A Simple General Method for (Radio)iodination of a Phenylalanine Residue in Peptides: Preparation of [D-Pen^2,4′-^{125}I-Phe^4,D-Pen^5]Enkephalin, a Peptide with Extraordinary Selectivity for δ-Opioid Receptors

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Introduction

The chloramin-T method and the Bolton–Hunter approach are the most widely practiced methods for the radioiodination of proteins.[1] The chloramin-T method involves the introduction of one or two radioiodine atoms at 3′ and 5′ aromatic positions of a tyrosine residue in the protein under oxidative conditions, while the latter involves conjugating a separately radioiodinated prosthetic group to a protein. The direct application of these methods for radioiodination of various neuropeptides and peptide hormones, although practiced widely, often is detrimental to their biological profiles. For example, the use of the chloramin-T method resulted in (a) complete loss of biological activity of an radioiodinated α-melanotropin hormone[2] and (b) misleading results in a radioreceptor binding assay for adrenocorticotropin hormone (ACTH) that were later rectified by the synthesis and use of another carefully designed radioiodinated ACTH analogue.[3] Furthermore, endogenous peptides often exhibit rather stringent stereochemical requirements for a given biological profile. Even minor structural changes, like the introduction of an iodine atom in a tyrosine residues in a peptide, can alter both potency and selectivity toward a particular receptor subtype.[4,5] This calls for the need not only to study the effect of iodinating peptides on their biological activity and receptor selectivity, but also to develop other approaches for radioiodination that are suited for peptides.

The effect of iodination on the biological activity and receptor subtype selectivity has been studied systematically for the opioid peptide analog [D-Pen^2,D-Pen^5]enkephalin (DPDPE) developed earlier in our laboratory.[6] This peptide is highly selective for the δ-opioid receptor (δ/μ selectivity ratio about 2000). Radioiodination of this peptide by chloramin-T method resulted in [3′-^{125}I-Tyr^1]DPDPE, which exhibited a 10–100-fold loss in potency (Toth, Burks, and Hruby, unpublished). However, introduction of an iodo group at the para position of the phenyl ring of Phe^4 residue in DPDPE improved the receptor selectivity without compromising the potency. This iodinated analogue, [D-Pen^2,4′-I-Phe^4,D-Pen^5]enkephalin ([4′-I-Phe^4]DPDPE), exhibited 17 400-fold selectivity for the δ over the μ receptor.[7] Thus, the presence of a iodine atom as a structural feature in [4′-I-Phe^4]DPDPE offered for the first time the possibility of synthesizing a potent radioiodinated enkephalin analogue with high δ receptor selectivity. Because of its unique biologically profile and high specificity activity (2200 Ci/mmol), if synthesized under carrier-free radioiodination conditios, [4′-^{125}I-Phe^4]DPDPE could serve as an indispensable ligand in the characterization of δ-opioid receptors. Thus, this peptide was taken as an example for the radioiodination studies described here.

A number of different methods of introducing a radioiodine atom into a phenyl ring have been documented in the literature. From a practical standpoint, the synthesis of radioiodinated ligands must be straightforward and convenient involving relatively simple and nonhazardous chemical species such as ^{125}I-labeled sodium iodide for the introduction of radioactive iodo group. In this respect, the studies of Moerlein and Coenen[8] and de Paulis and coworkers[9] on regiospecific electrophilic aromatic iododestannylation using Na^{125}I–dichloramin-T or Na^{125}I–chloramin-T reaction, which results in a high degree of radioiodine incorporation (about 56%) with high purity seemed promising. However, the application of this method to DPDPE would require a cumbersome synthesis of [4′-(trialkylstannyl)-Phe^4]DPDPE as well as selective blocking of the tyrosine phenolic group. The latter is essential to prevent the incorporation of radioiodine into the phenolic ring of tyrosine under the oxidative iododestannylation conditions. The requirement to selectively block the phenolic group before iododestannylation and then to deblock afterwards makes this method much less attractive for peptides having tyrosine residues. Also, Heindel's modification of the Sandmeyer–Wallach reaction[10] that involves iododediazonization of triazenes synthesized by trapping nascent aryldiazonium ions with pyrrolidine seems less attractive for peptides. The presence of a tyrosine residue in a peptide would make it difficult to synthesize the required (4′-pyrrolidinyltriazenyl)-Phe-substituted peptide needed in this method. Further, the iododediazonization conditions that require refluxing the triazene with Na^{125}I under acidic conditions for a few hours will not be suitable for the stability of the peptides in general.

Another general method utilizing Sandmeyer's reaction,[11] however, appeared attractive for its simplicity and compatibility with peptides having a tyrosine residue. This method utilizes a 4′-NH_2-Phe-substituted precursor which is diazotized and treated with Na^{125}I in the presence of cuprous cyanide. The main drawback of this method, however, lies in a rather low (1–2%) radioiodine incorporation under carrier-free conditions. Therefore, we have reinvestigated and successfully modified this method to significantly improve the yield of the radioiodinated product.

The precursor peptide [4′-NH_2-Phe^4]DPDPE[12] was diazotized in 2 N H_2SO_4. As judged by HPLC, the precursor peptide was found to be stable at room temperature in 2 N H_2SO_4, for more than the 5-h duration of the radioiodination reaction and purification steps. Similarly, another peptide containing acid-sensitive tryptophane residue, α-melanotropin (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-

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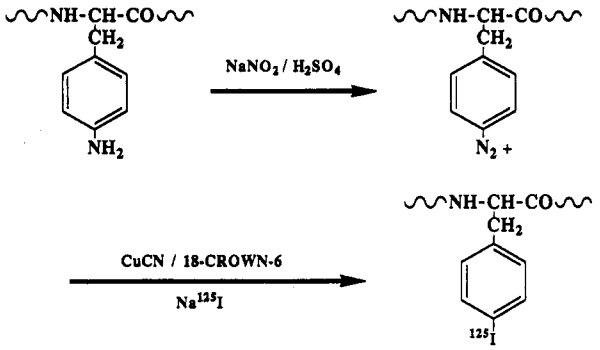
Scheme I. Synthetic Strategy for the Introduction of Radioiodine into a Phenylalanine Residue in a Peptide



Arg-Trp-Gly-Lys-Pro-Val-NH_2), was also found to be stable under identical conditions, suggesting general applicability of this method. As is evident from method 1 only 1-2% incorporation of the radioiodine was achieved in presence of CuCN as catalyst. The reaction here has employed a large excess of the precursor peptide over $Na^{125}I$, the radioiodinating species. The unreacted excess of the diazotized precursor peptide was separated from the product by HPLC during the purification step. As a modification to this method a crown ether, 18-crown-6, was also added to the reaction mixture (method 2). This caused a dramatic increase in the yield (25–27%) of the iodinated peptide. The radioactivity elution profile during HPLC exhibited no other major radioactive peak except the solvent peak (inorganic iodide) and the product peak. The purified product and separately prepared "cold" [4′-I-Phe⁴]DPDPE[7] when co-injected onto the HPLC column eluted together as a single peak.

It is well known that aromatic diazonium salts usually undergo direct aromatic nucleophilic substitution reaction with inorganic iodide via a S_N1 reaction.[13] The reaction with chloride or bromide anions, on the other hand, requires catalysis by the corresponding cuprous halide, which operates through a free-radical mechanism[13] (eq 1).

$$ArN_2^+X^- + CuX \rightarrow Ar^\bullet + N_2 + CuX_2 \qquad (1)$$

$$Ar^\bullet + CuX_2 \rightarrow ArX + CuX$$

However, under S_N1 conditions no detectable incorporation of radioiodine in the peptide was observed under carrier-free levels of radioiodide.[11] The presence of CuCN in the reaction led to low (1–2%) incorporation[11] (method 1). One of the reasons for this is likely due to the low concentrations (between 10 and 100 μM) of the radioiodinating species $Na^{125}I$ under these reaction conditions. Well-studied examples of Sandmeyer reaction, on the other hand, have employed concentrations in the 0.5–2 M range.[14-16] Further, in the presence of CuCN as catalyst, this reaction operates through a free-radical mechanism generating rather short-lived Ar• free radicals which might disintegrate by capturing some other free radical at the low concentrations of radioiodinating species ($Cu^{125}I$). In addition, the mechanism in eq 1 suggests that the reaction cannot go to completion if $^{125}I^-$ is the limiting reactant in the reaction mixture.

Furthermore, based on the results, the effectiveness of CuCN as catalyst also appeared questionable to us. While CuCN is reported to be insoluble in aqueous medium, the solubility of CuI is also very low (about 0.8 mg/100 mL in water[17]), suggesting that the primary catalytic species, $Cu^{125}I$, would always be present in the reaction mixture in low concentration. This led us to add a crown ether to the reaction mixture to help solubilize the cuprous ion in the aqueous medium (method 2) (see Scheme I), thereby giving a much higher yield (25–27%) of the radioiodinated product in a high degree of purity. It, therefore, appears that the solubility of the cuprous ions was a major factor responsible for low incorporations in method 1 and in the studies described by Escher.[11] The [4′-^{125}I-Phe⁴]DPDPE prepared by the method described here had properties identical with those of the cold peptide previously prepared[7] and exhibited excellent properties in radioreceptor assays.[18]

In conclusion, we have achieved a significant improvement in a method of introducing radioiodine in the aromatic ring of a phenylalanine residue. This simple and straightforward method using a crown ether should be applicable to many other similar systems. Utilizing this method we have recently synthesized another radioiodinated and biologically potent opioid peptide, Tyr-D-Ala-(4′-^{125}I)Phe-Glu-Val-Val-Gly-NH_2, ([4′-^{125}I-Phe³]deltorphin), in 30–35% yield from [4′-NH_2-Phe³]deltorphin (Hruby, Yamamura, and Davis, personal communication).

Materials and Methods

The precursor peptide [4′-NH_2-Phe⁴]DPDPE and the cold standard [4′-I-Phe⁴]DPDPE were synthesized as reported (refs 12 and 7, respectively). $Na^{125}I$ was obtained from Amersham Corporation (Arlington Heights, IL). The crown ether, 18-crown-6, was obtained from Aldrich Chemical Co. HPLC was carried out on a Spectra Physics system (Model SP 8700) using a C-18 reverse-phase analytical column (length 250 mm, internal diameter 4.6 mm) from Vydac (The Separation Group, Hesperia, CA). Individual fractions (1.5 mL each) were collected and an aliquot counted in a γ counter (LKB Model 1275 MINIGAMMA). The radiochemical yield was calculated after HPLC purification.

Method I. To a 10-μL aqueous solution of the precursor peptide (20 mg/mL) in a 1.5-mL polypropylene vial was added 12.5 μL of 2 N sulfuric acid. The resulting mixture was chilled in ice, and 2 μL of 1 M sodium nitrite was further mixed to it. After 5 min at 0 °C the reaction was quenched by the addition of 10 μL of 1 M sulfamic acid. The reaction mixture was transferred to a second polypropylene vial containing 1 mCi (10 μL) of $Na^{125}I$ at 0 °C. Immediately following, 2 μL of a cuperous cyanide suspension (100 mg/200 μL) was mixed in. The reaction mixture was placed in an ice bath that was allowed to warm to room temperature.

After 3 h, 10 μL of dimethylformamide (DMF) was added, and the reaction mixture was centrifuged. The [4′-^{125}I-Phe⁴]DPDPE was purified from the supernatant by HPLC using a linear gradient of 10–30% acetonitrile in 0.1% aqueous trifluroacetic acid that was completed in 30 min at a flow rate of 1.5 mL/min. The product eluted at 32.2 min under these conditions and was fully separated from the unreacted precursor peptide which elutes at 13.2 min. Alternatively and preferably, a linear gradient of 20–27% acetonitrile was used under the same conditions. In this case the product eluted at 26.7 min and the precursor peptide at 4.2 min. The radiochemical yield was 1–2% in several trials. The peptide was identical with the previously prepared [D-Pen²,4′-I-Phe⁴,D-Pen⁵]enkephalin.[7]

Method 2. One milligram each of CuCN and the crown ether, 18-crown-6, were added to a 1.5-mL polypropylene vial containing 10 μL of water. Then 10 μL (1 mCi) of $Na^{125}I$ was added, and the vial was placed in an ice bath. After 5 min a freshly diazotized

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and quenched mixture of the precursor peptide prepared as described in method 1 was added to the vial. The reaction was left in an ice bath with occasional shaking and was allowed to come to room temperature. After 3 h, 10 μL of DMF was added, the reaction mixture was centrifuged, and the supernatant was subjected to purification by HPLC as described above under method 1. The radiochemical yield was 25–27% in several trials. The peptide was identical with the previously prepared [D-Pen^2,4′-I-Phe^4,D-Pen^5]enkephalin.

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Vanadium(II)-Promoted Cyclization of 5,6-Enals or 5,6-Ynals. A Stereoselective Approach to *trans*-2-Alkyl- or *trans*-2-Alkylidenecyclopentanols

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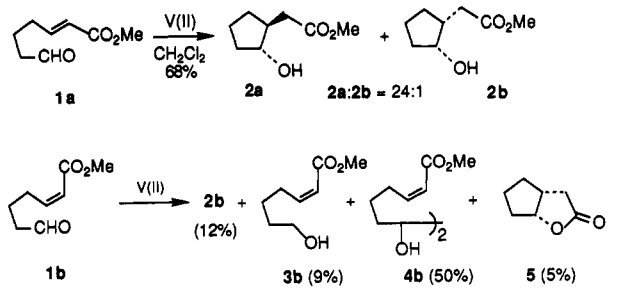
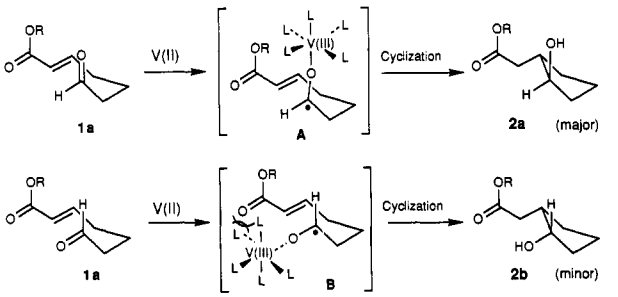
Reductive cyclization of δ,ε-enal or its keto version has found diverse applications in the synthesis of bioactive cyclopentanoids.[1] Low-valent metallic species such as zinc[2] and Sm(II)[3] as well as lithium naphthalenide[4] have been conveniently utilized for this purpose. Electroreduction in a divided cell system has also shown promise in the related conversion of an aldehyde–electron-deficient olefin[1a,5] or a ketone–normal olefin system.[6] Photoreduction is known to be effective for this purpose.[7] However, little attention has been paid to the diastereofacial selection of the process in spite of its potent applicability to the control of 1,2-relative stereochemistry. Recently, the bimetallic V(II) species, $[V_2Cl_3(THF)_6]_2[Zn_2Cl_6]$, has been introduced by Pedersen et al. to achieve the stereoselective cross coupling of two different alkanals under the mild conditions,[8] referred to as an intermolecular pinacol cross-coupling reaction. This encouraging development coupled with the propensity of this V(II) reagent to generate metalated aldehyde ketyls prompted us to envisage a synthesis of cyclopentanols with α-substituents in a stereoselective manner employing various δ,ε-enals. We now report that the cyclization of 5,6-unsaturated aldehydes is promoted with this reagent to provide 2-alkyl- or 2-alkylidenecyclopentanols, in which the kind of olefin and its geometry are responsible for the stereochemical outcome.

The treatment of methyl (*E*)-7-oxo-2-heptenoate (**1a**)[5] with 2–3 equiv of $[V_2Cl_3(THF)_6]_2[Zn_2Cl_6]$, freshly prepared from $VCl_3(THF)_3$ by the reduction with Zn,[8a] in dichloromethane at room temperature resulted in smooth cyclization to give **2a** in 68% yield. Relative stereochemistry at the C-1 and C-2 positions of newly constructed cyclopentanol was assigned to be **2a** with a ratio of 24:1 in favor of the trans isomer based on the ^{1}H NMR analysis.[9] The corresponding cis isomer was obtained as a mixture of the hydroxy ester **2b** and its lactone derivative. The similar conversion of **1a** into **2a** with the V(II) reagent

Scheme I



Scheme II



was conducted in the presence of *tert*-butyl alcohol as a proton donor to afford again the trans isomer **2a**, selectively (61%). However, in this case, the trans to cis ratio (**2a**:**2b**) decreased to 11:2, the cis isomer being obtained as the corresponding γ-lactone **5** (Scheme I).

It is noteworthy that the selectivity observed in this work is exceptionally high as compared with the previous reports in which a mixture of **2a** and **2b** was given in 69–70% yields with the ratios of 3.1:1 and 1.4:1 for the reduction of **1a** with Sm(II)[3] and the electroreduction of **1a** in a DMF–R_4NBF_4–(Pt)–(Hg) system,[5] respectively. Furthermore, trans selectivity increased when the methacrylate derivative **1c** was subjected to the present cyclization. Indeed, the formation of a cis isomer could not be detected at all by the ^{1}H NMR (500-MHz) analysis. A similar high level of trans selectivity was observed in the

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