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Sandmeyer reaction repurposed for the site-selective, non-oxidizing radioiodination of fully-protected peptides: Studies on the endogenous opioid peptide α -neoendorphin

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Abstract

Standard radioiodination methods lack site-selectivity and either mask charges (Bolton-Hunter) or involve oxidative reaction conditions (chloramine-T). Opioid peptides are very sensitive to certain structural modifications, making these labeling methods untenable. In our model opioid peptide, α -neoendorphin, we replaced a tyrosyl hydroxyl with an iodine, and in cell lines stably expressing mu, delta, or kappa opioid receptors, we saw no negative effects on binding. We then optimized a repurposed Sandmeyer reaction using copper(I) catalysts with non-redoxing/non-nucleophilic ligands, bringing the radiochemical yield up to around 30%, and site-selectively incorporated radioactive iodine into this position under non-oxidizing reaction conditions, which should be broadly compatible with most peptides. The ¹²⁵I- and ¹³¹I-labeled versions of the compound bound with high affinity to opioid receptors in mouse brain homogenates, thus demonstrating the general utility of the labeling strategy and of the peptide for exploring opioid binding sites.

Keywords

Peptide radioiodination; Opioid receptor binding; Endogenous opioid peptide; I-125; I-131

Opiates are the gold standard for treating moderate to severe pain, including the pain associated with cancer.^{1,2} Opioid receptors are G-protein coupled receptors, which mediate this relief primarily through the mu receptor. Three families of opioid receptors have been cloned: mu (MOR-1), delta (DOR-1), and kappa₁ (KOR-1). Subtypes of mu, delta, and

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kappa receptors have been proposed both on the basis of alternative splicing of the cloned receptors and using a variety of biochemical and pharmacological approaches.^{3–10} These sites may hold the key to separating the beneficial pain-relieving aspects of opiates from their negative—and potentially deadly—side effects.

Radiolabeled peptides have been indispensable in the characterization of opioid receptors. Tritiated probes of endogenous enkephalin analogs like [D-Ala²,MePhe⁴,Gly(ol)⁵]enkephalin (DAMGO) and [D-Pen²,D-Pen⁵]enkephalin (DPDPE) are commercially available mu- and delta-selective ligands, respectively. However, a tritiated probe has limitations for identifying high affinity sites expressed in low abundance, given its low efficiency of counting and a limitation of only 28.8 Ci/mmol per tritium incorporated.¹¹

To obviate these issues, we have synthesized a number of radioiodinated small molecules. Radioiodinated compounds have many uses, from tissue ablation and tumor imaging to autoradiography and binding assays.^{12–14} When studying heterogeneous receptor populations, a high specific activity, like that conferred by ¹²⁵I or ¹³¹I, has major advantages when visualizing small receptor subgroups. This is illustrated by our recent studies with [¹²⁵I]iodobenzoylnaltrexamide ([¹²⁵I]IBNtxA), which binds with high affinity a novel opioid target containing 6TM/E11 splice variants.^{15,16} To the best of our knowledge, there are no currently available iodinated small molecules to target delta or kappa₁ receptors, making an iodinated opioid peptide approach attractive. A previous study using [³H]α-neoendorphin revealed a complex binding pattern in rat brain homogenate, suggesting the possibility of a novel site in addition to the binding of the traditional kappa₁ site.¹⁷ A higher specific activity probe would allow further study of these sites. Isotopes of iodine are used in PET, SPECT, radioisotope therapy, and *in vitro* studies, and have sufficiently higher specific activities than tritium for our purposes.¹⁸ As such, we sought to label α-neoendorphin with radioactive iodine.

Chemical reactions with peptides are synthetically challenging, due to their sensitivity to heat and alkaline conditions, and the susceptibility of some amino acids to oxidation. Consequently, methods for the radioiodination of fully-formed, deprotected peptides are quite limited. Two of the most common methods for radioiodinating peptides are the conjugation of an iodinated Bolton-Hunter reagent via acylation of the ε-amine of lysines, and the incorporation of radioiodine *ortho* to the tyrosyl hydroxyl by electrophilic aromatic substitution of iodine monochloride formed by oxidation of iodide by chloramine-T.^{12,14,19–21} In addition to being nonselective, both methods have further limitations. The Bolton-Hunter reagent is bulky, and it removes the positive charge on the amine it acylates, thereby potentially altering the activity of the target molecule.¹⁴ Chloramine-T is a strong oxidizer, which can modify tryptophan, methionine, and cysteine residues, thus limiting the scope of substrates that are compatible with this chemistry.^{21–24} Iodogen is a milder oxidizing agent, but the electrophilic substitution method leaves many residues vulnerable to iodination, including potentially histidine, tryptophan, cysteine, methionine, and phenylalanine.^{14,19,25} Tyrosyl iodines are also reported to be dehalogenated *in vivo* leading to accumulation of radioactivity in the thyroid.^{26,27} Most opioid peptides—both endogenous and synthetic—contain a tyrosine at the one-position, which, when modified with an iodine, reduces binding affinity for the parent receptor between 10- and 100-fold.²⁸ Some, such as α-neoendorphin and β-endorphin, have multiple tyrosine residues, raising the issue of multiple substitutions on the same molecule (Fig. 1a).

Therefore, a non-oxidizing, site-selective method of radioiodination on a fully-formed, deprotected peptide is desired, and our model peptide of interest is α-neoendorphin. Sharma et al.²⁸ repurposed the Sandmeyer reaction to radioiodinate the fully deprotected opioid peptide, DPDPE. We decided to attempt this radioiodination protocol on α-neoendorphin at

the Tyr⁸ (Fig. 1 and Reaction Scheme). Like other opioid peptides, the α -neoendorphin analogs are sensitive to degradation from endogenous enzymes, leading us to investigate a D-alanine substitution at position 2. Prior work found that this substitution markedly enhanced the stability of the enkephalins, which share the same first amino acid sequence as Leu-enkephalin.²⁹

We needed to see if the proposed changes at position 2 and position 8 would alter the receptor specificity and binding affinity compared to the parent structure. To evaluate this, we characterized the unlabeled derivatives for activity at traditional opioid receptors expressed in CHO cell lines (Table 1). Incorporation of D-Ala² led to a small reduction in affinity for the KOR-1 receptor compared to α -neoendorphin and the 8-position analog. However, even with the 2-position replacement of Gly with D-Ala, and changing the 8-position from tyrosine to 4-iodophenylalanine, the compounds retained high affinity for the MOR-1, DOR-1, and KOR-1 binding sites. Thus, the [D-Ala²,4'-I-Phe⁸] α -neoendorphin analog retained high affinity for opioid receptors and behaved similarly to endogenous α -neoendorphin. In light of these findings, we radioiodinated the D-Ala² compound.

The fully deprotected α -neoendorphin analog (Fig. 1) was radioiodinated via a Sandmeyer-like dediazotization reaction.²⁸ The method involves incorporating a 4-aminophenylalanine into the peptide at the desired radioiodination location. The aryl amine is diazotized under acidic conditions with sodium nitrite, then substituted by iodide, generating a 4-iodophenylalanine (Fig. 1c and Reaction Scheme). Generation of the anticipated radiolabeled material was confirmed by reversed-phase HPLC co-injection of labeled peptide along with unlabeled reference standard and by assessment of its activity in radioreceptor binding assays.

When applied to [D-Ala²,4'-NH₂-Phe⁸] α -neoendorphin, the literature protocol gave very poor yields (range of 0.4–3.1%, average 1.6%; Table 2).²⁸ Radiochemical yields improved slightly to as high as 6.3% (range of 1.8–6.3%, average 3.0%; Table 2) by changing the catalyst from Cu(I)CN to Cu(I)Cl, possibly due to the nucleophilicity and redox potential of chloride being more favorable than that of cyanide in comparison to iodide.^{30,31} Around 60% of the radioactivity added to the reaction was lost to the filtered, insoluble salts, believed to be copper(I) [¹²⁵I]iodide, possibly explaining reduced yields.

A catalyst was not required for iododediazotization, but omitting the catalyst did not significantly improve the yield. Non-oxidizing acids such as hydrochloric acid, trimethanesulfonic acid, and p-toluenesulfonic acid gave yields similar to sulfuric acid.²⁸ Addition of ascorbic acid alongside the [¹³¹I]NaI prevented iodine incorporation, and its addition later in the reaction had no effect. Sodium thiosulfate also did not influence the reaction at any time of addition.

While the diazonium can be iodinated without a catalyst, the Sandmeyer reaction utilizes copper to install the anion to which it is coordinated, CuX. We seek to utilize copper catalysts coordinated to ligands that will not react, thus avoiding product mixtures and favoring iodine incorporation. By rational catalytic ligand choice, we were able to increase our yields. Copper catalysts with non-nucleophilic and non-reducing/oxidizing ligands (i.e. copper(I) trifluoromethanesulfonate benzene complex ([Cu(I)OTf]₂·C₆H₆), tetrakis(acetonitrile)copper(I) tetrafluoroborate (Cu(I)[CH₃CN]₄BF₄), copper(I) 3-methylsalicylate, and copper(II) sulfate (Cu(II)SO₄) gave radiochemical yields of approximately 30% (data not shown). Comparable yields were obtained with copper(II) sulfate—without any copper(I)—and one equivalent of ascorbic acid. This reaction gave a yield of 24.9%, while the copper triflate ([Cu(I)OTf]₂·C₆H₆) alone gave a 32.4% yield

(yields not decay corrected). Adding one equivalent of copper(II) sulfate to the copper(I) reactions did not alter the yield (data not shown).

Employing this chemistry, we synthesized an ^{131}I -radiolabeled version of α -neoendorphin, i.e. ^{131}I -[D-Ala²,4'-I-Phe⁸] α -neoendorphin, and examined its receptor binding. In mouse brain, it displayed saturable binding with an apparent K_D of 11 nM and B_{max} of 465 fmol/mg (Fig 2). Despite the likelihood that it was labeling multiple opioid classes, its binding was monophasic, implying similar affinities for the sites labeled. This is consistent with the affinity of the compound against the cloned mu and delta opioid receptors. The abundance of kappa₁ receptors in mouse brain is quite low and is not likely to make a contribution to the overall binding levels.

Endogenous α -neoendorphin competed these sites with an apparent K_i of 10 nM (Table 3), suggesting the radioligand bound opioid sites similarly as native α -neoendorphin. Based upon the high affinity of the analog to all three receptor types in competition studies against binding to the cloned MOR-1, DOR-1 and KOR-1 receptors, we anticipated that its binding in brain would be complex, comprised of sites from all three receptor families. This would not be unusual since other endogenous opioid peptide ligands, like dynorphin A (1–17) and dynorphin B, also show limited, if any, selectivity among the various sets of receptors. This would explain their ability to compete ^{131}I -[D-Ala²,4'- ^{131}I -Phe⁸] α -neoendorphin binding with moderate affinities (Table 3; 5.5 ± 2.6 nM and 6.7 ± 3.5 nM, respectively). α -Neoendorphin is generated from the same precursor peptide as dynorphin A and dynorphin B.³² The synthetically-derived opioid peptides DAMGO and DPDPE, which are MOR-1 and DOR-1-selective, respectively, also lowered binding with affinities (1.4 ± 0.56 nM and 3.8 ± 2.7 nM, respectively), similar to their affinities for their respective receptors.^{33,34} U50,488H is a small molecule with high selectivity for KOR-1. We were unable to determine an apparent K_i for U50,488H due in part to the low abundance of KOR-1 receptors relative to MOR-1 and DOR-1 in the mouse brain.

The mechanisms of dediazotization are complex.^{30,31,35–37} The first step is electron transfer by a reducing agent, followed by releasing N_2 and forming the aryl radical. Iodide has sufficient reduction potential to donate the electron, however, it cannot halogenate because the backwards reaction is too fast. Halogenation is accomplished by other forms of iodine: $\text{I}^\bullet$, I_2 , I_3^- , $\text{I}_2^{\bullet-}$. While iodide can initiate, other nucleophiles such as bromide, chloride, and cyanide, need a further reducing agent. This role can be played by Cu(I). Copper has dual roles, with Cu(I) [or Cu(0)] acting as an electron transfer agent, and Cu(II) acting as a ligand donor and oxidant, thus completing the redox cycle.

When the Sandmeyer reaction is performed competitively with equimolar chloride and bromide, more bromination occurs than chlorination.³⁷ Because the incoming substituent is in the form of a nucleophile, not a radical, one could expect that reducing the nucleophilicity of the other players should confer an advantage to the iodide.^{30,31,36} However, Hanson et al.³⁷ concluded that in the presence of Cu(II)X_2 , the aryl radical actually behaves like the nucleophile. Additionally, the rate of reaction is faster when the redox potential of Cu(II)X_2 is larger, which is the case for Cu(II)Br_2 over Cu(II)Cl_2 . The case would be even more favorable for Cu(II)I_2 , and more favorable than iodide alone. Copper also serves to coordinate the iodide, coupling the oxidation of iodide with the reduction of copper. In the copper-less case, with slight modification, this reaction has also been shown to work with $^{211}\text{At}^-$ with comparable if not greater yields than $^{131}\text{I}^-$.^{36,38} However, favorable redox potentials parallel favorable nucleophilicity for the halogens.

Galli points out that the dediazotization reaction runs most smoothly with 1eq Cu(I) and 1eq Cu(II). Side reactions are reduced by stabilization of the diazonium, often by addition of

crown ethers or formation of tetrafluoroborate salts. Electron donating substituents—like the α -carbon of phenylalanine—on the ring stabilize the diazonium, decreasing the likelihood of picking up an electron. Electron transfer is rate-limiting, so side products are minimized by utilizing the reducing agent with the lowest oxidation potential, in this case, Cu(I).³⁶

That we have found yields much higher in the presence of copper is not surprising. The recommended amount of iodide in a catalyst-free iododediazotization ranges from equimolar up to 10.7 equivalents.³⁵ In radiochemistry, however, these equivalents are impractical. An excess of radioactive iodide means reduced radiochemical yield, wasted isotope, and unnecessary exposure to radioactivity. For these reasons, the starting material is typically in excess, rather than the radioiodide. Also, the copper-catalyzed reaction can accept I^- , one more form of iodine than the copper-free reaction.

Copper(II) acts as a nucleophile transfer agent and oxidant, and the addition of one equivalent of copper(II), in addition to copper(I), has been shown to improve yield,³⁶ but we observed little difference. We also tested copper(II) sulfate, without any copper(I) catalyst, instead adding one equivalent of ascorbic acid as a reducing agent to reduce the copper(II) to copper(I), thus completing the copper redox cycle. We did not discern a substantial yield difference between this reaction and the three non-redoxing/non-nucleophilic copper(I) ligands.

Sharma et al.²⁸ attribute their increase in yield to 18-crown-6 dissolving Cu(I)CN, thus making more copper available for the reaction, as copper(I) salts have very low solubility in water. 18-Crown-6 has been shown to stabilize the diazonium.³⁹ Also, the crown ether could be pulling the Na^+ away from iodide, thus leaving it a stronger nucleophile. However, the abundance of H^+ available with which to pair may render this effect negligible. Increasing the association of iodide with the diazonium and/or copper(II) would likely increase the yield. We did not find any yield advantage by adding 18-crown-6.

In summary, we have developed a radioiodinated version of the endogenous α -neoendorphin. The chemistry utilized herein was previously applied to another opioid peptide, DPDPE, which suggests wide applicability.²⁸ The radioiodinated peptide bound all opiate receptors with an affinity similar to the endogenous α -neoendorphin. The sites in mouse brain bound by our peptide are competed by selective mu and delta ligands, as well as non-selective endogenous opioid peptides, all with reasonable affinities. This suggests our peptide is binding mu, delta, and kappa₁ sites, as would be expected based on the affinity of the compound in competition studies with the cloned MOR-1, DOR-1, and KOR-1 receptors. This ligand could prove useful in the future evaluation of opioid drug targets in mammalian brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- a) Tyr¹-Gly²-Gly³-Phe⁴-Leu⁵-Arg⁶-Lys⁷-Tyr⁸-Pro⁹-Lys¹⁰
- b) Tyr¹-D-Ala²-Gly³-Phe⁴-Leu⁵-Arg⁶-Lys⁷-*p*-NH₂-Phe⁸-Pro⁹-Lys¹⁰
- c) Tyr¹-D-Ala²-Gly³-Phe⁴-Leu⁵-Arg⁶-Lys⁷-*p*-I*-Phe⁸-Pro⁹-Lys¹⁰

Figure 1.

Structures of endogenous (a) and modified (b and c) α -neoendorphin.

Replacement of glycine by D-alanine reduces consumption by some proteases ²⁹.

Modification of the initial tyrosine alters binding behavior, thus iodination was focused on later aromatics.

Endogenous α -Neoendorphin: Modified α -Neoendorphin: **Figure 2.**

Homologous competition studies of ^{131}I -[D-Ala²,4'-I-Phe⁸] α -neoendorphin.

Homologous competition studies of ^{131}I -[D-Ala²,4'-I-Phe⁸] α -neoendorphin (0.17 nM; SA of [^{131}I]NaI ranged from 500–1000 Ci/mmol) were performed using unlabeled [D-Ala²,4'-I-Phe⁸] α -neoendorphin in CD-1 mouse brain membrane preparations. This figure is a representative experiment, and error bars represent the SEM of triplicate samples. Error bars that cannot be seen are smaller than the size of the symbol. The data were fit by one-site homologous competition using nonlinear regression analysis, constrained to empirically-determined nonspecific binding (GraphPad Prism, La Jolla, CA). Apparent K_D and B_{max} values are the means $\pm$ SEM of at least three independent determinations. Very high competitor concentrations over 100 nM fully competed specific binding and occasionally lowered total binding slightly below nonspecific levels, presumably a result of experimental variability.

Reaction Scheme.

Reaction conditions: a) while on ice, the peptide was dissolved in aqueous acid, NaNO₂ was added and reacted for five minutes, followed by quenching with sulfamic acid; b) to various catalysts and additives (typically in water), Na*I was added, followed by the freshly diazotized peptide. Reaction was held 2–3 hours, and either kept on ice or allowed to come to room temperature.

Table 1Competition by α -neoendorphin analogues against [125 I]IBNtxA in transfected cell lines

Peptide Analogue	K_i (nM) $\pm$ SEM		
	CHO-MOR-1	CHO-DOR-1	CHO-KOR-1
α -Neoendorphin	7.3 $\pm$ 1.6	1.7 $\pm$ 0.15	2.4 $\pm$ 0.53
[4'-I-Phe ⁸] α -neoendorphin	13 $\pm$ 3.8	3.0 $\pm$ 0.077	3.5 $\pm$ 0.42
[D-Ala ²] α -neoendorphin	9.3 $\pm$ 1.2	0.95 $\pm$ 0.24	13 $\pm$ 2.2
[D-Ala ² ,4'-I-Phe ⁸] α -neoendorphin	6.6 $\pm$ 2.3	2.1 $\pm$ 0.88	14 $\pm$ 4.4

Our modifications have not significantly altered the binding behavior as compared to the endogenous peptide. Competition studies in membranes from CHO cells stably transfected with the indicated opioid receptors were performed with [125 I]IBNtxA (75 pM) and K_i values determined by nonlinear regression analysis (GraphPad Prism, La Jolla, CA). Results are the means $\pm$ SEM of at least three independent replications.

Table 2

Radiochemical yields varying acid and copper ligand concentrations

Catalyst	Isotope	mCi [¹²⁵ I]NaI	Average Radiochemical Yield (%)
CuCN	I-125	3.1–5.5	1.6
CuCl	I-125	1.0–5.5	3.0
None	I-125	2.4	2.0
None	I-131	1.0–1.1	3.1
Cu(II)SO ₄ + Ascorbic Acid	I-131	0.13	32.4
Cu(I)(CH ₃ CN) ₄ BF ₄	I-131	0.12	24.9

Reactions using CuCN followed the published protocol ²⁸. Radiochemical yields were calculated by dividing the total millicuries of collected HPLC fractions that contain measureable specific activity in a binding assay by the total millicuries added to the reaction. Yields are not decay corrected. Copper-containing reactions were difficult to purify by HPLC due to closely retaining peaks, which was reduced in the copper-free reactions. Non-redoxing/non-nucleophilic copper catalysts gave the highest yields.

Table 3Competitions against ^{131}I -[D-Ala²,4'-I-Phe⁸] α -neoendorphin in CD-1 mouse brain

Competitor	K _i (nM) $\pm$ SEM
α -Neoendorphin	9.8 $\pm$ 0.83
U50,488	>500
Dynorphin A (1-17)	5.5 $\pm$ 2.6
Dynorphin B	6.7 $\pm$ 3.5
DAMGO	1.4 $\pm$ 0.56
DPDPE	3.8 $\pm$ 2.7

Competition studies were performed with the indicated compounds against ^{131}I -[D-Ala²,4'-I-Phe⁸] α -neoendorphin (0.35 nM, SA of [^{131}I]NaI ranged from 230–420 Ci/mmol) in membranes prepared from CD-1 mouse brain. The data were fit by one-site competition using nonlinear regression analysis (GraphPad Prism, La Jolla, CA). Apparent K_i values are the means $\pm$ SEM of at least two independent determinations.