Supporting Information

Towards the Development of a Potent and Selective Organoruthenium Mammalian Sterile 20 Kinase Inhibitor

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A) Kinase Assays

PI3K Kinase-Glo assay: Recombinantly expressed human PI3Kγ catalytic domain was preincubated with various concentration of inhibitors with a final DMSO concentration of 2% in reaction buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) for 1 hour at room temperature before this mixture was added to a solution of 0.1 mg/mL D-myo-phosphatidylinositol 4,5-biphosphate (PtdIns (4,5)P_2, Echelon Biosciences) and 10 μM ATP. The kinase reaction was carried out in a 50 μL final volume in a 96-well microliter plate at 37°C for 3 hours before 50 μL Kinase-Glo® (Promega) developing solution was added into the mixture to generate a luminescence signal. The signal was recorded using the Perkin Elmer Wallac 1420 luminometer using a luminescence filter. Data were processed and IC_{50} values derived using 2% DMSO and no kinase measurements as controls and a sigmoidal dose response curve fitting by GraphPad Prism.

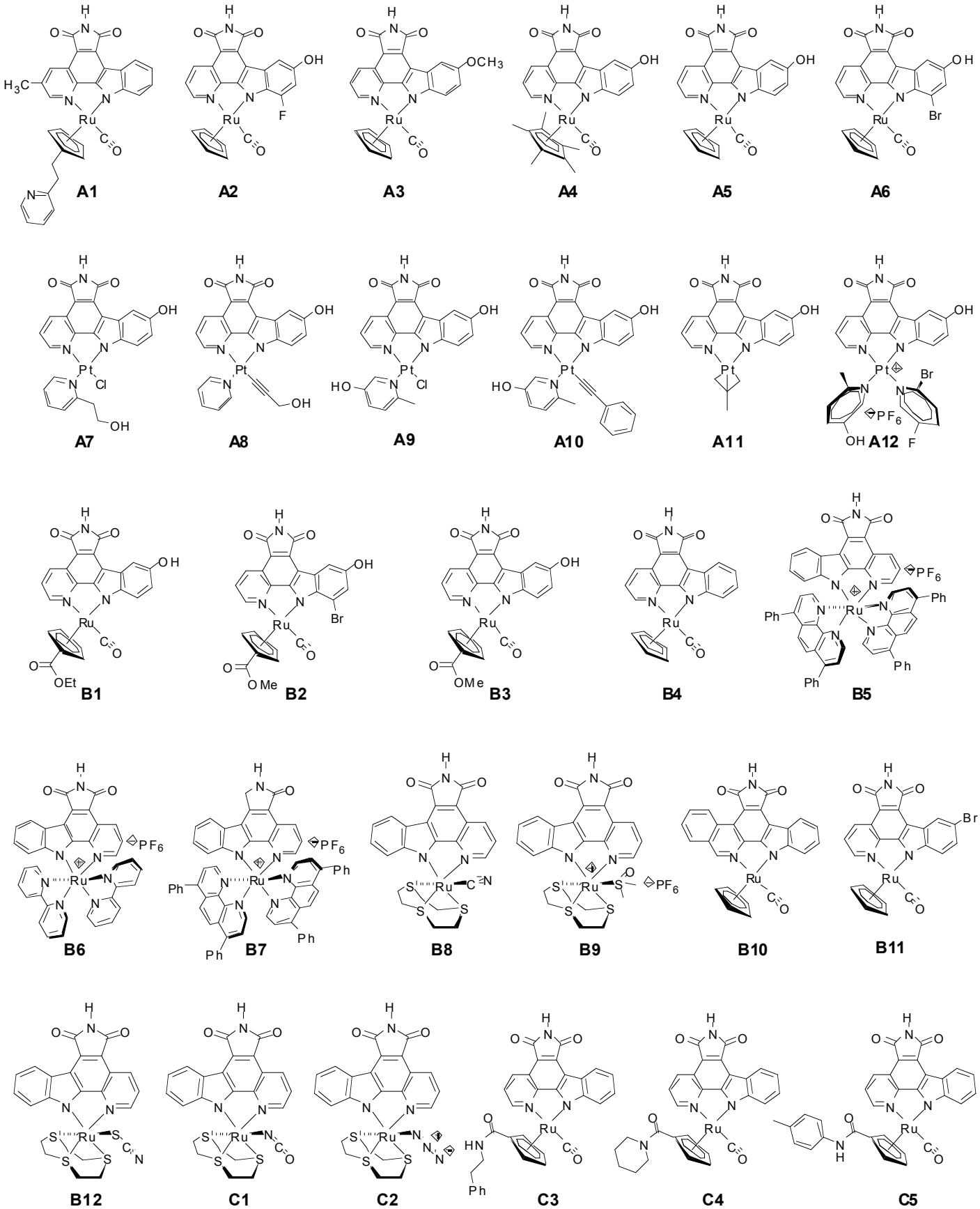
BRAF kinase assay: Recombinantly expressed GST-MEK diluted in TTBS buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% TWEEN-20) to 50 μg/mL in a volume of 100 μL was bound to the wells of a 96-well glutathione coated plate (Pierce Biotechnology). 1 μL of compound with 2x serial dilutions in a 100% DMSO stock solution were added to a mixture of 50 μL of a buffer containing 50 mM HEPES pH 7.0 with 0.7 pmoles of BRAF kinase. This mixture was incubated at room temperature for 1 hour before it was added into the GST-MEK bound wells of the 96-well plate. An additional 50 μL of phosphorylation buffer (50 mM HEPES pH 7.0, 200 mM NaCl, 10 mM $MgCl_2$, 200 μM ATP) was added into the well mixture to start the kinase reaction at 37°C for 30 min with intermittent shaking. The kinase reaction was stopped by extensive washing with TTBS buffer and a 1:5000 dilution of anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226) monoclonal antibody (Millipore) in TTBS was subsequently added into the wells and incubated for 1 hour with shaking. Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (BioRad Laboratories) in a

1:5000 dilution was added into the wells to incubate at room temperature with shaking. Finally, the SuperSignal ELISA Pico chemiluminescent substrate (Pierce Biotechnology) was added into the wells. The luminescence signal was recorded with a luminescence filter using a Wallac 1420 luminometer (PerkinElmer). These data were processed and IC_{50} values derived from fitting into a sigmoidal dose response model using GraphPad Prism.

PIM-1 kinase assay: Kinase assays were performed using labeled γ-P^{32} ATP and the incorporation of labeled phosphate onto substrate (S6 kinase/Rsk2 peptide) was monitored. Various concentrations of inhibitor were incubated at room temperature in 20 mM MOPS, 30 mM $MgCl_2$, 0.8 μg/μl BSA, 5% DMSO (resulting from the inhibitor stock solution), pH 7.0, in the presence of substrate (S6 kinase/Rsk2 Substrate Peptide 2: 50 μM) and PIM-1 kinase (0.16 nM). After 15 min, the reaction was initiated by adding ATP to a final concentration of 100 μM, including approximately 0.2 μCi/μl γ-P^{32} ATP. Reactions were performed in a total volume of 25 μL. After 30 minutes, the reaction was terminated by spotting 17.5 μL on a circular P81 phosphocellulose paper (diameter 2.1 cm, Whatman) followed by washing four times (five minutes each wash) with 0.75% phosphoric acid and once with acetone. The dried P81 papers were transferred to a scintillation vial and 5 ml of scintillation cocktail was added and the counts per minute (CPM) determined with a Packard 1500 Tri-Carb Liquid Scintillation Analyzer. IC_{50} values were defined to be the concentration of inhibitor at which the CPM was 50% of the control sample, corrected by the background.

GSK-3β kinase assay: Following the procedure as described for the PIM-1 kinase assay, but in the presence of substrate peptide phospho-glycogen synthase peptide-2 (20 μM), and GSK3β (0.2 nM for IC_{50} determinations).

B) Structures of the 58-Member Organometallic Library



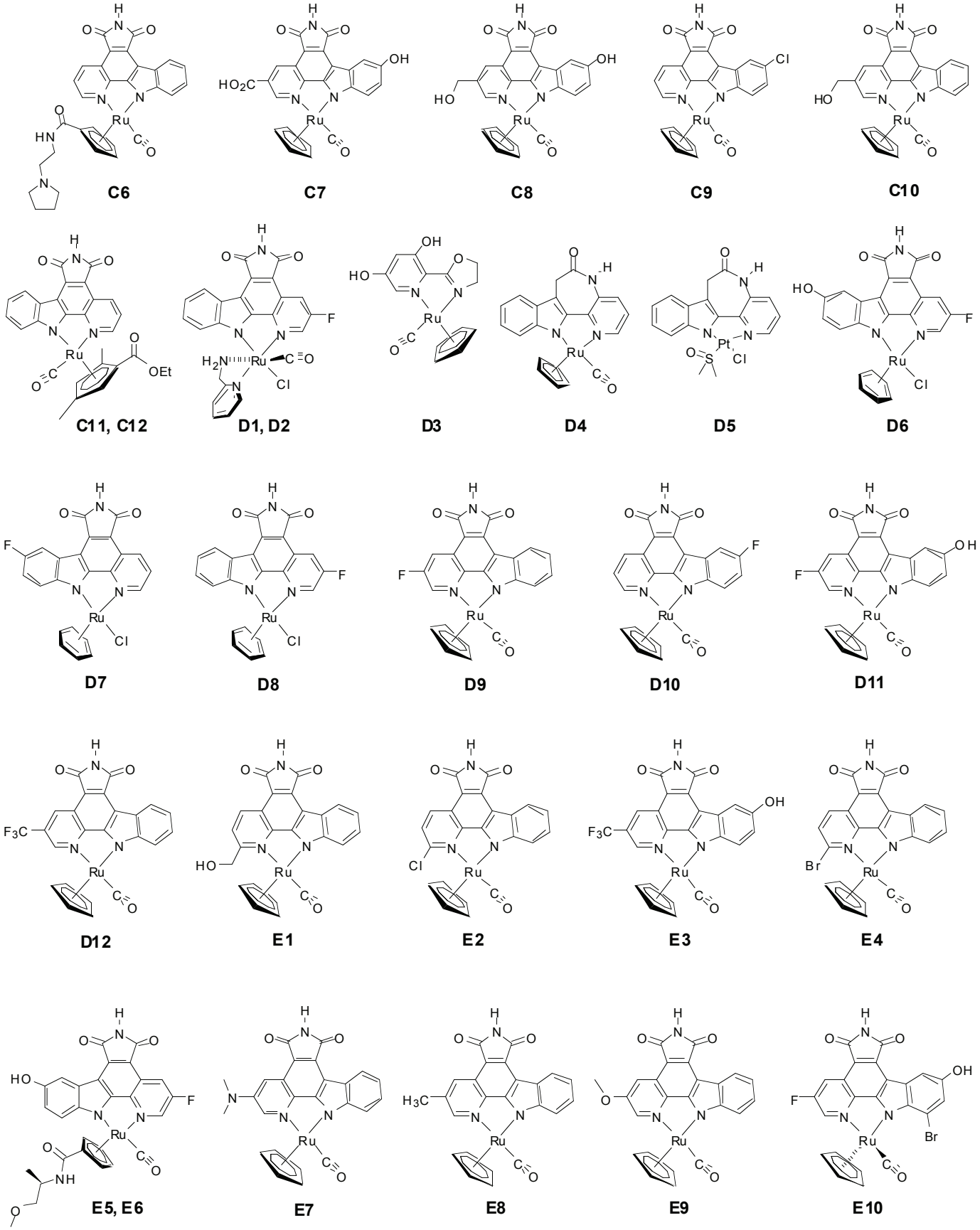


Figure S1. Structures of the 58-member organometallic library (see Figure 1A).

C) Structures of Primary Amines Used in the 44-Member Amide Library

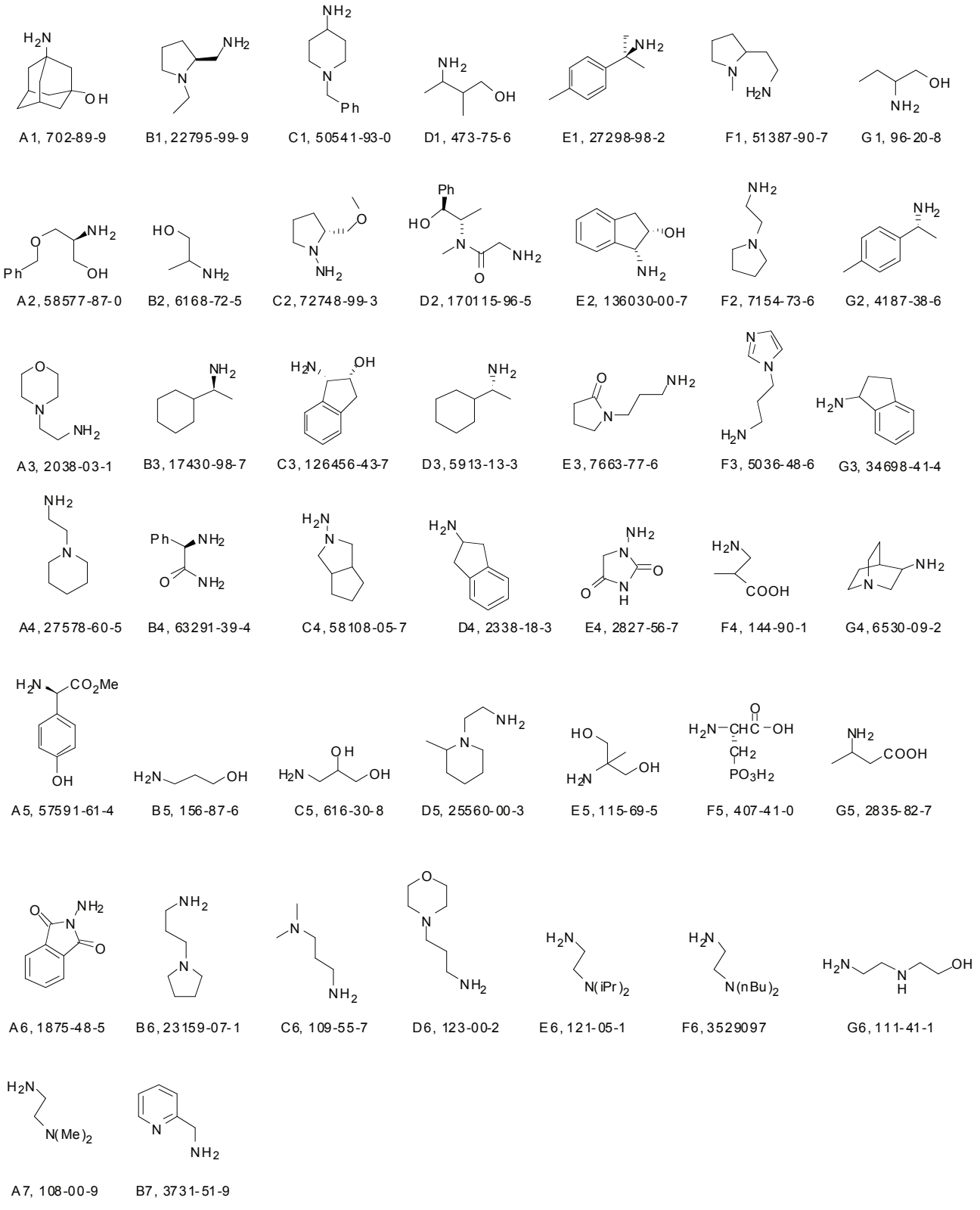


Figure S2. Structures of primary amines used in the 44-member amide library. Compare with histogram in Figure 3A.

D) Assignment of the Absolute Configurations of 9E1 and 9E2

The CD-spectra of the pure enantiomers **9E1** and **9E2** were compared with reference half-sandwich compounds. See for example:

1.) J. É. Debreczeni, A. N. Bullock, G. E. Atilla, D. S. Williams, H. Bregman, S. Knapp, E. Meggers, *Angewandte Chemie Int. Ed.* **2006**, *45*, 1580-1585.

2.) G. E. Atilla, D. S. Williams, H. Bregman, N. Pagano, E. Meggers, *ChemBioChem* **2006**, 1443-1450.

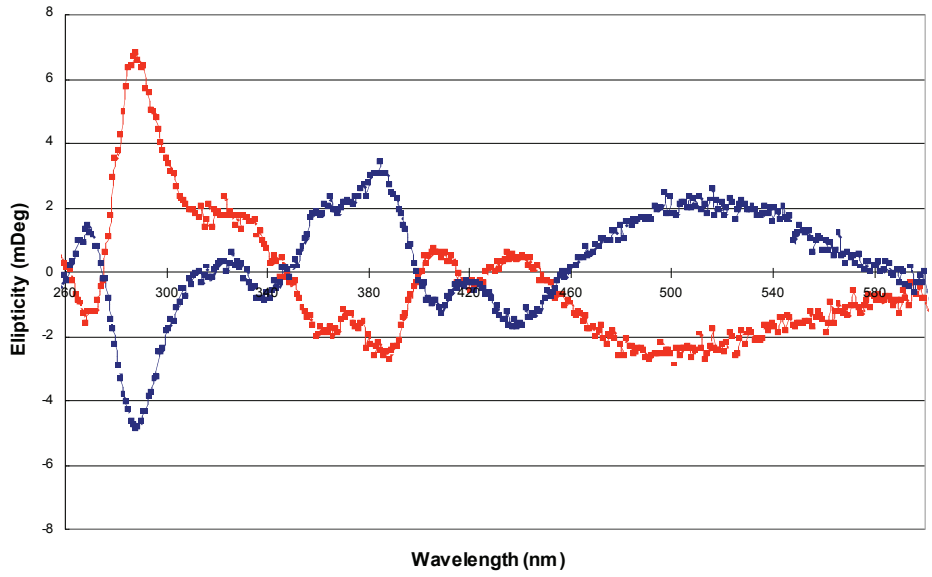


Figure S3. Assignment of absolute configuration of compound **9E1** by CD-spectroscopy.

E) Inhibitor Profile of 9E1 and Staurosporine Against 50 Kinases

	9E1 @ 10 nM	Staurosporine @ 10 nM
Bmx(h)	108	53
CaMKI(h)	93	33
CDK1/cyclinB(h)	133	34
CDK2/cyclinA(h)	134	32
CDK2/cyclinE(h)	103	30
CDK3/cyclinE(h)	100	40
CHK1(h)	106	8
CHK2(h)	94	58
cKit(h)	105	97
cSRC(h)	99	51
DAPK1(h)	108	68
EphA3(h)	102	80
EphA4(h)	97	95
EphA5(h)	112	84
EphB1(h)	85	93
EphB4(h)	105	104
ErbB4(h)	107	106
Fes(h)	101	16
FGFR4(h)	110	97
Fgr(h)	99	11
Flt1(h)	88	18
Flt4(h)	88	18
Fyn(h)	102	19
GRK5(h)	101	89
GSK3β(h)	44	57
HIPK2(h)	88	99
IRAK1(h)	101	91
Lyn(h)	109	13
MAPK1(h)	105	109
MAPK2(h)	109	104
MAPKAP-K3(h)	92	112
MELK(h)	81	10
MSK1(h)	84	22
MST1(h)	**81**	**2**
MST2(h)	95	38
PAK2(h)	96	46
PAK4(h)	103	66
Pim-1(h)	4	55
PKA(h)	88	10
PKBα(h)	105	59
PKBβ(h)	118	69
PKCα(h)	99	36
ROCK-I(h)	95	57
Ros(h)	105	71
SAPK2a(h)	107	105
SGK(h)	88	70
Snk(h)	101	86
Syk(h)	81	5
TAK1(h)	105	98
TrkA(h)	87	8
Yes(h)	96	13

Figure S4. Inhibitor profile of **9E1** and staurosporine against 50 kinases. Summary of percent of remaining activity using 10 nM of the respective inhibitor. These studies were carried out using the Millipore kinase profiler service. The MST1 substrate used for the Millipore studies was an Axltide peptide (KKSRGDYMTMQIG) derived from the mouse insulin receptor.

F) Co-crystal structure of PIM-1 with 14

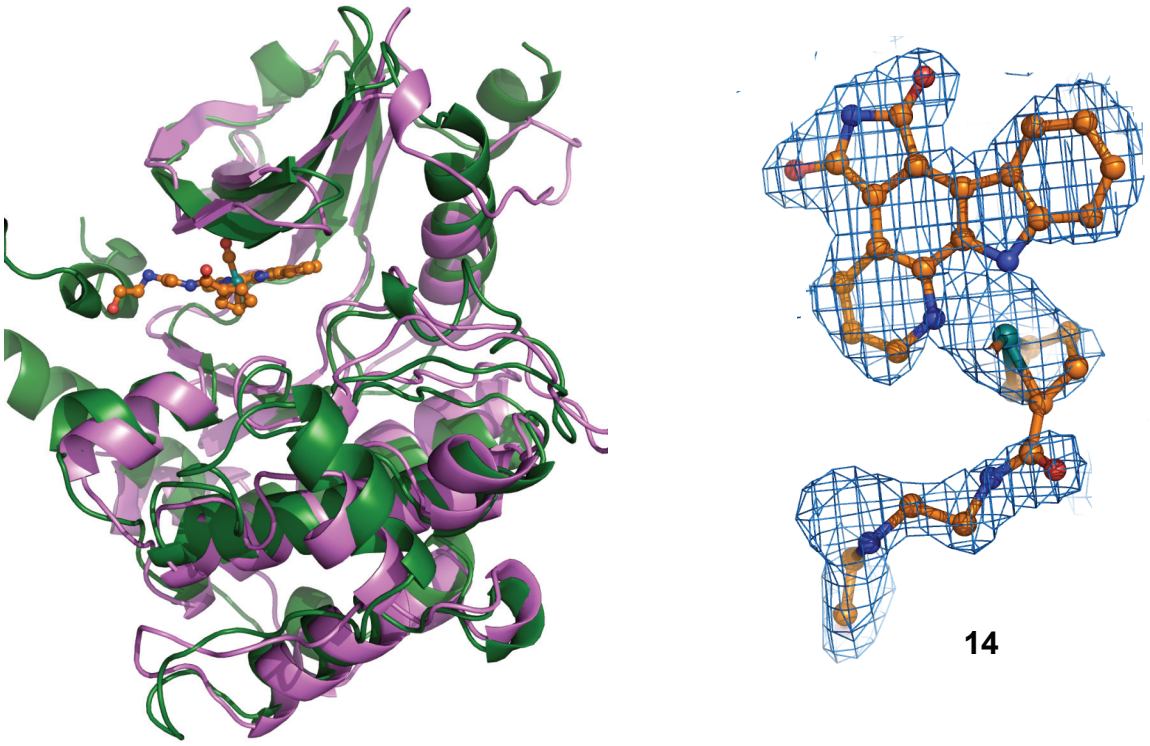


Figure S5. Co-crystal structure of PIM-1 with **14**. Left: Superposition of the modeled structure of MST1 (green) onto the crystal structure of Pim-1 (violet) bound to compound **14** shown in ball and stick**.** Right: Electron density of the compound **14** bound to PIM-1.

G) Figure S6 – Superposition of Kinases Assayed in this Study onto the PIM-1/14 Co-Crystal Structure.

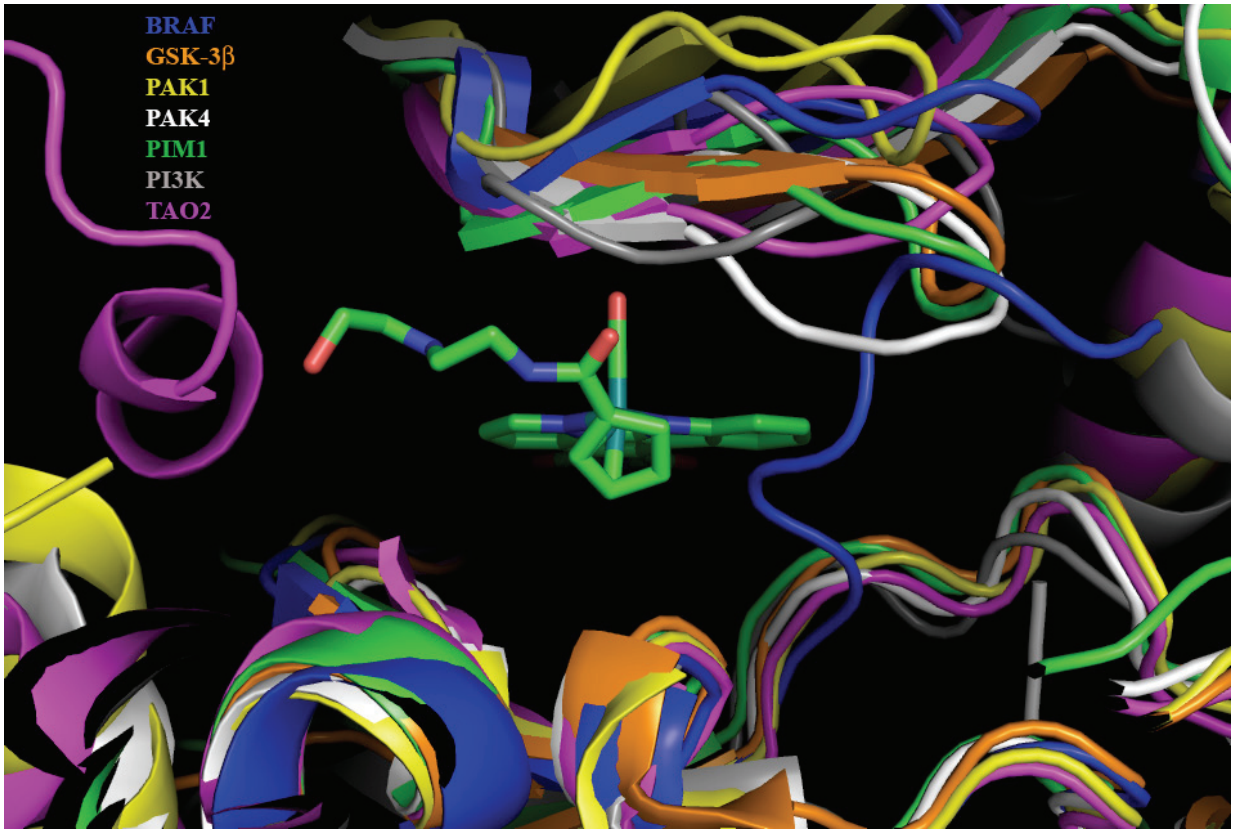


Figure S6. Superposition of Kinases Assayed in this Study onto the PIM-1/14 Co-Crystal Structure. Each of the superimposed kinases are shown in ribbon representation. The color-coding is as follows: BRAF, blue; GSK-3β, orange; PAK1, yellow; PAK4, white; PIM-1, green; PI3K, gray; TAO2, pink.

H) Table S1 - Crystallographic Data, Refinement and Statistics for the PIM-1/14 Co-Crystal Structure

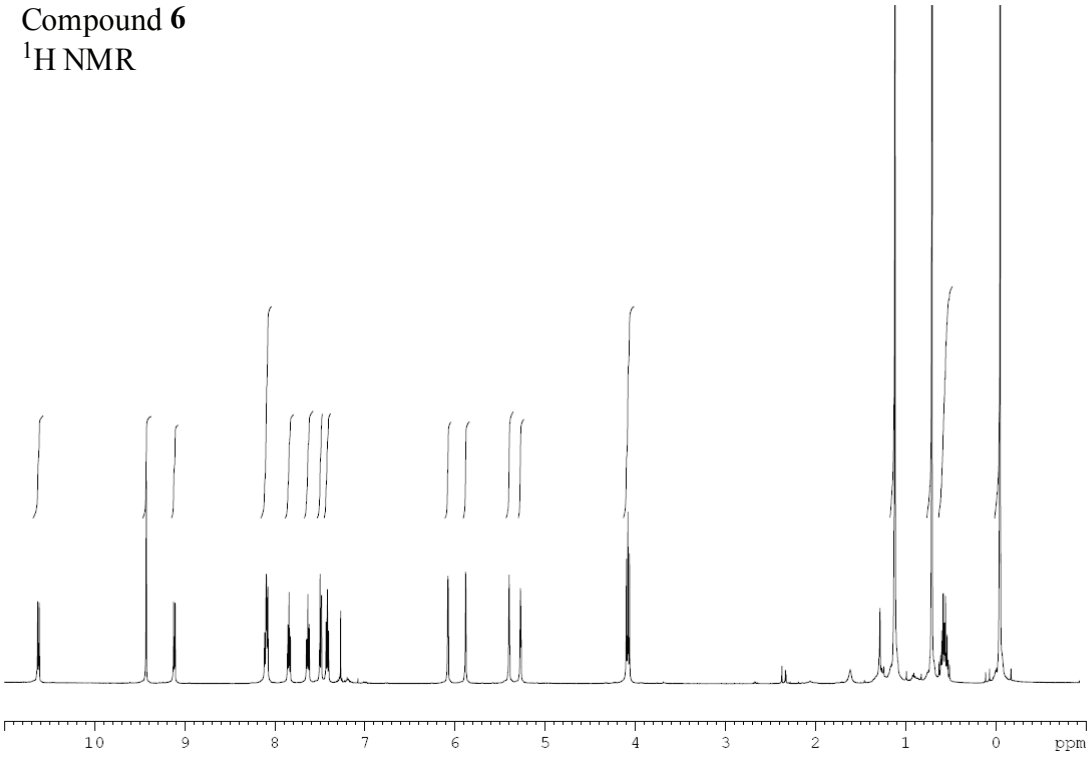
Crystallographic data and refinement statistics of compound **14** with PIM-1 [a]	
Parameters	
Space group	$P6_5$
Unit cell dimensions [Å]	a,b = 98.07, c = 80.57
Resolution [Å]	2.05
Total number of reflections (unique, redundancy)	213939 (27634, 7.7)
completeness (outer shell) [%]]	99.8 (100.0)
[a] R_{merge} (outer shell) [%]	8.7 (43.9)
I/σ (outer shell)	9.1 (2.7)
[b] R_{work} ([c] R_{free}) [%]	23.1 (26.7)
rmsd bond length [Å]	0.02
rmsd bond angle [°]	1.595
Ramachandran [%] (favoure / allowed/disallowed) d	92.4/ 7.6/ 0

[a] rmsd = root-mean-square deviation.
Values for the outer resolution shell are given in parentheses. [a] $R_{merge} = \Sigma\Sigma_i | I_I - \langle I \rangle | / \Sigma \langle I \rangle$, where <I> is the mean intensity of the N reflections intensities I_i and common indices *h,k,l* for the native and derivative crystals, respectively.

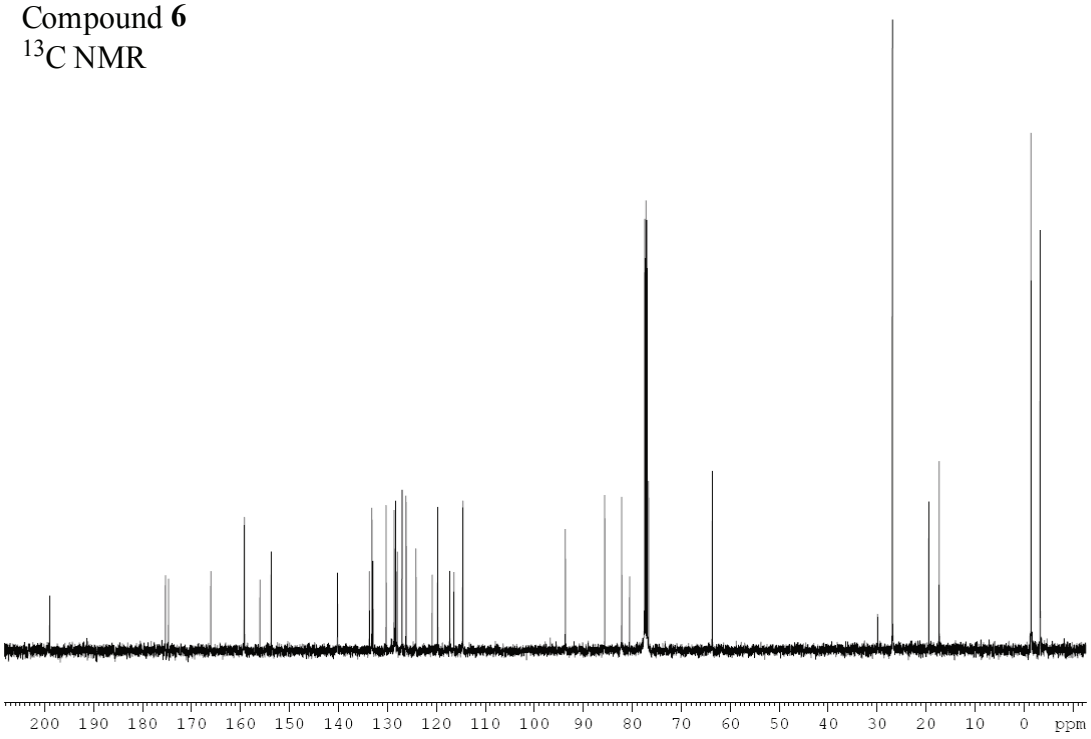
[b] R factor $\sum_{hkl} | | F_{obs} | - k | F_{cal} | | / \sum_{hkl} | F_{obs} |$, where F_{obs} and F_{cal} are observed and calculated structure factors, respectively. For [c] R_{free} the sum is extended over a subset of reflections (10%) that were excluded from all stages of refinement.

I) NMR Spectra

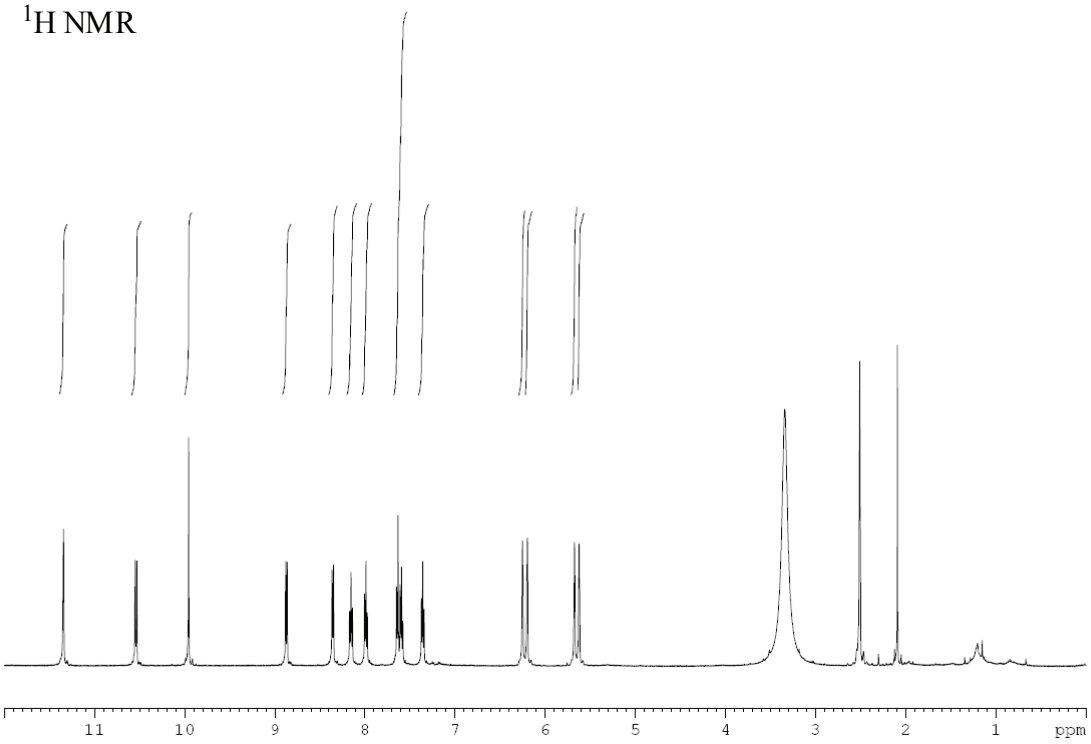
Compound **6**
^{1}H NMR



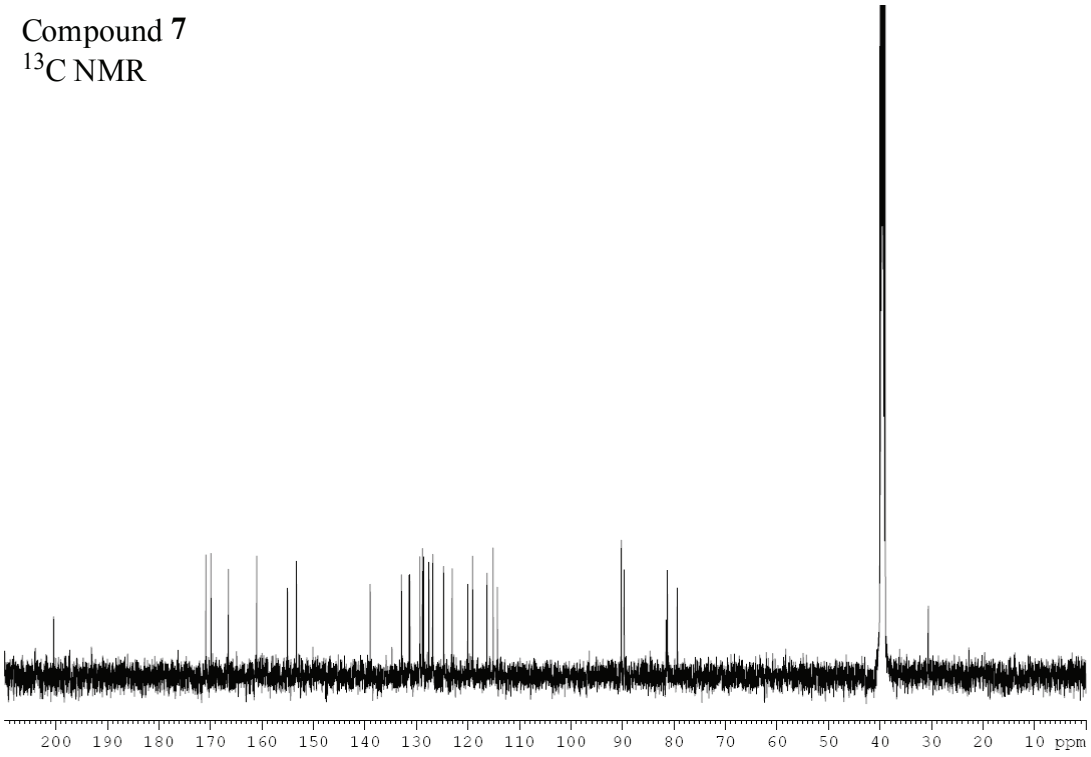
Compound **6**
^{13}C NMR



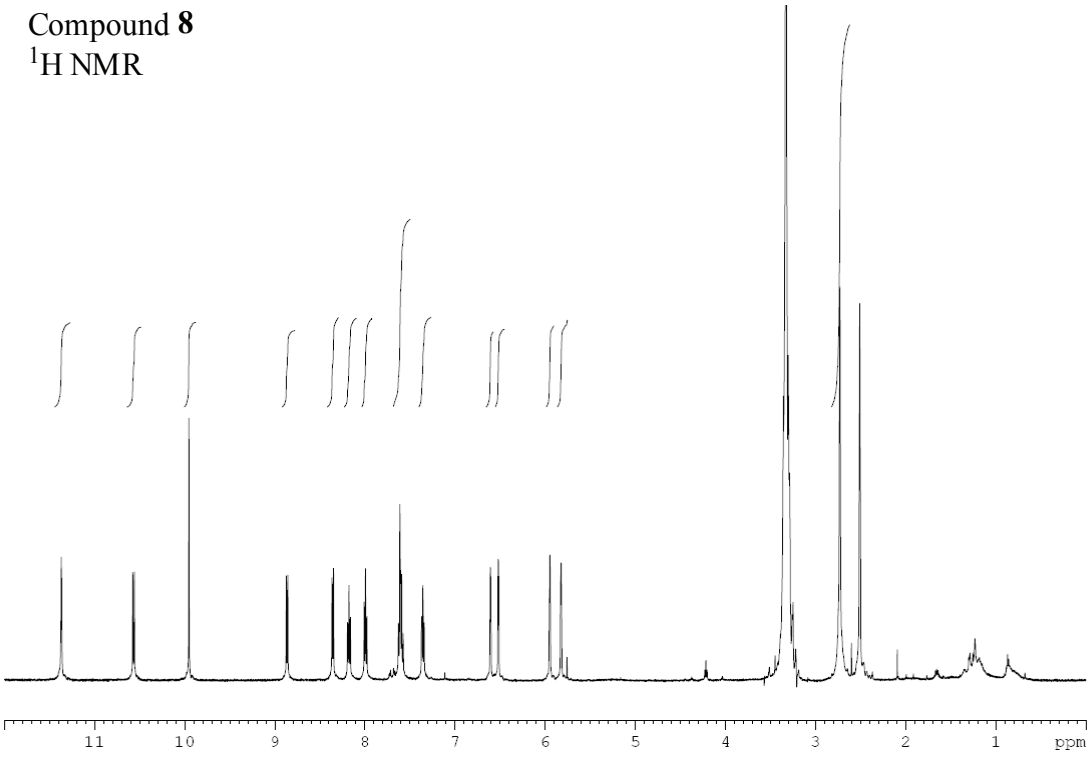
Compound **7**
^{1}H NMR



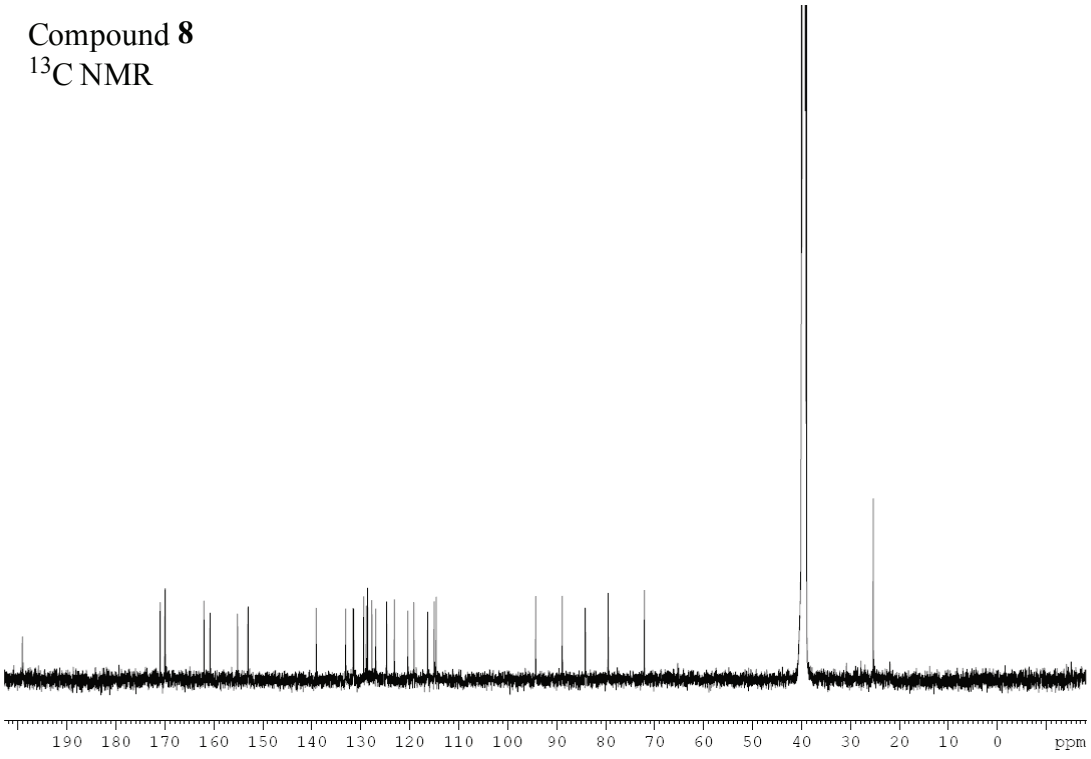
Compound **7**
^{13}C NMR



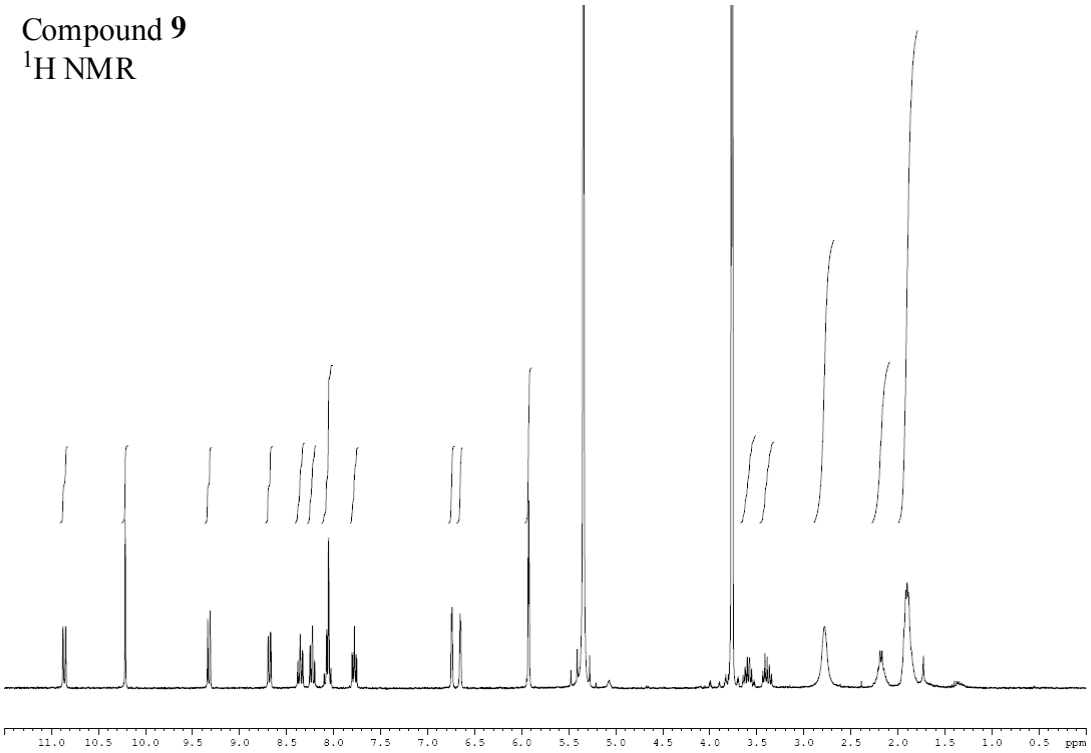
Compound **8**
^{1}H NMR



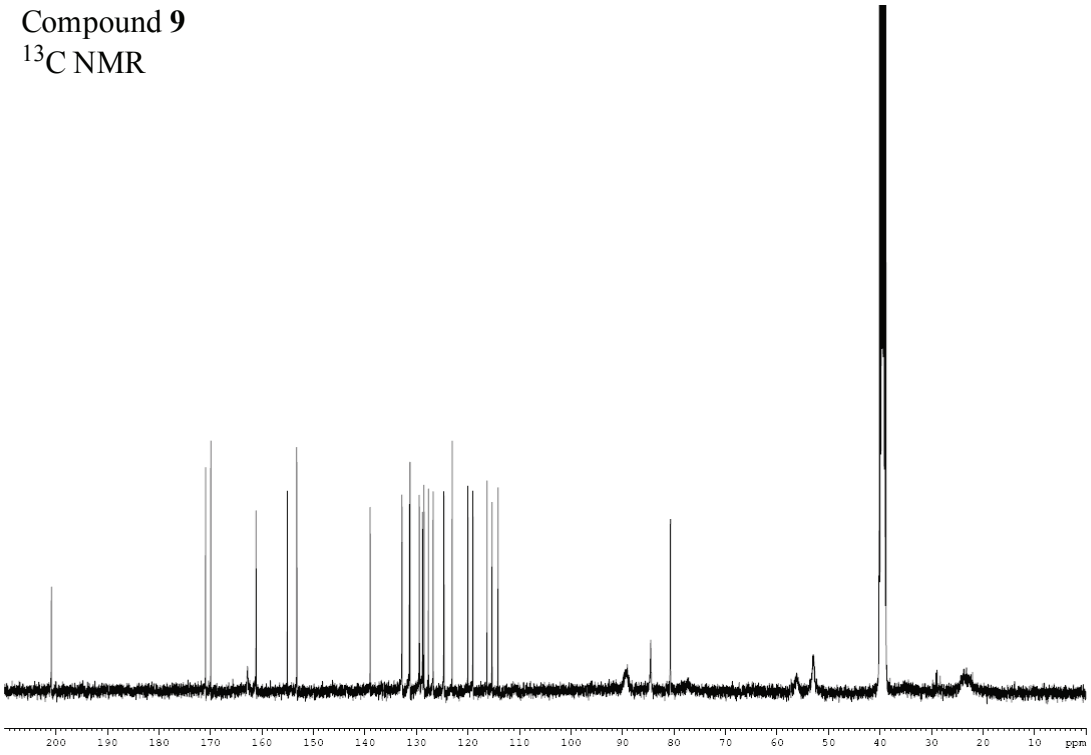
Compound **8**
^{13}C NMR



Compound **9**
^{1}H NMR



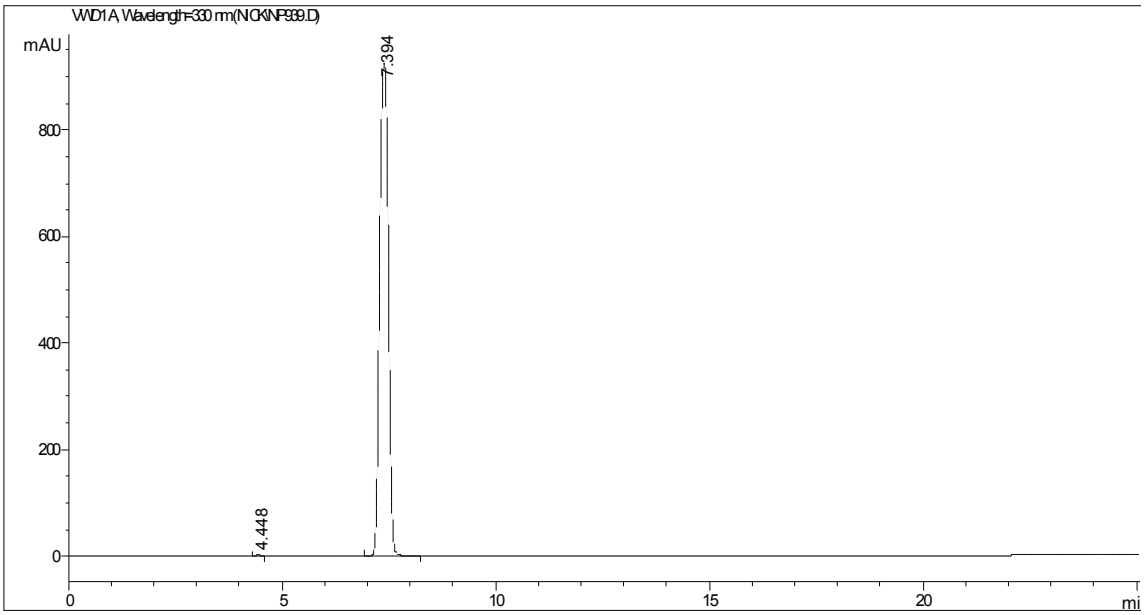
Compound **9**
^{13}C NMR



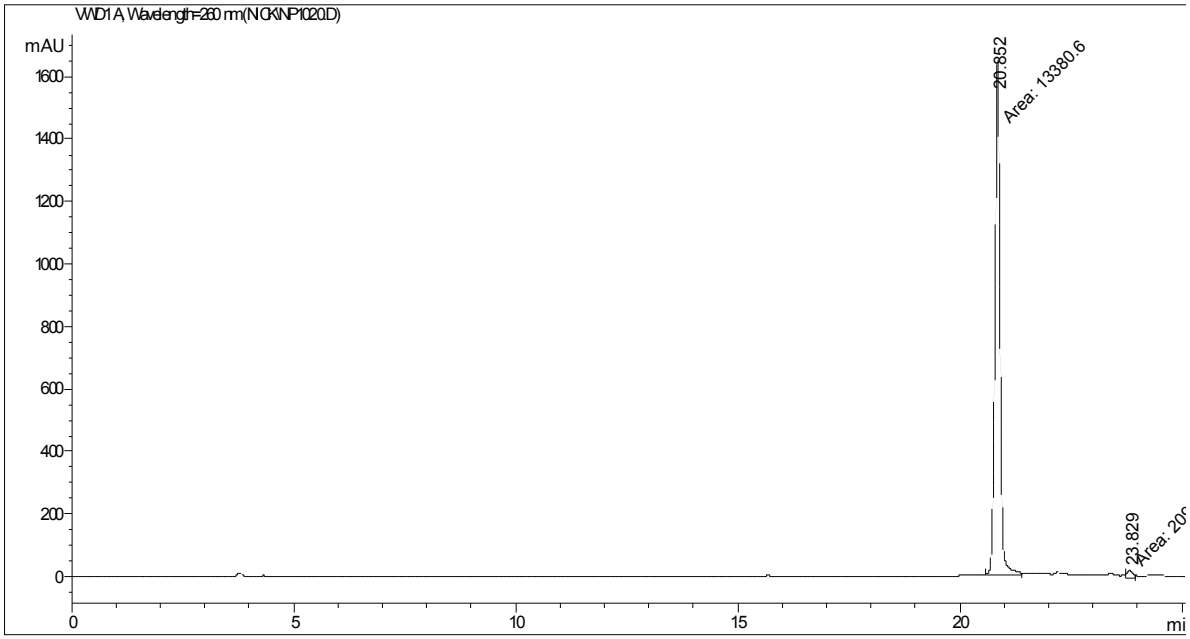
J) HPLC Chromatographs

Compounds **6** and **8** were eluted using a normal phase silica gel column (Merck Purospher STAR, 250-4.6 mm, Si (5µm)) whereas compounds **7** and **9** were eluted using a reverse phase C8 column (Merck Purospher STAR, 250-4.6, RP-8e (5µm)). Flow rate for all four experiments was 1 mL/min. Detection of compounds **6** and **8** was conducted at 330 nm and 260 nm for compounds **7** and **9**.

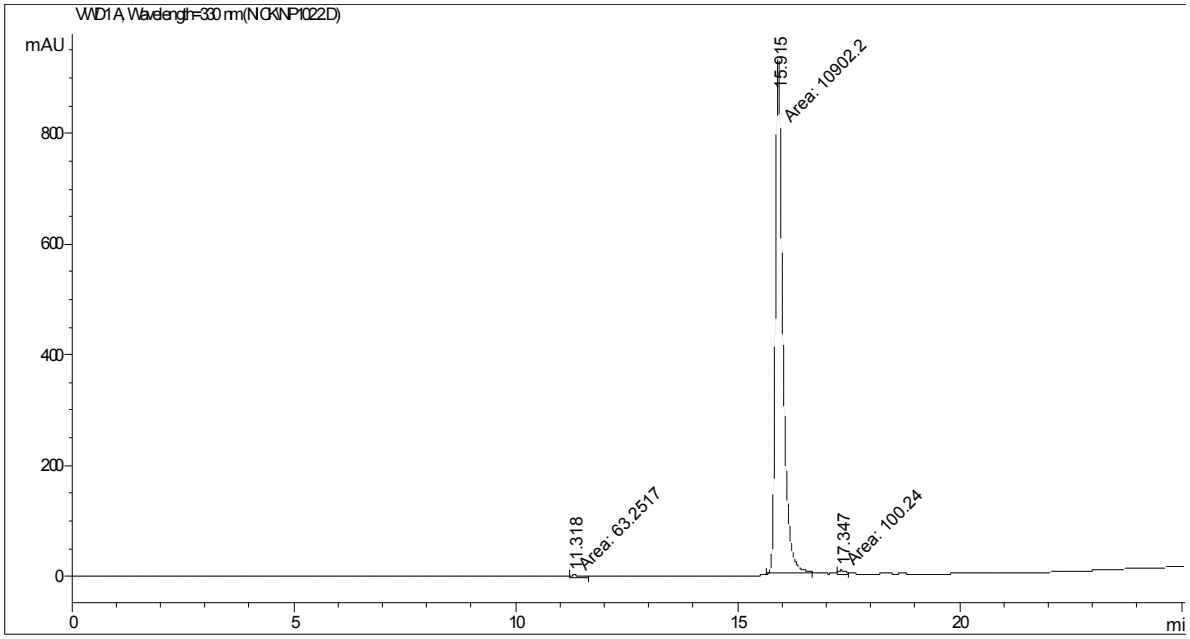
Compound **6**, Purity – **99%**
25% - 75% EtOAc in hexanes over 20 minutes



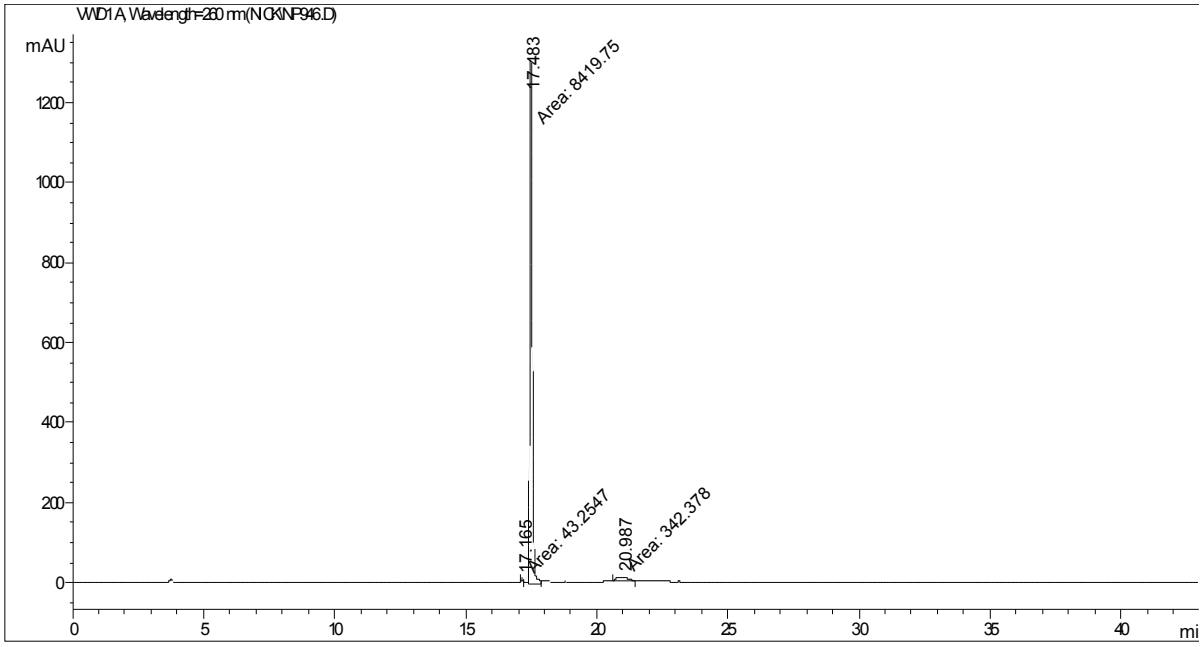
Compound **7**, Purity – **98%**
10% - 75% acetonitrile in water (0.1% TFA) over 20 minutes



Compound **8**, Purity – **99%**
25% - 95% EtOAc in hexanes over 20 minutes



Compound **9**, Purity – **96%**
10% - 75% acetonitrile in water (0.1% TFA) over 20 minutes



K) Enantiomer Separation of Compound 8

The racemic NHS-ester **8** was resolved to the individual enantiomers using an analytical chiral HPLC column (Chiral Pak 1B, Daicel/Chiral Technologies). Each injection was conducted isocratic using hexanes:EtOH (1:24) with a flow rate of 0.8 mL/min. Each enantiomer was then reacted separately with *N*-(2-aminoethyl)piperidine to provide **9E1** (from **8E1**) and **9E2** (from **8E2**).