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Solid Phase Synthesis and Application of Labeled Peptide Derivatives: Probes of Receptor-Opioid Peptide Interactions

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Abstract

Solid phase synthetic methodology has been developed in our laboratory to incorporate an affinity label (a reactive functionality such as isothiocyanate or bromoacetamide) into peptides (Leelasvatanakij, L. and Aldrich, J. V. (2000) *J. Peptide Res.* 56, 80), and we have used this synthetic strategy to prepare affinity label derivatives of a variety of opioid peptides. To date side reactions have been detected only in two cases, both involving intramolecular cyclization. We have identified several peptide-based affinity labels for δ opioid receptors that exhibit wash-resistant inhibition of binding to these receptors and are valuable pharmacological tools to study opioid receptors. Even in cases where the peptide derivatives do not bind covalently to their target receptor, studying their binding has revealed subtle differences in receptor interactions with particular opioid peptide residues, especially Phe residues in the N-terminal “message” sequences. Solid phase synthetic methodology for the incorporation of other labels (e.g. biotin) into the C-terminus of peptides has also been developed in our laboratory (Kumar, V. and Aldrich, J. V. (2003) *Org. Lett.* 5, 613). These two synthetic approaches have been combined to prepare peptides containing multiple labels that can be used as tools to study peptide ligand-receptor interactions. These solid phase synthetic methodologies are versatile strategies that are applicable to the preparation of labeled peptides for a variety of targets in addition to opioid receptors.

Keywords

affinity label; bromoacetamide; isothiocyanate; biotin; His tag; TIPP

Introduction

Labeled peptides can be valuable pharmacological tools to study receptors and receptor-ligand interactions (Baindur and Triggle, 1994). Typical labels incorporated into peptides are fluorescent groups, which can be used to study receptor localization and internalization (see for example (Gaudriault et al., 1997; Arttamangkul et al., 2000; Marinova et al., 2005)), or biotin which can be used in receptor purification (see for example (Eppler et al., 1992; Eppler

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et al., 1993)). In addition, reactive functionalities can be incorporated into peptides to yield derivatives that bind to their target covalently. These affinity label derivatives bind in a two-step process, first binding reversibly to the target followed by covalent attachment (Takemori and Portoghese, 1985). In order for the second step to occur the reactive group must be suitably located on the ligand where it can react with an appropriate functionality on the receptor. Affinity label ligands fall into two categories, those containing an electrophilic group (e.g. an isothiocyanate, haloacetamide, Michael acceptor, or nitrogen mustard) and ones containing a photoaffinity label (i.e. a precursor of a nitrene or carbene).

Our research focuses on the synthesis of labeled derivatives of opioid peptides as tools to study opioid receptors. As part of this research we have developed synthetic methodology for the preparation of both affinity label derivatives of opioid peptides and of analogs containing a label such as biotin or a fluorescent group incorporated at the C-terminus of the peptide. These modifications have been combined into dual labeled opioid peptide analogs that can be used to detect covalently modified opioid receptors.

Here we describe the solid phase synthetic methods developed to prepare these labeled peptide derivatives and the scope and limitations of these methodologies. In addition, insights about opioid peptide-receptor interactions that have been gained from studying the binding of these peptides to opioid receptors will be discussed.

Results and Discussion

Solid phase synthesis of peptides containing a reactive affinity label group

Early peptide-based affinity labels for opioid receptors were mainly photoaffinity labels containing Phe(*p*-N₃) (see (Takemori and Portoghese, 1985; Schiller, 1993; Aldrich and Vigil-Cruz, 2003) for reviews). However, the use of photoaffinity labels for opioid receptors has been limited because these receptors are susceptible to inactivation by UV irradiation (Glaser and Venn, 1981). When we began our research, the opioid peptide derivatives containing an electrophilic affinity label group were limited to a few compounds, mainly enkephalin derivatives, containing a C-terminal chloromethyl ketone derivative (Pelton et al., 1980; Venn and Barnard, 1981; Szücs et al., 1983a; Benyhe et al., 1987), the amino acid nitrogen mustard melphalan (Szücs et al., 1983b; Aldrich Lovett and Portoghese, 1987), a C-terminal cysteine residue (i.e. DALCE, [D-Ala², Leu⁵, Cys⁶]enkephalin (Bowen et al., 1987)), or a Cys residue protected with the 3-nitro-2-pyridinesulfonyl (Npys) group (Shimohigashi et al., 1992). Except for DALCE] these peptides were synthesized in solution.

Other reactive functionalities, particularly the isothiocyanate group (Rice et al., 1983; Burke et al., 1986; Portoghese et al., 1990) as well as the bromoacetamide group (Archer et al., 1983), have been used successfully in nonpeptide affinity labels for opioid receptors (see (Takemori and Portoghese, 1985; Portoghese, 1992; Aldrich and Vigil-Cruz, 2003) for reviews). Therefore we sought to develop a general synthetic methodology that could be used to efficiently incorporate various reactive moieties such as these into a target peptide and to prepare opioid peptide derivatives containing these electrophilic groups. Our initial syntheses of such peptide affinity label derivatives were performed in solution (Maeda et al., 2000a; Maeda et al., 2000b) as we investigated the compatibility of the reactive functionalities with reaction conditions used in the synthesis of these peptides. We subsequently adapted the strategy to solid phase synthesis (Leelasvatanakij and Aldrich, 2000), permitting much more rapid preparation of the peptides.

Scheme 1 shows the general solid phase synthetic strategy for the preparation of peptide-based affinity labels. The peptides are synthesized on standard resins used for Fmoc (fluorenylmethoxycarbonyl) solid phase synthesis, e.g. a Wang or PAC (peptide acid) resin for

acids or a PAL (peptide amide linker) resin for amide derivatives. The reactive affinity label group was generally introduced into the para position of the phenyl ring of a phenylalanine residue, either in the N-terminal “message” sequence (Chavkin and Goldstein, 1981) or in longer peptides also in the C-terminal “address” sequence (see Table 1). During the peptide chain assembly Fmoc-Phe(*p*-NHAlloc) (Alloc = allyloxycarbonyl) (Leelaswatanakij and Aldrich, 2000) was incorporated into the position where the affinity label group was desired. The peptide synthesis follows standard Fmoc procedures using *t*-butyl type protecting groups for the protection of other side chain functionalities; the N-terminal Tyr residue is protected as the Boc (*t*-butyloxycarbonyl) derivative. Following assembly of the complete peptide sequence the Alloc group is selectively removed using Pd(PPh₃)₄ (Leelaswatanakij and Aldrich, 2000) and the reactive functionality, i.e. an isothiocyanate or bromoacetamide group, introduced into the peptide using TCDI (thiocarbonyldiimidazole) or bromoacetic acid, respectively. The peptides are then cleaved from the resin using TFA (trifluoroacetic acid) with water as a scavenger, and the peptides purified by standard procedures.

We have used this synthetic strategy to successfully prepare potential affinity label derivatives of a variety of opioid peptides (Table 1). Recently we have also been exploring alternative positions for incorporation of the reactive affinity labeling group, including alternative positions on the phenyl ring of a phenylalanine residue (Wang et al., 2006) and alternative residues in the sequence (Dattachowdhury et al., 2008). The synthetic strategy has been applied successfully to peptides containing a variety of amino acids, including basic (Arg and Lys), acidic (Asp) and hydroxyl-containing (Tyr, Ser, and Thr) amino acids. To date we have only observed side reactions in two cases, both of which involved intramolecular cyclization. One involved the C-terminal alcohol of DAMGO ([D-Ala², NMePhe⁴, glyol]enkephalin) derivatives reacting with an affinity label group attached to the side chain of a D-diamino acid in position 2 (Dattachowdhury et al., 2008). While the bromoacetamide derivatives were successfully prepared, following cleavage from the resin the isothiocyanate derivatives underwent intramolecular cyclization with the C-terminal glyol to yield O-alkyl thiocarbamates. We had previously successfully prepared several peptides containing both an isothiocyanate group and a hydroxyl-containing amino acid (Table 1), indicating that the occurrence of this side reaction is dependent on the conformation of the final peptide. In the second case the bromoacetamide derivative of a peptide containing a C-terminal His₆ tag (see below) underwent a side reaction with one of the His residues (Peck et al., 2006).

Interaction of peptides containing a reactive affinity label group with opioid receptors

As noted in the introduction, in order for these peptides to alkylate opioid receptors two conditions must be met: first, they must maintain high affinity for the receptor in order to undergo reversible binding in the first step, and second, the electrophilic group on the peptide must be in the proper position for covalent attachment to the receptor to occur. Fulfilling both of these requirements and identifying peptide-based affinity labels for opioid receptors can be challenging.

The peptides are evaluated for their ability to interact with opioid receptors and to bind covalently to their target receptor in radioligand binding assays (Maeda et al., 2000b). The IC₅₀ values for the peptides are measured in standard binding assays to determine whether introduction of the reactive functionality affects interaction of the peptide with the receptor. In many cases, particularly for peptides targeting μ opioid receptors (MOR) (Choi et al., 2003a, c), introduction of a reactive functionality onto the Phe residue in the “message” sequence (i.e. Phe³ or Phe⁴, depending upon the peptide) resulted in large reductions in opioid receptor affinity. Peptides that retain reasonable affinity for their target receptor in these initial assays are then evaluated for wash-resistant inhibition of binding (WRIB) (Maeda et al., 2000b), typically at a concentration near their IC₅₀ value. Following preincubation of the cell

membranes containing the target receptor with the test peptide, the membranes are washed five times by dilution with buffer followed by centrifugation. Then the percent radioligand binding of the membranes treated with the peptide are determined relative to untreated control membranes. Binding that is significantly lower than the untreated control membranes is strong evidence that the peptide is an affinity label that binds covalently to its target receptor. A control compound that cannot bind covalently to the receptors (typically the corresponding peptide containing an amine in place of the reactive affinity label group) is included in these assays to assess the efficiency of the washing procedure to remove noncovalently bound peptide; such controls are particularly important for very hydrophobic or charged peptides.

To date we have been most successful in identifying peptide-based affinity labels for δ opioid receptors (DOR). This includes the peptide DOR antagonist [*N,N*-dibenzylTyr¹, Phe(*p*-NCS)⁴, Leu⁵]enkephalin (Maeda et al., 2000b), which was the first peptide-based affinity label for any receptor containing an isothiocyanate group as the electrophilic labeling moiety. Subsequently we identified three derivatives of the more potent DOR antagonist TIPP (Tyr-Tic-Phe-Phe, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) (Schiller et al., 1992) in which the affinity label was attached to the para position of the phenyl ring in either position 3 or 4 (Maeda et al., 2000a; Kumar et al., 2002). One of these derivatives [Phe(*p*-NHCOCH₂Br)⁴]TIPP, exhibits 85% WRIB at a concentration of only 2.5 nM (Kumar et al., 2002). We have also identified the first electrophilic affinity label derivative of one of the potent and selective amphibian opioid peptides, [D-Ala², Phe(*p*-NCS)⁴]deltorphin I (Aldrich et al., 2004); this peptide is also the first electrophilic affinity label derivative of an agonist containing the reactive functionality in the “message” sequence of the peptide. Recently we have successfully identified our first peptide-based affinity labels for MOR (manuscript in preparation).

Even in cases where peptides are not affinity labels for their target receptor, these binding studies can reveal subtle differences in receptor interactions of a given amino acid residue in closely related peptides. The differences in the affinities of three different enkephalin derivatives containing a para substituent on the phenyl ring of Phe⁴ illustrate these subtle differences. Thus the rank order of the DOR affinities of the isothiocyanate-, bromacetamide- and amine-containing peptides is very different for Leu-enkephalin vs. DTLET ([D-Thr², Leu⁵, Thr⁶]enkephalin) (Choi et al., 2003b). Thus the difference in the peptide sequences (i.e. D-Thr in position 2 in DTLET vs. Gly in position 2 in Leu-enkephalin) appear to change the orientation of Phe⁴ in the DOR binding site. The same substituents also have very different effects on the DOR affinity of the antagonist [*N,N*-dibenzylTyr¹, Leu⁵]enkephalin (Maeda et al., 2000b) as compared to the agonist Leu-enkephalin, suggesting that N-terminal dialkylation also alters the interaction of Phe⁴ with the DOR binding site. Such marked differences in DOR affinities have not been reported for other para-substituted phenylalanine derivatives in enkephalin analogs.

The effects of these substitutions can also reveal subtle differences in the binding pockets of different opioid receptors for the same residue in the same or closely related peptides. For example, while substitution with a para-isothiocyanate on the phenyl ring of Phe³ in the amphibian peptides is tolerated by DOR (Aldrich et al., 2004), this functionality causes large decreases in MOR affinity (Choi et al., 2003a). In the case of the dermorphin derivative, this resulted in a switch in receptor selectivity from MOR to DOR (Choi et al., 2003a). A similar pattern is observed for potential affinity labels derived from the DOR antagonist TIPP vs. the closely related MOR agonist endomorphin-2. Thus while affinity label groups on the para position of the phenyl ring of either Phe³ or Phe⁴ of TIPP are well tolerated and interact covalently with DOR (Maeda et al., 2000a; Kumar et al., 2002), the same substitutions in endomorphin-2 result in large decreases in MOR affinity, particularly for the Phe³-substituted peptides (Choi et al., 2003c).

Incorporation of biotin and other labels into opioid peptides

We have also developed solid phase synthetic methodology for the incorporation of a label such as biotin or a fluorescent group into opioid peptides (Kumar and Aldrich, 2003). Because of the importance of the N-terminus of opioid peptides for interaction with opioid receptors, such a label must be incorporated at the C-terminus of the peptide. Many labels of interest, particularly many fluorescent groups, are large bulky moieties that can interfere with receptor binding if incorporated too close to pharmacophoric functionalities in the peptide. Even a small labeling group such as biotin can decrease receptor affinity (Koman and Terenius, 1980). Therefore, particularly for small peptides, it is important to incorporate a spacer between the peptide and the label to separate the latter from important pharmacophoric groups. Like the label, the spacer itself must not interfere with receptor interaction. Particularly for hydrophobic peptides, such as several of the opioid peptides of interest, utilizing a hydrophilic spacer is preferred to avoid increasing the lipophilicity of the peptide, which could lead to increased nonspecific interactions and/or decreased aqueous solubility. Therefore we have utilized PEG (poly(ethylene glycol)) -like linkers to attach the label to the peptide.

The solid phase synthetic strategy was developed by synthesizing a labeled derivative of TIPP containing biotin attached to the peptide C-terminus via a PEG-like diamine spacer (Scheme 2) (Kumar and Aldrich, 2003). Since a free carboxylic acid in TIPP and its derivatives is important for maintaining selectivity for DOR over MOR (Schiller et al., 1992), TIPP was extended with an aspartic acid residue, with the α -carboxylic acid attached to the linker and label, while the side chain acid functionality was left free. In previous structure-activity relationship studies in our laboratory we demonstrated that such TIPP analogs extended with an acidic residue maintained DOR affinity and selectivity (Kumar et al., 2000).

The solid phase synthesis is performed on an aldehyde-containing resin that permits modifications of both ends of the diamine spacer (Scheme 2) (Kumar and Aldrich, 2003). The Alloc-monoprotected diamine is attached to the resin by reductive amination, followed by attachment of the label, e.g. biotin, to the resulting secondary amine. The peptide chain is then assembled on the primary amine of the spacer following deprotection of the Alloc group. The order of the reactions is critical to the success of the synthesis. If an Fmoc-protected amino acid (Fmoc-Asp(OtBu) in the case of the TIPP derivative) is first attached to the secondary amine and biotin then attached to the primary amine of the linker following Alloc deprotection, a side reaction occurs; the primary amine resulting from Alloc deprotection causes premature removal of the Fmoc group from the amino acid and subsequent formation of the dibiotinylated product (Kumar and Aldrich, 2003).

The resulting biotinylated TIPP derivative retains nanomolar DOR affinity ($K_i = 12$ nM) which is only slightly lower than that of the parent peptide TIPP ($K_i = 6$ nM), demonstrating the applicability of this design approach to prepare labeled opioid peptides. We are currently preparing labeled derivatives of other opioid peptides using these design and synthetic strategies.

Dual labeled opioid peptide derivatives

While affinity labels are useful pharmacological tools, a second label is required to detect the labeled receptors and take full advantage of this type of ligand. This typically has involved preparing radiolabeled derivatives of affinity labels, but dual labeled ligands containing an alternative second label (e.g. biotin) can be advantageous, both for ease of preparation and for application in various pharmacological assays. Prior to our research, affinity label derivatives of opioid receptor ligands containing a second nonradioactive functionality such as biotin for detection of labeled receptors had not been reported. This approach is particularly appealing for peptide derivatives, since the polymeric nature of peptides facilitates the incorporation of

both an affinity label and a second labeling functionality within a single molecule. Therefore we have pursued dual labeled opioid peptide derivatives containing an affinity label plus a second label, specifically either biotin or a histidine tag. Because of our success in identifying affinity label derivatives of TIPP, we initially explored the attachment of an additional label to the C-terminus of these analogs. The dual labeled peptides were synthesized by modifications of the strategies described above.

In the case of biotin-containing dual labeled TIPP derivatives, analogs with the affinity label incorporated at the para position of the phenyl ring of both Phe³ and Phe⁴ were initially synthesized. While the dual labeled peptides in which the affinity label was incorporated into Phe⁴ retain nanomolar DOR affinity similar to that of the corresponding tetrapeptide derivatives (manuscript in preparation), this was not the case for the dual labeled TIPP derivatives where the affinity label was attached to Phe³; the latter peptides exhibit drastic decreases in DOR affinity (IC₅₀ values typically > 1 μ M) regardless of the spacer and how the biotin was attached to the C-terminus of the peptide (manuscript in preparation). Thus C-terminal extension of the TIPP tetrapeptides appears to cause a subtle shift of Phe³ in the DOR binding pocket, interfering with receptor binding. The aromatic ring of Phe⁴ may be in a more flexible region of the receptor binding pocket such that C-terminal extension does not adversely affect its interaction with the receptor. The dual labeled TIPP derivatives containing the affinity label on Phe⁴ and biotin have been used to detect labeled DOR receptors on Western blots (manuscript in preparation).

We have also prepared dual labeled TIPP derivatives containing a 6 histidine tag along with an affinity label on Phe⁴ (Fig. 1) (Peck et al., 2006). Because the His tag consists of amino acids, these peptides can be synthesized using the strategy described for peptide-based affinity labels. As for the biotinylated peptides, a hydrophilic spacer was used to separate the His tag from the opioid peptide; the Fmoc-protected PEG-like ω -amino acid was synthesized by an efficient methodology developed in our laboratory (Aldrich and Kumar, 2006). While the synthesis of the isothiocyanate derivative proceeded smoothly, during the preparation of the bromoacetamide derivative an intramolecular side reaction occurred involving nucleophilic displacement of the bromine by an imidazole nitrogen to yield a cyclic peptide. The imidazole π -nitrogen of histidine is reactive toward alkyl bromides but not alkyl chlorides (Wieghardt and Goren, 1975), and therefore we were able to successfully prepare the dual labeled TIPP derivative containing a chloroacetamide and His₆ tag without the cyclization side reaction. These dual labeled TIPP derivatives also retain nanomolar affinity for DOR and are being used to study the labeled DOR receptors.

Concluding Remarks

Thus we have developed solid phase synthetic strategies for the preparation of two types of labeled peptides: peptide-based affinity labels and peptides containing a label such as biotin at their C-terminus. These strategies have been combined to prepare dual labeled peptides as tools to study peptide ligand-receptor interactions. These synthetic strategies are compatible with a variety of peptide sequences. To date we have encountered side reactions in only two cases, both involving intramolecular cyclization between an affinity label and another functionality on the peptide; these side reactions can be circumvented by either modifying the peptide or the reactive functionality. Thus these synthetic methodologies are general strategies that are applicable to the preparation of labeled peptides for a variety of targets in addition to opioid receptors.

We have identified several opioid peptide-based affinity label derivatives, particularly for DOR, that exhibit wash-resistant inhibition of receptor binding and thus appear to be binding covalently to their receptor. These peptides are useful pharmacological tools to study opioid

peptide-receptor interactions, studies that are currently ongoing in our laboratory. Even in cases where the peptides appear to only bind reversibly, the pharmacological results have revealed subtle differences in peptide-receptor interactions.

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Figure 1.

A dual labeled TIPP derivative containing both an affinity label (X) and a His₆ tag (Peck, et al., 2006).

Scheme 1.

The solid phase synthesis of peptide-based affinity labels, illustrated by the synthesis of [Phe (*p*-X)⁴]TIPP (Kumar et al, 2002). DIC = diisopropylcarbodiimide, TCDI = thiocarbonyldiimidazole

Scheme 2.

The solid phase synthesis of peptides containing a C-terminal label, illustrated by the synthesis of a biotinylated TIPP derivative (Kuman and Aldrich, 2003).

Affinity label derivatives of opioid peptides prepared by solid phase synthesis (X =-NCS or -NHCOCH₂Br). A check mark by the receptor type indicates that one or more of the derivatives exhibit wash-resistant inhibition of binding to the target receptor, indicative of covalent binding.

Table 1

Peptide	Sequence	Target opioid receptor	Reference
[D-Pro ¹]-Dynorphin A-(1-11)NH ₂	Tyr-Gly-Gly-Phe(<i>p</i> -X)-Leu-Arg-Arg-Ile- Arg-D-Pro-LysNH ₂	κ	(Leelaswatanakij and Aldrich, 2000)
TIPP ^b	Tyr-Tic-Phe(<i>p</i> -X)-PheOH ^d Tyr-Tic-Phe-Phe(<i>p</i> -X)OH ^a Tyr-Gly-Gly-Phe(<i>p</i> -X)-LeuOH Tyr-D-Thr-Gly-Phe(<i>p</i> -X)-Leu-ThrOH Tyr-D-Ala-Phe(<i>p</i> -X)-Asp-Val-Val-GlyNH ₂ Tyr-D-Ala-Phe-Asp-Phe(<i>p</i> -X)-Val-GlyNH ₂	δ √ δ √ δ δ δ √ δ	(Maeda et al., 2000a; Kumar et al., 2002) (Maeda et al., 2000a; Kumar et al., 2002) (Choi et al. 2003b) (Choi et al. 2003b) (Aldrich et al., 2004) (Aldrich et al., 2004)
Dermorphin	Tyr-D-Ala-Phe(<i>p</i> -X)-Gly-Tyr-Pro-SerNH ₂	μ	(Choi et al., 2003a)
Endormorphin-2	Tyr-D-Ala-Phe-Gly-Phe(<i>p</i> -X)-Pro-SerNH ₂ Tyr-Pro-Phe(<i>p</i> -X)-PheNH ₂ Tyr-Pro-Phe-Phe(<i>p</i> -X)NH ₂ Tyr-D-Ala-Gly-NMePhe(<i>p</i> -X)-glyol Tyr-D-aa(X)-Gly-NMePhe-Y D-aa = D-Orn or D-Lys Y = glyol or GlyNH ₂	μ μ μ μ μ μ	(Choi et al., 2003a) (Choi et al., 2003a) (Choi et al., 2003c) (Choi et al., 2003c) Manuscript in prep (Dattachowdhury et al., 2008)

^aThe initial isothiocyanate affinity label derivatives of TIPP were prepared by solution phase synthesis (Maeda et al, 2000a).

^b See text for discussion of dual labeled derivatives.

^c Derivatives of the peptide antagonist [*N,N*-dibenzy]Tyr¹, Leu⁵]enkephalin (Bz₂Tyr-Gly-Gly-Phe(*p*-X)-LeuOH) were prepared by solution phase synthesis (Maeda, et al., 2000b). The isothiocyanate derivative exhibits wash-resistant inhibition of binding.