Supporting Information

Rational Design and Optimization of a Novel Class of Macrocyclic ASK1 Inhibitors

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(i) Experimental conditions for the ASK1 in vitro biochemical and cellular assays

ASK1 Biochemical Assay

The protein kinase inhibitory activity of the compounds described herein were tested using the ASK1/MAP3K5 assay by Reaction Biology Corp. (Malvern, PA). The assay procedure follows (and is also available on the Reaction Biology Corp. website).

Base Reaction Buffer: 20 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/mL BSA, 0.1 mM Na_3VO_4, 2 mM DTT, 1% DMSO;

Substrate: 20 μM of myelin basic protein (MBP) and 100 μM ATP;

Protein kinase: ASK1/MAP3K5.

Reaction Procedure:

1. Prepare indicated substrate in freshly prepared Base Reaction Buffer.
2. Deliver any required cofactors to the substrate solution.
3. Deliver indicated kinase into the substrate solution and gently mix.
4. Deliver compounds in DMSO into the kinase reaction mixture by Acoustic; technology (Echo550; nanoliter range), incubate for 20 minutes at room temperature.
5. Deliver ^{33}P-ATP (specific activity 10 μCi/μL) into the reaction mixture to initiate the reaction.
6. Incubate kinase reaction for 2 hours at room temperature.
7. Reactions are spotted onto P81 ion exchange paper.
8. Detect kinase activity by filter-binding method.

ASK1 Autophosphorylation Assay. ASK1-T838 autophosphorylation was measured in HEK-293T cells using an MSD assay format. HEK 293T cells were seeded in 15cm plates at a density of 18 million cells in 20 mL DMEM with 10%FBS, Pen/Strep media. The plates were incubated at 37 ℃ overnight. The media on the plates was changed to OPTI-MEM and the cells were transfected with human ASK1-V5 tagged full length plasmid using Lipofectamine 2000 (Invitrogen), and incubated at 37 ℃ overnight. Twenty-four hours later, the cells were collected by Trypsin-EDTA (Invitrogen) and plated into 96 well tissue culture plates with 100,000 cells/well. The cells were then incubated for 4 h at 37 ℃ and then then the ASK1 compounds were added using a HP 300e. The compounds were tested at 20 μM with 3-fold, 10-point dilution points then incubated for 1 h at 37 ℃. The media from the cells was discarded and 120 μL of cold lysis buffer supplemented with protease and phosphatase inhibitor was added to the cells. The lysate was mixed using an Apricot liquid handler and 50 μL of cell lysates were transferred to a pre-coated MSD plates containing mouse anti-V5 antibody (Sigma) and washed 3x with MSD wash buffer (TBST) and blocked with a 3% BSA solution. The plates were then incubated on a plate shaker overnight at 4 ℃. The plates were then washed 3x with MSD wash buffer and 50 μL of rabbit anti-pASK1 T838 antibody (Cell Signaling) was added to the wells and incubated for 2 h at room temperature on a plate shaker. The plates were then washed and 50 μL of goat anti-rabbit sulfa-tag was added to the wells and incubated for 1 h at room temperature on a plate shaker. Plates were washed and read on a MSD reader S600. The data was analyzed using Graph Pad or Genedata, the data was normalized and plotted, % activity versus log of compound concentration. The IC_{50} values were obtained from a 4-parameter fit.

(ii) Experimental conditions for crystallization, collection and refinement statistics for compounds 4 and 11

ASK1 and compound **4** cocrystal structure generation. Crystallization was performed using commercially available human ASK1 construct encoding residues 667-939 with a C-terminal HIS_6 tag (Crelux) at 12 mg/mL with compound **4** added to 0.5 mM. Crystals grew in 0.1 Na Acetate pH 5.5, 2% PEG4K and 22% Polyacrylic Acid at 18 ℃ and X-ray diffraction data was collected at the Swiss Light Source facility (PSI, Xo6DA (PXIII)).

ASK1 and compound **11** cocrystal structure generation. Crystallization was performed using an E.coli expressed human ASK1 construct encoding residues 659-951 encoding a T838E mutation at 7 mg/mL with compound **11** added to 0.5mM. Crystals grew in 0.1M BisTRIS pH 5.5, 0.2M $MgCl_2$, 10mM Na cholate and 12% PEG3350 and X-ray diffraction data was collected at the Advanced Photon Source (LRL-CAT 31-ID-D)

Disclosures: This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at Sector 31 of the Advanced Photon Source was provided by Eli Lilly Company, which operates the facility.

Data Collection	ASK1 Compound **4**	ASK1 Compound **11**

PDB Code	6OYT	6OYW
Space Group	$P2_1$	$P2_12_12_1$
Cell Dimensions		
a (Å)	86.51	75.98
b (Å)	73.58	130.41
c (Å)	95.13	137.48
Wavelength (Å)	0.98	0.98
Resolution (Å)	50.0-2.85	50.0-2.60
R_{sym}[a]	0.10 (0.78)	0.187 (0.74)
I/σ	9.4 (1.4)	7.2 (2.3)
Multiplicity	1.8	7.1
Total No. reflections/ No. unique reflections	96,181/51,795	301,524/42,652
Completeness (%)	92.8 (86)	99.9 (100)
Rwork (Rfree)	23.21/28.96	20.78/25.52
$CC_{1/2}$	0.99(0.57)	0.98(0.67)
No. Molecules per asymmetric unit	4	4
R.m.s.d. bond distance (Å)	0.007	0.004
R.m.s.d bond angle (deg)	1.16	0.81
Total no. of non-H atoms in ASU	7,623	8,385
No. of solvent molecules	80	252
Avg. protein B-value ($Å^2$)	74.2	42.1
Avg. solvent B-value ($Å^2$)	49.1	36.4

Ramachandran Plot	94.64	95.67
Preferred	5.36	4.14
Generous	0.00	0.20
Disallowed		

*The value in parentheses is for the highest resolution bin (approximate interval, 0.1 A)

[a]Rsym = f°| f§hkl.< f§hkl >|/ f° f§hkl

[b]Rwork = f°f§hkl| |Fo . |Fc| | / f°f§hkl|Fo| for all data except 5% which is used for the Rfree calculation

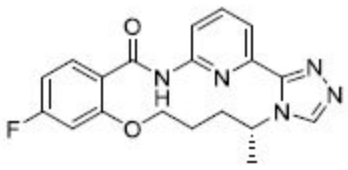
(iii) Calculated IR and VCD spectra to assign absolute stereochemistry of compound 18.

This analog was synthesized as the racemate and the enantiomers were separated. VCD experiments were carried out with the less potent enantiomer. Absolute configuration of the less potent enantiomer of **18** was predicted to be (*R*). Confidence level: 99%

Concentration	5 mg/0.15mL
Solvent	DMSO-d6
Resolution	4 cm^{-1}
PEM setting	1400 cm^{-1}
Number of scans/Measurement time	12 hours
Sample cell	BaF_2
Path length	100 µm
CALCULATION DETAILS	
Gaussian version	Gaussian 09
Total low-energy conformer used for Boltzmann sum	2
Methodology and basis set for DFT calculations	B3LYP/6-31G(d)
Enantiomer used for calculation	(*S*)
Total calculated conformers	2
Number of low-energy conformations shown in report	1

COMMENTS

The confidence level is a measure of the degree of congruence between a calculated and measured spectrum. If identical spectra are being compared the confidence level is 100%. The confidence level (CL) is not the likelihood that the assignment is correct. Rather it's a measure of quality or degree of agreement between calculated and measured spectra.



CompareVOA results:

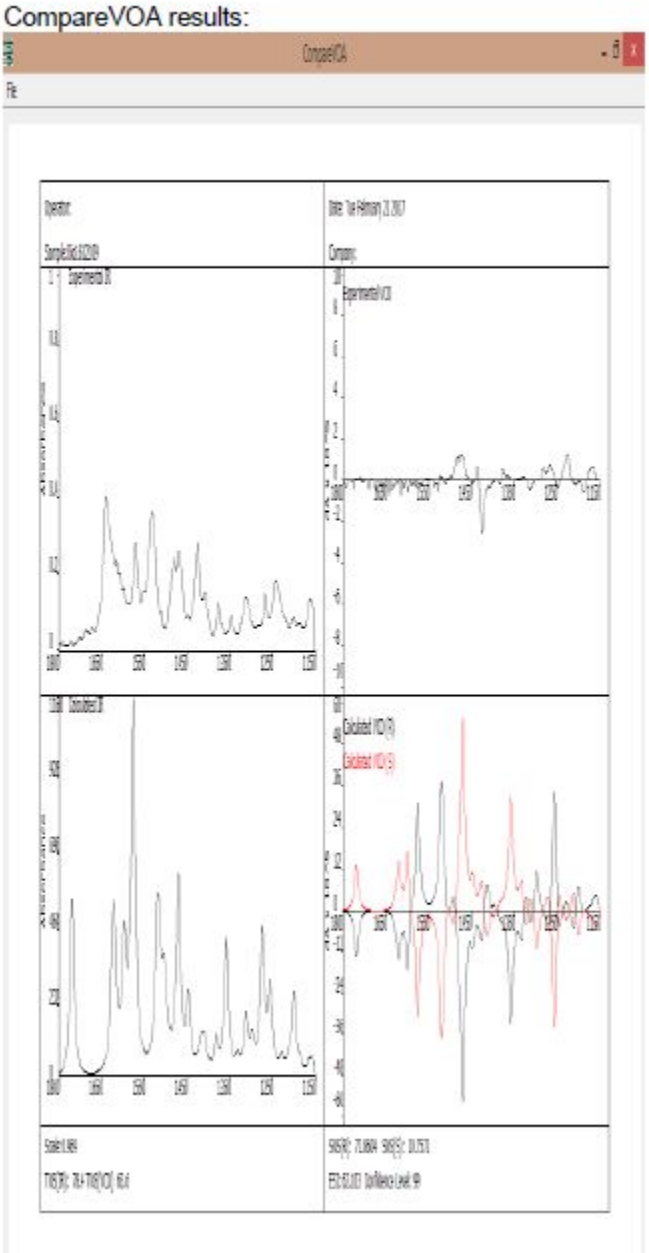
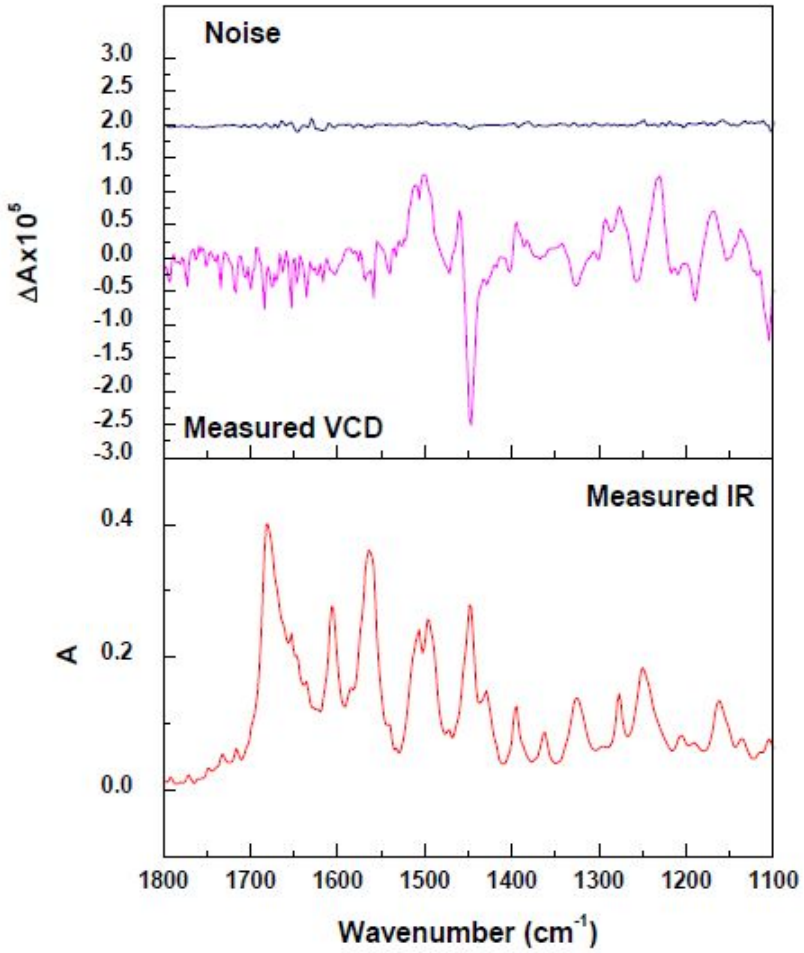


Table: Numerical comparison describing the similarity in the range of 1150-1800 cm^{-1} between the calculated IR and VCD spectra for the (R) enantiomer at the B3LYP/6-31G(d) level and the observed IR and VCD spectra for **18b**

Cal. (1150-1800 cm^{-1})	Numerical comparison	Observed Compound 18
	scaling factor	0.969
	IR similarity (%)	78.4
(*R*)	$^{a}\sum$ (%)	72.8604
	$^{b}\Delta$ (%)	62.103
	Confidence Level (%)	99

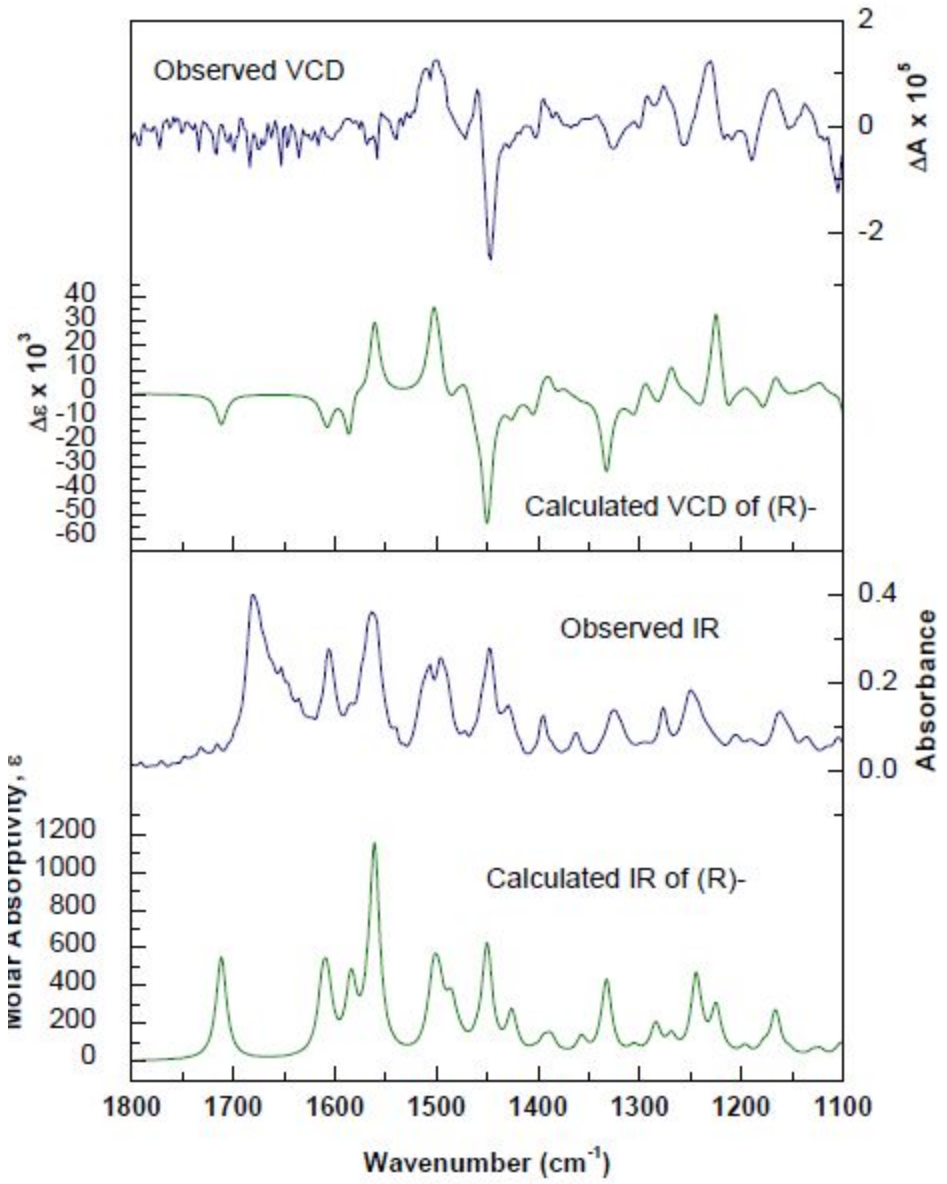
$^{a}\sum$: single VCD similarity, gives the similarity between the calculated and observed VCD spectra.
$^{b}\Delta$: enatiomeric similarity index, gives the difference between the values of $\sum$ for both enantiomers of a given diastereoisomer.

Compound **18b** in DMSO



IR (lower frame) and VCD (upper frame) spectra of CL**S28** in DMSO-d6 (5mg/0.15mL); 0.1mm path-length cell with BaF_2 windows; 12 h collection for samples and solvent; instrument optimized at 1400 cm^{-1}. Solvent-subtracted IR and VCD spectra are shown. Uppermost trace is the VCD noise spectra.

Compound **18b**-vs-calc-



IR (lower frame) and VCD (upper frame) spectra observed for compound **18** (right axes) compared with calculated Boltzmann-averaged spectra of the calculated conformations for the (*R*) – configuration, left (axes)

(iv) Calculated IR and VCD spectra to assign absolute stereochemistry of compound 19.

This analog was synthesized as the racemate and the enantiomers were separated. VCD experiments were carried out with the less potent enantiomer. Absolute configuration of the less potent enantiomer of **19** was predicted to be (*S*). Confidence level: 89%

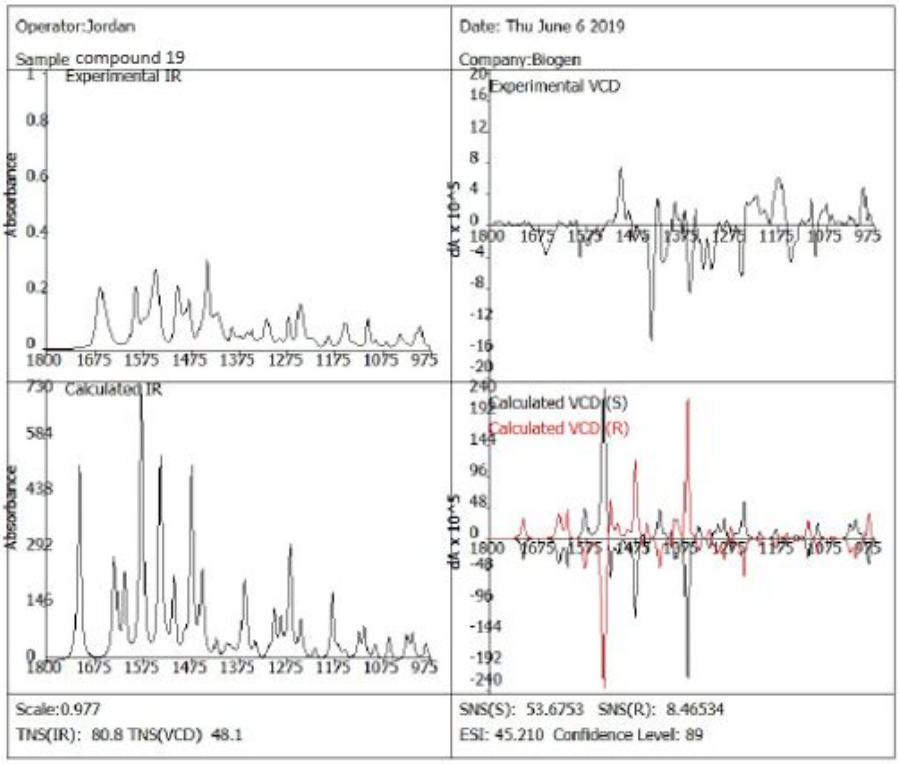
MEASUREMENT PARAMETERS	
Concentration	2.8mg/100uL
Solvent	$CDCl_3$
Resolution	4 cm^{-1}
PEM setting	1400 cm^{-1}
Number of scans/Measurement time	12 hours
Sample cell	BaF_2
Path length	100 µm

CALCULATION DETAILS	
Gaussian version	Gaussian 09
Total low-energy conformers used for Boltzmann sum	2
Methodology and basis set for DFT calculations	B3PW91/cc-pVTZ with cpcm (chloroform)
Enantiomer used for calculation	(S)
Total calculated conformers	2
Number of low-energy conformations shown in report	2

COMMENTS

The confidence level is a measure of the degree of congruence between a calculated and measured spectrum. If identical spectra are being compared the confidence level is 100%. The confidence level (CL) is not the likelihood that the assignment is correct. Rather it's a measure of quality or degree of agreement between calculated and measured spectra.

There is a small artifact in the measured VCD from the BaF_2 cell at 1175cm^{-1} – the confidence level in CompareVOA would likely be a bit higher if not for this.

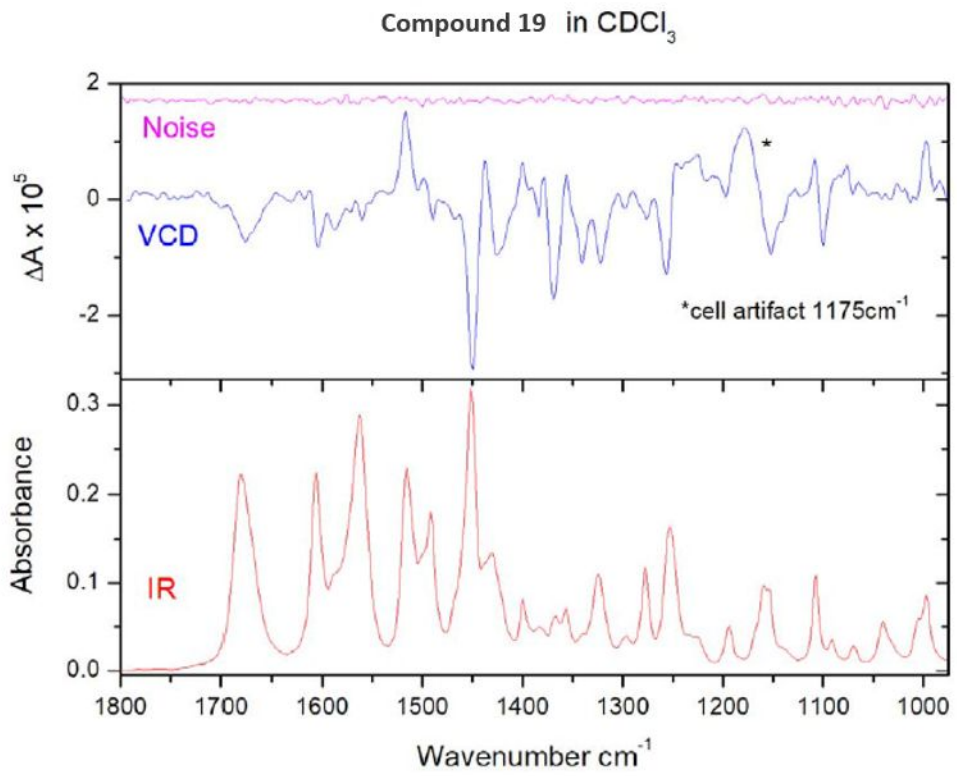


CompareVOA Results.
Please note: In this plot the frequency scaling factor is not applied.

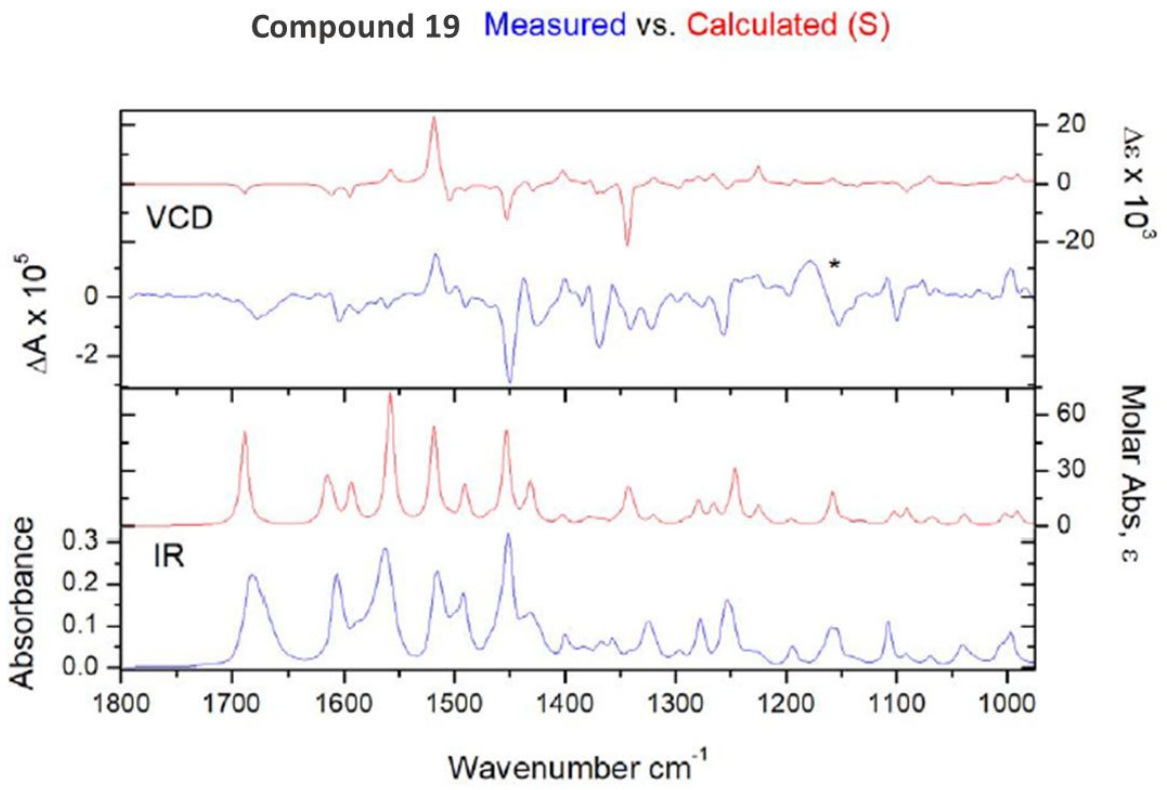
Table 1. Numerical comparison describing the similarity in the range of 975 - 1800 cm^{-1} between the calculated IR and VCD spectra for the **(S)** enantiomer at the B3PW91/cc-pVTZ with cpcm (chloroform) level and the observed IR and VCD spectra for **compound 19**

Cal. ($975\text{-}1800\ cm^{-1}$)	Numerical comparison	Observed **compound 19**
(S)	scaling factor	0.977
	IR similarity (%)	80.8
	$^{a}\sum$ (%)	53.6753
	$^{b}\Delta$ (%)	45.210
	Confidence Level (%)	89

$^{a}\sum$: single VCD similarity, gives the similarity between the calculated and observed VCD spectra.
$^{b}\Delta$: enatiomeric similarity index, gives the difference between the values of $\sum$ for both enantiomers of a given diastereoisomer.



IR (lower frame) and VCD (upper frame) spectra of **compound 19** in $CDCl_3$; 0.1mm path-length cell with BaF_2 windows; 12 h collection for enantiomer and solvent; instrument optimized at 1400 cm^{-1}. Solvent subtracted IR and VCD spectra are shown. Uppermost trace is the VCD noise spectrum. Note the small artifact at 1175cm^{-1} from the BaF_2 cell window.



IR (lower frame) and VCD (upper frame) spectra observed **compound 19** (left axes) compared with Boltzmann-averaged spectra of the calculated conformations for the **(S)** configuration, (right axes). Cell artifact is noted with *.

(v) Kinase selectivity table for compound 24.

Compound	DiscoveRx Gene Symbol	Entrez Gene Symbol	Percent Control	Compound Concentration (nM)
24	AAK1	AAK1	31	100
24	ABL1(E255K)-phosphorylated	ABL1	96	100
24	ABL1(F317I)-nonphosphorylated	ABL1	100	100
24	ABL1(F317I)-phosphorylated	ABL1	88	100
24	ABL1(F317L)-nonphosphorylated	ABL1	100	100
24	ABL1(F317L)-phosphorylated	ABL1	70	100

24	ABL1(H396P)-nonphosphorylated	ABL1	100	100
24	ABL1(H396P)-phosphorylated	ABL1	77	100
24	ABL1(M351T)-phosphorylated	ABL1	72	100
24	ABL1(Q252H)-nonphosphorylated	ABL1	74	100
24	ABL1(Q252H)-phosphorylated	ABL1	78	100
24	ABL1(T315I)-nonphosphorylated	ABL1	100	100
24	ABL1(T315I)-phosphorylated	ABL1	41	100
24	ABL1(Y253F)-phosphorylated	ABL1	88	100
24	ABL1-nonphosphorylated	ABL1	79	100
24	ABL1-phosphorylated	ABL1	78	100
24	ABL2	ABL2	96	100
24	ACVR1	ACVR1	98	100
24	ACVR1B	ACVR1B	74	100
24	ACVR2A	ACVR2A	100	100
24	ACVR2B	ACVR2B	100	100
24	ACVRL1	ACVRL1	100	100
24	ADCK3	CABC1	99	100
24	ADCK4	ADCK4	100	100
24	AKT1	AKT1	100	100
24	AKT2	AKT2	100	100
24	AKT3	AKT3	92	100
24	ALK	ALK	67	100
24	ALK(C1156Y)	ALK	72	100
24	ALK(L1196M)	ALK	76	100
24	AMPK-alpha1	PRKAA1	84	100
24	AMPK-alpha2	PRKAA2	100	100
24	ANKK1	ANKK1	32	100
24	ARK5	NUAK1	90	100
24	ASK1	MAP3K5	1.1	100
24	ASK2	MAP3K6	10	100
24	AURKA	AURKA	65	100
24	AURKB	AURKB	64	100
24	AURKC	AURKC	85	100
24	AXL	AXL	79	100
24	BIKE	BMP2K	66	100
24	BLK	BLK	88	100
24	BMPR1A	BMPR1A	86	100

24	BMPR1B	BMPR1B	73	100
24	BMPR2	BMPR2	23	100
24	BMX	BMX	100	100
24	BRAF	BRAF	85	100
24	BRAF(V600E)	BRAF	86	100
24	BRK	PTK6	94	100
24	BRSK1	BRSK1	100	100
24	BRSK2	BRSK2	61	100
24	BTK	BTK	77	100
24	BUB1	BUB1	74	100
24	CAMK1	CAMK1	84	100
24	CAMK1B	PNCK	96	100
24	CAMK1D	CAMK1D	96	100
24	CAMK1G	CAMK1G	95	100
24	CAMK2A	CAMK2A	74	100
24	CAMK2B	CAMK2B	85	100
24	CAMK2D	CAMK2D	91	100
24	CAMK2G	CAMK2G	77	100
24	CAMK4	CAMK4	100	100
24	CAMKK1	CAMKK1	78	100
24	CAMKK2	CAMKK2	56	100
24	CASK	CASK	59	100
24	CDC2L1	CDK11B	100	100
24	CDC2L2	CDC2L2	98	100
24	CDC2L5	CDK13	76	100
24	CDK11	CDK19	100	100
24	CDK2	CDK2	91	100
24	CDK3	CDK3	100	100
24	CDK4	CDK4	100	100
24	CDK4-cyclinD1	CDK4	59	100
24	CDK4-cyclinD3	CDK4	95	100
24	CDK5	CDK5	100	100
24	CDK7	CDK7	76	100
24	CDK8	CDK8	75	100
24	CDK9	CDK9	94	100
24	CDKL1	CDKL1	75	100
24	CDKL2	CDKL2	94	100
24	CDKL3	CDKL3	98	100
24	CDKL5	CDKL5	91	100
24	CHEK1	CHEK1	87	100
24	CHEK2	CHEK2	6.3	100

24	CIT	CIT	33	100
24	CLK1	CLK1	14	100
24	CLK2	CLK2	13	100
24	CLK3	CLK3	85	100
24	CLK4	CLK4	17	100
24	CSF1R	CSF1R	90	100
24	CSF1R-autoinhibited	CSF1R	89	100
24	CSK	CSK	97	100
24	CSNK1A1	CSNK1A1	13	100
24	CSNK1A1L	CSNK1A1L	22	100
24	CSNK1D	CSNK1D	34	100
24	CSNK1E	CSNK1E	36	100
24	CSNK1G1	CSNK1G1	27	100
24	CSNK1G2	CSNK1G2	9.5	100
24	CSNK1G3	CSNK1G3	7.6	100
24	CSNK2A1	CSNK2A1	73	100
24	CSNK2A2	CSNK2A2	8.5	100
24	CTK	MATK	89	100
24	DAPK1	DAPK1	83	100
24	DAPK2	DAPK2	53	100
24	DAPK3	DAPK3	85	100
24	DCAMKL1	DCLK1	68	100
24	DCAMKL2	DCLK2	77	100
24	DCAMKL3	DCLK3	59	100
24	DDR1	DDR1	88	100
24	DDR2	DDR2	84	100
24	DLK	MAP3K12	100	100
24	DMPK	DMPK	99	100
24	DMPK2	CDC42BPG	98	100
24	DRAK1	STK17A	58	100
24	DRAK2	STK17B	46	100
24	DYRK1A	DYRK1A	0.15	100
24	DYRK1B	DYRK1B	4.1	100
24	DYRK2	DYRK2	53	100
24	EGFR	EGFR	82	100
24	EGFR(E746-A750del)	EGFR	93	100
24	EGFR(G719C)	EGFR	92	100
24	EGFR(G719S)	EGFR	89	100
24	EGFR(L747-E749del, A750P)	EGFR	78	100
24	EGFR(L747-S752del, P753S)	EGFR	97	100
24	EGFR(L747-T751del,Sins)	EGFR	93	100

24	EGFR(L858R)	EGFR	97	100
24	EGFR(L858R,T790M)	EGFR	96	100
24	EGFR(L861Q)	EGFR	100	100
24	EGFR(S752-I759del)	EGFR	84	100
24	EGFR(T790M)	EGFR	100	100
24	EIF2AK1	EIF2AK1	100	100
24	EPHA1	EPHA1	100	100
24	EPHA2	EPHA2	100	100
24	EPHA3	EPHA3	98	100
24	EPHA4	EPHA4	100	100
24	EPHA5	EPHA5	96	100
24	EPHA6	EPHA6	96	100
24	EPHA7	EPHA7	85	100
24	EPHA8	EPHA8	90	100
24	EPHB1	EPHB1	96	100
24	EPHB2	EPHB2	100	100
24	EPHB3	EPHB3	100	100
24	EPHB4	EPHB4	100	100
24	EPHB6	EPHB6	99	100
24	ERBB2	ERBB2	74	100
24	ERBB3	ERBB3	98	100
24	ERBB4	ERBB4	92	100
24	ERK1	MAPK3	93	100
24	ERK2	MAPK1	100	100
24	ERK3	MAPK6	93	100
24	ERK4	MAPK4	100	100
24	ERK5	MAPK7	100	100
24	ERK8	MAPK15	12	100
24	ERN1	ERN1	69	100
24	FAK	PTK2	98	100
24	FER	FER	90	100
24	FES	FES	88	100
24	FGFR1	FGFR1	88	100
24	FGFR2	FGFR2	99	100
24	FGFR3	FGFR3	97	100
24	FGFR3(G697C)	FGFR3	100	100
24	FGFR4	FGFR4	96	100
24	FGR	FGR	96	100
24	FLT1	FLT1	87	100
24	FLT3	FLT3	76	100
24	FLT3(D835H)	FLT3	23	100

24	FLT3(D835V)	FLT3	3.4	100
24	FLT3(D835Y)	FLT3	20	100
24	FLT3(ITD)	FLT3	41	100
24	FLT3(ITD,D835V)	FLT3	1.1	100
24	FLT3(ITD,F691L)	FLT3	4.4	100
24	FLT3(K663Q)	FLT3	77	100
24	FLT3(N841I)	FLT3	43	100
24	FLT3(R834Q)	FLT3	68	100
24	FLT3-autoinhibited	FLT3	93	100
24	FLT4	FLT4	82	100
24	FRK	FRK	100	100
24	FYN	FYN	93	100
24	GAK	GAK	13	100
24	GCN2(Kin.Dom.2,S808G)	EIF2AK4	100	100
24	GRK1	GRK1	61	100
24	GRK2	ADRBK1	100	100
24	GRK3	ADRBK2	70	100
24	GRK4	GRK4	76	100
24	GRK7	GRK7	61	100
24	GSK3A	GSK3A	100	100
24	GSK3B	GSK3B	10	100
24	HASPIN	GSG2	83	100
24	HCK	HCK	98	100
24	HIPK1	HIPK1	41	100
24	HIPK2	HIPK2	12	100
24	HIPK3	HIPK3	55	100
24	HIPK4	HIPK4	99	100
24	HPK1	MAP4K1	100	100
24	HUNK	HUNK	99	100
24	ICK	ICK	78	100
24	IGF1R	IGF1R	98	100
24	IKK-alpha	CHUK	63	100
24	IKK-beta	IKBKB	70	100
24	IKK-epsilon	IKBKE	95	100
24	INSR	INSR	89	100
24	INSRR	INSRR	100	100
24	IRAK1	IRAK1	65	100
24	IRAK3	IRAK3	86	100
24	IRAK4	IRAK4	72	100
24	ITK	ITK	93	100
24	JAK1(JH1domain-catalytic)	JAK1	100	100

24	JAK1(JH2domain-pseudokinase)	JAK1	80	100
24	JAK2(JH1domain-catalytic)	JAK2	50	100
24	JAK3(JH1domain-catalytic)	JAK3	93	100
24	JNK1	MAPK8	80	100
24	JNK2	MAPK9	96	100
24	JNK3	MAPK10	100	100
24	KIT	KIT	94	100
24	KIT(A829P)	KIT	97	100
24	KIT(D816H)	KIT	64	100
24	KIT(D816V)	KIT	36	100
24	KIT(L576P)	KIT	81	100
24	KIT(V559D)	KIT	86	100
24	KIT(V559D,T670I)	KIT	91	100
24	KIT(V559D,V654A)	KIT	89	100
24	KIT-autoinhibited	KIT	86	100
24	LATS1	LATS1	100	100
24	LATS2	LATS2	99	100
24	LCK	LCK	100	100
24	LIMK1	LIMK1	100	100
24	LIMK2	LIMK2	100	100
24	LKB1	STK11	97	100
24	LOK	STK10	72	100
24	LRRK2	LRRK2	0.6	100
24	LRRK2(G2019S)	LRRK2	2.3	100
24	LTK	LTK	78	100
24	LYN	LYN	100	100
24	LZK	MAP3K13	91	100
24	MAK	MAK	84	100
24	MAP3K1	MAP3K1	77	100
24	MAP3K15	MAP3K15	48	100
24	MAP3K2	MAP3K2	74	100
24	MAP3K3	MAP3K3	78	100
24	MAP3K4	MAP3K4	100	100
24	MAP4K2	MAP4K2	25	100
24	MAP4K3	MAP4K3	76	100
24	MAP4K4	MAP4K4	77	100
24	MAP4K5	MAP4K5	92	100
24	MAPKAPK2	MAPKAPK2	84	100
24	MAPKAPK5	MAPKAPK5	51	100
24	MARK1	MARK1	66	100
24	MARK2	MARK2	85	100

24	MARK3	MARK3	100	100
24	MARK4	MARK4	78	100
24	MAST1	MAST1	93	100
24	MEK1	MAP2K1	70	100
24	MEK2	MAP2K2	70	100
24	MEK3	MAP2K3	47	100
24	MEK4	MAP2K4	35	100
24	MEK5	MAP2K5	0.2	100
24	MEK6	MAP2K6	100	100
24	MELK	MELK	85	100
24	MERTK	MERTK	77	100
24	MET	MET	81	100
24	MET(M1250T)	MET	89	100
24	MET(Y1235D)	MET	96	100
24	MINK	MINK1	29	100
24	MKK7	MAP2K7	100	100
24	MKNK1	MKNK1	100	100
24	MKNK2	MKNK2	100	100
24	MLCK	MYLK3	100	100
24	MLK1	MAP3K9	91	100
24	MLK2	MAP3K10	74	100
24	MLK3	MAP3K11	100	100
24	MRCKA	CDC42BPA	97	100
24	MRCKB	CDC42BPB	100	100
24	MST1	STK4	100	100
24	MST1R	MST1R	70	100
24	MST2	STK3	92	100
24	MST3	STK24	100	100
24	MST4	MST4	1.2	100
24	MTOR	MTOR	86	100
24	MUSK	MUSK	100	100
24	MYLK	MYLK	5.3	100
24	MYLK2	MYLK2	82	100
24	MYLK4	MYLK4	99	100
24	MYO3A	MYO3A	100	100
24	MYO3B	MYO3B	100	100
24	NDR1	STK38	78	100
24	NDR2	STK38L	100	100
24	NEK1	NEK1	100	100
24	NEK10	NEK10	39	100
24	NEK11	NEK11	100	100

24	NEK2	NEK2	97	100
24	NEK3	NEK3	84	100
24	NEK4	NEK4	90	100
24	NEK5	NEK5	100	100
24	NEK6	NEK6	100	100
24	NEK7	NEK7	100	100
24	NEK9	NEK9	80	100
24	NIK	MAP3K14	27	100
24	NIM1	MGC42105	100	100
24	NLK	NLK	100	100
24	OSR1	OXSR1	99	100
24	p38-alpha	MAPK14	100	100
24	p38-beta	MAPK11	87	100
24	p38-delta	MAPK13	79	100
24	p38-gamma	MAPK12	78	100
24	PAK1	PAK1	100	100
24	PAK2	PAK2	100	100
24	PAK3	PAK3	82	100
24	PAK4	PAK4	92	100
24	PAK6	PAK6	100	100
24	PAK7	PAK7	99	100
24	PCTK1	CDK16	71	100
24	PCTK2	CDK17	100	100
24	PCTK3	CDK18	95	100
24	PDGFRA	PDGFRA	73	100
24	PDGFRB	PDGFRB	66	100
24	PDPK1	PDPK1	99	100
24	PFCDPK1(P.falciparum)	CDPK1	73	100
24	PFPK5(P.falciparum)	MAL13P1.279	98	100
24	PFTAIRE2	CDK15	93	100
24	PFTK1	CDK14	93	100
24	PHKG1	PHKG1	95	100
24	PHKG2	PHKG2	76	100
24	PIK3C2B	PIK3C2B	95	100
24	PIK3C2G	PIK3C2G	100	100
24	PIK3CA	PIK3CA	100	100
24	PIK3CA(C420R)	PIK3CA	100	100
24	PIK3CA(E542K)	PIK3CA	82	100
24	PIK3CA(E545A)	PIK3CA	94	100
24	PIK3CA(E545K)	PIK3CA	62	100
24	PIK3CA(H1047L)	PIK3CA	100	100

24	PIK3CA(H1047Y)	PIK3CA	100	100
24	PIK3CA(I800L)	PIK3CA	91	100
24	PIK3CA(M1043I)	PIK3CA	92	100
24	PIK3CA(Q546K)	PIK3CA	100	100
24	PIK3CB	PIK3CB	100	100
24	PIK3CD	PIK3CD	88	100
24	PIK3CG	PIK3CG	73	100
24	PIK4CB	PI4KB	100	100
24	PIKFYVE	PIKFYVE	68	100
24	PIM1	PIM1	94	100
24	PIM2	PIM2	97	100
24	PIM3	PIM3	100	100
24	PIP5K1A	PIP5K1A	71	100
24	PIP5K1C	PIP5K1C	92	100
24	PIP5K2B	PIP4K2B	100	100
24	PIP5K2C	PIP4K2C	56	100
24	PKAC-alpha	PRKACA	92	100
24	PKAC-beta	PRKACB	69	100
24	PKMYT1	PKMYT1	100	100
24	PKN1	PKN1	100	100
24	PKN2	PKN2	93	100
24	PKNB(M.tuberculosis)	pknB	84	100
24	PLK1	PLK1	92	100
24	PLK2	PLK2	75	100
24	PLK3	PLK3	78	100
24	PLK4	PLK4	54	100
24	PRKCD	PRKCD	88	100
24	PRKCE	PRKCE	48	100
24	PRKCH	PRKCH	100	100
24	PRKCI	PRKCI	93	100
24	PRKCQ	PRKCQ	87	100
24	PRKD1	PRKD1	17	100
24	PRKD2	PRKD2	51	100
24	PRKD3	PRKD3	82	100
24	PRKG1	PRKG1	72	100
24	PRKG2	PRKG2	92	100
24	PRKR	EIF2AK2	61	100
24	PRKX	PRKX	81	100
24	PRP4	PRPF4B	100	100
24	PYK2	PTK2B	100	100
24	QSK	KIAA0999	0	100

24	RAF1	RAF1	93	100
24	RET	RET	100	100
24	RET(M918T)	RET	77	100
24	RET(V804L)	RET	70	100
24	RET(V804M)	RET	90	100
24	RIOK1	RIOK1	74	100
24	RIOK2	RIOK2	83	100
24	RIOK3	RIOK3	67	100
24	RIPK1	RIPK1	100	100
24	RIPK2	RIPK2	91	100
24	RIPK4	RIPK4	84	100
24	RIPK5	DSTYK	60	100
24	ROCK1	ROCK1	82	100
24	ROCK2	ROCK2	13	100
24	ROS1	ROS1	97	100
24	RPS6KA4(Kin.Dom.1-N-terminal)	RPS6KA4	71	100
24	RPS6KA4(Kin.Dom.2-C-terminal)	RPS6KA4	5.5	100
24	RPS6KA5(Kin.Dom.1-N-terminal)	RPS6KA5	100	100
24	RPS6KA5(Kin.Dom.2-C-terminal)	RPS6KA5	61	100
24	RSK1(Kin.Dom.1-N-terminal)	RPS6KA1	90	100
24	RSK1(Kin.Dom.2-C-terminal)	RPS6KA1	98	100
24	RSK2(Kin.Dom.1-N-terminal)	RPS6KA3	13	100
24	RSK2(Kin.Dom.2-C-terminal)	RPS6KA3	83	100
24	RSK3(Kin.Dom.1-N-terminal)	RPS6KA2	54	100
24	RSK3(Kin.Dom.2-C-terminal)	RPS6KA2	94	100
24	RSK4(Kin.Dom.1-N-terminal)	RPS6KA6	5.9	100
24	RSK4(Kin.Dom.2-C-terminal)	RPS6KA6	85	100
24	S6K1	RPS6KB1	85	100
24	SBK1	SBK1	91	100
24	SGK	SGK1	98	100
24	SgK110	SgK110	100	100
24	SGK2	SGK2	85	100
24	SGK3	SGK3	77	100
24	SIK	SIK1	89	100
24	SIK2	SIK2	100	100
24	SLK	SLK	88	100
24	SNARK	NUAK2	65	100
24	SNRK	SNRK	90	100

24	SRC	SRC	93	100
24	SRMS	SRMS	98	100
24	SRPK1	SRPK1	71	100
24	SRPK2	SRPK2	100	100
24	SRPK3	SRPK3	90	100
24	STK16	STK16	100	100
24	STK33	STK33	57	100
24	STK35	STK35	89	100
24	STK36	STK36	95	100
24	STK39	STK39	72	100
24	SYK	SYK	100	100
24	TAK1	MAP3K7	8.7	100
24	TAOK1	TAOK1	98	100
24	TAOK2	TAOK2	92	100
24	TAOK3	TAOK3	98	100
24	TBK1	TBK1	92	100
24	TEC	TEC	94	100
24	TESK1	TESK1	100	100
24	TGFBR1	TGFBR1	100	100
24	TGFBR2	TGFBR2	96	100
24	TIE1	TIE1	100	100
24	TIE2	TEK	88	100
24	TLK1	TLK1	100	100
24	TLK2	TLK2	100	100
24	TNIK	TNIK	62	100
24	TNK1	TNK1	81	100
24	TNK2	TNK2	72	100
24	TNNI3K	TNNI3K	100	100
24	TRKA	NTRK1	100	100
24	TRKB	NTRK2	100	100
24	TRKC	NTRK3	97	100
24	TRPM6	TRPM6	85	100
24	TSSK1B	TSSK1B	100	100
24	TSSK3	TSSK3	93	100
24	TTK	TTK	93	100
24	TXK	TXK	100	100
24	TYK2(JH1domain-catalytic)	TYK2	59	100
24	TYK2(JH2domain-pseudokinase)	TYK2	75	100
24	TYRO3	TYRO3	69	100
24	ULK1	ULK1	81	100
24	ULK2	ULK2	28	100

24	ULK3	ULK3	74	100
24	VEGFR2	KDR	79	100
24	VPS34	PIK3C3	95	100
24	VRK2	VRK2	74	100
24	WEE1	WEE1	100	100
24	WEE2	WEE2	100	100
24	WNK1	WNK1	89	100
24	WNK2	WNK2	82	100
24	WNK3	WNK3	93	100
24	WNK4	WNK4	98	100
24	YANK1	STK32A	100	100
24	YANK2	STK32B	100	100
24	YANK3	STK32C	100	100
24	YES	YES1	79	100
24	YSK1	STK25	100	100
24	YSK4	MAP3K19	3.2	100
24	ZAK	ZAK	100	100
24	ZAP70	ZAP70	78	100

(vi) Experimental details for K_{puu} experiments

To evaluate the CNS penetration of the compounds described herein, several compounds were selected for *in vivo* rat K_{puu} studies. In these experiments the compounds are administered *via* an IV infusion (using *N*,*N*-dimethylacetamide:ethanol:1,2-propylene glycol:water in a 1:1:3:5 ratio as the vehicle) in the carotid artery for a period of four hours (1 mg/kg, 0.1 mg/mL) to reach steady state. After this time the plasma and brain concentration levels are quantified, and the values are adjusted by the measured protein binding in plasma and brain homogenate to calculate the K_{puu} (see Di, L.; Kerns, E.H. *Blood-Brain Barrier in Drug Discovery* (Wiley)) according to the equation below.

$K_{puu} = C_{u,b} / C_{u,p}$

Wherein:

$C_{u,b}$ = Unbound concentration in brain (C x $f_{u,b}$). (C = concentration at steady state; $f_{u,b}$ = fraction unbound in brain)

And in which: $C_{u,p}$ = Unbound concentration in plasma (C x $f_{u,p}$). (C = concentration at steady state; $f_{u,p}$ = fraction unbound in plasma)

Plasma and brain protein binding values were generated *via* the Rapid Equilibrium Dialysis method. The compound of interest was incubated in K_2EDTA plasma and brain homogenate (homogenized 1:7 (w:v) in 1xPBS) purchased from BioIVT (Westbury, NY), opposite a buffered compartment of 100 mM Potassium phosphate/150 mM Sodium chloride, pH 7.4, at 1 µM for 4 hr and 6 hr respectively. At the conclusion of incubation samples were taken from both matrix and buffered compartments, matrix-matched using blank buffer and matrix, extracted with acetonitrile, diluted with water, and analyzed utilizing an Agilent RapidFire 365 high-throughput LC coupled with MS/MS detection *via* an AB Sciex 5500. Free fractions (f_u) were then calculated by comparing internal standard/analyte peak-area ratios of matrix and buffered compartments. Cross-species brain protein binding was considered to be equivalent

for the purposes of calculating free fraction (see Di, L., *et al.*, (2011a) *Species Independence in Brain Tissue Binding Using Brain Homogenates*, Drug Metab Dispos 39:1270–1277).

Total drug concentration in plasma and brain tissue was measured *via* well-established bioanalytical extraction (protein precipitation) and detection methods (LC-MS/MS). Brain tissues were homogenized 1:4 (w:v) with 1x PBS in MP Biomedicals Lysing Matrix D tubes *via* an MP Biomedicals FastPrep-24™ homogenizer and were then extracted alongside plasma samples by matrix-matching with blank K_2EDTA plasma (purchased from BioIVT), followed by protein crash/extraction with acetonitrile, supernatant dry down under nitrogen, and reconstitution with an acidified aqueous/organic mixture before being measured against a calibration curve of the compound of interest prepared in plasma, matrix-matched with blank brain homogenate (generated with brains purchased from BioIVT), and similarly extracted. Reconstituted extracts were then analyzed *via* LC-MS/MS (AB Sciex 5500) utilizing a binary HPLC setup (Shimadzu LC-20ADvp) and reverse-phase chromatography gradient (ACE 3 C18-AR). Peak area ratios and a $1/x^2$ regression fit were used to generate sample concentration values that, combined with plasma and brain protein binding values, were used to generate free drug concentration values and partitioning coefficient (K_{puu}).