Tetrahedron Vol. 44, No. 3, pp 697 to 710, 1988
Printed in Great Britain.

0040-4020/88 $3.00+.00
Pergamon Journals Ltd

A NEW APPROACH TO RECEPTOR LIGAND DESIGN: SYNTHESIS AND CONFORMATION OF A NEW CLASS OF POTENT AND HIGHLY SELECTIVE μ OPIOID ANTAGONISTS UTILIZING TETRAHYDROISOQUINOLINE CARBOXYLIC ACID

Wieslaw Kazmierski and Victor J. Hruby*

Chemistry Department, University of Arizona, Tucson, AZ 85721 U.S.A.

(*Received in USA 20 July* 1987)

Abstract: Investigations of the physiological functions of opioid receptors (μ, δ, κ and others) require potent and selective receptor ligands. Conformational constraint provides a useful approach to increase receptor selectivity of flexible peptides. This approach reduces the set of low energy conformations accessible for the ligand and thus can provide insight into topological features that may be responsible for high affinity to a particular receptor subtype. Using this approach, we describe a new class of potent and selective μ opioid receptor antagonists, and demonstrate a new approach for the design of receptor specific ligands by which a low affinity, "non-physiological" activity of a natural peptide hormone is converted to a high potency, receptor selective ligand for that receptor, and, at the same time, eliminates the activity at the natural receptors for the peptide.

Recently we reported the design and synthesis of a new class of μ opioid receptor selective antagonists, of which D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP) was found to be among the most potent and selective, and D-Pgl-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (PCTP) exhibited a sharp decrease of affinity for μ receptors (100 fold) and a modest increase (3 fold) in affinity for δ receptors. We now present ^1H NMR evidence which suggests a more folded conformation for the latter compound.

This result led to the design of further constrained analogues in which a methylene bridge is inserted between the α-amino group and the 2´position of the aromatic ring of D-Phe^1 in CTP. This analogue D-Tic-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (TCTP) was found to be the most μ vs. δ receptor selective ligand known (> 9000 fold selective), with very little somatostatin-like activity. NMR investigations have revealed that the side chain of D-Tic residue exists exclusively in a g^- conformation. Disconnection of this methylene bridge via synthesis of D-N-MePhe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 gave an analogue that exhibited low affinity for the μ opioid receptor and greatly reduced selectivity. NMR investigations have uncovered a large participation of g^+ and trans side chain conformations for the aromatic ring in the D-N-MePhe residue, and a more folded overall conformation. These results illustrate how constraint of side chain moieties of critical amino acid residues to a specific or "biased" conformation can provide important insights into the topological requirements for peptide-receptor interactions and can contribute to design of ligands for receptor mapping.

INTRODUCTION

Efforts to understand the precise structural, conformational and dynamic properties of peptide ligands which underlie their multiple biological effects is at the forefront of modern molecular biology approaches to develop a rational approach to drug design. Due to the inherent flexibility of most small peptide hormones and neurotransmitters, structure-activity studies by themselves rarely provide insights into the "bioactive conformation" (or reciprocally the receptor topology) of peptide hormones and neurotransmitters (the host-guest problem). In addition most peptide neurotransmitters interact with several different receptors. For example, the opioid receptors, the subject of this paper, exist as at least three different classes, μ, δ and κ. Specific ligands for each class are needed to determine the biological functions of the different receptors. As a consequence of these and other problems, attempts to rationally design potent and receptor selective ligands for peptides have been difficult and have obstructed efforts to understand the physiological roles of the multiple receptor systems found for most peptide hormones and neurotransmitters.

To help overcome these problems, the concept of conformational restrictions via pseudo-isosteric cyclization has been introduced[1,2] and can be used in conjunction with local constraints imposed by sterically constrained amino acids and peptido-mimetics[1,3] to aid in the rational design of peptide analogues for specific receptors. For the latter purpose, amino

acids such as α,β-dehydro-[4], N-methyl-[5], cyclopropyl[6], α,α-dialkyl-[7] and β,β-dialkyl-[8] amino acids have been used with considerable success. However much more work is needed to understand how to utilize these constraints best for precise application to peptide conformation, topology, and dynamics in relation to peptide-receptor interactions. In this regard, peptide hormone and neurotransmitter receptors are exquisitely sensitive hosts for examining such questions about hormones and transmitters. Thus, in conjunction with modern molecular pharmacological methods (e.g. binding assays and bioassays) on the one hand, and state of the art conformational analysis on the other (e.g. using NMR, various laser spectroscopies, etc.), a rational basis for peptide ligand design can be developed.

In this paper we provide an example of such an approach which has led to the design of a peptide which is the most μ opioid receptor selective ligand known to date. Since these peptides were derived from somatostatin, a peptide hormone and neurotransmitter which has its principal physiological receptors for quite different functions (inhibition of growth hormone, insulin, and glucagon release), we will provide a brief discussion of the background for this investigation. The idea of converting the potency and specificity of a peptide ligand from its natural, physiological, high affinity receptor(s), to a "non-physiological", very weakly binding receptor(s) for the native hormone provides a new approach in the design of these important compounds.

Table 1. Binding Potencies and Receptor Selectivities of Somatostatin and Somatostatin-Octapeptide Analogues in Opioid and Somatostatin Binding Assays With Rat Brain Homogenates

Compound	[^{3}H]-NAL IC_{50} (nM)	[^{3}H]-DADLE IC_{50} (nM)	[^{125}I]-CGP23996 IC_{50} (nM)	[^{3}H]-DADLE / [^{3}H] NAL	[^{125}I]-CGP / [^{3}H] NAL
1. SOMATOSTATIN	27,400	16,400	6	0.60	(0.0002)
2. D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-OH	4,500	5,000	300	0.90	(0.067)
3. D-Phe-Pen-Phe-D-Trp-Lys-Thr-Pen-Thr-OH	10,000	9,800	1,000	0.98	(0.10)
4. D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-OH	931	5,400	170	5.8	(0.18)
5. D-Phe-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr-OH	61,000	38,000	800	0.65	(0.013)
6. D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-OH	290	3,800	1,600	13.	(5.5)
7. D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP)	3.5	950	1,462	271	(395)

Compound	[^{3}H]CTOP IC_{50} (nM)	[^{3}H]DPDPE IC_{50} (nM)	[^{125}I]CGP23996 IC_{50} (nM)	[3H]DPDPE / [^{3}H]CTOP	[^{125}I]GCP / [^{3}H]CTOP
7. CTP	3.70	1,153	1,462	311.8	(395)
8.[Arg^5]CTP (CTAP)	2.10	5,314	8,452	2,530	(4,025)
9.[Orn^5]CTP (CTOP)	4.30	5,598	47,704	1,301	(11,094)
10.[Nle^5]CTP (CTNP)	9.64	5,570	N.D.	578	-

BACKGROUND

It has been observed that native somatostatin, H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, interacts very weakly with opioid receptors[9,10], and that a cyclic truncated analogue D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol) (SMS 201-995), a superpotent growth hormone release inhibitor, also is capable of antagonizing the excitatory effect of enkephalins in electrophysiological experiments[11]. These results suggested that somatostatin possesses topological features compatible with a weak interaction with opioid receptors. Conformational analysis suggested that the central tetrapeptide sequence -Phe-D-Trp-Lys-Thr- possessed a β-turn conformation that was critical to its biological activity at somatostatin receptors[12,13]. Therefore, we attempted to discriminate between opioid-like and somatostatin-like topologies by designing analogues which would enhance opioid receptor interaction, and suppress interactions with the somatostatin receptor. We began our studies with the carboxylate terminal analogue 2 (Table 1). Binding assays showed increased potency at opioid receptors relative to somatostatin but greatly reduced binding at somatostatin receptors[14].

Next, we examined the use of penicillamine (β,β-dimethylcysteine) as a replacement for cysteine 2 and/or 7 in 2. The conformational effects of this residue include an increased disulfide dihedral angle[15] to > 90°, and transannular effects[2], due to the geminal dimethyl groups of penicillamine in a cyclic medium-sized ring. Of 3 possible combinations (3, 4, 5, Table 1), only the Pen[7] analogue (4, Table 1) had increased binding at a μ opioid receptor (5 fold relative to 2, Table 1), while affinities for both the δ opioid and somatostatin receptors were practically unchanged, thus resulting in a μ opioid receptor selective ligand.

Since a Tyr residue is believed to be an essential pharmacophore for opioid ligands, we replaced Tyr for Phe in both the 1 and 3 positions, resulting in the former case in an analogue with greatly reduced binding[16] but in the latter case there was a further 3-fold increase in affinity for the μ receptor, little change at the δ receptor (6, Table 1, compared with 4), but a 10-fold reduction of binding (Table 1) to the somatostatin receptor[16]. Conversion of the C-terminal acid to a C-terminal amide (7, CTP, Table 1) enhanced the selectivity to μ vs. δ opioid receptors approximately 20 times. Similar μ opioid receptor selectivity upon substitution of a carboxamide for a carboxylate C-terminal group has been noted by Schiller et al.[17,18] and Mosberg et al.[19,20] for opioid peptide agonists. These results suggest that a decrease of overall negative charge of the molecule might be an important factor in μ receptor selection.[21]

To further reduce somatostatin-like activity, Lys in position 5 of 7 was substituted with Arg (8, Table 1) and Orn (9, Table 1) (these compounds are designated as CTAP and CTOP, (respectively)[22]. In both cases a drastic decrease of somatostatin receptor binding is accompanied by high affinity for μ and low affinity for δ opioid receptors, resulting in a very high μ opiate to somatostatin receptor selectivity, and μ vs. δ opioid receptor selectivity. Similar effects of Lys substitution on the somatostatin-like activities in a cyclic hexapeptide series have been reported by Nutt et al.[23]. All of our compounds such as CTP, CTOP, etc. have been characterized as potent and selective μ opioid receptor antagonists[24,25].

A basic premise of this research has been that the overall conformational properties of the "central" tetrapeptide sequence Phe-D-Trp-Lys-Thr would be retained and could serve as a topographical template for design of analogues for the opioid receptors. Thus conformational analysis has been an important component of this research. Indeed, utilizing high field ^{1}H and ^{13}C NMR studies in both aqueous[26] and DMSO[27] solution it has been determined that the "core" tetrapeptide of CTP (Tyr-D-Trp-Lys-Thr) does indeed possess a βII′ conformation. Furthermore, it was shown that the helicity of the disulfide bond is negative[27,28]. Interestingly, CTOP, an isomer of CTP with a D-Pen[7] rather than an L-Pen[7] residue was nearly inactive at opioid receptors (Tourwé, Sugg, Kazmierski, Van Binst, Shook, Yamamura, Burks and Hruby, manuscript in preparation) but had the same βII′ conformation as CTP. However, the helicity of the disulfide bond now was positive.

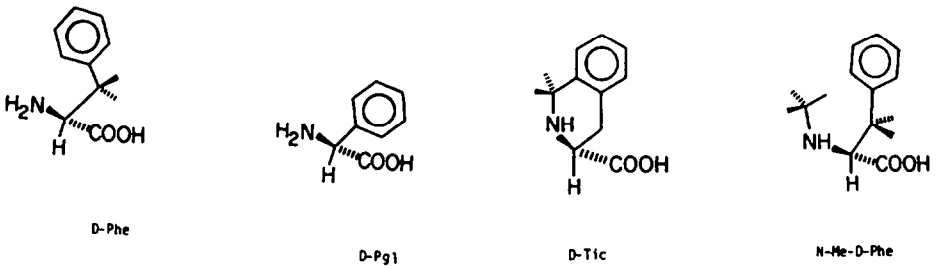


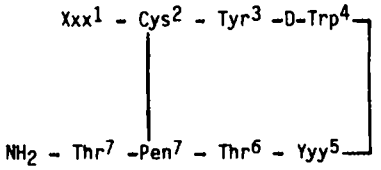
Fig. 1. Comparison of Structures of Amino Acids Used on First Position of Somatostatin Analogues

Thus, we have converted somatostatin from a peptide with high potency at somatostatin receptors to an analogue with high potency and receptor selectivity for the μ opioid receptor and little somatostatin potency using the tetrapeptide Tyr-D-Trp-Lys-Thr as a conformational template. These results led us to further investigate the conformational and structural requirements of the μ opioid receptor utilizing the conformationally stable CTP structure and constrained amino acid residues which possess specific or highly biased side chain conformations. We report here the design, synthesis and conformational analysis of three analogues which examine the conformational requirements for position 1 of CTP for the μ receptor.

RESULTS AND DISCUSSION

DESIGN AND SYNTHESIS OF ANALOGUES

The above results suggest that important topological and structural features for μ opioid receptor recognition of CTP molecules are in the vicinity of the disulfide bridge, and most probably involve both N- and C-terminal amino acids. The aforementioned inversion of helicity of the disulfide bond would generally change the spatial relationship of the terminal amino acid residues, as well as the Cys^2 and Pen^7 residues, and taken together these would appear to constitute the essential pharmacophore for the μ opioid receptor. Thus we focused our initial attention on exploring the structural and conformational requirements of the exocyclic positions and have synthesized and pharmacologically evaluated a number of further constrained and/or structurally modified analogues of CTP at the 1 and 8 positions. For the 1 position, three analogues have provided exquisite insight into the topographical and structural requirements of the μ receptor, namely those containing D-phenylglycine (D-Pgl, Figure 1), D-tetrahydroisoquinoline carboxylate (D-Tic, Figure 1) and D-N-methylphenylalanine (D-N-MePhe, Figure 1) in the 1 position (Figure 2). Each has their own unique properties.



Entry	Xxx^1	Yyy^5
11	D-Pgl	Lys
12	D-Tic	Lys
13	N-Me-D-Phe	Orn

Fig. 2. Structure of peptides investigated in this work.

Table 2. Binding Potencies to the μ and δ Rat Brain Opioid Receptors and Receptor Selectivity of the Analogues Described in This Work.

	[D-Pgl[1]]CTP (11)[a]	[D-Tic[1]]CTP (12)[b]	[D-N-MePhe[1]]CTOP (13)[b]
IC_{50} (nM) binding vs. [^{3}H]CTOP	350 ± 97[c]	1.15 ±.03	284 ±36
IC_{50} (nM) binding vs. [^{3}H]DPDPE	2800 ± 570	9,320 ± 546	10% at 10^{-5} M
Selectivity to μ vs. δ opioid receptors	8	8,080	> 35

[a]see reference 16; [b]see reference 40; [c] [^{3}H]NAL was used

Phenylglycine has two related conformational effects relative to Phe. First it has fewer rotational degrees of freedom, and second, the aromatic ring is spatially closer to the peptide backbone.

Analysis of the conformationally restricted amino acid Tic (Figure 1) indicates that it should be excellent for fixing the side chain conformation. This amino acid can be viewed as a Phe in which rotation around the C_α-C_β (χ^1) and C_β-C_γ (χ^2) bonds is greatly limited due to the methylene unit which bridges the 2' position in the aromatic ring and the α-nitrogen. Only 2 discrete side chain conformations are accessible, g^+ and g^- (Figure 3). Thus, this residue provides an opportunity to determine the preferred conformation of the 1 position side chain moiety for the μ receptor.

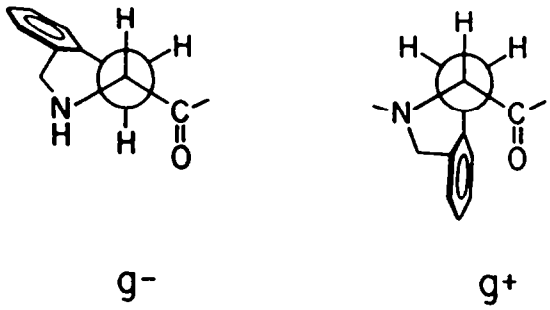


Fig. 3. The Possible Conformations for the Side Chain of D-Tetrahydroisoquinoline Carboxylic Acid

N-MePhe can be viewed topologically as a product of the disconnection between the 2' position of the aromatic ring and the N-CH_2 of Tic (Figure 1). Conformational analysis would suggest that the trans conformation would be most favored with lesser amounts of the g^- and g^+ conformers. Thus the bias is toward a trans conformation in this residue (whereas in Tic, this conformation is not accessible), which provides a unique opportunity to assess further specific topological requirements of the μ receptor.

ANALYSIS OF THE RELATIONSHIP OF THE BIOLOGICAL AND CONFORMATIONAL PROPERTIES OF [D-Pgl^1]CTP

In previous studies it was shown that [D-Pgl^1]CTP (11, PCTP Table 1) was about 100-fold less potent in binding to the μ receptor than CTP, but about 3 times more potent in binding to the δ receptor (Table 2)[16]. Thus a dramatic loss of receptor selectivity was observed.

This raises an immediate question. Is the observed pharmacological profile of 11 caused by the local conformational effects of D-Pgl or more global topological changes due to unfavored steric interactions of the D-Pgl aromatic ring and neighboring side chains? We have addressed this question by ^{1}H NMR investigations of the conformation of 11.

Unambiguous assignment of all ^{1}H signals in PCTP has been facilitated by use of phase sensitive 2D COSY experiments[29,30]. Homonuclear decoupling, combined with difference spectroscopy was used to selectively extract coupling constants out of crowded spectral regions. Additionally, COSY experiments with delays were used to obtain the long-range couplings[31] between the β protons and the ortho aromatic protons of the Tyr^3 and D-Trp^4 residues. All spectral assignments and coupling constants as well as temperature factors for the amide protons are listed in Table 3.

Table 3. Spectral Assignments, Coupling Constants, Temperature Factors for 11, 12, 13.

Residue		D-Pgl^1 (11)	D-Tic^1 (12)	N-Me-D-Phe^1 (13)
Xxx^1	NH	8.62	7.75	8.72
	α	5.19	4.25 (Jαβ=12.1,3.6)	4.30 (Jαβ=8.9,4.7)
	β	---	3.43/3.13 (J_g=16.3) N-CH_2 4.37	3.33/3.15 (J_g=14.3)
Cys^2	NH	9.36 (J=9.7, Δδ/Δτ = -5.1)	9.44 (J=9.9, Δδ/Δτ = -5.1)	9.35 (J=9.2, Δδ/Δτ =-5.9)
	α	5.66 (Jαβ=7.7,7.7)	5.64 (Jαβ=9.4,7.3)	5.53 (Jαβ=9.2,6.8)
	β	2.64	2.85 (J_g=N.D.)	2.75
Tyr^3	NH	8.69 (J=8.1, Δδ/Δτ = -5.0)	8.63 (J=7.2, Δδ/Δτ = -3.5)	8.48 (J=8.3, Δδ/Δτ=-3.6)
	α	4.61 (Jαβ=8.1,6.9)	4.58 (Jαβ=8.0,6.3)	4.57 (Jαβ=8.1,6.8)
	β	2.74	2.77/2.74 (J_g=14.7)	2.80/2.63 (J_g=13.6)
D-Trp^4	NH	8.90 (J=5.3, Δδ/Δτ = -5.1)	8.83 (J=5.7, Δδ/Δτ = -3.8)	8.83 (J=5.7, Δδ/Δτ =-5.8)
	α	4.17 (Jαβ=9.4,5.8)	4.20 (Jαβ=9.4,6.0)	4.22 (Jαβ=7.6/7.6)
	β	3.00/2.69	3.04/2.71 (J_g=13.8)	3.03/2.80 (J_g=14.3)
Lys^5 11,12	NH	8.23 (J=9.1, Δδ/Δτ=-2.0)	8.24 (J=9.3, Δδ/Δτ=-1.8	8.39 (J=9.1, Δδ/Δτ=-4.5)
	α	3.98 (Jαβ=11.2,2.7)	4.02 (Jαβ=10.9,3.1)	4.10 (Jαβ=10.3, 3.4)
	β	1.20/1.70	1.71/1.20	1.88/1.25
	γ	.64	0.68	1.10
	δ	1.28	1.32	2.60
	ε	2.54	2.54	----
	NH_3+	7.68	7.75	7.60
Thr^6	NH	7.66 (J=9.00, Δδ/Δτ = 0.0)	7.69 (J=8.5, Δδ/Δτ = 0.3)	7.66 (J=9.0, Δδ/Δτ = -.2)
	α	4.52 (Jαβ=7.2)	4.56 (Jαβ=6.8)	4.53 (Jαβ=6.8)
	β	3.95 (Jβγ=6.3)	4.00 (Jβγ=6.7)	4.02 (Jβγ=6.4)
	γ	1.06	1.04	1.04
Pen^7	NH	8.49 (J=9.8, Δδ/Δτ = -8.5)	8.49 (J=8.7, Δδ/Δτ = -6.5)	8.44 (J=9.6, Δδ/Δτ = -3.4)
	α	4.96	4.94	4.96
	γ	1.18/.95	1.27/1.13	1.26/1.21
Thr^8	NH	8.69 (J=8.1, Δδ/Δτ = -5.0)	8.39 (J=9.4, Δδ/Δτ = -4.3)	8.44/ J=8.4, Δδ/Δτ =-4.0
	α	4.40 (Jαβ=3.4)	4.30 (Jαβ=3.5)	4.36 (Jαβ=3.2)
	β	4.02 (Jβγ=6.3)	4.00 (Jβγ=6.7)	4.02 (Jβγ=6.3)
	γ	1.02	1.05	1.04

Chemical shifts in ppm, referred to the DMSO temperature independent signal at δ = 2.49 ppm; coupling constants in Hz; temperature coefficient in ppb/deg.

Phi(ϕ) angles consistent with the observed coupling constants were calculated by use of the Karplus-Bystrov[32] relationship and used to examine possible conformations consistent with the data. Vicinal $\alpha\beta$ coupling constants, assigned to pro-R and pro-S β-hydrogens[33], enabled us to calculate side chain rotamer populations[34,35] which are shown in Table 4. 2D NOESY studies were helpful to differentiate between the NH-Thr^8 and NH-Thr^6 resonances, due to an inter-residual NOE observed between Lys^5 CHα and Thr^6 NH as well as Pen^7 CHα and Thr^8 NH. Additionally, important information regarding the backbone conformation can be obtained (Table 5) from NOESY experiments. As the magnitude of magnetization transfer is inversely proportional to the sixth power of the distance between two protons, quantitative estimations of the NOE effects allows one to build up a 3 dimensional distribution of protons that can also be described by the ϕ, ψ, χ and ω angles[28] of the amino acid residues. In this regard, NOE experiments can identify and distinguish between well known structures such as the α-helix, β-turns, γ-turns, β-sheets, etc.[36]. Thus, type I β turns are characterized by NH^{i+1}/NH^{i+2} and NH^{i+2}/NH^{i+3} NOE signals[37], type II β turns by CH_{i+1}/NH_{i+2} and NH_{i+2}/NH_{i+3}[38], γ turns by CH_{i+1}/CH_{i+2}[39], etc. Analysis of the 2D NOE spectrum of 11 reveals very important interactions between the Lys^5 NH and Thr^6 NH protons as well as between D-Trp^4 α CH and Lys^5 NH protons (spectra not shown) suggesting the presence of a βII' turn[27]. This is supported by the lack of a temperature dependence of the Thr^6 amide chemical shift (Table 3), suggesting an intramolecular H-bond to the i+3(Thr^6) NH. It is noteworthy that the ϕ angles required for βII' turns are found among those available from coupling constants analysis of the data from Table 3 using the Bystrov relationship[32], which further validates our conformational considerations.

Table 4. Possible Dihedral Angles ϕ for 11,12,13.

Residue	D-Pgl 11	D-Tic 12	N-Me-D-Phe 13
Xxx[1]	N.D.	N.D.	N.D.
Cys[2]	-140/-135 -113/-94	-157/-130 -112/-92	-154/-140 -101/-86,48/72
Tyr[3]	-162/-152 -110/-94 38/82	-156/-146 -83/-76 32/42 78/92	-159/-140 -101/-86 48/72
D-Trp[4]	163/67,79/66 -19/-23, -92/-102	172/166, 75/68 -20/-28 -92/-102	172/163,76/68 -21/-28,-94/-105
Lys[5] 11,12 Orn[5] 13	-154/-141 -101/-88 47/74	-152/-140 -103/-86 52/71	-154/-141 -101/-88 47/74
Thr[6]	-156/-143, -100/-87, 47/75	-159/-149 -93/-82 40/80	-156/-143 -100/-87 47/75
Pen[7]	-150/-135 -112/-93	-156/-146 -94/-84 42/59	-145/-135 -105/-92
Thr[8]	-161/-150 -86/-83 38/82	-152/-140 -103/-89	-161/-150 -92/-83 40/82

§Calculated according to (32).

Wüthrich et al.[37] analyzed distance vs. Ψ angles for cis and trans isomers of the Pro-Pro, dipeptide, and showed that for peptides with trans peptide bonds, the distance between CH_α and $CH_{2\,\delta}$ reaches the minimum (and the NOE reaches the maximum) for Ψ_1 <+60 to +180>. In the case of the cis peptide bond, no Ψ_1 rotation can place the protons $CH\alpha^i$ and $CH_{2\,\delta}{}^{i+1}$ (NH^{i+1}) spatially within 4.2 Å where the NOE is observable. Cis peptide bonds occur rather rarely in linear or large cyclic peptides, except for N-substituted amino acids. Table 5 reveals that in 11 strong NOEs exist between CH^i and NH^{i+1} of the following peptide pairs 1/2, 2/3, 3/4, 4/5, 6/7, 7/8. It is thus reasonable to assume that all these peptide bonds are trans (±180°).

Table 5. Interresidual Relative Intensities for the NOEs of 11, 12, 13[a]

Residue	[D-Pgl^1]CTP 11	[D-Tic^1]CTP 12	[N-Me-D-Phe]CTOP 13
NH^i/NH^{i+1}			
Lys^5/Thr^6	+++	+++	
Orn^5/Thr^6			+++
CH^i/NH^{i+1}			
Xxx^1/Cys^2	+++	N.O.	+++
Cys^2/Tyr^3	+++	+++	+++
Tyr^3/D-Trp^4	+++	+++	+++
D-Trp^4/Lys^5	+++	+++	
D-Trp^4/Orn^5			+++
Lys^5/Thr^6	N.O.	+	
Orn^5/Thr^6			+
Thr^6/Pen^7	+++	+++	+++
Pen^7/Thr^8	+++	+++	+++
CH_α/CH_α			
Cys^2/Pen^7	+++	+++	+++

[a] + = weak; +++ = strong; N.O. = not observed

No Ψ angles can be directly determined from ^{1}H NMR coupling constant experiments. However, NOESY experiments suggest that for L-amino acids Ψ = 60° to 180° and for D-amino acids (D-Pgl^1, D-Trp^4) Ψ = -60° to -180°. The latter value is in the range expected for Ψ^{i+1} of a βII´ turn, which has an idealized value of -120°. It should be noted, that the existence of a strong NH_5/NH_6 NOE precludes a strong CH_5/NH_6 NOE. Thus, assuming a trans bond, Ψ_5 = -120° to 0°; this range is that expected for the Ψ_{1+2} of βII´ which has an idealized value of 0°.

Also, an interresidual NOE for the Cys^2 $C_\alpha H$ to Pen^7 $C_\alpha H$ is detected (Figure 4) which suggests a disulfide bond helicity similar to that of CTP (7). Comparison of spectral parameters for CTP[27] and 11, as well as their side chain populations (Tables 3-5), leads to the conclusion that both compounds have very similar backbone conformations as well as topologies of the aromatic side chain residues. However, there is a very substantial downfield shift of the D-Trp^4 αCH (+.39 ppm) and a less dramatic shift of the Thr^8 NH (+.14 ppm) in 11 compared with 7[27]. These results indicate that in 11 these protons are in the deshielding zone of the benzene ring of D-Pgl^1. This is completely consistent with the idea that the conformation and

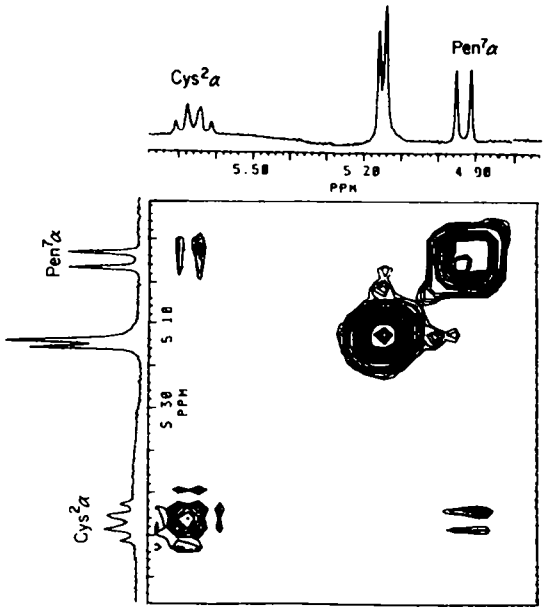


Fig. 4. Interresidue NOE for the Cys^2 α-CH and Pen^7 α-CH protons of 11.

topology for the cyclic 20-membered of 11 is practically identical with that of 7. Therefore, the only major difference between them is that the aromatic ring of D-Pgl^1 in 11 is positioned much closer to the βII´ turn, especially the Tyr^3 aromatic group (Figure 5, empty square structure). These closer spatial relationships results in a somewhat more folded conformation for 11 as compared with 7, which seems to be responsible for the observed loss of potency and selectivity at the μ opioid receptors. Thus, by proper topological adjustment of critical distances between these two pharmacophoric groups one can alter affinity for the μ receptor. A compact topology in this region by 11 leads to a large decrease of affinity for μ receptors and to a relative increas in selectivity for δ opioid receptors. In 7, the large participation of the g$^-$ side chain rotamer for D-Phe^{127} gives a more extended conformation which apparently is related to the high antagonist potency and selectivity for the μ opioid receptor. Qualitati-

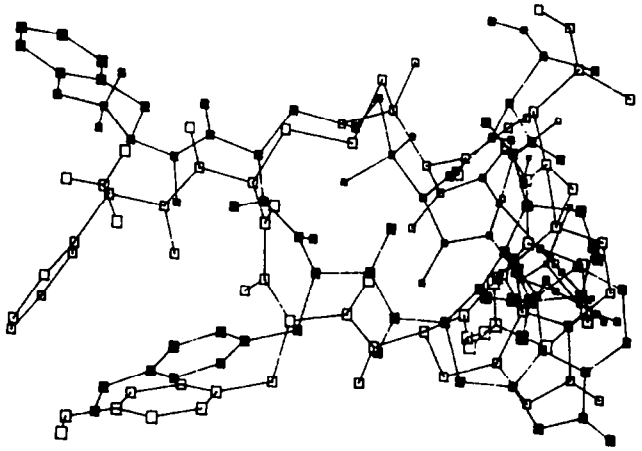


Fig.5. Superimposed Structures of 11 and 12 Reveal Essential Differences in The Orientation of The Aromatic Ring of The N-Terminal Amino Acid. Note The "Folded" Conformation of 11 vs. The Unfolded Conformation of 12

vely, therefore, one would expect that a further increase in the g^- population of the aromatic amino acid in the 1 position should result in an increase in μ-opioid receptor affinity and selectivity. The Tic residue was chosen to examine this hypothesis.

ANALYSIS OF THE RELATIONSHIP OF THE BIOLOGICAL AND CONFORMATIONAL PROPERTIES OF [D-Tic^1]CTP.

[D-Tic^1]CTP (12) was synthesized and indeed was found to be more potent and selective towards μ opioid receptors than CTP (Table 2)[40]. The immediate question arises as to which of the two allowed side chain conformations is present in this analogue, and whether the overall topology of the neuropeptide is perturbed due to rigidity and structual properties of Tic. Thus careful NMR studies have been made on this analogue. As with 11, all of the ^{1}H NMR spectral signals have been assigned (Table 3), the ϕ angles calculated (Table 4), and the side chain rotamer populations determined. NOESY cross peaks (Table 5) indicate a βII' conformation for the backbone Tyr-D-Trp-Lys-Thr residues of this peptide as well, and this was strongly supported by the presence of a H-bonded amide of Thr^6 (Table 3). Analysis of other spectral parameters, including side chain rotamer populations, decisively eliminates any major perturbation of the cyclic ring structure of the peptide due to the D-Tic amino acid. Moreover, the conformation of this peptide at the disulfide bridge is not altered by the changes at the exocyclic N-terminal position as deduced from a strong transannular Cys^2 $C_\alpha H$ to Pen $C_\alpha H$ NOE cross peak (Table 5). Most of the CH_i/NH_i interresidual NOEs found for 11 also are seen in 12 (Table 5), with the exception of the 1/2 NOE. As cis peptide bonds generally are not formed by α-amino acids, it is reasonable to assume a trans 1/2 peptide bond with a possible $\psi_1 \approx$ (0°;120°). Other ψ values are in the same range as for 11; all ω angles are $\pm$ 180°.

To determine the two conformations possible for Tic, g^- and g^+ (Figure 3), we examined the ^{1}H NMR splitting patterns for $J_{\alpha\beta}$ which easily distinguish the two conformations. Analysis of $\alpha\beta$ coupling constants of this amino acid residue (Table 3) demonstrates that in [D-Tic^1]CTP the Tic residue has a gauche(-) conformation about the $\alpha\beta$ (χ^1) bond. Thus the aromatic ring of D-Tic^1 becomes fixed in a conformation which places the aromatic ring in a g^- conformation, away from the rest of the molecule including the other important pharmacophore groups, especially the Tyr^3 residue (Figure 5, structure with solid squares). In light of the other NMR data, this topological feature seems to be exclusively responsible for the observed biological properties of 12. This relationship can be seen in Figures 5 and 6.

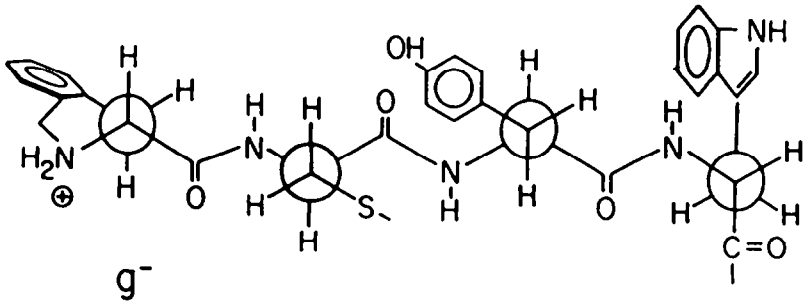


Fig. 6. Partial structure of [D-Tic^1]CTP 12 which emphasizes mutual spatial relationship of aromatic side chains (backbone conformation is not considered at this point). Notice extended conformation of the peptide.

The highly restricted nature of the D-Tic[1] moiety in 12 makes it highly likely that the g⁻ side conformation is an important element in the bioactive conformation of μ selective ligands. Since the aromatic ring of D-Tic[1] in 12 is parallel to the cyclic moiety (Figures 5, 6), it should shield the terminal amino acid Thr[8]. This effect is easily observable (Table 3), as there is a substantial upfield shift of the Thr[8] $C_\alpha H$ (+ .08 ppm) and the Thr[8] α NH (+ .16 ppm) in 12 compared to 7[27]. Also, chemical shift differences of both β,β-geminal methyl groups of Pen[7] are larger for 12 (.14 ppm) than for 7 (.08 ppm)[27] suggesting a more rigid conformation for the D-Tic residue in 12 than for the D-Phe residue in 7. This parameter is remarkably large for 11 (.23 ppm), consistent with the constrained nature of the peptide, and suggesting a close spatial proximity of D-Pgl aromatic ring to the cyclic moiety. A visual picture of the topological differences between 11 and 12 is given in Figure 5 where 11 and 12 are superimposed.

It should be noted that as a μ-selective ligand, [D-Tic[1]]CTP (12) is superior to CTP in both peripheral as well as central opioid receptor bioassays[40]. The fact that only a fraction of D-Phe side chain population in CTP[27] is in the g⁻ conformation may be attributed to the differences in potency and selectivity of 7 vs. 11 at the μ receptor. To further examine this, we have prepared [D-N-MePhe[1]]CTP.

ANALYSIS OF THE RELATIONSHIP OF THE BIOLOGICAL AND CONFORMATIONAL PROPERTIES OF [D-N-MePhe[1]]CTP

The synthesis and purification of [D-N-MePhe[1]]CTP (13) followed standard procedures. A dramatic change in the pharmacological properties of 13 was observed. This compound exhibited a very weak (~300 times less potent than 12) binding at μ receptors (Table 2). This result encouraged us to make a detailed conformational analysis of 13. Comparison of chemical shifts and coupling constants (Table 3), φ angles from calculations, the magnitude of the NOE effects (Table 5), and the population of the side chain rotamers (Table 4), suggests that 13 possesses a βII' conformation with a negative disulfide bond chirality, that is the same backbone conformation and side chain topology as in the 20-membered ring of 7[27], 11 and 12 (vide supra). As in 11 but not 12, the CH_α/NH^{i+1} interresidual NOE is strong for all but the 5/6 case (as a result of NH^5/NH^6 NOE), indicating a Ψ_1= -60° to -180°. Again, important differences can be noticed only at the 1 position. As calculated from Jαβ coupling constants, there is a large participation of the trans side chain rotamer, with lesser amounts of the g^+ and g⁻ forms in the side chain rotamer population of D-N-MePhe[1]. This effect can be explained in terms of nonbonded interactions between the N-methyl and phenyl groups which leads to destabilization of the usually energetically more favorable g⁻ conformation via distortion of χ^1 and/or χ^2 angles. In terms of conformation, the phenyl ring of residue 1 in 13 is more folded with respect to the cyclic 20-membered ring than in 12. Thus 13 is more similar to [D-Pgl[1]]CTP (11), making 13 a pharmacologically similar ligand to 11 (Table 2). Persistent, though not substantial, upfield shifts of NH and CH_α protons are consistent with this. Furthermore, the consistency of other parameters throughout the molecule relative to 7, 11 and 12 gives support to the suggestion that conformational changes occur only at the N-terminal position, suggesting that the observed pharmacological effects can be directly attributed to the relative spacial relationships of the position 1 amino acid with other pharmacophore elements of the ligand. One other observation is worth noting. It has been found for all compounds in this series (11, 12, 13) that the ^1H NMR signals of the two methyl groups of Pen[7] have very different intensities, the upfield signal being less intense. Since the integrated intensities of both peaks are exactly equal, their different heights clearly show that the upfield peak has a larger linewidth. Irradiation of the alpha hydrogen of Pen[7] renders the height of these two signals almost equal. Therefore, this broadening is due to long range CH_α-C-CH_3 coupling[41]. This coupling is most effective when the α-hydrogen and an "upfield" - but not "downfield" - methyl group are in an axial-axial relationship. Only t and/or g^+, but not g⁻ (considering N-C_α-C_β-S dihedral angle) side chain conformations can exhibit an axial-axial relationship between a methyl group and the alpha hydrogen, making long range

coupling(^{4}J) more effective. These considerations can be utilized to determine the possible range of χ^1 angles for the Pen side chain groups of 11, 12,and 13.

SUMMARY AND CONCLUSIONS

The solution conformations of the cyclic 20-membered ring of CTP analogues 11 and 12 and 13 are essentially identical to that previously reported for CTP (7)[27]. The βII' conformation of the core sequence and the negative disulfide helicity characterize the cyclic 20-membered ring of the three compounds.

Critical conformational differences at position 1 can explain the observed conformation-biological activity relationships. In particular, moving from D-Pgl^1 (folded conformation), through N-Me-D-Phe^1 (all rotational states g^-, t, g^+ available but t preferred), to D-Tic^1 (extended g^- conformation is exclusively obtained) results in a continuous relative unfolding of the peptide topology that can be more quantitatively described as an increase in the distance between the aromatic pharmacophore groups which make contact with the receptor. This conformational unfolding corresponds to a large increase in affinity and selectivity for μ opioid receptors, the largest effect occurring for 12 and the smallest for 11, with a decrease of affinity towards the δ receptor being smallest for 12 and largest for 11. The strong correlations in these highly constrained analogues supports our thesis that interresidue distances of aromatic rings are an important factor that allows the ligands to discriminate between μ and δ receptors.

Further work is in progress by using a combination of design and synthesis of conformationally restricted peptides, NMR analysis, and molecular mechanics and dynamics calculations on these and other analogues to further analyze those topological features that the ligand utilizes when selecting between μ and δ receptors.

We believe that this approach to receptor ligand design which correlates conformational (rather than just structural) and pharmacological data is fairly general and may be applied in other bioactive compounds. Of critical importance for such an approach to be successful is the presence of a rather conformationally stable backbone structure for some portion of the molecule (e.g. the βII' conformation of the examples reported here) that can serve as a template for further assessment of the topological relationships that are important to receptor selectivity and potency.

Finally, these investigations have shown that N-terminally located tetrahydroisoquinoline carboxylic acid exists exclusively in the g^- conformation (no g^+). This dependence can be used as a structural tool to design other bioactive molecules.

EXPERIMENTAL

The syntheses of [D-Pgl^1]CTP and CTP have been previously described[22]. Both 12 and 13 were prepared by solid phase synthetic techniques[42] using a Vega Model 250 or 1000 peptide synthesizer. Amino acid derivatives were either purchased from Bachem (Torrance, CA) or were prepared by described methods[43]. Carboxamide peptides were prepared by using a p-methylbenzhydrylamine (pMBHA) resin synthesized by published methods[44] (substitution of 1.0 mM/g of resin). A 1.5 M excess of preformed symmetrical anhydrides or a 3 M excess of N-hydroxybenzotriazole active esters was used for coupling reactions which were monitored by ninhydrin[45] or chloranil[46] tests.

N^{α}-Boc-Thr(O-Bzl) was coupled to pMBHA resin via its N-hydroxybenzotriazole active ester. Deprotection by two treatments of 50% TFA trifuroacetic acid (TFA) in dichloromethane (DCM) for 2 and 20 min each and neturalization with 10% diisopropylethylamine (DIEA) in DCM was followed by a sequence of couplings and deprotections of N^{α}-Boc-Pen(S-4-MeBzl), N^{α}-Boc-Thr(O-Bzl), N^{α}-Boc-Lys(N^{ϵ}-2-ClZ) for 12 (for 13 N^{α}-Boc-Orn(N^{δ}-Cbz) was used instead), N^{α}-Boc-D-Trp. Since the N^{α}-Boc-D-Trp was used without protection of the indole nitrogen, the TFA solution was modified to contain 20% methylsulfide and 10% dithioethane following incorporation of the D-Trp residue. Next, N^{α}-Boc-Tyr(O-2,6-Cl_2Bzl), was incorporated. Then N^{α}-Boc-D-Tic or N^{α}-Boc-D-N-Me Phe was coupled (for 11 and 12, respectively), the N^{α}-Boc protecting group was removed, the amino group neutralized with DIEA, and the peptide resin dried in vacuo. The peptide resins were then separately cleaved by liquid HF with addition of 10% anisole in 0°C for 45 min. Then the solvents were evaporated off, the dried product washed with ethyl ether (3 x 20 mL), and the peptide extracted with 10% acetic acid (3 x 30 mL), filtered and lyophilized. Next the disulfide bond was formed by dissolving 1 g of the linear peptide into 1.5 L of previously degassed water at pH 8.4 (adjusted with aqueous ammonia). Then the solution was titrated with 0.01 M of $K_3Fe(CN)_6$, stirred for 45 min, and then acidified to pH = 4.5 with HOAc, the excess of ferro- and ferricyanide was removed by the addition of 15 mL of Amberlite IRA-45 (mesh

HOAc as the eluting solvent. Final purification was made by RP-HPLC using gradient of 10-40% acetonitrile and 0.1% aqueous TFA.

D-Tic-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (12). The overall yield after purification was 15%. The synthesis of D-Tic was accomplished according to the method of Picted and Spengler[47] (78% yield, mp 308-310°C), and the N^{α}-Boc protecting group added by standard procedures[43] to give N^{α}-Boc-D-Tic (54%; mp 122-123°C; $[\alpha]_D$ = -18.6 (c = 1.0, CH_3OH). ^1H NMR (250 MHz, [$^2H_6$]-DMSO) of N^{α}-Boc-D-Tic: 13.0 ppm (br, ^1H, acid); 7.13 (m, 4H, aromatic); 4.84 (m, 0.5H, $C^{\alpha}H$); 4.5 (m, 2.5H, 0.5 $C^{\alpha}H$ + N-CH_2); 3.1 (m, 2H, $C^{\beta}H_2$); 1.39 and 1.46 (both singlets, 9H, t-Bu). TLC of N^{α}-Boc-D-Tic: R_f = 0.83 (AcOH/n-BuOH/Pyr/H_2O - 20/10/3/5, Baker Silica Gel Plates); R_f = 0.64 ($CHCl_3$/MeOH/AcOH 94/6/2, Baker Silica Gel Plates). TLC, HPLC, and FAB-MS data for 12 are presented in Table 6.

Table 6. Analytical Characteristics of 12 and 13

Peptide	Thin-layer chromatography[a] R_f Values I	II	III	IV	HPLC[b] k´	FAB-MS $[M+H]_{obs}$	$[M+H]_{calc}$
12	.45	.68	.77	.74	5.06	1088	1088
13	.43	.69	.77	.73	5.04	1076	1076

[a]Silica gel GF 250 microns (Analtech) glass plates were used. Following solvent system has been applied: (I) n-BuOH/AcOH/H_2O, 4:1:5 (v/v); (II) iPr-OH/NH_3/H_2O, 3 : 1 : 1 (v/v); (III) n-BuOH/AcOH/H_2O/Pyr, 6 : 1 : 5 : 6 (v/v); (IV) n-BuOH/AcOH/H_2O/Pyr 15 : 3 : 10 : 12 (v/v). [b]Vydac 218 TP 104 C_{18} column (25 cm x 4.6 mm), 0.1% TFA/CH_3CN 80:20, flowrate 2.5 ml/min, monitored at λ=214 nm.

D-N-MePhe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (13). The total yield was 18%, after purification. Synthesis of D-N-MePhe was accomplished by published methods[48], followed by N^{α}-Boc-D-N-MePhe formation as previously described. The HPLC, TLC, and FAB-MS data for 13 are given in Table 6.

The in vitro pharmacological characterization of [D-Tic^1]CTP, [D-N-MePhe^1]CTOP and related compounds will be reported in detail elsewhere[40].

The ^1H NMR spectra were acquired using a Bruker AM-250 spectrometer equipped with an Aspect 3000 computer. All spectra were recorded at 303°K. A 5 mg sample of the peptide (trifluoroacetate salt) was dried overnight in vacuo, dissolved in 0.3 mL of [$^2H_6$]DMSO (100% D atom, Aldrich), degassed and sealed.

The ^3J scalar connectivities were determined by double quantum filtered phase sensitive COSY experiments[29,30] using the Time-Proportional Phase Increments (TPPI) method. The pulse sequence was: D1-90-D0-90-D3-90-FID with D1 = 1.0s D0 = 0.000003s. For data manipulation: zero filling in F1, and sine-bell multiplication in both directions was applied prior to the FT. Homonuclear shift - correlated 2-D NMR with delay[31]; pulse sequence: D1-90-D0-90-D2-FID; D1 = 1.5s, D2 = 0.08s; data manipulation: zero filling in F1, squared sine-bell multiplication in both directions applied prior to FT. Homonuclear dipolar-correlated 2-D NMR; pulse sequence: D1-90-D0-90-D9-90-FID; D9 = 0.3s; D1 = 1.5s; data manipulation: zero filling in F1, squared sine-bell multiplication in both directions prior to FT. The temperature studies were carried out in the temperature range of 303-328°K, every 5 deg. DMSO was used as an internal standard, as no temperature influence on the chemical shift of $(CH_3)_2SO$ (δ = 2.49 ppm) occurs.

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Grant NS 19972 and DA 04248. We wish to thank Dr. Henry I. Yamamura and George Lui for the binding studies results which were provided to us prior to publication. The mass spectral determinations were performed by the Midwest Center for Mass Spectrometry, a National Science Foundation Regional Instrumentation Facility (Grant No. CHE 8211164).

REFERENCES

[1]V. J. Hruby, Life Sciences 31, 189-199 (1982) for a discussion and review.
[2]V. J. Hruby in Topics in Molecular Pharmacology, (A. S. V. Burgen and G. C. K. Roberts, eds.) pp. 100-126. Elsevier/North-Holland, Amsterdam (1981).

[3]M. Feigel, J. Am. Chem. Soc. 108, 181–182 (1986).
[4]T. J. Nitz, Y. Shimohigashi, T. Costa, H.-C. Chen and C. H. Stammer, Int. J. Peptide Protein Res. 27, 522–529 (1986).
[5]B. Vitoux, A. Aubry, M. T. Lung and M. Marraud, Int. J. Peptide Protein Res. 27, 617–632 (1986).
[6]C. Mapelli, H. Kimura and C. H. Stammer in Peptides: Structure and Function (C. M. Deber, V. J. Hruby and K. D. Kopple, eds.), pp. 503–506, Pierce Chemical Co., Rockford (1985).
[7]B. V. V. Prasad and P. Balaram, CRC Crit. Rev. Biochem. 307–348 (1984) for a review.
[8]J.-P. Meraldi, V. J. Hruby and A. T. R. Brewster, Proc. Natl. Acad. Sci. U.S.A., 74, 1373–1377 (1977).
[9]L. Terenius, Eur. J. Pharmacol. 38, 211–213 (1978).
[10]M. Rezek, V. Havlicek, L. Leybin, F.S. La Bella and H. Friesen, Can. J. Pharmacol. 56, 227–231 (1978).
[11]R. Maurer, B. H. Gaehwiler, H. H. Buescher, R. C. Hill and D. Roemer, Proc. Natl. Acad. Sci. USA 79, 4815–4817 (1982).
[12]D. F. Veber in Peptide Structure and Biological Function (D. Rich and E. Gross, eds.), pp. 685–694, Pierce Chemical Co., Rockford (1981).
[13]C. Wynants, G. Van Binst, A. Michel and J. Zanen, Int. J. Peptide Protein Res. 26, 561–567 (1985).
[14]J. T. Pelton, K. Gulya, V. J. Hruby, S. Duckles and H. I. Yamamura, Peptides 6, 159–163 (1985).
[15]V. J. Hruby, K. K. Dev, J. Fox, J. Bjorneeson and A. T. Tu, J. Biol. Chem. 253, 6060–6067 (1978)
[16]J. T. Pelton, K. Gulya, V. J. Hruby, S. P. Duckles and H. I. Yamamura, Proc. Natl. Acad. Sci. USA 82, 236–239 (1985).
[17]P. W. Schiller, B. Eggimann, J. DiMaio, C. Lemieux and T. M.-D. Nguyen, Biochem. Biophys. Res. Commun. 101, 3371 (1981).
[18]P. W. Schiller, J. DiMaio and T. M.-D. Nguyen in Proceedings of the 16th FEBS Congress, (Y. A. Ovchinnikov, ed.), pp.457–462, Utrecht, UNV Science Press (1985).
[19]H. I. Mosberg, R. Hurst, V. J. Hruby, J. J. Galligan, T. F. Burks, K. Gee, and H. I. Yammamura, Life Sciences 32, 2565–2569 (1983).
[20]H. I. Mosberg, R. Hurst, V. J. Hruby, K. Gee, H. I. Yamamura, J. J. Galligan and T. F. Burks, Proc. Natl. Acad. Sci. U.S.A. 80, 5871–5874 (1983).
[21]R. Schwyzer, Biochemistry 25, 6335–6342 (1986).
[22]J. T. Pelton, W. Kazmierski, K. Gulya, H. I. Yamamura and V. J. Hruby, J. Med. Chem. 29, 2370–2375 (1986).
[23]R. F. Nutt, D. F. Veber, P. E. Curley, R. Saperstein and R. Hirschmann, Int. J. Peptide Protein Res. 21, 66–73 (1983).
[24]K. Gulya, J. T. Pelton, V. J. Hruby and H. I. Yamamura, Life Sciences 38, 2221–2230 (1986).
[25]J. E. Shook, J. T. Pelton, W. S. Wire, L. D. Hirning, V. J. Hruby and T. F. Burks, J. Pharmacol. Exp. Therap. 240, 772–777 (1987).
[26]J. T. Pelton, T. Whalon, L. Cody and V. J. Hruby, Int. J. Peptide Protein Res. in press (1987).
[27]E. E. Sugg, D. Tourwé, W. Kazmierski, V. J. Hruby and G. Van Binst, Int. J. Peptide Protein Research, accepted for publication (1987).
[28]IUPAC-IUB Commission on Biochemical Nomenclature, Biochemistry 9, 3471–3479 (1970).
[29]D. Marion and K. Wüthrich, Biochem. Biophys. Res. Commun. 113, 967–974 (1983).
[30]M. Rance, O. W. Sorensen, G. Bodenhausen, G. Wagner, R. R. Ernst and K. Wüthrich, Biochem. Biophys. Res. Commun. 117, 471–478 (1983).
[31]A. Bax and R. Freeman, J. Magn. Res. 44, 542–561 (1981).
[32]V. F. Bystrov, Prog. NMR Spectroscopy 10, 41–81 (1976).
[33]J. Kobayashi, T. Higashijima, S. Sekido and T. Miyazawa, Int. J. Peptide Protein Res.17, 486–494 (1981).
[34]F. A. A. M. De Leeuw and C. Altona, Int. J. Peptide Protein Res. 20, 120–125 (1982).
[35]J. Feeney, J. Magn. Res. 21, 473–478 (1976).
[36]M. D. Shenderovich, G. V. Nikiforovich and G. I. Chipens, J. Magn. Res. 59, 1–12 (1984).
[37]K. Wüthrich, M. Billeter and W. Braun, J. Mol. Biol. 180, 715–740 (1984).
[38]B. N. N. Rao, A. Kumar, H. Balaram, A. Ravi and P. Balaram, J. Am. Chem. Soc. 105, 7432–7428 (1983).
[39]C. Grathwohl, A. Tun-Kyo, A. Bundi, R. Schwyzer and K. Wüthrich, Helv. Chim. Acta 58, 415–423 (1975).
[40]W. Kazmierski, J. E. Shook, R. J. Knapp, H. I. Yamamura, T. F. Burks and V. J. Hruby, submitted (1987).
[41]L. Lunazzi, G. Placucci and G. Cerioni, J. Chem. Soc. Perkin II, 1966–1969 (1977).
[42]D. A. Upson and V. J. Hruby, J. Org. Chem. 41, 1353–1358 (1976).
[43]J. M. Stewart and J. D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford, IL, 1984.
[44]R. C. Orlowski, R. Walter and D. Winkler, J. Org. Chem. 41, 3702–3705 (1976).
[45]E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, Anal. Biochem. 34, 595–598 (1970).
[46]T. Christensen in Peptides, Structure and Biological Function (E. Gross and J. Meienhofer, Eds.) pp.385–388, Pierce Chemical Co., Rockford, IL (1979).
[47]A. Picted and T. Spengler, Chem. Ber. 44, 2030–2036 (1911).
[48]J. R. McCermott and N. L. Benoiton, Can. J. Chem. 51, 1915–1919 (1973).