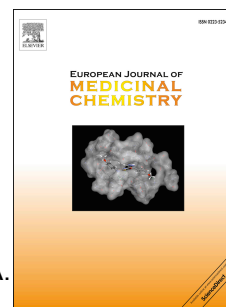


# Accepted Manuscript

Conformationally rigid derivatives of WAY-267,464: Synthesis and pharmacology at the human oxytocin and Vasopressin-1a receptors

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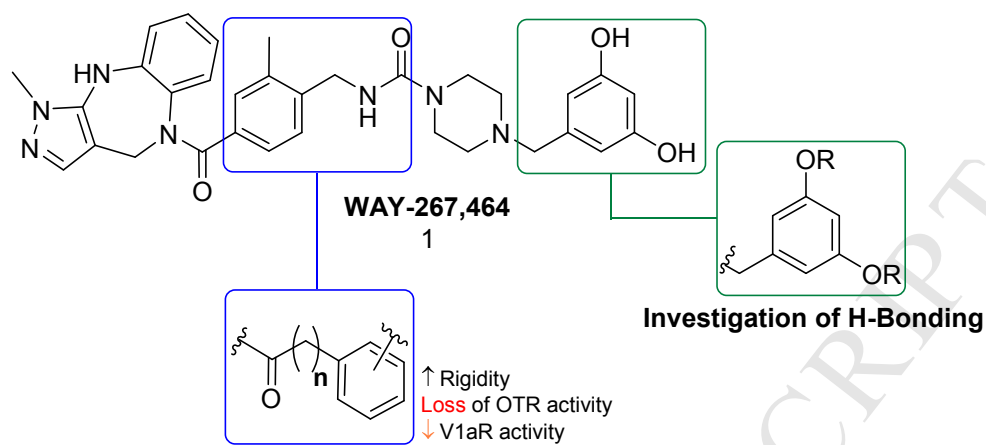
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## Investigation of Linker Conformation



# Conformationally Rigid Derivatives of WAY-267,464: Synthesis and Pharmacology at the Human Oxytocin and Vasopressin-1a Receptors

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**Keywords:** Oxytocin receptor; WAY-267,464; arginine vasopressin 1<sub>a</sub> receptor; diazepam.

## Abstract

WAY-267,464 (**1**) and twelve conformationally rigid analogues (**3a-f** – **4a-f**) were synthesised, characterised and evaluated in cellular assays with the aim of systematically exploring interactions with the oxytocin receptor (OTR). Each analogue was evaluated in radioligand binding displacement assays at both human OTR and arginine vasopressin 1a receptors (V<sub>1a</sub>R). Physiological characterisation was determined by whole cell IP1 accumulation assays on stably transfected human embryonic kidney (HEK) cells. Incorporation of the rigid, optionally substituted benzene ring abolished OTR activity and diminished V<sub>1a</sub>R pharmacology when compared to **1**. A general trend was observed in V<sub>1a</sub>R affinity for the propyl analogues (**3d-3f**) which identified the *ortho*-substituted analogue as the best in series (K<sub>i</sub> = 251 nM) followed by a decrease in affinity through the *meta* and *para*- derivatives (**3e**; K<sub>i</sub> = 874 nM and **3f**; K<sub>i</sub> = 1756 nM respectively). This study confirms the importance of the central pharmacophoric motifs of WAY-267,464 and illuminates the differences in the binding pocket of the highly conserved OTR and V<sub>1a</sub>R.

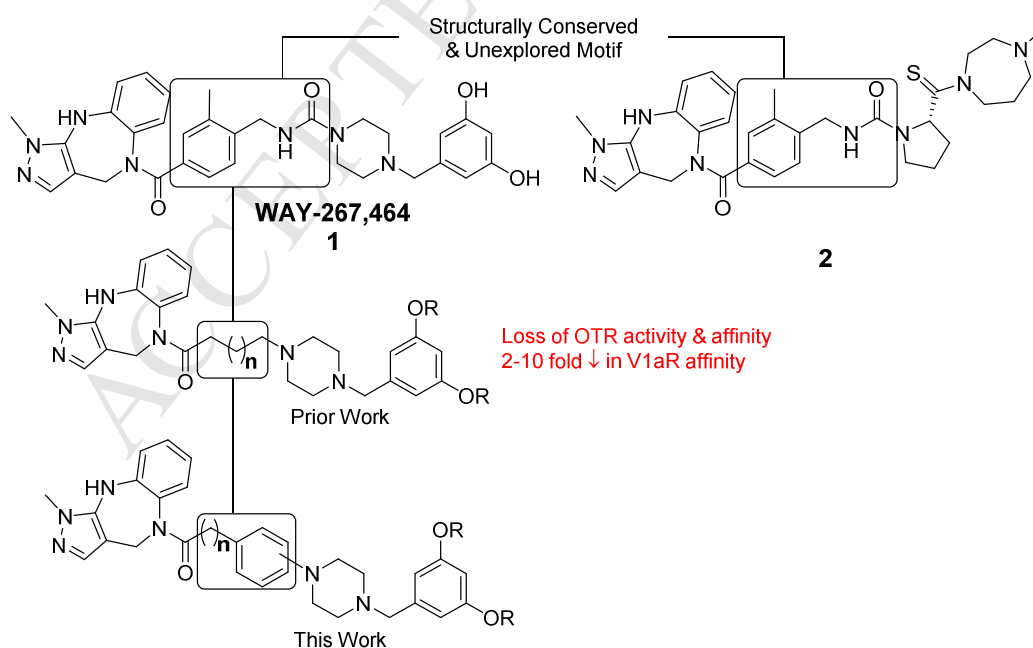
## 1. Introduction

Psychological disorders such as depression, anxiety and substance addiction often manifest concomitantly with severe social dysfunction.[1] This reveals a possible therapeutic paradigm through the use of pro-social modulators to treat such psychiatric illnesses. Surmounting evidence over the past two decades has implicated centrally located oxytocin (OT) receptors and arginine vasopressin (AVP) 1a receptors as viable targets for the improvement of social cognition.[2],[3] OT facilitates social bonding and pair attachment in humans and animals,[4] whereas male rats lacking functional V<sub>1a</sub> receptors (V<sub>1a</sub>R) display profound anxiety-like behaviour and a marked reduction in social recognition.[5] It follows that the development of a centrally penetrating, orally bio-available, selective ligand at the OT receptor (OTR) or V<sub>1a</sub>R represents an attractive research goal.

OT and AVP are structurally related cyclic neuropeptides which elicit their modulatory roles via interaction with four G-protein coupled receptors; the OT, V<sub>1a</sub>, V<sub>1b</sub> and V<sub>2</sub> receptors. Unfortunately, the highly conserved sequence homology across each receptor subtype,[6][7] coupled with the partial co-localisation of the OTR and V<sub>1a</sub>R within the CNS presents a significant challenge in the design of selective receptor ligands. Furthermore, appreciable crosstalk between the endogenous neuropeptides amongst the OT and vasopressin receptor families, coupled with the poor pharmacokinetic properties inherent to cyclic peptides (short half-life, poor CNS permeability and unknown metabolomics), limit their use as pharmacological tools. As such, the identification of selective, centrally penetrating OTR or V<sub>1a</sub>R ligands is of

paramount importance towards the development of molecular probes to differentiate the behavioural roles of each receptor subtype.[8] Our group has been interested in understanding the intricacies surrounding the functional activity of WAY-267,464 (**1**), one of the very few non-peptidic OTR agonists. We have previously published the behavioural profile of WAY-267,464 and found significant pro-social effects (IP, 100 mg/kg)[9] which were later in-part correlated to centrally located V<sub>1a</sub>R antagonism rather than its oxytocinergic effects.[10] In 2016, we reported the pharmacological binding and efficacy profile of **1** along with its first published structure-activity relationship. WAY-267,464 was shown to be a non-selective OTR agonist/V<sub>1a</sub>R antagonist (OTR K<sub>i</sub> = 230 nM; V<sub>1a</sub>R K<sub>i</sub> = 27 nM; OTR EC<sub>50</sub> = 420 nM; V<sub>1a</sub>R IC<sub>50</sub> = 613 nM).[11]

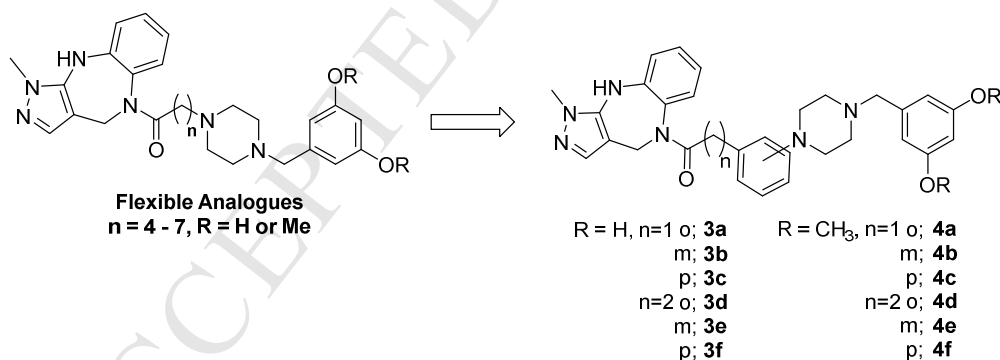
Whilst the initial disclosure of WAY-267,464 reported the synthesis of multiple analogues, derivatisation was focused on modifications to the tricyclic western component or the benzylpiperazine tail moiety. The central benzylurea linkage (**Figure 1**) was maintained across the compound library.[12] In 2010, Hubert and colleagues performed a structure activity relationship analysis of proline **2**, a weak OTR partial agonist which shares structural motifs, including the benzylurea linker, with WAY-267,464. Whilst their work focused on the truncation of **2** and elaboration through hybridisation with AVPR and OTR ligands, the benzylurea component was maintained. As such, the majority of our work has looked at identifying the functional necessity of this central, structurally conserved motif.



**Figure 1:** The conserved benzylurea moiety across non-peptide OTR agonists (**1** & **2**) and our efforts to explore its importance.

Previous work involved replacing the rigid benzylurea with flexible alkyl tethers of varying lengths. This modification abolished OTR activity and largely diminished the OTR binding affinity (Figure 1). Marginal receptor affinity was gained at increasing chain lengths similar in distance to the lead molecule **1**. Whilst deleterious to receptor pharmacology, these modifications were better tolerated at the V<sub>1a</sub>R and thus resulted in selective V<sub>1a</sub>R ligands. To exemplify the subtleties surrounding the OTR, removal of the hydrogen-bonding capabilities of the resorcinol functionality through methyl-ether incorporation resulted in a reversal of WAY-267,464 functional activity (resulting in a weak OTR antagonist; IC<sub>50</sub> = 4  $\mu$ M).[11]

Considering the lack of published structure-activity relationship (SAR) studies surrounding the WAY-267,464 molecule and the previously observed subtleties surrounding the flexible alkyl tethered analogues, we now report the synthesis and pharmacological evaluation of 12 conformationally restricted analogues. Several considerations were necessary in the design of these molecules. Shortened chain lengths limited the number of freely rotatable bonds, minimising overall molecular flexibility. Phenylpiperazine analogues, **3a-f** and **4a-f** were designed to methodically explore optionally restrained analogues of **1** in an attempt to re-establish the OTR activity which was lost upon the introduction of flexibility. For consistency, both methyl-ether and phenolic analogues were incorporated to better correlate with the previous work in a sustained effort towards the identification of a viable OTR pharmacophore (Figure 2).

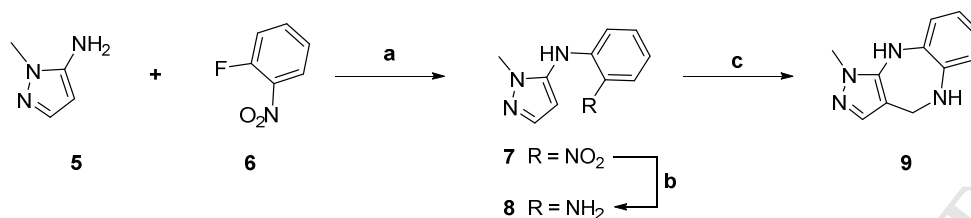


**Figure 2:** Re-introduction of rigidity *via* incorporation of an optionally substituted benzene ring.

## 2. Synthetic Chemistry

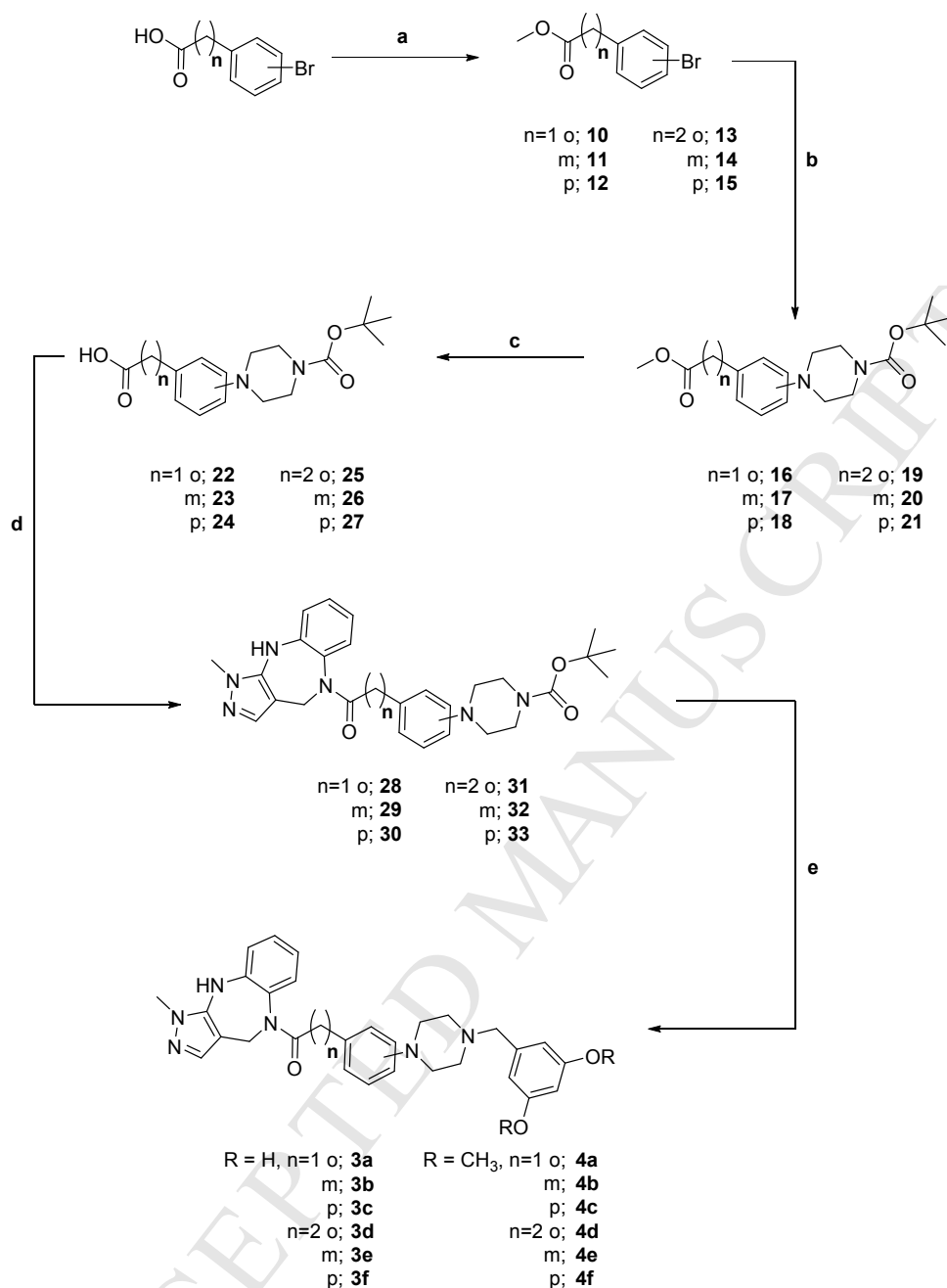
The synthetic protocols to generate WAY-267,464 on the gram-scale have previously been described.[11] Furthermore, we have recently reported a large-scale method for the production of pyrazolo[1,4]diazepine molecules through a novel, Pictet Spengler cyclisation (**Scheme 1**).[13] Alkoxide mediated nucleophilic aromatic substitution of arylfluoride **6** by 5-aminopyrazole generated nitrobenzene **7**. Palladium catalysed hydrogenation furnished the

Pictet-Spengler precursor **8** that, upon subjection to formaldehyde and catalytic quantities of acetic acid, produced the desired diazepine **9**.



**Scheme 1:** Reagents and conditions: (a) KO<sup>t</sup>Bu, THF, 0 °C → RT, 3 h, 77%; (b) Pd/C (10 mol%), H<sub>2</sub> (1 atm), EtOAc, RT, 18 h 88%; (c) HCHO, AcOH (cat), MeCN, RT, 18 h, 79%.

The synthesis of the desired analogues begins with the Fischer esterification of the appropriately substituted bromophenyl alkanolic acids to yield the methyl esters (**10 – 15**), followed by a Buchwald-Hartwig cross coupling amination with Boc-piperazine to furnish the desired phenylpiperazines (**16 – 21**). Hydroxide mediated hydrolysis in aqueous methanol allowed access to the alkanolic acid precursors and upon subjection to a PyBOP® mediated amidation with diazepine **9** generated the amide intermediates (**28 – 33**). An optimised one-pot carbamate deprotection-reductive alkylation with either 3,5-dihydroxybenzaldehyde or 3,5-dimethoxybenzaldehyde produced the desired rigid analogues in sufficient yield for pharmacological assays (**Scheme 2**).

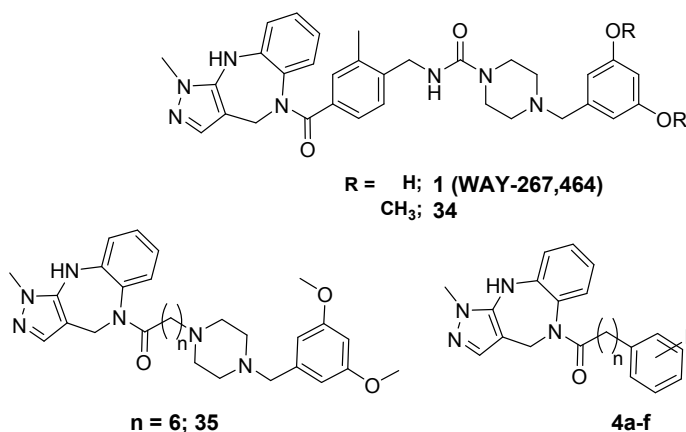


**Scheme 2:** (a)  $\text{H}_2\text{SO}_4$ , MeOH, reflux, 18 h, 90-100%; (b) Boc-piperazine, RuPhos<sup>®</sup>,  $\text{Cs}_2\text{CO}_3$ ,  $\text{Pd}_2(\text{dba})_3$ , PhMe, reflux, 18 h, 55-99%; (c) LiOH (aq), MeOH, reflux, 1 h, 90-97%; (d) PyBOP<sup>®</sup>, diazepine **9**,  $i\text{-Pr}_2\text{NEt}$ ,  $\text{CH}_2\text{Cl}_2$ , rt, 12 h, 67-81%; (e) *i.* HCl (1M in dioxane), MeOH, rt, 3 h. *ii.* 3,5-dihydroxybenzaldehyde or 3,5-dimethoxybenzaldehyde, NMM,  $\text{MgSO}_4$ ,  $\text{NaCNBH}_3$ , rt, 18 h, 43-100%.

### 3. Results & Discussion

The 12 derivatives synthesised were subjected to radioligand displacement assays to determine selectivity and receptor affinity utilising membranes from HEK 293 cells expressing hOTR and hV<sub>1a</sub>R. Comparison of the rigid dimethoxy analogues **4a-f** to the lead compound WAY-267,464 and dimethoxy analogue **34** as well as the best in series of the flexible analogues **35**, [11] is summarised in **Table 1**.



**Table 1:** Binding and functional activity of the conformationally restricted dimethoxy derivatives (general structure **4a-f**) compared to WAY-267,464 (**1**), diMeO-WAY (**34**), and the most potent dimethoxy flexible analogue (**35**).

#	n		OTR (nM ± SD)			V <sub>1a</sub> R (nM ± SD)		
			K <sub>i</sub>	EC <sub>50</sub>	IC <sub>50</sub>	K <sub>i</sub>	EC <sub>50</sub>	IC <sub>50</sub>
<b>1</b>	-	-	230 ± 31	420 ± 59	n/a	27 ± 3	>10,000	613 ± 206
<b>34</b>	-	-	801 ± 139	>10,000	4129 ± 645	62 ± 21	>10,000	1113 ± 180
<b>35*</b>	6		>10,000	>10,000	>10,000	194.7 ± 82.6	>10,000	>10,000
<b>4a</b>	1	<i>o</i>	>10,000	>10,000	>10,000	6807 ± 642.5	>10,000	>10,000
<b>4b</b>	1	<i>m</i>	>10,000	>10,000	>10,000	2095 ± 691.9	>10,000	>10,000
<b>4c</b>	1	<i>p</i>	>10,000	>10,000	>10,000	1910 ± 262.6	>10,000	>10,000
<b>4d</b>	2	<i>o</i>	>10,000	>10,000	>10,000	1062 ± 143.2	>10,000	>10,000
<b>4e</b>	2	<i>m</i>	>10,000	>10,000	>10,000	3343 ± 59.30	>10,000	>10,000
<b>4f</b>	2	<i>p</i>	>10,000	>10,000	>10,000	6250 ± 964.2	>10,000	>10,000

\*The strongest binder of the dimethoxy flexible alkyl analogues at the hV<sub>1a</sub>R.

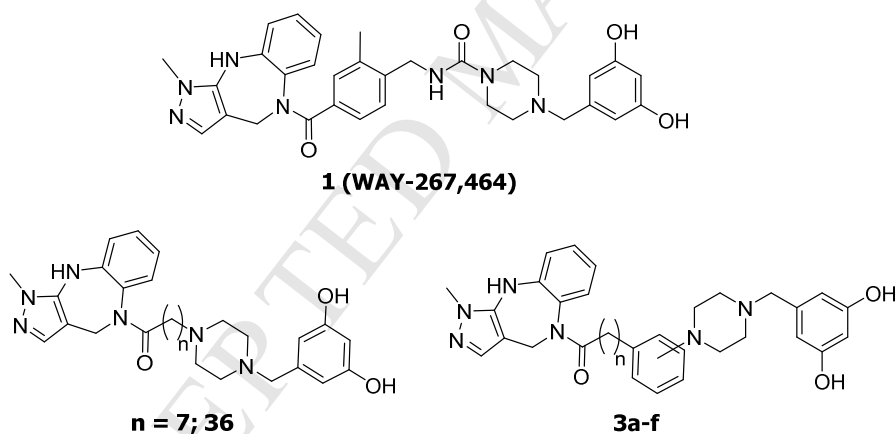
Reintroduction of conformity in the central linker of the WAY analogues did not reinstate functional efficacy or affinity at the OTR. It is likely that the central tolyl linker and/or urea moiety participate in favourable binding interactions at the OTR active site which are lacking in both the flexible and conformationally-restricted analogues. The methylation of the phenols did not generate OTR antagonists, in contrast to the effect seen with methylation of WAY-267,464.[11]

In regards to V<sub>1a</sub>R activity, restricting the number of freely rotatable bonds resulted in a decrease in radioligand displacement. This was observed for both the n = 1 and n = 2 alkyl *ortho/meta/para* derivatives (**Table 1**). In comparison to the dimethoxy flexible analogue (n = 6, **35**) which is the strongest V<sub>1a</sub>R binder of the dimethoxy alkyl analogues (K<sub>i</sub> = 195 nM), the

best dimethyl conformationally-restricted analogue **4d** incorporated an *ortho*-piperazine substitution pattern (with a binding affinity  $K_i = 1062$  nM). Although these molecules weakly bind to the  $V_{1a}R$  in a homogenised cell membrane assay, they do not have a functional effect on the  $G_q$  pathway of the  $V_{1a}R$ . These functional assays are conducted under different experimental conditions to the binding assays, and so the incongruous behaviour of the molecules between assays may be explained by a differing effect on receptors in the whole cell versus membrane environment, or at different temperatures.

It was anticipated that the *O*-desmethyl analogues would perform better in radioligand binding assessment considering the increase in binding affinity observed with the phenolic alkyl derivatives presented previously.[11] It was satisfying to confirm this prediction with a 2-3-fold increase in affinity at the  $V_{1a}R$  compared to the methylated analogues. This further justifies the requirement of H-bond donation for  $V_{1a}R$  affinity. In comparison to the previously synthesised flexible analogues, the rigid derivatives resulted in a dramatic loss (4-25 fold) in  $V_{1a}R$  binding affinity. This suggests flexibility is an important determinant of  $V_{1a}R$  pharmacology (**Table 2**).

**Table 2:** Binding and functional activity of the conformationally restricted resorcinolic derivatives (general structure **3a-f**) compared to WAY-267,464 (**1**) and the most potent dihydroxy flexible analogue (**36**).



#	n		OTR (nM $\pm$ SEM)			$V_{1a}R$ (nM $\pm$ SEM)		
			$K_i$	$EC_{50}$	$IC_{50}$	$K_i$	$EC_{50}$	$IC_{50}$
<b>1</b>	-	-	230 $\pm$ 31	420 $\pm$ 59	>10,000	27 $\pm$ 3	>10,000	613 $\pm$ 206
<b>36*</b>	7	-	8349 $\pm$ 664	>10,000	>10,000	64.1 $\pm$ 5.1	>10,000	4667 $\pm$ 823
<b>3a</b>	1	o	>10,000	>10,000	>10,000	1938 $\pm$ 519.3	>10,000	>10,000
<b>3b</b>	1	m	>10,000	>10,000	>10,000	642.8 $\pm$ 108.3	>10,000	>10,000
<b>3c</b>	1	p	>10,000	>10,000	>10,000	795.1 $\pm$ 228.0	>10,000	>10,000
<b>3d</b>	2	o	>10,000	>10,000	>10,000	250.7 $\pm$ 32.6	>10,000	>10,000
<b>3e</b>	2	m	>10,000	>10,000	>10,000	874.1 $\pm$ 142.4	>10,000	>10,000

<b>3f</b>	<b>2</b>	<b>p</b>	>10,000	>10,000	>10,000	1756 ± 481.9	>10,000	>10,000
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\*The strongest binder of the alkyl analogues at the hV<sub>1a</sub>R.

Interestingly, for the propyl chain analogues, a trend in the substitution pattern could be observed. The *ortho* substitution resulted in the highest binding affinity (**3d**; K<sub>i</sub> = 250 nM) followed by the *meta* substituted analogue (**3e**; K<sub>i</sub> = 874 nM) and finally the *para* derivative (**3f**; K<sub>i</sub> = 1756 nM). This suggests that optimal binding orientation at the V<sub>1a</sub>R is obtained through a highly kinked structural conformer which is not replicated at the OTR.

The highest binder of this series of compounds was the *O*-desmethyl, *ortho* substituted analogue **3d** which had a radioligand binding displacement affinity of 250 nM. Unfortunately, the functional inhibition of the Gq-pathway of the V<sub>1a</sub>R by these compounds was not observed in the current pharmacological assay.

#### 4. Conclusion

The primary focus of this study was to further explore the structural motifs associated with the unique oxytocinergic and vasopressinergic activity of the non-peptidic WAY-267,464 molecule. Prior work has shown that replacement of the central tolyl-urea linkage abolished OTR affinity and was detrimental to V<sub>1a</sub>R activity. Reducing the number of freely rotatable bonds between the tricyclic diazepine and the benzylpiperazine resulted in 12 conformationally rigid analogues. The rigidity of these molecules did not restore OTR binding activity or efficacy which confirms that the functionality within the WAY-267,464 linker is important for its receptor pharmacology.

Similarly to the alkyl tethered analogues, removal of the H-bond donating capabilities of the resorcinol functionality was detrimental to overall receptor pharmacology. Furthermore, a trend was observed at the V<sub>1a</sub>R regarding optimal substitution around the central aromatic ring incorporated to impart rigidity. The 2-piperazinyl substituted (*ortho*) compound, **3d** was the highest binder of this series. Unfortunately, these rigid analogues failed to produce an improved pharmacological profile compared to the lead compound in terms of affinity, potency or selectivity. This work has shown that the central linker components of WAY-267,464 are imperative for functional activity, efficacy and affinity at the OTR providing further insight into the OTR pharmacophore. Furthermore, modifications to this central component are better tolerated at the V<sub>1a</sub>R and as such, differences in key receptor-ligand interactions between these highly homologous receptors have been brought to light. Future work will look at the systematic introduction of the key functional groups that make up the linker moiety of WAY-267,464 to elaborate on the conclusions presented here.

## 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 diisopropyl ethylamine were 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 visualised 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<sup>-1</sup>). Nuclear magnetic resonance spectra were recorded at 298 K unless stated otherwise, using either a Bruker DRX400 (400.1 MHz) or AVANCE III 500 Ascend (500.1 MHz) spectrometer. The data is reported as the chemical shift ( $\delta$  ppm) relative to the solvent residual peak, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, etc.), coupling constant (*J* Hz), relative integral. Low resolution mass spectra (LRMS) were recorded using electrospray ionisation (ESI) on a Finnigan LCQ ion trap spectrometer. 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  $\mu$ L/hr) using a Cole Palmer syringe pump.

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  $\mu$ m column (2.1x150 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 minutes). 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 (**9**) was completed as previously reported.[13] All characterisation data matched with those previously reported.

### 5.2.1. 1-Methyl-*N*-(2-nitrophenyl)-1*H*-pyrazol-5-amine (**7**)

A magnetically stirred solution of amine **5** (10.0 g, 103 mmol) in THF (250 mL) was treated with potassium *tert*-butoxide (23.0 g, 205 mmol) at 0 °C. The resultant suspension was brought to room temperature and stirred for 1 h before the dropwise addition of *o*-fluoronitrobenzene (12 mL, 113 mmol). The reaction mixture was stirred for 3 h, quenched with water (300 mL) and extracted with EtOAc (3 x 150 mL). The combined organic extracts were dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. The crude oil was purified by flash column chromatography (silica, 3:5 v/v EtOAc:hexanes) and concentration of the relevant fractions ( $R_f$  = 0.42 in 1:1 v/v EtOAc:hexanes) afforded the title compound (16.9 g, 77%) as yellow crystals. <sup>1</sup>H NMR (300 MHz, Chloroform-*d*):  $\delta$  9.11 (s, 1H, NH), 8.25 (d, *J* = 8.1 Hz, 1H), 7.56 – 7.54 (m, 1H), 7.52 – 7.38 (m, 1H), 6.96 – 6.84 (m, 1H), 6.78 – 6.74 (m, 1H), 6.20 (d, *J* = 2.5 Hz, 1H), 3.76 (s, 3H). <sup>13</sup>C NMR (75 MHz, Chloroform-*d*):  $\delta$  142.7, 139.0, 136.8, 136.3, 133.4, 126.6, 118.7, 115.8, 101.8, 35.2. IR (diamond cell, neat)  $\nu_{\max}$ : 3297, 3094, 1609, 1572, 1509, 1490, 1340, 1221, 1145, 1075, 1043, 927, 750 cm<sup>-1</sup>. LRMS (+ESI) *m/z*: 219 [(M+H)<sup>+</sup>, 100%]. HRMS (+ESI) Found: (M+Na)<sup>+</sup>, 241.0697. C<sub>10</sub>H<sub>10</sub>N<sub>4</sub>O<sub>2</sub> requires (M+Na)<sup>+</sup>, 241.0696. MP: 133 – 135 °C.

### 5.2.2. *N*<sup>1</sup>-(1-Methyl-1*H*-pyrazol-5-yl)benzene-1,2-diamine (**8**)

A stirred suspension of 1-methyl-*N*-(2-nitrophenyl)-1*H*-pyrazol-5-amine (**7**) (5.50 g, 25.2 mmol) and Pd/C (10% w/w, 630 mg, 0.6 mmol) in MeOH (250 mL) was stirred under an atmosphere of hydrogen (1 atm) for 18 h after which time the reaction mixture was filtered through basic alumina and Celite® and residues washed with EtOAc (150 mL). The filtrate was concentrated under reduced pressure to give the title compound (4.15 g, 88%) as a pink crystalline solid.

<sup>1</sup>H NMR (300 MHz, Chloroform-*d*):  $\delta$  7.40 (d, *J* = 2.0 Hz, 1H), 6.86 (t, *J* = 7.5 Hz, 1H), 6.75 (d, *J* = 7.5 Hz, 1H), 6.63 (d, *J* = 7.8 Hz, 1H), 5.80 (d, *J* = 2.0 Hz, 1H), 5.33 – 5.28 (m, 1H), 4.00 – 3.36 (m, 3H). <sup>13</sup>C NMR (75 MHz, Chloroform-*d*):  $\delta$  142.1, 138.5, 136.6, 132.2, 122.9, 120.3, 117.9, 117.3, 96.7, 34.9. IR (diamond cell, neat)  $\nu_{\max}$ : 3326, 3213, 1554, 1498, 1430, 1384, 1281, 993, 927, 733, 627 cm<sup>-1</sup>. LRMS (+ESI) *m/z*: 211 [(M+Na)<sup>+</sup>, 80%], 189 [(M+H)<sup>+</sup>, 100%]. HRMS (+ESI) Found: (M+Na)<sup>+</sup>, 211.0955. C<sub>10</sub>H<sub>12</sub>N<sub>4</sub> requires (M+Na)<sup>+</sup>, 211.0954. MP: 133 – 135 °C.

### 5.2.3. 1-Methyl-1,4,5,10-tetrahydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepine (**9**)

A solution of aniline **99** (1.40 g, 7.84 mmol) and formaldehyde (0.334 mL of a 37% aq. solution, 8.6 mmol) in CH<sub>3</sub>CN (80 mL) was treated with acetic acid (0.09 mL, 20 mol%) and stirred at room temperature for 18 h. The resultant solution was concentrated under reduced pressure and taken up in NaHCO<sub>3</sub> (50 mL of a sat. aq. solution) and extracted with EtOAc (3 x 50 mL). The combined organics were washed with brine (50 mL), dried (MgSO<sub>4</sub>), filtered and solvent evaporated under reduced pressure. The crude oil was purified by flash column chromatography (silica, 1:9 v/v MeOH:CH<sub>2</sub>Cl<sub>2</sub>) and concentration of the relevant fractions ( $R_f$  = 0.43 in 1:9 v/v MeOH:CH<sub>2</sub>Cl<sub>2</sub>) yielded the title compound (1.24 g, 79%) as an off-white powder.

**<sup>1</sup>H NMR** (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  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). **<sup>13</sup>C NMR** (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  140.9, 139.5, 135.0, 133.0, 121.7, 120.8, 120.2, 118.9, 101.5, 43.4, 35.0. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3293, 1560, 1505, 1393, 1318, 761 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 223 [(M+Na)<sup>+</sup>, 40%],  $m/z$ : 201 [(M+H)<sup>+</sup>, 40%]. **HRMS** (+ESI) Found: (M+Na)<sup>+</sup>, 223.0958. C<sub>11</sub>H<sub>12</sub>N<sub>4</sub> requires (M+Na)<sup>+</sup>, 223.0954. **MP**: 205 – 207 °C.

#### 5.2.4. General procedure for the Fischer esterification of phenyl alkanolic acids[14]:

A stirred solution of the appropriate bromophenyl alkanolic acid (1 eq) in MeOH (25 mL) was treated with H<sub>2</sub>SO<sub>4</sub> (0.1 eq of a conc. aq. solution) and heated at reflux for 18 h. The resultant solution was cooled to room temperature and concentrated under reduced pressure. The resultant oil was diluted with NaHCO<sub>3</sub> (50 mL of a sat. aq. solution) and subsequently extracted with diethyl ether (3 x 25 mL). The combined organics were washed with brine (1 x 100 mL), dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure to give the desired methyl ester:

#### 5.2.5. Methyl 2-(2-bromophenyl)acetate (10)

Subjecting 2-bromophenyl acetic acid (1.05 g, 5 mmol) to the above general procedure gave the title compound (1.06 g, 93%) as a colourless oil.

**<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*):  $\delta$  7.58 (d,  $J$  = 8.0 Hz, 1H), 7.32 – 7.27 (m, 2H), 7.19 – 7.11 (m, 1H), 3.80 (s, 2H), 3.72 (s, 3H). **<sup>13</sup>C NMR** (101 MHz, Chloroform-*d*):  $\delta$  171.1, 134.3, 133.0, 131.6, 129.0, 127.7, 125.2, 52.3, 41.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 1734, 1471, 1435, 1340, 1219, 1158, 1026, 733, 660, 440 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 251/253 [(M+Na)<sup>+</sup>, 98/100%].

#### 5.2.6. Methyl 2-(3-bromophenyl)acetate (11)

Subjecting 3-bromophenyl acetic acid (1.05 g, 5 mmol) to the above general procedure gave the title compound (1.11 g, 97%) as a yellow oil.

**<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*):  $\delta$  7.44 (t,  $J$  = 1.6 Hz, 1H), 7.41 (dt,  $J$  = 6.7, 2.1 Hz, 1H), 7.25 – 7.15 (m, 2H), 3.70 (s, 3H), 3.59 (s, 2H). **<sup>13</sup>C NMR** (101 MHz, Chloroform-*d*):  $\delta$  171.4, 136.2, 132.5, 130.4, 130.2, 128.1, 122.7, 52.3, 40.8. **IR** (diamond cell, neat)  $\nu_{\max}$ : 1734, 1471, 1435, 1340, 1219, 1158, 1026, 733, 660, 440 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 251/253 [(M+Na)<sup>+</sup>, 94/100%].

#### 5.2.7. Methyl 2-(4-bromophenyl)acetate (12)

Subjecting 4-bromophenyl acetic acid (1.05 g, 5 mmol) to the above general procedure gave the title compound (1.07 g, 94%) as a colourless oil.

**<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*):  $\delta$  7.45 (d,  $J$  = 8.4 Hz, 2H), 7.15 (d,  $J$  = 8.4 Hz, 2H), 3.69 (s, 3H), 3.58 (s, 2H). **<sup>13</sup>C NMR** (101 MHz, Chloroform-*d*):  $\delta$  171.6, 133.0, 131.8, 131.1, 121.3, 52.3, 40.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 1734, 1471, 1435, 1340, 1219, 1158, 1026, 733, 660, 440 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 251/253 [(M+Na)<sup>+</sup>, 95/100%].



**5.2.8. Methyl 3-(2-bromophenyl)propanoate (13)**

Subjecting 2-bromophenyl propionic acid (0.560 g, 2.44 mmol) to the above general procedure gave the title compound (0.583 g, 97%) as a pale yellow oil.

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.52 (dd,  $J$  = 7.9, 1.1 Hz, 1H), 7.26 – 7.19 (m, 2H), 7.06 (ddd,  $J$  = 8.1, 6.7, 2.4 Hz, 1H), 3.67 (s, 3H), 3.06 (dd,  $J$  = 8.3, 7.4 Hz, 2H), 2.65 (dd,  $J$  = 8.4, 7.4 Hz, 2H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  173.2, 139.8, 133.0, 130.5, 128.2, 127.7, 124.4, 51.8, 34.0, 31.5. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2950, 1734, 1471, 1436, 1364, 1294, 1257, 1195, 1025, 781, 750, 656 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 265/267 [(M+Na)<sup>+</sup>, 95/100%].

**5.2.9. Methyl 3-(3-bromophenyl)propanoate (14)**

Subjecting 3-bromophenyl propionic acid (1.15 g, 5 mmol) to the above general procedure gave the title compound (1.15 g, 95%) as a pale yellow oil.

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.34 (d,  $J$  = 1.9 Hz, 1H), 7.31 (dt,  $J$  = 7.3, 2.0 Hz, 1H), 7.17 – 7.08 (m, 2H), 3.65 (s, 3H), 2.90 (t,  $J$  = 7.3 Hz, 2H), 2.60 (dd,  $J$  = 8.2, 7.3 Hz, 2H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  172.9, 142.9, 131.4, 130.1, 129.4, 127.0, 122.5, 51.7, 35.3, 30.5. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2950, 1732, 1596, 1567, 1475, 1436, 1363, 1297, 1196, 1165, 1071, 1028, 809, 780, 731, 687, 665, 428 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 265/267 [(M+Na)<sup>+</sup>, 95/100%].

**5.2.10. Methyl 3-(4-bromophenyl)propanoate (15)**

Subjecting 4-bromophenyl propionic acid (1.15 g, 5 mmol) to the above general procedure gave the title compound (1.10 g, 90%) as a pale yellow oil.

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.39 (d,  $J$  = 8.4 Hz, 2H), 7.06 (d,  $J$  = 8.5 Hz, 2H), 3.65 (s, 3H), 2.89 (t,  $J$  = 7.7 Hz, 2H), 2.60 (t,  $J$  = 7.7 Hz, 2H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  173.0, 139.5, 131.6, 130.1, 120.1, 51.7, 35.4, 30.3. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2951, 1733, 1488, 1435, 295, 1257, 1195, 1072, 1010, 908, 694, 614 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 265/267 [(M+Na)<sup>+</sup>, 95/100%].

**5.2.11. General procedure for the Buchwald-Hartwig amination of bromophenyl alkanates:**

A stirred solution of RuPhos® (10 mol%, w/w) and Pd<sub>2</sub>(dba)<sub>3</sub> (5 mol%, w/w) in dry, degassed PhCH<sub>3</sub> (25 mL) was treated with Boc-piperazine (2 eq), Cs<sub>2</sub>CO<sub>3</sub> (1.5 eq), the required bromophenyl alkanate (1 eq) and heated at reflux for 18 h. The reaction volume was filtered through a Celite® pad and the solids retained were washed with EtOAc (3 x 30 mL). The combined organics were concentrated under reduced pressure and the resultant crude oil was purified by flash chromatography (silica,  $R_f$  = 0.19-0.22 in a 1:5 v/v EtOAc/hexanes solution).

**5.2.12. *tert*-Butyl 4-(2-(2-methoxy-2-oxoethyl)phenyl)piperazine-1-carboxylate (16)**

Subjecting methyl 2-(2-bromophenyl)acetate (920 mg, 4 mmol) to the above general procedure produced the title compound (740 mg, 55%) as a waxy solid ( $R_f$  = 0.19 in a 1:5 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.29 – 7.24 (m, 2H), 7.14 – 7.10 (m, 2H), 3.71 (s, 2H), 3.69 (s, 3H), 3.53 (br s, 4H), 2.80 (br s, 4H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  172.8, 155.0, 151.7, 131.2, 131.1, 128.5, 125.1, 121.4, 79.9, 52.6, 52.0, 44.0 (br), 37.4, 28.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 1735, 1689, 1418, 1365, 1247, 1160, 1001, 913, 864, 767, 728, 647 cm<sup>-1</sup>. **LRMS** (+ESI)  $m/z$ : 357 [(M+Na)<sup>+</sup>, 100%], 691 [(2M+Na)<sup>+</sup>, 40%].

**5.2.13. *tert*-Butyl 4-(3-(2-methoxy-2-oxoethyl)phenyl)piperazine-1-carboxylate (17)**

Subjecting methyl 2-(3-bromophenyl)acetate (920 mg, 4 mmol) to the above general procedure produced the title compound (910 mg, 68%) as a waxy solid ( $R_f = 0.21$  in a 1:5 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.21 (td,  $J = 8.0, 2.7$  Hz, 1H), 6.91 – 6.73 (m, 3H), 3.74 – 3.62 (m, 3H), 3.62 – 3.45 (m, 6H), 3.24 – 3.03 (m, 4H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  172.1, 154.8, 151.6, 135.0, 129.5, 121.2, 117.6, 115.3, 80.0, 52.1, 49.4, 43.5 (br), 41.6, 28.5. **IR** (diamond cell, neat)  $\nu_{\max}$ : 1736, 1690, 1601, 1419, 1365, 1239, 1157, 997, 970, 912, 868, 768, 729, 646  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 357 [(M+Na)<sup>+</sup>, 100%], 691 [(2M+Na)<sup>+</sup>, 5%].

#### 5.2.14. *tert*-Butyl 4-(4-(2-methoxy-2-oxoethyl)phenyl)piperazine-1-carboxylate (18)

Subjecting methyl 2-(4-bromophenyl)acetate (920 mg, 4 mmol) to the above general procedure produced the title compound (897 mg, 67%) as a waxy solid ( $R_f = 0.22$  in a 1:5 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.18 (dd,  $J = 8.5, 2.6$  Hz, 2H), 6.88 (dd,  $J = 8.5, 2.6$  Hz, 2H), 3.68 (d,  $J = 2.4$  Hz, 3H), 3.60 – 3.50 (m, 6H), 3.11 (d,  $J = 5.5$  Hz, 4H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  172.5, 154.9, 150.4, 130.1, 125.7, 116.8, 80.1, 52.1, 49.5, 44.0 (br), 40.4, 28.5. **IR** (diamond cell, neat)  $\nu_{\max}$ : 1736, 1690, 1516, 1419, 1365, 1227, 1158, 1000, 915, 810, 768, 574  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 357 [(M+Na)<sup>+</sup>, 100%].

#### 5.2.15. *tert*-Butyl 4-(2-(3-methoxy-3-oxopropyl)phenyl)piperazine-1-carboxylate (19)

Subjecting methyl 3-(2-bromophenyl)propanoate (500 mg, 2 mmol) to the above general procedure produced the title compound (500 mg, 70%) as a yellow oil ( $R_f = 0.14$  in a 1:9 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.24 – 7.15 (m, 2H), 7.11 – 7.04 (m, 2H), 3.68 (s, 3H), 3.57 (br s, 4H), 3.08 – 2.97 (m, 2H), 2.83 (br s, 4H), 2.72 – 2.61 (m, 2H), 1.49 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  173.9, 155.0, 151.5, 136.2, 129.8, 127.5, 124.8, 120.9, 79.9, 52.9, 51.7, 44.9 (broad), 34.9, 28.6, 26.8. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2975, 2950, 1736, 1691, 1491, 1451, 1418, 1365, 1321, 1246, 1222, 1163, 1120, 1001, 922, 863, 765, 731  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 371 [(M+Na)<sup>+</sup>, 100%], 719 [(2M+Na)<sup>+</sup>, 15%].

#### 5.2.16. *tert*-Butyl 4-(3-(3-methoxy-3-oxopropyl)phenyl)piperazine-1-carboxylate (20)

Subjecting methyl 3-(3-bromophenyl)propanoate (972 mg, 4 mmol) to the above general procedure produced the title compound (1.01 g, 72%) as a yellow oil ( $R_f = 0.12$  in a 1:9 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.18 (dd,  $J = 9.0, 7.4$  Hz, 1H), 6.88 – 6.74 (m, 2H), 6.74 – 6.68 (m, 1H), 3.67 (s, 3H), 3.57 (t,  $J = 5.2$  Hz, 4H), 3.12 (t,  $J = 5.1$  Hz, 4H), 2.91 (t,  $J = 7.9$  Hz, 2H), 2.62 (dd,  $J = 8.5, 7.2$  Hz, 2H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  173.4, 154.8, 151.6, 141.7, 129.4, 120.3, 116.8, 114.6, 80.0, 51.7, 49.5, 43.6 (broad), 35.9, 31.4, 28.5. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2976, 1735, 1685, 1601, 1478, 1285, 1239, 1163, 1125, 1086, 907, 865, 727, 698, 646  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 371 [(M+Na)<sup>+</sup>, 100%], 719 [(2M+Na)<sup>+</sup>, 5%].

#### 5.2.17. *tert*-Butyl 4-(4-(3-methoxy-3-oxopropyl)phenyl)piperazine-1-carboxylate (21)

Subjecting methyl 3-(4-bromophenyl)propanoate (972 mg, 4 mmol) to the above general procedure produced the title compound (1.32 g, 95%) as a white solid ( $R_f = 0.21$  in a 1:5 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*):  $\delta$  7.09 (d,  $J = 8.6$  Hz, 2H), 6.85 (d,  $J = 8.6$  Hz, 2H), 3.65 (s, 3H), 3.56 (t,  $J = 5.2$  Hz, 4H), 3.08 (t,  $J = 5.2$  Hz, 4H), 2.87 (t,  $J = 7.8$  Hz, 2H), 2.58 (dd,  $J = 8.5, 7.2$  Hz, 2H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*):  $\delta$  173.5, 154.8, 149.8, 132.4, 129.1, 116.9, 79.9, 51.6, 49.7, 43.6 (broad), 36.0, 30.2, 28.5. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2813, 1727, 1677, 1515,



1454, 1414, 1366, 1333, 1305, 1282, 1191, 1174, 1078, 1044, 999, 981, 931, 913, 846, 827, 692, 542 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 371 [(M+Na)<sup>+</sup>, 100%]. **MP**: 88 – 91 °C.

### 5.2.18. General procedure for the saponification of the methyl esters (16 – 21).

A stirred solution of the appropriate alkanoate (1 eq) in MeOH:H<sub>2</sub>O (10 mL of a 9:1 v/v solution) was treated with LiOH (3 eq) and heated at reflux for 1 h. The MeOH was removed under reduced pressure and the resultant aqueous solution washed with ether (3 x 50 mL). The aqueous solution was acidified (pH 6) with HCl (4M aq. solution) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 25 mL), dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. The resultant solids were purified by column chromatography (silica, *R<sub>f</sub>* = 0.19-0.22 in a 1:1 v/v EtOAc/hexanes solution).

### 5.2.19. 2-(2-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)phenyl)acetic acid (22)

Subjecting ester **16** (800 mg, 2.4 mmol) to the above general procedure produced the title compound (704 mg, 91%) as white crystalline needles (*R<sub>f</sub>* = 0.21 in a 1:1 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*): δ 7.45 – 7.07 (m, 4H), 3.75 (s, 2H), 3.63 (br s, 4H), 2.96 (t, *J* = 5.2 Hz, 4H), 1.49 (s, 9H), OH signal not observed. **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*): δ 173.5, 154.7, 149.1, 132.1, 130.0, 129.2, 126.7, 121.4, 80.5, 52.6, 43.5 (broad), 40.4, 28.6. **IR** (diamond cell, neat) *v*<sub>max</sub>: 3200, 1733, 1661, 1518, 1438, 1371, 1317, 1255, 1133, 1043, 1002, 910, 804, 761, 651, 604, 529 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 343 [(M+Na)<sup>+</sup>, 100%]. **MP**: 141 – 142 °C.

### 5.2.20. 2-(3-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)phenyl)acetic acid (23)

Subjecting ester **17** (800 mg, 2.4 mmol) to the above general procedure produced the title compound (710 mg, 92%) as a white powder (*R<sub>f</sub>* = 0.19 in a 1:1 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*): δ 7.23 (dd, *J* = 15.5, 7.5 Hz, 1H), 6.87 – 6.78 (m, 3H), 3.62 – 3.54 (m, 6H), 3.13 (t, *J* = 4.7 Hz, 4H), 1.48 (d, *J* = 2.1 Hz, 9H), OH signal not observed. **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*): δ 176.9, 154.9, 151.6, 134.5, 129.6, 121.4, 117.8, 115.6, 80.0, 49.4, 43.7 (broad), 41.4, 28.6. **IR** (diamond cell, neat) *v*<sub>max</sub>: 3190, 1728, 1664, 1430, 1367, 1319, 1266, 1155, 1131, 1031, 1004, 935, 834, 770, 752, 678, 600, 535, 459 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 343 [(M+Na)<sup>+</sup>, 100%]. **MP**: 136 – 138 °C.

### 5.2.21. 2-(4-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)phenyl)acetic acid (24)

Subjecting ester **18** (800 mg, 2.4 mmol) to the above general procedure produced the title compound (710 mg, 92%) as yellow cubes (*R<sub>f</sub>* = 0.21 in a 1:1 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*) δ 7.20 – 7.13 (m, 2H), 6.98 – 6.71 (m, 2H), 3.75 – 3.42 (m, 6H), 3.11 (t, *J* = 4.6 Hz, 4H), 1.48 (s, 9H). OH signal not observed. **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*): δ 177.4, 154.9, 150.6, 130.3, 125.1, 116.9, 80.1, 49.5, 44.3 (broad), 40.2, 28.6. **IR** (diamond cell, neat) *v*<sub>max</sub>: 2977, 1733, 1644, 1433, 1367, 1275, 1241, 1161, 995, 971, 845, 779, 733, 693, 971, 599, 539, 428 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 343 [(M+Na)<sup>+</sup>, 100%]. **MP**: 121 – 122 °C.

### 5.2.22. 3-(2-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)phenyl)propanoic acid (25)

Subjecting ester **19** (300 mg, 0.86 mmol) to the above general procedure produced the title compound (224 mg, 78%) as a white crystalline powder (*R<sub>f</sub>* = 0.31 in a 1:1 v/v EtOAc/hexanes solution).

**<sup>1</sup>H NMR** (400 MHz, Methanol-*d*<sub>4</sub>): δ 7.23 – 7.19 (m, 1H), 7.19 – 7.15 (m, 1H), 7.12 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.04 (td, *J* = 7.3, 1.5 Hz, 1H), 3.56 (t, *J* = 4.9 Hz, 4H), 3.00 (dd, *J* = 8.5, 7.1 Hz, 2H), 2.82 (t, *J* = 5.0 Hz, 4H), 2.62 (dd, *J* = 8.5, 7.1 Hz, 2H), 1.48 (s, 9H), OH signal not observed. **<sup>13</sup>C NMR** (101

MHz, Methanol- $d_4$ ):  $\delta$  173.5, 154.7, 149.1, 132.1, 130.0, 129.2, 126.7, 121.4, 80.5, 52.6, 43.5 (broad), 40.4, 31.6, 28.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3217, 2972, 1729, 1640, 1490, 1364, 1323, 1167, 1148, 1130, 921, 758, 575  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 357 [(M+Na) $^+$ , 100%]. **MP**: 134 – 135  $^{\circ}\text{C}$ .

### 5.2.23. 3-(3-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)phenyl)propanoic acid (26)

Subjecting ester **20** (300 mg, 0.86 mmol) to the above general produced the title compound (271 mg, 94%) as a red oil ( $R_f$  = 0.22 in a 1:1 v/v EtOAc/hexanes solution).

**$^1\text{H}$  NMR** (400 MHz, Methanol- $d_4$ ):  $\delta$  7.15 (t,  $J$  = 7.9 Hz, 1H), 6.85 (t,  $J$  = 2.0 Hz, 1H), 6.80 (ddd,  $J$  = 8.2, 2.5, 0.9 Hz, 1H), 6.74 (dt,  $J$  = 7.7, 1.1 Hz, 1H), 3.55 (t,  $J$  = 5.1 Hz, 4H), 3.09 (t,  $J$  = 5.1 Hz, 4H), 2.86 (t,  $J$  = 7.7 Hz, 2H), 2.57 (t,  $J$  = 7.7 Hz, 2H), 1.48 (s, 9H), OH signal not observed.  **$^{13}\text{C}$  NMR** (101 MHz, Methanol- $d_4$ ):  $\delta$  176.7, 156.4, 152.9, 143.1, 130.2, 121.5, 118.1, 115.9, 81.3, 50.8, 44.9 (broad), 36.8, 32.3, 28.7. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2976, 1733, 1691, 1601, 1492, 1423, 1366, 1239, 1160, 997, 957, 836, 774, 734, 696  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 357 [(M+Na) $^+$ , 100%].

### 5.2.24. 3-(4-(4-(*tert*-Butoxycarbonyl)piperazin-1-yl)phenyl)propanoic acid (27)

Subjecting ester **21** (300 mg, 0.86 mmol) to the above general produced the title compound (224 mg, 78%) as an off-white powder ( $R_f$  = 0.22 in a 1:1 v/v EtOAc/hexanes solution).

**$^1\text{H}$  NMR** (400 MHz, Methanol- $d_4$ ):  $\delta$  8.67 (d,  $J$  = 8.6 Hz, 2H), 8.46 (d,  $J$  = 8.6 Hz, 2H), 5.11 (t,  $J$  = 5.1 Hz, 4H), 4.74 – 4.52 (m, 4H), 4.39 (t,  $J$  = 7.6 Hz, 2H), 4.10 (t,  $J$  = 7.6 Hz, 2H), 1.04 (s, 9H), OH signal not observed.  **$^{13}\text{C}$  NMR** (101 MHz, Methanol- $d_4$ ):  $\delta$  176.8, 156.4, 151.2, 134.3, 130.0, 118.3, 81.3, 51.1, 45.0 (broad), 36.9, 31.2, 28.7. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2970, 1692, 1616, 1517, 1478, 1419, 1390, 1364, 1287, 1159, 1116, 1085, 866, 819, 768, 596, 522  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 357 [(M+Na) $^+$ , 100%]. **MP**: 146 – 148  $^{\circ}\text{C}$ .

### 5.2.25. General procedure for the amidation of diazepine 9.

An ice cold magnetically stirred solution of the required phenylalkanoic acid (1 eq), amine **9** (1 eq) and  $\text{Pr}_2\text{NEt}$  (2 eq) in  $\text{CH}_2\text{Cl}_2$  (5 mL) was treated with PyBOP $^{\circ}$  (1 eq), allowed to warm to room temperature and stirring continued for 12 h. The solution was diluted with  $\text{CH}_2\text{Cl}_2$  (50 mL) and water (50 mL) and the separated organic phase was subsequently washed with  $\text{NaHCO}_3$  (25 mL of a sat. aq. solution) and brine (100 mL) before being dried ( $\text{MgSO}_4$ ), filtered, concentrated and purified *via* flash column chromatography (silica,  $R_f$  = 0.24–0.36 in EtOAc).

### 5.2.26. *tert*-Butyl 4-(2-(2-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)-2-oxoethyl)phenyl)piperazine-1-carboxylate (28)

Subjecting **22** (200 mg, 0.62 mmol) to the above general procedure in  $\text{CH}_2\text{Cl}_2$  (5 mL) produced the title compound (220 mg, 71%) as a white powder ( $R_f$  = 0.36 in EtOAc).

**$^1\text{H}$  NMR** (500 MHz, Chloroform- $d$ ):  $\delta$  7.22 – 7.10 (m, 3H), 7.08 (dd,  $J$  = 7.9, 1.5 Hz, 1H), 7.03 (dd,  $J$  = 7.7, 1.6 Hz, 1H), 6.98 – 6.82 (m, 4H), 5.90 (br s, 1H), 5.63 (d,  $J$  = 14.5 Hz, 1H), 3.80 (d,  $J$  = 14.5 Hz, 1H), 3.74 – 3.58 (m, 4H), 3.48 (d,  $J$  = 15.5 Hz, 1H), 3.31 (br s, 4H), 2.48 – 2.33 (m, 4H), 1.47 (s, 9H).  **$^{13}\text{C}$  NMR** (126 MHz, Chloroform- $d$ ):  $\delta$  171.2, 155.0, 151.3, 138.9, 138.7, 136.5, 131.6, 131.0, 130.5, 130.5, 129.0, 127.7, 124.2, 122.4, 120.1, 119.7, 101.8, 80.0, 52.1, 44.0 (broad), 43.5, 37.2, 34.7, 28.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3220, 1633, 1560, 1401, 1249, 1174, 1131, 846, 757, 556, 454  $\text{cm}^{-1}$ . **LRMS** (+ESI)  $m/z$ : 525 [(M+Na) $^+$ , 100%]. **MP**: 161 – 163  $^{\circ}\text{C}$ .

### 5.2.27. *tert*-Butyl 4-(3-(2-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)-2-oxoethyl)phenyl)piperazine-1-carboxylate (29)

Subjecting **23** (400 mg, 1.24 mmol) to the above general procedure in  $\text{CH}_2\text{Cl}_2$  (10 mL) produced the title compound (415 mg, 67%) as an off-white solid ( $R_f$  = 0.24 in EtOAc).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*): 7.23 (td, *J* = 8.0, 1.6 Hz, 1H), 7.19 (s, 1H), 7.14 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.05 – 6.95 (m, 2H), 6.82 (dd, *J* = 8.1, 1.4 Hz, 1H), 6.72 (dd, *J* = 7.9, 2.3 Hz, 1H), 6.47 (t, *J* = 2.0 Hz, 1H), 6.30 (d, *J* = 8.0 Hz, 1H), 5.55 (br s, 1H), 5.60 (d, *J* = 14.5 Hz, 1H), 3.81 (d, *J* = 14.5 Hz, 1H), 3.62 (s, 3H), 3.52 (br s, 4H), 3.42 (d, *J* = 5.5 Hz, 2H), 2.97 (br s, 4H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*): δ 170.7, 154.9, 151.2, 139.0, 138.8, 136.6, 135.9, 131.9, 130.8, 129.3, 128.9, 122.5, 121.0, 119.7, 116.9, 115.0, 101.6, 80.1, 49.4, 44.2 (broad), 43.6, 41.8, 34.7, 28.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3201, 1697, 1627, 1561, 1507, 1408, 1234, 1164, 1122, 1053, 973, 833, 765, 740, 704, 629, 534, 457 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 525 [(M+Na)<sup>+</sup>, 100%]. **MP**: 158 – 160 °C.

**5.2.28. *tert*-Butyl 4-(4-(2-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)-2-oxoethyl)phenyl)piperazine-1-carboxylate (30)**

Subjecting **24** (400 mg, 1.24 mmol) to the above general procedure in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) produced the title compound (520 mg, 81%) as an off-white solid (*R*<sub>f</sub> = 0.22 in EtOAc).

**<sup>1</sup>H NMR** (500 MHz, Chloroform-*d*): 7.28 – 7.24 (m, 1H), 7.17 (s, 1H), 7.14 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.02 (td, *J* = 7.5, 1.4 Hz, 1H), 6.92 (dd, *J* = 8.1, 1.4 Hz, 1H), 6.82 (d, *J* = 8.7 Hz, 1H), 6.73 (d, *J* = 8.7 Hz, 2H), 5.78 (br s, 1H), 5.59 (d, *J* = 14.6 Hz, 1H), 3.82 (d, *J* = 14.6 Hz, 1H), 3.64 (s, 3H), 3.55 (t, *J* = 5.2 Hz, 4H), 3.35 (s, 2H), 3.05 (t, *J* = 5.2 Hz, 4H), 1.47 (s, 9H). **<sup>13</sup>C NMR** (126 MHz, Chloroform-*d*): δ 171.0, 154.9, 150.1, 139.1, 138.9, 136.5, 132.0, 130.7, 130.0, 129.3, 126.8, 122.6, 119.9, 116.5, 101.6, 80.1, 49.7, 43.7, 43.6, 40.3, 34.7, 28.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3202, 1639, 1555, 1502, 1390, 1229, 1161, 839, 760, 556 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 525 [(M+Na)<sup>+</sup>, 100%]. **MP**: 161 – 163 °C.

**5.2.29. *tert*-Butyl 4-(2-(3-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)-3-oxopropyl)phenyl)piperazine-1-carboxylate (31)**

Subjecting **25** (200 mg, 0.6 mmol) to the above general procedure in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) produced the title compound (220 mg, 71%) as an off-white solid (*R*<sub>f</sub> = 0.21 in EtOAc).

**<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*) δ 7.23 – 7.16 (m, 2H), 7.11 (ddd, *J* = 8.0, 7.1, 1.7 Hz, 1H), 7.04 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.98 (dd, *J* = 8.2, 1.3 Hz, 1H), 6.97 – 6.84 (m, 4H), 6.32 (br s, 1H), 5.64 (d, *J* = 14.5 Hz, 1H), 3.80 (d, *J* = 14.5 Hz, 1H), 3.68 (s, 3H), 3.40 (t, *J* = 5.0 Hz, 4H), 2.91 – 2.75 (m, 2H), 2.64 (t, *J* = 4.8 Hz, 4H), 2.51 – 2.23 (m, 2H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (101 MHz, Chloroform-*d*): δ 172.1, 155.2, 151.4, 139.0(3), 139.9(9), 136.9, 136.5, 131.9, 130.4, 130.3, 129.1, 127.1, 124.5, 122.5, 120.6, 119.9, 101.7, 79.9, 52.6, 44.3 (broad), 43.2, 34.9, 34.5, 28.6, 27.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2817, 1635, 1557, 1503, 1392, 1365, 1277, 1246, 1163, 1123, 1087, 1035, 842, 760, 533 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 539 [(M+Na)<sup>+</sup>, 100%]. **MP**: 118 – 120 °C.

**5.2.30. *tert*-Butyl 4-(3-(3-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)-3-oxopropyl)phenyl)piperazine-1-carboxylate (32)**

Subjecting **26** (200 mg, 0.6 mmol) to the above general procedure in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) produced the title compound (241 mg, 78%) as an off-white solid (*R*<sub>f</sub> = 0.24 in EtOAc).

**<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*) δ 7.27 – 7.21 (m, 1H), 7.19 (s, 1H), 7.11 – 7.05 (m, 1H), 7.05 – 7.02 (m, 1H), 7.02 – 6.93 (m, 2H), 6.73 – 6.66 (m, 1H), 6.55 (t, *J* = 1.9 Hz, 1H), 6.50 (d, *J* = 7.5 Hz, 1H), 5.83 (br s, 1H), 5.65 (d, *J* = 14.5 Hz, 1H), 3.81 (d, *J* = 14.5 Hz, 1H), 3.70 (s, 3H), 3.53 (t, *J* = 5.2 Hz, 4H), 3.01 (t, *J* = 5.6 Hz, 4H), 2.80 – 2.63 (m, 2H), 2.41 – 2.22 (m, 2H), 1.48 (s, 9H). **<sup>13</sup>C NMR** (101 MHz, Chloroform-*d*): δ 171.8, 154.9, 151.4, 142.5, 139.1, 138.9, 136.5, 132.2, 130.6, 129.2, 129.1, 122.9, 120.4, 120.0, 117.0, 114.3, 101.6, 80.1, 49.5, 43.6 (broad), 43.1, 36.1, 34.8, 32.1, 28.6. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2817, 1637, 1600, 1557, 1502, 1391, 1365, 1285, 1240, 1161, 1126, 997, 840, 162, 696, 557 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 539 [(M+Na)<sup>+</sup>, 100%]. **MP**: 101 – 103 °C.

### 5.2.31. *tert*-Butyl 4-(4-(3-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)-3-oxopropyl)phenyl)piperazine-1-carboxylate (33)

Subjecting **27** (200 mg, 0.6 mmol) to the above general procedure in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) produced the title compound (241 mg, 78%) as an off-white solid (*R<sub>f</sub>* = 0.24 in EtOAc).

<sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 7.25 – 7.20 (m, 1H), 7.18 (s, 1H), 7.06 – 6.94 (m, 3H), 6.89 (d, *J* = 8.6 Hz, 2H), 6.74 (d, *J* = 8.6 Hz, 2H), 6.17 (s, 1H), 5.62 (d, *J* = 14.6 Hz, 1H), 3.81 (d, *J* = 14.6 Hz, 1H), 3.69 (s, 3H), 3.57 – 3.47 (m, 4H), 3.02 (t, *J* = 5.2 Hz, 4H), 2.87 – 2.62 (m, 2H), 2.41 – 2.21 (m, 2H), 1.47 (s, 9H). <sup>13</sup>C NMR (101 MHz, Chloroform-*d*): δ 172.0, 154.9, 149.6, 139.2, 139.1, 136.4, 133.1, 131.9, 130.4, 129.3, 129.2, 122.8, 120.0, 116.8, 101.6, 80.0, 49.8, 43.7 (broad), 43.2, 36.1, 34.8, 30.7, 28.6. IR (diamond cell, neat) *v*<sub>max</sub>: 3200, 1636, 1611, 1558, 1504, 1391, 1365, 1284, 1249, 1230, 1161, 1127, 1000, 839, 762, 557 cm<sup>-1</sup>. LRMS (+ESI) *m/z*: 539 [(M+Na)<sup>+</sup>, 100%]. MP: 121 – 123 °C.

### 5.2.32. General procedure for the one pot carbamate de-protection/reductive amination of the protected piperazines (28 – 33).

A magnetically stirred solution of the required 1-boc-Piperazine (1 eq) in MeOH (5 mL) was treated with HCl (1.5 eq of a 4M solution in dioxane) and stirring continued at room temperature for 3 h. The resultant solution was concentrated under a stream of nitrogen, followed by reduced pressure. The resultant foams were re-dissolved in MeOH (5 mL) and the pH adjusted (pH = 6) with *N*-methyilmorpholine (4-8 drops), treated with the appropriate benzaldehyde (2 eq), MgSO<sub>4</sub> (20% w/w) and magnetically stirred at room temperature for 1 h. The resulting suspension was then treated with NaCNBH<sub>3</sub> (1.2 eq) and stirred for a further 18 h. The solvent was then removed under reduced pressure and the residue dissolved in EtOAc (20 mL) and NaHCO<sub>3</sub> (20 mL of a sat. aq. solution). The separated organic phase was washed with brine (50 mL) before being dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure to give off-white solids. The crude solids were purified by flash column chromatography (silica, EtOAc followed by 5:95 v/v MeOH(sat. with ammonia)/CH<sub>2</sub>Cl<sub>2</sub> solution) (*R<sub>f</sub>* = 0.08-0.23 in EtOAc).

### 5.2.32. 2-(2-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)ethan-1-one (3a)

Subjecting **28** (100 mg, 0.2 mmol) to the above general procedure using 3,5-dihydroxybenzaldehyde (55 mg, 0.4 mmol) produced the title compound **3a** (64 mg, 61%) as a white powder (*R<sub>f</sub>* = 0.12 in EtOAc).

<sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>): δ 7.19 (ddd, *J* = 8.6, 7.2, 1.6 Hz, 1H), 7.16 (s, 1H), 7.13 (ddd, *J* = 8.6, 7.2, 2.0 Hz, 1H), 7.10 – 7.18 (m, 2H), 6.97 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.93 (dd, *J* = 7.6, 1.4 Hz, 1H), 6.90 (dd, *J* = 8.3, 1.6 Hz, 1H), 6.86 (td, *J* = 7.4, 1.2 Hz, 1H), 6.31 (d, *J* = 2.2 Hz, 2H), 6.21 (t, *J* = 2.2 Hz, 1H), 5.51 (d, *J* = 14.5 Hz, 1H), 3.78 (d, *J* = 14.5 Hz, 1H), 3.75 – 3.62 (m, 4H), 3.46 (d, *J* = 15.6 Hz, 1H), 3.43 – 3.36 (m, 2H), 2.67 – 2.55 (m, 4H), 2.41 (br s, 4H), OH signals and NH signal not observed. <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>): δ 173.7, 159.5, 152.7, 141.6, 140.3, 140.2, 137.3, 132.2, 131.6, 131.0(4), 130.9(5), 130.2, 128.7, 124.8, 123.0, 121.0, 120.9, 109.2, 102.6(5), 102.6(4), 64.0, 54.2, 52.8, 44.8, 37.9, 35.2. IR (diamond cell, neat) *v*<sub>max</sub>: 2920, 1595, 1552, 1494, 1449, 1391, 1292, 1156, 999, 842, 757, 696, 554, 454, 428 cm<sup>-1</sup>. LRMS (+ESI) *m/z*: 525 [(M+H)<sup>+</sup>, 100%], 547 [(M+Na)<sup>+</sup>, 29%]. HRMS (+ESI) Found: (M+H)<sup>+</sup>, 525.2609. C<sub>30</sub>H<sub>32</sub>N<sub>6</sub>O<sub>3</sub> requires (M+H)<sup>+</sup>, 525.2609. MP: 192 – 194 °C. HPLC purity: 99.69%, RT: 14.44 min.

### 5.2.33. 2-(3-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)ethan-1-one (3b)

Subjecting **29** (100 mg, 0.2 mmol) to the above general procedure using 3,5-dihydroxybenzaldehyde (55.0 mg, 0.4 mmol) produced the title compound **3b** (74 mg, 71%) as a white powder (*R<sub>f</sub>* = 0.13 in EtOAc).



**<sup>1</sup>H NMR** (500 MHz, DMSO-*d*<sub>6</sub>) δ 9.13 (br s, 2H), 8.35 (br s, 1H), 7.34 – 7.24 (m, 2H), 7.21 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.07 (s, 1H), 7.04 – 6.93 (m, 2H), 6.71 (dd, *J* = 8.3, 2.4 Hz, 1H), 6.39 (t, *J* = 2.0 Hz, 1H), 6.34 (d, *J* = 7.4 Hz, 1H), 6.22 (t, *J* = 1.7 Hz, 2H), 6.11 (d, *J* = 2.0 Hz, 1H), 5.40 (d, *J* = 14.5 Hz, 1H), 3.76 (d, *J* = 14.5 Hz, 1H), 3.66 (s, 3H), 3.32 (s, 2H), 3.31 – 3.22 (m, 2H), 2.99 (t, *J* = 5.3 Hz, 4H), 2.46 (br s, 4H). **<sup>13</sup>C NMR** (126 MHz, DMSO-*d*<sub>6</sub>) δ 169.4, 158.3, 158.1, 150.8, 139.4, 139.2, 136.1, 135.7, 131.1, 130.3, 128.8, 128.5, 121.2, 119.8, 119.6, 115.7, 113.6, 106.8, 106.7, 100.6, 62.3, 52.6, 48.2, 43.0, 40.0, 35.3. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3079, 1626, 1598, 1560, 1504, 1447, 1405, 1341, 1294, 1251, 1155, 995, 757, 740, 689, 450 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 525 [(M+H)<sup>+</sup>, 95%], 547 [(M+Na)<sup>+</sup>, 100%]. **HRMS** (+ESI) Found: (M+Na)<sup>+</sup>, 547.2428. C<sub>30</sub>H<sub>32</sub>N<sub>6</sub>O<sub>3</sub> requires (M+Na)<sup>+</sup>, 547.2428. **MP**: 192 – 195 °C. **HPLC** purity: 99.36%, RT: 15.49 min.

**5.2.34. 2-(4-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)ethan-1-one (3c)**

Subjecting **30** (100 mg, 0.2 mmol) to the above general procedure using 3,5-dihydroxybenzaldehyde (55.0 mg, 0.4 mmol) produced the title compound **3c** (104 mg, quant.) as a white powder (*R<sub>f</sub>* = 0.08 in EtOAc).

**<sup>1</sup>H NMR** (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.30 (t, *J* = 7.8 Hz, 1H), 7.20 (d, *J* = 7.8 Hz, 1H), 7.18 – 7.14 (m, 2H), 7.03 (t, *J* = 7.6 Hz, 1H), 6.74 (d, *J* = 8.2 Hz, 2H), 6.67 (d, *J* = 8.2 Hz, 2H), 6.34 (d, *J* = 2.2 Hz, 2H), 6.23 (d, *J* = 2.2 Hz, 1H), 5.47 (d, *J* = 14.4 Hz, 1H), 3.82 (d, *J* = 14.4 Hz, 1H), 3.64 (s, 3H), 3.45 (s, 2H), 3.34 (dd, *J* = 3.5, 1.8 Hz, 2H), 3.13 (d, *J* = 5.1 Hz, 4H), 2.63 (t, *J* = 5.1 Hz, 4H), OH signals and NH signal not observed. **<sup>13</sup>C NMR** (126 MHz, MeOD-*d*<sub>4</sub>) δ 173.4, 159.5, 151.3, 141.5, 140.5, 140.3, 137.3, 132.3, 131.1, 130.5, 130.4, 127.1, 122.8, 121.0, 117.1, 109.1, 102.7, 102.5, 64.0, 54.1, 50.1, 45.0, 41.2, 35.1. **IR** (diamond cell, neat)  $\nu_{\max}$ : 2922, 2336, 2158, 2016, 1695, 1545, 1499, 1447, 1389, 1345, 1230, 1156, 974, 824, 756, 535, 457 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 525 [(M+H)<sup>+</sup>, 60%], 547 [(M+Na)<sup>+</sup>, 100%]. **HRMS** (+ESI) Found: (M+Na)<sup>+</sup>, 547.2429. C<sub>30</sub>H<sub>32</sub>N<sub>6</sub>O<sub>3</sub> requires (M+Na)<sup>+</sup>, 547.2428. **MP**: 182 – 184 °C. **HPLC** purity: 98.67%, RT: 15.50 min.

**5.2.35. 3-(2-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)propan-1-one (3d)**

Subjecting **31** (100 mg, 0.19 mmol) to the above general procedure using 3,5-dihydroxybenzaldehyde (27 mg, 0.2 mmol) produced the title compound **3d** (100 mg, 93%) as a white powder (*R<sub>f</sub>* = 0.09 in EtOAc).

**<sup>1</sup>H NMR** (400 MHz, MeOD-*d*<sub>4</sub>) δ 7.30 (ddd, *J* = 8.5, 7.0, 1.6 Hz, 1H), 7.23 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.17 (s, 1H), 7.16 – 7.12 (m, 1H), 7.10 – 7.04 (m, 2H), 7.02 – 6.89 (m, 1H), 6.92 – 6.83 (m, 2H), 6.39 (d, *J* = 2.2 Hz, 2H), 6.30 (s, 1H), 5.52 (d, *J* = 14.5 Hz, 1H), 3.80 (d, *J* = 14.5 Hz, 1H), 3.74 – 3.70 (m, 5H), 2.85 (t, *J* = 7.1 Hz, 2H), 2.80 (br s, 8H), 2.47 (t, *J* = 7.3 Hz, 2H), OH signals and NH signal not observed. **<sup>13</sup>C NMR** (101 MHz, MeOD-*d*<sub>4</sub>) δ 174.4, 159.9, 152.0, 141.4, 140.3, 137.7, 137.2(2), 137.2(1), 132.5, 131.1(2), 131.1(0), 130.3, 128.3, 125.7, 123.1, 121.8, 121.2, 109.6, 103.6, 102.6, 63.3, 54.3, 52.4, 44.5, 35.6, 35.2, 28.2. **IR** (diamond cell, neat)  $\nu_{\max}$ : 3301, 2931, 1624, 1599, 1559, 1491, 1396, 1351, 1293, 1250, 1224, 1200, 1017, 764, 670, 488 cm<sup>-1</sup>. **LRMS** (+ESI) *m/z*: 539 [(M+H)<sup>+</sup>, 100%], 561 [(M+Na)<sup>+</sup>, 45%]. **HRMS** (+ESI) Found: (M+Na)<sup>+</sup>, 561.2587. C<sub>31</sub>H<sub>34</sub>N<sub>6</sub>O<sub>3</sub> requires (M+Na)<sup>+</sup>, 561.2585. **MP**: 201 – 203 °C. **HPLC** purity: 97.24%, RT: 15.70 min.

**5.2.36. 3-(3-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)propan-1-one (3e)**

Subjecting **32** (100 mg, 0.19 mmol) to the above general procedure using 3,5-dihydroxybenzaldehyde (27 mg, 0.2 mmol) produced the title compound **3e** (58 mg, 54%) as a white powder (*R<sub>f</sub>* = 0.07 in EtOAc).

**<sup>1</sup>H NMR** (400 MHz, MeOD-*d*<sub>4</sub>) δ 7.32 – 7.21 (m, 2H), 7.15 (s, 1H), 7.04 – 6.99 (m, 1H), 6.99 – 6.95 (m, 2H), 6.74 (dd, *J* = 8.1, 2.4 Hz, 1H), 6.51 (t, *J* = 2.0 Hz, 1H), 6.42 (d, *J* = 7.4 Hz, 1H), 6.33 (d, *J* =

2.2 Hz, 2H), 6.21 (t,  $J$  = 2.2 Hz, 1H), 5.50 (d,  $J$  = 14.5 Hz, 1H), 3.79 (d,  $J$  = 14.5 Hz, 1H), 3.70 (s, 3H), 3.44 (s, 2H), 3.12 – 2.99 (m, 4H), 2.68 (t,  $J$  = 7.1 Hz, 2H), 2.61 (t,  $J$  = 5.0 Hz, 4H), 2.47 – 2.18 (m, 2H), 2 OH signals and 1 NH signal not observed.  $^{13}\text{C}$  NMR (101 MHz, MeOD- $d_4$ ):  $\delta$  174.1, 159.6, 152.7, 142.9, 141.5, 140.6, 140.3, 137.2, 132.7, 131.1, 130.4, 130.0, 123.3, 121.3, 121.0, 117.4, 115.4, 109.1, 102.7, 102.3, 64.0, 54.1, 50.1, 44.5, 37.1, 35.2, 32.9. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2820, 1599, 1559, 1501, 1446, 1396, 1342, 1296, 1248, 1151, 1021, 996, 833, 763, 671, 628, 452  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 539 [(M+H) $^+$ , 100%], 561 [(M+Na) $^+$ , 28%]. HRMS (+ESI) Found: (M+H) $^+$ , 539.2767.  $\text{C}_{31}\text{H}_{34}\text{N}_6\text{O}_3$  requires (M+H) $^+$ , 539.2765. MP: 214 – 216 °C. HPLC purity: 97.48%, RT: 16.02 min.

**5.2.37. 3-(4-(4-(3,5-Dihydroxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)propan-1-one (3f)**

Subjecting **33** (100 mg, 0.19 mmol) to the above general procedure using 3,5-dihydroxybenzaldehyde (27 mg, 0.2 mmol) produced the title compound **3f** (92 mg, 85%) as a white powder ( $R_f$  = 0.12 in EtOAc).

$^1\text{H}$  NMR (400 MHz, MeOD- $d_4$ ):  $\delta$  7.34 – 7.22 (m, 2H), 7.15 (s, 1H), 7.02 – 6.96 (m, 2H), 6.81 (d,  $J$  = 8.7 Hz, 2H), 6.78 – 6.70 (m, 2H), 6.31 (d,  $J$  = 2.2 Hz, 2H), 6.19 (t,  $J$  = 2.2 Hz, 1H), 5.50 (d,  $J$  = 14.5 Hz, 1H), 3.79 (d,  $J$  = 14.5 Hz, 1H), 3.70 (s, 3H), 3.40 (s, 2H), 3.09 (t,  $J$  = 5.0 Hz, 4H), 2.66 (t,  $J$  = 5.0 Hz, 2H), 2.59 (t,  $J$  = 5.0 Hz, 4H), 2.47 – 2.18 (m, 2H). OH signals and NH signal not observed.  $^{13}\text{C}$  NMR (101 MHz, MeOD- $d_4$ ):  $\delta$  174.2, 159.5, 151.0, 141.5, 140.5(2), 140.5(0), 137.3, 133.7, 132.7, 131.1, 130.4, 129.8, 123.2, 121.2, 117.5, 109.0, 102.6, 102.4, 64.1, 54.1, 50.5, 44.5, 37.0, 35.2, 31.6. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2920, 1598, 1558, 1502, 1447, 1395, 1349, 1295, 1248, 1230, 1149, 998, 829, 761, 743, 706  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 539 [(M+H) $^+$ , 33%], 561 [(M+Na) $^+$ , 100%]. HRMS (+ESI) Found: (M+H) $^+$ , 539.2767.  $\text{C}_{31}\text{H}_{34}\text{N}_6\text{O}_3$  requires (M+H) $^+$ , 539.2765. MP: 191 – 193 °C. HPLC purity: 99.32%, RT: 15.80 min.

**5.2.38. 2-(2-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)ethan-1-one (4a)**

Subjecting **28** (100 mg, 0.2 mmol) to the above general procedure using 3,5-dimethoxybenzaldehyde (47.0 mg, 0.4 mmol) produced the title compound **4a** (64.0 mg, 43%) as a white powder ( $R_f$  = 0.21 in EtOAc).

$^1\text{H}$  NMR (500 MHz, Chloroform- $d$ ):  $\delta$  7.15 (d,  $J$  = 6.4 Hz, 1H), 7.13 – 7.04 (m, 3H), 7.00 – 6.95 (m, 1H), 6.92 – 6.87 (m, 2H), 6.85 – 6.80 (m, 2H), 6.52 (d,  $J$  = 2.3 Hz, 2H), 6.38 (t,  $J$  = 2.3 Hz, 1H), 6.15 (br s, 1H), 5.61 (d,  $J$  = 14.6 Hz, 1H), 3.87 – 3.75 (m, 7H), 3.73 – 3.60 (m, 2H), 3.59 (s, 3H), 3.52 – 3.43 (m, 2H), 2.67 – 2.58 (m, 4H), 2.52 – 2.22 (m, 4H).  $^{13}\text{C}$  NMR (126 MHz, Chloroform- $d$ ):  $\delta$  171.4, 160.9, 151.2, 139.1, 138.7, 136.4, 135.3, 131.5, 130.5, 130.3, 130.1, 128.9, 127.5, 123.6, 122.2, 119.7, 107.2, 106.9, 101.6, 99.3, 66.9, 55.5, 53.3, 51.8, 43.5, 36.9, 34.8. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2810, 1640, 1595, 1556, 1502, 1451, 1391, 1292, 1202, 1151, 1063, 1011, 912, 833, 728, 454  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 553 [(M+H) $^+$ , 100%], 575 [(M+Na) $^+$ , 20%]. HRMS (+ESI) Found: (M+Na) $^+$ , 575.2743.  $\text{C}_{32}\text{H}_{36}\text{N}_6\text{O}_3$  requires (M+Na) $^+$ , 575.2741. MP: 98 – 100 °C. HPLC purity: 98.81%, RT: 17.44 min.

**5.2.39. 2-(3-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)ethan-1-one (4b)**

Subjecting **29** (100 mg, 0.2 mmol) to the above general procedure using 3,5-dimethoxybenzaldehyde (47.0 mg, 0.4 mmol) produced the title compound **4b** (81.0 mg, 73%) as a white powder ( $R_f$  = 0.20 in EtOAc).

$^1\text{H}$  NMR (500 MHz, Chloroform- $d$ ):  $\delta$  7.19 (td,  $J$  = 8.1, 1.4 Hz, 1H), 7.16 (s, 1H), 7.12 (dd,  $J$  = 7.8, 1.5 Hz, 1H), 7.03 – 6.92 (m, 2H), 6.84 (dd,  $J$  = 8.1, 1.4 Hz, 1H), 6.69 (dd,  $J$  = 8.2, 2.5 Hz, 1H), 6.53

(d,  $J = 2.3$  Hz, 2H), 6.42 (t,  $J = 2.0$  Hz, 1H), 6.37 (t,  $J = 2.3$  Hz, 1H), 6.28 (d,  $J = 7.4$  Hz, 1H), 5.98 (br s, 1H), 5.58 (d,  $J = 14.5$  Hz, 1H), 3.79 (br s, 7H), 3.58 (s, 3H), 3.49 – 3.46 (m, 2H), 3.42 (s, 2H), 3.11 – 2.98 (m, 4H), 2.54 (t,  $J = 5.0$  Hz, 4H).  $^{13}\text{C}$  NMR (126 MHz, Chloroform- $d$ ):  $\delta$  170.8, 160.8, 151.3, 140.7, 139.1, 138.9, 136.4, 135.7, 131.7, 130.6, 129.2, 128.7, 122.2, 120.2, 119.8, 116.2, 114.4, 107.0, 101.5, 99.2, 63.1, 55.4, 53.2, 49.0, 43.6, 41.8, 34.7. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2811, 1632, 1596, 1558, 1502, 1446, 1389, 1316, 1246, 1202, 1150, 1061, 944, 834, 763, 694, 441  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 553 [(M+H) $^{+}$ , 100%], 575 [(M+Na) $^{+}$ , 40%]. HRMS (+ESI) Found: (M+Na) $^{+}$ , 575.2740.  $\text{C}_{32}\text{H}_{36}\text{N}_6\text{O}_3$  requires (M+Na) $^{+}$ , 575.2741. MP: 101 – 103  $^{\circ}\text{C}$ . HPLC purity: 99.49%, RT: 17.50 min.

**5.2.40. 2-(4-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)ethan-1-one (4c)**

Subjecting **30** (100 mg, 0.2 mmol) to the above general procedure using 3,5-dimethoxybenzaldehyde (47.0 mg, 0.4 mmol) produced the title compound **4c** (71.0 mg, 64%) as a white powder ( $R_f = 0.21$  in EtOAc).

$^1\text{H}$  NMR (500 MHz, Chloroform- $d$ ):  $\delta$  7.21 (td,  $J = 7.7, 1.6$  Hz, 1H), 7.16 (s, 1H), 7.11 (dd,  $J = 7.8, 1.6$  Hz, 1H), 6.98 (td,  $J = 7.5, 1.3$  Hz, 1H), 6.91 (dd,  $J = 8.1, 1.3$  Hz, 1H), 6.77 (d,  $J = 8.3$  Hz, 2H), 6.68 (d,  $J = 8.3$  Hz, 2H), 6.52 (d,  $J = 2.4$  Hz, 2H), 6.36 (t,  $J = 2.4$  Hz, 1H), 6.30 (br s, 1H), 5.58 (d,  $J = 14.5$  Hz, 1H), 3.81 (d,  $J = 14.5$  Hz, 1H), 3.78 (s, 6H), 3.57 (s, 3H), 3.48 (s, 2H), 3.34 (s, 2H), 3.10 (t,  $J = 5.0$  Hz, 4H), 2.56 (t,  $J = 5.0$  Hz, 4H).  $^{13}\text{C}$  NMR (126 MHz, Chloroform- $d$ ):  $\delta$  171.1, 160.8, 150.1, 140.6, 139.2, 139.1, 136.4, 131.8, 130.5, 129.7, 129.2, 125.9, 122.2, 119.9, 115.8, 107.0, 101.5, 99.1, 63.1, 55.4, 53.1, 49.3, 43.6, 40.3, 34.7. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2810, 1595, 1556, 1502, 1429, 1386, 1225, 1202, 1150, 1056, 993, 918, 833, 729  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 553 [(M+H) $^{+}$ , 100%], 575 [(M+Na) $^{+}$ , 80%]. HRMS (+ESI) Found: (M+Na) $^{+}$ , 575.2740.  $\text{C}_{32}\text{H}_{36}\text{N}_6\text{O}_3$  requires (M+Na) $^{+}$ , 575.2741. MP: 102 – 104  $^{\circ}\text{C}$ . HPLC purity: 95.41%, RT: 17.55 min.

**5.2.41. 3-(2-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)propan-1-one (4d)**

Subjecting **31** (100 mg, 0.19 mmol) to the above general procedure using 3,5-dimethoxybenzaldehyde (32 mg, 0.2 mmol) produced the title compound **4d** (103 mg, 93%) as a white powder ( $R_f = 0.19$  in EtOAc).

$^1\text{H}$  NMR (400 MHz, MeOD- $d_4$ ):  $\delta$  7.27 – 7.16 (m, 2H), 7.12 (s, 1H), 7.08 (ddd,  $J = 8.5, 6.6, 2.1$  Hz, 1H), 7.04 – 6.96 (m, 2H), 6.92 (ddd,  $J = 7.9, 6.6, 1.9$  Hz, 1H), 6.86 – 6.76 (m, 2H), 6.53 (d,  $J = 2.3$  Hz, 2H), 6.39 (t,  $J = 2.3$  Hz, 1H), 5.48 (d,  $J = 14.4$  Hz, 1H), 3.76 (s, 6H), 3.73 – 3.68 (m, 1H), 3.66 (s, 3H), 3.45 (d,  $J = 2.8$  Hz, 2H), 2.92 – 2.60 (m, 6H), 2.56 – 2.29 (m, 6H), 1 NH signal not observed.  $^{13}\text{C}$  NMR (101 MHz, MeOD- $d_4$ ):  $\delta$  174.3, 162.3, 152.6, 141.4, 140.6, 140.3, 137.6, 137.2, 132.5, 131.1, 131.0, 130.2, 128.2, 125.2, 123.0, 121.7, 121.1, 108.6, 102.6, 100.3, 64.2, 55.7, 54.7, 53.4, 44.4, 35.6, 35.2, 28.4. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2934, 1634, 1594, 1557, 1503, 1448, 1429, 1392, 1347, 1316, 1293, 1248, 1221, 1202, 1149, 1061, 1010, 992, 933, 832, 759, 745, 693, 487  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 567 [(M+H) $^{+}$ , 100%], 589 [(M+Na) $^{+}$ , 10%]. HRMS (+ESI) Found: (M+H) $^{+}$ , 567.3078.  $\text{C}_{33}\text{H}_{38}\text{N}_6\text{O}_3$  requires (M+H) $^{+}$ , 567.3078. MP: 104 – 106  $^{\circ}\text{C}$ . HPLC purity: 99.61%, RT: 17.76 min.

**5.2.42. 3-(3-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)propan-1-one (4e)**

Subjecting **32** (100 mg, 0.19 mmol) to the above general procedure using 3,5-dimethoxybenzaldehyde (32 mg, 0.2 mmol) produced the title compound **4e** (88 mg, 78%) as a white powder ( $R_f = 0.20$  in EtOAc).

$^1\text{H}$  NMR (400 MHz, MeOD- $d_4$ ):  $\delta$  7.31 – 7.21 (m, 2H), 7.13 (s, 1H), 7.03 – 6.97 (m, 1H), 6.97 – 6.93 (m, 2H), 6.75 – 6.68 (m, 1H), 6.58 – 6.51 (m, 2H), 6.50 (t,  $J = 2.1$  Hz, 1H), 6.40 (d,  $J = 2.1$  Hz, 2H),

5.49 (d,  $J = 14.5$  Hz, 1H), 3.78 – 3.70 (m, 7H), 3.69 (s, 3H), 3.50 (s, 2H), 3.11 – 2.98 (m, 4H), 2.68 (ddt,  $J = 16.4, 14.5, 7.3$  Hz, 2H), 2.59 (t,  $J = 5.0$  Hz, 4H), 2.45 – 2.16 (m, 2H), NH signal not observed.  $^{13}\text{C}$  NMR (101 MHz, MeOD- $d_4$ ):  $\delta$  174.1, 162.3, 152.6, 142.9, 141.4, 140.6, 140.5, 137.2, 132.7, 131.1, 130.4, 130.0, 123.2, 121.2, 121.0, 117.3, 115.4, 108.5, 102.3, 100.3, 64.0, 55.7, 54.1, 50.1, 44.5, 37.1, 35.2, 32.9. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2935, 2813, 1635, 1595, 1557, 1501, 1445, 1429, 1394, 1348, 1315, 1293, 1245, 1148, 1057, 993, 831, 761, 694, 664  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 567 [(M+H) $^+$ , 100%], 589 [(M+Na) $^+$ , 12%]. HRMS (+ESI) Found: (M+H) $^+$ , 567.3078.  $\text{C}_{33}\text{H}_{38}\text{N}_6\text{O}_3$  requires (M+H) $^+$ , 567.3078. MP: 100 – 102 °C. HPLC purity: 96.14%, RT: 18.00 min.

#### 5.2.43. 3-(4-(4-(3,5-Dimethoxybenzyl)piperazin-1-yl)phenyl)-1-(1-methyl-4,10-dihydrobenzo[*b*]pyrazolo[3,4-*e*][1,4]diazepin-5(1*H*)-yl)propan-1-one (4f)

Subjecting **33** (100 mg, 0.19 mmol) to the above general procedure using 3,5-dimethoxybenzaldehyde (32 mg, 0.2 mmol) produced the title compound **4f** (106 mg, 94%) as a white powder ( $R_f = 0.21$  in EtOAc).

$^1\text{H}$  NMR (400 MHz, MeOD- $d_4$ ):  $\delta$  7.35 – 7.20 (m, 2H), 7.14 (s, 1H), 7.00 – 6.95 (m, 2H), 6.80 (d,  $J = 8.7$  Hz, 2H), 6.78 – 6.65 (m, 2H), 6.53 (d,  $J = 2.3$  Hz, 2H), 6.39 (t,  $J = 2.3$  Hz, 1H), 5.49 (d,  $J = 14.5$  Hz, 1H), 3.76 (s, 7H), 3.70 (s, 3H), 3.50 (s, 2H), 3.09 (t,  $J = 5.1$  Hz, 4H), 2.65 (td,  $J = 7.4, 5.0$  Hz, 2H), 2.60 (t,  $J = 5.0$  Hz, 4H), 2.44 – 2.15 (m, 2H), NH signal not observed.  $^{13}\text{C}$  NMR (101 MHz, MeOD- $d_4$ ):  $\delta$  174.2, 162.3, 150.9, 141.5, 140.5, 137.3, 133.7, 132.7, 131.1, 130.4, 130.0, 129.9, 123.2, 121.2, 117.5, 108.5, 102.4, 100.3, 64.1, 55.7, 54.1, 50.4, 44.5, 37.0, 35.2, 31.6. IR (diamond cell, neat)  $\nu_{\text{max}}$ : 2933, 2814, 1635, 1307, 1557, 1513, 1502, 1453, 1430, 1393, 1350, 1316, 1295, 1241, 1226, 1202, 1149, 1056, 993, 919, 830, 760, 744, 695, 538  $\text{cm}^{-1}$ . LRMS (+ESI)  $m/z$ : 567 [(M+H) $^+$ , 40%], 589 [(M+Na) $^+$ , 100%]. HRMS (+ESI) Found: (M+Na) $^+$ , 589.2898.  $\text{C}_{33}\text{H}_{38}\text{N}_6\text{O}_3$  requires (M+Na) $^+$ , 589.2898. MP: 111 – 113 °C. HPLC purity: 95.56%, RT: 17.82 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  $V_{1a}$ R. pOG44 plasmids (Life Technologies) and pcDNA5/FRT plasmids containing human OT or  $V_{1a}$ R 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  $\mu\text{g}/\text{mL}$  ampicillin (Sigma-Aldrich, St. Louis, MO, USA). Ampicillin-resistant colonies were isolated and inoculated into 5 mL of LB broth containing 50  $\mu\text{g}/\text{mL}$  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  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich), penicillin-streptomycin (100U; Sigma-Aldrich) and zeocin™ selection reagent (100  $\mu\text{g}/\text{mL}$ ; Life Technologies). Cells were co-transfected with pcDNA5 plasmid containing human OT or  $V_{1a}$ R 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  $\mu\text{g}/\text{mL}$ ; Invitrogen). Surface receptor expression was verified using immunocytochemistry according to the method of Werry et



al.[15] 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.[16]

### 5.3.2. Membrane Preparation.

OTR and V<sub>1a</sub>R-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<sub>2</sub>, pH 7.4) prior to homogenization using an Ultra-Turrax homogeniser (IKA, Wilmington, NC, USA). Resulting homogenates were centrifuged twice at 48000 g, 4 °C for 30 min and membrane pellets resuspended in 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 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 [<sup>3</sup>H]-oxytocin or [<sup>3</sup>H]-vasopressin at K<sub>d</sub> concentrations. Membranes (50 µg/well) from OT or V<sub>1a</sub> receptor-expressing HEK293 cells were incubated in a final volume of 200 µL containing [<sup>3</sup>H]-oxytocin (10 nM) or [<sup>3</sup>H]-vasopressin (2 nM) alongside competing compounds (0.1 nM - 100 µM) in reaction buffer (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 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 Microbeta<sup>2</sup> 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.

OTR or V<sub>1a</sub>R-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<sup>3</sup> cells per well. Levels of receptor activation induced by compounds were assessed at concentrations ranging from 1 nM - 100 µM using the HTRF-IP-One kit (CisBio International, Bagnols-sur-Cze, France), according to the manufacturer's protocol. For agonist assessment, cells were incubated with compounds for 1 h prior to the addition of Ab-Cryptate and IP<sub>1</sub>-d2. The ligand concentration that induced a 50% maximal response (EC<sub>50</sub>) 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<sub>70</sub> 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 IP<sub>1</sub>-d2 added. The ligand concentration that inhibited 50% of vasopressin-induced response (IC<sub>50</sub>) was used to evaluate antagonistic functional effects across compounds.

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## Highlights:

- Synthesis of 12 analogues of WAY-267,464
- Investigated the ideal linker conformation for OTR and V<sub>1a</sub>R pharmacophore
- Modifications abolished OTR pharmacology and reduced V<sub>1a</sub>R affinity in comparison to lead
- All analogues are investigated in competitive binding assays and functionally evaluated.