CHEMMEDCHEM
Chemistry & Drug Discovery

Supporting Information

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Supporting material for

2,3-dihydro-1-benzofuran Derivatives as a Novel Series of Potent Selective Cannabinoid Receptor 2 Agonists: Design, Synthesis, and Binding Mode Prediction through Ligand-steered Modeling

Philippe Diaz,[a] Sharangdhar S. Phatak,[b] Jijun Xu,[c] Frank R. Fronczek,[d] Fanny Astruc-Diaz,[a] Charles M. Thompson,[a] Claudio N. Cavasotto,[b] and Mohamed Naguib*[c]

[a] Core Laboratory for Neuromolecular Production, Department of Biomedical and Pharmaceutical Sciences, The University of Montana, 32 Campus Drive, Missoula, MT 59812.

[b] School of Health Information Sciences, The University of Texas Health Science Center at Houston, 7000 Fannin, Suite 860B, Houston, TX 77030.

[c] Department of Anesthesiology and Pain Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

[d] Chemistry Department, Louisiana State University, Baton Rouge, LA 70803-1804.

*To whom correspondence should be addressed:

Department of Anesthesiology and Pain Medicine, Unit 409

The University of Texas M. D. Anderson Cancer Center

1400 Holcombe Boulevard, Houston, TX 77030

Phone: 713-745-4948

Fax: 713-792-7591

E-mail: Naguib@mdanderson.org

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1. LC-MS ANALYSES

Method A:

LC-MS analyses were performed on an Agilent HP1100-series liquid chromatography system equipped with a diode array detector and a Waters ZQ single quadrupole mass spectrometer (ionization type Electrospray). The liquid chromatrography conditions were as follow: a Phenomenex Gemini 3-um C18 110A 100x2 mm column was used, and it was eluted with a gradient made up of two solvent mixtures. Solvent A consisted of water and 0.2% formic acid. Solvent B consisted of methanol. The gradient was processed as follows:

Time	A%	B%	C%	D%	Flow (ml/min)	Pressure
0.00	90.0	10.0	0.0	0.0	0.300	400
1.00	90.0	10.0	0.0	0.0	0.300	400
7.00	0.0	100.0	0.0	0.0	0.300	400
9.00	0.0	100.0	0.0	0.0	0.300	400
10.00	90.0	10.0	0.0	0.0	0.300	400
14.00	90.0	10.0	0.0	0.0	0.300	400

Compound purity was assigned on the basis of 254-nM detection data assessed by comparing relative peak areas of the signals.

Method B:

LC-MS analyses were performed on a Waters/Micromass LCT, TOF equipped with an Alliance HT Waters 2795 liquid chromatography system equipped with a Waters 2487 dual absorbance detector. The liquid chromatrography conditions were as follow: a Zorbax Eclipse XBD-C18 3.5-um 50x2.1 mm column equipped with a rapid resolution cartridge Zorbax Eclipse XBD-C18 3.5-um 15x2.1 mm was used, and it was eluted with a gradient made up of two solvent mixtures. Solvent A consisted of water and 0.08% TFA. Solvent C consisted of acetonitrile. The gradient was processed as follows:

Time	A%	B%	C%	D%	Flow (ml/min)
0.00	90.0	0.0	10.0	0.0	0.300
1.00	90.0	0.0	10.0	0.0	0.300
8.00	5.0	0.0	95.0	0.0	0.300
10.00	5.0	0.0	95.0	0.0	0.300
13.00	5.0	0.0	95.0	0.0	0.300
20.00	90.0	0.0	10.0	0.0	0.300

Compound purity was assigned on the basis of 254-nM detection data assessed by comparing relative peak areas of the signals.

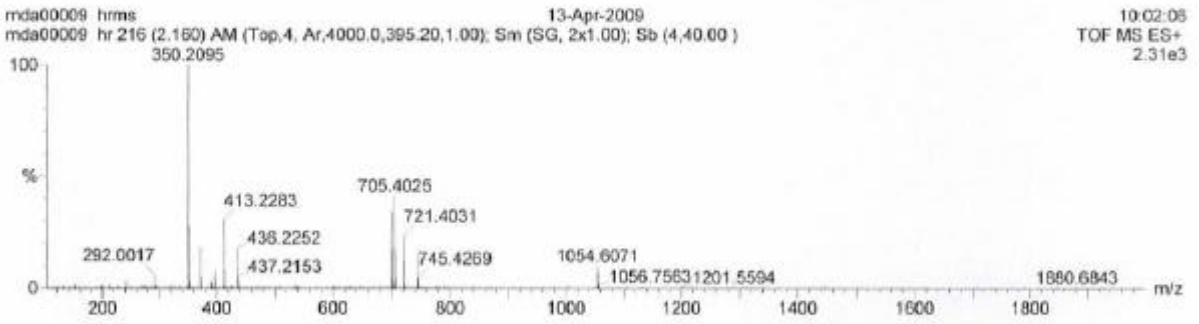
Compound	Retention Time (min)	Purity (%)	Method
14	9.71	100	A
	10.62	100	B
15	9.29	100	A
	10.25	100	B
16	11.35	94.55	A
17	9.71	100	A
	10.80	93.93	B
18	9.63	100	A
	10.29	100	B
19	9.97	98.77	A
	11.36	100	B
20	10.04	95.17	A
	11.54	98.00	B
21	10.53	100	A
22	9.17	96.01	A
23	10.90	100	A
24	9.14	99.21	A
27	10.01	95.27	A
	11.35	95.30	B
28	9.93	99.31	A
	11.01	99.00	B
29	9.92	94.00	A
	11.00	92.59	B
30	9.77	90.36	A
	10.71	92.60	B
31	10.20	99.00	A
32	7.00	99.01	A

2. ELEMENTAL COMPOSITION REPORT

Compound 14:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

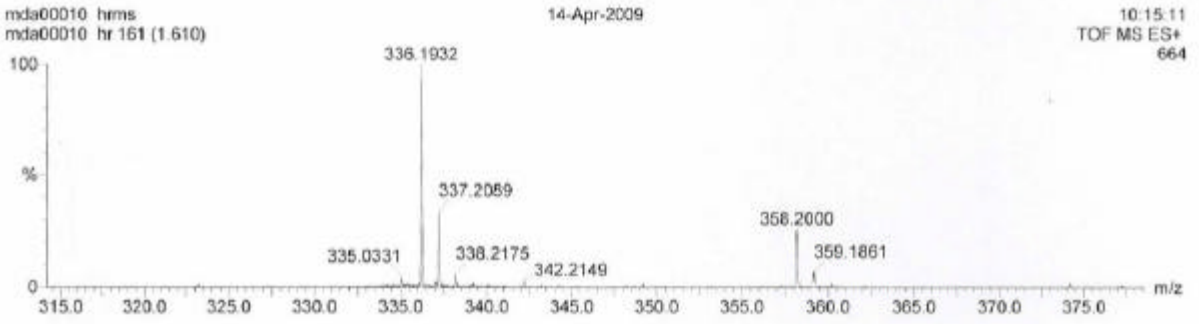


Minimum:		200.0	1000.0	-1.5 50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
350.2095	350.2120	-2.5	-7.2	10.5	1	C23 H28 N O2

Compound 15:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope matching not enabled

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

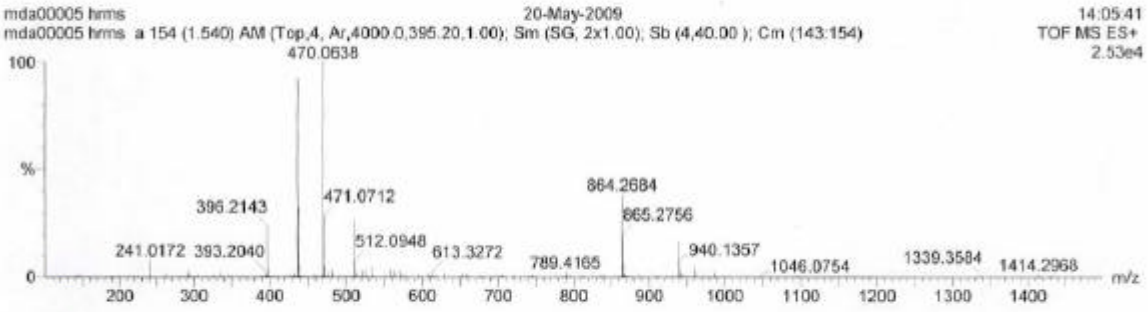


Minimum:		200.0	1000.0	-1.5 50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
336.1932	336.1964	-3.2	-9.4	10.5	C22 H26 N O2

Compound 16:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
4 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

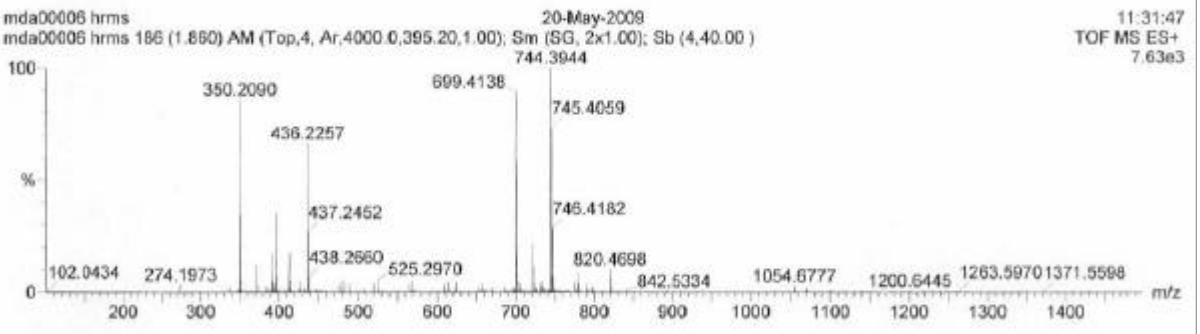


Minimum:			-1.5		
Maximum:		200.0	1000.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
470.0638	470.0617	2.1	4.5	13.5	1	C23 H21 N O2 I

Compound 17:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

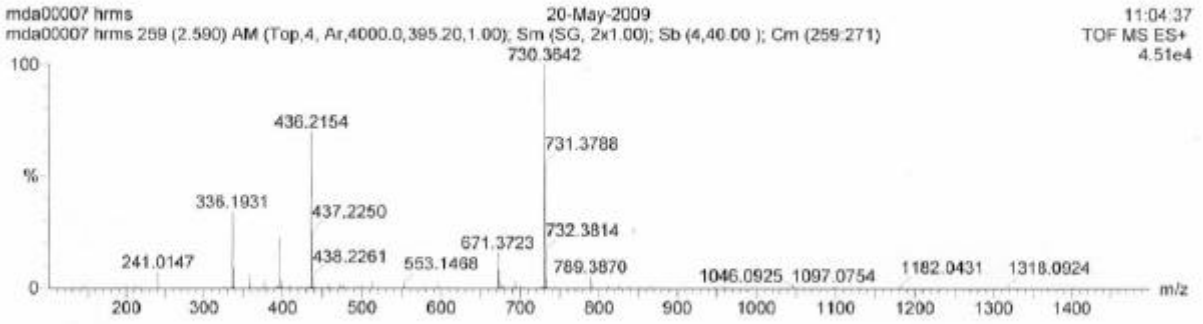


Minimum:			-1.5		
Maximum:		200.0	1000.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
350.2090	350.2120	-3.0	-8.6	10.5	1	C23 H28 N O2

Compound 18:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)



Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
336.1931	336.1964	-3.3	-9.7	10.5	1	C22 H26 N O2

Compound 19:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope matching not enabled

Monoisotopic Mass, Odd and Even Electron Ions
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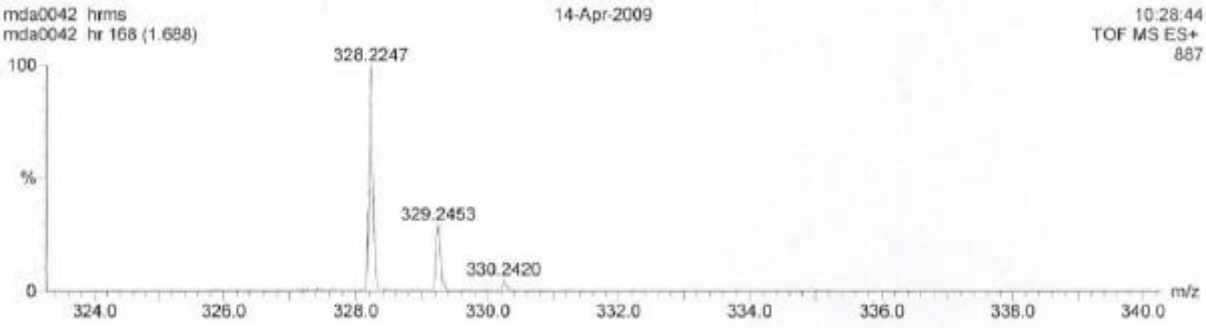
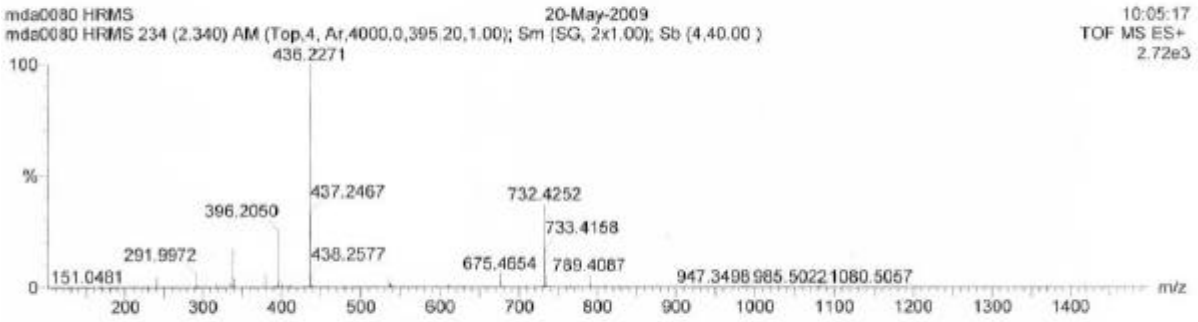


Minimum:			-1.5		
Maximum:		200.0	1000.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
328.2247	328.2277	-3.0	-9.0	7.5	C21 H30 N O2

Compound 21:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

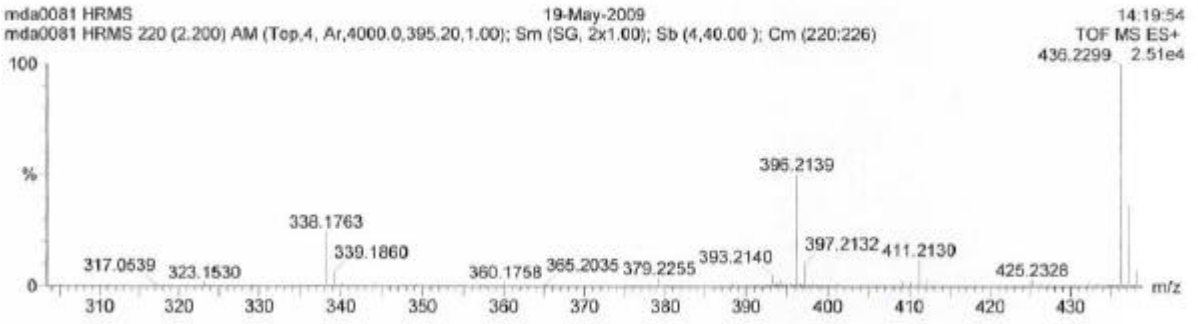


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
338.2148	338.2120	2.8	8.3	9.5	1	C22 H28 N O2

Compound 22:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
4 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

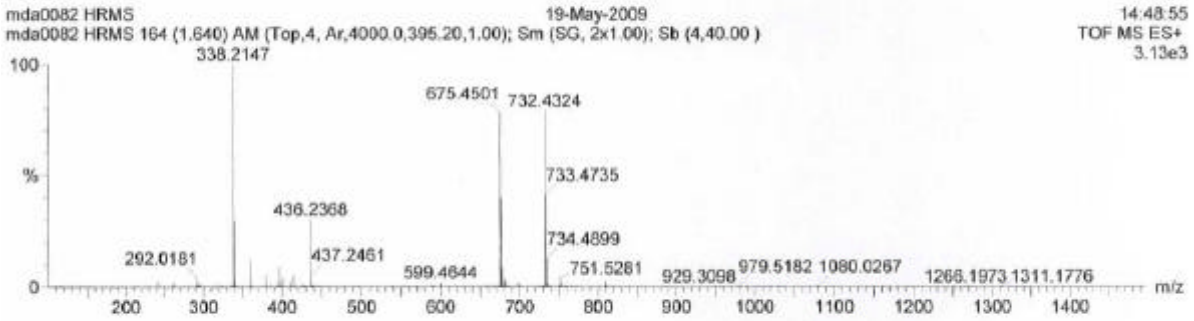


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
338.1763	338.1756	0.7	2.0	10.5	1	C21 H24 N O3

Compound 23:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

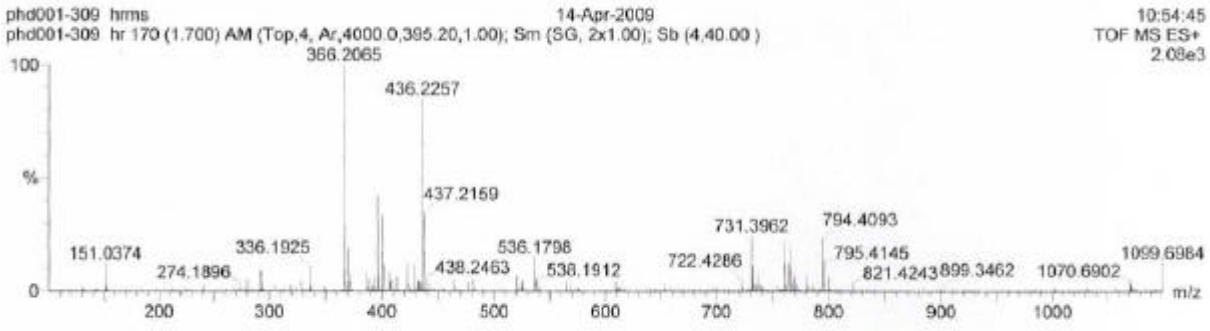


Minimum:			-1.5		
Maximum:		200.0	1000.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
338.2147	338.2120	2.7	8.0	9.5	1	C22 H28 N O2

Compound 24:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

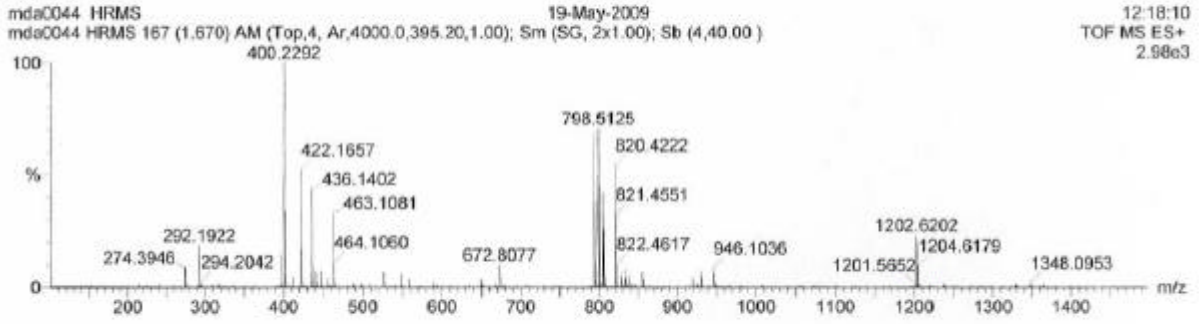


Minimum:			-1.5		
Maximum:		200.0	1000.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
366.2065	366.2069	-0.4	-1.1	10.5	1	C23 H28 N O3

Compound 27:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

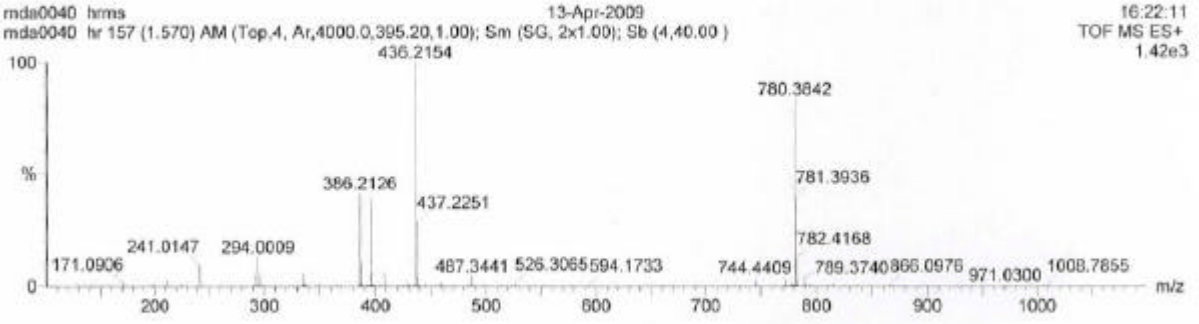


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
400.2292	400.2277	1.5	3.9	13.5	1	C27 H30 N O2

Compound 28:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

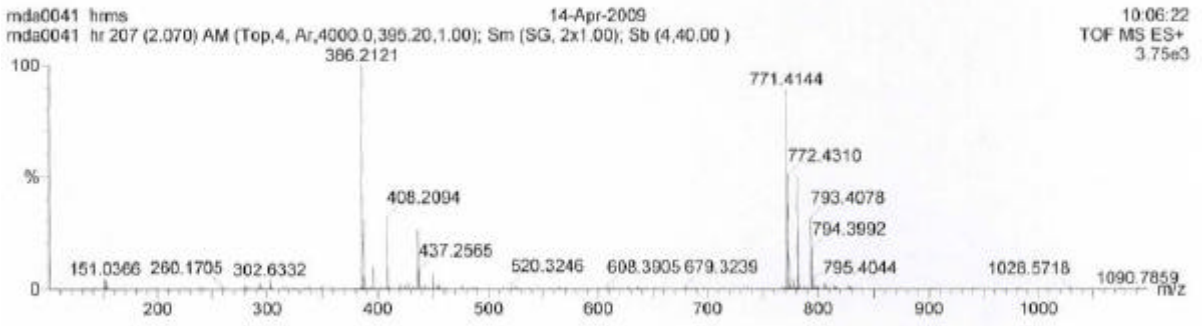


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
386.2126	386.2120	0.6	1.5	13.5	1	C26 H28 N O2

Compound 29:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

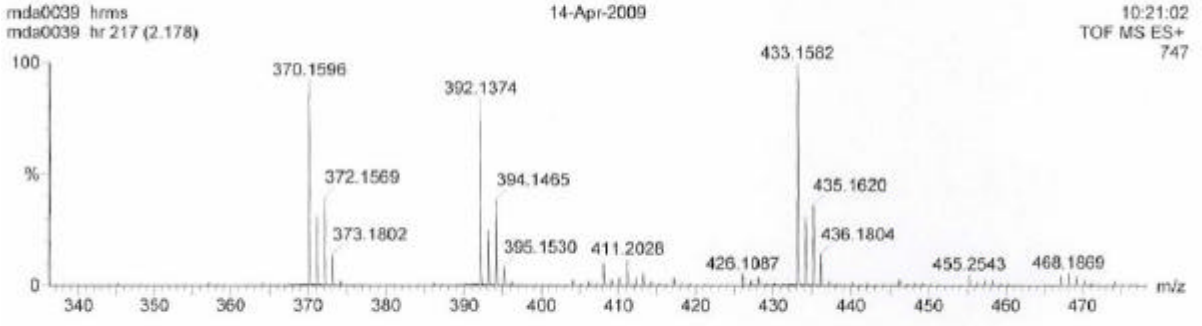


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
386.2121	386.2120	0.1	0.2	13.5	1	C26 H28 N O2

Compound 30:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope matching not enabled

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

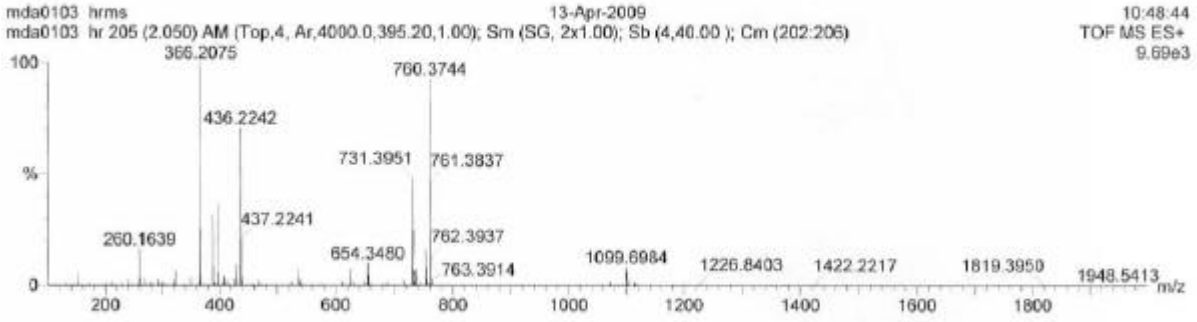


Minimum:				-1.5	
Maximum:		200.0	1000.0	50.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
370.1596	370.1574	2.2	6.0	10.5	C22 H25 N O2 Cl

Compound 31:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

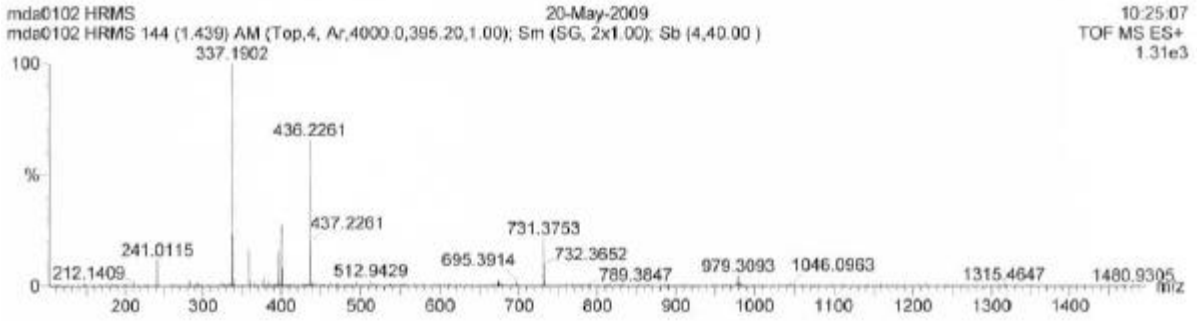


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
366.2075	366.2069	0.6	1.6	10.5	1	C23 H28 N O3

Compound 32:

Single Mass Analysis
Tolerance = 1000.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

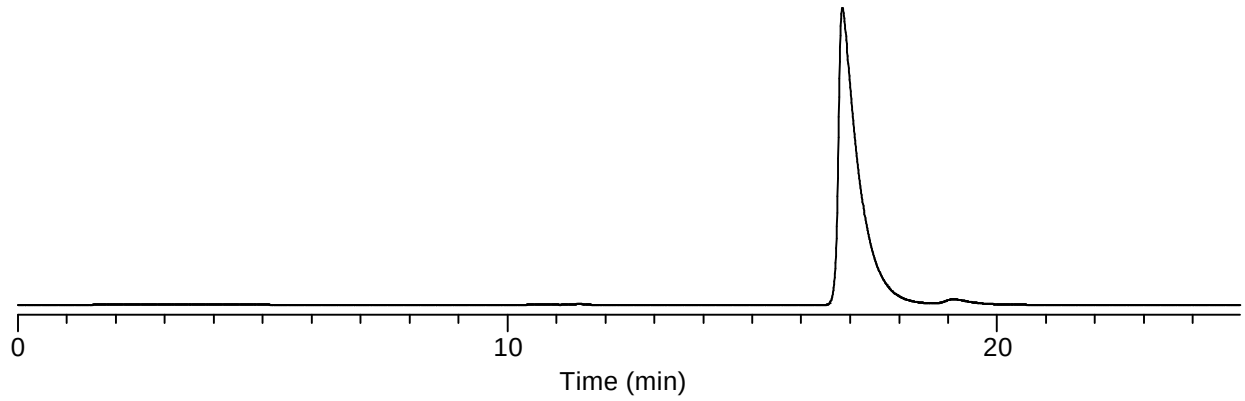


Minimum:				-1.5		
Maximum:		200.0	1000.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
337.1902	337.1916	-1.4	-4.2	10.5	1	C21 H25 N2 O2

3. ENANTIOMERIC PURITY RESULTS FOR COMPOUNDS 33 AND 34

Enantiomer 1 (compound 33):

UV



Conditions:

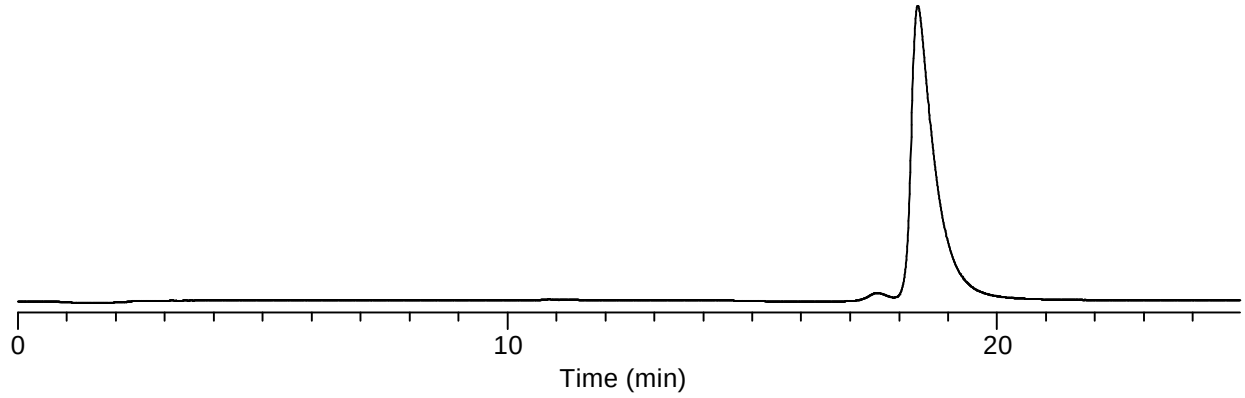
Column:	CYCLOBOND DMP, 25 cm x 4.6 mm I.D., 5 µm (20724AST)
Mobile Phase:	100:0.01:0.05, ACN:HOAc:TEA
Temperature:	10°C
Flow Rate:	0.3 mL/min
Detection:	UV at 288 nm
Injection Volume:	10 µL
Sample:	200 µg/mL solution in 100:0.01:0.05, ACN:HOAc:TEA

Peak 1 retention time (R_{t1}): 16.84 min.

Enantiomeric purity of Peak 1 = 97.3%.

Enantiomer 2 (compound 34):

UV



Conditions:

Column:	CYCLOBOND DMP, 25 cm x 4.6 mm I.D., 5 µm (20724AST)
Mobile Phase:	100:0.01:0.05, ACN:HOAc:TEA
Temperature:	10°C
Flow Rate:	0.3 mL/min
Detection:	UV at 288 nm
Injection Volume:	10 µL
Sample:	200 µg/mL solution in 100:0.01:0.05, ACN:HOAc:TEA

Peak 2 retention time (R_{t1}): 18.38 min.

Enantiomeric purity of Peak 2 = 97.5%.

4. PURITY ASSESSMENT FOR COMPOUND 18 USED IN ADME-TOX STUDIES

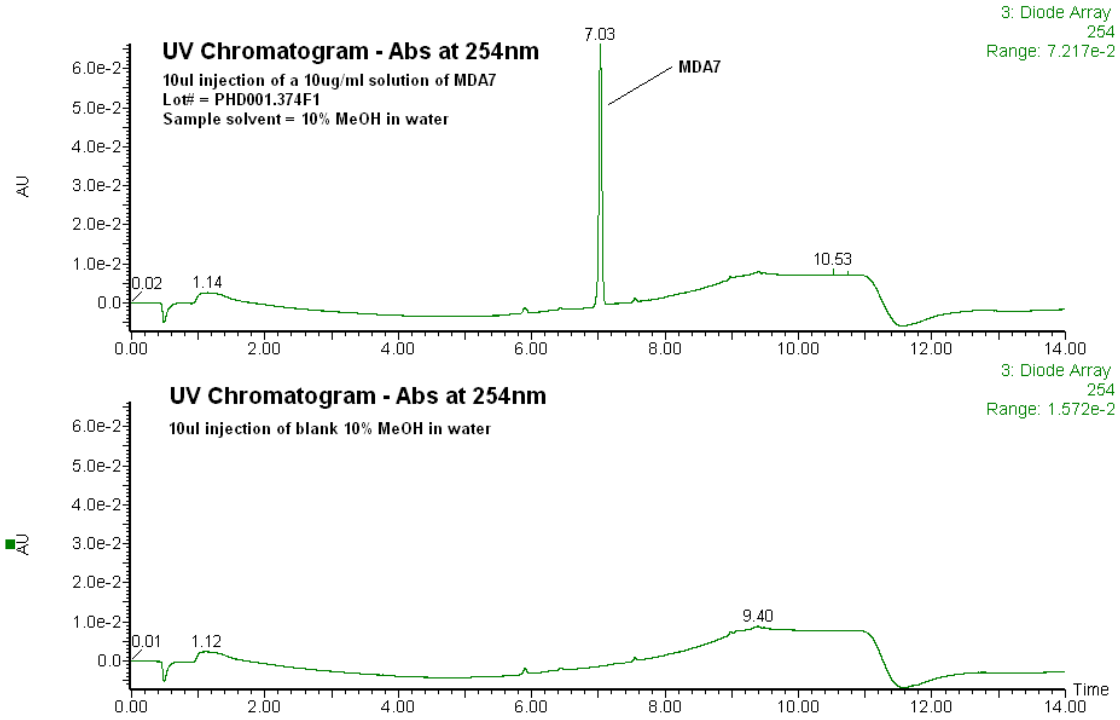


Figure 1a. UV absorption traces at ? = 254 nm. Purity is > 99%.

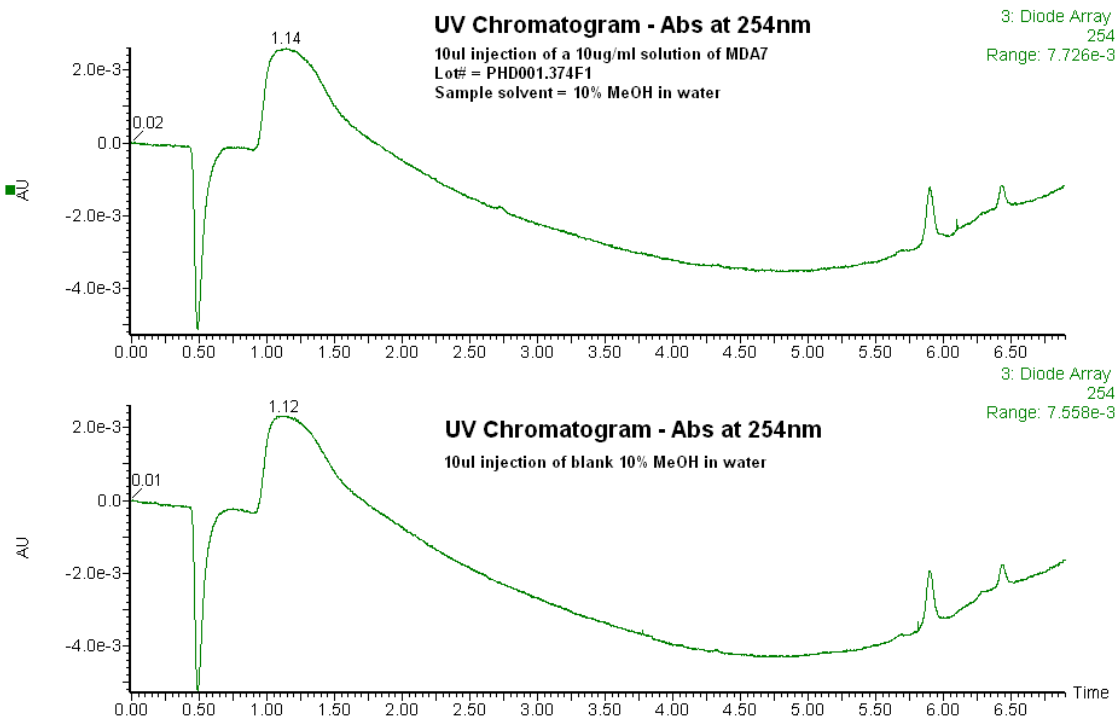


Figure 1b. UV absorption traces at ? = 254 nm expanded from 0 to 6.9 min. No differences/impurities observed. Purity is > 99%.

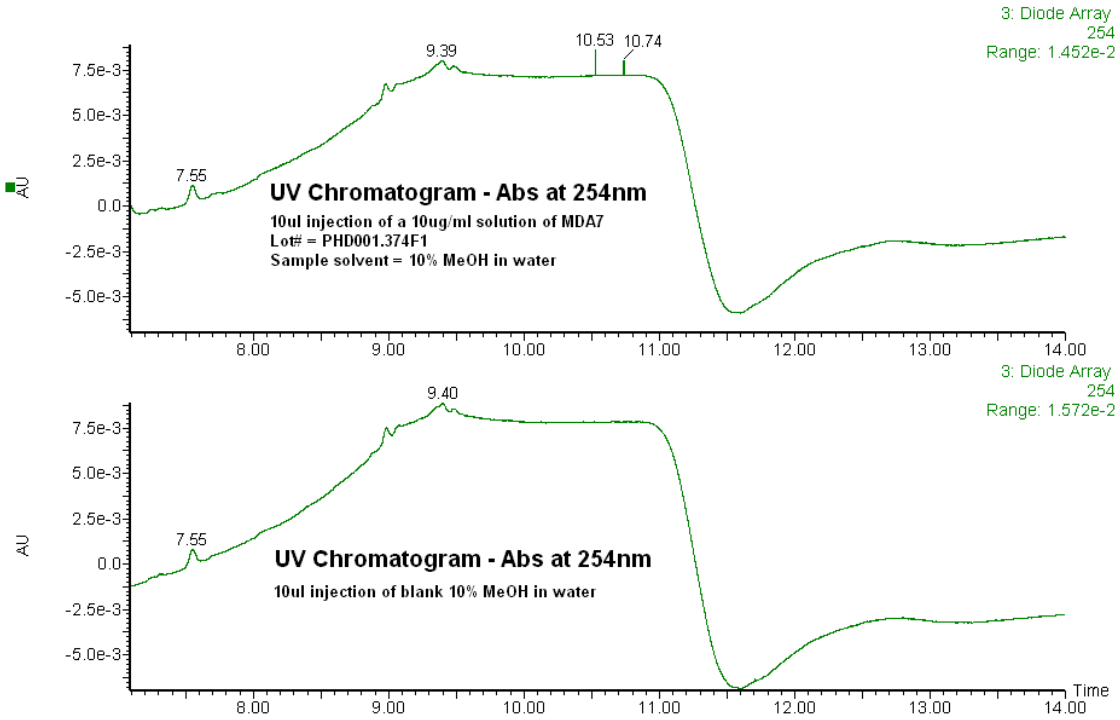


Figure 1c. UV absorption traces at ? = 254 nm expanded from 7.1 to 14 min. No differences/impurities observed. Purity is > 99%.

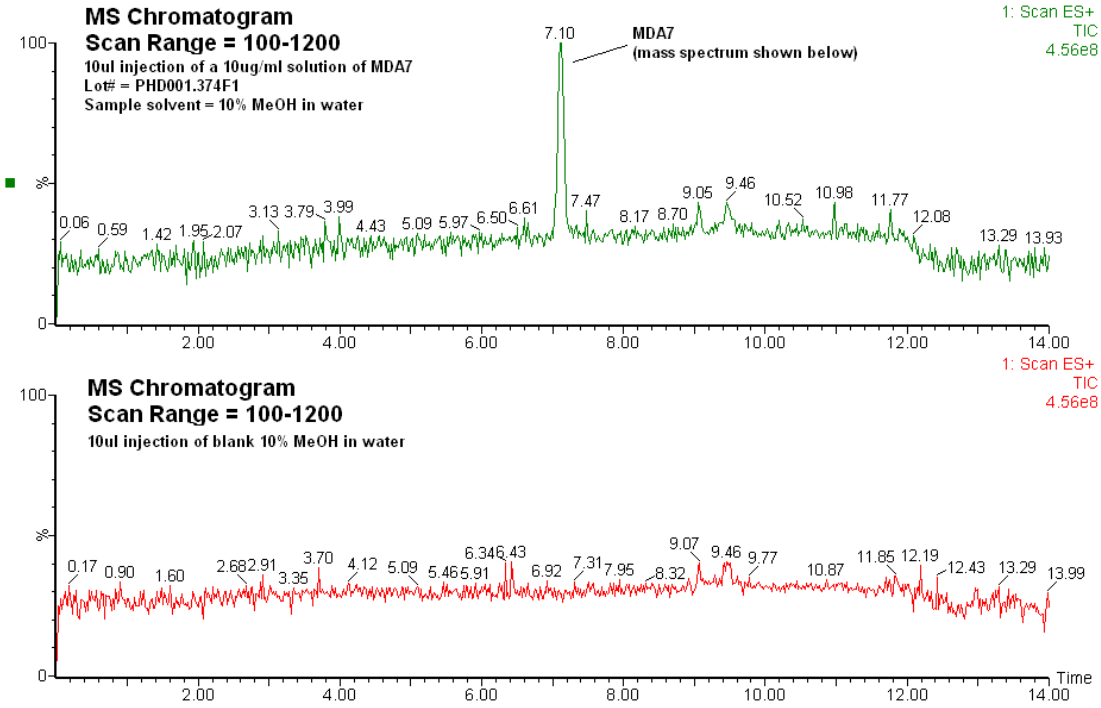


Figure 2. MS chromatograms. Scan range = 100-1200 *m/z*.

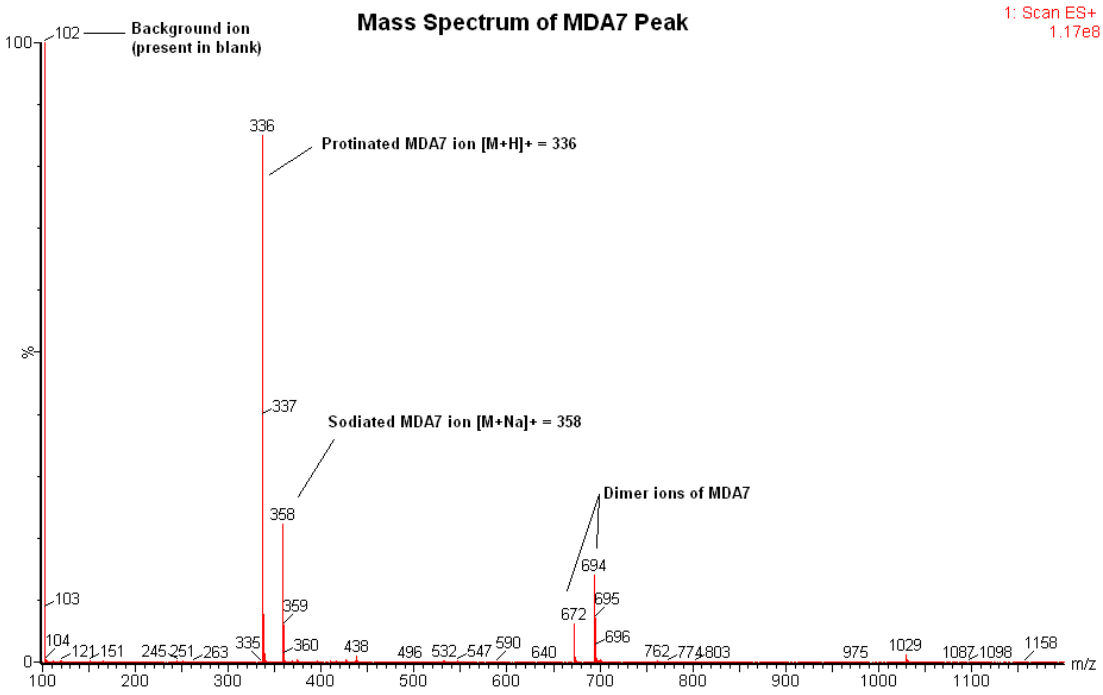


Figure 3. Mass spectrum of compound **18** peak in above MS chromatogram.

Procedure

Sample Preparation

First, a 1.0-mg/ml solution of compound **18** was made by weighing out 2.246 mg of solid compound **18** (Lot # PHD001.374F1), placing it in an amber vial, and then adding 2246 µl of methanol. This solution was then diluted 100x to a concentration of 10 µg/ml using a 10% methanol (in water) solution. Both a blank solution of 10% methanol and the 10 µg/ml solution of compound **18** were then analyzed by LC-UV-MS.

LC-UV-MS Methodology

The instrumentation consisted of a Waters Acquity LC system, equipped with a photodiode array (PDA) detector, interfaced to a Waters TQD mass spectrometer operated in ESI positive mode. The LC analysis was performed utilizing a reversed phase gradient system with the aqueous phase being 0.2% formic acid and the organic phase being methanol. The gradient timeline is outlined in the experimental details below. The LC column used was a Waters Acquity BEH C18 1.7 µm, 1.0x100mm column. The column was placed in a column heater that was maintained at 60°C. The samples were placed in a refrigerated autosampler that was maintained at 8°C. Ten microliters of sample was injected for each analysis. The UV range for the PDA was 210-500 nm. Purity was assessed at 254 nm. The mass spectrometer was scanned from 100 to 1200 *m/z*. All of the experimental details are outlined below.

Results

As is shown in Figures 1a through 1c, the UV trace of the compound **18** sample at 254 nm shows no additional peaks when compared to the blank solvent (10% MeOH) other than the compound **18** peak (UV retention time = 7.03 min). The purity was therefore determined to be > 99% by this method. The MS chromatogram shows the presence of a single major compound in the compound **18** sample (Figure 2), and this compound was identified as compound **18** based on the mass spectrum of this peak (Figure 3).

Experimental Details

LC/UV Parameters

Instrument:	Waters Acquity #1
Run Time:	14.00 min
Column:	Waters Acquity BEH C18 1.7 µm, 1.0x100mm
Flow Rate:	0.15 ml/min.
Solvent A:	Water w/ 0.2% FA
Solvent B:	Methanol
Seal Wash:	2.0 min
UV Range:	210-500 nm
UV Resolution:	1.2 nm
UV Sampling Rate:	20 points/sec
Column Temp:	60°C
Sample Temp:	8°C
Injection Volume:	10 µl

[Gradient Table]

	Time (min)	%A	%B
1.	Initial	90.0	10.0
2.	0.50	90.0	10.0
3.	8.00	5.0	95.0
4.	10.00	5.0	95.0
5.	11.00	90.0	10.0
6.	14.00	90.0	10.0

MS Parameters

Instrument	Waters TQD #1
Polarity	ES+
Capillary (kV)	3.20
Cone (V)	35.00
Extractor (V)	1.00
RF (V)	0.2
Source Temperature (°C)	125
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	20
Desolvation Gas Flow (L/Hr)	650
Collision Gas Flow (mL/Min)	Off
LM 1 Resolution	15.0
HM 1 Resolution	15.0
Ion Energy 1	0.5
MS Mode Entrance	50.00
MS Mode Collision Energy	3.00
MS Mode Exit	50.00
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy 2	1.0
Gain	1.00
Multiplier	-625.80
Mass Range	100 to 1200
Scan Time	0.5 sec

5. ADME-TOX DATA FOR COMPOUND 18

ADME-Tox: Aqueous Solubility and Plasma Protein Binding for Compound 18

5.1.1. Aqueous Solubility

Shake-flask method was used (Lipinski et al. Advanced Drug Delivery Reviews 2001;46:3-26, 2001). Reference compounds used were metoprolol, rifampicin, ketoconazole, phenytoin, haloperidol, simvastatin, diethylstilbestrol, and tamoxifen. Equilibration: 24 hours. Detection wavelength: 230 nm. Briefly, aqueous solubility (PBS Sigma, catalog number D-5652, pH 7.4) for the tested compound was determined by comparing the peak area of the principal peak in a calibration standard (200 µM in a 2% DMSO solution) containing organic solvent (methanol/water, 60/40, v/v) with the peak area of the corresponding peak in a buffer sample. In addition, chromatographic purity (%) was defined as the peak area of the principal peak relative to the total integrated peak area in the HPLC chromatogram of the calibration standard (Dionex). A chromatogram of the calibration standard of each test compound, along with a UV/VIS spectrum with labeled absorbance maxima, was generated.

5.1.2. Plasma Protein Binding with % Recovery

Equilibrium dialysis method was used (Banker et al. Journal of pharmaceutical sciences 2003;92:967-74). Three reference compounds were used: acebutolol, quinidine, and warfarin. Human plasma was purchased from Rockland, catalog number D519-06, pooled and mixed gender. 96-well dialysis apparatus: HTDialysis LLC (Gales Ferry, CT), part #1006. Incubation: 8 hours at 37°C in human plasma, 12-14K MWCO dialysis membrane 0.05 M phosphate buffer, pH 7.5. Briefly, the procedure is as follows: dialysis membrane strips are presoaked and rinsed prior to use. Following assembly of the 96-well dialysis apparatus, 0.15 mL of phosphate buffer is added to the dialysate side of each well. Plasma (unfiltered) is spiked with the test compound (10 µM), and 0.15 mL is added to the sample side of each well. The dialysis plate is sealed and incubated with shaking at 37°C until equilibrium is reached (8 hours). Equal volumes of sample are removed from the buffer and plasma sides of each well, diluted with acetonitrile, and then centrifuged. Also at this time, an additional sample is prepared (in duplicate) by spiking the test compound in plasma at 10 µM (1% DMSO) followed by sampling and diluting in acetonitrile/buffer in the same manner as the incubated plasma sample. This reference sample serves as the basis for a recovery determination. The supernatant of all samples is then analyzed by HPLC-MS/MS (Thermo Finnigan). The peak area of the test compound in the test buffer and plasma samples is used to calculate percent binding according to the following formula:

$$\text{Protein Binding}(\%) = \frac{(Area_{pe} - Area_{be}) \times \frac{V_{pe}}{V_{pi}}}{[(Area_{pe} - Area_{be}) \times \frac{V_{pe}}{V_{pi}}] + Area_{be}} \times 100$$

where $Area_{pe}$ = Peak area of analyte in plasma at equilibrium

$Area_{be}$ = Peak area of analyte in buffer at equilibrium

V_{pe} = Volume of plasma at equilibrium

V_{pi} = Initial volume of plasma

The sum of the peak areas of the test compound in the test buffer and plasma samples compared to the average peak area in the reference plasma samples is used to calculate percent recovery according to the following formula:

$$\text{Recovery (\%)} = \frac{(Area_{pe} + Area_{be})}{Area_{cs}} \times 100$$

where $Area_{pe}$ = Peak area of analyte in test plasma at equilibrium

$Area_{be}$ = Peak area of analyte in test buffer at equilibrium

$Area_{cs}$ = Average peak area of analyte in reference plasma sample

ADME-Tox: *In Vitro* Absorption

5.1.3. General Procedures

Assay	Cell	Passage Number	Days in Culture	Reference Compound	Bibliography
A-B Permeability *(*TC7*, pH 6.5/7.4)*	TC7	15 passages in culture between passages 20 and 40	13 to 25	propranolol, ranitidine, vinblastine, labetalol	Gres et al. Pharm Res 1998;15:726-33.
B-A Permeability (TC7*, pH 6.5/7.4)*	TC7	15 passages in culture between passages 20 and 40	13 to 25	propranolol, ranitidine, vinblastine, labetalol	Hunter et al. J Biol Chem 1993;268:14991-7.

Notes:
TC7 is a subclone of the Caco-2 cell line.

5.1.4. Experimental Conditions

Assay	***Test Concentration***	***Biological Conditions***	**Analytical Method**
A-B Permeability *(*TC7*, pH 6.5/7.4)*	10 µM in HBSS 1% DMSO	A-to-B flux at 37°C with shaking 96-well multiscreen plate	HPLC-MS/MS

	(n=2)	pH 6.5 in A and pH 7.4 in B Donor samples: time 0 and 60 min Receiver samples: time 60 min	
B-A Permeability (TC7, *pH 6.5/7.4)*	10 µM in HBSS 1% DMSO (n=2)	B-to-A flux at 37°C with shaking 96-well multiscreen plate pH 6.5 in A and pH 7.4 in B Donor samples: time 0 and 40 min Receiver samples: time 40 min	HPLC-MS/MS

Notes:
Multiscreen plate: 96-well plate, from Millipore, catalog number MACACO2S5

Abbreviations:
A: Apical side
B: Basolateral side
DMSO: Dimethylsulfoxide
HBSS: Hank's balanced salt solution, from Invitrogen, catalog number 11201, plus 5 mM MES, from Sigma, catalog number H 8652, pH 6.5
HBSS: Hank's balanced salt solution, from Invitrogen, catalog number 14065-056, plus 5 mM HEPES, from Sigma, catalog number H 3375, pH 7.4
HEPES: *N*-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
HPLC-MS/MS: HPLC coupled with tandem mass spectrometry (Instrumentation: Thermo Finnigan)
HPLC: High-performance liquid chromatography
MES: 2-(*N*-morpholino)-ethanesulfonic acid, from Sigma, catalog number M-8652

5.1.5. Analysis and Expression of Results

A-B Permeability

The apparent permeability coefficient (P_{app}) of the test compound in the apical to the basolateral direction is calculated as follows:

$$P_{app}(cm/s) = \frac{V_R \times C_{R60}}{\Delta t} \times \frac{1}{A \times (C_{D,mid} - C_{R,mid})}$$

where V_R is the volume of the receiver chamber; C_{R60} is the concentration of the test compound in the receiver chamber at time 60 minutes; Δt is the incubation time (60 minutes); A is the surface area of the cell monolayer; $C_{D,mid}$ is the calculated midpoint concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 minutes and the donor concentration at time 60 minutes; and $C_{R,mid}$ is the midpoint concentration of the test compound in the receiver side, which is one half of the receiver concentration at time 60 minutes. Concentrations of the test compound are expressed as peak areas of the test compound.

Recovery of the Test Compound from A-B Permeability Assay

The recovery of the test compound is calculated as follows:

$$Recovery(\%) = \frac{V_D \times C_{D60} + V_R \times C_{R60}}{V_D \times C_{D0}} \times 100$$

where V_D and V_R are the volumes of the donor and receiver chambers, respectively; C_{D60} is the concentration of the test compound in the donor sample at time 60 minutes; C_{R60} is the concentration of the test compound in the receiver sample at time 60 minutes; and C_{D0} is the concentration of the test compound in the donor sample at time zero. Concentrations of the test compound are expressed as peak areas of the test compound.

B-A Permeability

The apparent permeability coefficient (P_{app}) of the test compound in the basolateral to the apical direction is calculated as follows:

$$P_{app}(cm/s) = \frac{V_R \times C_{R40}}{\Delta t} \times \frac{1}{A \times (C_{D,mid} - C_{R,mid})}$$

where V_R is the volume of the receiver chamber; C_{R40} is the concentration of the test compound in the receiver chamber at time 40 minutes; Δt is the incubation time (40 minutes); A is the surface area of the cell monolayer; $C_{D,mid}$ is the calculated midpoint concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 minutes and the donor concentration at time 40 minutes; and $C_{R,mid}$ is the midpoint concentration of the test compound in the receiver side, which is one half of the receiver concentration at time 40 minutes. Concentrations of the test compound are expressed as peak areas of the test compound.

Recovery of the Test Compound from B-A Permeability Assay

The recovery of the test compound is calculated as follows:

$$Recovery(\%) = \frac{V_D \times C_{D40} + V_R \times C_{R40}}{V_D \times C_{D0}} \times 100$$

where V_D and V_R are the volumes of the donor and receiver chambers, respectively; C_{D40} is the concentration of the test compound in the donor sample at time 40 minutes; C_{R40} is the concentration of the test compound in the receiver sample at time 40 minutes; and C_{D0} is the

concentration of the test compound in the donor sample at time zero. Concentrations of the test compound are expressed as peak areas of the test compound.

Fluorescein assessment for TC7 Permeability assay

Fluorescein is used as the cell monolayer integrity marker. Fluorescein permeability assessment (in the A-B direction at pH 7.4 on both sides) is performed after the permeability assay for the test compound. A cell monolayer that has a fluorescein permeability of less than 0.5×10^{-6} cm/s is considered intact, and the permeability result of the test compound from intact cell monolayer is reported.

ADME-Tox: Genetic Toxicity for Compound 18

5.1.6. General Procedures

Assay	Cell Type	Reference Compound
Bacterial cytotoxicity Assay (strain TA98)	His-reverted Salmonella typhimurium Strain TA98	Mitomycin C
Bacterial cytotoxicity Assay (strain TA100)	His-reverted Salmonella typhimurium Strain TA100	Mitomycin C
Ames test (strain TA98)	Salmonella typhimurium Strain TA98	Aminoanthracene, mitomycin C, quercetin, streptozotocin
Ames test (strain TA98+S9)	Salmonella typhimurium Strain TA98 and rat liver S9 (0.2 mg/mL)	Aminoanthracene, mitomycin C, quercetin, streptozotocin
Ames test (strain TA100)	Salmonella typhimurium Strain TA100	Aminoanthracene, mitomycin C, quercetin, streptozotocin
Ames test (strain TA100+S9)	Salmonella typhimurium Strain TA100 and rat liver S9 (0.2 mg/mL)	Aminoanthracene, mitomycin C, quercetin, streptozotocin

5.1.7. Experimental Conditions

Assay	Substrate	Incubation	Reaction Product	Method of Detection
Bacterial cytotoxicity Assay (strain TA98)	Test compound (8 concentrations with dilutions from 100 *µM)* 1 % DMSO (n=3)	96 hours, 37°C	Growth (OD_{650})	Photometry
Bacterial cytotoxicity Assay (strain	Test compound (8 concentrations with dilutions from 100 *µM)*	96 hours, 37°C	Growth (OD_{650})	Photometry

TA100)	1 % DMSO (n=3)			
Ames test (strain TA98)	Test compound (5, 10, 50 and 100 *μM)* 1 % DMSO (n=48)	96 hours, 37°C	Bacterial growth due to mutation reversion	Photometry
Ames test (strain TA98+S9)	Test compound (5, 10, 50 and 100 *μM)* 1 % DMSO (n=48)	96 hours, 37°C	Bacterial growth due to mutation reversion	Photometry
Ames test (strain TA100)	Test compound (5, 10, 50 and 100 *μM)* 1 % DMSO (n=48)	96 hours, 37°C	Bacterial growth due to mutation reversion	Photometry
Ames test (strain TA100+S9)	Test compound (5, 10, 50 and 100 *μM)* 1 % DMSO (n=48)	96 hours, 37°C	Bacterial growth due to mutation reversion	Photometry

Analysis and Expression of Results

Bacterial Cytotoxicity

The results for cytotoxicity are expressed as percent of control growth (OD_{650}). Compounds with growth of less than 60 % of control are flagged and considered cytotoxic.

Ames Tests

Wells that display bacteria growth due to the reversion of the histidine mutation (as judged by the ratio of OD_{430}/OD_{570} being greater than 1.0) are counted and recorded as positive counts. The significance of the positive counts between the treatment (in the presence of test compound) and the control (in the absence of test compound) is calculated using the one-tailed Fisher's exact test.
Three significance levels are reported as follows:

Weak positive, if $0.01 = p < 0.05$, denoted as "+"
Strong positive, if $0.001 = p < 0.01$, denoted as "++"
Very strong positive, if $p < 0.001$, denoted as "+++"

Table 9. Bacterial cytotoxicity assay.

Assay	Compound	Test conc. (M)	Cytotoxicity (% of control)	Reference Compound	IC_{50} (M)	Ref
Strain TA98	**18**	$6.3E^{-07}$	97	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$1.3E^{-06}$	96	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$2.5E^{-06}$	92	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$5.0E^{-06}$	92	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$1.0E^{-05}$	97	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$2.5E^{-05}$	91	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$5.0E^{-05}$	89	Mitomycin C	$3.7E^{-08}$	
Strain TA98	**18**	$1.0E^{-04}$	89	Mitomycin C	$3.7E^{-08}$	
Strain TA100	**18**	$6.3E^{-07}$	100	Mitomycin C	$2.7E^{-08}$	
Strain TA100	**18**	$1.3E^{-06}$	99	Mitomycin C	$2.7E^{-08}$	
Strain TA100	**18**	$2.5E^{-06}$	96	Mitomycin C	$2.7E^{-08}$	

Strain TA100	**18**	$5.0E^{-06}$	97	Mitomycin C	$2.7E^{-08}$
Strain TA100	**18**	$1.0E^{-05}$	97	Mitomycin C	$2.7E^{-08}$
Strain TA100	**18**	$2.5E^{-05}$	97	Mitomycin C	$2.7E^{-08}$
Strain TA100	**18**	$5.0E^{-05}$	95	Mitomycin C	$2.7E^{-08}$
Strain TA100	**18**	$1.0E^{-04}$	95	Mitomycin C	$2.7E^{-08}$

Table 10. Ames test with TA98 and TA100.

Compound	**Assay**	**Test concentration (M)**	**Positive significance (- to +++)**	**Fisher Exact test (p value)**
18	Ames test (strain TA98)	$5.0E^{-06}$ to $1.0E^{-04}$	-	
18	Ames test (strain TA98+S9)	$5.0E^{-06}$ to $1.0E^{-04}$	-	
18	Ames test (strain TA100)	$5.0E^{-06}$ to $1.0E^{-04}$	-	
18	Ames test (strain TA100+S9)	$5.0E^{-06}$ to $1.0E^{-04}$	-	
Mitomycin C	Ames test (strain TA98)	$1.0E^{-05}$	-	1.0000
Streptozotocin	Ames test (strain TA98)	$1.5E^{-07}$	-	1.0000
Aminoanthracene	Ames test (strain TA98)	$3.0E^{-05}$	-	1.0000
Quercetin	Ames test (strain TA98)	$2.5E^{-06}$	+++	0.0000
Mitomycin C	Ames test (strain TA98+S9)	$1.0E^{-05}$	-	0.0062
Streptozotocin	Ames test (strain TA98+S9)	$1.5E^{-07}$	-	0.3795
Aminoanthracene	Ames test (strain TA98+S9)	$3.0E^{-05}$	+++	0.0000
Quercetin	Ames test (strain TA98+S9)	$2.5E^{-06}$	+++	0.0000
Mitomycin C	Ames test (strain TA100)	$1.0E^{-05}$	-	0.5000
Streptozotocin	Ames test (strain TA100)	$1.5E^{-07}$	+++	0.0000
Aminoanthracene	Ames test (strain TA100)	$3.0E^{-05}$	-	0.3085
Quercetin	Ames test (strain TA100)	$2.5E^{-06}$	-	0.1808
Mitomycin C	Ames test (strain TA100+S9)	$1.0E^{-05}$	-	0.0793
Streptozotocin	Ames test (strain TA100+S9)	$1.5E^{-07}$	+++	0.0000
Aminoanthracene	Ames test (strain TA100+S9)	$3.0E^{-05}$	+++	0.0000
Quercetin	Ames test (strain TA100+S9)	$2.5E^{-06}$	+	0.0163

ADME-Tox: Cardiac Toxicity

5.1.8. General Procedures

Assay: K^+ (hERG) (automated patch-clamp)
Cells: CHO-Kl cell line stably expressing hERG
Reference Compound: E-4031 from Wako, catalog number 052-06523.
E-4031 was tested as the reference compound concurrently with the test compound to ensure the sensitivity of the cell line.

5.1.9. Experimental Conditions

Assay: K^+ (hERG) (automated patch-clamp)
Incubation: 5 min with the test compound dosing solutions at room temperature cumulatively.
Conditions (mM): Intracellular Solution: 130 KCI, 10 NaCI, I $MgCl_2$, 10 EGTA, 5 MgATP, 10 HEPES (pH adjusted to 7.2 with 1 M KOH). Extracellular Solution: 137 NaCI, 4 KCI, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 D(+)-Glucose, 10 HEPES (pH adjusted to 7.4 with 1 M NaOH)
Method of Detection: Automated whole-cell patch clamp (by Qpatch 16)

Notes:
Qpatch 16 (by Sophion Bioscience, Denmark) is used in the automated patch clamp assay.

Abbreviations:
CHO-KI: Chinese hamster ovary cell line
EGTA: Ethylene glycol-bis(aminoethyl ether)-N, N, N', N'-tetraacetic acid
FBS : Fetal bovine serum
HEPES: *N*-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
hERG: Human ether-a-go-go related gene (gene encoding IKr)
IKr: Major repolarizing current in cardiac myocytes (rapid component of IK, the delayed rectifier current)
MgATP: Adenosine triphosphate, magnesium salt

hERG (automated patch-clamp)
CHO-Kl cells were originally obtained from American Tissue Culture Collection. hERG cDNA (OenBank sequence NM_000238) was subcloned into pSI vector (Promega). The CHO-Kl cells were co-transfected with this construct and pPUR (containing puromycin selective marker, BD Bioscience). After selection in puromycin for 10 days, single colonies were selected and verified with hERG potassium currents. Stably transfected cells were used in this study. The stably transfected cells were cultured in F-12 Kaighn's nutrient mixture medium (Invitrogen) + 10% FBS at 37°C for 1-3 days. Cells were kept at 30°C for 24 to 48 hours before patch clamp experiment in order to increase the hERO current amplitude. Subsequently the cells were harvested by trypsination and kept in serum-free medium for up to 6 hours at room temperature

before recording. The cells were washed and resuspended in extracellular solution before being applied to the patch clamp sites.

After whole cell configuration was achieved, the cell was held at -80 mV. A 50-ms pulse to -40 mV was delivered to measure the leaking current, which was subtracted from the tail current online. Then the cell was depolarized to +20 mV for 2 seconds, followed by a 1-s pulse to -40 mV to reveal hERG tail current. This paradigm was delivered once every 5 s to monitor the current amplitude. The extracellular solution (control) was applied first, and the cell was stabilized in extracellular solution for 5 minutes. Then the test compound was applied from low concentrations to high concentrations cumulatively. The cell was incubated with each test concentration for 5 minutes. During the incubation, the cell was repetitively stimulated using the voltage protocol described above, and the tail current amplitude was continuously monitored.

5.1.10. Analysis and Expression of Results

hERG (automated patch-clamp) Data were acquired and analyzed by Qpatch (Sophion Bioscience), and Excel (Microsoft) and are reported as mean and individual values. The degree of inhibition (%) was obtained by measuring the tail current amplitude before and after drug incubation (the difference current was normalized to control and multiplied by 100 to obtain the percent of inhibition).

The reference compound, E-4031, yielded comparable inhibition on hERG tail current (IC_{50} = 24.3 nM, n=2, 25.4 nM and 23.2 nM, respectively). The individual values for the effects of compound **18** are summarized below.

*Compound **18***	*% Inhibition of Tail current*		
Test Concentration	1^{st}	2^{nd}	Mean
$1.0E^{-07}$	2.5	6.0	4.2
$1.0E^{-06}$	8.5	15.2	11.9
$1.0E^{-05}$	28.5	25.8	27.0