Synthesis and biological evaluation of leucine enkephalin turn mimetics†

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A cyclic Leu-enkephalin mimetic containing a 7-membered ring, and two linear analogues, has been prepared on solid phase. In the cyclic mimetic the intramolecular (1–4) hydrogen bond found in crystalline Leu-enkephalin has been replaced by an ethylene bridge. In addition, the amide bond between Tyr1 and Gly2 has been replaced by a methylene ether isostere and Gly3 has been deleted. The two linear analogues both contain the methylene ether isostere instead of the Tyr1-Gly2 amide bond and the shorter of the two lacks Gly3. The three compounds, and a β-turn mimetic analogous to the 7-membered turn mimetic but with Gly3 included, were evaluated for specific binding to μ- and δ-opioid receptors in rat brain membranes. With the exception of the β-turn mimetic the three other Leu-enkephalin analogues all bound with varying affinity to the μ- and δ-opioid receptors. From the results it could be concluded that Leu-enkephalin binds in a turn conformation to the opiate receptors, but that this conformation is not a (1–4) β-turn.

Introduction

The opiate receptors, which belong to the family of G protein-coupled receptors (GPCR's), are divided into μ-, δ- and κ-receptor sub-types.[1] In the mid 1970s two endogenous opiate receptor ligands were discovered, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met).[2] They act as agonists at the opiate receptors and trigger a response cascade resulting in the relief of pain.

Soon after the discovery of the two enkephalins X-ray crystallography revealed that Leu-enkephalin formed a (1–4) β-turn in the solid state.[3] Based on this finding it was proposed that the flexible structures of the two enkephalins were stabilized in the bioactive conformation by forming an intramolecular hydrogen bond between the carbonyl oxygen atom in Tyr (i) and the amide NH in Phe (i+3), thereby generating a β-turn conformation.[4] Considerable efforts have been made to elucidate the bioactive conformation of the two ligands using spectral and computational techniques, including NMR spectroscopy in membrane mimicking environments.[5–7] However, in spite of all efforts a clear understanding of the bioactive conformation of the enkephalins has not yet been established.

Native peptides are usually not suitable as drugs intended for oral administration because of poor stability towards proteolysis, limited ability to cross membrane barriers, and rapid excretion. However, the important biological functions of peptides make the preparation of peptidomimetics highly interesting. Such mimetics should retain the biological effect of peptides simultaneously with presenting an improved pharmacokinetic profile obtained through molecular modifications. Consequently, strategies using endogenous receptor ligands as starting points for design of peptidomimetics are of great importance.[8] This ligand based drug design methodology has been utilized extensively in efforts to develop selective and potent opiate receptor ligands, at the same time as attempting to gain increased understanding of the bioactive conformation. The vast literature in the field contains several examples of β-turn mimetics, as well as non-cyclic mimetics of the enkephalines.[9–13]

We recently reported the incorporation of a β-turn mimetic based on a 10-membered ring in place of the first four residues of Leu-enkephalin (*cf.* **1**, Fig. 1).[14] Conformational studies based on ^{1}H NMR data for mimetic **1** showed that the β-turn mimetic was flexible, but resembled a type II β-turn at low temperature.[14] This low energy conformer also closely resembled the structure determined for crystalline Leu-enkephalin.[3] In view of studies suggesting the importance of different reverse turn conformations in biologically active conformations of Leu-enkephalin, our design was expanded to mimetic **2** (Fig. 2), which lacks one of the two

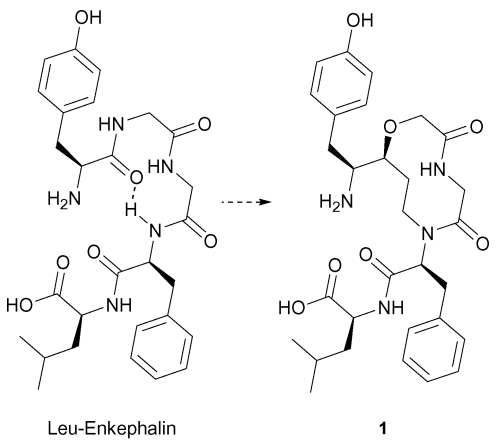

Fig. 1 Peptidomimetic **1** has been designed to contain a covalently bonded 10-membered ring that mimics the (1–4) β-turn found in crystalline Leu-enkephalin.

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† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra for **2**–**4**, **11**, **13**–**17**. See DOI: 10.1039/b515618a

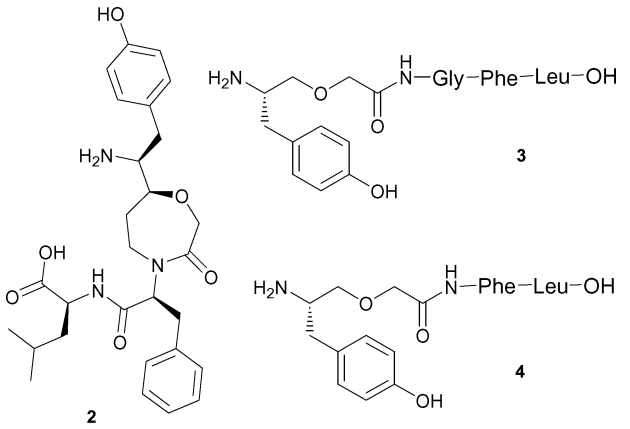


Fig. 2 Peptidomimetic **2** contains a 7-membered ring which induces a different turn conformation than for **1**. Linear peptidomimetics **3** and **4** were used to probe the effect of cyclization in binding of **1** and **2** to the opiate receptors.

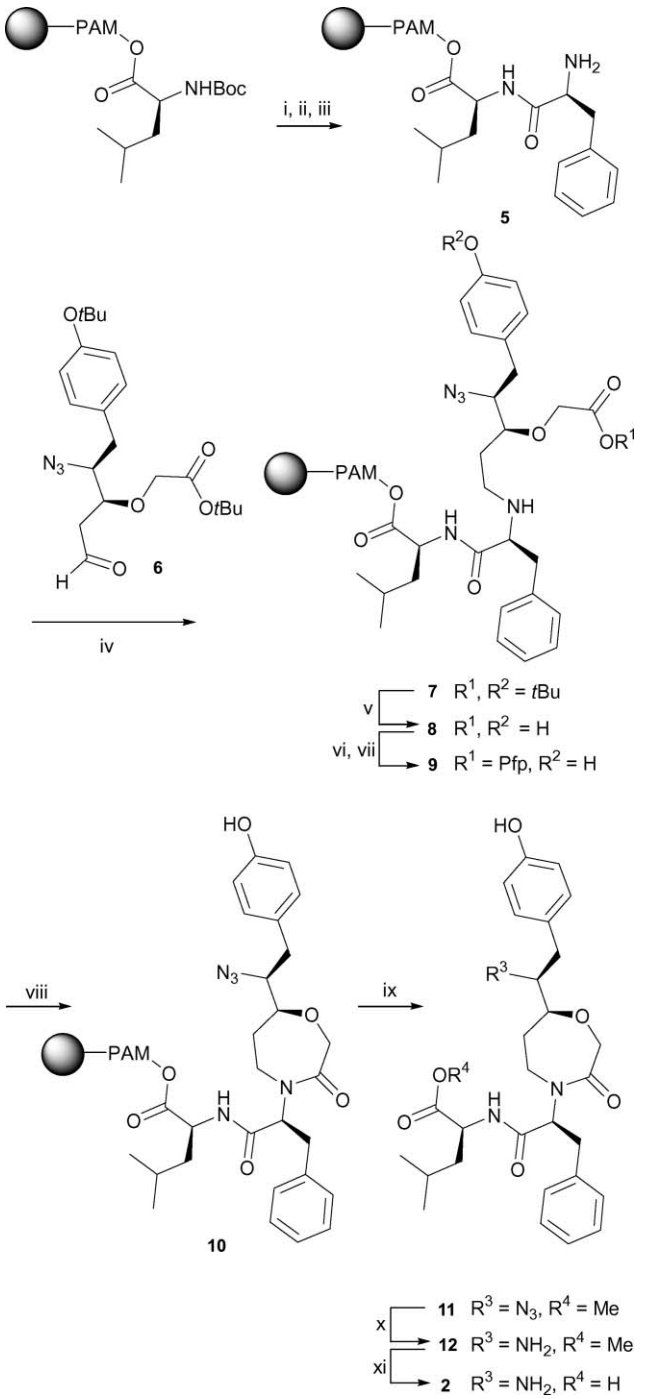
glycines in Leu-enkephalin. The 7-membered ring in **2** orients the critical side chains of Tyr and Phe to slightly different positions as compared to mimetic **1**. In both of mimetics **1** and **2** the intramolecular hydrogen bond found in a potential (1–4) β-turn has been replaced by an ethylene bridge. Simultaneously the amide bond between residues i and $i + 1$ has been replaced with a methylene ether isostere. This isostere was chosen because replacement of the Tyr-Gly amide bond in Leu-enkephalin by a methylene ether isostere, as in **3**, has been reported to be well tolerated at the opiate receptors.[15] Mimetics **1** and **2** are therefore well suited for investigation of the role played by (1–4) turns in interactions of Leu-enkephalin with the opiate receptors.

In this paper we describe the synthesis of turn mimetic **2**, as well as the linear analogues **3** and **4**. Compounds **3** and **4** contain the same side chains as found in the corresponding cyclized mimetics **1** and **2**, and a methylene ether isostere between residues i and $i + 1$, but they lack the ethylene bridge. We also describe affinity studies at the μ- and δ-opiate receptors for mimetics **1**–**4**.

Results and discussion

Synthesis of 7-membered Leu-enkephalin mimetic 2

In contrast to **1** which was prepared by a solution phase route,[14] it was decided to carry out the synthesis of mimetic **2** on a solid support. This was done so as to minimize oligomerization during formation of the 7-membered ring and to establish conditions that would allow the synthesis of libraries of turn mimetics. Synthesis of **2** started by deprotection of Boc-leucine attached to Tentagel resin by treatment with trifluoroacetic acid in dichloromethane (Scheme 1). Fmoc-phenylalanine was coupled to the resulting free amine by using diisopropylcarbodiimide under standard conditions,[16] after which the Fmoc group was removed with piperidine in DMF to give solid-phase bound dipeptide **5**. Reductive amination[17] of aldehyde **6**[14] with resin bound dipeptide **5**, using sodium triacetoxyborohydride as reducing agent in 1,2-dichloroethane, gave secondary amine **7**. At this stage solid-phase bound **7** contained the *C*-terminal leucine and phenylalanine residues connected to the Tyr-Gly moiety of the desired 7-membered ring Leu-enkephalin mimetic. The *tert*-butyl ester and



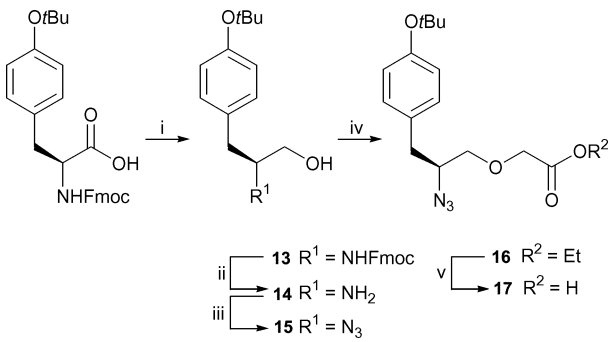
Scheme 1 i) TFA (30%) in CH_2Cl_2; ii) HOBt, DIC, bromophenol blue solution, Fmoc-Phe-OH, DMF; iii) piperidine (20%) in DMF; iv) **5**, $Na(OAc)_3BH$, NEt_3, CH_2ClCH_2Cl; v) TFA (30%) in CH_2Cl_2; vi) HCl (1 M) in 1,4-dioxane; vii) PfpOH, DIC, EtOAc; viii) NEt_3, 1,4-dioxane, reflux; ix) Na (0.22 M) in MeOH, 25% (over the solid phase synthesis); x) $SnCl_2$, PhSH, TEA, THF; xi) LiOH (0.1 M), THF, 56% (from **11**).

the phenolic *tert*-butyl ether functionalities of **7** were then cleaved simultaneously under acidic conditions, without affecting the PAM linker which attached **7** to the solid phase. Activation[18] of the carboxylic acid in **8** with diisopropylcarbodiimide and pentafluorophenol in ethyl acetate gave pentafluorophenyl ester **9**. Ring closure to 7-membered ring **10** was achieved by treatment

with triethylamine in refluxing dioxane. Polymerization problems during the analogous ring closure reaction in the solution phase synthesis of **1** required high dilution conditions,[14] but such problems were avoided by the solid phase strategy adopted for synthesis of **10**. The cyclized product was released from the solid phase by treatment with sodium methoxide in methanol. This gave protected turn mimetic **11** in 25% yield over the solid phase sequence, after purification by chromatography on silica gel. Finally, the *N*- and *C*-termini of **11** were deprotected in two steps. First the azide was reduced[19] using tin chloride, thiophenol and triethylamine in THF to give amine **12**. After filtration through silica gel the methyl ester in **12** was hydrolyzed with lithium hydroxide in THF, after which purification by reversed phase HPLC gave turn mimetic **2** in 56% yield over the two deprotection steps. Thus, mimetic **2** was prepared from aldehyde **6** and resin bound Boc-leucine in an eleven step sequence, nine steps of which were carried out in the solid phase, with a total yield of 14%.

Synthesis of the linear analogs 3 and 4

Synthesis of Tyr-Gly dipeptide mimetic building block **17** was performed in solution. The synthesis began with reduction[20] of Fmoc-Tyr(*t*Bu)–OH to alcohol **13** by activation of the carboxylic acid with isobutyl chloroformate in THF, followed by reduction with sodium borohydride (Scheme 2). Subsequent cleavage of the Fmoc group with morpholine gave amino alcohol **14** (81% from Fmoc-Tyr(*t*Bu)–OH). Compound **14** was converted to azido alcohol **15** by treatment[21,22] with a freshly prepared solution of triflyl azide in dichloromethane and a catalytic amount of cupric sulfate (72%). Alcohol **15** was deprotonated with potassium hydride and alkylated with ethyl bromoacetate at 0 °C to give ester **16** (89%). Hydrolysis of **16** was accomplished by treatment with an excess of sodium hydroxide in ethanol and water to give acid **17** (80%). In this way, Tyr-Gly dipeptide mimetic **17**, which contains a methylene ether isostere instead of the amide bond, was prepared over five steps in 42% total yield. Dipeptide mimetic **17** is the common intermediate for the solid phase synthesis of the linear analogs **3** and **4**.



Scheme 2 i) NMM, isobutylchloroformate, $NaBH_4$, MeOH, THF, −15 °C, 90%; ii) morpholine, 90%; iii) NaN_3, Tf_2O, H_2O, $CuSO_4$, DMAP, CH_2Cl_2, 0 °C to rt, 72%; iv) KH, NBu_4I, ethyl bromoacetate, THF, 0 °C, 89%; v) NaOH, EtOH, 80%.

Synthesis of **3** and **4** from building block **17** was carried out on solid phase (Scheme 3). Solid-phase bound leucine (TentagelS-PHB-Leu-Fmoc) was deprotected and coupled with Fmoc-protected phenylalanine to form dipeptide **18**, following a standard protocol.[16] The solid phase bound dipeptide **18** was then divided into two parts that were processed separately. One part of **18** was subjected to Fmoc-deprotection followed by coupling of Fmoc-protected glycine, to give tripeptide **19**. After Fmoc-deprotection resin bound tripeptide **19** was coupled with **17** using HATU and diisopropylethylamine in dichloromethane,[23,24] to generate *tert*-butyl-protected pentapeptide **22**. The progress of the reaction was monitored by IR spectroscopy directly on the resin through the appearance of an azide stretch at 2107 cm^{-1}. The azide functionality was then reduced[19] using tin chloride, thiophenol, and triethylamine in THF to give amine **23**, a reaction that was also monitored by IR spectroscopy on the solid phase. Finally, deprotection of the phenolic ether and cleavage from the solid phase using a mixture of trifluoroacetic acid and water containing scavengers, followed by purification with reversed phase HPLC, gave the linear Tyr-Gly-Gly-Phe-Leu analogue **3**[15] in 70% yield over the solid phase synthetic sequence. Application of the same reaction sequence to dipeptide **18** gave the linear Tyr-Gly-Phe-Leu analogue **4** in 62% yield.

Binding of peptide mimetics to the μ- and δ-opioid receptors

The binding affinities of Leu-enkephalin mimetics **1–4** to the μ- and δ-opioid receptor subtypes were measured using membrane bound receptors obtained from rat brain. Competition with radio labeled DAMGO (Fig. 3, Table 1) and DPDPE (Fig. 4, Table 2), ligands which are selective for the μ- and δ-receptor subtypes,

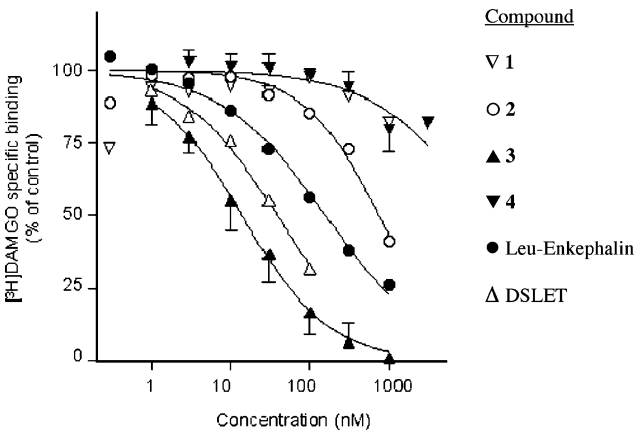
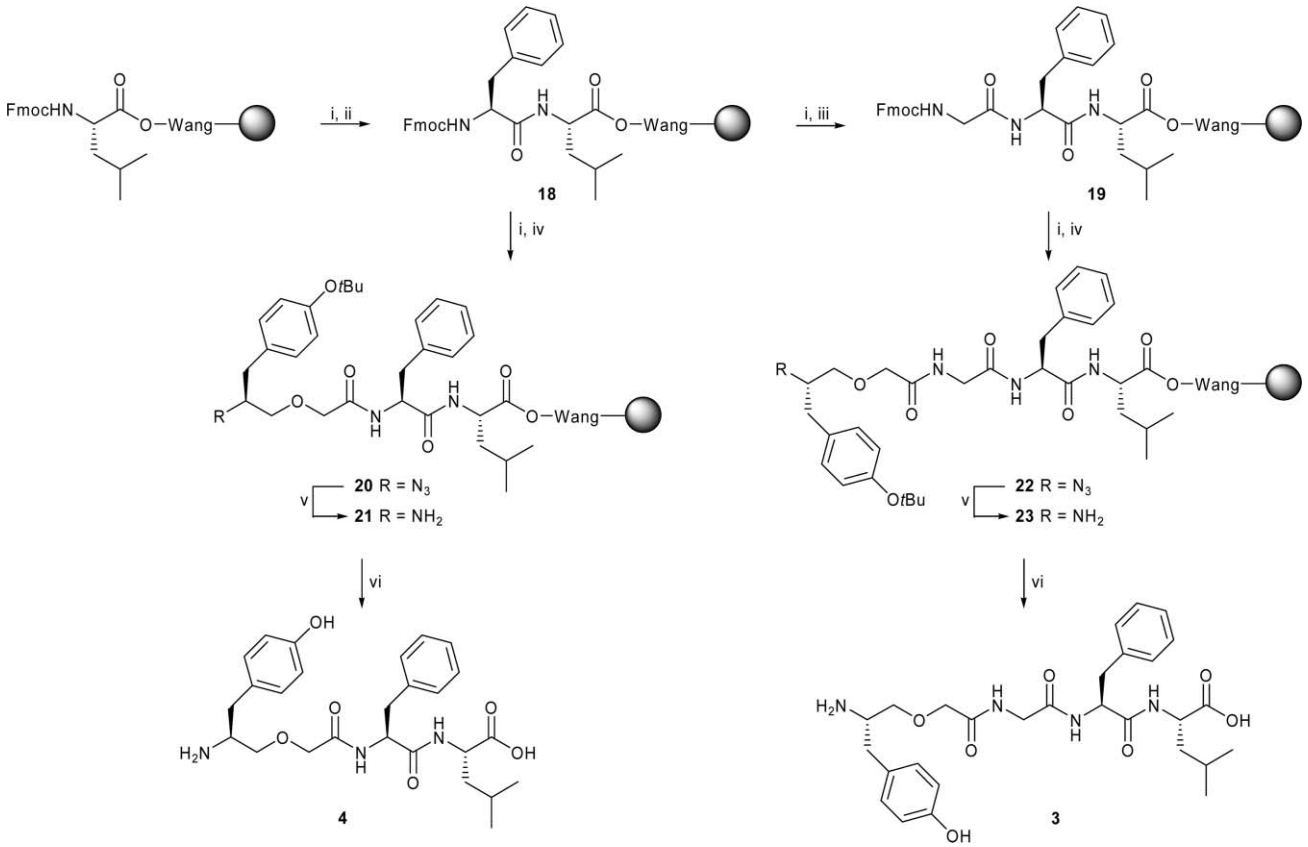


Fig. 3 Competitive inhibition curves for compounds **1–4**, DSLET and Leu-enkephalin at the rat μ-opiate receptor with [^{3}H]DAMGO as radioligand, corrected for unspecific binding using naloxone (for clarity only a few error bars are shown in the figure).

Table 1 Inhibitory potencies and Hill slopes for compounds **1–4**, Leu-enkephalin and DSLET at the rat μ-opiate receptor determined with [^{3}H]DAMGO as radioligand

Compound	pIC_{50}	IC_{50}/nM	n_h
1	<6	>1000	—
2	6.13 ± 0.12	740	0.90 ± 0.24
3	7.86 ± 0.09	14	0.80 ± 0.12
4	<5.5	>1000	—
Leu-enkephalin	6.81 ± 0.12	160	0.66 ± 0.12
DSLET	7.41 ± 0.07	39	0.74 ± 0.01



Scheme 3 i) Piperidine (20%) in DMF; ii) HOBt, DIC, bromophenol blue solution, Fmoc-Phe-OH, DMF; iii) HOBt, DIC, bromophenol blue solution, Fmoc-Gly-OH, DMF; iv) HATU, DIEA, **17**, CH_2Cl_2; v) $SnCl_2$, PhSH, TEA, THF; vi) H_2O, TFA, ethanedithiol, thioanisole, 62–70%.

Table 2 Inhibitory potencies and Hill slopes for compounds **1–4**, Leu-enkephalin and DSLET at the rat δ-opiate receptor determined with [^{3}H]DPDPE as radioligand

Compound	pIC_{50}	IC_{50}/nM	n_h
1	<6	>1000	—
2	6.79 ± 0.12	160	0.98 ± 0.26
3	8.88 ± 0.08	1.3	0.92 ± 0.14
4	6.07 ± 0.14	370	1.03 ± 0.37
Leu-enkephalin	8.44 ± 0.15	3.6	0.67 ± 0.15
DSLET	8.83 ± 0.16	1.5	0.58 ± 0.14

respectively, was used to determine selectivity, with correction for non-specific binding using naloxone.[25] All compounds were tested in triplicate at each concentration and on three different rat brain homogenates. The known ligands DSLET (D-Ser-Tyr-Gly-Gly-Phe-Leu-Thr) and Leu-enkephalin were included as reference compounds.

Among the tested peptidomimetics linear Leu-enkephalin analogue **3**[15] showed the highest affinity both for the μ- (IC_{50} = 14 nM) and δ-opioid receptor subtype (IC_{50} = 1.3 nM). In agreement with literature results,[15] these affinities were slightly higher or in the same range as for DSLET and Leu-enkephalin. This confirms that a methylene ether isostere can replace the amide bond between tyrosine and glycine in Leu-enkephalin without any reduction in the affinity for the two receptor subtypes. Linear Leu-enkephalin analogue **3** was also found to be a partial agonist at both receptor subtypes in a GTPγs assay (data not shown). Cyclic 10-membered

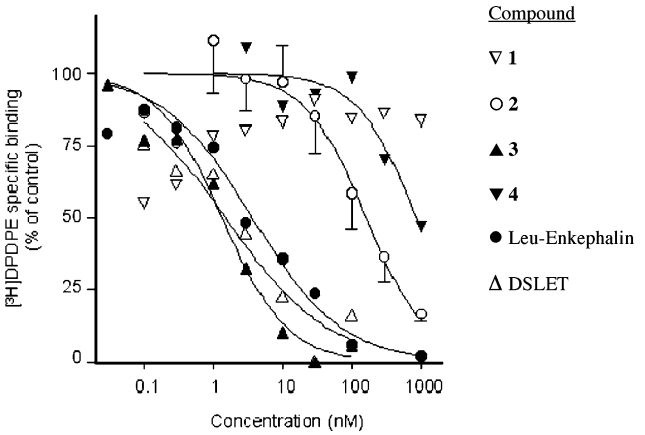


Fig. 4 Competitive inhibition curves for compounds **1–4**, DSLET and Leu-enkephalin at the rat δ-opiate receptor with [^{3}H]DPDPE as radioligand, corrected for unspecific binding using naloxone (for clarity only a few error bars are shown in the figure).

turn mimetic **1** showed no affinity (IC_{50} > 1000 nM) at either of the opioid receptors. The lack of affinity displayed by **1** can not be due to the replacement of the amide bond between the tyrosine and glycine moieties with a methylene ether isostere, as this modification is well tolerated in the corresponding linear analogue **3**. Instead the rigidity imposed by the cyclization appears to render **1** incapable of adopting the conformation required for interactions with the opioid receptors. Steric hindrance caused by

the incorporation of the ethylene bridge, or loss of a potential hydrogen bond involving the Phe NH, could also contribute to the loss of receptor affinity. However, the fact that mimetic **2**, which also contains the ethylene bridge and lacks the Phe NH, binds to both receptor subtypes (*cf.* below) shows that these two structural features do not alone explain the lack of receptor affinity displayed by **1**. Previously, conformational studies based on ^{1}H NMR data has shown that **1** adopts a flexible type II β-turn at low temperature.[14] Taken together with the ability of **1** to adopt a β-turn confirmation, and the lack of affinity of **1** at the μ- and δ-receptors, questions whether Leu-enkephalin binds as a (1–4) β-turn to the opioid receptors.

The 7-membered cyclic mimetic **2** displays significant affinity for both receptor subtypes (IC_{50} = 740 nM at the μ-receptor, IC_{50} = 160 nM at the δ-receptor), while the corresponding linear Leu-enkephalin analogue **4** showed almost no affinity for the μ-opioid receptor (IC_{50} > 1000 nM) and only low affinity for the δ-receptor (IC_{50} = 370 nM). The conformation imposed by cyclization to give **2** is thus favored for binding to both receptor subtypes, as compared to the flexible linear analogue **4**. It thus appears that linear **4**, as compared to cyclic **2**, pays a significant entropy cost upon adopting the required receptor bound conformation. Importantly, the fact that 7-membered turn mimetic **2** binds to both the μ- and δ-receptors with sub μM affinity does indicate that Leu-enkephalin binds to both receptor subtypes in some type of turn conformation. It should be emphasized that even though the interactions of **2** with the two opioid receptors are less favorable than for the linear analogue **3** and the two known agonists DSLET and Leu-enkephalin, 7-membered ring **2** constitutes a new drug-like scaffold with potential for use in ligand based drug development targeting the opiate receptors, as well as other GPCR's.

Experimental

Methyl *N*-((2*S*)-2-{(7*S*)-7-[(1*S*)-1-azido-2-(4-hydroxyphenyl)-ethyl]-3-oxo-1,4-oxazepan-4-yl}-3-phenylpropanoyl)-L-leucinate (11)

Tentagel PAM-Leu-Boc (0.50 g, capacity 0.42 mmol g^{-1}) was rinsed with DMF, toluene, EtOAc and CH_2Cl_2, then pre-swelled in CH_2Cl_2 and treated with 30% TFA in CH_2Cl_2 for 10 h to give PAM-Leu-NH_2. Fmoc-Phe-OH (0.33 g, 0.84 mmol), diisopropyl carbodiimide (130 μL, 0.82 mmol) and hydroxy benzotriazole (170 mg, 1.3 mmol) were stirred in DMF for 1 h and then added to the pre-swelled solid phase along with bromophenol blue (15 μL, 2 mM in DMF) and rotated for 8 h rinsed with DMF and CH_2Cl_2 to give Fmoc-protected **5**. The solid phase was treated with 20% piperidine in DMF (3 min continuous flow followed by 7 min rotation) to liberate resin bound amine **5**. Aldehyde **6** (0.087 g, 0.22 mmol) and triethylamine (40 μL, 0.27 mmol) were dissolved in 1,2-dichloroethane (3 mL) and added to the pre-swelled solid phase. After 1 hour $Na(OAc)_3BH$ (45 mg, 0.21 mmol) was added and the reaction was further rotated for 14 h washed with DMF and CH_2Cl_2 to afford resin bound secondary amine **7**. The resin was treated with 30% TFA in CH_2Cl_2 for 6 h to give resin bound acid **8**. The solid phase was rinsed with 1 M HCl in dioxane for 10 min to expedite a counterion exchange to the HCl-salt of **8**. Solid phase bound carboxylic acid **8** was activated as a pentafluorophenol ester by adding pentafluorophenol (45 mg, 0.21 mmol) and diisopropyl carbodiimide (35 μL, 0.21 mmol) in EtOAc (2 mL), after 5 h the solid phase was rinsed with DMF, EtOAc and CH_2Cl_2 to give **9**. Dioxane (6 mL) and NEt_3 (87 μL, 0.63 mmol) were then added to the solid phase which was heated to reflux for 9 h to give **10**. The ring closed product was cleaved from the solid phase by treatment with freshly prepared NaOMe (2 × 4 mL, sodium (20 mg) in CH_3OH (4 mL)) for 2 × 30 min. The solid phase was rinsed with EtOAc, H_2O was added to the organic layer and the two phases were separated. The aqueous phase was extracted with EtOAc and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc/heptane 1 : 2 → 2 : 1) to give 7-membered ring **11** (23 mg, 25% over the nine step solid phase synthesis) as a colorless solid. $[\alpha]_D^{20}$ = −91.7 (c = 0.71 in $CHCl_3$); IR 3300, 2106, 1741, 1637 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, $CHCl_3$) δ 7.33–7.16 (m, 5H), 7.02 (d, J = 8.3 Hz, 2H), 6.79 (d, J = 8.3 Hz, 2H), 6.56 (d, J = 8.0 Hz, 1H), 5.97 (bs, 1H), 5.22–5.16 (m, 1H), 4.51 (d, J = 15.6 Hz, 1H), 4.50–4.46 (m, 1H), 4.07 (d, J = 15.6 Hz, 1H), 3.77–3.65 (m, 1H), 3.68 (s, 3H), 3.37–3.24 (m, 3H), 3.21–3.15 (m, 1H), 3.10 (dd, J = 14.6 and 8.8 Hz, 1H), 2.83 (dd, J = 6.7 and 13.9 Hz, 1H), 2.73 (dd, J = 8.2 and 13.9 Hz, 1H), 1.68–1.55 (m, 3H), 1.54–1.44 (m, 2H), 0.87 (d, J = 5.9 Hz, 3H), 0.86 (d, J = 5.9 Hz, 3H); ^{13}C NMR (100 MHz, 25 °C, $CHCl_3$): δ 173.6, 172.7, 169.8, 154.9, 136.4, 130.3, 128.9, 128.8, 128.7, 126.9, 115.6, 81.4, 72.7, 66.1, 58.8, 52.3, 50.9, 42.5, 40.9, 35.3, 34.0, 31.9, 24.9, 22.7, 21.7; HRMS (FAB) calcd. for $C_{29}H_{37}N_5O_6Na$ (M + Na) 574.2642, found 574.2643.

N-((2*S*)-2-{(7*S*)-7-[(1*S*)-1-Amino-2-(4-hydroxyphenyl)ethyl]-3-oxo-1,4-oxazepan-4-yl}-3-phenylpropanoyl)-L-leucine (2)

7-Membered ring **11** (10 mg, 0.018 mmol) was treated with a mixture of $SnCl_2$ (0.022 g, 0.37 mmol), TEA (0.040 mL, 1.1 mmol) and thiophenol (0.040 mL, 1.5 mmol) in THF (1 mL). After 1 h the solvent was removed under reduced pressure, and the residue was filtered through a short path of silica (EtOH/toluene, 1 : 4 as eluent). After removal of the solvents amine **12** (0.018 mmol) was dissolved in THF (1.5 mL) followed by addition of 0.1 M aqueous LiOH (0.63 mL, 0.063 mmol). After 5 h the reaction was acidified with an excess of acetic acid (0.3 mL) and the solvent was removed with toluene as azeotrope. The residue was purified by reversed phase HPLC, lyophilized to give turn mimetic **2** (5 mg, 56% from **11**) as a colorless solid. IR(neat) 3700–2600, 1658, 1616, 1515 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD, 25 °C, MeOH) δ 8.18 (d, J = 8.2 Hz, 1H), 7.31–7.18 (m, 5H), 7.04 (d, J = 8.6 Hz, 2H), 6.79 (d, J = 8.6, 2H), 5.36 (dd, J = 10.2 and 6.1 Hz, 1H), 4.44–4.36 (m, 1H), 4.33 (d, J = 15.4 Hz, 1H), 4.25 (d, J = 15.4 Hz, 1H), 3.80–3.69 (m, 1H), 3.59–3.46 (m, 2H), 3.40 (dd, J = 14.9 and 6.2 Hz, 1H), 3.19–3.11 (m, 1H), 3.06 (dd, J = 14.9 and 10.1 Hz, 1H), 2.86 (dd, J = 14.6 and 6.5 Hz, 1H), 2.68 (dd, J = 14.6 and 7.5 Hz, 1H), 1.94–1.86 (m, 1H), 1.68–1.60 (m, 3H), 1.38–1.28 (m, 1H), 0.94 (d, J = 6.3 Hz, 3H), 0.90 (d, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD, 25 °C, MeOH) δ 174.3, 173.5, 171.3, 156.8, 137.0, 130.1, 128.6, 128.3, 126.5, 125.3, 115.6, 79.6, 71.9, 58.6, 56.2, 50.9, 42.1, 40.0, 34.5, 34.3, 24.7, 22.0, 20.4; HRMS (FAB) calcd for $C_{28}H_{38}N_3O_6$ (M + H) 512.2761, found 512.2756.

9*H*-Fluoren-9-ylmethyl [(1*S*)-1-(4-*tert*-butoxybenzyl)-2-hydroxyethyl]carbamate (13)

To Fmoc-Tyr(*t*-Bu)–OH (0.50 g, 1.1 mmol), dissolved in THF (6 mL), was added *N*-methylmorpholine (0.13 mL, 1.1 mmol). The temperature was lowered to −20 °C and isobutyl chloroformate (0.17 mL, 1.14 mmol) was added slowly and stirred for 20 minutes. The formed precipitate was removed by filtration and $NaBH_4$ (0.12 g, 3.3 mmol) was added in one portion to the THF solution followed by careful addition of MeOH (10 mL). After 1 h 2 N HCl (aq.) and EtOAc were added. After extraction with EtOAc the combined organic phases were washed with brine, dried over $NaSO_4$ and concentrated. The residue was purified by flash chromatography (EtOH/toluene, 1 : 20 → 1 : 10) to give alcohol **13** (0.44 g, 90%) as a colorless solid. $[\alpha]_D^{20} = -21.8$ (c = 0.5 in $CHCl_3$); IR (neat) 3316, 2973, 2933, 1687 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.76 (d, J = 7.4 Hz, 2H), 7.56 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 7.08 (d, J = 7.9 Hz, 2H), 6.91 (d, J = 7.9 Hz, 2H), 5.04 (d, J = 7.2 Hz, 1H), 4.46–4.32 (m, 2H), 4.19 (t, J = 6.7 Hz, 1H), 3.96–3.83 (m, 1H) 3.71–3.56 (m, 2H), 2.87–2.75 (m, 2H), 2.33 (bs, 1H), 1.32 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.4, 154.0, 143.8, 141.3, 132.3, 129.6, 127.7, 127.0, 125.0, 124.2, 119.9, 78.3, 66.6, 63.8, 54.1, 47.2, 36.6, 28.8; HRMS (FAB) calcd for $C_{28}H_{32}NO_4$ 446.2331 (M + H), found 446.2359.

(2*S*)-2-Amino-3-(4-*tert*-butoxyphenyl)propan-1-ol (14)

To alcohol **13** (0.42 g, 0.90 mmol) morpholine (15 mL) was added. The reaction was stirred for 3 h and then co-evaporated with toluene. The residue was purified by flash chromatography ($CHCl_3$/MeOH, 9 : 1 → 7 : 3) to furnish amino alcohol **14** (0.19 g, 90%) as a colorless oil. $[\alpha]_D^{20} = -8.3$ (c = 1.4 in $CHCl_3$); IR (neat) 3195, 2973, 2888, 1608 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.08 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 3.66 (dd, J = 3.7 and 10.9 Hz, 1H), 3.43 (dd, J = 7.2 and 10.9 Hz, 1H), 3.22–3.09 (m, 1H), 2.77 (dd, J = 6.1 and 13.5 Hz, 1H), 2.61 (dd, J = 8.0 and 13.5 Hz, 1H), 1.32 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.1, 132.8, 129.7, 124.4, 78.5, 65.2, 54.4, 39.2, 28.9; HRMS (FAB) calcd for $C_{13}H_{22}NO_2$ 224.1651 (M + H), found 224.1658.

(2*S*)-2-Azido-3-(4-*tert*-butoxyphenyl)propan-1-ol (15)

NaN_3 (5.8 g, 89 mmol) dissolved in a biphasic system of H_2O (13 mL) and CH_2Cl_2 (22 mL) was treated with triflic anhydride (3.0 mL, 18 mmol) at 0 °C under vigorous stirring. After 2 h the two phases were separated and the organic layer was washed with sat. $NaHCO_3$ (aq.) and dried over Na_2SO_4 (do not evaporate to dryness, explosions are reported![22]). The triflic azide solution was then added to amino alcohol **14** (0.90 g, 4.0 mmol) in CH_2Cl_2 (10 mL) containing *N*,*N*-dimethylaminopyridine (0.28 g, 2.3 mmol) and a catalytic amount of $CuSO_4$ (0.035 g, 0.22 mmol). After 2 h 10% citric acid (aq.) was added and the two phases were separated. The organic layer was washed with 10% citric acid (aq.), sat. $NaHCO_3$ (aq.), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2/Et_2O, 2 : 1) to yield azido alcohol **15** (0.72 g, 72%) as a colorless oil. $[\alpha]_D^{20} = -0.8$ (c = 1.2 in $CHCl_3$); IR (neat) 3411, 2975, 2933, 2102 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.12 (d, J = 8.3 Hz, 2H), 6.94 (d, J = 8.3 Hz, 2H), 3.74–3.65 (m, 2H), 3.58–3.50 (m, 1H), 2.85 (dd, J = 6.3 and 13.9 Hz, 1H), 2.79 (d, J = 7.4, 13.9 Hz, 1H), 2.19 (bs, 1H), 1.33 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.3, 131.8, 129.7, 124.4, 78.5, 65.5, 64.4, 36.5, 28.8; HRMS (FAB) calcd for $C_{13}H_{22}NO_2$ 249.1477 (M+), found 249.1489.

Ethyl {[(2*S*)-2-azido-3-(4-*tert*-butoxyphenyl)propyl]oxy}acetate (16)

Azido alcohol **15** (0.40 g, 1.6 mmol) was added to a suspension of KH (0.13 g, 3.2 mmol) in THF (7 mL) at 0 °C followed by addition of ethyl bromoacetate (0.23 mL, 2.1 mmol). After 2 h the reaction was quenched with sat. NH_4Cl (aq.). EtOAc was added, the two phases separated and the aqueous layer was extracted with EtOAc. The combined organic phases were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (heptane/EtOAc, 9 : 1 → 4 : 1) to give ester **16** (0.48 g, 89%) as a colorless oil. $[\alpha]_D^{20} = -2.7$ (c = 0.15 in $CHCl_3$); IR (neat) 2107, 1756 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.11 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.4 Hz, 2H), 4.21 (q, J = 7.1 Hz, 2H), 4.14 (d, J = 16.6 Hz, 1H), 4.09 (d, J = 16.6 Hz, 1H), 3.79–3.72 (m, 1H), 3.67 (dd, J = 3.9 and 9.7 Hz, 1H), 3.53 (dd, J = 6.7 and 9.7 Hz, 1H), 2.86 (dd, J = 6.0 and 14.0 Hz, 1H), 2.75 (dd, J = 8.1 and 14.0 Hz, 1H), 1.33 (s, 9H), 1.28 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 154.2, 131.9, 129.7, 124.3, 78.4, 73.3, 68.7, 62.9, 60.9, 36.5, 28.8, 14.1; HRMS (FAB) calcd for $C_{17}H_{25}N_3O_4$ 335.1845 (M+), found 335.1846.

{[(2*S*)-2-Azido-3-(4-*tert*-butoxyphenyl)propyl]oxy}acetic acid (17)

To ester **16** (0.10 g, 0.30 mmol) dissolved in EtOH (3 mL, 95%) NaOH(s) (0.073 g, 1.8 mmol) was added. After 6 h the reaction was quenched with AcOH. H_2O and EtOAc were added and the aqueous layer was extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2/MeOH/AcOH, 225 : 25 : 0 → 225 : 25 : 1) and gave acid **17** (0.074 g, 80%) as a colorless oil. $[\alpha]_D^{20} = +8.33$ (c = 0.12 in $CHCl_3$); IR (neat) 2923, 2113, 1737 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.11 (d, J = 8.6 Hz, 2H), 6.94 (d, J = 8.6 Hz, 2H), 4.20 (d, J = 17.1 Hz, 1H), 4.15 (d, J = 17.1 Hz, 1H), 3.81–3.73 (m, 1H), 3.68 (dd, J = 3.7 and 9.9 Hz, 1H), 3.54 (dd, J = 6.9 and 9.9 Hz, 1H), 2.86 (dd, J = 6.0 and 13.9 Hz, 1H), 2.77 (dd, J = 7.8 and 13.9 Hz, 1H), 1.31 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.3, 154.2, 131.6, 129.7, 124.4, 78.6, 73.3, 68.2, 62.8, 36.5, 28.8; HRMS (FAB) calcd for $C_{15}H_{21}N_3O_4$ 307.1532 (M+), found 307.1534.

***N*-({[(2*S*)-2-Amino-3-(4-hydroxyphenyl)propyl]oxy}acetyl)glycyl-L-phenylalanyl-L-leucine (3) and *N*-({[(2*S*)-2-Amino-3-(4-hydroxyphenyl)propyl]oxy}acetyl)-L-phenylalanyl-L-leucine (4)**

Tentagel Leu-Fmoc (0.60 g, 0.22 mmol g^{-1}) was washed with DMF and preswelled in DMF. The amine was liberated by treatment with 20% piperidine in DMF (3 min continuous flow and 7 min rotation) followed by washing with DMF and CH_2Cl_2. Fmoc-Phe-OH (0.20 g, 0.53 mmol) was preactivated in DMF (1.7 mL) with diisopropyl carbodiimide (78 μL, 0.50 mmol) and hydroxy benzotriazole (0.11 g, 0.80 mmol) for 10 min and added to the

preswelled solid phase along with bromophenol blue (80 μL, 2% in DMF). The reaction was rotated for 2.5 h washed with DMF and CH_2Cl_2 to give **18**. Solid phase bound **18** was then separated into two parts and one part (0.30 g, 0.22 mmol g^{-1}) was treated with 20% piperidine in DMF (3 min continuous flow and 7 min rotation) to liberate the amine followed by washing with DMF and CH_2Cl_2. Fmoc-Gly-OH (0.078 g, 0.26 mmol) was preactivated in DMF (1.7 mL) for 10 min with diisopropyl carbodiimide (39 μL, 0.25 mmol) and hydroxy benzotriazole (0.054 g, 0.40 mmol) and added to the preswelled solid phase along with bromophenol blue (40 μL, 2% in DMF). The reaction was rotated for 3 h and the solid phase was washed with DMF and CH_2Cl_2 to give resin bound tripeptide **19**. The second part of dipeptide **18** and tripeptide **19**, respectively (0.30 g, 0.22 mmol g^{-1}) were treated in different experiments with 20% piperidine in DMF (3 min continuous flow and 7 min rotation) to liberate the amine followed by washing with DMF and CH_2Cl_2. HATU (*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) (0.038 g, 0.10 mmol), diisopropylethylamine (29 μL, 0.17 mmol) and acid **17** (0.026 g, 0.085 mmol) in CH_2Cl_2 were added to the pre-swelled solid phase and rotated for 48 h, washed with DMF and CH_2Cl_2 to give solid phase bound peptidomimetics **20** and **22** respectively. The resin was pre-swelled in THF followed by treatment with $SnCl_2$ (0.080 g, 0.33 mmol), thiophenol (0.17 mL, 1.7 mmol) and triethyl amine (0.29 mL, 2.1 mmol) in THF (2 mL) for 24 h, then washed with DMF and CH_2Cl_2 to give amines **21** and **23**. This reduction procedure was repeated twice. The products were cleaved from the solid phase and the acid labile protection groups deprotected using a mixture of trifluoroacetic acid (26 mL), ethanedithiol (0.75 mL), thioanisole (1.5 mL) and H_2O (1.5 mL) for 3 h. The solvents were removed under reduced pressure and the residue was triturated with Et_2O. The remainder was purified by reversed phase HPLC, lyophilized to give **4** (20 mg, 62%) and **3** (25 mg, 70%) as colorless solids.

Compound **3** had: IR (neat) 3276–2856, 1652, 1515 cm^{-1}; $[\alpha]_D^{20} = +8.3$ ($c = 0.06$ in MeOH); ^{1}H NMR (400 MHz, CD_3OD, 25 °C, MeOH) δ 7.28–7.15 (m, 5H), 7.07 (d, $J = 8.6$ Hz, 2H), 6.77 (d, $J = 8.6$ Hz, 2H), 4.73–4.67 (m, 1H), 4.45–4.39 (m, 1H), 4.02 (s, 2H), 3.93 (d, $J = 16.6$ Hz, 1H), 3.84 (d, $J = 16.6$ Hz, 1H), 3.65 (dd, $J = 2.9$ and 10.1 Hz, 1H), 3.62–3.54 (m, 1H), 3.50 (dd, $J = 6.6$ and 10.1 Hz, 1H), 3.20 (dd, $J = 4.9$ and 14.0 Hz, 1H), 2.89 (dd, $J = 9.2$ and 14.0 Hz, 1H), 2.85 (d, $J = 7.2$ Hz, 2H), 1.75–1.61 (m, 3H), 0.95 (d, $J = 6.2$ Hz, 3H), 0.91 (d, $J = 6.2$ Hz, 3H);^{13}C NMR (100 MHz, CD_3OD, 25 °C, MeOH) δ 175.8, 173.4, 172.2, 171.1, 158.0, 138.3, 131.3, 130.4, 129.4, 127.8, 127.0, 116.8, 70.9, 55.7, 54.0, 52.3, 42.6, 41.7, 38.9, 35.7, 26.0, 23.3, 21.9; HRMS (FAB) calcd for $C_{28}H_{39}N_4O_7$ (M + H) 543.2819, found 543.2820.

Compound **4** had: IR (neat) 3388–2500, 1656, 1515 cm^{-1}; $[\alpha]_D^{20} = +24$ ($c = 0.05$ in MeOH); ^{1}H NMR (400 MHz, CD_3OD, 25 °C, MeOH) δ 7.30–7.16 (m, 5H), 7.03 (d, $J = 8.4$ Hz, 2H), 6.77 (d, $J = 8.4$ Hz, 2H), 4.84–4.78 (m, 1H), 4.48–4.42 (m, 1H), 3.96 (d, $J = 15.0$ Hz, 1H), 3.91 (d, $J = 15.0$ Hz, 1H), 3.57–3.48 (m, 2H), 3.46–3.40 (m, 1H), 3.24 (dd, $J = 4.9$ and 14.1 Hz, 1H), 2.92 (dd, $J = 9.2$ and 14.1 Hz, 1H), 2.88–2.76 (m, 2H), 1.78–1.62 (m, 3H), 0.97 (d, $J = 6.2$ Hz, 3H), 0.92 (d, $J = 6.2$ Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD, 25 °C, MeOH) δ 175.7, 173.6, 171.4, 158.0, 138.0, 131.3, 130.3, 129.5, 127.9, 127.0, 116.8, 70.7, 70.4, 54.9, 53.9, 52.3, 41.6, 39.0, 35.6, 26.0, 23.3, 21.8; HRMS (FAB) calcd for $C_{26}H_{36}N_3O_6$ 486.2604 (M + H), found 486.2614.

Radioligand binding assay

Rat brain membranes were prepared as previously described.[25] Briefly, rat brain, without cerebellum, was homogenized in Tris-HCl buffer (20 mL g^{-1}, 0.05% BSA, 50 mM, pH 7.4) at 0 °C. After centrifugation (25000 × *g*) for 40 min the supernatant was discarded and the pellet was homogenized in the same way once more, then put in 37 °C for 30 min followed by centrifugation (25000 × *g*) for 40 min at 4 °C. The resulting pellet was again homogenized, centrifuged (25000 × *g*) for 40 min at 4 °C and resuspended in Tris-HCl buffer (5 mL g^{-1}, 0.05% BSA) and stored at −80 °C until use. The protein concentration for each brain homogenate was determined with BSA as calibration protein, whereby the preparations varied in protein concentration between 5.13 and 9.31 μg mL^{-1}.

Compounds **1–4**, Leu-enkephalin and DSLET ([D-ser^2]Leu-enkephalin-Thr) were diluted in Tris-HCl buffer (0.05% BSA, 50 mM) at pH 7.4 to a concentration of 800 μM and stored at −80 °C until use. [^{3}H]DAMGO ([^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin), μ selective) or [^{3}H]DPDPE ([^{3}H][D-Pen^2, D-Pen^5]enkephalin, δ selective) were used as receptor selective radioligands. Unspecific binding was corrected with naloxone (a final well concentration of 1 μM), and degradation of peptides or peptide like compounds was inhibited by addition of bacitracin (40 μg $well^{-1}$). Compounds **1–4**, Leu-enkephalin and DSLET were added to the homogenized brain also containing bacitracin and radioligand to give a final well concentration ranging from 0.1 → 1000 nM in a final volume of 0.20 mL. Plates were incubated for 1 h at room temperature before radioactivity was measured. The well content was flushed through glass fibers, pre-soaked in polyethyleneimine, and the membrane bound radioactivity was counted by liquid scintillation spectrometry. All samples were tested as triplicates at each concentration, on three different brain homogenates at 7–8 different concentrations in a 96-well plate assay. Specific binding was calculated as total binding minus unspecific binding.

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