Supporting information:

BI-97C1, an Optically Pure Apogossypol Derivative as Pan-active Inhibitor 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 a cryogenic probe 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 One aliquot (100 µL) of the incubation mixture was withdrawn at 0, 15, 30, and 60 min and combined immediately with 200 µL of ACN/MeOH containing an internal standard.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 plasma 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.

Chemical Stability Studies.

Compounds in a 10 mM of DMSO solution were incubated at various temperatures for different time period. 1 µL of tested compound (10mM) was added to 99 µL of Acetonitrile to obtain a 100 µL final solution. The mixture 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 chemical stability.

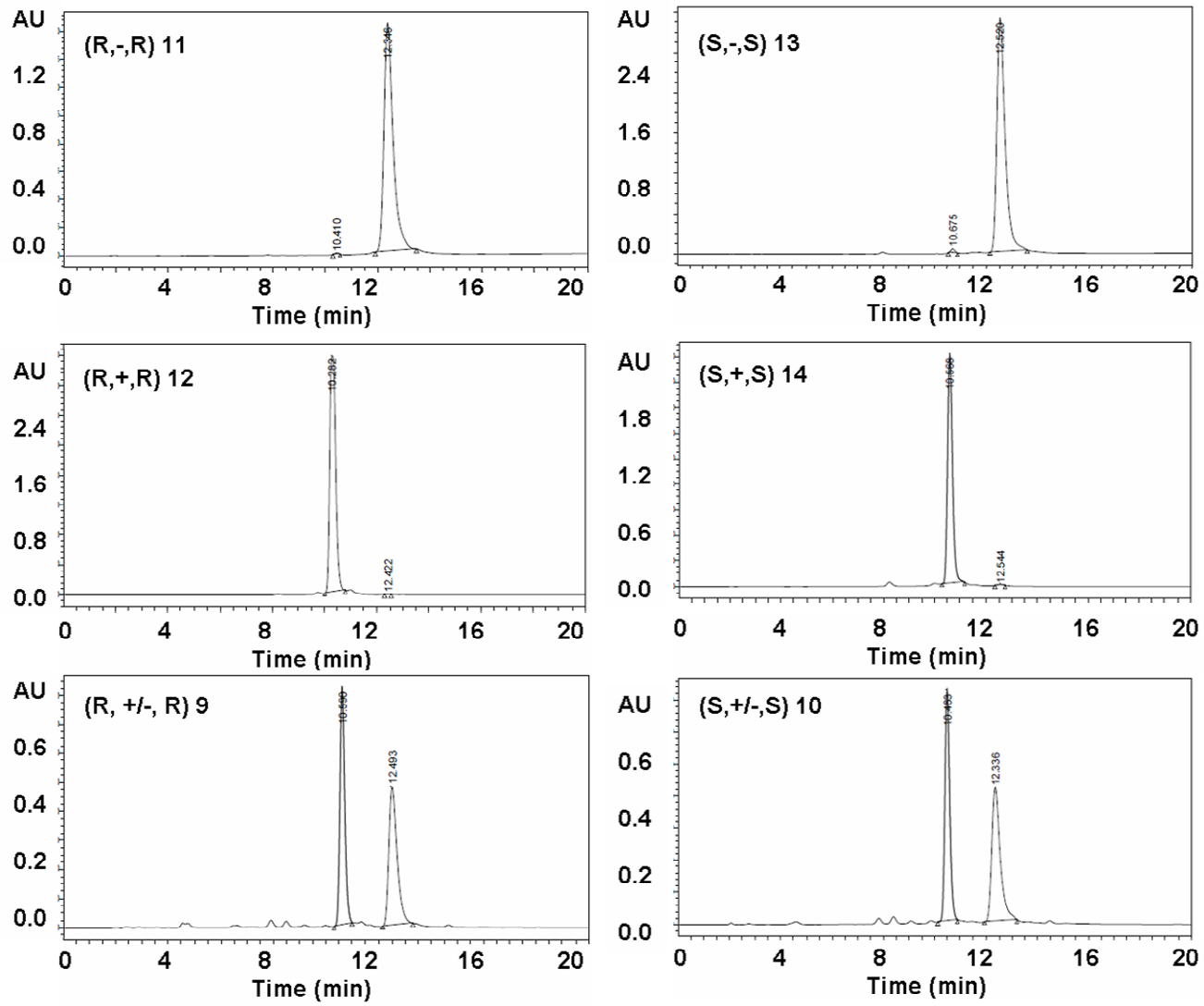
Toxicity Studies.

Young female Balb/c mice (7-weeks-old) were injected with 25 mg/kg and 10 mg/kg of compounds **(11-14)** intraperitoneally (one mouse per dose) and observed for survival and weight loss for 11 days. Compounds **(11-14)** were 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.

2. Supplementary Figures

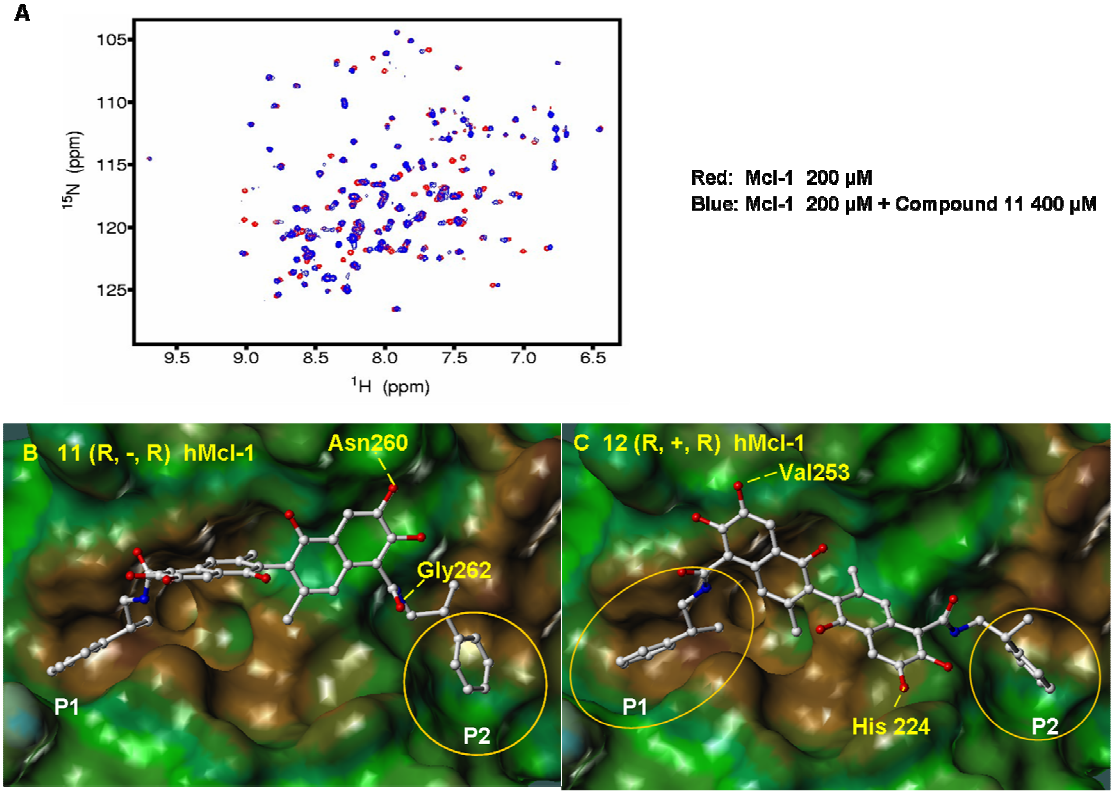
Supplementary Figure 1.

HPLC purity of isomers (9-14) using chiral column chromatography.



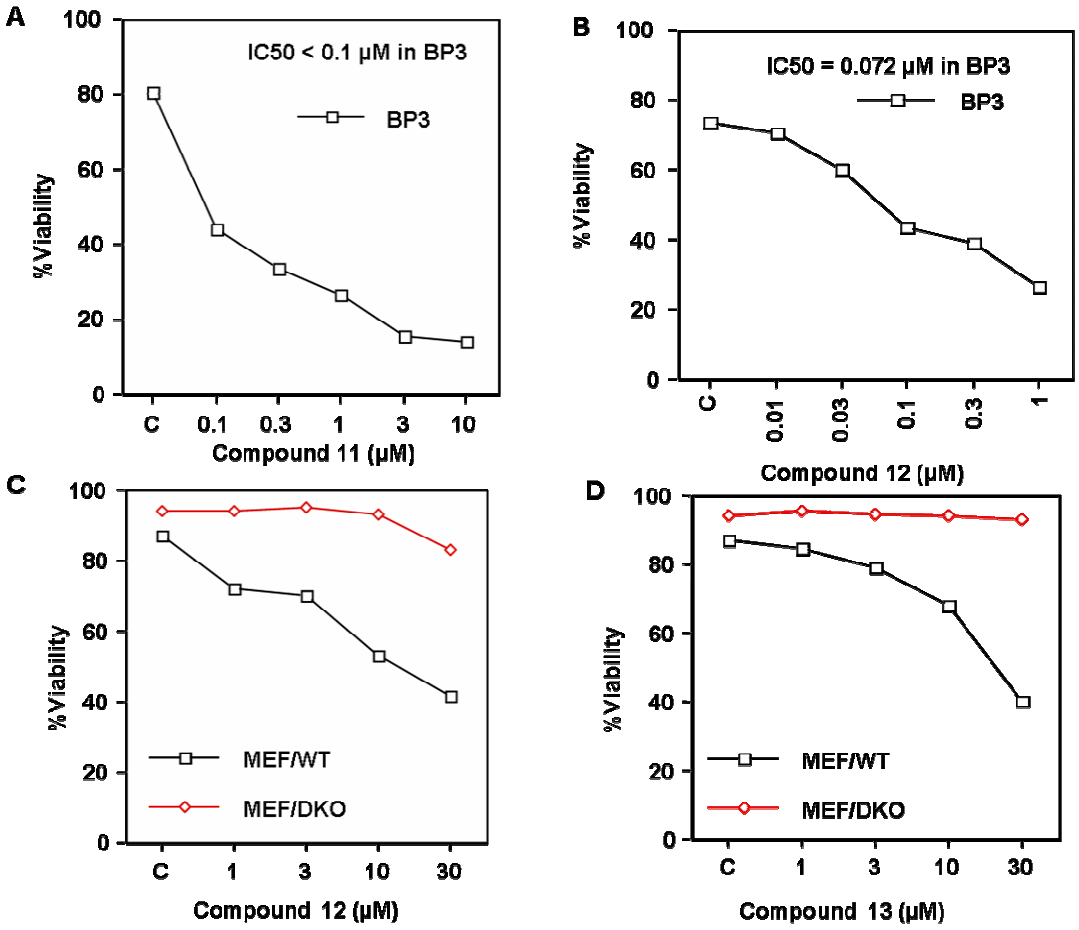
Supplementary Figure 2.

(A) Superposition of 2D [^{15}N,^{1}H]-TROSY spectra of hMcl-1 (200 µM; red) before and after addition of compound **11** (400 µM; blue). **(B)** and **(C)** Molecular docking studies. Stereo views of docked structures of compound **11** and compound **12** into hMcl-1 (PDB ID:2NL9). Molecular modeling suggested that the left pocket (P1) of Mcl-1 is shallow compared to the deeper pocket (P1) in Bcl-X_L (Figure 3) and the right pocket of Mcl-1 (P2) is more open (Supporting Information Figure 2B) in comparison to that seen in Bcl-X_L (Figure 3). The open right pocket (P2) and shallow left pocket (P1) of Mcl-1 are tolerant of different orientations of atropisomers **11** and **12**. Mcl-1 also have residues such as Asn260, Gly262, Val253 and His224 which can readily form two hydrogen bonds with both atropisomers **11** and **12** (Supporting Information Figure 2B and 2C).



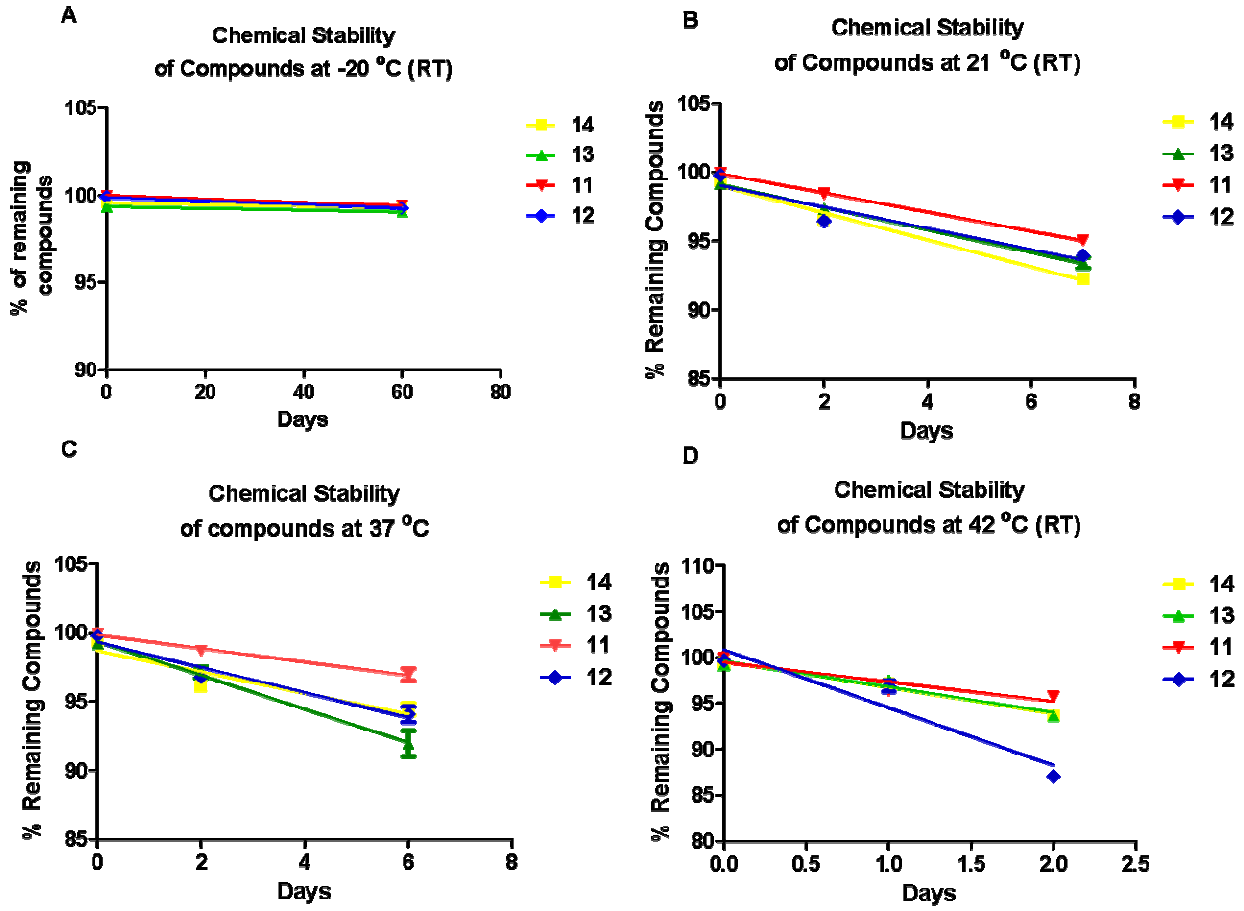
Supplementary Figure 3.

(A) and **(B)** Inhibition of cell growth by compound **11** and compound **12** in the human BP3 cell line. Apoptosis was monitored by Annexin V-FITC assays. **(C)** and **(D)** Mouse embryonic fibroblast cells with wild-type (MEF/WT; black square) or *bax*$^{-/-}$*bak*$^{-/-}$ (MEF/DKO; red square) double knockout genotypes were treated with compounds **12** and **13** at various concentrations and apoptosis was monitored by Annexin V-FITC assays.



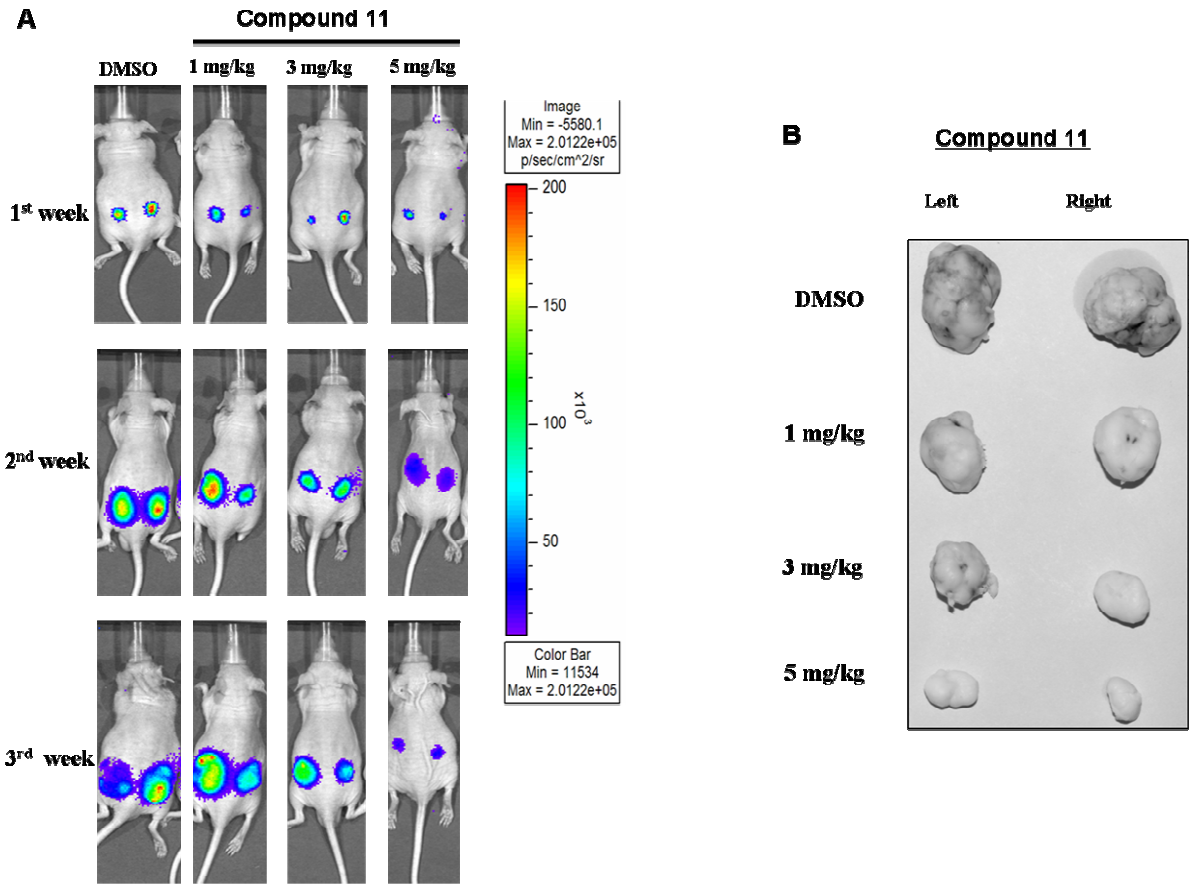
Supplementary Figure 4.

Chemical stabilities of compounds **11-14** in DMSO solution (10 mmol/L) were evaluated at different temperatures (21 ºC and 37 ºC) using LC-MS. Overall, compound **11** showed best chemical stability compared to compounds **12-14** at different temperatures.



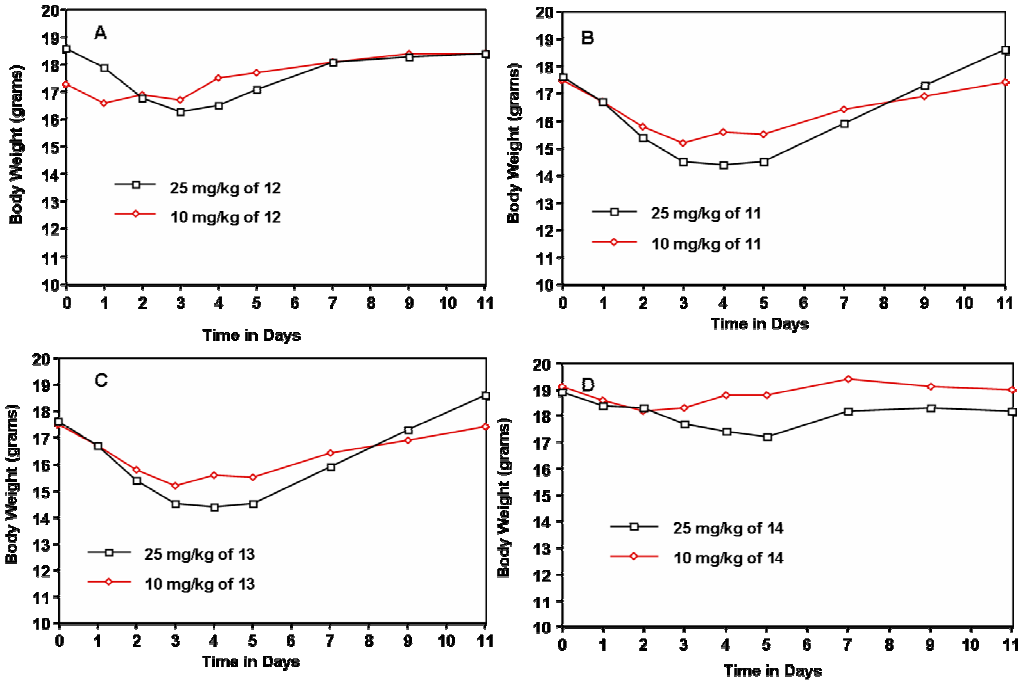
Supplementary Figure 5.

A) Tumor xenografts from M2182-Luc cells were established in athymic nude mice on the left and right flanks. After establishing visible tumors of ~75-mm^3, requiring ~5-6 days, intraperitoneal (i.p.) injections of DMSO or compound **11** (1 mg/kg, 3 mg/kg or 5 mg/kg) were given every two days (total of nine injections). For *in vivo* imaging the mice were anesthetized and injected i.p. with 150 mg/kg luciferin and light emitted from each tumor determined in a Xenogen system with CCD camera with an integration time of 1 min. B) Photograph of the M2182-Luc xenograft tumors at the termination of the study (3 weeks post-treatment).

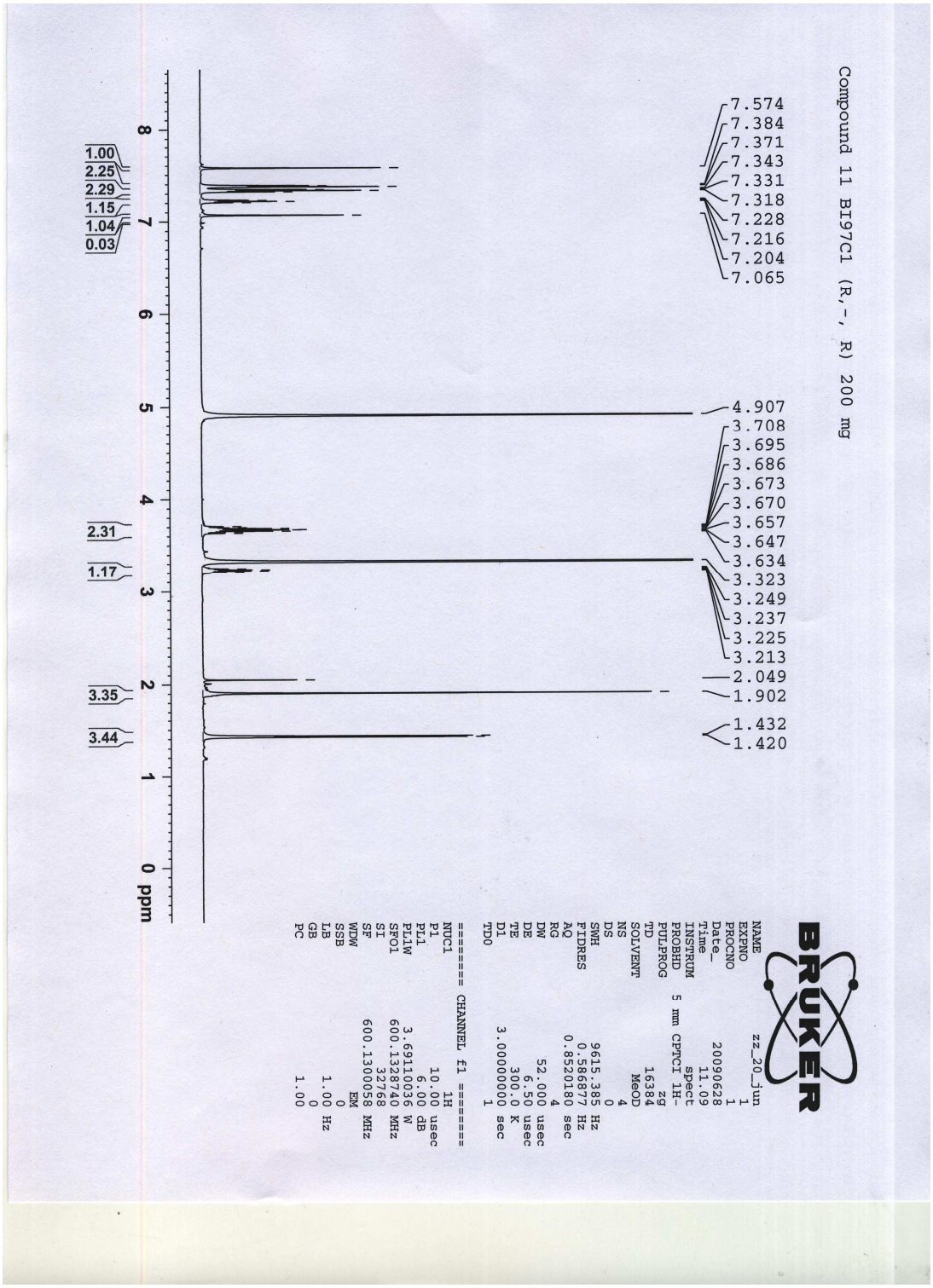


Supplementary Figure 6. Toxicity Studies.

Young female Balb/c mice (7-weeks-old) were injected with 25 mg/kg and 10 mg/kg of compounds **(11-14)** intraperitoneally (one mouse per dose) and observed for survival and weight loss for 11 days. Compounds **(11-14)** were 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.



3. Spectrums of key compounds (^{1}H-NMR, ^{13}C-NMR, HRMS and HPLC).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7.343
7.331
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7.228
7.216
7.204
7.065
4.907
3.708
3.695
3.686
3.673
3.670
3.657
3.647
3.634
3.323
3.249
3.237
3.225
3.213
2.049
1.902
1.432
1.420
1.00
2.25
2.29
1.15
1.04
0.03
2.31
1.17
3.35
3.44
8
7
6
5
4
3
2
1
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PROCNO 1
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PULPROG zg
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SOLVENT MeOD
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RG 4
DW 52.000 usec
DE 6.50 usec
TE 300.0 K
D1 3.00000000 sec
TD0 1
======== CHANNEL f1 ========
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P1 10.00 usec
PL1 6.00 dB
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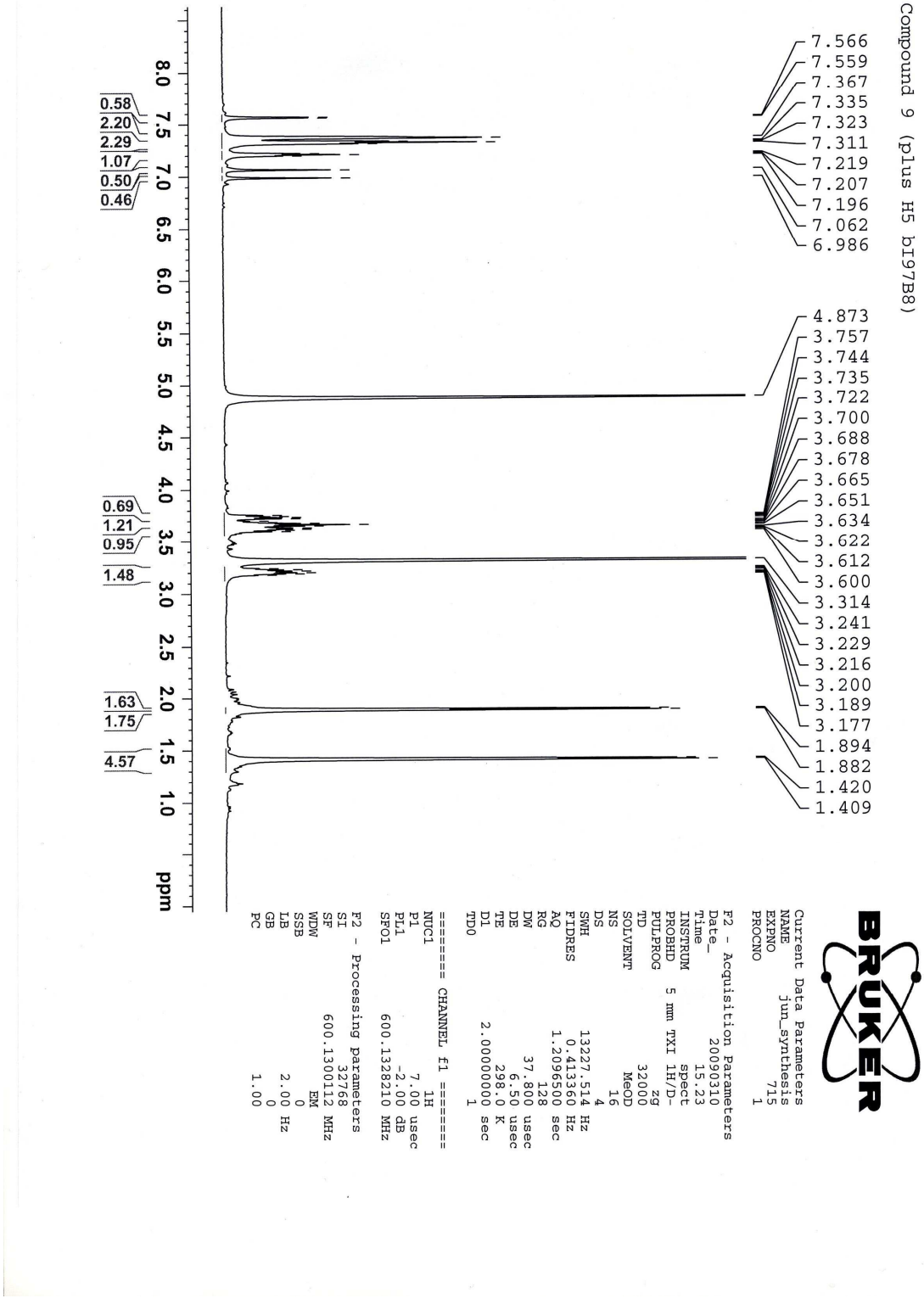
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7.219
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7.062
6.986
4.873
3.757
3.744
3.735
3.722
3.700
3.688
3.678
3.665
3.651
3.634
3.622
3.612
3.600
3.314
3.241
3.229
3.216
3.200
3.189
3.177
1.894
1.882
1.420
1.409
0.58
2.20
2.29
1.07
0.50
0.46
0.69
1.21
0.95
1.48
1.63
1.75
4.57
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
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TD 32000
SOLVENT MeOD
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TE 298.0 K
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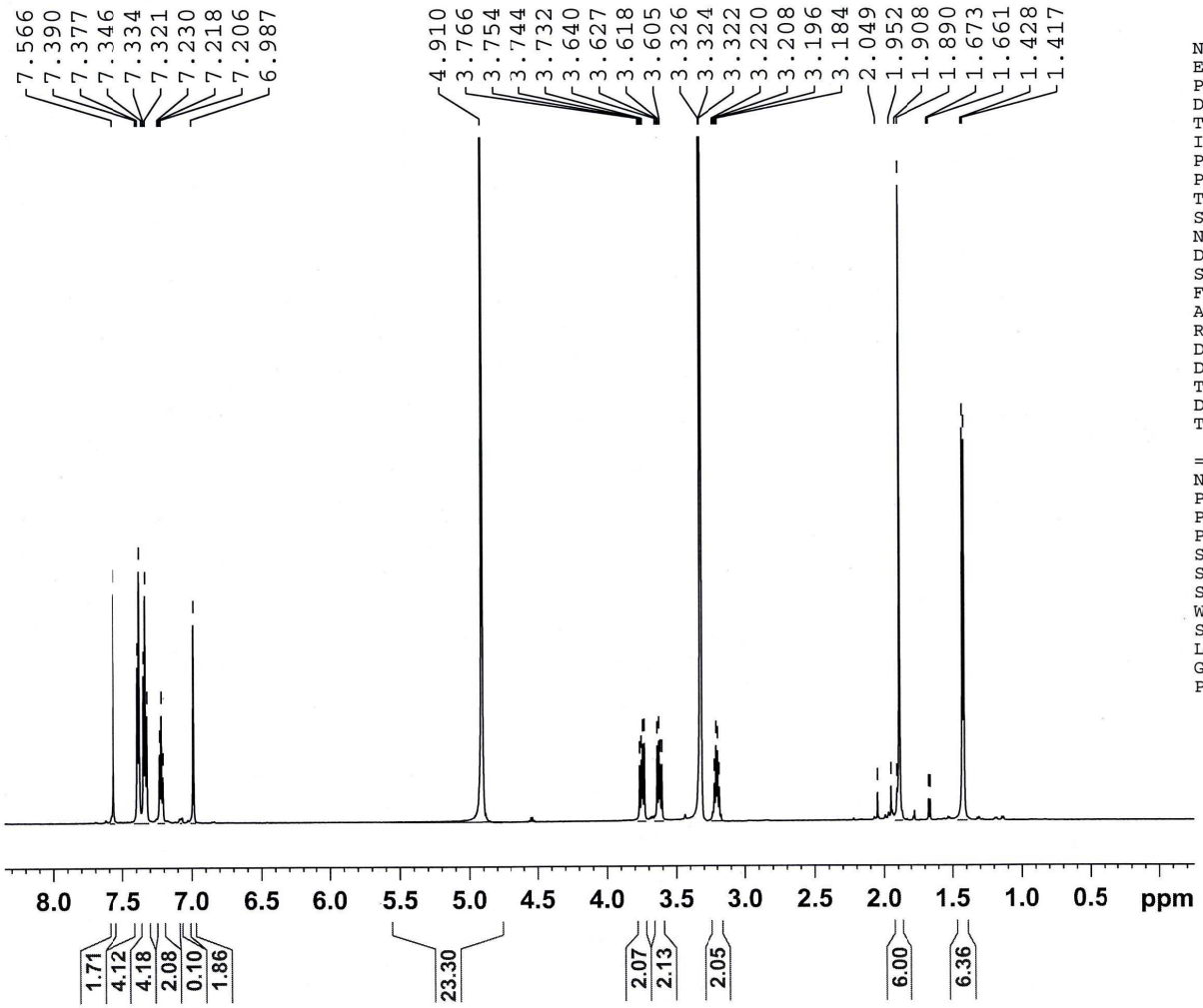
Compound 12 BI97C2 (R,R,+) H5



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burnham

Project Name: J20090707

Reported by User: System

Breeze

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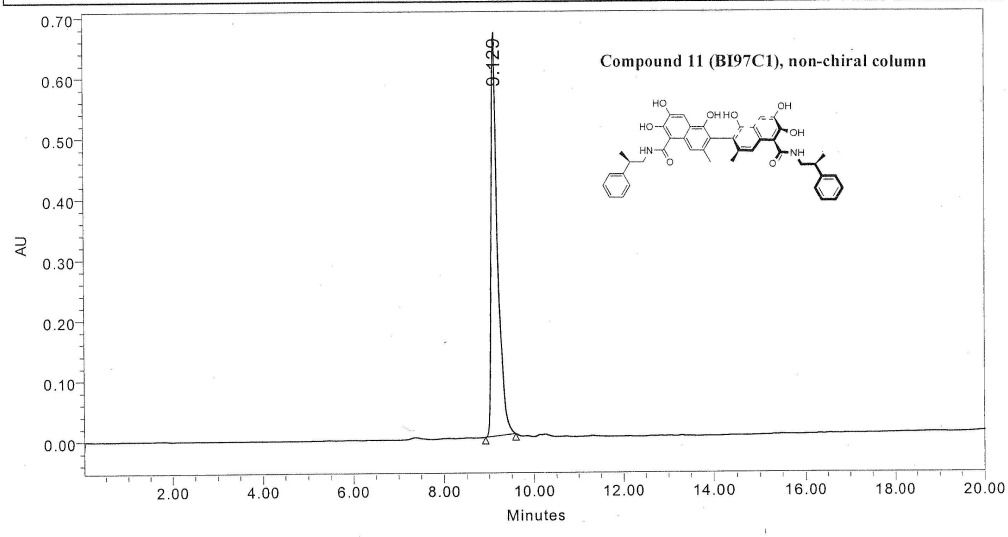
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	20.00 Minutes	Sample Set Name	M



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Final publication chiral column.

C1 = 11

burnham
Project Name: J20090707
Reported by User: System

'/Breeze

SAMPLE INFORMATION

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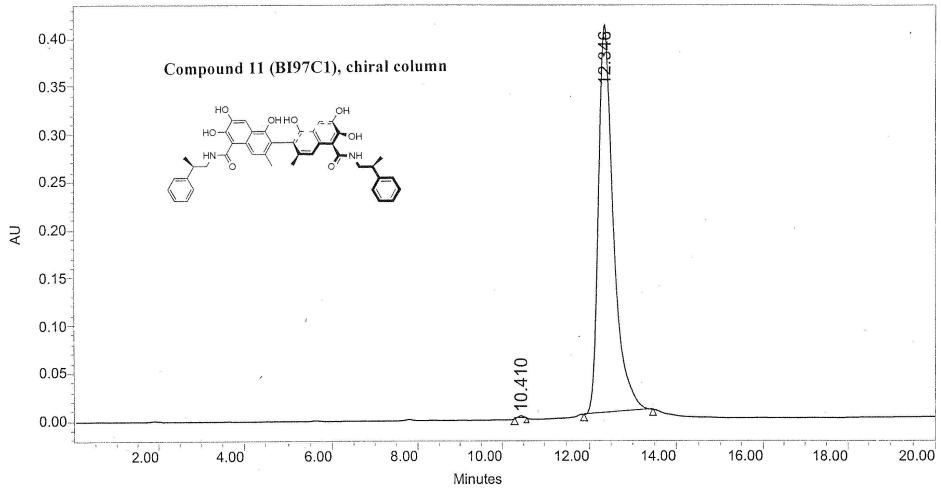

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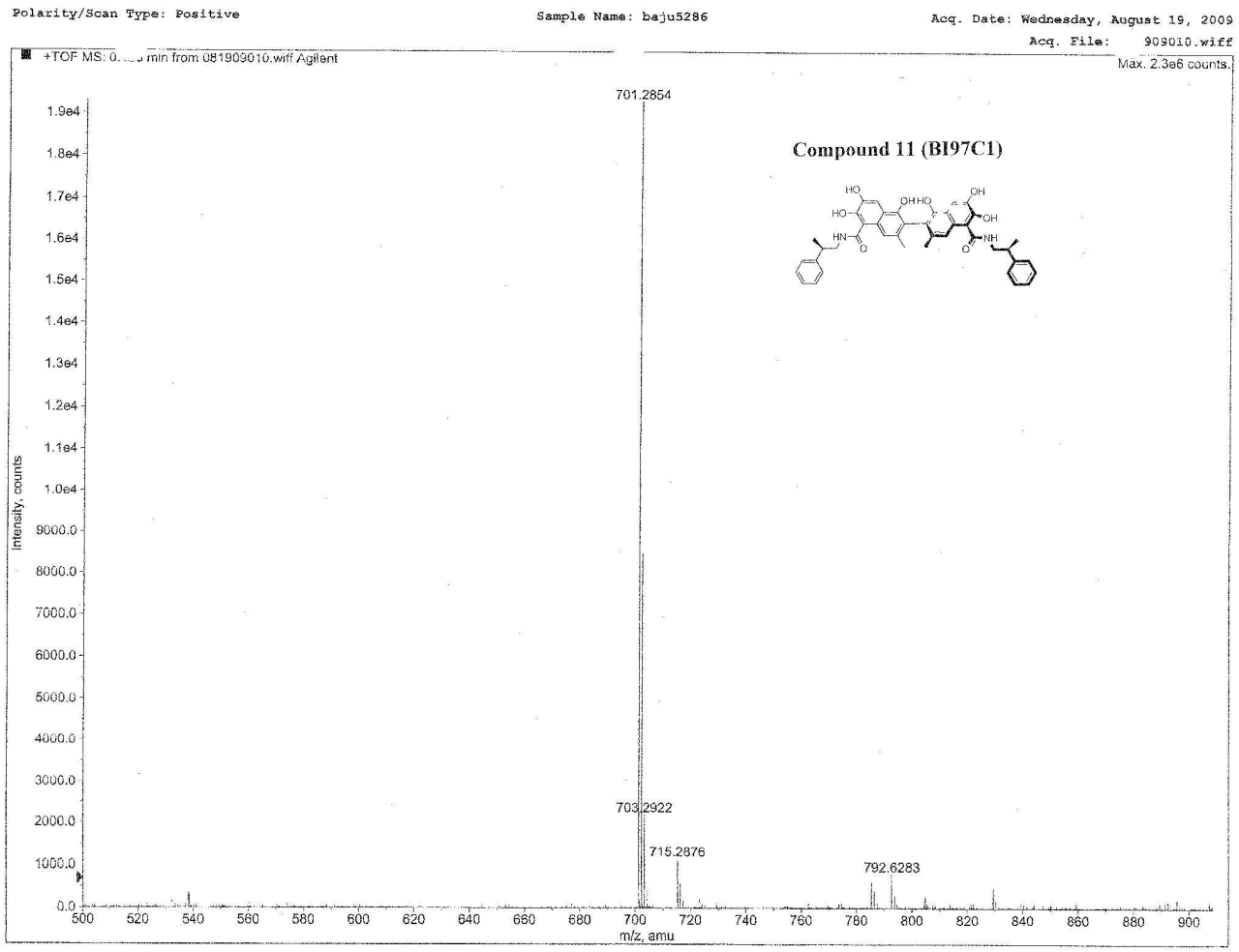
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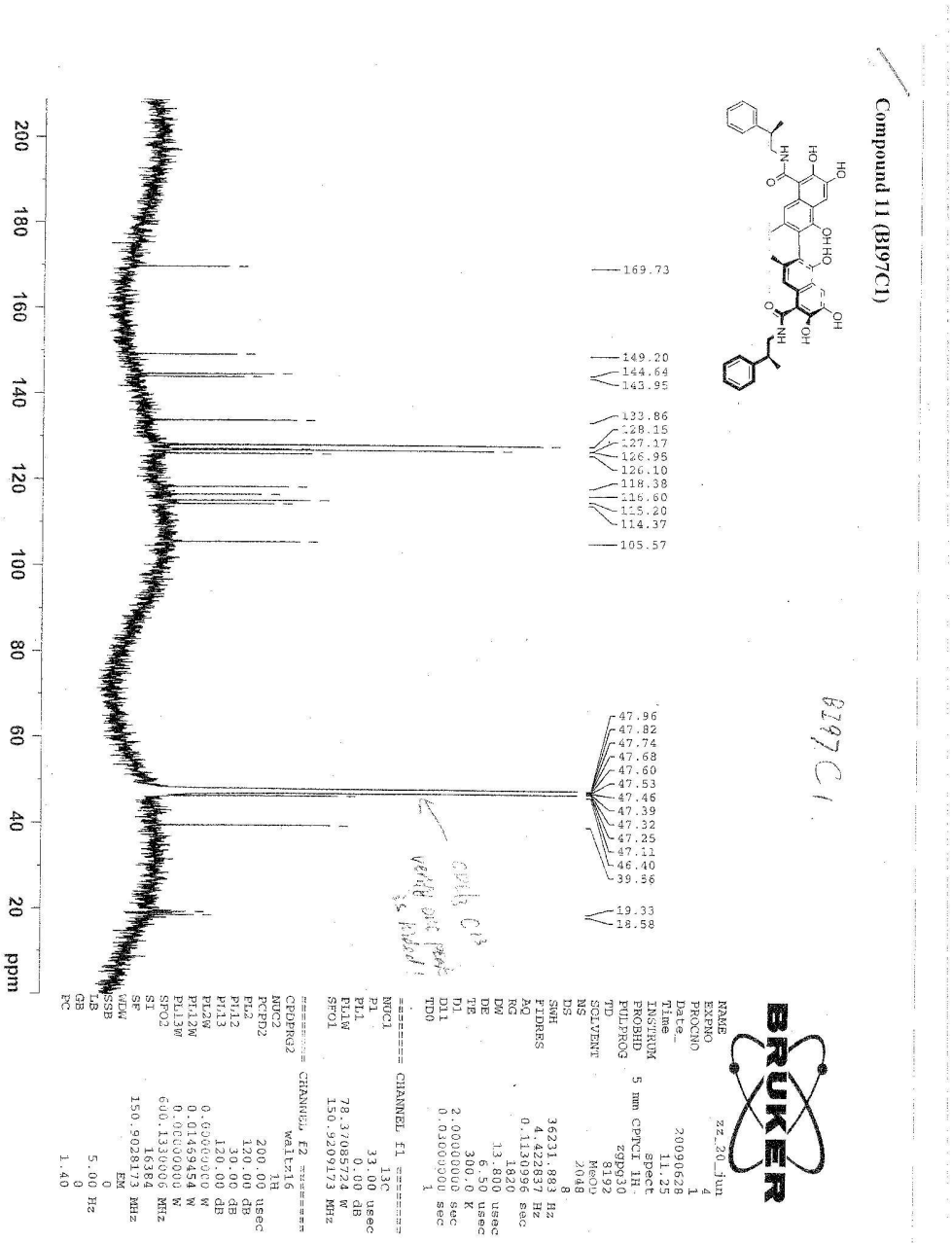
Intensity, counts
m/z, amu



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.