Synthesis and application of a new cleavable linker for "click"-based affinity chromatography

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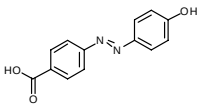
1 Chemical Synthesis

1.1 General methods

All non-aqueous reactions were carried out under an atmosphere of nitrogen using oven-dried glassware that was cooled in a dessicator prior to use. Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. Dichloromethane (DCM) and triethylamine (Et_3N) were distilled from and stored over calcium hydride under a nitrogen atmosphere. Saturated aqueous solutions of inorganic salts are represented as (volume, sat. aq.). ^{1}H and ^{13}C NMR spectra were obtained on Bruker instuments at the stated frequency. Infra-red spectra were recorded using a Biorad FTS-7 or Perkin-Elmer Paragon 1000 FT-IR spectrometer as thin films or KBr discs. Electrospray (ESI) and fast atom bombardment (FAB) mass spectra were obtained on a Kratos MS50TC mass spectrometer. Melting points were determined on a Gallenkamp Electrothermal Melting Point apparatus and are uncorrected. Flash chromatography was carried out using Merck Kieselgel 60 (Merck 9385) under positive pressure. Eluent compositions are quoted as $^{v}/_{v}$ ratios.

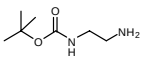
1.2 Linker synthesis

4-(4'-Hydroxy-phenylazo)-benzoic acid 2[1]



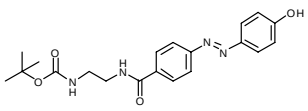
4-aminobenzoic acid (1.23 g, 9.00 mmol), was dissolved in a mixture of hydrochloric acid (1.2 ml, conc) and water (24 ml) and the suspension obtained was cooled to 0 °C. Sodium nitrite (1.04 g, 15.0 mmol) dissolved in water (5 ml) was added next and the pale yellow solution formed was stirred for 20 min. A second solution of phenol (667 mg, 7.20 mmol), sodium hydroxide (257 mg, 6.42 mmol) and potassium carbonate (1.33 g, 9.60 mmol) dissolved in water (20 ml) was prepared, cooled to 0 °C and added dropwise to the reaction mixture. The reaction was allowed to reach RT and stirred for 3 h. The brown suspension formed was acified with HCl (1 M, aq) and filtered. The filtrate was dissolved in MeOH, silica was added and the mixture dried *in vacuo*. Flash chromatography (dry loaded silica column, 15% MeOH in DCM) gave product **3** as an orange solid (1.57 g, 90% yield); $\mathbf{R_f}$ (5% MeOH in DCM = 0.25); **mp** 275-277 °C; **νmax** (neat)/cm^{-1} 3286 (OH), 1642 (CO), 1538 (NN); ^{1}**H NMR** δ (250 MHz, CD_3OD) 8.19 (2H, d, J = 8.5 Hz, Ar*H*), 7.92 (2H, d, J = 8.5 Hz, Ar*H*), 7.90 (2H, d, J = 8.8 Hz, Ar*H*), 7.17 (2H, d, J = 8.8 Hz, Ar*H*); ^{13}**C NMR** δ (62.9 MHz, CD_3OD) 169.23 (C), 162.88 (C), 156.84 (C), 147.58 (C), 133.00 (C), 131.81 (2 × CH), 126.46 (2 × CH), 123.21 (2 × CH), 116.87 (2 × CH); ***m/z*** (ESI–) 241 ([M–H]$^-$, 48%), 226 (12), 112 (100).
All spectroscopic data were in good agreement with the literature.[1]

(2-Aminoethyl)-carbamic acid *tert*-butyl ester



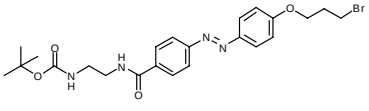
To a vigorously stirred solution of ethane-1,2-diamine (9.97 g, 167 mmol) in DCM (100 ml) at RT was added a solution of di-*tert*butylcarbonate (6.10 g, 28.0 mmol) in DCM (400 ml) over 5 h. The resulting solution was stirred for 24 h at RT and then concentrated *in vacuo* to obtain a colourless oil which was partitioned between Na_2CO_3 (20 ml, 2 M, aq) and DCM (20 ml). The aqueous layer was extracted with DCM (2 × 20 ml) and concentrated under reduced pressure to give the Boc-protected amine as a colourless oil (4.45 g, 99% yield); $\mathbf{R_f}$ (10% MeOH in DCM) = 0.1; $\mathbf{\nu_{max}}$ (neat)/cm^{-1} 3446 (NH), 1710 (CO); ^{1}**H NMR** δ (360 MHz, CD_3OD) 3.13 (2H, t, J = 6.2 Hz, CH_2NHCO), 2.73 (2H, t, J = 6.2 Hz, CH_2NH$_2$), 1.44 (9H, s, 3 × CH_3); ^{13}**C NMR** δ (90.6 MHz, CD_3OD), 158.68 (C), 80.18 (C), 43.26 (CH_2), 42.20 (CH_2), 28.79 (3 × CH_3); ***m/z*** (ESI+) 183 ([M+Na]$^+$, 8%), 161 ([M+H]$^+$, 58).
All spectroscopic data were in good agreement with the literature.[2]

2-[4'-(4''-Hydroxy-phenylazo)-benzoylamino]-ethyl-carbamic acid *tert*-butyl ester



To a solution of benzoic acid **2** (100 mg, 0.413 mmol), in DMF (3 ml), EDC (94.0 mg, 0.500 mmol) and HOBt (27.0 mg, 0.200 mmol) were added. After stirring for 15 min *N*-Boc-ethylenediamine (131 mg, 0.800 mmol) was added and the reaction stirred for 48 h at RT. The solvent was removed *in vacuo*, the crude material was dissolved in DCM (2 ml) and the solution washed with HCl (5 ml, 1 M aq). The solvent was removed *in vacuo*. The crude product was dissolved in MeOH (2 ml), silica was added and the mixture dried *in vacuo*. Flash chromatography (dry loaded silica column, 2% MeOH in DCM) yielded the desired amide as an orange solid (94 mg, 60% yield).
$\mathbf{R_f}$ (5% MeOH in DCM) = 0.31; **mp** 169-171 °C; $\mathbf{\nu_{max}}$ (neat)/cm^{-1} 3361 (NH), 1689 (CO), 1637 (CO), 1535 (NN); ^{1}**H NMR** δ (250 Mz, $(CD_3)_2CO$) 8.06 (2H, d, J = 8.6 Hz, Ar*H*), 7.90 (2H, d, J = 8.5 Hz, Ar*H*), 7.89 (2H, d, J = 8.8 Hz, Ar*H*), 7.04 (2H, d, J = 8.6 Ar*H*), 6.47 (1H, br s N*H*), 3.73-3.66 (2H, m, CH_2), 3.55-3.48 (2H, m, CH_2), 1.58 (9H, s, CH_3); ^{13}**C NMR** δ (62.9 MHz, $(CD_3)_2CO$) 167.11 (C), 162.20 (C), 161.66 (C), 155.17 (C), 147.27 (C), 137.13 (C), 129.18 (2 × CH), 126.15 (2 × CH), 123.03 (2 × CH), 116.91 (2 × CH), 79.02 (C), 41.63 (CH_2), 41.06 (CH_2), 28.71 (3 × CH_3); ***m/z*** (ESI-) 383 ([M-H]$^-$, 18%), 134 (100), 113 (72); **HRMS** (ESI+) [M+NH$_4$]$^+$ found 402.2132, $C_{20}H_{28}O_4N_5$ requires 402.2136.

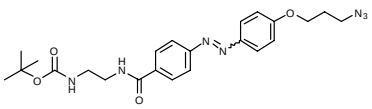
2-{4'''-[4''-(3'-Bromo-propoxy)-phenylazo]-benzoylamino}-ethyl-carbamic acid *tert*-butyl ester 3



To a stirred solution of the phenol (800 mg, 2.08 mmol) in CH_3CN (30 ml) was added potassium carbonate (430 mg, 16.6 mmol) and the mixture was heated to 70 °C. 1,3-Dibromopropane (1.70 ml, 16.6 mmol) was added and the reaction stirred at reflux for 5 h. The solvent was removed *in vacuo* to give the crude bromide. Purification by flash chromatography (5% MeOH in DCM) yielded the desired bromide **3** (710 mg, 67% yield) as a yellow solid; $\mathbf{R_f}$ (5% MeOH in DCM) = 0.58; **mp** 188-190 °C; $\mathbf{\nu_{max}}$ (neat)/cm^{-1} 1700 (CO), 1659 (CO); ^{1}**H NMR** δ (250 MHz, $CDCl_3$) 7.98-7.88 (6H, m, Ar*H*), 7.44 (1H, br s, N*H*), 7.26-7.21 (2H, m, Ar*H*), 5.12 (1H, br s, N*H*), 4.20 (2H, t, J = 6.0 Hz, CH_2), 3.64 (2H, t, J = 6.4 Hz, CH_2), 3.60-3.55 (2H, m, CH_2), 3.48-3.40 (2H, m, CH_2), 2.37 (2H, qn, J = 6.0 Hz, CH_2), 1.44 (9H, s, CH_3); ^{13}**C NMR** δ (62.9 MHz, $CDCl_3$) 175.55 (C), 175.27 (C), 167.57 (C), 162.00 (C), 154.70 (C), 135.24 (C), 128.40 (2 × CH), 125.50 (2 × CH), 122.96 (2 × CH), 115.21 (2 × CH), 80.49 (C), 66.06 (CH_2), 42.76 (CH_2), 40.28 (CH_2), 32.60 (CH_2), 30.14 (CH_2), 28.75 (3 × CH_3); ***m/z*** (ESI+) 529 ([^{81}BrM+Na]$^+$, 38%), 527 ([^{79}BrM+Na]$^+$, 36),

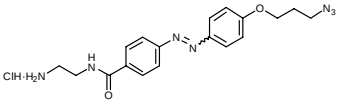
507 ([^{81}BrM+H]$^+$, 2), 505 ([^{79}BrM+H]$^+$, 2); **HRMS** (FAB, 3-NOBA) [M+H]$^+$ found 505.1461, $C_{23}H_{30}N_4O_4{}^{79}Br$ requires 505.1450.

2-{4'''-[4''-(3'-Azido-propoxy)-phenylazo]-benzoylamino}-ethyl-carbamic acid *tert*-butyl ester 4



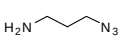
The Boc-protected bromide **3** (710 mg, 1.40 mmol) was dissolved in DMF (15 ml) and sodium azide (273 mg, 4.20 mmol) was added. The solution was stirred overnight (~18 h) at RT. The solvent was removed *in vacuo* and the crude material dissolved in diethyl ether (20 ml), washed with brine (20 ml) and water (20 ml) to give the azide **4** (588 mg, 90% yield) as a pale yellow solid (90:10, *E*:*Z*); $\mathbf{R_f}$ (5% MeOH in DCM) = 0.58; **mp** 173-174 °C; $\boldsymbol{\nu_{max}}$ (neat)/cm^{-1} 3366 (NH), 3317 (NH), 2100 (N_3), 1680 (CO), 1636 (CO); **^{1}H NMR** δ (250 MHz, $CDCl_3$), 7.96-7.86 (5.40 H (*E*), m, Ar*H*) 7.74 (0.20 H (*Z*), d, *J* = 8.5 Hz Ar*H*), 7.35 (0.90 H (*E*), br s, N*H*), 7.07-6.93 (1.80 H (*E*), m, Ar*H*), 6.87 (0.40 H (*Z*), m, ArH), 6.71 (0.20 H (*Z*), d, *J* = 8.8 Hz, ArH), 5.00 (0.90 H (*E*), br s, N*H*), 4.13 (1.80 H (*E*), t, *J* = 6.0 Hz, CH_2), 3.98 (0.20 H (*Z*), t, *J* = 6.0 Hz, CH_2), 3.66-3.34 (6H, m, 2 × CH_2NH+CH_2N$_3$), 2.14-1.94 (2H , m, $CH_2CH_2CH_2$), 1.42 (8.10 H (*E*), s, 3 × CH_3) 1.39 (0.90 H (*Z*), s, 3 × CH_3); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 167.35 (C), 161.79 (C), 157.95 (C), 154.62 (C), 147.34 (C), 135.53 (C), 128.21 (2 × CH), 125.32 (2 × CH), 122.80 (2 × CH), 115.00 (2 × CH), 80.40 (C), 65.09 (CH_2), 48.36 (CH_2), 42.68 (CH_2), 40.09 (CH_2), 28.94 (CH_2), 28.56 (3 × CH_3); ***m/z*** (ESI+) 957 ([2M+Na]$^+$, 27%), 490 ([M+Na]$^+$, 100), 390 (52), 71 (43); **HRMS** (FAB, 3-NOBA) found [M+H]$^+$ 468.2351, $C_{23}H_{30}N_7O_4$ requires 468.2359.

***N*-(2-Amino-ethyl)-4-{-2-[4-(3-azido-propoxy)-phenyl]-vinyl}-benzamide hydrochloride 1**



Acetyl chloride (10 ml) was added to dry MeOH (60 ml) at 0 °C and the solution was stirred for 30 min. Boc-protected aminoazide **4** (850 mg, 1.82 mmol) was dissolved in this solution and stirred for 1 h. Completion of the reaction was monitored by TLC. Diethyl ether (20 ml) was added and the amine hydrochloride salt precipitated as a yellow solid. The salt **1** was filtered and used without further purification (720 mg, 95% yield), (80:20, *E*:*Z*); $\mathbf{R_f}$ (20% MeOH in DCM = 0.42); **mp** 250 °C (decomposition); $\boldsymbol{\nu_{max}}$ (nujol)/cm^{-1} 3413 (NH), 3273 (NH), 2100 (N_3), 1639 (CO); **^{1}H NMR** δ (800 MHz, DMSO) 8.77 (0.80 H (*E*), br s, N*H*), 8.61 (0.20 H (*Z*), br s, N*H*), 8.06 (1.60 H (*E*), d, *J* = 8.5 Hz, Ar*H*), 7.95-7.93 (3.20 H (*E*), Ar*H*), 7.82 (0.40 H (*Z*), d, *J* = 8.5 Hz, Ar*H*), 7.17 (1.60 H (*E*), d, *J* = 8.8 Hz, Ar*H*), 6.94 (0.40 H (*Z*), d, *J* = 8.5 Hz, Ar*H*), 6.91-6.87 (0.80 H (*Z*), Ar*H*), 4.18 (1.60 H (*E*), t, *J* = 6.1 Hz, CH_2), 4.00 (0.40 H (*Z*), t, *J* = 6.1 Hz, CH_2), 3.56-3.52 (3.20 H (*E*), m, 2 × CH_2), 3.48-3.46 (0.80 H (*Z*), m, 2 × CH_2), 3.03 (1.60 H (*E*) br s, NH_2), 2.98 (0.40 H (*Z*) br s, NH_2), 2.03 (1.60 H (*E*), qn, *J* = 6.4 Hz, CH_2), 1.94 (0.40 H (*Z*), qn, *J* = 6.4 Hz, CH_2); **^{13}C NMR** δ (200 MHz, DMSO), 166.21 (C) (*E*), 166.04 (C) (*Z*), 161.60 (C) (*E*), 156.46 (C) (*Z*), 153.55 (C) (*E*), 146.25 (C) (*E*), 146.19 (C) (*Z*), 135.51 (C) (*E*), 131.88 (C) (*Z*), 128.69 (2 × CH) (*E*), 128.40 (2 × CH) (*Z*), 124.87 (2 × CH) (*E*), 123.21 (2 × CH) (*Z*), 121.98 (2 × CH) (*E*), 118.95 (2 × CH) (*Z*), 115.19 (2 × CH) (*E*), 114.36 (2 × CH) (*Z*), 65.27 (CH_2) (*E*), 64.91 (CH_2) (*Z*), 47.63 (CH_2) (*E*), 47.56 (CH_2) (*Z*), 38.65 (CH_2) (*E*), 37.20 (CH_2) (*E*), 37.10 (CH_2) (*Z*), 28.01 (CH_2) (*E*), 27.96 (CH_2) (*Z*); ***m/z*** (ESI+) 368 ([M+H]$^+$, 60%) **HRMS**, (FAB, 3-NOBA), found [M+H+], 368.1844, $C_{18}H_{22}N_7O_2$, Requires 368.1835.

3-Azido-1-propylamine

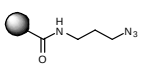


To a stirred solution of 3-chloropropylamine hydrochloride (560 mg, 4.31 mmol), dissolved in water (5 ml) sodium azide was added (840 mg, 12.0 mmol), and the mixture heated to 80 ºC. After 15 h KOH in pellets was added to basify the solution, followed by extraction with diethyl ether (3 × 5 ml). The combined organic phases were dried over magnesium sulphate and concentrated to give the desired amine as a colourless volatile oil (413 mg, 99% yield); $\boldsymbol{\nu_{max}}$ (neat)/cm^{-1} 2104 (N_3) **^{1}H NMR** δ (250 MHz, $CDCl_3$) 3.35 (2H, t, *J* = 6.8 Hz, CH_2NH), 2.90 (2H, t, *J* = 6.8 Hz CH_2N$_3$) 1.83 (2H, qn, *J* = 6.8 Hz $CH_2CH_2CH_2$) 1.35 (2H, br s, NH_2); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 49.12 (CH_2), 39.30 (CH_2), 32.43 (CH_2); ***m/z*** (ESI+) 101 ([M+H]$^+$, 68%), 76 (32), 58 (100).
All spectroscopic data were in good agreement with literature.[3]

Safety in the Handling of Sodium Azide and other Azides:[4] Sodium azide is toxic and can be absorbed through the skin. It decomposes explosively upon heating to above 275 °C. Sodium azide reacts vigorously with CS_2, bromine, nitric acid, dimethyl sulfate, and a series of heavy metals, including copper and lead. In reaction with water or Brønsted acids the highly toxic and explosive hydrogen azide is released. It has been reported that sodium azide and polymer-bound azide reagents form explosive di- and triazidomethane with CH_2Cl_2 and $CHCl_3$, respectively. Heavy-metal azides that are highly explosive under pressure or shock are formed when solutions of NaN_3 or HN_3 vapors come into contact with heavy metals or their salts. Heavy-metal azides can accumulate under certain circumstances, for example, in metal pipelines and on the metal components of diverse equipment (rotary evaporators, freeze drying equipment, cooling traps, water baths, waste pipes), and thus lead to violent explosions. Some organic and other covalent azides are classified as toxic and highly explosive, and appropriate safety measures must be taken at all times.

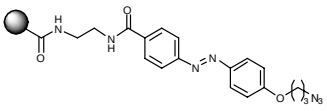
1.3 Solid support synthesis

Affi-Gel supported azide 5



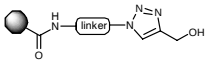
3-Azido-1-propylamine (562 mg, 5.62 mmol), was added to a solution of Affi-Gel 10 in isopropanol (25.0 ml, 0.375 mmol). The suspension was shaken gently for 48 h. The matrix was filtered, washed with MeOH (3 × 5 ml), water (3 × 5 ml), and stored in isopropanol (12.0 ml); ν_{max} (KBr)/cm^{-1} 2103 (N_3), 1656 (CO).

Affi-Gel supported azide 6



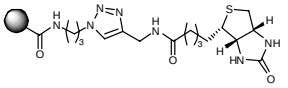
The hydrochloride salt of diazobenzene linker **1** (226 mg, 0.56 mmol) was dissolved in H_2O:acetonitrile (4 ml, 1:1) and Et_3N (81.0 µl, 0.56 mmol) was added. The mixture was added to a solution of Affi-Gel 10 in isopropanol (12.5 ml, 0.187 mmol) and the suspension was shaken gently for 24 h. The matrix was filtered, washed with MeOH (3 × 5 ml), water (3 × 5 ml) and stored in isopropanol (12.5 ml). ν_{max} (KBr)/cm^{-1} 2103 (N_3), 1660 (CO).

Affi-Gel supported alcohol 7



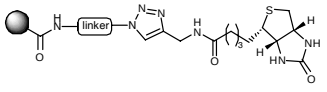
Affi-Gel supported azide **6** in isopropanol (10 ml, 0.150 mmol) was filtered, washed with MeOH (3 × 3 ml), water (3 × 3 ml) and resuspended in water/^{t}BuOH (10 ml, 1:1). Sodium ascorbate (6.0 mg, 0.03 mmol) and $CuSO_4$ (4.0 mg, 0.01 mmol) were dissolved in a minimal amount of H_2O and the solution was added to the suspended resin, followed by a solution of TBTA[5] (8.0 mg, 0.01 mmol) dissolved in ^{t}BuOH (0.5 ml). After stirring for 10 min propargyl alcohol (26 µl, 0.450 mmol) was added and the mixture was shaken gently for 48 h. The reaction was monitored by IR and stopped once the azide peak at 2100 cm^{-1} disappeared. The matrix was filtered, washed with MeOH (3 × 3 ml), H_2O (3 × 3 ml), EDTA (0.1 M, aq, 3 × 3 ml) and the matrix **7** was stored in isopropanol (12.5 ml); ν_{max} (KBr), 1626 (CO).

Affi-Gel supported biotin 9



Affi-Gel supported azide **5** in isopropanol (25.0 ml, 0.375 mmol) was filtered, washed with MeOH (3 × 5 ml), water (3 × 5 ml) and resuspended in water/^{t}BuOH (24.0 ml, 2:1). Propargyl biotin[6] (316 mg, 1.12 mmol), sodium ascorbate (14.8 mg, 0.075 mmol) and $CuSO_4$ (10.0 mg, 0.037 mmol) were dissolved in water (0.5 ml) and the solution was added to the suspended resin. The suspension was shaken gently for 48 h. The reaction was monitored by IR and stopped once the azide peak at 2100 cm^{-1} disappeared. The matrix was filtered, washed with MeOH (3 × 5 ml), water (3 × 5 ml), EDTA (0.1 M, aq, 3 × 5 ml) and matrix **9** was stored in isopropanol (25.0 ml); ν_{max} (KBr), 1638 (CO), 1618 (CO).

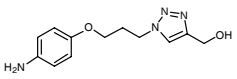
Affi-Gel supported biotin 10



Affi-Gel supported azide **6** in isopropanol (12.5 ml, 0.187 mmol) was filtered, washed with MeOH (3 × 5 ml), water (3 × 5 ml) and resuspended in water/^{t}BuOH (12 ml, 2:1). Sodium ascorbate (6.0 mg, 0.03 mmol) and $CuSO_4$ (4.0 mg, 0.01 mmol) were dissolved in water (0.5 ml) and the solution was added to the suspended resin, followed by a solution of TBTA[5] (8.0 mg, 0.01 mmol) dissolved in *t*BuOH (0.5 ml). After stirring for 10 min propargyl biotin was added (158 mg, 0.562 mmol) and the mixture was shaken gently for 48 h. The reaction was monitored by IR and stopped once the azide peak at 2100 cm^{-1} disappeared. The matrix was filtered, washed with MeOH (3 × 5 ml), water (3 × 5 ml), EDTA (0.1 M, aq, 3 × 5 ml) and matrix **10** was stored in isopropanol (12.5 ml); ν_{max} (KBr), 1651 (CO).

1.4 Dithionite release

{1-[3-(4-Amino-phenoxy)-propyl]-1*H*-1,2,3-triazol-4-yl}-methanol 8



Affi-Gel supported alcohol **7** in isopropanol (0.075 mmol, 5 ml) was filtered and resuspended in an aqueous solution of sodium dithionite, (3 ml, 0.3 M) and shaken gently for 10 min. The matrix was filtered and the procedure repeated twice. The cleavage was monitored by the change in colour of the matrix (from bright orange to colourless). The combined washings were extracted with ethyl acetate (3 × 5 ml) to give the aniline **8** as a colourless oil (13 mg, 70 % yield). **^{1}H NMR** δ (250 MHz, $CDCl_3$) 7.50 (1H, s, Ar*H*), 6.76 – 6.54 (4H, m, Ar*H*), 4.77 (2H, s, CH_2OH), 4.56 (2H, t, *J* = 6.8 Hz, CH_2O), 3.87 (2H, t, *J* = 6.0 Hz, CH_2N), 2.33 (2H, qn, *J* = 6.5 Hz, CH_2), 1.56 (2H, br s, NH_2); ***m/z*** (ESI+) 271 ([M+Na]$^+$, 30%), 249 ([M+H]$^+$, 100).

2 Preliminary Affinity Studies

2.1 Material and methods

SDS-PAGE materials and avidin were from Invitrogen; NuPAGE 4-12% Bis-Tris Gels (NP0321) were run according to manufacturers descriptions using 4×SDS loading buffer (NP0007) and 10×reducing agent (NP0004). Molecular weight markers were Precision Plus unstained standards from Bio-Rad (161-0363). Phosphate Buffered Saline (PBS) was prepared by dissolving PBS tablets from Oxoid (BR0014G) in distilled water. Avidin was from Invitrogen. All other reagents were from Sigma Aldrich and were of the highest available purity.

2.2 Competitive capture and release of avidin

Five affinity experiments were carried out in parallel as follows:
1: Compound **9** + avidin (classical elution method)
2: Compound **9** + avidin (chemical cleavage)
3: Compound **10** + albumin (classical elution method)
4: Compound **10** + avidin (classical elution method)
5: Compound **10** + avidin (chemical cleavage)

Matrix **9** or **10** in isopropanol (0.015 mmol, 1 ml) was transferred to a Falcon tube, pelleted by centrifugation (5 min at 3,000*g*) and the solvent was decanted. The matrix was washed with water (3 × 5 ml) and HEPES buffer (3 × 5 ml, 50 mM HEPES, pH 7.5, 150 mM NaCl). A solution of avidin (1 ml, 1 mg ml^{-1}) was added to matrix **9** (exp 1 & 2) and matrix **10** (exp 4 & 5), and a solution of albumin (1 ml, 1 mg ml^{-1}) was added to matrix **10** (exp 3). The matrix was further diluted with HEPES buffer (4 ml) and incubated for 30 min with gentle shaking. The matrix was pelleted and the supernatant collected and analysed by SDS-PAGE (Figure 2, Lanes A-E). The matrix was further washed with HEPES buffer (3 × 5 ml) prior to the elution protocol.
The matrix (exp 1, 3 & 4) was suspended in 2×SDS loading buffer (1 ml) and HEPES buffer (1 ml), heated for 10 min at 100 ºC, pelleted by centrifugation (2 min at 10,000*g*), and the supernatant collected. The supernatants were analysed by SDS-PAGE (Figure 2, Lanes F-H).
The matrix (exp 2 & 5) was divided into three aliquots which were treated with a freshly prepared solution of sodium dithionite (25 µl, 0.3 M) at three different pHs (6.5, 7.5 & 8.5, 50 mM KPO_4). The matrix was gently shaken for 10 min, pelleted by centrifugation (2 min at 10,000*g*) and the supernatant collected. The cleavage procedure was repeated and the supernatants were pooled, treated with 2×SDS loading buffer and heated for 10 min at 100 ºC. The supernatants were analysed by SDS-PAGE (Figure 2, Lanes I_1-J_3).

2.3 Capture and release of avidin from a protein mixture

Two affinity experiments were carried out in parallel as follows:
1: Compound **9** + avidin enriched FBS in PBS (classical elution method)
2: Compound **10** + avidin enriched FBS in PBS (chemical cleavage)

Matrix **9** or **10** in isopropanol (0.015 mmol, 1 ml) was transferred to a Eppendorf tube, pelleted by centrifugation (2 min at 10,000*g*) and the solvent was decanted. The matrix was washed with water (3 × 1.5 ml) and PBS buffer (3 × 1.5 ml). A solution of avidin enriched (100 µg ml^{-1}) 10 % FBS in PBS (1 ml) was added. The matrix was incubated with the protein mixture for 30 min with gentle shaking. The matrix was pelleted and the supernatant collected and analysed by SDS-PAGE (Figure 3, Lanes B & C). The matrix was washed with 4×PBS buffer (3 × 1.5 ml) prior to the elution protocol.
Matrix **9** was resuspended in 4×SDS loading buffer (100 µl) and 10×reducing agent (10 µl) and heated for 10 min at 100 ºC, centrifuged (2 min at 10,000*g*) and the supernatant collected. The supernatant was analysed by SDS-PAGE (Figure 3, Lane D).
Matrix **10** was incubated with a freshly prepared solution of sodium dithionite (25 µl, 0.3 M) at pH 6.5. The matrix was gently shaken for 10 min, pelleted by centrifugation (2 min at 10,000*g*) and the supernatant collected. The cleavage procedure was repeated and the supernatants were pooled, treated with 4×SDS loading buffer (25 µl) and 10×reducing agent (8 µl) and heated for 10 min at 100 ºC. The supernatants were analysed by SDS-PAGE (Figure 3, Lane E).

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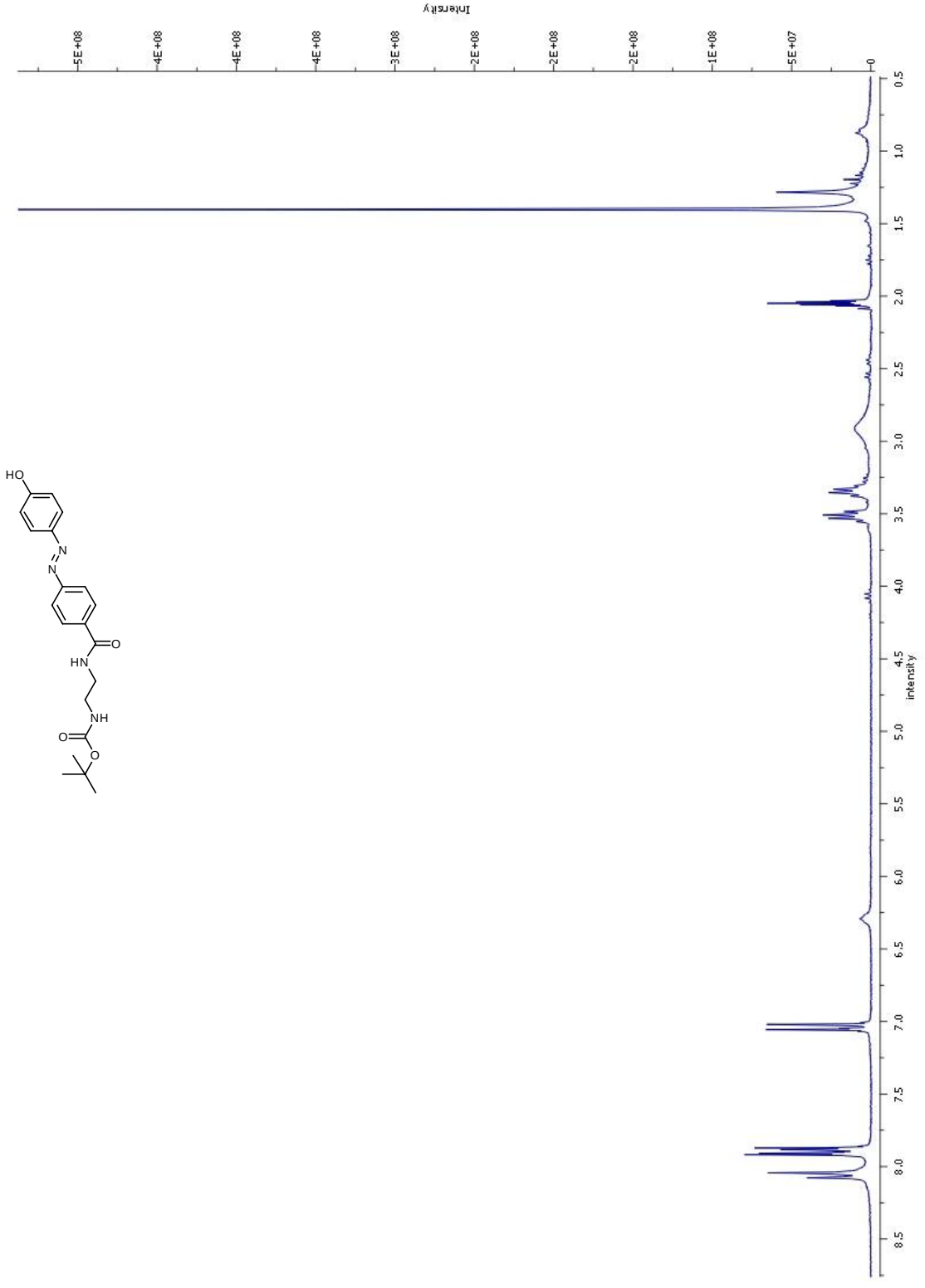
3 Spectra

2-[4’-(4’’-Hydroxy-phenylazo)-benzoylamino]-ethyl-carbamic acid *tert*-butyl ester



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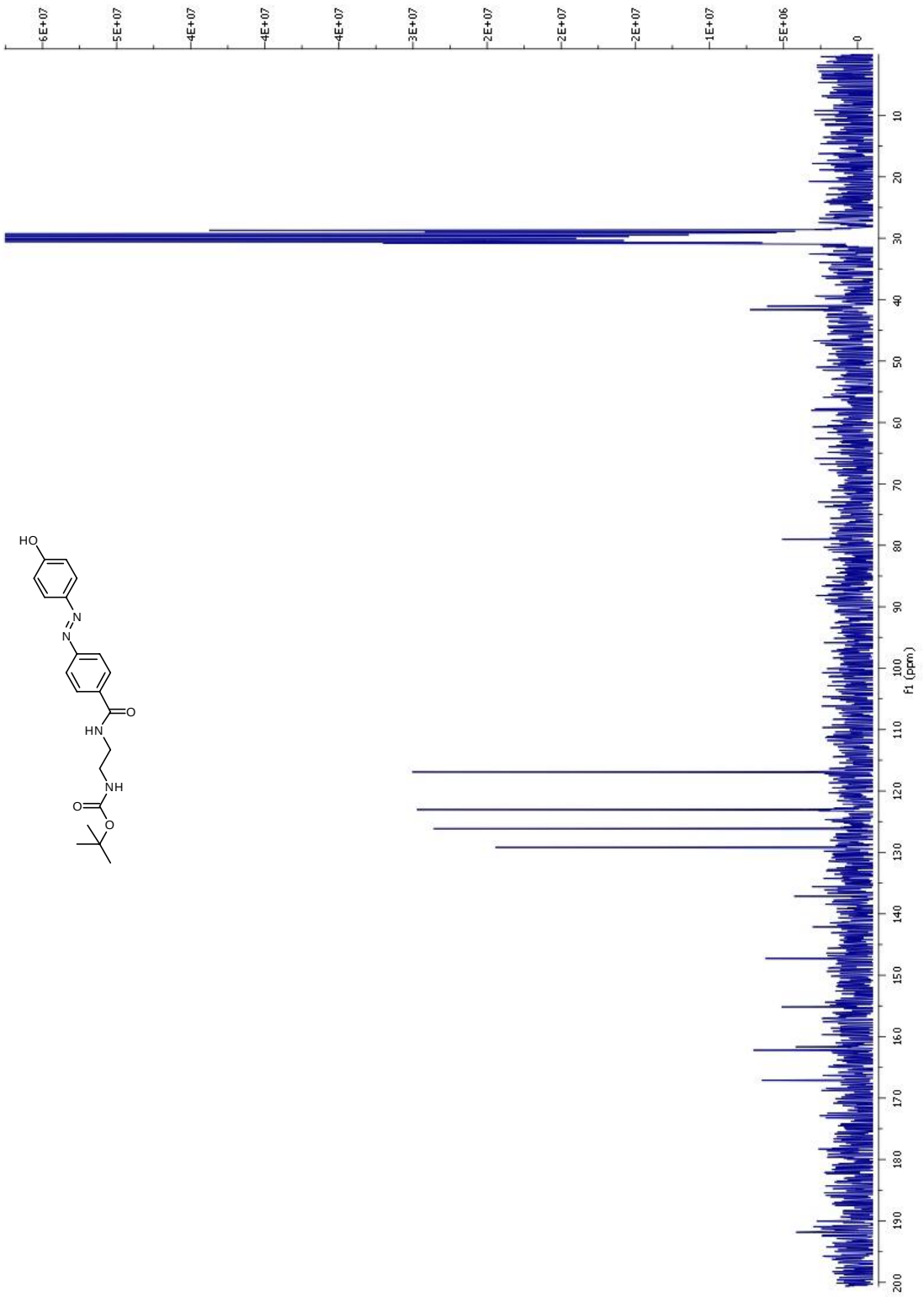
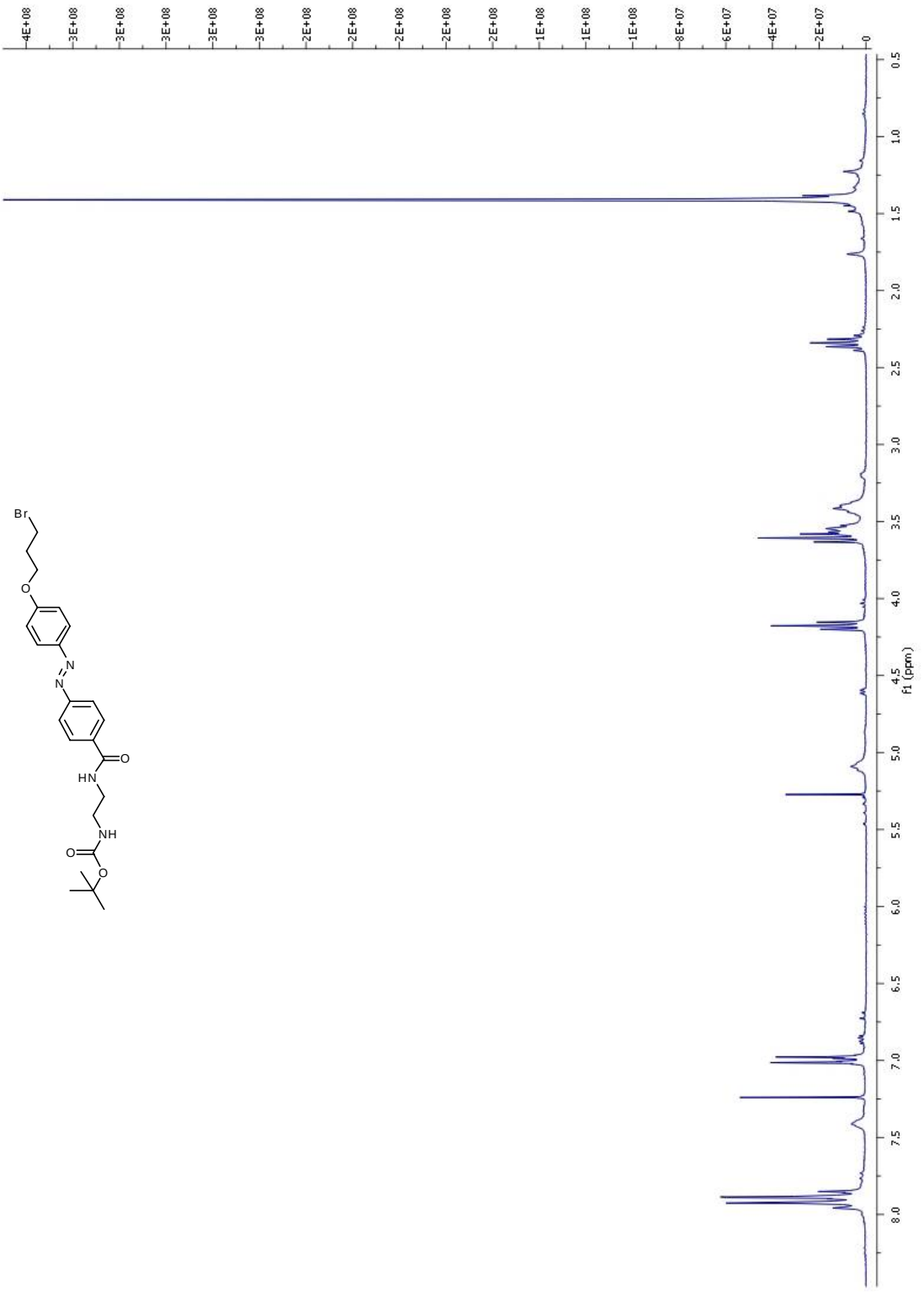


2-{4’’’-[4’’-(3’-Bromo-propoxy)-phenylazo]-benzoylamino}-ethyl)-carbamic acid *tert*-butyl ester **3**



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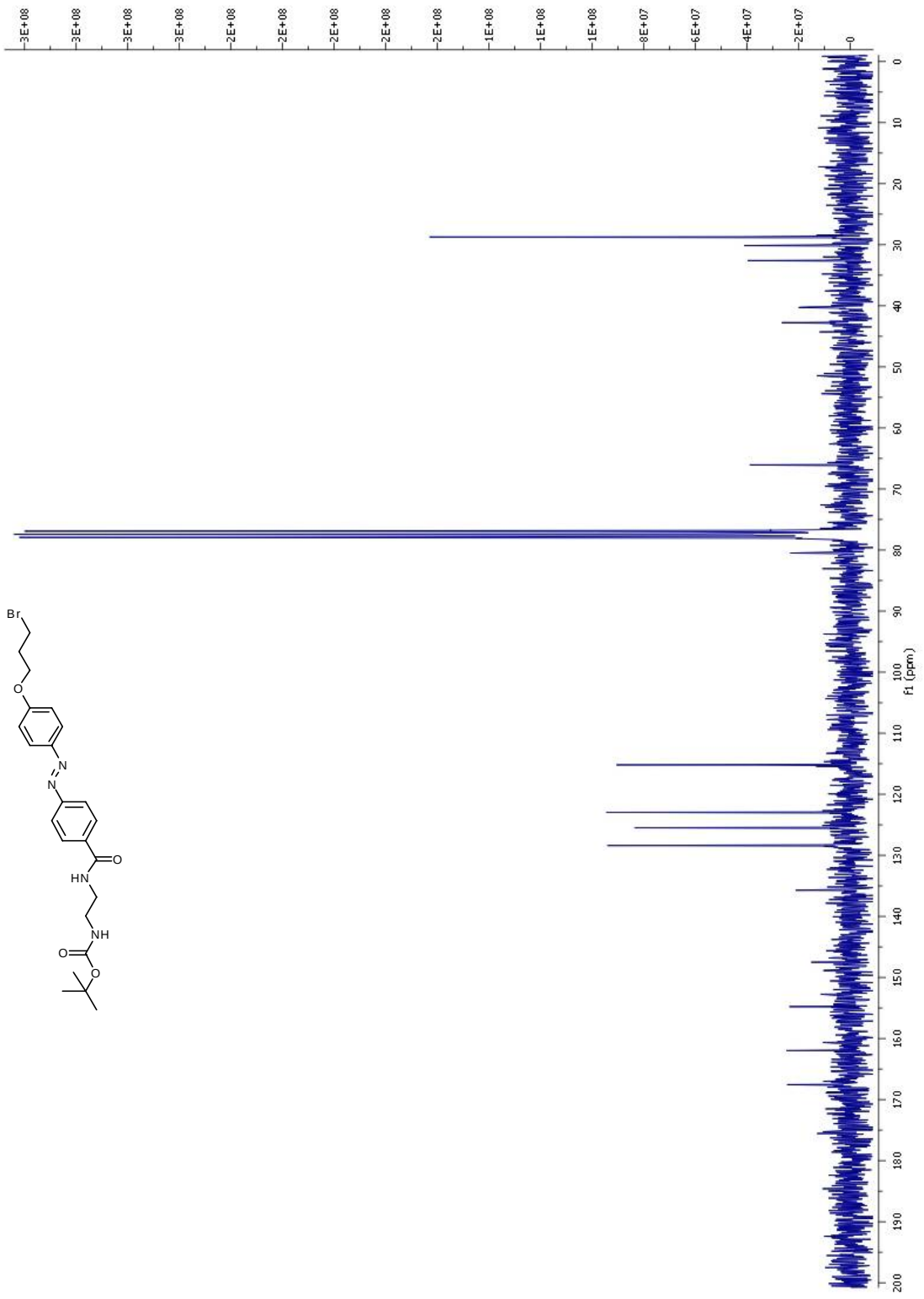
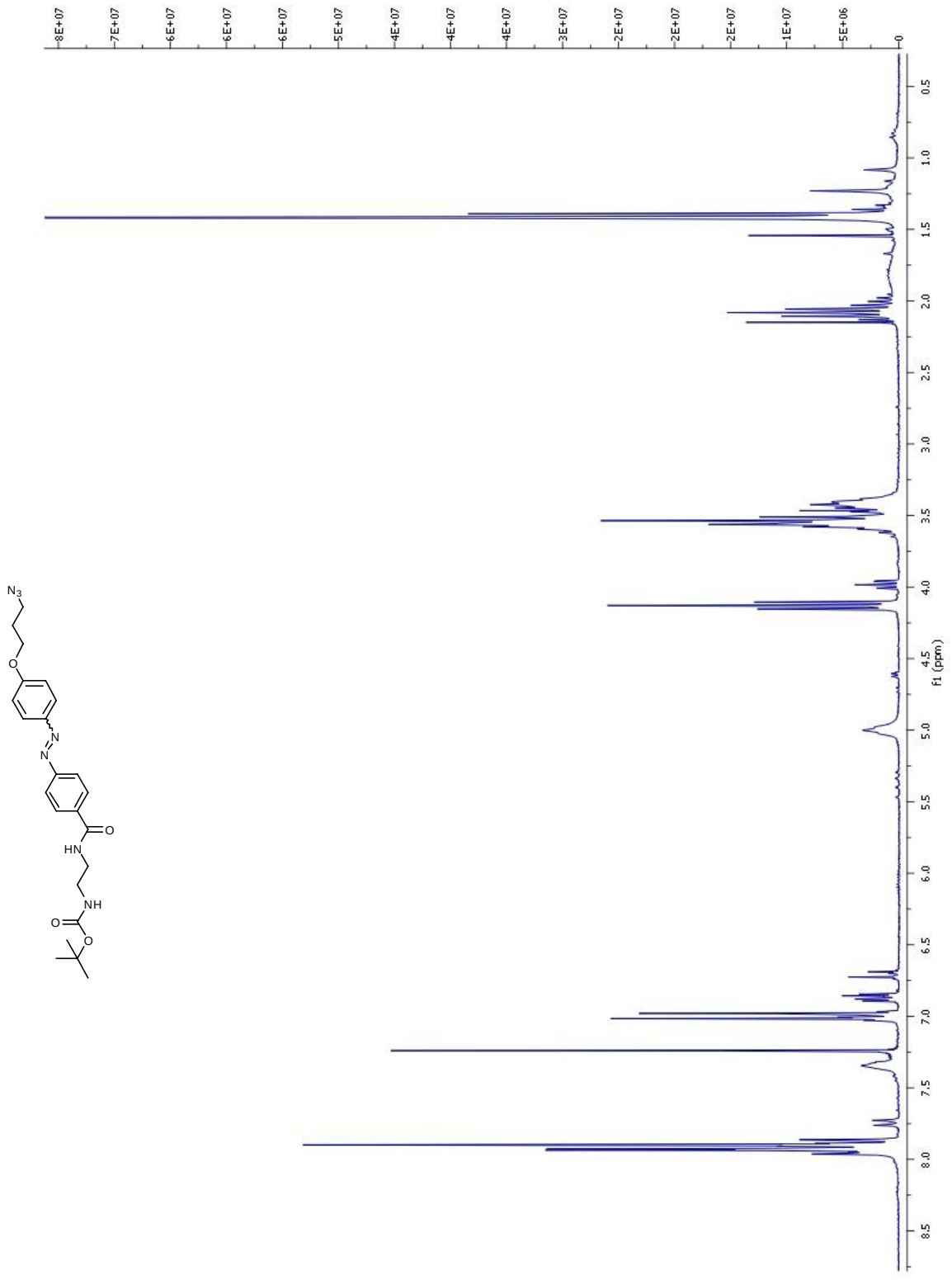
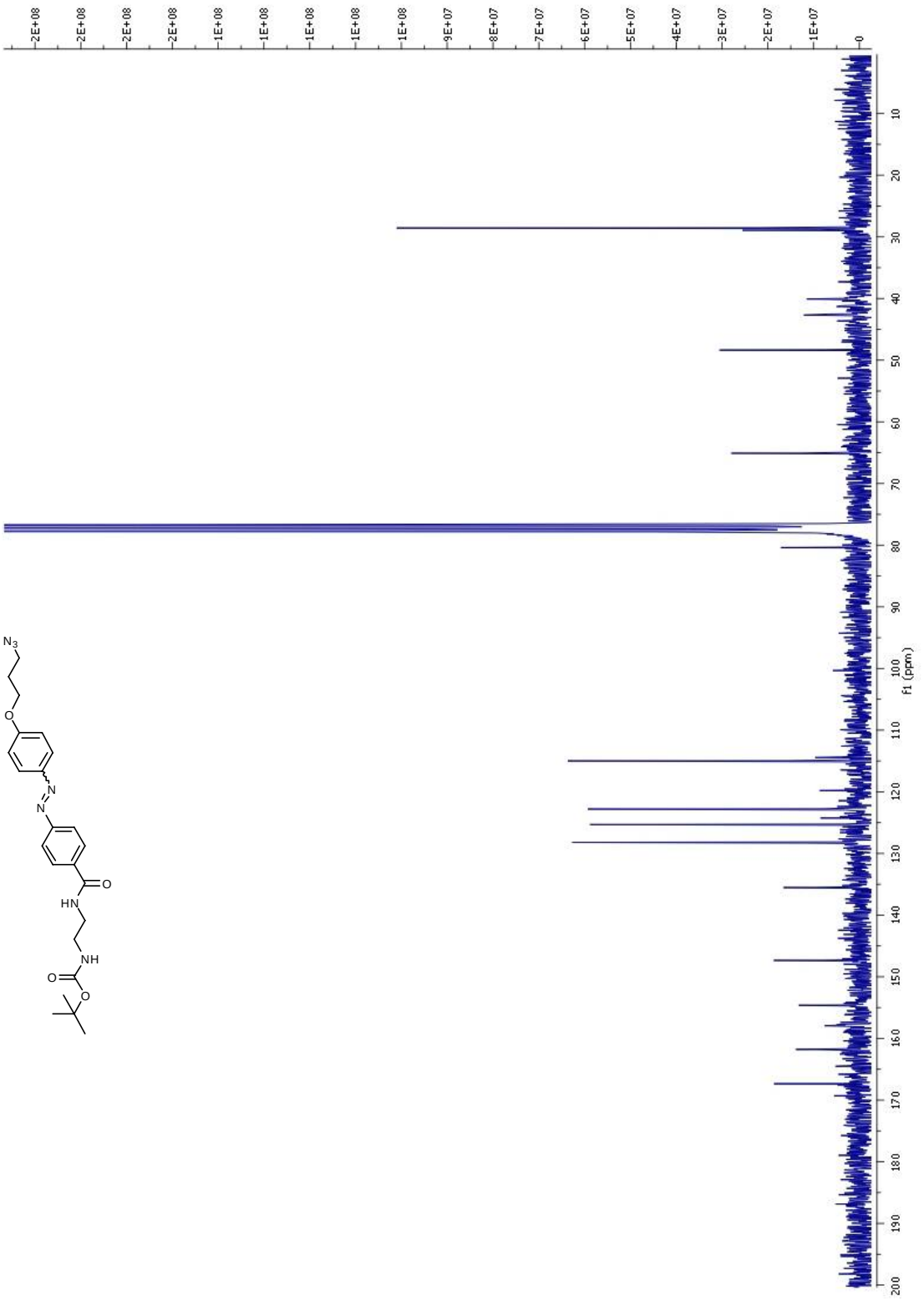


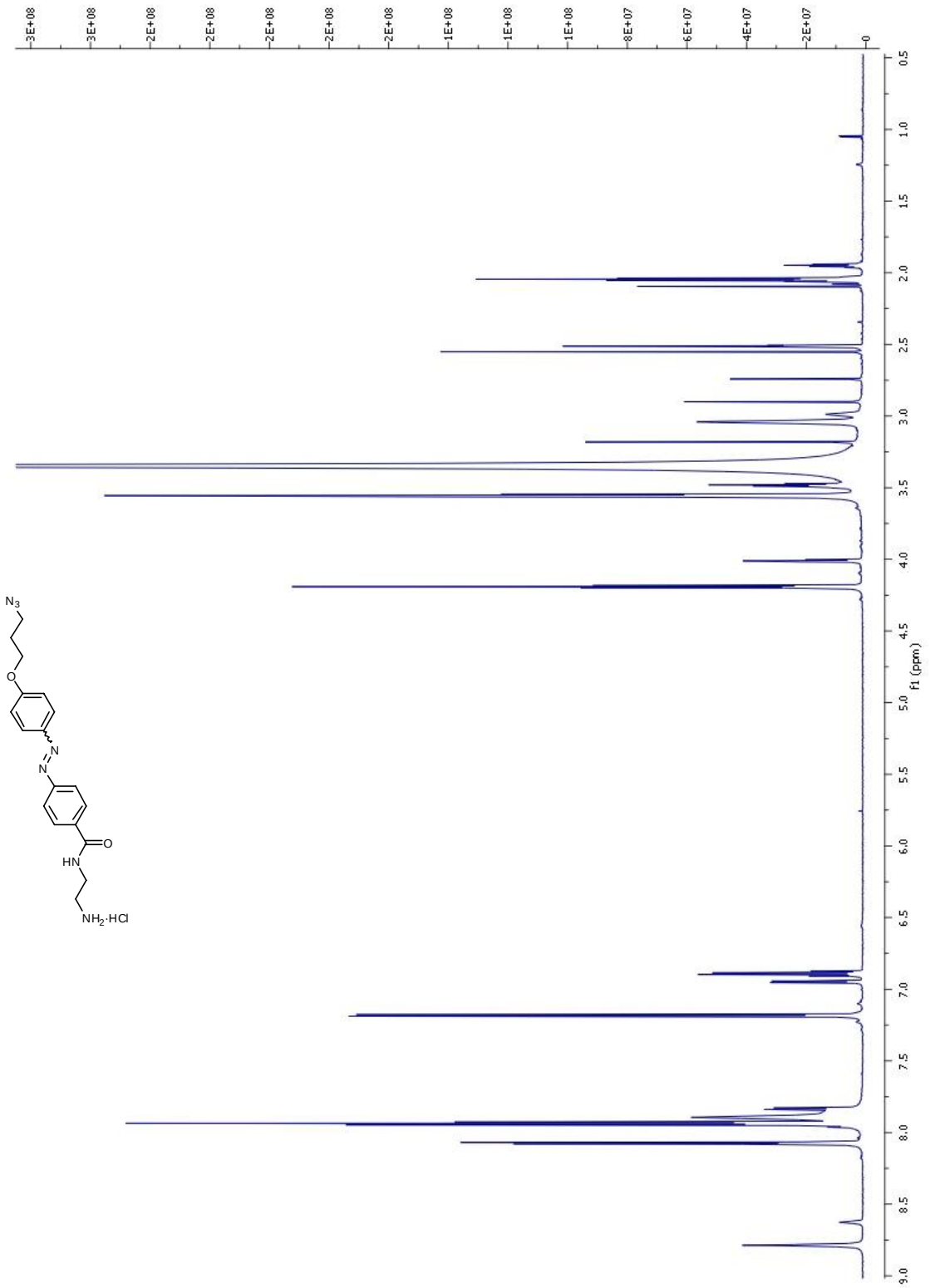
2-{4'''-[4''-(3'-Azido-propoxy)-phenylazo]-benzoylamino}-ethyl-carbamic acid tert-butyl ester **4**



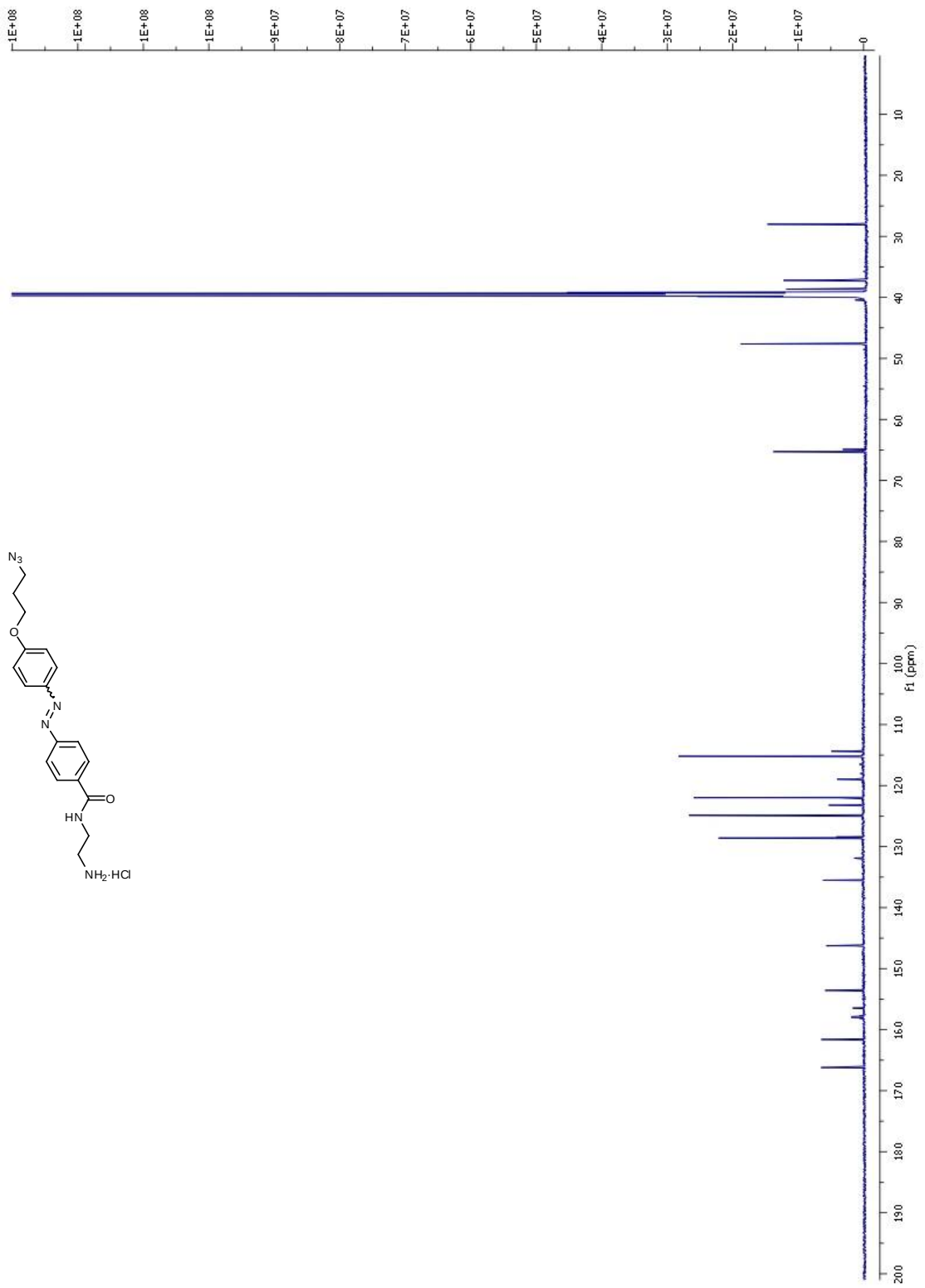
2-{4’’’-[4’’-(3’-Azido-propoxy)-phenylazo]-benzoylamino}-ethyl-carbamic acid tert-butyl ester **4**



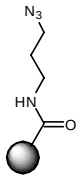
N-(2-Amino-ethyl)-4-{-2-[4-(3-azido-propoxy)-phenyl]-vinyl}-benzamide hydrochloride **1**

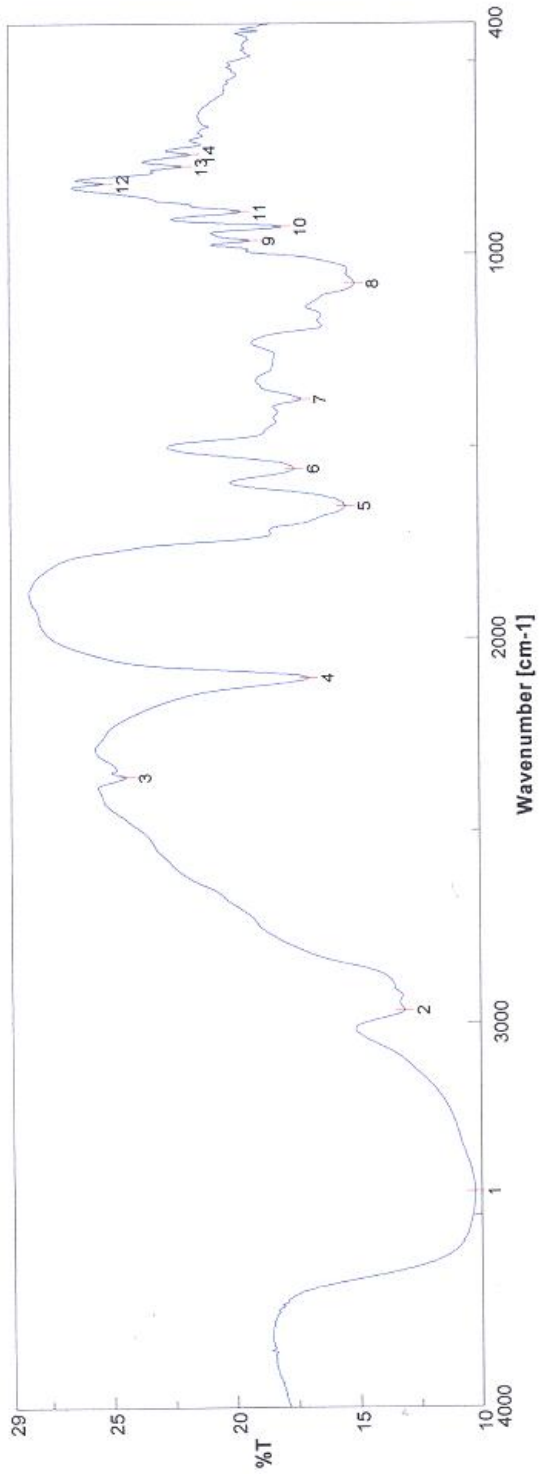


N-(2-Amino-ethyl)-4-{-2-[4-(3-azido-propoxy)-phenyl]-vinyl}-benzamide hydrochloride **1**



Affi-Gel supported azide **5**





Affi-Gel supported azide **6**