Supplementary information

"Click and go": simple and fast folic acid conjugation

Alexandre F. Trindade,[a,b] Raquel F.M. Frade,[b] Ermelinda M.S. Macoas,[a] Catia Graça,[a] Catarina A.B. Rodrigues,[a] José M.G. Martinho,[a] Carlos A.M. Afonso[b]

[a] *CQFM, Centro de Química-Física Molecular and IN - Institute of Nanosciences and Nanotechnology, Instituto Superior Técnico, 1049-001 Lisboa, Portugal. Fax: + 351 21 846 4455/7;*
[b] *iMed.UL, Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal. Fax: + 351 21 7946470;*

1 General Remarks

Synthesis

Dichloromethane was freshly distilled over calcium hydride prior to use, while diethyl ether, acetone and hexane were distilled once without drying agents. THF, DMSO and DMF were used without any purification. Folic acid was used in dehydrate form, purchased from Alpha-Aesar. N-hydroxysuccinimide DIPEA, fluorescein isothiocyanate, dansyl chloride were purchased for Aldrich. BCN N-hydroxysuccinimide carbamate was purchased from Synaffix, MeO-PEG_{350}-OH from Clariant and ethanolamine from Panreac. DCC was distilled in vacuum and stored in Schlenk under Argon. Rhodamine 6G (4-(chloromethyl)phenyl)methyl ether was synthethized according a reported procedure.[1]

The reactions involved in the preparation of the organic azides were analysed by TLC using ALUGRAM® SIL G/UV_{254} from MN (Ref. 818133, silica gel 60), and visualisation of TLC spots was effected using UV and phosphomolybdic acid solution, and flash chromatography were carried out on silica gel 60 M purchased from MN (Ref. 815381). NMR spectra were recorded in a Bruker AMX 400 and 300 MHz using $CDCl_3$ and DMSO-d^6 as solvent and $(CH_3)_4Si$ (^1H) as internal standard. Any coupling constants are expressed in Hz. High Pressure Liquid Chromatography for the determination of folate conjugation selectivity was carried out using a Dionex P680 pump equipped with a diode array detector Dionex UVD340S and Phenomenex Gemini 5u C18 110A. Acetonitrile and water employed were HPLC grade. Injection of samples dissolved in DMSO, 1 ml/min, 0 to 5.5min 100% water, linear gradient from 5.5min at 100% water to 15.5min at 100% acetonitrile. Electrospray ionization (ESI) mass spectra were recorded in a mass spectrometer (Micromass Quattro Micro API, Waters, Ireland) with a Triple Quadrupole (TQ) and with an electrospray ion source operating in positive and negative mode.

Biological essays

Hydrophobic, uncoated and sterile 1 -Slide 8 well (Ref.80821) from ibidi GmbH, sterile-filtered Poly-L-lysine solution mol wt 70,000-150,000 (Ref. P4707) from Sigma, staining WGA-Alexa594 (Ref. W11262) from Invitrogen, RPMI-1640 medium with (Ref. 8758) and without phenol red (Ref. 8755) from Sigma, inactivated fetal-bovine-serum (FBS) (LTID 10082-147) from Alfagene, antibiotic antimycotic solution (Ref. A5955) from Sigma and Trypsin-EDTA solution (Ref. T4174) from Sigma. Human non-small cell lung cancer (NCI-H460) and breast cancer (MCF-7) cell lines and non-tumoral kidney embryonic (HEK 293) cell line were purchased from ATCC.

Fluorescence Microscopy Imaging

Imaging was carried on in a Leica TCS-SP5 Multiphoton/Confocal Fluorescence Microscope equipped with a continuous Ar ion laser (458, 476, 488, 496 and 514 nm) and a Helium-Neon laser (633 nm). The images shown in the discussion are an overlaid of two images that where collected simultaneous by using the two excitation sources and collecting the emission at two different photomultiplying tube. Emission of either the folate targeting probe and the free probe was collected in the 530-580 nm region upon excitation at 476 nm, and the emission of the membrane marker was collected in the 670-730 nm region upon excitation at 633 nm. The emission band was selected by the spectrophotometer detector system in the microscope that uses a prism in combination with adjustable slits to limit the band path at the detector.

Spectroscopic Methods

The linear absorption spectra were recorded on a Jasco V-660 UV-vis spectrophotometer, and the fluorescence measurements were obtained on a Horiba Jobin Yvon Fluorolog 3-22 Spectrofluorimeter. The spectra were recorded in mol solutions prepared in spectroscopic grade DMSO. The time-resolved emission fluorescence measurements were performed by time-correlated the single photon timing technique counting (TCSPTC) using the second harmonic of a femtosecond Ti:Sapphire laser (350-470 nm) as an excitation source and a Hamamatsu R2809U-01 MCP-PMT (290-700nm).

[1] Carlos A. M. Afonso, V. Santhakumar, Alan Lough, Robert A. Batey, *Synthesis*, **2003**, 17, pp 2647

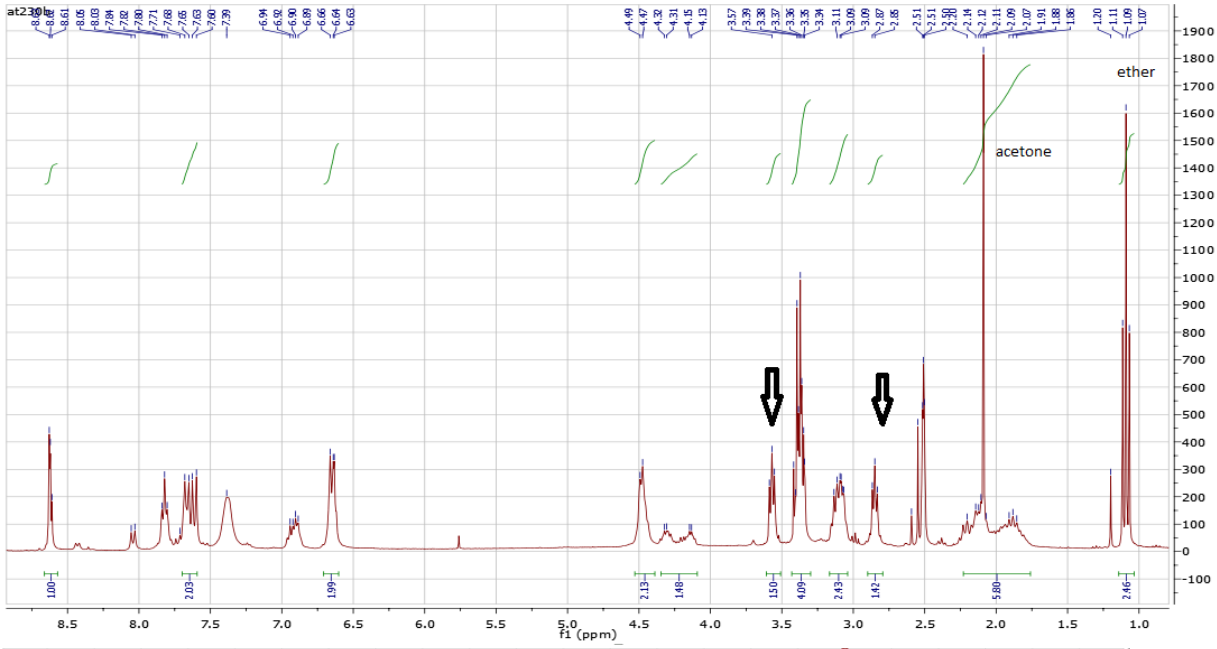
2 Selectivity screening in folic acid activation

In a round-bottomed flask was dissolved x eq. folic acid in 4 ml of DMSO. After the dissolution was complete (about 30 minutes with mild heating), Z eq. of N-hydroxysuccinimide and Y eq. of DCC were added successively. The reaction mixture was stirred for the given time at room temperature, after which the urea precipitates. Then, it was added 5 eq. of ethanol amine relatively to folic acid. The mixture was stirred 4 hours, filtered and a mixture of 20% acetone in diethylether was added. The formed thin yellow precipitated was carefully centrifuged and washed four times with acetone and two with diethyl ether and dried under vacuum. The sample was analyzed in RP-HPLC (injection of sample dissolved in water, 1 ml/min, 0 to 5 min 5% acetonitrile/water, linear gradient from 30 min at 50% acetonitrile/water, Gemini 5u 18 110A, 5umx4.6x250mm, Phenomenex column).

Spectra of isolated Folate-ethanolamine obtained after isolation from a reaction using two equivalents of ethanol amine.

^{1}H NMR (300 MHz, DMSO) δ: 8.63 (s, 1H, pterin), 8.05-7.82 (m, 2H), 7.68-7.60 (m, 2H, aromatic), 7.39-6.89 (m, 3H), 6.66-6.63 (d, 2H, aromatic), 4.49-4.47 (bs, 2H, benzylic), 4.32-4.13 (m, 1H, αH), 3.38- 3.34 (m, 2H, ethanolamine), 3.11-3.09 (m, 2H, ethanolamine), 2.20-1.86 (m,4H, glutamic moiety).

^{13}C NMR (75 MHz, DMSO) δ 175.93, 172.76, 172.38, 166.61, 165.91, 162.34, 156.82, 155.24, 151.04, 148.86, 148.67, 129.49, 129.00, 128.43, 122.57, 121.86, 111.77, 111.63, 60.37, 60.32, 58.35, 41.95, 41.85, 32.68, 28.22.



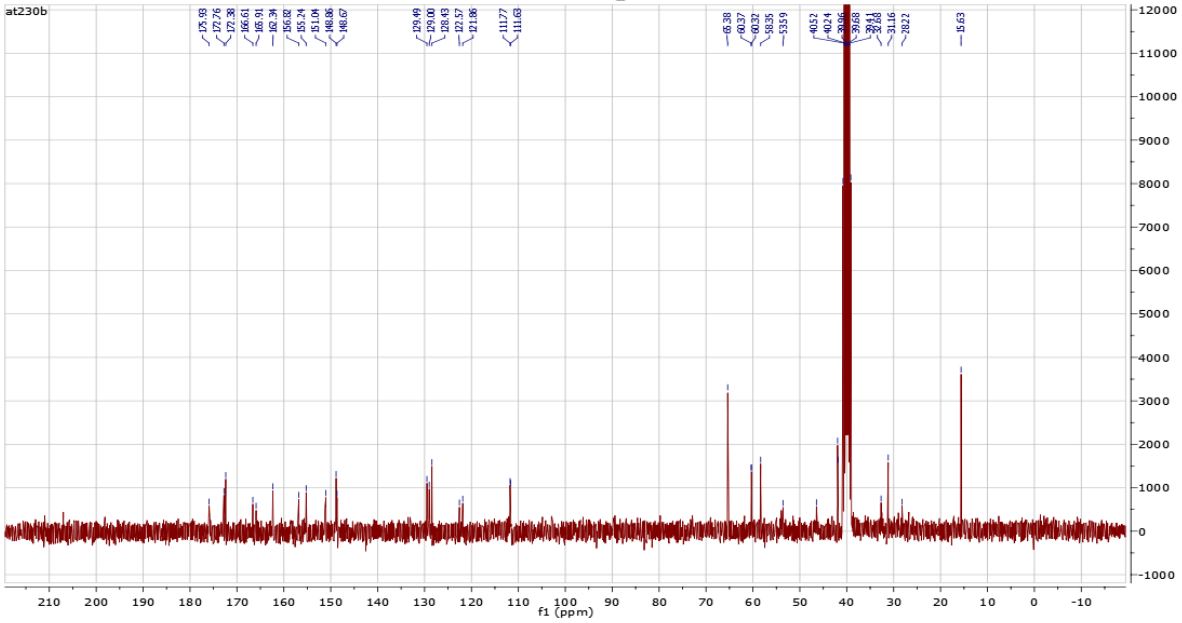
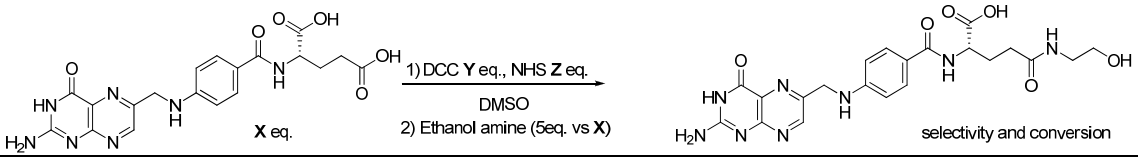


Table 1 – Optimization of folic acid conjugation to ethanol amine



Entry	Folic acid X eq.	DCC Y eq.	NHS Z eq.	Base (2 eq.)	Conversion[a]	Selectivity[s]
1[c]	1	1	1	-	67	88
2[c]	1	2	1	-	98	87
3[c]	1	2	2	-	96	92
4[c]	1	1	2	-	65	92
5[c]	2	2	1	-	48	88

6[c]	2	1	2	-	33	84
7[c]	2	1	1	-	27	85
8[d]	1	2	1	-	96	91
9[d]	1	2	0	-	89	48
10[d]	1	2	1	Pyridine (100 eq.)	96	92
11[d]				Pyridine	99	90
12[d]				TEA[e]	26	90
13[d]				DBU[f]	-[g]	-
14[d]				DIPEA[h]	21	94
15[d]				DMAP[i]	83	82
16[d]				DABCO[j]	42	73
17[d]				TOA[k]	92	88
18[d]				1,6-lutidine	94	84
19[d]				K_2CO_3	6	56
20[d]				KOH	74	87
21[d]				[t]BuOK	2	61
22[d]				MgO	85	86
23[d,l]				-	91	88

a) Determined by RP-HPLC (C18 Luna Phenomenex) and represents the percentage of new compounds formed relatively to initial folic acid amount; b) percentage of the γ-carboxylic amine obtained relatively to other products formed; c) 35ºC (temperature close to the ideal for protein functionalization); d) 25ºC; e) triethylamine; f) 1,8-Diazabicycloundec-7-ene; g) complex mixture was obtained; h) diisopropylethyl amine; i) 4-dimethylaminopyridine; j) 1,4-diazabicyclo[2.2.2]octane; k) trioctylamine; l) non-distilled DCC and without prior dissolution of folic acid.

Table 2 – Kinetic study in the activation of folic acid with DCC and NHS method

Entry	Time (h)	Conversion[a]	Selectivity[s]
1[c]	2	65	86
2[c]	4	84	86
3[c]	6	89	84
4[c]	17	96	91

a) Determined by RP-HPLC (C18 Luna Phenomenex) and represents the percentage of new compounds formed relatively to initial folic acid amount; b) percentage of the γ-carboxylic amine obtained relatively to other products formed; c) 25ºC, FA/DCC/NHS ratio of 1/2/1.

3 Kinetic studies

3.1 NMR studies on the conjugation of NHS-BCN mixed carbonate into folate ethylenediamine

To a solution of ethylenediamine-folate (3,5mg) in DMSO-d[6] was added diisopropylethyl amine (2.6ul, 2.5eq). ^{1}H NMR spectra was recorded. Then the solution of (1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (1.9 mg in 0.3 ml DMSO-d[6], 1.1 eq) was added to achieve the stoichiometry described below. ^{1}H NMR spectra were recorded after each addition.

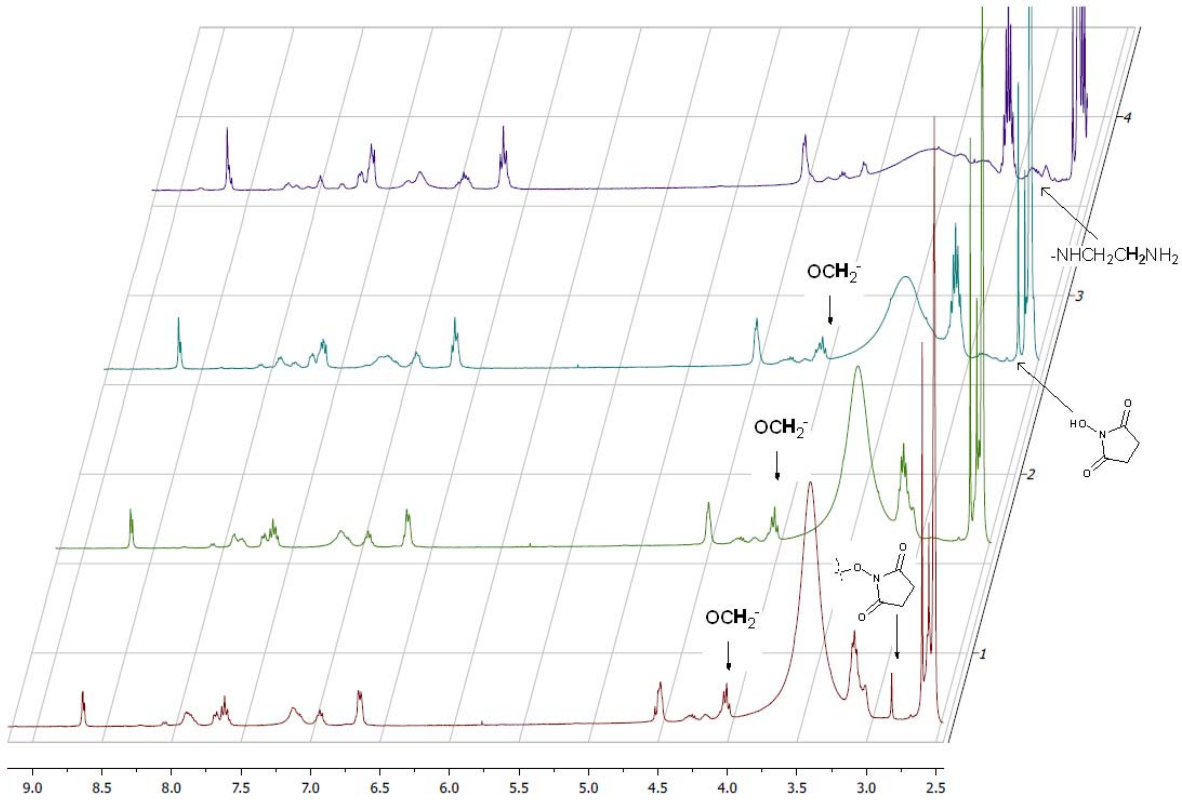


Figure 1 – from top to bottom: ethylene diamine folate + 3eq.DIPEA; ethylene diamine folate + 3eq.DIPEA + 0.5 eq BCN-CO-NHS; ethylene diamine folate + 3eq.DIPEA + 1 eq BCN-CO-NHS; ethylene diamine folate + 3eq.DIPEA + 1.05 eq BCN-CO-NHS. Peak at 4ppm $OC\underline{H}_2$ from BCN; broad multiplet at 2.84 ppm from $-NHCH_2C\underline{H}_2NH_2$; singlet at 2.80 ppm from aliphatic NHS protons in carbonate; singlet at 2.60 ppm from free aliphatic NHS protons.

3.2 NMR studies on the copper-free cycloaddition of benzylazide and N_3-PEG_{350}-OMe into folate-ethylenediamine-BCN at 25ºC

To the previous solutions was added at once 2 mg of benzyl azide or 3.5 mg of N_3-PEG_{350}-OMe (2.2 equivalents) at 25ºC. Several ^{1}H NMR spectra were collected to access when the cycloaddition reaction was complete. Below are the crude spectra when the yield of triazole reached >95 %.

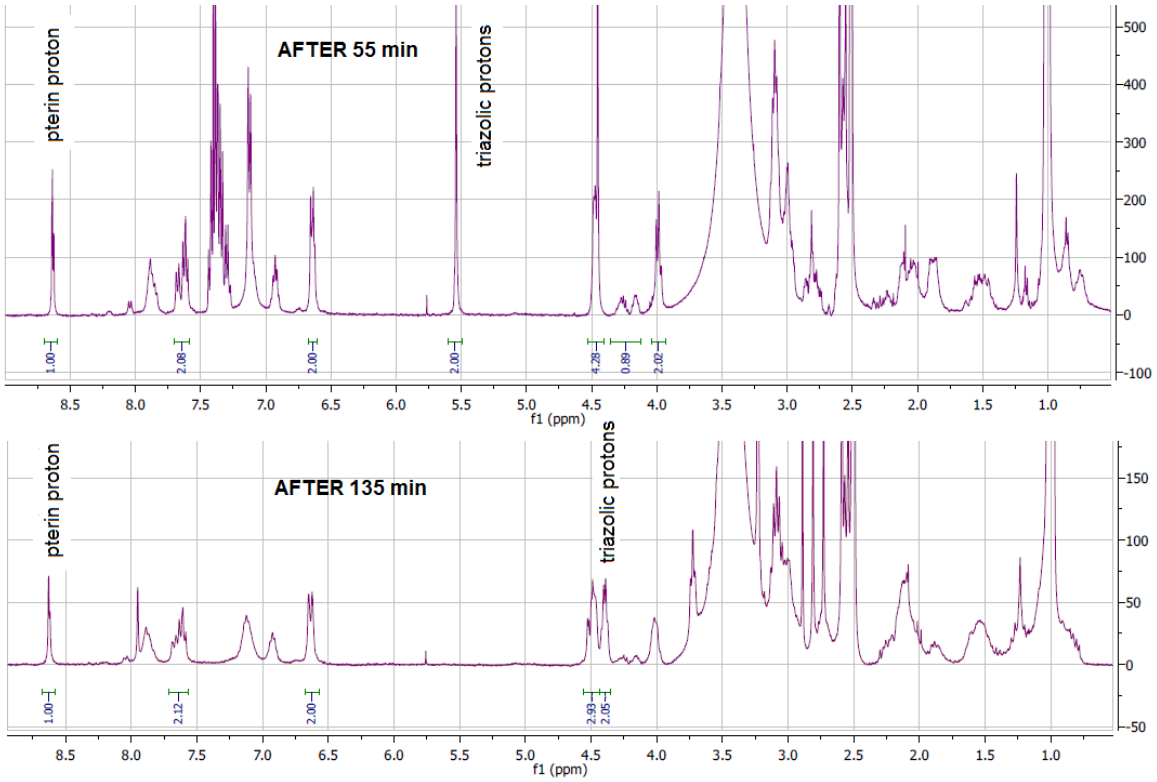


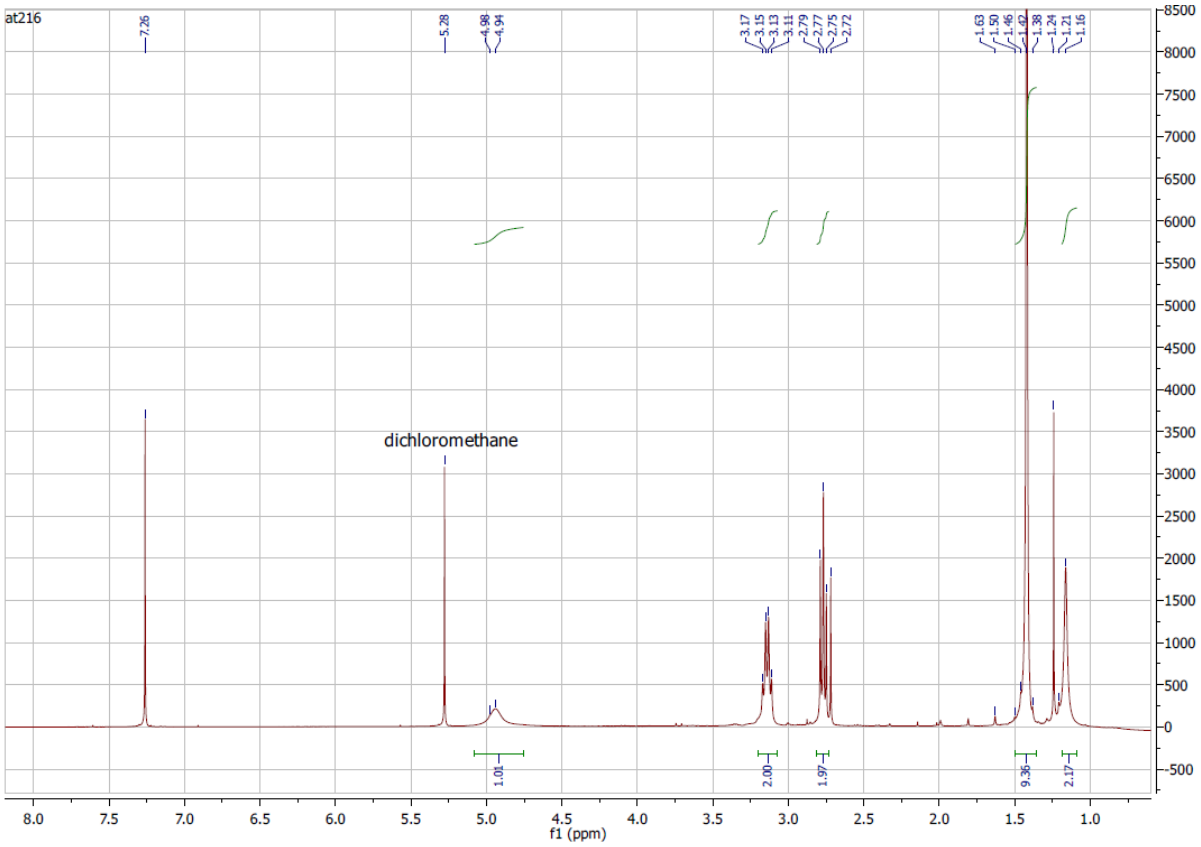
Figure 2 – top: crude spectrum **of** ethylene diamine folate after addition of 3eq.DIPEA + 1 eq BCN-CO-NHS followed by the addition of 2 eq of benzyl azide after 55 minutes; bottom: crude spectrum of ethylene diamine folate after the addition of 3eq.DIPEA + 1 eq BCN-CO-NHS followed by the addition of 2 eq. of N_3-PEG_{350}-OMe after 135 min.

4 Synthesis of folate conjugates

4.1 N-BOC ethylenediamine

In a round-bottomed flask was dissolved 4 ml (60 mmol, 10 eq.) of ethylene diamine in 40 ml of dry dichloromethane and stirred at 0ºC. Then, it was added drop-by-drop a solution of Boc anhydride (1.31g, 6 mmol, 1eq.) in 20 ml of dichloromethane. After the addition was finished the reaction mixture was stirred for 24 hours at room temperature, before the solvent was removed under low pressure to yield a viscous oil. It was redissolved in a solution of 2M $NaCO_3$ (60 ml) and extracted twice with 60 ml of dichloromethane. The organic phase was dried with $NaSO_4$, evaporated to dryness. N-Boc ethylenediamine (1,1 g, 92 % yield) was isolated as an uncolored oil with enough purity to be used in subsequent reactions.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.94 (bs, 1H), 3.17-3.11 (q, 2H), 2.79-2.72 (t, 2H), 1.42 (s, 9H), 1.16 (bs, 2 H)

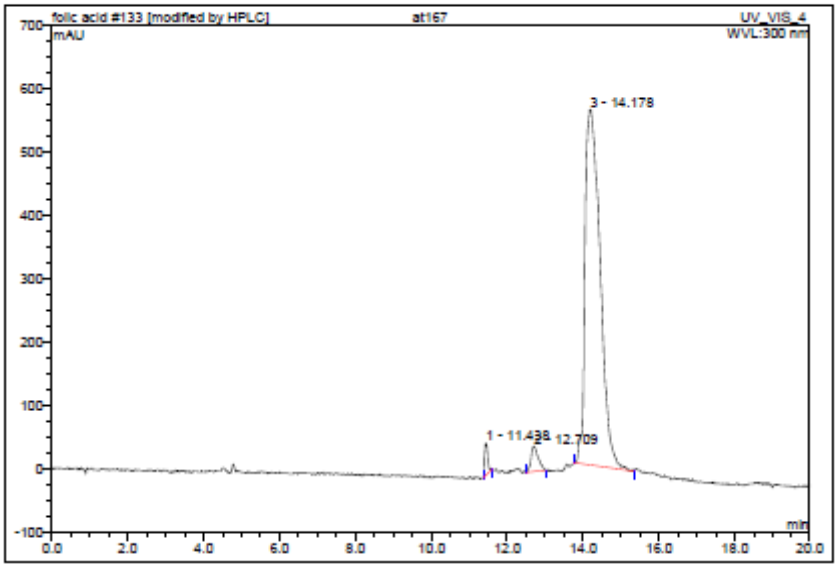


4.2 N-BOC ethylenediamine-folate

In a round-bottomed flask was dissolved 640 mg of folic acid (1.34 mmol, 1 eq. dehydrated powder) in 25 ml of DMSO. After the dissolution was complete (about 30 minutes with mild heating), 308 mg (2 eq.) of N-hydroxysuccinimide and 552 mg (2eq.) of DCC were added successively. The reaction mixture was stirred for 16h at room temperature, after which the urea precipitate was filtered off. Then, it was added 0.376 ml (2 eq.) of triethylamine followed by 429mg (2 eq.) of N-Boc-ethylene diamine dissolved in 5 ml of DMSO. The mixture was again stirred overnight, before was added to a mixture of 20% acetone in diethylether. The thin yellow precipitated was carefully centrifuged and washed four times with acetone and two with diethyl ether and dried under vacuum (658 mg, 73 % yield). The folic acid was conjugated with N-Bocethylene diamine almost exclusively in the terminal carboxylic acid as confirmed by RP-HPLC (injection of sample dissolved in DMSO, 1 ml/min, 0 to 5.5min 100% water, linear gradient from 5.5min at 100% water to 15.5min at 100% acetonitrile, Gemini 5u 18 110A, 5umx4.6x250mm, Phenomenex column) and ^{1}H NMR. The ^{1}H NMR spectra is consistent with the one available in the literature.

133 at167

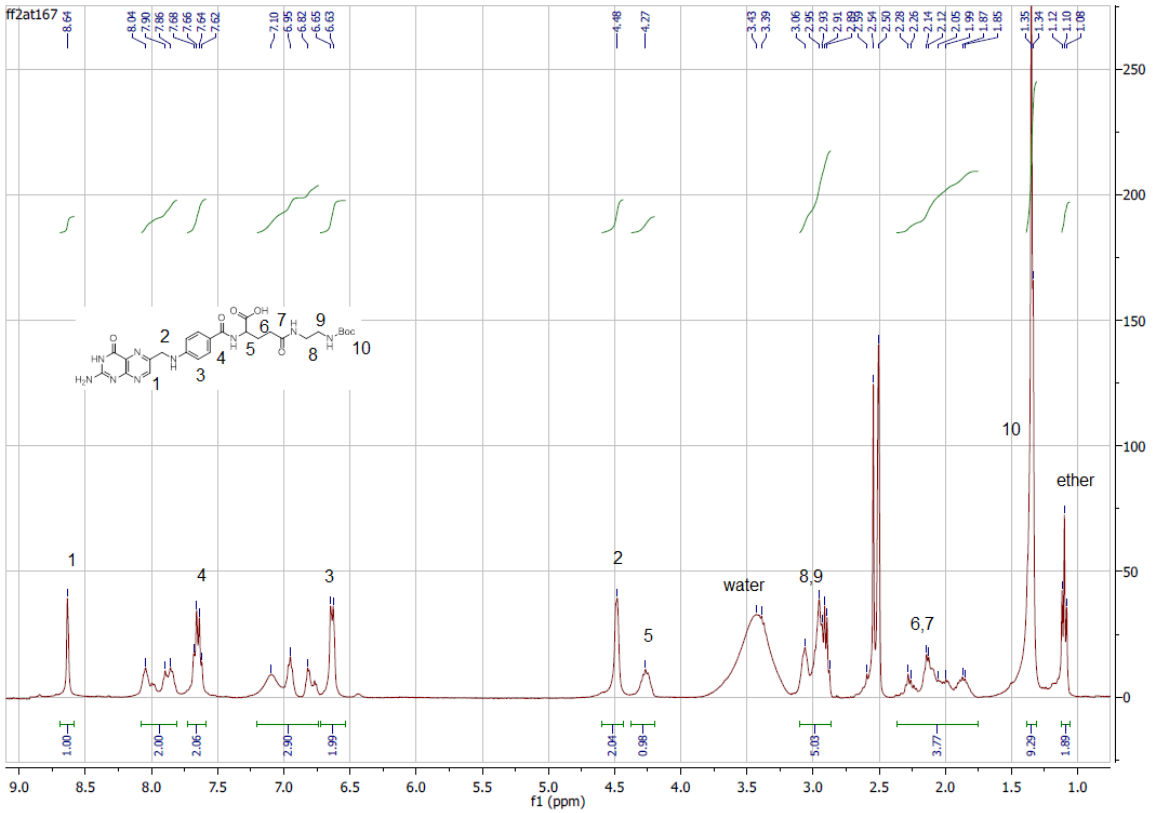
Sample Name:	**at167**	*Injection Volume:*	**1.0**
Vial Number:	**1**	*Channel:*	**UV_VIS_4**
Sample Type:	**unknown**	*Wavelength:*	**300**
Control Program:		*Bandwidth:*	**1**
Quantif. Method:	**default**	*Dilution Factor:*	**1.0000**
Recording Time:	**2/19/2013 14:42**	*Sample Weight:*	**1.0000**
Run Time (min):	**19.97**	*Sample Amount:*	**1.0000**

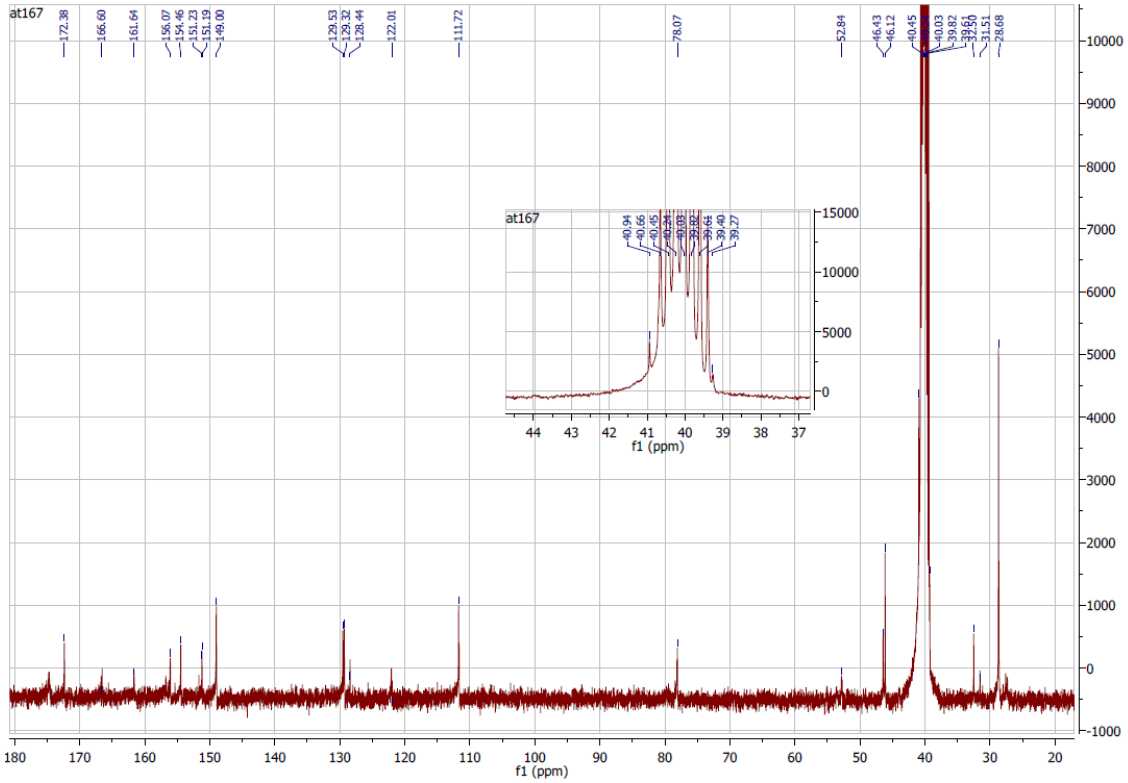


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	11.44	n.a.	50.897	4.588	1.69	n.a.	BMB*
2	12.71	n.a.	40.235	8.710	3.21	n.a.	BMB*
3	14.18	n.a.	561.649	258.345	95.10	n.a.	BMB*
Total:			652.782	271.643	100.00	0.000	

^{1}H NMR (400 MHz, DMSO) δ 8.64 (s, 1H, pterin), 8.04-7.86 (m, 2H), 7.68-7.62 (m, 2H, aromatic), 7.10-6.82 (m, 3H), 6.65-6.63 (d, 2H, aromatic), 4.48 (bs, 2H, benzylic), 4.27 (m, 1H, αH), 3.43 (bs, water) 3.06- 2.88 (m, 4H, ethylenediamine), 2.28-1.85 (m,4H, glutamic moiety), 1.35(s, 9H, Boc).

^{13}C NMR (100 MHz, DMSO) δ 172.38, 166.60, 161.64, 156.07, 154.46, 151.23, 151.19, 149.00, 129.53, 129.32, 128.44, 122.01, 111.72, 78.07, 52.84, 46.43, 46.12, 40.94, 39.27, 32.50, 31.51, 28.68, 10.14.



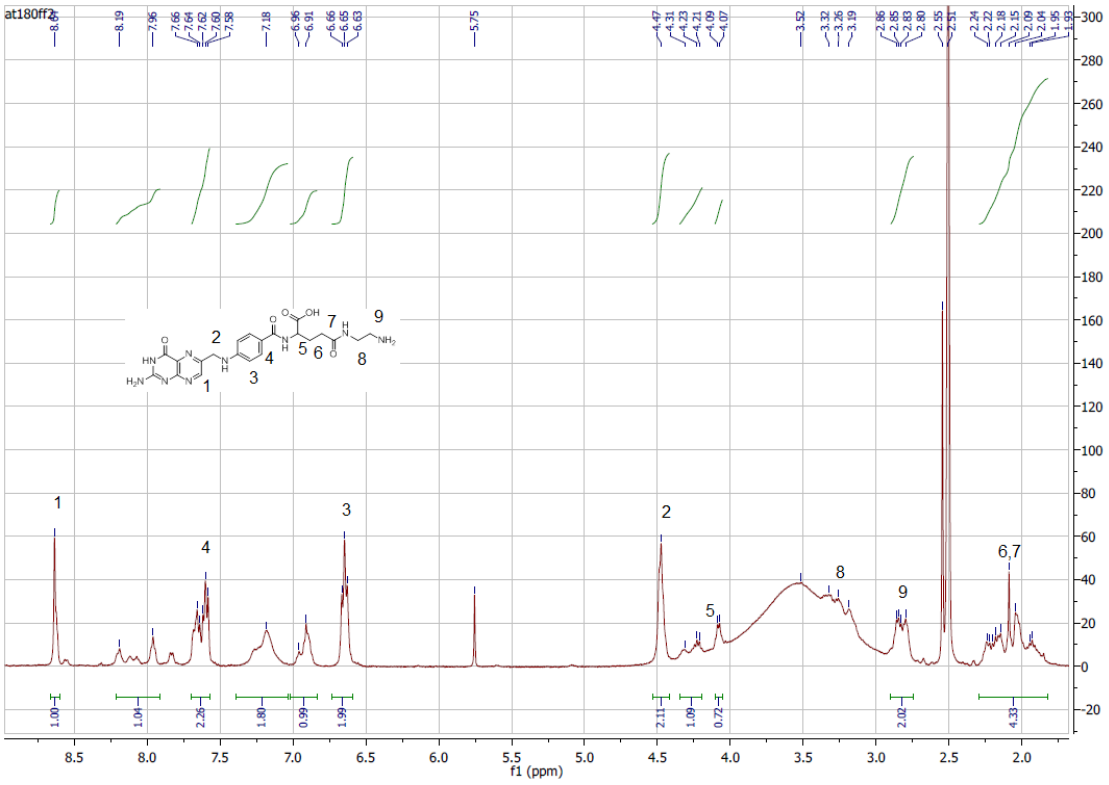


4.3 Ethylenediamine-folate

The ethylenediamine-folate conjugate was prepared according with a literature protocol. N-Boc-ethylenediamine folate (300 mg) was dissolved in 2 ml of trifluoroacetic acid and stirred

during two hours. The solvent was removed under pressure with aid of dichloromethane and the red-dark residue was dissolved in the minimal amount of dry DMF. The addition of triethylamine resulted in the precipitation of a yellow powder which was washed and centrifuged four times with acetone and two times with diethyl ether (207 mg, 83 % yield). The ^{1}H NMR spectra is consistent with the one available in the literature.

^{1}H NMR (400 MHz, DMSO) δ 8.64 (s, 1H, pterin), 8.19-7.96 (m, 1H), 7.66-7.58 (m, 2H, aromatic), 7.18-6.91 (m, 3H), 6.66-6.63 (m, 2H, aromatic), 4.47 (bs, 2H, benzylic), 4.31-4.07 (m, 1H), 3.52 (water), 3.32-3.19 (m, ethylene diamine), 2.86-2.80 (m, 2H, ethylene diamine), 2.24-1.93 (m, 4H, glutamic moiety).



4.4 BCN-Ethylenediamine-folate

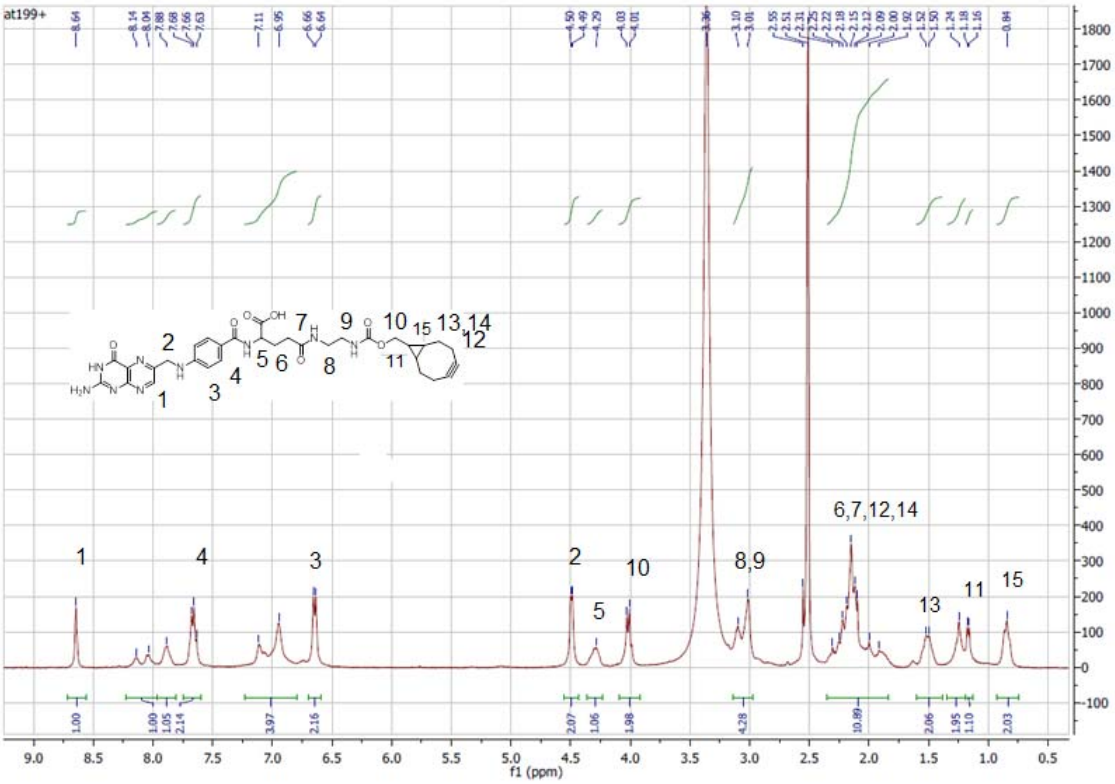
To a solution of ethylenediamine-folate (13 mg, 0.027 mmol) was added diisopropylethyl amine (17 ul, 0.1mmol, 4 eq) and (1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (12 mg, 0.038 mmol, 1.4 eq) and stirred until a clear solution is obtained (about 20 minutes). The reaction mixture was poured into amixture of 20% acetone in diethylether. The thin yellow precipitated was carefully centrifuged and washed two times with acetone and two times with diethyl ether and dried under vacuum (13mg, 73% yield).

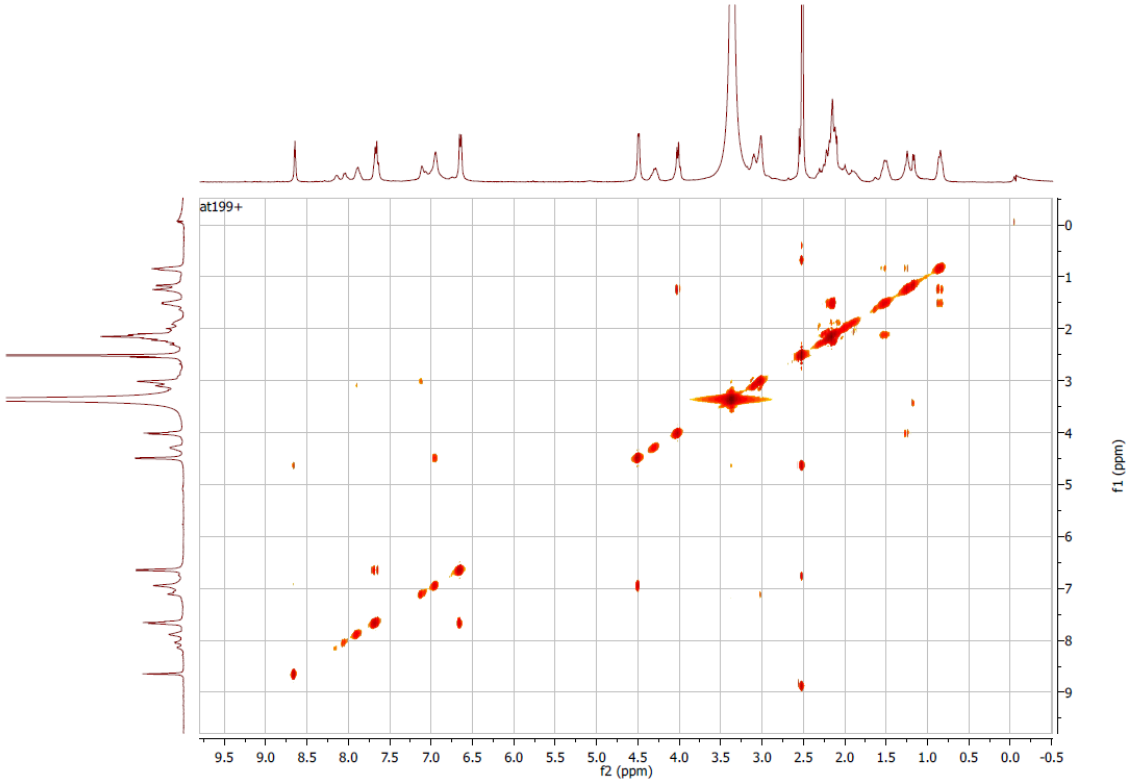
^{1}H NMR (400 MHz, DMSO) δ 8.64 (s, 1H, pterin), 8.14-7.88 (m, 2H), 7.68-7.63 (m, 2H, aromatic), 7.11-6.95 (m, 4H), 6.66-6.64 (m, 2H, aromatic), 4.50 (bs, 2H, benzylic), 4.29 (m, 1H, αH), 4.03-4.01 (m, 2H, -HNCO$_2$C**H**$_2$), 3.36 (water), 3.10-3.02 (m, 4H,O=C-NHC**H**$_2$C**H**$_2$), 2.22-1.92 (m, 10H, C**H**$_2$C**H**$_2$CO$_2$+ CH**H**-CH$_2$-alkyne-CH$_2$-CH**H**+ -C**H**$_2$-alkyne-C**H**$_2$-), 1.52 (m, 2H,CH**H**-CH$_2$-alkyne-CH$_2$-CH**H**), 1.24-1.16 (m, 3H, -HNCO$_2$CH$_2$C**H**), 0.84 (m, 2H, HNCO$_2$CH$_2$CH(C**H**)$_2$).

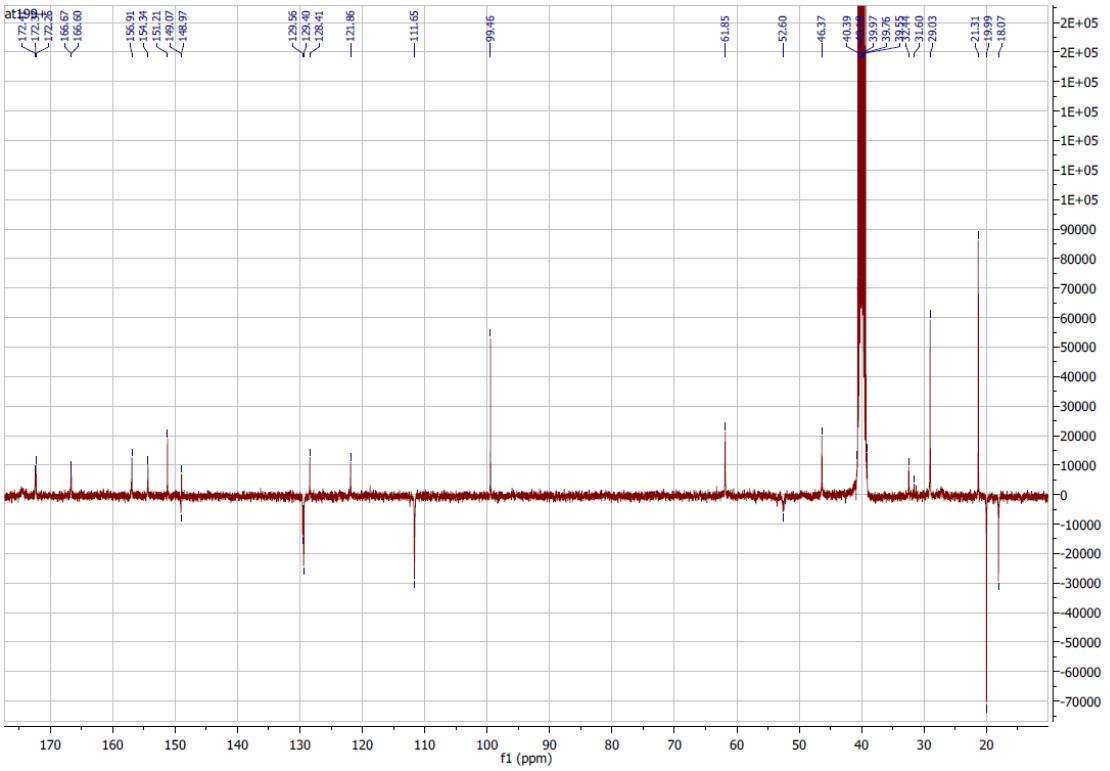
APT NMR (100 MHz, DMSO) δ 172.41, 172.37, 172.26, 166.67, 166.60, 156.91, 154.34, 151.21, 149.07, 148.97, 129.56, 129.40, 128.41, 121.86, 111.65, 99.46, 61.85, 52.60, 46.37, 40.70, 39.13, 32.44, 31.60, 29.03, 21.31, 19.99, 18.07.

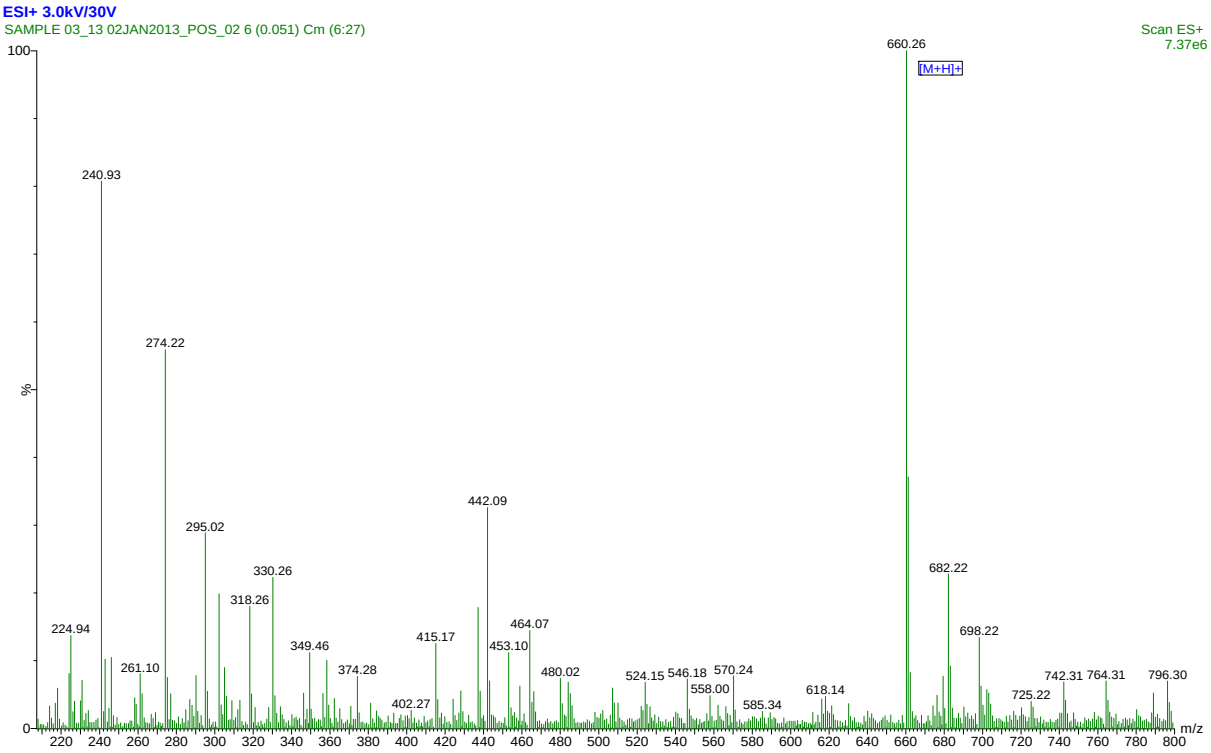
MS (ESI+) = 660.26 $(M+H)^{+}$

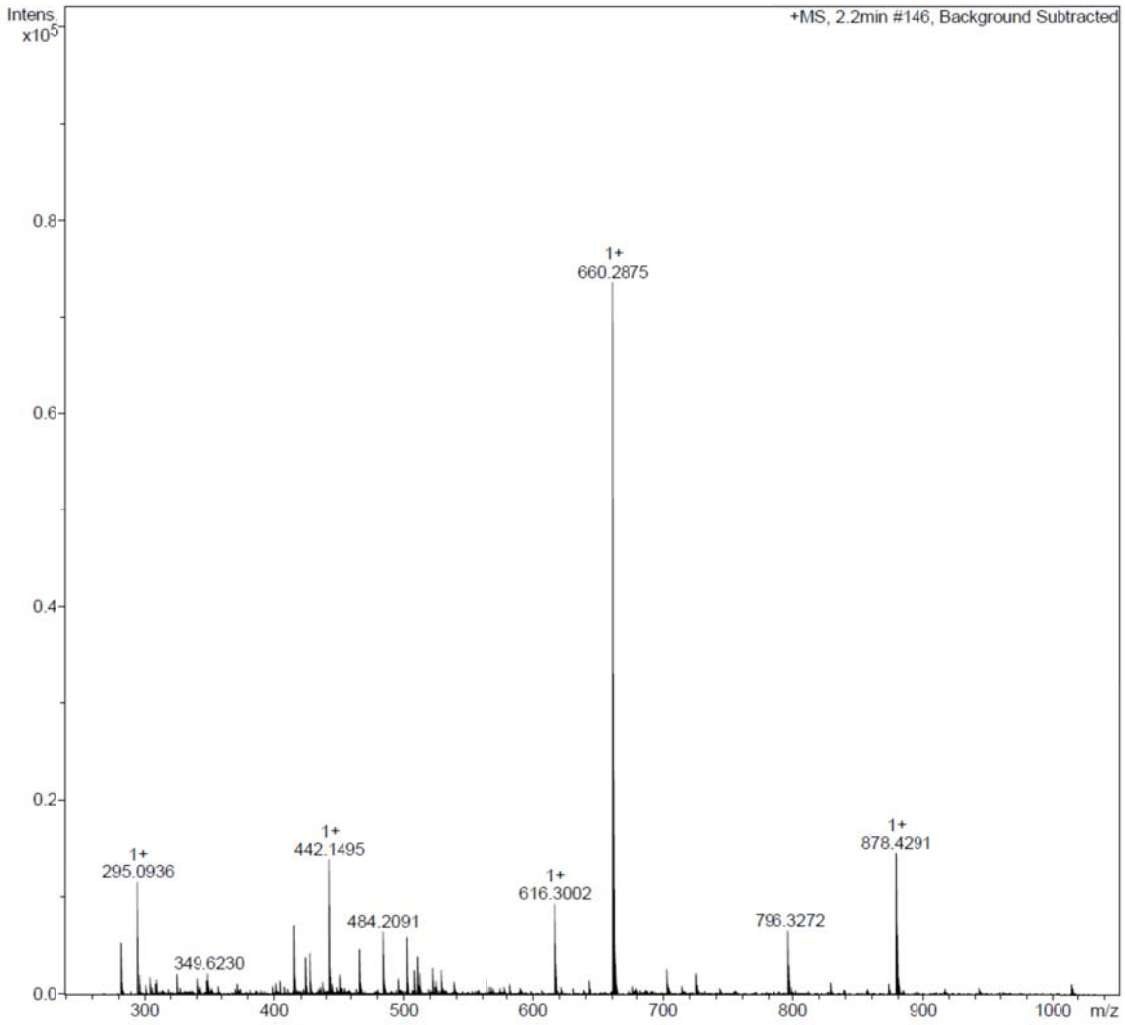
HRMS (FAB+) calc. = 660.2894, found = 660.2875 (ESI-FIA-TOF)


at199+
1
4
3
2
5
10
8,9
6,7,12,14
13
11
15
f1 (ppm)

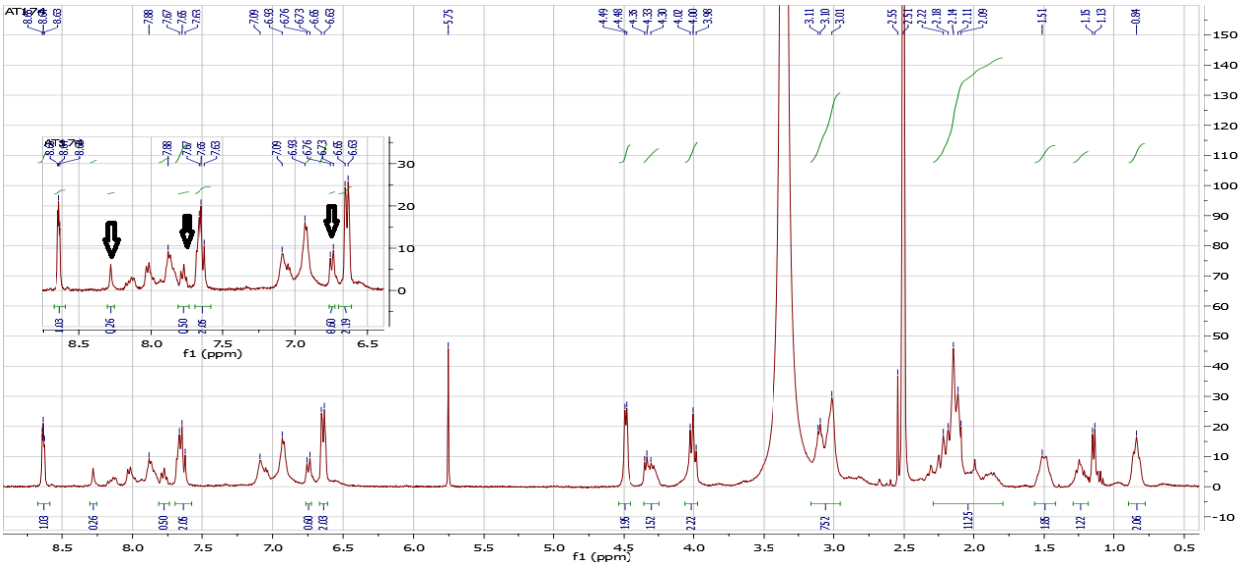

at199+
f2 (ppm)
f1 (ppm)


at198+c
172.13
172.58
166.67
166.60
156.91
154.34
151.21
149.07
148.97
129.56
129.40
128.41
121.86
111.65
99.46
61.85
52.60
46.37
40.39
39.97
39.76
32.44
31.60
29.03
21.31
18.99
18.07
2E+05
2E+05
1E+05
1E+05
1E+05
1E+05
1E+05
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
-10000
-20000
-30000
-40000
-50000
-60000
-70000
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)


ESI+ 3.0kV/30V
SAMPLE 03_13 02JAN2013_POS_02 6 (0.051) Cm (6:27)
Scan ES+
7.37e6
100
%
0
660.26
[M+H]+
240.93
274.22
442.09
295.02
330.26
682.22
318.26
224.94
464.07
698.22
415.17
453.10
349.46
261.10
374.28
480.02
524.15
546.18
570.24
558.00
742.31
764.31
796.30
618.14
585.34
725.22
402.27
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
520
540
560
580
600
620
640
660
680
700
720
740
760
780
800
m/z



^{1}H NMR spectra of BCN-Ethylenediamine-folate prepared overnight presenting 20 % of impurity (peaks highlighted by arrows).



5 Click reactions

5.1 BCN-Ethylenediamine-folate + benzylazide: synthesis of conjugate 7a

Method A

BCN-Ethylenediamine-folate (3.5 mg, 0.0051 mmol, 1 eq.) was dissolved in 1 ml of DMSO and added to a flask containing 2 mg of benzyl azide[2] (0,015 mmol, 3 eq.). The reaction was stirred for 2 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 95 % yield (4mg).

^{1}H NMR (400 MHz, DMSO) δ 11.58 (carboxylic acid), 8.65 (s, 1H, pterin), 8.15-8.02 (m, 1H), 7.88 (m, 1H), 7.68-7.66 (m, 2H, aromatic), 7.37-7.27 (m, 3H, phenyl), 7.14-6.94 (m, 2H + m, 4H), 6.66-6.64 (m, 2H, aromatic), 5.54 (s, 2H, benzylic H triazole), 4.50 (bs, 2H, benzylic), 4.29 4.29 (m, 1H, αH), 4.01-3.97 (m, 2H, -HNCO$_2$C**H**$_2$), 3.36 (water), 3.09-3.00 (m, 6H, O=C-NHC**H**$_2$C**H**$_2$ + -CH**H**-triazole-CH**H**-), 2.86-2.75 (m, 2H, -CH**H**-triazole-CH**H**-), 2.31- 1.90 (m, 6H, C**H**$_2$C**H**$_2$CO$_2$ + CH**H**-CH$_2$-triazole-CH$_2$-CH**H**), 1.57-1.46 (m, 2H, CH**H**-CH$_2$-triazole-CH$_2$-CH**H**), 1.08-1.05 (m, 1 H, -HNCO$_2$CH$_2$C**H**), 0.88-0.76 (m, 2 H, HNCO$_2$CH$_2$CH(C**H**)$_2$).

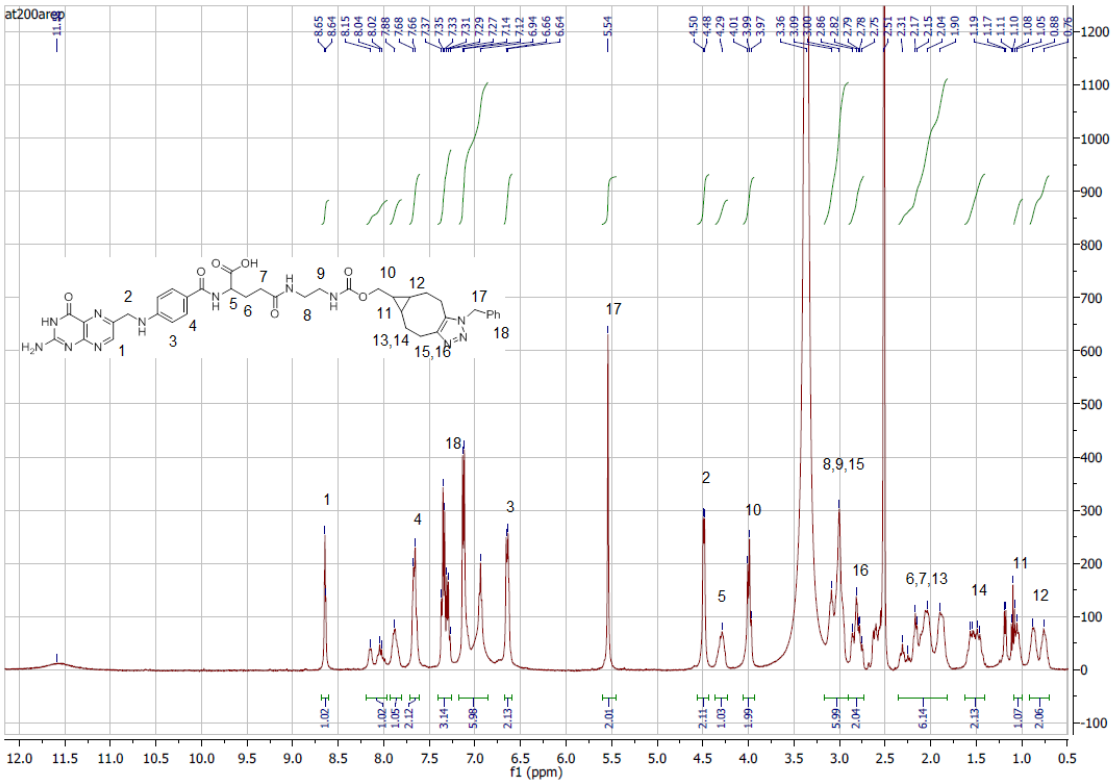
^{13}C NMR (100 MHz, DMSO) δ 172.40, 172.38, 172.25, 166.73, 166.66, 156.93, 156.84, 154.37, 151.21, 148.97, 144.37, 133.50, 129.55, 129.40, 128.41, 128.23, 127.42, 121.87, 111.65, 61.87, 51.11, 46.38, 39.13, 32.45, 25.93, 22.58, 22.09, 21.31, 19.16, 19.04, 17.60.

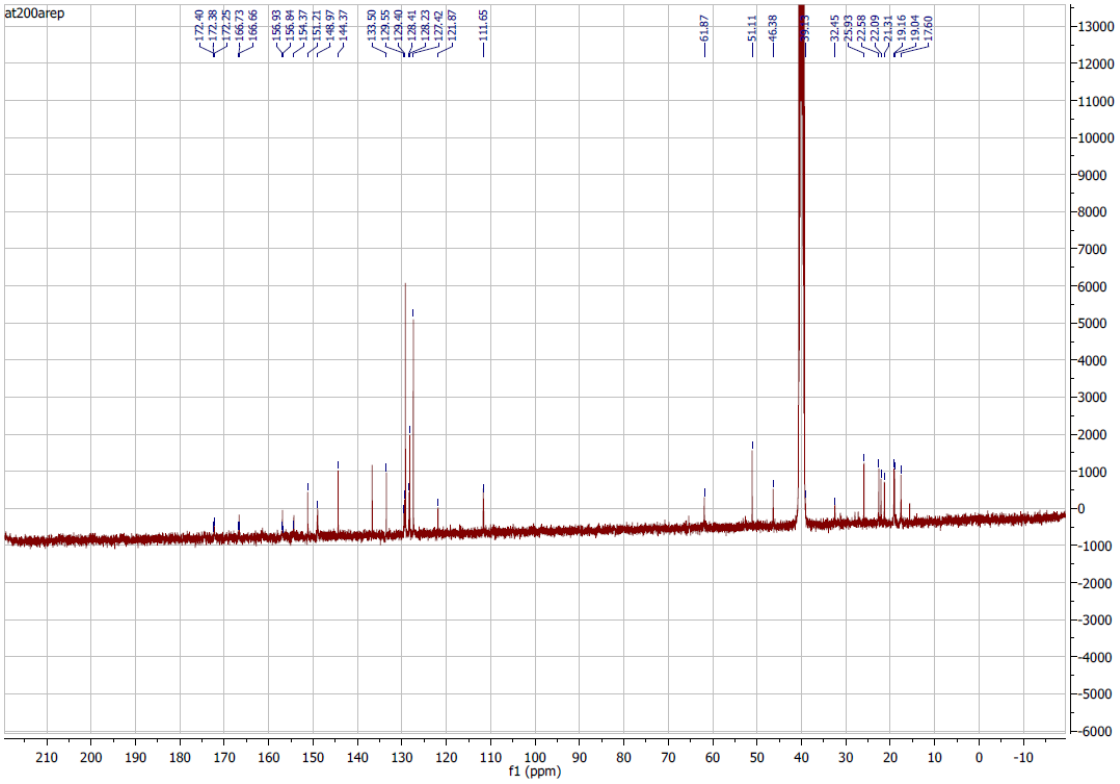
MS (ESI+) = 793.33(M+H)$^{+}$ HRMS (ESI-FIA-TOF) calc. = 793.3534, found = 793.3522

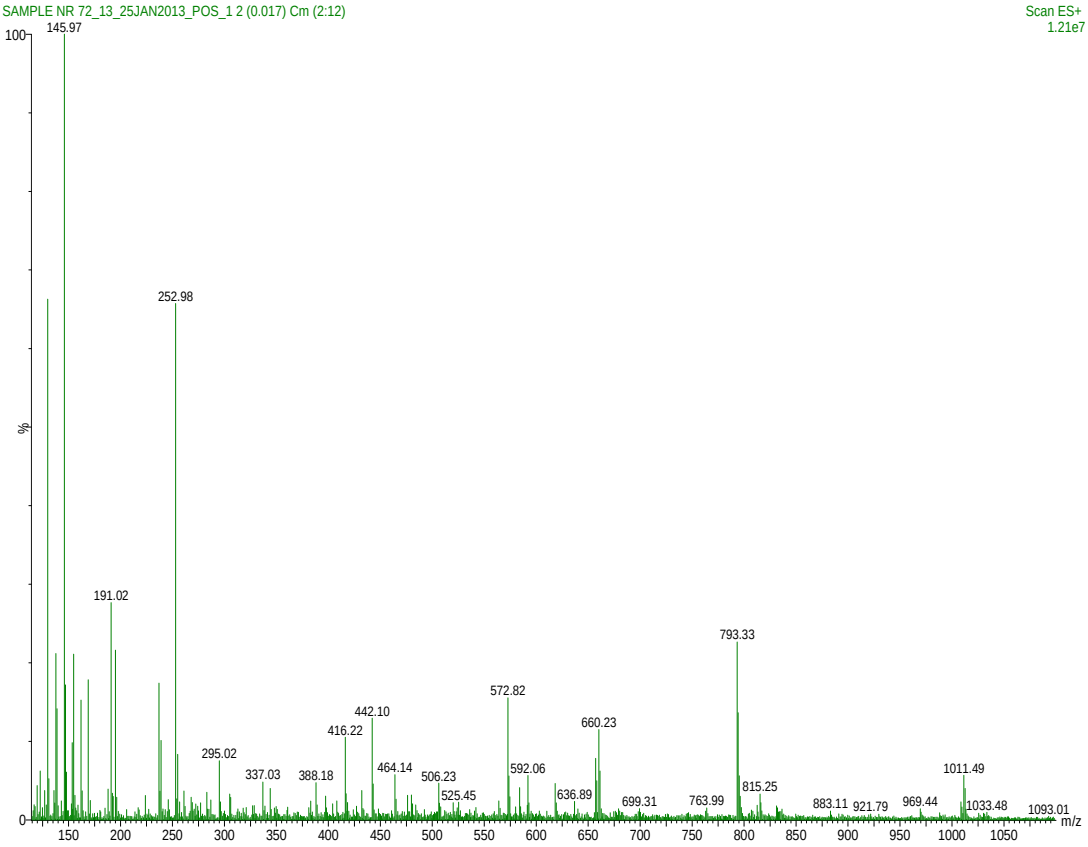
Method B

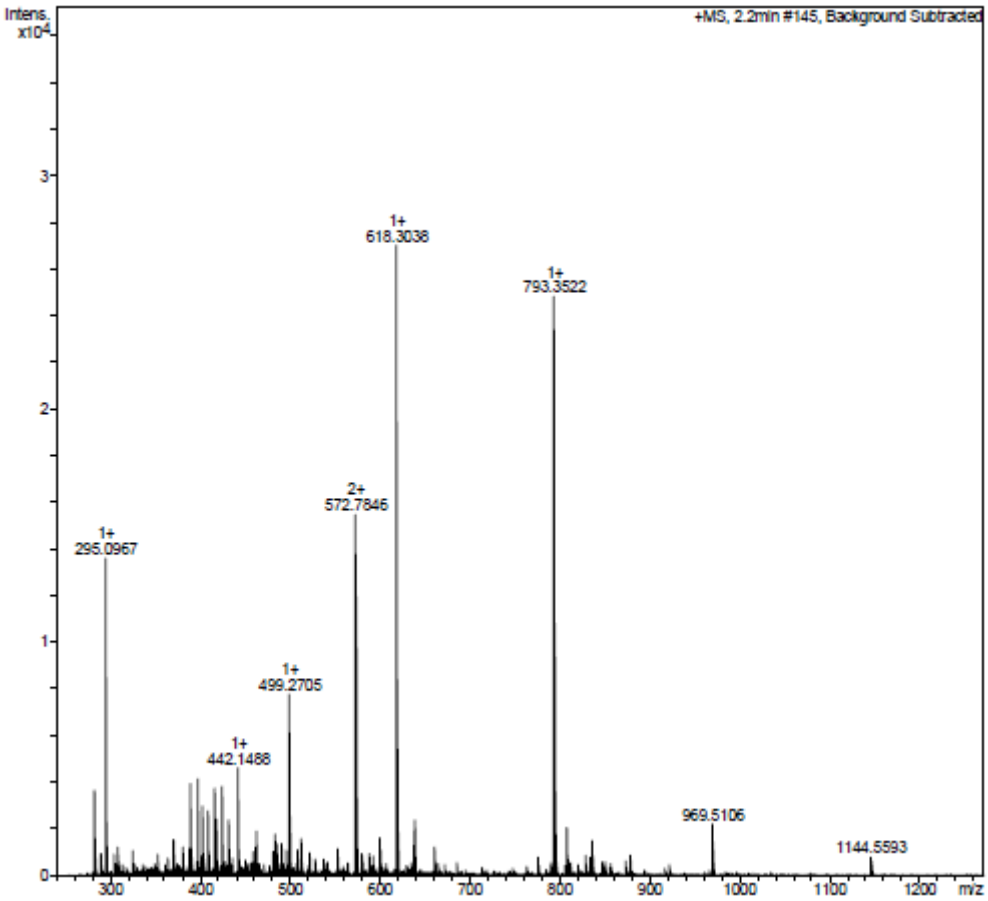
Ethylenediamine folate conjugate (10 mg, 0.021 mmol, 1 eq) was dissolved 2ml of DMSO, followed by the addition of diisopropylethyl amine (11ul, 0.063 mmol, 3 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (6.5 mg, 0.022 mmol, 1.05 eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of benzyl azide (5.6 mg, 0.042 mmol, 2 eq). The reaction was stirred for 2 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 86 % yield (14 mg).

[2] Amyes, Tina L.; Jencks, William P,. *J. Am. Chem. Soc.,* **1989** , vol. 111, 20 p. 7900 - 7909


at200arep
f1 (ppm)


at200arep
f1 (ppm)


SAMPLE NR 72_13_25JAN2013_POS_1 2 (0.017) Cm (2:12)
Scan ES+
1.21e7
100
%
0
145.97
252.98
191.02
793.33
572.82
442.10
660.23
416.22
295.02
337.03
388.18
464.14
506.23
525.45
592.06
636.89
699.31
763.99
815.25
883.11
921.79
969.44
1011.49
1033.48
1093.01
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050
m/z


Intens.
x10^4
+MS, 2.2min #145, Background Subtracted
3
2
1
0
1+
618.3038
1+
793.3522
2+
572.7846
1+
295.0967
1+
499.2705
1+
442.1488
969.5106
1144.5593
300
400
500
600
700
800
900
1000
1100
1200
m/z

5.2 BCN-Ethylenediamine-folate + PEG azide: synthesis of conjugate 7b

Method A

BCN-Ethylenediamine-folate (3.5 mg, 0.0051 mmol, 1 eq.) was dissolved in 1 ml of DMSO and added to a flask containing 6 mg of MeO-PEG_{350}-N_3[3] (0,015 mmol, 3 eq.). The reaction was stirred for 5 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 96 % yield (5 mg).

^{1}H NMR (400 MHz, DMSO) δ 8.65 (1H, s, N=C**H**-C=N), 8.16-8.05 (1H, bm, -**H**NCHCO_2H), 7.89 (1H, bm, O=C-N**H**CH_2CH_2), 7.68- 7.63 (2H, m, aromortho to amide), 7.13-6.94 (m, 4H), 6.66-6.64 (2H, d aromortho to amine), 4.50-4.48 (2H, d, NC**H**$_2$-Ar), 4.40 (2H, N=N-N-C**H**$_2$), 4.29 (1H, bm, -HNC**H**CO_2H), 4.03 (2H, bm, -HNCO_2C**H**$_2$), 3.74 (2H, bm, PEG), 3.50-3.47 (24H, PEG), 3.37 (water), 3.24 (3H, PEG, OC**H**$_3$), 3.10- 2.95 (6H, bm, O=C-NHC**H**$_2$C**H**$_2$+CH**H**-triazole-CH**H**), 2.77-2.71 (2H, bm, C**H**H-triazole-C**H**H), 2.55-1.92 (6H, bm, C**H**$_2$C**H**$_2$$CO_2$H+CH**H**-$CH_2$-triazole-$CH_2$-CH**H**), 1.55-1.52 (2H, bd, C**H**H-CH_2-triazole-CH_2-C**H**H)), 1.10- 1.05 (1H, bm, -HN$CO_2$$CH_2$C**H**), 0.92 (2H, bm, -HN$CO_2$$CH_2$CH(C**H**$)_2$).

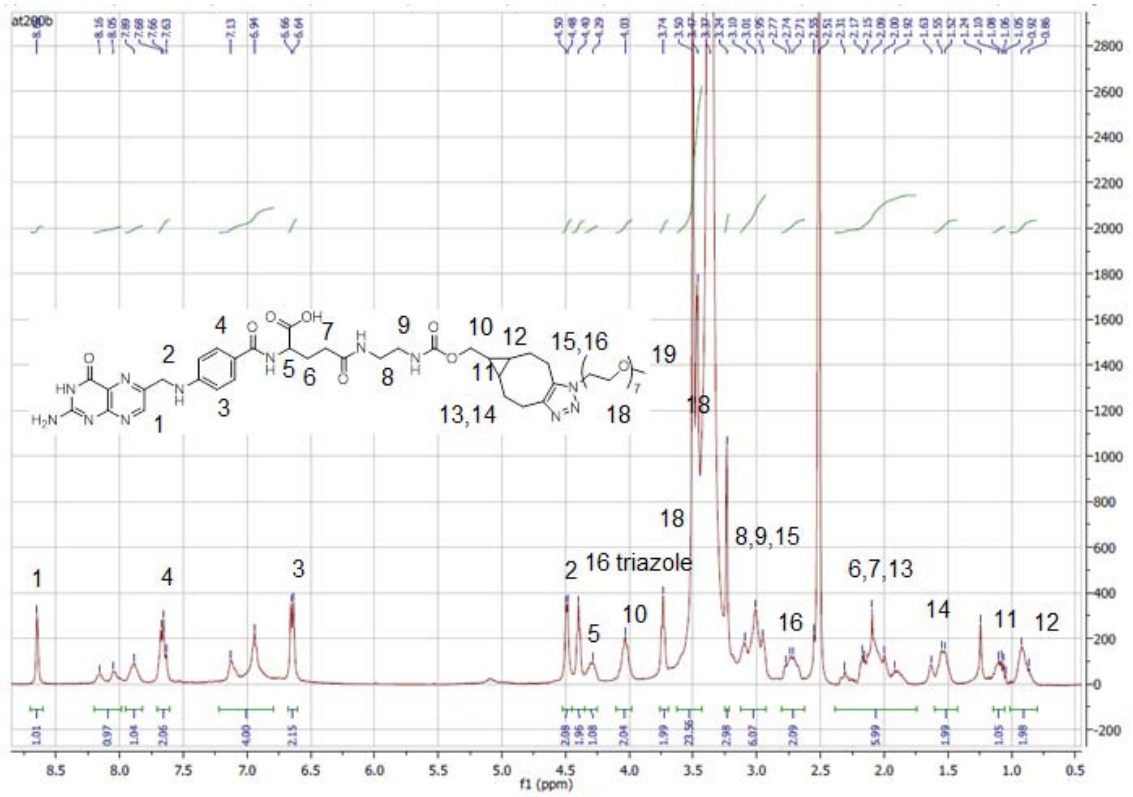
^{13}C NMR (75 MHz, DMSO) δ 172.24, 166.68, 156.89, 154.24, 151.20, 149.09, 149.02, 148.96, 143.64, 134.27, 129.57, 129.41, 128.38, 121.84, 111.63, 71.72, 70.22, 70.02, 69.82, 58.49, 55.37, 47.59, 46.33, 25.81, 22.68, 22.42, 21.72, 19.63, 19.08, 17.72.

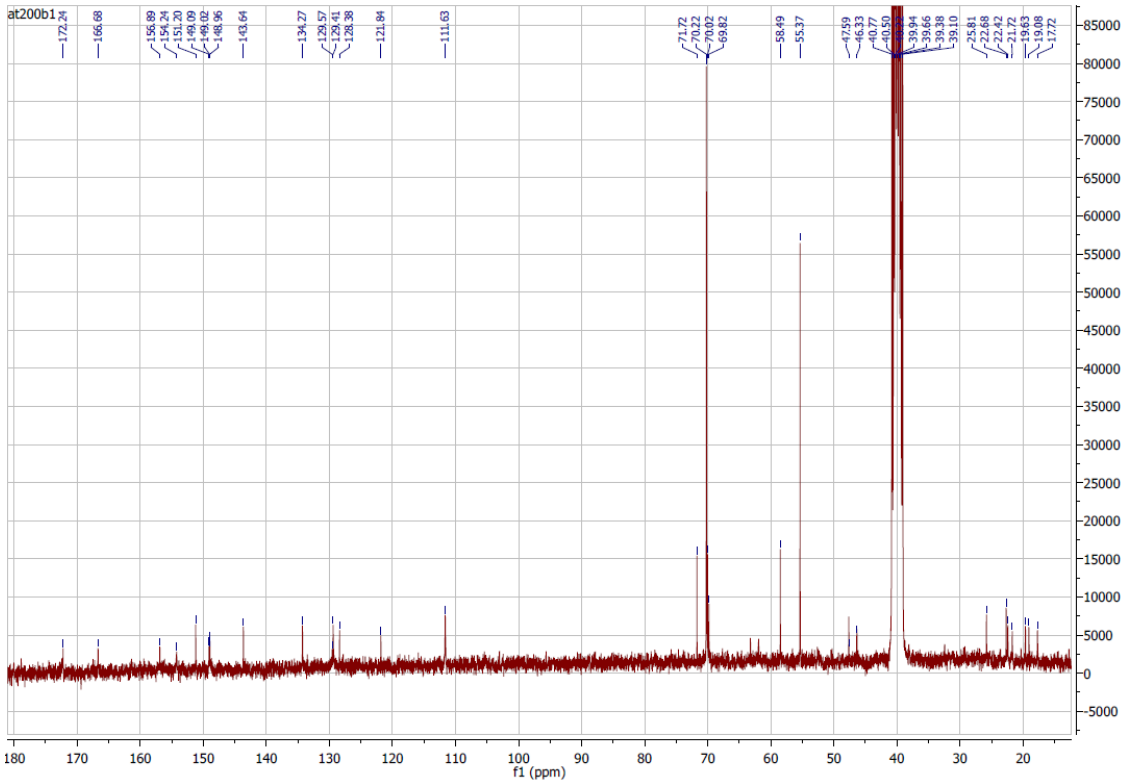
MS (ESI+) = 1025.5 mixture oligomers with and without Na.

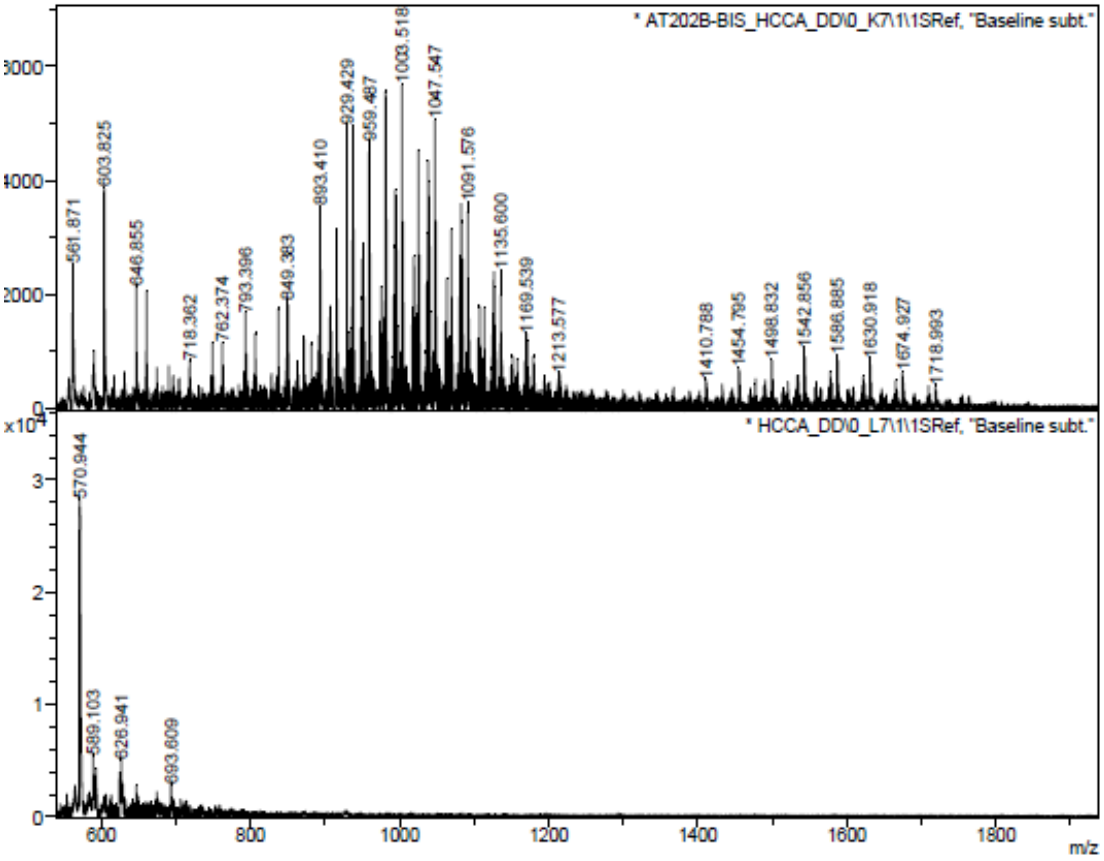
Method B

Ethylenediamine folate conjugate (10 mg, 0.021 mmol, 1 eq) was dissolved 2ml of DMSO, followed by the addition of diisopropylethyl amine (11ul, 0.063 mmol, 3 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (6.5 mg, 0.022 mmol, 1.05 eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of MeO-PEG_{350}-N_3[3] (15 mg, 0.042 mmol, 2 eq). The reaction was stirred for 5 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 88 % yield (19 mg).

[3] Pan, Xusong; Chen, Chao; Peng, Jiang; Yang, Yongan; Wang, Yinghan; Feng, Wen; Yuan, Lihua; Deng, Pengchi**,** *Chem.Commun.*, **2012** , vol. 48, # 76 p. 9510


at200b
1
4
3
2
16 triazole
5
10
18
8,9,15
16
6,7,13
14
11
12
f1 (ppm)


at200b1
f1 (ppm)



m/z	SN	Quality Fac.	Res.	Intens.	Area	m/z	SN	Quality Fac.	Res.	Intens.	Area
561.871	9.3	932	440	1704.84	3519	1035.452	4.5	357	3740	774.99	480
590.237	3.5	396	641	654.91	1061	1036.471	8.5	226	4929	1483.72	598
603.825	20.5	3242	883	3777.62	4356	1037.457	11.3	1202	4091	1974.00	1034
646.855	9.9	992	1406	1860.23	1427	1038.451	14.3	214	4935	2491.27	1007
660.495	9.3	2954	1854	1752.18	1017	1039.471	13.9	1611	3789	2402.46	1336
718.362	3.3	159	2094	630.98	410	1041.447	3.5	344	2222	608.93	580
749.249	5.3	861	2051	999.40	668	1047.547	28.1	13002	4030	4809.01	2542
762.374	5.4	433	2709	1027.39	511	1061.519	7.8	1741	4089	1331.97	741
793.396	7.6	1882	2426	1428.37	875	1063.522	10.5	1221	3953	1783.47	965
806.389	6.3	709	2651	1178.83	656	1067.526	6.9	592	3677	1164.99	714
837.534	7.3	1675	2133	1354.34	954	1069.523	16.2	20567	4049	2732.21	1481
849.383	8.8	1918	3088	1637.35	809	1081.484	14.4	832	4055	2428.44	1410
850.415	4.9	409	2622	905.54	590	1083.496	17.1	2749	3948	2872.44	1647
863.370	3.6	145	3744	660.32	293	1091.576	18.4	9001	3918	3075.29	1824
871.438	7.0	148	3584	1299.31	561	1105.531	5.8	720	3865	953.73	634
881.693	3.6	212	1649	654.71	615	1107.549	8.2	1514	4616	1352.81	699
893.410	19.1	2513	3564	3472.04	1573	1111.546	4.3	157	3761	702.68	477
906.375	8.6	152	3801	1565.62	705	1113.550	8.1	5914	4176	1341.13	772
908.376	5.1	743	3739	927.41	443	1123.507	4.9	69	4071	799.88	520
915.453	16.4	5369	3957	2962.64	1294	1125.499	7.6	656	3560	1232.42	935
929.429	4.1	113	3568	734.73	379	1126.541	8.5	156	6681	1396.31	478
931.434	6.1	160	4410	1103.58	448	1127.540	7.1	431	4458	1163.98	623
935.435	6.5	131	3877	1167.25	526	1135.600	10.9	4889	4091	1763.49	1098
936.399	5.5	113	4078	981.97	489	1149.561	3.2	160	4155	508.35	338
937.432	24.2	9320	3696	4354.92	2080	1151.561	3.8	170	4109	600.33	398
948.396	6.5	384	3978	1164.52	539	1157.576	4.0	390	4558	632.52	370
949.401	6.2	511	3734	1113.95	552	1169.539	6.9	394	4270	1068.35	708
950.412	8.7	173	3861	1564.89	704	1171.561	4.6	584	4507	721.42	405
951.414	11.0	790	3692	1977.38	967	1179.610	5.0	411	4294	767.38	513
959.487	24.8	10264	3858	4434.23	2110	1213.577	3.5	55	3428	523.77	459
973.473	6.9	743	4564	1231.43	503	1410.788	3.3	230	4362	393.19	359
975.446	10.1	1608	4148	1804.67	823	1454.795	5.3	561	4640	603.45	548
981.461	28.7	22034	3789	5094.61	2527	1476.760	3.0	202	4578	334.23	319
991.426	3.2	337	4290	561.78	264	1498.832	6.9	1641	4404	744.86	748
992.421	7.2	382	3652	1278.15	674	1533.754	4.1	330	3267	413.42	621
993.430	9.9	1212	3841	1752.94	893	1542.856	8.7	3998	4481	875.61	909
994.432	13.0	315	4376	2289.16	977	1577.798	6.0	256	4358	571.39	642
995.436	13.4	1647	3813	2384.09	1215	1586.885	8.9	5547	4427	838.06	934
997.415	3.2	295	2530	561.40	458	1621.824	4.4	133	4150	390.40	495
1003.518	29.9	14305	3955	5269.27	2669	1630.918	8.8	6452	4564	763.51	851
1017.490	8.9	3461	4315	1554.38	754	1665.848	4.5	571	4792	375.48	429
1019.487	12.7	2986	4288	2219.52	1043	1674.927	5.8	1961	4775	476.03	539
1023.492	9.9	703	4055	1731.94	882	1708.832	3.0	71	3715	229.33	363
1025.487	23.5	29622	3770	4092.15	2213	1718.993	3.8	485	4413	286.45	373

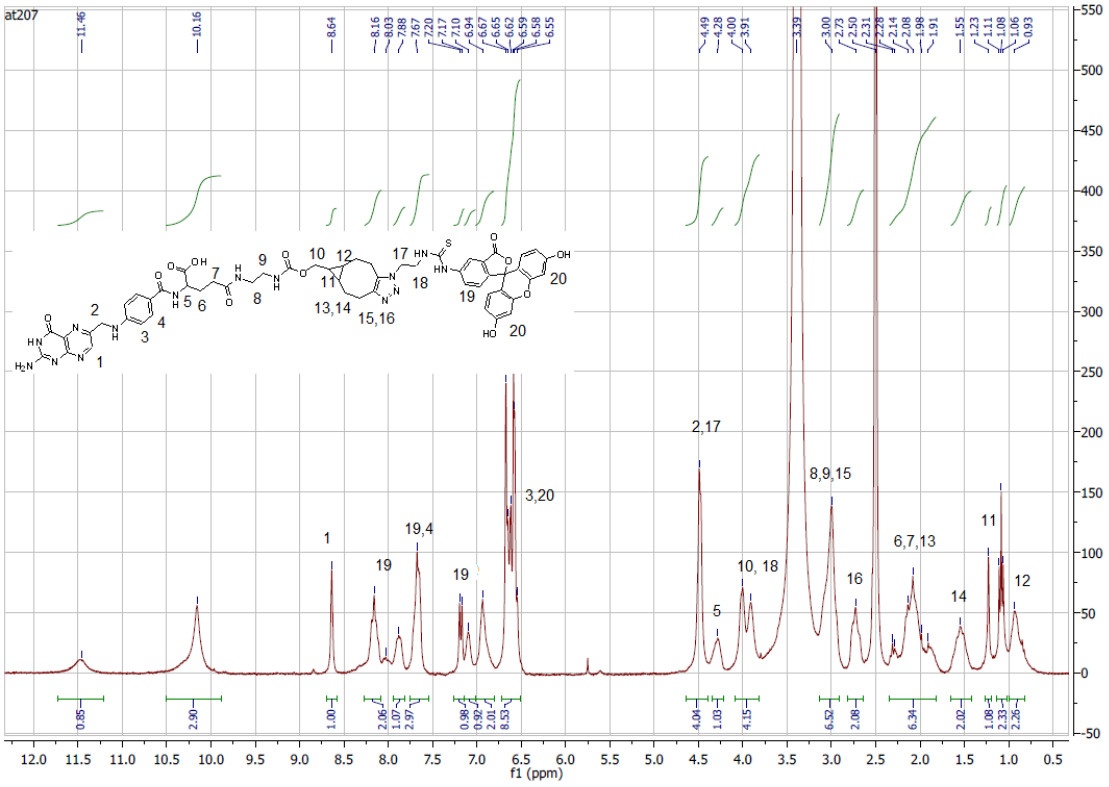
5.3 BCN-Ethylenediamine-folate + fluorescein isocyanate azide: synthesis conjugate 7d

BCN-Ethylenediamine-folate (6.9 mg, 0.01 mmol, 1 eq) was dissolved in 2 ml of DMSO and added to a flask containing 10 mg of 3-(1-(2-azidoethyl)thiourea) fluorescein[4] (0,021 mmol, 2 eq). The reaction was stirred for 4 hours before it was poured into a solution of 20 % acetone/diethyl ether. The light orange precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 88 % yield (10.5 mg) with 98 % purity.

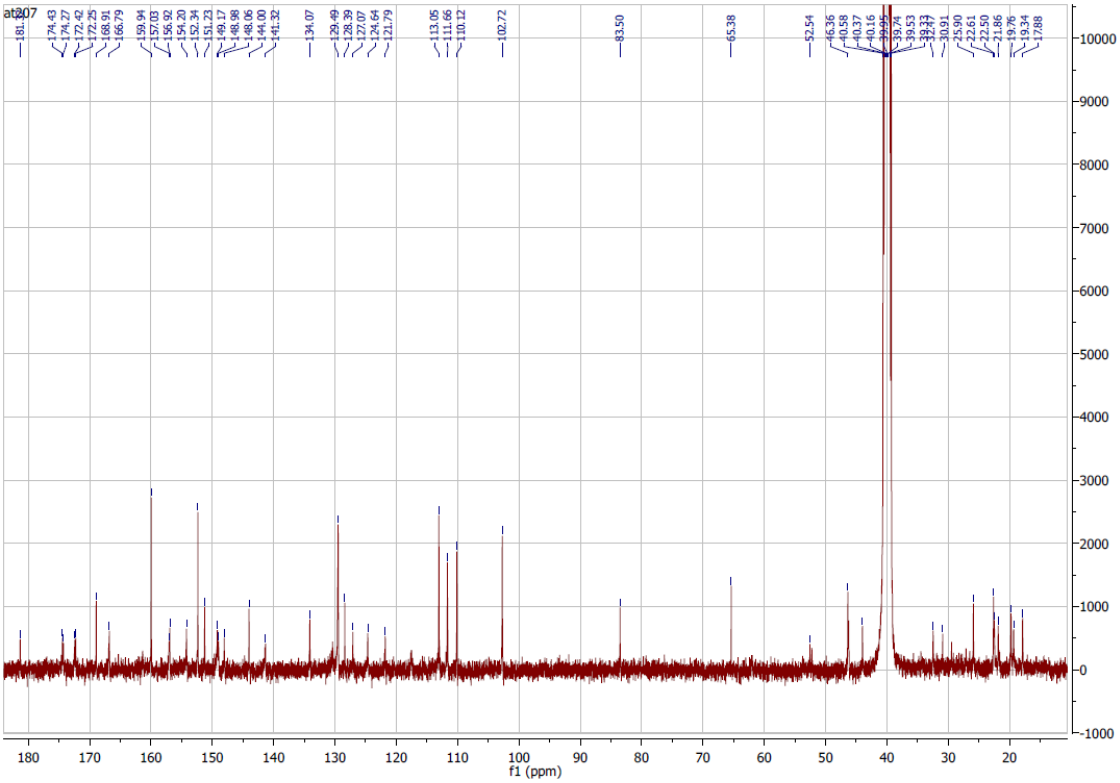
^{1}H NMR (300 MHz, DMSO) δ 11.46 (bs, 1H), 10.16 (bs, 3H), 8.64 (s, 1H, pterin), 8.16-7.88 (m, 3H, 1H fluorescein carboxylate ring), 7.67 (m, 3 H, 1H fluorescein carboxylate ring + 2H aromatic folate), 7.20-7.17 (d, 1H, 1H fluorescein carboxylate ring), 7.10-6.94 (m, 3H), 6.67-6.55 (m, 8H, 6H fluorescein carboxylate ring + 2H aromatic folate), 4.49(m, 4H, NC**H**$_2$-Ar + triazole-C**H**$_2$), 4.28 (m, 1H, -HNC**H**CO$_2$H), 4.00-3.91 (m, 4H,-HNCO$_2$C**H**$_2$ +triazole-CH$_2$-C**H**$_2$) 3.39 (H$_2$O), 3.00 (m, 6H,O=C-NHC**H**$_2$C**H**$_2$+CH**H**-triazole-CH**H**) 2.73 (m, 2H, C**H**H-triazole-C**H**H), 2.31-1.91 (m, 6H C**H**$_2$C**H**$_2$CO$_2$H+CH**H**-CH$_2$-triazole-CH$_2$-CH**H**), 1.55 (m, 2H, C**H**H-CH$_2$-triazole-CH$_2$-C**H**H), 1.23 (s, 1H, HNCO$_2$CH$_2$C**H**), 0.93 (bs, 2H, -HNCO$_2$CH$_2$CH(C**H**)$_2$).

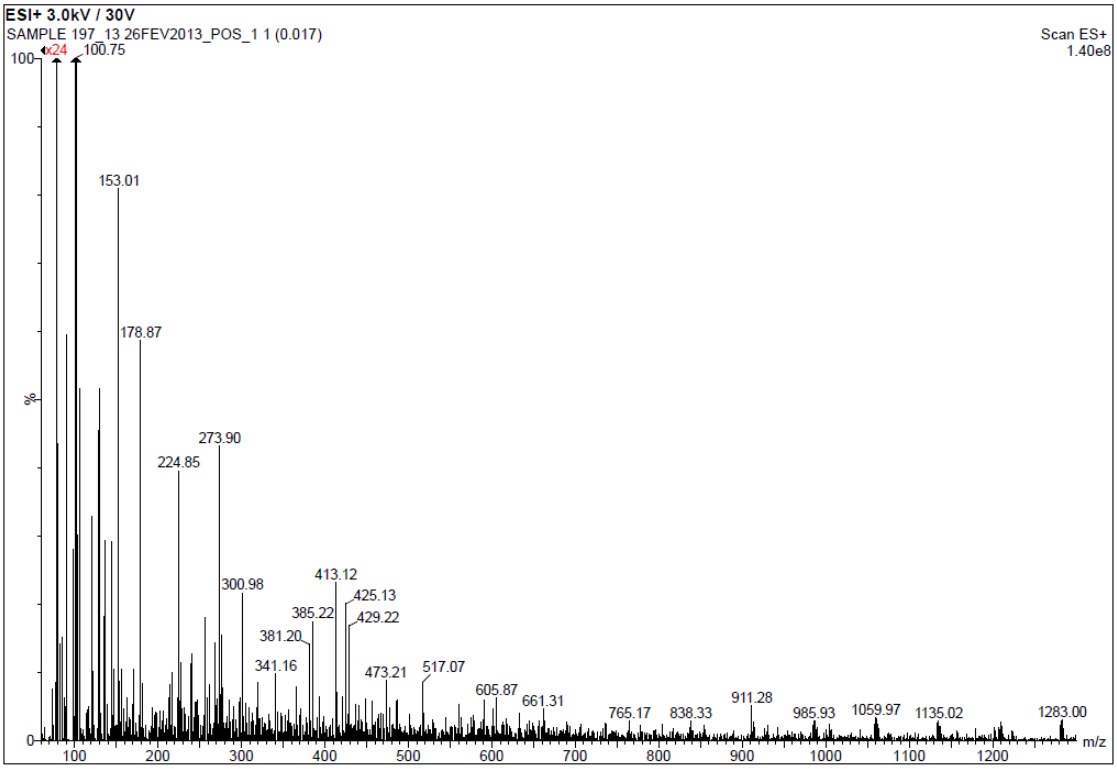
^{13}C NMR (101 MHz, DMSO) δ181.32, 174.43, 174.27, 172.42, 172.25, 168.91, 166.79, 159.94, 157.03, 156.92, 154.20, 152.34, 151.23, 149.17, 148.98, 148.06, 144.00, 141.32, 134.07, 129.49, 128.39, 127.07, 124.64, 121.79, 113.05, 111.66, 110.12, 102.72, 83.50, 65.38, 52.54, 46.36, 43.99, 40.58, 40.37, 40.16, 39.95, 39.74, 39.53, 39.33, 32.47, 30.91, 25.90, 22.61, 22.50, 21.86, 19.76, 19.34, 17.88.

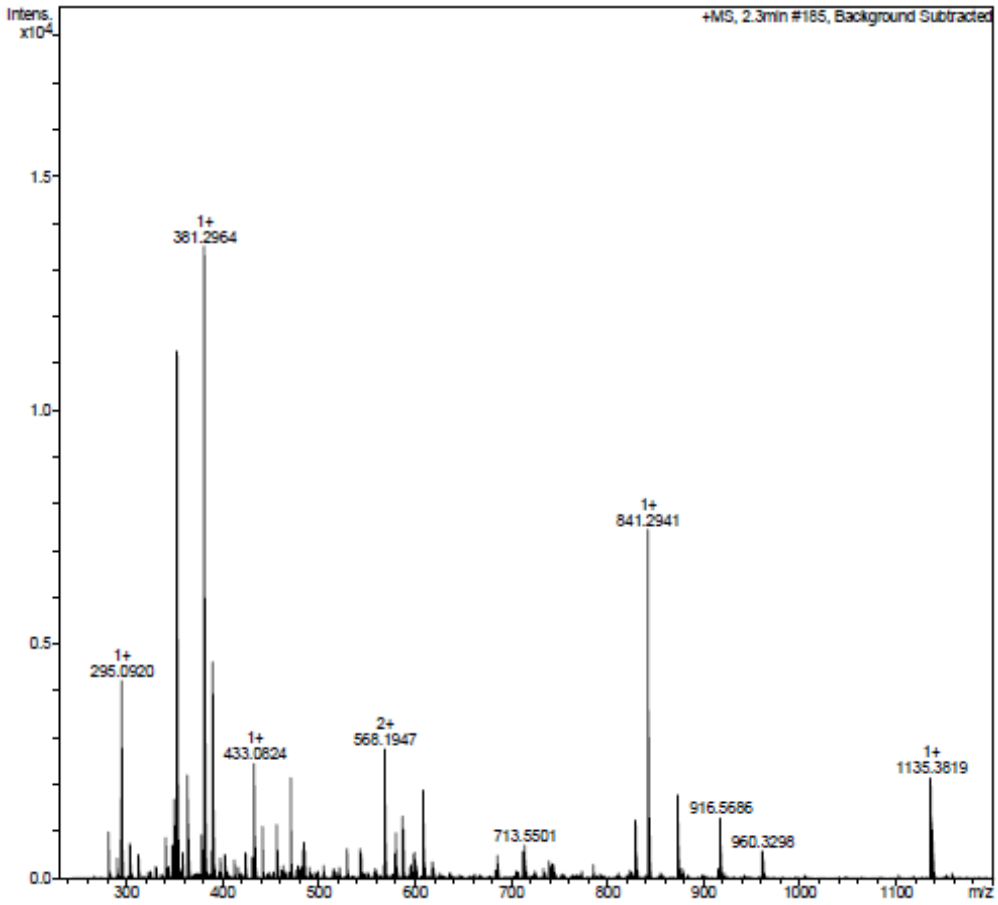
MS (ESI+, M+H) = 1135.02; HRMS (ESI-FIA-TOF) calc. = 1135.3844, found = 1135.3819



[4] JNC CORPORATION; Hosoya, Takamitsu; Kii, Isao; Yoshida, Suguru; Matsushita, Takeshi; US2013/11901 A1, **2013**

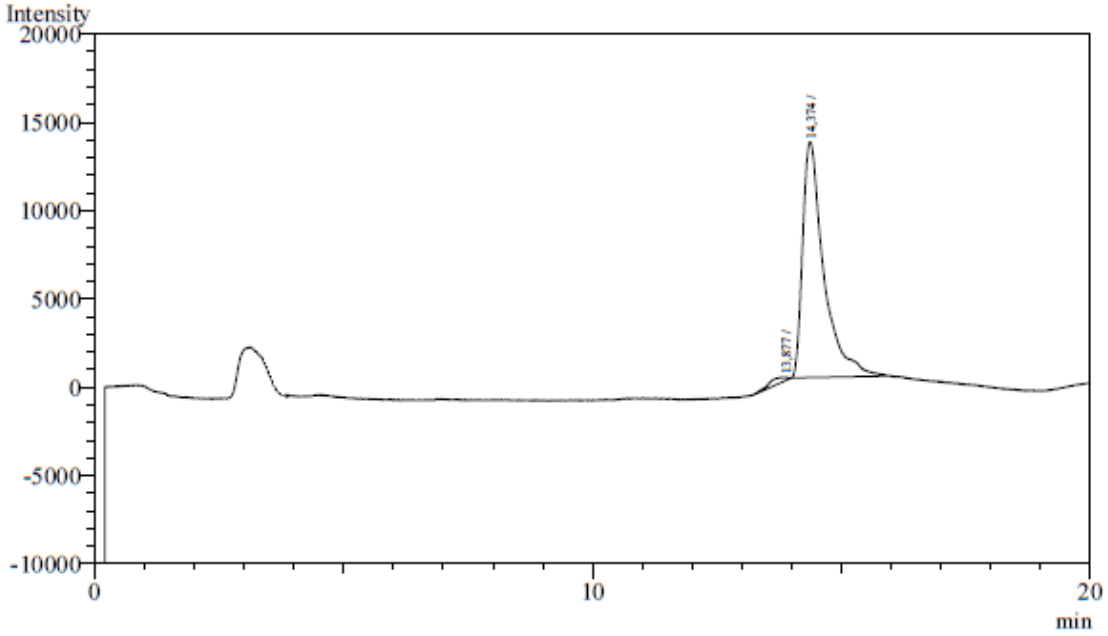

f1 (ppm)


ESI+ 3.0kV / 30V
SAMPLE 197_13 26FEV2013_POS_1 1 (0.017)
Scan ES+
1.40e8
x24
100.75
153.01
178.87
273.90
224.85
300.98
413.12
425.13
385.22
429.22
381.20
341.16
473.21
517.07
605.87
661.31
765.17
838.33
911.28
985.93
1059.97
1135.02
1283.00
%
m/z


Intens.
x10^4
+MS, 2.3min #185, Background Subtracted
1.5
1.0
0.5
0.0
1+
381.2964
1+
841.2941
1+
295.0920
1+
433.0824
2+
568.1947
1+
1135.3819
916.5686
713.5501
960.3298
300
400
500
600
700
800
900
1000
1100
m/z

Analysis Date & Time : 2/8/2014 8:23:21 PM
User Name : Admin
Vial# : 1
Sample Name : fafluo
Sample ID : fafluo
Sample Type : Unknown
Injection Volume :
ISTD Amount :

Data Name : C:\GCsolution\Data\AT folic acid\fafluo.gcd
Method Name : C:\GCsolution\Data\Project1\Untitled22.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit Mark	ID#	Cmpd Name
1	13.877	8464	194	2.096			
2	14.374	395409	13341	97.904			
Total		403873	13535				

5.4 BCN-Ethylenediamine-folate + dansylazide: synthesis of conjugate 7c

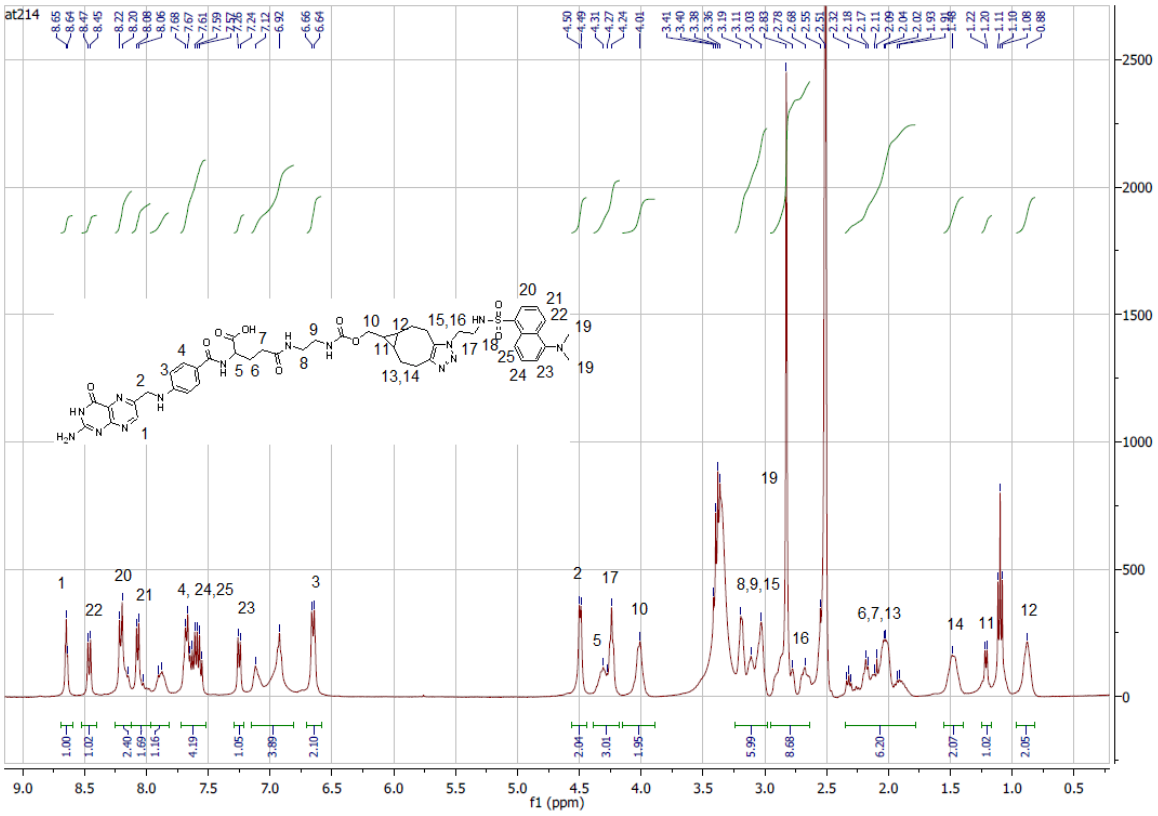
Ethylenediamine folate conjugate (10 mg, 0.021 mmol, 1 eq) was dissolved 2ml of DMSO, followed by the addition of diisopropylethyl amine (11ul, 0.063 mmol, 3 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (6.5 mg, 0.022mmol, 1.05eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of N-(2-azidoethyl) dansyl[5] (23 mg, 0.072 mmol, 3.4 eq). The reaction was stirred for 5 hours before it was poured into a solution of 20 % acetone/diethyl ether. The yellow precipitate was washed two times with acetone and diethyl ether to yield the respective triazole product in 70 % yield (14 mg) with 92% purity.

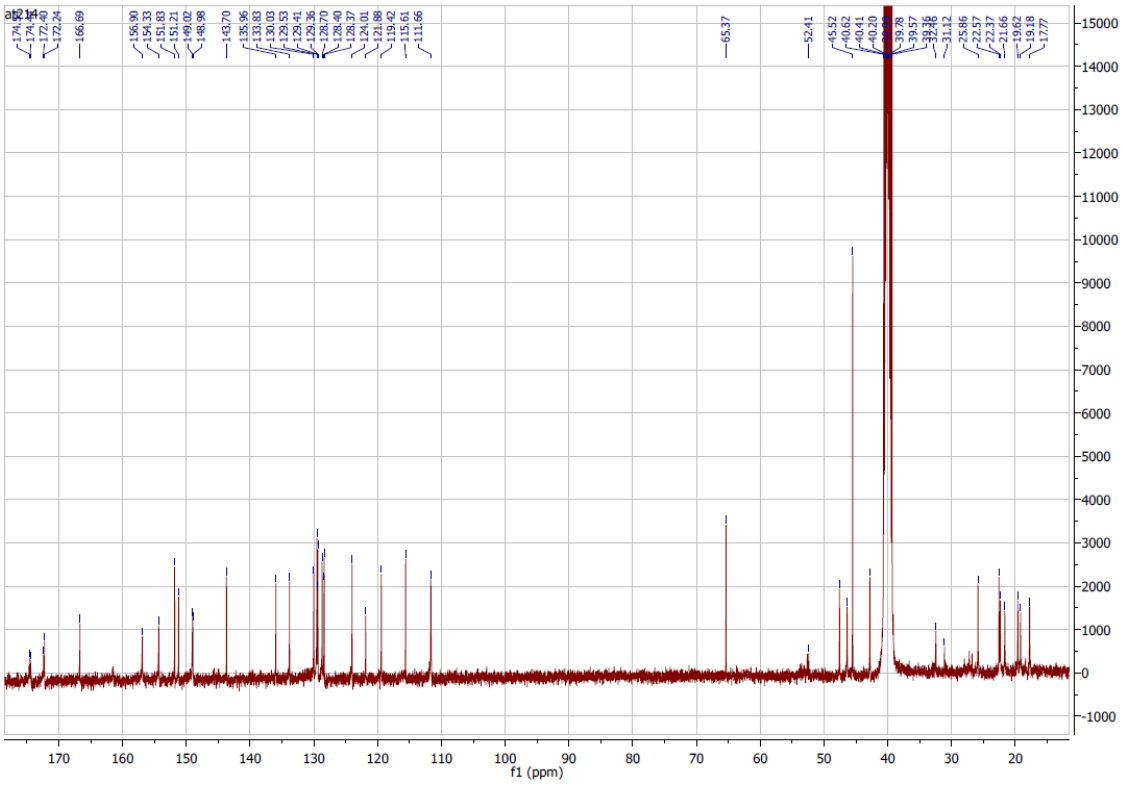
^{1}H NMR (400 MHz, DMSO) δ 8.65 (s, 1H, pterin), 8.47-8.45 (d, 1H, dansyl sulfonamide ring), 8.22-8.20 (d, 1H, dansyl sulfonamide ring), 8.08-8.03 (d, 1H, dansyl amine ring), 7.90-7.88 (bs 1H), 7.68-7.55 (m, 4H, 2H aromatic folate + 1H dansyl sulfonamide ring + 1H, dansyl amine ring), 7.26-7.24 (d, 1H, dansyl sulfonamide ring), 7.12-6.92 (m, 4H), 6.66-6.64 (d, 2H, aromatic folate), 4.50-4.49 (d, 2H, NC**H**$_2$-Ar), 4.31-4.24 (m, 3H, -HNC**H**CO$_2$H + triazole-C**H**$_2$), 4.01 (m, 2H, -HNCO$_2$C**H**$_2$), 3.36 (H$_2$O), 3.19-3.03 (m, 6H, O=C-NHC**H**$_2$C**H**$_2$+CH**H**-triazole-CH**H**), 2.83-2.68 (m, 8H, N(C**H**$_3$)$_2$ + C**H**H-triazole-C**H**H), 2.34-1.91 (m, 6H, C**H**$_2$C**H**$_2$CO$_2$H+CH**H**-CH$_2$-triazole-CH$_2$-CH**H**), 1.48 (m, 2H, C**H**H-CH$_2$-triazole-CH$_2$-C**H**H), 1.22-1.20 (m, 1H, HNCO$_2$CH$_2$C**H**), 0.88 (bs, 2H, -HNCO$_2$CH$_2$CH(C**H**)$_2$).

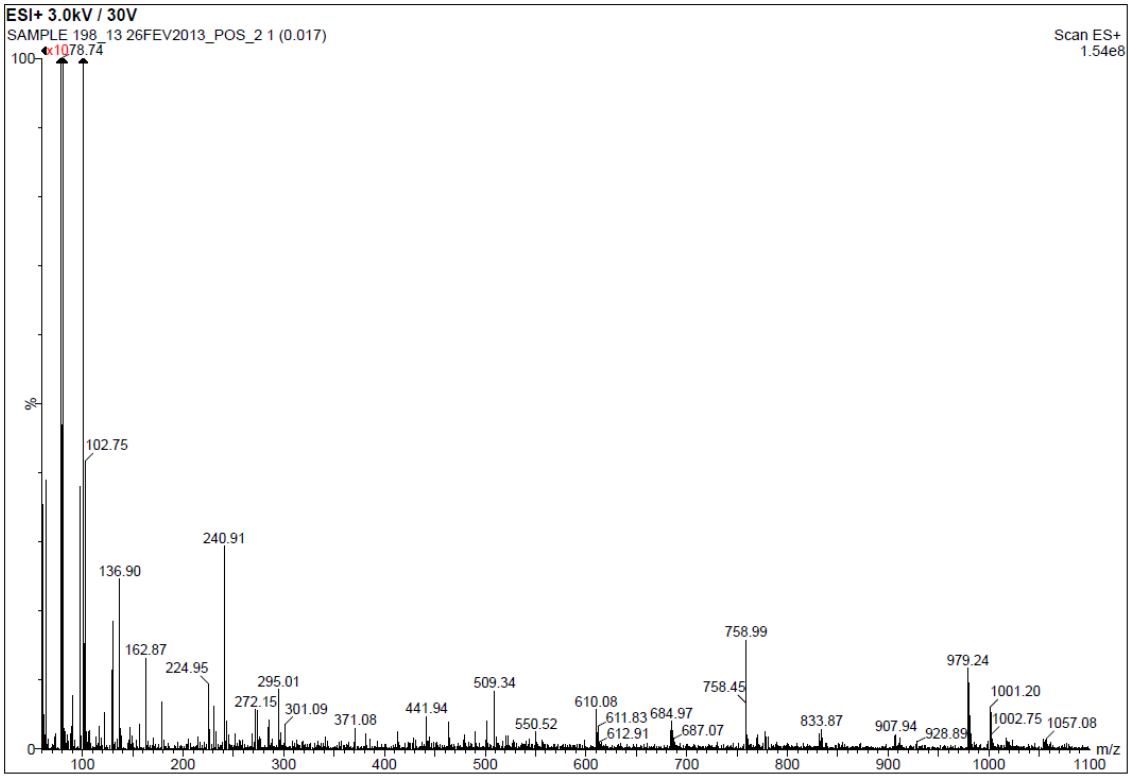
[5] Inverarity, Iain A.; Hulme, Alison N. *Org. Biomol. Chem.,* **2007** , vol. 5, 636

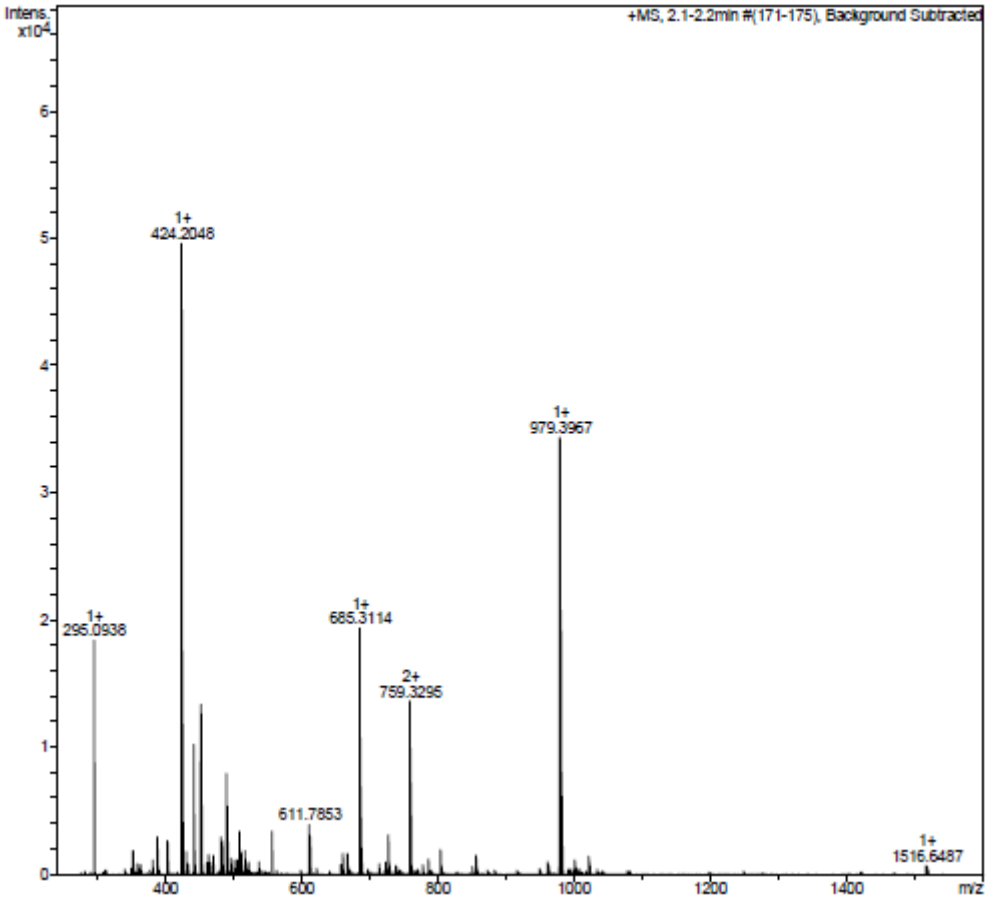
^{13}C NMR (100 MHz, DMSO) δ 174.54, 174.44, 172.40, 172.24, 166.69, 156.90, 154.33, 151.83, 151.21, 149.02, 148.98, 143.70, 135.96, 133.83, 130.03, 129.53, 129.41, 129.36, 128.70, 128.40, 128.37, 124.01, 121.88, 119.42, 115.61, 111.66, 65.37, 52.41, 47.57, 46.39, 45.52, 42.82, 40.62, 40.41, 40.20, 39.99, 39.78, 39.57, 39.36, 32.46, 31.12, 25.86, 22.57, 22.37, 21.66, 19.62, 19.18, 17.77.

MS (ESI+) = 979.24; HRMS (ESI-FIA-TOF) calc. = 979.3997, found = 979.3967



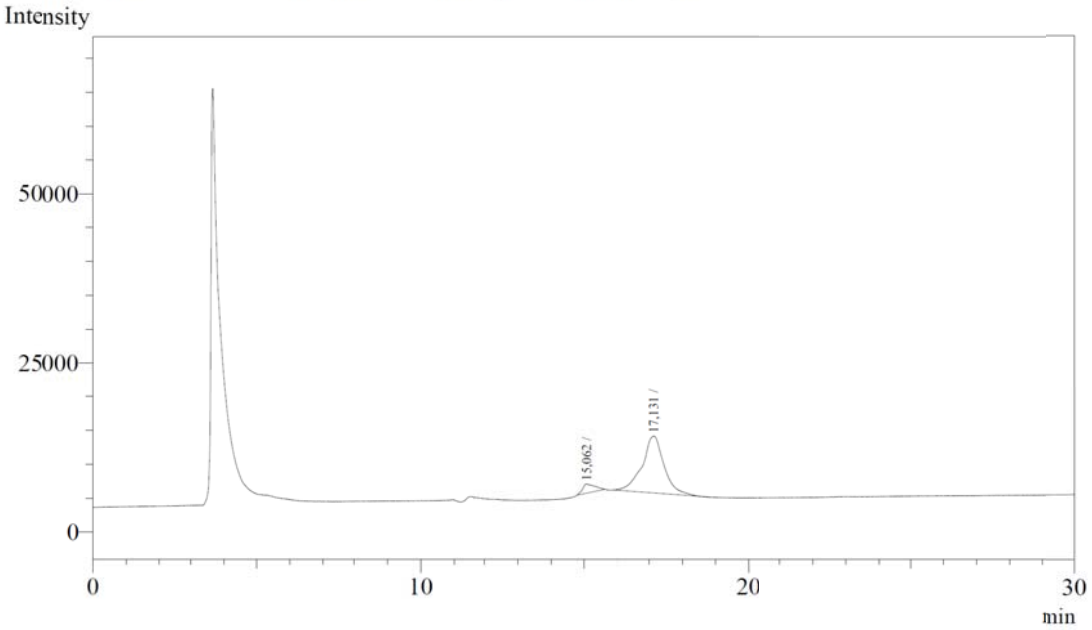

f1 (ppm)


ESI+ 3.0kV / 30V
SAMPLE 198_13 26FEV2013_POS_2 1 (0.017)
Scan ES+
1.54e8
1078.74
102.75
240.91
136.90
162.87
224.95
295.01
272.15
301.09
371.08
441.94
509.34
550.52
610.08
611.83
612.91
684.97
687.07
758.99
758.45
833.87
907.94
928.89
979.24
1001.20
1002.75
1057.08
m/z


Intens.
x10^4
+MS, 2.1-2.2min #(171-175), Background Subtracted
1+
424.2048
1+
979.3967
1+
685.3114
1+
295.0938
2+
759.3295
611.7853
1+
1516.6487
m/z

Analysis Date & Time : 2/8/2014 3:01:06 PM
User Name : Admin
Vial# : 1
Sample Name : fadansyl
Sample ID : fadansyl
Sample Type : Unknown
Injection Volume :
ISTD Amount :

Data Name : C:\GCsolution\Data\AT folic acid\fadansyl.gcd
Method Name : C:\GCsolution\Data\Project1\Untitled22.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit Mark	ID#	Cmpd Name
1	15.062	33809	1362	8.125			
2	17.131	382307	8360	91.875			
Total		416116	9722				

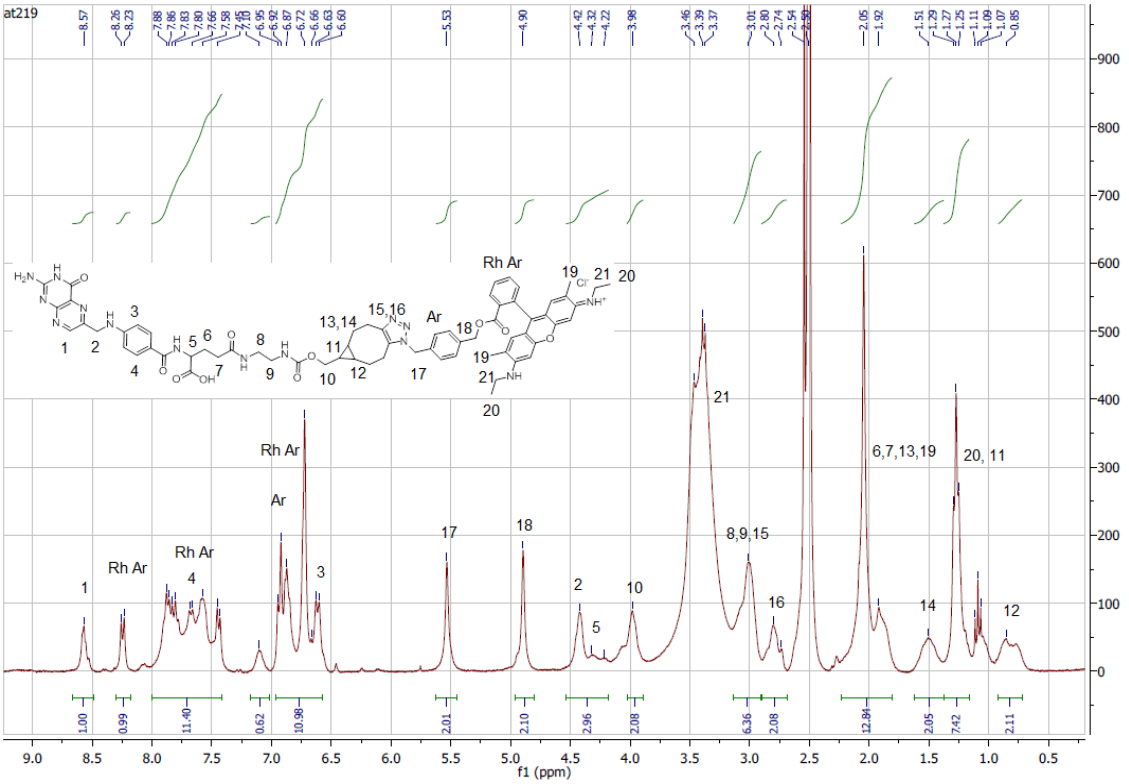
5.5 BCN-Ethylenediamine-folate + rhodamine 6G azide: synthesis of conjugate 7e

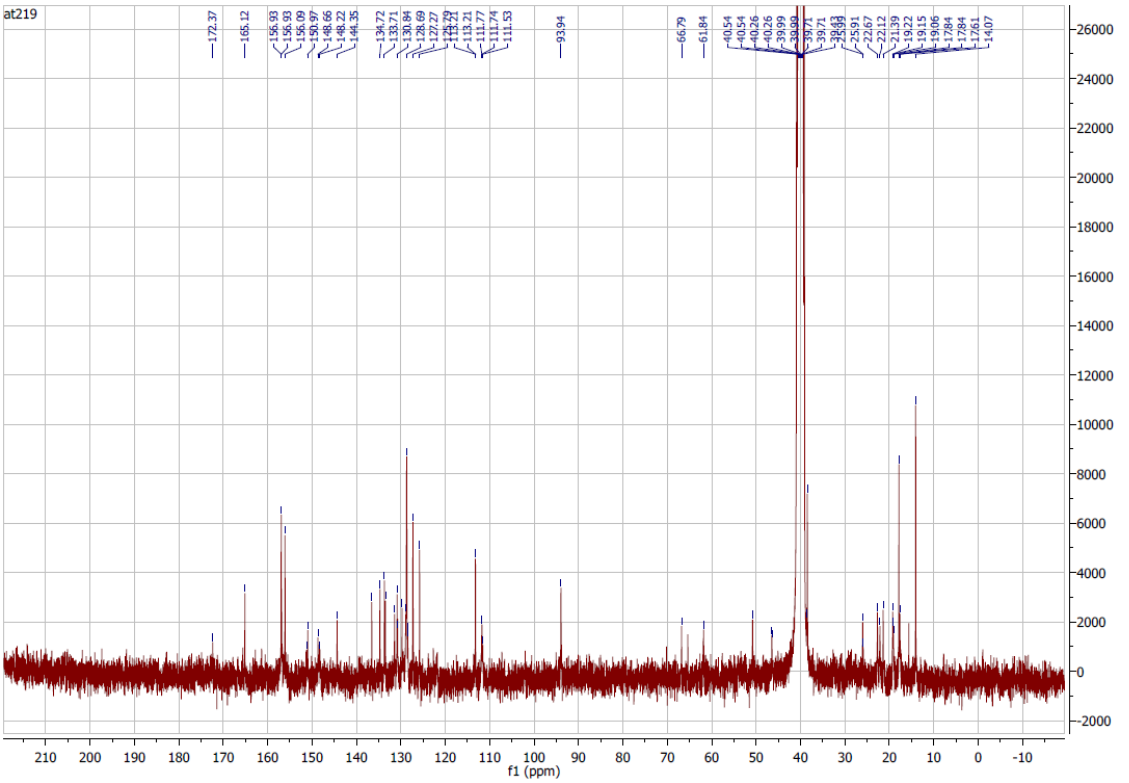
BCN-Ethylenediamine-folate (7 mg, 0.01 mmol, 1 eq) was dissolved in 2 ml of DMSO and added to a flask containing 14 mg of rhodamine 6G (4-(azidomethyl)phenyl)methyl ether (0.024 mmol, 2.4 eq). The reaction was stirred for 4 hours before it was poured into a solution of 20 % acetone/diethyl ether. The purple precipitate was washed four times with acetone and two times with diethyl ether to yield the respective triazole product in 93 % yield **(**13 mg).

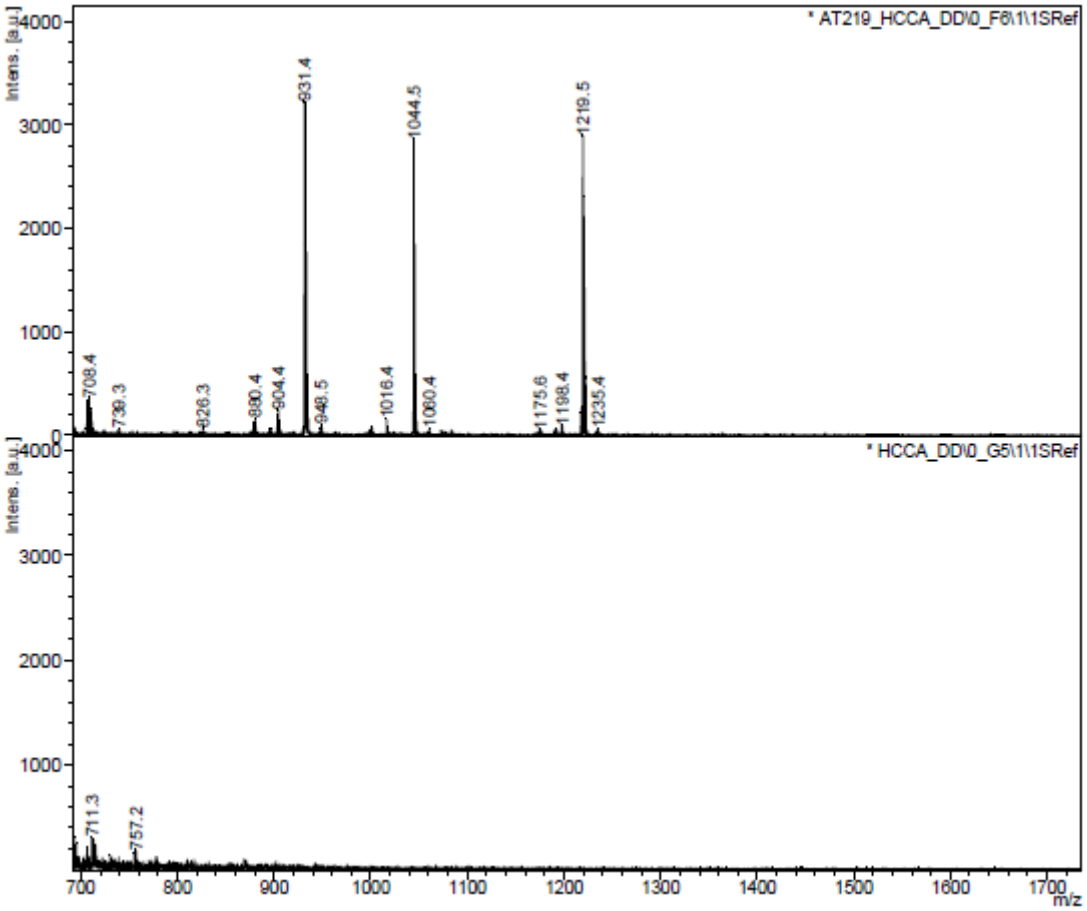
^{1}H NMR (400 MHz, DMSO) δ 8.57 (bs, 1**H**, pterin), 8.26-8.23 (d, 1H, ortho **H** ester rhodamine 6G), 7.88-7.45 (m, 11H, aromatic folate + rhodamine 6G), 7.10-6.60 (m, 12H, aromatic folate + rhodamine 6G + phenyl linker), 5.53 (s, 2**H**, triazole-C**H**$_2$-phenyl), 4.90 (s, 2H, ester-C**H**$_2$-phenyl), 4.42-4.22 (m, 3H, NC**H**$_2$-Ar + -HNC**H**CO$_2$H), 3.98 (m, 2H, -HNCO$_2$C**H**$_2$), 3.46-3.37 (water + C**H**$_2$rhodamine), 3.01 (m, 6H, O=C-NHC**H**$_2$C**H**$_2$+CH**H**-triazole-CH**H**), 2.80 (m, 2H, C**H**H-triazole-C**H**H), 2.05-1.92 (m, 12H, C**H**$_2$C**H**$_2$CO$_2$H + CH**H**-CH$_2$-triazole-CH$_2$-CH**H**, 2x benzylic C**H**$_3$), 1.51 (m, 2H, , C**H**H-CH$_2$-triazole-CH$_2$-C**H**H), 1.29-1.25 (m, 7H, C**H**$_3$rhodamine + HNCO$_2$CH$_2$C**H**), 0.85 (m, 2H, -HNCO$_2$CH$_2$CH(C**H**)$_2$).

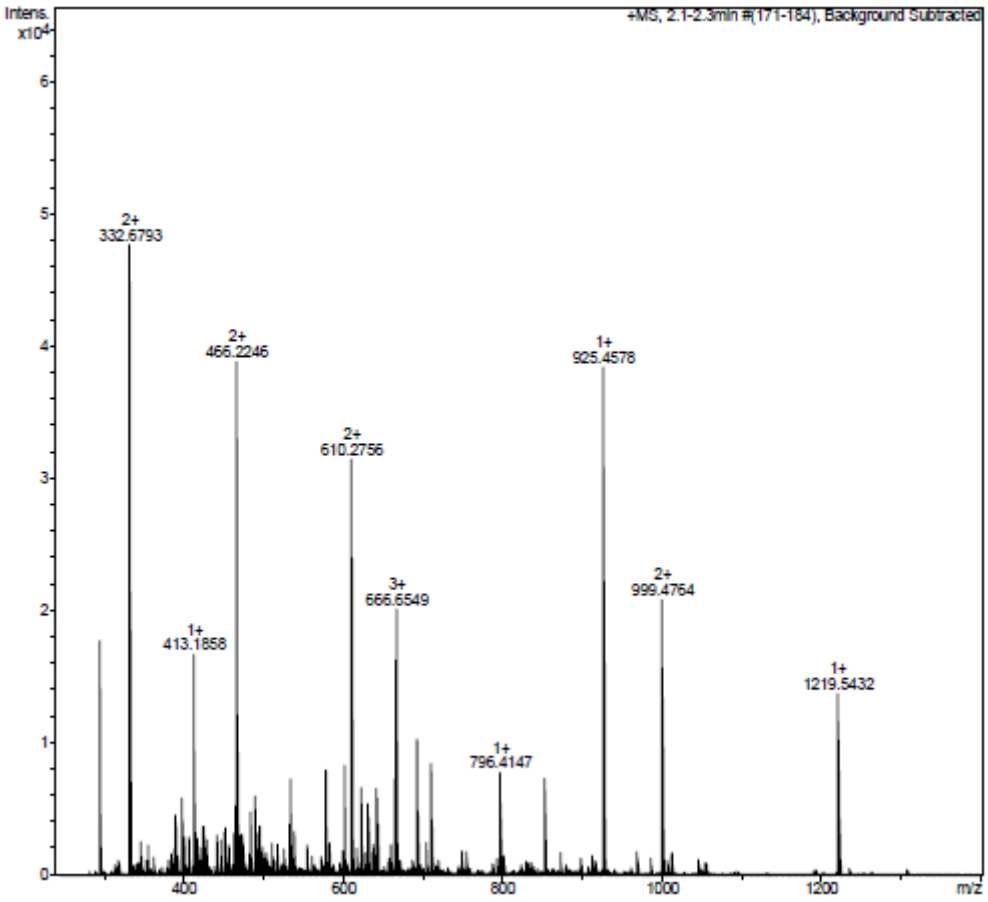
^{13}C NMR (75 MHz, DMSO) δ 172.37, 165.12, 156.93, 156.93, 156.09, 151.25, 150.97, 148.66, 148.22, 144.35, 136.56, 134.72, 133.71, 133.47, 131.42, 130.84, 130.76**,** 129.74, 128.88, 128.69, 128.49, 128.43, 127.27, 125.79, 113.21, 113.21, 111.77, 111.74, 111.53, 93.94, 66.79, 61.84, 50.80, 46.48, 46.42, 38.65, 38.47, 38.47, 25.99, 25.91, 22.67, 22.12, 2**1**.39, 19.22, 19.15, 19.06, 17.84, 17.84, 17.61, 14.07.

MS (ESI+) = 1219.5; HRMS (ESI-FIA-TOF) calc. = 1219.5477, found = 1219.543**2**


at219
Rh Ar
Ar
6,7,13,19
20, 11
8,9,15
f1 (ppm)


at219
f1 (ppm)


* AT219_HCCA_DD\0_F6\1\1\1SRef
Intens. [a.u.]
4000
3000
2000
1000
0
708.4
739.3
826.3
890.4
904.4
931.4
948.5
1016.4
1044.5
1060.4
1175.6
1198.4
1219.5
1235.4
* HCCA_DD\0_G5\1\1\1SRef
Intens. [a.u.]
4000
3000
2000
1000
711.3
757.2
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
m/z



5.6 Rhodamine 6G (4-(azidomethyl)phenyl)methyl ether

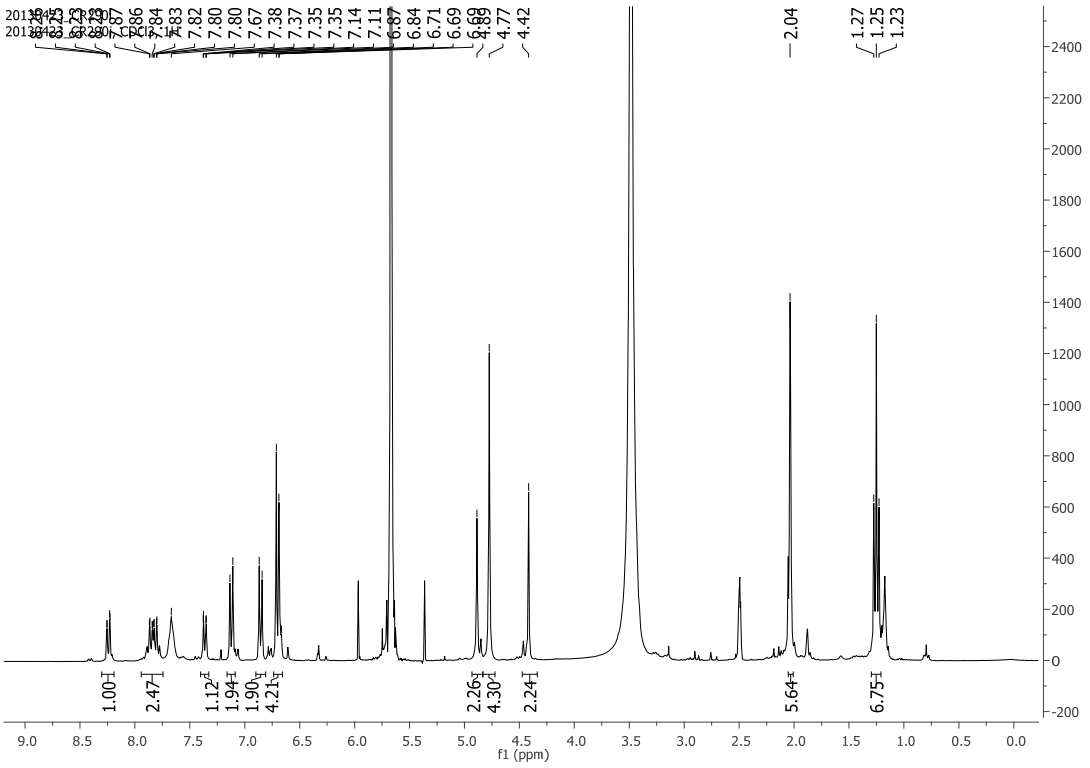
Rhodamine 6G 4-chloromethyl-1-phenylmethyl ester chloride derivative (30.4 mg, 1 eq.), NaN_3 (6.7 mg, 2 eq.) and NaI (cat.) were dissolved in a mixture of acetone (1 mL) and water (0.5 mL), the resulting mixture was stirred at room temperature during 19.5 hrs. The mixture was extracted with CH_2Cl_2. The organic fraction was dried with Na_2SO_4, filtered and the organics were removed under vacuum. The remaining solid was recrystallized from AcOEt, EtOH and Et_2O to give a greenish pink solid. (8.7 mg, yield= 28%)

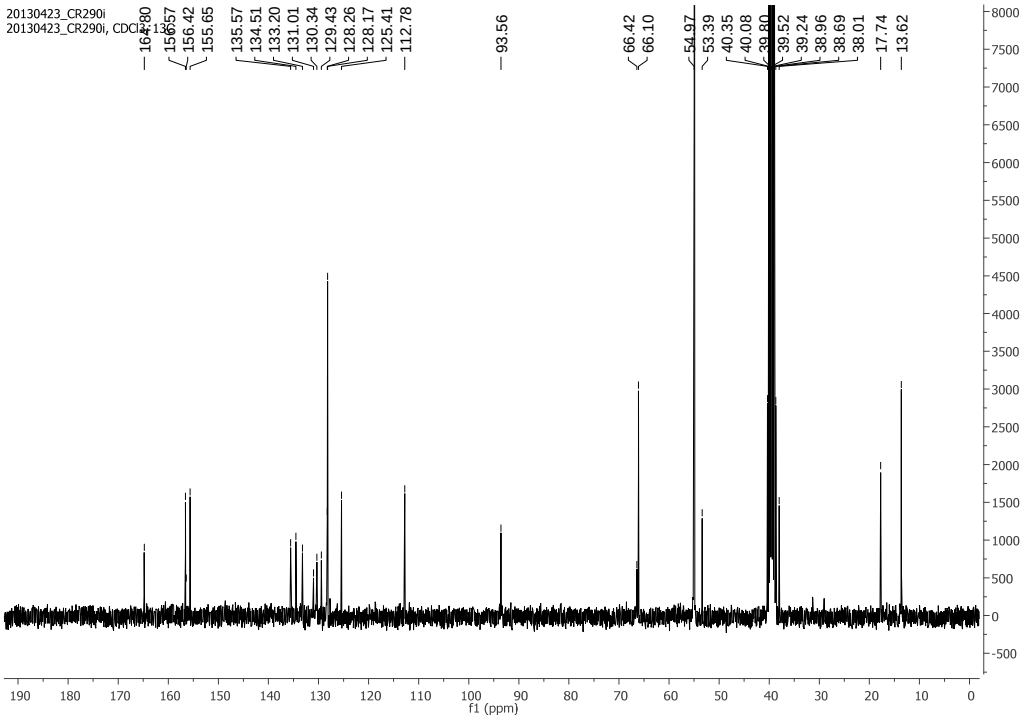
^{1}H NMR (300 MHz, DMSO) δ (ppm) 8.24 (dd, *J* = 7.6, 1.2 Hz, 1H, 1x$C\underline{H}_{Ar}$), 7.83 (dtd, *J* = 19.3, 7.5, 1.4 Hz, 2H, 2x$C\underline{H}_{Ar}$), 7.36 (dd, *J* = 7.4, 1.1 Hz, 1H, 1x$C\underline{H}_{Ar}$), 7.12 (d, *J* = 8.1 Hz, 2H, 2x$C\underline{H}_{Ar}$), 6.86 (d, *J* = 8.0 Hz, 2H, 2x$C\underline{H}_{Ar}$), 6.74 – 6.66 (m, 4H, 4x$C\underline{H}_{Ar}$), 4.89 (s, *J* = 10.5 Hz, 2H, $C\underline{H}_2N_3$), 4.77 (s, 4H, 2x$C\underline{H}_2CH_3$), 4.42 (s, *J* = 14.5 Hz, 2H, $C\underline{H}_2O$), 2.04 (s, 6H, 2x$C\underline{H}_3$), 1.25 (t, *J* = 7.1 Hz, 6H, 2x$CH_2C\underline{H}_3$).

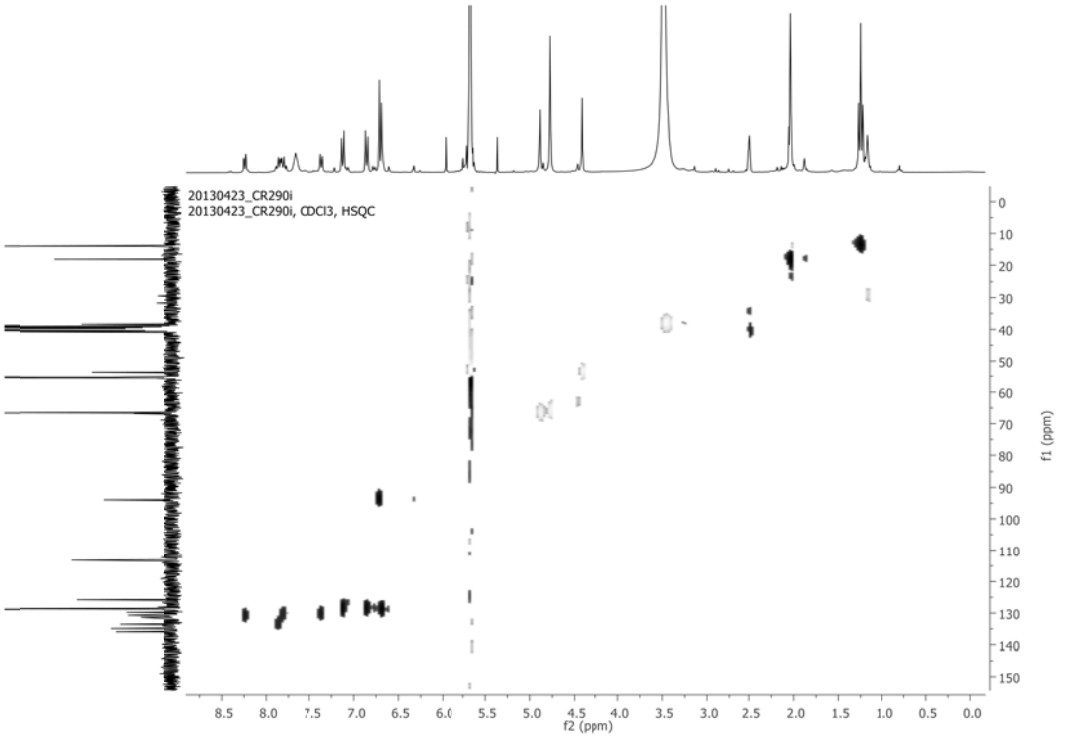
^{13}C NMR (75 MHz, DMSO) δ (ppm) 164.80, 156.57, 156.42, 155.65, 135.57, 134.51, 133.20 ($\underline{C}H_{Ar}$), 131.01 ($\underline{C}H_{Ar}$), 130.34 ($\underline{C}H_{Ar}$), 129.43 ($\underline{C}H_{Ar}$), 128.26 ($\underline{C}H_{Ar}$), 128.17 ($\underline{C}H_{Ar}$), 125.41, 112.78, 93.56 ($\underline{C}H_{Ar}$), 66.42 ($\underline{C}H_2N_3$), 66.10 ($\underline{C}H_2CH_3$), 54.97, 53.39 ($\underline{C}H_2O$), 38.01, 17.74 ($\underline{C}H_3$), 13.62 ($CH_2\underline{C}H_3$).

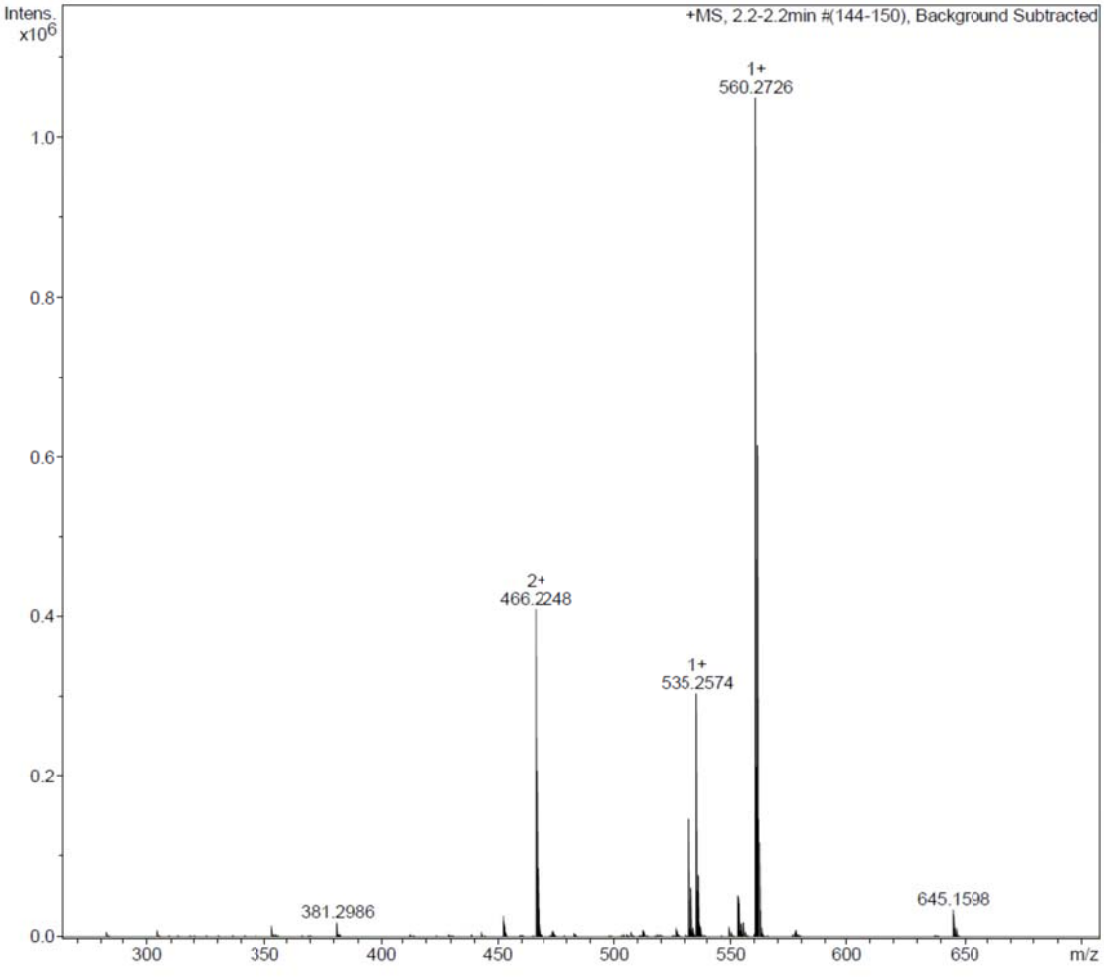
MS (ESI-Fia-TOF, cation) = 560.27

HRMS calc for $C_{34}H_{34}ClN_5O_3$: 560.2750, found: 560.2726 (ESI-Fia-TOF)


7.86
7.84
7.83
7.82
7.80
7.80
7.67
7.38
7.37
7.35
7.35
7.14
7.11
6.87
6.84
6.71
6.69
6.69
4.89
4.77
4.42
2.04
1.27
1.25
1.23
1.00
2.47
1.12
1.94
1.90
4.21
2.26
4.30
2.24
5.64
6.75
f1 (ppm)


20130423_CR290i
20130423_CR290i, CDCl3
164.80
156.57
156.42
155.65
135.57
134.51
133.20
131.01
130.34
129.43
128.26
128.17
125.41
112.78
93.56
66.42
66.10
54.97
53.39
40.35
40.08
39.80
39.52
39.24
38.96
38.69
38.01
17.74
13.62
f1 (ppm)


20130423_CR290i
20130423_CR290i, CDCl3, HSQC
f2 (ppm)
f1 (ppm)


Intens.
x10^6
+MS, 2.2-2.2min #(144-150), Background Subtracted
1+
560.2726
2+
466.2248
1+
535.2574
381.2986
645.1598
m/z

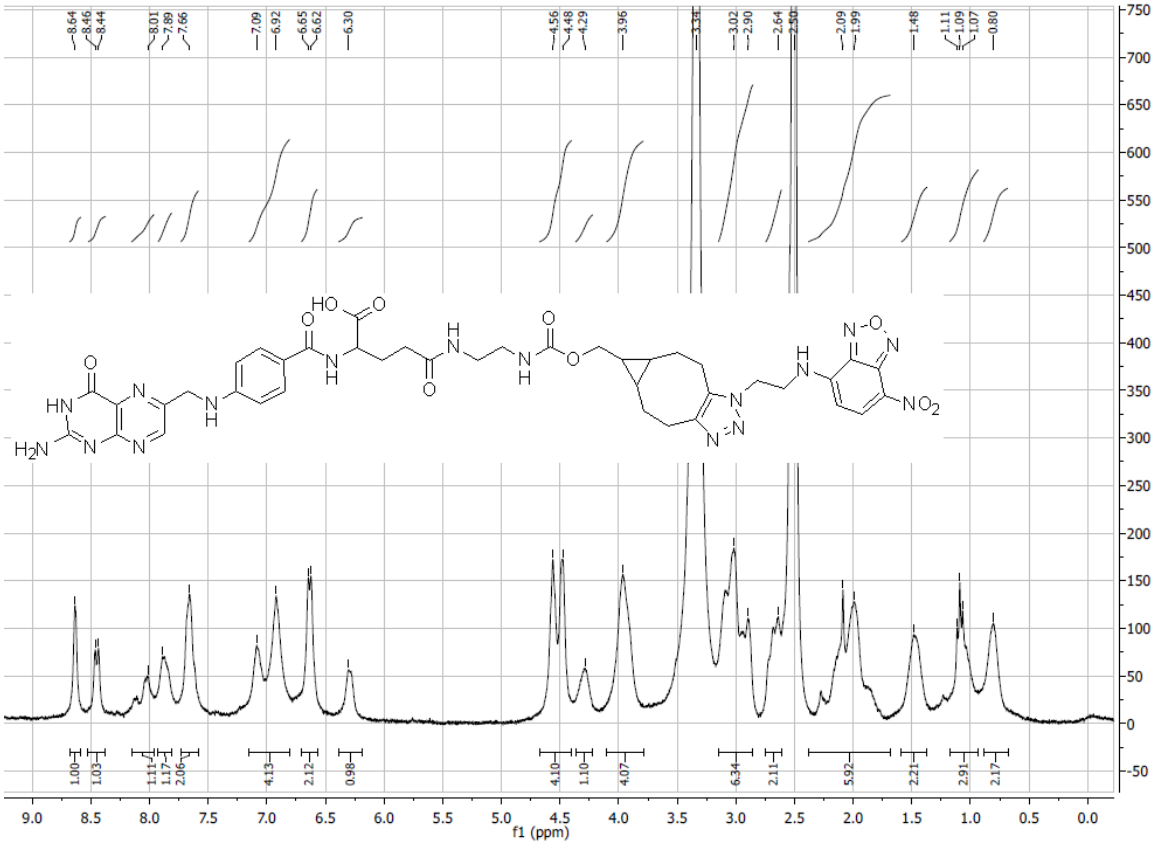
5.7 BCN-Ethylenediamine-folate + furazan azide: synthesis of conjugate 7f

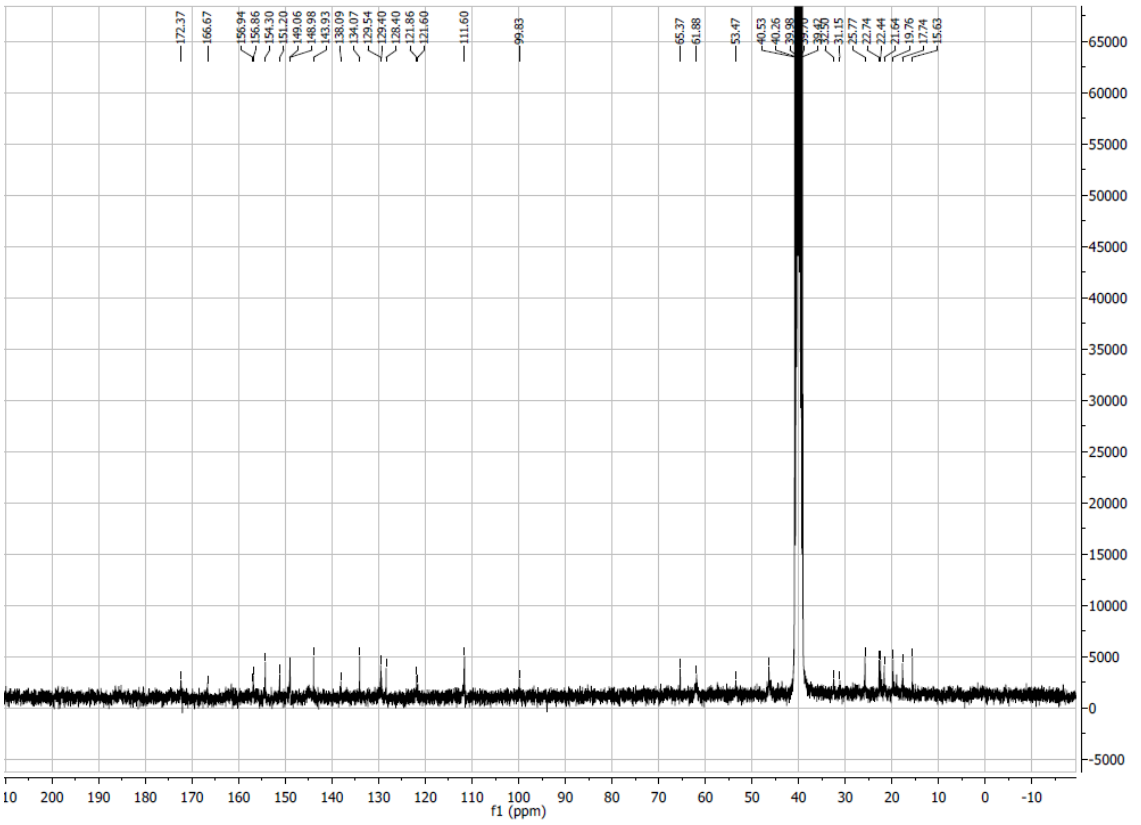
BCN-Ethylenediamine-folate (7 mg, 0.01 mmol, 1 eq) was dissolved in 2 ml of DMSO and added to a flask containing 5 mg of N-(2-azidoethyl)-7-nitro benzofurazan (0.02 mmol, 2 eq). The reaction was stirred for 4 hours before it was poured into a solution of 20 % acetone/diethyl ether. The brown precipitate was washed four times with acetone and two times with diethyl ether to yield the respective triazole product in 68 % yield (8 mg) with 96% purity.

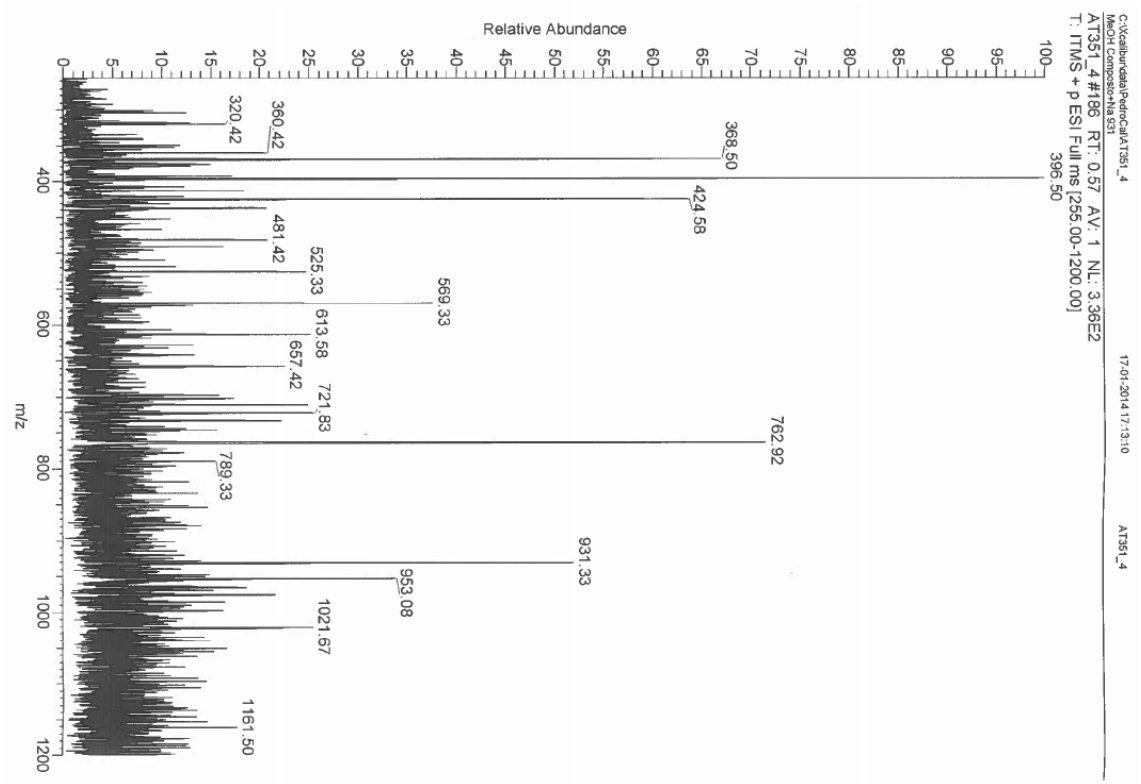
^{1}H NMR (300 MHz, DMSO) δ 8.64 (bs, 1H), 8.46-8.46 (d, 1H, furazan), 8.01-7.89 (m, 2H), 7.66 (m, 2H), 7.09-6.92 (m, 4H), 6.65-6.62 (m, 2H), 6.30 (d, 1H, furazan) 4.56-4.48 (m, 4H), 4.29 (bs, 1H), 3.96 (m, 3H), 3.02-2.90 (m, 6H), 2.64 (m, 2H), 2.09-1.99 (m, 6H), 1.48 (m, 2H), 1.11-1.07 (m, 3H), 0.8 (m, 2H).

^{13}C NMR (75 MHz, DMSO) δ 172.37, 166.67, 156.94, 156.86, 154.30, 151.20, 149.06, 148.98, 143.93, 138.09, 134.07, 129.54, 129.40, 128.40, 121.86, 121.60, 111.60, 99.83, 65.37, 61.88, 53.47, 46.37, 32.50, 31.15, 25.77, 22.74, 22.44, 21.64, 19.76, 17.74, 15.63.

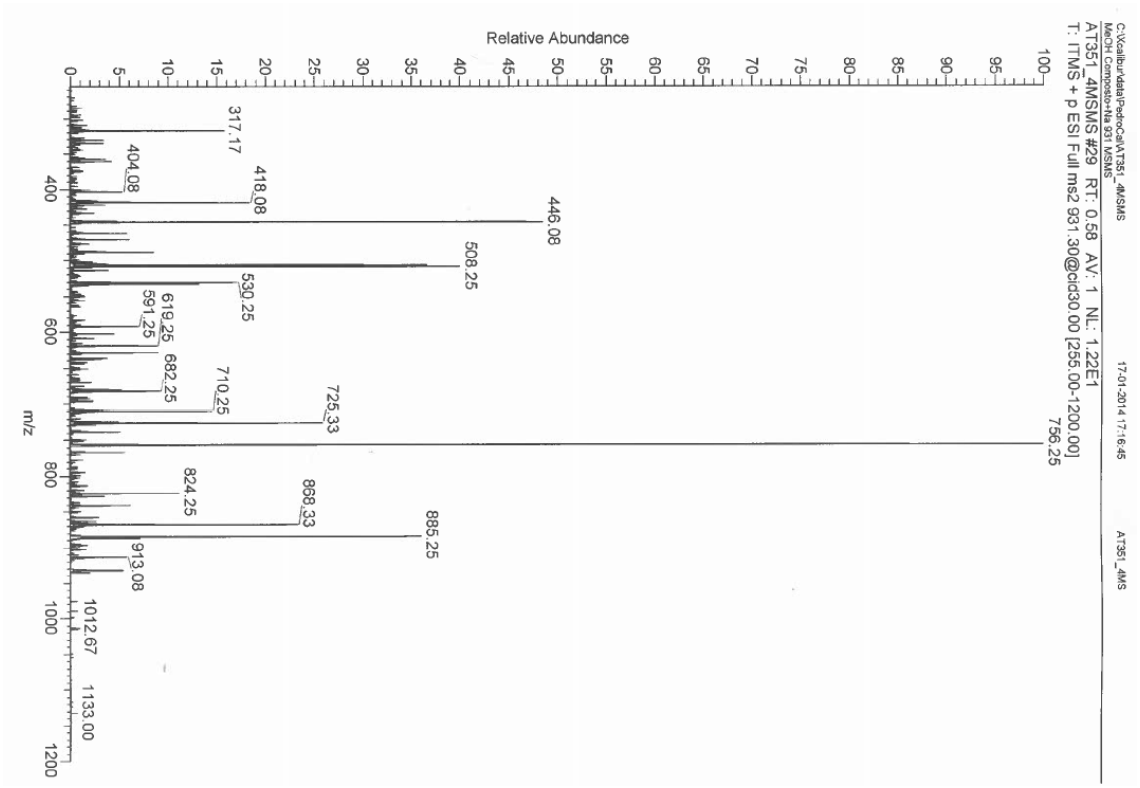
MS (ESI+, $C_{40}H_{44}N_{16}NaO_{10}$) = 931.33; HRMS (ESI-FIA-TOF, M+H) calc. = 909.3504, found = 909.3488

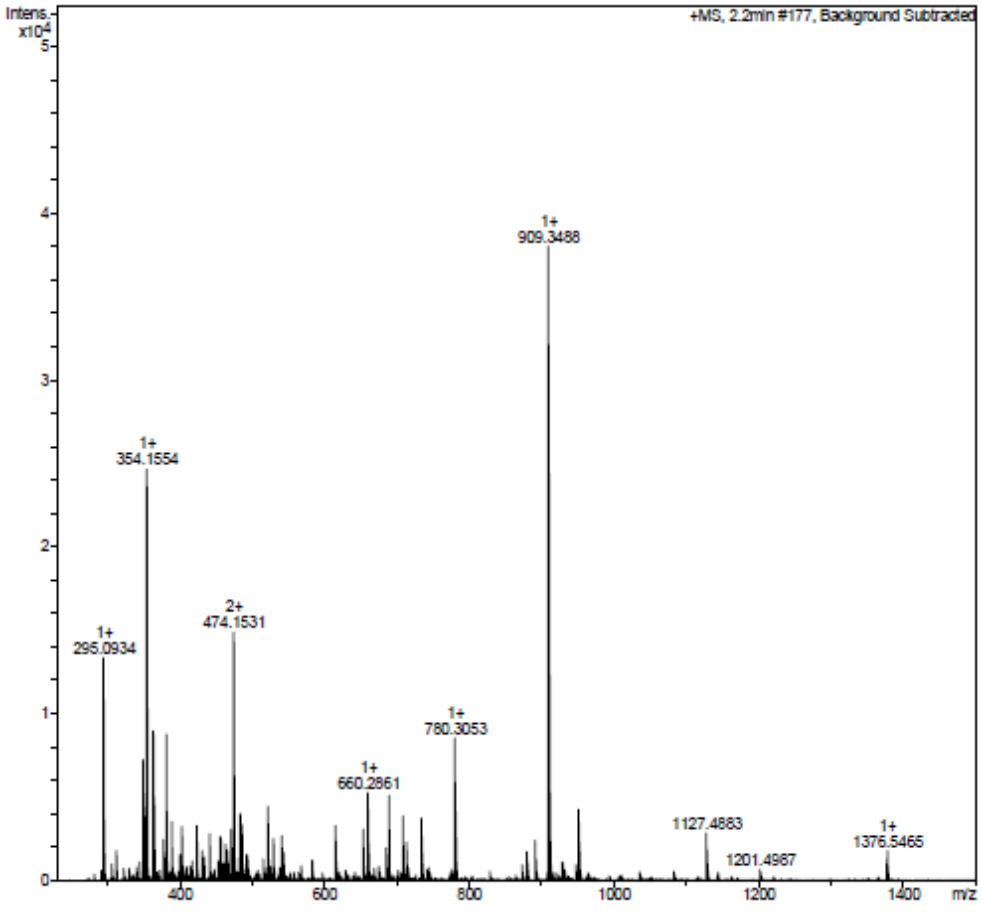



f1 (ppm)


Relative Abundance
m/z
C:\Xcalibur\data\Pedro\Cat\AT351_4
MeOH Composto+Na 931
AT351_4 #186 RT: 0.57 AV: 1 NL: 3.36E2
T: ITMS + p ESI Full ms [255.00-1200.00]
17-01-2014 17:13:10
AT351_4
320.42
360.42
368.50
396.50
424.58
481.42
525.33
569.33
613.58
657.42
721.83
762.92
789.33
931.33
959.08
1021.67
1161.50

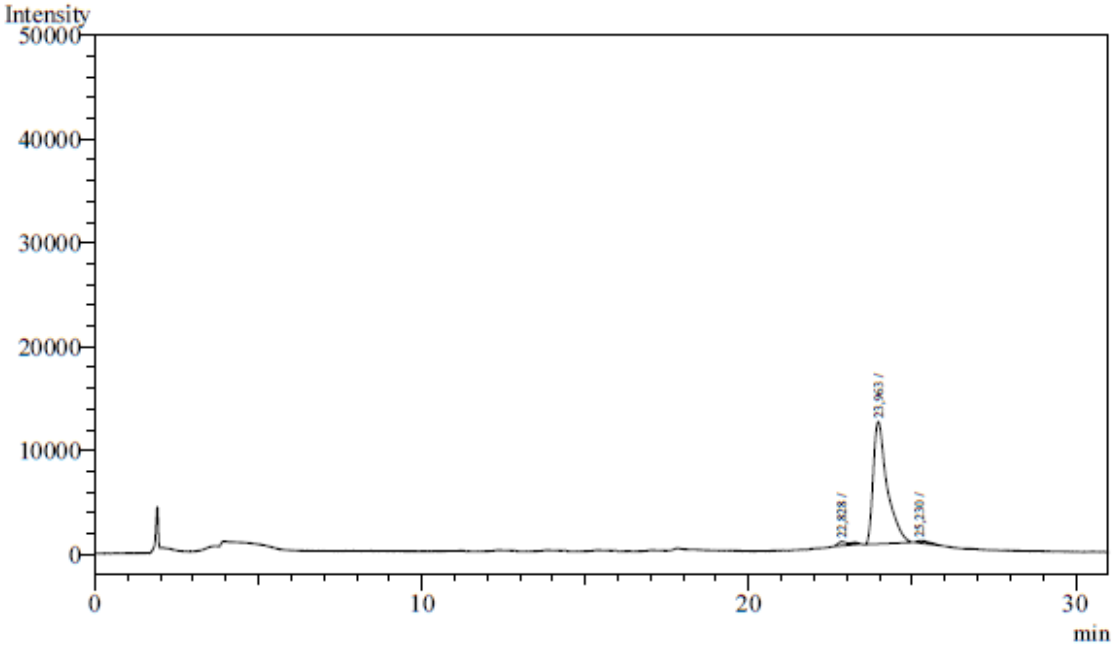
MS of 931.33:


Relative Abundance
m/z
317.17
404.08
418.08
446.08
508.25
530.25
591.25
619.25
682.25
710.25
725.33
756.25
824.25
868.33
885.25
913.08
1012.67
1133.00
C:\Xcalibur\data\PedroCal\AT351_4MSMS
MeOH Composto+Na 931 MSMS
AT351_4MSMS #29 RT: 0.58 AV: 1 NL: 1.22E1
T: ITMS + p ESI Full ms2 931.30@cid30.00 [255.00-1200.00]
17-01-2014 17:16:45
AT351_4MS


Intens.
x10^4
+MS, 2.2min #177, Background Subtracted
1+
909.3488
1+
354.1554
2+
474.1531
1+
295.0934
1+
780.3053
1+
660.2861
1127.4863
1201.4967
1+
1376.5465
m/z

Analysis Date & Time : 2/8/2014 5:57:20 PM
User Name : Admin
Vial# : 1
Sample Name : fafur
Sample ID : fafur
Sample Type : Unknown
Injection Volume :
ISTD Amount :

Data Name : C:\GCsolution\Data\AT folic acid\fafur.gcd
Method Name : C:\GCsolution\Data\Project1\Untitled22.gcm

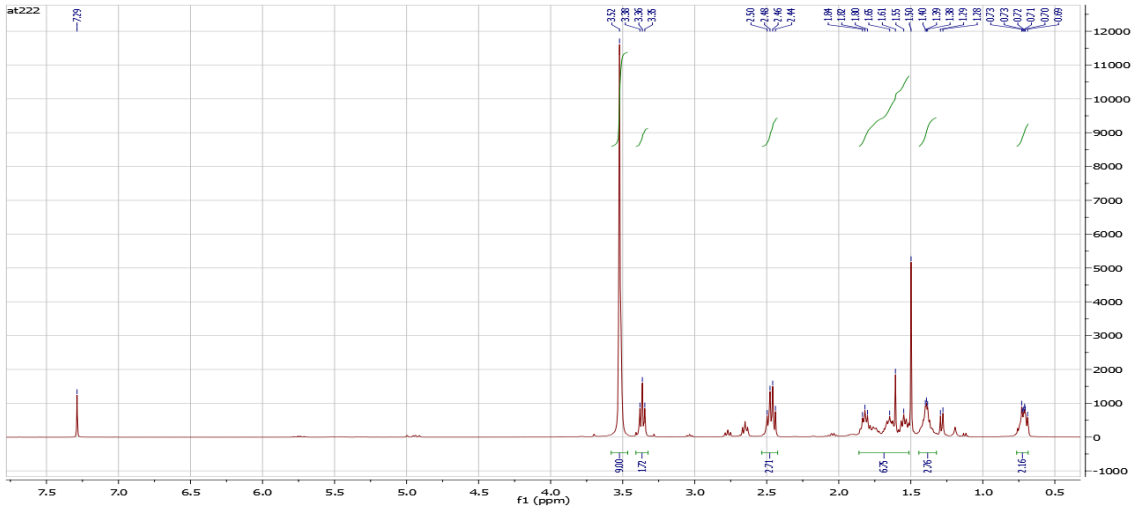


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	22,828	10034	409	2,743				
2	23,963	349546	11773	95,543				
3	25,230	6271	229	1,714				
Total		365851	12411					

5.8 BCN-Ethylenediamine-folate + silica functionalized with azide: synthesis of 7g

1st step – In a glass reactor, 3-mercaptopropyl trimethoxysilane (0.36g, 1.8mmol), 6-bromo-1-hexene (0.244g, 1.5mmol) and 2,2'-azo-bisisobutyronitrile (0.12g, 0.75mmol) were dissolved in 10 ml of chloroform. The reaction was heated for 24h at 80ºC, before the solvent been removed under reduced pressure. The crude was analyzed by 1H NMR, confirming the complete conversion of the alkene, and used in the next step without further purification.[6]

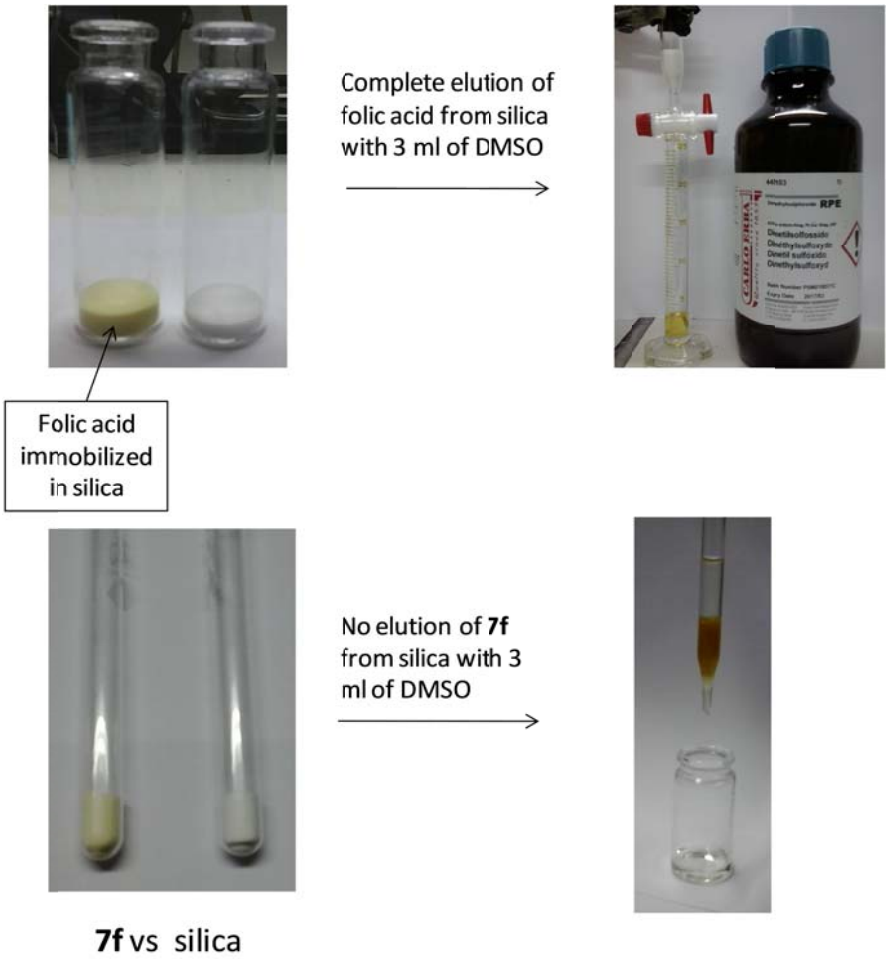
[6] Gill, Christopher S.; Venkatasubbaiah, Krishnan; Jones, Christopher W. *Adv. Synth. Catal.*, **2009**, vol. 351, # 9 p. 1344



2nd step – The crude thioether was refluxed in 15 ml of dry toluene in presence of flash chromatography grade silica-gel during 24h under Argon atmosphere. After filtrations and washings with dichloromethane, 1.9 g of functionalized silica-gel was isolated accounting for 95 % over two-steps.[6]

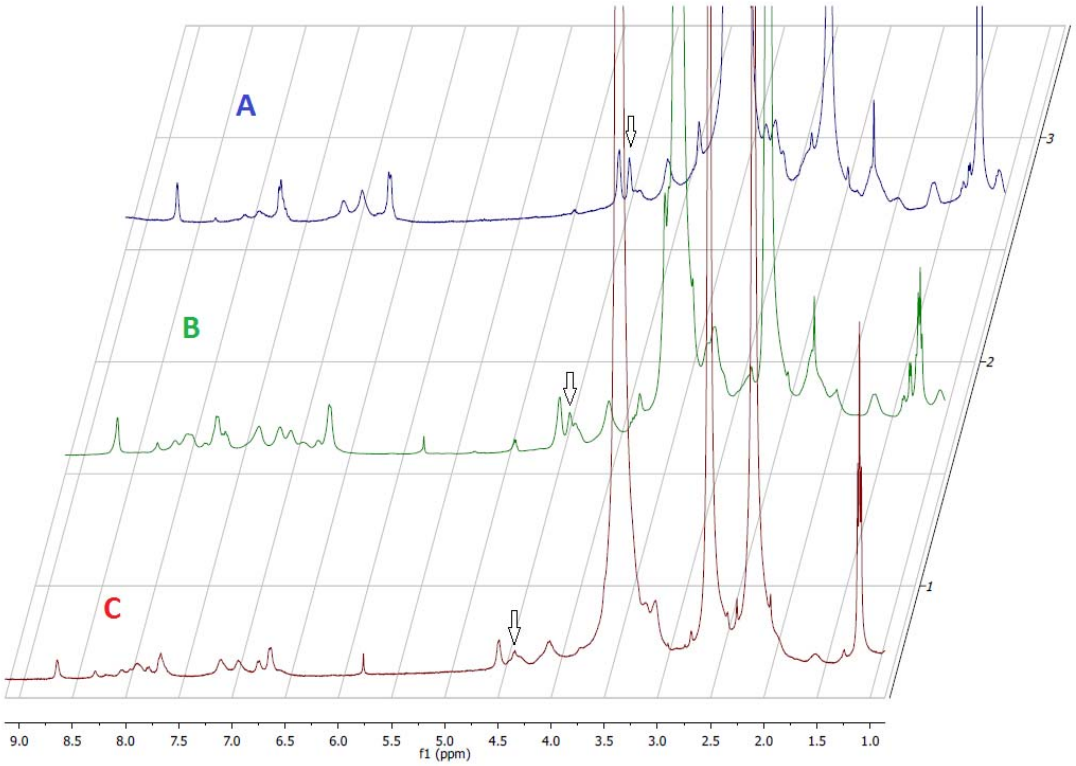
3rd step – The bromide functionalized silica-gel (maximum theoretical of 1.5 mmol) was reacted with 650mg of sodium azide (10mmol) in DMF during 72h at 80ºC. The crude solid was filtered and washed thoroughly with water to remove the inorganic by-products salts, yielding 1.4g of a white solid (72% isolated yield).

4th step – Ethylenediamine folate conjugate (20 mg, 0.041 mmol, 1 eq) was dissolved 4ml of DMSO, followed by the addition of diisopropylethyl amine (22ul, 0.12 mmol, 3 eq) and (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (13 mg, 0.044 mmol, 1.05eq). The reaction mixture was stirred until yellow solution is obtained (about 30 minutes), followed by the addition of azide-functionalized silica-gel (260mg, maximum theoretical 0.2mmol of azides). The reaction was stirred for 16h, centrifuged and the light yellow solid (275 mg) was washed thoroughly with acetone and ether. The yellow color was absent from the supernatant indicating fully immobilization. This yellow powder was further eluted with DMSO, and since no leaching of color to the mobile phase was detected it further confirmed the chemical immobilization. Folic acid when physically immobilized in silica-gel is eluted with DMSO. To immobilize physically folic acid in silica-gel, it was dissolved in DMSO, and stirred 30 minutes with silica. After, the DMSO was removed by washing with mixture of 20 % acetone/diethyl ether.



5.9 Click testing under biological compatible conditions

MeO-PEG_{350}-N_3 (2 eq., 3.4 mg) was dissolved in water and added to a solution of conjugate **3** (1 eq., 3 mg) in DMSO giving three solutions of (A) 1:1, (B) 1:9 and (C) 1:99 DMSO:water ratio ([**3**] = 5.625 mM, 1.125 mM and 112.5 µM, respectively). The solutions were placed in a bath at 37ºC and left to react for 1h (A), 2h (B) and 24h (C) without stirring. The folates were isolated by precipitation and analyzed by ^{1}H NMR. The imidazolic protons are evidenced by an arrow in the following spectra.



6 Cell staining procedure

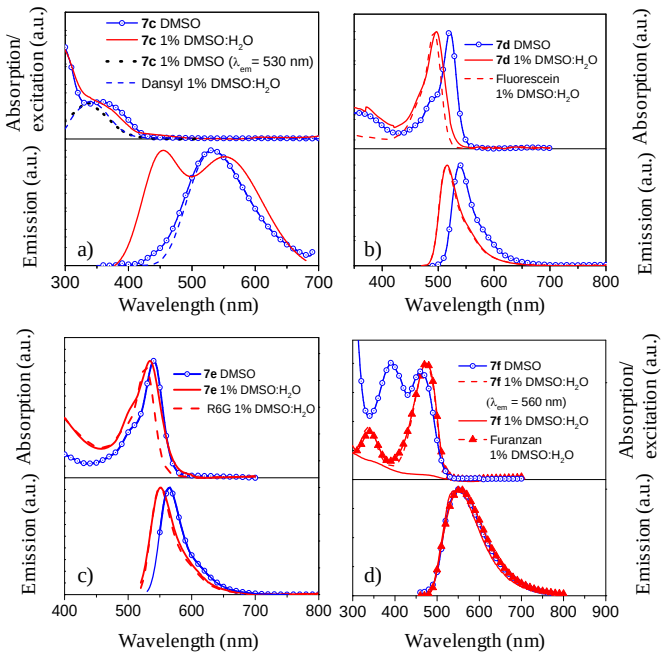
Cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotic antimycotic solution (100 units/ml penicillin, 0,1 mg/ml streptomycin and 0,25 mg/ml amphotericin B) and grown in an incubator at 37 ºC and under a 5% CO_2 atmosphere.

-Slide 8 well plates were coated with poly-L-Lysine for at least 30min and washed before seeding with the cells. After an incubation period of approximately 2 days, cells were treated for 1 hour with 5 µM of the probe to be tested. For conduction of the click reaction *in situ*, substrates (25 µM of **8** and 12.5 µM of **3**) were added to the cells medium and left incubating for a 24 hours period. After treatment, cells were washed with phenol-red free medium and treated with 5 µg/ml of the membrane staining WGA-Alexa594 that was kept 20-30 minutes in contact with the cells. After this staining treatment, cells were washed and fresh phenol-red free medium was added to the wells before imaging.

7 Spectroscopic characterization of folate dyes

Scheme 5 compares the absorption and emission spectra of the modified free dyes and the dye labeled folate conjugates in similar conditions to those used in the cell staining experiments (micromolar concentrations in a 1% DMSO in water solution). Linking different fluorescent probes to the folate residue has very little effects on the shape of the absorption and emission bands associated with transitions localized in the fluorophore. This is particularly evident when comparing the fluorescein (7d) and R6G (7e) labeled folate with the free dyes in Scheme 5b and c, where the shifts in the absorption maxima are only 6 nm and the emission spectra are completely overlapping. The spectra of the labeled folates are also shown in pure DMSO in Scheme 5. Solubility of the folate conjugates in DMSO is higher than in water. Thus, comparison of the spectra in the aqueous mixture with those observed in pure DMSO allows to evaluate possible aggregation effects. No new bands associated with aggregates are observed for any of the conjugates but a substantial decrease in absorption is observed for 7c and 7f,

suggesting formation of aggregates. For the fluorescein (7d) and R6G (7e) conjugates the aqueous mixture leads to a hypsochromic shift of the bands, which is typical of charged compounds in the ground state. It is worth noting that even though it is the neutral non-emissive lactone form of fluorescein that is linked to the folate residue, a prototropic equilibrium is gradually established in solution leading to a predominance of the dianionic form in the equilibrium. 52 In the aqueous mixture, the emission quantum yield of the 7d and 7e conjugates (7-9%) are about one order of magnitude lower than the quantum yields of the corresponding modified free dyes. The absorption spectra of the dansyl conjugate (7c) in DMSO and in the water mixture (Scheme 5a) are dominated by the contribution from the folate residue, which has its lowest energy transition at about 360 nm. This observation is associated with the comparatively low absorption intensity of the transition localized in the dansyl unit, which is observed in the free dyes at 340 nm. Nevertheless, this transition can be clearly observed in the excitation spectrum of 7c when emission is collected at 530 nm (excitation spectra in Scheme 5a). In DMSO, both the emission spectrum of 7c and the free dye are strongly overlapping, with maxima at 530 nm. In the aqueous mixture this band suffers a bathochromic shift (552 nm) and emission from an excited state localized in the folate unit of 7c can be simultaneously observed at 455 nm. Observation of the folate emission becomes possible due to the combined effect of lower absorption cross-section of the dansyl unit and quenching of the fluorescence quantum yield ($\phi F \sim 2\%$) by an order of magnitude in the aqueous solution. The photophysics of the benzofuranzan conjugate (7f) is somewhat similar to the dansyl conjugate (7c). The absorption spectrum of 7f (Scheme 5d) in the aqueous mixture is also dominated by the bands of the folate residue, but in 7f the weak absorption of the benzofuranzan can be observed at 470 nm. The corresponding excitation spectrum collected at 550 nm shows two bands at 340 and 475 nm typical of the benzofuranzan dye substituted by a nitro group at the position 7 and an amine group at position 4 [**DOI:** 10.1039/A904989D]. The nearly perfect overlap between the excitation spectrum of 7f and the absorption of the free dye gives support to this interpretation. Likewise, a nearly perfect overlap is observed between the emission of the 7f and the free dye in the aqueous mixture. The quantum yield of 7f is as low as that of 7c, but in the former it is possible to excite selectively the dye and separate its emission from emission of the folate residue.



Scheme 5 – Comparison between the normalized absorption and emission spectra of the modified free dyes and the corresponding folate conjugates in a 1% DMSO:H_2O mixture. The spectra of the folate conjugates in DMSO are also shown. a) **7c**

and N-(2-azidoethyl) dansyl, b) 7d and fluorescein, c) 7e and rhodamine 6G and d) 7f and furanzan. For 7c and 7f, the band overlap with absorption bands localized in the folate unit and the low absorption cross-section of the bands localized in the dye unit precludes a clear identification of the transitions localized in the dye, in the absorption spectra. Alternatively, a clear observation of these transitions is possible in the excitation spectra shown in panels a and d. The double band structure in the emission of 7c in 1% DMSO:H_2O shown in panel a) is due to a contribution from emission localized in the folate (λ_{em}= 455 nm) in addition to the emission band localized in the dansyl dye (λ_{em}= 532 nm).