ELECTRONIC SUPPLEMENTARY INFORMATION

TMTHSI, a superior 7-membered ring alkyne containing reagent for strain-promoted azide-alkyne cycloaddition reactions

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Analytical methods

GC/MS methods:

Methode A, Instrument: GC: Agilent 6890N, FID: Det. temp: 300 °C and MS: 5973 MSD, EI-positive, Det.temp.: 280 °C Mass range: 50-550; Column: RXi-5MS 20m, ID 180µm, df 0.18µm; Average velocity: 50 cm/s; Injection vol: 1 µl; Injector temp: 250 °C; Split ratio: 20/1; Carrier gas: He; Initial temp: 100 °C; Initial time: 1.5 min; Solvent delay: 1.3 min; Rate 75 °C/min; Final temp 250 °C; Final time 2.5 min.

LC/MS methods:

SC_ACID, Apparatus: Agilent 1260 Bin. Pump: G1312B, degasser; autosampler, ColCom, DAD: Agilent G1315D, 220-320 nm, MSD: Agilent LC/MSD G6130B ESI, pos/neg 100-1000, ELSD Alltech 3300 gas flow 1.5 ml/min, gas temp: 40 °C; column: Waters XSelectTM C18, 30x2.1mm, 3.5µ, Temp: 35 ºC, Flow: 1 mL/min, Gradient: t_0 = 5% A, $t_{1.6min}$ = 98% A, t_{3min} = 98% A, Posttime: 1.3 min, Eluent A: 0.1% formic acid in acetonitrile, Eluent B: 0.1% formic acid in water).

SC_BASE, Apparatus: Agilent 1260 Bin. Pump: G1312B, degasser; autosampler, ColCom, DAD: Agilent G1315C, 220-320 nm, MSD: Agilent LC/MSD G6130B ESI, pos/neg 100-1000; column: Waters XSelectTM CSH C18, 30x2.1mm, 3.5µ, Temp: 25 ^{o}C, Flow: 1 mL/min, Gradient: t_0 = 5% A, $t_{1.6min}$ = 98% A, t_{3min} = 98% A, Posttime: 1.3 min, Eluent A: acetonitrile, Eluent B: 10mM ammoniumbicarbonate in water (pH=9.5).

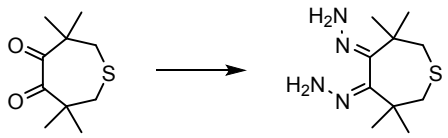
MS parameters

Source: ESI, Capillary voltage: 3000V, Drying gas flow: 12 L/min, Nebulizer Pressure 60 psig, Drying Gas Temp: 350 °C, Fragmentor 70, MS scan: MS range 100-1000 (positive and negative mode), scan speed: 0.84 sec/cycle.

^{1}H-NMR:

All ^{1}H-NMR spectra were recorded on a Bruker Avance-400 ultrashield NMR spectrometer, using CD_3OD, $CDCl_3$ or DMSO-d6 as solvent and are reported in ppm referenced to TMS (0 ppm) as an internal standard.

Synthesis of (3,3,6,6-Tetramethylthiepane-4,5-diylidene)bis(hydrazine) 10



9 **10**

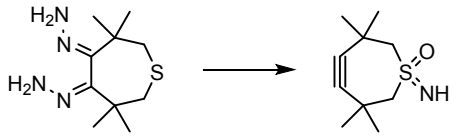
A 300 ml glass autoclave was charged with 3,3,6,6-tetramethylthiepane-4,5-dione (22.4 g, 112 mmol) **9** prepared according to the literature (refs 13, 14, 16 and 18 in the paper) and dissolved in 2-propanol (180 ml). Hydrazine monohydrate (33 ml, 678 mmol, 6 eq.) was added and the mixture was stirred for 20 min's, then pTsOH (42.5 g, 224 mmol, 2.0 eq.) was added, the autoclave was closed and heated at 90 °C in an oil bath for 5 days in which time a maximum pressure of 2 bar was reached. The autoclave was allowed to cool, the mixture was concentrated under reduced pressure and the residue was partitioned between saturated aqueous. $NaHCO_3$ soln (400 ml) and ethyl acetate (200 ml). The aqueous layer was extracted with ethyl acetate (3x200 ml). The combined organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure to afford 25 g of the crude product as a waxy solid. The residue was crystallised/triturated from hot heptane (180 ml, the product was not fully dissolved) and allowed to cool to room temperature. The solids were collected by filtration, washed with heptane and air dried to afford 19.05 g (74%) of the product as an off-white solid. The spectral data of **10** were in agreement with the literature (refs 13, 14, 15 and 18 in the paper).

GC/MS (Method_A) t_R 4.47 min, M^+= 228.

LC/MS (SC_BASE)): t_R 1.79 min, purity 99%, mass found $[M+H]^+$ 229.

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.24 (s, 4H), 2.54 (d, *J* = 14.4 Hz, 2H), 2.48 (d, *J* = 14.4 Hz, 2H), 1.34 (s, 6H), 1.21 (s, 6H)

Synthesis of 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide (TMTHSI) 12



10 **12**

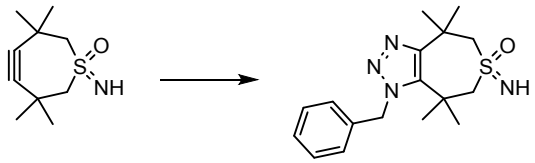
A three-necked roundbottom flask was charged with ammonium acetate (13.5 g, 175 mmol, 8 eq.), methanol (15 ml) and water (~1 ml). Then, (3,3,6,6-tetramethylthiepane-4,5-diylidene)bis(hydrazine) **10** (5.00 g, 21.9 mmol) was added and the mixture was cooled with ice-salt/water. A suspension/solution of iodobenzene diacetate (28.2 g, 88.0 mmol, 4 eq.) in dichloromethane (80 ml) was added at such a rate that a gentle flow of N_2 escapes from the reaction mixture. When the addition was complete, the mixture was allowed to reach room temperature and stirred for another 90 minutes. The mixture was quenched in

water (100 ml). The layers were separated and the aqueous layer extracted with dichloromethane (2x 50 ml). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to afford an orange oil. The residue was purified by flash column chromatography (silica, 40 g; 50-100% EtOAc in heptane) to afford 1.3 g (30%, pty 76 area%) as a waxy solid.

An amount of 217 mg was purified by preparative RP-MPLC (Reveleris-Prep, Xselect, 5-40% MeCN in water, 10mM NH_4HCO_3, pH=9.5) to afford 117 mg of an analytically pure sample **12**.

LCMS (SC_BASE): t_R 1.59 min, purity 100%, mass found $[M+H]^+$ 200; mp: 90-93 °C; TLC (EtOAc): R_f = 0.18; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.23 (d, J = 14.2 Hz, 2H), 3.14 (d, J = 14.1 Hz, 2H), 2.66 (s, 1H), 1.41 (s, 6H), 1.25 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 101.60, 71.07, 34.73, 27.65, 26.61; HRMS (m/z): $[M+H]^+$ calcd. for $C_{10}H_{17}NOS$, 200.11036; found, 200.11031; analysis (calcd., found for $C_{10}H_{17}NOS$): C (60.26, 57.58) H (8.60, 8.77) N (7.03, 6.64).

Click reaction: Synthesis of 1-benzyl-6-imino-4,4,8,8-tetramethyl-1,4,5,6,7,8-hexahydro-6 λ ^{4}-thiepino[4,5-d][1,2,3]triazole 6-oxide 13a,b



12 **13a,b**

An 8 ml screwcap vial was charged with 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1H-1λ^6-thiepine 1-oxide **12** (50 mg, 0.25 mmol) and dissolved in dichloromethane (1 ml). A solution of benzyl azide (38 µl, 0.30 mmol, 1.2 eq.) in dichloromethane (0.5 ml) was added and the mixture was stirred at room temperature for 15 minutes. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica 4 g; 50-100% EtOAc in heptane) to afford 53.6 mg (64%) of the product **13a**,**b** as a white sticky foam which solidified upon standing.

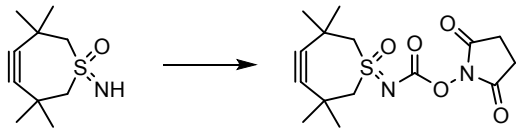
LCMS (SC_BASE): t_R 1.71 min, purity 99%, mass found $[M+H]^+$ 333; mp: 149-151 °C; TLC (EtOAc): R_f = 0.19; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38 – 7.28 (m, 3H), 7.03 (d, J = 6.6 Hz, 2H), 5.73 (s, 2H), 3.46 (s, 2H), 3.43 (s, 2H), 2.76 (s, 1H), 1.70 (s, 3H)*, 1.70 (s, 3H), 1.44 (s, 3H), 1.43 (s, 3H). *overlapping singlets. ^{13}C NMR (101 MHz, $CDCl_3$): δ 148.97, 137.37, 135.84, 129.13, 128.30, 126.47, 68.42, 67.43, 54.75, 34.18, 33.87, 30.86, 30.76, 29.49, 28.92; HRMS (m/z): $[M+H]^+$ calcd. for $C_{17}H_{24}N_4OS$, 333.17436; found, 333.17483; analysis (calcd., found for $C_{17}H_{24}N_4OS$): C (61.42, 61.43) H (7.28, 7.41) N (16.85, 16.64).

Comparison of reaction kinetics of a model click reaction between TMTHSI and BCNOH and benzyl azide

The alkyne (TMTSHI or BCN-OH) was reacted with 1.3 eq benzylazide and reaction kinetics were measured using NMR ($CDCl_3$). Conversion of TMTHSI with benzyl azide reached 79% after 225 seconds, the reaction with BCN-OH took about 40-fold longer (9312 seconds). The reaction conversion (%) is calculated based on triazole signals, taking the plateau of triazole

signal at the end of reaction as 100% level = when all TMTHSI or BCN-OH fully reacted with benzyl-azide. The results of the measurements are included in Figure 2 TMTHSI **12** (red curve) is significantly faster than BCN **3** (blue curve). For further details see pages S59 - S62

Synthesis of 2,5-dioxopyrrolidin-1-yl (3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepan-1-ylidene)carbamate 14



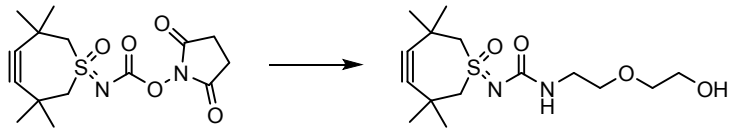
12 **14**

A 50 ml round bottom flask was charged with N,N'-disuccinimidyl carbonate (566 mg, 2.21 mmol, 1.08 eq.) was suspended in MeCN (10 ml). A solution of 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1H-1λ^6-thiepine-1-oxide **12** (509 mg, 80% purity 2.04 mmol) in acetonitrile (5 ml) was added via pipette. The suspension slowly dissolves and the mixture was stirred at room temperature for 90 minutes in which time a light yellow suspension is formed.

The mixture was concentrated under reduced pressure, the residue was purified over a plug of alumina (25 g) eluting in succession with 20% MeCN in DCM (50 ml), 30% MeCN in DCM (25 ml) and 40% MeCN in DCM (25 ml) to afford 486 mg of the product as a white solid. The residue was triturated with diethyl ether, filtered and air dried to afford 423 mg (60%) of the product **14** as a white powder.

LCMS (SC_ACID): t_R 1.80 min, purity 100%, mass found $[M+Na]^+$ 363, $[2xM+Na]^+$ 703; mp: 223 - 225 °C (decomp.); TLC (Heptane:EtOAc, 1:3 v/v): R_f = 0.33; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.93 (d, J = 14.2 Hz, 2H), 3.45 (d, J = 14.2 Hz, 2H), 2.81 (s, 4H), 1.52 (s, 6H), 1.29 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.87, 154.39, 101.43, 68.76, 34.23, 27.75, 26.71, 25.71; HRMS (m/z): $[M+Na]^+$ calcd. for $C_{15}H_{20}N_2O_5S$, 363.09851; found, 363.09823; analysis (calcd., found for $C_{15}H_{20}N_2O_5S$): C (52.93, 53.48) H (5.92, 6.12) N (8.23, 8.96).

Synthesis of 1-(2-(2-hydroxyethoxy)ethyl)-3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepin-1-ylidene)urea (15)



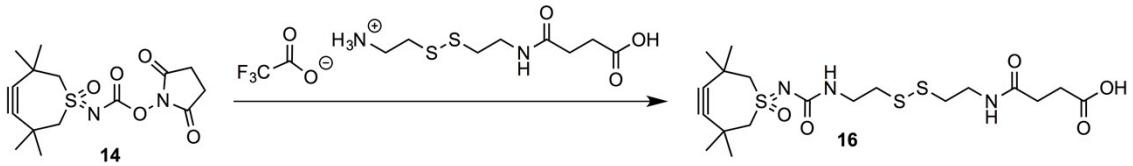
14 **15**

An 8 ml screwcap vial was charged with 2,5-dioxopyrrolidin-1-yl (3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepan-1-ylidene)carbamate **14** (50 mg, 0.15 mmol) and the material was suspended in acetonitrile (1 ml). A solution of 2-(2-amino-ethoxy)ethanol (74 µl, 0.73 mmol, 5 eq.) in acetonitrile (0.5 ml) was added and the mixture was stirred at room temperature for 90 minutes. The

reaction mixture was purified directly by preparative RP-MPLC (Reveleris-Prep, XSelect 10-50% MeCN in water, 10mM NH_4HCO_3, pH=9.5), the product fractions were combined and concentrated under reduced pressure. The aqueous residue was lyophilised overnight to afford 38 mg (78%) of product **15**.

LCMS (SC_BASE): t_R 1.67 min, purity 98%, mass found $[M+H]^+$ 331; TLC (EtOAc): R_f = 0.09; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.60 (t, J = 6.0 Hz, 1H), 3.80 (d, J = 14.1 Hz, 2H), 3.76 – 3.70 (m, 2H), 3.61 – 3.53 (m, 5H), 3.49 (d, J = 14.0 Hz, 2H), 3.43 – 3.34 (m, 2H), 1.49 (s, 6H), 1.27 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 160.14, 101.74, 72.35, 70.39, 68.49, 61.68, 40.21, 34.20, 27.78, 26.86. HRMS (m/z): $[M+H]^+$ calcd. for $C_{15}H_{26}N_2O_4S$, 331.1686; found, 331.16844.

Synthesis of 4-oxo-4-((2-((2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepin-1-ylidene)ureido)ethyl)disulfaneyl)ethyl)amino)butanoic acid (16)



Disulfide spacer:

A 100 ml roundbottom flask was charged with tert-butyl (2-((2-aminoethyl)disulfaneyl)ethyl)carbamate (1.28 g, 4.43 mmol) and the material was dissolved in dichloromethane (25 ml). TEA (0.68 ml, 4.87 mmol, 1.1 eq.) and DMAP (54 g, 0.44 mmol, 0.1 eq.) were added followed by succinic anhydride (0.46 g, 4.65 mmol, 1.05 eq.). The resulting mixture was stirred at room temperature for one hour. The mixture was quenched with aqueous 1N $KHSO_4$ solution (30 ml), the layers were separated and the organic layer was washed with aqueous 1N $KHSO_4$ solution (~30 ml). The combined aqueous layers were extracted with DCM (30 ml). The organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure to afford 1.47 g (94%) of the crude product as a solid.

LCMS (SC_ACID): t_R 1.67 min, purity 100%, mass found $[M-H]^-$ 351; mp: 75-76 °C; TLC (EtOAc): R_f = 0.21; ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.33 (s, 1H), 7.05 (t, J = 5.8 Hz, 1H), 5.16 (t, J = 6.2 Hz, 1H), 3.58 (q, J = 6.0 Hz, 2H), 3.50 – 3.31 (m, 2H), 2.85 (t, J = 6.1 Hz, 2H), 2.78 (t, J = 6.9 Hz, 2H), 2.70 (t, J = 6.5 Hz, 2H), 2.63 – 2.47 (m, 2H), 1.44 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 176.26, 173.01, 156.31, 80.07, 39.71, 38.51, 38.40, 37.74, 30.73, 29.94, 28.49; HRMS (m/z): $[M+H]^+$ calcd. for $C_{13}H_{24}N_2O_5S_2$, 353.11994; found, 353.11973.

Next, a 100 ml roundbottom flask was charged with 2,2-dimethyl-4,13-dioxo-3-oxa-8,9-dithia-5,12-diazahexadecan-16-oic acid (1.41 g, 4.00 mmol) was dissolved in dichloromethane (5 ml) and treated with trifluoroacetic acid (3.5 ml, 45.7 mmol, 11.4 eq.). The reaction was stirred at room temperature for approximately one hour. The mixture was quenched with water (10 ml). The layers were separated and the aqueous layer was washed with dichloromethane (2x 5 ml). The aqueous layer was lyophilised overnight. The residue still contained trifluoroacetic acid. The material was dissolved in water (20 ml) and

concentrated under reduced pressure, this was repeated two times. The resulting residue was redissolved in water and lyophilised overnight to afford 1.54 g in quantitative yield of the product as a glass.

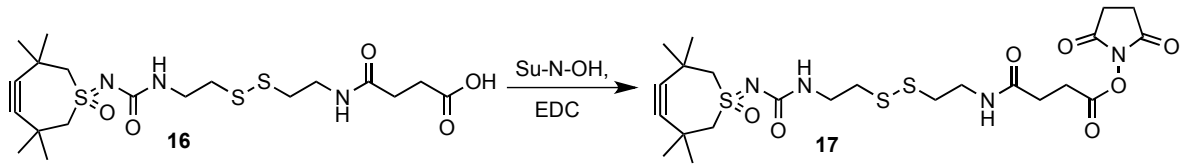
^{1}H NMR (400 MHz, CD_3OD): δ 3.50 (t, J = 6.7 Hz, 2H), 3.28 (t, J = 6.8 Hz, 2H, overlaps with CD_3OD residual peak), 2.97 (t, J = 6.8 Hz, 2H), 2.85 (t, J = 6.7 Hz, 2H), 2.60 (t, J = 6.8 Hz, 2H), 2.48 (t, J = 6.7 Hz, 2H); ^{13}C NMR (101 MHz, CD_3OD, TFA signal was not included): δ 176.32, 174.80, 39.48, 39.40, 38.23, 35.50, 31.41, 30.14; HRMS (m/z): $[M+H]^+$ calcd. for $C_8H_{16}N_2O_3S_2$, 253.06751; found, 253.06743.

*Synthesis of **16***

2,5-Dioxopyrrolidin-1-yl (3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepan-1-ylidene)carbamate (210 mg, 0.62 mmol) **14** was suspended in acetonitrile (3 ml) and DIPEA (270 µl, 1.55 mmol, 2.51 eq.) was added. A solution of 4-((2-((2-aminoethyl)disulfaneyl)ethyl)amino)-4-oxobutanoic acid TFA-salt (249 mg, 0.68 mmol, 1.1 eq.) in water (1 ml) was added and the mixture was stirred at room temperature for six hours in which time the solids slowly dissolved. The mixture was concentrated under reduced pressure. The residue was dissolved in a mixture of MeCN and water (<5 mL) and purified by preparative RP-MPLC (Reveleris-Prep, LUNA-C18, 10-50% MeCN in water, 0.1% (v/v) formic acid). The product fractions were combined and lyophilised overnight to afford 168 mg (57%) was obtained as a white fluffy solid. The residue was dissolved in MeCN and transferred to a 50 ml roundbottom flask and concentrated under reduced pressure, co-evaporated with diethylether to obtain white solid **16**.

LCMS (SC_ACID): t_R 1.72 min, purity 98%, mass found $[M-H]^-$ 477; mp: 137-139 °C; TLC (EtOAc): R_f =0.03; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.07 (s, 1H), 8.05 (t, *J* = 5.6 Hz, 1H), 6.91 (t, J = 5.8 Hz, 1H), 3.88 (d, J = 13.9 Hz, 2H), 3.63 (d, J = 13.9 Hz, 2H), 3.40 – 3.27 (m, 2H, overlaps with H_2O peak), 3.26 – 3.17 (m, 2H), 2.75 (q, J = 6.1, 5.7 Hz, 4H), 2.42 (t, J = 6.9 Hz, 2H), 2.31 (t, J = 6.9 Hz, 2H), 1.34 (s, 6H), 1.20 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6): δ 173.84, 171.15, 159.66, 102.09, 66.53, 38.06, 37.66, 37.30, 33.74, 29.98, 29.10, 26.81, 26.21. HRMS (m/z): $[M+H]^+$ calcd. for $C_{19}H_{31}N_3O_5S_3$, 478.14986; found, 478.14924; analysis (calcd., found for $C_{19}H_{31}N_3O_5S_3$): C (47.78, 47.59) H (6.54, 6.51) N (8.80, 8.98).

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-oxo-4-((2-((2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ6-thiepin-1-ylidene)ureido)ethyl)disulfaneyl) ethyl)amino)butanoate (17)



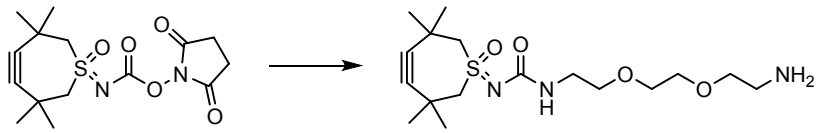
16 17

An 8 ml screwcap vial was charged with 4-oxo-4-((2-((2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ6-thiepin-1-ylidene)ureido)ethyl)disulfaneyl)ethyl) amino)butanoic acid (40 mg, 0.084 mmol) and dichloromethane (1 ml). N-Hydroxysuccinimide (14 mg, 0.12 mmol, 1.4 eq.) was added followed by the addition of EDC·HCl (23 mg, 0.12 mmol, 1.4 eq.). The mixture was stirred at room temperature for

approximately two hours. The mixture was concentrated under reduced pressure and the resulting white foam was purified by preparative RP-MPLC (Reveleris-Prep, LUNA-C18, 20-60% MeCN in water, 0.1% (v/v) formic acid). The product fractions were combined and lyophilised overnight. The freeze-dried material was dissolved in dichloromethane and transferred to an 4 ml brown glass vial, the solvent was evaporated under a stream of N_2 and co-evaporated with diethyl ether to afford 43 mg (89%) of the product **17**.

LCMS (SC_ACID): t_R 1.85 min, purity 85%, mass found $[M+H]^+$ 575; mp: 60-65 °C; TLC (EtOAc): R_f =0.22; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (t, J = 6.0 Hz, 1H), 5.37 (t, J = 6.2 Hz, 1H), 3.79 (d, J = 14.0 Hz, 2H), 3.58 (q, J = 6.1 Hz, 2H), 3.53 – 3.40 (m, 4H), 3.00 (t, J = 7.2 Hz, 2H), 2.92 – 2.76 (m, 8H), 2.67 (t, J = 7.1 Hz, 2H), 1.47 (s, 6H), 1.28 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.55, 169.10, 168.26, 160.15, 101.59, 68.56, 39.43, 38.42, 38.26, 37.85, 34.14, 30.49, 27.56, 26.85, 26.80, 25.59; HRMS (m/z): [M+H]+ calcd. for $C_{23}H_{34}N_4O_7S_3$, 575.16624; found, 575.16607.

Synthesis of 1-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-(3,3,6,6-tetramethyl-1-oxido—4,5-didehydro-2,3,6,7-tetrahydro-1λ⁶-thiepin-1-ylidene)urea. 18



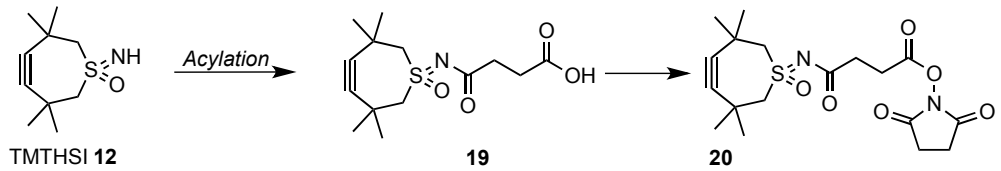
14 **18**

Under N_2-atmosphere, 2,5-dioxopyrrolidin-1-yl (3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepan-1-ylidene)carbamate (197 mg, 0.58 mmol) was dissolved in dichloromethane (12.5 ml) and 2,2'-(ethane-1,2-diylbis(oxy))bis(ethan-1-amine) (0.42 ml, 2.89 mmol, 5 eq.) was added. The reaction was stirred at room temperature for 2 hours. The reaction mixture was concentrated under reduced pressure. The residue was taken up in a 1:1 mixture of acetonitrile/water (4 ml) and purified (in two portions) by preparative RP-MPLC (Reveleris, XSelect 10-50% MeCN in water, 10mM NH_4HCO_3, pH=9.5). The product fractions were combined and lyophilised to afford 156 mg (72%) of the product **18** as a white fluffy solid.

LC/MS (SC_BASE) t_R 1.67 min, purity 99%, mass found $[M+H]^+$ 374.

^{1}H NMR (400 MHz, DMSO-d_6 + D_2O) δ 3.86 (d, *J* = 14.0 Hz, 2H), 3.61 (d, *J* = 13.9 Hz, 2H), 3.54 – 3.46 (m, 4H), 3.40 (q, *J* = 6.0 Hz, 4H), 3.15 – 3.04 (m, 3H), 2.67 (t, *J* = 5.7 Hz, 1H), 1.34 (s, 6H), 1.21 (s, 6H). HRMS (m/z): $[M+H]^+$ calcd. for $C_{17}H_{32}N_3O_4S$, 374.2108; found, 374.21026; analysis (calcd., found for $C_{17}H_{31}N_3O_4S$): C (54.67, 51.35) H (8.37, 8.06) N (11.25, 10.68).

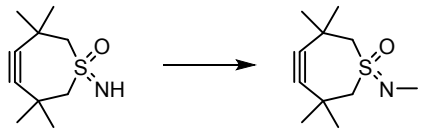
Synthesis of 2,5-dioxopyrrolidin-1-yl 4-oxo-4-((3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ⁶-thiepin-1-ylidene)amino)butanoate 20 *via* 19



A 100 mL roundbottom flask was charged with 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide **12** (300 mg, 1.51 mmol), dichloromethane (10 ml) and DIPEA (525 µl, 3.01 mmol, 2 eq.). Succinic anhydride (226 mg, 2.26 mmol, 1.5 eq.) was added and the mixture was stirred at room temperature for two hours, providing in situ generation of **19**. N-Hydroxysuccinimide (433 mg, 3.76 mmol, 2.5 eq.) was added, followed by a suspension of EDC·HCl (721 mg, 3.76 mmol, 2.5 eq.) in dichloromethane (~5 ml). The resulting mixture was stirred at room temperature for two hours. The mixture was quenched with aqueous 2M $KHSO_4$ solution and the organic phase was passed through a phase separator. The filtrate was concentrated under reduced pressure and residue was purified by flash column chromatography (silica 12 g, 50-75% EtOAc in heptane), the product fractions were pooled and concentrated under reduced pressure to afford 181 mg (30%) of the product **20** as a white solid.

LCMS (SC_ACID): t_R 1.86 min, purity 89%, mass found $[M+H]^+$ 397; mp: 174-176 °C; TLC (Heptane:EtOAc, 1:3 v/v): R_f = 0.25; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.77 (d, J = 14.1 Hz, 2H), 3.60 (d, J = 14.1 Hz, 2H), 2.98 – 2.88 (m, 2H), 2.86 – 2.79 (m, 4H), 2.78 – 2.70 (m, 2H), 1.52 (s, 6H), 1.26 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 178.54, 169.26, 168.53, 101.61, 67.59, 34.15, 33.77, 27.88, 27.68, 26.91, 25.71; HRMS (m/z): $[M+H]^+$ calcd. for $C_{18}H_{24}N_2O_6S$, 397.14278; found, 397.14262; analysis (calcd., found for $C_{18}H_{24}N_2O_6S$): C (54.53, 54.06) H (6.10, 6.14) N (7.07, 6.94).

Synthesis of 3,3,6,6-tetramethyl-1-(methylimino)-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide 21



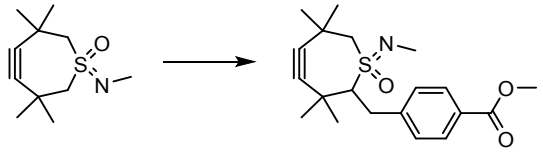
12 **21**

An 8 ml screwcap vial was charged with 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide **12** (100 mg, 0.50 mmol) and dissolved in tetrahydrofuran (2 ml). A solution of KOtBu in tetrahydrofuran (1M, 0.65 ml, 0.65 mmol, 1.3 eq.) was added and the mixture was stirred at room temperature for 5 minutes. Then iodomethane (60 µl, 0.96 mmol, 1.92 eq.) was added and the mixture was stirred at room temperature for two hours. The mixture was partitioned between water (10 ml) and diethylether (5 ml). The aqueous layer was extracted with diethylether (3x 5 ml). The organic layers were combined, washed with brine, dried Na_2SO_4 and concentrated under reduced pressure to afford 84 mg of

the crude product as an oily residue. The residue was purified by flash column chromatography (silica 4g; 50-100% EtOAc in heptane) to afford 66.1 mg (61%) of the product **21** as a white solid.

LCMS (SC_BASE): t_R 1.72 min, purity 98%, mass found $[M+H]^+$ 214; mp: 73-74 °C; TLC (EtOAc): R_f = 0.23; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.32 (d, J = 14.0 Hz, 2H), 3.07 (d, J = 14.0 Hz, 2H), 2.84 (s, 3H), 1.43 (s, 6H), 1.28 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 102.22, 67.59, 34.46, 29.04, 27.83, 26.75; HRMS (m/z): $[M+H]^+$ calcd. for $C_{11}H_{19}NOS$, 214.12601; found, 214.12597; analysis (calcd., found for $C_{11}H_{19}NOS$): C (61.93, 61.59) H (8.98, 8.90) N (6.57, 6.45).

Synthesis of methyl 4-((3,3,6,6-tetramethyl-1-(methylimino)-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ-thiepin-2-yl)methyl)benzoate 22



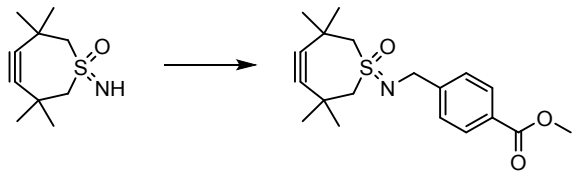
21 **22**

An 8 ml screwcap vial was charged with 3,3,6,6-tetramethyl-1-(methylimino)-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide (31 mg, 0.14 mmol) **21** and dissolved in anhydrous THF (1 ml). LiHMDS (1M in THF; 174 µl, 0.17 mmol, 1.2 eq.) was added and the mixture was stirred at room temperature for 15 minutes, then methyl 4-(bromomethyl)benzoate (50 mg, 0.22 mmol, 1.5 eq) was added and the mixture was stirred at room temperature for 20 hours. The mixture was quenched in water and extracted with diethylether (3x 10 ml). The combined organic layers were washed with brine and concentrated under reduced pressure to afford a semi-solid residue. The residue was purified by preparative RP-MPLC (Reveleris, LUNA-C18, 30-70% MeCN in water, 0.1% (v/v) formic acid). The product fractions were combined and lyophilised to afford 9 mg (17%) of the product **22** as a white fluffy solid.

LC/MS (SC_BASE) t_R 2.20 min, purity 97%, mass found $[M+H]^+$ 362.

^{1}H NMR (400 MHz, Chloroform-d) δ 7.97 (d, *J* = 8.2 Hz, 2H), 7.43 (d, *J* = 8.2 Hz, 2H), 6.72 (d, *J* = 15.7 Hz, 1H), 6.25 (d, *J* = 15.8 Hz, 1H), 3.91 (s, 3H), 3.81 – 3.69 (m, 1H), 3.02 (d, *J* = 12.8 Hz, 1H), 2.83 – 2.76 (m, 4H), 1.44 (s, 3H), 1.39 (s, 3H), 1.37 (s, 6H).

Synthesis of methyl 4-(((3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1l6-thiepin-1-ylidene)amino)methyl)benzoate (23)



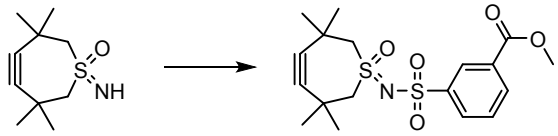
12 **23**

An 8 ml screwcap vial was charged with 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide (75 mg, 0.37 mmol) **12** and dissolved in anhydrous THF (1.5 ml). KOtBu (1.7 M soln in THF; 280 µl, 0.47 mmol, 1.26 eq.) was added and the resulting mixture was stirred at room temperature for 15 minutes. Methyl-4(bromomethyl)benzoate (129 mg, 0.56 mmol, 1.5 eq.) was added to the resulting suspension and the mixture was stirred at room temperature for 6 hours. The mixture was quenched in water and extracted with diethylether (3x 15 ml), the combined organic extracts were washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure to afford 128 mg of the crude product as a semi-solid residue. The residue was purified by preparative RP-MPLC (Reveleris, LUNA C18, 30-70% MeCN in water, 0.1% (v/v) formic acid). The product fractions were combined and lyophilised to afford 28 mg (21%) of the product **23** as an oily residue.

LC/MS (SC_BASE) t_R 2.14 min, purity 86%, mass found $[M+H]^+$ 348.

^{1}H NMR (400 MHz, Chloroform-d) δ 7.99 (d, *J* = 8.3 Hz, 2H), 7.46 (d, *J* = 8.0 Hz, 2H), 4.39 (s, 2H), 3.91 (s, 3H), 3.35 (d, *J* = 14.0 Hz, 2H), 3.18 (d, *J* = 14.1 Hz, 2H), 1.45 (s, 6H), 1.28 (s, 6H).

Synthesis of methyl 3-(N-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepin-1-ylidene)sulfamoyl)benzoate 24



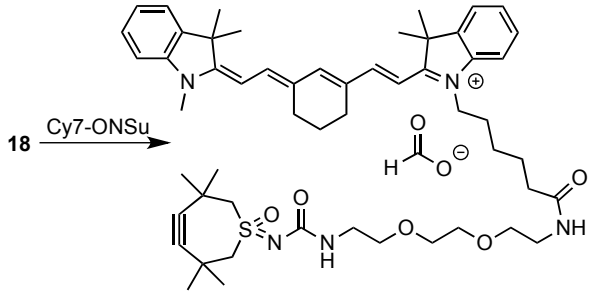
12 **24**

An 8 ml screwcap vial was charged with 1-imino-3,3,6,6-tetramethyl-4,5-didehydro-2,3,6,7-tetrahydro-1*H*-1λ^6-thiepine 1-oxide **12** (100 mg, 0.50 mmol) and dissolved in dichloromethane (2 ml). Pyridine (81 µl, 1.00 mmol, 2 eq.) was added and the mixture was stirred at room temperature for 5 minutes. A solution of methyl 3-(chlorosulfonyl)benzoate (152 mg, 0.65 mmol, 1.29 eq.) in dichloromethane (1 ml) was added slowly via pipette and the mixture was stirred at room temperature for 90 minutes. The mixture was quenched with aqueous 1N HCl solution and the mixture was passed over a phase separator. The aqueous residue was washed with DCM (1 ml, 3x) and the combined organic filtrates were concentrated under reduced pressure to afford 206 mg of the crude product as a yellow residue. The residue was purified by flash column chromatography (silica 4g, 25-50% EtOAc in heptane) to afford 70 mg (35%) of product **24** as a white solid.

LCMS (SC_BASE): t_R 2.09 min, purity 99%, mass found $[M+H]^+$ 398; mp: 164-165 °C, TLC (Heptane:EtOAc, 1:1 v/v); R_f = 0.36; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.63 (t, J = 1.9 Hz, 1H), 8.25 – 8.12 (m, 2H), 7.58 (t, J = 7.8 Hz, 1H), 4.08 (d, J = 14.2 Hz, 2H), 3.95 (s, 3H), 3.44 (d, J = 14.2 Hz, 2H), 1.49 (s, 6H), 1.28 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 165.90, 144.56, 133.14, 130.97, 130.83, 129.11, 127.89, 101.27, 71.22, 52.59, 34.17, 27.73,

26.57; HRMS (m/z): $[M+H]^+$ calcd. for $C_{18}H_{23}NO_5S_2$, 398.10904; found, 398.10883; analysis (calcd., found for $C_{18}H_{23}NO_5S_2$): C (54.39, 54.76) H (5.83, 5.98) N (3.52, 3.51).

Synthesis of 1-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepin-1-ylidene)urea-Cy7 adduct formate salt (25)



Cy7-TMTHSI **25**

An 8 ml screw cap vial was charged with Cy7 NHS ester (30 mg, 0.041 mol) and acetonitrile (1 ml), a solution of 1-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepin-1-ylidene)urea **18** (20 mg, 0.053 mmol, 1.31 eq.) in acetonitrile (1 ml) was added and the mixture was stirred at room temperature for two hours. The mixture was purified directly by preparative MPLC (Reveleris-Prep, LUNA-C18, 20-60% MeCN in water, 0.1% (v/v) formic acid), the product fractions were concentrated under reduced pressure and the residue was diluted with a little of MeCN and lyophilised overnight to afford 34 mg (87%) of product **25** as a dark green solid.

LC/MS (AN_ACID_DAY_UV800) t_R 3.46 min, purity 88%, mass found $[M]^+$ 905, $[M+H]^{2+}$ 453.

HRMS (m/z): $[M]^+$ calcd. for $C_{54}H_{74}N_5O_5S^+$, 904.54052; found, 904.53949.

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.37 (s, 1H), 8.02 - 7.95 (m, 0.5H), 7.75 (dd, *J* = 14.0, 8.5 Hz, 1.5H), 7.47 (d, *J* = 7.5 Hz, 2H), 7.43 – 7.34 (m, 1H), 7.31 – 7.20 (m, 3H), 7.15 – 6.92 (m, 1H), 6.81 – 6.34 (m, 2H), 6.24 – 6.09 (m, 1H), 4.11 (t, *J* = 7.3 Hz, 1H), 3.86 (d, *J* = 14.1 Hz, 2H), 3.66 – 3.55 (m, 8H), 3.55 – 3.45 (m, 5H), 3.39 – 3.32 (m, 3H), 3.29 – 3.21 (m, 2H), 2.57 (q, *J* = 5.6 Hz, 3H), 2.50 – 2.28 (m, 1H), 2.27 – 2.15 (m, 2H), 2.00 – 1.89 (m, 2H), 1.89 – 1.74 (m, 3H), 1.75 – 1.61 (m, 10H), 1.59 – 1.52 (m, 2H), 1.51 – 1.42 (m, 2H), 1.40 (s, 7H), 1.33 – 1.20 (m, 8H), 1.03 (s, 1H).

*Azide functionalised polymeric micelles (**26**)*

Azide-functionalized polymeric micelles **26** were manufactured essentially following the reported protocol (Hu et al. Biomaterials. 2015, 53 370-378), except that a fraction of azide-functionalized block copolymer (7.5 kDa, derivatized with 10 mol% crosslinker L2 (Hu et al. Biomaterials. 2015, 53 370-378), 5 w% of total block copolymer) was added next to the (95 w%) non-functionalized block copolymer (7.5 kDa, derivatized with 10 mol% crosslinker L2 95 w% of total block copolymer). These two block copolymers were synthesized following the same procedures, except that for the former (azide-PEG_{5000})$_2$-ABCPA initiator synthesized from azide-PEG_{5000}-OH was used

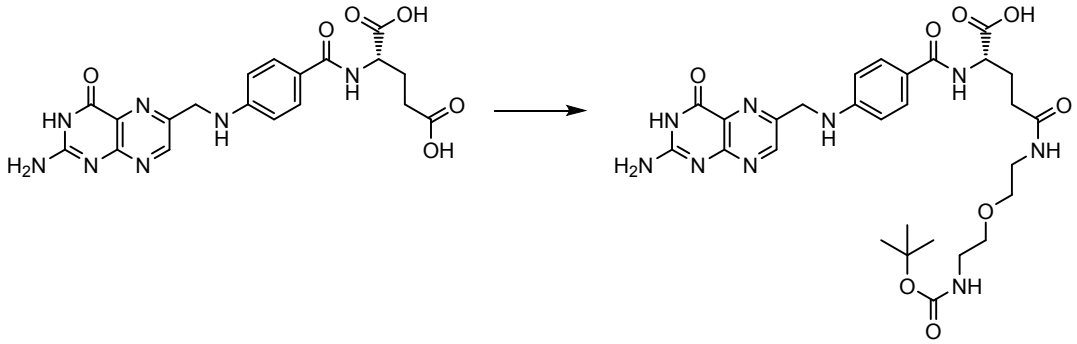
instead. The obtained azide-functionalized polymeric micelles were then purified in 20 mM ammonium acetate pH 5 buffer containing 130 mM NaCl, buffer swapped to 20 mM sodium phopshate pH 7.4 buffer containing 130 mM NaCl and concentrated to approx. 30 mg/mL polymer equiv. using Tangential Flow Filtration (TFF) equipped with a modified Polyethersulfone (mPES) 100 kDa module (Spectrumlabs).

*Cy7 bearing polymeric micelles (**27**)*

Polymeric micelles with 5 w% azide functionalization (**26**), concentrated to approx. 30 mg/mL polymer in 20 mM sodium phosphate pH 7.4 buffer and TMTHSI-functionalized Cy7 **25**, were dissolved in DMSO and mixed to a 66.7% aqueous reaction mixture. Depending on the desired surface coverage of Cy7, appropriate equivalents of **25** were added and 0.5 equivalents proved sufficient to yield a 1% Cy7 surface coverage. This reaction was monitored by measuring the decrease of the TMTHSI Cy7 concentration. After two hours, the reaction was complete and unreacted **25** was removed using centrifugation (Vivaspin) with a cut-off MW of 5 kDa. Residual TMTHSI Cy7 was measured using UPLC and determined to be <0.5%.

Synthesis of (*S*)-15-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino) benzamido)-2,2-dimethyl-4,12-dioxo-3,8-dioxa-5,11-diazahexadecan-16-oic acid) (28)

*Preparation of Boc-protected **28***



Folic acid (1.84 g, 4.17 mmol) was suspended in anhydrous dimethyl sulfoxide (15 ml) and the flask was placed in a sand bath of 100 °C until a clear solution was obtained. Then the solution was allowed to cool to room temperature and N-hydroxysuccinimide (503 mg, 4.37 mmol, 1.05 eq.) was added followed by a solution of N-Boc-2-(2-amino-ethoxy)-ethylamine (852 mg, 4.17 mmol) in anhydrous dimethyl sulfoxide (3 ml). The mixture was stirred for 10 minutes, then EDC (840 mg, 4.38 mmol, 1.05 eq.) was added and the mixture was stirred at room temperature for 17 hours.

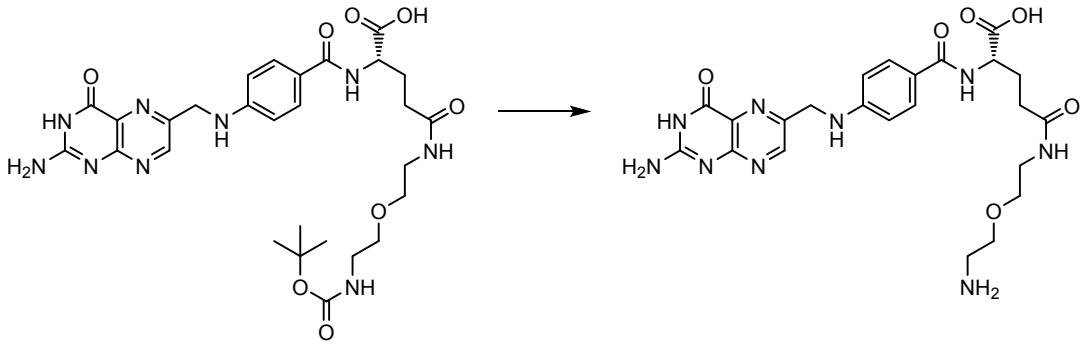
The reaction mixture was purified (in seven portions) by preparative RP-MPLC (Reveleris, XSelect 5-30% MeCN in water, 10mM NH_4HCO_3, pH=9.5). The product fractions were combined and partly concentrated under reduced pressure. The resulting residue was lyophilised to afford 0.91 g (34%) of the product probably consisting of a mixture of α and γ (predominantly) regio isomers.

LC/MS (SC_BASE) t_R 1.37min, purity 100%, mass found $[M-H]^-$ 626.

^{1}H NMR (400 MHz, DMSO-d_6 + D_2O) δ 8.66 (s, 1H), 7.99 (q, *J* = 4.9 Hz, 1H), 7.65 (dd, *J* = 11.6, 8.5 Hz, 2H), 6.75 (d, *J* = 6.1 Hz, 1H), 6.67 (dd, *J* = 9.0, 2.7 Hz, 2H), 4.52 (s, 2H), 4.33 (dd, *J* = 8.9, 4.8 Hz, 0.4H), 4.19 (dd, *J* = 8.1, 4.7 Hz, 0.6H), 3.45 – 3.31 (m, 4H), 3.26 – 3.13 (m, 2H), 3.11 – 3.01 (m, 2H), 2.26 (t, *J* = 7.1, 6.4 Hz, 1H), 2.15 (t, *J* = 8.0 Hz, 1H), 2.08 – 1.82 (m, 2H), 1.35 and 1.33 (2x s, combined 9H).

*Conversion of Boc-**28** to **28***

Synthesis of *N*^{2}-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino) benzoyl)-*N*^{5}-(2-(2-aminoethoxy)ethyl)-*L*-glutamine (28)



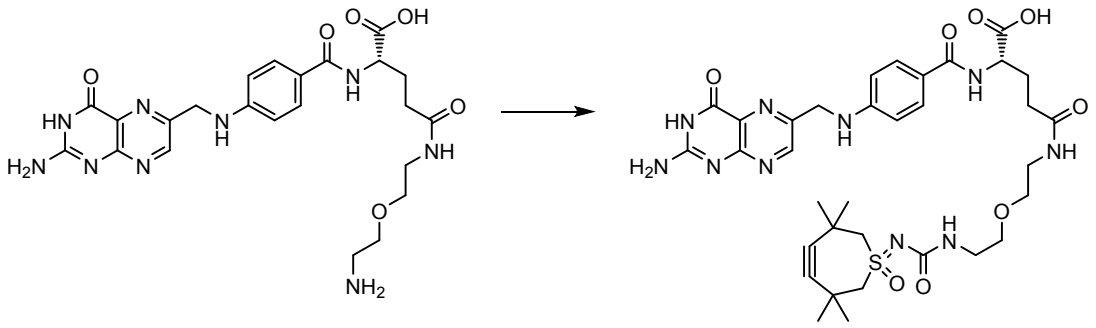
Boc-**28** **28**

(*S*)-15-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzamido)-2,2-dimethyl-4,12-dioxo-3,8-dioxa-5,11-diazahexadecan-16-oic acid (403 mg, 0.64 mmol) was dissolved in trifluoroacetic acid (2 ml, 26.0 mmol) and the resulting mixture was stirred at room temperature for 25 minutes. The reaction mixture was concentrated under reduced pressure, the residue was co-evaporated with dichloromethane (2x). The residue was taken up in N,N-dimethylformamide (1 ml) and warmed until fully dissolved, then allowed to cool to room temperature. The solution was treated with triethylamine (3x 200 □l) to give an orange precipitate. The mixture was diluted with acetone, the solids were collected by filtration. The residue was washed with acetone (3x) and air dried to afford 346 mg (97%) of the product as an orange solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.66 – 8.57 (m, 1H), 8.24 (t, *J* = 5.5 Hz, 0.6H), 8.07 (t, *J* = 5.6 Hz, 0.4H), 7.72 – 7.62 (m, 1H), 7.62 – 7.51 (m, 1H), 7.30 (bs, 2H), 6.91 (q, *J* = 6.1 Hz, 1H), 6.63 (t, *J* = 7.6 Hz, 2H), 4.52 – 4.38 (m, 2H), 4.30 (q, *J* = 6.3 Hz, 0.4H), 4.10 (q, *J* = 6.4 Hz, 0.6H), 3.55 (q, *J* = 6.2, 5.6 Hz, 3H), 3.43 (bs, 3H), 3.31 – 3.13 (m, 4H), 2.94 (q, *J* = 6.2, 5.8 Hz, 2H), 2.21 – 2.11 (m, 2H), 2.06 – 1.79 (m, 3H), 0.97 (t, *J* = 7.2 Hz, 1H.

NMR shows very broad HOD signal underneath signals between 4.5 and 2.5 ppm.

Synthesis of *N*^{2}-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino) benzoyl)-*N*^{5}-(2-(2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1□^{6}-thiepin-1-ylidene)ureido)ethoxy)ethyl)-*L*-glutamine (29).



28 **29**

N^2-(4-(((2-amino-4-oxo-3,4-dihydropteridin-6-yl)methyl)amino)benzoyl)-N^5-(2-(2-amino ethoxy)ethyl)-*L*-glutamine (90.3 mg, 0.13 mmol) **28** was dissolved in hot dimethyl sulfoxide (1.5 ml) and allowed to cool to room temperature. Triethylamine (107 µl, 0.77 mmol, 6 eq.) was added and the resulting mixture was added to 2,5-dioxopyrrolidin-1-yl (3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepan-1-ylidene)carbamate (52 mg, 0.15 mmol, 1.2 eq.). The reaction mixture was stirred at room temperature for 4 days.

The reaction mixture was purified by preparative RP-MPLC (Reveleris, XSelect 5-40% MeCN in water, 10mM NH_4HCO_3, pH=9.5). The product fractions were combined and lyophilised to afford 51 mg (53%) of product **29**.

LC/MS (SC_BASE) t_R 1.45 min, purity 99%, mass found $[M+H]^+$ 753.

^{1}H NMR (400 MHz, DMSO-d_6 + D_2O) δ 8.66 (s, 1H), 8.02 – 7.90 (m, 1H), 7.70 – 7.59 (m, 2H), 6.72 – 6.61 (m, 3H), 4.50 (s, 2H), 4.37 - 4.32 (m, 0.4H), 4.22 – 4.13 (m, 0.6H), 3.90 – 3.79 (m, 2H), 3.59 (dd, *J* = 14.0, 4.5 Hz, 2H), 3.44 – 3.30 (m, 5H), 3.25 – 3.14 (m, 2H), 3.09 (t, *J* = 5.8 Hz, 2H), 2.26 (t, *J* = 7.7 Hz, 1H), 2.15 (t, *J* = 7.6 Hz, 1H), 2.05 – 1.93 (m, 1H), 1.90 (d, *J* = 13.5 Hz, 1H), 1.33 (s, 6H), 1.19 (s, 6H).

Coupling of TMTHSI-containing linker Folic acid 29 to an azide containing nanoparticle to afford 30

Folic acid-labelled core-crosslinked polymeric micelles were generated by conjugating Folic acid to the surface of the polymeric micelles shell and used for targeting studies. To allow for Folic acid conjugation via click chemistry, azide-functionalized polymeric micelles were prepared as was described in the preparation of **27**. Next, Folic acid was conjugated to the concentrated azide-functionalized core-crosslinked polymeric micelles via copper-free click chemistry as folllows: at RT, DMSO (93 µL) was added to 200 µL of an aqueous solution of azide-functionalized modified core-crosslinked polymeric micelles (0.13 µmol azide equiv.) while stirring (300 rpm) in an amber UPLC vial. Upon dissipation of heat, Folic acid (1.0 eq., 0.13 µmol, 5 µL in DMSO) was added dropwise to the reaction mixture. The progress of the conjugation reaction was monitored by UPLC-UV for 4 hours after which the reaction was stopped. Based on UPLC, conversion of Folic Acid was determined to be 45%, which translates to 2.3% Folic acid labelled core-crosslinked polymeric micelles.

Coupling of TMTHSI-containing linker to HER2-peptide: Preparation of TMTHSI-succinyl-Fcycl[CGDGFYAC]YMDV (32)

An 8 ml screwcap vial was charged with **HER2 [**Fcycl[CGDGFYAC]YMDV] peptide **31** (20 mg, 13 µmol, prepared by Alamc) and dissolved in DMSO (1 ml). 2,5-Dioxopyrrolidin-1-yl 4-oxo-4-((3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ^6-thiepin-1-ylidene)amino)butanoate **20** (6.5 mg, 14 µmol, 1.04 eq.) was added followed by diisopropylethylamine (13 µl, 75 µmol, 5.6 eq.). The mixture was stirred at room temperature for 16 hours. The reaction mixture was purified directly by preparative RP-MPLC (Reveleris, LUNA-C18, 20-60% MeCN in water, 0.1% Formic acid), the product fractions were pooled and lyophilised to afford 16.6 mg (69%) of product **32**.

LC/MS (AN_BASE_M1800) t_R 2.57 min, purity 96%, mass found $[M-H]^-$ 1768, $[M-2H]^{2-}$ 883.

Coupling of TMTHSI-containing linker HER2-peptide 32 to an azide containing nanoparticle under physiological conditions: 26 to 33

HER2 peptide-labelled core-crosslinked polymeric micelles **33** were generated by conjugating TMTHSI functionalised HER2 peptide **32** to the surface of the polymeric micelles shell and used for targeting studies. To allow for HER2 peptide conjugation via click chemistry, azide-functionalized polymeric micelles **26** were manufactured prepared as described above in the preparation of **27**). Next, HER2 peptide was conjugated to the concentrated azide-functionalized core-crosslinked polymeric micelles via copper-free click chemistry as follows:

At RT, MeCN (86 µL) was added to 200 µL of azide-functionalized modified core-crosslinked polymeric micelles (0.4 µmol azide equiv.) while stirring (300 rpm) in an amber UPLC vial. HER2 peptide (1.0 eq., 0.4 µmol, 86 µL MeCN/H_2O 1/1) was added dropwise to the reaction mixture. The progress of the conjugation reaction was monitored by UPLC-UV for 24 hours after which the reaction was stopped. Based on UPLC, conversion of HER2 peptide was determined to be 43%, which translates to 2.2% HER2 peptide labelled core-crosslinked polymeric micelles. After conjugation reaction, HER2 peptide-labelled polymeric micelles were purified by TFF against 10 v% ethanol to remove unreacted HER2 peptide.

Coupling of TMTHSI-containing linker HER2-peptide 32 to an azide containing nanoparticle under acidic pH: 26 to 33

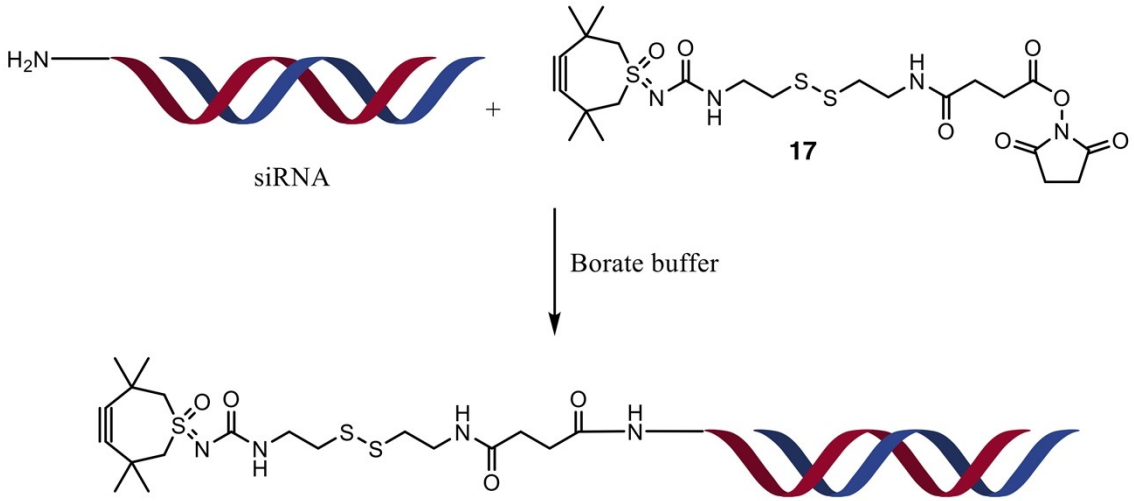
To allow for HER2 targeting peptide conjugation via click chemistry, azide-functionalized polymeric micelles were prepared above in the preparation of **27**. Next, at RT, 150 mM Ammonium Acetate pH 5 (130 µL) was added to 1500 µL of azide-functionalized modified core-crosslinked polymeric micelles (0.57 µmol azide equiv.) while stirring (300 rpm) in an amber UPLC vial. HER2 peptide (5.0 eq., 2.85 µmol, 842 µL 2mg/ml HER2-TMTH stock solution) was added dropwise to the reaction mixture. The progress of the conjugation reaction was monitored by UPLC-UV for 24 hours after which the reaction was stopped. Based on UPLC, conversion of HER2 peptide was determined to be 23%, which translates to 5% HER2 peptide

labelled core-crosslinked polymeric micelles. After conjugation reaction, HER2 peptide-labelled polymeric micelles were purified by TFF against 10 v% ethanol to remove unreacted HER2 peptide.

Summary of the synthesis steps of a cell penetrating peptide - siRNA biomolecular construct employing TMTHSI

For obtaining this molecular construct a 5'-amino modified oligonucleotide (siRNA) was first conveniently attached to TMTHSI-linker compound **16** using its hydroxy succinimide ester derivative **17** (**Step 1**) Next, a cell-penetrating peptide sequence (CPP: Z. Qian, A. Martyna, R. L Hard, J. Wang, G. Appiah-Kubi, C. Coss, M. A. Phelps, J. S. Rossman, D. Pei, *Biochem*. **2016,** *55*, 2601-2612.) having an N-terminal azido lysine residue and equipped with a acid-labile linker containing a polymerizable moiety, could be rapidly introduced by our strain-promoted click reaction (**Step 2**) In this way highly-functionalized biopolymer derivatives can be joined of which the functional groups (phosphorthioate) are incompatible with the application of the copper-catalyzed click reaction. In the last step (**3**) coupling of acid labile hydrazone linker to CPP-TMTHSI-siRNA Oligonucleotide is achieved.

Step 1. Coupling of TMTHSI - disulfide NHS ester to siRNA Oligonucleotide. Preparation of TMTHSI – NH-siRNA PLK1

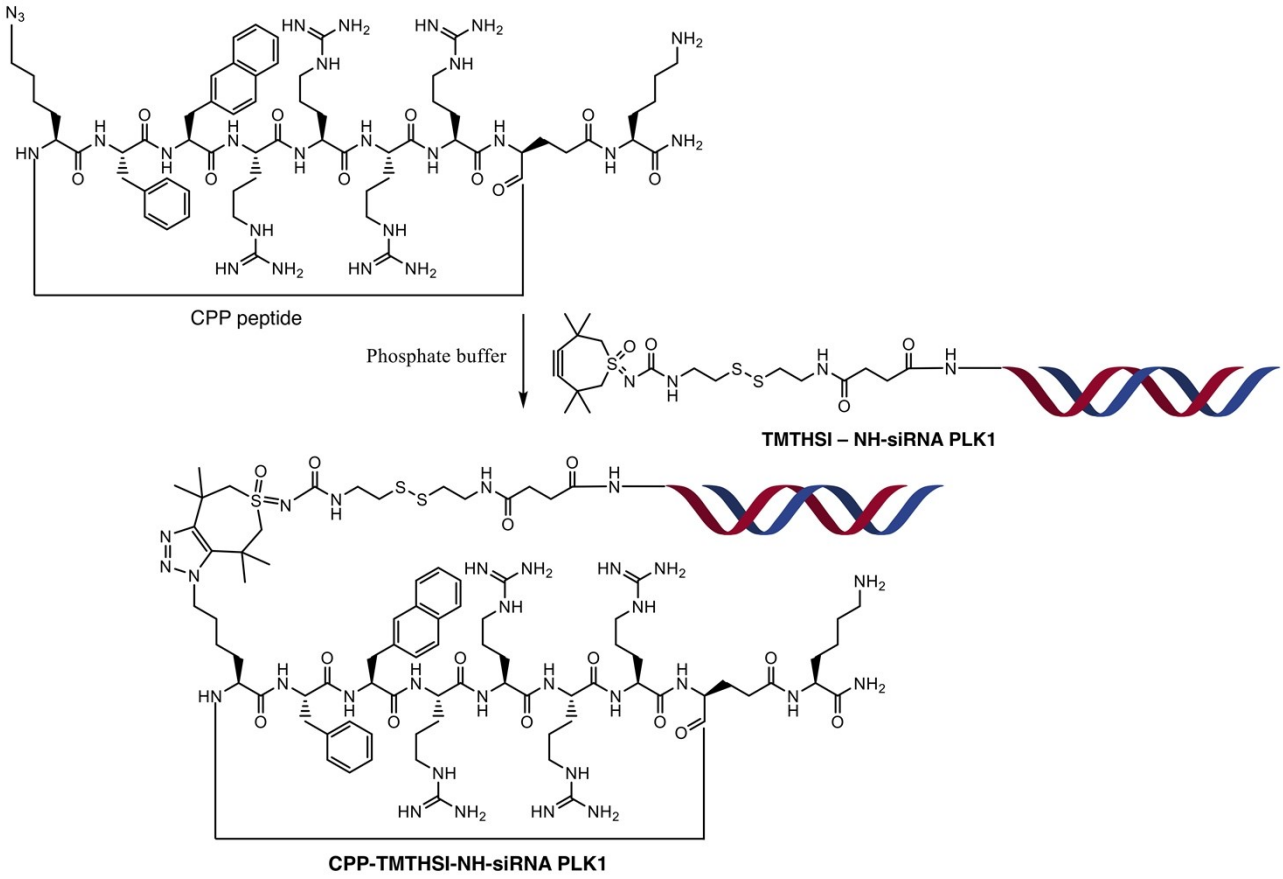


TMTHSI – NH-siRNA PLK1

A 2 mL UPLC vial was charged with 5'-amino C6 modified siRNA PLK1 oligonucleotide (5 mg, 0.36 µmol) and borate buffer pH 8.4 (250 µl) was added. A 20 mM stock solution of 2,5-dioxopyrrolidin-1-yl-4-oxo-4-((2-((2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ6-thiepin-1-ylidene)ureido)ethyl)disulfaneyl) ethyl)amino)butanoate in DMSO was prepared and 5 eq., (90 µl, 1.04 mg, 1.8 µmol) of the stock solution was added to the siRNA containing solution while stirring. The mixture was stirred at room temperature and monitored by UPLC. After addition of an extra 5 eq., of 2,5-dioxopyrrolidin-1-yl 4-oxo-4-((2-((2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ6-thiepin-1-ylidene)ureido)ethyl)disulfaneyl) ethyl)amino)butanoate (90 µl, 1.04 mg, 1.8 µmol) and additional stirring, the reaction showed >90% conversion towards TMTHSI – NH-siRNA PLK1. The final crude product was purified by PD-10 (buffer swap to

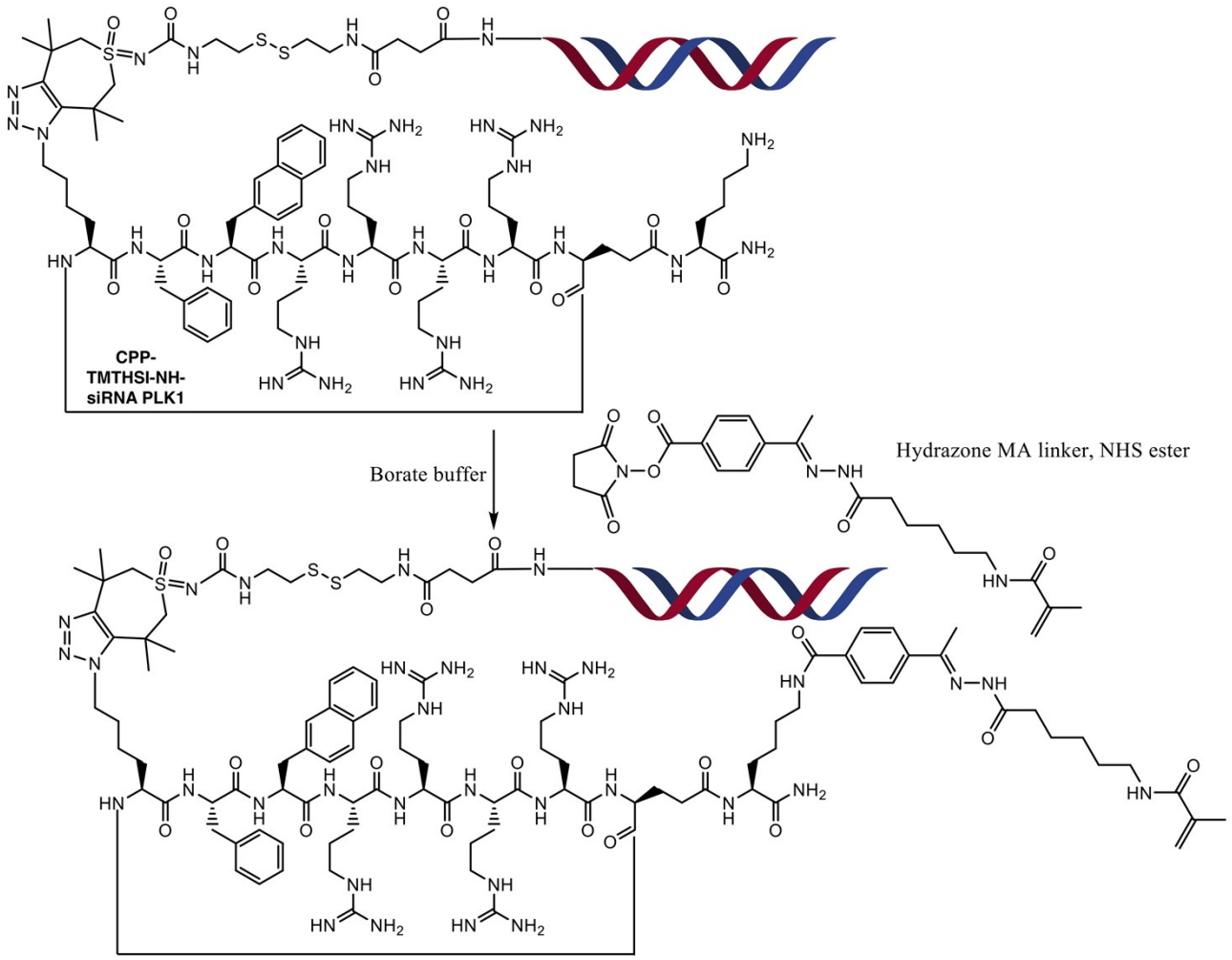
phosphate buffer pH 7.4) and Vivaspin (5000Da MWCO, 4000g, 3 times phosphate buffer pH 7.4, final concentration step) to remove excess of TMTHSI NHS 2,5-dioxopyrrolidin-1-yl 4-oxo-4-((2-((2-(3-(3,3,6,6-tetramethyl-1-oxido-4,5-didehydro-2,3,6,7-tetrahydro-1λ6-thiepin-1-ylidene)ureido)ethyl)disulfaneyl) ethyl)amino)butanoate. The final solution of TMTHSI-NH-siRNA PLK1 (1000µl) was transferred to a 2 mL UPLC vial and stored at 4 °C.

Step 2. Coupling of azide-CPP to TMTHSI-NH-siRNA Oligonucleotide by the Strain-promoted azide-alkyne cycloaddition click reaction. Preparation of CPP-TMTHSI-NH-siRNA PLK1 conjugate.



At first, a stock solution of azide-CPP was prepared (0.49 mg, 0.35 µmol) in acetonitrile/water (1:1, 100 µl volume). Initially 50 µl (0.5 eq., 0.25 mg, 0.18 µmol) of the stock solution was added to the 2 mL UPLC vial containing TMTHSI-NH-siRNA PLK1 (0.35 µmol in 1000 µl phosphate buffer pH 7.4). The mixture was stirred at room temperature and monitored by UPLC. After 30 minutes all azide-CPP peptide was converted and the second half of the azide-CPP stock solution was added (0.5 eq., 0.25 mg, 0.18 µmol). After an additional 30 minutes of stirring the reaction towards CPP-TMTHSI-NH-siRNA PLK1 reached full conversion. The final crude product was washed (5000Da MWCO, 4000g, 3 times phosphate buffer pH 7.4, final concentration step) and concentrated in a Vivaspin centrifugal tube. Final solution of CPP-TMTHSI-NH-siRNA PLK1 (750 µl) was transferred to a 2 mL UPLC vial.

Step 3. Coupling of acid labile hydrazone linker to CPP-TMTHSI-siRNA Oligonucleotide. Preparation of MA hydrazone Linker CPP-TMTHSI-NH-siRNA PLK1 conjugate

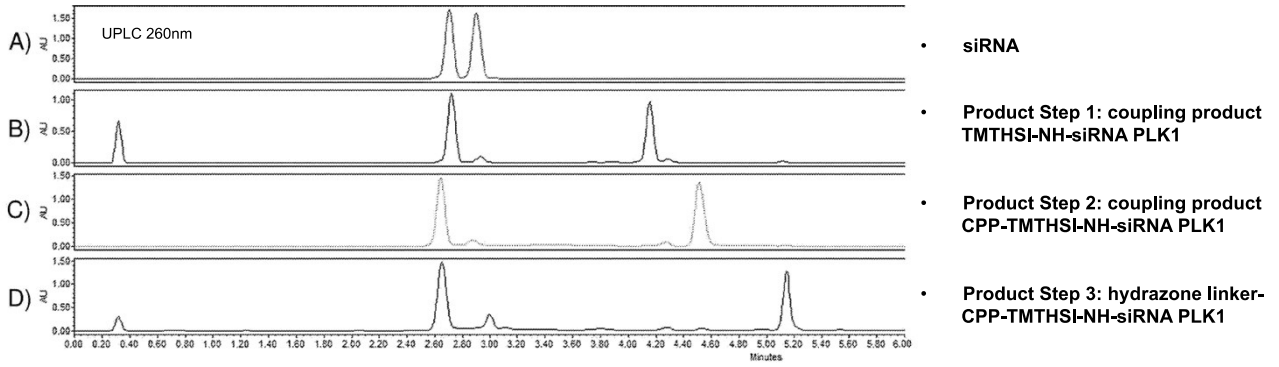


The solution of CPP-TMTHSI-NH-siRNA PLK1 (750 µl) was transferred to a vivaspin centrifugal tube and buffer swapped to borate buffer pH 8.4 (5000Da MWCO, 4000g, 3 times borate buffer pH 8.4, final concentration step). Final volume after Vivaspin treatment: 1200 µl. A 20 mM stock solution of hydrazone MA linker NHS ester (see figure) was prepared in DMSO. While stirring, 5eq., of hydrazone MA linker NHS ester stock solution in DMSO (66 µl, 0.73 mg, 1.6 µmol) was added to CPP-TMTHSI-siRNA (0.32 µmol) in borate buffer (1000 µl) at room temperature and monitored by UPLC. After 45 minutes the reaction showed full conversion to Linker 7 – CPP-TMTHSI - siRNA PLK1 conjugate. The final crude conjugate was purified by PD-10 (5000Da MWCO, 4000g, buffer swap to phosphate buffer pH 7.4) and Vivaspin (5000Da MWCO cut-off, 4000g, 3 times phosphate buffer pH 7.4, final concentration step) to remove excess of hydrazone MA linker NHS ester.

For monitoring the reaction steps leading to the final biomolecular construct a Waters Acquity H-class UPLC system was used with a Acquity OST C18 UPLC column (1.7 µm, 2.1 x 50 mm). Eluent A (100 mMol TEAA / MeOH (93:7 % v/v)) and eluent B (MeOH) were used ins the gradient indicated below. The sample tray temperature was 20 °C and column temperature was 80 °C and the flow 0.5 ml/min. Detection was by UV (258 nm) in a total run time of 10 min.

Gradient

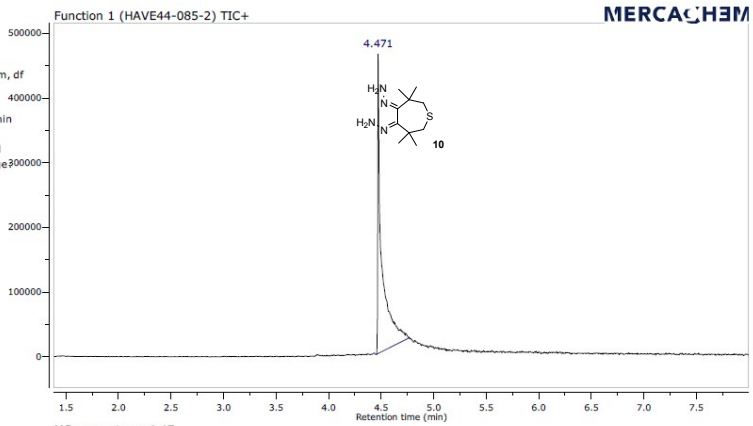
Time (min)	Flow rate (ml/min)	%A	%B	Curve
Initial	0.500	100.0	0.0	-
0.10	0.500	100.0	0.0	6
5.00	0.500	55.0	45.0	6
5.50	0.500	10.0	90.0	6
7.00	0.500	10.0	90.0	6
7.10	0.500	100.0	0.0	6
10.00	0.500	100.0	0.0	6



Legend: Monitoring the reaction steps leading toward **34**. Both sense and antisense strand of the starting siRNA (A) are visible on HLPC because separation was carried out at 80 °C , that is above the annealing temperature. The antisense strand equipped with an amine has reacted with the hydroxy succinic ester TMTHSI-construct **17** resulting in a shift in the retention time of the resulting **TMTHSI – NH-siRNA PLK1** construct (B). After this the construct was clicked by the strain-promoted azide-alkyne cycloaddition to azide-CPP leading to **CPP-TMTHSI-NH-siRNA PLK1** conjugate (C). In the last step the hydrazone linker was coupled to the CPP moiety leading to conjugate (D). Note that as expected the sense strand eluted at similar retention times in the UPLC traces of steps 1-3.

Analyses for compound 10

title	**HAVE44-085-2**
Method	A
Date acquired	20 Feb 18 3:38 pm
FileName	Analysis\GCMS2_0220_015.D
Instrument	Agilent 6890N / Column: RXi-5MS 20m, ID 180 µm, df 0.18 µm
Average velocity	50 cm/s / Carrier gas: He
Initial temp	100°C / Initial time: 1.5 min / Solvent delay: 1.3 min
Rate	75°C/min, Final temp: 250°C, Final time: 4.5 min
Split ratio	20:1 / Injector temp: 250°C Injection volume: 1 µl
Detection	MSD (EI-positive) / Det. temp.: 280°C / Mass range 50-550
Detection	FID / Detector temp: 300°C



Integrals spectrum TIC

rt (min)	height	area	area (%)
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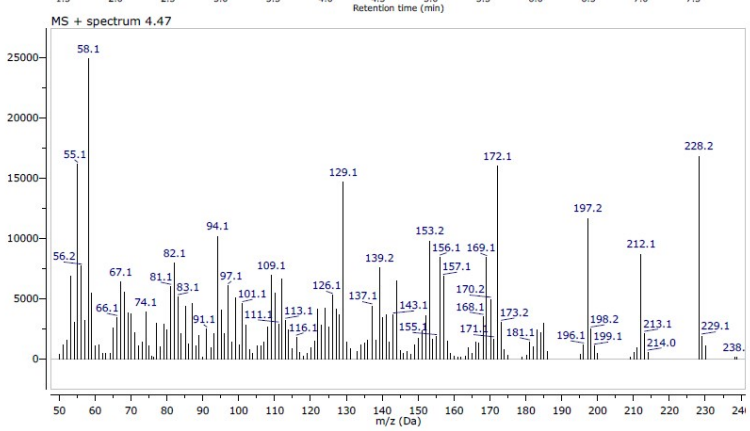
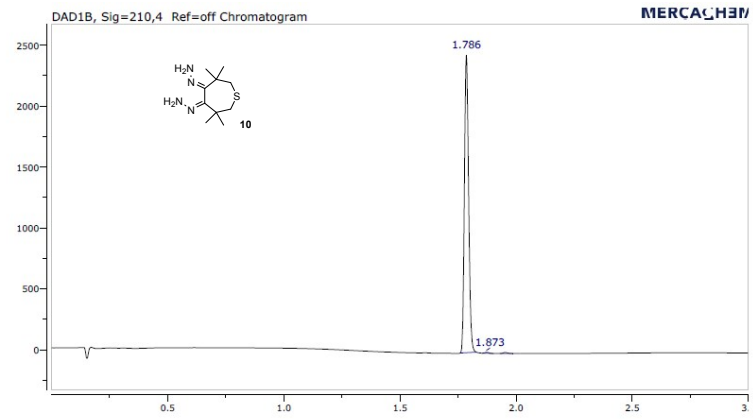
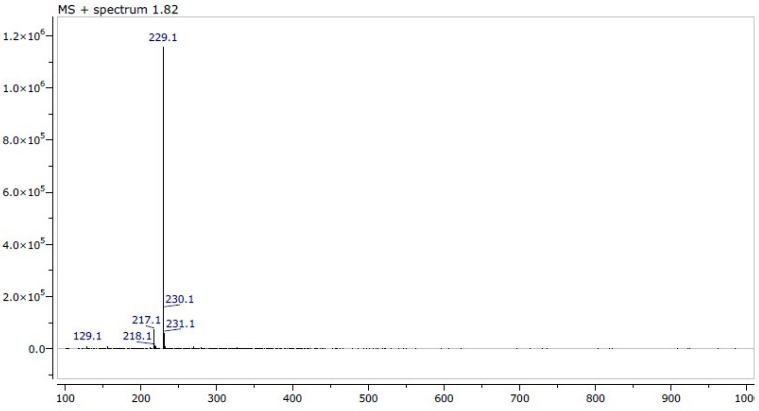


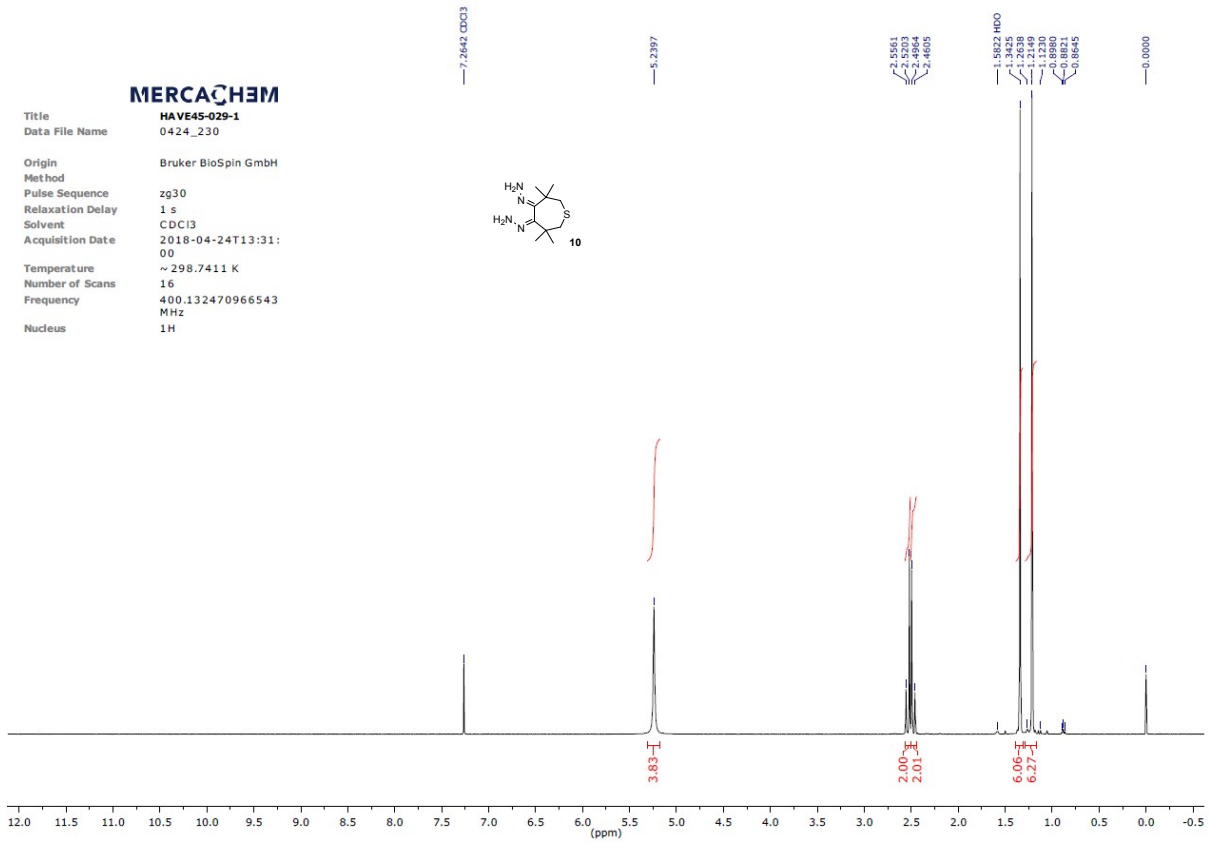
title	**HAVE46-083-2**
Method	SC_BASE.M
Date acquired	29-Jan-19, 09:56:19
FileName	Analysis\LCMS19_0129_028.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)



Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
1.79	2444	61263	99.34
1.87	8.122	186.9	0.30
1.95	9.079	219.5	0.36

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Title HAVE45-029-1
Data File Name 0424_230
Origin Bruker BioSpin GmbH
Method
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent CDCl3
Acquisition Date 2018-04-24T13:31:00
Temperature ~298.7411 K
Number of Scans 16
Frequency 400.132470966543 MHz
Nucleus 1H
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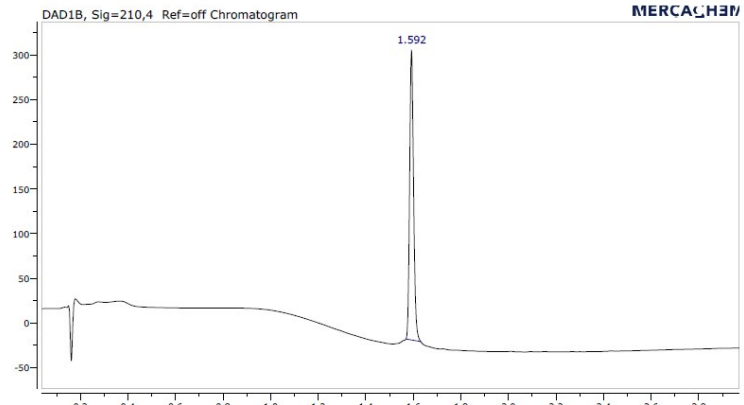
5.2397
2.5561
2.5203
2.4964
2.4605
1.5822 HDO
1.2425
1.2258
1.2149
1.1230
0.8980
0.8821
0.8645
0.0000
10
3.83
2.00
2.01
6.06
6.27
(ppm)

Analyses for compound 12

title	**HAVE48-039-3**
Method	SC_BASE.M
Date acquired	25-Sep-19, 16:12:13
FileName	Analysis\LCMS23_0925_091.D
Acq. method	SC_BASE
Column	XSelect CSH C18 (50x2.1mm 3.5µ)
Flow	1 ml/min, Column temp: 25°C
Eluent A	Acetonitrile
Eluent B	10mM Ammoniumbicarbonate in water
Lin. gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Postrun	1.2 min
Detection	DAD (210, 220 and 220-320nm)
Detection	PDA (210-320nm)
Detection	MSD (ESI pos/neg) mass range 100-1000



Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
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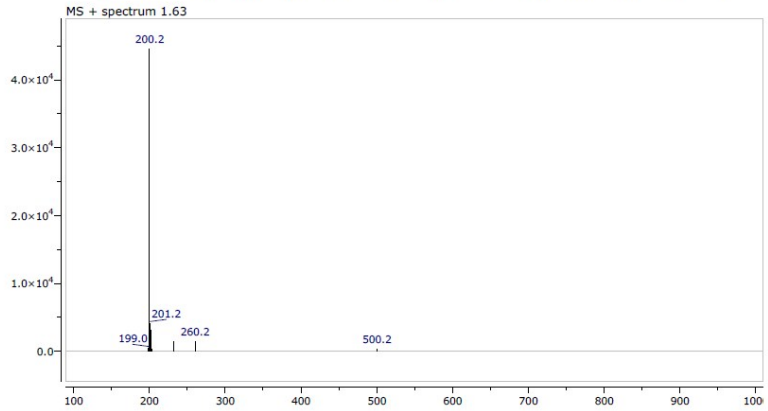

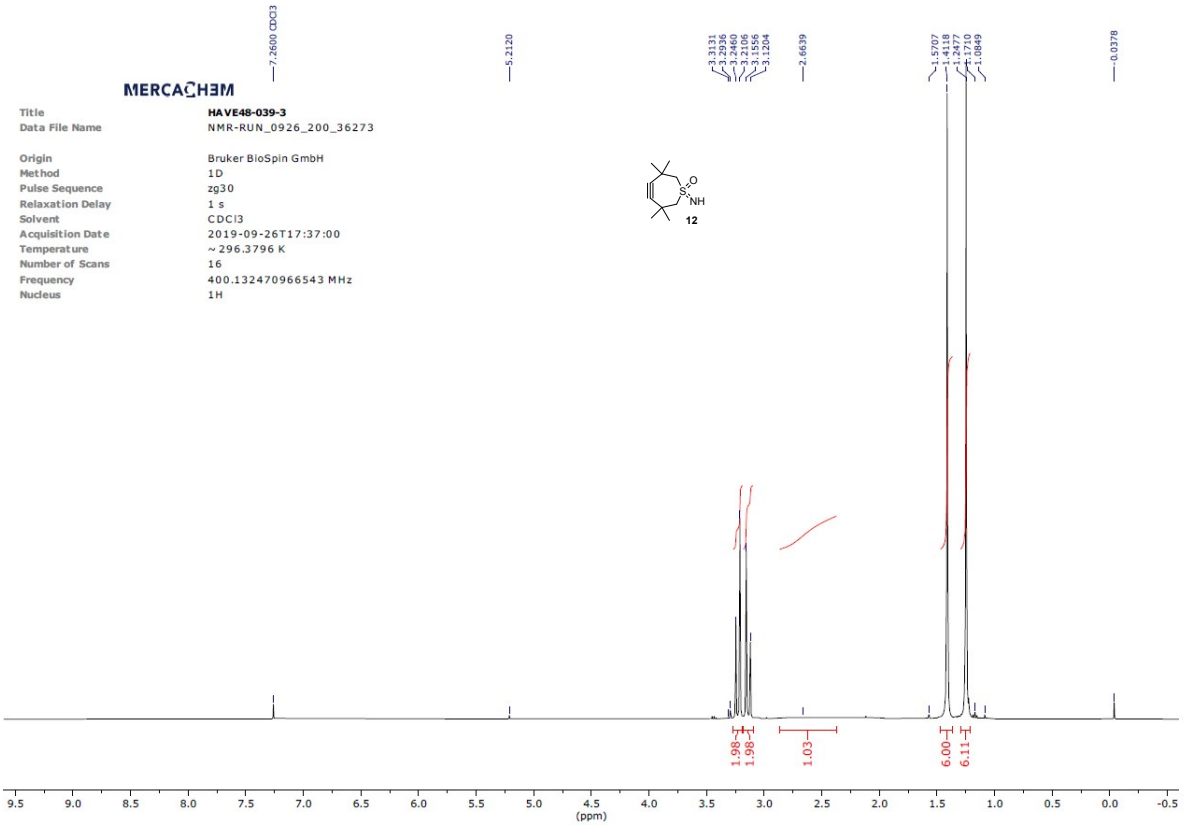

Title	**HAVE48-039-3**
Data File Name	NMR-RUN_0926_200_36273
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Method	1D
Pulse Sequence	zg30
Relaxation Delay	1 s
Solvent	CDCl3
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Temperature	~296.3796 K
Number of Scans	16
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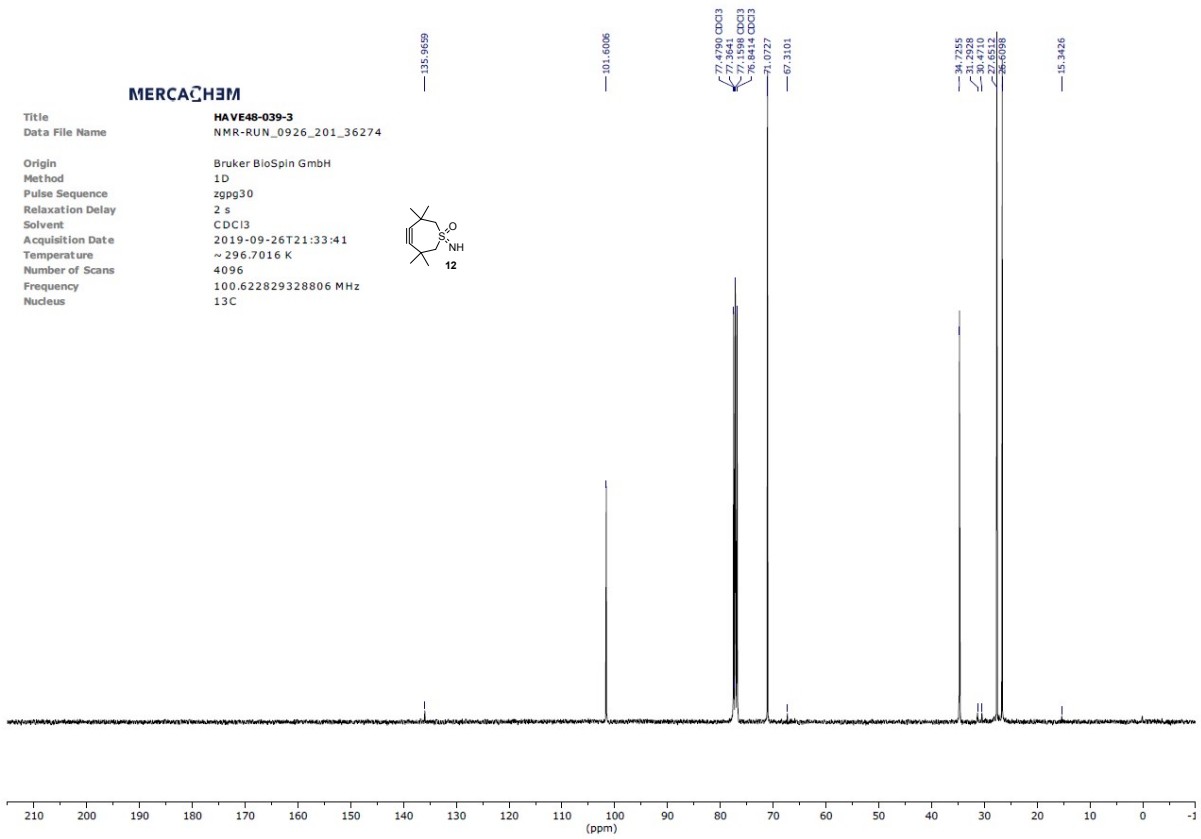
Nucleus	1H

12


MERCACHEM
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Data File Name NMR-RUN_0926_201_36274
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-09-26T21:33:41
Temperature ~296.7016 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
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135.9659
101.6006
77.4790 CDCl3
77.3641
77.1598 CDCl3
76.8414 CDCl3
71.0727
67.3101
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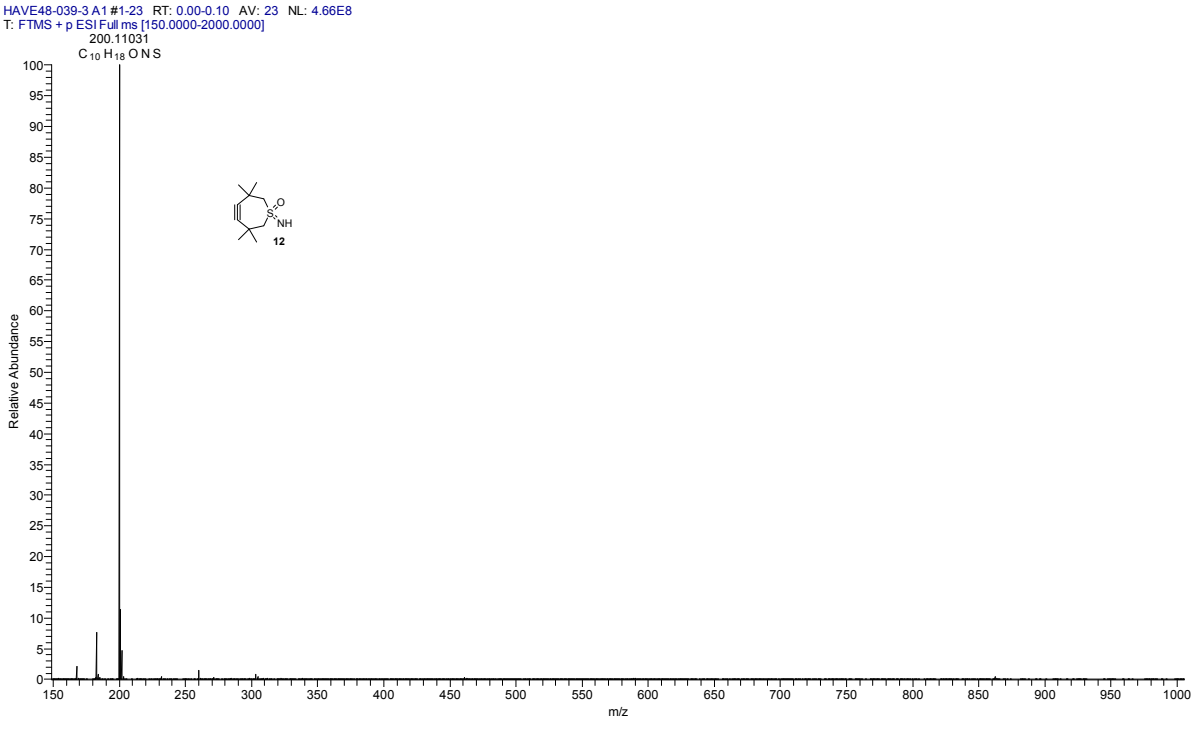
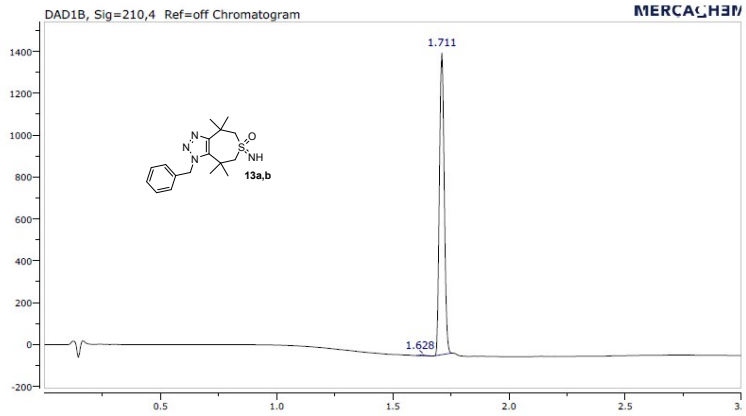
31.2928
30.4710
27.6512
26.6098
15.3426
(ppm)


HAVE48-039-3 A1 #1-23 RT: 0.00-0.10 AV: 23 NL: 4.66E8
T: FTMS + p ESI Full ms [150.0000-2000.0000]
200.11031
C10 H18 O N S
12
Relative Abundance
m/z

Analyses for compound 13a,b

title	**HAVE48-018-1**
Method	SC_BASE.M
Date acquired	26-Aug-19, 16:20:41
FileName	Analysis\LCMS19_0826_046.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)



Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
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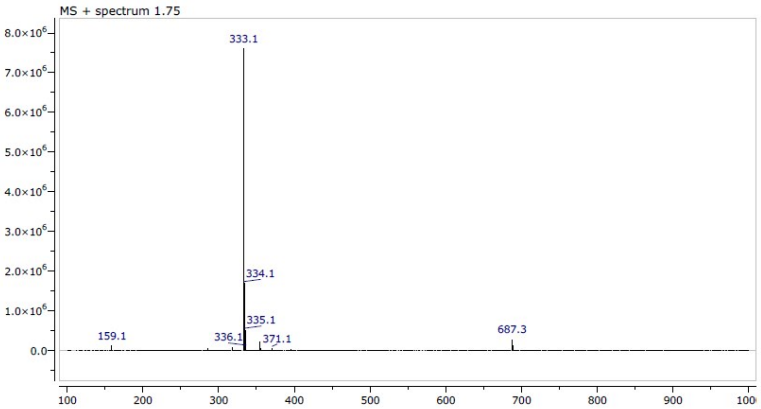
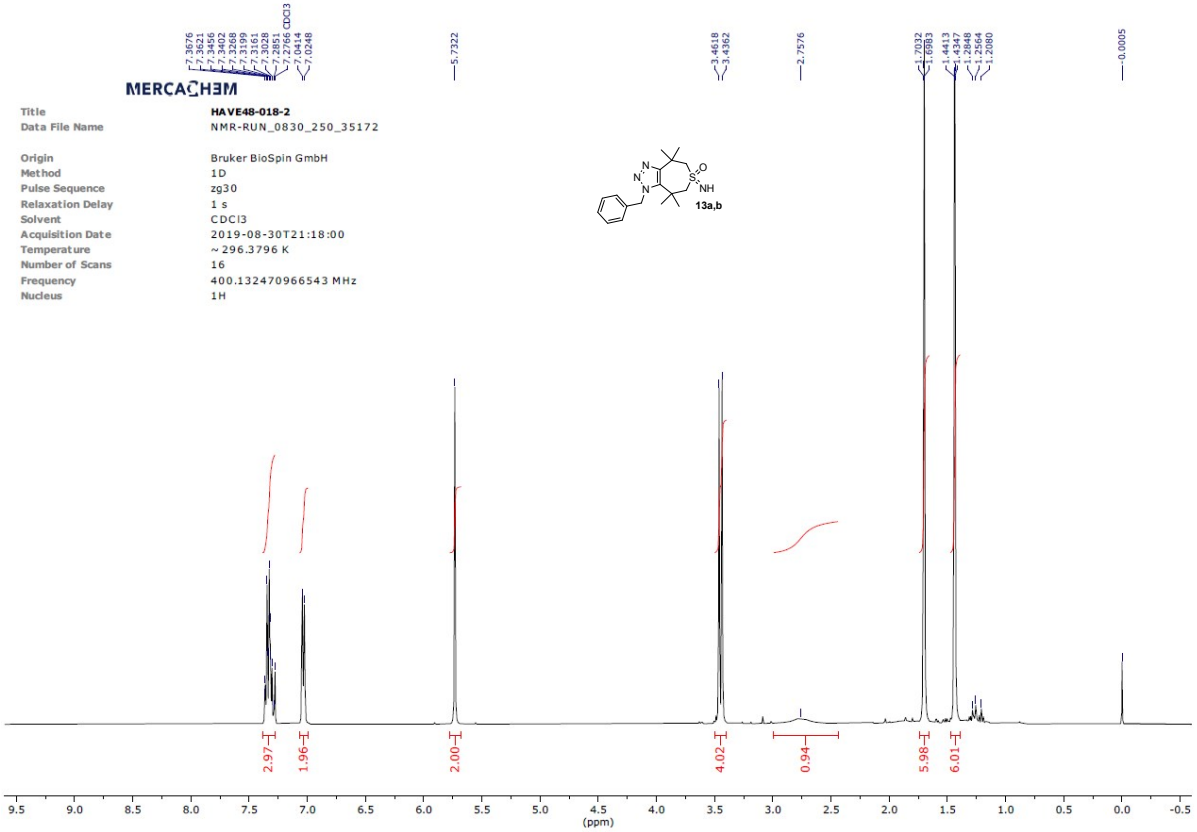
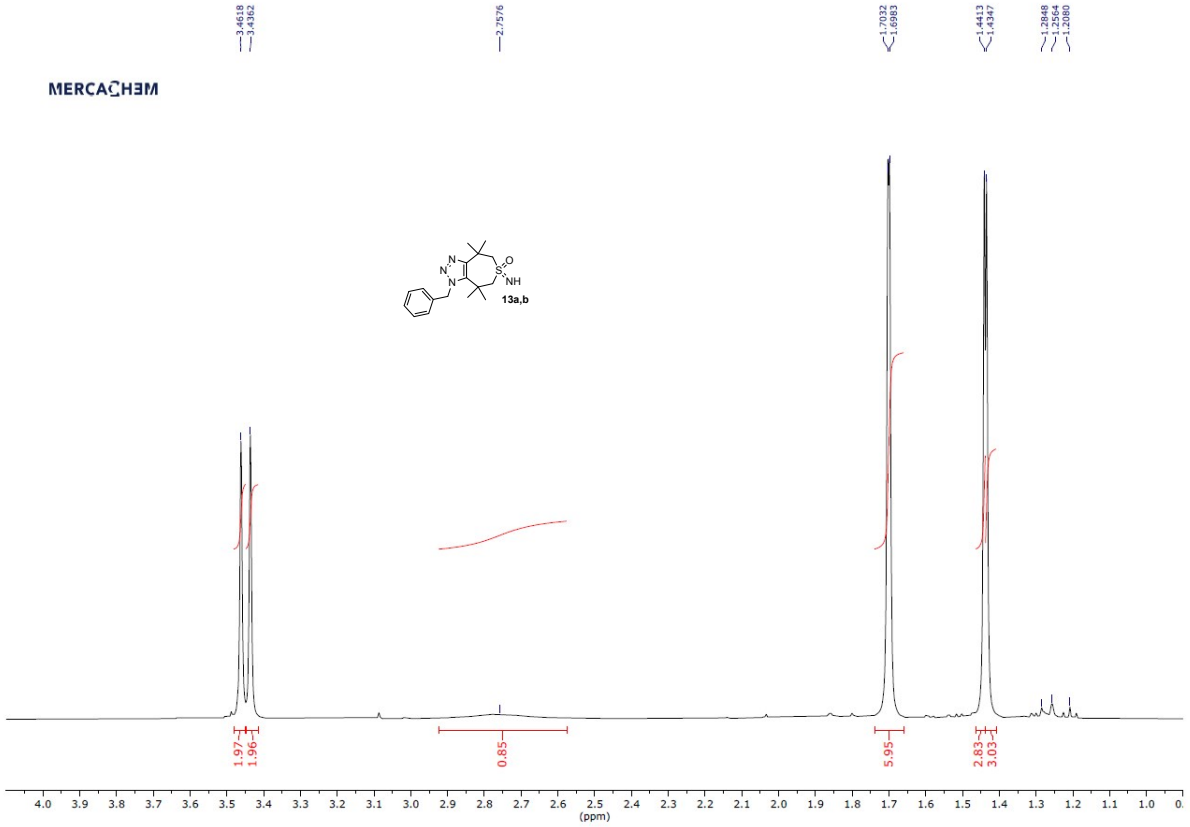
1.71	1442	41784	99.70



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1.4347
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MERCACHEM
13a,b
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0.85
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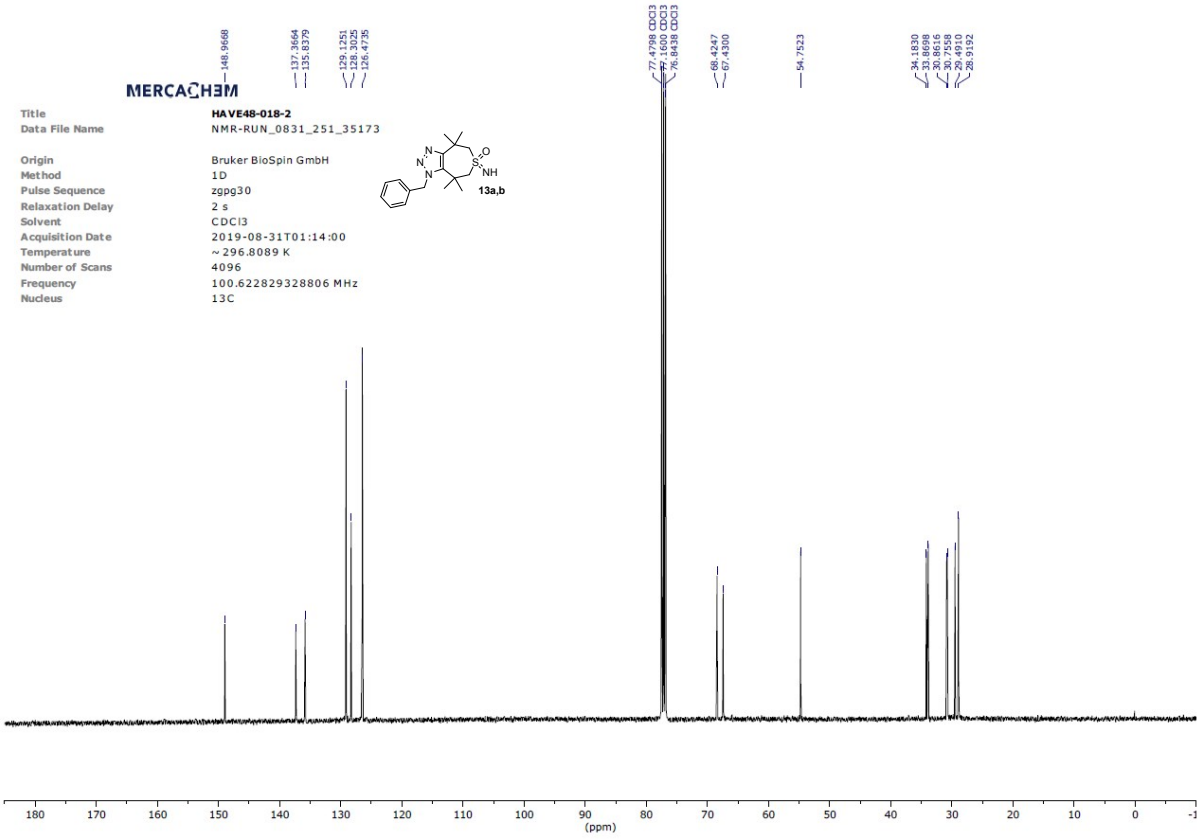
2.83
3.03
(ppm)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30.7558
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MERCACHEM
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Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
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Acquisition Date 2019-08-31T01:14:00
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13a,b
(ppm)

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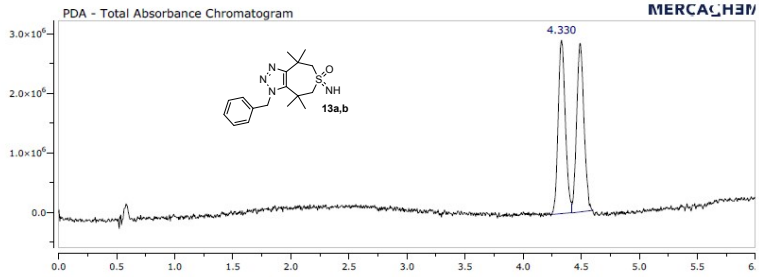
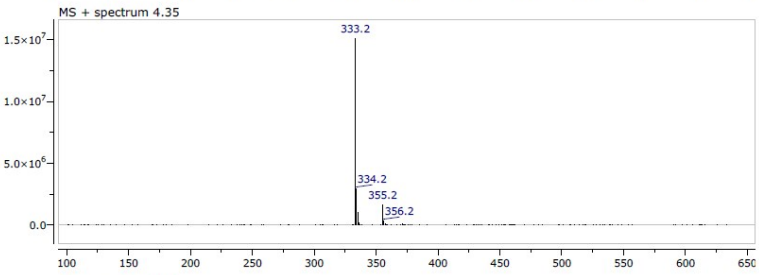
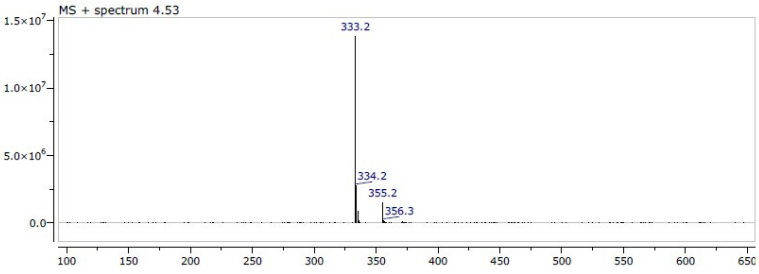
Date acquired	17-Sep-2019, 19:40:24
FileName	Analysis\SFC1_190917_HAVE_008.raw
UPLC	Waters UPC2
Acq. Method	5_IC_05-50_f25_P170_Amm_6min
Column	Chiralpak IC (100x4.6mm 5µm)
Flow	2.5 ml/min; Column temp: 35°C; BPR: 170 bar
Eluent A	CO2
Eluent B	Methanol + 20mM ammonia
Gradient	t=0 min 5% B, t=5 min 50% B, t=6 min 50% B
Posttime	0.5 min
Detection PDA	210-320nm
MS	QDa
Detection MS	ESI (scan)
Mass range	100-650 (pos), 1Hz
Time	6 min
Cone	15 V



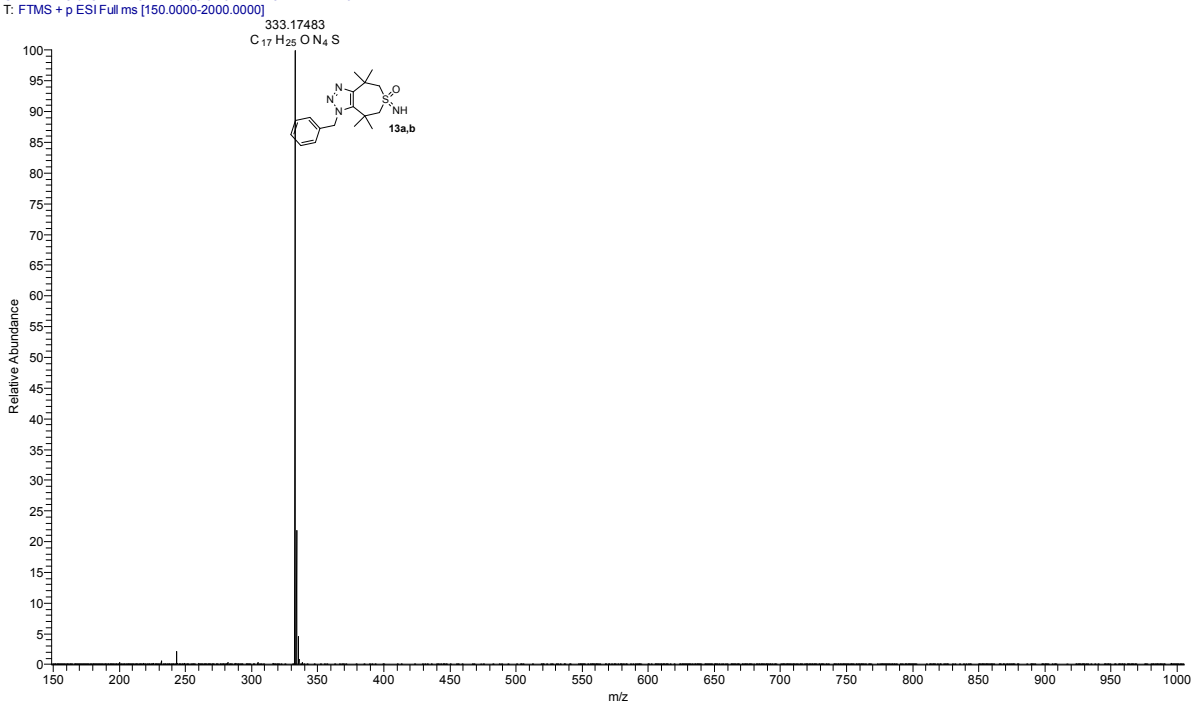




Integrals spectrum PDA - Total Absorbance Chromatogram

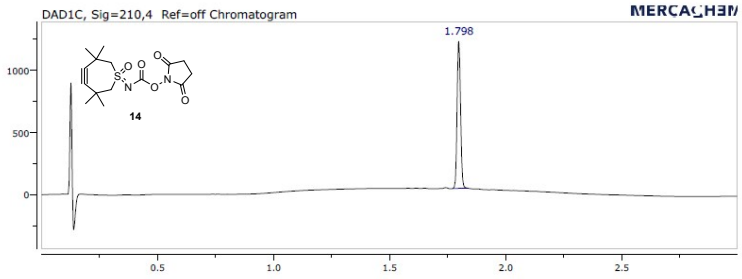
rt (min)	height	area	area (%)
4.33	2913737	250971557	50.27
4.49	2834996	248282095	49.73

C1 HAVE48-018-2 #1-23 RT: 0.00-0.10 AV: 23 NL: 1.44E9
T: FTMS + p ESI Full ms [150.0000-2000.0000]



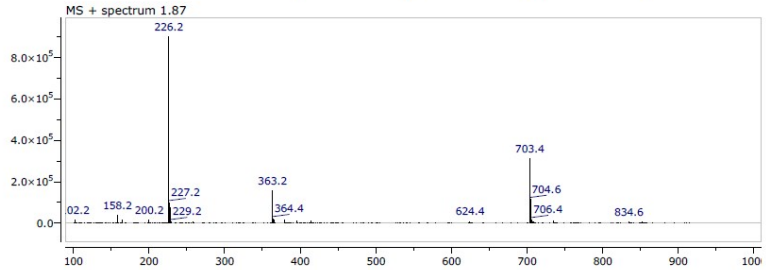
Analyses for compound 14

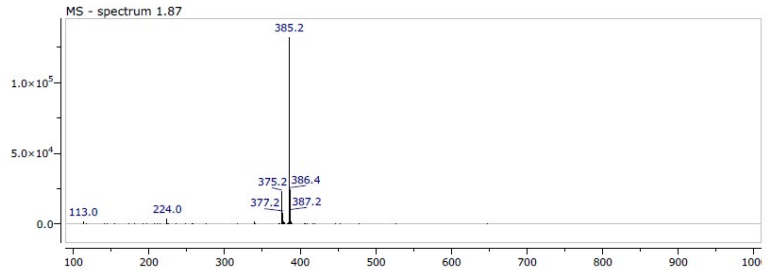
title	**HAVE48-036-1**
Method	SC_ACID.M
Date acquired	16-Sep-19, 13:10:27
FileName	Analysis\LCMS22_0916_062.D
Acq.method	SC_ACID
Column	XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min, Column temp.: 40°C
Eluent A	0.1% Formic acid in Acetonitrile
Eluent B	0.1% Formic acid in Water
Lin. gradient	t=0 min 5%A, t=1.6 min 98%A, t=3 min 98% A
Postrun	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range 100-1000
Detection	ELSD (Neb temp. 40°C, gassflow 1.5 ml/min)

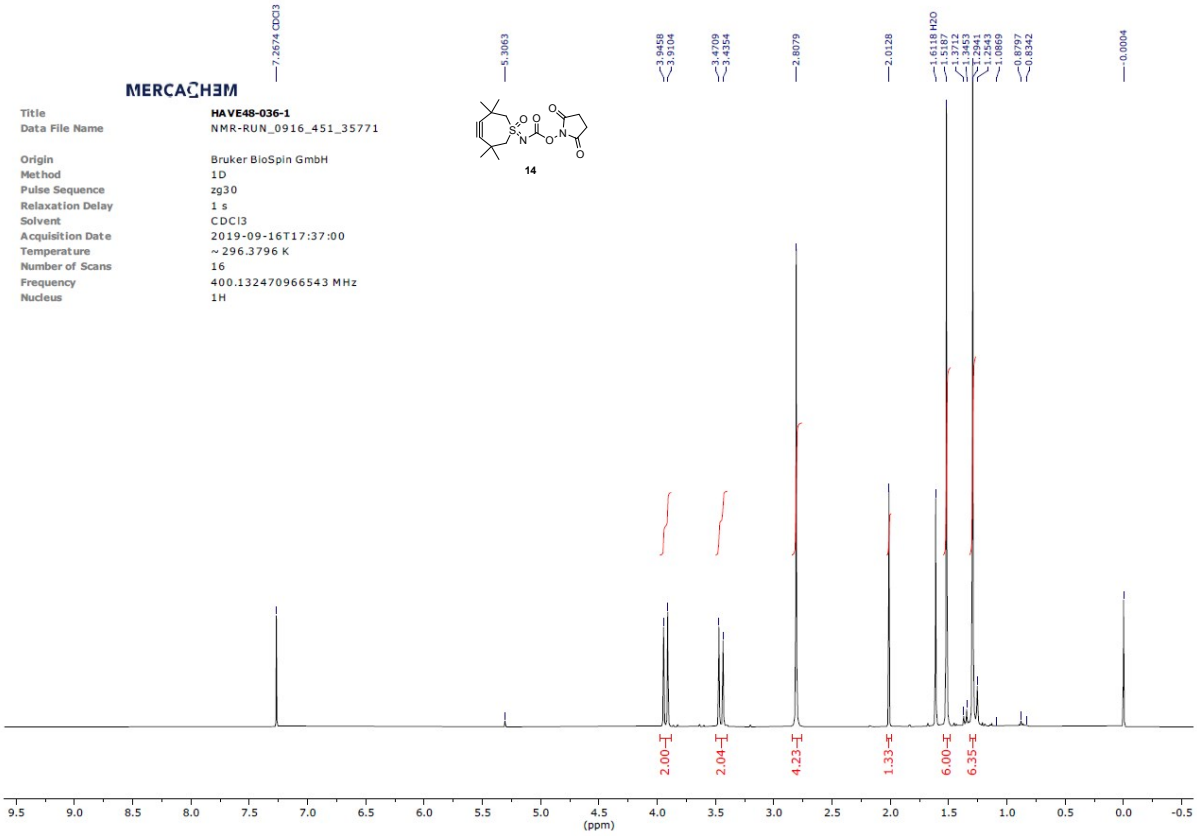


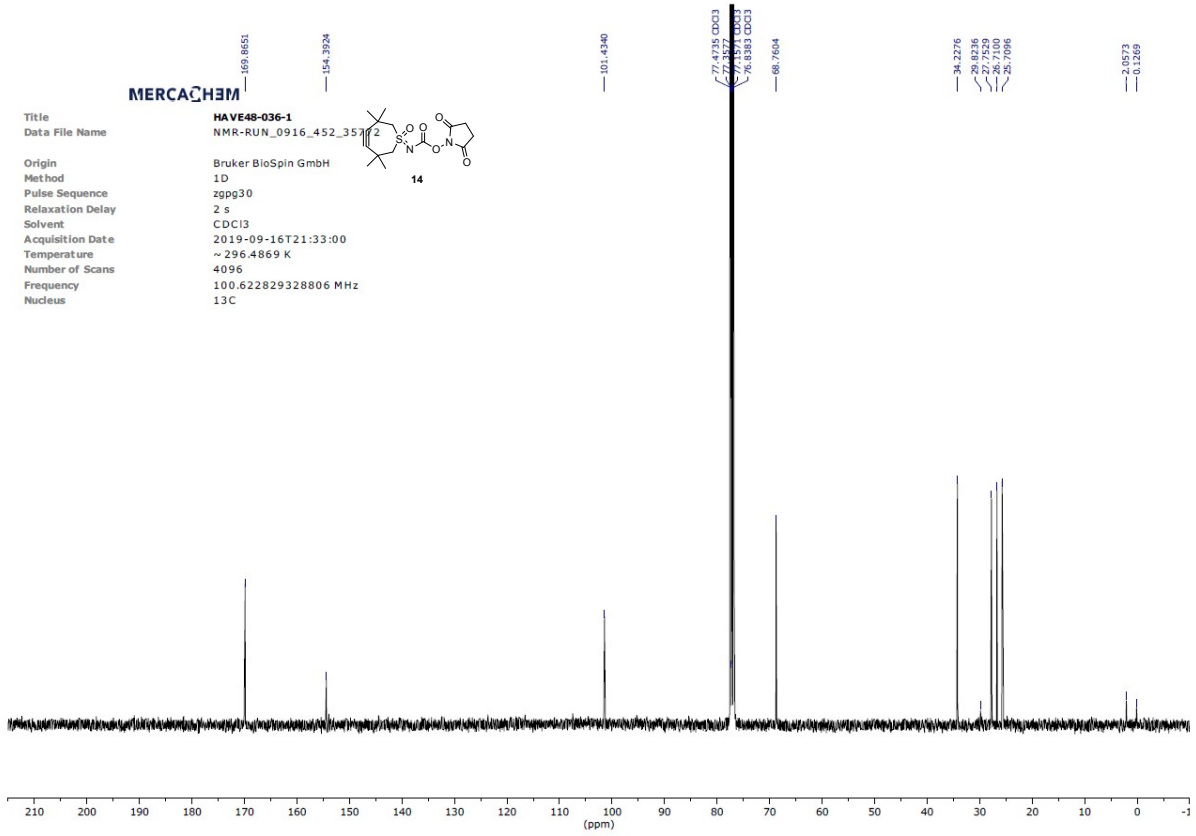
Integrals spectrum Chromatogram DAD1C, Sig=210,4 Ref=off

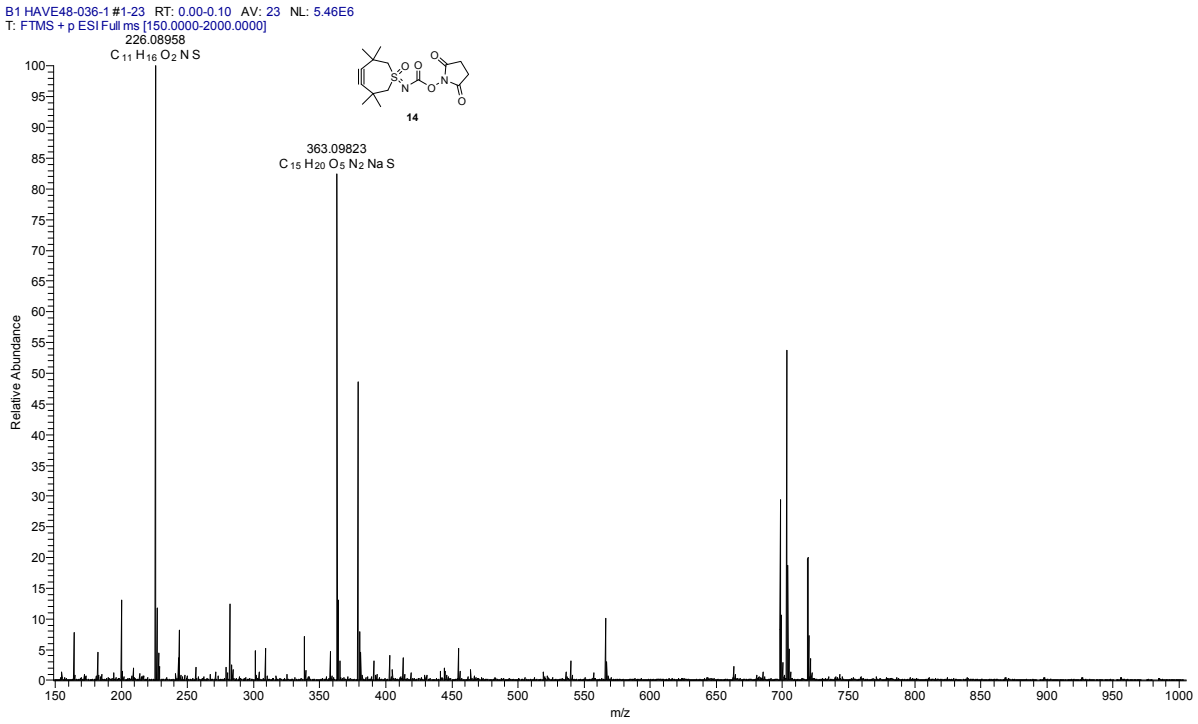
rt (min)	height	area	area (%)
1.80	1183	24495	100.00





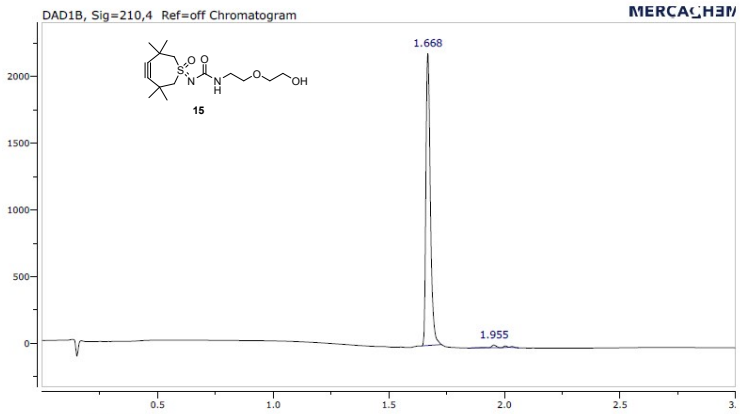



169.8651
154.3924
101.4340
77.4735 CDCl3
77.3577
77.1571 CDCl3
76.8383 CDCl3
68.7604
34.2276
29.8236
27.7529
26.7000
25.7096
2.0573
0.1269
MERCACHEM
Title HAVE48-036-1
Data File Name NMR-RUN_0916_452_357[2
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-09-16T21:33:00
Temperature ~ 296.4869 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
14
(ppm)


B1 HAVE48-036-1 #1-23 RT: 0.00-0.10 AV: 23 NL: 5.46E6
T: FTMS + p ESI Full ms [150.0000-2000.0000]
226.08958
C 11 H 16 O 2 N S
363.09823
C 15 H 20 O 5 N 2 Na S
14
Relative Abundance
m/z

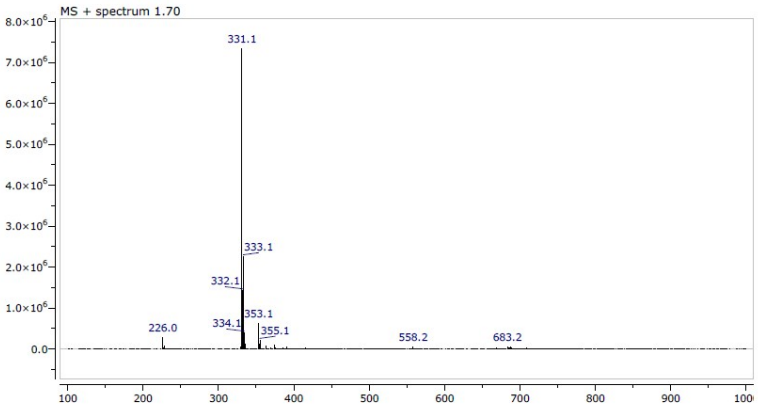
Analyses for compound 15

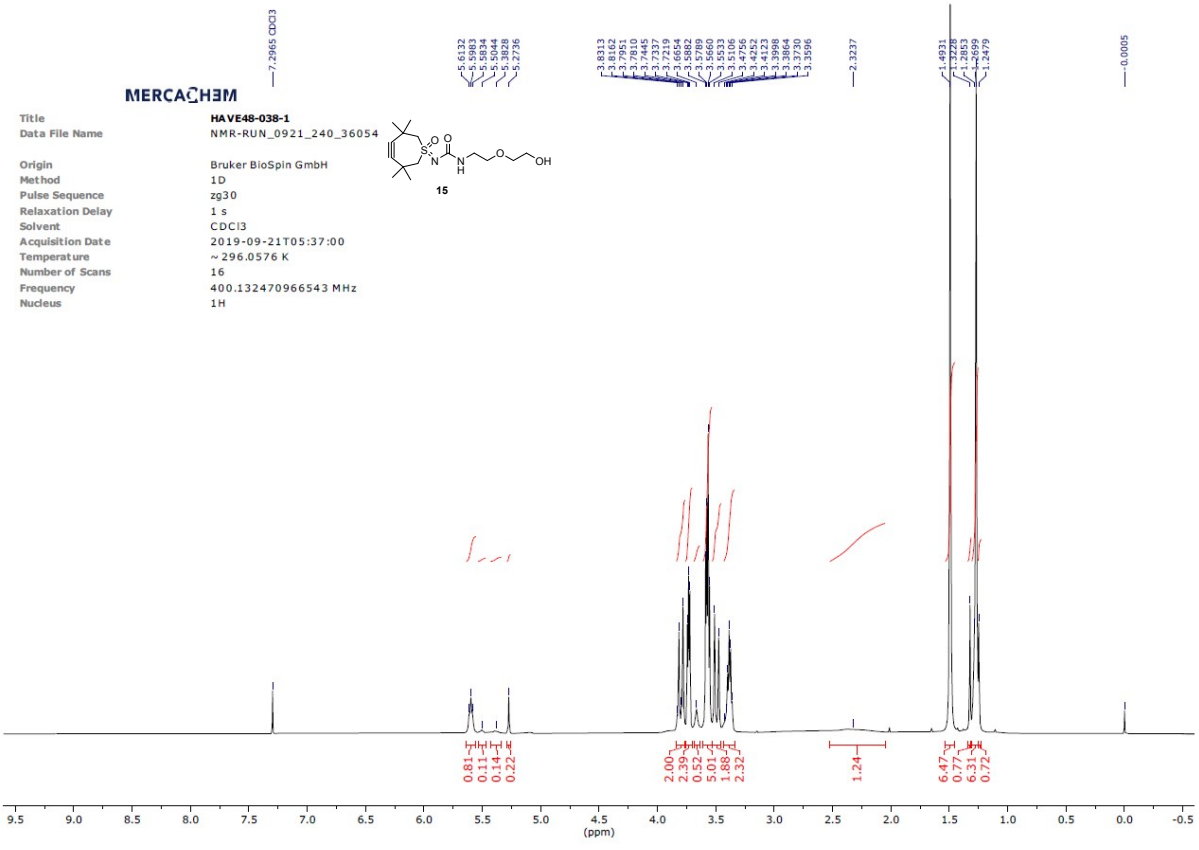
title	**HAVE48-038-1**
Method	SC_BASE.M
Date acquired	18-Sep-19, 16:28:43
FileName	Analysis\LCMS19_0918_071.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)

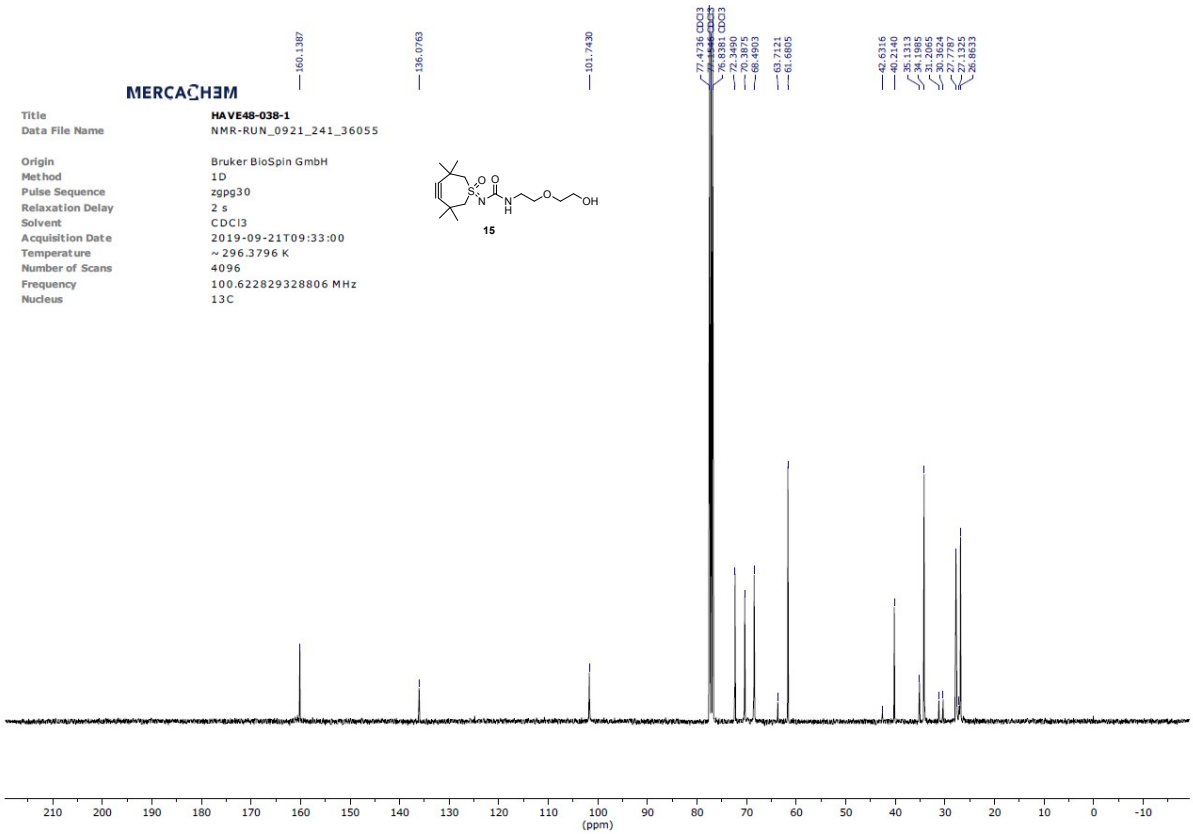


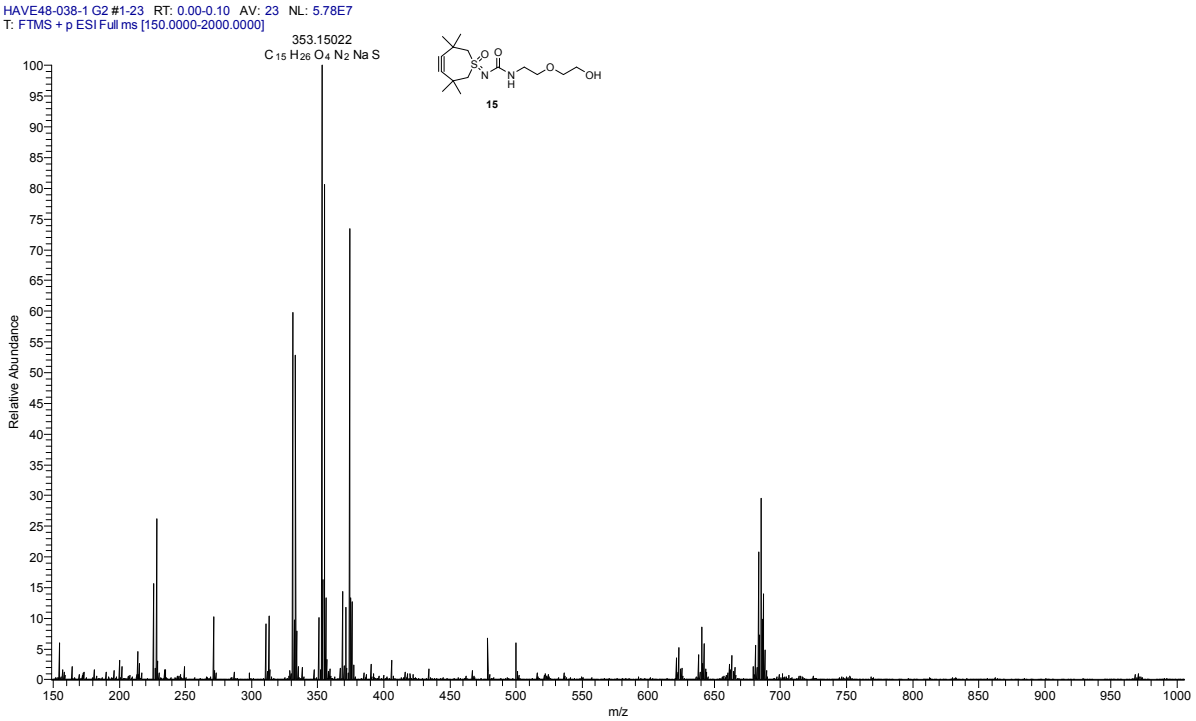
Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
1.67	2188	58724	98.18
1.91	3.699	169.6	0.28
1.96	20.89	550.0	0.92
2.00	11.52	218.6	0.37
2.03	7.214	148.0	0.25



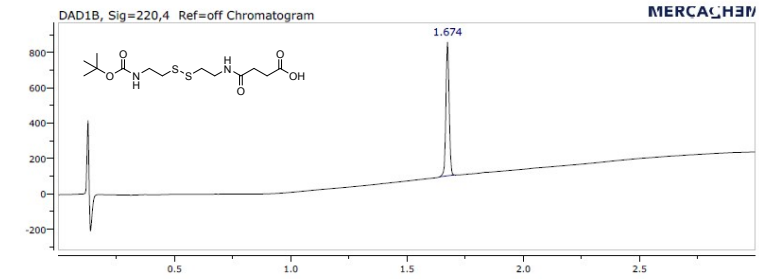



MERCACHEM
Title HAVE48-038-1
Data File Name NMR-RUN_0921_241_36055
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-09-21T09:33:00
Temperature ~296.3796 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
160.1387
136.0763
101.7430
77.4736 CDCl3
76.8381 CDCl3
73.3490
72.3875
68.4903
63.7121
61.6895
42.6316
40.2140
35.1313
34.1985
31.7055
30.3624
27.7787
27.1325
26.8633
15
(ppm)


HAVE48-038-1 G2 #1-23 RT: 0.00-0.10 AV: 23 NL: 5.78E7
T: FTMS + p ESI Full ms [150.0000-2000.0000]
353.15022
C 15 H26 O4 N2 Na S
15
Relative Abundance
m/z

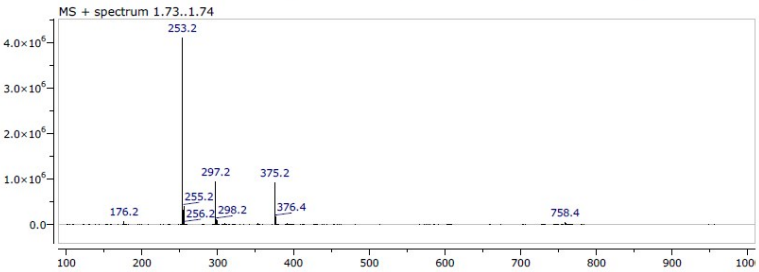
Analyses of compound 16 and its synthetic intermediates

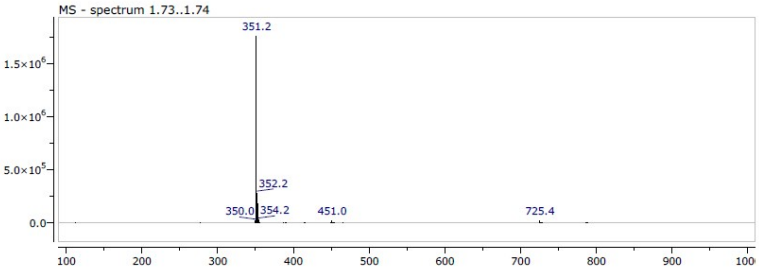
title	**HAVE48-024-1**
Method	SC_ACID.M
Date acquired	08-Oct-19, 09:40:53
FileName	Analysis\LCMS22_1008_027.D
Acq.method	SC_ACID
Column	XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min, Column temp.: 40°C
Eluent A	0.1% Formic acid in Acetonitrile
Eluent B	0.1% Formic acid in Water
Lin. gradient	t=0 min 5%A, t=1.6 min 98%A, t=3 min 98% A
Postrun	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range 100-1000
Detection	ELSD (Neb temp. 40°C, gassflow 1.5 ml/min)

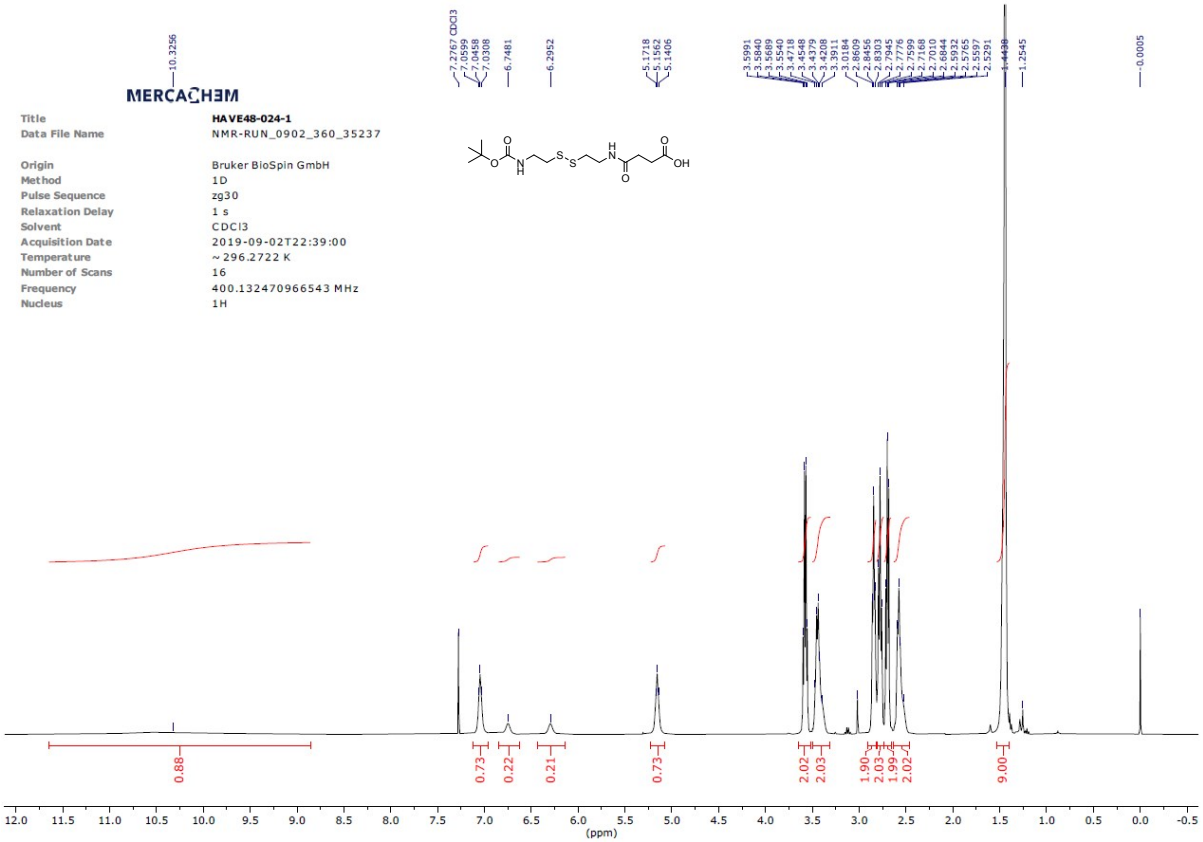


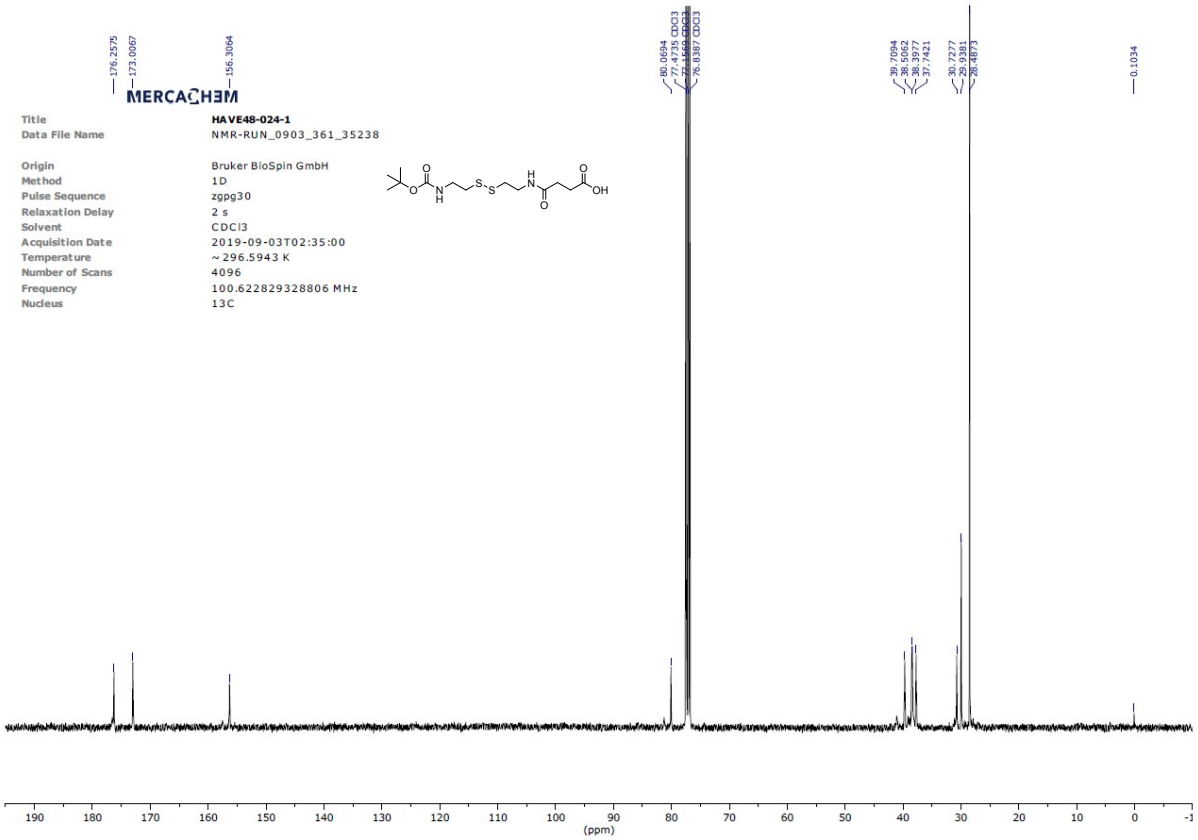
Integrals spectrum Chromatogram DAD1B, Sig=220,4 Ref=off

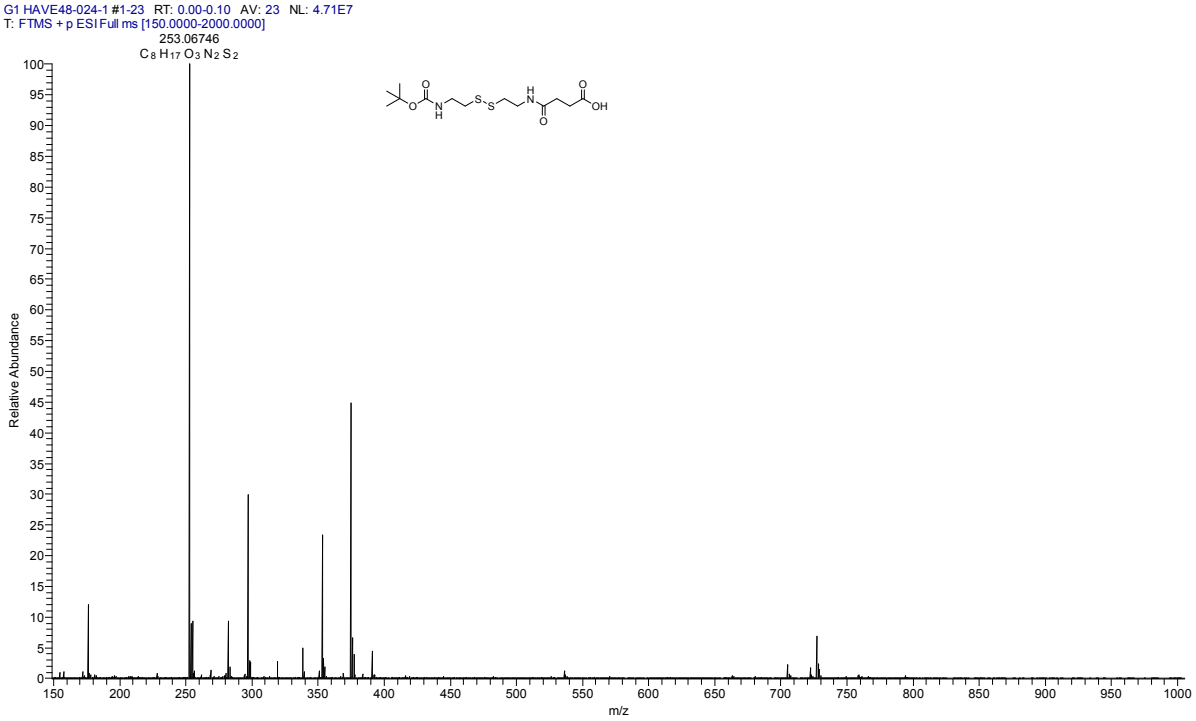
rt (min)	height	area	area (%)
1.67	756.7	14666	100.00

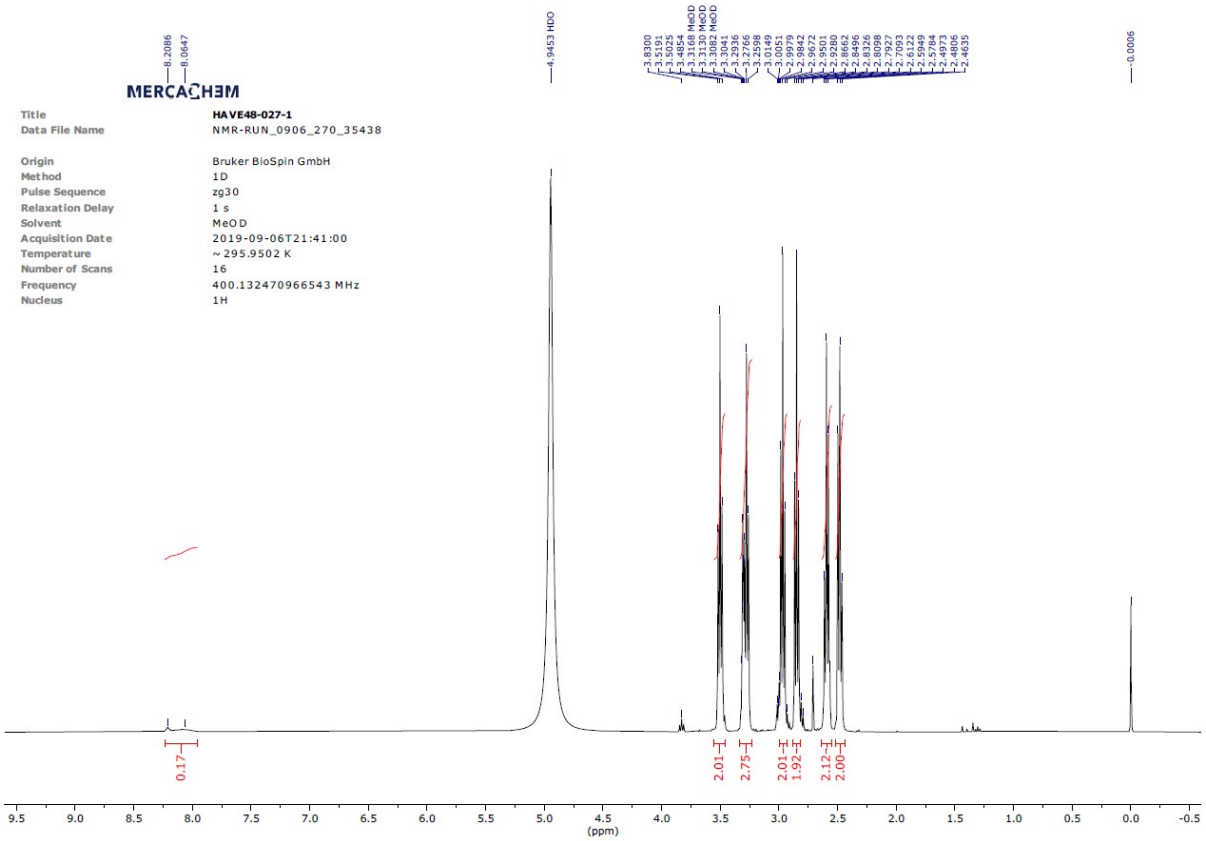


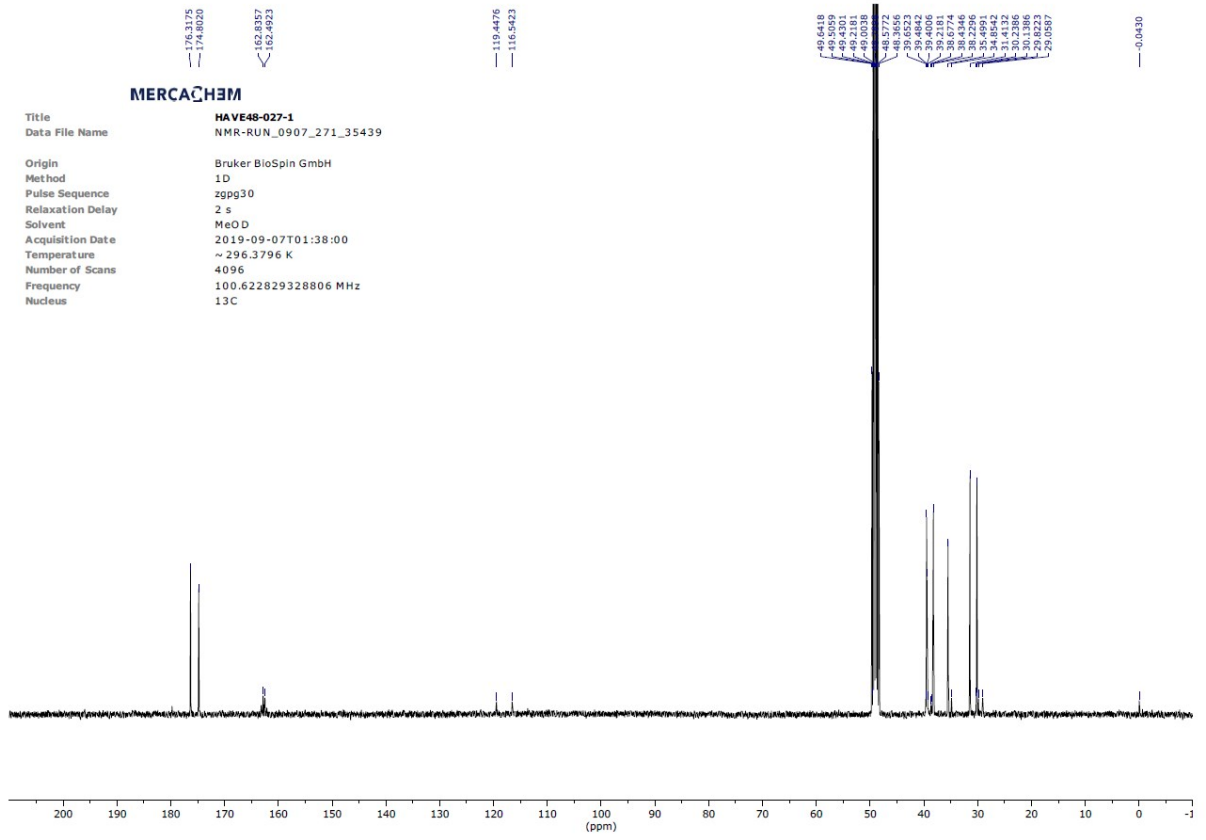


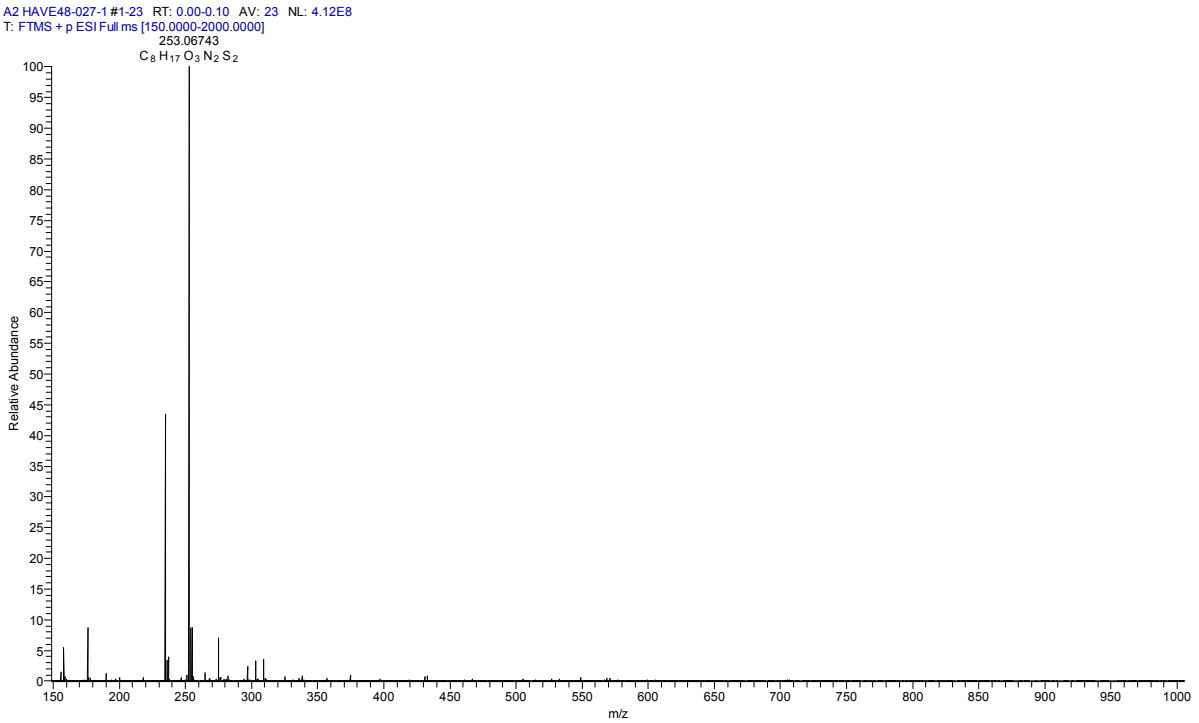



176.2575
173.0067
156.3064
80.0694
77.4735 CDCl3
77.1569 CDCl3
76.8387 CDCl3
39.7094
38.5062
38.3027
37.7421
30.7277
29.9381
28.4873
0.1034
MERCACHEM
Title HAVE48-024-1
Data File Name NMR-RUN_0903_361_35238
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-09-03T02:35:00
Temperature ~296.5943 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
(ppm)


G1 HAVE48-024-1 #1-23 RT: 0.00-0.10 AV: 23 NL: 4.71E7
T: FTMS + p ESI Full ms [150.0000-2000.0000]
253.06746
C8 H17 O3 N2 S2
Relative Abundance
m/z

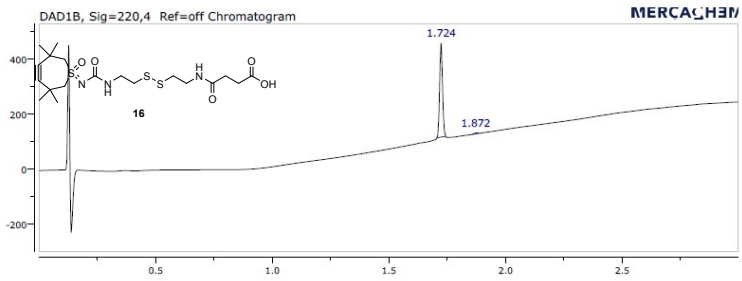

MERCACHEM
Title HAVE48-027-1
Data File Name NMR-RUN_0906_270_35438
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent MeOD
Acquisition Date 2019-09-06T21:41:00
Temperature ~ 295.9502 K
Number of Scans 16
Frequency 400.132470966543 MHz
Nucleus 1H
8.2086
8.0647
4.9453 HDO
3.8300
3.5191
3.5025
3.4844
3.3168 MeOD
3.3130 MeOD
3.3082 MeOD
3.3041
3.2936
3.2766
3.2598
3.0149
3.0051
2.9979
2.9842
2.9672
2.9501
2.8980
2.8662
2.8496
2.8326
2.8098
2.7927
2.7093
2.6122
2.5949
2.5784
2.4973
2.4806
2.4635
0.0006
0.17
2.01
2.75
2.01
1.92
2.12
2.00
(ppm)


MERCACHEM
Title HAVE48-027-1
Data File Name NMR-RUN_0907_271_35439
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent MeOD
Acquisition Date 2019-09-07T01:38:00
Temperature ~ 296.3796 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
176.3175
174.8020
162.8357
162.4923
119.4476
116.5423
49.6418
49.5059
49.4301
49.2181
49.0038
48.5772
48.3656
39.6523
39.4452
39.4006
39.2181
38.6774
38.4346
38.2196
35.4991
34.8542
31.4132
30.2286
30.1386
29.8223
29.0587
-0.0430
(ppm)



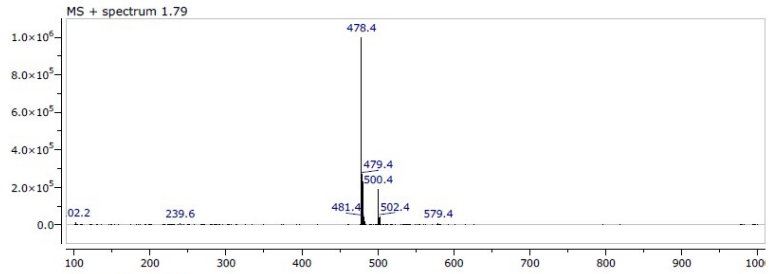
Analyses of compound 16

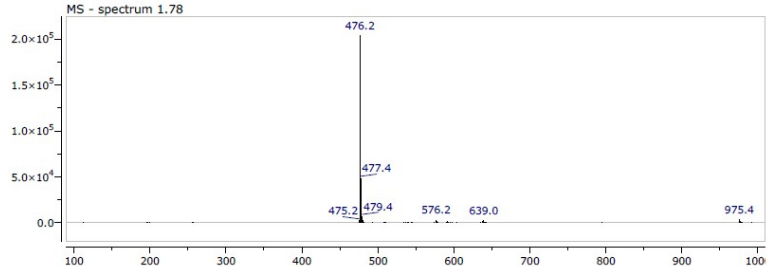
title	**HAVE48-042-2**
Method	SC_ACID.M
Date acquired	25-Sep-19, 13:56:23
FileName	Analysis\LCMS22_0925_089.D
Acq.method	SC_ACID
Column	XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min, Column temp.: 40°C
Eluent A	0.1% Formic acid in Acetonitrile
Eluent B	0.1% Formic acid in Water
Lin. gradient	t=0 min 5%A, t=1.6 min 98%A, t=3 min 98% A
Postrun	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range 100-1000
Detection	ELSD (Neb temp. 40°C, gassflow 1.5 ml/min)

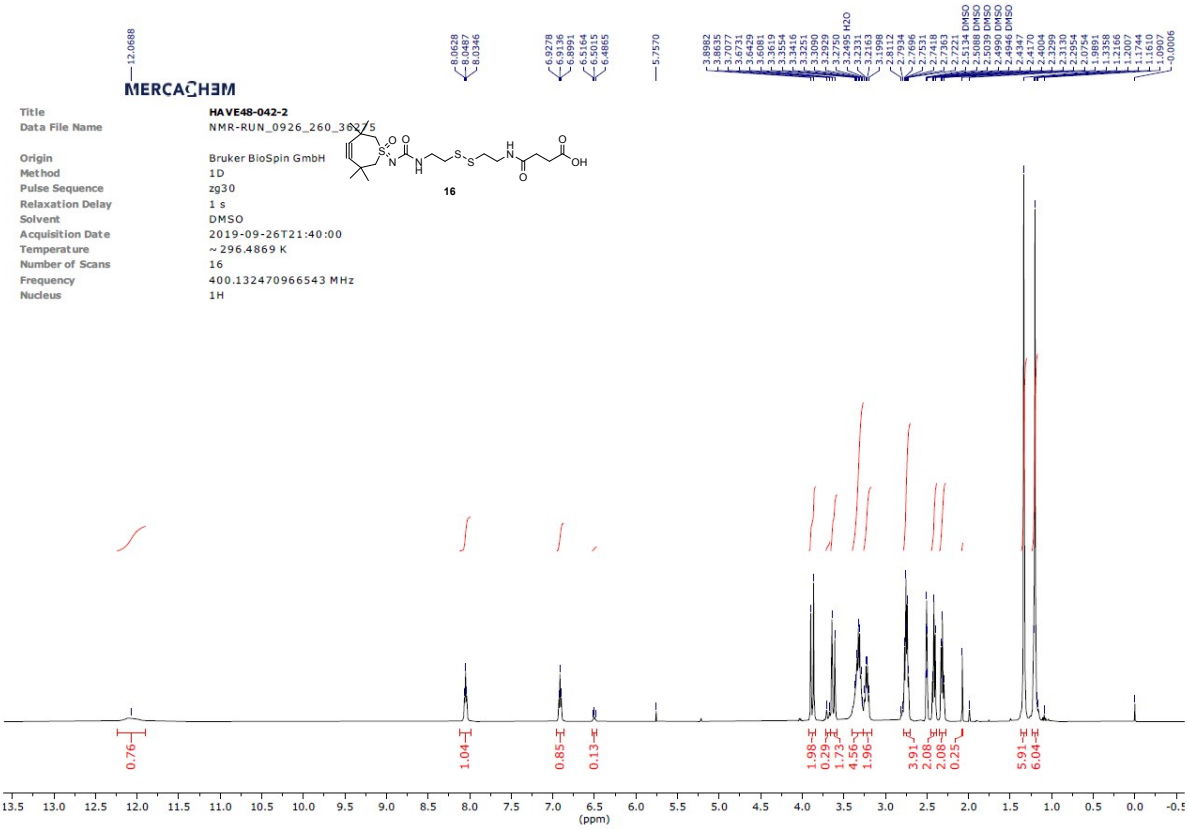


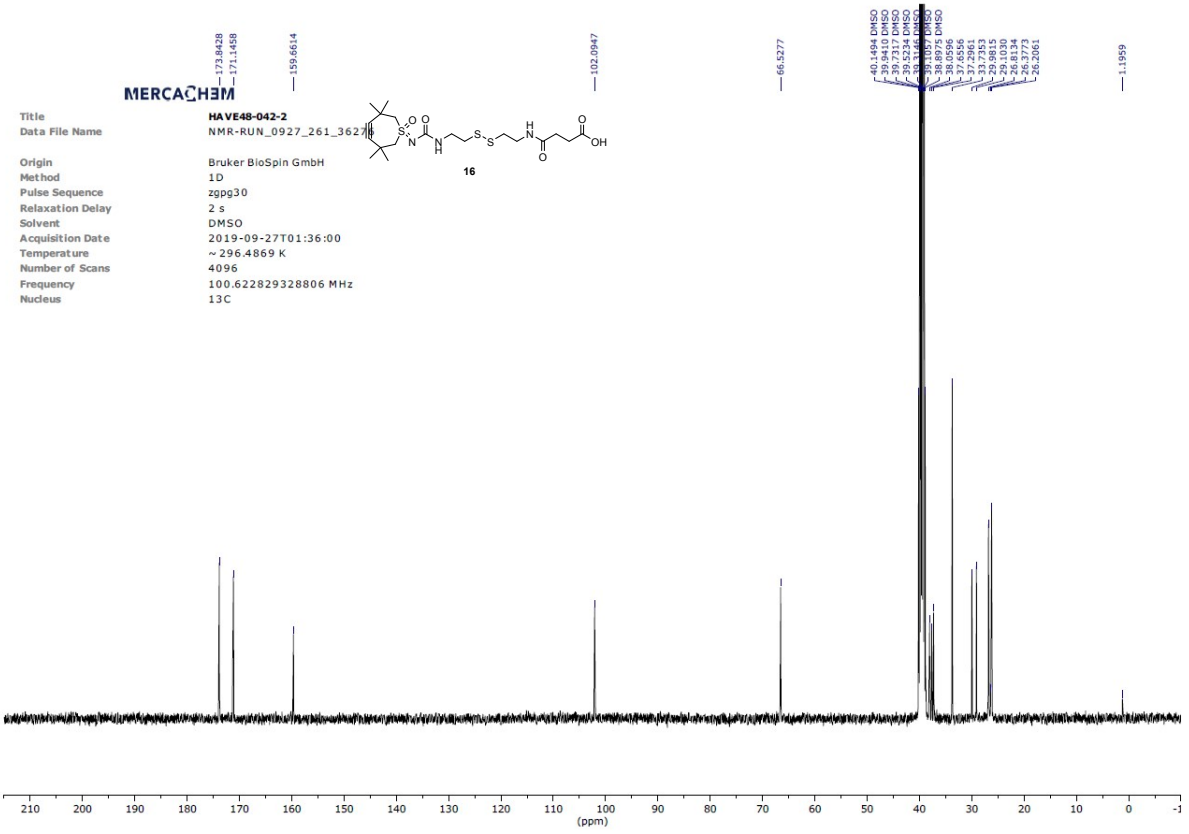
Integrals spectrum Chromatogram DAD1B, Sig=220,4 Ref=off

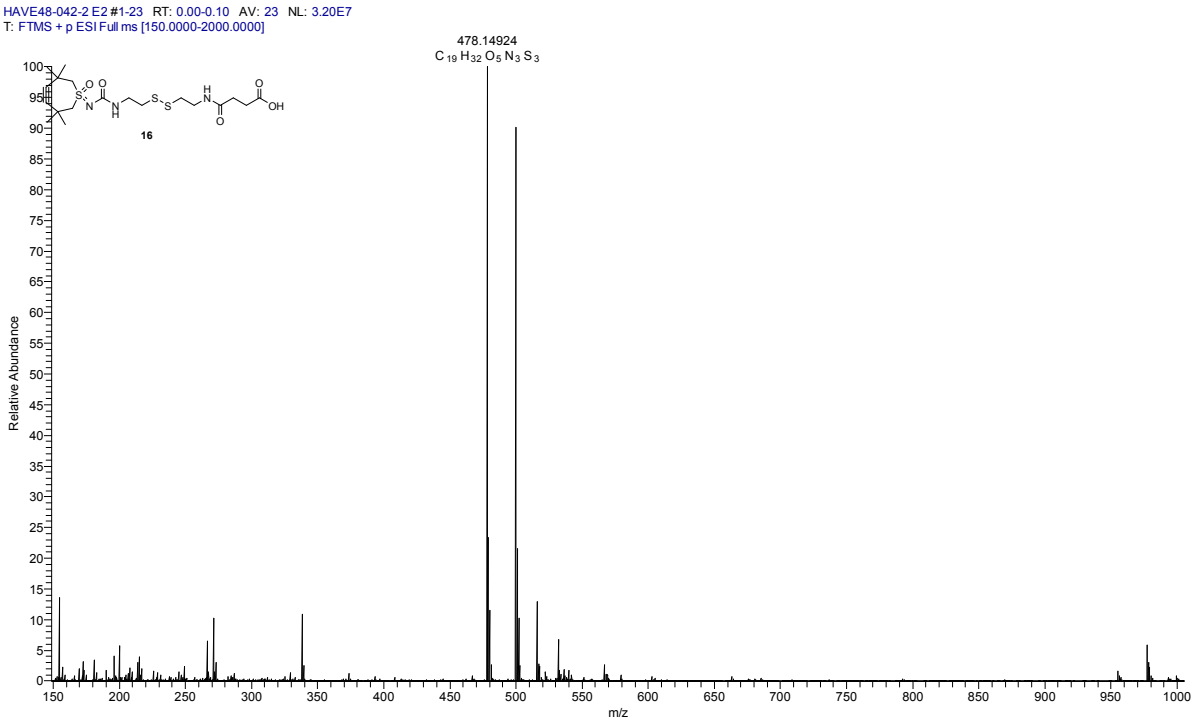
rt (min)	height	area	area (%)
1.72	341.2	5849	99.23
1.87	2.970	45.52	0.77





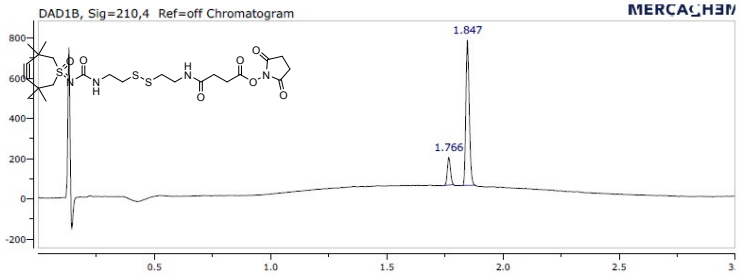

MERCACHEM
Title HAVE48-042-2
Data File Name NMR-RUN_0926_260_36275
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent DMSO
Acquisition Date 2019-09-26T21:40:00
Temperature ~ 296.4869 K
Number of Scans 16
Frequency 400.132470966543 MHz
Nucleus 1H
16
(ppm)


MERCACHEM
Title HAVE48-042-2
Data File Name NMR-RUN_0927_261_36276
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent DMSO
Acquisition Date 2019-09-27T01:36:00
Temperature ~ 296.4869 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
16
(ppm)


HAVE48-042-2 E2 #1-23 RT: 0.00-0.10 AV: 23 NL: 3.20E7
T: FTMS + p ESI Full ms [150.0000-2000.0000]
478.14924
C 19 H 32 O 5 N 3 S 3
16
Relative Abundance
m/z

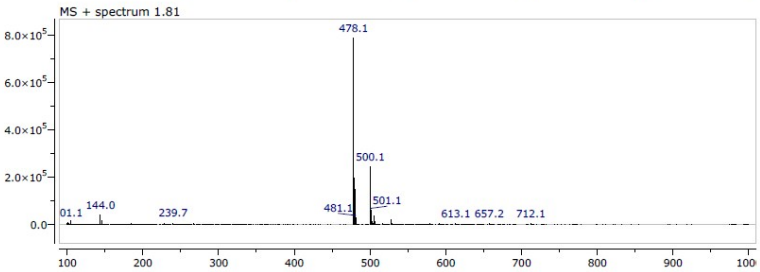
Analyses for OSu-ester 17

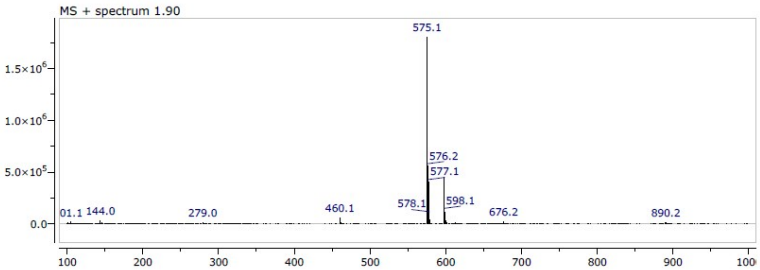
title	**HAVE48-052-1**
Method	SC_ACID.M
Date acquired	10-Oct-19, 11:36:16
FileName	Analysis\LCMS12_1010_042.D
Column	Waters XSelect CSH C18 (30x2.1mm, 3.5µ)
Flow	1 ml/min Column temp: 40°C
Eluent A	0.1% Formic acid in acetonitrile
Eluent B	0.1% Formic acid in water
Lin. Gradient	t=0min 5% A, t=1.6min 98% A, t=3min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range: 100-1000
Detection	ELSD (Alltech 3300): gasflow 1.5 ml/min, gas temp: 40°C



Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

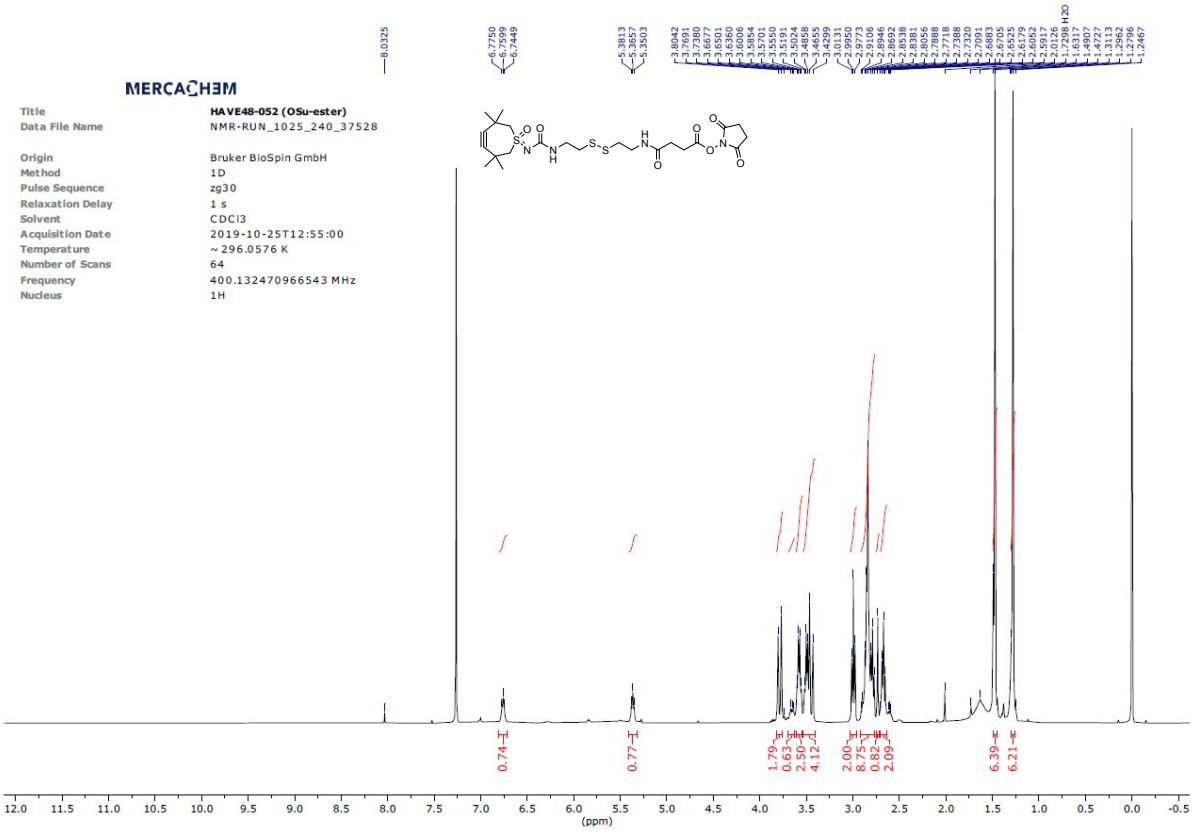
rt (min)	height	area	area (%)
1.77	139.4	2582	14.90
1.85	722.1	14747	85.10

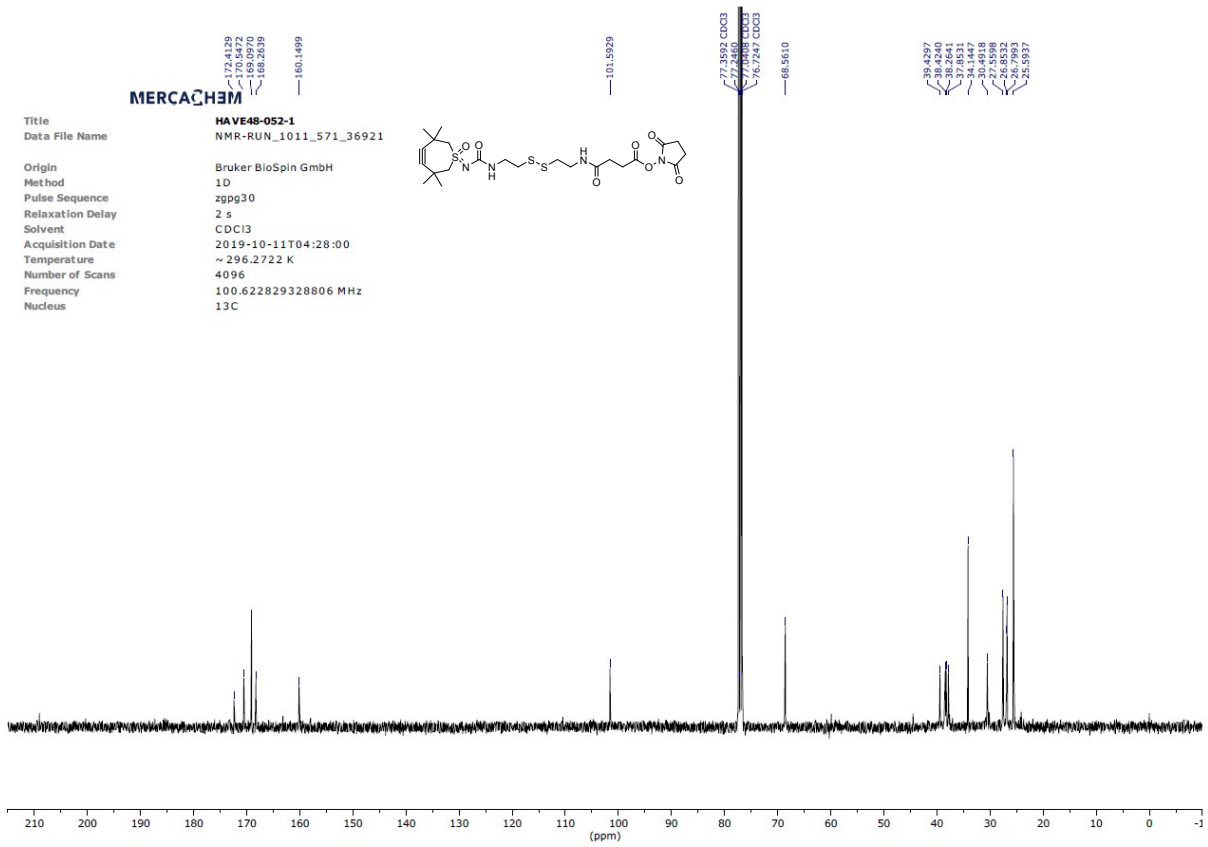




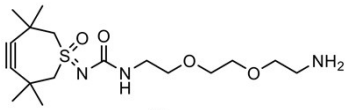
MERCACHEM

Title	**HAVE48-052 (OSu-ester)**
Data File Name	NMR-RUN_1025_240_37528
Origin	Bruker BioSpin GmbH
Method	1D
Pulse Sequence	zg30
Relaxation Delay	1 s
Solvent	CDCl3
Acquisition Date	2019-10-25T12:55:00
Temperature	~ 296.0576 K
Number of Scans	64
Frequency	400.132470966543 MHz
Nucleus	1H



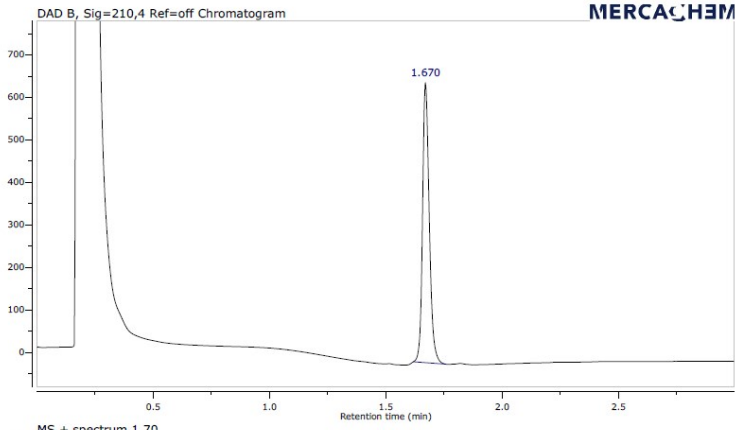

MERCACHEM
Title HAVE48-052-1
Data File Name NMR-RUN_1011_571_36921
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-10-11T04:28:00
Temperature ~ 296.2722 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
172.4129
170.5472
168.8970
168.2639
160.1499
101.5929
77.3592 CDCl3
77.2460
77.0308 CDCl3
76.7247 CDCl3
68.5610
39.4297
38.3240
38.1641
37.8531
34.1447
30.4918
27.1598
26.8532
26.7993
25.5937
(ppm)

Analyses for compound 18



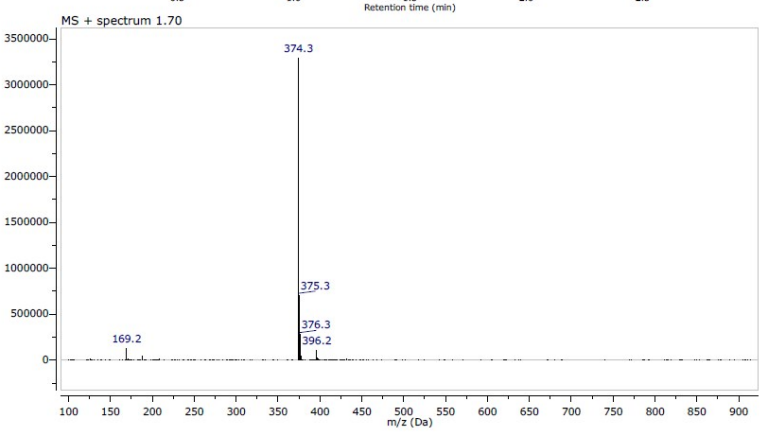
18

title	**robe28-44-1**
Method	SC_BASE.M
Date acquired	18-Jan-19, 13:59:14
FileName	Analysis\LCMS5_0118_090.D
Acq. method	SC_BASE valve: 6
Column	Waters XSelect CSH C18 (30x2.1mm, 3.5µ)
Flow	1 ml/min; Column temp: 25°C
Eluent A	100% acetonitrile
Eluent B	10mM ammoniumbicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range: 100-1000



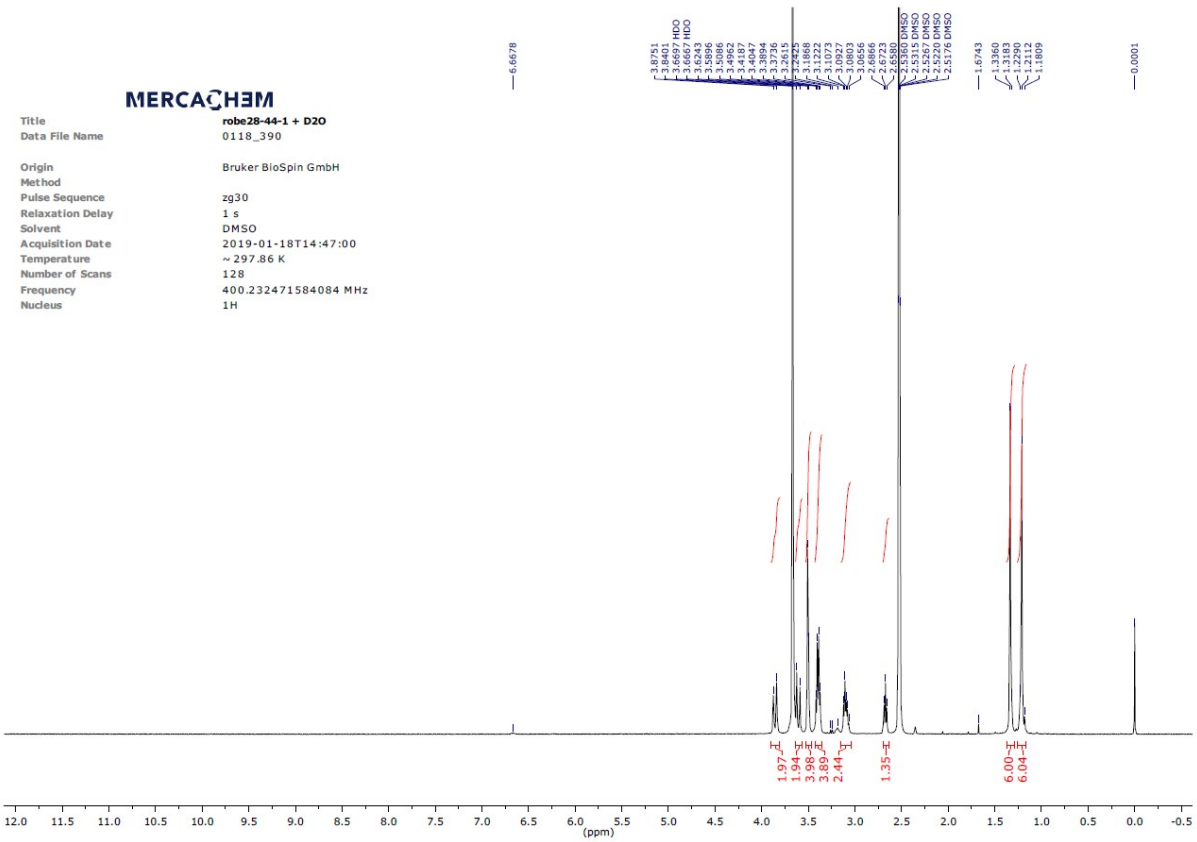
Integrals spectrum Chromatogram DAD B, Sig=210,4 Ref=off

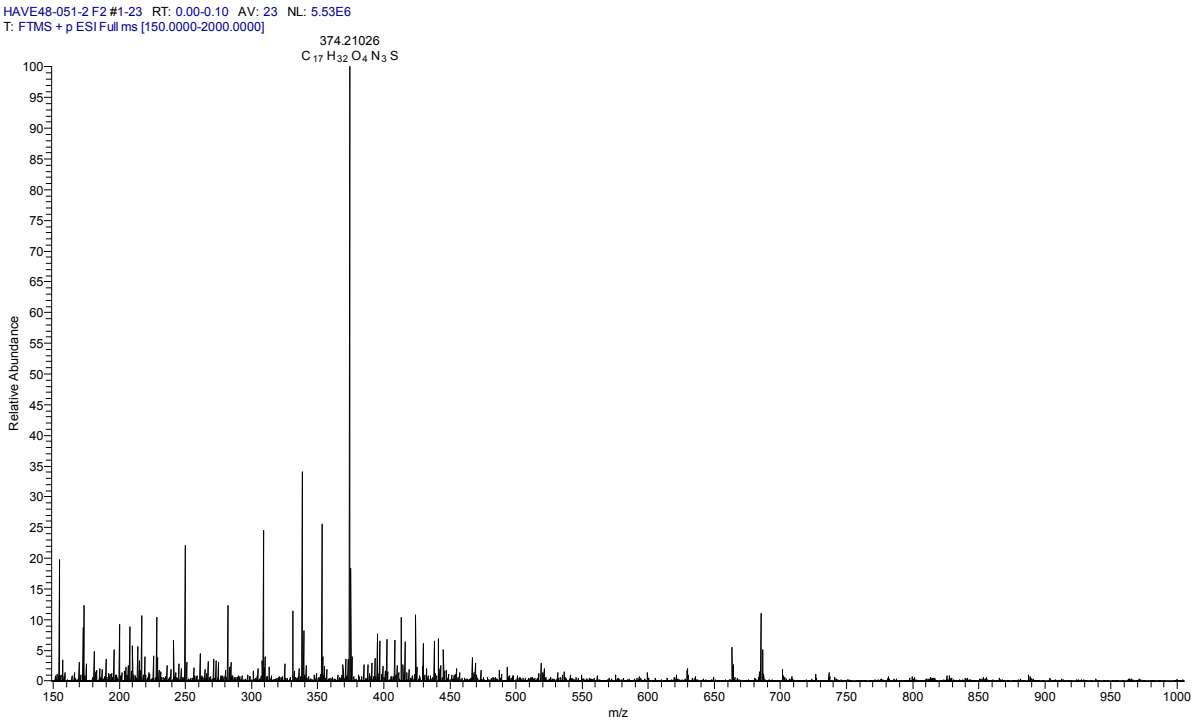
rt (min)	height	area	area (%)
1.67	659.0	22.02	100.00



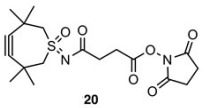
MERCACHEM

Title	**robe28-44-1 + D2O**
Data File Name	0118_390
Origin	Bruker BioSpin GmbH
Method	
Pulse Sequence	zg30
Relaxation Delay	1 s
Solvent	DMSO
Acquisition Date	2019-01-18T14:47:00
Temperature	~297.86 K
Number of Scans	128
Frequency	400.232471584084 MHz
Nucleus	1H



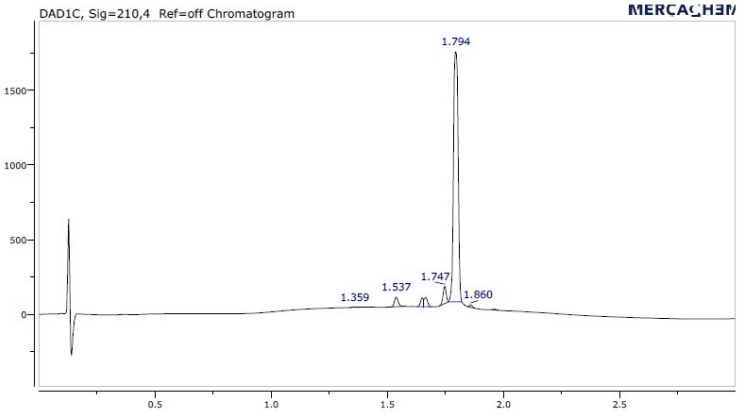


Analyses for compound 20



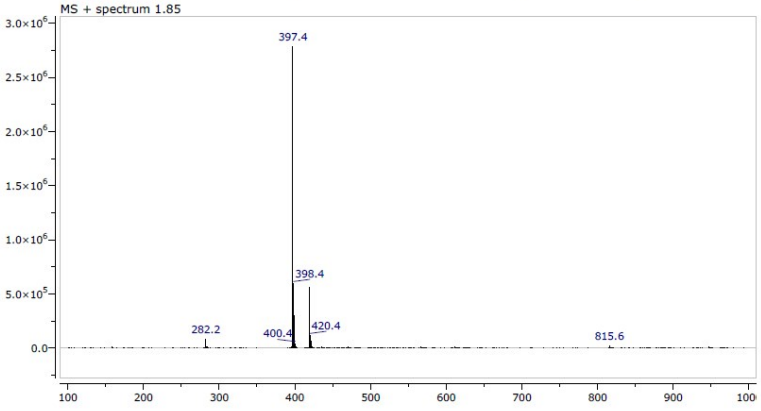
20

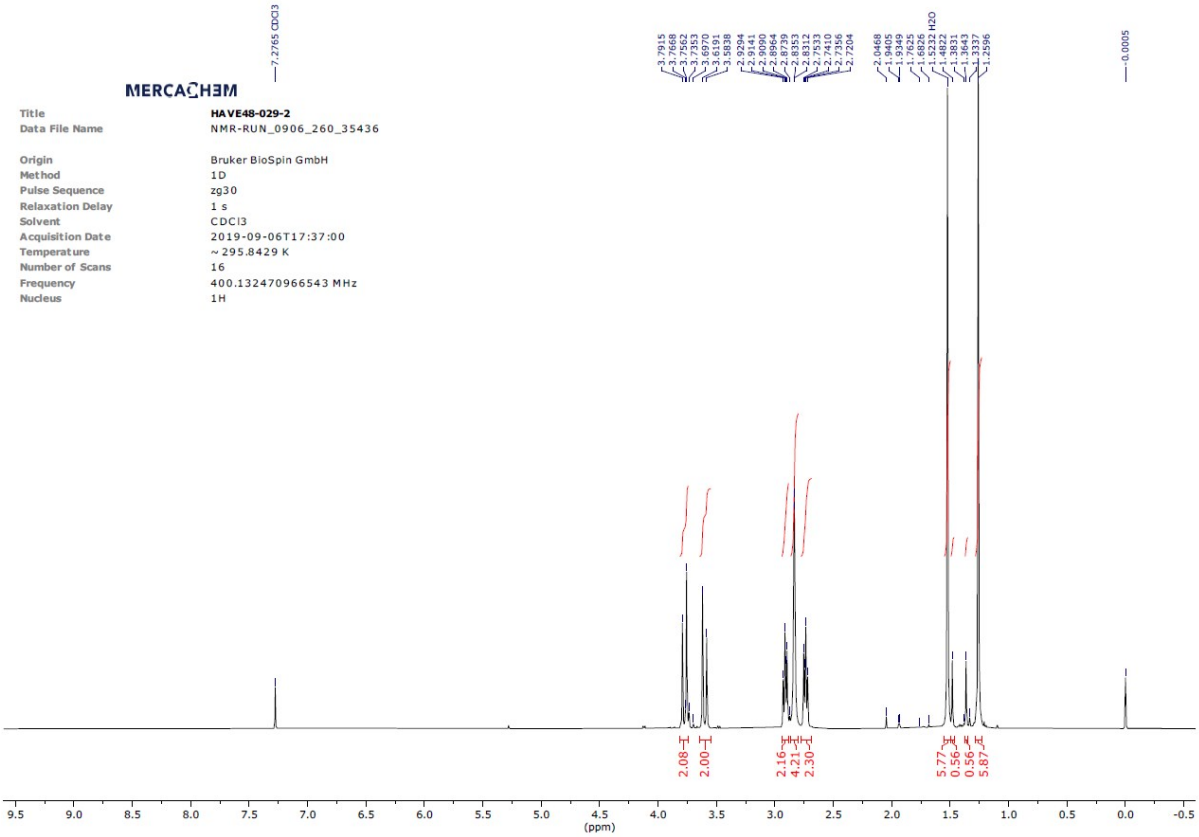
title	**HAVE48-029-2**
Method	SC_ACID.M
Date acquired	08-Oct-19, 09:45:50
FileName	Analysis\LCMS22_1008_028.D
Acq.method	SC_ACID
Column	XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min, Column temp.: 40°C
Eluent A	0.1% Formic acid in Acetonitrile
Eluent B	0.1% Formic acid in Water
Lin. gradient	t=0 min 5%A, t=1.6 min 98%A, t=3 min 98% A
Postrun	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range 100-1000
Detection	ELSD (Neb temp. 40°C, gassflow 1.5 ml/min)

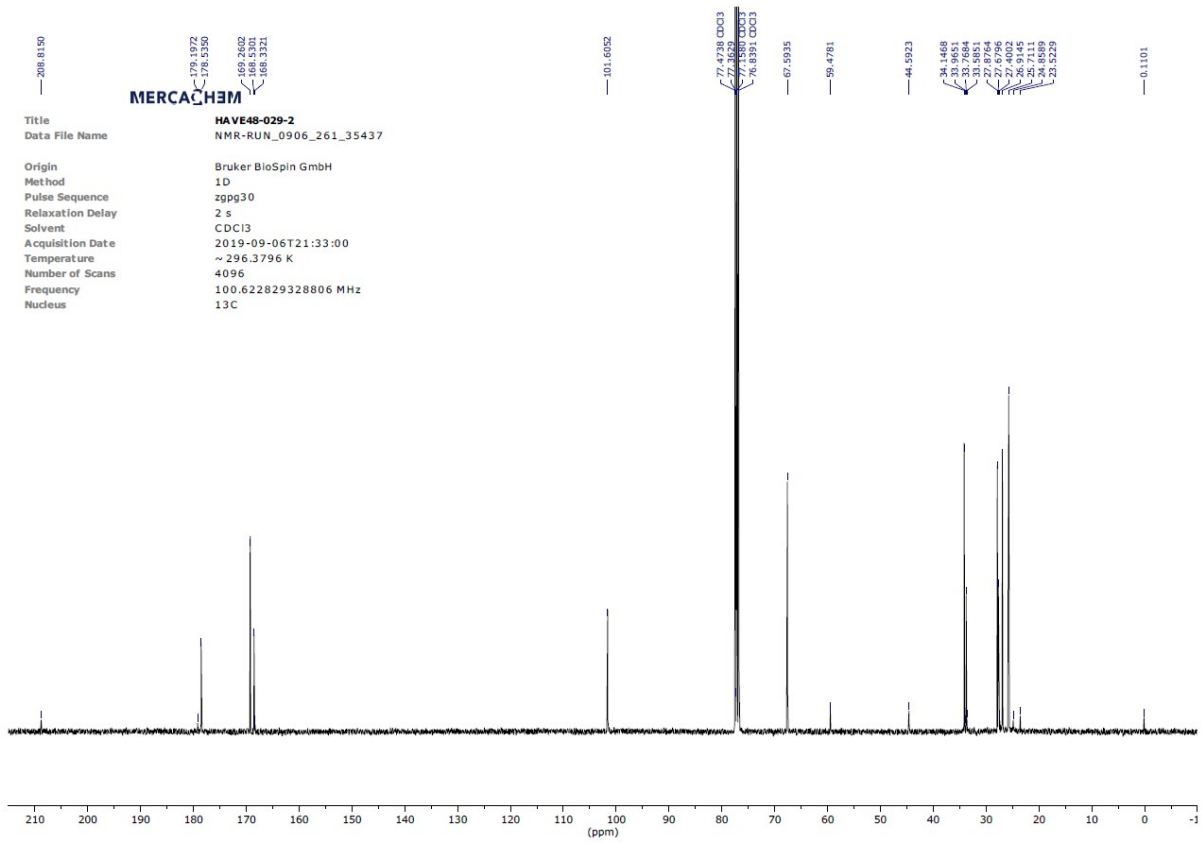


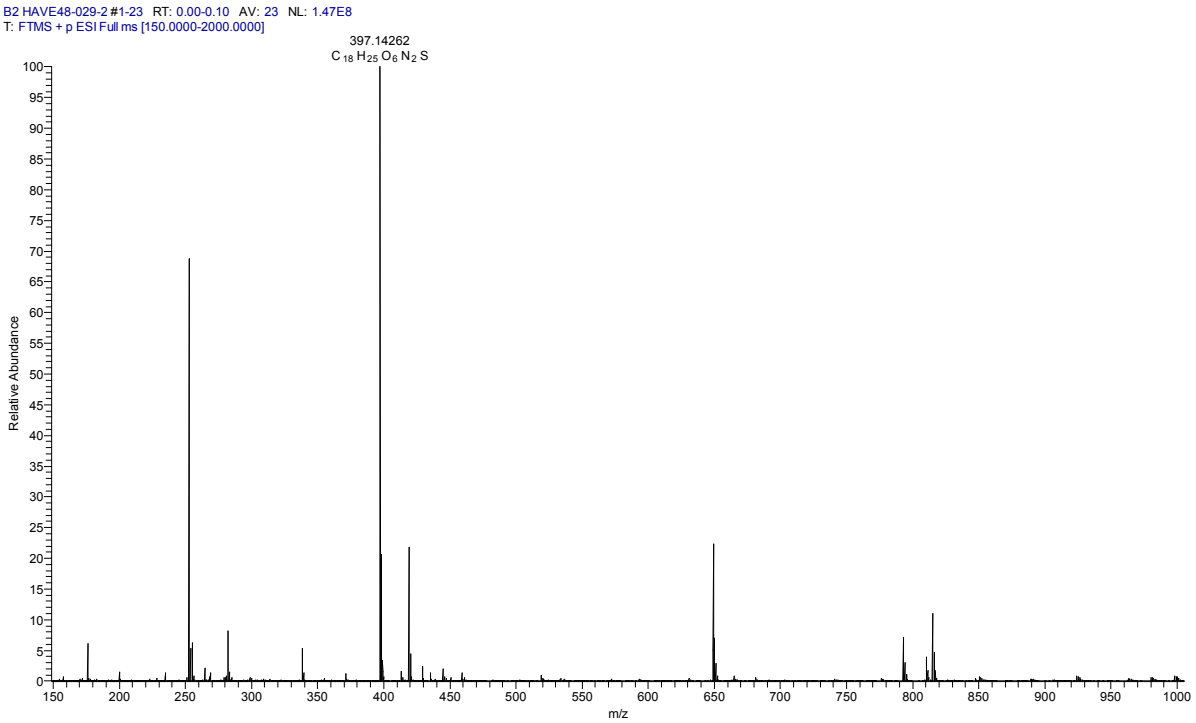
Integrals spectrum Chromatogram DAD1C, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
1.36	3.747	86.65	0.16
1.54	63.99	1415	2.69
1.65	59.52	1071	2.04
1.67	62.26	1200	2.28
1.75	115.9	2130	4.05
1.79	1672	46286	88.07
1.86	17.03	278.9	0.53
1.96	5.513	90.52	0.17




MERCACHEM
Title HAVE48-029-2
Data File Name NMR-RUN_0906_260_35436
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent CDCl3
Acquisition Date 2019-09-06T17:37:00
Temperature ~ 295.8429 K
Number of Scans 16
Frequency 400.132470966543 MHz
Nucleus 1H
7.2765 CDCl3
3.7915
3.7668
3.7562
3.7353
3.6970
3.6191
3.5838
2.9294
2.9141
2.9000
2.8964
2.8739
2.8553
2.8312
2.7533
2.7410
2.7356
2.7204
2.0468
1.9465
1.9349
1.7625
1.6826 H2O
1.4822
1.3831
1.3643
1.3337
1.2996
0.0005
2.08
2.00
2.16
4.21
2.30
5.77
0.56
0.56
5.87
(ppm)

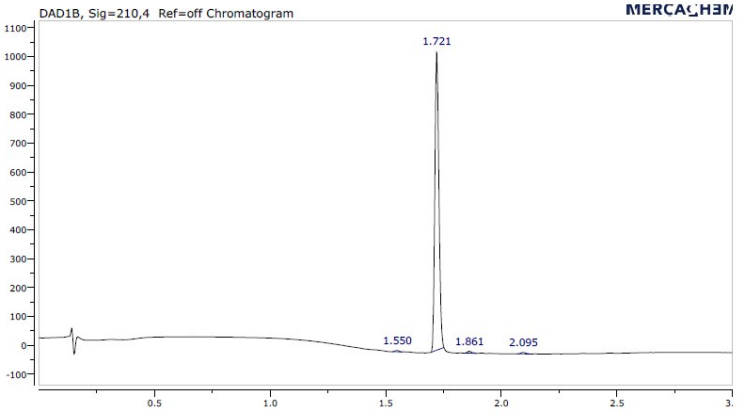

MERCACHEM
Title HAVE48-029-2
Data File Name NMR-RUN_0906_261_35437
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-09-06T21:33:00
Temperature ~ 296.3796 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
208.8150
179.1972
178.5350
169.2602
168.5301
168.3321
101.6052
77.4738 CDCl3
77.3629
77.1580 CDCl3
76.8391 CDCl3
67.5935
59.4781
44.5923
34.1468
33.9651
33.7684
33.5851
27.7264
27.6796
27.4002
26.9145
26.4711
24.6588
23.5229
0.1101
(ppm)


B2 HAVE48-029-2 #1-23 RT: 0.00-0.10 AV: 23 NL: 1.47E8
T: FTMS + p ESI Full ms [150.0000-2000.0000]
397.14262
C 18 H 25 O 6 N 2 S
Relative Abundance
m/z

Analyses for compound 21

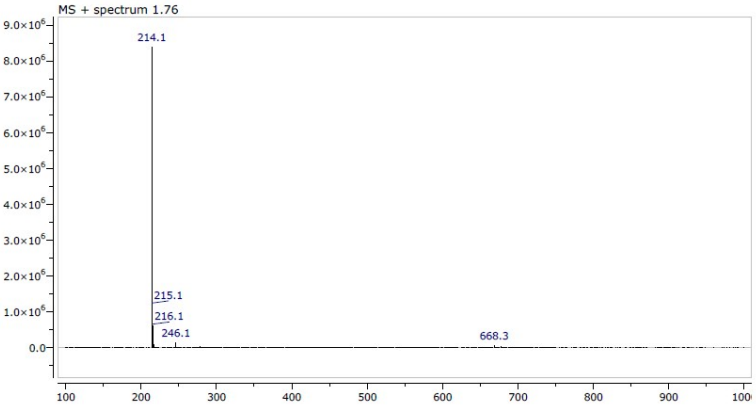
21

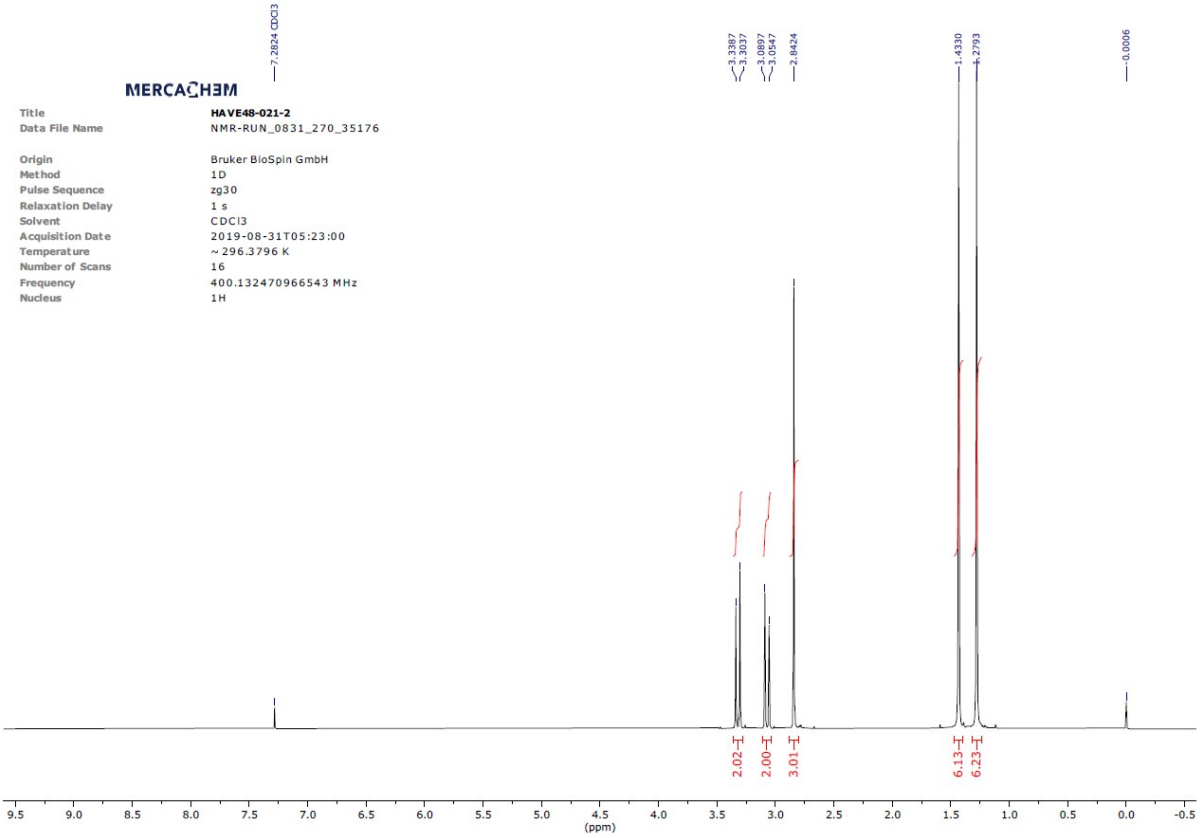
title	**HAVE48-021-2**
Method	SC_BASE.M
Date acquired	08-Oct-19, 10:14:09
FileName	Analysis\LCMS19_1008_031.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)



Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

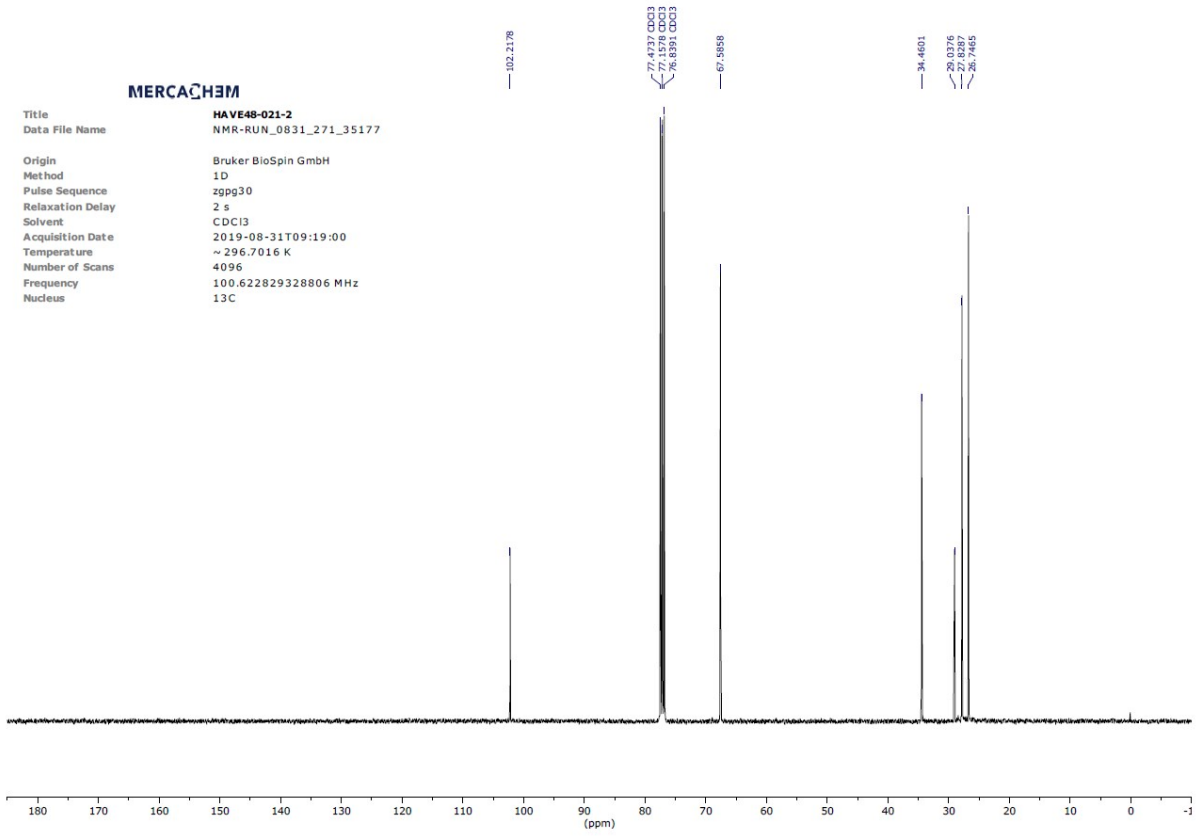
rt (min)	height	area	area (%)
1.55	4.822	114.9	0.45
1.72	1034	25053	98.24
1.86	7.117	177.9	0.70
2.09	5.411	156.6	0.61

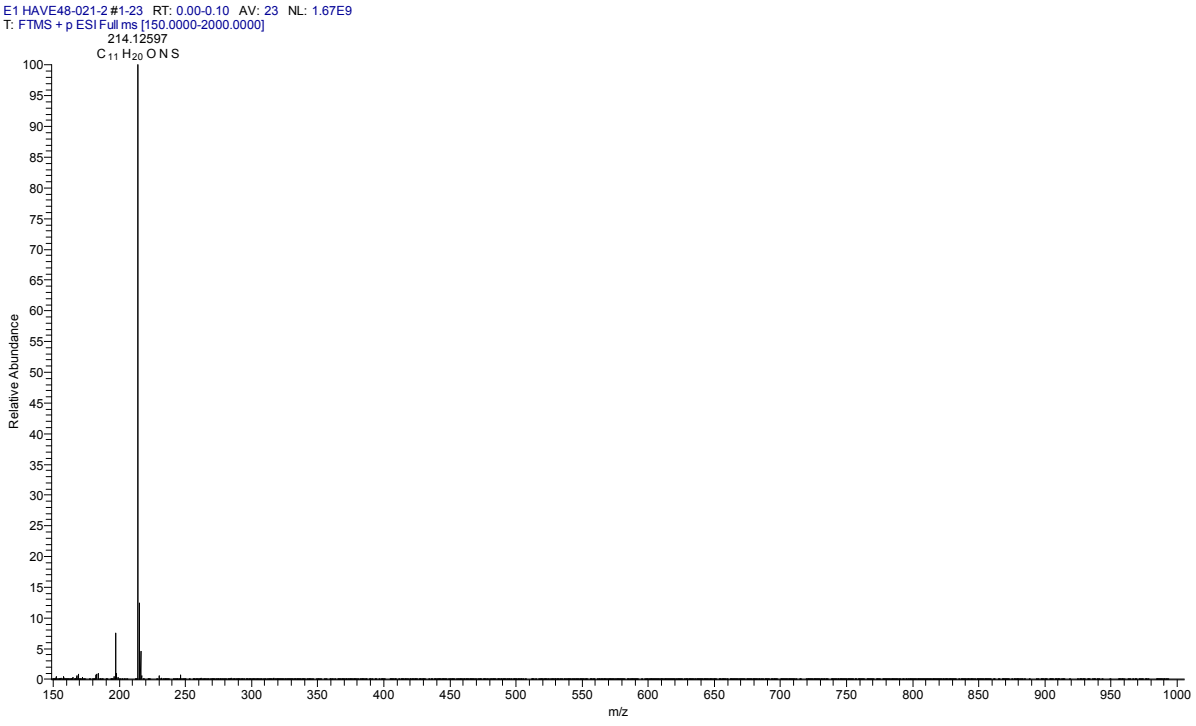




MERCACHEM

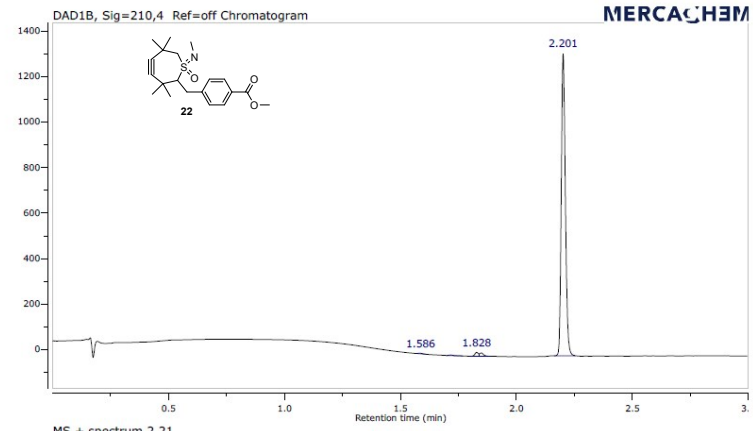
Title	**HAVE48-021-2**
Data File Name	NMR-RUN_0831_270_35176
Origin	Bruker BioSpin GmbH
Method	1D
Pulse Sequence	zg30
Relaxation Delay	1 s
Solvent	CDCl3
Acquisition Date	2019-08-31T05:23:00
Temperature	~296.3796 K
Number of Scans	16
Frequency	400.132470966543 MHz
Nucleus	1H


MERCACHEM
Title HAVE48-021-2
Data File Name NMR-RUN_0831_271_35177
Origin Bruker BioSpin GmbH
Method 1D
Pulse Sequence zgpg30
Relaxation Delay 2 s
Solvent CDCl3
Acquisition Date 2019-08-31T09:19:00
Temperature ~296.7016 K
Number of Scans 4096
Frequency 100.622829328806 MHz
Nucleus 13C
102.2178
77.4737 CDCl3
77.1579 CDCl3
76.8391 CDCl3
67.5858
34.4601
29.0376
27.8287
26.7465
(ppm)


E1 HAVE48-021-2 #1-23 RT: 0.00-0.10 AV: 23 NL: 1.67E9
T: FTMS + p ESI Full ms [150.0000-2000.0000]
214.12597
C11 H20 O N S
Relative Abundance
m/z

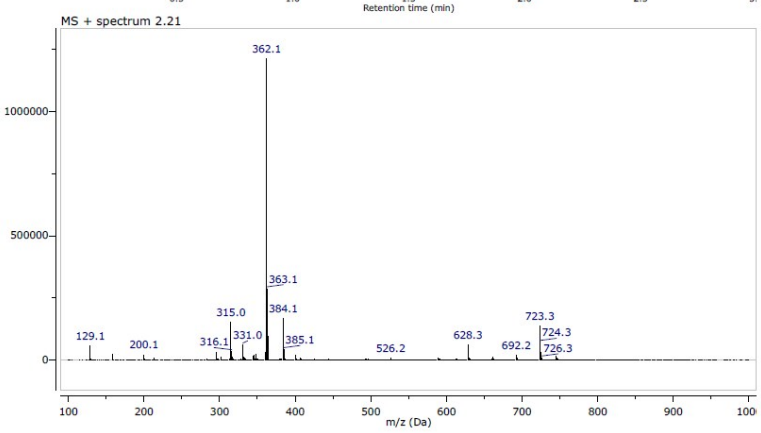
Analyses for compound 22

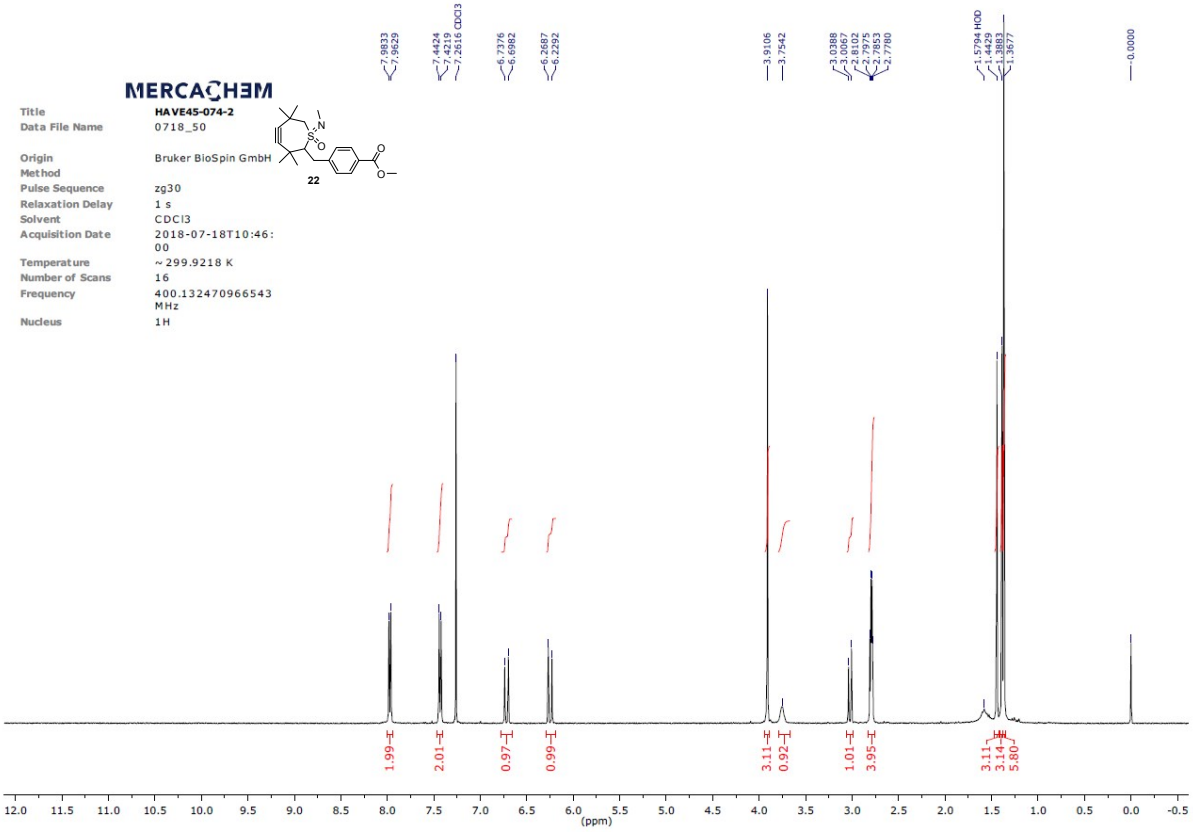
title	**HAVE45-074-2**
Method	SC_BASE.M
Date acquired	18-Jul-18, 09:51:55
FileName	Analysis\LCMS19_0718_027.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)



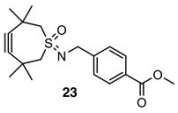
Integrals spectrum — Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
1.59	2.328	0.05112	0.18
1.72	2.797	0.06835	0.25
1.83	17.10	0.2916	1.05
1.85	14.34	0.2716	0.98
2.20	1328	27.00	97.53



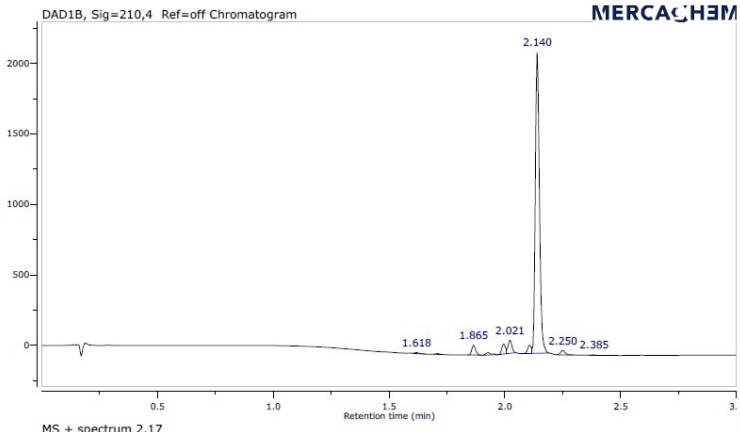


Analyses for compound 23



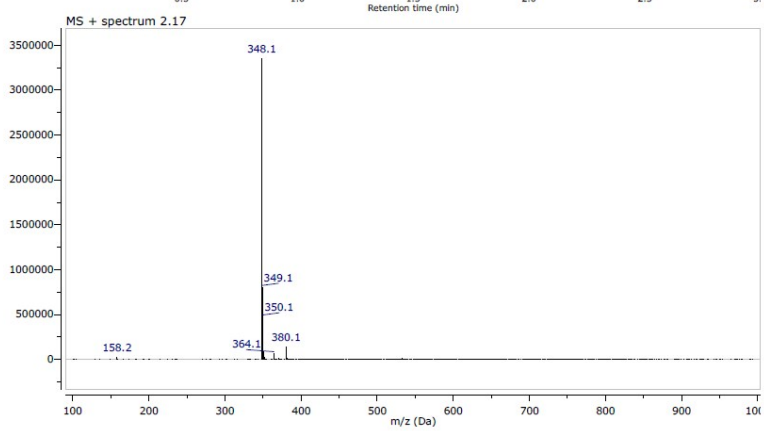
23

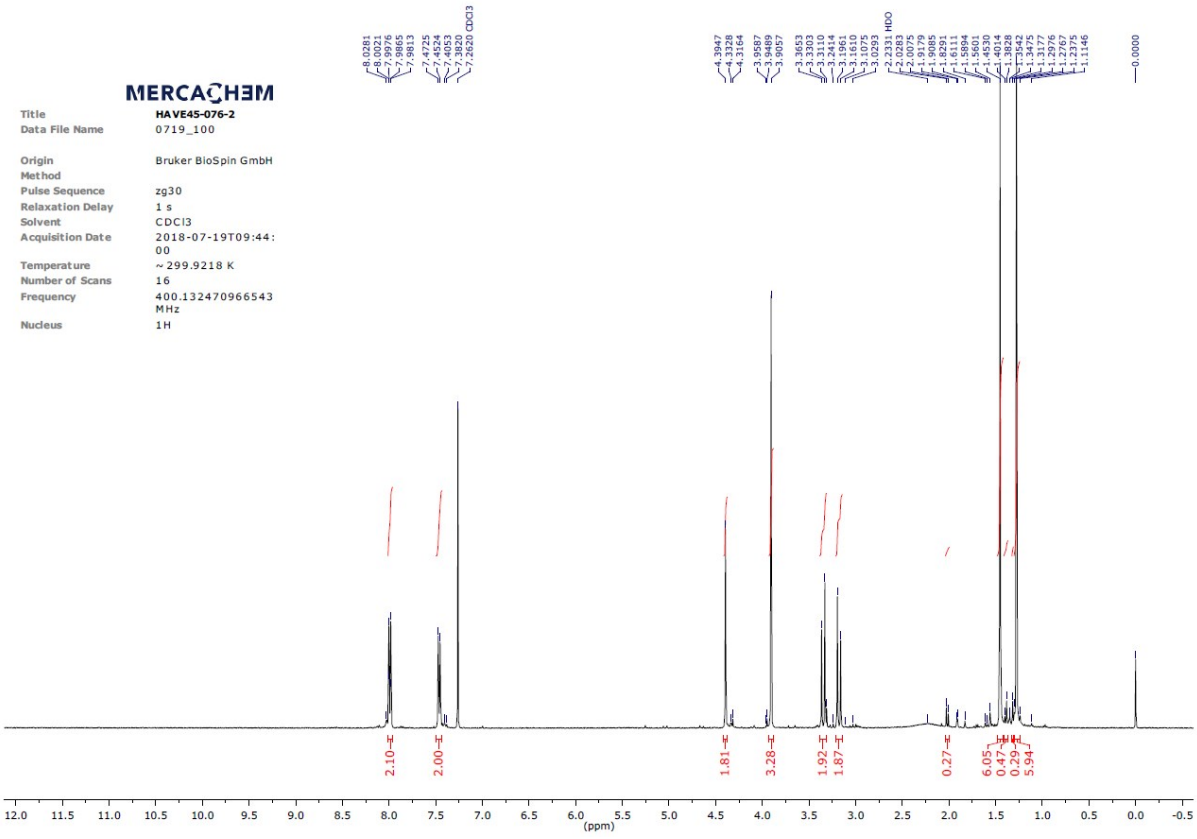
title	**HAVE45-076-2**
Method	SC_BASE.M
Date acquired	19-Jul-18, 09:27:59
FileName	Analysis\LCMS19_0719_009.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)



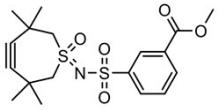
Integrals spectrum — Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
1.62	6.968	0.1380	0.27
1.71	5.519	0.1010	0.20
1.86	69.62	1.331	2.61
1.93	16.66	0.4714	0.93
1.99	70.85	1.332	2.62
2.02	92.75	1.767	3.47
2.11	59.48	1.088	2.14
2.14	2133	43.95	86.28
2.25	31.13	0.6947	1.36
2.38	3.458	0.06389	0.13



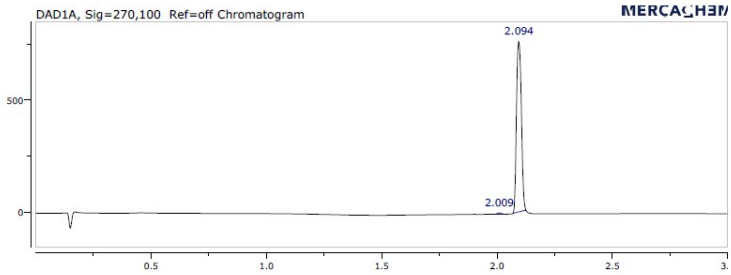


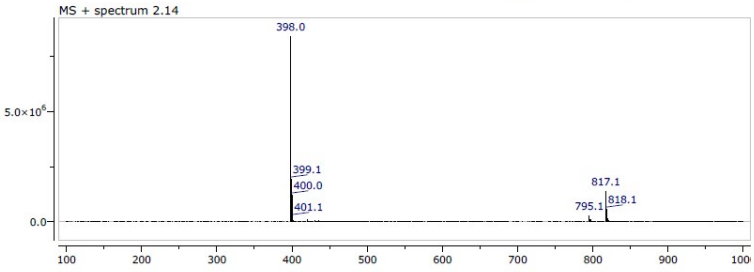
Analyses for compound 24

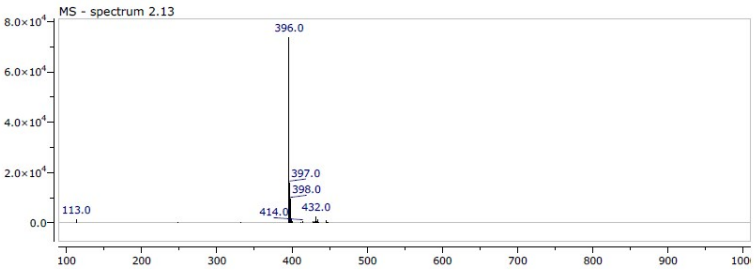


: **24**

title	**HAVE48-020-2**
Method	SC_BASE.M
Date acquired	08-Oct-19, 10:09:22
FileName	Analysis\LCMS19_1008_030.D
Column	Waters XSelect CSH C18 (30x2.1mm 3.5µ)
Flow	1 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 100-1000)

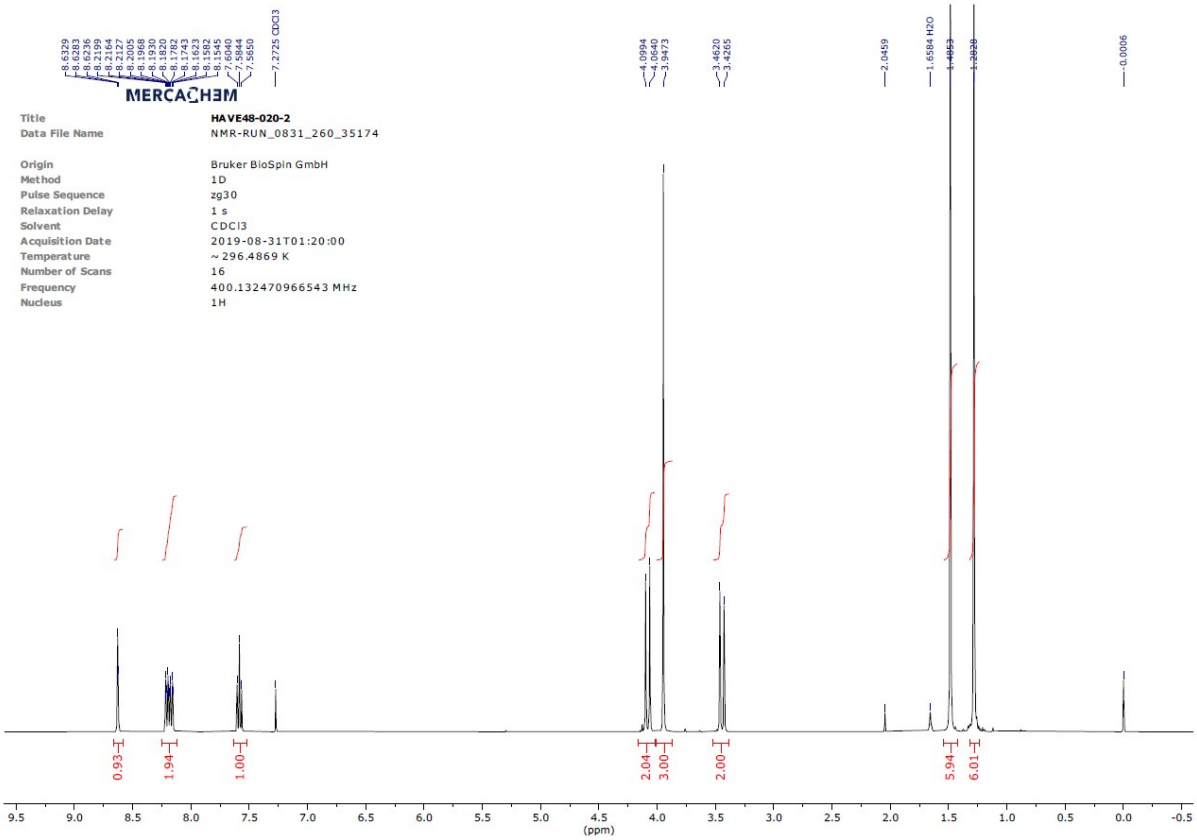




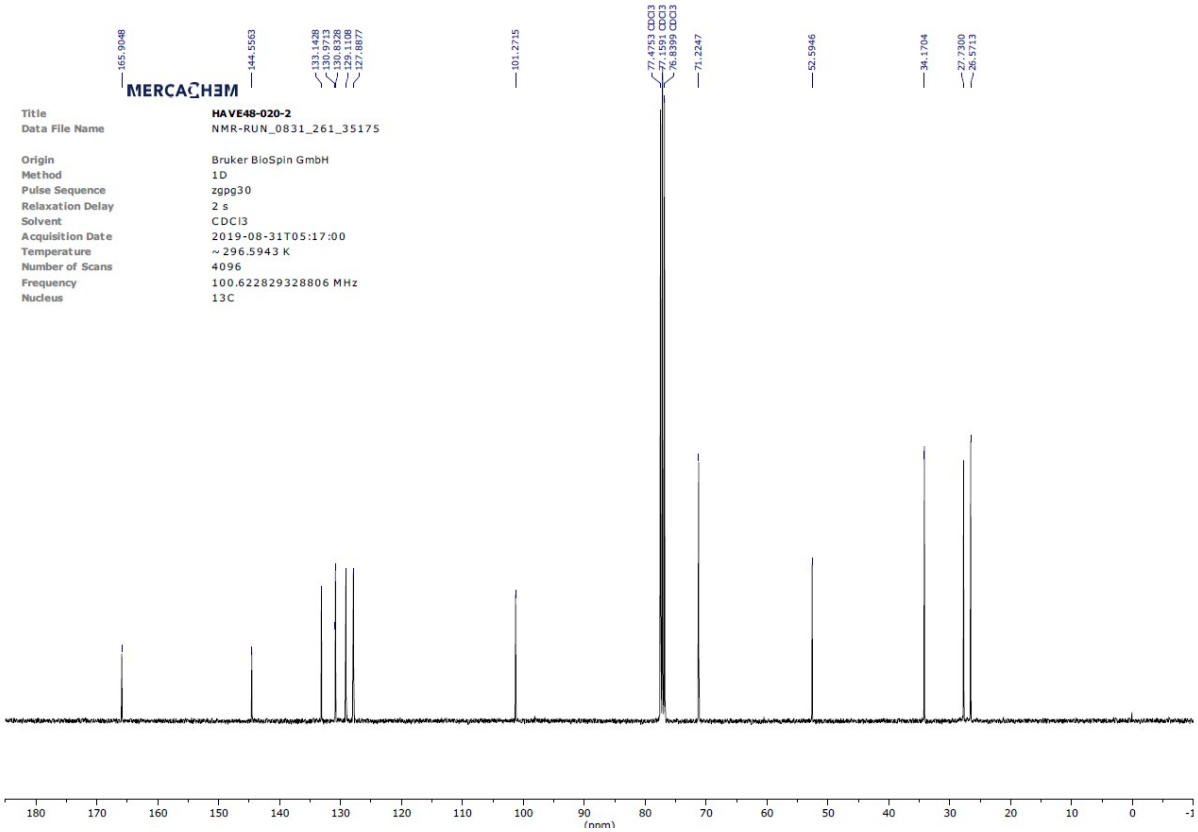


Integrals spectrum Chromatogram DAD1A, Sig=270,100 Ref=off

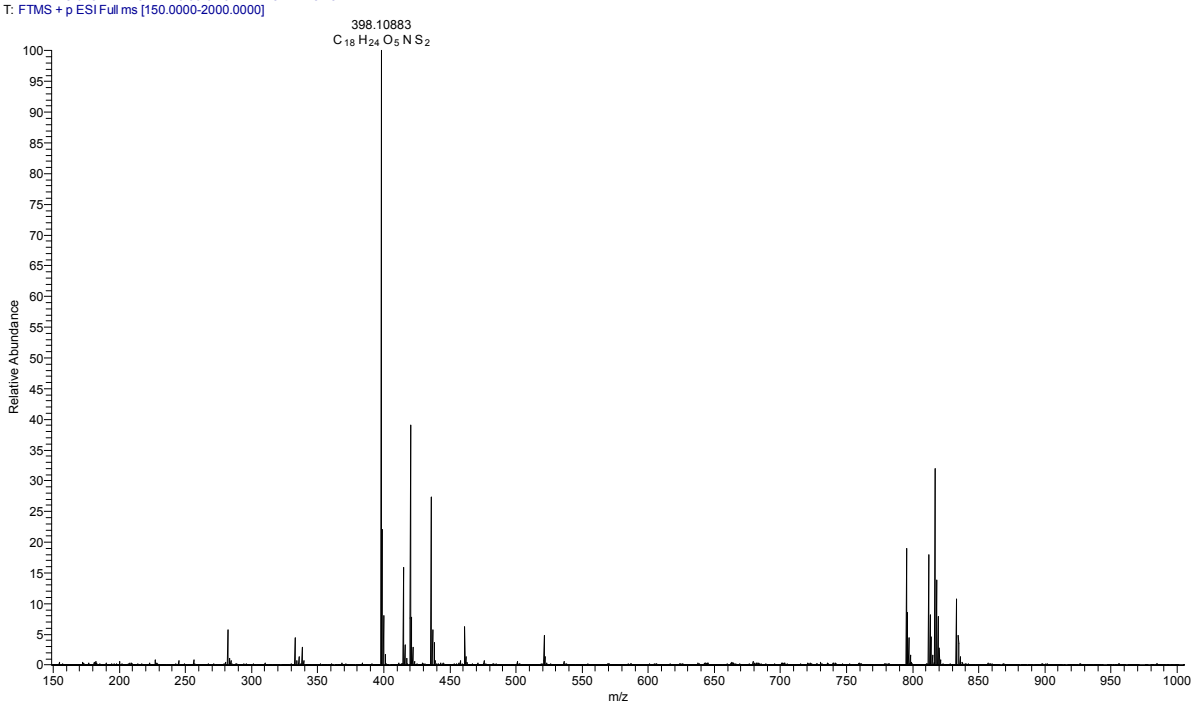
rt (min)	height	area	area (%)
2.01	6.324	150.4	0.67
2.09	764.5	22372	99.33



Title	**HAVE48-020-2**
Data File Name	NMR-RUN_0831_260_35174
Origin	Bruker BioSpin GmbH
Method	1D
Pulse Sequence	zg30
Relaxation Delay	1 s
Solvent	CDCl3
Acquisition Date	2019-08-31T01:20:00
Temperature	~296.4869 K
Number of Scans	16
Frequency	400.132470966543 MHz
Nucleus	1H

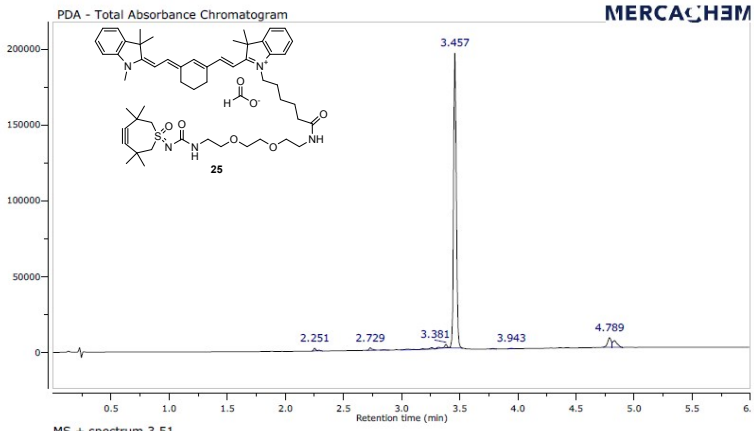


D1 HAVE48-020-2 #1-23 RT: 0.00-0.10 AV: 23 NL: 3.73E7
T: FTMS + p ESI Full ms [150.0000-2000.0000]



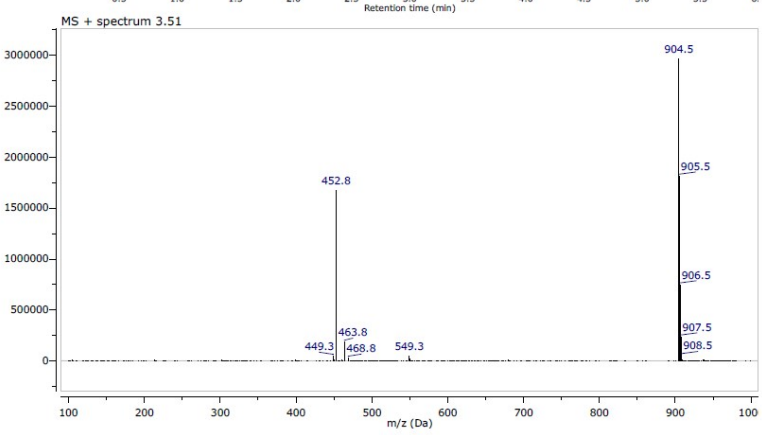
Analyses for compound 25

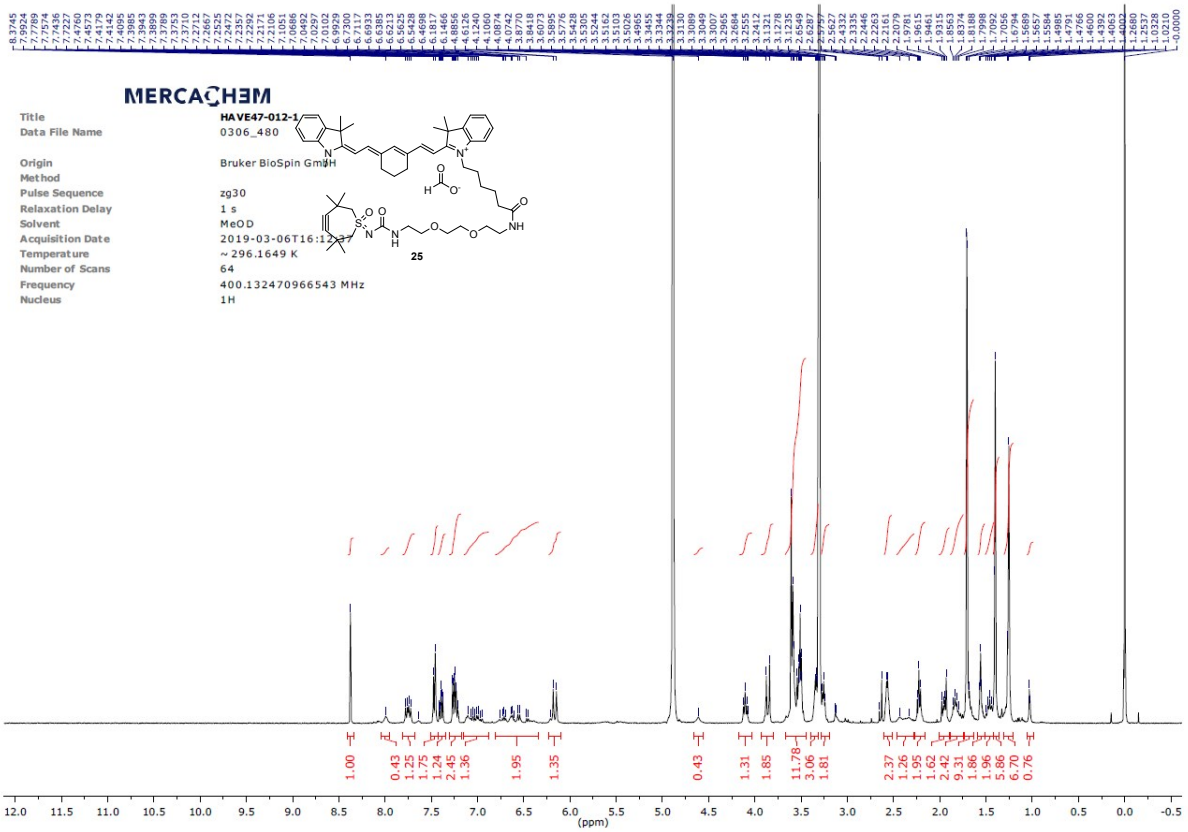
title	**HAVE47-012-1**
Method	AN_ACID_DAY_UV800.M
Date acquired	05-Mar-19, 13:35:46
FileName	Analysis\LCMS12_0305_047.D
Column	Waters XSelect CSH C18 (50x2.1mm, 3.5µ)
Flow	0.8 ml/min Column temp: 35°C
Eluent A	0.1% Formic acid in acetonitrile
Eluent B	0.1% Formic acid in water
Lin. Gradient	t=0min 5% A, t=3.5min 98% A, t=6min 98% A
Posttime	2 min
Detection	DAD (220-320 nm, 210 and 220 nm)
Detection	PDA (210-800 nm)
Detection	MSD (ESI pos/neg) mass range: 100-1000
Detection	ELSD (Alltech 3300): gasflow 1.5 ml/min, gas temp: 40°C

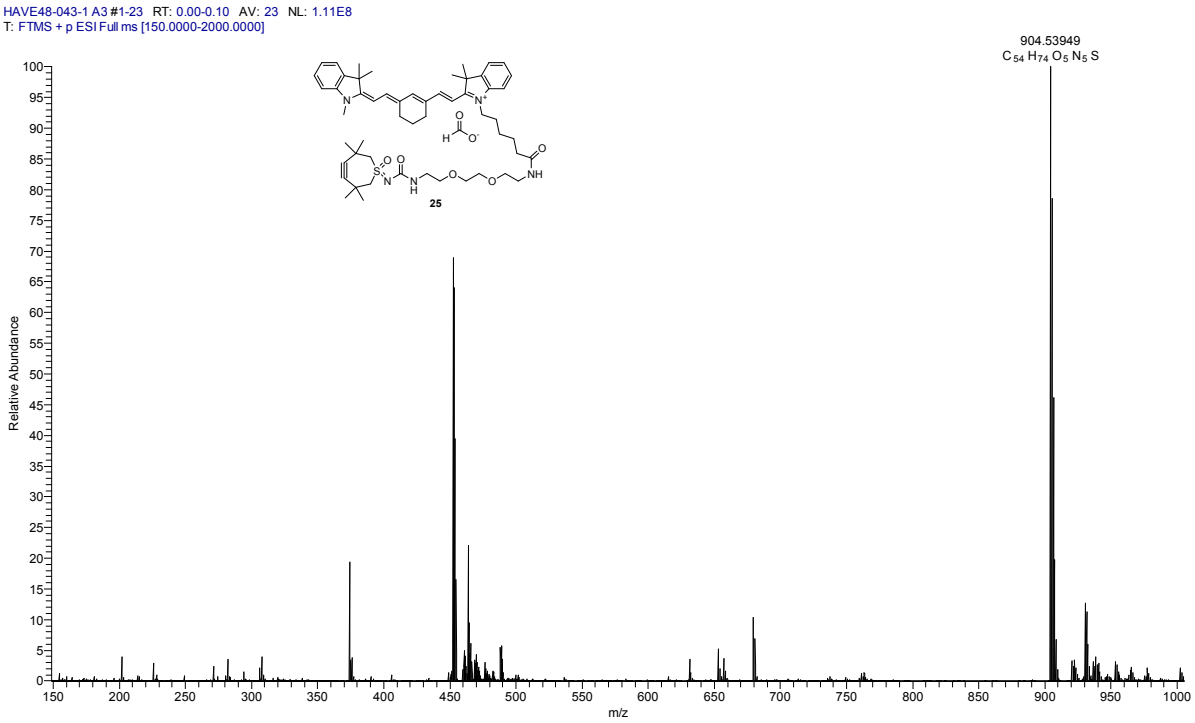


Integrals spectrum PDA - Total Absorbance Chromatogram

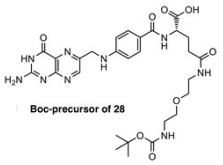
rt (min)	height	area	area (%)
2.25	1800	34.27	0.58
2.29	448.2	8.523	0.14
2.73	1778	48.34	0.82
2.84	281.7	9.385	0.16
3.05	477.4	25.15	0.42
3.18	427.3	13.28	0.22
3.26	910.7	28.51	0.48
3.32	740.2	22.61	0.38
3.38	2293	53.08	0.90
3.46	194356	5252	88.60
3.79	283.7	6.835	0.12
3.94	295.5	7.274	0.12
4.79	6343	229.4	3.87
4.83	4434	189.3	3.19






HAVE48-043-1 A3 #1-23 RT: 0.00-0.10 AV: 23 NL: 1.11E8
T: FTMS + p ESI Full ms [150.0000-2000.0000]
904.53949
C 54 H 74 O 5 N 5 S
25
Relative Abundance
m/z

Analyses for compound 29 and its synthetic intermediates

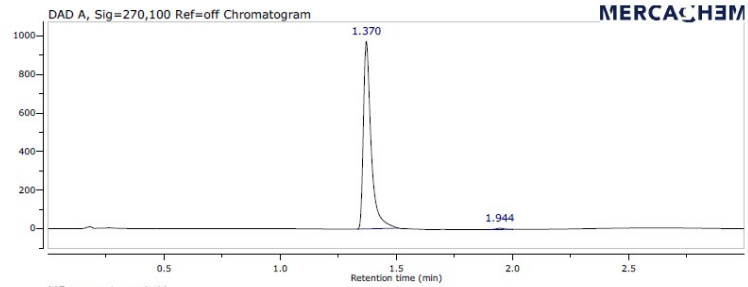


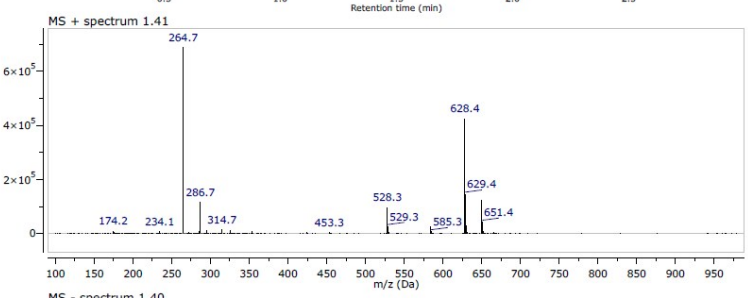
Boc-precursor of 28

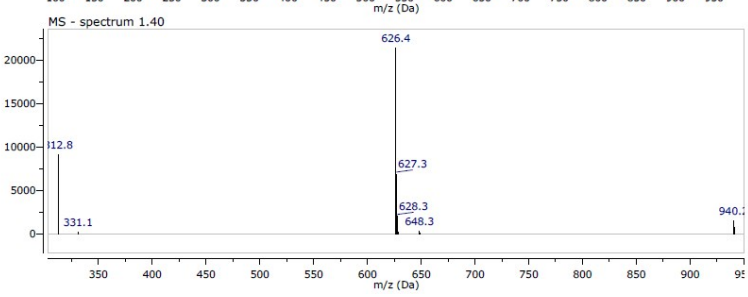
title	**ROBE28-31-3**
Method	SC_BASE.M
Date acquired	13-Dec-18, 16:43:05
FileName	Analysis\LCMS5_1213_101.D
Acq. method	SC_BASE valve: 6
Column	Waters XSelect CSH C18 (30x2.1mm, 3.5µ)
Flow	1 ml/min; Column temp: 25°C
Eluent A	100% acetonitrile
Eluent B	10mM ammoniumbicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range: 100-1000

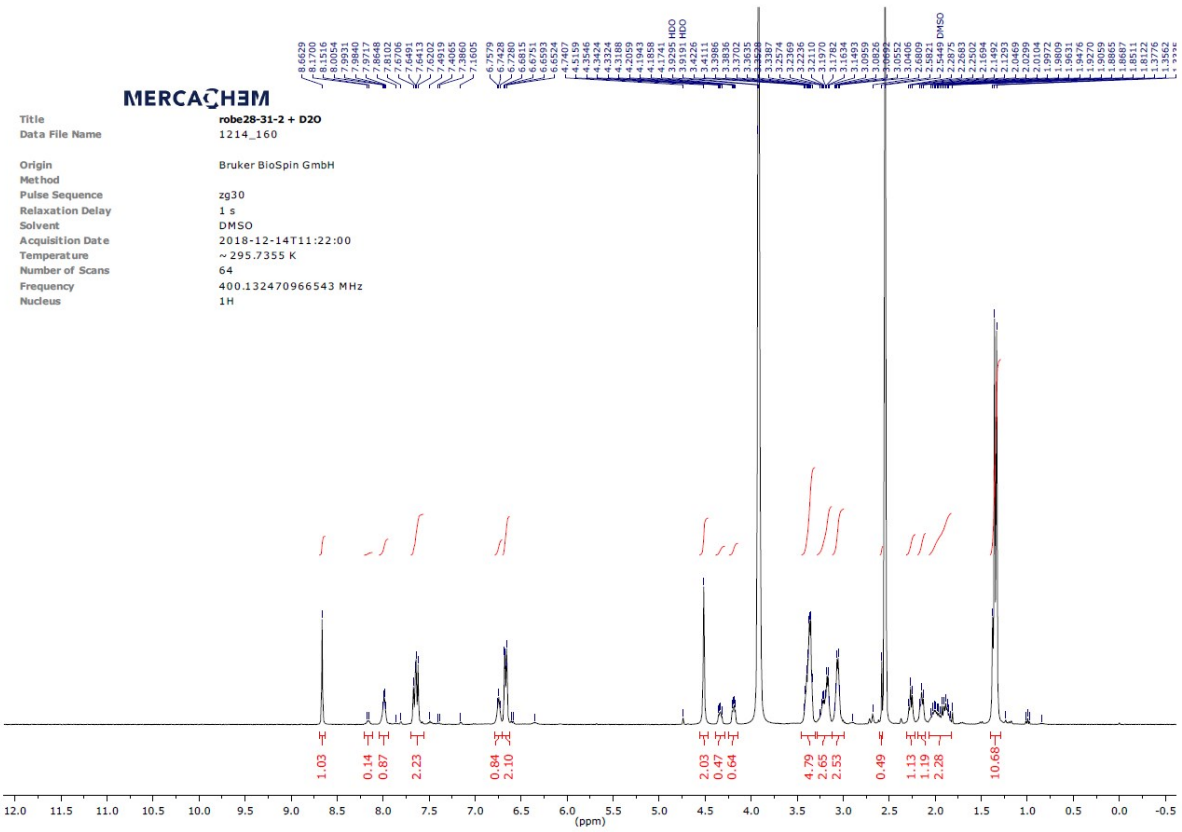
Integrals spectrum Chromatogram DAD A, Sig=270,100 Ref=off

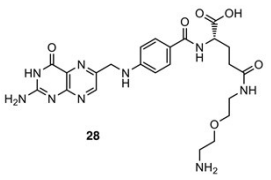
rt (min)	height	area	area (%)
1.37	973.5	37.57	99.34
1.94	6.414	0.2506	0.66

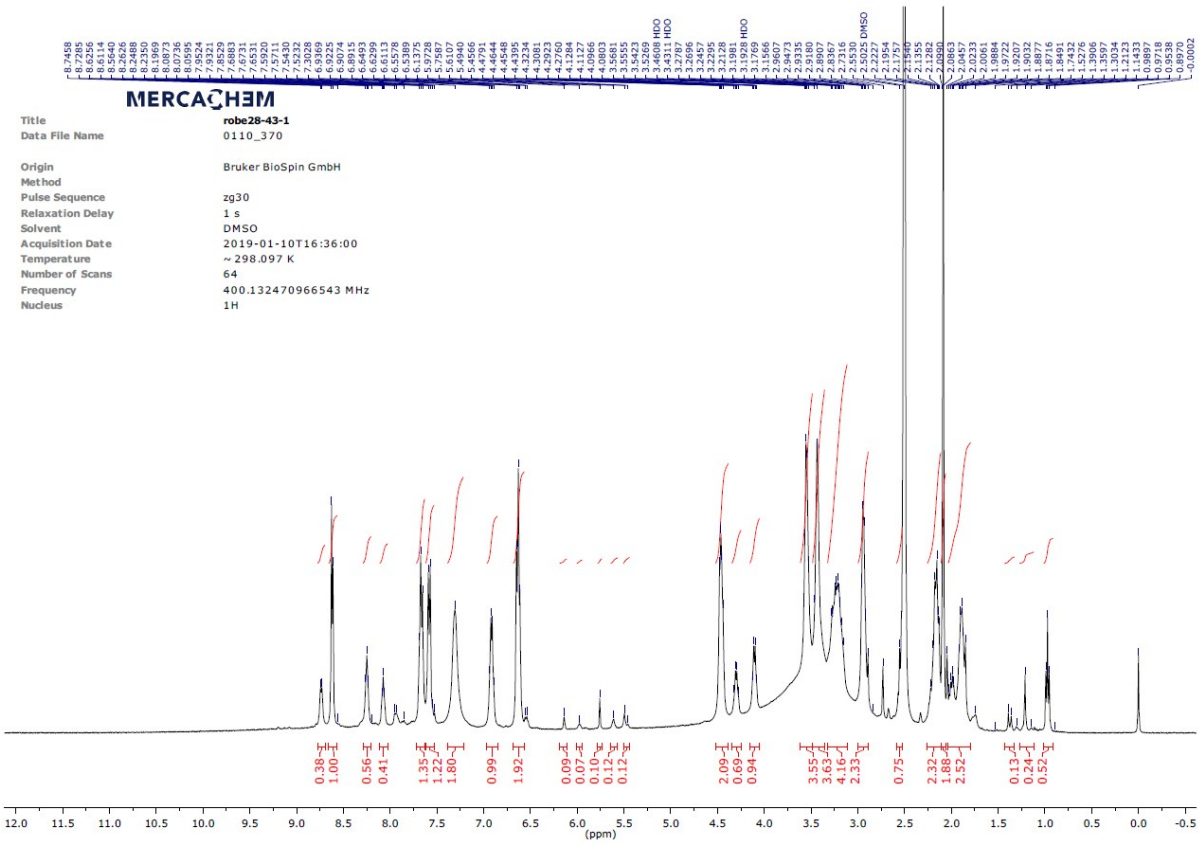


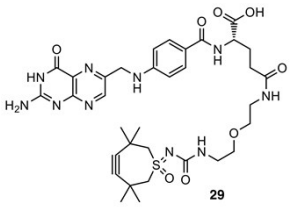





MERCACHEM
Title robe28-31-2 + D2O
Data File Name 1214_160
Origin Bruker BioSpin GmbH
Method
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent DMSO
Acquisition Date 2018-12-14T11:22:00
Temperature ~ 295.7355 K
Number of Scans 64
Frequency 400.132470966543 MHz
Nucleus 1H
HDO
DMSO
1.03
0.14
0.87
2.23
0.84
2.10
2.03
0.47
0.64
4.79
2.65
2.53
0.49
1.13
1.19
2.28
10.68
(ppm)

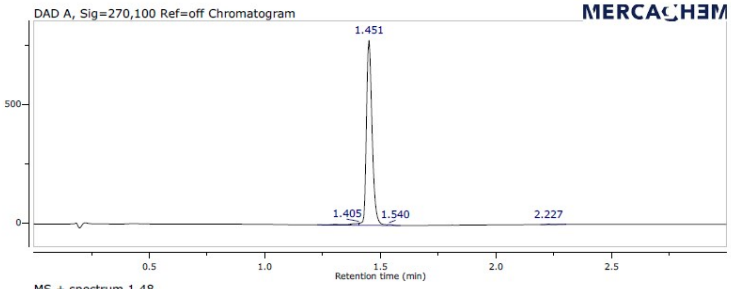

28

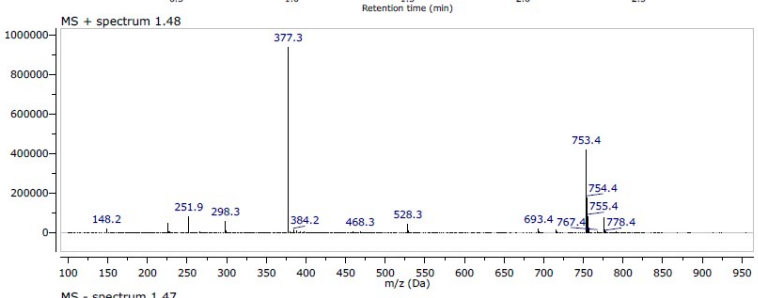

MERCACHEM
Title robe28-43-1
Data File Name 0110_370
Origin Bruker BioSpin GmbH
Method
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent DMSO
Acquisition Date 2019-01-10T16:36:00
Temperature ~ 298.097 K
Number of Scans 64
Frequency 400.132470966543 MHz
Nucleus 1H
HDO
DMSO
0.38
1.00
0.56
0.41
1.35
1.22
1.80
0.99
1.92
0.09
0.07
0.10
0.12
0.12
2.09
0.69
0.94
3.55
3.63
4.16
2.33
0.75
2.32
1.88
2.52
0.13
0.24
0.52
(ppm)

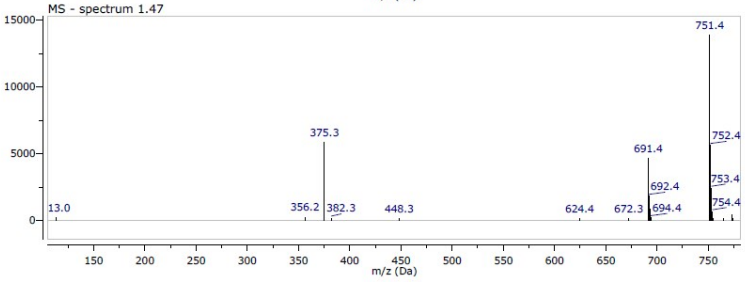


29

title	**robe28-42-1**
Method	SC_BASE.M
Date acquired	15-Jan-19, 09:57:58
FileName	Analysis\LCMS5_0115_035.D
Acq. method	SC_BASE valve: 6
Column	Waters XSelect CSH C18 (30x2.1mm, 3.5µ)
Flow	1 ml/min; Column temp: 25°C
Eluent A	100% acetonitrile
Eluent B	10mM ammoniumbicarbonate in water
Lin. Gradient	t=0 min 5% A, t=1.6 min 98% A, t=3 min 98% A
Posttime	1.3 min
Detection	DAD (210, 220 and 220-320 nm)
Detection	PDA (210-320 nm)
Detection	MSD (ESI pos/neg) mass range: 100-1000

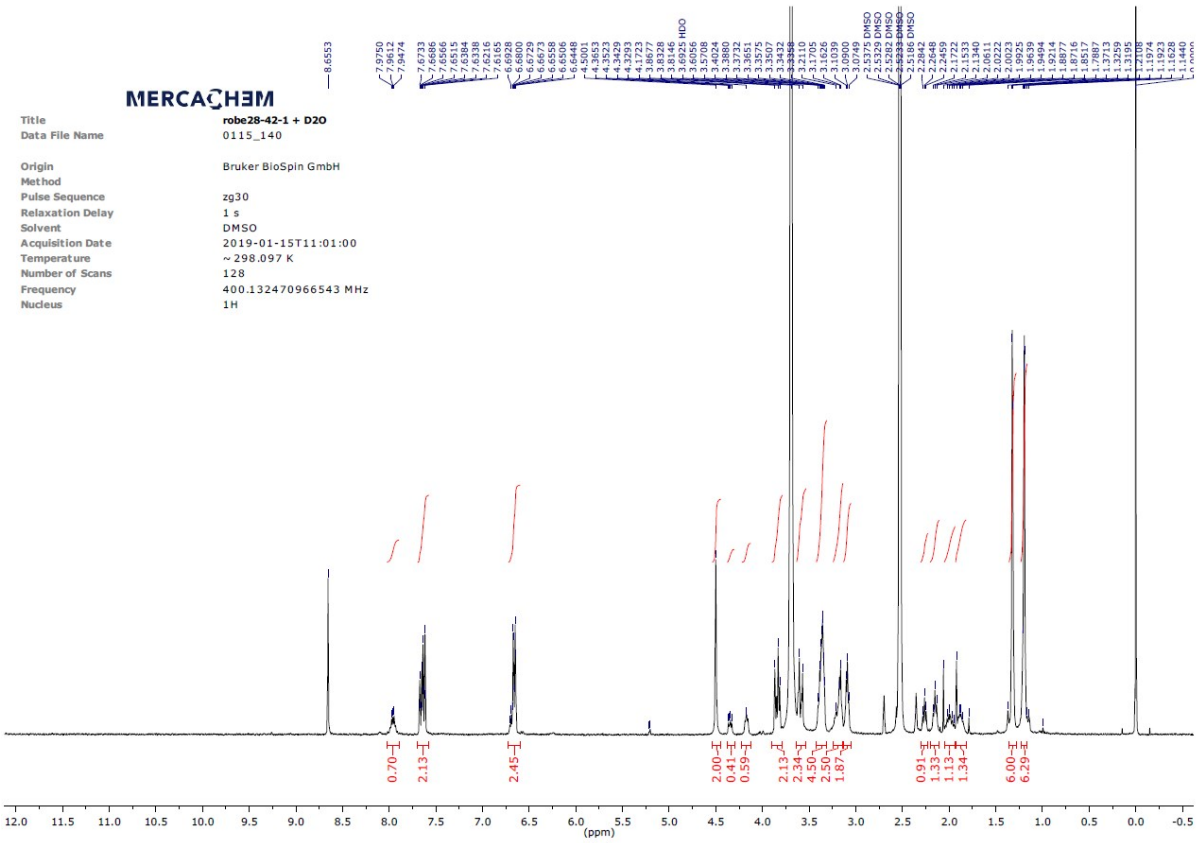






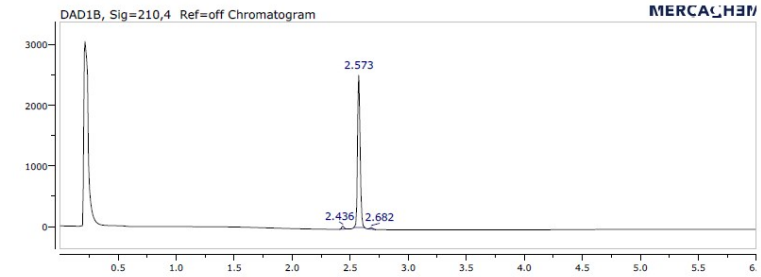
Integrals spectrum Chromatogram DAD A, Sig=270,100 Ref=off

rt (min)	height	area	area (%)
1.40	7.976	0.4595	2.02
1.45	781.1	22.18	97.33
1.54	3.719	0.1013	0.44
2.23	1.502	0.04766	0.21


MERCACHEM
Title robe28-42-1 + D2O
Data File Name 0115_140
Origin Bruker BioSpin GmbH
Method
Pulse Sequence zg30
Relaxation Delay 1 s
Solvent DMSO
Acquisition Date 2019-01-15T11:01:00
Temperature ~298.097 K
Number of Scans 128
Frequency 400.132470966543 MHz
Nucleus 1H
(ppm)

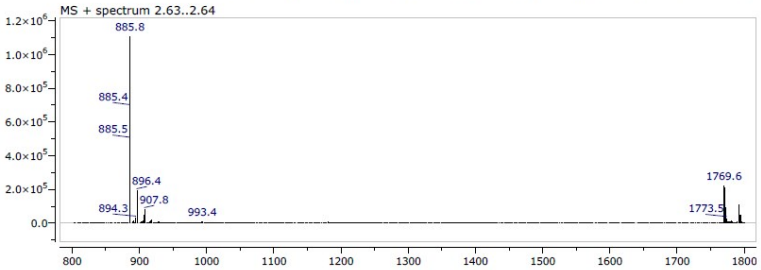
Analyses of compound HER2-TMTHSI 32

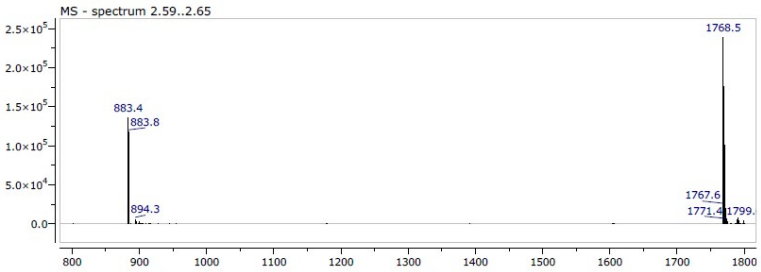
title	**HAVE45-034-1**
Method	AN_BASE_M1800.M
Date acquired	03-May-18, 16:50:54
FileName	U:\20180004\08 Publication\0503_055.D
Column	Waters XSelect CSH C18 (50x2.1mm 3.5µ)
Flow	0.8 ml/min; Column Temp: 25°C
Eluent A	acetonitrile
Eluent B	10 mM ammonium bicarbonate in water
Lin. Gradient	t=0 min 5% A, t=3.5 min 98% A, t=6 min 98% A
Posttime	2 min
Detection	DAD (220-320 nm, 210 and 220nm)
Detection	PDA (210-320 nm)
Detection	MSD ESI pos/neg (mass range 800-1800)



Integrals spectrum Chromatogram DAD1B, Sig=210,4 Ref=off

rt (min)	height	area	area (%)
2.44	49.03	1355	1.68
2.57	2515	78220	97.25
2.68	26.77	853.3	1.06





Detailed NMR-analysis of reaction of TMTHSI-derivatives with nanoparticles

A plot was generated (Figure S1) with overlayed 2D HMBC ^{15}N-^{1}H spectra of reference TMTHSI-PEG5000 click reacted TTz adduct in KPi 50 mM pH 7.1 D_2O, together with nanoparticle (NP749) azide reference at 7.6 mg/ml, and (NP739) TTz clicked NP 7.6 mg/ml after coupling to alkyne modified HER1 peptide.

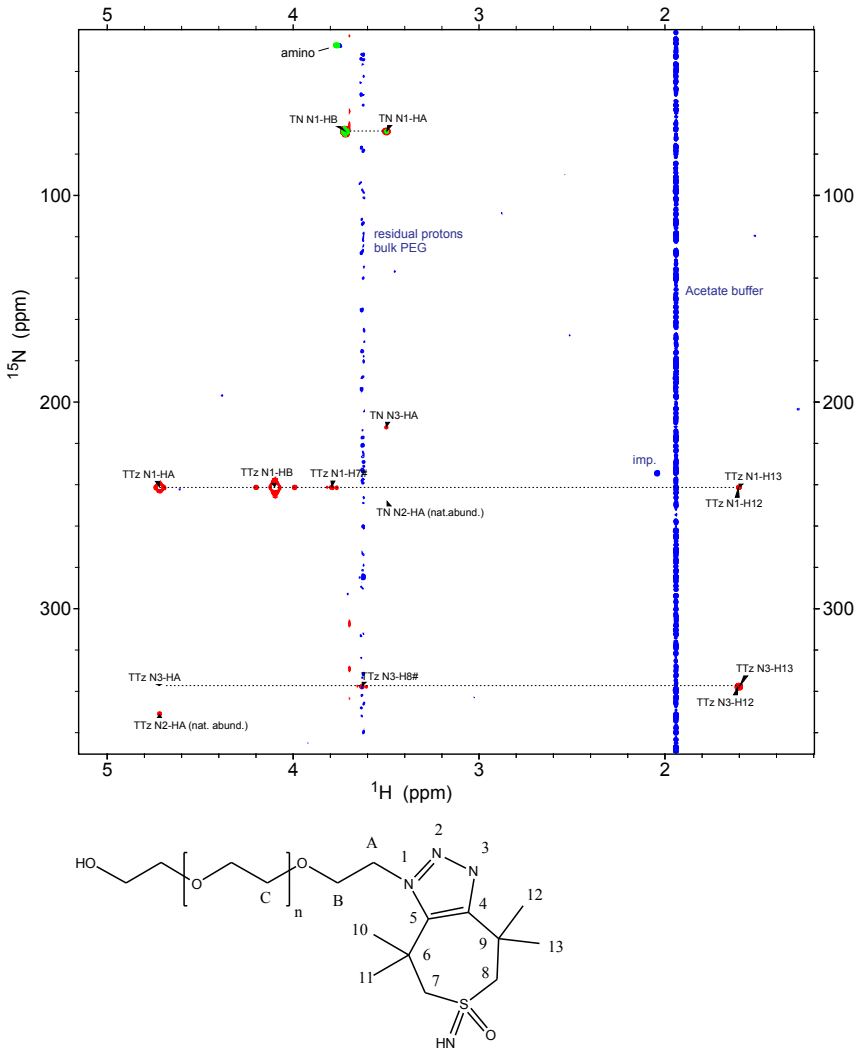
The sensitivity of the long-range ^{15}N HMBC experiment was greatly enhanced by making use of ^{15}N-N1 sodium azide as for preparation of the azide linker in PEG5000 and of the exterior PEG chains of NP particles. Effectively, this labelling strategy led to overall 50% ^{15}N-labelling on positions N1 and N3 in coupled azides and thus in formed triazole rings after the click reaction. The sensitivity of long-range ^{2}JNH and ^{3}JHN correlations to N1 and N3 was therefore ~160x fold higher than would be case using standard -unlabelled- azide. Correlations from proton to nitrogen N2 had a decreased sensitivity, because the ^{15}N-N2 nitrogen atom remained present at the natural abundance with this method.

Connection of ^{15}N-labelled azide to protons of neighbouring PEG-CH_2 was well detectable (even at a low load factor) when attached to the large NP. We contributed this efficient magnetization transfer largely to the favourable dynamic properties of the freely rotatable PEG chains with its azide end group. The percentage of conversion from azide to the triazole moeity was conveniently monitored by the gradual disappearance and final absence of these azide correlations in the HMBC spectrum.

We noted that newly formed CH_2-^{1}H to ^{15}N correlations in the HMBC spectrum corresponding to formation of the triazole-ring were well detected in the TMTHSI-PEG5000 model system, but also for the NPs functionalized with PEG-N_3. In contrast, the triazole-linked moiety becomes less mobile in the NP after the click reaction. Therefore, the peaks started to become broader and were less efficiently detected via their long-range ^{1}H-^{15}N couplings. For that reason triazole peaks were often missing in the spectra of the clicked NP, but conversion efficiency of the click reaction could be still estimated from the intensity loss against those from the original HMBC azide cross peaks to the intensities in the corresponding NP reference sample with unreacted azide groups.

Figure S1. Overlay of HMBC spectra of azido nanoparticles NP739-NP749 after the click reaction and TMTHSI-PEG5000 click adduct.

Overlay HMBC ^{15}N-^{1}H spectra NP739, NP 749 and THMTSI-PEG5000 reference



red = reference TMTHSI-PEG5000-N_3[^{15}N-N^1/N^3] click reacted triazole adduct (TTz)
plus slight excess unreacted PEG5000-N_3[^{15}N-N^1] (TN)

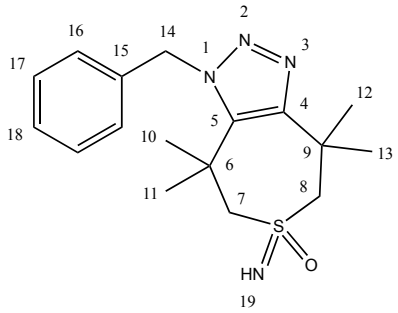
green = NP749 azide reference at 7.6 mg/ml
blue = NP739 triazole clicked NP with coupled HER1 peptide at 7.6 mg/ml

Determination of the reaction rates by real-time NMR spectroscopy: experimental details

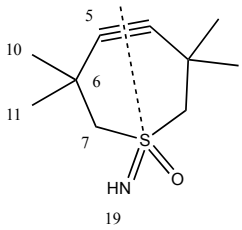
NMR spectra of TMTHSI, BCN-OH and its click reaction products were recorded on a Bruker Avance III HD 700 MHz, equipped with triple channel TCI cryoprobe. Full characterization of the compounds was carried out by a combination of 1D proton, 1D ^{13}C, and various 2D correlation spectra (DIPSI, NOESY, ROESY, ^{15}N-^{1}H HSQC, ^{15}N-^{1}H HMBC, ^{13}C-^{1}H HSQC, ^{13}C-^{1}H HMBC, for the assignments and chemical shifts, see the tables below). Monitoring in real-time of the reaction between TMTHSI at 5 mM and 6.6 mM Benzylazide (BA, slight molar excess) was carried out in $CDCl_3$. Small aliquots of concentrated stock solution of BA were instantly added to a pre-made TMTHSI solution in $CDCl_3$ that yielded final concentrations of 5 mM TMTHSI and 6.38 mM BA. After vigorously vortexing the Eppendorf tube with reaction components the reaction solution was quickly transferred into a 5 mm NMR tube for immediate data collection by means continuously recording repeated 1D proton

spectra at 25 °C. The first stable 1H 1D spectrum was normally obtained after an initial reaction time of +2m 45s. However, according to the NMR results, the click reaction of TMTHSI was quite fast and nearly complete after already three minutes. This only allowed for data collection up to four to five useful points during integration of proton signals, before chemical conversion is complete after ca. 5-6 minutes.

The same type of setup was used to determine the reference reaction rate between 5.3 mM BCN-OH and 6.7 mM BA (slight excess) under the same experimental conditions. BCN-OH has a limited solubility in chloroform, but still reaches 5 mM concentration under saturated solute conditions. The click reaction between BCN-OH and BA proceed much slower than in the case of TMTHSI and BA. Typically, the BCN-OH reaction was monitored over ca. 12 hours ($CDCl_3$) necessary for completion at 25 °C, thereby collecting sufficient number of time points for integration and analysis. The proton peak intensities of both starting molecules and reaction product could be fitted exponentially in order to determine the overall reaction rate (Figure S3: identical to the left part of Figure 2 in the paper).



TMTHSI to benzylazide adduct



TMTHSI

Figure S2. NMR-atom numbering in click product and in TMTHSI

Chemical shifts TMTHSI and TMTHSI - benzylazide adduct (in ppm rel. to TMS)
Concentration 17.5 mM in CDCl3 at 25 oC
Assignments based on 2D HSQC 13C-1H, HMBC 13C-1H, HMBC 15N-1H, DIPSI, NOESY
Atom numbering scheme, see the corresponding Chemdraw file

	Group	Atom	Nuc	Shift	SDev	Assignment
TMTHSI (free)	alkyne	C5	13C	101.539	0.006	4
	Cq	C6	13C	34.679	0.007	4
	CH2	C7	13C	71.131	0.004	4
	MeBottom	C10	13C	27.588	0.015	4
	MeTop	C11	13C	26.575	0.003	4
	NdH	N19	15N	98.782	0.000	1

^{15}N chemical shift are relative to the external reference neat nitromethane-d^3 (CD_3NO_2)

	Hbottom	H7a	1H	3.169	0.001	7
	Htop	H7b	1H	3.248	0.002	8
	MeBottom	H10	1H	1.286	0.001	7
	MeTop	H11	1H	1.449	0.001	7

ι

TTz-BA adduct	TMT	C10	13C	28.845	0.009	4
	TMT	C11	13C	29.427	0.010	4
	TMT	C12	13C	30.653	0.011	4
	TMT	C13	13C	30.787	0.011	4
	TMT	C14	13C	54.679	0.002	3
	TMT	C15	13C	135.740	0.003	2
	TMT	C16	13C	126.385	0.003	5
	TMT	C17	13C	129.016	0.004	3
	TMT	C18	13C	128.215	0.005	3
	TMT	C4	13C	148.862	0.008	3
	TMT	C5	13C	137.243	0.007	4
	TMT	C6	13C	33.798	0.003	3
	TMT	C7	13C	68.415	0.003	5
	TMT	C8	13C	67.431	0.002	5
	TMT	C9	13C	34.104	0.002	3
	TMT	H10	1H	1.444	0.001	10
	TMT	H11	1H	1.438	0.001	10
	TMT	H12	1H	1.705	0.001	9
	TMT	H13	1H	1.701	0.002	8
	TMT	H14	1H	5.731	0.004	15
	TMT	H16#	1H	7.036	0.001	7
	TMT	H17#	1H	7.345	0.001	5
	TMT	H18	1H	7.310	0.003	2
	TMT	H7#	1H	3.455	0.001	13
	TMT	H8#	1H	3.433	0.001	14
	TMT	HN19	1H	2.682	0.001	3
	TMT	N1	15N	242.487	0.028	2
	TMT	N2	15N	363.608	0.000	1
	TMT	N3	15N	350.063	0.041	2
	TMT	N19	15N	100.775	0.034	2

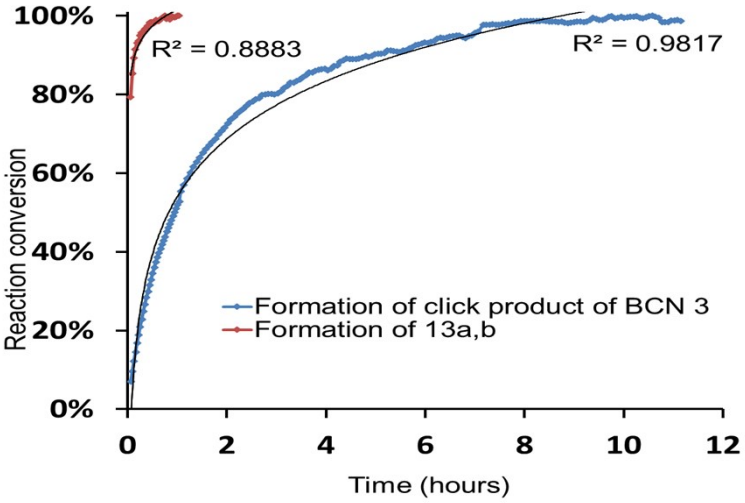


Figure S3. Determination of the reaction rate of the strain-promoted azide-alkyne cycloaddition reaction of TMTHSI **12** and benzyl azide by NMR. Reaction of 5 mM TMTHSI and 6.38 mM benzyl azide (1:1.3) in $CDCl_3$ at 25 °C as compared to the reaction of endo-BCN-OH 3 (4.75 mM) and benzyl azide (6.02 mM) determined by NMR in $CDCl_3$

Determination of the reaction rates by real-time mass spectrometric analysis: general considerations

Direct monitoring of the various species involved in a reaction may allow elucidating reaction mechanism and establishing its reaction kinetics. Typically, the reactivity of copper free click chemistry reagents (alkynes) was determined by nuclear magnetic resonance (NMR) to monitor the formation of the triazole product from the alkyne of choice and benzylazide (BA) over time. However, due to the relative long sample handling and inherent insensitivity of a standard 1H NMR experiment, this method is limited to monitor relatively slow reaction rates in a time range above say 1-3 minutes without resorting to special equipment. Other fluorescence and absorption-based spectroscopy methods are more suitable in terms of fast detection, but are more or less limited to either emission of light or having different absorption spectrum per chemical species (Johnson KA. Transient-State Kinetic Analysis of Enzyme Reaction Pathways, In: Sigman DS, (Ed) Academic Press, Inc; 1992:1-61; Poloukhtine AA, Mbua NE, Wolfert MA, Boons G-J, Popik VV. Selective Labeling of Living Cells by a Photo-Triggered Click Reaction. J. Am. Chem. Soc. 2009, 131, 15769-15776; Lang K, Davis L, Wallace S*, et al.* Genetic Encoding of Bicyclononynes and trans-Cyclooctenes for Site-Specific Protein Labeling in Vitro and in Live Mammalian Cells via Rapid Fluorogenic Diels–Alder Reactions. J. Am. Chem. Soc. 2012, 134, 10317-10320.).

Considering these limitations, there was a great interest to use a technique capable of monitoring entire mass spectrum of a sample in millisecond scale and detecting transient intermediates. High sensitivity and selectivity of mass spectrometry as well as structural information that could be obtained form ion mobility spectrometry (IMS) are the reasons to opt for IMS-MS technique for real-time monitoring of our fast reaction (Figure 2b). Applying the well-established method (Kolakowski BM, Simmons DA, Konermann L. Stopped-flow electrospray ionization mass spectrometry: a new method for studying chemical reaction kinetics in solution. *Rapid Comm. Mass Spec* 2000, 14, 772-776), the consumption of TMTHSI and the formation of product were spontaneously monitored with MS (Figure 2b - Supplementary Figure S1). By plotting the second-order reaction rate graph, the k value of the reaction was determined to be 0.8 $M^{-1}s^{-1}$ (Figure 2b). The same method was applied to monitor the reaction of BCN-OH with BA. The obtained k value was in satisfactory agreement with k=0.14 $M^{-1}s^{-1}$ that has been previously determined by IR-based measurements (see above references). In addition, the ion mobility of the product explored to identify possible isomeric compounds, nevertheless there was only one mobility species identified for the product (Figure S1c).

Determination of the reaction rates by real-time mass spectrometric analysis: experimental details

In order to monitor the reaction, 5mM of TMTHSI **12** and benzyl azide (1:1) were prepared in water/acetonitrile (1:3) and 0.01% acetic acid at 25 ^{o}C. A calibrated QTOF tandem mass spectrometer equipped with traveling wave ion mobility spectrometry (Synapt HDMS G2Si, Waters, Milford, MA, USA), were optimized for the chemical species of the interest. In order to initiate the reaction, 100µL of TMTHSI

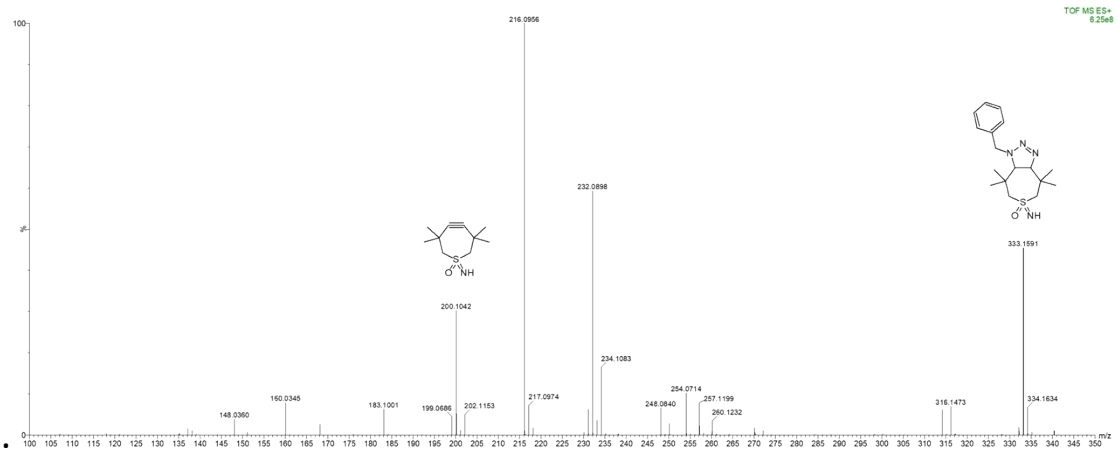
and BA rapidly mixed in an Eppendorf tube and immediately injected to mass spectrometer with electrospray ionization source to monitor the reaction in positive ion mode. The entire mass spectrum of the reaction monitored approximately 30 seconds after mixing of the reactants. The reaction carried out under second order condition according to the following equation:

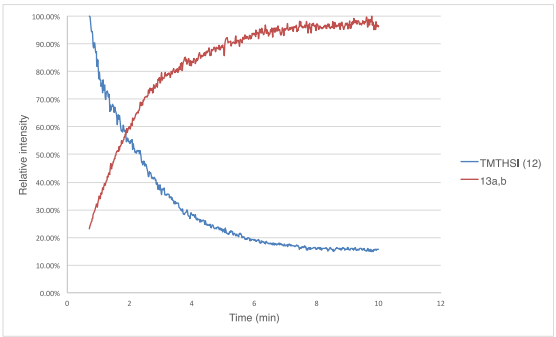
$$kt = \frac{1}{[B]0 - [A]0} \times \ln\frac{[A]0([B]0 - [P])}{([A]0 - [P])[B]0}$$

k = second order rate constant ($M^{-1}s^{-1}$), t = reaction time (s), [A]0 = the initial concentration of reagent 1 ($mmol.mL^{-1}$), [B]0 = the initial concentration of reagent 2 ($mmol.mL^{-1}$) and [P] = the concentration of product ($mol.L^{-1}$).

All of the experiments have been performed 3 times per day in two consecutive days.

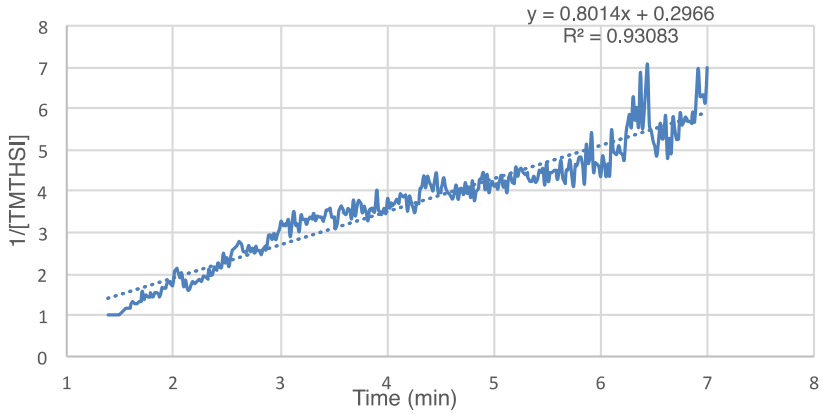
a.





b.

c.



d.

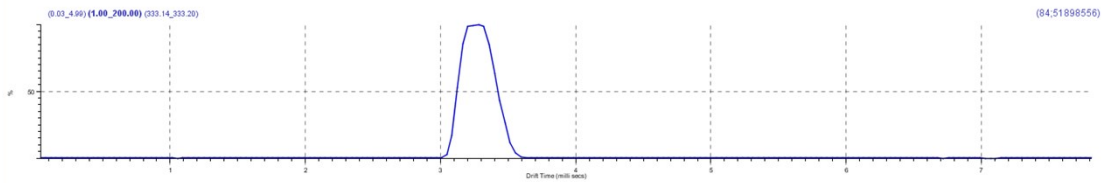


Figure S4. (a) Mass spectrum of the TMTHSI reaction with BA and the product; (b) The relative intensity of TMTSI **12** consumption and triazole product **13a,b** formation; (c) Determination of the reaction rate of TMTHSI consumption from the second order reaction graph; (d) Ion mobility spectrum of the product showing one mobility peak.

Cell culture and flow cytometric analysis of cellular uptake of Cy7 functionalized nanoparticles

A431 cells were cultured in 12-well plates (Cellstar; Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 37 °C 5% CO_2 in DMEM (BioWhittaker; Lonza, Breda, the Netherlands) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Zwijndrecht, the Netherlands) and 1% penicillin/streptomycin (Sigma-Aldrich). Cy7 labelled (1.7% surface coverage) and unlabeled nanoparticles (0.1 mg/mL polymer content: a) Q. Hu, C. J. Rijcken, R. Bansal, W. E. Hennink, G. Storm, J. Prakash, *Biomaterials*, **2015**, *53*, 370-378; b) Q. Hu,m C. F. rijcken, E. van Gaal, P. Brundel, H. Kostkova, T. Etrych, B. weber, M. Barz, F. Kiessling, J. Prakash, G. Storm, W. E. Hennink, T. Lammers, J. Contr. Rel. **2016**, *244*, part B, 314-325.) were added to the cells at 60-80% cell confluency and incubated for 2h or 24h. All experiments were carried out in triplicate. Samples were washed with PBS and harvested using trypsin (Sigma-Aldrich) and collected in 5 ml Polystrene Round-Bottom tubes (BD Biosciences, Breda, the Netherlands). Cells were re-suspended in PBS/5%FBS and cell-associated fluorescence (10,000 cells per sample) was detected using a FACSCanto II flow cytometer (BD Biosciences).

Visualisation of Cy7 functionalized nanoparticles uptake by cells

Cells were incubated with Cy7 functionalized nanoparticles **27**. After 2 hours and 24 hours of incubation, 69% and 93% of the cells, respectively, had taken up **27**. Also the level of uptake as indicated by the mean fluorescence intensity (MFI) increased in time from 576 after 2h to 1169 after 24h.

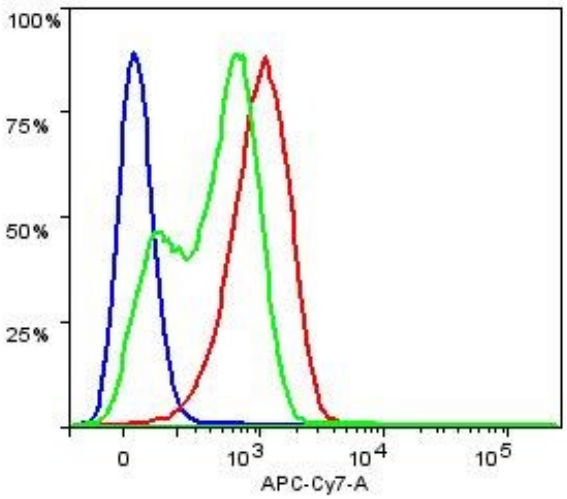


Figure S5. **Cy7 functionalized nanoparticles (27) uptake by cells**: Histogram plot showing cells treated for 24h with unlabelled CriPec (BLUE curve), and cells treated for 2h (GREEN curve) and 24h (RED curve) with **27**.