Identification of portimine B, a new cell permeable spiroimine that induces apoptosis in oral squamous cell carcinoma

Andrew M. Fribley [Φ,] *, Yue Xi [Φ], Christina Makris,[¥] Catharina Alves-de-Souza,[δ] Robert York , [δ] Carmelo Tomas, [δ] Jeffrey L.C. Wright, [¥Δ] Wendy K. Strangman[Δ]*

S2: Experimental

A clonal culture of *Vulcanodinium rugosum* strain CCFWC516 was grown in an Ika 10L photobioreactor. It was cultured at 22^{o}C with a photo period of 16:8, in L1 nutrient medium with a salinity of a 33 psu for 45 days. The culture was fed with additional L1 medium at days 15 and 30 and harvested via flowthough centrifugation before storage at -80^{o}C. The frozen cell pellet was then extracted two times with 500mL of 100% MeOH and filtered onto glass microfibre (VWR, 691) paper. A final extraction with 4:1 MeOH:H_2O was also filtered, combined, and dried *in vacuo*. The residue was then re-dissolved in 500mL of 90%MeOH and partitioned against hexane. The aq MeOH fraction was then dried to HP20 resins for desalting and primary fractionation. The extract-bound resins were washed with 100% water and the remaining de-salted extract was eluted with increasing concentrations of acetone. The dried desalted extract was then fractionated on a 10g C_{18} SPE cartridge with a stepwise elution of MeCN and H_2O (0.1% FA). LC-MS monitoring indicated that the spiroimines eluted in the 40% and 50% MeCN fractions. The portimine containing fractions were then purified by size-exclusion LH20 chromatography in 100% MeOH and finally with reversed phase HPLC (semi-prep YMC Pack ODS-AM 5μm x 12nm 250x10mm column) under isocratic conditions (30% MeCN: 70% H_2O (0.05% Formic acid) 2.5mL/ min (RT **1**: 10.8 min, RT **2**: 11.4 min). UV λmax was acquired with a Genesys 10s UV-Vis (1:1 MeCN:H_2O) λmax (**2**) 225 nm; IR was acquired on a Thermo scientific Nicolet iS5 FTIR equipped with an iD7 attenuated total reflection (ATR) diamond crystal accessory: IR ν_{max} (**2**) 3366, 2925, 1701, 1374, 1262, 1067 cm^{-1}.

NMR analyses were performed using a Bruker® Avance 1 500 MHz system run by TopSpin version 2.0. HPLC isolation was performed using a Waters Breeze HPLC system with a Waters dual wavelength detector (210 and 240 nm). LC-MS monitoring was performed on a Waters QDa mass detector tandem to a Waters I-Class UPLC PDA instrument. All solvents were of HPLC grade and were used without further purification. The HRMS spectrum was obtained on Waters UPLC I-Class system coupled to a Waters Xevo-G2XS QToF-MS mass spectrometer (Waters, Manchester, UK). The system was operated in electrospray positive mode (ESI^+) with the capillary voltage set at 2.5kV, source and desolvation temperatures at 100°C and 550°C respectively and desolvation gas flow at 800 L/h. MS-MS data were acquired with the same instrument under the same conditions with a set mass of 418.2 and a collision energy of 35eV. All solvents were of mass spectrometry grade and were used without further purification.

Metabolomics

UPLC chromatography was performed with a Waters Acquity BEH C18 2.1 x 100mm 1.7 μm column and gradient elution (flow rate 0.5 mL/min; mobile phase A: water (0.1% formic acid); mobile phase B: MeCN (0.1% formic acid); hold at 5% B for 1 minute, 5-65% B over 8 min., 65-100% B over 1 min., hold at 100% B for 1.5 min., re-equilibrate at 5% B for 4.5 minutes). Mass spectrometry data were acquired on a Qtof mass spectrometer in positive mode with simultaneous acquisition of a lockmass solution (leucine encephalin) in continuum mode with the MS^e function (mass range 50-1800 Da; scan time 0.1 scans/sec; function 1 CE off; function 2 CE ramp 45-50 V; lockmass data acquired but not applied). Non-lockmass corrected data were imported into Progenesis QI software (Nonlinear Dynamics, UK) for further analysis including spectra alignment, lockmass calibration, peak picking and principal component analysis (PCA). Alignment was performed on QC samples and defaults were applied for peak picking. The retention time was restricted to 1-10 minutes.

PAMPA Assay

Stock solutions for control compounds were prepared by creating a 10mM solution in DMSO. Extract stock solutions were prepared at 20mg/mL in DMSO. A 96 well donor and acceptor plate comprising the Corning Gentest kit were prepared by adding 300μL of 5%DMSO in PBS (PH=7.4) to each donor well and 200μL of the same solution to each of the acceptor wells. Donor well solutions of control compounds were prepared by diluting the stock solution to a final concentration of 100μM with 5%DMSO in PBS before addition to the donor plate. Donor well solutions of extracts were prepared by diluting 12μL of stock solution with 288μL of 5%DMSO in PBS. The acceptor plate was then placed on top of the donor plate so that the membrane was in contact with the donor solution. A lid was placed on the acceptor plate and the union was wrapped with teflon tape to prevent evaporation. The plate was allowed to sit at room temperature for 6 hours. After 6 hours, the donor and acceptor well solutions were removed to sealed mass spectrometry vials for analysis. UPLC-Mass Spectrometry data were acquired as described above. Extracted ion counts (EIC) were acquired in Masslynx. Percent permeability was calculated by dividing the EIC of the target compound in the acceptor well after the 6 hour incubation by the EIC of the compound in the donor well at 6 hours.

Cell lines

The laryngeal squamous cell carcinoma cell line UMSCC23 was derived from a primary lesion from a female patient (T2N0M0) and was provided by Dr. Thomas Carey at the University of Michigan. The tongue squamous cell carcinoma cell line WSU-HN12 was derived from a metastatic lymph node from a female patient and was provided by Dr. George Yoo at the Karmanos Cancer Center at Wayne State University. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum under standard tissue culture conditions.

Proliferation assay

The ATP-based Cell Titer-Glo Cell Viability Assay (Promega Corp., Madison, WI) was used according to the manufacturer's protocol. Briefly, 5000 cells were plated into 96 well white culture plates(50 μL final volume) overnight at 37 °C and 5% CO2. Cells were treated as indicated and at the termination of the experiment the luminescent substrate was added and relative luciferase units (RLU) were appreciated using a Flex Station 3 Multimode Plate Reader (Molecular Devices, San Jose, CA); data were expressed as the percent survival compared to DMSO vehicle controls. All experiments were performed at least three times with triplicate samples, error bars represent standard error of the mean.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was harvested from portimine A, portimine B or DMSO treated UMSCC23 and HN12 cultures using Cells to CT™ (Ambion; Life Technologies) as previously described [1]. cDNA libraries were then generated with SsoFast Supermix (Bio-Rad, Hercules, CA) and interrogated with TaqMan (Life Technologies) primer probes for gene expression analysis. Primer probes were as follows: *18S* (Hs99999901_s1), *DR5/TNFRSF1* (Hs00366 278_m1), *NOXA/PMAIP1* (Hs00560402_ m1), *BCL2* (Hs00153350_m1), *GADD45*α (Hs00169255_m1); the $^{\Delta\Delta}$Ct method was used to calculate fold change with respect to 18S. All experiments were performed with triplicate samples each measured three times; data are represented as standard error of the mean.

DNA Fragmentation

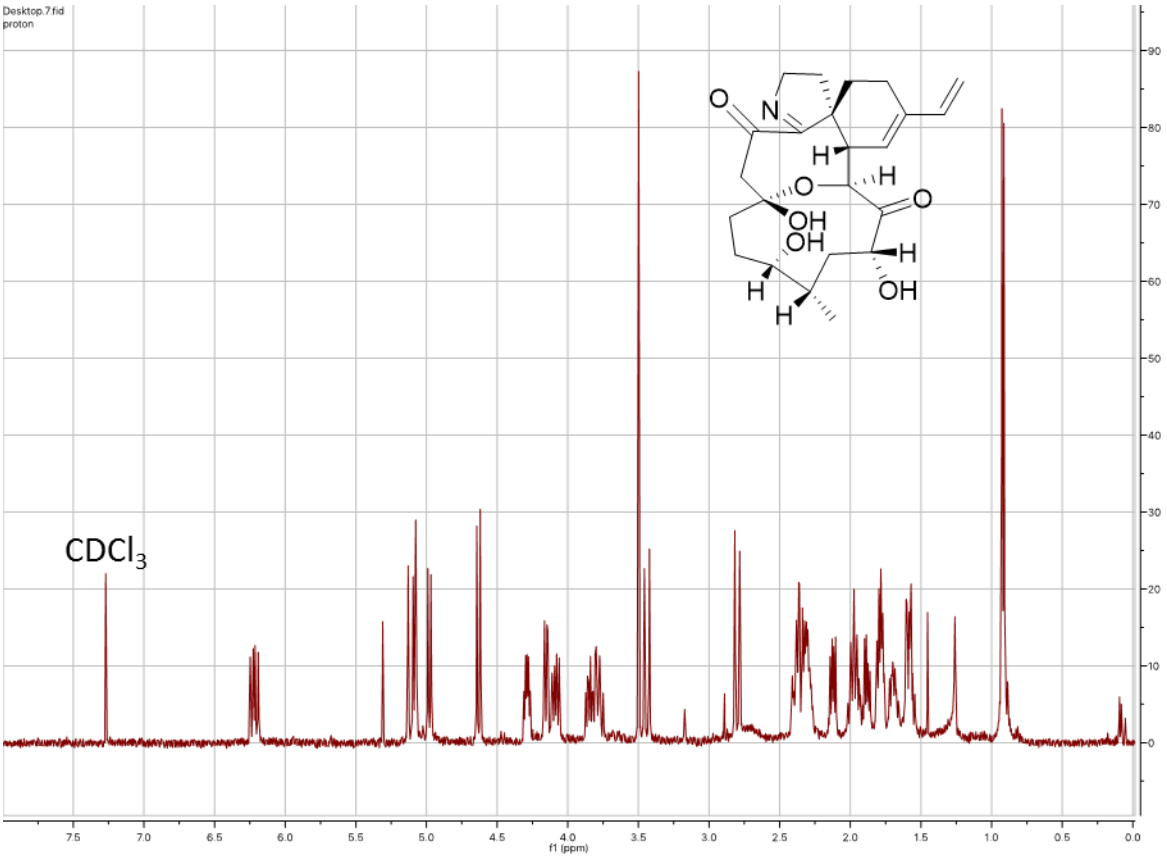
The ability of portimines to fragment genomic DNA, a hallmark of apoptosis, was determined according to our previously published protocol [1]. Briefly, HN12 cells were incubated with portimine A and B for 48h. Cells were collected by centrifugation and lysed; the soluble fraction was then extracted with phenol-chloroform. Ethanol precipitated DNA was electrophoretically resolved on a 1.5% agarose gel.

1. Fribley, A.M., et al., *Large-scale analysis of UPR-mediated apoptosis in human cells.* Methods Enzymol, 2011. **491**: p. 57-71.

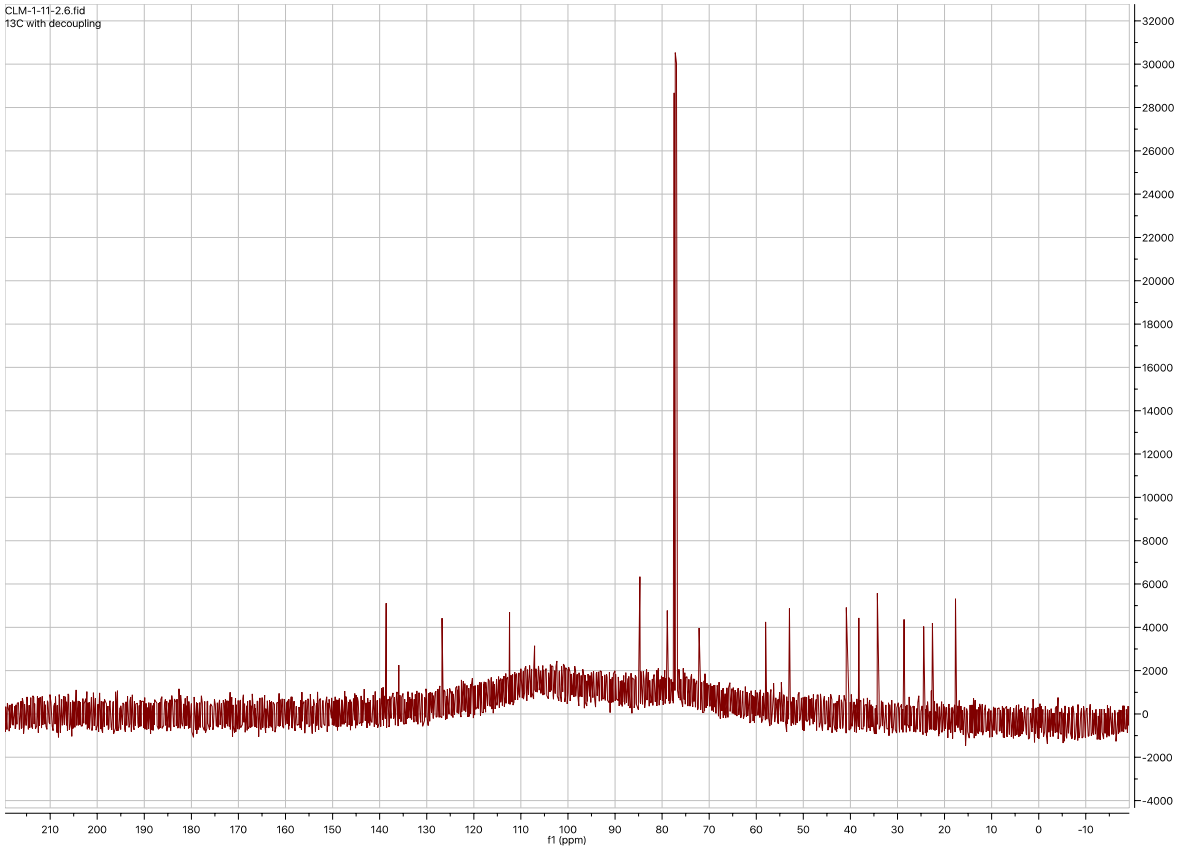
S5: NMR Assignments for portimine B (**1**) in $CDCl_3$ (500MHz for ^1H and 125 MHz for ^{13}C).

#	^1H	^{13}C	COSY	TOCSY	HMBC	NOESY
1	4.09, CH_2 3.85	58.4	1B, 2A,B 1A, 2A,B	1B, 2A,B 1A, 2A,B	2,3,4 3	1B, 2A 1A, 2B
2	1.99, CH_2 1.89	28.8	1A,B, 2B 1A,2A	1A,B, 2B 1A,B, 2A	20 3,4,20	1A, 15 1B
3		55.1				
4		182.9				
5		201.4				
6	3.43, CH_2 2.80	53.2	6B 6A	6B 6A	5,7,8 4,5,7,8	6B, 16 6A, 8B
7		107.2				
8	2.14, CH_2 1.58	38.5	8B, 9A 8A, 9B	8B, 9A,B, 10 8A, 9A,B, 10	7,9,10 7,9,10	8B, 9A,B 6B, 8A, 9B, 10
9	1.96, CH_2 1.79	24.6	10 8B, 10	8A,B, 9B, 10, 11, 12A,B, 23 8A,B, 9A, 10, 11, 12A,B, 23	8,10,11 7	8A, 23 8A,B, 10, 23
10	4.29, CH	85.0	9A,B, 11	8A,B, 9A,B, 11, 12A,B, 23	12	8B, 9B, 11, 23
11	2.33, CH	34.7	10, 12A,B, 23	9A,B, 10, 12A,B, 13, 23	9,10,13,23	10, 13, 23
12	2.38, CH_2 1.59	34.3	11, 13 11, 13	9A,B, 10, 11, 12B, 13, 23 9A,B, 10, 11, 12B, 13, 23	10,11,13,14,23 10,11,13,14,23	15 13, 23
13	4.16, CH	79.1	12 A,B	11,12A,B, 23	11	11, 12B
14		204.8				
15	4.63, CH	72.4	16	16, 17	7,14,16,17	2A, 12A, 16, 17
16	3.79, CH	41.2	15	15, 17		6A, 15, 17, 20B
17	5.09, CH	126.9		15, 16	3,15,16,19,21	15, 16, 21
18		135.8				
19	2.35, CH_2 2.35	22.8	20A,B	20A,B	3,17,18	22A
20	1.79, CH_2 1.71	28.8	19A,B, 20B 19A,B, 20A	19, 20B 19, 20A	3,16,18,19 2	 2B, 16
21	6.22, CH	138.8	22A,B	22A, B	17,18,19	17, 22B
22	5.12, CH_2 4.98	112.5	21, 22B 21, 22A	21, 22B 21, 22A	18,21	19, 22B 21, 22A
23	0.93, CH_3	18.0	11	9A,B, 10, 11, 12A,B, 13	10,11,12	9A,B, 10, 11, 12B

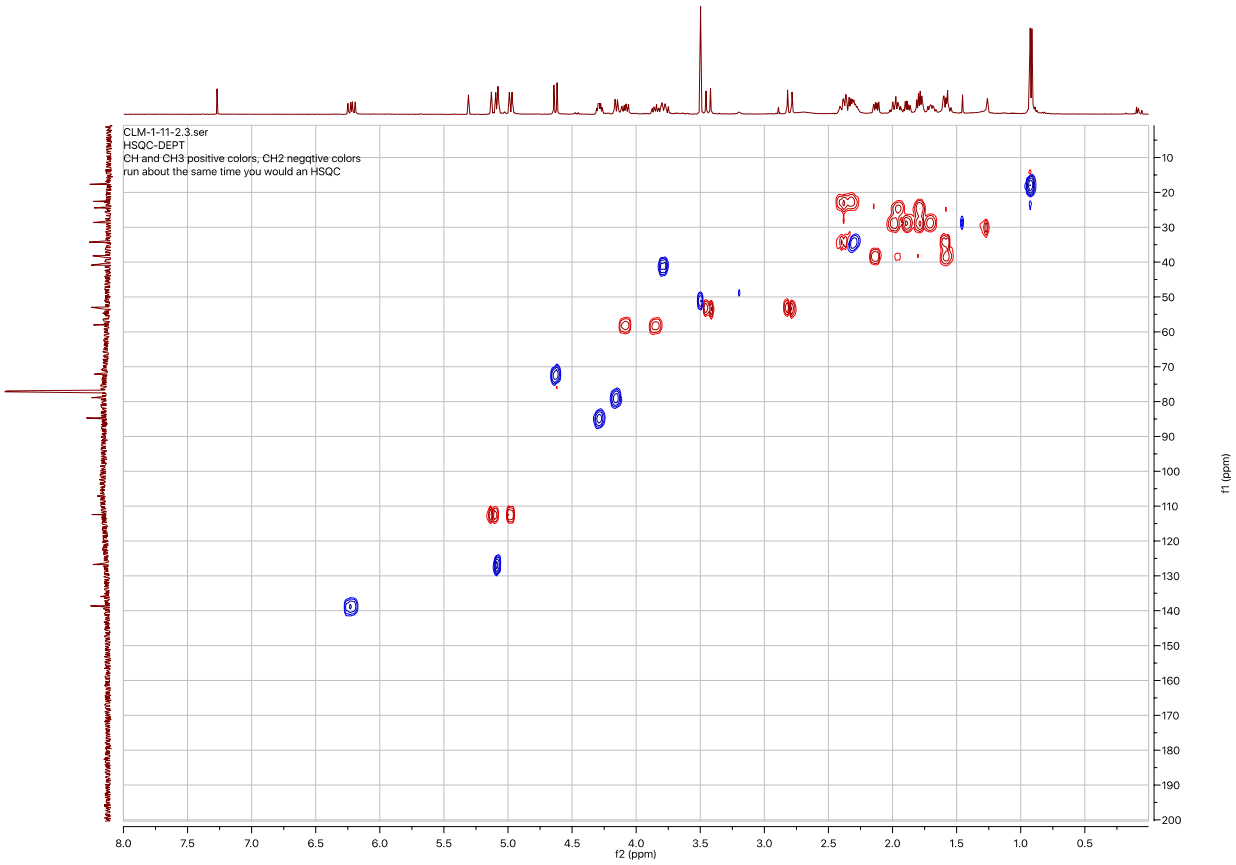
S6: ^{1}H NMR spectrum for portimine B (**2**)-recorded in $CDCl_3$at 500MHz



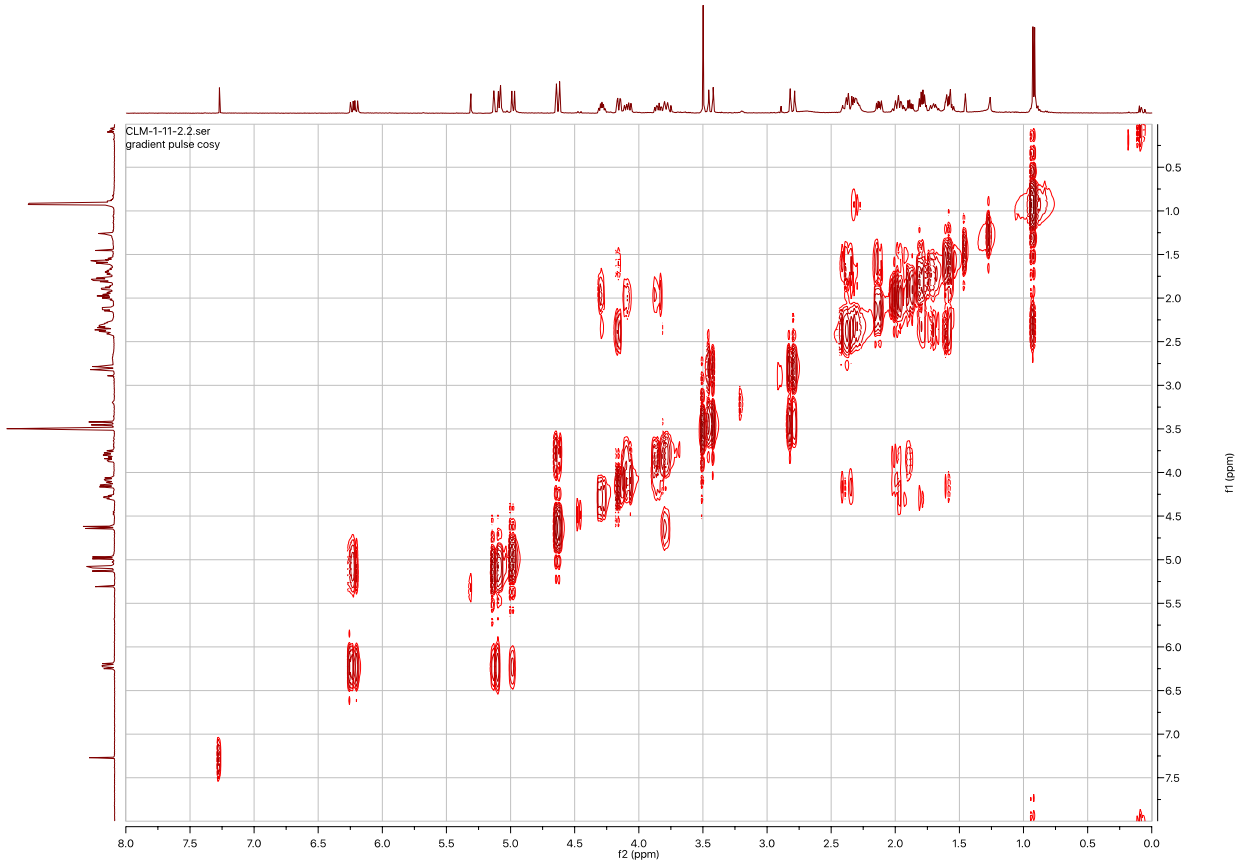
S7: ^{13}C NMR spectrum for portimine B (**2**)-recorded in $CDCl_3$ at 125MHz



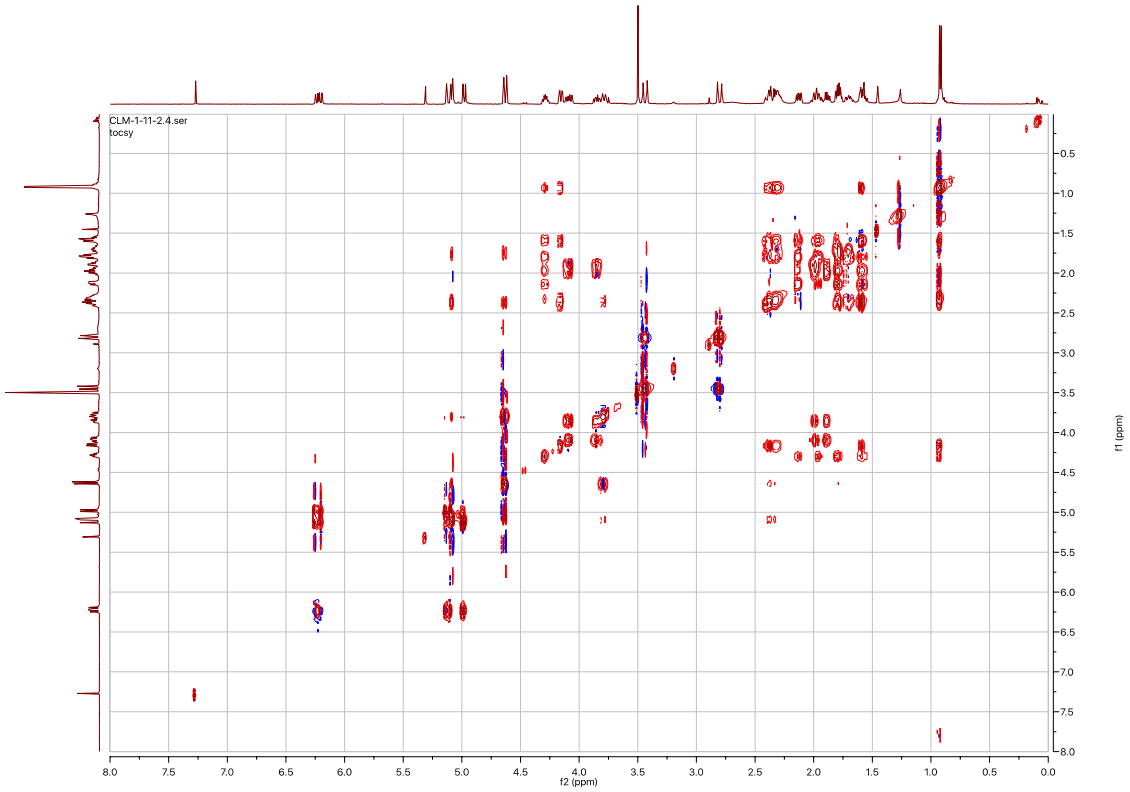
S8: HSQC-DEPT NMR spectrum for portimine B (**2**)-recorded in $CDCl_3$at 500MHz



S9: COSY NMR spectrum for portimine B (**2**)-recorded in $CDCl_3$at 500MHz

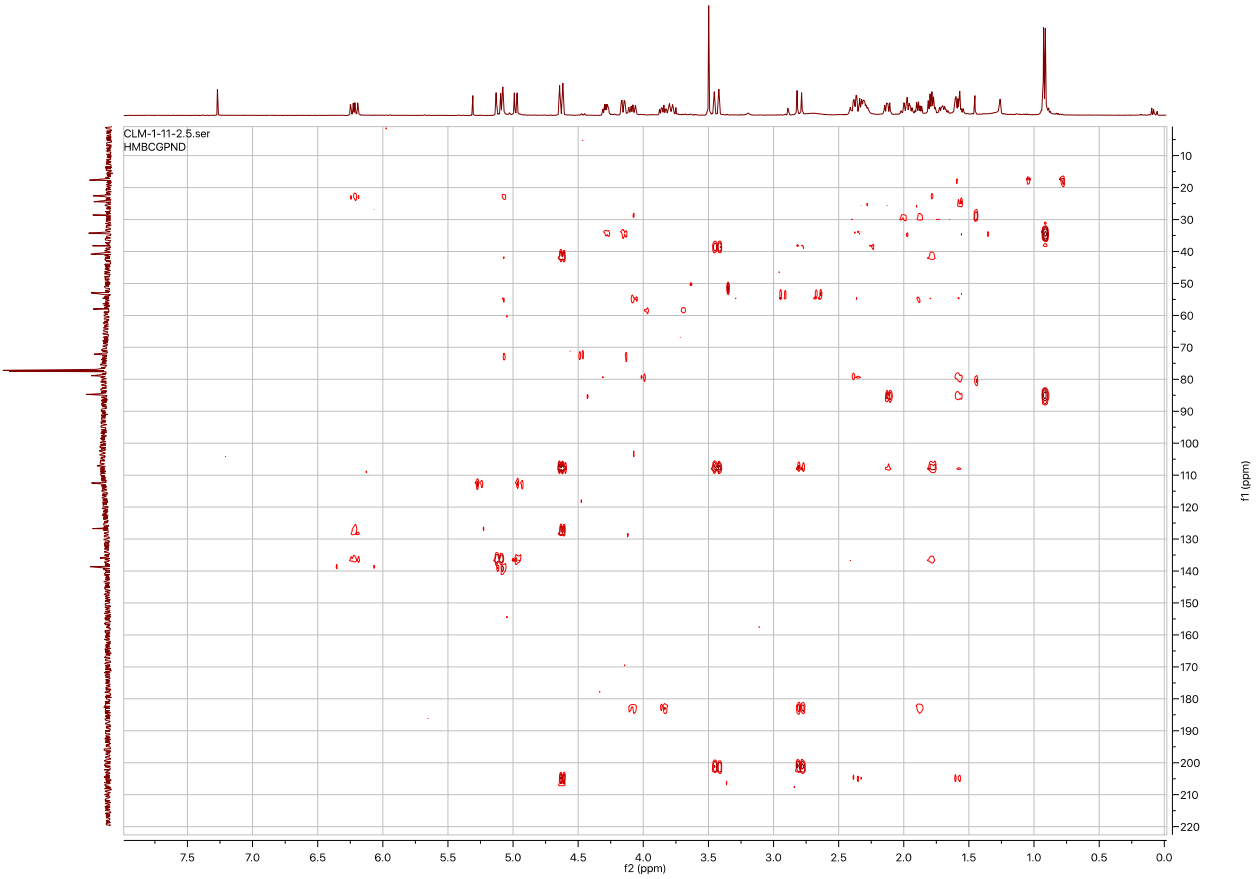


S10: TOCSY NMR spectrum for portimine B (**2**)-recorded in $CDCl_3$at 500MHz

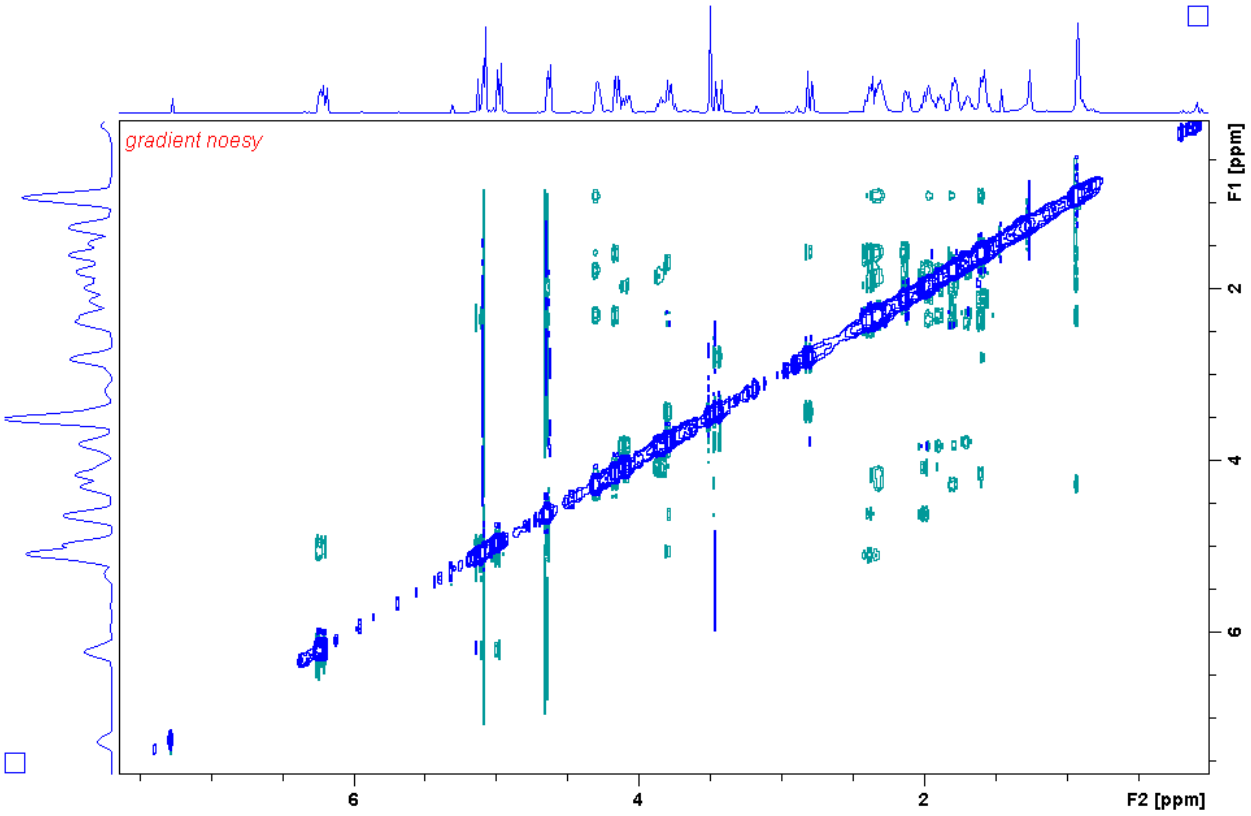


TOCSY 2-D NMR spectrum

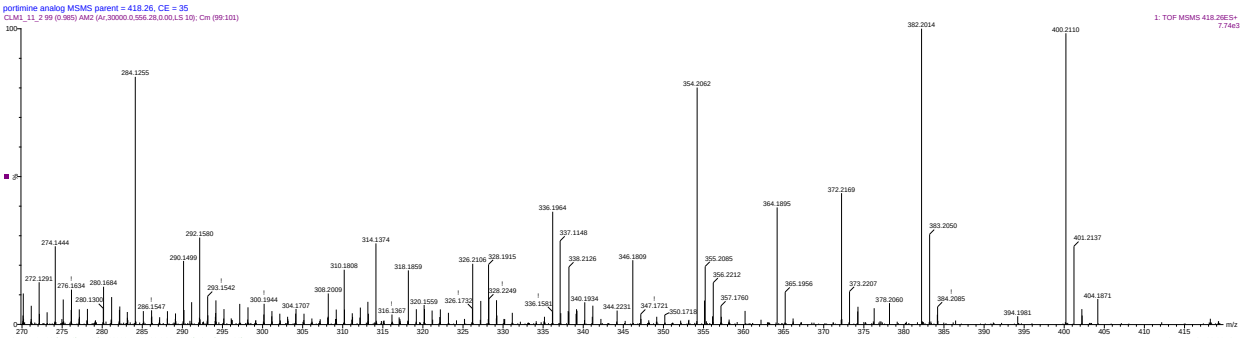
S11: HMBC NMR for portimine B (**2**)-recorded in $CDCl_3$at 500MHz



S12: NOESY NMR spectrum for portimine B (**2**)-recorded in $CDCl_3$ at 500MHz

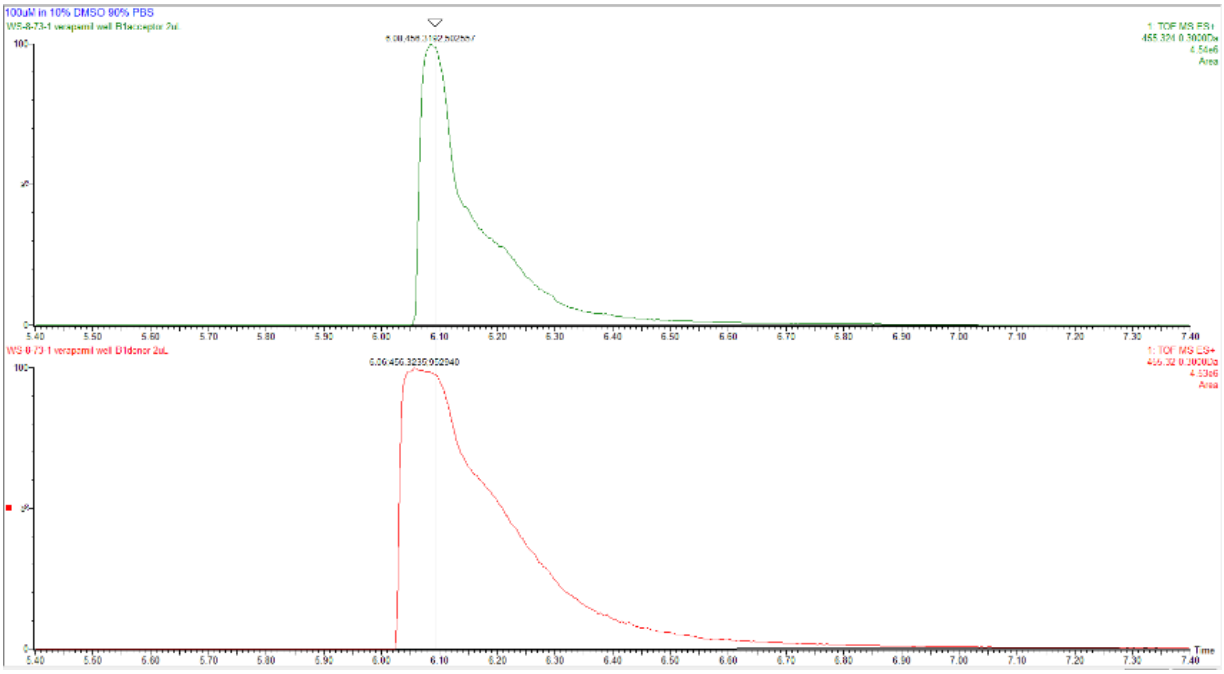


S13: HRESI-ToF fragmentation for portimine B (**2**)

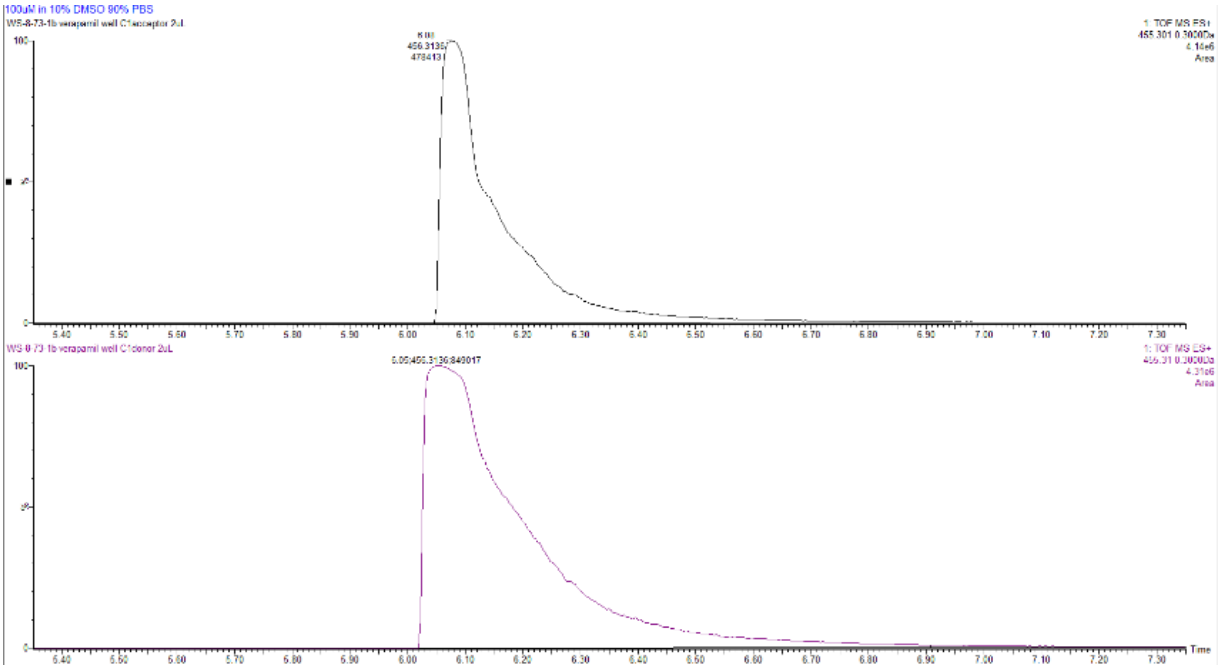


S14-S17 Extracted Ion chromatograms for controls and portimines A and B

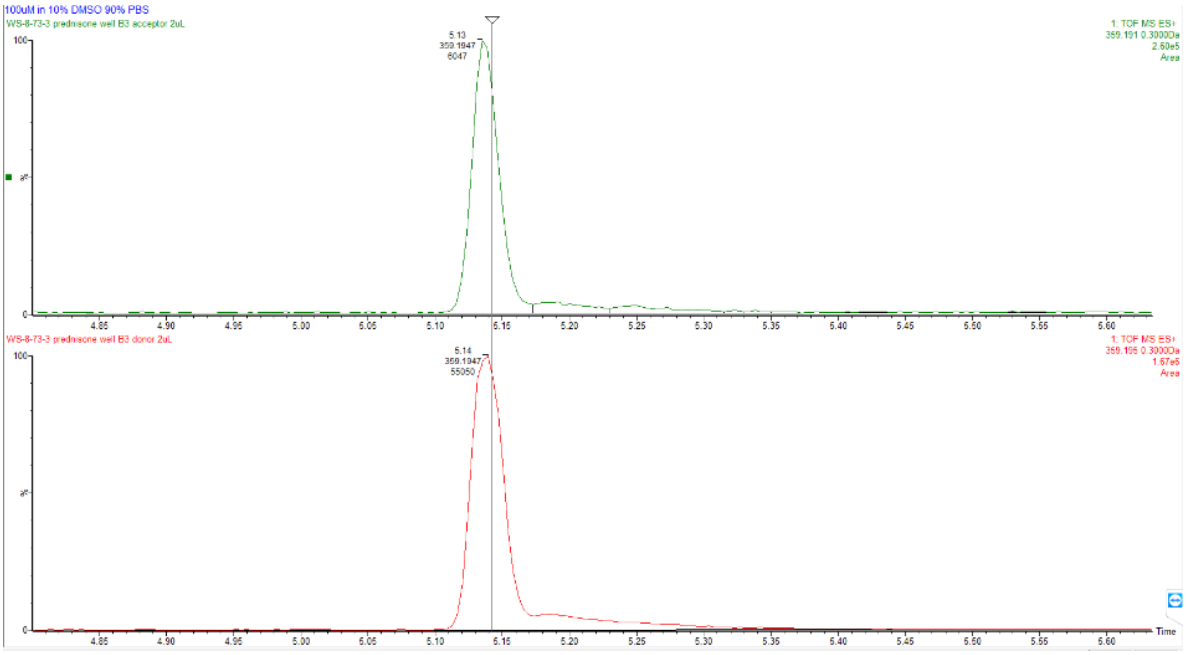
Verapamil replicate 1: relative permeability = 53%



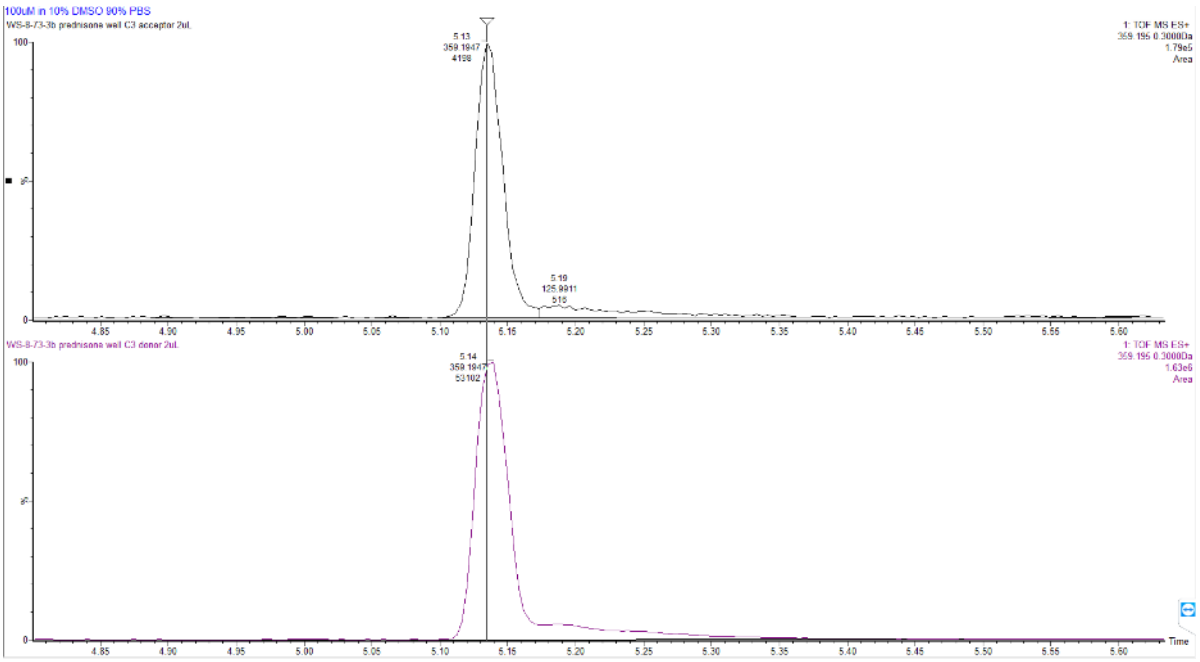
Verapamil replicate 2: relative permeability = 56%



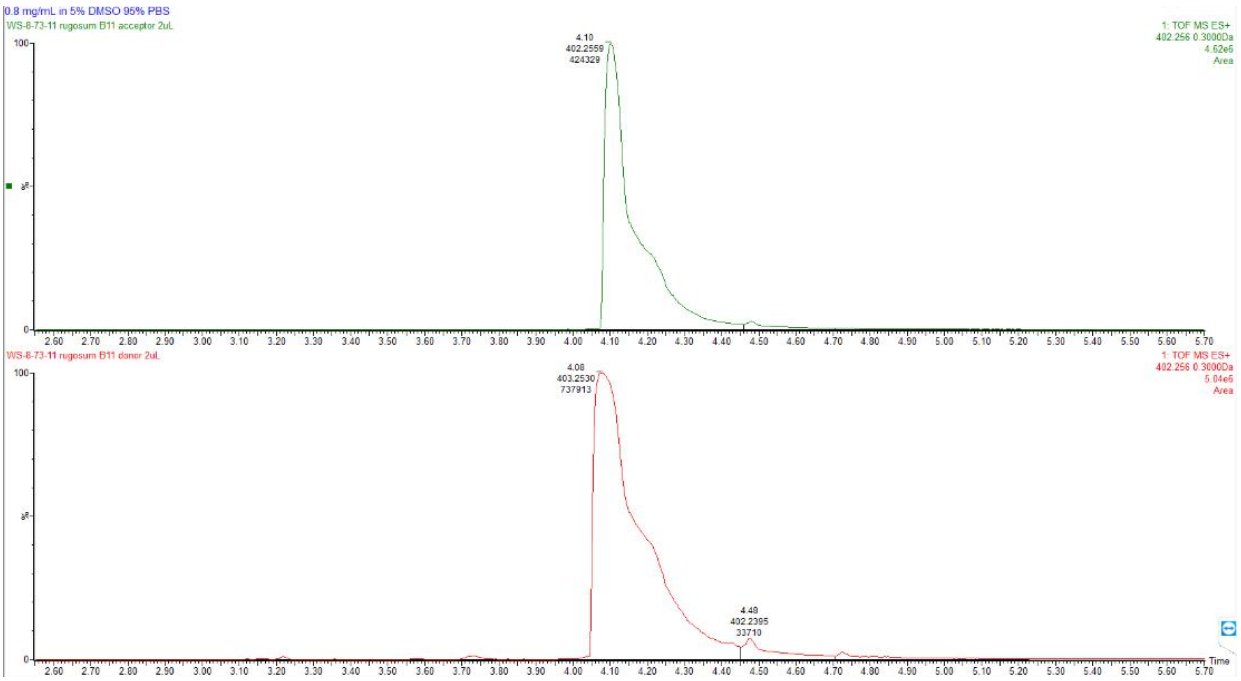
Prednisone replicate 1: relative permeability = 15%



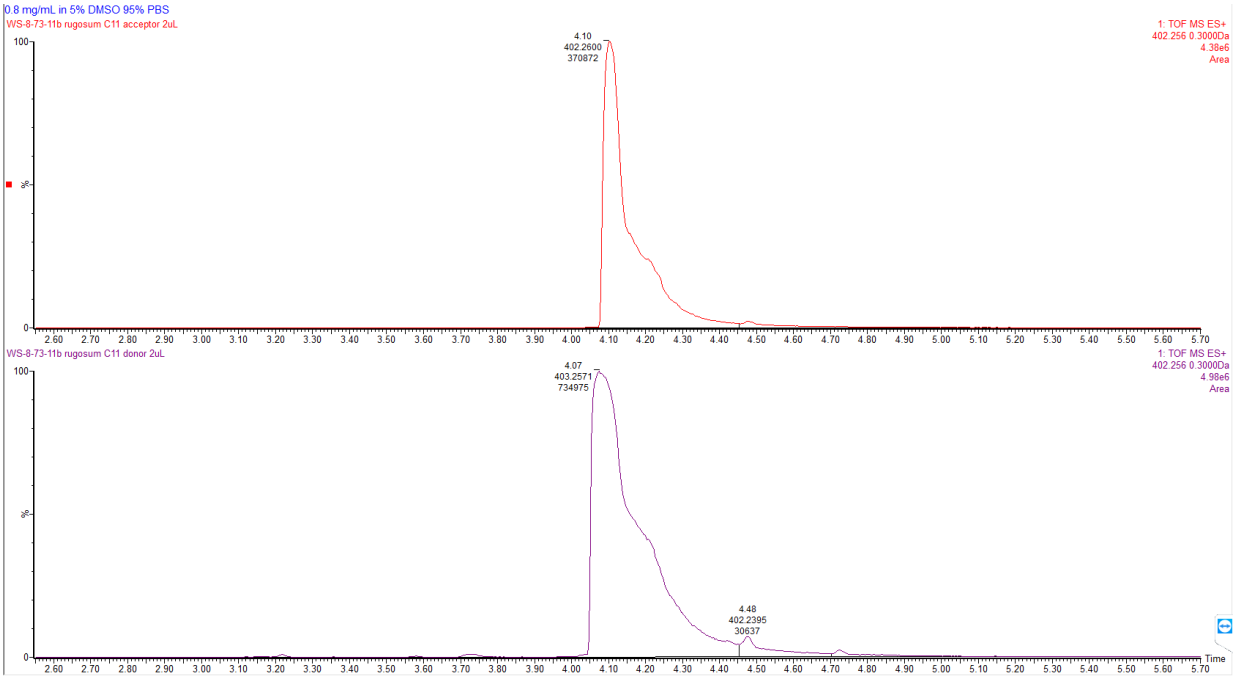
Prednisone replicate 2: relative permeability = 9%



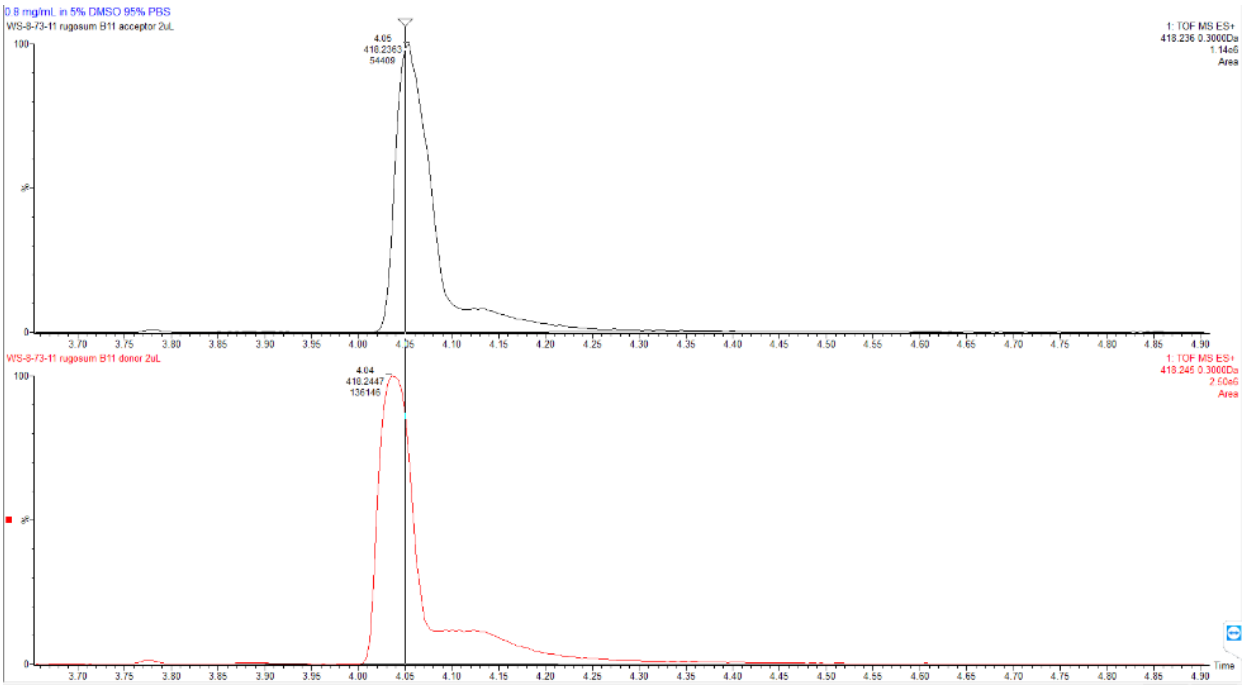
Portimine A replicate 1: relative permeability = 58%



Portimine A replicate 2: relative permeability = 50%



Portimine B replicate 1: relative permeability = 40%



Portimine B replicate 2: relative permeability = 29%