Minimum-Structure Enkephalin Analogues Incorporating L-Tyrosine, D(or L)-Phenylalanine, and a Diamine Spacer†

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In order to test the theory that high μ-activity of opioid peptides could be elicited by the presence of an amino-terminal L-Tyr residue and a Phe aromatic ring held in the proper relative spatial disposition, a novel series of hybrid retro peptides were prepared in which L-Tyr was linked to N-acyl Phe through a variety of diamine spacers. These compounds were evaluated for opioid agonist and antagonist activity in the guinea pig ileum (GPI) in vitro assay. Analogues containing a 1,2-ethanediamine spacer, which conferred a Tyr–Phe separation distance closest to that found in Phe^3 opioid peptides, were more potent agonists than the corresponding analogues containing a 1,3-propanediamine spacer. Agonist activity was observed for both L-Phe and D-Phe analogues, consistent with the known activity for both Phe stereochemistries for certain Phe^3 opioid peptide analogues. Concerning the diamine spacer, conformational constraints imposed by 4-aminopiperidine and 4-(aminomethyl)piperidine as well as the presence of a hydroxyl group eliminated activity, but the presence of *gem*-dimethyl substitution next to the nitrogen attached to Tyr increased activity substantially for the D-Phe derivatives. Removal of the *N*-acetyl group from Phe did not eliminate activity. Naloxone K_e values determined for six of the most potent analogues are indicative of predominantly μ-agonism, but the D-Phe compounds **3a** and **6a** (1.4–2.1 nM) appear to be more μ-selective than the L-Phe compounds **2b**, **3b**, **5b**, and **6b** (3.3–4.4 nM), even though the latter are more potent agonists. Compounds **3a** and **3b**, which were found to be 10 and 21 times more potent, respectively, than morphine in the GPI, are two of the most structurally simple yet potent opioid peptide analogues described to date.

It is well-established that there are at least three major opioid receptor subtypes in the central nervous system, designated μ, δ, and κ.[1,2] Although much effort has been directed at elucidating the structural factors which govern agonist and antagonist activity at each receptor subtype, these factors remain incompletely understood. For the originally identified endogenous opioid peptides (enkephalins and endorphins), the Tyr^1 tyramine and Phe^4 phenyl moieties were acknowledged to be the major contributors to recognition at opioid receptors in general.[3-5] The discovery several years ago that the naturally occurring (from frog skin) peptide dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) displays potent analgesic activity[6] suggested that the appearance of the critical Phe residue at position 4 of the peptide sequence was not a firm requirement for opioid activity. Another series of exogenous Tyr^1/Phe^3 peptides with potent opioid activity was found in hydrolysates of casein,[7] including the parent "casomorphin" (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), as well as the abbreviated tetrapeptide amide Tyr-Pro-Phe-Pro-NH_2 known as morphiceptin.[8]

The fact that Phe^3 peptides can have potent opioid activity was arrived at independently by Stewart and coworkers,[9-11] whose efforts to elucidate the "minimum structure" of enkephalins required for activity included the preparation of Gly^3-deletion analogues of metabolically stabilized D-Ala^2 enkephalins. They found that Tyr-D-Ala-Phe-Met-NH_2 and Tyr-D-Ala-Gly-Phe-Met-NH_2 had nearly identical activity in the guinea pig ileum (GPI) in vitro assay, and that a structure as simple as Tyr-D-Ala-$NHCH_2CH_2CH_2Ph$ retained greater than 50% of this activity.[10,11]

In these early studies, it soon became clear that the Phe^3 peptides had a much greater preference for μ- over δ-receptors, compared to typical Phe^4 enkephalin analogues. Subsequently, a number of additional synthetic Phe^3 analogues with potent μ-selective opioid activity were discovered.[12,13] These findings gave the impression that improved recognition at μ-opioid receptors resulted from a decreased Tyr–Phe distance. However, all potent Phe^3 opioids contained the α-helix-disrupting residues D-Ala or Pro at position 2, and switching from D-Ala^2 to L-Ala^2 resulted in a much greater decrease in activity for the Phe^3 peptides relative to the Phe^4 peptides.[9,12-14] This suggested that conformational factors governing the relative orientation of the Tyr tyramine and Phe phenyl moieties deserved at least as much focus as did simple distance factors. In recent years, this question has been explored through the synthesis of a wide range of cyclic Phe^3 and Phe^4 peptides with restricted conformational freedom.[15-21]

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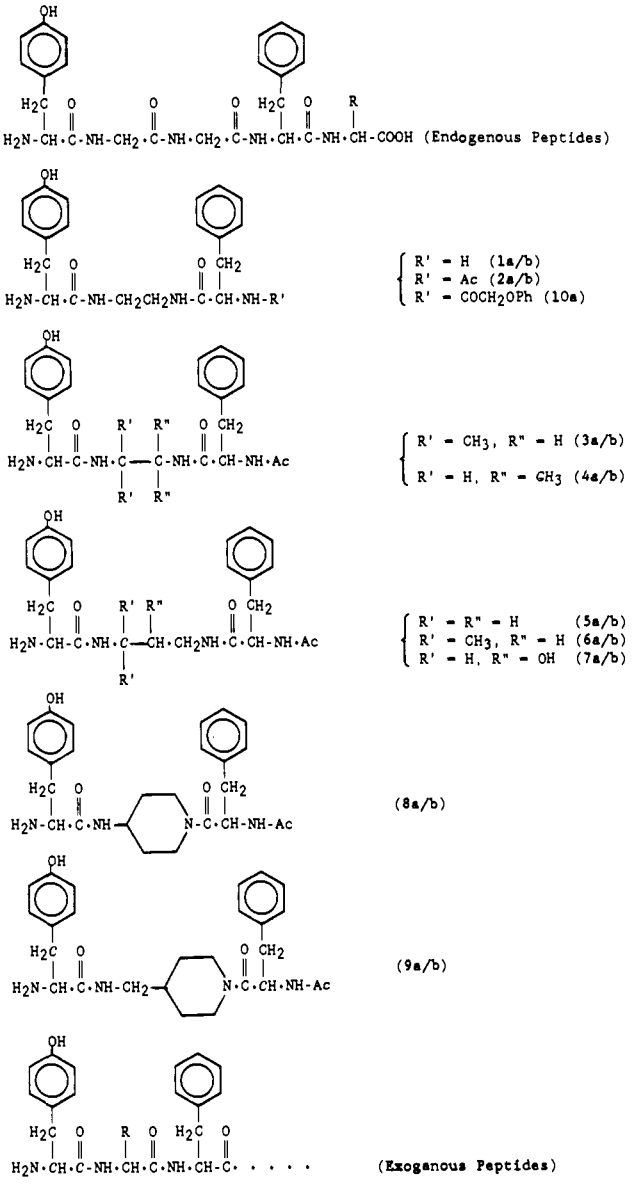

Figure 1. Structural relationship between the endogenous Phe[4] opioid peptides (e.g., enkephalins), the exogenous Phe[3] opioid peptides (dermorphins and casomorphins), and the hybrid analogues examined in this study.

A more far-reaching implication of the Phe[3]/Phe[4] ambiguity was that the role of the amino acid residues in between Tyr and Phe was strictly one of conformational control rather than one of presenting additional primary recognition contacts. If so, this suggested that active μ-ligands need contain only the crucial Tyr tyramine and Phe phenyl recognition elements held in the appropriate relative spatial orientation. In order to test this theory, we set out to design minimum-structure opioid peptides containing only two amino acids. This was accomplished by joining the amino-terminal L-Tyr through various diamine spacers to a *N*-acyl Phe residue present in a retro[22] sense, denoted as r-Phe (Figure 1). These novel hybrid analogues were evaluated for μ-opioid activity using the intact GPI preparation.[23]

Design Strategy

Since the Phe residue in our hybrid peptides was incorporated with reversed polarity from that of a normal peptide, biological activity would be expected to reside in our r-D-Phe analogues, which preserve the relative stereochemistry of the naturally occurring L-Phe peptides. However, the replacement of L-Phe[3] by D-Phe[3] in the casomorphins is known to increase activity.[24,25] Thus, we felt it was wise to synthesize both L-Tyr,r-D-Phe and L-Tyr,r-L-Phe analogues in our hybrid series, herewith abbreviated L,D and L,L. Our decision to incorporate the Phe residue in a retro fashion took into account the report that the presence of a retro amide bond to the "left" of the Phe residue in dermorphin analogues did not diminish either activity or μ-selectivity.[26]

The design strategy of our analogues involved several additional factors. First, the "linear distance" between Tyr and Phe was selected to vary between that of the natural Phe[3] and Phe[4] peptides (Figure 1). Second, the *gem*-dimethyl substitution in the diamine spacer of compounds **3** and **6** and the piperidine ring branch point in compound **8** correspond to the use of Aib (α-aminoisobutyric acid) and Ala, respectively, as the second amino acid residue in opioid peptides. Methyl substitution at C_α in the second residue of enkephalins is known to improve activity.[3,4] Third, since cyclization at nitrogen of Gly[2] of enkephalin (incorporation into an oxopiperazinyl ring) eliminates activity,[27] N-1 of the piperidine spacers was joined to retro Phe rather than to Tyr. Fourth, the absence of a free COOH terminus was expected to favor μ as opposed to δ-activity.[3-5] Fifth, the hybrid nature of these analogues (i.e., the lack of a COOH terminus and the presence of a non amino acid residue following Tyr[1]) was expected to confer significant resistance to the action of peptidases responsible for the normal rapid inactivation of opioid peptides.

The phenoxyacetyl compound **10a** was synthesized in response to the acknowledged benefit of an added hydrophobic group in the dermorphin "post-enkephalin" sequence.[28-31] Compounds **7a/b**, containing a hydroxyl group in the diamine spacer, were prepared in order to reinstate a H-bonding moiety in between the Tyr and Phe, in case removal of the normal amino acid linkage eliminated an important amide interaction with the receptor.[32]

Chemistry

The preparation of analogues **1a/b–9a/b** and **10a** was carried out according to standard solution-phase peptide synthesis and amine-protection methodologies as depicted in Schemes I–V. For **3a/b**, **4a/b**, and **6a/b**, positional

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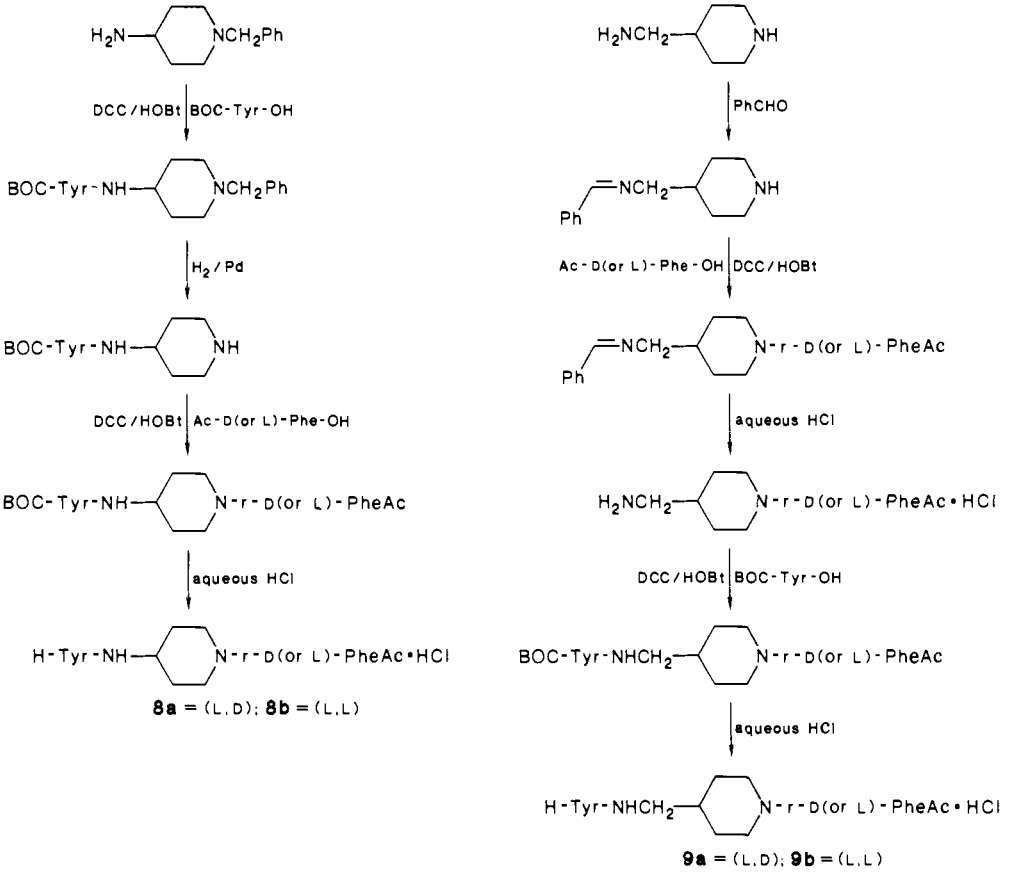
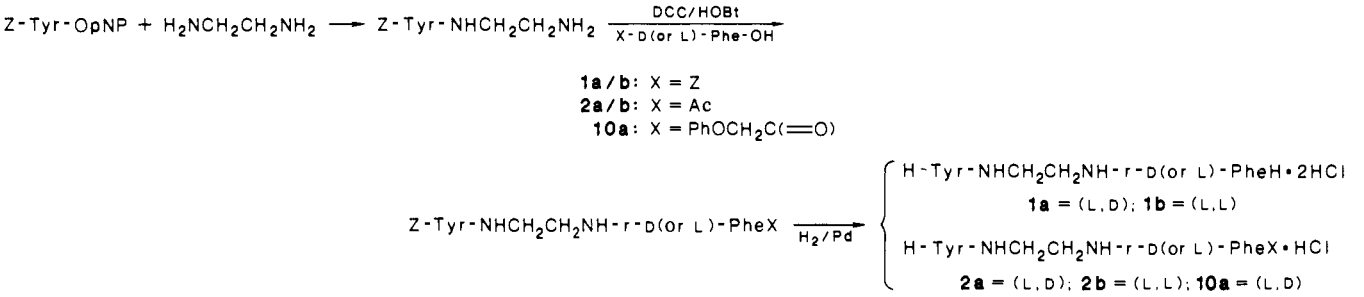
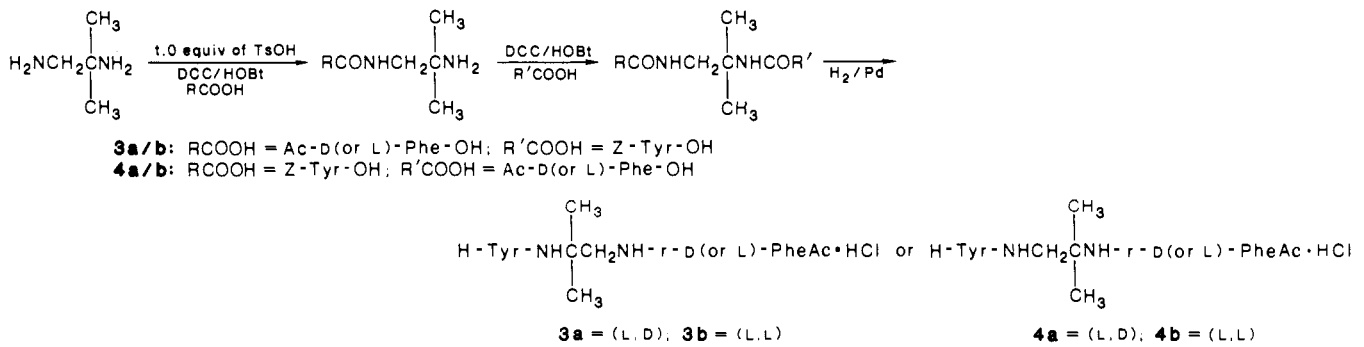
Scheme I. Synthesis of Piperidine-Based Analogues **8a/b** and **9a/b**



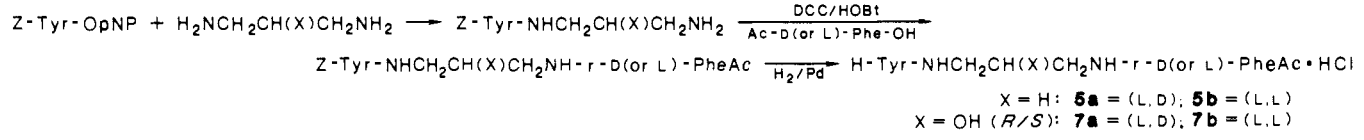
Scheme II. Synthesis of Ethylenediamine-Derived Analogues **1a/b**, **2a/b**, and **10a**



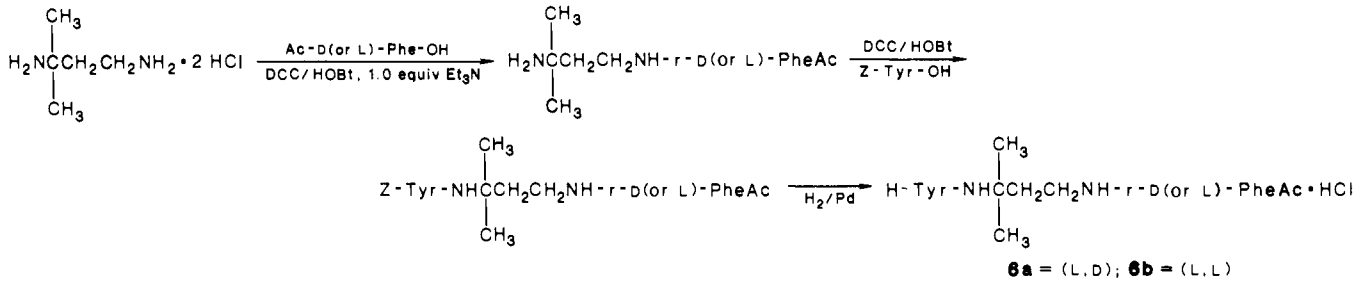
Scheme III. Synthesis of 1,2-Diamino-2-methylpropane-Derived Analogues **3a/b** and **4a/b**



Scheme IV. Synthesis of 1,3-Diaminopropane- and 1,3-Diamino-2-propanol-Derived Analogues **5a/b** and **7a/b**



Scheme V. Synthesis of 1,3-Diamino-3-methylbutane-Derived Analogues **6a/b**



control over the differential acylation of the unsymmetrical diamine spacers was achieved through an ordered sequential coupling strategy based on steric accessibility factors. All final target compounds were single optical isomers with the exception of **7a/b**, which incorporate a prochiral 1,3-diamino-2-propanol spacer. In the latter case, no attempt was made to control the diastereomeric purity at C-2 of the spacer, and the compounds tested were assumed to be mixtures of diastereomers, though neither ^{1}H NMR spectra at 200 MHz nor reverse-phase HPLC revealed the existence of mixtures. HPLC analysis of the D-Phe- and L-Phe-derived epimers in each case showed single (>95%) peaks eluting with similar but nonidentical retention times.

The ^{1}H NMR spectra of the compounds in D_2O revealed several interesting features which may relate to solution conformational issues. First, two (unequal) *N*-acetyl methyl singlets were observed for the analogues containing a piperidine ring in the diamine spacer (**8a/b** and **9a/b**). This phenomenon is commonly observed for unsymmetrical tertiary amides when *E/Z* rotational isomerism results in different magnetic environments. In the case of **8a/b** and **9a/b**, splitting of the *N*-acetyl methyl signal apparently reflects the fact that the two sides of the piperidine ring are diastereotopic. A small splitting of the upfield Tyr aryl doublet was observed for **3a**, **6b**, and **9a**, presumably also due to amide rotational isomerism of some type. Second, both the diastereotopic methyls and the diastereotopic methylene hydrogens in the two *gem*-dimethyl-substituted ethanediamine analogues **3a/b** and **4a/b** both appeared as distinct signals, separated on the average by 0.1 ppm. On the other hand, for the *gem*-dimethyl-substituted propanediamine analogues **6a/b**, distinct signals could be elucidated only for the diastereotopic methyls, with a small separation of 0.03 ppm. No observable splitting of diastereotopic methylene hydrogens in the diamine spacer was exhibited by the unsubstituted ethanediamine and propanediamine analogues **1a/b**, **2a/b**, **5a/b**, and **10a**.

Third, the four piperidine-containing compounds exhibited a downfield chemical shift of 0.5 ppm (δ ~5.0) for the Phe C_α hydrogen relative to the other analogues (δ ~4.5) (Table I). The assignment of these resonances to Phe was made on the basis that the higher field αCH resonance at δ ~4.1 is that expected for the amino terminus (Tyr). In fact, for the analogues lacking an *N*-acyl group on Phe (**1a/b**), the lower field resonance is missing and there are two αCH resonances in the amino-terminus range of δ ~4.1. The Phe αCH chemical shifts observed for the four piperidine compounds are downfield of the "random-coil" value (δ 4.66) expected in the absence of any preferred local conformation,[33] whereas the Phe αCH chemical shifts observed for the other analogues are upfield of the "random-coil" value.

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Table I. ^{1}H NMR Data[a] for Tyr and Phe C_α-H

compd	δ Tyr-C*H*	δ PheC*H*
1a	(4.11)[b]	(4.15)[b]
1b	(4.07)[b]	(4.13)[b]
2a	4.07	4.44
2b	4.06	4.44
3a	3.99	4.49
3b	3.95	4.47
4a	4.18	4.34
4b	4.14	4.39
5a	3.91	4.43
5b	4.06	4.43
6a	4.02	4.42
6b	4.02	4.42
7a	4.13	4.49
7b	4.15	4.50
8a	~4.02[c]	4.96
8b	~4.02[c]	4.98
9a	4.04	4.99
9b	4.05	4.99
10a	4.14	4.62

[a] Values listed are for the HCl salts in D_2O solvent relative to internal CH_2Cl_2 at δ 5.45 (referenced to DSS). [b] This assignment assumes that the more downfield signal is that due to the Phe C_α-H, but the two values may actually be switched. [c] Approximate value on account of overlap with one of the ring C_2-H resonances.

Table II. Agonist Activity in the GPI

compound	% inhibn at 1 μM[a]	% inhibn by morphine at 0.1 μM
1a, Tyr-NHCH$_2$CH$_2$NH-r-D-Phe	8.0	49.5[c]
1b, Tyr-NHCH$_2$CH$_2$NH-r-L-Phe	12.0	49.5[c]
2a, Tyr-NHCH$_2$CH$_2$NH-r-D-PheAc	10.0	49.5[c]
2b, Tyr-NHCH$_2$CH$_2$NH-r-L-PheAc	89.5	40.0
3a, Tyr-NHC(CH$_3$)$_2$CH$_2$NH-r-D-PheAc	79.3	47.0
3b, Tyr-NHC(CH$_3$)$_2$CH$_2$NH-r-L-PheAc	91.5	43.5
4a, Tyr-NHCH$_2$C(CH$_3$)$_2$NH-r-D-PheAc	16.3	47.0
4b, Tyr-NHCH$_2$C(CH$_3$)$_2$NH-r-L-PheAc	42.5	40.0
5a, Tyr-NHCH$_2$CH$_2$CH$_2$NH-r-D-PheAc	5.0[b]	47.0
5b, Tyr-NHCH$_2$CH$_2$CH$_2$NH-r-L-PheAc	29.5	47.0
6a, Tyr-NHC(CH$_3$)$_2$CH$_2$CH$_2$NH-r-D-PheAc	50.5	47.0
6b, Tyr-NHC(CH$_3$)$_2$CH$_2$CH$_2$NH-r-L-PheAc	53.0	40.0

[a] Compounds were tested in the presence of 1 μM bestatin. Values listed represent the average of two to four measurements. Compounds **3a/b**, **4a**, **5a/b**, and **6a** were also screened one time in the presence of the full cocktail of peptidases, as well as without any inhibitors present. Under both of these conditions, little variation (<5%) from the values listed above was observed. Compounds **7a/b**, **8a/b**, **9a/b**, and **10a** exhibited immeasurably low activity at 1 μM (<5% inhibition of twitch). [b] 8.0% inhibition at 2 μM. [c] At 0.5 μM.

Results

All of the analogues were initially screened at 10^{-6} M for their ability to display a naloxone-reversible inhibition of the electrically induced contractions in GPI in the presence of 1.0 μM bestatin, and the inhibitory activity compared to that of morphine at 10^{-7} M in the same tissue is listed in Table II. When some of these compounds were also tested in the presence of a full cocktail of peptidase inhibitors, the magnitude of inhibition was not altered. For the seven compounds which displayed >25% inhibition

Table III. Inhibitory Activity (GPI) and Naloxone K_e Data

compd	potency relative to morphine[a]	K_e, nM[b]
2b	1.6 ± 0.2	4.0 ± 0.2
3a	10.0 ± 2.2	1.4 ± 0.3[c]
3b	21.2 ± 1.0	4.2 ± 0.6[c]
4b	0.270 ± 0.005	N.D.
5b	0.0077 ± 0.0027	4.4 ± 0.9
6a	0.052 ± 0.015	2.1 ± 0.4[d]
6b	0.220 ± 0.002	3.3 ± 0.3[d]

[a] IC_{50}(morphine)/IC_{50}(test compound). IC_{50} values were interpolated from concentration–response curves generated for the test compound and morphine (before and after the test compound) in the same GPI preparation. Numbers represent the average of at least three experiments performed on ilea from different guinea pigs. The average morphine IC_{50} was 0.13 μM. In all cases, concentrations of 50–500 μM naloxone were able to completely block agonist activity. [b] Calculated as described in the Experimental Section from concentration–response curves generated in the absence and presence of 10 nM naloxone. N.D. = not determined. [c] $p < 0.005$. [d] $p < 0.05$.

of contractions at 1 μM, full concentration–response curves were generated, and inhibitory potencies relative to morphine are given in Table III.

The four analogues containing the piperidine ring (**8a/b** and **9a/b**) were found to be devoid of inhibitory activity at 10^{-6} M, as were the two analogues containing the 1,3-diamino-2-propanol bridge (**7a/b**) and the phenoxyacetyl-Phe derivative (**10a**). All other analogues were active agonists, some being considerably more potent than morphine. A comparison between appropriate analogue pairs (**5a** vs **2a**, **5b** vs **2b**, **6a** vs **3a**, **6b** vs **3b**) shows that the two-carbon diamine spacer results in greater activity than the three-carbon diamine spacer. For the two-carbon diamine spacer, the removal of the Phe *N*-acetyl group diminished the inhibitory activity in the case of L-Phe (**1b** vs **2b**), but was not deleterious in the case of D-Phe (**1a** vs **2a**).

It can be seen that *gem*-dimethyl substitution in the diamine spacer at the carbon nearest to Tyr increased agonist activity for analogues containing both (2)- and (3)-carbon diamine spacers, though this increase was more dramatic (5–10 times) in the case of the D-Phe analogues (**3a** vs **2a** and **6a** vs **5a**) than for the L-Phe analogues (**3b** vs **2b** and **6b** vs **5b**). In contrast, *gem*-dimethyl substitution at the carbon nearest to Phe (for the two-carbon diamine spacer) had little effect in the case of D-Phe (**4a** vs **2a**), and agonist activity in the case of L-Phe was diminished (**4b** vs **2b**). Our finding that the activity of these analogues was independent of the presence of aminopeptidase inhibitors indicates that the increased activity of the *gem*-dimethyl analogues is not likely due to a stabilization against aminopeptidases.

For the six most potent analogues, naloxone K_e values (Table III) were calculated from the observed IC_{50} shifts in the presence of 10 nM naloxone. K_e values less than 5 nM were observed for all six compounds, but the values for the L,D compounds **3a** and **6a** (1.5–2.0 nM) were lower by a statistically significant margin than those for the L,L compounds **2b**, **3b**, **5b**, and **6b** (3.3–4.4 nM).

All compounds which exhibited little or no agonist activity were also screened for their ability to antagonize morphine in the GPI. Only compound **8a** did so, and this was weak (10^{-6} M **8a** reduced the inhibitor effect of 10^{-7} M morphine by 13.5%).

Discussion

With the recognition that Phe[3] opioid peptides exhibit increased μ-activity and selectivity relative to the "traditional" Phe[4] enkephalin analogues,[6-11,28,29] the purpose of the present study was to test whether minimum-structure μ-ligands could be constructed by joining together Tyr and Phe with nonpeptidergic bridging moieties in a manner which conferred appropriate conformational control over the crucial tyramine and ancillary phenyl recognition elements.

Inactive Analogues. Molecular models indicate that the inactive piperidine-containing compounds **8a/b** and **9a/b** correspond to 2–2.5 atoms and 3–3.5 atoms, respectively, separating the two nitrogens of the diamine spacer. Since our analogues containing *acyclic* diamine spacers with this separation distance do exhibit opioid agonism, "linear" distance factors alone do not appear to be the cause of the inactivity of **8a/b** and **9a/b**. It is certainly possible that the tertiary amide nature of the Phe attachment and/or steric hindrance to binding by the additional ethylene bridge present in the piperidine analogues could be responsible for the lack of activity relative to the simple acyclic diamine spacer analogues. Alternatively, the lack of activity could be the result of an adverse conformational constraint imposed on the crucial tyramine and Phe aryl ring recognition elements.

The latter interpretation is supported by the distinctive ^{1}H NMR features of the piperidine-containing analogues discussed earlier, namely the low-field chemical shift of the Phe αCH (Table I) and the appearance of two Phe *N*-acetyl methyl singlets. We interpret the NMR data for compounds **8a/b** and **9a/b** to imply an extended conformation enforced by the pseudo-1,4-diequatorial stereochemical preference about the piperidine ring. Apparently, such extended conformation is deleterious to activity.

Demorphin contains a Tyr[5] residue, and studies on analogues of the type Tyr-D-Ala-Phe-Gly-NHR indicate that the presence of a large hydrophobic group R enhances μ-activity.[28-31] Compound **10a**, which is identical with **2a** except that the Phe-*N*-acetyl group is replaced by an *N*-phenoxyacetyl group, was prepared in an effort to access this ancillary hydrophobic binding domain. The observed inactivity of the compound probably reflects an incorrect stereo–spatial palcement of the added phenyl ring, resulting in steric inhibition of binding to the receptor. For compounds **7a/b**, which contain a hydroxyl group in the propanediamine spacer, the observed inactivity suggests that the receptor is unable to tolerate a polar H-bonding group at this position.

Active Analogues. As shown in Figure 1, our choice of two- and three-carbon diamine spacers resulted in a Tyr–Phe "linear" distance in between that found in the Phe[3] and Phe[4] peptides. The finding of greater activity for the shorter spacer suggests that the optimal Tyr–Phe "linear" distance corresponds more closely to the Phe[3] peptides, though this consideration is clearly dependent on conformational factors.

The fact that both D-Phe and L-Phe versions of our r-Phe analogues exhibit activity in the GPI contrasts the strict requirement for L-Phe[4] in enkephalins[3,4] but is consistent with the recognition of both Phe stereochemistries for certain Phe[3] peptides.[24,25] One possible explanation is that different binding domains on the receptor are involved for recognition of the aryl ring of Phe[3] peptides as opposed to Phe[4] peptides. This hypothesis was previously advanced by Schiller to explain the finding that *p*-NO_2 substitution on Phe affects activity of the Phe[3] peptides and Phe[4] peptides in different ways.[34] However, it is also possible that a single aryl ring binding domain

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is involved, if one assumes that the Phe^3 and Phe^4 analogues adopt distinct backbone conformations associated with different stereochemical requirements for the Phe aryl ring. Resolution of this question is difficult in view of the possibility that our analogues interact with multiple opioid receptor subtypes.

In the latter regard, we measured naloxone K_e values for the most active compounds (Table III), since the finding of significantly different K_e values is indicative of differential agonist interaction with receptor subtypes.[35,36] There is general consensus that the naloxone K_e value is 1–3 nM for μ-selective compounds, 10–20 nM for δ-selective compounds, and 15–40 nM for κ-selective compounds.[37-41] Our finding of K_e values less than 5 nM for all six analogues indicates that they are acting primarily at μ-receptors in GPI. However, the finding of significantly lower K_e values for the L,D analogues **3a** and **6a** than for the L,L analogues **2b**, **3b**, **5b**, and **6b** is suggestive of a more pure μ-agonist activity for the former and the mixing in of some non-μ-agonism for the latter. The GPI contains predominantly μ- and κ-receptors,[1,2] but δ-receptors have also been detected.[37] Since short enkephalin analogues have not been found to elicit significant κ-activity, the apparent non-μ-activity of our L,L analogues is unlikely to involve interaction with κ-receptors, and thus may be associated with δ-receptors. However, direct evidence for such δ-activity could only be provided by additional studies on a δ-rich tissue such as mouse vas deferens.

The finding that our L,D analogues, which possess the same relative Phe stereochemistry as that found in naturally occurring opioid peptides, are *more μ-selective but less potent* than our L,L analogues is consistent with the report that L-Phe^3-casomorphin is ~5 times more μ-selective but ~7 times less potent than D-Phe^3-casomorphin in the GPI.[24] The structural basis for this dissociation between potency and selectivity is not clear, but the fact that this property is shared by casomorphin and our hybrid peptide analogues is suggestive of a common stereo–structural relationship between the crucial Tyr and Phe residues.

Proposed Active Conformation. A multitude of studies on enkephalin analogues involving energy-minimization calculations, computer modeling, solution NMR investigations, and other spectroscopic measurements have been carried out in an effort to elucidate conformational factors associated with subtype-selective recognition at opioid receptors. The consensus at this time is that folded conformations are required for both μ- and δ-recognition[42-53] but that there is a difference in the overall compactness and relative disposition of the crucial tyramine and Phe aryl ring moieties. The most convincing evidence for a folded pharmacophore is that a large number of cyclic enkephalin analogues, which preclude an extended conformation of the peptide chain, have been found to exhibit enhanced activity and subtype-selectivity profiles.[15-21]

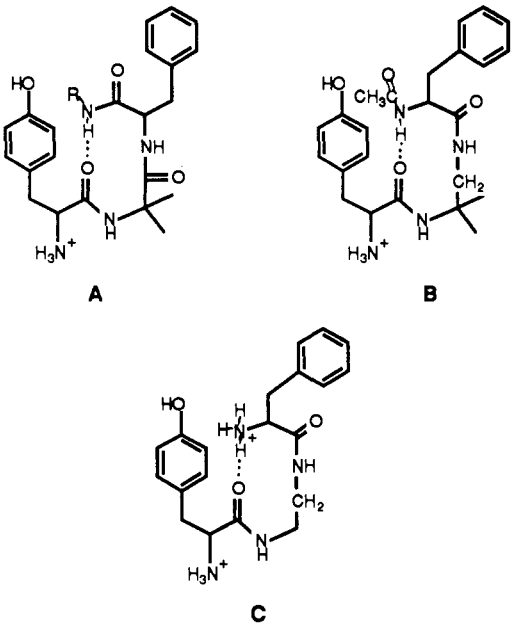

Figure 2. Schematic of proposed intramolecular H bonding in folded conformations of (A) Phe^3 opioid peptide with Aib at position 2, (B) two-carbon diamine spacer analogues (**3a**/**b** shown here), and (C) analogues **1a**/**b** lacking the Phe *N*-acetyl group.

For the acyclic opioid peptides, linking of the crucial Tyr and Phe recognition elements by the Gly^2-Gly^3 fragment in the enkephalins and by D-Ala^2 or Pro^2 in the dermorphins and casomorphins, respectively, encourages folding through induction of a β-turn, involving an intramolecular hydrogen-bonded 10-membered ring.[54] Evidence that the D-Ala^2 residue in dermorphin directs an active agonist conformation is provided by a NMR study[46] on the native peptide and its inactive[14] L-Ala^2 analogue, in which the Ala methyl signal in the D-Ala^2 case appeared δ 0.5 upfield of that in the L-Ala^2 case. The upfield shift was interpreted in terms of a sandwiching of this methyl between the Tyr and Phe aromatic rings in the proposed conformation.

Multiple β-turns (type III) in peptides are cooperatively stabilized through formation of 3_{10} helices.[54-57] None-

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theless, Balaram has provided spectroscopic evidence that a single type III β-turn structure is induced for simple *N*-acyl-tripeptides containing two or more β-turn-inducing residues (Aib or Pro).[58,59] Thus, the Phe[3] dermorphin and casomorphin tetrapeptides or tripeptide amides are likely to exist, at least in part, in a β-turn conformation involving H-bonding of the Tyr carbonyl oxygen through a 10-membered ring to the N–H following Phe[3] (Figure 2A).

Although our analogues are not tripeptides per se, they should be capable of adopting a similar family of secondary structures according to the retro–inverso concept.[22] For our ethylenediamine-bridged analogues, a 10-membered H-bonded ring can be formed between the Tyr carbonyl and the inverted Phe N–H (Figure 2B). This folded conformation would be encouraged by the *gem*-dimethyl substitution present in **3a/b** in analogy to the induction of β-turns by the Aib residue in peptides.[60,61] Our finding of increased activity for **3a** compared to **2a** and to some extent for **3b** compared to **2b** (Tables II and III) suggests that such folded structure is associated with increased activity. For the 1,3-propanediamine-based compounds **5a/b** and **6a/b**, the analogous intramolecular H-bond-stabilized conformation would require an 11-membered ring, the stability of which should not be significantly less than that for the 10-membered ring. Thus we propose a folded biologically active conformation for **5a/b** and **6a/b** as well, with increased potency of the latter arising from the β-turn-stabilizing effect of the *gem*-dimethyl group. For both two- and three-carbon diamine spacers, the potency-enhancing effect of *gem*-dimethyl substitution next to Tyr is more pronounced for the "more μ-selective" L,D analogues than for the "more potent" L,L analogues, suggesting that the postulated folded conformation is preferentially recognized at μ-receptors. The fact that *gem*-dimethyl substitution next to Phe (compounds **4a/b**) should also promote folding but does not enhance activity may indicate that folding in this case involves a β-turn of "opposite" directionality, which is deleterious to opioid recognition.

We were initially surprised to find that the analogs lacking the *N*-acetyl group on Phe (**1a/b**) retained significant activity, since there is no precedent for the ability of opioid receptors to accept a positive charge at the "C terminus". However, the N–H of the free ammonium group in **1a/b** could intramolecularly H bond to the Tyr carbonyl in place of the amide N–H in **2a/b**, forming the same 10-membered ring structure (Figure 2C) as that proposed for the *N*-acetyl-Phe case. Since this interaction would substantially delocalize the positive charge in question, it could explain the lack of a large deleterious effect on activity of removing the *N*-acetyl group in **2a/b**.

The finding that the Phe αCH resonances for the active analogues appear ~0.5 ppm upfield of those seen for the inactive piperidine-containing analogues may be indicative of folded conformations in which the Phe αCH is experiencing anisotropic shielding by the Tyr aryl ring.[62] In principle, direct evidence for the β-turn structures proposed for our active analogues (Figure 2) could be obtained through NOE measurements and studies on the solvent, concentration, and temperature dependence of the amide NH chemical shifts. However, such measurements are beyond the scope of the present study, and thus, the conformational proposals must be considered speculative at the present time.

Summary

The findings reported here suggest that a very simple molecule containing only two amino acids, L-Tyr and Phe, connected by an appropriate spacer, is sufficient for eliciting potent opioid activity, though the relative configuration and conformational aspects of the two aromatic rings appear to be important determinants of activity and μ-selectivity. Our results suggest that the optimal Tyr–Phe distance for eliciting activity in GPI corresponds most closely to Phe[3] peptides, in which case both L- and D-Phe can be accommodated, and that structural features which encourage a folded conformation can result in a substantial activity enhancement. Analogues **3a/b** described in this study are some of the structurally most simple yet potent opioid-like compounds reported to date.

Experimental Section

General Procedures. All melting points were determined using a Mel-Temp capillary block apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter (Na D line, 589 nm) using Gold-Label MeOH as solvent. ^{1}H NMR spectra were recorded on a Varian XL-200 (200 MHz, FT mode) instrument, and chemical shifts of the HCl salts in D_2O are reported relative to internal CH_2Cl_2 at δ 5.45 (referenced independently to DSS). Abbreviations used are s, singlet; d, doublet; t, triplet; q, quartet; p, pentuplet; m, multiplet; br, broad; app, apparent. A prefix of d indicates "doublet of", e.g., dd = doublet of doublets. Elemental analyses were determined by Galbraith Labs, Inc., Knoxville, TN, and were within 0.4% of the calculated values. Thin-layer chromatography was run on Kieselgel 60 F_{254} glass plates (0.25-mm thickness) with UV, I_2, and ninhydrin (when applicable) for visualization. The TLC eluant system EMA refers to EtOAc–MeOH 1:1, 1% NH_4OH.

HPLC was performed on a Shimadzu binary gradient system, composed of dual LC-6A pumps, SCL-6A controller, SPD-A variable-wavelength UV detector, and C-R3A integrator. Waters Associates μ-Bondapak C-18 columns were used for both analytical (3.9 mm × 150 mm) and semipreparative (19 mm × 150 mm) work. The analytical conditions for which relative retention time (k' values) are reported were as follows: flow rate = 0.1 mL/min; H_2O, 0.01% TFA, linear gradient, 0–35% CH_3CN (15 min), λ = 210 and 254 nm. Only HPLC grade (Mallinckrodt or Fisher Scientific) solvents were used. For aqueous systems, the H_2O was doubly distilled with a NY/COR still. All solvents were filtered (Gelman Sciences filters, 0.2-μm pore size) and degassed with He prior to use.

All inorganic chemicals were of ACS or reagent-grade quality and used without further purification. Diamines were purchased from Aldrich Chemical Co., and amino acids and derivatives were from Sigma Chemical Co. 1,3-Diamino-3-methylbutane was synthesized in our group (P. K. Arora and L. M. Sayre, unpublished). All amines were used after at least 24 h storage over NaOH pellets. Certain protected amino acids were independently synthesized[63] and were compared with authentic materials prior to usage. Solvents were AR grade were used without purification with the exception of THF, which was distilled under N_2 in the presence of Na/benzophenone. The drugs used (sources) were as follows: naloxone (Du Pont), morphine (Mallinckrodt), ethylketocycloazocine (Sterling-Winthrop), bestatin (Sigma), thiorphan (Peninsula), captopril (Squibb), and L-leucylleucine (Sigma).

No attempts were made to maximize the yields of the synthetic reactions. All evaporations were carried out in vacuo with a rotary evaporator.

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4-(L-Tyrosylamino)-1-(*N*-acetyl-D(or L)-phenylalanyl)-piperidine Hydrochloride (8a/b). To a cooled solution of CH_2Cl_2 (40 mL) was added 2.5 g (8.6 mmol) of BOC-Tyr-OH, 1.63 g (8.6 mmol) of 1-benzyl-4-aminopiperidine, and 1.31 g (8.6 mmol) of HOBt. After stirring for 5 min, 1.95 g (9.4 mmol) of DCC was added and the reaction was allowed to warm to room temperature overnight with mixing. After filtration, the mother liquor was washed with two 20-mL portions of half-saturated Na_2CO_3 and evaporated to dryness in vacuo. Yields of crude BOC-Tyr-NHCH(CH_2CH_2)$_2$NCH_2Ph were quantitative and TLC revealed a single spot (EMA, R_f = 0.67).

Debenzylation of 4.0 g (9.5 mmol) of the above material was accomplished by hydrogenation at 1 atm for 8 h in refluxing EtOH (25 mL) containing 2.5 mL of HOAc, using 0.4 g of 10% Pd/C as catalyst. Cooling, filtration of the catalyst, and evaporation yielded 3.05 g (87%) of 4-(BOC-tyrosylamino)piperidine, isolated as the HCl salt. TLC showed a single spot (EMA, R_f = 0.27) and this compound was used without further purification.

Coupling to Ac-D-Phe-OH (or Ac-L-Phe-OH) was accomplished under standard DCC/HOBt conditions in $CHCl_3$ with an equimolar amount of Et_3N. After removal of the DCU by filtration and extraction (0.6 N HCl, half-saturated Na_2CO_3, and distilled H_2O in succession), the protected compounds, 4-(BOC-tyrosylamino)-1-(*N*-acetyl-D(or L)-phenylalanyl)piperidine were purified by silica gel flash chromatography ($CHCl_3$–MeOH 9:1). Both diastereomers exhibited R_f = 0.52 with this eluant. The yields, after purification, were 1.1 g (49%) of the L,D isomer and 1.3 g (56%) of the L,L isomer.

Removal of the BOC group was accomplished by dissolving the protected peptide in a 4:1 mixture of dioxane and 4 N HCl, and allowing the resulting solution to stand overnight at room temperature. After removal of solvent in vacuo, the HCl salts were crystallized from anhydrous *i*-PrOH containing a small amount of MeOH.

8a: yield 403 mg (39%); mp 204–207 °C dec; $[\alpha]^{25}_D$ = 15.4° (*c* = 1.0, MeOH); HPLC (*k'* 3.37); ^{1}H NMR (D_2O) δ 0.33–1.73 (4 H, m, C-3 and C-5 CH_2 of ring), 1.96 (3 H, br s, Ac), 2.72 (1 H, m, one C-2/6 H_{ax} of ring), 2.93–3.01 (4 H, m, both $ArCH_2$), 3.12 (1 H, dt, *J* = 5.8, 13.2 Hz, one C-2/6 H_{ax} of ring), 3.51–3.71 (2 H, m, one C-2/6 H_{eq} and C-4 H of ring), 3.97–4.07 (2 H, m, Tyr C_α-H and one C-2/6 H_{eq} of ring), 4.96 (1 H, m, Phe C_α-H), 7.10 and 7.15 (2 H each, m, Tyr Ar), 7.28 (5 H, m, Phe Ar). Anal. ($C_{25}H_{32}N_4O_4$·HCl·H_2O·*i*-PrOH) C, H, N.

8b: yield 465 mg (38%); mp 209–213 °C dec; $[\alpha]^{25}_D$ = 35.8° (*c* = 1.0, MeOH); HPLC (*k'* = 3.28); ^{1}H NMR (D_2O) δ 0.93–1.73 (4 H, m, C-3 and C-5 CH_2 of ring), 1.96 (3 H, app d, Ac), 2.73 (1 H, m, one C-2/6 H_{ax} of ring), 2.93–3.01 (4 H, m, both $ArCH_2$), 3.12 (1 H, dt, *J* = 6.0, 13.4 Hz, one C-2/6 H_{ax} of ring), 3.52–3.72 (2 H, m, one C-2/6 H_{eq} and C-4 H of ring), 3.92–4.12 (2 H, m, Tyr C_α-H and one C-2/6 H_{eq} of ring), 4.98 (1 H, t, *J* = 7.8 Hz, Phe C_α-H), 6.88 and 7.17 (2 H each, d, *J* = 8.3 Hz, Tyr Ar), 7.29 (5 H, m, Phe Ar). Anal. ($C_{25}H_{32}N_4O_4$·HCl·2CH_3OH) C, H, N.

4-[(L-Tyrosylamino)methyl]-1-(*N*-acetyl-D(or L)-phenylalanyl)piperidine Hydrochloride (9a/b). To 8.0 g (70 mmol) of 4-(aminomethyl)piperidine in 70 mL of THF was added 7.8 mL of PhCHO and the reaction was stirred at room temperature under N_2 overnight. After removal of the solvent in vacuo, 40 mL of $CHCl_3$ was added, and the organic layer was extracted with two 30-mL portions of half-saturated aqueous Na_2CO_3 to remove the residual PhCOOH (from oxidation of PhCHO). The organic layer was dried and (Na_2CO_3) taken to dryness in vacuo, and the crude imine (10.0 g, 71%) was used without purification.

Coupling with Ac-D-Phe-OH (or Ac-L-Phe-OH) was as reported for 8, with workup consisting of filtering off DCU and extracting two times each with half-saturated aqueous Na_2CO_3 and then distilled H_2O. The organic layer was concentrated in vacuo, and the residue was resuspended in 0.6 N HCl–dioxane (1:1) and allowed to stand overnight at room temperature. The solvent was removed, and trituration of the thick residue with Et_2O afforded 2.5 g (72% for both steps) of 1-(*N*-acetyl-D-phenylalanyl)-4-(aminomethyl)piperidine and 2.3 g (67% for both steps) of the L-Phe isomer.

Coupling with BOC-Tyr-OH, using an equimolar amount of Et_3N in the DCC/HOBt reaction and deprotection of the BOC group were carried out as for 8, and the final compounds were purified as HCl salts crystallized from anhydrous *i*-PrOH containing a small amount of MeOH.

9a: yield 375 mg (48%); mp 195–201 °C dec; $[\alpha]^{25}_D$ = 12.2° (*c* = 1.1, MeOH); HPLC (*k'* 3.45); ^{1}H NMR (D_2O) δ 0.43–1.33 (5 H, m, both C-3/5 CH_2 and C-4 H of ring), 1.96 (3 H, app d, Ac), 2.37 (2 H, m, NHCH_2), 2.73 (1 H, m, one C-2 H_{ax} of ring), 2.86–2.96 (4 H, m, both $ArCH_2$), 3.14 (1 H, m, one C-2/6 H_{ax} of ring), 3.66 (1 H, br d, one C-2/6 H_{eq} of ring), 4.04 (1 H, m, Tyr C_α-H), 4.18 (1 H, br d, one C-2/6 H_{eq} of ring), 4.99 (1 H, m, Phe C_α-H), 6.86 and 7.13 (2 H each, app 2d and d, *J* = 8.6 Hz, Tyr Ar), 7.37 (5 H, m, Phe Ar). Anal. ($C_{26}H_{34}N_4O_4$·HCl·2CH_3OH) C, H, N.

9b: yield 250 mg (32%): mp 208–211 °C dec; $[\alpha]^{25}_D$ = 40.7° (*c* = 0.8, MeOH); HPLC (*k'* 3.46); ^{1}H NMR (D_2O) δ 0.43–1.33 (5 H, m, both C-3/5 CH_2 and C-4 H of ring), 1.96 (3 H, app d, Ac), 2.39 (2 H, m, NHCH_2), 2.69 (1 H, m, one C-2 H_{ax} of ring), 2.91–2.99 (4 H, m, both $ArCH_2$), 3.17 (1 H, m, one C-2/6 H_{ax} of ring), 3.65 (1 H, m, one C-2/6 H_{eq} of ring), 4.05 (1 H, dd, *J* = 5.6, 10.2 Hz, Tyr C_α-H), 4.18 (1 H, m, one C-2/6 H_{eq} of ring), 4.99 (1 H, m, Phe C_α-H), 6.86 and 7.13 (2 H each, d, *J* = 8.0 Hz, Tyr Ar), 7.34 (5 H, m, Phe Ar). Anal. ($C_{26}H_{34}N_4O_4$·HCl·H_2O·*i*-PrOH) C, H, N.

1-(L-Tyrosylamino)-2-[(D(or L)-phenylalanyl)amino]ethane Dihydrochloride (1a/b) and 1-(L-Tyrosylamino)-2-[(*N*-acetyl-D(or L)-phenylalanyl)amino]ethane Hydrochloride (2a/b). Ethylenediamine (25 mL, used as solvent) was cooled in an ice bath and 2.6 g (6.0 mmol) of Z-Tyr-OpNP was added as a solid in small portions over a 5-min period. The mixture was allowed to warm to room temperature, and the excess EDA was removed by repeated concentrations in vacuo after dilution with $PhCH_3$. The gummy residue was diluted with 0.6 N HCl (50 mL) and extracted twice with $CHCl_3$. The aqueous layer was evaporated to dryness, and the HCl salt was crystallized from CH_3CN–MeOH 10:1, yielding 2.0 g (85%) of Z-Tyr-EDA·HCl. Coupling of the latter to equimolar quantities of either *N*-Ac-D(or L)-Phe-OH (**1a/b**) or Z-D(or L)-Phe-OH (**2a/b**) was carried out as for 8. Removal of the Z protecting groups was accomplished by room-temperature hydrogenation at 1 atm using 10% Pd/C in H_2O containing a trace of HOAc.

1a: the dihydrochloride salt was crystallized from anhydrous *i*-PrOH–MeOH, giving 180 mg (29% overall yield); mp 188 °C dec; $[\alpha]^{25}_D$ = 0.95° (*c* = 1.9, MeOH); HPLC (*k'* 2.40); ^{1}H NMR (D_2O) δ 3.10 (8 H, m), 4.11 (1 H, t, *J* = 8.0 Hz, C_α-H), 4.15 (1 H, t, *J* = 7.6 Hz, C_α-H), 6.89 and 7.15 (2 H each, d, *J* = 8.0 Hz, Tyr Ar), 7.34 (5 H, m, Phe Ar). Anal. ($C_{20}H_{26}N_4O_3$·2HCl·CH_3OH·0.5H_2O) C, H, N.

1b: the dihydrochloride salt was crystallized from anhydrous *i*-PrOH–MeOH, giving 250 mg (41% overall yield); mp 174 °C dec; $[\alpha]^{25}_D$ = 78.0° (*c* = 0.7, MeOH); HPLC (*k'* 2.38); ^{1}H NMR (D_2O) δ 3.13 (8 H, m), 4.07 (1 H, t, *J* = 8.1 Hz, C_α-H), 4.13 (1 H, t, *J* = 7.4 Hz, C_α-H), 6.91 and 7.18 (2 H each, d, *J* = 8.3, Tyr Ar), 7.38 (5 H, m, Phe Ar). Anal. ($C_{20}H_{26}N_4O_3$·2HCl·CH_3OH) C, H, N.

2a: the HCl salt was crystallized from anhydrous *i*-PrOH, giving 190 mg (30% overall yield); mp 130–136 °C, $[\alpha]^{25}_D$ = 8.4° (*c* = 1.2, MeOH); HPLC (*k'* 3.15); ^{1}H NMR (D_2O) δ 1.92 (3 H, s, Ac), 3.08 (8 H, m), 4.07 (1 H, t, *J* = 7.6 Hz, Tyr C_α-H), 4.44 (1 H, t, *J* = 7.3 Hz, Phe C_α-H), 6.89 and 7.15 (2 H each, d, *J* = 8.1 Hz, Tyr Ar), 7.27 (5 H, m, Phe Ar). Anal. ($C_{22}H_{28}N_4O_4$·HCl·0.5H_2O·0.5*i*-PrOH) C, H, N.

2b: purification by silica gel flash chromatography ($CHCl_3$–MeOH 6:1, 0.5% NH_4OH, R_f = 0.42), conversion to the HCl salt, and recrystallization from *i*-PrOH gave 150 mg (20% overall yield); mp 108–110 °C; $[\alpha]^{25}_D$ = 43.7° (*c* = 0.9, MeOH); ^{1}H NMR (D_2O) δ 1.92 (3 H, s, Ac), 3.05 (8 H, m), 4.06 (1 H, t, *J* = 7.6 Hz, Tyr C_α-H), 4.44 (1 H, t, *J* = 7.2 Hz, Phe C_α-H), 6.89 and 7.13 (2 H each, d, *J* = 8.0 Hz, Tyr Ar), 7.32 (5 H, m, Phe Ar). Anal. ($C_{22}H_{28}N_4O_4$·HCl·*i*-PrOH·1.5H_2O) C, H, N.

2-(L-Tyrosylamino)-1-[(*N*-acetyl-D(or L)-phenylalanyl)amino]-2-methylpropane Hydrochloride (3a/b). The monoacylation of 1,2-diamino-2-methylpropane was accomplished by a modification of a known procedure[64] using 1.0 equiv of acid in the reaction mixture. To 15 mL of DMF was added 1.0 g (4.8 mmol) of Ac-D(or L)-Phe-OH, 426 mg (4.8 mmol) of the diamine, 920 mg (4.8 mmol) of TsOH·H_2O, and 652 mg (4.8 mmol) of HOBt.

(64) Large, M. S.; Smith, L. H. *J. Med. Chem.* **1980**, *23*, 112.

After allowing the mixture to mix for 5 min, 1.1 g (5.3 mmol, 1.1 equiv) of DCC was added and the reaction was mixed overnight at room temperature. The solvent was removed in vacuo and 50 mL of $CHCl_3$ was added. The mixture was then filtered and extracted with two 20-mL portions of half saturated aqueous Na_2CO_3. The organic layer was concentrated in vacuo giving 600 mg (46%) of Ac-D(or L)-Phe-$NHCH_2C(CH_3)_2NH_2$ as a white solid, which ran as a single spot on TLC (EMA, R_f = 0.45).

Coupling with BOC-Tyr-OH and the subsequent removal of the BOC protecting group was as for **8**. Both product isomers were purified as the free base with silica gel flash chromatography ($CHCl_3$–MeOH 3:1, 1% NH_4OH, R_f = 0.28 for both isomers) and then converted to the HCl salt (with EtOH azeotropic drying and recrystallization).

3a: yield 200 mg (20%); mp 163–165 °C; $[\alpha]^{25}_D$ = 23.8° (c = 1.2, MeOH); HPLC (k' 3.60); ^{1}H NMR (D_2O) δ 0.99 and 1.11 (3 H each, s, *gem*-dimethyl), 1.93 (3 H, s, Ac), 2.9–3.2 (5 H, m), 3.32 (1 H, app t, J_{app} = 13.5 Hz, one of CH_2CMe_2), 3.99 (1 H, t, J = 8.0 Hz, Tyr C_α-H), 4.49 (1 H, t, J = 7.7 Hz, Phe C_α-H), 6.89 and 7.15 (2 H each, app 2d and d, J = 8.6 Hz, Tyr Ar), 7.33 (5 H, m, Phe Ar). Anal. ($C_{24}H_{32}N_4O_4$·HCl·CH_3CH_2OH·2.5H_2O) C, H, N.

3b: yield 130 mg (13%); mp 172–180 °C; $[\alpha]^{25}_D$ = 36.7° (c = 2.4, MeOH); HPLC (k' 3.50); ^{1}H NMR (D_2O) δ 1.02 and 1.08 (3 H each s, *gem*-dimethyl), 1.91 (3 H, s, Ac), 2.9–3.1 (5 H, m), 3.25 (1 H, br s, one of CH_2CMe_2), 3.95 (1 H, t, J = 8.0 Hz, Tyr C_α-H), 4.47 (1 H, t, J = 7.8 Hz, Phe C_α-H), 6.98 and 7.13 (2 H each, d, J = 7.6 Hz, Tyr Ar), 7.28 (5 H, m, Phe Ar). Anal. ($C_{24}H_{32}N_4O_4$·HCl·CH_3CH_2OH) C, H, N.

1-(L-Tyrosylamino)-2-[(*N*-acetyl-D(or-L)-phenylalanyl)amino]-2-methylpropane Hydrochloride (4a/b). These were prepared as for **3a/b**, starting with the coupling of BOC-Tyr-OH to 1,2-diamino-2-methylpropane.

4a: purification by silica gel flash chromatography ($CHCl_3$–MeOH 9:1, 0.5% NH_4OH, R_f = 0.27) and conversion to the HCl salt (with EtOH azeotropic drying and recrystallization) gave 175 mg (17.5% overall yield): mp 128–133 °C; $[\alpha]^{25}_D$ = −15.8° (c = 3.4, MeOH); HPLC (k' 3.77); ^{1}H NMR (D_2O) δ 0.90 and 1.01 (3 H each, s, *gem*-dimethyl), 1.98 (3 H, s, Ac), 2.95–3.13 (5 H, m), 3.27 (1 H, br s, one of CH_2CMe_2), 4.18 (1 H, t, J = 7.6 Hz, Tyr C_α-H), 4.34 (1 H, t, J = 8.0 Hz, Phe C_α-H), 6.87 and 7.14 (2 H each, d, J = 8.4 Hz, Tyr Ar), 7.34 (5 H, m, Phe Ar). Anal. ($C_{24}H_{32}N_4O_4$·HCl·CH_3CH_2OH·0.5H_2O) C, H, N.

4b: the HCl salt was crystallized from anhydrous *i*-PrOH–MeOH giving 180 mg (18% overall yield): mp 160–164 °C; $[\alpha]^{25}_D$ 63.8° (c = 1.0, MeOH); ^{1}H NMR (D_2O) δ 0.96 and 1.01 (3 H each, s, *gem*-dimethyl), 1.96 (3 H, s, Ac), 2.97–3.23 (5 H, m), 3.43 (1 H, d, J = 13.6 Hz, one of CH_2CMe_2), 4.14 (1 H, dd, J = 6.8, 8.0 Hz, Tyr C_α-H), 4.39 (1 H, t, J = 7.8 Hz, Phe C_α-H), 6.87 and 7.14 (2 H each, d, J = 8.5 Hz, Tyr Ar), 7.34 (5 H, m, Phe Ar). Anal. ($C_{24}H_{32}N_4O_4$·HCl·*i*-PrOH·$^1/_3H_2O$) C, H, N.

1-(L-Tyrosylamino)-3-(*N*-acetyl-D(or L)-phenylalanyl)amino]propane Hydrochloride (5a/b). Synthesis of these compounds was as for **3a/b** but used solid Z-Tyr-OpNP and 1,3-diaminopropane as solvent. Coupling with Ac-Phe-OH was as for **8a/b**, and removal of the Z protecting group was as reported for **1a/b** and **2a/b**.

5a: purification by silica gel flash chromatography ($CHCl_3$–MeOH 3:1, 1% NH_4OH, R_f = 0.66) and conversion to the HCl salt (with EtOH azeotropic drying and recrystallization) gave 450 mg (42% overall yield): mp 125–131 °C; $[\alpha]^{25}_D$ = 12.8° (c = 1.4, MeOH); HPLC (k' 3.06); ^{1}H NMR (D_2O) δ 1.31 (2 H, p, J = 7.8 Hz, $CH_2CH_2CH_2$), 1.97 (3 H, s, Ac), 3.04 (8 H, m), 3.91 (1 H, dd, J = 6.2, 8.1 Hz, Tyr C_α-H), 4.43 (1 H, t, J = 7.9 Hz, Phe C_α-H), 6.88 and 7.11 (2 H each, d, J = 8.5 Hz, Tyr Ar), 7.28 (5 H, m, Phe Ar). Anal. ($C_{23}H_{30}N_4O_4$·HCl·CH_3CH_2OH) C, H, N.

5b: purification was by recrystallization of the HCl salt from MeOH–CH_3CN 1:10, giving 350 mg (33% overall yield): mp 180–185 °C; $[\alpha]^{25}_D$ = 33.2° (c = 0.6, MeOH); HPLC (k' 3.04); ^{1}H NMR (D_2O) δ 1.29 (2 H, p, J = 7.8 Hz, $CH_2CH_2CH_2$), 1.97 (3 H, s, Ac), 2.97 (8 H, m), 4.06 (1 H, dd, J = 6.5, 8.0 Hz, Tyr C_α-H), 4.43 (1 H, t, J = 8.2 Hz, Phe C_α-H), 6.86 and 7.13 (2 H each, d, J = 8.45 Hz, Tyr Ar), 7.32 (5 H, m, Phe Ar). Anal. ($C_{23}H_{30}N_4O_4$·HCl·0.5CH_3OH) C, H, N.

3-(L-Tyrosylamino)-1-[(*N*-acetyl-D(or L)-phenylalanyl)amino]-3-methylbutane Hydrochloride (6a/b). The synthesis of these compounds was carried out as described for **3a/b** starting with monoacylation of 1,3-diamino-3-methylbutane using Ac-D-Phe-OH (or Ac-L-Phe-OH), followed by acylation with Z-Tyr-OH. Removal of the Z protecting group was as for **1a/b** and **2a/b**.

6a: purification by silica gel flash chromatography ($CHCl_3$–MeOH 6:1, 0.5% NH_4OH, R_f = 0.10), conversion to the HCl salt, and recrystallization from *i*-PrOH–MeOH gave 195 mg (16% overall yield); mp 160–165 °C; $[\alpha]^{25}_D$ = 19.0° (c = 0.3, MeOH); ^{1}H NMR (D_2O) δ 1.09 and 1.12 (3 H each, s, *gem*-dimethyl), 1.48 (2 H, m, CH_2CMe_2), 1.97 (3 H, s, Ac), 2.73–3.21 (6 H, m), 4.02 (1 H, dd, J = 6.7, 8.8 Hz, Tyr C_α-H), 4.42 (1 H, t, J = 7.7 Hz, Phe C_α-H), 6.90 and 7.14 (2 H each, d, J = 8.0 Hz, Tyr Ar), 7.27 (5 H, m, Phe Ar). Anal. ($C_{25}H_{34}N_4O_4$·HCl·CH_3OH) C, H, N.

6b: purification by silica gel flash chromatography as for **6a** (R_f = 0.28), conversion to the HCl salt, and recrystallization from EtOH gave 180 mg (15% overall yield); mp 154–158 °C; $[\alpha]^{25}_D$ = 36.5° (c = 0.8, MeOH); ^{1}H NMR (D_2O) δ 1.10 and 1.13 (3 H each, s, *gem*-dimethyl), 1.51 (2 H, m, CH_2CMe_2), 1.96 (3 H, s, Ac), 2.73–3.21 (6 H, m), 4.02 (1 H, dd, J = 6.3, 9.0 Hz, Tyr C_α-H), 4.42 (1 H, t, J = 7.9 Hz, Phe C_α-H), 6.89 and 7.18 (2 H each, app 2d and d, J = 8.4 Hz, Tyr Ar), 7.33 (5 H, m, Phe Ar). Anal. ($C_{25}H_{34}N_4O_4$·HCl·CH_3CH_2OH) C, H, N.

1-(L-Tyrosylamino)-3-[(*N*-acetyl-D(or L)-phenylalanyl)amino]-(*R*,*S*)-2-propanol Hydrochloride (7a/b). Monoacylation of 1,3-diamino-2-propanol with Z-Tyr-OpNP was as above for **3a/b**. Purification was accomplished by conversion to the HCl salt and selectively crystallizing the dihydrochloride salt of the unreacted 1,3-diamino-2-propanol from absolute EtOH. After concentrating the mother liquor to dryness, TLC showed a single ninhydrin-positive spot (EMA, R_f = 0.26), and the Z-Tyr-$NHCH_2CHOHCH_2NH_2$ was used without further purification. Coupling with Ac-Phe-OH was as reported for **8**, and the subsequent removal of the Z group was as reported as for **1a/b** and **2a/b**. No attempt was made to separate the epimeric alcohols of either product diastereomer.

7a: purification by silica gel flash chromatography ($CHCl_3$–MeOH 5:1, 0.5% NH_4OH, R_f = 0.20), conversion to the HCl salt, and recrystallization from *i*-PrOH–MeOH gave 207 mg (38% overall yield); mp 190–196 °C; $[\alpha]^{25}_D$ = 15.0° (c = 2.9, MeOH); HPLC (k' 2.93); ^{1}H NMR (D_2O) δ 1.87 (3 H, s, Ac), 3.01 (8 H, m), 3.51 (1 H, m, J = 6.4 Hz, CHOH), 4.13 (1 H, t, J = 7.5 Hz, Tyr C_α-H), 4.49 (1 H, t, J = 8.1 Hz, Phe C_α-H), 6.88 and 7.15 (2 H each, d, J = 8.5 Hz, Tyr Ar), 7.27 (5 H, m, Phe Ar). Anal. ($C_{23}H_{30}N_4O_5$·HCl·1.5H_2O) C, H, N.

7b: purification by silica gel flash chromatography as for **7a** (R_f = 0.18) and conversion to the HCl salt (with EtOH azeotropic drying and recrystallization) gave 184 mg (33% overall yield): mp 160–164 °C; $[\alpha]^{25}_D$ = 30.5° (c = 1.9, MeOH); HPLC (k' 2.92); ^{1}H NMR (D_2O) δ 1.96 (3 H, s, Ac), 3.07 (8 H, m), 3.56 (1 H, br t, J = 5.0 Hz, CHOH), 4.15 (1 H, t, J = 7.2 Hz, Tyr C_α-H), 4.50 (1 H, t, J = 7.2 Hz, Phe C_α), 6.89 and 7.16 (2 H each, d, J = 8.3 Hz, Tyr Ar), 7.33 (5 H, m, Phe Ar). Anal. ($C_{23}H_{30}N_4O_5$·HCl·0.5CH_3CH_2OH) C, H, N.

1-(L-Tyrosylamino)-2-[(*N*-(phenoxyacetyl)-D-phenylalanyl)amino]ethane Hydrochloride (10a). The synthesis of this compound was carried out as described for **1** and **2**. The *N*-(phenoxyacetyl)-D-Phe-OH was prepared under Schotten–Baumann conditions in 77% yield, and exhibited $[\alpha]^{25}_D$ = −20.8° (c = 1.2, MeOH). This was coupled (DCC/HOBt) directly to Z-Tyr-EDA, and removal of the Z group was as reported above for **1** and **2**. The resulting material was converted to the HCl salt and crystallized from anhydrous *i*-PrOH–MeOH, giving 300 mg (41% overall yield); mp 201–206 °C; $[\alpha]^{25}_D$ = 19.4° (c = 3.8, MeOH); HPLC (k' 4.81); ^{1}H NMR (D_2O) δ 3.17 (8 H, m), 4.14 (1 H, t, J = 6.5 Hz, Tyr C_α-H), 4.31 (2 H, br s, CH_2OPh), 4.62 (1 H, br t, J = 7.9 Hz, Phe C_α-H), 6.73 and 6.87 (2 H, d, J = 8.3 Hz, Tyr Ar), 7.13 (10 H, m, Phe and PhO Ar). Anal. ($C_{28}H_{32}N_4O_5$·HCl·1.5H_2O) C, H, N.

Bioassay. Opioid-agonist activity of the analogues was assessed through determination of their ability to depress the magnitude of electrically evoked contractions of the intact GPI in a naloxone-reversible fashion,[65] as previously described.[23] Male albino guinea pigs (Hartley strain, Camm Co.) weighing 400–450 g were sacrificed and the terminal portion of the ileum was surgically

(65) Paton, W. D. M. *Br. J. Pharmacol.* **1957**, *12*, 119.

removed. The lumen was washed thoroughly with Krebs buffer. Approximately 3–4-cm segments were cut from the anal end and secured in a 3.5-mL jacketed organ bath containing Krebs buffer that was maintained at 37 ± 0.5 °C and bubbled continuously with 95% O_2–5% CO_2. With resting tension fixed at 1.0 g, the ileum was electrically stimulated transmurally with platinum electrodes (0.1 Hz, 0.2-ms duration, 2.0 V). Isometric contractions were recorded on a Gould dual-pen polygraph via a Grass FT-03 transducer.

All compounds were weighed out ±0.1 mg on a Sartorius microbalance and dissolved in distilled water. In general, stock solutions were made (and stored at 4 °C) such that 50 μL added to the 3.5-mL organ bath gave a 10^{-6} M final concentration of the drug. Experiments were run in the presence of bestatin (10 μM), captopril (10 μM), thiorphan (0.3 μM), and L-leucylleucine (10 mM) or just bestatin (10 μM), to protect the peptide analogs against proteolytic breakdown.[66] The potency of each agonist is described in terms of its ability to reduce the contraction heights and is compared with the potency of morphine obtained in the same preparation. Responses to synthetic opioids and morphine were determined in alternating fashion in each preparation. In order to compare the potency of each synthetic compound with that of morphine, inhibitory responses to each were determined at least twice in each preparation.

For the seven most potent agonists, concentration–response curves were determined, and a minimum of three experiments were performed, of which at least one represented responses to drug freshly weighed and diluted. For these compounds, it was also established that naloxone completely and reversibly blocked the observed agonist activity. Thus, the entire reduction of contraction height is mediated via an opioid-receptor mechanism.

For the K_e determinations,[67] agonist concentration–response curves were determined in two tissue preparations run in parallel, one serving as a control while the other was used to obtain the antagonist shift. The K_e was calculated as $C/(DR - 1)$, where DR (drug ratio) is the ratio of IC_{50} values in the presence and absence of antagonist at concentration C. The naloxone concentration C used in our case (10 nM) produced a parallel shift in the log (concentration) – response curves. The data were analyzed with a two-tailed Student's t test.

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Registry No. **1a** (free base), 120687-62-9; **1a**·2HCl, 120687-28-7; **1b** (free base), 120687-63-0; **1b**·2HCl, 120687-29-8; **2a** (free base), 120687-64-1; **2a**·HCl, 120687-30-1; **2b** (free base), 120687-65-2; **2b**·HCl, 120687-31-2; **3a** (free base), 120687-66-3; **3a**·HCl, 120687-32-3; **3b** (free base), 120687-67-4; **3b**·HCl, 120687-33-4; **4a** (free base), 120687-68-5; **4a**·HCl, 120687-34-5; **4b** (free base), 120687-69-6; **4b**·HCl, 120687-35-6; **5a** (free base), 120687-70-9; **5a**·HCl, 120687-36-7; **5b** (free base), 120687-71-0; **5b**·HC, 120687-37-8; **6a** (free base), 120687-72-1; **6a**·HCl, 120711-04-8; **6b** (free base), 120687-73-2; **6b**·HCl, 120687-38-9; (*R*)-**7a** (free base), 120786-52-9; (*R*)-**7a**·HCl, 120687-39-0; (*S*)-**7a** (free base), 120786-53-0; (*S*)-**7a**·HCl, 120849-25-4; (*R*)-**7b** (free base), 120786-54-1; (*R*)-**7b**·HCl, 120849-26-5; (*S*)-**7b** (free base), 120786-55-2; (*S*)-**7b**·HCl, 120849-27-6; **8a** (free base), 120687-74-3; **8a**·HCl, 120687-40-3; **8b** (free base), 120687-75-4; **8b**·HCl, 120687-41-4; **9a** (free base), 120687-76-5; **9a**·HCl, 120687-42-5; **9b** (free base), 120711-26-4; **9b**·HCl, 120687-43-6; **10a** (free base), 120687-77-6; **10a**·HCl, 120687-44-7; BOC-Tyr-OH, 3978-80-1; BOC-Tyr-NHCH$(CH_2CH_2)_2$NCH$_2$Ph, 120687-45-8; BOC-Tyr-NHCH$(CH_2CH_2)_2$NH·HCl, 120687-46-9; Ac-D-Phe-OH, 10172-89-1; Ac-Phe-OH, 2018-61-3; BOC-Tyr-NHCH$(CH_2CH_2)_2$N-r-D-Phe-Ac, 120687-47-0; BOC-Tyr-NHCH$(CH_2CH_2)_2$N-r-Phe-Ac, 120687-48-1; PhCHO, 100-52-7; PhCH=NCH$_2$CH$(CH_2CH_2)_2$NH, 71207-29-9; Ac-D-Phe-N$(CH_2CH_2)_2$CHCH$_2$N=CHPh, 120687-49-2; Ac-Phe-N$(CH_2CH_2)_2$CHCH$_2$N=CHPh, 120687-50-5; Ac-D-Phe-N(CH_2CH_2)CHCH$_2$NH$_2$·HCl, 120687-51-6; Ac-Phe-N(CH_2CH_2)-CHCH$_2$NH$_2$·HCl, 120687-52-7; BOC-Tyr-NHCH$_2$CH-$(CH_2CH_2)_2$N-r-D-Phe-Ac, 120687-53-8; BOC-Tyr-NHCH$_2$CH-$(CH_2CH_2)_2$N-r-Phe-Ac, 120687-54-9; Z-Tyr-OpNP, 3556-56-7; $H_2NCH_2CH_2NH_2$, 107-15-3; Z-Tyr-EDA·HCl, 120687-55-0; H_2N-$CH_2C(CH_3)_2NH_2$, 811-93-8; Ac-D-Phe-NHCH$_2$C$(CH_3)_2$NH$_2$, 120687-56-1; Ac-Phe-NHCH$_2$C$(CH_3)_2$NH$_2$, 120687-57-2; $H_2N(C-H_2)_3NH_2$, 109-76-2; $H_2NCH_2CH_2C(CH_3)_2NH_2$, 116473-67-7; Z-Tyr-OH, 1164-16-5; (±)-$H_2NCH_2CH(OH)CH_2NH_2$, 120687-58-3; Z-Tyr-NHCH$_2$-(*R*)-CH(OH)CH$_2$NH$_2$, 120687-59-4; Z-Tyr-NHCH$_2$-(*S*)-CH(OH)CH$_2$NH$_2$, 120687-60-7; PhOCH$_2$CO-D-Phe-OH, 88556-12-1; Z-Tyr-EDA, 120687-61-8; Z-D-Phe-OH, 2448-45-5; Z-Phe-OH, 1161-13-3; 1-benzyl-4-aminopiperidine, 50541-93-0; 4-(aminomethyl)piperidine, 7144-05-0.

(66) McKnight, A. T.; Corbett, A. D.; Kosterlitz, H. W. *Eur. J. Pharmacol.* **1983**, *86*, 393.
(67) Kosterlitz, H. W.; Watt, A. J. *Br. J. Pharmacol.* **1968**, *33*, 266.
(68) Abbreviations: Standard three-letter abbreviations are used for the amino acids, with Aib referring to α-aminoisobutyric acid. Retro nomenclature r-Phe indicates that the polarity of this residue is inverted relative to the standard N-to-C terminal convention of peptide notation. The following are abbreviations used throughout the paper: DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; Z, benzyloxycarbonyl; BOC, *tert*-butoxycarbonyl; EDA, ethylenediamine; Ac, acetyl; OpNP, *p*-nitrophenoxy; THF, tetrahydrofuran.