Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Click-enabled heterotrifunctional template for sequential bioconjugations.

David M. Beal*[§], Victoria E. Albrow, George Burlsem, Louisa Hitchen, Carla Fernandes, Cris Lapthorn, Lee R. Roberts, Matthew D. Selby, Lyn H. Jones[†]

Chemical Biology Group, Sandwich Laboratories, Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT13 9NJ

[†]Current address: WorldWide Medicinal Chemistry, Pfizer, 200 Cambridge Park Drive, Cambridge, MA 02140, USA.

[§]Current address: School of Biosciences, University of Kent, Canterbury, Kent, CT2 7NZ

E-mail: dmb38@kent.ac.uk

Supporting information

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General methods

All reagents were purchased from Sigma-Aldrich, Fluka, VWR, Expedion, Berry and Associates, Fluorous Technologies, Invitrogen, Pierce, and Charnwood Molecular, and used without further purification unless otherwise noted. Dry solvents were used as purchased from Sigma-Aldrich. Infrared (IR) spectra were obtained using a Nicolet Avatar 360 FTIR. Proton, carbon and fluorine nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR and ^{19}F NMR) were recorded on a Varian Mercury 400BB spectrometer with solvent resonance as the internal standard. ^{1}H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, BS = broad singlet, d = doublet, dd = doublet of doublets, dt = doublet of triplets, t = triplet, q = quartet, m = multiplet), coupling constants (Hz), and integration. LC-MS acquired on Waters ZQ ESCI single quadrupole LC-MS with an Agilent 1100 series consisting of a degasser, pump, column compartment and diode array detector using method A: 0.1 % formic acid in water; B: 0.1 % formic acid in acetonitrile; Column: Agilent Extend C18 phase 50 x 3mm with 3 micron particle size; Gradient: 95-0% A over 3.5 min, 1 min hold, 0.4 min re-equilibration, 1.2ml/min flow rate; UV: 210nm - 450nm DAD;Temperature: 50^{o}C. Accurate mass data was acquired on Thermo LTQ Orbitrap ESI LC-MS with an Agilent 1100 series consisting of a degasser, pump, column compartment and diode array detector - column: 360 µm OD, 75 µm ID pulled capillary column, packed with Reprosil C18 beads with a gradient from 95.5%, H_2O 0.5% acetic acid to 80% acetonitrile, 19.5% H_2O and 0.5% acetic acid. Mass spectra were acquired in positive ionization mode from 300 to 1850, using data dependent CID on the most intense ion from fullscan data. Bruker microTOF was also used according to manufacturer's guidelines for the collection of accurate mass data. Chromatographic purification was performed on ISCO Companion Combiflash using Redisep cartridges (normal phase 69-2203-304 etc and reverse phase 69-2203-410 etc. available from Teledyne ISCO) or using Merck silica gel 60 (0.040mm-0.63mm) 23-400 MESH, catalogue no. 1.09385 and the indicated solvent eluents. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} TLC glass plates. Visualisation was accomplished with UV light and aqueous potassium permanganate ($KMnO_4$) solution with heating. Yields refer to isolated yields of analytically pure material unless otherwise noted. The Western transfer was carried out using an Invitrogen i-blot (cat. IB1001) as per the supplier guidelines. Antibody staining of the gel was affected using Millipore SNAP i.d. system (cat. no. WBAVBASE) as per supplier guidelines. Size-exclusion chromatography was achieved using GE Healthcare illustra Nap 5 column (cat. No. 17-0853-01) following user instructions. Protein samples were concentrated using a Millipore Amicon ultra centrifugal filter device with a 3KDa cut-off (cat. no. UFC800324) following user instructions on a Beckman Coulter Allegra X-12R centrifuge. Protein conjugations were rolled using a Stuart Roller Mixer SRT6. The gels were fluorescently scanned using Fujifilm FLA-5000 according to manufacturer's instructions. Coomassie stained gel image was captured using an Amersham Biosciences Image Scanner according to the manufacturer's guidelines.

Abbreviations

DIPEA = Diisopropylethylamine

HATU = 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate Methanaminium

SATA = S-acetylthioacetic acid

TEA = Triethylamine

dPBS = Dulbecco's phosphate buffered saline

EDTA.2Na = Ethylenediamine tetraacetic acid disodium salt

SDS = Sodium dodecyl sulphate (used = NuPAGE LDS sample loading buffer 4x – NP0007, Invitrogen)

TCEP = Tris(2-carboxyethyl)phosphine

BSA = Bovine Serum Albumin

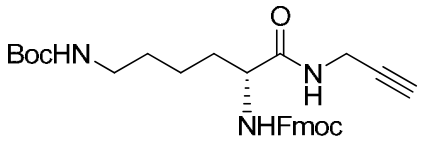
TFA = Trifluoro acetic acid

TIS = Triisopropylsilane

DMF = Dimethylformamide

Preparation of known intermediates

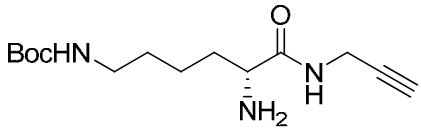
Fmoc-Lysine (Boc)-propargyl amide (**2**)



Known compound: M. Van Dijk, M. L. Nollet, P. Weijers, A. C. Dechesne, C. F. Van Nostrum, W. E. Hennink, D. T. S. Rijkers, and R. M. J. Liskamp *Biomacromolecules* **2008**, *9*, 2834-2843. Alternative method - ^{1}H NMR matches.

FmocLys(Boc)OH (20g, 1eq.) was dissolved in dichloromethane (214 ml), and stirred at ambient temperature under N_2. To this solution HATU (25.6g, 1.5eq.) was added followed by DIPEA (22.5ml, 3eq.), propargylamine (2.8g, 1.2eq.) and the reaction was stirred overnight. LC-MS analysis shows conversion to the desired product. The reaction mixture was washed with sat. $Na_2CO_{3(aq.)}$. The aqueous was backwashed with dichloromethane (x2) and the combined organics were dried over $MgSO_4$ and evaporated *in-vacuo* to leave an off white solid. This solid was preadsorbed onto silica and chromatographed on a 330g Redisep column eluting with a linear gradient from 1:1 heptane:ethyl acetate to ethyl acetate 100%. The appropriate fractions were evaporated *in-vacuo* to leave **2** as a white solid (13.7g, 60%). ^{1}H NMR consistent with literature values.

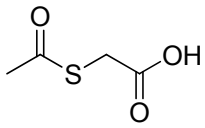
H-Lysine (Boc)-propargyl amide (**3**)



Known compound: M. Van Dijk, M. L. Nollet, P. Weijers, A. C. Dechesne, C. F. Van Nostrum, W. E. Hennink, D. T. S. Rijkers, and R. M. J. Liskamp *Biomacromolecules* **2008**, *9*, 2834-2843. Alternative method - ^{1}H NMR matches.

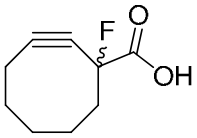
2 (5.8g, 1eq.) was partially dissolved in acetonitrile (150 ml) and stirred under N_2. 4-Methyl piperidine (10ml, 7.4eq.) was added, which was then stirred at ambient temperature under N_2 overnight. Stirring overnight caused the precipitation of a very fine white solid, the fullvene by-product from Fmoc deprotection. The resulting suspension was filtered and then concentrated *in-vacuo* to leave an off white solid. This material was further purified using a 100g plug of silica. Elution with ethyl acetate (~500ml) removed the residual fullvene. Subsequent elution with dichloromethane:methanol 4:1 gave the product. The appropriate fractions were then evaporated *in-vacuo* to leave an orange oil (2.5g, 91%), which was used without further purification.

2-(Acetylthio)acetic acid (SATA) (**18**)



Prepared according to *Eur. J. Org. Chem.* **2008**, 4277-4295. Identical by ^{1}H NMR. The material was distilled by Kugelrohr distillation before use.

1-Fluorocyclooct-2-ynecarboxylic acid (**19**)



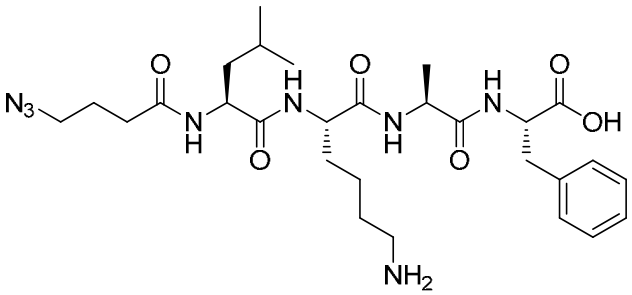
Prepared according to *Org. Lett.* **2010**, *12*, 2398-2401. ^{1}H NMR matches.

Preparation of Azide Reagents

Compounds **11** and **12** were synthesized according to the following general solid phase method.

In brief, the Fmoc protecting group of either Fmoc-Phe-Wang resin or Fmoc-Asp(OtBu)-Wang resin was removed by the treatment of 20% 4-methyl piperidine in DMF for 30 minutes, followed by three washes with DMF. A 0.16M solution of *N*-Fmoc-protected amino acid (4 eq.) and HATU (4 eq.) in DMF were added to the resin followed by DIPEA (4 eq.). The reactions were shaken for three hours and then washed three times with DMF. For each following step of the solid-phase peptide synthesis, the same deprotection and coupling reactions were performed. Capping of *N*-terminal amine was achieved by shaking the resin with a 0.16M solution of 4-azidobutanoic acid (4 eq.), HATU (4 eq.) and DIPEA (4 eq.) in DMF for 72 hours. The peptides were cleaved from the resin by treatment with a solution of 95% TFA / 2.5% TIS / 2.5% H_2O. After standing for one hour, the cleavage mixture was collected, and the resin was washed with the fresh cleavage solution. The combined fractions were evaporated to dryness and the product was purified using a 26g reverse phase RediSep cartridge eluting with a gradient from 99.9% H_2O + 0.1% formic acid to 99.9% acetonitrile + 0.1% formic acid. Fractions containing product were lyophilized to give white solids.

FAKL-N_3, Azidobutanamide-Leu-Lys-Ala-Phe-OH (**12**)



Starting scale = 0.4mmol

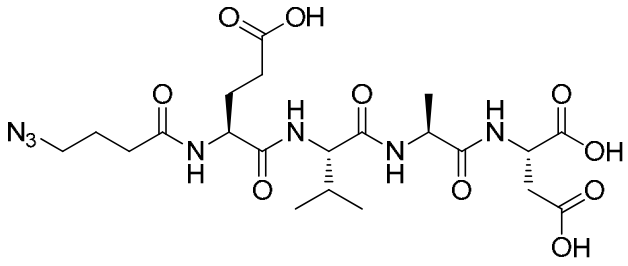
Yield = 38mg, 15%

Molecular Formula = $C_{28}H_{44}N_8O_6$

Monoisotopic Mass = 588.338381 Da

Observed – $[M+H]^+$ = 589.3445 Da $C_{28}H_{45}N_8O_6$ error = -1.8904ppm

DAVE-N_3, Azidobutanamide-Glu-Val-Ala-Asp-OH (**11**)



Starting scale = 0.4mmol

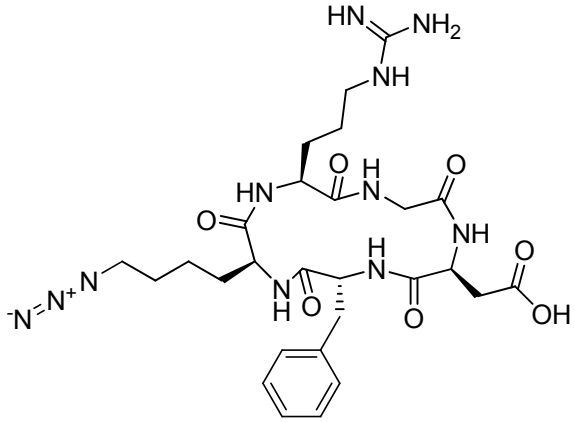
Yield = 63mg, 29%

Molecular Formula = $C_{21}H_{33}N_7O_{10}$

Monoisotopic Mass = 543.22889 Da

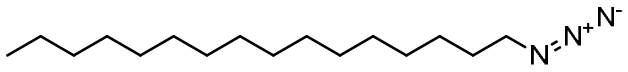
Observed – $[M+H]^+$ = 544.2355 Da $C_{21}H_{34}FN_7O_{10}$ error = -1.1612ppm

N-e-Azido cyclo(Arg-Gly-Asp-D-Phe-Lys) - RGD peptide (**10**)



Prepared according to *Org. and Biomolecular Chem*. **2007**, 5(6), 935-944

1-Azidohexadecane (**13**)

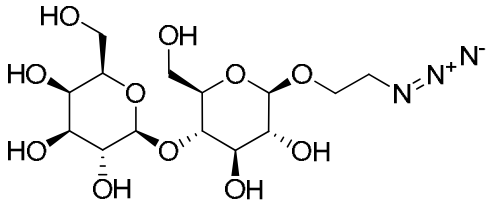


Hexadecylamine (Sigma-Aldrich, 445312, 205-596-8) (100mg, 0.414mmol) was dissolved in Methanol (3ml) and treated with K_2CO_3 (114mg, 0.828mmol) followed by the $CuSO_4.5H_2O$ (1mg, 0.004mmol). To this mixture Imidazole-1-sulfonyl Azide Hydrochloride[1] (104mg, 0.497mmol) dissolved in Methanol (2ml) was added and the reaction was stirred at ambient temperature under N_2 for two hours. The reaction was quenched by the addition of H_2O(3ml). This was acidified with acetic acid and then extracted into Heptane (2x4ml). The combined organics were dried over $MgSO_4$ and evaporated *in-vacuo* to leave a colourless gum (207mg, 93%)

1) Goddard-Borger, E. D.; Stick, R. V. *Org. Lett.* **2007** *9* 3797-3800

IR: (golden gate) 2921 (C-H), 2856 (C-H), 2092 (N=N$^+$=N$^-$), 1250 (N=N$^+$=N$^-$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ ppm 0.85 - 0.94 (m, 3 H, C**H$_3$**CH$_2$CH$_2$), 1.21 - 1.42 (m, 26 H, CH3C**H$_2$**C**H$_2$**.....), 1.56 - 1.69 (m, 2 H, CH$_2$C**H$_2$**CH$_2$N$_3$), 3.26 (t, *J*=6.9 Hz, 2 H, CH$_2$CH$_2$C**H$_2$**N$_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ ppm 14.1 (**C**H$_3$CH$_2$CH$_2$), 22.7 (CH$_3$**C**H$_2$CH$_2$), 26.7, 28.8, 29.2, 29.4, 29.5, 29.5, 29.6, 29.7 (br, unresolved), 31.9, 51.5 (CH$_2$CH$_2$**C**H$_2$N$_3$), all unassigned peaks attributed to CH_2 chain.

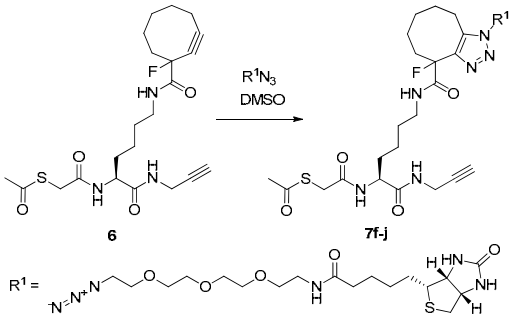
2-[2-(2-Azidoethoxy)ethoxy]ethyl a-D-Lactose (**14**)



Prepared according to *Synlett* **2006**, 3, 455-459. Identical by ^{1}H NMR

Click chemistry conditions

SPAAC conditions



Compound **7f –** from template **6** and compound **15**

Template **6** (10mg/ml concentration = 22mM, 100µl) in DMSO was treated with Biotin-TEG-azide **15** (available from Berry and Associates, BT 1085) in DMSO (1.1eq = 2.64µmoles, 54mM stock = 48µl) in DMSO. The reaction was rolled for six hours. LC-MS showed that the reaction had proceeded to completion. No purification was required before the next step was undertaken.

Molecular Formula = $C_{40}H_{62}FN_9O_9S_2$

Monoisotopic Mass = 895.409593 Da

Observed – $[M+H]^+$ = 896.4173 Da $C_{40}H_{63}FN_9O_9S_2$ error = 0.4762ppm

Compounds **7a**, **7b, 7e** and **7f** were prepared using these same conditions. Examples **7c, 7d, 7g, 7h, 7i, 7j** are repeats of these reactions. Examination of the HPLC traces for samples **7a** and **7e** shows a broadening and a change in the shape of the peaks. This change in peak shape is attributed to the sample being a mixture of the four products formed in the reaction. This alteration in shape does not allow for the determination of the ratios of the component products.

7a – Using Template **6** and compound **14**

Molecular Formula = $C_{36}H_{55}FN_6O_{15}S$

Monoisotopic Mass = 862.343013 Da

Observed –Electrospray ionisation$[M+H]^+$ = 86.60 Da and $[M+Na]^+$ = 885.60 Da and $[M-H]^-$ = 861.59 Da**,** Atmospheric pressure ionisation $[M+Na]^+$ = 885.21 Da and $[M-H]^-$ 861.76 Da

7b – Using template **6** and compound **10**

Molecular Formula = $C_{49}H_{69}FN_{14}O_{11}S$

Monoisotopic Mass = 1080.497497 Da

Observed – $[M+H]^+$ = 1081.5062 Da $C_{49}H_{70}FN_{14}O_{11}S$ error = 1.3420ppm

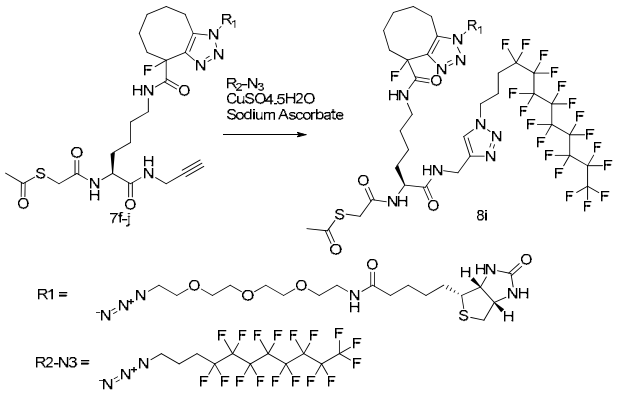
7e – using template **6** and compound **12**

Molecular Formula = $C_{50}H_{74}FN_{11}O_{10}S$

Monoisotopic Mass = 1039.532486 Da

Observed –Electrospray ionisation $[M+H]^+$ = 1040.77 Da and $[M+Na]^+$ = 1062.72Da and $[M-H]^-$ = 1038.82 Da**,** Atmospheric pressure ionisation $[M+H]^+$ = 1040.40 Da

CuAAC conditions



7f (34µl, 0.5µmoles) was treated with fluorous azide **16** (available from Flourous technologies BR017190) (1mg in 70µl, 2µmoles = 4eq.) was added followed by 0.5M sodium ascorbate (7µl = 7eq.) and 0.25M $CuSO_4.5H_2O$ (10µl = 5eq.). The reactions were rolled for two hours. On addition of the $CuSO_4.5H_2O$ the reaction turned bright yellow. LC-MS shows complete conversion to the desired product **8i**.

Molecular Formula = $C_{51}H_{68}F_{18}N_{12}O_9S_2$

Monoisotopic Mass = 1398.438619 Da

Observed –Electrospray ionisation $[M+H]^+$ = 1399.66.89 Da and $[M+Na]^+$ = 1421.65 Da

Examples **8i** and **8j** a larger excess of the second azide was used – due to the low solubility of the lipophilic azides in DMSO a 1,4-dioxane stock solution was used to solubilize the azides as well as increasing the molar equivalents used. For all other examples 2eq. of second azide was used.

8a – using **7a** and compound **10**

Molecular Formula = $C_{63}H_{94}FN_{17}O_{22}S$

Monoisotopic Mass = 1491.646406 Da

Observed – Atmospheric pressure ionisation $[M+H]^+$ = 1492.48 Da, Electrospray ionisation$[M+H]^+$ = 1492.89 Da and $[M-H]^-$ = 1490.86 Da

8b – using **7b/c/d** and compound **12**

Molecular Formula = $C_{77}H_{113}FN_{22}O_{17}S$

Monoisotopic Mass = 1668.835878 Da

Observed – $[M+2H]^{2+}$ =835.4240 Da $C_{77}H_{115}FN_{22}O_{17}S$ error = -1.5150ppm

8c - using **7b/c/d** and compound **17**

Molecular Formula = $C_{76}H_{96}FN_{20}O_{21}S_3$

Monoisotopic Mass = 1739.619955 Da

Observed – $[M+2H]^{2+}$ =870.3152 Da $C_{76}H_{97}FN_{20}O_{21}S_3$ error = 1.8028ppm

8d- using **7b/c/d** and compound **11**

Molecular Formula = $C_{70}H_{102}FN_{21}O_{21}S$

Monoisotopic Mass = 1623.726388 Da

Observed – $[M+2H]^{2+}$ =812.8704 Da $C_{70}H_{104}FN_{21}O_{21}S$ error = -0.0591ppm

8e - using **7e** and compound **11**

Molecular Formula = $C_{71}H_{107}FN_{18}O_{20}S$

Monoisotopic Mass = 1582.761376 Da

Observed – $[M+2H]^{2+}$ =792.3874 Da $C_{71}H_{109}FN_{18}O_{20}S$ error = -0.7251ppm

8f - using **7f/g/h/i/j** and compound **17**

Molecular Formula = $C_{67}H_{89}FN_{15}O_{19}S_4$

Monoisotopic Mass = 1554.53205 Da

Observed – $[M+2H]^{2+}$ =777.7711 Da $C_{67}H_{90}FN_{15}O_{19}S_4$ error = 1.7903ppm

8g - using **7f/g/h/i/j** and compound **10**

Molecular Formula = $C_{67}H_{101}FN_{20}O_{16}S_2$

Monoisotopic Mass = 1524.712985 Da

Observed – $[M+2H]^{2+}$ =763.3649 Da $C_{67}H_{103}FN_{20}O_{16}S_2$ error = 1.4389ppm

8h - using **7f/g/h/i/j** and compound **14**

Molecular Formula = $C_{54}H_{87}FN_{12}O_{20}S_2$

Monoisotopic Mass = 1306.558501 Da

Observed – $[M+2H]^{2+}$ =654.2855 Da $C_{54}H_{89}FN_{12}O_{20}S_2$ error = -1.5364ppm

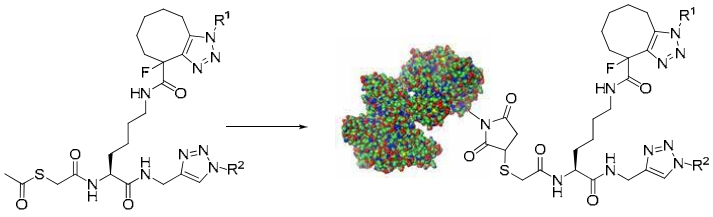
8j - using **7f/g/h/i/j** and compound **13**

Molecular Formula = $C_{56}H_{95}FN_{12}O_9S_2$

Monoisotopic Mass = 1162.677041 Da

Observed – $[M+H]^+$ =1163.93 Da and $[M+Na]^+$= 1185.93

Conjugation to BSA maleimide



8 **9**

BSA-Maleimide (Pierce – 77115, lot LG147033, SMCC activated to give 15-25 maleimide molecules per BSA molecule) 1mg was reconstituted in 100µl distilled water and then diluted to 1.5ml with dPBS. Aliquots of 75µl (74.5nmoles) were treated with 8.3µl of PBS+5M $NH_2OH.HCl$ +50mM EDTA.2Na (pH had been adjusted to pH4). To each aliquot 10µl of each DMSO template solution (**8a-8i**) was added and then the reaction was incubated on a roller for four hours.

The samples **9c** and **9f**, as representative samples of the array, were then subjected to size-exclusion chromatography using a GE Healthcare illustra Nap 5 column (cat. No. 17-0853-01), eluted with dPBS and then concentrated back to approximately 95µl volume using a Millipore Amicon ultra-centrifugal filter device with a 3KDa cut-off (cat. no. UFC800324).

Gel Analysis of conjugates

Analysis of the conjugates was then carried out using gel electrophoresis. The samples were treated with 30µl SDS + TCEP (10mM, aq.) and heated for five mins at 95°C. 15µl samples were run at 200V in MES buffer on

Invitrogen 4-12% NuPAGE bis-tris gel (NP0329), compared with fluorescent ladder (Invitrogen Benchmark™ Fluorescent standard, LC5928) and standard ladder (Novex® Sharp Protein Standard, LC5800).

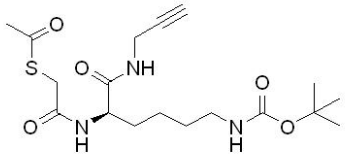
The gel was imaged using a fluorescent scanner prior to Coomassie staining.

Coomassie staining was affected with Expedion Instant Blue stain (cat. no. ISB1L), which was used according to the manufacturer's guidelines. The resulting gel was pictured using an Amersham Biosciences Image Scanner.

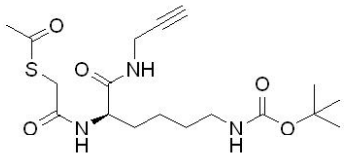
Fluorescent scan of gel: taken using Fujifilm FLA-5000 at 488nm.

Western blot: the gel electrophoresis was repeated for the biotinylated samples **9f**, **9g**, **9h**, **9i**, **9j.** These samples were run with five-fold dilution to the samples for the coomassie/fluorescence gel, under the same electrophoresis conditions, and were then subject to Western blot analysis. The gels were transferred onto membrane using Invitrogen i-blot (cat. No. IB1001) according to the manufacturer's instructions. The membrane was then blocked using skimmed milk powder and treated with Monoclonal Anti-Biotin Alkaline-Phosphatase antibody (produced in mouse-clone BN-34, purified immunoglobulin, buffered aqueous glycerol solution, Sigma-Aldrich, A6561) using Millipore Snap i.d. system (cat. No. WBAVBASE) according to manufacturer's instructions. Staining of membrane was then achieved using NBT-BCIP® solution (Sigma-Aldrich, cat.no. 72091,alkaline-phosphatase stain).

NMR data (^{1}H and ^{13}C)



Compound 4



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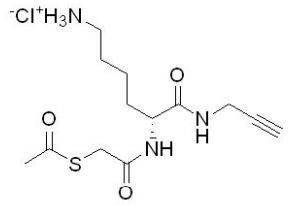
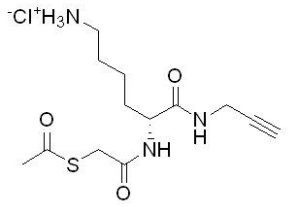
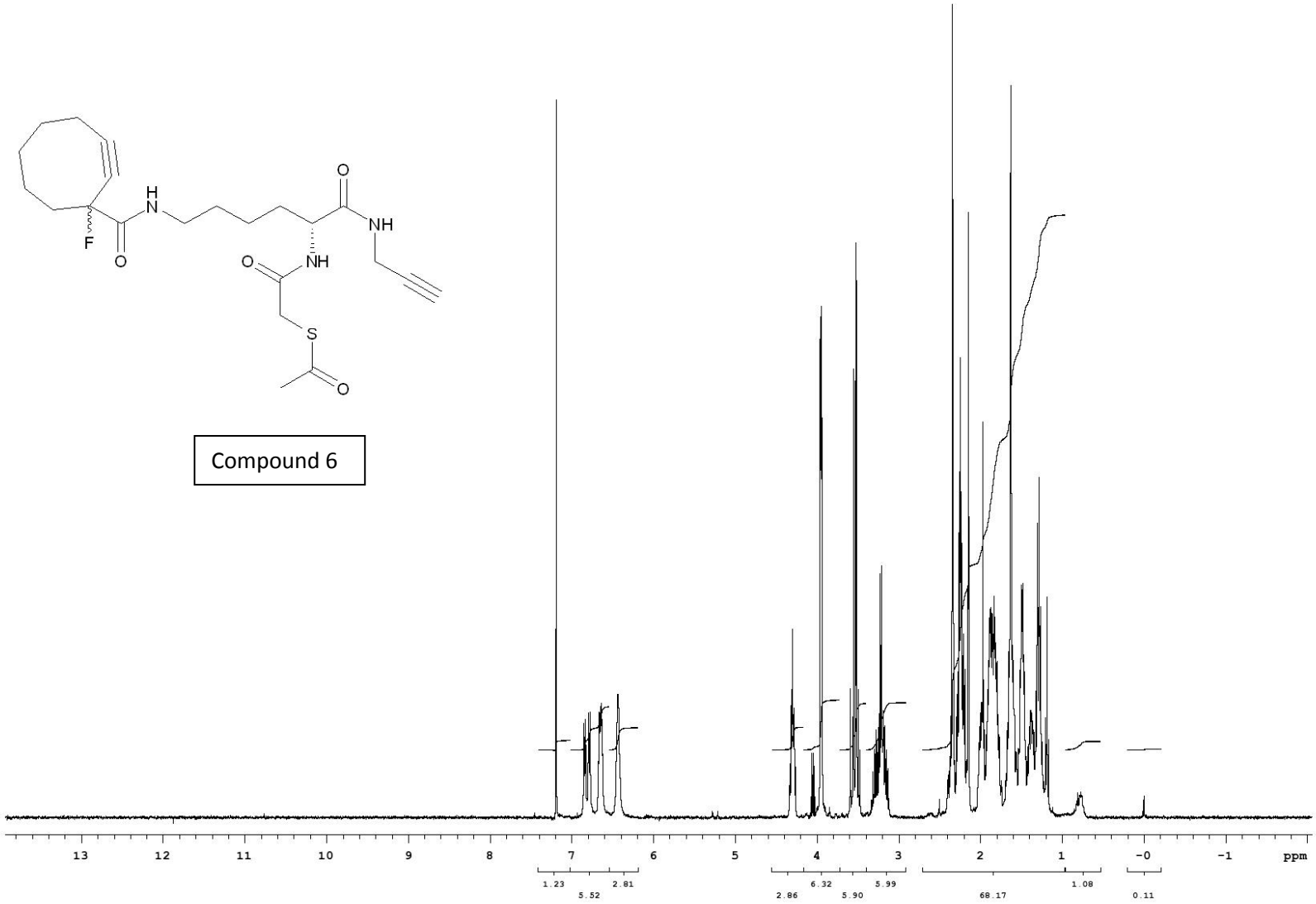


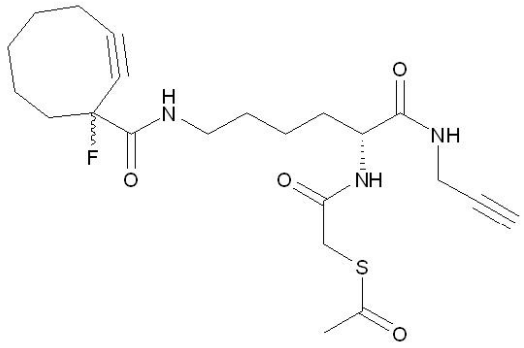
Compound 5



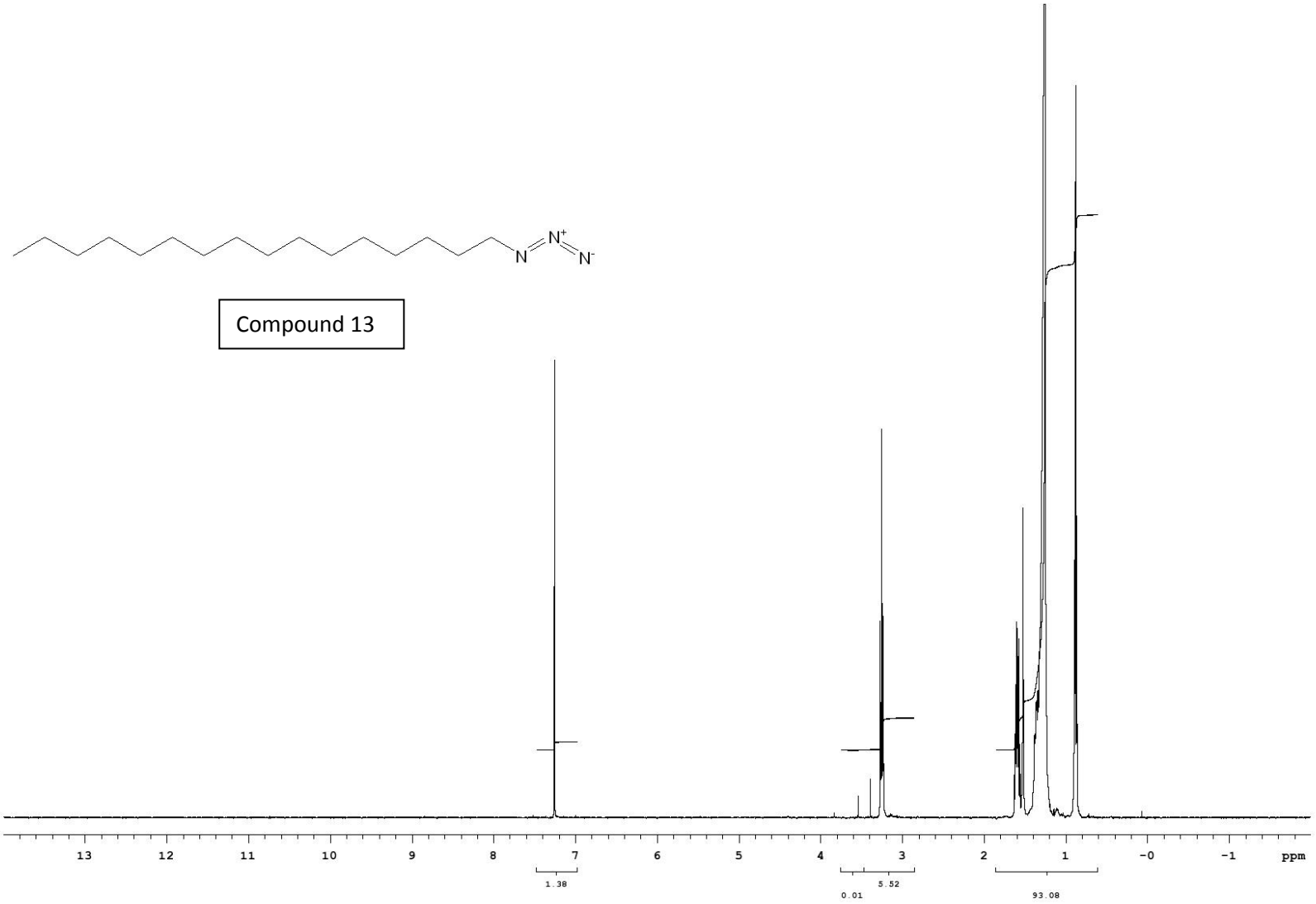
Compound 5



Compound 6



Compound 6



Compound 13