ration to give methyl 2-[(benzyloxycarbonyl)amino]-4-bromobutanoate as an oily solid, which was crystallized from hexane–ether to give 14.3 g (80–82%) of a white powder: mp 87–88 °C (lit.[28] mp 87–89 °C); 60-MHz NMR ($CDCl_3$) δ 2.0–2.55 (m, 2 H, CH_2CH_2Br), 3.4 (t, 2 H, J = 7 Hz, CH_2Br), 3.75 (s, 3 H, $COOCH_3$), 4.25–4.70 (m, 1 H, (MeOOC)(CBz(H)N)$CHCH_2$), 5.10 (s, 2 H, OCH_2Ph), 7.35 (s, 5 H, aromatic H). Anal. ($C_{13}H_{16}BrNO_4$).

Methyl 4-[Isopropoxy[(diisopropylphosphono)methyl]phosphinyl]-2-[(benzyloxycarbonyl)amino]butanoate (24a). Phosphonite **22** (1.0 g, 3.2 mM) and bromobutanoate **23** (1.1 g, 3.3 mM) were heated 110–112 °C for 1.5 h under argon. The evolution of a gas was observed, presumably isopropyl bromide. The reaction mixture was cooled and DMSO (25 mg, 3.3 mM) was added and the mixture heated to 60–65 °C for 2–3 h. The mixture was chromatographed on silica gel (methanol–ethyl acetate, 1:9) and gave 0.7 g (39%) of an oil: 360-MHz NMR ($CDCl_3$) δ 1.45–1.15 (m, 18 H, $OCH(CH_3)$), 1.84–2.45 (m, 6 H, $CH_2CH_2PCH_2P$), 3.7–3.78 (m, 3 H, $POCH(CH_3)$), 3.72 (s, 3 H, OCH_3), 4.3–4.4 (m, 1 H, $CH_2CH(NH)(COO)$), 5.08 (s, 2 H, OCH_2Ph), 5.95–5.98 (m, 1 H, NH), 7.32 (s, 5 H, aromatic H); mass spectrum, m/e (relative intensity) 535 (1), 477 (17), 435 (20), 418 (100), 393 (11). Anal. ($C_{23}H_{39}NO_9P_2$).

2-Amino-4-[(phosphonomethyl)hydroxyphosphinyl]butanoic Acid (9). To **24** (1.0 g, 1.9 mM) was added 40 mL of 6 N HCl and the mixture was refluxed for 30 h. The solution was then rotoevaporated and residue chromatographed on a 1.5 × 30 cm Dowex-50 X8 H+ (100–200 mesh) column eluted with water. Seventy (5 mL each) fractions were collected and the acidic and ninhydrin positive fractions were combined and lyophilized to give 350 mg (64%) of a white hygroscopic solid: 360-MHz NMR (D_2O) δ 1.7–1.85 (m, 2 H, $CHCH_2CH_2P$), 1.95–2.2 (m, 4 H, CH_2CH_2P), 3.76 (t, 1 H, (DOOC)(D_2N)$CHCH_2$). Anal. ($C_5H_{13}NO_7P_2 \cdot H_2O$) C, H, N.

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A 500-MHz Proton Nuclear Magnetic Resonance Study of μ Opioid Peptides in a Simulated Receptor Environment

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The structure–activity relationship of several μ selective opioid peptides has been evaluated on the basis of both experimental and theoretical approaches. The conformations of Tyr-D-Ala-Phe-Gly-NH_2, the tetrapeptide N-fragment of dermorphin, and two analogues have been studied in solution by ^1H NMR spectroscopy. The physicochemical environment inside the receptor has been simulated by complexing the peptides with a crown ether and dissolving the complexes in chloroform. The family of conformations derived from the NMR data possesses most of the features previously proposed for μ agonists and is fully consistent with an original model of the μ receptor based on the structures of many rigid opiates. As a simple test of this model, the synthesis of a linear peptide with significant μ activity in spite of the absence of Tyr[1] is reported.

A huge amount of work has been devoted to the structure–activity relationship of flexible opioid agonists, notably opioid peptides.[1,2] This work has not been decisive for our knowledge of the opioid receptors owing to the intrinsic difficulty of identifying the so-called "biologically active conformation" of a flexible molecule and also because it has been largely directed to the search of similarities between the conformations of flexible molecules and the rigid structure of a single opioid, i.e., morphine.[3–5]

This approach is understandable if one considers the historical importance of morphine, but it is not justified, at least in the case of some endogenous opioids, since these peptides interact preferentially with a different receptor (δ for enkephalins vs. μ for morphine). It is the goal of this paper to interpret the SAR of a series of μ opioid peptides. Thus it is essential to refer their conformation to a reliable μ receptor model. Several important features of the μ receptor have been already identified through comparisons of the structures of many opioid molecules.[6–10] Once again, however, some of these comparisons are biased by the attempt to fit the structures of even the most potent molecules to the three-dimensional shape of morphine, in spite of the fact that this molecule is *not* one of the most potent agonists.

Thus it seems useful to reexamine all existing evidence on the μ receptor site starting from two elementary considerations: (i) the "molecular molds" used to infer the shape of the site can only be the most active ones and their completely inactive homologues, but not compounds with intermediate potency; (ii) it is essential to use only conformationally rigid molecules or at least compounds in which a substantial portion of the molecule has a fixed conformation.

The identification of a likely biologically active conformation for μ opioid peptides was based on the NMR study of Tyr-D-Ala-Phe-Gly-NH_2 (the tetrapeptide N-fragment of dermorphin) in a lipophilic environment. Dissolution in $CDCl_3$ was made possible by complexation of the NH_3^+ group with a crown ether. This medium, although quite different from the natural receptor, is

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preferable to the very polar solvents usually employed in NMR studies, since it reproduces at least two features of the active site, i.e., a hydrophobic environment and the anchoring of the cation. The combination of these theoretical and experimental approaches gives a sound basis for the structure–activity relationship of several peptide opioids.

Basic Features of the μ Receptor

There are several compounds that present only minor modifications with respect to morphine and yet have an activity higher than (or at least comparable to) that of morphine. These compounds can be used to identify the minimal requirements of the morphine site:

(a) All morphine-like compounds are T-shaped, with the stem of the T formed by an aromatic ring and the head consisting of two fused cyclic alkanes or even a simple six-membered ring.

(b) The two ends of the head are limited by two basic groups, a hard base (henceforth called B_h) that in nearly all opioids is a tertiary amine and a soft base (henceforth called B_s) that may be OH, N_3, CO, etc. Attempts to define more precisely the relative positions of the aromatic ring and the hard base have led to contradictory results.[10] It is possible that the low directionality of the electrostatic interaction between the hard base and the complementary anionic subsite allows a large variability in the position of the T stem. A simple topological model may thus have more heuristic power than very rigid models.

(c) The aromatic ring contains one OH group, i.e., it is the phenolic moiety of tyramine.

The best way to improve this oversimplified view of the receptor can only be to resort to molecules whose structures resemble that of morphine alkaloids and that yet have activities orders of magnitude higher than that of morphine. A suitable group of such molecules is represented by fentanyl (400 times more active than morphine) and the related molecules sulfentanyl (×4500), R 30490 (×4600), and R 26800, i.e., methylfentanyl (×6700). Solid-state structures are available for these agonists.[11] The conformations in solution and (a fortiori) inside the receptor may be different, owing to the flexibility of some parts of these molecules, but their overall shape is dictated by the rigidity of the amide bond that assures a T-shaped structure to all four molecules, with a regular T instead of the skewed T typical of morphine, and the carbonyl oxygen acting as B_s.

The most important issue concerning the conformation of these molecules is the preference of the phenethyl (or thiophenethyl) moiety for the equatorial position with respect to the axial position. One of the various proposed receptor models[12] requires axial orientation if the phenethyl moiety has to play the role of the so-called F ring.

We chose to investigate this issue by means of a detailed conformational analysis of methylfentanyl, which is more active than its congeners and possibly even more rigid owing to the overcrowding imposed by the methyl group in the piperidyl ring.

Conformational Analysis

The method employed in the conformational analysis is based on the empirical evaluation of the internal energy arising from electrostatic, nonbonded atoms and intrinsic torsional, stretching, and bending contributions.

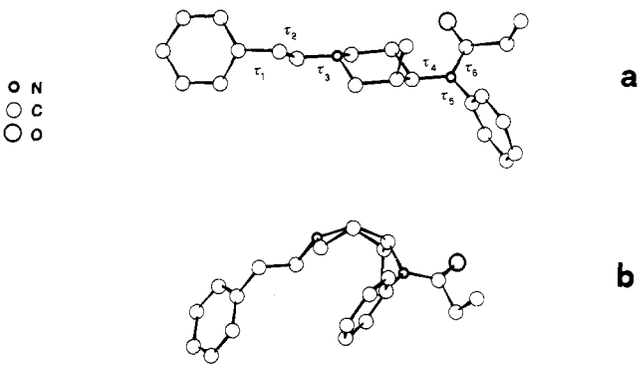

Figure 1. Molecular models of the minimum energy conformations of methylfentanyl with equatorial (a) and axial (b) N-substituent obtained from the full geometry minimization procedure. Only the torsion angles of the substituents are explicitly indicated.

The main results of the conformational analysis of the protonated form of methylfentanyl can be summarized as follows: the two aromatic moieties connected to the piperidine ring via bonds characterized by the τ_3 and τ_4 torsions (see Figure 1) have an essentially independent behavior, i.e., there is nearly no cross correlation in their motion; the two local conformations corresponding to τ_3 values of ca. 180 and ca. −60 are nearly isoenergetic (with differences in energy of less than 0.1 kcal/mol); conformations around τ_4 have minima corresponding to the ranges 5–10 and 110–115 with differences (in favor of the first range) of the order of 1–2 kcal/mol. Accordingly it can be said that the axial–equatorial preference of the phenethyl group is independent from the local conformations determined by τ_4 values and can be evaluated directly from the energy difference between local minima of conformations with two equatorial substituents (henceforth called $N_{eq}C_{eq}$) and with an equatorial substituent at carbon and an axial substituent at nitrogen (henceforth called $N_{ax}C_{eq}$).

The results of the energy minimization in the torsional subspace yield differences of internal energy between the axial–equatorial and the diequatorial conformers ($\Delta E_{ax,eq}$) of 77.7 and 48.4 kcal/mol respectively for the parameter sets of Hopfinger[13] and Lifson et al.[14]

The rather high absolute values reflect not only the preference for the equatorial conformation but also the nature of the functional dependence of the interaction potential from interatomic distances[15] and the lack of full geometry minimization.

Full geometry optimization has been performed by using AMBER[16] in the case of the most stable conformations containing equatorial and axial substituents. The energy difference between the two minima amounts to 6.1 kcal/mol, a figure that, although smaller than those found for fixed geometry, is large enough to prevent a significant population of the second conformer. It is essential to note that the smaller energy difference between axial and equatorial given by full geometry minimization (6.1 vs. 48.4

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Table I. Relevant Torsional Parameters of the Minimum Energy Conformations of Methylfentanyl and Corresponding Energy Differences (ΔE)

	force field	τ_1	τ_2	τ_3	τ_4	τ_5	τ_6	ΔE, kcal/mol
$N_{eq}C_{eq}$	*a*	−87.7	−171.3	71.0	9.8	103.9	179.9	
								77.7
$N_{ax}C_{eq}$	*a*	62.1	173.8	72.7	13.9	101.7	179.5	
$N_{eq}C_{eq}$	*b*	−88.9	−176.5	77.7	4.8	101.6	−177.3	
								48.4
$N_{ax}C_{eq}$	*b*	82.0	−179.3	79.0	13.2	99.7	180.6	
$N_{eq}C_{eq}$	*c*	−82.0	−175.4	64.8	−1.1	89.7	−178.2	
								6.1
twist-boat	*c*	82.0	150.4	188.0	−8.0	87.7	−178.2	

[a] Hopfinger, ref 13. [b] Lifson et al., ref 14. [c] Weiner et al., ref 16.

and 77.7 kcal/mol for Lifson and Hopfinger potentials, respectively) is obtained at the expense of a major departure of the piperidyl ring from the chair conformation.

In fact, the final conformation of the ring is very close to a twist-boat; accordingly we can consider the position of the phenethyl moiety as axial only from a topological point of view, i.e., in terms of its relationship with the methyl substituent in the chair conformation, but in the equilibrium conformation, the two bulkier substituents are both equatorial.

The final geometry of the equatorial conformer does not show relevant differences with respect to the conformations obtained in the cases of Lifson and Hopfinger parameters. Relevant torsional parameters are summarized in Table I. Figure 1 shows the corresponding molecular models.

It is reassuring to recall that the preference in favor of the equatorial orientation of the phenethyl group has been also established by an accurate conformational analysis of fentanyl[5] and by quantum mechanical calculations on phenethylmorphine and other opiates.[17] A recent experimental study lends further support in favor of the equatorial preference of the N-substituent even for opiates with the smallest possible substituent (i.e., a methyl group) like morphine and oxymorphone.[18]

μ Receptor Model

It is possible to use the shapes of fentanyl and its congeners to delineate the essential features of the μ receptor site, keeping in mind that both the ethyl group and the CH_2CH_2 moiety of the phenethyl group, however, are so flexible that their precise orientation inside the receptor cannot be inferred from any conformational analysis of isolated molecules. Thus, only the rigid parts of these molecules will be used for mapping whereas the other parts will be regarded as covering a larger volume around the positions determined by the conformational analysis. The model that emerges is the basic T-shaped structure of morphine-like compounds plus a hydrophobic subsite adjacent to the hard base of the T. Such a subsite is essentially equivalent to that proposed by Portoghese and co-workers[6] (i.e., their P subsite); but our model gives a more precise steric relationship between the P subsite and the T moiety.[19] Figure 2 shows a schematic drawing of the hypothetical opiate molecule resulting from a combination of the basic features of the receptor with the structures of the fentanyl-like molecules. In other words, we propose that highly active μ opioids can be characterized by essentially five features: a rigid T-shaped backbone (similar to that of morphine), a hard base, a soft base, a hydrogen bond donor on the stem of the T, and an aryl ring adjacent to the hard base (as in fentanyl). It seems correct to attribute the activity of opioid peptides to the possibility of using the aromatic rings of Tyr and Phe to interact with both the T and P subsites as postulated by Portoghese et al.[6] Even more significant is the observation of the higher μ agonism of dermorphin and of its N-fragments[20] with respect to enkephalins. In fact, the location of a Phe residue in the third position, as in dermorphin, favors the attainment of low-energy conformations in which the two aromatic rings are placed in a relative position very similar to that of the two aromatic rings of fentanyl (vide infra).

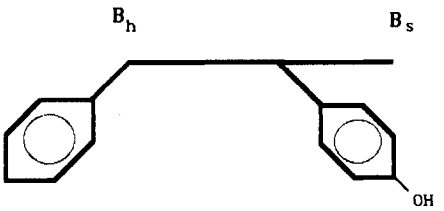

Figure 2. Schematic diagram of a hypothetical opioid agonist derived from a combination of the shapes of morphine-like molecules and the shapes of fentanyl-like molecules: the T shape is indicated by the heavier line; B_h and B_s indicate a hard base and a soft base, respectively; the phenyl ring represents the P subsite of Portoghese.[6]

On the other hand, enkephalins may use the Phe[4] ring to interact with the P subsite (but less efficiently than dermorphin owing to the larger separation between the rings) or as an F ring[12] to fit the δ receptor, with a conformation similar to that of oripavine, as originally suggested by Bradbury et al.[21]

It is worth mentioning that the phenethyl ring of fentanyl has been considered by some authors as the T ring of this opiate.[5] However, our identification of the anilino ring of fentanyl with the T stem has recently gained indirect support by the finding[22] that substitution of the phenethyl group of fentanyl with Tyr, Tyr-Gly, or Tyr-Gly-Gly deprives fentanyl of its activity. If the phenyl ring of the phenethyl group were the T stem, substitution with the phenolic ring of Tyr ought to increase the activity.

Conformation of μ Opioid Peptides

Owing to the extreme conformational flexibility of these peptides, it is not possible to try to identify one (minimum

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Table II. Chemical Shifts (δ) of the Studied Tetrapeptides Measured at 300 K

	P1			D-tetra		
	DMSO	$CDCl_3$	P2, DMSO	DMSO	$CDCl_3$	H_2O
F^1						
H_α	3.66	4.38	4.42			
H_β	2.77	3.20	3.08			
	2.53	2.86	2.78			
H_N	–	7.36	–			
Y^1						
H_α				[b]	4.23	3.52
H_β				2.77	2.94	2.85
				2.75	2.66	2.68
H_N				–	7.25	–
a^{2a}						
H_α	4.23	4.38	4.16	4.22	4.31	4.00
H_β	0.90	0.82	0.91	0.90	0.80	0.87
H_N	8.02	9.06	8.71	7.97	7.79	8.22
F^3						
H_α	4.50	4.66	4.42	4.49	4.61	4.59
H_β	3.10	2.98	2.95	3.09	3.20	3.19
	2.91	2.89	2.80	2.79	3.06	2.93
H_N	8.38	7.84	8.54	8.34	7.46	8.41
G^4						
H_α	3.37	3.92	3.66	3.68	3.84	3.82
			3.58		3.62	
H_N	8.02	8.22	8.37	8.26	8.37	8.34

[a] a = D-Ala. [b] Obscured by the water peak.

energy) active conformation by means of internal energy calculations for isolated molecules in vacuo. In fact conformational analyses[23] on the closely related molecules of enkephalins have only indicated broad classes of likely conformations. Most theoretical studies however consistently point to the relevance of folded conformations in which all hydrophobic side chains are exposed.[23] On the other hand, a preliminary NMR study of dermorphin and of its N-fragments in DMSO solution[24] indicated essentially random conformations. The main reason, besides the intrinsic flexibility, is that the solvent used does not favor the formation of folded structures. In fact DMSO is a well-known structure-breaking solvent for polypeptides, and it has been used in the past to study the so-called random coil conformation of many synthetic and natural poly-α-amino acids.[25] Owing to its high polarity, it can only be used to study the peptides in a state corresponding to that assumed in the transport medium (although it might be preferable to use water for this purpose).

The physiological environment in which the agonist–receptor interaction takes place can be inferred from the model previously described or from any of the models proposed by other authors:[6,9,12] it is invariably characterized by an anionic subsite and a hydrophobic cavity.

In order to approach the hypothetical physicochemical conditions inside the receptor we have looked for what might be called a "structured solvent medium", that is, a medium in which the terminal cation is anchored to a surface and the remaining part of the peptide is surrounded by apolar molecules. Such a situation can actually be achieved[26] by complexing the NH_3^+ group with 18-crown-6 ether and dissolving the complex in $CDCl_3$. Preliminary data on several peptides,[26] including one of those presented in this paper,[26c] showed that this solvent medium favors in all cases definite, nonrandom conformations. These conformations are not ipso facto bioactive forms; in fact they may well be artifacts due to complexation of the peptide with the crown ether. Nevertheless the lipophilic environment provided by $CDCl_3$ represents a better approximation to active-site environment than polar solvents. The choice of chloroform for the apolar environment was also motivated by the fact that it is the only solvent for which a detailed study on model peptides has furnished reliable numerical values for the temperature coefficients of the chemical shifts of NH protons involved in hydrogen bonds.[27] Besides Tyr-D-Ala-Phe-Gly-NH_2 (henceforth called D-tetra), the following two analogues were studied for comparison: Phe-D-Ala-Phe-Gly-NH_2 (henceforth called P1) and NH_2-C(=NH)-Phe-D-Ala-Phe-Gly-NH_2 (henceforth called P2), which were prepared in order to test the receptor model. P2 could not be studied as a complex of 18-crown-6 ether owing to its limited solubility in $CDCl_3$, probably due to the fact that the guanidinium ion is too large to be complexed efficiently.

All peptides were studied as trifluoroacetates in DMSO-d_6, and the corresponding crown ether complexes were studied in $CDCl_3$; D-tetra was also studied in H_2O, to compare the effects of the two polar environments on conformation.

Assignments in water and DMSO were based mainly on 1D experiments and on a comparison with literature values for similar peptides in the same solvents.

Assignments for the $CDCl_3$ solutions could not rely on any comparison with literature data since the spectra bear little resemblance even with those of the same compounds

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Table III. NH Temperature Coefficients (ppb/K)

	P1			D-tetra		
	DMSO	$CDCl_3$	P2, DMSO	DMSO	$CDCl_3$	H_2O
F^1	–	−0.67	–			
Y^1				–	−0.96	–
a^{2a}	−2.67	−3.30	0.20	−6.50	−2.82	−8.30
F^3	−4.97	−0.84	−4.00	−5.30	−3.06	−11.50
G^4	−4.86	−5.00	−4.20	−4.00	−5.36	−8.90

[a] a = D-Ala.

Table IV. α-CH Temperature Coefficients (ppb/K)

	P1			D-tetra	
	DMSO	$CDCl_3$	DMSO	DMSO	$CDCl_3$
F^1	2.10	0.36	0.86		
Y^1				–	2.23
a^{2a}	−0.90	0.50	0.22	0.00	−0.64
F^3	−0.34	0.36	0.86	0.21	1.00
G^4	−1.31	0.93	0.00	0.00	0.92

[a] a = D-Ala.

in other solvents and were based solely on 2D experiments (COSY, *J* resolved). All the assignments are summarized in Table II. The chemical shifts of both labile and nonlabile protons in DMSO-d_6 solutions have values typical of random conformations.[28] All systems showed NOE enhancements too small to be effectively used as conformational indicators. Nonetheless it was possible to gain conformational information from the temperature dependences of the labile protons.

Very small coefficients (i.e., of the order of 0–2 ppb K^{-1}) are usually taken as an indication that the corresponding amide proton is bound to an electronegative atom and remains bound in the temperature range examined, or at least that the proton is not accessible to solvent molecules.[29] No definite meaning can be attached, in most solvents, to coefficients higher than 2 ppb K^{-1}.

The coefficients in DMSO-d_6 solutions are close to an average value of −5 ppb K^{-1}, indicating that all protons are bound to solvent molecules, as could be expected from a collection of extended conformations. The behavior of D-tetra in H_2O is very similar to that in DMSO, thus confirming that DMSO solutions can effectively be used to study conformational preferences in a transport medium.

The $CDCl_3$ data are much more informative on the likely behavior of the peptides inside the receptor. A detailed study on model peptides[27] has shown that the NH protons exposed to $CDCl_3$ have temperature coefficients of the order of −2.4 ppb K^{-1}, NH's that are hydrogen bonded throughout the whole temperature range have coefficients smaller (in absolute value) than 2.4 ppb K^{-1}, and NH's that are bound at the lower temperatures but become free as a consequence of the increase in temperature have temperature coefficients larger (in absolute value) than 2.4 ppb K^{-1}. Table III summarizes the temperature coefficients of the NH (and NH_3^+ groups when observable) for all systems studied. Table IV shows the temperature coefficients of the α-CH groups, that were measured for comparison with those of the NH's of the corresponding residues. Abnormally large coefficients for nonlabile protons might reveal the presence of major conformational transitions; this is not the case for our compounds.

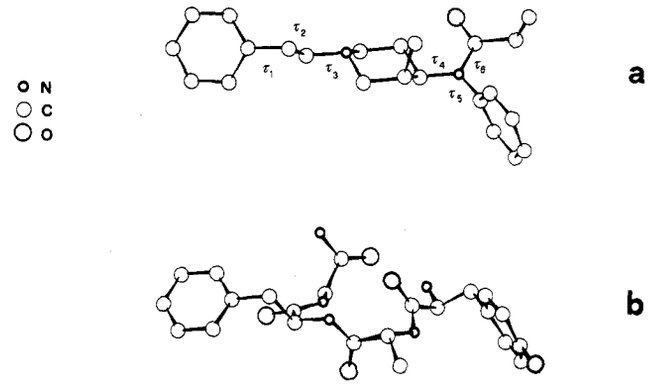

Figure 3. Comparison of the molecular models of methylfentanyl (a) and the type II′ β-turn conformation of D-tetra (b).

The coefficients of the terminal NH_3^+ groups of the crown ether complexes are all very small, indicating that the complexes remain stable throughout the temperature range. The other figures show that in both peptides at least one of the NH's is either inaccessible to the solvent or hydrogen bonded. In peptides of this size, however, all atoms are exposed to solvent to some degree,[23e] even when a folded conformation is adopted; accordingly we attributed all very small and very high values to hydrogen bonds. We considered only values differing by more than 50% from 2.4 ppb K^{-1} as clear indications of hydrogen-bonded NH's. The possibility of intermolecular hydrogen bonds was excluded by dilution studies and by working at rather low peptide concentrations. There is a single intramolecular hydrogen bond for D-tetra involving the NH of Gly^4. This data alone is not sufficient to determine the global conformation since Gly^4 NH may be linked to either $D\text{-}Ala^2$ or Tyr^1 carbonyls, leading to formation of a C_7 or a C_{10} ring, respectively, but elementary energetic considerations, based on solid-state studies,[30] favor a C_{10} ring involving the CO of Tyr^1.

In the case of P1 we have two intramolecular hydrogen bonds, but it is not possible to link both Gly^4 and Phe^3 NH's to carbonyl groups of the peptide. It seems much more likely that Gly^4 NH forms a C_{10} ring with Tyr^1 CO while Phe^3 NH binds to one of the crown oxygens; the NH chemical shifts however are close to those of D-tetra, a good indication that the conformations of these two peptides are similar. It is not possible to define the C_{10} rings more specifically in terms of different types of β-turns on the basis of our NMR data, but it is likely that P1 and D-tetra adopt a type II′ β-turn, owing to the influence of the chirality of the second residue.[30,23e]

SAR of Some μ Peptides

Figure 3 shows the comparison between the molecular model of methylfentanyl and that of D-tetra in the conformation imposed by formation of a type II′ β-turn stabilized by a hydrogen bond between the CO group of Tyr^1 and the NH group of Gly^4 (see Computational Methods). The similarity of the two models is striking and can form the basis for the interpretation of many apparently unrelated data both on analogues of D-tetra and on other opioids.

It has been observed that even a short lengthening and/or an increase of the flexibility of the backbone at the crucial position of the second residue of the tetrapeptide (e.g., substituting D-Ala with D- or L-*O*-Ala; *O*-Ala = 2-

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(aminooxy)propionic acid residue (-NHOCH(CH_3)CO-), leading to a destabilization of the β-turn, is paralleled by a 1000-fold decrease of the activity.[20b] That is, the relative μ potency measured by the guinea pig ileum test (GPI)[31] drops from 100 of Tyr-D-Ala-Phe-Gly-NH_2 to 0.1 of Tyr-D-*O*-Ala-Phe-Gly-NH_2. On the other hand, all modifications of the fourth residue (whose side chain has little influence on the stability of the β-turn) have little or no influence on activity.

Addition of a guanidino terminus always proved advantageous for activity; for instance, the relative GPI and mouse vas deferens (MVD, a preparation exhibiting particular sensitivity to δ receptor agonists[31]) potencies jump from 100 of the parent tetrapeptide to 460 and 582, respectively, in H_2N-C(=NH)-Tyr-D-Ala-Phe-Gly-NH_2.[32]

A simple explanation of this fact is that two extra single bonds between the positive nitrogen and the stem of the T make the structure closer to that of methylfentanyl with respect to the arrangement of all peptide opioids. However, it cannot be excluded that the increased polarizability of the cationic group also plays a role. It is possible that the different separation between the positive nitrogen and the T stem be compensated in opioid peptides by the presence of the OH group of Tyr and in part by other interactions in longer peptides. Thus, hydrophobic interactions are probably responsible for the fact that the relative GPI and MVD potencies of Tyr-D-Ala-Phe-Gly-NH-adamantyl become 2204 and 1127, respectively, and those of Tyr-D-Ala-Phe-Gly-NH-CH_2-adamantyl 4431 and 10 408, respectively.[32]

In this respect we find extremely significant the finding that, in a series of analogues of the pentapeptide N-fragment of dermorphin, substitution of Tyr^5 with more hydrophobic substituents can revert μ specificity into δ specificity. For instance, the δ selectivity ratio (calculated from IC_{50} (μ)/IC_{50} (δ)) in the parent pentapeptide N-fragment (Tyr-D-Ala-Phe-Gly-Tyr-NH_2) is only 0.06 but jumps to 2.77 when Tyr^5 is substituted with D-phenylglycine.[33]

The importance of the relative arrangement of the aromatic rings in these compounds is emphasized by the recent finding that cyclic peptides of general formula Tyr-D-Xxx-Phe-Yyy-NH_2, i.e., cyclic analogues of D-tetra, in which the rings are forced to stay on the same side, are the most selective μ receptor ligands reported to date.[34]

In order to substantiate these ideas we have devised a crucial test, i.e., we designed a *linear* peptide lacking Tyr^1 that ought to retain a substantial μ activity. According to our model receptor, the OH group of Tyr^1, although generally very important, can be absent provided the entire peptide structure is made rigid by cyclization or if the separation between the aromatic (T) ring and the hard base is increased with the insertion of two single bonds, to make the local arrangement closer to that of methylfentanyl. The case of cyclization is well-documented by the cyclic enkephalin analogue prepared by DiMaio et al.[35] The first linear peptide lacking Tyr^1 that retains a significant opioid activity was prepared by us in the course of the present investigation.[19]

It was predicted that Phe-D-Ala-Phe-Gly-NH_2 ought to lose activity considerably (with respect to the parent tetrapeptide), whereas H_2N-C(=NH)-Phe-D-Ala-Phe-Gly-NH_2, with two extra bonds at the N-terminal, should have a μ activity comparable to that of Tyr-D-Ala-Phe-Gly-NH_2. Indeed, when these compounds were synthesized and subjected to the GPI test, the relative potencies (referred to the value of 100 for the tetrapeptide N-fragment of dermorphin) were found to be 3 for Phe-D-Ala-Phe-Gly-NH_2 and 21 for H_2N-C(=NH)-Phe-D-Ala-Phe-Gly-NH_2, respectively. Since the conformational behavior in solution of these two compounds is similar to that of D-tetra, it seems fair to attribute most of the difference in biological activity to the different constitution, in particular to the increased separation between the T stem and B_h.

While this manuscript was in preparation, another linear opioid peptide lacking Tyr^1 was reported in the literature:[36] Pya^1-Enk-OMe. It is interesting to note that also in this case the bulkiness of the pyrenyl ring makes the overall distance from the nitrogen to the extreme of the aromatic system comparable to that of methylfentanyl.

Conclusions

Comparison of the structures of several rigid analogues of morphine with the conformation of fentanyl-like molecules indicates that the main topological features of the μ receptor can be identified with two hydrophobic pockets that interact with the two aromatic rings of fentanyl: one is coincident with the subsite interacting with the aromatic ring of the stem of the T structure of morphine, and the other is essentially identical with the P subsite proposed by Portoghese. The features of this model of the μ receptor, compared to the solution conformation of simple μ peptides in a solvent medium that simulates the environment of the receptor site, explain many apparently unrelated observations on peptide opioids. A comparative analysis of several synthetic opioid peptides shows that the presence of a bulky hydrophobic group in the region above the T (i.e., above the plane of the β-turn that is essential for high μ activity) increases the μ potency but also, to a larger extent, the δ potency, leading in extreme cases to a reversal of the receptor specificity. Even more significant, in our opinion, is the fact that the model suggested the design of a very simple *linear* peptide with significant μ activity in spite of the absence of Tyr^1.

Experimental Section

Synthesis of the Peptides. The synthesis of D-tetra was carried out as described in ref 20a.

The synthesis of the two new [Phe^1] analogues was carried out by classical solution methods, essentially as previously reported for analogous peptides.[20a,32] Boc-Phe-D-Ala-Phe-Gly-OH, mp 134–136 °C, $[\alpha]^{22}_D$ +17.8° (*c* 1.0, methanol), prepared by hydrogenation of the corresponding benzyl ester, mp 209–211 °C, $[\alpha]^{22}_D$ +9.3° (*c* 1.0, dimethylformamide), was coupled with ammonia as described in ref 32 to obtain the protected tetrapeptide amide Boc-Phe-D-Ala-Phe-Gly-NH_2, mp 140–142 °C, $[\alpha]^{22}_D$ +27.9° (c 1.0, methanol). The peptide Phe-D-Ala-Phe-Gly-NH_2 (acetate), mp 98–100 °C $[\alpha]^{22}_D$ +41.1° (c 1.0, methanol), amino acid ratios Gly 1.00, Ala 1.01, Phe 2.02, was obtained by trifluoroacetic acid deprotection and successive peptide elution (0.2 N acetic acid) through anion exchange resin DE 52 Whatman (acetate form). The guanidino derivative H_2N-C(=NH)-Phe-D-Ala-Phe-Gly-NH_2 (acetate), mp 128–130 °C, $[\alpha]^{22}_D$ +16.3° (*c* 1.0, methanol), amino acid ratios Gly 1.00, Ala 0.98, Phe 2.02, was prepared and purified

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exactly as described in a previous paper[32] for other guanidino derivatives. All compounds displayed correct elemental analyses.

The amino acid composition was determined with a Carlo Erba 3A 29 amino acid analyzer after acid hydrolysis in constant boiling 6 N HCl containing 1% phenol. Melting points were determined on a Tottoli apparatus in open capillary and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter with a 10-cm water-jacketed cell.

Stimulated Guinea Pig Ileum. The peptides were examined for their ability to inhibit the electrically induced contractions of guinea pig ileum (GPI).[31] Comparison of the relative agonist potency was made on the basis of IC_{50} values (dose causing a depression of 50% of the electrically evoked contraction). IC_{50}'s determined from the mean of at least three independent observations of H-Tyr-D-Ala-Phe-Gly-NH_2 (reference compound) and [Phe[1]] and [H_2N-C(=NH)-Phe] analogues were 45.2 ± 3.2, 1593.8 ± 103, and 215.1 ± 11.8 nM, respectively. Naloxone (1.4 nmol/L; i.e., the pA_2 value against dermorphin) was a potent antagonist of peptides tested at IC_{50} concentration.

NMR. Samples for the NMR spectra in DMSO solutions were prepared by dissolving ca. 1 mg of each peptide in 0.5 mL of 99.98% DMSO-d_6 (Farmitalia C. Erba, Milano, Italy) taken from sealed ampules immediately before the experiment, with the addition of 5 μL of TMS for referencing. The sample of D-tetra in water was prepared by dissolving 1.2 mg of peptide in 0.5 mL of 90% H_2O/10% D_2O and adjusting the pH to 2.89 with 0.1 M TFA. The samples of the complexes for 2D experiments were prepared by dissolving ca. 1 mg of each peptide in 0.5 mL of 99.96% $CDCl_3$ (Aldrich Chemical Co., Milwaukee, WI) already containing the stoichiometric amount of 18-crown-6 ether (Farmitalia C. Erba). One millimole/liter samples were used for most 1D experiments; control spectra were run on 0.3 mM samples to check the presence of possible aggregation phenomena.

All spectra were run at 500 MHz on a Bruker WM-500 spectrometer, equipped with an Oxford Instruments superconducting magnet and an Aspect 2000 computer. 1D spectra were acquired with 16K data points and transformed with 32K data points. 2D COSY spectra were acquired with 2K data points in the F_2 domain and 512 data points in the F_1 domain. *J*-Resolved spectra were acquired with 8K times 128 matrices. All 2D spectra were processed with standard Bruker programs from the DISNMR package.

Dynamic range problems, in the case of the water solution of D-tetra, were overcome by means of zero-excitation techniques; both 1-1[37] and 1-3-3-1[38] sequences were employed, but the final chemical shift data of Table II were recorded by using only the 1-3-3-1 sequence.

Computational Methods. The potential energy function chosen is given as a sum of strain energies and nonbond interaction terms:

$$E_{total} = \Sigma_{bonds}K_R(R - R_0)^2 + \Sigma_{angles}K_\vartheta(\vartheta - \vartheta_0)^2 + \Sigma_{dihedrals}V_n(1 + \cos(n\varphi - \gamma))/2 + E_{nb} \quad (1)$$

where R, ϑ, φ, and γ are the equilibrium bond distance, valence angle, intrinsic torsions (proper and improper), and relative phases for the torsion, respectively. Nonbond energy was based on pairwise summations over all van der Waals and Coulomb interactions from atoms separated by three or more bonds:

$$E_{nb} = \Sigma_{i<j}(A_{ij}/r_{ij}^n - B_{ij}/r_{ij}^6 + q_iq_j/Dr_{ij}) \quad (2)$$

where A_{ij} and B_{ij} are the repulsive and attractive terms, respectively, for a given atom pair i,j; q_i and q_j are the partial charges of atoms i and j; r_{ij} is the interatomic distance; D is the dielectric constant; and n is an integer that can assume the value 12 or 9 depending on the kind of potential chosen. In the case of simple energy computations and energy minimization in the torsional subspace, only the third and fourth terms of eq 1 have been retained.

Table V. Relevant Torsional Parameters of the Minimum Energy Conformation of Tyr-D-Ala-Phe-Gly-NH_2[a]

	φ_i	ψ_i	ω_i	χ_{1i}	χ_{2i}	χ_{3i}
Y[1]	174.8	158.1	−174.3	−176.7	77.6	178.0
a[2]	66.7	−114.6	−177.1	179.2		
F[3]	−83.0	−2.8	176.5	−177.3	71.3	
G[4]	131.4	−79.0	−179.2			

[a] E_{total} = −0.24 kcal/mol.

As for the values of A_{ij} and B_{ij}, different types of parameter sets have been used to gain a deeper insight in the role played by the potentials and by their variable parameters in determining the final minimum energy conformations. After several trial runs, our choice rested on three different parameter sets, that proposed by Hopfinger,[13] in view of its widespread use in conformational analysis of several classes of drug molecules, and those of Lifson et al.,[14] because its 9–6 potential has been shown to be more accurate in the repulsive part, a circumstance that can be critical whenever highly crowded molecules are concerned (i.e., the N-axial conformation of methylfentanyl). The starting molecular parameters of methylfentanyl were taken from the solid-state structures of fentanyl[5,11] and R 30490[11] and from standard literature values,[39] in particular for the aromatic rings. Partial charges calculated according to the method of Del Re[40] were employed when the Hopfinger parameters were used, whereas in the case of *full geometry* minimization using AMBER, net charges were derived from CNDO/2 calculations of molecular fragments.[41] In both cases a dielectric constant of 2.0 was used for the Coulombic term. In the case of the Lifson set, the charges suggested by the authors[14] were used without changes and a value of 1.0 was employed for the dielectric constant.

Bond distances and valence angles for the energy calculations of the tetrapeptide Tyr-D-Ala-Phe-Gly-NH_2 were those suggested by Momany et al.[42] In this case only the potential of Nemethy et al.[43] was employed. A type II′ β-turn was chosen as starting conformation of the backbone, mainly on the basis of the NMR studies. Several combinations of starting conformations of the side chains were chosen in order to find the best relative arrangement of Tyr and Phe. Energy minimization included all torsion angles with fixed bond lengths and valence angles. The relevant parameters of the minimum energy conformation are reported in Table V. It can be seen that the final backbone conformation is close to an ideal type II′ β-turn; the side chains of Tyr and Phe are oriented in a way that makes the relative positions of the two aromatic rings very similar to the corresponding positions of the aromatic rings of methylfentanyl (see Figure 3).

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Registry No. P1 (acetate salt), 110027-27-5; P2 (acetate salt), 110027-28-6; D-tetra, 78700-75-1; BOC-Phe-D-Ala-Phe-Gly-OH, 110027-24-2; BOC-Phe-D-Ala-Phe-Gly-OCH_2Ph, 110027-25-3; BOC-Phe-D-Ala-Phe-Gly-NH_2, 110027-26-4; methylfentanyl, 42045-86-3.

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