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A Solid-Phase Synthetic Strategy for Labeled Peptides: Synthesis of a Biotinylated Derivative of the δ Opioid Receptor Antagonist TIPP (Tyr-Tic-Phe-Phe-OH)

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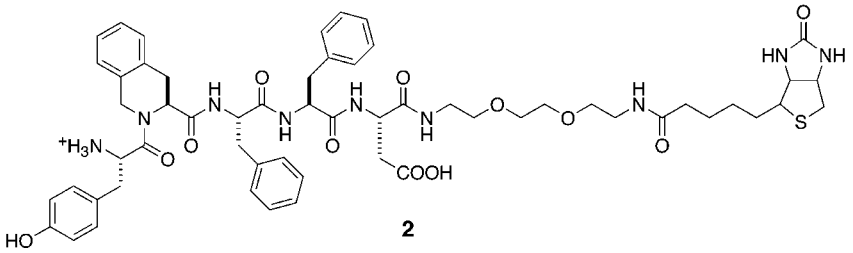
ABSTRACT



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A general solid-phase synthetic strategy for labeled peptides was developed and used to prepare a biotinylated derivative of the δ opioid receptor antagonist TIPP (Tyr-Tic-Phe-Phe-OH). A monoprotected hydrophilic diamine linker was attached to an aldehyde-containing solid-phase resin by reductive amination, followed by introduction of biotin and peptide synthesis to yield Tyr-Tic-Phe-Phe-Asp-NH($CH_2CH_2O)_2CH_2$-CH_2NH-biotin (2). The high δ receptor affinity and selectivity of 2 demonstrate the applicability of this design approach for labeled peptide derivatives.

Peptide derivatives containing a biotin or a fluorescent label can be useful pharmacological tools for studying receptors.[1] Biotinylated peptides can be used in receptor purification (see, for example, refs 2 and 3). Binding assays can use fluorescently labeled ligands in place of radioligands, thus eliminating issues related to using and disposing of radioactive material.[1] Fluorescently labeled ligands can also be used for studying receptor localization and internalization.[4,5]

Labels are typically attached to a free carboxylic acid, amine, or thiol in the peptide, either on a side chain of a noncritical residue in the sequence or by extending the peptide backbone.[1] In either case, the point of attachment must be chosen so that the label has a minimal influence on receptor binding. Many labeling functionalities (e.g., biotin and fluorescein) contain a carboxylic acid or other functionality that can react with an amine. Derivatives of these labels

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(1) Baindur, N.; Triggle, D. J. *Med. Res. Rev.* **1994**, *14*, 591−664.
(2) Eppler, C. M.; Zysk, J. R.; Corbett, M.; Shieh, H.-M. *J. Biol. Chem.* **1992**, *267*, 15603−15612.
(3) Eppler, C. M.; Hulmes, J. D.; Wang, J. B.; Johnson, B.; Corbett, M.; Luthin, D. R.; Uhl, G. R.; Linden, J. *J. Biol. Chem.* **1993**, *268*, 26447−26451.
(4) Gaudriault, G.; Nouel, D.; Dal Farra, C.; Beaudet, A.; Vincent, J.-P. *J. Biol. Chem.* **1997**, *272*, 2880−2888.
(5) Arttamangkul, S.; Alvarez-Maubecin, V.; Thomas, G.; Williams, J. T.; Grandy, D. K. *Mol. Pharmacol.* **2000**, *58*, 1570−1580.

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containing an amine or an electrophilic group (typically a maleimide or a haloacetamide) that can react with a carboxylic acid and thiol, respectively, have also been developed.[1]

A spacer between the peptide and the labeling functionality can be important to maintain the desired biological properties of the labeled peptide. Many labels of interest such as fluorescein are large bulky groups that can interfere with receptor binding if incorporated too close to pharmacophoric moieties in a peptide. Even a small labeling group such as biotin can decrease receptor affinity.[6] Therefore, particularly for small peptides, it is important to incorporate a spacer between the peptide and the label to separate the labeling functionality from important pharmacophoric groups. Like the label, the spacer itself must not interfere with the affinity or potency of the molecule. Particularly for hydrophobic peptides, it is preferable to utilize a hydrophilic spacer to avoid increasing the lipophilicity of the peptide, which could lead to decreased aqueous solubility and/or increased nonspecific interactions.

We are interested in developing potent and selectively labeled opioid peptides as pharmacological tools to study opioid receptor structure and function. Unlike other classes of peptides, the N-terminal amine is critical for the opioid receptor affinity of the endogenous opioid peptides and most of their analogues; other pharmacophoric groups (the N-terminal tyrosine and generally a second aromatic residue) are also located in the N-terminal sequence.[7] Thus a labeling moiety generally cannot be appended to the N-terminus of an opioid peptide but instead must be attached to an amino acid side chain or the C-terminus, making the design and synthesis of labeled derivatives more challenging.

Both approaches have been utilized to prepare labeled opioid peptide derivatives with mixed success. Labels have been attached to the side chain amine of a lysine that has been either part of the peptide sequence (in the longer peptides dynorphin[8] and β-endorphin[3,9] and in the μ-receptor-selective peptide DALDA, Tyr-D-Arg-Phe-Lys-NH_2[10]) or appended to the C-terminus (in the case of labeled enkephalin derivatives).[6,11] Labeling functionalities have also been attached to the C-terminus of enkephalin and dynorphin derivatives and to the amphibian opioid peptides [D-Ala^2]-deltorphin I and [Lys^7]dermorphin via a hydrophobic diamine spacer.[4,12−14] In two cases where multiple amino groups were present[4,9] multiple products were obtained that had to be separated by preparative HPLC and subsequently identified. Fluorescent derivatives of several opioid peptides ([D-Ala^2]-deltorphin I, dermorphin, endomorphin, and the δ opioid receptor antagonist TIPP, Tyr-Tic-Phe-Phe-OH) were recently reported that were prepared by attachment via an iodoacetamide or maleimide derivative of the fluorescent group to the thiol of a cysteine appended to the C-terminus of the peptide.[5]

At least part of the synthesis of the labeled opioid peptides reported to date has been performed in solution. Most of the smaller labeled opioid peptides were synthesized entirely in solution. For the longer peptides dynorphin, β-endorphin and the amphibian opioid peptides, generally the peptide chains were assembled by solid-phase synthesis, followed by introduction of the label into the peptide in solution. Only in the case of a biotinylated dynorphin A derivative, in which the preformed labeled lysine derivative Boc-Lys(biotin)-OH (Boc-biocytin) was incorporated into the peptide during the solid-phase synthesis,[8] was the labeled peptide synthesized entirely on a solid support.

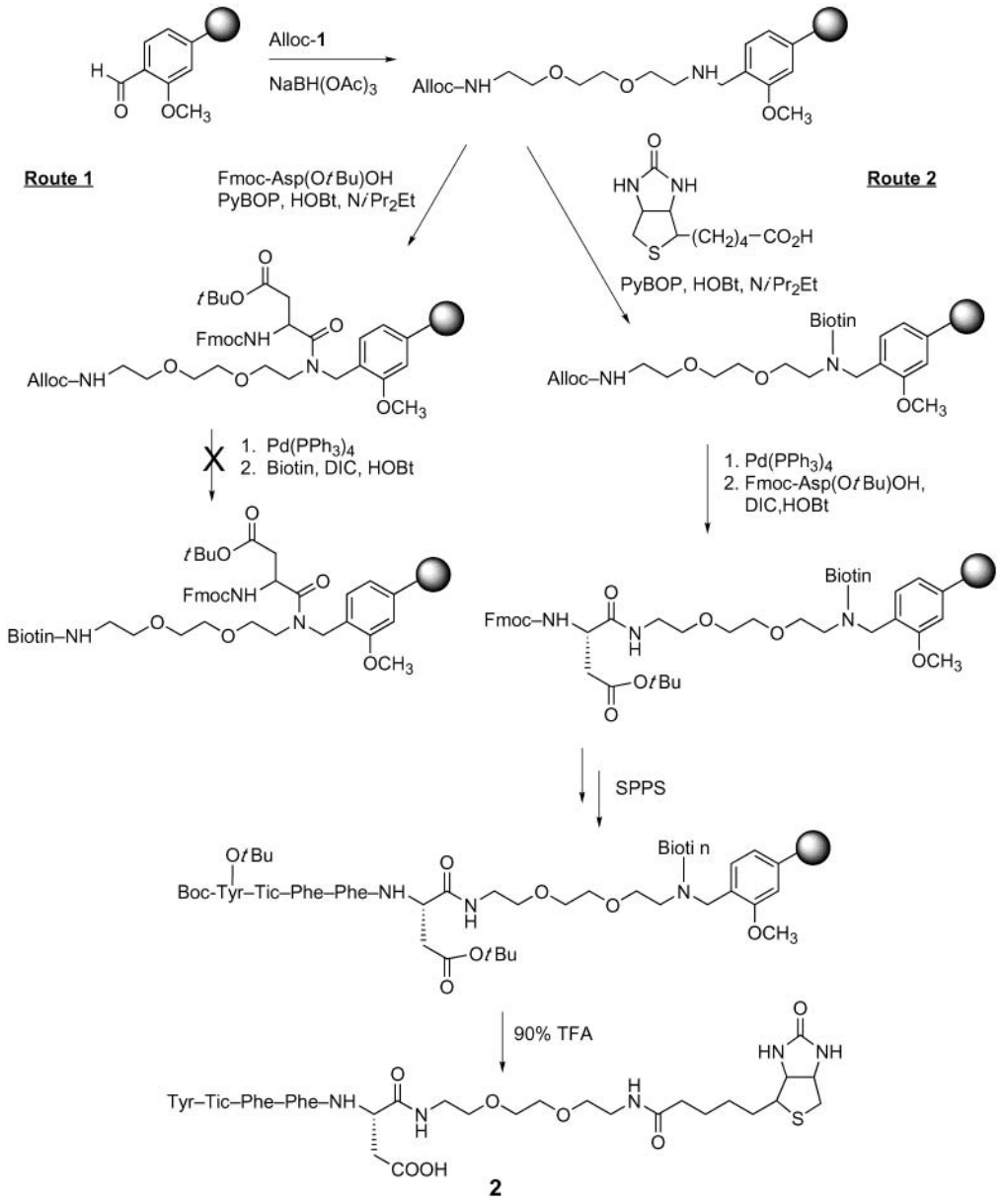
We wanted to develop a general synthetic strategy whereby peptides labeled selectively in the C-terminal region could be synthesized entirely on a solid support. We also wanted a strategy where a variety of labeling functionalities could be incorporated into the peptide without initially preparing labeled derivatives of an amino acid or other group (e.g., a spacer). Because we were interested in incorporating labels into short peptides, the label was attached via a hydrophilic spacer. As noted above, many labeling functionalities of interest (e.g., biotin and fluorescein) contain a carboxylic acid or other functionality that can react with an amine, and therefore a diamine spacer was used; the poly(ethylene glycol) (PEG)-like diamine $H_2N(CH_2CH_2O)_2CH_2CH_2NH_2$, **1**, was chosen because of its hydrophilic character.

The synthetic strategy was initially evaluated by designing and synthesizing a labeled derivative of TIPP.[15] TIPP, which contains a 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) residue at the 2-position, represents the prototype of a class of potent and highly selective δ opioid receptor antagonists.[15] TIPP exhibits nanomolar δ receptor affinity and high selectivity (K_i ratio (μ/δ) = 1410) in radioligand binding assays. It is also a potent antagonist against various δ agonists in the mouse vas deferens (MVD) assay (K_e = 3−5 nM) but does not display any μ or κ antagonist properties in the guinea pig ileum (GPI) assay at concentrations as high as 10 μM.[15]

In the labeled TIPP derivative **2**, biotin was attached to the C-terminus of the peptide via the diamine spacer. A free C-terminal carboxylic acid moiety in TIPP and its derivatives is important for maintaining their δ receptor selectivity.[15] Therefore, peptides were synthesized extended at the C-terminus with an aspartic acid residue; one of the carboxylic acid groups was then used to attach the label, while the other was left free. In an earlier report, we found that chain

(6) Koman, A.; Terenius, L. *FEBS Lett.* **1980**, *118*, 293−295.
(7) Aldrich, J. V.; Vigil-Cruz, S. C. In *Burger's Medicinal Chemistry*, 6th ed.; Abraham, D. J., Ed.; Wiley & Sons: New York, 2003; Vol. 6, in press.
(8) Hochhaus, G.; Patthy, A.; Schwietert, R.; Santi, D. V.; Sadee, W. *Pharm. Res.* **1988**, *5*, 790−794.
(9) Hochhaus, G.; Gibson, B. W.; Sadee, W. *J. Biol. Chem.* **1988**, *263*, 92−97.
(10) Bickel, U.; Yamada, S.; Pardridge, W. M. *J. Pharmacol. Exp. Ther.* **1994**, *268*, 791−796.
(11) Hazum, E.; Chang, K.-J.; Shechter, Y.; Wilkinson, S.; Cuatrecasas, P. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 841−846.
(12) Fournie-Zaluski, M. C.; Gacel, G.; Roques, B. P.; Senault, B.; Lecomte, J. M.; Malfroy, B.; Swerts, J. P.; Schwartz, J. C. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 300−305.
(13) Goldstein, A.; Nestor, J. J.; Naidu, A.; Newman, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7375−7379.
(14) Basak, A.; Dugas, H.; Lazure, C. *Bioconjugate Chem.* **1994**, *5*, 301−305.
(15) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, B. J.; Lemieux, C.; Chung, N. N. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11871−11875.

Scheme 1



extension of TIPP with Asp-NH_2 resulted in less than a 2-fold decrease in δ receptor affinity compared to TIPP,[16] and therefore the α-carboxylic acid of Asp could be used to attach the diamine spacer and biotin label.

The synthesis of labeled peptide **2** was first attempted using Fmoc-Asp-(O-Wang resin)-OH (prepared by loading Fmoc-Asp-OAllyl onto the Wang resin and subsequent allyl ester deprotection using $Pd(PPh_3)_4$[17] and attachment of the Alloc (allyloxycarbonyl)-protected derivative of **1** to the α-carboxylic acid of the Asp. The Alloc-monoprotected diamine was prepared by reacting a 10-fold excess of **1** with allyl chloroformate in CH_2Cl_2 for 4 h, and the product was purified by silica gel flash chromatography (4/1/0.1 CH_2Cl_2/MeOH/NEt_3) to give Alloc-**1** in 70% yield as a light yellow oil. Following deprotection of the Alloc group on the resin with $Pd(PPh_3)_4$,[17] biotin was coupled to the primary amine, and peptide chain elongation was attempted. However, the desired product was not obtained from this synthetic strategy, most likely due to a side reaction either during functionalization of the linker (see below) and/or to aspartimide formation by the nitrogen linker during Fmoc deprotection with piperidine.[18]

Therefore, we investigated the use of an aldehyde resin that permitted modifications at both ends of the diamine spacer. Reductive amination of the resin (4-(4-formyl-3-methoxyphenoxy)butyryl AM resin, 0.85 mmol/g, Novabio-

(16) Kumar, V.; Murray, T. F.; Aldrich, J. V. *J. Med. Chem.* **2000**, *43*, 5050−5054.

(17) Leelasvatanakij, L.; Aldrich, J. V. *J. Pept. Res.* **2000**, *56*, 80−87.

(18) Delforge, D.; Dieu, M.; Delaive, E.; Art, M.; Gillon, B.; Devreese, B.; Raes, M.; Van Beeumen, J.; Remacle, J. *Lett. Pept. Sci.* **1996**, *3*, 89−97.

chem, San Diego) with Alloc-**1** (9 equiv) and $NaBH(OAc)_3$ (10 equiv) in trimethylorthoformate/CH_2Cl_2 (1/2) was conducted for 16 h (Scheme 1). Once the resin was loaded with Alloc-**1**, the label could be attached to one end of the spacer, while the other amine could be used to extend the peptide chain.

There were two possible synthetic schemes starting from the Alloc-**1**-resin (Scheme 1), and the order of the initial reactions was critical to the success of the synthesis. Initially, Fmoc-Asp(O*t*Bu)-OH (5 equiv) was reacted with the secondary amine of the linker using the highly efficient coupling reagent benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), in the presence of hydroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (5/5/10)[19] in *N*,*N*-dimethylacetamide (DMA) for 6 h (Scheme 1, route 1). The Alloc protecting group was then removed from the primary amine using $Pd(PPh_3)_4$,[17] and biotin was attached to this amine using *N*,*N*-diisopropylcarbodiimide (DIC) and HOBt (5 equiv each) in a 1/1 mixture of dimethyl sulfoxide (DMSO) and (DMA) overnight. However, the subsequent attempt to deprotect the Fmoc group on Asp using 20% piperidine in DMA failed. Following cleavage of the product from the resin, mass spectrometric analysis indicated the formation of the dibiotinylated product (biotin-Asp-linker **1**-biotin). These results reinforced our earlier findings (Kumar and Aldrich, unpublished results) that the Fmoc group is labile in the presence of free amines on the solid support.

Therefore, the order of the reactions was reversed (Scheme 1, route 2) to avoid the premature loss of the Fmoc group. Biotin (5 equiv) was first attached to the secondary amine of the linker, using PyBOP, HOBt and N*i*Pr_2Et (5/5/10) in DMSO/DMA and reacting overnight; the chloranil test for secondary amines[20] was negative, suggesting the reaction was complete. Following removal of the Alloc group using $Pd(PPh_3)_4$,[17] Fmoc-Asp(O*t*Bu)-OH was attached to the resulting primary amine by reacting with DIC and HOBt (4 equiv each) in DMA overnight; quantitative Fmoc determination[21] indicated that the loading of the resin was essentially quantitative (0.51 mmol/g, 98%). Standard Fmoc peptide synthetic procedures were then used to assemble the peptide chain; DIC plus HOBt was used for the couplings of Fmoc-Phe-OH and Fmoc-Tic-OH, and PyBOP plus HOBt and N*i*Pr_2Et (4/4/8)[19] was used for coupling Boc-Tyr(O*t*Bu)-OH (4 equiv) to the secondary amine of Tic[2]. The resin was then cleaved using 90% aqueous trifluoroacetic acid (TFA), and after evaporation of the TFA and dissolution in 10% AcOH, the crude peptide was lyophilized. Analytical HPLC indicated a major peak plus three minor later peaks. Analysis by electrospray mass spectrometry (ESI-MS) indicated the formation of the desired molecule **2** (Scheme 1). Following purification,[22] the desired product was isolated along with the three later minor impurities. These minor impurities were identified by ESI-MS as dimers lacking biotin with the second peptide sequence complete or lacking one (Asp) or two (Asp-Phe) residues. These dimeric products, which totaled approximately 20−25% of the crude product based on the analytical HPLC, appear to have resulted from incomplete reaction of the secondary amine of the linker with biotin, despite the negative result with the chloranil test.

Purified peptide **2** was evaluated for its binding affinity for δ and μ opioid receptors in radioligand binding assays using cloned receptors stably expressed in CHO (Chinese hamster ovary) cells and [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]-enkephalin) and [^{3}H]DAMGO ([D-Ala^2,NMePhe^4,glyol^5]-enkephalin), respectively, as the radioligands.[23] Peptide **2** retained high δ receptor affinity ($K_i = 12.3 \pm 4.7$ nM), which was only slightly lower than that of TIPP ($K_i = 6.1 \pm 0.5$ nM) and the extended derivative TIPP-Asp-NH_2 ($K_i = 9.5 \pm 1.7$ nM);[16] all of these peptides exhibited negligible affinity for μ opioid receptors (<50% inhibition of binding at 10 μM). Thus, attachment of the spacer and biotin label to the C-terminus of the extended TIPP derivative did not interfere with interaction of the pharmacophoric groups in the peptide with the receptor. This is in sharp contrast to the TIPP derivative containing a fluorescent group attached to the thiol of a Cys residue appended to the C-terminus, which exhibited very low affinity for δ receptors ($K_i = 119$ nM).[5]

The aim of the current study was to develop a general synthetic scheme that could be used to attach different labels such as biotin or a fluorescent group via a hydrophilic spacer to lead peptides. The applicability of the synthetic strategy was demonstrated by preparing a biotinylated derivative of TIPP that retained high affinity for δ opioid receptors. The labeled peptide was successfully prepared by careful choice of the reaction sequence to avoid premature loss of the Fmoc protecting group from the growing peptide chain. The high affinity and selectivity of the resulting peptide **2** for δ opioid receptors illustrate the applicability of this strategy for designing labeled peptides. This peptide will be a useful pharmacological tool to study δ opioid receptors.

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(19) Coste, J.; Frérot, E.; Jouin, P. *J. Org. Chem.* **1994**, *59*, 2437−2446.
(20) Vojkovsky, T. *Pept. Res.* **1995**, *8*, 236−237.
(21) Weighed quantities of resin (approximately 5 mg) were treated with 0.4 mL piperidine and 0.4 mL CH_2Cl_2 for 30 min. MeOH (1.6 mL) was added and the solution diluted to 25 mL with CH_2Cl_2. The absorbance of the solution was read at 301 nm and the substitution calculated using an extinction coefficient of 7800 M^{-1} cm^{-1}. Milligen Technical Note 3.10.
(22) The peptide was purified to >98% homogeneity on a Vydac column 218-TP column (22 × 250 mm) with a Vydac guard cartridge using a linear gradient of 20 to 50% aqueous MeCN containing 0.1% TFA over 30 min at a flow rate of 20 mL/min. Analytical HPLC for purified **2** (Vydac 218-TP column (4.6 × 250 mm) equipped with a Vydac guard cartridge, flow rate 1 mL/min), $t_r = 18.4$ min (20−50% linear gradient of aqueous MeCN containing 0.1% TFA over 20 min); ESI-MS *m*/*z* 1107.1 ($M + H^+$).
(23) Arttamangkul, S.; Ishmael, J. E.; Murray, T. F.; Grandy, D. K.; DeLander, G. E.; Kieffer, B. L.; Aldrich, J. V. *J. Med. Chem.* **1997**, *40*, 1211−1218.