Published in final edited form as:

ChemMedChem. 2017 April 20; 12(8): 571–576. doi:10.1002/cmdc.201700103.

Synthesis and Opioid Activity of Tyr¹– $\psi[(Z)CF=CH]$ –Gly² and Tyr¹– $\psi[(S)/(R)-CF_3CH-NH]$ –Gly² Leu-enkephalin Fluorinated Peptidomimetics

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Abstract

We describe the design, synthesis, and opioid activity of fluoroalkene (Tyr¹– $\psi[(Z)CF=CH]$ –Gly²) and trifluoroethylamine (Tyr¹– $\psi[(S)/(R)-CF_3CH-NH]$ –Gly²) analogues of the endogenous opioid neuropeptide, Leu-enkephalin. The fluoroalkene peptidomimetic exhibited low nanomolar functional activity (5.0 ± 2 nM and 60 ± 15 nM for δ - and μ -opioid receptors, respectively) with a μ/δ -selectivity ratio that mimicked the natural peptide. However, the trifluoroethylamine peptidomimetics, irrespective of stereochemistry, did not activate the opioid receptors, which suggest that bulky CF₃ substituents are not tolerated at this position.

Table of Contents

New fluoroalkene and trifluoroethylamine analogues of the endogenous opioid neuropeptide, Leu-enkephalin are synthesized, and the functional activity of these new peptides is evaluated. The fluoroalkene peptidomimetic exhibited low nanomolar functional activity (5 ± 2 nM and 60 ± 15 nM for δ - and μ opioid receptors, respectively) with a μ/δ -selectivity ratio that mimicked the natural peptide.

Keywords

Opioids; Enkephalin; Amide Bonds; Fluorine; Peptidomimetics

Despite the development of various analgesics, opioids remain the most commonly prescribed class of medication to treat both acute and chronic pain,^[1] with nearly 245 million prescriptions dispensed by US pharmacies in 2014.^[2, 3] Although current opioids

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relieve pain quickly, their utility is hampered by various serious side effects, such as sedation, respiratory depression, constipation, nausea, and most notably tolerance and dependence which can lead to overdose-related deaths.^[1, 4, 5]

Most clinically employed opioids, including the gold-standard morphine, predominantly activate μ -opioid receptors (MOPRs). This selective activation of MOPRs is associated with the undesirable side effects seen in clinical opioid use.^[6] In contrast, two other opioid receptors, the δ - and κ -opioid receptors (DOPR and KOPR, respectively), have been indicated as possible therapeutic targets for treating pain with a reduced liability for side effects.^[7] Herein, we focus on the DOPR, because selective activation of DOPRs induces analgesia in persistent and chronic pain models, including inflammatory,^[8-10] neuropathic,^[11, 12] and cancer pains,^[13] without promoting the undesirable MOPR-related side effects.^[14-17] Further, administration of DOPR agonists in combination with MOPR agonists may enable lower dosing of the latter.^[18-21] Thus, DOPR agonists have potential to treat chronic and acute pain with minimal side effects.^[22]

Endogenous peptides activate opioid receptors, and are therefore promising leads for developing therapeutic candidates for managing pain.^[23] One such peptide, Leu-enkephalin (Tyr¹-Gly²-Gly³-Phe⁴-Leu), acts by interacting with DOPR (1–5 fold binding affinity over MOPR and >1000 fold over KOPR).^[24, 25] However, clinical administration of Leu-enkephalin is limited by a poor pharmacokinetic (PK) profile, including rapid proteolysis of Tyr¹-Gly² by aminopeptidase N in human plasma ($t_{1/2}$ = 0.69 min; 0.50 U/mL purified enzyme)^[26, 27] and of Gly³-Phe⁴ by angiotensin-converting enzyme at the BBB ($t_{1/2}$ = 130 min; 0.12 U/mL purified enzyme),^[28] both of which inhibit penetration into the CNS.^[29] To improve potency and selectivity, many cyclic and linear analogues of enkephalin have been developed that possess excellent affinities and selectivities for the DOPR,^[24, 30-32] and in some cases, the replacement of hydrolyzable amide bonds at various positions with peptidomimetics (e.g. *trans* alkene,^[24] thioamide,^[33] ester^[34] or *N*-methyl amide dipeptide isostere^[34]) have modulated stability and PK properties of enkephalins, without major loss of agonist activity at the DOPR. However, many of these analogues still lack appropriate physicochemical and biophysical properties for *in vivo* use. To generate analogues of enkephalin with improved drug like properties, we herein present new fluorinated Tyr¹-Gly² peptidomimetic analogues.

The strategic incorporation of fluorine onto a target molecule alters several biophysical properties including solubility, lipophilicity, conformation, and metabolic stability, which in turn affect transport across biological membranes, binding efficiency to biological target and clearance from the host (Figure 1).^[35-37] In fact, fluorinated peptidomimetics have been used to modulate properties of many peptide-based probes, including dipeptidyl peptidase,^[38] thermolysin,^[39] HIV-1 protease,^[40] antibiotic peptides and other protease inhibitors.^[41-43] Considering Leu-enkephalin, we envisioned using fluoroalkene (**2**) and β,β,β - trifluoroethylamine (**3** and **4**) peptidomimetics to modulate its physicochemical properties, while also increasing proteolytic stability. Using these substitutions, we specifically targeted the Tyr¹-Gly² linkage of Leu-enkephalin because it is the predominant site of metabolism that is responsible for its short half-life and limited tissue distribution.^[26-29, 44, 45] Additionally, this site tolerates substitution with *E* alkenes^[24, 46-48]

high suggests that other Tyr¹–Gly² substitutions might be tolerated. Therefore, we investigated the synthesis and opioid activity of Tyr¹– $\psi[(Z)CF=CH]$ –Gly² and Tyr¹– $\psi[(S)/(R)-CF_3CH-NH]$ –Gly² analogues of enkephalin (**6–8**, Figure 2).

To test our hypothesis, we first prepared Tyr¹– $\psi[(Z)CF=CH]$ –Gly²–Gly–Phe–Leu-enkephalin (**6**) using a diastereoselective Reformatsky–Honda condensation, an *E*-selective Horner–Wadsworth–Emmons olefination, and a SmI₂ mediated reduction as key steps (Scheme 1A and B). This strategy would rely on preparation of Boc–Tyr¹– $\psi[(Z)CF=CH]$ –Gly² (**14**) prior to incorporation into Leu-enkephalin fluorinated peptidomimetic (**6**). Introduction of the fluoroalkene moiety began with the synthesis of **11**, which was prepared from TIPS protected phenylacetaldehyde (**9**)^[49] and chiral amine (**10**) *via* rhodium catalyzed diastereoselective Reformatsky–Honda reaction.^[50] The chiral auxiliary was removed by hydrogenolysis using Pd(OH)₂/C–H₂ in EtOH followed by Boc protection to yield (*S*)- α,α -difluoro- β -amino ester (**12**) in 73% yield. After DIBAL–H reduction of **12**, the corresponding aldehyde underwent olefination reaction to provide (*E*)- γ,γ -difluoro- α,β -enoate (**13**) in 60% yield.^[50] Subsequently, SmI₂-mediated reductive isomerization of the alkene^[51] and saponification^[24] of **13** gave the desired dipeptide mimetic, Boc–Tyr¹– $\psi[(Z)CF=CH]$ –Gly² (**14**), in 78% isolated yield and >99% enantiopurity (determined by coupling to (*R*)-1-phenylethan-1-amine; See SI for details). Subsequent coupling of dipeptide isostere **14** with tripeptide **15**,^[52, 53] saponification of the methyl ester, Boc deprotection and HPLC purification afforded **6** in 45% yield and 98% UPLC purity (Scheme 1B).

Trifluoroethylamine peptidomimetic (*S*)-**7** was prepared using a chiral auxiliary–controlled diastereoselective nucleophilic addition of TMSCF₃ to (*R*)- α -amino *N*-*tert*-butanesulfinimine (**18**),^[54] as the key step towards accessing the Bn–Tyr¹– $\psi[(S)-CF_3CH-NH]$ –Gly² (**22**) dipeptide mimic (Scheme 2A–B). The synthesis of (*S,S*)-**22** initiated by condensing benzyl protected α -amino aldehyde (*S*)-**16**^[55] with (*R*)-(-)-2-methyl-2-propanesulfinamide (**17**), in the presence of titanium (IV) ethoxide to provide (*S,R*)- α -amino sulfinylimine (**18**) in 91% isolated yield (Scheme 2A).^[56] Subsequent, diastereoselective 1,2-addition of the Ruppert Prakash reagent (TMSCF₃) afforded (*S,S,R*)-**19** in 89% yield and > 99% dr, consistent with a matched substrate-auxiliary pair (determined by ¹H and ¹⁹F NMR, Scheme 2A).^[54] Removal of the *tert*-butanesulfinyl group with hydrochloric acid in refluxing methanol gave amine (*S,S*)-**20**,^[54] which was alkylated with ethyl bromoacetate to afford (*S,S*)-**21**, although the alkylation required harsh conditions (NaH, HMPA) due to the weak nucleophilicity of the trifluoroethylamine.^[57] The saponification of (*S,S*)-**21** with LiOH furnished the desired trifluoroethylamine dipeptide mimetic, Bn–Tyr¹– $\psi[(S)-CF_3CH-NH]$ –Gly² (**22**). Finally, EDC mediated coupling of (*S,S*)-**22** with tripeptide **15**,^[52, 53] deprotection with LiOH and Pd(OH)₂/C–H₂, followed by HPLC purification gave (*S*)-**7** in 37% yield and >99% UPLC purity (Scheme 2B).

Similarly, the synthesis of Leu-enkephalin trifluoroethylamine peptidomimetic, (*R*)-**8** relied on a chiral auxiliary–controlled nucleophilic addition of the TMSCF₃ to (*S*)- α -amino *N*-*tert*-butanesulfinimine (**18**) as a key step to generate the Bn–Tyr¹– $\psi[(R)-CF_3CH-NH]$ –Gly² fragment (**22**, Scheme 3A). The synthesis of (*S,R*)-**22** initiated by condensing benzyl

protected α -amino aldehyde (**S**)-**16**^[55] with (*S*)-(-)-2-methyl-2-propanesulfinamide (**17**) to provide (*S,S*)- α -amino sulfinylimines (**18**) in 90% isolated yield.^[56] Subsequent addition of Ruppert–Prakash reagent (TMSCF₃) afforded (*S,R,S*)-**19** in 67% isolated yield, but only in moderate selectivity for the desired diastereomer (2.7:1), suggesting a mismatched substrate-auxiliary pair.^[54] The absolute configuration of the major diastereomer was confirmed by chromatographic separation and X-ray crystallography (See SI for details). Removal of the *tert*-butanesulfinyl group afforded amine (*S,R*)-**20**, and subsequent alkylation with ethyl bromoacetate provided (*S,R*)-**21** (Scheme 3A).^[54, 57] Saponification of (*S,R*)-**21** provided desired dipeptide isostere, Bn-Tyr¹- ψ [(*R*)-CF₃CH-NH]-Gly² (**22**). Finally, EDC mediated coupling of (*S,R*)-**22** with tripeptide **15**,^[52, 53] deprotection, and HPLC purification provided (*R*)-**8** in 25% yield and 99% overall purity (Scheme 3B).

The opioid activity of fluorinated analogues **6–8** was assessed *in vitro* at the DOPR, MOPR and KOPR using CHO cells stably expressing one of the three receptors. Dose-dependent forskolin-induced cAMP accumulation was evaluated and compared to that of control agonist **5**. Amongst our new analogues, only fluoroalkene **6** retained activity at the DOPR, with an EC₅₀ value of 5.0 ± 2 nM. Although a 60-fold decrease from the parent compound **5**, this activity confirms that the Tyr¹-Gly² amide is not essential for binding to the DOPR.^[24] These results contrast the 6-fold decreased binding affinity in Gly³-Phe⁴ fluoroalkene peptidomimetic.^[58] At the MOPR, a similar activity trend emerged with **6** being the only active analog, albeit 45-fold less active than **5**. While the fluoroalkene modification did not impart a significant change to the μ/δ selectivity, this substructure will likely stabilize the probe toward proteolytic metabolism. Additionally, the lack of activity seen with (*S*)-**7** and (*R*)-**8** gives further insight into the SAR around the amide bond, specifically that the receptors do not tolerate the bulky CF₃ group, regardless of *S*- or *R*-configuration (Table 1), as previously demonstrated for trifluoroethylamine substitution at Gly³-Phe⁴ (30–110 fold decrease in affinity), and at Gly²-Gly³ (non-measurable affinity).^[59] Analogues **6–8** were also screened at the KOPR. Conflicting reports indicate that (1) **5** does not bind to the KOPR,^[60] but that (2) **5** has partial agonist activity.^[61] However, using the functional assay described above, **5** demonstrated full KOPR agonist activity with an EC₅₀ value of 80 ± 20 nM, 1000-fold less potent than at the DOPR. This result highlights the differences amongst screening methods and assays. In contrast to **5**, trifluoroethylamine derivatives, (*S*)-**7** and (*R*)-**8** had no activity at KOPR up to concentrations of 10 μ M.

In conclusion, we successfully synthesized fluoroalkene (Tyr¹- ψ [(*Z*)CF=CH]-Gly²) and trifluoroethylamine (Tyr¹- ψ [(*S*)/(*R*)-CF₃CH-NH]-Gly²) fluorinated peptidomimetics, and incorporated them into Leu-enkephalin. Amongst these peptidomimetics, the trifluoroethylamine analogues did not activate the opioid receptors, irrespective of the stereochemistry. However, fluoroalkene analogue **6** activated DOPR in the low nanomolar range, with similar μ/δ selectivity relative to the parent Leu-enkephalin, which suggests that this analog binds the DOPR and MOPR in a similar fashion to the parent compound. Future work will characterize the physicochemical and pharmacokinetic perturbations imparted by this peptidomimetic, which should provide improved proteolytic stability and drug-like properties relative to the endogenous peptide. Additional effort will focus on developing the

next generation of fluorinated peptidomimetic analogues of Leu-enkephalin with excellent drug-like properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute on Drug Abuse under Award Numbers DA036730 (R.A.A.) and DA018151 (T.E.P.), and by the National Institute of General Medical Sciences under Award Number GM008545 (R.S.C.). Support for the NMR instrumentation was provided by NIH Shared Instrumentation Grant # S10D016360. We thank Ben Neuenswander for HPLC purifications and Dr. Victor Day for X-ray crystallography. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1.
Fluorinated isoelectronic and isopolar peptidomimetics

Figure 2.
Target fluoroalkene **6** and trifluoroethylamine (*S*)-**7**/*R*)-**8** analogues of Leu-enkephalin

Scheme 1. Preparation of Tyr¹-ψ[(Z)CF=CH]-Gly² Leu-Enkephalin

Reagents and conditions: (a) 3 Å molecular sieves, THF, 0 °C, 4 h; (b) RhCl(PPh₃)₃ BrCF₂CO₂Et, Et₂Zn, 0 °C 30 min, 45%; (c) (Boc)₂O, 20% Pd(OH)₂/C, H₂, EtOH, rt, 48 h, 73%; (d) DIBAL-H, DCM, -78 °C; (e) a (EtO)₂P(O)CH₂CO₂Et, LiCl, DIPEA, CH₃CN, 0 °C→rt, 12 h, 60%; (f) SmI₂, THF:*t*-BuOH (3:1), 0 °C, 1 h, 75%; (g) LiOH, THF:H₂O (1:1), 0 °C→rt, 36 h, 78%; (h) DIPEA, HOBT, EDC.HCl, THF 0 °C→rt, 12 h, 65%. (i) LiOH, THF:H₂O (1:1), 0 °C→rt, 12 h, 75; (j) 4 N HCl in 1,4-Dioxane, 15 °C, 2 h, HPLC purification, 45%.

Scheme 2. Preparation of Tyr¹-ψ[(S)-CF₃CH-NH]-Gly² Leu-Enkephalin

Reagents and conditions: (a) Ti(OEt)₄, THF, rt, 6 h, 91%; (b) TMAF, TMSF₃, THF, – 35 °C, 2 h, 89%; (c) 4N HCl in 1,4-dioxane, MeOH, 70 °C, 2 h, 90%; (d) NaH, BrCH₂CO₂Et, HMPA, 48 h, 52%; (e) LiOH, THF:H₂O (1:1), 12 h, 88%; (f) DIPEA, HOBt, EDC.HCl, THF, 0 °C→rt, 12 h, 72 %; (g) LiOH, THF:H₂O (1:1) 0 °C→rt, 12 h, 80%; (h) Pd(OH)₂/C, methanol, 6 h, H₂ balloon, rt, HPLC purification, 37%

Scheme 3. Preparation of Tyr¹-ψ[(R)-CF₃CH-NH]-Gly² Leu-Enkephalin

Reagents and conditions: (a) Ti(OEt)₄, THF, rt, 6 h, 90%; (b) TMAF, TMSCF₃, THF, – 35 °C, 2 h, 67%; (c) 4N HCl in 1,4-dioxane, MeOH, 70 °C, 2 h, 95%; (d) NaH, BrCH₂CO₂Et, HMPA, 3 d, 70%; (e) LiOH, THF:H₂O (1:1), 12 h, 91%; (f) DIPEA, HOBT, EDC.HCl, THF, 0 °C→rt, 12 h, 69 %; (g) LiOH, THF:H₂O (1:1) 0 °C→rt, 12 h, 85%; (h) Pd(OH)₂/C, methanol, 6h, H₂ balloon, rt, HPLC purification, 25%

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Table 1
Activation of opioid receptors by fluoroalkene (**6**) and trifluoroethylamine Leu-enkephalin analogues (*S*)-**7**/*R*)-**8**

Compound	EC ₅₀ ± SEM <i>a, b</i> (nM)				Selectivity		
	δ	μ	κ	μ/δ	κ/δ	κ/δ	κ/δ
5	0.08 ± 0.01	1.3 ± 0.1	80 ± 20	16	1,000		
6	5.0 ± 2	60 ± 15	>10,000 <i>c</i>	12	>2,000		
(<i>S</i>)- 7	>10,000 <i>c</i>	>10,000 <i>c</i>	>10,000 <i>c</i>	–	–		
(<i>R</i>)- 8	>10,000 <i>c</i>	>10,000 <i>c</i>	>10,000 <i>c</i>	–	–		

[a] Mean ± standard error of the mean; n = 2 individual experiments run in triplicate.

[b] E_{max} = 100% unless otherwise noted.

[c] E_{max} = 0 % up to 10 μM.