Published in final edited form as:
Bioconjug Chem. 2009 February ; 20(2): 201–204. doi:10.1021/bc800420t.

Dual Labeled Peptides as Tools to Study Receptors: Nanomolar Affinity Derivatives of TIPP (Tyr-Tic-Phe-Phe) Containing an Affinity Label and Biotin as Probes of δ Opioid Receptors

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Abstract

A general strategy for the design of dual labeled peptides was developed and derivatives of the δ opioid receptor (DOR) selective antagonist TIPP (Tyr-Tic-Phe-PheOH) containing both an affinity label and biotin were prepared by solid phase synthesis. Tyr-Tic-Phe-Phe(*p*-X)-Asp-NH $(CH_2CH_2O)_2$-CH_2CH_2NH-biotin (where X = N=C=S or $NHCOCH_2Br$) exhibit nanomolar DOR affinity. The ability to detect receptors labeled with these peptides following solubilization and SDS-PAGE demonstrate the applicability of this design approach for dual labeled peptide derivatives.

Affinity labels, ligands that bind to their target in a nonequilibrium manner, are important pharmacological tools to study receptors and their biological effects. These irreversible ligands bind to their targets in a two-step process, first binding reversibly followed by formation of a covalent bond with their target (1). These ligands are useful pharmacological tools to study receptors (1), and, by determining their point of attachment to their target, can be used to examine receptor-ligand interactions at the molecular level (2,3).

While affinity labels without an additional functionality are useful pharmacological tools, a second label is required to detect the labeled receptors and take full advantage of this class of ligands. This typically has involved using radiolabeled derivatives of affinity labels (see, for example, (2,4,5)), but the preparation of radiolabeled ligands requires specialized facilities and handling to incorporate the radioactive atom. Biotin or a fluorescent group are attractive alternatives to a radioactive label for incorporation into ligands (6). Ligands containing a biotin or a fluorescent group in addition to a reactive affinity label can have several advantages, both in ease of preparation and application in various pharmacological assays. To date the only such ligands described for opioid receptors are “reporter affinity labels” (see (7)) in which an *o*-phthalaldehyde group on the ligand functions as an affinity label that reacts with an amine and a thiol on the receptor and yields a fluorescent isoindole group. This approach to affinity labeling is very limited, however, because it requires that two nucleophilic receptor residues be in close proximity to one another and to the phthalaldehyde on the ligand.

Our research focuses on the synthesis of labeled derivatives of opioid peptides as tools to study opioid receptors. We have been particularly successful in identifying electrophilic affinity label

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derivatives of several peptides selective for δ opioid receptors (DOR) (8-11), including analogs of the potent and selective DOR antagonist TIPP (Tyr-Tic-Phe-PheOH, where Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid)[1] (9,10). The [Phe(p-X)^4]TIPP derivatives (where X is an isothiocyanate or bromoacetamide group) have high affinity for DOR (IC_{50} = 5 nM) and inhibit binding in a wash-resistant manner (9,10).

We have also incorporated biotin into TIPP, attached to the C-terminus via a hydrophilic diamine linker to separate biotin from the small hydrophobic peptide (12). This maintains the basic N-terminal amine, which is important for the interaction of most opioid peptides with opioid receptors; Asp was also incorporated in the peptide to maintain an acidic C-terminal functionality, which is important for high DOR selectivity (12,13). The resulting peptide TIPP-Asp-NH$(CH_2CH_2O)_2CH_2CH_2$NH-biotin (**1a**) exhibits high affinity (K_i = 12 nM) for DOR (12).

Combining an affinity label and second labeling functionality into the same molecule is a particularly appealing approach for peptide derivatives because of their polymeric nature. This approach has been applied to label neurokinin receptors with substance P derivatives containing both a photoaffinity label and a biotin derivative and to study peptide ligand-receptor interactions (see (14)). Here we describe the synthesis and evaluation of the first dual labeled opioid peptide derivatives containing both an affinity label and a second nonradioactive labeling functionality as tools to study opioid receptors.

Based on our success with initial TIPP derivatives, we combined an affinity label and biotin in the same peptide to give the dual labeled peptides **1c** and **1d** (Figure 1). The corresponding amine-containing peptide **1b** was prepared as a reversible control compound for the pharmacological assays. The dual labeled TIPP derivatives were prepared by solid phase synthesis using a modification of the strategy developed in our laboratory to prepare the reversible biotinylated peptide **1a** (Scheme 1) (12). This strategy uses the Alloc (allyloxycarbonyl) group for orthogonal protection of the amine groups to which both the biotin and affinity label groups are attached. Following reductive amination of an an aldehyde-containing solid phase resin with the monoprotected hydrophilic diamine linker Alloc-NH $(CH_2CH_2O)_2CH_2CH_2NH_2$ (Alloc-**2**) and introduction of the biotin, the peptide was assembled on the resin by standard procedures (see Supplemental Information for detailed procedures). Following completion of the peptide chain, the Alloc group was removed from Phe(*p*-NHAlloc)^4 and the resin then treated with thiocarbonyl diimidazole (TCDI) or bromoacetic acid plus *N,N*-diisopropylcarbodiimide (DIC) to yield the isothiocyanate and bromoacetamide derivatives, respectively. The dual labeled peptides **1c** and **1d** plus the amine-containing peptide **1b** were cleaved from the resins and deprotected using 90% aqueous trifluoracetic acid (TFA). Mass spectrometric analysis verified the formation of the desired derivatives (see Table S1 in the Supplemental Information).

The purified peptides were initially evaluated for DOR and μ opioid receptor (MOR) affinities in radioligand binding assays under standard conditions (15) (Table 1). The dual labeled peptides **1c** and **1d** and the amine-containing analog **1b** retain nanomolar DOR affinity, with comparable, or in the case of **1d** higher, affinity to the parent biotinylated peptide **1a**. Thus the combination of an affinity label on Phe^4 and a C-terminal biotin is well tolerated by DOR. These results parallel those found for the TIPP affinity label derivatives without the C-terminal

[1]Abbreviations: Alloc, allyloxycarbonyl; CHO, Chinese hamster ovary; DAMGO, [D-Ala^2,NMePhe^4,glyol^5]enkephalin; DIC, *N,N*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMA, *N,N*-dimethylacetamide; Dmt, 2′,6′-dimethyltyrosine; DPDPE, *cyclo*[D-Pen^2,D-Pen^5]enkephalin; Fmoc, fluorenylmethoxycarbonyl; HOBt, hydroxybenzotriazole; HRP, horse radish peroxidase; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TCDI, thiocarbonyl diimidazole; TFA, trifluoracetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP, Tyr-Tic-Phe-PheOH;.

extension (9,10), although the DOR affinity of the dual labeled peptides containing biotin is somewhat lower (8- and 2-fold for **1c** and **1d**, respectively) than that of the corresponding affinity labeled tetrapeptides. Peptides **1a**-**1d** all exhibit negligible affinity for MOR.

The ability to combine the affinity label and biotin with retention of nanomolar affinity for DOR is dependent on the position of the affinity label. In contrast to the results described here, when the affinity label was incorporated in Phe^3, the DOR affinity of the resulting dual labeled derivatives decreased dramatically to micromolar concentrations (Aldrich, Kumar and Murray, manuscript in preparation), even though incorporation of an affinity label on Phe^3 is well tolerated in the shorter tetrapeptide TIPP (9).

The ability of the affinity label derivatives **1c** and **1d** to inhibit DOR binding in a wash-resistant manner was then assessed as previously described (8), with the amine-containing peptide **1b** serving as a reversible control (Figure 2). Incubation with peptides **1c** and **1d** at concentrations equal to their IC_{50} values for 90 min at room temperature, followed by extensive washing of the membranes, resulted in recovery of only 44 ± 13% and 31 ± 4%, respectively, of the binding to control membranes. Thus the C-terminal extension does not appear to alter the orientation of the reactive functionality on Phe^4 in the receptor site, so that, like the shorter TIPP derivatives (9,10), both **1c** and **1d** exhibit wash-resistant inhibition of binding. These peptides are much more potent DOR affinity labels than the enkephalin affinity label derivative DALCE ([D-Ala^2,Leu^5,Cys^6]enkephalin), which requires a concentration of 3 μM to cause 50% loss of DOR binding sites (16). Peptide **1d** appears to be comparable to FIT (fentanyl isothiocyanate), SUPERFIT ((+)-*cis*-3-methylfentanyl isothiocyanate) and NTII (5′-naltrindole isothiocyanate), which cause approximately 50% loss of DOR at concentrations of 1-10 nM (17-19), although differences in experimental procedures make it difficult to directly compare the results from the different studies.

Membranes treated with the reversible amine-containing peptide **1b** exhibited >90% of the control membrane binding, indicating that the washing procedure effectively removed noncovalently bound peptide. These results for **1b** are in marked contrast to the very lipophilic fluorescent Dmt-Tic derivative Dmt-Tic-Glu-NH$(CH_2)_5$NH-fluorescein (Dmt = 2′,6′-dimethyltyrosine), which cannot be removed from the mouse vas deferens preparation by extensive washing (20). This marked difference illustrates the advantage of our design strategy in which a hydrophilic linker is incorporated to decrease the hydrophobicity of our peptides, thereby minimizing nonspecific interactions.

The utility of the dual labeled peptides **1c** and **1d** was demonstrated by solubilizing labeled DOR, followed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 3). The cells were incubated with peptides **1b**-**1d** in the presence or absence of TIPP, and following solublization with RIPA+ buffer and immunoprecipitation according to procedures previously described (21), the proteins were subjected to SDS-PAGE. After Western blotting, proteins labeled with the biotinylated peptide were detected using a streptavidin-horse radish peroxidase (HRP) conjugate and a chemoluminescent substrate. Labeled proteins were detected when the cells were incubated with the affinity labels **1c** or **1d** alone, but not when the cells were incubated with peptides **1c** or **1d** in the presence of TIPP or with reversible peptide **1b** (Figure 3). These results clearly demonstrate that peptides **1c** and **1d** are binding covalently and specifically to DOR. To our knowledge this represents the first report where an affinity labeled opioid receptor has been detected on a gel without using a radiolabeled ligand and demonstrates the utility of the dual labeled peptides to directly detect labeled receptors using the biotin functionality.

Thus we have developed a strategy for the preparation of dual labeled peptides containing both an affinity label and a second label such as biotin. The applicability of the design and synthetic

strategies was demonstrated by the preparation of dual labeled TIPP derivatives containing both functionalities. The results for these TIPP derivatives show that an affinity label and biotin can be combined in the same peptide with retention of both high opioid receptor affinity and covalent binding of the affinity labels to the receptor. The detection of the labeled receptors following solubilization illustrates the immense potential for application of these derivatives as pharmacological tools to study opioid receptors; these studies are currently underway in our laboratories. This strategy can be applied to other peptides, including ligands for other biological targets as well as for opioid receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by a grant from the National Institute on Drug Abuse (R01 DA10035). The authors thank Jennif Chandler and B. Marinda Thomas for performing the radioligand binding assays.

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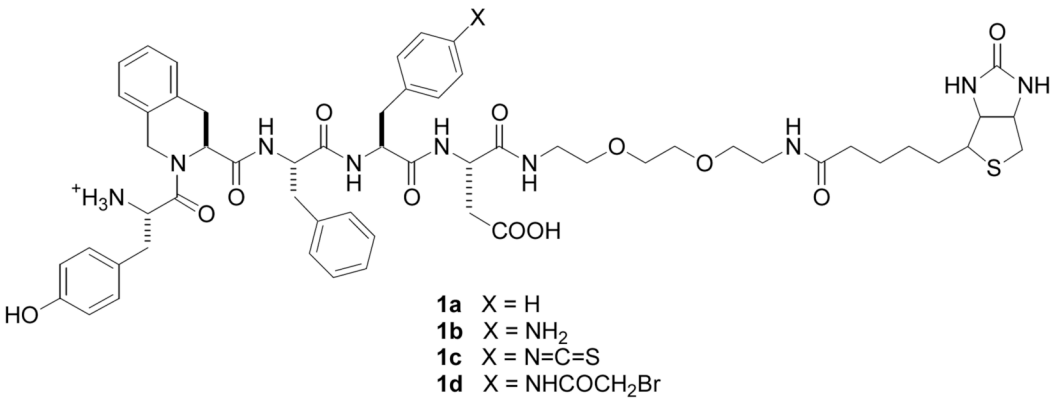


Figure 1.
Dual labeled TIPP derivatives

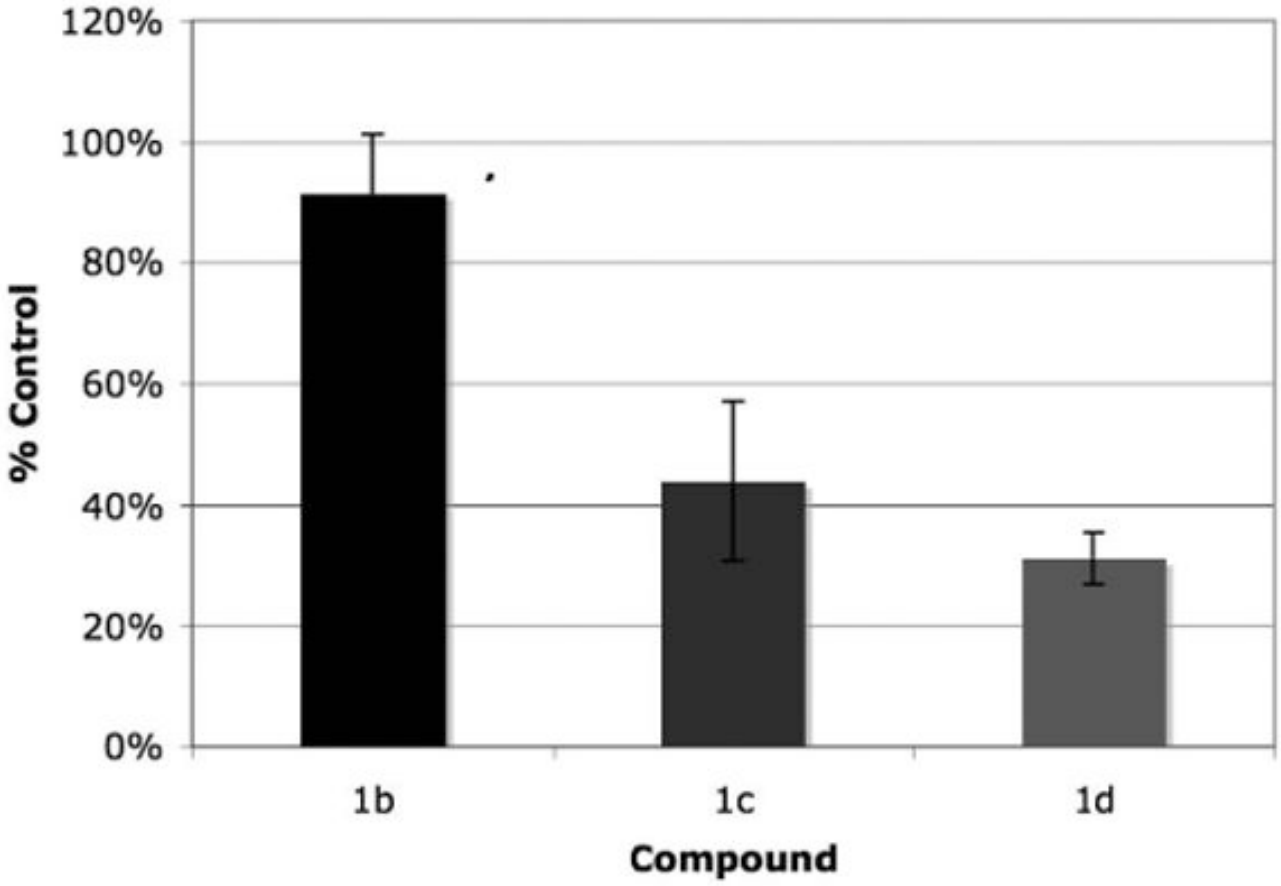


Figure 2.
Wash-resistant inhibition of [^{3}H]DPDPE binding to DOR by TIPP derivatives **1b-1d** compared to untreated control membranes following preincubation at 33, 40 and 10 nM, respectively, for 90 min. The results are the average of 3 independent experiments.

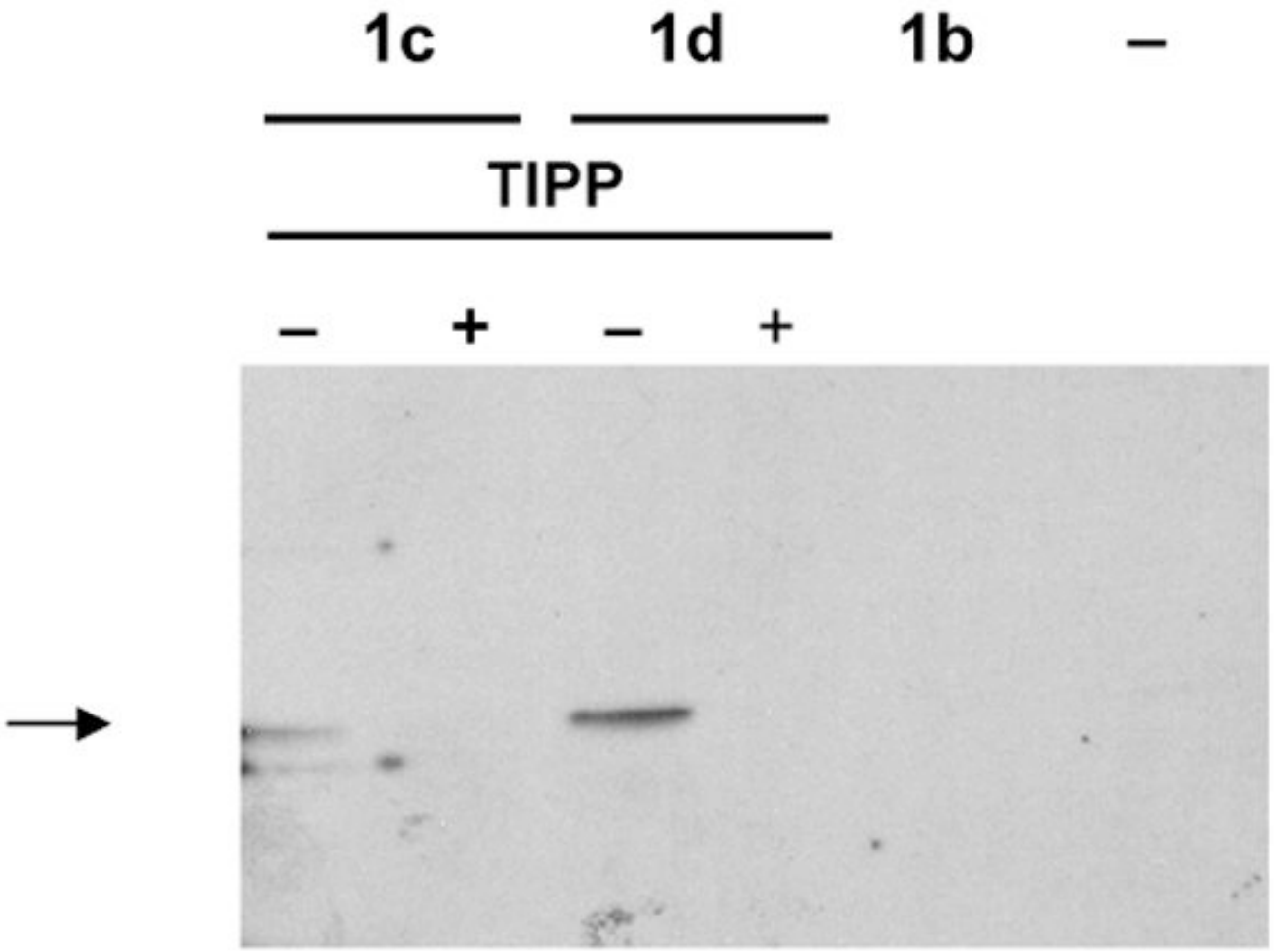
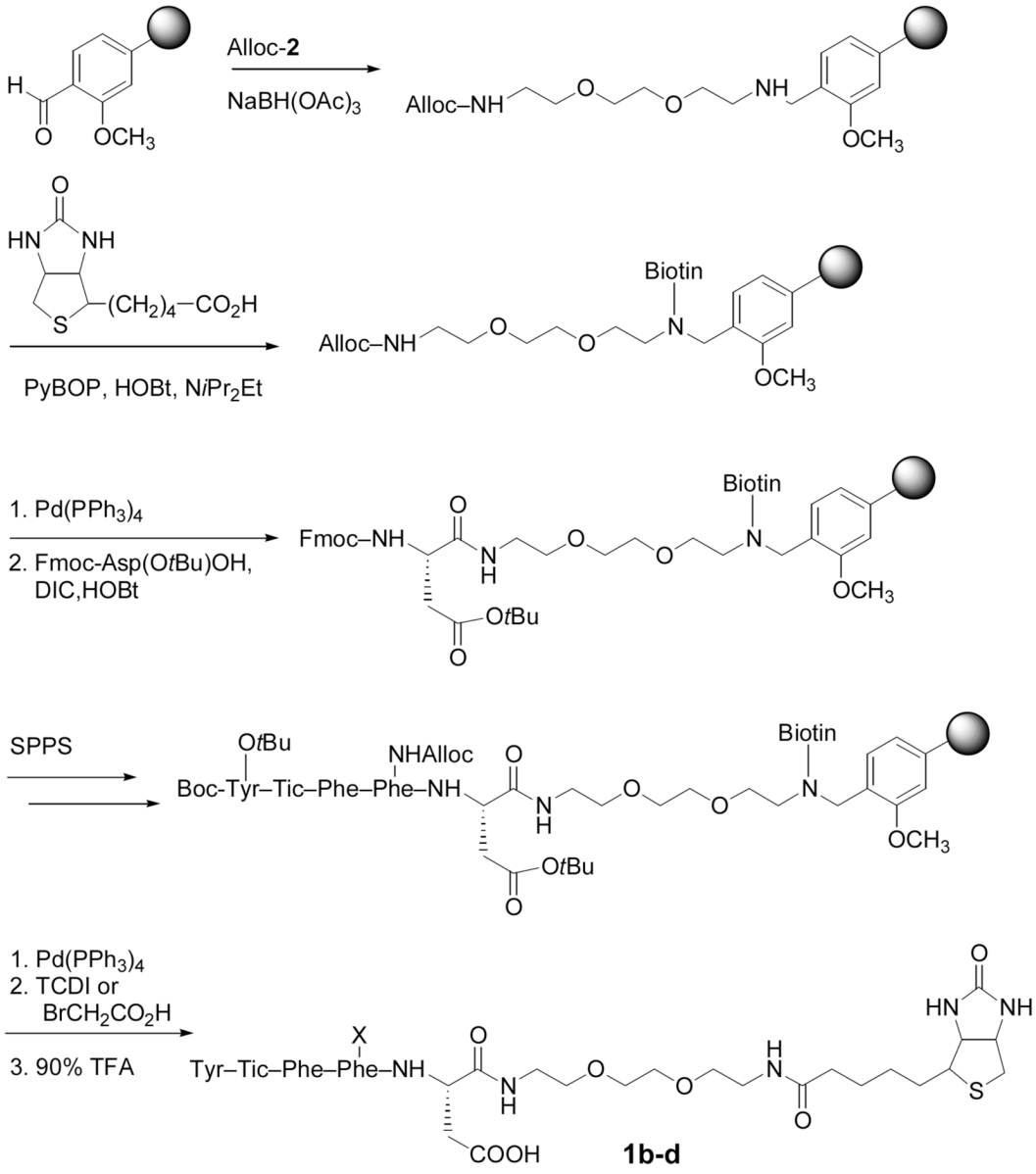


Figure 3.

Detection of labeled DOR by streptavidin-HRP conjugate following solubilization and SDS-PAGE. Myc-mδ/hμ CHO cells were incubated with the test peptide for 20 min at 25 °C at the following concentrations: 1) 200 nM **1c**, 2) 200 nM **1c** + 20 μM TIPP, 3) 50 nM **1d**, 4) 50 nM **1d** + 20 μM TIPP, 5) 165 nM **1b**, and 6) no peptide; when TIPP was included it was added 15 min prior to the test peptide. See the Supplemental Information for the isolation procedure. The arrow points to the band for the labeled DOR.



Scheme 1.

Table 1

DOR affinities of **1a-1d**.[a, b]

TIPP(*p*-X)-Asp-linker-Biotin[c]	**IC_{50} (nM) ± S.E.M**
X = H (**1a**)	24.8 ± 8.4
X = NH_2 (**1b**)	33.3 ± 8.4
X = N=C=S (**1c**)	40.5 ± 9.6
X = $NHCOCH_2Br$ (**1d**)	10.4 ± 2.0

[a] Using [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin) as the radioligand. Results are mean ± S.E.M of 3 independent experiments.

[b] IC_{50} > 10,000 nM at MOR for all compounds.

[c] Linker = -$NH(CH_2CH_2O)_2CH_2CH_2NH$-