Electronic Supplementary Information

Maleimide-based metal-free ligation with dienes: a comparative study

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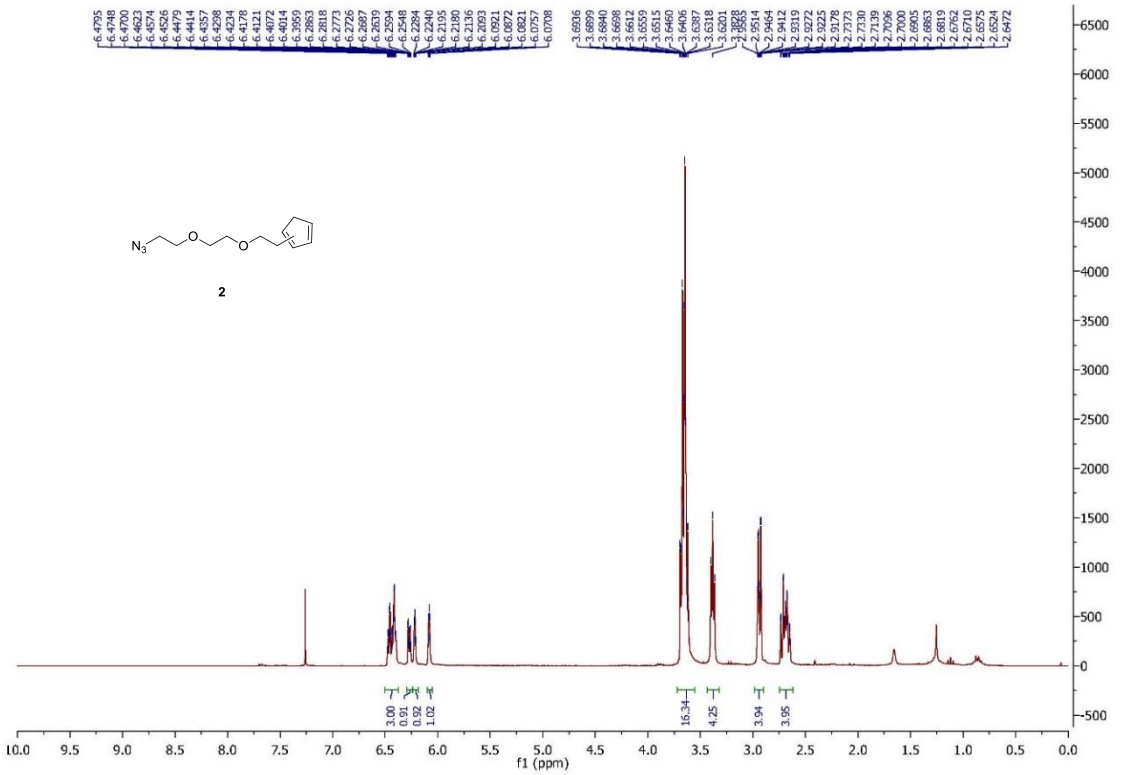
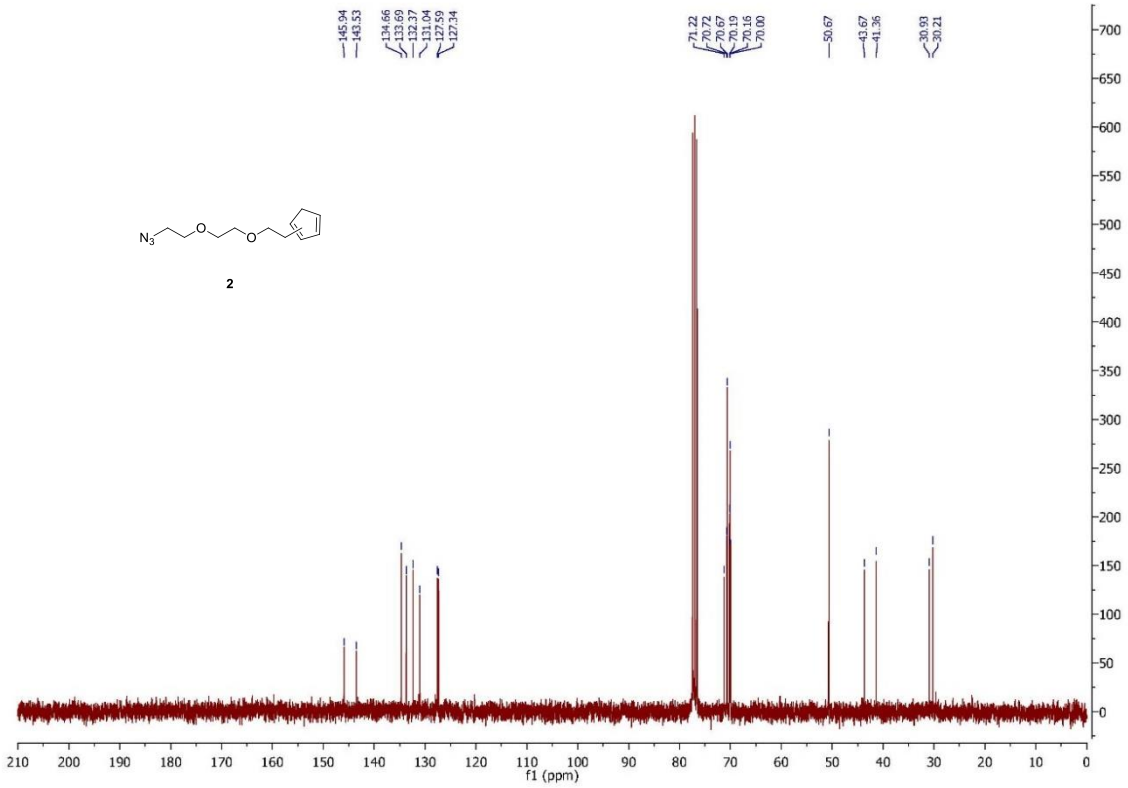
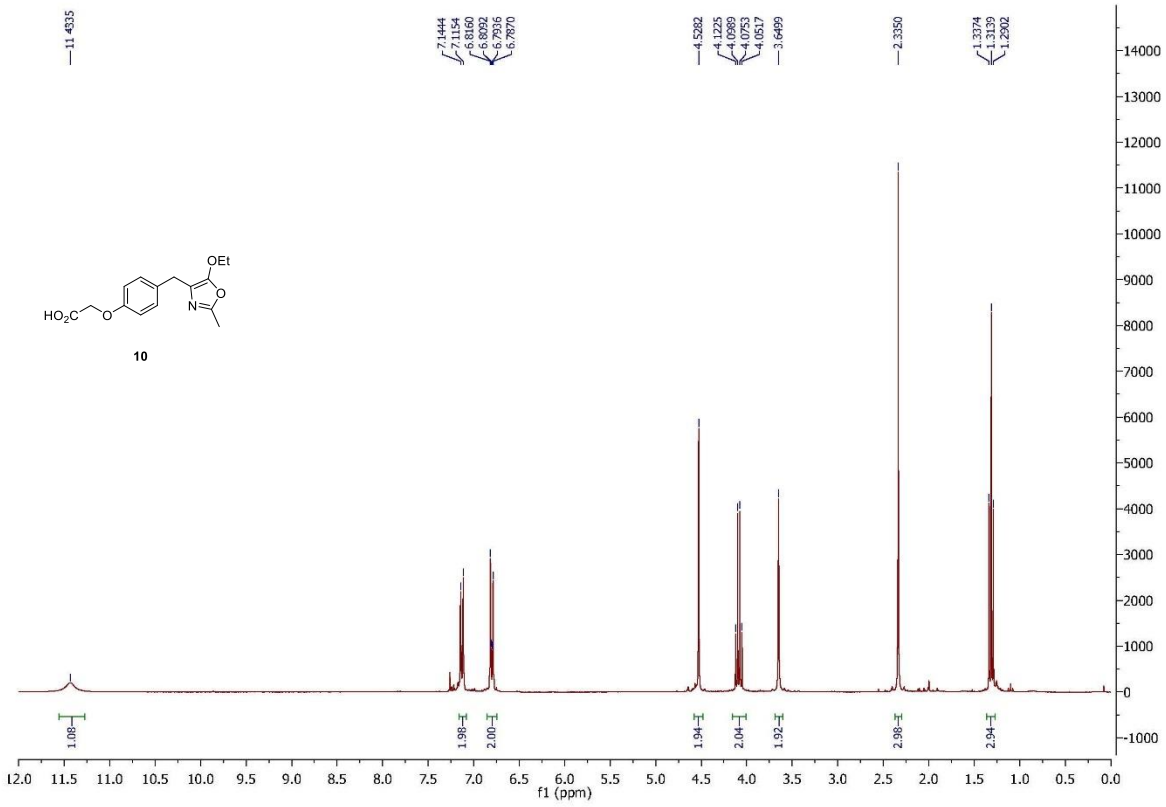
I.1. Copies of ^{1}H and ^{13}C NMR spectra



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4.0517
3.6499
2.3350
1.3374
1.3139
1.2902
OEt
HO2C
O
N
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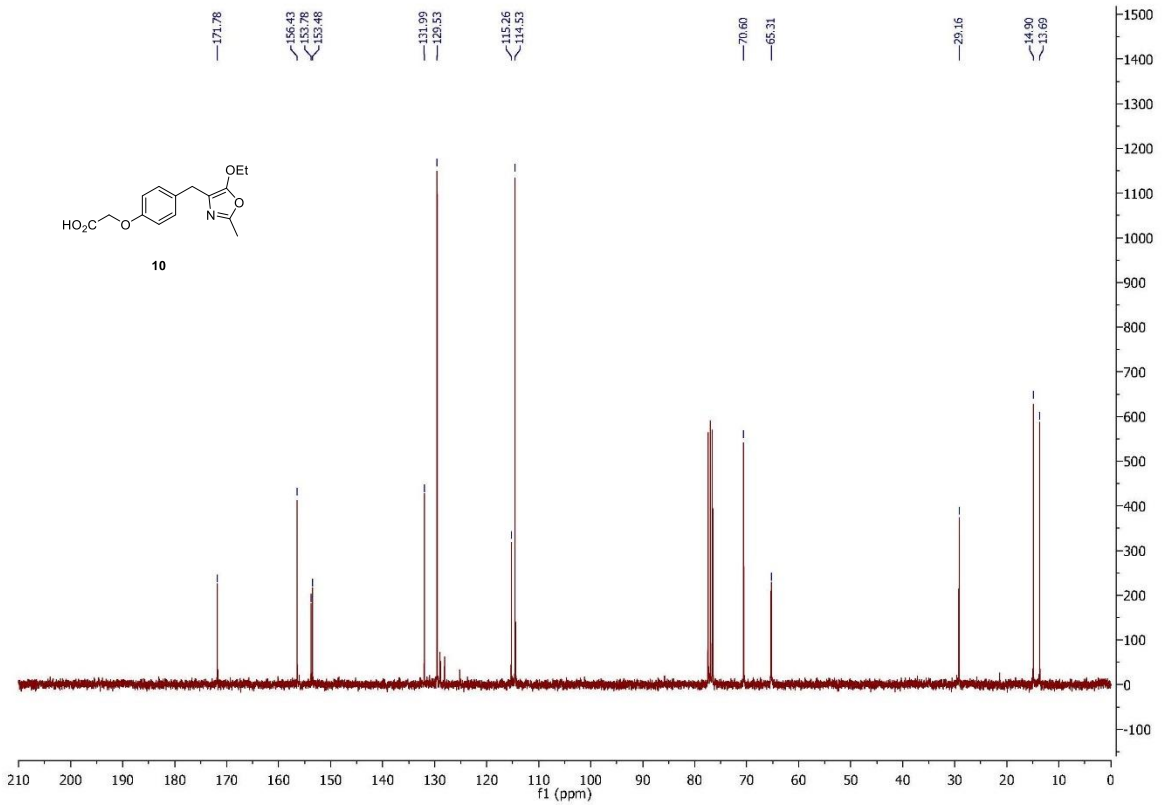
2.00
1.94
2.04
1.92
2.98
2.94
f1 (ppm)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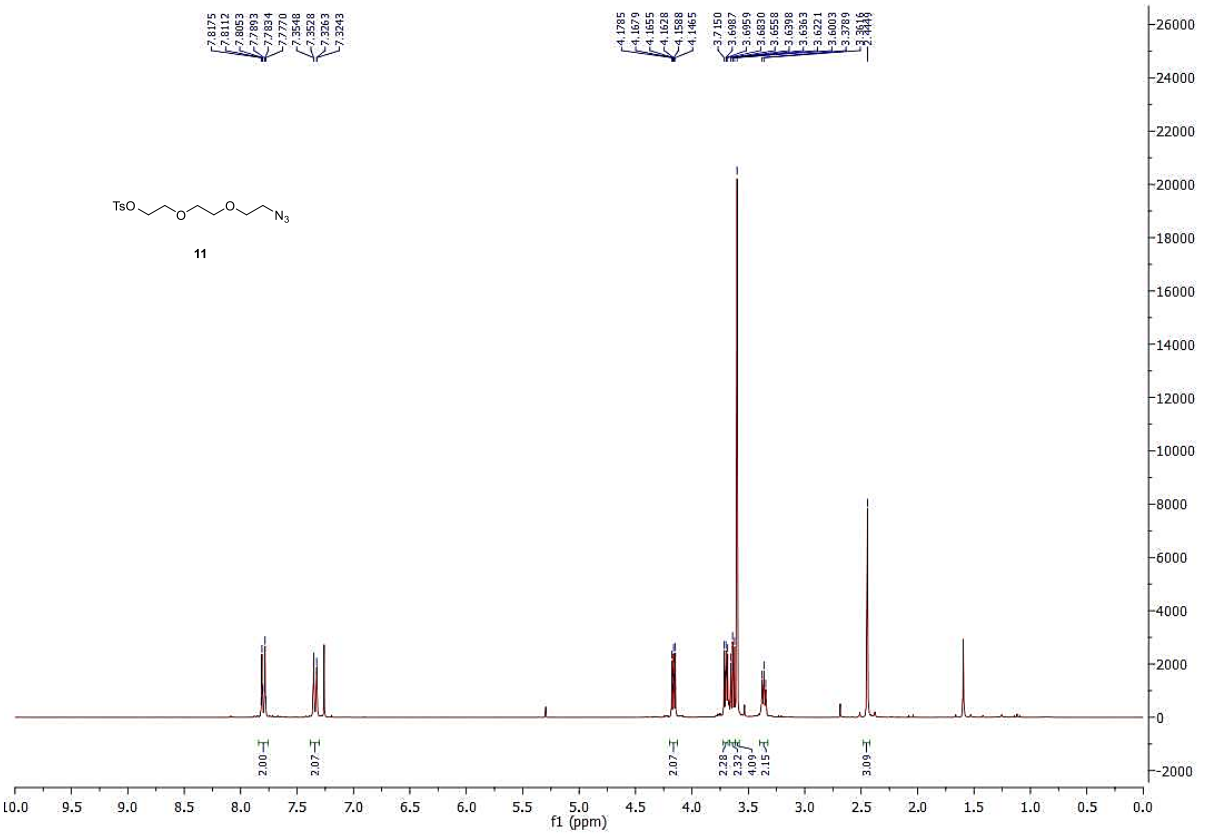
HO2C
O
N
10
f1 (ppm)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4.1679
4.1655
4.1628
4.1588
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3.7150
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3.6959
3.6830
3.6558
3.6398
3.6363
3.6221
3.6003
3.3789
3.3616
2.4440
TsO
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O
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3.09
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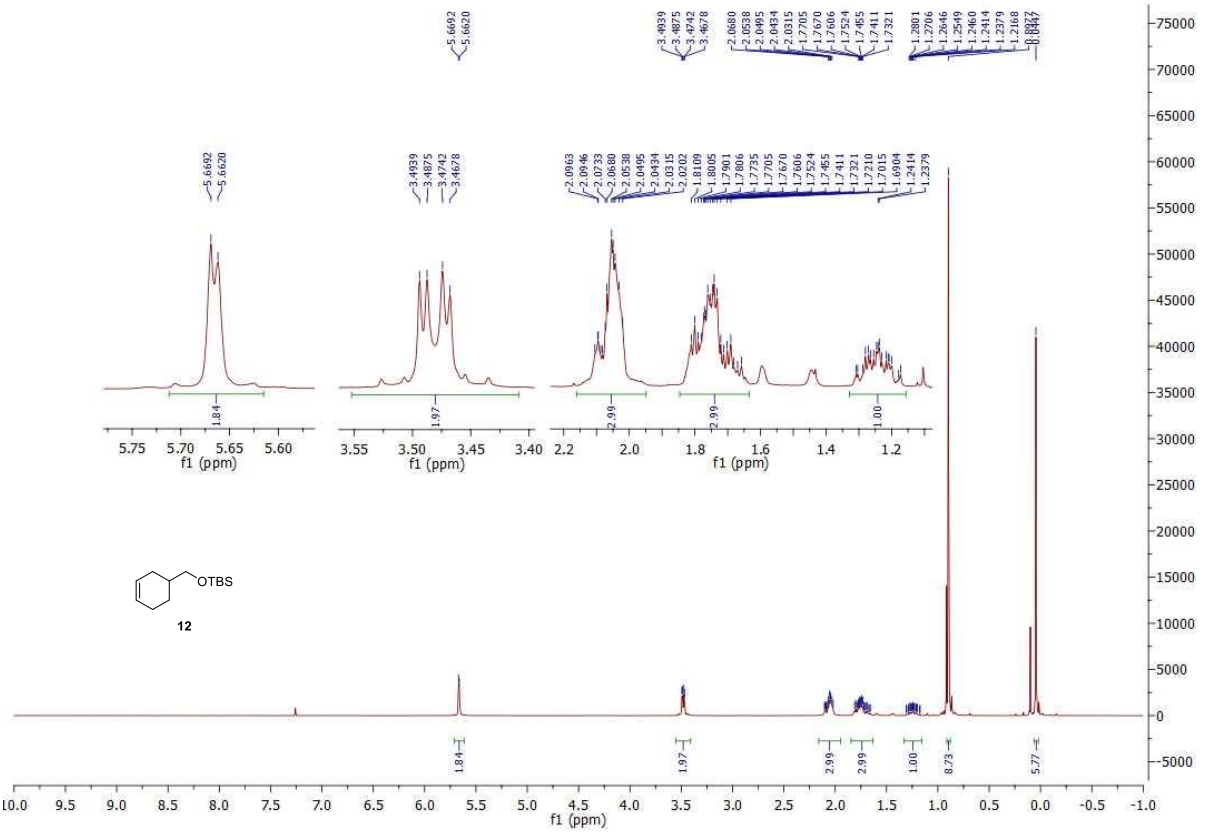
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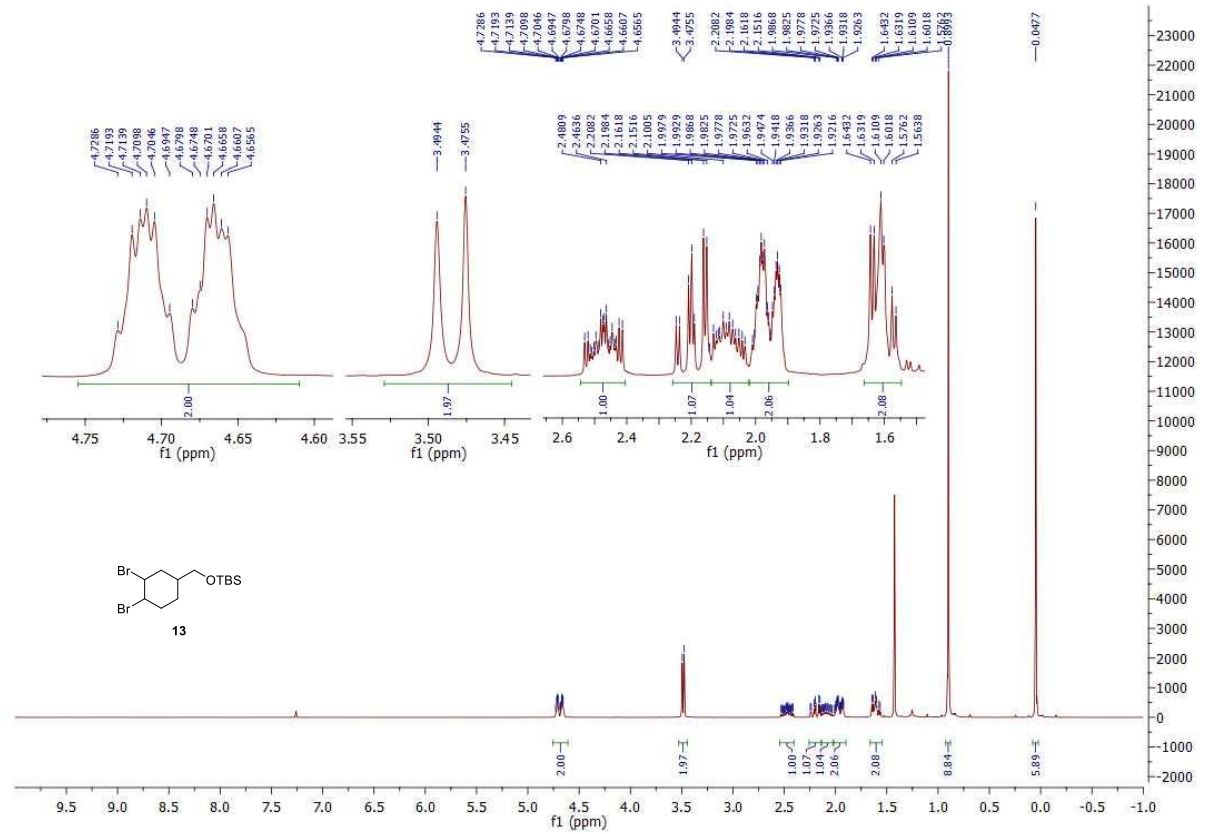
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Br
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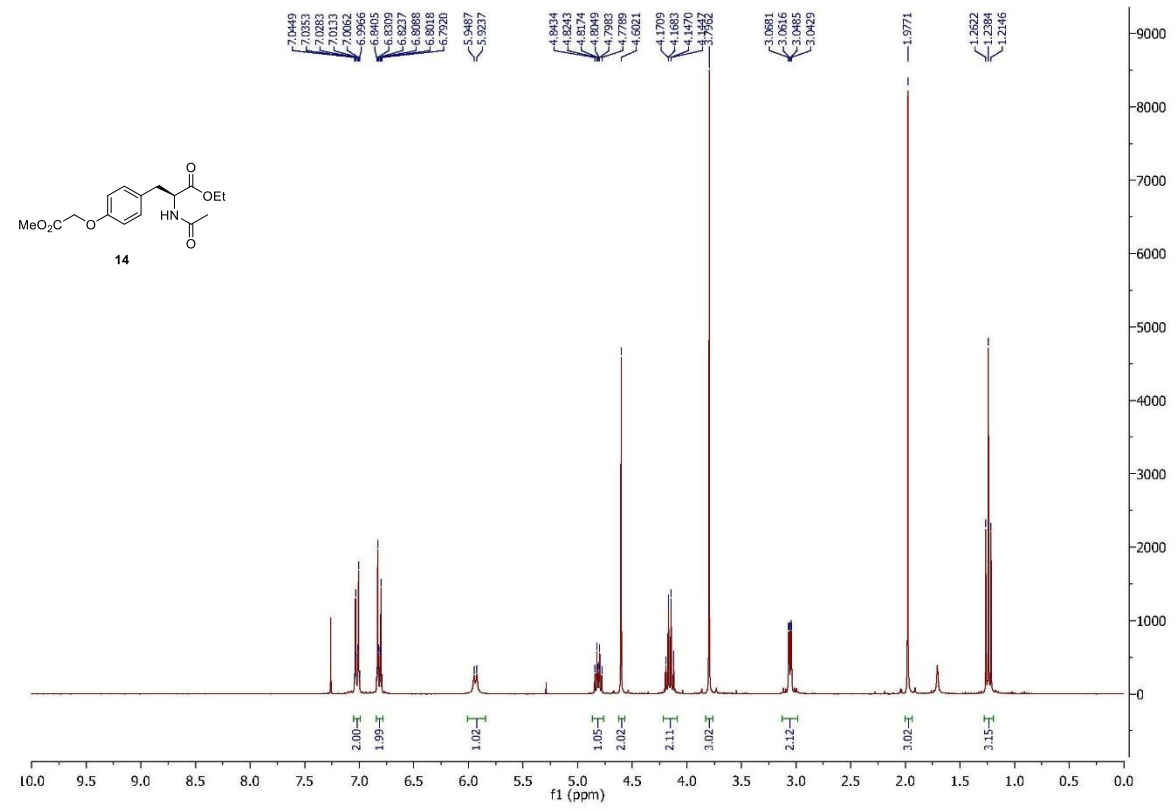
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5.9487
5.9237
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4.8243
4.8174
4.8049
4.7983
4.7789
4.6021
4.1709
4.1683
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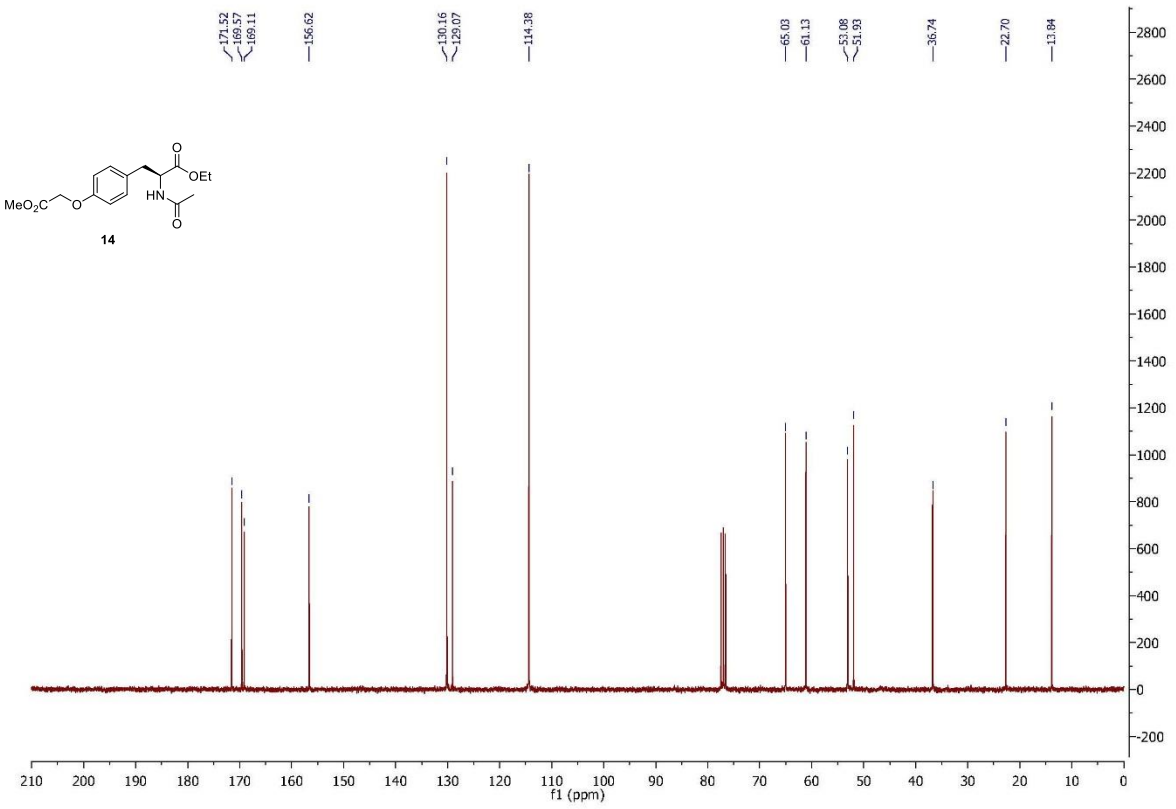
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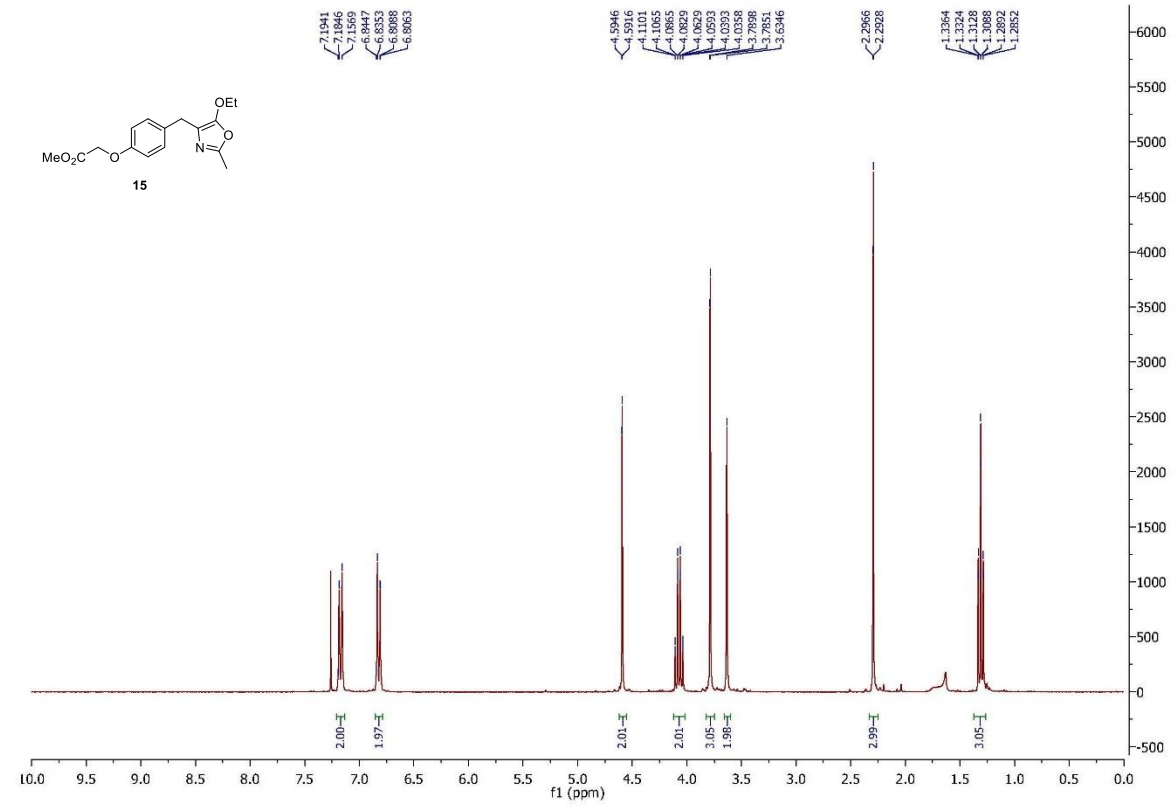
f1 (ppm)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4.0829
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4.0593
4.0358
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3.7651
3.6346
2.2966
2.2928
1.3364
1.3324
1.3128
1.3088
1.2692
1.2652
OEt
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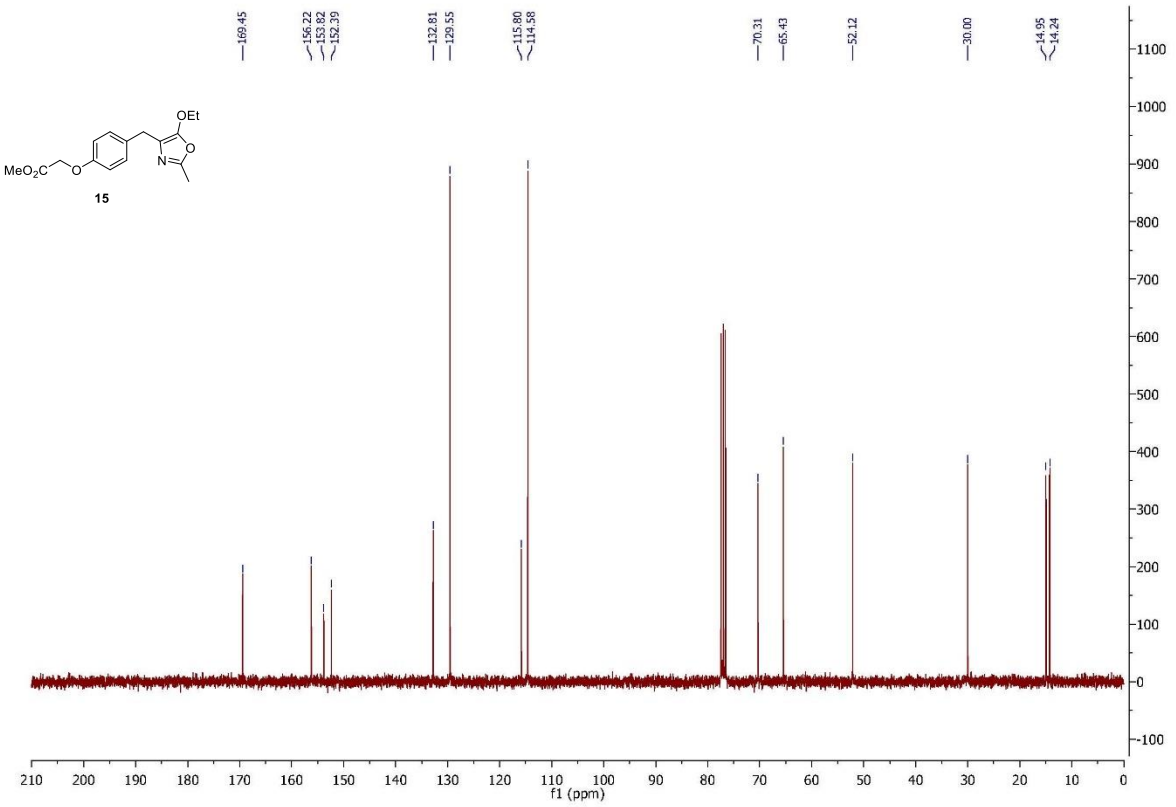
2.01
2.01
3.05
1.98
2.99
3.05
f1 (ppm)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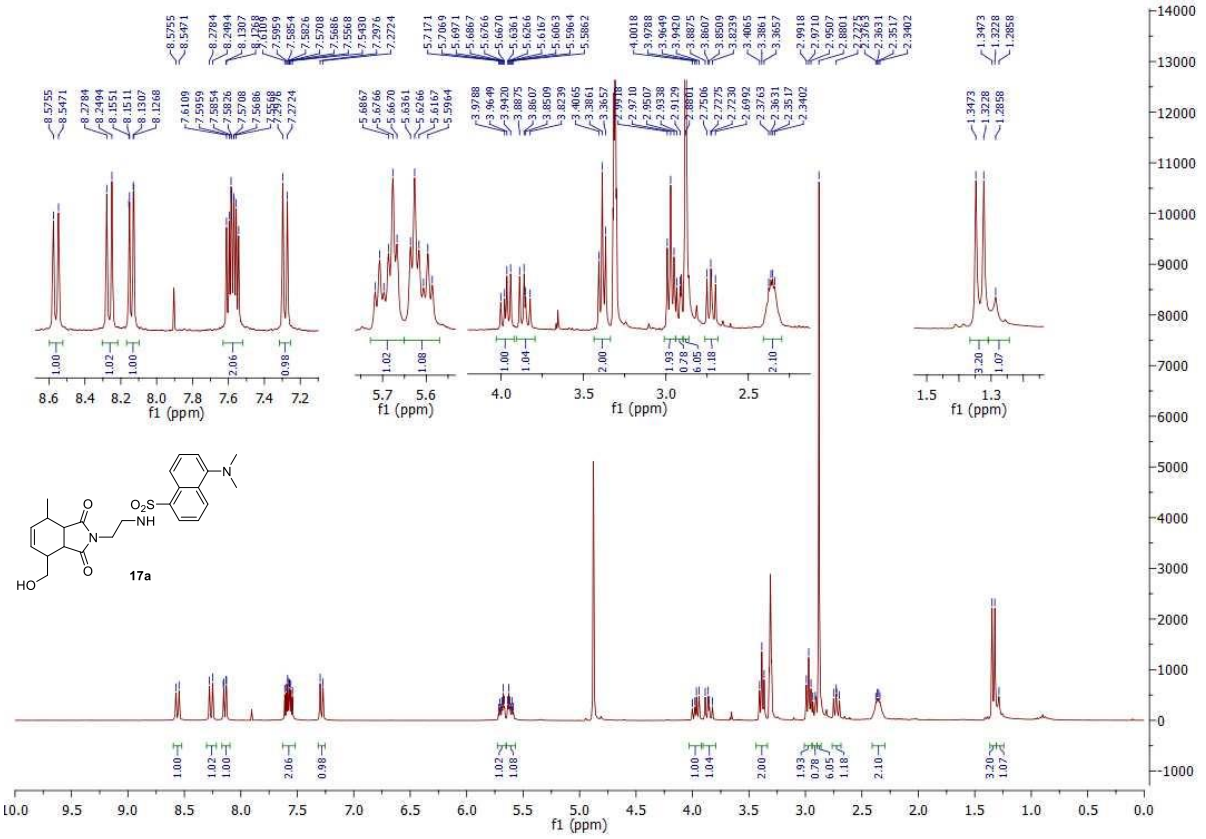
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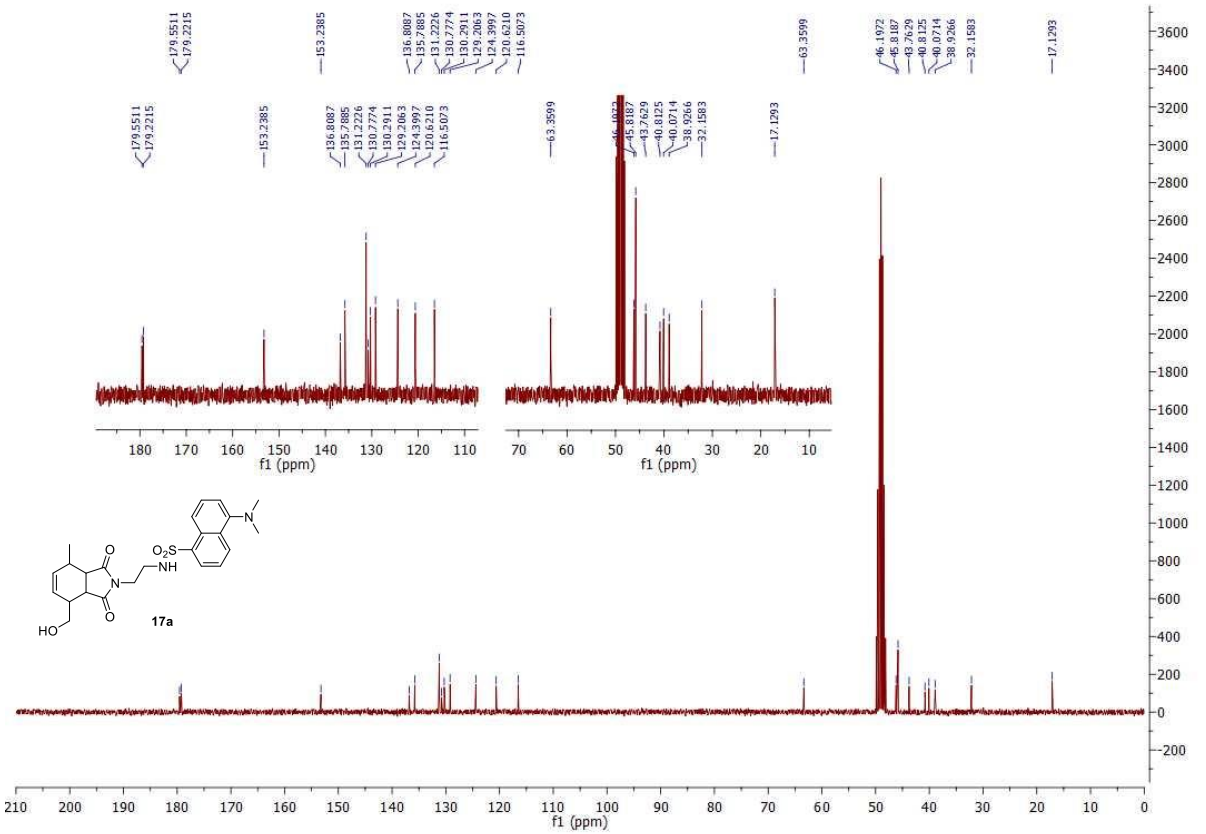
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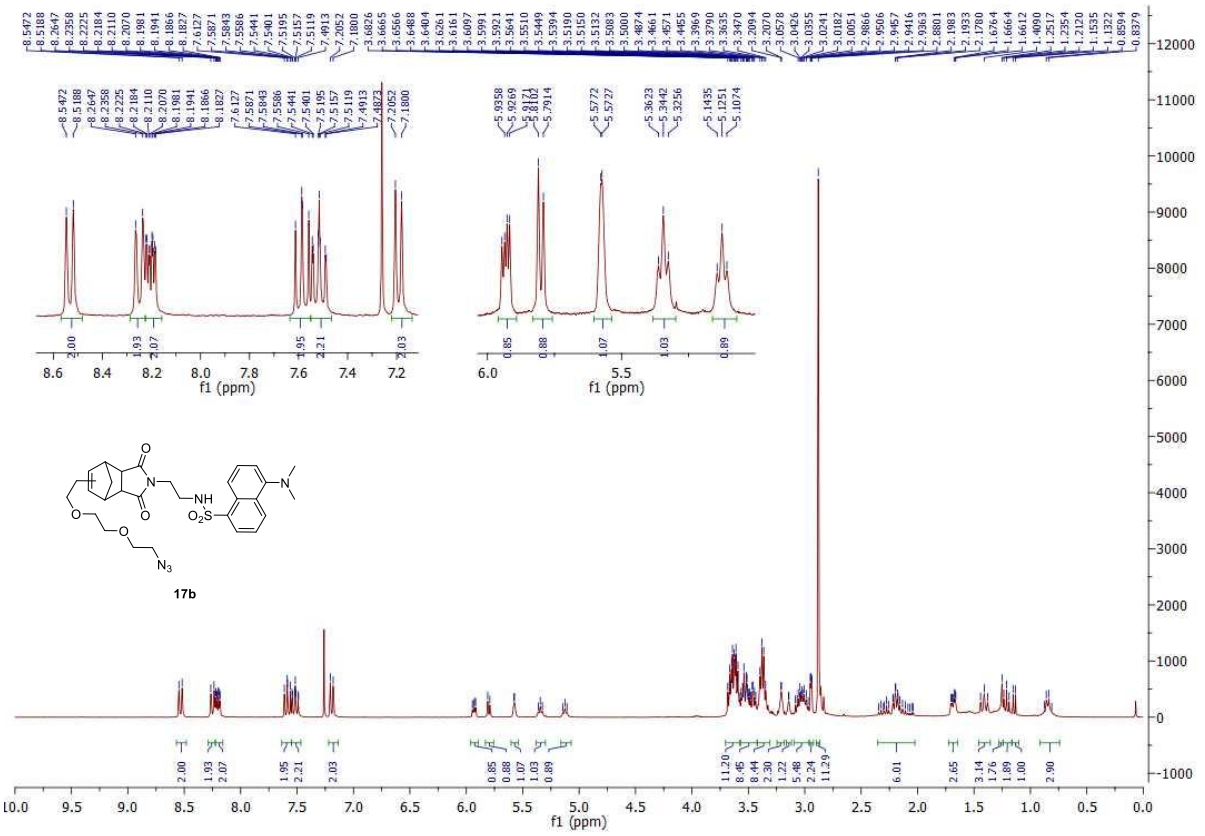
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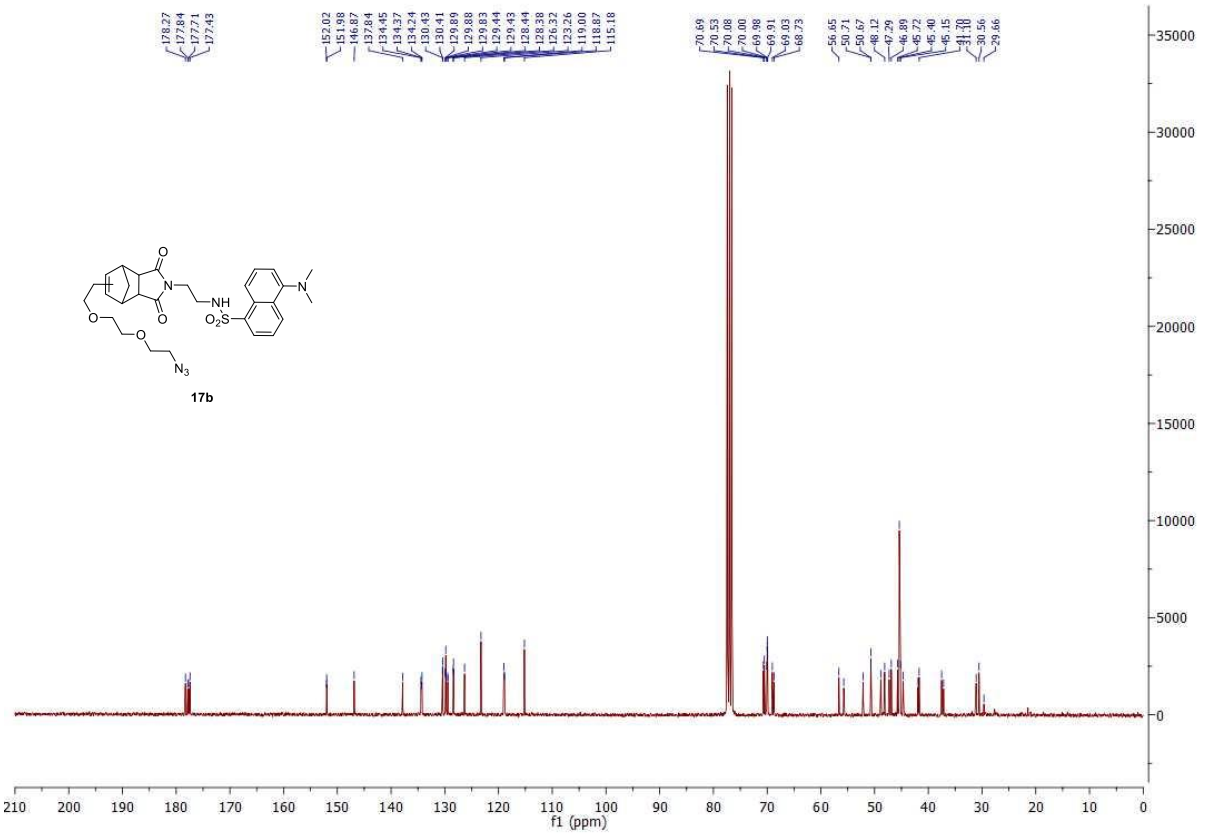
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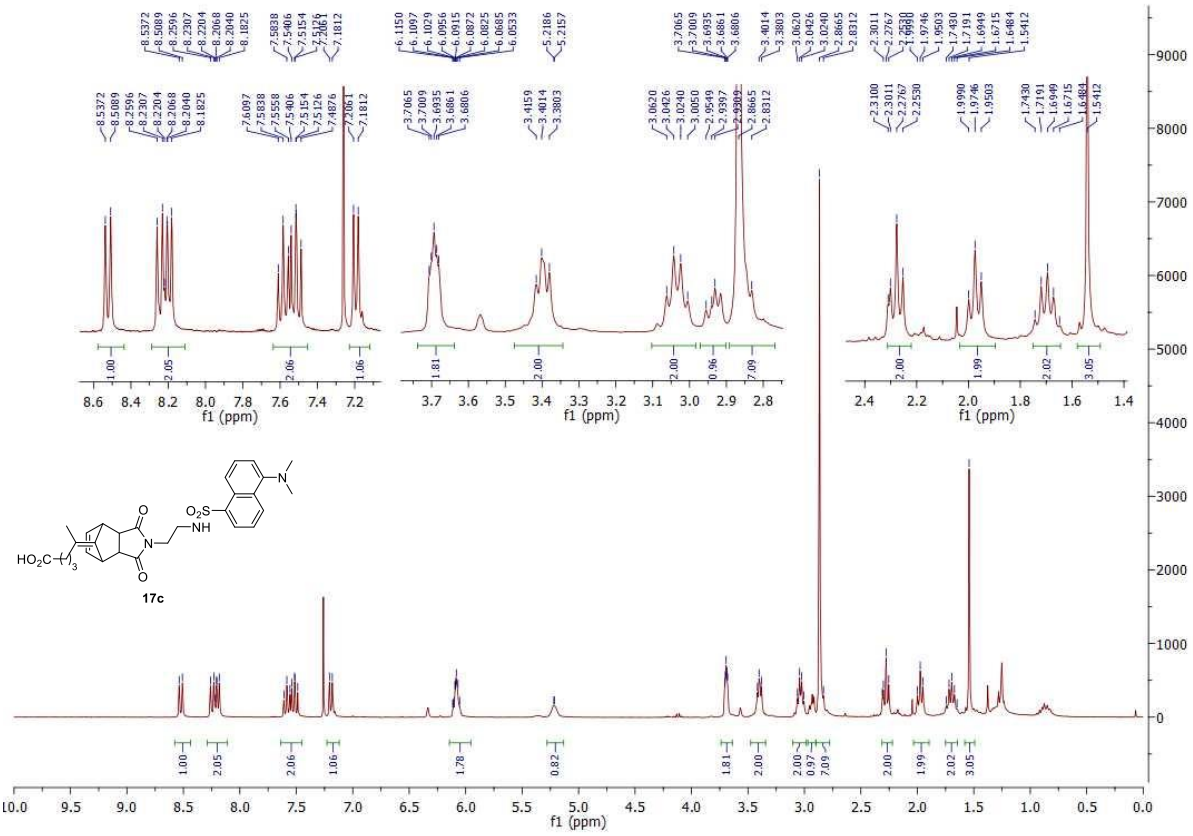
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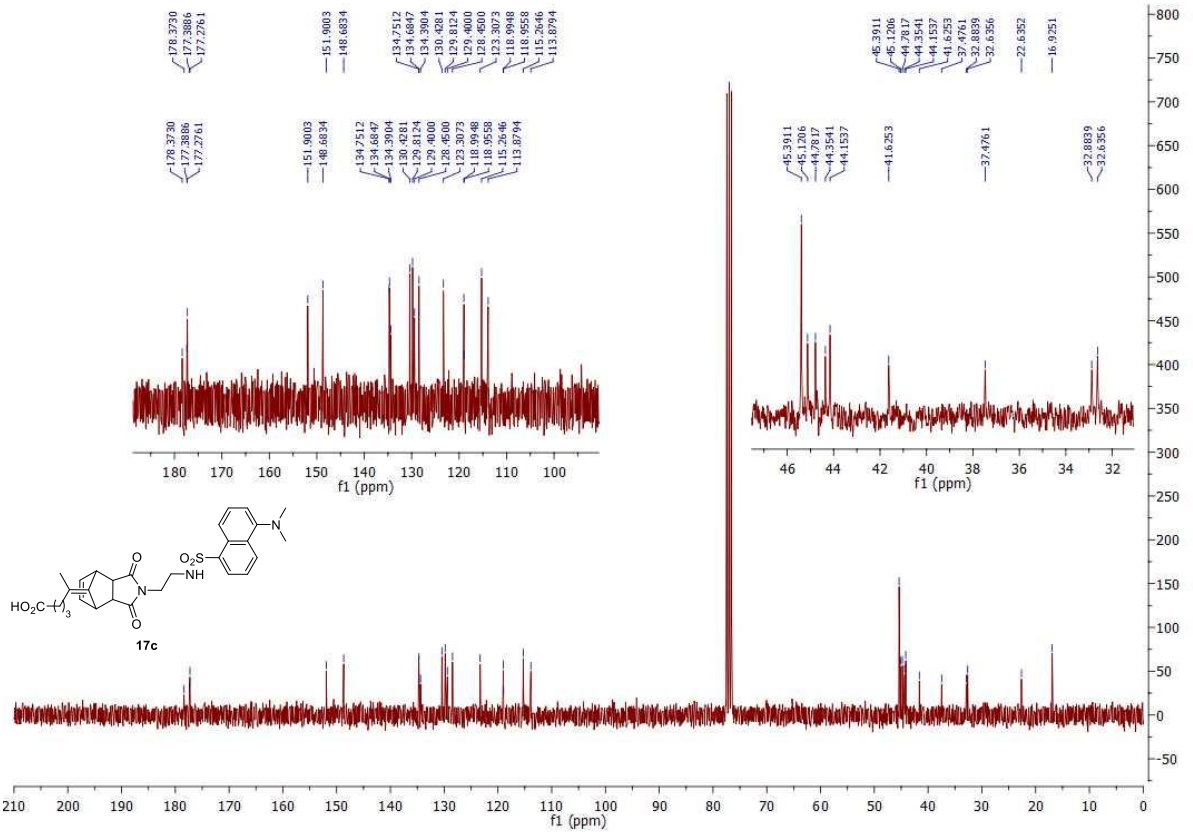
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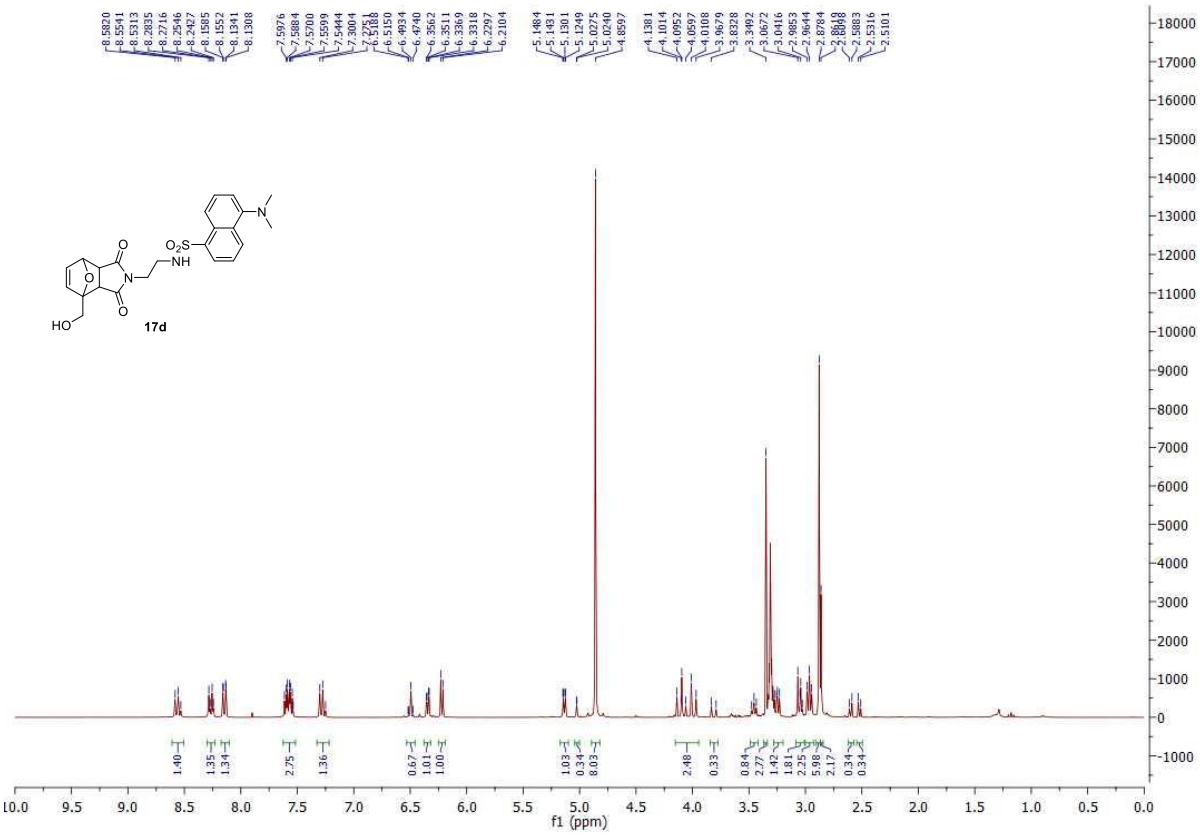
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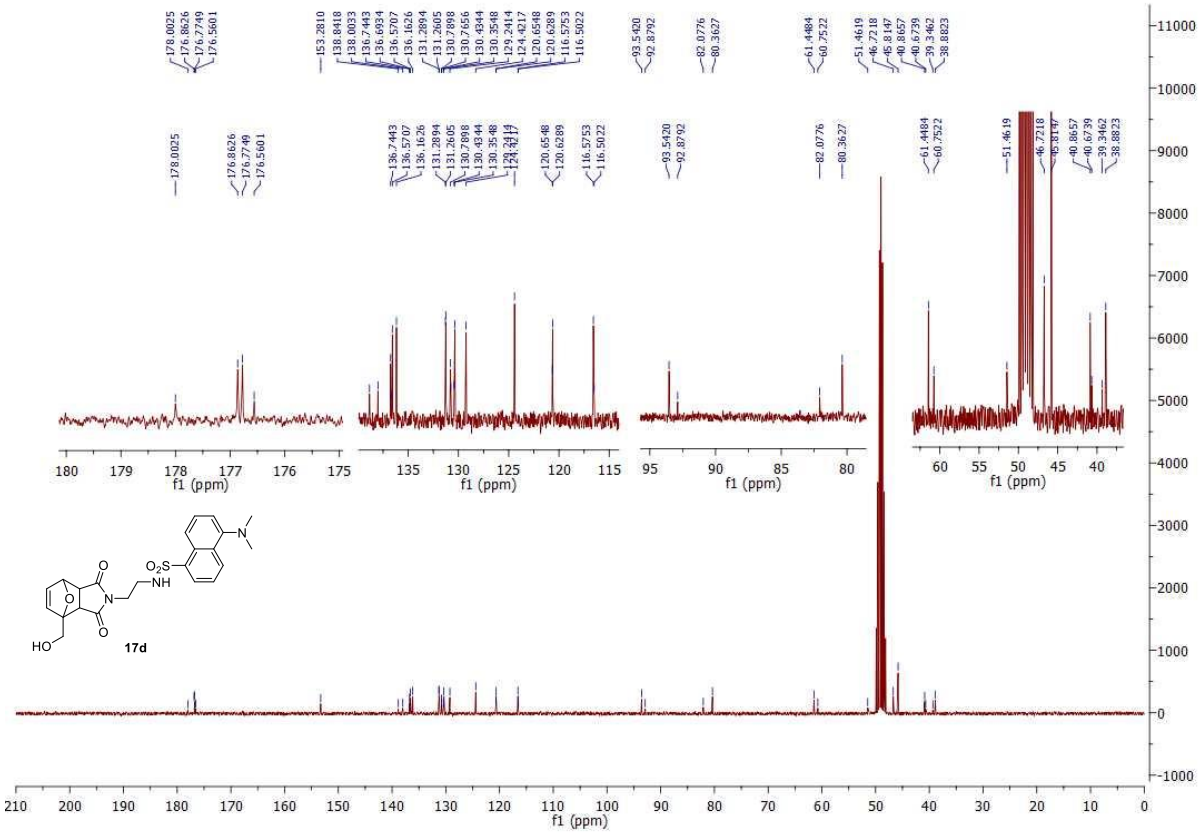
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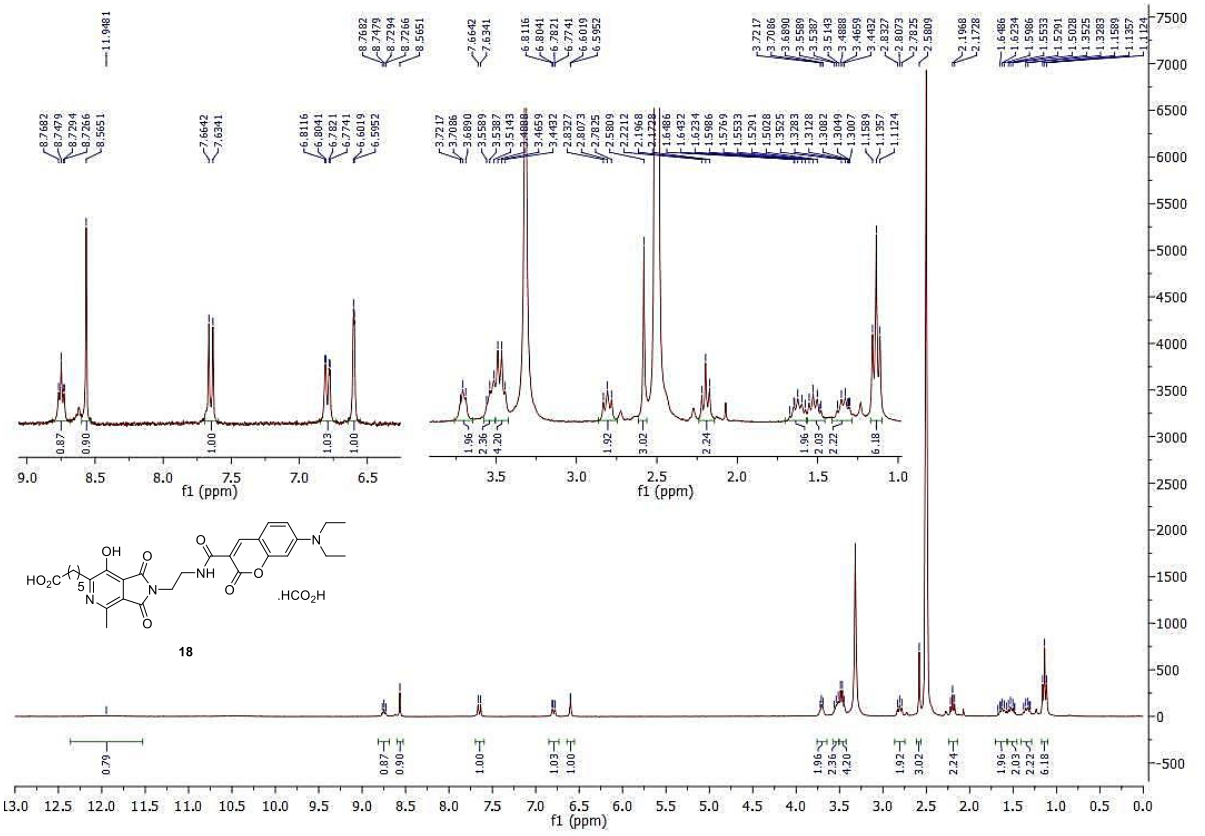
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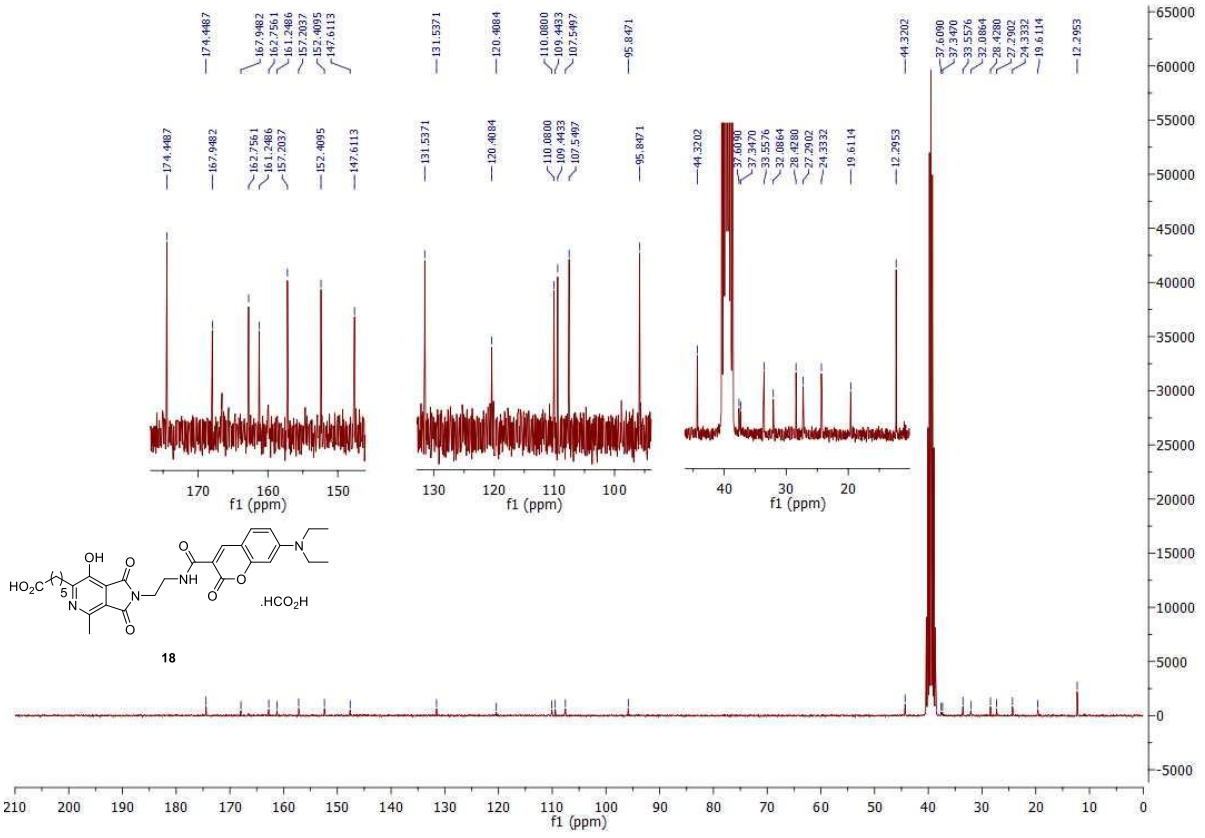
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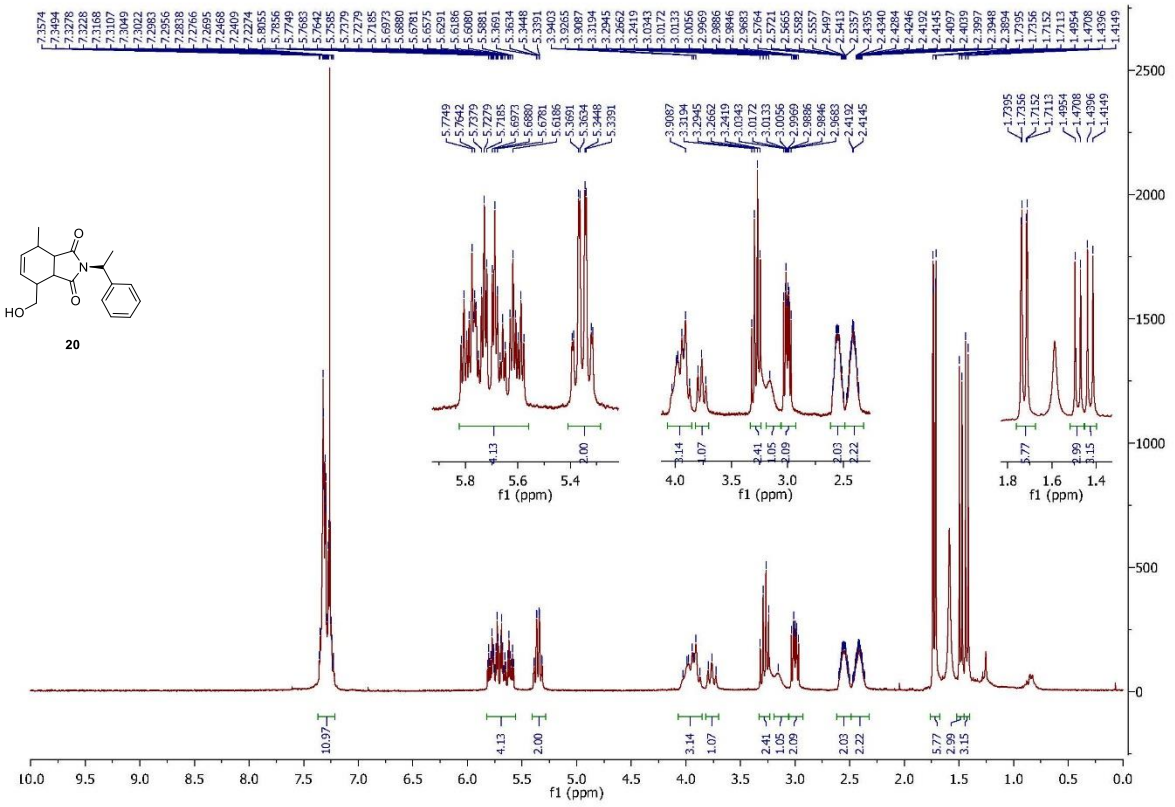
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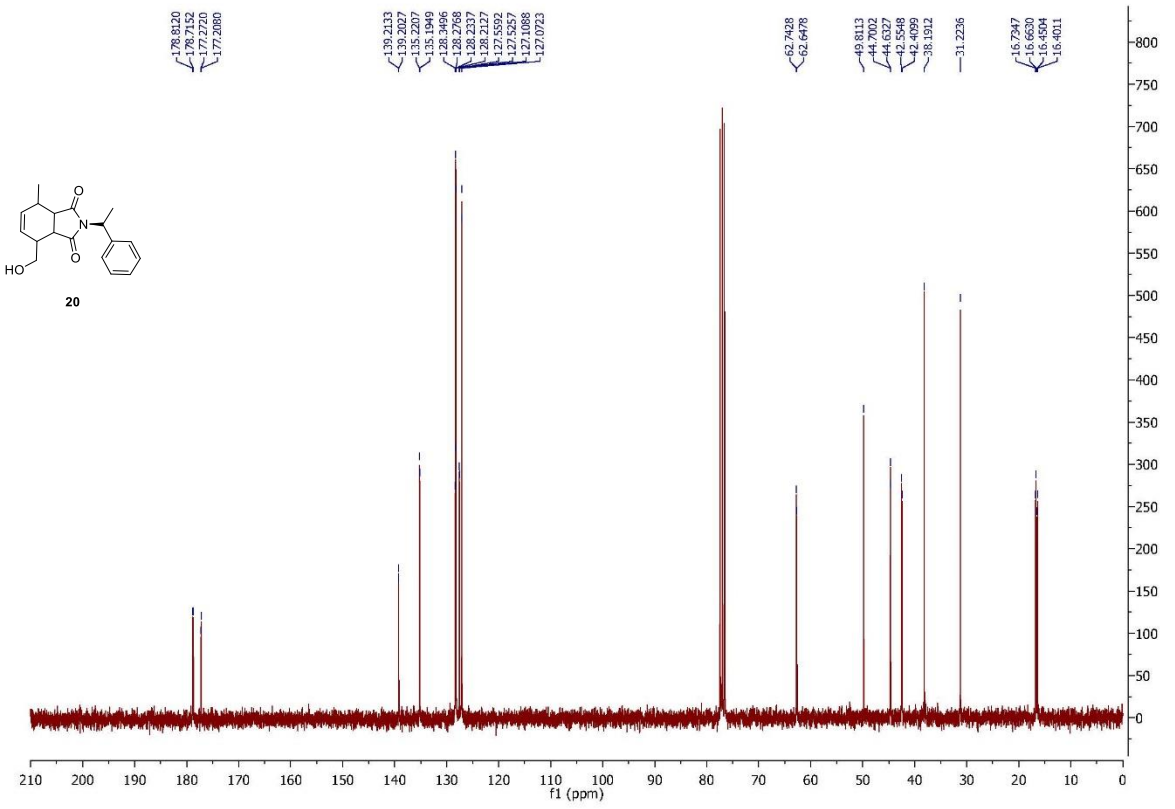
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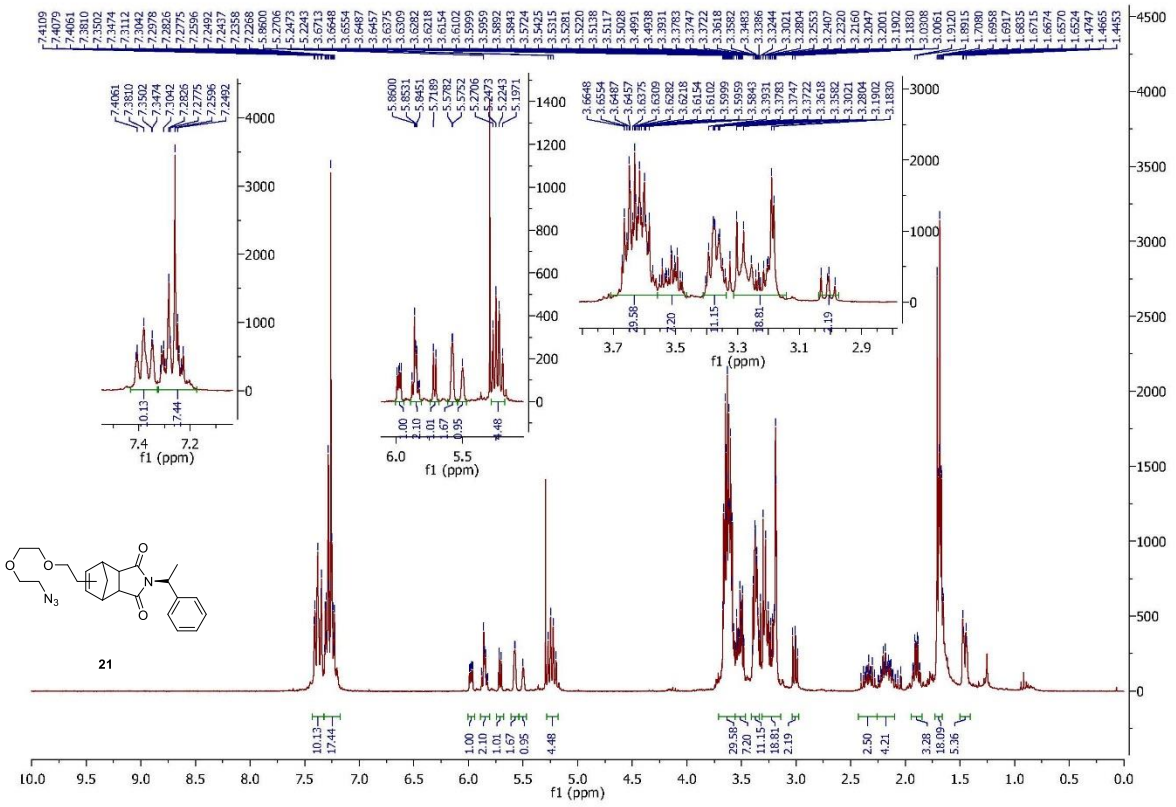
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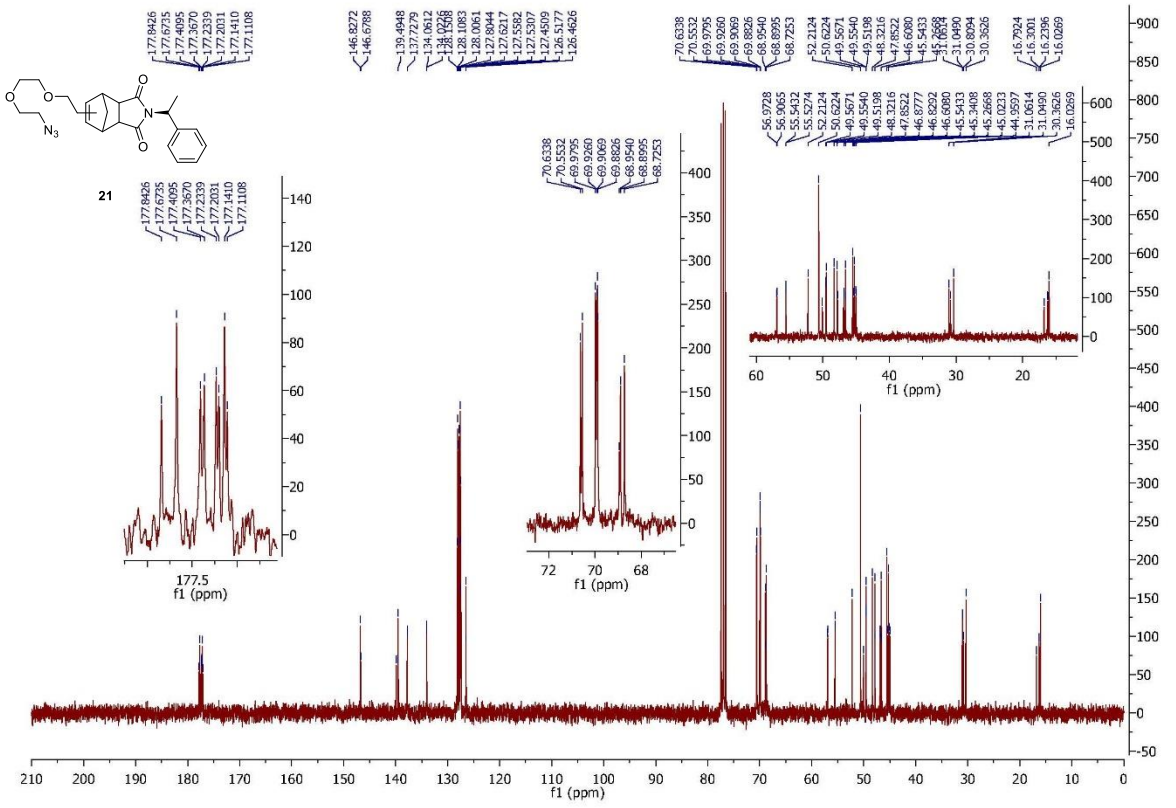
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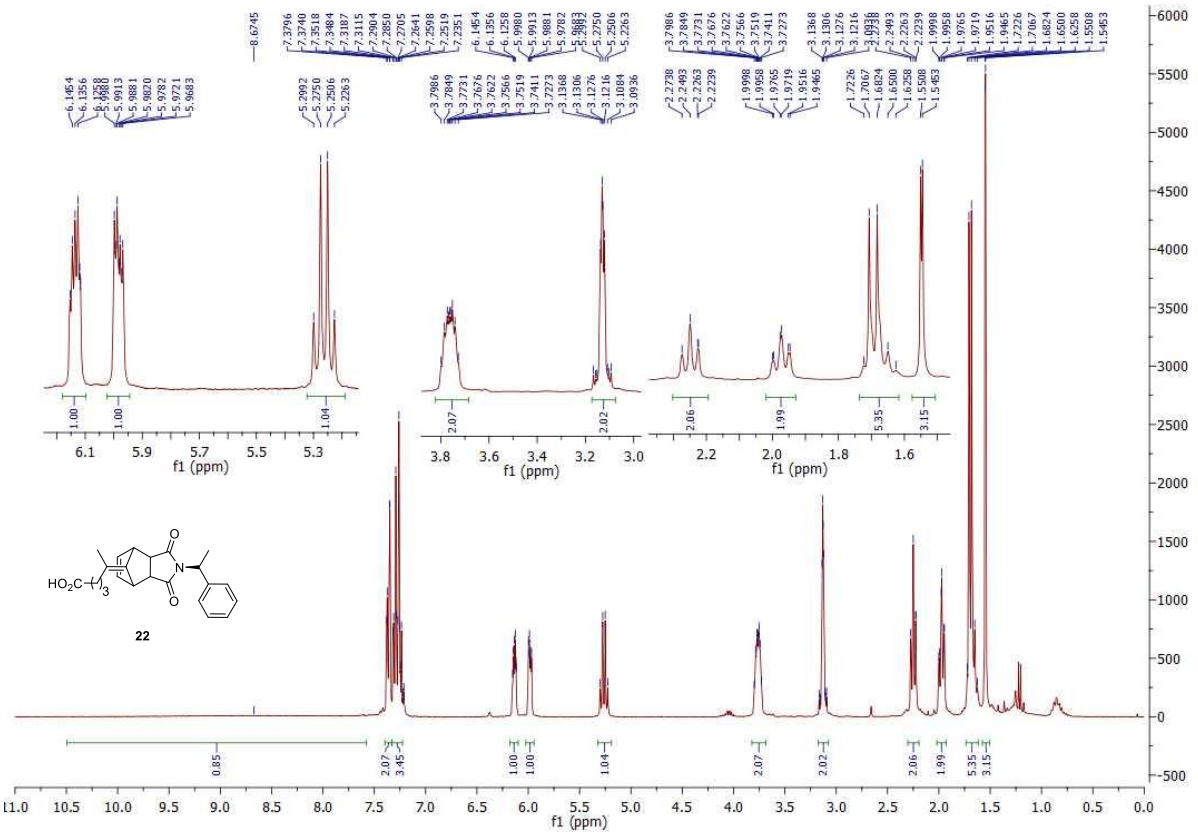
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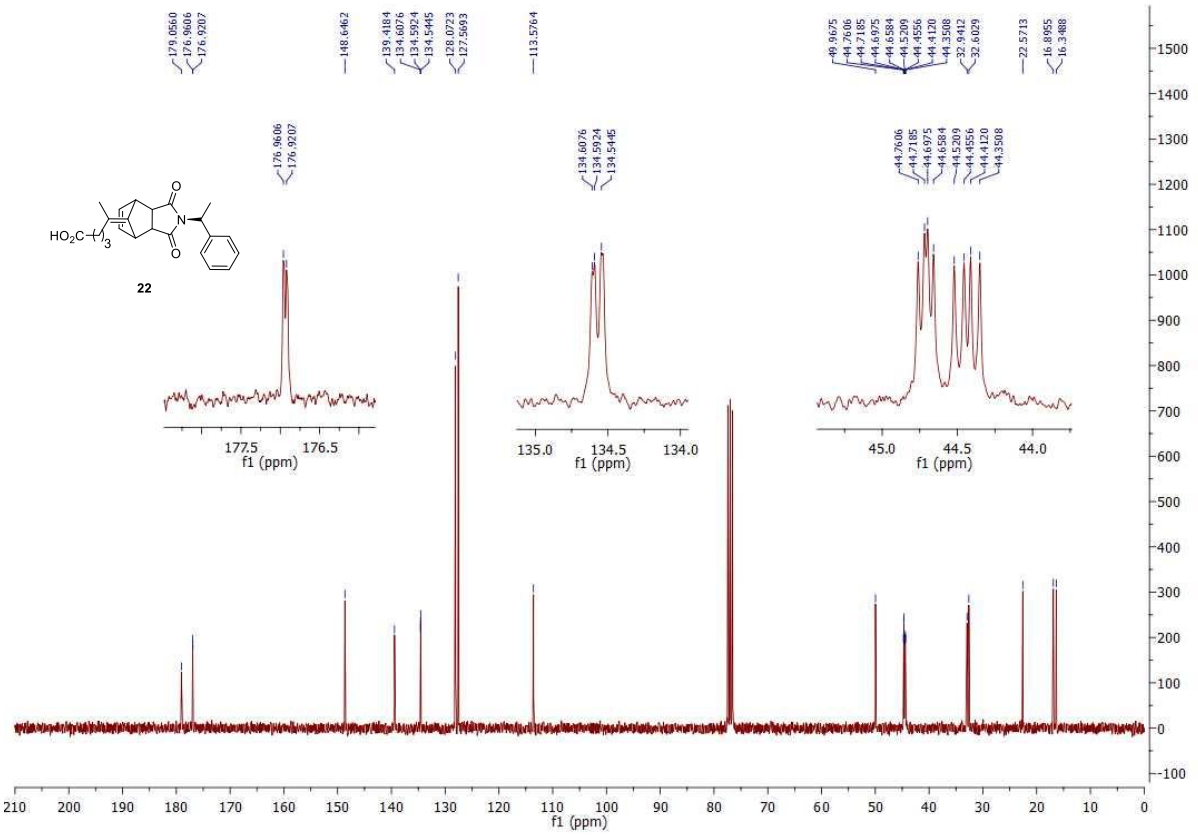
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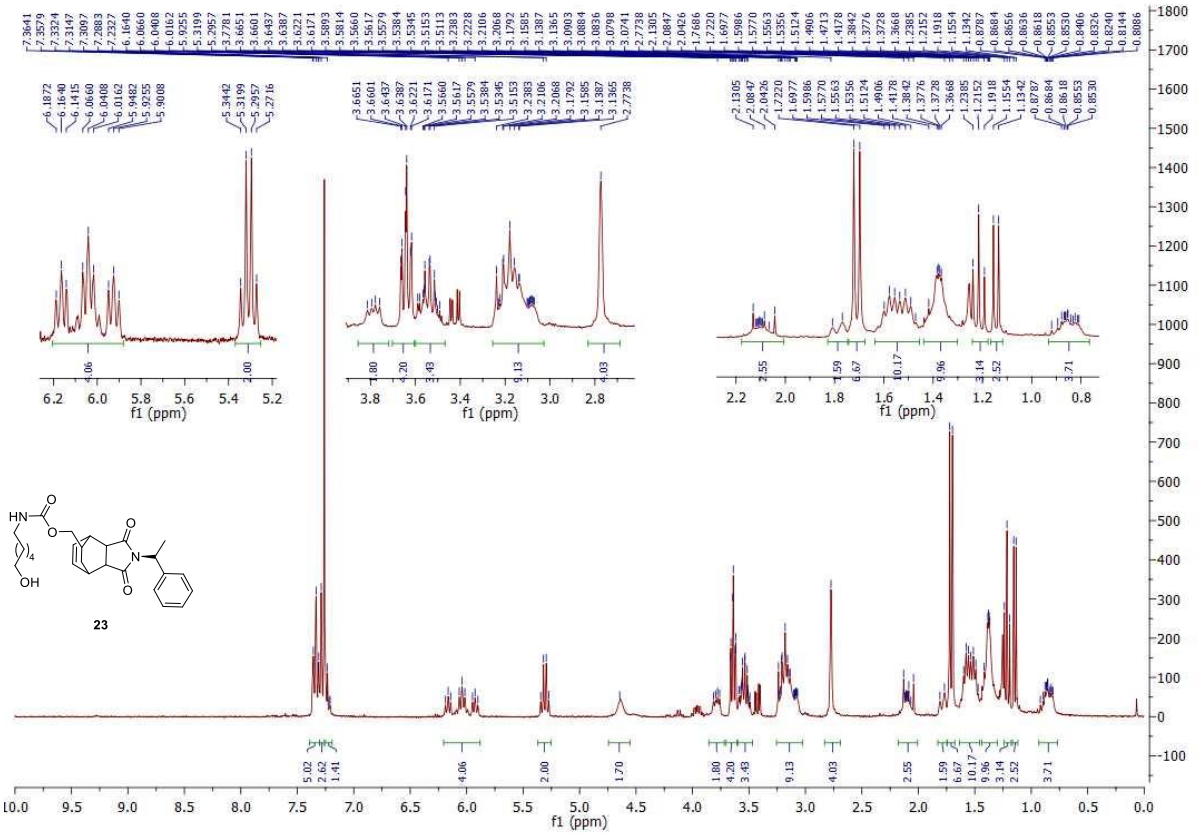
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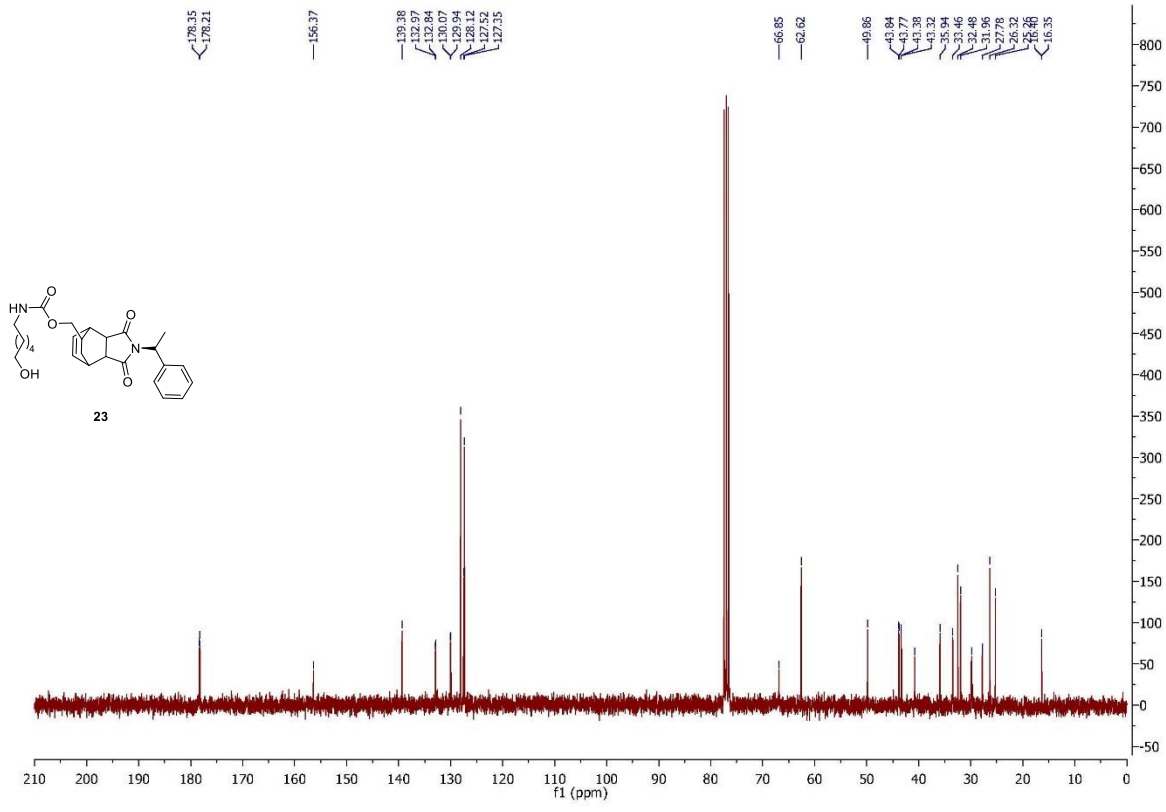
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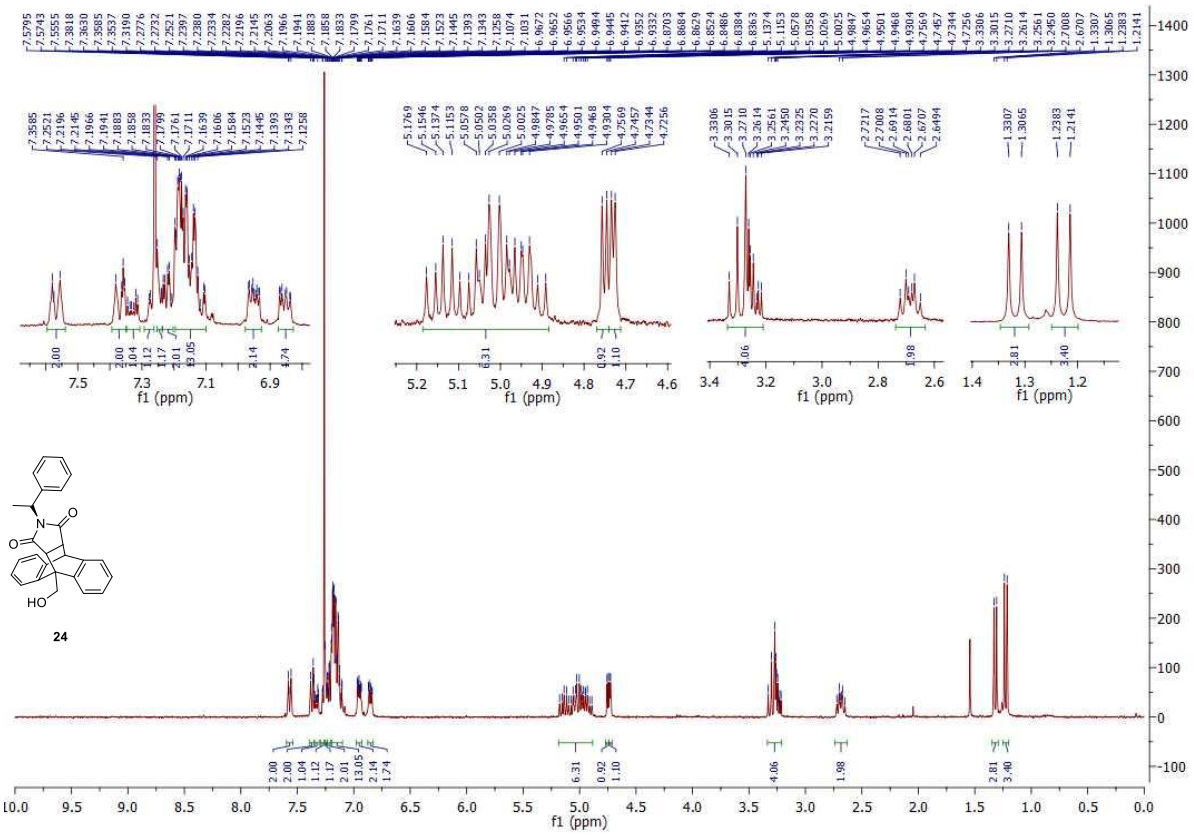
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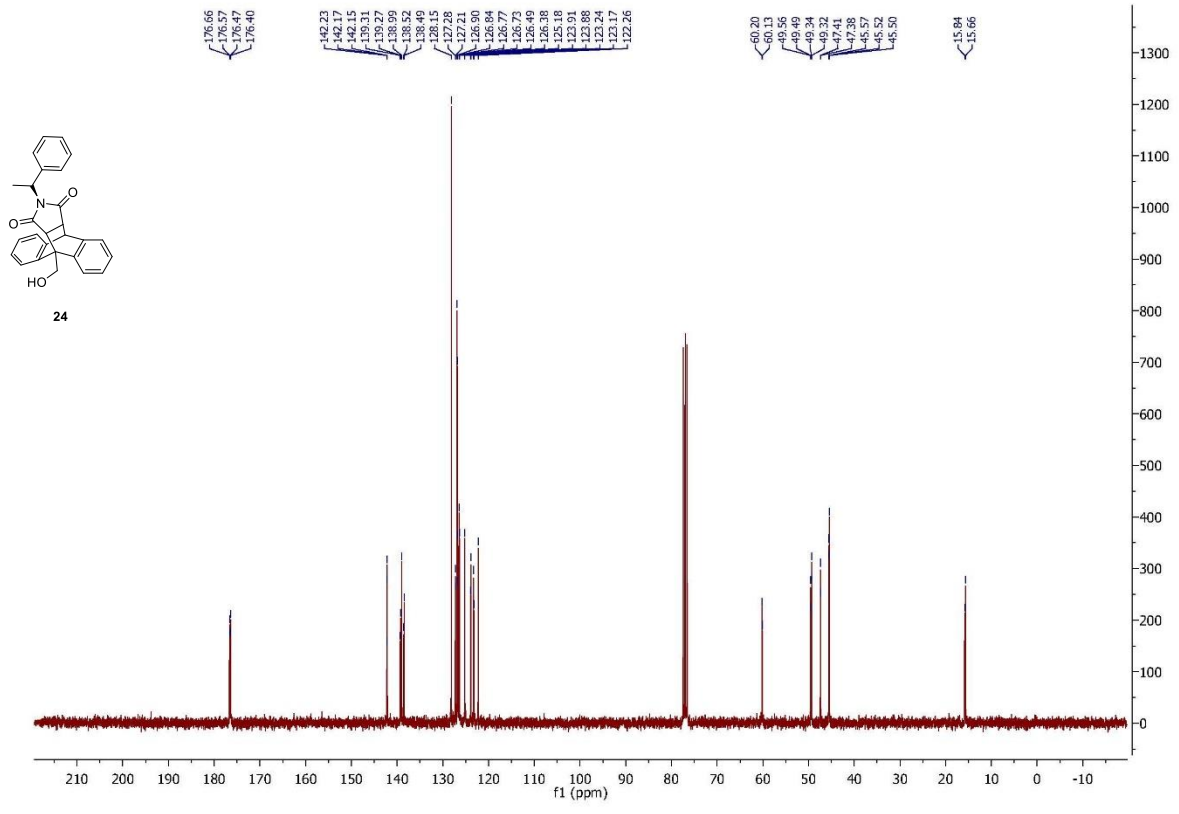
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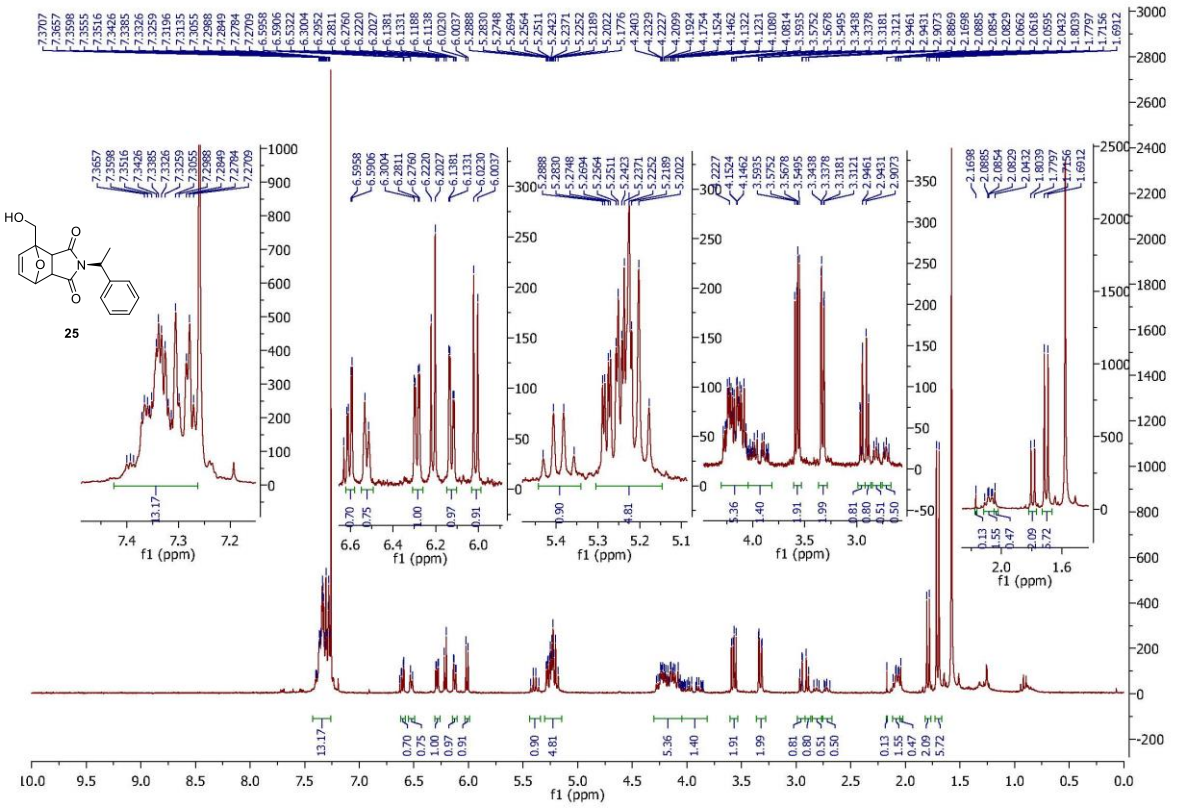
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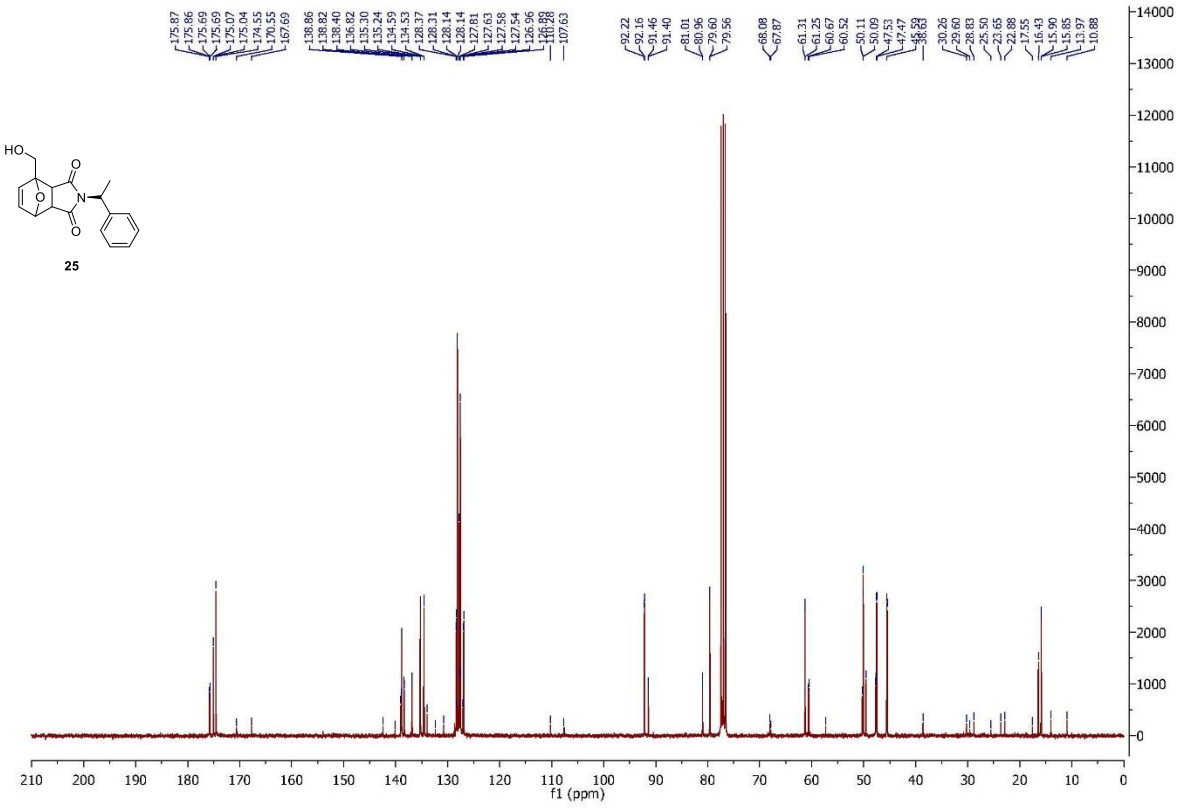
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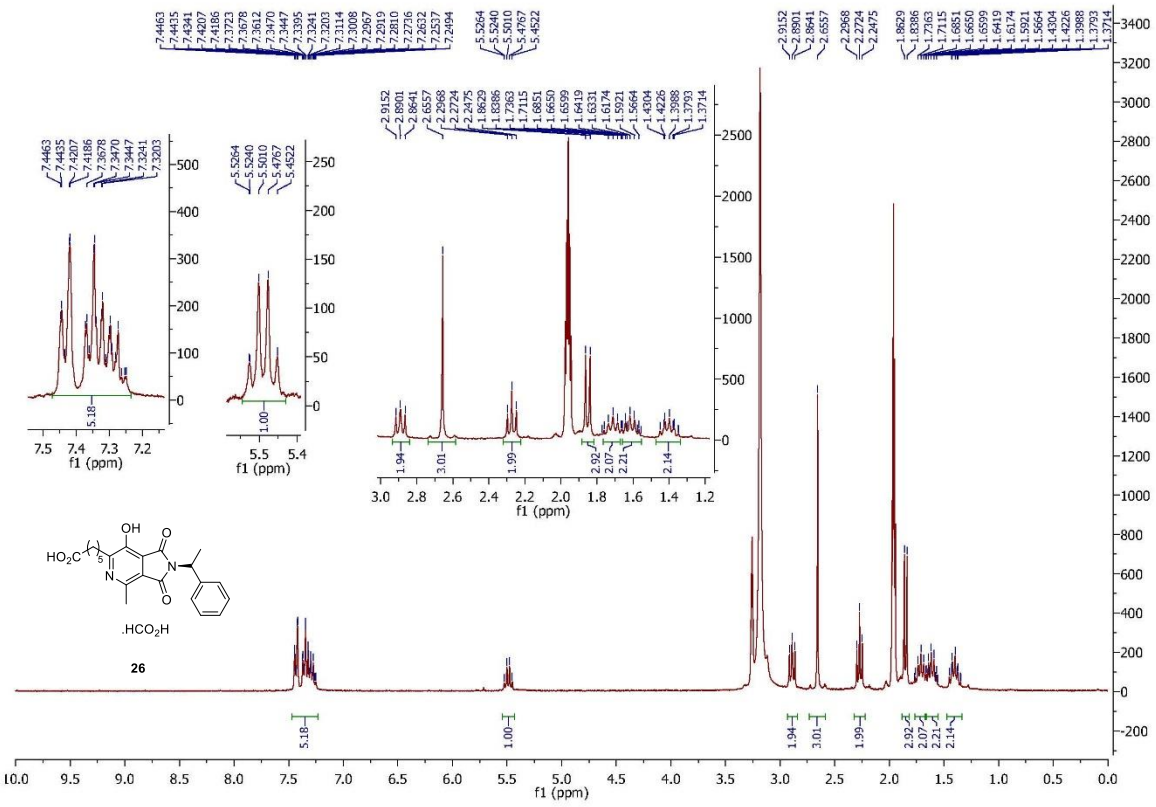
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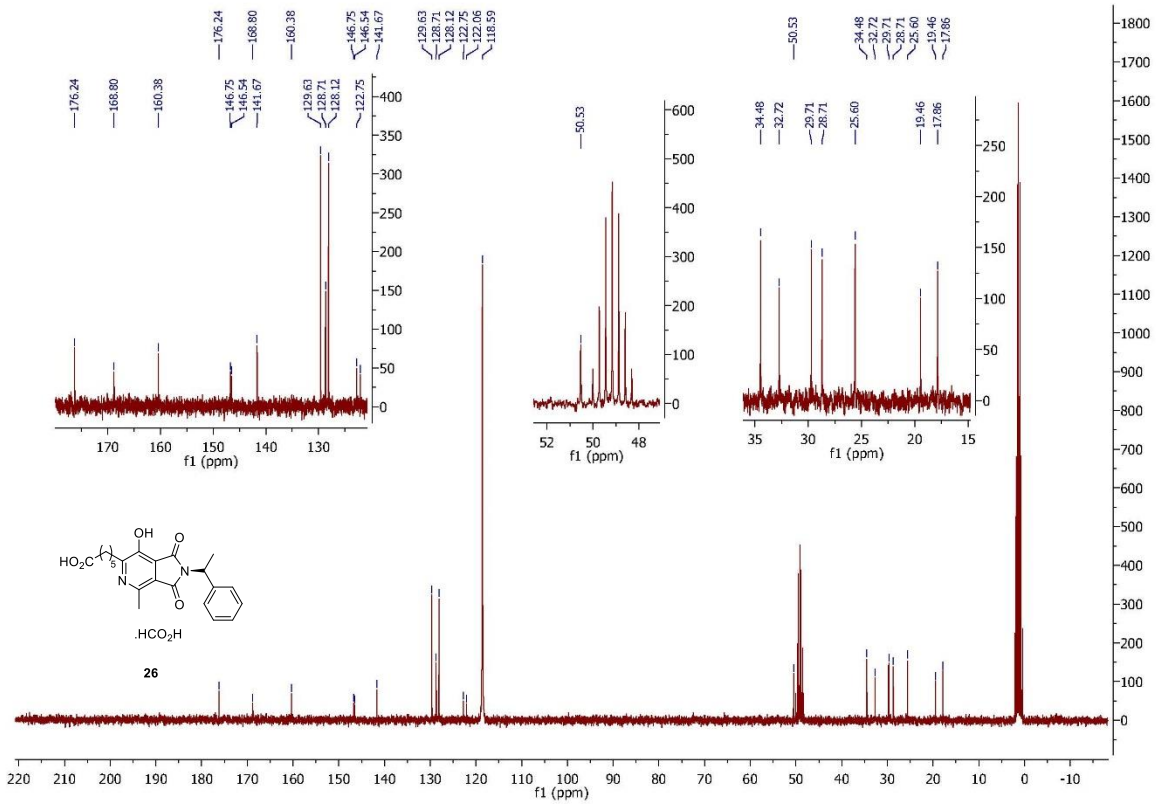
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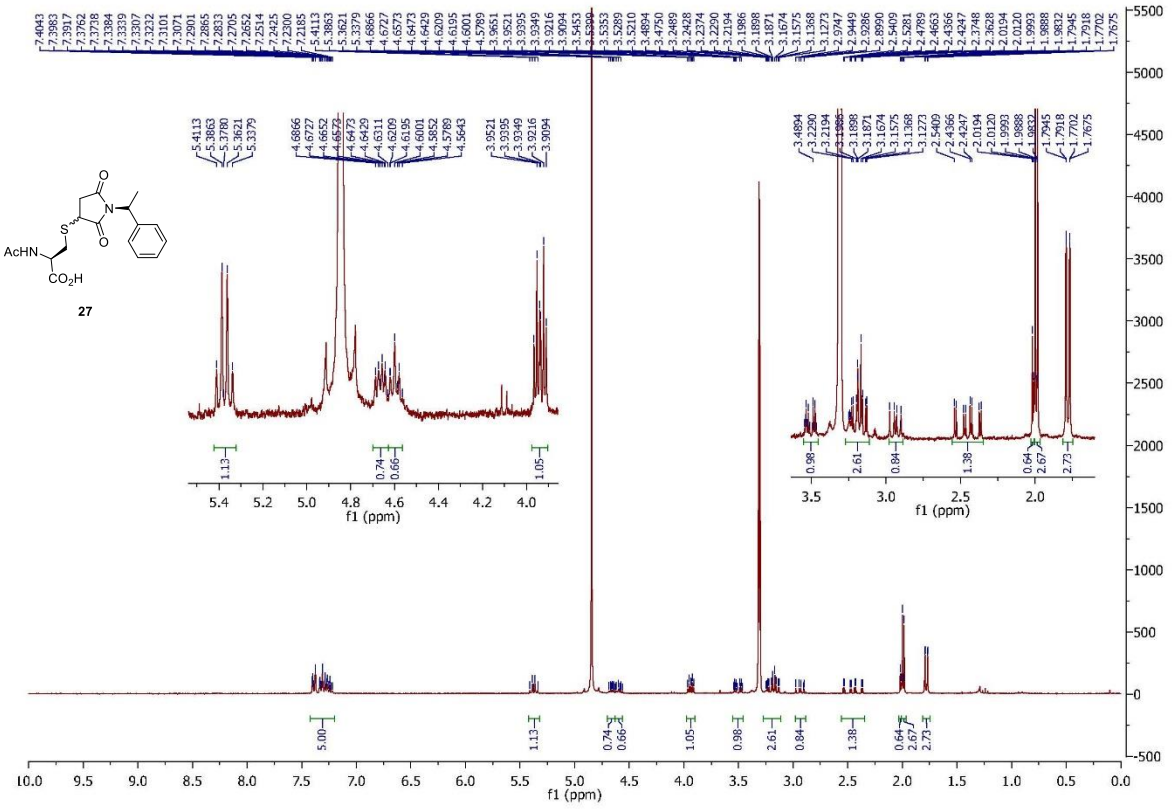
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CO2H
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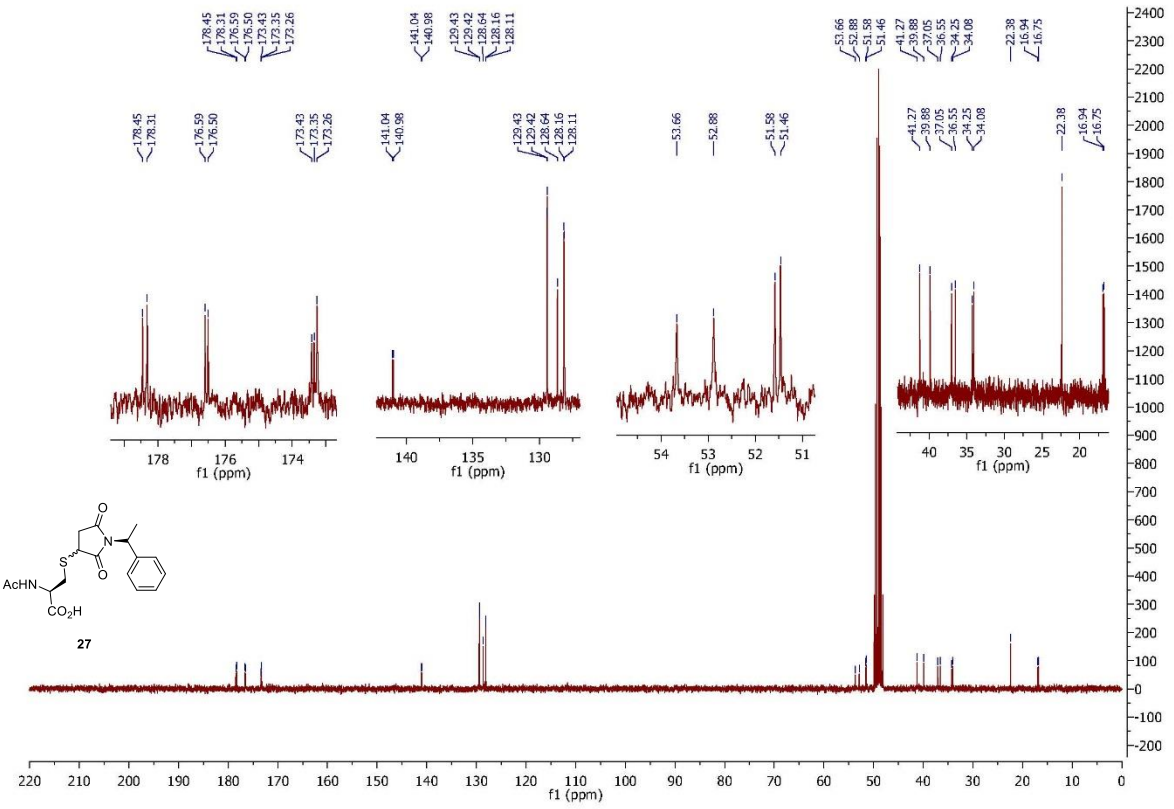
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CO2H
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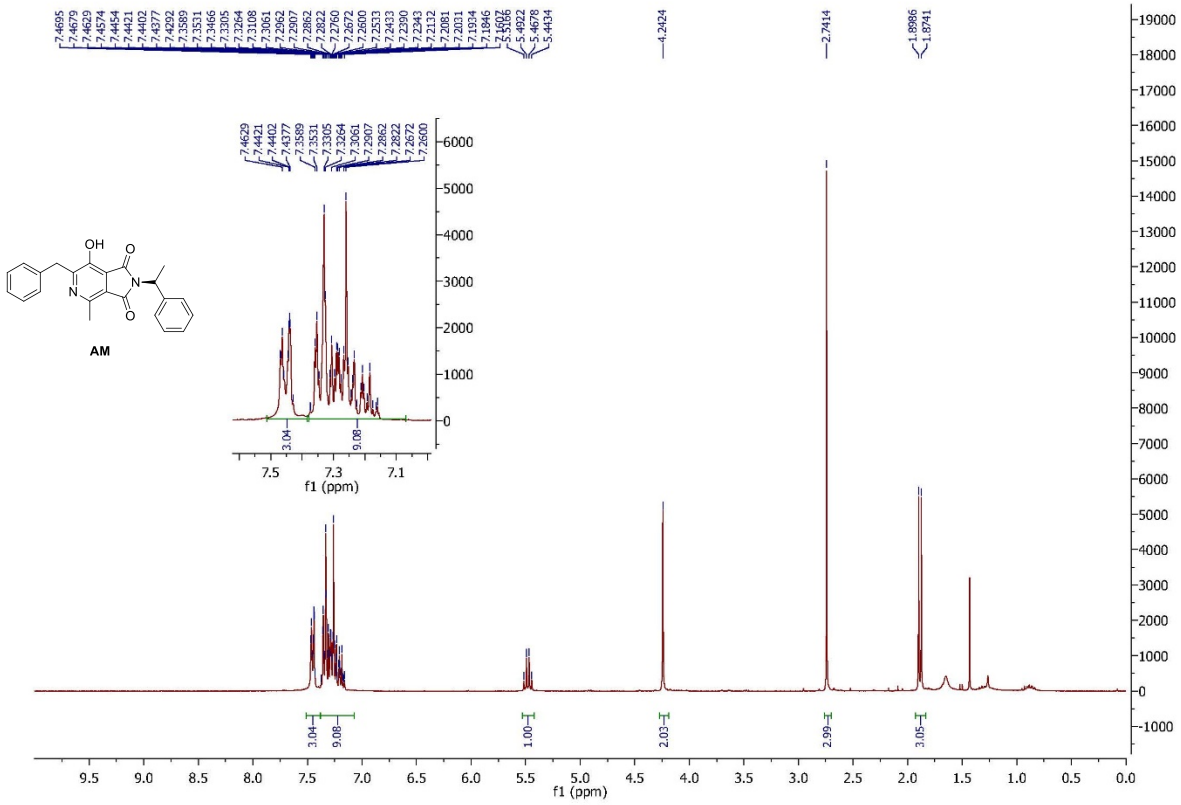
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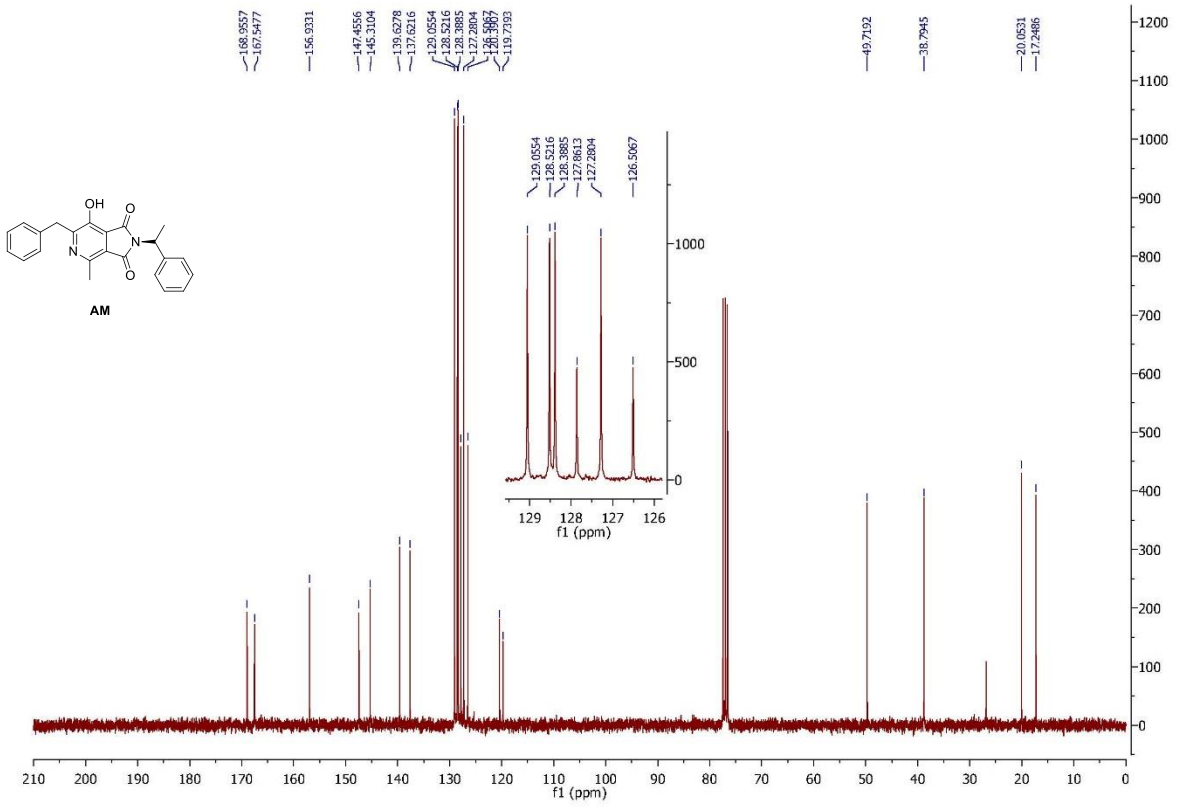
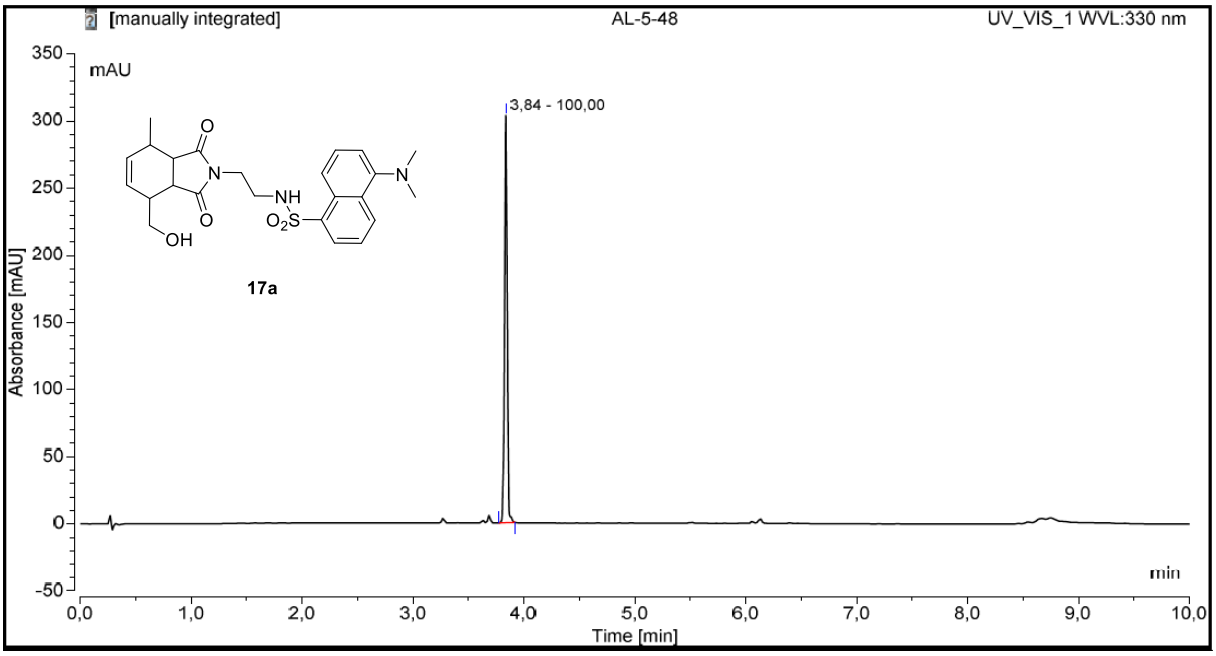

AM
f1 (ppm)

I.2. RP-HPLC chromatograms

HPLC analyses were performed according to described procedure in Materials and Methods, at λ = 254 and 330 nm. Compounds were dissolved in MeCN (300 - 400 µL) and then with milliQ H_2O (700 - 600 µL). Furfuryl alcohol was not visible by RP-HPLC. For every analyses, RP-HPLC system (A or B) are specified.

System B



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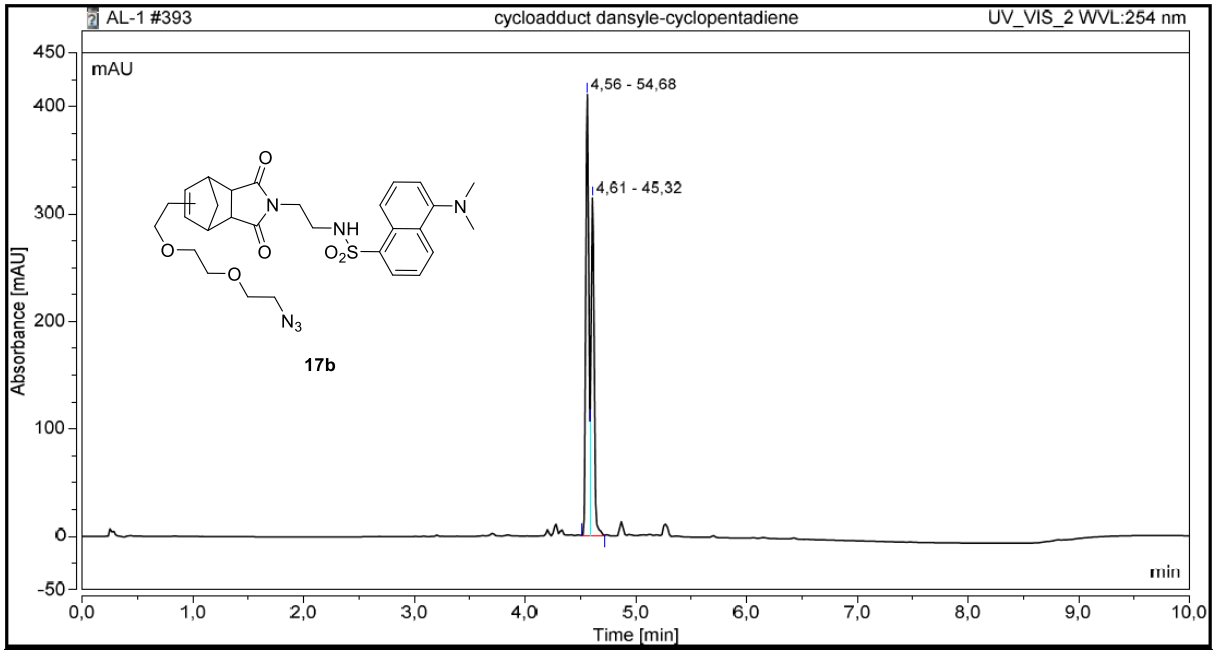

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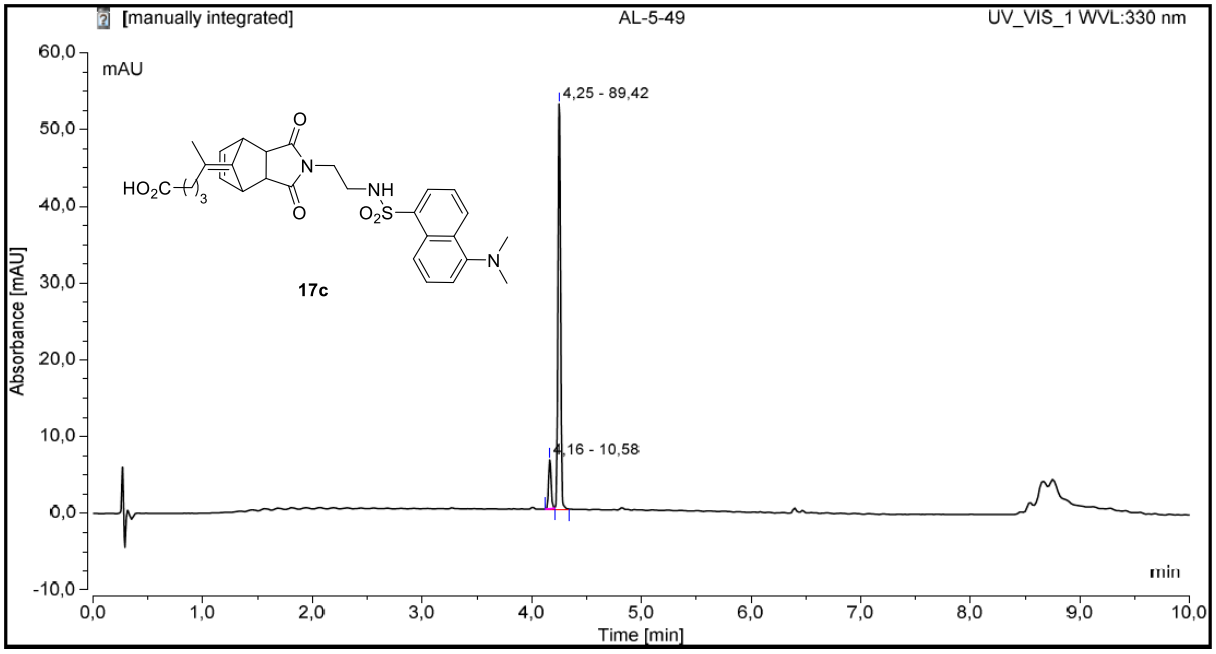

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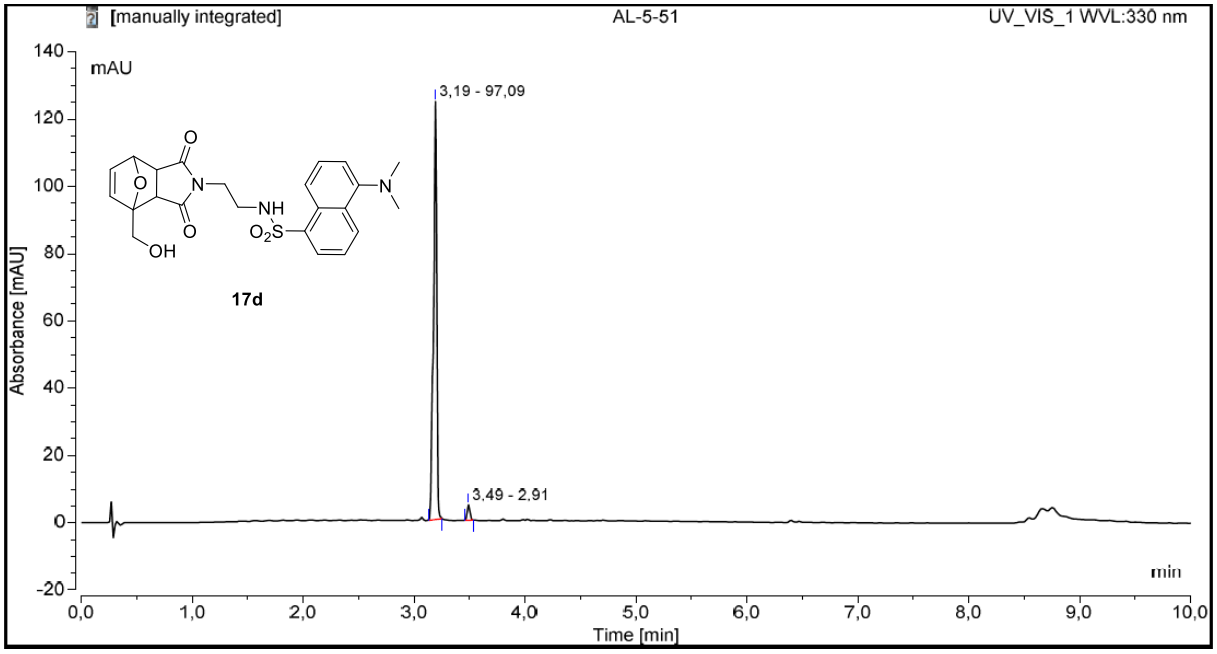

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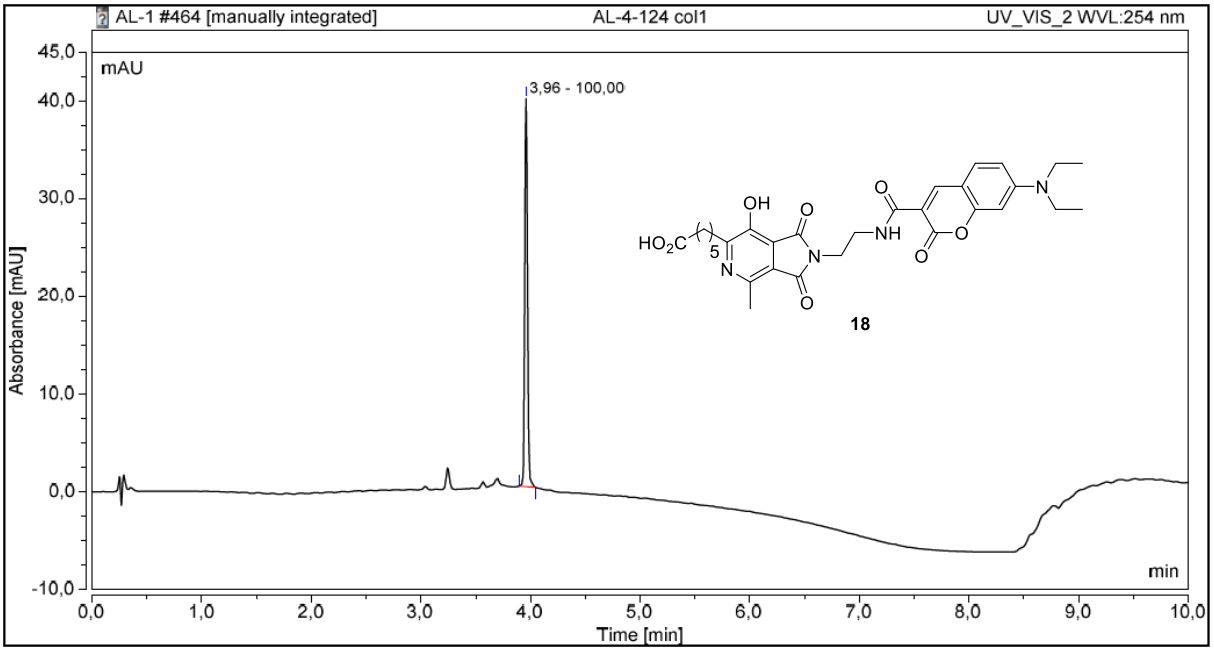

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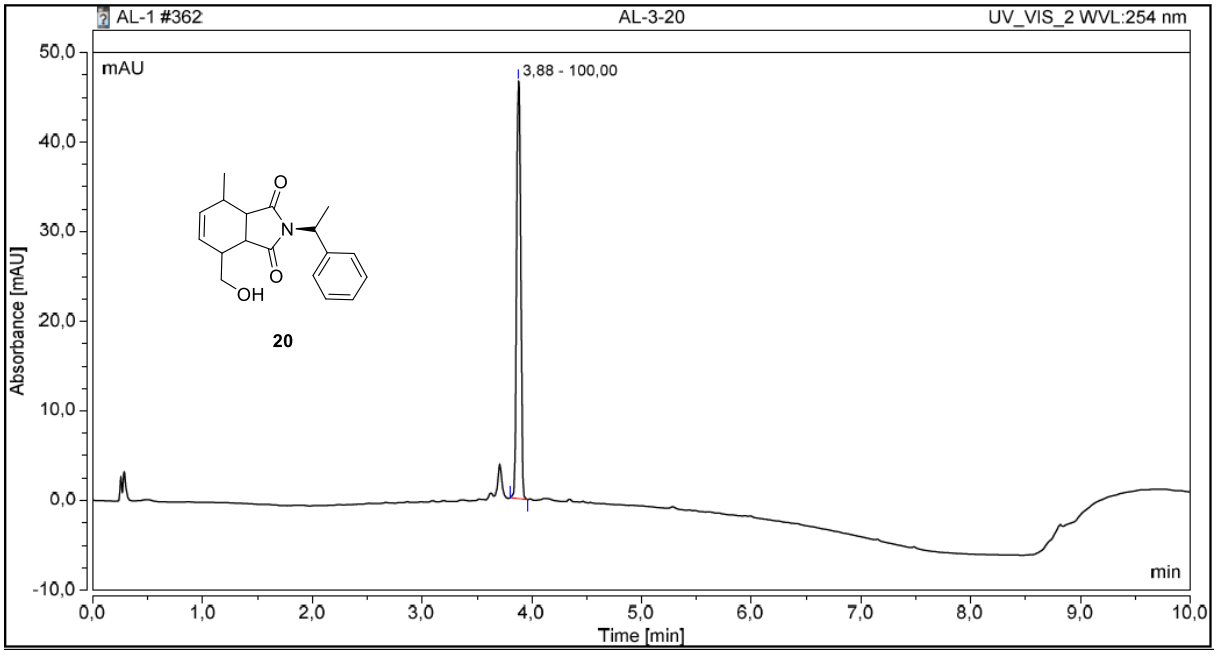
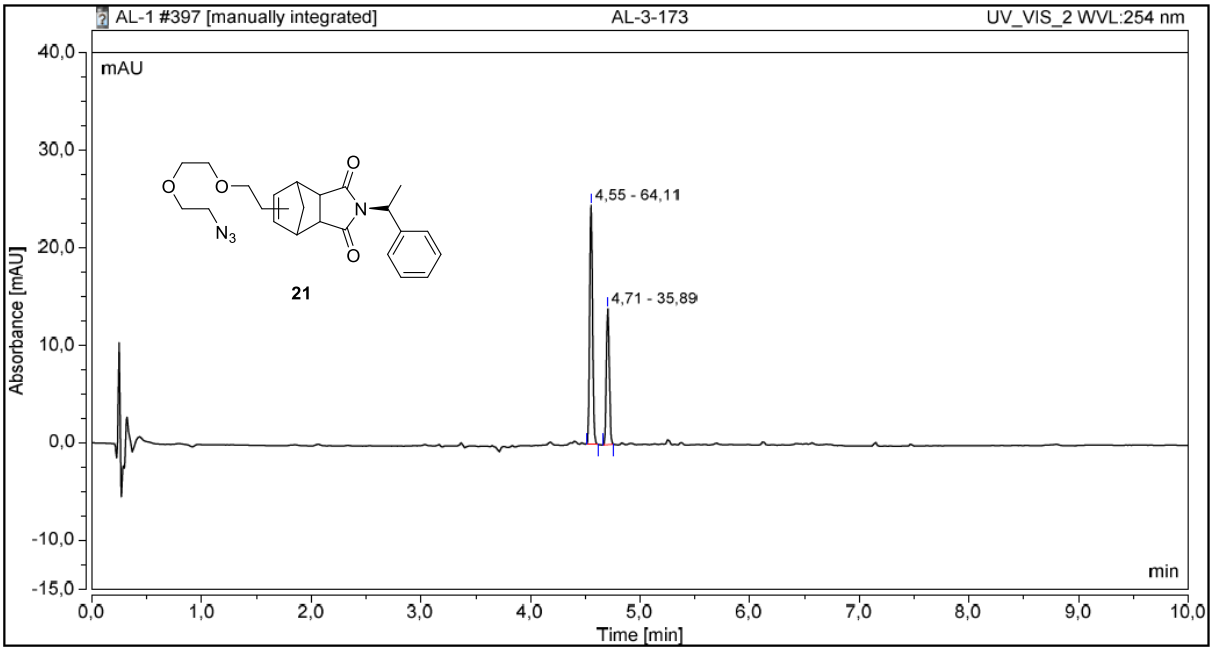
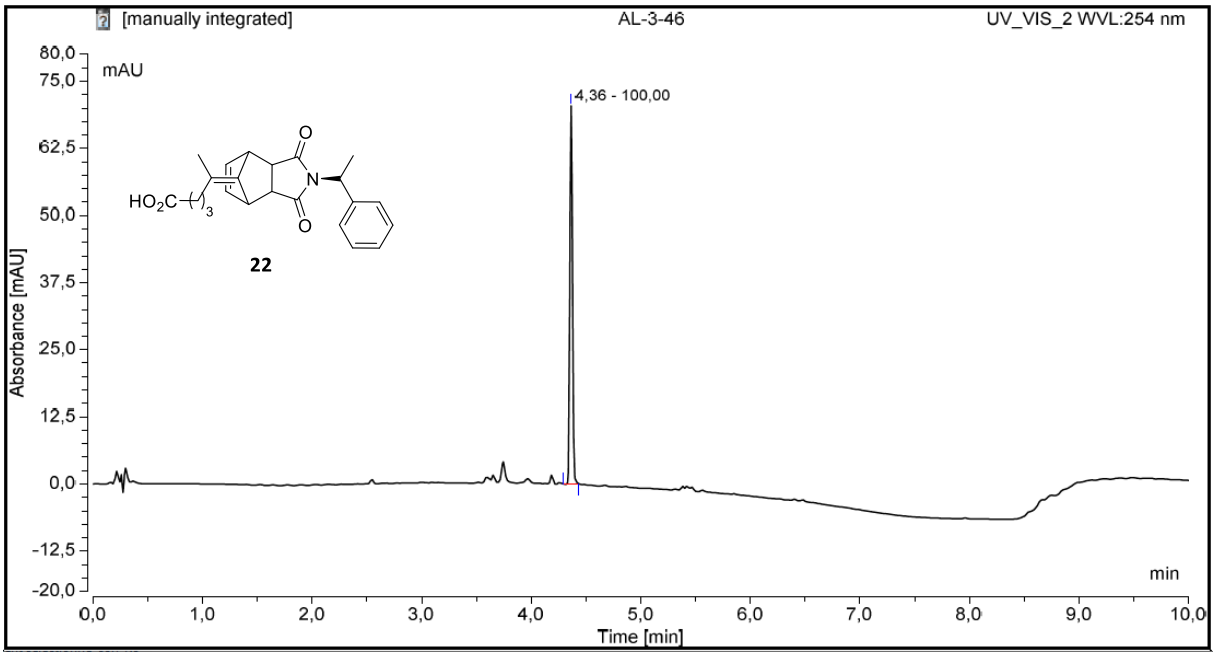


System A



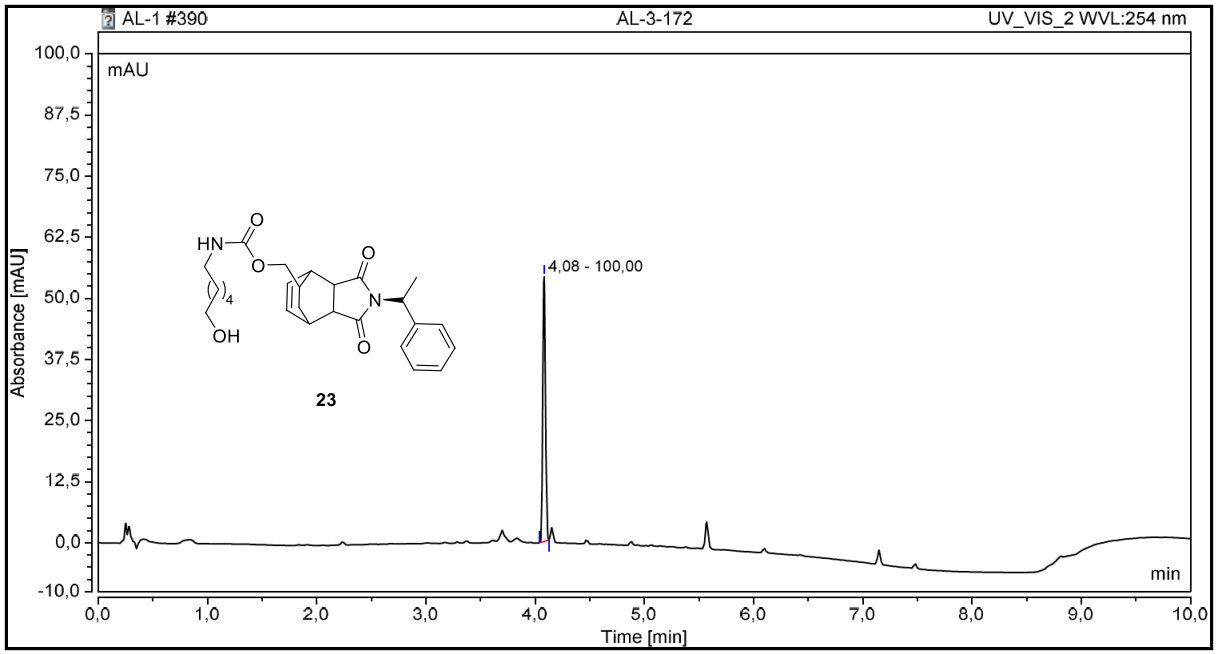
21

System B

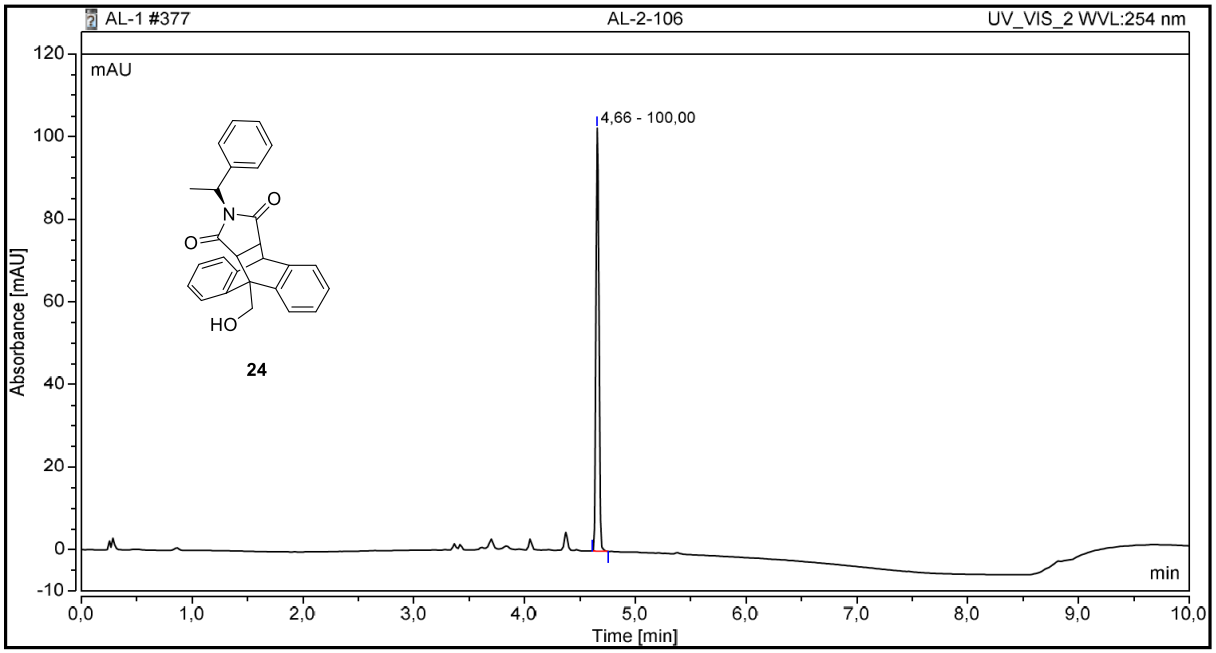


22

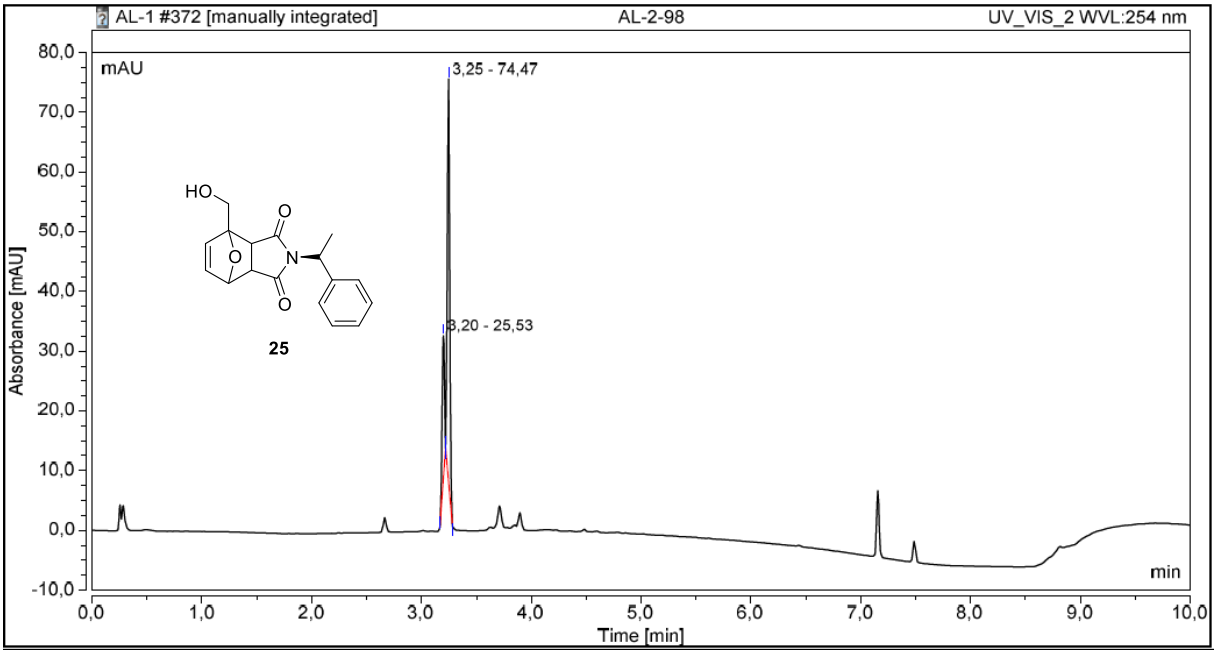
System A



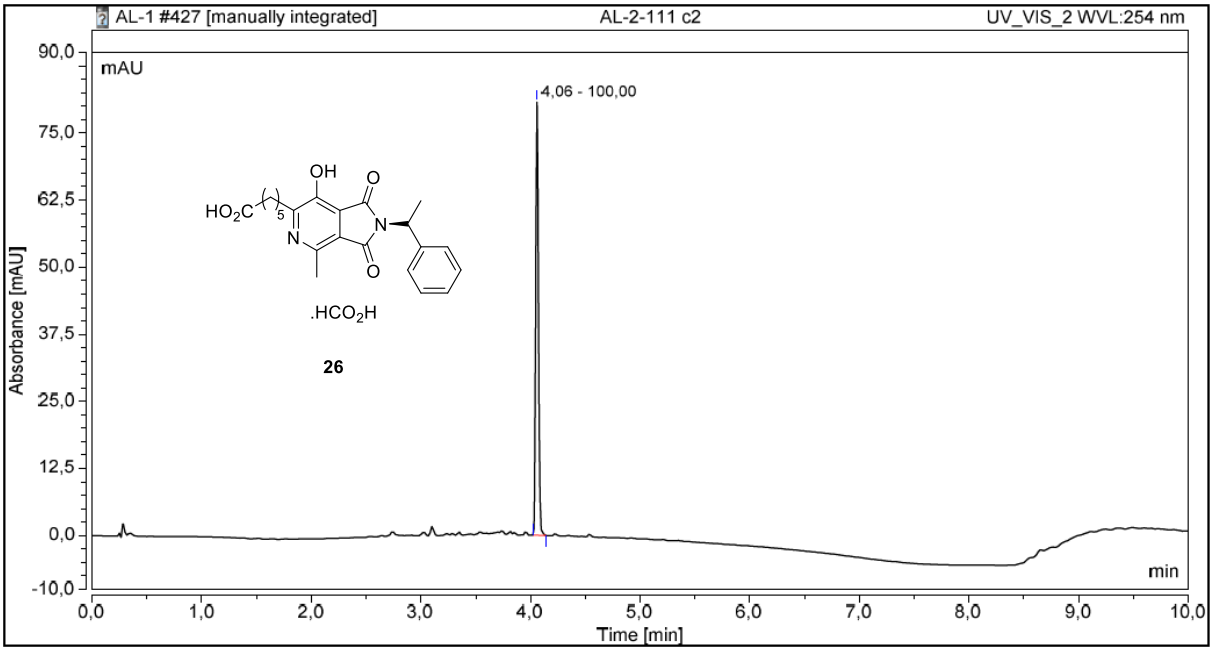
System A



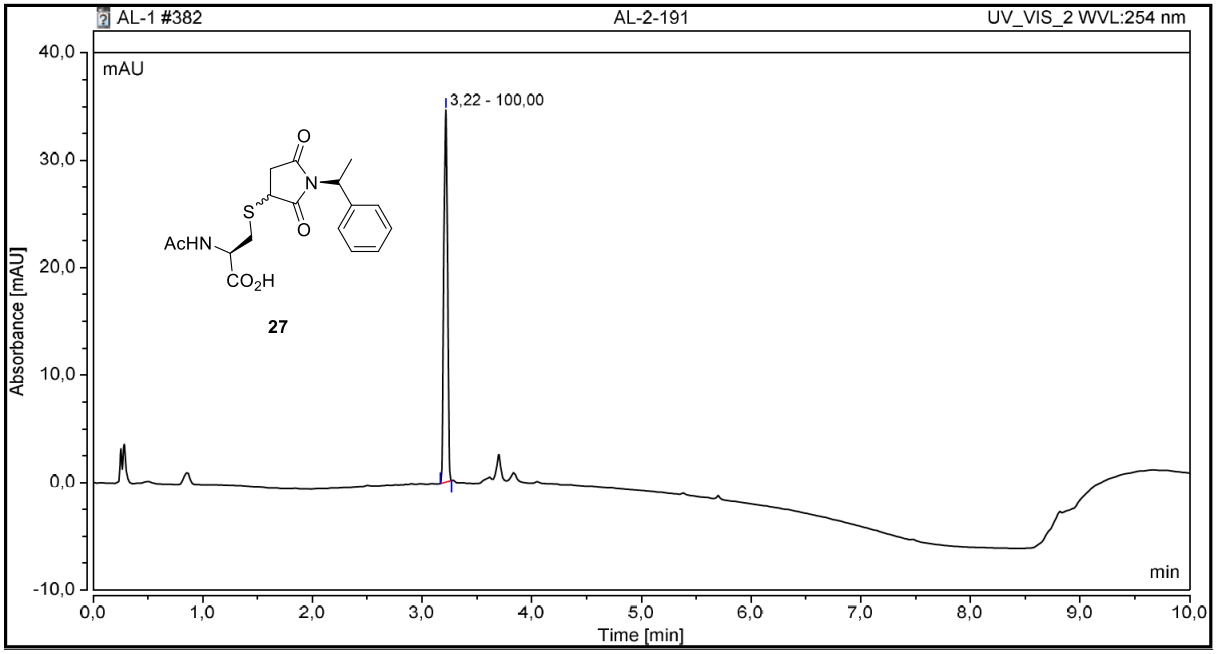
System A



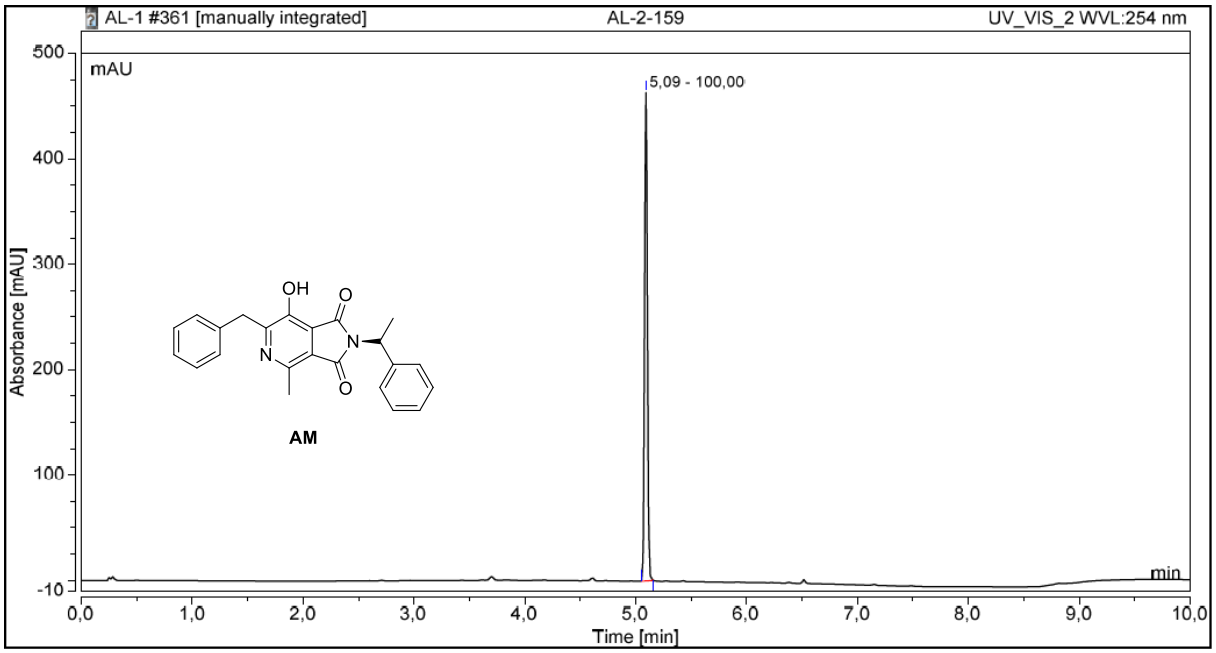
System A



System A



System A



I.3. Photophysical data for compounds **3** and **26**

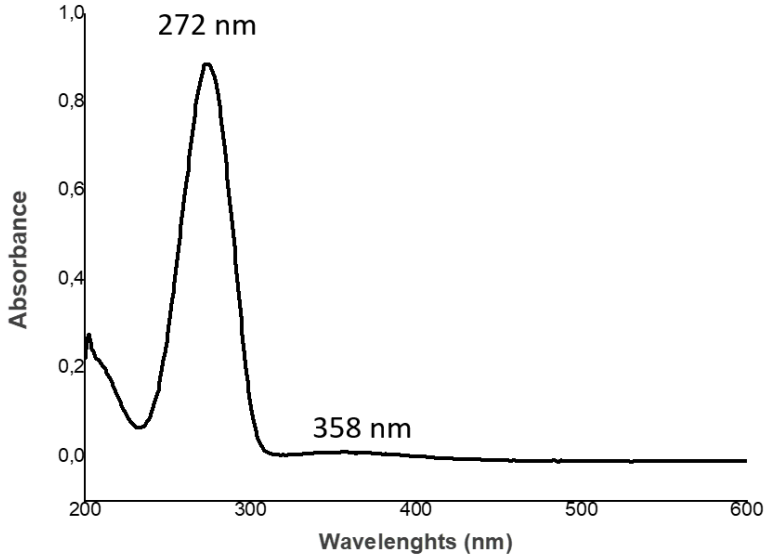


Figure S1: Absorption spectra for compound **3** (20 µM) in PBS pH 7.4/MeCN (90:10, v:v) at 20 °C.

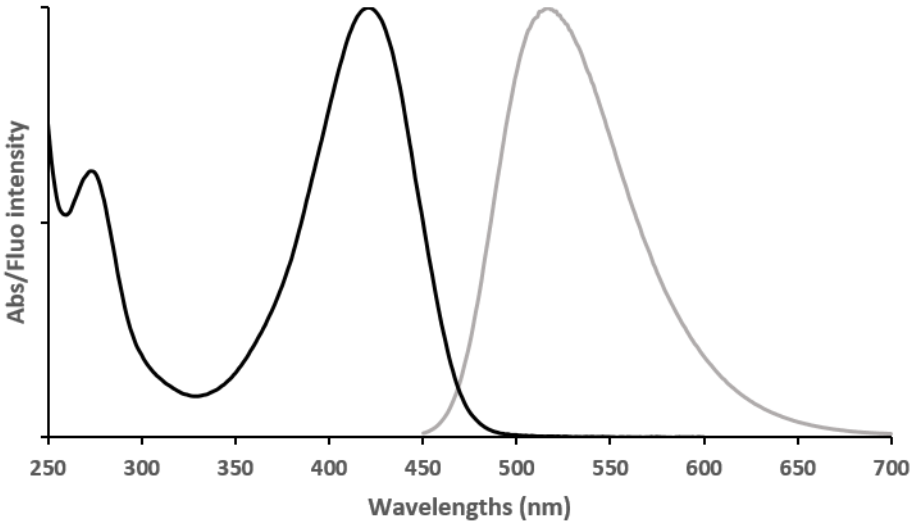
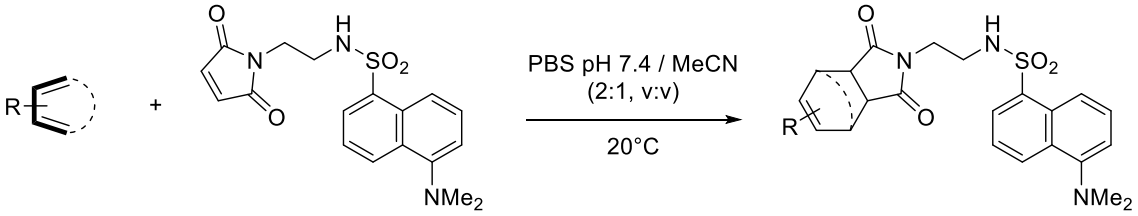


Figure S2: Normalized absorption (black line), emission (grey line, λ_{ex} = 422 nm) spectra for compound **26** in PBS pH 7.4 at 25 °C.

II. Kinetic experiments

II.1. Monitoring of fluorescence intensity change of Diels-Alder reactions

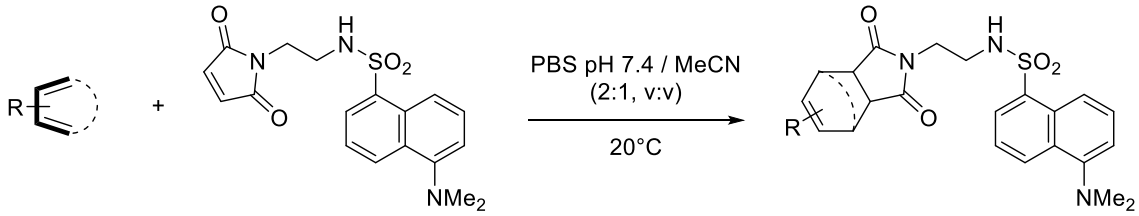
a) Probe **16** (1 mM) and dienes **1**-**10** (5 mM)



Diene 1 - 10 **16**

To a 1.25 mM solution of probe **16** in MeCN/PBS pH 7.4 (2.4 mL, 1:5, v:v) in a fluorescence cell, was added a 25 mM solution of the corresponding diene **1–10** in MeCN (0.6 mL). The resulting mixture was rapidly homogenized and introduced in the spectrophotometer. The resulting composition of the mixture at t = 0 min was as follows: MeCN/PBS pH 7.4 (3mL, 1:2, v:v); probe **16** (1 mM); diene (5 mM, 5 equiv.). Diels-Alder reactions were monitored by fluorescence spectroscopy (λ_{ex} = 330 nm ; λ_{ex} = 560 nm) at T = 20 °C.

b) Probe **16** (0.05 mM) and dienes **1**-**2**, **4**-**6** or **8** (4 mM)



Diene 1 - 5 and 7 **15**

To a 1.4 mM solution of probe **16** in MeCN (50 µL) in a fluorescence cell, were successively added MeCN (17 µL), a solution of PBS pH 7.4 (933 µL) and a 14 mM solution of the corresponding diene **1**-**2**, **4**-**6** or **8** in MeCN (0.4 mL). The resulting mixture was rapidly homogenized and introduced in the spectrophotometer. The resulting composition of the mixture at t = 0 min was as follows: MeCN/PBS pH 7.4 (1.4 mL, 1:2, v:v); probe **16** (0.05 mM); diene (4 mM, 80 equiv.). Diels-Alder reactions were monitored by fluorescence spectroscopy (λ_{ex} = 330 nm ; λ_{ex} = 560 nm) at T = 20 °C.

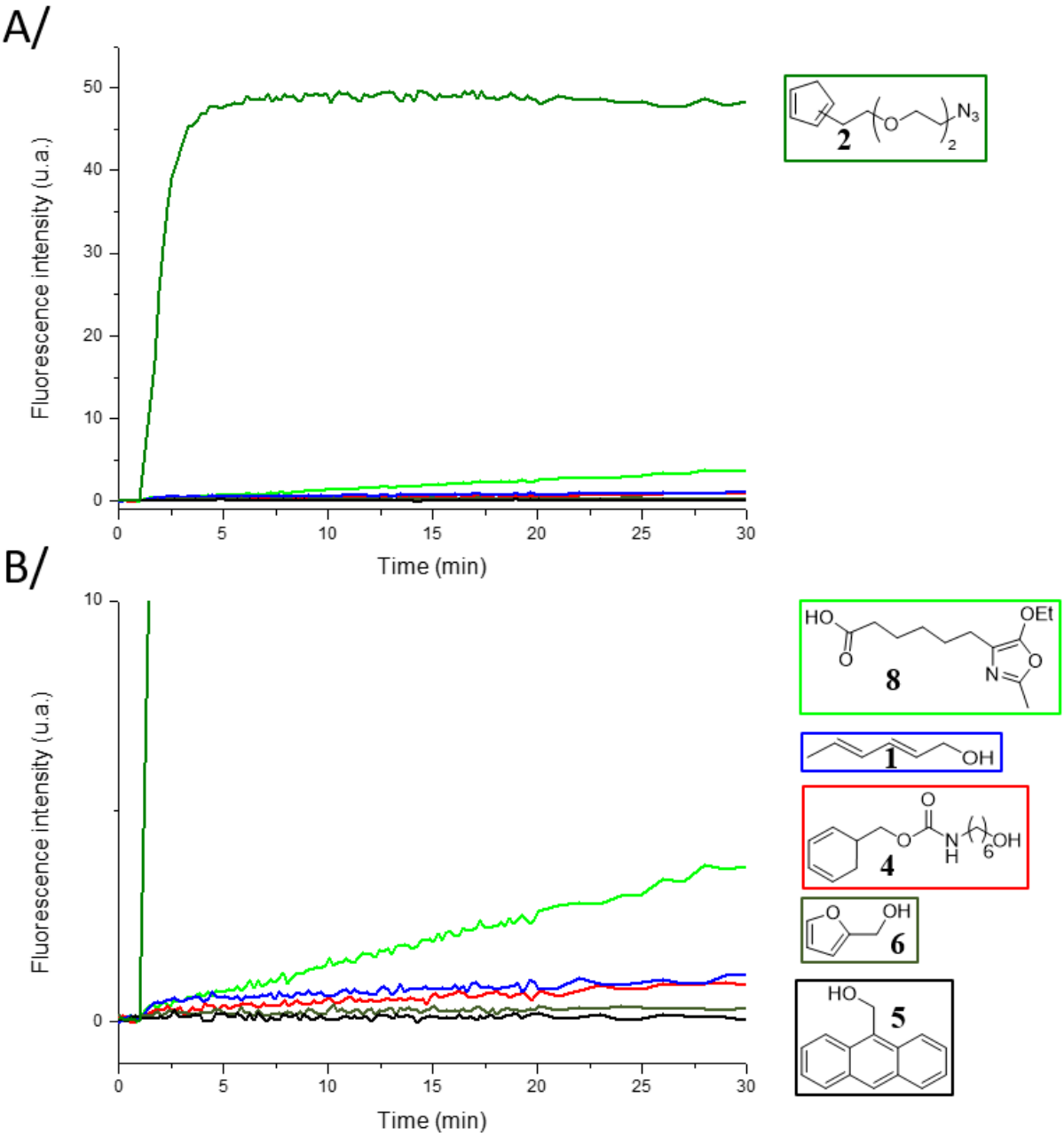


Figure S3. (A) Time-dependent fluorescence intensity changes of dansyl-based probe **16** (0.05 mM) in the presence of dienes **1**-**2**, **4**-**6** or **8** (4 mM, 80 equiv.) in MeCN/PBS pH 7.4 (1:2, v:v) recorded at λ_{em} 560 nm upon excitation at λ_{ex} 330 nm. (B) Enlarged view of Figure (A).

II.2. RP-HPLC monitoring of cycloadducts formation

Diels-Alder reactions carried out with probe **16** (1 mM) and dienes (5 mM) in PBS/MeCN (2:1, v:v) were also monitored by RP-HPLC at λ_{abs} = 330 nm after 1 h (except for diene **2** which was monitored after 10 min) in order to confirm the reaction progress of probe **16** which was observed by fluorescence spectroscopy. System A or B are specified for each experiment (sA or sB, respectively).

Legend:
- Compound **16** (t_R = 3.19 to 3.42 min) is labelled with green star (*).
- Dienes are labelled with a purple star (*) when detected at 330 nm.
- Cycloadducts are labelled with a blue star (*).

Note:
330 nm was selected as the detection wavelength because only dansyle scaffold absorbs, which is not the case for all cycloadduct scaffolds at this wavelength, thus allowing a reliable ratio determination between the dansyl-based maleimide and dansyl-based cycloadduct. Of note, only the fulvene **3** and anthracene **5** were able to absorb at 330 nm but their corresponding cycloadducts scaffolds do not (cf following RP-HPLC).

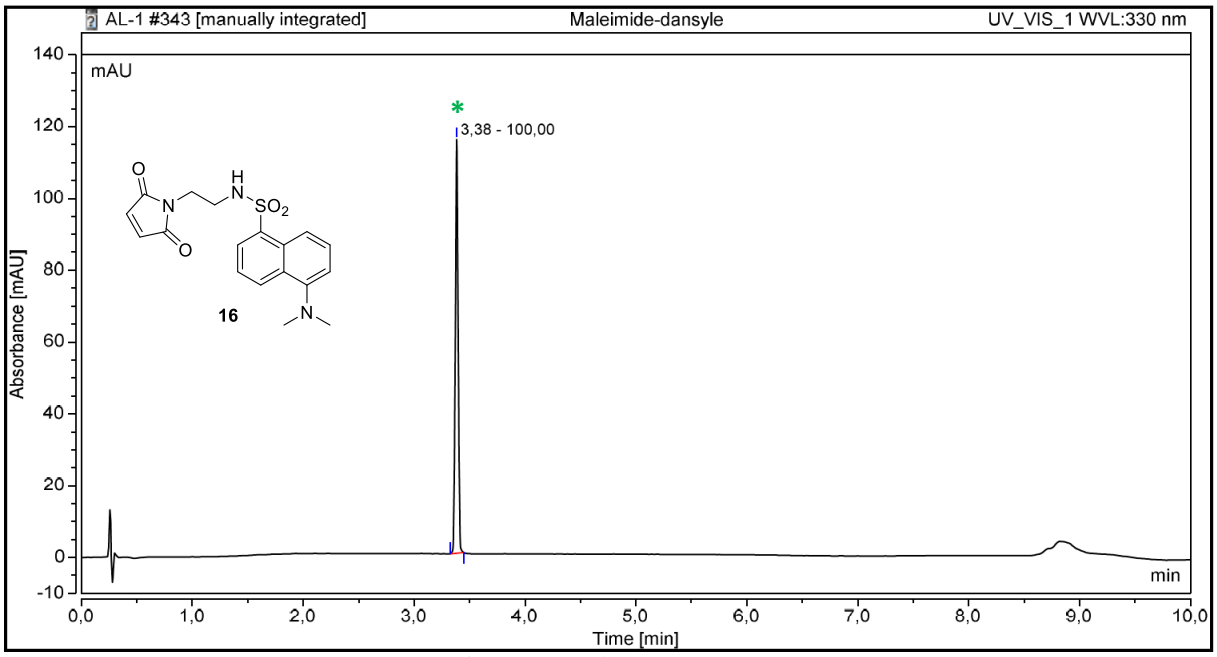


Figure S4. RP-HPLC chromatogram of probe **16** (λ = 330 nm, sA).

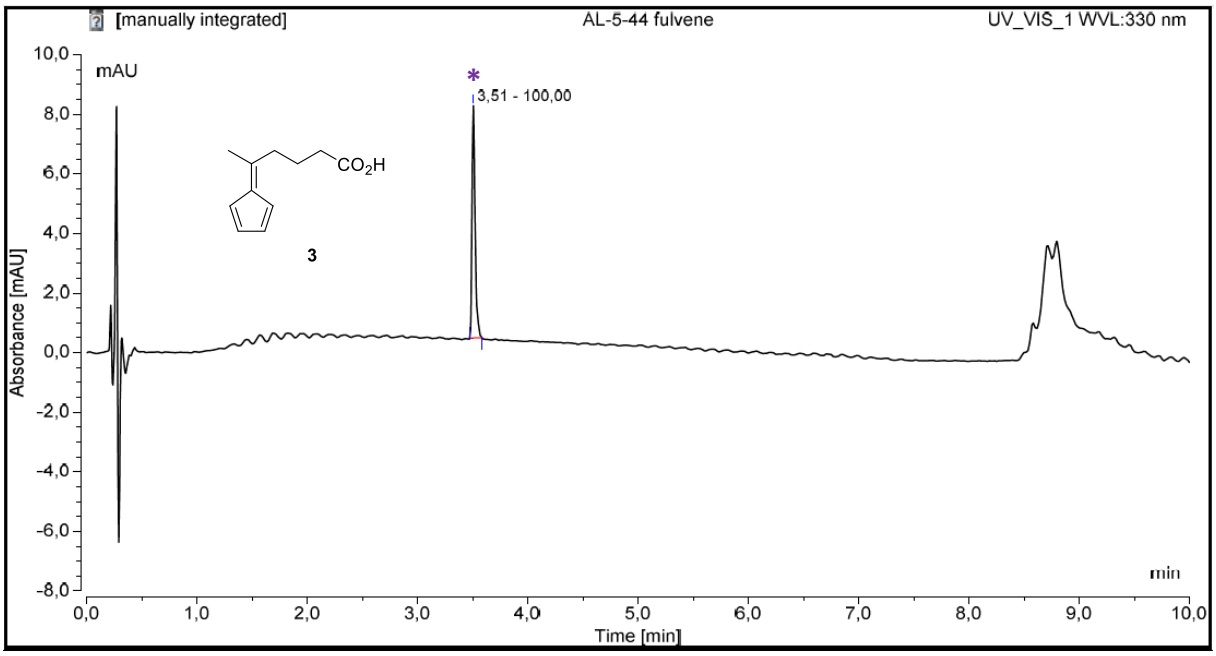


Figure S5. RP-HPLC chromatogram of fulvene **3** (λ = 330 nm, sB).

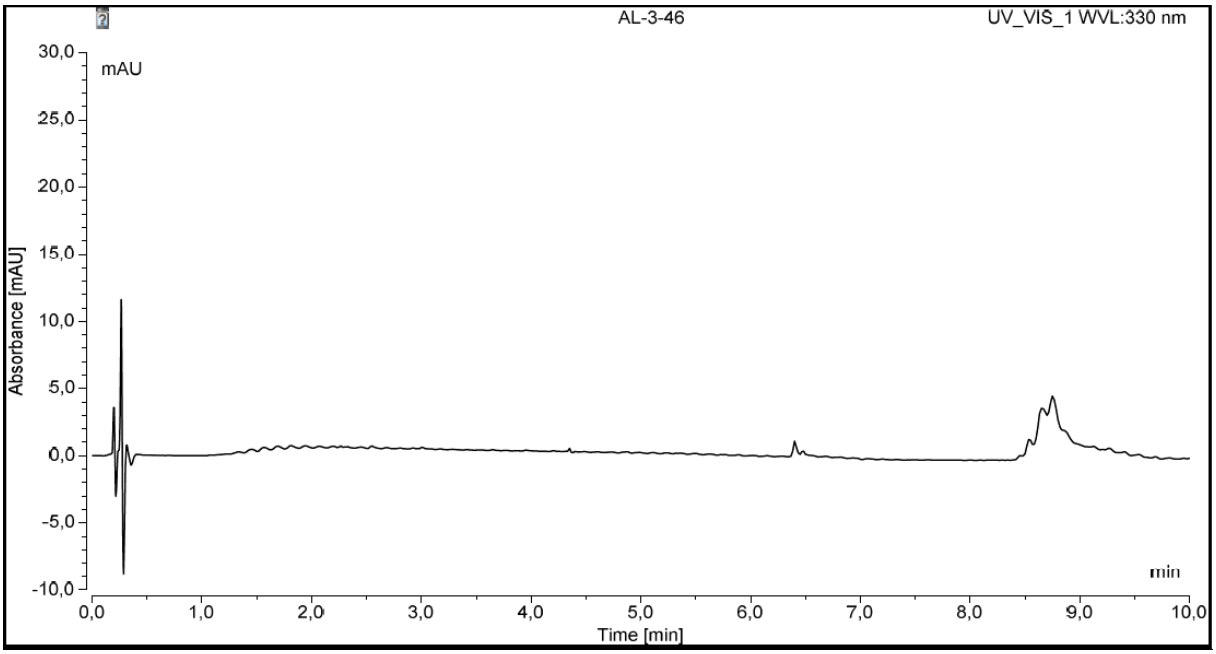


Figure S6. RP-HPLC chromatogram of fulvene-based cycloadduct **22** (λ = 330 nm, sB).

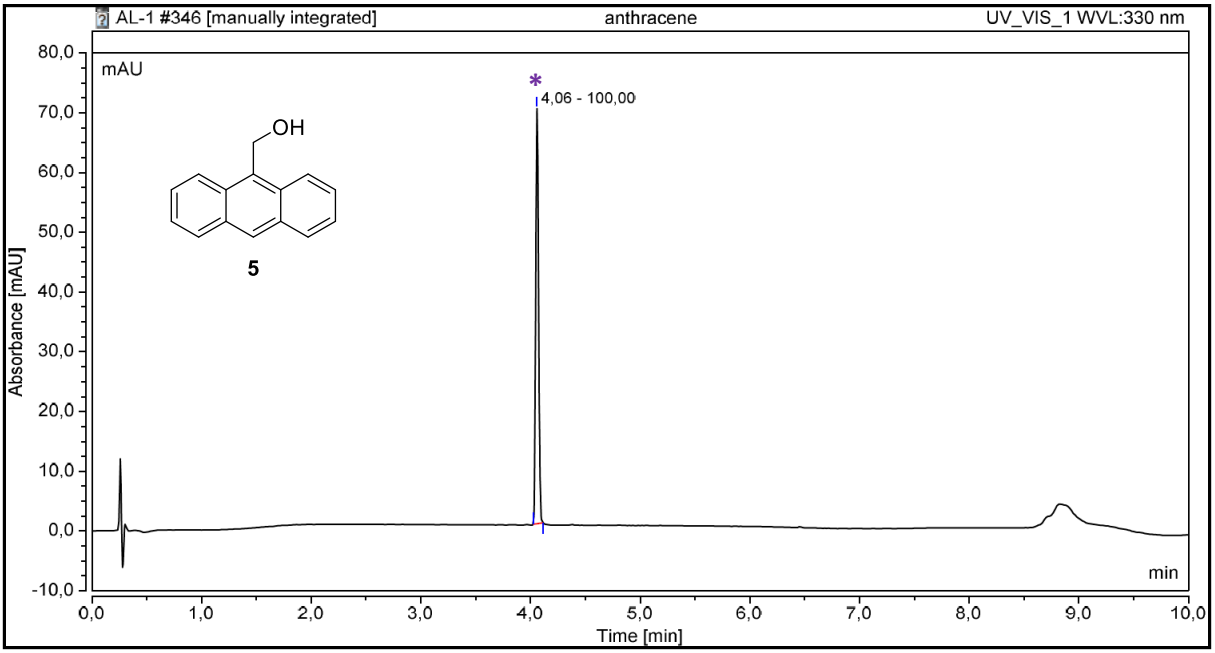


Figure S7. RP-HPLC chromatogram of anthracene **5** (λ = 330 nm, sA).

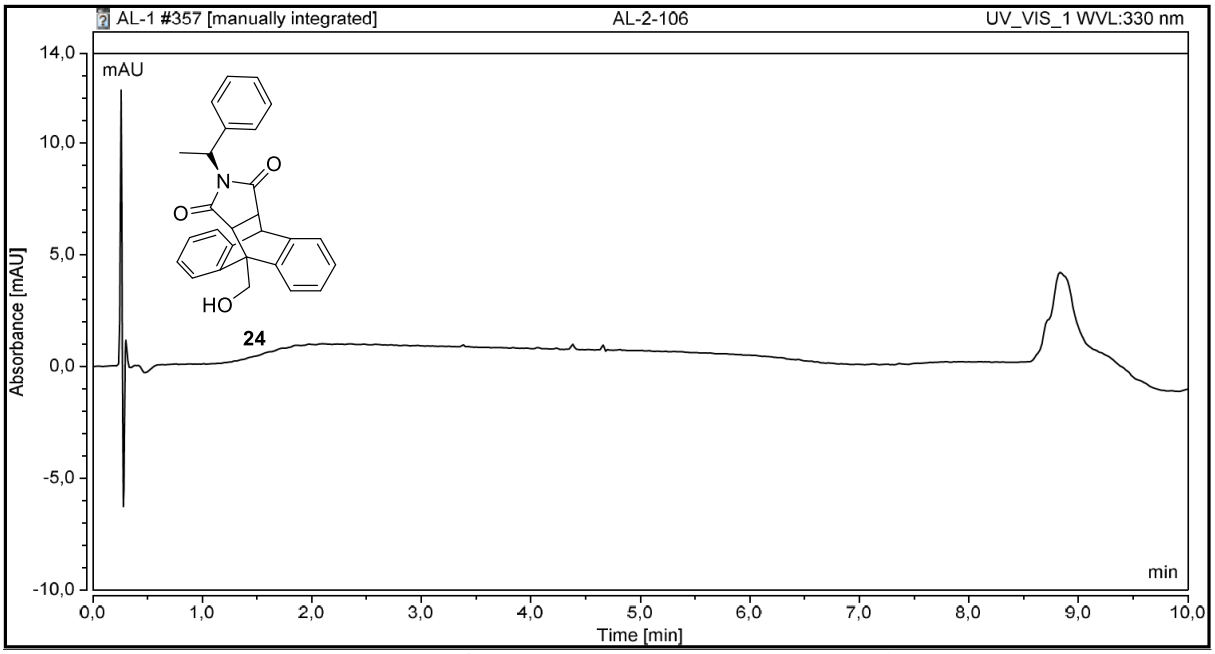
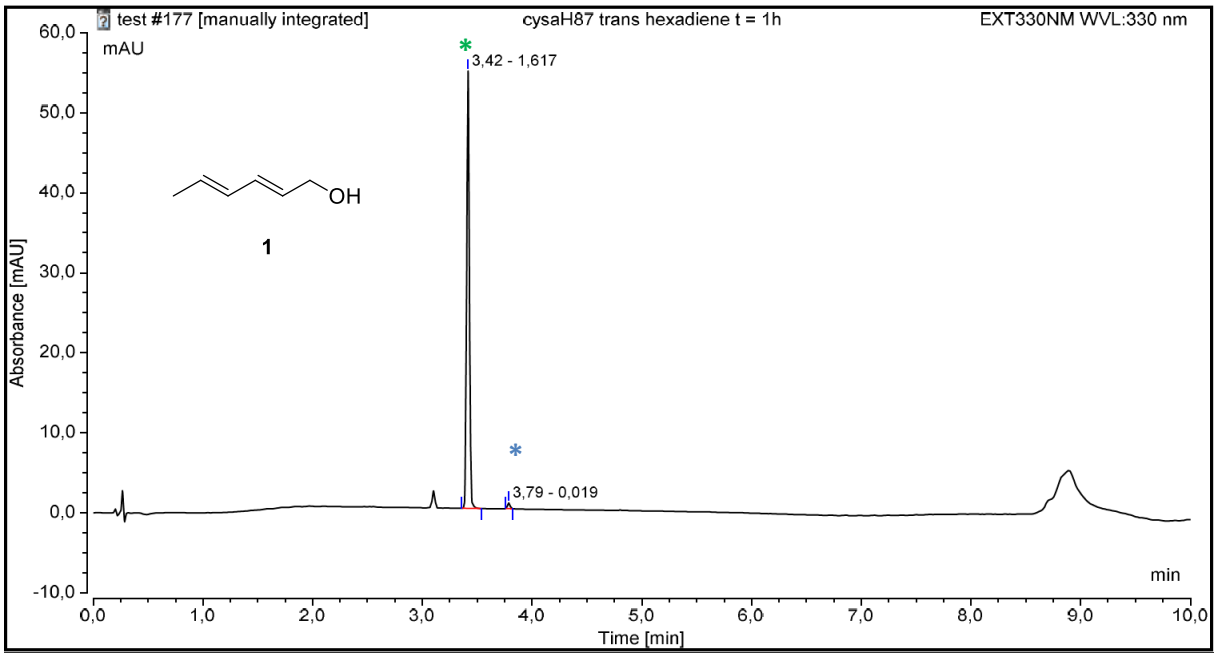


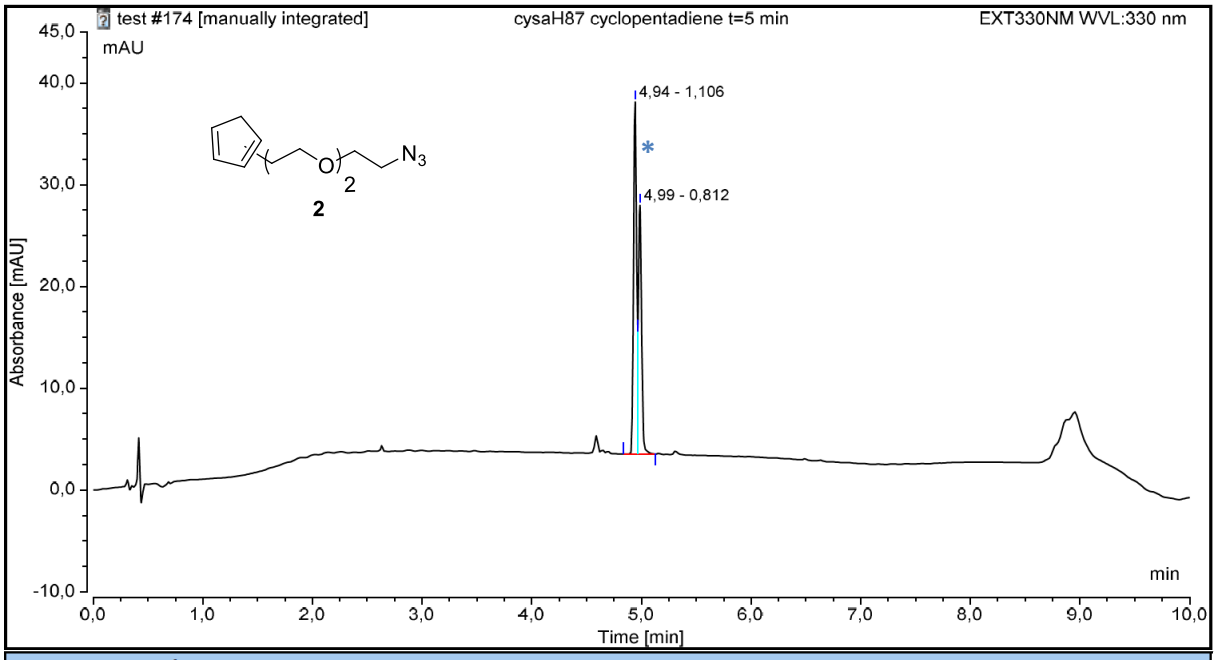
Figure S8. RP-HPLC chromatogram of anthracene-based cycloadduct **24** (λ = 330 nm, sA).



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,417	1,617	54,669	98,81	98,70	n.a.
2		3,787	0,019	0,721	1,19	1,30	n.a.
Total:			**1,636**	**55,390**	**100,00**	**100,00**	

Conversion of 16 = 1.2%

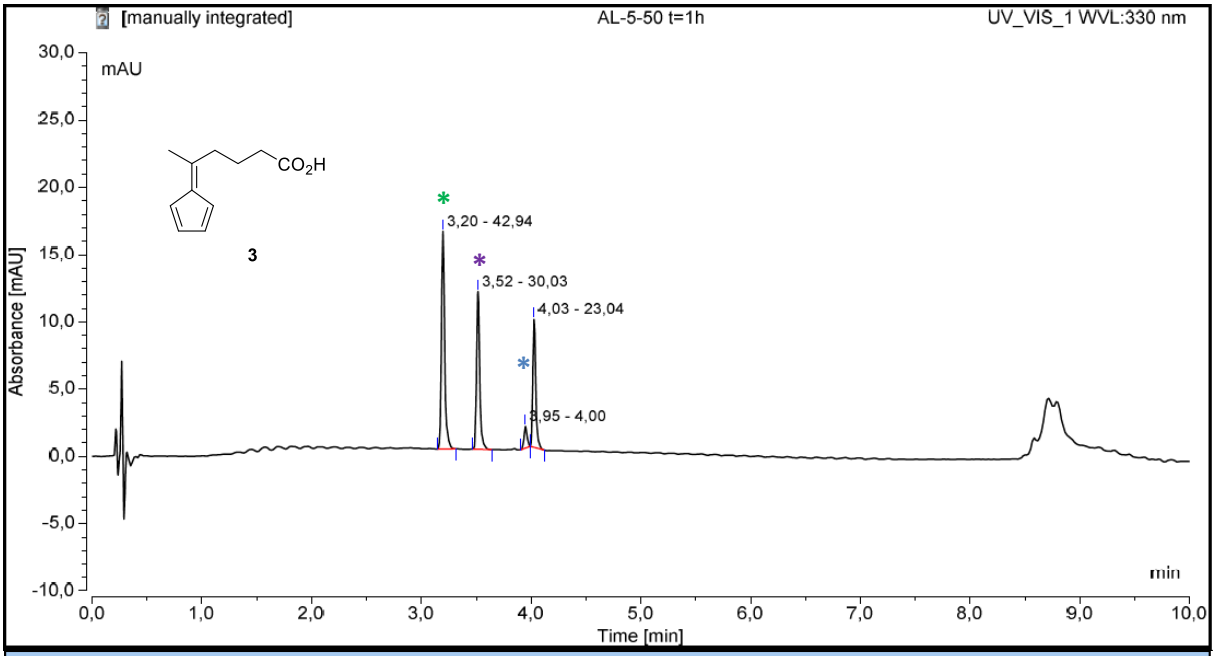
Figure S9. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **1** (λ = 330 nm, sA).



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		4,943	1,106	34,586	57,66	58,59	n.a.
2		4,987	0,812	24,446	42,34	41,41	n.a.
Total:			**1,918**	**59,031**	**100,00**	**100,00**	

Conversion of 16 = 100%

Figure S10. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **2** (λ = 330 nm, sA).

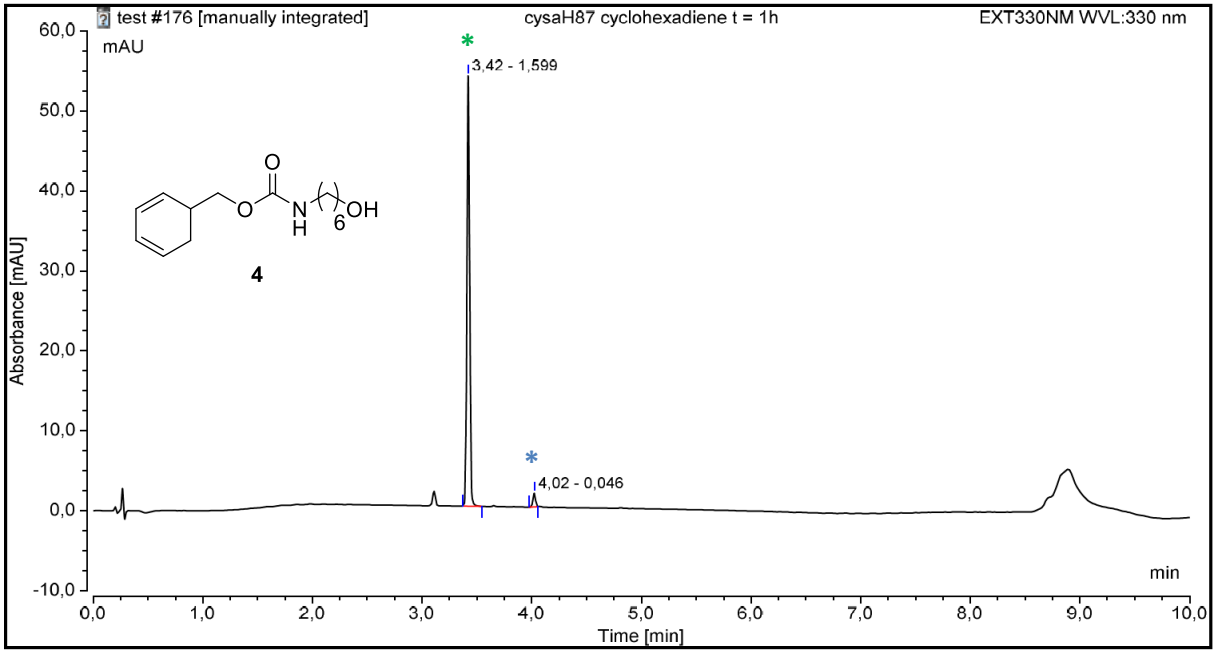


Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,197	0,554	16,182	42,94	41,45	n.a.
2		3,517	0,388	11,730	30,03	30,05	n.a.
3		3,947	0,052	1,615	4,00	4,14	n.a.
4		4,027	0,297	9,510	23,04	24,36	n.a.
Total:			**1,291**	**39,037**	**100,00**	**100,00**	

Conversion of 16 = 39%

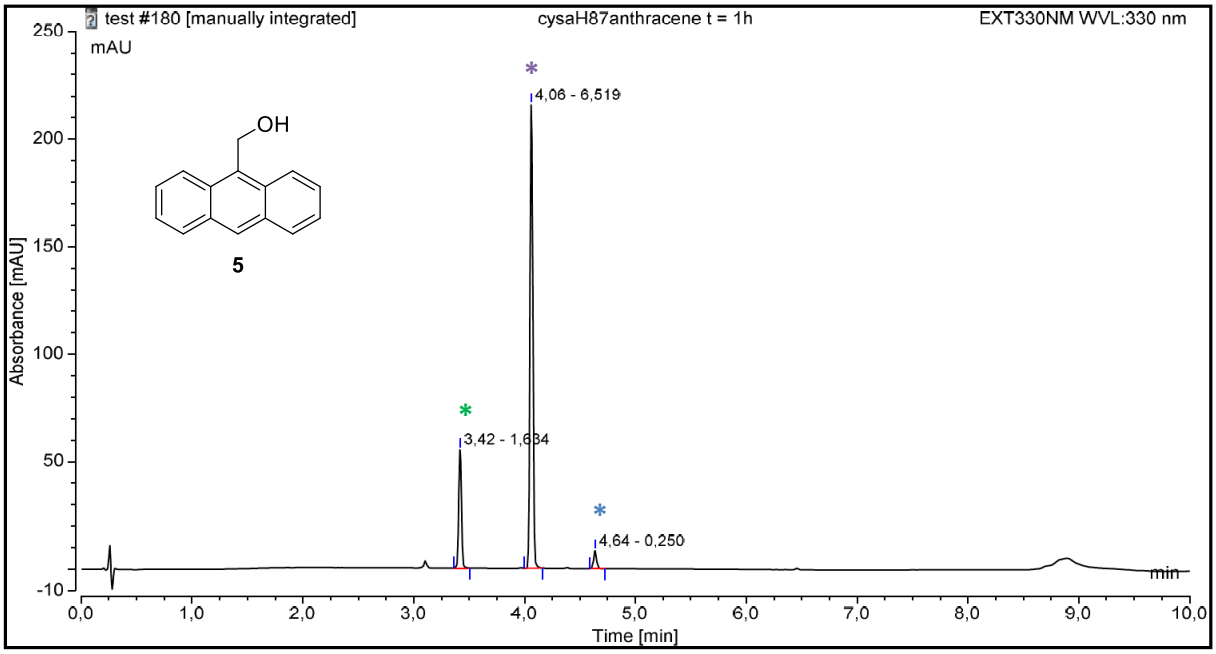
Figure S11. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **3** (λ = 330 nm, sB)



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,420	1,599	53,838	97,21	96,92	n.a.
2		4,023	0,046	1,709	2,79	3,08	n.a.
Total:			**1,645**	**55,547**	**100,00**	**100,00**	

Conversion of 16 = 2.8%

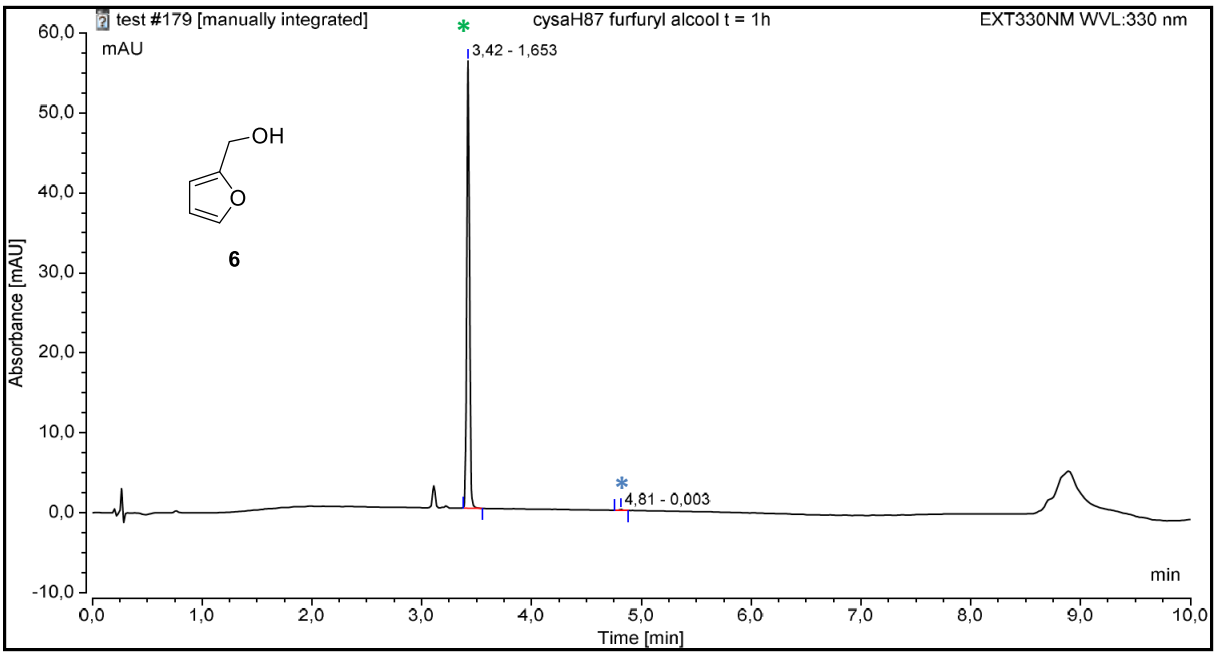
Figure S12. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **4** (λ = 330 nm, sA).



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,417	1,634	55,006	19,45	19,73	n.a.
2		4,060	6,519	215,430	77,58	77,28	n.a.
3		4,637	0,250	8,322	2,97	2,99	n.a.
Total:			**8,404**	**278,758**	**100,00**	**100,00**	

Conversion of 16 = 13.2%

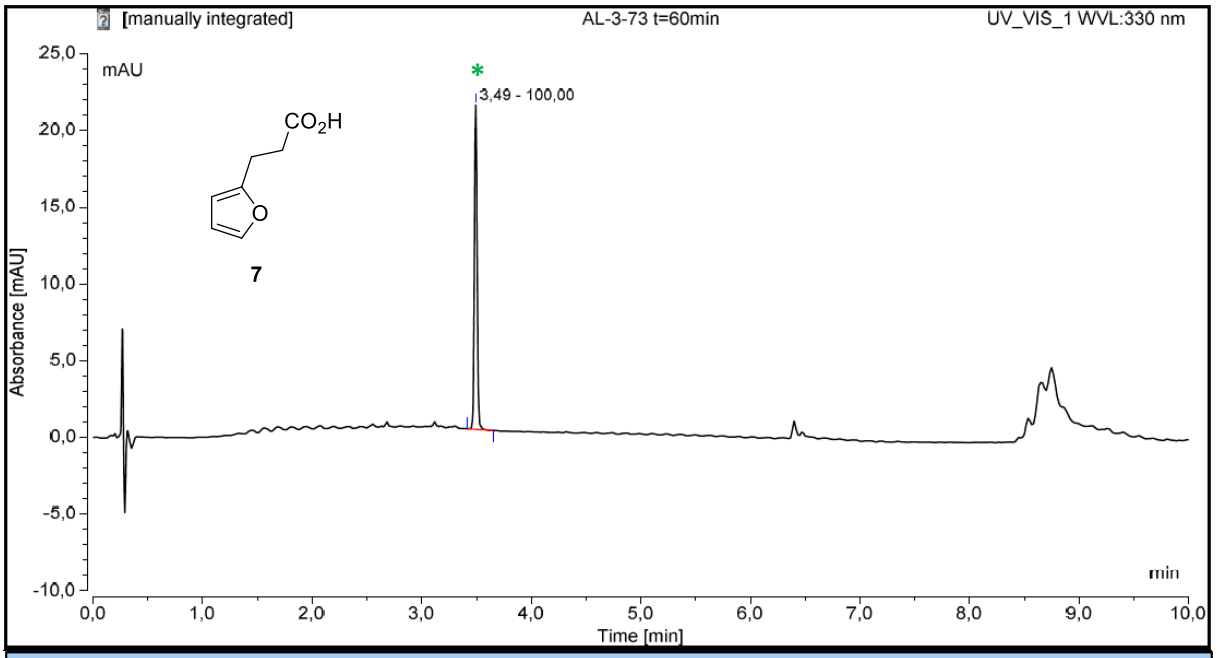
Figure S13. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **5** (λ = 330 nm, sA)



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,423	1,653	55,961	99,79	99,80	n.a.
2		4,813	0,003	0,110	0,21	0,20	n.a.
Total:			**1,656**	**56,071**	**100,00**	**100,00**	

Conversion of 16 = 0.2%

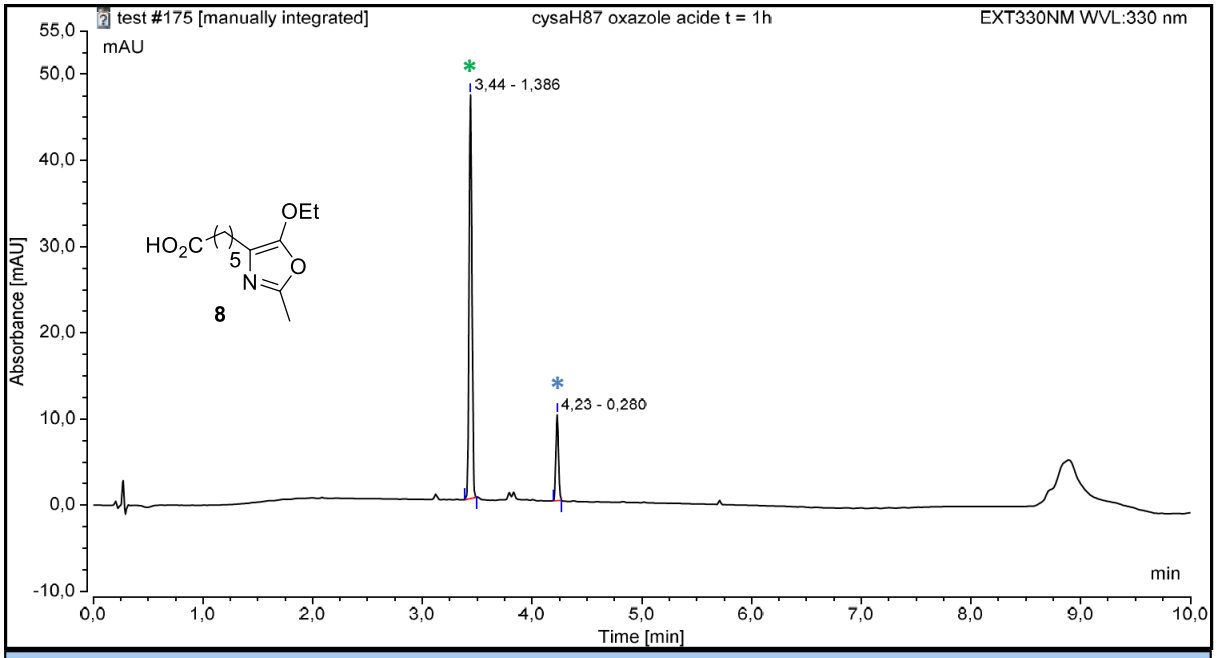
Figure S14. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **6** (λ = 330 nm, sA)



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,493	0,610	21,131	100,00	100,00	n.a.
Total:			**0,610**	**21,131**	**100,00**	**100,00**	

Conversion of 16 = not detectable

Figure S15. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **7** (λ = 330 nm, sB)

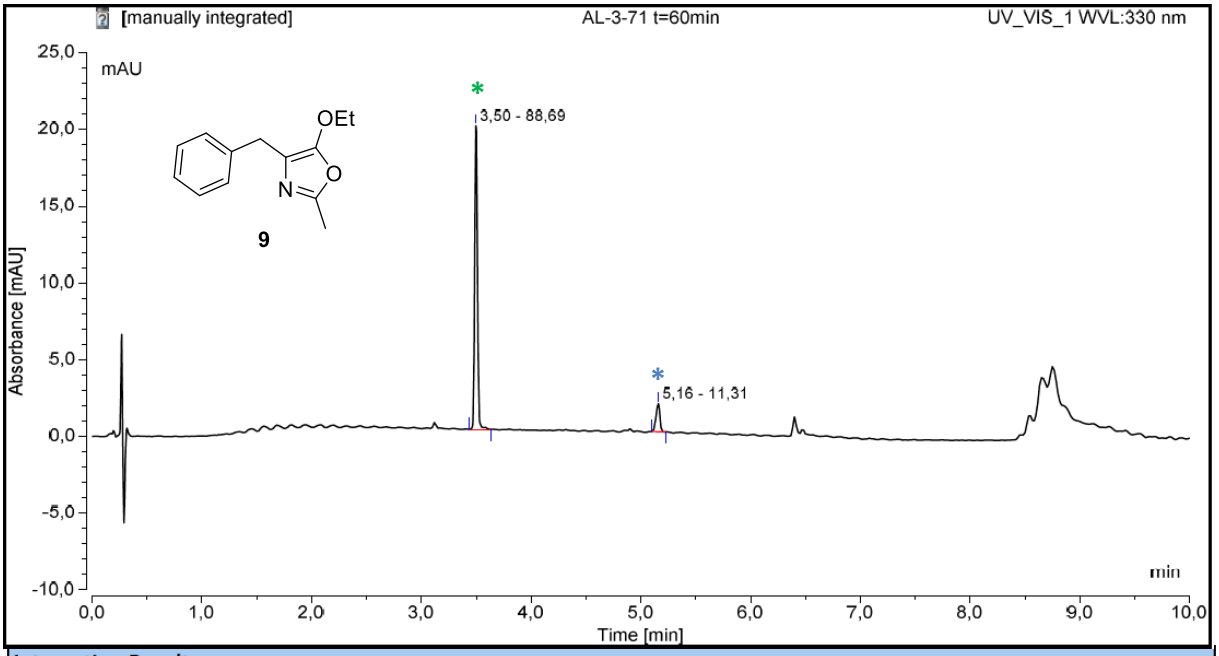


Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,440	1,386	46,845	83,21	82,49	n.a.
2		4,230	0,280	9,943	16,79	17,51	n.a.
Total:			**1,665**	**56,788**	**100,00**	**100,00**	

Conversion of 16 = 16.8%

Figure S16. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **8** (λ = 330 nm, sA)



Integration Results

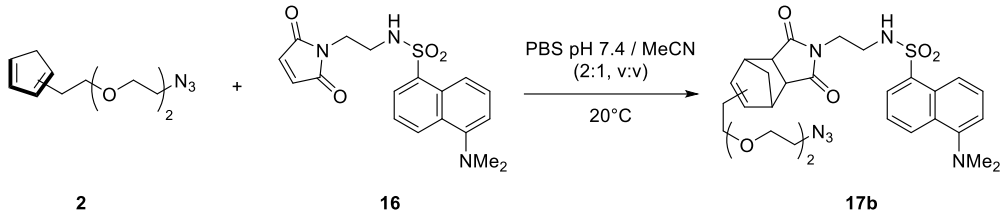
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,497	0,571	19,768	88,69	91,50	n.a.
2		5,160	0,073	1,836	11,31	8,50	n.a.
Total:			**0,644**	**21,604**	**100,00**	**100,00**	

Conversion of 16 = 11.3%

Figure S17. RP-HPLC of the Diels-Alder reaction of probe **16** with diene **9** (λ = 330 nm, sB)

II.3. Reaction rate determination of cycloaddition of probe **16** with dienes **1-3**, **6**, and **8-9**

*a) Reaction rate determination of cycloaddition of probe **16** with cyclopentadiene **2** in aqueous/organic medium*



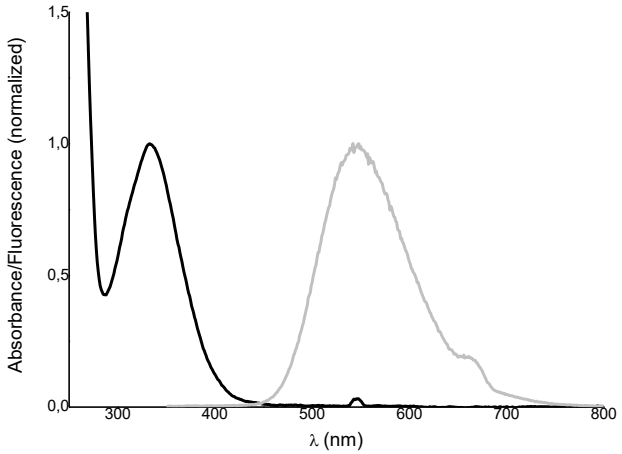


Figure S18. Normalized absorption (black line) and emission (grey line) spectra of **17b** in MeCN/PBS pH 7.4 (1:2, v:v) at T = 20 ° C and at λ_{exc}=330 nm

- Calibration curve between fluorescent intensity and concentration of cycloadduct 17.

From a 1.4 mM stock solution of **17b** in MeCN were prepared five diluted solutions (from 10 to 50 µM in PBS/MeCN, 2:1). A fluorescence emission spectrum was recorded for each diluted solution (λ_{ex} = 330 nm , T = 20 °C) in a 1.4 mL Hellma fluorescence cell. Then, the fluorescence intensity observed at λ_{em} = 560 nm for λ_{ex} = 330 nm was plotted against concentration to afford linear relation.

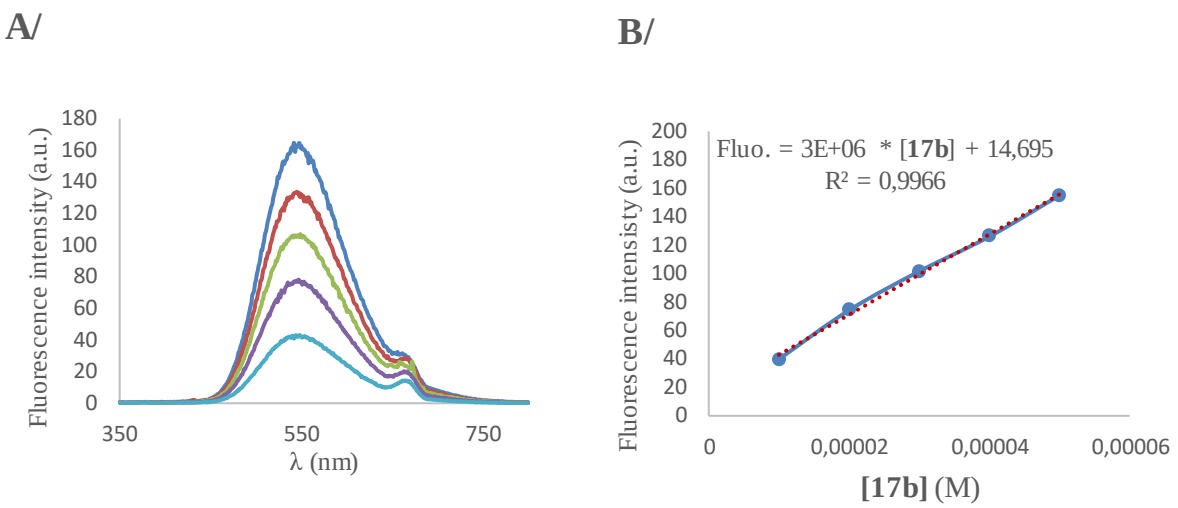


Figure S19. (A) Fluorescence intensity for **17b** at five concentrations (from 10 to 50 µM in PBS/MeCN, 2:1) for λ_{ex} = 330 nm. (B) Fluorescence intensity for **17b** plotted against concentration (λ_{ex} = 330, λ_{em} = 560 nm).

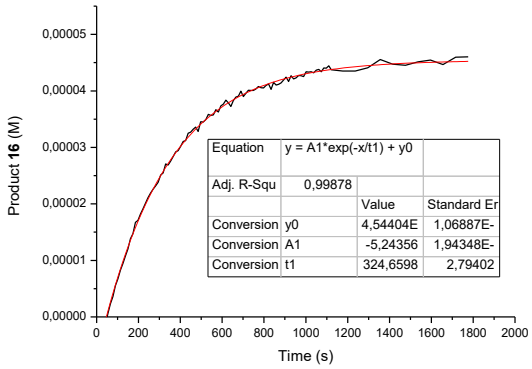
- Second order reaction rate k_2 determination *via* k_{obs}

To a 1.4 mM solution **16** in MeCN (50 µL) in a 1.4 mL fluorescence cell, were added MeCN (X µL, cf Table) and a solution of PBS pH 7.4 (933 µL). Then, a 14 mM solution of **2** in MeCN (Y µL, cf Table) was added and the reactional mixture was directly introduced into the spectrophotometer after homogenization. The resulting composition of the mixture at t = 0 min was as follows: MeCN/PBS pH 7.4 (1.4 mL, 1:2, v:v) ; [**16**] = 50 µM ; [**2**] = 500 µM, 750 µM, 875 µM or 1000 µM. The Diels-Alder reaction was monitored by fluorescence spectroscopy (λ_{ex} = 330 nm ; λ_{em} = 560 nm) at T = 20°C.

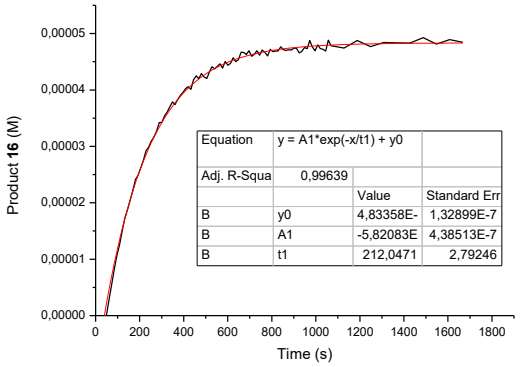
Final concentration of **2** (µM)	Volume X of MeCN (µL)	Volume Y of the 14 mM solution of **2** in MeCN (µL)
500	367	50
750	342	75
875	329.5	87.5
1000	317	100

Resulting fluorescence curves were converted to the conversion against time thanks to the calibration relation. Conversion curves were fitted with non-linear exponential correlation, which afforded t_1 values and then k_{obs} (= 1/ t_1). k_{obs} values were plotted against concentration of **2** and the line was fitted with linear regression, affording a slope = k_2 ($M^{-1}.s^{-1}$).

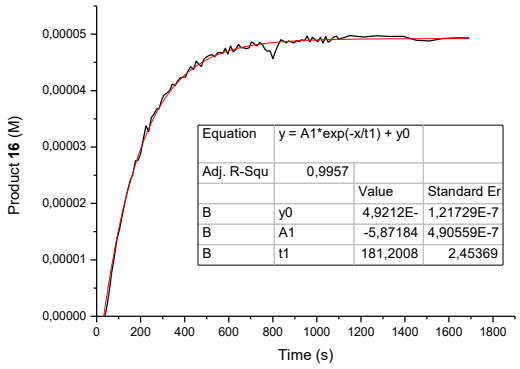
A/ [**2**] = 500 µM



B/ [**2**] = 750 µM



C/ [**2**] = 875 µM



D/ [**2**] = 1000 µM

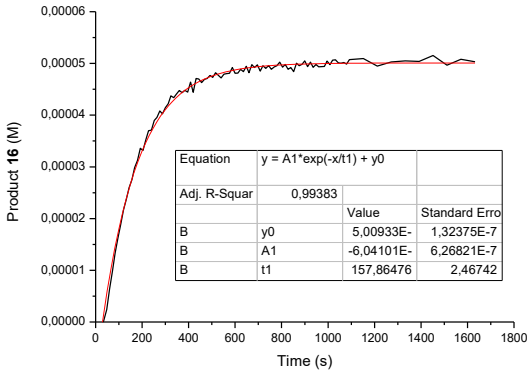


Figure S20. Time course formation of product **17b** at different concentrations of **2**: 500, 750, 875 and 1000 µM for (A), (B), (C), and (D), respectively.

[**2**] (µM)	t_1 (s)	k_{obs} (s^{-1})
500	324,6598	0.00308015
750	212,0471	0,00471593
875	181,20086	0,00551874
1000	157,86476	0,00633454

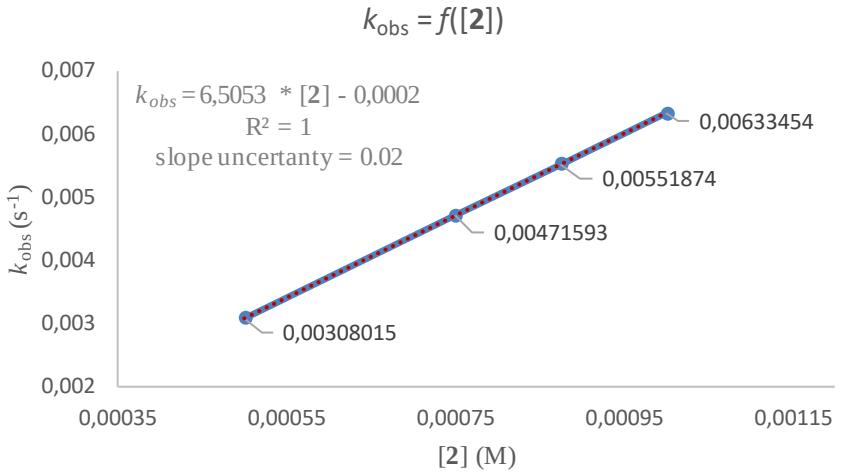


Figure S21. Plot of the k_{obs} values against the cyclopentadiene **2** concentration. The slope of the linear fit is the second-order rate constant.

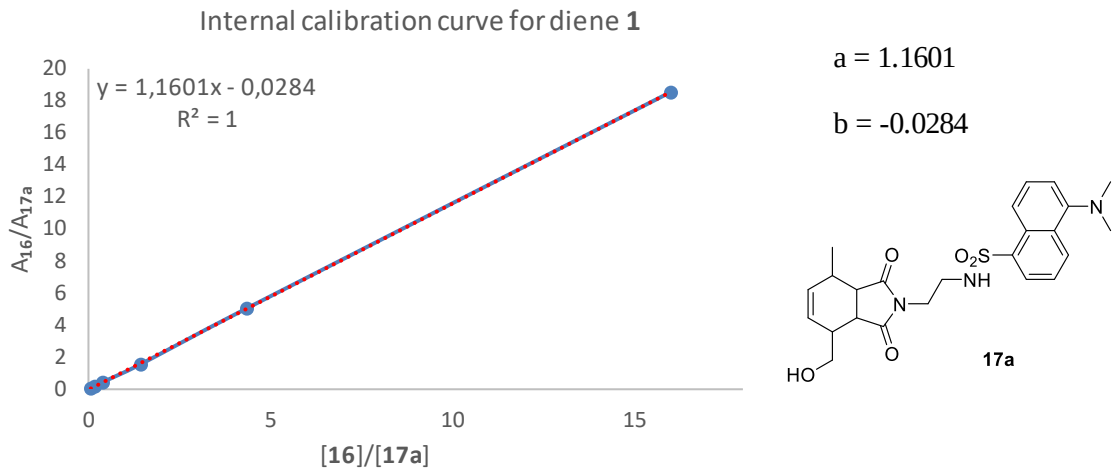
$$\text{Slope} = \boldsymbol{k_2 = 6.50 \pm 0.02\ M^{-1}.s^{-1}}$$

*b) Reaction rate determination of cycloaddition of probe **16** with diene **1, 3, 6, 8, 9** in aqueous/organic medium*

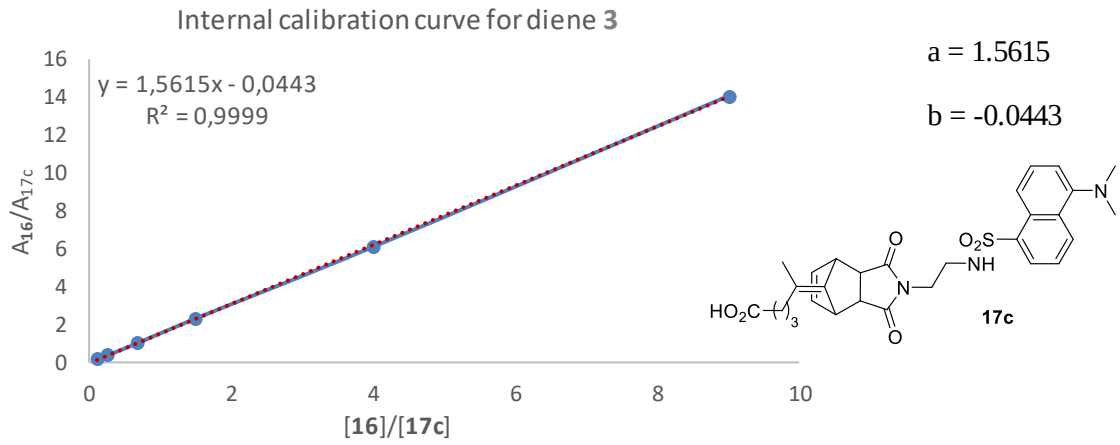
- Internal calibration procedure for kinetic HPLC monitoring

For each diene (**1**, **3** and **6**), a range of titrated solution containing the corresponding isolated dansyl-based cycloadduct (**17a**, **17c** and **17d**, respectively) and probe **16** was prepared in H_2O/MeCN (2:1, v:v). The range was established according to the solubility of diene and the kinetic experiment. Every titrated mixtures were analyzed by HPLC ($\lambda = 330$ nm, System B) and the ratios of HPLC areas ($A_{15}/A_{cycloadduct}$) were plotted against the corresponding concentration ratios ([**16**]/[**cycloadduct**]). Parameters a and b are employed for kinetic calculations.

A/



B/



C/

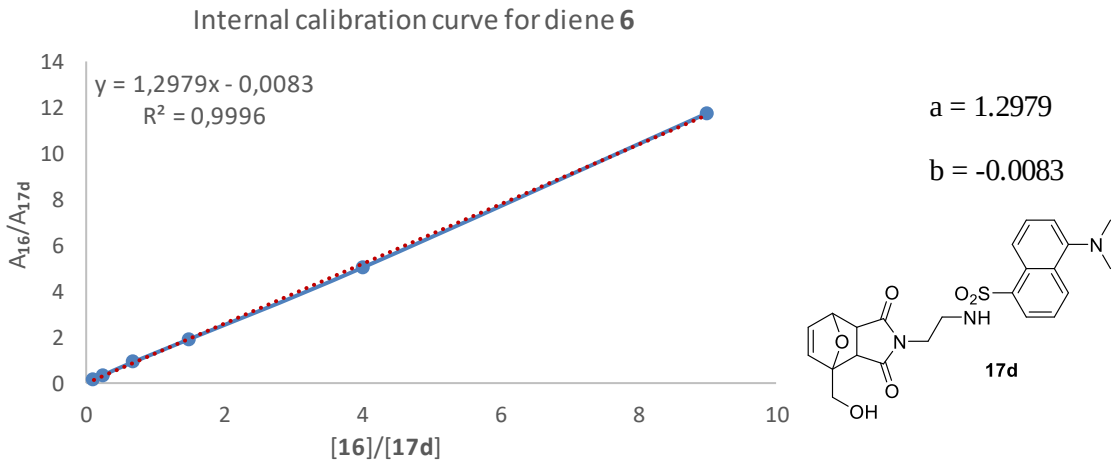


Figure S22. Internal calibration curves for (A) diene **1**; (B) diene **3**; and (C) diene **6**.

- External calibration procedure for kinetic HPLC monitoring

Corresponding cycloadducts for dienes **8** and **9** could not be isolated. A range of titrated solution containing the probe **16** and commercial **coumarin** (external HPLC reference, CAS number: 91-64-5) was prepared in H_2O/MeCN (2:1, v:v). The range was established according to the solubility of diene and the kinetic experiment, with [**coumarin**] = constant = 5.0 mM. Every titrated mixtures were analyzed by HPLC (λ = 330 nm, System B) and the ratios of HPLC areas ($A_{15}/A_{coumarin}$) were plotted against the corresponding concentration ratios (([**16**]/[**coumarin**]). Parameters a and b are employed for kinetic calculations.

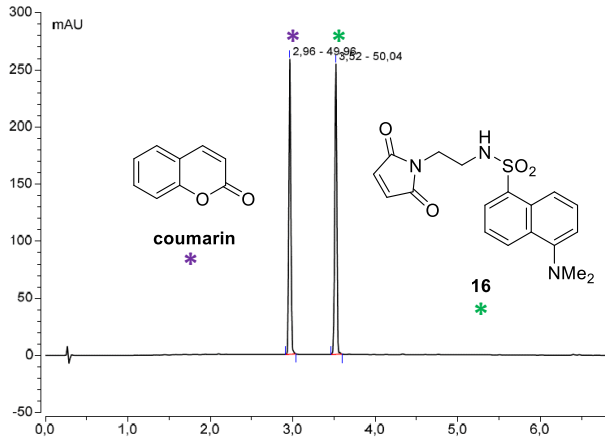
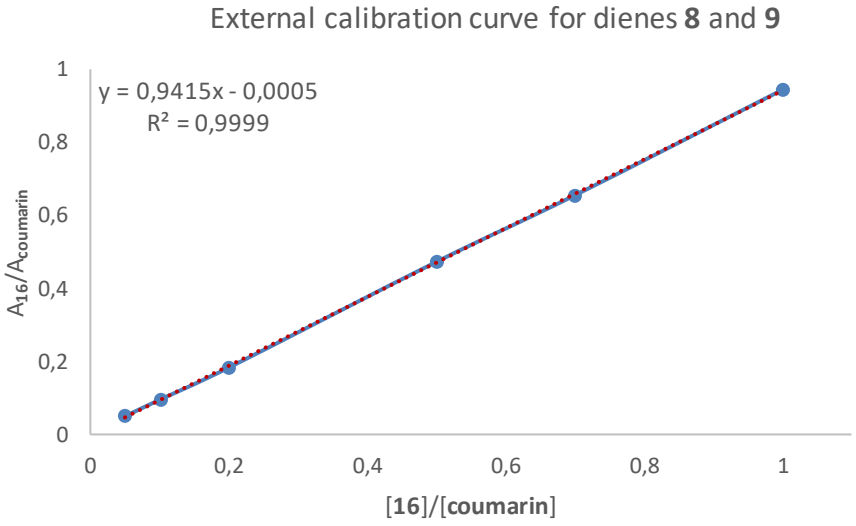


Figure S23. Chromatogram (λ = 330 nm, System B) of equimolar mixture of **coumarin** + probe **16**.

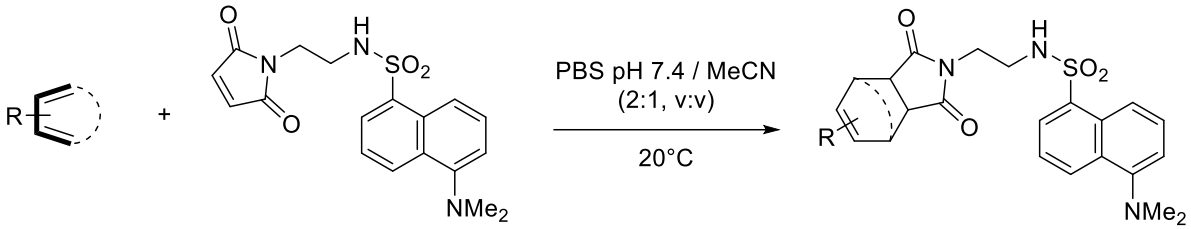


a = 0.9415

b = -0.0005

Figure S24. External calibration curve with **coumarin** for dienes **8** and **9**.

- Direct second order reaction rate k_2 determination.



Diene **1**, **3**, **6** and **8-9** **16** **cycloadduct 17**

In a flask containing a stirring bar were added PBS pH 7.4 (666 µL) and MeCN (volume calculation: $V_{MeCN} = 333 - V_{\mathbf{16}} - V_{diene}$ µL so as to reach a final ration PBS/MeCN, 2:1). For every experiments, titrated stock solutions of dienes and probe **16** were prepared in MeCN. Titrated stock solutions of diene (volume $V_{\mathbf{diene}}$) and probe **16** (volume $V_{\mathbf{16}}$) were sequentially added (final volume = 999 µL, PBS/MeCN, 2:1). Reaction time was immediately measured with a chronometer. Starting concentrations for T = 0 are specified for each experiments ($[\mathbf{16}]_0 = [\mathbf{diene}]_0$). The Diels-Alder reactions were monitored by HPLC (λ = 330 nm, System B) at T = 20 °C.

HPLC aliquots were prepared as follows:

- Internal calibration: 30 µL reactional mixture + 120 µL H_2O + 50 µL MeCN
- External calibration: 30 µL reactional mixture + 120 µL H_2O + 20 µL MeCN + 30 µL coumarin solution (5 mM in H_2O/MeCN, 2:1)

Equations for internal calibrations:

- Concentration of dansyl dye at any time:
  $[\mathbf{16}]_0 = [\mathbf{16}] + [\mathbf{cycloadduct}]$

- Linear relation from internal calibration (with a and b the parameters from calibration curves):
  $A_{\mathbf{16}}/A_{\mathbf{cycloadduct}} = a * ([\mathbf{16}]/[\mathbf{cycloadduct}]) + b$

- [**cycloadduct**] at any time with the previous relations:
  $[\mathbf{cycloadduct}] = [\mathbf{16}]_0 / ((((A_{\mathbf{16}}/A_{\mathbf{cycloadduct}})-b)/a)+1)$

- [**16**] at any time with the previous relations:
  $[\mathbf{16}] = [\mathbf{16}]_0 - [\mathbf{cycloadduct}]$

Equations for external calibration:

- Linear relation from external calibration (with a and b the parameters from calibration curves and [**coumarin**] = constant):
  $A_{\mathbf{16}}/A_{\mathbf{coumarin}} = a * ([\mathbf{16}]/[\mathbf{coumarin}]) + b$

- Concentration of probe **16** at any time with the previous relation:
  $[\mathbf{16}] = [\mathbf{coumarin}] * (((A_{\mathbf{16}}/A_{\mathbf{coumarin}})-b)/a)$

[**16**] in each kinetic experiments were calculated thanks to these correlations (internal and external calibrations) and plotted against time (s).
Typical kinetic equation for a second order reaction is:

- d[C]/dt = k_2.[**16**].[**diene**]

With [**16**] = [**diene**] (for $[\mathbf{16}]_0 = [\mathbf{diene}]_0$), the previous equation becomes:

- d[C]/dt = k_2.[**16**]²

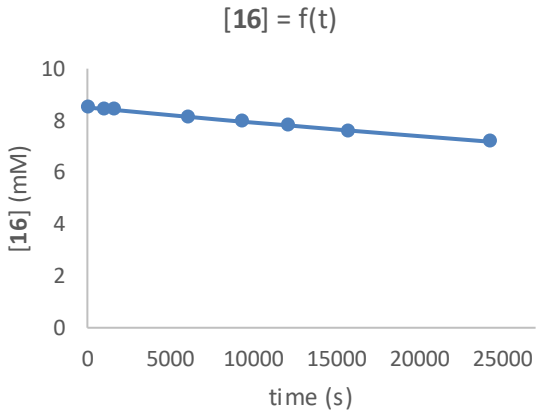
The following relation is found after integration:

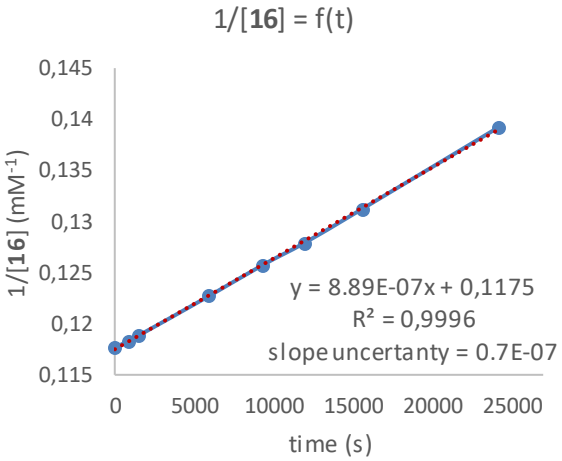
- $1/[\mathbf{16}] = k_2.t + 1/[\mathbf{16}]_0$

with:
- k_2 in $M^{-1}.s^{-1}$
- t in s
- [**16**], [**diene**] and [**coumarin**] in mM

1/[**16**] were plotted against time for each kinetic experiments. The slope of the resulting right affords the k_2 value.

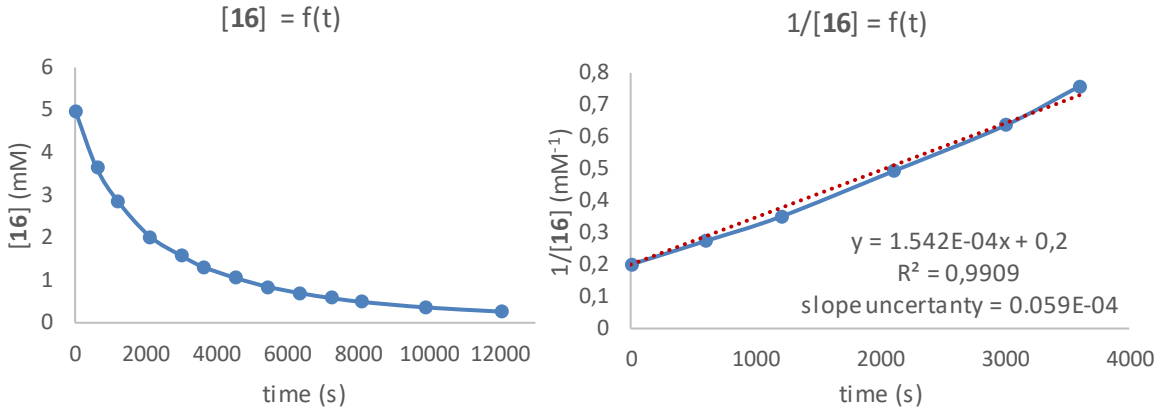
A/ Diene **1** ($[\mathbf{16}]_0 = [\mathbf{1}]_0 = 8.5$ mM)





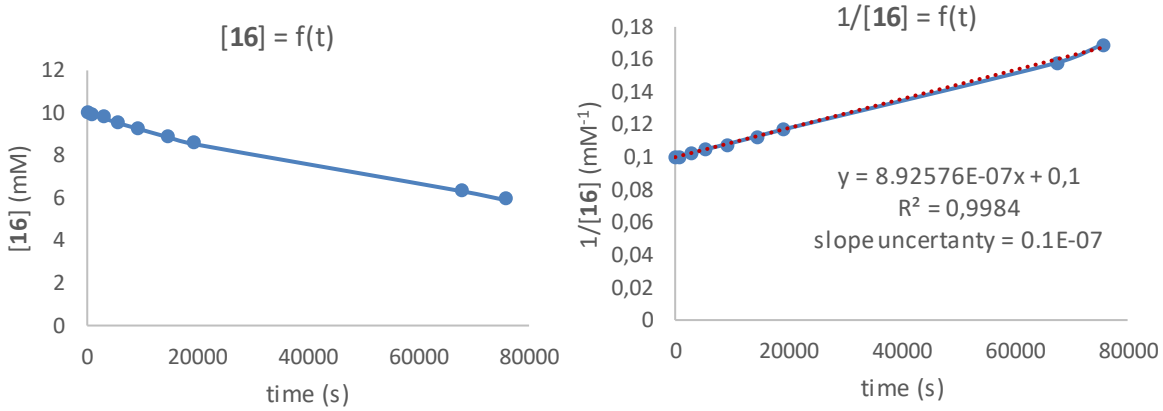
Slope = $\boldsymbol{k_2}$ **= (8.9 ± 0.7) * 10^{-3} M^{-1}.s^{-1}**

B/ Diene **3** ($[\mathbf{16}]_0 = [\mathbf{3}]_0 = 5$ mM)



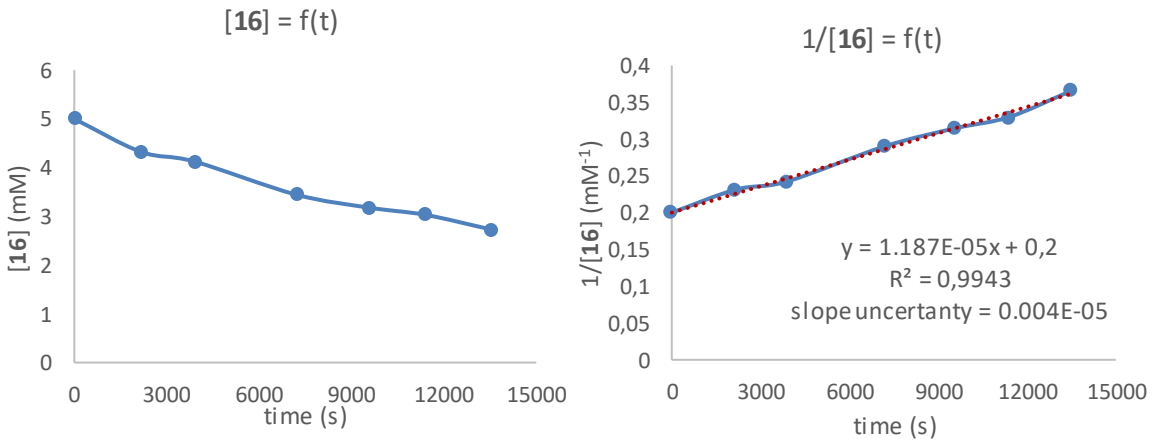
Slope = $\boldsymbol{k_2 = 0.15 \pm 0.01\ M^{-1}.s^{-1}}$

C/ Diene **6** ($[\mathbf{16}]_0 = [\mathbf{6}]_0 = 10$ mM)



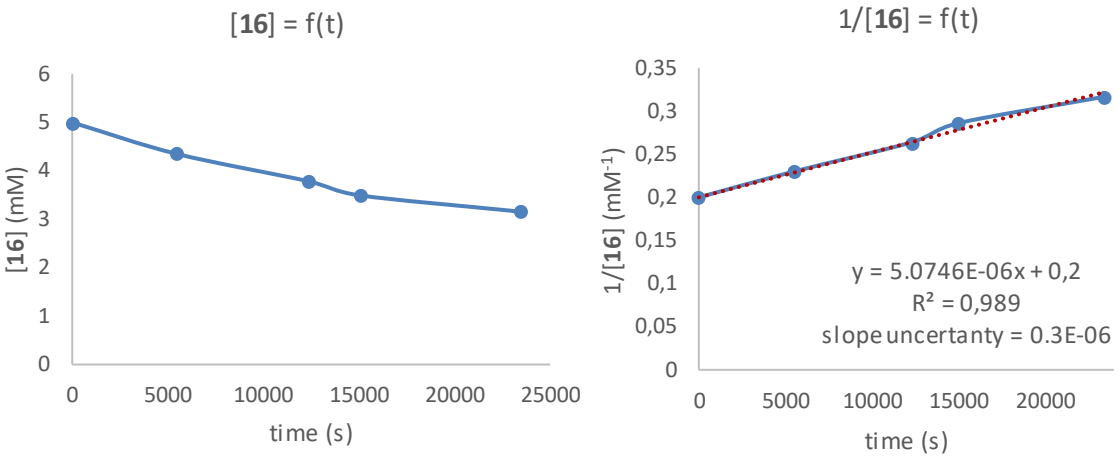
Slope = $\boldsymbol{k_2 = (8.9 \pm 0.1) * 10^{-4}\ M^{-1}.s^{-1}}$

D/ Diene **8** ($[\mathbf{16}]_0 = [\mathbf{8}]_0 = 5$ mM)



Slope = k_2 = **(1.2 ± 0.4) * 10^{-2} $M^{-1}.s^{-1}$**

E/ Diene **9** ($[\mathbf{16}]_0 = [\mathbf{9}]_0 = 5$ mM)



Slope = k_2 = **(5.1 ± 0.3) * 10^{-3} $M^{-1}.s^{-1}$**

Figure S25. Second order kinetic rate constant calculations for (A) diene **1**, (B) diene **3**, (C) diene **6**, (D) diene **8** and (E) diene **9**.

II.4. One-pot double labeling strategy with dienes **2** and **8**

a) Full experiment

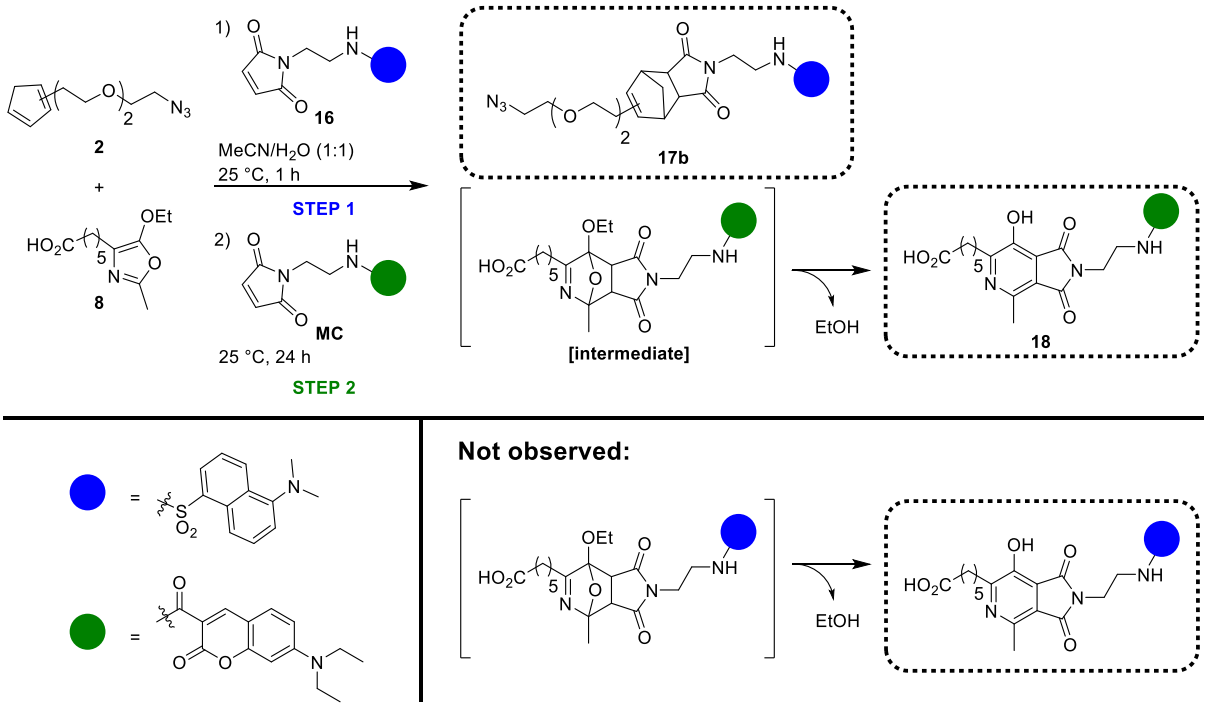


Figure S26. Sequential labeling experiment from dienes **2** and **8** and maleimides **16** and **MC**.

To a mixture of H_2O (250 µL) and MeCN (190 µL) were added a 25 mM solution of **2** (20 µL) and a 25 mM solution of **8** (20 µL). After 1 min of stirring, a 20 mM solution of **16** (25 µL, 1 equiv.) was added and the mixture was stirred at room temperature (T ≈ 25 °C). After completion of STEP 1 (1 h), maleimide-based coumarine **MC** (1 mg, 2.6 µmol, 5 equiv.) was added to the mixture for the STEP 2 and the reaction further stirred for 24 h at 25 °C. Diels-Alder reactions were monitored by RP-HPLC (system A).

Final concentration for time = 0:

- [**2**] = 1 mM (STEP 1)
- [**8**] = 1 mM (STEP 1 and 2)
- [**16**] = 1 mM (STEP 1)
- [**MC**] = 5 mM (STEP 2)
- 500 µL H_2O/MeCN (1:1, v:v)

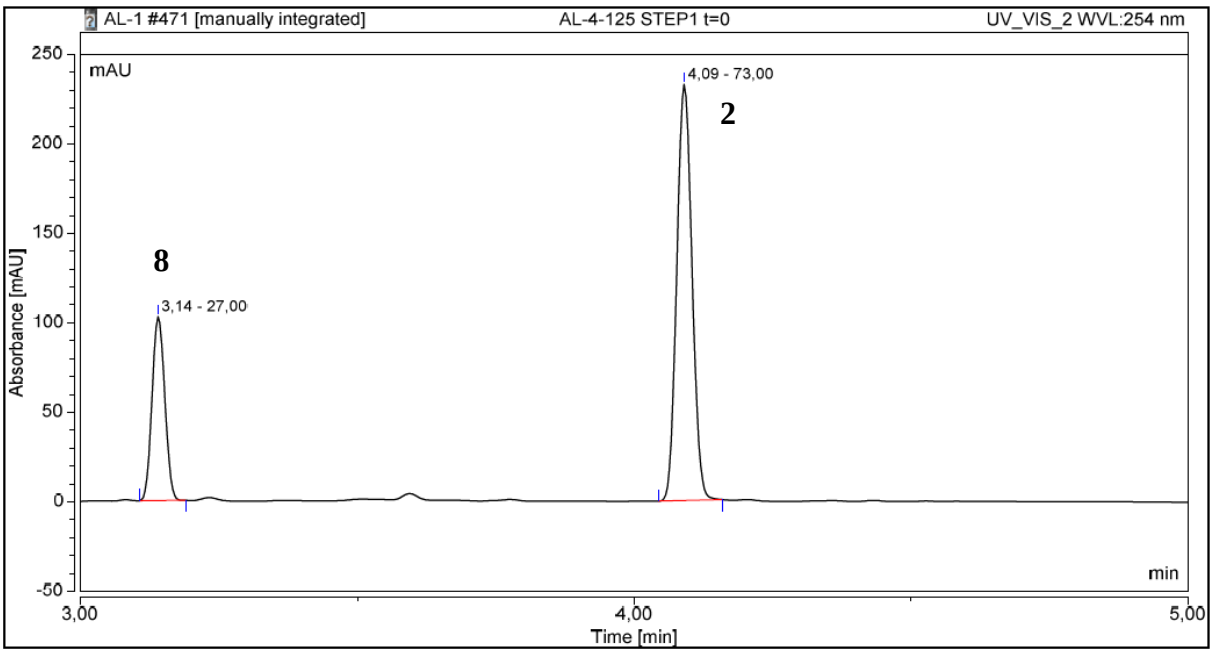


Figure S27. RP-HPLC chromatogram of a mixture of **2** and **8** (λ = 254 nm, sA) at T = 0.

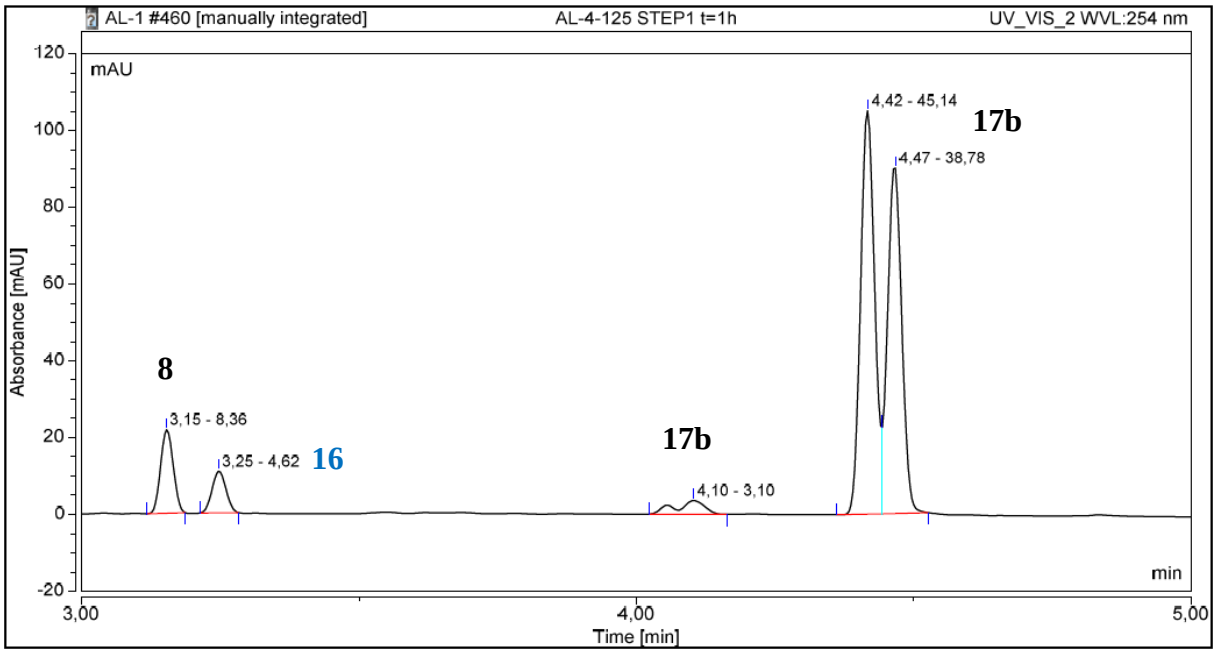


Figure S28. RP-HPLC chromatogram of a mixture of **2**, **8**, and **16** (λ = 254 nm, sA) at T = 1 h (STEP 1).

STEP 2, T = 8 h

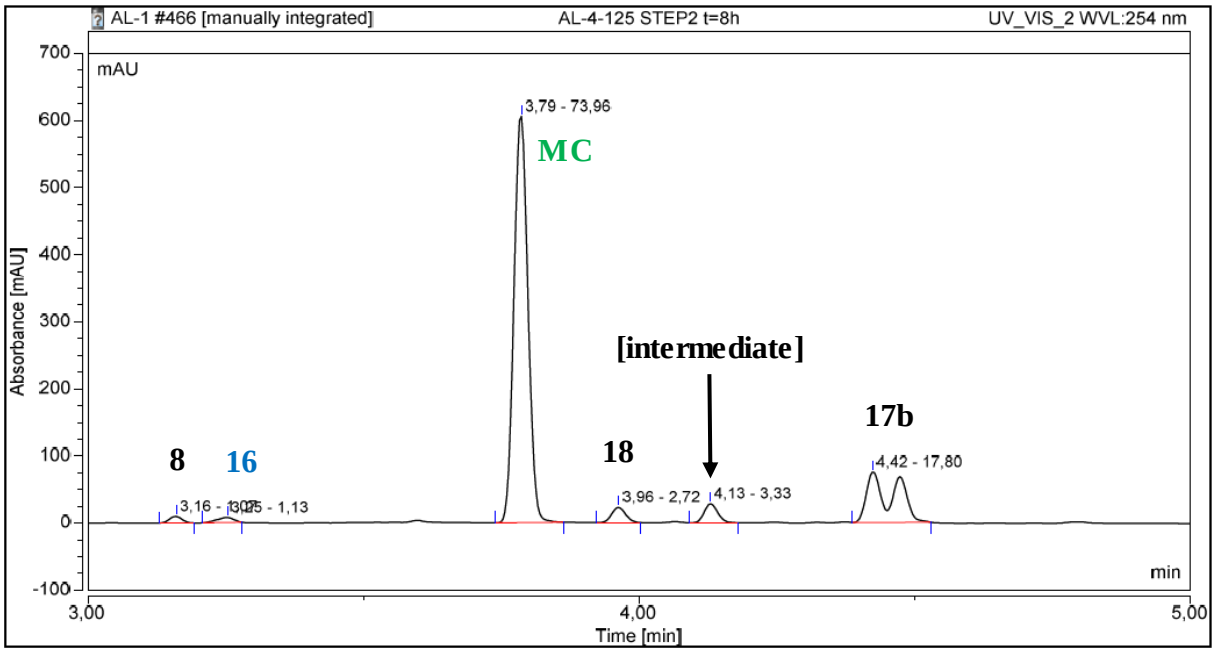


Figure S29. RP-HPLC chromatogram of a mixture of **2**, **8**, **16**, and **MC** (λ = 254 nm, sA) at T = 8 h (STEP 2).

STEP 2, T = 24 h

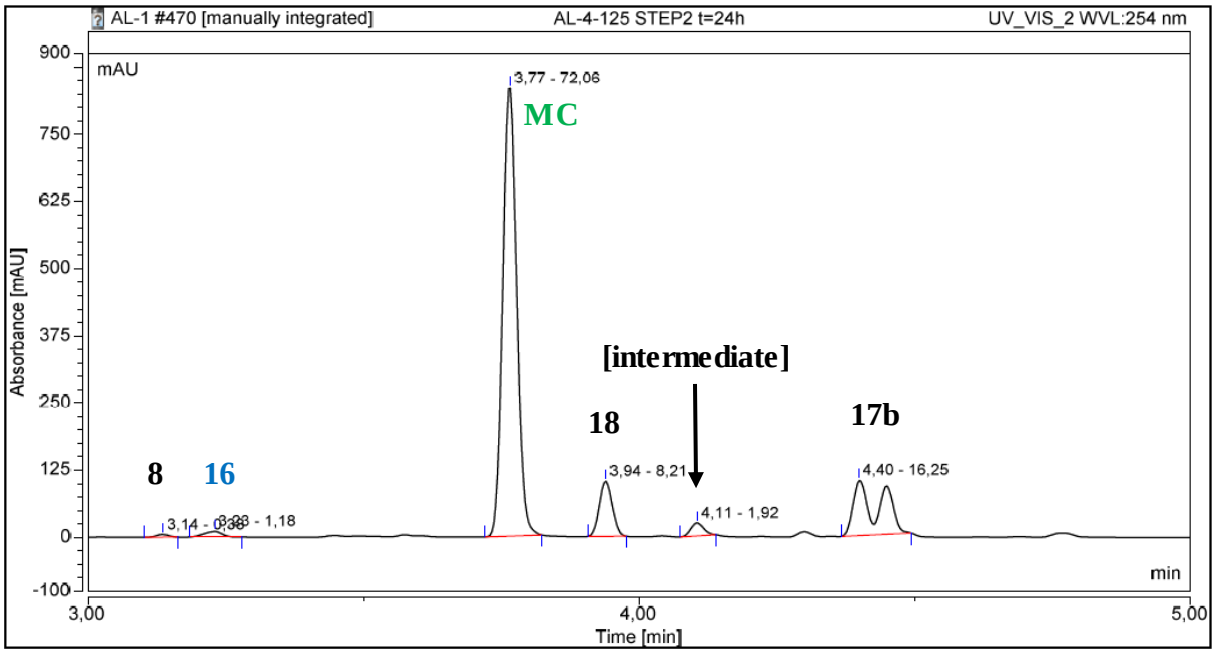


Figure S30. RP-HPLC chromatogram of a mixture of **2**, **8**, **16**, and **MC** (λ = 254 nm, sA) at T = 24 h (STEP 2).

b) Control experiment

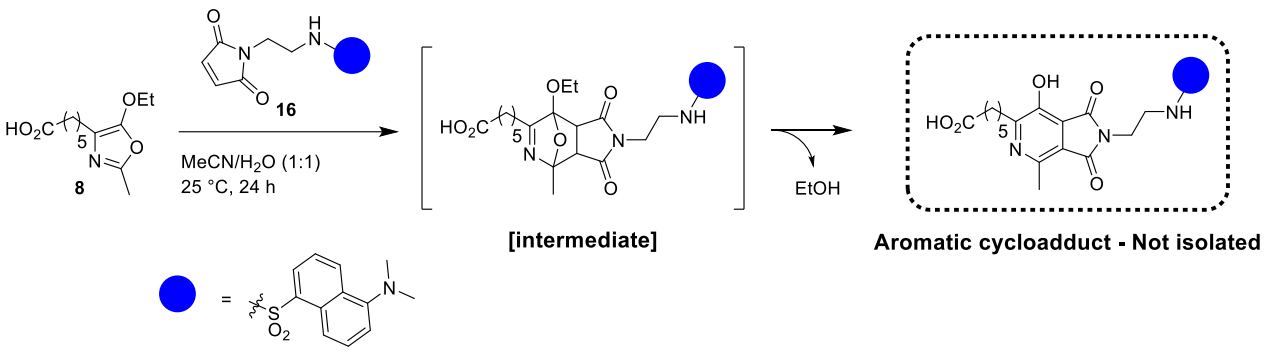


Figure S31. Control experiment regarding the plausible formation the azaphthalimide generated from the Diels-Alder reaction of **8** and **16**.

To a 25 mM solution of **8** (20 µL) in a solution of H_2O/MeCN (250:105 µL) was added a 20 mM solution of **16** (125 µL, 5 equiv.) and the mixture was stirred at room temperature (≈ 25 °C). Diels-Alder reactions were monitored by RP-HPLC after 5 and 24 h.

Final concentration for time = 0:

- [**8**] = 1 mM
- [**16**] = 5 mM
- 500 µL H_2O/MeCN (1:1, v:v)

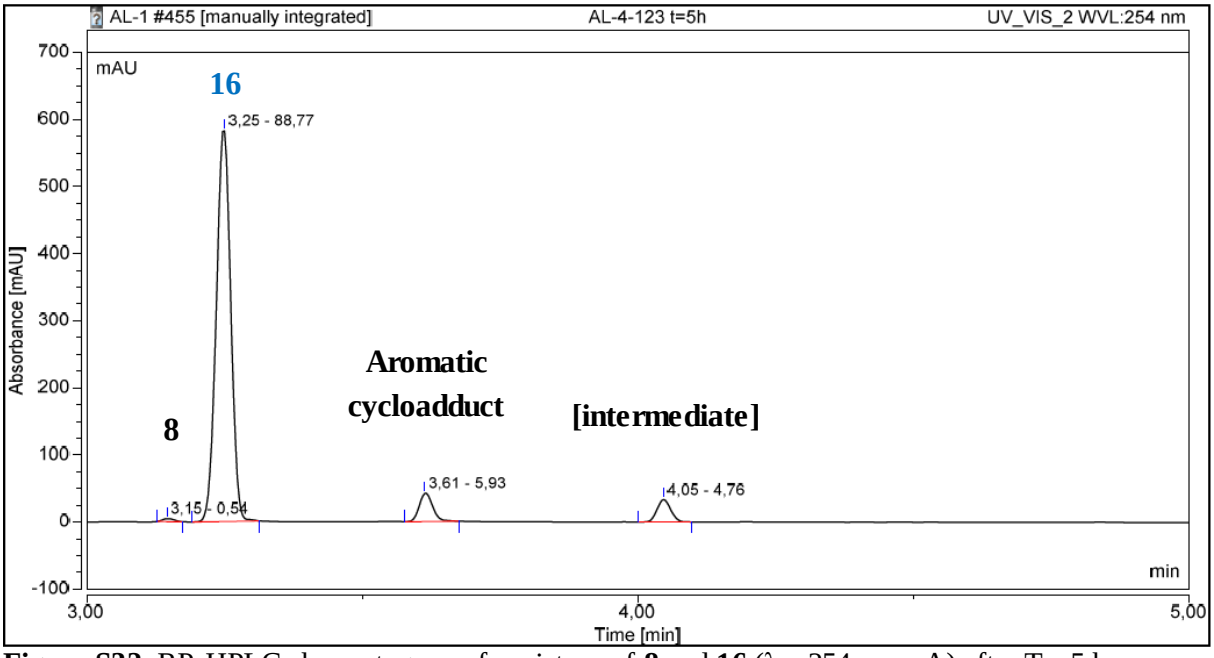


Figure S32. RP-HPLC chromatogram of a mixture of **8** and **16** (λ = 254 nm, sA) after T = 5 h.

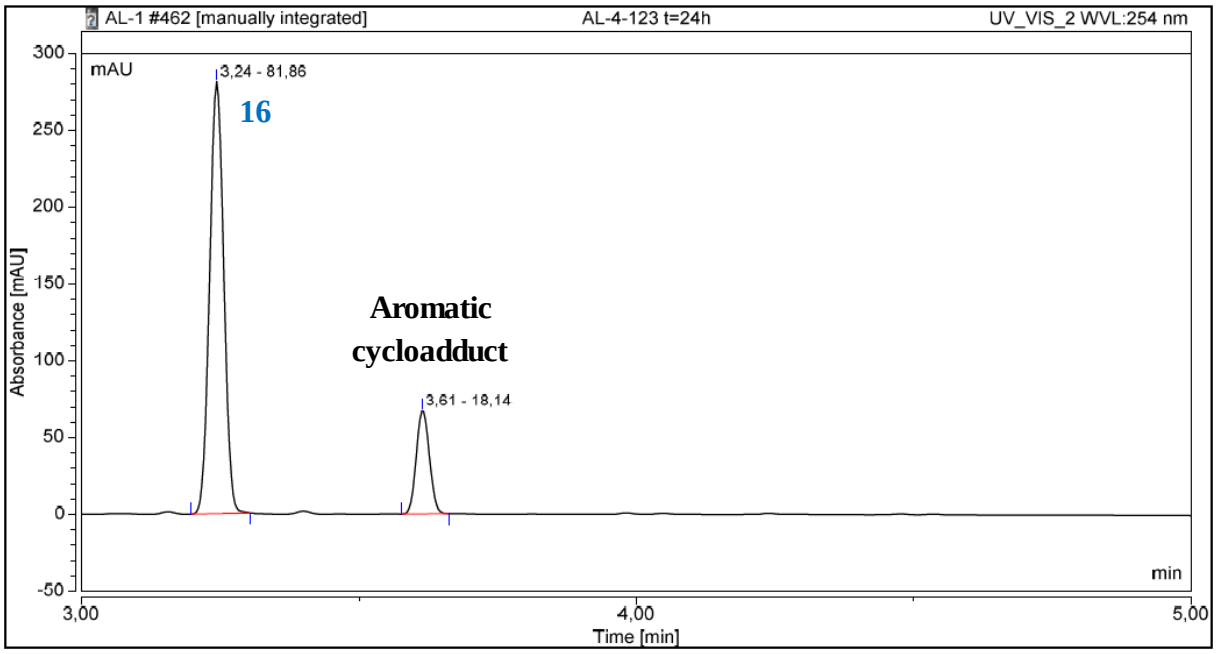


Figure S33. RP-HPLC chromatogram of a mixture of **8** and **16** (λ = 254 nm, sA) after T = 24 h.

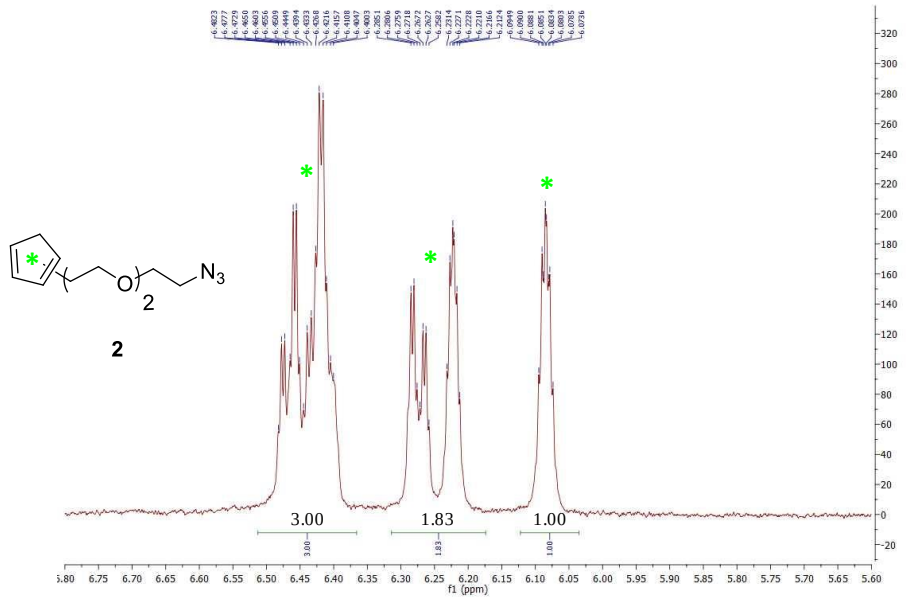
III. Study of stability of dienes 2, 3, 8, 9 and conjugates 19-24

III.1 Study of stability of dienes **2**, **3**, **8**, and **9**

All non-commercial dienes were conserved at -25 °C after their synthesis.

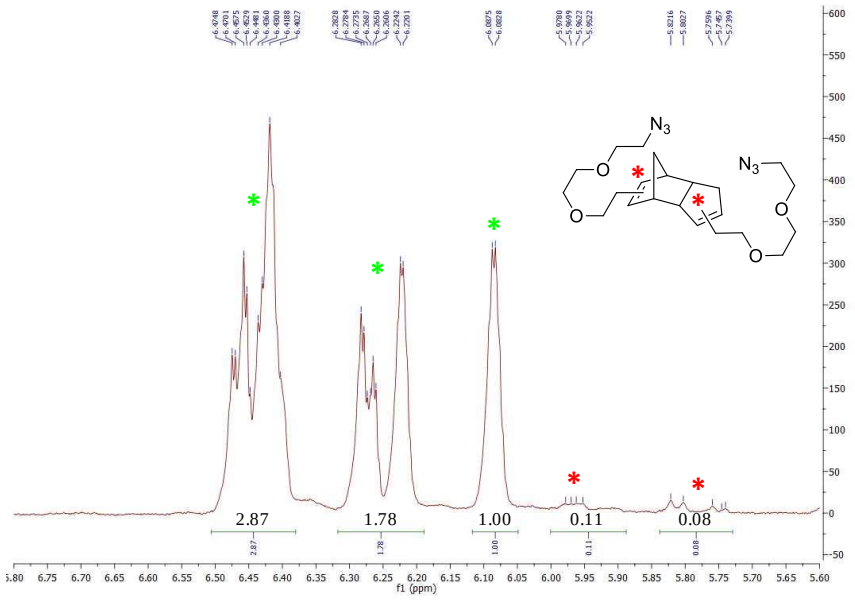
*a) Cyclopentadiene **2***

A/



2

B/



C/

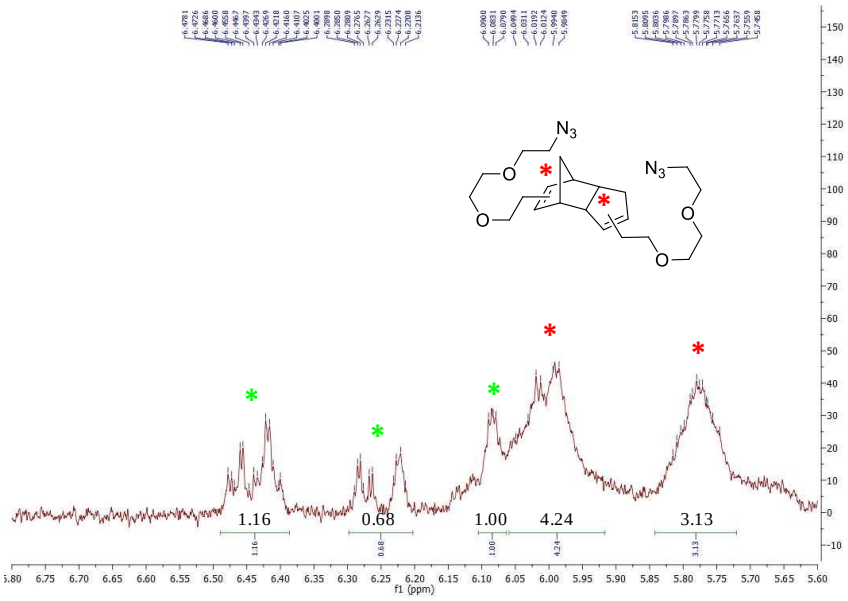


Figure S34. ^{1}H NMR analysis in $CDCl_3$ (300 MHz) of (A) isolated cyclopentadiene **2**; (B) cyclopentadiene **2** stored for 9 months at -25 °C; (C) cyclopentadiene **2** stored for 36 h at room temperature.

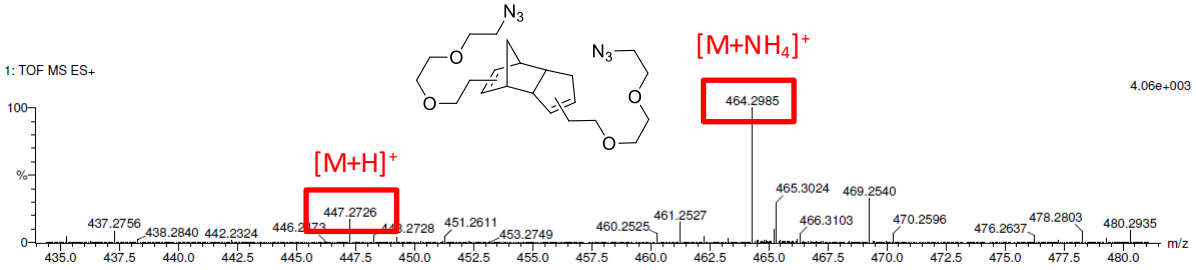
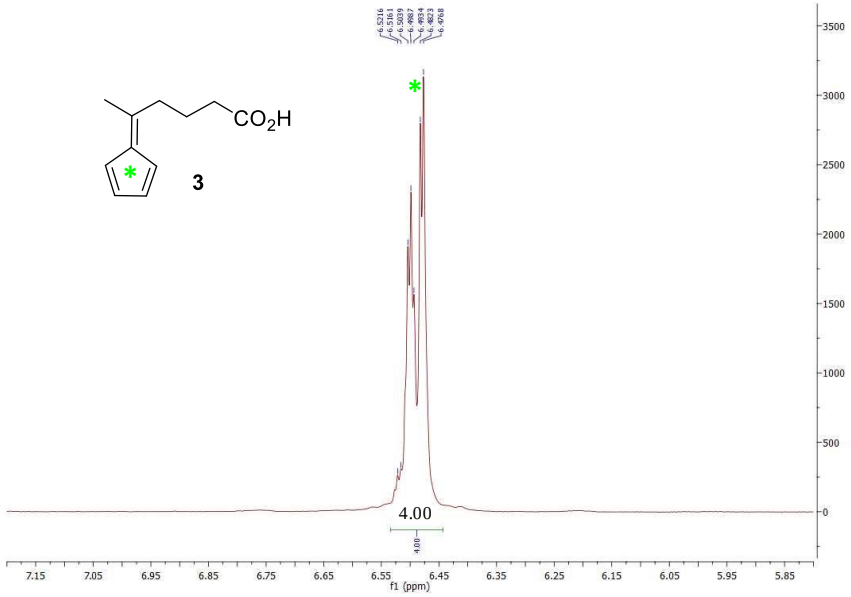


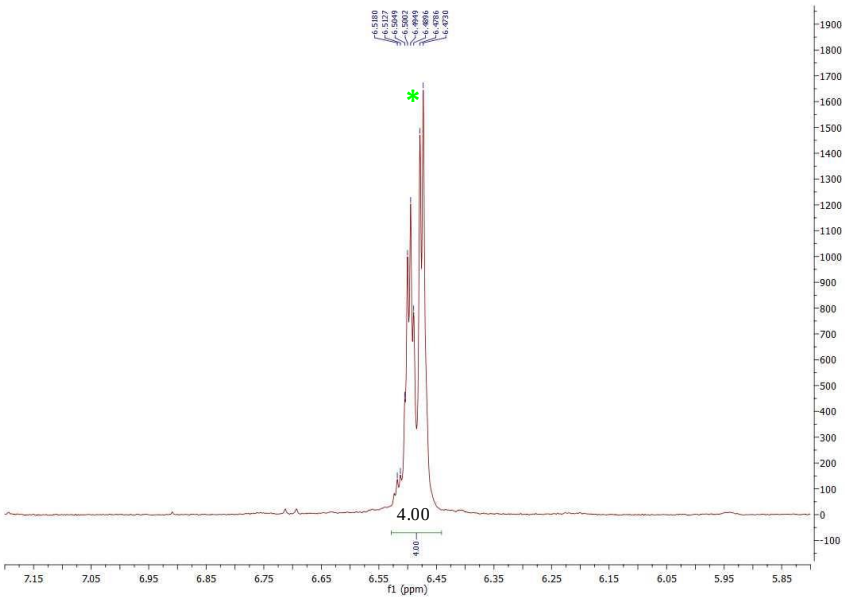
Figure S35. HRMS (ESI+) mass spectrum of compound **2** after 36 h at room temperature.

*b) Fulvene **3***

A/



B/



C/

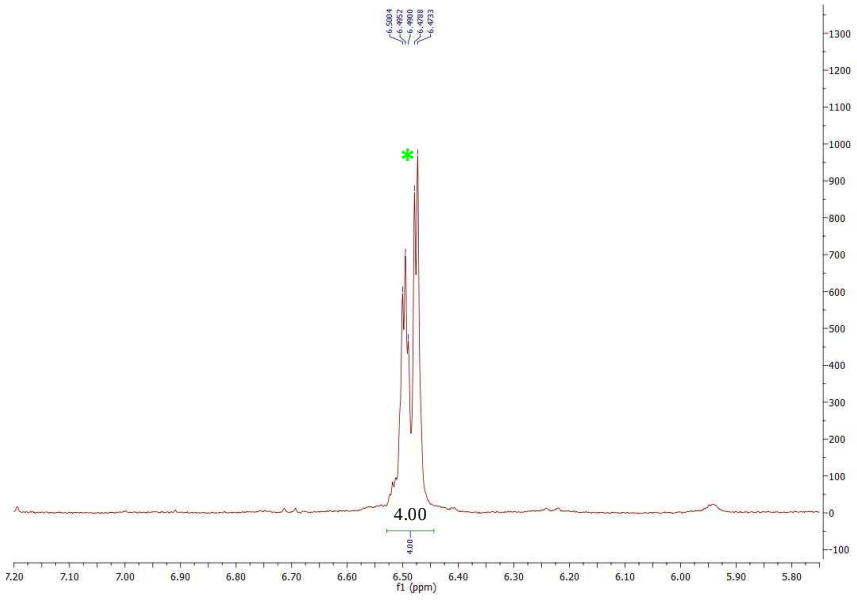
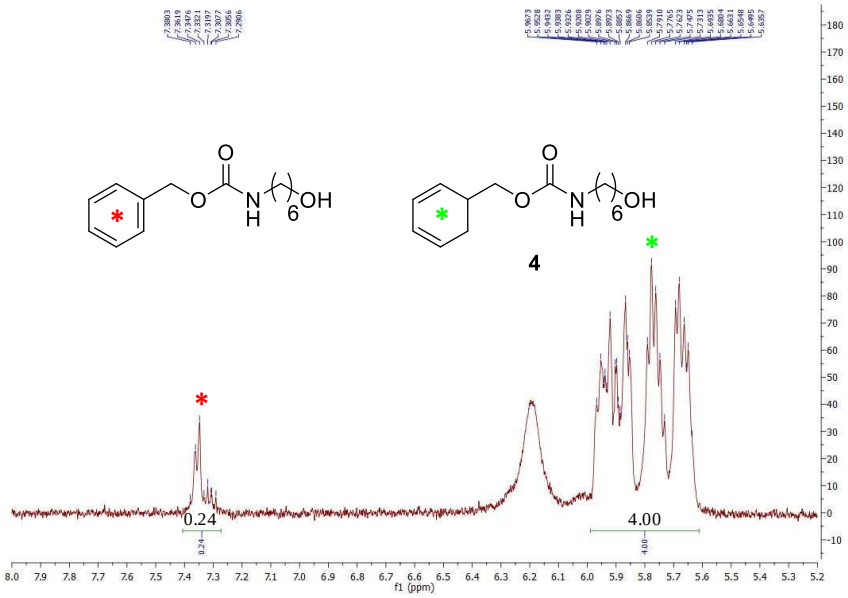


Figure S36. ^{1}H NMR analysis in $CDCl_3$ (300 MHz) of (A) isolated fulvene **3**; (B) fulvene **3** stored for 36 h at room temperature; (C) fulvene **3** stored for 16 days at room temperature.

*c) Cyclohexadiene **4***

A/



B/

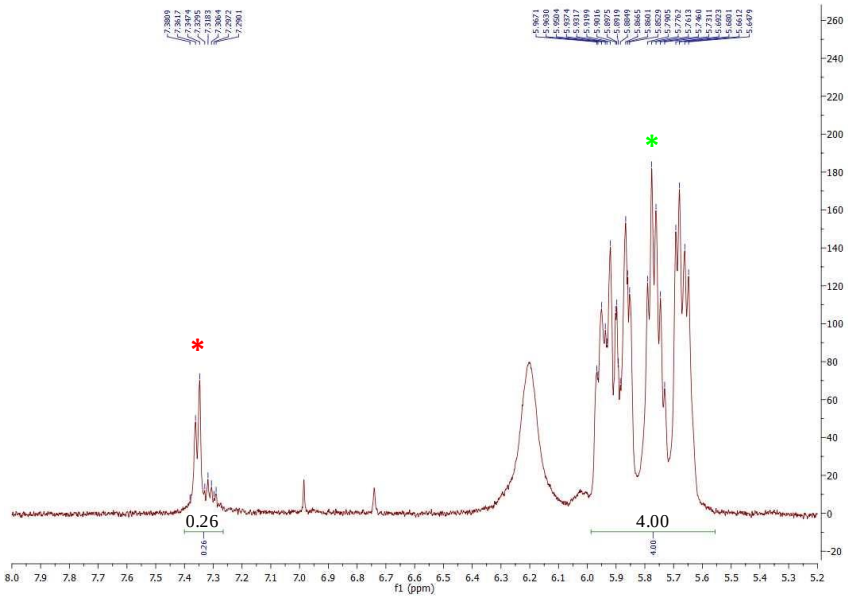
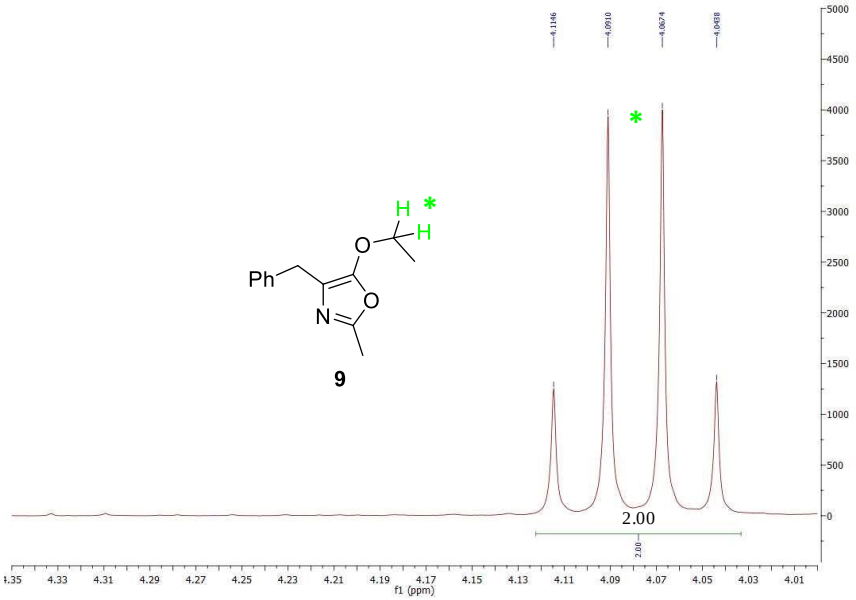


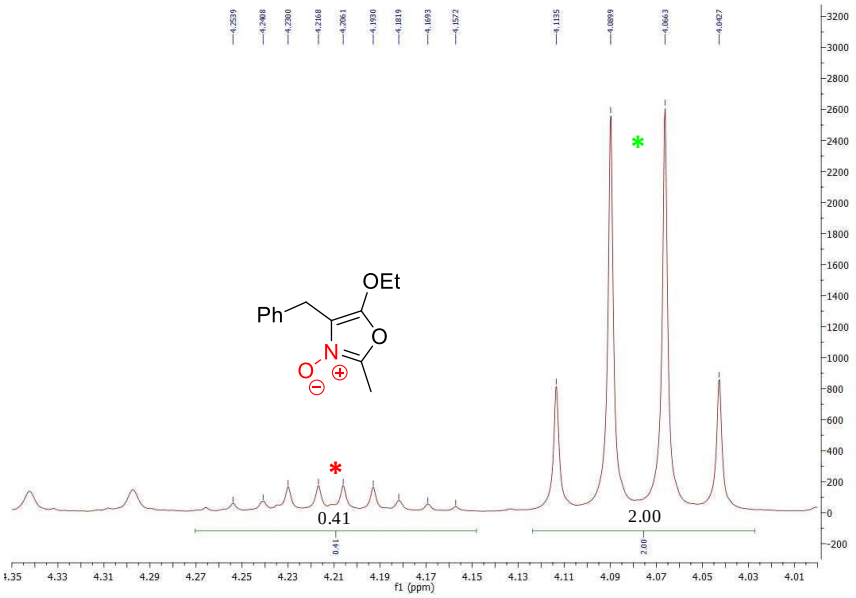
Figure S37. ^{1}H NMR analysis in $CDCl_3$ (300 MHz) of (A) isolated cyclohexadiene **4**; (B) cyclohexadiene **4** stored for 3 months at – 25 °C.

*d) Oxazole **9***

A/



B/



C/

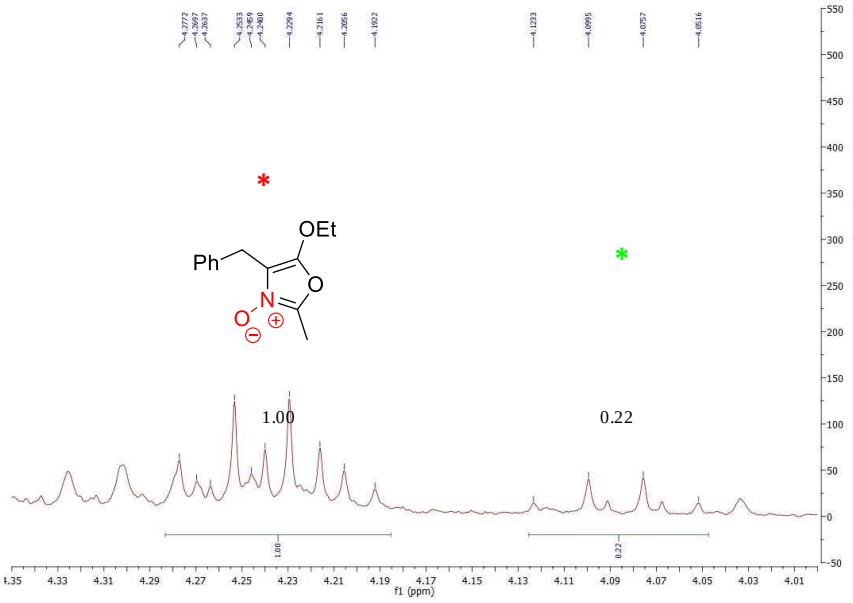
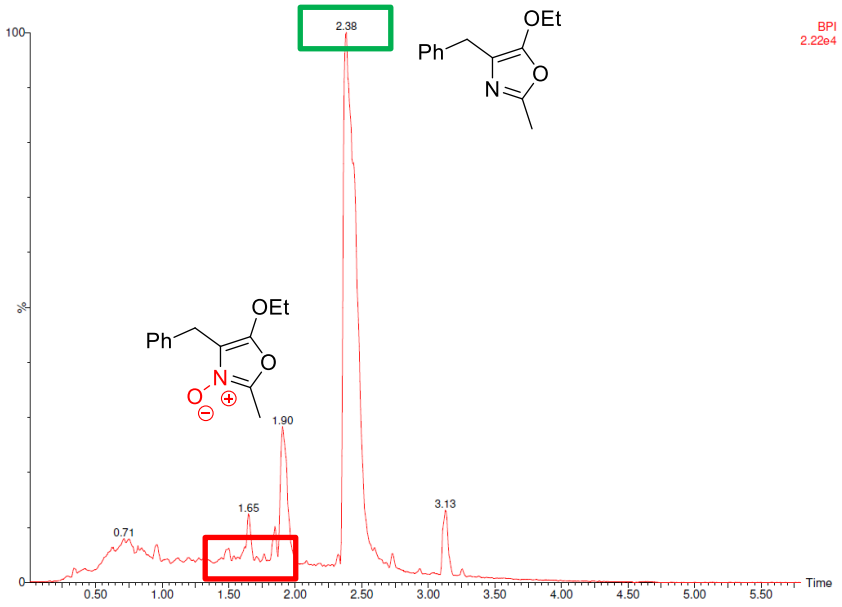
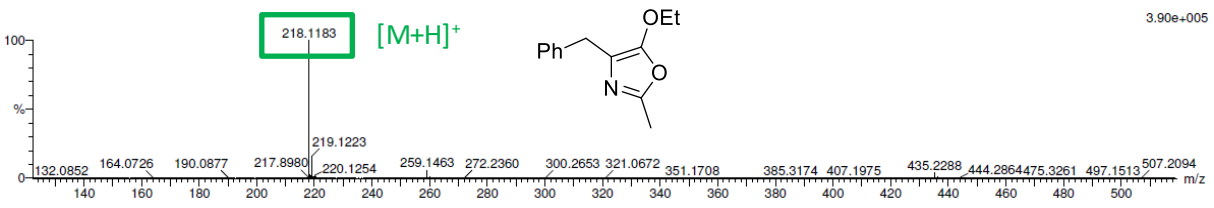


Figure S38. ^{1}H NMR analysis in $CDCl_3$ (300 MHz) of (A) isolated oxazole **9**; (B) oxazole **9** with AcOH (1 equiv.) stored for 9 months at – 25 °C; (C) oxazole **9** stored for 9 months at – 25 °C.

A/



B/



C/

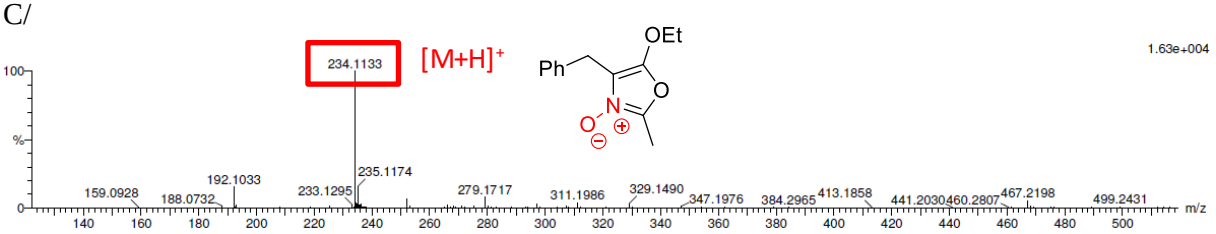
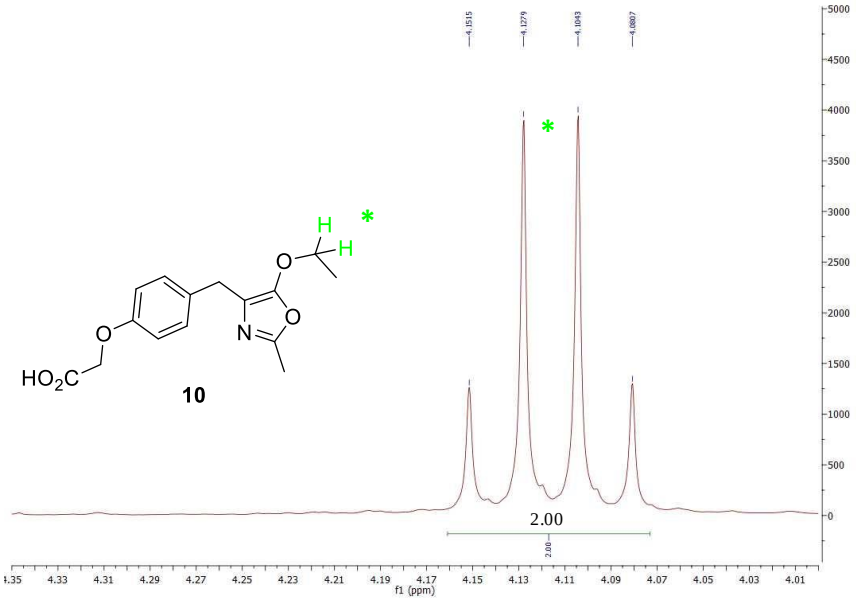


Figure S39. (A) TIC chromatogram of compound **9**, (B) HRMS (ESI+) mass spectrum of compound **9** at t = 2.38 min, (C) HRMS (ESI+) mass spectrum of compound **9** at t = 1.65 min, after 36 h at room temperature.

*e) Oxazole **10***

A/



B/

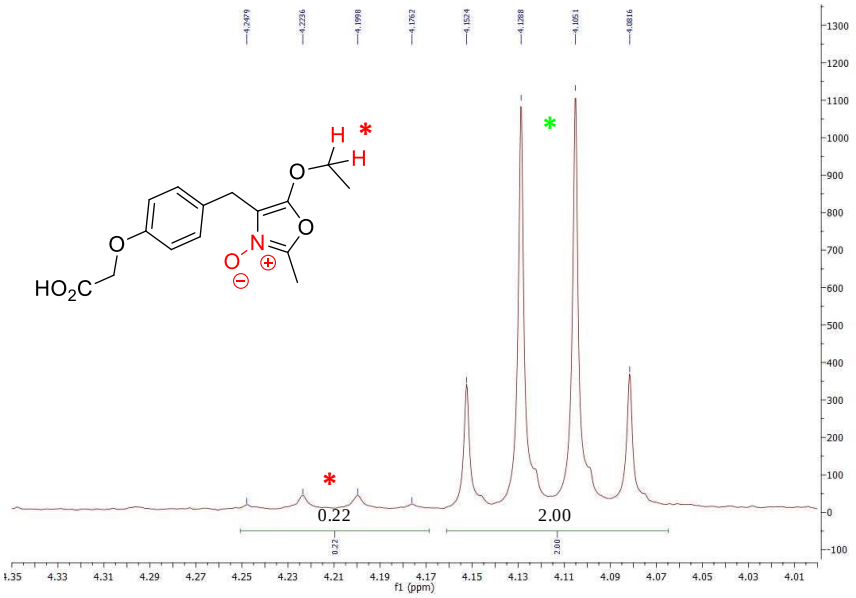


Figure S40. ^{1}H NMR analysis in $CDCl_3$ (300 MHz) of (A) isolated oxazole **10**; (B) oxazole **10** stored for 9 months at – 25 °C.

III.2. Study of stability of conjugates **20-26**

*a) LC-MS (System C) of starting conjugates **20-26***

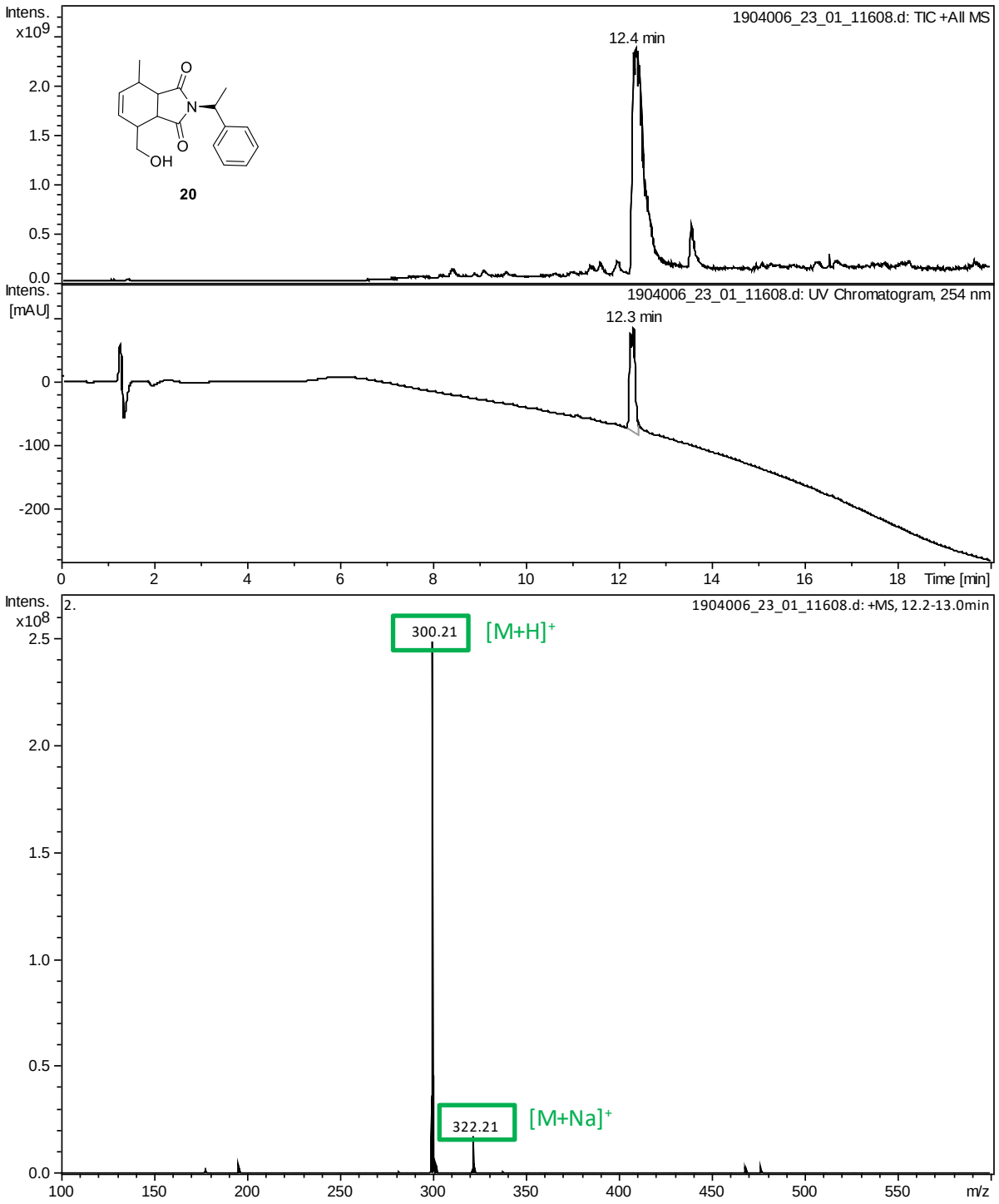
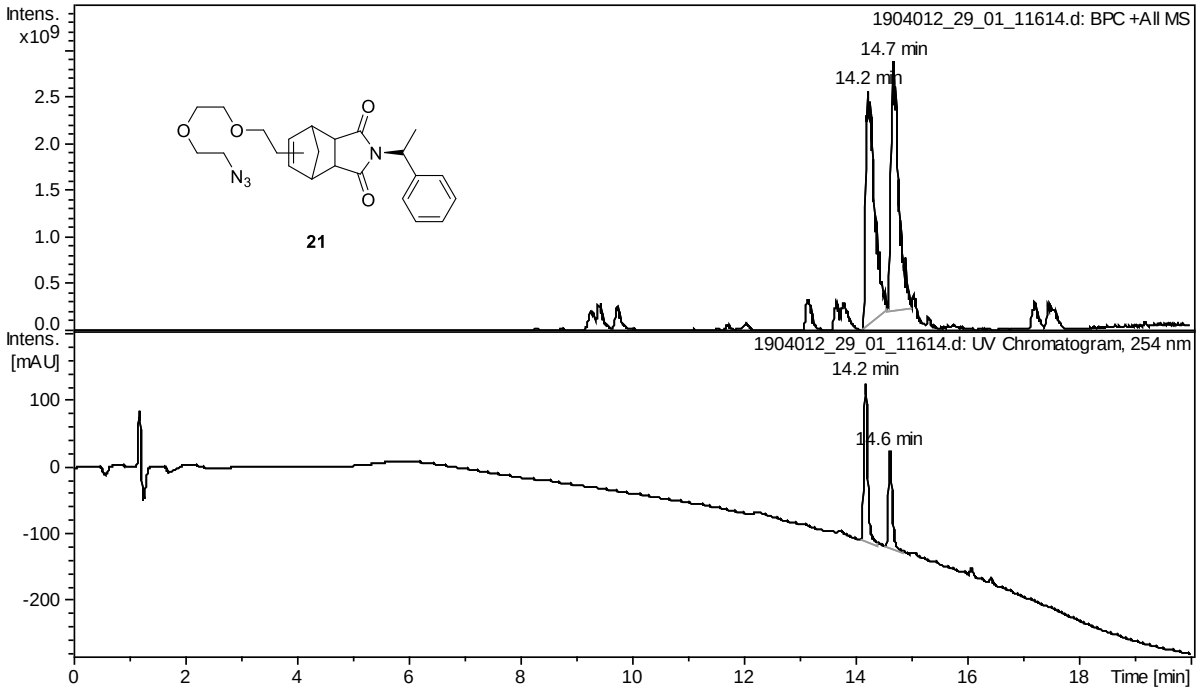
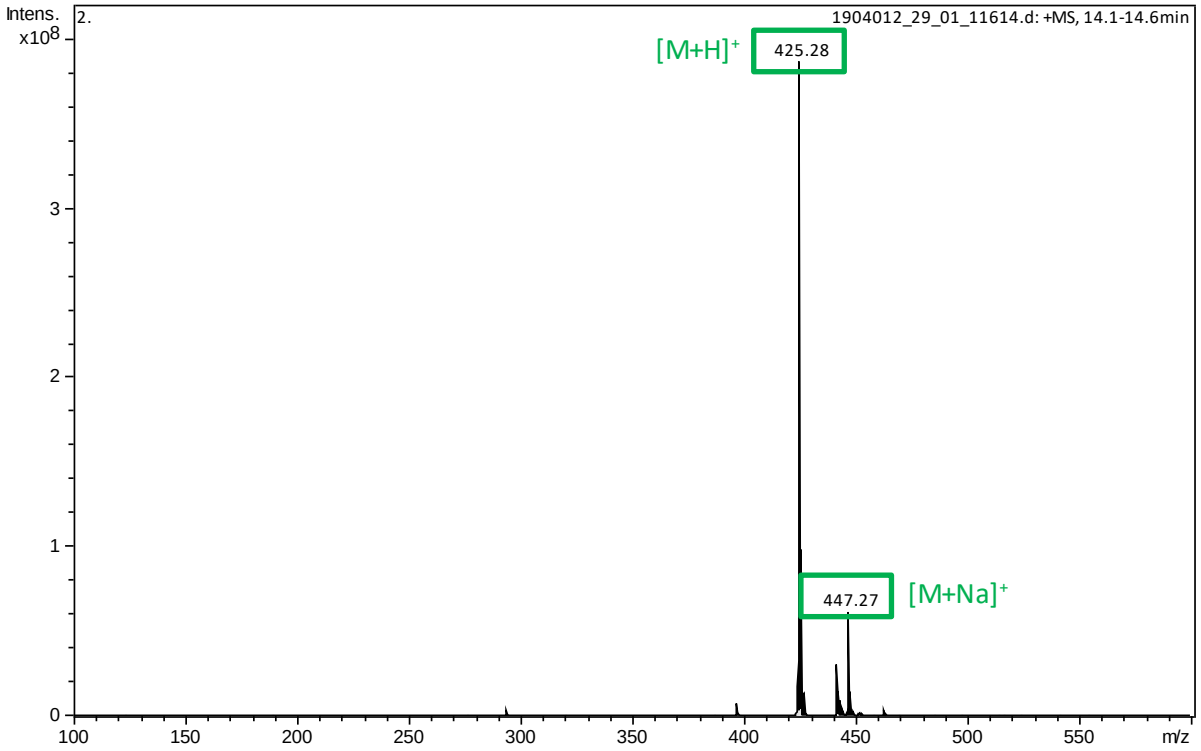


Figure S41. TIC, UV (254 nm) chromatograms and HRMS (ESI+) mass spectrum of compound **20**.


Intens.
x10^9
1904012_29_01_11614.d: BPC +All MS
14.7 min
14.2 min
21
Intens.
[mAU]
1904012_29_01_11614.d: UV Chromatogram, 254 nm
14.2 min
14.6 min
Time [min]


Intens.
x10^8
2.
1904012_29_01_11614.d: +MS, 14.1-14.6min
[M+H]+
425.28
447.27
[M+Na]+
m/z

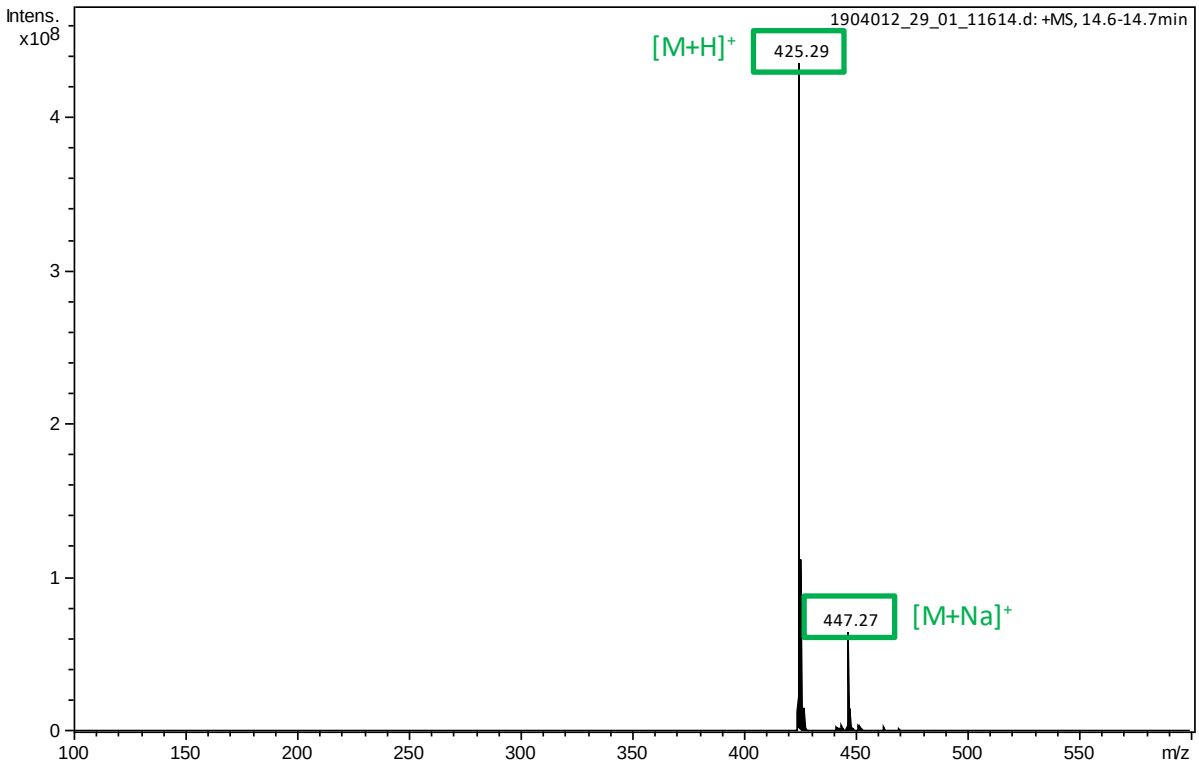


Figure S42. TIC UV (254 nm) chromatograms and HRMS (ESI+) mass spectra of compound **21**.

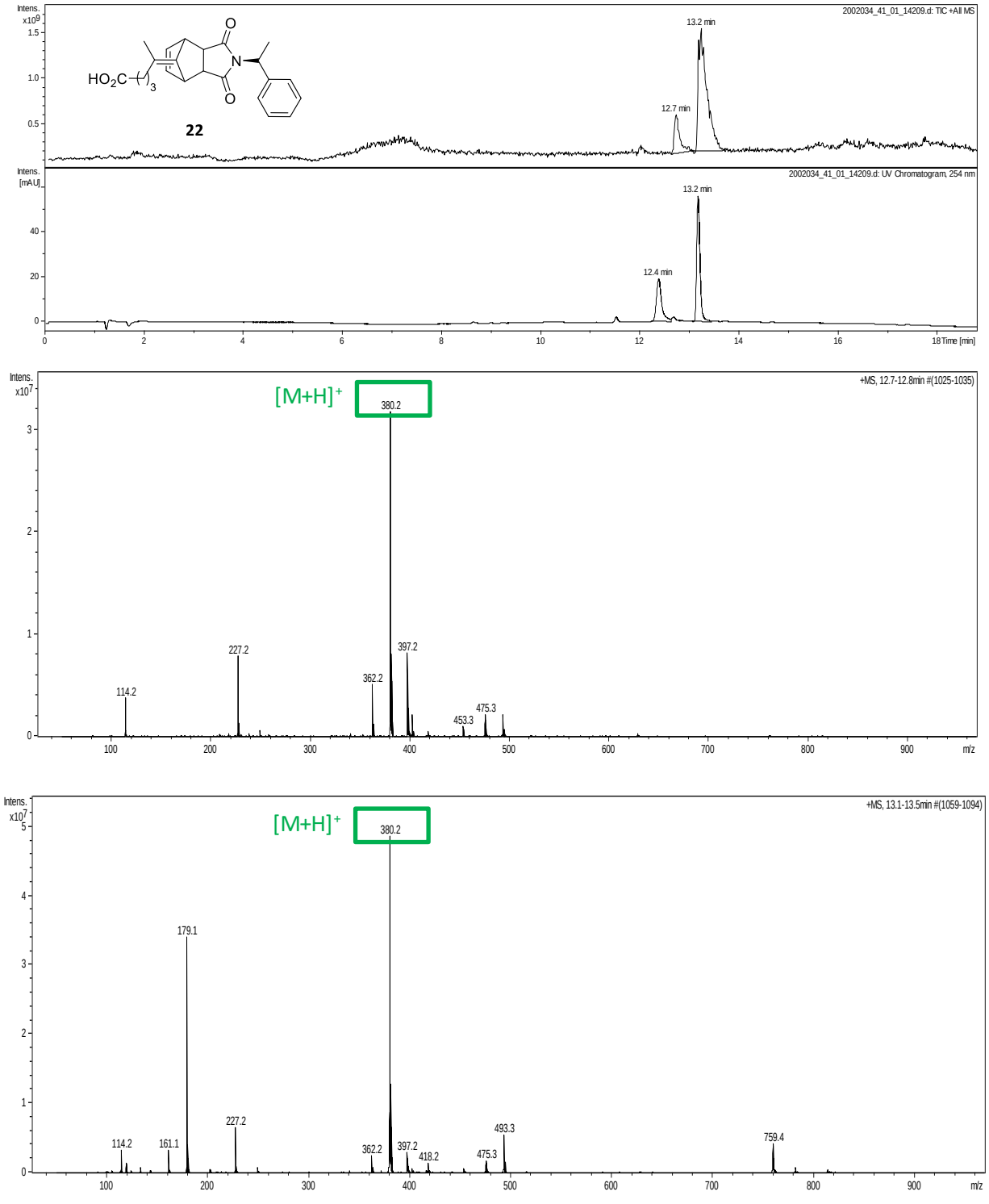
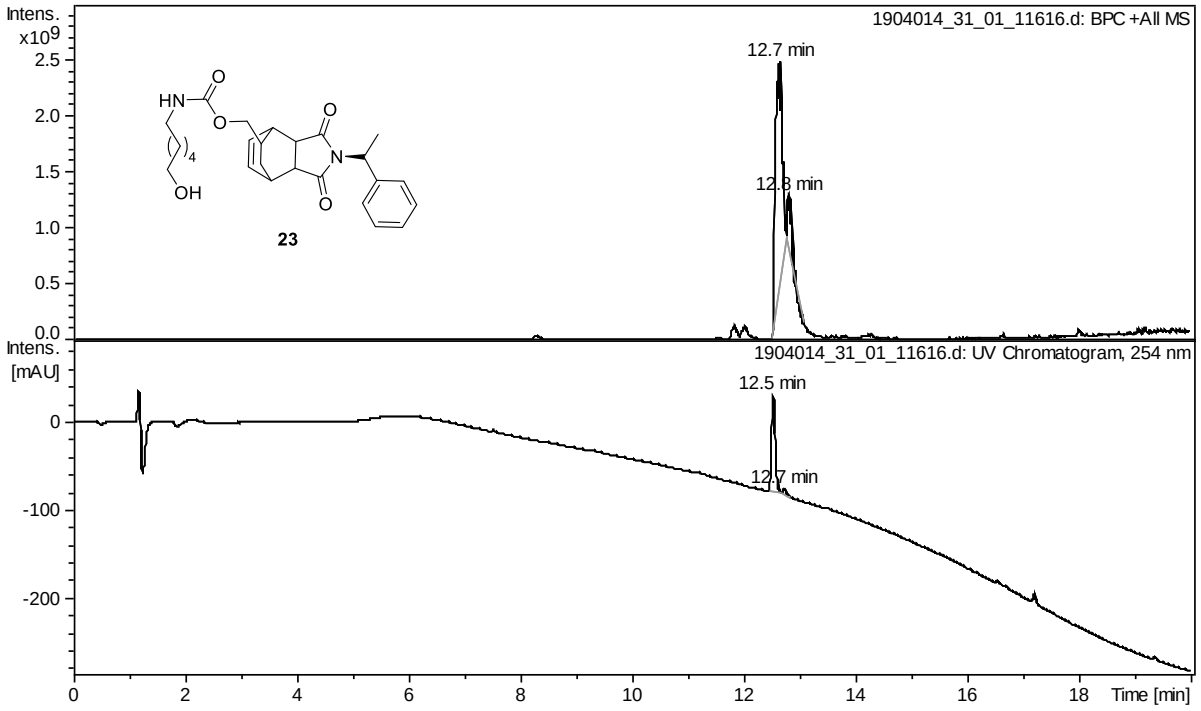
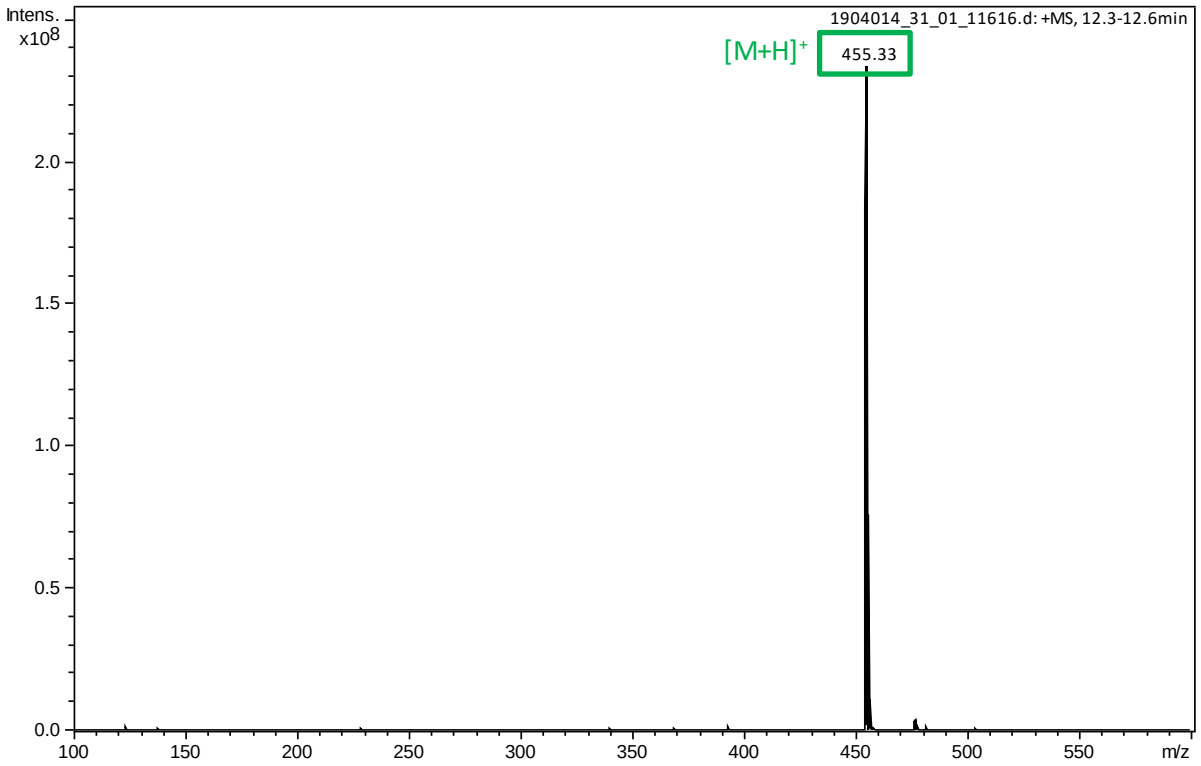


Figure S43. TIC UV (254 nm) chromatograms and HRMS (ESI+) mass spectra of compound **22**.


Intens.
x10^9
2.5
2.0
1.5
1.0
0.5
0.0
1904014_31_01_11616.d: BPC +All MS
12.7 min
12.8 min
HN
O
O
O
N
O
4
OH
23
Intens.
[mAU]
0
-100
-200
1904014_31_01_11616.d: UV Chromatogram, 254 nm
12.5 min
12.7 min
0
2
4
6
8
10
12
14
16
18
Time [min]


Intens.
x10^8
2.0
1.5
1.0
0.5
0.0
1904014_31_01_11616.d: +MS, 12.3-12.6min
[M+H]+
455.33
100
150
200
250
300
350
400
450
500
550
m/z

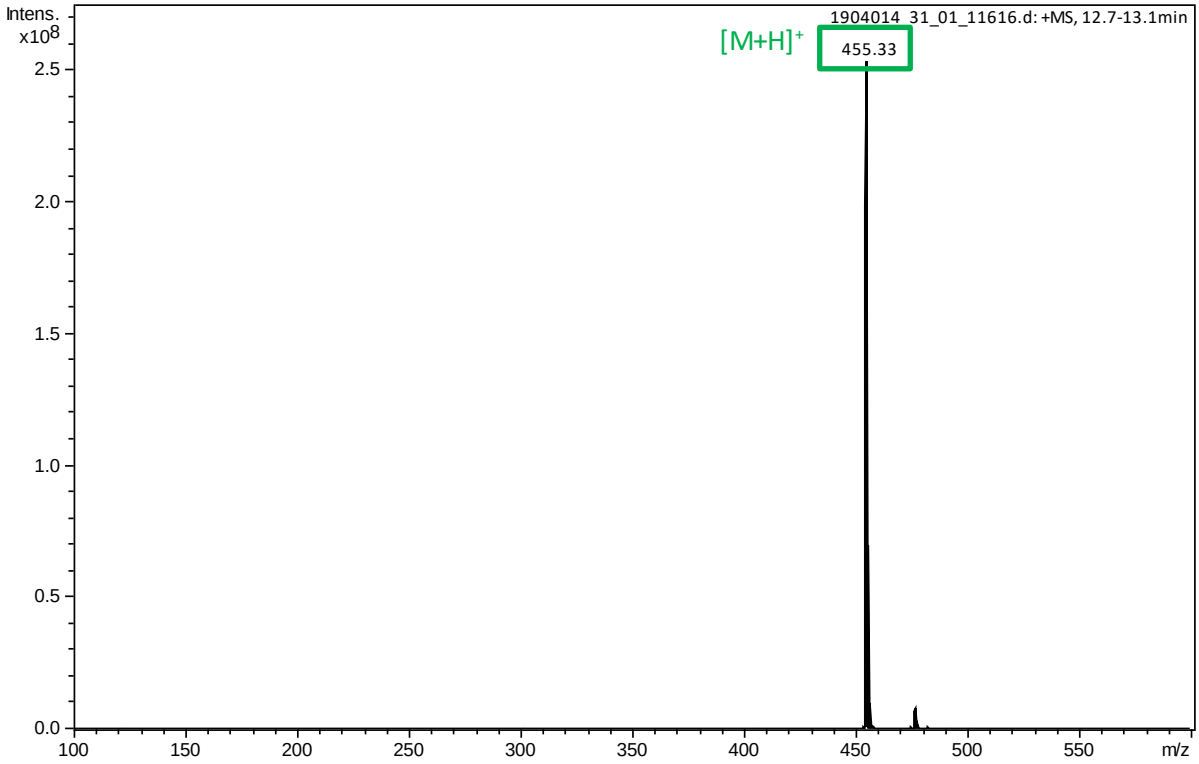


Figure S44. TIC, UV (254 nm) chromatograms and HRMS (ESI+) mass spectra of compound **23**.

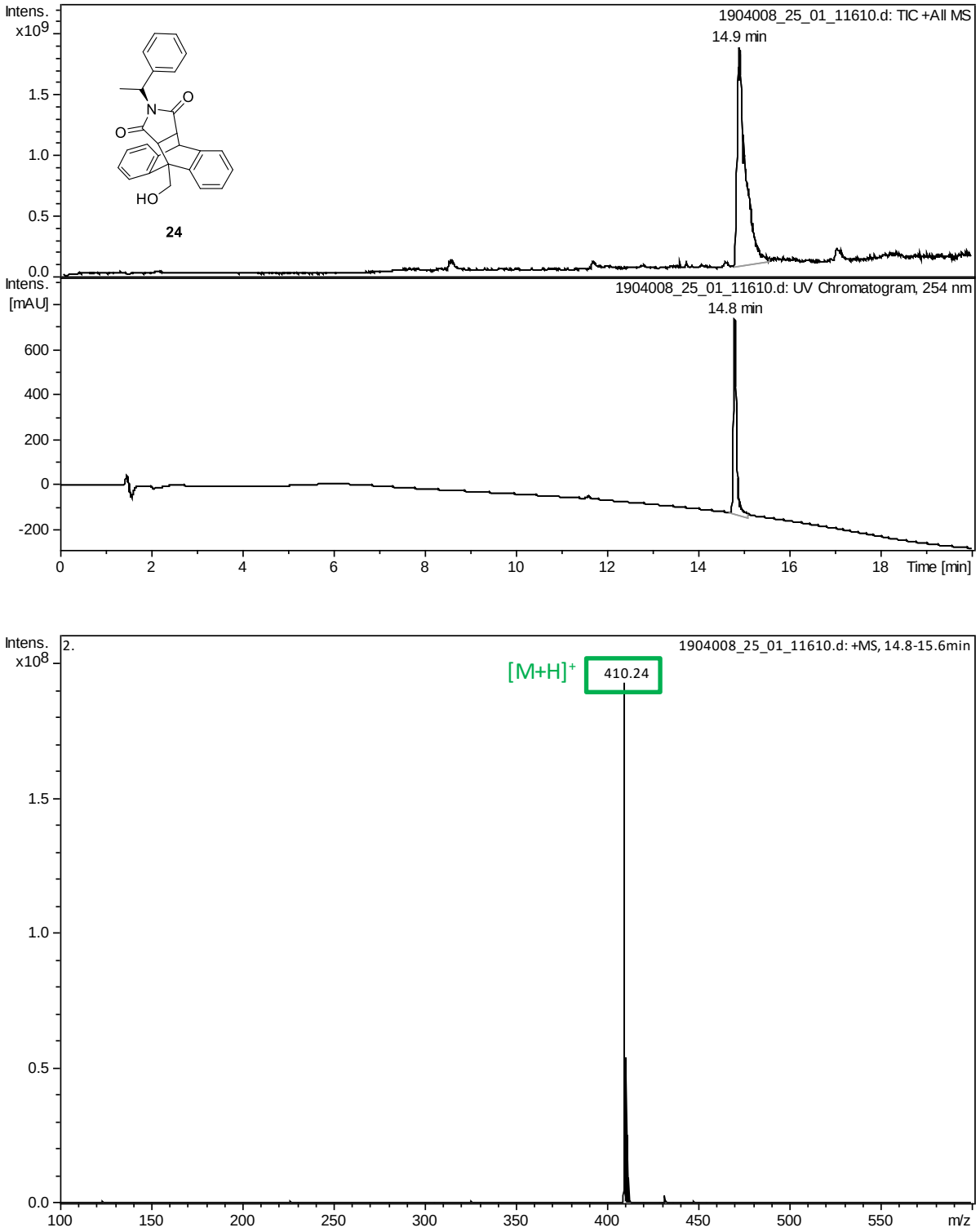


Figure S45. TIC, UV (254 nm) chromatograms and HRMS (ESI+) mass spectrum of compound **24**.

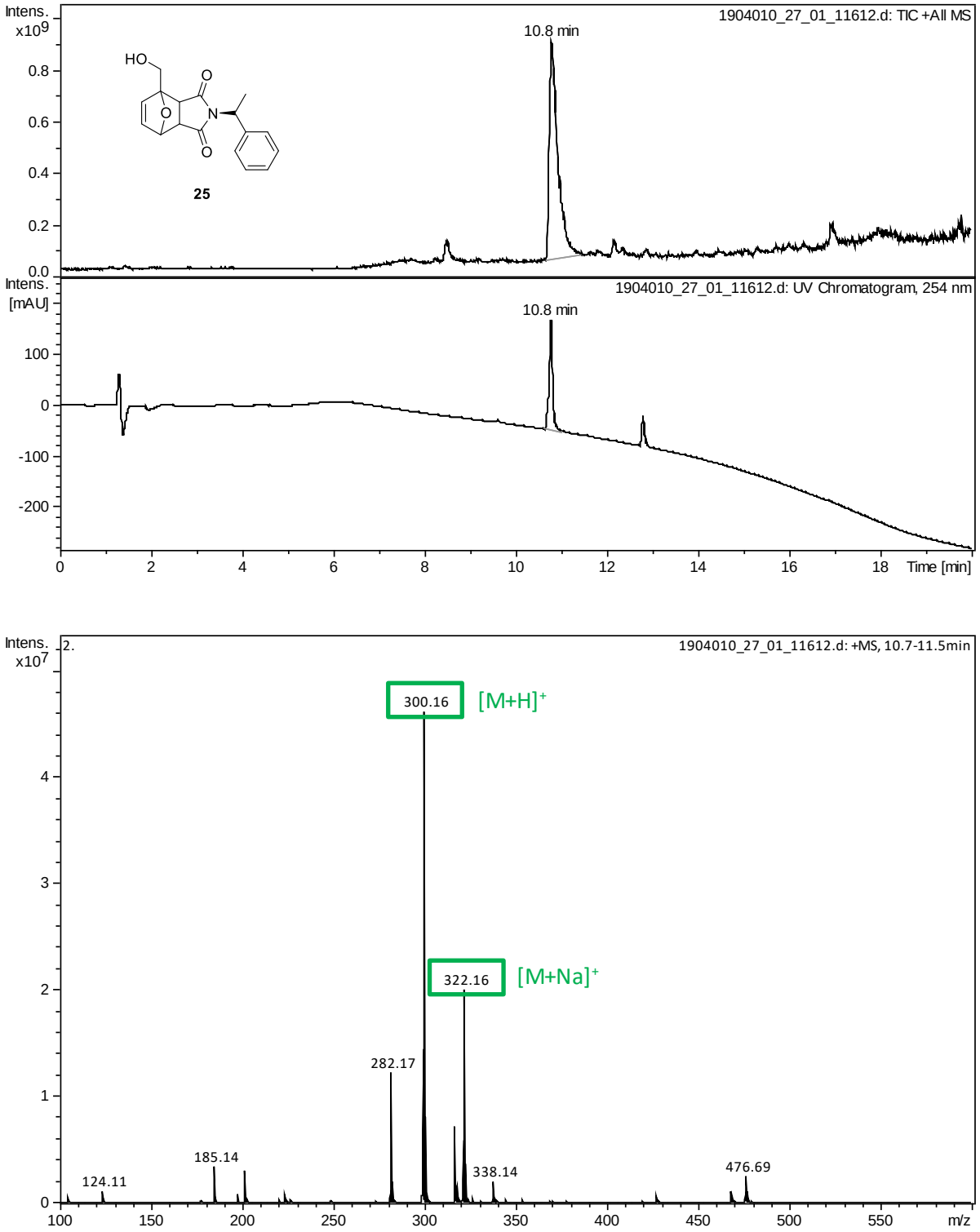


Figure S46. TIC, UV (254 nm) chromatograms and HRMS (ESI+) mass spectrum of compound **25**.

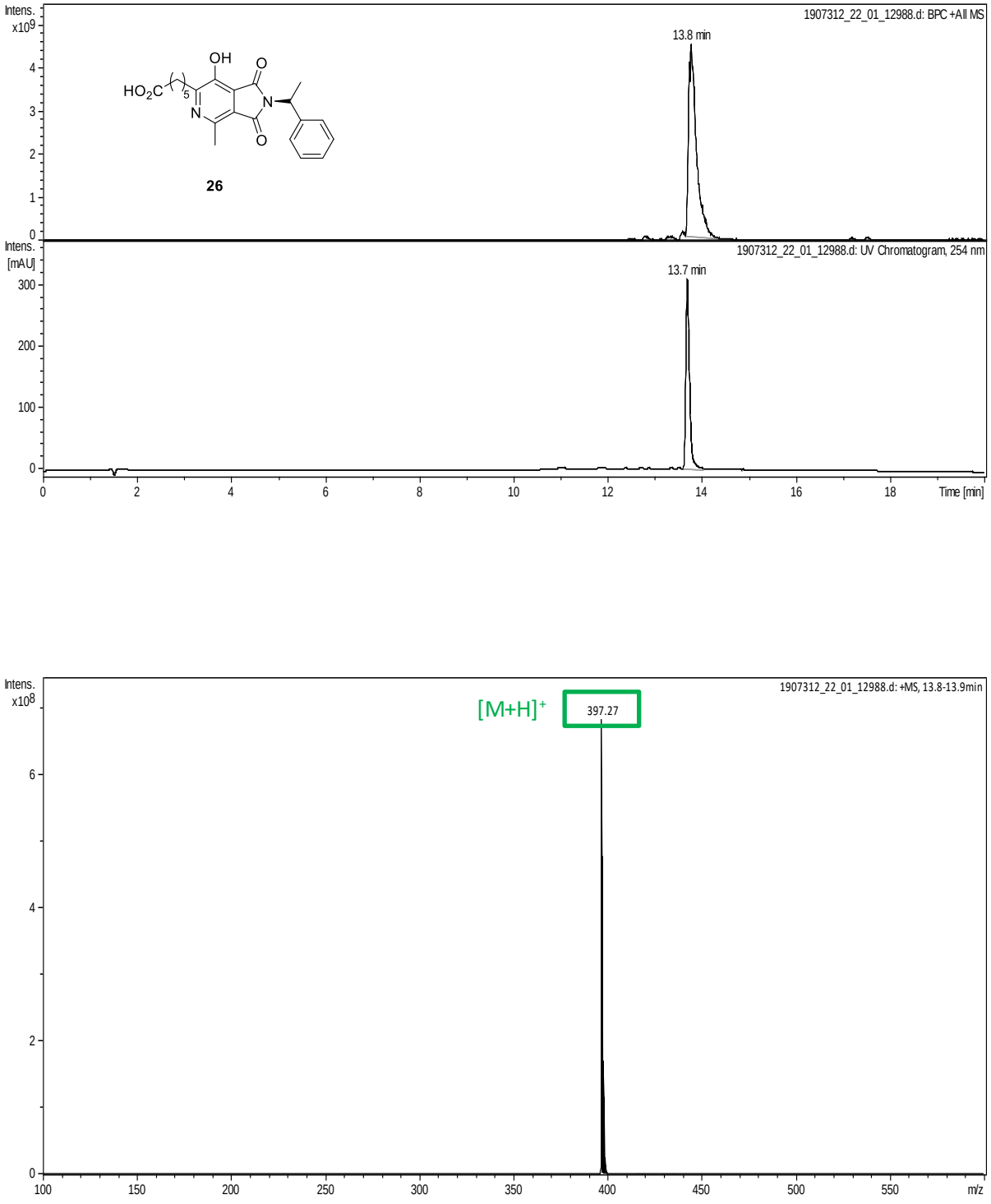


Figure S47. TIC, UV (254 nm) chromatograms and HRMS (ESI+) mass spectrum of compound **26**.

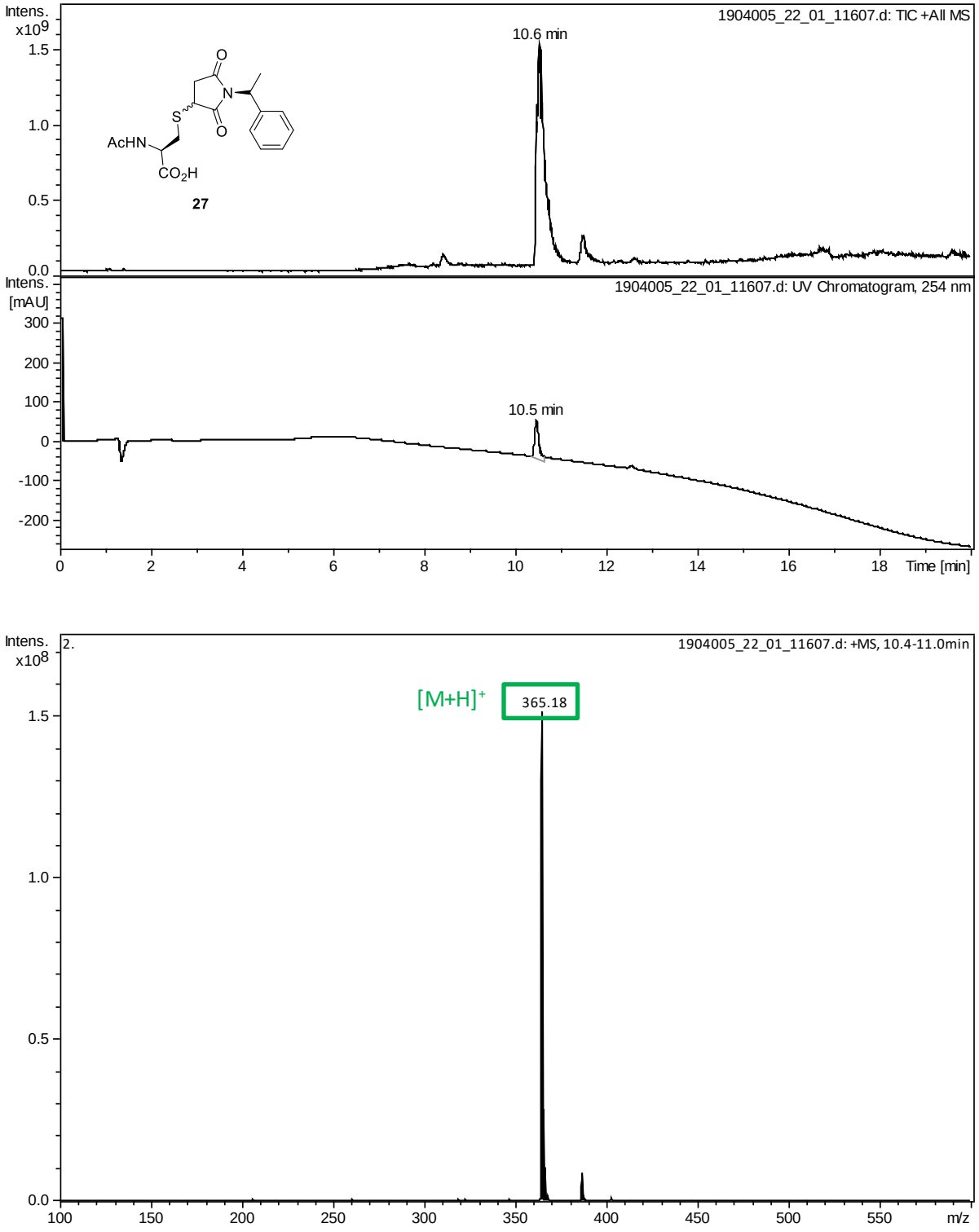


Figure S48. TIC, UV (254 nm) chromatograms and HRMS (ESI+) mass spectrum of compound **27**.

b) Study of retro-Diels-Alder reactions under physiological conditions

The reaction rate of Michael addition between *N*-acetylcysteine (NAC) and maleimide **16** was qualitatively studied following similar conditions as retro-Diels-Alder experiments. To a mixture of iPrOH/PBS pH 7.4 (1388 µL and 1174 µL respectively) at T = 37 °C was added a solution of probe **16** (112 µL, 26.8 mM in iPrOH). After 2 minutes of incubation, a solution of NAC (326 µL, 91.9 mM in PBS pH 7.4) was added. Final concentrations for t = 0 min were 1 mM for probe **16**, 10 mM (10 equiv.) for NAC and solvents ratio was iPrOH/PBS (1:1, v:v). The reaction was monitored by fluorescence spectroscopy and RP-HPLC after 8 minutes of reaction.

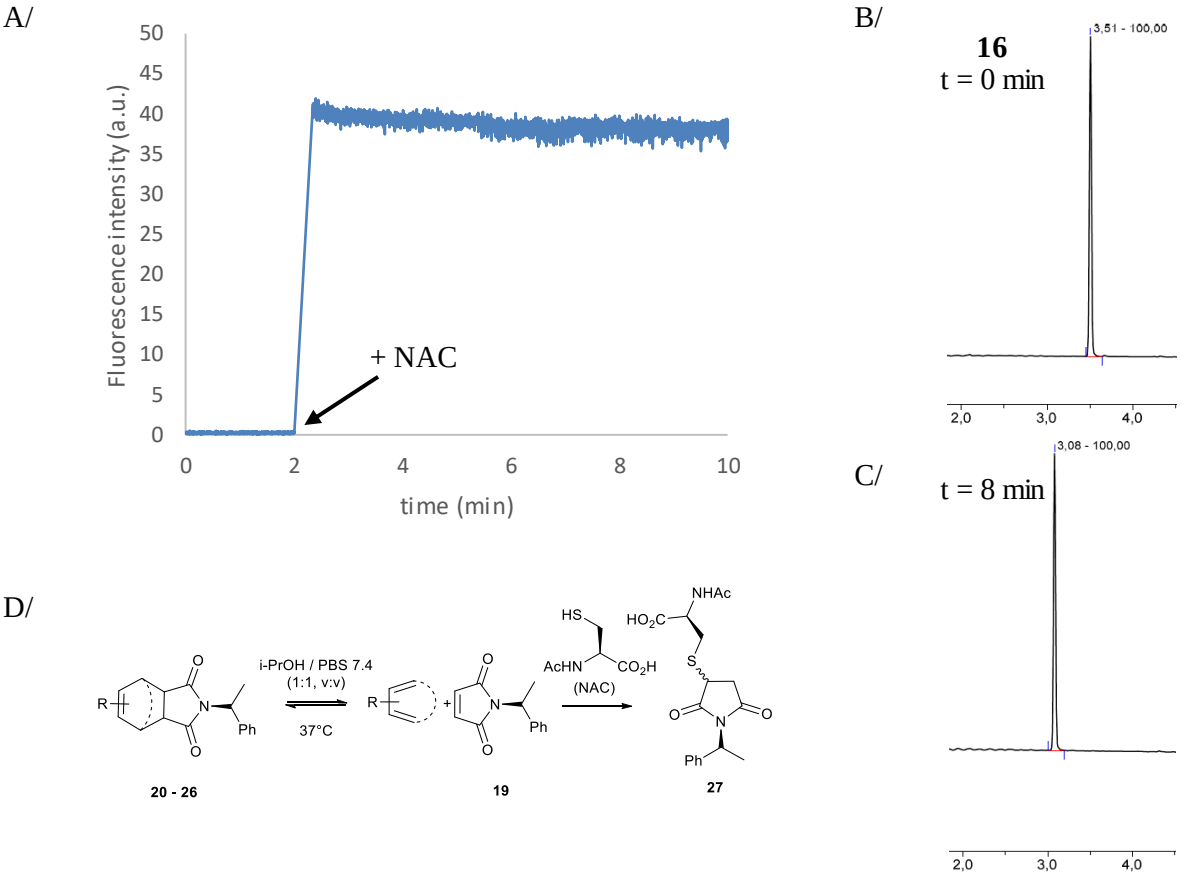


Figure S49. (A) Time-dependent fluorescence increase of probe **16** (1 mM) in the presence of NAC (10 mM, 10 equiv.) at λ_{em} = 560 nm and λ_{ex} = 330 nm. (B) RP-HPLC of isolated probe **16** (λ = 330 nm, sB). (C) RP-HPLC of the reactional mixture after 8 minutes of reaction (λ = 330 nm, sB). (D) rD-A study in the presence of NAC as fast maleimide-trapping agent.

500 µL of a stock solution of the conjugate **20-26** (2 mg of corresponding conjugate in 1 mL of iPrOH), were added to a 1 mL Eppendorf containing NAC (10 equiv.) in PBS pH 7.4 (500 µL). Then, the mixture was heated to 37 °C in a water-bath. Unless otherwise stated, aliquots for LC-MS analyses were prepared after 24 h as follows: to the reactional mixture (100 µL) were added iPrOH (50 µL) and H_2O (50 µL). Thioether **27** was only detected if maleimide **19** was formed from a retro-Diels-Alder process, and subsequently trapped by *N*-acetylcysteine through a thio Michael addition. All aliquots were stored at -25 °C pending LC-MS analyses (System C).

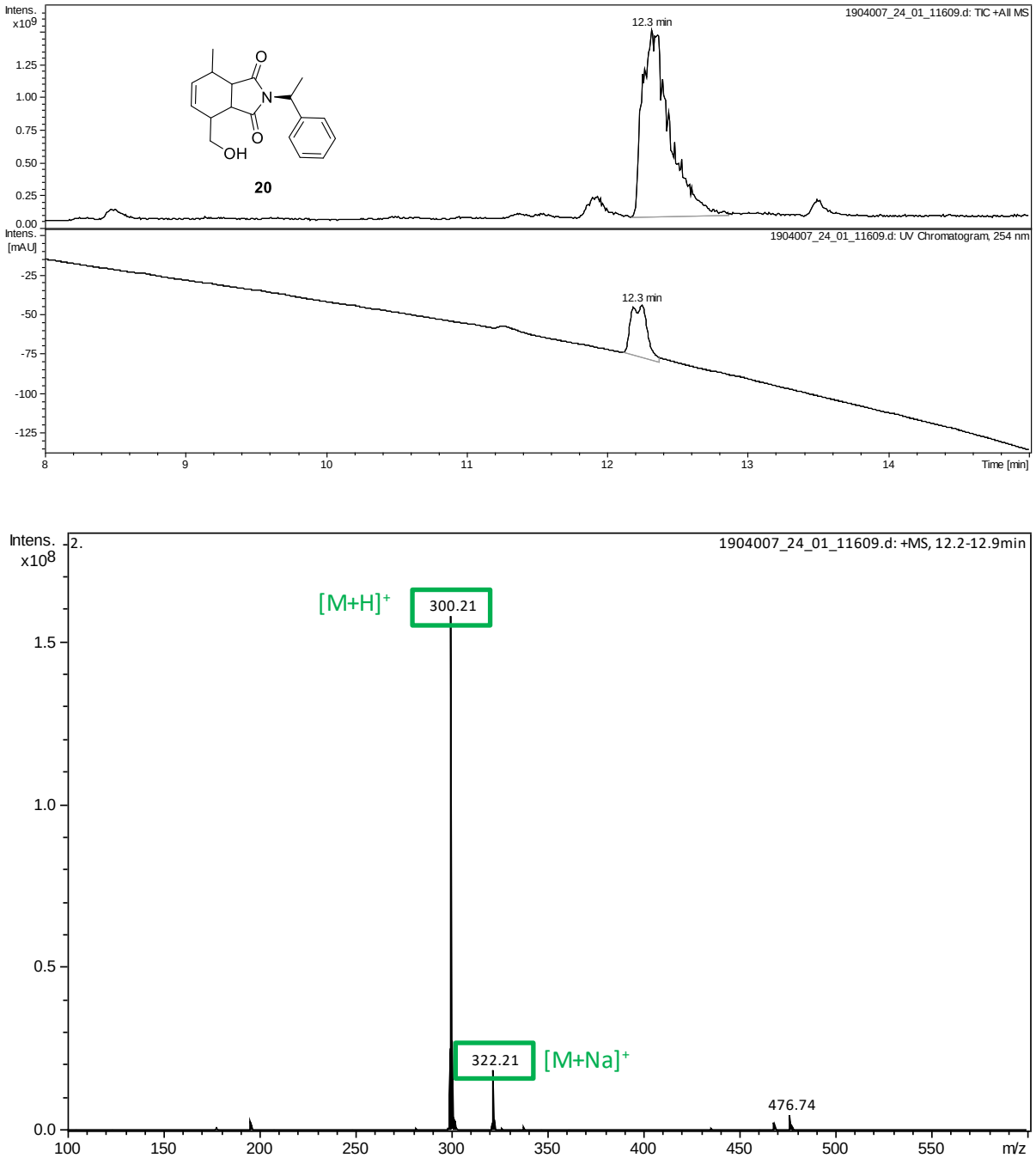
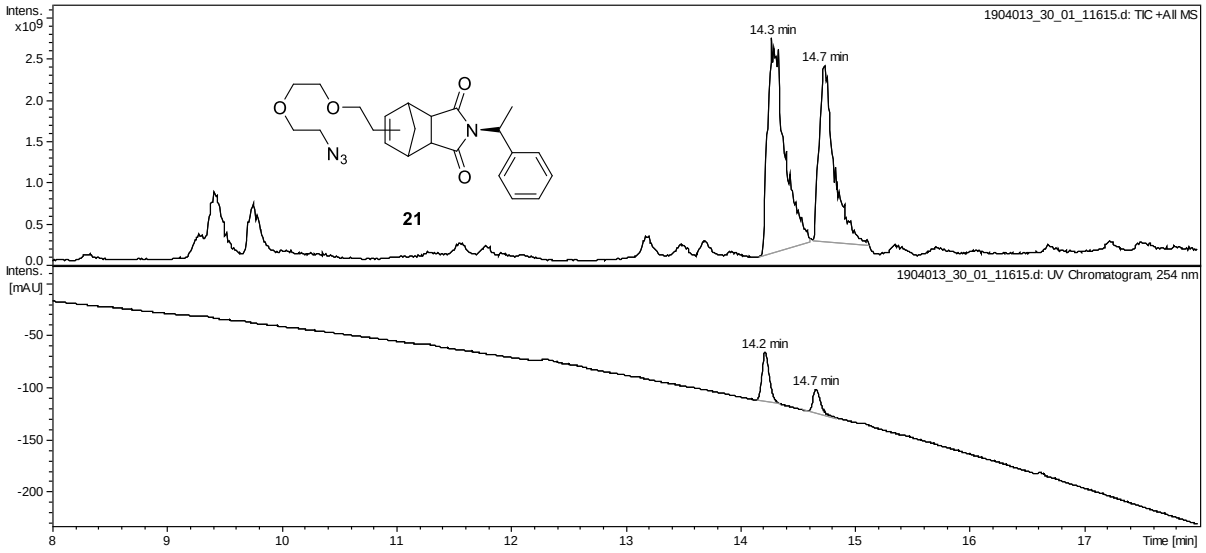
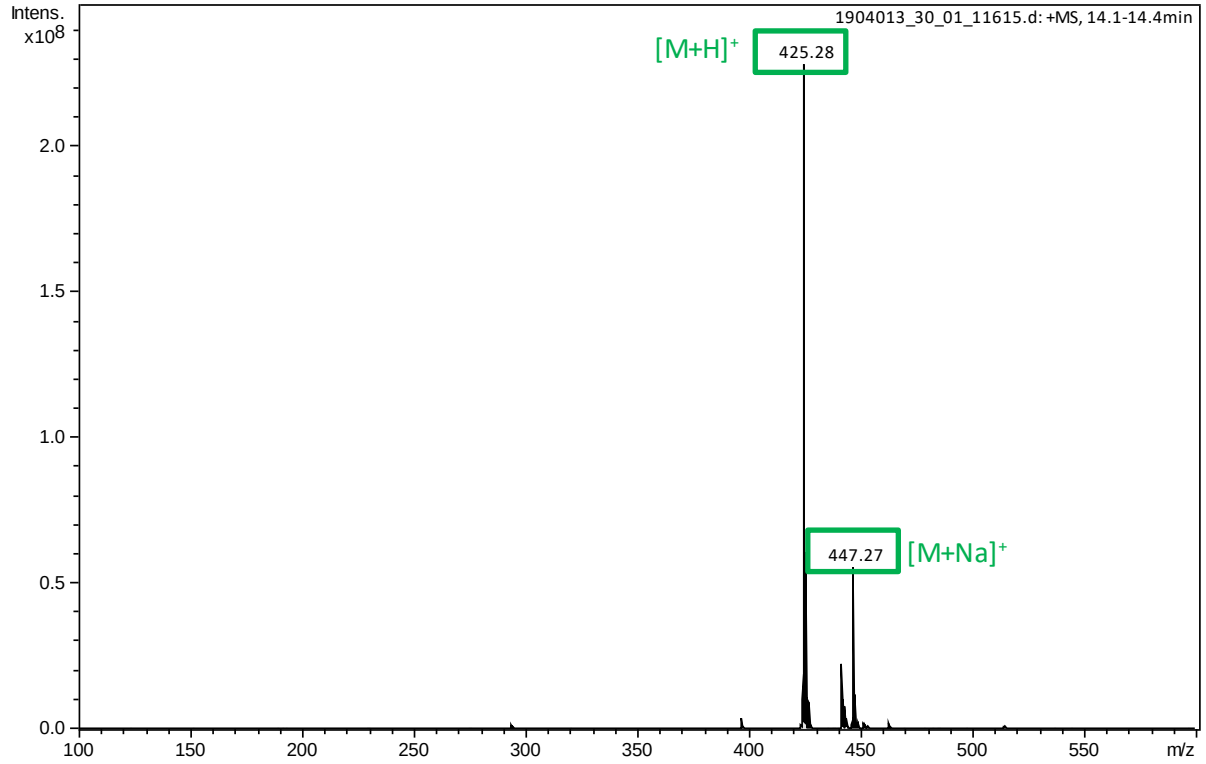


Figure S50. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectrum of r-D–A with **20**.


Intens.
x10^9
2.5
2.0
1.5
1.0
0.5
0.0
1904013_30_01_11615.d: TIC +All MS
14.3 min
14.7 min
O
O
O
N
N3
O
21
Intens.
[mAU]
-50
-100
-150
-200
1904013_30_01_11615.d: UV Chromatogram, 254 nm
14.2 min
14.7 min
8
9
10
11
12
13
14
15
16
17
Time [min]


Intens.
x10^8
2.0
1.5
1.0
0.5
0.0
1904013_30_01_11615.d: +MS, 14.1-14.4min
[M+H]+
425.28
447.27
[M+Na]+
100
150
200
250
300
350
400
450
500
550
m/z

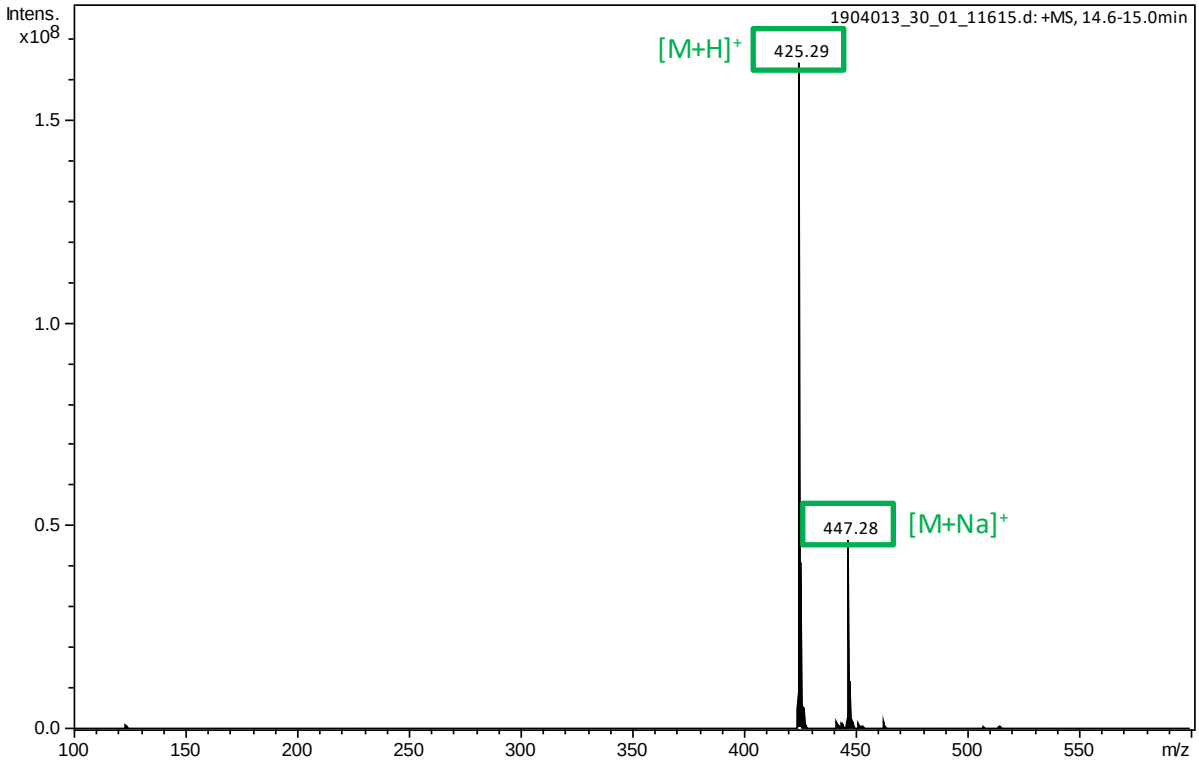


Figure S51. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of r-D–A with **21**.

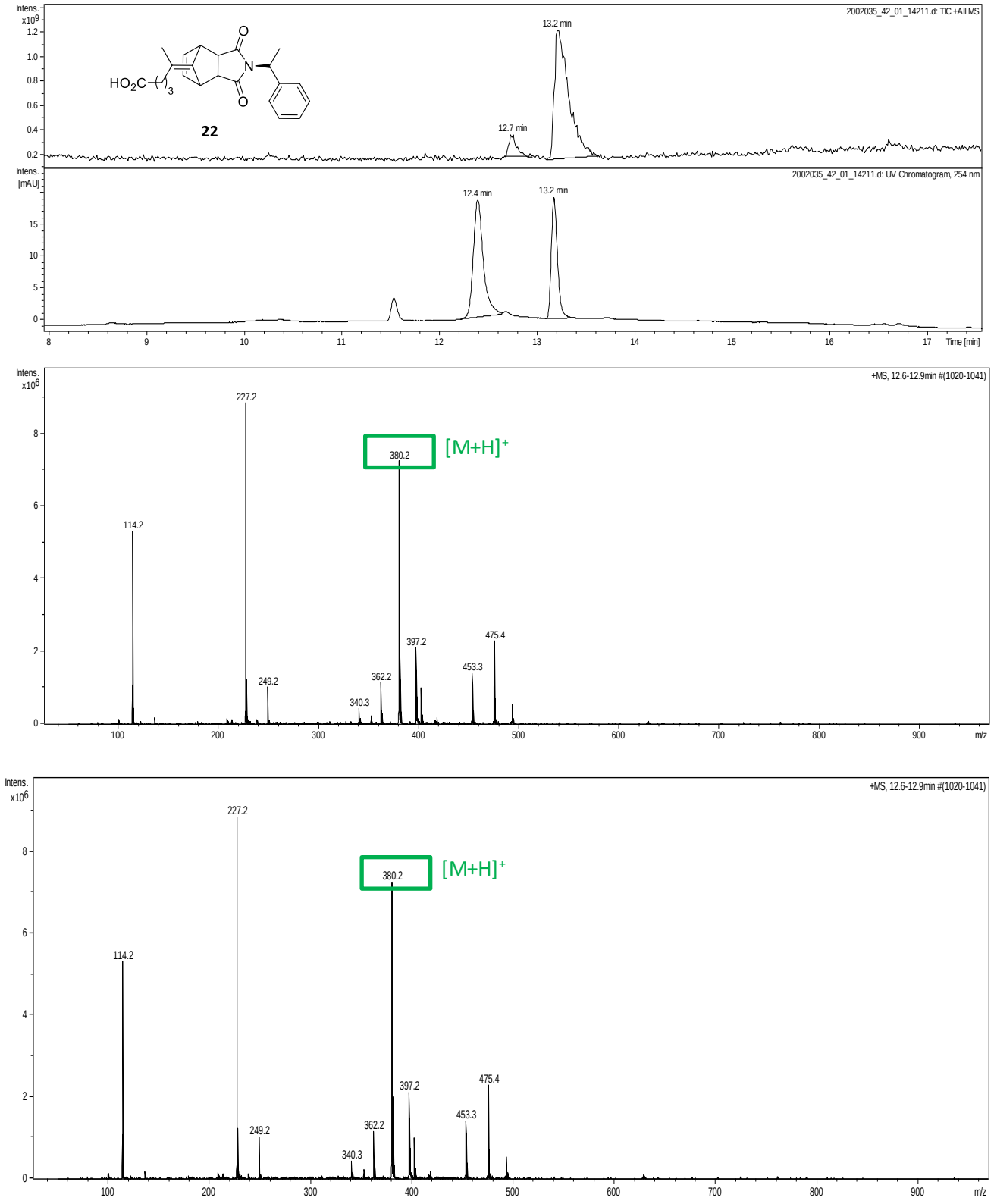
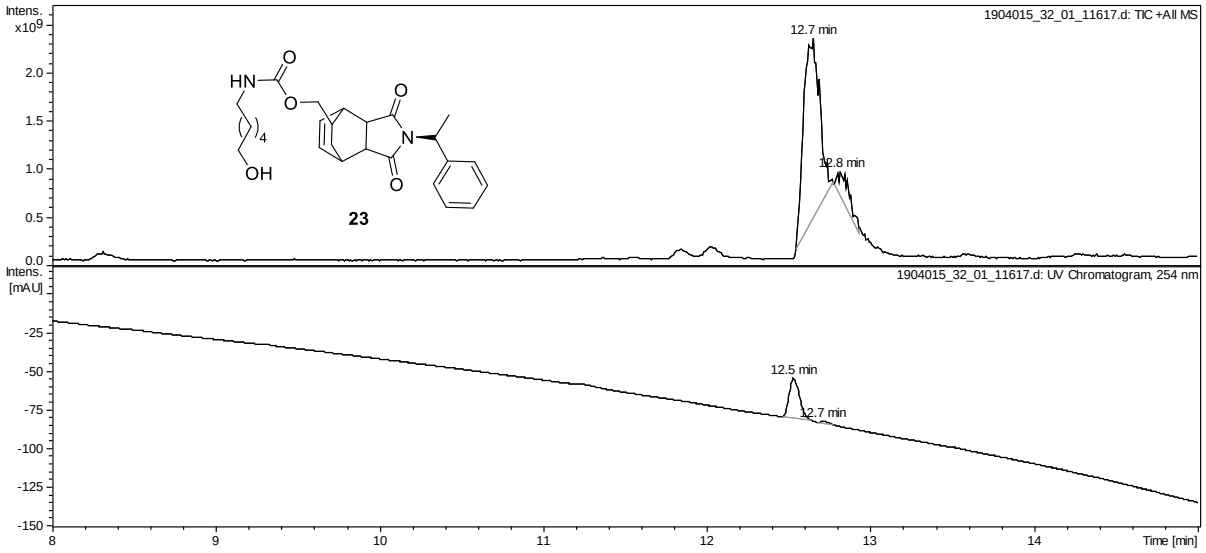
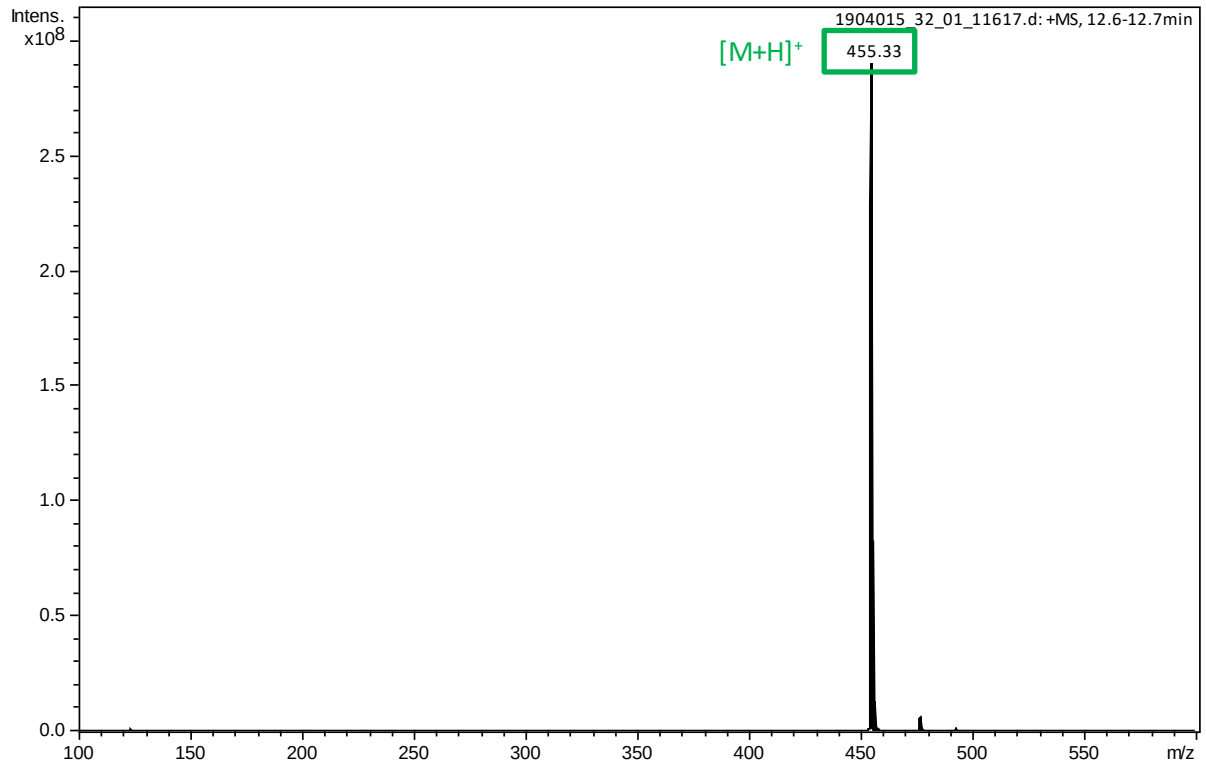


Figure S52. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of r-D–A with **22**.


Intens.
x10^9
2.0
1.5
1.0
0.5
0.0
1904015_32_01_11617.d: TIC +All MS
12.7 min
12.8 min
HN
O
O
O
N
O
4
OH
23
Intens.
[mAU]
-25
-50
-75
-100
-125
-150
1904015_32_01_11617.d: UV Chromatogram, 254 nm
12.5 min
12.7 min
8
9
10
11
12
13
14
Time [min]


Intens.
x10^8
2.5
2.0
1.5
1.0
0.5
0.0
1904015_32_01_11617.d: +MS, 12.6-12.7min
[M+H]+
455.33
100
150
200
250
300
350
400
450
500
550
m/z

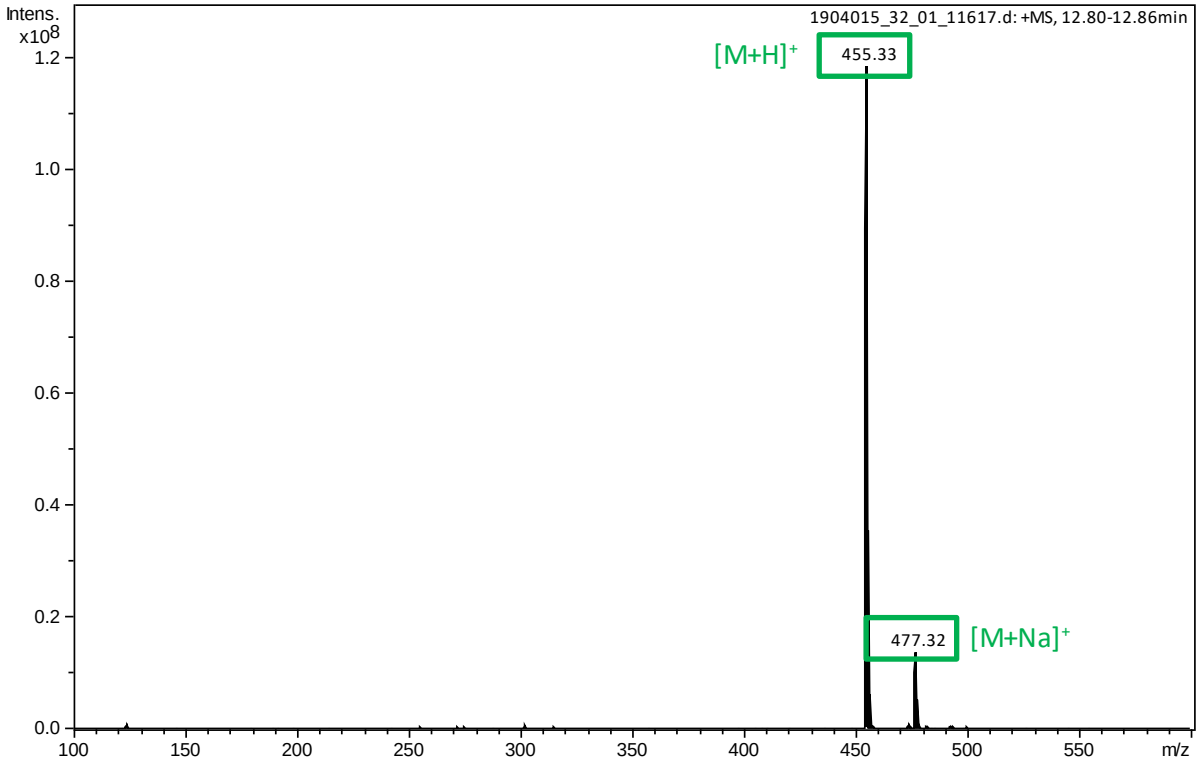


Figure S53. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of r-D–A with **23**.

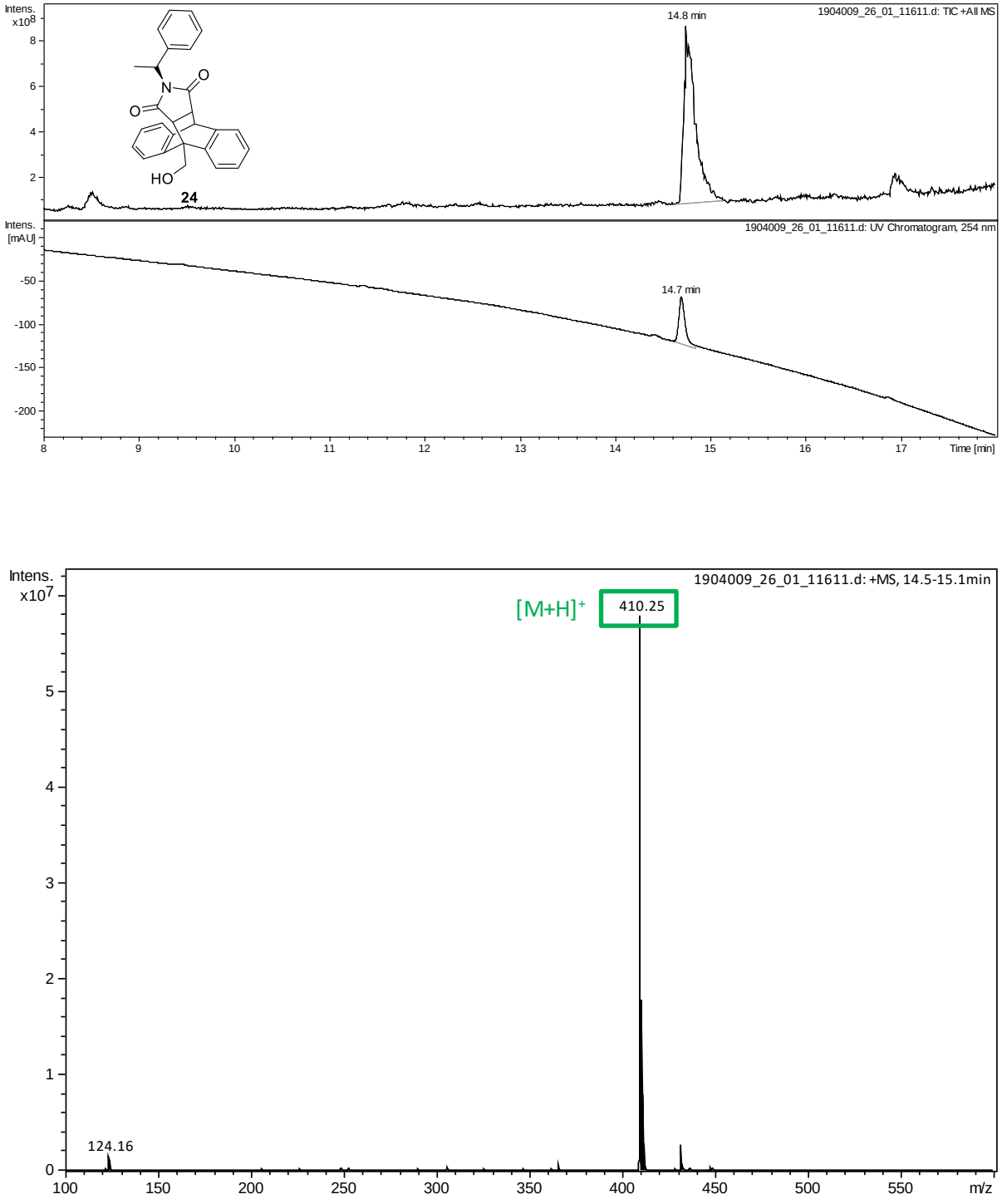
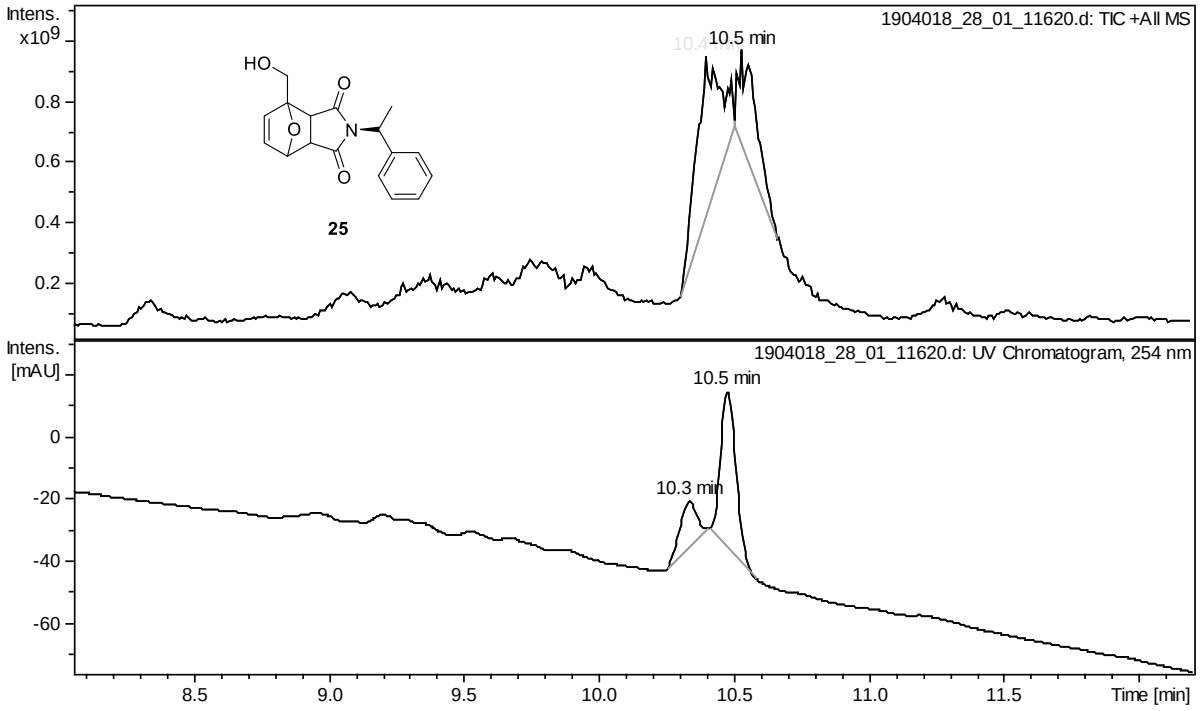
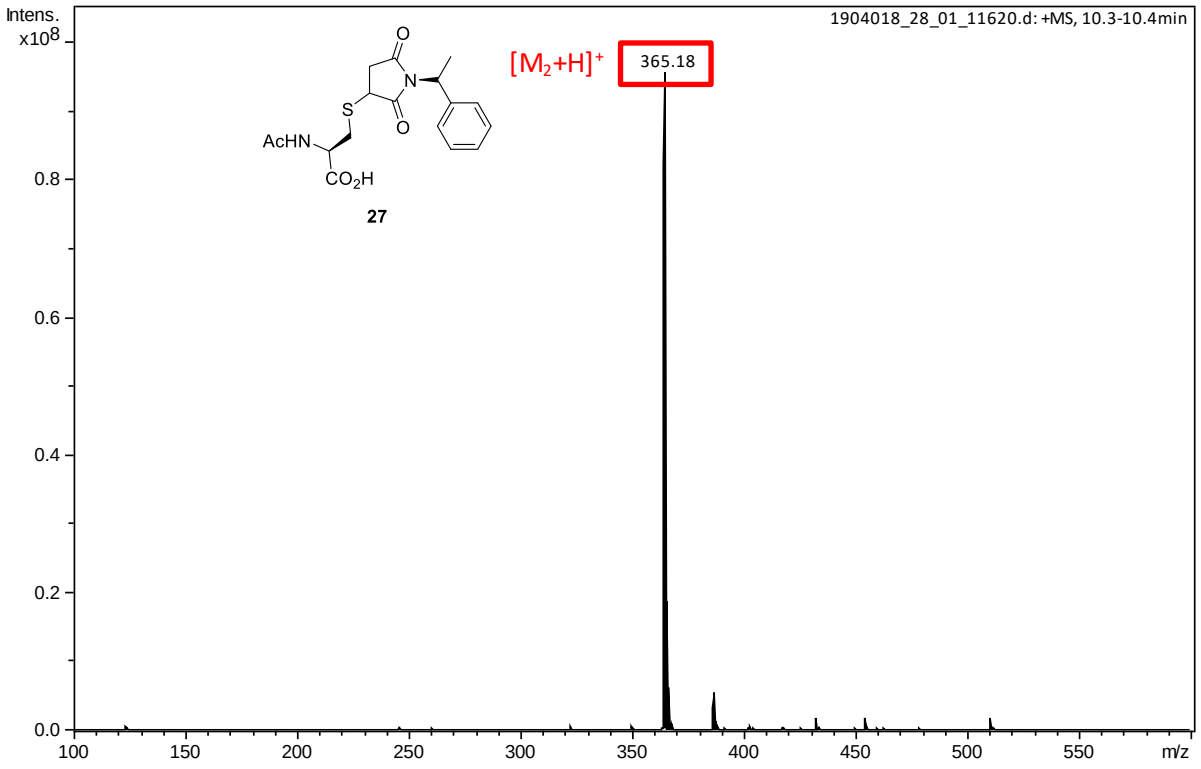


Figure S54. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectrum of r-D–A with **24**.


Intens.
x10^9
1904018_28_01_11620.d: TIC +All MS
10.5 min
HO
O
O
N
O
25
Intens.
[mAU]
1904018_28_01_11620.d: UV Chromatogram, 254 nm
10.5 min
10.3 min
Time [min]


Intens.
x10^8
1904018_28_01_11620.d: +MS, 10.3-10.4min
[M2+H]+
365.18
AcHN
CO2H
27
m/z

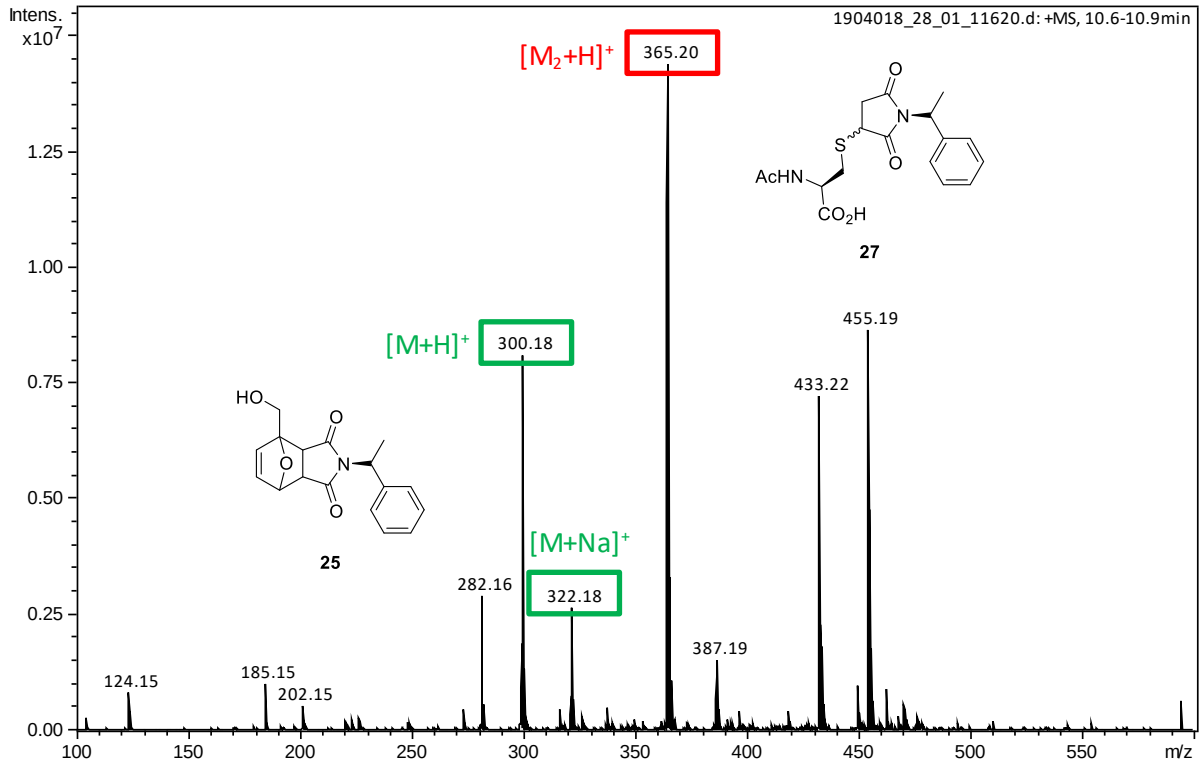
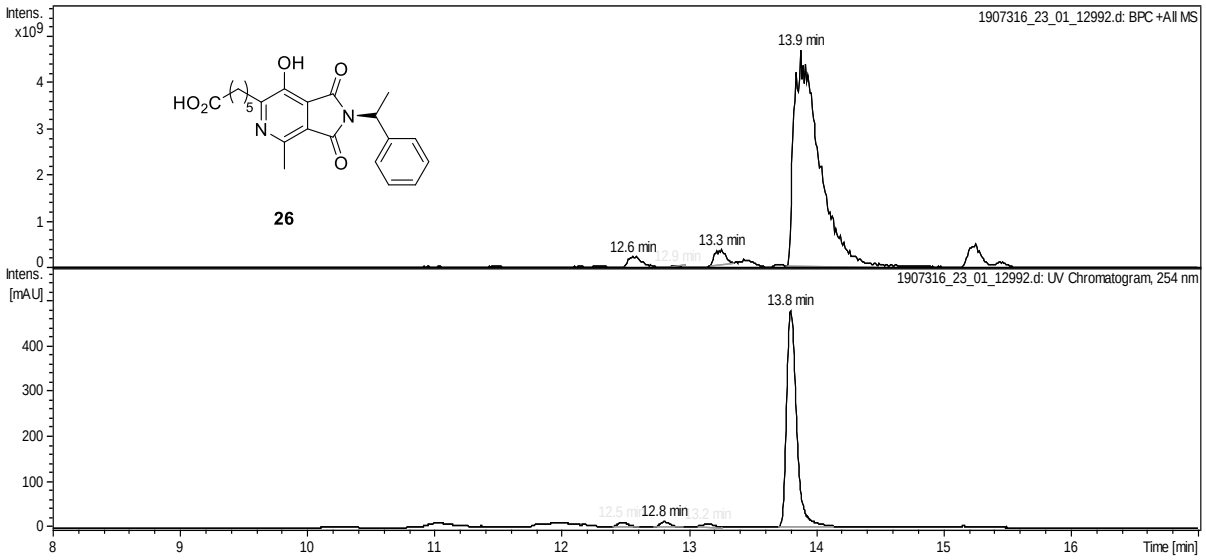
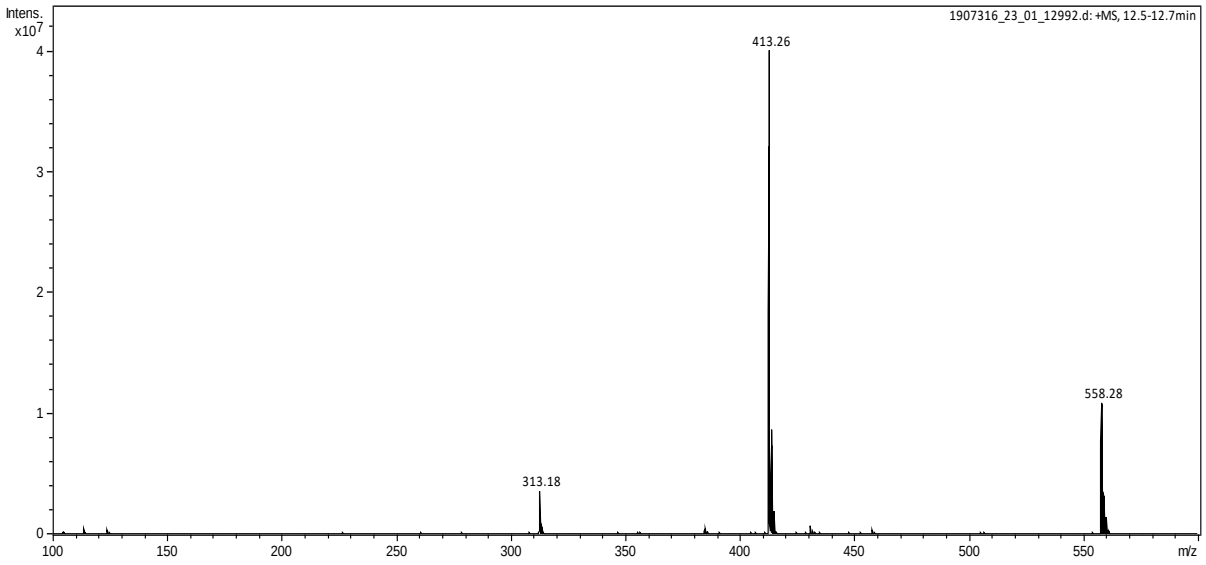
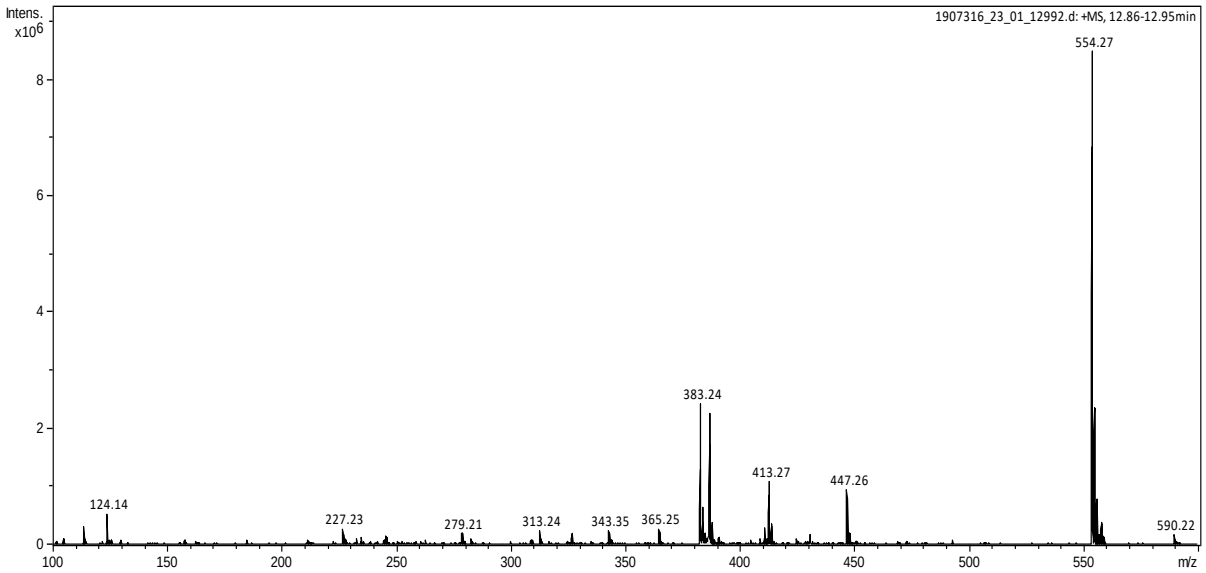


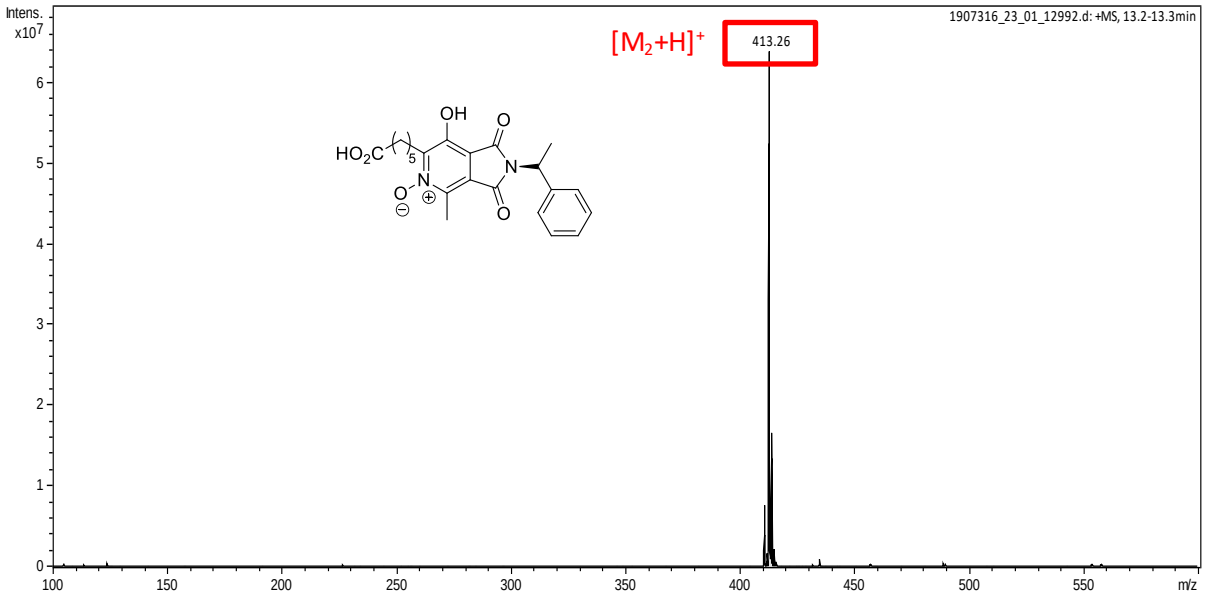
Figure S55. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of r-D–A with **25.**



Retention time (min)	% area 254 nm (%)
12.5	2.0
12.8	2.4
13.2	1.9
13.8	93.6




Intens.
x10^6
1907316_23_01_12992.d: +MS, 12.86-12.95min
554.27
383.24
413.27
447.26
124.14
227.23
279.21
313.24
343.35
365.25
590.22
m/z


Intens.
x10^7
1907316_23_01_12992.d: +MS, 13.2-13.3min
[M2+H]+
413.26
OH
O
HO2C
5
N
O
N
O
m/z

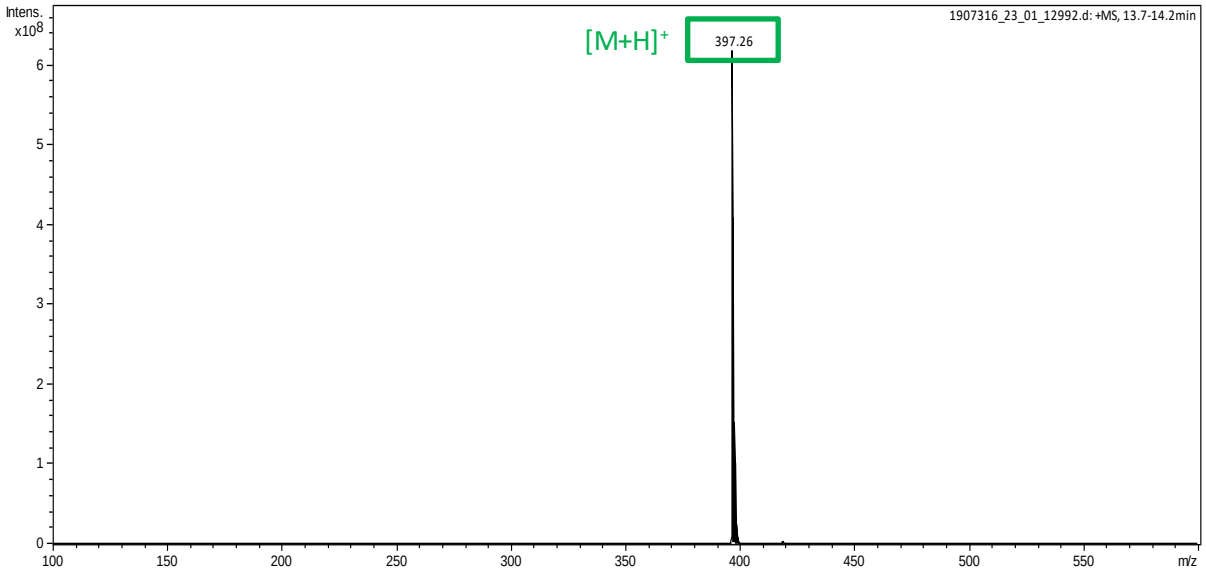


Figure S56. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of r-D–A with **26**.

*c) Study of conjugates **20-26** stability in human blood plasma (HBP)*

50 µL of a stock solution of the conjugate **20**-**26** (1 mg of corresponding conjugate in a solution of 100 µL of DMSO/iPrOH, 1:1, v:v) were added to an 2 mL Eppendorf containing HBP (500 µL). Then, the mixture was heated to 37 °C in a water-bath. After 24 h, the mixture was diluted with EtOH (500 µL) which led to the formation of a precipitate. The mixture was centrifuged for 30 min at 11 000 rotations/min. Then, the supernatant was withdrawn with a syringe and filtered through an HPLC filter (porosity: 0.45 µm). All aliquots were stored at -25°C pending LC-MS analyses (System C).

Blanks 1 and 2 from HBP were prepared following the same procedure without Diels-Alder product.

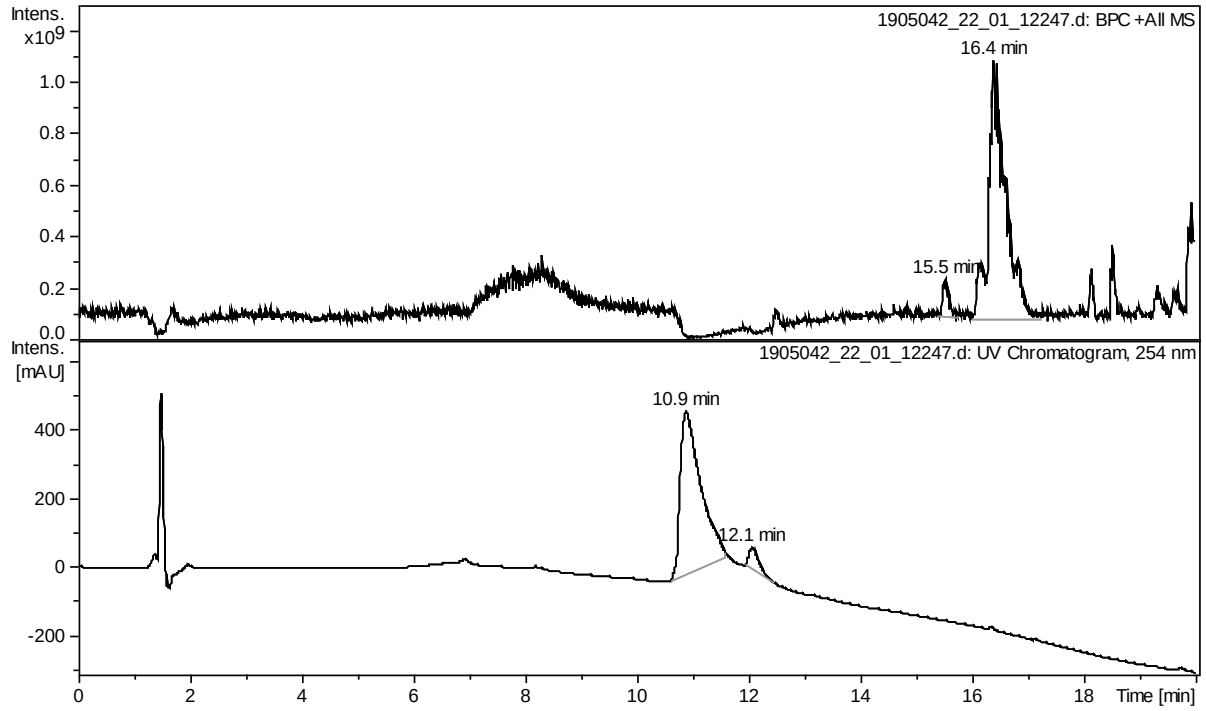


Figure S57. TIC, UV (254 nm) chromatograms of human blood plasma (Blank 1).

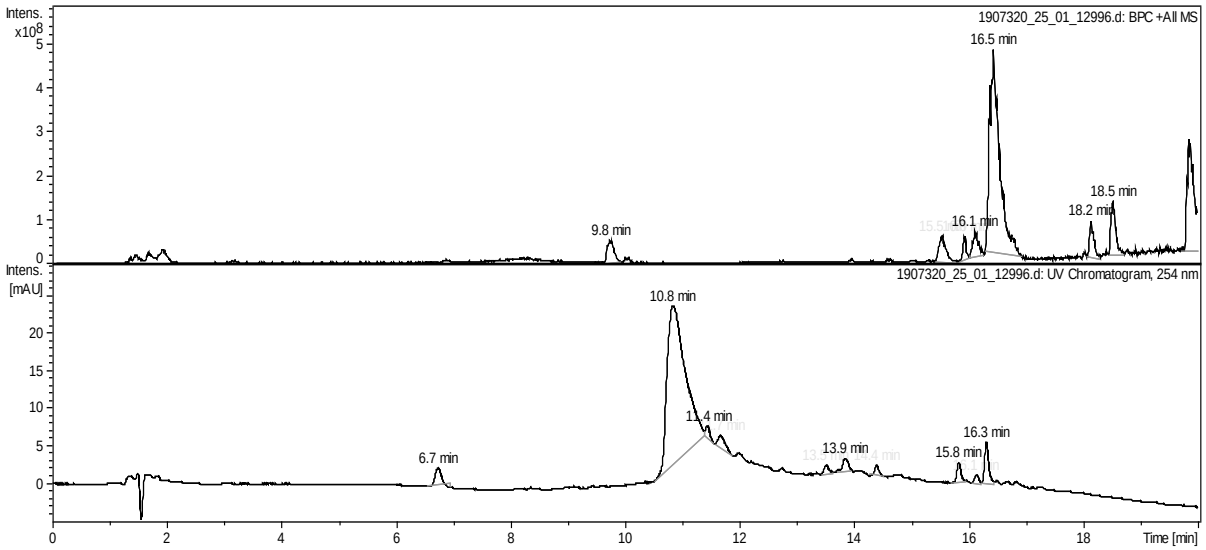
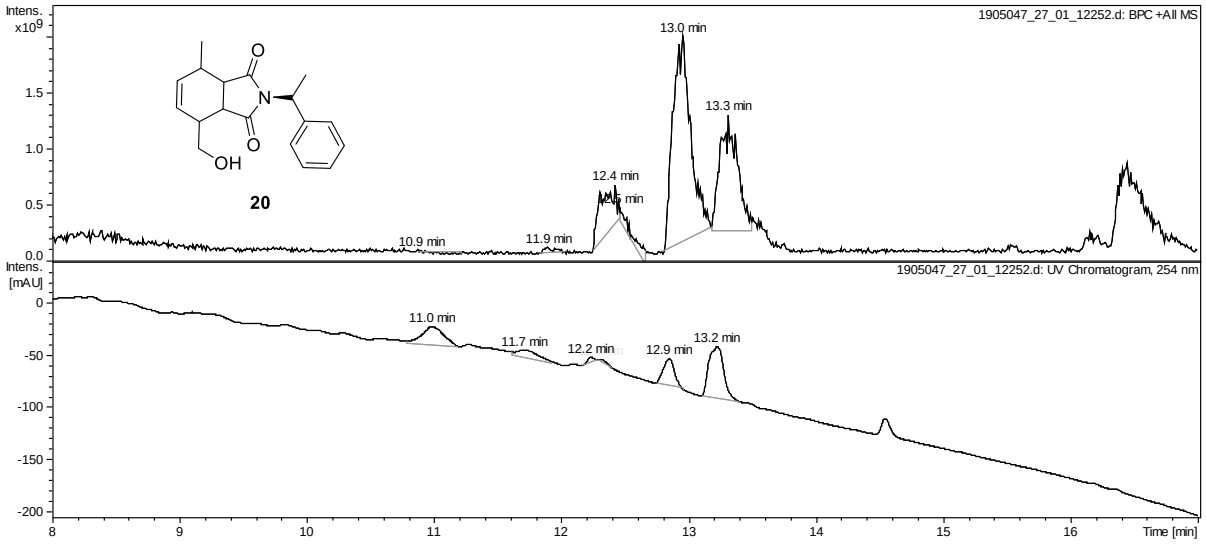
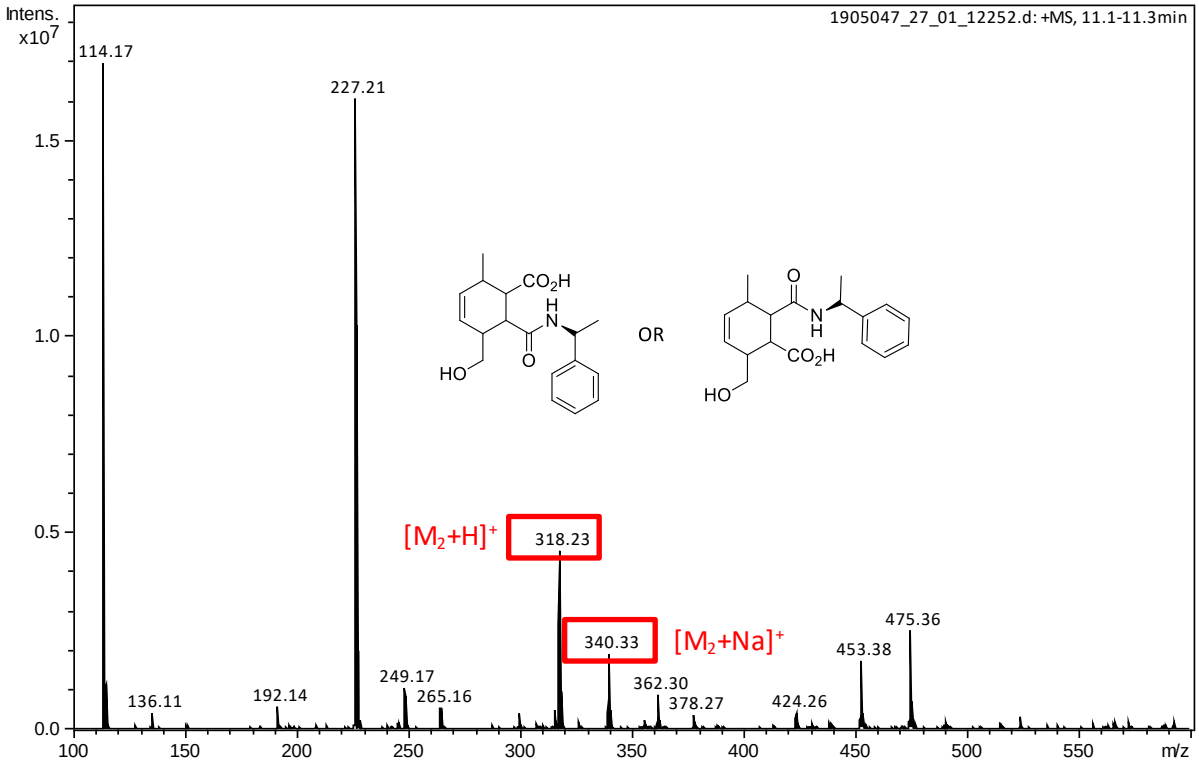
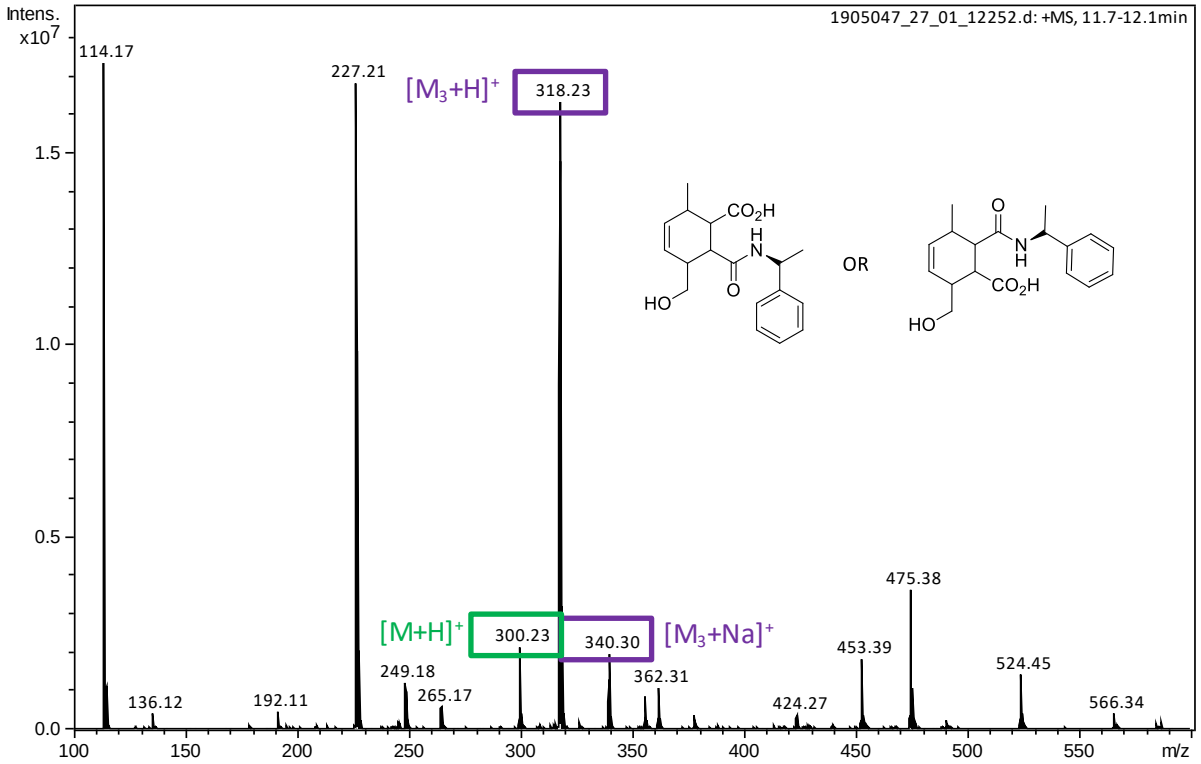


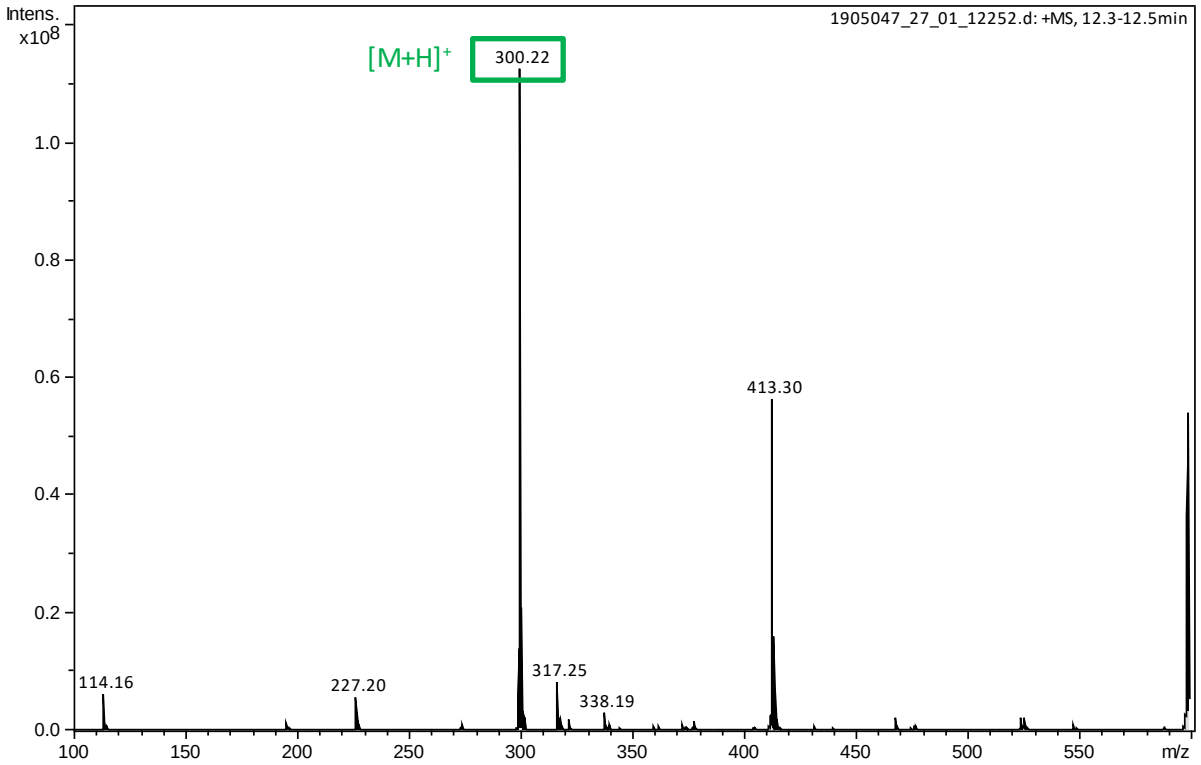
Figure S58. TIC, UV (254 nm) chromatograms of human blood plasma (Blank 2).



Retention time (min)	% area 254 nm (%)
11.0	24.3
11.7	10.7
12.2	1.7
12.3	0.8
12.9	17
13.2	45.5




Intens.
x10^7
1905047_27_01_12252.d: +MS, 11.7-12.1min
114.17
227.21
[M3+H]+
318.23
OR
[M+H]+
300.23
340.30
[M3+Na]+
136.12
192.11
249.18
265.17
362.31
424.27
453.39
475.38
524.45
566.34
m/z


Intens.
x10^8
1905047_27_01_12252.d: +MS, 12.3-12.5min
[M+H]+
300.22
413.30
114.16
227.20
317.25
338.19
m/z

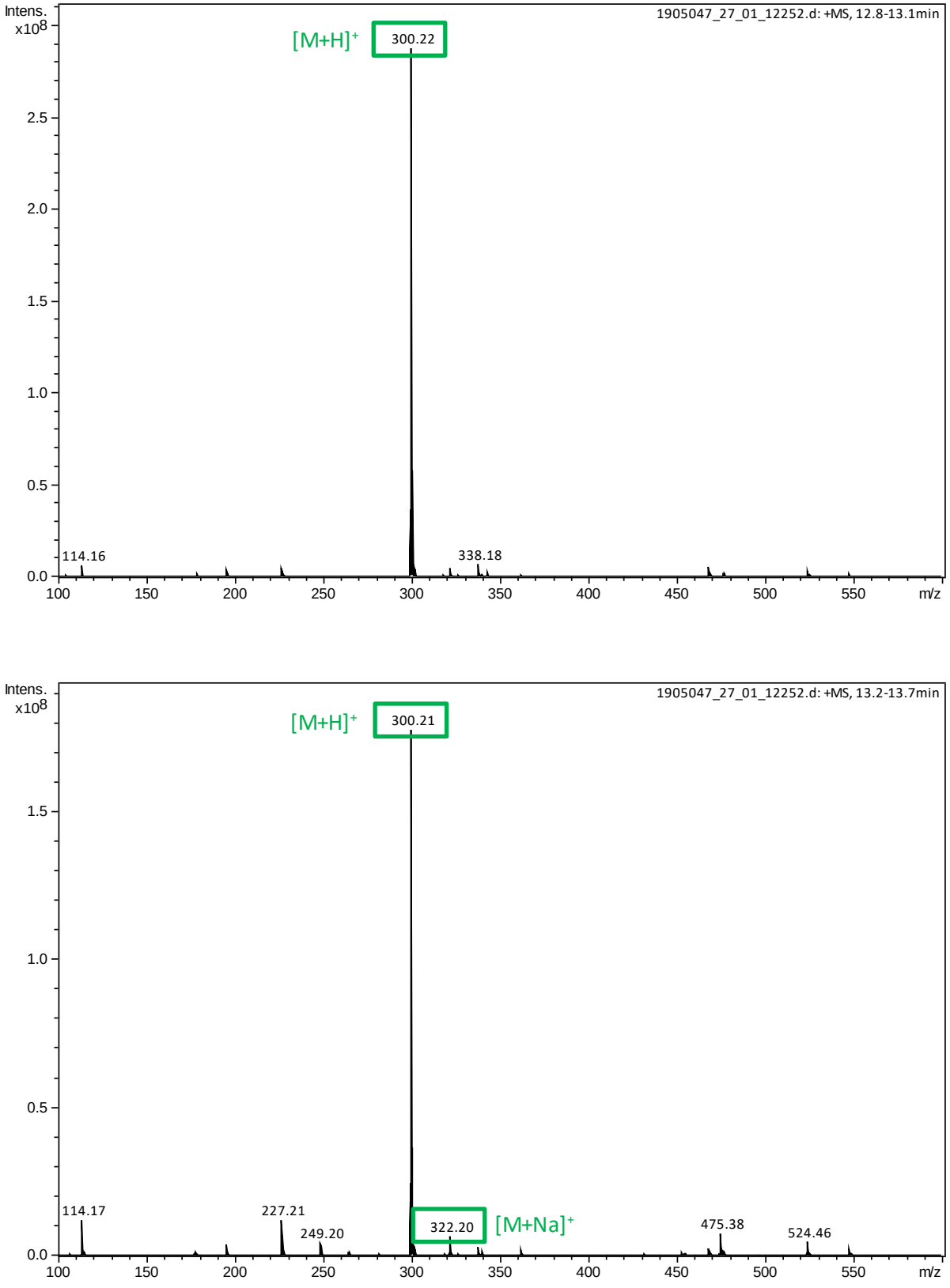
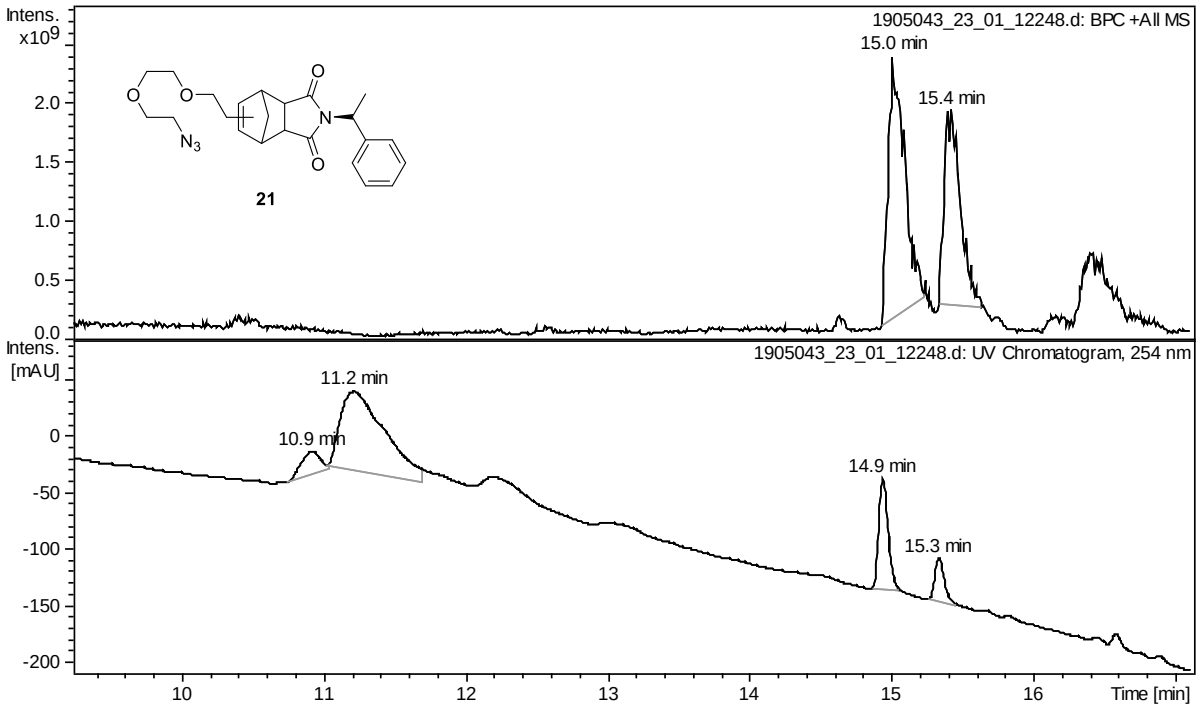
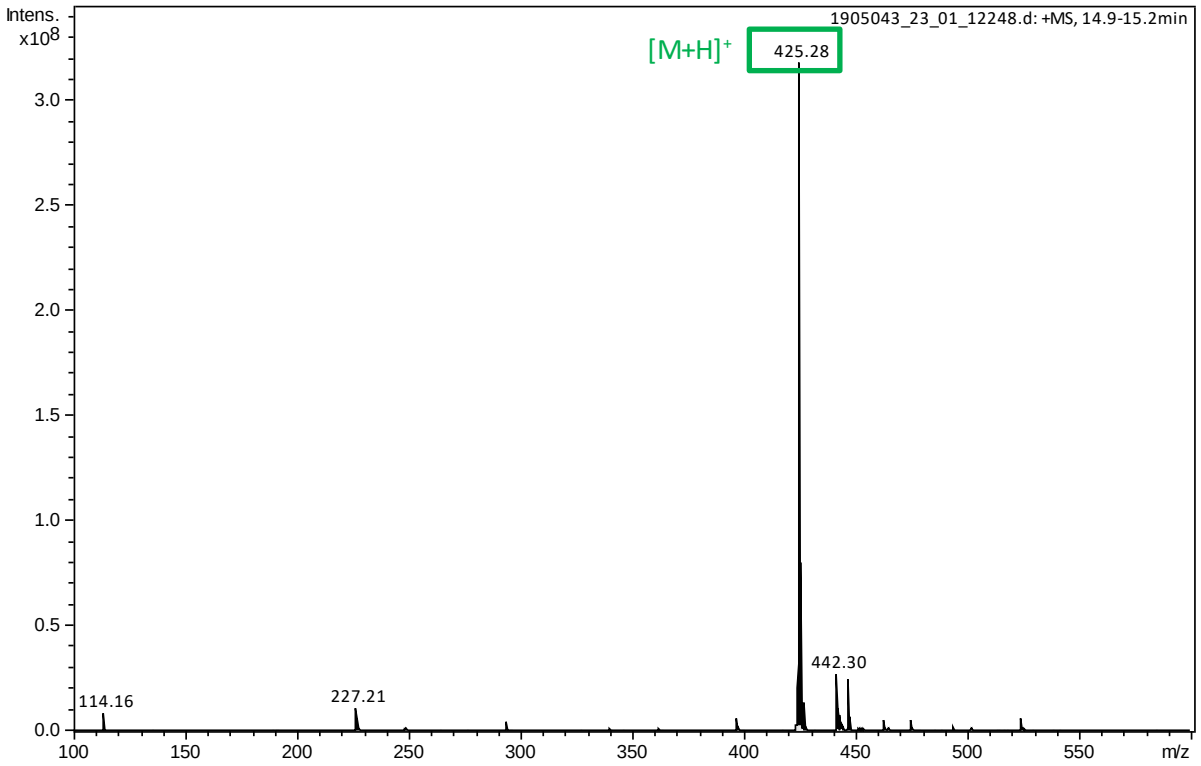


Figure S59. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of **20** after 24 h in human blood plasma.


Intens.
x10^9
2.0
1.5
1.0
0.5
0.0
1905043_23_01_12248.d: BPC +All MS
15.0 min
15.4 min
O
O
N
O
O
N3
21
Intens.
[mAU]
0
-50
-100
-150
-200
1905043_23_01_12248.d: UV Chromatogram, 254 nm
11.2 min
10.9 min
14.9 min
15.3 min
10
11
12
13
14
15
16
Time [min]


Intens.
x10^8
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1905043_23_01_12248.d: +MS, 14.9-15.2min
[M+H]+
425.28
442.30
114.16
227.21
100
150
200
250
300
350
400
450
500
550
m/z

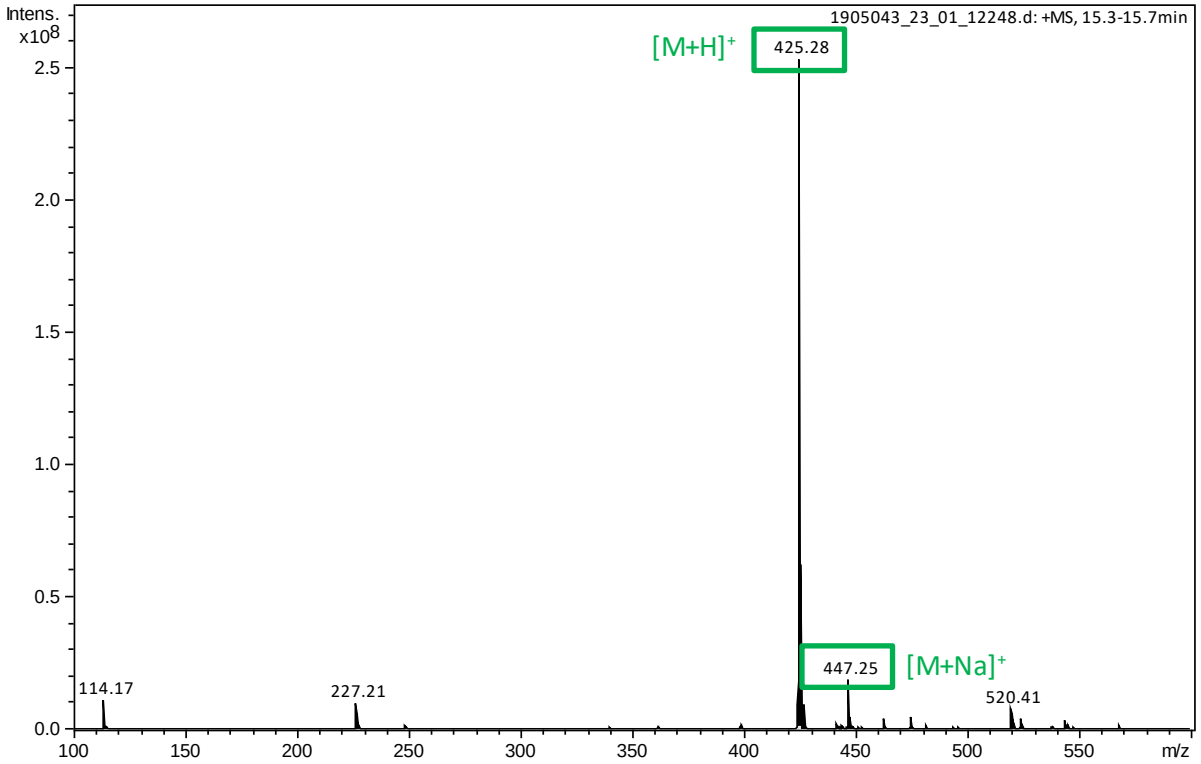


Figure S60. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of **21** after 24 h in human blood plasma.

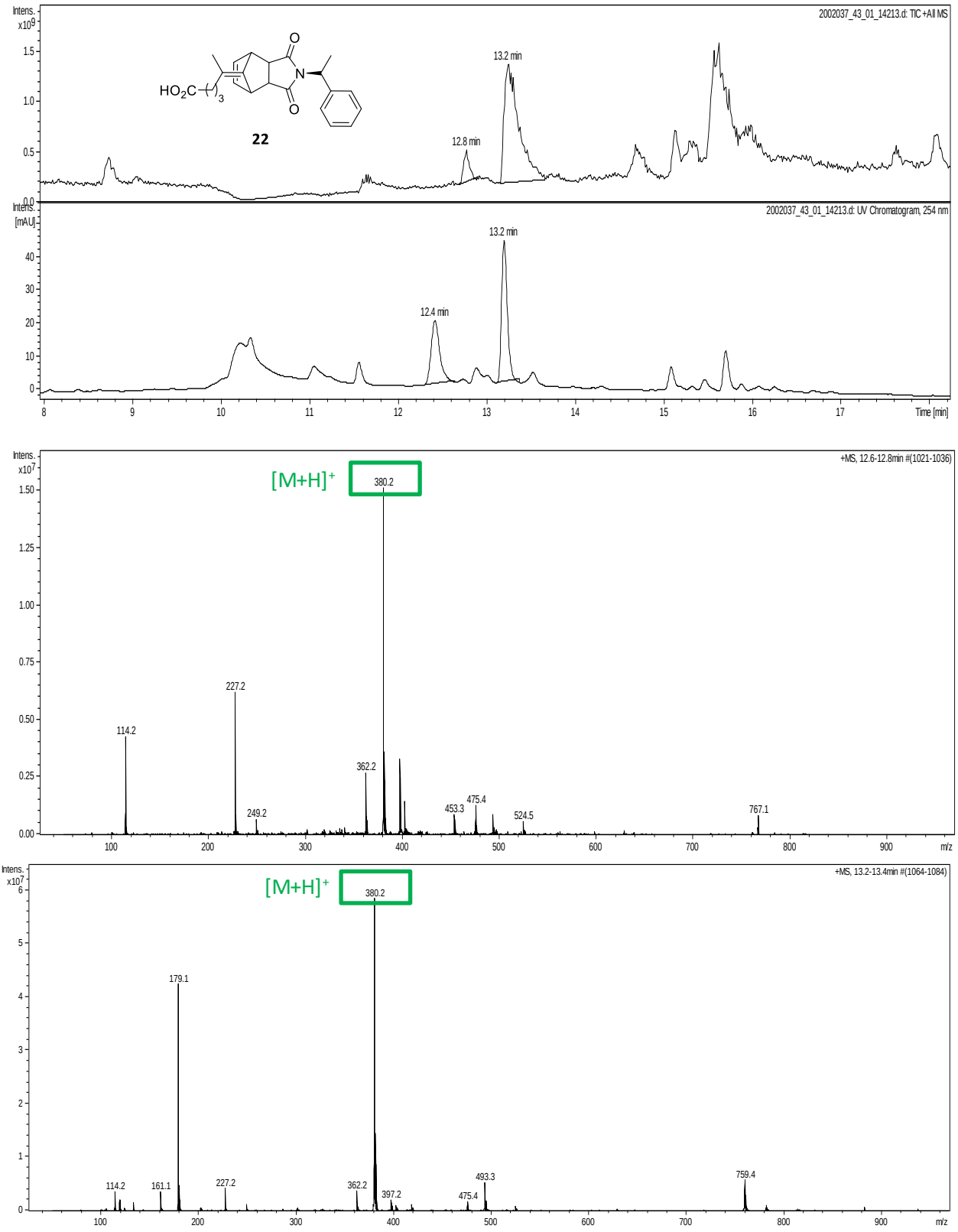
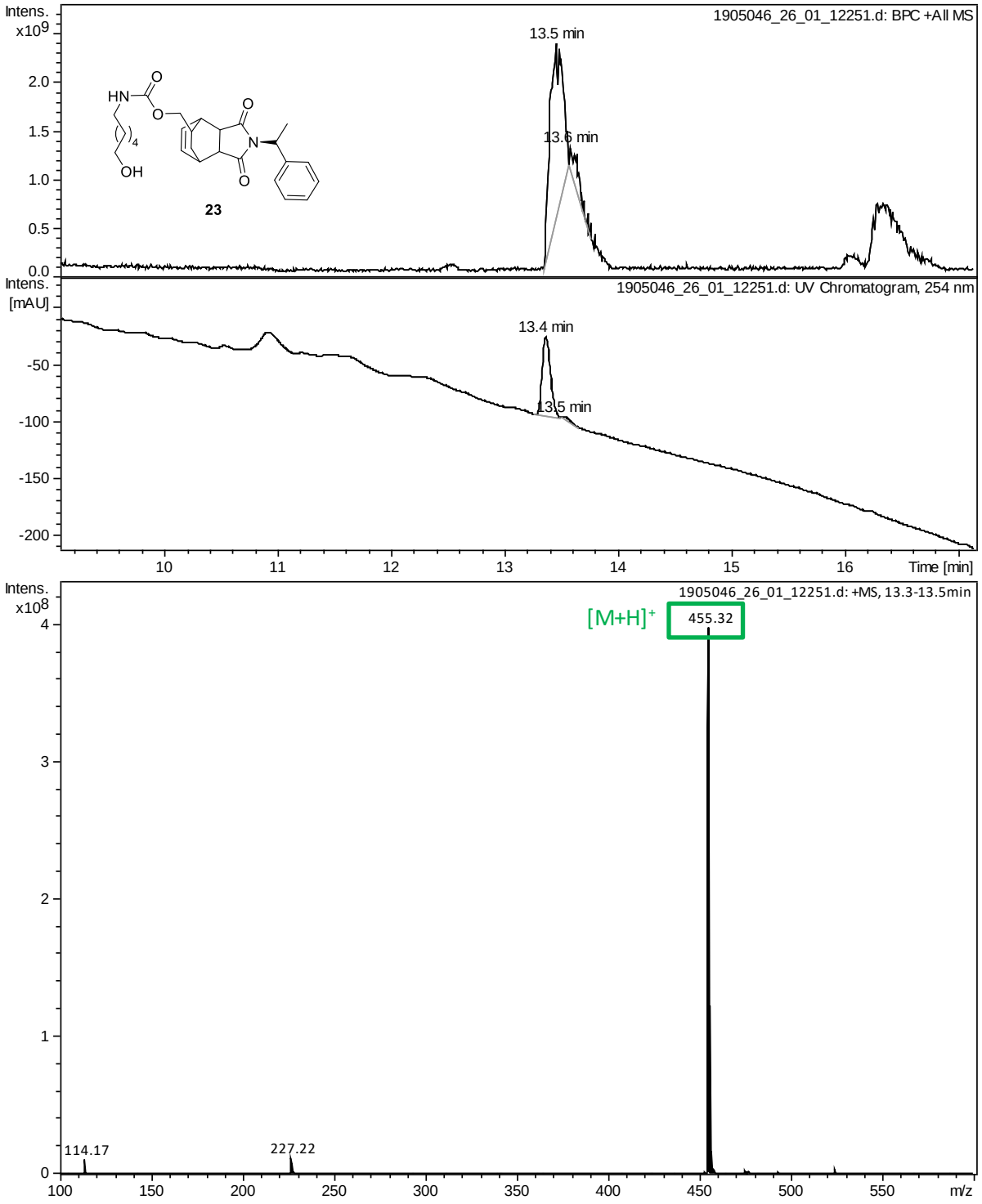


Figure S61. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of **22** after 24 h in human blood plasma.


Intens.
x10^9
2.0
1.5
1.0
0.5
0.0
1905046_26_01_12251.d: BPC +All MS
13.5 min
13.6 min
HN
O
O
4
OH
O
N
O
23
Intens.
[mAU]
-50
-100
-150
-200
1905046_26_01_12251.d: UV Chromatogram, 254 nm
13.4 min
13.5 min
10
11
12
13
14
15
16
Time [min]
Intens.
x10^8
4
3
2
1
0
1905046_26_01_12251.d: +MS, 13.3-13.5min
[M+H]+
455.32
114.17
227.22
100
150
200
250
300
350
400
450
500
550
m/z

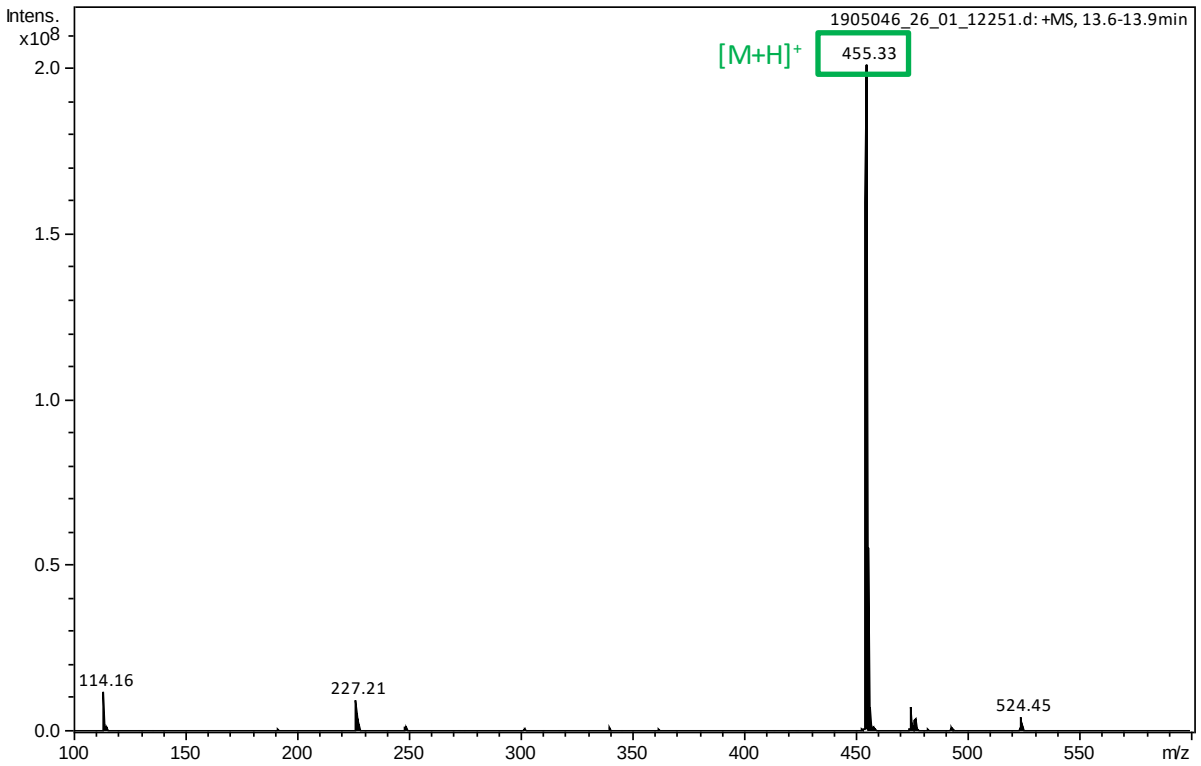


Figure S62. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of **23** after 24 h in human blood plasma.

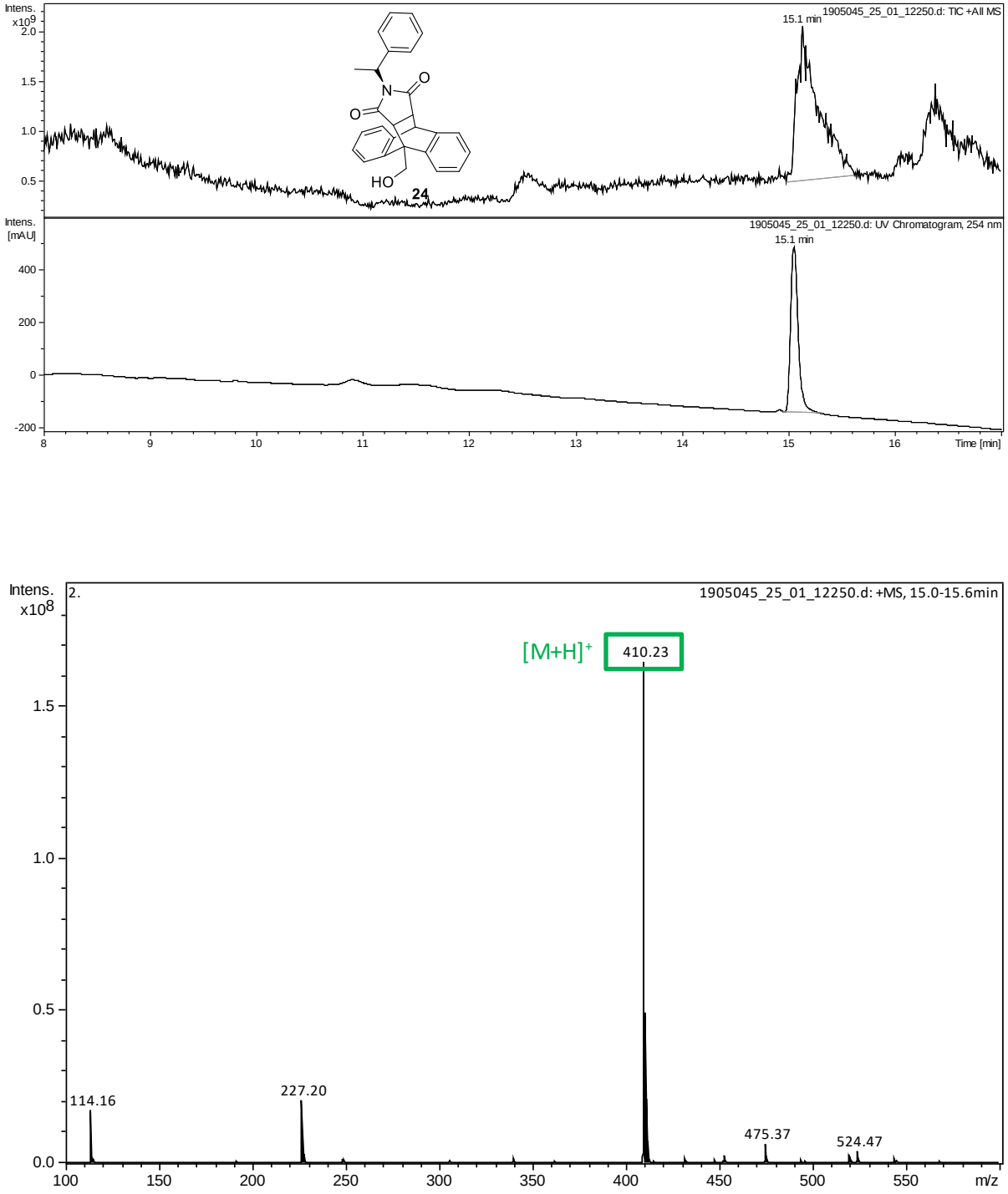
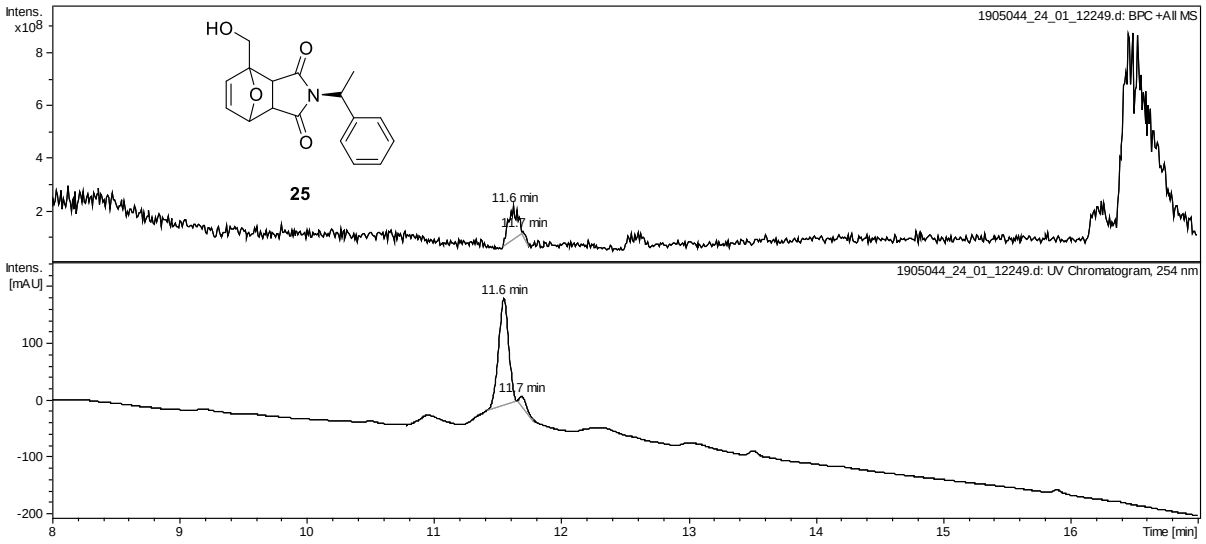


Figure S63. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of **24** after 24 h in human blood plasma.



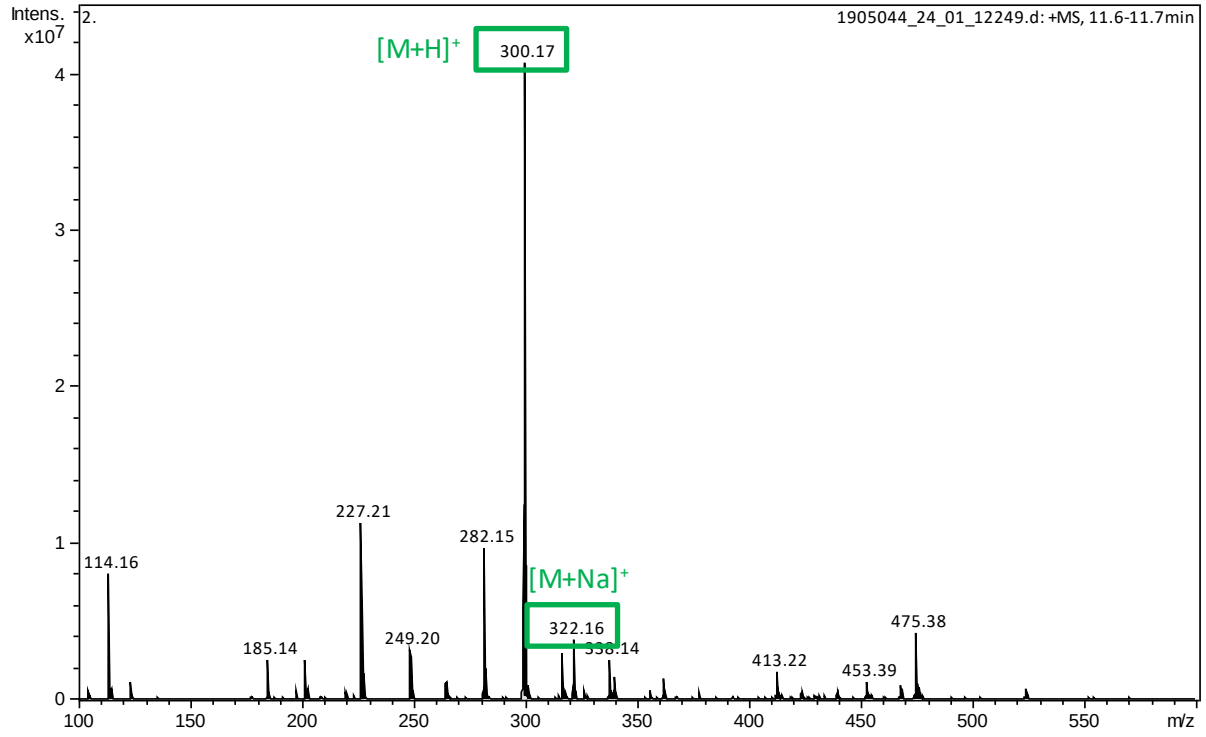
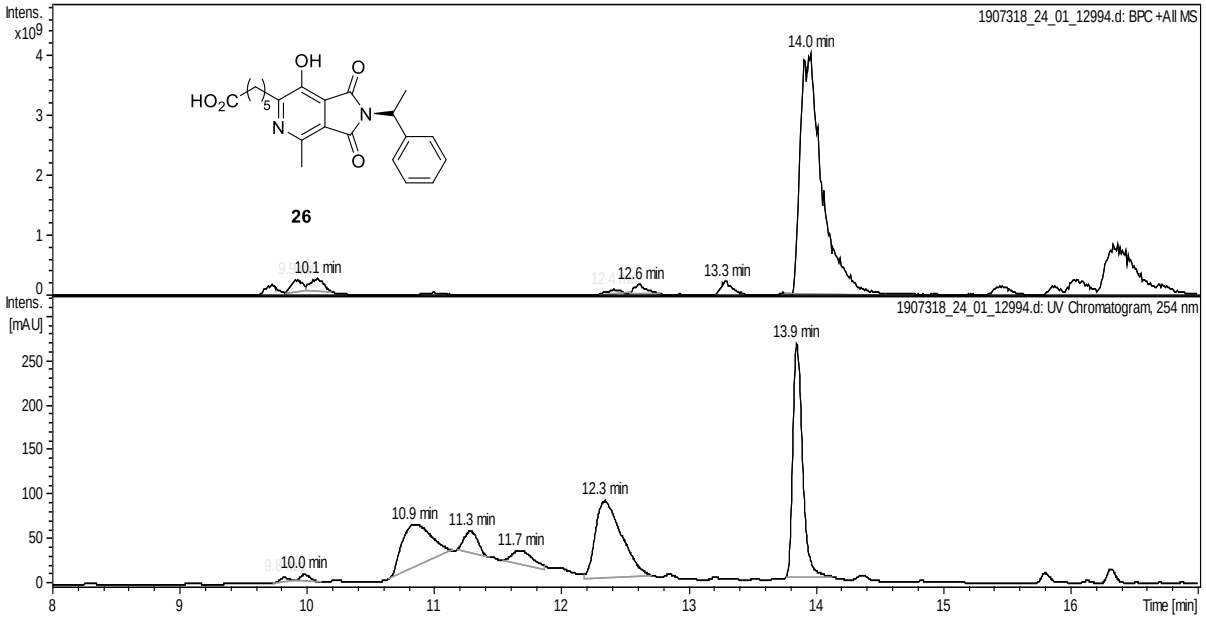
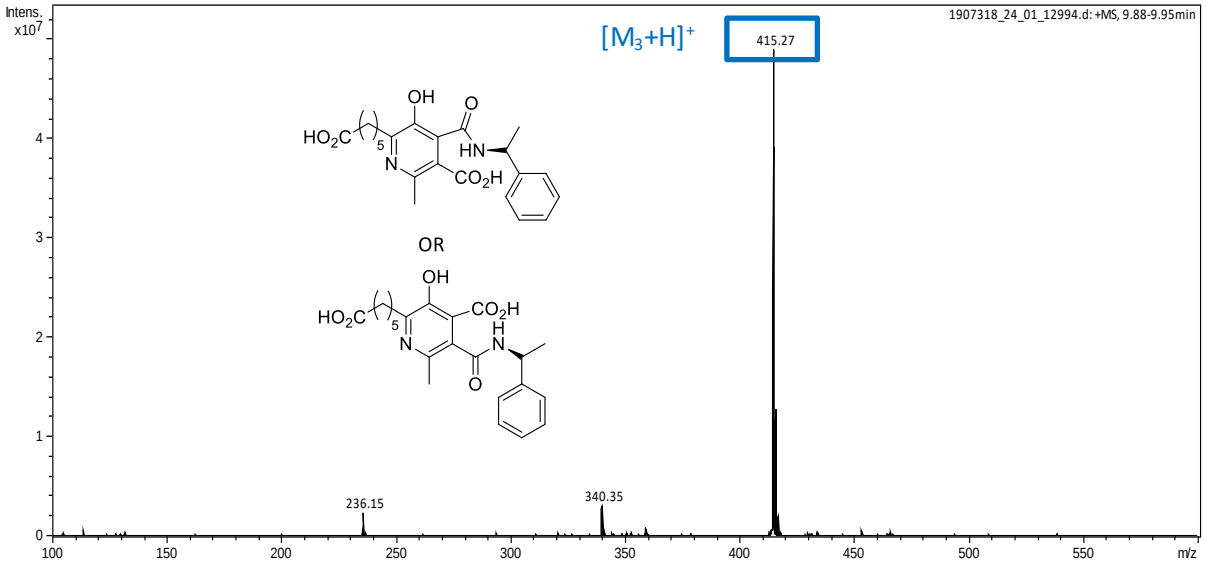
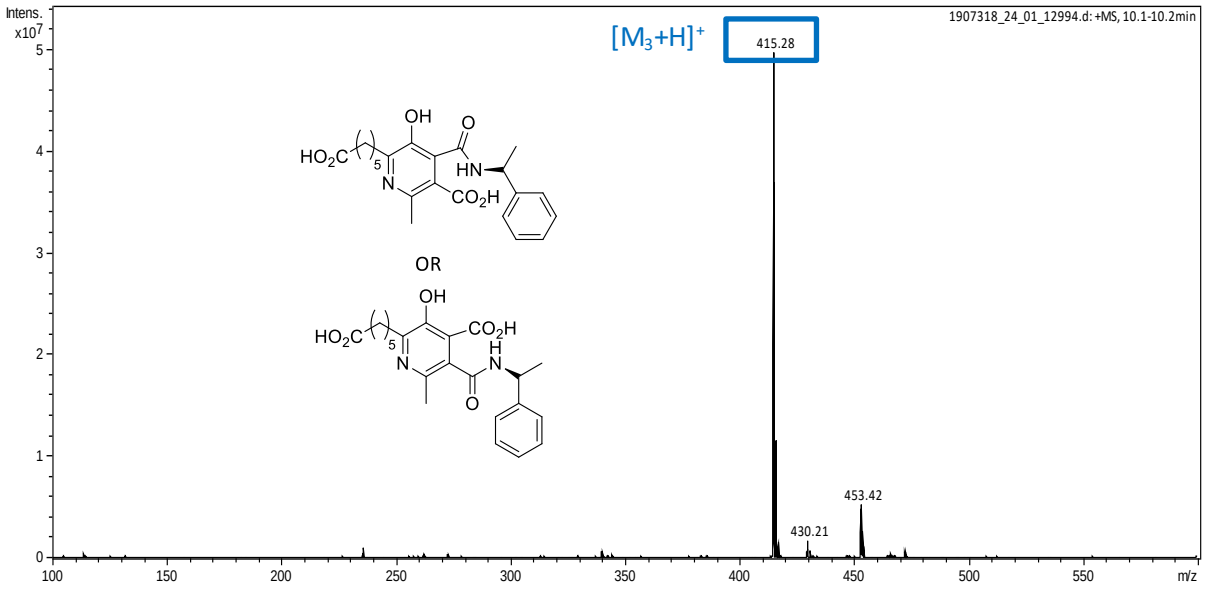


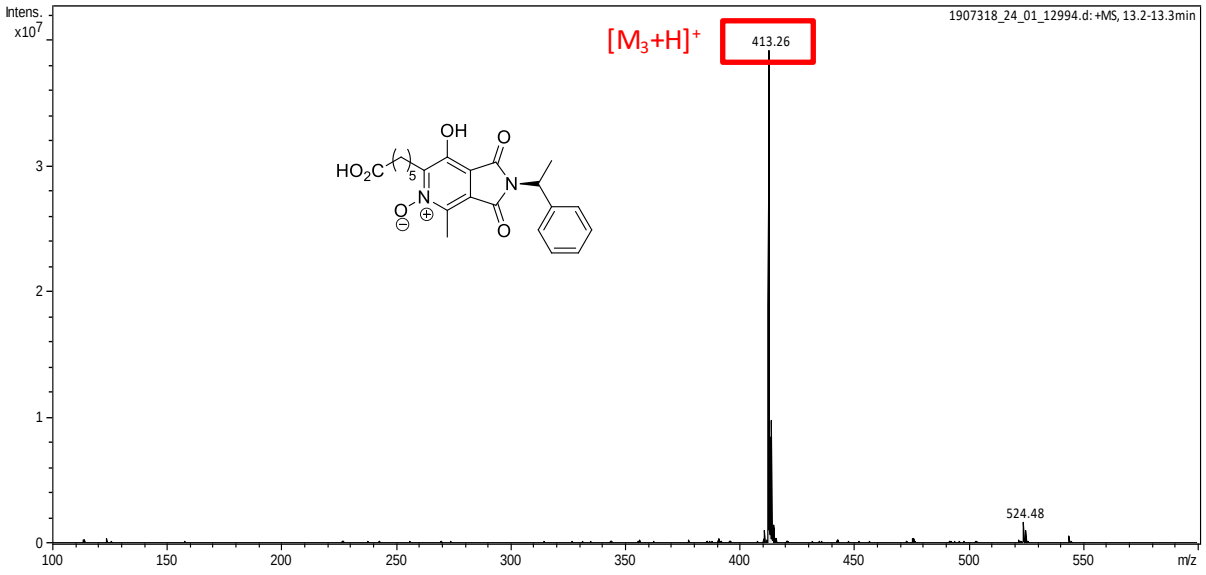
Figure S64. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectrum of **25** after 24 h in human blood plasma.



Retention time (min)	% area 254 nm (%)
9.8	2.7
10.1	1.6
13.3	traces
13.9	95.7




Intens.
x10^7
1907318_24_01_12994.d: +MS, 10.1-10.2min
[M3+H]+
415.28
OR
453.42
430.21
m/z


Intens.
x10^7
1907318_24_01_12994.d: +MS, 13.2-13.3min
[M3+H]+
413.26
524.48
m/z

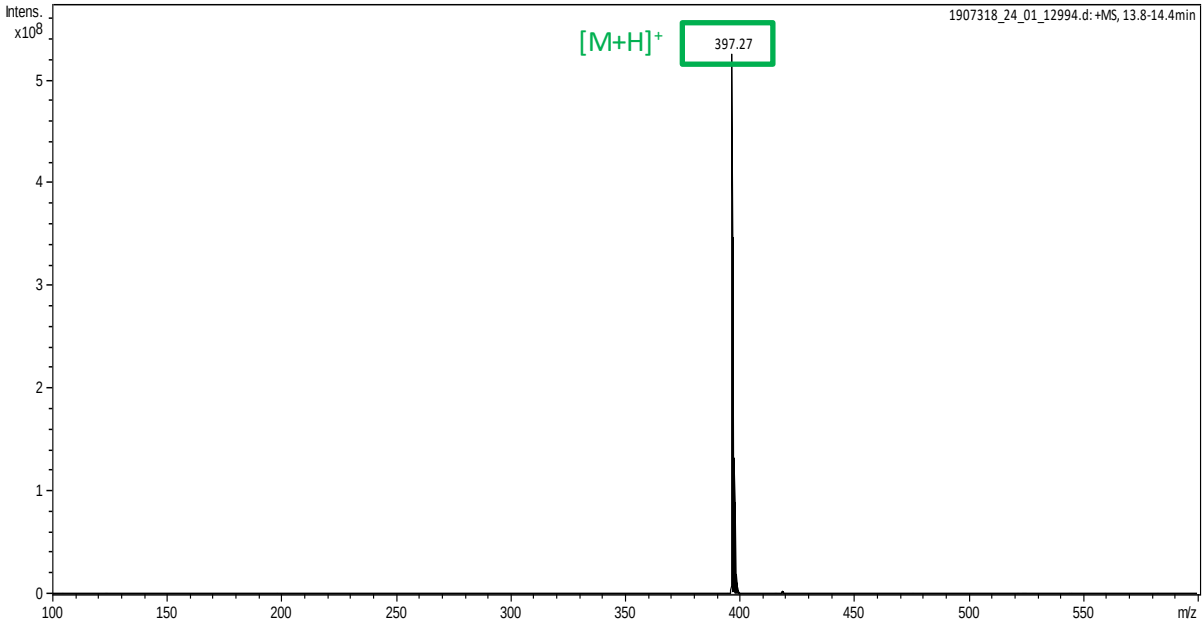


Figure S65. TIC, UV (254 nm) chromatograms and MS (ESI+) mass spectra of **26** after 24 h in human blood plasma.