Supplementary Material

Synthesis and Pharmacological Characterization of two Novel, Brain Penetrating $P2X_7$ Antagonists

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General Procedures

In obtaining the compounds described in the examples below and the corresponding analytical data, the following experimental and analytical protocols were followed unless otherwise indicated.

Unless otherwise stated, reaction mixtures were magnetically stirred at room temperature (rt) under a nitrogen atmosphere. Where solutions were "dried," they were generally dried over a drying agent such as Na_2SO_4 or $MgSO_4$. Where mixtures, solutions, and extracts were "concentrated", they were typically concentrated on a rotary evaporator under reduced pressure.

Normal-phase silica gel column chromatography (sgc) was performed on silica gel (SiO_2) using prepackaged cartridges, eluting with 2 M NH_3/MeOH in CH_2Cl_2 unless otherwise indicated.

Mass spectra (MS) were obtained on an Agilent series 1100 MSD using electrospray ionization (ESI) in positive mode unless otherwise indicated. Calculated (calcd.) mass corresponds to the exact mass.

Nuclear magnetic resonance (NMR) spectra were obtained on Bruker model DRX spectrometers. The format of the ^1H NMR data below is: chemical shift in ppm downfield of the tetramethylsilane reference (multiplicity, coupling constant J in Hz, integration).

Preparation of Intermediates and Final Products

(4-(4-phenylpiperazin-1-yl)tetrahydro-2H-pyran-4-yl)methanamine (10).

Step 1: 4-(4-Phenylpiperazin-1-yl)tetrahydro-2H-pyran-4-carbonitrile. To a 250 mL 2 neck flask equipped with nitrogen inlet and an outlet hooked up to a gas scrubber (with aq NaOH), 1-phenylpiperazine (7.64 mL, 50 mmol) was mixed with water (100 mL). The pH was then adjusted to 3 with 6 M HCl (~8 mL). To this mixture was added dihydro-2H-pyran-4(3H)-one (4.6 mL, 50 mmol) and KCN (3.26 g, 50 mmol) all at once and the reaction mixture was stirred at room temperature overnight. A white precipitate formed and the solids were filteredby vacuum filtration. The filter cake was rinsed two times with water. The solid was then partitioned between 75 mL DCM and 75 mL sat sodium bicarbonate. The layers were separated and the aqueous layer was extracted with 75 mL DCM. The combined organic layers were dried over magnesium sulfate, filtered, and the solvent was removed under vacuum to provide the title compound, (10.2 g, 75%). 1H NMR (600 MHz, $CDCl_3$) δ 7.30 – 7.26 (m, 2H), 6.96 – 6.91 (m, 2H), 6.91 – 6.86 (m, 1H), 4.09 – 4.00 (m, 2H), 3.73 – 3.64 (m, 2H), 3.29 – 3.18 (m, 4H), 2.88 – 2.77 (m, 4H), 2.18 – 2.09 (m, 2H), 1.84 – 1.73 (m, 2H).

Step 2: (4-(4-Phenylpiperazin-1-yl)tetrahydro-2H-pyran-4-yl)methanamine (**10**). To a solution of lithium aluminum hydride (1.0 M in THF, 48.9 mL) at 0 °C was added a suspension of **10** (10.2 g, 37.6 mmol) in THF (50 mL) (Note: the suspension was added in portions). The ice bath was removed and the reaction was stirred overnight at room temperature. Solid Na_2SO_4-10 H_2O (10 g) was then added portionwise while controlling the resulting exotherm with an ice/brine bath. The resulting mixture was stirred at room temperature overnight, filtered through Celite, and the filter cake was washed two times with THF (50 mL). The filtrate was concentrated to give the title compound as a white solid (10 g, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.23 (m, 2H), 6.96 – 6.89 (m, 2H), 6.89 – 6.82 (m, 1H), 3.93 – 3.81 (m, 2H), 3.67 – 3.55 (m, 2H), 3.21 – 3.12 (m, 4H), 2.92 – 2.77 (m, 6H), 1.91 – 1.78 (m, 2H), 1.63 – 1.50 (m, 2H).

N-((4-(4-phenylpiperazin-1-yl)tetrahydro-2H-pyran-4-yl)methyl)-2-(phenylthio)nicotinamide (7).

Step 1: 2-(phenylthio)nicotinoyl chloride (**9**). 2-(phenylthio)nicotinoyl chloride was prepared via the dropwise addition of oxalyl chloride (13.6 mL, 2.0 M in DCM) to a mixture of 2-(phenylthio)nicotinic acid (5g, 20.9 mmol) in DCM (60 mL) and DMF (5 mL). The mixture was stirred at room temperature for 5 minutes and then the solvent was removed under vac-

uum. The crude product was re-dissolved in DCM and the solvent was once again removed un-under vacuum. The resulting material was used without further purification.

Step 2: N-((4-(4-phenylpiperazin-1-yl)tetrahydro-2H-pyran-4-yl)methyl)-2-(phenylthio)nicotinamide (**7**). To a solution of **10** (5.83 g, 20.9 mmol) in DCM (30 mL) was added drop wise over 15 minutes the product of Step 1 in DCM (50 mL). This mixture was stirred at room temperature for 2 hours, then at 38 °C for 30 minutes. The white precipitate that formed was collectedusing vacuum filtration, washed with DCM and heptane, and the resulting solid was partitioned between DCM (100 mL) and saturated sodium bicarbonate (100 mL), and extracted. The DCM layer was collected, the aqueous layer was extracted with additional DCM (100 mL), the organic layers were combined, dried over magnesium sulfate, filtered and the solvent was removed under vacuum. The product was purified by sgc using EtOAc as solvent to give the title compound as a white solid (5.4 g, 53%). MS (ESI) mass calcd. $C_{28}H_{32}N_4O_2S$, 488.65; m/z found 489.1 [M+H]+. ^{1}H NMR (600 MHz, CDCl3) δ 8.37 (dd, *J* = 4.7, 1.8 Hz, 1H), 7.91 (dd, *J* = 7.7, 1.8 Hz, 1H), 7.50 - 7.43 (m, 2H), 7.38 - 7.32 (m, 3H), 7.30 - 7.22 (m, 3H), 7.09 (dd, *J* = 7.7, 4.7 Hz, 1H), 6.90 - 6.84 (m, 3H), 3.92 (dt, *J* = 11.8, 4.1 Hz, 2H), 3.79 (d, *J* = 5.0 Hz, 2H), 3.72 - 3.65 (m, 2H), 3.23 - 3.12 (m, 4H), 2.89 - 2.80 (m, 4H), 2.03 - 1.93 (m, 2H), 1.56 (d, *J* = 13.4 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.22, 156.34, 151.11, 151.08, 136.91, 134.55, 130.53, 130.16, 129.20, 129.11, 128.76, 120.30, 119.98, 116.08, 64.16, 56.33, 50.23, 44.68, 40.62, 29.89. Elemental Analysis: Calcd. For $C_{28}H_{32}N_4O_2S$ C, 68.82; H, 6.60; N, 11.47; O, 6.55: S, 6.56; found C, 68.66; H, 6.42; N, 11.35.

2-(tert-butoxycarbonyl)-1,2,3,4-tetrahydroisoquinoline-5-carboxylic acid (11).

2-(*ter*t-Butoxycarbonyl)-1,2,3,4-tetrahydroisoquinoline-5-carboxylic acid (**11**). 2-tert-butyl 5-methyl 3,4-dihydroisoquinoline-2,5(1H)-dicarboxylate (3.5 g, 12.0 mmol,) was dissolved in MeOH (10 mL) and water (10 mL) and was treated with NaOH (0.96 g, 20.0 mmol). The reaction was stirred at room temperature overnight, acidified to pH 3 with 1 N HCl and extracted with EtOAc. The EtOAc layer was washed with brine, dried over magnesium sulfate, and the solvent was removed under vacuum. The product was used without further purification. ^{1}H NMR (400 MHz, DMSO) δ 13.37 – 12.49 (s, 1H), 7.74 – 7.66 (m, 1H), 7.41 – 7.35 (d, *J* = 7.3 Hz, 1H), 7.32 – 7.24 (m, 1H), 4.56 – 4.50 (s, 2H), 3.55 – 3.47 (t, *J* = 5.7 Hz, 3H), 3.12 – 3.04 (m, 2H), 1.51 – 1.36 (s, 9H).

(1-(4-phenylpiperazin-1-yl)cyclohexyl)methanamine (12).

(1-(4-Phenylpiperazin-1-yl)cyclohexyl)methanamine (**12**) can be prepared in a manner analogous to Compound **10** substituting cyclohexanone for dihydro-2H-pyran-4(3H)-one in the first step. NMR data for 1-(4-phenylpiperazin-1-yl)cyclohexanecarbonitrile: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.24 (m, 2H), 6.95 – 6.90 (m, 2H), 6.90 – 6.84 (m, 1H), 3.27 – 3.19 (m, 4H), 2.83 (t, *J* = 5.0 Hz, 4H), 2.23 – 2.12 (m, 2H), 1.87 – 1.75 (m, 2H), 1.70 – 1.56 (m, 5H), 1.37 – 1.25 (m, 1H). NMR data for (1-(4-Phenylpiperazin-1-yl)cyclohexyl)methanamine (**12**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 – 7.23 (m, 2H), 6.95 – 6.90 (m, 2H), 6.84 (tt, *J* = 7.2, 1.0 Hz, 1H), 3.19 – 3.10 (m, 4H), 2.80 (t, *J* = 4.9 Hz, 4H), 2.74 (s, 2H), 1.70 – 1.56 (m, 4H), 1.51 – 1.40 (m, 4H), 1.41 – 1.33 (m, 2H).

tert-butyl 5-(((1-(4-phenylpiperazin-1-yl)cyclohexyl)methyl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (13).

tert-Butyl 5-(((1-(4-phenylpiperazin-1-yl)cyclohexyl)methyl)carbamoyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (**12**). Compound **11** (3.23 g, 11.6 mmol) and Compound **12** (3.19 g, 11.6 mmol) were dissolved in DCE (100 mL) and this solution was treated sequentially with TEA (4.9 mL, 34.9 mmol) and BOP (5.15 g, 11.6 mmol). The reaction was stirred at room temperature for 8 hours, then diluted with water (50 mL) and extracted with DCM. The DCM layer was collected, dried over sodium sulfate, and the solvent was removed under vacuum. The product was purified by sgc using EtOAc/hexanes as solvent to provide the title compound (4.75 g, 77%).

2-methyl-N-((1-(4-phenylpiperazin-1-yl)cyclohexyl)methyl)-1,2,3,4-tetrahydroisoquinoline-5-carboxamide (8).

Step 1: N-((1-(4-Phenylpiperazin-1-yl)cyclohexyl)methyl)-1,2,3,4-tetrahydroisoquinoline-5-carboxamide. Compound **13** (4.97 g, 9.33 mmol) was dissolved in DCM (100 mL) and treated with trifluoroacetic acid (14.3 mL, 197 mmol). The mixture was stirred overnight at room temperature then the solvent was removed under vacuum. The resulting mixture was diluted with DCM, washed with aqueous saturated sodium bicarbonate and the organic layer was concentrated to give the title compound which was used in the subsequent step without further purification. MS (ESI) mass calcd. $C_{27}H_{36}N_4O$, 432.29; m/z found 433.3 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO) δ 8.12 – 8.00 (m, 1H), 7.29 – 7.14 (m, 5H), 6.98 – 6.89 (d, *J* = 8.1 Hz, 2H), 6.82 – 6.68 (m, 1H), 4.12 – 4.03 (s, 2H), 3.63 – 3.10 (bs, 1H), 3.32 – 3.27 (d, *J* = 6.1 Hz, 2H), 3.15 – 3.04 (m, 6H), 2.94 – 2.86 (m, 2H), 2.84 – 2.76 (s, 4H), 1.86 – 1.71 (d, *J* = 13.7 Hz, 2H), 1.67 – 1.45 (m, 3H), 1.45 – 1.16 (m, 5H).

Step 2: 2-Methyl-N-((1-(4-phenylpiperazin-1-yl)cyclohexyl)methyl)-1,2,3,4-tetrahydro-isoquinoline-5-carboxamide (**8**). To a solution of the intermediate from Step 1 (**8**)(4.20 g, 9.71 mmol) in DCE (50 mL) and MeOH (10 mL) was added aqueous formaldehyde (3.15 mL, 38.8 mmol) and sodium triacetoxyborohydride (8.23 g, 38.8 mmol). The reaction mixture was stirred at room temperature for 5 hours. Water was then added and the reaction was washed with EtOAc. The pH of the aqueous layer was adjusted to 10 and the resulting suspension was extracted with EtOAc. The organic layers were combined, dried over sodium sulfate, and the solvent was removed under vacuum. The product was purified by sgc using DCM to 10% MeOH/0.1% NH_4OH/DCM as solvent to give the title compound (3.2 g, 74%). MS (ESI) mass calcd. $C_{28}H_{38}N_4O$, 446.6; m/z found 447.3 $[M+H]^+$. ^1H NMR (400 MHz, DMSO) δ 8.01 (t, *J* = 6.1 Hz, 1H), 7.23 – 7.09 (m, 5H), 6.94 – 6.88 (m, 2H), 6.75 (t, *J* = 7.3 Hz, 1H), 3.49 (bs, 2H), 3.31 – 3.26 (m, 2H), 3.08 (bs, 4H), 2.87 (app t, 2H), 2.83 – 2.76 (m, 4H), 2.55 (app t, 2H), 2.31 (s, 3H), 1.83 – 1.73 (m, 2H), 1.66 – 1.48 (m, 3H), 1.44 – 1.17 (m, 6H).

Pharmacological Assays

$P2X_7$ antagonism in human peripheral blood mononuclear cells (PBMCs):

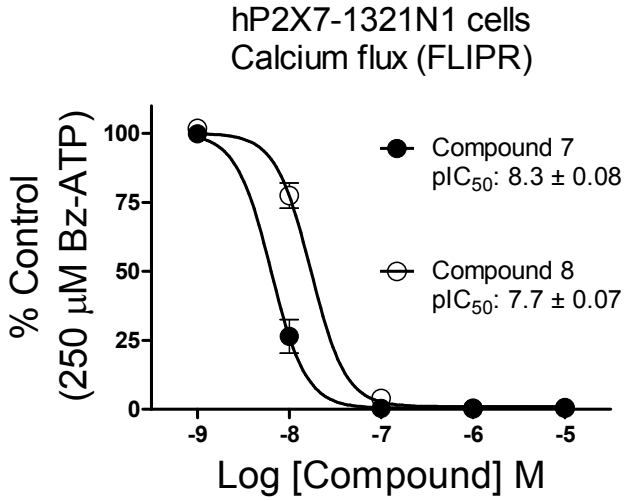
Human blood was collected using a blood donor program. PBMCs were isolated from blood using a Ficoll density gradient technique. Briefly, blood was laid on Ficoll solution and centrifuged at room temperature for 20 minutes at 2000 rpm. The buffer layer (between red blood cells and plasma) was carefully collected by aspiration, washed with PBS and centrifuged again at 1500 rpm for 15 minutes. The resulting cell pellet was washed and plated on 96 well-plates for experiments. LPS (30 ng/ml) was added to each well and incubated for 1 hour. Test compounds were then added and incubated for 30 minutes. The $P2X_7$ agonist, Bz-ATP was then added at a final concentration of 0.5 mM. Cells were incubated for an additional 1.5 hours. At that point, supernatant was collected and stored for IL-1β assay using manufacturer's protocol for ELISA. Data was expressed as percent control, where control is defined as the difference in IL-1β release in LPS+Bz-ATP samples and LPS only samples.

$P2X_7$ antagonism in recombinant $hP2X_7$ cells: (a) Ca^{2+} flux (b) radioligand binding:

(A) Ca^{2+} flux: 1321N1 cells expressing the recombinant human $P2X_7$ channel were cultured in HyQ DME/high glucose supplemented with 10% FBS and 500μg/ml G418. Cells were seeded at a density of 25000 cells/well (96-well clear bottom black walled plates) in 100 μl volume/well. On the day of the experiment, cell plates were washed with assay buffer, con-

taining (in mM): 130 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 glucose; pH 7.40 and 300 mOs. After the wash, cells were loaded with the Calcium-4 dye (Molecular Device) and incubated in the dark for 60 minutes. Test compounds were prepared at 250X the test concentration in neat DMSO. Intermediate 96-well compound plates were prepared by transferring 1.2 µL of the compound into 300 µL of assay buffer. A further 3X dilution occurred when transferring 50 µL/well of the compound plate to 100 µL/well in the cell plate. Cells were incubated with test compounds and dye for 30 minutes. Calcium dye fluorescence was monitored in FLIPR as the cells were challenged by adding 50 µL/well of BzATP (final concentration is 250 µM BzATP). The fluorescence change was measured 180 seconds after adding the agonist.

The Ca^{2+} flux data are presented on Table 1, for clarity the curves for Compounds 7 and 8 are also shown here.



(B) Radioligand binding: $hP2X_7$-1321N1 cells were collected and frozen @ 80 °C. On the day of the experiment, cell membrane preparations were made according to standard published methods. The total assay volume was 100 µl:10 µl compound (10x) + (b) 40 µl tracer (2.5x) + 50 µl membrane (2x). The tracer used for the assay was tritiated A-804598. The compound can be prepared described in Donnelly-Roberts, D. *Neuropharmacology* (2008), 56(1), 223-229. Final concentration of the tracer was 30 nM. Compounds, tracer and membranes were incubated for 1 hour @ 4 °C. The assay was terminated by filtration (GF/B filters presoaked with 0.3% PEI) and washed with washing buffer (Tris-HCl 50 mM).

Extracellular binding of ATP to $P2X_7$ that is expressed in the cell-membrane opens the ligand gated cation channel and allows Ca^{2+} entry into the cell. This ligand-induced Ca^{2+} flux was

measured in 1321N1 astrocytoma cells overexpressing hP2X$_7$ for the compounds of interest.

The calcium assay kit used (Molecular Devices, R8090), provides a Ca^{2+} sensitive dye together with a quenching dye. However, no specifications are given by the manufacturer. The kit most likely consists of a membrane permeable acetoxymethyl (AM) ester of a fluorescent Ca^{2+} indicator, such as fluo-4 or fluo-3. Upon cellular uptake, the AM esters get cleaved by esterases, liberating the Ca^{2+}-sensitive dye which can then bind calcium. The dyes have an absorption spectrum compatible with excitation at 488 nM by argon laser sources and a large fluorescence intensity increase in response to Ca^{2+} binding without an accompanying spectral shift. Emission wavelength is in the range of 510-560 nM.

The fluorescence, and thus the changes in intracellular [Ca^{2+}] in the 1321N1 cells was monitored in time, before and after addition of the agonist. The effect of the antagonist was measured as K_i.

The binding affinities of compounds at the human and rat serotonin transporter (SERT) were obtained via in vitro assay as described in PCT Int. Appl. WO 2006066197.

P2X$_7$ Autoradiography

All animal work done in this paper was in accordance with the Guide Care for and Use of Laboratory Animals adopted by the US National Institutes of Health. Animals were allowed to acclimate for 7 days after receipt. They were group housed in accordance with institutional standards, received food and water ad libitum and were maintained on a 12 hour light/dark cycle. Male Sprague Daley Rats approximately 300-400 grams in body weight were used. For time course studies 3 animals per time point over 7 time points were used. For dose response studies, three animals per dose over 7-10 doses were tested. Animals were euthanized with carbon dioxide and plasma and tissue removed. Tissue sections were prepared as previously described (Langlois et al, 2001 and Barbier et al, 2007). P2X$_7$ autoradiography was determined at room temperature with 30 nM [^{3}H] A-804598 in 50mM Tris HCl incubation buffer containing 0.1% BSA. The non-specific binding was measured with 100μM A-740003. Sections were incubated for 10 minutes, rinsed four times for five minutes in the 50mM Tris Buffer with 0.1% BSA, followed by 2 dips in ice cold water. Sections were allowed to dry before acquisition with β-Imager for 2 hours. Quantitative analysis was previously described (Langlois et al, 2001). SERT autoradiography was carried out as previously described (Barbier et al, 2007).

Liver Microsomal Stability.

Microsomal stability studies were conducted on a Biomek® FX Robotic Liquid Handling Workstation (Beckman Coulter, Brea, CA), which consists of a 96-channel pipette head, a 12-postion workstation deck, and a plate incubator. Test compounds (1 μM) were spiked in a reaction mix consisting of 100 mM potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$, and 0.5 mg/mL liver microsomes from mouse, rat, and human (BD Gentest). The reaction was brought to 37 °C and initiated by adding NADPH to a final concentration of 1 mM. After mixing on the plate-deck, 50 μL aliquots were excised from the reaction plate at 0, 5, 10, 20, 40, and 60 min and quenched with four volumes of acetonitrile spiked with 500 μg/nL of the internal standard phenytoin. Quenched plates were centrifuged at 5700 rpm for 10 min at 4 °C, and supernatant was diluted 1:3 in water before LC/MS/MS analysis.

The compound half-lives were derived from plots of the ln of percent remaining compound over time to determine the intrinsic clearance. The predicted hepatic clearance was derived from the intrinsic clearance value using equations from the well-stirred model (**Current Drug Metabolism, 2008, 9, 940-951**), where no correction was made plasma protein binding and the blood to plasma concentration ratio was assumed to be one. The extraction ratio (ER) was calculated by dividing the predicted hepatic clearance by species blood flow (Q), where Q is 90, 55, and 21.7 mL/min/kg for mouse, rat and human, respectively.

Plasma Protein Binding.

Plasma protein binding was determined by equilibrium dialysis using the RED device (Pierce, catalog # #89810), according to manufacturer's protocol. Compounds were prepared as 100 μM DMSO stocks and spiked into 1 mL of mouse, rat and human plasma (Bioreclamations) to make a final concentration of 1 μM. Plasma (300 μL) was dispensed into wells separated by an 8 KDa-permeable cellulose

membrane from wells containing 100 mM potassium phosphate, pH 7.4 (500 µL). Each compound was tested in triplicate. The RED devise was sealed and equilibrium was permitted for 6 h in a 37 °C incubator with gentle agitation at 100 RPM. After incubation, plasma samples were prepared by transferring 10 µL from plasma wells to 90 µL of fresh 100 mM potassium phosphate, pH 7.4, and buffer samples were prepared by transferring 90 µL from buffer wells to 10 µL of naïve plasma. In addition, a reference sample without equilibration was prepared in triplicate by mixing 10 µL of plasma containing 1 µM compound with 90 µL buffer in order to determine compound recovery from the assay. Two-volumes of 1:1 acetonitrile:methanol spiked with the internal standard phenytoin (0.2 µg/mL) were added to reference and samples. Precipitation of plasma protein binding was allowed for 15 min before reference and samples were centrifuge clarified. Supernatant (10 µL) was used for LC/MS/MS analyses.

Caco-2 Permeability Assay.

Caco-2 permeability assays were conducted at CEREP according to company's protocol. In brief, Caco-2 cells were seeded onto a 96-well Multiscreen plate™ (Millipore) at a cell density of 1 x 10^5 cells/cm^2 and cultured for at least 21 days before permeability studies were conducted. Test compounds were dissolved in DMSO and added to HBSS-HEPES, pH 7.4 culture media at a final concentration of 10 µM (1 % DMSO v/v). The working solution was applied to cells on the donor side and incubated at 37 ° C with gentle agitation for 60 and 40 min to determine the A to B and B to A permeability, respectively. Samples were extracted from the donor side at time zero and the end-point and from the receiver side at the end-point time. Samples were then processed for LC/MS/MS analyses to determine the apparent permeability coefficient (P_{app}) of the test compound in the A to B and B to A direction **(Expert Opin Drug Metab. Toxicol. (2005) 1 (2): 175 – 185**) as well as the percent recovery.

LC/MS/MS Methodology.

Compounds were quantified on an API4000 MS/MS System (Applied Biosystems, Concord, Ontario, Canada) interfaced with an Agilent 1100 Series HPLC. Samples were loaded onto a 2 x 50 mm, 5µ Zorbax SB-Phenyl column (Agilent, catalog # 860975-912) under a flow rate of 1.0 mL/min, using water and 0.1 % formic acid (A) as stationary phase and acetonitrile and 0.1 % formic acid (B) as mobile phase. After a 25 µL injection, the mobile phase was increased from 5 to 90 % B using a linear gradient for 1.5 min, held at 100 % B for 0.5 min, and equilibrated at 5% B for 0.5 min for an overall run-time of 2.5 min. Compound 7 was quantified by MS/MS in the positive ion mode by monitoring the transition of 489.25 to 214 m/z with the dwell time, declustering potential, collision energy, and collision cell exit potential set to 100 msec, 80 V, 40 V, and 12 V, respectively. Compound 8 was quantified by MS/MS in the positive ion mode by monitoring the transition of 447.25 to 285.5 m/z with the dwell time, declustering potential, collision energy, and collision cell exit potential set to 100 msec, 40 V, 40 V, and 12 V, respectively.