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Synthesis of Gly-ψ[(*Z*)CF=CH]-Phe, a fluoroalkene dipeptide isostere, and its incorporation into a Leu-enkephalin peptidomimetic

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TITLE RUNNING HEAD: Replacement of the third peptide bond of Leu-enkephalin by a fluoroalkene.

ABSTRACT

A new Leu-enkephalin peptidomimetic designed to explore the hydrogen bond acceptor ability of the third peptide bond has been prepared and studied. This new analog is produced by replacing the third amide of Leu-enkephalin with a fluoroalkene. An efficient and innovative synthesis of the corresponding dipeptide surrogate Fmoc-Gly-ψ[(*Z*)CF=CH]-Phe-OH is described. The key step involves the alkylation of a tin dienolate from the less hindered face of its chiral sulfonamide auxiliary derived from camphor. Once its synthesis was complete, its incorporation into the peptidomimetic sequence was achieved on a solid support with chlorotrityl resin and following the Fmoc strategy. The peptidomimetic was characterized using competition binding with [^{125}I]-deltorphin I on membrane extracts of HEK293 cells expressing the mouse delta opioid receptor (DOPr) and based on its abilities to inhibit the electrically induced contractions of the mouse *vas deferens* and to activate the ERK1/2 signaling pathway in DRGF11/DOPr-GFP cells. Together with our previous observations, our findings strongly suggest that the third amide bond of Leu-enkephalin primarily acts as a hydrogen bond acceptor in DOPr. Consequently, this amide bond can be successfully replaced by an ester, a thioamide, or a fluoroalkene without greatly impacting the binding or biological activity of the corresponding analogs. The lipophilicity ($LogD_{7.4}$) of the active analog was also measured. It appears that fluoroalkenes are almost as efficient at increasing the lipophilicity as normal alkenes.

Keywords: Delta opioid receptor, enkephalin, amide bonds, fluoroalkene, lipophilicity, H-bonds

INTRODUCTION

Opioids, which are widely used for treating moderate to severe pain, are currently the most powerful class of analgesics and, unlike nonsteroidal anti-inflammatory drugs (NSAIDs), are effective against neuropathic pain.[1,2] The major drawback of this class of compounds is undoubtedly the large array of unwanted effects accompanying acute and chronic treatments (e.g., sedation, constipation, nausea,

tolerance, dependence, addiction and respiratory depression). These effects are commonly associated with the activation of the mu opioid receptor (MOPr), a G protein-coupled receptor (GPCR) that acts as the biological target of most clinically used opioids, including morphine.[3,4] In animal studies, it has been observed that the activation of the delta opioid receptor (DOPr), another opioid receptor subtype (the third one being the kappa opioid receptor, KOPr) produces analgesia in preclinical chronic pain models.[5] Furthermore, the administration of DOPr agonists leads to less addiction and a smaller loss of gastrointestinal motility than the administration of MOPr agonists, and it has been postulated that the selective activation of DOPr could produce analgesia devoid of the undesirable effects commonly associated with opioids.[6] Indeed, SNC-80, a non-peptide selective DOPr agonist, produces analgesia and antidepressant effects while causing limited constipation, addiction or respiratory depression.[5] However, its therapeutic use is prevented by the receptor-mediated convulsions that are often observed with a variety of non-peptide DOPr agonists.[7]

Discovered in 1975 by Hughes and Kosterlitz,[8,9] Leu-enkephalin is a pentapeptide (Tyr-Gly-Gly-Phe-Leu) known to activate DOPr.[10] Despite presenting a good pharmacodynamic profile, this peptide does not produce analgesia when injected intravenously due to its poor pharmacokinetic properties. Indeed, because of their low lipophilicity, enkephalins cannot readily cross the blood-brain barrier to reach the opioid receptors in the central nervous system.[11] When injected intra-cerebroventricularly, however, Leu-enkephalin produces analgesic effects. Unfortunately, the analgesic effect of Leu-enkephalin has a very short duration ($t_{1/2} \approx 2$ min) because it is rapidly degraded by various peptidases (EC 3.4.24.11 enkephalinase and EC 3.4.11.2 alanyl aminopeptidase, among others).[12,13] These weaknesses are largely due to the peptidic nature of the enkephalins. Indeed, the peptide bonds (amide) that link the amino acids constituting these peptides are considered highly hydrophilic, making peptides generally unable to cross the blood-brain barrier and thus the target of peptidase enzymes. These two characteristics make these peptide bonds a strategic target for improving the pharmacokinetic profile of Leu-enkephalin. In the past, bioisosteres have been used extensively to obtain peptide analogs, called peptidomimetics,[14,15]

in which the amide bonds have been replaced by other functional groups, imparting the molecule with an improved pharmacokinetic profile while retaining its biological activity.[16]

By systematically replacing the amide bonds in Leu-enkephalin with various dipeptide isosteres, we and others have previously found that the first amide bond can be substituted by a *trans*-alkene dipeptide isostere,[11] that the second can be substituted by a thioamide,[17] and that the fourth can be substituted either by a thioamide,[17] an ester[13] or an *N*-methyl amide dipeptide isostere[13] without a major loss of affinity or activity toward DOPr. These observations suggest that the first amide bond acts as neither a hydrogen bond donor nor an acceptor, that the second plays a donor role, and that the fourth is an acceptor. Regarding the third amide bond, we observed that its substitution by an ester leads to some losses in affinity (~ 5-fold) and activity.[13] However, the fact that it appears to be somewhat tolerated (and that substitution by a thioamide is less tolerated)[17] could indicate that a hydrogen bond acceptor is important at this position. Substitution by an *N*-methylamide at the same position led to a considerable loss of activity, which may be due to the steric hindrance caused by the addition of a methyl group.[13] To further ascertain the acceptor role of the third amide, it may prove interesting to substitute it by another mimetic acting as a hydrogen bond acceptor that is small enough to avoid a loss of activity.

Geometrically, fluoroalkenes are isosteres for amide bonds. In an amide bond, the lone pair of electrons on the nitrogen is delocalized in the π system of the carbonyl, which provides the C-N bond with a strong double-bond character. The bond length, angles, and rigidity are very similar parameters in fluoroalkenes and in amide bonds.[18] Furthermore, the fluorine atom is able to mimic, to a certain extent, the oxygen atom of the carbonyl because they have similar van der Waals radii (1.47 Å for fluorine and 1.52 Å for oxygen). Thus, they are, to some degree, able to participate in hydrogen bonds as acceptors, although the interaction can be much weaker than with amides. Fluoroalkenes cannot act as hydrogen bond donors. Like many amide bioisosteres, they disturb α-helix and β-turn structures.[15,18,19] Fluoroalkenes acting as amide bioisosteres have been used in the synthesis of substance P analogs that present activity similar to that of the parent peptide and in the synthesis of dipeptidase inhibitors.[20,21] In

addition, fluoroalkenes are considered highly lipophilic, which makes them very interesting isosteres for enhancing membrane permeability.

Although many methods have been reported for the synthesis of fluoroalkene dipeptide isosteres containing a glycine,[22-24] the obtained compounds were either Gly-ψ[(*Z*)CF=CH]-Gly or, in one case, racemic Phe-ψ[(*Z*)CF=CH]-Gly. Thus far, no method has been reported for the synthesis of enantiomerically pure Gly-ψ[(*Z*)CF=CH]-Phe, the dipeptide isostere required for synthesizing a Leu-enkephalin peptidomimetic in which the third amide bond has been substituted with a fluoroalkene. A widely used and versatile method for the synthesis of fluoroalkene dipeptide isosteres (FADIs) involves, as a key step, the copper-mediated reduction of a conjugated alkene followed by a one-pot alkylation. The addition of a chiral auxiliary (such as one derived from camphor) allows the synthesis of *L* and *D* amino acid dipeptide isosteres.[25] This method has led to the synthesis of various fluoroalkene dipeptide isosteres, namely, Val-Gly, Val-Ala, Val-D-Ala, Val-Phe, Val-D-Phe, Val-Asp, Val-D-Asp and Val-D-Tyr.[25]

In this study, we describe the use of copper-mediated reduction followed by a one-pot alkylation of an alkene to synthesize a suitably *N*-protected Gly-Phe fluoroalkene **2** (Figure 1).

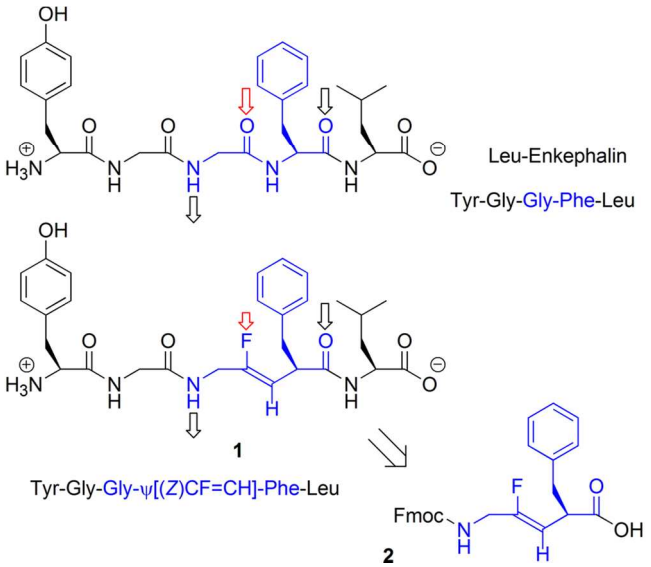


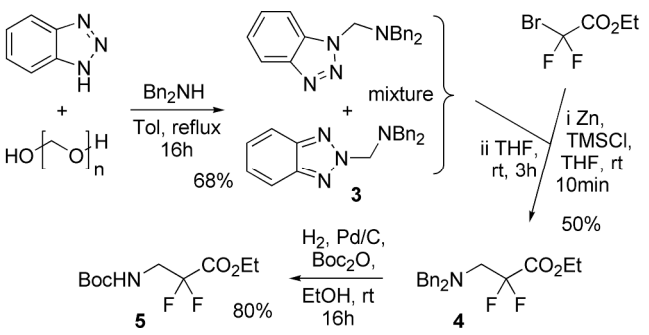
Figure 1. Relationship between Leu-enkephalin and its fluoroalkene peptidomimetic **1** that have a similar H-bond acceptor ability at the third amide position. The structure of the required Fmoc-protected FADI **2** is also shown.

In addition to applying the method for the synthesis of an FADI that has not been previously synthesized, we replaced hexamethylphosphoramide (HMPA), which has been proven to be carcinogenic, with 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU), a solvent with similar characteristics.[26] Furthermore, we introduced Gly-ψ[(*Z*)CF=CH]-Phe into a Leu-enkephalin peptidomimetic **1**. The lipophilicity ($LogD_{7.4}$) of this analog was measured, and its binding affinity and its ability to activate DOPr were also investigated.

RESULTS AND DISCUSSION

Chemistry

The Fmoc-protected fluoroalkene dipeptide isostere of Gly-Phe, Fmoc-Gly-ψ[(*Z*)CF=CH]-Phe **2**, was synthesized using liquid-phase chemistry before being incorporated into the Leu-enkephalin peptidomimetic **1** using solid-phase peptide synthesis. The condensation of benzotriazole, paraformaldehyde and benzylamine using a Dean-Stark apparatus led to the formation of the two isomers of 1,2,3-benzotriazolyl-*N*,*N*-dibenzylmethanamine as an inseparable mixture **3**, which was contaminated with benzotriazole (Scheme 1).[27] A Reformatsky-type reaction between ethyl bromodifluoroacetate and mixture **3** afforded compound **4**.[28]

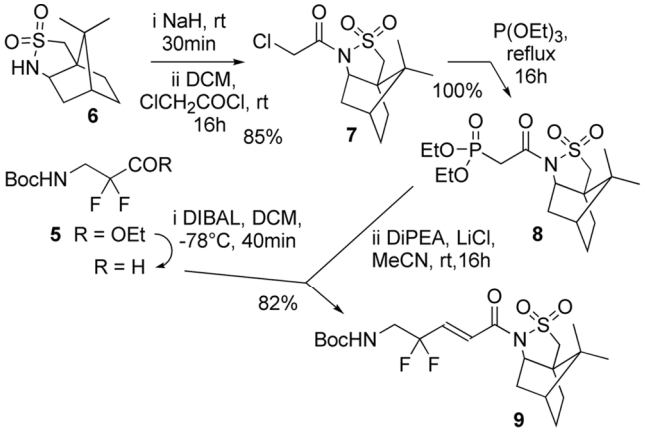


Scheme 1. Synthesis of ethyl 3-(*tert*-butoxycarbonylamino)-2,2-difluoropropanoate **5**.

Mixture **3** was not purified before this last step because the benzotriazole moiety acts as a leaving group in the Reformatsky-type reaction, and both isomers are able to play this role and lead to the same final product. Furthermore, because the reaction is irreversible, the presence of benzotriazole, a reaction product, in the starting mixture is not problematic. Compound **3** was therefore used directly after several washes.

The dibenzylated amine **4** was then hydrogenated using a palladium/carbon catalyst with *tert*-butoxycarbonyl anhydride to yield the Boc-protected amine **5**.[29] Because the use of methanol (as described in the literature)[29] as a solvent led to a mixture of methyl and ethyl esters of **5** (detected by ^{1}H NMR and LC-MS), ethanol was used in place of methanol, thereby avoiding transesterification.

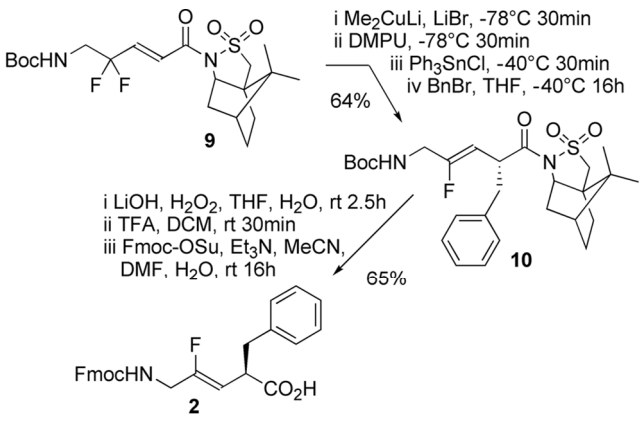
To allow the stereoselective alkylation necessary for the synthesis of Gly-ψ[(*Z*)CF=CH]-Phe, the camphor-based chiral auxiliary **8** was synthesized in two steps (Scheme 2), with a global yield of 85%. Thus, (1*S*)-(-)-camphor-10,2-sultam was deprotonated with sodium hydride and then alkylated using chloroacetyl chloride.[30] The resulting compound **7** then reacted with neat triethyl phosphite to yield phosphonoacetate **8**.[31]



Scheme 2. Synthesis of the conjugated alkene **9**.

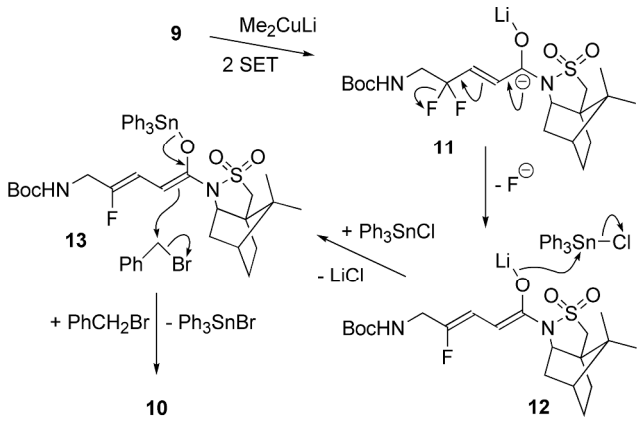
Ester **5** was reduced to the corresponding aldehyde (with DIBAL) before undergoing Horner-Wadsworth-Emmons olefination with phosphonoacetate **8** to yield the conjugated alkene **9**.[19] Lithium chloride was used to help stabilize the phosphonoacetate enolate and thus increase the acidity of the α

protons to the carbonyl, thereby facilitating their removal using a weak base such as *N*,*N*-diisopropylethylamine (DiPEA).[32] The conjugated alkene **9** was consecutively reduced and alkylated (in a one-pot reaction) to yield the deconjugated fluoroalkene **10** (Scheme 3).[19] As the final step of the synthesis, hydrolysis of the chiral auxiliary followed by deprotection of the Boc-protected amine and its subsequent protection with Fmoc yielded carboxylic acid **2** with an overall yield of 14% from ethyl bromodifluoroacetate.[19]



Scheme 3. Synthesis of the Fmoc-protected Gly-Phe fluoroalkene dipeptide isostere **2**.

The reduction of the conjugated alkene **9** occurs in two consecutive single-electron transfers (SETs) by Me_2CuLi (Scheme 4).[19] The resulting anion **11** then undergoes the elimination of a fluorine atom, leading to dienolate **12**. Studies performed with methyl iodide have indicated that this type of lithium dienolate is rather unreactive. Similar to HMPA, DMPU is a solvent known to activate the nucleophilic properties of lithium reagents.[33] DMPU was therefore used to trap the lithium cation and enable its substitution with a tin atom. The tin dienolate **13** then attacked benzyl bromide from its less hindered face (Figure 2) to yield the desired product **10** as a single isomer. The stereochemistry of compound **10** was confirmed by X-ray diffraction (Figure 3).



Scheme 4. Mechanism of the one-pot reduction/alkylation.

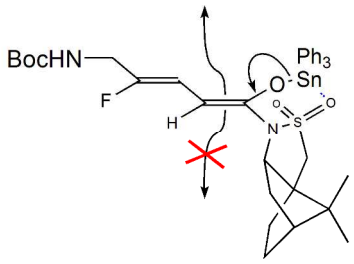


Figure 2. 3D representation of tin dienolate **13**, showing its preferential face (top) for nucleophilic attack. The Boc group is at the front position of the molecule, whereas the chiral camphor region is at the back.

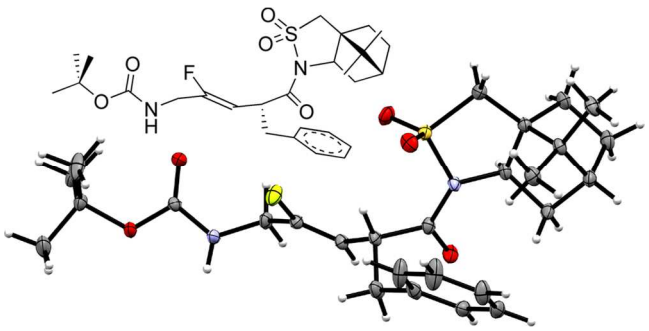
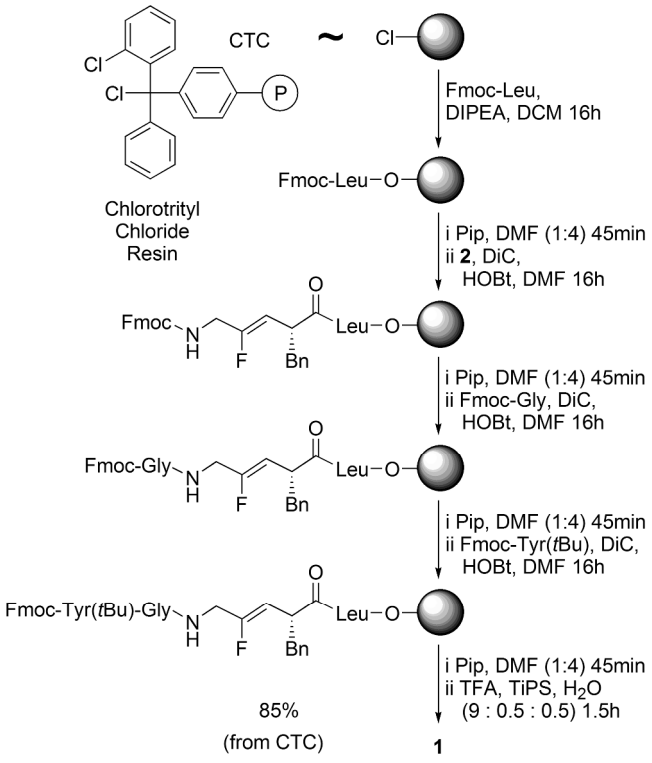


Figure 3. Crystal structure of compound **10**. ORTEP representation with 25% probability level for heavy atoms.

The synthesis of the Leu-enkephalin peptidomimetic was performed via solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin (CTC, Scheme 5). Fmoc-Leu-OH was coupled to CTC in the presence of DiPEA. The second, third and fourth couplings were conducted using compound **2**, Fmoc-Gly-OH and Fmoc-Tyr(*t*Bu)-OH, respectively, with N,N-diisopropylcarbodiimide (DiC) and 1-

hydroxybenzotriazole (HOBt) as coupling agents. Each coupling was followed by washes with DMF/MeOH/DCM and Fmoc deprotection using 20% piperidine (pip) in DMF.[34] Cleavage from the resin and deprotection of the phenol hydroxyl were conducted using a mixture of TFA, TiPS and water, yielding the Leu-enkephalin fluoroalkene peptidomimetic **1** H-Tyr-Gly-Gly-ψ[(*Z*)CF=CH]-Phe-Leu-OH with a yield of 85%, as calculated from the substitution level of the resin (measured by UV absorbance).



Scheme 5. Solid-phase peptide synthesis of the Leu-enkephalin fluoroalkene peptidomimetic **1**.

Binding properties and activity at DOPr

Following the synthesis of the Leu-enkephalin peptidomimetic **1**, its affinity for DOPr and its lipophilicity were evaluated. The affinities of Leu-enkephalin and of the peptidomimetic **1** toward DOPr were measured by competitive binding assays using iodinated deltorphin I ([^{125}I]-deltorphin I) on membrane preparations obtained from HEK293 cells expressing a FLAG-tagged version of the mouse DOPr (Figure 4). For all other peptidomimetics, competitive binding assays were performed with

tritiated deltorphin II ([^{3}H]-deltorphin II) on membrane preparations obtained from GH3 cells (note that Leu-enkephalin was also tested in this assay in our previous publications with similar results).[11,13]

Although compound **1** is still able to bind DOPr (K_i = 43 +/- 14 nM), the substitution of the third amide bond by a fluoroalkene is accompanied by a slight (6x) decrease in affinity compared to Leu-enkephalin (K_i = 6.9 +/- 2.3 nM). However, note that compound **1** shows a considerably higher affinity (>10x) than a peptidomimetic in which the third amide bond has been replaced by an alkene (K_i = 587 +/- 19 nM).[11] These results suggest that the fluoroalkene isostere and the third amide bond of Leu-enkephalin act as a hydrogen bond acceptor. These findings are in agreement with previous results, where the substitution of the third amide bond of Leu-enkephalin by an ester[13] or a thioamide isostere[17] also led to high-affinity DOPr ligands with K_i values of 34 +/- 17 nM and 47 +/- 17 nM, respectively. In summary, Leu-enkephalin and its ester, fluoroalkene, thioamide, and alkene isosteres at the third amide position have their affinities ranked in approximately the same order as their hydrogen bond acceptor abilities[18] (Figure 4), strongly supporting the hypothesis that the third peptide bond interacts with a hydrogen bond donor group inside the binding pocket of DOPr. This same peptide bond does not appear to partner with an acceptor because the ester and the fluoroalkene still retain a reasonable affinity for the receptor, although they have no donor ability. Moreover, the thioamide, which is a considerably stronger hydrogen bond donor that the amide,[18] does not show increased affinity.

As shown in Figure 5, peptidomimetic **1** also induces time- and concentration-dependent phosphorylation of the ERK1/2 signaling pathway. The phosphorylation of ERK1/2 reached a peak at 5 min of stimulation (Fig. 5A), and the effect was significant at 1 μM and 10 μM (Fig. 5B and C). The results of this assay suggest that the ability of peptidomimetic **1** to activate DOPr is similar to that of Leu-enkephalin. The mouse *vas deferens* (MVD) assay was also used to further quantify the potency of peptidomimetic **1**. In this assay, the concentrations of Leu-enkephalin and peptidomimetic **1** necessary to produce a 50% inhibition (EC_{50}) of the MVD contractions induced by electric field stimulation were found to be 30 +/- 2 nM and 226 +/- 21 nM, respectively. The efficacy (E_{max}) of peptidomimetic **1** was

identical to that of Leu-enkephalin. Indeed, at 10 μM, peptidomimetic **1** produced a maximal inhibition of the electric field-induced MVD contractions. These results confirm that although the affinity of peptidomimetic **1** is slightly lower than that of Leu-enkephalin, this compound is fully active on DOPr.

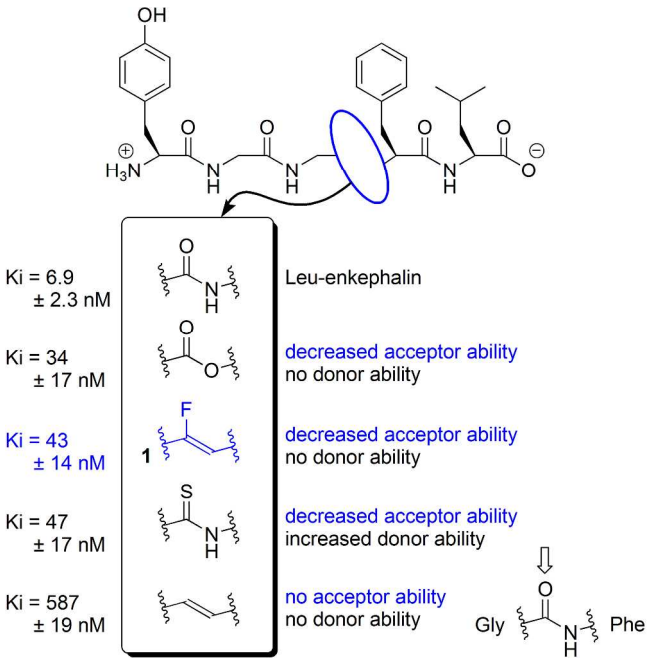


Figure 4. Structures and affinities (K_i) of five Leu-enkephalin analogs, including **1**, that have peptide bond surrogates at the third amide position.

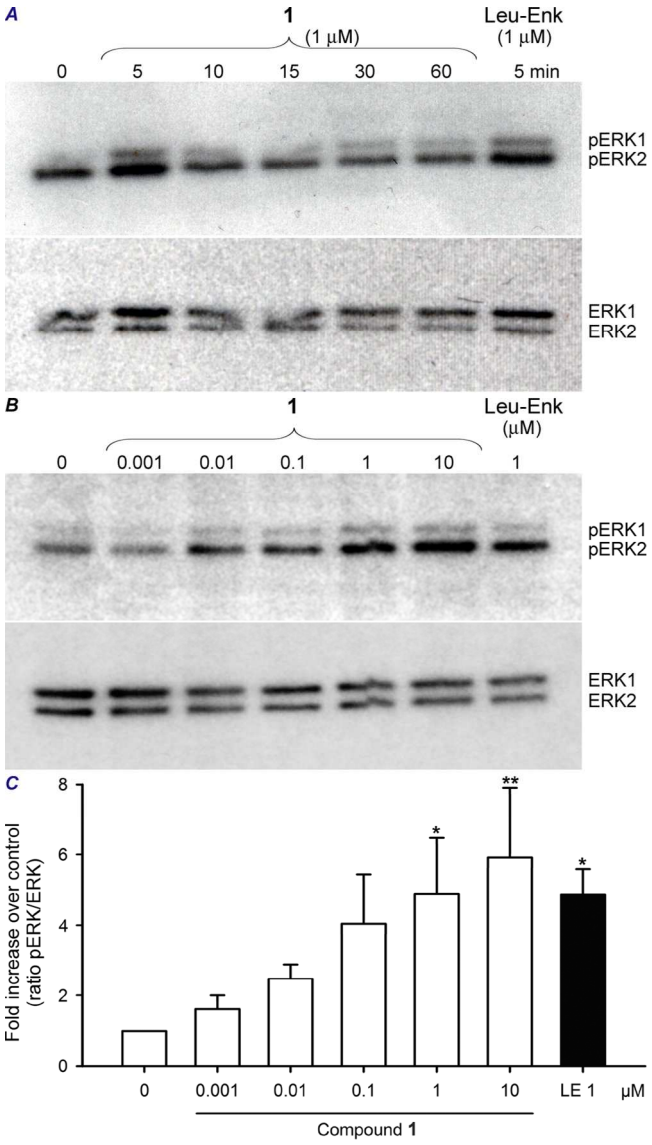


Figure 5. Compound **1** induces a rapid, transient, and dose-dependent phosphorylation (activation) of ERK1/2. *A*; Western blot analyses of phosphorylated ERK1/2 (pERK1 and pERK2) after 5 to 60 min of stimulation of DRGF11/DOPr-GFP cells with compound **1** (1 μM) or 5 min of stimulation with Leu-enkephalin (LE; 1 μM) are shown. The total amount of ERK1/2 was analyzed (bottom blot) and used as a loading control. Images are representative of 3 independent experiments. *B*; Compound **1** produces a dose-dependent phosphorylation of ERK1/2 after 5 min of stimulation. Images are representative of 4 independent experiments. *C*; Ratiometric quantification (ratio of pERK/ERK signals) of the dose-dependent phosphorylation of ERK1/2 induced by compound **1** is illustrated (n = 4). Data are expressed as fold-increase over control +/- standard error of the mean (S.E.M.). One-way ANOVA followed by

Kruskal-Wallis multiple comparison test; * $p < 0.05$ and ** $p < 0.01$ compared to control, non-stimulated cells.

Lipophilicity

The lipophilicity of compound **1** was measured using the $LogD_{7.4}$ (Table 1),[13] which represents the logarithm of the ratio of the compound's concentrations in octanol and in aqueous solution at physiological pH (7.4) after being shaken in a mixture of both solvents and is given by the equation

$$LogD_{7.4} = Log ([X_{octanol}] / [X_{water}])$$

where $[X_{octanol}]$ represents the compound's concentration in the octanol phase and $[X_{water}]$ represents the concentration in the aqueous phase.

Table 1. $LogD_{7.4}$ values for Leu-enkephalin and peptidomimetics containing alkene and fluoroalkene peptide bond isosteres at the third amide position.

Peptidomimetic	$LogD_{7.4}$
Tyr-Gly-Gly-Phe-Leu	-0.954 ± 0.006
Tyr-Gly-Gly//Phe-Leu	-0.065 ± 0.003
Tyr-Gly-Gly*F*//Phe-Leu (**1**)	-0.109 ± 0.007

Gly//Phe and Gly*F*//Phe stand for Gly-ψ[(*E*)CH=CH]-Phe and Gly-ψ[(*Z*)CF=CH]-Phe, respectively.

The substitution of the third amide bond by a fluoroalkene leads to a 0.845 increase in $LogD_{7.4}$, which is 0.044 lower than the value for the compound in which one amide bond has been substituted by an alkene bond (represented by Tyr-Gly-Gly//Phe-Leu).[11] The $LogD_{7.4}$ of compound **1** is still substantially lower than the minimum suggested value (2) obtained by a statistical analysis of drugs that show good permeability toward the blood-brain barrier.[35]

Conclusions

The asymmetric synthesis of the Gly-Phe fluoroalkene dipeptide isostere (Gly-ψ[(*Z*)CF=CH]-Phe) has been accomplished using a one-pot reduction of a conjugated alkene followed by an enantioselective alkylation of the resulting tin dienolate bearing a camphor-based chiral auxiliary, with DMPU as a solvent. The dipeptide isostere was then incorporated into a Leu-enkephalin peptidomimetic using solid-phase peptide synthesis (SPPS). The affinity of peptidomimetic **1** for DOPr was slightly lower than that of Leu-enkephalin, although the loss of affinity was less than one order of magnitude, supporting the hypothesis that a hydrogen bond acceptor is necessary at the third peptide position for optimal complementarity with DOPr. The peptidomimetic also showed greater lipophilicity than Leu-enkephalin, thus demonstrating that the substitution of amide bonds by fluoroalkenes in peptides can be a good approach to improve their pharmacokinetic profiles.

To pursue research on Leu-enkephalin peptidomimetics in the same vein, it would be interesting to sequentially and systematically substitute all the peptide's amide bonds (1, 2 and 4) by a fluoroalkene. The method used in this paper involves a chiral auxiliary for the formation of a chiral center on the C-terminus amino acid of the dipeptide isostere. Another method that uses L-phenylglycine (or its enantiomer) for the formation of the N-terminus amino acid's chiral center[36] could be used to synthesize the Tyr-Gly and Phe-Leu fluoroalkene dipeptide isosteres. The substitution of amides 1 and 4 could be of particular interest because we have shown that the former can be substituted by an alkene and the latter by a hydrogen bond acceptor (ester, thioamide, or *N*-Me amide), both without a substantial loss of affinity or activity for DOPr.[11,13]

EXPERIMENTAL SECTION

Chemistry

Commercial-grade reagents (Sigma-Aldrich and VWR International for organic synthesis and Chem-Impex International for SPPS) were used without further purification. When necessary, the solvents

(Fisher Scientific) were purified and dried prior to use. Peptide purification by HPLC was performed using preparative reverse-phase HPLC, detecting at 280 nm, with a VYDAC 218TP C18 column (Grace Discovery Sciences, Salaberry-de-Valleyfield, Canada) and using an acetonitrile (MeCN) gradient in a 0.1% TFA aqueous solution (from 0% to 60%) with a flow rate of 7 mL/min over 2 h. The purity of all fractions was analyzed using an Agilent 1100 series analytical HPLC, detecting at 214 nm, with a Phenomenex 5 μm, 4.6 × 100 mm C18 column using a gradient of MeCN in 0.1% TFA aqueous solution. Fractions with a purity greater than 95% were pooled and freeze-dried. Optical rotation measurements were performed using a Perkin-Elmer 241 Polarimeter and are reported in units of 10^{-1} deg cm^2 g^{-1}. Infrared spectra were recorded on a Perkin-Elmer Spectrum 1600 FTIR. ^1H and ^{13}C NMR spectra were recorded in deuterated solvents on Bruker AC 300 NMR and Bruker AscendTM 400 spectrometers. The ^1H and ^{13}C NMR chemical shifts are reported in ppm (parts per million). The residual solvent peaks were used as an internal reference. All coupling constants (*J*) are in hertz. The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), qt (quintet), m (multiplet) and br (broad). Mass spectra were recorded on a VG Micromass ZAB-2F or MALDI-TOF or on an ESI-Q-Tof (Maxis).

Ethyl 3-(dibenzylamino)-2,2-difluoropropanoate (4)

A solution containing benzotriazole (18.9 g, 159 mmol), dibenzylamine (30.5 mL, 159 mmol) and paraformaldehyde (4.77 g, 159 mmol) in toluene (400 mL) was heated under reflux with a Dean-Stark trap until the theoretical amount of water (2.86 mL) was collected. The solvent was evaporated under reduced pressure, and the residue was dissolved in ethyl acetate and then washed six times with 1 M aqueous NaOH. The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure, and the resulting residue was triturated with diethyl ether to yield a mixture of isomers (1H-1,2,3-benzotriazol-1-yl-N,N-dibenzylmethanamine and 2H-1,2,3-benzotriazol-2-yl-N,N-dibenzylmethanamine) **3** as a white solid (35.6 g, 68%), which was used in the next step without further purification.

TMSCl (13.8 mL, 108.5 mmol) was added to a suspension of zinc dust (14.2 g, 217 mmol) in anhydrous THF (100 mL) under an argon atmosphere. After stirring for 10 min, $BrCF_2CO_2Et$ (8.97 mL, 70.0 mmol) was slowly added; the reaction appeared to be slightly exothermic. After stirring for 10 min, a mixture of 1H- and 2H-1,2,3-benzotriazol-1-yl-N,N-dibenzylmethanamine **3** (35.6 g, 108.5 mmol) in anhydrous THF (150 mL) was added. The resulting solution was stirred at room temperature for 3 h, after which saturated aqueous $NaHCO_3$ (150 mL) was added, and the mixture was then filtered using diatomaceous earth. The aqueous phase was extracted with EtOAc (3 × 200 mL). The combined organic phases were washed with 1 N aqueous HCl, dried ($MgSO_4$) and then concentrated under reduced pressure. The crude product was purified using dry-column chromatography on silica gel, eluting with ethyl acetate and hexane (1:19) to yield the title compound (11.4 g, 50%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 7.34-7.25 (m, 10H), 4.18 (q, 2H, *J*=7.0 Hz), 3.68 (s, 4H), 3.15 (t, 2H, *J*=13.0 Hz), 1.22 (t, 3H, *J*=7.0 Hz). **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm): 163.8 (t, *J*=32 Hz), 138.1, 129.2, 128.3, 127.3, 116.2 (t, *J*=253 Hz), 62.7, 58.5, 55.2 (t, *J*=26 Hz), 13.8. **IR** (NaCl) ν (cm^{-1}) 3029, 2834, 1767, 1660, 1448, 1057. **Exact mass:** calculated for $C_{19}H_{21}F_2NO_2Na^+$: 356.1433, found: 356.1436.

Ethyl 3-(*tert*-butoxycarbonylamino)-2,2-difluoropropanoate (5)

A solution containing ethyl 3-(dibenzylamino)-2,2-difluoropropanoate (**4**) (11.1 g, 33.4 mmol) and Boc_2O (11.1 g, 50.9 mmol) in EtOH (350 mL) was added to Pd/C (10%, 2.13 g, 2.00 mmol). Gaseous hydrogen (five balloons of approximatively 15 cm in diameter, for a total of approximatively 9 L) was bubbled through the solution while stirring vigorously, and the suspension was stirred overnight at room temperature under a hydrogen atmosphere. The mixture was filtered using diatomaceous earth and rinsed with EtOH. The solvent was evaporated under reduced pressure, and the crude product was purified using flash chromatography on silica gel, eluting with ethyl acetate and hexane (3:17 to 1:3 to 7:13) to yield the title compound (6.72 g, 80%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 4.97 (br, 1H), 4.29 (q, 2H, *J*=7.0 Hz), 3.72 (td, 2H, *J*=13.5, 7.0 Hz), 1.40 (s, 9H), 1.32 (t, 3H, *J*=7.0 Hz).

^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 163.0 (t, *J*=32 Hz), 155.3, 113.3 (t, *J*=252 Hz), 80.3, 63.2, 43.4 (t, *J*=28 Hz), 28.1, 13.8. **IR** (NaCl) ν (cm^{-1}) 3572-3201 (br), 2980, 2941, 1767, 1705, 1516, 1247, 1157, 1124. **Exact mass:** calculated for $C_{10}H_{17}F_2NO_4Na^+$: 276.1018, found: 276.1022.

Chloroacetyl-(*S*)-camphor-10,2-sultam (7)

To a solution of (1*S*)-(-)-camphor-10,2-sultam (5.58 g, 25.9 mmol) in DCM (235 mL) was slowly added NaH (60% suspension in mineral oil, 1.04 g, 25.9 mmol), and the suspension was stirred for 30 min at room temperature under an argon atmosphere. The temperature was lowered to -60 °C, and chloroacetyl chloride (2.48 mL, 31.07 mmol) was added dropwise to the mixture, which was then stirred overnight at room temperature. Saturated aqueous ammonium chloride solution and water were added to the mixture, and the layers were separated. The aqueous phase was extracted twice with DCM. The combined organic layers were washed with saturated aqueous sodium bicarbonate and then dried using anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure. The crude product was purified using flash chromatography on silica gel, eluting with ethyl acetate and hexane (1:7) to yield the title compound (6.40 g, 85%) as a crystalline white solid. **mp** 131-132 °C. **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 4.50 (s, 2H), 3.92 (dd, 1H, *J*=7.5, 5.0 Hz), 3.53 (d, 1H, *J*=14.0 Hz), 3.46 (d, 1H, *J*=14.0 Hz), 2.22-2.05 (m, 2H), 1.99-1.87 (m, 3H), 1.48-1.37 (m, 2H), 1.14 (s, 3H), 0.98 (s, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm): 165.6, 65.4, 52.6, 49.2, 47.9, 44.5, 42.4, 38.0, 32.7, 26.4, 20.7, 19.8. **IR** (NaCl) ν (cm^{-1}) 3750-3020 (br), 3005, 2962, 1655, 1325. **$[\alpha]^{20}_D$** -113 (c=9.06, $CHCl_3$). **Exact mass:** calculated for $C_{12}H_{18}ClNO_3SNa^+$: 314.0588, found: 314.0600.

Diethylphosphonylacetyl-(*S*)-camphor-10,2-sultam (8)

A solution containing chloroacetyl-(*S*)-camphor-10,2-sultam (**7**) (6.25 g, 21.4 mmol) in triethyl phosphite (95 mL, 554 mmol) was heated overnight under reflux under an argon atmosphere, and the triethyl phosphite was removed under reduced pressure. The crude product (still containing some triethyl phosphite) was purified using flash chromatography on silica gel, eluting with ethyl acetate and

hexane (2:3 to 1:1 to 3:2) to yield the title compound (8.42 g, 100%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 4.24-4.08 (m, 4H), 3.85 (dd, 1H, *J*=7.5, 5.0 Hz), 3.59-3.36 (m, 3H), 3.17 (1H, dd, *J*= 22.5, 15.5 Hz), 2.19-1.97 (m, 2H), 1.96-1.82 (m, 3H), 1.46-1.20 (m, 8H), 1.14 (s, 3H), 0.93 (s, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm): 163.6 (d, *J*=7 Hz), 65.2, 62.8 (d, *J*=6 Hz), 62.6 (d, *J*=6 Hz), 52.9, 48.1 (d, *J*=38 Hz), 44.6, 38.2, 35.8, 34.0, 32.8, 26.5, 20.7, 19.9, 16.4 (d, *J*=4 Hz), 16,3 (d, J=3 Hz). **IR** (NaCl) ν (cm^{-1}) 3757-3024 (br), 2985, 1638, 1325, 1051, 1018. **Exact mass:** calculated for $C_{16}H_{28}NO_6PSNa^+$: 416.1267, found: 416.1278. **$[\alpha]^{20}_D$** -60.5 (c=7.54, $CHCl_3$).

(*E*)-5-(*tert*-Butoxycarbonyl)amino)-4,4-difluoropent-2-enoyl-(*S*)-camphor-10,2-sultam (9)

To a solution of ethyl-3-(*tert*-butoxycarbonylamino)-2,2-difluoropropanoate (**5**) (4.86 g, 19.2 mmol) in anhydrous DCM (200 mL) at -78 °C under an argon atmosphere was slowly added diisobutylaluminum hydride (1 M in toluene, 30.8 mL, 30.8 mmol). The reaction was closely monitored by thin-layer chromatography on silica gel, and after 40 min, saturated aqueous citric acid and water were added to the mixture, which was then extracted with diethyl ether (3 × 200 mL). The organic phase was washed with brine and dried using anhydrous magnesium sulfate, and the solvent was evaporated under reduced pressure to yield the aldehyde, which was used in the next step without further purification.

To a solution of diethylphosphonylacetyl-(*S*)-camphor-10,2-sultam (**8**) (8.31 g, 21.1 mmol) in MeCN (140 mL) at 0 °C under an argon atmosphere was added LiCl (1.06 g, 25.0 mmol) and DiPEA (4.35 mL, 25.0 mmol). After stirring for 30 min at 0 °C, a solution of the above aldehyde in MeCN (45 mL) was added, and the mixture was stirred for 2 h at 0 °C and overnight at room temperature. Saturated aqueous ammonium chloride was added, and the mixture was extracted with diethyl ether (3 × 200 mL). The organic phase was washed with brine and dried using anhydrous magnesium sulfate, and the solvent was evaporated under reduced pressure. The crude product was purified using flash chromatography on silica gel, eluting with ethyl acetate and hexane (1:3 to 7:13) to yield the title compound (7.03 g, 82%) as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 7.01 (dt, 1H, *J*=15.5, 2.0 Hz), 6.93-6.81 (m,

1H), 4.81 (t, 1H, *J*=6.0 Hz), 3.94 (dd, 1H, *J*=6.5, 6.5 Hz), 3.64 (td, 2H, *J*=13.5, 6.5 Hz), 3.53 (d, 1H, *J*=13.8 Hz), 3.46 (d, 1H, *J*=13.8 Hz), 2.16-2.07 (m, 2H), 2.01-1.82 (m, 3H), 1.53-1.29 (m, 2H), 1.43 (s, 9H), 1.16 (s, 3H), 0.98 (s, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm): 162.1, 155.4, 137.5 (t, *J*=26 Hz), 125.4 (t, *J*=8 Hz), 118.4 (t, *J*=242 Hz), 80.3, 65.1, 53.0, 48.7, 47.8, 45.2, 44.7, 38.3, 32.9, 28.2, 26.4, 20.8, 19.9. **IR** (NaCl) ν (cm^{-1}) 3493-3288 (br), 2963, 1705, 1683, 1515, 1331, 1163, 1135. **Exact mass:** calculated for $C_{20}H_{30}F_2N_2O_5SNa^+$: 471.1736, found: 471.1746. **$[\alpha]^{20}_D$** -55.0 (c=7.64, $CHCl_3$).

(2*R*,*Z*)-2-Benzyl-5-(N-(*tert*-Butoxycarbonyl)-amino)-4-fluoropent-3-enoyl-(*S*)-camphor-10,2-sultam (10)

To a suspension of CuI (3.43 g, 18.0 mmol) in anhydrous THF (55 mL) was slowly added a solution of MeLi.LiBr complex in diethyl ether (1.5 M, 24.0 mL, 36.0 mmol), using a syringe that was cooled with dry ice for 1 min. The resulting mixture was stirred for 10 min at 0 °C. At this point, the solution has to be colorless (or very slightly pink, but not yellowish, even slightly) for the next step to be successful. The temperature was lowered to -78 °C, and a solution of (*E*)-5-(*tert*-Butoxycarbonyl)amino)-4,4-difluoropent-2-enoyl-(*S*)-camphor-10,2-sultam (**9**) (1.96 g, 4.38 mmol) in anhydrous THF (82 mL) was added dropwise. After stirring for 30 min at -78 °C, DMPU (8.67 mL, 72.0 mmol) was added to the mixture. After stirring for 30 min at -78 °C, a solution of triphenyltin chloride (3.48 g, 9.02 mmol) in anhydrous THF (82 mL) was added to the mixture. After stirring for 30 min at -40 °C, benzyl bromide (4.28 mL, 36.0 mmol) was slowly added to the mixture, which was then stirred overnight at -40 °C. Due to the sensitive nature of the reaction, it should not be monitored by TLC until the next day. The reaction was quenched by the addition of saturated aqueous ammonium chloride and 28% aqueous ammonium hydroxide (1:1). The mixture was then stirred at room temperature for 30 min, after which it was extracted with diethyl ether (3 × 200 mL). The organic phase was washed with brine and dried using anhydrous magnesium sulfate, and the solvent was evaporated under reduced pressure. The crude product was purified using flash chromatography on silica gel, eluting with ethyl acetate and hexane (3:7) to yield the title compound (1.45 g, 64%) as a white solid (all the following data have been

recorded from the solid collected after chromatography). **mp** 54-58 °C **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 7.27-7.06 (m, 5H), 4.97 (dd, 1H, *J*=35.5, 9.5 Hz), 4.69 (br, 1H), 4.45-4.37 (m, 1H), 3.83- 3.59 (m, 3H), 3.41 (d, 1H, *J*=14.0 Hz), 3.34 (d, 1H, *J*=14.0 Hz), 3.09 (dd, 1H, *J*=13.0, 8.0 Hz), 2.77 (dd, 1H, *J*=13.0, 7.5 Hz), 2.08-1.70 (m, 5H), 1.42 (s, 9H), 1.32-1.14 (m, 2H), 0.87 (s, 3H), 0.73 (s, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm): 172.2, 156,9 (d, *J*=261 Hz), 155.3, 137.4, 129.5, 128.2, 126.7, 104.0 (d, *J*=11 Hz), 79.8, 65.1, 53.0, 48.2, 47.6, 44.6, 43.1, 41.1 (d, *J*=33 Hz), 40.3, 38.3, 32.8, 28.3, 26.4, 20.5, 19.8. **IR** (NaCl) ν (cm^{-1}) 3773-2874 (br), 2097, 1638, 1325, 1163. **Exact mass:** calculated for $C_{27}H_{37}FN_2O_5SNa^+$: 543.2299, found: 543.2310. **$[\alpha]^{20}_D$** -57.0 (c=6.75, $CHCl_3$).

(*R*,*Z*)-5-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-2-benzyl-4-fluoropent-3-enoic acid (2)

To a solution of (2*R*,*Z*)-2-benzyl-5-(N-(*tert*-butoxycarbonyl)-amino)-4-fluoropent-3-enoyl-(*S*)-camphor-10,2-sultam (**10**) (117 mg, 0.225 mmol) in THF (3.3 mL) and H_2O (0.6 mL) was added 30% H_2O_2 (133 μL, 1.30 mmol) and LiOH (0.45 mL, 1 N in water, 0.45 mmol). The mixture was stirred at room temperature for 2.5 h, and 1 N aqueous HCl was added until a pH of 2 was reached. The mixture was then extracted with EtOAc (2 × 10 mL). The organic layers were combined and dried using anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure. The crude residue was then dissolved in DCM (10 mL) and TFA (2.7 mL). The solution was stirred at room temperature for 30 min, the solvent was evaporated under reduced pressure, and the resulting residue was dissolved in MeCN (6.7 mL), DMF (6.0 mL) and H_2O (0.7 mL). At a temperature of 0 °C, Fmoc-OSu (106 mg, 0.315 mmol) and Et_3N (62.8 μL, 0.450 mmol) were added, and the mixture was stirred overnight at room temperature. Aqueous 1 N HCl was added until a pH of 2 was reached, and the mixture was extracted with EtOAc (3 × 10 mL). The organic layers were combined and dried using anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel, eluting with EtOAc, hexane and acetic acid (35:65:1 to 40:60:1 to 45:55:1) to yield the title compound (65 mg, 65%) as a white solid. **mp** 80-84 °C **^{1}H NMR** (300 MHz, $CDCl_3$) δ (ppm): 7.77 (d, 2H, *J*=7.5 Hz), 7.61 (d, 2H, *J*=7.5 Hz), 7.41 (dd, 2H, *J*=7.5, 7.5 Hz), 7.32 (dd,

2H, *J*=7.5, 7.5 Hz), 7.26-7.09 (m, 5H), 6.2-5.4 (br, 1H), 4.99-4.71 (m, 2H), 4.44 (d, 2H, *J*=7.0 Hz), 4.21 (t, 1H, *J*=7.0 Hz), 3.87-3.75 (m, 3H), 3.12 (dd, 1H, *J*=13.5, 6.5 Hz), 2.83 (dd, 1H, *J*=13.5, 8.0 Hz). **^{13}C NMR** (75 MHz, $CDCl_3$) δ (ppm): 177.8, 156.8 (d, *J*=259 Hz), 156.2, 143.7, 141.3, 137.7, 129.1, 128.4, 127.8, 127.1, 126.7, 125.0, 120.1, 104.6 (d, *J*=12 Hz), 67.1, 47.1, 42.3, 41.5 (d, *J*=30 Hz), 38.3. **IR** (NaCl) ν (cm^{-1}) 3694-3103 (br), 3063, 3024, 1711, 1521, 1443, 1247, 749, 738. **Exact mass:** calculated for $C_{27}H_{24}FNO_4Na^+$: 468.1582, found: 468.1593. **$[\alpha]^{20}_D$** -29.3 (c=6.58, $CHCl_3$).

(*S*)-2-((*R*,*Z*)-5-(2-((*S*)-2-Amino-3-(4-hydroxyphenyl)propanamido)acetamido)-2-benzyl-4-fluoropent-3-enamido)-4-methylpentanoic acid (1)

H-Tyr-Gly-Gly*F*//Phe-Leu-OH

CTC resin loading: The loading of the resin was determined by UV quantification of Fmoc release following a first coupling with Fmoc-Leu-OH (see details in the next paragraph). An aliquot (precise mass M ~ 10 mg) of resin was dried under vacuum. A mixture of piperidine (1 mL) and DMF (1 mL) was added, and the suspension was agitated in a shaker for 30 min. A portion of the supernate (0.5 mL) was diluted in DCM (4.5 mL), and the UV absorbances (at 290 nm and 301 nm) of the resulting solution were recorded (A_1^{290} and A_1^{301}). The reference absorbances (A_0^{290} and A_0^{301}) were measured in the same way from a blank solution consisting of a mixture of piperidine (0.25 mL), DMF (0.25 mL) and DCM (4.5 mL). The loading of the resin ($mmol.g^{-1}$) was then calculated as the average of the two values $[20{,}000 \times (A_1^{290} - A_0^{290}) / (5{,}800 \times M)]$ and $[20{,}000 \times (A_1^{301} - A_0^{301}) / (7{,}800 \times M)]$, where M is the quantity of the resin aliquot in mg.

SPPS: A small amount of 2-chlorotrityl chloride resin (285 mg, loading of 0.38 mmol/g) was used. Fmoc-Leu-OH (165 mg, 0.456 mmol), DiPEA (517 μL, 2.85 mmol) (the resin was stirred for 5 min with the first 207 μL, 1.14 mmol, before adding the remaining 310 μL, 1.71 mmol) and DCM (5 mL) were used in the first coupling. The free sites were subsequently protected using MeOH (1 mL), DiPEA (0.25 mL) and DCM (4 mL) and agitating in a shaker for 1 h. Fmoc deprotection was performed using

piperidine and DMF (1:4), agitating for 45 min in a shaker. The same Fmoc deprotection procedure was applied after each coupling using the following Fmoc-protected amino acids: compound **2**, Fmoc-Gly-OH and Fmoc-Tyr(*t*Bu)-OH. (*R*,*Z*)-5-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-2-benzyl-4-fluoropent-3-enoic acid (**2**) (288 mg, 0.648 mmol), DiC (102 µL, 0.648 mmol), HOBt.H_2O (99 mg, 0.648 mmol) and DMF (5 mL) were used in the second coupling. Fmoc-Gly-OH (321 mg, 1.08 mmol), DiC (169 µL, 1.08 mmol), HOBt.H_2O (165 mg, 1.08 mmol) and DMF (5 mL) were used in the third coupling. Fmoc-Tyr(*t*Bu)-OH (496 mg, 1.08 mmol), DiC (169 µL, 1.08 mmol), HOBt.H_2O (165 mg, 1.08 mmol) and DMF (5 mL) were used in the fourth coupling. After removal of the N-terminal Fmoc group, cleavage of the final peptide off the resin was performed in a glass vial using TFA, water and TiPS (95:2.5:2.5) and agitating for 1.5 h. After cleavage, the mixture was filtered on cotton into a large amount of water (20 mL). The remaining TFA was evaporated *in vacuo*, and the aqueous solution was freeze-dried. HPLC purification yielded the title peptide as a white solid (51 mg, 85%). **^{1}H NMR** (400 MHz, D_2O) δ (ppm): 7.34 (t, 2H, *J*=7.1 Hz), 7.30-7.23 (m, 3H), 7.17 (d, 2H, *J*=8.6 Hz), 6.89 (d, 2H, *J*=8.6 Hz), 4.98 (dd, 1H, *J*=36.2, 9.3 Hz), 4.29 (dd, 1H, *J*=8.1, 7.9 Hz), 4.25 (t, 1H, *J*= 7.9 Hz), 3.88 (m, 4H), 3.76 (q, 1H, *J*= 7.9 Hz), 3.15 (d, 2H, *J*=7.4 Hz), 3.01 (dd, 1H, *J*=13.6, 8.1 Hz), 2.85 (dd, 1H, *J*=13.6, 7.4 Hz), 1.65-1.45 (m, 3H), 0.88 (d, 3H, *J*=6.3 Hz), 0.82 (d, 3H, *J*=6.2 Hz). **^{1}H NMR** (300 MHz, DMSO-d_6) δ (ppm): 12.42 (br, 1H), 9,33 (s, 1H), 8.70 (t, 1H, *J*=5.5 Hz), 8.24-8.20 (m, 2H), 8.02 (br, 3H), 7.23-7.10 (m, 5H), 7.02 (d, 2H, *J*=8.5 Hz), 6.67 (d, 2H, *J*=8.5 Hz), 4.91 (dd, 1H, *J*=38.0, 9.5 Hz), 4.15-4.08 (m, 1H), 3.95 (br, 1H), 3.84-3.66 (m, 4H), 3.59-3.51 (m, 1H), 3.00-2.90 (m, 2H), 2.77 (dd, 1H, *J*=14.0, 8.5 Hz), 2.55 (dd, 1H, *J*=14.0, 8.5 Hz), 1.62-1.37 (m, 3H), 0.84 (d, 3H, *J*=6.5 Hz), 0.78 (d, 3H, *J*=6.5 Hz). **^{13}C NMR** (101 MHz, D_2O) δ (ppm): 176.2, 175.3, 170.5, 169.7, 156.3 (d, *J*=258 Hz), 155.2, 138.4, 130.8, 129.1, 128.5, 126.7, 125.4, 115.9, 105.1 (d, *J*=12 Hz), 54.5, 51.4, 43.3, 42.3, 39.2 (d, *J*=30 Hz), 37.7, 35.9, 24.3, 22.1, 20.5. **^{13}C NMR** (75 MHz, CD_3OD) δ (ppm): 174.4, 172.4, 168.9, 168.5, 157.9, 156.2 (d, *J*=255 Hz), 139.2, 130.9, 129.5, 128.4, 126.5, 125.2, 115.8, 105.9 (d, *J*=11 Hz), 54.2, 50.8, 43.0, 42.2, 40.5 (d, J=30 Hz), 39.2, 36.7, 24.7, 23.2, 21.8. **^{19}F NMR** (377 MHz,

D_2O) δ (ppm): -75.59 (s, 3F from TFA), 112.51 (dt, *J*=36.2, 12.7 Hz, 1F). **IR** (NaCl) ν (cm^{-1}) 3748, 2971, 2932, 2364, 2339, 1654, 1565. **Exact mass:** calculated for $C_{29}H_{37}FN_4O_6Na^+$: 579.2589, found: 579.2604. $\mathbf{[\alpha]^{20}_D}$ -1.5 (c=3.47, MeOH).

Cell culture

GH3 (a rat pituitary tumor cell line) and HEK293 (human embryonic kidney) cells stably expressing the mouse DOPr and DRGF11 (mouse neuroblastoma / embryonic rat dorsal root ganglia neuron hybridoma) cells stably expressing mouse DOPr-eGFP were grown at 37 °C in DMEM supplemented with 10% fetal bovine serum and 10 IU penicillin, 100 mg/L streptomycin and 2 mM glutamine. The cells were maintained in a humidified atmosphere of 95% air and 5% CO_2.[11]

Competitive binding assays

We evaluated the affinity (K_i) of the different compounds for DOPr using membrane extracts from either GH3 (thioamide) or HEK293 cells (Leu-enkephalin and compound **1**) expressing the mouse DOPr.

Leu-enkephalin and compound **1**: 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 sec before returning to ice. The cells were then harvested in ice-cold buffer A (tris-HCl 20 mM, $MgCl_2$ 5 mM, NaCl 150 mM, 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 resuspended in 1 mL of buffer A. The protein concentration was determined with Bio-Rad DC™ Protein Assay reagents (Bio-Rad Laboratories, Mississauga, ON, Canada), and the pellet was further diluted in buffer A containing 0.1% BSA and 0.01% bacitracin and distributed in 96-well plates. [^{125}I]-Deltorphin I (specific activity: ~2000 Ci/mmol) was used to determine the binding affinity of Leu-enkephalin and peptidomimetic **1** in a competitive binding assay. Experiments were performed using a membrane concentration of 150-200 µg of proteins/mL and 200 000 cpm of the radiolabeled ligand. Non-specific binding was determined using 10 µM non-radioactive deltorphin II. Incubations were performed for 60

min at 37 °C with increasing concentrations of compound ranging from 10 μM to 1 pM, and 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 Wizard^2 Automatic Gamma Counter (PerkinElmer Canada). Data were analyzed using a nonlinear fitting analysis (SigmaPlot 7.0), and K_i values were calculated from the IC_{50} determination using the Cheng-Prusoff equation[37], $K_i = IC_{50} / (1 + ([L]/K_d))$, where K_i represents the inhibition constant, IC_{50} represents the required concentration to inhibit 50% of the radioligand binding, [L] represents the radioligand concentration and K_d represents the dissociation constant of the radioligand (K_d for [^{125}I]-deltorphin I was determined experimentally = 0.9 nM). K_i are expressed as the mean +/- S.E.M. from 3 independent experiments, each performed in duplicate.

Thioamide: After being harvested in phosphate-buffered saline, the GH3 cells were obtained by centrifugation. The resulting pellet was resuspended in a 10 mM potassium phosphate buffer pH 7.2 (buffer B) and centrifuged for 10 min at 40,000 g. The cell pellet was resuspended in buffer B and left on ice for 20 min. The suspension was then centrifuged three times at 800 g for 5 min, the supernatants were saved, and the pellets were resuspended in buffer B. The supernatants were pooled and centrifuged for 10 min at 40,000 g. The resulting pellet was finally resuspended in buffer B supplemented with 0.32 M sucrose and 5 mM EDTA and stored at -80 °C until use. The protein concentration was determined with Bio-Rad DC™ Protein Assay reagents. [^{3}H]-Deltorphin II (specific activity: 30-60 Ci/mmol; PerkinElmer, Waltham, MA, USA) was used to determine the binding affinity of the thioamide in a competitive binding assay. Experiments were performed using a membrane concentration of 100 μg of proteins/mL and a concentration of the radiolabeled ligand near the K_d value previously obtained in saturation binding assays (1 nM). Non-specific binding was determined using 10 μM non-radioactive deltorphin II. Experiments were conducted with tris buffer solution pH 7.4 in 5 mL polypropylene tubes for a final volume of 0.5 mL. Incubations were performed for 60 min at 37 °C, and the reaction was stopped by filtration using ice-cold buffer B on a Whatman GF/C filter (GE Healthcare Life Sciences). Filters were placed in vials containing 8 mL of Ready Gel scintillation cocktail (Beckman Coulter

Canada, Inc., Mississauga, ON, Canada), and the radioactivity was determined using a Beckman Coulter LS-6500 scintillation counter (Beckman Coulter Canada, Inc.). Data were analyzed using a nonlinear fitting analysis (SigmaPlot 7.0), and K_i values were calculated as described above from the IC_{50} determination using the Cheng-Prusoff equation. For [^{3}H]-deltorphin II, the dissociation constant (K_d) was 1 nM. K_i are expressed as the mean +/- S.E.M. from 3 independent experiments, each performed in duplicate.

Mouse *vas deferens* assay

Mice were anesthetized with isoflurane and sacrificed by a cervical dislocation. Their *vas deferens* were dissected out, the semen was ejected, and the tissues were bathed in modified Krebs solution (mM): NaCl 118.1, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and D-glucose 11.1, gassed with 95% O_2 and 5% CO_2 and kept at 37 °C. Longitudinal contractions were recorded isometrically at a constant tension of 0.5 g using a Radnoti force displacement transducer (Radnoti, LLC, Monrovia, CA, USA), amplified by a Powerlab 8/30 and displayed on LabChart 8 (AD Instruments, Colorado Springs, CO, USA). Electric stimulation trains were generated by a Grass S48 square pulse stimulator (Astro-Med, Inc.) interfaced through a stimulus power booster (Stimu-Splitter II; Med-Lab Instruments, Loveland, CO, USA) and connected to a universal coil platinum electrode (Radnoti, LLC, Monrovia, CA, USA). Trains were repeated every 20 sec. The trains consisted of 6 impulses of 20 V for 1 ms with 9 ms intervals. A volume of 150 µL of the tested ligand was added to the 15 mL bath to obtain final concentrations ranging from 10^{-11} to 10^{-5} M. After recording for 5 min, the preparation was washed twice with 15 mL of fresh medium. As a control, Leu-enkephalin was added at a final concentration of 10^{-5} M. The data were analyzed with a nonlinear fitting analysis, and the concentration required to produce a 50% inhibition (EC_{50}) of the contraction was determined. Data are expressed as the mean +/- S.E.M. of 4 different MVDs.

ERK1/2 phosphorylation

ERK1/2 phosphorylation/activation was measured using DRGF11 cells stably expressing green fluorescent protein (GFP)-tagged mDOPr (DRGF11/DOPr-GFP).[11] After 2-3 days in culture, the cells were stimulated for periods ranging from 5 to 60 min with 1 μM of either Leu-enkephalin or compound **1** or for 5 min with increasing concentrations (10^{-9}-10^{-5} M) of compound **1**. At the end of the incubation, the cells were washed with Hanks' buffered saline (HBS; (mM): NaCl 130, KCl 3.5, $CaCl_2 \cdot 2H_2O$ 2.3, $MgCl_2 \cdot 6H_2O$ 0.98, HEPES 5, EGTA 0.5) and stabilized for 10 min in ice-cold HBS containing 0.1 μM staurosporine, 1 mM sodium orthovanadate and Complete™ protease inhibitor (Roche Diagnostics, Laval, Canada). The cells were then lysed for 30 min in 50 mM HEPES pH 7.8 containing 1% Triton X-100, 0.1 μM staurosporine, 1 mM sodium orthovanadate, and Complete™ protease inhibitor. The lysates were then centrifuged at 13,500 g for 10 min at 4 °C, and the supernatants were stored at -20 °C. Proteins (15 μg per sample) were separated on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes. Membranes were then blocked for 1 h in tris-buffered saline (TBS) containing 1% gelatin and incubated for 2 h with rabbit polyclonal anti-phosphorylated $p42/44^{mapk}$ (1:1000; #9101, Cell Signaling Technology, Danvers, MA, USA) or anti-$p42/44^{mapk}$ (1:1000; #9102, Cell Signaling Technology) antibodies. After three washes with TBS containing 0.05% Tween 20 (TBS-T), the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:2000; #NA934, GE Healthcare, Mississauga, ON, Canada), and the proteins were detected using an enhanced chemiluminescence detection system (Amersham ECL western blotting detection reagents, GE Healthcare Life Sciences). Signal intensities were obtained by densitometry using NIH ImageJ software (1.47v).

Log $D_{7.4}$ determination

The distribution coefficient (LogD) was determined using a modified version of the shake-flask method. Before the experiment, octanol and phosphate buffer (PBS pH 7.4) were mixed together for 24 h to allow the saturation of each solution. The mixture was allowed to rest, and the phases were separated

and used as solvents in the coefficient measurement. The experiment was performed at room temperature using triplicates for each measurement. Each peptide (0.1 mg) was placed in a vial to which saturated PBS (1 mL) and octanol (0.5 mL) were added, and the vial was then mechanically shaken for 30 min. The mixture was allowed to rest for 15 min or until the phase separation was complete. Aliquots of both phases were collected and injected into an HPLC (10 μL of each aliquot was injected in an Agilent 1100 series HPLC, column: Agilent Eclipse XD8 C-18 column, 250 mm × 4.6 mm, 5 μm; solvent A, 0.1% TFA in water; solvent B 0.1% TFA in acetonitrile; 10-70% B in A over 20 min; flow rate: 0.2 mL/min; UV detection at 210 nm). The retention time of each peptide was already known from the HPLC purity analysis of each peptide. The octanol peak did not interfere with the experiment. The area under the curve (AUC) of the corresponding peak was integrated for each phase injected. The LogD for each peptide was calculated as follows: $LogD_{7.4} = \log_{10}$ (AUC octanol phase / AUC PBS phase).

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SUPPORTING INFORMATION AVAILABLE.

PDF file containing the following information: ^{1}H NMR spectra for compounds **1**, **2**, **4**, **5**, and **7**-**10**; ^{13}C NMR spectra for compounds **1**, **2**, and **10**; ^{19}F NMR spectrum for compound **1**; HPLC-UV chromatograms of compound **1** and crystal structure report for compound **10**. Crystallographic information file (cif) for compound **10**. These files are available free of charge via the internet at: To be completed by the journal.

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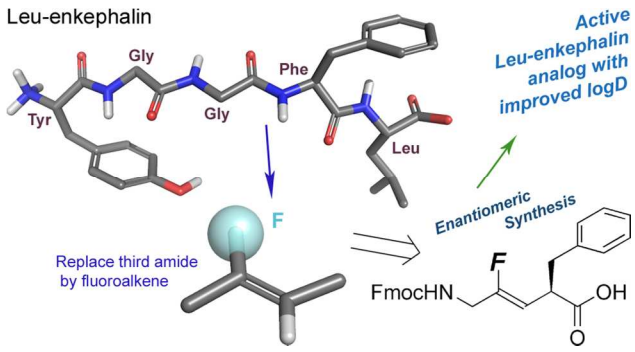
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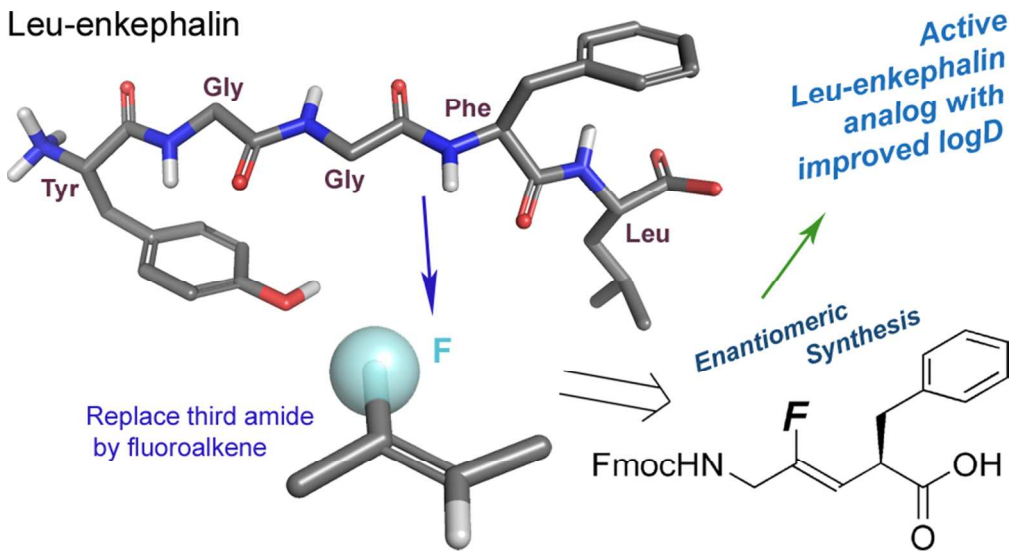

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