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Article

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Selective allosteric antagonists for the G protein-coupled receptor GPRC6A based on the 2-phenylindole privileged structure scaffold

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Abstract.

G protein-coupled receptors (GPCRs) represent a biological target class of fundamental importance in drug therapy. The GPRC6A receptor is a newly deorphanized class C GPCR that we recently reported the first allosteric antagonists for based on the 2-arylindole privileged structure scaffold (e.g. **1-3**). Herein, we present the first structure-activity relationship study for the 2-arylindole antagonist **3**, comprising the design, synthesis and pharmacological evaluation of a focused library of 3-substituted 2-arylindoles. In a FRET-based inositol mono-phosphate (IP_1) assay we identified compounds **7**, **13e** and **34b** as antagonists at the GPRC6A receptor in the low micromolar range and show that **7** and **34b** display >9 fold selectivity for the GPRC6A receptor over related GPCRs, making **7** and **34b** the most potent and selective antagonists for the GPRC6A receptor reported to date.

Introduction

G protein-coupled receptors (GPCRs), constitute the largest superfamily of membrane-bound proteins encoded in the human genome,[1,2] and represent the most common target class in drug therapy.[3-5] Based on phylogenetic analyses, the human GPCRs have been divided into a number of classes of which class A (rhodopsin family) is by far the most numerous, diverse with respect to endogenous ligands, and common as drug target.[1,5,6] The Class C (Glutamate family) is a significantly smaller group of dimeric GPCRs comprising eight metabotropic glutamate (mGlu) receptors, three taste 1 (T1R1-3) receptors, two γ-aminobutyric acid type B ($GABA_B$) receptors, the calcium-sensing (CaSR) receptor, the G protein-coupled receptor class C, group 6, subtype A (GPRC6A) and seven orphan receptors.[7-9] Class C GPCRs are generally activated by nutrients (e.g. amino acids, cations and sugar molecules), and display characteristic topological features such as a large extracellular and ligand-binding *N*-terminal domain ('Venus flytrap domain') in addition to the generic transmembrane helical domain present in all GPCRs.[9] Furthermore, class C GPCRs have proven viable as drug targets, exemplified by two marketed drugs; the CaSR positive allosteric modulator cinacalcet and the $GABA_B$ receptor agonist baclofen used in the treatment of primary hyperparathyroidism and spasticity, respectively.[10]

In 2004 our research group reported the first cloning, expression and sequencing of the GPRC6A receptor.[11] Subsequently the receptor was deorphanized as a promiscuous L-amino acid-sensing receptor with preference for basic amino acids (e.g. arginine, lysine and ornithine), and displaying wide tissue expression in humans.[12,13] Efforts by our group and others to elucidate the physiological roles of the GPRC6A receptor using knock-out mouse models have yielded partly contradictory

findings,[14-18] but generally link the receptor to metabolism and energy homeostasis.[19] In an attempt to shed new light on the physiological functions of the GPRC6A receptor, we initiated a medicinal chemistry program aimed at identifying new pharmacological tool compounds for studying the GPRC6A receptor. Recently, we reported the chemogenomic discovery of the first allosteric antagonists with modest preference for the GPRC6A receptor.[20] In a focused screening based on the GPCR privileged structure[21,22] 2-phenylindole, we identified allosteric antagonists **1**-**3** (Fig. 1) binding in the transmembrane domain of the mouse GPRC6A receptor and displaying inhibitory activity in the micromolar range in an inositol phosphate turnover assay. The 2-phenylindole scaffold of allosteric antagonists **1-3** were proposed to bind to the receptor with hydrogen bonding, hydrophobic and aromatic-aromatic interactions in a hydrophobic pocket, and the binding mode was validated by receptor mutagenesis and ligand modifications.[20]

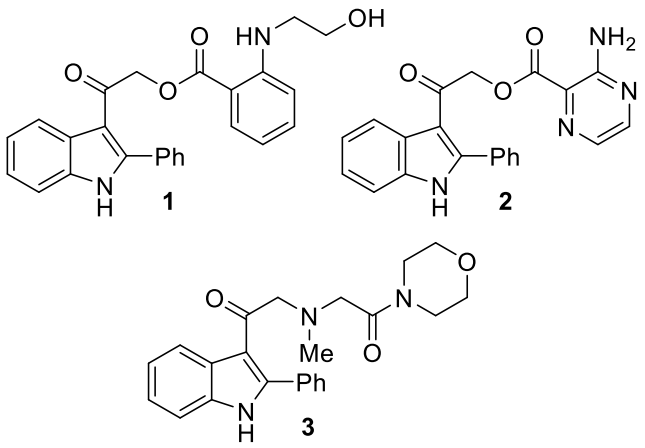


Figure 1. 3-Substituted 2-phenylindoles **1**-**3** acting as allosteric antagonists at the GPRC6A receptor.[20]

The identification of indoles **1**-**3** as the first allosteric antagonists targeting the GPRC6A receptor provided a good starting point for ligand optimization. However, GPCR privileged structure scaffolds are inherently promiscuous GPCR binders, and if

not equipped with unique extensions, ligands containing such structural elements may consequently display activity at a range of GPCRs. Indeed, besides the GPRC6A receptor ligands **2** and **3** were found to also antagonize the class A muscarinic acetylcholine M_1 and M_3 receptors and the metabotropic glutamate receptor subtype 5 (mGluR5). However, we were encouraged by the fact that ligand **1** displayed selectivity for the GPRC6A receptor over six related GPCRs that all contain the 2-arylindole allosteric binding motif, demonstrating that this class of privileged structures can indeed be made selective by appropriate modification.[20] Compounds **1** and **2** contained a potentially labile ester moiety and displayed significantly lower aqueous solubility than compound **3** (5-10 fold).[20] Despite initial attempts to address these issues, such as replacing the ester of compound **2** with an amide,[23] the compounds generally suffered from poor aqueous solubility. Consequently, compounds **1** and **2** were deselected for further ligand development at this stage. Instead, indole **3** was chosen as our lead structure and we set out to improve the selectivity for the GPRC6A receptor over other GPCRs, in particular the muscarinic acetylcholine receptors that had previously proven problematic.[20] By an empirical approach, we envisioned that a focused structure-activity relationship study based on indole **3**, in combination with a selectivity screening over two related class C (CaSR and mGluR5) and two distant class A (M_3 and serotonin 5-HT_{2C}) GPCRs would reveal structural determinants for receptor potency and selectivity, and thereby provide a structural basis for further ligand optimization. We chose four different structural regions of indole **3** as target for our chemical modifications: 1) the indole scaffold, 2) the 2-phenyl substituent, 3) the 3-carbonyl moiety and 4) the side chain in the indole-3-position (Fig. 2). Only the 3- and the 5-positions of the 2-arylindole were substituted in the present study. These represent the most frequently substituted

positions among known 2-arylindole GPCR ligands, and have access to adjacent binding cavity sub sites in our previously reported GPRC6A model.[20] Furthermore, hydrogen bonding between the indole nitrogen and the receptor has been validated as a determinant receptor-ligand interaction,[20] and therefore no substitutions were carried out at this position in this study.

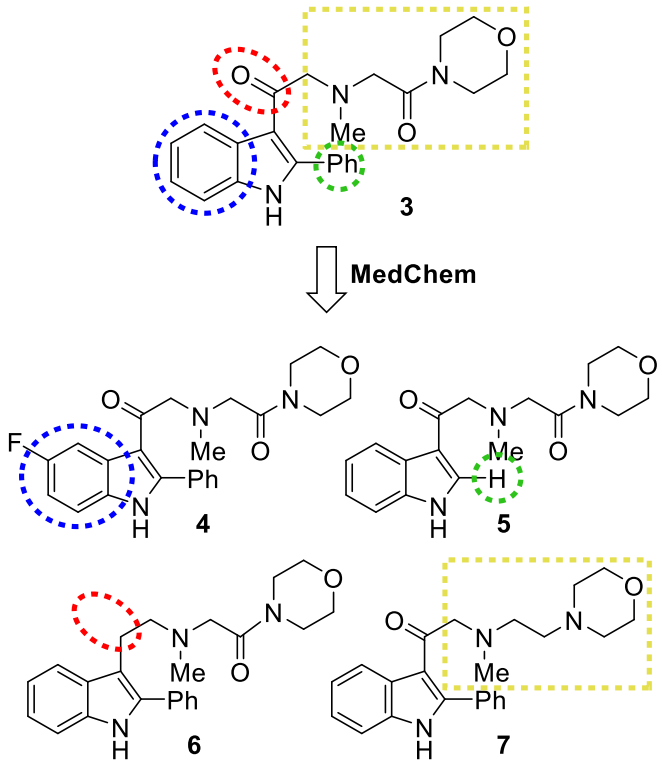
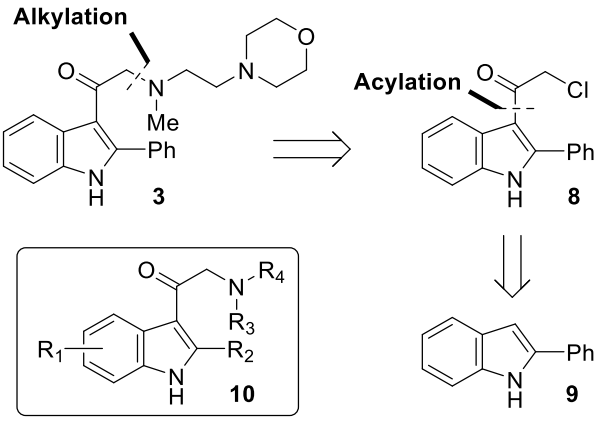


Figure 2. The four structural regions of indole **3** subject to chemical modifications are highlighted in dashed shapes and exemplified with target molecules **4** (indole scaffold, blue), **5** (2-phenyl substituent, green), **6** (3-carbonyl moiety, red) and **7** (side chain in the indole-3-position, yellow).

We have previously published the synthesis of antagonist **3** via a synthetic route based on the alkylation of a secondary amine with chloro-acetylated 2-phenylindole **8** that in turn is obtained by acylation of 2-phenylindole **9** (Scheme 1).[20,23] We envisioned that

this route could be applied to a broader range of substituted indoles and amines to obtain a series of analogs of antagonist **3**, i.e. indoles **10** (Scheme 1).

Scheme 1. The optimized synthesis route to antagonist **3** goes via acylation of 2-phenylindole **9** followed by alkylation of a secondary amine.[20,23] It was proposed as a general route to obtain analogs of **3** bearing various substituent on the indole scaffold (R_1 and R_2), and the amine functionality (R_3 and R_4).



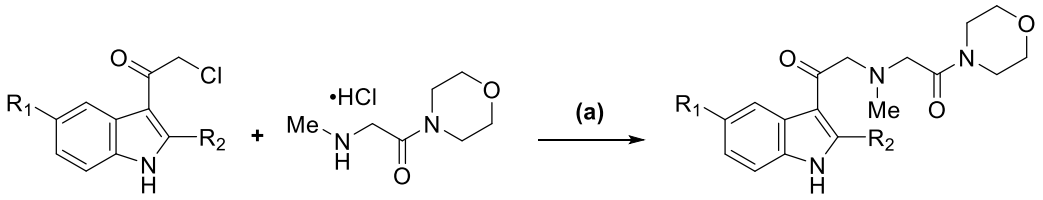
Herein, we report the design, synthesis and pharmacological evaluation of a focused library of 3-substituted 2-arylindole analogs of antagonist **3** targeting the GPRC6A receptor, as well as the first structure-activity relationship (SAR) study conducted for this receptor.

Results and Discussion.

Ligand synthesis. We first set out to explore the impact on ligand binding by introducing substituents at the 5-position of the indole scaffold and by varying the substituent in the indole-2-position e.g. using electron-withdrawing and donating groups, alkyl and hydroxyalkyl substituents and heterocycles. As part of our medicinal chemistry program targeting GPCRs we have previously reported the

synthesis of a number of 3-acylated 2-arylindole building blocks e.g. **11a-f**[24] that we employed in the present study for alkylation of secondary amine **12**[20] (Scheme 2).

Scheme 2. Synthesis of analogs **4**, **5**, **13b**, and **13d-f** via alkylation of secondary amine **12** using indole building blocks **11a-f**.[a]



11a R_1 = F, R_2 = Ph
11b R_1 = OMe, R_2 = Ph
11c R_1 = H, R_2 = H
11d R_1 = H, R_2 = 4-F-Ph
11e R_1 = H, R_2 = 4-OMe-Ph
11f R_1 = H, R_2 = 2-Furanyl

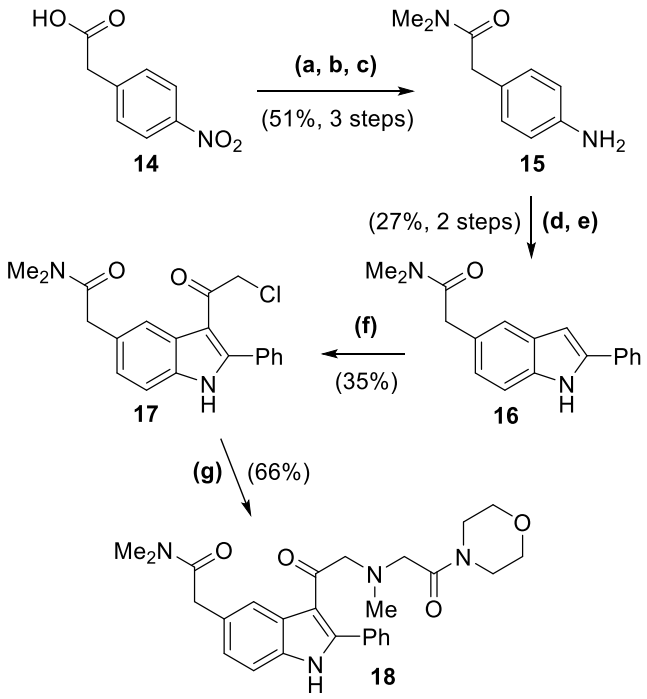
12

4 R_1 = F, R_2 = Ph (80%)
13b R_1 = OMe, R_2 = Ph (39%)
5 R_1 = H, R_2 = H (62%)
13d R_1 = H, R_2 = 4-F-Ph (26%)
13e R_1 = H, R_2 = 4-OMe-Ph (42%)
13f R_1 = H, R_2 = 2-Furanyl (25%)

[a] Reagents and conditions: (a) NaI, $NaHCO_3$, MeCN or acetone, 60 °C.

The alkylation of secondary amine **12** was generally uncomplicated, giving analogs of antagonist **3** lacking the 2-phenyl group (**5**), bearing electron-withdrawing substituents (**4** and **13d**), electron-donating substituents (**13b** and **13e**) and a heterocycle (**13f**) in moderate to good yields. Lower yields were generally associated with the generation of small amounts of side-products that were challenging to remove by column chromatography (e.g. **13d** and **13f**). With these analogs in hand we went on to investigate the impact of introducing larger or more polar substituents onto the indole scaffold; substituents that would challenge the hypothesis of the 2-arylindole scaffold binding to a relatively small, hydrophobic pocket in the transmembrane domain of the GPRC6A receptor.[20] The 5-carboxamidomethyl-substituted analogue **18** was selected as a target molecule with new hydrogen bonding opportunities (Scheme 3).

Scheme 3. Synthesis of 5-carboxamidomethyl-substituted analog **18** from carboxylic acid **14**.[a]

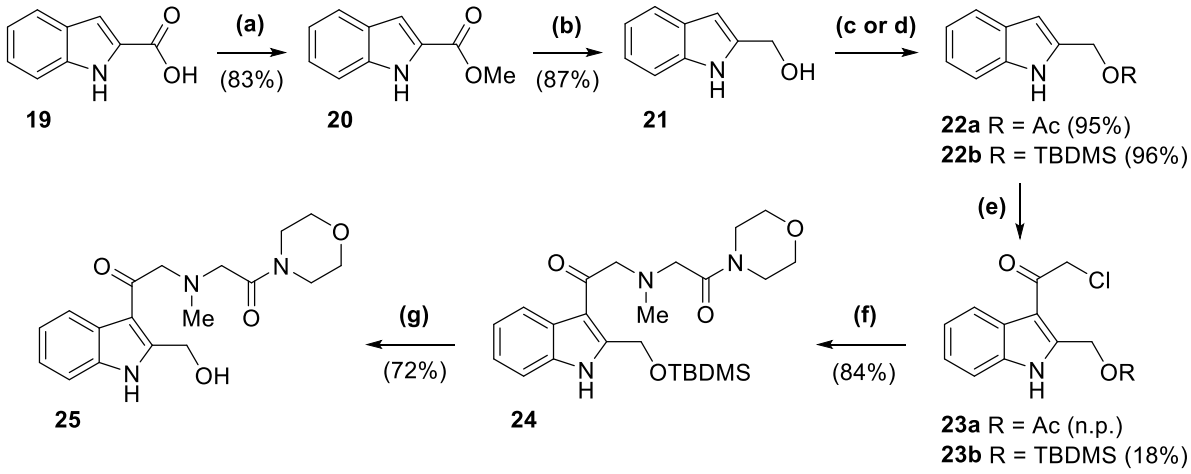


[a] Reagents and conditions: (a) $(COCl)_2$, DMF, CH_2Cl_2, 0 °C – rt; (b) 7.9 M aqueous Me_2NH, THF, 0 °C – rt; (c) Pd/C, H_2, EtOH, 0 °C – rt; (d) acetophenone, 3Å MS, PhMe, rt – 75 °C; (e) *n*-Bu_4NBr, $Pd(OAc)_2$, O_2, DMSO, 60 °C; (f) chloroacetyl chloride, pyridine, PhMe, 1,4-dioxane, CH_2Cl_2, rt – 65 °C; (g) **12**, NaI, $NaHCO_3$, MeCN, 60 °C.

Aniline **15** was obtained from carboxylic acid **14** in three steps without purification of intermediates. Condensation of **15** with acetophenone gave an imine that underwent Pd(II)-catalyzed oxidative cyclization under the conditions reported by Wei *et al.*[25] Indole **16** was acylated in the indole-3-position using chloroacetyl chloride and pyridine to give alkyl chloride **17**, and subsequent alkylation of secondary amine **12** gave the target molecule in good yield.

Finally, we decided to complete the targeted set of analogs bearing substituents on the indole scaffold by replacing the aryl group in the indole 2-position. We replaced the aryl group with a polar substituent (hydroxymethyl group) to explore the effect on the ligands' physicochemical properties and potency. Moreover, our previous mutagenesis study suggests that the indole-NH forms a crucial hydrogen bonding interaction to the receptor.[20] Thus, the introduction of a hydroxy-group in this position may give rise to an additional stabilizing hydrogen bonding interaction. Starting from 2-carboxyindole **19**, 2-hydroxymethyl-substituted analog **25** was synthesized as outlined in Scheme 4.

Scheme 4. Synthesis of 2-hydromethyl-substituted analog **25** from indole **19**.[a]



[a] Reagents and conditions: (a) $SOCl_2$, MeOH, 0 °C – reflux; (b) $LiAlH_4$, THF, 0 °C; (c) Ac_2O, pyridine, 0 °C – rt; (d) TBDMS-Cl, imidazole, DMF, 0 °C – rt; (e) AcOH, chloroacetyl chloride, Ac_2O, 60 °C; (f) **12**, NaI, $NaHCO_3$, MeCN, 60 °C; (g) TBAF, THF, 0 °C.

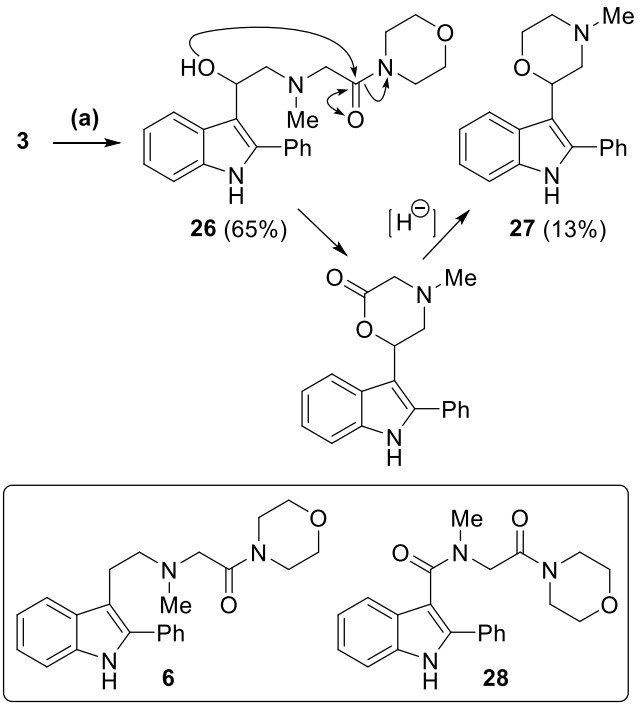
Esterification and reduction of carboxylic acid **19** provided alcohol **21** in good yield followed by acetylation to give **22a**. However, the following acylation at the indole 3-

position to give indole **23a** was complicated. Chloroacetylation under basic conditions using chloroacetyl chloride and pyridine in toluene only gave complex product mixtures, and attempts of using Lewis acid catalysis with $SnCl_4$ and chloroacetyl chloride in dichloromethane and nitromethane according to the method of Ottoni *et al.*[26] failed to produce the desired product. We suspected that the acetyl-protection group was causing problems during the acetylation. Thus, *tert*-butyldimethylsilyl-protected (TBDMS) indole **22b** was synthesized but again, acylation under basic conditions only gave trace amounts of product and Lewis acidic conditions made no improvement. Ultimately, 3-chloroacetylation of indole **22b** was achieved using a mixture of chloroacetyl chloride and acetic acid in acetic acid anhydride, inspired by the method of Slätt *et al.*[27] The acidic conditions produced significant amounts of side-products, but allowed isolation of the required acyl chloride **23b**. In order to improve the stability of the starting material TBDMS was replaced with the less acid labile *tert*-butyldiphenylsilyl-protection group but to no avail. Alkylation of secondary amine **12** using alkyl chloride **23b** and subsequent silyl ether deprotection produced the desired analog **25** in good yield.

Next, we turned our attention to modification of the ketone moiety of antagonist **3**. As mentioned above, hydrogen bonding of the ligand indole-NH is required for activity.[20] We hypothesized that the ketone at the 3-position might play a critical role through direct interactions with the receptor (e.g. hydrogen bonding), or indirectly by electron delocalization from the indole nitrogen over the carbonyl. To address this we decided to include analogs devoid of the ketone moiety or with significantly altered electronic properties. In a previous communication we have reported the synthesis of analogs **6** and **28** (Scheme 5).[23] Analog **28** is a truncated amide analogue of antagonist **3**, and in

analog **6** the carbonyl has been replaced by a methylene unit. In addition, secondary alcohol **26** was synthesized by direct reduction of antagonist **3** (Scheme 5).

Scheme 5. Reduction of antagonist **3** gave analogue **26** and cyclized side-product **27**.[a]



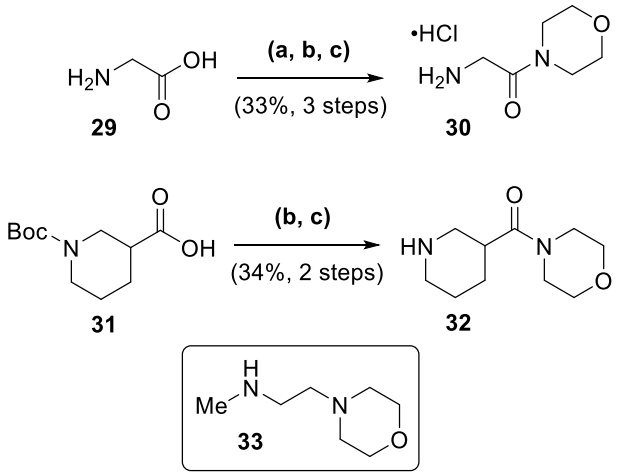
[a] Reagents and conditions: (a) $NaBH_4$, $CeCl_3 \times 7H_2O$, MeOH, 0 °C.

Initial attempts at reducing ketone **3** using $NaBH_4$ in MeOH did not go to completion, however by switching to Luche conditions ($CeCl_3$ and $NaBH_4$ in MeOH)[28,29] the desired alcohol **26** was obtained in good yield. The reduction also gave rise to morpholine side product **27** that was isolated in a low yield. The formation of **27** is rationalized by cyclization of the formed alcohol with the amide carbonyl, expulsion of morpholine and subsequent reduction.

Being a privileged scaffold, 2-arylindoles display activities at a number of biological targets,[20,30,31] and receptor potency and selectivity often arises from variation in the

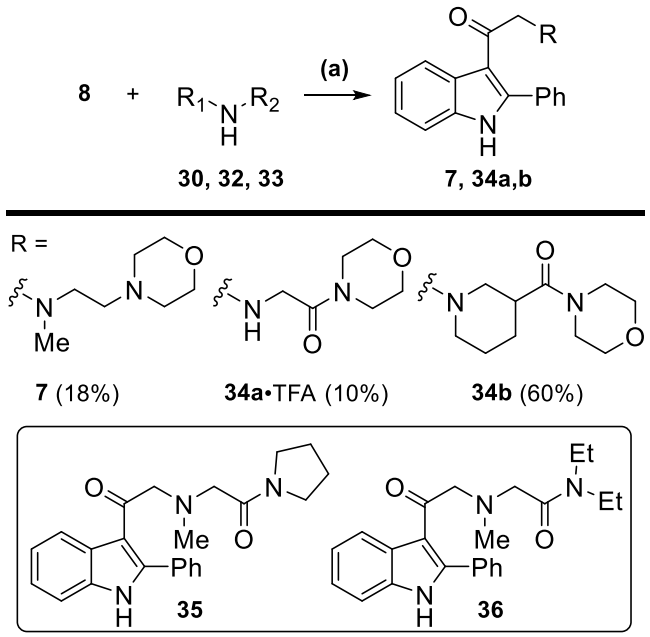
structure of the substituent in the indole 3-position.[32] Consequently, the final structural modifications of antagonist **3** undertaken in this study was the alteration of the substituent in the indole 3-position, trying to identify structural determinants for receptor potency and selectivity. We modified the tertiary amine, the amide moiety, and the length and shape/flexibility of the 3-substiutent, implementing a synthesis route based on alkylation of suitable amines **30**, **32** and **33** with alkyl chloride **8** (Schemes 6 and 7). While amine **33** was purchased, **30** and **32** were synthesized from the corresponding *N*-Boc-protected amino acid via amide coupling and subsequent Boc-deprotection (Scheme 6).

Scheme 6. Synthesis of amines **30** and **32** from carboxylic acids **29** and **31**.[a]



[a] Reagents and conditions: (a) Boc_2O, 3.8 M aq NaOH, THF, 0 °C; (b) morpholine, *N*,*N*-diisopropylethylamine, EDCI × HCl, DMAP, CH_2Cl_2, rt; (c) 4 M aq HCl, 1,4-dioxane, rt.

Scheme 7. Synthesis of analogs **7**, **34a** and **34b** via alkylation of secondary amines **30**, **32** and **33** using alkyl chloride **8**. R_1 = H or alkyl; R_2 = alkyl.[a]



[a] Reagents and conditions: (a) NaI, $NaHCO_3$, MeCN, 60 °C.

The synthesis and isolation of analogs **7**, **34a**, and **34b** proved challenging due to their high polarity and closely eluting side products. However, ultimately all three ligands were isolated in high purity in low to moderate yield. In addition, to analogs **7**, **34a** and **34b**, two commercially available analogs **35** and **36** (Scheme 7, >95% purity) were purchased to deliver a focused library of 16 structurally similar analogs of antagonist **3**, covering chemical modifications in four structural regions; the indole scaffold (**4**, **13b**, **18**), the 2-phenyl substituent (**5**, **13d-f**, **25**), the 3-carbonyl moiety (**6**, **26**, **28**), and the substituent in the indole 3-position (**7**, **34a**, **34b**, **35**, **36**; Table 1).

Pharmacology. All compounds were tested on the mouse GPRC6A receptor using a recently described time-resolved Förster resonance energy transfer (FRET) based inositol monophosphate assay protocol.[33] The reason for choosing the mouse rather than the human GPRC6A ortholog was that the latter is not cell surface expressed or functional in recombinant cell lines.[11-13] The Ile759 residue, shown to be important

for binding of compound **3**,[20] is conserved in both human, mouse, rat and goldfish GPRC6A orthologs[13] indicating the compound series will be active across species. The compounds were tested up to concentrations of 400 µM, with exception of **13e**, which was limited by its solubility to 100 µM. Compounds **7**, **13e** and **34b**, were found to be more potent than **3** at the GPRC6A receptor (Table 1 and Fig. 3) and compound **13a** to be equipotent. Notably, compound **5**, **18**, **25**, **26** and **28** were found to posses none to little inhibitory activity at 400 µM. To examine the selectivity of the new potent compounds, **7**, **13e** and **34b** were tested on recombinant rat CaSR, human 5-HT_{2C}, native human M_3 receptors expressed in Human Embryonic Kidney 293T (HEK-293T) cells and recombinant rat mGluR5 in Chinese Hamster Ovary (CHO) cells (Table 2). Choosing these receptor subtypes and species enabled comparison with our previous results on the compound **3** series.[20]

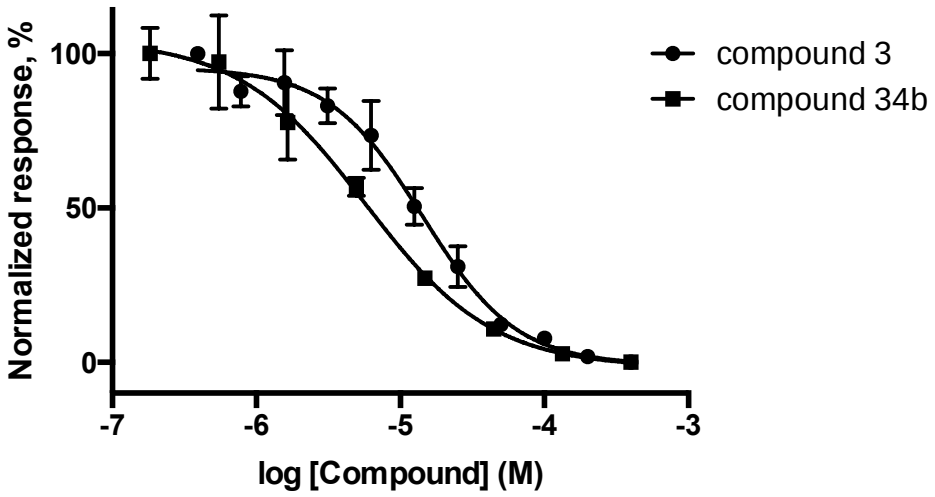
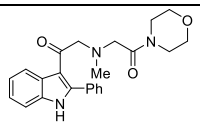
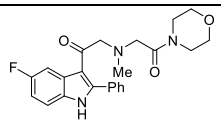
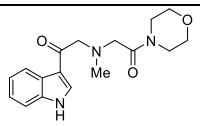
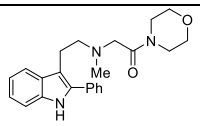
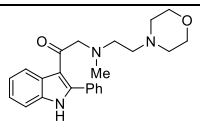
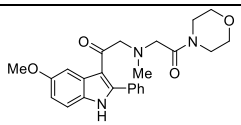
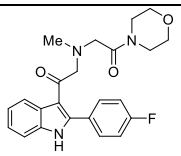
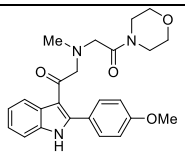
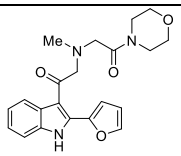
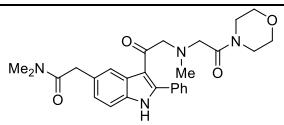
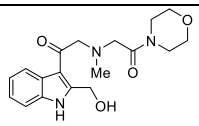
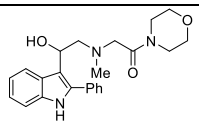
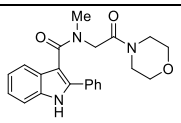
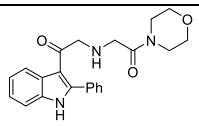
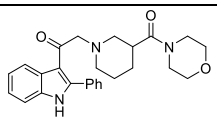
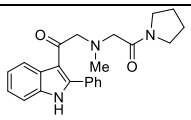
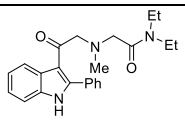


Figure 3. Representative concentration-response curves showing the inhibitory effect of compound **3** and **34b** on the response elicited by 500 µM L-ornithine on HEK-293T transfected with mouse GPRC6A receptor and Gq_{G66D}. The data was obtained via a FRET-based inositol monophosphate assay. The response for each concentration has been determined in triplicate and is shown as the mean±SD.

Table 1. Pharmacological evaluation of lead compound **3** and the focused library for *in vitro* antagonistic effect at the mouse GPRC6A receptor, in a FRET-based inositol monophosphate assay.

Cmpd	Structure	pIC_{50} ± SEM	IC_{50} (μM)	Relative potency (%)[a]	n
3		4.66 ± 0.05	21.9	100	7
4		4.40 ± 0.06	39.8	55	3
5[c]		-	>400	< 6	2
6[b]		3.88 ± 0.08	132	17	4
7		5.21 ± 0.01	6.17	355	5
13b[b]		4.18 ± 0.09	66.1	33	3
13d		4.67 ± 0.05	21.3	103	3
13e		5.03 ± 0.15	9.33	235	4

13f[b]		3.61 ± 0.07	248	9	4
18[c]		-	>400	< 6	2
25[c]		-	>400	< 6	2
26[c]		-	>400	< 6	2
28[c]		-	>400	< 6	2
34a[b]		3.59 ± 0.04	256	9	3
34b		5.26 ± 0.03	5.50	398	5
35[b]		4.24 ± 0.04	57.3	38	3
36[b]		3.94 ± 0.06	116	19	3

n = Number of independent experiments. [a] The relative antagonistic potency of the analogs in relation to lead compound **3** was calculated as IC_{50} (**3**)/IC_{50} (**cmpd**) × 100. [b] Due to low solubility/potency, the fitted concentration-response curve was obtained by restraining lower-plateau to buffer control values. [c] Little to no inhibition seen at compound concentrations of 40 µM and 400 µM.

Table 2. Activity of compound **3**, **7**, **13e** and **34b** at the mouse GPRC6A receptor and human 5-HT_{2C}, rat CaSR, human M_3 and rat mGluR5. All the receptors couple via a G_q mediated signaling pathway. A FRET-based assay was used to measure the generation of accumulated inositol monophosphate from receptor activation, in the presence of the following agonists: 500 μM L-ornithine, 1 μM serotonin, 20 mM Ca^{2+}, 1 mM carbachol and 100 μM L-glutamate for the mouse GPRC6A, human 5-HT_{2C}, rat CaSR, human M_3 and rat mGluR5 receptors, respectively.

Receptor	**mouse GPRC6A**	**human 5-HT_{2C}**	**rat CaSR**	**human M_3**	**rat mGluR5**
Cmpd	pIC_{50}±SEM/IC_{50} (μM)/Independent experiments (n)				
3	4.66±0.05	3.67±0.11[a]	3.50±0.04[a]	4.56±0.03[a]	3.65±0.06[a]
	21.9	214	316	27.5	224
	7	3	3	3	3
7	5.21±0.01	3.57±0.07[a]	3.46±0.03[a]	4.24±0.03[a]	3.57±0.04[a]
	6.17	269	347	58	269
	5	3	3	3	3
13e	5.03±0.15	4.29±0.07[a]	4.25±0.06[a]	5.38±0.13	4.38±0.03[a]
	9.33	51.3	56.2	4.17	41.7
	4	3	3	7	3
34b	5.26±0.03	3.68±0.03[a]	3.62±0.05[a]	4.29±0.04[a]	3.58±0.02[a]
	5.50	209	240	51.3	263
	5	3	3	3	3

[a] Due to low solubility and potency, the bottom half of the concentration-response curve was obtained by restraining lower-plateau to buffer level.

Structure Activity Relationship. The data in Table 1 shows that the substitution pattern on the indole scaffold has a large impact on receptor inhibition. Introducing substituents in the indole 5-position causes 2-fold and 3-fold loss of activity for analogs **4** and **13b** respectively, with the bulkiest analog **18** being completely inactive at the GPRC6A receptor. However, introducing substituents on the 2-phenyl group appears advantageous for activity. 2-(4-Fluorophenyl)indole **13d** retains the activity and the electron-donating and bulkier methoxy-substituted analog **13e** showed a 2-fold increase in activity compared to **3**. Switching to the smaller 2-furanyl-substituent (**13f**) caused significant loss of activity and removal of the 2-substituent (**5**) or introducing the polar hydroxymethyl group (**25**) rendered the analogs completely inactive at the GPRC6A receptor. These findings support the previously proposed hypothesis[20,32] that the privileged 2-phenylindole part of the ligands bind to a relatively small and hydrophobic pocket in the GPCR transmembrane domain, clearly demonstrates the importance of the 2-phenyl group as a prerequisite for ligand activity, and provides incentives for exploring the effects of substitution on the 2-phenylindole scaffold further.

Analogs **6**, **26**, and **28** were synthesized in order to evaluate the role of the ketone in the indole 3-position. The delocalization of electrons from the indole nitrogen over the carbonyl group in the indole 3-position is absent in analogs **6** and **26**, which may influence ligand hydrogen bonding at the indole nitrogen. Furthermore, analog **28** can act as a hydrogen bond acceptor at the carbonyl group, whereas analog **26** features both hydrogen bond donor and acceptor abilities and analog **6** lacks such abilities. Noteworthy, reduction of **3** to give alcohol **26** or replacement with the shorter amide-analog **28** results in complete loss of activity at the GPRC6A receptor while the 3-alkylsubsituted analog **6** shows some activity (6-fold less than **3**). These findings

suggest that the ketone does not merely function as a hydrogen bond acceptor but that delocalization of electrons from the indole nitrogen over the ketone carbonyl may also be an important feature of the pharmacophore.

The last SAR sub study was to alter the substituent in the indole 3-position. Removal of the *N*-methyl group (**34a**) resulted in more than a 10-fold loss of activity, while rigidifying and extending the side chain by a single methylene (**34b**), or entirely removing the amide carbonyl group (**7**) resulted in more than a 3-fold gain in potency. Analogue **36**, which lacks the morpholine oxygen atom, displays approximately 6-fold lower potency at the GPRC6A receptor, some of which could be regained for the cyclized analog **35** (3-fold less than **3**). These results show that small structural changes in the side chain at the indole 3-position can have a major influence on ligand potency. The morpholine ring appears to be important for binding, possibly by hydrogen bonding interactions with the receptor and we speculate that the structural changes in analogs **7** and **34b** could allow the morpholine moiety to be positioned more favorably. In the case of ligand **7** removal of the amide carbonyl leads to protonation of the nitrogen (morpholine pK_a ~ 8) and produces a more flexible side chain, while structural constraints restricts ligand **34b** into what appears to be a biologically relevant conformation. Low energy conformations were indeed found in which the **34b** and **3** amide carbonyls overlay very well (data not shown). The reduced analog **7** and the constrained analog **34b** represent the two most potent leads, and new hybrid ligands also incorporating other heterocycles will be explored in future optimization. Importantly, it should be noted that ligand **34b** was tested as a racemic mixture and that likely there will be a difference in the potency between the two enantiomers. Furthermore, in a follow-up study we have initiated work on a new GPRC6A model based on the first Class C crystal structures, the metabotropic

mGluR1[34] and mGlu5[35] receptors, which will form the basis for a mutagenesis study to further elucidate the molecular requirements for allosteric antagonistic activity.

The pharmacological evaluation of the focused set of analogs presented in Table 1, identified **7**, **13e**, and **34b** as the most potent antagonists at the GPRC6A receptor reported to date. Antagonist **3** has previously been reported to display activity at the muscarinic acetylcholine M_1 and M_3 receptors (class A GPCRs) and the mGluR5 receptor (a class C GPCR) in addition to the GPRC6A activity.[20] This was confirmed for the M_3 receptor in the present study. Improving the selectivity of ligand **3** for the GPRC6A receptor is critical for developing a useful pharmacological tool compound, and it was the main objective of the present study to do so while also improving potency. Consequently, the three most potent antagonists discovered in the present study, **7**, **13e** and **34b**, were tested for activity at the class A GPCRs 5-hydroxytryptamine receptor 5-HT_{2C} and muscarinic acetylcholine receptor M_3, as well as the related class C GPCRs CaSR and mGluR5 receptor (Table 2). As previously reported ligand **3** displays highest potency at M_3 amongst these receptors, which in the present study is close to equipotent with the GPRC6A receptor (Table 2). Ligand **13e** in which the side chain in the indole 3-position is unaltered displays even poorer selectivity than ligand **3** being equipotent at M_3 and GPRC6A and showing moderate and similar potencies at the three remaining receptors. Gratifyingly, the two remaining ligands **7** and **34b** show a significantly improved selectivity profile. Both ligand **7** and **34b** display 9-fold selectivity for GPRC6A over the M_3 receptor and only show weak potencies at the remaining three receptors (>38-fold selectivity). To assess the prospects of obtaining selectivity over the off-targets, we identified the most similar ligands for the off-target receptors (5-HT_{2C}, CaSR, M_3, and $mGluR_5$), as available in ChEMBL (SI, Table S1). Only the 5-HT_{2C} ligand displays a complete 2-

phenylindole scaffold and a high structural similarity (Tanimoto Similarity of 0.78). However, it lacks the morpholinoethan-1-one moiety, which could explain the selectivity we observe for GPRC6A. Given the privileged structure scaffold it is evident that the antagonists could have activities at other GPCRs than those tested here. Going forward it is thus our aim to first develop even more potent compounds with nanomolar potency, and subsequently test them on a much broader set of GPCRs.

Conclusion

We recently identified 3-substituted 2-phenylindoles **1**-**3** as the first allosteric antagonists with preference for the GPRC6A receptor, and a medicinal chemistry program was initiated aimed at developing potent and selective inhibitors for this receptor. Such inhibitors would constitute valuable pharmacological tool compounds for elucidating the physiological functions of the GPRC6A receptor. Antagonist **3** had the best physicochemical profile and was selected as our lead compound for further optimization. Herein, we report a profiling of the pharmacophore and a preliminary structure-activity relationship study to improve the ligands selectivity towards the GPRC6A receptor. Pharmacological evaluation of antagonistic effect at the GPRC6A receptor was carried out for 16 new ligands that each had been modified systematically in one of the four regions of the pharmacophore. We show that substituted aryl groups in the indole 2-position and modifications in the structure of the substituent in the indole 3-position improve the ligand antagonistic effect and report antagonists **7**, **13e** and **34b** as the most potent allosteric antagonists at the GPRC6A receptor to date. Moreover, antagonists **7** and **34b** display an improved selectivity profile for the GPRC6A receptor over related class A and C GPCRs (>9-

fold). Thus, it is clear from the present study that it is indeed possible to improve the selectivity for the GPRC6A receptor over related receptors that bear the same 2-arylindole binding motif. Moreover, the present study has demonstrated that potency can be increased by modifying the 2-arylindole scaffold (e.g. **13e**) and that receptor selectivity can be achieved by modification of the side chain at the indole 3-position (e.g. **7** and **34b**). These findings provide an important structural basis for further ligand optimization and hold promise that antagonists for the GPRC6A receptor with even higher pharmacological utility can be obtained in the near future.

Experimental section.

General procedure for amine alkylation (GP-Alkylation)

A microwave vial was charged with an alkyl chloride (1.0 equiv), $NaHCO_3$ (1.8 to 3.0 equiv), NaI (0.2 to 0.3 equiv) and a free amine or amine hydrochloric salt (1.0 equiv). Anhydrous MeCN was added and the vial was sealed and stirred at 60 °C in a heating block until TLC indicated full conversion of the alkyl chloride (2-7.5 h). The solids were removed by filtration, washed with MeCN and the combined organic phases were concentrated *in vacuo* to afford the crude tertiary amine. Purification by column chromatography (MeOH, aqueous NH_3 in CH_2Cl_2 or *n*-heptane in EtOAc) typically gave a colorless film that was dissolved in CH_2Cl_2 or Et_2O and concentrated *in vacuo* to give an amorphous solid.

1-(5-Fluoro-2-phenyl-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethanone (4)

Synthesized according to the general procedure **GP-Alkylation** using indole **11a** (0.101 g, 0.35 mmol), NaI (0.010 g, 0.07 mmol), $NaHCO_3$ (0.088 g, 1.05 mmol), amine hydrochloride **12** (0.068 g, 0.35 mmol) and anhydrous MeCN (1.75 mL).

Stirred at 60 °C for 2 h 15 min. Purification by column chromatography (3% MeOH, 0.2% aqueous NH_3 in CH_2Cl_2, v/v) gave indole **4** (0.114 g, 80%) as a white, amorphous solid. **TLC** R_f = 0.35 (3% MeOH, 0.2% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 6.17 min (Method A); **IR** (neat) ν_{max} = 3196, 2856, 1630, 1446, 1114; **^{1}H NMR** (400 MHz, DMSO-d_6): δ 12.22 (br s, 1H; NH), 7.82 (dd, $^3J_{HF}$ = 10.5 Hz, $^4J_{HH}$ = 2.5 Hz, 1H; indole-H4), 7.64-7.60 (m, 2H; PhH), 7.58-7.55 (m, 3H; PhH), 7.43 (dd, $^3J_{HH}$ = 9 Hz, $^4J_{HF}$ = 5 Hz, 1H; indole-H7), 7.09 (ddd, $^3J_{HF}$ = 9 Hz, $^3J_{HH}$ = 9 Hz, $^4J_{HH}$ = 2.5 Hz, 1H; indole-H6), 3.48-3.31 (m, 10H; 4 × morpholine-CH_2, Ar(C=O)CH_2), 3.12 (s, 2H; CH_2(C=O)N), 2.12 (s, 3H; CH_3); **^{13}C NMR** (151 MHz, DMSO-d_6): δ 194.2 (ketone-C=O), 167.9 (amide-C=O), 158.5 (d, $^1J_{CF}$ = 233 Hz; CF), 145.9 (indole-C2), 132.4 (Ph-C1), 132.1 (indole-C7a), 129.7 (Ph-C), 129.5 (Ph-C4), 128.5 (Ph-C), 127.5 (d, $^3J_{CF}$ = 11 Hz, indole-C3a), 113.0 (d, $^4J_{CF}$ = 4 Hz; indole-C3), 112.9 (d, $^3J_{CF}$ = 10 Hz; indole-C7), 111.0 (d, $^2J_{CF}$ = 26 Hz; indole-C6), 106.3 (d, $^2J_{CF}$ = 25 Hz; indole-C4), 66.2, 66.1 ((CH_2)$_2$O), 64.4 (Ar(C=O)*C*H_2), 58.8 (*C*H_2(C=O)N), 45.3 ((C=O)N*C*H_2), 42.3 (CH_3), 41.5 ((C=O)N*C*H_2); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{23}H_{25}FN_3O_3^+$ 410.1874, found 410.1857.

2-((2-(1*H*-Indol-3-yl)-2-oxoethyl)(methyl)amino)-1-morpholinoethanone (5)

A microwave vial was charged with indole **11c** (0.062 g, 0.32 mmol), NaI (0.010 g, 0.06 mmol), $NaHCO_3$ (0.081 g, 0.96 mmol), amine hydrochloride **12** (0.062 g, 0.32 mmol), and acetone (1.5 mL). The vial was capped and heated at 60 °C for 2 h, then allowed to cool. The mixture was filtered and the solids were washed with acetone. The combined organic fractions were concentrated *in vacuo* to afford the crude product as an orange film. Purification by column chromatography (4% MeOH, 0.3% aqueous NH_3 in CH_2Cl_2, v/v) gave indole **5** (0.063 g, 62%) as an off-white, amorphous solid. **TLC** R_f = 0.3 (4% MeOH, 0.3% aqueous NH_3 in CH_2Cl_2, v/v);

HPLC t_R = 5.34 min (A); **IR** (neat) ν_{max} = 3213, 2855, 1638, 1432; **^{1}H NMR** (400 MHz, DMSO-d_6): δ 11.91 (br s, 1H; NH), 8.52 (d, J = 3 Hz, 1H; indole-H2), 8.18-8.16 (m, 1H; indole-H4), 7.48-7.45 (m, 1H; indole-H7), 7.23-7.15 (m, 2H; indole-H6, H5), 3.72 (s, 2H; Ar(C=O)CH_2), 3.61-3.59 (m, 2H; (C=O)NCH_2) 3.56-3.53 (m, 4H; $(CH_2)_2O$), 3.45-3.43 (m, 2H; (C=O)NCH_2), 3.37 (s, 2H; CH_2(C=O)N), 2.32 (s, 3H; CH_3); **^{13}C NMR** (100 MHz, DMSO-d_6): δ 192.9 (ketone-C=O), 168.2 (amide-C=O), 136.2 (indole-C7a), 134.3 (indole-C2), 125.5 (indole-C3a), 122.7 (indole-C6), 121.7 (indole-C5), 121.2 (indole-C4), 114.9 (indole-C3), 112.0 (indole-C7), 66.3, 66.2 ($(CH_2)_2O$), 63.8 (Ar(C=O)*C*H_2), 59.2 (*C*H_2(C=O)N), 45.5 ((C=O)N*C*H_2), 42.6 (CH_3), 41.6 ((C=O)N*C*H_2); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{17}H_{22}N_3O_3^+$ 316.1656, found 316.1667.

2-(Methyl(2-morpholinoethyl)amino)-1-(2-phenyl-1*H*-indol-3-yl)ethan-1-one (7)

Synthesized according to **GP-Alkylation** using indole **8** (0.300 g, 1.11 mmol), $NaHCO_3$ (0.168 g, 2.00 mmol), NaI (0.045 g, 0.3 mmol), *N*-methyl-2-morpholinoethan-1-amine **33** (0.160 g, 1.11 mmol) and MeCN (8 mL). The mixture was stirred at 60 °C for 5 h. Purification by column chromatography (2.5% MeOH, 0.2% aqueous NH_3 in CH_2Cl_2, v/v) gave indole **7** (0.075 g, 18%) as an orange-colored film. **TLC** R_f = 0.15 (4% MeOH, 0.3% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 5.85 min (A); **IR** (neat) ν_{max} = 3177, 3062, 2954, 2805, 1637, 1449, 1115; **^{1}H NMR** (600 MHz, $CDCl_3$): δ 8.43 (br s, 1H; NH), 8.31-8.29 (m, 1H; indole-H4), 7.57-7.55 (m, 2H; Ph-H2, H2'), 7.52-7.49 (m, 3H; Ph-H3, H3', H4), 7.40-7.37 (m, 1H; indole-H7), 7.31-7.28 (m, 2H; indole-H6, H5), 3.62 (t, J = 5 Hz, 4H; $((CH_2)_2O)$, 3.44 (s, 2H; (C=O)CH_2), 2.56-2.54 (m, 2H; NC*H*$_2$$CH_2$), 2.36-2.34 (m, 6H; (C*H*$_2$N(C*H*$_2$$CH_2$)$_2$O), 2.25 (s, 3H; CH_3); **^{13}C NMR (DEPTQ)** (150 MHz, $CDCl_3$): δ 195.7 (C=O), 143.5 (indole-C2), 135.3 (indole-C7a), 133.1 (Ph-C1), 129.9 (Ph-C4), 129.7 (Ph-C2, C2'),

129.0 (Ph-C3, C3'), 127.5 (indole-C3a), 123.8 (indole-C6), 122.8 (indole-C5), 122.5 (indole-C4), 114.6 (indole-C3), 110.9 (indole-C7), 67.0 ($(CH_2)_2O$), 66.0 ((C=O)CH_2), 57.0 (NCH_2CH_2), 54.13 ($(CH_2CH_2)_2O$), 54.07 (NCH_2CH_2), 42.9 (CH_3); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{23}H_{27}N_3NaO_2^+$ 400.1995, found 400.2005.

1-(5-Methoxy-2-phenyl-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethanone (13b)

Synthesized according to the general procedure **GP-Alkylation** using indole **11b** (0.105 g, 0.35 mmol), NaI (0.010 g, 0.07 mmol), $NaHCO_3$ (0.088 g, 1.05 mmol), amine hydrochloride **12** (0.068 g, 0.35 mmol) and MeCN (1.75 mL). Stirred at 60 °C for 3 h. Purification by column chromatography (3.5% MeOH, 0.3% aqueous NH_3 in CH_2Cl_2, v/v) gave indole **13b** (0.058 g, 39%) as a light-yellow, amorphous solid. **TLC** R_f = 0.2 (4% MeOH, 0.2% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 6.05 min (A); **IR** (neat) ν_{max} = 3217, 3054, 2855, 1626, 1443, 1114; **^{1}H NMR** (400 MHz, DMSO-d_6): δ 11.97 (br s, 1H; NH), 7.63 (d, *J* = 2.5 Hz, 1H; indole-H4), 7.61-7.54 (m, 5H; PhH), 7.32 (d, *J* = 9 Hz, 1H; indole-H7), 6.87 (dd, *J* = 9, 2.5 Hz, 1H; indole-H6), 3.80 (s, 3H; OCH_3), 3.48-3.33 (m, 10H; 4 × morpholine-CH_2, Ar(C=O)CH_2), 3.13 (s, 2H; CH_2(C=O)N), 2.12 (s, 3H; NCH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 194.3 (ketone-C=O), 167.9 (amide-C=O), 155.3 (indole-C5), 144.6 (indole-C2), 132.9 (Ph-C1), 130.4 (indole-C7a), 129.7 (Ph-C), 129.2 (Ph-C4), 128.5 (Ph-C), 127.8 (indole-C3a), 112.8 (indole-C3), 112.6 (indole-C6), 112.4 (indole-C7), 103.2 (indole-C4), 66.2, 66.1 ($(CH_2)_2O$), 64.4 (Ar(C=O)CH_2), 58.9 (CH_2(C=O)N), 55.3 (OCH_3), 45.3 ((C=O)NCH_2), 42.3 (NCH_3), 41.5 ((C=O)NCH_2); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{24}H_{28}N_3O_4^+$ 422.2074, found 422.2070.

1-(2-(4-Fluorophenyl)-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethan-1-one (13d)

Synthesized according to **GP-Alkylation** using indole **11d** (0.150 g, 0.50 mmol), amine hydrochloride **12** (0.100 g, 0.52 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol), NaI (0.020 g, 0.13 mmol) and MeCN (3 mL). The mixture was heated at 60 °C for 7.5 h. DMSO (0.5 mL) was added and the mixture was stirred for an additional 30 minutes, then allowed to cool to room temperature. The solids were removed by filtration and washed with EtOAc (3 × 5 mL). The solution was washed with saturated aqueous $NaHCO_3$ (25 mL), brine (15 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to give the crude product as a yellow oil. Purification by column chromatography (1% MeOH, 0.1% aqueous NH_3 in EtOAc, v/v) gave indole **13d** (0.055 g, 26%) as a white, amorphous solid. **TLC** R_f = 0.3 (1% MeOH, 0.1% aqueous NH_3 in EtOAc, v/v). **HPLC** t_R = 6.11 min (A); **IR** (neat) ν_{max} = 3215, 1639, 1439; **^{1}H NMR** (400 MHz, DMSO-d_6): δ 12.11 (s, 1H; NH), 8.09-8.06 (m, 1H; indole-H4), 7.70-7.66 (m, 2H; Ph-H2, H2'), 7.44-7.37 (m, 3H; indole-H7, Ph-H3, H3'), 7.25-7.18 (m, 2H; indole-H6, H5), 3.48-3.43 (m, 10H; $N(CH_2CH_2)_2O$, $Ar(C{=}O)CH_2$), 3.16 (s, 2H; $CH_2(C{=}O)N$), 2.17 (s, 3H; CH_3); **^{13}C NMR** (100 MHz, DMSO-d_6) δ 194.2 (ketone-C=O), 167.9 (amide-C=O), 162.6 (d, $^1J_{CF}$ = 245 Hz; Ph-C4), 143.1 (indole-C2), 135.4 (indole-C7a), 132.0 (d, $^3J_{CF}$ = 8 Hz; Ph-C2, C2'), 129.1 (Ph-C1), 126.7 (indole-C3a), 122.8 (indole-C6), 121.7 (indole-C5), 121.3 (indole-C4), 115.4 (d, $^2J_{CF}$ = 22 Hz; Ph-C3, C3'), 112.9 (indole-C3), 111.7 (indole-C7), 66.16, 66.12 ($(CH_2)_2O$), 64.8 ($Ar(C{=}O)CH_2$), 58.9 ($CH_2(C{=}O)N$), 45.4 ($(C{=}O)NCH_2$), 42.4 (CH_3), 41.5 ($(C{=}O)NCH_2$). **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{23}H_{25}FN_3O_3^+$ 410.1874, found 410.1862.

1-(2-(4-Methoxyphenyl)-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethan-1-one (13e)

Synthesized according to **GP-Alkylation** using indole **11e** (0.060 g, 0.20 mmol),

$NaHCO_3$ (0.050 g, 0.60 mmol), NaI (0.006 g, 0.04 mmol), amine hydrochloride **12** (0.039 g, 0.20 mmol) and MeCN (2 mL). Stirred at 60 °C for 3.5 h. Purification by column chromatography (5% MeOH in CH_2Cl_2, v/v) gave indole **13e** as a white solid. Drying on a freeze-dryer caused contamination and partial protonation of the amine, but basic work-up using CH_2Cl_2 (75 mL), saturated aqueous $NaHCO_3$ (50 mL) and drying over Na_2SO_4, filtration and concentration *in vacuo* gave the free amine as a white solid. Dispersion and sonication of the solid in diethyl ether removed residual CH_2Cl_2 and gave indole **13e** (0.035 g, 42%) as a white solid. **TLC** R_f = 0.35 (5% MeOH in CH_2Cl_2, v/v); **HPLC** t_R = 6.11 min (A); **IR** (neat) ν_{max} = 3237, 2870, 1631, 1434; **^{1}H NMR** (600 MHz, DMSO-d_6): δ 11.98 (s, 1H; NH), 8.09-8.06 (m, 1H; indole-H4), 7.57-7.54 (m, 2H; Ph-H2, H2'), 7.42-7.40 (m, 1H; indole-H7), 7.22-7.17 (m, 2H; indole-H6, H5), 7.13-7.09 (m, 2H; Ph-H3, H3'), 3.85 (s, 3H; OCH_3), 3.47-3.33 (m, 10H; 4 × morpholine-CH_2, Ar(C=O)CH_2), 3.16 (s, 2H; CH_2(C=O)N), 2.15 (s, 3H; NCH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 194.5 (ketone-C=O), 167.9 (amide-C=O), 160.1 (Ph-C4), 144.3 (indole-C2), 135.4 (indole-C7a), 131.1 (Ph-C2, C2'), 127.0 (indole-C3a), 124.7 (Ph-C1), 122.6 (indole-C6), 121.6 (indole-C5), 121.2 (indole-C4), 113.9 (Ph-C3, C3'), 112.6 (indole-C3), 111.5 (indole-C7), 66.2, 66.1 ((CH_2)$_2$O), 64.6 (Ar(C=O)*C*H_2), 59.0 (*C*H_2(C=O)N), 55.3 (OCH_3), 45.3 ((C=O)N*C*H_2), 42.3 (NCH_3), 41.5 ((C=O)N*C*H_2); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{24}H_{28}N_3O_4^+$ 422.2074, found 422.2072.

1-(2-(Furan-2-yl)-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethanone (13f)

Synthesized according to the general procedure **GP-Alkylation** using indole **11f** (0.085 g, 0.33 mmol), NaI (0.010 g, 0.065 mmol), $NaHCO_3$ (0.082 g, 0.98 mmol), amine hydrochloride **12** (0.064 g, 0.33 mmol) and anhydrous MeCN (2.0 mL). Stirred

at 60 °C for 2 h. Purification by column chromatography (2% MeOH, 0.2% aqueous NH_3 to 4% MeOH, 0.3% aqueous NH_3 in CH_2Cl_2, v/v) gave indole **13f** (0.031 g, 25%) as a light-brown, amorphous solid. **TLC** R_f = 0.2 (2% MeOH, 0.2% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 5.83 min (A); **IR** (neat) ν_{max} = 3225, 1630, 1420, 1113; **^{1}H NMR** (600 MHz, DMSO-d_6): δ 12.30 (br s, 1H; NH), 7.97 (dd, J = 2, 0.5 Hz, 1H; furan-H5), 7.91 (d, J = 8 Hz, 1H; indole-H4), 7.58 (dd, J = 3.5, 0.5 Hz, 1H; furan-H3), 7.50 (ddd, J = 8, 1, 0.5 Hz, 1H; indole-H7), 7.24 (ddd, J = 8, 6.5, 1 Hz, 1H; indole-H6), 7.20 (ddd, J = 8, 6.5, 1 Hz, 1H; indole-H5), 6.74 (dd, J = 3, 2 Hz, 1H; furan-H4), 3.87 (s, 2H; Ar(C=O)CH_2); 3.48-3.45 (m, 4H; (C=O)NCH_2, CH_2O), 3.37-3.36 (m, 4H; (C=O)NCH_2, CH_2O), 3.30 (br s, 2H; CH_2(C=O)N), 2.32 (s, 3H; CH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 193.8 (ketone-C=O), 168.0 (amide-C=O), 145.6 (furan-C2), 144.4 (furan-C5), 135.6 (indole-C7a), 132.5 (indole-C2), 125.9 (indole-C3a), 123.0 (indole-C6), 121.7 (indole-C5), 121.2 (indole-C4), 113.6 (furan-C3), 112.4 (furan-C4), 112.1 (indole-C7), 111.9 (indole-C3), 66.2, 66.1 ((CH_2)$_2$O), 65.8 (Ar(C=O)CH_2), 59.3 CH_2(C=O)N), 45.4 ((C=O)NCH_2), 42.4 (CH_3), 41.5 ((C=O)NCH_2); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{21}H_{24}N_3O_4^+$ 382.1761, found 382.1749.

2-(4-Aminophenyl)-*N*,*N*-dimethylacetamide (15)

Acid chloride formation: 2-(4-Nitrophenyl)-acetic acid (3.00 g, 16.6 mmol) was dispersed in anhydrous CH_2Cl_2 (55 mL). The mixture was cooled to 0 °C using an ice-water bath and oxalyl chloride (1.42 mL, 21.5 mmol) was added followed by 5 drops of DMF. After 5 min the cooling bath was removed and the mixture was stirred at ambient temperature for 2 h 15 min. The solvent was removed under reduced pressure and the residue was re-dissolved in CH_2Cl_2 and concentrated *in vacuo* once

more to afford the crude acid chloride as a yellow oil. The crude product was used without further purification.

Amide formation: The crude acid chloride was dissolved in anhydrous THF (50 mL) and the mixture was cooled to 0 °C using an ice-water bath. Commercial 7.9 M aqueous dimethylamine (5.2 mL, 41.1 mmol) was added in portions over 5 min and the mixture was stirred for another 5 min. The cooling bath was removed and the mixture was stirred at ambient temperature over night. After stirring for 16 h in total the mixture was concentrated *in vacuo* and the resulting crude orange solid was taken up in sulfate buffer (150 mL) and EtOAc (50 mL) and transferred to a separation funnel. The phases were separated and the aqueous phase was extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (2 × 60 mL), brine (60 mL), dried (Na_2SO_4), filtered and concentrated *in vacuo* to give the crude amide (2.15 g) as a light-yellow solid. The crude amide was used without further purification.

Reduction: The crude amide (2.15 g, 10.3 mmol) was dissolved in anhydrous EtOH (50 mL) under nitrogen and the mixture was cooled to 0 °C using an ice-water bath. 10 wt% Pd/C (1.10 g, 1.03 mmol Pd) was added and the flask was pump-filled with hydrogen gas. The cooling bath was removed and the mixture was stirred vigorously under hydrogen for 3 h. The reaction mixture was filtered through a plug of Celite and the solids were washed with MeOH (75 mL). The combined organic phases were concentrated *in vacuo* to afford the crude product as an orange solid. EtOAc (40 mL) was added and the mixture was heated to reflux. The hot solution was filtered through a filter paper to remove insoluble solids, and the solution was concentrated *in vacuo*. Recrystallization of the afforded solid from EtOAc/*n*-heptane gave aniline **15** (1.50 g, 51% over 3 steps) as white needles in two crops. **TLC** R_f = 0.1 (20% *n*-heptane in

EtOAc, v/v); **HPLC** t_R = 2.56 min (A); **IR** (neat) ν_{max} = 3428, 3343, 3226, 2932, 1611, 1515; **mp** 99-101 °C (EtOAc/*n*-heptane; Lit.[36] 98-100 °C); **^{1}H NMR** (600 MHz, DMSO-d_6): δ 6.86-6.84 (m, 2H; Ph-H2, H2'), 6.50-6.48 (m, 2H; Ph-H3, H3'), 4.89 (s, 2H; NH_2), 3.46 (s, 2H; CH_2), 2.95 (s, 3H), 2.80 (s, 3H) (2 × CH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 170.9 (C=O), 147.0 (Ph-C4), 129.2 (Ph-C2, C2'), 122.5 (Ph-C1), 113.9 (Ph-C3, C3'), 39.1 (CH_2), 37.1, 34.9 (2 × CH_3); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{10}H_{15}N_2O^+$ 179.1179, found 179.1173.

***N*,*N*-Dimethyl-2-(2-phenyl-1*H*-indol-5-yl)acetamide (16)**

Imine formation: A flask was charged with aniline **15** (1.07 g, 6.0 mmol), acetophenone (0.70 mL, 6.0 mmol), 3Å molecular sieves (4.2 g) and anhydrous toluene (10 mL), and the mixture was stirred under nitrogen at room temperature for 24 h, then stirred at 75 °C for 76 h until TLC analysis indicated virtually full conversion. The mixture was allowed to cool and CH_2Cl_2 (50 mL) was added. The mixture was filtered through a plug of Celite and the solids were washed with EtOAc. The combined organic phases were concentrated *in vacuo* to afford the crude product as an orange-colored oil.

Oxidative cyclization: Similar to the method reported by Wei *et al.*[25] the crude imine (1.55 g, 5.5 mmol), *n*-Bu_4NBr (3.56 g, 11.1 mmol) and $Pd(OAc)_2$ (0.124 g, 0.55 mmol) were dissolved in anhydrous DMSO (28 mL), and the mixture was stirred at 60 °C under O_2 for 24 h. Sulfate buffer (125 mL) was added and the mixture was extracted with EtOAc (7 × 30 mL). Purification by column chromatography (30% *n*-heptane in EtOAc to 100% EtOAc, v/v) followed by recrystallization from EtOH gave indole **16** (0.44 g, 27%) as shiny, light-yellow flakes in two crops. **TLC** R_f = 0.4 (20% *n*-heptane in EtOAc, v/v); **HPLC** t_R = 8.37 min (A); **IR** (neat) ν_{max} = 3267, 2923, 1626, 1405; **mp** 192.5-194 °C (EtOH); **^{1}H NMR** (600 MHz, DMSO-d_6): δ 11.45 (br

s, 1H; NH), 7.84 (dd, *J* = 8.5, 1 Hz, 2H; Ph-H2, H2'), 7.45 (dd, *J* = 8, 7 Hz, 2H; Ph-H3, H3'), 7.36-7.35 (m, 1H; indole-H4), 7.33-7.29 (m, 2H; indole-H6, Ph-H4), 6.98 (dd, *J* = 8, 1 Hz, 1H; indole-H7), 6.85 (dd, *J* = 2.5, 1 Hz, 1H; indole-H3), 3.71 (s, 2H; CH_2), 3.00 (s, 3H), 2.83 (s, 3H) (2 × CH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 170.9 (C=O), 137.8, 135.9, 132.2 (3 × C), 128.9 (Ph-C3, C3'), 128.8 (C), 127.3 (Ph-C4), 126.5 (C), 124.9 (Ph-C2, C2'), 122.8 (indole-C7), 119.8 (indole-C4), 111.1 (indole-C6), 98.5 (indole-C3), 40.1 (CH_2), 37.2, 35.0 (2 × CH_3); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{18}H_{19}N_2O^+$ 279.1492, found 279.1495.

2-(3-(2-Chloroacetyl)-2-phenyl-1*H*-indol-5-yl)-*N*,*N*-dimethylacetamide (17)

Indole **16** (0.40 g, 1.44 mmol) was suspended in anhydrous toluene (10 mL) and heated to 60 °C. Pyridine (0.139 mL, 1.73 mmol) was added followed by additional toluene (3 mL) and 1,4-dioxane (5 mL) to slightly improve the solubility of the starting material. Chloroacetyl chloride (0.126 mL, 1.58 mmol) was added. After 20 min anhydrous CH_2Cl_2 (2 mL) was added to give full dissolution of the reaction mixture. After 2 h the temperature was raised to 65 °C and additional pyridine (0.07 mL, 0.8 mmol), and chloroacetyl chloride (0.065 mL, 0.8 mmol) was added. The mixture was stirred for an additional 2.5 h. Sulfate buffer (50 mL) was added and the aqueous mixture was extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL), dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford the crude product as a brown solid. Purification by column chromatography (15% *n*-heptane in EtOAc, v/v) and recrystallization from EtOAc (50 mL) gave indole **17** (0.18 g, 35%) as gray needles that were washed with *n*-heptane and dried. **TLC** R_f = 0.25 (15% *n*-heptane in EtOAc, v/v); **HPLC** t_R = 8.02 min (A); **IR** (neat) ν_{max} = 3143, 3062, 2932, 1619, 1434; **mp** 191.5-193 °C (EtOAc); **^{1}H NMR** (600 MHz, DMSO-d_6): δ 12.26 (br s, 1H;

NH), 8.04 (br s, 1H; indole-H4), 7.66-7.64 (m, 2H; Ph-H2, H2'), 7.60-7.58 (m, 3H; Ph-H3, H3', H4), 7.38 (d, J = 8.5 Hz, 1H; indole-H7), 7.14 (dd, J = 8.5, 1 Hz, 1H; indole-H6), 4.28 (s, 2H; Ar(C=O)CH_2), 3.80 (s, 2H; Ar-CH_2), 3.02 (s, 3H), 2.84 (s, 3H) (2 × CH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 186.8 (ketone-C=O), 170.7 (amide-C=O), 145.6 (indole-C2), 134.4 (indole-C7a), 132.2 (Ph-C1), 129.8 (Ph-C4), 129.79 (indole-C5), 129.78 (Ph-C2, C2'), 128.7 (Ph-C3, C3'), 127.1 (indole-C3a), 124.6 (indole-H6), 121.5 (indole-H4), 111.6 (indole-H7), 111.5 (indole-C3), 48.0 (CH_2Cl), 40.3 (Ar-CH_2), 37.2, 35.0 (2 × CH_3); **HRMS** (ESI+) m/z $[M+H]^+$ Calcd for $C_{20}H_{20}ClN_2O_2^+$ 355.1208, found 355.1217.

***N,N*-Dimethyl-2-(3-(2-(methyl(2-morpholino-2-oxoethyl)amino)acetyl)-2-phenyl-1*H*-indol-5-yl)acetamide (18)**

Synthesized according to the general procedure **GP-Alkylation** using indole **17** (0.089 g, 0.25 mmol), NaI (0.007 g, 0.05 mmol), $NaHCO_3$ (0.063 g, 0.75 mmol), amine hydrochloride **12** (0.049 g, 0.25 mmol), and anhydrous MeCN (2 mL). Stirred at 60 °C for 3 h. Purification by column chromatography (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v) to give a colorless film. Addition of Et_2O and concentration *in vacuo* gave indole **18** (0.078 g, 66%) as an off-white solid. **TLC** R_f = 0.3 (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 5.85 min (A); **IR** (neat) ν_{max} = 3283, 2923, 1630, 1432, 1113; **^{1}H NMR** (600 MHz, DMSO-d_6): δ 12.04 (br s, 1H; NH), 7.97 (br s, 1H; indole-H4), 7.61-7.59 (m, 2H; Ph-H2, H2'), 7.56-7.55 (m, 3H; Ph-H3, H3', H4), 7.35 (d, J = 8.5 Hz, 1H; indole-H7), 7.10 (dd, J = 8.5, 2 Hz, 1H; indole-H6), 3.78 (s, 2H; Ar-CH_2), 3.47-3.33 (m, 10H; 4 × morpholine-CH_2, Ar(C=O)CH_2), 3.13 (s, 2H; CH_2(C=O)N), 3.01 (s, 3H), 2.84 (s, 3H) (N(CH_3)$_2$), 2.11 (s, 3H; NCH_3); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 194.4 (ketone-C=O), 170.8 ((*C*=O)N(CH_3)$_2$), 167.9 ((C=O)N), 144.5 (indole-C2), 134.3 (indole-C7a), 132.8 (Ph-C1), 129.8 (Ph-

C2, C2'), 129.3 (Ph-C4), 129.2 (indole-C5), 128.5 (Ph-C3, C3'), 127.1 (indole-C3a), 124.2 (indole-C6), 121.4 (indole-C4), 112.7 (indole-C3), 111.4 (indole-C7), 66.2, 66.1 ($(CH_2)_2O$), 64.5 ($Ar(C=O)CH_2$), 58.9 ($CH_2(C=O)N$), 45.4 (½ × $N(CH_2CH_2)_2$), 42.3 (NCH_3), 41.5 (½ × $N(CH_2CH_2)_2$), 40.3 ($ArCH_2$), 37.2, 35.0 ($(C=O)N(CH_3)_2$); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{27}H_{33}N_4O_4^+$ 477.2496, found 477.2486.

Methyl 1*H*-indole-2-carboxylate (20)

Indole 2-carboxylic acid **19** (0.286 g, 1.77 mmol) was dissolved in anhydrous MeOH (10 mL) and cooled to 0 °C using an ice-water bath under nitrogen. Thionyl chloride (0.26 mL, 3.6 mmol) was added and the cooling bath was removed. The solution was stirred at ambient temperature for 1 h then heated under reflux for 4 h. The mixture was allowed to cool and the pH was raised to pH 8 with saturated aqueous $NaHCO_3$. The mixture was extracted with CH_2Cl_2 and the combined organic phases were dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude product as a white solid. Recrystallization from EtOAc gave indole **20** (0.26 g, 83%) as off-white needles. **TLC** R_f = 0.65 (40% EtOAc in *n*-heptane, v/v); **HPLC** t_R = 8.69 min (A); **mp** 151-152.5 °C (EtOAc; Lit.[37] 152.5-153 °C); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.93 (br s, 1H; NH), 7.70 (dq, *J* = 8, 1 Hz, 1H; indole-H4), 7.43 (dq, *J* = 8.5, 1 Hz, 1H; indole-H7), 7.33 (ddd, *J* = 8, 7, 1 Hz, 1H; indole-H6), 7.23 (dd, *J* = 2, 1 Hz, 1H; indole-H3), 7.16 (ddd, *J* = 8, 7, 1 Hz, 1H; indole-H5), 3.97 (s, 3H; CH_3); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 162.5 (C=O), 136.9, 127.5, 127.1 (3 × C), 125.5 (CH), 122.7 (CH), 120.9 (CH), 112.0 (CH), 108.9 (CH), 52.2 (CH_3); **LRMS** (ESI+) *m/z* found: 176.1 $[M+H]^+$. The data is in agreement with that reported by others.[38]

(1*H*-Indol-2-yl)methanol (21)

$LiAlH_4$ (0.18 g, 4.5 mmol) was dispersed in THF (15 mL) at 0 °C. A solution of indole **20** (0.225 g, 1.28 mmol) in THF was added dropwise over 15 min and the

mixture was stirred under nitrogen at 0 °C for 1 h. H_2O (10 mL) was carefully added, the cooling bath was removed and the mixture was stirred at ambient temperature for 15 min. A mixture of MeOH and CH_2Cl_2 (1:8, 25 mL) was added and the afforded emulsion was stirred vigorously for 30 min then extracted with CH_2Cl_2 (× 5). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude product as a slightly yellow oil. Purification by column chromatography (40% EtOAc in *n*-heptane, v/v) gave indole **21** as a white solid (0.16 g, 87%). **TLC** R_f = 0.3 (40% EtOAc in *n*-heptane, v/v); **HPLC** t_R = 5.24 min (B); **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.37 (br s, 1H; NH), 7.58 (dq, J = 7.5, 1 Hz, 1H; indole-H4), 7.34 (dq, J = 8, 1 Hz, 1H; indole-H7), 7.19 (ddd, J = 8, 7, 1 Hz, 1H; indole-H6), 7.11 (ddd, J = 8, 7, 1 Hz, 1H; indole-H5), 6.41-6.40 (m, 1H; indole-H3), 4.82 (d, J = 1 Hz, 2H; CH_2), 1.94 (br s, 1H; OH); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 137.7 (C), 136.5 (C), 128.2 (C), 122.3 (CH), 120.8 (CH), 120.1 (CH), 111.1 (CH), 100.7 (CH), 58.9 (CH_2); **LRMS** (ESI+) *m/z* found: 148.1 $[M+H]^+$. The analytical data is in agreement with that reported by others.[39,40]

(1*H*-Indol-2-yl)methyl acetate (22a)

According to the method by Keller *et al.*[41] indole **21** (0.80 g, 5.4 mmol) was dissolved in anhydrous pyridine (2 mL) and cooled to 0 °C with an ice-water bath. Acetic anhydride (0.7 mL, 7.1 mmol) was added under nitrogen. After 10 min the cooling bath was removed and the solution was stirred for 2 h at ambient temperature. Sulfate buffer was added, and the aqueous mixture was extracted with EtOAc (× 3). The combined organic phases were washed with sulfate buffer and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude product. Purification by DCVC[42] (0 to 40% EtOAc in *n*-heptane, v/v, 5% increments up to 20% EtOAc, then 2.5% increments) gave indole **22a** (0.98 g, 95%) as a white solid. **TLC** R_f = 0.6

(40% EtOAc in *n*-heptane, v/v); **HPLC** t_R = 6.30 min (B); **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.61 (br s, 1H; NH), 7.62 (dq, J = 8, 1 Hz, 1H; indole-H4), 7.36 (dq, J = 8, 1 Hz, 1H; indole-H7), 7.30 (ddd, J = 8, 7, 1 Hz, 1H; indole-H6), 7.13 (ddd, J = 8, 7, 1 Hz, 1H; indole-H5), 6.56 (m, 1H; indole-H3), 5.25 (s, 2H; CH_2), 2.12 (s, 3H; CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 172.4 (C=O), 136.7, 133.1, 127.6 (3 × C), 122.9, 121.0, 120.1, 111.2, 104.0 (5 × CH), 59.9 (CH_2), 21.1 (CH_3); **LRMS** (ESI+) *m/z* found: 130.1 $[M\text{-}OAc]^+$ (100%). The analytical data is in agreement with that reported by others.[40,43]

2-(((*tert*-Butyldimethylsilyl)oxy)methyl)-1*H*-indole (22b)

Indole **21** (0.30 g, 2.0 mmol) was dissolved in anhydrous DMF (0.5 mL) and cooled to 0 °C using an ice-water bath. *tert*-Butyldimethylsilyl chloride (0.37 g, 2.45 mmol) was added under nitrogen followed by imidazole (0.35 g, 5.1 mmol). The cooling bath was removed and the mixture was stirred at ambient temperature for 3.5 h. Water (20 mL) was added and the aqueous mixture was extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude product as a yellow solid. Purification by column chromatography (5% EtOAc in *n*-heptane, v/v) gave indole **22b** (0.51 g, 96%) as an off-white solid. **TLC** R_f = 0.25 (5% EtOAc in *n*-heptane, v/v); **HPLC** t_R = 8.38 min (B); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.27 (br s, 1H; NH), 7.58-7.53 (m, 1H; indole-H4), 7.37 (dq, J = 8, 1 Hz, 1H; indole-H7), 7.16 (ddd, J = 8, 7, 1 Hz, 1H; indole-H6), 7.08 (ddd, J = 8, 7, 1 Hz, 1H; indole-H5), 6.31 (dt, J = 2, 1 Hz, 1H; indole-H3), 4.89 (d, J = 0.5 Hz, 2H; CH_2), 0.96 (s, 9H; $C(CH_3)_3$), 0.13 (s, 6H; $Si(CH_3)_2$); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 138.2, 135.9, 128.5 (3 × C), 121.6, 120.4, 119.8, 110.9, 98.9 (5 × CH), 59.4 (CH_2), 26.2 ($C(CH_3)_3$), 18.6

($C(CH_3)_3$), −5.0 ($Si(CH_3)_2$); **LRMS** (ESI+) *m/z* found: 262.4 $[M+H]^+$. The data is in agreement with that reported by others.[44]

1-(2-(((*tert*-Butyldimethylsilyl)oxy)methyl)-1*H*-indol-3-yl)-2-chloroethanone (23b)

A 50 mL flask was charged with acetic acid anhydride (10 mL). Acetic acid (0.015 mL, 0.19 mmol), and chloroacetyl chloride (0.17 mL, 2.1 mmol) was added under nitrogen. The temperature was raised to 60 °C and indole **22b** (0.50 g, 1.91 mmol) was added. After stirring at 60 °C for 2.5 h the reaction mixture was taken up in EtOAc and washed with saturated aqueous $NaHCO_3$ (× 3) and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude product. Purification by two consecutive rounds of column chromatography (10% EtOAc in *n*-heptane, v/v, then 10% acetone in *n*-heptane, v/v) gave indole **23b** (0.11 g, 18%) as an off-white solid. **TLC** R_f = 0.25 (10% EtOAc in *n*-heptane, v/v); **HPLC** t_R = 11.62 min (A); **^{1}H NMR** (500 MHz, $CDCl_3$): δ 9.29 (br s, 1H; NH), 7.75 (d, J = 8 Hz, 1H; indole-H4), 7.49 (ddd, J = 7.5, 1, 0.5 Hz, 1H; indole-H7), 7.32 (ddd, J = 8, 7, 1 Hz, 1H; indole-H5), 7.28 (ddd, J = 8, 7, 1 Hz, 1H; indole-H6), 5.26 (s, 2H; CH_2O), 4.77 (s, 2H; CH_2Cl), 1.00 (s, 9H; $C(CH_3)_3$), 0.20 (s, 6H; $Si(CH_3)_2$); **^{13}C NMR** (125 MHz, $CDCl_3$): δ 186.7 (C=O), 150.9, 134.4, 125.8 (3 × C), 122.9, 122.8, 120.2, 112.2 (4 × CH), 109.4 (C), 61.2 (CH_2O), 49.2 (CH_2Cl), 26.1 ($C(CH_3)_3$), 18.6 ($C(CH_3)_3$), −5.2 ($Si(CH_3)_2$); **HRMS** (ESI+) *m/z* $[M+Na]^+$ Calcd for $C_{17}H_{24}ClNNaO_2Si^+$ 360.1157, found 360.1150.

1-(2-(((*tert*-Butyldimethylsilyl)oxy)methyl)-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethanone (24)

Synthesized according to the general procedure **GP-Alkylation** using indole **23b** (0.108 g, 0.32 mmol), NaI (0.014 g, 0.096 mmol), $NaHCO_3$ (0.081 g, 0.96 mmol),

amine hydrochloride **12** (0.062 g, 0.32 mmol), and anhydrous MeCN (2 mL). The mixture was stirred at 60 °C for 4 h. Purification by column chromatography (1 to 10% MeOH, 0.1 to 0.8% aqueous NH_3 in EtOAc, v/v) gave indole **24** (0.12 g, 84%) as an orange-colored oil. **TLC** R_f = 0.3 (1% MeOH, 0.1% aqueous NH_3 in EtOAc, v/v); **HPLC** t_R = 5.82 min (B); **^{1}H NMR** (400 MHz, $CDCl_3$): δ 9.18 (br s, 1H; NH), 7.82 (d, J = 8 Hz, 1H; indole-H4), 7.46-7.44 (m, 1H; indole-H7), 7.30-7.23 (m, 2H; indole-H5, H6; overlaps with solvent peak), 5.24 (s, 2H; CH_2O), 4.02 (s, 2H; Ar(C=O)CH_2) 3.71-3.56 (m, 8H; 4 × morpholine-CH_2), 3.52 (s, 2H; CH_2(C=O)N), 2.58 (s, 3H; NCH_3), 1.00 (s, 9H; $C(CH_3)_3$), 0.20 (s, 6H; $Si(CH_3)_2$); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 193.4 (ketone-C=O), 168.9 (amide-C=O), 149.4, 134.3, 126.1 (3 × C), 122.54, 122.51, 121.0, 111.9 (4 × CH), 110.9 (C), 67.15, 67.10, 66.0, 61.1, 59.7 (5 × CH_2), 46.1 ((C=O)NCH_2), 43.6 (NCH_3), 42.3 ((C=O)NCH_2), 26.1 ($C(CH_3)_3$), 18.6 ($C(CH_3)_3$), −5.2 ($Si(CH_3)_2$); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{24}H_{38}N_3O_4Si^+$ 460.2626, found 460.2615.

1-(2-(Hydroxymethyl)-1*H*-indol-3-yl)-2-(methyl(2-morpholino-2-oxoethyl)amino)ethanone (25)

Indole **24** (0.115 g, 0.25 mmol) was dissolved in anhydrous THF (5 mL) and the mixture was cooled to 0 °C using an ice-water bath. TBAF (1M in THF; 0.26 mL, 0.26 mmol) was added under nitrogen and the mixture was stirred at 0 °C for 1.5 h. Saturated aqueous $NaHCO_3$ was added and the mixture was extracted with EtOAc (5 × 25 mL). The combined organic phases were washed with water and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude product as a yellow film. Purification by column chromatography (10% MeOH, 0.8% aqueous NH_3 in EtOAc, v/v) gave a slightly yellow film. Addition of $CHCl_3$ and concentration *in vacuo* gave **25** (0.062 g, 72%) as an off-white, amorphous solid. **TLC** R_f = 0.25

(10% MeOH, 0.8% aqueous NH_3 in EtOAc, v/v); **HPLC** t_R = 1.35 min (B); **IR** (neat) ν_{max} = 3248, 2857, 1630, 1457, 1113; **^{1}H NMR** (400 MHz, $CDCl_3$): δ 10.17 (br s, 1H; NH), 7.91-7.89 (m, 1H; indole-H4), 7.40 (ddd, J = 7.5, 1.5, 1 Hz, 1H; indole-H7), 7.24-7.16 (m, 2H; indole-H6, H5), 5.00 (s, 2H; CH_2OH), 3.95 (s, 2H; Ar(C=O)CH_2), 3.62-3.52 (m, 8H; 4 × morpholine-CH_2), 3.44 (s, 2H; CH_2(C=O)N), 2.55 (s, 3H; NCH_3); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 194.0 (ketone-C=O), 168.7 (amide-C=O), 148.6, 135.0, 126.4 (3 × C), 123.0, 122.6 (indole-C5, C6), 121.3 (indole-C4), 112.07 (indole-C7), 111.97 (indole-C3), 66.9 (Ar(C=O)CH$_2$), 66.8 ((CH_2)$_2$O), 59.0 (CH_2OH), 58.6 (CH$_2$(C=O)N), 45.7 ((C=O)NCH$_2$), 43.8 (CH_3), 42.3 ((C=O)NCH$_2$); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{18}H_{24}N_3O_4^+$ 346.1761, found 346.1767.

2-((2-Hydroxy-2-(2-phenyl-1*H*-indol-3-yl)ethyl)(methyl)amino)-1-morpholinoethanone (26)

A 25 mL flask was charged with indole **3** (0.10 g, 0.26 mmol), MeOH (2 mL) and the solution was cooled to 0 °C with an ice-water bath. $CeCl_3$ heptahydrate (0.047 g, 0.13 mmol) and sodium borohydride (0.029 g, 0.77 mmol) were added and the mixture was stirred for 1.5 h. Water (10 mL) was added and the mixture was extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give the crude product as an off-white, amorphous solid. Purification by column chromatography (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v) gave alcohol **26** (0.066 g, 65%) and morpholine **27** (0.010 g, 13%) as white solids. **TLC** R_f = 0.3 (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 4.75 min (B); **IR** (neat) ν_{max} = 3271, 3055, 2855, 1628, 1456; **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.08 (br s, 1H; NH), 7.90 (d, J = 8 Hz, 1H; indole-H4), 7.56-7.36 (m, 6H; PhH, indole-H7), 7.23-7.18 (m, 1H; indole-H6), 7.15-7.10 (m, 1H; indole-H5), 5.15 (dd, J = 10, 4 Hz, 1H; CHOH), 3.72-3.43 (m, 9H; N(CH_2CH_2)$_2$O,

OH), 3.38 (s, 2H; CH_2C=O), 3.28 (dd, *J* = 13, 10 Hz, 1H; CHC*H*$_a$H$_b$), 2.84 (dd, *J* = 13, 4 Hz, 1H; CHCH$_a$*H*$_b$), 2.47 (s, 3H; CH_3); **^{13}C NMR** (150 MHz, DMSO-*d*$_6$): *δ* 168.2 (C=O), 136.2 (indole-C7a), 135.1 (indole-C2), 132.8 (Ph-C1), 128.7 (Ph-C2, C2'), 128.5 (Ph-C3, C3'), 127.6 (Ph-C4), 127.0 (indole-C3a), 121.2 (indole-C6), 120.8 (indole-C4), 118.5 (indole-C5), 114.2 (indole-C3), 111.1 (indole-C7), 65.9 ($(CH_2)_2O$), 64.5 (OCH), 62.9 (CH*C*H$_2$), 60.6 (*C*H$_2$C=O), 45.2 (½ × (C=O)N(*C*H$_2$)$_2$), 42.4 (CH_3), 41.4 (½ × (C=O)N(*C*H$_2$)$_2$); **LRMS** (ESI+) *m/z* found: 809.2 $[M+M+Na]^+$. **HRMS** (ESI+) *m/z* $[M-18+H]^+$ Calcd for $C_{23}H_{26}N_3O_2^+$ 376.2020, found 376.2020 (A high resolution mass spectrum providing the correct mass could not be obtained).

4-Methyl-2-(2-phenyl-1*H*-indol-3-yl)morpholine (27)

TLC R_f = 0.2 (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 4.19 min (B); **^{1}H NMR** (500 MHz, $CDCl_3$): *δ* 8.16 (br s, 1H; NH), 7.93-7.90 (m, 1H; indole-H4), 7.54-7.52 (m, 2H; Ph-H2, H2'), 7.49-7.45 (m, 2H; Ph-H3, H3'), 7.42-7.39 (m, 1H; Ph-H4), 7.37 (ddd, *J* = 8, 1.1, 0.8 Hz, 1H; indole-H7), 7.21 (ddd, *J* = 8, 7, 1 Hz, 1H; indole-H6), 7.14 (ddd, *J* = 8, 7, 1 Hz, 1H; indole-H5), 5.20 (dd, *J* = 10.5, 4 Hz, 1H; OCH), 3.71 (ddd, *J* = 11.5, 7, 4 Hz, 1H; OC*H*$_a$H$_b$), 3.65 (ddd, *J* = 11.5, 6, 4.5 Hz, 1H; OCH$_a$*H*$_b$), 3.33 (dd, *J* = 13, 10.5 Hz, 1H; OCHC*H*$_a$H$_b$), 2.75 (ddd, *J* = 13, 7, 4.5, 1H; OCH$_2$C*H*$_a$H$_b$), 2.68 (dd, *J* = 13, 3.5 Hz, 1H; OCHCH$_a$*H*$_b$), 2.65 (ddd, *J* = 13, 6, 4 Hz, 1H; OCH$_2$CH$_a$*H*$_b$), 2.41 (s, 3H; CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$): *δ* 136.3, 136.2, 132.7 (3 × C), 129.0, 128.8, 128.4 (5 × Ph-CH), 127.3 (indole-C), 122.6, 121.0, 120.2 (3 × indole-CH), 112.8 (indole-C), 111.2 (indole-CH), 65.0 (OCH), 63.4 (OCH_2), 59.7, 59.4 ($N(CH_2)_2$), 42.5 (CH_3); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{19}H_{21}N_2O^+$ 293.1648, found 293.1665.

2-Amino-1-morpholinoethanone hydrochloride (30)

N-protection: Glycine (0.30 g, 4.0 mmol) was dissolved in 3.8 M aqueous NaOH (2.4 mL) and THF (2.0 mL) and cooled to 0 °C with an ice-water bath. Boc_2O (0.88 g, 4.0 mmol) was dissolved in THF (1 mL) and added dropwise. The cooling bath was removed and the mixture was stirred at ambient temperature for 23 h. The mixture was cooled with an ice-water bath and pH was adjusted to pH ≈ 3 using 2M aqueous HCl. The mixture was extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the crude *N*-Boc-protected amino acid (0.51 g) as a white solid.

Amide coupling: The crude *N*-Boc-protected amino acid was dissolved in CH_2Cl_2 (7 mL). *N*,*N*-diisopropylethylamine (1.95 mL, 11.0 mmol), morpholine (0.31 mL, 3.52 mmol), DMAP (0.46 g, 3.8 mmol), and EDCI hydrochloride (0.68 g, 3.52 mmol) was added and the mixture was stirred at ambient temperature for 40 h. CH_2Cl_2 was added and the organic layer was washed with sulfate buffer, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give the crude amide (0.39 g) as a white solid.

Boc-deprotection: The crude amide was dissolved in dioxane (4 mL) and 4M aqueous HCl (4 mL) was added. The mixture was stirred vigorously at ambient temperature for 3.5 h. Toluene was added and the solvents were removed under reduced pressure to afford the crude amine hydrochloric salt. Recrystallization from absolute EtOH/Et_2O gave amine **30** (0.24 g, 33% over 3 steps) as a white solid that was collected by filtration, washed with diethyl ether and dried under vacuum. **TLC** R_f = 0.25 (10% MeOH, 0.8% aqueous NH_3 in CH_2Cl_2, v/v); **^{1}H NMR** (300 MHz, DMSO-d_6) δ 8.14 (s, 3H; NH_3^+) 3.85 (s, 2H; $CH_2C{=}O$), 3.60-3.55 (m, 4H; $(CH_2)_2O$), 3.50-3.46 (m, 2H; $(C{=}O)NCH_2$), 3.39-3.36 (m, 2H; $(C{=}O)NCH_2$); **^{13}C NMR** (125 MHz, DMSO-d_6) δ 164.8 (C=O), 65.90, 65.86 ($(CH_2)_2O$), 44.5 ($CH_2C{=}O$), 41.8, 39.9

($N(CH_2)_2$); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_6H_{13}N_2O_2^+$ 145.0972, found 145.0966.

Morpholino(piperidin-3-yl)methanone (32)

Amide formation: 1-(*tert*-Butoxycarbonyl)piperidine-3-carboxylic acid (0.500 g, 2.18 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and *N*,*N*-diisopropylethylamine (1.15 mL, 6.5 mmol), DMAP (0.052 g, 0.44 mmol), EDCI hydrochloride (0.48 g, 2.5 mmol), and morpholine (0.21 ml, 2.4 mmol) were added. The mixture was stirred at ambient temperature under nitrogen for 26 h. CH_2Cl_2 (30 mL) was added and the organic phase was washed with sulfate buffer (2 × 30 mL), saturated aqueous $NaHCO_3$ (30 mL), brine (30 mL), dried over Na_2SO_4 and concentrated *in vacuo* to afford the crude amide (0.5 g) as a colorless gum. **TLC** R_f = 0.55 (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v).

Boc-deprotection: The crude amide was dissolved in 1,4-dioxane (10 mL) and 4M aqueous HCl (5 mL) was added. The solution was stirred at ambient temperature for 4.5 h then concentrated *in vacuo* together with toluene to afford a colorless oil. Saturated aqueous $NaHCO_3$ (30 mL) was added and the aqueous phase was extracted with CH_2Cl_2 (6 × 20 mL), $CHCl_3$/EtOH (3:1, 3 × 25 mL) and $CHCl_3$/EtOH (2:1) until no more amine was detected in the aqueous phase. The combined organic phases were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo* to afford the crude product as a colorless oil. Purification by column chromatography (10% MeOH, 0.8% aqueous NH_3 in CH_2Cl_2, v/v) gave amine **32** (0.173 g, 34% over 2 steps) as a white solid. **TLC** R_f = 0.20 (8% MeOH, 0.6% aqueous NH_3 in CH_2Cl_2, v/v); **IR** (neat) ν_{max} = 3428, 2930, 2856, 1618, 1437, 1112; **^{1}H NMR** (400 MHz, $CDCl_3$): δ 3.66-3.49 (m, 8H; 4 × morpholine-CH_2), 3.06-2.97 (m, 2H; 2 × HNC$H_a$$H_b$), 2.86 (dd, *J* = 12.5, 9.5 Hz, 1H; HNC$H_a$$H_b$CH), 2.69-2.60 (m, 2H; HNC$H_a$$H_b$, CH), 2.26 (br s, 1H; NH),

1.86-1.79 (m, 1H), 1.75-1.64 (m, 2H), 1.56-1.44 (m, 1H) (4 × alkane-H); **^{13}C NMR** (100 MHz, $CDCl_3$): δ 173.0 (C=O), 67.1, 67.0 ($(CH_2)_2O$), 49.2 (NCH_2CH), 46.5 (NCH_2CH_2), 46.1, 42.0 (C=O)N($CH_2)_2$), 39.6 (CH), 28.0, 25.6 ($CH_2(CH_2)_2CH$); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{10}H_{19}N_2O_2^+$ 199.1441, found 199.1435.

1-Morpholino-2-((2-oxo-2-(2-phenyl-1*H*-indol-3-yl)ethyl)amino)ethanone trifluoroacetic acid salt (34a)

Synthesized according to the general procedure **GP-Alkylation** using indole **8** (0.080 g, 0.30 mmol), NaI (0.015 g, 0.1 mmol), $NaHCO_3$ (0.071 g, 0.85 mmol), amine hydrochloride **30** (0.054 g, 0.30 mmol) and MeCN (4 mL). Stirred at 60 °C for 3 h. Purification by column chromatography (10% *n*-heptane, 0.4% Et_3N in EtOAc, v/v, then 10% MeOH, 0.4% Et_3N in EtOAc) and preparative HPLC gave indole **34a** (0.015 g, 10%) as the TFA salt. **TLC** R_f = 0.55 (10% MeOH, 0.8% aqueous NH_3 in CH_2Cl_2); **HPLC** t_R = 6.00 min (A); **IR** (neat) ν_{max} = 3071, 1655, 1452, 1199; **^{1}H NMR** (600 MHz, DMSO-d_6): δ 12.51 (br s, 1H; indole-NH), 9.02 (br s, 2H; NH_2^+), 8.16-8.15 (m, 1H, indole-H4), 7.70-7.68 (m, 2H; Ph-H2, H2'), 7.62-7.57 (m, 3H; Ph-H3, H3', H4), 7.50-7.48 (m, 1H; indole-H7), 7.32-7.29 (m, 2H; indole-H5, H6), 4.05 (s, 2H; Ar(C=O)CH_2), 4.02 (s, 2H; CH_2(C=O)N), 3.58-3.56 (m, 4H; $(CH_2)_2O$; the peak overlaps with the water signal), 3.47 (t, J = 5 Hz, 2H; the peak overlaps with the water signal), 3.31 (t, J = 5 Hz, 2H) ((C=O)N$(CH_2)_2$); **^{13}C NMR** (150 MHz, DMSO-d_6): δ 186.1 (ketone-C=O), 163.8 (amide-C=O), 157.9 (q, $^2J_{CF}$ = 32 Hz; CF_3C=O), 146.4 (indole-C2), 135.7 (indole-C7a), 131.6 (Ph-C1), 129.9 (Ph-C4), 129.8 (Ph-C2, C2'), 128.6 (Ph-C3, C3'), 126.4 (indole-C3a), 123.5, 122.6 (indole-C6, C5), 121.1 (indole-C4), 116.9 (q, $^1J_{CF}$ = 298 Hz; CF_3), 112.2 (indole-C7), 110.7 (indole-C3), 65.9, 65.8 ($(CH_2)_2O$), 53.4 (Ar(C=O)CH_2), 46.8 (CH_2(C=O)N), 44.5, 41.7

((C=O)N(CH_2)$_2$); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{22}H_{24}N_3O_3^+$ 378.1812, found 378.1807.

2-(3-(Morpholine-4-carbonyl)piperidin-1-yl)-1-(2-phenyl-1*H*-indol-3-yl)ethanone (34b)

Synthesized according to the general procedure **GP-Alkylation** using indole **8** (0.125 g, 0.46 mmol), NaI (0.014 g, 0.093 mmol), $NaHCO_3$ (0.097 g, 1.16 mmol), amine **32** (0.092 g, 0.46 mmol) and MeCN (4 mL). Stirred at 60 °C for 7 h. Purification by column chromatography (3% MeOH, 0.2% aqueous NH_3 in CH_2Cl_2, v/v) gave indole **34b** as an off-white solid (0.121 g, 60%). **TLC** R_f = 0.3 (5% MeOH, 0.4% aqueous NH_3 in CH_2Cl_2, v/v); **HPLC** t_R = 6.35 min (A); **IR** (neat) ν_{max} = 3211, 2937, 2857, 1630, 1449, 1433; **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.94 (br s, 1H; NH), 8.32-8.28 (m, 1H; indole-H4), 7.55-7.44 (m, 5H; PhH), 7.42-7.38 (m, 1H; indole-H7), 7.32-7.26 (m, 2H; indole-H5, H6), 3.61-3.44 (m, 6H; 6 × morpholine-H), 3.36-3.23 (m, 4H; 2 × morpholine-H, Ar(C=O)CH_2), 2.78-2.62 (m, 3H), 2.02 (t, *J* = 11 Hz, 1H), 1.85-1.78 (m, 1H), 1.68-1.64 (m, 1H), 1.59-1.53 (m, 2H), 1.44-1.34 (m, 1H) (9 × piperidine-H); **^{13}C NMR** (150 MHz, $CDCl_3$): δ 194.7 (ketone-C=O), 172.8 (amide-C=O), 143.8 (indole-C2), 135.3 (indole-C7a), 133.1 (Ph-C1), 129.9 (Ph-C4), 129.6 (Ph-C2, C2'), 128.9 (Ph-C3, C3'), 127.6 (indole-C3a), 123.9 (indole-C6), 122.9 (indole-C5), 122.5 (indole-C4), 114.4 (indole-C3), 110.9 (indole-C7), 67.1 ((CH_2)$_2$O), 66.9 (Ar(C=O)*C*H_2), 56.4, 54.1 (Ar(C=O)CH_2N(*C*H_2)$_2$), 46.0, 42.0 ((C=O)N(*C*H_2)$_2$), 39.3 (*C*H(C=O)), 27.3, 24.9 (2 × piperidine-CH_2); **HRMS** (ESI+) *m/z* $[M+H]^+$ Calcd for $C_{26}H_{30}N_3O_3^+$ 432.2282, found 432.2260.

Cell culturing

HEK-293T cells were maintained in GlutaMAX-I Dulbecco's modified medium (DMEM) containing 10% (v/v) dialyzed fetal bovine serum and 5% (v/v) penicillin (100 U/mL)/streptomycin (100 mg/mL). A previously described CHO cell-line stably expressing rat mGluR5[20] were maintained in GlutaMAX-I DMEM medium, supplemented with 10% (v/v) dialyzed fetal bovine serum, 1% (w/v) L-proline and 5% (v/v) penicillin (100 U/mL)/streptomycin (100 mg/mL). All cells were grown at 37 °C at 5% CO_2 in a humidified incubator.

Transfection and cell work

In brief, HEK-293T cells were transiently transfected with a plasmid encoding the receptor of interest using Lipofectamine® 2000 (Life Technologies, Nærum, Denmark), in accordance with manufactures protocol, deviations from standard Lipofectamine®:DNA ratios are noted. Cells were transfected with either mouse GPRC6A, human 5-HT_{2C} or rat CaSR plasmid DNA. For GPRC6A and 5-HT_{2C}, 0.25 µg/0.25 µL DNA:Lipofectamine® per well were used. For CaSR this was reduced to 0.03 µg/0.25 µL DNA:Lipofectamine® per well to prevent overstimulation during the assay. To enable efficient coupling to the phospholipase C pathway, cells transfected with the GPRC6A receptor were also co-transfected with Gq_{G66D} (1:1 transfection ratio) as previously described.[20] For experiments involving the M_3 receptor, HEK-293T cells were transfected with empty pCIneo plasmid DNA for consistency, as HEK-293T cells natively express this receptor.[45] Transfections were carried out 48 hours prior to the assay in clear 96-well plates (Corning, Corning, NY, USA) pre-treated with 50 µL/well of 30 µg/mL poly-D-lysine. 12,500 HEK-293T cells were seeded per well. To test mGluR5 activity a previously described CHO cell-line stably

expressing rat mGluR5[20] was used. These cells were not further transfected, 25,000 cells/well were seeded 24 hours prior to the assay.

FRET-based inositol monophosphate accumulation assay

Prior to ligand addition the cells were washed twice with a two hours interval at 37 ^{o}C in Wash Buffer (Hank's Balanced Salt Solution (HBSS) + 20 mM HEPES + 1 mg/mL Bovine Serum Albumine, pH adjusted to 7.4).

Dilutions of all compounds to be tested were done in dimethylsulfoxide (DMSO) to prevent precipitation. Ligand Buffer (HBSS + 1 mM $CaCl_2$ + 1 mM $MgCl_2$ + 40 mM $LiCl_2$, pH adjusted to 7.4) containing either 500 µM L-ornithine, 1 µM serotonin, 20 mM Ca^{2+}, 1 mM carbachol or 100 µM L-glutamate was then added to dilute the DMSO-compound solutions to the desired concentrations. This yielded a final concentration of 2% DMSO in all samples, which was shown to not affect the measured response in a significant way (data not shown). The resultant compound solutions were added to the cells after a wash with Ligand Buffer and allowed to stimulate for 1 hour at 37 $^{\circ}$C. Compound solutions were removed and 30 µL Conjugation & Lysis buffer (IP-One HTRF® kit, Cisbio, Codolet, France) was added and allowed at least 30 minutes to lyse the cells. For cells expressing CaSR, Ligand Buffer without Ca^{2+} was used and cells were stimulated for 30 minutes instead of 1 hour, to prevent pre-stimulation and overstimulation respectively.

10 µL cell lysate was transferred to a 384-well plate and 10 µL Assay Buffer (HBSS + 1 mM $MgCl_2$ + 1 mM $CaCl_2$) containing 2.5% (v/v) anti-IP_1 cryptate Tb and 2.5% (v/v) IP_1-d2 (IP-One HTRF® kit, Cisbio, Codolet, France) was added and allowed to incubate in darkness for 1 hour. The light emitted at 615 nm and 665 nm was then measured on an EnVision plate reader (PerkinElmer Life and Analytical Sciences,

Waltham, MA) after excitation at 340 nm. The FRET ratios (665 nm/615 nm) were converted to concentrations of IP_1 using the provided standard.

Pharmacological data analysis

Concentration-response experiments were performed in triplicate of at least three independent experiments. Single concentration tests were performed in triplicate with two independent experiments. Curve fitting, determination of pIC_{50} values and statistical analysis was done using the non-linear regression curve-fitting program Prism v. 6.0d (GraphPad, San Diego, CA, USA). Compounds with low potency (see footnotes in Table 1 and Table 2) had the lower plateau of the curve restrained to levels corresponding to 1 µM of the specific G_q inhibitor UBO-QIC for GPRC6A experiments and buffer controls for other receptors tested.

Associated content.

Supporting information.

Equipment and general experimental information, ^{1}H and ^{13}C NMR spectra for all synthesized compounds, including selected 2D NMR spectra and HPLC chromatograms for all synthesized and UV-active compounds.

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Notes.

The authors declare no competing financial interest.

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Abbreviations used.

5-HT_{2C} = 5-Hydroxytryptamine receptor subtype 2C; Boc_2O = Di-*tert*-butyl dicarbonate; CaSR = Calcium-Sensing Receptor; CHO = Chinese Hamster Ovary; DMEM = Dulbecco's Modified Eagle Medium; DMSO = Dimethylsulfoxide; EDCI = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; FRET = Förster resonance energy transfer; $GABA_B$ = γ-aminobutyric acid type B receptor; GPCR = G-protein coupled receptor; GPRC6A = G protein-coupled receptor, class C, group A, subtype 6; HBSS = Hank's Balanced Salt Solution; HEK-293T = Human Embryonic Kidney 293T;

HEPES = 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; IP_1 = inositol monophosphate; M_3 = muscarinic acetylcholine receptor subtype 3; mGluR5 = Metabotropic Glutamate Receptor subtype 5; SAR = structure activity relationship; TBDMS = *tert*-butyldimethylsilyl; TFA = Trifluoroacetic acid; UBO-QIC = L-Threonine,(3*R*)-*N*-acetyl-3-hydroxy-L-leucyl-(a*R*)-a-hydroxybenzenepropanoyl-2,3-idehydro-*N*-methylalanyl-L-alanyl-*N*-methyl-L-alanyl-(3*R*)-3-[[(2*S*,3*R*)-3-hydroxy-4-methyl-1-oxo-2-[(1-oxopropyl)amino]pentyl]oxy]-L-leucyl-*N*,*O*-dimethyl-,(7→1)-lactone (9CI).

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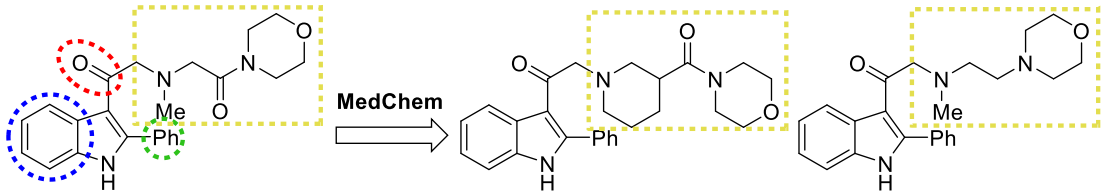
Table of Contents Graphic.



GPRC6A IC_{50} = 22 μM
selectivity (3-fold)

GPRC6A IC_{50} = 5.5 μM
selectivity (9-fold)

GPRC6A IC_{50} = 6.2 μM
selectivity (9-fold)