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Short communication

Flexible analogues of WAY-267,464: Synthesis and pharmacology at the human oxytocin and vasopressin 1_a receptors

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ABSTRACT

A previously identified, non-peptidic oxytocin (OT) receptor agonist WAY-267,464 (**1**) and nine novel derivatives (**3, 4a-7a, 4b-7b**) were synthesised and evaluated *in vitro* with the aim of systematically exploring hydrogen bonding interactions and ligand flexibility. All analogues were subjected to competition radioligand binding assays at human oxytocin (OT) and arginine vasopressin 1_a (V_{1a}) receptors. Physiological activity was determined using whole cell IP1 accumulation assays. Under these conditions, WAY-267,464 had higher affinity for the V_{1a} receptor compared to the OT receptor (8.5x more selective) with poor functional selectivity (2x selective for OT receptor agonism over V_{1a} receptor antagonism). Methylation of the resorcinol moiety (**3**) reversed the OT receptor pharmacological profile, removing agonist activity and inducing antagonist activity, without altering V_{1a} receptor pharmacology. All flexible tethered derivatives removed OT receptor affinity and activity resulting in the generation of highly selective V_{1a} receptor ligands.

1. Introduction

A vast majority of psychological disorders present with a fundamental disruption in social behaviour. Two emerging drug targets that have shown potential as pro-social modulators of behaviour are centrally located oxytocin (OT) and arginine vasopressin (AVP) receptors [1]. Intracerebral administration of OT in animal studies is anxiolytic [2,3] which has led to the clinical evaluation of OT in the involvement of a range of neurological disorders which often present with socially-withdrawn behaviour (for review see Ref. [4]).

AVP is structurally related to OT and exerts its endogenous actions via three G protein coupled receptors, V_{1a}, V_{1b} and V_2 [5]. In the brain, the V_{1a} receptor is the predominant subtype with V_{1a} receptor-deficient mice characterised by a significant reduction in anxiety-like behaviour [6], suggesting that V_{1a} antagonism could result in anxiolysis. This idea is supported by studies in which intracerebral administration of a V_{1a} receptor antagonist resulted in decreased anxiety-like behaviour in rats [7] and a reduction in passive coping behaviour [8]; similar behavioural effects to those of clinically approved anti-depressant drugs.

Unfortunately, the development of selective ligands for the OT and AVP receptors is hindered by the highly conserved sequence homology between the four receptors [9,10]. The endogenous ligands, OT and AVP participate in appreciable cross-talk between each receptor subtype however, OT displays poor binding affinity at the V2 receptor [11]. The lack of selectivity of current peptidergic ligands coupled with the poor physiochemical properties inherent with cyclic peptides - low oral bioavailability, short half-life, and poor blood brain barrier penetration - highlights the need for the development of selective, centrally active, non-peptide receptor ligands. The discovery of such compounds would serve as molecular probes to further identify the specific behavioural roles of the OT and AVP receptor subtypes.

Despite the behavioural evidence for the involvement of the OT receptor in neurological disorders, there is not a single, selective, non-peptidic receptor agonist currently available. Although the

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high sequence homology between the OT and AVP receptors is considered problematic when developing selective OT ligands, the first non-peptidic OT receptor agonists were identified through a high throughput screen of AVP receptor ligands against the OT receptor. These two OT receptor agonists were WAY-267,464 (**1**) and the structurally similar proline derivative **2** (Fig. 1) [12,13]. Further analysis of molecule **2** indicated poor functional selectivity as it was an agonist at the OT receptor (K_i = 147 ± 11 nM and EC_{50} = 667 ± 68 nM) and an antagonist at the V_{1a} receptor (K_i = 330 ± 38 nM and IC_{50} = 271 ± 61 nM) [14]. Truncation and chimeric hybridization of various OT and AVP receptor ligands, published by Frantz et al. provided further evidence of the difficulty in differentiating binding profiles of the OT and AVP receptors [14].

To the best of our knowledge, the biochemical profile of WAY-267,464 at the V_{1a} receptor has not previously been reported. The original disclosure of WAY-267,464 presented the following pharmacological results including OT receptor agonist activity (K_i = 58 ± 11 nM and EC_{50} = 61 ± 5 nM) measured via whole cell radioligand displacement assay upon CHO–K1 cell lines transfected with human OT receptors. Functional responses at the human OT receptor were reported as elevation of intracellular Ca^{2+} in comparison to OT within the same assay (OT results: K_i = 0.8 ± 0.03 nM, EC_{50} = 3.0 ± 1.7 nM) [13].

Prior behavioural and pharmacological work published by our group suggests that WAY-267,464 is both an OT receptor agonist and a V_{1a} ligand, which is not surprising considering the synthetic origin of the molecule [15]. Additional research has suggested that some of the behavioural effects of WAY-267,464 could be through an AVP receptor mediated pathway as OT receptor antagonism did not reduce the pro-social cognitive effects presented with WAY-267,464 administration [16].

Few structure-activity relationship studies have been reported for WAY-267,464 in the peer-reviewed literature. Herein we report the synthesis and pharmacological data of 8 alkyl chain tethered derivatives, **4a**-**7a** and **4b**-**7b**, designed to systematically explore alterations in the distance between the pyrazolo [1,4]diazepine and the benzyl piperazine of WAY-267,464 to provide information regarding the size of the binding pocket. Substitution of the central aromatic group and urea linkage with these alkyl linkers assesses the need for such structural motifs in receptor binding while allowing for increased flexibility. Finally, to determine the hydrogen bonding interactions of the resorcinol moiety within WAY-267,464, both hydroxyl and methoxy derivatives were synthesised (see Fig. 2). In this effort we attempt to shed light on the functional motifs required for receptor affinity, functional activity, and selectivity between the OT and V_{1a} receptors.

2. Synthetic chemistry

The synthesis of WAY-267,464 and novel analogues begins with the large scale production of the pyrazolo [1,4]diazepine (Scheme 1) previously reported by our group [17]. The base promoted nucleophilic aromatic substitution between pyrazole **8** and 2-nitroaniline (**9**) produced the nitroarene **10** which was then subjected to palladium catalysed reductive cyclisation conditions to afford the pyrazolo [1,4]diazepine **11** in an overall yield of 50%.

The WAY-267,464 molecule was synthesised according to previously reported methods and was easily adapted for the first synthesis of analogue **3** [17,18]. Acid chloride mediated amidation between the pyrazolo [1,4]diazepine and benzoyl chloride **12** furnished the corresponding benzamide **13**, the nitrile of which was selectively reduced with Raney nickel to give the benzylamine **14**. A carbonylative coupling between **14** and the appropriately 3,5-disubstituted benzyl piperazines (**15a** or **15b**) gave WAY-267,464 (**1**) and the 3,5-dimethoxy analogue **3** (Scheme 2).

The synthesis of alkyl chain derivatives **4ab-7ab** followed a similar procedure whereby amidation of the tricyclic **11** with bromoalkanoyl chlorides **16**–**19** of variable length produced compounds **20**–**23**. Due to problems associated with stability of the alkyl bromides (**20**–**23**) they were immediately subjected to base-promoted nucleophilic substitution by benzyl piperazine **15b** to furnish the dimethoxy WAY analogues **4b-7b**. Alternatively, bromide substitution with benzyl piperazine **15c** afforded the benzyl protected analogues **24**–**27** that underwent hydrogenolysis to produce the dihydroxy analogues **4a-7a** (Scheme 3).

3. Results and discussion

The alkyl chain analogues **4ab** – **7ab**, WAY-267,464 (**1**) and the dimethoxy derivative **3** were evaluated for OT and V_{1a} receptor affinity using membranes from HEK293 cells expressing transfected human OT or V_{1a} receptors. Displacement of either [^{3}H]-OT or [^{3}H]-AVP was measured. Functional activity was determined using a homogenous time-resolved fluorescence (HTRF) inositol monophosphate (IP1) accumulation assay. Agonist functional activity was assessed as the ligand concentration that induced IP1 levels that were 50% of the maximum seen with each compound (EC_{50}). Antagonist functional activity was assessed as the ligand concentration that halved IP1 levels stimulated by an EC_{70} concentration of oxytocin or vasopressin (IC_{50}). The binding affinities and functional activities are summarised in Tables 1 and 2.

The observed binding affinity of WAY-267,464 (**1**) at human OT receptor was measured as 230 ± 31 nM which is 4-fold lower than the originally reported binding affinity, 58 ± 11 nM [13]. The originally reported affinity was derived using whole cell radioligand binding in a different binding buffer, so these technical differences may account for this discrepancy. Furthermore, our affinity falls within the range of published binding affinities for WAY-267,464 [13,15]. Although data was not presented in the original disclosure of WAY-267,464 (**1**), it was stated that the lead compound had

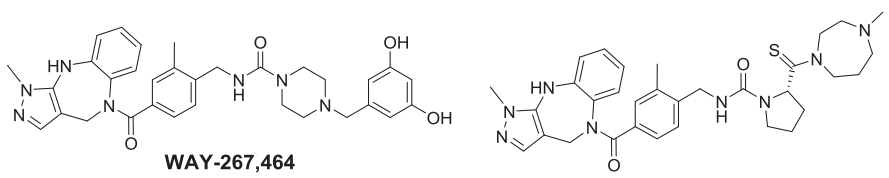


Fig. 1. The molecular structures of WAY-267,464 and compound **2** identified from the original high throughput screen of a vasopressin antagonist library with included pharmacological data [13,14].

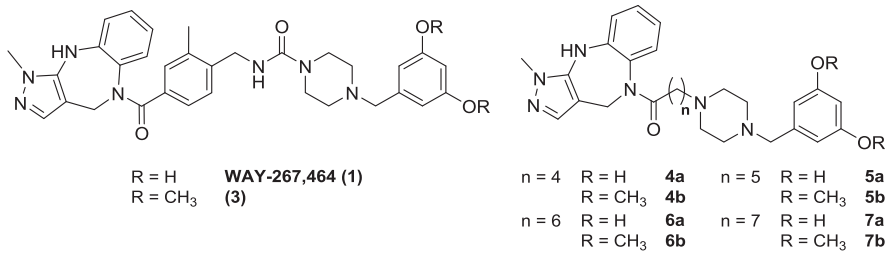
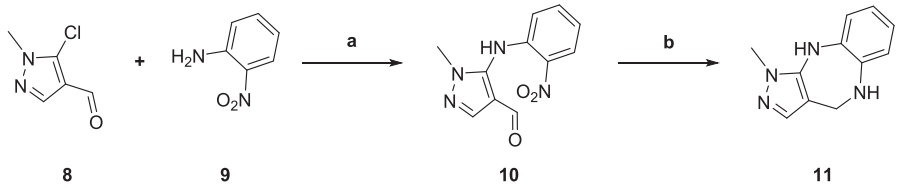
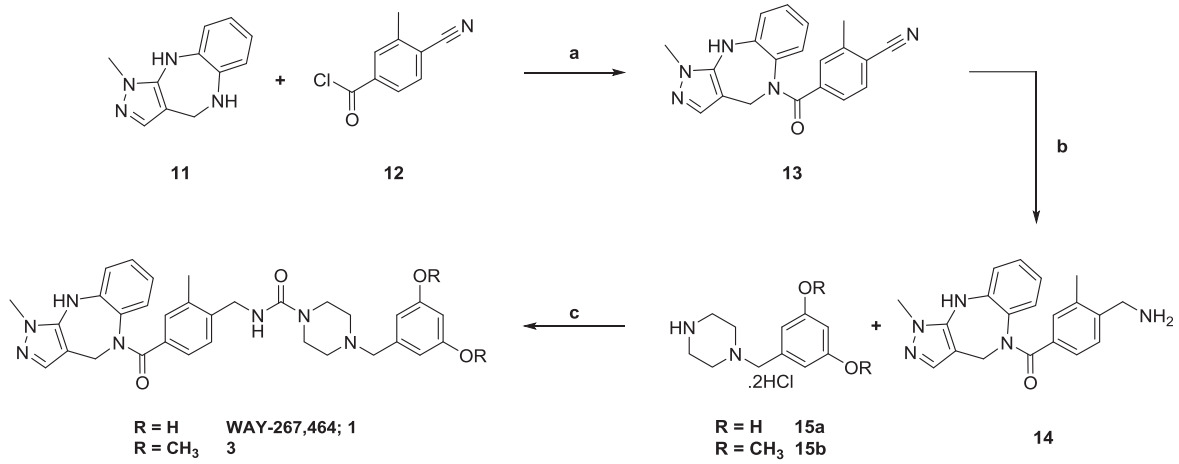


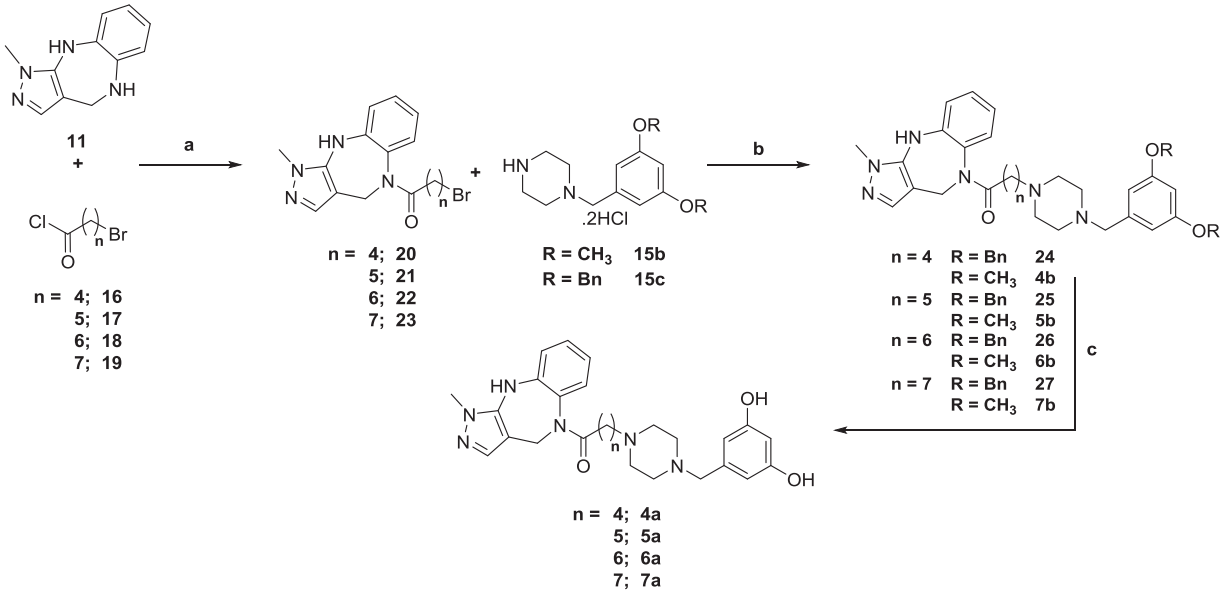
Fig. 2. Non-peptidic lead compounds **1** and **3** with structurally modified alkyl derivatives **4ab-7ab**.



Scheme 1. Reagents and conditions: (a) **9** (3 equiv), KOH (2 equiv), DMF, 100 °C, 4 h, 80%; (b) Pd/C (10 mol%), H_2 (1 atm), MeOH, 14 h, 95%.



Scheme 2. Reagents and conditions: (a) **12** (1.2 equiv), NEt_3 (2.2 equiv), CH_2Cl_2, 0 °C - rt, 8 h, 76%; (b) Raney Ni (1 equiv), H_2 (1 atm), MeOH (saturated with NH_3), 65 °C, 18 h, 51%; (c) **15a** or **15b** (1.3 equiv), CDI (1.3 equiv), $^iPr_2NEt$ (2.4 equiv), DMF, rt, 51%–65%.



Scheme 3. Reagents and conditions: (a) 16–19 (1.2 equiv), NEt_3 (2.2 equiv), CH_2Cl_2, 0 °C – rt, 8 h, 24–39%; (b) 15b-15c (1.3 equiv)), $^iPr_2NEt$ (2.4 equiv), KI (cat.), DMF, 80 °C, 18 h, 33–82%; (c) Pd/C (10 mol %), H_2 (1 atm), MeOH, rt, 12 h, 90–96%.

Table 1
Binding affinities and functional activities of WAY-267,464 and derivative *3* given as (nM ± SD).

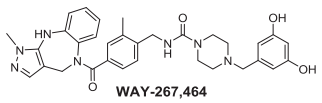
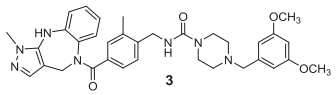
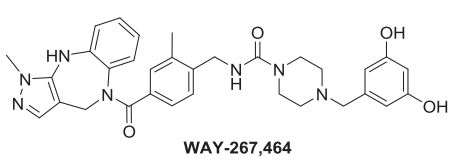
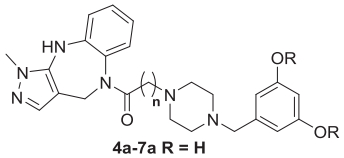
	OTR			$V_{1a}R$		
	K_i (nM ± SD)	EC_{50} (nM ± SD)	IC_{50} (nM ± SD)	K_i (nM ± SD)	EC_{50} (nM ± SD)	IC_{50} (nM ± SD)
 WAY-267,464 **1**	230 ± 31	420 ± 59	>10,000	27 ± 3	>10,000	613 ± 206
 **3**	801 ± 139	>10,000	4129 ± 645	62 ± 21	>10,000	1113 ± 180

Table 2
Binding affinity and functional activity of alkyl derivatives **4ab-7ab**.


WAY-267,464
lead compound (1)


4a-7a R = H
4b-7b R = CH_3

			OTR (nM ± SD)			$V_{1a}R$ (nM ± SD)		
#	n	R	K_i	EC_{50}	IC_{50}	K_i	EC_{50}	IC_{50}
4a	4	H	>10,000	>10,000	>10,000	466 ± 61	>10,000	>10,000
5a	5	H	>10,000	>10,000	>10,000	449 ± 38	>10,000	>10,000
6a	6	H	>10,000	>10,000	>10,000	130 ± 2	>10,000	>10,000
7a	7	H	8349 ± 664	>10,000	>10,000	64 ± 5	>10,000	4667 ± 823
4b	4	CH_3	>10,000	>10,000	>10,000	409 ± 68	>10,000	>10,000
5b	5	CH_3	7452 ± 190	>10,000	>10,000	266 ± 6	>10,000	8203 ± 2399
6b	6	CH_3	>10,000	>10,000	>10,000	195 ± 83	>10,000	>10,000
7b	7	CH_3	>10,000	>10,000	>10,000	248 ± 71	>10,000	7674 ± 461

greater than 100-fold selectivity in both binding and functional activity at the OT receptor over AVP receptors [13]. This could not be replicated in the current study; in line with our previous report [15] we found WAY-267,464 (**1**) bound to the V_{1a} receptor with greater than 8-fold selectivity ($K_i = 27 \pm 3$ nM). It is interesting that despite having a better binding affinity at the V_{1a} receptor, WAY-267,464 (**1**) displayed greater functional activity at the OT receptor (OTR EC_{50} = 420 ± 59 nM compared to $V_{1a}R$ IC_{50} = 613 ± 206 nM) (Table 1). Although this is not uncommon in receptor pharmacology, and could be due to differing experimental conditions between binding and functional assays, it adds significant complexity to the understanding of OT/V_{1a} receptor binding sites as binding affinity and functional activity are non-correlating. It has previously been suggested that the behavioural observations of WAY-267,464 (**1**) are likely due to V_{1a} receptor inactivation [16]. To the best of our knowledge, the functional activity of WAY-267,464 (**1**) at the V_{1a} receptor has not previously been reported. This study presents the first, quantified V_{1a} receptor inhibition of WAY-267,464 (**1**).

Removal of the hydrogen bond-donating ability of the resorcinol moiety in the lead compound via methyl ether incorporation (Table 1, compound **3**) displayed a preference in binding towards the V_{1a} receptor (K_i = 63 ± 21 nM) compared to the OT receptor (K_i = 801 ± 139 nM). This preference for V_{1a} receptor affinity is similarly observed by the lead compound **1**, however loss of the hydroxyl groups decreased affinity (2.5-fold at V_{1a} receptor and 3.5 fold at OT receptor). Although the binding results are similar between the compounds, the functional activity is not. Interestingly, functional activity at the OT receptor is reversed, agonistic activity is entirely removed and weak antagonism is gained (IC_{50} = 4129 ± 645 nM). A two fold decrease in inhibition of the V_{1a} receptor is also observed with an IC_{50} of 1113 ± 180 nM. This data implies that hydrogen bond donation (seen within WAY-267,464 (**1**)) influences OT receptor activation and is slightly detrimental to V_{1a} receptor inhibition.

Pharmacological evaluation of the alkyl chain derivatives represents, to our knowledge, the first published structure-activity relationship of WAY-267,464 (**1**) in the peer reviewed literature. Several interesting trends were observed and are summarised in Table 2. Efficacy at the OT receptor was almost completely removed, which suggests that the aromatic tolyl benzamide plays a role in OT receptor activity. For both the **a** hydroxyl, and **b**, methoxy benzylamine series', marginal OT receptor affinity was gained at increasing chain lengths which can be attributed to the distance between the pyrazolo [1,4]diazepine and the piperazine moiety approaching a similar distance to that in the lead molecule.

A similar trend in binding affinity was also observed at the V_{1a} receptor. The *O*-desmethyl derivatives (**4a-7a**) performed better than the methylated analogues (**4b-7b**), which is expected considering the decreased binding affinity of compound **3** over WAY-267,464 (**1**). This suggests that the tolyl urea linkage present in WAY-267,464 (**1**) is important for OT binding whereas increasing alkyl chain length and flexibility improves V_{1a} receptor selectivity. Derivative **7a** consisting of a heptyl chain linkage between the tricyclic benzodiazepine and piperazine functional group bound to the V_{1a} receptor with a K_i of 64 ± 5 nM (130 fold selective over OT receptor binding). Functional activity was lost at the OT receptor

and significantly diminished at the V_{1a} receptor for all analogues. This loss of binding affinity could be attributed to several structural features present in WAY-267,464 (**1**) and absent from the alkyl analogues. The decreased distance between the benzyl piperazine and pyrazolo [1,4]benzodiazepine could be sufficient in preventing favourable binding interactions and is supported by the restoration of binding affinity in derivative **7a** which contained the longest alkyl chain. Further research would be required to determine if the flexibility, removal of the urea or the tolyl linker contributed to the markedly reduced binding affinity and functional activity at the OT receptor. The restored binding affinities at the V_{1a} receptor, observed in derivatives **6a** and **7a** resulted in no functional inhibition at the doses examined. This information suggests that the flexibility gained due to increasing the alkyl chain length, allows for the competitive displacement of tritiated AVP from the V_{1a} receptor binding site, although further evidence would be required to determine if these structural motifs simply do not perform as well in whole cell pharmacological assays compared to membrane-based radioligand binding assays due to factors such as desensitisation and differing ionic environments which is often seen with oxytocin. Therefore, despite being involved in the activation of OT receptors, the resorcinol moiety is not required for V_{1a} receptor antagonism and is also evidenced by the previously reported pharmacology of compound **2** [14] which lacks the resorcinol group entirely. Similarly, additional research would be required to determine the importance of the tolyl functionality in V_{1a} receptor inhibition.

4. Conclusions

Above we have detailed the synthesis and pharmacological evaluation of nine novel analogues of WAY-267,464 (**1**). These results suggest that hydrogen bond donation of the resorcinol moiety in **1** plays a role in OT receptor functional activity. Methyl ether formation at these functional groups (analogue **3**), completely reverses the agonistic activity at OT receptors. All alkyl chain analogues decreased or removed affinity at the OT and V_{1a} receptors. The comparable binding affinity at the V_{1a} receptor observed in the dihydroxyheptyl analogue **7a** did not restore the antagonistic profile observed in WAY-267,464 (**1**). This implies that functional activity is a result of favourable binding interactions between the aromatic ring and urea linkage which is absent in the alkyl chain analogues. This work presents the generation of novel, V_{1a} receptor selective ligands and insight into the subtleties surrounding the OT receptor pharmacophore. Future work will look at the synthesis and biological evaluation of analogues which have restricted flexibility via incorporation of tolyl amides at the pyrazolo [1,4]diazepine. Furthermore, in order to assess the overall importance of the benzylamine urea linkage in WAY-267,464 (**1**), modifications at this functional group will look at removing each nitrogen and ketone moiety systematically to confirm the tentative conclusions presented here and shed light on the pharmacophores pertaining to the OT and V_{1a} receptors.

5. Experimental procedures

5.1. General experimental

Unless otherwise stated, reactions were conducted under positive pressure of a dry nitrogen or argon atmosphere. Temperatures of 0 °C and −10 °C were obtained by the use of a water/ice bath or salt/ice bath respectively. Reaction mixture temperatures were reported according to the oil bath/cooling bath temperature unless otherwise stated. Anhydrous dichloromethane, triethylamine and diisopropylethylamine was obtained by distillation from calcium hydride. Anhydrous DMF, methanol, THF, and acetonitrile were obtained from a PureSolv MD 7 solvent purification system (Innovative Technology, Inc.). Unless noted otherwise, commercially obtained reagents were used as purchased without further purification. Analytical thin-layer chromatography (TLC) was performed using Merck aluminium backed silica gel 60 F254 (0.2 mm) plates which were visualized with shortwave (254 nm) and/or longwave (365 nm) ultraviolet (UV) light. Non UV-active products were visualised with potassium permanganate, vanillin, *p*-anisaldehyde, ninhydrin or cerium molybdate ("Goofy's Dip") stains. Flash chromatography was performed using Grace Davisil silica gel, pore size 60 Å, 230–400 mesh particle size. Solvents for flash chromatography were distilled prior to use, or used as purchased for HPLC grade, with the eluent mixture reported as the volume/volume ratio (v/v).

Melting points were measured with open capillaries using a Stanford Research Systems (SRS) MPA160 melting point apparatus with a ramp rate of 0.5–2.0 °C/min and are uncorrected. Infrared absorption spectra were recorded on a Bruker ALPHA FT-IR spectrometer, and the data are reported as vibrational frequency (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 298 K unless stated otherwise, using either a Bruker AVANCE DRX200 (200 MHz), DRX300 (300 MHz), DRX400 (400.1 MHz), or AVANCE III 500 Ascend (500.1 MHz) spectrometer. The data is reported as the chemical shift (δ ppm) relative to the solvent residual peak, relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, etc.), coupling constant (*J* Hz). Low resolution mass spectra (LRMS) were recorded using electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) recorded on a Finnigan LCQ ion trap spectrometer, or by electron impact gas chromatography mass spectrometry (GC/MS). High resolution mass spectra were run on a Bruker 7T Apex Qe Fourier Transform Ion Cyclotron resonance mass spectrometer equipped with an Apollo II ESI/APCI/MALDI Dual source by the Mass Spectrometry Facility of the School of Chemistry at The University of Sydney. Samples run by ESI were directly infused (150 μL/h) using a Cole Palmer syringe pump. Samples run by APCI were injected (5 μL) into a flow of methanol (0.3 mL/min) by HPLC (Agilent 1100) coupled to the mass spectrometer.

Analytical HPLC purity traces were taken on a Waters 2695 Separations module equipped with Waters 2996 Photodiode Array detector (set at 230, 254 and 271 nm). All samples were eluted through a Waters SunFire™ C18 5 μm column (2.1 × 150 mm) using a flow rate of 0.2 mL/min of Solvent A: MilliQ water (+0.1% trifluoroacetic acid or 0.1% formic acid) and Solvent B: acetonitrile (+0.1% trifluoroacetic acid or 0.1% formic acid). This method consisted of gradient elution (0–100% Solvent A:B over 30 min). Data acquisition and processing was performed with the Waters Empower 2 software. Reported data for all compounds are based on the 254 nm channel.

5.2. Synthetic procedures

The synthesis of 1-Methyl-1,4,5,10-tetrahydrobenzo[*b*]pyrazolo [3,4-*e*] [1,4]diazepine (**11**) was completed as previously reported [17] with the following modifications. All characterisation data matched with those previously reported.

5.2.1. 5-Chloro-1-methyl-1H-pyrazole-4-carbaldehyde (*8*)

A magnetically stirred solution of methyl 3-methoxyacrylate (20.0 g, 172.2 mmol) in methanol (100 mL) was treated slowly with *N*-methylhydrazine (10.0 mL, 189.4 mmol) then heated at reflux for 8 h. The resulting mixture was cooled to room temperature then concentrated under a stream of nitrogen followed by

high vacuum pump to afford an orange solid that was dissolved in DMF (19.9 mL, 258.4 mmol) and cooled to 0 °C. The resulting mixture was treated dropwise with phosphoryl chloride (48.1 mL, 516.6 mmol) then heated at 80 °C for 12 h before being cooled and neutralised via the slow addition of ice cold NaOH (10 M, 300 mL). The aqueous solution was then extracted with a dichloromethane/isopropanol mixture (9:1, 5 × 100 mL) and the combined organic phases were washed with chilled water (3 x 100 mL), brine (1 × 100 mL) before being dried ($MgSO_4$), filtered and concentrated under reduced pressure to afford an orange solid. Recrystallisation (dichloromethane/hexane) afforded the title pyrazole **8** (16.31 g, 66%) as yellow crystals, m.p. 77–78 °C, (R*f* = 0.44 in 1:1 ethyl acetate/hexane).

^{1}H NMR (Chloroform-*d*, 300 MHz): δ 9.82 (s, 1H), 7.95 (s, 1H), 3.89 (s, 3H) ppm; **^{13}C NMR** (Chloroform-*d*, 75 MHz): δ 182.5, 140.1, 132.5, 119.1, 36.2 ppm; **IR** (diamond cell, thin film) ν_{max}: 1683, 1529, 1424, 1390, 1196, 814, 770 cm^{-1}; **LRMS**: (+ESI) *m/z*: 145 [$(M + H)^+$, 100%].

5.2.2. 1-Methyl-5-((2-nitrophenyl)amino)-1H-pyrazole-4-carbaldehyde (**10**)

A magnetically stirred solution of pyrazole **8** (7.67 g, 53.1 mmol) and 2-nitroaniline (22 g, 159.2 mmol) in DMF (50 mL) was treated with powdered KOH (5.96 g, 106.2 mmol) then heated at 100 °C for 18 h. The resulting mixture was cooled and diluted with NH_4Cl (500 mL of a saturated aqueous solution) and extracted with ethyl acetate (3 × 200 mL). The combined organic phases were washed with brine (1 × 100 mL) before being dried ($MgSO_4$), filtered and concentrated under reduced pressure to afford a yellow oil. Subjection of this residue to medium pressure liquid chromatography (MPLC) using a Teledyne ISCO CombiFlash® Rf + system (silica, 80% hexane/ethyl acetate 40 min followed by 50% hexane/ethyl acetate gradient elution) and concentration of the relevant fractions (R*f* = 0.33 in 1:1 v/v ethyl acetate/hexane) afforded the title pyrazole **10** (10.43 g, 80%) as a yellow, crystalline solid, m.p. 102–105 °C.

^{1}H NMR (Chloroform-*d*, 300 MHz): δ 9.74 (s, 1H), 9.35 (s, 1H), 8.22 (d, *J* = 8.4 Hz, 1H), 7.97 (s, 1H), 7.46 (t, *J* = 8.1 Hz, 1H), 6.98 (t, *J* = 8.1 Hz, 1H), 6.60 (d, *J* = 8.4 Hz, 1H), 3.72 (s, 3H) ppm. **^{13}C NMR** (Chloroform-*d*, 75 MHz): δ 183.2, 140.9, 140.3, 140.1, 136.1, 134.6, 126.6, 120.2, 116.2 (C and CH, two overlapping signals), 35.8 ppm. **IR** (diamond cell, thin film) ν_{max}: 3340, 1679, 1613, 1579, 1506, 1338, 1271, 1226, 1149, 742 cm^{-1}; **LRMS** (+ESI) *m/z*: 269 [$(M + Na)^+$, 100%]. **HRMS** (+ESI) Found: $(M + Na)^+$, 269.0646. $C_{11}H_{10}N_4O_3$ requires $(M + Na)^+$, 269.0651.

5.2.3. 1-Methyl-1,4,5,10-tetrahydrobenzo[b]pyrazolo[3,4-e][1,4]diazepine (**11**)

A suspension of pyrazole **10** (8.08 g, 16.0 mmol) and Pd/C (100 mg of 10% w/w) in methanol (200 mL) was stirred magnetically at room temperature under an atmosphere of H_2 (1 atm) for 16 h. The resulting mixture was filtered through Celite™ and the solids thus retained were washed with methanol (3 × 20 mL). The combined filtrates were concentrated under reduced pressure to afford the title diazepine **11** (6.0 g, 95%) as a yellow powder, m.p. 205–207 °C, (R*f* = 0.43 in 1:9 v/v methanol/dichloromethane) and used as is without further purification. An analytically pure sample can be obtained via crystallization from dichloromethane/hexane to give **11** as an off white solid.

^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.99 (s, 1H), 7.05 (d, *J* = 7.6 Hz, 1H), 6.97 (s, 1H), 6.81 (d, *J* = 7.6 Hz, 1H), 6.73 (t, *J* = 7.6 Hz, 1H), 6.64 (t, *J* = 7.6 Hz, 1H), 5.36 (s, 1H), 3.86 (s, 2H), 3.67 (s, 3H) ppm. **^{13}C NMR** (DMSO-d_6, 75 MHz): δ 140.9, 139.5, 135.0, 133.0, 121.7, 120.8, 120.2, 118.9, 101.5, 43.4, 35.0 ppm. **IR** (diamond cell, neat) ν_{max}: 3293, 1560, 1505, 1393, 1318, 761 cm^{-1}. **LRMS** (+ESI) *m/z*: 201 [$(M + H)^+$, 100%].

The synthesis of WAY-267,464 was carried out as previously reported [17] with the following modifications.

5.2.4. 2-Methyl-4-(1-methyl-1,4,5,10-tetrahydrobenzo[b]pyrazolo[3,4-e][1,4]diazepine-5-carbonyl)benzonitrile (**13**)

Acid chloride **12** was prepared by combining the parent acid (3.40 g, 21.1 mmol) and DMF (1 drop) in dichloromethane (25 mL) at 0 °C and treating the mixture with oxalyl chloride (3.60 mL, 42.2 mmol) and stirring continued at room temperature for 2 h followed by concentration under a stream of nitrogen to afford **12** as a yellow solid. This residue was dissolved in dichloromethane (15 mL) then added, dropwise, to a magnetically stirred solution of diazepine **11** (3.25 g, 16.2 mmol) and NEt_3 (4.5 mL, 32.5 mmol) in dichloromethane (28 mL) at 0 °C. Following addition the reaction mixture was warmed to room temperature and stirred for a further 8 h then concentrated under reduced pressure. The residue was taken up in dichloromethane (50 mL) and $KHSO_4$ (100 mL of a 0.3 M aqueous solution) and the separated aqueous phase extracted with a mixture of chloroform and 2-propanol (2 × 100 mL of an 8:2 mixture). The combined organic layers were washed with $NaHCO_3$ (1 × 100 mL of a saturated aqueous solution) before being dried ($MgSO_4$), filtered and concentrated under reduced pressure to yield a residue that was triturated with ethyl acetate (20 mL). The resulting solid was filtered and washed with ethyl acetate (2 × 50 mL) and the solid thus obtained dried under high vacuum to give the title compound **13** (4.23 g, 76%) as a pale-yellow powder, m.p. 253–255 °C, (R_f = 0.51 in 1:9 v/v methanol/dichloromethane). The spectral data for this compound were identical, in all respects, with those previously reported [14].

5.2.5. (4-(Aminomethyl)-3-methylphenyl) (1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)methanone (**14**)

A magnetically stirred mixture of nitrile **13** (6.70 g, 19.5 mmol) and Raney nickel (approximately 1 g, 2 mmol) in methanol (20 mL, saturated with ammonia) was maintained under an atmosphere of H_2 (1 atm) at reflux for 18 h. The resulting mixture was cooled then filtered through Celite™ and the solids thus retained were washed with methanol (3 x 10 mL). The combined filtrates were concentrated under reduced pressure and purified by flash chromatography (silica 9:1 dichloromethane/methanol (saturated with ammonia)) and concentration of the relevant fractions afforded the title amine **14** (3.43 g, 51%) as a white powder. The spectral data for this compound were identical, in all respects, with those previously reported [14].

5.2.6. 3,5-Bis(benzyloxy)benzaldehyde (**28**)

A stirred suspension of 3,5-dihydroxybenzaldehyde (690 mg, 5 mmol), potassium carbonate (3.46 g, 25 mmol) and potassium iodide (166.0 mg, 1 mmol) in acetone (15 mL) was treated dropwise with benzyl bromide (1.18 mL, 10 mmol) and stirring continued for 4 h at room temperature. The resulting suspension was concentrated under reduced pressure and the crude mass subjected to flash column chromatography (silica, 4:1 v/v hexane/ethyl acetate) and concentration of the relevant fractions (R_f = 0.58 in 4:1 v/v hexane/ethyl acetate) afforded the title compound as a white powder (1.3 g, 82%) m.p. 80–81 °C.

^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.91 (s, 1H), 7.48–7.44 (m, 4H), 7.43–7.38 (m, 4H), 7.37–7.32 (m, 2H), 7.17 (d, *J* = 2.3 Hz, 2H), 7.01 (t, *J* = 2.3 Hz, 1H), 5.17 (s, 4H) ppm. **^{13}C NMR** (DMSO-d_6, 126 MHz): δ 192.6, 159.9, 138.3, 136.6, 128.4, 127.9, 127.7, 108.2, 108.1, 69.6 ppm. **IR** (diamond cell, neat) ν_{max}: 1686, 1591, 1447, 1381, 1349, 1296, 1170, 1048, 829, 739, 719, 694, 675, 629 cm^{-1}. **LRMS** (+ESI) *m/z*: 319 [$(M + H)^+$, 100%].

*5.2.7. General procedure for the synthesis of **15a-c***

Step i: A solution of 1-boc-piperazine (2.58 g, 13.8 mmol) and the appropriate benzaldehyde (13.8 mmol) in methanol/acetic acid (40 mL of a 99:1 mixture) was stirred magnetically at room temperature for 1 h then treated, in portions, with $NaCNBH_3$ (1.13 g, 17.9 mmol). The resulting mixture was stirred for a further 18 h then concentrated under reduced pressure and the residue dissolved in ethyl acetate (100 mL) and $NaHCO_3$ (20 mL of a 10% aqueous solution). The separated organic phase was washed with brine (50 mL) before being dried ($MgSO_4$), filtered and concentrated under reduced pressure to give off white solids. The crude solids were purified by flash column chromatography (4:1 v/v hexane/ethyl acetate) and used immediately in the subsequent step.
Step ii: A solution of the appropriate tert-butyl carboxylate (5 mmol) in methanol (15 mL) was treated with HCl (5.0 mL of a 4 M solution in 1,4-dioxane, 20.3 mmol) and stirred magnetically for 1 h. The resulting mixture was concentrated under reduced pressure and azeotroped with toluene (10 mL) to give:

*5.2.8. 5-(Piperazin-1-ylmethyl)benzene-1,3-diol dihydrochloride (**15a**)*

The title compound was obtained as a white powder (2.91 g, 75% over two steps), m.p. 278–280 °C, (R_f = 0.0 in 1:9 v/v methanol/dichloromethane).

^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.87–9.57 (complex m, 4H), 6.44 (s, 2H), 6.36 (s, 1H), 5.36 (s, 1H), 4.16 (s, 2H), 3.55–3.24 (complex m, 8H). **^{13}C NMR** (DMSO-d_6, 75 MHz): δ 158.7, 130.4, 109.4, 103.8, 58.6, 47.1, 39.5 ppm. **IR** (diamond cell, thin film) ν_{max}: 3330, 2970, 1738, 1598, 1365, 1216, 1175, 1009, 946, 859 cm^{-1}. **LRMS** (+ESI) *m/z*: 209 [$(M + H)^+$ as a free base, 100%].

*5.2.9. 1-(3,5-Dimethoxybenzyl)piperazine dihydrochloride (**15b**)*

The title compound was obtained as a white powder (3.1 g, 73% over two steps), m.p. 239–240 °C, (R_f = 0.02 in 1:9 v/v methanol/dichloromethane).

^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.0 (br s, 2H), 6.92 (s, 2H), 6.54 (s, 1H), 4.31 (s, 2H), 3.76 (s, 6H), 3.48–3.16 (m, 9H) ppm. **^{13}C NMR** (DMSO-d_6, 75 MHz): δ 160.5, 131.1, 109.2, 101.2, 58.5, 55.4, 48.6, 47.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 2566, 2434, 1598, 1430, 1350, 1300, 1208, 1253, 1055, 819, 590, 550 cm^{-1}. **LRMS** (+ESI) *m/z*: 237 [$(M + H)^+$ as a free base, 100%].

*5.2.10. 1-(3,5-Bis(benzyloxy)benzyl)piperazine dihydrochloride (**15c**)*

The title compound was obtained as a white powder (4.8 g, 96% over two steps), m.p. 210–212 °C decomp., (R_f = 0.05 in 1:1 v/v ethyl acetate/hexane).

^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.35–7.92 (complex m, 10H), 6.71 (s, 2H), 6.55 (m, 1H), 5.05 (s, 4H), 2.89 (s, 2H), 2.67 (br s, 4H), 2.25 (br s, 4H) ppm NH signals not observed. **^{13}C NMR** (DMSO-d_6, 75 MHz): δ 159.3, 140.8, 137.0, 128.3, 127.7, 127.6, 107.6, 100.3, 69.2, 62.8, 53.9, 45.5 ppm. **IR** (diamond cell, thin film) ν_{max}: 2801, 1589, 1450, 1289, 1152, 1055, 1010, 747, 695 cm^{-1}. **LRMS** (+ESI) *m/z*: 389 [$(M + H)^+$ as a free base, 100%].

*5.2.11. 4-(3,5-Dihydroxybenzyl)-N-(2-methyl-4-(1-methyl-1,4,5,10-tetrahydrobenzo[b]pyrazolo[3,4-e][1,4]diazepine-5-carbonyl) benzyl)piperazine-1-carboxamide or WAY 267,464 (**1**)*

A magnetically stirred solution of carbonyldiimidazole (588 mg, 3.63 mmol) and diisopropylethylamine (632 μL, 3.63 mmol) in DMF (9 mL) was treated over 2 h via syringe pump with a solution of compound **14** (1.05 g, 3.02 mmol) in DMF (9 mL) then stirred for a further 1 h. The resulting mixture was then added over 2 h via syringe pump to a solution of compound **15a** (1.02 g, 3.63 mmol) and diisopropylethylamine (632 μL, 3.63 mmol) in DMF (10 mL). The resulting mixture was stirred for a further 16 h then concentrated under reduced pressure. Subjection of the residue to flash column chromatography (silica, 1:9:90 v/v 28% aqueous ammonia/methanol/dichloromethane elution) and concentration of the relevant fractions (R_f = 0.10 in 1:9:90 v/v/v 28% aqueous ammonia/methanol/dichloromethane elution) afforded the title compound **1** (901 mg, 51%) as a white powder, m.p. 204–207 °C.

^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.11 (s, 2H), 8.60 (s, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.17 (s, 1H), 7.11 (t, J = 7.4 Hz, 1H), 7.00 (s, 1H), 6.90 (m, 3H), 6.73–6.62 (complex m, 2H), 6.17 (s, 2H), 6.09 (s, 1H), 5.68 (d, J = 14.5 Hz, 1H), 4.11 (br s, 2H), 3.89 (d, J = 14.5 Hz, 1H), 3.77 (s, 3H), 3.36–3.27 (complex m, 6H), 2.28 (m, 4H), 2.11 (s, 3H). **^{13}C NMR** (DMSO-d_6, 75 MHz) δ 168.1, 158.2, 157.3, 140.1, 140.0, 139.9, 138.9, 135.6, 134.4, 134.2, 132.6, 130.3, 129.1, 128.2, 125.5, 124.8, 121.4, 119.6, 106.7, 101.2, 100.3, 62.3, 52.5, 43.5, 43.2, 41.0, 35.3, 18.4 ppm. **IR** (diamond cell, thin film) ν_{max}: 3293, 1703, 1603, 1558, 1504, 1363, 1298, 1148, 998, 839, 761 cm^{-1}. **LRMS** (+ESI) *m/z*: 582 [$(M + H)^+$, 100%].

*5.2.12. 4-(3,5-Dimethoxybenzyl)-N-(2-methyl-4-(1-methyl-1,4,5,10-tetrahydrobenzo[b]pyrazolo[3,4-e][1,4]diazepine-5-carbonyl)benzyl)piperazine-1-carboxamide (**3**)*

A magnetically stirred solution of carbonyldiimidazole (56 mg, 0.35 mmol) and diisopropylethylamine (60 μL, 0.35 mmol) in DMF (1.5 mL) was treated dropwise with a solution of **14** (100 mg, 0.28 mmol) in DMF (1.5 mL) then stirred for a further 1 h. The resulting mixture was then added dropwise to a solution of compound **15b** (105 mg, 0.35 mmol) and diisopropylethylamine (180 μL, 1.05 mmol) in DMF (2 mL). The resulting mixture was stirred for a further 8 h then concentrated under reduced pressure. Subjection of the residue to flash column chromatography (silica, 1:9:90 v/v 28% aqueous ammonia/methanol/dichloromethane elution) and concentration of the relevant fractions (R_f = 0.17 in 1:9:90 v/v/v 28% aqueous ammonia/methanol/dichloromethane elution) afforded the title compound **3** (115 mg, 65%) as a white powder, m.p. 138–140 °C.

^{1}H NMR (MeOD-d_4, 300 MHz) δ 7.22 (s, 2H), 7.11 (t, J = 7.2 Hz, 1H), 7.03 (s, 1H), 7.00 (s, 2H), 7.62 (d, J = 7.2 Hz, 1H), 6.66 (t, J = 7.5 Hz, 1H), 6.52 (s, 2H), 6.39 (s, 1H), 5.79 (d, J = 14.4 Hz,1H), 4.23 (s, 2H), 3.97 (d, J = 24.4 Hz, 1H), 3.81 (d, 3H), 3.76 (s, 6H), 3.46 (s, 2H), 3.41 (m, 4H), 2.42 (br s, 4H), 2.15 (s, 3H) ppm 2 NH signals not observed. **^{13}C NMR** (MeOD-d_4, 75 MHz) δ 171.7, 162.4, 159.9, 142.1, 141.1, 140.8, 140.2, 137.3, 136.6, 135.7, 134.2, 131.5, 130.5, 129.8, 127.3, 126.4, 123.1, 120.7, 108.4, 100.3, 64.0, 55.7, 53.9, 44.8, 44.7, 42.8, 35.2, 18.9 ppm. **IR** (diamond cell, thin film) ν_{max}: 2948, 2553, 2366, 1597, 1569, 1432, 1262, 1204, 1153, 1052, 944, 839, 762, 701, 557 cm^{-1}. **LRMS** (+ESI) *m/z*: 611 [$(M + H)^+$, 100%]. **HRMS** (+ESI) Found: $(M + Na)^+$, 632.2962. $C_{34}H_{39}N_7O_4$ requires $(M + Na)^+$, 632.2961. **HPLC** purity: 97.26%, RT: 18.55 min.

*5.2.13. General procedure for the synthesis of **4b-7b** and **24-27***

Step i: Acid chlorides **16**–**19** were prepared by combining the parent acids (10 mmol) and DMF (1 drop) in dichloromethane (10 mL) at 0 °C and treating the mixture with oxalyl chloride (1.0 mL, 11 mmol) and stirring continued at room temperature for 2 h then concentrating under a stream of nitrogen to afford acyl chlorides **16**–**19** as yellow oils and used immediately in the following step.
Step ii: A magnetically stirred suspension of **11** (400 mg, 2 mmol) and triethylamine (556 μL, 4 mmol) was treated with

a solution of the appropriate bromoalkanoyl chlorides (**16**–**19**) (10 mmol) in dichloromethane (10 mL) dropwise at 0 °C. The reaction mixture was then warmed to room temperature and stirring continued for 8 h. The resultant solution was diluted with dichloromethane (25 mL) and $NaHCO_3$ (25 mL of a saturated aqueous solution), the separated organic phase was subsequently washed with $NaHCO_3$ (25 mL of a saturated aqueous solution) and brine (50 mL) before being dried ($MgSO_4$), filtered and concentrated under reduced pressure to give crude oils (**20**–**23**) which were purified via flash chromatography (silica, 0.25:2.25:97.5 to 0.5:4.5:95 v/v/v 28% aqueous ammonia/methanol/dichloromethane gradient elution). Compounds **20**–**23** decomposed rapidly and became discoloured over 2 h and thus were used immediately in the following step.

*5.2.14. 5-Bromo-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)pentan-1-one (**20**)*

Obtained as a white foam (175 mg, 24%) (R_f = 0.44, 1:9 v/v methanol/dichloromethane).

*5.2.15. 6-Bromo-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)hexan-1-one (**21**)*

Obtained as a white foam (196 mg, 26%) (R_f = 0.44, 1:9 v/v methanol/dichloromethane).

*5.2.16. 7-Bromo-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)heptan-1-one (**22**)*

Obtained as a pale yellow foam (204 mg, 26%) (R_f = 0.51, 1:9 v/v methanol/dichloromethane).

*5.2.17. 8-Bromo-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)octan-1-one (**23**)*

Obtained as a yellow gummy solid (315 mg, 39%) (R_f = 0.55, 1:9 v/v methanol/dichloromethane).

Step iii: A stirred suspension of the appropriate alkyl bromides (**20**–**23**) (0.11 mmol), diisopropylethylamine (0.33 mmol) and potassium iodide (0.01 mmol) in DMF (5 mL) were treated with the appropriate benzyl piperazine (**15b** or **15c**) (0.11 mmol) and heated to 80 °C for 18 h. The resultant solution was then allowed to cool to room temperature and diluted with water (50 mL). The aqueous suspension was extracted with ethyl acetate (3 x 25 mL) and the combined organics were subsequently washed with water (3 x 50 mL) followed by brine (1 x 100 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give brown oils which were purified by flash column chromatography (silica, 0.5:4.5:95 v/v/v 28% aqueous ammonia/methanol/dichloromethane elution):

*5.2.18. 5-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)pentan-1-one (**4b**)*

Treating **20** with piperazine **15b** according to the above general procedure gave the title compound as a colourless wax (47 mg, 82%) (R_f = 0.2, 5:95 v/v methanol/dichloromethane).

^{1}H NMR (Chloroform-*d*, 400 MHz) δ 7.26–7.24 (m, 2H), 7.19 (s, 1H), 7.15–7.13 (m, 1H), 7.04 (t, J = 7.5 Hz, 1H), 6.47 (s, 2H), 6.35 (s, 1H), 5.64 (d, J = 15 Hz, 1H), 3.83 (d, J = 15 Hz, 1H), 3.76 (s, 6H), 3.74 (s, 3H), 3.43 (s, 2H), 2.48 (br, 8H), 2.25–2.21 (m, 3H), 2.17–2.13 (m, 1H), 1.54–1.46 (m, 2H), 1.37–1.33 (m, 2H) ppm 1 NH signal not observed. **^{13}C NMR** (Chloroform-*d*, 101 MHz) δ 172.5, 160.7, 140.5, 139.2, 139.0, 136.6, 132.5, 130.5, 129.6, 122.9, 120.4, 107.1, 101.6, 99.2, 63.1, 58.2, 55.5, 53.1, 52.7, 43.1, 35.1, 35.5, 25.8, 23.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 3383, 2944, 2439, 1598, 1568, 1501, 1434, 1398, 1360, 1320, 1301, 1262, 1235, 1205, 1154, 1062, 1024, 948, 850, 740, 631, 538 cm^{-1}. **LRMS** (+ESI) m/z: 519 [(M + H)$^+$, 100%]. **HRMS** (+ESI) Found: (M + H)$^+$, 519.3073. $C_{29}H_{38}N_6O_3$ requires (M + H)$^+$, 519.3078. **HPLC** purity: 97.26%, RT: 13.90 min.

*5.2.19. 6-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)hexan-1-one (**5b**)*

Treating **21** with piperazine **15b** according to the above general procedure gave the title compound as an off white sticky oil (41 mg, 70%) (R_f = 0.2, 5:95 v/v methanol/dichloromethane).

^{1}H NMR (Chloroform-*d*, 500 MHz) δ 7.27–7.24 (m, 2H), 7.19 (s, 1H), 7.15–7.13 (m, 1H), 7.10–7.00 (m, 1H), 6.48 (d, J = 2.3 Hz, 2H), 6.34 (t, J = 2.3 Hz, 1H), 5.63 (d, J = 14.6 Hz, 1H), 3.81 (d, J = 14.5 Hz, 1H), 3.77 (s, 6H), 3.72 (s, 3H), 3.43 (s, 2H), 2.47 (br s, 8H), 2.25 (td, J = 7.0, 2.7 Hz, 2H), 2.13 (dt, J = 15.2, 7.4 Hz, 1H), 1.93 (dt, J = 15.3, 7.5 Hz, 1H), 1.53–1.49 (m, 2H), 1.34 (p, J = 7.5 Hz, 2H), 1.15–1.10 (m, 2H) ppm 1 NH signal not observed. **^{13}C NMR** (Chloroform-*d*, 126 MHz) δ 172.5, 160.8, 140.7, 139.2, 139.0, 136.6, 132.3, 130.5, 129.2, 122.8, 120.0, 107.1, 101.7, 99.1, 63.2, 58.6, 55.4, 53.3, 52.9, 43.1, 34.9, 33.8, 27.1, 26.4, 25.1. **IR** (diamond cell, thin film) ν_{max}: 2941, 2438, 1654, 1598, 1568, 1500, 1434, 1320, 1154, 1064, 949, 844, 768, 700 cm^{-1}. **LRMS** (+ESI) m/z: 533 [(M + H)$^+$, 100%]. **HRMS** (+ESI) Found: (M + H)$^+$, 533.3231. $C_{30}H_{40}N_6O_3$ requires (M + H)$^+$, 533.3235. **HPLC** purity: 99.16%, RT: 14.04 min.

*5.2.20. 7-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)heptan-1-one (**6b**)*

Treating **22** with piperazine **15b** according to the above general procedure gave the title compound as a pale yellow oil (38 mg, 63%) (R_f = 0.11, 5:95 v/v methanol/dichloromethane).

^{1}H NMR (MeOD-d_4, 300 MHz) δ 7.29–7.27 (m, 2H), 7.20–7.17 (m, 1H), 7.16 (s, 1H), 7.06–7.00 (m, 1H), 6.50 (d, J = 2.3 Hz, 2H), 6.37 (t, J = 2.4 Hz, 1H), 5.49 (d, J = 14.6 Hz, 1H), 3.80 (d, J = 14.5 Hz, 2H), 3.75 (s, 6H), 3.72 (s, 3H), 3.44 (s, 2H), 2.48 (br s, 8H), 2.26–2.21 (m, 2H), 2.13–2.00 (m, 2H), 1.45–1.40 (m, 4H), 1.15 (br s, 4H) ppm 1 NH signal not observed. **^{13}C NMR** (MeOD-d_4, 76 MHz) δ 174.9, 162.3, 141.5, 140.7, 140.7, 137.4, 132.9, 131.1, 130.4, 123.3, 121.3, 108.4, 102.6, 100.3, 64.0, 59.6, 55.7, 53.9, 53.6, 44.5, 35.2, 34.7, 28.9, 28.2, 27.2, 26.2 ppm. **IR** (diamond cell, thin film) ν_{max}: 2930, 2450, 1598, 1566, 1501, 1434, 1319, 1205, 1154, 1063, 946, 768, 700 cm^{-1}. **LRMS** (+ESI) m/z: 547 [(M + H)$^+$, 100%]. **HRMS** (+ESI) Found: (M + H)$^+$, 547.3387. $C_{31}H_{42}N_6O_3$ requires (M + H)$^+$, 547.3391. **HPLC** purity: 97.81%, RT: 14.36 min.

*5.2.21. 8-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)octan-1-one (**7b**)*

Treating **23** with piperazine **15b** according to the above general procedure gave the title compound as pale yellow oil (40 mg, 78%) (R_f = 0.1, 5:95 v/v methanol/dichloromethane).

^{1}H NMR (Chloroform-*d*, 400 MHz) δ 7.26–7.24 (m, 2H), 7.20 (s, 1H) 7.14–7.12 (m, 1H), 7.03–7.01 (m, 1H), 6.48 (d, J = 2.4 Hz, 2H), 6.36 (s, 1H), 5.64 (d, J = 14.5 Hz, 1H), 3.78 (s, 10H), 3.47 (s, 2H), 2.61 (br s, 8H) 2.43 (t, J = 9.5 Hz, 2H), 2.13–2.07 (m, 1H), 2.02–1.96 (m, 1H), 1.52–1.47 (m, 4H), 1.21 (d, J = 19.8 Hz, 6H) ppm. **^{13}C NMR** (Chloroform-*d*, 101 MHz) δ 172.7, 160.9, 140.4, 139.4, 139.2, 136.6, 132.3, 130.5, 129.1, 122.6, 120.2, 107.1, 101.6, 99.3, 62.9, 58.5, 55.5, 53.1, 52.1, 43.1, 35.3, 33.6, 28.9, 28.7, 27.2, 26.0, 25.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 2931, 2433, 1598, 1567, 1501, 1433, 1319, 1205, 1154, 1063, 948, 768, 700, 548 cm^{-1}. **LRMS** (+ESI) m/z: 561 [(M + H)$^+$, 100%]. **HRMS** (+ESI) Found: (M + H)$^+$, 561.3548. $C_{32}H_{44}N_6O_3$ requires (M + H)$^+$, 561.3548. **HPLC** purity: 96.41%, RT: 14.78 min.

*5.2.22. 5-(4-(3,5-Bis(benzyloxy)benzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl) pentan-1-one (**24**)*

Treating **20** (170 mg, 0.47 mmol) with piperazine **15c** according to the above general procedure gave the title compound as a colourless oil (110 mg, 35%) (R_f = 0.52, 1:9 v/v methanol/dichloromethane).

^{1}H NMR (Chloroform-*d*, 500 MHz) δ 7.41 (dd, J = 8.2, 1.6 Hz, 4H), 7.39–7.34 (m, 4H), 7.33–7.28 (m, 2H), 7.24 (dd, J = 7.5, 1.5 Hz, 1H), 7.18 (s, 1H), 7.14 (dd, J = 7.8, 1.5 Hz, 1H), 7.06–6.99 (m, 2H), 6.58 (d, J = 2.3 Hz, 2H), 6.54–6.48 (m, 2H), 5.64 (d, J = 14.5 Hz, 1H), 5.02 (s, 4H), 3.82 (d, J = 14.4 Hz, 1H), 3.69 (s, 3H), 3.41 (s, 2H), 2.61–2.30 (m, 8H), 2.12–2.14 (m, 3H), 1.99–1.93 (m, 1H), 1.62–1.42 (m, 2H), 1.41–1.17 (m, 2H) ppm. **^{13}C NMR** (Chloroform-*d*, 126 MHz) δ 172.4, 159.9, 140.7, 139.2, 139.1, 137.0, 136.5, 132.2, 130.4, 129.1, 128.6, 128.0, 127.6, 122.7, 120.1, 108.2, 101.6, 100.8, 70.1, 63.0, 58.2, 53.2, 52.9, 43.1, 34.9, 33.6, 26.1, 23.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 3339, 2943, 1648, 1564, 1559, 1503, 1448, 1397, 1156, 1209, 835, 740, 698 cm^{-1}. **LRMS** (+ESI) m/z: 671 [(M + H)$^+$, 100%].

*5.2.23. 6-(4-(3,5-Bis(benzyloxy)benzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl) hexan-1-one (**25**)*

Treating **21** (180 mg, 0.45 mmol) with piperazine **15c** according to the above general procedure gave the title compound as a colourless oil (104 mg, 33%) (R_f = 0.38, 1:9 v/v methanol/dichloromethane).

^{1}H NMR (Chloroform-*d*, 500 MHz) δ 7.46–7.39 (m, 4H), 7.39–7.35 (m, 4H), 7.34–7.29 (m, 2H), 7.27–7.23 (m, 1H), 7.19 (s, 1H), 7.14 (dd, J = 7.8, 1.5 Hz, 1H), 7.06 (dd, J = 8.1, 1.3 Hz, 1H), 7.02 (td, J = 7.6, 1.4 Hz, 1H), 6.57 (d, J = 2.3 Hz, 2H), 6.52 (t, J = 2.3 Hz, 1H), 6.41 (br s, 1H), 5.64 (d, J = 14.6 Hz, 1H), 5.02 (s, 4H), 3.81 (d, J = 14.6 Hz, 1H), 3.73 (s, 3H), 3.43 (s, 2H), 2.46 (br s, 8H), 2.27 (dt, J = 10.7, 5.9 Hz, 2H), 2.13 (ddd, J = 15.1, 8.1, 6.7 Hz, 1H), 1.94 (ddd, J = 15.3, 8.3, 6.7 Hz, 1H), 1.57–1.46 (m, 2H), 1.38–1.34 (m, 2H), 1.21–1.02 (m, 2H) ppm. **^{13}C NMR** (Chloroform-*d*, 126 MHz) δ 172.5, 160.0, 140.6, 139.2, 139.1, 137.1, 136.5, 132.3, 130.5, 129.2, 128.7, 128.1, 127.7, 122.7, 120.1, 108.2, 101.6, 100.9, 70.2, 63.0, 58.5, 53.2, 52.7, 43.1, 35.0, 33.7, 27.0, 26.2, 25.1 ppm. **IR** (diamond cell, thin film) ν_{max}: 3290, 2945, 2812, 1637, 1594, 1558, 1503, 1447, 1395, 1348, 1313, 1290, 1156, 1506, 835, 737, 697 cm^{-1}. **LRMS** (+ESI) m/z: 685 [(M + H)$^+$, 100%].

*5.2.24. 7-(4-(3,5-Bis(benzyloxy)benzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl) heptan-1-one (**26**)*

Treating **22** (200 mg, 0.51 mmol) with piperazine **15c** according to the above general procedure gave the title compound as a colourless oil (127 mg, 36%) (R_f = 0.35, 1:9 v/v methanol/dichloromethane).

^{1}H NMR (Chloroform-*d*, 500 MHz) δ 7.44–7.39 (m, 4H), 7.39–7.34 (m, 4H), 7.33–7.28 (m, 2H), 7.23 (td, J = 7.7, 7.2, 1.5 Hz, 1H), 7.19–7.16 (m, 2H), 7.13 (dd, J = 7.8, 1.5 Hz, 1H), 7.00 (td, J = 7.5, 1.5 Hz, 1H), 6.93–6.77 (br s, 1H), 6.58 (d, J = 2.3 Hz, 2H), 6.52 (t, J = 2.3 Hz, 1H), 5.63 (d, J = 14.5 Hz, 1H), 5.02 (s, 4H), 3.81 (d, J = 14.5 Hz, 1H), 3.73 (d, J = 1.1 Hz, 3H), 3.44 (s, 3H), 2.53 (br m, 8H), 2.33–2.30 (m, 2H), 2.06 (dq, J = 40.0, 7.5 Hz, 2H), 1.72–1.34 (m, 4H), 1.21–0.99 (m, 4H) ppm. **^{13}C NMR** (Chloroform-*d*, 126 MHz) δ 172.68, 156.0, 140.5, 139.4, 139.3, 137.0, 136.5, 132.1, 130.4, 129.0, 128.6, 128.0, 127.6, 122.4, 120.1, 108.2, 101.5, 100.9, 70.1, 62.9, 58.5, 53.1, 52.3, 43.1, 35.2, 33.7, 28.9, 27.0, 26.1, 25.2 ppm. **IR** (diamond cell, thin film) ν_{max}: 3288, 2933, 2813, 1594, 1559, 1503, 1447, 1395, 1347, 1290, 1154, 1057, 952, 835, 738, 697 cm^{-1}. **LRMS** (+ESI) m/z: 699 [(M + H)$^+$, 100%].

*5.2.25. 8-(4-(3,5-Bis(benzyloxy)benzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl) octan-1-one (**27**)*

Treating **23** (150 mg, 0.38 mmol) with piperazine **15c** according to the above general procedure gave the title compound as a colourless oil (141 mg, 52%) (R_f = 0.38, 1:9 v/v methanol/dichloromethane).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 7.49–7.21 (m, 10H), 7.21–7.10 (m, 3H), 7.02 (dt, J = 7.4, 3.8 Hz, 2H), 6.74–6.12 (m, 3H), 5.49 (d, J = 14.0 Hz, 1H), 5.02 (s, 4H), 3.70 (s, 3H), 3.79 (d, J = 14.4 Hz, 1H), 3.41 (d, J = 2.5 Hz, 2H), 2.42 (br s, 8H), 2.25 (dt, J = 7.7, 3.7 Hz, 2H), 2.13–2.01 (m, 2H), 1.57–1.33 (m, 4H), 1.13 (br s, 6H) ppm. NH peak not observed. **^{13}C NMR** (MeOD-d_4, 101 MHz) δ 175.0, 161.3, 141.5, 140.7, 140.7, 138.7, 137.4, 132.9, 131.1, 130.4, 129.5, 128.8, 128.6, 123.3, 121.3, 109.7, 102.6, 102.4, 71.0, 63.9, 59.6, 53.9, 53.5, 44.5, 35.2, 34.7, 30.0, 30.0, 28.4, 27.3, 26.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 3292, 2930, 1593, 1558, 1503, 1448, 1317, 1153, 1056, 950, 835, 736, 696 cm^{-1}. **LRMS** (+ESI) m/z: 713 [(M + H)$^+$, 100%].

*5.2.26. General procedure for the synthesis of **4a-7a***

A magnetically stirred suspension of the appropriate benzyl protected resorcinol **24**–**27** (0.1 mmol) and Pd/C (20 mg of 10% w/w) in methanol (25 mL) was placed under an atmosphere of H_2 (1 atm) for 8 h. The resulting mixture was filtered through Celite™ and the solids thus retained were washed with methanol (3 × 20 mL). The combined filtrates were concentrated under reduced pressure and purified by flash column chromatography (0.1:0.9:9 v/v/v 28% aqueous ammonia/methanol/dichloromethane):

*5.2.27. 5-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)pentan-1-one (**4a**)*

Subjecting **24** (120 mg, 0.16 mmol) to the above general procedure produced the title compound (**4a**) as a white powder, mp 147–149 °C (77 mg, 95%) (R_f = 0.08, 0.1:0.9:9 v/v/v 28% aqueous ammonia/methanol/dichloromethane).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 7.29–7.27 (m, 2H), 7.22–7.18 (m, 1H), 7.16 (s, 1H), 7.04 (dt, J = 8.0, 1.6 Hz, 1H), 6.26 (s, 2H), 6.17 (s, 1H), 5.51 (d, J = 14.5 Hz, 1H), 3.82 (d, J = 14.5 Hz, 1H), 3.72 (s, 3H), 3.35 (s, 2H), 2.43 (br s, 8H), 2.20–2.15 (m, 3H), 2.11–2.00 (m, 1H), 1.45 (t, J = 8.1 Hz, 2H), 1.30 (q, J = 8.0, 7.5 Hz, 2H) ppm. NH and 2 OH peaks not observed. **^{13}C NMR** (MeOD-d_4, 101 MHz) δ 174.6, 159.5, 141.5, 140.6, 140.4, 137.3, 132.9, 131.1, 130.5, 123.4, 121.6, 109.0, 102.6, 102.5, 63.9, 59.1, 53.7, 53.5, 44.5, 35.2, 34.5, 26.6, 24.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 3150, 2815, 1594, 1556, 1502, 1444, 1390, 1287, 1147, 997, 832, 760, 571, 450 cm^{-1}. **LRMS** (+ESI) m/z: 491 [(M + H)$^+$, 100%]. **HRMS** (+ESI) Found: (M + H)$^+$, 491.2761. $C_{27}H_{34}N_6O_3$ requires (M + H)$^+$, 491.2765. **HPLC** purity: 97.06%, RT: 13.02 min.

*5.2.28. 6-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)hexan-1-one (**5a**)*

Subjecting **25** (90 mg, 0.13 mmol) to the above general procedure produced the title compound (**5a**) as a white powder, mp 153–155 °C (60 mg, 90%) (R_f = 0.08, 0.1:0.9:9 v/v/v 28% aqueous ammonia/methanol/dichloromethane).

^{1}H NMR (MeOD-d_4, 500 MHz) δ 7.32–7.27 (m, 2H), 7.22 (d, J = 7.7 Hz, 1H), 7.16 (s, 1H), 7.05 (t, J = 7.5 Hz, 1H), 6.27 (s, 2H), 6.18 (s, 1H), 5.50 (d, J = 14.4 Hz, 1H), 3.83 (d, J = 14.5 Hz, 1H), 3.73 (s, 3H), 3.38 (s, 2H), 2.51 (br s, 8H), 2.30 (br s, 2H), 2.09 (dq, J = 49.4, 7.5 Hz, 2H), 1.46 (br s, 2H), 1.35 (br s, 2H), 1.24–0.98 (m, 2H) ppm. 1 NH and 2 OH peaks not observed. **^{13}C NMR** (MeOD-d_4, 126 MHz) δ 174.8, 159.6, 141.5, 140.7, 140.2, 137.4, 132.9, 131.1, 130.5, 123.4, 121.3, 109.0,

102.7, 102.6, 63.8, 59.2, 53.7, 53.2, 44.5, 35.2, 34.6, 27.9, 26.7, 26.0 ppm. **IR** (diamond cell, thin film) ν_{max}: 3149, 2814, 1592, 1556, 1502, 1446, 1390, 1288, 1146, 997, 832, 760, cm^{-1}. **LRMS** (+ESI) *m/z*: 505 [$(M + H)^+$, 100%]. **HRMS** (+ESI) Found: $(M + H)^+$, 505.2918. $C_{28}H_{36}N_6O_3$ requires $(M + H)^+$, 505.2922. **HPLC** purity: 95.54%, RT: 13.40 min.

5.2.29. 7-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)heptan-1-one (**6a**)

Subjecting **26** (120 mg, 0.17 mmol) to the above general procedure produced the title compound (**6a**) as a white powder, mp 122–124 °C (84 mg, 94%) (R_f = 0.09, 0.1:0.9:9 v/v/v 28% aqueous ammonia/methanol/dichloromethane).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 7.31–7.27 (m, 2H), 7.20–7.17 (m, 1H), 7.16 (s, 1H), 7.06–7.01 (m,1H), 6.29 (d, J = 2.2 Hz, 2H), 6.20 (s, 1H), 5.50 (d, J = 13.9 Hz, 1H), 3.81 (d, J = 14.5 Hz, 1H), 3.72 (s, 3H), 3.36 (s, 2H), 2.47 (br s, 8H), 2.26–2.20 (m, 2H), 2.13–2.00 (m, 2H), 1.41 (dt, J = 29.7, 7.2 Hz, 4H), 1.12–1.11 (m, 4H) ppm. NH and 2 OH signals not observed. **^{13}C NMR** (MeOD-d_4, 101 MHz) δ 175.0, 159.5, 141.5, 140.6, 140.4, 137.4, 132.9, 131.0, 130.4, 123.3, 121.3, 109.0, 102.6, 102.5, 64.0, 59.6, 53.8, 53.5, 44.5, 35.2, 34.7, 29.9, 28.1, 27.1, 26.2 ppm. **IR** (diamond cell, neat) ν_{max}: 3270, 2973, 2938, 2872, 1603, 1560, 1504, 1448, 1351, 1251, 1157, 1087, 1045, 834, 571 cm^{-1}. **LRMS** (+ESI) *m/z*: 519 [$(M + H)^+$, 100%]. **HRMS** (+ESI) Found: $(M + H)^+$, 519.3071. $C_{29}H_{38}N_6O_3$ requires $(M + H)^+$, 519.3078. **HPLC** purity: 96.98%, RT: 13.85 min.

5.2.30. 8-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)-1-(1-methyl-4,10-dihydrobenzo[b]pyrazolo[3,4-e][1,4]diazepin-5(1H)-yl)octan-1-one (**7a**)

Subjecting **27** (130 mg, 0.18 mmol) to the above general procedure produced the title compound (**7a**) as a white powder, mp 80–82 °C (93 mg, 96%) (R_f = 0.07, 0.1:0.9:9 v/v/v 28% aqueous ammonia/methanol/dichloromethane).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 7.29–7.26 (m, 2H), 7.19 (dd, J = 7.9, 1.3 Hz, 1H), 7.16 (s, 1H), 7.04 (dt, J = 7.2, 1.6 Hz, 1H), 6.28 (s, 2H), 6.17 (s, 1H), 5.50 (d, J = 14.5 Hz, 1H), 3.82 (d, J = 14.5 Hz, 1H), 3.72 (s, 3H), 3.36 (s, 2H), 2.49 (br s, 8H), 2.36–2.27 (m, 2H), 2.15–2.00 (m, 2H), 1.46–1.39 (m, 4H), 1.17–1.12 (m, 6H) ppm 1NH and 2 OH signals not observed. **^{13}C NMR** (MeOD-d_4, 101 MHz) δ 175.1, 159.5, 141.6, 140.7, 140.4, 137.4, 132.9, 131.1, 130.5, 123.3, 121.3, 109.0, 102.6, 102.6, 64.0, 59.7, 53.9, 53.5, 44.5, 35.2, 34.7, 30.0, 29.9, 28.4, 27.3, 26.3 ppm. **IR** (diamond cell, thin film) ν_{max}: 3270, 2974, 2936, 2875, 1603, 1561, 1504, 1444, 1350, 1291, 1252, 1155, 1087, 1045, 1022, 833, 760, 571 cm^{-1}. **LRMS** (+ESI) *m/z*: 533 [$(M + H)^+$, 100%]. **HRMS** (+ESI) Found: $(M + H)^+$, 533.3235. $C_{30}H_{40}N_6O_3$ requires $(M + H)^+$, 533.3235. **HPLC** purity: 96.26%, RT: 14.35 min.

5.3. Pharmacological evaluation

5.3.1. Generation of OT and V_{1a} receptor-expressing human embryonic kidney (HEK) cells

The Flp-In™ system (Life Technologies, Carlsbad, CA, USA) was used to establish cell lines expressing the human OT and V_{1a} receptor. pOG44 plasmids (Life Technologies) and pcDNA5/FRT plasmids containing human OT or V_{1a} receptor cDNA were synthesised by GenScript and were propagated in endonuclease and recombinase-deficient E. coli (BIO-85027, Bioline, London, UK). Competent E. coli were transformed with plasmid DNA (50 ng) by heat shock at 42 °C. Cultures were then incubated for 16 h at 37 °C on Luria-bertani (LB) agar plates (Life Technologies) containing 100 μg/mL ampicillin (Sigma–Aldrich, St. Louis, MO, USA). Ampicillin-resistant colonies were isolated and inoculated into 5 mL of LB broth containing 50 μg/mL ampicillin. This culture was incubated for 16 h at 37 °C, and pcDNA5/FRT and pOG44 DNA purified using the Plasmid Midiprep System (Promega, Madison, WI, USA) according to manufacturer's protocols. Plasmid integrity was verified by performing a restriction enzyme digest using the restriction endonuclease FspI (New England Biolabs, Ipswich, MA, USA) (Appendix 1). Flp-In™ T-REx™ HEK293 cells used for transfection were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen), blasticidin (15 μg/mL; Sigma–Aldrich), penicillin-streptomycin (100U; Sigma–Aldrich) and zeocin™ selection reagent (100 μg/mL; Life Technologies). Cells were co-transfected with pcDNA5 plasmid containing human OT or V_{1a} receptor cDNA sequences, alongside pOG44 plasmid containing Flp-recombinase cDNA. Transfections were undertaken using the non-liposomal transfection reagent FugeneHD® (Promega) according to manufacturer's protocols. Selection of receptor-positive clones was achieved by treatment with the antibiotic hygromycin (80 μg/mL; Invitrogen). Surface receptor expression was verified using immunocytochemistry according to the method of Werry et al. [19] Stably transfected cell lines were subsequently maintained in 10% DMEM containing hygromycin (80 μg/mL), penicillin/streptomycin (100U) and blasticidin (15 μg/mL). To induce receptor expression, cells were incubated with tetracycline (2 μg/mL) for 48 h prior to membrane preparation and functional assays. This concentration of tetracycline was selected based on previously demonstrated efficacy in inducing receptor expression [20].

5.3.2. Membrane preparation

OT and V_{1a} receptor-expressing HEK293 cells were detached from culture dishes using PBS with 5 mM EDTA and centrifuged at 1200 g for 5 min. The supernatant was removed and cells resuspended in homogenization buffer (50 mM HEPES, 5 mM EDTA, 5 mM $MgCl_2$, pH 7.4) prior to homogenization using an Ultra-Turrax homogeniser (IKA, Wilmington, NC, USA). Resulting homogenates were centrifuged twice at 48,000 g, 4 °C for 30 min and membrane pellets resuspended in 50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4. Final protein concentration was calculated using the BCA protein-assay method (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocols. Membranes were stored at −80 °C.

5.3.3. Competition radioligand binding

Binding affinity of WAY-267,464 and derivatives was indexed by competitive displacement of [^{3}H]-oxytocin or [^{3}H]-vasopressin at K_d concentrations. Membranes (50 μg/well) from OT or V_{1a} receptor-expressingHEK293 cells were incubated in a final volume of 200 μL containing [^{3}H]-oxytocin (10 nM) or [^{3}H]-vasopressin (2 nM) alongside competing compounds (0.1 nM - 100 μM) in reaction buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4). Reactions were incubated for 90 min at 4 °C to reach equilibrium, and terminated by rapid filtration over glass fibre filters (Whatman GF/A 1.6 μM), and washing with ice-cold reaction buffer. Radioactivity was detected after soaking filters in Microscint 0 using a Microbeta2 2450 microplate-reader (Perkin Elmer). Nonspecific binding was determined in the presence of 1 μM cold oxytocin or vasopressin (Sigma–Aldrich), respectively.

5.3.4. HTRF-IP1 accumulation assays

OT or V_{1a} receptor-expressing-HEK293 cells were seeded onto clear, poly-L-lysine (100 μg/mL)-coated 384 well plates at a density of 8.75 x 10^3 cells per well. Levels of receptor activation induced by compounds were assessed at concentrations ranging from 1 nM to 100 μM using the HTRF-IP-One kit (CisBio International, Bagnols-sur-Cze, France), according to manufacturer's protocol. For agonist

assessment, cells were incubated with compounds for 1 h prior to the addition of Ab-Cryptate and IP1-d2. The ligand concentration that induced a 50% maximal response (EC50) was used to evaluate functional effects across compounds. For antagonist assessment, cells were pre-incubated with test compounds (1 nM - 100 μM) solubilized in stimulation buffer for 30 min prior to the addition of an EC_{70} concentration of vasopressin (25 nM) mixed with compounds or DMSO control (0.1%). Cells were then incubated for a further 1 h, and Ab-Cryptate and IP1-d2 added. The ligand concentration that inhibited 50% of vasopressin-induced response (IC_{50}) was used to evaluate antagonistic functional effects across compounds.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.11.050.

References

[1] Y. Yang, H. Li, R. Ward, L. Gao, J.-F. Wei, T.-R. Xu, Novel oxytocin receptor agonists and antagonists: a patent review (2002 – 2013), Expert Opin. Ther. Pat. 24 (2014) 29–46.
[2] R.J. Windle, N. Shanks, S.L. Lightman, C.D. Ingram, Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats, Endocrinology 138 (1997) 2829–2834.
[3] A. Blume, O.J. Bosch, S. Miklos, L. Torner, L. Wales, M. Waldherr, I.D. Neumann, Oxytocin reduces anxiety via ERK1/2 activation: local effect within the rat hypothalamic paraventricular nucleus, Eur. J. Neurosci. 27 (2008) 1947–1956.
[4] M. Marazziti, M.C. Dell'Osso, The role of oxytocin in neuropsychiatric disorders, Curr. Med. Chem. 15 (2008) 698–704.
[5] H.H. Zingg, Vasopressin and oxytocin receptors, Bailliere Clin. Endoc. 10 (1996) 75–96.
[6] I.F. Bielsky, S.-B. Hu, K.L. Szegda, H. Westphal, L.J. Young, Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice, Neuropsychopharmacology 29 (2003) 483–493.
[7] G. Liebsch, C.T. Wotjak, R. Landgraf, M. Engelmann, Septal vasopressin modulates anxiety-related behaviour in rats, Neurosci. Lett. 217 (1996) 101–104.
[8] K. Ebner, C.T. Wotjak, R. Landgraf, M. Engelmann, Forced swimming triggers vasopressin release within the amygdala to modulate stress-coping strategies in rats, Eur. J. Neurosci. 15 (2002) 384–388.
[9] B. Chini, M. Manning, Agonist selectivity in the oxytocin/vasopressin receptor family: new insights and challenges, Biochem. Soc. Trans. 35 (2007) 737–741.
[10] G. Gimpl, F. Fahrenholz, The oxytocin receptor system: structure, function and regulation, Physiol. Rev. 81 (2001) 626–683.
[11] M. Manning, S. Stoev, B. Chini, T. Durroux, B. Mouillac, G. Guillon, D.N. Inga, L. Rainer, Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents, in: Prog Brain Res, Elsevier, 2008, pp. 473–512.
[12] D.M. Ashworth, A.R. Batt, A.J. Baxter, P. Broqua, R.M. Haigh, P. Hudson, C.M.S. Heeney, R. Laporte, A. Penson, G.R.W. Pitt, P.A. Robson, D.P. Rooker, A.L. Tartar, C.M. Yea, M.B. Roe, Nonpeptide oxytocin agonists, Drug Future 31 (2006) 345–353.
[13] R.H. Ring, L.E. Schechter, S.K. Leonard, J.M. Dwyer, B.J. Platt, R. Graf, S. Grauer, C. Pulicicchio, L. Resnick, Z. Rahman, S.J. Sukoff Rizzo, B. Luo, C.E. Beyer, S.F. Logue, K.L. Marquis, Z.A. Hughes, S. Rosenzweig-Lipson, Receptor and behavioral pharmacology of WAY-267464, a non-peptide oxytocin receptor agonist, Neuropharmacology 58 (2010) 69–77.
[14] M.C. Frantz, J. Rodrigo, L. Boudier, T. Durroux, B. Mouillac, M. Hibert, Subtlety of the structure-affinity and structure-efficacy relationships around a non-peptide oxytocin receptor agonist, J. Med. Chem. 53 (2010) 1546–1562.
[15] C. Hicks, W. Jorgensen, C. Brown, J. Fardell, J. Koehbach, C.W. Gruber, M. Kassiou, G.E. Hunt, I.S. McGregor, The nonpeptide oxytocin receptor agonist WAY 267,464: receptor-binding profile, prosocial effects and distribution of c-Fos expression in adolescent rats, J. Neuroendocrinol. 24 (2012) 1012–1029.
[16] C. Hicks, L. Ramos, T. Reekie, R. Narlawar, M. Kassiou, I. McGregor, WAY 267,464, a non-peptide oxytocin receptor agonist, impairs social recognition memory in rats through a vasopressin 1A receptor antagonist action, Psychopharmacology (2015) 1–9.
[17] T.A. Reekie, I.S. McGregor, M. Kassiou, Pyrazolo[1,4]diazepines as non-peptidic probes of the oxytocin and vasopressin receptors, Tetrahedron Lett. 55 (2014) 4568–4571.
[18] P. Hudson, G. Pitt, R. Batt, M. Roe, Piperazines as oxytocin agonists, in: Patent fetcher, B.V. Ferring (Eds.), PCT, International Application, 2005.
[19] E.L. Werry, G.J. Liu, M.R. Bennett, Glutamate-stimulated ATP release from spinal cord astrocytes is potentiated by substance P, J. Neurochem. 99 (2006) 924–936.
[20] A. Knapman, F. Abogadie, P. McIntrye, M. Connor, A real time, fluorescence-based assay for measuring μ-opioid receptor modulation of adenylyl cyclase activity in Chinese Hamster Ovary cells. J. Biomol. Screen. 19 (2014) 223–231.