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Synthesis, Opiate Receptor Affinity, and Conformational Parameters of [4-Tryptophan]enkephalin Analogues[1]

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A series of analogues of the opioid peptide enkephalin with tryptophan substituted for phenylalanine in position 4 was synthesized by the solid-phase method. The [Trp[4]]enkephalin analogues and the corresponding [Phe[4]]enkephalin analogues displayed nearly parallel affinities in the opiate receptor binding assay throughout the series. In a conformational study fluorescence parameters were measured and intramolecular Tyr-Trp distances were estimated on the basis of resonance energy transfer experiments. No gross conformational differences were observed between analogues with widely differing opiate receptor affinity; however, small but significant changes in the intramolecular distance between the phenol ring and the indole moiety and/or in their relative orientation became apparent in some compounds. Identical intramolecular distances of 9.3 ± 0.2 Å between the two aromatic rings were obtained with [Trp[4],Met[5]]enkephalin, [Trp[4],Leu[5]]enkephalin, and the N-terminal tetrapeptide comprised in the latter two analogues, indicating the existence of folded conformations in 2×10^{-3} M aqueous solution and demonstrating conformational analogy between these three peptides. The conformational parameters are discussed in relation to the observed affinities and the putative opiate receptor topography.

Since the recently discovered opioid peptides [Met[5]]-enkephalin (H·Tyr-Gly-Gly-Phe-Met·OH) and [Leu[5]]-enkephalin (H·Tyr-Gly-Gly-Phe-Leu·OH) compete with morphine and its derivatives for opiate binding sites,[2] the enkephalin–opiate receptor system is uniquely suited for studying conformational aspects of a polypeptide–receptor interaction. The multitude of structure–activity relationships established with opiates has provided clues regarding the chemical functions which are critical for binding to the receptor and for activity. On the basis of these studies and taking into account the relatively rigid structure of morphine-derived ligands, descriptions of the opiate receptor topography in terms of binding sites have been put forward.[3-6] It is thus of considerable interest to demonstrate correspondence between critical chemical functions in morphine derivatives and in enkephalin and to establish their relative spatial disposition in the peptide.

In Figure 1 the structural formulas of morphine and a potent derivative of the oripavine family, 7α-[1-(*R*)-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetrahydrooripavine (PEO), are compared with that of [Met[5]]enkephalin. Omission[7,8] and acetylation[9,10] of the α-amino group in enkephalin produce an almost complete loss of activity both in the binding assay and in the guinea pig ileum bioassay. Methylation of the α-amino group to the secondary amine corresponding to the situation in normorphine results in an analogue with still good activity and affinity.[11,12] Finally, N-allylation of the tyrosyl residue induces partial antagonist properties[13] in analogy to the strong antagonism observed with *N*-allyl derivatives of morphine (e.g., naloxone). Substitution of phenylalanine for tyrosine leads to a drastic reduction in activity[8] and affinity,[14] which demonstrates the importance of the phenolic hydroxyl group for the interaction with the receptor. This finding is in qualitative agreement with the reduced analgesic activity of nonphenolic benzomorphans compared to the corresponding phenolic compounds.[15] O-Methylation of the tyrosine hydroxyl group engenders a drastic drop in activity[11] as is the case with O-methylated derivatives of morphine (e.g., codeine). Clearly, the ensemble of these results supports the idea of a correspondence between the tyrosine moiety in enkephalin and the phenol ring and tertiary nitrogen in morphine-related compounds. Furthermore, these findings suggest similar

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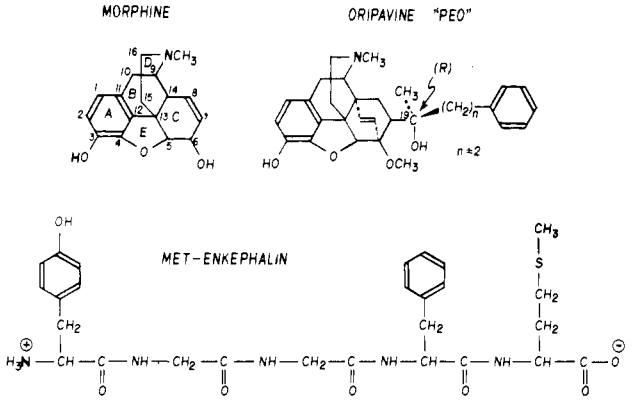

Figure 1. Structural formulas of morphine, oripavine (PEO), and [Met^5]enkephalin.

modes of binding[4] for morphine and enkephalin. In addition, evidence from a theoretical conformational analysis[16] and from an X-ray diffraction study[17] suggests that the carboxyl terminus of enkephalin and the C-6 hydroxyl (methoxy) group of morphine and its derivatives might have identical functions. Two hypotheses regarding the role of the phenylalanine residue in position 4 have been promulgated. The first proposal is based on studies with analogues in the oripavine family which had shown that attachment of a phenylethyl group to carbon-19 (Figure 1) increases potency by three orders of magnitude relative to morphine.[5] This observation had led to the proposition of a second lipophilic binding site at a distance of approximately 10 Å from the binding site of the phenol ring. It has been suggested that the phenylalanine residue might correspond to the carbon-19 substituent in oripavine, whereby its correct spatial disposition relative to the tyramine moiety would be brought about by 4 → 1 or 5 → 2 hydrogen-bonded β-bend conformations[18,19] or other types of low-energy conformers.[16] In an alternative proposal carbon atoms 5 and 6 of the C ring in morphine are considered as an additional binding element which would be provided by the meta and para positions of the phenyl ring of enkephalin in a computer-generated pharmacophoric conformation.[20] The latter conformation is characterized by a close proximity of the two aromatic rings (~5 Å) in contrast to the phenol–phenyl distance of about 10 Å in the β bend models. The experimental determination of this intramolecular distance is thus of considerable interest.

Theoretical conformational analyses produced several folded conformers of low energy, including the 4 → 1 and the 5 → 2 hydrogen-bonded β_I bends,[21] the II'β bend centered on Gly^3-Phe^4,[22] and other compact structures.[16] In the crystalline state the presence of a β bend centered on Gly^2-Gly^3 with antiparallel hydrogen bonding between tyrosine and phenylalanine was revealed by X-ray diffraction.[17] NMR studies indicate the existence of a preferred, rigid backbone conformation both in Me_2SO and in aqueous solution.[19,23,24] The results of two NMR investigations have been interpreted in favor of orientational freedom of all three side chains relative to the peptide backbone,[19,23] while a third study suggests restricted motion for the tyrosyl side chain.[24] Some of the conflicting results obtained from NMR experiments have been attributed to pH-dependent conformational changes[25] and to dimerization at high concentrations.[26]

Conformational studies with [Met^5]enkephalin and the active analogue [Trp^4,Met^5]enkephalin have been performed using fluorescence techniques.[11,27] Fluorescence spectroscopy permits the measurement of conformational parameters in aqueous solution at low concentrations (~10^{-5} M) where enkephalin presumably exists in the monomeric form. The results of these experiments permitted the following conclusions.

(a) Conformers with the tyrosyl hydroxyl engaged in a hydrogen bond are not predominant in aqueous solution. This finding is in contrast to reports[22,26] indicating the existence of a hydrogen bond between the phenolic hydroxyl and a backbone carbonyl group in either position 3 or 4.

(b) On the basis of a resonance energy transfer experiment, an average intramolecular distance of about 10 Å between the two aromatic rings in [Trp^4,Met^5]enkephalin was determined.

(c) Very similar, if not identical conformations or distributions of conformations are observed at pH 1.5 and 5.5. This finding indicates that titration of the C-terminal carboxyl group does not induce a conformational change and provides evidence against the proposal[23,25] of a NH_3^+–COO^- interaction as an important factor in stabilizing the conformation at physiological pH.

Fluorescence techniques are of particular value for conformational comparisons in a series of related peptides. In the present paper conformational parameters of enkephalin analogues with widely differing opiate receptor affinity are presented in an attempt to find a possible correlation between solution conformation and receptor binding.

Energy Transfer Experiments. In singlet–singlet resonance energy transfer between a donor fluorophore and an acceptor chromophore, the intramolecular donor–acceptor separation, r, is related to the transfer efficiency, E, by eq 1, where the Forster critical distance, R_0, is defined

$$E = R_0^6/(r^6 + R_0^6) \quad (1)$$

as that donor–acceptor separation[28] where 50% of the donor excitation energy is transferred to the acceptor. R_0, measured in centimeters, is related to various measurable spectroscopic parameters by eq 2, which permits its computation.

$$R_0 = [(8.79 \times 10^{-25})(\kappa^2/n^4)\phi_D^0 J_{AD}]^{1/6} \quad (2)$$

κ^2 = dipole–dipole orientation factor, n = refractive index, ϕ_D^0 = donor fluorescence quantum yield in absence of the acceptor, and $J_{AD} = \int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda$ = spectral overlap integral between the molar decadic absorption coefficient of the acceptor (ϵ_A) and the spectral distribution of the donor fluorescence (F_D), normalized to unity and modified by the wavelength factor λ^4. The spectroscopic properties of phenol (donor) and indole (acceptor) in aqueous solution permit the measurement of intramolecular Tyr-Trp distances in peptides in a range from 8 to 15 Å. The transfer efficiency can be determined experimentally by measurement of ϕ_D^0 and the donor fluorescence quantum yield in the presence of the acceptor, ϕ_D, on the basis of eq 3.

$$E = 1 - (\phi_D/\phi_D^0) \quad (3)$$

In the case of a peptide with the donor–acceptor pair tyrosine–tryptophan, ϕ_D^0 is best determined with a related peptide containing phenylalanine in place of tryptophan. Phenylalanine, while similar to tryptophan, does not participate in energy transfer at an excitation wavelength of 270 nm. Thus, for the computation of the Tyr-Trp intramolecular distance in [Trp^4,Met^5]enkephalin (Tyr-Gly-Gly-Trp-Met), ϕ_D^0 is best determined by measurement of the tyrosine fluorescence quantum yield in [Met^5]-enkephalin [Tyr-Gly-Gly-Phe-Met). For the determination of ϕ_D, the tyrosine emission of [Trp^4,Met^5]enkephalin is quantified through normalization at 370 nm of its fluorescence emission spectra obtained with excitation at

270 and 293 nm and subsequent subtraction of the normalized spectra. The transfer efficiency can also be determined from the relative enhancement of the acceptor fluorescence[29,30] by comparison of the fluorescence excitation and absorption spectrum of [Trp^4,Met^5]enkephalin with the excitation spectrum of [Phe^1,Trp^4,Met^5]enkephalin (Phe-Gly-Gly-Trp-Met). For the transfer of excitation energy from the phenol ring of tyrosine to the indole moiety of tryptophan in [Trp^4,Met^5]enkephalin, the determination of the transfer efficiency from donor quenching and from the relative enhancement of the acceptor fluorescence yielded values of $E = 0.70 \pm 0.015$ and 0.54 ± 0.10, respectively.[11] This result indicates that most of the tyrosine fluorescence quenching due to the presence of the acceptor tryptophan is indeed caused by energy transfer. Aside from the distance, the transfer efficiency is also dependent on the relative orientation of the transition dipoles of the donor and acceptor chromophores, which is taken into account by the orientation factor (κ^2) in Förster's equation. The implications of the orientation factor on the measurement of intramolecular distances by energy transfer have recently been discussed to great extent.[31] In the case of dynamic random orientation κ^2 assumes a value of $^2/_3$. The results of a recent theoretical study by Haas et al.[32] indicate that even for fixed orientations the problem of the orientation factor is markedly alleviated if the donor and/or the acceptor fluorophore show mixed polarizations in the wavelength region of spectral overlap. Fluorophores displaying mixed polarizations contain two or three transition dipole moments and their polarizations, p, are low. Thus, the electronic transition of tryptophan in the spectral region of overlap with the tyrosine fluorescence spectrum is characterized by two dipole moments and, therefore, a low polarization ($p = 0.10 \pm 0.05$) is observed. According to the analysis of Haas et al.,[32] this value of polarization results in an error of less than 20% for the estimated distance in a situation of fixed orientations if a value of $\kappa^2 = ^2/_3$ is used. In the case of [Met^5]enkephalin, segmental motion relative to the peptide backbone and the existence of three populations of rapidly interconverting rotamers has been demonstrated for the aromatic side chains in positions 1 and 4 by NMR spectroscopy.[23,25] Obviously, this orientational freedom of the fluorophores reduces the error in the distance even further.

While energy transfer experiments provide a valuable tool to estimate absolute intramolecular distances with an accuracy of about 20%, their potential in studies aiming at conformational comparisons is perhaps of greater interest. Transfer efficiencies in conjunction with fluorescence quantum yields represent sensitive parameters permitting the detection of conformational differences in a series of peptide analogues containing the same donor–acceptor pair. Among the various parameters contributing to R_0, ϕ_D^0 can assume different values in a series of analogues, while n and J_{AD} are usually constant in a given solvent. In the case where the same values of ϕ_D^0 are observed for two analogues, the transfer efficiency in both compounds is only a function of r and κ^2 (eq 4), where

$$E = S_0^6\kappa^2/(r^6 + S_0^6\kappa^2) \quad (4)$$

$S_0 = [(8.79 \times 10^{-25})(1/n^4)\phi_D^0 J_{AD}]^{1/6}$ = constant. If, in addition, identical values of E are also observed in the same two analogues, this is a strong indication for an analogous spatial disposition of the donor and acceptor fluorophores in the two peptides. The possibility that different values of r and κ^2 in each peptide compensate each other to produce exactly the same value of E is extremely unlikely.

Table I. Opiate Receptor Affinities of Enkephalin Analogues[a]

	analogue	rel receptor affinities, % ([Met^5]enkephalin = 100%) X = Phe	X = Trp
I	Tyr-Gly-Gly-X-Met	100	129 ± 30
II	Tyr-Gly-Gly-X-Leu	38 ± 6	32 ± 7
III	Tyr-Gly-Gly-X	2 ± 0.2	2 ± 0.2
IV	Tyr-Gly-Gly-X-Met-Thr	84 ± 16	59 ± 14
V	Tyr-D-Ala-Gly-X-Met	54 ± 14	34 ± 5
VI	Tyr-L-Ala-Gly-X-Met	5 ± 2	2 ± 1
VII	Tyr-Gly-Gly-D-X-Met	<1	<0.1

[a] Mean of three determinations ± SEM.

Results and Discussion

The relative affinities of various enkephalin analogues and the corresponding [Trp^4]enkephalin analogues are presented in Table I. Since the binding experiments were performed at 4 °C, enzymatic degradation was minimized[33] and the obtained values are likely to reflect true molar affinities. The similar affinities of [Trp^4,Met^5]enkephalin and [Met^5]enkephalin indicate that expansion of the aromatic system from phenyl to indole in the side chain of position 4 does not alter the binding properties. Similarly, the introduction of a chloro substituent in the para position of phenylalanine led only to a slight reduction in potency in the guinea pig ileum bioassay,[34] in contrast to the low affinities observed with analogues containing histidine[35] and tyrosine[36] substituted in position 4. In the latter case the phenolic hydroxyl group might interact with a dipolar site on the receptor, thereby causing a misalignment of the molecule. In close agreement with the results of others,[14,36] [Leu^5]enkephalin was found to possess a three times lower affinity than [Met^5]enkephalin. An almost identical drop in affinity is observed with [Trp^4,Leu^5]enkephalin. While most authors find a lower activity for [Leu^5]enkephalin compared to [Met^5]enkephalin both in the binding assay and in the bioassay, results indicating the reverse relationship have been presented in a recent report.[35] This discrepancy could be due to the different assay conditions used. Omission of the methionine residue in position 5 or extension of the chain at the C-terminus with L-threonine according to the sequence of β-lipotropin lowers the affinity to 2 and 59%, respectively, in qualitative agreement with recently published data.[14,36] Nearly parallel drops in affinity are observed with the corresponding [Trp^4] analogues (Table I). The analogue [D-Ala^2,Met^5]enkephalin has been tested in various assay systems. A seven- to tenfold increase in potency was observed in bioassays,[35,37] while the activity in an in vivo analgesic test was enhanced by two orders of magnitude.[38] In contrast to several reports of enhanced affinity,[35,37,38] our results indicate an affinity of only 54% for [D-Ala^2,Met^5]enkephalin relative to [Met^5]enkephalin in confirmation of the data obtained by Pert et al.[39] with the same assay system. Our results imply that the increased activity in the bioassay and in in vivo analgesic tests is brought about by high resistance toward enzymatic degradation rather than by enhanced affinity for the receptor. A similar reduction in affinity is observed with [D-Ala^2,Trp^4]enkephalin. Low affinities are obtained with [L-Ala^2,Met^5]enkephalin and [L-Ala^2,Trp^4,Met^5]enkephalin, while [D-Phe^4,Met^5]enkephalin and [D-Trp^4,Met^5]enkephalin do not seem to bind at all. Since the [Phe^4]-enkephalin analogues and the corresponding [Trp^4]-enkephalin analogues display nearly parallel affinities in all cases (Table I), it appears that tryptophan is a perfect substitute for phenylalanine in position 4 and the two

Table II. Intramolecular Tyr-Trp Distances in [Tryptophan]enkephalin Analogues

	analogue[a]	ϕ_D^0	E	R_0, Å	r, Å[b]
I	Tyr-Gly-Gly-Trp-Met	0.027	0.70 ± 0.015	10.7	9.3 ± 0.2
II	Tyr-Gly-Gly-Trp-Leu	0.027	0.69 ± 0.01	10.7	9.4 ± 0.1
III	Tyr-Gly-Gly-Trp	0.028	0.71 ± 0.01	10.8	9.3 ± 0.1
IV	Tyr-Gly-Gly-Trp-Met-Thr	0.032	0.61 ± 0.02	11.0	10.2 ± 0.2
V	Tyr-D-Ala-Gly-Trp-Met	0.022	0.62 ± 0.05	10.3	9.5 ± 0.3
VI	Tyr-L-Ala-Gly-Trp-Met	0.026	0.81 ± 0.05	10.6	8.3 ± 0.4
VII	Tyr-Gly-Gly-D-Trp-Met	0.023	0.58 ± 0.01	10.4	9.8 ± 0.1

[a] Peptide concentration = 2×10^{-5} M; solvent = H_2O. [b] Experimental errors reflect uncertainties in E.

series of analogues are thus well suited for a conformational study by energy transfer.

Within the accuracy of the measurements, the same values of E and ϕ_D^0 are observed for [Trp^4,Met^5]enkephalin and for [Trp^4,Leu^5]enkephalin in the energy transfer experiments (Table II). It can thus be concluded that the same average distance and orientation between the phenol ring and the indole moiety is present in the two analogues. This result is in harmony with theoretical conformational analyses which demonstrated a close similarity between the conformations of [Met^5]enkephalin and [Leu^5]enkephalin.[22,40] If random donor–acceptor orientation is assumed ($\kappa^2 = {}^2/_3$), an average intramolecular distance of 9.3 ± 0.2 Å between the two aromatic rings is obtained both for [Trp^4,Met^5]enkephalin and for [Trp^4,Leu^5]enkephalin. This distance is compatible with β-bend conformations, where intramolecular phenol–indole distances of 9–11 Å are observed in CPK models. The results of recent NMR studies[19,23,41] have been interpreted in favor of the existence of a 5 → 2 hydrogen-bonded β_I turn in Me_2SO and in aqueous solution, while others found no indication for a conformational stabilization by intramolecular hydrogen bonding.[24,42] It is quite conceivable that other types of folded conformations, stabilized by solvent interactions, could also accommodate the two aromatic rings at the observed distance. The result of an X-ray diffraction study recently performed[17] with [Leu^5]enkephalin permits another interesting comparison. The latter investigation established the existence of two similar conformations in the crystal which are both characterized by antiparallel hydrogen bonding between tyrosine and phenylalanine but differ in the orientation of the tyrosine side chain. The intramolecular distances of 10.67 and 11.28 Å between the centers of the two aromatic rings observed[43] in the two conformations are slightly larger than the Tyr-Trp distance determined with [Trp^4,Leu^5]enkephalin in the present study. On the basis of a theoretical conformational analysis, an equilibrium of extended and folded conformations was proposed and mean intramolecular distances of 8.7 and 8.6 Å between the centers of the aromatic rings were calculated for [Met^5]enkephalin and [Leu^5]enkephalin, respectively.[40] If an equilibrium situation did indeed exist, the latter distances could not be directly compared with our distance obtained from energy transfer experiments, which would represent a value for $\langle r^{-6} \rangle_{av}^{-1/6}$ and not a linear average.[44] However, NMR experiments[19,23,24] favor the existence of a preferred, rigid backbone conformation over an equilibrium of significantly different conformers in solution. The intramolecular distance of 9.3 Å is similar to the phenol–phenyl distance in PEO (8–10.5 Å). Under the assumption of a correspondence between the side chain of phenylalanine in enkephalin and the phenylethyl substituent in PEO,[18] this result would indicate that the solution conformation is similar to the receptor-bound conformation and that only minor conformational adjustments[16,17] might be necessary for the formation of the peptide–receptor complex. If, on the other hand, a recently published[20] model of the receptor-bound conformation of enkephalin were correct, our results would imply a change of the order of 5 Å in the intramolecular distance between the two aromatic rings in the course of the binding process. Such a drastic conformational change is somewhat difficult to conceive.

Values of ϕ_D^0 and E identical with those of [Trp^4,Met^5]enkephalin and [Trp^4,Leu^5]enkephalin were also obtained for the tetrapeptide H·Tyr-Gly-Gly-Trp·OH (Table II). It can thus be concluded that the addition of the fifth residue has no influence on the average conformation of the N-terminal tetrapeptide sequence in the pentapeptides [Trp^4,Met^5]enkephalin and [Trp^4,Leu^5]enkephalin. Since the tetrapeptide displays low affinity (Table I), this finding suggests a role for the fifth residue as an additional binding element. This observation is in contrast to the results of a conformational analysis recently performed with the tetrapeptide H·Tyr-Gly-Gly-Phe·OH by NMR spectroscopy at high concentration (10^{-2} M) in Me_2SO and by X-ray diffraction in the crystalline state.[45] On the basis of this study, different types of bent structures were proposed for the tetrapeptide and for the native enkephalins, and a conformational key role was ascribed to the fifth residue. However, these results do not reflect the situation at low concentrations in aqueous solution and their interpretation may be complicated by aggregation phenomena and intermolecular interactions in the crystal.

Values of ϕ_D^0 and E differing from those of the first three peptides (I–III) were determined with the hexapeptide H·Tyr-Gly-Gly-Trp-Met-Thr·OH (Table II). This finding is indicative of small, but significant changes in either the intramolecular distance and/or orientation between the two aromatic rings, which may be of relevance to the observed decrease in affinity. Significant differences in E and ϕ_D^0 were also detected in the analogues with D-alanine and L-alanine substituted for glycine in position 2 and in the analogue with the D configuration in position 4 (Table II). If, in all cases, random orientation is assumed ($\kappa^2 = {}^2/_3$), larger deviations in the value of r compared to that of [Trp^4,Met^5]enkephalin are obtained for analogues with low affinity ([L-Ala^2,Trp^4,Met^5]enkephalin and [D-Trp^4,Met^5]enkephalin) than, e.g., for [D-Ala^2,Trp^4,Met^5]enkephalin which still displays relatively high affinity. However, since small differences in donor–acceptor orientation between the various analogues cannot be entirely excluded, it is more appropriate to state that subtle differences in tyrosine quantum yields and in the intramolecular distance and/or orientation between the phenol and indole rings are observed with analogues IV–VII relative to analogues I–III (Table II). Differences of similar magnitude in the mean distance between the aromatic rings in a series of analogues were obtained from a theoretical analysis,[40] whereby in the case of [D-Ala^2,Met^5]enkephalin a small decrease in r relative to [Met^5]enkephalin was obtained in contrast to the increase observed in our study. Whether or not these differences are related to the observed receptor affinities remains to be established, but they are reminiscent of the situation existing in the oripavine family, where the intramolecular distance

Table III. Tryptophan Quantum Yields of Enkephalin Analogues

	analogue	ϕ^{Trp}
I	Tyr-Gly-Gly-Trp-Met	0.058 ± 0.002
II	Tyr-Gly-Gly-Trp-Leu	0.057 ± 0.001
III	Tyr-Gly-Gly-Trp	0.051 ± 0.001
IV	Tyr-Gly-Gly-Trp-Met-Thr	0.051 ± 0.001
V	Tyr-D-Ala-Gly-Trp-Met	0.059 ± 0.001
VI	Tyr-L-Ala-Gly-Trp-Met	0.054 ± 0.002
VII	Tyr-Gly-Gly-D-Trp-Met	0.088 ± 0.001

and relative orientation between the two aromatic rings in PEO are of crucial importance for activity (maximal activity is observed with carbon-19 in the *R* configuration and with two methylene groups as spacers between carbon-19 and the phenyl ring).[5] A further argument for the importance of the correct spatial disposition between the tyramine moiety and the phenyl ring in position 4 of enkephalin is the low activity observed with an analogue[35] containing phenylglycine substituted for phenylalanine.

Both the quantum yield and the position of the emission maximum of tryptophan fluorescence are very sensitive to the polarity of the microenvironment of the indole ring. For all [Trp^4]enkephalin analogues very similar tryptophan fluorescence quantum yields were determined (ϕ_{av}^{Trp} = 0.055 ± 0.004) (Table III) with the interesting exception of [D-Trp^4,Met^5]enkephalin which shows an increase of 60% in this parameter (ϕ^{Trp} = 0.088 ± 0.001). The enhanced quantum yield in the latter analogue is accompanied by a blue shift of 2 nm relative to the maximum at 350 nm observed with the other analogues. This finding reflects a slightly more hydrophobic environment of the indole ring due to its different orientation and interaction with hydrophobic groups (e.g., the side chain of methionine) in [D-Trp^4,Met^5]enkephalin. A similarly enhanced quantum yield (ϕ^{Trp} = 0.086) and the same blue shift in the emission spectrum of the tryptophan fluorescence were detected in the C-terminal heptapeptide of cholecystokinin.[46]

Recently, a strong concentration dependence of NMR, CD, and UV parameters in the concentration range from 10^{-1} to 10^{-4} M was reported[26] and taken as evidence for molecular association at high concentrations. The measurement of tryptophan fluorescence quantum yields should provide a sensitive tool for monitoring dimerization or oligomerization processes which are likely to bring about a concomitant change in the polarity of the microenvironment of the indole ring. No change in the tryptophan fluorescence quantum yield of [Trp^4,Met^5]enkephalin was observed in the concentration range from 10^{-5} to 5×10^{-7} M in aqueous solution. The absence of a concentration effect provides evidence, even though no definite proof, that molecular association does not take place at these low concentrations and that we are dealing with [Trp^4,Met^5]enkephalin in its monomeric state. The observation[26] of an associated form of enkephalin at concentrations higher than 10^{-4} M and the absence of a concentration effect observed in this study at concentrations below 10^{-5} M can be explained with a monomer–dimer equilibrium characterized by a dissociation constant of the order of 10^{-3}–10^{-4} M. The concentration range above 10^{-5} M is not accessible to fluorescence spectroscopy because of self-quenching.

A more meaningful interpretation of the differences in fluorescence parameters in relation to the observed affinities requires information on the relationship between the average solution conformation and the receptor-bound conformation. Binding experiments and energy transfer experiments involving analogues with restricted conformational freedom might help to define the pharmacophoric conformation.

Experimental Section

Peptide Synthesis. Peptides were synthesized by the solid-phase technique[47] with the aid of a Beckman Model 990 peptide synthesizer. Protection of the α-amino group with the *tert*-butyloxycarbonyl group (Boc) was used with all amino acids. *tert*-Butyloxycarbonyl-D-alanine, -D-phenylalanine, and -D-tryptophan were prepared according to an established procedure.[48] Other *tert*-butyloxycarbonylamino acids were purchased from Bachem, Inc., Torrance, Calif. Benzyl protection was used for the reactive hydroxyl groups of tyrosine and threonine. The protected C-terminal amino acids were reacted with chloromethyl resin[49] (Bio-Beads S-X1, 200–400 mesh, 1.25 mequiv of Cl/g), whereby yields of 0.3–0.5 mmol of *tert*-butyloxycarbonylamino acid per gram of resin were obtained. Couplings of the subsequent *tert*-butyloxycarbonylamino acids were performed following a protocol described elsewhere.[11] Boc protection was removed at each step by treatment with 25% F_3Ac in $CHCl_3$. For tryptophan-containing peptides 1 N HCl in AcOH containing 2% mercaptoethanol was used[50] instead of F_3Ac–$CHCl_3$ in order to prevent oxidation of the indole ring. After completion of the last cycle, the peptides were cleaved from the resin and completely deprotected by treatment with HF.[51] The reaction was carried out for 1 h at 0 °C with 20 mL of HF and in the presence of 1 mL of anisole and 1 mL of ethyl methyl sulfide as scavengers per gram of resin. In the case of tryptophan-containing peptides, 200 mg of skatole/g of resin was added as an additional scavenger for protection of the indole ring.[11] After evaporation of the HF, the resin was extracted several times with diethyl ether and subsequently with 90% acetic acid. Lyophilization of the latter extract yielded the crude peptide in solid form.

All peptides were purified by a two-step procedure. Approximately 200 mg of crude product dissolved in 0.1 N ammonium acetate in 1 N acetic acid was applied to a SP-Sephadex C-25 column (2.5 × 100 cm) and elution was performed using a linear gradient with the final buffer reaching a concentration of 0.4 N ammonium acetate in 1 N acetic acid. The fractions containing the major product were combined and lyophilized. The resulting product was purified further by partition chromatography on a Sephadex G-25 column (2.5 × 100 cm) with the system 1-butanol–acetic acid–H_2O (4:1:5). All peptide analogues were obtained as lyophilisates. Purity was established by ascending thin-layer chromatography on precoated plates (silica gel G, 250 μm, Analtech, Inc.) in the following systems: (a) 1-butanol–acetic acid–H_2O (BAW) (4:1:5, organic phase), (b) 1-butanol–pyridine–acetic acid–H_2O (BPAW) (15:10:3:12), and (c) *sec*-butyl alcohol–3% ammonium hydroxide (SH) (100:44). Spots on plates were stained with the chlorine peptide spray. All peptides showed a single major spot and in some cases a second faint spot with a smaller R_f indicating the presence of a small fraction (<5%) of methionine sulfoxide peptide. Amino acid analyses were performed on a Beckman Model 121 C amino acid analyzer equipped with a system AA computing integrator on samples which were hydrolyzed (110 °C, 24 h) in 6 N HCl containing 5% thioglycolic acid.[52] Modest yields (10–30%) were obtained for most peptides because the cuts made in the purifications on the basis of thin-layer chromatography of the individual fractions emphasized purity more than yields in view of the high-purity requirements for fluorometric studies.

H-Tyr-Gly-Gly-Phe-OH: amino acid analysis gave Tyr 1.00, Gly 2.02, Phe 1.04; TLC R_f = 0.41 (BAW), R_f = 0.67 (BPAW), R_f = 0.23 (SH).

H-Tyr-Gly-Gly-Trp-OH: amino acid analysis gave Tyr 1.00, Gly 1.95, Trp 1.01; TLC R_f = 0.45 (BAW), R_f = 0.54 (BPAW), R_f = 0.22 (SH).

H-Tyr-Gly-Gly-Phe-Leu-OH: amino acid analysis gave Tyr 0.93, Gly 2.08, Phe 1.06, Leu 1.00; TLC R_f = 0.51 (BAW), R_f = 0.71 (BPAW), R_f = 0.28 (SH).

H-Tyr-Gly-Gly-Trp-Leu-OH: amino acid analysis gave Tyr 1.00, Gly 1.88, Trp 1.12, Leu 1.10; TLC R_f = 0.64 (BAW), R_f = 0.70 (BPAW), R_f = 0.28 (SH).

H-Tyr-Gly-Gly-Phe-Met-Thr-OH: amino acid analysis gave Tyr 1.00, Gly 1.97, Phe 0.98, Met 1.05, Thr 0.90; TLC R_f = 0.40 (BAW), R_f = 0.65 (BPAW), R_f = 0.24 (SH).

H·Tyr-Gly-Gly-Trp-Met-Thr·OH: amino acid analysis gave Tyr 1.00, Gly 2.05, Trp 0.96, Met 1.08, Thr 1.01; TLC R_f = 0.48 (BAW), R_f = 0.65 (BPAW), R_f = 0.18 (SH).

H·Tyr-D-Ala-Gly-Phe-Met·OH: amino acid analysis gave Tyr 1.00, Ala 0.95, Gly 1.04, Phe 1.03, Met 0.93; TLC R_f = 0.53 (BAW), R_f = 0.68 (BPAW), R_f = 0.27 (SH).

H·Tyr-D-Ala-Gly-Trp-Met·OH: amino acid analysis gave Tyr 0.98, Ala 0.99, Gly 1.00, Trp 0.97, Met 1.03; TLC R_f = 0.57 (BAW), R_f = 0.68 (BPAW), R_f = 0.30 (SH).

H·Tyr-L-Ala-Gly-Phe-Met·OH: amino acid analysis gave Tyr 1.01, Ala 0.99, Gly 1.00, Phe 1.02, Met 0.99; TLC R_f = 0.44 (BAW), R_f = 0.62 (BPAW), R_f = 0.27 (SH).

H·Tyr-L-Ala-Gly-Trp-Met·OH: amino acid analysis gave Tyr 1.09, Ala 1.00, Gly 1.11, Trp 0.87, Met 1.09; TLC R_f = 0.54 (BAW), R_f = 0.67 (BPAW), R_f = 0.26 (SH).

H·Tyr-Gly-Gly-D-Phe-Met·OH: amino acid analysis gave Tyr 0.93, Gly 2.09, Phe 1.00, Met 1.05; TLC R_f = 0.41 (BAW), R_f = 0.62 (BPAW), R_f = 0.24 (SH).

H·Tyr-Gly-Gly-D-Trp-Met·OH: amino acid analysis gave Tyr 1.00, Gly 2.00, Trp 0.91, Met 1.03; TLC R_f = 0.45 (BAW), R_f = 0.62 (BPAW), R_f = 0.30 (SH).

Opiate Receptor Binding Assay. Opiate receptor affinities were determined by a modified version of an assay described in the literature.[53] Male Sprague–Dawley rats (300–350 g) of the Canadian Breeding Laboratories were decapitated and after excision of the cerebellum the brain tissue was homogenized in 30 vol of ice-cold standard buffer (50 mM Tris–HCl, pH 7.7). After centrifugation at 30 000g for 30 min at 4 °C, the membranes contained in the pellet were resuspended in the original volume of fresh standard buffer. Incubation at 37 °C for 30 min, centrifugation, and subsequent reconstitution of the membranes in the initial volume of fresh standard buffer yielded the final membrane suspension. Aliquots (2 mL) of the membrane preparation were incubated for 1 h at 0 °C[14] with 1 mL of standard buffer containing the peptide to be tested and [^{3}H]naloxone (17.7 Ci/mmol; New England Nuclear Corp.) at a final concentration of 0.5 nM. The incubation was terminated by filtration through Whatman GF/B filters under vacuum at 4 °C. Subsequently, the filters were washed with two 5-mL portions of ice-cold standard buffer, transferred to scintillation vials, and treated with 1 mL of Soluene (Packard) for 30 min. After addition of 0.5 mL of acetic acid and 10 mL of Aquasol (New England Nuclear Corp.) the vials were shaken for 30 min and then counted at an efficiency of 40–45%. Stereospecific binding as determined by displacement of [^{3}H]naloxone with excess (10 μmol) cold [Met^5]enkephalin accounted for 70–80% of total binding. Values of half-maximal inhibition (IC_{50}) of stereospecific binding were obtained graphically from semilogarithmic plots. Each analogue was tested at least three times and [Met^5]enkephalin was included in each binding experiment as the reference compound.

Spectroscopic Measurements. Absorption spectra were obtained with a Beckman Model 25 spectrophotometer. Uncorrected fluorescence emission spectra were recorded on a Hitachi Perkin-Elmer fluorescence spectrophotometer MPF-3L. Temperature was maintained constant at 25 °C through thermostating of the cell block with a Haake FK2 circulating water bath. Approximately 2×10^{-5} M aqueous solutions of peptides were used in all fluorescence experiments. Quantum yields of tyrosine and tryptophan fluorescence in peptides were determined through comparison with emission spectra of L-tyrosine and L-tryptophan in aqueous solution, whose quantum yields, ϕ_{AA}, were taken from the literature[54] as 0.14 and 0.13, respectively. Excitation wavelengths for tyrosine and tryptophan were 275 and 293 nm, respectively. Relative fluorescence intensities were determined through integration of the spectral area and the quantum yield of the peptide fluorophore, ϕ_{PP}, was calculated with eq 5, where

$$\phi_{PP} = \phi_{AA} (I_{PP}A_{AA}/I_{AA}A_{PP}) \quad (5)$$

I_{PP} and I_{AA} are the fluorescence intensities and A_{PP} and A_{AA} the absorbances of the peptide fluorophore and the reference fluorescent amino acid, respectively.

In the computation of R_0, random donor–acceptor orientation was assumed ($\kappa^2 = {}^2/_3$) and the refractive index was taken as 1.5. The value of $\phi_D{}^0$ was determined in each case with the corresponding [Tyr^1,Phe^4]enkephalin analogue and the value of J_{AD} = 4.8×10^{-16} M^{-1} cm^6 was found in the literature.[55] Transfer efficiencies were determined from donor (tyrosine) fluorescence quenching by comparison of the emission spectra of the [Tyr^1,Phe^4]enkephalin analogues with the corresponding [Tyr^1,Trp^4]enkephalin analogues as described above.

Acknowledgment. This research was supported by an operating grant of the Medical Research Council of Canada (MA-5655). We thank J. Chevrier for excellent technical assistance. Thanks are also due to Drs. G. D. Smith and J. F. Griffin for the communication of conformational parameters resulting from the X-ray diffraction study and to Dr. E. Haas for a preprint of the paper dealing with the orientation factor.

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Psychotropic Agents. 3.[1] 4-(4-Substituted piperidinyl)-1-(4-fluorophenyl)-1-butanones with Potent Neuroleptic Activity

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A series of 1-(4-fluorophenyl)-4-(1-piperidinyl)-1-butanones substituted with benzimidazole, benzotriazole, or quinoxaline at the 4 position of the piperidine ring was synthesized and subjected to neuroleptic tests. Neuroleptic activities of several compounds were comparable to those of haloperidol. In particular, 4-[4-(2,3-dihydro-2-thioxo-1*H*-benzimidazol-1-yl)-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone (**10**) was characterized by having a potent neuroleptic activity with less liability to the extrapyramidal side effect.

Haloperidol,[2] which is the prototype of a series of chemically related butyrophenones, has been used widely in the therapy of psychic disorder, especially of schizophrenia. Two members of this series, spiroperidol and benperidol,[2] are the most potent neuroleptics known at the present time. In the previous paper,[1] we described that some modification of the side chain of haloperidol or benperidol lowered neuroleptic activity of the parent drugs. In the present study, 1-(4-fluorophenyl)-4-(1-piperidinyl)-1-butanones substituted with benzimidazole, benzotriazole, or quinoxaline derivatives at the 4 position of the piperidine ring and related compounds were prepared in order to search for new antipsychotic drugs. The structure–activity relationships of these derivatives are discussed herein and pharmacological properties of two selected compounds are described.

Chemistry. Various butanone derivatives (**4–28**) and 1,1-diphenylbutane derivatives (**30–33**) were synthesized by the general route outlined in Scheme I and listed in Table I. An intermediate, *o*-phenylenediamine (**3**), was prepared by reaction of 4-(2-nitroanilino)piperidine[3] (**1**) with 2-(3-chloropropyl)-2-(4-fluorophenyl)-1,3-dioxolane, followed by catalytic hydrogenation over Raney nickel of the resulting butanone derivative **2**. Cyclization of **3** with carboxylic acids or benzoyl chloride (method A), carbon disulfide (method B), sodium nitrite (method G), and pyruvic acid or oxalic acid (method H) gave benzimidazoles **4–9**, benzimidazolinethione (**10**), benzotriazole (**26**), and quinoxaline derivatives **27** and **28**, respectively. Alkylation of **10** with dimethyl sulfate (method C), alkyl halides (method D), ethylene oxide (method E), or acyl chlorides (method F) gave **11–25** in good yield. 1,1-Diphenylbutanes **30** and **31** were also synthesized from an *o*-phenylenediamine derivative[3] **29** as described above (methods A and B), and alkylation of **31** according to methods C and E gave 2-(alkylthio)benzimidazole derivatives **32** and **33**, respectively.

Structure–Activity Relationships. Table I shows experimental results expressed as ED_{50} values estimated from dose–response curves of the compounds tested. Of the compounds in which alkyl groups were introduced at the 2 position of the benzimidazole ring (**4–7**), **5** was the most potent in inhibiting SMA and MGT. This compound was almost equally effective to haloperidol, although inhibition of SMA with the former was of shorter duration than that with the latter. Compounds with a phenyl or

0022-2623/78/1821-1116$01.00/0