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PAPER

Design and synthesis of a bivalent ligand to explore the putative heterodimerization of the mu opioid receptor and the chemokine receptor CCR5†

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The bivalent ligand approach has been utilized not only to study the underlying mechanism of G protein-coupled receptors dimerization and/or oligomerization, but also to enhance ligand affinity and/or selectivity for potential treatment of a variety of diseases by targeting this process. Substance abuse and addiction have made both the prevention and the treatment of human immunodeficiency virus (HIV) infection more difficult to tackle. Morphine, a mu opioid receptor (MOR) agonist, can accelerate HIV infection through up-regulating the expression of the chemokine receptor CCR5, a well-known co-receptor for HIV invasion to the host cells and this has been extensively studied. Meanwhile, two research groups have described the putative MOR–CCR5 heterodimers in their independent studies. The purpose of this paper is to report the design and synthesis of a bivalent ligand to explore the biological and pharmacological process of the putative MOR–CCR5 dimerization phenomenon. The developed bivalent ligand thus contains two distinct pharmacophores linked through a spacer; ideally one of which will interact with the MOR and the other with the CCR5. Naltrexone and Maraviroc were selected as the pharmacophores to generate such a bivalent probe. The overall reaction route to prepare this bivalent ligand was convergent and efficient, and involved sixteen steps with moderate to good yields. The preliminary biological characterization showed that the bivalent compound **1** retained the pharmacological characteristics of both pharmacophores towards the MOR and the CCR5 respectively with relatively lower binding affinity, which tentatively validated our original molecular design.

Introduction

The negative co-operativity among β-adrenergic receptors in frog erythrocyte membranes[1] and occurrence of opioid receptors in clusters on the neuroblastoma cell surface[2] were attributed to be the earliest reports of receptor dimerization–oligomerization.[3,4] The exact term "dimerization" was actually coined by Gregory and co-workers in 1982,[5] yet it took more than another decade for more convincing evidence to be recognized. Among the evidence, the most significant pieces are the co-expression studies with mutant muscarinic–adrenergic receptors conducted by Maggio *et al.*[6]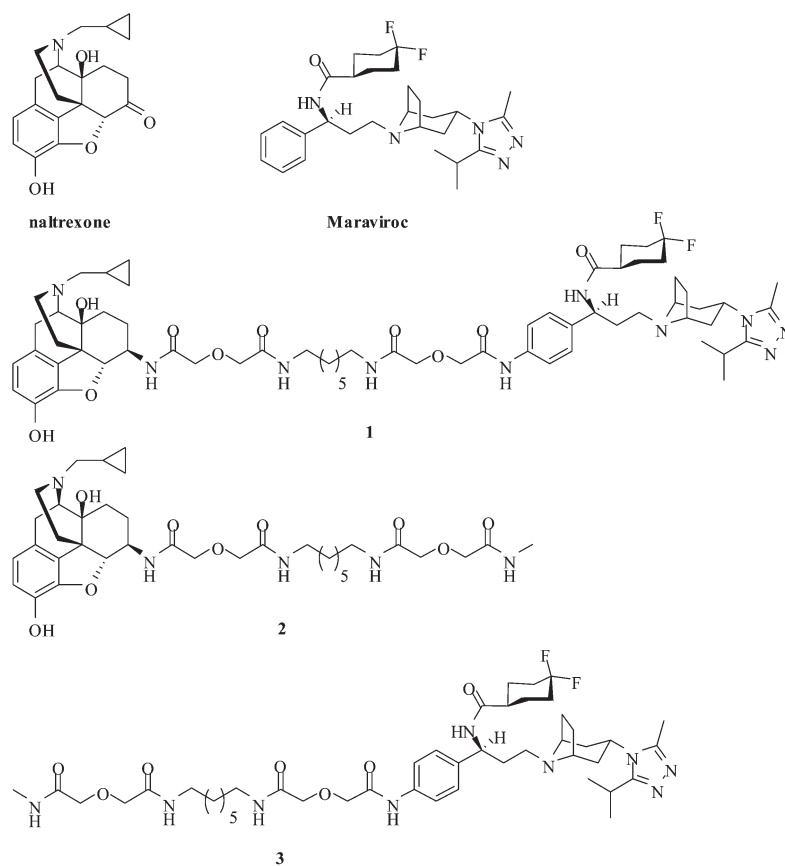 and a co-immunoprecipitation approach utilized by the Bouvier group for β2-adrenergic receptor.[7] The "dimerization–oligomerization" concept for G protein-coupled receptors (GPCRs) was widely accepted by the end of 1990s based on the research on GABA$_B$ receptor from several groups,[8] and was further supported by the X-ray crystal structures of some others later on.[9] The dimerization–oligomerization of GPCRs poses a differentiated pharmacology from the monomers.[10] In this regard, a number of bivalent ligands have been synthesized to explore the underlying biology and pharmacology mechanisms of GPCRs dimerization–oligomerization, as well as to develop prospective agents with enhanced affinity and/or selectivity to treat different disorders and diseases by targeting this "novel" mechanism.[4a,11]

Since acquired immunodeficiency syndrome (AIDS) was identified three decades ago,[12] the global prevalence of AIDS has become stable at 0.8%, with over 33 million people infected with human immunodeficiency virus (HIV) in 2007.[13] There are almost 16 million people who are injecting drug users (IDUs) worldwide and nearly 10% of HIV infection was attributed to injecting drug use through contaminated needles.[14] Statistics showed IDUs account for approximately 13% of the total HIV

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Fig. 1 Chemical structures of naltrexone, Maraviroc, designed bivalent (**1**) and monovalent ligands (**2**, **3**).

infection in thirty-four US states during 2004 to 2007.[15] Not only driving HIV transmission among IDUs, the abused substances, such as opioids, cocaine, and alcohol also accelerate the progression of AIDS and complicate the treatment of this disease.[16] Moreover, HIV infection seems to increase drug addiction vulnerability as well.[17] Needle-exchange programs (NEPs) have shown appreciable outcome on reducing HIV prevalence among IDUs for over a decade.[18] The current available treatment for opioid-dependent HIV patients also adopts opioid substitution therapy (OST), *i.e.* methadone, buprenorphine and buprenorphine–naloxone, into HIV management.[19] Although opioid maintenance therapy have shown to improve patients adherence and promising outcome for HIV treatment, the adverse drug–drug interaction between methadone, buprenorphine and antiretroviral agents compromise the overall effects.[20] New agents and remedies are still highly demanded for the treatment of these patients.

Opiates and alcohol abuse–addiction liability is mainly associated with the mu opioid receptor (MOR),[21] which is also involved in different immunomodulatory activities induced by opioids.[22] The chemokine receptor CCR5 was identified as a major co-receptor for HIV in 1996,[23] and is largely expressed on activated memory CD45RO+ T cells, monocyte–macrophages, dendritic cells, granulocyte precursors, and natural killer cells.[24] Both receptors belong to the seven-transmembrane GPCR superfamily. Several research groups have shown that MOR agonists, such as morphine, methadone, and DAMGO, can increase

CCR5 expression, thus enhance and facilitate HIV infection and replication both *in vitro* and *in vivo*.[25] In light of this observation, as well as the fact that the opioid receptors and the CCR5 are all present on immune cells, Suzuki and co-workers studied the interactions between these GPCRs through a co-immunoprecipitation approach.[26] Their research demonstrated for the first time that CCR5 and opioid receptors form oligomers and the oligomerization modulates the function of such a complex.[26] Two years later, Chen *et al.* reported the heterodimerization and cross-desensitization between the MOR and the CCR5 in co-expressed Chinese hamster ovary (CHO) cells.[27] The authors proposed that the MOR–CCR5 heterodimers may contribute to the observed cross-desensitization. Despite these fundamental studies, a chemical probe that is capable of interacting with both receptors simultaneously has never been developed to facilitate the study of the biological and pharmacological process of MOR–CCR5 dimerization. Herein, we report the design and synthesis of a bivalent ligand **1** (Fig. 1) as a lead compound in order to test our hypothesis and to reveal the underlying mechanism of MOR–CCR5 heterodimers, eventually.

Bivalent ligand rational design

Receptor antagonists serve as important pharmacological probes to uncover the probable involvement of a receptor mechanism.[28] Therefore, it seemed ideal to build a bivalent ligand containing

a MOR-antagonist moiety as well as a CCR5-antagonist one, linked through an appropriate spacer. Naltrexone[29] (Fig. 1) was selected as the moiety to interact with the MOR based on the following considerations: first, naltrexone has been successfully used to investigate the dimerization of opioid receptors previously;[30] second, it represents an ideal treatment for alcohol and opiate addiction and has been successfully used to treat alcoholism clinically.[31] Maraviroc[32] (Fig. 1) is the only CCR5 antagonist that has been approved for HIV treatment by the FDA so far[33] and thus became our first choice as the CCR5 pharmacophore. Meanwhile, both of these two ligands showed high affinity and reasonable selectivity toward the MOR and the CCR5 respectively.

It has been proved that the loci for tethering two pharmacophores through a spacer affect the binding affinities of the resulted bivalent ligands.[34] In addition, the overall chemical modification of these two pharmacophores for spacer attachment should also be designed from a synthetic point of view, that is, chemical reactions should be readily accomplished. Thus, based on the successful cases from Portoghese group,[30a,b] the C6-position of naltrexone was selected as the attaching locus after transforming its carbonyl group to the 6β-amino group (Fig. 1). Whereas the discovery process of Maraviroc revealed that both of the difluorocyclohexyl moiety and the *exo*-1,2,4-triazole substituted tropane core are essential to its potent antiviral activity and weak hERG inhibition.[35] Additionally, an interactive docking study of Maraviroc to a rhodopsin-based CCR5 homology model demonstrated the interactions between Glu283 and the tropane core, as well as Ile198 and the difluorocyclohexyl moiety within the proposed binding pocket.[36] Hence, the *para*-position of the phenyl ring in Maraviroc was first chosen as the linking site to avoid severe impacts to the above interactions. Since EDCI–HOBt mediated coupling reaction between carboxylic acid and amine can be easily accomplished, an amino group was then chosen as the functional group on this *para*-phenyl ring to hook Maraviroc up with the spacer. Thus, pharmacophore 6 (Scheme 1) was designed as the precursor of the CCR5 antagonist.

Several studies indicated that a spacer with 16 to 22 atoms might be beneficial for targeting GPCR dimers, ideally with 21 atoms when both pharmacophores are antagonists of their respective receptors.[30a,b,37] Therefore, the 21-atom-spacer was adopted as an initial lead in the current study. The design rationale of such a spacer is to keep a favorable balance between hydrophobicity and hydrophilicity as well as to possess a reasonable rigidity, high stability and low toxicity.[38] Hence, one alkyldiamine moiety and two diglycolic units were employed to build up the spacer. Monovalent ligands 2 and 3 (Fig. 1) were also designed as controls to clarify the potential effects of the spacer to the binding affinity and potency of the bivalent ligand.

Chemistry and biological studies

The retrosynthetic analysis of bivalent ligand 1 revealed three major fragments, 6β-naltrexamine 4, diacid spacer 5, and the CCR5 antagonist precursor 4-NH₂-maraviroc 6 (Scheme 1). Among them, 6β-naltrexamine 4 can be conveniently prepared

from naltrexone following the reported procedure,[39] whereas nucleophilic reaction of 1,7-diaminoheptane with diglycolic anhydride[30b] can readily afford the diacid spacer 5. Similarly to Maraviroc, retrosynthetic analysis of 6 identified three key fragments: a 4,4-difluorocyclohexanecarboxylic acid 8, a β-phenyl-alanine ester 9, and a triazole-substituted tropane 10 (Scheme 1). As the preparation of difluoro acid 8 appeared to be challenging,[40] a strategy that enables a later introduction of this fragment was sought. In order to avoid the tedious reduction–oxidation procedure as well as to improve the overall yields, an amide coupling strategy instead of reductive amination[40,41] was postulated to generate 7 by coupling 9 with 10. Several papers have reported the highly stereoselective introduction of an amino group through Michael addition with lithium (*R*)-*N*-benzyl-*N*-α-methylbenzylamine in high yields.[42] Hence, the same method was adopted to prepare fragment 9. Two cinnamic acids are commercially available to synthesize the substrate 11 for a later Michael addition: 4-nitrocinnacid and 4-bromocinnacid. However, the conversion of the nitro group to the amino group poses an issue for the overall synthetic route since other functional groups, such as double bond, ester, benzyl, and amide, are present in the same molecule and reducing agents such as Na₂S and SnCl₂ are not environment-friendly. Therefore, 4-bromocinnacid was chosen as the starting material.

The overall synthesis of the precursor 6 is illustrated in Schemes 2 and 3. The carboxylic group protection was performed by refluxing 4-bromocinnacid in isopropanol with a few drops of concentrated sulfuric acid to give a moderate yield of 12.[43] The bromide 12 was then converted to aniline 13 using lithium hexamethyldisilazane (LHMDS) catalyzed by Pd₂(dba)₃ and P(*t*-Bu)₃,[44] which upon heating with di-*tert*-butyl dicarbonate furnished compound 14 in a good yield.[45] The diastereoselective Michael addition of 14 was achieved with lithium (*R*)-*N*-benzyl-*N*-α-methylbenzylamine prepared *in situ*.[42e] The stereoselectivity was confirmed by comparing with the literature reported data.[42f] The following saponification of the conjugate adduct 15 gave acid 16, which was then coupled with 3-(3-isopropyl-5-methyl-4H-1,2,4-triazol-4-yl)-*exo*-8-azabicyclo[3.2.1]octane[41a] 10 through HOBt–EDCI method to yield 17. The reduction of 17 with either BH₃·THF or LiAlH₄ at ambient temperature did not give any amide reduced product 18. Heating 17 with BH₃·THF resulted a mixture of complexes, with the loss of Boc and/or two benzyl groups. The steric hindrance generated by the two benzyl groups might complicate the reduction process. Hence, the catalytic hydrogenolysis of 17 with 10% Pd–C was conducted to produce intermediate 19 instead. Although reaction of 19 with BH₃·THF did afford compound 7, the majority of the product formed a complex with tetrahydrofuran (1 : 1), which requires acid to release the free amine.[46] However, the Boc group may be sensitive to such acidic conditions. Replacement of BH₃·THF with LiAlH₄, which only needs water to decompose the intermediate formed after the reaction,[47] provided 7 in a reasonable yield (Scheme 2). Reaction of 7 with 4,4-difluorocyclohexanecarboxylic acid[48] 8 was mediated by HOBt–EDCI and the coupling product 20 was subsequently converted to the CCR5 antagonist precursor 6 with TFA/DCM (1 : 10) at ambient temperature[49] (Scheme 3).

Then the bivalent ligand 1 was prepared following a linear synthetic route as shown in Scheme 4. Reaction of
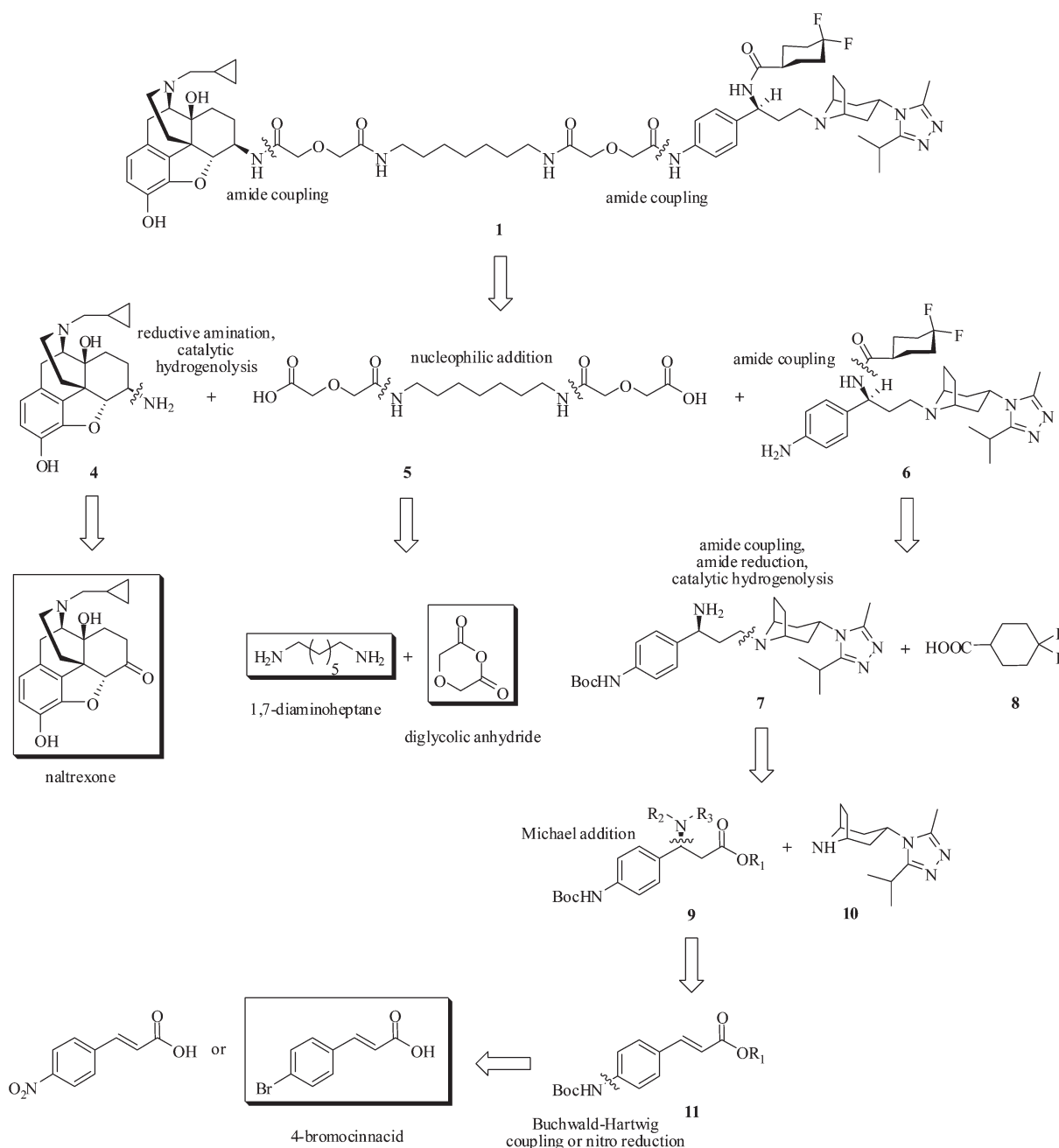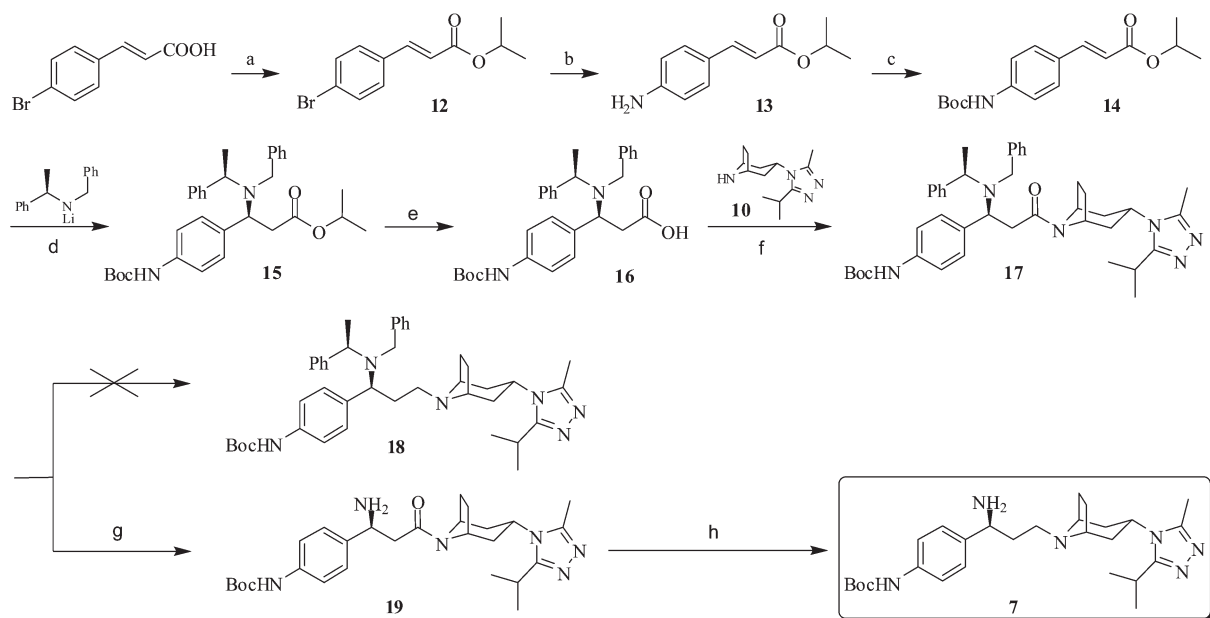
Scheme 1 Retrosynthetic analysis of the bivalent ligand **1**.

1,7-diaminoheptane with 0.9 equivalent of benzyl chloroformate under ice-water bath generated mono-Cbz protected intermediate **21**,[50] which was further condensed with diglycolic anhydride to give compound **22**.[30b] Intermediate **23** was prepared by coupling **22** with **4** (6β-naltrexamine[39]) utilizing HOBt–EDCI method. Hydrogenation–deprotection of **23** with 10% Pd–C catalyst yielded amine **24**. Condensation of **24** with a second molecule of diglycolic anhydride provided acid **25**, which was then coupled with the CCR5 antagonist precursor **6** via HOBt–EDCI mediation to furnish bivalent ligand **1**.

Monovalent ligand **2** was conveniently synthesized by coupling the intermediate **24** with **26**[30b] via HOBt–EDCI peptide coupling method (Scheme 5).

From an efficient synthesis perspective, monovalent ligand **3** was prepared according to Scheme 6, considering it only involved three steps and all the reactions can be simply monitored by UV. Thus, HOBt–EDCI-mediated coupling of **22** with precursor **6** afforded intermediate **27**, which underwent catalytic hydrogenolysis to yield amine **28**. Monovalent ligand **3** was then obtained by coupling **28** with **26** employing the HOBt–EDCI method.

All three ligands were then preliminarily further characterized for their binding affinity and functional activity. In a calcium mobilization assay with CCR5–MOLT-4 cells,[51] compound **1** showed no agonism and its antagonist properties are indicated by its calcium flux inhibition IC_{50} value of 231 ± 88 nM. Compared

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Scheme 2 Synthesis of intermediate **7**. Regents and conditions: (a) i-PrOH, H_2SO_4 (conc.), reflux, 80%; (b) i) LHMDS, $Pd_2(dba)_3$, $P(t\text{-Bu})_3$, toluene, rt; ii) 1N HCl, rt, 90%; (c) Boc_2O, THF, reflux, 85%; (d) THF, −78 °C; (e) LiOH, MeOH–H_2O (2/1), reflux, 85%, two steps; (f) EDCI, HOBt, TEA, **10**, 4Å MS, DCM, 0 °C to rt, 73%; (g) 10% Pd–C, 60 psi, MeOH, 84%; (h) i) $LiAlH_4$, THF, 0 °C to rt; ii) H_2O, NaOH, 71%.
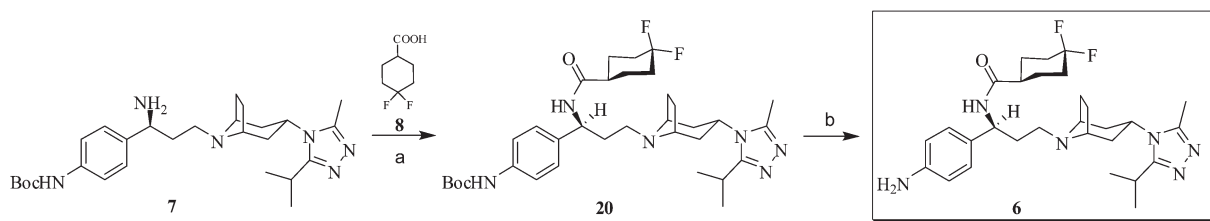
Scheme 3 Synthesis of 4-NH_2-Maraviroc (**6**). Regents and conditions: (a) EDCI, HOBt, TEA, **8**, 4Å MS, DCM, 0 °C to rt, 85%; (b) CF_3COOH, DCM, 0 °C to rt, 95%.

with the calcium flux inhibition IC_{50} value of Maraviroc under the same experimental conditions, which was 1.57 ± 0.32 nM, apparently the introduction of the long chain spacer seemed to be influential to the binding affinity of compound **1** to the receptor CCR5, as indicted by its significant decrement of functional activity of calcium flux inhibition. This was further supported by the even lower functional property of the control compound **3**, of which the calcium flux inhibition IC_{50} value was 833 ± 150 nM. A couple of reasons could lead to such results. First, the bulkiness of the spacer might influence the binding affinity to the receptor directly. Second, the substitution position of the spacer on the tailing aromatic ring system of Maraviroc might not be the most suitable one in preventing serious steric hindrance effect on the binding affinity. Currently the syntheses of new ligands with spacers attached at different positions of this tailing ring system are underway.

Similarly, in ^{35}S-GTP[γS] binding assays in MOR–CHO cells,[52] compound **1** showed very little apparent agonism ($E_{max} = 11.7 \pm 1.2\%$) compared to the full agonist DAMGO ($100 \pm 9.2\%$, $EC_{50} = 13.7 \pm 1.6$ nM), while its binding affinity to the mu opioid receptor as indicated by K_i value was 51.8 ±

7.9 nM, which was lower than naltrexone's binding affinity (K_i value was 0.71 ± 0.08 nM) under the same experimental condition. Correspondingly the control compound **2** also showed somewhat lower binding affinity as indicated by the K_i value of 9.18 ± 3.44 nM.

These preliminary biological activity results supported our original molecular design that the bivalent ligand did reserve the original antagonist properties from both pharmacophores while its relatively lower affinity to the each corresponding receptor compared to the parent pharmacophores, which was not unusual based on previous reports from others,[34c,37e] certainly requires more extensive structural modification and further syntheses effort.

Conclusions

In conclusion, a bivalent ligand with 21-atom spacer was designed and synthesized as a molecular probe to study the biological and pharmacological mechanisms of the putative hetero-dimerization between the mu opioid receptor and the chemokine
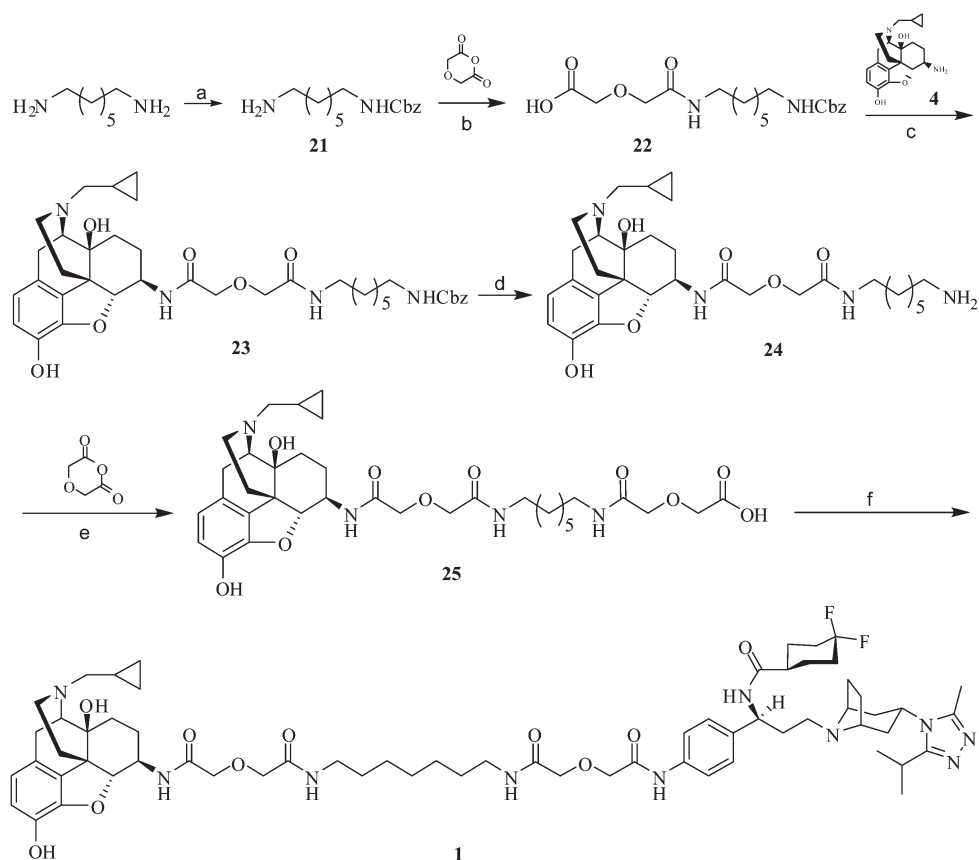
Scheme 4 Synthesis of bivalent ligand **1**. Regents and conditions: (a) CbzCl, DCM, MeOH, 5 °C, 32%; (b) THF, diglycolic anhydride, rt, 85%; (c) EDCI, HOBt, TEA, **4**·2HCl, 4Å MS, DMF, 0 °C to rt, 76%; (d) 10% Pd–C, 60 psi, MeOH, 99%; (e) DMF, diglycolic anhydride, rt, 82%; (f) EDCI, HOBt, TEA, **6**, 4Å MS, DMF, 0 °C to rt, 50%.
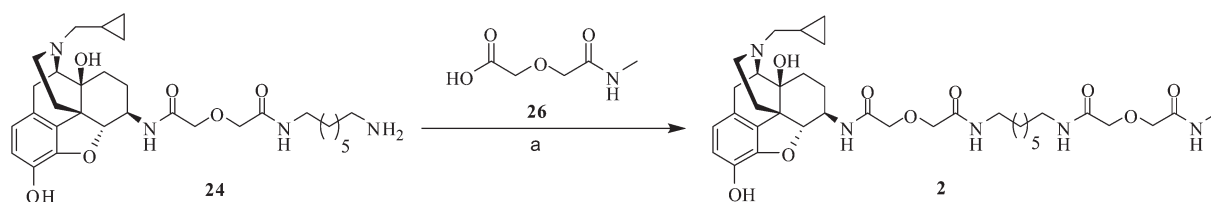
Scheme 5 Synthesis of monovalent ligand **2**. Regents and conditions: (a) EDCI, HOBt, TEA, **26**, 4Å MS, DMF, 0 °C to rt, 65%.
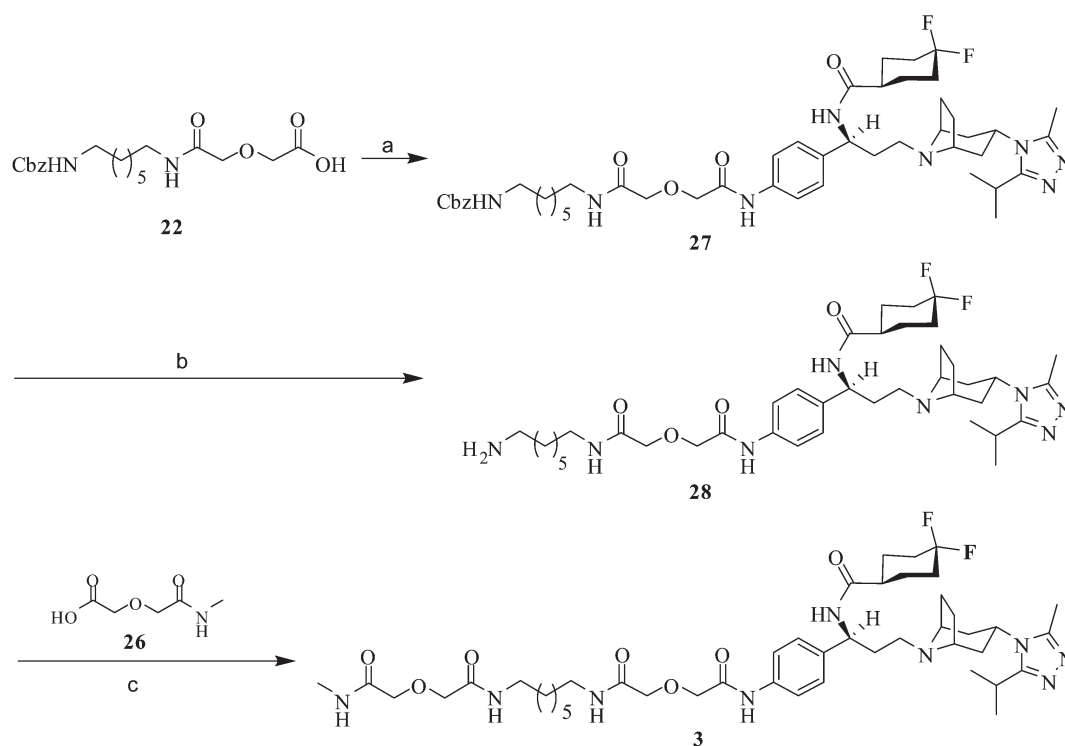
receptor CCR5. The overall 16-step synthetic route was efficient and convergent with reasonable yields. The preliminary biological data from the calcium mobilization assay and MOR–CHO binding assay showed that the bivalent ligand **1** retained the characteristics of its pharmacophores, antagonizing MOR and/or CCR5 respectively, with relatively lower binding affinity. Further characterization of these ligands and synthesis of ligands with different length of spacer and linkage at different position are undergoing right now. Based on the current pilot study, it is believed that such a bivalent ligand with a favorable length of spacer and an optimized linkage site may serve as a pharmacological probe to study the function of the putative MOR–CCR5 dimerization and help to understand the mechanism of such protein-protein interactions in various neuronal-immuno diseases, for example, HIV-infected opiate/alcohol abuse and addiction.

Experimental

Synthesis

General methods. All reagents were purchased from Sigma-Aldrich or as otherwise stated. TLC analyses were carried out on Analtech Uniplate F254 plates. Chromatographic purification was accomplished on silica gel columns (230–400 mesh, Merck). Melting points were obtained with a Fisher scientific micro melting point apparatus without further correction. All IR spectra were recorded on a Nicolet iS10 FT-IR Instrument.

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Scheme 6 Synthesis of monovalent ligand **3**. Regents and conditions: (a) EDCI, HOBt, TEA, **6**, 4Å MS, DMF, 0 °C to rt, 72%; (b) 5% Pd–C, 60 psi, MeOH, 51%; (c) EDCI, HOBt, TEA, **26**, 4Å MS, DMF, 0 °C to rt, 81%.

Proton (400 MHz) and Carbon-13 (100 MHz) nuclear magnetic resonance (NMR) spectra were acquired at ambient temperature with tetramethylsilane as the internal standard on a Bruker Ultrashield 400 Plus spectrometer. MS analysis was performed on an Applied Bio Systems 3200 Q trap with a turbo V source for TurbolonSpray. HPLC analysis of the final compounds was achieved on Varian ProStar 210 system on Microsorb-MV 100-5 C18 column (250 mm × 4.6 mm) at 254 (**1** and **3**) or 210 (**2**) nm eluting with acetonitrile (0.1% TFA)–water (50 : 50) at 1 mL min^{-1} over 10 min. Elemental analysis of the final compounds was conducted in Atlantic Microlab, Inc.

General procedure for amide coupling. In an ice-water bath EDCI (1.5 eq.), HOBt (1.5 eq.), molecular sieves, and TEA (4.0 eq.) with N_2 protection were added to a solution of acid in either DCM or DMF (3 mL). After 15 min, a solution of amine (1.0 eq.) in DMF or DCM (1 mL) was added dropwise. The resulted mixture was allowed to warm up to ambient temperature gradually. After completion of the reaction as monitored by TLC, the reaction mixture was filtered through celite. When DMF was used as the reaction solvent, the filtrate was concentrated in vacuum to remove DMF and the residue was then purified with column chromatography to afford the coupling product, whereas when DCM was the solvent, the filtrate was washed with brine, dried over Na_2SO_4, concentrated and the crude product was purified by either crystallization or column chromatography.

Bivalent ligand 1. The title compound was prepared according to the general amide coupling procedure by reacting acid **25** with amine **6** in DMF for 7 days. The crude product was purified by

column chromatography using CH_2Cl_2–MeOH (10 : 1) as eluent to give 87 mg white solid, in 50% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.60 (d, *J* = 8.56 Hz, 2H), 7.33 (d, *J* = 8.56 Hz, 2H), 6.67 (d, *J* = 8.08 Hz, 1H), 6.62 (d, *J* = 8.20 Hz, 1H), 5.03 (t, *J* = 7.38 Hz, 1H), 4.58 (d, *J* = 7.64 Hz, 1H), 4.39 (m, 1H), 4.20 (s, 2H), 4.12 (s, 2H), 4.05 (s, 2H), 4.04 (s, 2H), 3.74 (m, 1H), 3.43 (m, 2H), 3.28–3.23 (m, 5H), 2.90–2.74 (m, 3H), 2.50 (s, 3H), 2.48–2.41 (m, 2H), 2.41–2.32 (m, 2H), 2.30–2.20 (m, 2H), 2.19–2.05 (m, 4H), 2.04–1.69 (m, 15H), 1.68–1.46 (m, 8H), 1.37 (m, 6H), 1.34 (d, *J* = 6.84 Hz, 6H), 0.94 (m, 1H), 0.64–0.58 (m, 2H), 0.29 (m, 2H); ^{13}C NMR (100 MHz, CD$_3$OD): δ 176.63, 171.70, 171.55, 171.50, 170.10, 161.47, 161.43, 143.77, 143.44, 140.34, 138.06, 137.93, 128.22, 121.89, 120.63, 120.43, 119.03, 92.56, 71.99, 71.79, 71.62, 71.58 (×2), 63.99, 60.76, 60.24, 57.25, 52.46, 52.40, 49.51, 49.30, 44.31, 43.69, 40.06, 38.17, 36.74, 36.08, 35.89, 33.88 (*J* ^{13}C–^{19}F 23 Hz), 31.20, 30.34, 30.31, 29.95, 28.03, 27.84, 27.82, 27.29, 27.19, 27.07, 27.00, 26.76, 25.17, 23.91, 22.06, 15.72, 12.45, 3.93. IR *ν* (diamond, cm^{-1}): 3275, 1652, 1532, 1128, 1107. mp 158.5–160 °C. Anal. Calcd for C$_{64}$H$_{92}$F$_2$N$_{10}$O$_{11}$: C 63.24, H 7.63, N 11.52; Found: C 63.15, H 7.57, N 11.28. MS (ESI) *m/z* found 1198.4 (M + H)$^+$, 1220.5 (M + Na)$^+$.

Monovalent ligand 2. The title compound was prepared according to the general amide coupling procedure by reacting acid **26** with amine **24** in DMF for 8 h. The crude product was purified with chromatography using CH_2Cl_2–MeOH (20 : 1) as eluent to give 32 mg white solid, in 65% yield. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 9.03 (brs, 1H), 8.22 (d, *J* = 8.44 Hz, 1H), 8.04–7.96 (m, 3H), 6.59 (d, *J* = 8.08 Hz, 1H), 6.53 (d, *J* = 8.12 Hz, 1H), 4.90 (brs, 1H), 4.60 (d, *J* = 7.80 Hz, 1H), 3.95

(s, 2H), 3.94 (s, 2H), 3.91 (m, 4H), 3.57–3.48 (m, 1H), 3.19–3.09 (m, 4H), 3.03–2.99 (m, 2H), 2.66 (d, J = 4.68 Hz, 3H), 2.63–2.58 (m, 2H), 2.40–2.29 (m, 2H), 2.32–2.09 (m, 1H), 1.99 (dt, J_1 = 3.43 Hz, J_2 = 11.92 Hz, 1H), 1.84–1.75 (m, 1H), 1.48–1.42 (m, 6H), 1.33–1.24 (m, 8H), 0.86 (m, 1H), 0.48 (m, 2H), 0.13 (m, 2H); ^{1}H NMR (400 MHz, CD$_3$OD): δ 6.67 (d, J = 8.12 Hz, 1H), 6.60 (d, J = 8.12 Hz, 1H), 4.56 (d, J = 7.56 Hz, 1H), 4.10 (s, 2H), 4.09 (s, 2H), 4.08 (s, 2H), 4.06 (s, 2H), 3.82–3.78 (m, 1H), 3.30 (q, J = 7.34 Hz, 4H), 3.18–3.13 (m, 2H), 2.82 (s, 3H), 2.73–2.64 (m, 2H), 2.48–2.40 (m, 2H), 2.32–2.25 (m, 1H), 2.18 (dt, J_1 = 3.04 Hz, J_2 = 11.81 Hz, 1H), 1.94 (m, 1H), 1.60–1.58 (m, 6H), 1.48–1.32 (m, 8H), 0.95 (m, 1H), 0.56 (m, 2H), 0.19 (m, 2H); ^{13}C NMR (100 MHz, CD$_3$OD): δ 172.10, 171.51, 171.43, 171.38, 143.71, 141.88, 132.49, 125.45, 120.06, 118.55, 92.89, 71.68, 71.57, 71.53, 71.45, 71.41, 63.72, 60.27, 52.45, 48.88, 45.38, 45.24, 40.02, 31.98, 31.22, 30.35, 29.99, 27.84, 25.87, 25.46, 23.52, 10.29, 4.45, 4.21. IR ν (diamond, cm^{-1}): 3291, 1652, 1544, 1124. mp 74–76 °C. Anal. Calad for C$_{36}$H$_{55}$N$_5$O$_{10}$: C 60.23, H 7.72, N 9.76; Found: C 60.22, H 7.74, N 9.57. MS (ESI) m/z found 701.0 (M + H)$^+$, 722.9 (M + Na)$^+$.

Monovalent ligand 3. The title compound was prepared according to the general amide coupling procedure by reacting acid **26** with amine **28** in DMF overnight. The crude product was purified with chromatography using CH$_2$Cl$_2$–MeOH (8 : 1) as eluent to give 106 mg white solid, in 81% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.60 (d, J = 8.52 Hz, 2H), 7.33 (d, J = 8.56 Hz, 2H), 5.03 (t, J = 7.32 Hz, 1H), 4.44–4.35 (m, 1H), 4.20 (s, 2H), 4.12 (s, 2H), 4.02 (m, 4H), 3.42 (m, 2H), 3.30–3.20 (m, 5H), 2.78 (s, 3H), 2.50 (s, 3H), 2.47–2.40 (m, 2H), 2.39–2.30 (m, 1H), 2.30–2.17 (m, 2H), 2.15–2.03 (m, 4H), 2.02–1.95 (m, 2H), 1.90–1.65 (m, 10H), 1.60–1.45 (m, 4H), 1.40–1.30 (m, 12H); ^{13}C NMR (100 MHz, CD$_3$OD): δ 176.61, 172.13, 171.68, 171.46, 170.08, 161.42, 152.61, 140.33, 138.08, 128.22, 121.89, 71.97, 71.77, 71.48, 71.44, 60.71, 60.22, 52.40, 44.55, 43.70, 40.07, 40.03, 36.75, 36.10, 33.86 (J [13]C–[19]F 23 Hz), 30.38, 30.35, 29.99, 27.86, 27.20 (J [13]C–[19]F 9 Hz), 27.07, 27.02, 26.98, 26.76, 25.89, 22.06, 12.44. IR ν (diamond, cm^{-1}): 3272, 1652, 1532, 1107. mp 79–81 °C. Anal. Calcd for C$_{45}$H$_{73}$F$_2$N$_9$O$_9$: C 58.61, H 7.98, N 13.67; Found: C 59.29, H 7.96, N 13.47. MS (ESI) m/z found 887.2 (M + H)$^+$, 909.3 (M + Na)$^+$.

6'β-Naltrexamine hydrochloride salt (4·2HCl). The title compound was prepared following the reported procedure[39] in 62% yield for two steps (lit.,[39] 71%). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 9.58 (s, 1H, exchangeable), 8.91 (brs, 1H, exchangeable), 8.43 (m, 3H, exchangeable), 6.80 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 8.0 Hz, 1H), 6.40 (brs, 1H), 4.68 (d, J = 7.2 Hz, 1H), 3.90 (d, J = 4.8 Hz, 1H), 3.33 (m, 2H), 3.04 (dd, J_1 = 6.0 Hz, J_2 = 18.8 Hz, 2H), 2.90–2.70 (m, 2H), 2.50–2.40 (m, 2H), 1.99 (q, J = 12.5 Hz, 1H), 1.82 (d, J = 14.4 Hz, 1H), 1.78–1.70 (m, 1H), 1.46 (d, J = 8.8 Hz, 1H), 1.32 (m, 1H), 1.06 (m, 1H), 0.67 (m, 1H), 0.59 (m, 1H), 0.51 (m, 1H), 0.41 (m, 1H).

5,15-Dioxo-3,17-dioxa-diazanonadecane-1,19-dioic acid (5). To the solution of 1,7-diaminoheptane (1.3 g, 10 mmol) in THF (4 mL) at 0 °C was added diglycolic anhydride (2.44 g, 21 mmol) in one portion. The resultant mixture was stirred at the same temperature for 15 min and allowed to warm to ambient temperature and stirred overnight. After removal of THF under

reduced pressure, the residue was crystallized by EtOAc–hexane to give 3.470 g white solid as first crop, in 96% yield. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 12.78 (brs, 2H), 7.81 (t, J = 5.70 Hz, 2H), 4.10 (s, 4H), 3.94 (s, 4H), 3.08 (q, J = 6.76 Hz, 4H), 1.41 (m, 4H), 1.25 (m, 6H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 171.47, 168.60, 70.12, 67.85, 38.07, 28.98, 28.36, 26.24. IR ν (diamond, cm^{-1}): 3306, 1699, 1646, 1548, 1247, 1151, 1136, 711. mp 64–67 °C. MS (ESI) m/z found 363.5 (M + H)$^+$.

4,4-Difluoro-cyclohexanecarboxylic acid {1-(4-amino-phenyl)-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3,2,1]oct-8-yl]-propyl}-amide (6). On ice-water bath, to the solution of **20** (165 mg, 0.262 mmol) in DCM (5 mL) was added TFA (0.5 mL) dropwise. The resultant mixture was allowed to warm to ambient temperature within 15 min and stirred at the same temperature for 1.5 h. The mixture was cooled to 0 °C, and saturated Na$_2$CO$_3$ was added. The aqueous layer was adjusted to pH = 12, and taken up with DCM (20 mL × 3). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$, evaporated and dried in vacuum to afford 131 mg white solid, which is pure enough for the next step, in 95% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.05 (d, J = 8.28 Hz, 2H), 6.62 (d, J = 8.24 Hz, 2H), 6.38 (brs, 1H), 4.98 (q, J = 7.17 Hz, 1H), 4.27 (m, 1H), 3.36 (m, 2H), 2.78 (seq, J = 6.84 Hz, 1H), 2.48 (s, 3H), 2.39 (m, 2H), 2.26–2.02 (m, 8H), 1.94–1.73 (m, 6H), 1.70–1.50 (m, 5H), 1.36 (d, J = 6.72 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 173.28, 159.23, 150.75, 145.98, 131.65, 127.67, 122.74 (J [13]C–[19]F 239 Hz), 115.34, 58.98, 58.42, 51.59, 48.16, 47.38, 42.95, 35.59, 35.50, 34.84, 32.91 (J [13]C–[19]F 24 Hz), 26.84, 26.78, 26.06 (J [13]C–[19]F 9.7 Hz), 25.93, 21.75, 13.21. mp 109–110 °C. IR ν (diamond, cm^{-1}): 3334, 3230, 1636, 1517, 1105, 1031, 962, 831. MS (ESI) m/z found 529.6 (M + H)$^+$.

(4-{1-Amino-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3,2,1]oct-8-yl]-propyl}-phenyl)-carbamic acid tert-*butyl ester (7).* On ice-water bath, a solution of **19** (764 mg, 1.538 mmol) in dry THF (10 mL) was added dropwise to a suspension of LiAlH$_4$ (292 mg, 7.692 mmol) in dry THF (10 mL). The resultant mixture was stirred at the same temperature for 15 min and then 3 h at ambient temperature. The mixture was cooled in an ice bath again, and the complex was decomposed by dropwise addition of 2.4 mL H$_2$O, 2.4 mL 4 N NaOH, and 4.8 mL H$_2$O cautiously. The resulting white suspension was continued to stir for 1 h at ambient temperature, then filtered. The filtrate cake was washed with THF (20 mL × 3), diethyl ether (20 mL × 3). The combined filtrates were concentrated under reduced pressure and the residue was purified by silica gel using DCM–MeOH (6 : 1) to give 523 mg white solid, in 71% yield. ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.39 (d, J = 8.52 Hz, 2H), 7.28 (d, J = 8.60 Hz, 2H), 4.38 (m, 1H), 3.95 (t, J = 6.90 Hz, 1H), 3.42 (m, 2H), 3.25 (seq, J = 5.83 Hz, 1H), 2.45 (s, 3H), 2.43 (m, 1H), 2.42–2.32 (m, 1H), 2.24–2.15 (m, 2H), 2.05–1.96 (m, 3H), 1.94–1.81 (m, 1H), 1.73 (m, 4H), 1.51 (s, 9H), 1.33 (d, J = 6.84 Hz, 6H). ^{13}C NMR (100 MHz, CD$_3$OD): δ 161.38, 155.32, 152.56, 140.28, 139.81, 128.02, 120.01, 80.86, 60.17, 60.07, 55.62, 49.97, 38.02, 36.37, 36.30, 28.76, 27.13, 26.98, 26.76, 22.05, 12.39. mp 103–105 °C. IR ν (diamond, cm^{-1}): 3252, 1713, 1522, 1240, 1159, 838. MS (ESI) m/z found 483.7 (M + H)$^+$.

4,4-Difluoro-cyclohexanecarboxlic acid (8). The title compound was prepared as described by Mackenzie *et al.*,[48] except that the

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ester was purified by silica gel using EtOAc–hexane (80 : 1) as eluent. The total yield is 52%. ^{1}H NMR (400 MHz, CDCl$_3$): δ 2.48 (m, 1H), 2.16–2.09 (m, 2H), 2.06–2.02 (m, 2H), 1.94–1.83 (m, 4H). ^{19}F NMR (400 MHz, CDCl$_3$): δ −94.45–95.09 (d, 1F), −99.19–99.80 (d, 1F). mp 98.5–99.5 °C (lit.,[48] 105.9 °C).

3-(3-Isopropyl-5-methyl-[1,2,4]triazol-4-yl)-exo-8-aza-bicyclo [3.2.1]octane (10). The title compound and its precursors were synthesized following the same procedure by Haycock-Lewandowski *et al.*[41a] The crude product (free base) was crystal-lized from hexane to give 1.2 g **10**, in 97% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 4.33 (seq, J = 6.01 Hz, 1H), 3.74 (m, 2H), 3.02 (seq, J = 6.86 Hz, 1H), 2.53 (s, 3H), 2.18 (dt, J_1 = 12.6 Hz, J_2 = 2.66 Hz, 2H), 1.94 (m, 2H), 1.76 (m, 4H), 1.39 (d, J = 6.88 Hz, 6H). mp 190–191 °C.

3-(4-Bromo-phenyl)-acrylic acid isopropyl ester (12). To the sol-ution of 4-bromocinnacid (1.135 g, 5 mmol) in isopropanol (50 mL) was added several drops of concentrated sulfuric acid. The mixture was heated to reflux for 48 h. After cooled down, the residue was worked up with ethyl acetate. The ethyl acetate layer was washed with sat. NaHCO$_3$ aqueous solution, dried over Na$_2$SO$_4$. After filtration and concentration, the resulting crude product was purified by silica column using hexane and ethyl acetate (from 100 : 1 to 75 : 1 then 50 : 1) as eluent to give 1.08 g white solid, in 80% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.59 (d, J = 16.0 Hz, 1H), 7.51 (d, J = 8.48 Hz, 2H), 7.38 (d, J = 8.44 Hz, 2H), 6.40 (d, J = 16.0 Hz, 1H), 5.14 (seq, J = 6.26 Hz, 1H), 1.31 (d, J = 6.24 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 166.38, 143.04, 133.63, 132.26, 129.53, 124.51, 119.70, 68.14, 22.07. mp 64–66 °C. IR ν (diamond, cm^{-1}): 1703, 1637, 1304, 1172, 1104, 981, 817. MS (ESI) *m/z* found 268.9 (M + H)$^+$, 271.1 (M + 2 + H)$^+$.

3-(4-Amino-phenyl)-acrylic acid isopropyl ester (13). The mixture of **12** (1.53 g, 5.68 mmol), Pd$_2$(dba)$_3$ (260 mg, 5% mmol), P(*t*-Bu)$_3$ (0.23 mL, 1 M in toluene, 4% mmol) in dry toluene (30 mL) was stirred under N$_2$ protection for 15 min. Then a solution of LHMDS in toluene (6.2 mL, 1 M in toluene, 6.2 mmol) was added dropwise. After stirred at ambient temp-erature overnight, the resultant dark color suspension was added 1 N hydrochloric acid (8 mL) slowly. The resulting mixture was stirred at ambient temperature for 2 h. Then the suspension was filtered through celite and the filtrate was diluted with dichloro-methane (70 mL). The organic layer was washed with saturated NaHCO$_3$ aqueous solution, brine, and dried over Na$_2$SO$_4$. After filtered and concentrated, the crude product was purified by silica gel column using hexane and ethyl acetate (2 : 1) as eluent to give 1.03 g light yellow solid, in 90% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.58 (d, J = 15.9 Hz, 1H), 7.35 (d, J = 8.48 Hz, 2H), 6.65 (d, J = 8.56 Hz, 2H), 6.22 (d, J = 15.9 Hz, 1H), 5.12 (seq, J = 6.24 Hz, 1H), 3.91 (brs, 2H), 1.30 (d, J = 6.24 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 167.20, 148.56, 144.55, 129.80, 124.88, 114.84, 114.35, 67.32, 22.00. mp 78–80 °C. IR ν (diamond, cm^{-1}): 3417, 3335, 1673, 1592, 1513, 1264, 1168, 1104, 980, 823. MS (ESI) *m/z* found 206.2 (M + H)$^+$.

3-(4-tert-Butoxycarbonylamino-phenyl)-acrylic acid isopropyl ester (14). The solution of **13** (1.351 g, 6.58 mmol) and Boc$_2$O (1.58 g, 7.24 mmol) in dry tetrahydrofuran (30 mL) was heated to reflux overnight. After cooled down and concentrated, the residue was crystallized from DCM/hexane to give 1.711 g white

solid, in 85% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.60 (d, J = 16.0 Hz, 1H), 7.46 (d, J = 8.68 Hz, 2H), 7.38 (d, J = 8.64 Hz, 2H), 6.55 (brs, 1H), 6.32 (d, J = 16.0 Hz, 1H), 5.13 (seq, J = 6.25 Hz, 1H), 1.52 (s, 9H), 1.30 (d, J = 6.24 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 166.77, 152.41, 143.81, 140.30, 129.17, 129.00, 118.33, 117.03, 80.95, 67.63, 28.29, 21.95. mp 159–159.5 °C. IR ν (diamond, cm^{-1}): 3307, 1725, 1688, 1586, 1521, 1150, 1106, 981, 830. MS (ESI) *m/z* found 306.2 (M + H)$^+$.

3-[Benzyl-(1-phenyl-ethyl)-amino-3-(4-tert-butoxycarbonylamino-phenyl)-propionic acid isopropyl ester (15). In an ice-water bath, under N$_2$ protection, n-butyllithium (3.47 mL, 2.5 M in hexane) was added dropwise to a solution of *R*-(+)-*N*-benzyl-α-methyl-benzylamine (1.88 mL, 9.02 mmol) in dry THF. The resulting purple solution was stirred for 30 min, then cooled down to −78 °C, then the solution of **14** (1.06 g, 3.47 mmol) in dry THF was added dropwise. Then the dark red solution was stirred for 2 h at −78 °C. Saturated ammonium chloride aqueous was added to quench the reaction. The resultant yellow solution was allowed to warm to ambient temperature within 30 min. After worked up with ethyl acetate, the organic layer was dried, con-centrated. The residue was crystallized with ethyl acetate to give 20 mg **15** as colorless crystal. The filtrate was then concentrated and dried on vacuum. Purification of the crude compound by silica gel column chromatography gave mixtures of the excess *R*-(+)-*N*-benzyl-α-methylbenzylamine and the product. Hence, the following procedure was performed to transfer the excess amine to the amide to facilitate the purification process. Benzyl chloride (644 μL, 5.55 mmol) was added dropwise into a mixture of the above filtrate and triethylamine (1.543 mL, 11.1 mmol) in 30 mL CH$_2$Cl$_2$ at 0 °C. After stirred for 2 h, the reaction mixture was washed with brine. The organic layer, containing the product **15** and *N*-benzyl-*N*-(1-phenyl-ethyl)-benzamide, was dried, concentrated and used for next step without further purification. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.40 (d, J = 7.2 Hz, 2H), 7.35–7.15 (m, 12H), 6.43 (brs, 1H), 4.79 (seq, J = 6.25 Hz, 1H), 4.38 (dd, J_1 = 5.2 Hz, J_2 = 9.66 Hz, 1H), 3.97 (q, J = 6.75 Hz, 1H), 3.66 (s, 2H), 2.56 (dd, J_1 = 5.12 Hz, J_2 = 14.68 Hz, 1H), 2.50 (dd, J_1 = 14.58 Hz, J_2 = 9.82 Hz, 1H), 1.51 (s, 9H), 1.25 (d, J = 6.76 Hz, 3H), 1.05 (d, J = 6.24 Hz, 3H), 1.00 (d, J = 6.20 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 171.36, 152.77, 144.15, 141.63, 137.37, 136.34, 128.75, 128.13 (×2), 128.00, 127.84, 126.83, 126.55, 118.16, 80.47, 67.51, 59.06, 57.05, 50.82, 37.71, 28.37, 21.62, 16.46. mp 172–174 °C. IR ν (diamond, cm^{-1}): 1724, 1595, 1522, 1154, 1105, 1051, 697. MS (ESI) *m/z* found 517.5 (M + H)$^+$.

3-[Benzyl-(1-phenyl-ethyl)-amino-3-(4-tert-butoxycarbonylamino-phenyl)-propionic acid (16). Lithium hydroxide (831 mg, 34.7 mmol) was added to the above mixture of crude product **15** and *N*-benzyl-*N*-(1-phenyl-ethyl)-benzamide in methanol (30 mL) and water (15 mL). The resulting suspension was heated to reflux for 48 h. After it had cooled down, the mixture was concentrated to remove methanol. The water layer was taken up with CH$_2$Cl$_2$ (30 mL × 3). The organic layer was dried and concentrated. The residue was purified by silica gel column using hexane and ethyl acetate (2 : 1) as eluent to give 1.406 g white solid, in 85% yield for two steps. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.44 (d, J = 8.52 Hz, 2H), 7.37–7.22 (m, 12H), 6.55 (brs, 1H), 4.43 (dd, J_1 = 4.46 Hz, J_2 = 11.32 Hz, 1H), 4.15

(q, J = 6.87 Hz, 1H), 3.98 (d, J = 13.68 Hz, 1H), 3.64 (d, J = 13.68 Hz, 1H), 2.92 (dd, J_1 = 11.34 Hz, J_2 = 16.94 Hz, 1H), 2.41 (dd, J_1 = 4.48 Hz, J_2 = 16.96 Hz, 1H), 1.54 (s, 9H), 1.28 (d, J = 6.88 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 174.39, 152.82, 141.25, 138.44, 137.70, 132.56, 129.22, 128.80, 128.62, 128.60, 128.19, 127.81, 127.53, 118.52, 80.86, 57.96, 57.82, 50.61, 36.29, 28.34, 15.68. mp 93–95 °C. IR ν (diamond, cm^{-1}): 3307, 1699, 1594, 1081, 698. MS (ESI) m/z found 475.6 (M + H)$^+$.

(4-{1-[Benzyl-(1-phenyl-ethyl)-amino]-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3,2,1]oct-8-yl]-3-oxo-propyl}-phenyl)-carbamic acid tert-*butyl ester (17).* The title compound was prepared according to the general amide coupling procedure by reacting acid **16** with amine **10** in DCM for 4 h. The crude product was crystallized using DCM–hexane to give 986 mg white solid as first crop, in 73% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.88 (d, J = 8.32 Hz, 1H), 7.68 (d, J = 8.28 Hz, 1H), 7.46 (d, J = 7.60 Hz, 2H), 7.41–7.35 (m, 10H), 7.33–7.12 (m, 14H), 6.91 (brs, 1H), 6.66 (brs, 1H), 4.68 (m, 1H), 4.62 (m, 1H), 4.53 (dd, J_1 = 5.54 Hz, J_2 = 8.10 Hz, 1H), 4.40–4.28 (m, 3H), 4.03–3.96 (m, 2H), 3.82–3.62 (m, 6H), 2.76 (m, 2H), 2.54 (m, 4H), 2.21 (s, 3H), 2.11 (s, 3H), 2.06–1.96 (m, 4H), 1.96–1.55 (m, 12H), 1.51 (s, 9H), 1.50 (s, 9H), 1.30 (m, 12H), 1.28–1.24 (m, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 167.31, 166.99, 158.87, 152.80, 150.35, 144.43, 142.17 (142.05), 137.78, 136.61, 136.32, 80.46, 61.06, 59.34, 56.53 (56.40), 53.82 (53.43), 51.11 (50.80), 50.56 (50.49), 46.67, 38.87 (38.27), 37.60 (37.46), 35.67, 28.34, 26.86 (26.61), 25.79, 21.63 (21.56), 14.59, 13.80, 12.99. mp 128–130 °C. IR ν (diamond, cm^{-1}): 2966, 1721, 1637, 1545, 1436, 1242, 1165, 742, 705. MS (ESI) m/z found 691.5 (M + H)$^+$.

(4-{1-Amino-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3,2,1]oct-8-yl]-3-oxyl}-phenyl)-carbamic acid tert-*butyl ester (19).* A solution of **17** (500 mg, 0.725 mmol) in methanol (35 mL) was treated with palladium carbon (100 mg, 10 wt%), and the resultant slurry was shaken under an atmosphere of hydrogen at 60 psi for 4 days at ambient temperature. The reaction mixture was filtered through celite. The filtrate cake was washed with methanol and the combined filtrates were concentrated and purified by silica gel using DCM–MeOH (20 : 1) as eluent to give 304 mg white solid **19**, in 84% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.34 (d, J = 5.88 Hz, 8H), 6.46 (brs, 2H), 4.88 (m, 2H), 4.57–4.47 (m, 4H), 4.34–4.23 (m, 2H), 2.93 (seq, J = 7.03 Hz, 2H), 2.73–2.50 (m, 4H), 2.46 (s, 3H), 2.36 (s, 3H), 2.33–1.95 (m, 8H), 1.85–1.71 (m, 8H), 1.52 (s, 18H), 1.40–1.37 (m, 12H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 167.71 (167.41), 158.99 (158.93), 152.86, 150.51 (150.38), 139.43, 137.83 (137.73), 126.95 (126.86), 118.94 (118.88), 80.56, 53.89 (53.71), 52.36 (52.07), 50.85 (50.80), 46.85 (46.76), 43.77 (43.58), 37.67, 35.95 (35.88), 28.64 (28.56), 28.36, 26.97 (26.92), 25.92, 21.73, 21.66 (21.63), 13.13 (13.08). mp 121–122.5 °C. IR ν (diamond, cm^{-1}): 3273, 1713, 1609, 1521, 1413, 1158, 1028, 837. MS (ESI) m/z found 497.3 (M + H)$^+$.

(4-{1-[(4,4-Difluoro-cyclohexancarbonyl)-amino-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3,2,1]oct-8-yl]-propyl}-phenyl)-carbamic acid tert-*butyl ester (20).* The title compound was prepared according to the general amide coupling procedure by reacting acid **8** with amine **7** in DCM for 4 h. The crude product was purified by silica gel using DCM–MeOH (18 : 1) to

give 269 mg white solid, in 85% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.34 (d, J = 8.40 Hz, 2H), 7.11 (d, J = 8.48 Hz, 2H), 6.67 (brs, 1H), 6.54 (d, J = 7.56 Hz, 1H), 5.07 (q, J = 7.01 Hz, 1H), 4.29 (m, 1H), 3.37 (m, 2H), 2.98 (seq, J = 6.48 Hz, 1H), 2.49 (s, 3H), 2.40 (t, J = 6.62 Hz, 2H), 2.26–2.13 (m, 5H), 2.06–1.94 (m, 6H), 1.93–1.63 (m, 8H), 1.51 (s, 9H), 1.38 (d, J = 6.76 Hz, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 173.24, 159.12, 152.82, 150.56, 137.75, 136.34, 127.11, 122.51 (J ^{13}C–^{19}F 250 Hz), 118.92, 80.63, 58.87, 58.18, 51.61, 47.78, 47.28, 42.86, 35.43, 35.29, 34.66, 32.79 (J ^{13}C–^{19}F 25.5 Hz), 28.32, 26.80, 26.76, 25.94 (J ^{13}C–^{19}F 8 Hz), 25.85, 21.64, 13.11. mp 234–235 °C. IR ν (diamond, cm^{-1}): 3272, 1716, 1650, 1236, 1159, 1106, 963, 836. MS (ESI) m/z found 629.6 (M + H)$^+$.

(7-Amino-heptyl)-carbamic acid benzyl ester (21). On an ice-water bath, to the solution of 1,7-diaminoheptane (1.433 g, 11 mmol) in CH$_2$Cl$_2$–MeOH (125 mL : 125 mL) was added the solution of CbzCl (1.71 g, 10 mmol) in CH$_2$Cl$_2$ (250 mL) drop-wise within 12 h while keeping the temperature below 5 °C. The mixture was allowed to stir at the same temperature for another half of an hour before concentrated under reduced pressure to remove most of the MeOH. Water (150 mL) was then added, and the aqueous layer was adjusted to pH = 2 using 6 N HCl. The layers were separated. The aqueous layer was washed with DCM (50 mL × 3), then adjusted to pH = 12 with 10 N NaOH and extracted with DCM (50 mL × 3). The combined organic layers were dried over Na$_2$SO$_4$, concentrated and purified by flash column using DCM–MeOH (9 : 1) to give 856 mg white solid in 32% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.35–7.29 (m, 5H), 5.09 (s, 2H), 4.73 (brs, 1H), 3.18 (q, J = 6.64 Hz, 2H), 2.67 (t, J = 6.94 Hz, 2H), 1.55–1.44 (m, 2H), 1.44–1.40 (m, 2H), 1.32 (m, 6H). ^{13}C NMR (100 MHz, CDCl$_3$): δ 156.40, 136.70, 128.50, 128.09, 128.06, 66.55, 42.18, 41.06, 33.71, 29.91, 29.09, 26.76, 26.68. mp 78–80 °C. IR ν (diamond, cm^{-1}): 3327, 1686, 1532, 1263, 1144. MS (ESI) m/z found 264.8 (M + H)$^+$.

[(7-Benzyloxycarbonylamino-heptylcarbamoyl)-methoxy]-acetic acid (22). To the solution of **21** (350 mg, 1.324 mmol) in THF (4 mL) was added diglycolic anhydride (161 mg, 1.39 mmol) in one portion. The resultant mixture was stirred at ambient temperature for 12 h. After removed THF under reduced pressure, the residue was crystallized by EtOAc–hexane to give 429 mg white solid as first crop, in 85% yield. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 12.79 (brs, 1H), 7.81 (t, J = 5.52 Hz, 1H), 7.34–7.29 (m, 5H), 7.21 (t, J = 5.46 Hz, 1H), 5.00 (s, 2H), 4.10 (s, 2H), 3.94 (s, 2H), 3.08 (q, J = 6.56 Hz, 2H), 2.97 (q, J = 6.28 Hz, 2H), 1.42–1.37 (m, 4H), 1.24 (m, 6H). ^{13}C NMR (100 MHz, CDCl$_3$): δ 171.43, 168.53, 156.06, 137.30, 128.27, 127.65, 70.18, 67.88, 65.04, 40.22, 38.08, 29.65, 29.00, 28.38, 26.28, 26.14. mp 74–74.5 °C. IR ν (diamond, cm^{-1}): 3374, 3331, 1726, 1688, 1608, 1548, 1249, 1236, 1135, 956, 701. MS (ESI) m/z found 381.4 (M + H)$^+$.

6'β-(3,13-Dioxo-1-phenyl-2,15-dioxa-4,12-diazaheptadecanamido) morphinan (23). The title compound was prepared according to the general amide coupling procedure by reacting acid **22** with amine **4**·2HCl in DMF overnight. The crude product was purified with chromatography using CH$_2$Cl$_2$–MeOH (40 : 1) as eluent to give 339 mg white solid, in 76% yield. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 9.01 (brs, 1H), 8.21 (d, J = 8.36 Hz, 1H), 8.01 (t, J = 5.60 Hz, 1H), 7.37–7.30 (m, 5H), 7.19 (m, 1H), 6.58 (d, J = 8.04 Hz, 1H), 6.52 (d, J = 8.04 Hz, 1H), 4.99

(s, 2H), 4.88 (brs, 1H), 4.59 (d, J = 7.64 Hz, 1H), 3.94 (s, 2H), 3.93 (s, 2H), 3.53–3.45 (m, 1H), 3.18–3.07 (m, 2H), 3.01–2.94 (m, 4H), 2.60–2.56 (m, 2H), 2.38–2.28 (m, 2H), 2.15 (dt, J_1 = 4.89 Hz, J_2 = 12.29 Hz, 1H), 1.98 (m, 1H), 1.79 (m, 1H), 1.46–1.37 (m, 6H), 1.26 (m, 8H), 0.84 (m, 1H), 0.46 (m, 2H), 0.12 (m, 2H); ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.57 (d, J = 9.24 Hz, 1H), 7.35–7.32 (m, 5H), 6.90 (t, J = 5.66 Hz, 1H), 6.72 (d, J = 8.12 Hz, 1H), 6.55 (d, J = 8.16 Hz, 1H), 5.09 (s, 2H), 4.88 (m, 1H), 4.44 (d, J = 5.48 Hz, 1H), 4.02 (m, 4H), 3.24 (m, 2H), 3.18 (AB, J = 6.64 Hz, 2H), 3.10 (d, J = 5.84 Hz, 1H), 3.03 (d, J = 18.48 Hz, 1H), 2.62 (m, 2H), 2.36 (m, 2H), 2.19 (m, 2H), 1.79 (m, 1H), 1.64 (m, 1H), 1.54–1.48 (m, 7H), 1.32–1.26 (m, 7H), 0.80 (m, 1H), 0.53 (m, 2H), 0.13 (m, 2H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 168.63, 168.45, 156.51, 143.19, 139.38, 136.64, 130.56, 128.53 (×2), 128.10, 124.66, 119.23, 117.77, 92.27, 77.23, 70.89, 70.13, 66.63, 62.38, 59.40, 49.41, 47.20, 43.88, 41.02, 39.04, 31.87, 29.85, 29.50, 28.92, 28.81, 26.78, 26.55, 23.17, 22.61, 9.41, 4.00, 3.81. mp >300 °C. IR ν (diamond, cm^{-1}): 3670, 1700, 1560, 1136. MS (ESI) m/z found 705.5 (M + H)$^+$.

6'β-{2-[2-(7-aminoheptylamino)-2-oxoethoxy]acetamido}morphinan (24). A solution of **23** (120 mg, 0.167 mmol) in methanol (20 mL) was hydrogenated in the presence of 10% Pd–C (12 mg) under a H$_2$ atmosphere (60 psi) at room temperature for 48 h. The mixture was filtered, and the filtrate was concentrated and purified by silica gel with DCM–MeOH (7 : 1) to give **24** as white foam (110 mg, 99% yield). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 8.22 (d, J = 8.40 Hz, 1H), 8.03 (t, J = 5.74 Hz, 1H), 6.58 (d, J = 8.04 Hz, 1H), 6.52 (d, J = 8.12 Hz, 1H), 4.59 (d, J = 7.36 Hz, 1H), 3.94 (s, 2H), 3.93 (s, 2H), 3.56–3.48 (m, 1H), 3.19–3.09 (m, 2H), 3.10 (d, J = 5.52 Hz, 1H), 2.97 (d, J = 18.8 Hz, 1H), 2.70 (m, 2H), 2.61–2.55 (m, 2H), 2.39–2.28 (m, 2H), 2.18–2.10 (m, 1H), 1.98 (dt, J_1 = 3.56 Hz, J_2 = 11.92 Hz, 1H), 1.84–1.74 (m, 1H), 1.47–1.44 (m, 6H), 1.32–1.23 (m, 8H), 0.86 (m, 1H), 0.47 (m, 2H), 0.11 (m, 2H); ^{1}H NMR (400 MHz, CD$_3$OD): δ 6.62 (d, J = 8.08 Hz, 1H), 6.55 (d, J = 8.12 Hz, 1H), 4.51 (d, J = 7.56 Hz, 1H), 4.06 (m, 2H), 4.05 (m, 2H), 3.76 (m, 1H), 3.26 (t, J = 7.08 Hz, 2H), 3.10 (d, J = 5.96 Hz, 1H), 3.07 (d, J = 20.64 Hz, 1H), 2.66 (m, 2H), 2.58 (m, 2H), 2.40 (m, 2H), 2.27–2.11 (m, 2H), 1.90 (m, 1H), 1.60–1.51 (m, 6H), 1.49–1.32 (m, 8H), 0.85 (m, 1H), 0.54 (m, 2H), 0.16 (m, 2H); ^{13}C NMR (100 MHz, CD$_3$OD): δ 171.54, 171.38, 143.90, 142.52, 132.41, 125.06, 120.09, 118.88, 92.80, 71.74, 71.60, 71.55, 63.77, 60.32, 52.54, 48.93, 47.95, 45.28, 40.07, 32.05, 31.27, 30.43, 30.28, 28.38, 27.92, 25.51, 23.55, 22.12, 10.36, 4.49, 4.27. mp 83–85 °C. IR ν (diamond, cm^{-1}): 3278, 3075, 1652, 1548, 1128, 1035. MS (ESI) m/z found 571.6 (M + H)$^+$.

19-(6'β-morphinanamino)-5,15-19-trixox-3,17-dioxa-6,14-diaza-nonadecan-1-oic acid (25). Diglycolic anhydride (23 mg, 0.198 mmol) was added to the solution of **24** (113 mg, 0.198 mmol) in DMF (2 mL) within 15 min. The resultant mixture was stirred at ambient temperature for 2 h. After removal of DMF under reduced pressure, the residue was crystallized by EtOAc/hexane to give 112 mg light yellow solid as first crop, in 82% yield. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 9.22 (brs, 1H), 8.28 (d, J = 8.32 Hz, 1H), 8.06 (t, J = 5.62 Hz, 1H), 7.99 (m, 1H), 6.65 (d, J = 8.12 Hz, 1H), 6.59 (d, J = 8.12 Hz, 1H), 4.68 (d, J = 7.80 Hz, 1H), 4.06 (s, 2H), 3.95 (s, 2H), 3.94 (m, 4H), 3.56–3.47 (m, 1H), 3.24–3.06 (m, 6H), 2.89–2.83

(m, 2H), 2.65 (m, 1H), 2.33–2.28 (m, 2H), 1.87–1.78 (m, 1H), 1.59 (m, 1H), 1.49–1.40 (m, 6H), 1.27 (m, 8H), 0.98 (m, 1H), 0.58–0.52 (m, 2H), 0.30 (m, 2H); ^{1}H NMR (400 MHz, CD$_3$OD): δ 6.72 (d, J = 8.20 Hz, 1H), 6.70 (d, J = 8.40 Hz, 1H), 4.65 (d, J = 7.80 Hz, 1H), 4.07 (s, 2H), 4.06 (s, 2H), 3.98 (s, 2H), 3.93 (s, 2H), 3.77–3.71 (m, 2H), 3.31–3.22 (m, 4H), 3.14–2.99 (m, 3H), 2.94–2.89 (m, 1H), 2.78 (dd, J_1 = 7.34 Hz, J_2 = 13.30 Hz, 1H), 2.59–2.46 (m, 2H), 2.03–1.92 (m, 1H), 1.73–1.61 (m, 2H), 1.59–1.50 (m, 6H), 1.37 (m, 6H), 1.06 (m, 1H), 0.77–0.63 (m, 2H), 0.42 (m, 2H); ^{13}C NMR (100 MHz, CD$_3$OD): δ 177.25, 172.58, 171.61, 171.56, 143.81, 142.81, 131.26, 128.73, 120.70, 119.42, 92.23, 72.25, 71.67, 71.58, 71.47, 71.39, 64.21, 59.23, 52.37, 49.30, 47.97, 40.10, 39.96, 31.20, 30.26, 30.21, 29.95, 29.76, 27.81, 27.79, 24.91, 24.24, 7.78, 5.67, 3.68. mp 193 °C dec. IR ν (diamond, cm^{-1}): 3271, 3069, 1732, 1651, 1548, 1125, 1033. MS (ESI) m/z found 687.4 (M + H)$^+$.

Methylcarbamoylmethoxy-acetic acid (26). The title compound was prepared using the same procedure as that described by Zheng *et al.*,[30b] except that white solid instead of oil was obtained. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 12.77 (brs, 1H), 7.77 (brs, 1H), 4.10 (s, 2H), 3.94 (s, 2H), 2.62 (d, J = 4.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d$_6$): δ 171.32, 169.08, 70.09, 67.77, 25.11. mp 33–33.5 °C.

(7-{2-[[4-{1-[(4,4-Difluoro-cyclohexanecarbonyl)-amino]-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3.2.1]oct-8yl]-propyl}-phenylcarbamoyl)-methoxy]-acetylamino}-heptyl)-carbamic acid benzyl ester (27). The title compound was prepared according to the general amide coupling procedure by reacting acid **22** with amine **6** in DMF overnight. The crude product was purified with chromatography using CH$_2$Cl$_2$–MeOH (13 : 1) as eluent to give 260 mg white foam, in 72% yield. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.96 (s, 1H), 7.57 (d, J = 8.48 Hz, 2H), 7.35–7.27 (m, 5H), 7.24 (d, J = 8.48 Hz, 2H), 6.75 (m, 1H), 6.70 (m, 1H), 5.12–5.08 (m, 3H), 4.91 (m, 1H), 4.29 (m, 1H), 4.14 (s, 2H), 4.09 (s, 2H), 3.40 (m, 1H), 3.37 (m, 1H), 3.30 (q, J = 6.68 Hz, 2H), 3.15 (q, J = 6.71 Hz, 2H), 2.99 (m, 1H), 2.48 (s, 3H), 2.43 (t, J = 6.48 Hz, 2H), 2.30–2.04 (m, 6H), 2.01–1.71 (m, 8H), 1.70–1.46 (m, 9H), 1.376 (d, J = 6.80 Hz, 3H), 1.373 (d, J = 6.84 Hz, 3H), 1.35–1.31 (m, 6H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 173.50, 168.64, 167.15, 159.17, 156.55, 150.59, 138.31, 136.66, 136.51, 128.54, 128.12, 128.00, 127.12, 120.51, 77.24, 71.66, 71.50, 66.60, 58.90, 58.30, 51.68, 47.77, 47.24, 42.83, 40.98, 39.14, 35.33, 34.59, 32.82 (J ^{13}C–^{19}F 25.7 Hz), 29.82, 29.36, 28.71, 26.75 (J ^{13}C–^{19}F 10 Hz), 26.48, 25.87, 21.65, 13.15. mp 65–67 °C. IR ν (diamond, cm^{-1}): 3273, 1656, 1529, 1515, 1251, 1106, 697. MS (ESI) m/z found 891.9 (M + H)$^+$.

4,4-Difluoro-cyclohexanecarboxylic acid {1-(4-{2-[(7-amino-heptylcarbamoyl)-meth-oxy]-acetylamino}-phenyl)-3-[3-(3-isopropyl-5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3.2.1]oct-8-yl]-propyl}-amide (28). A solution of **27** (260 mg, 0.291 mmol) in methanol (15 mL) was hydrogenated in the presence of 5% Pd–C (26 mg) under a H$_2$ atmosphere (60 psi) at room temperature for 48 h. The mixture was filtered, and the filtrate was concentrated to give **28** as white foaming (113 mg, 51% yield). ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.80 (s, 1H), 7.58 (d, J = 8.48 Hz, 2H), 7.25 (d, J = 8.48 Hz, 2H), 6.61 (d, J = 7.48 Hz, 1H), 6.47 (m, 1H), 5.10 (q, J = 7.00 Hz, 1H), 4.30 (m, 1H), 4.17 (s, 2H), 4.12 (s, 2H), 3.40–3.30 (m, 4H), 2.98 (seq, J = 6.90 Hz, 1H),

2.68 (t, J = 6.84 Hz, 2H), 2.48 (s, 3H), 2.43 (t, J = 6.62 Hz, 2H), 2.26–2.12 (m, 5H), 2.08–1.53 (m, 16H), 1.44 (qu, J = 6.72 Hz, 2H), 1.383 (d, J = 6.76 Hz, 3H), 1.382 (d, J = 6.80 Hz, 3H); 1.34–1.30 (m, 6H); ^{1}H NMR (400 MHz, CD$_3$OD): δ 7.60 (d, J = 8.52 Hz, 2H), 7.33 (d, J = 8.56 Hz, 2H), 5.03 (t, J = 7.34 Hz, 1H), 4.39 (m, 1H), 4.20 (s, 2H), 4.12 (s, 2H), 3.41 (m, 2H), 3.30–3.21 (m, 3H), 2.69 (t, J = 7.32 Hz, 2H), 2.50 (s, 3H), 2.46–2.41 (m, 2H), 2.40–2.30 (m, 1H), 2.30–2.05 (m, 2H), 2.05–2.03 (m, 4H), 2.02–1.96 (m, 2H), 1.89–1.65 (m, 10H), 1.60–1.45 (m, 4H), 1.36–1.34 (m, 12H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 176.59, 171.68, 170.07, 161.41, 152.60, 140.39, 138.07, 128.21, 123.91 (J ^{13}C–^{19}F 239 Hz), 121.88, 71.95, 71.75, 60.69, 60.19, 52.41, 43.69, 42.03, 40.07, 36.77, 36.16, 33.90 (J ^{13}C–^{19}F 24 Hz), 33.87 (J ^{13}C–^{19}F 24 Hz), 32.28, 30.38, 30.11, 27.86, 27.78, 27.20(J ^{13}C–^{19}F 9 Hz), 27.05(J ^{13}C–^{19}F 9 Hz), 26.76, 22.07, 12.45. mp 100–102 °C. IR ν (diamond, cm^{-1}): 3256, 1651, 1538, 1515, 1261, 1103, 743. MS (ESI) m/z found 757.9 (M + H)$^+$.

Calcium mobilization assay. The ligands were first tested with various doses (in the range of 0.1 nM to 1 µM) for possible agonist activity. The protocol was the same for the following antagonism study, except no CCL5 (RANTES) was added.

MOLT-4–CCR5 cells were plated in black 96-well plates with transparent bottom (Greiner Bio-one) at 100,000 cells per well in 50 : 1 HBSS : HEPES assay buffer. They were incubated for 1 h at 37 °C and 5% CO$_2$ with control buffer or varying concentration of ligand for a total volume of 130 µL per well. Cells were then incubated with 50 µL of Fluo-4-AM loading buffer (40 µL 2 µM Fluo-4 dye, 100 µL 2.5 mM probenacid, in 5 mL assay buffer) for an additional hour. Then 20 µL 200 nM RANTES solution in assay buffer or assay buffer alone were added to the wells right before changes in Ca^{2+} concentration were monitored by RFU for 90 seconds using a microplate reader (FlexStation3, Molecular Devices). Peak values were obtained using SoftMaxPro software (Molecular Devices) and non-linear regression curves were generated using Prism (GraphPad) to calculate IC$_{50}$ values.

The mu opioid receptor binding and functional assay. MOR–CHO cell culture and membrane homogenate preparation followed the literature report.[52]

Opioid Receptor Binding. Saturation binding was performed by incubating membranes for 90 min at 30 °C with 0.5–15 nM [^{3}H] naloxone in assay buffer in a 0.5 mL volume. Non-specific binding was determined with 5 µM naltrexone. For competition assays, membranes were incubated as above with 2 nM [^{3}H] naloxone and various concentrations of unlabeled ligand, to determine competitor IC$_{50}$ for MOR. The reaction was terminated by rapid filtration through Whatman GF–B glass fiber filters, followed by 3 washes with 3 mL ice-cold Tris buffer. Bound radioactivity will be determined by liquid scintillation spectrophotometry at 45% efficiency for [^{3}H].

[^{35}S]GTPγS Binding. Membranes (10 µg protein) were incubated in assay buffer at 30 °C for 90 min with various drugs, 10 µM GDP (cells) and 0.1 nM [^{35}S]GTPγS in 0.5 mL total volume for appropriate times. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured with 10 µM unlabeled GTPγS. The reaction was terminated by rapid filtration as described above. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency.

Data analysis. For competition binding assay (agonists or antagonists), Hill plots linear regression analysis and the Cheng–Prusoff equation were applied to determine the IC$_{50}$ and K_i values. In [^{35}S]GTPγS binding assays, agonist concentration effect curves were fit by non-linear regression to obtain E_{max} and EC$_{50}$ values; antagonist inhibition of agonist-stimulated [^{35}S] GTPγS binding was analyzed by Hill analysis and AD$_{50}$ values were corrected to K_i values using the Cheng-Prusoff equation. All analyses were using Prism 4.0.

Acknowledgements

Notes and references

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