Fluorescently Labelled Morphine Derivatives for Bioimaging Studies

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ABSTRACT: Opioids, like morphine, are the mainstay analgesics for the treatment and control of pain. Despite this, they often exhibit severe side effects that limit dose; patients often become tolerant and dependent on these drugs, which remains a major health concern. The analgesic actions of opioids are primarily mediated via the μ-opioid receptor, a member of the G protein-coupled receptor superfamily. Thus far, development of small molecule fluorescent ligands for this receptor has resulted in antagonists, somewhat limiting the use of these probes. Herein, we describe our work on the development of a small molecule fluorescent probe based on the clinically used opiate morphine, and initial characterization of its behavior in cell-based assays.

■ INTRODUCTION

Opioids represent a useful class of drugs for the control and management of pain. Morphine and codeine, the active ingredients in opium from the plant *Papaver somniferum*, have been used for this purpose since early history.[1] More recently, semi-synthetic opioids have also become important therapeutic agents, with drugs such as oxycodone being useful therapeutic agents. Semi-synthetic opioid antagonists such as naloxone and naltrexone are also important therapeutic agents to treat opioid overdose. Despite their clinical effectiveness, opioid agonists suffer from significant drawbacks; dose-limiting side effects such as constipation, sedation and respiratory depression are common.[1] In addition to this, opioid drugs tend to be addictive and require higher doses as the patient develops a tolerance for them, further compounding the problem. In the United States alone, the CDC reported that opioid related deaths accounted for 61% of drug overdose related deaths between 2010 to 2015.[2] Furthermore, the number of incidents has increased dramatically, with death rates tripling during this period compared to 1999.

Opioids act via the three opioid receptor subtypes, namely the μ, κ and δ opioid receptors (MOR, KOR, and DOR respectively). The more recently discovered nociception/orphanin FQ peptide receptor (NOPr) is another member of the opioid receptor family, but does not bind these clinically used morphinan opioids.[3] Herein, we will only discuss the MOR, as this receptor is the primary target of the most commonly used analgesics,[4] and therefore holds significant clinical interest for the development of future analgesics.

Binding of an agonist to MOR typically activates $G_{i/o}$ proteins, resulting in inhibition of adenylate cyclase, and therefore a reduction in cyclic adenosine monophosphate (cAMP) levels.[1] This results in an influx of K^+ via G protein-mediated K^+ channels, causing neuron hyperpolarization and blockade of voltage-gated Ca^{2+} channels.[5] Hyperpolarization reduces firing potential, and therefore blockade of pain signaling, giving opioids their analgesic effects. Upon

prolonged activation, β-arrestin 2 recruitment to the MOR terminates signaling and induces internalization of the receptor.

However, not all opioids recruit arrestin and internalize with the same efficacy and, indeed, β-arrestin biased ligands have been discovered which give different pharmacological outcomes.[6] The prototypical opiate morphine causes very little β-arrestin 2 recruitment following MOR receptor activation, while also inducing significant dependency and tolerance in the patient.[7] Herkinorin, like morphine, also provides analgesia with little β-arrestin 2 recruitment, but shows very low dependence and tolerance liabilities in animal models.[8] DAMGO, a peptidic agonist, induces β-arrestin 2 recruitment while having reduced dependence and tolerance liabilities.[7] PZM21, a more recent example, appears to be biased towards G_i signaling, lacking any significant β-arrestin 2 recruitment.[6] Analgesia in mice has been demonstrated with fewer side effects compared to morphine. Just from these few examples, it can be seen that different agonists have different pharmacological outcomes despite all acting at MOR. Furthermore, Bohn *et al.* have demonstrated that tolerance and dependence are not necessarily correlated, as β-arrestin 2 knockout mice still develop a dependence towards morphine, but lack the expected tolerance.[9]

Understanding the mechanisms behind the different abilities of opioid ligands to promote arrestin recruitment and regulate receptor signaling poses a significant challenge. The development of fluorescent ligands to visualize G protein-coupled receptors (GPCRs) in live cells represents a major advantage that circumvents the use of recombinant cells and allows the study of these receptors in natively expressing systems. One of the earliest examples of a fluorescent opioid is described by Fournie-Zaluski *et al*. in which enkephalins were conjugated to a fluorescent dansyl sulfonyl group.[10] This particular ligand has seen limited use, perhaps due to the UV excitation maximum and poor quantum yield of the dansyl sulfonyl group not being particularly suitable to modern live cell imaging techniques. The same was true of probes described by Mihara *et al*. who conjugated enkephalins to the fluorophore *L*-1-pyrenylalanine.[11] In both cases, introduction of the fluorescent moiety resulted in a reduction in binding affinity compared to the native ligand. More

recent variants of these peptidic conjugates have been described by Arttamangkul *et al*. using more modern fluorophores.[12] These new fluorescent ligands have been used to study MOR internalization, desensitization and recycling.[13–15]

Small molecule fluorescent opioids have also been described. Kolb *et al.* described the synthesis of several fluorescent antagonists based on naloxone and naltrexone.[16] Another opioid probe bearing 7-nitrobenzo-2-oxa-1,3-diazole fluorescent label was described by Archer *et al.*[17] quickly followed by another example from Emmerson *et al.*[18] Most recently, Schembri *et al*. synthesized a series of orvinols based on the buprenorphine scaffold and conjugated them to a range of fluorophores.[19] However, in all these cases, the fluorescent probes described were antagonists. Although fluorescent antagonists are still useful to visualize receptors at the plasma membrane and to assess receptor binding, by definition, they do not allow the study of events that follow receptor activation. Herein, we describe our work on the synthesis and evaluation of small molecule fluorescent opioid partial agonists.

■ RESULTS

Model Morphine Congeners. Our aim was to develop fluorescent derivatives of morphine, as this clinically used drug is of particular interest. The C-6 position was chosen as our linking point, as the body of prior literature suggests that modifications at this point have minimal effect on ligand activity compared to alternative positions.[20,21] In order to assess whether our linking modifications would affect ligand activity, we first assessed the proposed structural modifications. To this end, we designed and synthesized model compounds comprising the targeting ligand with short chain linker modifications. Several linking methods were proposed and these model compounds are illustrated in Figure 1. Importantly, these compounds and their syntheses were designed such that the C-6 stereocenter would be preserved as the native *S* configuration.

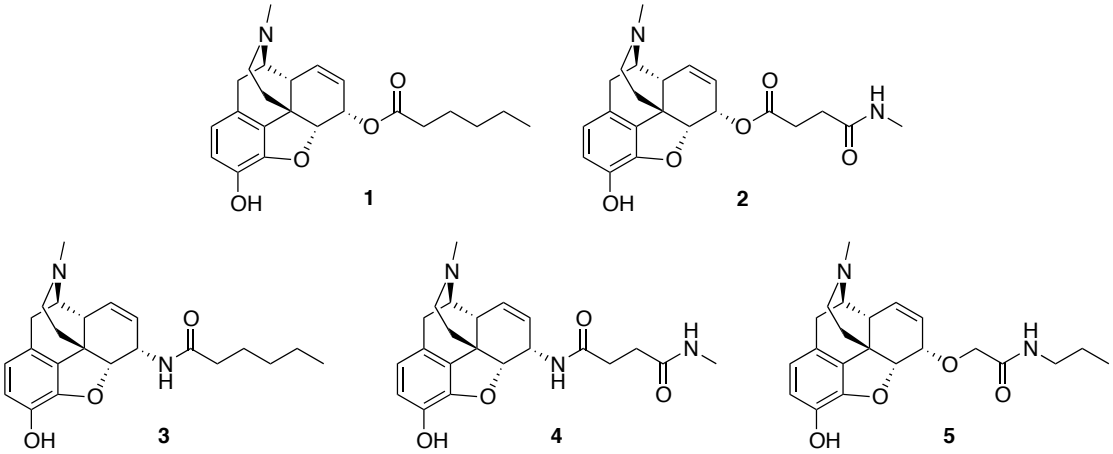
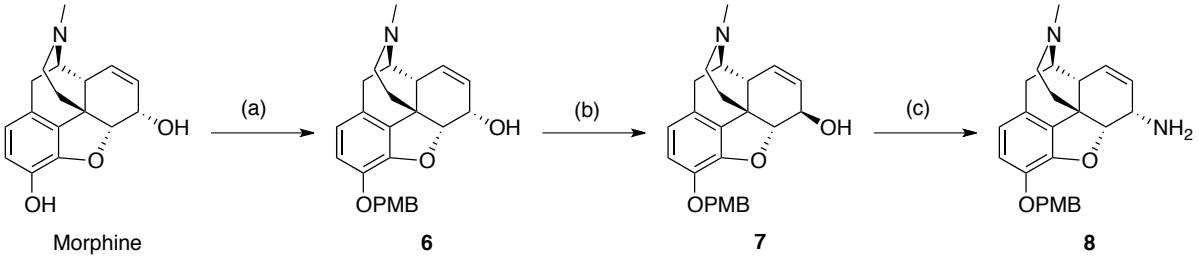


Figure 1. Pre-congeners synthesized for assessing linker suitability. Note preservation of the C-6 stereocenter.

Synthesis of our model compounds employed morphine as the starting material. This approach necessitated initial protection of the C-3 phenol, for which we chose the *para*-methoxybenzyl (PMB) group, and this was installed in modest yield to afford **6** (Scheme 1). This material formed the basis for all further synthetic manipulations.

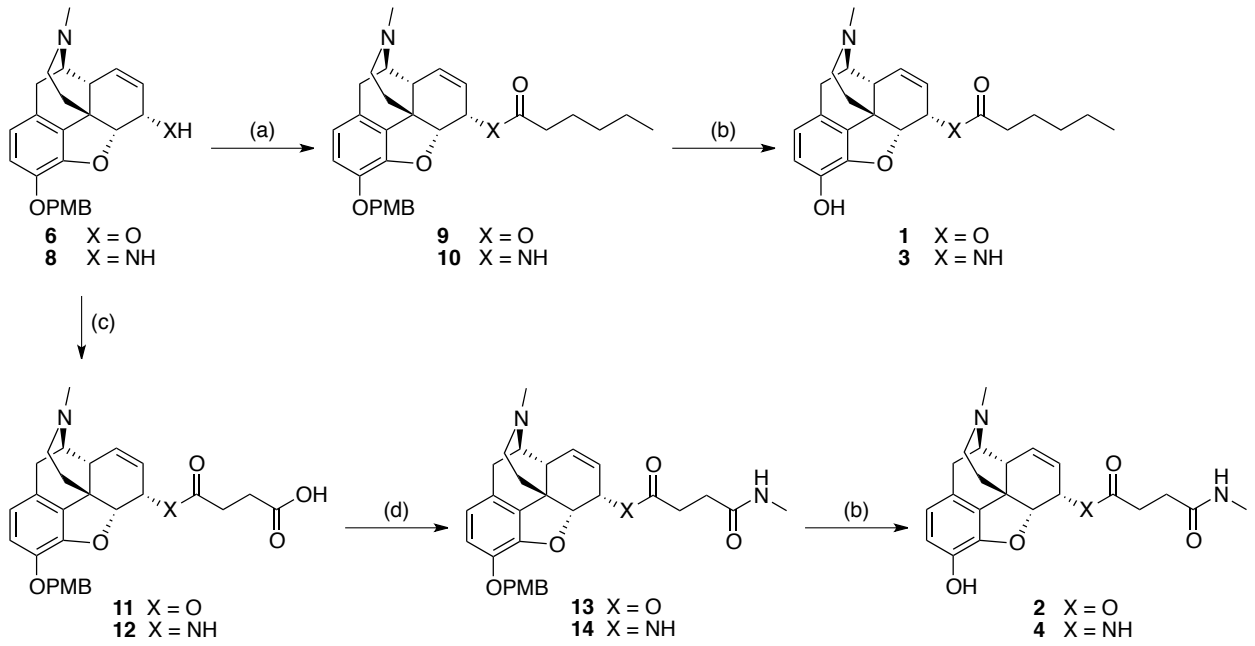
Scheme 1. Synthesis of C-6 position amine.



Reagents and conditions: (a) 3 M KOH in MeOH, PMB-Cl, DMF, RT, 4 h, 46%; (b) (i) benzoic acid, PPh_3, DIAD, toluene, 0 ^oC to RT, 4 h; (ii) 1:1 EtOH/1 M KOH, reflux, 20 min, 78%; (c) (i) phthalimide, PPh_3, DIAD, toluene, 0 ^oC to RT, 4 h; (ii) EtOH, $N_2H_4.H_2O$, reflux, 1 h, 34%.

An amine moiety was subsequently introduced into the 6-position to provide a point of attachment which retains the capacity to act as a hydrogen bond donor as per the 6-OH of morphine after the linker has been attached. As we also wished to maintain the native stereochemistry of the C-6 position a double Mitsunobu approach was employed to install this amine, noting that the Mitsunobu reaction has previously been reported to invert this stereocenter in closely related opiates.[22-25] Accordingly, 6-OH was first inverted using benzoic acid under Mitsunobu conditions followed by saponification of the resultant ester to give the 6-isomorphine derivative **7**. A comparison of the NMR spectra of compounds **6** and **7** (Figure S1) demonstrated stereospecific inversion of this chiral center. Compound **7** was subsequently converted to the desired *S*-stereoisomer via a phthalimide promoted Mitsunobu reaction. Treatment of the phthalimide group with hydrazine hydrate afforded **8**, allowing for linking and elongation to the desired model compounds (Scheme 1).

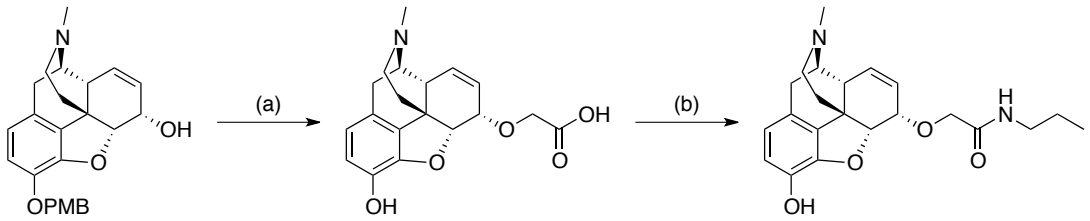
Scheme 2. Synthesis of C-6 position ester and amide pre-congeners.



Reagents and conditions: (a) Hexanoyl chloride, pyridine, 70 °C, 18 h, 49-80%; (b) triethylsilane, TFA, DCM, RT, 20 min, 16-39%; (c) succinic anhydride, DMAP, TEA, MeCN, 65 °C, 16 h, 28-77%; (d) EDC.HCl, HOBt, TEA, methylamine (2 M in THF), MeCN or DMF, RT, 3 h, 63-84%.

Two different approaches for ester and amide conjugation were trialed. The first, using an acid chloride, yielded compounds **1** and **3** following phenolic deprotection (Scheme 2). Fluorophore conjugation via acyl chloride chemistry is not a viable synthetic strategy and we therefore designed a method which would facilitate milder reaction conditions. Ring opening of succinic anhydride via the C-6 amine or alcohol provided a convenient handle for further elongation and subsequent fluorophore conjugation under milder conditions. Methylamine capping of this handle, followed by phenolic deprotection gave model compounds **2** and **4** (Scheme 2).

Scheme 3. Synthesis of C-6 position ether pre-congener.



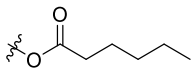
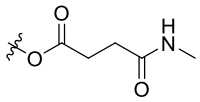
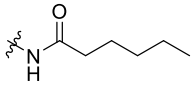
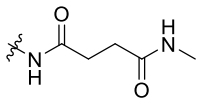
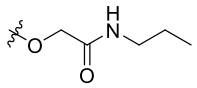
6 **15** **5**

Reagents and conditions: (a) (i) NaH, *tert*-butyl bromoacetate, THF, 0ºC-RT, 1 h; (ii) triethylsilane, TFA, DCM, RT, 1 h, 58%; (b) HCTU, TEA, propylamine, DMF, RT, 19 h, 17%.

As an alternative linking method, an ether linker was also proposed. Using *tert*-butyl bromoacetate to give the C-6 ether, followed by global deprotection gave **15**, again possessing a convenient handle for further linker elongation. This handle was capped with propylamine to provide the final model amide **5** (Scheme 3).

Model compounds were functionally assessed and compared to morphine using a bioluminescence resonance energy transfer (BRET)-based cAMP sensor using YFP-Epac-RLuc assay (CAMYEL) in HEK293 cells expressing the human MOR (hMOR) and normalized against forskolin (Table 1). All compounds were less potent than morphine, however the ester linked model compounds (**1** and **2**) maintained potencies in the mid nM range, while the amide-linked model compounds (**3** and **4**) showed a significant loss in potency beyond the μM range. The ether linked model compound **5** however maintained the same level of maximum activity and was the most potent model congener.

Table 1. *p*EC_{50} of model compounds as assessed in a cAMP CAMYEL BRET assay against hMOR.

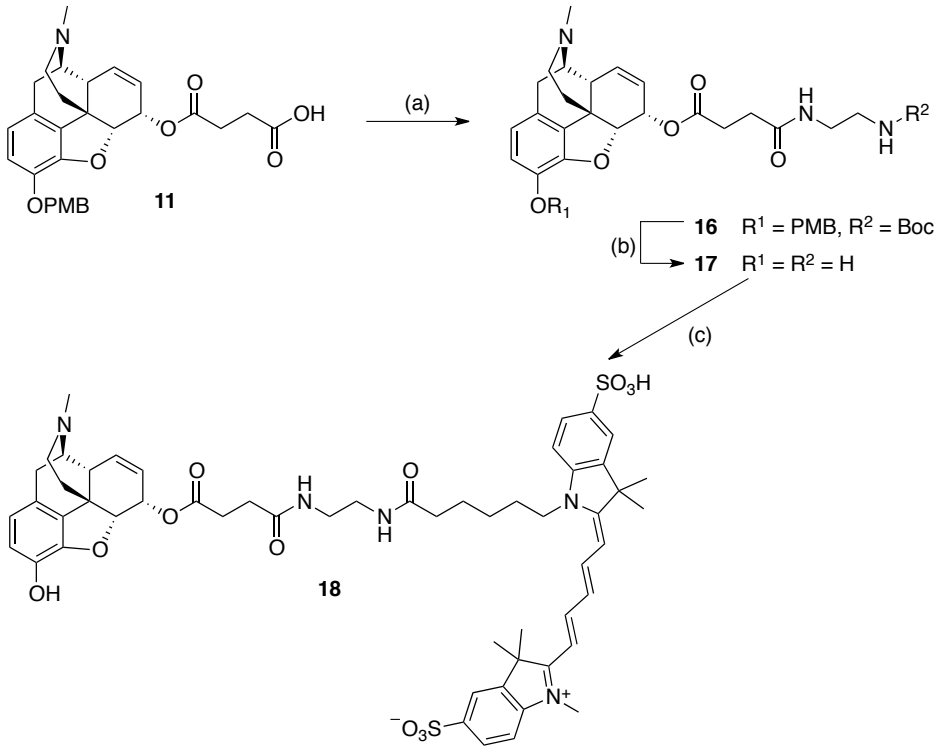
Cpd	6-Substituent	*p*EC_{50} ± SEM [a]	Relative potency [b]	E_{max} ± SEM [a,c]	Relative efficacy [d]
Morphine	-OH	7.07 ± 0.13	-	35.7 ± 1.7	-
1		6.46 ± 0.11	0.24	37.1 ± 1.9	1.04
2		6.45 ± 0.11	0.24	28.6 ±1.4	0.80
3		5.98 ± 0.11	0.08	28.9 ± 1.9	0.81
4		5.35 ± 0.71	0.02	23.3 ± 11.7	0.65
5		6.81 ± 0.14	0.55	37.5 ± 2.4	1.05

[a] Results presented are the average of 3 independent experiments.
[b] Relative to morphine. Relative potency was calculated by dividing EC_{50}(morphine) by EC_{50}(compound).
[c] E_{max} is the % inhibition of Forskolin-induced cAMP.
[d] Relative to morphine. Relative efficacy was calculated by dividing E_{max}(compound) by E_{max}(morphine).

Morphine-Cy5 Conjugates. We then proceeded to synthesize two fluorescent probes based on linker designs **2** and **5**. Sulfo-Cy5 was chosen as the fluorescent tag, as previous work by Schembri *et al.* suggested that this particular fluorophore had the least potential for inducing non-specific binding to the cell membrane.[19] The functional handles on **11** and **15** were elongated with a simple Boc-protected diamine chain, followed by global deprotection and chemoselective conjugation to sulfo-Cy5 to give the final fluorescent morphine probes **18** and **21** (Schemes 4 and 5, respectively). In the case of **18**, the coupling reaction with sulfo-Cy5 NHS ester failed to proceed to completion after 7 hours. As it was thought this may have resulted from the hydrolysis of the NHS ester to the corresponding carboxylic acid, HCTU was added to drive the reaction to completion (Scheme 4).

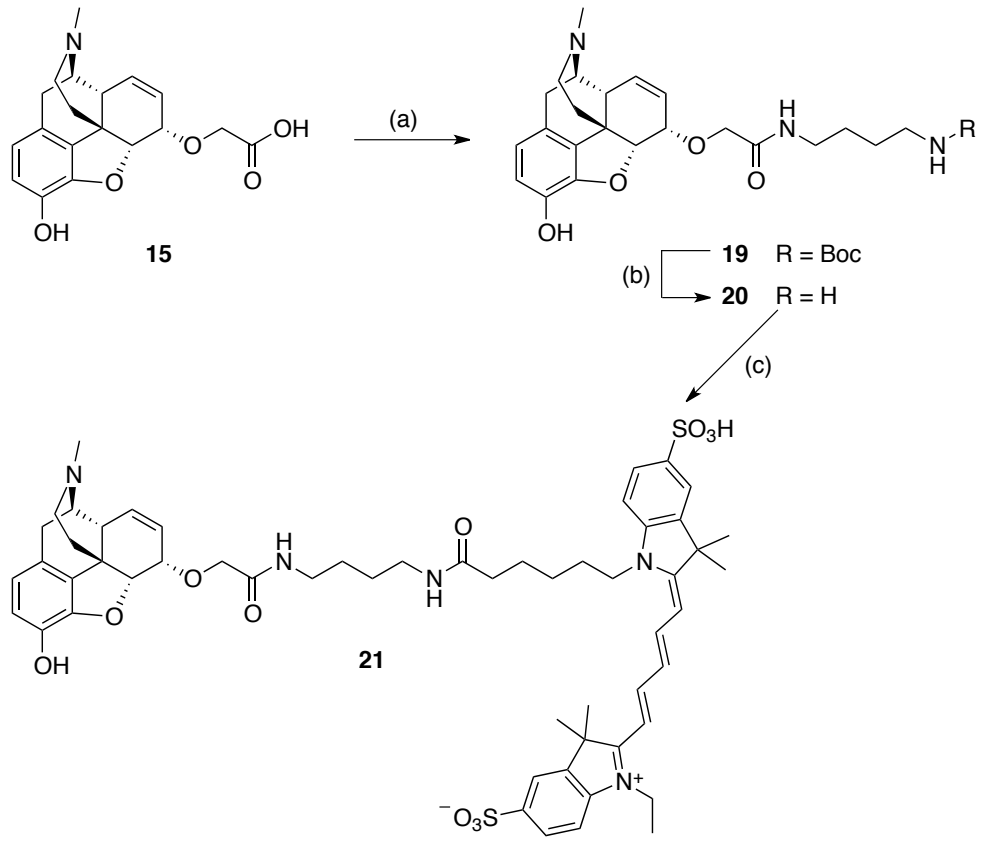
In the case of **21**, sulfo-Cy5 free acid was used in place of the NHS ester and was installed under standard HCTU coupling conditions (Scheme 5).

Scheme 4. Synthesis of ester linked morphine-Cy5 probe **18.**



Reagents and conditions: (a) *N*-Boc-diaminoethane, HCTU, TEA, DMF, RT, 19 h, 83%; (b) triethylsilane, TFA, DCM, RT, 1 h, 51%; (c) Sulfo-Cy5 NHS ester (Lumiprobe®), HCTU, TEA, DMF, RT, 21 h, 33%.

Scheme 5. Synthesis of ether linked morphine-Cy5 probe **21.**



Reagents and conditions: (a) *N*-Boc-diaminobutane, EDC.HCl, HOBt, TEA, DMF, RT, 17 h, 36%; (b) TFA, $CHCl_3$, RT, 1 h, quantitative; (c) Sulfo-Cy5, HCTU, TEA, DMF, RT, 18 h, 40%.

Functional assessment of **18** and **21** was conducted in the same manner as the model conjugates (Table 2). Although both sulfo-Cy5 derivatives were still able to activate MOR with potencies in the nM range, they showed a significant loss of efficacy (E_{max}). This loss in activity is consistent with previous literature compounds, where attachment of a fluorescent tag typically results in decreased activity.[12,26] A comparison with DAMGO in the same assay revealed that **21** clearly behaves as a partial agonist, albeit with a lower efficacy compared to the parent compound morphine (Figure 2).

Table 2. *p*EC_{50} assessment of morphine-Cy5 probes.

Probe	*p*EC_{50} ± SEM [a]	Relative potency [b]	E_{max} ± SEM [a,c]	Relative efficacy [d]
Morphine	7.07 ± 0.13	--	35.7 ± 1.7	--
18	7.34 ± 0.37	1.85	10.7 ± 1.2	0.30
21	6.30 ± 0.44	0.17	15.0 ± 3.2	0.42

[a] Results presented are the average of 3 independent experiments.

[b] Relative to morphine. Relative potency was calculated by dividing EC_{50}(morphine) by EC_{50}(compound).

[c] E_{max} is the % inhibition of Forskolin-induced cAMP.

[d] Relative to morphine. Relative efficacy was calculated by dividing E_{max}(compound) by E_{max}(morphine).

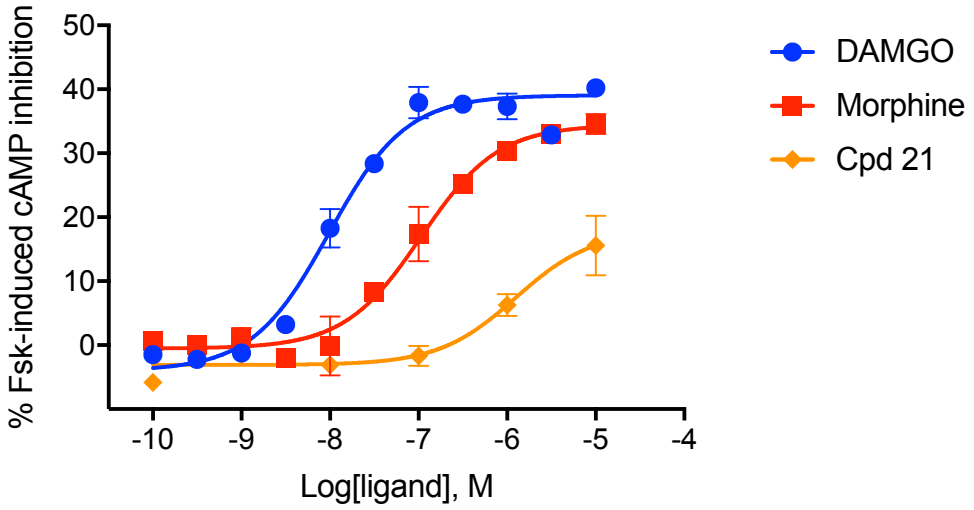


Figure 2. CAMYEL assay comparing DAMGO, morphine and **21**. The probe compound **21** is clearly a partial agonist compared to the full agonist DAMGO. Morphine is known to be a partial agonist, but may give differing levels of efficacy depending on the assay used. Results for DAMGO and morphine are consistent with those previously reported.[27,28] Results presented are an average of 3 independent experiments, with error bars representing SEM.

We then assessed whether compounds **18** and **21** were suitable for receptor visualization using confocal microscopy. Initial experiments were conducted to determine whether **18** and **21** bound specifically to the MOR. HEK293 stably expressing SNAP-tagged hMOR (SNAP-hMOR) were pre-incubated with cell impermeable SNAP-Surface® 488 (BG-488, New England BioLabs®, Figure 3) to label the cell surface SNAP-hMOR, then incubated in the presence or absence of the antagonist naloxone and before exposure to the fluorescent probes. Despite having a higher apparent pEC_{50} value, **18** showed no specific binding to the cell surface receptor population (Figure 4, panel A). On the other hand, **21** was observed to localize at the surface of cells expressing SNAP-hMOR (Figure 4, panel B). **21** binding was abolished by pre-incubation with naloxone (Figure 4, panel C).

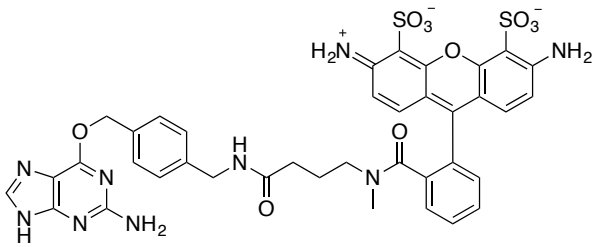


Figure 3. SNAP-Surface® 488 (New England BioLabs®), a cell impermeable dye targeted to the SNAP domain of SNAP-tagged receptors. This dye enables exclusive labelling of cell surface receptor populations.

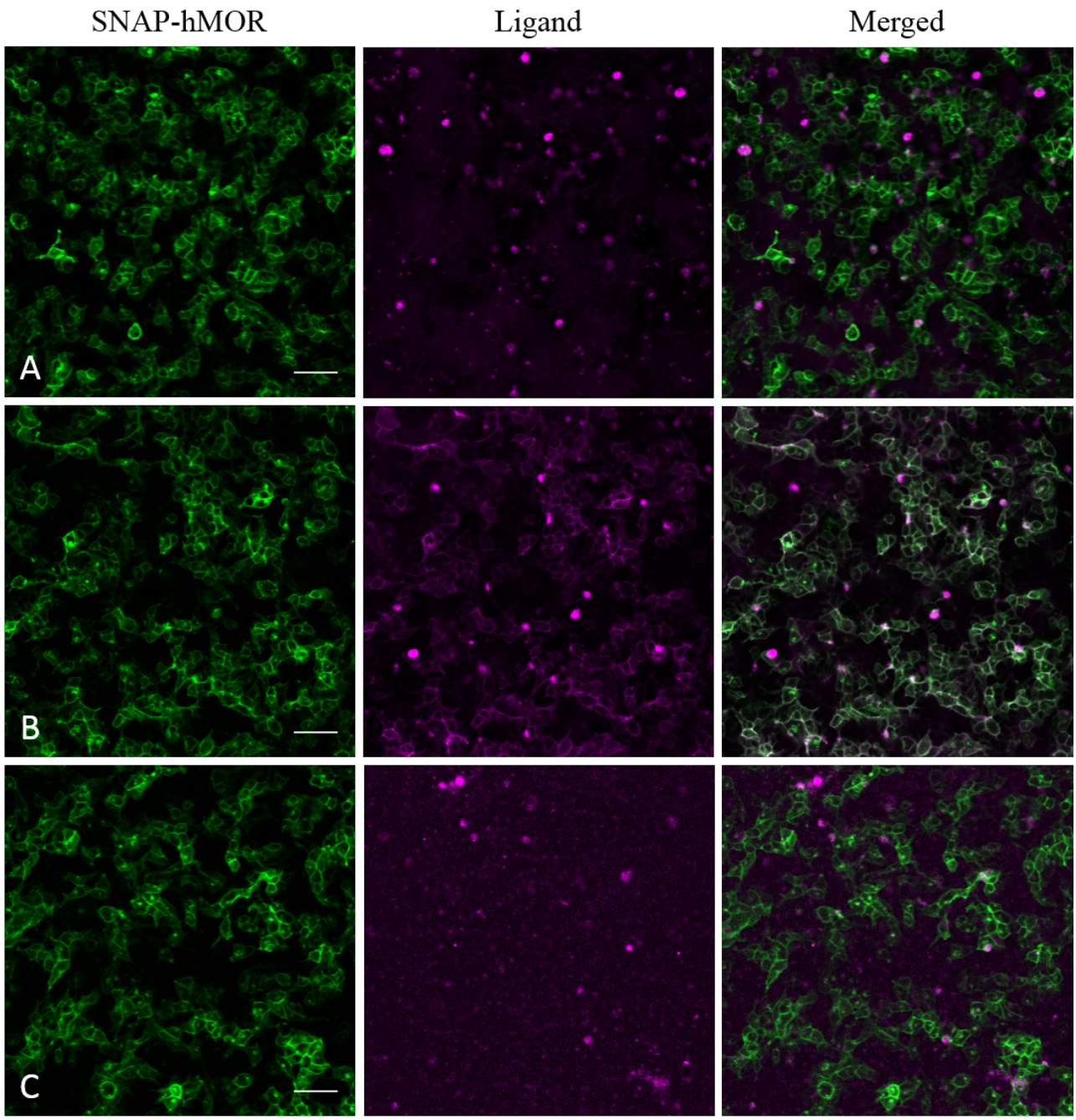


Figure 4. Initial high-content imaging of probes with HEK293 cells expressing SNAP-hMOR. Images are representative of 3 independent experiments. A) **18** at 3 μM, no specific binding of the probe to the cell surface was observed; B) **21** at 1 μM, binding of the probe to the cell surface was observed; C) **21** at 1 μM pre-incubated with naloxone (1 μM, 30 min, 37°C), complete blockade of probe binding was observed. Scale bars 50 μm.

Having demonstrated that **21** could be used to visualize specific binding to the MOR on HEK293 cell membranes, this probe was selected for subsequent study. In HEK293 cells stably

expressing SNAP-hMOR and pre-incubated with BG-488, it was found that **21** co-localized with SNAP-hMOR predominantly on the cell membrane (Figure 5, panels A - B), which was prevented by pre-incubation with naloxone, confirming the membrane binding of **21** was to the hMOR (Figure 5, panel C). The bulk of the co-localized fluorescence remained at the cell surface. Although some co-localized signals within the cells were present, the signal from intracellular compartments was very weak compared to that at the plasma membrane. These data are in keeping with literature data, which suggests that morphine only poorly induces internalization of the receptor-ligand complex.[7] Since incubation with BG-488 only labels cell surface populations of SNAP-hMOR, any internalized receptor population must therefore have previously been on the cell surface. In keeping with prior literature, it was found that a wash step removed bound ligand (Figure 5, panel D).[19] This is consistent with the fast k_{off} rate of non-peptide opioids and the known fast k_{off} rate of morphine, so this behavior is not unexpected. In any case, a wash step is typically not included in confocal microscopy experiments, particularly where there is no significant background from extracellular ligand, as in this case.[19,29,30,31]

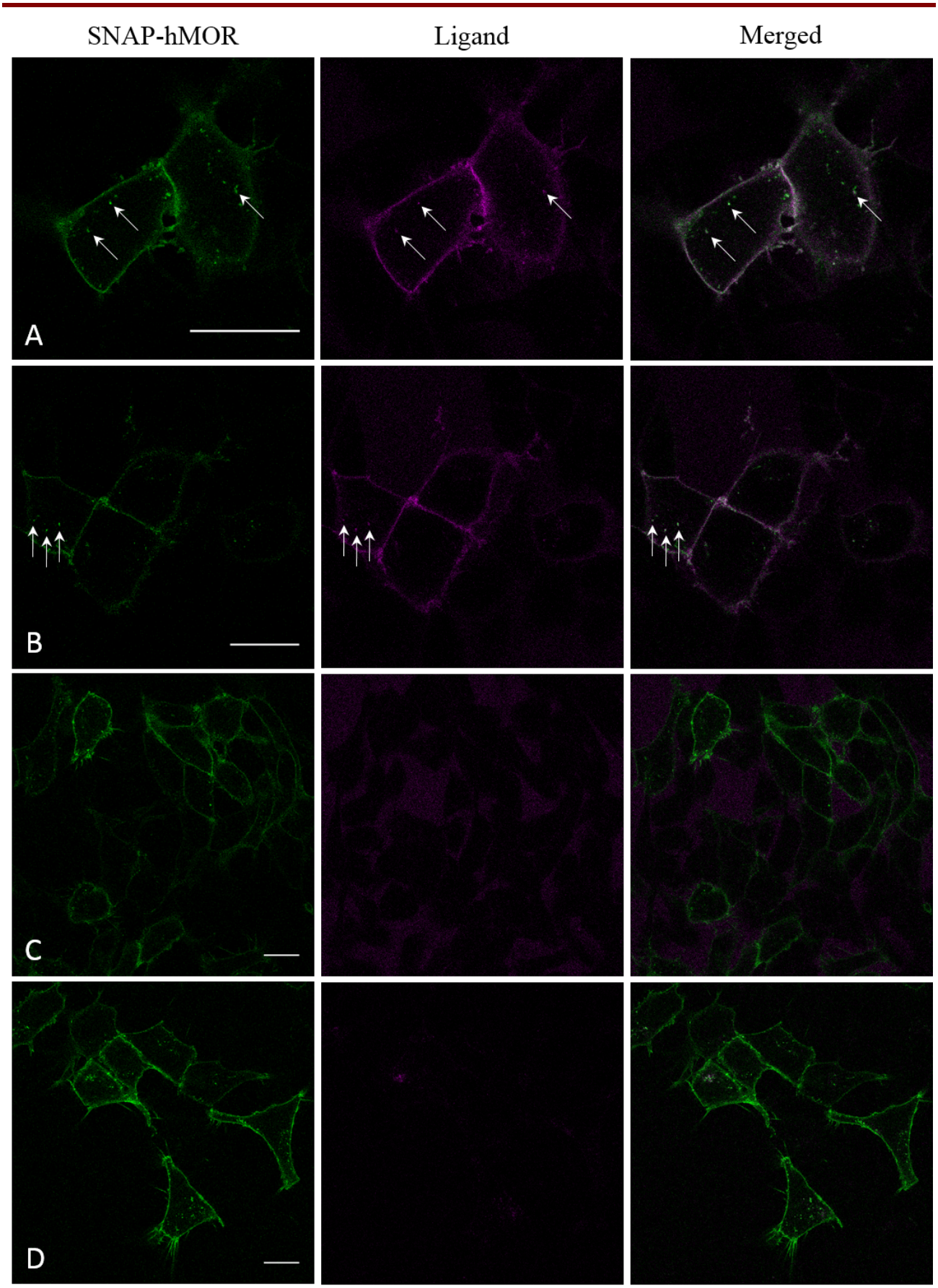

SNAP-hMOR
Ligand
Merged
A
B
C
D

Figure 5. Live cell confocal imaging of **21** in cells expressing SNAP-hMOR. Images are representative of 3 independent experiments. In all cases, **21** was incubated at 500 nM for 30 min at 37°C following pre-treatment (with or without naloxone). A, B) Typical results obtained from confocal microscopy, the white arrows indicate areas where co-localization of intracellular receptor populations with the fluorescent ligand were observed; C) **21** following pre-incubation with naloxone (1 μM, 30 min, 37°C), indicating specific binding of the probe to hMOR; D) Loss of ligand signal following a post-incubation wash. This is typical of non-peptide opioids, which tend to have a fast k_{off} rate. Scale bars 20 μm.

In the previous experiments, the EC_{50} concentration of 500 nM was used, thus we wanted to assess the behavior of **21** above this concentration. The use of 10 μM of **21** necessitated a wash step to remove background fluorescence from any excess ligand (Figure 6, panel A). As demonstrated, the level of observed co-localization between **21** and SNAP-hMOR in the intracellular compartment is significantly increased, with minimal cell surface co-localization due to the wash step as expected. Furthermore, this signal could be removed by blockade with naloxone, further demonstrating the lack of non-specific binding of **21** even at this higher concentration (Figure 6, panel B).

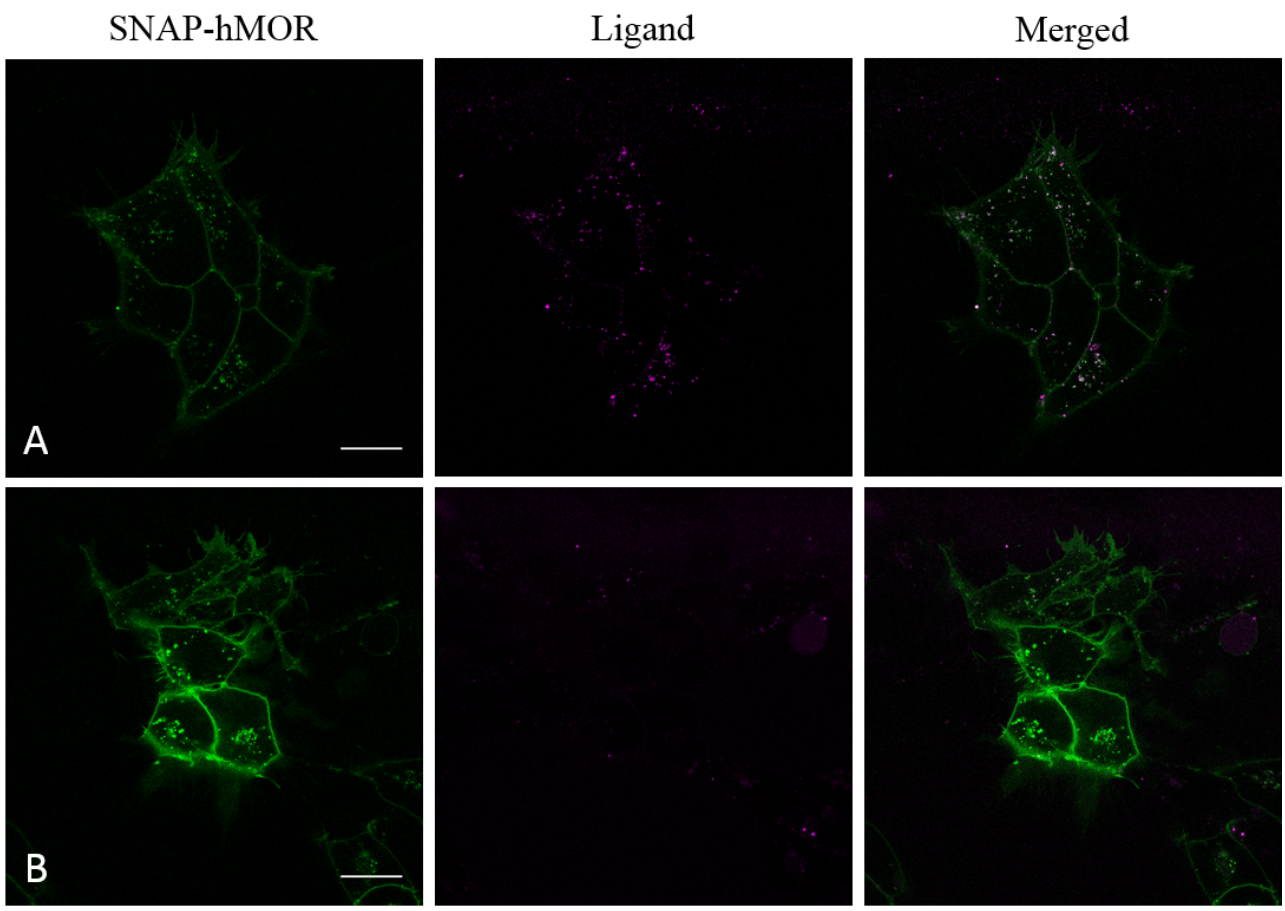


Figure 6. Live cell confocal imaging of **21** at increased concentration in cells expressing SNAP-hMOR. Images are representative of 3 independent experiments. A) **21** at 10 μM, note the increased level of internalized receptor populations compared to the data presented in Figure 4; B) **21** at 10 μM following pre-incubation with naloxone (1 μM, 30 min, 37 ^{o}C), demonstrating specific displacement of **21** with little background fluorescence. Scale bars 20 μm.

To directly compare, experiments were conducted using the parent compound morphine as the ligand (Figure 7). Obviously the lack of a fluorescent tag prevents direct visualization of the ligand, but observation of the tagged receptor fluorescence is still of value, as it enables visual comparisons to be made between the behavior of our fluorescently tagged probe and its parent compound. A baseline level of receptor internalization was first determined in cells with no ligand present (Figure 7, panel A). These data show that a low constitutive level of receptor internalization is present, even without stimulation. Morphine at 500 nM does not induce any significant receptor internalization, with levels similar to that of the baseline level (Figure 7, Panel B). Furthermore,

these levels of internalization are similar to when 500 nM of **21** was used (Figure 5, Panel A, B). This reinforces the idea that morphine is only weakly able to induce internalization of the MOR. Furthermore, at 10 μM morphine, the level of internalization was comparable to that observed with 10 μM of **21** (Figure 7, Panel C compared to Figure 6, Panel A). Taken together, these data suggest that our probe behaves very similarly to morphine, which may make it a useful tool in the study of morphine pharmacology.

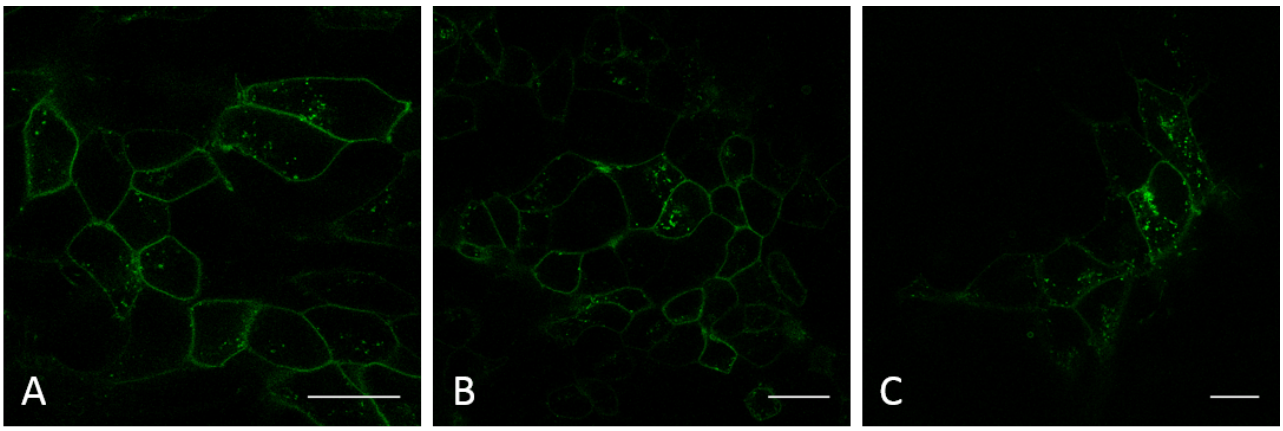


Figure 7. Live cell confocal imaging of SNAP-hMOR in cells exposed to morphine for comparison to **21**. Images are representative of 3 independent experiments. A) Cells with no stimulation, note the constitutive receptor turnover, even in the absence of any external internalizing stimuli; B) 500 nM morphine, the level of internalization is similar to that observed when no stimuli is applied, which is in keeping with literature data suggesting morphine's inability to induce internalization; C) 10 μM morphine, significantly more receptor populations are observed to internalize, and therefore can be attributed to the ligand stimulus. Scale bars 20 μm.

■ DISCUSSION AND CONCLUSIONS

Herein, we have described the design and synthesis of a fluorescent partial agonist probe for MOR based on the commonly used analgesic morphine. Our studies have shown that probe compound **21** is able to bind to hMOR on cell membranes, and behaves in a similar manner to the parent compound morphine. At EC_{50} concentrations, the probe does not induce internalization of the receptor, but is present in intracellular compartments at levels consistent with constitutive turnover. At higher concentrations, the probe is able to induce internalization at similar levels to morphine. These data suggest that the probe compound **21** may be a used as a tool compound to simulate the behavior of morphine, much in the same way DERM-A594 is used as a tool compound for the study of endogenous opioid peptides.[15] It is clear that morphine has significantly different signaling profile compared to peptides,[23,24] and therefore small molecule-based fluorescent probes may lead to different outcomes compared to studies where fluorescent peptides have been utilized. This new tool compound could also be used in conjunction with the previously described small-molecule fluorescent opioid antagonists to study the differences in ligand-receptor complexes in natively expressing systems, rather than in transfected systems.[19]

■ EXPERIMENTAL SECTION

Chemistry. Chemicals and solvents were purchased from standard suppliers and used without further purification. Davisil® silica gel (40-63 μm) for flash column chromatography was supplied by Grace Davison Discovery Sciences (Victoria, Australia) and deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (USA, distributed by Novachem PTY. Ltd, Victoria, Australia). Davisil® reverse phase silica gel (C18, 10–14 μm) for reverse phase flash column chromatography was supplied by Grace Davison Discovery Sciences (Victoria, Australia) and run using the following buffers; buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in MeCN.

Reactions were monitored by thin layer chromatography on commercially available pre-coated aluminium-backed plates (Merck Kieselgel 60 F_{254}). Visualization was done by examination under UV light (254 and 366 nm). General staining was carried out with $KMnO_4$ or phosphomolybdic acid. Organic solvents were evaporated *in vacuo* at ≥ 40 °C (water bath temperature). Purification using preparative layer chromatography (PLC) was carried out on Analtech preparative TLC plates (200 mm × 200 mm × 2 mm).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance Nanobay III 400 MHz Ultrashield Plus spectrometer at 400.13 MHz and 100.62 MHz respectively. Chemical shifts (δ) are recorded in parts per million (ppm) with reference to the chemical shift of the deuterated solvent. Coupling constants (*J*) are recorded in Hz and the significant multiplicities described by singlet (s), doublet (d), triplet (t), quadruplet (q), broad (br), multiplet (m), doublet of doublets (dd), doublet of triplets (dt).

LC-MS were run to verify reaction outcome and purity using an Agilent 6120 Series Single Quad coupled to an Agilent 1260 Series HPLC. The following buffers were used; buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in MeCN. The following gradient was used with a Poroshell 120 EC-C18 50 × 3.0 mm 2.7 micron column, and a flow rate of 0.5 mL/min and total run time of 5 min; 0–1 min 95% buffer A and 5% buffer B, from 1-2.5 min up to 0% buffer A and 100% buffer B, held at this composition until 3.8 min, 3.8–4 min 95% buffer A and 5% buffer B, held until 5 min at this composition. Mass spectra were acquired in positive and negative ion mode with a scan range of 100 – 1000 *m/z*. UV detection was carried out at 214 and 254 nm.

Preparative HPLC was performed using an Agilent 1260 infinity coupled with a binary preparative pump and Agilent 1260 FC-PS fraction collector, using Agilent OpenLAB CDS software (Rev C.01.04), and an Altima 5 μM C8 22 × 250 mm column. The following buffers were used; buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in MeCN, with sample being run at a

gradient of 5% buffer B to 100% buffer B over 15 min, at a flow rate of 15 mL/min. All screening compounds were of > 95% purity.

High resolution mass spectrometry – time of flight (HRMS TOF) was conducted using an Agilent 6224 TOF LC-MS mass spectrometer coupled to an Agilent 1290 Infinity. Chromatographic separation was performed using an Agilent Zorbax SB-C18 Rapid Resolution HT 2.1 × 50 mm, 1.8 µm column using a gradient of 5 – 100% buffer B in buffer A over 3.5 min at 0.5 mL/min; where buffers are as defined for LC-MS. All mass data was acquired and reference mass corrected via a dual-spray ESI source. Mass spectra were created by averaging scans across each peak and background subtracting against the first 10 seconds of the total ion count. Acquisition was performed using the Agilent Mass Hunter Data Acquisition software, and analysis performed using Mass Hunter Qualitative Analysis software. The mass spectrometer was run using the following conditions: drying gas flow 11 L/min, nebulizer 45 PSI, drying gas temperature 325 ^{o}C, capillary voltage 4000 V, fragmentor 160 V, skimmer 65 V, OCT RFV 750 V, scan range acquired 100 – 1500 *m/z*, internal reference ions positive ion mode = *m/z* = 121.050873 & 922.009798.

General Deprotection Procedure A. The protected compound was dissolved in dry DCM (typically 5 mL) under an N_2 atmosphere. Triethylsilane (15 eq.) and TFA (20 eq.) were added to the reaction and the vessel purged with N_2. The reaction was allowed to stir for 30 - 60 min, then reduced *in vacuo*. The resulting residue could then be purified via chromatography.

6-*O*-Hexanoylmorphine (1). Compound **9** (67 mg, 0.13 mmol, 1 eq.) was deprotected using general deprotection procedure A. The resulting oil was purified by flash column chromatography (94:5:1 $CHCl_3$/MeOH/TEA), and the resulting yellow oil was further purified by prep-HPLC to give the product as a solid white TFA salt (12 mg, 17%). ^{1}H NMR (DMSO-d_6) δ 10.04 (s, 1H), 9.17 (s, 1H), 6.58 (d, *J* = 8.1 Hz, 1H), 6.50 (d, *J* = 8.1 Hz, 1H), 5.71–5.59 (m, 1H), 5.52–5.39 (m, *J* = 22.7, 9.8 Hz, 1H), 5.26–5.14 (m, 1H), 5.12–5.00 (m, *J* = 20.5, 6.6 Hz, 1H), 4.13 (s, 1H), 3.31 (d, *J* = 9.0 Hz, 1H), 3.19 (d, *J* = 19.7 Hz, 1H), 3.06 (d, *J* = 4.5 Hz, 1H), 2.98 (s, 1H), 2.92–2.78 (m, *J* =

41.2 Hz, 3H), 2.71 (dd, *J* = 20.0, 6.7 Hz, 1H), 2.44–2.29 (m, 2H), 2.21 (td, *J* = 13.5, 4.6 Hz, 1H), 1.98–1.70 (m, *J* = 46.3, 12.5 Hz, 1H), 1.64–1.51 (m, 2H), 1.34–1.21 (m, 4H), 0.87 (t, *J* = 6.9 Hz, 3H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{23}H_{29}NO_4^+$ calc. 384.2169, found 384.2174.

6-*O*-(4-Methylamino-4-oxobutanoyl)morphine (2). Compound **13** (26 mg, 0.050 mmol, 1 eq.) was deprotected using general deprotection procedure A. The resulting oil was purified by prep-HPLC to give the product as a solid white TFA salt (4 mg, 16%). ^{1}H NMR (DMSO-d_6) δ 10.04 (s, 1H), 9.23 (s, 1H), 7.85 (d, *J* = 4.4 Hz, 1H), 6.61–6.56 (m, *J* = 8.0, 3.1 Hz, 1H), 6.54–6.48 (m, *J* = 8.1, 4.7 Hz, 1H), 5.65 (d, *J* = 10.0 Hz, 1H), 5.52–5.39 (m, *J* = 24.1, 10.0 Hz, 1H), 5.24–5.14 (m, 1H), 5.09–4.99 (m, *J* = 22.2, 6.7 Hz, 1H), 4.13 (s, 1H), 3.34–3.25 (m, *J* = 8.5 Hz, 1H), 3.23–3.13 (m, *J* = 19.7 Hz, 1H), 3.06 (d, *J* = 4.8 Hz, 1H), 2.98 (s, 1H), 2.89 (d, *J* = 3.1 Hz, 2H), 2.82–2.67 (m, 2H), 2.62–2.58 (m, *J* = 6.9, 4.1 Hz, 2H), 2.57–2.55 (m, *J* = 4.5 Hz, 3H), 2.40 (t, *J* = 6.9 Hz, 2H), 2.21 (td, *J* = 13.4, 4.4 Hz, 1H), 1.94–1.74 (m, *J* = 46.2, 11.8 Hz, 1H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{22}H_{27}N_2O_5^+$ calc. 399.1914, found 399.1922.

***N*-((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-hydroxy-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)hexanamide (3).** Compound **10** (39 mg, 0.10 mmol, 1 eq.) was deprotected using general deprotection procedure A. The resulting oil was purified by prep-HPLC to give the product as a solid white TFA salt (12 mg, 31%). $[\alpha]_D^{25}$ -21.4 (*c* 1.21 mg/mL, MeOH); ^{1}H NMR (DMSO-d_6) δ 10.11–9.88 (m, 1H), 9.28 (s, 1H), 8.20–8.05 (m, *J* = 25.3, 8.5 Hz, 1H), 5.73 (dt, *J* = 10.3, 3.1 Hz, 1H), 5.58 (d, *J* = 10.5 Hz, 1H), 5.16–5.02 (m, *J* = 25.4 Hz, 1H), 3.85 (t, *J* = 8.7 Hz, 1H), 3.74 (s, 1H), 3.27–3.20 (m, *J* = 7.0 Hz, 1H), 3.18 (s, 1H), 3.12–3.02 (m, *J* = 19.8, 6.3 Hz, 1H), 2.98 (s, 1H), 2.89 (s, 3H), 2.77–2.56 (m, *J* = 48.6, 9.6 Hz, 2H), 2.40 (d, *J* = 8.1 Hz, 1H), 2.17–1.97 (m, 3H), 1.87 (d, *J* = 11.8 Hz, 1H), 1.56–1.43 (m, 2H), 1.31–1.14 (m, 4H), 0.85 (t, *J* = 7.0 Hz, 3H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{23}H_{31}N_2O_3^+$ calc. 383.2329, found 383.2324.

***N*^{1}-((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-hydroxy-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)-*N*^{4}-methylsuccinamide (4).** Compound **14** (19 mg, 0.037 mmol, 1 eq.) was deprotected using general deprotection procedure A. The resulting oil was

purified by prep-HPLC to give the product as a solid white TFA salt (7 mg, 39%). $[\alpha]_D^{25}$ -21.9 (*c* 0.78 mg/mL, MeOH); ^{1}H NMR (DMSO-d_6) δ 10.08–9.73 (m, *J* = 44.4 Hz, 1H), 9.25 (s, 1H), 8.28–8.06 (m, *J* = 29.6, 9.0 Hz, 1H), 7.79 (d, *J* = 4.6 Hz, 1H), 5.78–5.68 (m, 1H), 5.65–5.57 (m, 1H), 5.10 (t, *J* = 13.6 Hz, 1H), 3.94 (d, *J* = 4.6 Hz, 1H), 3.86 (t, *J* = 8.5 Hz, 1H), 3.22 (s, 1H), 3.17 (d, *J* = 19.5 Hz, 1H), 3.10–2.96 (m, 2H), 2.89 (d, *J* = 4.6 Hz, 2H), 2.79–2.65 (m, 1H), 2.55 (d, *J* = 4.6 Hz, 3H), 2.43–2.21 (m, 5H), 2.11–1.97 (m, 1H), 1.92–1.67 (m, 1H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{22}H_{28}N_3O_4^+$ calc. 398.2074, found 398.2079.

6-*O*-(2-oxo-2-(propylamino)ethyl)morphine (5). Compound **15** (54 mg, 0.12 mmol, 1 eq.), HCTU (59 mg, 0.14 mmol, 1.2 eq.) and TEA (50 µL, 0.36 mmol, 3 eq.) were dissolved in dry DMF (800 µL) under an N_2 atmosphere and allowed to stir at room temperature for 30 min. A solution of propylamine (12 µL, 0.14 mmol, 1.2 eq.) in DMF (200 µL) was then added and the resulting solution allowed to stir at room temperature for 19 h. The solvent was then removed *in vacuo* and the residue taken up in buffer B and again reduced *in vacuo*. The residue was purified by flash column chromatography (74:25:1 MeOH/$CHCl_3$/NH_4OH), the solvent removed *in vacuo* and purified again by prep-HPLC to give the product as a solid white TFA salt (10 mg, 17%). $[\alpha]_D^{25}$ -106.1 (*c* 1.14 mg/mL, MeOH); ^{1}H NMR (DMSO-d_6) δ 9.81 (s, 1H), 9.18 (s, 1H), 7.74 (t, *J* = 5.7 Hz, 1H), 6.62–6.52 (m, 1H), 6.52–6.43 (m, 1H), 5.82 (d, *J* = 9.7 Hz, 1H), 5.41–5.24 (m, 1H), 5.18–5.02 (m, 1H), 4.16–3.97 (m, 4H), 3.29 (s, 1H), 3.20 (d, *J* = 19.8 Hz, 2H), 3.14–3.04 (m, 3H), 2.97 (s, 1H), 2.90 (d, *J* = 3.9 Hz, 3H), 2.83 (d, *J* = 16.0 Hz, 2H), 2.74 (dd, *J* = 19.8, 6.6 Hz, 1H), 2.18 (td, *J* = 13.8, 4.9 Hz, 1H), 2.03–1.79 (m, 1H), 1.45 (h, *J* = 7.3 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{22}H_{29}N_2O_4^+$ calc. 385.2122, found 385.2130.

3-*O*-(4-Methoxybenzyl)morphine (6). Morphine (394 mg, 1.4 mmol, 1 eq.) was dissolved in DMF (1 mL) and KOH (3 M in MeOH, 552 µL, 1.7 mmol, 1.2 eq.) was added. The resulting mixture was stirred for 10 min, then *para*-methoxybenzylchloride (215 µL, 1.6 mmol, 1.15 eq.) was added dropwise. After stirring for 4 h at room temperature, aqueous citric acid (1 M, 10 mL) was added. The resulting solution was washed successively with EtOAc (3 × 10 mL) and aqueous

NH_4OH (28%, 10 mL) was added to the aqueous phase. The resulting turbid solution was extracted with 3:1 $CHCl_3$/*i*PrOH (5 × 10 mL). The combined organic layers were backwashed with brine, dried over Na_2SO_4 and the solvent removed *in vacuo*. The resulting yellow oil was purified by flash column chromatography (98:1:1 DCM/MeOH/TEA). The resulting clear oil was redissolved in EtOAc and evaporated again to give the product as a white solid (259 mg, 46%). ^{1}H NMR ($CDCl_3$) δ 7.33 (d, *J* = 8.4 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.71 (d, *J* = 8.2 Hz, 1H), 6.53 (d, *J* = 8.1 Hz, 1H), 5.67 (d, *J* = 9.7 Hz, 1H), 5.27 (d, *J* = 9.7 Hz, 1H), 5.04 (dd, *J* = 28.7, 11.4 Hz, 1H), 4.87 (d, *J* = 6.3 Hz, 1H), 4.15 (d, *J* = 2.5 Hz, 1H), 3.80 (s, 2H), 3.36 (d, *J* = 2.4 Hz, 1H), 3.03 (d, *J* = 18.7 Hz, 1H), 2.86 (s, 1H), 2.69 (s, 1H), 2.61 (dd, *J* = 11.8, 3.9 Hz, 1H), 2.44 (s, 1H), 2.39 (dd, *J* = 12.2, 3.1 Hz, 1H), 2.30 (dd, *J* = 18.6, 6.0 Hz, 1H), 2.08 (td, *J* = 12.4, 4.8 Hz, 1H), 1.87 (d, *J* = 11.7 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.5, 147.1, 141.3, 133.6, 131.3, 129.5, 129.4, 128.2, 127.7, 119.7, 116.0, 114.0, 91.3, 71.7, 66.5, 59.0, 55.4, 46.6, 43.1, 42.9, 40.7, 35.8, 20.6; LC-MS *m/z* $[M+H]^+$ $C_{25}H_{28}NO_4^+$ calc. 406.2, found 406.2.

(4*R*,4a*R*,7*R*,7a*R*,12b*S*)-9-((4-Methoxybenzyl)oxy)-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-ol (7). Compound **6** (229 mg, 0.57 mmol, 1 eq.), benzoic acid (104 mg, 0.85 mmol, 1.5 eq.) and triphenylphosphine (223 mg, 0.85 mmol, 1.5 eq.) were dissolved in anhydrous toluene (4 mL) and cooled to 0 °C. DIAD (167 μL, 0.85 mmol, 1.5 eq.) in anhydrous toluene (0.5 mL) were then added dropwise over 2 min. The reaction was then warmed to room temperature and allowed to stir for 4 h, after which the solvent was removed *in vacuo*. The resulting residue was purified by flash column chromatography (59:40:1 DCM/EtOAc/TEA) to give a white solid and oil mixture. This residue was dissolved in a 1:1 mixture of EtOH and 1 M aqueous KOH (6 mL) and heated to reflux for 20 min. The reaction was then reduced *in vacuo* and 1 M citric acid (20 mL) was added to the residue. The aqueous phase was then washed with EtOAc (3 × 20 mL), then neutralized with aqueous NH_4OH (28%, 20 mL). The resulting solution was extracted with 3:1 $CHCl_3$/*i*PrOH (3 × 20 mL). The combined organic layers were backwashed with brine (20 mL), dried over Na_2SO_4 and the solvent removed *in vacuo*

to give a white solid as the pure product (179 mg, 78%). ^{1}H NMR ($CDCl_3$) δ 7.36–7.31 (m, 1H), 6.90–6.85 (m, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 6.50 (d, *J* = 8.2 Hz, 1H), 6.00–5.93 (m, 1H), 5.60 (dd, *J* = 9.8, 1.9 Hz, 1H), 5.05 (q, *J* = 11.4 Hz, 1H), 4.79 (s, 1H), 4.20 (d, *J* = 5.5 Hz, 1H), 3.79 (s, 1H), 3.40 (dd, *J* = 5.6, 3.2 Hz, 1H), 3.17 (s, 1H), 3.03 (d, *J* = 18.7 Hz, 1H), 2.66 (dd, *J* = 12.2, 3.9 Hz, 1H), 2.48 (s, 1H), 2.45–2.33 (m, 1H), 2.18 (td, *J* = 12.5, 4.9 Hz, 1H), 1.85 (dd, *J* = 12.7, 2.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 159.5, 146.5, 141.3, 132.8, 131.4, 130.6, 129.6, 129.5, 127.2, 119.0, 116.4, 113.9, 94.2, 71.7, 68.0, 59.3, 55.4, 47.2, 43.9, 42.9, 39.6, 35.5, 20.7; LC-MS *m/z* $[M+H]^+$ $C_{25}H_{28}NO_4^+$ calc. 406.2, found 406.3.

(4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-((4-Methoxybenzyl)oxy)-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-amine (8). Compound **7** (174 mg, 0.43 mmol, 1 eq.), phthalimide (79 mg, 0.64 mmol, 1.5 eq.) and triphenylphosphine (169 mg, 0.64 mmol, 1.5 eq.) were dissolved in anhydrous toluene (4 mL) and cooled to 0 °C. DIAD (127 μL, 0.64 mmol, 1.5 eq.) in anhydrous toluene (0.5 mL) was then added dropwise over 2 min. The reaction was then warmed to room temperature and allowed to stir for 4 h, after which the solvent was removed *in vacuo*. The resulting residue was purified by flash column chromatography (59:40:1 DCM/EtOAc/TEA) to give a white solid and oil mixture. To this residue, EtOH (10 mL) and $N_2H_4.H_2O$ (0.5 mL) were added and the mixture heated to reflux for 1 h. After cooling, the resulting precipitate was filtered and washed with EtOH. The filtrate was evaporated *in vacuo*, redissolved in $CHCl_3$ and the resulting precipitate removed. The filtrate was again evaporated *in vacuo* and the residue purified by flash column chromatography (89:10:1 $CHCl_3$/MeOH/TEA) to give the product as a yellow oil (59 mg, 34%). ^{1}H NMR ($CDCl_3$) δ 7.35–7.30 (m, 1H), 6.89–6.80 (m, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 6.55 (d, *J* = 8.2 Hz, 1H), 5.79–5.60 (m, 1H), 5.13–4.99 (m, 1H), 4.94 (d, *J* = 2.3 Hz, 1H), 3.78 (s, 2H), 3.59 (d, *J* = 4.2 Hz, 1H), 3.03 (d, *J* = 18.7 Hz, 1H), 2.73 (d, *J* = 9.1 Hz, 1H), 2.55 (dd, *J* = 12.1, 3.6 Hz, 1H), 2.48–2.37 (m, 2H), 2.29 (td, *J* = 12.1, 3.7 Hz, 1H), 2.03 (s, 1H), 1.99–1.93 (m, 1H), 1.83–1.77 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 159.4, 145.0, 142.2,

139.3, 130.1, 129.6, 129.5, 127.7, 123.9, 118.9, 116.3, 113.9, 87.4, 71.5, 56.2, 55.3, 49.2, 46.9, 46.3, 43.2, 40.9, 35.5, 20.0; LC-MS *m/z* $[M+H]^+$ $C_{25}H_{29}N_2O_3^+$ calc. 405.5, found 405.2.

6-*O*-Hexanoyl-3-*O*-(4-methoxybenzyl)morphine (9). Compound **6** (68 mg, 0.17 mmol, 1 eq.) and hexanoyl chloride (70.4 μL, 0.50 mmol, 3 eq.) were dissolved in pyridine (1 mL) and heated at 70°C in a sealed vessel for 19 h. Water (2 mL) and MeOH (1 mL) were added and the resulting mixture evaporated. MeCN (5 mL) was added and the mixture was again evaporated to give a yellow oil. Saturated aqueous $NaHCO_3$ (10 mL) was added and the resulting solution extracted with 3:1 $CHCl_3$/*i*PrOH (3 × 10 mL). The combined organic layers were backwashed with brine, dried over Na_2SO_4 and the solvent removed *in vacuo* to give a yellow oil. Ether was added and the resulting precipitate filtered. The filtrate was evaporated and purified by flash column chromatography (94:5:1 EtOAc/MeOH/TEA) to give the product as a yellow oil (67 mg, 80%). ^{1}H NMR ($CDCl_3$) δ 7.35–7.31 (m, 1H), 6.89–6.84 (m, 1H), 6.67 (d, *J* = 8.2 Hz, 1H), 6.49 (d, *J* = 8.2 Hz, 1H), 5.67–5.61 (m, 1H), 5.46–5.39 (m, 1H), 5.19 (ddd, *J* = 8.1, 5.1, 2.9 Hz, 1H), 5.12 (dd, *J* = 6.7, 0.8 Hz, 1H), 5.06 (s, 1H), 3.79 (s, 1H), 3.77 (dd, *J* = 3.6, 2.0 Hz, 1H), 3.40 (dd, *J* = 5.6, 3.2 Hz, 1H), 3.02 (d, *J* = 18.7 Hz, 1H), 2.83 (s, 1H), 2.64 (dd, *J* = 12.0, 3.9 Hz, 1H), 2.47 (s, 2H), 2.42–2.28 (m, 2H), 2.10 (td, *J* = 12.5, 4.9 Hz, 1H), 1.88 (dd, *J* = 12.7, 1.8 Hz, 1H), 1.69–1.58 (m, 1H), 1.35–1.20 (m, 3H), 0.92–0.80 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 173.4, 159.4, 147.6, 141.2, 130.9, 129.7, 129.5, 129.2, 128.9, 119.4, 117.3, 113.8, 88.0, 71.8, 68.0, 59.3, 55.3, 43.0, 42.5, 40.4, 35.2, 34.1, 31.4, 24.6, 22.4, 20.7, 14.02; LC-MS *m/z* $[M+H]^+$ $C_{31}H_{37}NO_5^+$ calc. 504.3, found 504.3.

***N*-((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-((4-Methoxybenzyl)oxy)-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)hexanamide (10).** Compound **8** (65 mg, 0.16 mmol, 1 eq.) and hexanoyl chloride (67 μL, 0.48 mmol, 3 eq.) were dissolved in pyridine (2 mL) and heated at 70 °C for 18 h. After cooling, the solvent was removed *in vacuo* and the resulting residue was purified by flash column chromatography (94:5:1 EtOAc/MeOH/TEA) to give the product as a yellow oil (39 mg, 49%). ^{1}H NMR ($CDCl_3$) δ 7.37–7.30 (m, 2H), 6.89–6.84 (m, 2H), 6.72 (d, *J* = 8.2 Hz, 1H), 6.59 (d, *J* = 8.2 Hz, 1H), 5.76 (ddd, *J* = 10.3, 3.5, 2.8 Hz, 1H), 5.60 (dd, *J*

= 10.3, 1.1 Hz, 1H), 5.40 (d, *J* = 9.6 Hz, 1H), 5.09–5.00 (m, 2H), 4.94–4.92 (m, 1H), 4.23–4.14 (m, 1H), 3.79 (s, 3H), 3.24 (dd, *J* = 5.8, 2.7 Hz, 1H), 2.99 (d, *J* = 18.8 Hz, 1H), 2.73 (dd, *J* = 18.8, 6.1 Hz, 1H), 2.56–2.49 (m, 1H), 2.39 (s, 3H), 2.27 (td, *J* = 11.7, 4.5 Hz, 1H), 2.18–2.12 (m, 2H), 2.10 (dd, *J* = 10.2, 2.8 Hz, 1H), 1.92–1.79 (m, 2H), 1.64–1.56 (m, 2H), 1.36–1.22 (m, 5H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 173.1, 159.4, 144.9, 142.2, 134.7, 129.6, 129.5, 129.1, 128.2, 126.2, 119.5, 116.5, 113.9, 86.6, 71.5, 56.7, 55.4, 47.7, 47.0, 44.3, 43.3, 41.1, 36.9, 35.6, 31.5, 25.5, 22.5, 20.0, 14.1; LC-MS *m/z* $[M+H]^+$ $C_{31}H_{39}N_2O_4^+$ calc. 503.3, found 503.4.

6-*O*-(3-Carboxypropanoyl)-3-*O*-(4-methoxybenzyl)morphine (11). Compound **6** (41 mg, 0.10 mmol, 1 eq.), succinic anhydride (15 mg, 0.15 mmol, 1.5 eq.), DMAP (19 mg, 0.15 mmol, 1.5 eq.) and TEA (29 µL, 0.20 mmol, 2 eq.) were dissolved in dry MeCN (1 mL) under N_2 and heated at 65 °C for 15.5 h. The mixture was then evaporated and the residue purified via flash column chromatography (79:20:1 $CHCl_3$/MeOH/NH_4OH) to give the product as a white foam (40 mg, 77%). ^{1}H NMR ($CDCl_3$) δ 9.98 (brs, 1H), 7.36–7.31 (m, 2H), 6.89–6.84 (m, 2H), 6.68 (d, *J* = 8.2 Hz, 1H), 6.49 (d, *J* = 8.2 Hz, 1H), 5.62 (d, *J* = 10.0 Hz, 1H), 5.34–5.29 (m, 1H), 5.25–5.18 (m, *J* = 13.8, 9.3, 4.7 Hz, 2H), 5.09 (s, 2H), 3.78 (s, 4H), 3.16 (s, 1H), 3.13–3.05 (m, 1H), 2.99 (d, *J* = 19.3 Hz, 1H), 2.75 (ddd, *J* = 17.4, 10.0, 3.8 Hz, 1H), 2.63 (s, 3H), 2.62–2.53 (m, *J* = 10.5, 5.7 Hz, 3H), 2.49–2.38 (m, 2H), 1.89 (d, *J* = 11.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 177.6, 172.9, 159.5, 147.8, 141.6, 130.3, 129.7, 129.5, 129.5, 127.6, 124.7, 119.6, 118.3, 113.9, 87.9, 72.0, 67.8, 59.1, 55.4, 46.6, 41.5, 41.1, 37.6, 33.1, 30.8, 30.0, 21.3; LC-MS *m/z* $[M+H]^+$ $C_{29}H_{32}NO_7^+$ calc. 506.2, found 506.2.

4-(((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-((4-Methoxybenzyl)oxy)-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)amino)-4-oxobutanoic acid (12). Compound **8** (71 mg, 0.18 mmol, 1 eq.), succinic anhydride (26 mg, 0.26 mmol, 1.5 eq.), DMAP (32 mg, 0.26 mmol, 1.5 eq.) and TEA (49 µL, 0.35 mmol, 2 eq.) were dissolved in MeCN (3 mL) and heated to 70 °C for 18 h. The solvent was then removed *in vacuo* and the resulting solid was triturated with EtOAc. The resulting material was suspended in hot EtOH and the resulting precipitate filtered to

give the product as a white solid (25 mg, 28%). ^{1}H NMR (DMSO-d_6) δ 7.35–7.29 (m, 2H), 6.95–6.89 (m, 2H), 6.78 (d, *J* = 8.2 Hz, 1H), 6.61 (d, *J* = 8.2 Hz, 1H), 5.69–5.63 (m, 1H), 5.56 (dd, *J* = 10.4, 1.2 Hz, 1H), 5.01–4.96 (m, 3H), 3.74 (s, 3H), 3.16 (s, 1H), 3.13 (dd, *J* = 5.7, 2.6 Hz, 1H), 2.87 (d, *J* = 18.7 Hz, 1H), 2.46 (d, *J* = 7.2 Hz, 1H), 2.44–2.39 (m, 3H), 2.34–2.30 (m, 1H), 2.28 (s, 3H), 2.15 (dd, *J* = 10.5, 2.7 Hz, 1H), 2.13–2.05 (m, 2H), 1.83 (td, *J* = 12.3, 4.7 Hz, 1H), 1.60 (d, *J* = 11.1 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.1, 171.3, 159.0, 147.4, 144.4, 141.6, 136.0, 129.7, 129.4, 129.2, 128.0, 125.0, 118.9, 115.6, 113.7, 106.8, 86.2, 70.2, 55.3, 55.1, 46.2, 43.4, 42.8, 38.8, 34.9, 30.0, 29.7, 29.2, 19.6; LC-MS *m/z* $[M+H]^+$ $C_{29}H_{33}N_2O_6^+$ calc. 505.2, found 505.3.

3-*O*-(4-Methoxybenzyl)-6-*O*-(4-Methylamino-4-oxobutanoyl)morphine (13). Compound **11** (40 mg, 0.079 mmol, 1 eq.), EDC.HCl (23 mg, 0.12 mmol, 1.5 eq.), anhydrous HOBt (16 mg, 0.12 mmol, 1.5 eq.) and TEA (22 µL, 0.16 mmol, 2 eq.) were dissolved in MeCN (2 mL) and stirred at room temperature for 1 h. Methylamine (2 M solution in THF, 79.2 µL, 0.1584 mmol, 2 eq.) was then added and stirred for an additional 2 h. The reaction mixture was then evaporated and purified by flash column chromatography (0.5 – 6% MeOH in $CHCl_3$ with 1% TEA) to give the product as a slightly yellow oil (26 mg, 63%). ^{1}H NMR ($CDCl_3$) δ 7.36–7.28 (m, 2H), 6.90–6.84 (m, 2H), 6.71 (d, *J* = 8.2 Hz, 1H), 6.52 (d, *J* = 8.2 Hz, 1H), 5.80 (d, *J* = 4.0 Hz, 1H), 5.63–5.57 (m, 1H), 5.47–5.38 (m, 1H), 5.17 (ddd, *J* = 8.9, 5.0, 3.0 Hz, 1H), 5.09 (dd, *J* = 6.9, 0.8 Hz, 1H), 5.04–4.97 (m, 2H), 3.79 (s, 3H), 3.37 (dd, *J* = 5.7, 3.2 Hz, 1H), 3.05 (dd, *J* = 8.8, 5.9 Hz, 1H), 3.02–2.98 (m, 1H), 2.78 (s, 1H), 2.73–2.67 (m, 2H), 2.60 (d, *J* = 4.8 Hz, 3H), 2.45 (s, 3H), 2.43–2.27 (m, 4H), 2.07 (td, *J* = 12.4, 5.0 Hz, 1H), 1.86 (dd, *J* = 12.7, 1.6 Hz, 1H), 1.37 (t, *J* = 7.3 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 172.1, 172.1, 159.7, 147.2, 141.2, 130.9, 129.7, 129.4, 129.1, 128.5, 127.7, 119.5, 116.0, 114.0, 87.9, 71.7, 68.0, 59.3, 55.4, 46.8, 45.9, 43.1, 42.5, 40.4, 35.3, 31.5, 30.4, 26.4, 20.5, 9.0; LC-MS *m/z* $[M+H]^+$ $C_{30}H_{35}N_2O_6^+$ calc. 519.3, found 519.2.

***N*^{1}-((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-((4-methoxybenzyl)oxy)-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)-*N*^{4}-methylsuccinamide (14).** Compound **12** (24 mg, 0.48 mmol, 1 eq.), EDC.HCl (14 mg, 0.072 mmol, 1.5 eq.), anhydrous HOBt (10 mg, 0.072

mmol, 1.5 eq.) and TEA (13 µL, 0.096 mmol, 2 eq.) were dissolved in dry DMF (1 mL) and stirred at room temperature for 1 h. Methylamine (2 M in THF, 48 µL, 0.096 mmol, 2 eq.) was then added and the reaction allowed to stir for 19 h. Additional EDC.HCl (18 mg, 0.094 mmol, 2 eq.) and HOBt (11 mg, 0.084 mmol, 1.7 eq.) were added, and following 30 min of stirring, further methylamine (2 M in THF, 48 µL, 0.096 mmol, 2 eq.) were added. The reaction was then allowed to stir at room temperature for a further 24 h and the solvent removed *in vacuo*. The resulting brown oil was purified by PLC (89:10:1 $CHCl_3$/MeOH/TEA) to give the product as a white solid (21 mg, 84%). ^{1}H NMR ($CDCl_3$) δ 7.35–7.30 (m, 2H), 6.89–6.83 (m, 2H), 6.71 (d, *J* = 8.2 Hz, 1H), 6.58 (d, *J* = 8.2 Hz, 1H), 6.30 (d, *J* = 9.5 Hz, 1H), 6.06 (d, *J* = 4.2 Hz, 1H), 5.75 (ddd, *J* = 10.3, 3.5, 2.9 Hz, 1H), 5.59 (dd, *J* = 10.3, 1.1 Hz, 1H), 5.08–5.00 (m, 2H), 4.95–4.91 (m, 1H), 4.16–4.07 (m, 1H), 3.79 (s, 3H), 3.23 (dd, *J* = 5.9, 2.7 Hz, 1H), 2.97 (d, *J* = 18.7 Hz, 1H), 2.76 (d, *J* = 4.8 Hz, 3H), 2.65 (d, *J* = 6.1 Hz, 1H), 2.55–2.44 (m, 5H), 2.39 (s, 3H), 2.27 (td, *J* = 12.0, 3.7 Hz, 1H), 2.17 (dd, *J* = 10.3, 2.8 Hz, 1H), 1.88 (td, *J* = 12.2, 4.8 Hz, 1H), 1.84–1.76 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 172.4, 159.4, 145.0, 142.3, 134.6, 129.6, 129.5, 129.2, 128.1, 126.1, 119.4, 116.5, 113.9, 86.7, 71.6, 56.5, 55.4, 47.0, 47.0, 46.2, 44.6, 43.2, 41.0, 35.5, 32.0, 31.7, 26.5, 20.1, 10.7; LC-MS *m/z* $[M+H]^+$ $C_{30}H_{36}N_3O_5^+$ calc. 518.3, found 518.4.

6-*O*-Carboxymethylmorphine (15). Compound **6** (93 mg, 0.23 mmol, 1 eq.) was dissolved in dry THF (3 mL) and cooled to 0°C under an N_2 atmosphere. To this solution, a suspension of prewashed NaH (60%, 37 mg, 0.92 mmol, 4 eq.) in THF (1 mL) was added dropwise. The solution was then allowed to warm to room temperature and stir for 15 min. A solution of *tert*-butyl bromoacetate in THF (1 mL) was then added dropwise and allowed to stir at room temperature for 1 h. The reaction was then quenched with saturated NH_4Cl (2 mL), then MeCN (2 mL) was added. The resulting solution was reduced *in vacuo* and saturated $NaHCO_3$ (20 mL) was added and extracted with $CHCl_3$ (3 × 20 mL). The combined organic phases were backwashed with brine (20 mL), dried over Na_2SO_4, and the solvent removed *in vacuo* to give a brown oil. The crude oil was dissolved in DCM (3 mL) under an N_2 atmosphere. Triethylsilane (1.1 mL, 6.9 mmol, 30 eq.), TFA

(1 mL) were then added and the reaction vessel purged with N_2. The reaction was allowed to stir for 1 h, then reduced *in vacuo*. The resulting oil was purified by prep-HPLC to give the product as a solid white TFA salt (61 mg, 58%). ^{1}H NMR (DMSO-d_6) δ 10.13 (s, 1H), 9.16 (s, 1H), 6.56 (d, *J* = 8.1 Hz, 1H), 6.46 (d, *J* = 8.1 Hz, 1H), 5.76 (d, *J* = 9.9 Hz, 2H), 5.35 (d, *J* = 9.7 Hz, 2H), 5.14–5.04 (m, 2H), 4.19 (s, 3H), 4.12 (dd, *J* = 16.4, 3.9 Hz, 3H), 3.30 (d, *J* = 12.7 Hz, 2H), 3.20 (d, *J* = 19.8 Hz, 1H), 3.02 (d, *J* = 26.9 Hz, 2H), 2.89 (s, 3H), 2.82 (s, 2H), 2.74 (dd, *J* = 19.1, 7.3 Hz, 1H), 2.19 (td, *J* = 13.6, 4.1 Hz, 1H), 1.95 (d, *J* = 12.5 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 171.6, 146.2, 139.3, 131.9, 128.8, 125.9, 121.9, 119.3, 117.2, 87.1, 73.4, 65.4, 59.6, 46.5, 41.4, 40.5, 38.2, 32.8, 20.8; LC-MS m/z $[M+H]^+$ $C_{19}H_{22}NO_5^+$ calc. 344.2, found 344.2.

3-*O*-PMB-(6-*O*-(4-((2-*tert*-butoxycarbonyl)amino)ethyl)amino-4-oxobutanoyl)morphine (16). Compound **11** (96.5 mg, 0.19 mmol, 1 eq.), HCTU (95 mg, 0.23 mmol, 1.2 eq.) and TEA (54 μL, 0.38 mmol, 2 eq.) were dissolved in dry DMF (1.5 mL) under an N_2 atmosphere and was allowed to stir at room temperature for 30 min. *tert*-Butyl (4-aminoethyl)carbamate (37 mg, 0.23 mmol, 1.2 eq.) was then added as a solution in dry DMF (0.5 mL) and allowed to stir at room temperature for 19 h. EtOAc (20 mL) was then added and the resulting solution was washed with half-saturated $NaHCO_3$ (3 × 10 mL), saturated $NaHCO_3$ (20 mL), half saturated brine (2 × 10 mL) and brine (20 mL). The organic phase was then dried over Na_2SO_4 and the solvent removed *in vacuo*. The resulting residue was purified by flash column chromatography (98:1:1 $CHCl_3$/MeOH/TEA) to give the product as a clear glass (102 mg, 83%). ^{1}H NMR ($CDCl_3$) δ 7.30 (d, *J* = 8.6 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 6.72 (d, *J* = 8.2 Hz, 1H), 6.54 (d, *J* = 8.2 Hz, 1H), 6.31 (t, *J* = 5.5 Hz, 1H), 5.59 (d, *J* = 10.1 Hz, 1H), 5.47–5.39 (m, 1H), 5.30 (s, 1H), 5.18–5.08 (m, 2H), 4.98 (s, 2H), 3.77 (s, 3H), 3.33 (dd, *J* = 5.5, 3.3 Hz, 1H), 3.30–3.23 (m, 1H), 3.11–2.97 (m, 3H), 2.96–2.87 (m, 1H), 2.73 (s, 1H), 2.70–2.61 (m, 2H), 2.57 (dd, *J* = 12.2, 3.8 Hz, 1H), 2.41 (s, 3H), 2.40–2.34 (m, 1H), 2.29 (ddd, *J* = 18.1, 9.0, 4.5 Hz, 3H), 2.07–1.97 (m, 1H), 1.84 (d, *J* = 11.1 Hz, 1H), 1.33 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.9, 171.8, 159.8, 156.4, 146.5, 141.2, 130.8, 129.9,

129.5, 128.6, 128.4, 127.7, 119.6, 114.8, 114.0, 87.7, 71.3, 67.7, 59.2, 55.4, 46.8, 43.1, 42.4, 40.6, 40.4, 39.9, 35.2, 31.8, 30.5, 28.4, 20.3; LC-MS *m/z* $[M+H]^+$ $C_{36}H_{46}N_3O_8^+$ calc. 648.3, found 648.4.

6-*O*-(4-((2-Aminoethyl)amino)-4-oxobutanoyl)morphine (17). Compound **16** (102 mg, 0.16 mmol, 1 eq.) was deprotected using general deprotection procedure A. The resulting residue was purified by prep-HPLC to give the product as a solid white mono-TFA salt (43 mg, 51%). ^{1}H NMR (DMSO-d_6) δ 10.01 (s, 1H), 9.22 (s, 1H), 8.10 (t, *J* = 5.7 Hz, 1H), 7.75 (s, 3H), 6.59 (d, *J* = 8.1 Hz, 1H), 6.51 (dd, *J* = 8.0, 4.2 Hz, 1H), 5.65 (d, *J* = 10.0 Hz, 1H), 5.52–5.39 (m, 1H), 5.25–5.14 (m, 1H), 5.09–4.98 (m, 1H), 4.13 (s, 1H), 3.27 (dd, *J* = 12.2, 6.2 Hz, 2H), 3.19 (d, *J* = 19.8 Hz, 1H), 3.06 (d, *J* = 4.7 Hz, 1H), 2.97 (s, 1H), 2.89 (s, 2H), 2.84 (dd, *J* = 12.1, 6.0 Hz, 2H), 2.71 (dd, *J* = 20.0, 6.7 Hz, 1H), 2.65–2.59 (m, 2H), 2.45 (t, *J* = 6.8 Hz, 2H), 2.20 (dd, *J* = 13.4, 9.1 Hz, 1H), 1.84 (dd, *J* = 46.3, 11.7 Hz, 1H); LC-MS *m/z* $[M+H]^+$ $C_{23}H_{30}N_3O_5^+$ calc. 428.2, found 428.2.

2-((1*E*,3*E*,5*Z*)-5-(1-(6-((2-(4-(((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-hydroxy-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)oxy)-4-oxobutanamido)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-indol-1-ium-5-sulfonate (18). Compound **17** (7.1 mg, 0.013 mmol, 2 eq.), Sulfo-Cy5 NHS ester (Lumiprobe, 5 mg, 0.0066 mmol, 1 eq.) and TEA (2.8 μL, 0.020 mmol, 3 eq.) was dissolved in dry DMF (150 μL) under an N_2 atmosphere and allowed to stir at room temperature for 7 h. HCTU (2.7 mg, 0.0066 mmol, 1 eq.) was then added and the reaction stirred for an additional 16 h. Water (20 mL) and MeCN (1 mL) were then added and the solvent removed by freeze-drying. The resulting residue was purified by reverse phase chromatography (5 – 30% buffer B in buffer A, 1% jump per column volume). Clean fractions were collected and freeze-dried to give the product as a dark blue solid TFA salt (2.6 mg, 33%). ^{1}H NMR (DMSO-d_6) δ 9.82 (s, 1H), 9.20 (s, 1H), 8.35 (t, *J* = 13.1 Hz, 1H), 7.91 (s, 1H), 7.81 (s, 2H), 7.77 (s, 1H), 7.64 (dd, *J* = 7.6, 3.9 Hz, 2H), 7.31 (d, *J* = 8.4 Hz, 2H), 6.62–6.44 (m, 5H), 6.28 (dd, *J* = 18.3, 13.9 Hz, 2H), 5.59 (d, *J* = 9.9 Hz, 1H), 5.41 (d, *J* = 9.7 Hz, 1H), 5.12–4.93 (m, 2H), 4.08 (s, 4H), 3.59 (s, 2H), 3.16–3.02 (m, 7H), 2.89 (s, 4H), 2.60–2.54 (m, 3H), 2.39 (t, *J* = 6.6 Hz, 3H), 2.03 (t, *J* = 7.1

Hz, 2H), 1.68 (s, 12H), 1.58–1.48 (m, 2H), 1.29 (ddd, *J* = 17.8, 13.8, 8.6 Hz, 5H), 1.17 (t, *J* = 7.3 Hz, 2H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{55}H_{66}N_5O_{12}S_2^+$ calc. 1052.4144, found 1052.4119, $[M+2H]^{2+}$ $C_{55}H_{67}N_5O_{12}S_2^+$ calc. 526.7108, found 526.7113.

6-*O*-((4-((*tert*-Butoxycarbonyl)amino)butyl)carbamoyl)morphine (19). Compound **15** (130 mg, 0.28 mmol, 1 eq.), anhydrous HOBt (42 mg, 0.31 mmol, 1.1 eq.), EDC.HCl (60 mg, 0.31 mmol, 1.1 eq.) and TEA (119 μL, 85 mmol, 3 eq.) were dissolved in DMF (2 mL) under an N_2 atmosphere and allowed to stir at room temperature for 1 h. *tert*-Butyl (4-aminobutyl)carbamate (64 mg, 0.34 mmol, 1.2 eq.) was then added as a solution in dry DMF (1 mL) and the reaction stirred at room temperature for 17 h. The solvent was then removed *in vacuo* and the residue purified by reverse phase chromatography (6–35% buffer B in buffer A, 1% jump per column volume). Clean fractions were collected and freeze dried to give the product as a light-orange solid TFA salt (65 mg, 36%). ^{1}H NMR (MeOD) δ 6.65 (dd, *J* = 8.1, 1.4 Hz, 1H), 6.58 (d, *J* = 8.1 Hz, 1H), 5.92–5.83 (m, 1H), 5.42 (d, *J* = 9.9 Hz, 1H), 5.19–5.12 (m, 1H), 4.91 (s, 9H), 4.28–4.10 (m, 4H), 3.39 (dd, *J* = 13.3, 3.7 Hz, 1H), 3.34 (d, *J* = 6.5 Hz, 1H), 3.26 (d, *J* = 7.3 Hz, 1H), 3.20 (d, *J* = 20.2 Hz, 1H), 3.12–2.94 (m, 7H), 2.87 (dd, *J* = 19.8, 6.8 Hz, 1H), 2.34 (td, *J* = 13.6, 5.0 Hz, 1H), 2.12 (d, *J* = 13.9 Hz, 1H), 1.75–1.62 (m, 3H), 1.60–1.47 (m, 2H), 1.42 (s, 3H); ^{13}C NMR (MeOD) δ 172.6, 147.4, 132.9, 129.9, 126.6, 121.2, 118.7, 89.0, 75.3, 69.8, 69.7, 62.3, 42.9, 41.7, 40.3, 40.0, 39.7, 39.1, 34.3, 28.8, 28.3, 27.7, 27.4, 25.8, 22.2; LC-MS *m/z* $[M+H]^+$ $C_{28}H_{40}N_3O_6^+$ calc. 514.3, found 514.3.

6-*O*-((4-Aminobutyl)carbamoyl)morphine (20). Compound **19** (65 mg, 0.10 mmol, 1 eq.) was dissolved in $CHCl_3$ (1 mL) and TFA (0.5 mL) and the resulting solution allowed to stir for 1 h. The solvent was then removed *in vacuo*, the resulting residue dissolved in buffer A and freeze-dried to give the product as a brown oil TFA salt (60 mg, quantitative). ^{1}H NMR (MeOD) δ 6.65 (d, *J* = 8.1 Hz, 1H), 6.58 (d, *J* = 8.2 Hz, 1H), 5.86 (d, *J* = 10.0 Hz, 1H), 5.41 (d, *J* = 9.8 Hz, 1H), 5.15 (d, *J* = 5.7 Hz, 1H), 4.28–4.10 (m, 4H), 3.38 (dd, *J* = 13.2, 3.8 Hz, 1H), 3.33 (d, *J* = 6.7 Hz, 1H), 3.27 (d, *J* = 20.0 Hz, 1H), 3.18 (d, *J* = 12.0 Hz, 1H), 3.07 (dd, *J* = 13.3, 3.5 Hz, 1H), 3.04–2.93 (m, 6H),

2.87 (dd, *J* = 19.9, 6.7 Hz, 1H), 2.36 (td, *J* = 13.6, 4.6 Hz, 1H), 2.09 (d, *J* = 12.0 Hz, 1H), 1.77–1.58 (m, 4H); ^{13}C NMR (101 MHz, MeOD) δ 172.5, 147.4, 141.0, 132.8, 129.9, 126.7, 123.2, 121.2, 118.7, 89.0, 75.4, 69.7, 62.2, 42.9, 41.7, 40.3, 39.8, 39.1, 34.2, 27.3, 26.8, 25.8, 22.2; LC-MS *m/z* $[M+H]^+$ $C_{23}H_{32}N_3O_4^+$ calc. 414.2, found 414.3.

1-Ethyl-2-((1*E*,3*E*,5*Z*)-5-(1-(6-((4-(2-(((4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-hydroxy-3-methyl-2,3,4,4a,7,7a-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl)oxy)acetamido)butyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3*H*-indol-1-ium-5-sulfonate (21). Sulfo-Cy5 TFA salt (6.0 mg, 7.8 μmol, 1 eq.), HCTU (3.9 mg, 9.3 μmol, 1.2 eq.), TEA (10 μL) were dissolved in DMF (600 μL) under an N_2 atmosphere and stirred at room temperature for 1 h. Compound **20** (8.2 mg, 16 μmol, 2 eq.) was then added as a solution in DMF (200 μL) and allowed to stir at room temperature for 18 h. Buffer A was added and the resulting solution freeze-dried. The residue was purified by reverse phase chromatography (5–30% buffer B in buffer A, 1% jump per column volume). Clean fractions were collected and freeze-dried to give the product as a dark blue solid TFA salt (3.6 mg, 40%). ^{1}H NMR (DMSO-d_6) δ 10.22 (s, 1H), 9.19 (s, 1H), 8.35 (t, *J* = 12.6 Hz, 2H), 7.82 (s, 2H), 7.78–7.71 (m, 2H), 7.68–7.61 (m, 2H), 7.34–7.30 (m, 2H), 7.18 (s, 1H), 7.05 (s, 1H), 6.59–6.55 (m, 2H), 6.46 (d, *J* = 8.1 Hz, 1H), 6.30 (d, *J* = 14.0 Hz, 1H), 5.77 (dd, *J* = 10.2, 2.2 Hz, 1H), 5.28 (d, *J* = 10.0 Hz, 1H), 5.07 (d, *J* = 5.9 Hz, 1H), 4.15–3.93 (m, 10H), 3.19 (d, *J* = 19.3 Hz, 1H), 3.14–3.04 (m, 5H), 3.02–2.96 (m, 3H), 2.89 (s, 3H), 2.82 (s, 1H), 2.75–2.66 (m, 1H), 2.23–2.13 (m, 1H), 2.02 (t, *J* = 6.9 Hz, 2H), 1.95–1.89 (m, 1H), 1.58–1.48 (m, 3H), 1.43–1.29 (m, 8H), 1.25 (t, *J* = 7.3 Hz, 6H), 1.17 (t, *J* = 7.3 Hz, 2H); ESI-TOF HRMS *m/z* $[M+H]^+$ $C_{56}H_{70}N_5O_{11}S_2$+ calc. 1052.4508, found 1052.4545, $[M+Na]^+$ $C_{56}H_{69}N_5O_{11}S_2Na^+$ calc. 1074.4327, found 1074.4338.

Pharmacology. *cAMP BRET CAMYEL assay.* HEK293 cells were transfected with 2 μg hMOR pEF5/FRT construct cDNA and 3 μg CAMYEL sensor cDNA. The cDNA was mixed with 30 μg polyethylenimine and made up to 250 μL with 0.15 M NaCl, then allowed to incubate for 15–20 min at room temperature. This mixture was then added to the cells dropwise and gently mixed.

The cells were then incubated for 24 h in PTM, after which they were resuspended in 15 mL PTM and replated onto white poly-*D*-lysine coated plates at 100 μL per well and maintained for 24 h. The media was then aspirated and the cells washed once with 100 μL/well HBSS at 37 °C. HBSS (70 μL/well) was then added back and the cells incubated for 30 min at 37 °C in the absence of CO_2. Coelenterazine H (10 μL, 5 μM final) was added and the cells incubated in the dark for 10 min at 37 °C. The drugs (10 μL) were then added at the desired concentration, followed immediately by forskolin (10 μL, 30 μM final), then incubated for 5 min inside a LUMIstar Omega plate reader set at 37 °C. Luminescence for RLuc8 (480 nm) and fluorescence for YFP (530 nm) were then measured. Baseline and vehicle control values were subtracted and the BRET signal was normalized as a percentage of forskolin response.

Confocal Microscopy. HEK293 cells stably expressing *N*-terminal SNAP-hMOR were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1 mg/mL G418. Cells were grown in poly-*D*-lysine–coated 8-well Labtek borosilicate chambered-cover glasses (Nalgene Nunc International, Fisher Scientific). On the day of experimentation, SNAP tag labelling was performed by incubating cells in 500 nM BG-488 in fresh cell culture media for 30 min at 37 °C. Cells were washed in pre-warmed HEPES-buffered saline solution (HBS) containing 4.5 mM *D*-glucose and incubated for 30 min at 37 °C in the presence or absence of 1 μM naloxone prior to addition and incubation with the relevant concentration of **21** for 30 min at 37 °C. Where the concentration of **21** was 10 μM, the cells were washed with HBS prior to imaging. Confocal images were captured using a Zeiss LSM710 laser scanning microscope using a Plan-Apochromat 63 × 1.3NA oil-immersion objective lens (1024 × 1024 pixels, averaging at four frames). The 633 nm HeNe and 488 nm argon lasers were used to excite sulfo-Cy5 and BG-488 respectively. Emission was captured at 638 – 759 nm and 493 – 628 nm respectively. Confocal settings for laser power, offset and gain were kept constant throughout acquisition. Images were processed in Zeiss Zen 2011, with any linear adjustments made equally cross all images within a dataset.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website:

Molecular formula strings

Comparison of the NMR spectra of compound **6** and **7**

■ AUTHOR INFORMATION

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■ ABBREVIATIONS USED

BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; DIAD, diisopropyl azodicarboxylate; DMAP, 4-dimethylaminopyridine; DOR, δ opioid receptor; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; GPCR, G protein-coupled receptor; HCTU, *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; KOR, κ opioid receptor; MOR, μ opioid receptor; NOPr, nociception/orphanin FQ peptide receptor; PMB, *para*-methoxybenzyl; NHS, *N*-hydroxysuccinimide; TEA, triethylamine; TFA, trifluoroacetic acid.

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