Synthesis, Biological, and Structural Explorations of New Zwitterionic Derivatives of 14-O-Methyloxymorphone, as Potent \( \mu/\delta \) Opioid Agonists and Peripherally Selective Antinociceptives

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ABSTRACT: Herein, the synthesis and pharmacological characterization of an extended library of differently substituted N-methyl-14-O-methylmorphinans with natural and unnatural amino acids and three dipeptides at position 6 that emerged as potent \( \mu/\delta \) opioid receptor (MOR/DOR) agonists with peripheral antinociceptive efficacy is reported. The current study adds significant value to our initial structure–activity relationships on a series of zwitterionic analogues of \( 1 \) (14-O-methyloxymorphone) by targeting additional amino acid residues. The new derivatives showed high binding and potent agonism at MOR and DOR in vitro. In vivo, the new 6-amino acid- and 6-dipeptide-substituted derivatives of \( 1 \) were highly effective in inducing antinociception in the writhing test in mice after subcutaneous administration, which was antagonized by naloxone methiodide demonstrating activation of peripheral opioid receptors. Such peripheral opioid analgesics may represent alternatives to presently available drugs for a safer pain therapy.

INTRODUCTION

Adequate treatment of acute and chronic severe pain remains a global medical and socioeconomic challenge at the beginning of the third millennium.¹ Current analgesics are either ineffective in a large proportion of patients or associated with significant adverse effects.¹² Effective pain relief can be achieved with opioid analgesics, but undesirable side effects following acute administration (i.e., respiratory depression, constipation, sedation, dizziness, nausea) and prolonged use (i.e., tolerance, dependence, abuse liability) limit their clinical usefulness.³–⁵ In the last years, the huge rise in prescription opioids resulted in increased opioid-related deaths, and consequently a substantial public concern.⁵,⁶

Opioid receptors have key functions in modulating pain and other behavioral responses.⁷–¹⁴ They are G protein-coupled receptors with seven transmembrane helices ⁷–⁹ and are localized in the central and peripheral nervous systems (CNS and PNS).¹⁰–¹² Activation of \( \mu \) (MOR), \( \delta \) (DOR), and \( \kappa \) (KOR) opioid receptors mediates analgesic effects of opioids.⁷,⁸ Currently, most opioids used in clinical practice are agonists at MOR causing severe CNS (i.e., respiratory depression, sedation, tolerance) and intestinal (i.e., constipation) side effects, and are mostly misused and abused.³–⁵,¹⁴ The imperative need for safer pain medications continues to drive the search for new lead molecules. Furthermore, the complexity of pain syndromes requires tailored pharmacological interventions and efficient drugs to fully control pain.¹⁵,¹⁶ Diverse strategies in designing better opioid analogues are considered, including targeting peripheral opioid receptors,¹⁷,¹⁸–²⁰ ligands acting at multiple opioid receptors,²¹ G protein-biased agonism,²²–²⁴ and abuse-deterrent formulations of existing opioids.²⁵,²⁶

Targeting peripheral opioid receptors as effective means of treating pain and avoiding the CNS-mediated side effects has been a research area of substantial and continuous attention in the past years.¹⁷,¹⁸,²² Preclinical and clinical studies have established that opioid agonists that are not able to enter the CNS produce analgesia by activating opioid receptors in the periphery in a variety of pain conditions with a more favorable side effect profile. Increasing the hydrophilicity of opioids to limit their access to the CNS and thus to minimize the incidence of undesirable CNS effects includes diverse chemical alterations.¹⁷,¹⁸,²⁷–³⁰ Earlier works to generate peripheral opioids targeted the quaternization of the nitrogen in morphine, oxymorphone, and naloxone.²⁷,³¹ Such quaternary compounds have decreased BBB permeability, while they have low binding...
affinity to opioid receptors. Quaternization also diminishes in vivo activity; therefore, alternative strategies were pursued. Polar or ionizable substituents are able to increase polarity and inhibit the cross of the blood–brain barrier (BBB). Morphinans having hydrophilic groups attached to the C6 position were prepared from β-oxymorphamine, β-naltrexamine, β-funtalrexamine, and 14-O-methylmorphine. The emerged molecules had reduced ability to enter the CNS, without substantially decreased in vitro and in vivo opioid activity. Small molecules, such as 5-pyrrolidinylquinoxalines, were also designed with limited BBB penetration and peripheral antinociceptive effects in animal models.

In the present study, we targeted additional derivatization of compound 1 through introduction of a number of amino acid residues at C6 position to the centrally acting MOR agonist 14-O-methylmorphoxymorphine (1, Figure 1). The first series of 6-amino acid-substituted derivatives (i.e., Gly, L-Ala, and L-Phe, compounds 2–5, Figure 1), as zwitterionic molecules, displayed high affinities at MOR and potent agonism, were more hydrophilic than 1, and therefore restricted BBB penetration. They were very effective as antinociceptive agents in several pain models in rodents after systemic subcutaneous (sc), intraperitoneal (ip), oral, and local (intraplantar) administration by activating peripheral opioid receptors.

In the present study, we report on the synthesis, pharmacological characterization, and peripheral antinociceptive efficacy of novel 6-amino acid- and 6-dipeptide-substituted derivatives of 1, and emerged structure–activity relationships (SAR).

Figure 1. Structures of compound 1 and previous 6-amino acid-substituted derivatives 2–5. Ph, phenyl.

Figure 2. Structures of new 6-amino acid (6–21)- and 6-dipeptide-substituted derivatives (22a/b and 23a). Ph, phenyl.

## RESULTS AND DISCUSSION

### Chemistry

Compound 1 was prepared as described earlier. Reductive amination of 1-HBr was performed using amino acid tert-butyl ester hydrochlorides or dipeptide benzyl ester hydrochlorides and NaBH₃CN in CH₃OH at 21 °C. Medium-pressure liquid chromatography (MPLC) was used to
separate the diastereoisomers providing esters 24a/24b–41a/b (Figure 3). Typically, the ratio of 6β-amin to 6α-

Figure 3. Structures of new 6-amino acid (24–39) - and 6-dipeptide-substituted esters (40a/b and 41a/b). t-Bu, tert-butyl; Ph, phenyl.

amino epimers was found to be between 4:1 to 2:1. The coupling constants (J(f6,s)) between H-C(f) and H-C(s) were used for assignment of configuration at C(6). The 6α-amino epimers have smaller J(f6,s) values (i.e., 3–4 Hz) than the 6β-
amino epimers (6.5–7.8 Hz).45,49,51,52 The results for compounds 24a/b–41a/b are in agreement with the earlier findings. The amino acid derivatives 6–21 were obtained through ester cleavage of the tert-butyl derivatives in dioxane/ HCl. Catalytic hydrogenation of the benzyl esters 40 and 41 in CH2OH using 10% Pd/C catalyst provided the dipeptides 22a/b and 23, respectively (see Supporting Information for details).

Pharmacology. In vitro binding affinity and functional activity of the new 6-amino acid (6–21) - and 6-dipeptide-substituted derivatives (22a/b and 23a) were evaluated at MOR, DOR, and KOR (Tables 1 and 2). Competition binding assays using rat brain (MOR and DOR) and guinea-pig brain (KOR) membranes (Table 1) were performed according to published procedures.44,49 Opioid-binding profiles of 6–23 were compared to those of compound 1 and earlier reported 6-
amino acid-substituted analogues 2–5.45,49 In addition, we have assessed binding at the human opioid receptors stably transfected in Chinese hamster ovary (CHO) cells (Table S1).

As shown in Table 1, all compounds, except 14b, have high binding affinity in the subnanomolar or low nanomolar range (K(i) = 0.19–4.62 nM) at MOR in rat brain membranes. Similar observations were made at the cloned human MOR expressed in CHO cells. Compared to 1, the 6α-L-Lys derivative 8a had similar MOR affinity (K(i) 0.10 nM for 1 vs 0.19 nM for 8a), whereas the next best compound was the 6α-L-Trp-substituted 10a, having a K(i) value of 0.36 nM at MOR. Moreover, 8a and 10a also exhibited increased affinity at MOR than the earlier described derivatives 2–44 (P < 0.05, analysis of variance (ANOVA)) and comparable MOR affinity to 5a and 5b49 (P > 0.05, ANOVA). Most 6-amino acid-substituted derivatives (exception being 13a/b, 15a/b, 18a, 19a, 20a/b, and 21a) of the new series have binding affinities in the low nanomolar range (K(i) = 1.02–9.03 nM) at DOR in rat brain membranes, being augmented or comparable to DOR affinity of 1 (K(i) = 4.80 nM), consequently resulting in decreased or even a complete loss of MOR vs DOR selectivity (Table 1). Binding affinities at the DOR in the rat brain correlate with affinity data obtained at the human DOR expressed in CHO cells (Table S1). Generally, the attachment of amino acid residues at C6 position in 1 caused an increased binding at DOR. The 6α-L-Abu derivative 19a was found to be the most MOR-selective with respect to DOR, with a DOR/MOR selectivity ratio of 49 that was similar to the ratio of 48 calculated for 1 (Table 1). Binding at KOR in guinea-pig brain membranes was generally decreased for the 6-amino acid (6–21)- and 6-dipeptide-substituted derivatives (22a/b and 23a) compared to 1 (K(i) = 10.2 nM), thus causing an increase in MOR vs KOR selectivity. Among them, the 6β-L-Val-L-Tyr-substituted 22b was the most MOR-selective with respect to KOR, with a KOR/MOR selectivity ratio of 886 (Table 1).

Introduction of unnatural amino acids at position 6, D-Ala, D-Val, d-Phe, L-Chg, L-Abu, β-Ala, and GABA, in 1 led to 15a/b, 16a/b, 17a/b, 18a/b, 19a/b, 20a/b, and 21a/b, respectively, all generally showing high binding MOR affinities equivalent to compounds with natural amino acids (Tables 1 and S1). We also compared the 6-D-amino acid-substituted derivatives with their corresponding 6-L-amino acid analogues. No major changes in the rat and human MOR affinity were observed upon substitution of 6-L-Ala in the previously reported 3a/b44 with the 6-D-Ala residue in 15a/b, and the replacement of the 6-L-Phe in 4a/b44 increased ca. 2 times MOR affinity only for the 6β-D-Phe analogue 17b (P < 0.05, ANOVA). An increase (2–3 times) in MOR affinity was also produced when the 6-L-Val residue in 7a/b was replaced by 6-D-Val in 16a/b (P < 0.05, ANOVA), while MOR selectivity remained unaffected (Table 1).

In this study, we also report on the high affinities at MOR of 6-dipeptide-substituted derivatives 22a/b and 23a. The 6β-L-Val-L-Tyr-substituted derivatives, 22a and 22b, showed 4 to 7 times increased MOR binding affinity in the rat brain than their single 6-amino acid-substituted derivatives with 6-L-Val (7a and 7b) and 6β-L-Tyr residues (9b) (Table 1). Very good binding was also displayed by 22a and 22b at the human MOR (Table S1). Compared to the 6α-L-Gly-substituted derivative 2a, the 6α-L-Gly-Gly-substituted analogue (23a) of 1 displayed about 5 times lower affinity at MOR, and thus decreased MOR selectivity (Table 1).

When evaluating the effect of α/β orientation of the amino acid residue at C6 position on the interaction with MOR and DOR, it was observed that binding affinities of α- vs β-epimers were largely comparable (Table 1), with few exceptions, where the 6α-L-Glu-substituted 14a (K(i) = 1.45 nM) showed 8 times increased rat MOR affinity than the 6β-L-Glu analogue 14b (K(i) = 12.2 nM).
Table 1. Binding Affinities at the Opioid Receptors and Calculated Physicochemical Properties of 6-Amino Acid (2–21)- and 6-Dipeptide-Substituted Derivatives (22a/b and 23a), and Reference Compound 1

| compd | amino acid substitution at position 6 | opioid receptor binding $K_i$ (nM) | physicochemical properties$^b$ | clog $D_2$ |
|-------|--------------------------------------|------------------------------------|-------------------------------|---------|
|       | MOR | DOR | KOR | $K_i$ ratio MOR/DOR/KOR |         |
| 1a    | α-Gly | 4.80 ± 0.22 | 10.2 ± 2.0 | 1/48/102 | 0.48 |
| 2a    | β-Gly | 15.4 ± 1.4 | 43.2 ± 7.0 | 1/77/49 | -3.35 |
| 3a    | α-L-Ala | 26.9 ± 0.8 | 142 ± 43 | 1/35/184 | -2.81 |
| 4a    | α-L-Phe | 7.96 ± 0.64 | 44.8 ± 0.1 | 1/95/54 | -3.35 |
| 5a    | β-Gly | 3.67 ± 0.32 | 28.5 ± 4.2 | 1/39/30 | -1.13 |
| 6a    | α-L-Ser | 0.10 ± 0.01 | 0.81 ± 0.03 | 1/12/51 | -0.85 |
| 7a    | β-L-Val | 5.32 ± 0.39 | 196 ± 24 | 1/24/89 | -3.89 |
| 8a    | α-L-Asn | 5.29 ± 0.31 | 152 ± 15 | 1/25/71 | -3.89 |
| 9a    | β-L-Pro | 3.91 ± 0.30 | 325 ± 19 | 1/12/103 | -1.94 |
| 10a   | α-L-Leu | 3.52 ± 0.04 | 305 ± 39 | 1/12/100 | -1.94 |
| 11a   | α-L-Trp | 1.27 ± 0.42 | 12.6 ± 1.3 | 1/67/66 | -5.57 |
| 12a   | β-L-Lys | 3.34 ± 0.46 | 33.7 ± 4.1 | 1/63/64 | -5.57 |
| 13a   | α-L-Tyr | 2.18 ± 0.98 | 39.5 ± 0.2 | 1/26/48 | -1.41 |
| 14a   | β-L-Tyr | 3.89 ± 0.64 | 186 ± 33 | 1/12/58 | -1.41 |
| 15a   | α-L-Asp | 1.02 ± 0.12 | 25.1 ± 9.3 | 1/28/70 | -1.03 |
| 16a   | β-L-Glu | 1.19 ± 0.40 | 8.66 ± 0.64 | 1/16/13 | -1.03 |
| 17a   | β-L-Val | 3.37 ± 0.21 | 74.0 ± 9.7 | 1/29/63 | -4.29 |
| 18a   | β-L-Gln | 2.25 ± 0.11 | 103 ± 10 | 1/18/82 | -4.29 |
| 19a   | β-L-Asn | 5.13 ± 0.52 | 351 ± 66 | 1/16/108 | -4.04 |
| 20a   | β-L-Val | 4.87 ± 0.76 | 390 ± 3 | 1/20/117 | -4.04 |
| 21a   | β-L-Glu | 14.6 ± 3.6 | 50.2 ± 9.2 | 1/11/37 | -5.64 |
| 22a   | β-L-Met | 22.6 ± 2.2 | 351 ± 43 | 1/66/103 | -5.64 |
| 23a   | β-L-Glu | 9.03 ± 0.70 | 87.2 ± 4.2 | 1/62/60 | -5.39 |
|       |       |       |       | 745 ± 70 | 1/07/108 | -5.39 |

$^a$Determined in competition radioligand binding assays using rat brain membranes (MOR and DOR) and guinea-pig brain membranes (KOR). Values represent the mean ± SEM of three to four independent experiments each performed in duplicate. $^b$Calculated using the MarvinSketch 18.8 (ChemAxon). $^c$Data from ref 44. $^d$Data from ref 49.

11.6 nM), the 6α-L-Chg-substituted 18a ($K_i = 14.3$ nM) showed 11 times decreased rat DOR affinity than the 6β-L-Chg analogue 18b ($K_i = 1.30$ nM), and the 6α-L-Abu-substituted 19a ($K_i = 37.5$ nM) showed 29 times decreased DOR affinity than the 6β-L-Abu analogue 19b ($K_i = 1.30$ nM) (all $P < 0.05$ ANOVA). Similar observations were made at the human MOR and DOR expressed in CHO cells (Table S1). Concerning the influence of α/β orientation on the interaction with KOR in the guinea-pig brain, only the 6α-L-Glu-substituted 14a ($K_i = 87.2$ nM) showed 14 times increased KOR affinity than the 6β-L-Glu analogue 14b ($K_i = 1252$ nM), and in the case of the 6-L-Val-L-Tyr-substituted derivatives 22a and 22b, the 6β analogue displayed about 6 times lower KOR affinity (Table 1).

In vitro functional activity at the opioid receptors was assessed for 6-amino acid (6–21)- and 6-dipeptide-substituted derivatives (22a/b and 23a) in the guanosine-5′-O-(3-thio)-triphosphate ([35S]GTPγS) binding assays with membrane preparations from CHO cells expressing the human opioid receptors as described previously. Potencies (ED$_{50}$) and efficacies (% stimulation) are presented in Table 2. We have
Table 2. In Vitro Agonist Activity at the Opioid Receptors of 6-Amino Acid (2–21)- and 6-Dipeptide-Substituted Derivatives (22a/b and 23a), and Reference Compound 1

| compd | amino acid substitution at position 6 | MOR EC50 (nM) | % stim. | DOR EC50 (nM) | % stim. | KOR EC50 (nM) | % stim. |
|-------|-------------------------------------|--------------|--------|--------------|--------|--------------|--------|
| 1     |                                     |              |        |              |        |              |        |
| 2a    | α-Gly                               | 3.83 ± 0.95  | 97 ± 3 |              |        |              |        |
| 2b    | β-Gly                               | 3.78 ± 0.73  | 98 ± 9 |              |        |              |        |
| 3a    | α-L-Ala                              | 1.34 ± 0.17  | 97 ± 8 |              |        |              |        |
| 3b    | β-L-Ala                              | 6.24 ± 0.79  | 87 ± 6 |              |        |              |        |
| 4a    | α-L-Phe                              | 0.38 ± 0.19  | 93 ± 8 |              |        |              |        |
| 4b    | β-L-Phe                              | 6.76 ± 3.16  | 99 ± 8 |              |        |              |        |
| 6a    | α-L-Ser                              | 1.60 ± 0.59  | 87 ± 5 |              |        |              |        |
| 6b    | β-L-Ser                              | 3.56 ± 0.76  | 101 ± 9|              |        |              |        |
| 7a    | α-L-Val                              | 10.5 ± 4.0   | 95 ± 8 |              |        |              |        |
| 7b    | β-L-Val                              | 11.7 ± 5.7   | 84 ± 4 |              |        |              |        |
| 8a    | α-L-Lys                              | 2.25 ± 0.07  | 90 ± 8 |              |        |              |        |
| 8b    | β-L-Lys                              | 6.85 ± 1.39  | 90 ± 7 |              |        |              |        |
| 9a    | α-L-Tyr                              | 1.87 ± 0.44  | 92 ± 2 |              |        |              |        |
| 9b    | β-L-Tyr                              | 24.7 ± 9.8   | 93 ± 7 |              |        |              |        |
| 10a   | α-L-Trp                              | 0.51 ± 0.34  | 93 ± 9 |              |        |              |        |
| 10b   | β-L-Trp                              | 1.64 ± 0.43  | 101 ± 11|             |        |              |        |
| 11a   | α-L-Asn                              | 0.83 ± 0.17  | 99 ± 0.2|            |        |              |        |
| 11b   | β-L-Asn                              | 2.04 ± 0.44  | 96 ± 14|             |        |              |        |
| 12a   | α-L-Gln                              | 2.27 ± 0.11  | 90 ± 8 |              |        |              |        |
| 12b   | β-L-Gln                              | 9.54 ± 1.15  | 98 ± 4 |              |        |              |        |
| 13a   | α-L-Asp                              | 4.10 ± 1.29  | 90 ± 7 |              |        |              |        |
| 13b   | β-L-Asp                              | 1.45 ± 0.01  | 74 ± 3 |              |        |              |        |
| 14a   | α-L-Glu                              | 3.11 ± 1.32  | 105 ± 3|             |        |              |        |
| 14b   | β-L-Glu                              | 12.7 ± 4.7   | 98 ± 4 |              |        |              |        |
| 15a   | α-D-Ala                              | 1.44 ± 0.49  | 100 ± 8|              |        |              |        |
| 15b   | β-D-Ala                              | 15.4 ± 8.4   | 102 ± 9|             |        |              |        |
| 16a   | α-D-Val                              | 4.51 ± 2.35  | 105 ± 4|             |        |              |        |
| 16b   | β-D-Val                              | 2.38 ± 0.77  | 101 ± 5|             |        |              |        |
| 17a   | α-D-Phe                              | 0.77 ± 0.24  | 96 ± 4 |              |        |              |        |
| 17b   | β-D-Phe                              | 0.68 ± 0.29  | 78 ± 5 |              |        |              |        |
| 18a   | α-D-Chg                              | 2.88 ± 1.21  | 86 ± 8 |              |        |              |        |
| 18b   | β-D-Chg                              | 5.33 ± 0.89  | 86 ± 9 |              |        |              |        |
| 19a   | α-D-Abu                              | 5.12 ± 0.09  | 88 ± 2 |              |        |              |        |
| 19b   | β-D-Abu                              | 5.47 ± 1.18  | 83 ± 2 |              |        |              |        |
| 20a   | α-D-Ala                              | 3.52 ± 1.65  | 99 ± 7 |              |        |              |        |
| 20b   | β-D-Ala                              | 5.74 ± 0.47  | 78 ± 6 |              |        |              |        |
| 21a   | α-D-GABA                             | 2.88 ± 0.82  | 103 ± 4|              |        |              |        |
| 21b   | β-D-GABA                             | 12.3 ± 6.8   | 86 ± 7 |              |        |              |        |
| 22a   | α-D-Val-Val-Tyr                     | 0.89 ± 0.04  | 84 ± 7 |              |        |              |        |
| 22b   | β-D-Val-Val-Tyr                     | 0.16 ± 0.06  | 73 ± 5 |              |        |              |        |
| 23a   | β-Gly-Gly                            | 4.39 ± 0.54  | 85 ± 9 |              |        |              |        |

*[^35]S*GTPγS binding assays were performed with membranes from CHO stably expressing the human opioid receptors. Percentage stimulation (% stim.) is presented relative to the reference full agonists DAMGO (MOR), DPDPE (DOR), and U69,593 (KOR). Values represent the mean ± SEM of three to four independent experiments each performed in duplicate.

reported earlier on the potent agonistic activity of 2–4 in the mouse vas deferens bioassay41 and for 2b also in the rat vas deferens bioassay.42 In this study, SAR outcomes of opioid receptor-induced G protein activation upon ligand binding are described. Efficacies are presented as percentage stimulation relative to the standard full agonists, [b-Ala]-N-Me-Phe4,Glyol enkephalin (DAMGO for MOR),15β-[b-Pen]-enkephalin (DPDPE for DOR)14 and N-methyl-2-phenyl-N-[5R,7S,8S]-7-(pyrrolidin-1-yl)-1-oxaspiro[4,5]dec-8-yl]-acetamide (U69,593 for KOR).55

Based on the functional activities at MOR, most derivatives were very potent agonists (EC50 = 0.16–9.54 nM) with high efficacy behaving as full agonists (≥85% of the response to DAMGO), with the most potent agonists being 4a, 10a, 11a, 17a/b, and 22a/b (EC50 < 1 nM) (Table 2). Most of the new 6-amino acid- and 6-dipeptide-substituted derivatives mainly maintained the high agonist potency and full efficacy of 1 at MOR. Only compounds 7a/b, 9b, 14b, 15b, and 21b showed lower potencies at MOR (EC50 = 12.3–24.7 nM). Derivatives 7b, 17b, 19b, 20b, and 22a exhibited high efficacies at MOR of around 80%, whereas 13b and 22b were highly potent partial agonists at MOR with efficacies of around 70%. The most potent agonist was the 6β-L-Val-1-Tyr-substituted 22b with a 24 times increased MOR potency than 1 (EC50 of 3.83 nM for 1 vs 0.16
nM for 22b), while 22b also displayed a distinct functional activity profile as a partial agonist vs full MOR agonism of 1 (Table 2).

All compounds 2–23 were full agonists at DOR (≥85% of the response to DPDPE) similar to 1, with EC_{50} values ranging between 0.39 and 152 nM (Table 2). All, except 8a, showed much increased DOR agonist potencies than 1 (EC_{50} = 37.3 nM); hence, the functional MOR selectivity was greatly decreased or even shifted to DOR selectivity in several cases. Particularly, the 6β-L-Phe-substituted 4b was a very potent, selective, and full agonist at DOR, with a 14 times increased potency at DOR vs MOR. In our earlier report on competition binding studies in rat brain preparations and current data at the human opioid receptors, 4b exhibits preference for DOR over MOR (P < 0.05, ANOVA) (Tables 1 and S1). Moreover, the functional profile of 4b at human MOR and DOR established in the present study using [35S]GTPγS binding assays supports and extends our previous findings in the mouse vas deferens bioassay. An increase, although less pronounced (2–4 times), in agonist potency at DOR vs MOR was also observed for other 6-amino acid-substituted derivatives, mainly for the 6β-epimers, 6β-L-Val (7b), 6β-L-Tyr (9b), 6β-L-Gln (12b), 6β-L-Glu (14b), 6β-D-Ala (15b), 6β-L-Abu (19b), and 6β-GABA (21b) (P > 0.05, ANOVA, except for 9b vs 9a, P < 0.05). Only the 6α-epimer 6α-D-Ala-substituted 16a showed ca. 4 times increased potency, although not statistically significant (P > 0.05, ANOVA), at DOR over MOR (Table 2). At KOR, most derivatives were partial agonists (<85% of the response to U69,593) similar to 1, but with reduced potencies than 1 (Table 2). Only compounds 2a, 6b, 10b, 15b, 16a/b, 17b, and 21b were full agonists at KOR, while they displayed lower potencies compared to 1. Generally, the functional MOR vs KOR selectivity was notably increased.

We also examined in vitro functional activities at the opioid receptors of 6-L-Ala (3a/b), 6-L-Phe (4a/b), and 6-L-Val (7a/b)-substituted derivatives in correlation to their corresponding 6-D-amino acid analogues, 15a/b, 17a/b, and 16a/b, respectively. Similar to our observations from binding studies (Tables 1 and S1), replacement of L-amino acids with D-amino acids at position 6 led to MOR agonist potencies unchanged or caused an increase, but largely retained with some exceptions MOR full agonism (Table 2). Exchange of the 6β-L-Phe with the 6β-D-Phe residue converted a full agonist to a partial agonist at MOR (99% for 4b vs 78% for 17b, P < 0.05, ANOVA), while substitution of 6β-L-Val with a 6β-D-Val residue converted a partial agonist to a full agonist (84% for 7b vs 101% for 16b, P < 0.05, ANOVA). The full agonism at DOR was not affected by the replacement of L- with D-amino acids, while some alterations in the functional activity at KOR were noted, specifically changing the partial agonist profile of 3b, 4a, 7a, and 7b to full agonists 15b, 17a, 16a, and 16b, respectively (all P < 0.05, ANOVA) (Table 2).

The two 6-L-Val-6-L-Tyr-substituted derivatives, 22a and 22b, were highly potent MOR partial agonists and very potent DOR full agonists, with 22a lacking selectivity, while 22b was 10 times more selective for MOR over DOR. The 6β-Gly-Gly-substituted 23a was less potent and a full agonist at MOR and DOR, with slight preference for DOR, although not statistically significant (P > 0.05, ANOVA). All three 6-dipeptide-substituted derivatives showed much reduced potencies at KOR and were partial agonists at this receptor (Table 2).

Following the pattern of SAR, we evaluated the effect of α/β orientation of the amino acid residue at C6 position on the in vitro functional profile at the opioid receptors. While the α-epimers were frequently favored for MOR by strongly activating this receptor, the β-epimers showed augmented interaction with potent activation of DOR. Few exceptions were detected with lower potencies at MOR for α-epimers over β-epimers, although not statistically significant, specifically for 6α/β-L-Asp analogues 13a and 13b (P > 0.05, ANOVA), 6α/β-L-Val analogues 16a and 16b (P > 0.05, ANOVA), and 6α/β-L-Val-6-L-Tyr analogues 22a and 22b (P < 0.05, ANOVA). A decrease in potency at DOR was noted for the 6β-L-Tyr-substituted 9b when comparing to its 6β-counterpart 9a (P > 0.05, ANOVA). While efficacies at MOR were altered in some cases by the α/β orientation of the 6-amino acid residue, with β-epimers becoming partial agonists, efficacies at DOR remained unchanged. Potencies at KOR were significantly lowered independent of the α- or β-substitution, whereas efficacies were mostly unaffected with only few exceptions (Table 2).

Evaluation of pharmacokinetic properties represents a key feature in today’s drug discovery and development, particularly in predicting response profiles in vivo of bioactive molecules. In this study, we have targeted opioid agonists from the class of N-methylmorphinans substituted with different amino acids and dipeptides at position 6 as analogues activating peripheral opioid receptors. We have used the coefficient of distribution, log D at the physiological pH of 7.4 (log D_{7.4}) as a major physicochemical factor describing the capability of a drug to pass lipophilic membranes. The calculation of the log D_{7.4} (c log D_{7.4}) of 6-amino acid (2–21) and 6-dipeptide-substituted derivatives (22a/b and 23a) (Figures 1 and 2) was performed with the software MarvinSketch 18.8 (ChemAxon), and the values are included in Table 1. The c log D_{7.4} values were ranging between −5.64 and −0.85, indicating the poor capability to enter the CNS. In contrast, the c log D_{7.4} of compound 1 is 0.48. Based on the c log D_{7.4} values, all compounds (2–23) showed much higher hydrophilicity than 1. The presence of amino acid residues as ionizable functional groups increases the polarity and therefore limits the BBB penetration.

The restricted capability to enter the CNS of compounds 2–5 was earlier demonstrated experimentally employing a commonly used pharmacological approach. Antinociceptive effects of 2–5 in pain models of thermal nociception (tail-flick test) and inflammatory hyperalgesia in the rat (the formalin test and carrageenan-induced hyperalgesia) after systemic sc administration were completely blocked by sc naloxone methiodide, an opioid antagonist that does not cross the BBB. Furthermore, naloxone methiodide completely antagonized antinociceptive effects of 6β-Gly-substituted 2b in the mouse eye wash behavioral test for trigeminal nociception following systemic ip administration. We also reported that antihyperalgesic effects of 2b given orally to rats with inflammatory hyperalgesia was reversed by sc administered naloxone methiodide. In the current study, as mentioned below, we also established the limited ability to pass the BBB for the new compounds 6–23.
in Table 3, and were compared to those of morphine and compound 1. We present first antinociceptive data in the acetic

Table 3. Antinociceptive Potencies of 6-Amino Acid (2–21)- and 6-Dipeptide-Substituted Derivatives (22a and 23a), Reference Compound 1 and Morphine in the Writhing Assay in Mice after sc Administrationa

| compd | amino acid substitution at position 6 | ED50 (μg/kg, sc) (95% CI) |
|-------|-----------------------------------|--------------------------|
| morphine |                                   | 437 (249–768)           |
| 1      |                                   | 3.26 (1.24–8.52)        |
| 2a     | α-Gly                             | 35.7 (15.2–84.0)        |
| 2b     | β-Gly                             | 27.5 (11.0–68.7)        |
| 3a     | α-L-Ala                           | 16.0 (5.45–47.0)        |
| 3b     | β-L-Ala                           | 86.3 (49.0–152)         |
| 4a     | α-L-Phe                           | 31.1 (14.3–67.7)        |
| 4b     | β-L-Phe                           | 579 (268–1252)          |
| 5a     | α-Gly                             | 36.1 (15.1–84.4)        |
| 5b     | β-Gly                             | 41.6 (15.5–112)         |
| 6a     | α-L-Ser                           | 32.1 (15.8–65.1)        |
| 7a     | β-L-Val                           | 117 (44.1–312)          |
| 7b     | α-L-Lys                           | 20.6 (10.1–41.8)        |
| 8a     | α-L-Tyr                           | 14.6 (5.15–41.7)        |
| 9a     | α-L-Trp                           | 92.7 (37.5–229)         |
| 10a    | β-L-Trp                           | 14.0 (5.90–33.0)        |
| 11a    | α-L-Asn                           | 15.2 (6.89–33.3)        |
| 13a    | α-L-Asp                           | 38.2 (16.4–88.9)        |
| 14a    | α-L-Glu                           | 36.1 (16.3–79.6)        |
| 16b    | β-L-Val                           | 14.0 (5.90–33.0)        |
| 17a    | α-L-Phe                           | 18.1 (8.37–39.2)        |
| 17b    | β-L-Phe                           | 250 (120–517)           |
| 18a    | α-L-Chg                           | 20.6 (4.33–98.2)        |
| 19a    | α-L-Abu                          | 17.5 (5.13–59.6)        |
| 20a    | α-L-Ala                           | 31.2 (13.2–73.9)        |
| 21a    | α-L-GABA                          | 41.9 (22.6–77.8)        |
| 22a    | β-L-Val-L-Tyr                     | 178 (58.0–545)          |
| 23a    | β-L-Gly-Gly                       | 104 (58.5–185)          |

aGroups of mice were administered sc test compound or saline (control), and evaluated in the acetic acid-induced writhing assay. Each compound was tested in at least three doses (n = 6–7 mice per dose). Inhibition of the writhing response was assessed 30 min after drug administration, and ED50 values and 95% confidence intervals (CI in parentheses) were calculated from dose–response curves.

Acid-induced writhing assay for the previously developed 6-amino acid-substituted 2a, 3a/b, 4a/b, and 5a/b (Figure 1 and Table 3), as well as for the new series of compounds (Figure 2 and Table 3). We have earlier reported on the high efficacy in inhibiting the writhing response of 6β-Gly-substituted 2b after sc administration, being 24 times more potent than morphine.48 Our present results are in line with these findings.

As shown in Table 3, all compounds, except 4b, were more effective in inducing an antinociceptive response than morphine (ED50 = 437 µg/kg), while they showed generally lower potencies compared to 1 (ED50 = 3.26 µg/kg) in the writhing assay. The most potent compounds were 3a, 9a, 11a, 16b, 17a, and 19a with ED50 < 20 µg/kg. The 6β-Gly-substituted 2a displayed comparable antinociceptive potency to its 6β counterpart 2b, while the 6α-epimers with an L-Ala and L-Phe residue (3a and 4a, respectively) had 5 and 19 times increased potencies than their corresponding 6β-epimers, 3b and 4b, a finding that correlates to the lower in vitro agonist potency of 3b and 4b (Table 2). The same pattern on significantly reduced potency for the 6β-epimer was observed when evaluating 6α and 6β derivatives substituted with the unnatural amino acid d-Phe, 17a and 17b, respectively, likely as a result of diminished efficacy at MOR of 17b (Table 2). We also examined antinociceptive profiles of 6-L-Phe (4a/b)- and 6-L-Val (7a/b)-substituted derivatives in association with their 6-d-amino acid analogues, 17a/b and 16a/b, respectively. Replacement of 6-L-Phe (4a/b) and 6β-L-Val residues (7b) with d-α-amino acids at position 6, 17a/b and 16b, respectively, produced an increase in antinociceptive potency, particularly for 16b (8 times vs 7a, Table 3), a profile that supports the results from in vitro binding and agonist activity (Tables 1 and 2). The 6-dipeptide-substituted derivatives 22a (6α-L-Val-L-Tyr-substituted) and 23a (6α-L-Val-L-Tyr-substituted) were also effective in inducing an antiwrithing response, with 2.5 and 4 times, respectively, higher potency than morphine (Table 3).

To determine whether targeted 6-α-amino acid and 6-dipeptide analogues inhibit writhing behavior in mice through peripheral opioid receptors activation, we used naloxone methiodide.27 Six 6-α-amino acid-substituted derivatives were selected, including three derivatives with a natural amino acid, 2b (6β-L-Gly), 8a (6β-L-Lys), and 10b (6β-L-Trp), two with an unnatural amino acid, 18a (6α-L-Chg) and 19a (6α-L-Abu), and two dipeptides, 22a (6β-L-Val-L-Tyr) and 23b (6β-Gly-Gly) (Figure 4). Subcutaneous co-administration of these opioid agonists with naloxone methiodide reversed their antinociceptive effect in the writhing assay indicative of activation of peripheral opioid receptors, due to increased polarity, and therefore restricted BBB penetration (Table 1). The results on the peripheral site of action of 2b corroborate and extend our earlier observations in nociceptive and inflammatory pain models in rats.45,46 Using the writhing assay in mice, we have demonstrated previously the lack of 2b to enter the CNS as intracerebroventricular administration of d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP),65 an MOR-selective antagonist, did not reverse the antinociceptive effects of systemic 2b.48

Conclusions

In summary, we have extended the SAR on our earlier 6-amino acid-substituted derivatives of 1 by targeting additional amino acid residues of the L- and/or D-series at position 6. In this study, we reported on the synthesis and pharmacological characterization of a library of differently substituted N-methyl-14-O-methylmorphinans with natural amino acids, i.e., Ser, Val, Lys, Tyr, Trp, Asn, Gln, Asp, and Glu (6–14), unnatural amino acids, i.e., d-Ala, d-Val, d-Phe, L-Chg, L-Abu, β-Ala, and GABA (15–21), and three dipeptides (22a/b and 23) at C6 position, which emerged as potent MOR/DOR agonists producing antinociception via activation of peripheral opioid receptors. On the basis of the SAR studies comprising results on in vitro binding and functional profiles and antinociceptive activities, the potent MOR/DOR agonist profile and reduced interaction and activation of KOR for most compounds was established. Derivatives with unnatural amino acids at position 6 generally showed high MOR and DOR binding affinities and agonism, similar to compounds with natural amino acids. Substituting L-amino acids by d-amino acids left MOR binding affinities and agonist potencies unchanged or caused an increase, but largely retained MOR full agonism. The full agonism at DOR was not affected by the replacement of L- with d-amino acids, while conversion from partial agonists to full agonists at KOR was observed. While the α-epimers were often favored for MOR by strongly activating this receptor, the β-epimers showed increased binding and potent activation of DOR. We also
presented opioid activities of 6-dipeptide-substituted analogues 22a and 22b as highly potent MOR partial agonists and very potent DOR full agonists, while 23a was less potent and an MOR/DOR full agonist. In vivo, the new 6-amino acid- and 6-dipeptide-substituted derivatives of 1 were highly effective in inducing antinociception in the acetic acid-induced writhing assay in mice, which was antagonized by sc co-administration with naloxone methiodide (NLXM, 1 mg/kg) in the acetic acid-induced writhing assay in mice at 30 min after drug administration. Values are shown as mean of writhes ± SEM (n = 6−7 mice per group). ***P < 0.001 vs control group; *P < 0.05, **P < 0.01 and ###P < 0.001 vs agonist treated group, ANOVA with Tukey’s post hoc test.

Figure 4. Systemic sc administration of 6-amino acid- and 6-dipeptide-substituted derivatives produces antinociception via activation peripheral receptors in the writhing assay in mice. Antinociceptive effects of 2b (50 μg/kg), 8b (50 μg/kg), 10b (200 μg/kg), 18b (50 μg/kg), 19b (50 μg/kg), and 23a (200 μg/kg) were antagonized by sc co-administration with naloxone methiodide (NLXM, 1 mg/kg) in the acetic acid-induced writhing assay in mice at 30 min after drug administration. Values are shown as mean of writhes ± SEM (n = 6−7 mice per group). ***P < 0.001 vs control group; *P < 0.05, **P < 0.01 and ###P < 0.001 vs agonist treated group, ANOVA with Tukey’s post hoc test.

### EXPERIMENTAL SECTION

Chemistry. General Methods. The starting material thebaine was obtained from Tasmanian Alkaloids Ltd., Westbury, Tasmania, Australia. All other 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. 1H NMR spectra were obtained on a Varian Gemini 200 (200 MHz) spectrometer using tetramethylsilane (TMS) as internal standard for CDCl3 and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) for D2O. Coupling constants (J) are given in Hz. IR spectra were taken on a Mattson Galaxy FTIR series 3000 in KBr pellets (in cm−1). Mass spectra were recorded on a Bruker-Esquire 3000+ 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. TLC was performed on silica gel plates Polygram SIL G/UV254 (Macherey-Nagel, Germany) with CH2Cl2/CH3OH/NH4OH 90:9:1 as eluent. The elemental analysis values were found to be within ±0.4% of the calculated values, indicating a purity of the tested compounds of >95%.
(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
phinan-6α-y-yl)-amino]-3-(1H-indolo-3-yl)propionic Acid Dihydro-
chloride (18a 2HCl). Colorless crystals from EtOH (80%); Mp > 230
°C (dec.); IR (KBr): 1734 (C=O); 170 N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar.
H), 6.73 (d, J = 8.4, 1 ar. H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m,
MeN); MS (ESI): m/z 445.7 (M+ + 1). Anal. (C23H31N3O6
·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
phinan-6α-y-yl)-amino]-3-(1H-indolo-3-yl)propionic Acid Dihydro-
chloride (18b 2HCl). Amorphous solid (81%); Mp > 230 ºC (dec.); IR (KBr):
1735 (C=O). 1 H N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 433.8
(M+ + 1). Anal. (C22H29N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
phinan-6β-y-yl)-amino]-3-(1H-indolo-3-yl)propionic Acid Dihydro-
chloride (18c 2HCl). Colorless crystals from EtOH (80%); Mp > 230 ºC (dec.);
1 H N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 445.7
(M+ + 1). Anal. (C23H31N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
phinan-6α-y-yl)-amino]-3-(1H-indolo-3-yl)propionic Acid Dihydro-
chloride (18b 2HCl). Colorless crystals from EtOH (80%); Mp > 230 ºC (dec.);
IR (KBr): 1734 (C=O); 170 N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 445.7
(M+ + 1). Anal. (C23H31N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
phinan-6β-y-yl)-amino]-3-(1H-indolo-3-yl)propionic Acid Dihydro-
chloride (18c 2HCl). Colorless crystals from EtOH (80%); Mp > 230 ºC (dec.);
IR (KBr): 1734 (C=O); 170 N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 445.7
(M+ + 1). Anal. (C23H31N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
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chloride (18c 2HCl). Colorless crystals from EtOH (80%); Mp > 230 ºC (dec.);
IR (KBr): 1734 (C=O); 170 N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 445.7
(M+ + 1). Anal. (C23H31N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
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chloride (18c 2HCl). Colorless crystals from EtOH (80%); Mp > 230 ºC (dec.);
IR (KBr): 1734 (C=O); 170 N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 445.7
(M+ + 1). Anal. (C23H31N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.

(25) 2-(4S,5a-Epoxy-3-hydroxy-14β-methoxy-17-methylmor-
phinan-6β-y-yl)-amino]-3-(1H-indolo-3-yl)propionic Acid Dihydro-
chloride (18c 2HCl). Colorless crystals from EtOH (80%); Mp > 230 ºC (dec.);
IR (KBr): 1734 (C=O); 170 N M R (D2O): δ 6.78 (d, J = 8.4, 1 ar. H), 6.73 (d, J = 8.4, 1 ar.
H), 4.95 (d, J = 3.6, H-C(5)), 3.27 (m, MeO), 2.86 (m, MeN); MS (ESI): m/z 445.7
(M+ + 1). Anal. (C23H31N3O6·2HCl·2H2O·1.0CH3OH) C, H, N.
(25)-2-[[4,5α-Epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-y]lamino][propionic Acid Dihydrochloride (19a 2HCl). Colorless crystals from EtOH (82%). Mp >290 °C (dec.); IR (KBr): 1724 (C=O) cm⁻¹; 1H NMR (D₂O): δ 6.68 (d, J = 8.4, 1 ar. H), 6.78 (d, J = 8.4, 1 ar. H), 7.01 (d, J = 7.8, H-C(3)), 3.25 (s, MeO), 2.85 (s, MeN); MS (ESI): m/z 388.8 (M⁺ + 1). Anal. (C₂₇H₃₂N₂O₅·2HCl·2H₂O) C, H, N.

(25)-2-[[4,5α-Epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-y]lamino][propionic Acid Dihydrochloride (20b 2HCl). Colorless crystals (78%). Mp >270 °C (dec.); IR (KBr): 1724 (C=O) cm⁻¹; 1H NMR (D₂O): δ 6.83 (d, J = 8.4, 1 ar. H), 6.78 (d, J = 8.4, 1 ar. H), 7.01 (d, J = 7.8, H-C(3)), 3.25 (s, MeO), 2.85 (s, MeN); MS (ESI): m/z 388.8 (M⁺ + 1). Anal. (C₂₇H₃₂N₂O₅·2HCl·2H₂O) C, H, N.

Calculation of Physicochemical Properties. Coefficient of distribution, log Dₐ, as log D at pH 7.4, was calculated with MarvinSketch 18.8 (ChemAxon, www.chemaxon.com).

Pharmacology: Drugs and Chemicals. Cell culture media and supplements were obtained from Sigma-Aldrich Chemicals (St. Louis, MO) or Life Technologies (Carlsbad, CA). Radioligands [³H]-DAMGO, [³H]-diprenorphine, [³H]-U69,593, and [³H]-GTTPyS were purchased from PerkinElmer (Boston, MA). [³H]-deltorphin II was obtained from the Institute of Isotopes Co. Ltd. (Budapest, Hungary). DAMGO, [α-Pen₉,β-Pen₉]-enkephalin (DPDPE), diprenorphine, U69,593, naloxone, naloxone methiodide, tris(hydroxymethyl) aminomethane (Tris), 2-[(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), unlabeled GTPyS, and guanosine diphosphate (GDP) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Morphine hydrochloride was obtained from Gatt-Koller GmbH (Innsbruck, Austria). All other chemicals were of analytical grade and obtained from standard commercial sources. Test compounds were prepared as 1 mM stocks in water and further diluted to working concentrations in the appropriate medium.
determined in the absence of test ligand. Samples are binding experiments were performed in duplicate and repeated at least three times with 5 mL of ice-cold 50 mM Tris-HCl buffer filtration through Whatman glass GF/C. Whatman glass GF/B fiber filters and counted as described for binding assays. All experiments were performed in duplicate and repeated at least three times.

**Drug Administration.** Solutions of test compounds were prepared in sterile physiological 0.9% saline and further diluted to working doses in saline solution. Test compounds or saline (control) were administered by sc route in a volume of 10 μL/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.

**Acetate Acid-Induced Writhing Assay.** Writhing was induced in male CD1 mice by ip injection of a 0.6% acetic acid aqueous solution as described previously.

Groups of mice were administered different doses of test compound or saline (control), and 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 × [(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. For the antagonism study, naloxone methiodide was sc co-administered with the respective opioid agonist, and writhing was assessed as described above.

**Data and Statistical Analysis.** Data were analyzed and graphically processed using the GraphPad Prism 5.0 Software (GraphPad Prism Software Inc., San Diego, CA). For in vitro assays, inhibition constant (Kᵢ in nM), potency (EC₅₀ in nM), and efficacy (% stimulation) values were determined from concentration–response curves by nonlinear regression analysis. The Kᵢ values were determined by the method of Cheng and Prusoff.

In the [35S]GTPγS binding assays, efficacy was determined relative to the reference full opioid agonists, DAMGO (MOR), DPDPE (DOR), and U69,593 (KOR). In vitro data are presented as mean ± SEM of three to four independent experiments each performed in duplicate. For the writhing assay, dose–response relationship of percentage inhibition of writhing was constructed, and the doses necessary to produce a 50% effect (ED₅₀) and 95% confidence intervals (95% CI) were calculated. Each experimental group included six to seven mice. Data were statistically evaluated using one-way ANOVA with Tukey’s post hoc test for multiple comparisons 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.8b01327.

Additional chemical and pharmacological information: general procedure for the synthesis of 6-amino acid esters 24−41; binding affinities of 6-amino acid (2a/b, 3a/b, 4a/b, 14a/b, 15a/b, 17a/b, and 19a/b) and 6-dipeptide substituted derivatives (22a/b) at the human opioid receptors (PDF)

Molecular formula strings (CSV)

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S.R. and T.B.H. contributed equally to this work.
The authors acknowledge Tasmanian Alkaloids Pty. Ltd., Westbury, Tasmania, Australia, for the generous gift of thebaine. The authors also thank Andrea Kaltschi and Beatrice Jungwirth for technical assistance. This research was supported by the Austrian Science Fund (FWF: P21350, TRP16-B18 and TRP19-B18), Tyrolean Research Fund (TWF-UNI-0404/94), and the University of Innsbruck.

**ABBREVIATIONS**

BBB, blood–brain barrier; CHO, Chinese hamster ovary; CNS, central nervous system; DAMGO, (δ-Ala², N-Me-Phe⁷, Gly-ol³)enkephalin; DOR, δ opioid receptor; DPDP, (δ-Pen³, δ-Pen⁵)enkephalin; GABA, γ-aminobutyric acid; MOR, μ opioid receptor; i.p., intraperitoneal; KOR, κ opioid receptor; i.t., i.t.-2-amino butyric acid; i.Chg, l-cyclohexylglycine; NLXm, naloxone methide; SAR, structure–activity relationships; sc, subcutaneous; U69,593, N-methyl-2-phenyl-N-[[(5R,7S,8S)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide

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