SUPPORTING INFORMATION

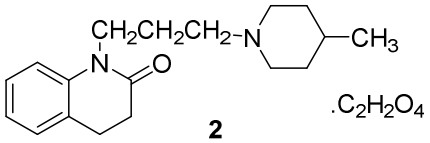
1-[3-(4-Butylpiperidin-1-yl)propyl]-1,2,3,4-tetrahydroquinolin-2-one (77-LH-28-1) as a Model for the Rational Design of a Novel Class of Brain Penetrant Ligands with High Affinity and Selectivity for Dopamine D_4 Receptor

Fabio Del Bello, Alessandro Bonifazi, Gianfabio Giorgioni, Carlo Cifani, Maria Vittoria Micioni Di Bonaventura, Riccardo Petrelli, Alessandro Piergentili, Stefano Fontana, Valerio Mammoli, Hideaki Yano, Rosanna Matucci, Giulio Vistoli, and Wilma Quaglia

Table of Contents: Elemental analysis results for compounds **2**-**4** and **8**-**14**. Experimental details of radioligand binding assays at α_{1a}-, α_{1b}- and α_{1d}-AR, β_1- and β_2-AR, σ_1 receptor, DAT and SERT. Calibration standard and quality control report for **12**.

Table 1S. Elemental analysis results for compounds **2**-**4** and **8**-**14**.

Compd	Formula	Calcd			Found		
		C%	H%	N%	C%	H%	N%
2	$C_{18}H_{26}N_2O.H_2C_2O_4$	63.81	7.50	7.44	63.67	7.38	7.60
3	$C_{20}H_{30}N_2O.H_2C_2O_4$	65.32	7.97	6.93	65.46	8.07	6.81
4	$C_{22}H_{34}N_2O.H_2C_2O_4$	66.64	8.39	6.48	66.79	8.24	6.61
8	$C_{23}H_{28}N_2O.H_2C_2O_4$	68.47	6.90	6.39	68.24	6.80	6.23
9	$C_{24}H_{30}N_2O.H_2C_2O_4$	69.01	7.13	6.19	68.88	7.30	6.10
10	$C_{25}H_{32}N_2O.H_2C_2O_4$	69.51	7.35	6.00	69.24	7.19	6.09
11	$C_{20}H_{31}N_3O.H_2C_2O_4$	62.99	7.93	10.02	63.25	7.90	10.19
12	$C_{22}H_{27}N_3O.H_2C_2O_4$	65.59	6.65	9.56	65.27	6.54	9.36
13	$C_{23}H_{29}N_3O.H_2C_2O_4$	66.21	6.89	9.27	66.41	7.02	9.09
14	$C_{24}H_{31}N_3O.HCl$	69.63	7.79	10.15	69.88	7.88	10.07



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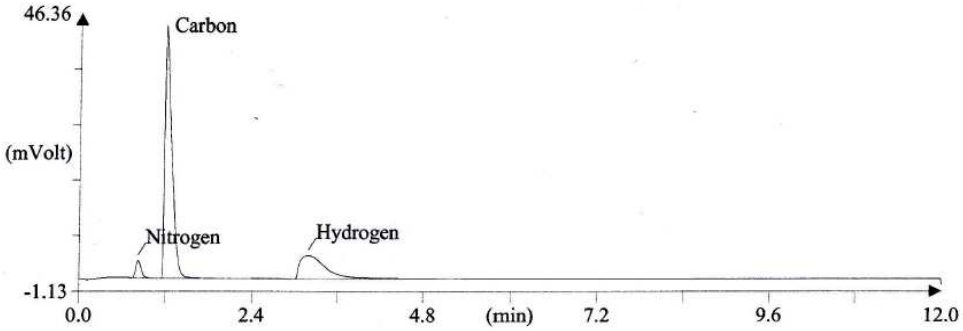

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Sample weight:	1.001

Element Name	%	Ret. Time
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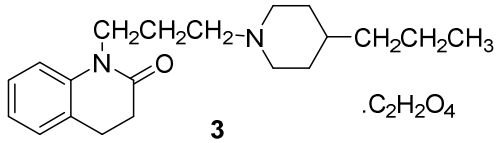
Carbon	63.6728	71
Hydrogen	7.3760	192
Totals	78.6495	

Figure 1S. Results sheet of elemental analysis of compound **2**.



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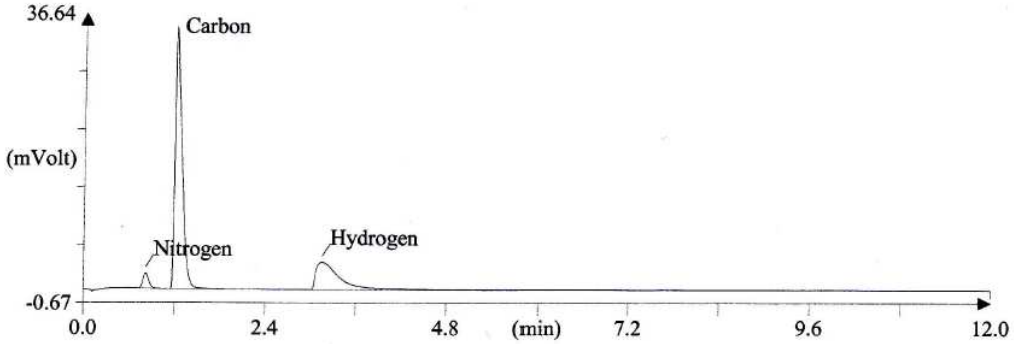

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Chromatogram filename:	Del Bello 2015 SS14.dat
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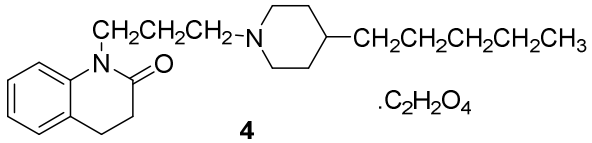
Element Name	%	Ret. Time
Nitrogen	6.8088	49
Carbon	65.4630	72
Hydrogen	8.0665	189
Totals	80.3383	

Figure 2S. Results sheet of elemental analysis of compound **3**.



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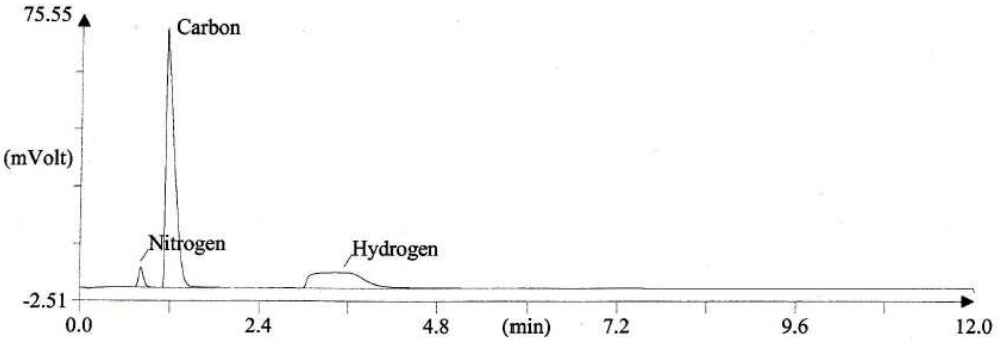

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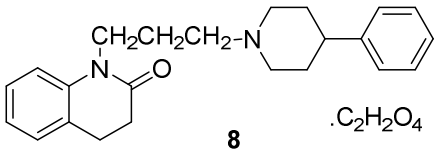
Analysis type:	UnkNown
Chromatogram filename:	Del Bello 2016 SS15.dat
Calibration method:	K Factors
Sample weight:	1.746

Element Name	%	Ret. Time
Nitrogen	6.6076	50
Carbon	66.7905	70
Hydrogen	8.2442	204
Totals	81.6423	

Figure 3S. Result sheets of elemental analysis of compound **4**.



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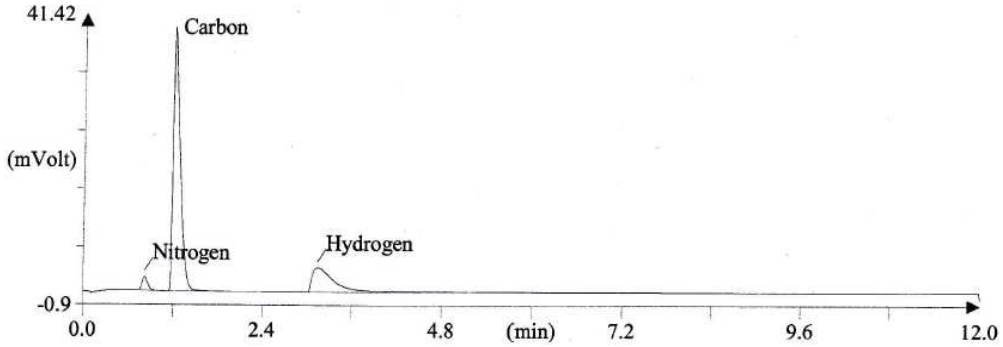

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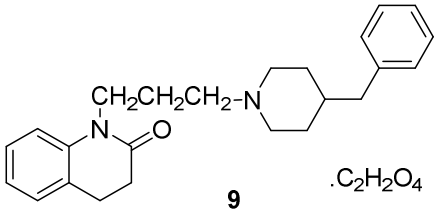
Chromatogram filename:	Del Bello 2015 SS12.dat
Calibration method:	K Factors
Sample weight:	.771

Element Name	%	Ret. Time
Nitrogen	6.2306	49
Carbon	68.2369	72
Hydrogen	6.7977	189
Totals	81.2652	

Figure 4S. Results sheet of elemental analysis of compound **8**.



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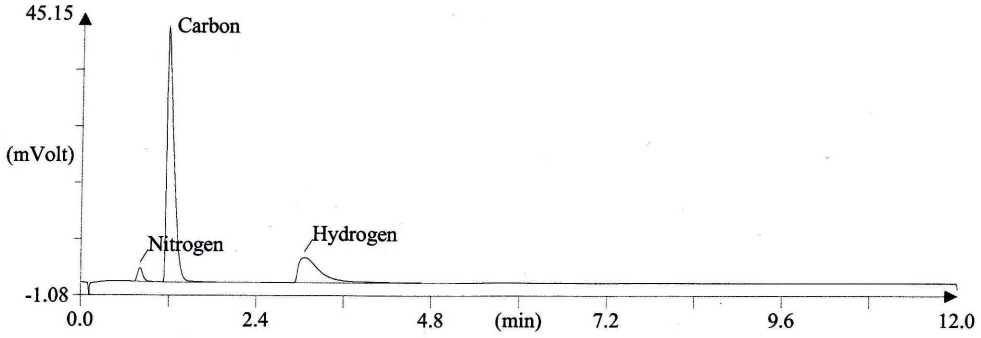

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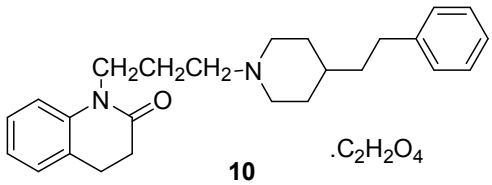
Chromatogram filename:	Del Bello 2015 SS7.dat
Calibration method:	K Factors
Sample weight:	.86

Element Name	%	Ret. Time
Nitrogen	6.1003	49
Carbon	68.8823	70
Hydrogen	7.3047	184
Totals	82.2873	

Figure 5S. Results sheet of elemental analysis of compound **9**.



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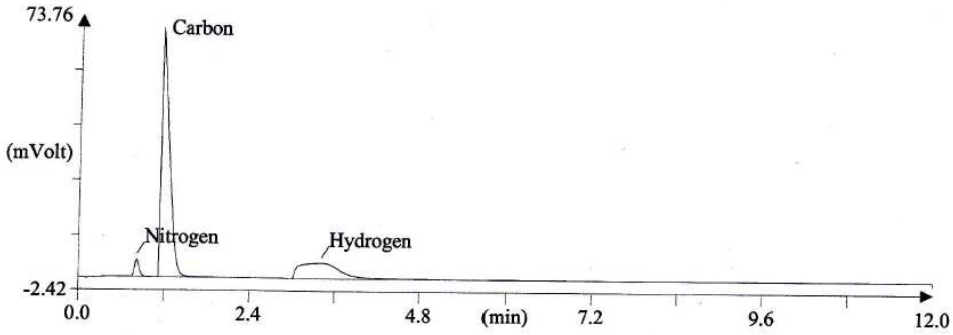

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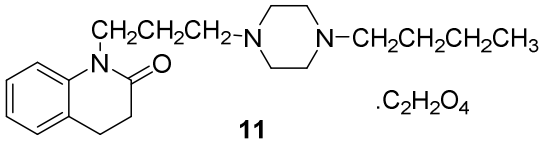
Element Name	%	Ret. Time
Nitrogen	6.0852	49
Carbon	69.2402	69
Hydrogen	7.1858	205
Totals	82.5112	

Figure 6S. Results sheet of elemental analysis of compound **10**.



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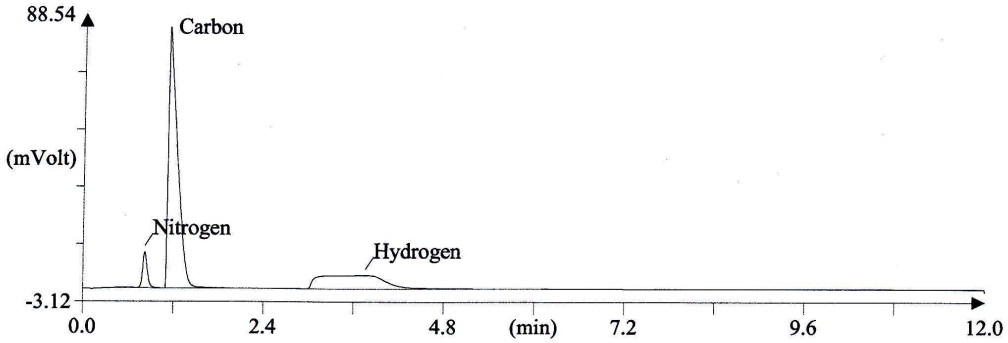

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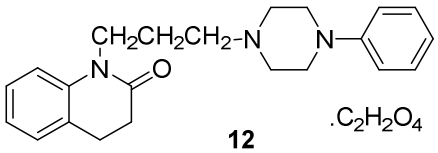
Chromatogram filename:	Del Bello 2015 07-AF-46.dat
Calibration method:	K Factors
Sample weight:	2.636

Element Name	%	Ret. Time
Nitrogen	10.1863	49
Carbon	63.2529	68
Hydrogen	7.8982	226
Totals	81.3374	

Figure 7S. Results sheet of elemental analysis of compound **11**.



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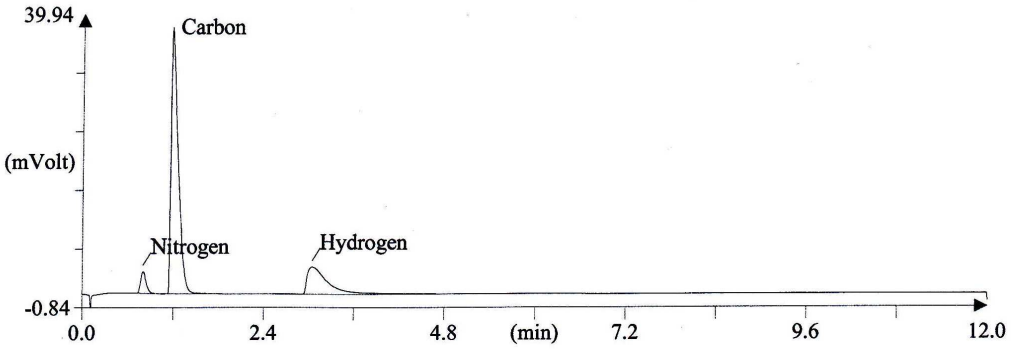

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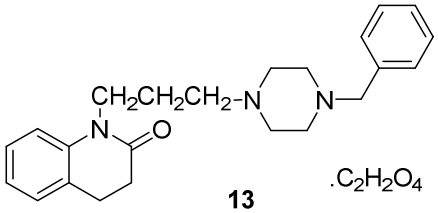
Chromatogram filename:	Del Bello 2015 MC8.dat
Calibration method:	K Factors
Sample weight:	.773

Element Name	%	Ret. Time
Nitrogen	9.3641	48
Carbon	65.2680	71
Hydrogen	6.5351	183
Totals	81.1672	

Figure 8S. Results sheet of elemental analysis of compound **12**.



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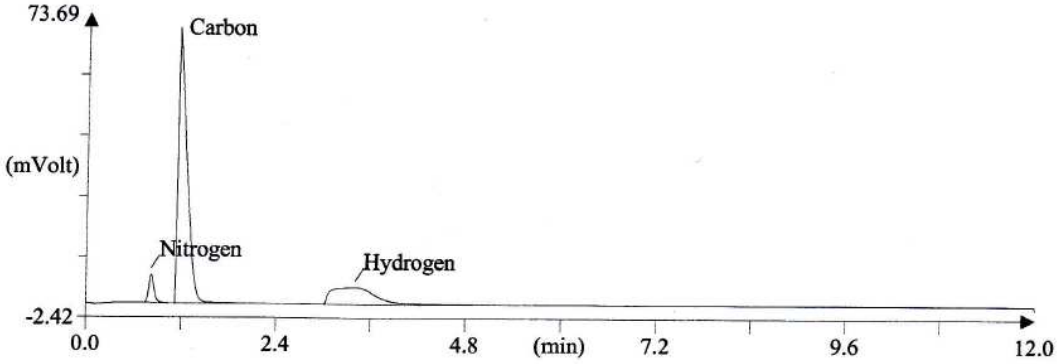

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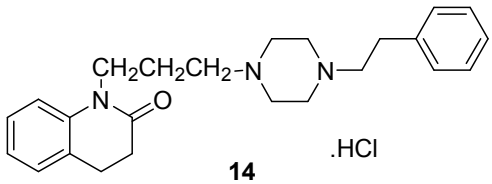
Chromatogram filename:	Del Bello 2016 MC16.dat
Calibration method:	K Factors
Sample weight:	1.89

Element Name	%	Ret. Time
Nitrogen	9.0919	49
Carbon	66.4090	69
Hydrogen	7.0211	204
Totals	82.5220	

Figure 9S. Results sheet of elemental analysis of compound **13**.



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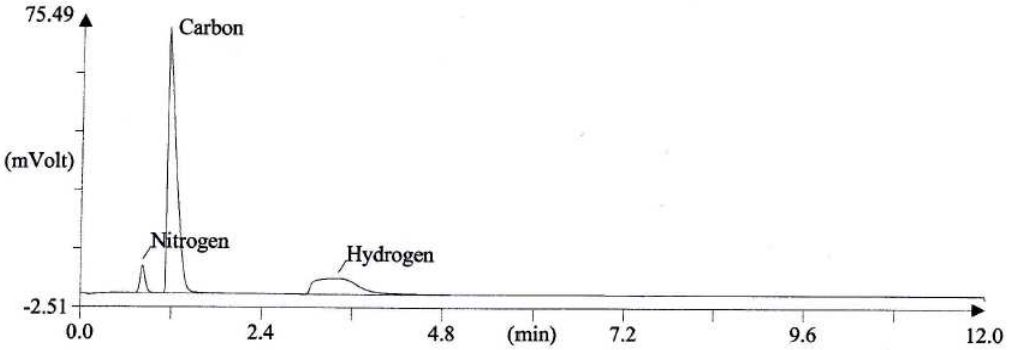

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Analysis type:	UnkNown
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Calibration method:	K Factors
Sample weight:	1.957

Element Name	%	Ret. Time
Nitrogen	10.0723	49
Carbon	69.8759	69
Hydrogen	7.8807	205
Totals	87.8289	

Figure 10S. Results sheet of elemental analysis of compound **14**.

Off-targets binding assays details

α-ARs radioligand binding in CHO cells. Binding to recombinant human α_1-AR subtypes was performed in membranes from CHO cells transfected by electroporation with DNA expressing the gene encoding each α_1-AR subtype. CHO cell membranes (70 μg of protein) were incubated in 50 mM Tris (pH 7.4) with 0.1–0.4 nM [^{3}H]prazosin, in a final volume of 0.32 mL for 30 min at 25 °C, in the absence or presence of competing drugs (1 pM to 1 μM). Nonspecific binding was determined in the presence of 10 μM prazosin. After an incubation of 30 min at 25 °C, bound radioligand was separated by vacuum filtration through glass fibre filters (UniFilter GF/B, Perkin-Elmer Life and Analytical Science, Monza, Italy). The filters were rapidly washed with milliQ water and the membrane-bound radioactivity was quantified by liquid scintillation counting (TopCount, Perkin-Elmer Life and Analytical Science, Monza, Italy)**.** IC_{50} values for each compound were determined from inhibition curves and K_i values were calculated using the Cheng-Prusoff equation. These analyses were performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA). K_i values were determined from at least 3 independent experiments and are reported as mean ± SEM.

Curves were fitted to the individual data points of sets of 3–5 separate experiments by computer-aided nonlinear regression (software: GraphPad Prism, version 5.02, GraphPad Software, San Diego, CA). Results are expressed as means ± S.E.M.

β-ARs radioligand binding in HEK293 cells. Binding to recombinant human β-AR subtypes was performed with HEK293 cells expressing human β_1 or β_2 receptors. The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/ml each of penicillin G and streptomycin (Sigma–Aldrich, Milano, Italy), 4 mM glutamine (Sigma–Aldrich, Milano, Italy) and puromycin (1.5 μg/ml) and maintained at 37°C in a 5% CO_2 in

humidified incubator. Cells were detached from the growth dishes at 95% confluence by a cell scraper, pelletted and homogenized in ice-cold buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 7.4) with an Ultra-Turrax (IKA Labortechnik, Staufen, Germany) twice for 20 s at half speed. The homogenate was centrifuged (50,000 g, 10 min, 4°C) and the pellet was resuspended in a small volume of ice-cold buffer and immediately frozen and stored at -80 °C until use. The protein content was measured according to the method of Bradford using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Munchen, Germany). Competition binding studies were carried out using a range of concentrations of test compounds, 0.2 μM [^{3}H]CGP12177 and about 30-40 μg/well of membrane suspension in a final volume of 250 μl in a 96 multiwell plate. Nonspecific binding was determined in the presence of 10 μM of propranolol. After an incubation of 90 min at 25 °C, bound radioligand was separated by vacuum filtration through glass fibre filters (UniFilter GF/B, Perkin-Elmer Life and Analytical Science, Monza, Italy). Plates were washed several times with ice-cold milliQ water and the radioactivity remained on the filter was counted in a β-counter (TopCount, Perkin-Elmer Life and Analytical Science, Monza, Italy) with Microscint 20 (TopCount, Perkin-Elmer Life and Analytical Science, Monza, Italy) as scintillation cocktail. IC_{50} values for each compound were determined from inhibition curves and K_i values were calculated using the Cheng-Prusoff equation. These analyses were performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA). K_i values were determined from at least 3 independent experiments and are reported as mean ± SEM.

σ_1 receptor radioligand binding in guinea pig cortex. Male Hartley guinea pig cortices were dissected from freshly harvested brains (shipped cold in PBS buffer from BioReclamation IVT) and frozen at -80 °C for future use. On test day, thawed guinea pig cortices were suspended and homogenized in 20 volumes (w/v) of ice cold buffer (10 mM Tris.HCl, 0.32M Sucrose, pH 7.4 at 25 °C) with a glass-teflon apparatus and centrifuged (~1,200 rpm) for 10 min at 4 °C. The supernatant was collected in a clean tube and the pellet re-suspended in 10 ml of cold buffer and

centrifuged again (~1,200 rpm) for 10 min at 4 °C. The supernatants were pooled together and centrifuged (20,000 rpm) for 15 min at 4 °C. The final pellet was suspended in ice-cold binding buffer at 50 mg/ml concentration (original wet weight). All test compounds were freshly diluted in 30% DMSO and 70% H_2O to a stock concentration of 1 mM or 100 μM. To assist the solubilization of free-base compounds, 10 μl of glacial acetic acid was added along with the DMSO (in place of 10 μl final H_2O volume). Each test compound was then diluted into 10 half-log serial dilutions using 30% DMSO as the vehicle. Radioligand competition experiments were conducted in 96-well plates containing 300 μl fresh binding buffer, 50 μl of diluted test compound, 100 μl of tissue preparation, and 50 μl of radioligand diluted in binding buffer ([^{3}H]-(+)-Pentazocine: 3 nM final concentration, ARC, Saint Louis, MO). Aliquots of [^{3}H]-(+)-pentazocine solution were also quantified accurately to determine how much radioactivity was added. Nonspecific binding was determined using 10 μM (+)-pentazocine and total binding was determined with 30% DMSO vehicle. All compound dilutions were tested in triplicate and the competition reactions started with the addition of the tissue preparation and incubated for 120 min at room temperature. The reaction was terminated by filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 120 min in 0.05% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed 3 times with 3 ml (3 x 1 ml/well) of ice cold binding buffer. 65 μL Perkin Elmer MicroScint 20 Scintillation Cocktail was added to each well and filters were counted using a Perkin Elmer MicroBeta Microplate Counter (efficiency: 33%). IC_{50} values for each compound were determined from inhibition curves and K_i values were calculated using the Cheng-Prusoff equation. K_d values for [^{3}H]-(+)-Pentazocine (σ_1 receptor: 5.18 nM) were determined via separate homologous competitive binding experiments. These analyses were performed using GraphPad Prism version 6.00 for Macintosh (GraphPad Software, San Diego, CA). K_i values were determined from at least 3 independent experiments and are reported as mean ± SEM.

SERT radioligand binding in rat midbrain. Frozen brain stems dissected from male Sprague-Dawley (supplied in ice cold PBS buffer from BioreclamationIVT (Hicksville, NY)) rat brains were homogenized in 10-20 volumes (w/v) of 50 mM ice cold Tris buffer (120 mM NaCl and 5mM KCl, adjusted to pH 7.4 at 25 ^{O}C) using a Brinkman Polytron (two cycles at setting 6 for 10 s each). The tissue was centrifuged at 20,000 rpm for 10 min at 4 ^{O}C. The pellet was suspended in cold buffer and centrifuged again. The resulting pellet was resuspended in cold buffer at a concentration of 20 mg/mL original wet weight. On test day, all test compounds were diluted, from an initial concentration of 1 mM or 100 µM, into 10 half-log serial dilutions using 30% DMSO vehicle. To assist the solubilization of free-base compounds, 10 µl of glacial acetic acid was added along with the DMSO (in place of 10 µl final H_2O volume). Radioligand competition experiments were conducted in 96-well plates containing 50 µL of diluted test compound, 300 µl of fresh binding buffer, 50 µl of radioligand diluted in binding buffer ([^{3}H]-citalopram HCl: 1.5 nM final concentration; American Radiolabeled Chemicals, Saint Louis, MO;) and 100 µl of tissue preparation (2 mg of brain stem membranes per well). Aliquots of [^{3}H]-citalopram solution were also quantified accurately to determine how much radioactivity was added. Non-specific binding was determined using 10 µM fluoxetine HCl and total binding was determined with 30% DMSO vehicle. The reaction was started with the addition of the tissue. All compound dilutions were tested in triplicate and the reaction incubated for 60 min at room temperature. The reaction was terminated by filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 60 min in 0.3% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed three times with ice cold binding buffer (1 mL each time). 65 µL Perkin Elmer MicroScint 20 Scintillation Cocktail was added to each well and filters were counted using a Perkin Elmer MicroBeta Microplate Counter (efficiency: 30.4%). IC_{50} values for each compound were determined from dose-response curves and K_i values were calculated using the Cheng-Prusoff equation. K_d value for [^{3}H]-citalopram HCl (6.68 nM) was determined via separate homologous competitive binding experiments. These analyses were performed using

GraphPad Prism version 6.00 for Macintosh (GraphPad Software, San Diego, CA). K_i values were determined from at least 3 independent experiments and are reported as mean ± SEM.

DAT radioligand binding in rat striatum. Frozen brain striata dissected from male Sprague-Dawley rat brains (supplied in ice cold PBS buffer from BioreclamationIVT (Hicksville, NY)) were homogenized in 10-20 volumes (w/v) of modified sucrose phosphate buffer (0.32M Sucrose, 7.74 mM Na_2HPO_4, 2.26mM NaH_2PO_4 adjusted to pH 7.4 at 25 C) using a Brinkman Polytron (two cycles at setting 6 for 10 s each). The tissue was centrifuged at 20,000 rpm for 10 min at 4 ^{O}C. The pellet was suspended in cold buffer and centrifuged again. The resulting pellet was resuspended in cold buffer at a concentration of 15 mg/mL OWW (original wet weight). On test day, all test compounds were freshly diluted in 30% DMSO and 70% H_2O to a stock concentration of 1 mM or 100 µM. To assist the solubilization of free-base compounds, 10 µl of glacial acetic acid was added along with the DMSO (in place of 10 µl final H_2O volume). Each test compound was then diluted into 10 half-log serial dilutions using 30% DMSO vehicle. Radioligand competition experiments were conducted in 96-well plates containing 50 µL of diluted test compound, 300 µl of fresh binding buffer, 50 µl of radioligand diluted in binding buffer ([^{3}H]-WIN35,428: 1.5 nM final concentration; ARC, Saint Louis, MO) and 100 µl of tissue preparation (1.5 mg of brain striatum membranes per well). Aliquots of [^{3}H]-WIN35,428 solution were also quantified accurately to determine how much radioactivity was added. Non-specific binding was determined using 10 µL Indatraline and total binding was determined with 30% DMSO vehicle. The reaction was started with the addition of the tissue. All compound dilutions were tested in triplicate and the reaction incubated for 120 min at 4 ^{O}C. The reaction was terminated by filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 120 min in 0.05% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed 3 times with 3 mL (3 x 1 mL/well) of ice cold binding buffer. 65 µL Perkin Elmer MicroScint20 Scintillation Cocktail was added to each well and filters were counted using a Perkin Elmer MicroBeta

Microplate Counter (efficiency: 32.3%). IC_{50} values for each compound were determined from dose-response curves and K_i values were calculated using the Cheng-Prusoff equation. K_d value for [^{3}H]- WIN35,428 (28.1 nM) was determined via separate homologous competitive binding experiments. These analyses were performed using GraphPad Prism version 6.00 for Macintosh (GraphPad Software, San Diego, CA). K_i values were determined from at least 3 independent experiments and are reported as mean ± SEM.

Calibration standard and quality control report for **12**.

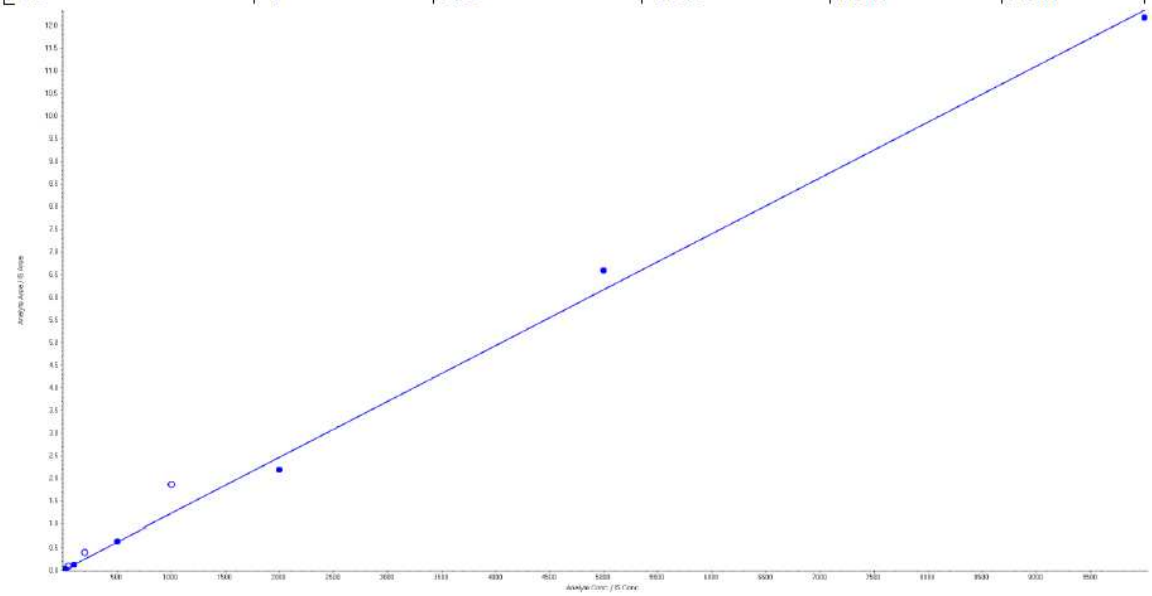
Created with Analyst Reporter
Printed: 26/01/2018 1:45:39 PM

Analyte Name: 12
Internal Standard: Rolipram

Data File	VDD6336_vm3_mo_pl_br\VDD6336_vm3_mo_br.wiff	**Result Table**	VDD6336_brain.rdb
Acquisition Date	26/01/2018 09:59:41	**Algorithm Used**	MQL
Acquisition Method	VDD6336_C1_a1b1.dam	**Instrument Name**	API 4000
Project	Unicam		

Regression Equation: y = 0.00123 x + 0.00293 (r = 0.9985)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
10000	1	9870.27	98.7	NaN	NaN
5000	1	5340.24	106.8	NaN	NaN
2000	1	1772.54	88.6	NaN	NaN
500	1	519.17	103.8	NaN	NaN
100	1	98.00	98.0	NaN	NaN
5	1	4.39	87.7	NaN	NaN
2.5	1	2.91	116.3	NaN	NaN



Data File	VDD6336_vm3_mo_pl_br\VDD6336_vm3_mo_br.wiff	**Result Table**	VDD6336_brain.rdb
Acquisition Date	26/01/2018 09:59:41	**Algorithm Used**	MQL
Acquisition Method	VDD6336_C1_a1b1.dam	**Instrument Name**	API 4000
Project	Unicam		

QC Summary at Concentration: 1000 ng/mL

Analyte Peak Name (MRM Transition)	Mean Calculated Conc (ng/mL)	Std. Deviation (ng/mL)	% CV	Number of Values Used	Mean Accuracy (%)
12 (350.200/160.200 Da)	929.545	96.434	10.37	5	92.95

QC Details

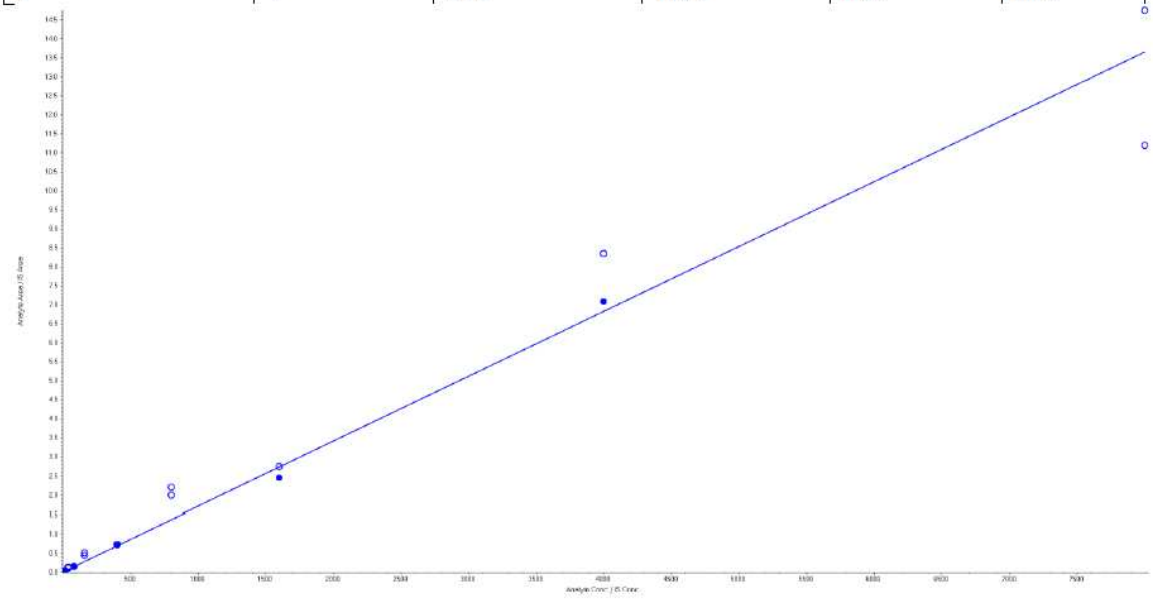
Analyte Peak Name	Sample Name (Index)	Calculated Conc (ng/mL)	Analyte RT (min)	Accuracy (%)
12	qc (17)	1000.000	0.60	100.0
12	qc (18)	804.000	0.60	80.4
12	qc (19)	847.000	0.60	84.7
12	qc (45)	712.000	0.60	71.2
12	qc (46)	1010.000	0.59	101.0
12	qc (47)	987.000	0.60	98.7

Analyte Name: 12a
Internal Standard: Rolipram

Data File	VDD6336_vm3_mo_pl_br\VDD6336_vm3_mo_pl.wiff	**Result Table**	VDD6336_plasma.rdb
Acquisition Date	26/01/2018 07:40:07	**Algorithm Used**	MQL
Acquisition Method	VDD6336_C1_a1b1.dam	**Instrument Name**	API 4000
Project	Unicam		

Regression Equation: y = 0.00171 x + 0.00906 (r = 0.9981)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
4000	1	4152.09	103.8	NaN	NaN
1600	1	1438.55	89.9	NaN	NaN
400	1	410.26	102.6	NaN	NaN
80	1	79.96	100.0	NaN	NaN
16	1	13.69	85.5	NaN	NaN
8	1	9.46	118.2	NaN	NaN



Data File	VDD6336_vm3_mo_pl_br\VDD6336_vm3_mo_pl.wiff	**Result Table**	VDD6336_plasma.rdb
Acquisition Date	26/01/2018 07:40:07	**Algorithm Used**	MQL
Acquisition Method	VDD6336_C1_a1b1.dam	**Instrument Name**	API 4000
Project	Unicam		

QC Summary at Concentration: 800 ng/mL

Analyte Peak Name (MRM Transition)	Mean Calculated Conc (ng/mL)	Std. Deviation (ng/mL)	% CV	Number of Values Used	Mean Accuracy (%)
12a (350.200/160.200 Da)	844.116	82.245	9.74	9	105.51

QC Details

Analyte Peak Name	Sample Name (Index)	Calculated Conc (ng/mL)	Analyte RT (min)	Accuracy (%)
12a	qc (17)	709.000	0.60	88.7
12a	qc (18)	857.000	0.60	107.0
12a	qc (19)	822.000	0.60	103.0
12a	qc (45)	740.000	0.59	92.5
12a	qc (46)	918.000	0.59	115.0
12a	qc (47)	910.000	0.59	114.0
12a	qc (65)	795.000	0.59	99.4
12a	qc (66)	922.000	0.59	115.0
12a	qc (67)	923.000	0.59	115.0