Chemistry

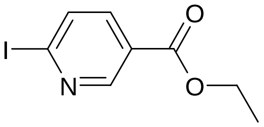
General

Reagents and solvents were commercially available and were used without purification unless otherwise noted. Chromatographic purifications were performed on a Biotage FlashMaster™ system or a Teledyne Isco CombiFlash® Companion® system using pre-packed silica gel columns and gradient elution with heptane and ethyl acetate.

Analytical LC-MS was performed using a Shimadzu LC8/SLC-10A (used for method 1) or Shimadzu LC10AD/SLC-10A (used for method 2) LC system equipped with UV and ELS detectors, and a PESciex API 150EX mass spectrometer with an APPI source operated in positive ion mode. Waters Symmetry C18 columns, 30 x 4.6 mm, with 3.5 µm particles were used with gradient elution using the following solvent systems: A = water/trifluoroacetic acid (100:0.05) and B = water/acetonitrile/trifluoroacetic acid (5:95:0.035). The following two methods were used: **method 1**: gradient elution with A/B 90:10 v/v to 100% B in 2.4 min, flow rate 3.3 ml/min, column temperature 40 °C; **method 2**: same as method 1, except that the column temperature was 60 °C.

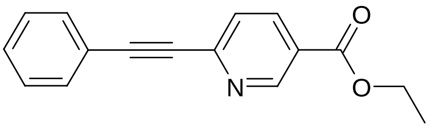
^{1}H NMR spectra were recorded at 500.13 MHz on a Bruker Avance AV500 instrument. TMS was used as internal reference standard. Chemical shift values are expressed in ppm.

*6-Iodo-nicotinic acid ethyl ester (**6**)*



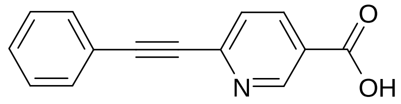
To a suspension of 6-iodonicotinic acid **5** (5.09 g, 20.4 mmol) (*see* Newkome, G. R. *et al.*, *J. Org. Chem.* **1986**, *51*, 953) in absolute ethanol (50 mL) was carefully added concentrated H_2SO_4 (2.3 mL, 41 mmol). *CAUTION! Highly exothermic!* The mixture was refluxed under N_2 for 3 h after which it was cooled to room temperature and poured into saturated Na_2CO_3 solution (150 mL). The aqueous layer was extracted with ethyl acetate (100 + 2 x 50 mL) and the combined organic layers were washed with brine (2 x 100 mL), dried over Na_2SO_4, and evaporated onto silica gel. CombiFlash purification afforded the title compound as a pale yellow oil which solidified on standing at room temperature (3.57 g, 63%). ^{1}H-NMR (DMSO-d_6) δ 1.33 (t, J=7.1 Hz, 3H); 4.34 (q, J=7.1 Hz, 2H); 7.94 (dd, J=2.5, 8.1 Hz, 1H); 8.04 (dd, J=0.8, 8.2 Hz 1H); 8.83 (dd, J=0.8, 2.5 Hz, 1H).

*6-Phenylethynyl-nicotinic acid ethyl ester (**7**)*



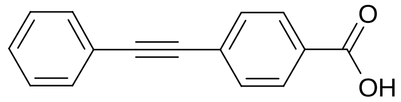
A Biotage microwave vial was loaded with 6-iodo-nicotinic acid ethyl ester **6** (500 mg, 1.80 mmol), $PdCl_2(PPh_3)_2$ (63 mg, 0.090 mmol) and triethylamine (3 mL). To the resulting suspension were added CuI (17 mg, 0.090 mmol) and phenylacetylene (237 µL, 2.16 mmol). The vial was capped and heated at 140 °C for 10 minutes using a Biotage microwave synthesizer. After cooling, the mixture was evaporated onto celite and purified on a FlashMaster system to yield the title compound (438 mg, 96%). ^{1}H-NMR (DMSO-d_6) δ 1.35 (t, *J*=7.1 Hz, 3H); 4.37 (q, *J*=7.1 Hz, 2H); 7.46-7.54 (m, 3H); 7.64-7.68 (m, 2H); 7.80 (dd, *J*=0.9, 8.1 Hz, 1H); 8.33 (dd, *J*=2.2, 8.2 Hz, 1H); 9.09 (dd, J=0.9, 2.2 Hz, 1H).

6-Phenylethynyl-nicotinic acid ***(8)***



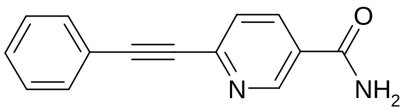
To a solution of 6-phenylethynyl-nicotinic acid ethyl ester **7** (500 mg, 1.99 mmol) in THF (18 mL) was added a solution of LiOH (429 mg, 17.9 mmol) in water (9 mL) and the resulting mixture was stirred at room temperature for 2.5 hours. It was then acidified to pH = 1 by the addition of 10% aqueous $KHSO_4$ solution (170 mL). The aqueous layer was extracted with ethyl acetate (2 x 100 mL) and the combined organic layers were dried over Na_2SO_4, filtered and evaporated to yield the title compound as a brown solid (326 mg, 73%). ^{1}H-NMR (DMSO-d_6) δ 7.46-7.53 (m, 3H); 7.64-7.67 (m, 2H); 7.78 (dd, *J*=0.9, 8.1 Hz, 1H); 8.30 (dd, *J*=2.2, 8.1 Hz, 1H); 9.08 (dd, *J*=0.8, 2.2 Hz, 1H); 13.54 (br s, 1H).

4-Phenylethynyl-benzoic acid ***(10)***



To a solution of 4-phenylethynyl-benzaldehyde **9** (1.00 g, 4.85 mmol), 2-methyl-2-butene (24.2 mL, 48.5 mmol, 2 M solution in THF), and tert-butanol (220 mL) in THF (120 mL) was added a solution of $NaClO_2$ (1.75 g, 19.4 mmol) and KH_2PO_4 (1.65 g, 12.1 mmol) in water (50 mL). The resulting mixture was vigorously stirred at room temperature for 1.5 hours after which volatiles were evaporated and the residue was partitioned between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated to yield the title compound (1.07 g, 99%). ^{1}H-NMR (DMSO-d_6) δ 7.44-7.48 (m, 3H); 7.58-7.62 (m, 2H); 7.67 (d, *J*=8.0 Hz, 2H); 7.98 (d, *J*=8.5 Hz, 2H).

6-Phenylethynyl-nicotinamide ***(11a)***

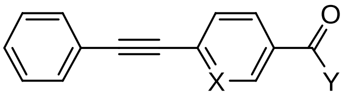


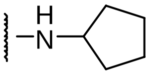
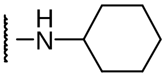
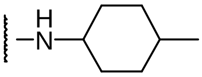
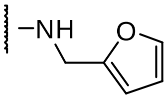
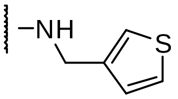
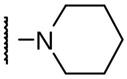
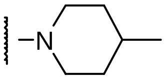
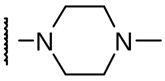
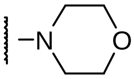
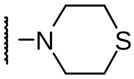
6-Phenylethynyl-nicotinic acid ethyl ester (14.8 mg, 0.059 mmol) and sodium cyanide (0.3 mg, 0.006 mmol) was dissolved in 4 N ammonia in methanol (1.5 mL). The reaction was heated at 50°C for 16 hours. After cooling sat. sodium bicarbonate (4 mL) was added. The aqueous layer was extracted with ethyl acetate (4 mL). The organic layer was washed with brine (4 mL), dried over Na_2SO_4, filtered and evaporated. The residue was purified by preparative LC-MS to afford the title compound. LC-MS *m*/*z* calcd for $[M+H]^+$ 223.1, found 223.1, t_R 1.01 min (method 1), UV Purity 97.9%, ELS purity 98.0%.

General procedure for the synthesis of compounds ***11b-11o***

Bohdan 48-well MiniBlock® fritted tube reactors were loaded with PS-carbodiimide resin (88 mg, 0.113 mmol, 1.29 mmol/g loading). Into each reactor was added a solution of 6-phenylethynyl-nicotinic acid **8** (8.4 mg, 0.038 mmol) or 4-phenylethynyl-benzoic acid **10** (8.4 mg, 0.038 mmol) in 1,2-dichloroethane (100 µL) followed by a solution of the primary or secondary amine (0.038 mmol) in 1,2-dichloroethane (620 µL). The resulting mixture was heated to 50 °C with shaking overnight. After cooling to room temperature, the resin was removed by filtration, washed with 1,2-dichloroethane (3 x 1 mL) and the combined washings and filtrates were evaporated. The residue was purified by preparative LC-MS to afford the desired product.

Analytical data for compounds ***11b-11o***.



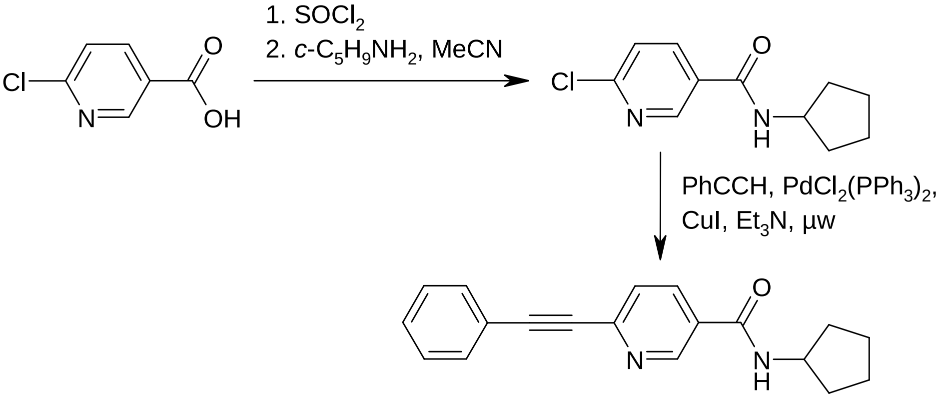
			m/*z*			% Purity	
Compound	X	Y	Calcd [a]	Found	t_R [b]	UV	ELS
11b	N	$HNCH_2CH_2CH(CH_3)_2$	293.2	293.1	1.61	86.3	100.0
11c	N		291.1	291.2	1.39 [c]	97.9	98.9
11d	N		305.2	305.3	1.60	93.7	100.0
11e	N		319.2	319.2	1.74	99.3	100.0
11f	N		303.1	303.1	1.40	99.9	100.0
11g	CH		318.1	318.1	1.79	98.1	100.0
11h	N		319.1	319.0	1.49	86.0	96.8
11i	N		291.1	291.2	1.43	98.8	100.0
11j	CH		304.2	304.1	1.98	99.7	100.0
11k	N		305.2	305.1	1.59	100.0	99.7
11l	CH		305.2	305.3	0.97	98.8	98.8
11m	N		306.2	306.1	0.77	99.0	99.5
11n	N		293.1	293.0	1.16	99.0	100.0
11o	N		309.1	309.2	1.38	99.4	99.6

[a] Calculated *m*/*z* for $[M+H]^+$.

[b] Retention time in minutes by LC-MS method 1 unless otherwise noted.

[c] Retention time in minutes by LC-MS method 2.

*N-Cyclopentyl-6-phenylethynyl-nicotinamide (**11c**) alternative method of preparation*

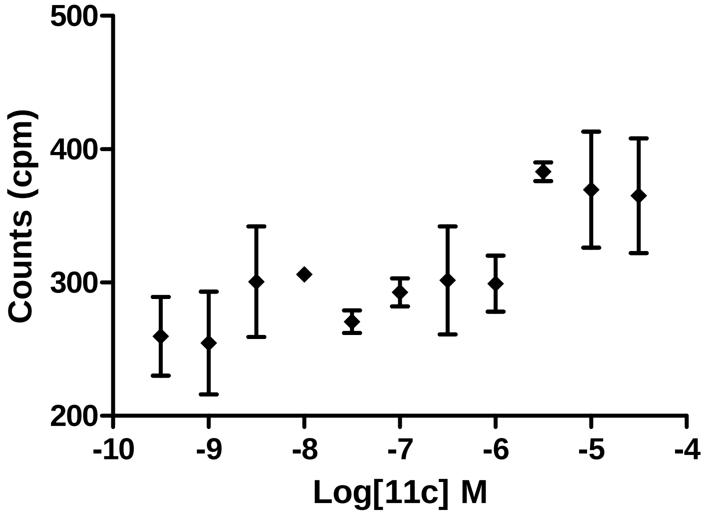


11c

A mixture of 6-chloronicotinic acid (5.00 g, 31.7 mmol) and thionyl chloride (5.0 mL, 68 mmol) was refluxed under argon for 3 h, yielding a homogeneous solution. This was co-evaporated with toluene to remove excess thionyl chloride and the residue was dissolved in acetonitrile (25 mL). The solution was added with stirring to a solution of cyclopentylamine (4.70 mL, 47.6 mmol) in acetonitrile (40 mL). The resulting mixture was stirred at r.t. for 1 h and then at reflux for 1 h after which it was treated with sat. $NaHCO_3$ solution (80 mL) for 20 minutes at r.t. Extraction with ethyl acetate (2 x 250 mL), washing the extract with brine (100 mL), drying over Na_2SO_4 and evaporation gave a crude product, which was purified by recrystallization from ethyl acteate/heptane to yield *6-chloro-N-cyclopentyl-nicotinamide* (4.39 g, 62%). ^{1}H-NMR (DMSO-d_6) δ 1.49-1.58 (m, 4H); 1.65-1.74 (m, 2H); 1.85-1.93 (m, 2H); 4.18-4.26 (m, 1H); 7.63 (dd, *J*=0.7, 8.3 Hz, 1H); 8.24 (dd, *J*=2.5, 8.3 Hz, 1H); 8.56 (br d, *J*=6.9 Hz, 1H); 8.82 (dd, *J*=0.7, 2.5 Hz, 1H).

A modification of the method of Takahashi *et al.*, *Synthesis* **1980**, 627, was used for the Sonogashira coupling. Three 20 mL Biotage microwave vials were each loaded with 6-chloro-*N*-cyclopentyl-nicotinamide (1.46 g, 6.51 mmol), $PdCl_2(PPh_3)_2$ (228 mg, 0.325 mmol) and CuI (62 mg, 0.325 mmol). Triethylamine (13 mL), which was deoxygenated by bubbling argon gas through for 5 min, was then added followed by phenylacetylene (1.07 mL, 9.76 mmol). The vials were capped and heated under argon at 150 °C for 10 minutes using a Biotage microwave synthesizer. After cooling, the reaction mixtures were combined, partitioned between water (100 mL) and ethyl acetate (200 mL), and the organic layer was evaporated onto silica gel. FlashMaster purification followed by two recrystallizations from ethyl acetate (*ca.* 20 mL) afforded *N-cyclopentyl-6-phenylethynyl-nicotinamide* (**11c**) pure as a tan solid (1.85 g, 33%). ^{1}H-NMR (DMSO-d_6) δ 1.51-1.59 (m, 4H); 1.66-1.74 (m, 2H); 1.86-1.94 (m, 2H); 4.20-4.29 (m, 1H); 7.46-7.52 (m, 3H); 7.62-7.67 (m, 2H); 7.75 (dd, *J*=0.9, 8.2 Hz, 1H); 8.24 (dd, *J*=2.3, 8.2 Hz, 1H); 8.57 (br d, *J*=7.0 Hz, 1H); 9.01 (dd, *J*=0.9, 2.3 Hz, 1H).

[^{3}H]-Quisqualic acid binding



Raw data from a [^{3}H]-quisqualic acid binding experiment using membranes from cells recombinantly expressing human mGluR5 (see below for details), showing that **11c** does not displace this orthosteric ligand, but instead potentiates its binding slightly (*ca*. 30%) at **11c** concentrations above 1 µM.

mGluR5 Functional Assay

Transient cell line

Standard molecular cloning techniques (Maniatis *et al.* Molecular Cloning, A Laboratory Manual (1989)) were used to generate a cell line of Baby Hamster Kidney (BHK) cells transient expressing human metabotropic glutamate receptor 5 (mGluR5). The cDNA encoding the mGluR5 corresponds to the amino acid sequence published in NCBI Genbank Nucleotide database, accession number NM_000842. The cell line was grown in DMEM + glucose (4.5 g/L) + Glutamax-I + pyruvate (Gibco cat.no. 31966-021) + 5% dialyzed FBS, 1% penicillin - streptomycin (Gibco cat. # 15140-122), and 1.0 µ/ml G418 (Gibco cat. # 11811-064).

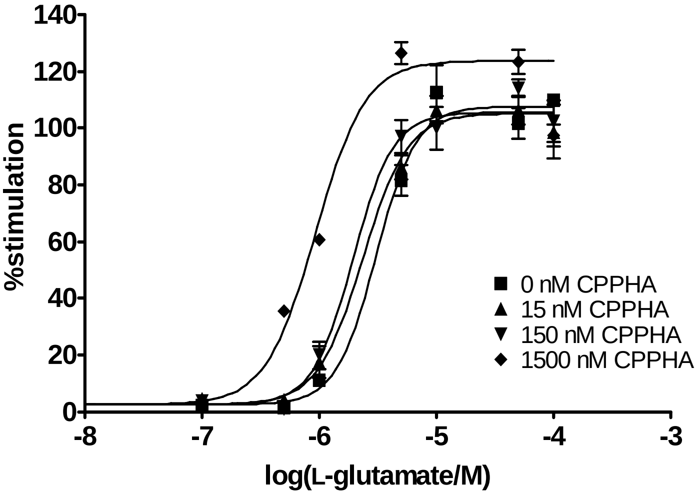
Methods

BHK cells transiently expressing human mGluR5 receptor were plated in growth medium at a density of 17.500 cells/well in clear-bottomed, cell-culture coated 96-well plates (Corning, Costar #3904) and grown for 48 h at 37 °C in the presence of 5% CO_2. Before assay, the cells were washed once with 100 µL of assay buffer (Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} (Gibco cat. # 14025-050) containing 20 mM HEPES at pH 7.4. The cells were incubated with a calcium-sensitive fluorescent dye, Calcium assay kit R8033 (100 µl/well, half concentration relative to manufacturers instruction, Molecular Devices, Inc.) with 2.5 mM Probenecid (Sigma #P-8761) for 50 min at 37 °C followed by 10 min at room temperature. Calcium flux was measured using a FLIPR 384: Fluorometric Imaging Plate Reader (Molecular Devices). The cells were excited by 488 nm laser light and emitted fluorescent light passed through a 510-570 nm bandpass interference filter and was detected by a cooled CCD camera. Test compounds were diluted from 2 mM stock solutions in 100% DMSO in assay buffer. Agonist activity was assayed by addition of test compounds to the cells (first addition: 25 µL test compound to 100 µL in assay well). Ten different concentrations were tested for each compound. Fluorescence readout was measured for 5 minutes starting just prior to compound addition. Positive modulation activity was assayed by further addition of EC_{20} (300 nM) of L-glutamate, (second addition: 25 uL of 1800 nM L-glutamate to 125 µL cells, final concentration 300 nM L-glutamate). Subsequently, the calcium flux assay was also established using a stable mGluR5 expressing BHK cell line and in 384-well assay format on a Hamamatsu FDSS7000 reader. The experimental conditions were similar to those described above.

Data analysis

The fluorescence readout was calculated as max-min response, *i.e.* maximum fluorescence reading after and before liquid addition during the 5 minute measuring time. Positive modulation activity was calculated from the fluorescence max-min data normalized to yield responses for no modulation (EC_{20} response) and full stimulation (1 µM L-glutamate) of 0% and 100% modulation, respectively. Data were collected from at least three independent experiments. Concentration-response data were fitted to the four-parameter logistic equation to estimate compound potency (EC_{50}) and efficacy (E_{max}) (see Harvey Motulsky, Arthur Christopoulos, Fitting Models to Biological Data Using Linear and Nonlinear Regression (A Practical Guide to Curve Fitting), ISBN: 0195171802, Oxford University, 2004).

Concentration-response curves at varying concentrations of CPPHA



Concentration-response curves of L-glutamate alone and together with three fixed concentrations of the reference PAM CPPHA at the human mGlu5 receptor are shown above.

Plasma and brain exposure analysis

Blood samples and brains were collected in order to determine plasma (ng/ml) and brain (ng/g) levels of compound **11c** in mice and rats. Under isoflurane anaesthesia, cardiac blood was obtained in EDTA-coated tubes and centrifuged for 10 min at 4 °C after which plasma was drawn off. Following decapitation, the brain was removed and brain homogenate was prepared by homogenizing the whole brain with 70%

acetonitrile (1:4 v/v) followed by centrifugation and collection of the supernatant. Plasma and brain supernatant samples were frozen at -80°C until analysis. Concentrations of compound **11c** were determined in plasma and brain homogenate using turboflow chromatography (dual column, focus mode, Cohesive Technologies, UK) followed by tandem mass spectrometry (MS/MS) detection in positive-ion electrospray ionization mode (Sciex API-3000 MS, Applied Biosystems, the Netherlands). Samples of plasma and brain homogenate were prepared by adding an equal amount of 10% methanol with internal standard included (escitalopram) and after centrifugation (6000 g, 5 °C, 20 min) 10 µl were injected into the turboflow system. The mobile phase consisted of water/methanol with 0.1% ammonium hydroxide pumped as a gradient through an XTerra analytical column (MS C8, 2.1 x 20 mm, Waters Corp., MA, USA). Detection was performed at a parent>daughter molecular mass of 291.1>223.1 AMU. Retention time was 4 min. The lower limit of quantification was 1.0 ng/ml in plasma and 5 ng/g in brain (peak S/N > 6).