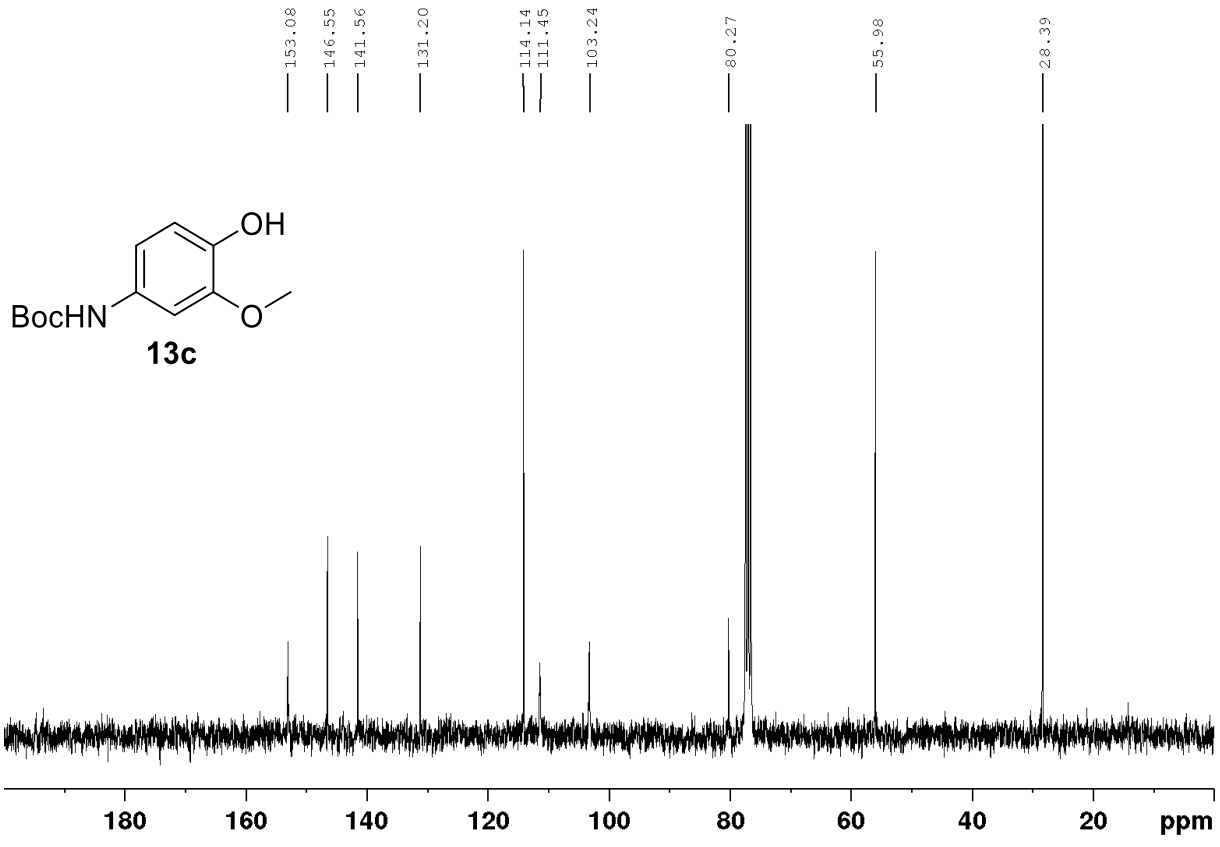
3.09
9.38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BocHN
O
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80
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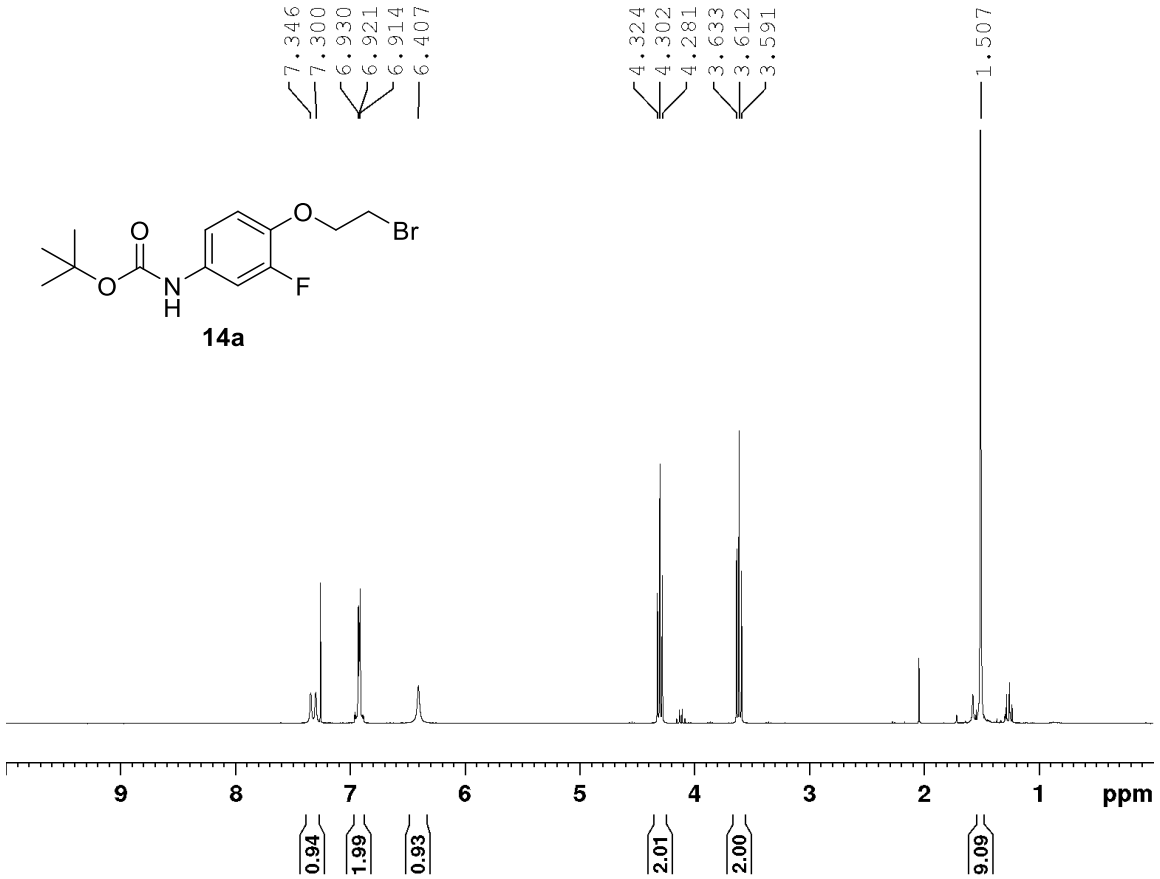
40
20
ppm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6.914
6.407
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4.281
3.633
3.612
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1.507
O
O
N
H
O
Br
F
14a
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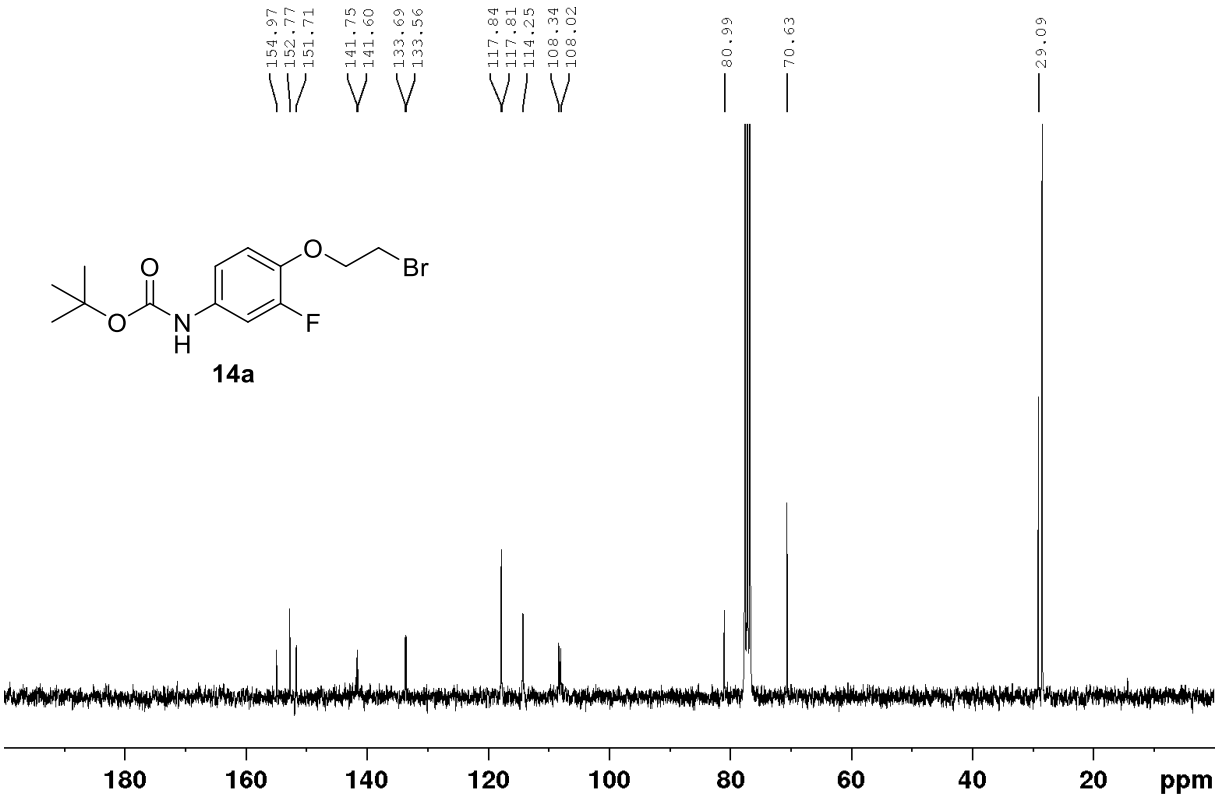
2.00
9.09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114.25
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70.63
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Br
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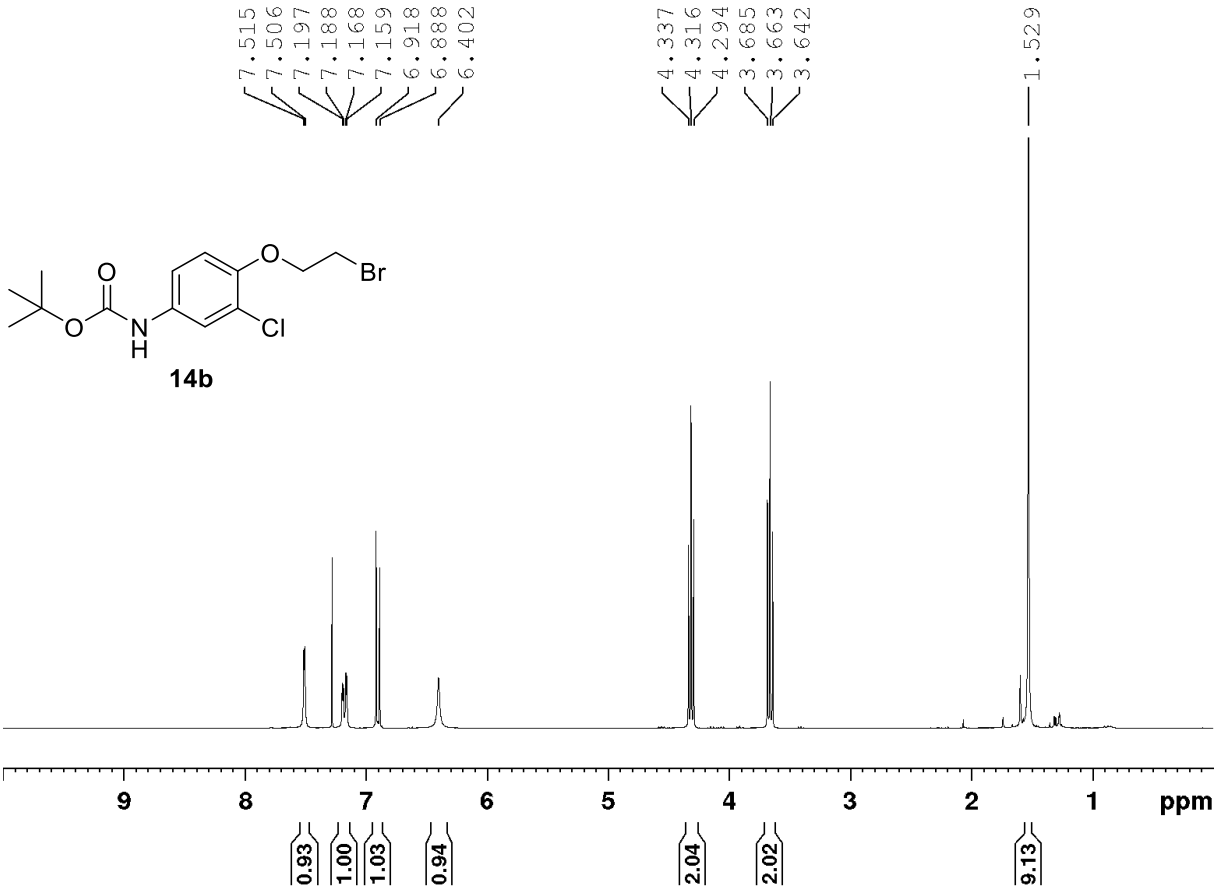
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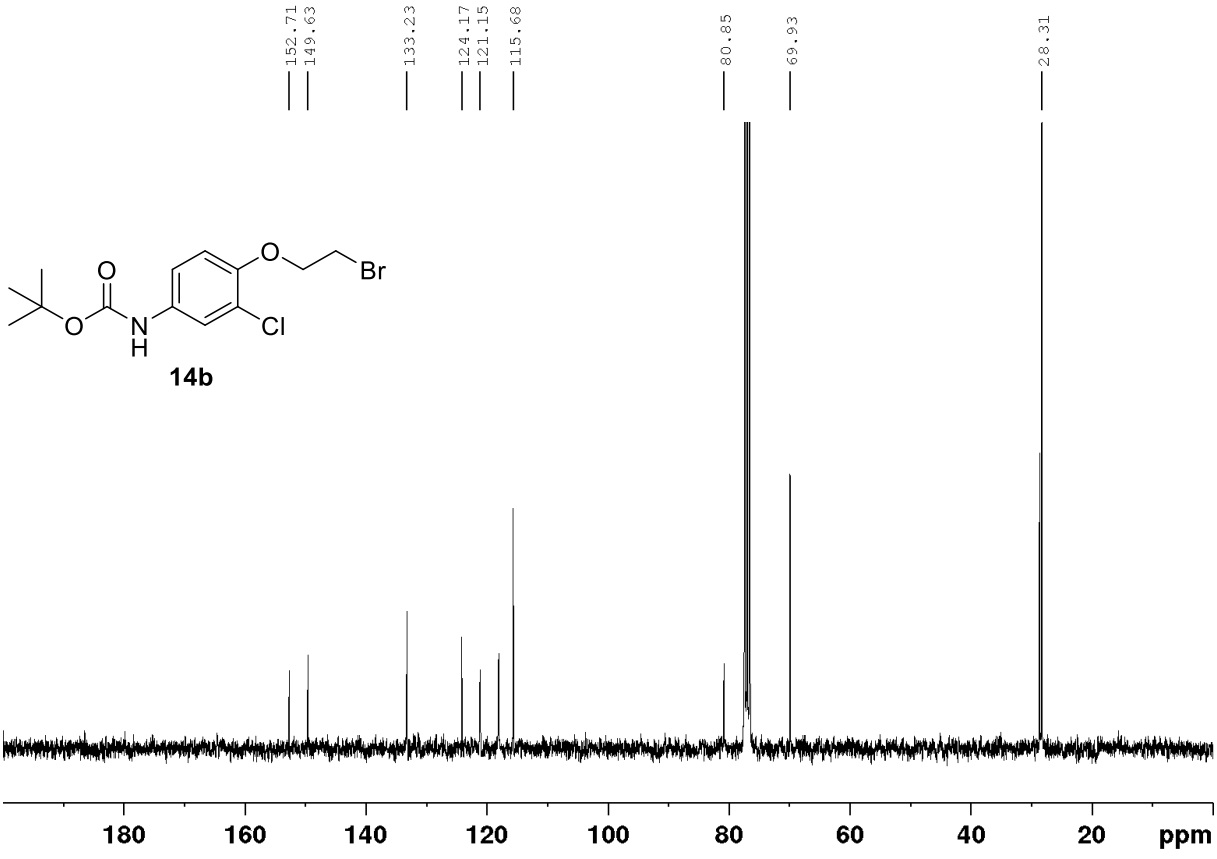
3.642
1.529
O
Br
O
N
H
Cl
14b
9
8
7
6
5
4
3
2
1
ppm
0.93
1.00
1.03
0.94
2.04
2.02
9.13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Br
O
N
H
Cl
14b
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140
120
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80
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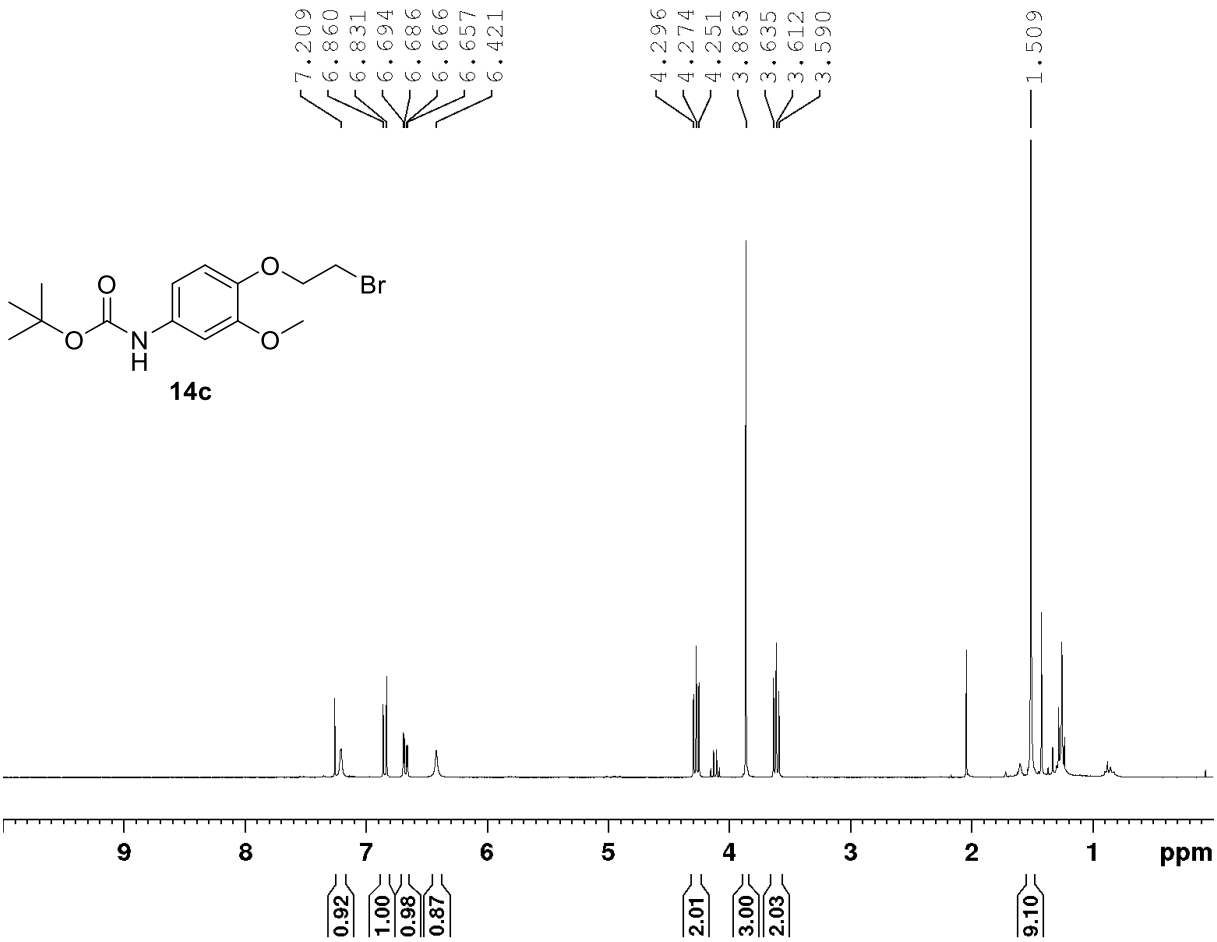
40
20
ppm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6.421
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4.274
4.251
3.863
3.635
3.612
3.590
1.509
O
O
N
H
O
Br
O
14c
9
8
7
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0.87
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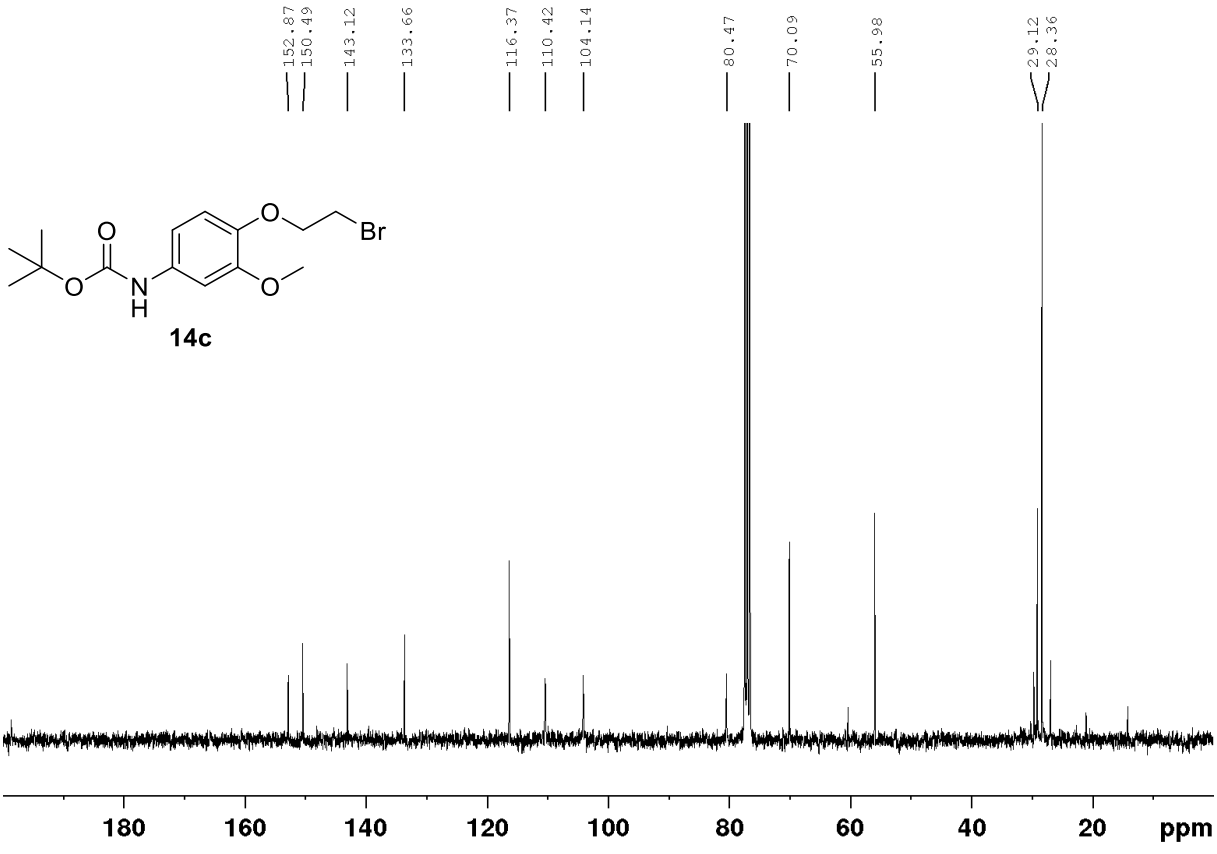
3.00
2.03
9.10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29.12
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N
H
O
Br
O
14c
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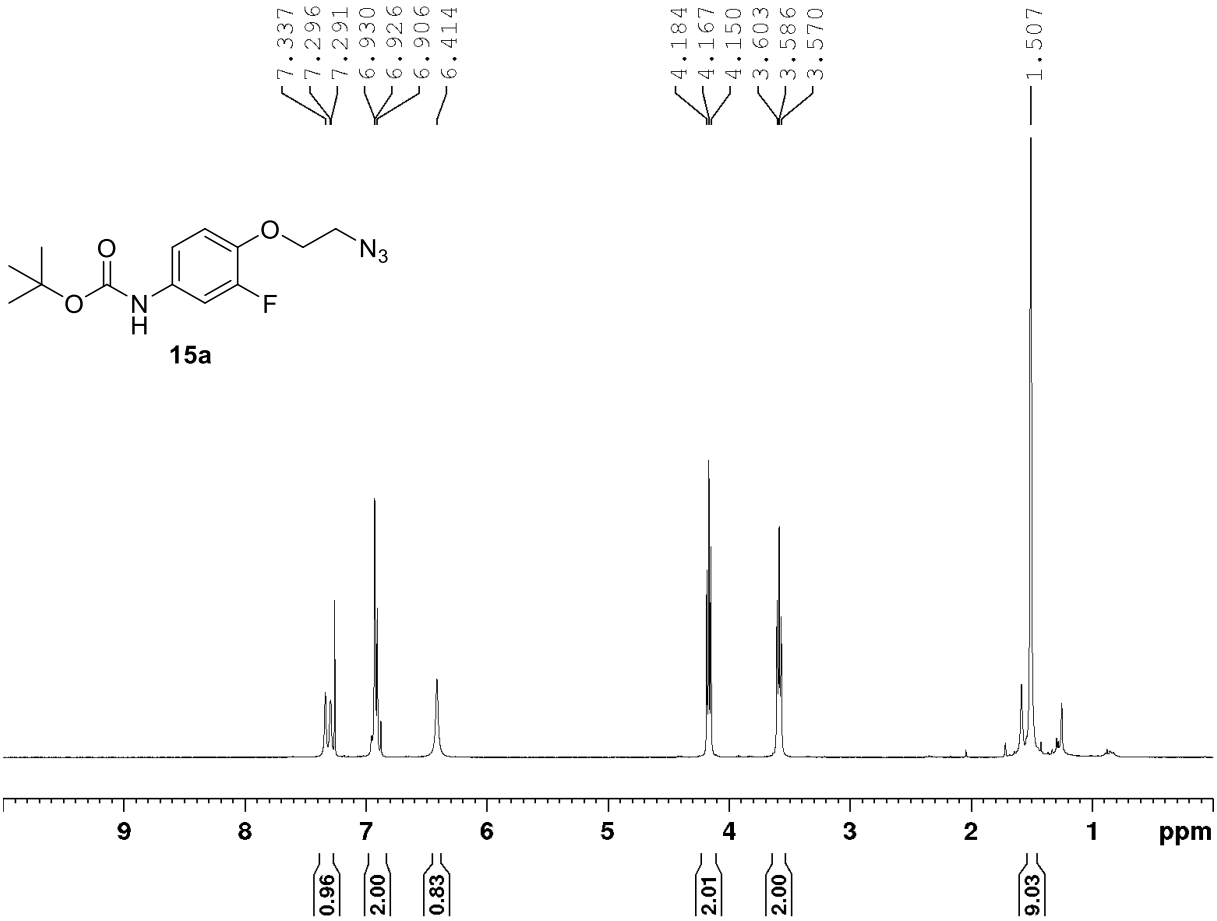
60
40
20
ppm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4.150
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3.586
3.570
1.507
O
O
N
H
O
N3
F
15a
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8
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4
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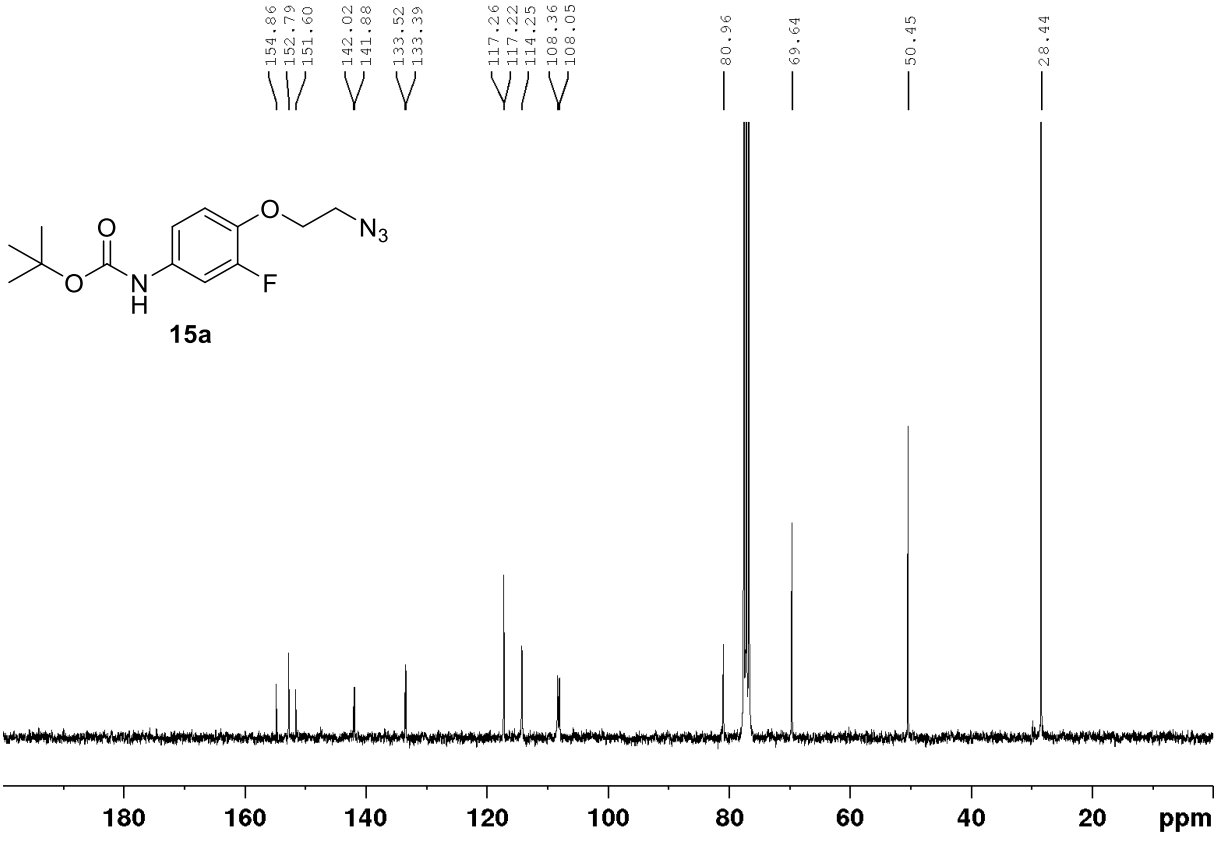
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133.39
117.26
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114.25
108.36
108.05
80.96
69.64
50.45
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N
H
O
N3
F
15a
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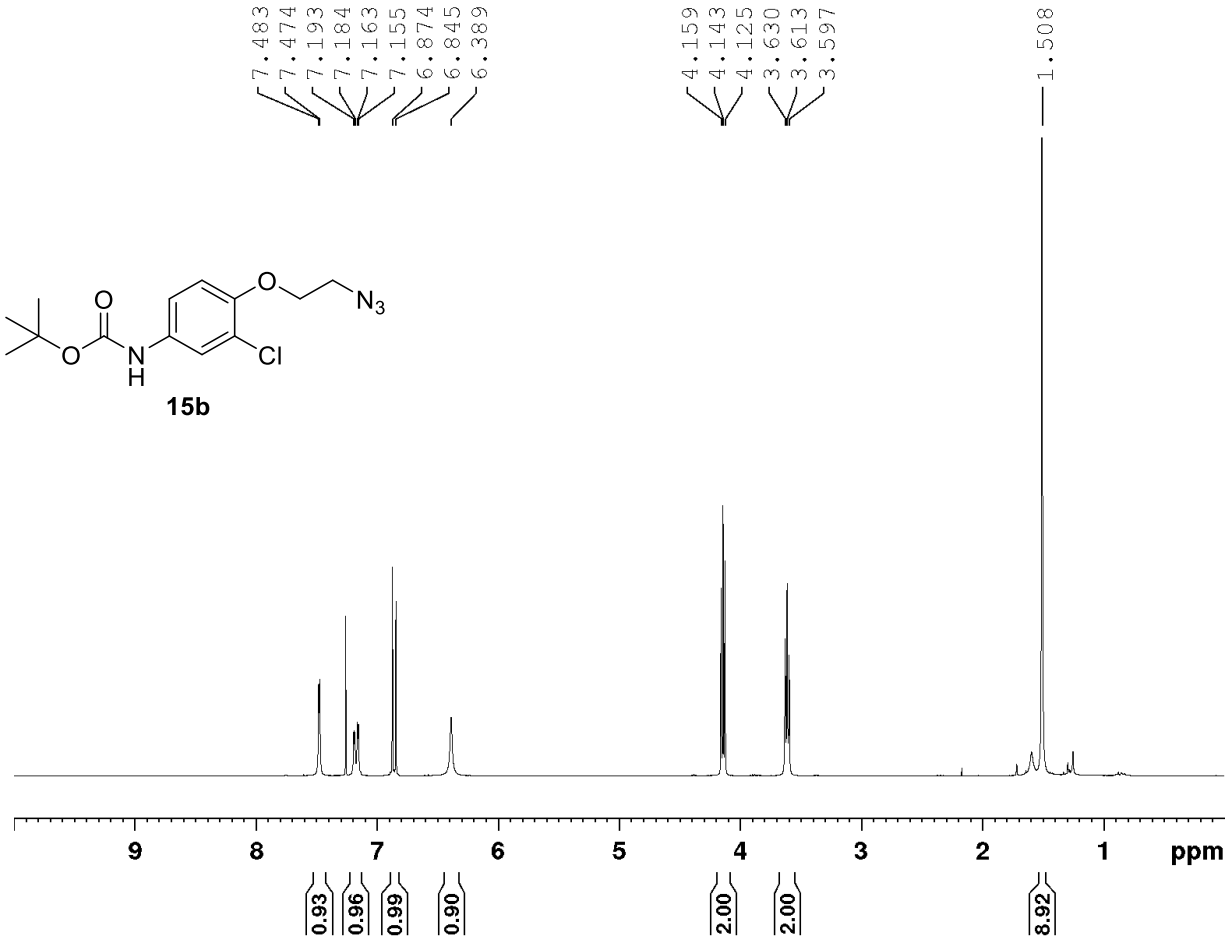
140
120
100
80
60
40
20
ppm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7.155
6.874
6.845
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4.143
4.125
3.630
3.613
3.597
1.508
O
N3
O
O
N
H
Cl
15b
9
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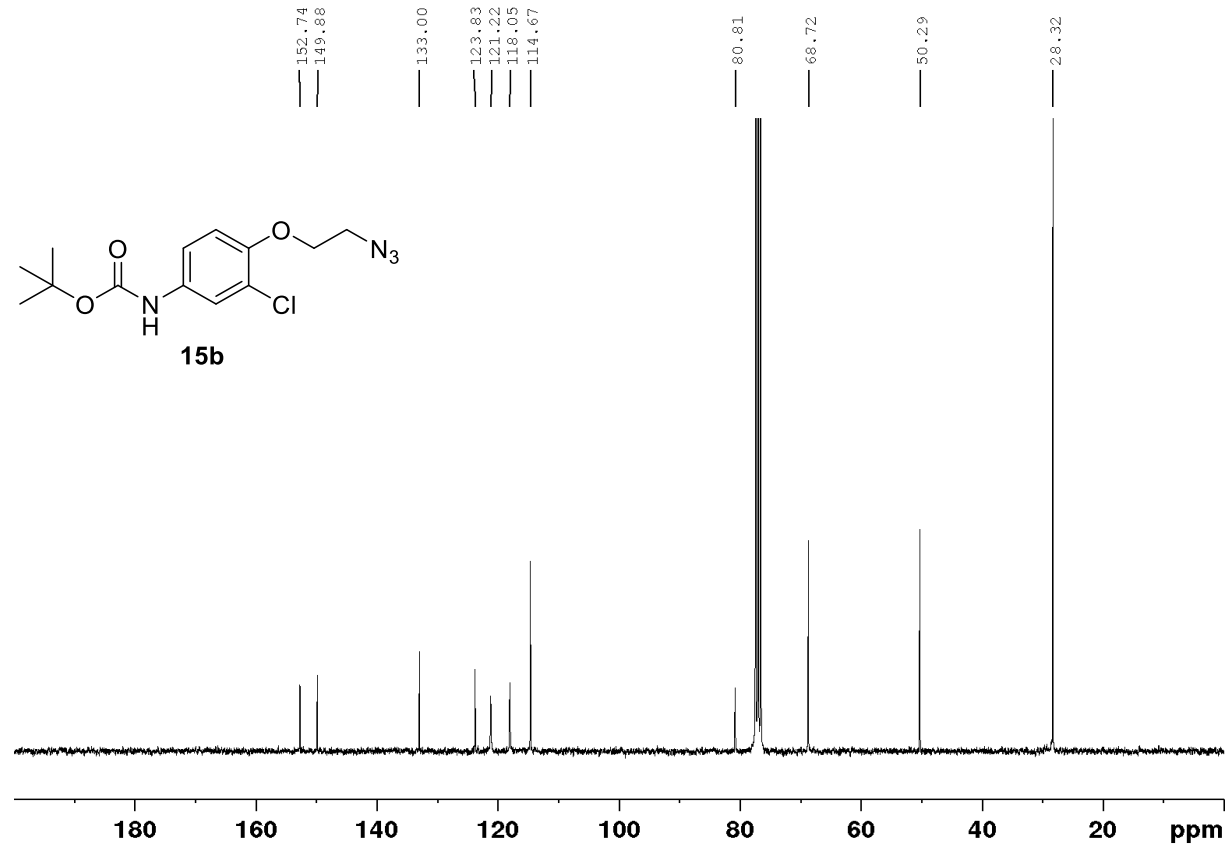
2.00
8.92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O
N
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Cl
15b
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120
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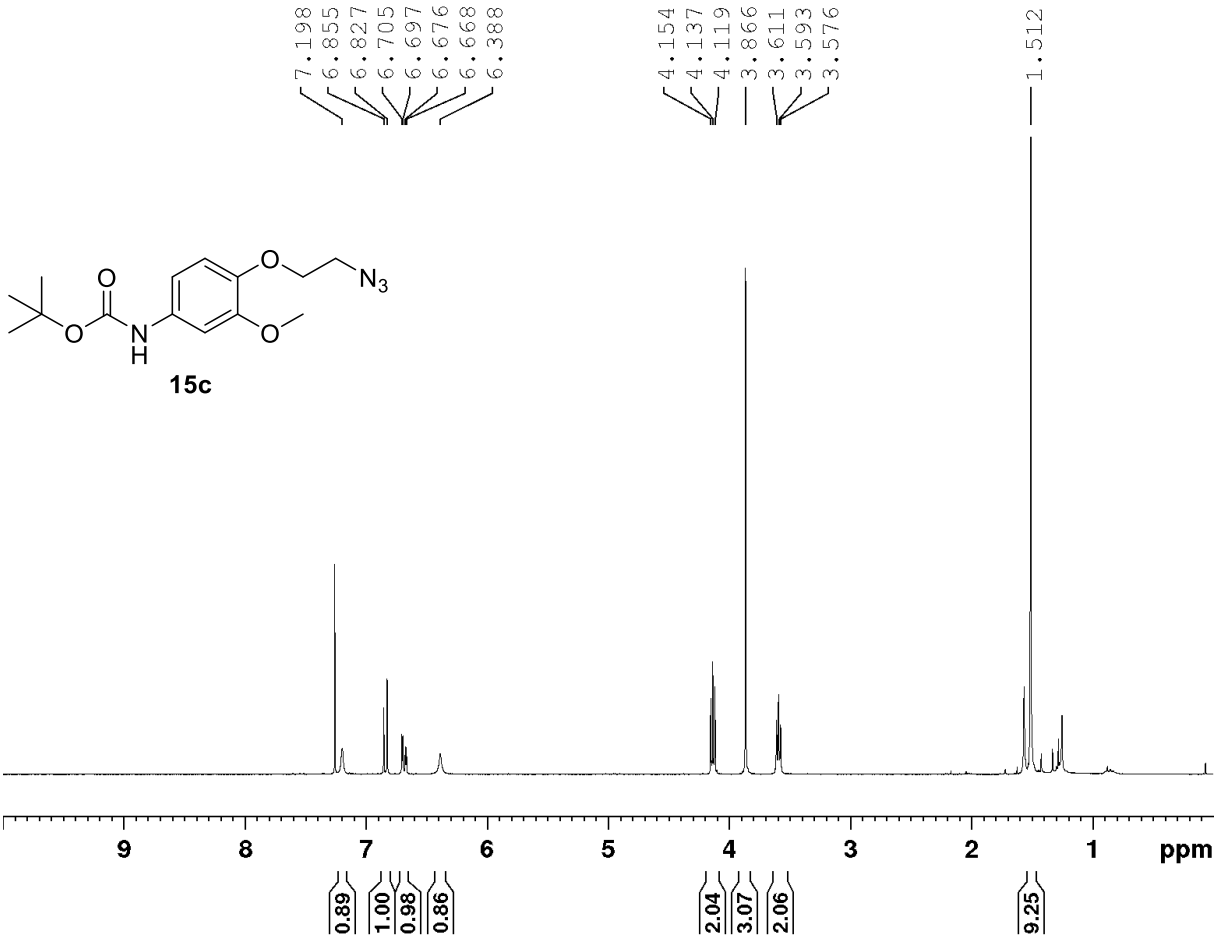
60
40
20
ppm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6.668
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4.137
4.119
3.866
3.611
3.593
3.576
1.512
O
O
N
H
O
O
N3
15c
9
8
7
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0.86
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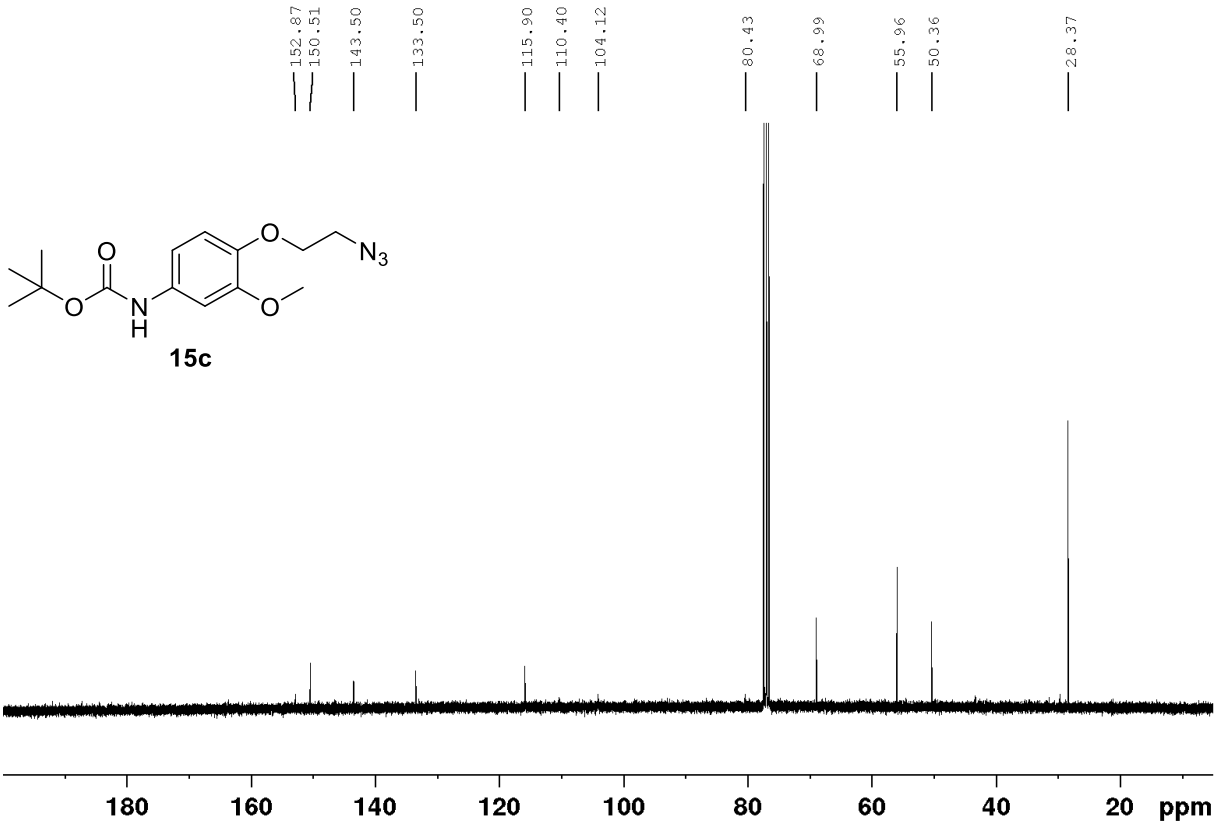
2.06
9.25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110.40
104.12
80.43
68.99
55.96
50.36
28.37
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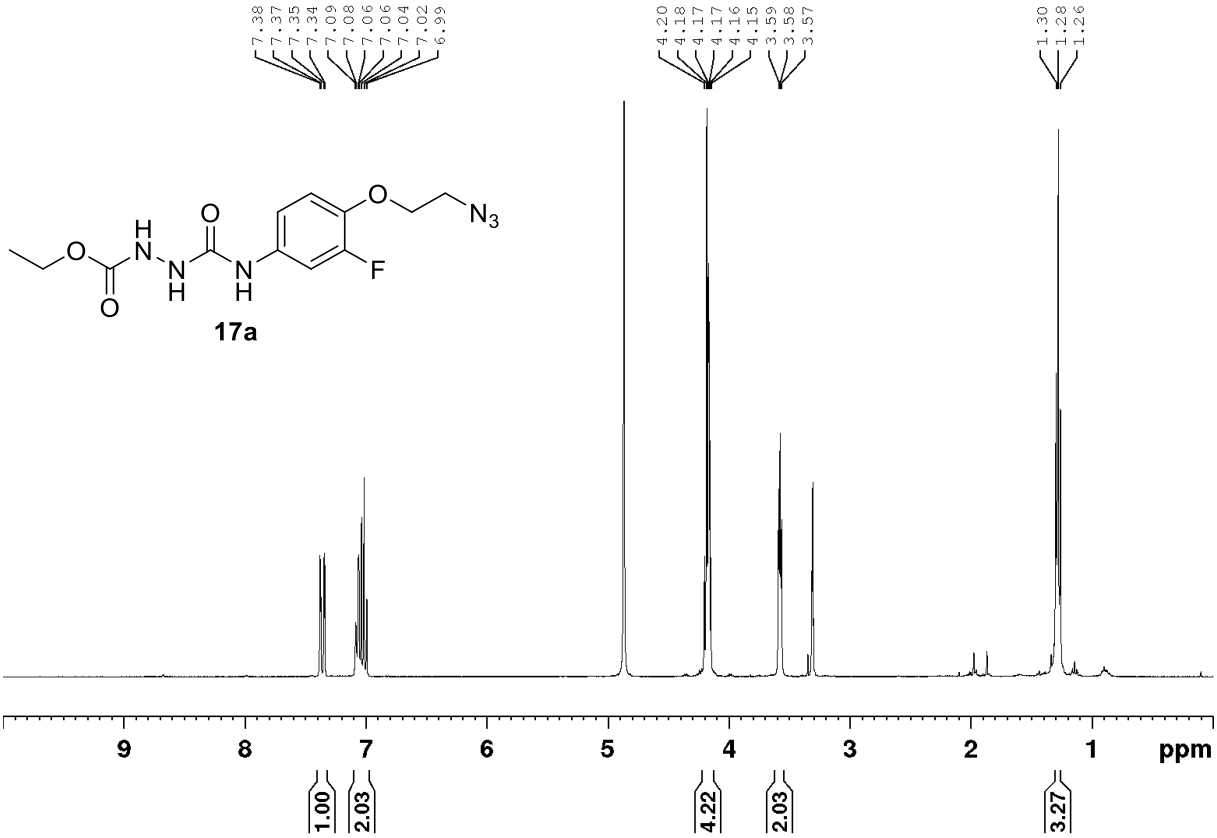
O
N
H
O
O
N3
15c
180
160
140
120
100
80
60
40
20
ppm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7.35
7.34
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7.08
7.06
7.06
7.04
7.02
6.99
4.20
4.18
4.17
4.17
4.16
4.15
3.59
3.58
3.57
1.30
1.28
1.26
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7
6
5
4
3
2
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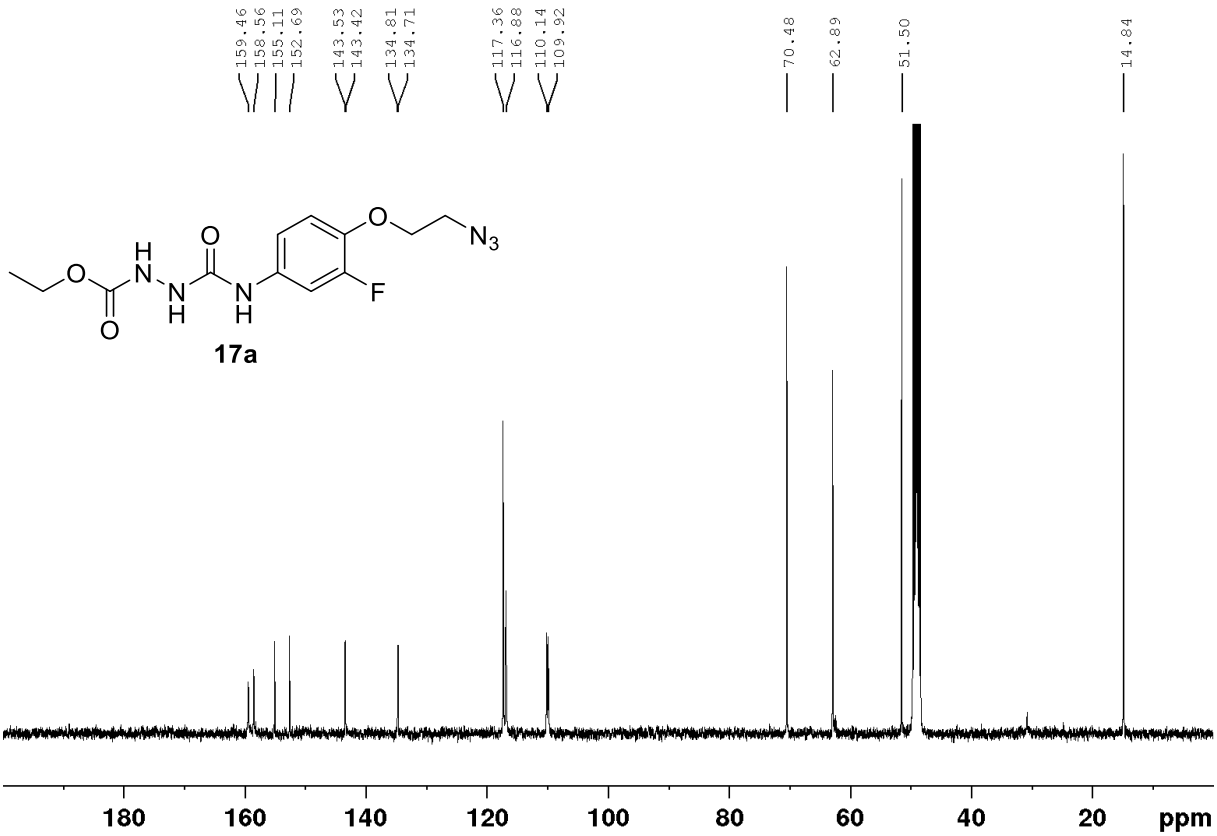
ppm
1.00
2.03
4.22
2.03
3.27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143.42
134.81
134.71
117.36
116.88
110.14
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62.89
51.50
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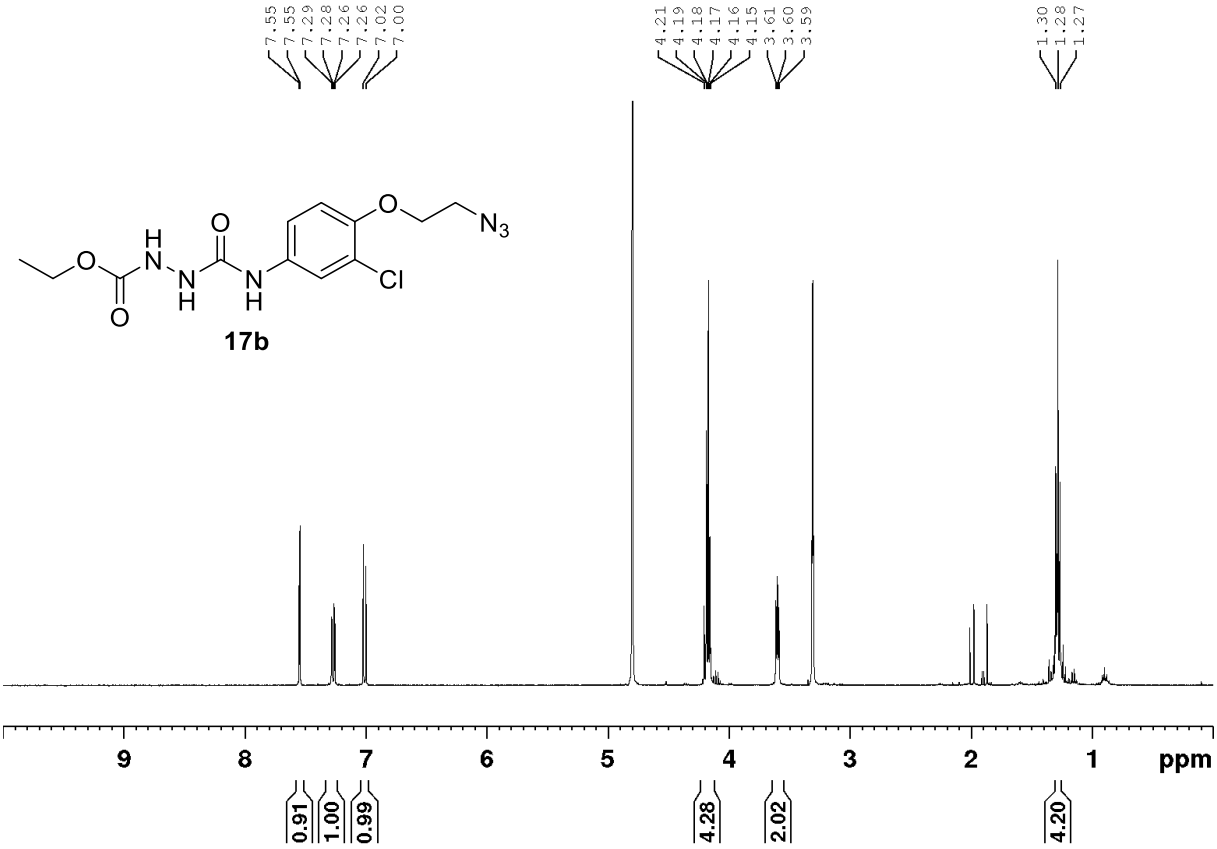
140
120
100
80
60
40
20
ppm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7.29
7.28
7.26
7.26
7.02
7.00
4.21
4.19
4.18
4.17
4.16
4.15
3.61
3.60
3.59
1.30
1.28
1.27
17b
9
8
7
6
5
4
3
2
1
ppm
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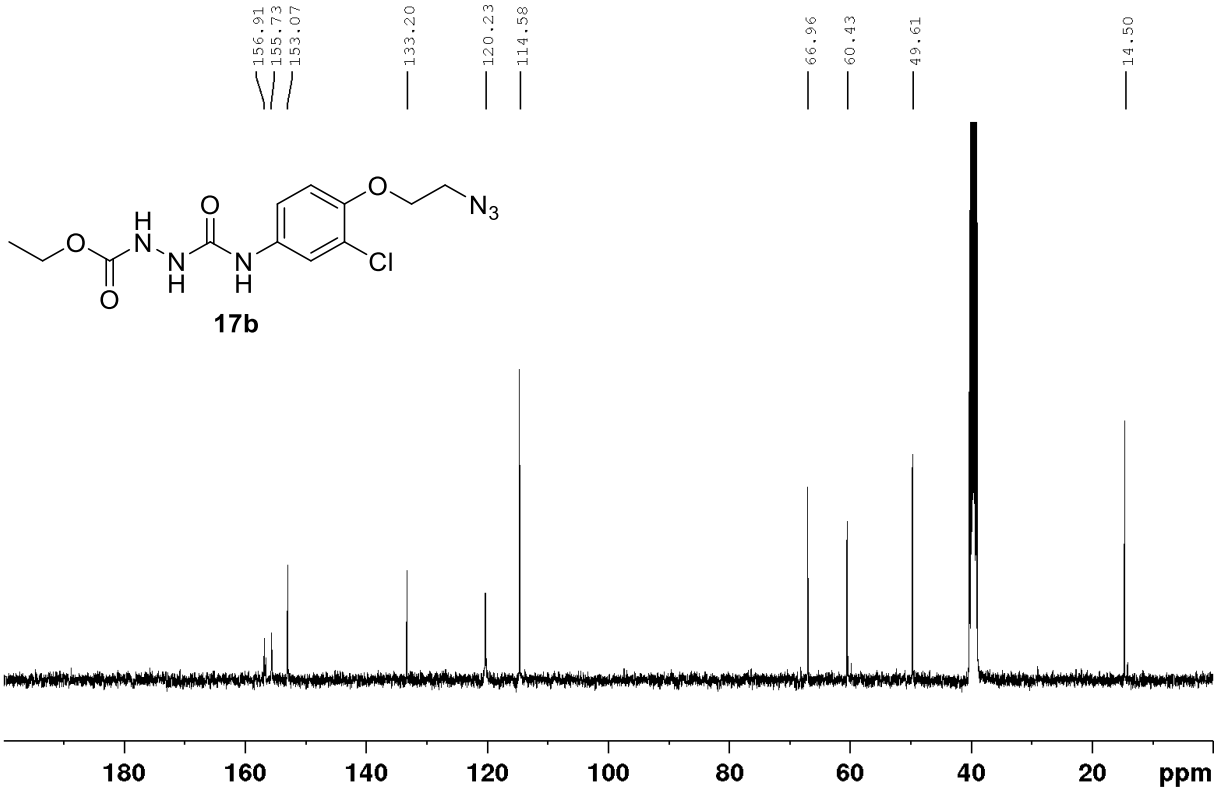
1.00
0.99
4.28
2.02
4.20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120.23
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60.43
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14.50
17b
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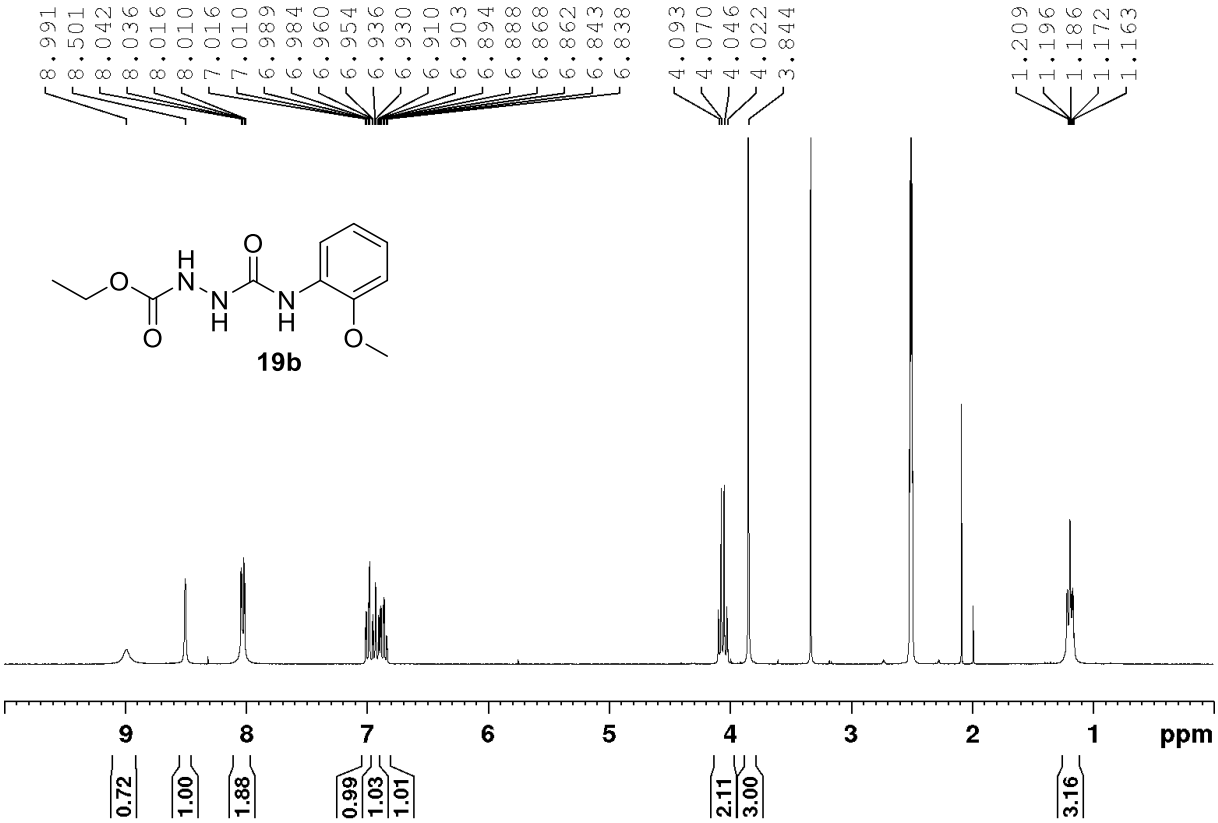
140
120
100
80
60
40
20
ppm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8.010
7.016
7.010
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6.954
6.936
6.930
6.910
6.903
6.894
6.888
6.868
6.862
6.843
6.838
4.093
4.070
4.046
4.022
3.844
1.209
1.196
1.186
1.172
1.163
19b
9
8
7
6
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ppm
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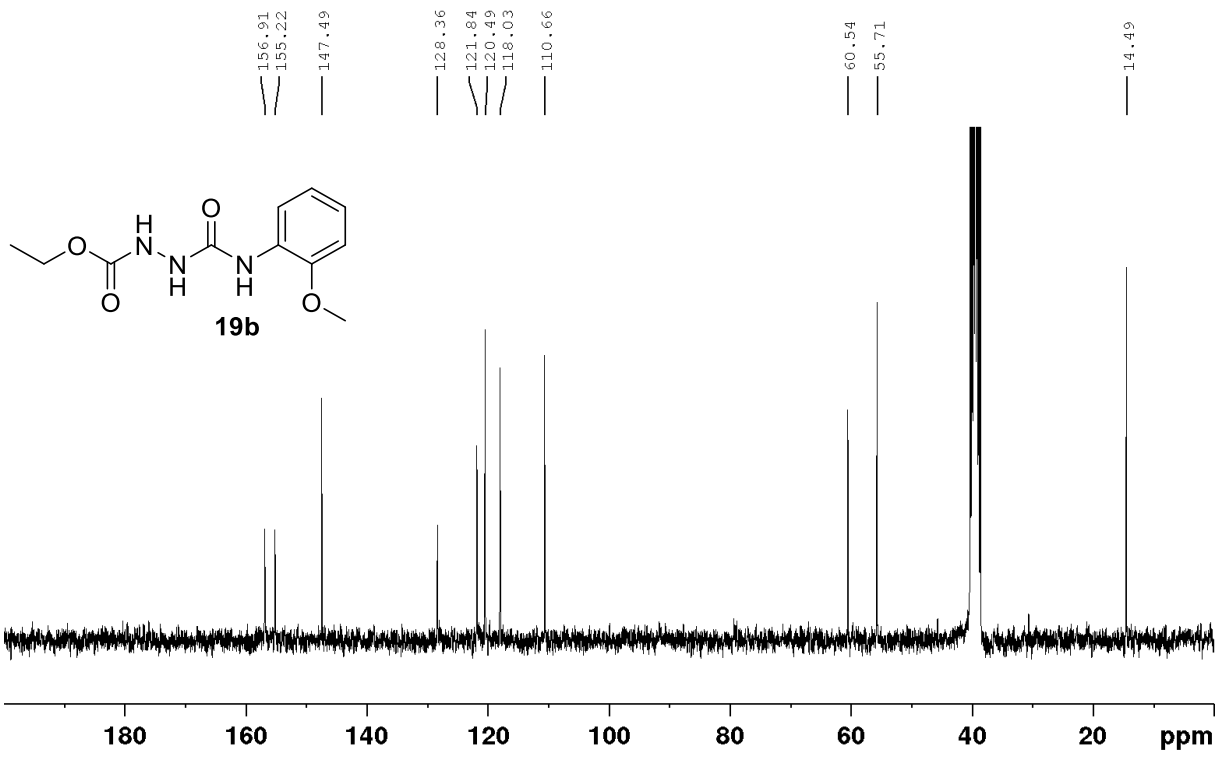
3.00
3.16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120.49
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60.54
55.71
14.49
19b
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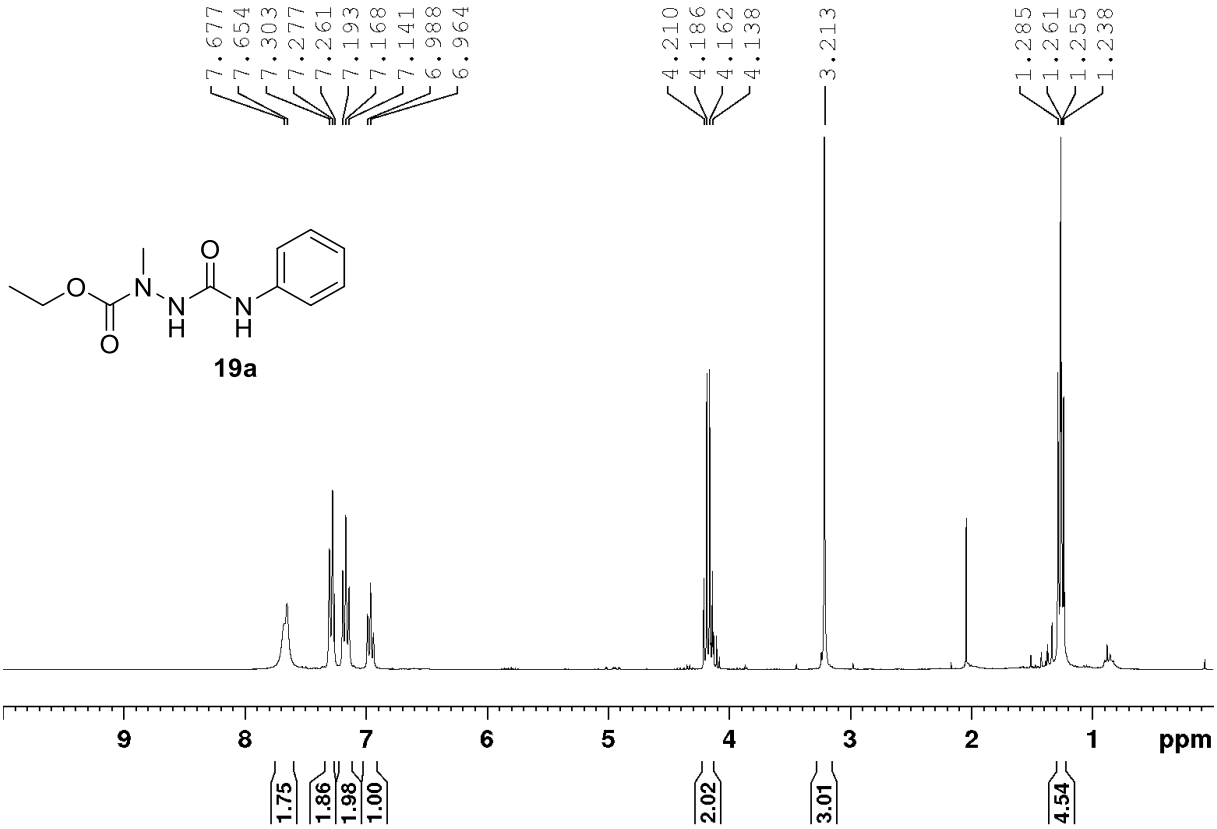
160
140
120
100
80
60
40
20
ppm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7.168
7.141
6.988
6.964
4.210
4.186
4.162
4.138
3.213
1.285
1.261
1.255
1.238
19a
9
8
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6
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4
3
2
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ppm
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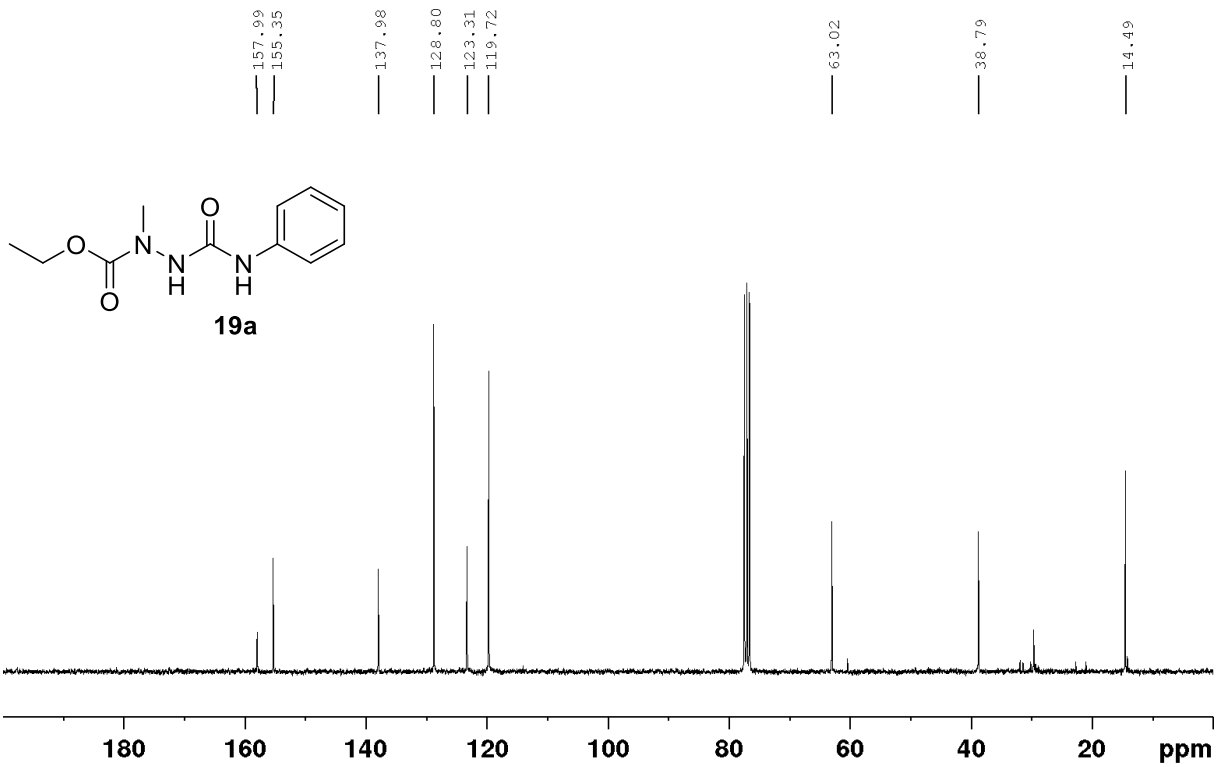
1.98
1.00
2.02
3.01
4.54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128.80
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63.02
38.79
14.49
19a
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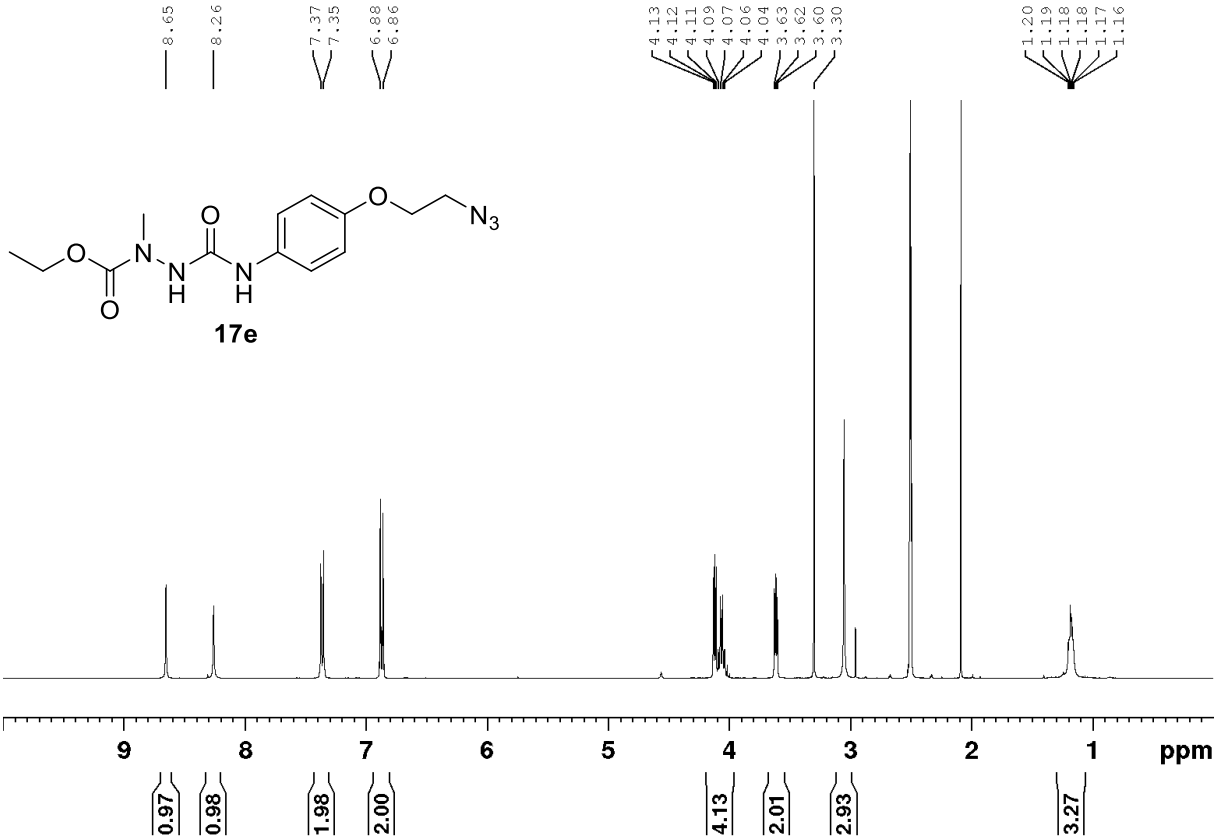
140
120
100
80
60
40
20
ppm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8.26
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6.86
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4.12
4.11
4.09
4.07
4.06
4.04
3.63
3.62
3.60
3.30
1.20
1.19
1.18
1.17
1.16
17e
9
8
7
6
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2
1
ppm
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2.00
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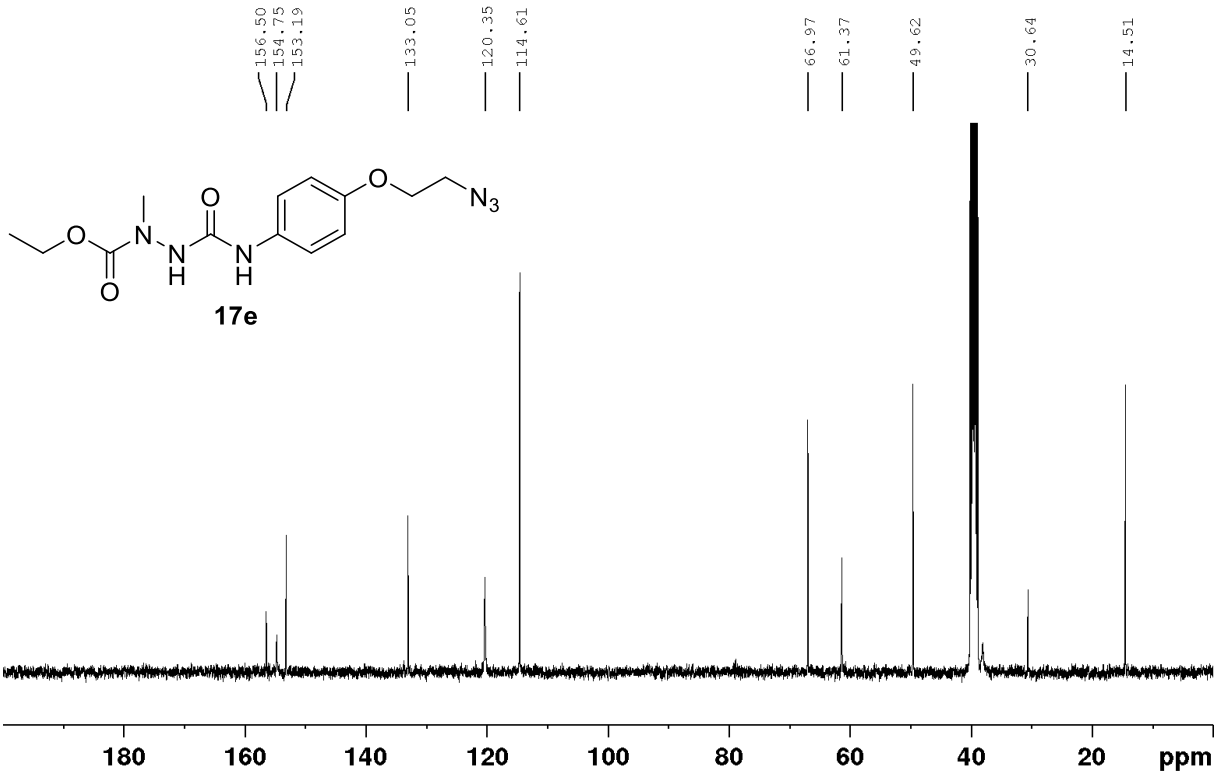
2.01
2.93
3.27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114.61
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30.64
14.51
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140
120
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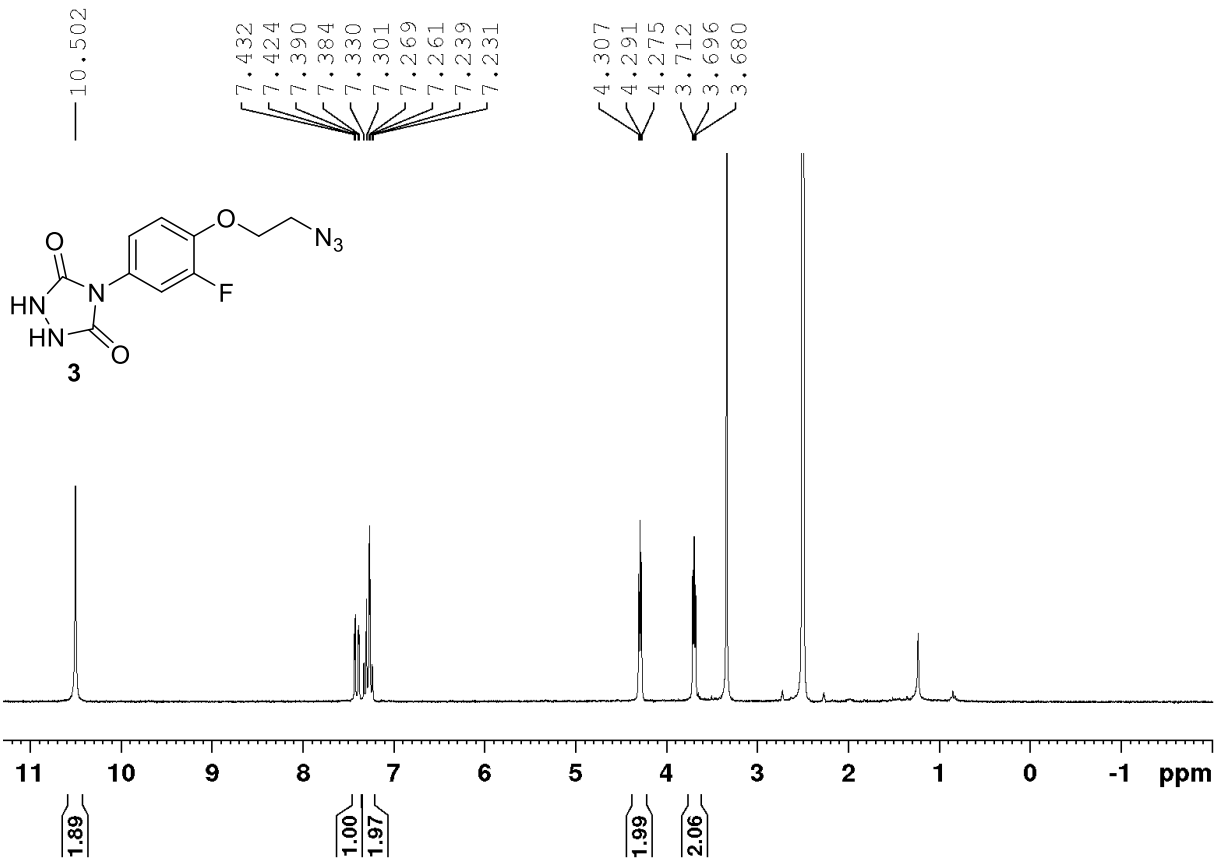
60
40
20
ppm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7.239
7.231
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4.291
4.275
3.712
3.696
3.680
O
N3
F
HN
N
HN
O
3
11
10
9
8
7
6
5
4
3
2
1
0
-1
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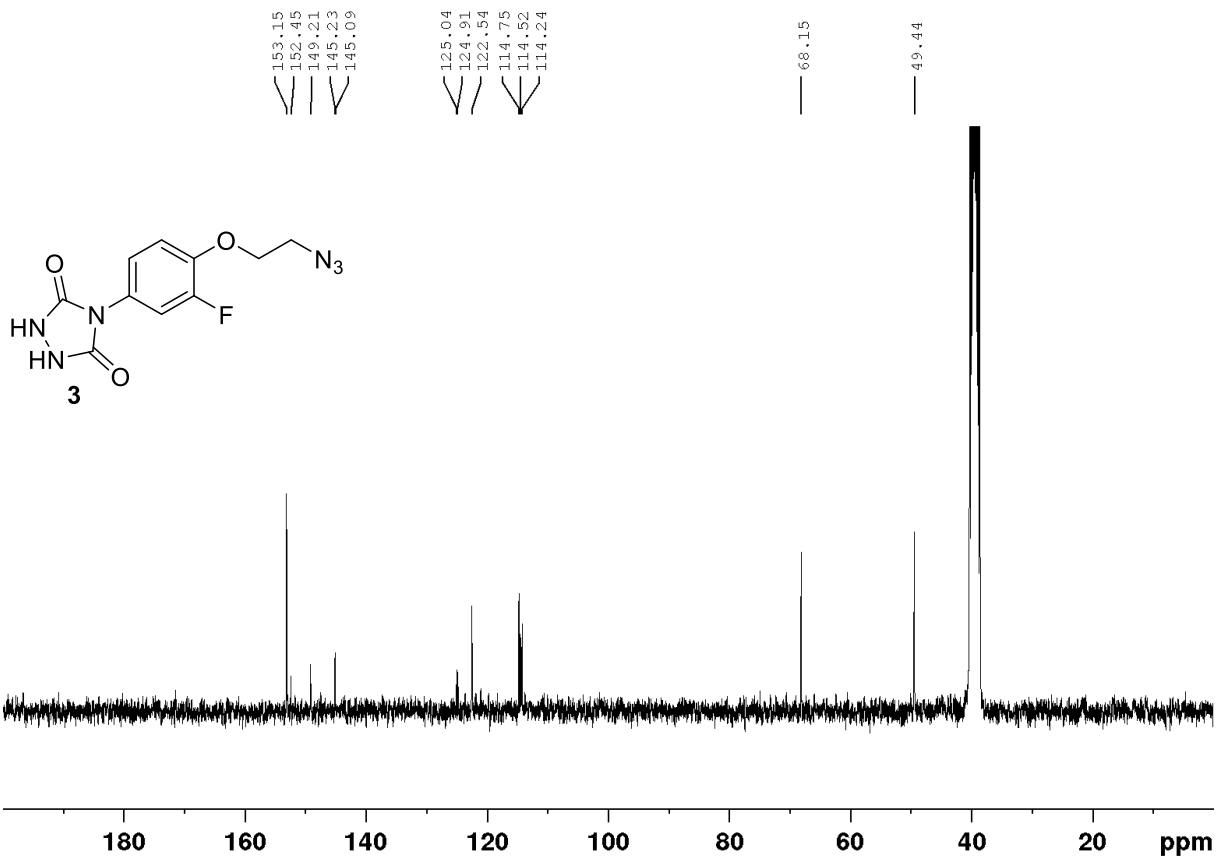
2.06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145.09
125.04
124.91
122.54
114.75
114.52
114.24
68.15
49.44
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N3
F
HN
N
HN
O
3
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140
120
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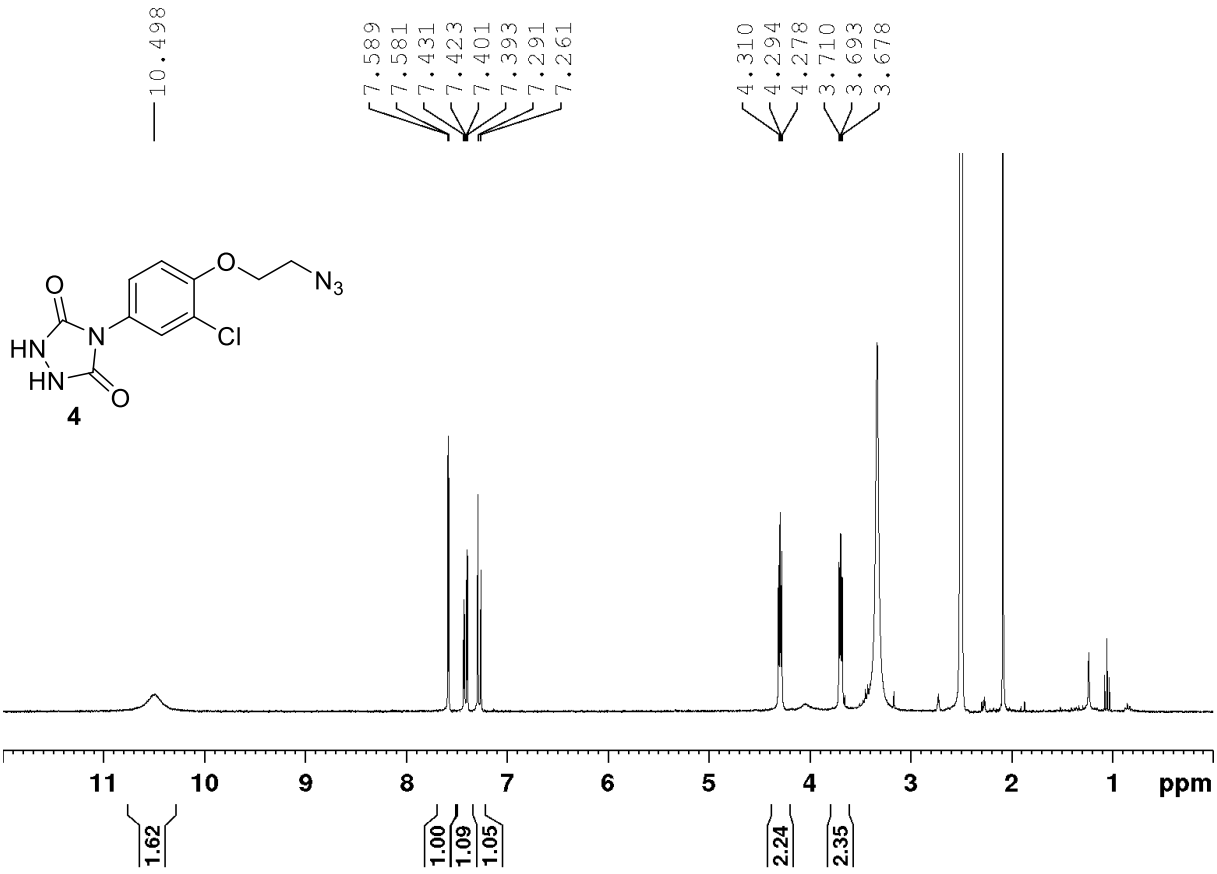
80
60
40
20
ppm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7.401
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7.291
7.261
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4.294
4.278
3.710
3.693
3.678
O
N3
Cl
HN
N
HN
O
O
4
11
10
9
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6
5
4
3
2
1
ppm
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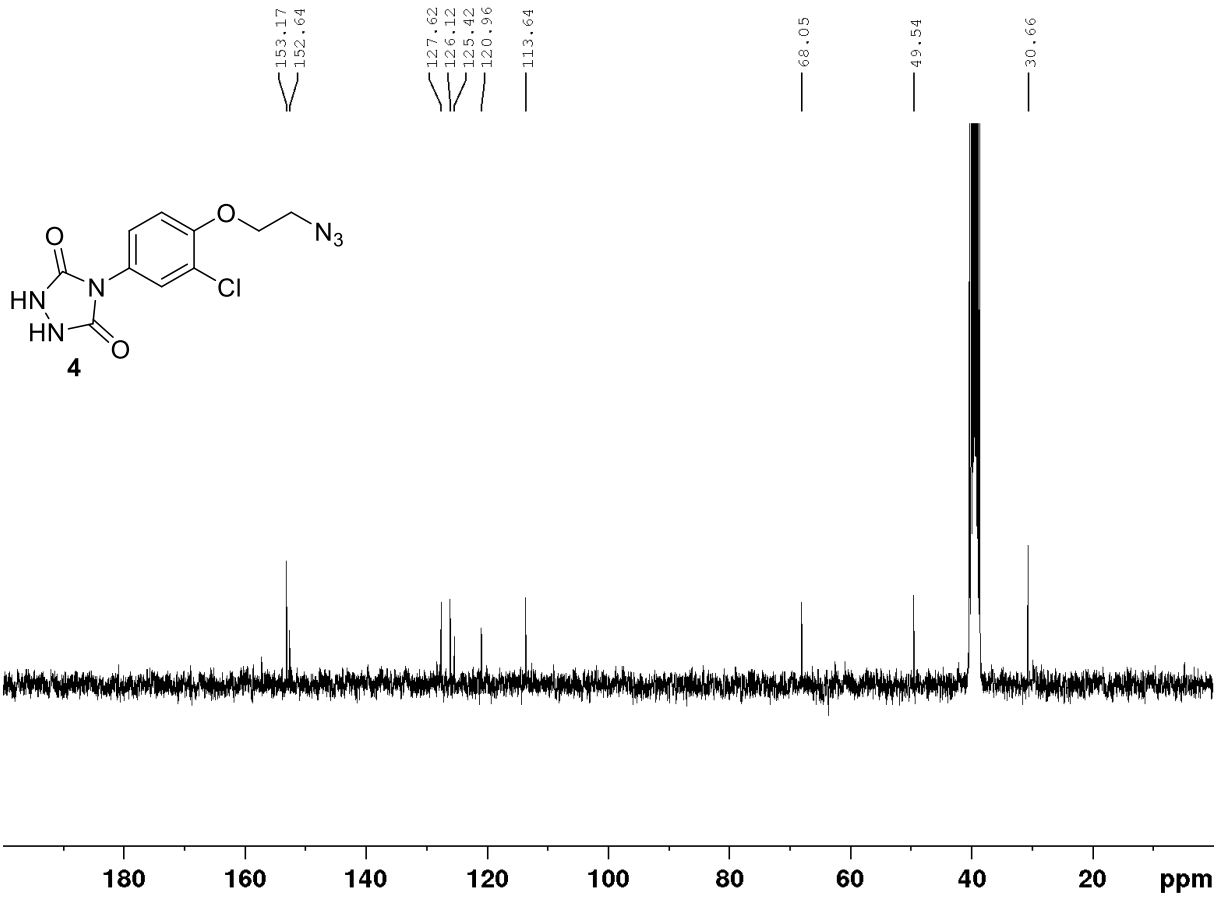
1.00
1.09
1.05
2.24
2.35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68.05
49.54
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Cl
HN
N
HN
O
O
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140
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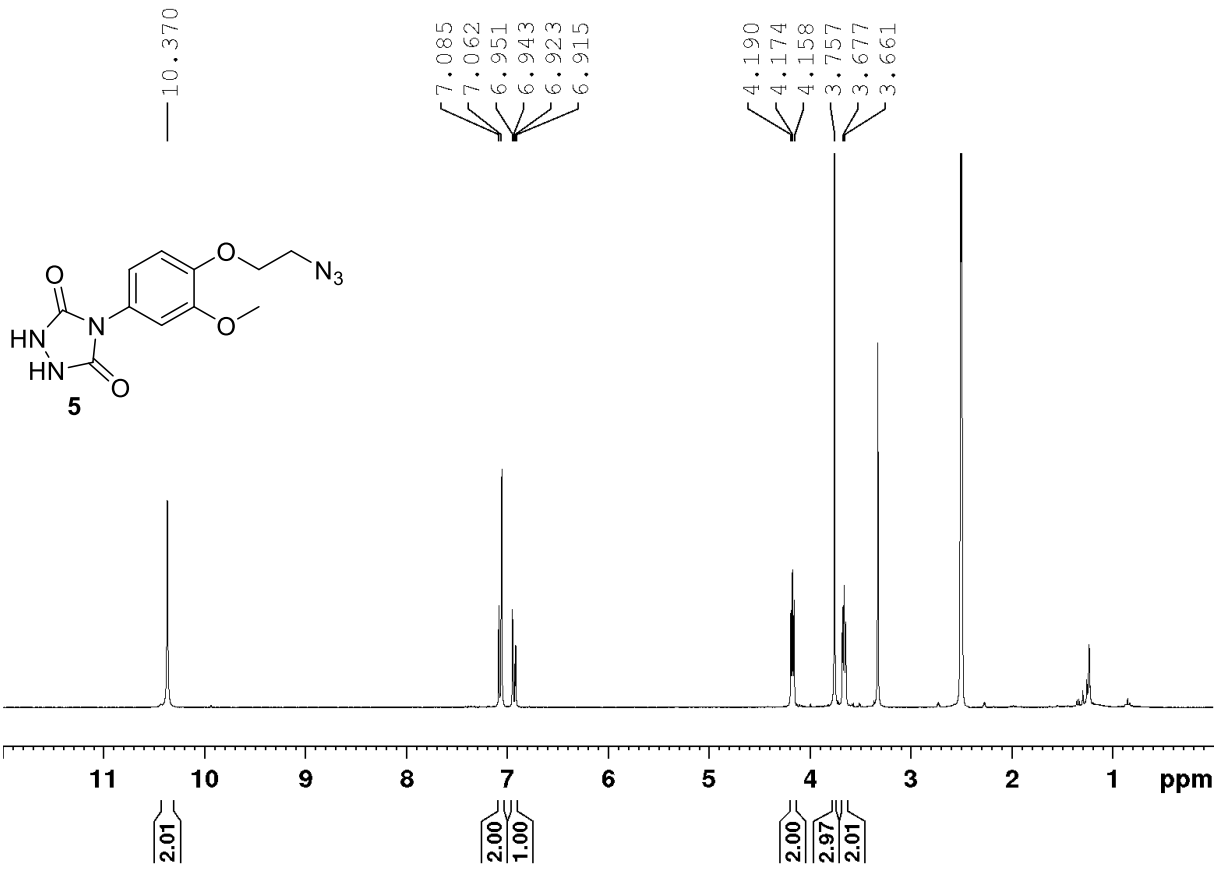
60
40
20
ppm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N3
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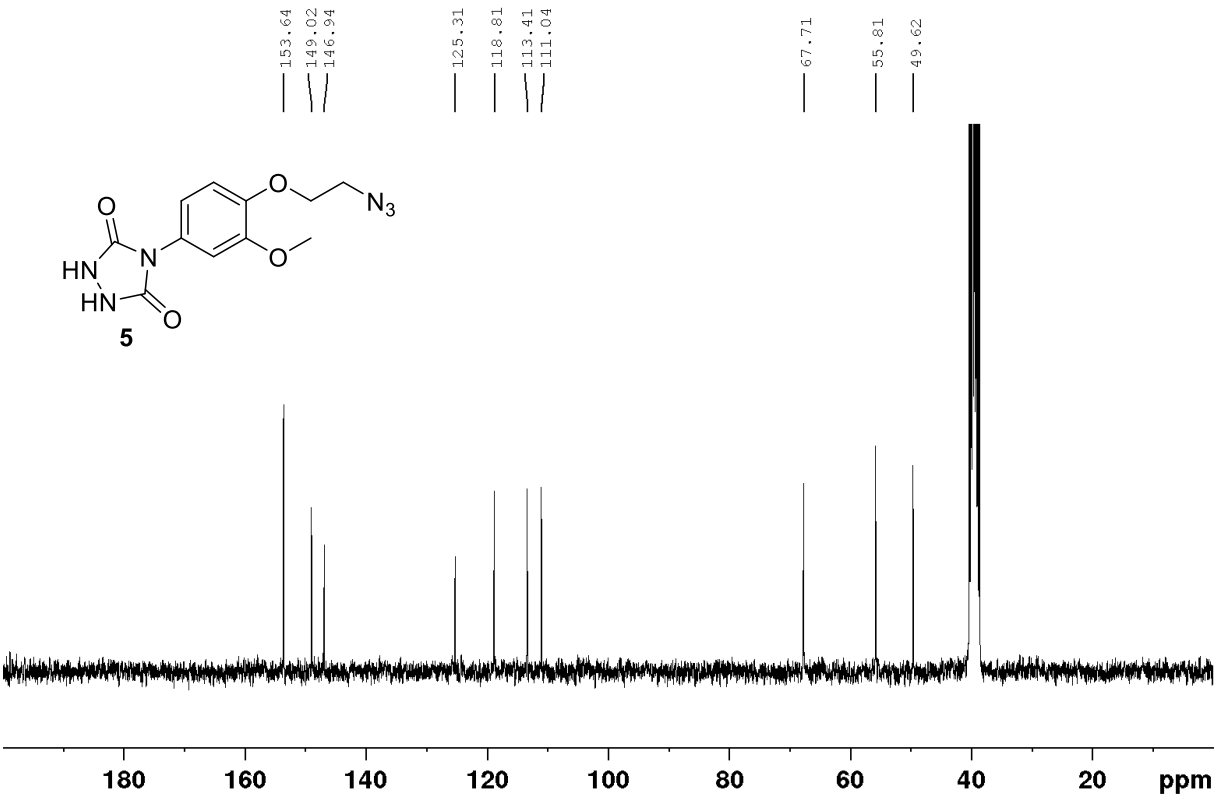
2.01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67.71
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N
HN
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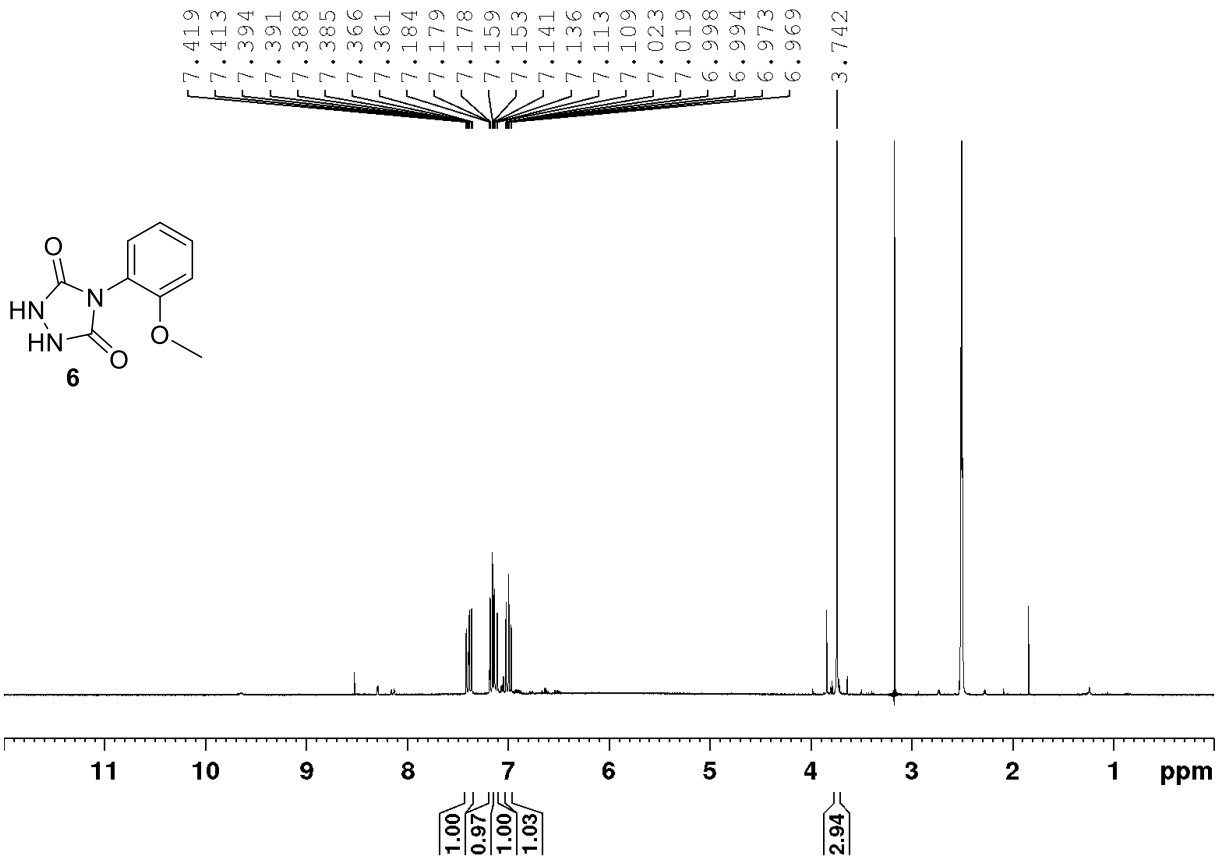
20
ppm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7.385
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7.361
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7.179
7.178
7.159
7.153
7.141
7.136
7.113
7.109
7.023
7.019
6.998
6.994
6.973
6.969
3.742
HN
HN
N
O
O
O
6
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7
6
5
4
3
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1
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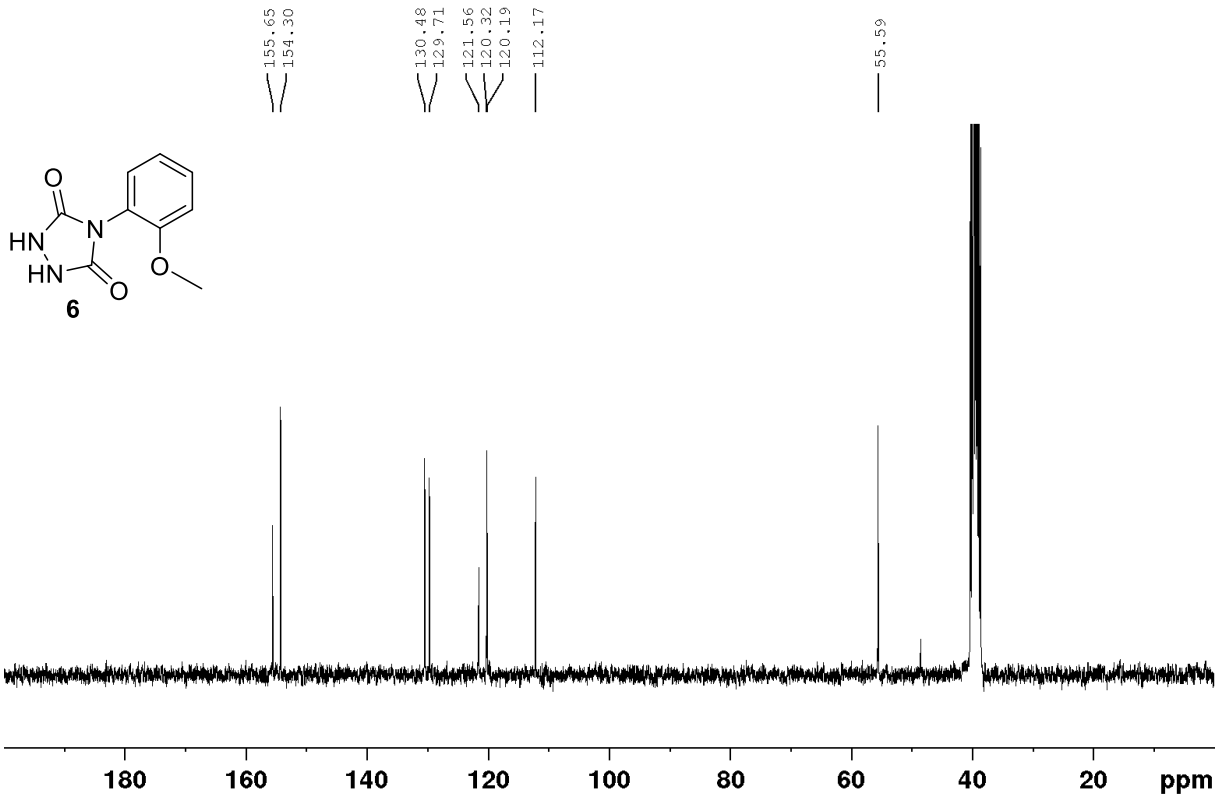
1.03
2.94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120.32
120.19
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HN
N
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O
O
6
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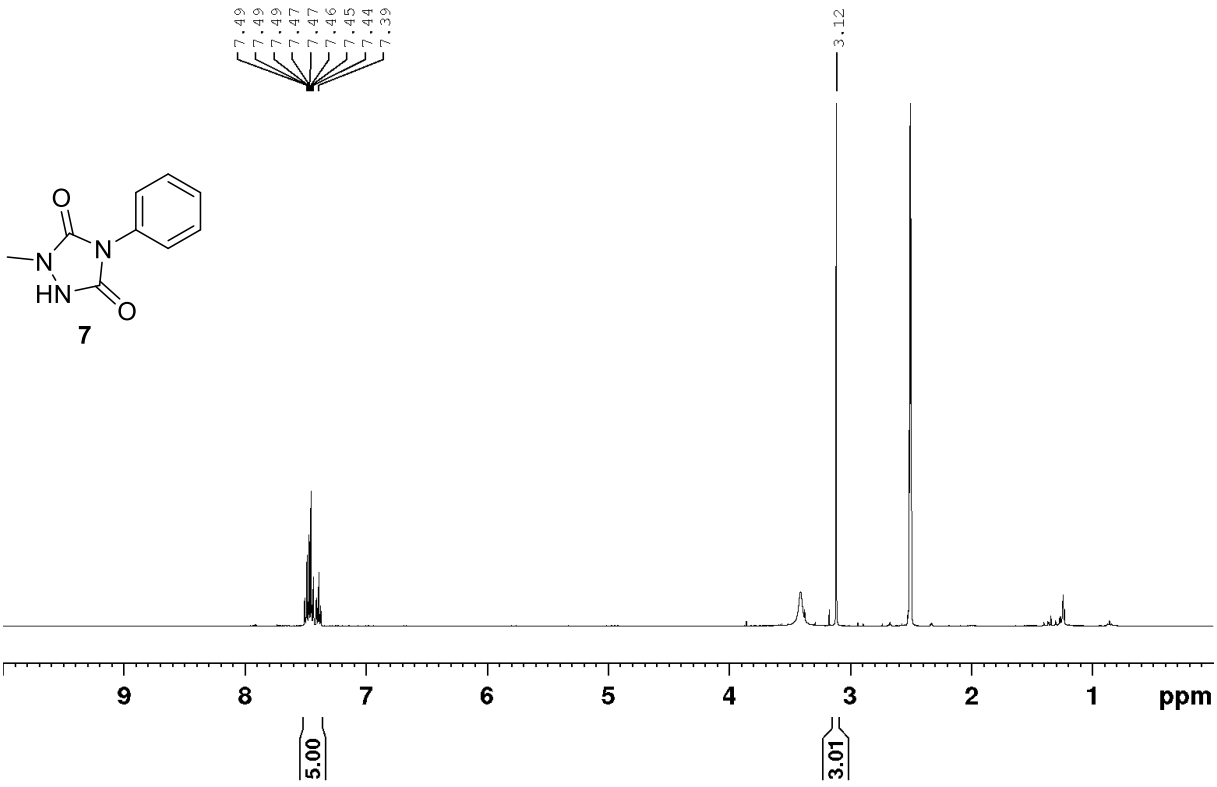
160
140
120
100
80
60
40
20
ppm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7.45
7.44
7.39
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HN
O
7
9
8
7
6
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4
3
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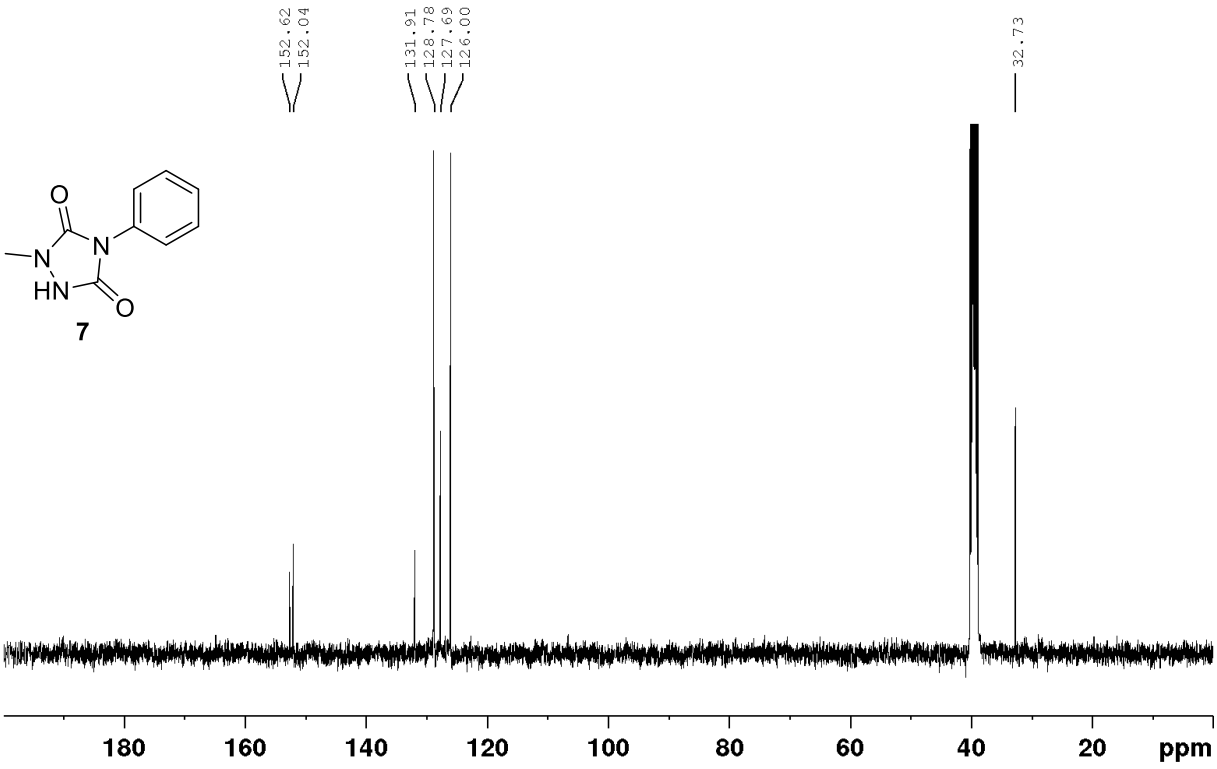
1
ppm
5.00
3.01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128.78
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140
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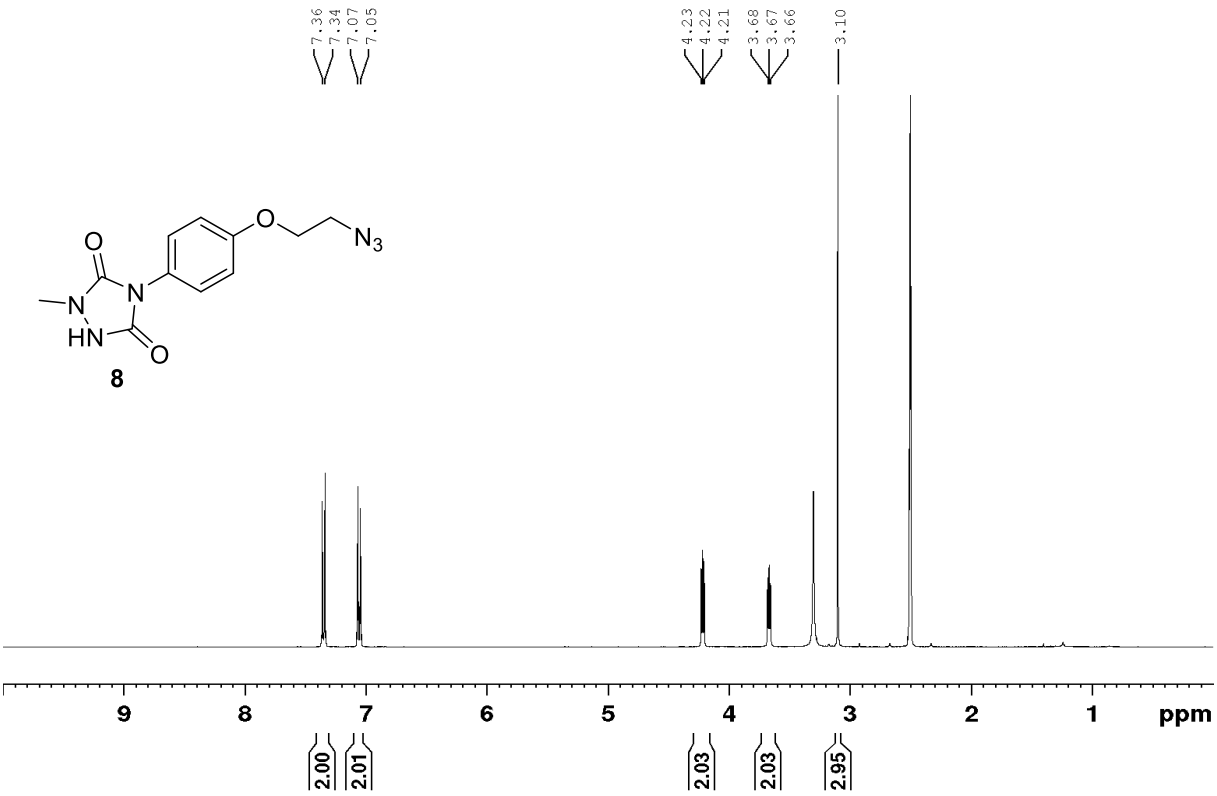
80
60
40
20
ppm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7.05
4.23
4.22
4.21
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3.66
3.10
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N3
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HN
O
O
8
9
8
7
6
5
4
3
2
1
ppm
2.00
2.01
2.03
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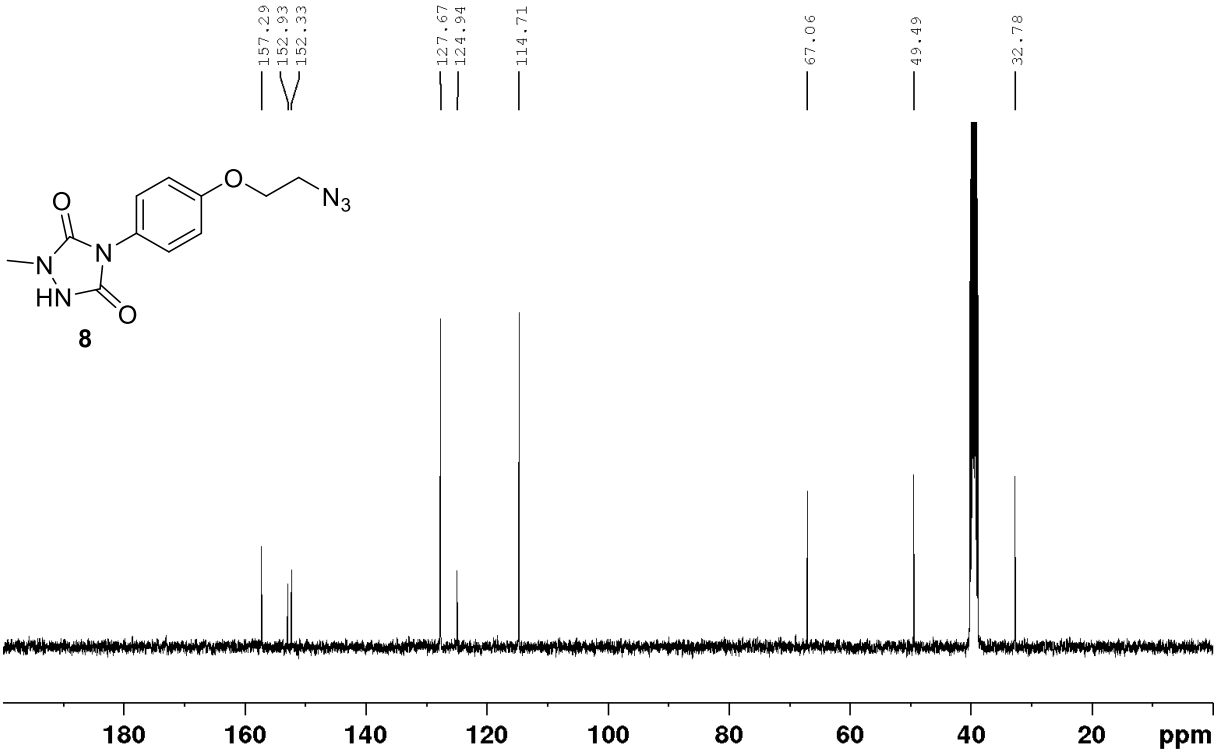
2.95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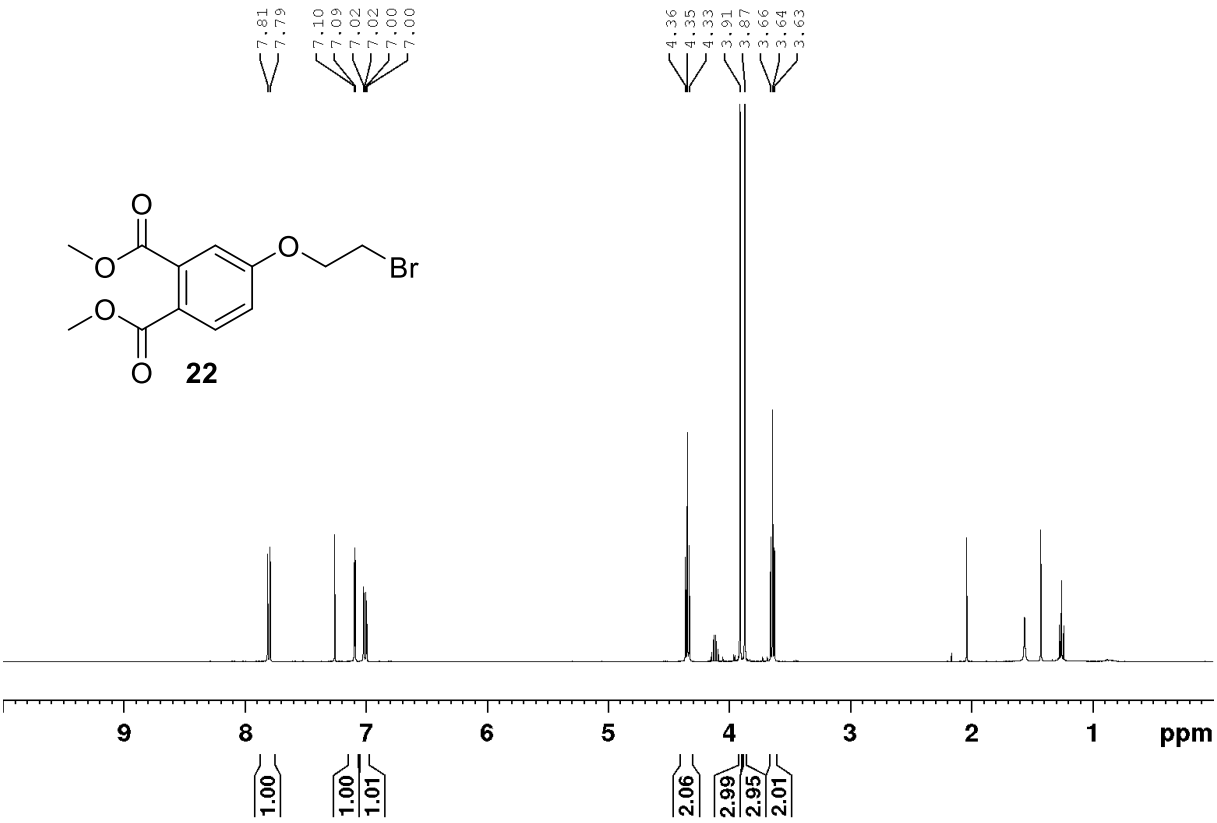
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3.87
3.66
3.64
3.63
O
O
O
O
O
Br
22
9
8
7
6
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4
3
2
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ppm
1.00
1.00
1.01
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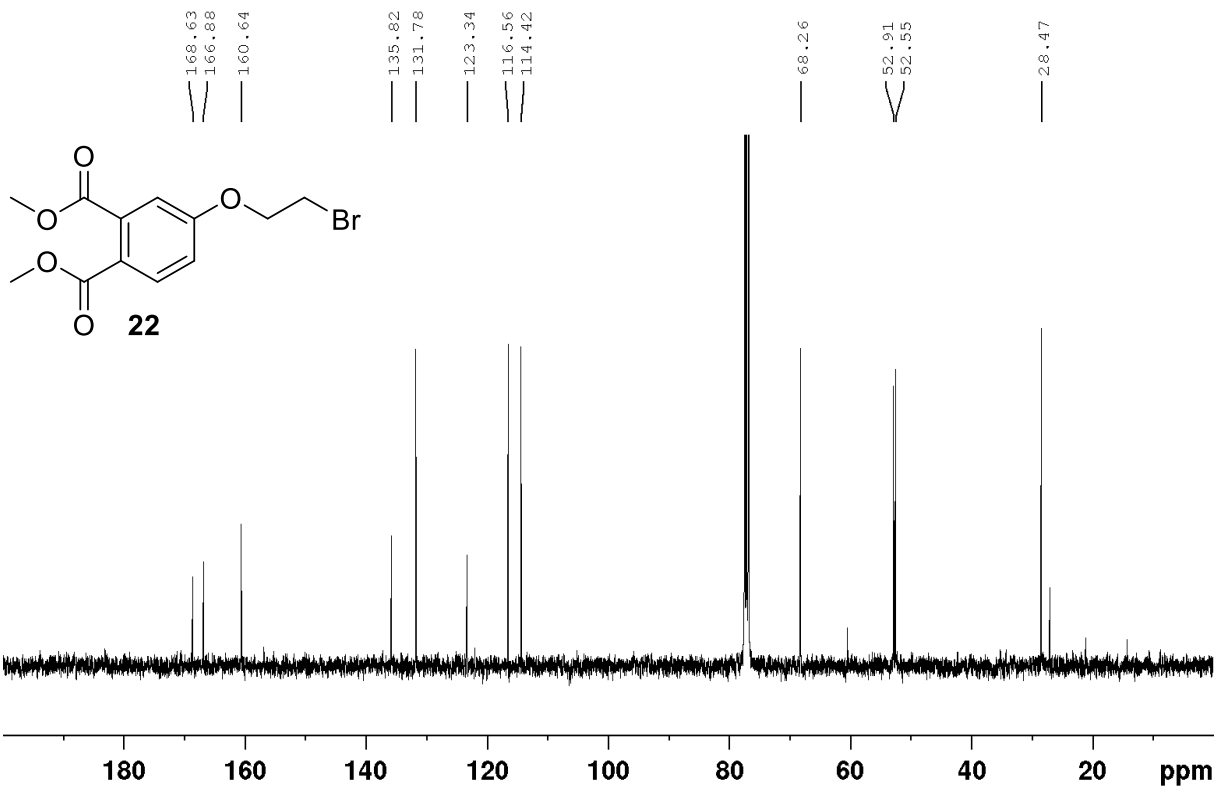
2.01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Br
22
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80
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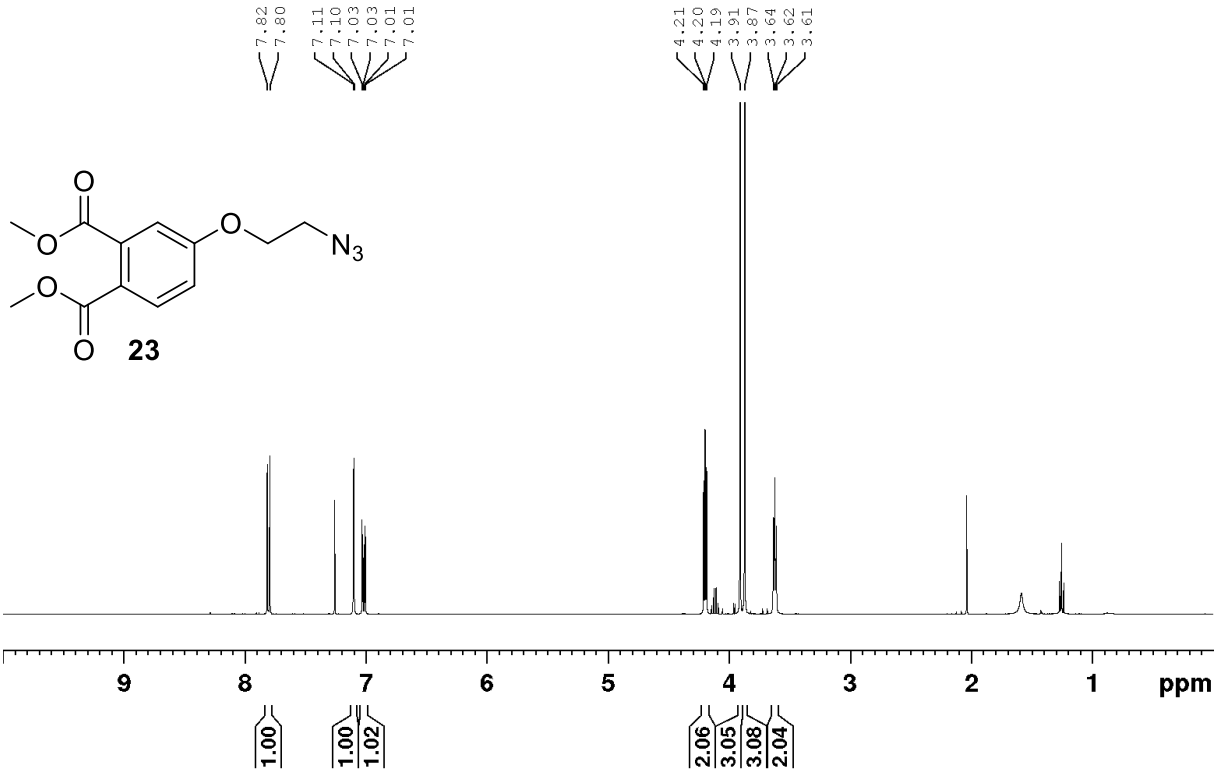
ppm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3.87
3.64
3.62
3.61
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O
O
O
O
N3
23
9
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7
6
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3
2
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ppm
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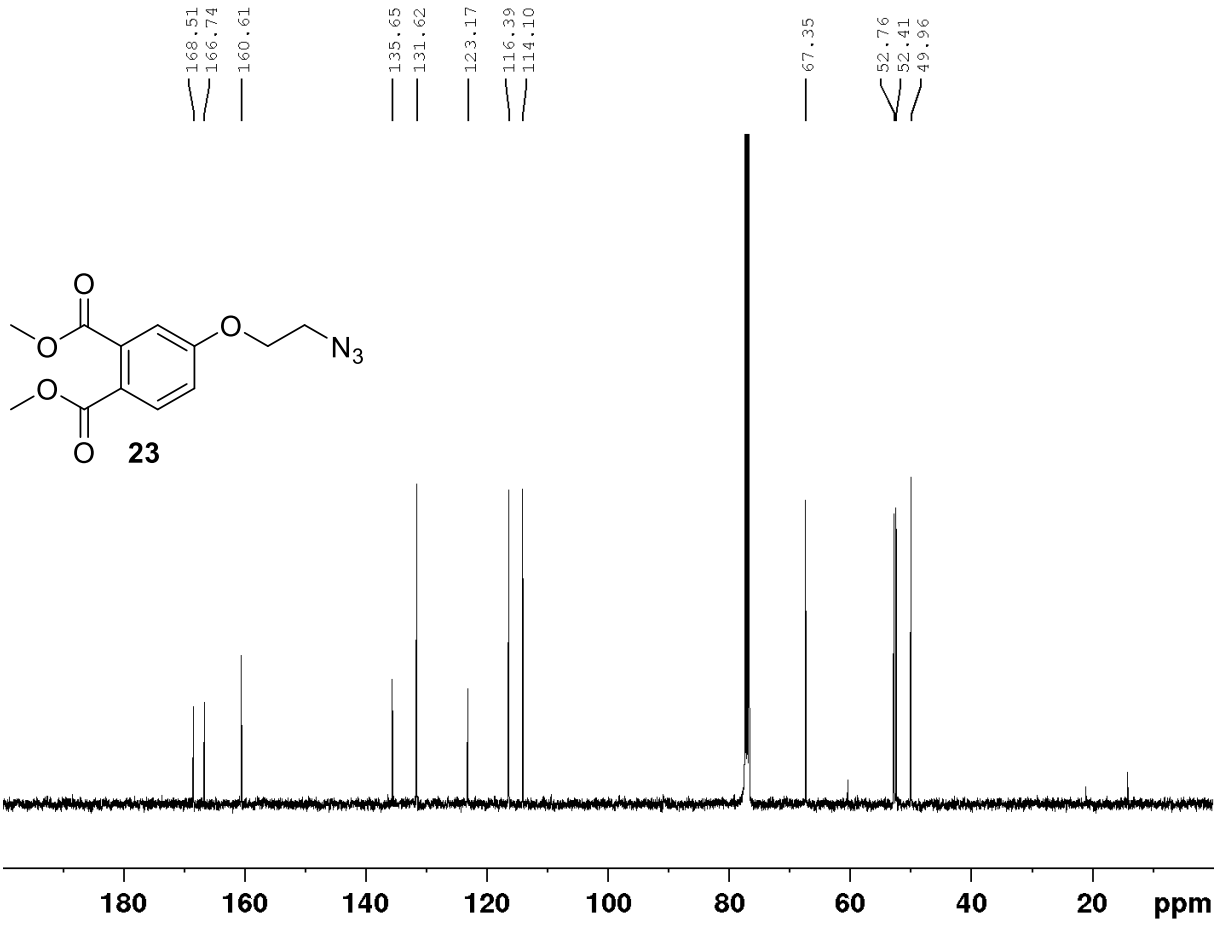
1.00
1.02
2.06
3.05
3.08
2.04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O
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N3
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140
120
100
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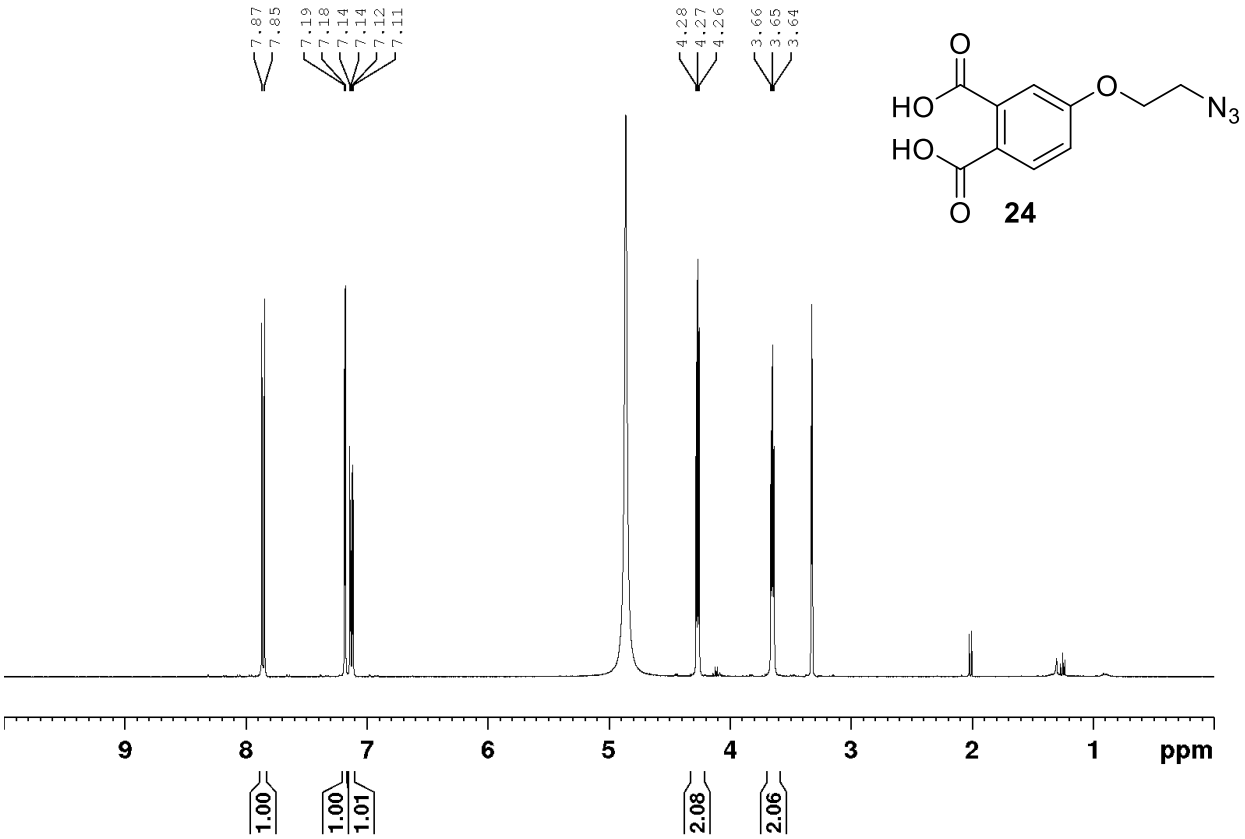
20
ppm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3.65
3.64
HO
HO
O
O
O
N3
24
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8
7
6
5
4
3
2
1
ppm
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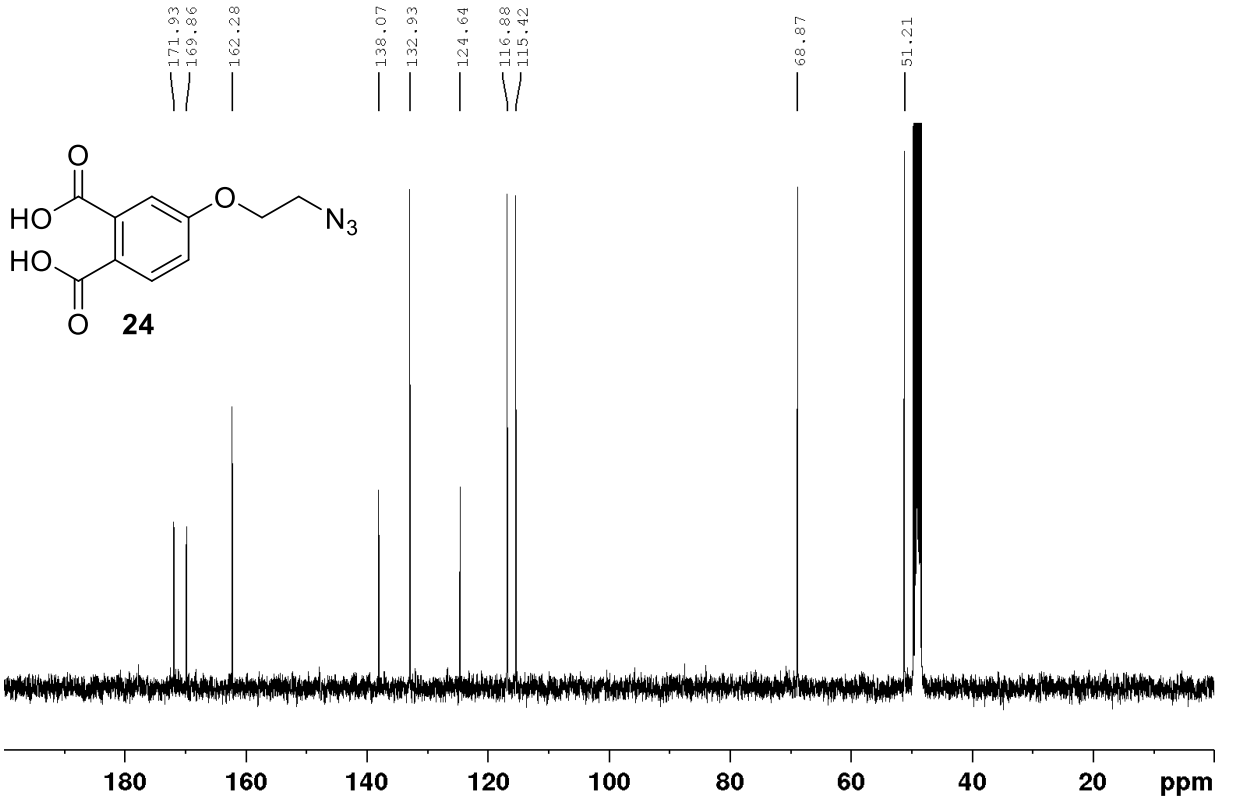
1.00
1.01
2.08
2.06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162.28
138.07
132.93
124.64
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115.42
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51.21
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HO
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O
O
N3
24
180
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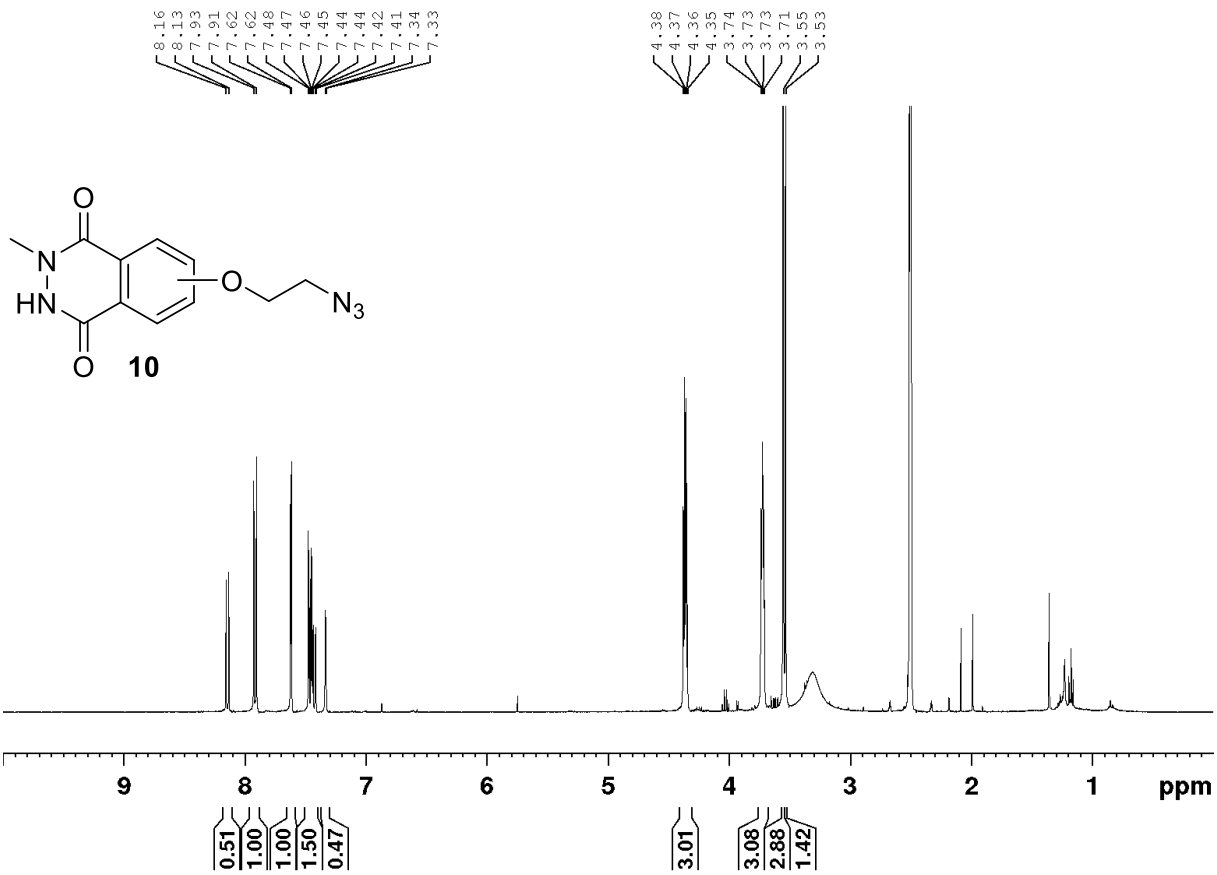
140
120
100
80
60
40
20
ppm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8.13
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7.62
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7.47
7.46
7.45
7.44
7.44
7.42
7.41
7.34
7.33
4.38
4.37
4.36
4.35
3.74
3.73
3.73
3.71
3.55
3.53
O
N
HN
O
O
N3
10
9
8
7
6
5
4
3
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1
ppm
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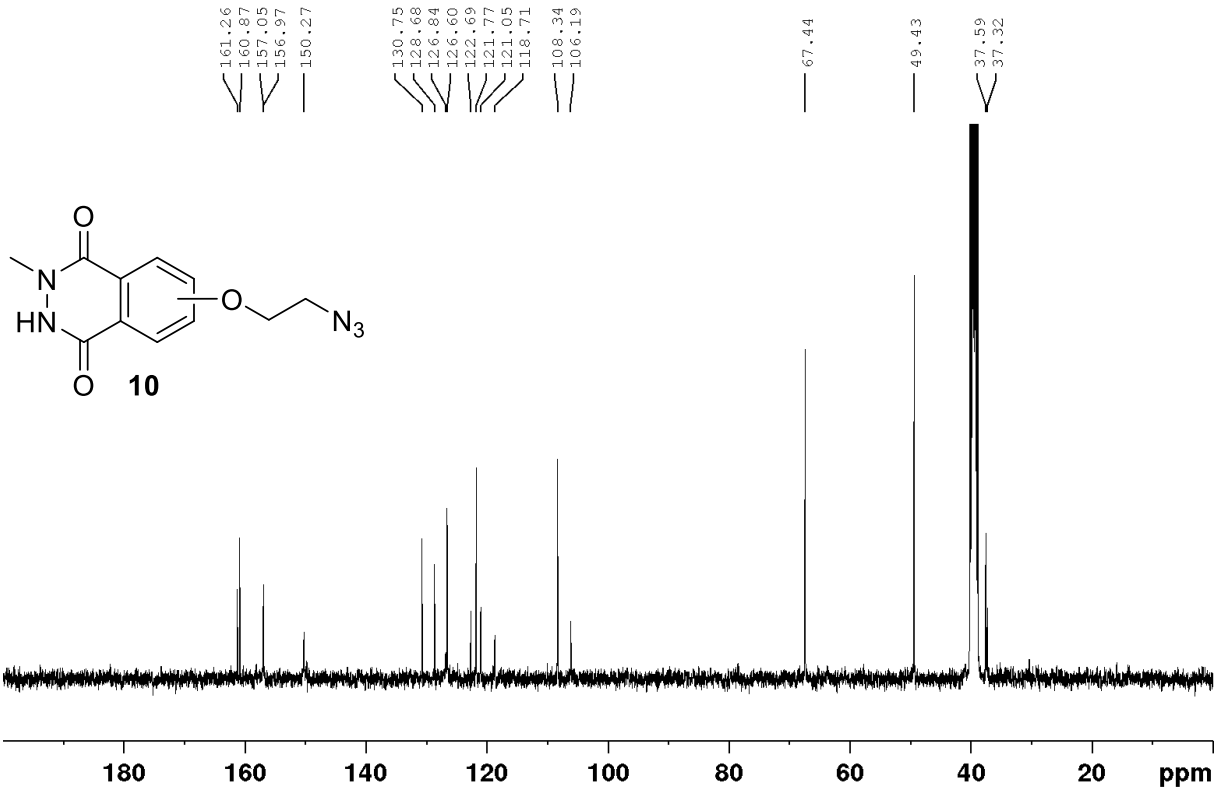
1.00
1.50
0.47
3.01
3.08
2.88
1.42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121.77
121.05
118.71
108.34
106.19
67.44
49.43
37.59
37.32
O
N
HN
O
O
N3
10
180
160
140
120
100
80
60
40
20
ppm

VI. References

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2 S. Sato, K. Nakamura and H. Nakamura, *ACS Chem. Biol.*, 2015, **10**, 2633–2640.