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Tuned-Affinity Bivalent Ligands for the Characterization of Opioid Receptor Heteromers

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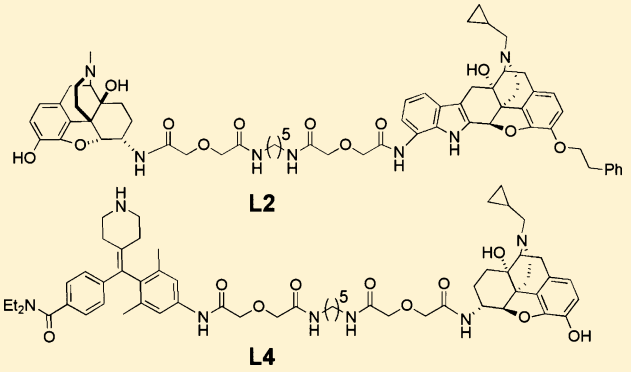
Supporting Information

ABSTRACT: Opioid receptors, including the μ- and δ-opioid receptors (MOR and DOR), are important targets for the treatment of pain. Although there is mounting evidence that these receptors form heteromers, the functional role of the MOR/DOR heteromer remains unresolved. We have designed and synthesized bivalent ligands as tools to elucidate the functional role of the MOR/DOR heteromer. Our ligands (L2 and L4) are comprised of a compound with low affinity at the DOR tethered to a compound with high affinity at the MOR, with the goal of producing ligands with "tuned affinity" at MOR/DOR heteromers as compared to DOR homomers. Here, we show that both L2 and L4 demonstrate enhanced affinity at MOR/DOR heteromers as compared to DOR homomers, thereby providing unique pharmacological tools to dissect the role of the MOR/DOR heteromer in pain.



KEYWORDS: *bivalent ligand, opioid receptors, heteromers, chronic pain*

Opioid receptors are important targets for the treatment of acute and chronic pain, but side effects, including respiratory suppression, constipation, analgesic tolerance, and dependence, limit their utility. The three types of opioid receptor, μ-opioid receptor (MOR), δ-opioid receptor (DOR), and κ-opioid receptor (KOR), associate with each other both in vitro and in vivo, producing heteromeric receptors with novel properties.[1−4] Additionally, the crystal structure of the MOR indicates that it is a dimer.[5] Opioid receptor heteromers may represent novel therapeutic targets, but their functions have not been fully elucidated, in part due to a lack of heteromer-specific ligands. There has been particular interest in ligands for the MOR/DOR dimer that show agonism at the MOR and antagonism at the DOR and/or the MOR/DOR heteromer, since antagonism, knock down, and knock out of the DOR improve the side effect profiles of classic opioid analgesics.[6−9]

We have designed a series of "tuned-affinity" bivalent ligands that specifically target the MOR/DOR heteromer to provide tools to evaluate its functional role. The design, synthesis, and binding affinity of these ligands are reported here. Although many bivalent opioid receptor ligands have been characterized previously,[10−13] none were designed to show unique activity on heteromeric (MOR/DOR) versus homomeric (DOR/DOR or MOR/MOR) receptors. Specifically, previous bivalent opioid receptor ligands were comprised of two pharmacophores, each with high affinity for both homomeric and heteromeric receptor complexes. Consequently, evaluating the role of the MOR/DOR heteromer was hindered by a lack of specificity. Moreover, characterizing the affinity of these bivalent ligands at heteromers has not been possible, since cells coexpressing MOR and DOR contain not only MOR/DOR heteromers but also DOR (and MOR) homomers, all of which have high affinity for both pharmacophores in the previously reported bivalent ligands.

Our ligands (L2 and L4, Figure 1) were designed to have novel or "tuned" affinities at MOR/DOR heteromers as compared to DOR homomers. Discrimination between DOR and MOR/DOR was accomplished by tethering a low affinity DOR ligand to a high affinity MOR ligand. These ligands were predicted to have a low affinity at DOR homomers and high affinity at MOR/DOR heteromers where binding of the high affinity MOR pharmacophore would increase the effective molarity of the tethered, low affinity DOR ligand—effectively "tuning" its affinity in favor of the heteromer.

The ligands presented here are comprised of a high affinity MOR compound, either an agonist or an antagonist, tethered to a low affinity DOR compound, again, either an agonist or an antagonist. This approach was used to determine whether binding of agonist or antagonist at the MOR in the MOR/DOR heteromer is more effective for tuning the affinity of the DOR ligand. One bivalent ligand, L2, is comprised of the high affinity MOR agonist oxymorphone and the low affinity DOR

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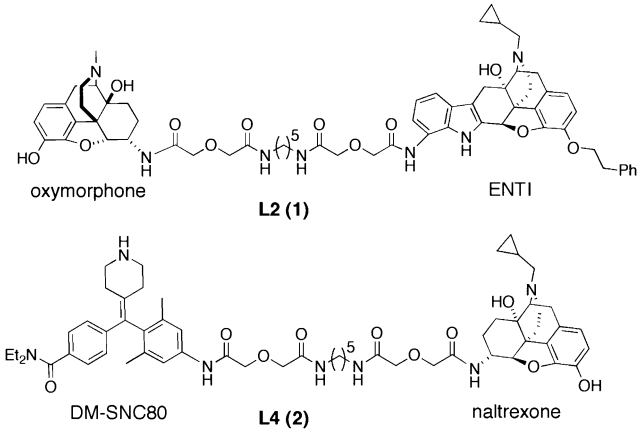
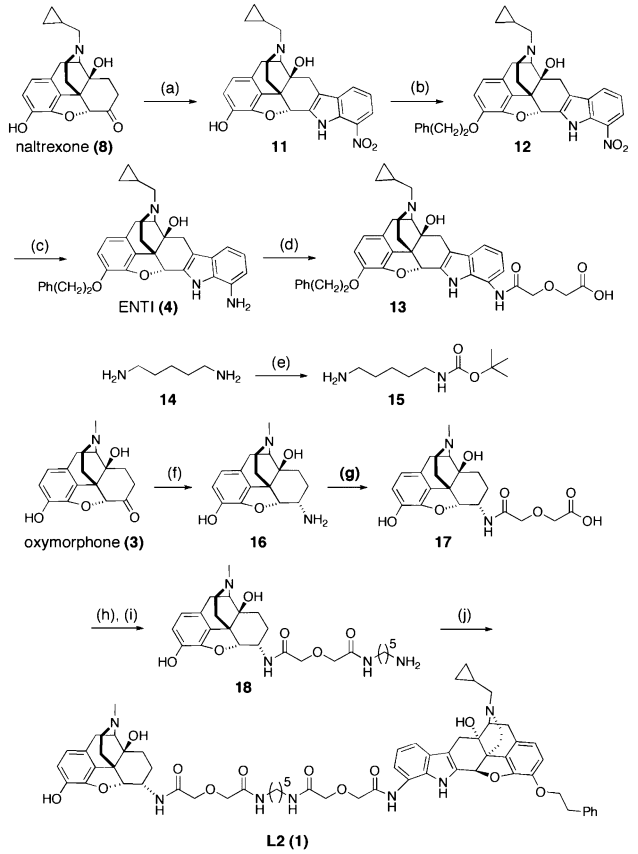


Figure 1. Tuned-affinity bivalent ligands.

antagonist ENTI.[14] The second, L4, features the high affinity MOR antagonist, naltrexone, and the low affinity DOR agonist, DM-SNC80[15] (Figure 1). The linker used here has been used extensively in previous bivalent ligands, where its composition and length were optimized for the heteromer.[10,16] Controls for these ligands are shown in Figure 2. Linker-attached controls were used when available.

L2 and L4 were prepared from their component fragments (antagonist, linker, and agonist) as shown in Schemes 1 and 2. For L2, Fisher indole synthesis of naltrexone (**8**) with 2-nitrophenyl hydrazine[10] followed by alkylation of the 3-phenol, reduction of the 7′-nitro group, and acylation of the resulting amine [ENTI (**4**)] with diglycolic anhydride produced the antagonist portion of the molecule (**13**). Next, the linker (**15**)[10] was synthesized via mono-Boc protection of cadaverine (**14**). The agonist synthesis is a known sequence[10] involving the selective reduction of oxymorphone (**3**),[17] attachment of diglycolic anhydride, peptide coupling with monoboc cadaverine, and deprotection to yield the agonist and linker portion of the molecule (**18**). Finally, the agonist linker (**18**) and antagonist (**13**) were combined in a peptide coupling to give L2 (**1**).

Scheme 1. Synthesis of L2[a]



[a](a) (2-Nitrophenyl)hydrazine, HCl, CH_3CO_2H, 90 °C, 2 h, 50%. (b) (2-Bromoethyl)benzene, K_2CO_3, DMF, 90 °C, 24 h, 92%. (c) 5% Pd/C, MeOH, H_2, 40 psi, 2 h, 92%. (d) Diglycolic anhydride, THF, 1 h, 95%. (e) *tert*-Butyl phenyl carbonate, EtOH, 65 °C, 16 h, 53%. (f) (i) H_2O, Na_2CO_3, 1 h; (ii) benzene, benzylamine, *p*-toluenesulfonic acid, 10 h; (iii) EtOH, $NaBH_4$, 3 h, H_2, 40 psi, 5% Pd/C, 72 h, 72%. (g) Diglycolic anhydride, THF, 1 h, 33%. (h) Compound **15**, DIC, HOBt, DIEA, THF, 60 °C, 2 h, 30%. (i) 4 N HCl, dioxane, 2 h, 99%. (j) Compound **13**, DIC, HOBt, DIEA, THF, 60 °C, 2 h, 27%.

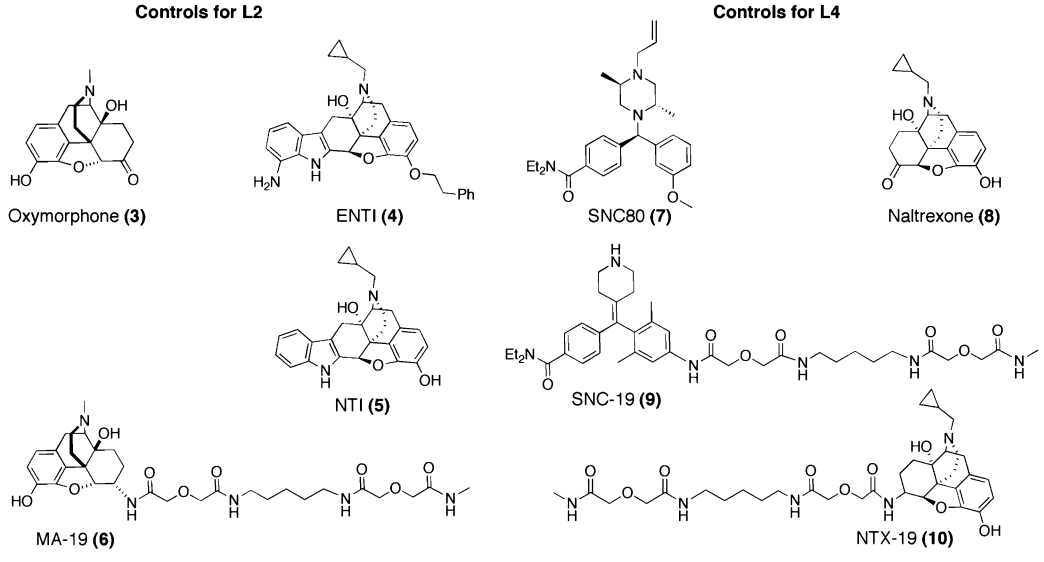


Figure 2. Monovalent control ligands for L2 and L4.

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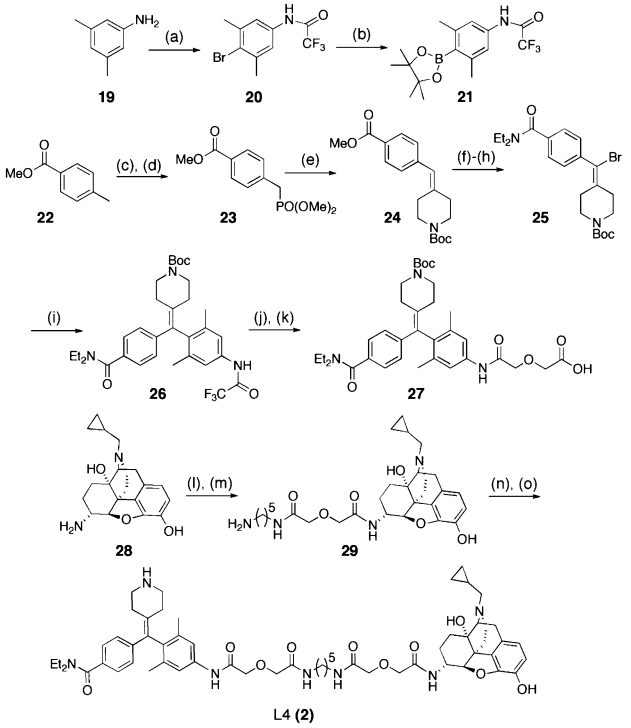
Scheme 2. Synthesis of L4[a]



[a](a) TFAA, Br_2, 0 °C, 1.5 h, 77%. (b) Bis(pinacolato)diboron, AcOK, $PdCl_2$(dppf), DMF, 80 °C, 16 h, 35%. (c) NBS, AIBN, CCl_4, reflux, 7 h, 99%. (d) $P(OMe)_3$, reflux, 5 h, 99%. (e) *tert*-Butyl 4-oxopiperidine-1-carboxylate, LDA, THF, 12 h, −78 °C → rt, 32%. (f) Br_2, K_2CO_3, CH_2Cl_2, 0 °C → rt, 1.5 h, 78%. (g) NaOH, MeOH, 40 °C, 3 h, 87%. (h) DMAP, $SOCl_2$, CH_2Cl_2, Et_2NH, −20 °C → rt, 12 h, 68%. (i) Compound **21**, K_2CO_3, THF/H_2O, $Pd(PPh_3)_2Cl_2$, 80 °C, 0.5 h, 41%. (j) NaOH, H_2O, MeOH, 12 h, 99%. (k) diglycolic anhydride, 0 °C → rt, 12 h, 93%. (l) Compound **15**, EDCI, HOBt, DMF, 12 h, 82%. (m) HCl/EtOAc, 12 h, 99%. (n) Compound **27**, EDCI, HOBt, DIEA, DMF, 12 h, 44%. (o) HCl/EtOAC, iPrOH, 10 h, 99%.

The synthesis of L4 required dimethyl amino diarylmethylenepiperidine (**26**), which was made via Suzuki coupling. The first coupling partner synthesis began with 3,5-dimethylaniline (**19**), which was protected, brominated, and transformed into the boronic ester (**21**). Next, methyl-4-methylbenzoate (**22**) was subjected to radical bromination and heating with triphenyl phosphite to give **23**.[15] Subsequent Wittig−Horner olefination with *N*-*tert*-butyoxy-carbonyl-4-piperidone gave **24**, which was brominated and transformed to the amide to yield the second coupling partner (**25**). Suzuki coupling of **21** with **25** followed by deprotection and reaction with diglycolic anhydride produced the agonist portion of L4 (**27**). Synthesis of the antagonist portion began with 6*β*-naltrexamine (**28**),[17] which was coupled to mono-Boc-cadaverine (**15**), deprotected and coupled to **27**.[10] A final Boc deprotection gave L4 (**2**).

We next examined whether these ligands showed tuned affinity at MOR/DOR heteromers as compared to DOR homomers. L2, L4, and their controls were analyzed in whole cell radioligand competition binding assays to evaluate affinity at DOR, MOR/DOR, and MOR. For both ligands, we expected high affinity at MOR, low affinity at DOR, and increased affinity at MOR/DOR as compared to DOR.

We first evaluated affinity at DOR in competition with the DOR radioligand ^{3}H [D-Pen2,D-Pen5]-enkephalin (DPDPE) in cells expressing only DOR and in cells expressing both MOR and DOR (which will contain a mixture of MOR homomers, DOR homomers, and MOR/DOR heteromers). In these conditions, both L2 and L4 show enhanced affinity at MOR/DOR as compared to DOR. L4 shows a significant (10×) shift in affinity (pK_i = 8.60 at MOR/DOR as compared to pK_i = 7.70 at DOR) (Table 1). Importantly, the L4 monovalent controls,

Table 1. Competition Binding

	^{3}H DPDPE (20 nM) pK_i ± SEM	
	MOR/DOR	DOR
L2	7.34 ± 0.06	6.69 ± 0.18
Oxy	6.29 ± 0.10	6.14 ± 0.09
ENTI	7.46 ± 0.09	7.04 ± 0.21
NTI	8.26 ± 0.15	8.58 ± 0.23
L4	8.60 ± 0.07	7.70 ± 0.11
SNC-19	5.78 ± 0.14	5.54 ± 0.20
SNC80	8.11 ± 0.13	7.78 ± 0.23
NTX-19	7.79 ± 0.11	7.51 ± 0.10
	^{3}H DAMGO (10 nM) pK_i ± SEM	
	MOR	MOR/DOR
L2	8.01 ± 0.05	8.21 ± 0.01
Oxy	8.30 ± 0.03	8.33 ± 0.07
MA-19	7.65 ± 0.06	6.90 ± 0.11
ENTI	6.92 ± 0.16	6.94 ± 0.08
NTI	7.78 ± 0.12	8.46 ± 0.14
L4	>9	>9
SNC-19	6.26 ± 0.15	5.92 ± 0.07
NTX-19	9.49 ± 0.11	8.38 ± 0.04
naltrexone	9.19 ± 0.23	9.06 ± 0.32

NTX-19 and SNC-19, do not show this increase in affinity for the heteromer. L2 also shows a change in affinity, but it did not reach statistical significance (pK_i = 7.34 at MOR/DOR and pK_i = 6.69 at DOR). Neither oxymorphone nor naltrindole hydrochloride (NTI) show a change between MOR/DOR and DOR. However, ENTI, the L2 low-affinity DOR control, shows a nonsignificant shift in affinity similar to L2. These data suggest that tuning may be achieved more productively by binding of an antagonist.

We also assessed the affinity of L2 and L4 at the MOR in cells expressing MOR alone or both MOR and DOR using the MOR radioligand ^{3}H [DAla2,NMe-Phe4,Gly-ol5]-enkephalin (DAMGO), where we do not expect tuned affinity. Indeed, L4 shows high affinity in both cell lines (pK_i > 9 nM) (Table 1). Naltrexone shows high affinity that is unchanged between the cell lines, and naltrexone with the linker attached, NTX-19, has a similar affinity to naltrexone on MOR but a decreased affinity at MOR/DOR. SNC-19 shows low affinity at MOR, as expected. L2 also shows a high "untuned" affinity in both cell lines, similar to that of its high affinity MOR component, oxymorphone (Table 1). Attachment of the linker to oxymorphone decreased its affinity at MOR, as shown by MA-19.[10] However, while the affinity of MA-19 decreased on MOR/DOR as compared to MOR cells, L2 shows the opposite trend, indicating that tethering these two compounds has a beneficial effect on affinity that overcomes adverse effects of the linker. Finally, NTI, predominantly a DOR ligand, shows higher affinity at MOR/DOR than at MOR.

Because the MOR/DOR cell line expresses a mixture of MOR homomers, DOR homomers, and MOR/DOR hetero-

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mers, the observed affinity of L2 and L4 is a mixture of the affinity at the three different receptor types. We evaluated the relative amount of heteromers and homomers expressed on the surface in our cell line by serial coimmunoprecipitation (Figure 3). The cells express substantially more DOR homomers than

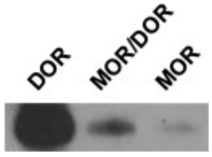

Figure 3. Relative expression of MOR, DOR, and MOR/DOR. HEK293 cells expressing both FLAG-MOR and HA-DOR were biotinylated to label surface receptors, and the receptors were serially immunoprecipitated to separate MOR, DOR, and MOR/DOR heteromers.

MOR/DOR heteromers and few MOR homomers. Consequently, we are likely significantly underestimating the shifts in affinity for L2 and L4.

MOR/DOR heteromers may be important targets for pain management, especially under chronic conditions such as stress, alcoholism, or long-term opiate use.[4,18] However, existing bivalent ligands have equivalent affinity for the heteromer and its constituent monomers, preventing validation of this target. We developed our "tuned-affinity" ligands as tools to distinguish between the MOR/DOR heteromer and the DOR homomers.

We are specifically interested in the MOR/DOR heteromer because of the untapped potential of DOR receptors in controlling pain.[19] The role of DORs is not fully understood, in part because of conflicting evidence regarding their function. Under some conditions, activation of DORs alleviates pain, while in others, the DORs exacerbate pain or oppose analgesia. For example, in acute conditions, DOR agonists are poor analgesics for thermal pain[20,21] and good analgesics in mechanical nociception.[22] However, in chronic conditions, DOR agonists can become potent analgesics.[23,24] Yet, in morphine-tolerant animals, DOR *antagonists* enhance antinociception.[25] Additionally, disruption or knockout of the DOR gene diminishes morphine tolerance.[6,26,27] These findings have led to the design of mixed and bivalent MOR agonist/DOR antagonist ligands as potential analgesics with reduced side effects. One such bivalent ligand, MDAN,[10] designed to target a MOR/DOR heteromer, showed analgesia with reduced tolerance and dependence and did not support conditioned place preference (CPP) in mice.[16] MDAN is comprised of a high affinity MOR agonist (oxymorphone) tethered to a high affinity DOR antagonist (naltrindole) and, thus, has high affinity at MOR homomers, DOR homomers, and the MOR/DOR heteromer. Therefore, it is not possible to determine if it is MDANs specific activity at the heteromer, nor what type of activity it is (agonism or antagonism) that is responsible for its biological action in vivo.

Indeed, it remains unclear whether heteromer activation increases or decreases pain perception, whether its actions are specific for thermal or mechanical nociception, and if its activity opposes that of the DOR homomer. Finally, when both an agonist and an antagonist are bound to a heteromeric receptor, it remains unclear which signal predominates (i.e., "who wins"). We designed our tuned-affinity ligands to address these questions.

We predicted that L2 and L4 would show low affinity at DOR alone and enhanced affinity at MOR/DOR, and this is, in fact, what we see. With L4, we see a significant change in affinity that occurs only for the bivalent ligand and not for the controls. Thus, we can postulate that L4 is bridging a heteromer and that tethering is important for its enhanced affinity at MOR/DOR. With L2, the change in affinity is not significant, and ENTI also shows enhanced affinity at MOR/DOR. Consequently, we cannot rule out that the shift in L2 is due to a shift in ENTI affinity, rather than receptor bridging. Intriguingly, ENTI shows equal affinity for both MOR and DOR homomers while retaining a higher affinity for the heteromer, suggesting that a nonbivalent ligand could itself show a preference for a heteromer.

It is noteworthy that our results were obtained in a cell line with a vastly greater population of DORs as compared to MOR/DORs (Figure 3), reflecting the fact that DORs have a higher affinity for one another than for the MOR.[28] Consequently, we are likely greatly underestimating the change in affinity of L2 and L4 on the heteromer.

In conclusion, we have successfully designed and synthesized compounds that specifically target the MOR/DOR heteromer. L2 and L4 represent a novel class of bivalent ligand that interacts differently with the heteromer, even in the presence of its constituent monomers. These ligands, L4 in particular, can be used as tools to characterize the heteromer as a novel therapeutic target.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures (for ENTI, L2, L4, SNC-19, and NTX-19) and biological assay details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.H.H. designed experiments, conducted experiments, and wrote the paper; D.H.L. conducted experiments; P.E. designed experiments; and J.L.W. designed experiments and wrote the paper.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; HEK, human embryonic kidney; DPDPE, [D-

Pen2,D-Pen5]-enkephalin; NTI, naltrindole hydrochloride; DAMGO, [DAla2,NMe-Phe4,Gly-ol5]-enkephalin; HA, hemagglutinin

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