Herkinorin Analogues with Differential β-Arrestin-2 Interactions

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Salvinorin A is a psychoactive natural product that has been found to be a potent and selective κ opioid receptor agonist in vitro and in vivo. The activity of salvinorin A is unusual compared to other opioids such as morphine in that it mediates potent κ opioid receptor signaling yet leads to less receptor downregulation than observed with other κ agonists. Our initial chemical modifications of salvinorin A have yielded one analogue, herkinorin (**1c**), with high affinity at the μOR. We recently reported that **1c** does not promote the recruitment of β-arrestin-2 to the μOR or receptor internalization. Here we describe three new derivatives of **1c** (**3c**, **3f**, and **3i**) with similar properties and one, benzamide **7b**, that promotes recruitment of β-arrestin-2 to the μOR and receptor internalization. When the important role μ opioid receptor regulation plays in determining physiological responsiveness to opioid narcotics is considered, μ opioids derived from salvinorin A may offer a unique template for the development of functionally selective μ opioid receptor–ligands with the ability to produce analgesia while limiting adverse side effects.

Introduction

Increasing evidence indicates that chemically distinct ligands can elicit different receptor regulation pathways.[1] For example, the opioids morphine, methadone, and fentanyl each promote μ opioid receptor (μOR) coupling to G proteins, but they differ in their ability to direct receptor trafficking.[2,3] This may be due to differences in agonist-induced receptor conformations, resulting in different degrees of phosphorylation, arrestin recruitment, and vesicular trafficking. Such differences in μOR regulation and trafficking may be physiologically relevant as mice lacking β-arrestin-2 display enhanced antinociception, decreased tolerance, and greatly diminished side effects (constipation and respiratory depression) following morphine treatment.[4–7] Therefore, an opioid agonist conferring nonconventional receptor conformations may yield novel analgesics with reduced potential to produce unwanted side effects.

Currently, there are no selective pharmaceutical or biochemical inhibitors of G-protein couple receptor (GPCR) desensitization nor are there specific inhibitors of the GRKs or β-arrestins. A therapeutic approach in which β-arrestins or GRKs were individually inhibited might produce unwanted alterations of the function of other GPCRs. Furthermore, because arrestins regulate >1000 different GPCRs,[8,9] it will be exceedingly difficult to produce receptor-selective effects using this approach. An alternate approach would be to selectively target μOR regulation by designing ligands that confer μOR conformations that allow for signaling yet disrupt receptor regulation.

Salvinorin A (**1a**, Figure 1) is a neoclerodane diterpene isolated from *Salvia divinorum*, a member of the Lamiaceae family native to Oaxaca, Mexico.[10,11] *S. divinorum* has been used as a vision-inducing plant by the Mazatec Indians in their divination rituals for centuries.[12] Previous studies have shown that **1a** is a potent and selective κ opioid receptor agonist in vitro and in vivo.[13–20] Interestingly, **1a** activates κ opioid receptor signaling with less receptor internalization than observed with other κ agonists.[21] These studies suggest that the κOR conformation induced by **1a** binding is conducive to G-protein mediated signal transduction but resistant to internalization-mediated regulation. Recent biochemical and site-directed mutagenesis studies indicate that **1a** has a unique binding epitope at κORs.[22–24] These findings support a novel mode by which subtype selectivity for GPCR ligands is induced by a change in the topology of conserved residues within a common binding pocket.[23,24]

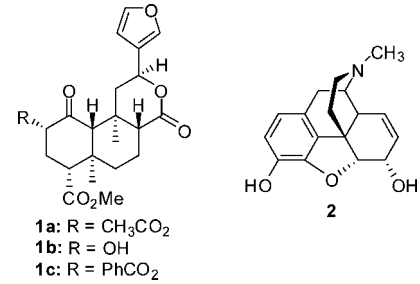

Figure 1. Structures of salvinorin A (**1a**), salvinorin B (**1b**), herkinorin (**1c**), and morphine (**2**).

Our initial chemical modifications of **1a** yielded several ligands, some agonists and some antagonists at μ, δ, or κ ORs.[25–27] In particular, herkinorin (**1c**) was identified as the first non-nitrogenous μ opioid receptor agonist and does not lead to receptor internalization under any conditions tested but, more interestingly, it does not promote the recruitment of β-arrestin-2 to the μOR.[28] As part of our ongoing program to develop analgesics with reduced propensity to induce tolerance and dependence, we synthesized several analogues of **1c**. These

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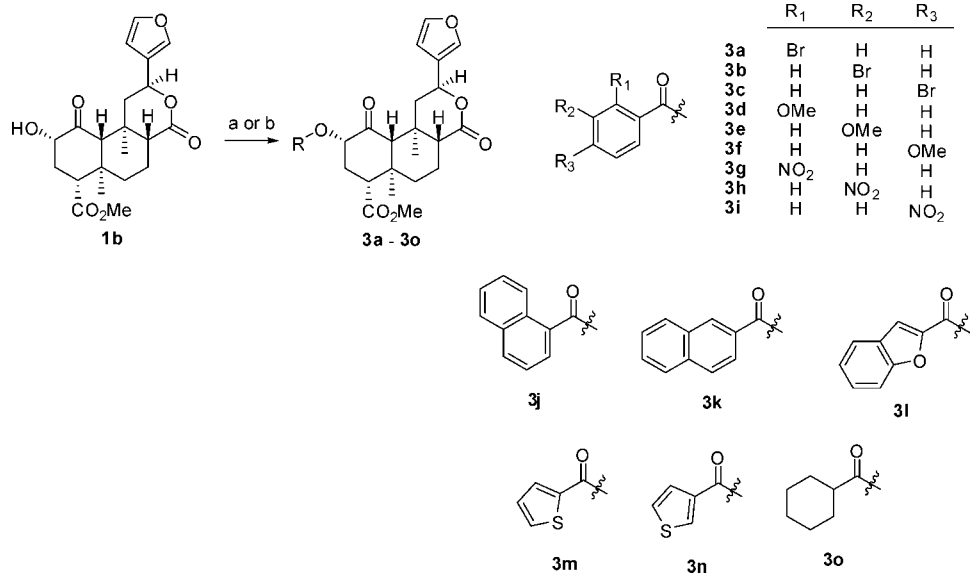
Scheme 1[a]



[a] Reagents and conditions: (a) appropriate acid chloride, DMAP, NEt_3, CH_2Cl_2; (b) appropriate acid, EDCI, HOBT, CH_2Cl_2.

analogues were prepared to further elucidate the role of structure on μOR affinity, activity, and regulatory pathways.

Chemistry

We synthesized compounds **3d–3o**, **7a**, **7b**, **8a**, **8b**, **9a**, and **9b**, as described in Schemes 1 and 2. Diterpene **1a** was isolated from *S. divinorum* and then converted to salvinorin B (**1b**), as described previously.[29] The reaction of **1b** with the appropriate acid halide or acid under basic conditions afforded compounds **3d–3o**.[30,31] Alternately, the reaction of **1b** with CBr_4 and PPh_3 afforded a mixture of **4**[32] (59%) and its C2 epimer (14%). However, addition of the PPh_3 in two portions afforded almost exclusively the β isomer. This method results in higher yields than previously described methods using $SOBr_2$.[32] The reaction of **4** with sodium azide in DMF was unsuccessful. However, if the reaction was conducted in a mixture of acetic acid and DMF,[33] azide **5**[32] was formed in 86% yield, a higher yield than previously described.[32] Interestingly, when the C2 epimer of **4** was subjected to identical conditions, azide **5** was also formed. Reduction of **5** using Zn metal and NH_4Cl[34] afforded amine **6**[35] in 36% yield. Staudinger reduction (PPh_3, H_2O)[36] of **5** was also attempted but led mainly to decomposition of starting material.[35] The treatment of amine **6** with acetic anhydride or benzoyl chloride under basic conditions and in the presence of a catalytic amount of DMAP afforded amides **7a**[32,35] and **7b**, respectively. The reaction of amine **6** with methanesulfonyl chloride or benzenesulfonyl chloride using similar conditions afforded **8a** and **8b**. Finally, the reaction of the potassium salt of thioacetic acid or thiobenzoic acid with **4** gave **9a**[32,37] and **9b**, respectively.

Results

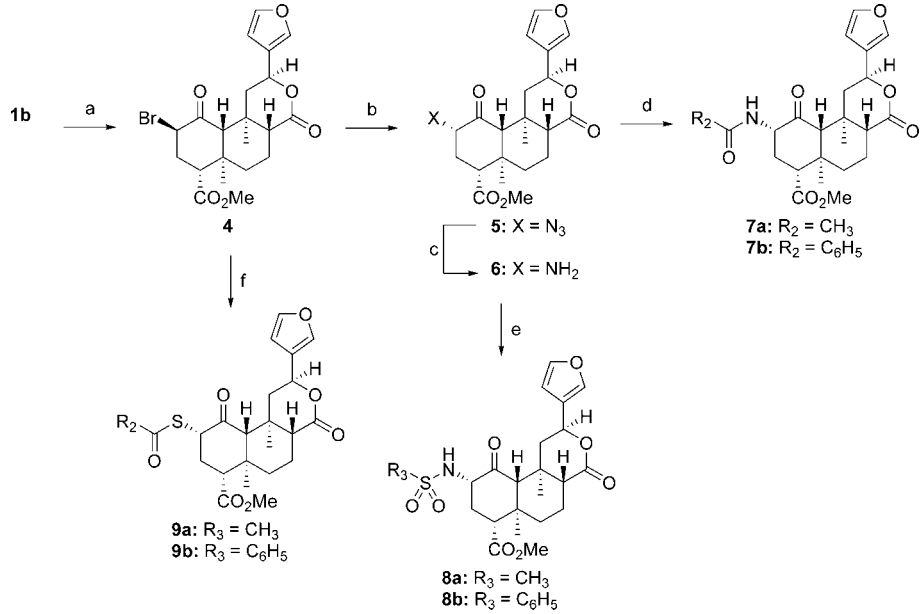
Newly synthesized compounds **3d–3o**, **7b**, **8a**, **8b**, and **9b** were then evaluated for affinity at opioid receptors using methodology previously described (Table 1).[38] These analogues were prepared to give insight as to the nature of the high affinity and selectivity of **1a** and **1c** for κ and μ receptors, respectively. Recently, we investigated the effects of the addition of a bromo group to **1c** (i.e., **3a–3c**).[30] It was found that substitution of the bromo group in the 4-position (**3c**) retained high affinity at μ receptors. This modification also increased μ/κ selectivity compared to **1c**. Given the clear effects of ring substitution, we sought to probe additional modifications of the benzene ring.

The addition of a 2-methoxy group (**3d**) decreased affinity for μ receptors over 130-fold compared to **1c** (K_i = 1640 vs 12 nM). Introduction of a methoxy group in the 3-position of the benzene ring (**3e**) also decreased affinity for μORs and κORs compared to **1c** (K_i = 30 vs 12 nM and K_i = 550 vs 90 nM, respectively). This modification, however, increased affinity 55-fold for μORs compared to **3d** (K_i = 30 vs 1640 nM) and improved selectivity for μORs over κORs compared to **1c** (μ/κ = 0.05 vs μ/κ = 0.13). The presence of a 4-methoxy group (**3f**) leads to an approximately 6-fold decrease in affinity (K_i = 70 vs 12 nM) and similar selectivity (μ/κ = 0.12 vs μ/κ = 0.13) for μORs compared to **1c**. This observation and our previous finding that **3c** has equal affinity when compared to **1c**[30] suggest that an electron-withdrawing group in the 4-position is more favorable for μOR affinity.

The introduction of a 2-nitro group (**3g**) decreased affinity for μORs over 600-fold compared to **1c** (K_i = 7550 vs 12 nM). This modification was better tolerated at κORs where only a 10-fold loss in affinity was observed (K_i = 900 vs 90 nM). This result, coupled with those observed for **3a**, **3d**, and **3g**, would indicate that factors other than electronics are likely involved in the binding of 2-position analogues. Substitution of a 3-nitro group (**3h**) abolished affinity at μORs (K_i > 10000) and decreased affinity approximately 10-fold at κORs compared to **1c** (K_i = 800 vs 90 nM). Finally, a 4-nitro group (**3i**) was also explored. This modification decreased affinity over 20-fold for μORs and over 6-fold for κORs compared to **1c** (K_i = 260 vs 12 nM and K_i = 570 vs 90 nM, respectively). This result, coupled with those observed for **3c** and **3f**, would indicate that factors other than the strength of the electron withdrawing group are likely involved in the binding of 4-position analogues.

We then sought to further explore the size requirements for the aromatic substituent. First, we annulated an additional benzene ring onto the 2 and 3 positions (**3j**).[31] This modification resulted in a roughly 1000-fold loss of affinity at μOR compared to **1c** (K_i > 10000 vs 12 nM). This change, however, was better tolerated at κORs with roughly a 5-fold loss in affinity compared to **1c** (K_i = 410 vs 90 nM). This is interesting given the observation that replacement of the acetoxy group in **1** with an

Scheme 2[a]



[a] Reagents and conditions: (a) CBr_4, PPh_3, CH_2Cl_2; (b) NaN_3, DMF, AcOH; (c) Zn, NH_4Cl, MeOH, CH_2Cl_2; (d) appropriate acid chloride or anhydride, DMAP, NEt_3, CH_2Cl_2; (e) appropriate sulfonyl chloride, DMAP, CH_2Cl_2; (f) RCOSK, CH_3CN.

Table 1. Binding Affinities of Salvinorin A Analogues at Opioid Receptors Using [^{125}I]IOXY as Radioligand[39,40]

	$K_i \pm$ SD, nM			selectivity	
cmpd	μ	δ	κ	μ/κ	δ/κ
1a[a]	>1000[b]	5790 ± 980	1.9 ± 0.2	>526	3050
1b	>10000	>10000	280 ± 20	>35	>35
1c[a]	12 ± 1	1170 ± 60	90 ± 2	0.13	12
3a[c]	110 ± 1	>10,000	90 ± 7	1.2	>100
3b[c]	110 ± 1	>10000	70 ± 7	1.6	>100
3c[c]	10 ± 1	1410 ± 80	740 ± 40	0.01	2
3d	1640 ± 90	>10000	230 ± 20	7	>43
3e	30 ± 2	1140 ± 60	550 ± 30	0.05	2
3f	70 ± 4	1860 ± 140	540 ± 40	0.12	3
3g	7550 ± 970	>10000	900 ± 50	8	>11
3 h	>10000	>10000	800 ± 50	>12	>12
3i	260 ± 210	>10000	570 ± 40	13	0.45
3j	>10000	>10000	410 ± 40	>24	>24
3k	180 ± 20	>10000	5490 ± 640	0.03	>2
3l	10 ± 1	580 ± 30	70 ± 2	0.14	8
3m[c]	10 ± 2	1380 ± 130	260 ± 20	0.04	5
3n	10 ± 1	690 ± 30	80 ± 3	0.16	9
3o	1030 ± 80	>10000	2010 ± 110	0.5	>5
7a	4180 ± 310	>10000	30 ± 2	13	>330
7b	3.1 ± 0.4	810 ± 30	7430 ± 880	0.0004	0.11
8a	>10000	>10000	260 ± 30	>38	>38
8b	>10000	>10000	1400 ± 110	>7	>7
9a	4370 ± 310	3990 ± 290	5.7 ± 0.4	767	700
9b	290 ± 70	1930 ± 70	1410 ± 80	0.21	1.4

[a] Data from ref 25. [b] Partial inhibitor. [c] Data from ref 30. All results are $n = 3$.

1-naphthoate abolishes affinity for κORs (K_i > 10000 nM).[31] This difference is likely due to the different radioligands used ([^{3}H]bremazocine vs [^{125}I]IOXY) or the possibility of misidentification because these compounds were not rigorously evaluated for purity.[31] Annulation of the benzene ring into the 3 and 4 positions (**3k**) reduced affinity at μORs approximately 10-fold compared to **1c** (K_i = 180 vs 12 nM). This modification also decreased affinity for κORs greater than 50-fold (K_i = 5490 vs 90 nM). This suggests that a β,γ-annulated system increases selectivity for μORs over κORs. To probe this, we prepared 2-benzofuran **3l** as an analogue that possesses a β,γ-annulated system. Somewhat surprisingly, **3l** had equal affinity at μORs compared to **1c** (K_i = 10 vs 12 nM). However, it retained selectivity for μORs over κORs. Previously, we showed that bioisosteric replacement of the benzene ring with a 2-thiophene (**3m**) retained affinity at μORs.[30] We were curious if the point of attachment might play a role in its affinity. To probe this, we synthesized the corresponding 3-thiophene (**3n**). Compound **3n** had similar affinity to **1c** for μORs and κORs (K_i = 10 vs 12 nM and K_i = 80 vs 90 nM, respectively), indicating that the point of attachment on the thiophene ring does not play a role in μOR binding. This change, however, increases affinity 3-fold for κORs (K_i = 80 vs 260 nM). Finally, we sought to further confirm the role of the aromatic moiety in the selectivity of **1c**. To address this, we prepared cyclohexyl analogue (**3o**). As expected, **3o** had reduced affinity for μORs and κORs compared to **1c** (K_i = 1030 vs 12 nM and K_i = 2010 vs 90 nM, respectively). This change also decreased selectivity for μORs over κORs (μ/κ = 0.5 vs μ/κ = 0.13).

While our studies were in progress, several groups reported the effects of bioisosteric replacement of the 2-acetoxy group in **1a** with an acetamido group (**7a**).[32,35] Consistent with those reports, we found this change resulted in a loss in affinity at κ receptors (K_i = 30 vs 1.9 nM). However, **7a** was found to have affinity for μORs (K_i = 4180 nM). Given our previous finding that introduction of a benzene ring increases μ affinity,[25] we synthesized benzamide **7b**. As expected, introduction of the benzene ring resulted in a decreased affinity at κ receptors and increased affinity at μ receptors. To our delight, **7b** has 4-fold higher affinity than **1c** (K_i = 3.1 vs 12 nM) and is more selective for μ receptors over κ receptors (κ/μ = 0.0004 vs κ/μ = 0.13. To further explore these developments, we synthesized sulfonamides **8a** and **8b**.

Previously, we showed that the addition of a methanesulfonyl group retained high affinity and activity at κORs.[25] The replacement of the acetamido group with a methanesulfonylamino group (**8a**) decreased affinity approximately 9-fold for κORs compared to **7a** (K_i = 260 vs 30 nM). This change also abolished affinity for μORs (K_i > 10000 nM). The addition of a benzene ring to **8a** (**8b**) decreased affinity approximately 5-fold for κORs (K_i = 1400 vs 260 nM). The loss of affinity at κORs

Table 2. Results from [^{35}S]GTP-γ-S Functional Assay Carried out in CHO Cells Containing DNA for Human μ and κ Receptors[39,40]

cmpd	μ EC_{50} ± SD, nM	μ E_{max}[a] ± SD	κ EC_{50} ± SD, nM	κ E_{max}[a] ± SD
1a	NT[b]	NT[b]	40 ± 10	120 ± 2
1c	500 ± 140	130 ± 4	1320 ± 150	140 ± 2
3c	4890 ± 980	108 ± 8[c]	NT[b]	NT[b]
3e	1670 ± 250	72 ± 3[c]	3590 ± 550	97 ± 2[c]
3f	830 ± 100	94 ± 3[c]	2610 ± 470	106 ± 5[c]
3i	1370 ± 230	46 ± 2[c]	NT[b]	NT[b]
3l	1680 ± 250	104 ± 5[c]	1120 ± 170	109 ± 5[c]
3m	1150 ± 250	95 ± 3[c]	NT[b]	NT[b]
3n	690 ± 60	108 ± 3[c]	840 ± 210	95 ± 8[c]
7a	NT[b]	NT[b]	120 ± 20	108 ± 3[c]
7b	360 ± 60	134 ± 5	NT[b]	NT[b]
(−)-U50,488	4840 ± 890	35 ± 2	30 ± 6	100 ± 4
DAMGO	40 ± 4	100 ± 4	NT[b]	NT[b]

[a] E_{max} is % compound that stimulates binding compared to DAMGO (10 μM) at μ and (−)-U50,488 (500 nM) at κ receptors, respectively; [b] Not tested. [c] $P < 0.05$ when compared to the E_{max} of **1c** at μ and κ receptors (Student's *t*-test).

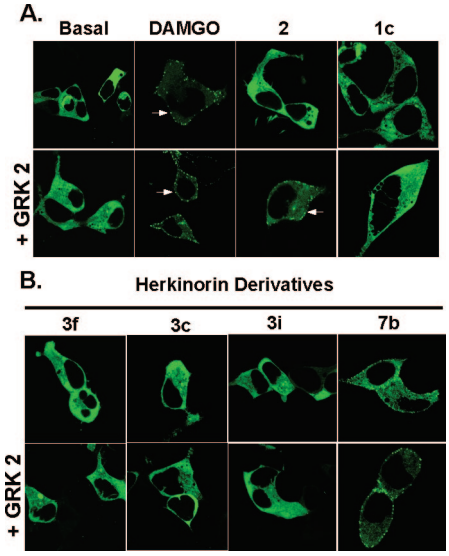


Figure 2. Agonist-induced β-arrestin-2-GFP translocation. HEK-293 cells transfected with MOR1 and βarr2-GFP and with or without GRK2 overexpression were treated with the indicated drugs. Representative cells of at least three independent experiments are shown in which several cells were imaged. (A) DAMGO induces robust translocation of βarr2-GFP (puncta; arrows) to the plasma membrane. Morphine, however, can only induce translocation when GRK2 is overexpressed. Ester **1c** is unable to induce robust βarr2-GFP translocation to the plasma membrane even in the presence of GRK2 overexpression. (B) Amide **7b** is the only herkinorin derivative that induces βarr2-GFP translocation in the absence or presence of GRK2 overexpression.

may be due to the increased ionizability of a sulfonamide compared to a sulfonyl ester. Recently, it has been shown that a tertiary amide has higher affinity for κORs than a secondary amide.[35] This data would seem to confirm this observation, as well as, our previous finding that sulfonyl esters of **1a** are not binding in an identical manner to alkyl esters.[30]

Finally, we probed the replacement of the 2-acetoxy group with a 2-acetylthio group. As seen previously,[37] this change resulted in a slight reduction in affinity at κ receptors ($K_i = 5.7$ vs 1.9 nM). However, **9a** was found to have low affinity for μORs ($K_i = 4370$ nM). The addition of a benzene ring to **9a** (**9b**) lead to an increase in affinity at μORs ($K_i = 290$ vs 4370 nM). However, this change lead to a 24-fold decrease in affinity compared to **1c**, indicating an ester or amide linkage is preferential for binding at μORs.

To test the hypothesis that μ opioids derived from **1a** have functional activity at opioid receptors, several analogues were then evaluated in a [^{35}S]GTP-γ-S assay (Table 2).[39,40] The introduction of a 4-bromo substituent (**3c**) resulted in an approximately 10-fold decrease in activity compared to **1c** ($EC_{50} = 4890$ vs 500 nM). This modification also reduced the efficacy compared to **1c** ($E_{max} = 108$ vs 130), but **3c** is just as efficacious as DAMGO ($E_{max} = 108$ vs 100). The presence of a 3-methoxy group (**3e**) resulted in an approximately 3-fold loss in activity at μORs compared to **1c** ($EC_{50} = 1670$ vs 500 nM). A similar effect was seen at κORs. Interestingly, **3e** is not as efficacious as **1c** ($E_{max} = 72$ vs 130) and appears to be a partial agonist when compared to DAMGO ($E_{max} = 72$ vs 100). A 4-methoxy group (**3f**) had similar activity compared to **1c** ($EC_{50} = 830$ vs 500 nM). However, **3f** is not as efficacious at μORs as **1c** ($E_{max} = 94$ vs 130) but is approximately as efficacious as DAMGO ($E_{max} = 94$ vs 100). A 4-nitro group (**3i**) decreased activity at μORs approximately 3-fold compared to **1c** ($EC_{50} = 1370$ vs 500 nM). This change, however, resulted in a large decrease in efficacy compared to **1c** and DAMGO ($E_{max} = 46$ vs 130 and $E_{max} = 46$ vs 100).

Substitution of the benzene ring in **1c** with a 2-benzofuran (**3l**) resulted in an approximately 3-fold loss in activity and decreased efficacy at μORs compared to **1c** ($EC_{50} = 1680$ vs 500 nM and $E_{max} = 104$ vs 130). However, **3l** is still a full agonist when compared to DAMGO ($E_{max} = 104$ vs 100). Benzofuran **3l** had similar activity at κORs compared to **1c** ($EC_{50} = 1120$ vs 1320 nM). Strikingly, **3l** was less efficacious as an agonist at κORs compared to **1a** ($E_{max} = 109$ vs 140), but more efficacious than U50,488H ($E_{max} = 109$ vs 100). Bioisosteric replacement of the benzene ring in **1c** with a 2-thiophene (**3m**) reduced activity and efficacy at μORs compared to **1c** ($EC_{50} = 1150$ vs 500 nM and $E_{max} = 95$ vs 130). Substitution of a 3-thiophene (**3n**) had little effect on activity at μORs ($EC_{50} = 690$ nM vs 500 nM) and decreased efficacy ($E_{max} = 108$ vs 130). Compound **3n**, however, is roughly as efficacious as DAMGO ($E_{max} = 108$ vs 100) at μORs.

Replacement of the 2-acetoxy group in **1a** with a 2-acetamido group (**7a**) resulted in a 3-fold loss in activity at κORs compared to **1a** ($EC_{50} = 120$ vs 40 nM). This change, however, had little effect on efficacy ($E_{max} = 108$ vs 120). Replacement of the 2-benzoyloxy group in **1c** with a 2-benzoylamino group (**7b**) resulted in a slight increase in activity and no change in efficacy ($EC_{50} = 360$ vs 500 nM and $E_{max} = 134$ vs 130).

To better understand the role of drug structure on μOR regulation pathways, we examined the ability of **3c**, **3f**, **3i**, and **7b** to induce β-arrestin-2-GFP translocation HEK-293 cells (Figure 2). The effects of DAMGO, morphine, and **1c** are shown for comparison.[3,28] DAMGO induces robust translocation of βarr2-GFP to the plasma membrane. Morphine, however, can only induce translocation when GRK2 is overexpressed. Compounds **3c**, **3f**, and **3i**, like **1c**, are unable to induce robust βarr2-GFP translocation to the plasma membrane even in the presence of GRK2 overexpression. Amide **7b** induces robust βarr2-GFP translocation under both conditions.

To further support the conclusion that μ opioids derived from **1a** have altered receptor regulation, we examined the ability of **3c**, **3f**, **3i**, and **7b** to induce μOR-YFP internalization in HEK-293 cells (Figure 3). The effects of DAMGO, morphine, and **1c** are shown for comparison.[3,28] DAMGO induces robust internalization of μOR-YFP. Morphine, however, can only induce μOR-YFP internalization when GRK2 is overexpressed. Unlike DAMGO and morphine and similar to **1c**, **3c**, **3f**, and **3i** are unable to induce robust μOR-YFP internalization even in

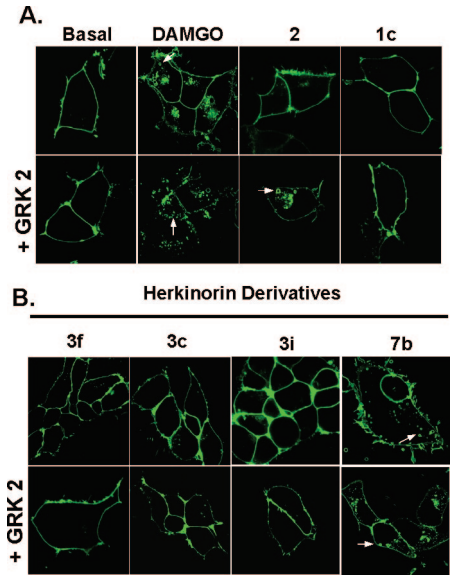


Figure 3. Agonist-induced MOR1-YFP internalization. HEK-293 cells stably transfected with MOR1-YFP were treated with the indicated drugs with or without GRK2 overexpression. Representative cells of at least three independent experiments are shown in which several cells were imaged. (A) DAMGO induces robust internalization of MOR1-YFP (vesicles; arrows). Morphine, however, can only induce robust MOR1-YFP internalization when GRK2 is overexpressed. Ester **1c** is unable to induce robust MOR1-YFP internalization even in the presence of GRK2 overexpression. (B) Amide **7b** is the only herkinorin derivative that can induce MOR1-YFP internalization, even in the in the presence of GRK2 overexpression.

the presence of GRK2 overexpression. However, **7b** induces robust μOR-YFP internalization in HEK-293 cells under both conditions.

To further assess agonist activity in parallel with the current studies, we used the phosphorylation of the downstream MAP kinases (ERK1/ERK2) as an indicator of receptor activation. Compounds **3c**, **3f**, **3i**, and **7b** were examined for their ability to activate ERK in hμOR-CHO cells. In Figure 4, **3c**, **3f**, **3i**, and **7b** are similar to DAMGO in that they are able to induce a μOR1-mediated, dose-dependent increase in ERK phosphorylation that is blocked by naloxone.[28]

Taken together, these data indicate that **3c**, **3f**, and **3i** are able to induce receptor conformations that are able to activate both G protein coupling and MAP kinase activation pathways, yet have unique properties compared to the morphine or DAMGO bound μOR rendering the receptor resistant to β-arrestin interactions or internalization. Amide **7b** appears to induce receptor conformations that are different than other derivatives of **1c** and produces effects similar to other opioids such as DAMGO.

Discussion

Our results indicate that the structure–activity relationships for affinity and activity at μ opioid receptors are not identical to those for receptor regulation. Addition of substituents to the aromatic ring of **1c** results in agonists and partial agonists at μORs and similar receptor regulation to **1c**. These changes do not affect the unique receptor regulation properties of **1c**. Analogues **3c**, **3f**, **3i** are unable to induce robust βarr2-GFP translocation and μOR-YFP internalization even in the presence of GRK2 overexpression in HEK-293 cells. Replacement of the ester linkage in **1c** with an amide linkage (**7b**) increases affinity at μORs compared to **1c**. Amide **7b** has been identified as the most potent neoclerodane μ agonist described to date. However, this change promotes β-arrestin translocation and receptor internalization in HEK-293 cells. The discovery of two compounds with nearly identical chemical structure and similar binding affinity and efficacies that elicit differential signaling at the cellular level would suggest that not only receptor conformation but also ligand structure contribute to signaling events. Future studies of the effect of chemical alterations of **1c** on the activation of cellular pathways may serve as a basis for the development of compounds that can selectively activate or block β-arrestin-receptor interactions may determine specific physiological responses.

The differences in affinity and receptor regulation between **1c** and **7b** are interesting. One potential explanation is that these two molecules, while very similar in structure are not binding in an identical manner at the μOR. This type of phenomenon has been seen previously with other opioids.[41] Another explanation is that the benzene rings in **1c** and **7b** may have different orientations relative to the A ring of the salvinorin core. X-ray crystallographic studies[26,30] indicate that the benzene ring in **1c** is out of the plane of the A ring of the salvinorin core. Preliminary molecular modeling indicates that the benzene ring in **7b** is in the plane of the A ring. This orientation of **7b** may be responsible for the increased affinity and activity at μORs compared to **1c**. However, the out of the plane orientation of the benzene ring in **1c** and esters **3c**, **3f**, and **3i** may be required for the lack of the β-arrestin translocation and receptor internalization. Conformationally constrained analogues will need to be prepared to further delineate the role of the benzene ring on affinity, activity, and receptor regulation pathways.

An alternate explanation for the differences seen in affinity and receptor regulation is that ester **1c** hydrolyzes too rapidly in media to cause internalization and other chronic effects. Amide **7b** would be expected to be more stable in serum, as recently shown for **7a**.[42] Additional stability studies of **1c** and **7b** will be necessary to further investigate the role of metabolism in the differences seen in receptor regulation pathways. However, **1b**, the likely metabolism product of ester **1c**, has no affinity for μORs (K_i > 10000 nM),[43] and after 30 min, **1c** still produces a 3.5-fold increase in ERK phosphorylation, demonstrating a persisting agonistic activity.[28] Moreover, cells treated with DAMGO will internalize the μOR in approximately 10–15 min, therefore, the compound, which is still active at 30 min in the ERK activation assay, should be sufficiently potent to induce internalization. Furthermore, chronic treatment of **1c** produces desensitization in cells, suggesting that it is active long enough to induce some yet undescribed mode of receptor desensitization.[44]

The molecular basis for the unique signaling properties of **1c** is not clear at this time. A likely explanation is that they are the result of a unique binding mode at the μOR relative to other opioids. Most nonpeptide opioid ligands, which contain a basic nitrogen atom, interact with aspartate 147 in TM III.[45] Given the structure of **1c**, this interaction is unlikely. This explanation is further supported by recent studies, indicating that **1a** utilizes unique residues in binding to κORs.[22–24] Ester **1c** and related analogues may have a similar mode of binding at μORs. The exact nature of the interaction of **1c** with the μOR will have to be confirmed through site-directed mutagenesis and/or affinity labeling experiments.

With regards to chemical structure, **1a** and **1c** have an interesting structural motif for GPCR ligands. The neoclerodane nucleus is not considered to be a *privileged structure*, which is defined as a selected substructure that is able to provide high-

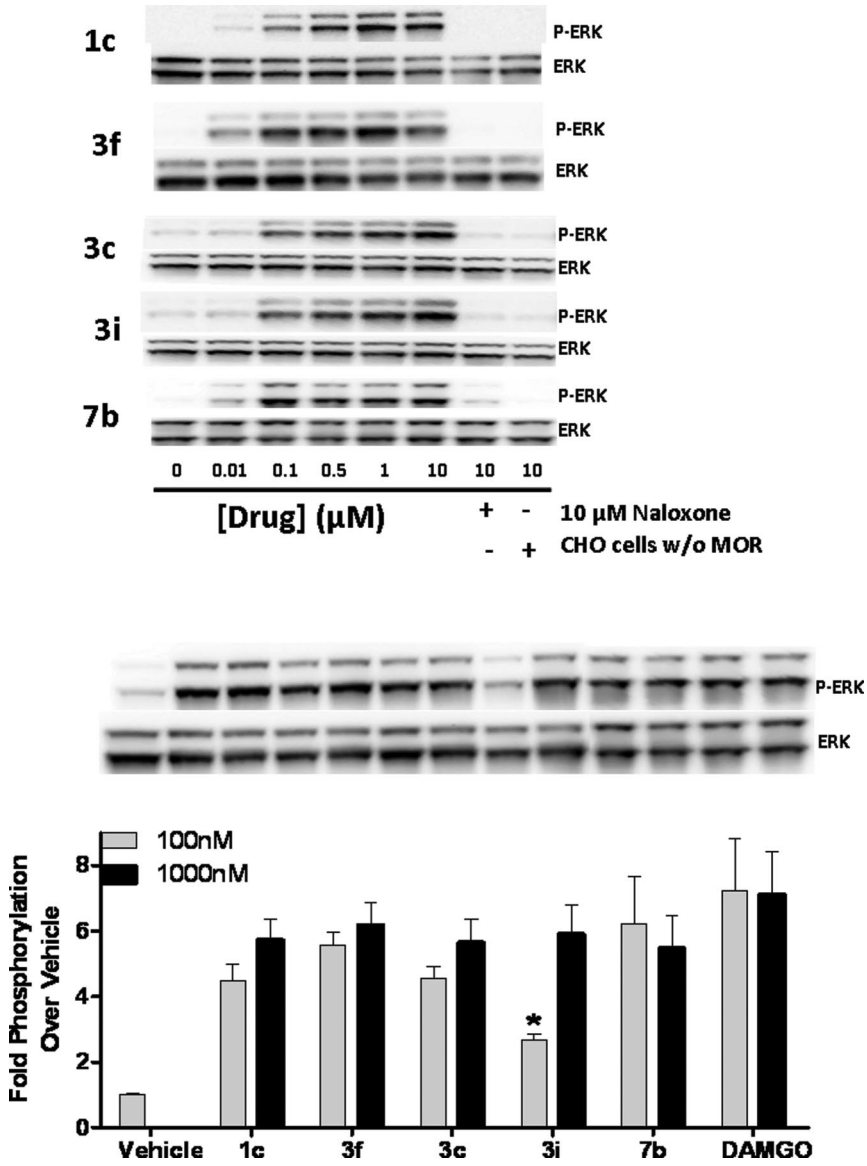


Figure 4. Herkinorin and its four derivatives induce dose-dependent, MOR1-mediated ERK activation. CHO cells stably expressing the human MOR1 were treated with the indicated drugs for 10 min. Top: Representative concentration–response data of **1c**, **3c**, **3f**, **3i**, and **7b** are shown. Experiments were performed at least three times in triplicate. Bottom: Densitometric analysis of two experiments done in triplicate compare efficacy of **1c**, **3c**, **3f**, **3i**, and **7b**. Bar graph shows means and SEM for the densitometric analysis (Student's *t*-test $p < 0.0001$ vs vehicle for all treatments; * $p < 0.05$ vs **3i** (100 nM) for all other treatments) Representative immunoblots of a single experiment are shown.

affinity ligands for more than one type of receptor.[46,47] However, natural products, such as **1a**, can be viewed as a population of privileged structures selected by evolutionary pressures to interact with a wide variety of proteins and other biological targets for specific purposes.[48] Finding additional molecules that have unique receptor regulation pathways for GPCRs may require examining additional natural products or natural product-like libraries.[49]

The life cycle of a GPCR is to reside at the cell surface and, upon activation, become phosphorylated, desensitized, internalized, and then either degraded or recycled. While internalized, the GPCR may also take part in activating signaling cascades.[50,51] Usual drug discovery efforts for GPCRs are to develop agonists, antagonists, or inverse agonists for the GPCR of interest. In our case, this is the μ opioid receptor. Our results illustrate a novel drug discovery strategy that seeks to develop a series of compounds that retain signaling properties at a GPCR but avoid typical regulation pathways. This has a clear impact on the development of novel opioids with reduced side effects and GPCR drug discovery because this finding illustrates the ability of selecting or designing novel agents that differentially activate regulation pathways of a single receptor. This has the potential to optimize therapeutic action in vivo by alleviating unwanted side effects.

Experimental Section

General Methods. Unless otherwise indicated, all reagents were purchased from commercial suppliers and are used without further purification. All melting points were determined on a Thomas-Hoover capillary melting apparatus and are uncorrected. The ^{1}H NMR spectra were recorded at 300 MHz on a Bruker Avance-300 spectrometer using $CDCl_3$ as solvent, δ values in ppm (TMS as internal standard), and J (Hz) assignments of ^{1}H resonance coupling. Thin-layer chromatography (TLC) was performed on 0.25 mm plates Analtech GHLF silica gel plates using *n*-hexanes/EtOAc, 1:1 as the solvent system. Spots on TLC visualized with vanillin/H_2SO_4 in ethanol. Column chromatography was performed with silica gel (32–63 μ particle size) from Bodman Industries (Atlanta, GA). Analytical HPLC was carried out on an Agilent 1100 Series

Capillary HPLC system with diode array detection at 254.8 nm on an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) with isocratic elution in 70% CH_3CN/30% H_2O at a flow rate of 5.0 mL/min.

General Procedure A. A solution of **1b** (1 equiv), appropriate acid chloride (1–3 equiv), NEt_3 (3 equiv), and a catalytic amount of DMAP in CH_2Cl_2 was stirred at room temperature. Absolute MeOH was added and the solvent was removed under reduced pressure. CH_2Cl_2 was added to the residue and the solution was washed with 10% HCl (3 × 20 mL) and saturated NaCl (3 × 20 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure afforded a crude solvent that was purified by column chromatography (eluent: *n*-hexanes/EtOAc) to yield the desired compound.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(4-Bromobenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho-[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3c). Compound **3c** was synthesized from **1b** using general procedure A and 4-bromobenzoyl chloride to afford 0.083 g (57%) as a white solid, mp 190–192 °C. ^{1}H NMR ($CDCl_3$): δ 1.17 (s, 3H), 1.46 (s, 3H), 1.65 (m, 3H), 1.83 (dd, J = 3.3, 9.9 Hz, 1H), 2.10 (dd, J = 2.7, 11.4 Hz, 1H), 2.17 (s, 1H), 2.20 (m, 1H), 2.50 (m, 3H), 2.83 (dd, J = 11.1, 11.7 Hz, 1H), 3.75 (s, 3H), 5.38 (dd, J = 9.9, 10.2 Hz, 1H), 5.52 (dd, J = 5.1, 11.7 Hz, 1H), 6.38 (dd, J = 0.9, 1.8 Hz, 1H), 7.41 (m, 2H), 7.61 (m, 2H), 7.94 (m, 2H).

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(2-Methoxybenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3d). Compound **3d** was synthesized from **1b** using general procedure A and 2-anisoyl chloride to afford 0.010 g (14%) as a white solid, mp 105–107 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.16 (s, 3H), 1.47 (s, 3H), 1.65 (m, 3H), 1.84 (m, 1H), 2.14 (m, 2H), 2.27 (s, 1H), 2.43 (m, 2H), 2.55 (dd, J = 5.1, 13.2 Hz, 1H), 2.83 (dd, J = 8.4, 8.7 Hz, 1H), 3.74 (s, 3H), 3.90 (s, 3H), 5.38 (dd, J = 9.9, 9.9 Hz, 1H), 5.52 (dd, J = 5.4, 11.7 Hz, 1H), 6.38 (s, 1H), 7.00 (dd, J = 7.5, 8.1 Hz, 2H), 7.40 (m, 2H), 7.51 (ddd, J = 1.8, 7.5, 8.1 Hz, 1H), 7.95 (d, J = 7.5 Hz, 1H). HRMS (m/z): [M^+] calcd for $C_{29}H_{32}O_9$, 525.2125; found, 525.2117. HPLC t_R = 4.43 min; purity = 97.76%.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(3-Methoxybenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3e). Compound **3e** was synthesized from **1b** using general procedure A and 3-anisoyl chloride to afford 0.017 g (26%) as a white solid, mp 200–202 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.17 (s, 3H), 1.46 (s, 3H), 1.65 (m, 3H), 1.82 (dd, J = 2.4, 9.9 Hz, 1H), 2.14 (m, 2H), 2.27 (s, 1H), 2.46 (m, 2H), 2.54 (dd, J = 5.4, 13.8 Hz, 1H), 2.84 (dd, J = 6.3, 10.5 Hz, 1H), 3.75 (s, 3H), 3.86 (s, 3H), 5.39 (dd, J = 9.6, 10.5 Hz, 1H), 5.51 (dd, J = 5.1, 11.7 Hz, 1H), 6.39 (d, J = 0.9 Hz, 1H), 7.13 (ddd, J = 0.9, 0.9, 7.1 Hz, 1H), 7.40 (m, 3H), 7.58 (dd, J = 1.5, 2.4 Hz, 1H), 7.69 (dt, J = 0.9, 0.9, 7.5 Hz, 1H). HRMS (m/z): [M^+] calcd for $C_{29}H_{32}O_9$, 525.2125; found, 525.2140. HPLC t_R = 5.14 min; purity = 98.16%.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(4-Methoxybenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3f). Compound **3f** was synthesized from **1b** using general procedure A and 4-anisoyl chloride to afford 0.083 g (60%) as a white solid, mp 185–187 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.17 (s, 3H), 1.46 (s, 3H), 1.65 (m, 3H), 1.83 (dd, J = 2.7, 11.7 Hz, 1H), 2.15 (m, 2H), 2.25 (s, 1H), 2.45 (m, 2H), 2.55 (dd, J = 5.1, 13.2 Hz, 1H), 2.83 (dd, J = 7.8, 8.7 Hz, 1H), 3.74 (s, 3H), 3.87 (s, 3H), 5.37 (dd, J = 9.6, 10.2 Hz, 1H), 5.52 (dd, J = 5.1, 11.7 Hz, 1H), 6.38 (dd, J = 0.9, 1.8 Hz, 1H), 6.93 (dt, J = 2.1, 3.0, 8.7 Hz, 2H), 7.39 (dd, J = 1.8, 1.8 Hz, 1H), 7.41 (dd, J = 0.9, 1.5 Hz, 1H), 8.04 (dt, J = 2.1, 3.0, 9.0 Hz, 2H). Anal. ($C_{29}H_{32}O_9$): C, H.

2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(2-Nitrobenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3g). Compound **3g** was synthesized from **1b** using general procedure A and 2-nitrobenzoyl chloride to afford 0.103 g (75%) as a white solid, mp 144–146 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.15 (s, 3H), 1.48 (s, 3H), 1.64 (m, 3H), 1.83 (dd, J = 2.7, 11.7 Hz, 1H), 2.15 (m, 2H), 2.27 (s, 1H), 2.40 (m, 2H), 2.55 (dd, J = 5.4, 12.3 Hz, 1H), 2.83 (dd, J = 3.6, 13.2 Hz, 1H), 3.75 (s, 3H), 5.42 (dd, J = 7.5, 12.6 Hz, 1H), 5.54 (dd, J = 5.1, 11.4 Hz, 1H), 6.41 (dd, J = 0.9, 1.8 Hz, 1H), 7.42 (dd, J = 1.5, 1.8 Hz, 1H), 7.45 (dd, J = 0.9, 1.5 Hz, 1H), 7.69 (td, J = 1.8, 7.8 Hz, 1H), 7.74 (td, J = 1.5, 7.5 Hz, 1H), 7.92 (dd, J = 1.8, 7.8 Hz, 1H), 8.00 (dd, J = 1.8, 7.5 Hz, 1H). Anal. ($C_{28}H_{29}NO_{10}\cdot 0.25H_2O$): C, H, N.

2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(3-Nitrobenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3 h). Compound **3h** was synthesized from **1b** using general procedure A and 3-nitrobenzoyl chloride to afford 0.110 g (80%) as a white solid, mp 148–150 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19 (s, 3H), 1.46 (s, 3H), 1.64 (m, 3H), 1.85 (dd, J = 2.7, 9.9 Hz, 1H), 2.15 (m, 2H), 2.28 (s, 1H), 2.52 (m, 3H), 2.85 (dd, J = 5.1, 11.7 Hz, 1H), 3.76 (s, 3H), 5.43 (dd, J = 8.4, 11.7 Hz, 1H), 5.53 (dd, J = 5.1, 11.7 Hz, 1H), 6.39 (dd, J = 0.9, 1.8 Hz, 1H), 7.41 (m, 2H), 7.69 (t, J = 8.1, 8.1 Hz, 1H), 8.41 (dt, J = 1.5, 1.5, 7.5 Hz, 1H), 8.46 (ddd, J = 0.9, 2.4, 8.1 Hz, 1H), 8.91 (t, J = 2.1, 2.1 Hz, 1H). Anal. ($C_{28}H_{29}NO_{10}\cdot 0.5H_2O$) C, H, N.

2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(4-Nitrobenzoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3i). Compound **3i** was synthesized from **1b** using general procedure A and 4-nitrobenzoyl chloride to afford 0.093 g (67%) as a white solid, mp 195–200 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18 (s, 3H), 1.46 (s, 3H), 1.66 (m, 3H), 1.84 (dd, J = 3.0, 9.9 Hz, 1H), 2.15 (m, 2H), 2.27 (s, 1H), 2.51 (m, 3H), 2.85 (dd, J = 6.9, 15.9 Hz, 1H), 3.76 (s, 3H), 5.42 (dd, J = 9.3, 10.8 Hz, 1H), 5.53 (dd, J = 5.1, 11.7 Hz, 1H), 6.39 (dd, J = 0.9, 1.8 Hz, 1H), 7.40 (dd, J = 1.8, 1.8 Hz, 1H), 7.42 (dd, J = 0.9, 1.8 Hz, 1H), 8.25 (dt, J = 1.8, 2.1, 9.3 Hz, 2H), 8.31 (dt, J = 1.8, 2.1, 9.0 Hz, 2H). Anal. ($C_{28}H_{29}NO_{10}$) C, H, N.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(1-Naphthoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3j). Compound **3j** was synthesized from **1b** using general procedure A and 1-naphthoyl chloride to afford 0.044 g (63%) of **3j** as a white solid, mp 155–160 °C. ^{1}H NMR ($CDCl_3$): δ 1.19 (s, 3H), 1.49 (s, 3H), 1.66 (m, 3H), 1.84 (dd, J = 2.7, 9.9 Hz, 1H), 2.15 (m, 2H), 2.30 (s, 1H), 2.55 (m, 3H), 2.87 (dd, J = 8.1, 8.4 Hz, 1H), 3.75 (s, 3H), 5.53 (m, 2H), 6.40 (dd, J = 0.9, 1.8 Hz, 1H), 7.40 (dd, J = 1.8, 1.8 Hz, 1H), 7.43 (d, J = 0.9 Hz, 1H), 7.56 (m, 3H), 7.89 (dd, J = 1.2, 7.8 Hz, 1H), 8.05 (d, J = 8.1 Hz, 1H), 8.32 (dd, J = 1.5, 7.5 Hz, 1H), 8.86 (d, J = 8.7 Hz, 1H).

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(2-Naphthoyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3k). Compound **3k** was synthesized from **1b** using general procedure A and 2-naphthoyl chloride to afford 0.020 g (29%) as a white solid, mp 155–160 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19 (s, 3H), 1.48 (s, 3H), 1.67 (m, 3H), 1.85 (dd, J = 2.7, 9.9 Hz, 1H), 2.15 (m, 2H), 2.29 (s, 1H), 2.56 (m, 3H), 2.86 (dd, J = 6.0, 10.8 Hz, 1H), 3.76 (s, 3H), 5.44 (m, 1H), 5.55 (dd, J = 5.4, 12.0 Hz, 1H), 6.39 (dd, J = 0.9, 1.8 Hz, 1H), 7.40 (dd, J = 1.8, 3.0 Hz, 1H), 7.42 (dd, J = 0.9, 1.8 Hz, 1H), 7.59 (m, 2H), 7.90 (m, 2H), 7.97 (d, J = 8.1 Hz, 1H), 8.08 (dd, J = 1.8, 10.2 Hz, 1H), 8.67 (m, 1H). HRMS (m/z): [M^+] calcd for $C_{32}H_{32}O_8Cs$, 677.1152; found, 677.1150. HPLC t_R = 7.38 min; purity = 98.22%.

Benzofuran-2-carboxylic Acid (2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-7-Carbomethoxy-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-9-yl Ester (3l). A solution of **1b** (0.10 g, 0.26 mmol), benzofuran 2-carboxylic acid (0.08 mg, 0.51 mmol), HOBT (0.07 g, 0.51 mmol), and EDCI (0.120 g, 0.64 mmol) in CH_2Cl_2 (20 mL) was stirred at room temperature for 4 d. The mixture was washed with 2 N HCl (3 × 15 mL), saturated $NaHCO_3$ (3 × 15 mL), and H_2O (3 × 15 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure afforded a crude product that was purified by column chromatography (eluent: *n*-hexanes/EtOAc, 1:1) to afford 0.05 g of **1b** and 0.016 g (22%)

of **3l** as a white solid, mp 226–227 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.19 (s, 3H), 1.48 (s, 3H), 1.64 (m, 3H), 1.84 (dd, J = 2.8, 10.5 Hz, 1H), 2.19 (m, 2H), 2.28 (s, 1H), 2.60 (m, 3H), 2.86 (1H, dd, J = 5.25, 10.5 Hz, 1H), 3.77 (s, 3H), 5.48 (dd, J = 9.3, 10.8 Hz, 1H), 5.57 (dd, J = 5.1, 11.7 Hz, 1H), 6.40 (dd, J = 0.9, 1.2 Hz, 1H), 7.36 (s, 1H), 7.43 (m, 2H), 7.52 (m, 1H), 7.66 (m, 2H), 7.73 (dd, J = 0.6, 7.8 Hz, 1H). Anal. ($C_{30}H_{30}O_9$): C, H.

Thiophene-3-carboxylic Acid (2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-7-Carbomethoxy-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-9-yl Ester (3m). Compound **3m** was synthesized from **1b** using general procedure A and 3-thiophenoyl chloride to afford 0.056 g (44%) as a white solid, mp 211–212 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18 (s, 3H), 1.47 (s, 3H), 1.69 (m, 3H), 1.82 (dd, J = 2.7, 10.0 Hz, 1H), 2.18 (m, 3H), 2.27 (s, 1H), 2.42 (m, 2H), 2.52 (dd, J = 5.1, 13.2 Hz, 1H), 2.84 (dd, J = 7.5, 12.6 Hz, 1H), 3.76 (s, 3H), 5.39 (m, 1H), 5.56 (dd, J = 5.1, 11.7 Hz, 1H), 7.36 (dd, J = 3.0, 5.1 Hz, 1H), 7.42 (m, 2H), 7.57 (dd, J = 1.0, 4.6 Hz, 1H), 8.22 (dd, J = 0.6, 2.7 Hz, 1H). Anal. ($C_{26}H_{28}0_8S$): C, H.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(1-Cyclohexanecarbonyloxy)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (3o). Compound **3o** was synthesized from **1b** using general procedure A and cyclohexane carbonyl chloride to afford 0.091 g (71%) as a white solid, mp 104–107 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.12 (s, 3H), 1.28 (m, 4H), 1.51 (m, 2H), 1.60 (m, 4H), 1.79 (m, 3H), 1.94 (m, 1H), 2.02 (m, 1H), 2.08 (m, 1H), 2.16 (m, 1H), 2.19 (s, 1H), 2.29 (dd, J = 8.7, 9.8 Hz, 2H), 2.42 (tt, J = 3.6, 11.3 Hz, 1H), 2.51 (dd, J = 5.1, 13.5 Hz, 1H), 2.76 (dd, J = 7.5, 9.3 Hz, 1H), 3.73 (s, 3H), 5.14 (dd, J = 9.8, 10.4 Hz, 1H), 5.52 (dd, J = 5.3, 11.6 Hz, 1H), 6.38 (dd, J = 0.8, 1.7 Hz, 1H), 7.32 (dd, J = 1.4, 1.4 Hz, 1H), 7.42 (m, 1H). Anal. ($C_{28}H_{36}O_8 \cdot 0.5H_2O$): C, H.

(2*S*,4a*R*,6a*R*,7*R*,9*R*,10a*S*,10b*R*)-9-(Bromo)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (4a) and (2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Bromo)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (4b). A mixture of salvinorin B (**1b**)[29] (0.15 g, 0.38 mmol), triphenylphosphine (0.21 g, 0.80 mmol), and carbon tetrabromide (0.15 g, 0.45 mmol) in CH_2Cl_2 (30 mL) was stirred at room temperature overnight. TLC indicated that starting material was still present after 16 h, thus additional triphenylphosphine (0.11 g, 0.42 mmol) and carbon tetrabromide (0.07 g, 0.21 mmol) were added and the mixture was stirred for an additional 3 h. The solvent was removed under reduced pressure affording a crude residue. The residue was purified by column chromatography (eluent: 30% EtOAc/ *n*-hexanes) to afford 0.10 g (59%) of **4a** as a white solid, mp 170–173 °C (lit.[32] 156–158 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.15 (s, 3H), 1.48 (s, 3H), 1.60 (m, 3H), 1.81 (dd, J = 2.7, 9.9 Hz, 1H), 1.95 (dd, J = 13.2, 26.1 Hz, 1H), 2.1 (m, 2H), 2.27 (s, 1H), 2.47 (dd, J = 4.8, 13.2 Hz, 1H), 2.66 (m, 1H), 2.80 (dd, J = 3.3, 13.2 Hz, 1H), 3.70 (s, 3H), 3.89 (d, J = 2.4 Hz, 2H), 4.45 (m, 1H), 5.55 (dd, J = 4.8, 11.7 Hz, 1H), 6.38 (dd, J = 0.9, 1.5 Hz, 1H), 7.4 (m, 2H).

A more polar spot was isolated to afford 0.02 g (14%) of **4b** as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.14 (s, 3H), 1.48 (s, 3H), 1.52 – 1.73 (m, 4H), 1.80 (dd, J = 3.0, 9.6 Hz, 1H), 2.08 (dd, J = 3.0, 11.4 Hz, 1H), 2.18 (m, 1H), 2.24 (s, 1H), 2.57 (dd, J = 5.1, 13.2 Hz, 1H), 2.63–2.70 (m, 2H), 2.76 (dd, J = 3.3, 12.9 Hz, 1H), 3.73 (s, 3H), 4.60 (dd, J = 7.8, 12.3 Hz, 1H), 5.56 (dd, J = 5.1, 11.7 Hz, 1H), 6.38 (dd, J = 0.9, 0.9 Hz, 1H), 7.41 (m, 1H).

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Azido)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (5). A solution of **4a** (0.10 g, 0.22 mmol), sodium azide (0.05 g, 0.77 mmol), and glacial acetic acid in DMF (3 mL) was stirred at room temperature for 4 h. H_2O (30 mL) was added and the mixture was extracted with EtOAc (20 mL). The EtOAc solution was washed with H_2O (2 × 20 mL) and saturated NaCl (20 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure afforded a crude solid. The crude solid was purified by column chromatography (eluent: 30% EtOAc/*n*-hexanes) to afford 0.08 g (86%) of **5** as a white solid, mp 200–203 °C (lit.[32] 179–181 °C; EtOAc/*n*-hexanes). ^{1}H NMR spectra was in agreement with that previously reported.[32]

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Amino)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (6). A mixture of **5** (0.21 g, 0.50 mmol), Zn dust (0.33 g, 5.0 mmol), and NH_4Cl (0.27 g, 5.0 mmol) in a mixture of CH_2Cl_2/MeOH (1:4, 10 mL) was stirred at room temperature for 3 h. The mixture was filtered and the filtrate was concentrated to dryness under reduced pressure. NaOH (2 N, 30 mL) was added to the residue and the mixture was extracted with CH_2Cl_2 (2 × 20 mL). The combined CH_2Cl_2 portion was washed with H_2O (30 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure afforded 0.07 g (36%) of **6** as an orange solid, mp 237–240 °C (EtOAc/ *n*-hexanes). The ^{1}H NMR spectra was in agreement with that previously reported.[35]

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Acetylamino)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (7a). Compound **7a** was prepared from **6** using a method similar to that previously described[35] to afford 0.04 g (58%) as a white solid, mp 222–224 °C (lit.[32] 137–138 °C; EtOAc/*n*-hexanes). The ^{1}H NMR spectra was in agreement with that previously reported.[35] Anal. ($C_{23}H_{29}NO_7$): C, H, N.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Benzoylamino)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (7b). A solution of **6** (0.10 g, 0.26 mmol), benzoyl chloride (0.11 g, 0.78 mmol), and DMAP (0.08 g, 0.78 mmol) in CH_2Cl_2 (20 mL) was stirred at room temperature for 2 h. Absolute MeOH (15 mL) was added and the solvent was removed under reduced pressure. CH_2Cl_2 (25 mL) was added to the residue, and the solution was washed with 10% HCl (2 × 20 mL), H_2O (3 × 20 mL), and saturated NaCl (3 × 20 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure afforded 0.09 g (67%) of **7b** as a white crystalline solid, mp 155–157 °C (EtOAc/*n*-hexanes). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.44 (s, 3H), 1.50 (s, 3H), 1.63 (m, 3H), 1.82 (dd, J = 2.1, 10.5 Hz, 1H), 2.0 (m, 1H), 2.12 (dd, J = 2.7, 8.4 Hz, 1H), 2.17 (m, 1H), 2.32 (s, 1H), 2.48 (dd, J = 5.4, 13.2 Hz, 1H), 2.79 (dd, J = 3.3, 6.9 Hz, 1H), 2.87 (dd, J = 2.7, 13.5 Hz, 1H), 3.71 (s, 3H), 4.69 (m, 1H), 5.55 (dd, J = 5.1, 11.4 Hz, 1H), 6.37 (dd, J = 0.9, 1.8 Hz, 1H), 7.1 (d, J = 6.0 Hz, 1H), 7.39 (t, J = 1.8 Hz, 1H), 7.41 (dd, J = 0.9, 1.8 Hz, 1H), 7.46 (m, 1H), 7.53 (tt, J = 1.5, 2.7, 7.2 Hz, 1H), 7.80 (t, J = 2.4 Hz, 1H), 7.82 (t, J = 1.2 Hz, 1H). Anal. ($C_{28}H_{31}NO_7 \cdot 0.5H_2O$): C, H, N.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Methanesulfonylamino)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (8a). A solution of **6** (0.10 g, 0.26 mmol), methanesulfonyl chloride (0.08 mL, 1.03 mmol), NEt_3 (0.04 mL, 0.28 mmol), and a catalytic amount of DMAP in CH_2Cl_2 (50 mL) was stirred at room temperature for 2 h. The mixture was washed with 2 N HCl (30 mL), 2 N NaOH (30 mL), and H_2O (30 mL) and dried (Na_2SO_4). Removal of the solvent under reduced pressure afforded a crude solid. The crude solid was purified by column chromatography (eluent: 2% MeOH/ CH_2Cl_2) to afford 0.7 g (56%) of **8a** as a white crystalline solid, mp 262–265 °C (EtOAc/*n*-hexanes). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.09 (s, 3H), 1.46 (s, 3H), 1.60 (m, 3H), 1.79 (dd, J = 2.7, 9.6 Hz, 1H), 2.07 (m, 2H), 2.18 (m, 1H), 2.21 (s, 1H), 2.50 (m, 2H), 2.75 (dd, J = 3.6, 13.2 Hz, 1H), 2.99 (s, 3H), 3.72 (s, 3H), 4.15 (m, 1H), 5.34 (d, J = 5.4 Hz, 1H), 5.55 (dd, J = 5.1, 11.4 Hz, 1H), 6.38 (dd, J = 0.9, 1.2 Hz, 1H), 7.41 (dd, J = 1.5, 1.8 Hz, 1H), 7.43 (dd, J = 0.9, 1.5 Hz, 1H). Anal. ($C_{22}H_{29}NO_8S$): C, H, N.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Benzenesulfonylamino)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (8b). A solution of **6** (0.08 g, 0.21 mmol), benzenesulfonyl chloride (0.07 g, 0.42 mmol), triethylamine (0.06 g, 0.63 mmol), and a catalytic amount of DMAP in CH_2Cl_2 (40 mL) was stirred at room temperature for 18 h. Absolute MeOH was then added and the solution was washed with 10% HCl (3 × 25 mL) and saturated NaCl (2 × 25 mL),

dried (Na_2SO_4), filtered, and concentrated under reduced pressure to yield a crude solid. The solid was purified by flash column chromatography (eluent: *n*-hexanes/EtOAc, 1:1). Removal of the solvent under reduced pressure gave 0.11 g (97%) of **8b** as a white solid, mp 271–273 °C (EtOAc/*n*-hexanes). ^{1}H NMR (300 MHz, acetone-d_6): δ 0.98 (s, 3H), 1.29 (s, 3H), 1.52 (m, 2H), 1.65 (m, 1H), 1.70 (ddd, J = 3.0, 3.0, 12.6 Hz, 1H), 1.82 (ddd, J = 1.8, 5.1, 13.5 Hz, 1H), 1.95 (ddd, J = 6.3, 6.3, 10.2 Hz, 1H), 2.09 (d, J = 13.2 Hz, 1H), 2.22 (dd, J = 2.7, 11.7 Hz, 1H), 2.29 (ddd, J = 3.3, 6.9, 13.5 Hz, 1H), 2.62 (s, 1H), 2.96 (dd, J = 3.5, 13.4 Hz, 1H), 3.66 (s, 3H), 4.19 (m, 1H), 5.47 (dd, J = 5.4, 12.0 Hz, 1H), 6.53 (dd, J = 0.9, 1.5 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 7.38 (m, 1H), 7.40 (d, J = 6.9 Hz, 1H), 7.44 (dd, J = 2.1, 3.0 Hz, 1H), 7.60 (m, 2H), 7.80 (m, 2H). ^{13}C NMR (acetone-d_6): δ 15.7, 16.8, 19.4, 35.5, 36.4, 39.1, 43.4, 44.3, 51.5, 52.2, 54.8, 61.2, 64.7, 72.4, 110.0, 127.5, 128.2, 130.1, 133.5, 141.2, 142.4, 145.1, 171.7, 173.0, 205.3. Anal. ($C_{27}H_{31}NO_8S\cdot H_2O$): C, H, N.

(2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Benzoylthio)-2-(3-furanyl)-dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester (9b). A solution of **4** (0.10 g, 0.22 mmol) and the potassium salt of thiobenzoic acid (0.194 g, 1.10 mmol) was stirred in acetonitrile at room temperature for 3 h. Solvent was removed under reduced pressure and it was then redissolved in DCM (30 mL). The DCM solution was washed with H_2O (3 × 30 mL) and saturated NaCl (2 × 30 mL) and dried (Na_2SO_4). Removal of solvent under reduced pressure affored 0.74 g (66%) of **9b** as a white solid, mp 212–215 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.16 (s, 3H), 1.47 (s, 3H), 1.63 (m, 3H), 1.80 (m, 1H), 2.12 (dd, 1H, J = 1.8, 11.1 Hz), 2.17 (m, 1H), 2.36 (m, 1H), 2.39 (s, 1H), 2.49 (m, 1H), 2.57 (dd, 1H, J = 5.1 13.5 Hz), 2.90 (dd, 1H, J = 3.3, 12.9 Hz), 3.72 (s, 3H), 4.52 (dd, J = 6.9, 13.2 Hz, 1H), 5.54 (dd, J = 5.1, 11.4 Hz, 1H), 6.39 (d, J = 0.9 Hz, 1H), 7.39 (dd, J = 1.5, 1.8 Hz, 1H), 7.42 (m, 1H), 7.46 (m, 2H), 7.60 (m, 1H), 7.96 (m, 2H). HRMS (*m*/*z*): [M^+] calcd for $C_{28}H_{30}O_7S$, 511.1791; found, 511.1781. HPLC t_R = 6.34 min; purity = 98.94%.

In Vitro Pharmacology. Cell culture, [^{35}S]GTP-γ-S binding assay, and [^{125}I]IOXY binding assays proceeded as described elsewhere.[43,52,53] Recombinant CHO cells (hMOR-CHO, hDOR-CHO, and hKOR-CHO) were produced by stable transfection with the respective human opioid receptor cDNA and provided by Dr. Larry Toll (SRI International, CA).

***β*-Arrestin-2 Translocation.** HEK-293 cells stably expressing the μ opioid receptor (~1000 fmol/mg membrane protein) were transiently transfected with 2 μg of β-arrestin-2 tagged on the C-terminus with Green Fluorescent Protein (βarr2-GFP) and 1.5 μg G-protein receptor kinase 2 (GRK2). Experiments were also done in HEK-293 cells transiently transfected with MOR1. After incubation at 37 °C for 24 to 36 h, cells were serum-starved for 30 min. Basal βarr2-GFP images were obtained, followed by drug treatment for 10 min. Drugs included DAMGO (1 μM), morphine (10 μM), and **1c** and its derivatives (10 μM). Cells were monitored each minute throughout the 10 min drug treatment. Representative cells at 5 min are shown. Images were taken using an Olympus Fluoview 300 confocal microscope and Olympus Fluoview imaging software version 4.3.

MOR-YFP Internalization. HEK-293 cells stably expressing MOR1 tagged with Yellow Fluorescent Protein at the C-terminus (MOR1-YFP) were transiently transfected with GRK2. After incubation at 37 °C for 24 to 36 h, cells were serum-starved for 30 min. Basal MOR1-YFP images were obtained, followed by drug treatment for 2 h. Drugs included DAMGO (1 μM), morphine (10 μM), and **1c** and its derivatives (10 μM). Cells were monitored every 15 min throughout the 2 h drug treatment. In some experiments, cells were left to incubate at 37 °C during the hour treatment time, and this did not result in different internalization profiles. Representative cells at 60 min are shown. Images were taken using the Olympus 300 confocal microscope and Olympus Fluoview imaging software version 4.3.

ERK Activation. CHO cells stably expressing the human MOR1 (~800 fmol/mg membrane protein) were serum-starved for 30 min at 37 °C. Cells were treated with **1c** or derivative for 10 min. Where indicated, naloxone was included during serum-starvation and drug treatment. After washing with PBS on ice, cells were collected in lysis buffer (20 mM Tris HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 0.25% dioxycholate, 1 mM sodium orthovanedate, 1 mM PMSF, 1 mM NaF, and protease inhibitor cocktail (Roche)) and centrifuged at 20000 × g for 30 min. Supernatants were quantified using Bio-Rad D_c protein assay and diluted to equal concentrations with 4 × XT sample buffer (Bio-Rad; 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue) with 5% β-mercaptoethanol and boiled at 95 °C for 3 min. Samples were subjected to SDS-PAGE and transferred to PVDF membranes. Blots were first probed with an antibody specific to total ERK1/2 (cell signaling; 1:1000). Blots were stripped and reblotted for Phospho-ERK (Tyr204; Santa Cruz; 1:2000). Bands were detected using secondary antibodies (Amersham; antirabbit IgG 1:2000 and antimouse IgG 1:5000, respectively) conjugated to horseradish peroxidase and Supersignal West Pico Chemiluminescent Substrate (Pierce). Densitometric analysis was performed on Kodak 1D imaging software. Phospho-ERK bands were normalized to corresponding total ERK bands. Statistical analysis was performed using GraphPad Prism software.

Statistics. Statistical analyses were performed using Prism software (GraphPad Software), and the specific tests used are presented in the figure legends.

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Supporting Information Available: HPLC analysis of compounds **3d**, **3e**, **3k**, and **9b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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