Supporting information:

Apogossypol Derivatives as Pan-active Inhibitors of Anti-apoptotic B-cell lymphoma/leukemia-2 (Bcl-2) Family Proteins

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1. Experimental Section.

NMR Experiments.

NMR-based binding assays have been conducted by acquiring one-dimensional ^{1}H experiments with 500 μL solution of Bcl-X_L at 25 μM concentration, in absence and presence of added compounds, each at 200 μM concentration. By observing the aliphatic region of the spectra, binding could be readily detected due to chemical shift changes in active site methyl groups of Ile, Leu, Thr, Val or Ala (region between -0.8 and 0.3 ppm).[1] All experiments were performed with a 600 MHz spectrometer Bruker Avance 600 equipped with four rf channels and z-axis pulse-field gradients.

Isothermal Titration Calorimetry Assays (ITC).

Titrations were performed using a VP-ITC or ITC200 calorimeter from Microcal (Northampton, MA). Bcl-X_L was used at concentrations between 25 and 100 μM in 20 mM sodium phosphate buffer (pH 7.4) and 5-10% DMSO. Titrants were used at concentrations 10 to 15 fold that of the protein in the same buffer. Titrations were carried out at 25 ^{o}C. Data were analyzed using Microcal Origin software provided by the ITC manufacturer (Microcal, Northampton, MA).

In Vitro ADME Studies.

Liver Microsomal Stability. Pooled rat liver microsomes (BD Biosciences, # 452701) were preincubated with test compounds at 37.5 °C for 5 min in the absence of NADPH. The reaction was initiated by addition of NADPH and then incubated under the same conditions. The final incubation concentrations were 4 μM test compound, 2 mM NADPH, and 1 mg/mL (total protein) liver microsomes in phosphate-buffered saline (PBS) at pH 7.4. One aliquot (100 μL) of the incubation mixture was withdrawn at 0, 15, 30, and 60 min and combined immediately with 200 μL of ACN/MeOH containing an internal standard. After mixing, the sample was centrifuged at approximately 13,000 rpm for 12 min. The supernatant was transferred into an autosampler vial and the amount of test compound was quantified using the Shimadzu LCMS 2010EV mass spectrometer. The change of the AUC (area under the curve) of the parent compound as function of time was used as a measure of microsomal stability.

Plasma Stability. A 20 μL aliquot of a 10 mM solution in DMSO of the test compound was added to 2.0 mL of heparinized rat plasma (Lampire, P1-150N) to obtain a 100 μM final solution. The mixture was incubated for 1 h at 37.5 °C. Aliquots of 100 μL were taken (0, 30 min, 1 h) and diluted with 200 μL of MeOH containing internal standard. After mixing, the sample was centrifuged at approximately 13,000 rpm for 12 min. The supernatant was transferred into an autosampler vial and the amount of test compound was quantified using the Shimadzu LCMS-2010EV system. The change of the AUC (area under the curve) of the parent compound as function of time was used as a measure of microsomal stability.

PAMPA (parallel artificial membrane permeation assay). A 96-well microtiter plate (Millipore, # MSSACCEPTOR) was completely filled with aqueous buffer solution (pH 7.2) and covered with a microtiter filterplate (Millipore, # MAPBMN310). The hydrophobic filter material was impregnated with a 10% solution of hexadecane in hexane and the organic solvent was allowed to completely evaporate. Permeation studies were started by the transfer of 200 μL of a 100 μM test compound solution on top of the filterplate. In general phosphate buffer at pH 7.2 buffer was used. The maximum

DMSO content of the stock solutions was <5%. In parallel, an equilibrium solution lacking a membrane was prepared using the exact concentrations and specifications but lacking the membrane. The concentrations of the acceptor and equilibrium solutions were determined using the Shimadzu LCMS-2010EV and AUC methods. The permeation of a compound through the membrane layer is described by the percentage permeation (% flux). The flux values were calculated considering the concentration of the acceptor compartment after 8 h and that of a reference well with the same concentration containing no membrane barrier.

Maximum Tolerated Dose (MTD).

Young female Balb/c mice (7-weeks-old) were injected with 100 mg/kg, 75 mg/kg, 50 mg/kg and 25 mg/kg of compound **8r** intraperitoneally (one mouse per dose) and observed for survival, vital signs, weight loss, etc. for 14 days, in compliance with MTD general protocol proposed by Developmental Therapeutics Program (DTP) at NCI. Compound **8r** was first dissolved in 100% ethanol, supplemented by Cremophore EL and saline, just before injection, with a ratio of Ethanol: Cremophore EL: Saline = 10:10:80. Upon conclusion of the study, mice were euthanized by CO2, and vital organs were harvested and fixed with z-FIX solution for 3 days at room temperature, rinsed in PBS three times, for further histological evaluation.

NMR data for compounds (7a-7t and **11a-11c)**

Following same synthetic procedure of compounds **7r** and **11e** and appropriate starting materials and reagents used; compounds (**7a-7t** and **11a-11c**) were synthesized.

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl- N^5,$N^{5'}$-diphenyl-2,2'-binaphthyl-5,5'-dicarboxamide (7a). Yield, 45%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.76 (d, J = 7.8 Hz, 4H), 7.59 (s, 2H), 7.52 (s, 2H), 7.40 (t, $J_1 = J_2$ = 7.8 Hz, 4H), 7.18 (s, 2H), 4.04 (s, 6H), 4.00 (s, 6H), 3.63 (s, 6H), 2.11 (s, 6H).

N^5,$N^{5'}$-Dicyclopentyl-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7b). Yield, 40%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.52 (s, 2H), 7.45 (s, 2H), 4.47 (m, 2H), 3.98 (s, 6H), 3.96 (s, 6H), 3.60 (s, 6H), 2.11 (m, 10H), 1.79 (s, 4H), 1.68 (s, 8H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl-N^5,$N^{5'}$-bis(4-phenoxyphenyl)-2,2'-binaphthyl-5,5'-dicarboxamide (7c). Yield, 46%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.76 (m, 6H), 7.59 (m, 2H), 7.53 (m, 2H), 7.35 (m, 2H), 7.11 (m, 2H), 7.03 (m, 8H), 4.00 (s, 6H), 4.00 (s, 6H), 3.63 (s, 6H), 2.12 (s, 6H).

N^5,$N^{5'}$-Bis(3-ethylphenyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7d). Yield, 47%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.62 (s, 2H), 7.58 (m, 4H), 7.52 (s, 2H), 7.30 (m, 2H), 7.05 (m, 2H), 4.04 (s, 6H), 3.99 (s, 6H), 3.63 (s, 6H), 2.54 (q, $J_1 = J_2 = 8.4$ Hz, 4H), 2.11 (s, 6H), 1.28 (t, $J_1 = J_2 = 8.4$ Hz, 6H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl- N^5,$N^{5'}$-bis(3-(trifluoromethyl)phenyl)-2,2'-binaphthyl-5,5'-dicarboxamide (7e). Yield, 50%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.88 (s, 2H), 7.77 (d, $J = 6.6$ Hz, 2H), 7.60 (m, 4H), 7.54 (s, 2H), 7.36 (s, 2H), 4.77 (s, 4H), 3.99 (s, 6H), 3.94 (s, 6H), 3.58 (s, 6H), 2.05 (s, 6H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl-N5,N5'-bis(1-phenylpropyl)-2,2'-binaphthyl-5,5'-dicarboxamide (7f). Yield, 46%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.50 (m, 6H), 7.38 (m, 4H), 7.26 (m, 4H), 5.12 (s, 2H), 4.01 (s, 6H), 4.00 (s, 6H), 3.89 (s, 6H), 3.58 (s, 3H), 3.55 (s, 3H), 1.95 (m, 10H), 1.10 (s, 6H).

N^5,$N^{5'}$-Dibenzyl-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7g). Yield, 49%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.53 (m, 6H), 7.38 (m, 6H), 7.30 (m, 2H), 4.68 (s, 4H), 4.00 (s, 6H), 3.91 (s, 6H), 3.57 (s, 6H), 2.02 (s, 6H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl- N^5,$N^{5'}$-bis(3-methylbenzyl)-2,2'-binaphthyl-5,5'-dicarboxamide (7h). Yield, 43%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.52 (s, 2H), 7.36 (d, $J = 7.8$ Hz, 4H), 7.29 (d, $J = 7.8$ Hz, 2H), 7.26 (t, $J_1 = 7.8$ Hz, $J_2 = 7.2$ Hz, 2H), 7.11 (d, $J = 7.2$ Hz, 2H), 4.64 (s, 4H), 4.00 (s, 6H), 3.92 (s, 6H), 3.57 (s, 6H), 2.37 (s, 6H) , 2.02 (s, 6H).

N^5,$N^{5'}$-Bis(3-chlorobenzyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7i). Yield, 46%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.61 (s, 2H), 7.53 (s, 2H), 7.42 (d, $J = 6.6$ Hz, 2H), 7.36 (m, 4H), 7.31 (d, $J = 7.2$ Hz, 2H), 4.68 (s, 4H), 4.00 (s, 6H), 3.98 (s, 6H), 3.58 (s, 6H) , 2.07 (s, 6H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl-N^5,$N^{5'}$-bis(2,4,6-trimethylbenzyl)-2,2'-binaphthyl-5,5'-dicarboxamide (7j). Yield, 40%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.48 (s, 2H), 7.41 (s, 2H), 6.96 (s, 2H), 6.88 (s, 2H), 3.92 (s, 6H), 3.87 (s, 6H), 3.55 (s, 6H), 3.39 (s, 6H), 2.46 (s, 6H) , 2.27 (s, 6H), 2.05 (s, 6H).

N^5,$N^{5'}$-Bis(1-(4-chlorophenyl)ethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7k). Yield, 49%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.52 (m, 6H), 7.39 (m, 4H), 7.24 (s, 1H), 7.25 (s, 1H), 5.36 (m, 2H), 4.01 (s, 3H), 4.00 (s, 3H), 3.90 (s, 3H), 3.89 (s, 3H), 3.57 (s, 3H), 3.56 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.58 (s, 3H), 1.57 (s, 3H).

N^5,$N^{5'}$-Bis(cyclopropylmethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7l). Yield, 46%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.52 (s, 2H), 7.49 (s, 2H), 4.00 (s, 6H), 3.96 (s, 6H), 3.59 (s, 6H), 3.37 (d, J = 6.9 Hz, 4H), 2.10 (s, 6H), 1.2 (m, 2H), 0.59 (m, 4H), 0.37 (m, 4H).

N^5,$N^{5'}$-Bis(cyclohexylmethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7m). Yield, 50%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.52 (s, 2H), 7.45 (s, 2H), 4.02 (s, 6H), 3.94 (s, 6H), 3.59 (s, 6H), 3.33 (d, J = 17.4 Hz, 4H), 2.09 (s, 6H), 1.94 (d, J = 12.0 Hz, 4H), 1.80 (d, J = 12.0 Hz, 4H), 1.72 (d, J = 10.6 Hz, 4H), 1.39-1.07 (m, 10H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl- N^5,$N^{5'}$-diphenethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7n). Yield, 51%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.51 (s, 2H), 7.36 (d, J = 7.2 Hz, 4H), 7.30 (m, 6H), 7.22 (t, $J_1 = J_2$ = 7.2 Hz, 2H), 4.00 (s, 6H), 3.89 (s, 6H), 3.78 (t, J_1 = 7.2 Hz, J_2 = 6.6 Hz, 4H), 3.57 (s, 6H), 3.02 (t, J_1 = 6.6 Hz, J_2 = 7.2 Hz, 4H), 2.04 (s, 6H).

1,1',6,6',7,7'-Hexamethoxy-3,3'-dimethyl-N^5,$N^{5'}$-bis(3-methylphenethyl)-2,2'-binaphthyl-5,5'-dicarboxamide (7o). Yield, 50%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.51 (s, 2H), 7.28 (s, 2H), 7.23 (m, 4H), 7.12 (m, 4H), 3.97 (s, 6H), 3.89 (s, 6H), 3.75 (s, 6H) , 3.58 (m, 4H), 2.97 (m, 4H), 2.29 (s, 6H), 2.02 (s, 6H).

N^5,$N^{5'}$-Bis(3-chlorophenethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7p). Yield, 45%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.51 (s, 2H), 7.39 (s, 2H), 7.30 (d, J

= 4.2 Hz, 4H), 7.25 (m, 4H), 4.03 (s, 6H), 3.95 (s, 6H), 3.78 (m, 4H), 3.55 (s, 6H), 3.02 (t, $J_1 = J_2$ = 6.6 Hz, 4H), 2.04 (s, 6H).

N^5,$N^{5'}$-Bis(4-ethylphenethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7q). Yield, 47%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.52 (s, 2H), 7.27 (s, 2H), 7.23 (d, *J* = 7.8 Hz, 4H), 7.15 (d, *J* = 7.8 Hz, 4H), 4.02 (s, 6H), 3.92 (s, 6H), 3.91 (m, 4H), 3.49 (s, 6H), 3.01 (t, $J_1 = J_2$ = 6.6 Hz, 4H), 2.61 (q, $J_1 = J_2$ = 7.8 Hz, 6H), 2.11 (s, 6H), 1.21 (t, $J_1 = J_2$ = 7.8 Hz, 6H).

N^5,$N^{5'}$-Bis(2,3-dihydro-1H-inden-2-yl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl-5,5'-dicarboxamide (7s). Yield, 47%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.50 (s, 2H), 7.45 (s, 2H), 7.26 (m, 4H), 7.15 (m, 4H), 4.94 (m, 2H), 3.99 (s, 6H), 3.90 (s, 6H), 3.56 (s, 6H), 3.43 (m, 4H), 3.07 (m, 4H), 2.08 (s, 6H).

5,5'-Diisobutyl-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (11a). Yield, 85%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 (s, 2H), 7.41 (s, 2H), 3.99 (s, 6H), 3.90 (s, 6H), 3.57 (s, 6H), 2.97 (d, *J* = 7.2 Hz, 4H), 2.19 (s, 6H), 2.12 (m, 2H),1.03 (t, $J_1 = J_2$ = 6.0 Hz, 12H).

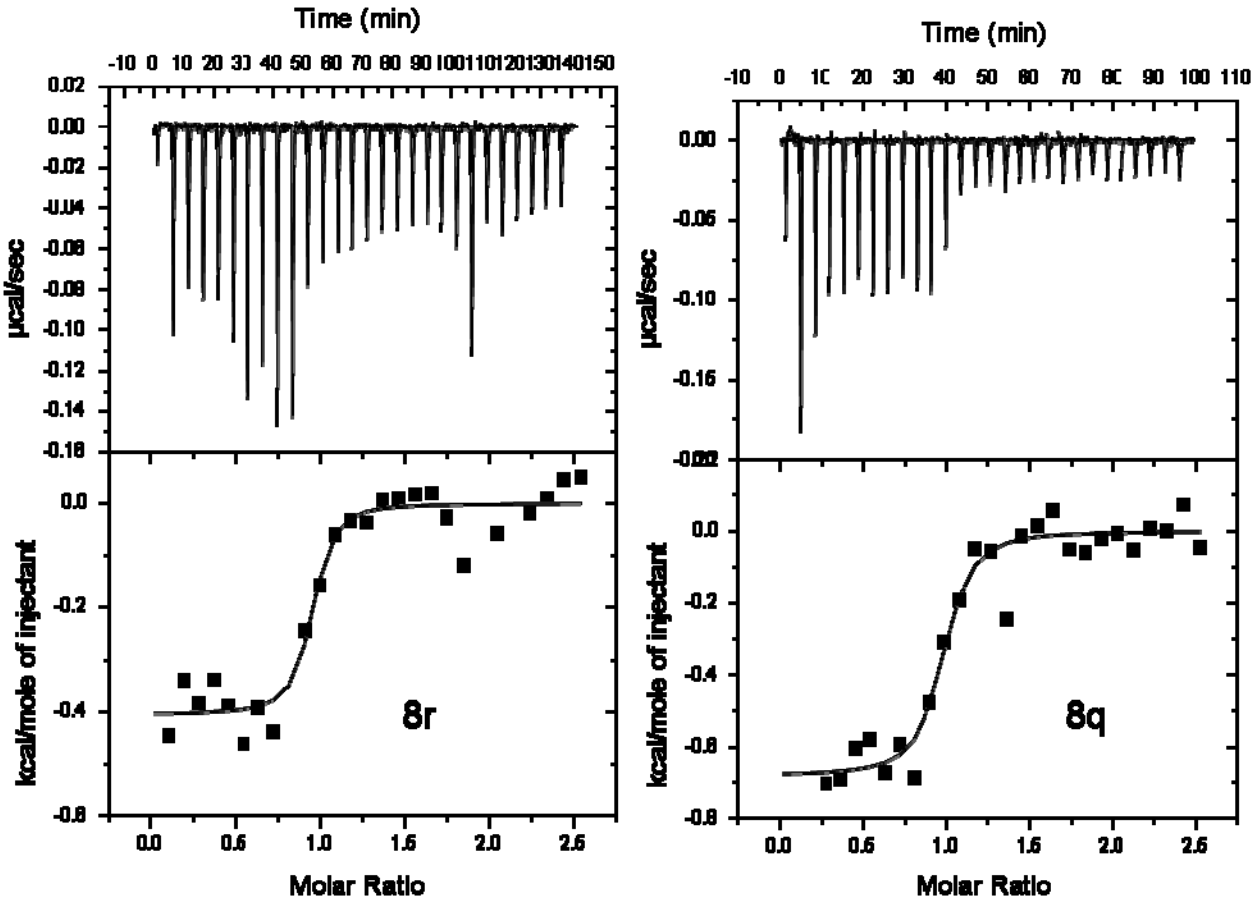
5,5'-Diisopentyl-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (11b). Yield, 81%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.63 (s, 2H), 7.38 (s, 2H), 3.99 (s, 6H), 3.96 (s, 6H), 3.59 (s, 6H), 3.08 (m, 4H), 2.2 (s, 6H), 1.80 (m, 2H), 1.29 (m, 4H), 1.06 (m, 12H).

5,5'-Bis(cyclopentylmethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (11c). Yield, 80%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.65 (s, 2H), 7.40 (s, 2H), 3.99 (s, 6H), 3.90 (s, 6H), 3.58 (s, 6H), 3.09 (d, *J* = 7.2 Hz, 4H), 2.38 (m, 2H), 2.20 (s, 6H), 1.73 (m, 8H), 1.54 (m, 8H).

2. Supplementary Figures

Supplementary Fig. 1.

ITC studies of 5, 5' substituted compound 2 derivatives

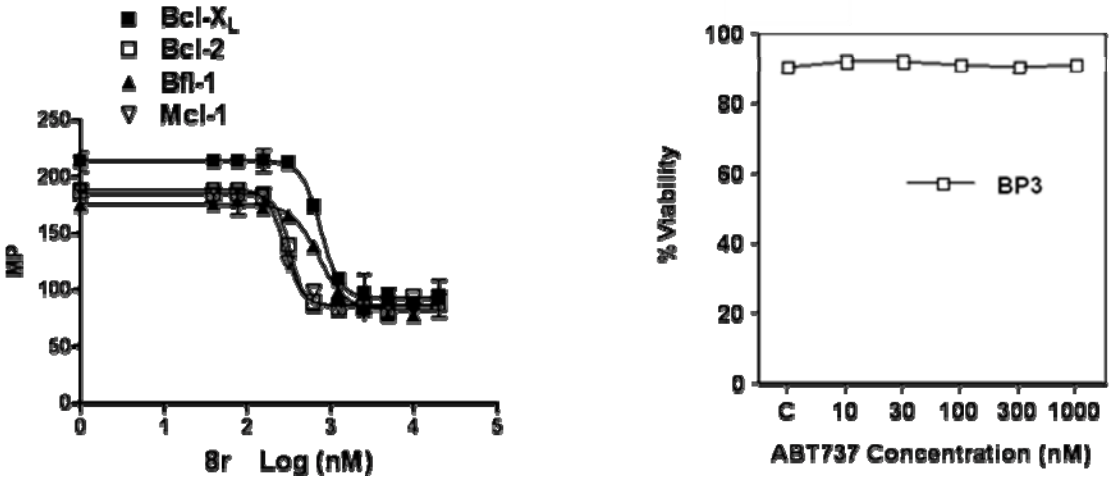


Compound	n (stoichiometry)	K_d (nM)	ΔH (kcal/mol)	ΔS (kcal/mol·K)
8q	0.95	121	-680	29
8r	0.91	109	-405	31

Supplementary Figure 2:

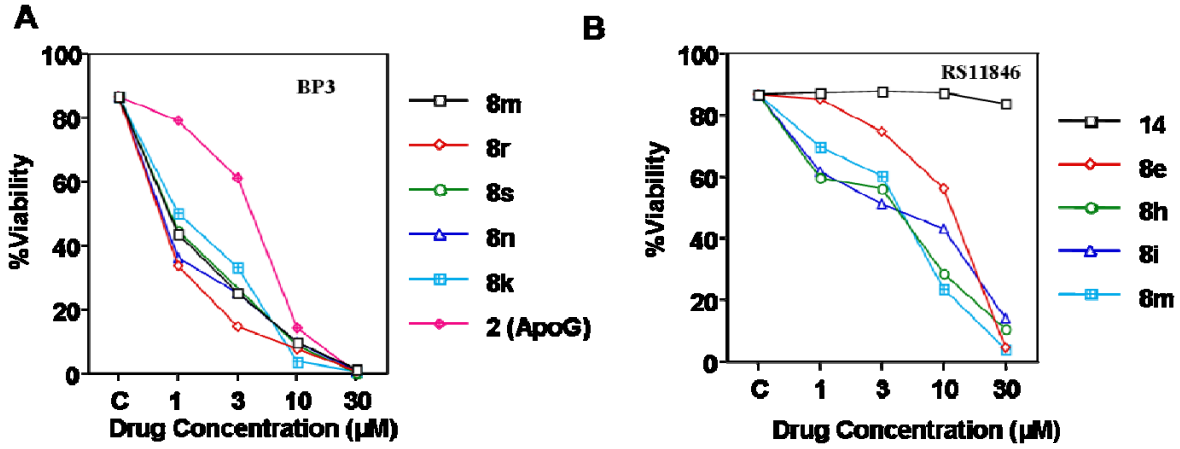
(A) Compound 8r competes with the binding of Bcl-2 family proteins to FITC-Bim BH3 peptide.

(B) Cytotoxicity assays of ABT-737 against BP3 using Annexin V-FITC and propidium iodide assay



Supplementary Figure 3.

Cytotoxicity assays of 5, 5' substituted compound 2 derivatives against (A) BP3 cell and (B) RS11846 cancer cell lines using Annexin V-FITC and propidium iodide assay.



3. Supplementary Tables

Supplementary Table 1. Summary of Western blot analysis of BP3 and RS4;11 cancel cell line.

	Mcl-1	Bcl-2	Bcl-xl	Bfl-1
BP3	+++	No	+	+++
RS4;11	No	++++	+	No

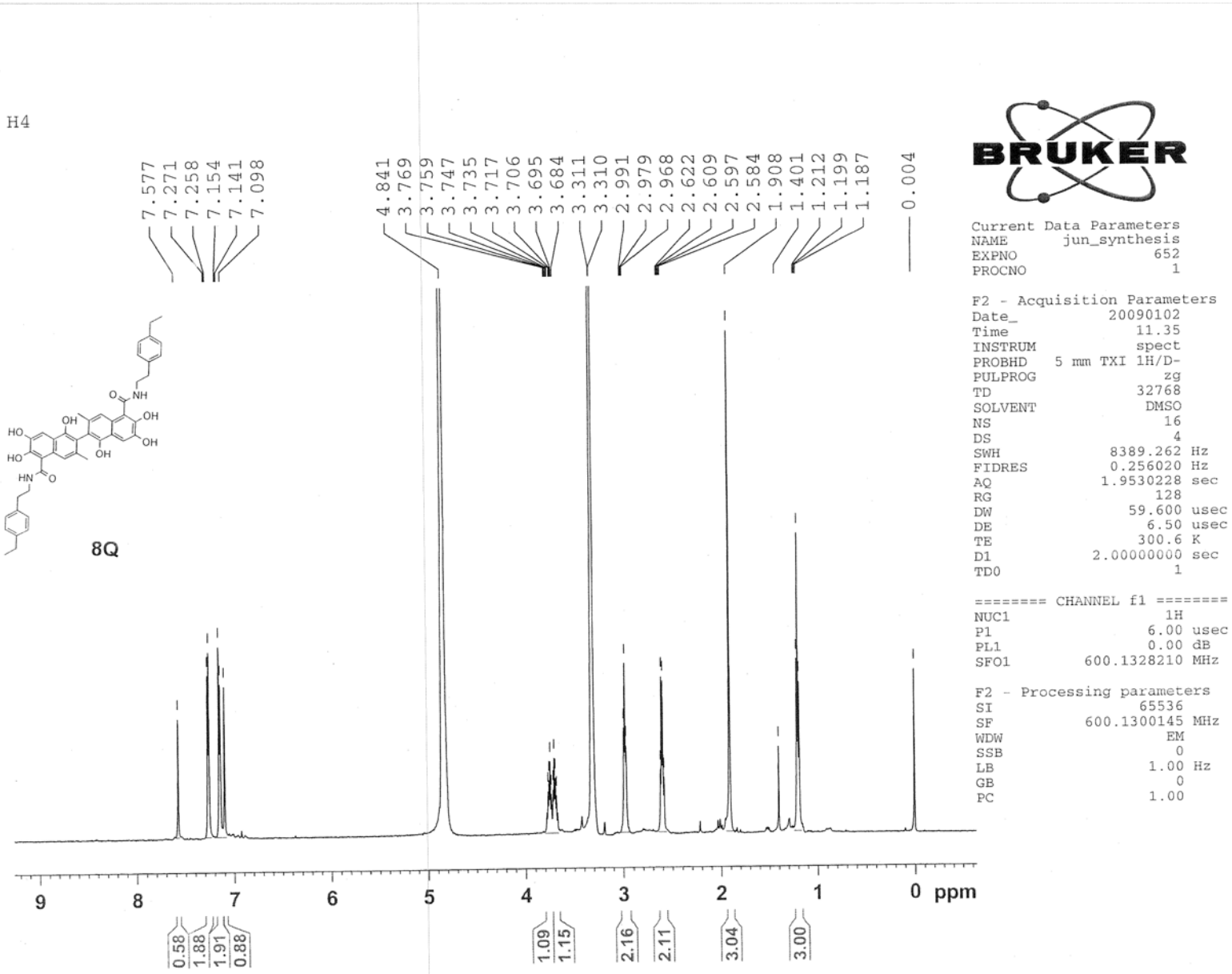
4-point rating scale for western data:
++++: Very high level
+++: High level
++: Medium level
+: Low
No: Not Detectable

Supplementary Table. 2 Efficacy/Toxicity studies of 5, 5' substituted compound 2 derivatives against B6/Bcl-2 transgenic mice.

	2	1	13	12e	8k	8m	8p	8q	8r	12c
Effica	PR	PR	NR	PR	CR	PR	CR	PR	CR	PR
Tox	0	2+	0	0	4+	4+	4+	4+	3+	1+

Toxicity Rating Scale: 4+ (lethal), 3+: severe, 2+: moderate, 1+: mild, 0: No toxicity

4. Spectrums of key compounds (^{1}HNMR and HPLC).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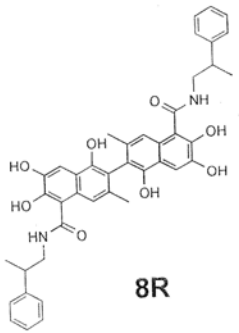

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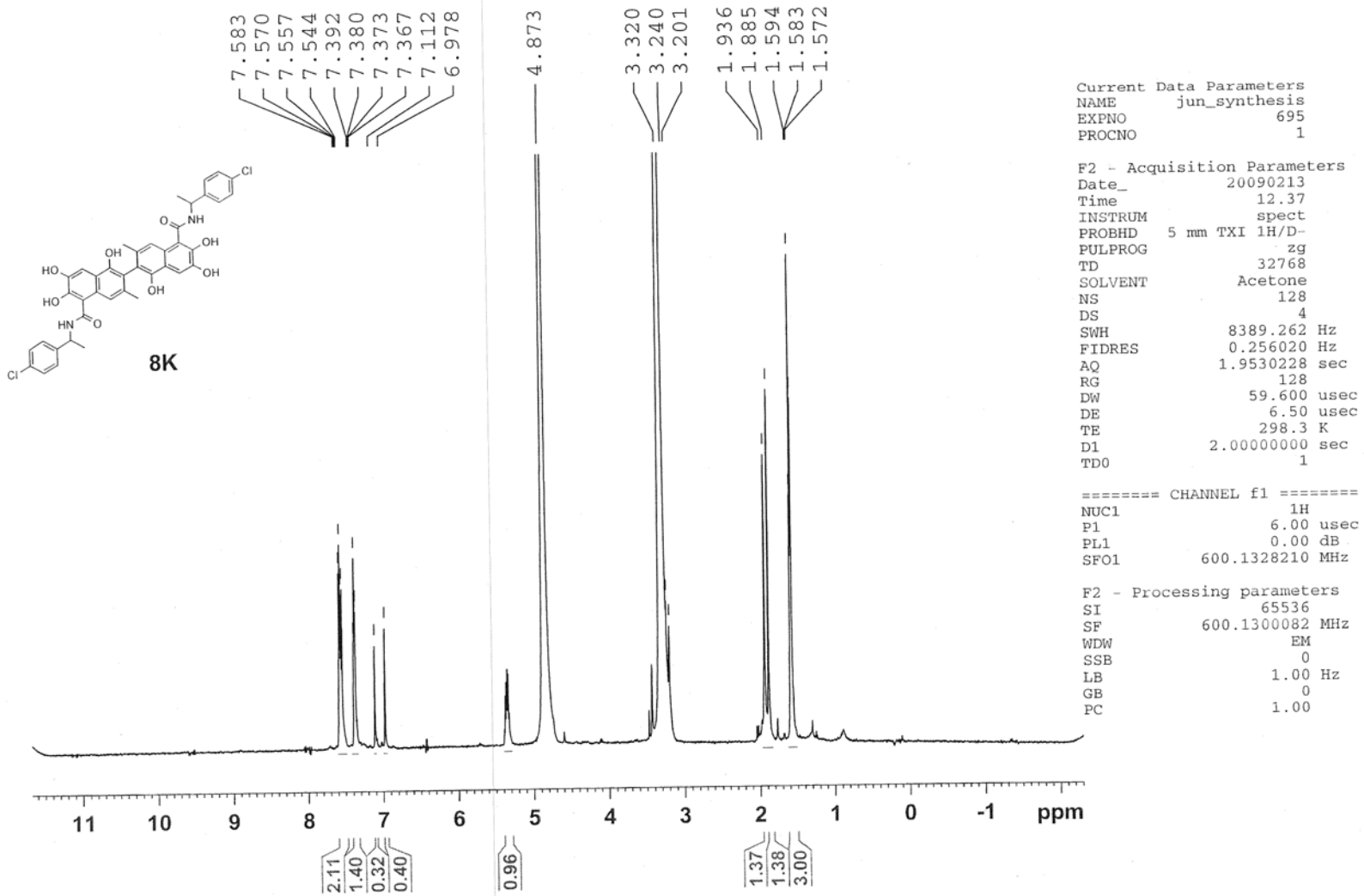


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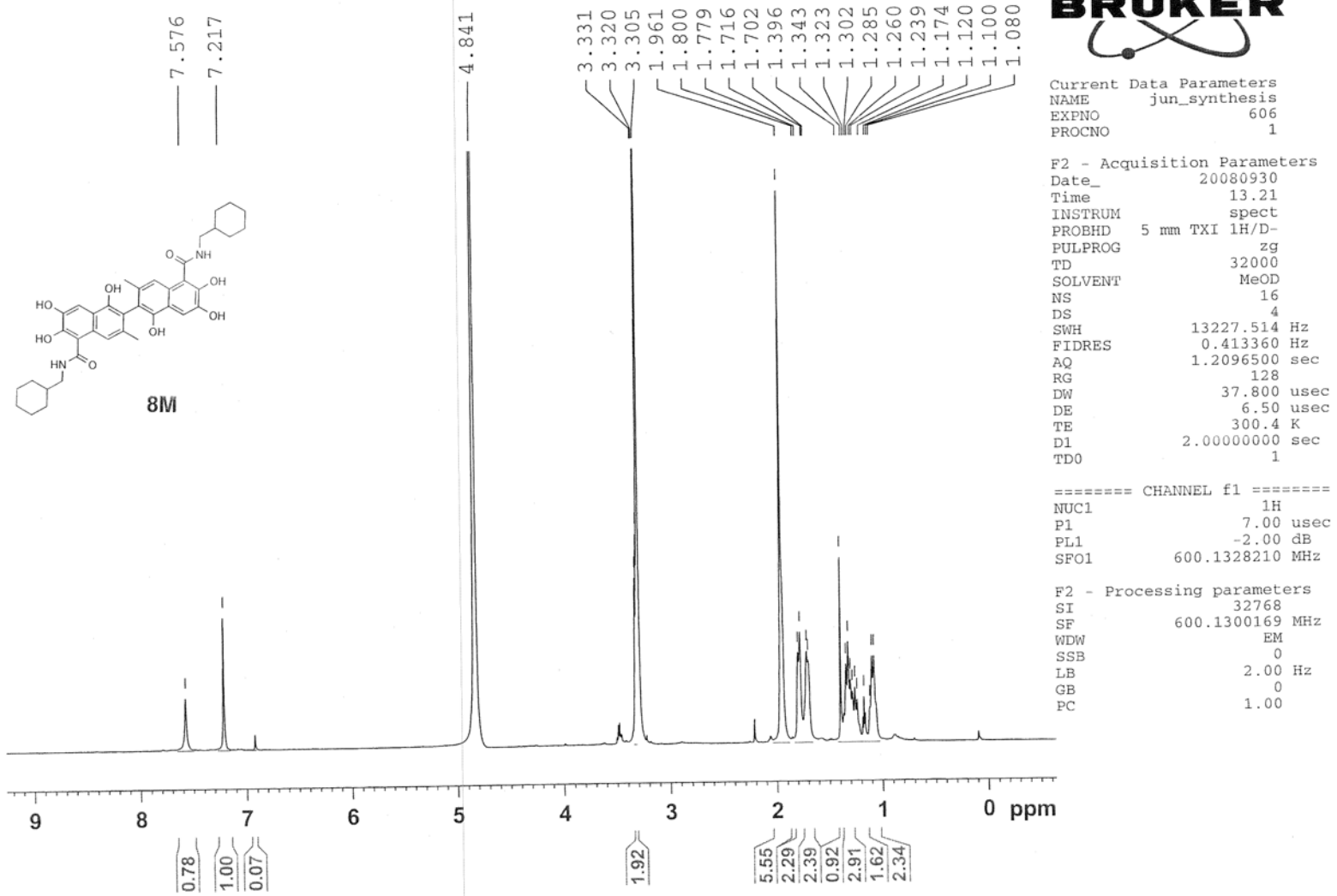

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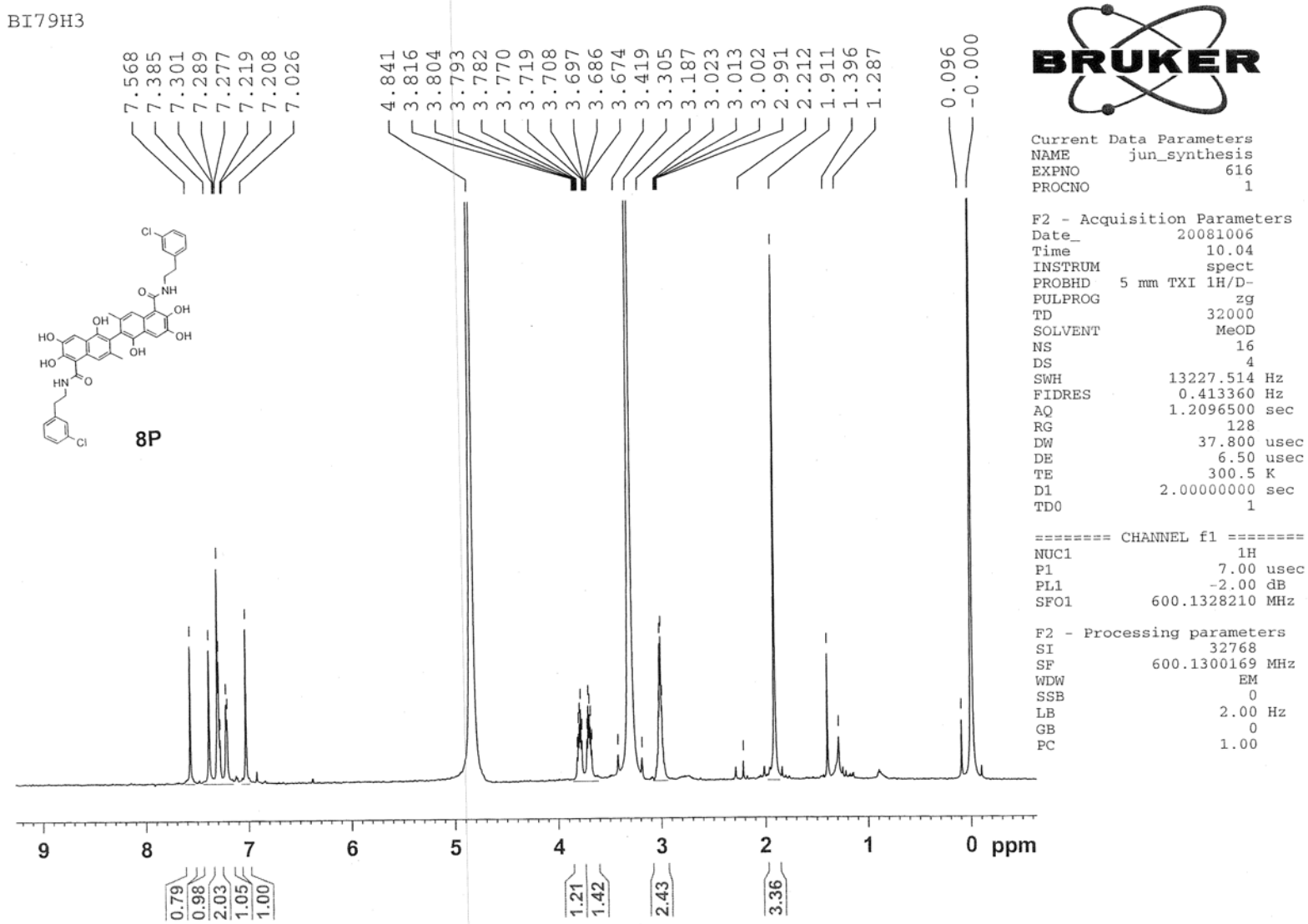
GB 0
PC 1.00



BRUKER

```
Current Data Parameters
NAME       jun_synthesis
EXPNO                616
PROCNO                 1

F2 - Acquisition Parameters
Date_           20081006
Time               10.04
INSTRUM            spect
PROBHD   5 mm TXI 1H/D-
PULPROG              zg
TD                32000
SOLVENT            MeOD
NS                   16
DS                    4
SWH           13227.514 Hz
FIDRES         0.413360 Hz
AQ             1.2096500 sec
RG                  128
DW                37.800 usec
DE                 6.50 usec
TE                300.5 K
D1             2.00000000 sec
TD0                   1

======== CHANNEL f1 ========
NUC1                  1H
P1                 7.00 usec
PL1               -2.00 dB
SFO1         600.1328210 MHz

F2 - Processing parameters
SI                32768
SF           600.1300169 MHz
WDW                  EM
SSB                   0
LB                 2.00 Hz
GB                    0
PC                 1.00
```

burnham

Project Name: Defaults

Reported by User: System

Breeze

SAMPLE INFORMATION

Sample Name: H5 PREP FINAL

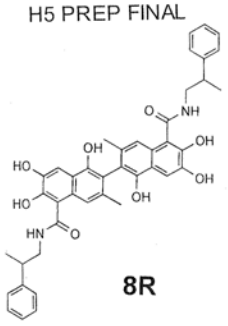
Sample Type:

Vial:

Injection #:

Injection Volum

Run Time:



8R

Acquired By: System

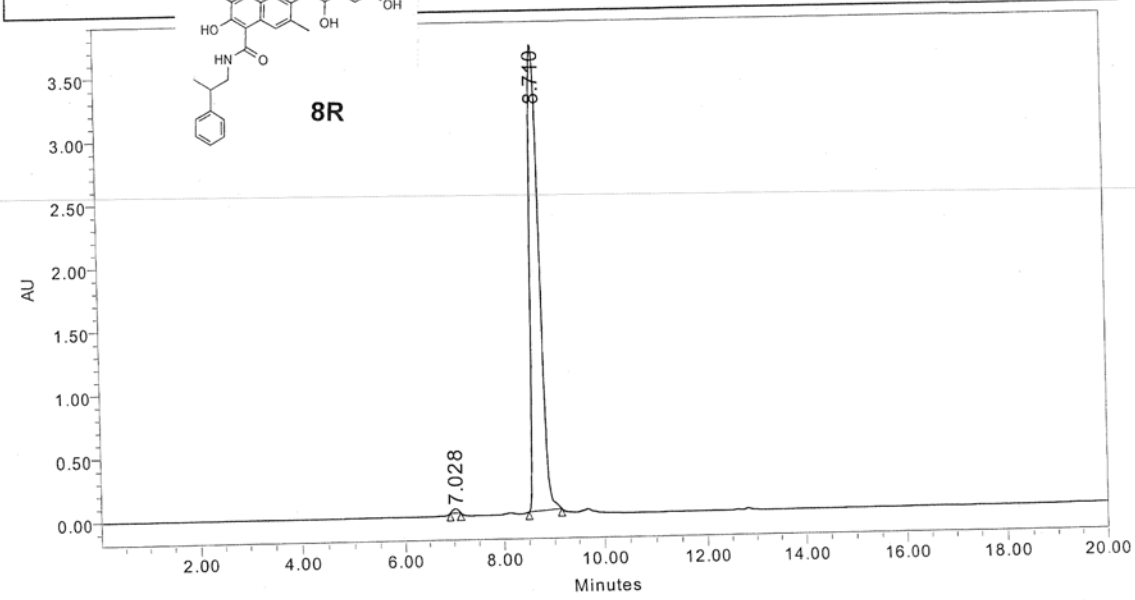
Date Acquired: 2/17/2009 11:23:58 PM

Acq. Method: Burnham 50_95%BACN 220

Date Processed: 3/21/2009 7:22:11 PM

Channel Name: 2487Channel 1

Sample Set Name M



	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	7.028	273747	0.60	35036	0.94
2	8.710	45448782	99.40	3702932	99.06

Report Method: Untitled

Printed 7:22:22 PM3/21/2009

Page: 1 of 1

burnham

Project Name: Defaults

Reported by User: System

Breeze

SAMPLE INFORMATION

Sample Name: H4

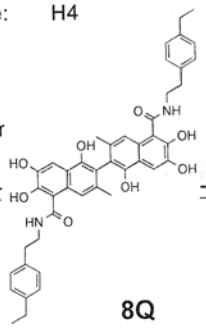
Sample Type:

Vial:

Injection #:

Injection Volum

Run Time:



Acquired By: System

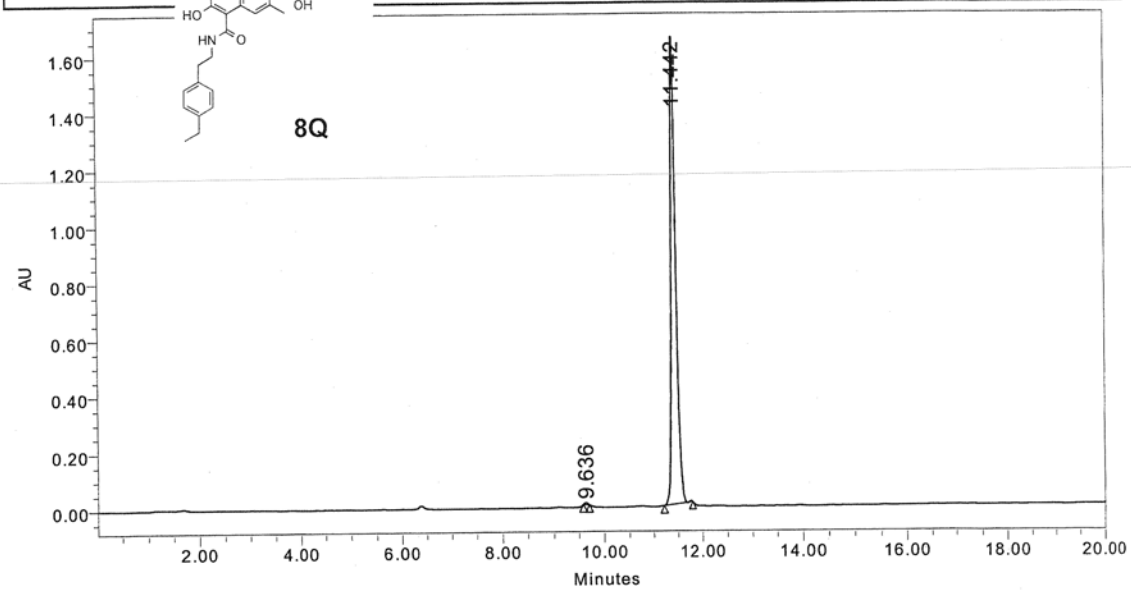
Date Acquired: 12/22/2008 11:08:25 AM

Acq. Method: Burnham 50_95%BACN 220

Date Processed: 12/22/2008 1:02:24 PM

Channel Name: 2487Channel 1

Sample Set Name H



	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	9.636	29469	0.25	6959	0.42
2	11.442	11802246	99.75	1669406	99.58

Report Method: Untitled

Printed 1:03:02 PM 12/22/2008

Page: 1 of 1

burnham

Project Name: Defaults

Reported by User: System

Breeze

SAMPLE INFORMATION

Sample Name: A1

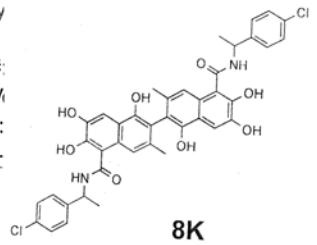
Sample Ty

Vial:

Injection #:

Injection V

Run Time:



8K

Acquired By: System

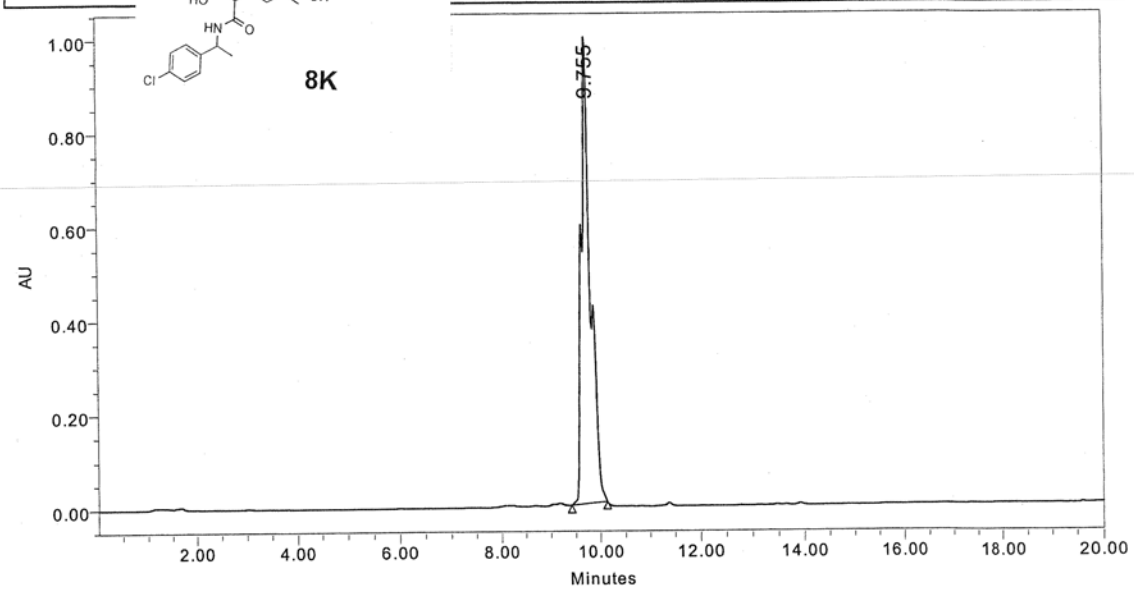
Date Acquired: 12/22/2008 12:02:50 PM

Acq. Method: Burnham 50_95%BACN 220

Date Processed: 12/22/2008 1:01:51 PM

Channel Name: 2487Channel 1

Sample Set Name H



	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height
1	9.755	12578951	100.00	991172	100.00

Report Method: Untitled

Printed 1:03:29 PM12/22/2008

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References:

1. Rega, M. F.; Leone, M.; Jung, D.; Cotton, N. J.; Stebbins, J. L.; Pellecchia, M. Structure-based discovery of a new class of Bcl-xL antagonists. *Bioorg Chem* 2007, 35, 344-53.