Supplementary Information for

Copper-free Click – A Promising Tool for Pre-Targeted PET Imaging

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Contents

General experimental procedures, materials and Instrumentation

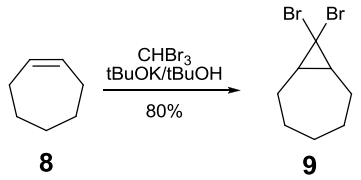
All reactions were performed under anhydrous conditions and an atmosphere of nitrogen in flame-dried glassware unless otherwise stated. Yields refer to chromatographically and spectroscopically (^{1}H-NMR) homogenous materials.

Solvents and reagents: All solvents were purified and dried according to standard methods prior to use. All chemicals were handled in accordance with COSHH regulations. All reagents were used as commercially supplied.

Flash chromatography (FC) was always performed on silica gel (Merck Kieselgel 60 F_{254} 320-400 mesh) according to the method of W. C. Still, unless otherwise stated. Thin Layer Chromatography (TLC) was performed on Merck aluminium-backed plated pre-coated with silica (0.2 mm, 60 F_{254}) which were visualised either by quenching of ultraviolet fluorescence (λ = 254 and 366 nm) or by charring with 10% $KMnO_4$ in 1M H_2SO_4. ^{1}H NMR spectra: These were recorded at 400 MHz on a Bruker AV-400 or on a Bruker AV-500 instrument. Chemical shifts (δ_H) are quoted in parts per million (ppm), referenced to the appropriate residual solvent peak. Coupling constants (*J*) are reported to the nearest 0.5 Hz. ^{13}C NMR spectra: These were recorded at 100 MHz on a Bruker AV-400 or on a Bruker AV-500 instrument. Chemical shifts (δ_C) are quoted in ppm, referenced to the appropriate residual solvent peak. ^{19}F NMR: These were recorded at 400 MHz on Bruker DRX-400 instrument. Chemical shifts (δ_F) are quoted in ppm, referenced to fluorobenzene at -113.5 ppm. Mass spectra: Low resolution mass spectra (*m/z*) were recorded on either a VG platform II or VG AutoSpec spectrometers, with only molecular ions (M^+, MH^+, MNa^+, MK^+, MNH_4^+) and major peaks being reported with intensities quoted as percentages of the base peak. Analytical reverse-phase HPLC was carried out on a Beckmann Pump 127 instrument using a Phenomenex Gemini C18 column (150 mm x 4.6 mm) with a gradient of acetonitrile and water. Semi-

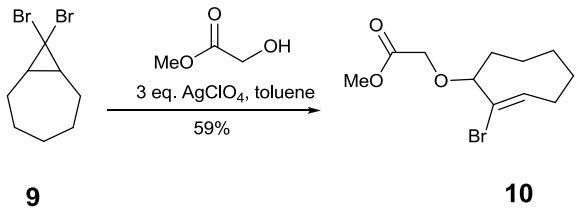
preparative reverse-phase HPLC was carried out on a Beckmann Pump 127 instrument using a Phenomenex Luna C18 column (100 mm x 10 mm) with an isocratic method of ethanol and water. Laura 3 software was used for processing all HPLC chromatograms.

Synthesis of cyclooct-1-yn-3-glycolic acid (9)



8,8-Dibromobicyclo[5.0.1]octane (9)[1]

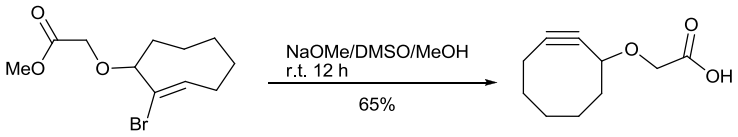
Potassium *tert*-butoxide (10.8 g, 145.6 mmol) was stirred in pentane (20 mL) at room temperature for 15 minutes. Cycloheptene **8** (8.4 mL, 72.8 mmol) was added and the mixture was cooled to -10 °C. Bromoform (9.4 mL, 109.2 mmol) was added dropwise over ~20 mins and the mixture turned pale brown. The reaction was allowed to warm to room temperature once the addition was complete and was stirred over night, after which time H_2O (100 mL) was added, and the mixture was acidified using HCl (2M). The product was extracted with pentane (2 x 100 mL) and the combined organic layers were washed with H_2O (2 x 50 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give crude material as an orange oil. The crude material was purified by silica gel chromatography, eluting with 5 % EtOAc in petroleum ether, to yield pure product **9** (16.69 g, 58.7 mmol, 80 %) as a colourless oil. δ_H (400 MHz, $CDCl_3$) 1.14-1.27 (m, 3H); 1.34-1.45 (m, 2H); 1.69-1.77 (m, 2H); 1.82-1.93 (m, 3H); 2.25-2.31 (m, 2H) [lit., δ_H (400 MHz, $CDCl_3$) 1.05-1.22 (m, 3H); 1.28-1.40 (m, 2H); 1.62-1.72 (m, 2H); 1.76-1.92 (m, 3H); 2.25-2.28 (m, 2H)]. δ_C (100 MHz, $CDCl_3$) 28.0; 28.9; 32.2; 34.7; 40.7 [lit., δ_C (100 MHz, $CDCl_3$) 27.9; 28.9; 32.2; 34.8; 40.7].



Methyl 2-bromocyclooct-1-en-3-glycolate (10)[1]

Silver perchlorate (667 mg, 3.2 mmol) was added portionwise to a stirred solution of 8,8-dibromobicyclo[5.0.1]octane **9** (467 mg, 1.6 mmol) and methyl glycolate (1.09 mL, 14.4 mmol) in anhydrous toluene (1 mL) under Nitrogen. The reaction vessel was protected from light by use of aluminium foil. The reaction mixture was stirred at room temperature for 1.5 hours, after which time the silver salts were removed by filtration and the residue was

purified straight away by column chromatography on Alumina eluting with 5-10 % EtOAc/pet. ether to afford product **10** as a pale yellow oil (260 mg, 59 %). δ_H (400 MHz, $CDCl_3$) 0.75-0.93 (m, 1H); 1.27-1.89 (m, 7H); 2.13-2.30 (m, 2H); 3.75 (s, 3H); 3.95 (d, *J* = 16.5 Hz, 1H); 4.10 (dd, *J* = 5.0, 10.0 Hz, 1H); 4.23 (d, *J* = 16.5 Hz, 1H); 6.35 (dd, *J* = 4.0-11.0, 5.0 Hz, 1H). [lit., (200 MHz, $CDCl_3$) 0.7-0.9 (m, 1H); 1.2-1.9 (m, 7H); 2.24-2.35 (m, 2H); 3.72 (s, 3H); 3.94 (d, *J* = 16.5 Hz, 1H); 4.10 (dd, *J* = 5.0-10.0 Hz, 1H); 4.23 (d, *J* = 16.5 Hz, 1H); 6.35 (dd, *J* = 4.0-11, 5Hz, 1H). δ_C (100 MHz, $CDCl_3$) 23.6; 27.0; 29.2; 30.3; 33.7; 51.8; 53.5; 60.6; 84.6; 126.3; 135.9; 170.7. [lit., (50 MHz, $CDCl_3$) 26.2; 28.0; 33.4; 36.5; 39.3; 51.8; 53.48; 65.4; 84.8; 131.4; 133.0; 170.7].

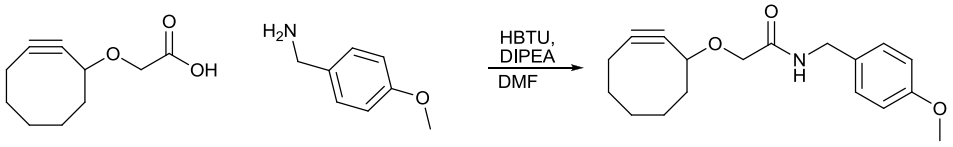


10 **4**

Cyclooct-1-yn-3-glycolic acid (4)[1]

To a solution of NaOMe in MeOH (0.5 M, 13 mL) and dry DMSO (0.65 mL) was added methyl 2-bromocyclooct-1-en-3-glycolate **10** (260 mg, 0.94 mmol) and the resultant mixture was stirred at room temperature for 12 hours. The solvent was evaporated *in vacuo* to yield an orange gel-like solid. CH_2Cl_2 (10 mL) was added and the mixture was acidified to pH ~ 2 by cautious addition of HCl (2 M). The product was extracted with CH_2Cl_2 (3 x 15 mL), and the combined organic layers were washed with H_2O (10 mL), dried over $MgSO_4$ and concentrated *in vacuo* to obtain crude material as a yellow oil. The crude material was purified by silica gel column chromatography eluting first with 2 % MeOH/DCM, then with 5 % MeOH/DCM, to obtain pure product **4** as a pale yellow solid after drying (110 mg, 65 %). δ_H (400 MHz, $CDCl_3$) 1.3-2.3 (m, 10H); 4.32-4.45 (m, 1H); 4.45 (d, *J* = 17.0 Hz, 1H); 4.58 (d, *J* = 17.0 Hz, 1H); 8.12 (s, 1H). [lit., 1.3-2.3 (m, 10H); 4.45 (d, *J* = 17.0 Hz, 1H); 4.32-4.45 (m, 1H); 4.58 (d, *J* = 17.0 Hz, 1H); 8.12 (s, 1H). δ_C (100 MHz, $CDCl_3$) 20.5; 26.0; 29.3; 33.7; 41.8; 65.5; 72.6; 91.2; 101.6; 173.5. [lit., (100 MHz, $CDCl_3$) 20.3; 25.9; 29.3; 33.9; 41.8; 65.3; 72.6; 90.7; 101.3; 174.1]. *m/z* (CI^+) calcd. for 182.2 found: 182.2.

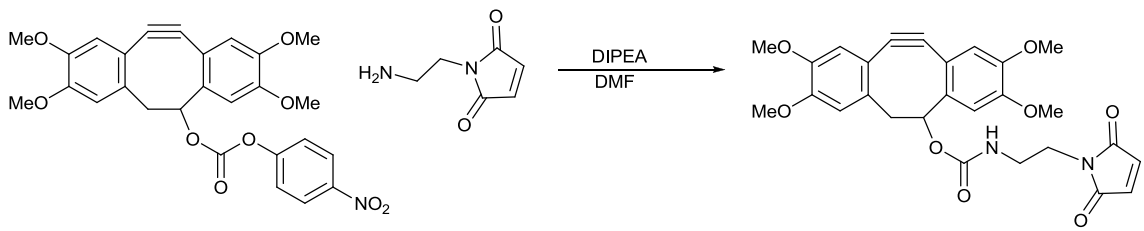
Synthesis of new compounds



4 **6**

2-(Cyclooct-2-yn-1-yloxy)-*N*-(4-methoxybenzyl)acetamide (6)

To a stirred solution of cyclooct-1-yn-3-glycolic acid **4** (12.5 mg, 0.07 mmol) in anhydrous DMF (1 mL) was added DIPEA (13.4 µL, 0.014 mmol) and HBTU (53 mg, 0.014 mmol). The resultant mixture was stirred at room temperature for 30 minutes, after which time 4-methoxy benzylamine (121.0 µL, 0.084 mmol) was added and the mixture was stirred at room temperature for 16 hours. TLC (1:1 EtOAC/pet. ether) indicated that the reaction had gone to completion. The reaction was stopped and the product was extracted with CH_2Cl_2 (2 x 10 mL) and washed with brine (2 x 10 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated *in vacuo* to yield crude material as an orange oil. The crude material was purified by silica gel chromatography eluting with 50-70 % EtOAc/pet. ether to yield amide **6** as a colourless oil (15.7 mg, 74 %). δ_H (400 MHz, $CDCl_3$) 1.30-2.11 (m, 10H); 3.69 (s, 3H); 3.89 (d, *J* = 15.0 Hz, 1H); 4.03 (d, *J* = 15.0 Hz, 1H); 4.33-4.43 (m, 3H); 6.84 (d, *J* = 8.5 Hz, 2H); 6.93 (bs, 1H); 7.20 (d, *J* = 8.5 Hz, 2H). δ_C (100 MHz, $CDCl_3$) 23.5; 27.0; 29.2; 31.4; 33.7; 42.3; 55.3; 68.3; 114.0; 129.0; 130.2; 135.6. *m/z* (EI^+) ($[M+ H]^+$) calcd. for 302.1756, found: 302.1760.



5 **7**

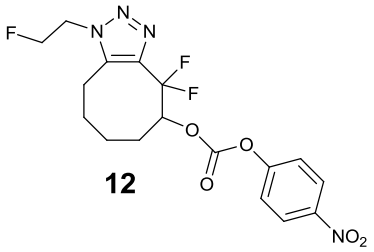
Carbamate-2',3',2",3"-tetramethoxy-7,8-didehydro-1,2:5,6-dibenzocyclocta-1,5,7-triene-3-yl *N*-(2-Aminoethyl)maleimide (7)

To a stirred solution of TMDIBO (**5**) (5 mg, 0.01 mmol) in DMF (1 mL) was added DIPEA (2.1 µL, 0.01 mmol). The resultant mixture was stirred at room temperature for 15 minutes, after which time *N*-(2-aminoethyl)maleimide (5.1 mg, 0.02 mmol) was added and the mixture was stirred at room temperature for 16 hours. TLC (1:1 EtOAC/pet. ether) indicated that the

reaction had gone to completion. The reaction was stopped and the product was extracted with EtOAc (2 x 10 mL) and washed with H_2O (2 x 10 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated *in vacuo* to yield crude material as a yellow oil. The product was purified by silica gel chromatography, eluting with 50-70% EtOAc/pet.ether to yield maleimide **7** as a yellow powder (5 mg, 99 %). δ_H (400 MHz, $CDCl_3$) 2.92 (s, 2H); 2.99 (s, 2H); 3.07-3.11 (m, 1H); 3.45-3.48 (m, 1H); 3.82-3.98 (m, 12H); 5.44 (m, 1H); 6.83-6.85 (d, *J* = 6.0 Hz, 2H); 6.92-6.94 (d, *J* = 9.0 Hz, 2H); 7.03-7.06 (d, *J* = 12.0 Hz, 2H). δ_C (100 MHz, $CDCl_3$) 30.9; 31.5; 36.5; 40.3; 46.7; 56.1; 68.2; 108.2; 108.7; 109.1; 112.5; 114.1; 115.6; 126.2; 128.8; 130.9; 134.2; 145.2; 147.8; 148.8; 150.2; 161.7; 162.4; 207.0. *m/z* (EI^+) ($[(M\text{-maleimide})+ H]^+$) calcd. for 341.1384, found: 341.1390.

General method for the copper-free 'click' reaction with cyclooctynes

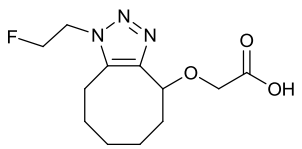
2-fluoroethyl azide (0.02 mmol) was added to a stirred solution of cyclooctyne (0.005 mmol) in $CDCl_3$ (0.6 mL). The resultant mixture was stirred at 55 °C for 2 h. When complete, the reaction was stopped and the solvent evaporated *in vacuo*. The crude material was re-dissolved in $CDCl_3$ and the ^{19}F, ^{1}H and ^{13}C NMR were taken. These crude compounds, as mixtures of regioisomers, were used as references only. The ratio of regioisomers was determined using a combination of ^{18}F HPLC traces and ^{19}F NMR spectra. The characterisation of these compounds is listed below.



12

4,4-Difluoro-1-(2-fluoroethyl)-4,5,6,7,8,9-hexahydro-1*H*-cycloocta[*d*][1,2,3]triazol-5-yl 4-nitrophenyl carbonate (12) as a mixture of regioisomers in ratio 2:1

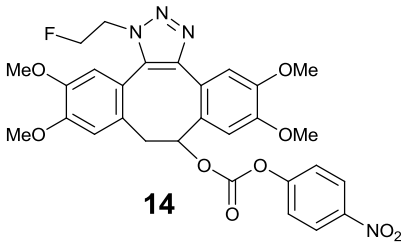
δ_H (400 MHz, $CDCl_3$) 1.18-1.37 (m, 1.5H); 1.73-1.86 (m, 1.5H); 2.30-2.39 (m, 6H); 2.43-2.47 (m, 3H); 4.57 (dm, *J* = 22.5 Hz, 3H); 4.92 (dm, *J* = 26.0 Hz, 3H); 5.35-5.38 (m, 1.5H); 7.19 (d, *J* = 8.0 Hz, 2H); 7.83 (d, *J* = 8.0 Hz, 2H). δ_c (100 MHz, $CDCl_3$) 19.7; 24.1; 29.1; 29.7; 32.6; 50.6 (d, J = 20.0 Hz); 80.4 (d, J = 176.0 Hz); 82.0; 85.1; 111.8; 112.4; 113.1; 113.7; 115.6; 121.8; 126.0; 128.6. δ_F (400 MHz, $CDCl_3$) -223.7 (2); -223.5 (1); -107.8; -107.5; -104.9; -101.3; -100.5; -93.7. *m/z* (EI^+) ($[M+H]^+$) calcd. for 415.1224, found: 415.1220.



13

{[1-(2-Fluoroethyl)-4,5,6,7,8,9-hexahydro-1*H*-cycloocta[*d*][1,2,3]triazol-4-yl]oxy}acetic acid (13) as a mixture of regioisomers in ratio 2:1.

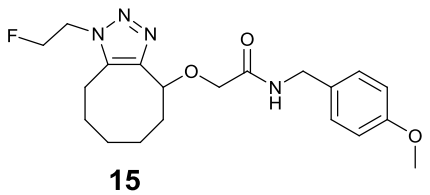
δ_H (400 MHz, $CDCl_3$) 1.25-2.46 (m, 15H); 3.30-3.40 (m, 4.5H); 4.26 (d, *J* = 27.0 Hz, 3H); 4.56 (d, *J* = 47.0 Hz, 3H); 8.06 (s, 1H). δ_F (400 MHz, $CDCl_3$) -224.5; -222.4. *m/z* (EI^+) ($[M+H]^+$) calcd. for 272.1405, found: 272.1405.



14

1-(2-fluoroethyl)-Carbonic acid-2',3',2",3"-tetramethoxy-7,8-didehydro-1,2:5,6-dibenzocyclocta-1,5,7-triene-[1,2,3]triazolyl 4-nitrophenyl ester (14) as a mixture of regioisomers in ratio 2:1

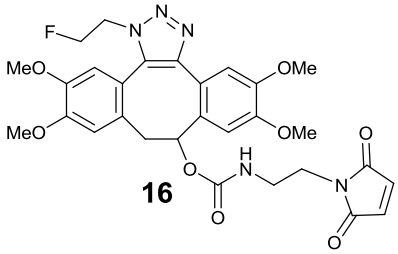
δ_H (400 MHz, $CDCl_3$) 2.89 (as, 1H); 2.96 (as, 1H); 3.10 (as, 0.5H); 3.21 (as, 0.5H); 3.70- 4.01 (m, 18H); 4.53-4.79 (m, 6H); 5.34-5.72 (m, 1H); 6.57-6.63 (m, 1H); 6.68-6.72 (m, 1H); 6.81-6.83 (m, 1H); 6.91 (d, *J* = 9.0 Hz, 2H); 6.99-7.01 (m, 1H); 7.07-7.10 (m, 1H); 8.07 (d, *J* = 9.0 Hz, 1H); 8.14 (d, *J* = 9.0 Hz, 2H). δ_c (100 MHz, $CDCl_3$) 41.0, 45.6, 50.8 (d, *J* = 24.0 Hz) 55.86, 55.92, 56.03, 56.04, 81.9 (d, *J* = 171.0 Hz), 107.4; 107.2; 109.3; 111.7; 113.0; 113.7; 114.7; 115.5; 115.7; 124.8; 125.9; 141.1; 142.5; 143.3; 146.5; 148.6; 149.7; 150.2; 150.7; 152.8; 154.3. δ_F (400 MHz, $CDCl_3$) -222.3; -222.4. *m/z* (EI^+) ($[M+2H]^{2+}$) calcd. for 298.0954 found: 298.1001.



15

{[1-(2-Fluoroethyl)-4,5,6,7,8,9-hexahydro-1*H*-cycloocta[*d*][1,2,3]triazol-4-yl]oxy} *N*-(4-methoxybenzyl)acetamide (15) as a mixture of regioisomers in ratio 2:1

δ_H (400 MHz, $CDCl_3$). 1.21-1.28 (m, 3H); 1.60-1.80 (m, 2H); 2.08 (s, 1H); 2.08-2.20 (m, 2H); 6.83-6.85 (d, *J* = 9.0 Hz, 2H); 7.17-7.18 (d, *J* = 9.0 Hz, 2H). δ_c (100 MHz, $CDCl_3$) 19.9; 23.0; 23.7; 24.2; 27.0; 29.1; 30.4; 33.3; 38.7; 48.6; 49.3 (d, *J* = 25.0 Hz); 48.6; 54.3; 68.2; 81.6 (d, *J* = 168.0 Hz); 125.9; 228.8; 131.0; 132.5; 165.6; 167.8. δ_F (400 MHz, $CDCl_3$) -222.5; -225.5. *m/z* (EI^+) ($[M+K]^+$) calcd. for 429.1699, found: 429.1701.

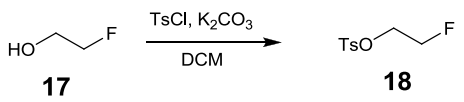


16

1-(2-fluoroethyl)-Carbonic acid-2',3',2",3"-tetramethoxy-7,8-didehydro-1,2:5,6-dibenzocyclocta-1,5,7-triene-[1,2,3]triazolyl *N*-(2-aminoethyl)maleimide (16) as a mixture of regioisomers in ratio 7:1

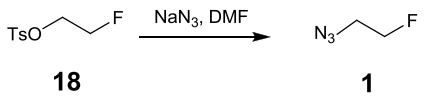
δ_H (400 MHz, $CDCl_3$). 2.44 (s, 1H); 2.60 (s, 1H); 2.72 (s, 1H); 2.80 (s, 1H); 3.00 (s, 1H); 3.07 (s, 1H); 4.16-4.23 (m, 12H); 4.25 (dm, *J* = 27.0 Hz, 2H); 4.56 (dm, *J* = 47.0 Hz, 2H); 5.28-5.61 (m, 1H); 7.33 (s, 1H); 7.35 (s, 1H); 7.50-7.52 (m, 2H); 7.67-7.69 (m, 2H). δ_c (100 MHz, $CDCl_3$) . 28.9; 29.7; 30.4; 36.5; 40.3; 46.1; 56.0; 67.1 (d, *J* = 26.5 Hz); 68.1, 80.6 (d, *J* = 174.5 Hz); 107.8; 108.7; 109.5; 112.0; 113.7; 114.1; 115.5; 128.0; 128.8; 130.8; 132.5; 145.1; 147.8; 148.5; 150.6; 151.1; 161.0; 162,6; 167.8. δ_F (400 MHz, $CDCl_3$) -225.2. *m/z* (EI^+) ($[(M\text{-maleimide})+H]^+$) calcd. for 430.1773, found: 430.1775.

2-Fluoroethyl azide synthesis



2-Fluoroethyl tosylate (18)

To a stirred solution of 2-fluoroethanol **17** (0.55 mL, 7.8 mmol) in CH_2Cl_2 (15 mL) was added tosylchloride (2.23 g, 11.7 mmol) and DIPEA (1.52 mL, 15.8 mmol) and the resultant mixture was stirred at room temperature under nitrogen for 16 hours. CH_2Cl_2 (30 mL) was added and was washed with H_2O (2 x 10 mL). The solvent was evaporated *in vacuo* to yield crude 2-fluoroethyl tosylate **18** (1.83 g), which was carried through to the next step without further purification.

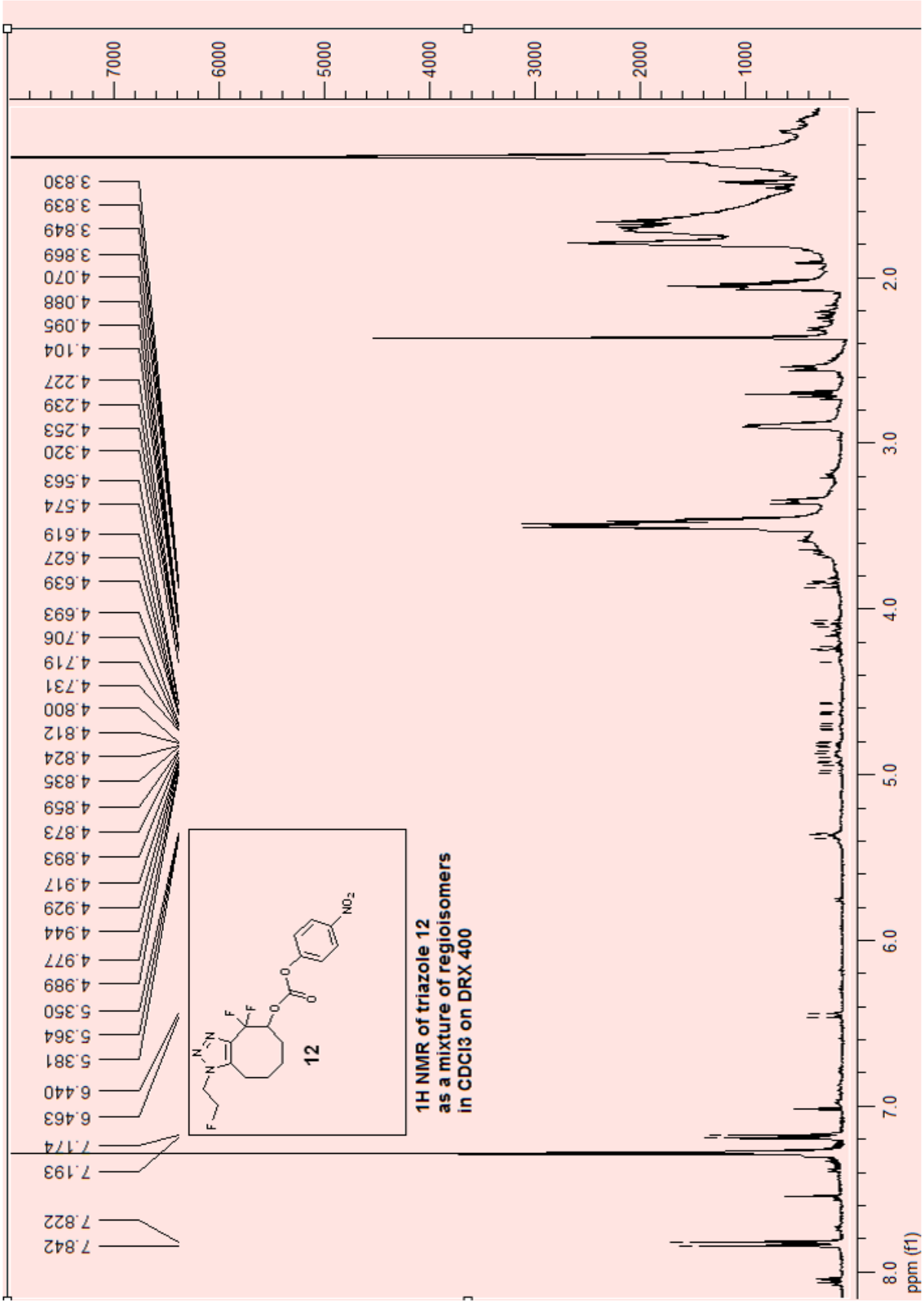


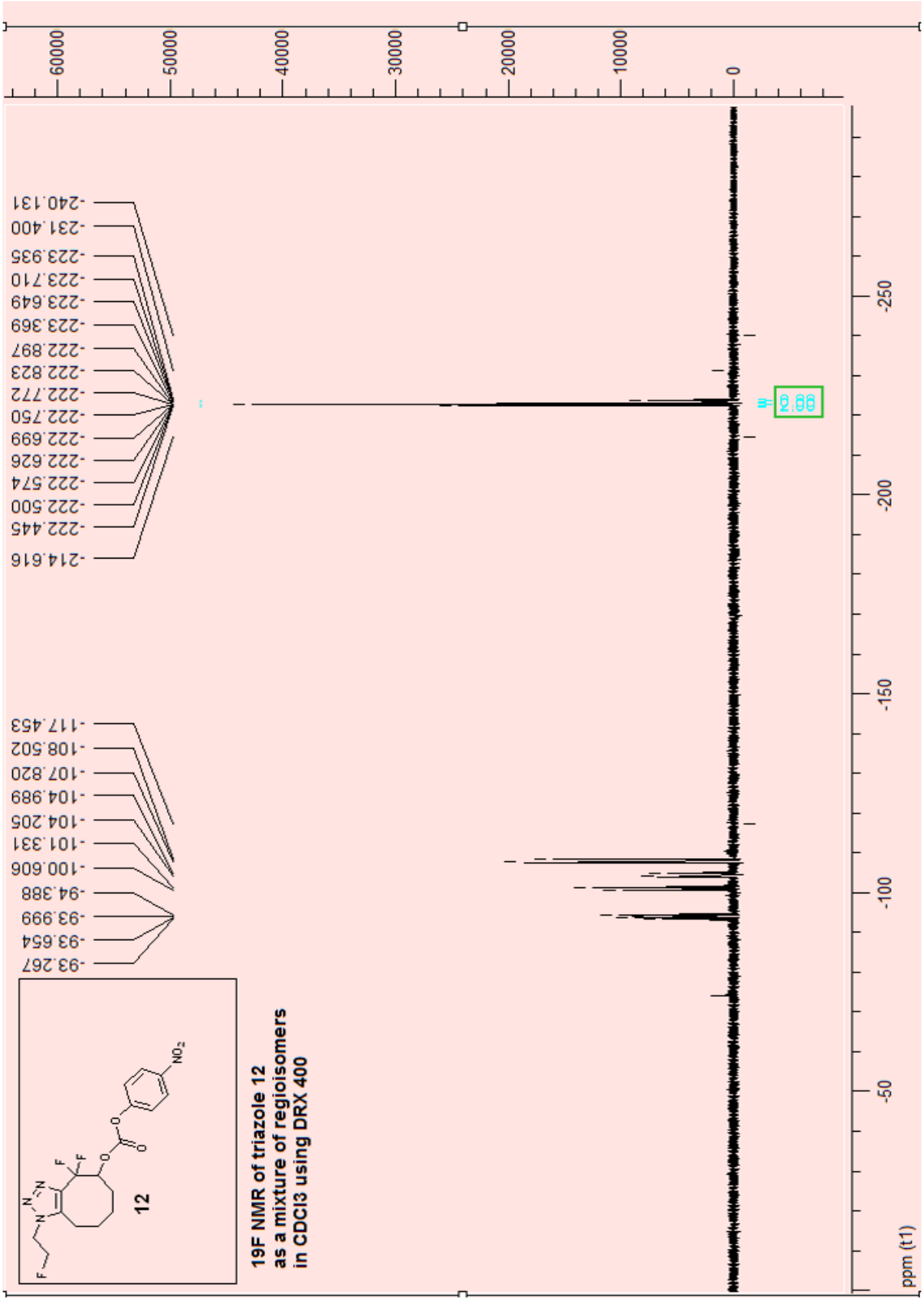
2-Fluoroethyl azide (1)

To a stirred solution of 2-fluoroethyl tosylate **16** (200mg, 0.92 mmol) in DMF (5 mL) was added sodium azide (178.6 mg, 2.75 mmol) and the resultant mixture was stirred at room temperature under nitrogen for 48 hours. TLC (1:1 EtOAc/Hex) showed complete consumption of the starting material. The reaction mixture was then filtered to remove the white precipitate. The filtrate was used for the next step without work-up or purification. δ_F (400 MHz, $CDCl_3$) -224.

Radiosynthesis of [^{18}F]2-fluoroethylazide (**[^{18}F]1**) was carried out using the method described by Glaser *et al*.[2] The product was detected using analytical radio- HPLC.

Selected NMR spectra




12
19F NMR of triazole 12
as a mixture of regioisomers
in CDCl3 using DRX 400
-93.267
-93.654
-93.999
-94.388
-100.606
-101.331
-104.205
-104.989
-107.820
-108.502
-117.453
-214.616
-222.445
-222.500
-222.574
-222.626
-222.699
-222.750
-222.772
-222.823
-222.897
-223.369
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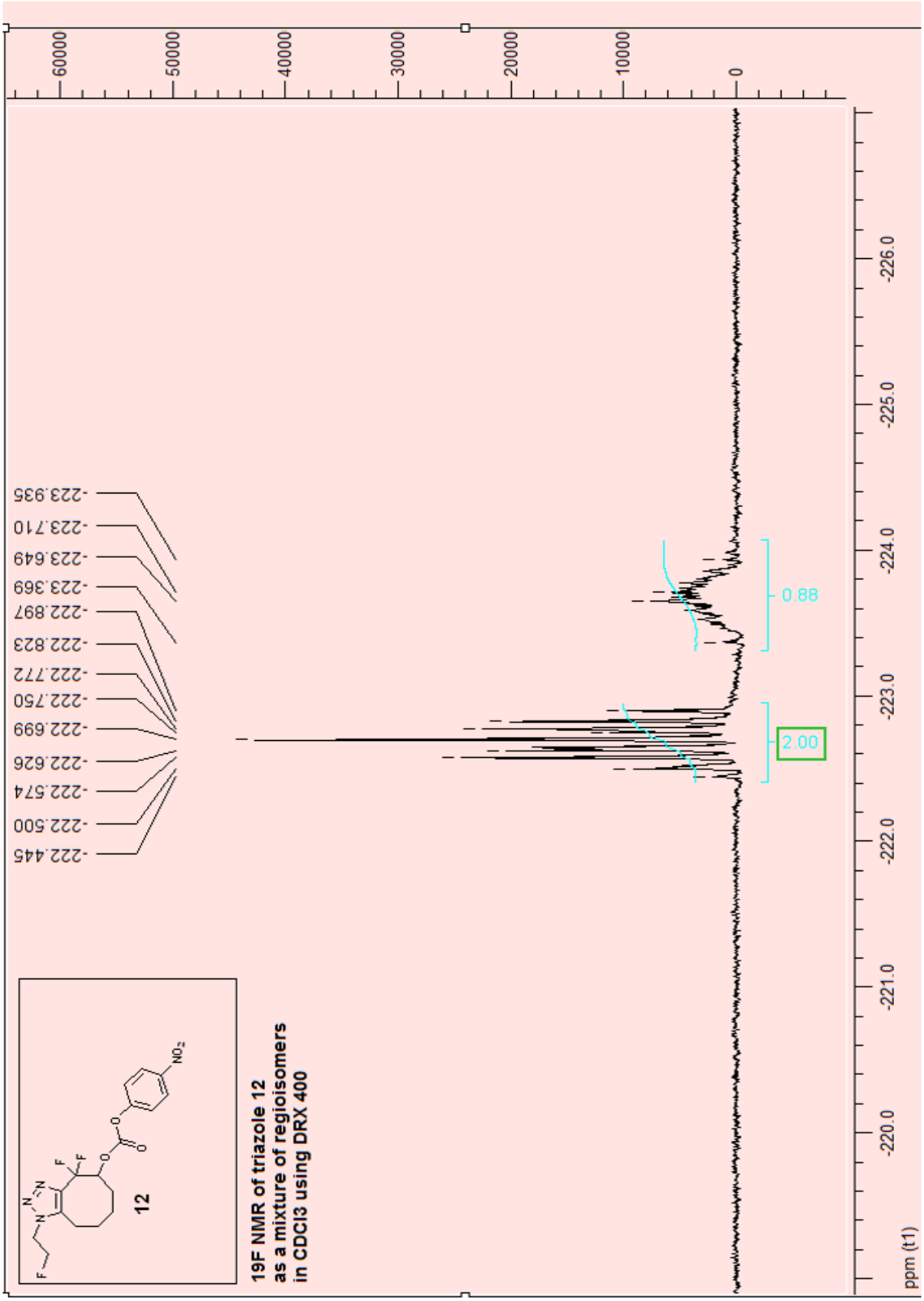
-223.710
-223.935
-231.400
-240.131
0.00
ppm (t1)


60000
50000
40000
30000
20000
10000
0
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-223.710
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-223.369
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-222.750
-222.699
-222.626
-222.574
-222.500
-222.445
0.88
2.00
-226.0
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-224.0
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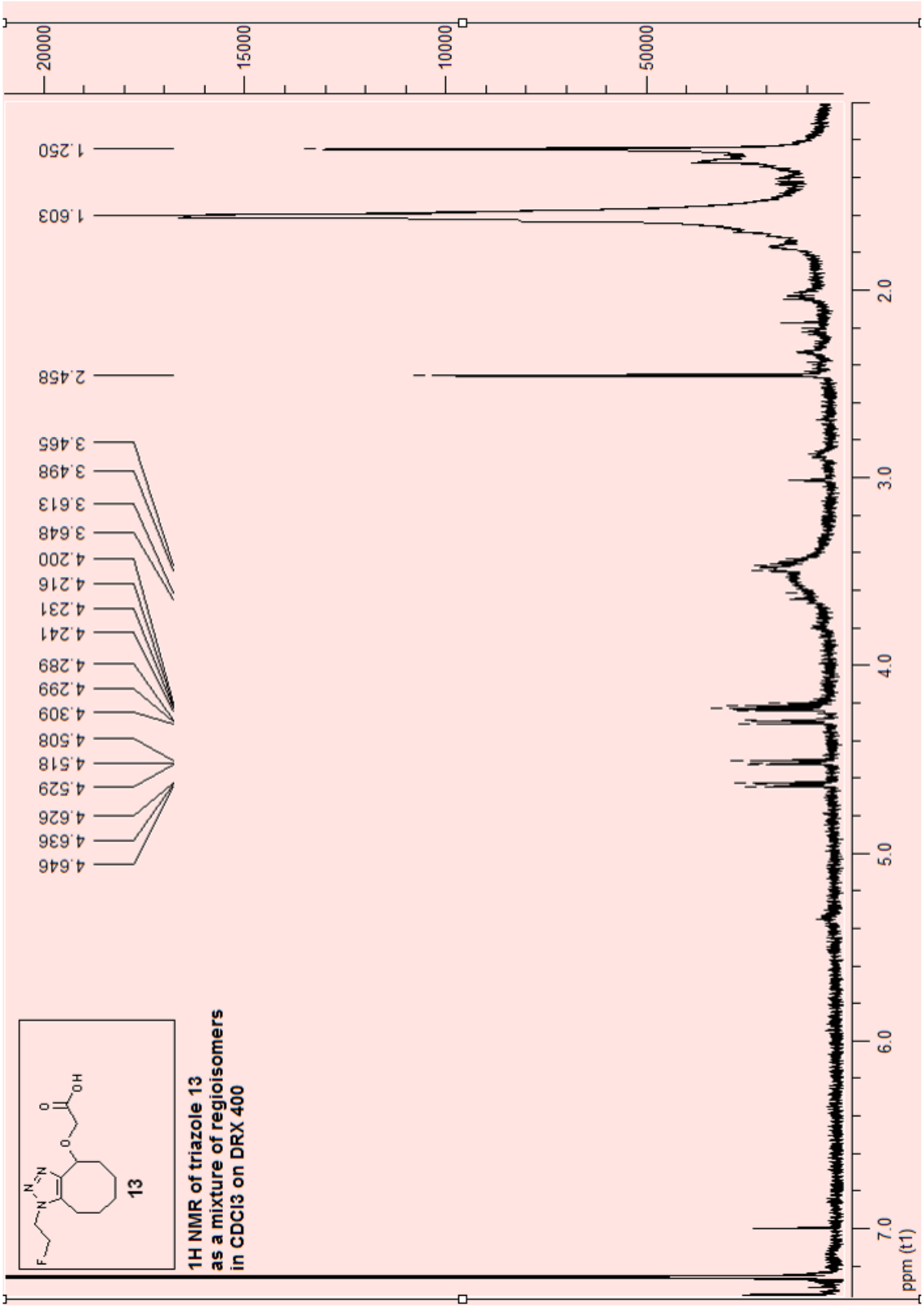
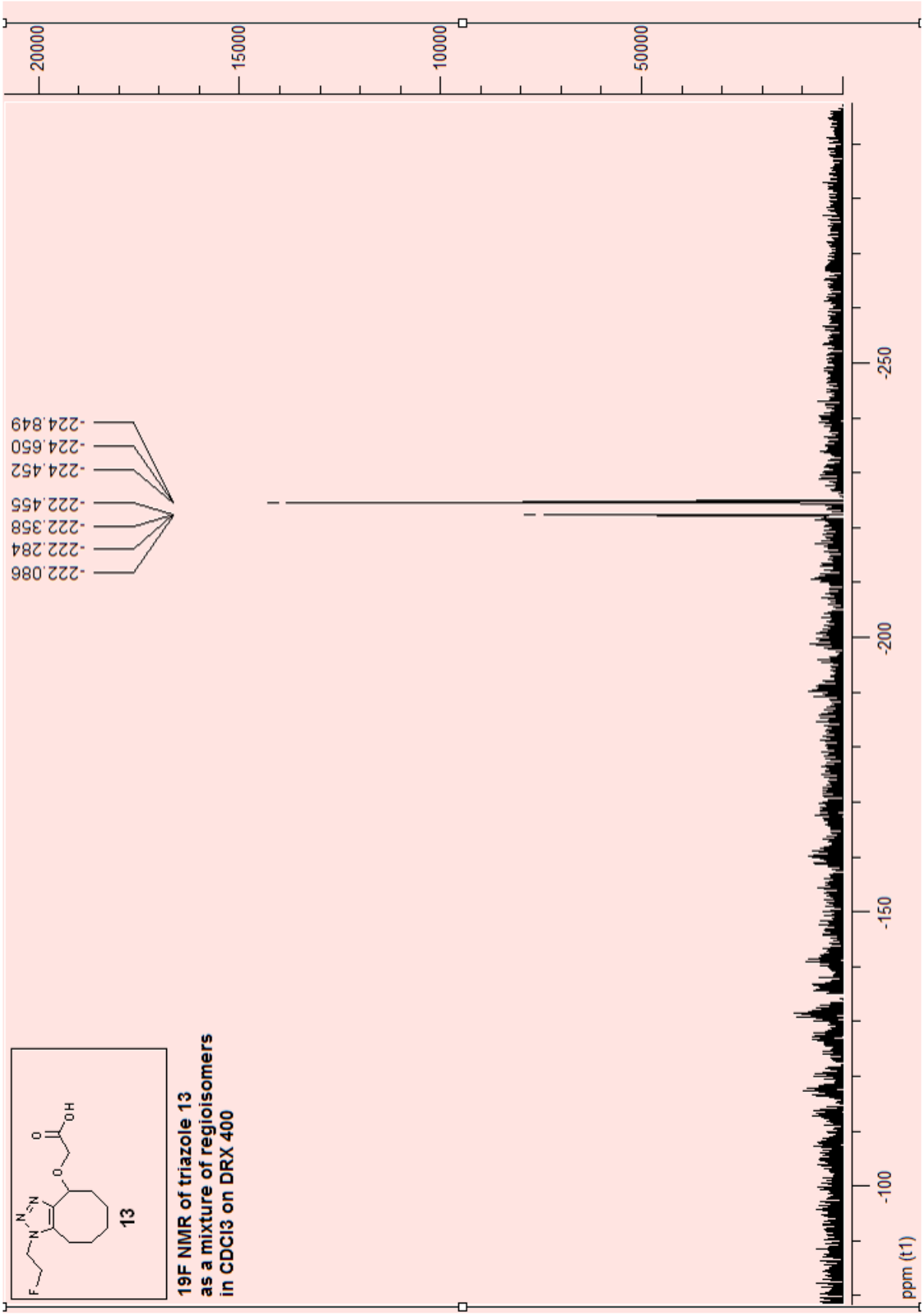
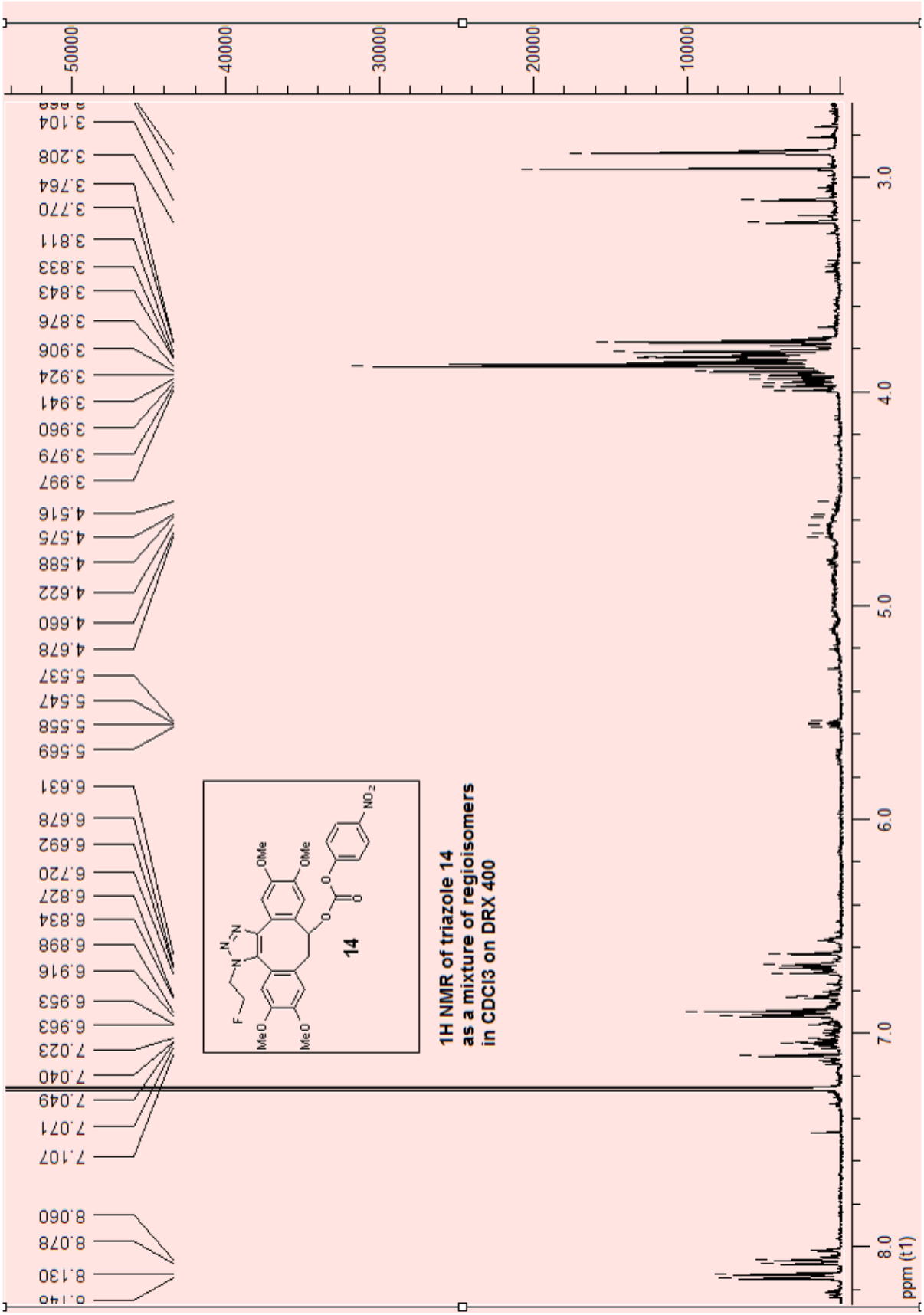
-222.0
-221.0
-220.0
ppm (t1)
12
19F NMR of triazole 12
as a mixture of regioisomers
in CDCl3 using DRX 400

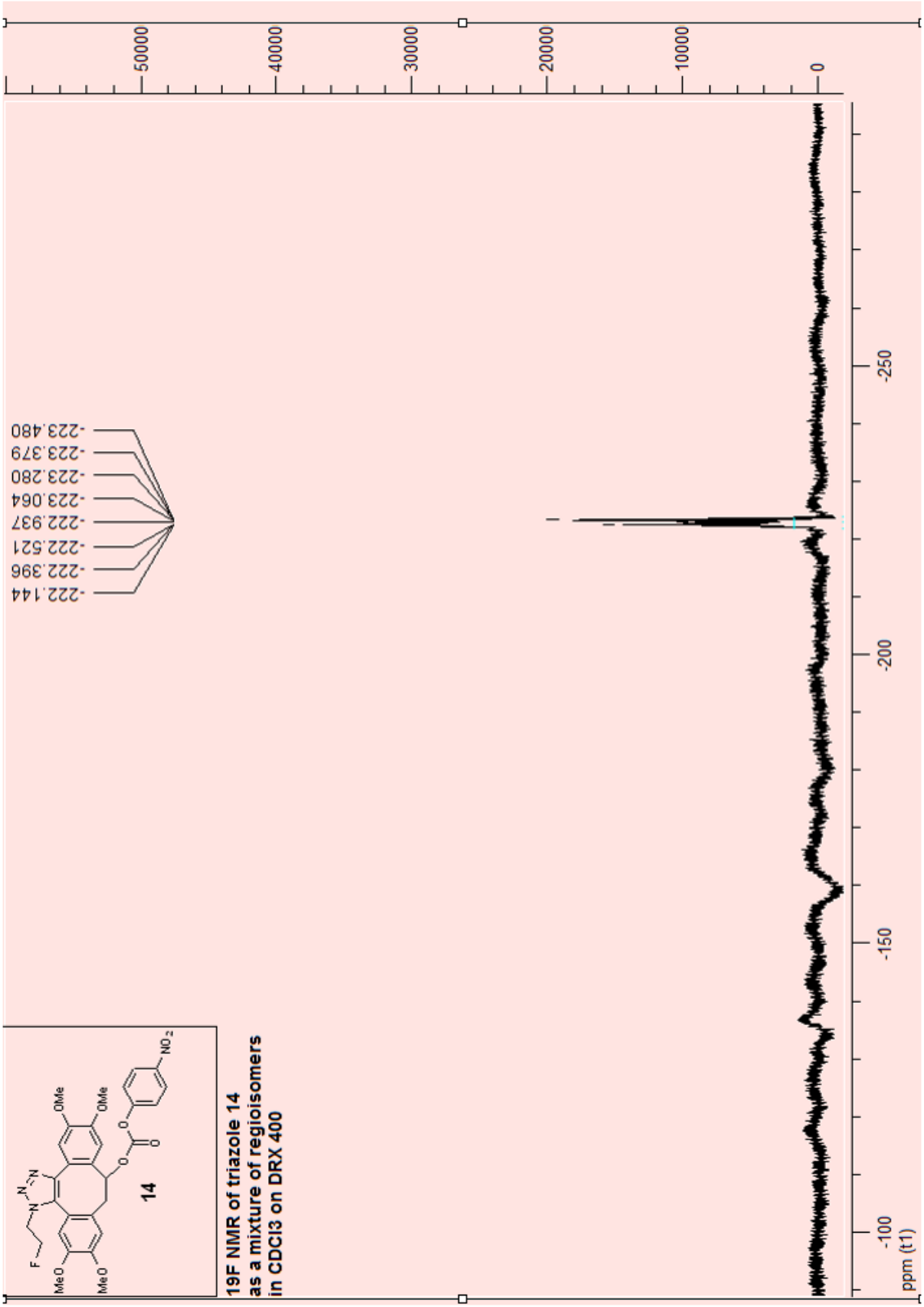


1H NMR of triazole 13 as a mixture of regioisomers in CDCl3 on DRX 400

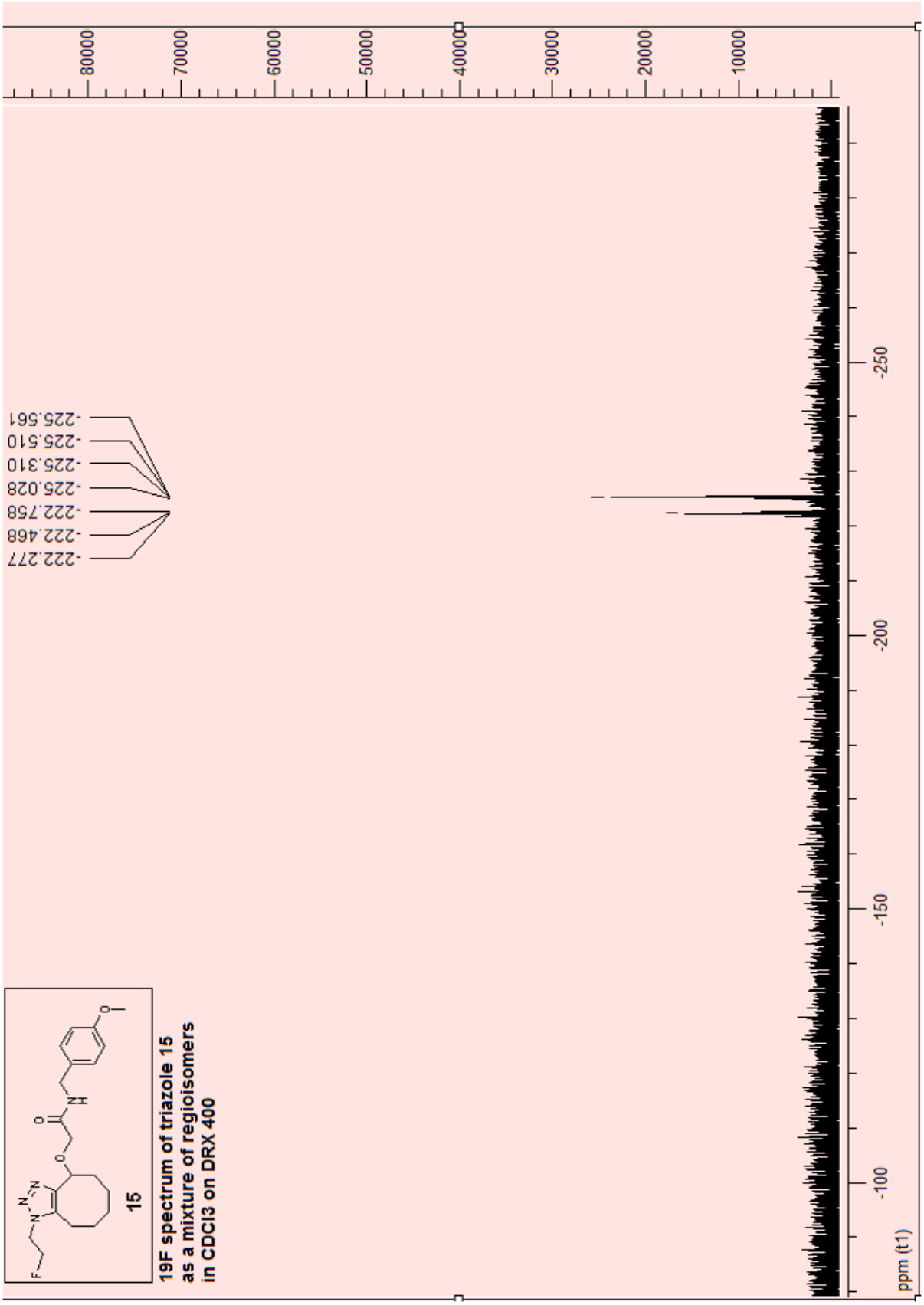


19F NMR of triazole 13 as a mixture of regioisomers in CDCl3 on DRX 400

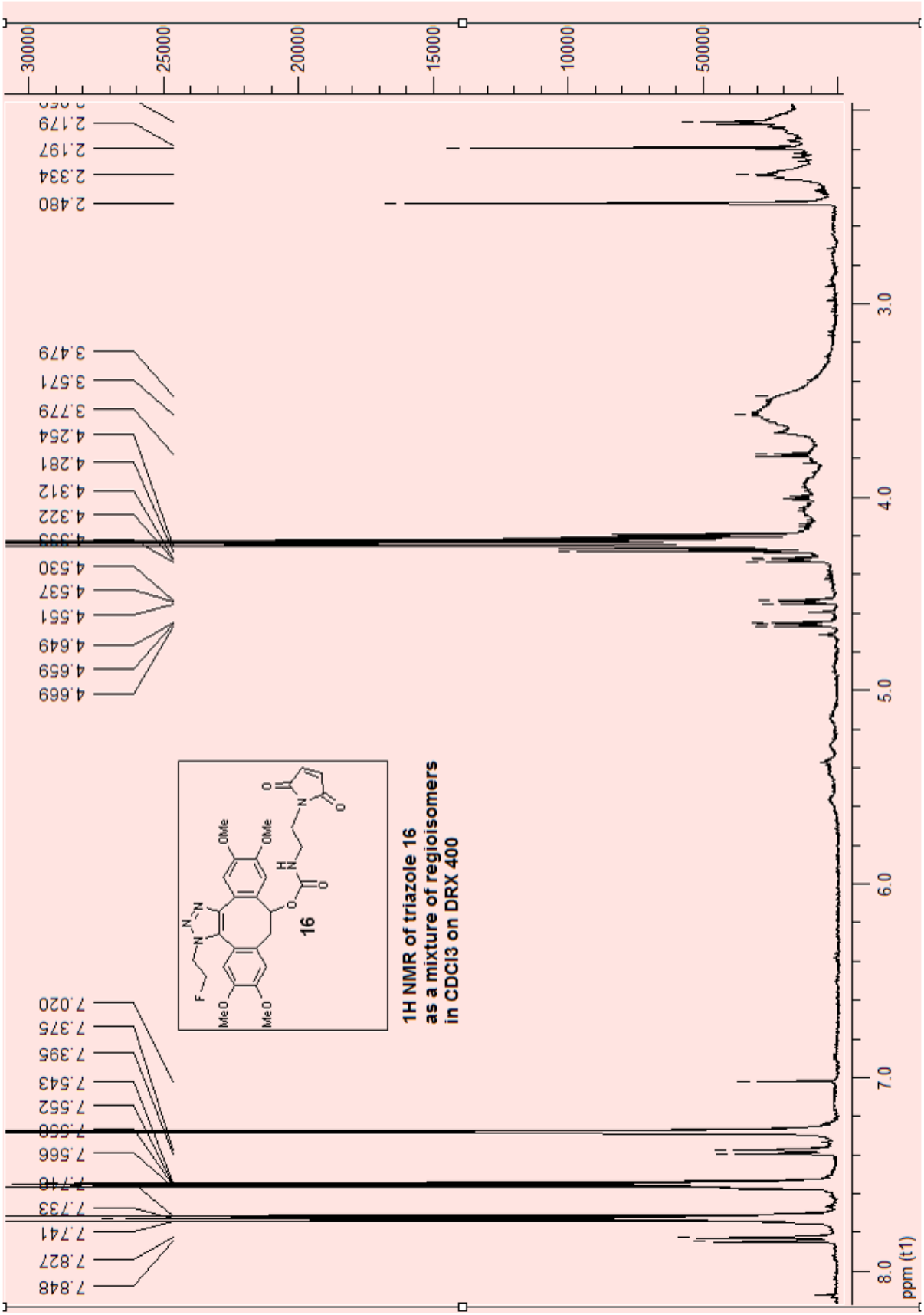

14
1H NMR of triazole 14
as a mixture of regioisomers
in CDCl3 on DRX 400
ppm (t1)



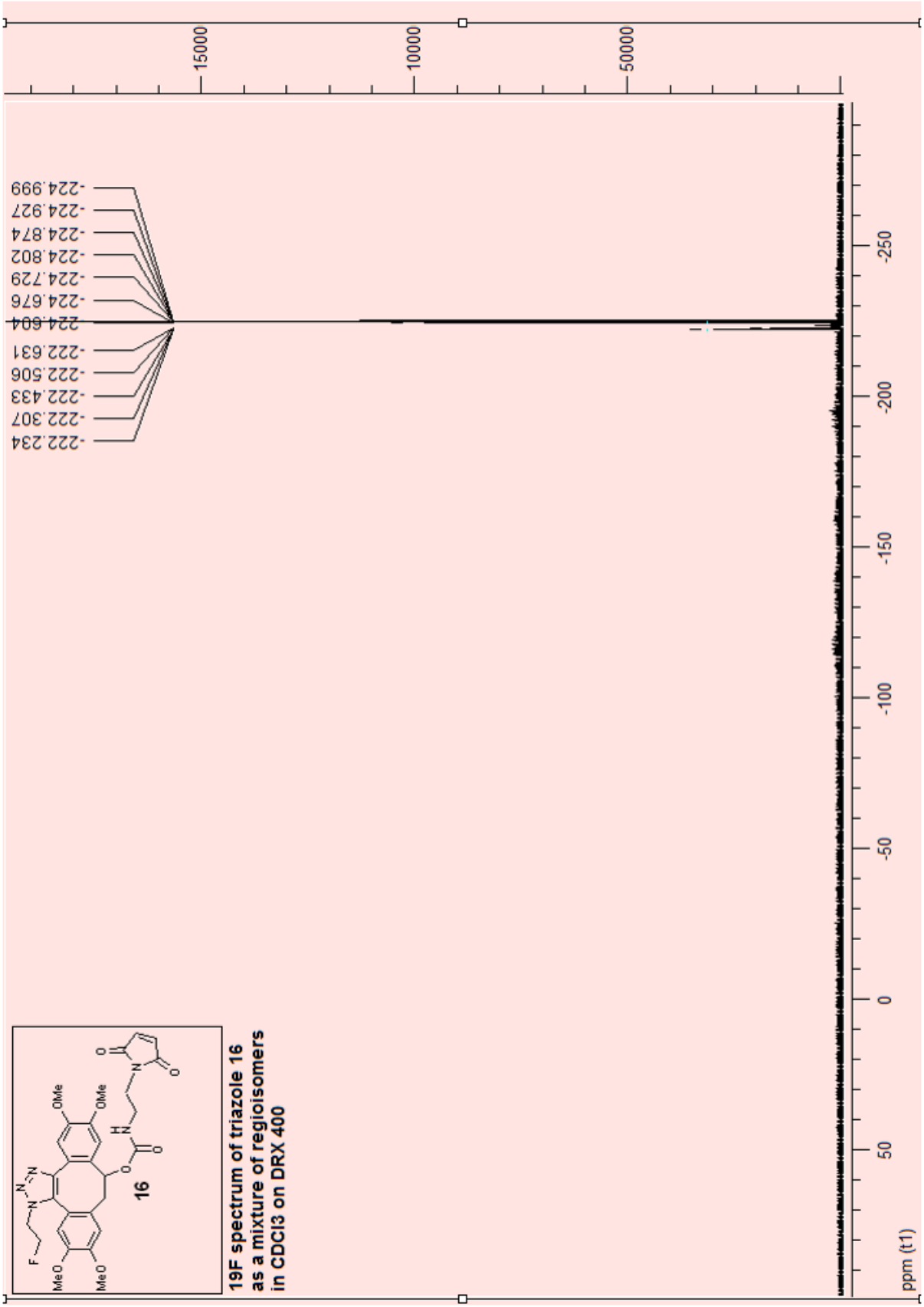
19F NMR of triazole 14 as a mixture of regioisomers in CDCl3 on DRX 400



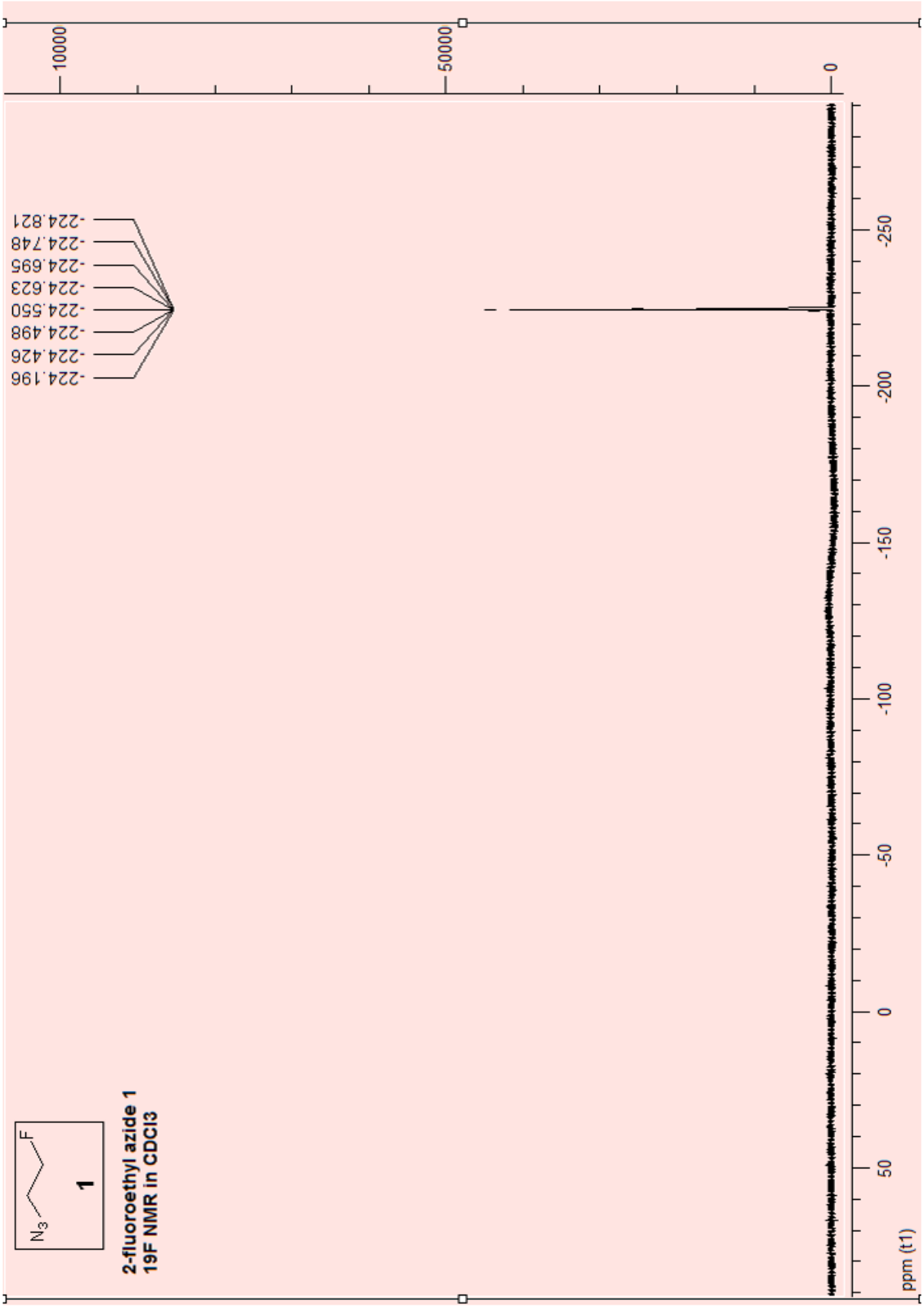
19F spectrum of triazole 15 as a mixture of regioisomers in CDCl3 on DRX 400



1H NMR of triazole 16 as a mixture of regioisomers in CDCl3 on DRX 400



19F spectrum of triazole 16 as a mixture of regioisomers in CDCl3 on DRX 400


2-fluoroethyl azide 1
19F NMR in CDCl3
N3
F
1
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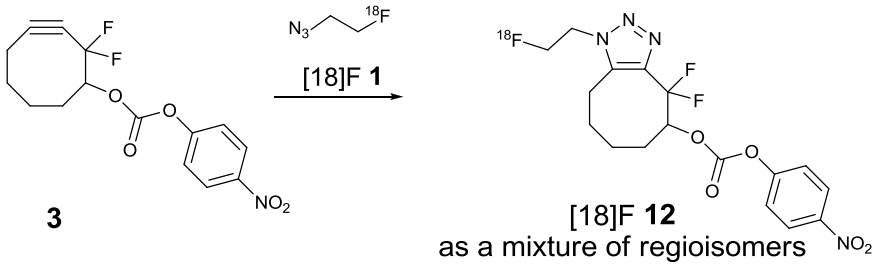
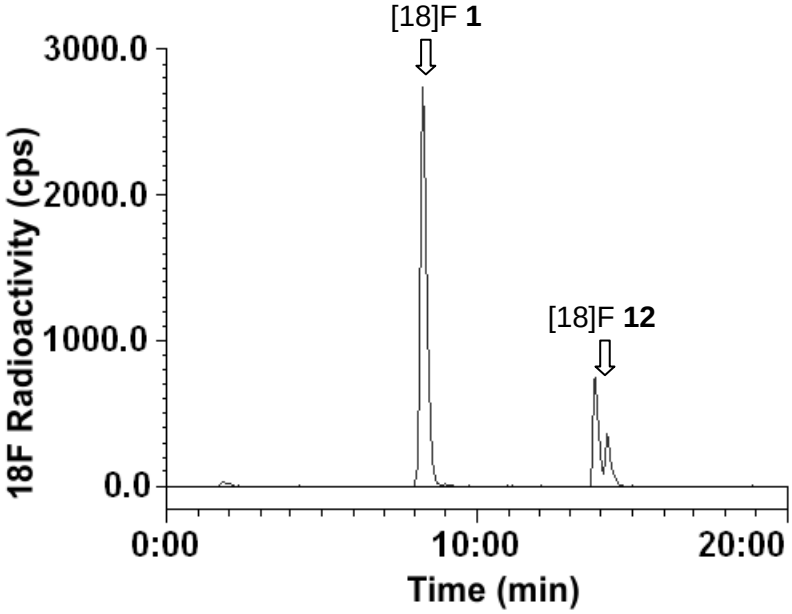
-224.426
-224.498
-224.550
-224.623
-224.695
-224.748
-224.821
10000
50000
0
-250
-200
-150
-100
-50
0
50
ppm (t1)

^{18}F HPLC traces for radiolabelling study

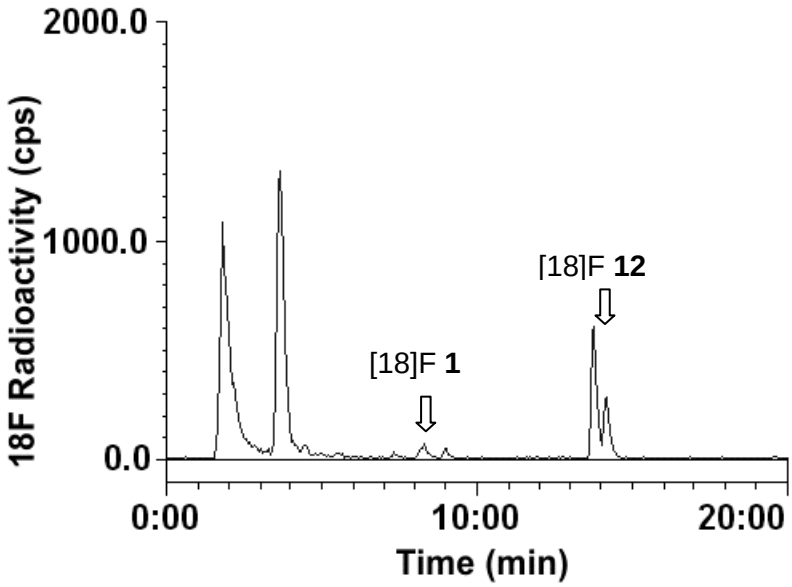
DIFO (**3**)



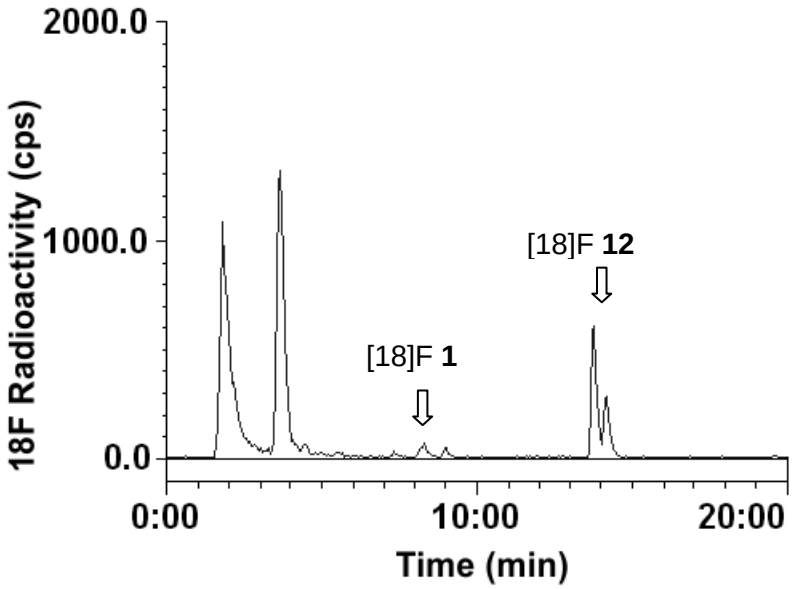
Conditions: 40°C, H_2O, 15 min



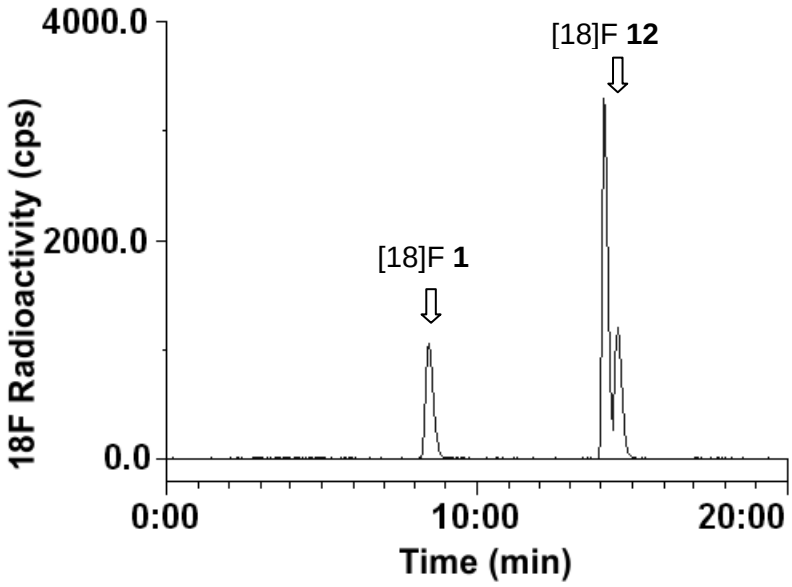
Conditions: 40°C, H_2O, 1h



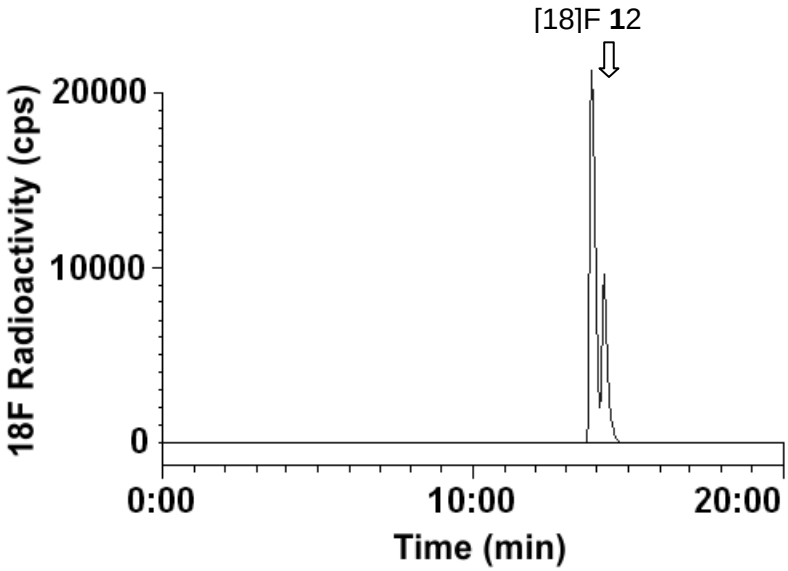
Conditions: 90°C, H_2O, 15 min



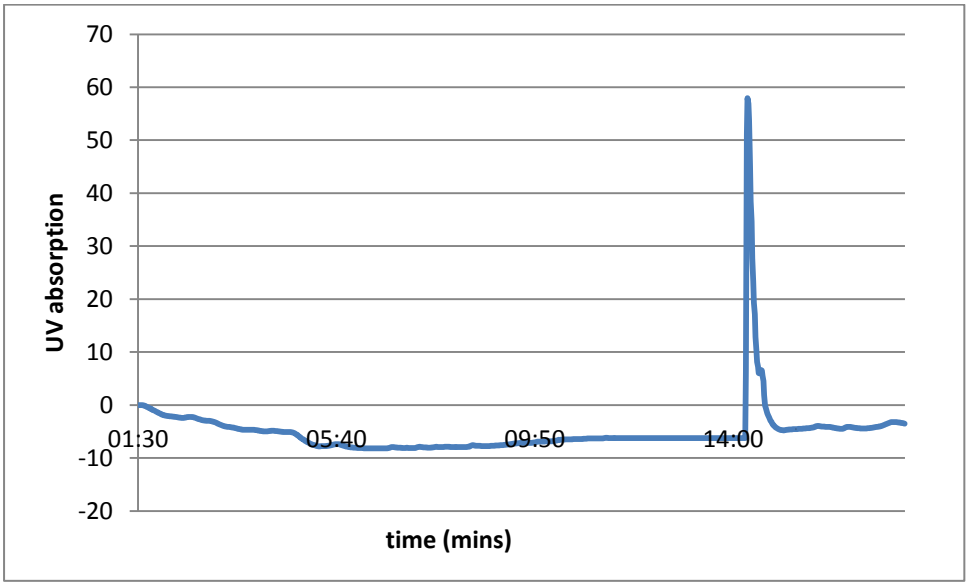
Conditions: 40°C, MeCN, 15 min



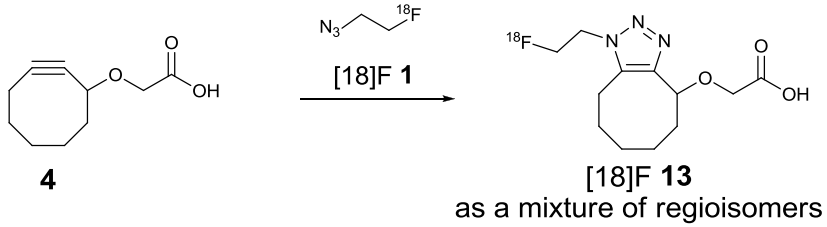
Conditions: 90°C MeCN, 15 min



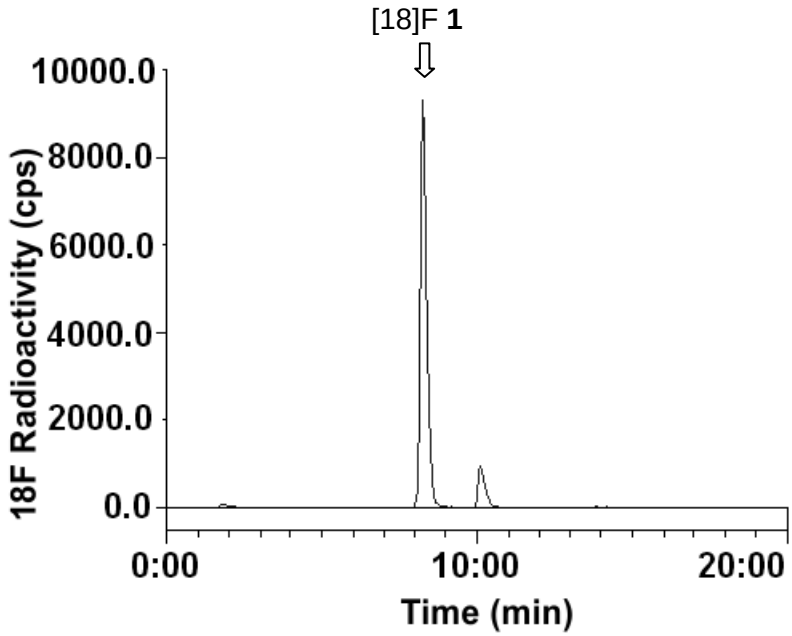
'Cold' Reference Compound



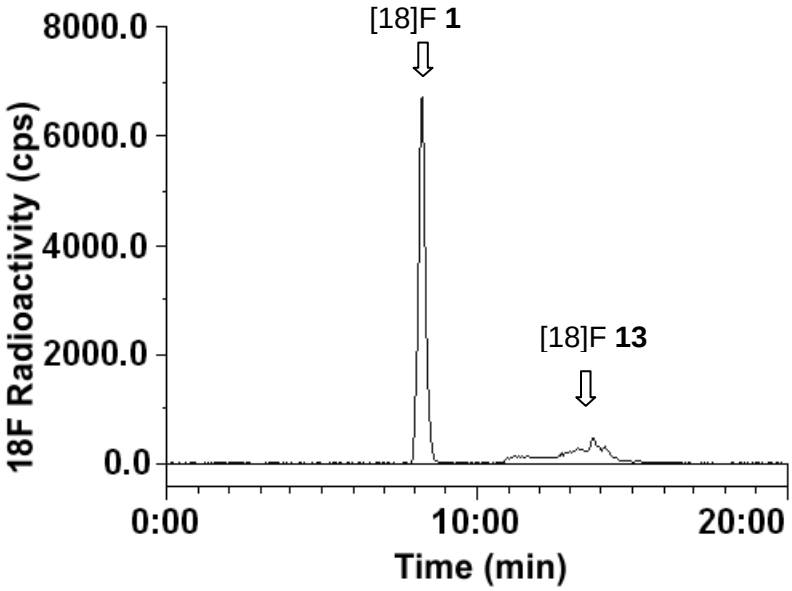
OCT1 (**4**)



Conditions: 40°C, H_2O, 15 min



Conditions: 90°C, MeCN, 15 min



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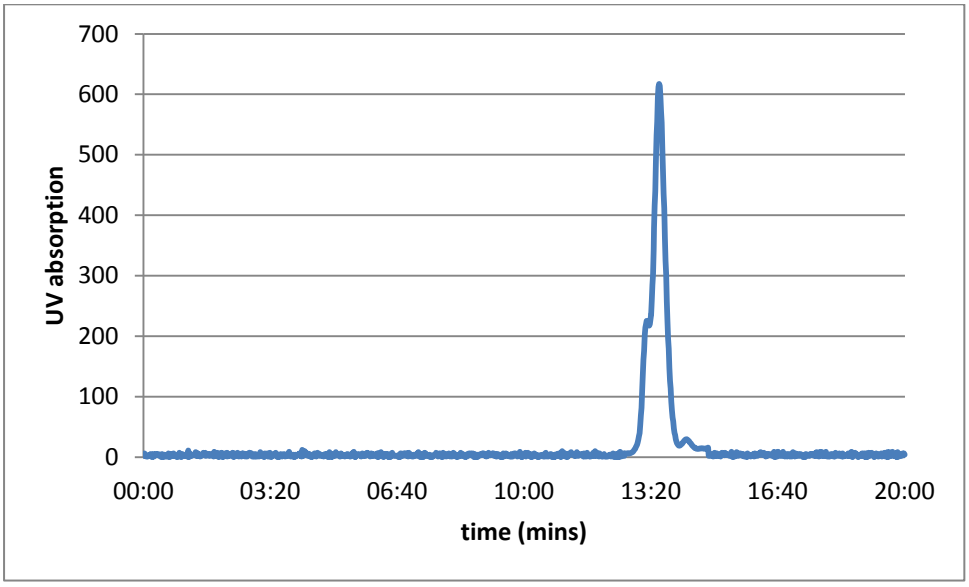
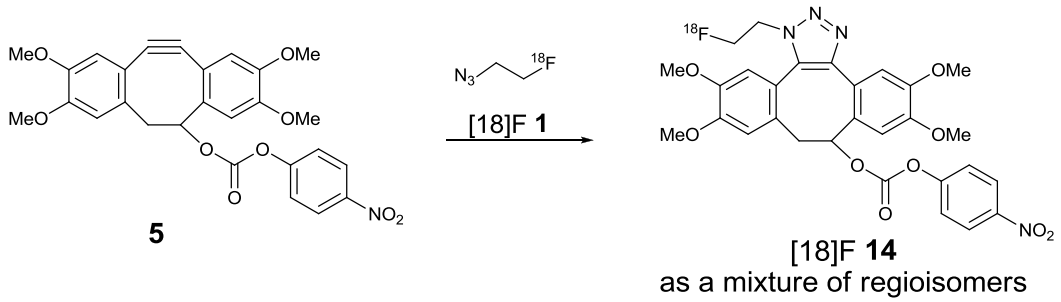
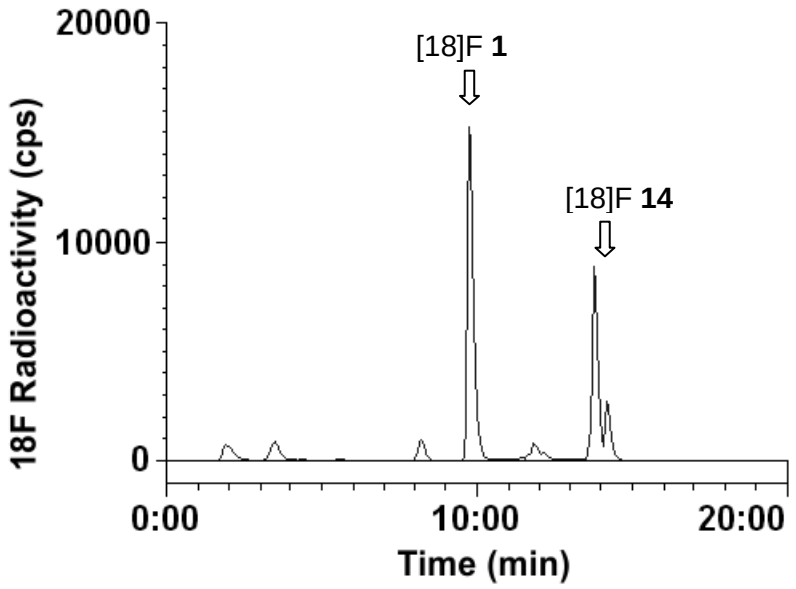


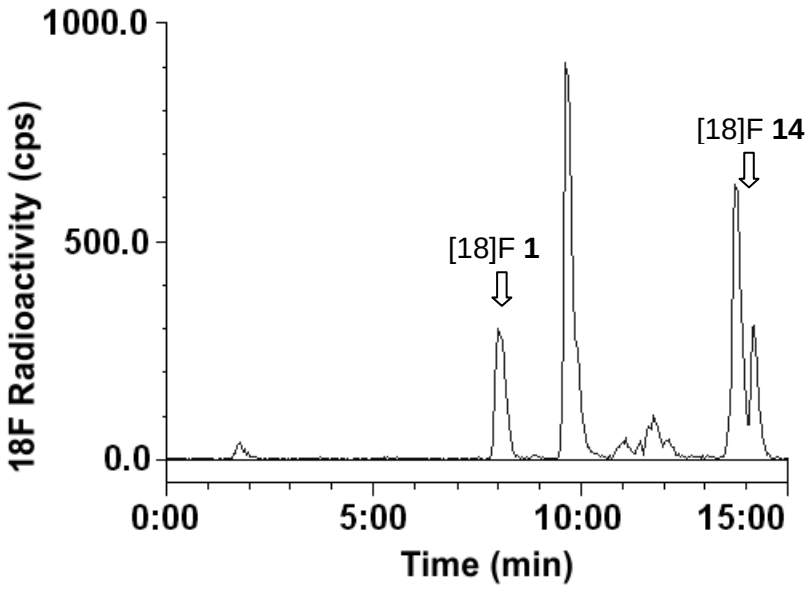
TMDIBO (**5**)



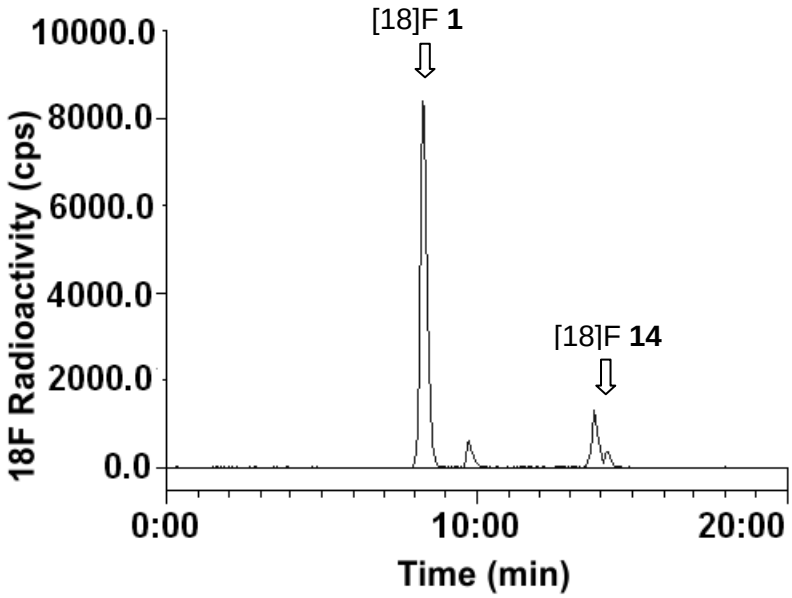
Conditions: 40°C, H_2O, 15 min



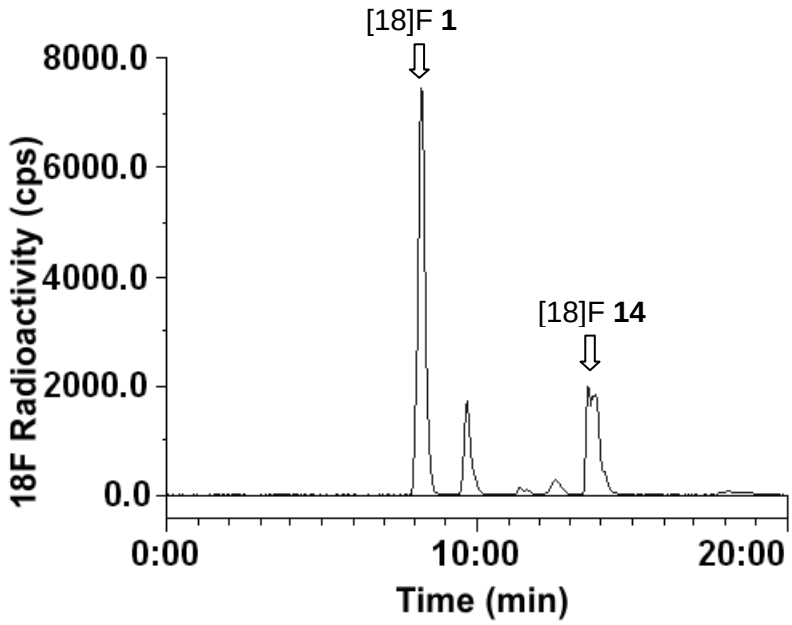
Conditions: 40°C, H_2O, 1h



Conditions: 40°C, MeCN, 15 min



Conditions: 90°C, MeCN, 15 min



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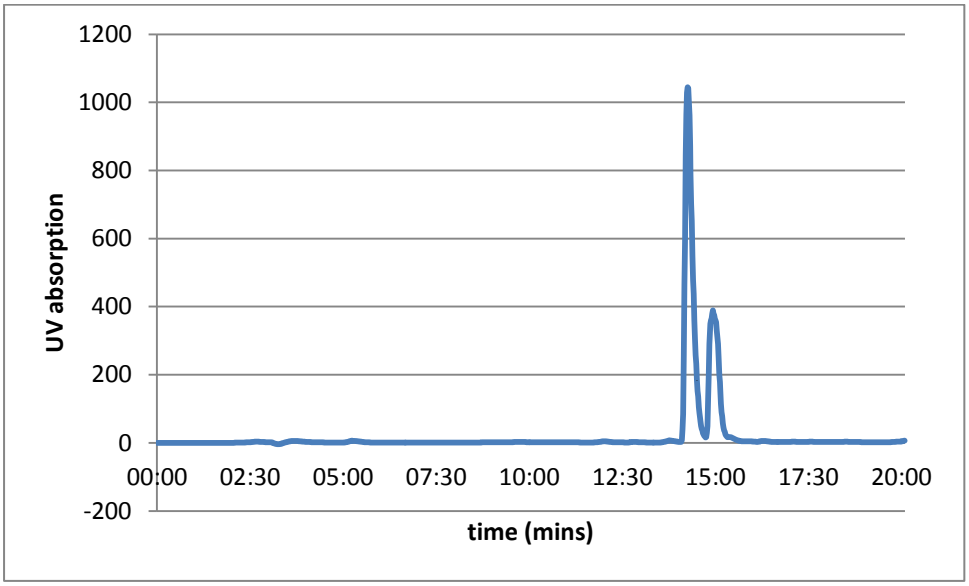
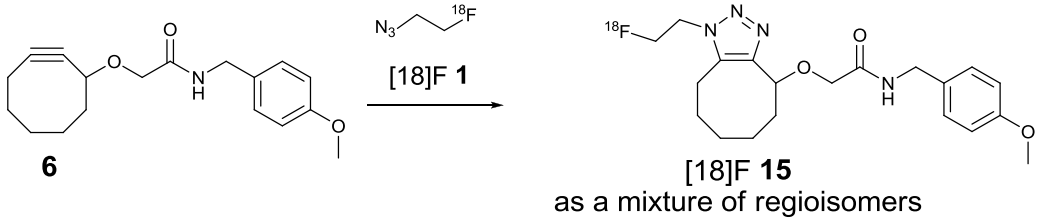
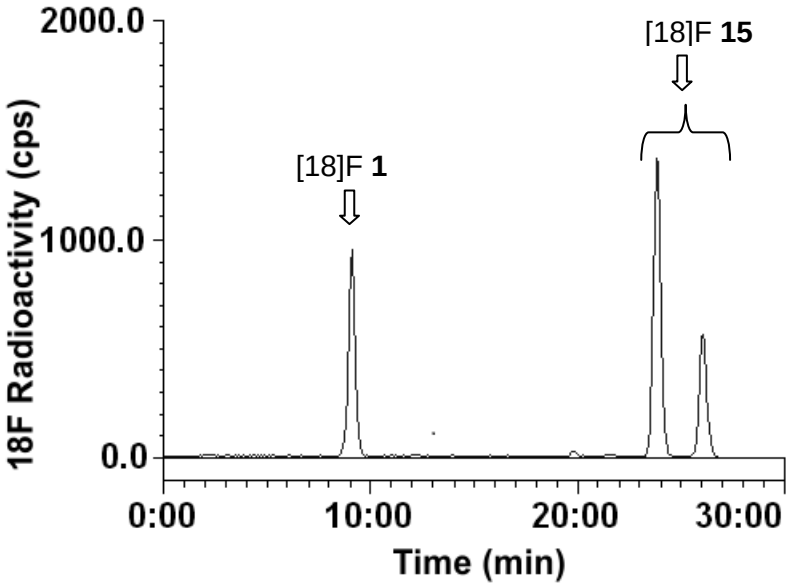


OCT-Am (**6**)



Conditions: 90°C, MeCN, 15 min



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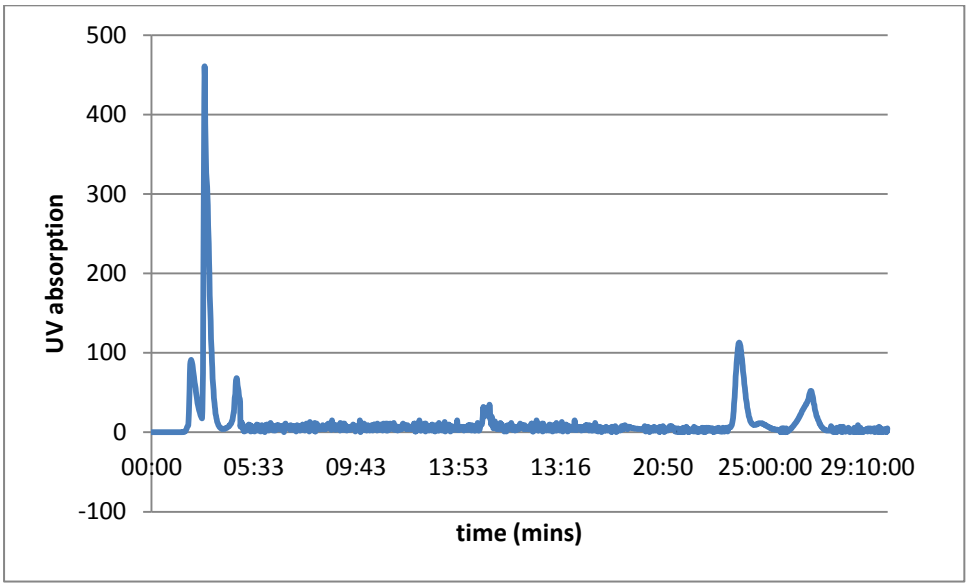
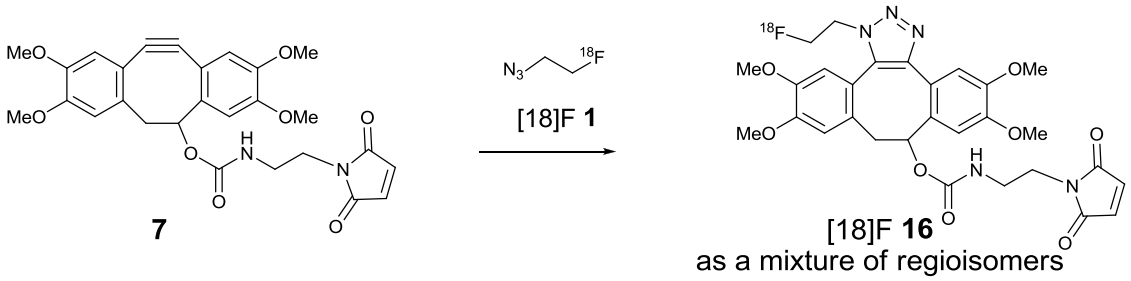
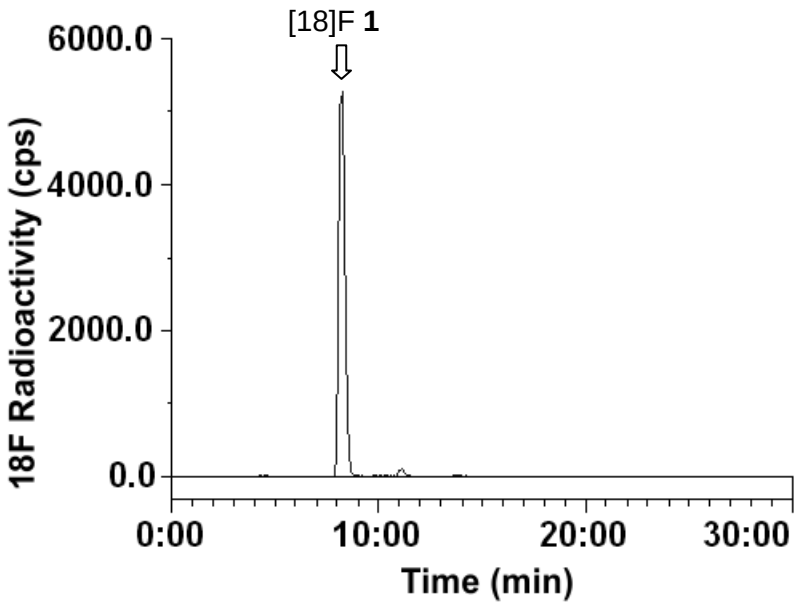


TMDIBO-mal (**7**)



Conditions: 40°C, MeCN, 15 min



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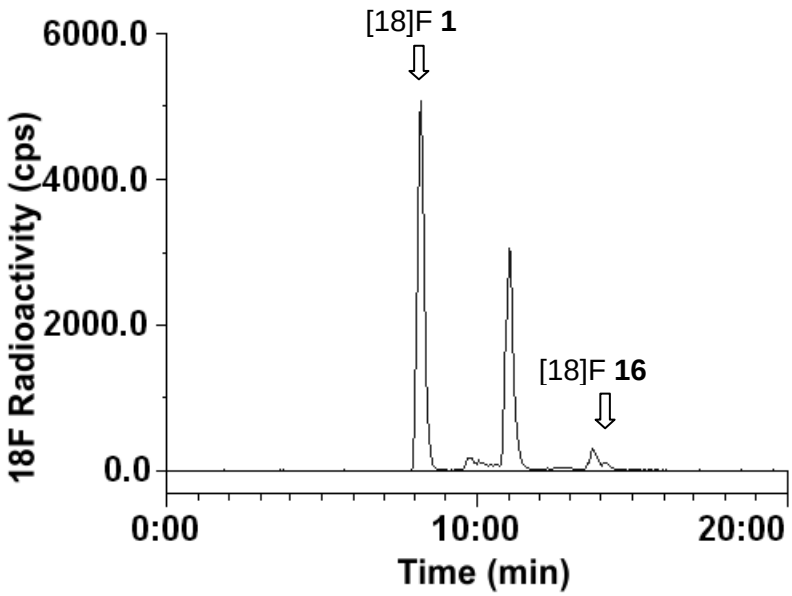

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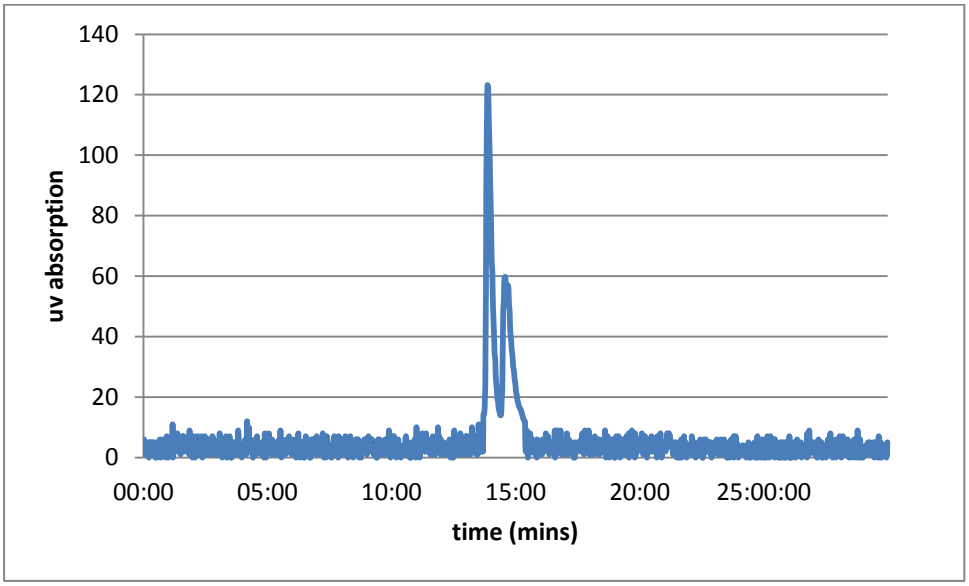

[^{18}F]2-Fluoroethylazide small animal PET imaging and tissue biodistribution.

BALB/c mice (n=3, Harlan, UK) were scanned on a dedicated small animal PET scanner (Siemens Multimodality Inveon, Siemens Molecular Imaging Inc., Knoxville, USA) following a bolus *i.v.* injection of ~3.7 MBq of [^{18}F]2-fluoroethylazide. All animal experiments were done in accordance with the United Kingdom Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 (HMSO, London, United Kingdom, 1990) and within guidelines set out by the United Kingdom National Cancer Research Institute Committee on Welfare of Animals in Cancer Research[3]*. Dynamic emission scans were acquired in list-mode format over 60 minutes. The acquired data were then sorted into 0.5 mm sinogram bins and 19 time frames for image reconstruction, which was done by filtered back projection. Direct [^{18}F]2-fluoroethylazide tissue biodistribution was assessed subsequent to the PET scan. For this, blood was taken by cardiac puncture from the animals and tissues were excised. The tissues were weighted and immediately counted for fluorine-18 radioactivity. Data were expressed as percentage injected dose per gram (%ID/ g).

Tissues	%ID/g
Plasma	3.55 ± 0.30
Blood	2.90 ± 0.23
Heart	2.43 ± 0.15
Lung	2.34 ± 0.06
Liver	3.74 ± 0.21
Gallbladder	3.47 ± 0.20
Stomach	2.22 ± 0.52
Duodenum	3.11 ± 0.22
Jejunum	2.85 ± 0.32
Caecum	3.73 ± 0.26
Colon	2.92 ± 0.33
Rectum	3.38 ± 1.01
Spleen	2.47 ± 0.08
Kidney	3.92 ± 0.18
Muscle	1.96 ± 0.09
Bone	7.11 ± 1.17
Brain	3.16 ± 0.24
Urine	46.33 ± 16.54

Table 1. [^{18}F]2-Fluoroethylazide tissue biodistribution in BALB/c mice.

Cell viability assay using TMDIBO (5)

A sulforhodamine B cell viability assay was carried out in order to assess the toxicity of the TMDIBO cyclooctyne. On day one HCT116 colon cancer cells were seeded in a 96-well plate at a density of 2,000 cells per well in a volume of 150 µl of RPMI supplemented with 1 % penicillin/streptomycin and 10 % FBS. On day two cells were treated by addition of 50 µL of TMDIBO at a range of concentrations between 0 and 20 µM. After 72 hours, cells were fixed by addition of 50 % trichloroacetic acid and stained with a 0.4 % solution of sulforhodamine B in 1 % acetic acid. Finally, 10 mM tris base buffer was used to solubilise the stained protein and absorbance was measured at 540 nm.

The cell viability *versus* compound concentration curve was plotted as a percentage of non-treated control cells. The experiment was carried out on three separate occasions with n=6 replicates for each concentration.

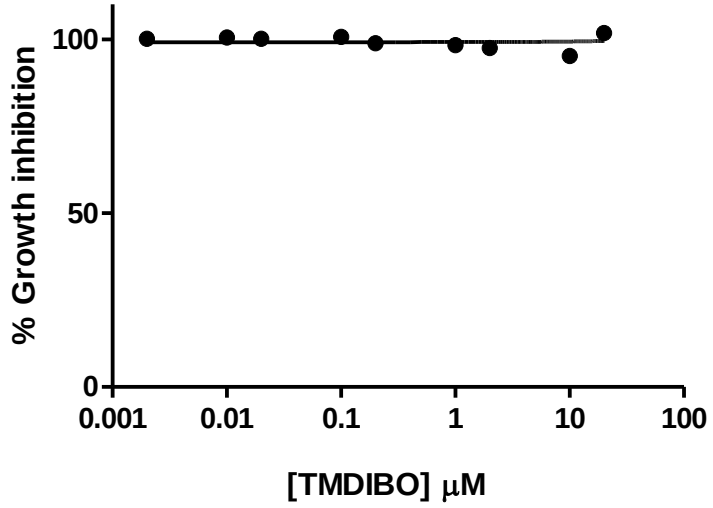

Figure 1. Cell viability assay

Growth inhibition of HCT116 cells after 72 hours incubation with an increasing concentration of TMDIBO **5**.

References

[1] A. Bernardin, A. l. Cazet, L. Guyon, P. Delannoy, F. o. Vinet, D. Bonnaffé and I. Texier, *Bioconjugate Chemistry* **2010**, *21*, 583-588.
[2] M. Glaser and E. Årstad, *Bioconjugate Chemistry* **2007**, *18*, 989-993.

[3] P. Workman, E. O. Aboagye, F. Balkwill, A. Balmain, G. Bruder, D. J. Chaplin, J. A. Double, J. Everitt, D. A. Farningham, M. J. Glennie, L. R. Kelland, V. Robinson, I. J. Stratford, G. M. Tozer, S. Watson, S. R. Wedge and S. A. Eccles, *British Journal of Cancer* **2010**, *102*, 1555-1577.