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(4-Carboxamido)phenylalanine is a surrogate for tyrosine in opioid receptor peptide ligands

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Abstract—(*S*)-4-(Carboxamido)phenylalanine (Cpa) is examined as a bioisosteric replacement for the terminal tyrosine (Tyr) residue in a variety of known peptide ligands for the μ, δ, and κ opioid receptors. The Cpa-containing peptides, assayed against cloned human opioid receptors, display comparable binding affinity (K_i), and agonist potency (EC_{50}) to the parent ligands at the three receptors. Cpa analogs of δ selective peptides show an increase in δ selectivity relative to the μ receptor. Cpa is the first example of an amino acid that acts as a surrogate for Tyr in opioid peptide ligands, challenging the long-standing belief that a phenolic residue is required for high affinity binding.

The discoveries of endogenous opioid peptides[1] and the existence of three opioid receptor types (μ, δ, and κ)[2] occurred nearly 30 years ago. These discoveries triggered an explosion of basic research in both academic and industrial laboratories to acquire a deeper understanding of the medicinal chemistry, pharmacology, and physiology of the opioid receptors and their ligands and to capitalize on the potential commercial windfall of type selective agents as superior analgesics. This explosion in basic research was characterized, in part, by the synthesis and evaluation of hundreds of opioid peptide analogs that established structure–activity relationships (SARs) against the receptor types.[3] Two salient SAR features, encompassing both acyclic and cyclic peptide analogs and common to all opioid receptor types, are the requirements of an appropriately spatially-oriented basic nitrogen and a hydroxylated phenyl ring.[3a,b] The necessity of a terminal tyrosine residue satisfying both of these minimal SAR requirements for high affinity peptide binding may arguably be regarded as dogma.[4,5]

In a recent disclosure from our laboratories, *trans*-3,4-dimethyl-4-(3-carboxamidophenyl)piperidines (e.g., **1**) were identified as a novel class of selective μ receptor antagonists.[6] These agents were discovered during a search for a bioisosteric replacement of the phenolic OH in the *trans*-3,4-dimethyl-4-(3-hydroxyphenyl)piperidine class of μ antagonists (e.g., **2**).[7] This research complemented a disclosure by Wentland et al., who demonstrated that (8-carboxamido)cyclazocine **3** possessed opioid receptor binding and functional agonist activity comparable in potency to cyclazocine **4** itself.[8] Bioisosteric $CONH_2$ substitution has been expanded to include morphine and naltrexone,[9a] and morphinan derivatives[9b] with analogous results. The success of the carboxamido residue as a surrogate for the phenolic OH in these nonpeptide ligands is attributed to its ability to also act as a hydrogen-bond donor.[8,9] The tertiary nitrogen and the phenolic OH residue in nonpeptide opioid ligands, and tyrosine's OH and NH_2 residues in peptide ligands constitute a common 'message' of the message-address concept of opioid ligand–receptor interaction.[10] Comparative molecular modeling and conformational analysis of δ ligands have been developed, assuming common three-dimensional arrangements of pharmacophore elements in peptide and nonpeptide ligands.[11] Site-directed mutagenesis and homology modeling studies suggest that the phenolic group (peptide or nonpeptide) may engage a hydrogen bond to a Lys ε-amino group or a His NH group in the putative active site of the receptor.[12] Although previous

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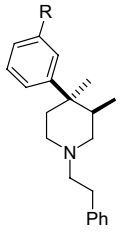
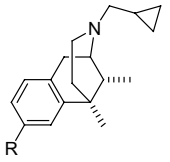
attempts to modify the phenol in **5** led to less potent ligands,[3] it was these compelling observations that led us to challenge conventional understanding. In this report, (*S*)-(4-carboxamido)phenylalanine **6** (Cpa) is examined as a potential surrogate for the N-terminal Tyr residue, [Tyr[1]], in a representative cross-section of classical opioid peptides (**i** → **ii**).

Phenolic OH to $CONH_2$ exchange in nonpeptide opioid ligands:[6,8]

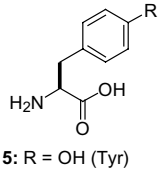


1: R = $CONH_2$; K_i (μ) = 2 nM (antagonist)[6]
2: R = OH; K_i (μ) = 2 nM (antagonist)

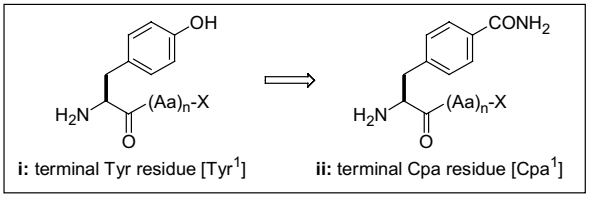


3: R = $CONH_2$; K_i (μ) = 0.5 nM (agonist)[8]
4: R = OH; K_i (μ) = 0.4 nM (agonist)

Phenolic OH to $CONH_2$ (Tyr → Cpa) exchange in peptide opioid ligands: *present study*



5: R = OH (Tyr)
6: R = $CONH_2$ (Cpa)



i: terminal Tyr residue [Tyr[1]] **ii:** terminal Cpa residue [Cpa[1]]

The first peptide targeted for modification was [Leu[5]]-enkephalin **7** (Table 1). This endogenous opioid peptide has been reported to be a δ selective peptide with an $IC_{50} = 318$ nM in the guinea pig ileum assay (GPI; rich in μ receptors) and an $IC_{50} = 13$ nM in the mouse vas deferens assay (MVD; rich in δ receptors): GPI/MDV (μ/δ) = 24.[13] In the cloned human receptor binding assays, **7** displayed a $K_i = 50$ nM (μ), 1.1 nM (δ), and >10,000 nM (κ), consistent with the δ selectivity ($\mu/\delta = 45$) observed in the in vitro rodent tissue strip assays. Replacement of [Tyr[1]] in **7** with Cpa gave peptide **8**, [Cpa[1], Leu[5]]-enkephalin.[14] Binding affinity and selectivity obtained for **8** revealed that it was nearly equivalent to the parent ligand: $K_i = 110$ nM (μ), 1.9 nM (δ), and >10,000 nM (κ); $\mu/\delta = 58$. In addition, the potency of **8** as an agonist against δ, as measured by its ability to stimulate the binding of [^{35}S]GTPγS to the cloned receptor, was also equivalent: $EC_{50} = 15$ nM for **8** versus 13 nM for **7**. Encouraged by this result, the corresponding amides **9**, [Leu[5]]-enkephalinamide, and **10**, [Cpa[1], Leu[5]]-enkephalinamide, were synthesized. In this peptide pair, the K_i and EC_{50} values for the δ receptor remained unchanged ($K_i = 5.3$ and 3.3 nM; $EC_{50} = 39$ and 74 nM, respectively) with a modest 3-fold improvement in selectivity over the μ receptor ($\mu/\delta = 13$ for **10** versus 4.7 for **9**). The affinity for **10** ($K_i = 240$) at the κ receptor was within 2-fold of **9**.

D-Amino acids at the 2 position in the opioid peptides are known to enhance potency and selectivity for the μ receptor.[3] Several pairs of [Tyr[1]]/[Cpa[1]] peptides were prepared with D-amino acids in the 2 and/or 5-position and evaluated against the cloned human receptors. These included the peptide pairs DADLE (**11/12**), its amide (**13/14**) and [ala[2], Met[5]]-enkephalin (**15/16**). In general, [Cpa[1]] was well tolerated at both the δ and μ receptors for all of the peptides but somewhat better tolerated at the δ receptor. For example, DADLE amide **13** had a μ/δ ratio of 5.0 while this ratio was 29 in the corresponding Cpa analog **14**.[15] The most potent and selective Cpa analog in the enkephalin series was [Cpa[1], ala[2], Met[5]]enkephalin **16**. It possessed a sub-nanomolar binding constant ($K_i = 0.72$ nM), a μ/δ ratio = 150, and agonist action ($EC_{50} = 1.3$ nM) comparable to its corresponding parent peptide **15**.

Endomorphin-1 (**17**),[16] TAPP (**19**),[17] and DAMGO (**21**),[18] well-characterized μ selective peptides, were subsequently selected for Cpa analog modification. The μ selectivity of the peptides was confirmed against the cloned human opioid receptors. As gleaned from Table 1, the corresponding Cpa-endomorphin **18** and -TAPP **20** analogs retained their μ receptor binding affinity within a ⩽2-fold window. As observed with the other Cpa peptides, this was accompanied by a modest (4-fold increase) in δ receptor binding. Although following the trend, Cpa-DAMGO analog **22** showed greater opposing effects on affinities at the μ and δ receptors. For the μ receptor, the K_i for **21** was 27 nM versus 97 nM for **22**, while for the δ and κ receptors **22** showed ⩾20× increases in affinities resulting in a reduction in selectivity of nearly 50-fold.

Exchange of [Tyr[1]] for [Cpa[1]] in a κ selective peptide, dynorphin (1–11) (**23**),[19] was also carried out. Peptide **23** possessed a $K_i = 0.26$ nM against κ and μ/κ and δ/κ selectivity ratios of 235 and 35. The corresponding Cpa analog **24** displayed affinity for the κ receptor

Table 1. Opioid receptor (μ, δ, and κ) binding and functional data of Cpa-containing peptides

Peptide sequence (no.)	Common name	K_i (nM)[a]				EC_{50} (nM)[b]		
		μ	δ	κ	μ/δ	μ	δ	κ
H-Tyr-Gly-Gly-Phe-Leu-OH (**7**)	Leu-enkephalin	50	1.1	[c]	45	141	13	[e]
H-Cpa-Gly-Gly-Phe-Leu-OH (**8**)		110	1.9	[c]	58	[d]	15	[e]
H-Tyr-Gly-Gly-Phe-Leu-NH_2 (**9**)	Leu-enkephalinamide	25	5.3	440	4.7	610	38.9	[e]
H-Cpa-Gly-Gly-Phe-Leu-NH_2 (**10**)		43	3.3	240	13	1043	74.3	[e]
H-Tyr-ala-Gly-Phe-leu-OH (**11**)[f]	DADLE	35	0.9	[c]	39	333	11.8	[e]
H-Cpa-ala-Gly-Phe-leu-OH (**12**)		119	8.2	[c]	15	640	134	[e]
H-Tyr-ala-Gly-Phe-leu-NH_2 (**13**)	DADLE amide	24	4.8	[c]	5.0	239	746	[e]
H-Cpa-ala-Gly-Phe-leu-NH_2 (**14**)		240	8.4	[c]	29	[d]	420	[e]
H-Tyr-ala-Gly-Phe-Met-OH (**15**)	[ala^2]-Met-enkephalin	14	0.35	[c]	40	210	1.0	[e]
H-Cpa-ala-Gly-Phe-Met-OH (**16**)		110	0.72	[c]	150	321	1.3	[e]
H-Tyr-Pro-Trp-Phe-NH_2 (**17**)	Endomorphin-1	45	1900	[c]	0.02	936	[e]	[e]
H-Cpa-Pro-Trp-Phe-NH_2 (**18**)		23	495	[c]	0.05	333	[e]	[e]
H-Tyr-ala-Phe-Phe-NH_2 (**19**)	TAPP	58	1213	1293	0.05	[d]	[d]	[e]
H-Cpa-ala-Phe-Phe-NH_2 (**20**)		85	285	[c]	0.29	330	[d]	[e]
H-Tyr-ala-Gly-(N-Me)Phe-Gly-ol (**21**)	DAMGO	27	330	[c]	0.05	710	[d]	[e]
H-Cpa-ala-Gly-(N-Me)Phe-Gly-ol (**22**)		97	24	420	4.0	584	220	[e]
H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-lle-Arg-Pro-Lys-OH (**23**)	Dynorphin (1-11)	61	9.1	0.26	—	[d]	3000	34
H-Cpa-Gly-Gly-Phe-Leu-Arg-Arg-lle-Arg-Pro-Lys-OH (**24**)		[c]	6.3	0.52	—	[d]	310	42
H-Tyr-ser-Gly-Phe-Leu-Thr-OH (**25**)	DSLET	140	1.1	[c]	130	149	5.1	[e]
H-Cpa-ser-Gly-Phe-Leu-Thr-OH (**26**)		370	1.2	[c]	308	365	9.9	[e]

[a] The binding affinities (K_i) of the peptides were determined by testing the ability of a range of concentrations of each peptide to inhibit the binding of the nonselective opioid antagonist, [^{3}H]diprenorphine, to cloned human μ, δ, and κ opioid receptors expressed in separate cell lines.[21] K_i values are the geometric means computed from at least three separate determinations.

[b] The potencies (EC_{50}) of the peptides were determined by testing the ability of a range of concentrations of each peptide to stimulate the binding of [^{35}S]GTPγS to cloned human μ, δ, and κ opioid receptors expressed in separate cell lines.[21] EC_{50} values are the geometric means computed from at least three separate determinations.[22]

[c] $K_i > 10,000$ nM.

[d] $EC_{50} > 1000$ nM.

[e] Not determined.

[f] D-Amino acids are indicated by all lower case letters.

($K_i = 0.52$ nM) essentially equal to **23** with a rather dramatic increase in δ selectivity versus μ (μ/κ >20,000).

The observation of the tendency of Cpa to skew μ/δ selectivity in favor of the δ opioid receptor by a factor of 2- to 50-fold in several [Tyr^1]/[Cpa^1] peptide pairs led to the hypothesis that incorporating the Cpa residue into a known δ opioid peptide may lead to greater selectivity. The peptide DSLET (**25**)[20] was chosen to test this hypothesis. Exchange of the terminal Tyr residue by Cpa afforded the new peptide **26** that was indeed found equipotent to DSLET at the δ receptor (K_i values = 1.1 and 1.2 nM; EC_{50} values = 5.1 and 9.9, respectively) but with a 2.6-fold higher K_i value at the μ receptor (K_i = 370 nM) resulting in 300-fold selectivity over the μ receptor.

In summary, (*S*)-4-(carboxamido)phenylalanine (Cpa) is the first example of an amino acid that acts as a surrogate for the terminal Tyr residue in opioid peptides. Previous attempts to modify the Tyr phenolic residue yielded inactive agents.[3,15] In the case of δ, μ and κ selective peptides, the Cpa-containing analogs display comparable binding affinities (K_i), and potencies (EC_{50}) with their parent ligands. The carboxamido bioisostere is more readily accommodated by the δ receptor as the novel ligands, in general, have enhanced selectivity for the δ receptor relative to the μ receptor. Affinity and potency for κ was also demonstrated in those peptides that bind to this receptor. The impetus for the [Tyr^1] → [Cpa^1] investigation was derived from recently reported SAR data for nonpeptide opioid agonists and antagonists.[6,8] This is a very rare example of successfully translating *nonpeptide* SAR to *peptide* SAR and strengthens the proposed commonality of nonpeptide/peptide phenolic OH residues in opioid receptor binding interactions.[10–12] Further studies of other potential bioisosteres for [Tyr^1] and the incorporation of Cpa into cyclic opioid peptide ligands will be reported subsequently.

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4. This point is further underscored upon inspection of the tables presented in the comprehensive review by Hruby and Gehrig (Ref. 3a) in which over 500 opioid peptides are listed, each one possessing a N-terminal tyrosine.
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14. (a) Fmoc- and Boc-protected Cpa may be derived via Pd-catalyzed carboamidation of the corresponding *O*-tyrosine triflate as the key step or purchased from RSP amino acids, Boston, MA. (b) Cpa-containing peptides were prepared on Wang or Rink resin using classical Fmoc chemistry with commercially available appropriately protected amino acids wherein the N-Boc protected **6** was used in the final coupling cycle. Acid-mediated cleavage of the penultimate resin resulted in simultaneous removal of the Boc-protecting group. Peptides were purified to >98% purity by HPLC and exhibited physical and spectroscopic properties consistent with their structure.
15. By way of control, the [Phe^1]-DADLE amide analog H-Phe-ala-Gly-Phe-leu-NH_2 **27** was prepared to demonstrate that indeed Tyr and Cpa (vida infra) were preferred N-terminal residues for opioid peptides against the cloned *human* receptors. Peptide **27** had the following binding data: $K_i(\mu) = 1200\,\text{nM}$; $(\delta) = 420\,\text{nM}$; $(\kappa) > 10,000\,\text{nM}$. This is consistent with previous SAR observations where modification of the phenol leads to a dramatic loss in binding affinity (Ref. 3).
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