Synthesis and Pharmacological Characterization in Vitro of Cyclic Enkephalin Analogues: Effect of Conformational Constraints on Opiate Receptor Selectivity[1]

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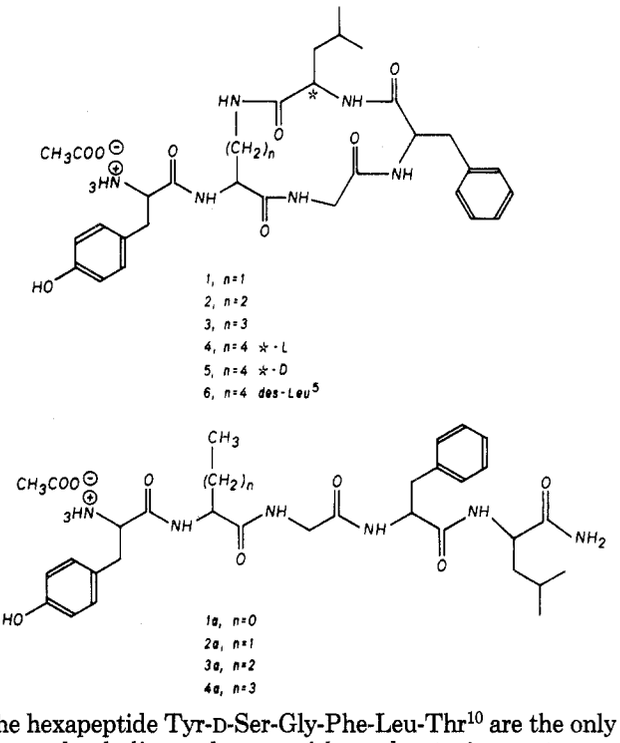
Using a combination of solid-phase and solution methods, we synthesized a series of cyclic [Leu^5]enkephalin analogues by substitution of D-α,ω-diamino acids in position 2 of the enkephalin sequence and cyclization of the ω-amino group to the C-terminal carboxy group of leucine. Cyclic analogues containing D-α,β-diaminopropionic acid (**1**), D-α,γ-diaminobutyric acid (**2**), D-ornithine (**3**), or D-lysine (**4**) in position 2 and the [D-Leu^5] and [des-Leu^5] analogues of **4** (**5** and **6**) showed, in general, high potency in the guinea pig ileum (GPI) assay and low potency in the mouse vas deferens (MVD) assay. IC_{50} (MVD)/IC_{50} (GPI) ratios ranging from 3.1 to 29.4 were obtained, indicating the preference of the cyclic analogues for μ receptors over δ receptors. With two exceptions, preferential affinity for μ receptors is reflected in the K_i ratios determined in parallel binding assays using [^{3}H]naloxone and [^{3}H] [D-Ala^2,D-Leu^5]enkephalin as μ and δ receptor selective radioligands, respectively. Comparison of the pharmacological profiles of the cyclic analogues **1–4** with those of their corresponding open-chain analogues, [D-Ala^2,Leu^5]enkephalinamide (**1a**), [D-Abu^2,Leu^5]enkephalinamide (**2a**), [D-Nva^2,Leu^5]enkephalinamide (**3a**), and [D-Nle^2,Leu^5]enkephalinamide (**4a**), revealed that the pronounced μ character of compounds **1–4** is a direct consequence of the conformational constraints introduced by cyclization. This finding is in agreement with the concept of different conformational requirements of μ- and δ-opiate receptors and raises the possibility of manipulating opiate receptor selectivity by varying the type and degree of conformational restriction.

Pharmacological studies performed in recent years with various types of opiates and opioid peptides have led to the concept of opiate receptor heterogeneity.[2,3] The existence of at least three different subclasses, μ, δ, and κ receptors, is now widely accepted. The physiological roles of the individual receptor subtypes have not yet been clearly established. In studies with opioid peptides, the major focus has been on μ- and δ-receptor interactions. Standard tests for opiate activity determinations in vitro are based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD). The opiate effect in the GPI preparation is mainly mediated by μ receptors, whereas the predominant receptors of the MVD are of the δ type. While morphine-related opiates are μ-receptor selective, the natural enkephalins show preference for δ receptors. It has been suggested,[4] but not proven unequivocally, that in the central nervous system μ receptors might mediate analgesia and that δ receptors may be implicated in eliciting behavioral effects.

Based on the enkephalin sequence [Tyr-Gly-Gly-Phe-Met(or Leu)] as a starting point, various peptide analogues with either μ- or δ-receptor selectivity have been prepared in recent years. The first analogues with considerable μ-receptor selectivity, [D-Ala^2,Met^5]enkephalinamide[5] and Tyr-D-Ala-Gly-MePhe-Met(O)-ol,[6] originated from attempts to stabilize the enkephalin molecule against enzymatic degradation. More recent analogues with good μ-receptor selectivity include substituted tetrapeptide amides[7] (e.g., Tyr-D-Ala-Gly-MePhe-Gly-ol) and substituted tripeptide amides.[8] [D-Ala^2,D-Leu^5]enkephalin[9] and the hexapeptide Tyr-D-Ser-Gly-Phe-Leu-Thr[10] are the only two enkephalin analogues with moderate δ-receptor selectivity that have been reported to date.

Chart I. Structural Formulas of Cyclic Enkephalin Analogues (1–6) and of the Corresponding Open-Chain Analogues (1a–4a)



Incorporation of conformational constraints (e.g., cyclic structures) into a linear peptide represents a promising new approach toward the goal of obtaining receptor selectivity. The conformational restriction introduced may be favorable for the interaction with one receptor subtype but incompatible with the conformational requirements of another subtype whereby receptor selectivity would result. Recently, we described[11] a cyclic enkephalin analogue,

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(2) W. R. Martin, C. C. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert, *J. Pharmacol. Exp. Ther.*, **197**, 517 (1976).
(3) J. A. Lord, A. A. Waterfield, J. Hughes, and H. W. Kosterlitz, *Nature (London)*, **267**, 495 (1977).
(4) S. H. Snyder, *Science*, **209**, 976 (1980).
(5) C. B. Pert, A. Pert, J. K. Chang, and B. T. W. Fong, *Science*, **194**, 330 (1976).
(6) D. Roemer, H. H. Buescher, R. C. Hill, J. Pless, W. Bauer, F. Cardinaux, A. Closse, D. Hauser, and R. Hugenin, *Nature (London)*, **268**, 547 (1977).
(7) B. K. Handa, A. C. Lane, J. A. H. Lord, B. A. Morgan, M. J. Rance, and C. F. C. Smith, *Eur. J. Pharmacol.*, **70**, 531 (1981).
(8) G. Gacel, M.-C. Fournié-Zaluski, E. Fellion, and B. P. Roques, *J. Med. Chem.*, **24**, 1119–1124 (1981).
(9) K. J. Chang, B. R. Cooper, E. Hazum, and P. Cuatrecasas, *Mol. Pharmacol.*, **16**, 91 (1979).
(10) G. Gacel, M.-C. Fournié-Zaluski, and B. P. Roques, *FEBS Lett.*, **118**, 245 (1980).

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Scheme I

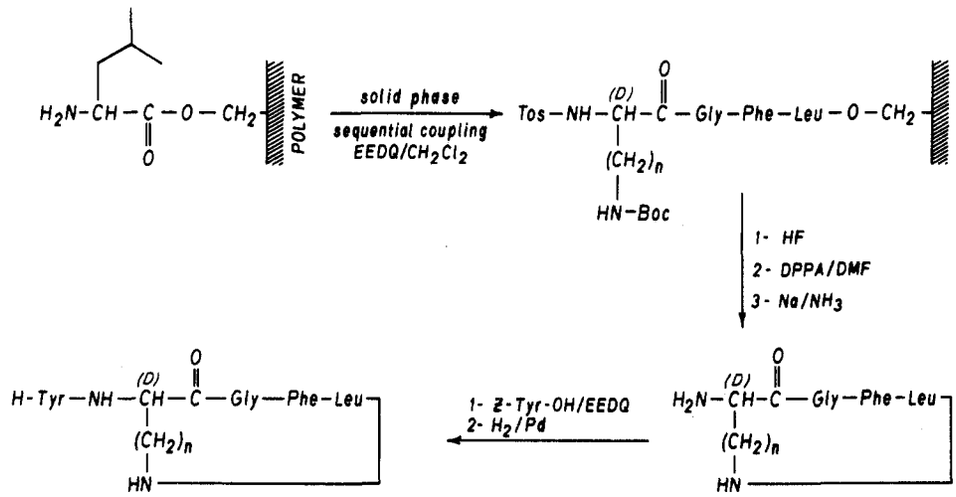

Table I. Inhibitory Potencies (IC_{50}) and Sensitivities to Naloxone (K_e) of Peptide Analogues, [Leu^5]enkephalin, and Levorphanol in the Guinea Pig Ileum and Mouse Vas Deferens Assays

no.	compound	IC_{50},[a] nM		IC_{50} (MVD)/IC_{50}	K_e,[a] nM	
		GPI	MVD	(GPI)	GPI	MVD
1	H-Tyr-c[-N^{β}-D-A_2pr-Gly-Phe-Leu-]	23.4 ± 4.2	73.1 ± 14.5	3.12	0.275 ± 0.065	0.387 ± 0.096
1a	H-Tyr-D-Ala-Gly-Phe-Leu-NH_2	7.63 ± 0.06	8.26 ± 1.32	1.08	0.401 ± 0.015	2.36 ± 0.37
2	H-Tyr-c[-N^{γ}-D-A_2bu-Gly-Phe-Leu-]	14.1 ± 2.9	81.4 ± 5.8	5.77	0.122 ± 0.028	0.576 ± 0.044
2a	H-Tyr-D-Abu-Gly-Phe-Leu-NH_2	28.7 ± 1.3	45.6 ± 9.1	1.59	0.181 ± 0.033	1.50 ± 0.19
3	H-Tyr-c[-N^{δ}-D-Orn-Gly-Phe-Leu-]	48.0 ± 4.3	475 ± 99	9.90	0.671 ± 0.116	0.216 ± 0.048
3a	H-Tyr-D-Nva-Gly-Phe-Leu-NH_2	23.2 ± 1.5	21.0 ± 3.4	0.905	0.227 ± 0.030	3.52 ± 0.42
4	H-Tyr-c[-N^{ϵ}-D-Lys-Gly-Phe-Leu-]	4.80 ± 1.79	141 ± 28	29.4	0.416 ± 0.092	0.360 ± 0.068
4a	H-Tyr-D-Nle-Gly-Phe-Leu-NH_2	24.6 ± 1.6	25.2 ± 7.9	1.02	0.729 ± 0.087	3.19 ± 0.11
5	H-Tyr-c[-N^{ϵ}-D-Lys-Gly-Phe-D-Leu-]	2.39 ± 0.57	17.5 ± 5.7	7.32	0.553 ± 0.151	2.37 ± 0.30
6	H-Tyr-c[-N^{ϵ}-D-Lys-Gly-Phe-]	7.09 ± 1.49	33.8 ± 1.3	4.77	0.875 ± 0.087	0.606 ± 0.120
7	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.046	1.53 ± 0.43	5.86 ± 0.90
8	levorphanol	17.0 ± 3.0	278 ± 56	16.4	0.127 ± 0.038	0.587 ± 0.165

[a] Mean of three determinations ± SEM.

Tyr-c[-N^{γ}-D-A_2bu-Gly-Phe-Leu-] (Chart I, compound **2**), which showed high potency at the μ receptor, as well as high stability against enzymatic degradation. The structure of this prototype permits subtle variation in conformational restriction by either shortening or lengthening the side chain in position 2. In the present paper, we therefore describe the synthesis of homologues of **2**, which contain D-diaminopropionic acid (**1**), D-ornithine (**3**), and D-lysine (**4**) in position 2 (Chart I). To evaluate the effect of cyclization upon opiate receptor interactions, we compared the potencies of the cyclic analogues with those of appropriate linear correlates that were obtained as analogues of [Leu^5]enkephalinamide with D-alanine, D-2-aminobutyric acid, D-norvaline, and D-norleucine substituted for glycine in position 2 of the peptide sequence (compounds **1a**, **2a**, **3a**, and **4a**). In addition, the diastereomer of **4** with Leu^5 in the D configuration (compound **5**) and an analogue lacking the leucine residue altogether (compound **6**) were also prepared in order to investigate the role of the 5-position residue in opiate receptor interactions. To determine a possible selectivity for either μ- or δ-opiate receptors, analogues were tested in the GPI and MVD assay and in binding assays based on displacement of a μ-receptor-selective radioligand, [^{3}H]naloxone, or a δ-receptor-selective radiolabel, [^{3}H][D-Ala^2,D-Leu^5]enkephalin.

Chemistry. A combination of the solid-phase technique and classical methods in solution was employed for the synthesis of compounds **1–6**, as shown in Scheme I. The C-terminal tetrapeptide or tripeptide sequence was assembled by the solid-phase method with *tert*-butyloxycarbonyl (Boc) protection and 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ)[12] as coupling agent. At this stage, the α- and ω-amino groups of the N-terminal residue were protected by the *p*-toluenesulfonyl (Tos = tosyl) group and the benzyloxycarbonyl (Z) or Boc group, respectively. The possibility of racemization during coupling of the N^{α}-tosyl amino acids with EEDQ can be assessed by comparing the in vitro activity of Tyr-c[-N^{γ}-D-A_2bu-Gly-Phe-Leu-] (**2**) with that of its diastereomer, Tyr-c[-N^{γ}-L-A_2bu-Gly-Phe-Leu-], which had previously been synthesized by an analogous route.[11] In the [^{3}H]-naloxone binding assay, Tyr-c[-N^{γ}-L-A_2bu-Gly-Phe-Leu-] was found to be 200 times less potent than **2** under conditions that exclude enzymatic degradation.[11] On the basis of this finding it can be concluded that less than 0.5% racemization occurred during coupling of N^{α}-Tos-N^{γ}-Boc-L-A_2bu. Treatment of the resin with HF at 0 °C for 1 h yielded the tetrapeptide with intact tosyl protection. Cyclization was performed with diphenyl phosphorazidate (DPPA)[13] at high dilution (0.1 mM). Ring closure usually occurred smoothly, except in the case of the precursor for compound **1**, which required a longer reaction time. Removal of the tosyl group with sodium in liquid ammonia provided the cyclic tetrapeptides or tripeptide in depro-

(11) J. DiMaio and P. W. Schiller, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7162 (1980).

(12) B. Belleau and G. Malek, *J. Am. Chem. Soc.*, **90**, 1651 (1968).

(13) T. Shioiri, K. Ninomiya, and S. Yamada, *J. Am. Chem. Soc.*, **94**, 6203 (1972).

Table II. Inhibitory Effects of Peptide Analogues and [Leu^5]enkephalin on the Binding of [^{3}H]Naloxone and [^{3}H][D-Ala^2,D-Leu^5]enkephalin in Rat Brain Homogenates[a]

		[^{3}H]naloxone		[^{3}H][D-Ala^2,D-Leu^5]enkephalin		
no.	compound	IC_{50}, nM	K_i, nM	IC_{50}, nM	K_i, nM	$K_i(\delta)/K_i(\mu)$
1	H-Tyr-c[-N^β-D-A_2pr-Gly-Phe-Leu-]	95.8 ± 18.4	53.2 ± 10.2	118 ± 39	53.6 ± 17.7	1.01
1a	H-Tyr-D-Ala-Gly-Phe-Leu-NH_2	3.97 ± 0.08	2.21 ± 0.04	5.51 ± 1.73	2.50 ± 0.78	1.13
2	H-Tyr-c[-N^γ-D-A_2bu-Gly-Phe-Leu-]	24.9 ± 6.2	13.8 ± 3.4	253 ± 36	115 ± 16	8.33
2a	H-Tyr-D-Abu-Gly-Phe-Leu-NH_2	10.1 ± 0.2	5.61 ± 0.11	8.94 ± 3.21	4.06 ± 1.46	0.72
3	H-Tyr-c[-N^δ-D-Orn-Gly-Phe-Leu-]	56.6 ± 12.2	31.4 ± 6.7	221 ± 83	100 ± 38	3.18
3a	H-Tyr-D-Nva-Gly-Phe-Leu-NH_2	5.89 ± 0.17	3.27 ± 0.09	6.81 ± 3.95	3.10 ± 1.80	0.95
4	H-Tyr-c[-N^ϵ-D-Lys-Gly-Phe-Leu-]	22.4 ± 4.1	12.4 ± 2.7	32.2 ± 8.8	14.6 ± 4.0	1.18
4a	H-Tyr-D-Nle-Gly-Phe-Leu-NH_2	4.08 ± 0.51	2.27 ± 0.28	9.57 ± 3.36	4.35 ± 1.53	1.92
5	H-Tyr-c[-N^ϵ-D-Lys-Gly-Phe-DLeu-]	8.51 ± 1.05	4.73 ± 0.58	47.8 ± 1.3	21.7 ± 0.6	4.59
6	H-Tyr-c[-N^ϵ-D-Lys-Gly-Phe-]	5.58 ± 0.19	3.10 ± 0.11	98.3 ± 5.5	44.7 ± 2.5	14.4
7	[Leu^5]enkephalin	49.8 ± 10.2	27.7 ± 5.7	17.7 ± 2.1	8.05 ± 0.96	0.291

[a] Mean of three determinations ± SEM.

tected form. Coupling to Z-L-Tyr-OH, followed by hydrogenolysis, afforded the final products **1–6**.

Results and Discussion

The results of the GPI and MVD bioassays are presented in Table I. In the GPI assay, all cyclic analogues are much more potent than [Leu^5]enkephalin. With the exception of compound **3**, increasing potency is observed with growing length of the side chain in position 2. Highest potency (100 times that of [Leu^5]enkephalin) is displayed by analogue **5** which contains Leu^5 in the D configuration; the cyclic des-Leu^5-analogue (**6**) was also found to be very potent. The open-chain analogues **2a**, **3a**, and **4a** uniformly showed about 10 times higher potency than [Leu^5]enkephalin, whereas **1a** was somewhat more potent. Comparison of the cyclic analogues **1–4** with their linear correlates (**1a–4a**) reveals that in the case of compounds **2** and **4** cyclization enhances potency on the GPI, while analogues **1** and **3** are less potent than their linear counterparts. The effect of all cyclic and linear analogues was completely naloxone reversible. Apparent dissociation constants (K_e) for naloxone as antagonist were found to be relatively uniform, with all values lying between those observed with levorphanol (0.127 ± 0.038 nM) and with [Leu^5]enkephalin (1.53 ± 0.43 nM).

In the MVD assay, all cyclic analogues show significantly lower potency than [Leu^5]enkephalin, and the cyclic compounds **1–4** are all less potent than their corresponding open-chain analogues (**1a–4a**). As in the GPI assay, highest potency within the series of cyclic compounds is observed with analogue **5**, which contains Leu^5 in the D configuration. The effects of all analogues on the MVD are naloxone reversible, and dissociation constants (K_e) for naloxone as antagonist were again determined. Interestingly, the cyclic analogues (**1–4**) showed K_e values similar to that of the μ-type agonist levorphanol, whereas with their linear counterparts (**1a–4a**), K_e values approaching that of [Leu^5]enkephalin are observed. As a possible interpretation of this finding, it can be suggested that the cyclic analogues interact preferentially with μ receptors on the MVD, while the linear peptides selectively bind to δ receptors in this preparation (cf. ref 7). Alternatively, different modes of binding to a single receptor[14] could be the reason for the difference in the naloxone K_e values observed between the cyclic and linear analogues. However, recently performed cross-protection studies[15,16] provided evidence for the existence of at least two distinct classes of opiate receptor in brain tissue.

The ratio of the IC_{50} values obtained in the MVD and GPI assays is usually taken as a measure to indicate μ- or δ-receptor selectivity.[3] The cyclic analogues **1–4** show increasing IC_{50} (MVD)/IC_{50} (GPI) ratios with growing length of the side chain in position 2, and the ratio obtained for analogue **4** is twice as high as that determined for the typical μ-receptor agonist levorphanol. No receptor selectivity is observed with the open-chain analogues (**1a–4a**), which all show ratios close to unity.

In the binding assay based on displacement of the μ-receptor-selective radiolabel [^{3}H]naloxone from rat brain membranes (Table II), the cyclic analogues show the same rank order of potency as in the GPI assay, with the exception of the analogue lacking Leu^5 (**6**), which is the most potent in this assay. However, in contrast to the GPI assay, potencies relative to [Leu^5]enkephalin are not dramatically increased and in two cases (compounds **1** and **3**) even slightly lower. The observation that in the [^{3}H]-naloxone binding assay all cyclic analogues are significantly less potent than the corresponding linear analogues constitutes another discrepancy with the results obtained in the GPI assay. This is exemplified most typically by Tyr-c[-N^ϵ-D-Lys-Gly-Phe-Leu-] (**4**), which in the [^{3}H]naloxone binding assay is five times less potent than its linear correlate, Tyr-D-Nle-Gly-Phe-Leu-NH_2 (**4a**), whereas in the GPI assay an exactly reversed potency relationship between the two compounds is observed. In qualitative agreement with the MVD assay, all cyclic analogues were found to be less potent than [Leu^5]enkephalin in the binding assay using [^{3}H][D-Ala^2,D-Leu^5]enkephalin as radioligand. Furthermore, the cyclic analogues **1–4** showed 3 to 30 times lower affinity than their linear counterparts **1a–4a** in this δ-receptor-selective binding assay. Evaluation of the binding data in terms of inhibition constants (K_i)[17] and calculation of the K_i (δ)/K_i (μ) ratios reveals preference for μ receptors over δ receptors in the case of most cyclic analogues, with the exception of compounds **1** and **4**, whereas the linear analogues (**1a–4a**) again show no such preference.

It is difficult to present a definite explanation for the several discrepancies observed between the bioassays and the binding assays. The fact that two of the cyclic analogues, compounds **1** and **4**, show moderate affinity in the [^{3}H]naloxone binding assay but high potency in the GPI assay, while their linear correlates (**1a** and **4a**) display high affinity and moderate to low potency on the GPI, might indicate that in these two analogues cyclization decreases affinity but *enhances efficacy* dramatically. The high

(14) P. S. Portoghese, *Acc. Chem. Res.*, 11, 21 (1978).
(15) L. E. Robson and H. W. Kosterlitz, *Proc. R. Soc. London, Ser. B*, **205**, 425 (1979).
(16) J. R. Smith and E. J. Simon, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 281 (1980).
(17) Y. C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, **22**, 3099 (1973).

efficacies of 1 and 4 might be brought about by formation of a more productive peptide–receptor complex through induction of a conformational change in the receptor molecule. Thus, the differences between the results of the bioassay and the binding assay could be explained by a divergent effect of cyclization on affinity and efficacy. As an alternative explanation, the possibility that some of the cyclic analogues could interact with yet a different receptor distinct from the μ or δ type has also to be considered. Future binding studies with radiolabeled cyclic analogues may shed light on this issue.

Surprisingly, inversion of the configuration of the leucine residue in position 5 produces an analogue (**5**) with 2–3 times higher potency in the GPI assay and in the [^{3}H]-naloxone binding assay than its diastereomer with Leu^5 in the L configuration. This is in contrast to the situation with linear analogues, where inversion of the configuration in position 5 produces a decrease in potency in μ-receptor-selective assays. On the other hand, in the MVD assay, **5** shows the highest potency among the cyclic analogues but is still less potent than [Leu^5]enkephalin and also less potent than [Leu^5]enkephalin and 4 in the [^{3}H][D-Ala^2,D-Leu^5]enkephalin binding assay. The des-Leu^5 analogue (**6**) was found to be the most potent of all cyclic compounds in the [^{3}H]naloxone binding assay but, in general, showed a pharmacological profile similar to analogues **4** and **5**. The similar results obtained with analogues **4–6** in the binding assays and bioassays indicate that inversion of the configuration of the residue in position **5** or its omission has little effect on the overall μ character observed with this type of cyclic analogue.

Conclusions

Compared to [Leu^5]enkephalin, all cyclic analogues show considerable μ-receptor selectivity. In particular, compound **4** has a higher IC_{50} (MVD)/IC_{50} (GPI) ratio than any of the substituted tripeptide amides[8] or tetrapeptide amides.[7] The only opioid peptide known to date with a higher IC_{50} ratio than **4** is morphiceptin,[18] which, however, is a relatively weak agonist in the GPI assay. The observed μ-receptor selectivity is obviously a consequence of the low affinity of the cyclic analogues for the δ receptors, which is reflected in the low potencies obtained in the MVD assay. On the other hand, μ-receptor affinity comparable to or higher than that of [Leu^5]enkephalin is maintained by these compounds.

It is interesting to note that the open-chain analogues (**1a–4a**) show almost uniform behavior in all assays and, most importantly, none of them displays any preference for either μ or δ receptors. Linear enkephalins are flexible molecules, and the interpretation of a theoretical conformational analysis of enkephalin analogues[19] in relation to conformational studies by fluorescence energy transfer measurements[20] provided evidence for the existence of a conformational equilibrium involving a number of quite different conformers in aqueous solution. Because of their inherent flexibility, the linear enkephalins can adapt equally well to the μ- and δ-receptor surface by undergoing a conformational change in the course of the binding process. Evidence for such a conformational change has been obtained from kinetic studies of opiate receptor binding.[21] Comparison of compounds 1–4 with compounds **1a–4a** is of considerable interest, since in each case the cyclic analogue and its linear correlate are distinguished from each other by the opening or closing of a single carbon–nitrogen bond. The obtained results indicate that the high μ-receptor selectivity of some of the cyclic analogues is a direct consequence of the conformational restriction obtained by ring closure and, furthermore, permits the unambiguous conclusion that μ- and δ-opiate receptors have different conformational requirements.[22] Obviously, the conformational constraint in the cyclic analogues is quite compatible with the μ-receptor topography, whereas it leads to an unfavorable interaction with δ receptors.

The demonstrated difference in opiate receptor topography permits variation in receptor selectivity by changing the type or degree of conformational restriction. Thus, substitution of D-cysteine in position 2 and of D- or L-cysteine in position 5 of enkephalin, followed by disulfide bond formation, resulted in two cyclic analogues showing very high potency but less receptor selectivity.[23] Obviously, the cystine analogues contain a different ring structure of a somewhat more flexible type. The cyclic analogues 1–4 of the present series are distinguished from one another by a growing number of methylene groups in the bridging side chain in position 2 and, therefore, by an increasing flexibility of the peptide backbone, which allows for subtle variation in the positioning of the important side chain of phenylalanine in position 4 relative to the tyramine moiety in position 1. On the other hand, within the series of the open-chain peptides (**1a–4a**), all analogues can assume the same backbone conformation; therefore, the same spatial disposition of the Phe^4 side chain relative to Tyr^1 can be obtained. This may explain the uniformity in the pharmacological profile observed with the latter series. Among the cyclic analogues, the conformational constraint is most severe in compounds 1 and 2, which contain a 13- and 14-membered ring structure, respectively. Thus, with the latter structures, formation of a 4 → 1 or 5 → 2 hydrogen-bonded β_I bend[24-26] and of the β bend[27] stabilized by two antiparallel H bonds between Tyr^1 and the Phe^4 is not possible. However, in the analogues with larger ring size (**3** and **4**), formation of these various bends becomes feasible.

In conclusion, the preparation of cyclic enkephalin analogues has several interesting implications, among which the enhanced stability against enzymolysis and the obtained insight into the receptor-bound conformation have already been mentioned.[11] Perhaps the most important aspect revealed in this study is the possibility of manipulating opiate receptor selectivity through subtle variation in conformational restriction.

Experimental Section

Chemistry. All compounds had NMR and IR spectra consistent with their respective structure. ^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer at 90 MHz in $CDCl_3$, D_2O, or D_2O/AcOH-d_6, and chemical shifts are reported in parts

(18) K.-J. Chang, A. Killian, E. Hazum. P. Cuatrecasas, and J.-K. Chang, *Science*, **212**, 77 (1981).
(19) J.-P. Demonte, R. Guillard, and A. Englert, *Int. J. Pept. Protein Res.*, **18**, 478 (1981).
(20) P. W. Schiller, C. F. Yam, and J. Prosmanne, *J. Med. Chem.*, **21**, 1110 (1978).
(21) R. Simantov, S. R. Childers, and S. H. Snyder, *Eur. J. Pharmacol.*, **47**, 319 (1978).
(22) P. W. Schiller and J. DiMaio, *Nature (London)*, **297**, 74 (1982).
(23) P. W. Schiller, B. Eggimann, J. DiMaio, C. Lemieux, and T. M.-D. Nguyen *Biochem. Biophys. Res. Commun.*, **101**, 337 (1981).
(24) A. F. Bradbury, D. G. Smyth, and C. R. Snell, *Nature (London)*, **260**, 165 (1976).
(25) B. P. Roques, C. Garbay-Jaureguiberry, R. Oberlin, M. Anteunis, and A. K. Lala, *Nature (London)*, **262**, 778 (1976).
(26) C. R. Jones, W. A. Gibbons, and V. Garsky, *Nature (London)*, **262**, 779 (1976).
(27) G. D. Smith and J. F. Griffin, *Science*, **199**, 1214 (1978).

per million (δ) downfield from Me_4Si or 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (TSP) as internal standard. IR spectra were obtained with a Beckman IR 4240 spectrophotometer by the KBr pellet technique. Mass spectra were recorded on a AEI-MS-902 spectrometer at 70 eV (direct-inlet method). Optical rotations were measured with a Perkin-Elmer 141 polarimeter in solvents and at concentrations specified below. Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. HPLC experiments were performed on a Waters LC instrument with a μ-Bondapak C_{18} column. For TLC, precoated plates (silica gel G, 250 μm, Analtech, Newark, DE) were used in the following solvent systems (all v/v): (1) *n*-BuOH/AcOH/H_2O (BAW) (4:1:5, organic phase), (2) *n*-BuOH/pyridine/AcOH/H_2O (BPAW) (15:10:3:12), and (3) *s*-BuOH/3% NH_4OH (SH) (100:44). For amino acid analysis, peptides were hydrolyzed in 6 N HCl for 24 h at 110 °C in deaerated tubes, and the hydrolysates were analyzed on a Beckman Model 121C amino acid analyzer equipped with a system AA computing integrator. Elemental analyses were performed by Canadian Microanalytical Service Ltd., Vancouver, B.C., Canada. The C, H, N values obtained for all peptide analogues were considerably lower than the calculated ones. Fluorine analysis of **6** revealed the presence of two trifluoroacetic acid molecules in addition to the acetate counterion. It thus appears that after HPLC purification in methanol/0.01 M trifluoroacetic acid (see below) all peptide analogues were isolated as either bis- or tris(trifluoroacetate)s. Salt formation between the amide nitrogens and trifluoroacetate represents a reasonable explanation for these adducts. In similar observations it has been found that amide nitrogens are basic enough to form salts with HBr,[28] and that the cyclic urea group of biotin forms a trifluoroacetate salt.[29]

N^{α}-*p*-Toluenesulfonyl-N^{β}-(*tert*-butyloxycarbonyl)-D-diaminopropionic Acid. N^{α}-Tos-D-A_2bu (1.5 g, 5.8 mmol), obtained by Hofmann rearrangement of N^{α}-Tos-D-Asn as described,[30] was added to 25 mL of 2 N NaOH, and the solution was diluted with 25 mL of dioxane. Five milliliters of Boc azide in 5 mL of dioxane was added while maintaining the pH at approximately 9. After a reaction time of 18 h, the bulk of the dioxane was evaporated in vacuo, and the concentrate was diluted with 100 mL of cold H_2O. The solution was extracted with ether and, subsequently, the aqueous extract was acidified with cold 1 N HCl and extracted with chloroform. The combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo. Crystallization from ether afforded 1.0 g (50% yield) of a white solid: mp 123–124 °C; $[\alpha]^{20}_D$ +68.5° (*c* 4.0, 1 N NaOH) [lit.[31] L enantiomer: mp 125–129 °C; $[\alpha]^{22}_D$ −71.1° (*c* 4.0, 1 N NaOH)]; NMR ($CDCl_3$) δ 1.2 [s, 9 H, $(CH_3)_3C$], 2.3 (s, 3 H, CH_3), 3.0 (br, 2 H, CH_2), 3.8 (m, 1 H, CH), 5.8 (br, 1 H, NH), 6.7 (br, 1 H, NH), 7.5 (q, 4 H, aromatic). Anal. ($C_{15}H_{22}N_2O_6S$) C, H, N.

N^{α}-*p*-Toluenesulfonyl-N^{γ}-(*tert*-butyloxycarbonyl)-D-diaminobutyric Acid. N^{α}-Tos-D-A_2bu (1.5 g, 5.5 mmol), prepared by Hofmann degradation of N^{α}-Tos-D-Gln (cf. ref 30), was treated as described above to give 1 g (50% yield) of a solid after crystallization from ether: mp 139–141 °C; $[\alpha]^{20}_D$ +48° (*c* 2.0, 1 N NaOH) [the corresponding L enantiomer had mp 136–140 °C and $[\alpha]^{20}_D$ −47.1° (*c* 2.0, 1 N NaOH)]; NMR ($CDCl_3$) δ 1.3 [s, 9 H, $(CH_3)_3C$], 1.9 (br, 2 H, CH_2C), 2.3 (s, 3 H, CH_3), 3.2 (br, 2 H, CH_2N), 3.8 (m, 1 H, CH), 5.8 (d, 1 H, NH), 6.7 (br, 1 H, NH), 7.5 (q, 4 H, aromatic). Anal. ($C_{16}H_{24}N_2O_6S$) C, H, N.

N^{α}-*p*-Toluenesulfonyl-N^{δ}-(benzyloxycarbonyl)-D-ornithine. N^{δ}-Z-D-Orn (2.66 g, 10 mmol), obtained by the copper complex method,[32] was dissolved in 200 mL of 1 N NaOH, and after cooling to 0 °C, 2.5 g of tosyl chloride in 100 mL of ether was added. Another addition of 2.5 g of tosyl chloride was made after 18 h. After a total reaction time of 24 h, the layers were separated, and the aqueous phase was extracted with ether. The aqueous phase was chilled, acidified with 1 N HCl, and extracted further with ethyl acetate. The combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The residue was recrystallized from ether/petroleum ether to afford 2 g of a solid (52% yield): mp 118–120 °C; $[\alpha]^{20}_D$ + 39.5° (*c* 2.0, 1 N NaOH); NMR ($CDCl_3$) δ 1.6 (m, 4 H, CH_2CH_2), 2.3 (s, 3 H, CH_3), 3.1 (m, 2 H, CH_2N), 3.8 (m, 1 H, CH), 5.0 (s, 2 H, CH_2), 5.7 (d, 1 H, NH), 7.3 (s, 5 H aromatic), 7.4 (q, 4 H aromatic), 8.7 (br, 1 H, COOH). Anal. ($C_{20}H_{24}N_2O_6S$) C, H, N.

N^{α}-*p*-Toluenesulfonyl-N^{ϵ}-(benzyloxycarbonyl)-D-lysine. N^{ϵ}-Z-D-Lys (150 mg, 0.54 mmol), prepared by the copper complex method,[32] was suspended in 50 mL of H_2O and brought into solution by the addition of 2 N NaOH. Treatment and workup as described above afforded 180 mg of a white solid (78% yield): mp 119–121 °C; $[\alpha]^{20}_D$ +41° (*c* 2.4, 1 N NaOH); NMR ($CDCl_3$) δ 1.0–1.7 [m, 6 H $(CH_2)_3$], 2.2 (s, 3 H, CH_3), 3.0 (br, 2 H, CH_2N), 3.8 (q, 1 H, CH), 5.1 (s, 2 H, CH_2), 5.6 (d, 1 H, NH), 7.2 (s, 5 H, aromatic), 7.4 (q, 4 H, aromatic), 9.6 (s, 1 H, COOH). Anal. ($C_{21}H_{26}N_2O_6S$), C, H, N.

Boc-D-αAbu, Boc-D-Nva, and Boc-D-Nle were prepared by the method of Schwyzer et al.[33] All three products were obtained as oils in 80–90% yield. Other Boc amino acids were purchased from Bachem, Inc., Torrance, CA.

H-Tyr-cyclo[-N^{β}-D-A_2pr-Gly-Phe-Leu-] (1). N^{α}-Tos-D-A_2pr-Gly-Phe-Leu-OH was prepared by the solid-phase method. Reaction of the cesium salt of Boc-Leu with chloromethyl resin[34] (1% cross-linked, 1–2 mequiv of Cl/g, Pierce, Rockford, IL) resulted in a substitution of 0.50 mmol of Boc-Leu per gram of resin. The protocol of the synthesis was the following: (1) CH_2Cl_2, 1 min × 3, EtOH, 1 min; (2) trifluoroacetic acid (50% in CH_2Cl_2), 1 h; (3) CH_2Cl_2, 1 min × 3, EtOH, 1 min; (4) diisopropylethylamine (10% in CH_2Cl_2), 15 min, EtOH, 1 min; (5) CH_2Cl_2, 1 min × 4, EtOH, 1 min; (6) CH_2Cl_2 and addition of Boc (or Tos) amino acid (10% excess) and EEDQ (10% excess), mixing for 15 h; (7) CH_2Cl_2, 1 min × 3, EtOH, 1 min. Treatment of the tetrapeptide resin with HF (20 mL per gram of resin) at 0 °C in the presence of anisole (1 mL per gram of resin), extraction with 15% AcOH, and lyophilization afforded 560 mg of a white fluff. The product was purified by ion-exchange chromatography on a SP-Sephadex G-25 column (2 × 70 cm) using a linear gradient of 0.1–0.4 N NH_4OAc in 1 N AcOH. Subsequent gel filtration on a Sephadex G-25 column (2 × 70 cm) provided 370 mg of a homogeneous product (66% yield). An NMR spectrum revealed that the tosyl group had remained intact.

The product (0.66 mmol), dissolved in 50 mL of dry DMF, was added over a period of 3 h to a cold solution (dry ice bath) of 0.2 mL of triethylamine and 0.3 mL of DPPA in 500 mL of DMF. The cyclization reaction was monitored by TLC and judged to be complete after 24 h. The solution was then evaporated in vacuo, and the residue was partitioned between 500 mL of ethyl acetate and 100 mL of 1 N HCl. The organic phase was extracted sequentially with 100 mL of 1 N HCl × 3, 100 mL of 5% $NaHCO_3$ × 3 and 100 mL of H_2O. After the extract was dried (Na_2SO_4) and the organic phase was evaporated, trituration with anhydrous ether yielded 200 mg of a white solid. Subsequently, the product was taken up in 150 mL of liquid NH_3, and Na chips were added until the appearing blue color persisted for at least 5 min. Evaporation of the ammonia left a residual solid that was subjected to ion-exchange chromatography as described above. Fractions corresponding to the major peak were lyophilized, and the resulting product was desalted by reversed-phase chromatography on an octadecasilyl column[35] with a linear gradient of 20–80% methanol in 1% trifluoroacetic acid. Lyophilization provided 60 mg (20% yield) of a fluffy powder, which was judged homogeneous by TLC [R_f 0.45 (BAW)].

The cyclic tetrapeptide (46 mg, 0.1 mmol), dissolved in 20 mL of DMF, was treated with 16 μL (0.13 mmol) of triethylamine, 32 mg (0.13 mmol) of EEDQ, and 40 mg (0.13 mmol) of N^{α}-Z-L-Tyr. After a reaction time of 18 h, the solution was evaporated in vacuo. The residue was dissolved in 100 mL of ethyl acetate

(28) M. Bodanszky and V. DuVigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
(29) M. Bodanszky and D. T. Fagan, *J. Am. Chem. Soc.*, **99**, 235 (1977).
(30) J. Rudinger, K. Poduska, and M. Zaoral, *Collect. Czech. Chem. Commun.*, **25**, 2022 (1960).
(31) W. Broadbent, J. S. Morley, and B. E. Stone, *J. Chem. Soc. C*, 2632 (1967).
(32) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).
(33) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).
(34) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(35) P. Böhlen, F. Castillo, N. Ling, and R. Guillemin, *Int. J. Pept. Protein Res.*, **16**, 306 (1980).

and extracted sequentially with 100mL portions of 2 N HCl × 3, 5% $NaHCO_3$ × 3, and H_2O. The organic phase was dried (Na_2SO_4) and evaporated in vacuo. The obtained residue crystallized upon addition of anhydrous ether. The product (60 mg) was hydrogenated for 10 h in 50% AcOH with 5% Pd/C at 20 psi H_2. Following filtration of the catalyst and lyophilization, final purification was achieved by HPLC (20–50% methanol (linear gradient)) in 0.01 M trifluoroacetic acid. After a final desalting step on a Sephadex G-25 column, 20 mg (31% yield) of compound 1 was obtained as a homogeneous white fluff: TLC R_f 0.67 (BAW), 0.70 (BPAW), 0.55 (SH). Amino acid analysis gave Tyr, 0.88; A_2pr, 0.91; Gly, 1.00; Phe, 1.01; Leu, 1.03. Anal. Calcd for $C_{29}H_{38}N_6O_6 \cdot CH_3COOH \cdot 3CF_3COOH$: C, 45.87; H, 4.68; N, 8.67. Found: C, 46.73; H, 5.72; N, 8.20.

H-Tyr-cyclo[-N^{γ}-D-A_2bu-Gly-Phe-Leu-] (2). N^{α}-Tos-D-A_2bu-Gly-Phe-Leu-OH was prepared in 80% yield with the same resin and protocol as in the synthesis of 1. Cyclization in the usual manner and deprotection (Na/NH_3), followed by similar purification as in the case of 1, provided 100 mg (27% yield) of homogeneous cyclic tetrapeptide. The obtained material (47.7 mg, 0.1 mmol) was coupled to Z-L-Tyr in the usual manner to give 30 mg (50% yield) of homogeneous compound 2 after hydrogenolysis and purification: MS, m/e 580 (M^+); TLC R_f 0.63 (BAW), 0.70 (BPAW) 0.64 (SH). Amino acid analysis gave Tyr, 0.93; A_2bu, 1.03; Gly, 1.00; Phe, 1.00; Leu, 1.03. Anal. Calcd for $C_{30}H_{40}N_6O_6 \cdot HCl \cdot 2CF_3COOH$: C, 48.32; H, 5.13; N, 9.94. Found: C, 47.23; H, 5.43; N, 9.62.

H-Tyr-cyclo[-N^{δ}-D-Orn-Gly-Phe-Leu-] (3). Starting with 2.5 g of Boc-Leu-resin, N^{α}-Tos-D-Orn-Gly-Phe-Leu-OH was prepared in 77% yield as described for 1. Cyclization and deprotection, followed by purification, afforded 200 mg (48% yield) of cyclic tetrapeptide. This material (49 mg, 0.1 mmol) was coupled to Z-L-Tyr to afford 30 mg (50% yield) of product 3 after hydrogenolysis and purification: MS, m/e 594 (M^+); TLC R_f 0.68 (BAW), 0.70 (BPAW), 0.63 (SH). Amino acid analysis gave Tyr, 0.86; Orn, 1.12; Gly, 1.07; Phe, 1.00; Leu, 1.07. Anal. Calcd for $C_{31}H_{42}N_6O_6 \cdot CH_3COOH \cdot 3CF_3COOH$: C, 46.99; H, 4.95; N, 8.43. Found: C, 46.83; H, 5.71; N, 8.68.

H-Tyr-cyclo[-N^{ϵ}-D-Lys-Gly-Phe-Leu-] (4). N^{α}-Tos-D-Lys-Gly-Phe-Leu-OH was prepared in 80% yield from 2 g of the same Boc-Leu-resin described above. Cyclization and deprotection, followed by purification, provided 150 mg (40% yield) of cyclic tetrapeptide. Coupling of this material to Z-L-Tyr, followed by hydrogenolysis and purification, gave 80 mg (42% yield) of homogeneous compound 4: MS, m/e 608 (M^+); TLC R_f 0.67 (BAW), 0.70 (BPAW), 0.56 (SH). Amino acid analysis gave Tyr, 0.92, Lys, 1.00; Gly, 1.02; Phe, 0.95; Leu, 1.01. Anal. Calcd for $C_{32}H_{44}N_6O_6 \cdot CH_3COOH \cdot 3CF_3COOH$: C, 47.53; H, 5.09; N, 8.31; Found: C, 47.49; H, 5.70; N, 8.81.

H-Tyr-cyclo[-N^{ϵ}-D-Lys-Gly-Phe-D-Leu-] (5). N^{α}-Tos-D-Lys-Gly-Phe-D-Leu-OH was prepared in 70% yield from 2.3 g of Boc-D-Leu-resin (0.50 mmol Boc-D-Leu per gram of resin). Cyclization, deprotection, and purification provided 190 mg of cyclic tetrapeptide in 50% yield. This material (170 mg, 0.36 mmol) was coupled to Z-L-Tyr in the usual manner. Hydrogenolysis of the product, followed by purification, provided 110 mg (50% yield) of analogue 5 in solid form: MS, m/e 608 (M^+); TLC R_f 0.63 (BAW), 0.70 (BPAW), 0.58 (SH). Amino acid analysis gave Tyr, 0.86; Lys, 1.01; Gly, 1.00; Phe, 0.93; Leu, 1.02. Anal. Calcd for $C_{32}H_{44}N_6O_6 \cdot CH_3COOH \cdot 3CF_3COOH$: C, 47.53; H, 5.09; N, 8.31. Found: C, 47.42; H, 5.71; N, 8.76.

H-Tyr-cyclo[-N^{ϵ}-D-Lys-Gly-Phe-] (6). N^{α}-Tos-D-Lys-Gly-Phe-OH was prepared in 42% yield starting with 2 g of Boc-Phe-resin (0.42 mmol Boc-Phe per gram of resin). This material was processed as described for 1, to give 90 mg (68% yield) of cyclic tripeptide. Coupling to Z-L-Tyr, hydrogenolysis, and purification in the usual manner resulted in 45 mg (33% yield) of a homogeneous fluffy powder: MS, m/e 495 (M^+); TLC R_f 0.64 (BAW), 0.70 (BPAW), 0.60 (SH). Amino acid analysis gave Tyr, 0.96; Lys, 0.99; Giy, 1.00; Phe, 1.02. Anal. Calcd for $C_{26}H_{33}N_5O_5 \cdot CH_3COOH \cdot 2CF_3COOH$: C, 49.04; H, 5.02; N, 8.94; F, 14.55; Found: C, 48.95; H, 5.70; N, 9.43; F, 15.42.

[D-Ala^2,Leu^5]enkephalinamide (**1a**) was purchased from Peninsula Laboratories, San Carlos, CA. [D-Abu^2,Leu^5]enkephalinamide (**2a**), [D-Nva^2,Leu^5]enkephalinamide (**3a**), and [D-Nle^2,Leu^5]enkephalinamide (**4a**) were prepared by the solid-phase technique with benzhydrylamine resin (2% cross-linked, 0.4 mM titratable amine per gram of resin, Pierce, Rockford, IL). Peptide chains were assembled according to a protocol published elsewhere,[36] and HF cleavage was performed as described above. Crude products were purified by gel filtration on a Sephadex G-10 column and by subsequent HPLC [0.05 M NH_4OAc, pH 6/50% (v/v) MeOH]. After desalting by a second passage through the Sephadex G-10 column, products were obtained as homogeneous white lyophilisates in yields ranging from 20 to 30%.

[D-Abu^2,Leu^5]enkephalinamide (2a): TLC R_f 0.61 (BAW), 0.74 (BPAW), 0.51 (SH). Amino acid analysis gave Tyr, 0.87, Abu, 0.92; Gly, 1.02; Phe, 1.00; Leu, 1.06. Anal. Calcd for $C_{30}H_{42}N_6O_6 \cdot CH_3COOH \cdot 2CF_3COOH$: C, 49.66; H, 5.56; N, 9.65; Found: C, 48.79; H, 6.08; N, 10.25.

[D-Nva^2,Leu^5]enkephalinamide (3a): TLC R_f 0.63 (BAW), 0.76 (BPAW), 0.44 (SH). Amino acid analysis gave Tyr, 0.97; Nva, 1.09; Gly, 1.00; Phe, 1.01; Leu, 1.04. Anal. Calcd for $C_{31}H_{44}N_6O_6 \cdot CH_3COOH \cdot 2CF_3COOH$: C, 50.23; H, 5.70; N, 9.50; Found: C, 48.11; H, 6.13; N, 9.51.

[D-Nle^2,Leu^5]enkephalinamide (4a): TLC R_f 0.66 (BAW), 0.77 (BPAW), 0.57 (SH). Amino acid analysis gave Tyr, 0.95; Nle, 1.08; Gly, 1.00; Phe, 0.94; Leu, 1.05; Anal. Calcd for $C_{32}H_{46}N_6O_6 \cdot CH_3COOH \cdot 2CF_3COOH$: C, 50.78; H, 5.83; N, 9.35; Found: C, 49.74; H, 6.30; N, 10.03.

Bioassays. The assay based on inhibition of electrically induced contractions of the GPI[37] was carried out as reported in detail elsewhere.[38]

The MVD assay was performed essentially as described in the literature.[39] Briefly, adult, male albino mice (Swiss Webster, 30–50 g: Canadian Breeding Laboratories, Montreal) were killed by cervical dislocation, and the vasa deferentia were dissected out. After removal of extraneous fat and connective tissue, the vas was stripped of its associated blood vessel, and the semen was gently expressed from the lumen. The vas was then mounted under 0.5-g tension in a 5 mL organ bath containing warmed (37 °C), oxygenated (95% O_2, 5% CO_2), Mg^{2+}-free Krebs solution of the following composition [mM]: NaCl, 118; $CaCl_2$, 2.54; KCl, 4.75; KH_2PO_4, 1.19; $NaHCO_3$, 25; glucose, 11; L-tyrosine, 0.2. A modified Harvard apparatus stimulator delivered repetitive field stimulation through platinum wire ring electrodes at the top and bottom of the bath, consisting of twin, rectangular pulses (80 V, 0.15 Hz, 10-ms delay, 1.0-ms duration). Contractions of the muscle were recorded via a Hewlett-Packard Model FTA-1-1 force transducer connected to a Hewlett-Packard 7702B recorder. Determination of the reduction in the twitch height at various doses permitted the construction of log dose–response curves.

A log dose–response curve was determined with [Leu^5]enkephalin as standard for each ileum or vas preparation, and IC_{50} values of the enkephalin analogues being tested were normalized according to a published procedure.[40] K_e values for naloxone as antagonist were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed naloxone concentration.[41]

Binding studies with rat brain membrane preparations were carried out as reported elsewhere.[38] [^{3}H]Naloxone and [^{3}H][D-Ala^2,D-Leu^5]enkephalin at respective concentrations of 0.4 and 0.96 nM were used as radioligands, and incubations were performed at 0 °C for 1 h. K_i values were calculated based on Cheng and Prusoff's equation[17] using values of 0.5 and 0.8 nM for the dissociation constants of [^{3}H]naloxone and [^{3}H][D-Ala^2,D-Leu^5]enkephalin, respectively.

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(36) P. W. Schiller, C. F. Yam, and M. Lis, *Biochemistry*, **16**, 1831 (1977).
(37) W. D. M. Paton, *Br. J. Pharmacol.*, **12**, 119 (1957).
(38) P. W. Schiller, A. Lipton, D. F. Horrobin, and M. Bodanszky, *Biochem. Biophys. Res. Commun.*, **85**, 1332 (1978).
(39) G. Henderson, J. Hughes, and H. W. Kosterlitz, *Br. J. Pharmacol.*, **46**, 764 (1972).
(40) A. A. Waterfield, F. M. Leslie, J. A. H. Lord, N. Ling, and H. N. Kosterlitz, *Eur. J. Pharmacol.*, **58**, 11 (1979).
(41) H. W. Kosterlitz, and A. J. Watt, *Br. J. Pharmacol.*, **33**, 266 (1968).

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Conformational Requirements for Norepinephrine Uptake Inhibition by Phenethylamines in Brain Synaptosomes. Effects of α-Alkyl Substitution

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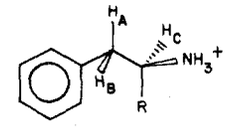
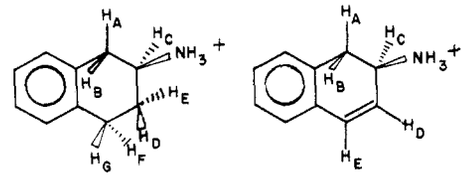
Amphetamine is a strong competitive antagonist of brain synaptosomal [^{3}H]norephinephrine ([^{3}H]NE) uptake. Its α-ethyl analogue is much less active, while 2-aminotetralin and 1,2-dihydro-2-aminonaphthalene, in which the α-ethyl group is tied to the aromatic ring, possess about the same inhibitory potency as amphetamine. The conformational properties of these compounds in solution were studied by ^{1}H and ^{13}C NMR methods. Only small differences between amphetamine and α-ethylphenethylamine hydrochlorides were observed in the relative rotamer populations due to rotation around the C_α–C_β bond of the side chain. In D_2O the gauche conformation is slightly favored, while in $CDCl_3$ the trans conformation is the predominant one. Conformational analysis of the α-ethyl group in α-ethylphenethylamine showed that this group exists in two equally populated conformations in both solvents. It is suggested that these conformations hinder the approach of α-ethylphenethylamine to the brain synaptosomal NE uptake sites.

Several phenethylamine analogues have been shown to inhibit the uptake of [^{3}H]norepinephrine (NE) by either brain synaptosomes[1,2] or heart tissue.[3] Amphetamine (**1**)



1 R = CH_3
2 R = C_2H_5



3 **4**

is known to be a strong competitive antagonist of synaptosomal [^{3}H]NE uptake. This inhibitory ability can vary drastically with relatively small structural modifications and is governed by strict stereoelectronic requirements.[1-7] Evidence from studies with rigid analogues suggests that the pharmacophoric conformation for interaction with the synaptosomal NE uptake sites is one in which the protonated amino group of **1** exists in a trans position with respect to the phenyl ring.[4-7]

In the present study we have examined the effect of α-alkyl substitution on the ability of the drug to inhibit [^{3}H]NE uptake in brain synaptosomes. We have investigated the conformations of α-ethylphenethylamine (**2**) 2-aminotetraline (**3**), and 1,2-dihydro-2-aminonaphthalene (**4**) (Figure 1) using ^{1}H NMR spectroscopy and have compared the results with already published data on the conformation of **1**.[8] The molecular flexibility of these compounds in D_2O was also examined by ^{13}C spin–lattice relaxation time (T_1) measurements.

Results and Discussion

Conformational Analysis. Amphetamine and α-Ethylphenethylamine. The three possible perfectly staggered conformers arising from rotation around the C_α–C_β bond for **1** and **2** are shown in Figure 1. An important ambiguity in the conformational analysis of α-substituted phenethylamines was the uncertainty in the assignment of the diastereotopic benzylic H_A and H_B protons in the ^{1}H NMR spectra. This problem was recently resolved for compound **1** through the stereospecific substitution of each of the two benzylic protons with a deuterium atom.[8] The ^{1}H NMR spectra of the hydrochlorides of the two β-*d* diastereomers in $CDCl_3$ showed that the H_A proton was more downfield than H_B. This was

(1) J. E. Harris and R. J. Baldessarini, *Neuropharmacology*, **12**, 669 (1973).
(2) G. M. Marquardt, V. DiStefano, and L. L. Ling, *Biochem. Pharmacol.*, **27**, 1497 (1978).
(3) L. L. Iversen in "The Uptake and Storage of Noradrenaline in Sympathetic Nerves", Cambridge University Press, Cambridge, 1967, p 161.
(4) A. S. Horn and S. H. Snyder, *J. Pharmacol. Exp. Ther.*, **1980**, 523 (1972).
(5) J. Tuomisto, L. Tuomisto, and T. L. Pazdernik, *J. Med. Chem.*, **19**, 725 (1976).
(6) M. Bartholow, L. E. Eiden, J. A. Ruth, G. L. Grunewald, J. Siebert, and C. O. Rutledge, *J. Pharmacol. Exp. Ther.*, **202**, 532 (1977).
(7) G. E. Komiskey, F. L. Hus, F. J. Bossart, J. W. Fowble, D. D. Miller, and P. N. Patil, *Eur. J. Pharmacol.*, **52**, 37 (1978).
(8) J. J. Knittel and A. Makriyannis, *Tetratedron Lett.*, **22**, 4631 (1981).
(9) L. A. Najjar, M. J. Blake, Ph. A. Benoit, and M. C. Lu, *J. Med. Chem.*, **57**, 1401 (1978).
(10) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nulcear Magnetic Resonance Spectroscopy", Academic Press, New York, p 103.
(11) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy", Vol. 1, Pergamon Press, Oxford, p 280.
(12) A. Allerhand, D. Doddrell, and R. Komoroski, *J. Chem. Phys.*, **55**, 189 (1971).

0022-2623/82/1825-1438$01.25/0