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14-Alkoxy- and 14-Acyloxypyridomorphinans: μ Agonist/δ Antagonist Opioid Analgesics with Diminished Tolerance and Dependence Side Effects

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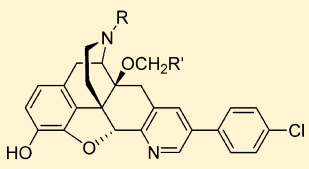
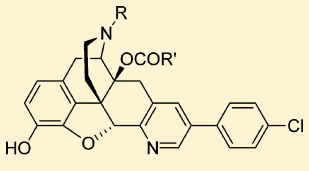
ABSTRACT: In the search for opioid ligands with mixed functional activity, a series of 5′-(4-chlorophenyl)-4,5α-epoxypyridomorphinans possessing alkoxy or acyloxy groups at C-14 was synthesized and evaluated. In this series, the affinity and functional activity of the ligands were found to be influenced by the nature of the substituent at C-14 as well as by the substituent at N-17. Whereas the incorporation of a 3-phenylpropoxy group at C-14 on *N*-methylpyridomorhinan gave a dual MOR agonist/DOR agonist **17h**, its incorporation on *N*-cyclopropylmethylpyridomorphinan gave a MOR agonist/DOR antagonist **17d**. Interestingly, **17d**, in contrast to **17h**, did not produce tolerance or dependence effects upon prolonged treatment in cells expressing MOR and DOR. Moreover, **17d** displayed greatly diminished analgesic tolerance as compared to morphine upon repeated administration, thus supporting the hypothesis that ligands with MOR agonist/DOR antagonist functional activity could emerge as novel analgesics devoid of tolerance, dependence, and related side effects.



R = Me, CPM
R' = H, Ph, CH=CHPh, CH_2CH_2Ph



R = Me, CPM
R' = Ph, CH_2Ph, CH_2CH_2Ph

INTRODUCTION

Opioids analgesics are the most effective and widely used drugs for the treatment of moderate-to-severe pain. The clinical usefulness of opioid analgesics, however, is limited by side effects such as respiratory depression, constipation, analgesic tolerance, physical dependence, and addiction liabilities. The development of analgesic tolerance significantly diminishes the analgesic effectiveness upon repeated administration, and physical dependence necessitates continued opioid administration to avoid withdrawal symptoms. The development of tolerance and concerns over the risk of developing physical dependence and addiction significantly hamper the optimal use of opioids in the treatment of chronic pain conditions.[1−4] Analgesic effects of opioids are mediated by three major types of opioid receptors, the μ-, δ-, and κ-opioid receptors (MORs, DORs and KORs, respectively).[5−8] The analgesic activity as well as the side effects such as euphoria, respiratory depression, tolerance, physical dependence, and opioid-induced bowel dysfunction produced by morphine and related clinically effective opioids are primarily mediated by MORs.[9−11] Whereas the mechanisms underlying the development of tolerance and other side effects are complex, the interactions between MOR and DOR in particular have been implicated as playing a key role in modulating analgesic activity as well as the side effects associated with opioids.[12−15]

On the basis of the findings that (i) DOR null mice do not develop analgesic tolerance to morphine,[16] (ii) administration of DOR antisense oligonucleotides attenuates morphine dependence,[17,18] and (iii) DOR antagonists such as naltrindole[19] and TIPP[ψ][20] are able to prevent the development of morphine tolerance and dependence, we and others have been pursuing the concept of developing ligands possessing mixed MOR agonist/DOR antagonist properties as analgesic agents potentially devoid of tolerance, dependence, and related side effects.[21−23] In our initial effort,[24] we found that annulation of an arylpyridine such as 3-(4-chlorophenyl)pyridine onto the C-ring of the opioid antagonist naltrexone (**1**, Chart 1) gave a ligand **6** with potent antagonist activity at DOR and agonist activity at MOR in ex vivo assays using guinea pig ileum and mouse vas deferens tissues. This compound, however, did not display MOR agonist activity in functional assays using cells expressing MOR.[25] The strategy of annulation of chlorophenylpyridine ring to the morphinan C-ring when applied to naloxone (**2**), 14-deoxynaltrexone (**3**), oxymorphone (**4**), and hydromorphone (**5**) gave ligands **7**−**10**. Of these pyridomorphinans, the hydromorphone-derived compound **10** displayed the desired MOR agonist/DOR antagonist profile of activity.

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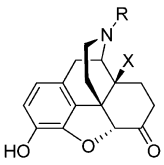
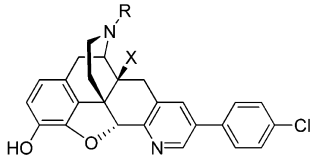
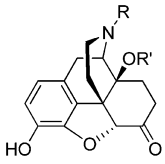
Chart 1[a]



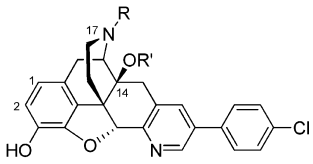
1, R = CPM, X = OH
2, R = Allyl, X = OH
3, R = CPM, X = H
4, R = Me, X = OH
5, R = Me, X = H



6, R = CPM, X = OH
7, R = Allyl, X = OH
8, R = CPM, X = H
9, R = Me, X = OH
10, R = Me, X = H



11, R = Me, R' = CH_2Ph
12, R = Me, R' = CH_2-1-Naphthyl
13, R = CPM, R' = $CH_2CH_2CH_2Ph$
14, R = Allyl, R' = $CH_2CH_2CH_2Ph$
15, R = CPM, R' = CO-Aryl
16, R = CPM, R' = COCH=CHPh



17, R = CPM or Me, R' = alkyl
18, R = CPM or Me, R' = acyl

[a]CPM = cyclopropylmethyl.

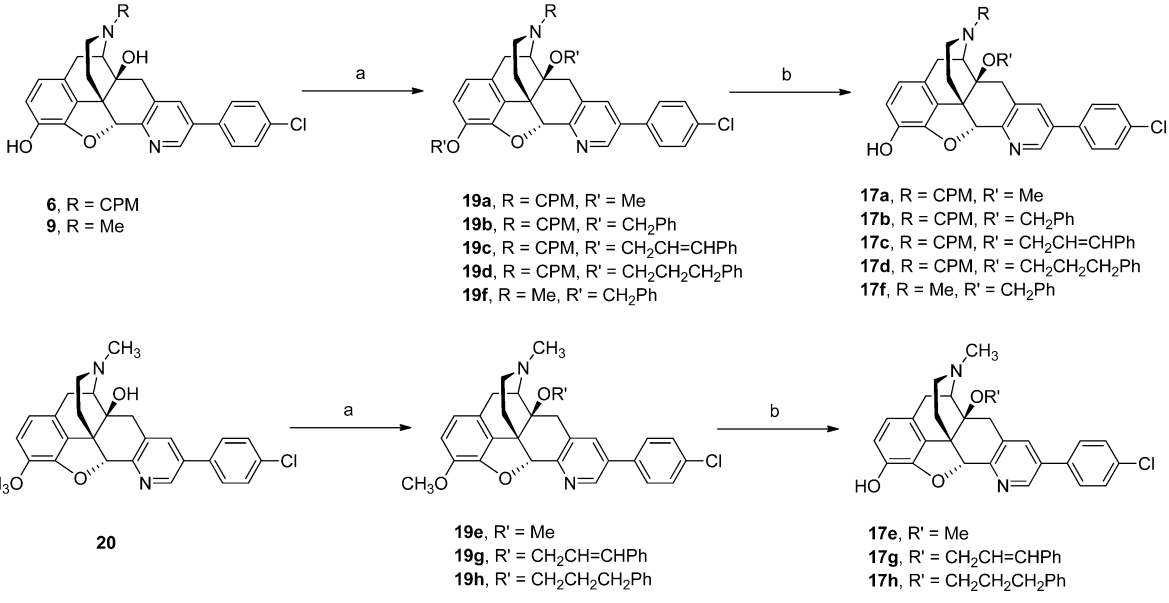
Although the compound displayed only modest agonist activity at MOR, its propensity to induce antinociceptive tolerance was found to be lower than that of morphine.[26]

Morphinan-6-one ligands possessing a methyl group on the morphinan nitrogen in general are known to display opioid agonist activity whereas those possessing a cyclopropylmethyl (CPM) group display opioid antagonist activity.[1,27,28] Among structural variations at the 14-position of the morphinan-6-ones, of particular interest is the incorporation of alkoxy and arylalkoxy ether functions explored extensively by Schmidhammer and co-workers.[29−35] Their studies have shown that incorporation of groups such as benzyloxy or 1-naphthylmethoxy on agonist templates led to ligands such as **11** and **12** that displayed considerably higher antinociceptive potencies.[34] Interestingly, their studies also revealed that incorporation of an alkoxy group such as 3-phenylpropoxy not only increased the binding affinities at all three opioid receptor types but also converted antagonists such as **1** and **2** into agonist ligands (**13** and **14**) possessing potent analgesic activity and no measurable antagonist activity in vivo.[33,34]

In addition to morphinan-6-ones possessing an ether function at the 14-position, ligands possessing an ester function such as **15** and **16** have also been investigated. Recently, Zhang and co-workers reported on the synthesis and activity of a series of aromatic and heteroaromatic esters of naltrexone.[36] Most of these ligands were found to be MOR antagonists in the [^{35}S]GTP-γ-S binding assay. Husbands and co-workers studied cinnamoyl ester derivatives such as **16**, derived from the antagonist **1**, to compare the activity profile with the more extensively studied 14-cinnamoyl amides.[37] They found that **16** displayed primarily MOR antagonist activity in in vitro isolated tissue assays. Whereas the compound showed no agonist activity in the warm-water tail-withdrawal assay, it produced substantial inhibition of acetic acid-induced writhing. Results from agonist selectivity experiments showed that the antinociceptive effects of **16** are primarily mediated through its agonist activity at MOR and DOR.[37]

In view of these observations indicating that an alkoxy or acyloxy substituent at the 14-position of the morphinan framework can have a substantial influence on the binding and functional activity at opioid receptor subtypes, it was of interest to explore the effect of incorporating such groups in the pyridomorphinan scaffold in our search for new ligands possessing MOR agonist/DOR antagonist activity. The target compounds that we explored, generically represented by **17** and **18**, included the *N*-methyl or *N*-CPM group at the morphinan nitrogen. Presented herein are the results of the investigation that led to the identification of potent dual MOR/DOR

Scheme 1. Synthesis of 14-Alkoxypyridomorphinans 17a−h[a]



6, R = CPM
9, R = Me

19a, R = CPM, R' = Me
19b, R = CPM, R' = CH_2Ph
19c, R = CPM, R' = CH_2CH=CHPh
19d, R = CPM, R' = $CH_2CH_2CH_2Ph$
19f, R = Me, R' = CH_2Ph

17a, R = CPM, R' = Me
17b, R = CPM, R' = CH_2Ph
17c, R = CPM, R' = CH_2CH=CHPh
17d, R = CPM, R' = $CH_2CH_2CH_2Ph$
17f, R = Me, R' = CH_2Ph

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19e, R' = Me
19g, R' = CH_2CH=CHPh
19h, R' = $CH_2CH_2CH_2Ph$

17e, R' = Me
17g, R' = CH_2CH=CHPh
17h, R' = $CH_2CH_2CH_2Ph$

[a]Reagents and conditions: (a) NaH, DMF, Me_2SO_4 or R′Br, 0 °C to rt; (b) BBr_3, CH_2Cl_2, −78 °C to rt.

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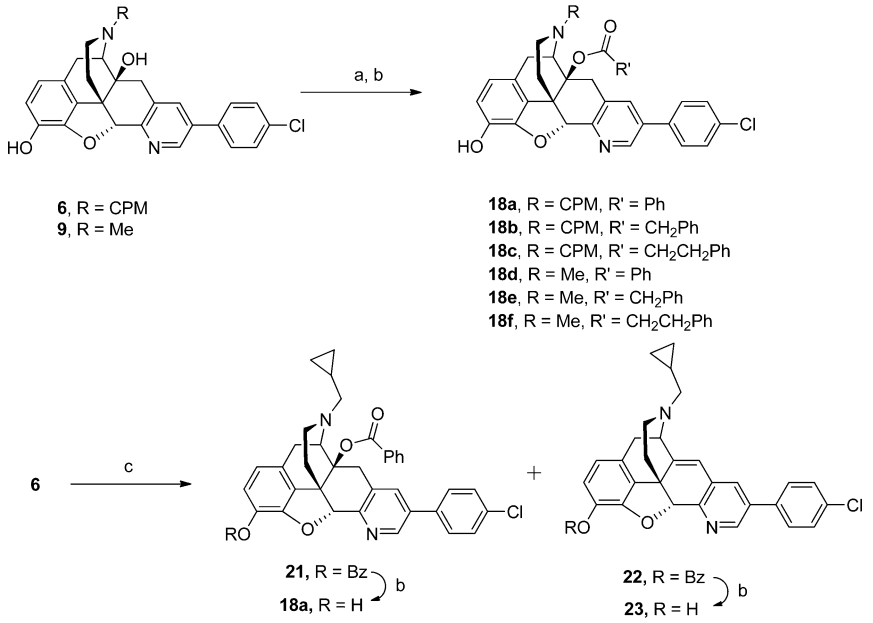
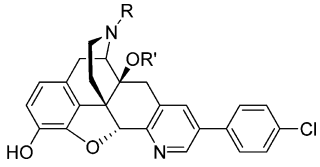
Scheme 2. Synthesis of 14-Acyloxypyridomorphinans 18a–f and 21–23[a]



[a]Reagents and conditions: (a) R′COCl, Et_3N, DMF or PhMe; (b) K_2CO_3, MeOH–H_2O, rt; (c) PhCOCl, Et_3N, DMF.

Table 1. Binding Affinities of the Pyridomorphinans at DOR, MOR, and KOR



			K_i ± SEM (nM)			selectivity ratio	
compd	R	R′	DOR[a]	MOR[b]	KOR[c]	MOR/DOR	KOR/DOR
17a	CPM	Me	1.95 ± 0.14	41.9 ± 2.8	5.37 ± 0.48	22	2.8
17b	CPM	$C_6H_5CH_2$	1.91 ± 0.08	8.42 ± 0.29	5.33 ± 0.27	4.4	2.8
17c	CPM	$C_6H_5CH{=}CHCH_2$	3.74 ± 0.32	3.03 ± 0.38	3.31 ± 0.30	0.81	0.89
17d	CPM	$C_6H_5CH_2CH_2CH_2$	1.20 ± 0.12	0.66 ± 0.06	1.82 ± 0.11	0.55	1.5
17e	Me	Me	1.63 ± 0.08	7.89 ± 0.33	27.99 ± 1.59	4.8	17
17f	Me	$C_6H_5CH_2$	1.78 ± 0.15	4.69 ± 0.17	49.0 ± 6.0	2.6	28
17g	Me	$C_6H_5CH{=}CHCH_2$	1.14 ± 0.11	0.41 ± 0.04	1.55 ± 0.09	0.36	1.4
17h	Me	$C_6H_5CH_2CH_2CH_2$	1.04 ± 0.08	0.54 ± 0.04	1.53 ± 0.10	0.52	1.5
18a	CPM	C_6H_5CO	87 ± 8	192 ± 21	117 ± 6	2.2	1.3
18b	CPM	$C_6H_5CH_2CO$	1.33 ± 0.10	1.04 ± 0.25	3.10 ± 0.19	0.78	2.3
18c	CPM	$C_6H_5CH_2CH_2CO$	4.13 ± 0.29	2.58 ± 0.12	12.0 ± 13	0.62	2.9
18d	Me	C_6H_5CO	17.0 ± 1.0	84.0 ± 7.0	112 ± 4	4.9	6.6
18e	Me	$C_6H_5CH_2CO$	0.97 ± 0.04	1.43 ± 0.08	17.0 ± 1.0	1.5	18
18f	Me	$C_6H_5CH_2CH_2CO$	0.96 ± 0.05	0.94 ± 0.05	4.41 ± 0.23	0.98	4.6
23	CPM	—	2.71 ± 0.11	13.0 ± 0.4	5.97 ± 0.19	4.8	2.2
6[d]	CPM	H	2.20 ± 0.20	51.0 ± 8.0	20.0 ± 1.0	23	9
9[d]	Me	H	3.90 ± 0.20	230 ± 10	468 ± 17	59	120

[a]Displacement of [^{3}H]DADLE from CHO cell membrane expressing hDOR. [b]Displacement of [^{3}H]DAMGO from CHO cell membranes expressing hMOR. [c]Displacement of [^{3}H]U69,593 from CHO cells expressing hKOR. [d]Data from refs 24 and 26 using brain tissue membranes included for comparison.

agonists and mixed MOR agonist/DOR antagonist ligands along with their analgesic effects and propensity to induce tolerance and dependence.

RESULTS AND DISCUSSION

Synthesis. For the synthesis of the desired target compounds, the previously reported 17-cyclopropylmethyl- and 17-methyl-3,14-dihydroxypyridomorphinans **6**[24] and **9**[26] served as suitable starting materials. For the synthesis of 14-alkoxy target compounds, we found it convenient to perform dialkylation at the phenolic hydroxyl at the 3-position and the tertiary alcohol at the 14-position followed by selective dealkylation of the phenolic ether function. Thus, dimethylation of **6** with dimethyl sulfate or dialkylation of **6** or **9** with

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Table 2. Functional Activity of the Pyridomorphinans at DOR, MOR, and KOR

	antagonist activity			agonist activity					
compd	DOR K_e (nM)	MOR K_e (nM)	KOR K_e (nM)	DOR EC_{50} (nM)	DOR E_{max} (%)	MOR EC_{50} (nM)	MOR E_{max} (%)	KOR EC_{50} (nM)	KOR E_{max} (%)
17a	*a*	*a*	*a*	no stim[b]	na[c]	no stim	na	*a*	*a*
17b	0.64 ± 0.11	11 ± 1	13 ± 3	no stim	na	no stim	na	27 ± 9	15 ± 1
17c	*a*	*a*	*a*	no stim	na	no stim	na	*a*	*a*
17d	0.091 ± 0.01	*d*	1.35 ± 0.28	no stim	na	1.74 ± 0.20	72 ± 2	no stim	na
17e	1.60 ± 0.35	*d*	17 ± 0.13	no stim	na	379 ± 50	118 ± 4	633 ± 108	36 ± 2
17f	4.54 ± 0.81	*d*	83 ± 14	11 ± 1	52 ± 1	301 ± 28	119 ± 3	215 ± 54	32 ± 2
17g	*e*	*e*	*e*	0.59 ± 0.05	84 ± 2.0	4.27 ± 0.57	130 ± 3	*e*	*e*
17h	*e*	*e*	6.9 ± 0.02	0.27 ± 0.03	85 ± 2.0	2.15 ± 0.21	125 ± 2	no stim	na
18a	218 ± 2	261 ± 36	126 ± 13	no stim	na	no stim	na	no stim	na
18b	0.37 ± 0.04	0.48 ± 0.06	7 ± 2	no stim	na	no stim	na	26 ± 9	34 ± 2
18c	1.86 ± 0.21	13 ± 1.7	14 ± 2	no stim	na	no stim	na	13 ± 4	25 ± 1
18d	45 ± 6	1273 ± 166	426 ± 47	no stim	na	4623 ± 1439	55 ± 8	no stim	na
18e	3.38 ± 0.34	*d*	198 ± 20	19 ± 6	27 ± 3	87 ± 10	122 ± 3	109 ± 24	36 ± 2
18f	1.64 ± 0.30	*d*	18 ± 0.2	5.52 ± 0.53	59 ± 2	48 ± 5	105 ± 2	136 ± 60	16 ± 2
23	0.58 ± 0.10	8.0 ± 0.89	19 ± 3.0	no stim	na	no stim	na	no stim	na

[a]Not tested because of lack of agonist activity at MOR. [b]No stimulation of [^{35}S]GTP-γ-S binding at 10 μM. [c]Not applicable. [d]Not tested because of full agonist activity at MOR. [e]Not tested because of agonist activity at both MOR and DOR.

appropriate alkyl bromides using sodium hydride as the base yielded the corresponding dialkyl derivatives **19a–d** and **19f**. Treatment of these with boron tribromide led to selective removal of the alkyl group from the ether function at C-3, yielding the target compounds **17a–d** and **17f**. For the preparation of **17e**, **17g**, and **17h**, the oxycodone-derived pyridomorphinan **20**[26] was used as the starting material. Alkylation of **20** with the appropriate alkylating agent followed by 3-*O*-demethylation of the resulting diethers **19e**, **19g**, and **19h** delivered the desired target compounds (Scheme 1).

The starting materials **6** and **9** were treated with an excess of the appropriate acid chloride, and the resulting intermediates were treated with aqueous base to remove the acyl group from the phenolic hydroxyl group to obtain the desired 14-*O*-acylated target compounds **18a–f**. The yields of the final products in these acylation reactions were only modest possibly due to elimination reactions setting in as side reactions. For example, isolation of the products from the reaction of **6** with benzoyl chloride after a 5 h reaction time gave the dibenzoate **21** and the elimination product **22** in 60:40 ratio. When the reaction was allowed to proceed for a longer period of time (16 h), the elimination product became the main product with a distribution ratio of 26:74 for **21** and **22**. These benzoates **21** and **22** could be converted to the free phenolic compounds **18a** and **23**, respectively, by treatment with K_2CO_3 in aqueous methanol (Scheme 2).

Ligand Binding at the Opioid Receptors. All target compounds were evaluated for binding affinities at DOR, MOR, and KOR using a radioligand displacement assay with membranes prepared from CHO cells stably expressing these receptors. The radioligands [^{3}H]DADLE, [^{3}H]DAMGO, and [^{3}H]U69,593 were used for labeling the DOR, MOR, and KOR sites, respectively. These evaluations were performed as previously described.[38−40] The affinity and selectivity data for the target compounds are given in Table 1. With the exception of **18a** and **18d**, all of the ligands displayed high affinity binding at DOR with K_i <5 nM. In general, all of the ligands displayed relatively nonselective binding profiles at all three opioid receptor subtypes. The 14-methoxy compound **17a** arising from 14-O-methylation displayed a binding profile somewhat similar to that of the parent compound **6**. In contrast, methylation of **9** produced the ligand **17e** that displayed markedly improved binding affinity at MOR and KOR. Among the *N*-CPM compounds, installation of the arylalkyl groups such as benzyl, cinnamyl, and 3-phenylpropyl on the oxygen at C-14 (compounds **17b**, **17c**, and **17d**) consistently increased the binding affinity at MOR. A similar trend of increasing MOR affinity is seen among the *N*-Me compounds **17f**, **17g**, and **17h**. Placement of a benzoyloxy group at C-14 is generally not well tolerated. The two benzoyloxy compounds **18a** and **18d** are 45- and 10-fold weaker in binding to DOR compared to their 14-benzyloxy counterparts, **17b** and **17f**, respectively. These benzoyloxy ligands also displayed a comparable decrease in affinity at MOR and KOR, indicating a general unfavorable interaction trend among all receptor subtypes. In contrast, installation of phenylacetyl and phenylpropionyl groups (**18b**, **18c**, **18e**, and **18f**) gave ligands with moderate to high affinity at all three receptors. In fact the phenylpropoxy and phenylpropionyl ligands possessing three-atom separation between 14-*O* and the pendant phenyl group (**17d** vs **18c** and **17h** vs **18f**) displayed somewhat comparable affinity profiles. The relatively high affinity of these ligands at all three receptors may be attributable to the conformational flexibility afforded by the longer chain to position the pendant phenyl group to occupy a suitable binding pocket for favorable hydrophobic or aryl-π interactions at the ligand binding pocket. The binding profile of compound **23** lacking an ether function with unsaturation between C-8 and C-14 resembled that of the saturated (**6**) or the methoxy (**17a**) analogues exhibiting lower affinity at MOR compared to affinities at DOR and KOR.

In Vitro Functional Activity at the Opioid Receptors. Compound selections and in vitro functional activity determinations were performed with the primary aim of identifying ligands possessing the desired MOR agonist/DOR antagonist activity. The agonist efficacy (E_{max}) and potency (EC_{50}) values were determined using previously described [^{35}S]GTP-γ-S binding assays with cells expressing MOR, DOR, or KOR. The agonist E_{max} values were normalized to the stimulation produced by the standard agonists DAMGO, DADLE, and U69,593 at MOR, DOR, and KOR, respectively.

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The antagonist potency of the ligands was determined using a [^{35}S]GTP-γ-S binding assay by measuring the shift in EC_{50} value of standard agonists. The functional activity data thus obtained are presented in Table 2. As might be expected, with the exception of **18d**, all of the compounds **17e−h**, **18e**, and **18f** possessing the classical MOR agonist *N*-Me structural feature displayed full agonist efficacy at MOR with E_{max} values >100. The weak MOR agonist potency and partial efficacy of **18d** is in conformity with its poor binding affinity at MOR. In terms of agonist potency, whereas the methyl and benzyl ethers were weak (**17e**, EC_{50} = 379 nM; **17f**, EC_{50} = 301 nM), the cinnamyl (**17g**) and the 3-phenylpropyl (**17h**) ethers were nearly 100-fold more potent with EC_{50} values of 4.27 nM and 2.15 nM, respectively. Compared to these ethers, the esters displayed diminished MOR agonist potencies (**18e** EC_{50} = 87 nM; **18f** EC_{50} = 48 nM). All of the esters (**18a−c**) as well as the unsaturated compound **23** possessing the classical antagonist *N*-CPM structural feature did indeed turn out to be antagonists at MOR. Similarly, the methyl, benzyl, and cinnamyl ethers **17a−c** possessing the *N*-CPM group also displayed a nonagonist profile at MOR. Most interestingly, however, the phenylpropyl ether **17d** possessing the *N*-CPM group displayed an agonist profile at MOR (E_{max} = 72%, EC_{50} = 1.74 nM). This transformative influence on the functional activity at MOR brought about by installation of a 3-phenylpropoxy group at the 14-position of the 17-cyclopropylmethyl-4,5-epoxypyridomorphinan is similar to the effect of such a group on 17-cyclopropylmethyl-4,5-epoxy-6-oxomorphinans.[33]

We had earlier demonstrated that pyridomorphinans in general, and those possessing an aryl group such as the 4-chlorophenylgroup at the 5′-position on the pyridine ring in particular, showed a nonagonist functional profile at DOR, irrespective of whether the ligands possessed a MOR-agonist methyl group (**9** and **10**, Chart 1) or a MOR-antagonist CPM group (**6** and **8**, Chart 1) on the morphinan nitrogen. The functional activity profile of the current series of compounds at DOR, however, is influenced by the nature of the substituent at C-14. In the current series of compounds, all of the ligands possessing an *N*-CPM group were antagonists at DOR including **17d**. However, ligands possessing *N*-methyl group (**17f**, **17g**, **17h**, **18e**, and **18f**) displayed weak to potent partial agonist activity at DOR. Among the *N*-methyl compounds, the 14-methoxy compound **17e** is the only exception retaining antagonist activity at DOR. Interestingly, the *N*-CPM containing MOR agonist ligand **17d** also turned out to be the most potent DOR antagonist with a K_e of 0.091 nM.

At KOR, most of the tested ligands displayed weak partial agonist activity (E_{max} <36%) with varying potencies as antagonists. The phenylpropoxy compound **17d** was devoid of agonist activity at KOR. Thus, the incorporation of the phenylpropoxy group on a pyridomorphinan possessing the *N*-CPM group did not induce agonist activity at DOR or KOR as it did at MOR. This is in contrast to the results from incorporation of a phenylpropoxy group on 6-oxomorphinans reported by Schmidhammer and co-workers, who found that introduction of a phenylpropoxy group at the C14-position of **1** yielded the agonist **13**, devoid of any antagonist activity.[33] The findings of the present study, together with the structure−activity relationships observed in earlier studies,[24,26,41,42] indicate that the substituted pyridine moiety fused to the C-ring of morphinans and the CPM substituent at the morphinan nitrogen serve as pharmacophoric elements that stabilize the inactive conformation of DOR and KOR regardless of the nature of the substituent at C-14. In contrast, installation of a group such as phenylpropoxy at C-14, even on frameworks possessing the *N*-CPM and pyridomorphinan groups, is capable of imparting agonist interaction at MOR. Among the compounds of the present study, **17d** emerged as a compound of particular interest as it displayed a balanced profile of potent agonist activity at MOR coupled with potent antagonist activity at DOR and KOR.

In Vitro Studies on Tolerance and Dependence. Having identified **17d** as a MOR agonist/DOR antagonist, we conducted two experiments using CHO cells coexpressing MOR and DOR (dimer cells) to determine the effect of **17d** on the development of tolerance and dependence, respectively. As a control, we included the structurally similar MOR/DOR dual agonist **17h** in these experiments. Dimer cells were treated for 20 h with medium (control), morphine (1 μM), **17d** (30 nM), or **17h** (30 nM). These concentrations were chosen to be approximately 25-fold greater than the corresponding EC_{50} values for stimulation of [^{35}S]GTP-γ-S binding to membranes prepared from dimer cells (EC_{50} for **17d** = 1.2 ± 0.1 nM, EC_{50} for **17h** = 0.34 ± 0.02 nM). As reported in Figure 1, chronic

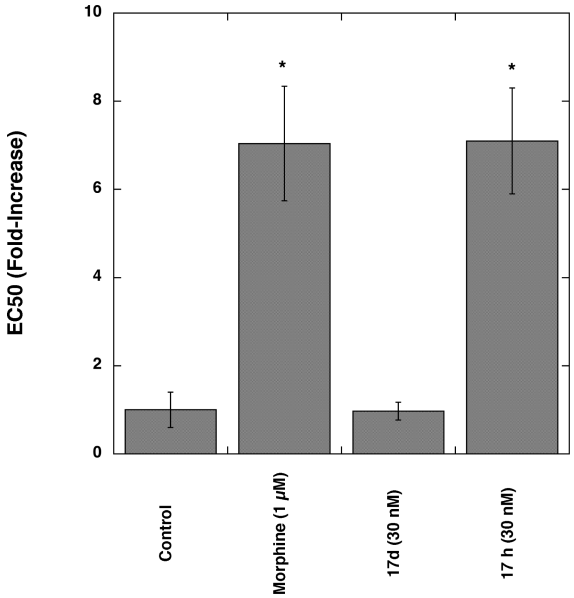

Figure 1. Comparison of the effects of chronic drug treatment on DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation in MOR/DOR dimer cells: MOR/DOR dimer cells were treated for 20 h with morphine (1 μM), or **17d** (30 nM), or **17h** (30 nM), respectively. DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation was performed as described in Experimental Section. Data are presented as the EC_{50} ratio (fold over control). Each value is the mean ± SEM (n = 3). *p < 0.05 when compared with the control cells (two-tailed Student's t test).

morphine and chronic **17h** resulted in an ∼7-fold increase in the EC_{50} value for DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation. Unlike morphine, **17d** and **17h** decreased the E_{max} value for DAMGO-mediated inhibition of forskolin-stimulated cAMP by 40% and 20%, respectively. The mechanism(s) underlying the decreased efficacy observed with chronic **17d** and **17h** treatment is currently under investigation.

In the "dependence" experiments (Figure 2), dimer cells were treated chronically as described above. After 20 h treatment, the cells were washed to remove drugs, and the

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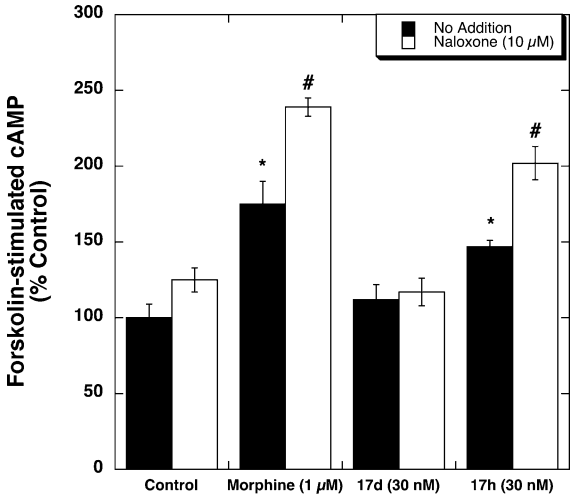

Figure 2. Comparison of the effects of chronic drug treatment on spontaneous and naloxone (10 μM)-induced cAMP overshoot in MOR/DOR dimer cells: MOR/DOR dimer cells were treated for 20 h with morphine (1 μM), or **17d** (30 nM), or **17h** (30 nM), respectively. The forskolin-stimulated cAMP accumulation was assessed as described in Experimental Section. Each value is the mean ± SEM (n = 3). *p < 0.05 when compared with no addition condition of the control cells. #p < 0.05 when compared with no addition condition of the morphine-treated or **17h** treated cells (two-tailed Student's t test).

degree of cAMP accumulation produced by forskolin/IBMX (100 μM/500 μM) was determined in the absence and presence of 10 μM naloxone. Chronic morphine produced a significant increase in forskolin-stimulated cAMP accumulation, a phenomenon called "cAMP superactivation".[6] The combination of forskolin plus naloxone produced a further increase in cAMP accumulation, a phenomenon called "naloxone-induced cAMP overshoot". The MOR agonist/DOR agonist compound (**17h**) produced effects similar to that of morphine, whereas the MOR agonist/DOR antagonist compound (**17d**) did not.

Chronic treatment of cells that express MOR with MOR agonists produce a variety of cellular adaptations that together produce tolerance and dependence.[6] In this study we assessed three such changes: (1) tolerance, as determined by shifts in the DAMGO dose–response curve for inhibition of forskolin-stimulated cAMP accumulation, (2) cAMP superactivation, and (3) the naloxone-induced cAMP overshoot. These latter two measures are generally considered as cellular signs related to dependence. The cAMP overshoot arises from chronic-morphine-induced formation of constitutively active receptors, which are receptors that activate G proteins in the absence of an agonist. The constitutively active MORs inhibit forskolin-stimulated cAMP. Because naloxone is an inverse agonist, it decreases the activity of the constitutively active MORs. This relieves the inhibitory effect of the constitutively active receptors on forskolin-stimulated cAMP, resulting in a further increase in forskolin-stimulated cAMP accumulation.[40,43]

The results observed here with chronic morphine treatment of dimer cells are similar to what we observed previously using CHO cells that stably express the cloned human MOR.[40] In contrast, chronic treatment of dimer cells with the MOR agonist/DOR antagonist **17d** did not produce either tolerance, as defined as an increase in the EC_{50} value for DAMGO-mediated inhibition of forskolin-stimulated cAMP, or dependence, as defined by the presence of cAMP superactivation or a naloxone-induced cAMP overshoot. The MOR agonist/DOR agonist **17h** had similar effects to that of morphine.

Numerous studies have replicated the original observations in whole animals that DOR antagonists reduce or block the development of morphine tolerance and dependence.[19,44] This is the first study that we are aware of that demonstrates this phenomenon at the cellular level, and this cell-based system should be useful for investigating the involvement of delta receptors in the development of morphine tolerance and dependence. Moreover, these data support the hypothesis that the mu/delta heterodimer may be a crucial mediator of tolerance and dependence.[45]

Analgesic Activity and Tolerance Studies in Mice. The analgesic activity of selected ligands were tested in mice using the 55 °C warm-water tail-withdrawal test as described previously.[46] Compounds that were evaluated in this study were **17d**, **17e**, **17g**, and **17h**. These compounds were administered by the intracerebroventricular (icv) route. All of the tested compounds produced dose-related antinociception in the 55 °C tail-flick assay. Morphine, **17e**, **17g**, and **17h** produced maximal or near-maximal agonist effects at the highest doses tested. Antinociceptive activity of **17d** exhibited a maximum at approximately 75% (a 10 nmol dose did not

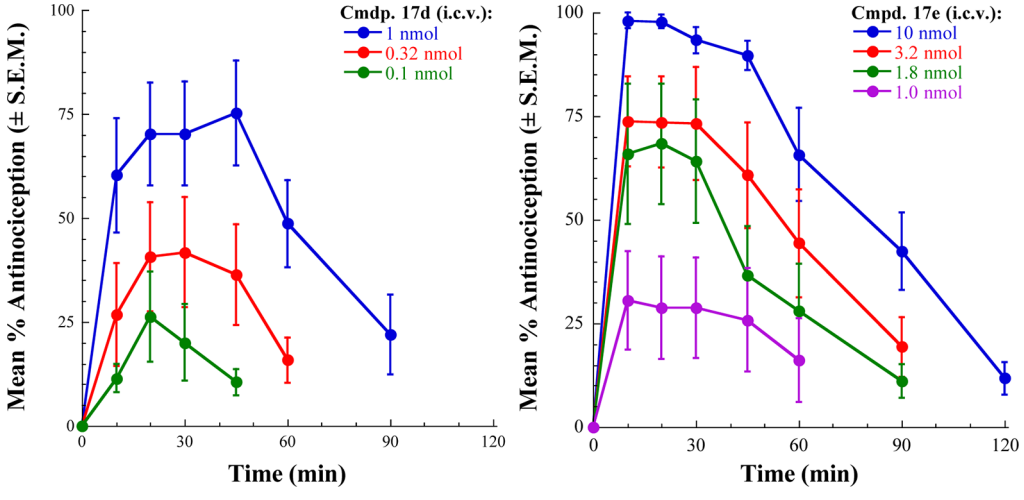

Figure 3. Antinociceptive dose– and time–response curves for **17d** and **17e** in the 55 °C warm-water tail-withdrawal assay.

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Table 3. Analgesic Activity of Selected Ligands in the Mouse Warm-Water Tail-Withdrawal Assay[a]

compd	maximal antinociception, %	dose for maximal antinociception, nmol	antinociceptive A_{50} values, nmol	95% confidence limits, nmol
17d	75	1	0.35	0.18−0.69
17e	98	10	1.44	1.02−2.03
17g	91	1	0.23	0.16−0.33
17h	92	1	0.23	0.18−0.30
morphine	100	10	0.43	0.38−0.51

[a]Compounds were administered icv and the A_{50} values calculated at time of peak effect.

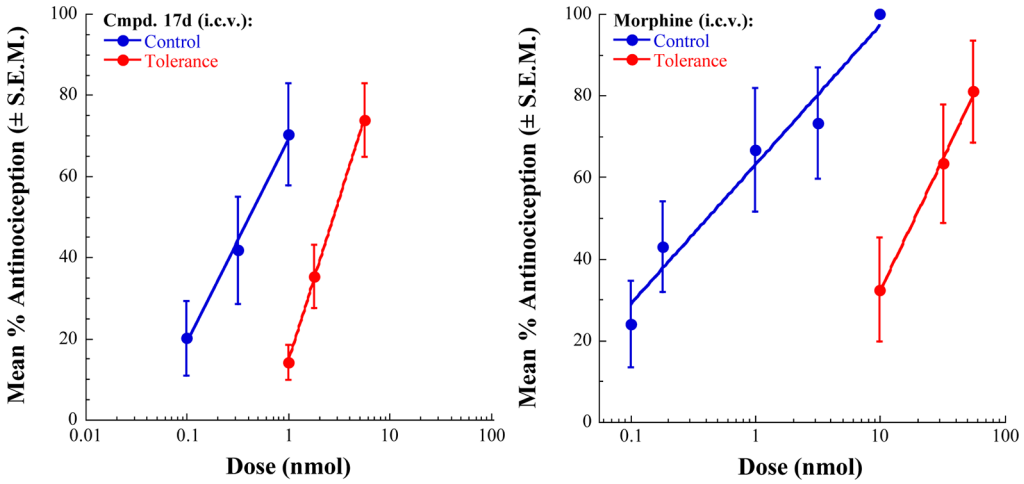

Figure 4. Antinociceptive dose−response curves for naive control mice and mice injected repeatedly with A_{90} doses of **17d** (left panel) or morphine (right panel) icv twice daily for 3 days.

produce an additional effect). The antinociceptive effects of these compounds were blocked by naloxone pretreatment, confirming that the analgesic activity of these compounds is mediated through opioid receptors. The antinociceptive dose−response curves for **17d** and **17e** are shown in Figure 3. The calculated antinociceptive A_{50} values of all the tested compounds and the morphine control are listed in Table 3. In this assay, compounds **17d**, **17g**, and **17h** displayed potency equivalent to or better than that of morphine. This is attributable to potent agonist activity of these ligands at MOR (**17d**) or at both MOR and DOR (**17g** and **17h**) as determined in the [^{35}S]GTP-γ-S assays. Despite the very weak MOR agonist potency displayed by **17e** in the [^{35}S]GTP-γ-S assay (MOR agonist EC_{50} = 379 nM), this compound produced significant analgesic effect and was only 6-fold weaker than other tested ligands. As evaluated in the in vitro tolerance assays, it was of interest to determine the propensity of the two compounds, **17d**, a MOR agonist/DOR antagonist and **17h**, a MOR-DOR dual agonist, to induce analgesic tolerance. The studies were carried out using the tolerance development assay involving repeated injection of the test compound (twice daily for 3 days). The degree of tolerance development is indicated by the fold-shift in the antinociceptive A_{50} values when tested in naive control mice and in the repeated injection paradigm. In this repeated administration paradigm, morphine produces a significant development of tolerance inducing a 44-fold shift in A_{50} value (control A_{50} = 0.43 nmol, tolerance A_{50} = 19.04 nmol). In contrast, **17d** displayed only a 7.9-fold shift in antinociceptive potency (control A_{50} = 0.35 nmol, tolerance A_{50} = 2.78 nmol) upon repeated administration, thus confirming that the MOR agonist/DOR antagonist ligand indeed produces significantly less tolerance than morphine (Figure 4). On the basis of the results from the in vitro tolerance study, the MOR-DOR dual agonist ligand **17h** might be expected to display robust tolerance development in the repeated injection paradigm. Unfortunately, undue toxicity displayed by **17h** as well as the alternative MOR-DOR dual agonist **17g** upon repeated administration precluded the determination of induction of analgesic tolerance by these dual agonists. In the [^{35}S]GTP-γ-S binding assay, the two compounds **17d** and **17h** displayed functional antagonism at KOR. While KOR-mediated effects may have contributed to the responses observed with these compounds in vivo, it is more likely that the MOR−DOR interactions are primarily responsible for tolerance- and dependence-related activities of these compounds based on the results from the in vitro tolerance and dependence experiments using MOR-DOR dimer cells devoid of KOR.

SUMMARY AND CONCLUSIONS

Several lines of evidence suggest physical or functional interactions between MOR and DOR and that activation of MOR while simultaneously inhibiting DOR can produce analgesic effects with diminished propensity for developing tolerance. With the goal of identifying novel small molecule ligands with mixed MOR agonist/DOR antagonist profiles, the current investigation focused on 4,5α-epoxypyridomorphinans possessing an ether or ester function at C-14. The observed structure−activity relationships clearly indicate that the intrinsic activity of such ligands is influenced by the nature of the substituent on the morphinan nitrogen (Me or CPM) as well as the group at C-14. Remarkably, the installation of a 3-phenylpropoxy group at C-14 on the framework containing a CPM group on the morphinan nitrogen led to a selective transformation of functional activity to that of an agonist at MOR with retention of antagonist activity at DOR and KOR,

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thus producing a ligand with the desired MOR agonist/DOR antagonist profile. Although the structural basis for this selective transformation of functional activity remains to be elucidated, it is likely that the placement of a group such as phenylpropoxy on suitable morphinan antagonist frameworks could transform them to mixed function ligands possessing agonist activity at MOR and antagonist activity at DOR and KOR.

Pharmacological evaluations with the MOR agonist/DOR antagonist ligand **17d** demonstrated that the mixed function ligand indeed produces diminished tolerance and dependence effects in a cellular model system as compared to the MOR/DOR dual agonist ligand **17h**. Moreover, the MOR agonist/DOR antagonist **17d**, when tested using the repeated administration procedure in mice, produced greatly diminished tolerance development as compared to morphine. These results suggest that developing small molecules with a mixed MOR agonist/DOR antagonist profile could be a fruitful approach in the search for analgesics devoid of some of the side effects associated with currently available opioid drugs.

EXPERIMENTAL SECTION

General Methods. Melting points were determined in open capillary tubes with a Mel-Temp melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Nicolet 300NB spectrometer operating at 300.635 MHz. Chemical shifts are expressed in parts per million downfield from tetramethylsilane. Spectral assignments were supported by proton decoupling. Mass spectra were recorded on a Varian MAT 311A double-focusing mass spectrometer in the fast atom bombardment (FAB) mode or on a Bruker BIOTOF II in electrospray ionization (ESI) mode. Elemental analyses were performed by Atlantic Microlab, Inc. (Atlanta, GA) or by the Spectroscopic and Analytical Laboratory of Southern Research Institute. Analytical results indicated by elemental symbols were within ±0.4% of the theoretical values. Thin layer chromatography (TLC) was performed on Analtech silica gel GF 0.25 mm plates. Flash column chromatography was performed with E. Merck silica gel 60 (230−400 mesh). Yields are of purified compounds and were not optimized. On the basis of NMR and combustion analysis data, all final compounds reported in the manuscript are >95% pure.

5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-methoxypyrido[2′,3′:6,7]morphinan (17a). *Step 1.* Sodium hydride (0.096 g, 4.0 mmol, 60% dispersion in mineral oil, washed with hexanes) was added to a stirred solution of **6** (0.487 g, 1.0 mmol) in DMF (7 mL) at 0−5 °C. The mixture was stirred at 0 °C for 10 min, treated dropwise with dimethyl sulfate (0.277 g, 2.2 mmol), and then allowed to warm to room temperature. The mixture was stirred at room temperature overnight and then was quenched by addition of small pieces of ice. The mixture was diluted with water (20 mL) and extracted with $CHCl_3$ (2 × 25 mL). The combined extracts were washed with water and brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to yield 0.3 g (58%) of 5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-3,14-dimethoxy-4,5α-epoxypyrido[2′,3′:6,7]morphinan (**19a**). ESI MS *m*/*z* 515 $(MH)^+$. The crude product thus obtained was used in the next step without further purification.

Step 2. A solution of **19a** (0.26 g, 0.5 mmol) in anhydrous CH_2Cl_2 (7 mL) was cooled to −78 °C and treated dropwise with boron tribromide (0.75 g, 3.0 mmol). The mixture was stirred at −78 °C for 1 h and then allowed to come to room temperature. After the reaction was quenched by addition of drops of ice-cold water, the mixture was extracted with $CHCl_3$ (2 × 50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified over silica gel column using $CHCl_3$−MeOH−NH_4OH (98:1.5:0.5) to yield 0.18 g (72%) of desired product **17a**: mp 170−173 °C; TLC R_f 0.39 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.12−0.54 (2 m, 4H, cyclopropyl CH_2CH_2), 0.84−0.88 (m, 1H, cyclopropyl C*H*), 1.43−1.47 (m, 1H, C-15 H), 2.16−2.68 (m, 7H, C-15 H, C-8 H, C-10 H, C-16 H_2, NCH_2), 3.01 (d, 1H, *J* = 17.2 Hz, C-8 H), 3.11 (m, 1H, C-10 H), 3.17 (s, 3H, OCH_3), 3.6 (m, 1H, C-9 H), 5.32 (s, 1H, C-5 H), 6.52 (s, 2H, C-2 H, C-1 H), 7.53−7.56 (m, 2H, C-3″ H, C-5″ H), 7.71−7.77 (m, 3H, C-4′ H, C-2″ H, C-6″ H), 8.76 (s, 1H, C-6′ H), 9.04 (s, 1H, C-3 OH). ESI MS *m*/*z* 501 $(MH)^+$. Anal. ($C_{30}H_{29}ClN_2O_3·H_2O$) C, H, N.

14-(Benzyloxy)-5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxypyrido[2′,3′:6,7]-morphinan (17b). *Step 1.* A solution of **6** (0.487 g, 1.0 mmol) in DMF (5.0 mL) was reacted with sodium hydride (0.12 g, 3.0 mmol, 60% dispersion in mineral oil, washed with hexane) and benzyl bromide (0.35 g, 2.2 mmol) as described in step 1 for the preparation of **17a**. Purification of the crude product by chromatography over a column of silica using $CHCl_3$−MeOH, 99:1 yielded 0.46 g (69%) of 3,14-bis(benzyloxy)-5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxypyrido[2′,3′:6,7]morphinan (**19b**). ESI MS *m*/*z* 667 $(MH)^+$.

Step 2. A solution of **19b** (0.46 g, 0.7 mmol) in anhydrous CH_2Cl_2 (5.0 mL) was cooled to −78 °C and treated dropwise with boron tribromide (3.0 mL of 1 M solution in CH_2Cl_2, 3.0 mmol). The mixture was stirred at −78 °C for 1 h and then allowed to attain room temperature. The reaction was quenched by the addition of water (10 mL). The mixture was extracted with $CHCl_3$ (2 × 50 mL), dried with anhydrous sodium sulfate, and filtered, and the solvent was removed under reduced pressure. The residue was purified over the column of silica gel using EtOAc−hexane (1:1) as the eluent. The product obtained was crystallized from EtOAc to afford 0.098 g (25%) of **17b**: mp 130−132 °C; TLC R_f 0.42 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.08−0.19 and 0.44−0.54 (m, 4H, cyclopropyl CH_2CH_2), 0.84−0.96 (m, 1H, cyclopropyl C*H*), 1.50 (d, 1H, *J* = 10.6 Hz, C-15 H), 2.20−2.28 (m, 1H, C-15 H), 2.34−2.82 (m, 7H, C-16 H_2, C-10 H, C-8 H_2, NCH_2-cyclopropyl), 3.10−3.23 (m, 1H, C-10 H), 3.82 (d, 1H, *J* = 5.7 Hz, C-9 H), 4.35 (dd, 2H, *J* = 11.1 and 11.4 Hz, OCH_2), 5.38 (s, 1H, C-5 H), 6.52−6.57 (m, 2H, C-1 H, C-2 H), 7.10−7.45 (m, 5H, C_6H_5), 7.53−7.56 (m, 2H, C-3″ H, C-5″ H), 7.70−7.74 (m, 3H, C-4′ H, C-2″ H, C-6″ H), 8.79 (d, 1H, *J* = 2.2 Hz, C-6′ H), 8.96 (s, 1H, C-3 OH). ESI MS *m*/*z* 577 $(MH)^+$. Anal. ($C_{36}H_{33}ClN_2O_3·H_2O$) C, H, N.

5′-(4-Chlorophenyl)-14-cinnamyloxy-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxypyrido[2′,3′:6,7]-morphinan (17c). *Step 1.* To a stirred solution of **6** (0.974 g, 2.0 mmol) in DMF (15 mL) was added sodium hydride (60% dispersion in mineral oil, 0.288 g, 6.0 mmol) at 0−5 °C. After the mixture was stirred at 0 °C for 10 min, cinnamyl bromide (0.871 g, 4.4 mmol) was added dropwise. The mixture was stirred at room temperature overnight, and the reaction was quenched by careful addition of small pieces of ice. The mixture was diluted with water and extracted with $CHCl_3$ (2 × 50 mL). The combined organic extracts were washed with water and brine and dried over anhydrous sodium sulfate. The crude product obtained after removal of the solvent under reduced pressure was purified over a column of silica using EtOAc−hexane 20:80 to yield 0.524 g (36%) of 5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-3,14-(dicinnamyloxy)-6,7-didehydro-4,5α-epoxypyrido[2′,3′:6,7]-morphinan (**19c**). ESI MS *m*/*z* 719 $(MH)^+$. The product thus obtained was used in the next step without further purification.

Step 2. A solution of **19c** (0.524 g, 0.73 mmol) in anhydrous CH_2Cl_2 (7 mL) was cooled to −78 °C. Boron tribromide (1.50 g, 6.0 mmol) was added dropwise. After being stirred for 1 h, the reaction mixture was allowed to attain room temperature. The reaction mixture was quenched by addition of drops of ice-cold water. After dilution with water, the crude product was extracted with $CHCl_3$ (2 × 100 mL), dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The crude product was purified by column chromatography over silica using EtOAc−hexane 25:75 as the eluent to obtain 0.268 g (61%) of **17c**: mp 138−140 °C; TLC R_f 0.35 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.06−0.23 (m, 2H, cyclopropyl CH_2), 0.43−0.57 (2 m, 2H, cyclopropyl CH_2), 0.83−0.99 (m, 1H, cyclopropyl C*H*), 1.43−1.53 (m, 1H, C-15 H), 2.12−2.76 (m,

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7H, C-16 H_2, C-8 H, C-10 H, NCH_2-cyclopropyl, C-15 H), 2.99 (m, 2H, C-8 H, C-10 H), 3.70 (d, 1H, J = 5.61 Hz, C-9 H), 4.05−4.39 (m, 2H, OCH_2), 5.41 (s, 1H, C-5 H), 6.05−6.35 (m, 2H, CH=CH), 6.50 (s, 2H, C-2 H, C-1 H), 6.88−7.18 (m, 5H, C_6H_5), 7.45−7.78 (m, 5H, C-5″ H, C-3″ H, C-4′ H, C-2″ H, C-6″ H), 8.8 (d, 1H, J = 2.2 Hz, C-6′ H), 9.06 (s, 1H, C-3 OH). ESI MS m/z 603 $(MH)^+$. Anal. ($C_{38}H_{35}ClN_2O_3 \cdot 0.5H_2O$) C, H, N.

5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-(3-phenylpropoxy)pyrido[2′,3′:6,7]-morphinan (17d). *Step 1.* To a stirred solution of **6** (1.948 g, 4.0 mmol) in DMF (40 mL) was added sodium hydride (0.96 g, 24 mmol, 60% dispersion in mineral oil, washed with hexanes) at 0−5 °C. After the mixture was stirred for 10 min, 3-phenylpropyl bromide (1.752 g, 8.8 mmol) was added dropwise. The reaction mixture was allowed to come to room temperature and stirred for 2 days. Excess of sodium hydride was decomposed with drops of ice-cold water, the mixture was then diluted with water and extracted with $CHCl_3$ (2 × 100 mL). The organic extracts were washed with water and brine and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by chromatography over a column of silica gel using EtOAc−hexane 20:80 as the eluent to obtain 5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-bis(3-phenylpropoxy)pyrido[2′,3′:6,7]morphinan (**19d**). Yield 0.96 g (34%). ESI MS m/z 723 $(MH)^+$.

Step 2. A solution of **19d** (0.92 g, 1.27 mmol) in anhydrous CH_2Cl_2 (25 mL) was cooled to −78 °C. Boron tribromide (3.18 g, 12.7 mmol) was added dropwise, and the mixture was stirred for 1 h. The mixture was then allowed to come to room temperature, and the reaction was quenched by addition of drops of ice-cold water. The mixture was diluted with water and extracted with $CHCl_3$. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure, and the residue was purified by chromatography over a column of silica using EtOAc−hexane 1:1 to obtain 0.23 g (28%) of the desired product (**17d**): mp 118−120 °C; TLC R_f 0.36 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.06−0.23 (m, 2H, cyclopropyl CH_2), 0.43−0.57 (2 m, 2H, cyclopropyl CH_2), 0.83−0.99 (m, 1H, cyclopropyl CH), 1.43−1.53 (m, 1H, C-15 H), 2.12−2.76 (m, 7H, C-16 H_2, C-8 H, C-10 H, NCH_2, C-15 H), 2.99 (m, 2H, C-8 H, C-10 H), 3.70 (d, 1H, J = 5.61 Hz, C-9 H), 4.05−4.39 (m, 2H, OCH_2), 5.41 (s, 1H, C-5 H), 6.05−6.35 (m, 4H, CH_2CH_2), 6.50 (s, 2H, C-2 H, C-1 H), 6.88−7.18 (m, 5H, C_6H_5) 7.45−7.78 (m, 5H, C-5″ H, C-3″ H, C-4′ H, C-2″ H, C-6″ H), 8.80 (d, 1H, J = 2.2 Hz, C-6′ H), 9.06 (s, 1H, C-3 OH). ESI MS m/z 605 $(MH)^+$. Anal. ($C_{38}H_{37}ClN_2O_3$) C, H, N.

5′-(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-methoxy-17-methylpyrido[2′,3′:6,7]morphinan (17e). *Step 1.* A stirred solution of **20** (0.69 g, 1.5 mmol) in DMF (15 mL) was cooled to 0−5 °C and sodium hydride (0.21 g, 45.25 mmol, 60% dispersion in mineral oil, washed with hexanes) was added. The mixture was stirred at 0 °C for 10 min and then treated dropwise with dimethyl sulfate (0.277 g, 1.8 mmol). The mixture was stirred at room temperature overnight and excess sodium hydride was destroyed by addition of ice-cold water. The mixture was diluted with water, and the product was extracted with $CHCl_3$ (2 × 25 mL). The organic extracts were washed with water and brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was purified over a column of silica using $CHCl_3$−MeOH−NH_4OH 97:2.5:0.5 as the eluent to obtain 0.29 g (41%) of the 3,14-dimethoxy compound **19e**: mp 290−294 °C (decomp); TLC R_f 0.35 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 1.42−1.46 (m, 1H, C-15 H), 2.16−2.63 (m, 5H, C-8 H_2, C-10 H, C-15 H, C-16 H), 2.33 (s, 3H, NCH_3), 3.01 (d, 1H, J = 17.12 Hz, C-16 H), 3.11 (s, 3H, C-14 OCH_3), 3.23−3.42 (m, 2H, C-10 H, C-9 H), 3.67 (s, 3H, C-3 OCH_3), 5.36 (s, 1H, C-5 H), 6.65 (d, 1H, J = 8.2 Hz, C-2 H), 6.70 (d, 1H, J = 8.2 Hz, C-1 H), 7.52−7.57 (m, 2H, C-2′ H, C-6″ H), 7.71−7.74 (m, 2H, C-3′ H, C-5″ H), 7.77 (d, 1H, J = 2.1 Hz, C-4′ H), 8.78 (d, 1H, J = 2.1 Hz, C-6′ H). ESI MS m/z 475 $(MH)^+$.

Step 2. The dimethoxy compound **19e** (0.360 g, 0.76 mmol) was dissolved in anhydrous CH_2Cl_2 (15 mL), cooled to −78 °C, and treated dropwise with boron tribromide (1 M solution in CH_2Cl_2, 1.5 mL, 1.5 mmol). After the mixture was maintained at −78 °C for 1 h, it was allowed to warm to room temperature. The reaction was quenched by addition of ice cold water. The crude product was extracted with $CHCl_3$ and dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the crude product was purified by column chromatography over silica using $CHCl_3$−MeOH−NH_4OH 97.5:2:0.5 to obtain 0.12 g (35%) of **17e**: mp 296−299 °C (decomp); TLC R_f 0.23 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 1.42−1.46 (m, 1H, C-15 H), 1.70−2.63 (m, 5H, C-8 H_2, C-10 H, C-15 H, C-16 H), 2.35 (s, 3H, NCH_3), 3.00 (d, 1H, J = 17.0 Hz, C-16 H), 3.10 (s, 3H, C-14 OCH_3), 3.14−3.50 (m, 2H, C-10 H, C-9 H), 5.32 (s, 1H, C-5 H), 6.49−6.56 (m, 2H, C-2 H, C-1 H), 7.52−7.57 (m, 2H, C-2″ H, C-6″ H), 7.71−7.74 (m, 2H, C-3″ H, C-5″ H), 7.77 (m, 1H, C-4′ H), 8.78 (d, 1H, J = 1.98 Hz, C-6′ H), 9.05 (s, 1H, C-3 OH). ESI MS m/z 461 $(MH)^+$. Anal. ($C_{27}H_{25}ClN_2O_3 \cdot 0.25H_2O$) C, H, N.

14-Benzyloxy-5′-(4-chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methylpyrido[2′,3′:6,7]morphinan (17f). *Step 1.* To a stirred solution of **9** in DMF (10 mL) at 0 °C was added sodium hydride (0.288 g, 6.0 mmol, 60% dispersion in mineral oil, washed with hexane). After the mixture was stirred at 0 °C for 20 min, benzyl bromide (0.425 g 6.0 mmol) was added, and the mixture was further stirred at room temperature overnight. The reaction mixture was cautiously treated with ice-cold water, diluted with water, and extracted with $CHCl_3$. The organic extracts were dried over anhydrous sodium sulfate and concentrated, and the residue obtained after removal of the solvent was purified over a column of silica using $CHCl_3$−MeOH 99:1 to obtain 0.57 g (46%) of 3,14-bis(benzyloxy)-5′-(4-chlorophenyl)-6,7-didehydro-4,5α-epoxy-17-methylpyrido-[2′,3′:6,7]morphinan (**19f**).

Step 2. A solution of **19f** (0.54 g, 1.0 mmol) in anhydrous CH_2Cl_2 (10 mL) was cooled to −78 °C and treated dropwise with boron tribromide (3.0 mL, 1 M solution in CH_2Cl_2, 3.0 mmol). After the mixture was stirred at −78 °C for 1 h, it was allowed to warm to room temperature. The mixture was quenched by addition of water (10 mL), extracted with $CHCl_3$ (2 × 50 mL), dried, and concentrated. The crude product thus obtained was purified by chromatography over a column of silica using $CHCl_3$−MeOH 98:2 as the eluent. The product was crystallized from ethyl acetate to yield 0.12 g (23%) of the desired product (**17f**): mp 228−232 °C; TLC R_f 0.5 ($CHCl_3$−MeOH, 92.5:7.5); ^{1}H NMR ($CDCl_3$), δ 1.49−1.52 (m, 1H, C-15 H), 2.21−2.28 (m, 1H, C-15 H), 2.37 (s, 3H, NCH_3), 2.45−2.61 (m, 4H, C-16 H_2, C-10 H, C-8 H), 3.12 (d, 1H, J = 17.1 Hz, C-10 H), 3.25 (s, 1H, C8 H), 3.52 (d, 1H, J = 5.5 Hz, C-9 H), 4.37 (dd, 2H, J = 11.5 and 11.4 Hz OCH_2), 5.32 (s, 1H, C-5 H), 6.52−6.57 (m, 2H, C-1 H, C-2 H), 7.07−7.18 (m, 5H, C_6H_5), 7.53−7.59 (m, 2H, C-3″ H, 5″ H), 7.69−7.73 (m, 3H, C-4′ H, C-2″ H, 6″ H), 8.79 (d, 1H, J = 1.95 Hz, C-6′ H), 9.05 (s, 1H, C-3 OH). ESI MS m/z 537 $(MH)^+$. Anal. ($C_{33}H_{29}ClN_2O_3 \cdot 0.25 \cdot H_2O$) C, H, N.

5′-(4-Chlorophenyl)-14-cinnamyloxy-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methylpyrido[2′,3′:6,7]morphinan (17g). *Step 1.* Compound **20** (0.460 g, 1.0 mmol) was reacted with sodium hydride (0.144 g, 6.0 mmol, 60% dispersion in mineral oil) and cinnamyl bromide (0.202 g, 1.1 mmol) in DMF (15 mL) as described in step 1 for **17e** to obtain 0.18 g (31%) of 5′-(4-chlorophenyl)-14-cinnamyloxy-6,7-didehydro-4,5α-epoxy-3-methoxy-17-methylpyrido-[2′,3′:6,7]morphinan (**19g**): mp 210−212 °C; TLC R_f 0.46 ($CHCl_3$−MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 1.46−1.51 (m, 1H, C-15 H), 2.15−2.63 (2 m, 5H, C-15 H, C-8 H, C-10 H, C-16 H_2), 2.36 (s, 3H, NCH_3), 3.05 (d, 1H, J = 17.1 Hz, C-8 H), 3.25−3.28 (m, 1H, C-10 H), 3.45−3.49 (m, 1H, C-9 H), 3.68 (s, 3H, C-3 OCH_3), 4.07−4.25 (m, 2H, CH_2CH=), 5.45 (s, 1H, C-5 H), 6.08−6.30 (m, 2H, −CH=CH−), 6.67 (d, 1H, J = 8.5 Hz, C-2 H), 6.71 (d, 1H, J = 8.2 Hz, C-1 H), 7.12−7.25 (m, 5H, phenyl), 7.50 (dd, 2H, J = 6.7 and 6.7 Hz, C-3″ H, C-5″ H), 7.60 (m, 2H, C-2″ H, C-6″ H), 7.71 (d, 1H, J = 2.0 Hz, C- 4′ H), 8.75 (s, 1H, C-6′ H). ESI MS m/z 577 $(MH)^+$.

Step 2. Compound **19g** (0.288 g, 0.5 mmol) was O-demethylated using boron tribromide, and the product obtained after column chromatography using EtOAc−hexane (75:25) was crystallized from EtOAc to yield 0.102 g (57%) of the desired product **17g**: mp 148−

150 °C; TLC R_f 0.33 ($CHCl_3$–MeOH, 90:10); ^{1}H NMR (DMSO-d_6) δ 1.46–1.51 (m, 1H, C-15 H), 2.20–2.66 (2 m, 5H, C-15 H, C-8 H, C-10 H, C-16 H_2), 2.36 (s, 3H, NCH_3), 3.05 (d, 1H, J = 17.1 Hz, C-8 H), 3.22–3.28 (m, 1H, C-10 H), 3.43–3.49 (m, 1H, C-9 H) 4.07–4.25 (m, 2H, CH_2CH═), 5.41 (s, 1H, C-5 H), 6.08–6.29 (m, 2H, CH═CH), 6.50–6.57 (m, 2H, C-2 H, C-1 H), 7.10–7.25 (m, 5H, C_6H_5), 7.46–7.52 (m, 2H, C-3″ H, C-5″ H), 7.62–7.68 (m, 2H, C-2″ H, C-6″ H), 7.71 (d, 1H, J = 2.0 Hz, C-4′ H), 8.78 (s, 1H, C-6′ H), 9.07 (s, 1H, C-3 OH). ESI MS m/z 563 $(MH)^+$. Anal. ($C_{35}H_{31}ClN_2O_3$·0.75H_2O) C, H, N.

5′-(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methyl-14-(3-phenylpropoxy)pyrido[2′,3′:6,7]morphinan (17h). *Step 1.* Compound **20** (0.96 g, 2.0 mmol) was reacted with sodium hydride (0.320 g, 4.0 mmol, 60% dispersion in mineral oil) and 3-phenylpropyl bromide (0.46 g, 2.6 mmol) in DMF (20 mL) as described in step 1 for **17e** to obtain 0.28 g (24%) of 5′-(4-chlorophenyl)-14-cinnamyloxy-6,7-didehydro-4,5α-epoxy-3-methoxy-17-methyl-14-(3-phenylpropoxy)pyrido[2′,3′:6,7]morphinan (**19h**). ESI MS m/z 579 $(MH)^+$.

Step 2. Compound **19h** (0.15 g, 0.26 mmol) was O-demethylated using boron tribromide to obtain 0.09 g (62%) of the desired product (**17h**): mp 128–130 °C; TLC R_f 0.37 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 1.39–1.53 (m, 1H, C-15 H), 1.52–1.66 (m, 2H, CH_2CH_2Ph), 2.09–2.66 (m, 7H, CH_2Ph, C-16 H_2, C-8 H, C-10 H, C-15 H), 2.49 (s, 3H, NCH_3), 2.95 (d, 1H, J = 16.81 Hz, C-8 H), 3.15–3.72 (m, 4H, OCH_2, C-10 H, C-9 H), 5.38 (s, 1H, C-5 H), 6.50 (s, 2H, C-2 H, C-1 H), 6.88–7.18 (m, 5H, C_6H_5), 7.48–7.58 (m, 2H, C-5″ H, C-3″ H), 7.65–7.75 (m, 3H, C-4′ H, C-2″ H, C-6″ H), 8.8 (d, 1H, J = 2.09 Hz, C-6′ H), 9.04 (s, 1H, C-3 OH). ESI MS m/z 565 $(MH)^+$. Anal. ($C_{35}H_{33}ClN_2O_3$·0.25H_2O) C, H, N.

14-Benzoyloxy-5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxypyrido[2′,3′:6,7]-morphinan (18a). To a solution of **6** (0.486 g, 1.0 mmol) in anhydrous DMF (10 mL) were added benzoyl chloride (0.421 g, 3.0 mmol) and triethylamine (0.42 mL). The reaction mixture was heated at 100 °C for 5 h under argon. The mixture was concentrated under reduced pressure, diluted with water, and extracted with $CHCl_3$. The organic extracts were dried over anhydrous sodium sulfate and filtered, and the filtrate was evaporated to dryness. The residue obtained was dissolved in methanol (24 mL) and treated with saturated aqueous K_2CO_3 to adjust the pH of the mixture to 9–10. The basic solution was stirred at room temperature for 3.5 h. The mixture was then concentrated under reduced pressure, diluted with water, and extracted with $CHCl_3$. Workup of the extract and purification of the crude product on a column of silica using $CHCl_3$–MeOH 98:2 yielded 0.192 g (32%) of the desired product **18a**: mp 158–162 °C; TLC R_f 0.56 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.07–0.17 and 0.32–0.35 (m, 4H, cyclopropyl CH_2CH_2), 0.58–0.62 (m, 1H, cyclopropyl CH), 1.67–1.71 (m, 1H, C-15 H), 2.17–2.45 (m, 3H, NCH_2-cyclopropyl, C-15 H), 2.63–2.82 (m, 4H, C-16 H_2, C-10 H, C-8 H), 3.15 (d, 1H, J = 18.5 Hz, C-10 H), 3.54 (d, 1H, J = 17.9 Hz, C-8 H), 4.68 (d, 1H, J = 6.0 Hz, C-9 H), 5.71 (s, 1H, C-5 H), 6.58 (s, 2H, C-1 H, C-2 H), 7.43–7.89 (m, 10H, C_6H_5, C-3″ H, C-5″ H, C-4′ H, C-2″ H, C-6″ H), 8.82 (d, 1H, J = 2.1 Hz, C-6′ H), 9.15 (s, 1H, C-3 OH). ESI MS m/z 591 $(MH)^+$. Anal. ($C_{36}H_{31}ClN_2O_4$·0.5H_2O) C, H, N.

5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-(phenylacetoxy)pyrido[2′,3′:6,7]-morphinan (18b). This compound was prepared using the method described above for the preparation of **18a** using toluene as the solvent instead of DMF. The reaction of **6** (0.486 g, 1.0 mmol), phenylacetyl chloride (0.50 g, 3.0 mmol), and triethylamine (0.6 mL) in toluene (10 mL) followed by basic workup of the reaction mixture and purification over a column of silica using EtOAc–hexane 60:40 yielded 0.101 g (17%) of the desired product **18b**: mp 118–120 °C; TLC R_f 0.46 ($CHCl_3$–MeOH, 92.5:7.5); ^{1}H NMR (DMSO-d_6) δ 0.33–0.47 (m, 4H, cyclopropyl CH_2CH_2), 0.64–88 (m, 1H, cyclopropyl CH), 1.51 (d, 1H, J = 10.3 Hz, C-15 H), 2.08–2.72 (m, 7H, C-16 H_2, C-15 H, C-10 H, C-8 H, NCH_2), 3.05 (d, 1H, J = 18.8 Hz, C-10 H), 3.55–3.70 (m, 3H, CH_2CO, C-8 H), 4.51 (d, 1H, J = 6.0 Hz, C-9 H), 5.34 (s, 1H, C-5 H), 6.53 (s, 2H, C-1 H, C-2 H), 6.92–7.06 (m, 5H, C_6H_5), 7.53 (m, 2H, C-3″ H, C-5″ H), 7.62 (d, 1H, J = 2.1 Hz, C-4′ H), 7.62–7.72 (m, 2H, C-2″ H, C-6″ H), 8.81 (d, 1H, J = 2.0 Hz, C-6′ H), 9.14 (s, C-3 OH). ESI MS m/z 605 $(MH)^+$. Anal. ($C_{37}H_{33}ClN_2O_4$·H_2O) C, H, N.

5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-(3-phenylpropionyloxy)pyrido-[2′,3′:6,7]morphinan (18c). This compound was prepared by using the method similar to that employed for the preparation of **18a**. Yield 19%. mp 96–98 °C; TLC R_f 0.47 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.08–0.11 and 0.39–0.48 (m, 4H, cyclopropyl CH_2CH_2), 0.67–71 (m, 1H, cyclopropyl CH), 1.57 (d, 1H, J = 10.7 Hz, C-15 H), 2.15–2.25 (m, 2H, CH_2CH_2Ph), 2.37–2.80 (m, 9H, C-16 H_2, C-15 H, C-10 H, C-8 H, NCH_2, CH_2CH_2Ph), 3.08 (d, 1H, J = 18.9 Hz, C-10 H), 3.54 (d, 1H, J = 17.6 Hz, C-8 H), 4.48 (d, 1H, J = 5.9 Hz, C-9 H), 5.46 (s, 1H, C-5 H), 6.55 (s, 2H, C-1 H, C-2 H), 6.98–7.16 (m, 5H, C_6H_5), 7.53–7.57 (m, 2H, C-3″ H, 5″ H), 7.71–7.75 (m, 3H, C-4′ H, C-2″ H, C-6″ H), 8.86 (d, 1H, J = 2.0 Hz, C-6′ H), 9.11 (s, 1H, C-3 OH). ESI MS m/z 618 $(MH)^+$. Anal. ($C_{38}H_{35}ClN_2O_4$·0.5H_2O) C, H, N.

14-Benzoyloxy-5′-(4-chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methylpyrido[2′,3′:6,7]morphinan (18d). This compound was prepared by reacting **9** (0.446 g, 1.0 mmol) with benzoyl chloride (0.425 g, 3.0 mmol) in the presence of triethylamine (0.62 mL, 5.0 mmol) in toluene (7 mL). Basic workup and purification of the reaction mixture yielded 0.068 g (12%) of the desired product: mp 280–284 °C; TLC R_f 0.39 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR ($CDCl_3$) δ 1.68 (d, 1H, J = 10.3 Hz, C-15 H), 2.25 (s, 3H, NCH_3), 2.90 (d, 1H, J = 3.3 Hz, C-15 H), 2.60–2.83 (m, 4H, C-16 H_2, C-10 H, C-8 H), 3.26 (s, 1H, C-10 H), 3.71 (d, 1H, J = 17.4 Hz, C-8 H), 4.36 (d, 1H, J = 6.1 Hz, C-9 H), 5.72 (s, 1H, C-5 H), 6.59–6.64 (m, 2H, C-1 H, C-2 H), 7.40–7.52 (m, 4H, 3″ H, 5″ H, *m*-protons of Bz), 7.54–7.62 (m, 1H, *p*-proton of Bz), 7.56–7.75 (m, 2H, C-2″ H, C-6″ H), 7.79 (d, 1H, J = 1.94 Hz, C-4′ H), 7.84–7.89 (m, 2H, *o*-protons of Bz), 8.81 (d, 1H, J = 2.1 Hz, C-6′ H), 9.16 (s, 1H, C-3 OH). ESI MS m/z 551 $(MH)^+$. Anal. ($C_{33}H_{27}ClN_2O_4$) C, H, N.

5′-(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methyl-14-(phenylacetoxy)pyrido[2′,3′:6,7]morphinan (18e). Prepared as described above for **18d** using phenylacetyl chloride as the reagent. Yield 16%. mp 194–196 °C; TLC R_f 0.68 ($CHCl_3$–MeOH, 92.5:7.5); ^{1}H NMR ($CDCl_3$), δ 1.58 (d, 1H, J = 10.3 Hz, C-15 H), 2.25 (s, 3H, NCH_3) 1.20–2.83 (m, 5H, C-16 H_2, C-15 H, C-10 H, C-8 H), 3.26 (s, 1H, C-10 H), 3.17 (d, 1H, J = 18.5 Hz, C8 H), 3.42–3.71 (m, 3H, CH_2Ph, C-8 H), 4.15 (d, 1H, J = 6.0 Hz, C-9 H), 5.32 (s, 1H, C-5 H), 6.53 (s, 2H, C-1 H, C-2 H), 6.93–7.06 (m, 5H, C_6H_5), 7.53 (d, 2H, J = 2.0 Hz, C-3″ H, C-5″ H), 7.61 (d, 1H, J = 1.94 Hz, C-4′ H), 7.68 (d, 2H, J = 6.6 Hz, C-2″ H, C-6″ H), 8.81 (d, 1H, J = 2.0 Hz, C-6′ H). ESI MS m/z 565 $(MH)^+$. Anal. ($C_{34}H_{29}ClN_2O_4$·0.25H_2O) C, H, N.

5′-(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methyl-14-(3-phenylpropionyloxy)pyrido[2′,3′:6,7]-morphinan (18f). Prepared as described above for **18d** using phenylpropionyl chloride as the reagent. Yield (22%): mp 242–244 °C; TLC R_f 0.63 ($CHCl_3$–MeOH, 92.5:7.5); ^{1}H NMR ($CDCl_3$), δ 1.55–1.59 (m, 1H, C-15 H), 2.15–2.22 (m, 1H, C-15 H), 2.25 (s, 3H, NCH_3), 2.38–2.76 (m, 8H, C-16 H_2, C-15 H, C-8 H, CH_2CH_2Ph), 3.18 (d, 1H, J = 19.8 Hz, C-10 H), 3.50 (d, 1H, J = 18.5 Hz, C-8 H), 4.17 (d, 1H, J = 6.0 Hz, C-9 H), 5.45 (s, 1H, C-5 H), 6.53 (s, 2H, C-1 H, C-2 H), 6.98–7.10 (m, 5H, C_6H_5), 7.54–7.57 (m, 2H, C-3″ H, C-5″ H), 7.71–7.76 (m, 3H, C-4′ H, C-2″ H, C-6″ H), 8.85 (d, 1H, J = 2.1 Hz, C-6′ H), 9.11 (s, 1H, C-3 OH). ESI MS m/z 579 $(MH)^+$. Anal. ($C_{35}H_{31}ClN_2O_4$) C, H, N.

3,14-Dibenzoyloxy-5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxypyrido[2′,3′:6,7]-morphinan (21) and 3-Benzoyloxy-5′-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7,8,14-tetradehydro-4,5α-epoxypyrido-[2′,3′:6,7]morphinan (22). To a solution of **6** (0.972 g, 2.0 mmol) in anhydrous DMF (15 mL) and benzoyl chloride (1.2 g, 6.0 mmol) was added triethylamine (1.67 mL, 12.0 mmol). The reaction mixture was heated at 100 °C for 5 h under argon. The mixture was cooled and diluted with H_2O (150 mL), and the product was extracted with

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$CHCl_3$. The organic extracts were washed with water and brine and dried over anhydrous sodium sulfate. The residue obtained after the removal of the solvent under reduced pressure was chromatographed over a column of silica using EtOAc–hexane 60:40 as the eluent. Collection of fractions containing the faster moving component and workup gave **21**: Yield 0.67 g (48%). TLC R_f 0.74 ($CHCl_3$–MeOH, 97.5:2.5); ^{1}H NMR (DMSO-d_6) δ 0.02–0.08 and 0.34–0.40 (m, 4H, cyclopropyl CH_2CH_2), 0.55–0.67 (m, 1H, cyclopropyl C*H*), 1.74–1.85 (m, 1H, C-15 H), 2.21–2.48 (m, 1H, C-15 H), 2.22–2.48 (m, 2H, NCH_2-cyclopropyl), 2.72–2.96 (m, 4H, C-16 H_2, C-10 H, C-8 H), 3.28–3.40 (s, 1H, C-8 H), 3.84 (d, 1H, J = 17.6 Hz, C-10 H), 4.78 (d, 1H, J = 6.0 Hz, C-9 H), 5.89 (s, 1H, C-5 H), 6.87 (d, 1H, J = 8.2 Hz, C-1 H), 7.02 (d, 1H, J = 6.2 Hz, C-2 H), 7.42–7.57 (m, 4H, *m*-protons of 3-Bz, C-3″ H, C-5″ H), 7.52–7.66 (m, 3H, *m*- and *p*-protons of 14-Bz), 7.72–7.82 (m, 3H, *p*-protons of 14-Bz, C-2″ H, C-6″ H), 7.86 (d, 1H, J = 1.7 Hz C-4′ H), 7.90–7.94 (m, 2H, *o*-protons of 14-Bz), 8.09–8.20 (m, 2H, *o*-protons of C-3 Bz), 8.81 (m, 1H, C-6′ H). ESI MS m/z 695 $(MH)^+$ Anal. ($C_{43}H_{35}ClN_2O_5 \cdot 0.25H_2O$) C, H, N. Elution and workup of the slower moving component gave **22**: Yield 0.48 g (35%). mp 132–134 °C; TLC R_f 0.58 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.15–0.19 and 0.50–0.54 (m, 4H, cyclopropyl CH_2CH_2), 0.85–0.91 (m, 1H, cyclopropyl C*H*), δ 1.77 (d, J = 11.7 Hz, 1H, C-15 H), 2.30–2.37 (m, 1H, C-15 H), 2.50–2.58 (m, 2H, NCH_2-cyclopropyl), 2.76–2.94 (m, 3H, C-16 H_2, C-10 H), 3.46 (s, 1H, C-10 H), 4.41 (d, 1H, J = 7.0 Hz, C-9 H), 5.88 (s, 1H, C-5 H), 6.31 (s, 1H, C-8 H), 6.73 (d, 1H, J = 8.3 Hz, C-2 H), 6.95 (d, 1H, J = 8.2 Hz, C-1 H), 7.55–7.78 (m, 7H, *m*- and *p*-protons of C-3 Bz and C-3″ H, C-5″ H, C-2″ H, C-6″ H), 7.85 (d, 1H, J = 2.2 Hz, C-4′ H), 8.10–8.11 (m, 2H, *o*-protons of C3-Bz), 8.72 (d, 1H, J = 2.1 Hz, C-6′ H). ESI MS m/z 573 $(MH)^+$. Anal. ($C_{36}H_{29}ClN_2O_3 \cdot 0.75H_2O$) C, H, N.

5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-3-hydroxy-6,7,8,14-tetradehydro-4,5α-epoxypyrido[2′,3′:6,7]morphinan (23). A solution of **22** (0.42 g, 0.73 mmol) was dissolved in MeOH (14 mL), and saturated aqueous K_2CO_3 was added dropwise to adjust the pH of the solution to 9–10. The mixture was stirred at room temperature for 3.5 h, diluted with H_2O, and extracted with $CHCl_3$. The organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue obtained was purified by chromatography over a column of silica using $CHCl_3$–MeOH 98:2 as the eluent to obtain 0.25 g (74%) of **23**: mp 164–166 °C; TLC R_f 0.38 ($CHCl_3$–MeOH, 95:5); ^{1}H NMR (DMSO-d_6) δ 0.01–0.17 and 0.47–0.54 (m, 4H, cyclopropyl CH_2CH_2), 0.82–0.89 (m, 1H, cyclopropyl C*H*), 1.70 (d, 1H, J = 12.1 Hz, C-15 H), 2.23–2.25 (m, 1H, C-15 H), 2.45–2.47 (m, 2H, C-8 H2), 2.70–2.89 (m, 3H, C-16 H_2, C-10 H), 3.16–3.21 (d, 1H, J = 18.0 Hz, C-10 H), 4.04–4.10 (m, 1H, C-9 H), 5.74 (s, 1H, C-5 H), 6.20 (s, 1H, C-8 H), 6.45–6.52 (m, 2H, C-1 H, C-2 H), 7.55–7.57 (m, 2H, C-3″ H, C-5″ H), 7.71–7.75 (m, 3H, C-4′ H, C-2″ H, C-6″ H), 8.76 (d, 1H, J = 2.2 Hz, C-6′ H), 9.13 (s, 1H, C-3 OH). ESI MS m/z 469 $(MH)^+$. Anal. ($C_{29}H_{25}ClN_2O_2 \cdot 0.75H_2O$) C, H, N.

Opioid Binding Assays. As described earlier,[38] the 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). The cells were grown on plastic flasks in DMEM (90%) (hDOR-CHO and hKOR-CHO) or DMEM/F-12 (45%/ 45%) medium (hMOR-CHO) containing 10% FetalClone II (HyClone) and geneticin (G-418: 0.10–0.2 mg/mL) (Invitrogen) under 95% air/5% CO_2 at 37 °C. Cell monolayers were harvested and frozen at −80 °C. The hKOR-CHO, hMOR-CHO, and hDOR-CHO cells were used for opioid binding experiments. For the [^{35}S]GTP-γ-S binding experiments, hKOR-CHO and hMOR-CHO cells were used for assaying KOR and MOR receptor function. Excellent signal-to-noise ratio were obtained using the NG108-15 neuroblastoma × glioma cell for the DOR [^{35}S]GTP-γ-S binding assay. Thus, hDOR-CHO cells were used for DOR binding assays, and the NG108-15 cells were used for the DOR [^{35}S]GTP-γ-S binding assay.

Radioligands [^{3}H][D-Ala^2-MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, SA = 44–48 Ci/mmol), [^{3}H][D-Ala^2, D-Leu^5]enkephalin ([^{3}H]DADLE, SA = 40–50 Ci/mmol) and [^{3}H](−)-U69,593 (SA = 50 Ci/mmol) were used to label MOR, DOR, and KOR binding sites, respectively. On the day of the assay, cell pellets were thawed on ice for 15 min and then homogenized with a polytron in 10 mL/pellet of ice-cold 10 mM Tris-HCl, pH 7.4. Membranes were then centrifuged at 30 000*g* for 10 min, resuspended in 10 mL/pellet ice-cold 10 mM Tris-HCl, pH 7.4 and again centrifuged 30 000*g* for 10 min. Membranes were then resuspended in 25 °C 50 mM Tris-HCl, pH 7.4 (~100 mL/pellet hMOR-CHO, 50 mL/pellet hDOR-CHO and 120 mL/pellet hKOR-CHO). All assays took place in 50 mM Tris-HCl, pH 7.4, with a protease inhibitor cocktail [bacitracin (100 μg/mL), bestatin (10 μg/mL), leupeptin (4 μg/mL), and chymostatin (2 μg/mL)], in a final assay volume of 1.0 mL. All drug dilution curves were made up with buffer containing 1 mg/mL BSA. Nonspecific binding was determined using 20 μM levallorphan ([^{3}H]DAMGO and [^{3}H]DADLE) and 1 μM (−)-U69,593 (for [^{3}H]U69,593 binding). [^{3}H]Radioligands were used at ~2 nM concentrations. Triplicate samples were filtered with Brandel Cell Harvesters (Biomedical Research & Development Inc., Gaithersburg, MD), over Whatman GF/B filters, after a 2 h incubation at 25 °C. The filters were punched into 24-well plates to which was added 0.6 mL of LSC-cocktail (Cytoscint). Samples were counted, after an overnight extraction, in a Trilux liquid scintillation counter at 44% efficiency. Opioid binding assays had ~30 μg protein per assay tube. Inhibition curves were generated by displacing a single concentration of radioligand by 10 concentrations of drug.

[^{35}S]GTP-γ-S Binding Assays. The [^{35}S]GTP-γ-S assays were conducted as described elsewhere.[38] In this description, buffer "A" is 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and buffer "B" is buffer A plus 1.67 mM DTT and 0.15% BSA. On the day of the assay, cells were thawed on ice for 15 min and homogenized using a polytron in 50 mM Tris-HCl, pH 7.4, containing 4 μg/mL leupeptin, 2 μg/mL chymostatin, 10 μg/mL bestatin, and 100 μg/mL bacitracin. The homogenate was centrifuged at 30 000*g* for 10 min at 4 °C and the supernatant discarded. The membrane pellets were resuspended in buffer B and used for [^{35}S]GTP-γ-S binding assays. [^{35}S]GTP-γ-S binding was determined as described previously. Briefly, test tubes received the following additions: 50 μL of buffer A plus 0.1% BSA, 50 μL of GDP in buffer A/0.1% BSA (final concentration = 40 μM), 50 μL of drug in buffer A/0.1% BSA, 50 μL of [^{35}S]GTP-γ-S in buffer A/0.1% BSA (final concentration = 50 pM), and 300 μL of cell membranes (50 μg of protein) in buffer B. The final concentrations of reagents in the [^{35}S]GTP-γ-S binding assays were as follows: 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 40 μM GDP, and 0.1% BSA. Incubations proceeded for 3 h at 25 °C. Nonspecific binding was determined using GTP-γ-S (40 μM). Bound and free [^{35}S]GTP-γ-S were separated by vacuum filtration (Brandel) through GF/B filters. The filters were punched into 24-well plates to which was added 0.6 mL of LSC-cocktail (Cytoscint). Samples were counted, after an overnight extraction, in a Trilux liquid scintillation counter at 27% efficiency.

Data Analysis and Statistics. These methods are described elsewhere.[39,40] For opioid binding experiments, the pooled data of three experiments (typically 30 data points) are fit to the two-parameter logistic equation for the best-fit estimates of the IC_{50} and N values: $Y = 100/(1 + ([\text{INHIBITOR}]/IC_{50})^N)$, where Y is the percent of control value. K_i values for test drugs are calculated according to the standard equation: $K_i = IC_{50}/(1 + [\text{radioligand}]/K_d])$. For the [^{3}H]radioligands, the following K_d values (nM ± SD, n = 3) were used in the K_i calculation: [^{3}H]DAMGO (0.93 ± 0.04), [^{3}H]DADLE (1.9 ± 0.3) and [^{3}H](−)-U69,593 (11 ± 0.6). The corresponding B_{max} values were (fmol/mg protein ± SD, n = 3): [^{3}H]DAMGO (1912 ± 68), [^{3}H]DADLE (3655 ± 391) and [^{3}H](−)-U69,593 (3320 ± 364).

For the [^{35}S]GTP-γ-S binding experiments, the percent stimulation of [^{35}S]GTP-γ-S binding was calculated according to the following formula: $(S - B)/B \times 100$, where B is the basal level of [^{35}S]GTP-γ-S binding and S is the stimulated level of [^{35}S]GTP-γ-S binding. Agonist dose–response curves (ten points/curve) were generated, and the data of several experiments, three or more, were pooled. The EC_{50} values

(the concentration that produces 50% maximal stimulation of [^{35}S]GTP-γ-S binding) and E_{max} were determined using either the program MLAB-PC (Civilized Software, Bethesda, MD), KaleidaGraph (Version 3.6.4, Synergy Software, Reading, PA), or Prism 4.0 (GraphPad Software, Inc., San Diego, CA). In most cases, the percent stimulation of the test compound is reported as a percent of the maximal stimulation of 1000 nM DAMGO, 500 nM SNC80 or 500 nM (−)-U50,488 in the appropriate cell type. For determination of K_e values using the "shift" experimental design, agonist (DAMGO, (−)-U50,488 or SNC80) dose−response curves were generated, using the appropriate cell type, in the absence and presence (ten points/curve) of a test compound. The data of several experiments, three or more, were pooled, and the K_e values were calculated according to the equation: [test drug]/($EC_{50\text{-}2}/EC_{50\text{-}1}$ − 1), where $EC_{50\text{-}2}$ is the EC_{50} value in the presence of the test drug and $EC_{50\text{-}1}$ is the value in the absence of the test drug.

Cell Culture and cAMP Assay. CHO cells coexpressing cloned MOR and DOR (cMyc-mδ-HμCHO cells) were produced by stable transfection with the mouse DOR N-cMyc tag and human MOR cDNA and were originally provided by Dr. J. B. Wang (University of Maryland Baltimore Campus, Baltimore, MD). Cells were grown on plastic flasks in F-12 Nutrient Mixture (HAM, GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 400 μg/mL hygromycin B (for DOR selection), and 400 μg/mL geneticin (for MOR selection) under 95% air/5% CO_2 at 37 °C. After 80% confluence, cell monolayers were plated in 24-well plates and grown in F-12 Nutrient Mixture (HAM, GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 400 μg/mL hygromycin B, and 400 μg/mL geneticin under 95% air/5% CO_2 at 37 °C. On the day of the experiment, cells (control or drug-treated) were washed three times with serum-free medium and incubated with serum-free medium containing IBMX (500 μM). After a 20-min incubation at 37 °C, medium was removed and then cells were incubated with fresh serum-free medium containing IBMX (500 μM) and forskolin (100 μM) and appropriate agonist or antagonist for 15 min (assay for opioid inhibition of cAMP accumulation) or 10 min (assay for naloxone-induced cAMP overshoot) at 37 °C. The reaction was terminated by aspiration of the medium and the addition of 0.5 mL of 0.1 N HCl. After plates were chilled at 4 °C for at least 1 h, 0.4 mL was removed, neutralized, vortexed, and centrifuged at 13 000 rpm for 5 min, and the supernatants were used for cAMP assay. This assay monitored inhibition of [^{3}H]cAMP binding to cAMP-dependent protein kinase. Assays took place in 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 5 mM EDTA. After a 2 h incubation at 4 °C (protected from light), bound and free [^{3}H]cAMP were separated by vacuum filtration through Whatman GF/B filters with two 4 mL washes with ice-cold 10 mM Tris-HCl, pH 7.4. Filters were punched into wells of plate to which was added 0.6 mL of LSC-cocktail (CytoScint) and counted in a liquid scintillation counter at 44% efficiency.

Data Analysis and Statistics. The amount of cAMP in the samples was quantitated from a cAMP standard curve ranging from 0.25 to 256 pmol of cAMP/assay. Forskolin (100 μM)-stimulated cAMP formation in the absence of agonist was defined as 100%. The EC_{50} (the concentration of agonist that produces 50% inhibition of forskolin-stimulated cAMP formation) and E_{max} (% of maximal inhibition of forskolin-stimulated cAMP) were calculated using program Prism version 4 (GraphPad Software, San Diego, CA). Data from three experiments were analyzed using Prism. Results are presented as the mean ± SEM.

Sources. [^{3}H]cAMP (adenosine 3′5′-cyclic phosphate, [2,8-^{3}H] (SA = 43 Ci/mmol)) was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). Forskolin, 3′5′-cyclic AMP (cAMP), 3-isobutyl-1-methylxanthine (IBMX), and protein kinase 3′5′-cyclic-AMP binding protein were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained from sources reported eariler.[47]

Antinociceptive Studies. Male ICR mice (Harlan) were used for all evaluations. Mice were housed in a temperature- and humidity-controlled vivarium on a 12:12 h light:dark cycle with unlimited access to food and water prior to the formal procedures. Graded doses of morphine or the test compounds were injected intracerebroventricularly (icv) under light ether anesthesia.[46] Morphine sulfate was dissolved in distilled water and injected in a volume of 5 μL. All test compounds were evaluated as free bases. The test compounds were dissolved in 100% DMSO and injected in a volume of 5 μL. Antinociceptive assays were performed at various times after injection.

Warm-Water Tail-Withdrawal Assay. Naive mice were baselined in the 55 °C warm-water tail-withdrawal test as previously described.[46,48] Doses of morphine or the test compound were injected icv, and antinociception was assessed at 10, 20, 30, 45, 60, 80, 120, and 180 min postinjection. Percent antinociception was calculated using the following formula: %MPE (maximal possible effect) = 100 × (test − control)/(cutoff − control) where control is the predrug observation, test is the postdrug observation, and cutoff is the maximal length of stimulus allowed (10 s for 55 °C tail-withdrawal). Antinociceptive A_{50} values and 95% confidence intervals were determined using linear regression software (FlashCalc). Opioid activity of the test compounds was assessed by pretreating animals with naloxone (10 mg/kg ip, −10 min) followed by an icv injection of an approximate A_{90} dose of test compound. If a compound did not produce a full agonist effect, then the dose that produced the greatest antinociceptive effect was used. Antinociception was assessed in the 55 °C warm-water tail-withdrawal test at 10, 20, and 30 min. A positive response to a fixed dose of naloxone was indicated when greater than 80% reduction in the antinociceptive effect of the agonist was observed.

Tolerance Regimen. Mice were injected twice daily (8 a.m. and 8 p.m.) with an approximate A_{90} dose of morphine or A_{90} dose of **17d** for 3 days. Antinociceptive dose−response curves in the warm-water tail-withdrawal assay were generated on the morning of the fourth day using the procedures outlined above.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CHO, Chinese hamster ovary; CPM, cyclopropylmethyl; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAMGO, [D-Ala^2,Me-Phe,Gly-ol^5]enkephalin; DOR, δ opioid receptor; [^{35}S]GTP-γ-S, guanosine-5′-*O*-(3-[^{35}S]thio-triphosphate; KOR, κ opioid receptor; MOR, μ opioid receptor; TIPP[ψ], H-Tyr-Ticψ-[CH_2−NH]-Phe-Phe-OH

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