Selective Ligands for the \( \mu \), \( \delta \), and \( \kappa \) Opioid Receptors Identified from a Single Mixture Based Tetrapeptide Positional Scanning Combinatorial Library\

(Received for publication, February 17, 1998, and in revised form, April 13, 1998)

Colette T. Dooley, Phibun Ny, Jean M. Bidlack†, and Richard A. Houghten§

From the Torrey Pines Institute for Molecular Studies, San Diego, California 92121 and the §Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York 14642

A combinatorial library of 6,250,000 tetrapeptides in the mixture based positional scanning format was screened in binding assays for the three opioid receptors, \( \mu \), \( \delta \), and \( \kappa \). Three different binding profiles were found. Individual peptides were synthesized representing all possible combinations of the active amino acids identified from the screening data. New, highly active peptides selective for each of the three receptors were chosen. This study demonstrates the power of mixture-based combinatorial libraries to identify distinctly different ligands for closely related receptors.

The opioid receptors represent a convenient system to investigate the power of combinatorial libraries to identify distinctly different ligands for related receptors. There are three primary opioid receptors: \( \mu \) (\( \mu \)), delta (\( \delta \)), and kappa (\( \kappa \)). All three receptors have recently been cloned, and they belong to the seven-transmembrane G-protein-coupled family of receptors and have approximately 60% amino acid sequence homology. Screening of the same combinatorial library in separate assays selective for each of the three receptors provides not only new ligands for these receptors but yields insights into the ability of combinatorial libraries to discriminate between closely related receptors.

A combinatorial library of 6,250,000 tetrapeptides, made using 50 different amino acids, was prepared in the positional scanning format (6, 7). The use of positional scanning synthetic combinatorial libraries (PS-SCLs)\(^1\) enables the most active amino acids at each position of a peptide or non-peptide to be determined directly from the initial screening data. This information can then be used to synthesize highly active individual compounds. A PS-SCL of tetrapeptide amides used in the current study consists of four separate sublibraries, each having a single defined position (\( O \)) and three mixture positions (\( X \)) as follows: \( Q, XX-\text{NH}_2, \quad X, XX-\text{NH}_2, \quad XXXO, XX-\text{NH}_2, \) and \( XXXO-\text{NH}_2 \). The defined positions of the mixtures making up each of the four separate sublibraries address a single position in the tetrapeptide. It should be noted that each of the four positional sublibraries are made up of the same 6,250,000 tetrapeptides. Screening the four sets of mixtures in the three separate opiate specific assays yielded information about the most important amino acids of each position in the tetrapeptide and led to the identification of three different series of active individual tetrapeptides selective for the \( \mu \), \( \delta \), and \( \kappa \) receptors.

**EXPERIMENTAL PROCEDURES**

**Preparation of the Tetrapeptide PS-SCL**

The PS-SCL used in this study is composed of 6,250,000 tetrapeptides and contains four sublibraries, in which one of the four positions is defined with a single amino acid (\( O \)) and the three remaining positions are a mixture of 50 different L-, D-, and unnatural amino acids (\( X \)). The tetrapeptides were synthesized using the solid phase simultaneous multiple peptide synthesis approach (8) on methylbenzhydrylamine resin.

\(^1\) The abbreviations used are: PS-SCLs, positional scanning synthetic combinatorial libraries; DAMGO, [\( \text{H} \)-\( \text{D}-\text{Ala}^2,\text{MePhe}^4,\text{Gly}^5\)-ol-\text{enkephalin}; DSLET, [\( \text{D}-\text{Ser}^3,\text{Leu}^4,\text{Thr}^6\)]-\text{enkephalin}; Boc, butoxycarbonyl; p-\( \text{Cha} \), p-\text{cyclohexylalanine}; l-\( \text{Cha} \), l-\text{cyclohexylalanine}; p-\text{Nle}, p-\text{norleucine}; l-\text{Nal}, l-\text{naphthylalanine}; p-\text{Nal}, p-\text{naphthylalanine}; p-\text{Nve}, p-\text{norvaline}; l-\text{Nve}, l-\text{norvaline}; y, \text{d-tyrosine}; w, \text{d-tryptophan}; r, \text{d-arginine}; f, \text{d-phenylalanine}; i, \text{l-leucine}; a\(\text{Aba}, \text{l-\text{o-nanobutylacrylic acid.}

---

* This work was funded by National Institutes of Health Grants DA08410 (to R. A. H.) and DA03742 (J. M. B.) and by Trega Biosciences, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Torrey Pines Institute for Molecular Studies, 3550 General Atomics Ct., San Diego, CA 92121 Tel.: 619-455-3803; Fax: 619-455-3804; E-mail: houghten@tpims.org.
polystyrene resin using t-butoxycarbonyl-protected amino acids. Mix-
ture resins (X) were prepared using mixtures of t-butoxycarbonyl-pro-
tected amino acids at each coupling step. Each of the amino acids was
present in a concentration that yielded close to equimolar coupling of
each amino acid. The ratio of the concentrations of the individual amino
acids used to yield this approximate equimolar coupling was pre-deter-
mined using reverse phase-high pressure liquid chromatography to
compare mixture profiles relative to standard mixtures synthesized
using the divide, couple, and recombine method (1) as detailed in Ref. 9.
Coupling completion was determined using Kaiser’s ninhydrin test (10).
Side chain deprotection and cleavage from the resin support were
achieved using low hydrogen fluoride (11) and high hydrogen fluoride

FIG. 1. Screening of the non-acetylated tetrapeptide PS-SCL for the ability to inhibit the binding of selective radiolabels to the μ, δ, and κ receptors. The μ receptor was labeled using [3H]DAMGO, and the δ receptor was labeled using [3H]DSLET. Both assays were carried out using rat brain homogenates. The κ receptor was labeled using [3H]U69,593 and guinea pig brain homogenates. Each panel represents one of the four positional SCLs (i.e. position one SCL is O1XXX-NH₂). Each bar within a panel represents percent inhibition by a peptide mixture defined in the O position with one of 50 amino acids. Amino acids are listed in the footnote to Table II. While these graphs illustrate active peptide mixtures, the choice of amino acids for the synthesis of individual peptides was based on IC₅₀ values.
Selective Opiate Ligands Identified from the Same Library

Receptor Assay—

Counter (Piscataway, NJ). The filters were subsequently counted as above. Unlabeled U50,488 was used as a competitor to generate a standard curve and determine nonspecific binding.

\( \text{total volume of 0.65 ml. Assay tubes were incubated for 2.5 h at 25 °C. The assay was terminated, filtered, and counted as above. Unlabeled DSLET was used as a competitor to generate a standard curve and determine nonspecific binding.} \)

Adenylyl Cyclase Assay

The human SH-SY5Y neuroblastoma cell line was a generous gift from Dr. David K. Grandy (Vollum Institute for Advanced Biomedical Research, Portland, OR). The R1.G1 mouse thymoma cell line was obtained from ATCC (Rockville, MD) and has been shown to express the \( \kappa \) opioid receptor but not the \( \mu \) or \( \delta \) receptors (17). The cells were cultured in RPMI 1640 medium, buffered with 12.5 mM HEPES, pH 7.2, and containing 300 \( \mu \)g/ml l-glutamine, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, 50 \( \mu \)g 2-mercaptoethanol, 60 \( \mu \)g 2-ethanolamine, and 10% iron-supplemented bovine calf serum in 5% CO\(_2\) at 37 °C. SH-SY5Y neuroblastoma cells were cultured in media containing 10 \( \mu \)g retinoic acid for 6 days before harvesting in order to differentiate the cells as described previously (18). Cell membranes were prepared for use in the adenylyl cyclase assays as described previously (19). After the initial centrifugation at 200 \( \times \) g for 15 min at 4 °C, the cells were resuspended in sucrose buffer (0.32 M sucrose, 40 mM HEPES, 2 mM EGTA, pH 7.6). Cells were centrifuged again at 200 \( \times \) g and then homogenized in sucrose buffer with five strokes of a Dounce homogenizer. Membranes were centrifuged at 22,000 \( \times \) g for 20 min at 4 °C, followed by resuspension in sucrose buffer. The protein concentration was determined by the method of Bradford (15) using bovine serum albumin as standard. Cell membranes at a protein concentration of 1–4 mg/ml were stored at −80 °C until use.

Membranes were incubated in a final volume of 100 \( \mu \)l of 40 mM HEPES, containing 15 units of creatine phosphokinase, 20 mM phosphocreatine, 1 mM 1,10-o-phenanthroline, 60 \( \mu \)M isobutylmethylxanthine, 50 \( \mu \)M ATP, 50 \( \mu \)M GTP, 3 mM MgCl\(_2\), and 100 mM NaCl. Agonists and antagonists were included at final concentrations as stated in the text. Naloxone, ICI 174,864, and nor-binaltorphimine were used to block \( \mu \), \( \delta \), and \( \kappa \) opioid receptors, respectively. The reaction was initiated by the addition of 36 \( \mu \)g of membrane protein. After 15 min at 30 °C, the reaction was stopped by the addition of 40 \( \mu \)l of cold 30% potassium bicarbonate, and then the membranes were centrifuged at 12,000 \( \times \) g for 4 min at 4 °C in a microcentrifuge.

The amount of cAMP present in 100 \( \mu \)l of the supernatant,
\textbf{Selective Opiate Ligands Identified from the Same Library}

The affinities at the \( \mu \) receptor of 32 tetrapeptides, representing all possible combinations of the amino acids chosen, are given. Binding conditions are detailed under "Experimental Procedures." Peptides are ranked by activity.

\begin{table}[h]
\centering
\caption{Ki values for individual peptides identified from the tetrapeptide PS-SCL in an assay selective for the \( \mu \) receptor}
\label{table:peptides}
\begin{tabular}{llllll}
\hline
Rank & Peptide & \( K_i \) & S.E. & Rank & Peptide & \( K_i \) & S.E. \\
\hline
1 & Y(D-Nve)-L(Nal)-NH\(_2\) & 0.4 & 0.1 & 17 & Y(D-Nle)-GWL-NH\(_2\) & 3.5 & 1.4 \\
2 & Y(D-Nve)-GW-NH\(_2\) & 1.1 & 0.2 & 18 & YG(L-Nal)-NH\(_2\) & 3.6 & 0.7 \\
3 & Y(A-L-Nal)-NH\(_2\) & 1.2 & 0.4 & 19 & Y(D-Nve)-[W(L-Nal)]-NH\(_2\) & 3.9 & 1.9 \\
4 & Y(D-Nve)-PF-NH\(_2\) & 1.2 & 0.3 & 20 & YF(W-NH\(_2\)) & 4.2 & 0.6 \\
5 & Y(D-Nve)[A(L-Nal)]-NH\(_2\) & 1.2 & 0.3 & 21 & YF(W-NH\(_2\)) & 4.3 & 0.2 \\
6 & YG(W-NH\(_2\)) & 1.3 & 0.2 & 22 & YG([L-Nal])-NH\(_2\) & 4.7 & 0.7 \\
7 & YAW(NH\(_2\)) & 1.3 & 0.1 & 23 & YG(W-NH\(_2\)) & 5.2 & 1.7 \\
8 & YY(W-NH\(_2\)) & 1.4 & 0.3 & 24 & YY(W-NH\(_2\)) & 6.1 & 0.5 \\
9 & Y(D-Nle)-GWL-NH\(_2\) & 1.5 & 0.6 & 25 & YY(W-NH\(_2\)) & 6.5 & 0.7 \\
10 & Y(D-Nle)-PF(L-Nal)-NH\(_2\) & 1.5 & 0.5 & 26 & YF[L-Nal]-NH\(_2\) & 7.3 & 1.2 \\
11 & Y(D-Nle)-AW-NH\(_2\) & 1.6 & 0.3 & 27 & YF[L-Nal]-NH\(_2\) & 7.8 & 0.6 \\
12 & Y(D-Nle)-[A(L-Nal)]-NH\(_2\) & 1.7 & 0.7 & 28 & YY(W-[L-Nal])-NH\(_2\) & 8.3 & 1.3 \\
13 & YYAW(NH\(_2\)) & 1.8 & 0.7 & 29 & Y(D-Nle)-[F(L-Nal)]-NH\(_2\) & 9.5 & 4.0 \\
14 & Y(D-Nve)-WW(NH\(_2\)) & 2.3 & 0.7 & 30 & YYW-[L-Nal]-NH\(_2\) & 11.7 & 1.9 \\
15 & Y(D-Nle)-AW(NH\(_2\)) & 2.4 & 0.3 & 31 & Y(D-Nle)-WW-NH\(_2\) & 12.4 & 5.8 \\
16 & Y(D-Nve)-PF(NH\(_2\)) & 2.7 & 1.2 & 32 & Y(D-Nle)-[W[L-Nal]]-NH\(_2\) & 13.5 & 4.5 \\
\hline
\end{tabular}
\end{table}

\* The amino acids defined at each position of the library and the single letter or abbreviated codes are as follows: A, L-alanine; F, L-phenylalanine; G, glycine; I, L-isoleucine; K, L-lysine; L, L-leucine; M, L-methionine; N, L-asparagine; P, L-proline; Q, L-glutamine; R, L-arginine; S, L-serine; T, L-threonine; V, L-valine; W, L-tryptophan; Y, L-tyrosine; a, D-alanine; f, D-phenylalanine; i, D-isoleucine; k, D-lysine; l, D-leucine; n, D-asparagine; p, D-proline; q, D-glutamine; r, D-arginine; s, D-serine; t, D-threonine; v, D-valine; w, D-tryptophan; y, D-tyrosine; (aAba), L- \(-\)aminobutyric acid; (aAlb), L- \(-\)aminobutyric acid; (aA), L-aminooxybenzylalanine; (aAe), L-aminooxyphenylalanine; (aAe), L-aminooxybenzylalanine; (aAe), L-aminooxyphenylalanine (IC 50 = 1378 nM); (aAe), L-aminooxybenzylalanine (IC 50 = 1507 nM) (where y indicates D-tyrosine and r indicates D-arginine).

**RESULTS**

The tetrapeptide PS-SCL was screened in each of the three separate opioid receptor binding assays described above. Each mixture contained 125,000 tetrapeptides (50). The mixtures were initially screened at a fixed concentration (0.08 mg/ml). The most active mixture found was 1546 nM. The second most active mixture was defined as YXOX-NH\(_2\) (IC 50 = 690 nM) was the most active mixture found. The second, third, and fourth most active mixtures were within a 2-fold difference in activity: X(0-Nle).XX-NH\(_2\) (IC 50 = 1112 nM), yXXX-NH\(_2\) (IC 50 = 1378 nM), and rXXX-NH\(_2\) (IC 50 = 1507 nM) (where y indicates D-tyrosine and r indicates D-arginine).

IC 50 values were calculated for seven mixtures in which the third position was defined (XOXO-NH\(_2\)). Six of the mixtures exhibited activities below 2,000 nM, and all six contained L-amino acids in the defined position. The three most active mixtures found were XXFX-NH\(_2\) (IC 50 = 690 nM), XGXX-NH\(_2\) (IC 50 = 1119 nM), and XXWX-NH\(_2\) (IC 50 = 1227 nM). IC 50 values were calculated for seven mixtures in which the fourth position was defined (XXYX-NH\(_2\)). The most active mixture found was XXY(L-Nal)-NH\(_2\) (IC 50 = 279 nM). The second most active mixture was 3-fold less active (XXXW-NH\(_2\); IC 50 = 850 nM) than the most active mixture, and the third most active mixture found (XXFX-NH\(_2\); IC 50 = 1545 nM) was over 5-fold less active than the most active mixture. The amino acids chosen to make 32 (1 × 4 × 4 × 2) individual peptides are listed in Table I. IC 50 values obtained for these peptides in the \( \mu \) receptor binding assay are given in Table II. All 32 of the IC 50 values were found to have high affinity for the \( \mu \) receptor (K 2 values were < 15 nM). Three of the amino acids in the most active peptide Tyr-[D-Nve]-Gly-(L-Nal)-NH\(_2\) (K 2 = 0.4 nM) were the most active amino acids found for their particular position, whereas glycine in the third position was the second most active amino acid found for that position. The four \( \delta \) amino acids chosen for the second position were found to be replaceable (i.e., peptides which differed only by the amino acid at this position had very similar activities). Whereas all four amino acids chosen in the third position yielded active peptides, those with small amino acid side chains (glycine and alanine) were more active than those with aromatic side chains. L-Naphthylalanine and L-tryptophan were found to be replaceable at the fourth position. The general motif of active peptides identified at the \( \mu \) receptor was Tyr-[D-amino acid]-[L-amino acid with small side chain] (L-aromatic)-NH\(_2\). Since all of the peptides synthesized were found to be active, additional \( \mu \)-selective peptides are likely to be identified from this library. The selectivity ratios (K 2 of peptide at \( \delta \) or \( \kappa \) receptor/K 2 of peptide at the
Selective Opiate Ligands Identified from the Same Library

μ receptor (of the 32 peptides are illustrated in Fig. 3A. All 32 peptides were found to be μ selective; none of the ratios were less than 1. The most μ-selective peptide found was YrAW-NH₂ (Rank 13 in Table II). All peptides with ß-arginine at the second position exhibited excellent μ selectivity. These μ-selective peptides were generally more active at the δ receptor than at the κ receptor.

δ Receptor—After an initial screening (0.08 mg/ml) in a δ-selective receptor binding assay using [³H]DSLET as radioligand, the tetrapeptide PS-SCL was screened again at a 10-fold lower concentration (Fig. 1). IC₅₀ values were calculated for those mixtures that inhibited >60% of [³H]DSLET binding for positions 1 and 2 and >70% of [³H]DSLET binding for positions 3 and 4 (Fig. 4). The most active of the 10 mixtures tested at position 1 included XXXX-NH₂ (IC₅₀ = 2468 nM), WXXX-NH₂ (IC₅₀ = 6250 nM), wXXX-NH₂ (IC₅₀ = 7906 nM; w indicates D-tryptophan). The most active mixture was approximately 3-fold more active than the second most active mixture. Although the active mixtures identified were similar to those found in the μ receptor assay, they were less active, e.g., the mixture XXXX-NH₂ was 4-fold more active in the μ versus the δ receptor assay.

Active mixtures with defined amino acids in the second position were also found to be similar to those identified in the μ assay. IC₅₀ values were determined for 11 mixtures. The five most active mixtures found contained ß-amino acids: XyXN-NH₂ (IC₅₀ = 4990 nM), XXXX-NH₂ (IC₅₀ = 7830 nM), XXNX-NH₂ (IC₅₀ = 8099 nM) Xδ(Nve)XX-NH₂ (IC₅₀ = 9970 nM), and Xδ(Ncl)XX-NH₂ (IC₅₀ = 11795 nM).

The 10 mixtures tested in which the third position was defined were found to be less active than those of the remaining three positions, and no mixture was found to have an IC₅₀ value lower than 5,000 nM. The four most active mixtures were XXX(aAb)(-NH₂ (IC₅₀ = 6474 nM), XXG-NH₂ (IC₅₀ = 7115 nM), XX(T-Cha)(-NH₂ (IC₅₀ = 8482 nM), and XXMX-NH₂ (IC₅₀ = 9085 nM). There was very little difference in affinity between the most active mixtures found at the third position; the 10 mixtures had IC₅₀ values between 6,000 and 10,000 nM.

IC₅₀ values were calculated for nine mixtures in which the fourth position was defined. Unlike the results found in the μ receptor assay, the two most active amino acids found at the fourth position were positively charged: XXXX-NH₂ (IC₅₀ = 4529 nM) and XXXX-NH₂ (IC₅₀ = 9026 nM). Mixtures ranked third and fourth at this position were aromatics, as was also found in the μ receptor assay [XXXW-NH₂ (IC₅₀ = 6966 nM) and XXXF-NH₂ (IC₅₀ = 7385 nM)]. The amino acid combinations chosen for the synthesis of 60 individual peptides are listed in Table III. Peptides that had K values below 500 nM in the δ-selective assay are shown in Table IV. Only three of the peptides were found to have activity under 10 nM. The δ selectivity of the peptides is shown in Fig. 3B. Twelve of the peptides had greater activity in the μ assay than in the δ-selective assay (ratio of less than 1). This is not altogether surprising as many of the mixtures with defined amino acids chosen for the δ peptides were more active in the μ receptor assay. It is also not surprising that the most δ-selective peptide found, Wy(αAba)R-NH₂ (Rank 2 in Table III) contained ß-arginine in the fourth position, since this amino acid was ranked first in the δ receptor screening and ranked 17 in the μ receptor screening. Many of the peptides tested were virtually inactive at the κ receptor at the highest concentration tested (10,000 nM). These data points are indicated by an asterisk in Fig. 3B.

κ Receptor—For the κ receptor, the library was screened at an initial concentration of 0.08 mg/ml in guinea pig brain homogenates using [³H]U69,593 as radioligand (Fig. 1). IC₅₀ values were subsequently calculated for those mixtures which
inhibited >90% of \[^3\text{H}\]U69,593 binding for positions 1–4 (Fig. 5). The most active mixtures found in the \(\kappa\)-selective assay do not bear any resemblance to those found in the \(\mu\) and \(\delta\) receptor assays. The 12 mixtures tested in which the first position was defined ranged in IC\(_{50}\) value from 3,000 to 22,000 nM. The four most active mixtures contained D-amino acids at this position (fXXX-NH\(_2\) (IC\(_{50}\) = 5,3615 nM); (D-Cha)XXX-NH\(_2\) (IC\(_{50}\) = 4,045 nM); (D-Nle)XXX-NH\(_2\) (IC\(_{50}\) = 5,936 nM); and iXXX-NH\(_2\) (IC\(_{50}\) = 6,910 nM)).

Eleven mixtures were tested in which the second position was defined. As observed for the first position, D-amino acids were also favored. The most active mixture, X(d-Nal)XX-NH\(_2\) (IC\(_{50}\) = 1,526 nM), was 4-fold more active than the second most active mixture XXX(d-Cha)-NH\(_2\) (IC\(_{50}\) = 4,904 nM)). The amino acids chosen for inclusion in the synthesis of individual peptides are shown in Table V. Twenty four peptides were synthesized, and their \(K_i\) values are given in Table VI. Fourteen of the 24 peptides had \(K_i\) values below 50 nM, 11 of which were below 10 nM. The most active peptide was found to be ff(D-Nle)r-NH\(_2\) (\(K_i\) = 1.2 nM). D-Phenylalanine and D-norleucine were replaceable at the first position. Surprisingly, D-phenylalanine and D-naphthylalanine were replaceable at the second position. D-Norleucine and D-isoleucine were replaceable at the third position; however, none of the peptides containing L-tryptophan in the third position had a \(K_i\) value below 1000 nM. This suggests the existence of another family of \(\kappa\) ligands containing L-tryptophan in the third position. The active peptides (those ranked 1–16) were also highly selective for the \(\kappa\) receptor (Fig. 3C). The most \(\kappa\) selective peptides found had \(\mu/\kappa\) and \(\mu/\delta\) ratios of greater than 5,000.

Adenylyl Cyclase Assay—Opioid agonists inhibit adenylyl cyclase activity, resulting in reduced levels of cyclic AMP (cAMP) (21, 22). An adenylyl cyclase assay using SH-SY5Y neuroblastoma or R1G1 thymoma cell line membranes was used to rapidly determine whether a peptide was an opioid agonist or antagonist. The opioid receptors expressed on the SH-SY5Y cell line after culturing in the presence of 10 \(\mu\text{M}\) retinoic acid are approximately 85% of the \(\mu\) type and 15% of the \(\delta\) type (18).2

2 J. M. Bidlack, unpublished results.
receptor subtype. A reduction of cyclic AMP levels to less than 70% of the basal cAMP levels was regarded as being indicative of an opioid agonist effect, provided that the inhibition of cAMP was blocked by an opioid antagonist. Naloxone was used for \( \mu \) receptors and nor-binaltorphimine was used for \( \kappa \) receptors. Their ability to inhibit the accumulation of cAMP was similar to that of DAMGO. Inhibition of cAMP was antagonized by naloxone but not the \( d \)-specific antagonist ICI 174,864, indicating that the reduction in cAMP was mediated by \( \mu \) receptors. The most active peptides found for the \( \mu \) and \( \kappa \) assays have been tested for ability to inhibit cAMP accumulation (Table VII). All peptides tested were found to be agonists at their respective receptor, indicating that their efficacy at the receptor is similar to standard ligands.

**DISCUSSION**

The opiate receptor systems used in the current study have been described in our earlier work (4, 7, 16, 23). In this laboratory studies involving combinatorial libraries for the identification of opioid ligands have focused on the \( \mu \) receptor. These studies have a dual purpose as follows: first to find new ligands and expand our knowledge of the opioid receptors, and second to explore the use of combinatorial libraries made up of large mixtures. The ability to identify highly active individual li-
The affinities at the \( \kappa \) receptor of 24 peptides, representing all possible combinations of the amino acids chosen, are presented. Binding conditions are detailed under “Experimental Procedures.” Peptides are ranked by affinity.

### TABLE VI

| Rank | Peptide* | \( K_i \) (nM) | S.E. |
|------|----------|----------------|------|
| 1    | ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 1.2 | 0.6 |
| 2    | ((D-Nle)(D-Nle)(D-Nle)(D-Cha)) -NH\(_2\) | 1.5 | 0.8 |
| 3    | ((D-Nle)(D-Nle)(D-Nle)(D-Cha)) -NH\(_2\) | 2.3 | 0.8 |
| 4-8  | ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 2.4 | 0.8 |
| 9-12 | ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 3.6 | 1.0 |
| 13   | ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 4.2 | 0.6 |
| 14   | ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 7.1 | 5.4 |
| 15-16| ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 9.3 | 3.8 |
| 17-18| ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 26 | 13 |
| 19-20| ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 27 | 11 |
| 21-22| ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 33 | 10 |
| 23-24| ((D-Nle)(D-Nle)(D-Cha)(D-Cha)) -NH\(_2\) | 53 | 24 |

*See Table II for abbreviations.

YmPG-NH\(_2\) was previously identified from a tetrapeptide library using an iterative deconvolution process (25) but was not identified here because \( \delta \)-methionine was not included in this library. The most \( \mu \)-selective peptides found in this study contain \( \delta \)-arginine at the second position and are similar to peptides reported previously (YrFK-NH\(_2\) (DALDA) (26)). There are other tetrapeptides with high affinity for the \( \mu \) receptor that were not identified in this report (e.g., YPWF-NH\(_2\) (27) and WWPR-NH\(_2\) (5)). YPWF-NH\(_2\) (27) would have been identified using a tetrapeptide library made up only of 20 \( \alpha \)-amino acids or if more amino acids were chosen at each position. We are currently working on deconvolution strategies that would minimize the peptides required to be synthesized while maximizing the number of amino acids chosen at each position. In all three assays, the number of amino acids chosen for the synthesis of individual peptides was restricted since the number of combinations rises exponentially with the number of amino acids at each position (i.e., 81 tetrapeptides for 3 amino acids are chosen at each position, 256 tetrapeptides if 4 amino acids are chosen at each position, and 625 tetrapeptides if 5 amino acids are chosen at each position). This restriction clearly results in a limitation in the identification of additional active sequences. This can be seen in the current report in which active sequences for the \( \mu \) receptor were identified when the same library was screened against the \( \delta \) receptor. It should be noted that these sequences would have been identified from the \( \mu \) screening data if a greater number of combinations had been synthesized. Furthermore, due to limited resources a subjective choice of amino acids is involved. In choosing the amino acids at each position, one endeavors to compromise between covering the greatest range in chemical diversity and avoiding the choice between two similar amino acids which are not replaceable.

YmPG-NH\(_2\) was previously identified from a tetrapeptide library using an iterative deconvolution process (25) but was not identified here because \( \delta \)-methionine was not included in this library. The most \( \mu \)-selective peptides found in this study contain \( \delta \)-arginine at the second position and are similar to peptides reported previously (YrFK-NH\(_2\) (DALDA) (26)). There are other tetrapeptides with high affinity for the \( \mu \) receptor that were not identified in this report (e.g., YPWF-NH\(_2\) (27) and WWPR-NH\(_2\) (5)). YPWF-NH\(_2\) (27) would have been identified using a tetrapeptide library made up only of 20 \( \alpha \)-amino acids or if more amino acids were chosen at each position. We are currently working on deconvolution strategies that would minimize the peptides required to be synthesized while maximizing the number of amino acids chosen at each position. In all three assays, the number of amino acids chosen for the synthesis of individual peptides was restricted since the number of combinations rises exponentially with the number of amino acids at each position (i.e., 81 tetrapeptides for 3 amino acids are chosen at each position, 256 tetrapeptides if 4 amino acids are chosen at each position, and 625 tetrapeptides if 5 amino acids are chosen at each position). This restriction clearly results in a limitation in the identification of additional active sequences. This can be seen in the current report in which active sequences for the \( \mu \) receptor were identified when the same library was screened against the \( \delta \) receptor. It should be noted that these sequences would have been identified from the \( \mu \) screening data if a greater number of combinations had been synthesized. Furthermore, due to limited resources a subjective choice of amino acids is involved. In choosing the amino acids at each position, one endeavors to compromise between covering the greatest range in chemical diversity and avoiding the choice between two similar amino acids which are not replaceable.

The majority of the combinations identified from the data for the \( \delta \)-selective assay were found to be more active in the \( \mu \) receptor assay. A common motif for \( \delta \)-selective peptides was...
Selective Opiate Ligands Identified from the Same Library

WOOR-NH₂, as can be seen in Table VIII, shows the effect on $K_i$ values at the $\mu$ and $\delta$ receptors of substitution analogs of the $\delta$-selective peptide Wy(3AbaR)-NH₂. There is no simple correlation between chemical similarities in amino acids and selectivity. Replacement of tryptophan at position 1 by tyrosine retains selectivity, but replacement of tyrosine by tryptophan at position 2 results in a substantial reduction of selectivity. Replacement of L-norvaline at position 3 by L-cyclohexyalanine results in reduction of selectivity, whereas a double replacement of tyrosine at position 1 and by L-cyclohexylalanine at position 3 completely reverses the selectivity, yielding a $\mu$-selective peptide. This illustrates the dangers inherent in making broad statements on the replaceability of amino acids of similar chemical character.

The similarities between the peptides found to have activity $\mu$ and $\delta$ receptors appear to indicate a closer relationship between the two receptors, as opposed to the peptides found to be active at the $\kappa$ receptor. DSLET is also known to bind to $\mu$ receptors, but we have included 100 nM Ac-RFWINK-NH₂ (16), a highly selective $\mu$ peptide, in the assay buffer in order to adequately block the radioligand from binding to $\mu$ receptors. Furthermore, the library was also screened using [3H]Naltrexone as a $\delta$-selective radiolabel, and no significant library profiles differences were observed.

The sequences found from screening the tetrapeptide PS-SCL in an assay selective for the $\kappa$ receptor are perhaps the most interesting of this study. They are unlike any peptides reported to have activity at $\kappa$ receptors and would not have been identified by classical structure-activity studies. A general motif is not easily identified. The sequence clearly appears to favor $\delta$-amino acids at all four positions. Also, any peptide synthesized containing L-tryptophan at position 3 had poor activity. The first position accepts an aromatic side chain, $\delta$-phenylalanine, or an aliphatic side chain, $\delta$-norleucine, but whether this may be generalized to all similar amino acids is not known. Peptides with $K_i$ values below 10 nM were found with $\delta$-arginine in the fourth position. Sequences that differed only in the fourth position were always more active with $\delta$-arginine than with $\delta$-cyclohexylalanine. Active peptides identified for the kappa receptor were highly selective. A study involving the synthesis of 1,000–3,000 individual tetrapeptides based on the data presented in this study is underway.

In this study, highly active individual compounds and highly $\mu$- and $\kappa$-selective compounds were rapidly identified from a large mixture-based positional scanning combinatorial library. All of the most active peptides tested were found to be agonists. It has yet to be determined if these peptides are capable of crossing the blood-brain barrier, but it is expected that the presence of $\delta$-amino acids in their sequences will prolong their biological half-lives. The $\kappa$ peptides identified may prove useful for pain management, as $\kappa$ compounds have come into focus in recent reports as having greater efficacy in analgesia for women (28). On the other hand, if these $\kappa$ peptides do not cross the blood-brain barrier, they may be useful in attenuating the pain and/or progression of adjugant arthritis (29). This study illustrates not only the power of the positional scanning concept for the rapid identification of new ligands but also how distinct ligands may be rapidly identified for closely related receptors.

Acknowledgments—We thank Amy Bower, David Dale, Christa Schoner, and Kevin Hill for technical assistance, and Eileen Weiler for editorial assistance.

REFERENCES
1. Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T., and Cuervo, J. H. (1991) Nature 354, 84–86
2. Leung, S. M., Salmon, S. L., Braby, V. J., Kazmerski, W. M., and Knapp, R. J. (1991) Nature 354, 82–84
3. Geysen, H. M., Rodda, S. J., and Mason, T. J. (1986) Mol. Immunol. 23, 799–815
4. Houghten, R. A., and Dooley, C. T. (1993) Biomed. Chem. Lett. 3, 405–412
5. Dooley, C. T., Kaplan, R. A., Chung, N. N., Schiller, P. W., Bidlack, J. M., and Houghten, R. A. (1995) Pept. Res. 8, 124–137
6. Pinilla, C., Appel, J. R., Blanc, P., and Houghten, R. A. (1992) BioTechniques 13, 901–905
7. Houghten, R. A., and Houghten, R. A. (1993) Life Sci. 52, 1509–1517
8. Houghten, R. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5313–5315
9. Ostresh, J. M., Winkle, J. H., Hamashin, V. T., and Houghten, R. A. (1994) Biopolymers 34, 1681–1689
10. Kaiser, E. T., Culecott, R. L., Blossinger, C. D., and Cook, P. I. (1970) Anal. Biochem. 34, 590–598
11. Tam, J. P., Heath, W. F., and Merrifield, R. B. (1983) J. Am. Chem. Soc. 105, 6442–6455
12. Houghten, R. A., Bray, M. K., DeGraw, S. T., and Kirby, C. J. (1986) Int. J. Pept. Protein Res. 27, 673–678
13. Pokorny, V., Mudra, P., Jechnicka, J., Zenisek, K., Pavlik, M., Voburka, Z., Rinovova, M., Stieranova, A., Lacka, A. W., Eichler, J., Houghten, R. A., and Lebl, M. (1994) in Innovation and Perspectives in Solid Phase Synthesis-Peptides, Proteins and Nucleic Acids (Epton, R., ed) pp. 643–648, Mayflower Worldwide Ltd., Birmingham, UK
14. Eichler, J., Houghten, R. A., and Lebl, M. (1996) J. Pept. Sci. 2, 240–244
15. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
16. Dooley, C. T., Chung, N. N., Wilkes, B. C., Schiller, P. W., Bidlack, J. M., Pulcinelli, G. W., and Houghten, R. A. (1994) Science 266, 2019–2022
17. Lawrence, D. M. P., Joseph, D. B., and Bidlack, J. M. (1995) Biochem. Pharmacol. 49, 81–89
18. Yu, V. C., and Sadee, W. (1988) J. Pharmacol. Exp. Ther. 245, 350–355
19. Lawrence, D. M. P., and Bidlack, J. M. (1998) J. Pharmacol. Exp. Ther. 266, 1678–1683
20. Tovey, K. C., Oldham, K. G., and Whelan, J. A. M. (1974) Clin. Chim. Acta 56, 221–234
21. Childers, S. R. (1991) Life Sci. 49, 1991–2003
22. Law, P. Y., Hom, D. S., and Leh, H. H. (1983) Mol. Pharmacol. 23, 26–35
23. Dooley, C. T., Chung, N. N., Schiller, P. W., and Houghten, R. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10811–10815
24. Charpentier, S., Sagan, S., Delfour, A., and Nicolas, P. (1991) Biochem. Biophys. Res. Commun. 179, 1161–1168
25. Dooley, C. T., Hope, S. K., and Houghten, R. A. (1995) in Peptides 94: Proceedings of the 23rd European Peptide Symposium (Maia, H. L. S., ed) pp. 805–806, ESCOM Science Publishers B.V., Leiden
26. Schiller, P. W., Nguyen, T. M.-D., Chung, N. N., and Lemieux, C. (1989) J. Med. Chem. 32, 698–703
27. Zadina, J. E., Hackler, L., Ge, L.-J., and Kastin, A. J. (1997) J. Pharmacol. Exp. Ther. 281, 499–502
28. Gear, R. W., Miaskowski, C., Gordon, N. C., Paul, S. M., Heller, P. H., and Levine, J. D. (1996) Nat. Med. 2, 1248–1250
29. Walker, J. S., Howlett, C. R., and Nayanar, V. (1995) Life Sci. 57, 371–378