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Highly Potent and Selective New Diphenethylamines Interacting with the κ-Opioid Receptor: Synthesis, Pharmacology, and Structure−Activity Relationships

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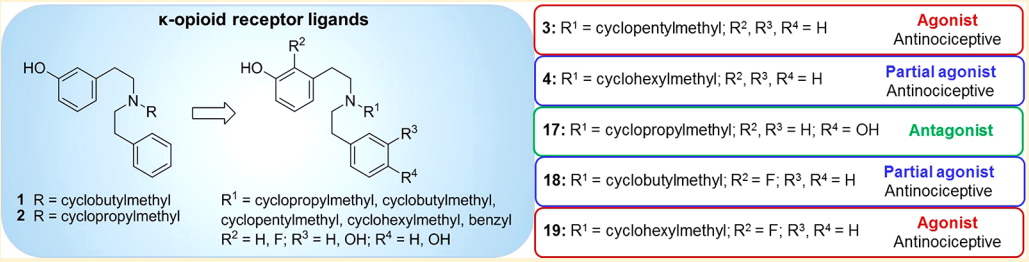
Supporting Information



ABSTRACT: We previously reported on a series of small molecules targeting the κ-opioid (KOP) receptor featuring a diphenethylamine scaffold and showed the promise of these ligands as effective analgesics with reduced liability for adverse effects. This study expands the structure−activity relationships on our original series by presenting several modifications in the lead compounds **1** (HS665) and **2** (HS666). A library of new diphenethylamines was designed, synthesized, and pharmacologically evaluated. In comparison with **1** and **2**, the KOP receptor affinity, selectivity, and agonist activity were modulated by introducing bulkier N-substituents, a 2-fluoro substitution, and additional hydroxyl groups at positions 3′ and 4′. Several analogues showed subnanomolar affinity and excellent KOP receptor selectivity acting as full or partial agonists, and one as an antagonist. The new diphenethylamines displayed antinociceptive efficacies with increased potencies than U50,488, **1** and **2** in the writhing assay and without inducing motor dysfunction after sc administration in mice.

INTRODUCTION

The κ-opioid (KOP) receptor belongs to the large family of the seven transmembrane GPCRs and is a key member of the opioid neuromodulatory system.[1] Activation of the KOP receptor by specific endogenous neuropeptides, the dynorphins,[1] initiates complex signaling events.[2] The elucidated KOP receptor crystal structure[3] offers a valuable platform for inquiry into receptor function and ligand−receptor interactions.[4] The downstream effects of KOP receptor agonism vary greatly and include beneficial (antinociception) and nonbeneficial actions (dysphoria, sedation, psychotomimesis, diuresis, and motor dysfunction). Growing preclinical and clinical evidence indicates that the KOP receptor/dynorphin system contributes to symptom clusters that are shared by many neuropsychiatric conditions and thus to their high comorbidity (i.e., pain and addiction, pain and depression, addiction and depression).[5] Differential modulation of the KOP receptor is nowadays regarded as a promising strategy for developing therapies for pain, drug addiction, mood disorders (e.g., depression and anxiety), neurological conditions (e.g., epilepsy), and itching skin and inflammatory diseases by either activating or blocking the receptor.[5,6] Hence, there have been significant efforts to generate ligands with distinct pharmacological properties including agonists, partial agonists, antagonists, allosteric modulators as well as biased agonists that selectively activate G protein signaling while not engaging the β-arrestin2 pathway.[7] Earlier and current drug development strategies target natural, naturally derived, and synthetic ligands to the KOP receptor, as small molecules or peptides, with short or long-acting pharmacokinetics, and central or peripheral site of action. Accumulated literature into the field is presented in extended reviews over the years.[7,8]

Recent observations from our laboratory on 3-hydroxy substituted diphenethylamines revealed that the character of the N-substituent plays an important role on the binding and activation of the KOP receptor.[9] An *N*-cyclopropylmethyl (*N*-CPM) and particularly an *N*-cyclobutylmethyl (*N*-CBM) substitution is more favorable for the interaction with the KOP receptor than *n*-alkyl groups by causing a significant increase in KOP receptor affinity and selectivity as well as in agonist potency and efficacy.[9a] Switching the hydroxyl group from position 3 to 4 resulted in reduced KOP receptor binding.[9b] The *N*-CBM substituted **1** (HS665)[9a] (Figure 1) is a

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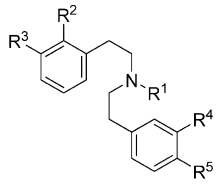


	R^1	R^2	R^3	R^4	R^5
1 (HS665)	CBM	H	OH	H	H
2 (HS666)	CPM	H	OH	H	H
3	CPeM	H	OH	H	H
4	CHM	H	OH	H	H
5	Bn	H	OH	H	H
6	CB	H	OH	H	H
7	isoamyl	H	OH	H	H
8	CBM	H	OH	OH	H
9	CPM	H	OH	OH	H
10	allyl	H	OH	OH	H
11	CHM	H	OH	OH	H
12	CPeM	H	OH	OH	H
13	isoamyl	H	OH	OH	H
14	CBM	H	OH	H	OH
15	CHM	H	OH	H	OH
16	allyl	H	OH	H	OH
17	CPM	H	OH	H	OH
18	CBM	F	OH	H	H
19	CHM	F	OH	H	H
20	CBM	F	OH	OH	H
21	CBM	F	OH	H	OH
22	CBM	H	H	H	H

Figure 1. Structures of reference diphenethylamines **1** and **2** and the new derivatives **3**–**22**. Bn, benzyl; CB, cyclobutyl; CBM, cyclobutylmethyl; CHM, cyclohexylmethyl; CPeM, cyclopentylmethyl; CPM, cyclopropylmethyl.

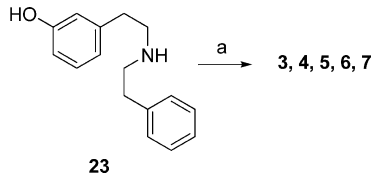
highly selective and full KOP receptor agonist,[9a,c,d] complemented by high antinociceptive potency and efficacy in vivo after subcutaneous (sc) administration comparable to 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*R*,2*R*)-2-pyrrolidin-1-ylcyclohexyl]acetamide (U50,488).[9a] Its *N*-CPM analogue **2** (HS666)[9a] (Figure 1) is a selective KOP partial agonist.[9a,d] We established lately that **1** and **2** produce dose-dependent antinociception in the 55 °C warm-water tail-withdrawal assay in mice after central intracerebroventricular (icv) administration mediated by the KOP receptor.[9d] Compound **1** was slightly more potent (<2-fold) than **2** in inducing an antinociceptive response. When compared to U50,488, **1** displayed a 2-fold greater antinociceptive potency, whereas **2** was equipotent. We found that these selective KOP ligands display in vitro varying bias signaling toward G protein activation consistent with a reduced liability profile, reflected by the lack of sedation and absence of conditioned place aversion in mice for **2**.[9d]

In our search for active molecules with distinct KOP receptor activation profiles, we utilized in the current study the 3-hydroxy substituted diphenethylamine scaffold to design, synthesize, and pharmacologically evaluate new derivatives carrying a variety of bulkier N-substituents, e.g., cyclopentylmethyl (CPeM), cyclohexylmethyl (CHM), benzyl, and isoamyl (**3**–**7**, Figure 1). For the purpose of probing the consequence of a second hydroxyl group in positions 3 (**8**–**13**, Figure 1) or 4 (**14**–**17**, Figure 1) at the second aromatic ring, we introduced essentially the aforementioned N-substituents. In this work, to extend the structure–activity relationships (SAR) within this class of compounds, the corresponding 2-fluoro substituted analogues (**18**–**21**, Figure 1) were prepared and biologically characterized. For comparison reasons, the aromatic unsubstituted *N*-CBM derivative (**22**) was synthesized.

RESULTS AND DISCUSSION

Chemistry. The new diphenethylamines were synthesized in a straightforward manner using five synthetic routes as depicted in Schemes 1–5.[10] The first route (Scheme 1) was

Scheme 1. Synthesis of Compounds 3–7[a]

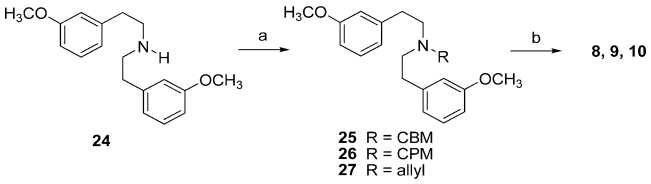


[a]Reagents and conditions: (a) respective alkyl or allyl bromide or cyclobutyl tosylate, $NaHCO_3$, CH_3CN, reflux.

used for 3-monohydroxy derivatives **3**–**7**, which were prepared from 3-[2-(phenylethylamino)ethyl]phenol (**23**)[9a] by *N*-alkylation with the respective alkyl or allyl bromide or cyclobutyl tosylate in the presence of $NaHCO_3$ in CH_3CN. This alkylation step was performed according to the earlier described procedure.[9a,b]

The synthesis of the 3,3′-dihydroxy derivatives **8**–**10** started from *N*-(3-methoxyphenethyl)-2-(3-methoxyphenyl)-ethanamine (**24**)[11] which was alkylated with the respective alkyl bromides or allyl bromide in the presence of K_2CO_3 in DMF to afford **25**–**27**. Ether cleavage with sodium ethanethiolate in DMF yielded the 3,3′-dihydroxy substituted diphenethylamines **8**–**10** (Scheme 2).

Scheme 2. Synthesis of Compounds 25–27 and 8–10[a]



[a]Reagents and conditions: (a) respective alkyl or allyl bromide, K_2CO_3, DMF, N_2, 80 °C; (b) sodium ethanethiolate, DMF, N_2, 130 °C.

3-Hydroxyphenylacetic acid (**28**) or 4-hydroxyphenylacetic acid (**29**) were reacted with 3-methoxyphenylethylamine (**30**) in CH_2Cl_2 in the presence of EDC and HOAt to provide amides **31** and **32**. BH_3 reduction in THF gave amines **33** and **34**, which were *N*-alkylated in CH_3CN with the respective alkyl bromides or allyl bromide in the presence of $NaHCO_3$ to yield compounds **35**–**41**. Ether cleavage of **35** and **37**–**40** with sodium ethanethiolate in DMF provided the final products **11** and **13**–**16**, while ether cleavage of **36** and **41** using BBr_3 in CH_2Cl_2 yielded compounds **12** and **17** (Scheme 3).

The 2-fluoro substituted diphenethylamines **18**–**21** were synthesized similarly to compounds **11**–**17**. Amides **46**–**48** were prepared from 2-fluoro-3-methoxyphenylacetic acid (**42**) by reaction with 2-phenylethylamine (**43**), 2-(3-methoxyphenyl)ethylamine (**44**), or 2-(4-hydroxyphenyl)-ethylamine (**45**) in the presence of EDC and HOAt in CH_2Cl_2. BH_3 reduction in THF gave amines **46**–**48**, which were *N*-alkylated in CH_3CN with the respective alkyl bromides

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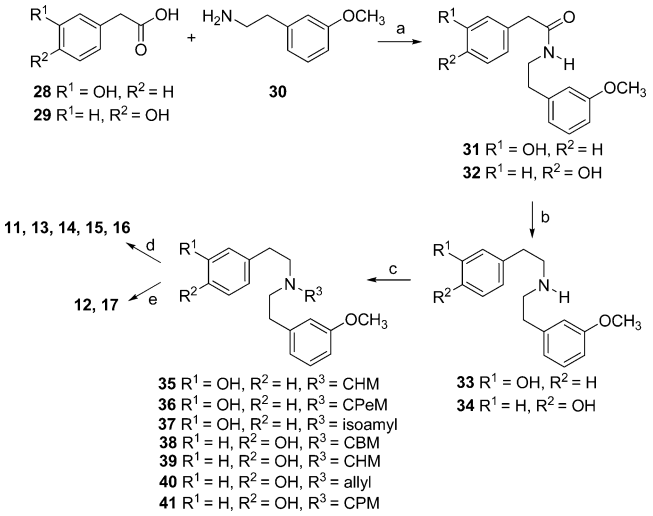
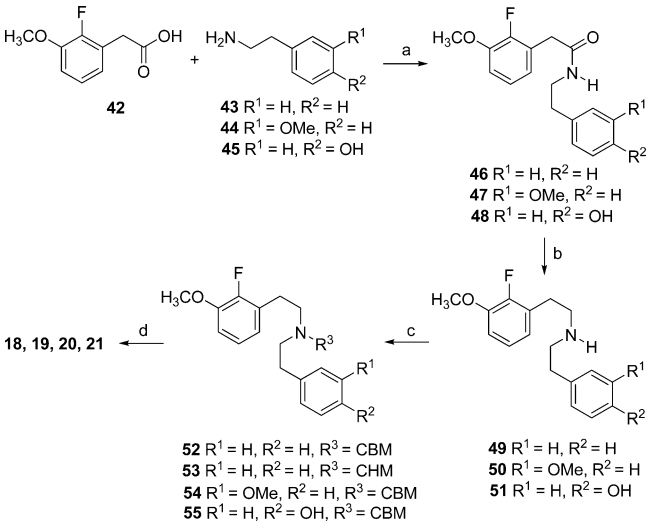
Scheme 3. Synthesis of Compounds 31−41 and 11−17[a]



[a]Reagents and conditions: (a) EDC and HOAt in CH_2Cl_2, N_2, rt; (b) BH_3·THF 1 M in THF, N_2, reflux; (c) respective alkyl or allyl bromide, $NaHCO_3$, CH_3CN, N_2, reflux; (d) sodium ethanethiolate, DMF, N_2, 130 °C; (e) BBr_3 1 M CH_2Cl_2 solution in CH_2Cl_2, −15 °C.

in the presence of $NaHCO_3$ to afford compounds **52−55**. Ether cleavage with BBr_3 in CH_2Cl_2 provided compounds **18−21** (Scheme 4).

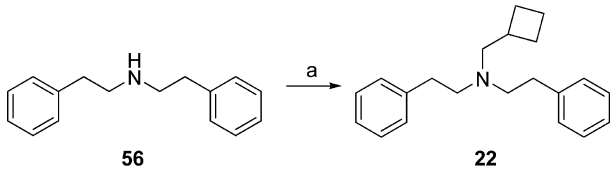
Scheme 4. Synthesis of Compounds 46−54 and 18−21[a]



[a]Reagents and conditions: (a) EDC and HOAt in CH_2Cl_2, N_2, rt; (b) BH_3·THF 1 M in THF, N_2, reflux; (c) respective alkyl bromide, $NaHCO_3$, CH_3CN, N_2, reflux; (d) BBr_3 1 M CH_2Cl_2 solution in CH_2Cl_2, −15 °C.

Diphenethylamine **22** was prepared from *N*,*N*-bis(2-phenylethyl)amine (**56**) by alkylation with cyclobutylmethyl bromide in CH_3CN in the presence of $NaHCO_3$ (Scheme 5).

Scheme 5. Synthesis of Compound 22[a]



[a]Reagents and conditions: (a) cyclobutylmethyl bromide, $NaHCO_3$, CH_3CN, reflux.

Pharmacology. Binding affinities at the KOP, μ-opioid (MOP), and δ-opioid (DOP) receptors of the new diphenethylamines **3−22** (Figure 1) were first determined in in vitro competition binding assays using membranes from Chinese hamster ovary (CHO) cells stably expressing one of the recombinant human opioid receptors (CHO-hKOP, CHO-hMOP, and CHO-hDOP cells) (Table 1), according to the described procedures.[9] The in vitro opioid binding profiles of **3−22** were compared with those of previously described analogues **1** and **2**.[9a]

To begin our expanded SAR, we first introduced diverse bulkier N-substituents. Within the new 3-monohydroxy series (**3−7**), introduction of *N*-CPeM (**3**) and *N*-CHM (**4**) substituents led to the largest increase in the KOP receptor affinity and selectivity, noticed as being considerably enhanced when compared to the lead compounds **1** and **2** (Table 1). The *N*-benzyl substituted **5** showed a KOP affinity similar to **1**, paralleled by a very high selectivity for KOP vs MOP and DOP receptors, whereas binding of *N*-cyclobutyl (**6**) and *N*-isoamyl (**7**) substituted diphenethylamines was in the range of the earlier reported *N*-CPM analogue **2**.[9a] Thus, it is evident that *N*-CPeM and *N*-CHM substitutions are highly favorable in terms of interaction with the KOP receptor.

Within the 3,3′-dihydroxy series (**8−13**), a *N*-CBM (**8**), *N*-CHM (**11**), and *N*-CPeM (**12**) substitution afforded the highest KOP receptor affinity and selectivity (Table 1). We observed that introduction of an additional hydroxyl group at position 3′ into **1** and **2** was without major alterations in both affinity and selectivity for the KOP receptor of the resulting analogues **8** and **9**, respectively. In this 3,3′-dihydroxy series, the *N*-CHM substituted **11** showed about 2-fold lower KOP affinity than **4** and somewhat reduced KOP receptor selectivity. A greater decrease in KOP affinity and selectivity was noticed for **12** when compared to its *N*-CPeM derivative **3** (Table 1). The 3,3′-dihydroxy *N*-isoamyl substituted **13** displayed similar KOP binding affinity and selectivity when compared to its counterpart **7**. We also observed that an *N*-allyl substitution (**10**) was less favorable for the interaction with the KOP receptor. The consequence of shifting the 3′-hydroxyl group to position 4′ was a decrease in both binding affinity and selectivity to the KOP receptor, specifically for compounds **14−16** when compared to their 3,3′-dihydroxy analogues (Table 1). Only the *N*-CPM derivative **17** showed a KOP affinity in the range of **9** and an increase in selectivity for KOP vs DOP receptors.

Within the series of 2-fluorinated diphenethylamines with a single 3-hydroxyl group, the *N*-CBM (**18**) and *N*-CHM (**19**) analogues showed very high KOP affinities in the picomolar range and an extraordinary KOP receptor selectivity. Compound **19** was identified as the most selective ligand for KOP vs MOP and DOP receptors in this new series of differently substituted diphenethylamines (**3−22**) (Table 1). In the case of an *N*-CBM substitution, introduction of a 2-fluoro substituent (**18**) enhanced both KOP receptor affinity and selectivity remarkably, in comparison to its counterpart reference compound **1**. An increase in the KOP receptor selectivity was also found when comparing the 2-fluorinated *N*-CHM analogue **19** with compound **4**, while both showed very good KOP binding affinity. Introduction of a fluoro substituent

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Table 1. Binding Affinities and Functional Activities at the Human Opioid Receptors and Calculated Physicochemical Properties of New Diphenethylamines 3−22 and Reference Compounds 1 and 2

	receptor binding (K_i, nM)[a]					functional activity[b]		physicochemical properties[c]	
	affinity			selectivity		[^{35}S]GTPγS KOP			
compd	KOP	MOP	DOP	MOP/KOP	DOP/KOP	EC_{50} or K_e (nM)	% stim	clogP	$clogD_{7.4}$
1[d]	0.49 ± 0.20	542 ± 239	>10000	1106	>20000	3.62 ± 1.87	90.0 ± 3.7	5.04	2.04
2[d]	5.90 ± 3.00	826 ± 98	>10000	140	>1700	35.0 ± 5.3	53.4 ± 8.1	4.64	1.73
3	0.017 ± 0.002	274 ± 20	2268 ± 625	16118	133471	3.87 ± 1.44	82.8 ± 3.7	5.43	2.34
4	0.061 ± 0.027	537 ± 172	2139 ± 346	8803	35066	0.23 ± 0.08	61.9 ± 8.1	5.83	2.73
5	0.71 ± 0.25	463 ± 95	1862 ± 501	652	2623	4.65 ± 1.63	79.5 ± 9.8	5.71	3.16
6	10.3 ± 1.2	670 ± 174	3539 ± 38	65	344	46.1 ± 13.9	50.7 ± 5.0	4.72	1.96
7	2.69 ± 0.43	259 ± 86	2743 ± 677	96	1020	22.1 ± 3.1	74.7 ± 3.6	5.47	2.61
8	0.38 ± 0.09	230 ± 43	3340 ± 844	605	8789	4.44 ± 1.36	71.1 ± 5.2	4.75	1.81
9	4.62 ± 0.11	631 ± 215	2850 ± 720	137	617	20.6 ± 0.6	51.3 ± 7.6	4.35	1.51
10	19.1 ± 0.6	358 ± 28	738 ± 16	19	39	154 ± 62	37.5 ± 0.6	4.39	2.49
11	0.14 ± 0.04	167 ± 52	1432 ± 506	1193	10229	17.6 ± 7.1	91.1 ± 3.6	5.54	2.50
12	0.31 ± 0.04	584 ± 119	2775 ± 545	1884	8952	13.7 ± 3.5	80.4 ± 5.8	5.15	2.11
13	2.10 ± 0.26	211 ± 17	1469 ± 147	100	699	16.6 ± 0.7	65.6 ± 3.4	5.18	2.39
14	3.43 ± 0.75	16.1 ± 4.7	428 ± 93	5	125	22.2 ± 3.6	76.4 ± 1.1	4.75	1.77
15	1.85 ± 0.07	234 ± 112	1637 ± 557	126	885	22.2 ± 7.2	84.3 ± 2.7	5.54	2.46
16	43.5 ± 1.5	192 ± 17	1099 ± 485	4	25	248 ± 89	29.8 ± 3.4	4.39	2.44
17	3.56 ± 0.94	460 ± 127	>10000	129	>2800	24.3 ± 1.5[e]		4.35	1.46
18	0.072 ± 0.027	398 ± 57	>10000	5529	>138000	6.90 ± 3.35	66.1 ± 10.6	5.17	2.56
19	0.040 ± 0.006	851 ± 292	>10000	21275	>250000	2.77 ± 0.29	88.9 ± 4.6	5.97	3.28
20	0.12 ± 0.01	557 ± 198	>10000	4642	>83000	1.49 ± 0.04	57.5 ± 4.4	4.89	2.32
21	3.37 ± 1.54	523 ± 33	1312 ± 281	155	389	36.7 ± 14.0	69.1 ± 1.9	4.89	2.29
22	79.1 ± 12.5	1065 ± 263	2178 ± 881	13	28	359 ± 116	91.9 ± 4.7	5.32	2.26

[a]Determined in competition binding assays using CHO cell membranes stably expressing human opioid receptors (CHO-hKOP, CHO-hMOP, or CHO-hDOP cells). [b]Determined in the [^{35}S]GTPγS binding assay using CHO-hKOP cell membranes. Percentage stimulation (% stim) relative to the KOP full agonist U69,593. [c]Calculated log *P* (clogP) and calculated log *D* at pH 7.4 ($clogD_{7.4}$) using MarvinSketch 17.10 (ChemAxon). [d]Data from ref 9a. [e]Antagonist K_e value at the KOP receptor against U69,593 determined in the [^{35}S]GTPγS binding assay. Values are means ± SEM of at least three independent experiments.

in position 2 into the 3,3′-dihydroxy *N*-CBM derivative **8** resulted in about 3-fold increase in affinity to the KOP receptor as well as a considerable increase in the KOP receptor selectivity for **20**. While the 3,4′-dihydroxy *N*-CBM derivative **21** showed similar KOP affinity to **14**, the presence of a 2-fluoro substituent in **21** increased selectivity for KOP vs MOP and DOP receptors by 31- and 3-fold, respectively. On this basis, a fluorine substitution in position 2 in this class of diphenethylamines is highly advantageous regarding binding and selectivity for the KOP receptor.

The *N*-CBM compound **22**, unsubstituted at both aromatic rings, showed much lower KOP receptor binding affinity and selectivity than its 3-hydroxy substituted analogue **1** (Table 1). The current results on the new series of targeted diphenethylamines indicate that the presence of a 3-hydroxyl group gives rise to favorable interaction with the KOP receptor in vitro.

Functional opioid activity of diphenethylamines **3**−**22** at the human KOP receptor was next evaluated where ligand-induced stimulation of guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding to membranes from CHO cells expressing the human KOP receptor was measured (Table 1), as described earlier.[9] Efficacies are presented as percentage stimulation (% stim) relative to the prototypical KOP full agonist *N*-methyl-2-phenyl-*N*-[(5*R*,7*S*,8*S*)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide (U69,593).[7d,12] The in vitro functional activity profiles of **3**−**22** were compared with data obtained for previously reported analogues **1** and **2**.[9a] On the basis of the functional activities, several derivatives (**3**, **5**, **11**, **12**, **15**, **19**, and **22**) showed high efficacy at the KOP receptor acting as full agonists (≥80% of the response to U69,593), with the most potent agonists being **3**, **5**, and **19** (Table 1). The majority of the new diphenethylamines (**2**, **4**, **6**−**10**, **13**, **14**, **16**, **18**, **20**, and **21**) were partial agonists at the KOP receptor with different levels of potencies (range 0.23−248 nM) and efficacies (range 29.8−74.7% of U69,593). Derivative **17** was found to display KOP antagonism in vitro. The *N*-CPeM (**3**), *N*-CHM (**4** and **19**), and the *N*-CBM (**18**) substituted derivatives, established in competition binding studies to have the highest KOP affinity and to be the most selective KOP ligands, were also highly potent in inducing G protein activation. Compounds **3** and **19** largely maintained the high KOP agonist potency and efficacy of **1**. Analogue **4** showed a 16-fold increased agonist potency than **1**, while **18** exhibited similar potency, with both ligands displaying KOP partial agonism (efficacies of 61.9% and 71.4% of U69,593, respectively).

Within the series of *N*-CBM substituted diphenethylamines, the new derivatives (**8**, **14**, **18**, **20**, and **21**) showed distinct functional activity properties to the lead compound **1**, described earlier as a potent full KOP agonist,[9a] with all derivatives except **22** acting as KOP partial agonists. Compound **20** showed the highest agonist potency, while potencies of **8** and **18** were in the range of **1**. The *N*-CBM substituted **22** had high efficacy but also substantially reduced potency at the KOP receptor compared to **1**, correlating with its low binding affinity to the KOP receptor (Table 1). The same observation on the low

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agonist potency was made for the *N*-allyl substituted **10** and **16** due to their decreased KOP affinity. Furthermore, **10** and **16** also showed the lowest KOP receptor efficacies (37.5% and 28.5% of U69,593, respectively) (Table 1). Comparison of the 3,3′-dihydroxy *N*-CBM substituted diphenethylamine **9** with the earlier reported KOP partial agonist **2**[9a] revealed equivalent potency and efficacy at the KOP receptor. Notable was the observation on the alteration of the functional activity of **2** from a potent KOP partial agonist to an antagonist **17** (K_e = 24.3 nM) upon introduction of an additional hydroxyl group in position 4′.

Lipophilicity is an important property for blood−brain barrier (BBB) penetration of bioactive compounds. The calculated log *P* (clogP) of compounds **1**−**22** are ranging between 4.35 and 5.97, while the calculated log $D_{7.4}$ (clog$D_{7.4}$) are ranging between 1.46 and 3.28 (Table 1), being similar to that of the brain penetrant KOP ligand U50,488 (clogP = 3.91 and clog$D_{7.4}$ = 1.66). These values are indicative for good capability to enter the CNS of the investigated KOP ligands from the class of diphenethylamines.

We have earlier reported on the antinociceptive activity of the *N*-CBM substituted diphenethylamine **1** after systemic sc administration in the mouse acetic acid-induced writhing assay[9a] with a potency equivalent to that of U50,488. It was also established to be KOP receptor-mediated based on the antagonism by *nor*-binaltorphimine (nor-BNI) of the antiwrithing response of **1**.[9a] Recently, we showed that the full KOP agonist **1** produces antinociception in a mouse model of acute thermal nociception, the 55 °C warm-water tail-withdrawal test, after central icv administration, with **1** displaying a 2-fold greater potency than U50,488.[9d] The *N*-CPM substituted analogue **2** also exhibited antinociceptive effects in the tail-withdrawal assay after icv administration, with **2** having a similar potency to U50,488.[9d] Using genetic approaches, the in vivo KOP receptor selectivity of the antinociceptive activity was demonstrated, as the effect of both **1** and **2** given icv, was absent in KOP receptor-knockout mice but still detected in the MOP receptor-knockout mice.[9d] In the current study, first investigations on the antinociceptive efficacy of the KOP partial agonist **2** in the writhing assay after sc administration are presented. Dose-dependent inhibition of the writhing response in mice was produced by **2** (Figure 2) with an antinociceptive ED_{50} value of 3.23 mg/kg, which was less than 2-fold lower when compared to **1** (Table 2). The antinociceptive effect of **2** was blocked by nor-BNI (Figure 2), indicating a KOP receptor-mediated mechanism and thus corroborating our recent observations in the tail-withdrawal assay after icv administration of **2** to KOP receptor-knockout mice.[9d]

On the basis of their in vitro profiles (Table 1), compounds **3**−**5**, **7**−**9**, **11**−**15**, and **18**−**21** were selected for in vivo studies of antinociceptive activity after sc administration in the writhing test. Dose-dependent inhibition of writhing was produced by all investigated compounds (Figure 3 and Supporting Information, Figure S1) with antinociceptive potencies (ED_{50} and 95% C.I.) listed in Table 2. The *N*-CPeM derivative **3** was the most active in inducing an antinociceptive response showing an increased potency by 4- and 7-fold than the lead compounds **1** and **2**, respectively, whereas compared to the reference U50,488, **3** was about 3-fold more potent. Its *N*-CHM analogue **4** was also highly effective as an antinociceptive agent, however, being about 2-fold less potent, possibly due to its decreased agonist KOP receptor efficacy (Table 2). The antiwrithing response of **3** and **4** was antagonized by the KOP antagonist nor-BNI

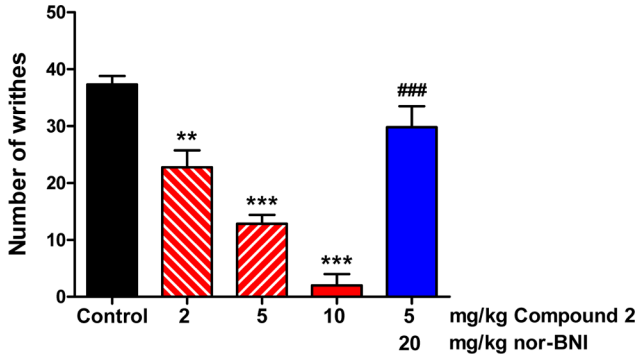

Figure 2. Dose-dependent and antagonism by nor-BNI of the antinociceptive effect of compound **2** after sc administration in the acetic acid-induced writhing test in CD1 mice. Groups of mice received sc control (saline) or different doses of **2**, and the number of writhes were counted at 30 min after drug administration for a period of 10 min. Nor-BNI was administered sc 24 h before **2**. Data are shown as the mean ± SEM (n = 5−6 mice per group). $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group; $^{\#\#\#}P < 0.001$ vs compound **2** (5 mg/kg)-treated group; one-way ANOVA followed by Tukey's post hoc test.

Table 2. Antinociceptive Potencies of New Diphenethylamines 3−22 and Reference Compounds 1, 2, and U50,488 in the Acetic Acid-Induced Writhing Assay in Mice after sc Administration

compd	ED_{50} (mg/kg, sc) (95% CI)[a]
U50,488[b]	1.54 (0.74−3.20)
1[b]	1.91 (1.02−3.58)
2	3.23 (1.43−7.29)
3	0.49 (0.092−2.64)
4	1.01 (0.30−3.45)
5	1.21 (0.45−3.27)
7	2.78 (1.08−7.15)
8	1.71 (0.67−4.34)
9	4.73 (1.33−16.8)
11	0.95 (0.38−2.35)
12	1.19 (0.44−3.26)
13	2.63 (0.75−9.18)
14	1.73 (0.83−3.62)
15	1.90 (0.52−6.84)
18	2.64 (0.77−9.04)
19	1.33 (0.48−3.65)
20	2.25 (0.58−8.70)
21	2.14 (0.63−7.28)

[a]Groups of CD1 mice were administered sc test compounds or saline (control), and evaluated in the acetic acid-induced writhing assay. Each drug was tested in at least three doses (n = 5−6 mice per dose). Inhibition of the writhing response was assessed at 30 min after drug administration, and antinociceptive ED_{50} values and 95% confidence intervals (CI in parentheses) were calculated from dose−response curves. [b]Data from ref 9a.

(Figure 4). A reduction in the in vivo antinociceptive potency by about 2-fold was showed by the 3,3′-dihydroxy *N*-CPeM derivative **12** compared to **3**, an observation that is in line with a decrease by about 3-fold in in vitro agonist potency of **12** (Table 1). The full KOP agonists **11** and **19**, with an *N*-CHM substituent, were equipotent to **4** in the writhing asay in mice after sc administration. The 2-fluorinated *N*-CBM substituted **18** was slightly less active than its analogue **1**, likely as a result of its diminished in vitro agonist potency and efficacy (Table

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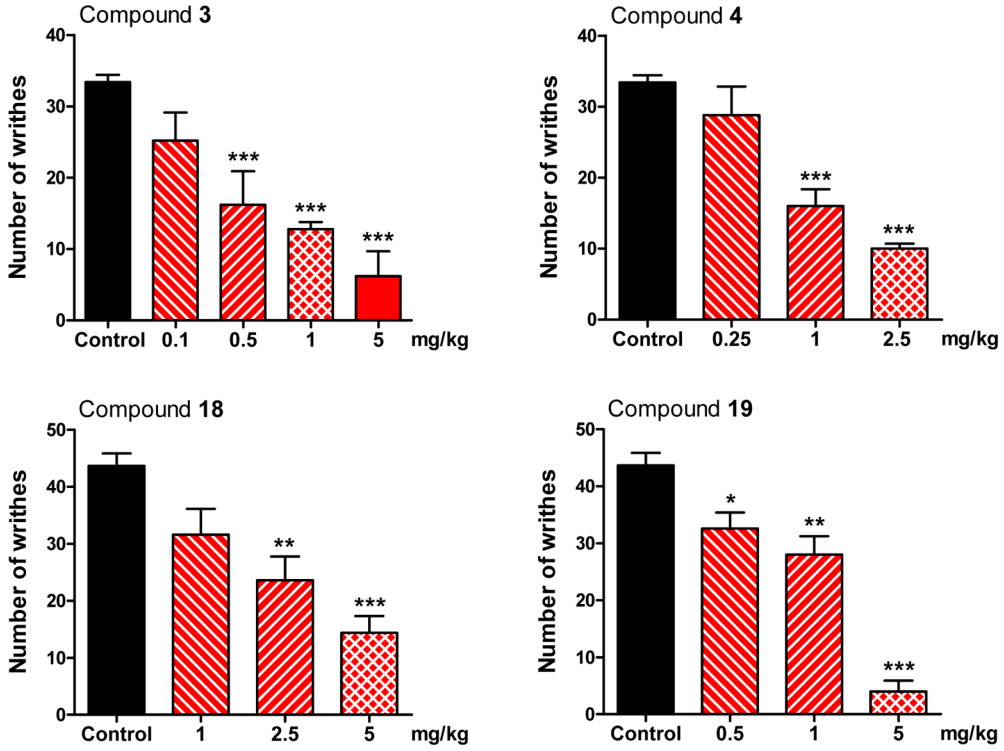

Figure 3. Dose-depedent antinociceptive effects of compounds **3**, **4**, **18**, and **19** after sc administration in the acetic acid-induced writhing test in mice. Groups of mice received sc saline (control) or different doses of test compounds, and the number of writhes were counted at 30 min after drug administration for a period of 10 min. Data are shown as the mean ± SEM (n = 5−6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001 vs control group; one-way ANOVA followed by Tukey's post hoc test.

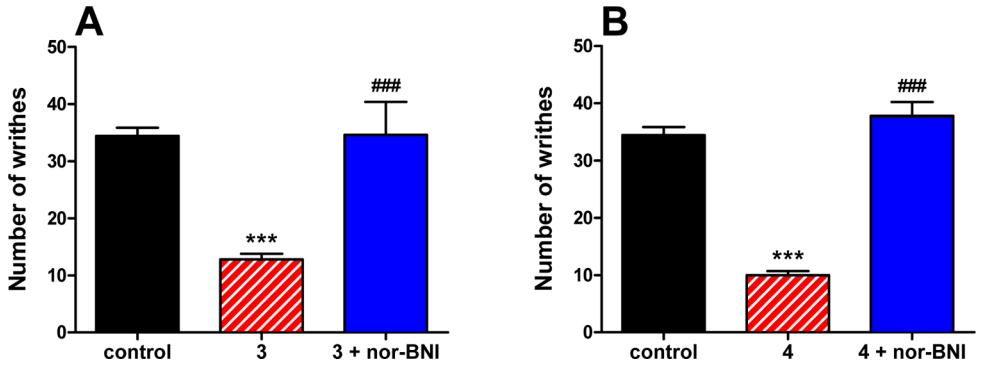

Figure 4. Antagonism by nor-BNI of the antinociceptive effect of (A) **3** and (B) **4** after sc administration in the acetic acid-induced writhing test in CD1 mice. Groups of mice received sc control (saline), **3** (1 mg/kg), or **4** (2.5 mg/kg), and the number of writhes were counted at 30 min after drug administration for a period of 10 min. Nor-BNI (20 mg/kg) was administered 24 h before **3** or **4**. Data are shown as the mean ± SEM (n = 5−6 mice per group). ***P < 0.001 vs control group; $^{\#\#\#}P$ < 0.001 vs agonist-treated group; one-way ANOVA followed by Tukey's post hoc test.

1). Moreover, it can be assumed that BBB penetration may be slightly restricted due to the fluoro substituent in the proximity to the 3-hydroxyl group, which results in a lower calculated pK_a value of the 3-OH group of **18** in comparison to the calculated pK_a value of the 3-OH group of **1** (cpK_a = 8.35 for **18**, and 9.77 for **1**; MarvinSketch 17.10, ChemAxon). We found that the other 2-fluorinated analogues **20** and **21** produced an equivalent antinociception to the *N*-CBM derivative **1**. The lowest antinociceptive potency in the series was shown by the *N*-CPM substituted **9**, somewhat less than its analogue **2** (Table 2).

We investigated the 3,4′-dihydroxy *N*-CPM derivative **17**, found to be a KOP receptor antagonist in vitro, for in vivo antagonism of U50,488-induced antinociception in the writhing test. Pretreatment of mice with **17** (10 mg/kg, sc) produced a complete reversal of U50,488-induced analgesia (Figure 5). Moreover, in vivo evaluation of **17** confirmed the lack of agonist activity, as it did not affect pain behavior in mice, with no alterations in chemical sensitivity of animals receiving sc **17**, when compared to control mice.

Activation of the KOP receptor is well-recognized to induce sedative effects that can be readily observed in animals by a marked decrease in the locomotor activity.[13] In this study, to further address the behavioral consequences of the KOP agonist profile exhibited by the lead compounds (**1** and **2**) and selected analogues (**3**, **4**, **18**, and **19**) after sc administration, the effect on motor coordination was assessed in mice using the rotarod test, according to the previously described protocols.[14]

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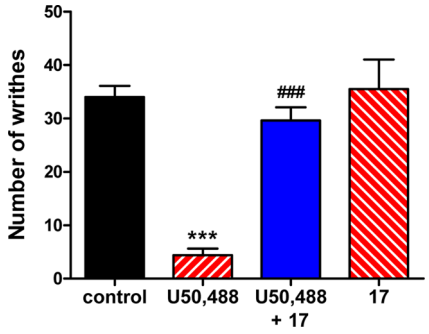

Figure 5. Antagonism by compound **17** of the antinociceptive effect of U50,488 after sc administration in the acetic acid-induced writhing test in CD1 mice. Groups of mice received sc control (saline), U50,488 (2 mg/kg), or **17** (10 mg/kg), and the number of writhes were counted at 30 min after drug administration for a period of 10 min. Compound **17** was administered 15 min before U50,488. Data are shown as the mean ± SEM (n = 5–6 mice per group). ***P < 0.001 vs control group; ###P < 0.001 vs U50,488-treated group; one-way ANOVA followed by Tukey's post hoc test.

The potential to depress motor activity was compared to that of U50,488. Dose-dependent effects on motor performance produced by compounds **1**, **2**, and U50,488 in the mouse rotarod test are shown in Figure 6. Mice were administered the test compounds, corresponding to 3- and 5-fold the antinociceptive ED_{50} dose (Table 2). U50,488 caused a significant deficit in rotarod performance via the KOP receptor activation (Figure 6C), whereas **1** and **2** did not affect the evoked locomotor activity of mice (Figure 6A,B). This finding is in line with the recent observation that **1** and **2** elicit marked antinociception with no sedation/motor incoordination after icv administration in mice.[9d] We also established that sc administration of the new diphenethylamines (**3**, **4**, **18**, and **19**) produced no changes in the motor behavior of mice, in which no significant alterations in rotarod latencies were observed at tested doses equivalent to 5-fold the antinociceptive ED_{50} dose (Figure 7).

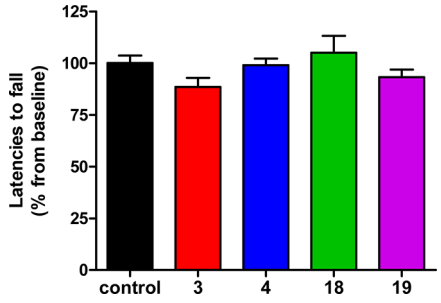

Figure 7. Evoked locomotor activity of new diphenethylamines **3**, **4**, **18**, and **19** after sc administration in the mouse rotarod assay. Mice were tested 30 min after sc administration of control (saline), **3** (2.5 mg/kg), **4** (5 mg/kg), **18** (15 mg/kg), or **19** (7.5 mg/kg). Data depicts latencies to fall from the rotarod as the mean percent changes from baseline performance ± SEM (n = 5–7 mice per group); one-way ANOVA followed by Tukey's post hoc test.

CONCLUSIONS

In the present study, we expanded the SARs on our original series of diphenethylamines by presenting targeted modifications in the lead compounds **1** and **2**. We have described the design, synthesis, and pharmacological evaluation of a library of new diphenethylamine derivatives (**3**–**22**) with substantially improved activities at the KOP receptor over the previously described analogues **1** and **2**. The KOP receptor affinity,

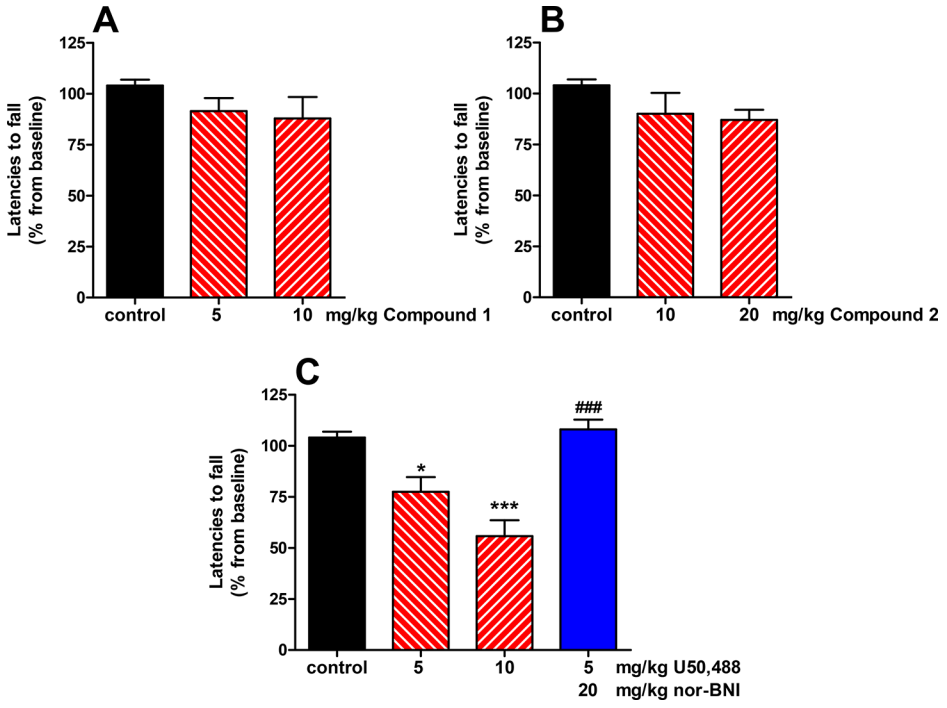

Figure 6. Evoked locomotor activity of (A) **1**, (B) **2**, and (C) U50,488 after sc administration in the mouse rotarod assay. Mice were tested 30 min after sc administration of control (saline) or test compound. Data depicts latencies to fall from the rotarod as the mean percent changes from baseline performance ± SEM (n = 5–7 mice per group). *P < 0.05, ***P < 0.001 vs control (saline) group; ###P < 0.001 vs U50,488 (5 mg/kg)-treated group; one-way ANOVA followed by Tukey's post hoc test.

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selectivity, and agonist activity were modulated by introducing bulkier N-substituents, a 2-fluoro substitution, and additional hydroxyl groups at positions 3′ and 4′. Notably, four ligands, the *N*-CPeM and *N*-CHM substituted **3** and **4**, respectively, and the 2-fluoro substituted **18** and **19** had the highest affinities and excellent selectivity for the KOP receptor in the series, paralleled by high potency acting as full or partial agonists in vitro. The 3,4′-dihydroxy *N*-CBM derivative **17** was a high affinity and selective KOP ligand with in vitro and in vivo antagonism. The new compounds with KOP full agonism or partial agonism displayed in vivo efficacy as antinociceptives with a KOP receptor-mediated mechanism of action and an increased potencies than U50,488, **1** and **2** in the writhing assay after sc administration in mice. Behavioral studies established the lack of sedation/motor impairment by investigated compounds **3**, **4**, **18**, and **19**. The emerged SAR findings highlight the value of the diphenethylamine scaffold for the discovery of new small molecules acting on the KOP receptor. Overall, a number of the currently reported compounds are suitable candidates that merit further investigation as prospective therapeutics for the treatment of pain as well as other human disorders (i.e., mood disorders, drug addiction, epilepsy, pruritus), where the KOP receptor/dynorphin system plays a role in their etiology. Selective KOP partial agonists are of particular interest with potential pharmacotherapeutic effects in mood, anxiety, or addictive states.

EXPERIMENTAL SECTION

Chemistry: General Methods. All chemicals used were of reagent grade and obtained from standard commercial sources. Melting points were determined on a Kofler melting point microscope and are uncorrected. ^{1}H NMR (200 MHz) were recorded on a Varian Gemini 200 spectrometer using tetramethylsilane (TMS) as internal standard for $CDCl_3$. IR spectra were taken on a Bruker Alpha FT-IR spectrometer (for detection, an ATR sensor was used). Mass spectra were recorded on a Varian MAT 44 S apparatus. Elemental analyses were performed at the Microanalytic Laboratory of the University of Vienna, Austria. For column chromatography (MPLC), silica gel 60 (0.040−0.063 mm, Fluka, Switzerland) was used. Compounds **3**−**21** were used as hydrochloride salts and compound **22** as a base for testing. The elemental analysis values were found to be within ±0.4% of the calculated values, indicating a purity of the tested compounds of >95%.

Procedure for the Synthesis of 3−7. A mixture of 3-[2-(phenylethylamino)ethyl]phenol (**23**)[9a] (300 mg, 1.24 mmol), the respective alkyl or allyl bromide or cyclobutyl tosylate (1.71 mmol), and $NaHCO_3$ (229 mg, 2.73 mmol) was refluxed for 48 h with a catalytic amount of KI in CH_3CN (8 mL). The mixture was cooled and filtered, the filtrate was evaporated to dryness, and the crude product was purified by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 98:1:1) to give the desired products (compounds **3**−**9** as oils). This alkylation step was performed according to the earlier described procedure.[9a,b] The resulting oils were converted into the hydrochloride salts by using the following procedure: A part of the obtained oil was dissolved in Et_2O and treated with HCl/Et_2O. The precipitate was isolated and recrystallized from acetone/Et_2O to afford the respective hydrochloride salt.

*3-[2-[(Cyclopentylmethyl)(phenethyl)amino]ethyl]phenol Hydrochloride (**3·HCl**).* Yield 40% (transparent oil of **3**). ^{1}H NMR ($CDCl_3$): δ 7.24−7.02 (m, 6 arom H), 6.67−6.54 (m, 3 arom H), 2.67−2.63 (m, 8 H, 4 CH_2), 2.25 (d, J = 7 Hz, CH_2-cyclopentyl),1.69−0.73 (m, 9 H, cyclopentyl). A part of the obtained oil of **3** was converted into **3·HCl** as a white solid; mp 154−156 °C. IR (ATR) 3080 cm^{-1} (OH). MS (ESI) m/z 323.31 [M + 1]$^+$. Anal. ($C_{22}H_{29}NO$·HCl) C, H, N.

*3-[2-[(Cyclohexylmethyl)(phenethyl)amino]ethyl]phenol Hydrochloride (**4·HCl**).* Yield 17% (yellow oil of **4**). ^{1}H NMR ($CDCl_3$): δ 7.33−7.09 (m, 6 arom H), 6.74−6.62 (m, 3 arom H), 2.81−2.63 (m, 8 H, 4 CH_2), 2.34 (d, J = 7 Hz, CH_2-cyclohexyl), 1.77−0.81 (m, 11 H, cyclohexyl). A part of the obtained oil of **4** was converted into **4·HCl** as slightly beige solid; mp 150−151 °C. IR (ATR) 3062 cm^{-1} (OH). MS (ESI) m/z 284.14 [M + 1]$^+$. Anal. ($C_{19}H_{26}NO$·HCl·0.3CH_2Cl_2) C, H, N.

*3-[2-[(Benzyl)(phenethyl)amino]ethyl]phenol Hydrochloride (**5·HCl**).* Yield 22% (yellow oil of **5**). ^{1}H NMR ($CDCl_3$): δ 7.32−7.07 (m, 11 arom H), 6.70−6.51 (m, 3 arom H), 3.72 (s, 2 H, CH_2-benzyl), 2.79−2.74 (m, 8 H, 4 CH_2). A part of the obtained oil of **5** was converted into **5·HCl** as a slightly beige solid; mp 177−178 °C. IR (ATR) 3072 cm^{-1} (OH). MS (ESI) m/z 332.26 [M + 1]$^+$. Anal. ($C_{23}H_{26}NO$·HCl) C, H, N.

*3-[2-[(Cyclobutyl)(phenethyl)amino]ethyl]phenol Hydrochloride (**6·HCl**).* Yield 32% (yellow oil of **6**). ^{1}H NMR ($CDCl_3$): δ 7.21−7.07 (m, 6 arom H), 6.65−6.60 (m, 3 arom H), 2.90−2.71 (m, 8 H, 4 CH_2), 2.51 (d, J = 6.6 Hz, CH-cyclobutyl), 0.88−0.78 (m, 2 H, cyclobutyl), 0.49−0.43 (m, 2 H, cyclobutyl), 0.12−0.07 (m, 2 H, cyclobutyl). A part of the obtained oil of **6** was converted into **6·HCl** (slightly brownish solid); mp 100−101 °C. IR (ATR) 3161 cm^{-1} (OH). MS (ESI) m/z 296.16 [M + 1]$^+$. Anal. ($C_{20}H_{26}NO$·HCl·0.1CH_2Cl_2·0.3Et_2O) C, H, N.

*3-[2-[(Isoamyl)(phenethyl)amino]ethyl]phenol Hydrochloride (**7·HCl**).* Yield 36% (yellow oil of **7**). ^{1}H NMR ($CDCl_3$): δ 7.28−7.11 (m, 6 arom H), 6.73−6.68 (m, 3 arom H), 2.87−2.72 (m, 8 H, 4 CH_2), 2.69−2.64 (m, 2 H, CH_2-isoamyl), 1.51−1.41 (m, 3 H, CH + CH_2-isoamyl), 0.90 (d, J = 6.6 Hz, 6 H, ($(CH_3)_2$-isoamyl). A part of the obtained oil of **7** was converted into **7·HCl** as a slightly yellow solid; mp 149−150 °C. IR (ATR) 3095 cm^{-1} (OH). MS (ESI) m/z 312.26 [M + 1]$^+$. Anal. ($C_{21}H_{30}NO$·HCl·0.6Et_2O) C, H, N.

Procedure for the Sodium Ethanethiolate Ether Cleavage of 25−27 to Afford 8−10. A mixture of **25**, **26**, or **27** (0.7 mmol) and sodium ethanethiolate (942 mg, 11.2 mmol) in anhydrous DMF (4 mL) was stirred under N_2 at 130 °C for 20 h. After cooling, the mixture was poured on 80 mL of saturated NH_4Cl solution. The resulting mixture was acidified with 2 N HCl to a pH of 2−3 and then alkalinized with diluted NH_4OH solution to a pH of ca. 10 and extracted with CH_2Cl_2 (3 × 15 mL). The organic phase was washed with H_2O (5 × 15 mL), brine (15 mL), dried over Na_2SO_4, and evaporated. The resulting oils were purified by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 97:2:1) to give the desired products as yellow oils.

*3,3′-[2,2′-(Cyclobutylmethylazanediyl)bis(ethane-2,1-diyl)]diphenol Hydrochloride (**8·HCl**).* Yield 44% (oil of **8**). ^{1}H NMR ($CDCl_3$): δ 7.14 (t, J = 8 Hz, 2 arom H), 6.71−6.64 (m, 6 arom H), 2.74−2.64 (m, 10 H, 5 CH_2), 2.06−1.69 (m, 7 H, cyclobutyl). A part of the obtained oil of **8** was converted into **8·HCl** (beige solid) in the above-described way; mp 100−101 °C. IR (ATR) 3202 cm^{-1} (OH). MS (ESI) m/z 326.27 [M + 1]$^+$. Anal. ($C_{21}H_{27}NO_2$·HCl·0.6Et_2O) C, H, N.

*3,3′-[2,2′-(Cyclopropylmethylazanediyl)bis(ethane-2,1-diyl)]diphenol Hydrochloride (**9·HCl**).* Yield 44% (oil of **9**). ^{1}H NMR ($CDCl_3$): δ 7.12 (t, J = 8 Hz, 2 arom H), 6.70−6.66 (m, 6 arom H), 2.96−2.89 (m, 4 H, 2 CH_2), 2.80−2.71 (m, 4 H, 2 CH_2), 2.56 (d, J = 6.6 Hz, CH_2-cycloprop), 0.92−0.90 (m, 1 H, cycloprop), 0.57−0.48 (m, 2 H, cycloprop), 0.19−0.12 (m, 2 H, cycloprop). A part of the obtained oil of **9** was converted into **9·HCl** (beige solid) in the above-described way; mp 105−107 °C. IR (ATR) 3190 cm^{-1} (OH). MS (ESI) m/z 312.23 [M + 1]$^+$. Anal. ($C_{20}H_{25}NO_2$·HCl·0.1CH_2Cl_2) C, H, N.

*3,3′-[2,2′-(Allylazanediyl)bis(ethane-2,1-diyl)]diphenol Hydrochloride (**10·HCl**).* Yield 38% (oil of **10**), ^{1}H NMR ($CDCl_3$): δ 7.12 (t, J = 8 Hz, 2 arom H), 6.69−6.63 (m, 6 arom H), 6.01−5.81 (m, 1 H olef), 5.27−5.16 (m, 2 H, olef), 3.28 (d, J = 6.6 Hz, CH_2-olef), 2.78−2.76 (m, 8 H, 4 CH_2). A part of the obtained oil of **10** was converted into **10·HCl** (beige solid) in the above-described way; mp 104−106 °C. IR (ATR) 3197 cm^{-1} (OH). MS (ESI) m/z 298.17 [M + 1]$^+$. Anal. ($C_{19}H_{23}NO_2$·HCl·0.4Et_2O) C, H, N.

Procedure for the Sodium Ethanethiolate Ether Cleavage of 35, 37−40 to Afford 11, 13−16. A mixture of **35**, **37**, **38**, **39**, or **40** (1.03 mmol) and sodium ethanethiolate (550 mg, 6.53 mmol) in anhydrous DMF (5 mL) was stirred under N_2 at 130 °C (bath

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temperature) for 20 h. After cooling, the mixture was poured on 50 mL of a saturated NH_4Cl solution. The resulting mixture was acidified with 2 N HCl to a pH of 2−3 and then alkalinized with diluted NH_4OH solution to a pH of ca. 10 and extracted with CH_2Cl_2 (3 × 15 mL). The organic phase was washed with H_2O (5 × 15 mL) and brine (15 mL), dried over Na_2SO_4, and evaporated. The resulting oils were purified by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 97:2:1) to give the desired compounds as transparent oils.

*3,3′-[2,2′-(Cyclohexylmethylazanediyl)bis(ethane-2,1-diyl)]diphenol Hydrochloride (**11·HCl**).* Yield 20% (oil of **11**). ^1H NMR ($CDCl_3$): δ 7.03−6.96 (m, 2 arom H), 6.60−6.48 (m, 6 arom H), 2.54 (br s, 8 H, 4 CH_2), 2.16 (d, J = 6.6 Hz, CH_2-cyclohexyl), 1.59−0.65 (m, 11 H, cyclohexyl). A part of the obtained oil of **11** was converted into **11·HCl** (white solid) as described above; mp 157−158 °C. IR (ATR) 2921 cm^{-1} (OH). MS (ESI) m/z 354.4 $[M + 1]^+$. Anal. ($C_{23}H_{31}NO_2$·HCl) C, H, N.

*3,3′-[2,2′-(Isoamylazanediyl)bis(ethane-2,1-diyl)]diphenol Hydrochloride (**13·HCl**).* Yield 25% (oil of **13**). ^1H NMR ($CDCl_3$): δ 7.15−7.07 (m, 2 arom H), 6.69−6.63 (m, 6 arom H), 2.76−2.60 (m, 10 H, 5 CH_2), 1.44−1.24 (m, 3 H, CH + CH_2-isoamyl), 0.86 (d, J = 6.2 Hz, $(CH_3)_2$-isoamyl). A part of the obtained oil of **13** was converted into **13·HCl** (yellowish solid) as described above; mp 71−72 °C. IR (ATR) 3191 cm^{-1} (OH). MS (ESI) m/z 328.4 $[M + 1]^+$. Anal. ($C_{22}H_{29}NO_2$·HCl·0.3CH_2Cl_2) C, H, N.

*3-[2-[(Cyclobutylmethyl)(4-hydroxyphenethyl)amino]ethyl]phenol Hydrochloride (**14·HCl**).* Yield 36% (oil of **14**). ^1H NMR ($CDCl_3$): δ 7.15 (t, J = 7.8 Hz, 2 arom H), 7.06−7.03 (m, 2 arom H), 6.78−6.62 (m, 4 arom H), 2.71−2.62 (m, 10 H, 5 CH_2), 2.09−1.27 (m, 7 H, cyclobutyl). A part of the obtained oil of **14** was converted into **14·HCl** (yellowish solid) as described above; mp 102−103 °C. IR (ATR) 3332 cm^{-1} (OH). MS (ESI) m/z 326.4 $[M + 1]^+$. Anal. ($C_{21}H_{27}NO_2$·HCl·0.2CH_2Cl_2) C, H, N.

*3-[2-[(Cyclohexylmethyl)(4-hydroxyphenethyl)amino]ethyl]phenol Hydrochloride (**15·HCl**).* Yield 31% (oil of **15**). ^1H NMR ($CDCl_3$): δ 7.13 (t, J = 7.6 Hz, 2 arom H), 7.04 (d, J = 8.4 Hz, 2 arom H), 6.77−6.58 (m, 4 arom H), 2.66 (br s, 8 H, 4 CH_2), 2.29 (d, J = 6.8 Hz, CH_2-cyclohexyl), 1.75−0.79 (m, 11 H, cyclohexyl). A part of the obtained oil of **15** was converted into **15·HCl** (white solid) as described above; mp 155−157 °C. IR (ATR) 2924 cm^{-1} (OH). MS (ESI) m/z 354.4 $[M + 1]^+$. Anal. ($C_{23}H_{31}NO_2$·HCl) C, H, N.

*3-[2-[(Allyl)(2-hydroxyphenethyl)amino]ethyl]phenol Hydrochloride (**16·HCl**).* Yield 23% (oil of **16**). ^1H NMR ($CDCl_3$): δ 7.14 (t, J = 7.6 Hz, 2 arom H), 7.03 (d, J = 8 Hz 2 arom H), 6.77−6.61 (m, 4 arom H), 5.97−5.83 (m, 1 H, olef), 5.30−5.14 (m, 2 H, olef), 3.25 (d, J = 6.2 Hz, CH_2-olef), 2.73 (br s, 8 H, 4 CH_2). A part of the obtained oil of **16** was converted into **16·HCl** (white solid) as described above; mp 103−105 °C. IR (ATR) 3195 cm^{-1} (OH). MS (ESI) m/z 298.2 $[M + 1]^+$. Anal. ($C_{19}H_{23}NO_2$·HCl) C, H, N.

Procedure for the BBr_3 Ether Cleavage of 36 and 41 to Afford 12 and 17. To a solution of **36** or **41** (60 mg, 0.17 mmol) in CH_2Cl_2 (4 mL) under N_2 atmosphere was added 1 M BBr_3 solution in CH_2Cl_2 (1.02 mL, 1.02 mmol) at −15 °C. After 30 min, ice (15 g) and concentrated NH_4OH were added (pH ca. 10) and the mixture was stirred at 0 °C for further 30 min. The organic phase was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried over Na_2SO_4 and evaporated. The resulting yellow oil was purified by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 97:2:1) to give the desired product as transparent oils.

*3,3′-[2,2′-(Cyclopentylmethylazanediyl)bis(ethane-2,1-diyl)]diphenol Hydrochloride (**12·HCl**).* Yield 69% (oil of **12**). ^1H NMR ($CDCl_3$): δ 7.19−7.10 (m, 3 arom H), 6.76−6.65 (m, 5 arom H), 2.75−2.71 (m, 8 H, 4 CH_2), 2.45 (d, J = 7.4 Hz, CH_2-cyclopentyl), 1.62−1.25 (m, 9 H, cyclopentyl). A part of the obtained oil of **12** was converted into **12·HCl** (beige solid) as described above; mp 112−114 °C. IR (ATR) 2952 cm^{-1} (OH). MS (ESI) m/z 339.4 $[M + 1]^+$. Anal. ($C_{21}H_{29}NO_2$·HCl) C, H, N.

*3-[2-[(Cyclopropylmethyl)(4-hydroxyphenethyl)amino]ethyl]phenol Hydrochloride (**17·HCl**).* Yield 52% (oil of **17**). ^1H NMR ($CDCl_3$): δ 7.28−7.03 (m, 4 arom H), 6.78−6.65 (m, 4 arom H), 2.85−2.74 (m, 8 H, 4 CH_2), 2.52 (d, J = 6.2 Hz, CH_2-cyclopropyl), 0.87 (br s, 2H, cyclopropyl), 0.53−0.52 (m, 2 H, cyclopropyl), 0.17−0.14 (m, 1 H, cyclopropyl). A part of the obtained oil of **17** was converted into **17·HCl** (beige solid) as described above; mp 104−105 °C. IR (ATR) 3168 cm^{-1} (OH). MS (ESI) m/z 312.4 $[M + 1]^+$. Anal. ($C_{20}H_{25}NO_2$·HCl·0.5CH_2Cl_2·0.2MeOH) C, H, N.

Procedure for the BBr_3 Ether Cleavage of 52−55 to Afford 18−21. To a solution of **52**, **53**, **54**, or **55** (0.54 mmol) in CH_2Cl_2 (12 mL) under N_2 atmosphere was added 1 M BBr_3 solution in CH_2Cl_2 (3.23 mL, 3.23 mmol) at −15 °C. After 30 min, ice (40 g) and concentrated NH_4OH were added (pH ca. 10) and the mixture was stirred at 0 °C for further 30 min. The organic phase was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and evaporated. The resulting oils were purified by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 97:2:1) to give the desired products as transparent oils.

*3-[2-[(Cyclobutylmethyl)(phenethyl)amino]ethyl]-2-fluorophenol Hydrochloride (**18·HCl**).* Yield 70% (oil of **18**). ^1H NMR ($CDCl_3$): δ 7.34−7.18 (m, 5 arom H), 6.99−6.92 (m, 2 arom H), 6.77−6.70 (m, 1 arom H), 2.98 (br s, 8 H, 4 CH_2), 2.90−2.87 (m, 2 H), 2.15−1.26 (m, 7 H, cyclobutyl). A part of the obtained oil of **18** was converted into **18·HCl** (beige solid) as described above; mp 122−124 °C. IR (ATR) 2978 cm^{-1} (OH). MS (ESI) m/z 328.3 $[M + 1]^+$. Anal. ($C_{21}H_{26}FNO$·HCl·0.1CH_2Cl_2) C, H, N.

*3-[2-[(Cyclohexylmethyl)(phenethyl)amino]ethyl]-2-fluorophenol Hydrochloride (**19·HCl**).* Yield 62% (oil of **19**). ^1H NMR ($CDCl_3$): δ 7.32−7.16 (m, 5 arom H), 6.96−6.78 (m, 2 arom H), 6.71−6.63 (m, 1 arom H), 2.74 (br s, 8 H, 4 CH_2), 2.33 (d, J = 6.6 Hz, 2 H), 1.74−0.80 (m, 11 H, cyclohexyl). A part of the obtained oil of **19** was converted into **19·HCl** (beige solid) as described above; mp 129−130 °C. IR (ATR) 2924 cm^{-1} (OH). MS (ESI) m/z 356.4 $[M + 1]^+$. Anal. ($C_{23}H_{30}FNO$·HCl·0.2CH_3OH) C, H, N.

*3-[2-[(Cyclobutylmethyl)(3-hydroxyphenethyl)amino]ethyl]-2-fluorophenol Hydrochloride (**20·HCl**).* Yield 54% (oil of **20**). ^1H NMR (DMSO-d_6): δ 7.23−7.10 (m, 1 arom H), 6.92−6.79 (m, 2 arom H), 6.71−6.59 (m, 3 arom H), 3.48 (br s, 4 H, 2 CH_2), 3.08−2.97 (m, 4 H, 2 CH_2), 2.64 (br s, 2 H, CH_2), 2.10−1.60 (m, 7 H, cyclobutyl). A part of the obtained oil of **20** was converted into **20·HCl** (beige solid) as described above; mp 130−133 °C. IR (ATR) 2932 cm^{-1} (OH). MS (ESI) m/z 344.3 $[M + 1]^+$. Anal. ($C_{21}H_{26}FNO_2$·HCl·0.3CH_2Cl_2) C, H, N.

*3-[2-[(Cyclobutylmethyl)(4-hydroxyphenethyl)amino]ethyl]-2-fluorophenol Hydrochloride (**21·HCl**).* Yield 48% (oil of **21**). ^1H NMR (DMSO): δ 7.14−7.07 (m, 2 arom H), 6.96−6.88 (m, 1 arom H), 6.80−6.71 (m, 3 arom H), 3.28−3.08 (m, 8 H, 4 CH_2), 2.89 (br s, 2 H, CH_2), 2.09−1.86 (m, 7 H, cyclobutyl). A part of the obtained oil of **21** was converted into **21·HCl** (beige solid) as described above; mp 135−136 °C. IR (ATR) 3153 cm^{-1} (OH). MS (ESI) m/z 344.3 $[M + 1]^+$. Anal. ($C_{21}H_{26}FNO_2$·HCl·0.2CH_2Cl_2·0.2CH_3OH) C, H, N.

*N-(Cyclobutylmethyl)-N-phenethyl-2-phenylethanamine (**22**).* A mixture of *N*,*N*-bis(2-phenylethyl)amine (**56**) (700 mg, 3.1 mmol), cyclobutylmethyl bromide (0.49 mL, 4.35 mmol), and $NaHCO_3$ (573 mg, 6.82 mmol) was refluxed for 48 h with a catalytic amount of KI in CH_3CN (20 mL). The mixture was cooled and filtered, the filtrate was evaporated to dryness, and the crude product was purified by column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 98:1:1) to give 300 mg (31%) of **22** as slightly orange solid; mp 96−98 °C. IR (ATR) 2933 cm^{-1} (CH). ^1H NMR ($CDCl_3$): δ 7.33−7.15 (m, 10 arom H), 2.83−2.75 (m, 8 H), 2.74−2.65 (m, 2 H, CH_2-cyclobutyl), 2.11−1.81 (m, 7 H, cyclobutyl). MS (ESI) m/z 294.21 $[M + 1]^+$. Anal. ($C_{21}H_{27}NO$·0.8CH_2Cl_2) C, H, N.

Calculation of Physicochemical Properties. Physicochemical parameters represented by pK_a, log P, and log $D_{7.4}$ (as log D at a pH of 7.4) were calculated (cpK_a, clogP, and clog$D_{7.4}$) for compounds **1**−**22** with MarvinSketch 17.10 (ChemAxon, www.chemaxon.com).

In Vitro Pharmacology: Materials. Cell culture media and supplements were obtained from Sigma-Aldrich Chemicals (St. Louis, MO), or Life Technologies (Carlsbad, CA). Radioligands [^{3}H]-U69,593, [^{3}H][D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO), [^{3}H]diprenorphine, and guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate

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([^{35}S]GTPγS) were purchased from PerkinElmer (Boston, MA). All other chemicals were of analytical grade and obtained from standard commercial sources. Test compounds **3**–**21** (as hydrochloride salts) and **22** (as base) were prepared as 1 mM stocks in 1% DMSO or 0.5% in acetic acid, respectively, and further diluted to working concentrations in the appropriate medium.

Cell Culture. CHO cells stably expressing recombinant human KOP, MOP, or DOP receptors (CHO-hKOP, CHO-hMOP, and CHO-hDOP cell lines) were kindly provided by Dr. Lawrence Toll (SRI International, Menlo Park, CA). The CHO-hKOP cell line was maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM), and geneticin (400 μg/mL). The CHO-hMOP and CHO-hDOP cell lines were maintained in DMEM/Ham's F-12 medium supplemented with FBS (10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM), and geneticin (400 μg/mL). Cell cultures were maintained at 37 °C in 5% CO_2 humidified air.

Radioligand Binding Assays for KOP, MOP, and DOP Receptors. Binding assays were conducted on human opioid receptors stably transfected into CHO cells according to the published procedures.[9a,b] Cell membranes from CHO-hKOP, CHO-hMOP, and CHO-hDOP cells were prepared as described previously and stored at −80 °C until use.[9] Protein content of cell membrane preparations was determined by the method of Bradford using bovine serum albumin as the standard.[15] Binding assays were conducted using [^{3}H]U69,593 (1 nM), [^{3}H]DAMGO (1 nM), or [^{3}H]diprenorphine (0.2 nM) for labeling KOP, MOP, or DOP receptors, respectively. Nonspecific binding was determined using 1–10 μM of the unlabeled counterpart of each radioligand. Assays were performed in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 mL. Cell membranes (15–20 μg) were incubated with the appropriate radioligand and various concentrations of test compound for 60 min at 25 °C. After incubation, reactions were terminated by rapid filtration through Whatman glass fiber filters. Filters were washed three times with 5 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Brandel M24R cell harvester (Gaithersburg, MD). Radioactivity retained on the filters was counted by liquid scintillation counting using a Beckman Coulter LS6500 (Beckman Coulter Inc., Fullerton, CA). All binding experiments were performed in duplicate and repeated at least three times. The inhibitory constant K_i values (in nM) were calculated from the competition binding curves by nonlinear regression analysis and the Cheng–Prusoff equation.[16]

[^{35}S]GTPγS Functional Assay for the KOP Receptor. The binding of [^{35}S]GTPγS to membranes from CHO cells stably expressing human KOP receptors (CHO-hKOP cells) was conducted according to the published procedures.[9a,b] CHO-hKOP cell membranes were prepared in buffer A (20 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.4). Cell membranes (8–15 μg) in buffer A were incubated with 0.05 nM [^{35}S]GTPγS, 10 μM GDP, and various concentrations of test compound in a final volume of 1 mL for 60 min at 25 °C. Nonspecific binding was determined using 10 μM GTPγS, and the basal binding was determined in the absence of test ligand. Samples are filtered over glass fiber filters and counted as described for binding assays. The increase in [^{35}S]GTPγS binding above the basal activity was used to determine potency (EC_{50}, in nM) and efficacy (as % stimulation of maximum stimulation with respect to the reference KOP full agonist, U69,593, which was set as 100%), from concentration–response curves by nonlinear regression analysis. Compounds that demonstrate no agonist activity were tested as antagonists. To determine the KOP antagonist activity, a concentration–response curve for U69,593 was obtained by assessing the [^{35}S]GTPγS binding to CHO-hKOP cell membranes in the presence or absence of test compound, as previously described.[9b] For each compound, the Schild analysis was conducted, utilizing a full agonist–dose response curve in the presence of at least two concentrations of the antagonist. The equilibrium dissociation constant (K_e) was calculated from the equation $K_e = [a]/(DR - 1)$, where "a" is the concentration of antagonist and DR the virtual shift of the agonist concentration–response curve to the right in the presence of a given concentration of antagonist. All experiments were performed in duplicate and repeated at least three times.

In Vivo Pharmacology: Animals and Drug Administration. Male CD1 mice (30–35 g, 6–8 weeks old) were obtained from the Center of Biomodels and Experimental Medicine (CBEM) (Innsbruck, Austria) or Charles River (Sulzfeld, Germany). Mice were group-housed in a temperature controlled room with a 12 h light/dark cycle and with free access to food and water. All animal studies were conducted in accordance with ethical guidelines and animal welfare standards according to Austrian regulations for animal research and were approved by the Committee of Animal Care of the Austrian Federal Ministry of Science and Research. Solutions of test compounds were prepared as 1% DMSO solutions in sterile physiological 0.9% saline and further diluted to working doses in saline solution. Test compounds or vehicle (saline) were administered by sc route in a volume of 10 μL/1 g of body weight. All doses are expressed in terms of salts. Separate groups of mice received the respective dose of compound, and individual mice were only used once for behavioral testing. Each experimental group included at least five animals.

Acetic Acid-Induced Writhing Assay. Writhing was induced in male CD1 mice by intraperitoneal (ip) injection of a 0.6% acetic acid aqueous solution as described previously.[9a] Groups of mice were administered sc different doses of test compound or saline (control), and then 5 min prior to testing (25 min after drug or saline) each animal received an ip injection of acetic acid solution. Each mouse was placed in individual transparent Plexiglas chambers, and the number of writhes was counted during a 10 min observation period. Antinociceptive activity, as percentage decrease in number of writhes compared to the control group, was calculated according to the following formula: % inhibition of writhing = $100 \times [(C - T)/C]$, where C is the mean number of writhes in control animals, and T is the number of writhes in drug-treated mice. Dose–response relationships of percentage inhibition of writhing were constructed, and the dose necessary to produce a 50% effect (ED_{50}) was calculated according to the method of Litchfield and Wilcoxon.[17] For the antagonism studies, nor-BNI (20 mg/kg) was sc administered 24 h before **2** (5 mg/kg) and **17** (10 mg/kg) was sc administered 15 min before U50,488 (2 mg/kg).

Rotarod Test. Possible motor dysfunction or sedative effects of test compounds were assessed in male CD1 mice using the rotarod test as described previously.[14] The accelerating rotarod treadmill (Acceler Rota-Rod 7650, Ugo Basile srl, Varese, Italy) for mice (diameter 3.5 cm) was used. Animals were habituated to the equipment in two training sessions (30 min apart) 1 day before testing. On the experimental day, mice were placed on the rotarod and the treadmill was accelerated from 4 to 40 rpm over a period of 5 min. The time spent on the drum was recorded for each mouse before (baseline) and at 30 min after sc administration of saline (control) or test compound. Decreased latencies to fall in the rotarod test indicate impaired motor performance. A 300 s cutoff time was used. Percentage (%) changes from the rotarod latencies obtained before (baseline, B) and after drug administration (test, T) were calculated as $100 \times (T/B)$.

Data Analysis. Experimental data were analyzed and graphically processed using the GraphPad Prism 5.0 Software (GraphPad Prism Software Inc., San Diego, CA) and are presented as means ± SEM. Data were statistically evaluated using one-way ANOVA with Tukey's multiple comparison post hoc test, with significance set at $P < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00981.

Additional chemical and biological information (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BBB, blood–brain barrier; CBM, cyclobutylmethyl; CHM, cyclohexylmethyl; CHO, Chinese hamster ovary; CPeM, cyclopentylmethyl; CPM, cyclopropylmethyl; DOP receptor, δ-opioid receptor; EDC, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide methiodide; HOAt, 1-hydroxy-7-azabenzotriazole; KOP receptor, κ-opioid receptor; MOP receptor, μ-opioid receptor; nor-BNI, nor-binaltorphimine

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