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Discovery of Dermorphin-Based Affinity Labels with Subnanomolar Affinity for Mu Opioid Receptors†

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Abstract: A series of potent electrophilic affinity labels (IC_{50} = 0.1−5 nM) containing either a bromoacetamide or isothiocyanate based on the μ opioid receptor (MOR) selective peptide dermorphin were prepared. All four analogues exhibited wash resistant inhibition of [^{3}H]DAMGO binding at subnanomolar to nanomolar concentrations, suggesting that these analogues bind covalently to MOR. To our knowledge, these peptides are the highest affinity peptide-based affinity labels for MOR reported to date.

Narcotic analgesics produce pain relief generally through activation of μ opioid receptors (MOR),[a] but the use of these analgesics is limited by their side effects, namely respiratory depression, tolerance, constipation, and physical dependence.[1] Therefore there is an ongoing need to develop novel analgesics with fewer side effects. Understanding receptor−ligand interactions at the molecular level can facilitate the design of novel opioid ligands. Since the cloning of the three major opioid receptors, MOR,[2,3] δ opioid receptors (DOR), and κ opioid receptors (KOR), in the 1990s and determination of their sequences, there have been considerable advancements in understanding opioid receptor−ligand interactions. These studies have utilized chimeric receptors (such as MOR/KOR chimeras, etc.) and site-directed mutagenesis.[4] Although these approaches have provided considerable information regarding receptor−ligand interactions, interpreting the results can be complicated by changes in the secondary and/or tertiary structures of the proteins.[4] Also while these approaches provide information about which residues in the receptor may interact with the ligand, they often do not provide information about what portions of the ligand are involved in these interactions.

Because pain relief is mediated mainly through MOR, it is important to understand the interactions between MOR ligands and the receptor. The endogenous ligands of opioid receptors are peptides, and studies of chimeric opioid receptors and site-directed mutagenesis suggest that peptide ligands may interact differently with opioid receptors than nonpeptide ligands.[4] Therefore information about peptide ligands interactions with opioid receptors is complementary to that obtained for nonpeptide ligands.

Affinity labels, which are ligands that interact with their target in a nonequilibrium manner,[5] can provide detailed information about specific receptor−ligand interactions,[6,7] and the information obtained from affinity labels can compliment results obtained from molecular biology and computational methods. The interaction of affinity labels with the receptor occurs in a two-step manner.[5] In the first step, the ligand binds reversibly to its receptor. In the second step, which can further increase the selectivity of the ligand for its receptor, the ligand binds irreversibly, provided an appropriate nucleophile in the receptor is in close proximity to the reactive group in the ligand. Affinity labels can be either photoaffinity or electrophilic affinity labels. The electrophilic affinity label naltrexamine derivative β-funaltrexamine (β-FNA), a well studied affinity label for MOR, was the first affinity label (and one of only two affinity labels[8]) for opioid receptors whose covalent attachment point (Lys^{233} in MOR) has been successfully determined.[7]

Although a number of nonpeptide affinity labels for opioid receptors have been reported in the literature,[1,5] until recently peptide-based affinity labels have been mostly limited to photoaffinity labels.[5] A disadvantage of using azido photoaffinity labels is that short wavelength UV irradiation generally used to generate the reactive species can inactivate opioid receptors.[9] Alkylation of the receptor by electrophilic affinity labels, on the other hand, depends on the selectivity and chemical reactivity of the electrophile, and thus is not subject to the receptor inactivation that can occur with photoaffinity labels. Examples of peptide-based electrophilic affinity labels, selective for DOR, that have been reported include [D-Ala^2,Cys^6]enkephalin (DALCE),[10] the chloromethyl ketone of [D-Ala^2,Leu^5]enkephalin,[11] and isothiocyanate and bromoacetamide-containing derivatives of TIPP (Tyr-Tic-Phe-Phe) and other DOR opioid peptides discovered in our laboratory (see ref 12). There have been very few reports of electrophilic peptide-based affinity labels selective for MOR. The chloromethyl ketone of Tyr-D-Ala-Gly-NMePhe (IC_{50} = 1−5 μM for concentration-dependent irreversible inhibition of [^{3}H]naloxone binding)[13] and Tyr-D-Ala-Gly-Phe-Leu(CH_2SNpys) (Npys = 3-nitro-2-pyridinesulphenyl, IC_{50} = 19 nM for concentration-dependent inhibition of [^{3}H]DAMGO binding)[14] are the only examples of peptide-based electrophilic affinity labels for MOR reported in the literature. Previous attempts in our group to prepare affinity labels for MOR by incorporating an electrophilic functionality such as bromoacetamide or isothiocyanate at the para position of either Phe^3 or Phe^4 in endomorphin-2 (Tyr-Pro-Phe-PheNH_2) were unsuccessful because the modified analogues exhibited large (40- to 80-fold) decreases in MOR binding affinity compared to endomorphin-2.[15]

Dermorphin (Figure 1), an endogenous peptide from South American frog skin,[16] was selected as the parent ligand for further modification in the present study. Dermorphin is a highly selective MOR ligand that has 100-fold higher affinity than morphine for MOR.[16] The characteristic feature of frog skin peptides are their N-terminal Tyr-D-aa-Phe sequence, which constitutes the "message" domain[17] of these peptides.

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[a] Abbreviations: Aloc, allyloxycarbonyl; DOR, δ opioid receptor; ss-FNA, β-FNA, β-funaltrexamine; KOR, κ opioid receptor; MOR, μ opioid receptor; Npys, 3-nitro-2-pyridinesulphenyl.

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The D-configuration at position 2 of dermorphin is critical for MOR binding and opioid activity.[16] Previously, we modified the para position of Phe[3] or a Phe in position 5 of dermorphin and [Lys[7]]dermorphin to introduce an electrophilic functionality, i.e., a bromoacetamide or an isothiocyanate group.[18] Modification in the "message" domain (Phe[3]) resulted in >1000-fold decrease in MOR affinity. While modification of a Phe in position 5 in the "address" domain of dermorphin and [Lys[7]]dermorphin was well tolerated and the peptides retain nanomolar affinity for MOR, none of these modified analogues exhibited wash-resistant inhibition of binding (WRIB) to MOR and therefore are not affinity labels for these receptors.[18]

Therefore, in the present study, we chose an alternative location in the "message" sequence, position 2, to incorporate a reactive functionality. Larger D-amino acids are tolerated at this position in peptides by MOR,[16] suggesting that introduction of a functionality such as an affinity label into the side chain of this residue would not interfere with the binding of these ligands to the receptor. In the present study, D-Ala at position 2 was replaced by either D-Orn or D-Lys. The free amine on the side chain of these amino acids was used as a suitable handle to incorporate the electrophilic bromoacetamide or isothiocyanate functionalities (Figure 1). This strategy also permits varying the length of the amino acid side chain to optimize binding of the affinity label to its receptor. For these series of analogues, [D-Orn($COCH_3$)[2]]- and [D-Lys($COCH_3$)[2]]dermorphin served as reversible control peptides for the respective series of compounds in the pharmacological assays.

Solid phase synthesis of the peptides was carried out on the PAL-PEG-PS (peptide amide linker-poly(ethylene glycol)-polystyrene) resin using Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids, except for the N-terminal Tyr residue, which was protected with the Boc (*t*-butyloxycarbonyl) group. The peptides were synthesized according to methods previously developed in our laboratory[12,19] (see Supporting Information). The side chains of Tyr and Ser were protected with the *t*Bu group, and the side chain of D-Orn or D-Lys was protected with the Aloc (allyloxycarbonyl) group. Once the protected full-length peptide was assembled, the Aloc group was selectively deprotected using tetrakis- (triphenylphosphine) palladium(0) and phenyl silane,[19,20] and the resins were then divided into three equal parts. The free amine in each part was treated with either bromoacetic acid, thiocarbonyldiimidazole, or acetic anhydride to obtain the bromoacetamide, isothiocyanate, or acetylated derivatives, respectively (see Supporting Information for details). The completion of the reactions was confirmed by the qualitative ninhydrin test. The final peptides were cleaved from the resins using 95% trifluoroacetic acid and 5% water for 2 h, and the peptides were purified using reversed phase preparative HPLC. The molecular weights of the peptides were confirmed by electrospray ionization mass spectrometry analysis, and the purity of the final peptides was verified using two HPLC systems (see Supporting Information).

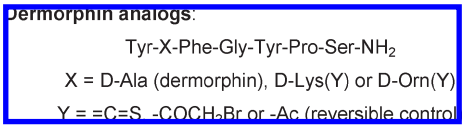

Figure 1. Affinity label derivatives for MOR and the corresponding reversible control peptides based on the parent peptide dermorphin.

The binding affinities of these peptides for opioid receptors were initially measured in radioligand binding assays using Chinese hamster ovary cells stably expressing MOR and DOR, with [^{3}H]DAMGO ([D-Ala[2],MeNPhe[4],glyol]enkephalin) and [^{3}H]DPDPE (*cyclo*[D-Pen[2],D-Pen[5]]enkephalin) as the radioligands, respectively, under standard conditions[21] (see Supporting Information). All of the compounds retain subnanomolar to nanomolar affinity for MOR (Table 1). Of the analogues prepared, **1**, **2**, and **4** exhibit the highest affinities for MOR (subnanomolar IC_{50} values), affinities that are markedly higher compared to the previously prepared Phe[3] substituted analogues (IC_{50} = 40−6050 nM).[18] In addition, these three potential affinity labels exhibit equal or higher affinity (7 and 2 times higher for analogues **2** and **4**, respectively) than the parent peptide dermorphin.

The isothiocyanate-containing affinity labels in the two series (D-Orn and D-Lys) exhibit similar binding affinities for MOR, while the affinity of the bromoacetamide derivative in the D-Orn series **2** is 48 times higher than the corresponding D-Lys derivative **5**. Similarly, the acetylated control compound in the D-Orn series, **3**, exhibits significantly higher affinity than the corresponding control compound **6** in the D-Lys series. Clearly, the different lengths of the side chains in D-Lys and D-Orn as well as the identity of the attached functionality play important roles in determining the affinities of the dermorphin analogues for MOR. In the case of the bromoacetamide analogues and the control compounds, the extra methylene group in the side chain of D-Lys is probably causing unfavorable steric interactions, resulting in decreases in MOR affinity. In contrast, the isothiocyanate analogues in the two series do not differ substantially in MOR binding affinity. The smaller size of the isothiocyanate group compared to the acetamide and bromoacetamide probably counterbalances the size increases due to the extra methylene group in the side chain of D-Lys that resulted in unfavorable interactions with MOR.

Table 1. Binding Affinities of Dermorphin Derivatives for MOR and DOR[a]

	IC_{50} (nM ± SEM)			
dermorphin analogues	MOR	DOR	relative MOR affinity[b]	IC_{50} ratio[c] (IC_{50} (DOR)/IC_{50} (MOR))
1 [D-Orn(=C=S)[2]]	0.81 ± 0.29	23.8 ± 2.1	0.89	29
2 [D-Orn($COCH_2Br$)[2]]	0.11 ± 0.02	342 ± 20	6.54	3110
3 [D-Orn($COCH_3$)[2]]	4.25 ± 0.35	272 ± 23	0.17	64
4 [D-Lys(=C=S)[2]]	0.38 ± 0.08	97.1 ± 4.9	1.89	255
5 [D-Lys($COCH_2Br$)[2]]	5.23 ± 2.31	382 ± 22	0.14	73
6 [D-Lys($COCH_3$)[2]]	29.8 ± 7.6	436 ± 34	0.02	15
Dermorphin[d]	0.72 ± 0.07	197 ± 28	1.0	274

[a] Determined using 1 nM [^{3}H]DAMGO and 0.15 nM [^{3}H]DPDPE as the radioligands for MOR and DOR, respectively. [b] Relative to dermorphin. [c] IC_{50} (DOR)/IC_{50} (MOR). [d] From ref 21.

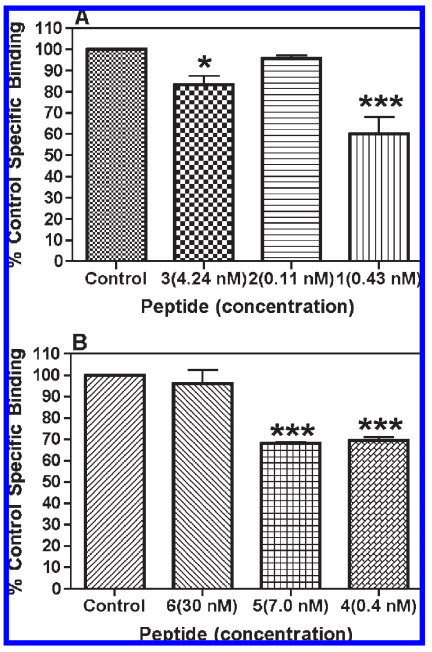

Figure 2. (A) WRIB by [D-Orn^2]dermorphin and (B) [D-Lys^2]dermorphin derivatives. The concentrations of the peptides in the incubations, which are approximately equal to their IC_{50} values, are indicated in parentheses. *$p < 0.05$, *** $p < 0.001$ compared to control.

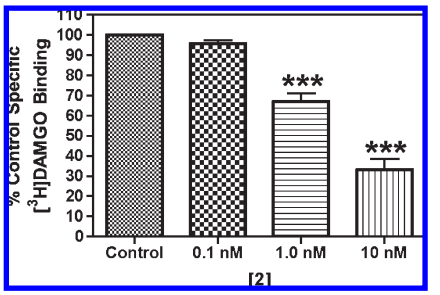

Figure 3. Concentration-dependent WRIB by [D-Orn(COCH$_2$Br)^2]dermorphin (2). *** $p < 0.001$ compared to control.

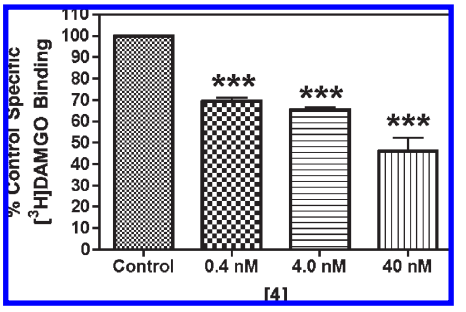

Figure 4. Concentration-dependent WRIB by [D-Lys(C=S)^2]dermorphin (**4**). ***$p < 0.001$ compared to control.

Comparing the affinities of these peptides for DOR, the isothiocyanate derivative in the D-Orn series, **1**, exhibits the highest affinity for DOR, four times higher than the affinity of the corresponding analogue **4** in the D-Lys series. The bromoacetamide analogues and acetylated control compound in both series (compounds **2**, **3**, **5**, and **6**) show lower affinity for DOR compared to dermorphin; no other major differences in DOR affinities were observed between the two series.

Except for the isothiocyanate derivatives, the D-Orn series of compounds are more selective for MOR over DOR than the corresponding D-Lys compounds. The affinity label derivative with the highest selectivity is [D-Orn(COCH$_2$Br)^2]-dermorphin, **2**, which exhibits a >3000-fold difference in the IC_{50} values for MOR vs DOR, is 49-fold more selective than the reversible control **3** and 11-fold more selective than the parent peptide (Table 1). In contrast, [D-Lys(COCH$_2$Br)^2]-dermorphin **5** exhibits 4-fold lower selectivity for MOR compared to dermorphin due to the large decrease in MOR affinity. For the isothiocyanate derivatives, however, the trend in selectivity is reversed. The D-Orn(=C=S)^2 derivative **1** is 9-fold less selective for MOR than dermorphin and also [D-Lys(=C=S)^2]-dermorphin, **4**. The selectivities were calculated using IC_{50} values, which vary as a function of the radioligand concentration used; therefore comparison of the selectivities for these peptides to those reported in other studies should be made with caution.

Because all four potential affinity labels showed subnanomolar to nanomolar affinity for MOR, they were examined to determine whether they may bind to MOR covalently. WRIB of [^{3}H]DAMGO by these four analogues, **1**, **2**, **4**, and **5**, at concentrations approximately equal to their IC_{50} values, was determined according to the procedure described previously[22] (see Supporting Information). The acetylated derivatives **3** and **6** were included as reversible controls to verify that the washing procedure completely removed noncovalently bound compound; the washing procedure removed >80% of both reversible control peptides. In the D-Orn series, [D-Orn(=C=S)^2]dermorphin (**1**) at a subnanomolar concentration caused 40 ± 8% inhibition of [^{3}H]DAMGO binding compared to control ($P < 0.001$) even after extensive washing of the membranes (Figure 2), suggesting that this peptide is binding covalently to a nearby nucleophile in the binding site of MOR. [D-Orn(COCH$_2$Br)^2]dermorphin (**2**), which shows the highest MOR affinity of all the compounds tested, did not exhibit WRIB to MOR when initially tested at a concentration equal to its IC_{50} (0.11 nM, Figure 2) but was effectively removed by the washing procedure. However, when the WRIB experiments were repeated at higher concentrations this analogue (**2**) did show concentration-dependent WRIB that was statistically significant compared to the control ($P < 0.001$) (Figure 3).

In the D-Lys series, both the bromoacetamide and isothiocyanate derivatives exhibit statistically significant ($P < 0.001$) inhibition of [^{3}H]DAMGO binding after extensive washing when evaluated at their IC_{50} values (Figure 2). The inhibition of [^{3}H]DAMGO binding by these ligands **4** and **5** were 31 ± 2% and 32 ± 1%, respectively (Figure 2). Moreover, [D-Lys(=C=S)^2]dermorphin (**4**) exhibits concentration-dependent inhibition of [^{3}H]DAMGO binding (Figure 4) when WRIB experiments of **4** were performed at higher concentrations of 4 and 40 nM ($P < 0.001$).

Comparison of the binding affinities reported for previous MOR selective affinity labels and the analogues discovered in the present study indicate that the dermorphin-based affinity labels have substantially higher MOR affinity. Previously Tyr-D-Ala-Gly-Phe-Leu(CH$_2$SNpys) was reported to be the highest affinity peptide-based electrophilic affinity label for MOR (IC_{50} = 19 nM for irreversible binding); however, it lacks selectivity and also shows nanomolar affinity for DOR (IC_{50} = 12 nM).[14] Importantly, three of the four affinity label derivatives reported here, [D-Orn(=C=S)^2]- (**1**), [D-Orn(−COCH$_2$Br)^2]- (**2**), and [D-Lys(=C=S)^2]dermorphin

(**5**), appear to have higher affinity (approximately 3- to 20-fold) than the well-studied nonpeptide MOR affinity label β-FNA (IC_{50} = 2.2 nM).[6,23]

In conclusion, we have successfully identified a series of dermorphin-based affinity label analogues that show exceptionally high affinity (IC_{50} = 0.1−5 nM) for MOR. These analogues were designed by modifying position 2 of dermorphin, which is a new strategy for designing peptide-based affinity label derivatives of opioid peptides that has not been previously reported. This resulted in a substantial improvement in binding affinity (between 10- to 100-fold) compared to the previous dermorphin-based analogues synthesized in our laboratory in which the para position of Phe^3 or a Phe in position 5 of dermorphin or [Lys^7]dermorphin were modified.[18] All four potential affinity labels in the present study show subnanomolar to nanomolar affinity for MOR in standard binding assays, indicating favorable interactions of the side chains in [D-Orn$(X)^2$]dermorphin and [D-Lys$(X)^2$]dermorphin (X = $-COCH_2Br$ or =C=S) with the binding pocket of MOR. [D-Orn$(COCH_2Br)^2$]dermorphin (**2**) shows exceptional selectivity for MOR over DOR, and [D-Lys$(C{=}S)^2$]dermorphin (**4**) exhibits selectivity comparable to the parent peptide dermorphin. All four potential affinity labels also exhibit WRIB to MOR, suggesting that these compounds are electrophilic affinity labels that bind covalently to MOR. Three of the four affinity label peptides exhibit WRIB to MOR at ≤1 nM. Thus we have identified peptide-based electrophillic affinity labels with exceptionally high affinity for MOR. These novel dermorphin analogues will be valuable tools to study MOR and the interactions of the peptides with this receptor. The next step will be to use these peptide-based electrophilic affinity labels to characterize MOR. These studies are currently underway in our laboratory.

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Supporting Information Available: Detailed experimental procedures for the synthesis and pharmacological evaluation of the peptide analogues and analytical (HPLC and mass spectral) data for peptides **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Aldrich, J. V.; Vigil-Cruz, S. C. Narcotic Analgesics. In *Burger's Medicinal Chemistry and Drug Discovery*, 6th ed.; John Wiley, Inc.: New York, 2003; Vol. 6, pp 329−481.
(2) Chen, Y.; Mestek, A.; Liu, J.; Hurley, J. A.; Yu, L. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol. Pharmacol.* **1993**, *44*, 8–12.
(3) Fukuda, K.; Kato, S.; Mori, K.; Nishi, M.; Takeshima, H. Primary structures and expression from cDNAs of rat opioid receptor delta- and mu-subtypes. *FEBS Lett.* **1993**, *327*, 311–314.
(4) Law, P. Y.; Wong, Y. H.; Loh, H. H. Mutational analysis of the structure and function of opioid receptors. *Biopolymers* (*Pept. Sci.*) **1999**, *51*, 440–455.
(5) Takemori, A. E.; Portoghese, P. S. Affinity labels for opioid receptors. *Annu. Rev. Pharmacol. Toxicol.* **1985**, *25*, 193–223.
(6) Chen, C.; Xue, J. C.; Zhu, J.; Chen, Y. W.; Kunapuli, S.; de Riel, J. K.; Yu, L.; Liu-Chen, L. Y. Characterization of irreversible binding of beta-funaltrexamine to the cloned rat mu opioid receptor. *J. Biol. Chem.* **1995**, *270*, 17866–17870.
(7) Chen, C.; Yin, J.; de Riel, J. K.; DesJarlais, R. L.; Raveglia, L. F.; Zhu, J.; Liu-Chen, L. Y. Determination of the amino acid residue involved in [^{3}H]beta-funaltrexamine covalent binding in the cloned rat mu-opioid receptor. *J. Biol. Chem.* **1996**, *271*, 21422–21429.
(8) Zhang, Y.; McCurdy, C. R.; Metzger, T. G.; Portoghese, P. S. Specific cross-linking of Lys^{233} and Cys^{235} in the mu opioid receptor by a reporter affinity label. *Biochemistry* **2005**, *44*, 2271–2275.
(9) Glasel, J. A.; Venn, R. F. The sensitivity of opiate receptors and ligands to short wavelength ultraviolet light. *Life Sci.* **1981**, *29*, 221–228.
(10) Bowen, W. D.; Hellewell, S. B.; Kelemen, M.; Huey, R.; Stewart, D. Affinity labeling of delta-opiate receptors using [D-Ala^2,Leu^5,Cys^6]enkephalin. Covalent attachment via thiol-disulfide exchange. *J. Biol. Chem.* **1987**, *262*, 13434–13439.
(11) Szucs, M.; Belcheva, M.; Simon, J.; Benyhe, S.; Toth, G.; Hepp, J.; Wollemann, M.; Medzihradszky, K. Covalent labeling of opioid receptors with ^{3}H-D-Ala^2-Leu^5-enkephalin chloromethyl ketone. I. Binding characteristics in rat brain membranes. *Life Sci.* **1987**, *41*, 177–184.
(12) Aldrich, J. V.; Kumar, V.; Dattachowdhury, B.; Peck, A. M.; Wang, X.; Murray, T. F. Solid phase synthesis and application of labeled peptide derivatives: probes of receptor−opioid peptide interactions. *Int. J. Pept. Res. Ther.* **2008**, *14*, 315–321.
(13) Benyhe, S.; Hepp, J.; Simon, J.; Borsodi, A.; Medzihradszky, K.; Wollemann, M. Tyr-D-Ala-Gly-(Me)Phe-chloromethyl ketone: a mu specific affinity label for the opioid receptor. *Neuropeptides* **1987**, *9*, 225–235.
(14) Shimohigashi, Y.; Takada, K.; Sakamoto, H.; Matsumoto, H.; Yasunaga, T.; Kondo, M.; Ohno, M. Discriminative affinity labelling of opioid receptors by enkephalin and morphiceptin analogues containing 3-nitro-2-pyridinesulphenyl-activated thiol residues. *J. Chromatogr.* **1992**, *597*, 424–428.
(15) Choi, H.; Murray, T. F.; Aldrich, J. V. Synthesis and evaluation of potential affinity labels derived from endomorphin-2. *J. Pept. Res.* **2003**, *61*, 58–62.
(16) Melchiorri, P.; Negri, L. The dermorphin peptide family. *Gen. Pharmacol.* **1996**, *27*, 1099–1107.
(17) Chavkin, C.; Goldstein, A. Specific receptor for the opioid peptide dynorphin: structure−activity relationships. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6543–6547.
(18) Choi, H.; Murray, T. F.; Aldrich, J. V. Dermorphin-based potential affinity labels for mu-opioid receptors. *J. Pept. Res.* **2003**, *61*, 40–45.
(19) Leelasvatanakij, L.; Aldrich, J. V. A solid-phase synthetic strategy for the preparation of peptide-based affinity labels: synthesis of dynorphin A analogs. *J. Pept. Res.* **2000**, *56*, 80–87.
(20) Balse-Srinivasan, P.; Grieco, P.; Cai, M.; Trivedi, D.; Hruby, V. J. Structure−activity relationships of novel cyclic alpha-MSH/beta-MSH hybrid analogues that lead to potent and selective ligands for the human MC3R and human MC5R. *J. Med. Chem.* **2003**, *46*, 3728–3733.
(21) Arttamangkul, S.; Ishmael, J. E.; Murray, T. F.; Grandy, D. K.; DeLander, G. E.; Kieffer, B. L.; Aldrich, J. V. Synthesis and opioid activity of conformationally constrained dynorphin A analogues. 2. Conformational constraint in the "address" sequence. *J. Med. Chem.* **1997**, *40*, 1211–1218.
(22) Maeda, D. Y.; Ishmael, J. E.; Murray, T. F.; Aldrich, J. V. Synthesis and evaluation of *N*,*N*-dialkyl enkephalin-based affinity labels for delta opioid receptors. *J. Med. Chem.* **2000**, *43*, 3941–3948.
(23) Tam, S. W.; Liu-Chen, L. Y. Reversible and irreversible binding of beta-funaltrexamine to mu, delta and kappa opioid receptors in guinea pig brain membranes. *J. Pharmacol. Exp. Ther.* **1986**, *239*, 351–357.