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Oxa-azaspiro Derivatives: a Novel Class of Triple Re-uptake Inhibitors

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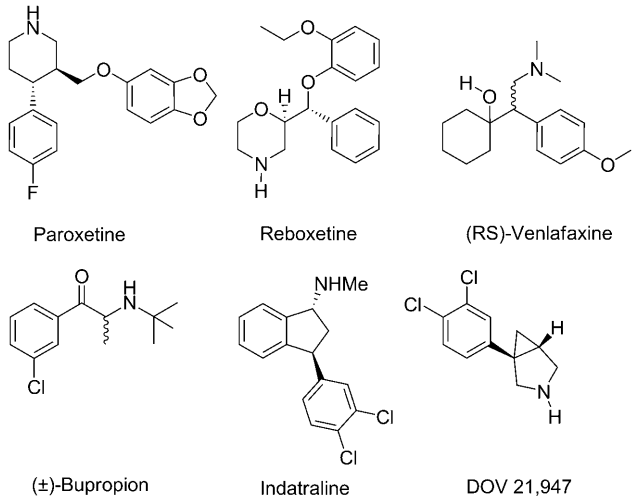
Drugs able to interfere with either the uptake or the metabolism of endogenous monoamines have been used for many years to treat depression. The first drugs of this type, such as the monoamine oxidase (MAO) inhibitors and the tricyclic antidepressants, achieved wide diffusion in the market, but are unfortunately associated with side effects that may influence patient compliance.[1] Over the last few years, compounds acting through the selective blockade of neurotransmitter re-uptake have demonstrated their efficacy as successful antidepressant agents. This blockade can take place in either serotoninergic neurons (SSRI; e.g., paroxetine) or noradrenergic neurons (SNRI; e.g., reboxetine). Moreover, drugs known as "dual uptake" inhibitors, which act on both serotoninergic and noradrenergic transporters (e.g., venlafaxine) or on both noradrenergic and dopaminergic transporters (e.g., bupropion), have also demonstrated good efficacy.[1]



More recently, compounds acting as "triple re-uptake" inhibitors (TRUI),[2] such as indatraline and DOV 21,947, have been disclosed. Given the extensive experience of investigators at GlaxoSmithKline (GSK) with selective, dual and triple re-uptake inhibitors, new TRUI scaffolds were rationally planned exploiting a pharmacophore model developed in house

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More precisely, three specific pharmacophore models for the monoamine transporters, SERT (serotonin transporter), NET (norepinephrine transporter) and DAT (dopamine transporter), were built using structurally rigid and selective derivatives. These compounds were derived both from the GSK proprietary collection and from the public domain (e.g., derivatives described in Reference [3]). The structures were modeled within the Maestro modeling environment.[4] Conformational analyses were carried out for the three structures with BatchMin[5] using the following parameters: 1000 steps/rotatable bond, OPLS_2005 FF, implicit water model of solvation, 5000 minimization steps with PRCG. Conformations lying within a 3 kcal mol^{-1} energy window were kept.

A single-ligand pharmacophore was then built over these structures using the Phase[6] module within Maestro, and selecting the most relevant pharmacophoric features. The three pharmacophores were finally merged together to create the triple re-uptake inhibitor pharmacophore. Average coordinates for the common pharmacophoric features were taken to determine their three-dimensional arrangement, as illustrated in Figure 1.

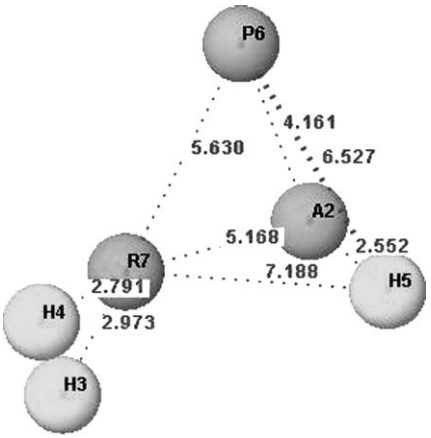
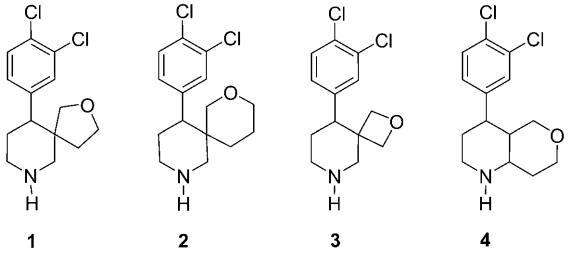


Figure 1. The triple re-uptake inhibitor pharmacophore. Coding of features: P6 sphere, positive ionizable; A2 sphere, H-bond acceptor; H3, H4, and H5 spheres, hydrophobic; R7 sphere, aromatic ring. Distances between the pharmacophoric points are given in Å.

The aim of the work described herein was twofold: the first objective was to set up the chemistry to prepare a new series of oxa-spiro derivatives; the second was to validate the capability of the pharmacophore model to predict the rank order of affinity of the new derivatives.

It was hypothesized that the introduction of an appropriately located tetrahydrofuran (**1**) or a tetrahydropyran (**2**) moiety on the 4-phenyl piperidine scaffold should have satisfied at



1 2 3 4

least five out of the six pharmacophoric points in the model as depicted in Figure 2, suggesting that the proposed derivatives should be endowed with good affinity at the three monoamine transporters, namely SERT, NET and DAT.

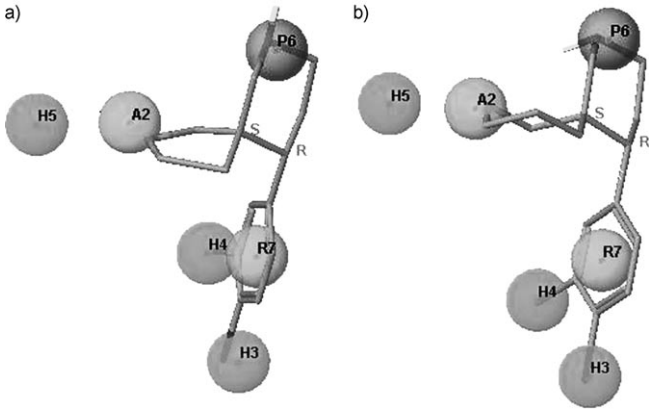


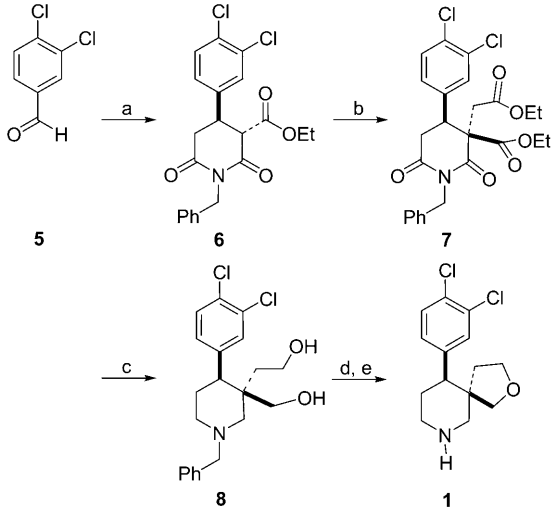
Figure 2. The proposed scaffolds a) **1** and b) **2** fitted in the TRUI pharmacophore model.

Such model compounds were meant to provide a rapid reply to the working hypothesis before embarking on a more complex synthesis of final scaffolds designed to fully fit all six of the pharmacophoric points.

From an in silico point of view, both derivatives **1** and **2** provided excellent fitting in the TRUI pharmacophore, with **2** being slightly superior in its ability to fit the desired features (Figure 2). However, it is evident that the sixth feature (i.e., the H5 hydrophobic region) is not satisfied by either derivative **1** or **2**, but that the introduction of an appropriate substituent able to reach this area might further increase the potential affinity of the molecules.

Given these promising theoretical results, the necessary synthetic steps to prepare compounds **1** and **2** were put in place and their preparation is reported in Scheme 1 and 2, respectively (n.b., relative stereochemistry shown).

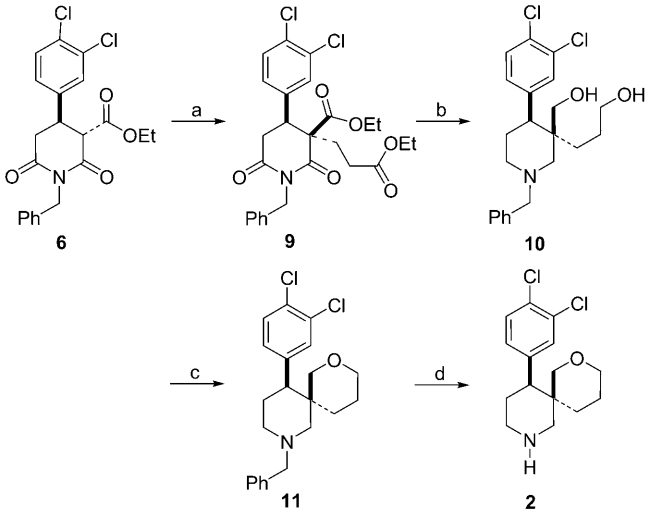
3,4-Dichlorobenzaldehyde was reacted with methyl 3-oxo-3-[(phenylmethyl)amino]propanoate under basic conditions[7] to provide the versatile intermediate **6** in 55% yield as a racemate. This derivative was alkylated with ethyl bromoacetate under thermodynamic conditions leading to the isolation of a single diastereoisomer **7** in 58% yield. The stereochemistry of the reaction is probably controlled by the electrophile approaching the reacting centre from the opposite side to the bulky aromatic group.



Scheme 1. *Reagents and conditions*: a) NaOEt, AcOEt/THF, RT, methyl 3-oxo-3-[(phenylmethyl)amino]propanoate, multistep; b) Ethyl bromoacetate, K_2CO_3, 85 °C, 1.5 h; c) BH_3 ·THF, 65 °C; d) PPh_3, DEAD, CH_2Cl_2; e) ACE-Cl, 1,2-dichloroethane, reflux, 5 h.

The subsequent exhaustive reduction using borane·THF complex led to the isolation of derivative **8** in 87% yield. The final cyclization was performed under Mitsunobu's conditions[8] and the target derivative **1** was isolated after deprotection of the benzyl group with 1-chloroethyl chloroformate[9] in 43% overall yield. Derivative **1** was tested in biological assays as the racemate.

As described in Scheme 2, intermediate **6** was reacted under Michael reaction conditions with ethyl acrylate to obtain, as previously, a single diastereoisomer (**9**) in 71% yield. Subsequently, this intermediate was reacted with borane·THF com-



Scheme 2. *Reagents and conditions*: a) Ethyl acrylate, K_2CO_3, acetone, overnight, RT; b) BH_3 ·THF, 60 °C, 8 h; c) PPh_3, DEAD, CH_2Cl_2; d) ACE-Cl, 1,2-dichloroethane, reflux, 5 h.

plex to obtain the diol **10** in 29% yield. Cyclization under Mitsunobu's conditions[8] led to the N-benzylated derivative **11** in 21% yield. Deprotection with 1-chloroethyl chloroformate led to the desired product **2** in 65% yield. As for derivative **1**, compound **2** was tested in biological assays as the racemate.

Both compounds were evaluated for their ability to bind to the three monoamine transporters (SERT, NET and DAT) in scintillation proximity assay (SPA)-binding assays on membranes from cells transiently transduced with BacMam virus. Commercially available indatraline was used as a reference compound. Results are reported in Table 1.

Table 1. Binding at the three transporters (SERT, NET, DAT).

Entry	pK_i hSERT SPA[a]	hNET SPA[a]	hDAT SPA[a]
DOV 21,947	7.8	7.2	7.1
Indatraline	9.7	8.8	9.1
1	8.3	7.1	7.4
2	9.2	8.0	7.7
3	7.2	6.8	6.8
4	7.5	7.3	7.4
20	4.3	4.5	5.6
21[b]	8.9	7.2	7.5
22[c]	9.5	8.4	7.9

[a] SEM for hSERT/NET/DAT data sets is $\pm$0.1. [b] **21** is the enantiomer 1 of derivative **2**. [c] **22** is the enantiomer 2 of derivative **2**

As can be clearly appreciated, and in agreement with the suggestions provided by the pharmacophore model, derivative **2** showed better affinity than **1** towards SERT and NET, while the affinity of the two molecules towards DAT was comparable. Interestingly, both derivatives were endowed with similar or superior affinity for these transporters to those reported in literature for the clinical candidate DOV 21,947.[2b]

To further increase the confidence in the ability of the pharmacophore model to rank order the affinity of the derivatives in this specific series, and therefore to reinforce the original hypothesis, derivatives **3** and **4** were also prepared. Figure 3 shows that these two model systems were endowed with an inferior "theoretical" ability to fit the pharmacophore. Despite the poor two-dimensional depiction of a three-dimensional reality, it can be appreciated that the overlap of the oxetane ring of derivative **3** with the A2 region of the pharmacophore is poor and inferior to the 5- and 6- membered spiro systems of compounds **1** and **2**, respectively. In derivative **4**, the oxygen atom is behind the A2 sphere and only a minimal overlap is present with this region.

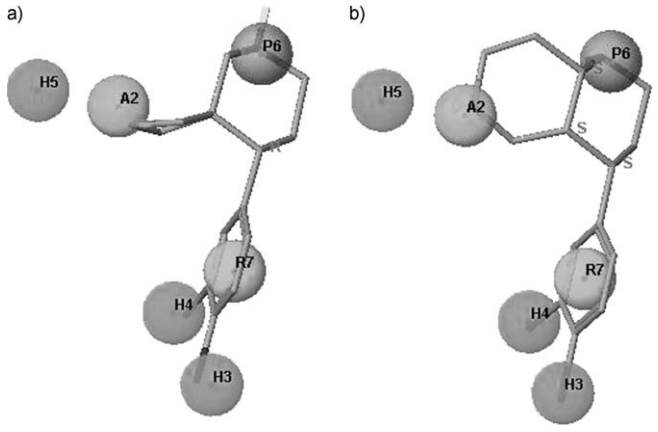
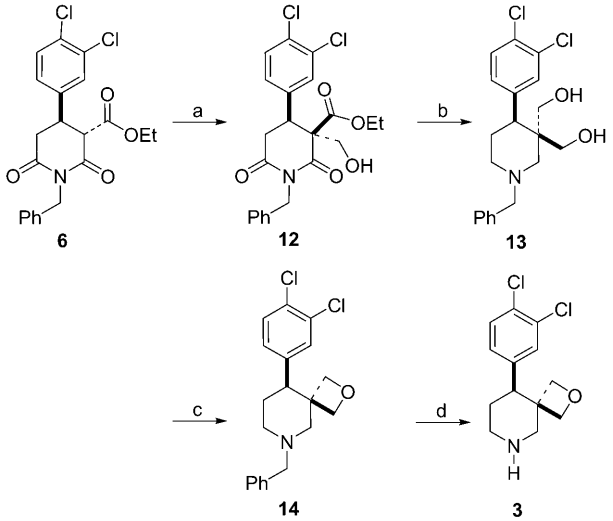


Figure 3. The proposed model scaffolds a) **3** and b) **4** fitted in the TRUI pharmacophore model. The overlap of the oxetane ring with the H-bond acceptor region A2 is the least pronounced in the series of oxa-spiro derivatives.

The synthesis of compound **3** from the versatile intermediate **6** is described in Scheme 3. The electrophile used, in this case, was an aqueous solution of formaldehyde, which allowed

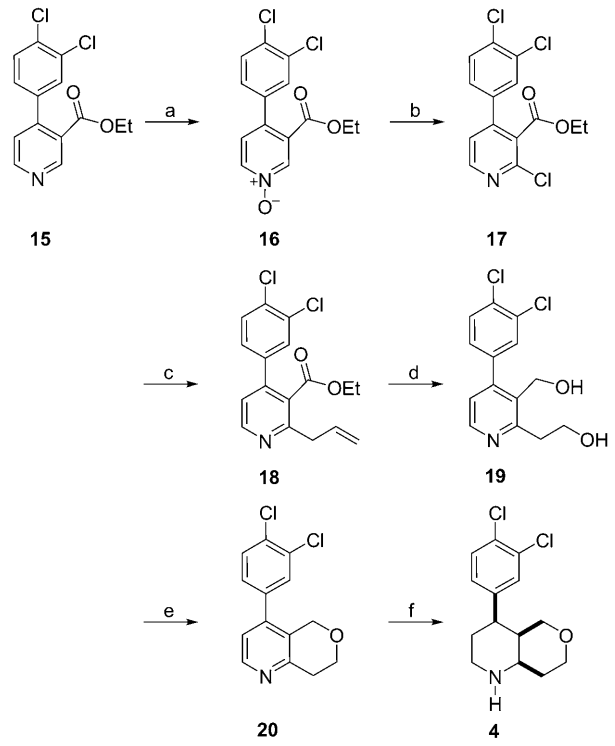


Scheme 3. *Reagents and conditions*: a) Formaline 37%, Et_3N, dioxane, 1.5 h, RT; b) BH_3·THF, 60 °C, 8 h; c) MsCl/Py, 0 °C; d) THF, NaOH, reflux, 6 h; e) ACE-Cl, 1,2-dichloroethane, reflux, 5 h.

the isolation of intermediate **12** in almost quantitative yield. The subsequent reaction with borane·THF complex led to derivative **13** in 91% yield. A two-step cyclization, through the formation of a mesylate intermediate, allowed the isolation of **14** in 30% yield. The final deprotection to derivative **3** was inefficient due to formation of a series of by-products, leading to an isolated yield of just 10%.

Concerning derivative **4**, the synthetic route to the desired fused bicyclic system had to be carefully evaluated so as to obtain the appropriate stereochemistry to fit the pharmacophore model. Actually, in this novel, substituted octahydro-2*H*-pyrano[4,3-b]pyridine template, a *cis* junction of the two six-membered rings led to a better fit in the pharmacophore model than the *trans* junction. For this specific reason, a hydrogenation step was considered as the key, last step in the synthesis.

The desired compound **4** was prepared from ethyl 4-(3,4-dichlorophenyl)-3-pyridinecarboxylate **15** (Scheme 4) by treating it with 3-chloro perbenzoic acid at room temperature to give intermediate **16** in quantitative yield. Reaction with phosphorus oxychloride allowed the preparation of the intermediate **17**



Scheme 4. *Reagents and conditions*: a) mCPBA, CH_2Cl_2, overnight, RT; b) $POCl_3$, overnight, 80 °C; c) allyl triethyl stannane, $Pd(PPh_3)_4$, toluene, reflux, 5 h; d) O_3, EtOH, −78 °C followed by $NaBH_4$ from −78 °C to RT, overnight; e) P_2O_5, toluene, dioxane, 130 °C, 5 h; f) PtO_2, H_2, EtOH, RT, 2 h.

in 45% yield, which was then subjected to a Stille coupling[10] to give the desired allyl derivative **18** in 40% yield. Ozonolysis of this compound at low temperature, followed by reduction with excess sodium borohydride, allowed the isolation of the diol **19** in quantitative yield. The subsequent cyclization step proved difficult; neither of the previously employed cyclization procedures worked for this system. Mitsunobu's conditions stopped at the activated intermediate stage, while an attempt to pass through a mesylate intermediate only led to degradation products. In the end, cyclization to the desired intermediate **20** was achieved in low yield (10%) through dehydrating conditions (P_2O_5 at high temperature). Despite the potential interference of the chlorine atoms, the planned hydrogenation step with platinum dioxide worked very well, giving the desired product **4** in 90% yield, with just 10% formation of dehalogenated by-products. The desired *cis* junction of the two rings and the reported stereochemistry was confirmed by NMR experiments.

Derivatives **3** and **4** were biologically evaluated along with intermediate **20**. Results for these derivatives are reported in Table 1. As predicted by the pharmacophore model, their affinity was inferior to derivatives **1** and **2**. The almost complete inactivity of intermediate **20** was also expected. In fact, this compound only fitted the lipophilic regions of the pharmacophore marginally and it was not endowed with the basic sp^3 positively ionizable feature identified by the P6 sphere (Figure 4).

Given that derivative **2** showed a good binding profile at the three monoamine transporters, the two enantiomers were

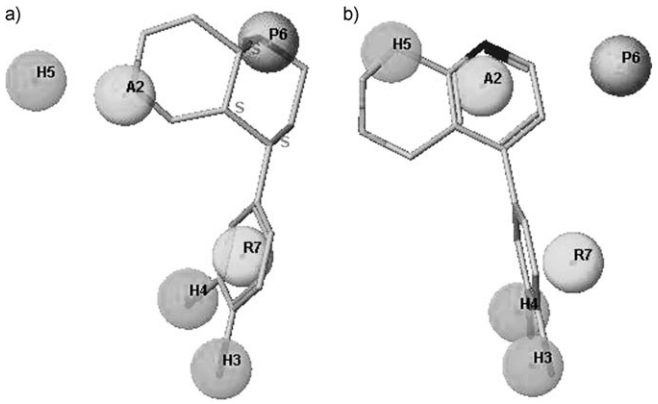


Figure 4. The scaffold a) **4** and b) intermediate **20** fitted in the TRUI pharmacophore model.

isolated. Accordingly, compounds **21** (**2**, enantiomer 1) and **22** (**2**, enantiomer 2) were obtained through chiral chromatography and tested for their binding affinity. Results are reported in Table 1.

Derivative **22** showed high affinity for the three transporters. For this reason, the ability of **22** to block [^{3}H]5-HT, [^{3}H]NE and [^{3}H]DA uptake was then tested in functional uptake SPA assays using LLCPK cells stably transfected with human SERT, NET or DAT. The results of these experiments are reported in Table 2.

Table 2. Ability of **22** to inhibit the uptake of all of the three monoamines.

Entry[a]	$pIC_{50} \pm SEM$		
	hSERT SPA[b]	hNET SPA[b]	hDAT SPA[b]
DOV 21,947	7.2	7.6	7.1
Indatraline	8.5	8.4	8.8
22	8.6	8.9	8.0

[a] DOV 21,947 and indatraline were included as reference compounds. [b] SEM for hSERT/NET/DAT data sets is ±0.2

The functional pIC_{50} value observed in the DAT assay was in line with the affinity of compound **22** determined in the binding assay, while the functional pIC_{50} value observed at NET was slightly higher. Conversely, the functional potency of **22** at SERT was lower than its binding affinity for this transporter. In the latter case, it is important to note that a difference was expected since the SERT uptake-SPA assay is known to be particularly sensitive to test conditions (e.g., cell numbers) and, from previous experience in house, most SERT blockers tested in uptake assays showed pIC_{50} values lower than the corresponding affinity in the binding assay.

Given its excellent in vitro profile, both in the binding and uptake assays, derivative **22** was submitted to CYPEX bactosome P450 inhibition assays and rat and human liver microsomes in vitro clearance assays. This further step was performed to validate the potential for development of such a scaffold. The compound showed an excellent CYP450 inhibition profile demonstrating IC_{50} values greater than 10 μM

against all isoforms, with the exception of CYP2D6 (IC_{50} = 4 μM). The rat and human intrinsic clearance values proved to be relatively low (0.6 and 1.5 mL min^{-1} g^{-1} of protein, respectively), further confirming the tractability of this template. On the basis of these promising in vitro results, the in vivo pharmacokinetic profile of compound **22** was determined in rats.

The overall blood clearance of compound **22** was estimated to be moderate (34 mL min^{-1} kg^{-1}), while its distribution volume was in the moderate to high range (4.9 L kg^{-1}). The resulting half-life was relatively long (2.2 h) and, importantly, the compound was demonstrated to readily cross the blood–brain barrier (Brain/Blood AUC ratio = 9.6). Notwithstanding its modest clearance, derivative **22** was prone to efficient metabolism during the absorption process. After oral administration, despite a good absorption (fraction absorbed = 91%), high hepatic extraction (EH = 90%) led to an overall bioavailability of 9%.

However, this potential drawback might be appropriately fixed when a rational decoration will be used for the scaffolds designed to fully fit the six features of the pharmacophore model as stated at the beginning of the manuscript.

Considering the declared objective of the work, the experimental results described here can be considered to be very positive. The pharmacophore model proved to be proficient in rank ordering the potency of the scaffolds designed in this novel series of spiro derivatives. The synthetic routes designed were successful in preparing the new oxa-spiro scaffolds. The oxa-spiro template **2** proved to be a potent TRUI scaffold, and the potential exists to further increase its affinity and develop its druglike characteristics by appropriately decorated final derivatives.

Experimental Section

Chemistry

DAD chromatographic traces, mass chromatograms and mass spectrums were taken on a on a UPLC/MS Acquity system coupled with a Micromass ZQ mass spectrometer operating in ESI positive or negative. Mobile phases: A) H_2O/MeCN (95:5) +0.1% TFA; B) H_2O/MeCN (5:95) +0.1% TFA. Gradient: t = 0 min) 95% A, 5% B; t = 0.25 min) 95% A, 5% B; t = 3.30 min) 100% B; t = 4.0 min) 100% B; followed by 1 min of reconditioning.

10-(3,4-Dichlorophenyl)-2-oxa-7-azaspiro[4.5]decane (1): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41–7.37 (d, 1H, J = 8.3 Hz), 7.31–7.28 (d, 1H, J = 2.0 Hz), 7.08–7.03 (dd, 1H, J = 8.0, 2.0 Hz), 4.00–3.94 (d, 1H, J = 9.0 Hz), 3.77–3.70 (d, 1H, J = 9.0 Hz), 3.70–3.60 (m, 1H), 3.27–3.20 (m, 1H), 3.22–3.15 (m, 1H), 3.11–3.05 (d, 1H, J = 12.0 Hz), 2.81–2.70 (m, 2H), 2.69–2.63 (d, 1H, J = 12.0 Hz), 1.92–1.78 (m, 2H), 1.74–1.67 (m, 1H), 1.52–1.42 ppm (m, 1H); Relative stereochemistry confirmed on the basis of nOe experiments; MS (ESI): m/z (%): 287 (100) $[M+H]^+$.

11-(3,4-Dichlorophenyl)-2-oxa-8-azaspiro[5.5]undecane (2): EtOAc (3.1 mmol) was added to a solution of **6** (2.4 mmol) in acetone (5 mL) containing K_2CO_3 (2.4 mmol). The reaction was stirred overnight at RT. The solvent was removed in vacuo and compound **9** was purified by chromatography (silica cartridge, 10→30% EtOAc/cyclohexane). Only one diastereoisomer was isolated (71%). Compound **9** (2.114 mmol) was then treated with 1.0 M solution of borane–THF complex in THF (23.25 mmol) and stirred at 60 °C for 8 h. The reaction mixture was cooled to RT and then MeOH (5 mL) was added, followed by 1.0 M HCl in Et_2O (21 mL); the resulting mixture was stirred at RT overnight. The solvent was removed in vacuo and the residue purified by SCX cartridge (100% MeOH then 2.0 N NH_3 in MeOH) to give intermediate **10** as a colorless oil. Compound **10** (0.6 mmol) was dissolved in CH_2Cl_2 and PPh_3 (0.70 mmol) was added, followed by dropwise addition of diethyl azodicarboxylate (40% in 0.86 mmol). The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo and the residue purified by SCX cartridge (100% MeOH then 2.0 N NH_3 in MeOH) to give compound **11** as colorless oil. This intermediate (0.13 mmol) was dissolved in 1,2-dichloroethane (10 mL), 1-chloroethyl chloroformate (1.3 mmol) was added and the mixture was refluxed for 5 h. It was then cooled to RT and MeOH (5 mL) added. The mixture refluxed for 2 h, cooled to RT and the residue purified by SCX cartridge (100% MeOH then 2.0 N NH_3 in MeOH) to give derivative **2**. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41–7.33 (d, 1H, J = 8.3 Hz), 7.21–7.18 (d, 1H, J = 2.0 Hz), 6.97–6.92 (dd, 1H, J = 8.3, 2.0 Hz), 4.36–4.29 (dd, 1H, J = 11.0, 2.4 Hz), 3.87–3.80 (dd, 1H, J = 11.0, 4.6 Hz), 3.74–3.67 (d, 1H, J = 12.2 Hz), 3.27–3.17 (m, 1H), 3.11–3.05 (d, 1H, J = 11.0 Hz), 3.07–2.95 (m, 1H), 2.78–2.67 (m, 1H), 2.49–2.40 (dd, 1H, J = 13.2, 3.7 Hz), 2.31–2.24 (d, 1H, J = 12.4 Hz), 2.05–1.92 (m, 1H), 1.85–1.72 (m, 1H), 1.54–1.47 (m, 1H), 1.47–1.36 (m, 1H), 1.35–1.22 ppm (m, 2H); Relative stereochemistry confirmed on the basis of nOe experiments; MS (ESI): m/z (%): 301 (100) $[M+H]^+$.

The enantiomeric purity of each enantiomer of compound **2**, obtained after preparative chromatography on a chiral column (Chiralpak AD-H, 25×2.0 cm; UV: 225 nm; 23 mg in EtOH per injection; mobile phase: n-hexane/EtOH+0.1% iPrNH$_2$, 70:30; flow rate = 13 mL min^{-1}), was verified on an analytical column (Chiralpak AD-H; DAD = 210–340 nm; CD = 230 nm; mobile phase: n-hexane/EtOH+0.1% iPrNH$_2$, 70:30; flow rate = 0.8 mL min^{-1}; RT = 16.746 min). Enantiomeric excess (ee) was determined for the separated enantiomers by means of SFC analytical techniques using a Chiralpak AD-H column (25×0.46 cm) with EtOH (+0.1% iPrNH$_2$) 15% as modifier, flow rate = 2.5 mL min^{-1}, P = 180 bar at 35 °C with detection at 220 nm. ee = 97%.

9-(3,4-Dichlorophenyl)-2-oxa-6-azaspiro[3.5]nonane (3): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.48–7.46 (d, 1H, J = 8.0 Hz), 7.37–7.36 (d, 1H, J = 2.0 Hz), 7.12–7.09 (dd, 1H, J = 8.0, 2.0 Hz), 4.63–4.62 (dd, 1H, J = 6.4, 1.6 Hz), 4.46–4.44 (d, 1H, J = 6.4 Hz), 4.38–4.36 (d, 1H, J = 6.0 Hz), 4.11–4.09 (d, 1H, J = 6.0 Hz), 3.63–3.60 (d, 1H, J = 12.8 Hz), 3.13–2.91 (m, 1H), 2.87–2.84 (m, 1H), 2.84–2.83 (m, 1H), 2.75–2.72 (m, 1H), 2.72–2.60 (m, 1H), 1.70–1.68 ppm (m, 2H); MS (ESI): m/z (%): 273 (100) $[M+H]^+$.

4-(3,4-Dichlorophenyl)octahydro-2*H*-pyrano[4,3-*b*]pyridine (4): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.40–7.35 (d, 1H, J = 8.3 Hz), 7.26–7.23 (d, 1H, J = 2.0 Hz), 7.03–6.96 (dd, 1H, J = 8.3, 2.0 Hz), 3.84–3.76 (m, 1H), 3.76–3.68 (m, 1H), 3.68–3.60 (m, 1H), 3.39–3.26 (m, 1H), 3.19–3.13 (m, 1H), 3.13–3.06 (dd, 1H, J = 11.0, 4.6 Hz), 2.91–2.77 (m, 2H), 2.20–2.09 (m, 1H), 2.06–1.97 (m, 1H), 1.96–1.85 (m, 1H), 1.58–1.50 (m, 1H), 1.49–1.38 (m, 1H); MS (ESI): m/z (%): 287 (100) $[M+H]^+$.

Biology

The research described here was conducted in compliance with company policy, and with national and European legislation on the care and use of animals in scientific experiments, and related codes of practice.

SPA-binding assay: The affinity of the compounds towards the human transporters has been assessed with radioligand displacement binding of [^{3}H]citalopram, [^{3}H]nisoxetine and [^{3}H]WIN-35,428 for SERT, NET, and DAT in BacMam-recombinant human SERT, NET and DAT membranes with SPA technology. The SPA beads (product number: RPNQ0260) were obtained from GE HealthCare (www.gehealthcare.com). Briefly, 0.3 μL of test compound in DMSO were added to 30 μL of the SPA mixture containing: 1 mg mL^{-1} SPA beads (SERT) or 2 mg mL^{-1} SPA beads (NET and DAT); 6, 40 or 20 μg mL^{-1} of SERT, NET or DAT BacMam membranes; 0.02% Pluronic F-127; 3 nM [^{3}H]citalopram, 10 nM [^{3}H]nisoxetine or 10 nM [^{3}H]WIN-35,428 for SERT/NET/DAT-binding SPA in the assay buffer (20 mM HEPES, 145 mM NaCl, 5 mM KCl, pH 7.4). Incubation was performed overnight at room temperature. Bound radioactivity was measured with a ViewLux™ instrument. Data analysis was performed using a four-parameter logistic equation with ActivityBase software (version 5.4; ID Business Solutions Ltd., UK), and pK_i values were calculated from the pIC_{50} values using the Cheng–Prusoff equation.[11]

Functional uptake SPA assay: The ability of the test compounds to block the [^{3}H]serotonin, [^{3}H]noradrenalin and [^{3}H]dopamine uptake was evaluated in a functional uptake SPA assay on LLCPK cells stably transfected with human SERT, NET or DAT. Briefly, 0.2 μL of test compound were added to 10 μL of the bead/cell SPA mixture containing: 1.5 mg mL^{-1} SPA beads (SERT and NET) or 2 mg mL^{-1} of SPA beads (DAT); 25000, 50000 or 75000 cell per well of SERT, NET, or DAT LLCPK cells; 0.02% Pluronic F-127 in the assay buffer (20 mM HEPES, 145 mM NaCl, 5 mM KCl, pH 7.4). The uptake was initiated by the addition of substrate: 10 μL of 35 nM [^{3}H]serotonin, 45 nM [^{3}H]noradrenalin or 75 nM [^{3}H]dopamine for SERT, NET or DAT uptake SPA. Incubation was performed at room temperature for 1 h. Uptake was measured with a ViewLux™ instrument and data analysis was performed as described above.

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