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ORIGINAL ARTICLE

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Exploration of the fifth position of leu-enkephalin and its role in binding and activating delta (DOP) and mu (MOP) opioid receptors

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Abstract

Enkephalins are pentapeptidic endogenous ligands that regulate nociception by binding to mu (MOP) and delta (DOP) opioid receptors. To further explore the role of the leucine residue of Leu-enkephalin, 12 peptidomimetic analogs were synthesized by systematically replacing this residue with non-natural amino acids. The analogs were tested for their ability to bind DOP and MOP. We also investigated the potency of these analogs to inhibit cAMP production and to recruit β-arrestin 2 via both receptors. We found that replacement of the leucine residue by substituted non-natural amino acid derivatives of alanine, cycloleucine, or isoleucine was generally well tolerated. By contrast, substituting leucine with homoproline greatly reduced the affinity for DOP and, to a lesser extent, for MOP. Interestingly, when compared to Leu-enkephalin, analogs containing either aza-β-homoleucine or cycloleucine showed a bias toward inhibition of cAMP production through the activation of DOP but not MOP. By contrast, derivatives containing 4,5-dehydroleucine or D-allo-isoleucine conferred a bias toward β-arrestin 2 at MOP, but not DOP. Our results suggest that position 5 in Leu-enkephalin analogs can be further exploited to develop compounds with the potential to produce bias toward G protein or β-arrestin 2.

KEYWORDS
biased signaling, enkephalins, opioid receptors, peptidomimetics, solid-phase synthesis

1 | INTRODUCTION

Opioids are among the most effective drugs used for the treatment of chronic pain.[1,2] They bind and activate three G protein-coupled receptors (GPCRs) named mu (MOP), delta (DOP), and kappa (KOP) opioid receptors.[3] Commonly used opioids mainly produce analgesia through the activation of MOP.[3,4] However, morphine, the golden standard in pain management, produces multiple adverse effects such

as sedation, myosis, euphoria, nausea, constipation, respiratory depression, and a high risk of addiction. As these adverse effects are mostly produced by the activation of MOP,[4] the other opioid receptor-subtypes (ie DOP and KOP) represent interesting alternatives for the development of novel analgesics.[5] If activation of KOP is accompanied by nausea, dysphoria, stress, and hallucinations, recent studies have shown that DOP agonists have the potential to induce analgesia with less or no important adverse effects.[5–7]

Endogenous opioids include endomorphins, endorphins, dynorphins, and enkephalins.[8,9] It is known that endomorphins are selective to MOP and dynorphins to KOP. However, enkephalins and endorphins bind both DOP and MOP with a similar affinity. Despite this fact, enkephalins are generally considered as the endogenous DOP ligands. The enkephalins (Leu-enkephalin and Met-enkephalin) are five amino acid peptides diverging only in their last residue (Tyr–Gly–Gly–Phe–Leu and Tyr–Gly–Gly–Phe–Met). They are produced by proteolysis of the pro-peptide precursor proenkephalin and were first isolated in 1975 by Hughes and colleagues.[10] Similar to most peptides naturally produced in the central nervous system, the enkephalins have a poor pharmacokinetic profile and cannot be used as analgesics. When injected in the peripheral system, hydrophilic opioid peptides are unable to cross the blood–brain barrier (BBB) to reach the receptors in the central nervous system and they are particularly sensitive to enzymatic degradation.[9,11,12]

In the message-address concept first introduced by Robert Schwyzer in 1977,[13] the "message" portion of the peptide is responsible for the pharmacological activity, whereas the "address" is responsible for the selectivity of the molecule for its target. In Leu- and Met-enkephalin the "message" contains the pharmacophore residues tyrosine and phenylalanine linked together by two glycine residues. The remaining leucine is viewed as the residue responsible for the specificity (ie the "address"; Figure 1).[9] We have shown previously that a systematic replacement of the amide bonds contained in Leu-enkephalin was useful to explore and understand their role and involvement in the interaction of Leu-enkephalin with DOP.[12,14–16] In the present study, we investigated the role of the fifth position of Leu-enkephalin in binding and activating DOP and MOP. The systematic replacement of the fifth residue of Leu-enkephalin with non-natural amino acids was therefore used to further explore the lipophilic pocket of MOP and DOP receiving the "address" portion of Leu-enkephalin.

2 | EXPERIMENTAL PROCEDURES

2.1 | General procedure for peptide synthesis on solid support

Between each step, the resin was washed using DMF (3×), *i*PrOH (3×) and DCM (3×), unless stated otherwise.

(A) For each synthesis, the loading of the Wang resin was determined as follows: Resin (1 g, 0.07 mmol/g supplier loading) was placed in a sintered glass peptide synthesis vessel. 2 eq of the first amino acid, 2 eq of 2,6-dichlorobenzoyl chloride, and 4 eq of pyridine were added. The suspension was agitated in a shaker for 16 h. The resin was washed. Amine loading was determined by UV quantization of Fmoc release: An aliquot (10 mg) of resin was dried under vacuum; a mixture of piperidine (5 mL) and DMF (5 mL) was added, and the suspension was agitated in a shaker for 30 min; a portion of the solution (0.5 mL) was diluted in DCM (4.5 mL) and its absorbance was read with a UV spectrometer. Loading of the resin (mmol/g) = Absorbance (301 nm) × 10^3 × 20/(7800 × weight of the aliquot in mg).

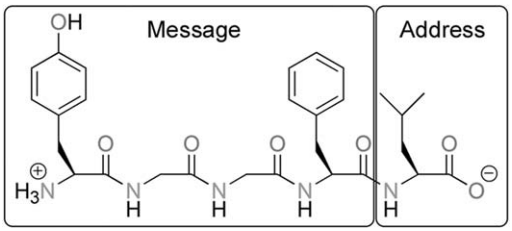


FIGURE 1 The structural component of Leu-enkephalin

(B) After the initial loading, the remaining free sites were protected using equal amounts of benzoyl chloride and pyridine. The solution was agitated in a shaker for 3 h.

(C) For Fmoc deprotection, the resin was treated with 50% piperidine in DMF, and the suspension was agitated in a shaker for 30 min.

(D) All couplings were performed using 6 eq of protected amino acid, and 6 eq of HATU (or COMU to couple Fmoc-Phe during the syntheses of **5** and **10**) with 12 eq of NMM in a minimum volume of DMF and the suspension was agitated in a shaker. When other coupling procedures were used, they are described in the "Experimental" section. All coupling procedures were stopped after 16 h or after negativity of the Kaiser's test.

(E) All the final peptides were cleaved from their resin in a glass vial, and the suspension was stirred for 1.5 h with a magnetic stirrer. All cleavage solutions were done with TFA/TIPS/H_2O (95:2.5:2.5) with a total amount of 10 mL/g of resin. After cleavage, the mixtures were filtered on cotton and dropped in a large amount of water (20 mL). The remaining solvents were concentrated under vacuum, and the aqueous solution was frozen and lyophilized.

(F) Purification and purity requirements: All crude peptides were purified using prepara+tive reverse-phase HPLC, detected at 280 nm, with a VYDAC 218TP C18 column and using ACN gradient in a 0.1% TFA aq solution (from 1:9 to 2:3), with a flow rate of 5 mL/min over 1 h. The purity of all fractions was analyzed using an Agilent 1100 series analytical HPLC, detection was performed at 214, 254, and 275 nm, with an Agilent 5 μm 3.0 × 50 mm C-18 column using ACN gradient in a 0.1% TFA aq solution (from 0:1 to 1:0) over 40 min, with a flow rate of 1 mL/min. The fractions with purities of 95% or higher were combined, frozen and lyophilized.

2.2 | Cell culture

HEK293 (human embryonic kidney) cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum and 10 IU penicillin, 100 mg/L streptomycin and 2 mM glutamine. Cells stably expressing the mouse Flag-DOP obtained from Dr. Richard Howells (New Jersey Medical

School, Newark, NJ) or human Flag-MOP obtained from Dr. Mark von Zastrow (University of California, San Francisco, CA) were grown in the presence of 250 mg/L geneticin.

2.3 | Radioligand competition binding studies

Stably transfected HEK293 cells grown to confluence in 150-mm Petri dishes were frozen at −80°C until use. On the day of the experiment, the cells were submitted to a heat shock by placing the Petri dishes at 37°C for 60 s before returning on ice. The cells were then harvested in ice-cold buffer A (20 mM Tris-HCl, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4) using a cell scraper and centrifuged at 3200*g* for 15 min at 4°C. The pellet containing the membrane extract was re-suspended in 1 mL of buffer A. The protein concentration was determined with Bio-Rad DC™ Protein Assay reagents (Bio-Rad Laboratories, Mississauga, ON, Canada) on a TECAN M200 plate reader (TECAN, Grödig, Austria) and the pellet was further diluted in buffer A containing 0.1% BSA. Experiments were performed using 10 μg of the cell preparation and 150 000 cpm of the radiolabeled ligand (we used [^{125}I]-Deltorphin I (specific activity 472 Ci/mmol) for DOP or [^{125}I]-DAMGO (specific activity 2200 Ci/mmol) for MOP) in a total volume of 200 μL per well in 96-well plates. Non-specific binding was determined using 10 μM non-radioactive Deltorphin II or DAMGO. Incubation was performed for 60 min at room temperature with increasing concentrations of Leu-enkephalin or compound **1**–**12** (1 pM to 10 μM). After the incubation period, the reaction was stopped by filtration using ice-cold buffer A on filtered 96-well plates. Filters were placed in 5 mL tubes, and the radioactivity was determined using a Wizard2 Automatic Gamma Counter (PerkinElmer, Woodbridge, ON, Canada). Data were analyzed using a nonlinear fitting analysis, and the K_i values were determined using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). K_i is expressed as the mean ± SEM (nM) from three independent experiments, each performed in duplicate.

2.4 | Rluc2-GFP10 BRET2-based biosensor assays

For BRET assays, HEK293 cells were transfected with the receptor and the adequate biosensor. Briefly, one day before transfection cells were washed with PBS at room temperature, trypsinized, and seeded at 3 000 000 cells in a 100-mm Petri dish. For Rluc2-EPAC-GFP10 transfection, 6000 ng of pcDNA3-Flag-ratDOP with 600 ng of Rluc2-EPAC-GPF10 or 3000 ng of pcDNA3-3HA-ratMOP with 150 ng of Rluc2-EPAC-GPF10 was added to 600 μL of 150 mM NaCl containing 27 μg of PEI. ssDNA was added to complement to 9 μg the total amount of DNA used in each transfection. For Arr2-GFP10/receptor-Rluc2 transfection, 600 ng of pcDNA3-Flag-ratDOP-Rluc2 or pcDNA3-3HA-ratMOP-Rluc2 with 12 000 ng Arr2-GFP10 was added to 600 μL of 150 mM NaCl containing 37.8 μg PEI. The mixture was incubated for 20 min before to being added to the cultured cells. At 24 h post-transfection, cells were washed with PBS, trypsinized and plated in 96-well white plates (75 000 cells/well) and grown for another 24 h. The cells were then equilibrated at room temperature for at least 1 h with 70 μL stimulation buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, pH 7.4). Coelenterazine 400A (an Rluc2 substrate) was added to a final concentration of 5 μM, 10 min before stimulation. For the EPAC assay, cells were first stimulated with 3 μM forskolin to increase cAMP. Cells were stimulated with ligand concentrations ranging from 1 pM to 10 μM (DOP) or 100 pM to 1 mM (MOP) and incubated for 10 min prior to the signal acquisition. BRET2 signals were measured using a TECAN M1000 fluorescence reader (TECAN). RLuc2 and GFP10 emissions were collected in the 400–450 nm window (RLuc2) and 500–550 nm window (GFP10). The BRET2 signal was calculated as the ratio of light emitted by the acceptor GFP10 over the light emitted by the donor RLuc2. All data were analyzed using the nonlinear curve fitting equation in GraphPad Prism 7.0 to estimate the pEC_{50} values of the curves for the different pathways. Results are expressed as the mean ± SEM (nM or μM) of four to five independent experiments, each performed in triplicate.

2.5 | Transduction coefficients and bias factors determination

Transduction coefficients and bias factors (BF) were derived using the method developed by Kenakin et al.,[17] as detailed by van der Westhuizen and colleagues.[18] Briefly, transduction coefficients ($\log(\tau/K_A)$), where τ (*tau*) represents ligand efficacy to induce a response of interest and K_A its "functional affinity," were first derived using the operational model equation. To this end, the equation allowing calculation of consolidated $\log(\tau/K_A)$ ratios[18] (here, all compounds were full agonists) was introduced in GraphPad Prism 7.0. Leu-enkephalin, the natural DOP agonist, was taken as the reference compound to yield $\Delta\log(\tau/K_A)$ values and were used to calculate differential ligand efficiency to engage cAMP signaling versus β-arrestin 2 recruitment by subtracting $\Delta\log(\tau/K_A)$ values of one signaling pathway from the $\Delta\log(\tau/K_A)$ of the other. BF correspond to antilogarithm base 10 of $\Delta\Delta\log(\tau/K_A)$ values.

2.6 | Homoleucine analog (1)

1.00 g of resin with a loading of 0.669 mmol/g was used. The title peptide was obtained as a white solid (115 mg, 30%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 8.50 (d, 2H, $J = 8.0$ Hz), 7.90 (d, 2H, $J = 8.0$ Hz), 7.29–7.12 (m, 5H), 7.11 (d, 2H, $J = 3.0$ Hz), 6.82 (d, 2H, $J = 3.0$ Hz), 4.76–4.71 (m, 1H), 4.36–4.30 (m, 1H), 4.09 (dd, 2H, $J = 6.5$ and 8.5 Hz), 3.95–3.72 (m, 4H), 3.17–3.14 (m, 2H), 3.00 (dd, 2H, $J = 9.0$ and 12.0 Hz), 1.89–1.83 (m, 1H), 1.75–1.70 (m, 1H), 1.59–1.52 (m, 1H), 1.31–1.23 (m, 2H), 0.93 (d, 6H, $J = 6.5$ Hz). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 173.8, 172.3, 170.0, 169.6, 156.9, 136.9, 130.1, 129.0, 128.0, 126.3, 124.5, 115.5, 54.7, 54.2, 52.6, 42.5, 41.7, 37.5, 36.2, 34.5, 29.1, 27.5, 21.5, 21.2. IR (NaCl) ν (cm^{-1}) 3600–3100 (br), 1660, 1518. HRMS: calculated for $C_{29}H_{39}O_7N_5$: 570.2922, found: 570.2922. $[\alpha]^{20}D$ +33.6 ($c = 5.99$, MeOH).

2.7 | Cyclopropyl alanine analog (2)

1.00 g of resin with a loading of 0.651 mmol/g was used. The title peptide was obtained as a white solid (100 mg, 28%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.28–7.16 (m, 5H), 7.07 (d, 2H, $J = 8.5$ Hz), 6.76 (d,

2H, $J = 8.5$ Hz), 4.70 (dd, 1H, $J = 5.0$ and 10.0 Hz), 4.40 (dd, 1H, $J = 5.5$ and 8.0 Hz), 4.04 (dd, 2H, $J = 6.5$ and 8.5 Hz), 3.97–3.68 (m, 4H), 3.18–3.11 (m, 2H), 2.99–2.90 (m, 2H), 1.69–1.62 (m, 2H), 0.85–0.70 (m, 1H), 0.46–0.41 (m, 2H), 0.13–0.05 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm)173.6, 172.1, 170.1, 169.6, 156.9, 136.9, 130.1, 128.0, 127.9, 126.3, 124.5, 115.4, 54.7, 54.3, 52.9, 47.3, 42.4, 41.7, 37.5, 36.3, 7.1, 3.7, 3.2. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1653, 1518. HRMS: calculated for $C_{28}H_{35}O_7N_5$: 554.2609, found: 554.2610. $[\alpha]^{20}$D +21.8 ($c = 5.67$, MeOH).

2.8 | Cyclohexylalanine analog (3)

1.00 g of resin with a loading of 0.143 mmol/g was used. The title peptide was obtained as a white solid (163 mg, 100%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 8.52 (d, 2H, $J = 8.0$ Hz), 7.84 (d, 2H, $J = 8.0$ Hz), 7.27–7.10 (m, 5H), 7.08 (d, 2H, $J = 2.0$ Hz), 6.76 (d, 2H, $J = 2.0$ Hz), 4.81–4.67 (m, 1H), 4.42–4.39 (m, 1H), 4.05 (dd, 1H, $J = 6.5$ and 8.5 Hz), 3.96–3.67 (m, 5H), 3.18–3.12 (m, 2H), 2.94 (dd, 2H, $J = 9.5$ and 14.0 Hz), 1.79–1.58 (m, 9H), 1.32–1.14 (m, 2H), 1.01–0.83 (m, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 174.3, 172.3, 170.0, 169.7, 169.6, 156.9, 136.9, 130.1, 129.0, 128.0, 126.3, 124.5, 115.4, 54.7, 54.2, 50.1, 42.5, 41.7, 38.6, 37.5, 36.2, 33.9, 33.3, 31.8, 25.9. IR (NaCl) ν (cm^{-1}) 3600-3300 (br), 1655, 1517, 1206, 803. HRMS: calculated for $C_{31}H_{41}O_7N_5$: 596.3079, found: 596.3091. $[\alpha]^{20}$D +17.3 ($c = 6.54$, MeOH).

2.9 | 4,5-Dehydro leucine analog (4)

1.00 g of resin with a loading of 0.7mmol/g was used. The title peptide was obtained as a white solid (58.6 mg, 15%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.30–7.15 (m, 5H), 7.09 (d, 2H, $J = 8.5$ Hz), 6.77 (d, 2H, $J = 8.5$ Hz), 4.74–4.59 (m, 1H), 4.58–4.42 (m, 1H), 4.11–4.06 (m, 1H), 4.07 (s, 3H), 3.95 (d, 2H, $J = 9.5$ Hz), 3.72–3.59 (m, 4H), 3.21–3.08 (m, 2H), 3.01–2.87 (m, 2H), 2.63–2.540 (m, 1H), 2.44–2.30 (m, 1H), 2.12–1.99 (m, 1H), 1.78 (s, 2H), 1.49 (s, 1H), 1.41 (s, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 156.9, 140.6, 136.8, 130.1, 128.9, 128.1, 127.9, 126.4, 126.3, 116.7, 115.4, 112.9, 82.3, 54.3, 50.8, 46.7, 41.9, 39.2, 37.5, 36.3, 27.6, 25.8, 20.8. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1664, 1515, 836. HRMS: calculated for $C_{28}H_{35}O_7N_5$: 554.2209, found: 554.2615. $[\alpha]^{20}$D +18.4 ($c = 7.24$, MeOH).

2.10 | Fmoc carbamate of L-neopentylglycine

The unnatural amino acid L-neopentylglycine (692 mg, 4.77 mmol) and $NaHCO_3$(s) were dissolved in an aqueous solution of H_2O/THF (7:13, 70 mL) at room temperature. Fmoc-Cl (1.85 g, 7.15 mmol) was added to the previous solution slowly under stirring at 0°C. The reaction was allowed to reach rt and stirred for 5 h. The aqueous phase was acidified with 1 N HCl to reach pH 2 after the addition of H_2O (100 mL) and extracted with EtOAc (3 × 30 mL). The organic phases were collected together, dried ($MgSO_4$) and concentrated under vacuum. The title product was obtained as a white solid (1.06 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.76 (d, 2H, $J = 7.0$ Hz), 7.58 (d, 2H, $J = 7.0$ Hz), 7.43–7.26 (m, 4H), 5.15 (br, 2H), 4.43 (d, 1H, $J = 2.0$ Hz), 4.24 (t, 1H, $J = 7.0$ Hz), 1.52–1.49 (m, 2H), 1.02 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 178.5, 155.9, 143.6, 141.3, 127.7, 127.1, 125.0, 120.0, 67.1, 51.6, 47.2, 45.8, 30.8, 17.6. IR (NaCl) ν (cm^{-1}) 3500–3300 (br), 3069, 2958, 1720, 1447. HRMS: calculated for $C_{22}H_{25}O_4N$: 367.1784, found: 367.1775. $[\alpha]^{20}$D -17.0 ($c = 8.80$, $CHCl_3$).

2.11 | Neopentylglycine analog (5)

1.00 g of resin with a loading of 0.656 mmol/g was used. The title peptide was obtained as a white solid (143 mg, 42%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm), 8.60 (d, 2H, $J = 8.0$ Hz), 7.80 (d, 2H, $J = 8.0$ Hz), 7.33–7.26 (m, 5H), 7.12 (d, 2H, $J = 8.0$ Hz), 6.77 (d, 2H, $J = 8.5$ Hz), 4.72–4.59 (m, 1H), 4.40–4.35 (m, 1H), 4.06 (dd, 2H, $J = 6.5$ and 8.0 Hz), 3.97–3.67 (m, 4H), 3.20–3.07 (m, 2H), 2.99–2.85 (m, 2H), 1.83 (dd, 1H, $J = 3.0$ and 14.5 Hz), 1.65 (dd, 1H, $J = 9.0$ and 14.5 Hz), 0.92 (s, 9H), 0.46–0.41 (m, 2H), 0.13–0.05 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm)174.6, 171.9, 170.0, 169.6, 156.9, 136.9, 130.1, 128.9, 127.9, 126.3, 124.5, 115.4, 54.7, 54.2, 48.1, 47.6, 47.0, 46.7, 44.4, 42.5, 41.2, 37.4, 36.3, 30.1, 28.5. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1669, 1517, 1372. HRMS: calculated for $C_{29}H_{39}O_7N_5$: 570.2922, found: 570.2935. $[\alpha]^{20}$D +15.3 ($c = 5.30$, MeOH).

2.12 | Azabeta-leucine analog (6)

1.00 g of resin with a loading of 0.691 mmol/g was used. The title peptide was obtained as a white solid (171 mg, 43%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.25–7.18 (m, 5H), 7.05 (d, 2H, $J = 8.0$ Hz), 6.75 (d, 2H, $J = 8.0$ Hz), 4.55–4.40 (m, 1H), 4.05 (dd, 2H, $J = 6.5$ and 8.0 Hz), 3.86–3.79 (m, 4H), 3.24–3.08 (m, 2H), 3.07–2.96 (m, 3H), 2.48–2.39 (m, 3H), 1.40–1.25 (m, 1H), 0.96–0.76 (m, 6H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 172.0, 170.1, 169.6, 156.9, 136.3, 130.1, 129.0, 128.2, 126.5, 124.5, 115.4, 64.1, 58.1, 54.7, 53.4, 48.1, 47.7, 46.9, 42.3, 41.7, 37.4, 36.3, 2537, 19.5. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1668, 1518, 1370, 996. HRMS: calculated for $C_{28}H_{38}O_7N_6$: 571.2875, found: 571.2877. $[\alpha]^{20}$D +15.1 ($c = 6.78$, MeOH).

2.13 | Isoleucine analog (7)

1.00 g of resin with a loading of 0.849 mmol/g was used. The peptide was obtained as a white solid (16.8 mg, 3%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 8.35 (d, 1H, $J = 8.0$ Hz), 7.87 (d, 1H, $J = 8.0$ Hz), 7.21–7.13 (m,5H), 7.04 (d, 2H, $J = 6.0$ Hz), 6.74 (d, 2H, $J = 6.0$ Hz), 4.76 (dd, 1H, $J = 5.0$ and 9.0 Hz), 4.32–4.28 (m, 1H), 4.08 (dd, 1H, $J = 6.5$ and 8.0 Hz), 3.96–3.68 (m, 4H), 3.17–3.10 (m, 2H), 2.98–2.90 (m, 2H), 1.89–1.81 (m, 1H), 1.53–1.45 (m, 1H), 1.28–1.21 (m, 1H), 0.95–0.86 (m, 6H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 189.5, 170.1, 169.9, 169.7, 152.8, 136.9, 130.1, 128.9, 128.0, 126.3, 115.4, 54.8, 54.5, 42.7, 41.8, 37.4, 37.1, 36.3, 24.8, 14.7, 10.5. IR (NaCl) ν (cm^{-1}) 3706–2822 (br), 1664, 1534, 1197. HRMS: calculated for $C_{28}H_{37}N_5O_7$: 578.2585, found: 578.2570. $[\alpha]^{20}$D +23.9 ($c = 3.89$, MeOH).

2.14 | D-isoleucine analog (8)

1.00 g of resin with a loading of 0.665 mmol/g was used. The title peptide was obtained as a white solid (31.9 mg, 9%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.30–7.26 (m, 5H), 7.08 (d, 2H, $J = 8.5$ Hz), 6.76 (d, 2H, $J = 8.5$ Hz), 4.22–4.18 (m, 1H), 4.15–4.01 (m, 1H), 3.99–3.92 (m, 5H), 3.20–3.03 (m, 2H), 2.99–2.85 (m, 2H), 1.77–1.60 (m, 1H), 0.84–0.77 (m, 8H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 169.5, 130.1, 129.0, 128.1, 126.4, 124.5, 115.5, 54.7, 54.3, 48.2, 48.0, 47.9, 47.7, 47.6, 47.5, 47.3, 47.1, 46.8, 42.5, 37.9, 36.7, 36.2, 24.6, 14.4. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1666, 1520, 1443. HRMS: calculated for $C_{28}H_{37}O_7N_5$: 556.2766, found: 556.2767. $[\alpha]^{20}$D +16.6 ($c = 5.60$, MeOH).

2.15 | D-alloisoleucine analog (9)

1.00 g of resin with a loading of 0.7 mmol/g was used. The title peptide was obtained as a white solid (107 mg, 28%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.40–7.17 (m, 5H), 7.10 (d, 2H, $J = 8.0$ Hz), 6.77 (d, 2H, $J = 8.0$ Hz), 4.87–4.72 (m, 1H), 4.40 (d, 1H, $J = 5.0$ Hz), 4.10 (dd, 1H, $J = 6.5$ and 8.0 Hz), 3.99–3.78 (m, 4H), 3.28–3.04 (m, 2H), 3.01–2.87 (m, 2H), 1.95–1.79 (m, 1H), 1.19–0.93 (m, 2H) 0.87–0.72 (m, 6H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 173.4, 172.1, 170.2, 169.6, 156.9, 136.7, 130.1, 128.9, 128.1, 126.4, 124.5, 115.4, 55.4, 54.7, 54.3, 47.2, 45.2, 41.9, 37.9, 36.7, 36.2, 25.6, 13.5, 10.6. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1660, 1514, 1443. HRMS: calculated for $C_{28}H_{37}O_7N_5$: 556.2766, found: 556.2770. $[\alpha]^{20}$D +17.1 ($c = 5.75$, MeOH).

2.16 | Cycloleucine analog (10)

500 mg of resin (1.5 mmol/gr factory loading) with an arbitrary loading of 1.5 mmol/g was used. The title peptide was obtained as a white solid (6.80 mg, 2%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 8.45 (s, 2H), 7.82 (d, 2H, $J = 8.0$ Hz), 7.30–7.28 (m, 5H), 7.14 (d, 2H, $J = 8.0$ Hz), 6.81 (d, 2H, $J = 8.0$ Hz), 4.69–4.61 (m, 1H), 4.11 (dd, 1H, $J = 6.5$ and 8.0 Hz), 3.95–3.72 (m, 4H), 3.18–3.10 (m, 2H), 3.01–2.93 (m, 2H), 2.20–2.02 (m, 2H), 1.98–1.79 (m, 2H), 1.74–1.57 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 174.1, 173.5, 140.8, 134.1, 133.0, 131.9, 130.3, 128.4, 119.4, 58.7, 57.9, 52.1, 51.8, 51.5, 51.3, 50.9, 50.7, 46.5, 45.8, 40.2, 40.1, 27.9. IR (NaCl) ν (cm^{-1}) 3600–3300 (br), 1655, 1517, 1206, 803. HRMS: calculated for $C_{28}H_{35}O_7N_5$: 554.2609, found: 554.2607. $[\alpha]^{20}$D +24.8 ($c = 2.55$, MeOH).

2.17 | Homocycloleucine analog (11)

1.00 g of resin with a loading of 0.258 mmol/g was used. The title peptide was obtained as a white solid (19.0 mg, 13%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 8.20 (s, 2H), 7.75 (d, 2H, $J = 8.0$ Hz), 7.31–7.15 (m, 5H), 7.12 (d, 2H, $J = 8.0$ Hz), 6.81 (d, 2H, $J = 8.0$ Hz), 4.80–4.75 (m, 1H), 4.05 (dd, 1H, $J = 6.0$ and 8.5 Hz), 3.95–3.60 (m, 4H), 3.16–3.01 (m, 2H), 2.99–2.80 (m, 2H), 2.03–1.81 (m, 2H), 1.80–1.65 (m, 2H), 1.60–1.23 (m, 6H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 176.2, 171.7, 170.1, 169.5, 156.9, 136.9, 130.1, 129.0, 128.0, 126.3, 124.5, 115.5, 58.9, 54.7, 54.0, 42.6, 41.7, 37.6, 36.2, 31.8, 31.6, 24.9, 20.9. IR (NaCl) ν (cm^{-1}) 3500–3200 (br), 1665, 1517, 1452. HRMS: calculated for $C_{29}H_{37}O_7N_5$: 568.2766, found: 568.2762. $[\alpha]^{20}$D +8.55 ($c = 6.49$, MeOH).

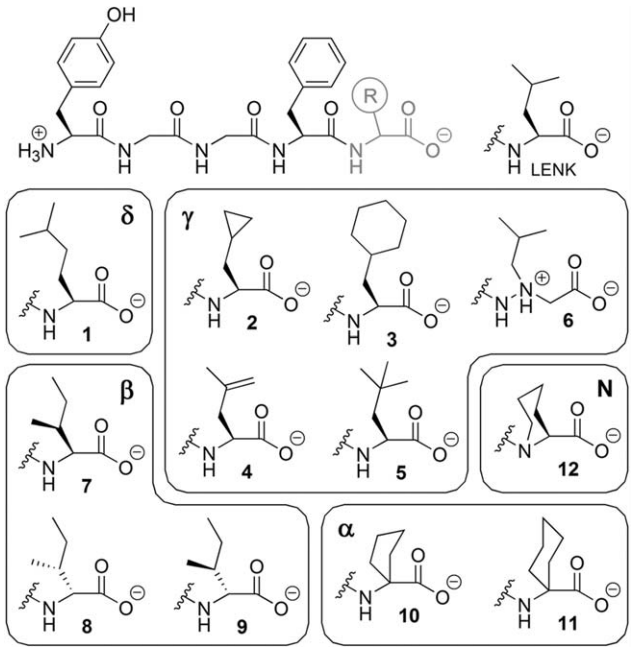


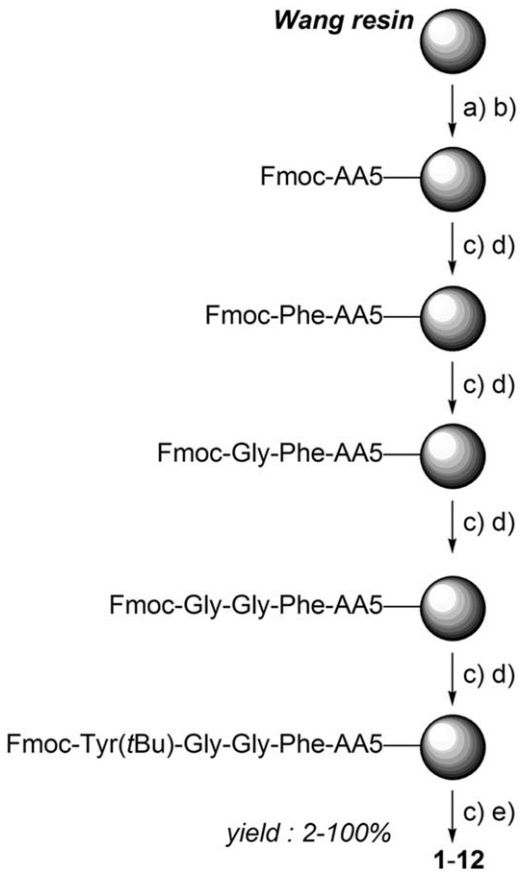
FIGURE 2 Non-natural amino acids replacing the 5th residue of the Leu-enkephalin. They are classified as families (δ, γ, β, α, N) according to their branching positions

2.18 | Homoproline analog (12)

1.00 g of resin with a loading of 0.59 mmol/g were used. The title peptide was obtained as a white solid (13.0 mg, 4%). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.28–7.19 (m, 5H), 7.09 (d, 2H, $J = 8.5$ Hz), 6.76 (d, 2H, $J = 8.5$ Hz), 4.59–4.51 (m, 1H), 4.06 (dd, 1H, $J = 6.5$ and 8.0 Hz), 3.99–3.70 (m, 5H), 3.46–3.33 (m, 3H), 3.18–3.04 (m, 3H), 2.98–2.88 (m, 3H), 2.37 (td, 2H, $J = 6.5$ and 2.5 Hz), 1.50–0.80 (m, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 174.1, 171.9, 170.2, 169.6, 156.9, 136.8, 130.1, 128.9, 128.0, 126.4, 124.5, 115.5, 54.7, 42.4, 41.9, 39.1, 37.6, 36.3, 34.9, 32.9. IR (NaCl) ν (cm^{-1}) 3500–3100 (br), 1665, 1443, 1213. HRMS: calculated for $C_{28}H_{35}O_7N_5$: 553.2537, found: 553.2543. $[\alpha]^{20}$D +2.33 ($c = 1.13$, MeOH).

3 | RESULTS AND DISCUSSION

As already stated, Leu-enkephalin binds to both DOP and MOP with similar affinity. Aiming to further explore the "address" portion of Leu-enkephalin, this is to say the fifth amino acid residue, a total of 12 analogs were synthesized and tested for their potency and efficacy at DOP and MOP. These pentapeptides share the same Tyr–Gly–Gly–Phe "message" as natural enkephalins, whereas the amino acid Leu forming the "address" region has been replaced by similar lipophilic residues that is non-natural amino acid derivatives of leucine, alanine, or proline (Figure 2). The analogs can be classified into five distinct families, namely δ, γ, β, α, and N according to their branching positions. The



SCHEME 1 SPPS of compounds **1**–**12** using the Fmoc strategy with the Wang resin. Reaction conditions: (a) Initial coupling, (b) protection of the remaining free sites of the resin, (c) Fmoc deprotection (Pip/DMF 1:1), 30 min at room temperature (r.t.) (d) protected amino acid (AA) (6 eq); HATU or COMU (6 eq); NMM (12 eq) DMF, 16 h at r.t., and (e) TFA/TIPS/H2O (38:1:1), 1.5 h at r.t

branching pattern of the most numerous γ group (**2**–**6**) is the same as Leu in Leu-enkephalin and the fifth residue in **1** and **3** are, respectively, as long or slightly longer than a Met residue. For the β, α and N families, residues were chosen because the length of their side chains is either equal (**7**–**9** and **11**) or slightly shorter (**10** and **12**) than that of a Leu residue. Thus, all amino acid residues were selected in order to explore the steric requirements of the hydrophobic pocket naturally filled by the side chains of Leu and Met. Additionally, the effect of a positive charge in the address was studied in the case of the aza-β-homoleucine derivative **6**.

3.1 | Chemistry

The twelve analogs (**1**–**12**) and Leu-enkephalin were synthesized on solid support using the Fmoc strategy on Wang resin (Scheme 1). Neopentylglycine was purchased and Fmoc protected before being used to obtain the corresponding analog (**5**). Fmoc-aza-β-leucine was synthesized and attached to the Wang resin to produce analog (**6**). All other residues were purchased N-protected with an Fmoc group.

3.2 | Synthesis

The classical methodology of solid phase peptide synthesis (SPPS) was used unless otherwise stated. For the analogs **5** and **10**, (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), an excellent alternative to 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) in the case of sterically demanding peptide bonds,[19,20] was used for the coupling between phenylalanine and the non-natural residue.

3.3 | Binding affinity of leu-enkephalin analogs

To evaluate the affinity of the synthesized analogs for DOP and MOP, we performed competitive binding assays using HEK293/DOP and HEK293/MOP cell membrane preparations with radiolabeled ^{125}I-Deltorphin I (DOP-selective ligand) and ^{125}I-DAMGO (MOP-selective ligand).

As shown in Table 1, most compounds, with the exception of analogs **8** and **9**, bind MOP with a slightly better affinity than DOP. Analog **12**, containing a homoproline in position 5 displays very low affinity for both receptors. This low affinity is likely the consequence of detrimental conformational constraints or unfavorable interactions imposed by the ring since we have previously show that the *N*-methylated analog Tyr–Gly–Gly–Phe–*N*Me–Leu has a comparable affinity for DOP when compared to Leu-enkephalin.[12] When compared to the other compounds, analog **6** was found to have a slightly lower affinity for both receptors. The geometry of the modified residue substantially differs from Leu and bears a positive charge that might induce unfavorable

TABLE 1 In Vitro binding affinity of compounds 1–12 at DOP and MOP

Family	Analog	K_i δ (nM)	K_i μ (nM)
γ	LENK	0.64 ± 0.29	0.20 ± 0.09
δ	Homoleucine **1**	0.71 ± 0.20	0.30 ± 0.08
γ	Cyclopropylalanine **2**	0.95 ± 0.18	0.22 ± 0.07
	Cyclohexylalanine **3**	0.43 ± 0.08	0.15 ± 0.06
	4,5-dehydroleucine **4**	2.71 ± 0.42	0.67 ± 0.18
	Neopentylglycine **5**	2.53 ± 0.57	0.57 ± 0.21
	Aza-β-homoleucine **6**	16.4 ± 3.5	4.6 ± 1.6
β	Isoleucine **7**	0.58 ± 0.13	0.44 ± 0.05
	D-isoleucine **8**	0.63 ± 0.17	0.95 ± 0.22
	D-allo-isoleucine **9**	1.19 ± 0.33	1.17 ± 0.98
α	Cycloleucine **10**	1.86 ± 0.49	0.82 ± 0.53
	Homocycloleucine **11**	1.62 ± 0.52	0.90 ± 0.37
N	Homoproline **12**	2220 ± 220	69 ± 17

The K_i value of each compound was determined using [^{125}I]-Deltorphin I (K_d: 0.6 nM; specific activity: 472 Ci/mmol), [^{125}I]-DAMGO (K_d: 0.12 nM; specific activity: 2200 Ci/mmol) and stable HEK293 cell lines expressing mouse DOP or human MOP. K_i are the means ± SEM (nM) of three independent experiments each performed in duplicate.

TABLE 2 In Vitro potency of analogs 1–12 on DOP and MOP

	DOP				MOP			
	EPAC		βArr2		EPAC		βArr2	
Analog	(EC_{50}; nM)	Emax (% ref)	(EC_{50}; nM)	Emax (% ref)	(EC_{50}; nM)	Emax (% ref)	(EC_{50}; μM)	Emax (% ref)
LENK	0.24 ± 0.09	100 ± 0	13.2 ± 4.1	100 ± 0	1.2 ± 0.2	100 ± 0	0.7 ± 0.2	100 ± 0
Homoleucine **1**	1.54 ± 0.03	98 ± 1	8.2 ± 1.8	92 ± 7	1.4 ± 0.6	95 ± 3	0.8 ± 0.2	93 ± 3
Cyclopropylalanine **2**	0.62 ± 0.09	96 ± 6	27.8 ± 3.6	77 ± 5	1.6 ± 0.7	98 ± 9	0.8 ± 0.3	102 ± 5
Cyclohexylalanine **3**	0.14 ± 0.06	95 ± 4	4.8 ± 1.7	73 ± 5	1.9 ± 0.7	96 ± 10	0.8 ± 0.4	95 ± 4
4,5-déhydroleucine **4**	1.71 ± 0.57	89 ±4	61 ± 18	90 ± 7	3.7 ± 1.7	95 ± 6	1.0 ± 0.1	84 ± 8
Neopentylglycine **5**	0.39 ± 0.14	93 ± 2	50.5 ± 6.8	95 ± 4	19.9 ± 7.1	98 ± 2	5.9 ± 1.5	87 ± 8
Aza-β-homoleucine **6**	4.0 ± 1.6	90 ± 8	814 ± 64	64 ± 6	39.4 ± 10.3	95 ± 3	15.1 ± 7.3	81 ± 9
Isoleucine **7**	0.51 ± 0.19	86 ± 4	15.3 ± 4.3	90 ± 5	2.6 ± 0.7	88 ± 2	0.6 ± 0.2	88 ± 6
D-isoleucine **8**	0.42 ± 0.11	98 ± 3	22.2 ± 4.6	82 ± 4	19.4 ± 8.3	95 ± 4	6.5 ± 2.9	101 ± 16
D-allo-isoleucine **9**	1.1 ± 0.7	95± 1	41.6 ± 10.9	79 ± 7	13.2 ± 4.3	92 ± 2	3.3 ± 0.7	84 ± 7
Cycloleucine **10**	0.57 ± 0.13	103 ± 4	95.2 ± 10.2	85 ± 6	9.9 ± 4.0	93 ± 3	3.7 ± 0.9	84 ± 3
Homocycloleucine **11**	1.34 ± 0.41	89 ± 3	48.7 ± 10.8	78 ± 2	9.9 ± 2.9	96 ± 6	3.4 ± 1.7	97 ± 6
Homoproline **12**	276 ± 111	69 ± 6	No signal	No signal	324 ± 179	86 ± 4	No signal	No signal

The potency (EC_{50}; nM or μM) of compounds **1**–**12** to inhibit the forskolin-induced cAMP production or to recruit β-Arrestin 2 following activation of DOP or MOP was respectively assayed using Rluc2-EPAC-GPF10 biosensor or βArr2-GPF10 biosensor with a Rluc2 tagged version of DOP or MOP. Values are the means ± SEM (nM or μM) of at least three independent experiments each performed in triplicate. The efficacy of each analog (Emax) was also determined as the percentage of the maximum activation achieved by the reference compound, LENK (% ref).

interactions with the receptor. Indeed, the natural residues Leu and Met at position 5 are less hydrophilic than aza-β-homoleucine. The δ-branched analog **1** retains a very good affinity for the opioid receptors, so does the cyclohexylalanine analog **3**, containing a γ-branched residue. These peptides confirm that there is a fairly large available space in the corresponding region of DOP and MOP. This observation is in agreement with the slightly higher affinity of Met-enkephalin, for which the side chain has a similar length as homoleucine and cyclohexylalanine. The three remaining γ-branched residues cyclopropylalanine, 4,5-dehydroleucine and neopentylglycine are highly similar to the leucine residue. However, only analog **2**, containing a cyclopropyl side chain, mimics a locked *i*-propyl group as found in Leu-enkephalin. Interestingly, the affinity and the potency of this analog for DOP and MOP are similar to those of Leu-enkephalin (Tables 1, 2). The slightly less bulky analog **4** sees its affinity decreased by ∼ 3- to 4-fold for both DOP and MOP when compared to Leu-enkephalin. This is presumably the consequence of suboptimal van der Waals interactions with some residues in the receptor pocket. The same remark applies to analog **5** containing a *t*-butyl side chain. In this particular case, the decreased affinity is likely due to excessive bulk and steric clashes. As for the β-branched residues, it was shown that replacing leucine with a valine residue at position 5 of the enkephalin leads to very low affinity analogs.[21] The poor affinity of such analogs has been attributed to their inability to reach Leu300$^{7.35}$ residue of DOP and develop a stabilizing van der Waals interaction. On the other hand, the longer β-branched residue Ile, found in analog **7**, has a similar affinity for DOP as does Leu-enkephalin. The extra methyl group (vs. Val) is likely to reach the bottom of the pocket partially defined by Leu300$^{7.35}$. The exploration was pursued with two *D* isomers of Ile, namely *D*-isoleucine and *D*-allo-isoleucine, incorporated in analogs **8** and **9**, respectively. These analogs proved to be very effective with respective binding affinity (K_i) values of 0.63 and 1.19 nM for DOP and 0.95 and 1.17 nM for MOP. Finally, the two α-branched analogs **10** and **11** bind DOP and MOP with a slightly decreased affinity when compared to Leu-enkephalin. It is worth mentioning that, under our experimental conditions (using ^{125}I-Deltorphin I, ^{125}I-DAMGO and stable HEK293 cell lines expressing DOP or MOP), Leu-enkephalin was found to bind MOP with a slightly better affinity than DOP.

We then evaluated the potency of all the synthesized analogs using EPAC and βArr2 BRET biosensors. When an agonist binds to an opioid receptor, complex signaling pathways are activated.[22,23] Among these pathways, agonist-stimulated opioid receptors couple to Gi heterotrimeric proteins inhibiting adenylyl cyclase and reducing cAMP levels. Moreover, opioid receptors also interact with β-arrestins, which leads to receptor internalization.[22,23] The EPAC BRET biosensor was used to measure the inhibition of cAMP production, while the investigation of the β-arrestin pathway was assessed with βArr2 BRET biosensor (Table 2). As compared to peptides **1**–**11**, compound **12** shows a much lower potency in inhibiting cAMP production through DOP and MOP. Despite having different potencies, all analogs but **12** were considered full agonists in this pathway as they all induced a maximal effect (Emax) similar to that of Leu-enkephalin (Table 2). As for the β-arrestin pathway, all compounds but **12** were found to recruit β-arrestin 2 upon DOP and MOP activation. Again, all compounds but **12** (for which no BRET signal was detected) produced a near maximal

TABLE 3 Transduction coefficient and bias factor determination for compounds 1–12 following activation of DOP

	EPAC		$\beta_2$arr		EPAC/$\beta_2$arr	
	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\Delta\Delta\log(\tau/K_A)$	BF
LENK	10.02 ± 0.34	0.00 ± 0.48	8.15 ± 0.11	0.00 ± 0.16	0.00 ± 0.49	1.00
Homoleucine **1**	9.92 ± 0.12	−0.10 ± 0.36	8.13 ± 0.10	−0.03 ± 0.15	−0.08 ± 0.39	0.84
Cyclopropylalanine **2**	9.36 ± 0.05	−0.67 ± 0.34	7.46 ± 0.09	−0.70 ± 0.14	0.03 ± 0.38	1.07
Cyclohexylalanine **3**	9.83 ± 0.17	−0.19 ± 0.38	8.40 ± 0.09	0.24 ± 0.15	−0.43 ± 0.39	0.37
4,5-déhydroleucine **4**	8.91 ± 0.14	−1.12 ± 0.36	7.09 ± 0.10	−1.06 ± 0.15	−0.06 ± 0.39	0.87
Neopentylglycine **5**	9.13 ± 0.15	−0.90 ± 0.37	7.29 ± 0.14	−0.87 ± 0.18	−0.03 ± 0.41	0.93
Aza-β-homoleucine **6**	8.27 ± 0.17	−1.75 ± 0.38	5.69 ± 0.08	−2.46 ± 0.14	0.71 ± 0.39	5.16
Isoleucine **7**	9.58 ± 0.08	−0.45 ± 0.35	7.79 ± 0.09	−0.37 ± 0.15	−0.08 ± 0.38	0.83
D-isoleucine **8**	9.34 ± 0.19	−0.68 ± 0.39	7.33 ± 0.12	−0.82 ± 0.17	0.14 ± 0.42	1.38
D-allo-isoleucine **9**	9.13 ± 0.21	−0.90 ± 0.40	7.11 ± 0.10	−1.05 ± 0.15	0.15 ± 0.42	1.41
Cycloleucine **10**	9.19 ± 0.13	−0.84 ± 0.36	7.02 ± 0.11	−1.14 ± 0.16	0.30 ± 0.39	2.00
Homocycloleucine **11**	9.19 ± 0.17	−0.83 ± 0.38	7.23 ± 0.08	−0.92 ± 0.14	0.09 ± 0.41	1.24
Homoproline **12**	5.80 ± 0.15	−4.23 ± 0.37	nd	nd	nd	nd

Data presented in Table 2 were further analyzed by nonlinear regression using the Operational Model equation as described in the methods to determine the transduction coefficients ($\log(\tau/K_A)$) and bias factors (BF) with LENK as the reference compound. $\Delta\Delta\log(\tau/K_A)$ and BF values were calculated as described in the methods. Data are the mean ± SEM of 3–6 independent experiments each performed in triplicate.

β-arrestin 2 response and were therefore considered full agonists in this pathway as well.

Interestingly, although there was no significant gain in DOP selectivity by replacing the fifth residue of Leu-enkephalin with various non-natural amino acids, functional assays allowed us to reveal that several of the new compounds displayed receptor-specific cAMP versus β-arrestin 2 signaling bias. In particular ligand efficiency to evoke response at each of these pathways was evaluated using the operational model of Black and Leff[24] to calculate $\log(\tau/K_A)$ transduction coefficients (refer to the Experimental procedures for further details). Transduction coefficients for each ligand at either DOP or MOP are respectively reported in Tables 3 and 4 along with ($\Delta\log(\tau/K_A)$) values

TABLE 4 Transduction coefficient and bias factor determination for compounds 1–12 following activation of MOP

	EPAC		$\beta_2$arr		EPAC/$\beta_2$arr	
	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\Delta\Delta\log(\tau/K_A)$	BF
LENK	9.00 ± 0.06	0.00 ± 0.09	6.21 ± 0.15	0.00 ± 0.22	0.00 ± 0.24	1.00
Homoleucine **1**	8.80 ± 0.19	−0.20 ± 0.20	6.20 ± 0.19	−0.01 ± 0.24	−0.19 ± 0.32	0.65
Cyclopropylalanine **2**	8.64 ± 0.13	−0.36 ± 0.15	6.12 ± 0.18	−0.09 ± 0.24	−0.27 ± 0.28	0.54
Cyclohexylalanine **3**	8.77 ± 0.27	−0.23 ± 0.28	6.00 ± 0.16	−0.21 ± 0.23	−0.02 ± 0.36	0.95
4,5-déhydroleucine **4**	7.99 ± 0.32	−1.00 ± 0.32	5.76 ± 0.16	−0.45 ± 0.22	−0.56 ± 0.39	0.28
Neopentylglycine **5**	8.05 ± 0.20	−0.95 ± 0.21	5.11 ± 0.09	−1.10 ± 0.18	0.15 ± 0.27	1.42
Aza-β-homoleucine **6**	7.44 ± 0.10	−1.56 ± 0.12	4.59 ± 0.14	−1.62 ± 0.21	0.06 ± 0.24	1.15
Isoleucine **7**	8.54 ± 0.06	−0.46 ± 0.09	5.72 ± 0.20	−0.49 ± 0.25	0.02 ± 0.27	1.05
D-isoleucine **8**	7.99 ± 0.24	−1.01 ± 0.25	5.29 ± 0.21	−0.92 ± 0.26	−0.09 ± 0.36	0.80
D-allo-isoleucine **9**	7.78 ± 0.19	−1.22 ± 0.20	5.42 ± 0.12	−0.79 ± 0.20	−0.44 ± 0.28	0.11
Cycloleucine **10**	7.92 ± 0.21	−1.08 ± 0.22	5.33 ± 0.12	−0.88 ± 0.20	−0.20 ± 0.29	0.63
Homocycloleucine **11**	8.17 ± 0.20	−0.83 ± 0.21	5.34 ± 0.25	−0.87 ± 0.30	0.03 ± 0.36	1.08
Homoproline **12**	6.29 ± 0.35	−2.71 ± 0.35	nd	nd	nd	nd

Data presented in Table 2 were further analyzed by nonlinear regression using the Operational Model equation as described in the methods to determine the transduction coefficients ($\log(\tau/K_A)$) and bias factors (BF) with LENK as the reference compound. $\Delta\Delta\log(\tau/K_A)$ and BF values were calculated as described in the methods. Data are the mean ± SEM of 3–6 independent experiments each performed in triplicate.

normalized to Leu-enkephalin. The extent to which each ligand preferentially evoked G protein-dependent modulation of cAMP production versus β-arrestin 2 recruitment was obtained by subtracting corresponding $\Delta\log(\tau/K_A)$ values to yield a bias factor ($\Delta\Delta\log(\tau/K_A)$). For the purpose of this study, we considered a compound biased when the bias factor (BF) was ≥2.00 (bias toward G protein-dependent cAMP inhibition) or ≤0.50 (biased toward β-arrestin 2 recruitment). As shown in Table 3, only compounds **6** (BF = 5.16) and **10** (BF = 2.00) were biased toward preferential modulation of cAMP production following DOP activation while only compound 3 (BF = 0.37) showed β-arrestin 2 recruitment bias at this receptor. This is of a particular interest when one considers that opioid ligands are thought to produce analgesia through activation of the G protein dependent-signals and unwanted effects such as constipation and respiratory depression have been associated to β-arrestin recruitement.[25–28] Compounds **6** and **10** therefore suggest that slight modifications within the side chain of the fifth amino acid residue has the potential to induce bias toward G protein coupling. By contrast, results from Table 4 reveal that none of the compounds are biased toward the modulation of cAMP production when MOP activation is considered. In fact, two compounds are rather biased toward β-arrestin 2 signaling following activation of MOP. Indeed, compounds **4** and **9** have BF values ≤0.50 and therefore their activation of MOP can be considered biased toward β-arrestin 2 versus G protein-mediated signaling.

4 | CONCLUSION

The current study on the optimal shape of the fifth residue of enkephalins revealed that its side chain has to be long enough to be able to develop positive interactions with the receptors. However, even when this condition is met, we observed that selectivity toward MOP or DOP remains unachieved with the chosen non-natural amino acids. Interestingly, the analogs displaying a high affinity for DOP also potently inhibited the cAMP production. The replacement of the leucine residue by aza-β-homoleucine or cycloleucine in position 5 resulted in peptidomimetics with significantly lower ability to recruit β-arrestin 2 toward DOP suggesting that this position has the potential to produce G protein biased signaling at this receptor. By contrast, derivatives containing 4,5-dehydroleucine or D-allo-isoleucine were more efficient at recruiting β-arrestin 2 than inhibiting cAMP production following MOP activation.

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AUTHOR CONTRIBUTIONS

DBN and VB have contributed equally to this work. SB synthesized the non-natural amino acid in **8** while ICS and JFN synthesized the analog **9**. DBN and APG synthesized the analogs **1**–**8** and **10**–**12**. RL and GP provided the expertise on the BRET biosensors. BG, LG, and YLD designed the study. DBN, VB, WR, and BH performed the experiments and wrote the manuscript. All authors have read and approved the final version of the manuscript.

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