Supporting information:

Synthesis and Biological Evaluation of Apogossypolone Derivatives as Pan-active Inhibitors of Anti-apoptotic B-Cell Lymphoma/Leukemia-2 (Bcl-2) Family Proteins

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Contents:

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 15 or 20 µM concentration, in absence and presence of added compounds, each at 150 or 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] The binding mode has also been characterized by recording [^{15}N, ^{1}H]-HSQC experiments with 500 µL solution of uniformly ^{15}N-labeled Bcl-X_L (200 µM concentration) in absence and presence of added compounds, each at 200 µM concentration. ^{15}N and unlabeled Bcl-X_L samples were prepared and purified as described previously.[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 One aliquot (100 μL) of the incubation mixture was withdrawn at 0, 15, 30, and 60 min and combined immediately with 200 μL of ACN/MeOH containing an internal standard.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.

NMR data for compounds (15b-i and **2b-2m)**

Following above mentioned procedure of compounds **15f** and **2f** and the appropriate starting materials and reagents used; compounds **15b-i** and **2b-2m** were synthesized. The syntheses of compounds **15b-c, 2b-c, 2e, 2i** and **2l** have been previously described have been previously described. [2, 3]

5,5'-bis(2-cyclohexylethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (15d). Yield, 58%;^{1}H NMR (600 MHz, CD_3OD) δ 7.62 (s, 2H), 7.40 (s, 2H), 4.00 (s, 6H), 3.97 (s, 6H), 3.58 (s, 6H), 3.10 (t, $J_1 = J_2 = 7.2$ Hz, 4H), 2.21 (s, 6H), 1.94 (d, $J = 14.0$ Hz, 4H), 1.79-0.87 (m, 22H).

1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-5,5'-diphenethyl-2,2'-binaphthyl (15e). Yield, 55%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.65 (s, 2H), 7.45 (s, 2H), 7.36 (m, 4H), 4.00 (s, 6H), 3.94 (s, 6H), 3.58 (s, 6H), 3.40 (t, $J_1 = 8.4$ Hz, $J_2 = 7.8$ Hz, 4H), 3.02 (t, $J_1 = 8.4$ Hz, $J_2 = 7.8$ Hz, 4H), 2.21 (s, 6H).

1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-5,5'-bis(3-phenylpropyl)-2,2'-binaphthyl (15g). Yield, 60%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.42 (s, 2H), 7.39 (s, 2H), 7.31 (m, 8H), 7.21 (m, 2H), 3.98 (s, 6H), 3.90 (s, 6H), 3.54 (s, 6H), 3.10 (t, $J_1 = 7.2$ Hz, $J_2 = 7.8$ Hz, 4H), 2.86 (t, $J_1 = 7.2$ Hz, $J_2 = 7.8$ Hz, 4H), 2.12 (s, 6H), 2.20 (m, 4H).

1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-5,5'-bis(3-methyl-3-phenylbutyl)-2,2'-binaphthyl (15h). Yield, 65%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.55 (d, $J = 7.2$ Hz, 4H), 7.41 (t, $J_1 = 7.2$ Hz, $J_2 = 7.8$ Hz,

4H), 7.35 (s, 2H), 7.26 (m, 4H), 3.96 (s, 6H), 3.84 (s, 6H), 3.53 (s, 6H), 2.80 (m, 4H), 2.10 (s, 6H), 2.01 (t, J_1 = 7.2 Hz, J_2 = 7.8 Hz, 4H), 1.26 (s, 12H).

5,5'-dibenzyl-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (15i). Yield, 60%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (s, 2H), 7.41 (s, 2H), 7.21 (m, 8H), 7.11 (m, 2H), 4.44 (s, 4H), 3.95 (s, 6H), 3.78 (s, 6H), 3.50 (s, 6H), 2.03 (s, 6H).

5,5'-bis(4-chlorobenzyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (15l). Yield, 70%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.52 (s, 2H), 7.49 (s, 2H), 7.24 (m, 8H), 4.46 (s, 4H), 4.03 (s, 6H), 3.85 (s, 6H), 3.57 (s, 6H), 2.10 (s, 6H).

5,5'-bis(biphenyl-4-ylmethyl)-1,1',6,6',7,7'-hexamethoxy-3,3'-dimethyl-2,2'-binaphthyl (15m). Yield, 62%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.64 (s, 2H), 7.58 (d, J = 7.8 Hz, 4H), 7.51 (m, 6H), 7.42 (t, J_1 = 7.2 Hz, J_2 = 7.8 Hz, 4H), 7.38 (d, J = 7.8 Hz, 4H), 7.32 (m, 2H), 4.55 (s, 4H), 4.04 (s, 6H), 3.89 (s, 6H), 3.59 (s, 6H), 2.13 (s, 6H).

5,5'-bis(2-cyclohexylethyl)-3,3'-dimethyl-2,2'-binaphthyl-1,1',6,6',7,7'-hexaol (2d). Yield, 80%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.41 (s, 2H), 7.34 (s, 2H), 3.04 (dd, J_1 = 7.2 Hz, J_2 = 13.8 Hz, 4H), 2.05 (s, 6H), 1.93 (t, J_1 = J_2 = 13.2 Hz, 4H), 1.76 (d, J = 12.0 Hz, 4H), 1.69 (d, J = 12.6 Hz, 2H), 1.55 (q, J_1 = J_2 = 6.6 Hz, 4H), 1.34 (m, 8H), 1.04 (m, 4H). ^{13}C NMR (600 MHz, CD_3OD) δ 148.94, 143.58, 143.38, 132.26, 128.69, 120.00, 118.27, 114.26, 113.42, 101.55, 37.68, 36.80, 32.84, 25.73, 21.89, 19.5. HPLC purity 98.0%, t_R = 15.28 min (Method B). HRMS calcd for $C_{38}H_{46}O_6$ 599.3367 (M + H), found 599.3366.

3,3'-dimethyl-5,5'-bis(3-phenylpropyl)-2,2'-binaphthyl-1,1',6,6',7,7'-hexaol (2g). Yield, 85%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.31 (s, 2H), 7.28 (m, 6H), 7.18 (t, J_1 = J_2 = 6.6 Hz, 2H), 7.14 (s, 2H), 3.07 (m, 4H), 2.81 (dd, J_1 = 7.2 Hz, J_2 = 7.8 Hz, 4H), 2.01 (m, 4H), 1.97 (s, 6H). ^{13}C NMR (600 MHz, CD_3OD) δ 149.28, 143.96, 142.54, 132.74, 129.07, 128.18, 127.84, 125.24, 119.71, 118.63, 114.59, 113.85, 102.13, 35.68, 31.18, 24.31. HPLC purity 98.6%, t_R = 12.36 min (Method B). HRMS calcd for **$C_{40}H_{38}O_6$** 615.2741 (M + H), found 615.2765.

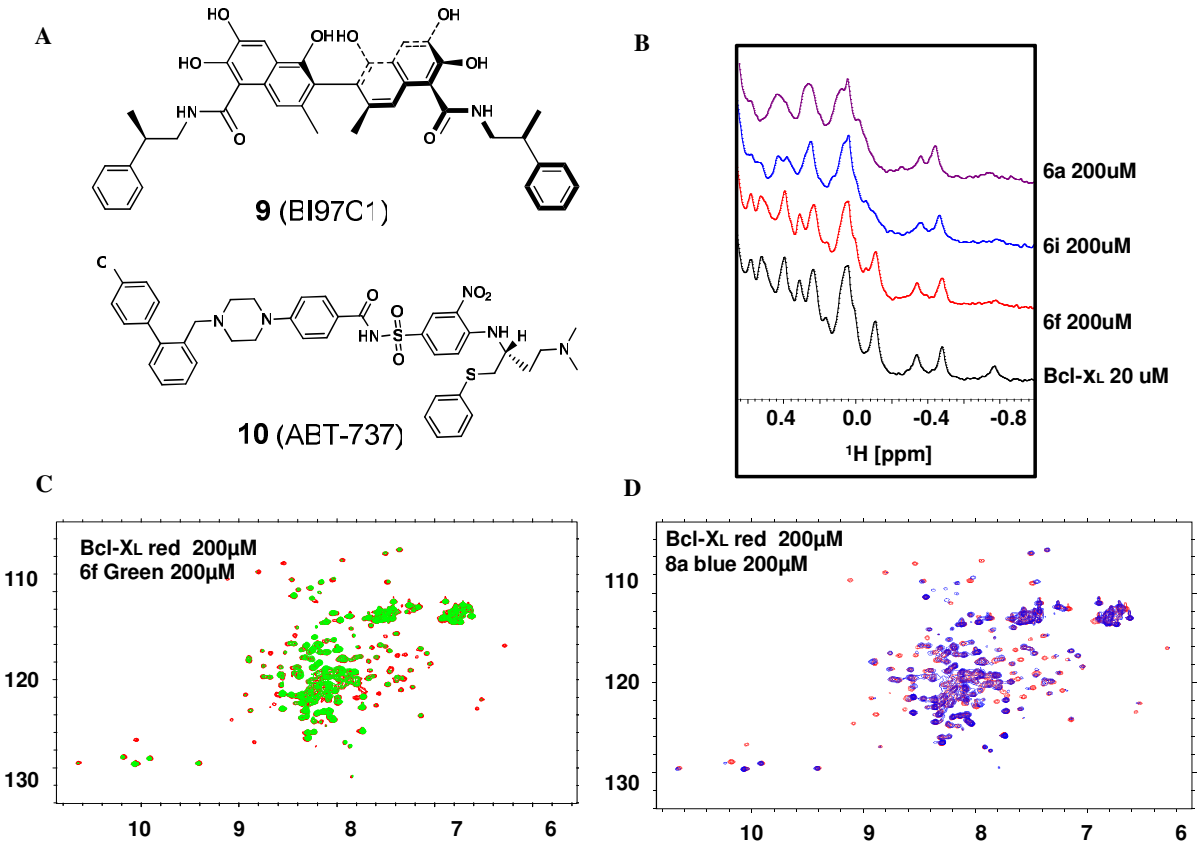
3,3'-dimethyl-5,5'-bis(3-methyl-3-phenylbutyl)-2,2'-binaphthyl-1,1',6,6',7,7'-hexaol (2h). Yield, 80%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.55 (d, J = 7.2 Hz, 4H), 7.40 (m, 6H), 7.23 (t, $J_1 = J_2$ = 7.2 Hz, 2H), 6.87 (s, 2H), 2.75 (dd, J_1 = 4.2 Hz, J_2 = 10.8 Hz, 4H), 1.96 (m, 4H), 1.95 (s, 6H), 1.48 (s, 6H), 1.47 (s, 6H). ^{13}C NMR (600 MHz, CD_3OD) δ 149.21, 148.95, 143.93, 143.72, 132.61, 128.94, 127.75, 125.82, 125.17, 120.13, 118.57, 114.46, 113.74, 101.92, 43.67, 37.66, 27.96, 20.43. HPLC purity 98.0%, t_R = 16.08 min (Method B). HRMS calcd for **$C_{44}H_{46}O_6$** 671.3367 (M + H), found 671.3367.

5,5'-bis(4-chlorobenzyl)-3,3'-dimethyl-2,2'-binaphthyl-1,1',6,6',7,7'-hexaol (2l). Yield, 80%; ^{1}H NMR (600 MHz, CD_3OD) δ 7.48 (s, 2H), 7.28 (m, 6H), 7.18 (d, J = 7.8 Hz, 4H), 4.44 (d, J = 15 Hz, 2H), 4.33 (d, J =15.6 Hz, 2H), 1.94 (s, 6H). HPLC purity 99.3%, t_R = 14.95 min (Method A). HRMS calcd for **$C_{40}H_{28}Cl_2O_6$** 627.1336 (M + H), found 627.1340.

2. Supplementary Figures

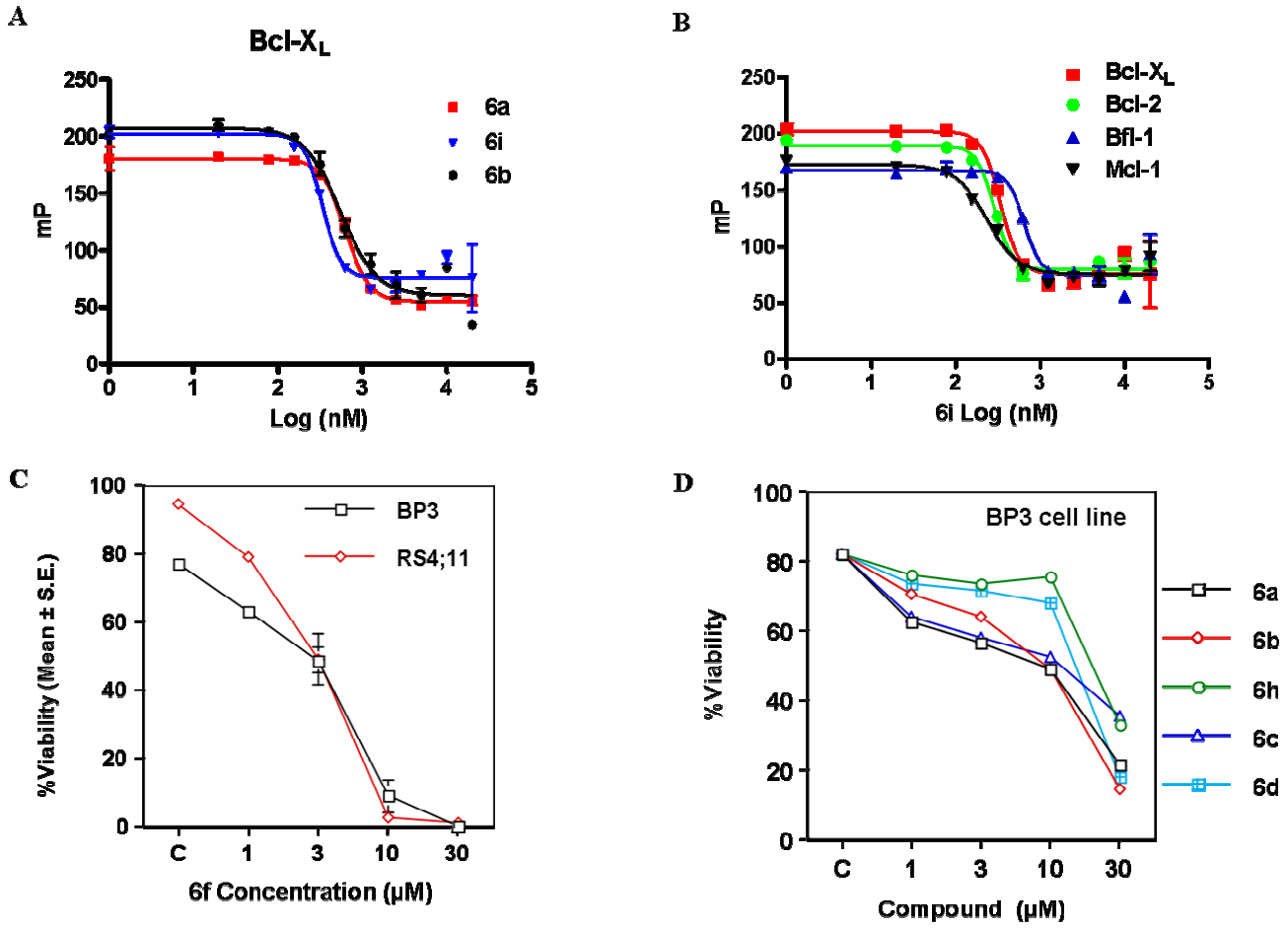
Supplementary Fig. 1.

NMR binding studies. **(A)** Structures of compounds **9** and **10**. **(B)** Aliphatic region of the ^{1}H-NMR spectrum of Bcl-X_L (20 µM, black) and Bcl-X_L in the presence of compound **6a** (200 µM, purple), compound **6i** (200 µM, blue) and compound **6f** (200 µM, red). **(C)** Superposition of [^{15}N, ^{1}H]-TROSY spectra of free Bcl-X_L (200 µM, red) after addition of compound **6f** (green, 200 µM) **(D)** Superposition of [^{15}N, ^{1}H]-TROSY spectra of free Bcl-X_L (200 µM, red) after addition of compound **8a** (blue, 200 µM).



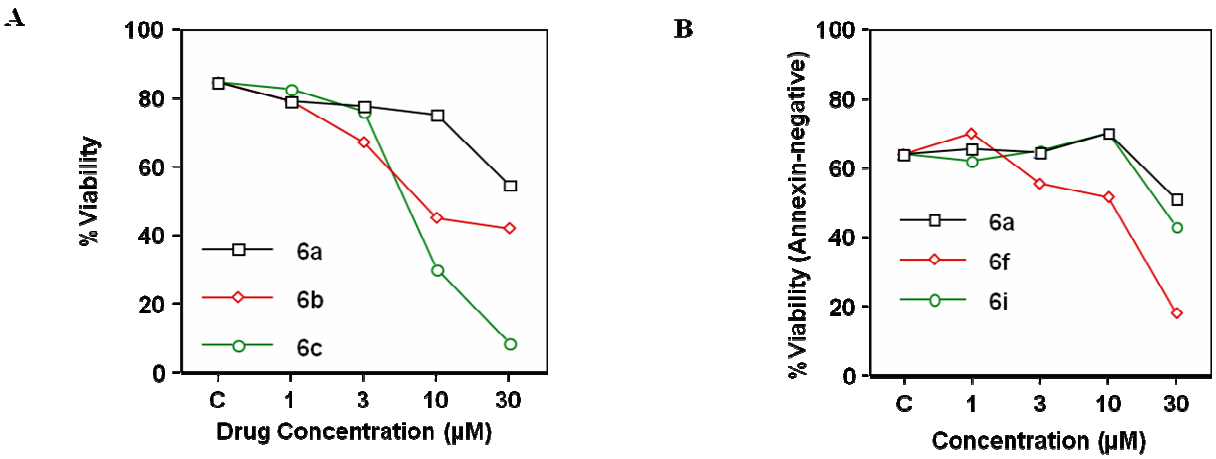
Supplementary Figure 2:

(A) Fluorescence polarization-based competitive binding curves of **6a** (red square), **6i** (blue down triangle) and **6b** (dark dot) using Bcl-X_L. **(B)** Fluorescence polarization-based competitive binding curves of **6i** using Bcl-X_L (red square), Bcl-2 (green dot), Bfl-1 (blue up triangle) and Mcl-1 (dark down triangle). **(C)** Cytotoxicity assays of **6i** against BP3 and RS4;11 using Annexin V-FITC and propidium iodide assay. **(D)** Inhibition of cell growth by **6a** and its derivatives in the BP3 cell line. Cells were treated for 1 days and cell viability was evaluated using Annexin V-FITC and propidium iodide assay.



Supplementary Figure 3.

(A) Cytotoxicity assays of **6a, 6b, 6c** against primary CLL using Annexin V-FITC and propidium iodide assay. **(B)** Cytotoxicity assays of **6a, 6f, 6i** against primary CLL using Annexin V-FITC and propidium iodide assay..



3. Supplementary Table

Supplementary Table 1. Summary of cytotoxicity assay of 6f against primary CLL cells using Annexin V-FITC and propidium iodide assay.

	CLL Pt#1	CLL Pt#2	CLL Pt#3	CLL Pt#4	CLL Pt#5	CLL Pt#6	CLL Pt#7
CRC ID#	TJK263	TJK287	TJK484	TJK688	TJK896	TJK898	TJK578
IC50	5.56 µM	7.98 µM	2.2 µM	12.44 µM	9.13 µM	16.9 µM	1.0 µM

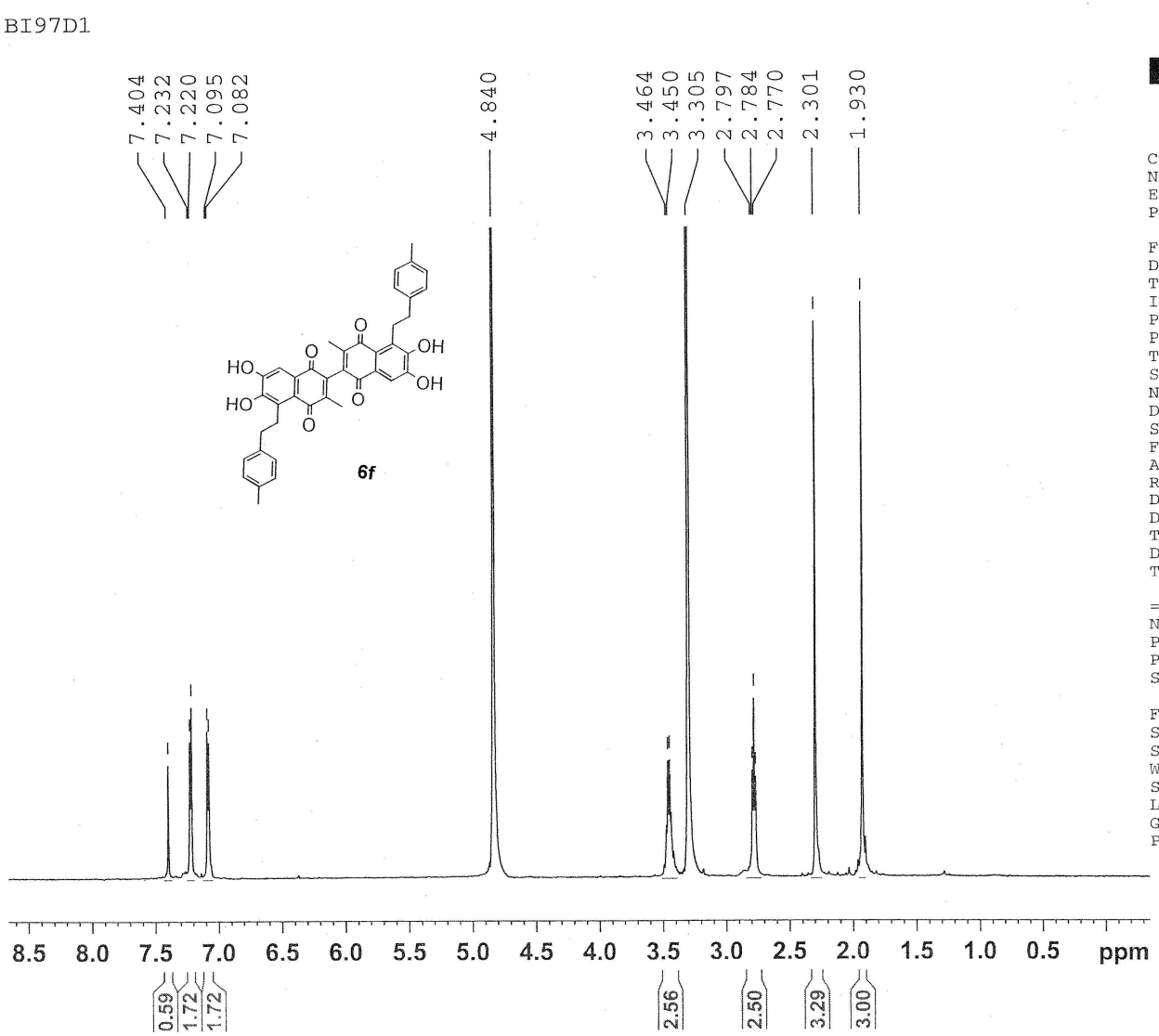
Supplementary Table 2. Summary of K_i values of selected 5, 5' 6a derivatives in our FP assay.

Compound	K_i (µM) FPA			
	Bcl-X_L	Bcl-2	Bfl-1	Mcl-1
6a	0.42	0.25	1.47	0.43
6b	0.37	0.17	0.93	0.31
6f	2.07	2.08	9.33	1.37
6i	0.23	0.19	0.43	0.16
6c	2.0	1.53	ND	2.07
6d	8.4	4.47	ND	3.93
6e	1.2	1.73	6.47	0.87
6g	0.96	1.45	3.53	0.45
6l	0.11	0.23	0.47	0.27
7	0.24	0.15	0.46	0.23
8a	0.21	0.15	0.47	0.31
8c	0.16	0.14	0.87	0.21

ND[a*] = Not determined

4. Spectrums of key compounds (^{1}HNMR, ^{13}CNMR and HPLC).

BI97D1



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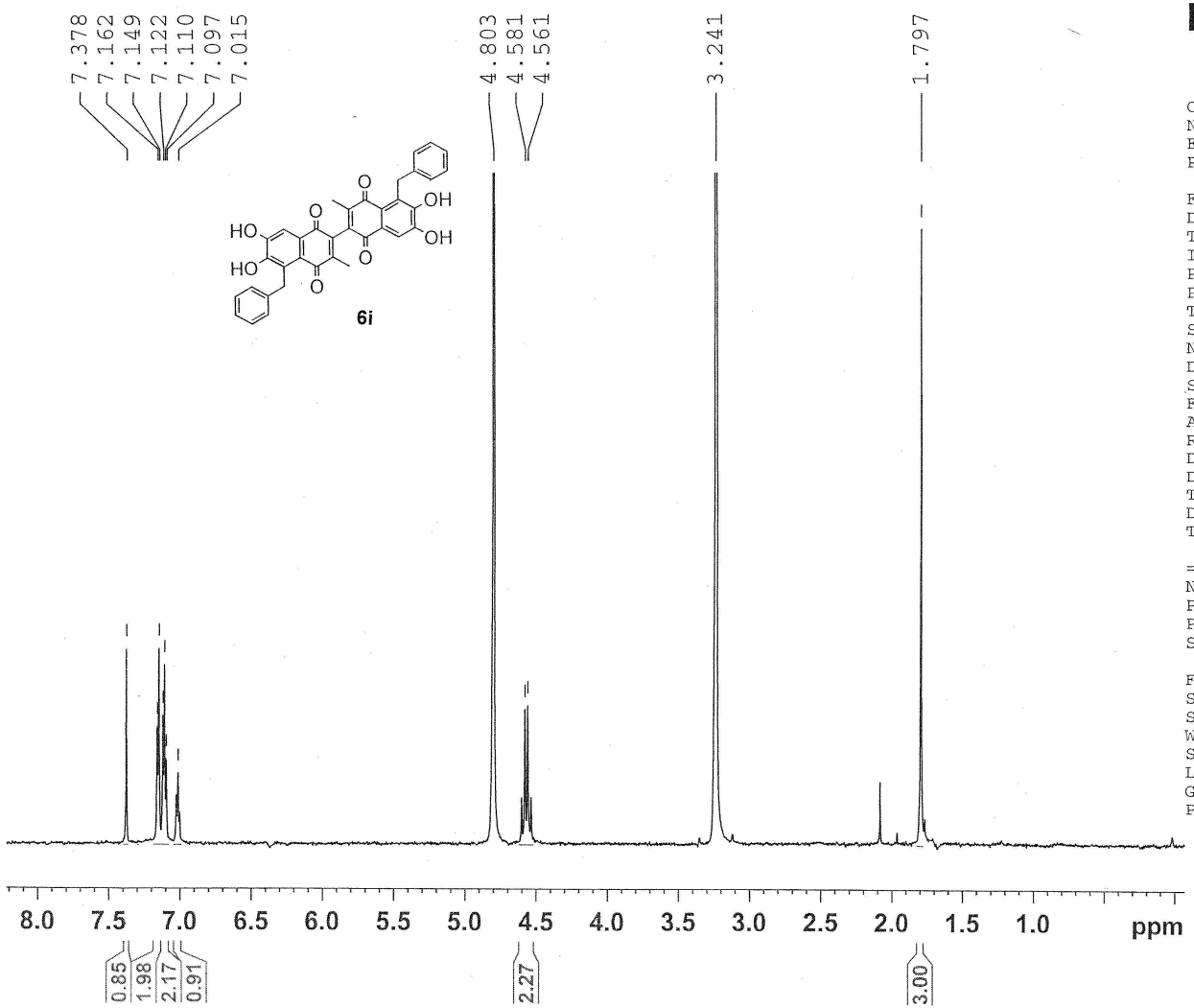

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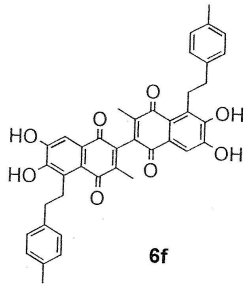

6f

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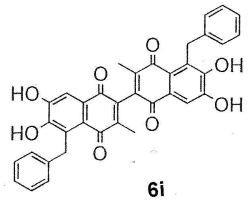
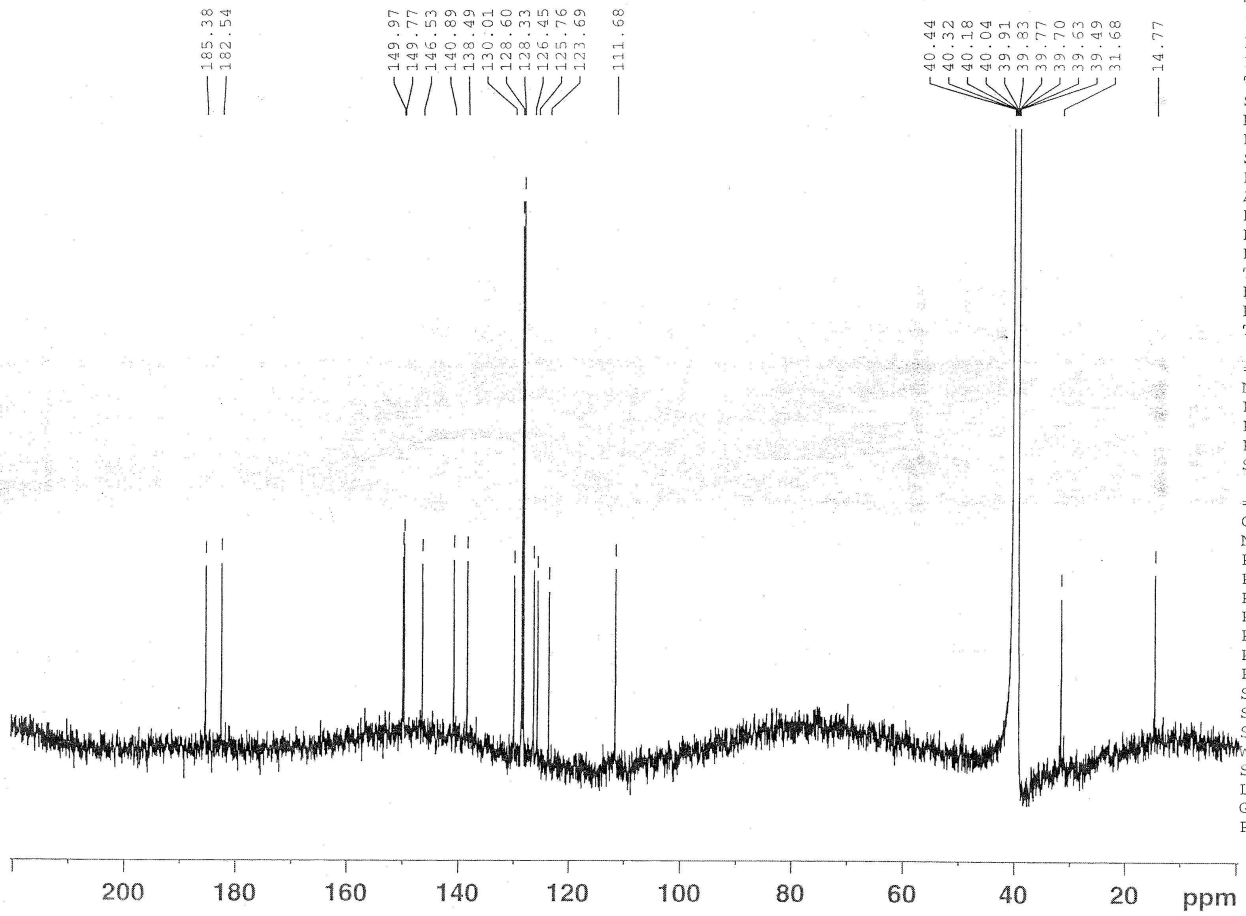
BI97 D6



6i



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burnham
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Breeze

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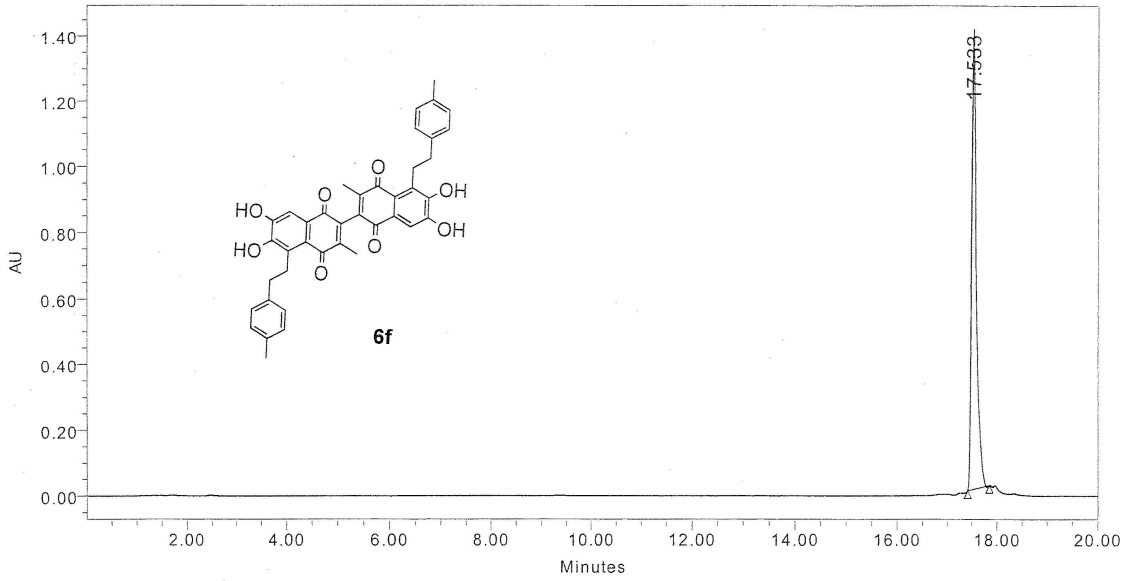

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burnham
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Breeze

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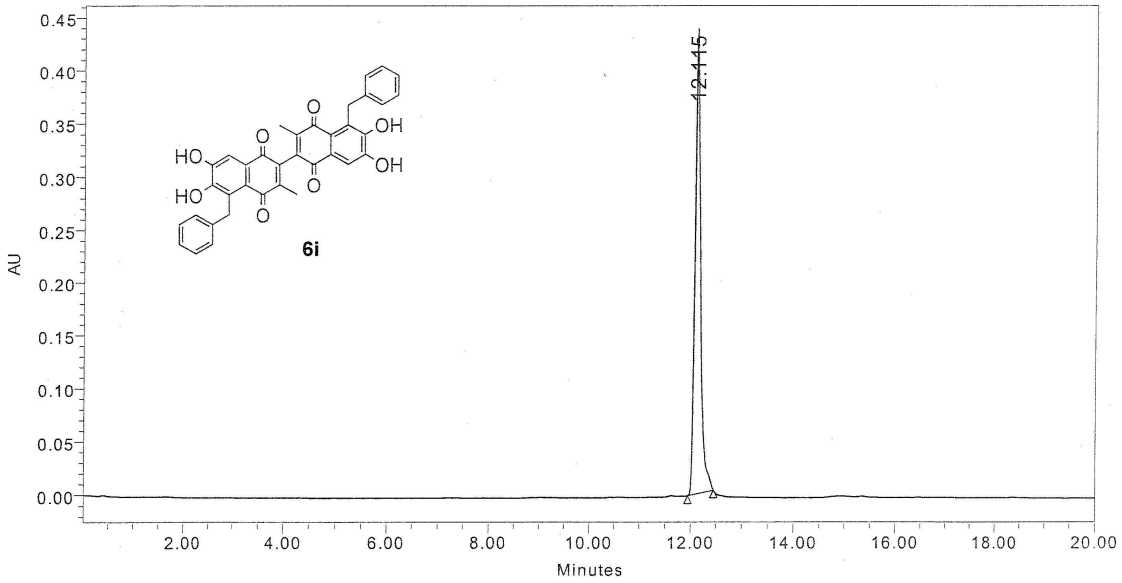

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References:

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