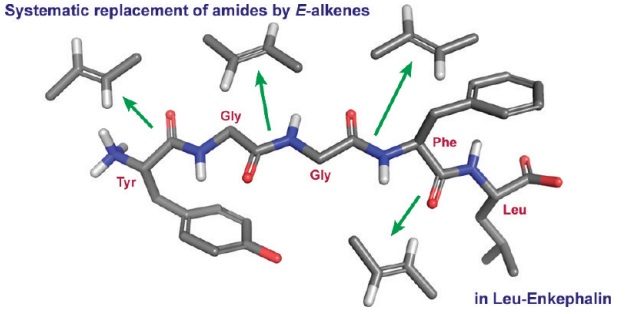
Exploring the Backbone of Enkephalins To Adjust Their Pharmacological Profile for the δ-Opioid Receptor

Arnaud Proteau-Gagné,[†] Véronique Bournival,[‡] Kristina Rochon,[‡] Yves L. Dory,*[,†] and Louis Gendron*[,‡]

[†]Laboratoire de synthèse supramoléculaire, Département de chimie, and [‡]Département de physiologie et biophysique, Institut de Pharmacologie, Université de Sherbrooke, 3001, 12e avenue nord, Sherbrooke, Qc J1H 5N4, Canada

Abstract



The role of each of the four amide bonds in Leu[5]-enkephalin was investigated by systematically and sequentially replacing each with its corresponding *trans*-alkene. Six Leu[5]-enkephalin analogs based on six dipeptide surrogates and two Met[5]-enkephalin analogs were synthesized and thoroughly tested using a δ-opioid receptor internalization assay, an ERK1/2 activation assay, and a competition binding assay to evaluate their biological properties. We observed that an *E*-alkene can efficiently replace the first amide bond of Leu[5]- and Met[5]-enkephalin without significantly affecting biological activity. By contrast, the second amide bond was found to be highly sensitive to the same modification, suggesting that it is involved in biologically essential intra- or intermolecular interactions. Finally, we observed that the affinity and activity of analogs containing an *E*-alkene at either the third or fourth position were partially reduced, indicating that these amide bonds are less important for these intra- or intermolecular interactions. Overall, our study demonstrates that the systematic and sequential replacement of amide bonds by *E*-alkene represents an efficient way to explore peptide backbones.

Keywords: Enkephalin, δ-opioid receptor, *trans*-olefin, *E*-alkene, dipeptide isostere, amide bond

Despite the development of numerous new analgesics, alkaloids are still among the most effective agents for the treatment of both chronic and acute pain. Morphine (Figure 1) and its derivatives bind to and activate at least three types of G-protein coupled receptors (GPCRs), namely, the μ (MOPR), δ (DOPR), and κ (KOPR) opioid receptors (*1*). To date, most clinically used analgesics target MOPR (*2*). However, this receptor is also responsible for the majority of undesirable side effects associated with narcotics. Several recent data indicate that the selective activation of DOPR does not lead to these side effects (*3*−*6*). It is thus generally accepted that, under certain circumstances, a selective DOPR agonist would induce analgesia in the absence of unwanted side effects (*7*). The enkephalins are endogenous pentapeptides [Met[5]-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu[5]-enkephalin (Tyr-Gly-Gly-Phe-Leu)] that exhibit selectivity for the DOPR (1−5× toward MOPR and >1000× toward KOPR). However, the enkephalins and other related peptide agonists are limited in their therapeutic potential by poor pharmacokinetic profiles. In addition to being quickly metabolized by peptidases (mainly the enkephalinase EC 3.4.24.11 and the aminopeptidase M EC 3.4.11.2) (*8*), these compounds are unable to cross the blood−brain barrier (BBB) to reach opioid receptors located in the central nervous system (*9*, *10*). Consequently, a great deal of current research is devoted to the identification of novel nonpeptide DOPR agonists. For example, compounds such as SIOM, SB213698, TAN67, BW373U86, and SNC80 are useful pharmacological tools for the study of DOPR (Figure 1). However, these types of synthetic molecules generally exhibit deleterious side effects (e.g., convulsions), which prevent their use in the clinic (*11*−*13*).

Interestingly, some novel synthetic chemically related small molecules seem to produce fewer unwanted side effects (*14*−*16*). Aside from small molecules, it has also been shown that several linear peptides similar to the enkephalins (i.e., DADLE (Tyr-D-Ala-Gly-Phe-D-Leu) and deltorphin II (Tyr-Ala-Phe-Glu-Val-Val-Gly-NH_2)) and that some cyclic peptides topologically similar to enkephalins (DPDPE, JOM13) exhibit excellent affinity and selectivity for DOPR (Figure 2) (*17*−*20*).

It is likely that the structures of these potent cyclic compounds closely resemble the bioactive conformation

Received Date: August 11, 2010
Accepted Date: September 8, 2010
Published on Web Date: September 17, 2010

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

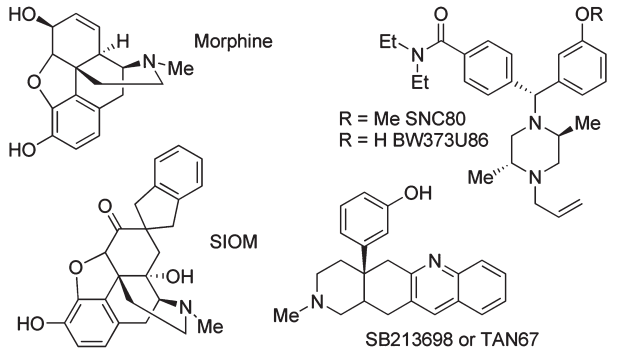


Figure 1. Structures of some nonpeptide opioid receptor agonists. Chemical structures of morphine and various DOPR selective, nonpeptide agonists.

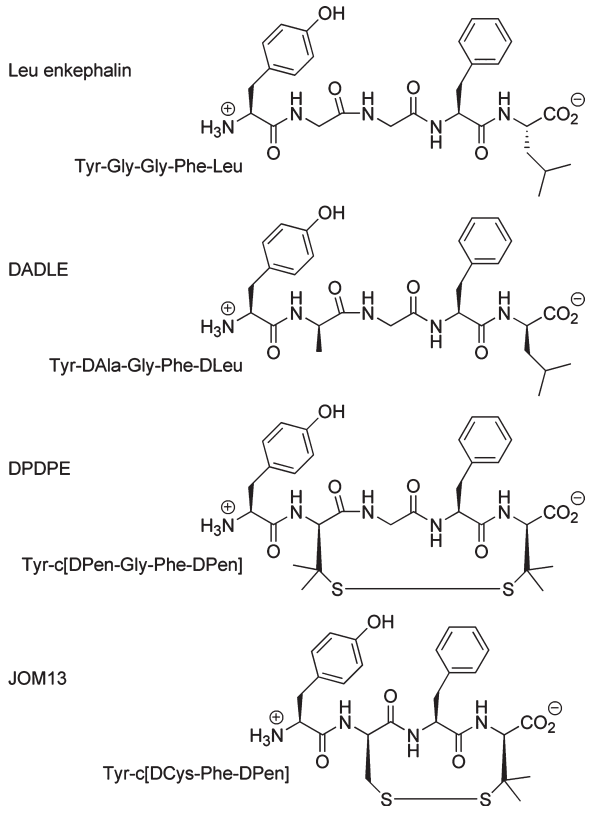


Figure 2. Structures of selected peptide DOPR agonists. Chemical structures of Leu^5-enkephalin and three commonly used DOPR-selective peptide agonists.

of enkephalins. However, the active conformation of enkephalins remains unresolved. Indeed, four different enkephalin crystals have been isolated (*21*−*24*). The present study aimed to address this challenging problem through the systematic investigation of the roles of the amide bonds in enkephalins. In medicinal chemistry, a simple and effective strategy to identify the peptide residues important for biological activity consists of systematically replacing each residue in a given peptide chain with an alanine, a process known as alanine scanning (*25*). Similarly, backbone amides can be scanned by systematically replacing them with topologically equivalent *trans*-olefins. Amides can stabilize active conformations by means of intramolecular hydrogen bonds or be directly involved in binding to receptors via either hydrogen bonds or electrostatic interactions. It is also possible that the amide bonds in enkephalins act simply to hold the peptide chain in a certain conformation without directly interacting with the receptor. The exchange of amide bonds that are not involved in these crucial weak interactions with *trans*-alkenes will likely not result in significant loss in the affinity of enkephalins for DOPRs (*26*). We hypothesized that this method would prove effective in the elucidation of the importance of each enkephalin amide bond.

Being pentapeptides, enkephalins contain four amide bonds. One analysis of crystallographic data has shown the presence of a β-turn with amides 1 and 3 (Figure 3) for Leu^5-enkephalin (*23*). However, there is no evidence indicating that this crystal conformation corresponds to the active geometry of enkephalins. Indeed, induced fit requirements can dictate shapes that are quite distinct from crystal conformations (*27*). In light of this fact, it is possible that there is no β-turn in the active conformation of enkephalins or that a β-turn between amides 2 and 4 might have been selected by DOPR (*28*).

Results and Discussion

We have synthesized and thoroughly tested six Leu^5-enkephalin analogs (**1**−**6**) and two Met^5-enkephalin analogs (**7** and **8**; Figure 4).

These analogs (**1**−**6**) are based on six dipeptide surrogates that have been named Tyr//Gly, Gly//Gly, Gly//Phe, Gly//D-Phe, Phe//Leu, and Phe//D-Leu for reasons of simplicity (*29*−*32*). Each isostere Xxx//Yyy corresponds to the dipeptide Xxx−Yyy in which the amide has been replaced by a *trans*-alkene moiety (*33*, *34*). These six isosteres cover the entire range of potential molecules formed by substitutions at positions α and δ. Thus Gly//Gly is substituted at neither of these positions, Gly//Phe (and its enantiomer Gly//D-Phe) and Tyr//Gly are monosubstituted at positions α and δ, respectively. In contrast, Phe//Leu and its diastereomer Phe//D-Leu are substituted at both of these positions. As shown in Figure 5, each of these four situations required different routes of synthesis.

Synthesis

The first dipeptide surrogate **14** (Tyr//Gly) was synthesized from tyrosine in seven steps with an overall yield of 17% (Scheme 1).

Tyrosine was first esterified as its methyl ester (*35*), then its amine was protected as a *t*-butyl carbamate (*36*). Finally, its phenol was transformed into its corresponding PMB ether, **9** (*36*). The ester was reduced to the aldehyde **10** with dibutyl aluminum hydride; **10** was directly used without additional purification in the subsequent

DOI: 10.1021/cn1000759 | ACS Chem. Neurosci. (2010), 1, 757–769

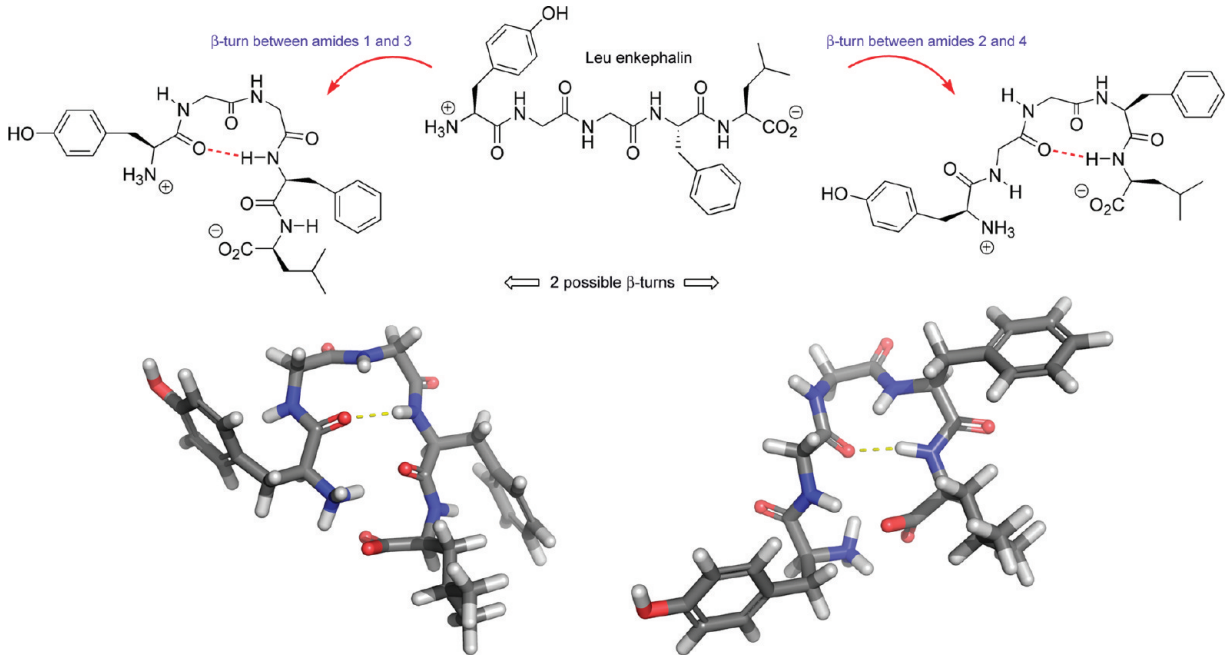


Figure 3. β-turn possibilities for Leu^5-enkephalin. Leu^5-enkephalin could potentially be stabilized by adopting two different β-turns. β-turns created by a hydrogen bond between amides 1 and 3 (left) and between amides 2 and 4 (right) are illustrated. Note that the orientations of the side chains are not meaningful.

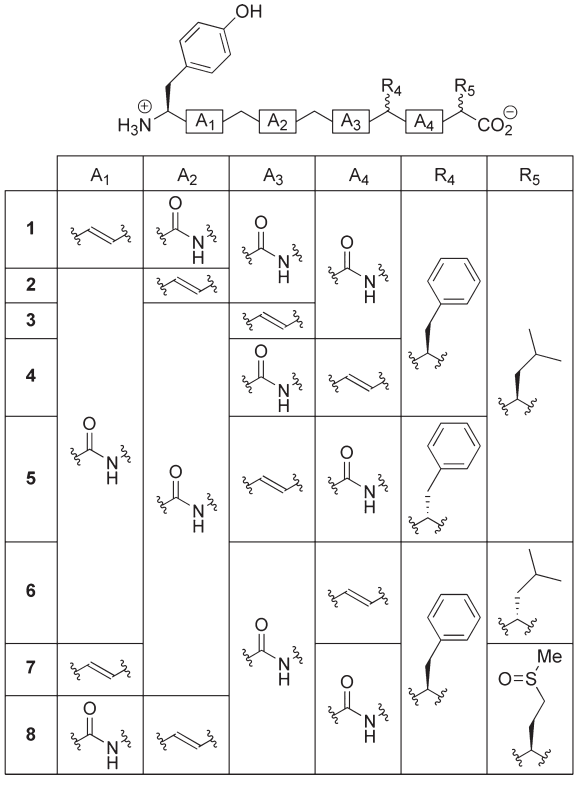


Figure 4. Structures of compounds **1**−**8**.

Wittig reaction to yield the alkene **11**. Both alkenes **11** and **12** were coupled in a metathesis reaction to give fully protected Tyr//Gly, **13** (*37*). Hydrolysis of the benzyl ester of **13** produced **14** in a ready-to-use form as a normal protected amino acid in solid-phase peptide synthesis (SPPS).

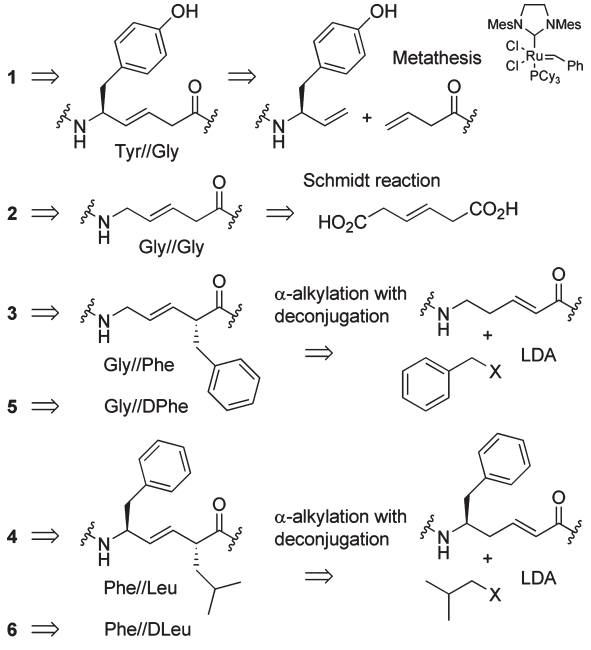
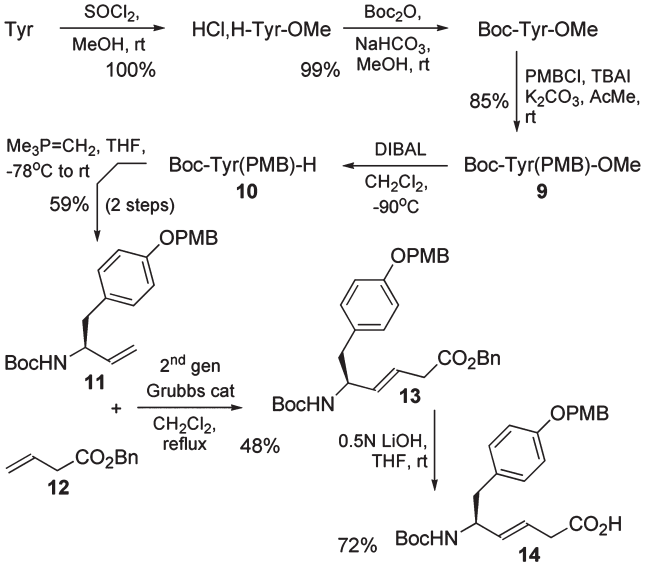


Figure 5. Strategies to synthesize **1**−**6**.

The simplest dipeptide alkene equivalent, Gly//Gly, **16**, was prepared in a single step by simply protecting the amino acid **15** (*38*) as its corresponding fluorenylmethyl carbamate. This synthesis reaction resulted in a yield of 90% (Scheme 2).

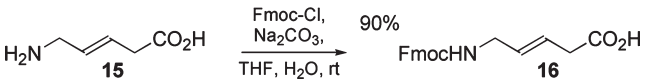
DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

Scheme 1. Synthesis of Dipeptide Surrogate **14**[a]

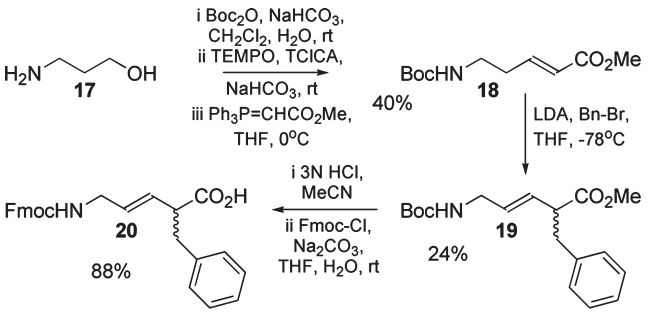


[a] Abbreviations: PMB, p-methoxybenzyl; TBAI, tetrabutyl ammonium iodide; DIBAL, diisobutyl aluminum hydride.

Scheme 2. Synthesis of Dipeptide Surrogate **16**



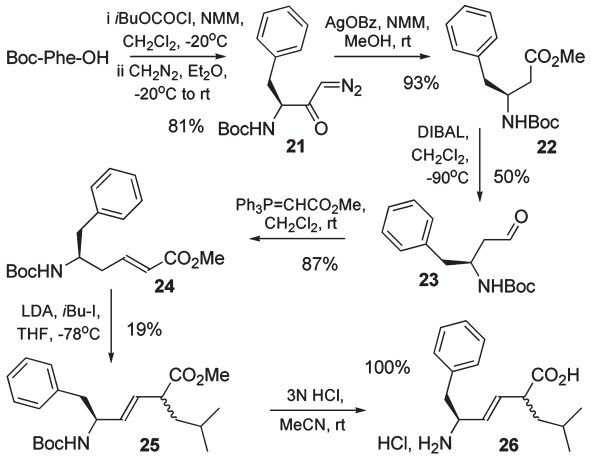
Scheme 3. Synthesis of Dipeptide Surrogate **20**[a]



[a] Abbreviations: TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; TClCA, trichloroisocyanuric acid; LDA, lithium diisopropylamide.

The Gly//Phe dipeptide surrogate **20** was obtained in three steps (Scheme 3) from amino alcohol **17** (overall yield 8%). The amine of **17** was protected as its *t*-butyl carbamate; the alcohol was oxidized to the aldehyde, which was immediately allowed to react with the proper ylide to afford conjugated δ amino acid methyl ester **18**. Treatment of **18** with LDA produced the kinetic conjugated enolate to which benzyl bromide was added to give the desired Gly//Phe dipeptide methyl ester **19** as a racemate. This Gly//Phe dipeptide methyl ester **19** was transformed into **20** by first cleaving all protecting groups with HCl and subsequently introducing an Fmoc group as protection for the amine site.

Scheme 4. Synthesis of Dipeptide Surrogate **26**



The last dipeptide surrogate, Phe//Leu, **26**, was synthesized in six steps from Boc phenylalanine with an overall yield of 6% (Scheme 4). Diazomethane was added to the mixed anhydride that resulted from the addition of isobutyl chloroformate to Boc-Phe to yield the diazoketone **21** (*39*). Wolf rearrangement of **21** gave the ester **22**, which was then reduced to the aldehyde **23**. Addition of the appropriate ylide produced the conjugated δ amino acid methyl ester **24**. Formation of the kinetic enolate followed by addition of isobutyl iodide afforded **25** as an inseparable mixture of diastereomers. Both the methyl ester and the *t*-butyl carbamate were cleaved with HCl to give the amino acid **26**.

With all the dipeptide equivalents (**14**, **16**, **20**, and **26**) in hand, the SPPS of the desired enkephalins could be carried out (Scheme 5). Fmoc-Leu and Fmoc-Met were coupled to Wang resin. From then on, standard procedures were used with Fmoc-Phe, Fmoc-Gly, Fmoc-Tyr(*t*Bu), **14**, and **16** to yield pure Leu^5-enkephalin analogs **1** and **2** and oxidized Met^5-enkephalin analogs **7** and **8**. Dipeptide equivalent **20** was similarly coupled with Leu-Wang resin followed by Fmoc-Gly then Fmoc-Tyr(*t*Bu). Cleavage of the resin with TFA yielded both diastereomers **3** and **5** as a separable mixture (both compounds were actually separated by HPLC and all fractions over 95% in purity were combined). The identity of these molecules was indirectly inferred from their respective affinities (cf. Table 1) for DOPR (Figures 7 and 8). Diastereomer **5**, being virtually inactive, was assigned the Tyr-Gly-Gly//D-Phe-Leu stereochemistry like its inactive parent (Tyr-Gly-Gly-D-Phe-Leu). In contrast, diastereomer **3** retains some affinity or activity for DOPR (*17*, *18*). Compounds **4** and **6** were prepared by a slightly different procedure because the first coupling to the resin would require too much of the dipeptide surrogate Phe//Leu. Instead, Fmoc-Gly, and Boc-Tyr(*t*Bu) were successively coupled to a chlorotrityl resin. Cleavage of the resin with 1% TFA afforded the tripeptide **27**, which was activated as its Pfp ester **28**

DOI: 10.1021/cn1000759 | ACS Chem. Neurosci. (2010), 1, 757–769

Scheme 5. Solid-Phase Synthesis of Enkephalin Analogs **1**−**8**

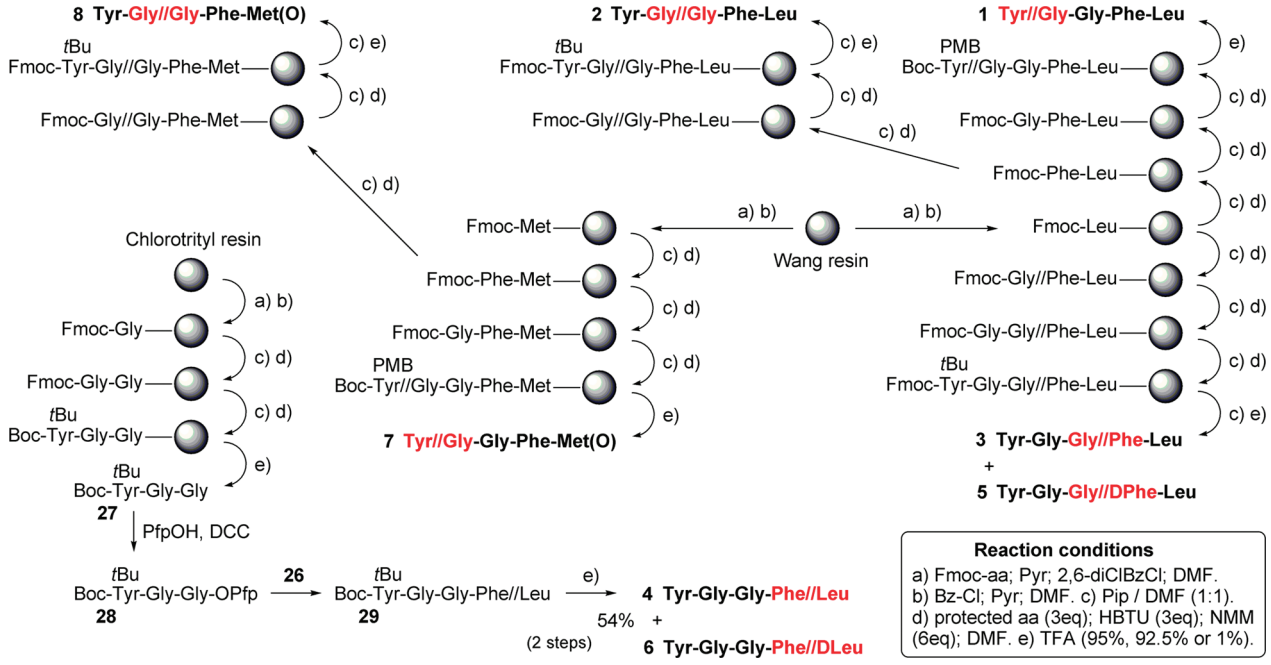


Table 1. *In Vitro* Binding Properties of Leu^5-Enkephalin (LE) and Compounds **1**−**6**

compound no.	name	K_i[a] (nM)
	LE	6.3 ± 0.9
1	Tyr//Gly	13.1 ± 6.2
2	Gly//Gly	761 ± 32
3	Gly//Phe	587 ± 19
4	Phe//Leu	196 ± 29
5	Gly//D-Phe	> 5000
6	Phe//D-Leu	116 ± 31

[a] Binding affinities (K_i) of the compounds were determined by their ability to inhibit the binding of [^{3}H]deltorphin II, a selective DOPR agonist (K_d = 1 nM), to GH3 cell membrane extracts expressing the cloned mouse DOPR. K_i values are the means ± SEM of three to four separate experiments each performed in triplicate.

prior to coupling with **26** in solution. Finally, all *t*Bu protecting groups were cleaved with 50% TFA to give **4** and **6** from **28** in yields of 15% and 39%, respectively. The Tyr-Gly-Gly-Phe//D-Leu stereochemistry was attributed to **6** because it is slightly more active than **4**, similar to Tyr-Gly-Gly-Phe-D-Leu, which is more active toward DOPR than Leu^5-enkephalin (cf. Table 1) (*17*, *18*). Admittedly, this assumption is only based on comparative binding and activity with previous studies and remains to be confirmed by X-ray analysis.

Agonist-Induced Internalization of DOPR

Following agonist stimulation, DOPR undergoes rapid internalization (*40*, *41*). We used this property of DOPR to rapidly screen the putative agonist activity of each compound. Using DRGF11 cells stably expressing the carboxy-terminal GFP-tagged mouse DOPR (DRGF11/DOPR−GFP), we first confirmed that deltorphin II, a potent selective DOPR agonist, was able to induce DOPR internalization. As shown in Figure 6, treatment of DRGF11/DOPR−GFP cells with deltorphin II (100 nM) induced a robust internalization of DOPR in vesicle-like structures. This effect was apparent as soon as 5 min after deltorphin II application (not shown) and almost complete after 35 min, a time point at which minimal fluorescence signal remains at the plasma membrane. Interestingly, at 100 nM, compound **1** produced levels of DOPR internalization comparable to deltorphin II (Figure 6). By contrast, equimolar concentrations of compounds **2**−**6** failed to induce any detectable internalization of DOPR. We therefore tested the effect of a higher concentration (10 μM) of compounds **2**−**6** on DOPR internalization (Figure 7). At this high dose, compound **6** did produce robust internalization of DOPR. Compounds **3** and **4** also triggered DOPR internalization, but this effect was less pronounced than that observed with compound **1** or deltorphin II. The partial efficacy of compounds **3** and **4** was demonstrated by subsequent incubation of compound **3**- or **4**-pretreated DRGF11/DOPR−GFP cells with 100 nM deltorphin II, which induced a more robust internalization of DOPR. Even at 10 μM, compounds **2** and **5** were ineffective at producing DOPR internalization. Our observations therefore suggest that most of our newly synthesized compounds can activate DOPR, with the exception of compounds **2** and **5**. Thus, enkephalin derivatives containing an *E*-alkene in lieu of an amide bond can promote DOPR internalization. Admittedly, internalization

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

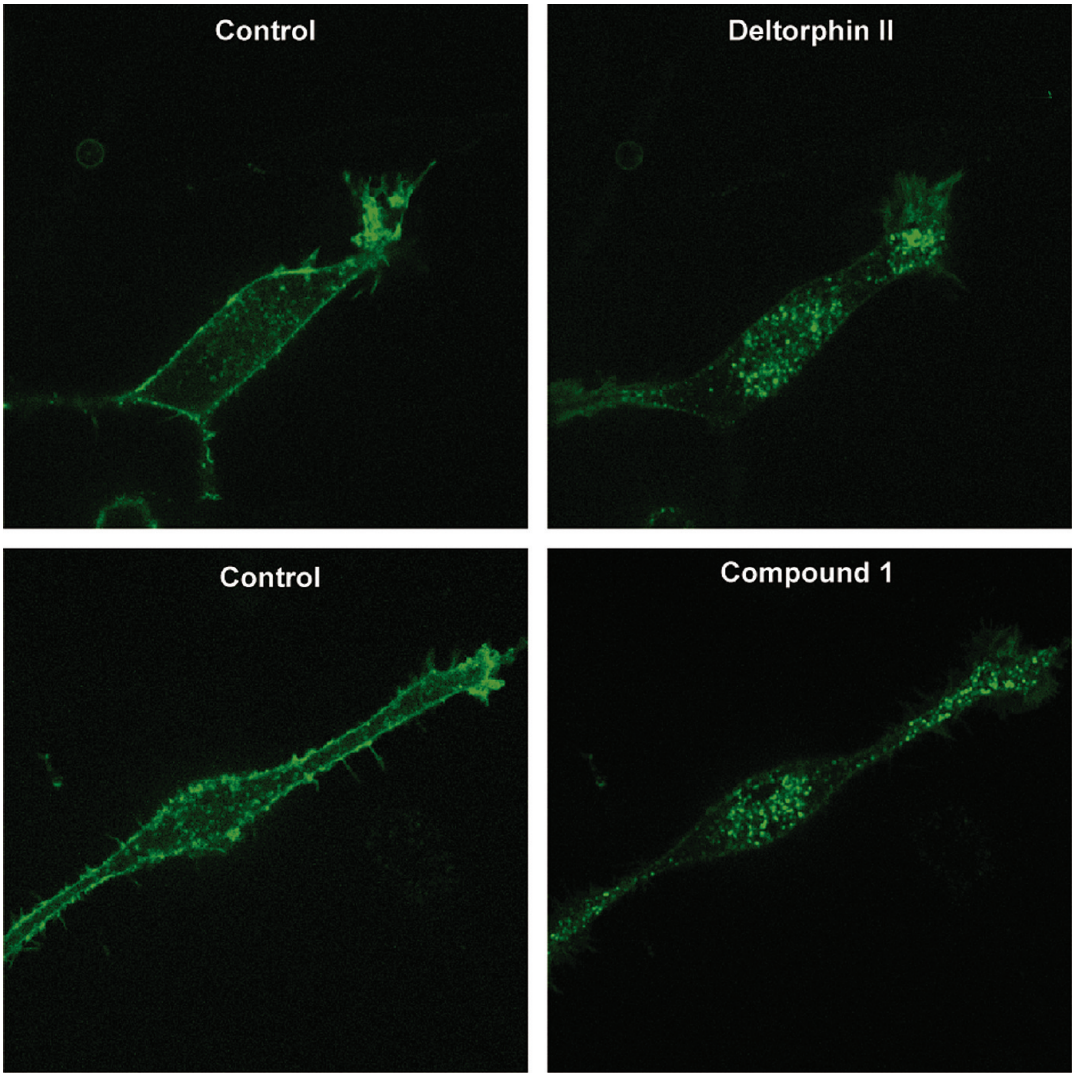


Figure 6. Deltorphin II and compound **1** induced internalization of GFP-tagged DOPR. Following agonist stimulation, DOPR is known to undergo rapid internalization. Confocal microscopy images taken before (control) and 35 min after treatment of DRGF11/DOPR−GFP cells with 100 nM deltorphin II or compound **1** are shown. Deltorphin II and compound **1** both induced robust internalization of DOPR. Indeed, while membrane labeling is decreased, fluorescent vesicle-like structures are clearly visible inside the cells following incubation with either deltorphin II or compound **1**.

assays do not allow for the differentiation between a ligand that binds to the receptor without inducing its internalization and a ligand that does not bind the receptor. Moreover, this technique is not sufficient to determine whether a given compound is an agonist or an antagonist for a receptor. In some cases, opioid agonists fail to induce receptor internalization (*42*). Indeed, morphine only poorly induces MOPR internalization unless GRK2 is overexpressed (*43*), and AR-M100390, a close relative of SNC-80 with potent δ agonist properties, does not induce significant DOPR internalization (*44*, *45*). Therefore, internalization assays are not sufficient to conclude that compounds **2** and **5** are not DOPR agonists. However, the fact that incubation of DRGF11/DOPR−GFP cells with 10 μM of either compound **2** or **5** for 35 min did not interfere with the ability of deltorphin II (100 nM) to promote DOPR internalization suggests these enkephalin analogs have a very low affinity for DOPR.

DOPR-Mediated ERK1/2 Activation

Recent research has established that different ligands can promote diverse receptor-mediated signaling pathways, a phenomenon referred to as "ligand-biased signaling" (*46*). Therefore, a ligand that fails to induce receptor internalization might very well be able to stimulate other signaling cascades. Indeed, some DOPR agonists have been shown to promote extracellular signal-regulated kinase (ERK1/2) activation through internalization-independent mechanisms (*47*, *48*). For this reason, we decided to evaluate the ability of each compound to activate ERK1/2. The classical DOPR-mediated signaling

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

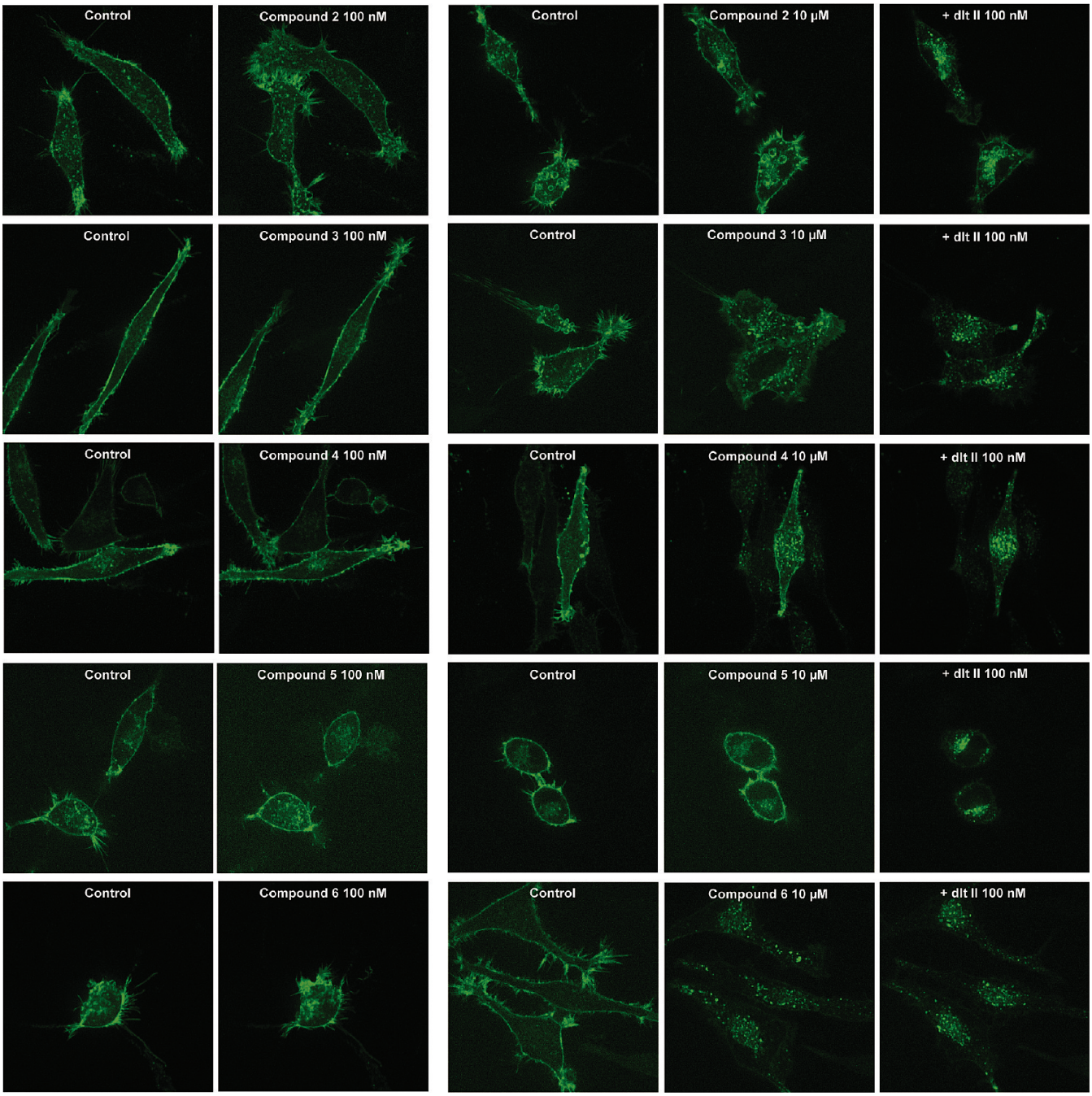


Figure 7. Compounds **2**−**6** differentially affected internalization of GFP-tagged DOPR. The ability of compounds **2**−**6** to promote DOPR internalization in DRGF11/DOPR−GFP cells was studied. Confocal microscopy images taken before (control) and 35 min after treatment of cells with compounds **2**−**6** (100 nM and 10 μM) are shown. At 100 nM, compounds **2**−**6** were unable to induce significant internalization of GFP-tagged DOPR. By contrast, compounds **3**, **4**, and **6** at 10 μM induced different levels of internalization of GFP-tagged DOPR, while compounds **2** and **5** at 10 μM were still unable to produce internalization. Thirty-five minutes following application of compounds **2**−**6**, deltorphin II (100 nM) was added, and images were acquired 35 min later. These images reveal that internalization of GFP-tagged DOPR induced by compounds **3** and **4** was not maximal. Results further suggest that compounds **2** and **5** did not bind DOPR, since they did not reduce deltorphin II-induced internalization of GFP-tagged DOPR.

cascade is well described (*49*). δ-Opioid receptor is coupled to a heterotrimeric G protein having the subunit $G_{\propto i2}$, which induces a decrease in intracellular cyclic AMP levels via inhibition of adenylyl cyclase. Stimulation of DOPR also induces, via the $\beta\gamma$ subunits of the G protein, the rapid and transient activation of ERK1/2. In order to determine whether the enkephalin derivatives synthesized here can lead to ERK1/2 activation, the Western blot technique was used to determine the ability of each compound to induce phosphorylation of

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

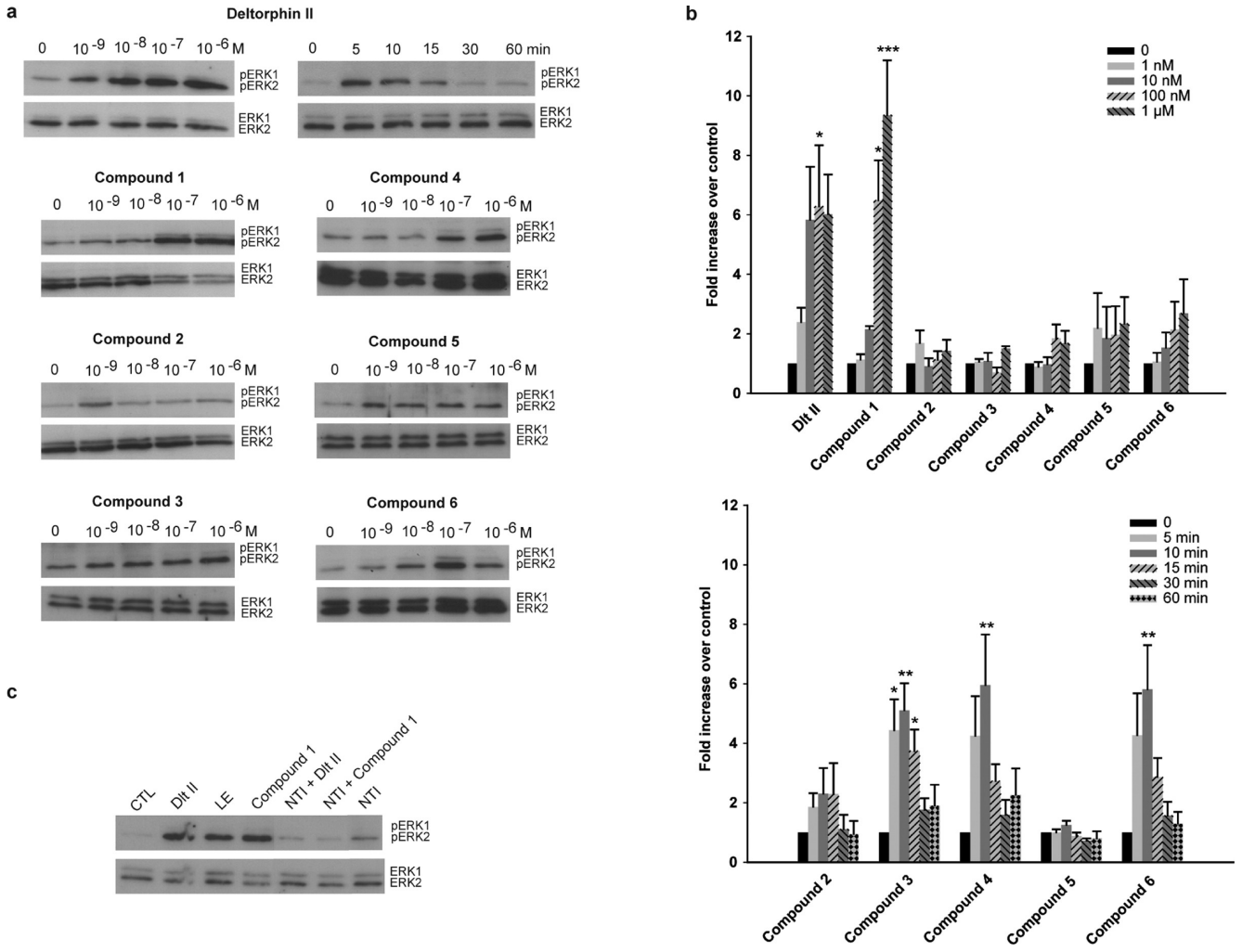


Figure 8. DOPR agonists induced ERK1/2 phosphorylation. Stimulation of DOPR with an agonist induces rapid and transient phosphorylation (activation) of ERK1/2 proteins. (a) Western blot analysis of phosphorylated ERK1/2 (pERK1/2) protein after dose-dependent stimulations (5 min stimulation) of DRGF11/DOPR−GFP cells with deltorphin II and compounds **1**−**6**. Western blot analysis of ERK1/2 phosphorylation after time-dependent stimulation with deltorphin II (100 nM) is also shown, demonstrating transient activation of ERK1/2 with a maximum at 5 min. (b) Densitometric analyses of Western blot results (pERK/ERK ratio compared with control) for dose-dependent responses of deltorphin II and compounds **1**−**6** (top) and for time-dependent responses of compounds **2**−**6** at 10 μM (bottom) are shown. At 100 nM and 1 μM, only deltorphin II and compound **1** induced significant ERK1/2 phosphorylation. Compounds **3**, **4**, and **6** were found to produce significant time-dependent ERK1/2 phosphorylation at a concentration of 10 μM, while compounds **2** and **5** had no effect. Data are the means ± SEM as compared with nontreated cells (0) (one-way ANOVA followed by Dunnett's test) of three to six independent experiments: ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$; . (c) Effects of preincubation with naltrindole (15 min; 1 μM), a selective DOPR antagonist, on ERK1/2 phosphorylation are shown.

residues Thr^{202} and Tyr^{204} of ERK1/2 (*50*−*52*). As shown in Figure 8a, application of deltorphin II to the DRGF11 cell line induced a dose- and time-dependent increase of ERK1/2 phosphorylation (maximal at 5 min). Five minutes after their application to DRGF11 cells at concentrations up to 1 μM, only compound **1** significantly induced phosphorylation of ERK1/2 (Figures 8a,b). This effect was mediated by DOPR since a 15-min preincubation of DRGF11 cells with 1 μM naltrindole (NTI), a DOPR selective antagonist, abolished both deltorphin II and compound **1** induced ERK1/2 phosphorylation (Figure 8c). At a higher concentration (10 μM), compounds **3**, **4**, and **6** produced small but significant increases in ERK1/2 phosphorylation with maximal effects ranging between 5 and 10 min. By contrast, compounds **2** and **5** were unable to stimulate ERK1/2. These results therefore suggest that compound **1** and, to a lesser extent, compounds **3**, **4**, and **6** display agonist properties at DOPR, while compounds **2** and **5** are inactive. Observations made with these compounds revealed that the first amide bond in Leu^5-enkephalin can be replaced by an *E*-alkene without significantly affecting its biological activity, but the replacement of the second amide bond abolishes any interactions with DOPR and, consequently, inhibits the biological activity of enkephalins. As a proof of concept,

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

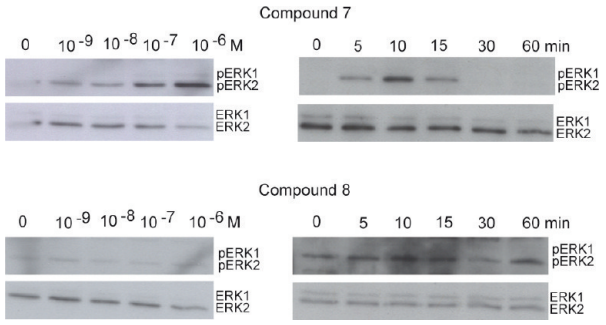


Figure 9. Met[5]-enkephalin derivatives induced similar DOPR-mediated ERK1/2 phosphorylation. Western Blot analysis of phosphorylated ERK1/2 (pERK1/2) protein after dose-dependent (5 min stimulation) and time-dependent stimulations (concentration of 100 nM) in DRGF11/DOPR−GFP cells with compounds **7** and **8** (Met[5]-enkephalin derivatives equivalent to Leu[5]-enkephalin compounds **1** and **2**, respectively) are shown. Compound **7** was found to induce phosphorylation of ERK1/2 in a dose- and time-dependent manner, whereas compound **8** was ineffective. Illustrations are representative of three independent experiments.

we also synthesized compounds **7** and **8**, the Met[5]-enkephalin derivatives equivalent to compounds **1** and **2**, respectively, and measured DOPR-induced ERK1/2 activation. Like compound **1**, compound **7** was found to strongly activate ERK1/2 in a time- and dose-dependent manner (Figure 9). By contrast, compound **8** was inactive, a result that highlights the importance of the second amide bond for the biological activity of enkephalins (Figure 9).

Binding Properties of Enkephalin Derivatives

To further characterize our compounds, we measured their affinity at DOPR. As shown in Table 1, compound **1** has a K_i of 13.1 ± 6.2 nM for DOPR. By comparison, we found a K_i of 6.3 ± 0.9 nM for the unmodified Leu[5]-enkephalin. Compounds **2**−**6** displayed lower affinity than Leu[5]-enkephalin and compound **1**. Interestingly, the K_i values for compounds **1**−**6** are in accordance with the observations previously made with the receptor internalization and ERK1/2 assays. Indeed, compounds **2** and **5** have poor affinity for DOPR. Compounds **3**, **4**, and **6** were found to bind DOPR with modest affinity, a finding consistent with our observation that these compounds were less efficient than Leu[5]-enkephalin and compound **1** at promoting DOPR internalization (Figures 6 and 7) and ERK1/2 phosphorylation (Figure 8). Compound **5**, the epimer of compound **3** at the Phe[4] position, was unable to bind DOPR. This result is consistent with previous research showing that the replacement of L-Phe[4] with D-Phe[4] in Leu[5]-enkephalin strongly reduces its affinity and activity for DOPR (*17*, *18*). In fact, it is known that the phenyl side chain of the L-Phe[4] residue of Leu[5]-enkephalin is critical for its biological activity (*17*) and that Leu[5]-enkephalin containing the hindered amino acid dibenzylglycine (Dbg) at position 4 is a potent DOPR agonist (*53*). Based on this information, we concluded that compound **3** contains L-Phe[4] while compound **5** contains D-Phe[4]. Compound **6** is an epimer of compound **4** at the Leu[5] position. In this case, both L-Leu[5]- and D-Leu[5]-enkephalin were shown to bind and activate DOPR similarly (*17*, *18*). Still, the slightly better affinity of compound **6** led us to hypothesize that this compound contains a D-Leu at position 5 while compound **4** contains an L-Leu at the same position.

Conclusions

Recently, multiple strategies have been used to generate enkephalin analogs containing modified amide bonds. For example, Leu[5]-enkephalin analogs containing an α-amino squaric acid (*54*) or a cyclopropane (*55*) have been synthesized. In addition, cyclic analogs have been prepared to evaluate Leu[5]-enkephalin turn mimetics (*56*). Although these analogs remained active on MOPR, DOPR, or both, such modifications significantly affected their affinity. In the present study, we demonstrated that *E*-alkene can efficiently replace the first amide bond of Leu[5]- and Met[5]-enkephalin. By contrast, the second amide bond was found to be highly sensitive to the same modification. These results are in accordance with work from other groups that introduced *trans*-olefinic dipeptides into enkephalins (*57*−*59*). Our observations with enkephalin analogs containing a modified third or fourth amide bond are of particular interest. Indeed, although the affinity and the activity of these analogs were decreased following the introduction of an *E*-alkene at either of these positions, our results suggest that these amide bonds are involved in weaker and less important intramolecular (such as those necessary for enkephalin to adopt a biologically active conformation) or intermolecular interactions (i.e., with DOPR). Further studies and modifications are required to identify the precise nature of these interactions. Nonetheless, the systematic and sequential replacement of amide bonds by *E*-alkene represents an efficient way to explore the backbone of peptides and to further understand the role and nature of interactions involving these amide bonds.

Methods

Chemicals

The following reagents were used for this study: horseradish peroxidase (HRP)-conjugated anti-rabbit antibody from Amersham Pharmacia Biotech (Oakville, ON, Canada); bovine serum albumin from BioShop (Burlington, ON, Canada); naltrindole hydrochloride (NTI) from Cedarlane (Burlington, ON, Canada); DMEM, fetal bovine serum, and Geneticin from Gibco BRL (Burlington, ON, Canada); anti-phosphorylated

DOI: 10.1021/cn1000759 | ACS Chem. Neurosci. (2010), 1, 757–769

p42/p44^{mapk} and anti-p42/p44^{mapk} rabbit antibodies from New England Biolab (Beverly, MA); [^{3}H]deltorphin II from PerkinElmer (Boston, MA); hygromycin, complete protease inhibitor, polyvinylidene difluoride (PVDF) membranes, and enhanced chemiluminescence (ECL) system from Roche (Montréal, QC, Canada); gentamicin from Sandoz (Boucherville, QC, Canada); deltorphin II, Leu^5-enkephalin, and poly(L-lysine) from Sigma (St. Louis, MO). Note that experimental procedures and spectral characterization data are available as Supporting Information.

Cell Culture

GH3 cells expressing the mouse DOPR (*60*) were kindly provided by Dr. Paul L. Prather (University of Arkansas for Medical Sciences, Little Rock, AR). GH3/DOPR cells were cultured in DMEM supplemented with 10% FBS and 50 mg/L gentamicin at 37 °C in poly(L-lysine) (25 mg/mL) coated Petri dishes in a humidified atmosphere of 95% air and 5% CO_2.

DRGF11 cells were transfected with a DOPR−GFP construct kindly provided by Dr. Rüdiger Schulz (University of Munich, Germany) (*61*). DRGF11/DOPR−GFP cells were cultured in DMEM supplemented with 10% FBS and 50 mg/L gentamicin at 37 °C in Petri dishes, in a humidified atmosphere of 95% air and 5% CO_2.

Receptor Internalization Assay

DRGF11/DOPR−GFP cells were grown on 35-mm glass bottom dishes (MatTek Corporation) for 2−3 days. For internalization assays, medium was first replaced with Earle's buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.2% BSA, and 0.09% glucose). For each assay, three different sites of each dish were observed with a IX81 Olympus microscope fitted with a ProScan II motorized stage system (Prior scientific) and a CSU-X1 confocal scanner unit (Yokogawa). Images were acquired (at interval of 30 s) at room temperature using a 60× objective and a QuantEM: 512SC camera (Photometrics) and processed with MetaMorph software (Molecular Devices). Approximately 3 min of baseline recording was performed prior to the addition of tested compounds. Each compound was first tested at a concentration of 100 nM. For compounds that induced no observable internalization within 35 min, an additional set of experiments was performed using a concentration of 10 μM for 35 min, followed by stimulation with deltorphin II (100 nM) for another 35 min.

MAPK Activity Measurements

DRGF11/DOPR−GFP cells were grown for 3 days in DMEM medium containing 10% FBS and then stimulated for various time intervals (5−60 min) with deltorphin II (100 nM) or other compounds of interest (100 nM). The reaction was stopped by aspiration of the medium and the addition of ice-cold Hanks' balanced salt solution containing 0.1 μM staurosporine and 1 mM sodium orthovanadate. Cells were then left on ice for 30 min and lysed 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 cell lysates were centrifuged at 8000*g* for 15 min at 4 °C, and the supernatants were stored at −20 °C until use. For each lysate, equal amounts of protein (25 μg) were separated on 10% SDS−polyacrylamide gels and transferred to PVDF membranes. PVDF membranes containing proteins were incubated for 2 h at room temperature with anti-phosphorylated p42/p44^{mapk} (1:1000) or anti-p42/p44^{mapk} (1:1000) rabbit antibodies, followed by three washes with Tris-buffered saline/Tween 20. Detection of immunoreactive proteins was accomplished using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:2000) and an enhanced chemiluminescence detection system.

Competitive Binding Assay

Binding assays used to establish affinities for DOPR were performed using membranes of GH3/DOPR cells. Cells were harvested in phosphate-buffered saline and collected by centrifugation. Cell pellets were then washed by resuspension in 10 mM potassium phosphate buffer, pH 7.2 (buffer A), followed by centrifugation at 40 000*g* for 10 min. The resulting pellet was resuspended in buffer A, incubated on ice for 20 min, and centrifuged at 800*g* for 5 min, saving the supernatant. The low-speed pellet was resuspended in buffer A again, and the last step was repeated two more times. The low-speed supernatants were pooled and centrifuged at high speed (40 000*g*, 10 min). The resulting pellets were resuspended in buffer A containing 0.32 M sucrose and 5 mM EDTA (buffer B) and stored at −80 °C. Protein concentrations were determined using the protein assay kit from Sigma Aldrich. [^{3}H]Deltorphin II was used to selectively label DOPR expressed at high levels in the cell membrane preparations. Binding experiments were performed in 50 mM Tris buffer, pH 7.4, in 5-mL polypropylene tubes (final volume of 0.5 mL). Incubations were carried out for 60 min at 37 °C. Nonspecific binding was determined using deltorphin II at 10^{-5} M. The reactions were terminated by filtration using cold assay buffer (3 × 2 mL) on a GF/C. The radioactivity present on the disks was determined by liquid scintillation counting using a Beckman LS6500 beta counter. Experiments were performed using a membrane concentration of 100 μg protein/mL and radioligand concentrations close to the experimental K_d value (2.2 ± 0.2 nM) obtained from saturation studies. The data obtained from competition binding experiments were analyzed with nonlinear fitting analysis. The K_i values were determined from IC_{50} determinations using the Cheng−Prusoff equation (*62*).

DOI: 10.1021/cn1000759 | ACS Chem. Neurosci. (2010), 1, 757–769

Acknowledgment

Authors are grateful to Dave Dubois who transfected the DRGF11 cells.

Supporting Information Available

Experimental procedures and spectral characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Information

Corresponding Author

* To whom correspondence should be addressed. E-mail addresses: Yves.Dory@USherbrooke.ca or Louis.Gendron@USherbrooke.ca.

Author Contributions

Authors A.P.-G. and V.B. contributed equally to this work.

Funding Sources

Until December 2009, this work was financially supported by Grant No. DG-218405 from the Natural Sciences and Engineering Research Council of Canada (NSERC) to Y.L.D. and Grant No. MOP-84538 from the Canadian Institute for Health Sciences (CIHR) awarded to L.G. Since January 2010, this work has been supported by Grant No. MOP-102612 awarded to L.G. and Y.L.D. A.P.-G and K.R. are the recipients of the 2009 Dominico-Regoli/Institut de Pharmacologie de Sherbrooke fellowship and the 2010 Centre des Neurosciences/Institut de Pharmacologie de Sherbrooke fellowship, respectively. L.G. is the recipient of a Junior 1-salary support from the Fonds de la Recherche en Santé du Québec.

References

1. Kieffer, B. L. (1999) Opioids: First lessons from knockout mice. *Trends Pharmacol. Sci. 20*, 19–26.

2. Bodnar, R. J., and Klein, G. E. (2005) Endogenous opiates and behavior: 2004. *Peptides 26*, 2629–2711.

3. Beaudry, H., Proteau-Gagne, A., Li, S., Dory, Y., Chavkin, C., and Gendron, L. (2009) Differential noxious and motor tolerance of chronic delta opioid receptor agonists in rodents. *Neuroscience 161*, 381–391.

4. Gallantine, E. L., and Meert, T. F. (2005) A comparison of the antinociceptive and adverse effects of the mu-opioid agonist morphine and the delta-opioid agonist SNC80. *Basic Clin. Pharmacol. Toxicol. 97*, 39–51.

5. Mika, J., Przewlocki, R., and Przewlocka, B. (2001) The role of delta-opioid receptor subtypes in neuropathic pain. *Eur. J. Pharmacol. 415*, 31–37.

6. Petrillo, P., Angelici, O., Bingham, S., Ficalora, G., Garnier, M., Zaratin, P. F., Petrone, G., Pozzi, O., Sbacchi, M., Stean, T. O., Upton, N., Dondio, G. M., and Scheideler, M. A. (2003) Evidence for a selective role of the delta-opioid agonist [8R-(4bS*,8aalpha,8abeta, 12bbeta)]7,10-Dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro[3,2-e]pyrrolo[2,3-g]iso quinoline hydrochloride (SB-235863) in blocking hyperalgesia associated with inflammatory and neuropathic pain responses. *J. Pharmacol. Exp. Ther. 307*, 1079–1089.

7. Vanderah, T. W. (2010) Delta and kappa opioid receptors as suitable drug targets for pain. *Clin. J, Pain 26* (Suppl 10), S10–15.

8. Roques, B. P., and Noble, F. (1995) Dual inhibitors of enkephalin-degrading enzymes (neutral endopeptidase 24.11 and aminopeptidase N) as potential new medications in the management of pain and opioid addiction. *NIDA Res. Monogr. 147*, 104–145.

9. Cornford, E. M., Braun, L. D., Crane, P. D., and Oldendorf, W. H. (1978) Blood-brain barrier restriction of peptides and the low uptake of enkephalins. *Endocrinology 103*, 1297–1303.

10. Pardridge, W. M., and Mietus, L. J. (1981) Enkephalin and blood-brain barrier: Studies of binding and degradation in isolated brain microvessels. *Endocrinology 109*, 1138–1143.

11. Broom, D. C., Jutkiewicz, E. M., Folk, J. E., Traynor, J. R., Rice, K. C., and Woods, J. H. (2002) Convulsant activity of a non-peptidic delta-opioid receptor agonist is not required for its antidepressant-like effects in Sprague-Dawley rats. *Psychopharmacology* (*Berlin*) *164*, 42–48.

12. Broom, D. C., Nitsche, J. F., Pintar, J. E., Rice, K. C., Woods, J. H., and Traynor, J. R. (2002) Comparison of receptor mechanisms and efficacy requirements for delta-agonist-induced convulsive activity and antinociception in mice. *J. Pharmacol. Exp. Ther. 303*, 723–729.

13. Comer, S. D., Hoenicke, E. M., Sable, A. I., McNutt, R. W., Chang, K. J., De Costa, B. R., Mosberg, H. I., and Woods, J. H. (1993) Convulsive effects of systemic administration of the delta opioid agonist BW373U86 in mice. *J. Pharmacol. Exp. Ther. 267*, 888–895.

14. Codd, E. E., Carson, J. R., Colburn, R. W., Stone, D. J., Van Besien, C. R., Zhang, S. P., Wade, P. R., Gallantine, E. L., Meert, T. F., Molino, L., Pullan, S., Razler, C. M., Dax, S. L., and Flores, C. M. (2009) JNJ-20788560 [9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide], a selective delta opioid receptor agonist, is a potent and efficacious antihyperalgesic agent that does not produce respiratory depression, pharmacologic tolerance, or physical dependence. *J. Pharmacol. Exp. Ther. 329*, 241–251.

15. Le Bourdonnec, B., Windh, R. T., Ajello, C. W., Leister, L. K., Gu, M., Chu, G. H., Tuthill, P. A., Barker, W. M., Koblish, M., Wiant, D. D., Graczyk, T. M., Belanger, S., Cassel, J. A., Feschenko, M. S., Brogdon, B. L., Smith, S. A., Christ, D. D., Derelanko, M. J., Kutz, S., Little, P. J., DeHaven, R. N., DeHaven-Hudkins, D. L., and Dolle, R. E. (2008) Potent, orally bioavailable delta opioid receptor agonists for the treatment of pain: Discovery of *N*,*N*-diethyl-4-(5-hydroxyspiro[chromene-2,4′-piperidine]-4-yl)benzamide (ADL5859). *J. Med. Chem. 51*, 5893–5896.

16. Le Bourdonnec, B., Windh, R. T., Leister, L. K., Zhou, Q. J., Ajello, C. W., Gu, M., Chu, G. H., Tuthill, P. A., Barker, W. M., Koblish, M., Wiant, D. D., Graczyk, T. M., Belanger, S., Cassel, J. A., Feschenko, M. S., Brogdon, B. L.,

Smith, S. A., Derelanko, M. J., Kutz, S., Little, P. J., DeHaven, R. N., DeHaven-Hudkins, D. L., and Dolle, R. E. (2009) Spirocyclic delta opioid receptor agonists for the treatment of pain: Discovery of *N*,*N*-diethyl-3-hydroxy-4-(spiro[chromene-2,4′-piperidine]-4-yl) benzamide (ADL5747). *J. Med. Chem. 52*, 5685–5702.

17. Beddell, C. R., Clark, R. B., Hardy, G. W., Lowe, L. A., Ubatuba, F. B., Vane, J. R., and Wilkinson, S. (1977) Structural requirements for opioid activity of analogues of the enkephalins. *Proc. R. Soc. London, Ser. B: Biol. Sci. 198*, 249–265.

18. Beddell, C. R., Clark, R. B., Lowe, L. A., Wilkinson, S., Chang, K. J., Cuatrecasas, P., and Miller, R. (1977) A conformational analysis for leucine-enkephalin using activity and binding data of synthetic analogues. *Br. J. Pharmacol. 61*, 351–356.

19. Lazarus, L. H., Bryant, S. D., Cooper, P. S., and Salvadori, S. (1999) What peptides these deltorphins be. *Prog. Neurobiol. 57*, 377–420.

20. Mosberg, H. I., Hurst, R., Hruby, V. J., Gee, K., Akiyama, K., Yamamura, H. I., Galligan, J. J., and Burks, T. F. (1983) Cyclic penicillamine containing enkephalin analogs display profound delta receptor selectivities. *Life Sci. 33* (Suppl 1), 447–450.

21. Camerman, A., Mastropaolo, D., Karle, I., Karle, J., and Camerman, N. (1983) Crystal structure of leucine-enkephalin. *Nature 306*, 447–450.

22. Griffin, J. F., Langs, D. A., Smith, G. D., Blundell, T. L., Tickle, I. J., and Bedarkar, S. (1986) The crystal structures of [Met5]enkephalin and a third form of [Leu5]enkephalin: Observations of a novel pleated beta-sheet. *Proc. Natl. Acad. Sci. U.S.A. 83*, 3272–3276.

23. Smith, D., and Griffin, J. F. (1978) Conformation of [Leu5]enkephalin from X-ray diffraction: features important for recognition at opiate receptor. *Science 199*, 1214–1216.

24. Aubry, A., Birlirakis, N., Sakarellos-Daitsiotis, M., Sakarellos, C., and Marraud, M. (1988) Relationship of the crystal and molecular structure of leucine-enkephalin trihydrate to that of morphine. *J. Chem. Soc., Chem. Commun.*963–964.

25. Falciani, C., Lozzi, L., Pini, A., and Bracci, L. (2005) Bioactive peptides from libraries. *Chem. Biol. 12*, 417–426.

26. Garbe, D., Sieber, S. A., Bandur, N. G., Koert, U., and Marahiel, M. A. (2004) Enzymatic cyclisation of peptidomimetics with incorporated (E)-alkene dipeptide isosteres. *ChemBioChem 5*, 1000–1003.

27. Catoire, L. J., Damian, M., Giusti, F., Martin, A., van Heijenoort, C., Popot, J. L., Guittet, E., and Baneres, J. L. (2010) Structure of a GPCR ligand in its receptor-bound state: Leukotriene B4 adopts a highly constrained conformation when associated to human BLT2. *J. Am. Chem. Soc. 132*, 9049–9057.

28. Tyndall, J. D., Pfeiffer, B., Abbenante, G., and Fairlie, D. P. (2005) Over one hundred peptide-activated G protein-coupled receptors recognize ligands with turn structure. *Chem. Rev. 105*, 793–826.

29. Gardner, R., Liang, G., and Gellman, S. (1999) β-Turn and β-hairpin mimicry with tetrasubstituted alkenes. *J. Am. Chem. Soc. 121*, 1806–1816.

30. Li, Y., Luthman, K., and Hacksell, U. (1992) Novel L-Phe-Gly mimetics. *Tetrahedron Lett. 33*, 4487–4490.

31. Oishi, S., Kamano, T., Niida, A., Odagaki, Y., Hamanaka, N., Yamamoto, M., Ajito, K., Tamamura, H., Otaka, A., and Fujii, N. (2002) Diastereoselective synthesis of new ψ[(*E*)-CH═CMe]- and ψ[(*Z*)-CH═CMe]-type alkene dipeptide isosteres by organocopper reagents and application to conformationally restricted cyclic RGD peptidomimetics. *J. Org. Chem. 67*, 6162–6173.

32. Spatola, A. (1983) *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. 7, Marcel Dekker, New York.

33. Borg, S., Luthman, K., Nyberg, F., Terenius, L., and Hacksell, U. (1993) 1,2,4-Oxadiazole derivatives of phenylalanine: Potential inhibitors of substance P endopeptidase. *Eur. J. Med. Chem. 28*, 801–810.

34. Jenmalm, A., Luthman, K., Lindeberg, G., Nyberg, F., Terenius, L., and Hacksell, U. (1992) Novel peptidomimetics: Inhibitors of substance P endopeptidase. *Bioorg. Med. Chem. Lett. 2*, 1693–1698.

35. White, J. D., and Amedio, J. C. J. (1989) Total synthesis of geodiamolide A, a novel cyclodepsipeptide of marine origin. *J. Org. Chem. 54*, 736–738.

36. Lygo, B., and Rudd, C. N. (1995) Synthesis of Xaa-Gly-Xaa′ keto-methylene tri-peptide isosteres incorporating phenylalanine, tyrosine, and valine units. *Tetrahedron Lett. 36*, 3577–3580.

37. Bélanger, D., Tong, X., Soumaré, S., Dory, Y. L., and Zhao, Y. (2009) Cyclic peptide−polymer complexes and their self-assembly. *Chem.—Eur. J. 15*, 4428–4436.

38. Baillargeon, P., Bernard, S., Gauthier, D., Skouta, R., and Dory, Y. (2007) Efficient synthesis and astonishing supramolecular architectures of several symmetric macrolactams. *Chem.—Eur. J. 13*, 9223–9235.

39. Gordon, E. M., Godfrey, J. D., Delaney, N. G., Asaad, M. M., Von Langen, D., and Cushman, D. W. (1988) Design of novel inhibitors of aminopeptidases. Synthesis of peptide-derived diamino thiols and sulfur replacement analogues of bestatin. *J. Med. Chem. 31*, 2199–2211.

40. Gaudriault, G., Nouel, D., Dal Farra, C., Beaudet, A., and Vincent, J. P. (1997) Receptor-induced internalization of selective peptidic mu and delta opioid ligands. *J. Biol. Chem. 272*, 2880–2888.

41. Ko, J. L., Arvidsson, U., Williams, F. G., Law, P. Y., Elde, R., and Loh, H. H. (1999) Visualization of time-dependent redistribution of delta-opioid receptors in neuronal cells during prolonged agonist exposure. *Brain Res. Mol. Brain Res. 69*, 171–185.

42. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) Morphine activates opioid receptors without causing their rapid internalization. *J. Biol. Chem. 271*, 19021–19024.

43. Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. R., Laporte, S. A., Law, P. Y., and Caron, M. G. (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc. Natl. Acad. Sci. U.S.A. 95*, 7157–7162.

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769

44. Marie, N., Landemore, G., Debout, C., Jauzac, P., and Allouche, S. (2003) Pharmacological characterization of AR-M1000390 at human delta opioid receptors. *Life Sci. 73*, 1691–1704.

45. Pradhan, A. A., Becker, J. A., Scherrer, G., Tryoen-Toth, P., Filliol, D., Matifas, A., Massotte, D., Gaveriaux-Ruff, C., and Kieffer, B. L. (2009) In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One 4*, No. e5425.

46. Galandrin, S., Oligny-Longpre, G., and Bouvier, M. (2007) The evasive nature of drug efficacy: Implications for drug discovery. *Trends Pharmacol. Sci. 28*, 423–430.

47. Kramer, H. K., and Simon, E. J. (2000) mu and delta-opioid receptor agonists induce mitogen-activated protein kinase (MAPK) activation in the absence of receptor internalization. *Neuropharmacology 39*, 1707–1719.

48. Trapaidze, N., Gomes, I., Cvejic, S., Bansinath, M., and Devi, L. A. (2000) Opioid receptor endocytosis and activation of MAP kinase pathway. *Brain Res. Mol. Brain Res. 76*, 220–228.

49. Quock, R. M., Burkey, T. H., Varga, E., Hosohata, Y., Hosohata, K., Cowell, S. M., Slate, C. A., Ehlert, F. J., Roeske, W. R., and Yamamura, H. I. (1999) The delta-opioid receptor: Molecular pharmacology, signal transduction, and the determination of drug efficacy. *Pharmacol. Rev. 51*, 503–532.

50. Gendron, L., Cote, F., Payet, M. D., and Gallo-Payet, N. (2002) Nitric oxide and cyclic GMP are involved in angiotensin II AT(2) receptor effects on neurite outgrowth in NG108-15 cells. *Neuroendocrinology 75*, 70–81.

51. Gendron, L., Laflamme, L., Rivard, N., Asselin, C., Payet, M. D., and Gallo-Payet, N. (1999) Signals from the AT2 (angiotensin type 2) receptor of angiotensin II inhibit p21ras and activate MAPK (mitogen-activated protein kinase) to induce morphological neuronal differentiation in NG108−15 cells. *Mol. Endocrinol. 13*, 1615–1626.

52. Gendron, L., Oligny, J. F., Payet, M. D., and Gallo-Payet, N. (2003) Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II AT2 receptor signaling pathway in NG108-15 cells. *J. Biol. Chem. 278*, 3606–3614.

53. Cotton, R., Hardy, P. M., and Langran-Goldsmith, A. E. (1986) Peptides containing diacylglycines. *Int. J. Pept. Protein Res. 28*, 245–253.

54. Shinada, T., Ishida, T., Hayashi, K.-H., Yoshida, Y., Shigeri, Y., and Ohfune, Y. (2007) Synthesis of leucine-enkephalin analogs containing α-amino squaric acid. *Tetrahedron Lett. 48*, 7614–7617.

55. Martin, S. F., Dwyer, M. P., Hartmann, B., and Knight, K. S. (2000) Cyclopropane-derived peptidomimetics. Design, synthesis, and evaluation of novel enkephalin analogues. *J. Org. Chem. 65*, 1305–1318.

56. Blomberg, D., Kreye, P., Fowler, C., Brickmann, K., and Kihlberg, J. (2006) Synthesis and biological evaluation of leucine enkephalin turn mimetics. *Org. Biomol. Chem. 4*, 416–423.

57. Cox, M. T., Gormley, J. J., Hayward, C. F., and Peter, N. F. (1980) Incorporation of trans olefinic dipeptide isostere into enkephaline and substance P analogs. *J. Chem. Soc., Chem. Commun*.800–802.

58. Hann, M. M., and Sammes, P. G. (1980) On double bond isosteres of the peptide bond; an enkephalin analogue. *J. Chem. Soc., Chem. Commun*.234–235.

59. Hann, M. M., Sammes, P. G., Kennewell, P. D., and Taylor, J. B. (1982) On the double bond isostere of the peptide bond: preparation of an enkephalin analogue. *J. Chem. Soc., Perkin Trans*.307–314.

60. Prather, P. L., Song, L., Piros, E. T., Law, P. Y., and Hales, T. G. (2000) delta-Opioid receptors are more efficiently coupled to adenylyl cyclase than to L-type Ca(2+) channels in transfected rat pituitary cells. *J. Pharmacol. Exp. Ther. 295*, 552–562.

61. Schulz, R., Wehmeyer, A., and Schulz, K. (2002) Opioid receptor types selectively cointernalize with G protein-coupled receptor kinases 2 and 3. *J. Pharmacol. Exp. Ther. 300*, 376–384.

62. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol. 22*, 3099–3108.

DOI: 10.1021/cn1000759 | *ACS Chem. Neurosci.* (2010), *1*, 757–769