Supporting Information

Janus Kinase 2 Inhibitors. Synthesis and Characterization of a Novel Polycyclic Azaindole

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Contents: (1) Synthetic procedures, ^{1}H NMR data for compounds **5**, **6**, **7**, and **9A** and **B** and LC/MS data for all compounds and HRMS data for both atropisomers **9A** and **B**, (2) assay protocols for K_i and IC_{50} determinations, (3) protocols for cloning, expression and purifying and crystalization of JAK2, (4) data collection and refinement statistics for JAK2 + Compound **9** (5) assay protocols for the colony forming assays, (6) HPLC and LC-MS traces for compounds **9A** and **B** as well as for the equilibrium mixture of compound **9.**

General experimental

Proton NMR spectra were recorded on a Bruker Advance instrument with a QNP probe using TMS as the internal standard in the indicated deuterated solvent. LC−MS analyses were performed on a Waters ZQ or ZMD or QuatroII mass spectrometer using the electrospray (ESI) ionization technique. Samples were introduced into the mass spectrometer using chromatography. All commercially available reagents were used without further purification. Purity assessment for compound **9** was based on orthogonal HPLC methods. Methods (LC-MS) consisted of the following: column, 4.6 mm × 50 mm Waters YMC Pro-C18 column, 5 μm, 120A. Mobile phases are as follows: (Method A): A, H_2O with 0.1% formic acid; B, acetonitrile with 0.1% formic acid; gradient, 10% B to 90% B in 3 min with 5 min run time. Flow rate is 1.5 mL/min. (Method B): A, H_2O with 0.1% formic acid; B, methanol with 0.1% formic acid; gradient, 10% B to 90% B in 3 min with 5 min run time. Flow rate is 1.5 mL/min. (Method C): A, H_2O with 10 mM ammonium formate; B, methanol with 10 mM ammonium formate; gradient, 2% B to 90% B in 3 min with 5 min run time. Flow rate is 1.5 mL/min. Analytical HPLC method consisted of the following: column: 2.0 mm × 50 mm YMC Pro C18 column, 5 μm, 120A. Mobile phases are as follows: A, H_2O with 0.1% TFA; B, ACN with 0.1% TFA; gradient, 0% B to 100% B in 4 min with 6 min run time. Flow rate is 0.8 mL/min. As used herein, the term "t_R" refers to the HPLC retention time, in minutes, associated with the compound. HRMS analysis was performed on a Bruker 9.4T AS FT Mass Spectrometer.

Preparation of Compound 5:

4-bromo-1H-pyrrolo[2,3-b]pyrydine (11.6g, 59.2 mmol) and 2-ethanamidophenylboronic acid (11.8 g, 65.2 mmol) was dissolved in about 50 mL DME followed by addition of Tetrakis(triphenylphodphine)palladium(0) (3.34 g, 2.9 mmol, 5 %mol) and 20 mL

saturated aqueous Na_2CO_3. The reaction mixture was purged with nitrogen and refluxed overnight under nitrogen. The reaction was cooled to room temperature and extracted with ethyl acetate (200 mL x 2). The combined organic layers were washed with brine, dried over Na_2SO_4. The solvent was removed and the resulting crude material was purified using a short plug of silica gel. The resulting material (a brown solid) was dissolved in about 200 mL toluene and 6N aq. HCl (20 mL) was added. The mixture was refluxed for 12 hours and the reaction was cooled to room temperature. A solution of 6N aq. NaOH was added to adjust the pH to 8. The mixture was extracted with DCM (200 mL x 2). The combined organic layers were washed with brine, dried over Na_2SO_4. The solvent was removed and crude material was purified on silica gel to afford 8.04 g (65 %) desired product as a beige solid.

1H NMR (300 MHz, DMSO) δ 11.69 (s, 1H), 8.26 (d, J = 4.9 Hz, 1H), 7.48 (dd, J = 8.2, 5.3 Hz, 1H), 7.19 – 7.08 (m, 2H), 7.06 (d, J = 4.9 Hz, 1H), 6.88 – 6.76 (m, 1H), 6.68 (td, J = 7.4, 1.0 Hz, 1H), 6.26 (dd, J = 3.4, 1.8 Hz, 1H), 4.80 (s, 2H). LC-MS (method A) t_R = 2.47 min., $(M + H^+)$ 210.3.

Preparation of Compound 6:

Compound **5** (3.13 g, 15.0 mmol) and methyl 2-(hydroxyphenyl)-2-oxoethanoate (2.78 g, 15.0 mmol, this was prepared according to the procedure in J. Med. Chem. **2003,** *45*, 3946-3952.) was dissolved in 100 mL MeOH and 30 mL 4M HCl in dioxane was added into the reaction. The reaction vessel was sealed and heated at 100 °C overnight. The reaction was cooled to room temperature and the solvent was removed. The resulting crude material was purified on silica gel to afford 3.5 g (63 %) desired product as a yellow solid.

1H NMR (300 MHz, DMSO) δ 12.33 (s, 1H), 8.42 (s, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.67 (s, 1H), 7.36 (d, J = 7.8 Hz, 1H), 7.27 (t, J = 7.4 Hz, 1H), 7.08 – 6.93 (m, 4H), 6.66 (d, J = 8.6 Hz, 2H), 6.49 (s, 1H), 5.75 (s, 1H), 3.63 (s, 3H). LC-MS (method A) t_R = 2.67 min., $(M + H^+)$ 372.7, $(M - H^+)$ 370.7.

Preparation of Compound 7:

Compound **6** (2.1 g, 5.7 mmol) was dissolved in 20 mL MeOH followed by addition of 3 mL aq. 6N HCl and catalytic amount of Palladium (0) on carbon (10% weight). The reaction was placed under hydrogen atmosphere (50 PSI). The reaction was shaken on the apparatus for 72 hours. The catalyst was filtered and pH was adjusted to 8. The mixture was extracted using DCM (30 mL x 2) and the combined organic layers were washed with brine, dried over Na_2SO_4. The solvent was removed and crude material was purified on silica gel to afford 1.85 g (88 %) desired product as a yellow solid.

1H NMR (300 MHz, DMSO, mixture of rotamers) δ 11.66 and 11.55 (two d, J = 2.0 Hz, 1H), 9.31 and 9.20 (two s, 1H), 8.23 and 8.21 (two d, J = 4.8 Hz, 1H), 7.17 (d, J = 2.4 Hz, 0.5 H), 6.95 – 6.73 (complex m, 3.5H), 6.70 – 6.45 (complex m, 5H), 4.92 and 4.80 (two s, 1H), 4.52 and 4.48 (two s, 2H), 3.45 and 3.25 (two s, 3H). LC-MS (method A) t_R = 2.68 min., $(M + H^+)$ 374.6, $(M - H^+)$ 372.7.

Preparation of Compound 8:

Compound **7** (1.8 g, 5.0 mmol) was dissolved in 20 mL aq. 6N HCl and the mixture was refluxed for 6 hours. The reaction was cooled to room temperature and pH was carefully adjusted to 6~7. The mixture was extracted with DCM (20mlx5) and the combined organic layers were washed with brine, dried over Na_2SO_4. The solvent was removed and crude material 1.54 g (86 %) was taken forward without further purification.

(atropisomers) LC-MS (method A) t_R = 2.3 min. and 2.7min., (M + H^+) 360.4, (M - H^+) 358.2.

Preparation of Compound 9:

Compound **8** (1.2 g, 3.3 mmol) was dissolved in 5ml DMF. Triethylamine (0.91 ml 6.6 mmol) was added to mixture followed by the addition of HATU (1.90g, 5.0 mmol). The reaction was heated at 60 °C for 30 min. The reaction was cooled to room temperature and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4. The solvent was removed and crude material was purified on silica gel to afford two forms of atropisomers **9A** (major, 340 mg, 35 %) and **9B** (minor, 168mg, 15 %).

Analytical HPLC of the equilibrated samples showed only the presence of the two atropisomer peaks (**9A**: t_R = 2.49 min. and **9B**: t_R = 1.72 min.) and indicated a combined purity of >95%.

Major atropisomer **9A**: ^{1}H NMR (500 MHz, Methanol-d4) 8.27 (d, J = 5.1 Hz, 1 H), 7.64 (dd, J = 1.9, 7.3 Hz, 1 H), 7.60 - 7.54 (m, 2 H), 7.46 (d, J = 8.6 Hz, 2 H), 7.34 (dd, J = 1.6, 7.4 Hz, 1 H), 7.09 (d, J = 5.1 Hz, 1 H), 6.94 (s, 1 H), 6.80 (d, J = 8.6 Hz, 2H), 5.27 (s, 1 H); LC-MS (method A) t_R = 2.0 min., (M + H^+) 342.3, (M - H^+) 340.4.; LC-MS (method B) t_R = 3.0 min., (M + H^+) 342.19.; LC-MS (method C) t_R = 3.39 min., (M + H^+) 342.19.; HRMS (M + H^+) calculated for ($C_{21}H_{16}N_3O_2$): 342.1237, found: 342.12346 with a deviation of only 1.24 ppm.

Minor atropisomer **9B**: ^{1}H NMR (500 MHz, Methanol-d4) 8.49 (d, J = 6.1 Hz, 1 H), 7.84 (s, 1 H), 7.48 (d, J = 6.1 1 Hz, H), 7.36 (dd, J = 1.3, 7.7 Hz, 1 H), 7.23 - 7.17 (m, 2 H), 6.84 - 6.82 (m, 1 H), 6.59 (d, J = 8.2 Hz, 2 H), 6.34 (d, J = 8.6 Hz, 2 H), 5.24 (s, 1 H); LC-MS (method A) t_R = 1.7 min., (M + H^+) 342.3, (M - H^+) 340.4.; LC-MS (method B) t_R = 2.38 min., (M + H^+) 342.19.; LC-MS (method C) t_R = 2.96 min., (M + H^+) 342.19, (M - H^+) 340.16.; HRMS (M + H^+) calculated for ($C_{21}H_{16}N_3O_2$): 342.1237, found: 342.12328 with a deviation of only 0.72 ppm.

K_i Determination.

Materials: ATP and polyE4Y were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ^{33}P-γ-ATP, GF/B filter plates, and Ultima Gold™ scintillant were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). JAK2 and JAK3 used in Vertex assays were expressed and purified by the Gene Expression and Protein Biochemistry groups,

respectively, at Vertex Pharmaceuticals Incorporated using standard recombinant methods[1].

Methods: The inhibitory activity of **9** against JAK2 was determined by following the residual kinase activity of JAK2 using a radiometric assay. The final concentration of the components in the assay were as follows: 100 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 0.01% BSA, 0.6 nM JAK2, 0.5 mg/ml polyE4Y, and 12 μM ^{33}P-γ-ATP. A stock solution of inhibitor was made up in DMSO from which additional dilutions were made in DMSO; a 1.5 μL aliquot of DMSO or inhibitor in DMSO was added to each well. 50 μL of a 2x substrate mixture (100 mM HEPES, 10 mM $MgCl_2$, 1.0 mg/mL polyE4Y, and 24 μM ^{33}P-γ-ATP) was added and mixed with the inhibitor/DMSO. The reaction was initiated by the addition of 50 μL of a 2x enzyme mixture (100 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 0.02% BSA, 1.0 nM JAK2). After 15 min, the reaction was quenched with 50 μL of 20% TCA. The quenched reaction was transferred to the GF/B filter plates and washed 3 times with 5% TCA. Following the addition of Ultimate Gold™ scintillant (50 μL), the samples were counted in a Packard TopCount. The radioactivity trapped is a measure of the residual JAK2 kinase activity. JAK3 assays were performed in a similar manner except that 0.1 nM JAK3, 0.25 mg/ml polyE4Y, and 5 μM ^{33}P-γ-ATP were used. From the activity vs. inhibitor concentration, the Ki value was determined by fitting the data to an equation for competitive tight binding inhibition kinetics[2] using Prism software, version 4.0, San Diego, CA, USA.

Literature References:

(1) Fox T.; Coll J. T.; Ford P. J.; Germann U. A.; Porter M. D.; Pazhanisamy S.; Fleming M. A.; Gulullo V.; Su M.-S.; and Wilson K. P.; *Protein Sci*. **1998**, 7, 2249-2255.

(2) Morrison J. F.; and Stone S. R.; *Comments Mol. Cell. Biophys*. **1985**, *2*, 347-368.

IC50 determinations.

Materials: TF-1 cells and HT-2 cells were obtained from American Type Culture Collection, Manassas, VA and cultured according to the provider's instructions in the presence of variable concentrations of compounds or DMSO.

Methods: JAK2-STAT5 signaling was stimulated with the addition of 2 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D systems, Minneapolis, MN) for fifteen minutes to TF-1 cells. JAK3-STAT5 signaling was stimulated with the addition of 10 ng/mL IL-2 (R&D systems, Minneapolis, MN) for twenty minutes to HT-2 cells. Stimulated cells were fixed with the addition of 4% formaldehyde and permeabilized with 90% methanol. Phospho-STAT5 (pSTAT5) was quantified by flow cytometry in a Guava PCA-96 system (Guava Technologies, Hayward, CA) using an anti-STAT5 monoclonal antibody conjugated to phycoerythrin (BD Biosciences, San Jose, CA). IC50 values were calculated with Softmax Pro (Molecular Devices, Sunnyvale CA).

Crystallography.
Cloning, expression, and purification.

A fragment of human JAK2 (residues T842-G1132; GenBank reference NP_004963), encompassing the kinase domain, was generated by PCR amplification and inserted into the dual bacterial/baculoviral expression vector pBEV1 [1], incorporating an N-terminal hexahistidine purification tag and thrombin cleavage site. Thrombin cleavage of the purified protein yields the Jak2 fragment with an N-terminal extension composed of the amino acid sequence GSHNM-.

Sf9 insect cells were infected with recombinant baculovirus expressing JAK2 T842-G1132. Frozen Sf9 insect cell paste was thawed in 5 volumes of lysis buffer (50 mM Hepes, pH 8.0, 0.5 M NaCl, 20% (v/v) glycerol, 0.2% (v/v) Tween 20, 5 mM imidizole, 0.05% (v/v) mM β-mercaptoethanol) containing protease inhibitors (5 μg/ml leupeptin, 10 μM DFP, 3 mM benzamidine, and 25 μL/L Benzonase) and were mechanically lysed by passing through a 110Y microfluidizer (Microfluidics, Corp., Newton, MA). The lysate was centrifuged for 1h at 54,000 x g and the resulting supernatant was incubated with Talon resin (Invitrogen, Corp., Carlsbad, CA) overnight at 4°C with gentle rocking. After extensive washing with lysis buffer, protein was eluted with lysis buffer containing 100 mM imidizole, readjusted to pH 8. The elution pool was sized on Superdex S200 (50 mM Hepes, pH 8.0, 500 mM NaCl, 20 % (v/v) glycerol, 5 mM DTT, 0.05 % (w/v) beta-octylglucopyranoside) and the JAK2 containing fractions were identified by SDS-PAGE analysis. The pooled protein was adjusted to a final concentration of 20 mM MgCl2 and loaded onto a γ-phenyl ATP-Sepharose column [2] pre-equilibrated with ATP Buffer (50 mM Hepes pH 8.0, 20 % (v/v) glycerol, 0.5 M NaCl, 20 mM $MgCl_2$, 0.05 % β-octylglucopyranoside and 5 mM DTT). After washing with 10 column volumes of ATP Buffer, JAK2 was eluted from the column with 10 mM ADP in ATP Buffer and the fractions containing JAK2 were pooled based on SDS-PAGE analysis. The his-tag was cleaved by treatment with 10 units/mg thrombin (Calbiochem, La Jolla, CA) for 20h at 4 °C and the thrombin removed with benzamidine sepharose (GE Healthcare, Piscataway, NJ). The elution pool was treated with thrombin to cleave the his-tag followed by benzamidine-sepharose to remove the thrombin. A final polishing step was performed on Superdex 75 to remove aggregated protein (50 mM Hepes, pH 8.0, 0.5 M NaCl, 5 % (v/v) glycerol, 5mM DTT, 0.05 % (w/v) beta-octylglucopyranoside). The final protein solution was adjusted to 0.5 mM ADP and 1 mM MgCl2.

Crystal structure determination.

The kinase domain of JAK2 was expressed and purified as described above and concentrated to 10 mg/mL using a Vivaspin 10 kDa MWCO membrane (Sartorius Stedim Biotech, Goettingen, Germany). Well diffracting crystals of compound **9** bound to JAK2 were grown by hanging-drop vapor diffusion in 24-well plates using repeated seeding. The reservoir contained 0.5 mL of 1.7 to 2.1 M DL-malic acid, pH 7.0 and 2 mM dithiothreitol. The crystallization drop contained 1 μL protein containing 1 mM of compound 9 dissolved in DMSO + 0.5 μL resevoir solution + 0.5 μL water. Drops were then immediately seeded. The crystals grew in space group $C2_12_12_1$ with unit cell dimensions a = 95.1 Å, b = 101.2 Å, c = 68.5 Å. For data collection the crystal were transferred to a drop containing crystallization buffer that was 25% glycerol, and then flash frozen in liquid nitrogen. Data were collected at the Advanced Light Source,

Beamline 5.02, using an ADSC Quantum q315 detector at 100 K. Images were processed with d*TREK[3]. The structure was solved by difference Fourier methods using a previously determined structure, and refinement and model building were performed with REFMAC5[4] and COOT[5], respectively. Data collection and refinement statistics appear in Table 1.

Table 1. Data collection and refinement statistics JAK2 + Compound **9**

Data Collection	
Resolution (Å)	47.6-2.10 (2.18-2.10)[a]
Total reflections	69462
Unique reflections	19678
Completeness (%)	94.7 (94.7)
Rmerge	0.061 (0.305)
I/σ(I)	12.2 (2.5)
Refinement	
Resolution (Å)	20-2.10
Reflections used	17112
R/Rfree[b]	0.195 / 0.271
Average B-value (Å2)	29.0
Number of protein atoms	2372
Number of ligand atoms	26
Number of solvent atoms	176
r.m.s.d. bonds (Å)	0.022
r.m.s.d. angles (º)	2.0

[a] Numbers in parentheses refer to the highest resolution shell

[b] $R = \Sigma||Fo| - |Fc||/ \Sigma Fo$, where R and R_{free} are calculated using the working and test reflection sets, respectively.

(1) Chambers S. P.; Austen D. A.; Fulghum J. R.; Kim W. M.; Protein Expression and Purification **2004**, *36*(1), 40-47.

(2) Haystead C. M.; Gregory P.; Sturgill T. W.; Haystead T. A.; Gamma-phosphate-linked ATP-sepharose for the affinity purification of protein kinases. Rapid purification to homogeneity of skeletal muscle mitogen-activated protein kinase kinase. *Eur J Biochem* **1993**, *214*, 459-467.

(3) Pflugrath, J.W. The finer things in X-ray diffraction data collection. *Acta Cryst.* **1999**, *D55*, 1718-1725.

(4) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J.; Refinement of Macromolecular Structures by the Maximum-Likelihood Method. *Acta Cryst.* **1997**, *D53*, 240-255.

(5) Emsley, P.; Cowtan, K.; Coot: model-building tools for molecular graphics. *Acta Crystallographica Section D-Biological Crystallography* **2004**, *60* (12), 2126-2132.

Colony Forming Assays.

1.1 Specimens

Bone marrow was collected from three patients with polycythemia vera (PV) presenting for routine clinical evaluation. All samples were collected with patient or donor informed consent and in accordance with an approved local ethics review board at the clinical sites. Samples were assigned a unique identifier for the protection of patient privacy. Each patient was previously treated by phlebotomy prior to sample collection.

1.2 Cell Preparation and Freezing

Bone marrow aspirates (2-3 mL) were collected in sterile 5 mL EDTA Vacutainer tubes at the site and shipped on ice packs to Mosaic Laboratories. Specimens were processed within 48 hours of collection by density gradient separation using Ficoll-Paque™ Plus at 400 *g* for 30 minutes at room temperature without break. Bone marrow mononuclear cells (MNCs) were removed from the interface between density solution and plasma and washed with Phosphate Buffered Saline (PBS) followed by Iscove's Modified Dulbecco's Medium (IMDM) + 2% Fetal Bovine Serum (FBS). Cells were counted and assessed for viability using 0.4% Trypan Blue and a hemocytometer.

After the second wash in IMDM + 2% FBS, cells were resuspended at 5 x 10^6 cells/mL in freezing medium containing 70% IMDM, 20% FBS, and 10% DMSO. Cryovials were placed in a Nalgene temperature regulator containing 100% isopropyl alcohol and stored in an ultra-low freezer (–65 - –85 °C) for at least 4 hours after which the vials were transferred to a liquid nitrogen freezer ($\leq$–150 °C) for long-term storage.

1.3 Colony Forming Assays

Cryopreserved MNCs were thawed rapidly in a 37°C water bath and slowly resuspended 10-fold in warm IMDM + 2% FBS. Cells were centrifuged at 100 *g* for 10 minutes, washed in IMDM + 2% FBS, and resuspended in IMDM + 2% FBS. Specimen ML0605005 required the addition of 500 U DNase I (2000 U/mL) due to clumping.

Four hundred (400) µL of diluted cells were added to each tube of medium containing 4 mL of MethoCult and additional components. Each tube was vortexed and bubbles were allowed to dissipate before 1.1 mL of cell mixture was dispensed for each of three 35 mm dishes. A new sterile 5 mL luer-lock syringe and 16-gauge blunt end needle was used for each condition. The cell mixture was evenly distributed across the surface of each 35 mm dish.

To maintain humidity, one dish containing 3-4 mL sterile water was placed next to two dishes containing cell mixture and the three dishes were placed in a 100 mm Petri dish. This was repeated until all dishes were accounted for.

For optimization of the assay, final seed density was 1 x 10^4 or 2 x 10^4 MNCs per 35 mm culture dish. For further testing, final seed density was 1 x 10^4 per 35 mm culture dish. Plates were incubated at 37 °C, 5% CO_2, and full humidity for 14 days.

1.4 Scoring of Erythroid Colonies

On day 14, all erythroid colonies were scored by *in situ* observation with an inverted microscope on the basis of their red hemoglobinized color and characteristic colony

morphology [1]. Each 35 mm Petri dish, centered in a 60 mm gridded tissue culture dish in order to provide columns for ease of tracking, was scanned vertically at 40X. CFU-E colonies and BFU-E colonies were enumerated for each condition.

1.5 Data Analysis

Dose-response curves were generated in Microsoft Excel. The percent inhibition of each concentration was calculated for each compound tested at each concentration. IC_{50} values were calculated using a linear spline model in XLfit (IDBS, Alameda, CA).

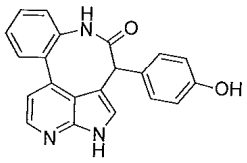
(1) Eaves C, Lambie K. Atlas of human hematopoietic colonies: StemCell Technologies, Inc.; 1995.

Re-sequencing of JAK2 exon 14:

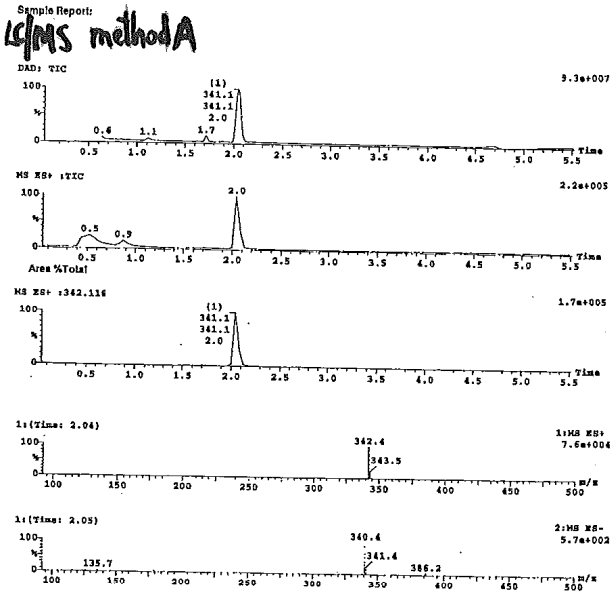
Materials: Frozen samples of bone marrow aspirate from patients diagnosed with polycythemia vera were obtained from Mosaic Labs, Lake Forest, CA.

Methods: Genomic DNA was extracted from each bone marrow aspirate with the QIAamp DNA mini kit (Qiagen, Valencia, CA). A 417 nucleotide PCR product encompassing exon 14 of JAK2 (NCBI Reference Sequence: NG_009904.1) was amplified from 100 ng of each genomic DNA sample using standard PCR protocols and the following primers: 5'-TGCTCTCTCTCACTTTGATCTCCATATTCC-3' and 5'-AGTTTACACTGACACCTAGCTGTGATCCTG-3'. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA), and both strands were sequenced

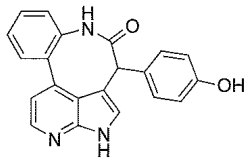
LC-MS trace (method A) of the major atropisomer (**9A**):



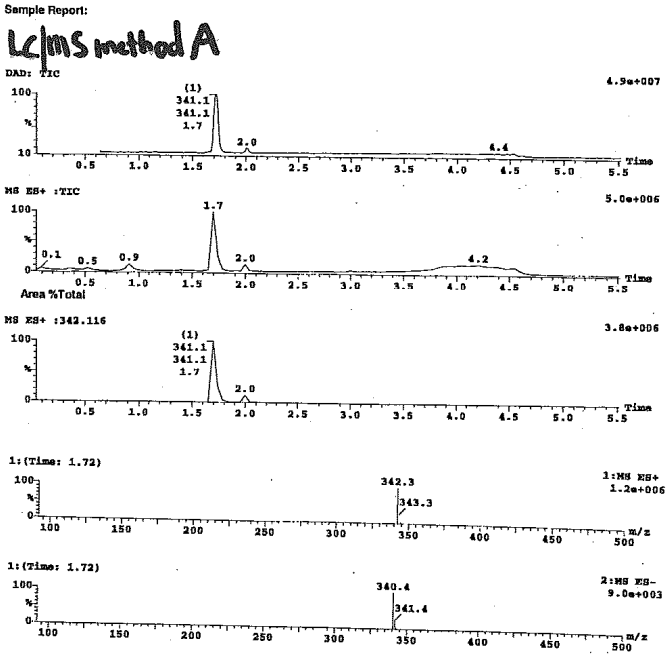
compound **9 A**



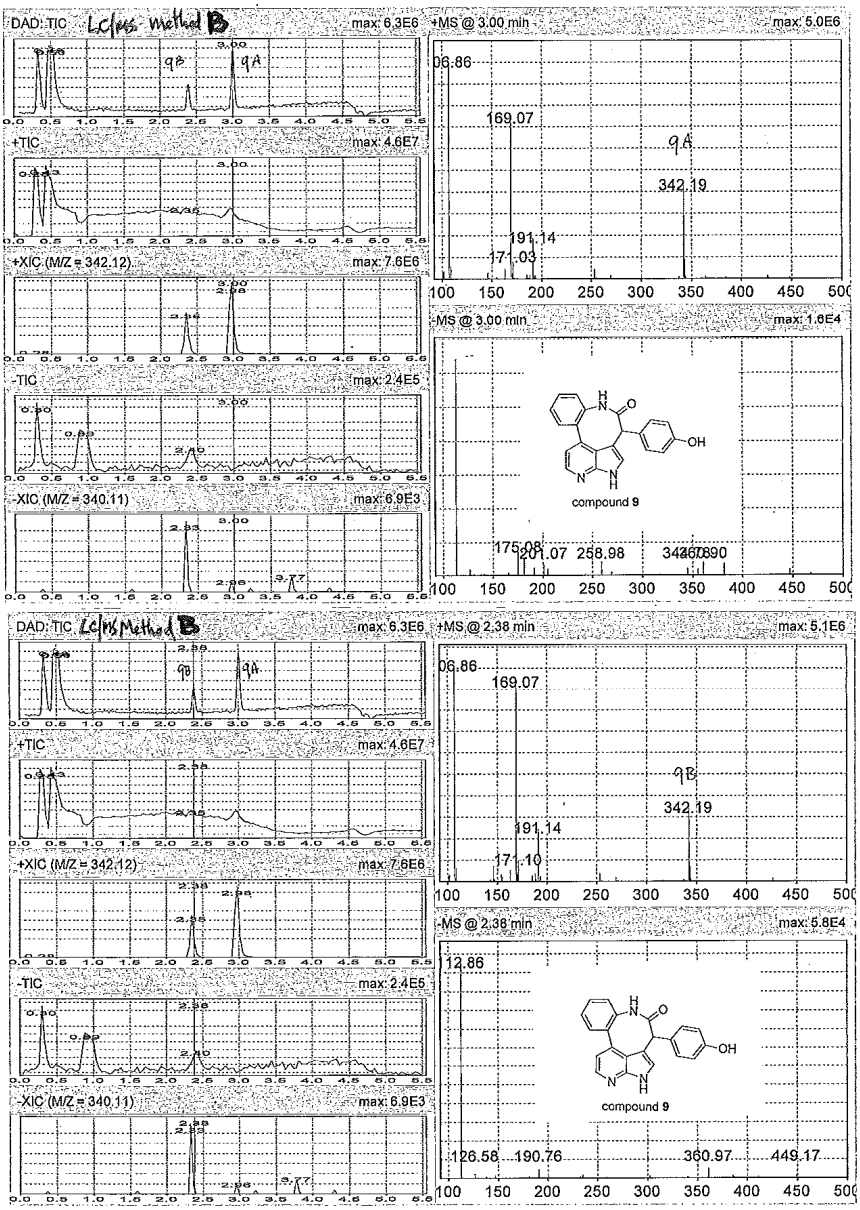
LC-MS trace (method A) of the minor atropisomer (**9B**):



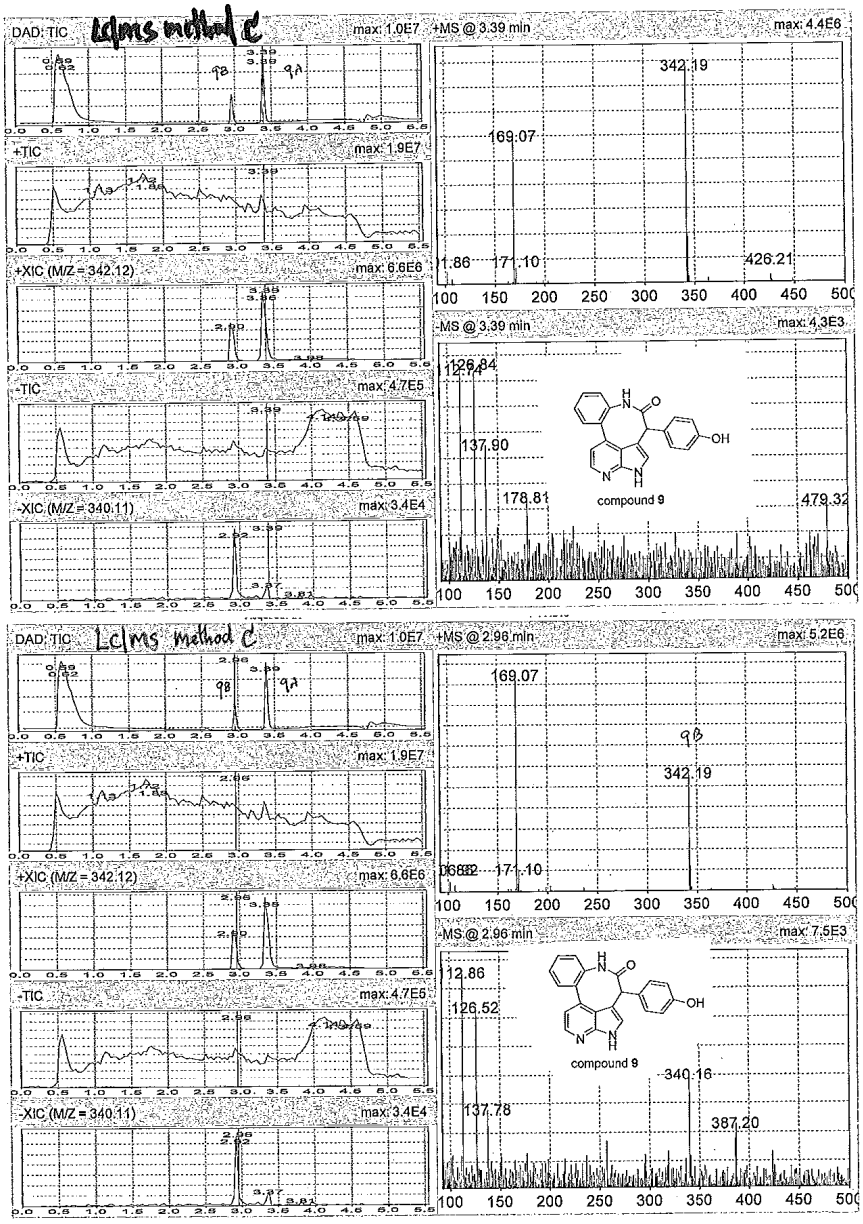
compound **9 B**



LC-MS trace (method B) of the equilibrium mixture of atropisomers:



LC-MS trace (method C) of the equilibrium mixture of atropisomers:



Analytical HPLC trace & HRMS ($M + H^+$) of the equilibrium mixture of atropisomers: