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Probing the Shape of a Hydrophobic Pocket in the Active Site of δ-Opioid Antagonists

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Abstract: The change of selectivity and the induction of antagonism by the insertion of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) in the second position of several opioid peptides have led to the interpretation of Tyr-Tic as a specific message domain for δ-opioid antagonists and to the discovery of dipeptides with substantial opioid activity. Selectivity and activity increase enormously when Tyr is substituted by 2′,6′-dimethyl tyrosine (Dmt), hinting that the side chain of Dmt fits a hydrophobic cavity of the receptor very tightly and precisely. We have investigated the specificity of this fit by systematic changes of the substituents on the aromatic ring of Tyr. Mono- and disubstitutions different from 2′,6′- invariably lead to catastrophic decreases of activity. The only substitution compatible with retention of substantial antagonism is 2′-methyl. An analysis of the conformational properties of all analogues reveals that substitutions do not affect the global shape of the molecule significantly. Accordingly, it is possible to use the shape of the different side chains to map the hydrophobic cavity of the receptor. The resulting complementary image is funnel shaped. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antagonists; dipeptides; opioids

INTRODUCTION

Opioid antagonists are molecules with a chemical constitution very similar to that of the corresponding agonists. They are generally obtained by replacement of one or more protons of the basic nitrogen, common to all opioids, with an appropriate substituent, e.g. an allyl or a cyclopropylmethyl group. The only exceptions with respect to nitrogen derivatization are a series of peptides containing 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, commonly dubbed Tic [1–4].

The *N*-terminal Tyr-Tic sequence behaves as an effective 'message' for δ-selective antagonism, since even the simple H-Tyr-Tic-NH_2 dipeptide behaves as a δ-selective antagonist [2] and insertion of Tic in the second position of typical agonists induces antagonism [2,3]. When Tyr was substituted by 2′,6′-dimethyl tyrosine (Dmt), we obtained ultraselective antagonists (H-Dmt-Tic-NH_2 and H-Dmt-Tic-Ala-NH_2) with activity comparable to that of naltrindole [5]. The enormous increase of both the activity and selectivity observed in going from H-Tyr-Tic-NH_2 to H-Dmt-Tic-NH_2 is not paralleled by significant conformational changes. A detailed conformational analysis [6] in solution of H-Dmt-Tic-NH_2 showed that its lowest energy conformers (i.e. *c*/T/T and *t*/T/T for *cis* and *trans* conformations, respectively) have a compact shape, with a relative arrangement of the two aromatic rings similar to that of the corresponding conformers of H-Tyr-Tic-NH_2 (C1b+ and T1b+) that had inspired the design of the first

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opioid dipeptides [2] and reminiscent of that of naltrindole [7]. It is interesting to note that the structural similarities in the pharmacophores of δ-agonists and antagonists are also the basis of a convergent model that was developed on entirely different premises [8–10].

The similarity of the bioactive conformations hints that the main cause of the increase in activity in going from H-Tyr-Tic-NH_2 to H-Dmt-Tic-NH_2 can be attributed to a very tight and precise fit of the side chain of Dmt in the hydrophobic cavity of the receptor. We have investigated the specificity of this fit by systematic changes of the number and position of substituents (methyl and hydroxyl groups) on the aromatic ring of Tyr in dipeptides of general formula H-*n*Me-Tyr-Tic-OH. In particular, the amino acids used in the peptide synthesis were the following: D,L-2′,3′-dimethyltyrosine); D,L-2′,5′-dimethyltyrosine; D,L-2′-methyltyrosine; 3-(2′,4′-dimethyl-3′-hydroxyphenyl)-D,L-alanine; D,L-2′,4′-dimethylphenylalarune.

MATERIAL AND METHODS

Chemistry

The racemic mixtures of the substituted aromatic amino acids were prepared according to the method of Abrash *et al.* [11] *ad hoc* modified (Scheme 1).

The starting materials (**1a**–**4a**) were reacted with ethyl chlorocarbonate to obtain the corresponding *O*-carbethoxy intermediates (**1b**–**4b**). The chloromethylation of the *O*-carbethoxy derivatives (**1b**–**4b**) by CH_2O/HCl gave always the 4-chloromethyl derivatives (**1c**, **2c**, **3c**) except for the case of *O*-carbethoxy-2,6-dimethylphenol (**4b**). In this case, we obtained the intermediate substituted in the 3-position, namely *O*-carbethoxy-2,6-dimethyl-3-chioromethylphenol (**4c**). In addition, when the chloromethylation was applied to *m*-xylene (**5b**) we obtained the derivative 2,4-dimethylbenzylchloride (**5c**). The intermediates **1c**–**5c** were reacted with diethyl acetamidomalonate in the presence of EtOH/Na to afford the corresponding benzyl malonate derivatives (**1d**–**5d**) which were successively hydrolysed and decarboxylated by treatment with 6 N HCl to give the desired amino acids (**1e**–**5e**). The amino acids (**1e**–**5e**) were treated by di-*tert*-butyldicarbonate in *tert*-butylalcohol (*t*Bu-OH) to obtain the corresponding Boc-derivatives (**1f**–**5f**). All dipeptides were prepared by standard solution methods by coupling the appropriate Boc-D,L-amino acid with H-L-Tic-O*t*Bu (Bachem, Bubendorf, Switzerland) *via* 1-ethyl-3-(3′dimethylaminopropyl)carbodiimmide·HCl/1-hydroxybenzoriazole·H_2O (WSC·HCl/HOBt) [12]. The obtained Boc-dipeptides were treated with trifluoroacetic acid (TFA) and precipitated with diethyl ether. The extent of diketopiperazine formation appeared to be essentially modest. Nevertheless, we isolated the cyclic derivatives when their yields were significant (**IId** and **IVd**). Crude diastereoisomers were purified by preparative reversed-phase HPLC.

Homogeneity of the products were assessed by analytical reversed-phase HPLC with a Vydac C18-column (5 μm, 4.6 × 250 mm, spherical) connected to a Rheodyne model 7725 injector, an Altex 420 HPLC system using two Altex 100A pumps, a Waters 486 tunable absorbance detector set to 220 nm, and a SE-120 strip chart recorder. Analytical determinations were carried out by two solvent systems: A: 10% (v/v), acetonitrile in 0.1% TFA; B: 60% (v/v), acetonitrile in 0.1% TFA (linear gradient from 100% A to 100% B over 25 min, UV detection at 220 nm, flow rate 1 ml/min).

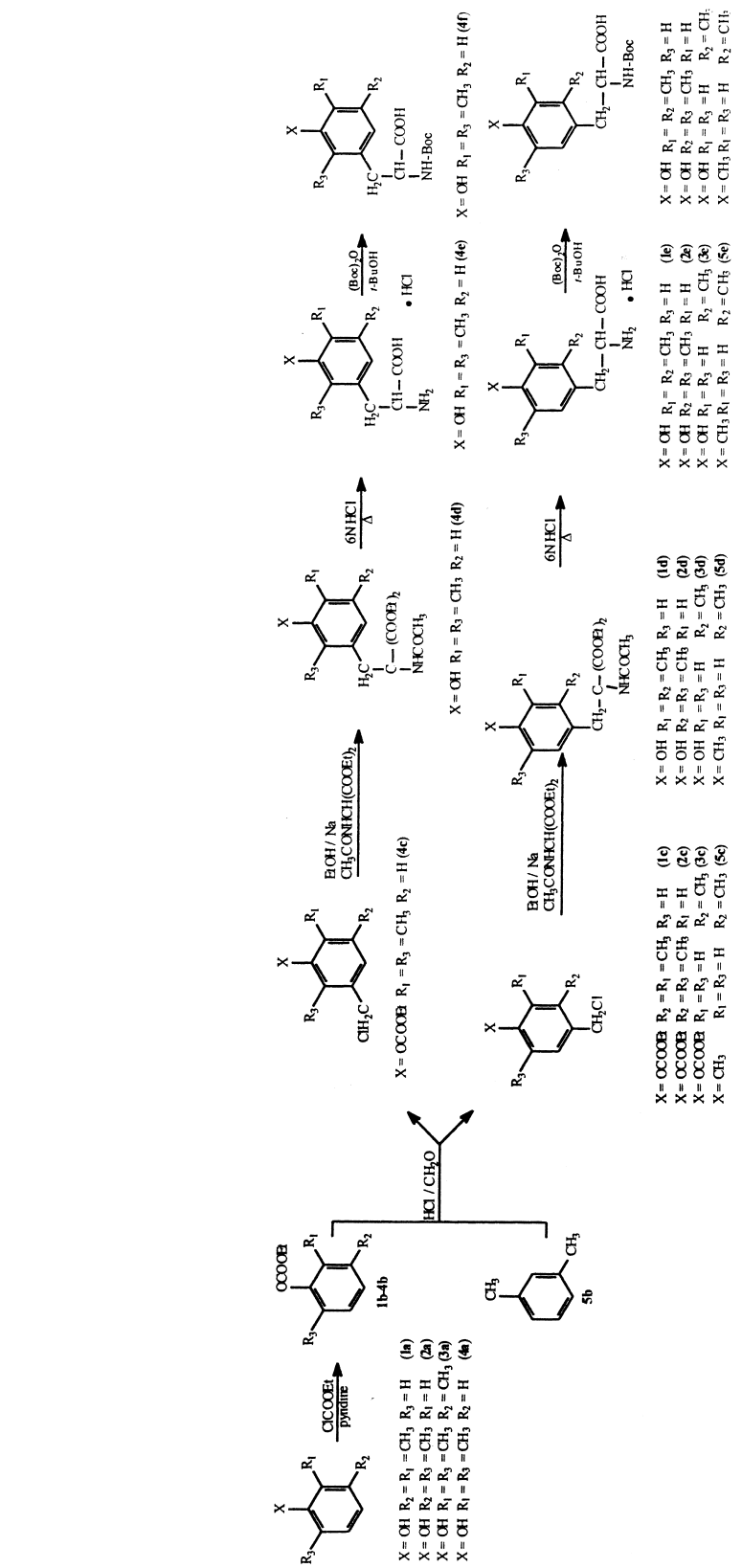
All analogues showed less than 1% impurities when monitored at 220 nm. TLC was performed on precoated silica gel Kieselgel 60F254 (Merck, Darmstadt, Germany) plates. The compounds were detected on TLC plates by UV light and either chlorination followed by a solution of 1% starch-15 KI (1:1, v/v) or ninhydrin. All solvents were purchased from C. Erba (Rodano, Milan, Italy). Extraction solvents were dried over sodium sulphate. Solvents used for reactions were dried over 3 Å molecular sieves. All solvents were filtered and degassed prior to use Reagent grade materials were purchased from Bachem and Aldrich (Milan, Italy) and were used without further purification.

All of the synthetic intermediates obtained in the amino acids synthesis were characterized by ^{1}H-NMR, except the protected dipeptides which were characterized by mass spectrometry (LCQ Thermoquest-Ion Trap). The final products (**I**–**V**, **IId** and **IVd**) were characterized by mass spectrometry (LCQ Thermoquest-Ion Trap).

Table 1 summarizes the schematic molecular formulas of the linear peptides and of two cyclic analogues.

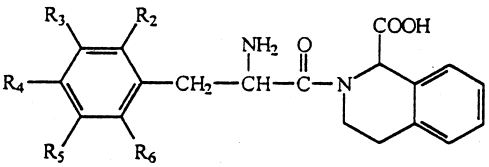
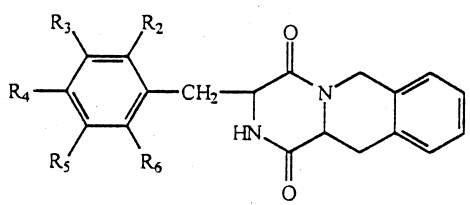
Biological Material

[^{3}H]-Naltrindole (33 Ci/mmol) was purchased from NEN Life Science Product (Boston, MA). Soybean trypsin inhibitor, [D-Ala^2, D-Leu^5]-enkephalin



Scheme 1

Table 1 Molecular Formulas of the Linear Peptides and the Two Cyclic Analogues

	R_2	R_3	R_4	R_5	R_6
					
H-Dmt-Tic-OH	$-CH_3$	–H	–OH	–H	$-CH_3$
I	$-CH_3$	$-CH_3$	–OH	–H	–H
II	$-CH_3$	–H	–OH	$-CH_3$	–H
III	$-CH_3$	–H	–OH	–H	–H
IV	$-CH_3$	–OH	$-CH_3$	–H	–H
V	$-CH_3$	–H	$-CH_3$	–H	–H
					
IId	$-CH_3$	–H	–OH	$-CH_3$	–H
IVd	$-CH_3$	–OH	$-CH_3$	–H	–H

(DADLE) and [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAGO) were obtained from Sigrna (St. Louis, MO). Bacitracin and phenylmethylsulfonyl fluoride (PMSF) were products of Fluka (Buchs, Switzerland), while bestatin was from Boehringer-Mannheim (Roche Diagnostic, Hoffinarm-La Roche, Basel, Switzerland). 7-Benzylidenenaltrexone (BNTX) was purchased form Tocris Cookson (Bristol, UK). Other agents and reagents were from standard commercial sources.

General Procedures for the Synthesis of the Amino Acids

The unconventional amino acids [D,L-2′,3′-dimethyltyrosine, D,L-2′,5′-dimethyltyrosine, D,L-2′-methyltyrosine, 3-(2′,4′-dimethyl-3-hydroxyphenyl)-D,L-alanine and D,L-2′,4′-dimethylphenylalanine] were synthesized in accordance with the method of Abrash *et al.* [11] appropriately modified as reported above. Details of the general method of synthesis are given for D,L-2′,3′-dimethyltyrosine. An analogous procedure was followed for the other derivatives.

***O*-Carbethoxy-2,3-dimethylphenol (1b)**. To a solution of 14 g of 2,3-dimethyiphenol (**1a**) in 85 ml of benzene and 9.2 ml of pyridine were added dropwise and with stirring 12.4 g of ethyl chlorocarbonate. The reaction mixture was stirred for 12 h at room temperature, then was filtered and the filtrate was evaporated. The residue was dissolved in $CHCl_3$ and washed with 2 N NaOH and citric acid (10%). The organic phase was dried and evaporated to an oily residue: yield 16.1 g (73%). ^{1}H-NMR ($CDCl_3$) $\delta = 1.30–1.33$ (m, 3H), 2.06 (s, 3H), 2.2 (s, 3H), 4.22–4.26 (m, 2H), 6.87, 6.89 (d, 1H), 6.97, 6.98 (d, 1H), 7.01–7.04 (m, 1H).

***O*-Carbethoxy-2,3-dimethyl-4-chloromethylphenol (1c)**. A mixture of 16.1 g of *O*-carbethoxy-2,3-dimethylphenol (**1b**), 17 ml of 37% aqueous formaldehyde, and 80.5 ml of concentrated HCl was kept at 50–60°C and anhydrous HCl was added to the reaction mixture for 4 h. The cooled reaction mixture was taken up in $CHCl_3$. The organic phase was dried over anhydrous sodium sulphate, the solvent was evaporated and the oily residue was purified on a silica gel column using hexane/diethyl ether (9:1) as eluant: yield 6.3 g (32%). ^{1}H-NMR (MeOD) $\delta =$ 1.38–1.41 (m, 3H), 2.2 (s, 3H), 2.3 (s, 3H), 4.30–4.33 (m, 2H), 4.6 (s, 2H), 6.96, 6.97 (d, 1H), 7.18, 7.19 (d, 1H).

Diethyl acetamido-(2′,3′-dimethyl-4′-hydroxybenzyl)malonate (1d). A solution of sodium ethoxide was prepared by adding 0.55 g of sodium to 150 ml of absolute EtOH. To this solution were added 5.2 g of diethyl acetamidomalonate and then after 10 min 5.8 g of *O*-carbethoxy-2,3-dimethyl-4-chloromethylphenol (**1c**). The mixture was refluxed for 2 h, cooled, and filtered, and the filtrate was evaporated *in vacuo* to a colourless oil, which crystallized when triturated with diethyl ether: yield 6.5 g (77%). ^{1}H-NMR (MeOD) $\delta = 1.27–1.30$ (m, 6H), 2.00 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 3.58 (s, 2H), 4.18–4.29 (m, 4H), 6.54, 6.56 (d, 1H), 6.64, 6.66 (d, 1H).

H-D,L-2′,3′-dimethyltyrosine-OH·HCl (1e). A mixture of 6.5 g of diethyl acetamido(2′,3′-dimethyl-4′-hydroxybenzyl)-malonate (**1d**) and 44.5 ml of 6 N HCl was refluxed for 12 h. The reaction mixture was cooled, and the crystalline product was collected and dried to give 2.2 g of D,L-2′,3′-dimethyltyrosine hydrochloride. An additional 1.2 g was obtained from the mother liquor: yield 3.4 g (75%). ^{1}H-NMR (MeOD) $\delta = 1.94$ (s, 3H), 2.04 (s, 3H), 2.68–2.78 (m, 1H), 3.10–3.21 (m, 1H), 3.78–3.84 (m, 1H), 6.39–6.43 (d, 1H), 6.63, 6.66 (d, 1H).

Boc-D,L-2′,3′-dimethyltyrosine-OH (1f). To a solution of 1.6 g of H-D,L-2′,3′-dimethyltyrosine-OH·HCl (**1e**) in 27 ml of *t*BuOH/H_2O (2:1) were added 1 N NaOH (13 ml) and 1.6 g of di-*tert*-butyldicarbonate at 0°C. The reaction mixture was stirred for 3 h at 0°C and 24 h at room temperature. Then, H_2O (40 ml) and solid citric acid (9 g) were added. The product was extracted with EtOAc, dried over sodium sulphate and evaporated to dryness. The residue was crystallized with diethyl ether/hexane: yield 1.5 g (75%). ^{1}H-NMR (MeOD) $\delta = 1.22$ (s, 9H), 1.90 (s, 3H), 2.10 (s, 3H), 2.68–2.78 (m, 1H), 3.10–3.21 (m, 1H), 3.79–3.86 (m, 1H), 6.41, 6.45 (d, 1H), 6.71, 6.74 (d, 1H).

Using the procedure described above for the preparation of **1b** the following additional intermediates were synthesized:

O-Carbethoxy-2,5-dimethylphenol (2b). Yield 70%. ^{1}H-NMR ($CDCl_3$) $\delta = 1.20–1.38$ (m, 3H), 2.19 (s, 3H), 2.30 (s, 3H), 4.27–4.32 (m, 2H), 6.90 (s, 1H), 7.00–7.08 (d, 1H), 7.10, 7.13 (d, 1H).

O-Carbethoxy-3-methylphenol (3b). Yield 91%. ^{1}H-NMR ($CDCl_3$) $\delta = 1.30–1.35$ (m, 3H), 2.3 (s, 3H), 4.21–4.28 (m, 2H), 6.87–6.89 (d, 1H), 6.92 (s, 1H), 6.98,7.02 (d, 1H), 7.28–7.33 (m, 1H).

O-Carbethoxy-2,6-dimethylphenol (4b). Yield 80%. ^{1}H-NMR ($CDCl_3$) $\delta = 1.12–1.26$ (m, 3H), 2.20 (s, 6H), 4.30–4.36 (m, 2H), 6.6l–6.71 (m, 1H), 6.89, 6.93 (d, 2H).

Using the procedure described above for the preparation of **1c** the following additional intermediates were synthesized.

O-Carbethoxy-2,5-dimethyl-4-chloromethylphenol (2c). Yield 30%. ^{1}H-NMR ($CDCl_3$) $\delta = 1.20–1.38$ (m, 3H), 2.16 (s, 3H), 2.38 (s, 3H), 4.27–4.32 (m, 2H), 4.63 (s, 2H), 6.96 (s, 1H), 7.23 (s, 1H).

O - Carbethoxy - 3 - methyl - 4 - chloromethylphenol (3c). Yield 33%. $\delta = 1.31–1.37$ (m, 3H), 2.43 (s, 3H), 4.27–4.31 (m, 2H), 4.66 (s, 1H),7.05 (s, 1H), 7.36,7.37 (d, 1H).

O - Carbethoxy - 2,6 - dimethyl - 3 - chloromethylpheno1 (4c). Yield 41%. ^{1}H-NMR ($CDCl_3$) $\delta = 135–145$ (m, 3H), 2.18 (s, 3H), 2.25 (s, 3H), 4.30–4.36 (m, 2H), 4.58 (s, 2H), 7.04, 7.06 (d, 1H), 7.12, 7.14 (d, 1H).

2,4-Dimethylbenzylchloride (5c). Yield 69%. ^{1}H-NMR (MeOD) $\delta = 2.29$ (s, 3H), 2.37 (s, 3H), 4.60 (s, 2H), 6.96, 6.97 (d, 1H), 7.00 (s, 1H), 7.15, 7.17 (d, 1H).

Using the procedure described above for the preparation of **1d** the following additional intermediates were synthesized.

Diethylacetamido-(2′,5′-dimethyl-4′-hydroxybenzyl)malonate (2d). Yield 83%. ^{1}H-NMR (MeOD) $\delta =$ 1.22–1.26 (m, 6H), 1.95 (s, 3H), 2.05 (s, 3H), 2.15 (s, 3H), 3.45 (s, 2H), 4.15–4.25 (m, 4H), 6.5 (s, 1H), 6.65 (s, 1H).

Diethylacetamido-(2′-methyl-4′-hydroxybenzyl)-malonate (3d). Yield 69%. ^{1}H-NMR (MeOD) $\delta = 1.2–1.4$ (m, 6H), 2.05 (s, 3H), 2.30 (s, 3H), 3.65 (s, 2H), 4.20–4.40 (m, 4H), 6.62, 6.67 (d, 1H), 6.72 (s, 1H), 6.98, 7.01 (d, 1H).

Diethylacetamido-(2′4′-dimethyl-3′-hydroxybenzyl)malonate (4d). Yield 78%. ^{1}H-NMR (MeOD) $\delta =$ 1.10–1.14 (m, 6H), 1.82 (s, 3H), 1.88 (s, 3H), 1.97 (s, 3H), 3.48 (s, 2H), 4.02–4.06 (m, 4H), 6.67, 6.68 (d, 1H), 6.85, 6.87 (d, 1H).

Diethylacetamido-(2′,4′-dimethylbenzyl)malonate (5d). Yield 90%. ^{1}H-NMR (MeOD) $\delta = 1.26–1.32$ (m, 6H), 2.20 (s, 3H), 2.26 (s, 3H), 2.31 (s, 3H), 3.60 (s, 2H), 4.18–4.29 (m, 4H), 6.85, 6.87 (d, 1H), 6.90, 6.92 (d, 1H), 6.98 (s, 1H).

Using the procedure described above for the preparation of le the following additional intermediates were synthesized.

D,L-2′,5′-Dimethyltyrosine (2e). Yield 55%. ^{1}H-NMR (MeOD) $\delta = 2.10$ (s, 3H), 2.30 (s, 3H), 3.10–3.13 (m, 1H), 3.26–3.31 (m, 1H), 4.17–4.19 (m, H), 6.92 (s, 1H), 6.20 (s, 1H).

D,L-2′-Methyltyrosine (3e). Yield 58%. ^{1}H-NMR (MeOD) $\delta = 2.31$ (s, 3H), 3.02–3.07 (m, 1H), 3.23–3.27 (m, 1H), 4.15–4.18 (m, 1H), 6.63, 6.65 (d, 1H), 6.70 (s, 1H), 6.98, 7.00 (d, 1H).

3-(2′,4′-Dimethyl-3′-hydroxyphenyl)-D,L-alanine (4e). Yield 68%. ^{1}H-NMR (MeOD) $\delta = 1.87$ (s, 3H), 1.91 (s, 3H), 3.06–3.16 (m, 1H), 3.45–3.49 (m, 1H), 3.84–3.90 (m, 1H), 6.69, 6.70 (d, 1H), 6.87, 6.89 (d, 1H).

D,L-2′,4′-Dimethylphenylalanine (5e). Yield 76%. ^{1}H-NMR (MeOD) $\delta = 2.18$ (s, 3H), 2.28 (s, 3H), 3.11–3.15 (m, 1H), 3.28–3.32 (m, 1H), 4.19–4.21 (m, 1H), 6.82, 6.88 (d, 1H), 6.92, 6.96 (d, 1H), 7.00 (s, 1H).

Using the procedure described above for the preparation of if the following additional intermediates were synthesized.

Boc-D,L-2′,5′-dimethyltyrosine-OH (2f). Yield 87%. ^{1}H-NMR (MeOD) $\delta = 1.20$ (s, 9H), 2.12 (s, 3H), 2.35 (s, 3H), 3.10–3.13 (m, 1H), 2.28–3.35 (m, 1H), 4.19–4.22 (m, 1H), 6.90 (s, 1H), 7.20 (s, 1H).

Boc-D,L-2′,-methyltyrosine-OH (3f). Yield 60%. ^{1}H-NMR (MeOD) $\delta = 1.28$ (s, 9H), 2.30 (s, 3H), 3.00–3.05 (m, 1H), 3.23–3.27 (m, 1H), 4.10–4.13 (m, 1H), 6.61, 6.64 (d, 1H), 6.68 (s, 1H), 6.98, 7.01 (d, 1H).

Boc-3-(2′,4′-dimethyl-3′-hydroxyphenyl)-D,L-alanine-OH (4f). Yield 70%. ^{1}H-NMR (MeOD) $\delta =$ 1.26 (s, 9H), 1.95 (s, 3H), 2.01 (s, 3H), 3.06–3.16 (m, 1H), 3.40–3.44 (m, 1H), 3.81–3.87 (m, 1H), 6.71, 6.72 (d, 1H), 6.88, 6.90 (d, 1H).

Boc-D,L-2′,4′-dimethylphenylalanine (5f). Yield 60%. ^{1}H-NMR (MeOD) $\delta = 1.20$ (s, 9H), 2.15 (s, 3H), 2.20 (s, 3H), 3.08–3.10 (m, 1H), 3.25–3.28 (m, 1H), 4.15–4.18 (m, 1H), 6.80, 6.85 (d, 1H), 6.90–6.93 (d, 1H), 6.98 (s, 1H).

Peptide Synthesis

The main procedures used for the preparation of the dipeptides reported in Table 1 varied little in the individual steps and are therefore summarized in the following general form.

General procedure for condensation of Boc-D,L-amino acid-OH (1f–5f) with H-L-Tic-OtBu. To a solution of Boc-D,L-amino acid-OH (1 mmol) and H-L-Tic-O*t*Bu (1 mmol) in DMF at 0°C were added HOBt (1.1 mmol), WSC (1.1 mmol), and trietylamine (TEA) (1.1 mmol).

The reaction mixture was stirred for 3 h at 0°C and 24 h at room temperature. After evaporation of DMF the residue was solubilized in EtOAc and washed with citric acid (10%), $NaHCO_3$ (5%) and brine. The organic phase was dried and evaporated to dryness. The residue was crystallized from diethyl ether and characterized by MS (LCQ-Thermoquest-Ion Trap). The MS data were as follows:

Boc-D,L-2′,3′-dimethyltyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid-t-butyl ester. M 524.66 Calc.; MH^+ 525.90 Found.

Boc-D,L-2′,5′-dimethyltyrosyl-1,2,3,4-tetrahydroisoquinohne-3-carboxylic acid-t-butyl ester. M 524.66 Calc.; MH^+ 525.85 Found.

Boc-D,L-2′-methyltyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid-t-butyl ester. M 510.63 Calc.; MH^+ 511.85 Found.

Boc-3-(2′,4′-dimethyl-3′-hydroxyphenyl)-D,L-1,2,3,4-tetrahydroisoquinohne-3-carboxylic acid-t-butyl ester. M 524.66 Calc.; MH^+ 525.90 Found.

Boc-D,L-2′,4′-dimethylphenylalanyl-1,2,3,4-tetrahydroisoqrnnoline-3-carboxy1ic acid-t-butyl-ester. M 508.66 Calc.; MH^+ 509.85 Found.

General procedure for deprotection. The terminally protected peptide was treated with TFA (2 ml) for 30 min at room temperature. Diethyl ether was added to the solution until the product precipitated.

TFA·H-D,L-2′,3′-dimethyltyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (I). Yield 70%. M 368.43 Calc.; MH^+ 369.65 Found. R_t 14.91.

TFA·H-D,L-2′,5′-dimethyltyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (II). Yield 75%. M 368.43 Calc.; MH^+ 369.70 Found. R_t 15.01.

TFA·H-D,L-2′-methyltyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (III). Yield 73%. M 354.40 Calc.; MH^+ 355.75 Found. R_t 14.49.

TFA·H-3-(2′,4′-dimethyl-3′-hydroxyphenyl)-D,L-alanyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (IV). Yield 70%. M 368.43 Calc.; MH^+ 369.80 Found. R_t 16.31.

TFA·H-D,L-2′,4′-dimethylphenylalanyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (V). Yield 77%. M 352.43 Calc.; MH^+ 353.65 Found. R_t 12.44.

Diketopiperazines. **IId**: M 350.41 Calc.; MH^+ 351.65 Found. R_t 17.30.

IVd: M 350.41 Calc.; MH^+ 351.70 Found. R_t 18.40.

Membrane Preparation

Male Sprague–Dawley rats (150–200 g) were sacrificed by cervical dislocation. Tissue membranes were prepared as described [13] with some modifications. Briefly, the whole brain without cerebellum was rapidly removed and homogenized using a Polytron homogenizer at 0°C in 20 volumes of 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ (buffer A). The membrane homogenate was centrifuged at 48000 × *g* for 15 min at 4°C. The resulting pellet was resuspended in 20 volumes of 50 mM Tris–HCl (pH 7.4) and 1 mM EDTA, 100 mM NaCl (buffer B) containing 0.05 mg/ml of soybean trypsin inhibitor, 0.1 mM PMSF and100 μM GDP and incubated at 25°C for 30 min. This treatment is required to remove endogenous opioid peptides and saturate α-subunits of G proteins with GDP. After preincubation, membranes were centrifuged and resuspended three times in buffer A to remove excess of Na^+ and GDP and to induce the receptor population into the high-affinity state [13]. The final pellet was stored in aliquots at −80°C until the time of assay. Membrane protein concentration was determined by the method of Lowry *et al*. [14] using bovine serum albumin (BSA) as a standard.

Radioligand Binding Assay

Brain membranes (0.2 mg of proteins) were incubated with 0.1–0.2 nM [^{3}H]naltrindole in 1 ml of buffer A containing 1 mg/ml BSA, 50 μg/ml bacitracin, 30 μM bestatin and 0.1 mM PMSF at 25°C for 3 h. Non-specific binding was measured in the presence of 10 μM BNTX. Incubation was terminated by filtration through GF/C glass fiber filters (Whatman, Springfield Mill, UK), previously treated with 0.1% polyethylenimine for more than 1 h. Then, filters were washed three times with 4.0 ml of ice-cold buffer A and radioactivity was measured using a liquid scintillation cocktail by a Packard TRI-CARB 1600 scintillation counter (Packard Instrument, Meriden, CT).

For saturation studies, membranes were incubated in buffer A with ten different concentrations of [^{3}H]-naltrindole ranging from 25 pM to 1 nM. Competition studies were carried out by incubating membranes in buffer A with 0.1 nM [^{3}H]-naltrindole and up to eight to 12 concentrations of BNTX (0.01–5 nM), DADLE (0.1–50 nM), DAGO (0.025–5 μM), a reference (0.1 nM to 1 μM) or newly synthesized peptides (1 nM to 10 μM).

Data Analysis

Data were analysed by non-linear least-square fitting, using the GraphPad Prism Version 3.0 computer program (Graphpad Software, San Diego, CA). Single- and multiple-site models were statistically compared to determine the best fit and differences among models were tested by comparing the residual variance, using a partial *F* test and a significance level of $p < 0.05$. The IC_{50} values obtained from displacement curves were converted to K_i values by the Cheng and Prusoff equation [15]. Values represent the mean ± S.E. of three experiments except when otherwise stated.

Energy Calculations

Energy minimization was performed using the SANDER module of the AMBER 5.0 package [16]. The 1991 version of the all-atom force field was used [17] with a distance-dependent dielectric constant $\varepsilon = r_{ij}$. In order to reduce the artifacts which can arise during *in vacuo* simulation, the charge of the ionizable groups was reduced to 20% of its full value. A distance cut-off of 8 Å was used in the evaluation of non-bonded interactions.

RESULTS AND DISCUSSION

[^{3}H]-Naltrindole binds to rat brain membranes in a specific manner. Saturation experiments demonstrated that specific binding is saturable. Analysis of saturation data showed that the best fit obtained is for a one-site model, indicating the presence of a homogenous population of binding sites (data not shown). In addition, transformation of these data according to Scatchard produced linear plots (data not shown). The derived K_D and B_{max} values were 62.3 pM and 63 fmol/mg protein ($n = 2$), respectively. This K_D value is very similar to that reported for [^{3}H]-naltrindole binding to mouse brain membranes [13].

Displacement curves of [^{3}H]-naltrindole binding by BNTX, DAGO and DADLE were monophasic and represented by a one-site model (data not shown).

The selective δ_1-opioid receptor antagonist BNTX inhibited specific [^{3}H]-naltrindole binding to membranes with a K_i value of 1.7 ± 0.1 nM ($n = 3$), while the K_i values for inhibition by the selective δ-(DADLE) and μ-(DAGO) opioid receptor agonists were 3.1 ± 0.3 nM ($n = 3$) and 168.2 ± 0.3 nM ($n = 3$), respectively. The reference opioid dipeptide H-Dmt-Tic-OH also displaces [^{3}H]-naltrindole binding with a monophasic curve (Figure 1), which is fitted by a one-site model. The derived K_i value is 1.6 ± 0.3 nM ($n = 3$). This value, although comparable with that of typical non-peptidic antagonists, is considerably lower than that previously reported [5], reflecting the variability intrinsic to the preparation of tissues in this type of experiments.

In the screening of a series of analogues of H-Dmt-Tic-OH, we decided to use diastereomeric mixtures since binding should not be much affected by the homogeneity of the products. The crucial residue for antagonism is the second one: we have in fact shown in the past that only peptides containing L-Tic confer δ-antagonism [2,3], whereas those containing D-Tic are μ-agonists [1]. In all the new peptides described in the manuscript the second residue is L-Tic. As for the first residue, we showed recently that D-Dmt, combined with either an L- or D- second aromatic residue, confers some μ-antagonism [18]. Thus, we argued that the effect of the D,L components of the mixtures could only somehow subtract from δ-antagonism. Since the net result is essentially negative, albeit interesting, i.e. all analogues are far less effective than Dmt (*vide infra*), we proceeded to a more accurate measurement of the binding constant for only two compounds.

Table 2 shows the inhibitory effects of the newly synthesized peptides. Only peptide **III** inhibited specific [^{3}H]-naltrindole binding more than 50% at a concentration of 1 μM. Therefore, displacement studies by various concentrations of this peptide were carried out. The competition curve was monophasic and represented by a one-site model (Figure 1) with a K_i value of 60.7 ± 0.3 nM ($n = 3$). In our competition assays peptide **III** showed a binding affinity for δ-opioid receptors in rat brain membranes 20-fold lower and threefold higher than those obtained for DADLE and DAGO, respectively. In addition, this compound has 40-fold less affinity than the reference compound H-Dmt-Tic-OH. The cyclic peptide analogue **IVd**, chosen as representative of a chargeless cyclic antagonist, in spite of its low inhibition activity (Table 2), also displayed a monophasic competition curve fitted by a one site-model (Figure 1). This curve was right-shifted as compared to those of the other peptides. Indeed, the

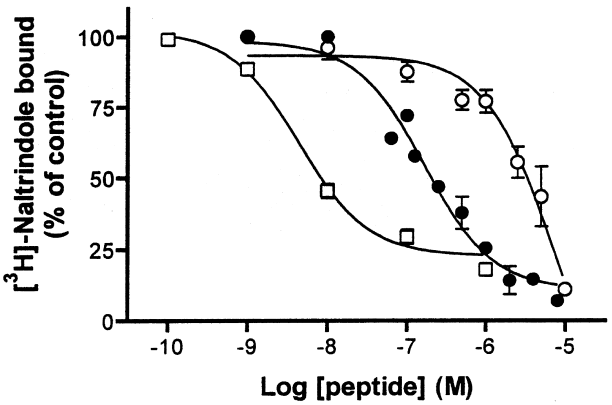


Figure 1 Displacement of [^{3}H]-naltrindole binding to rat brain membranes by peptide analogues. Membranes (0.2 mg of proteins) were incubated with 0.1 nM [^{3}H]-naltrindole and various concentrations of dipeptide H-Dmt-Tic-OH (0.1 nM to 1 μM), peptide **III** (1 nM to 7.9 μM), or cyclic peptide analogue **IVd** (1 nM to 10 μM) as described in 'Material and Methods'. Values are mean ± S.E. of three independent experiments, each performed in duplicate. The IC_{50} values were determined by fitting the data as one-site competition curves with the GraphPad Prism version 3.0 computer program. The K_i values were calculated from the IC_{50} values by the Cheng and Prusoff equation [15]. □, H-Dmt-Tic-OH; ●, peptide **III**; ○, peptide **IVd**.

Table 2 Inhibition of Specific [^{3}H]-Naltrindole Binding to Rat Brain Membranes by Dipeptide Antagonists and Binding Constants of Selected Compounds

Analogue	% of binding inhibition at 1 μM	K_i (nM)
I	30.7 ± 0.7[a]	
II	47.0 ± 4.6[c]	
III	70.7 ± 7.3[a]	60.7 ± 0.3[a]
IV	31.0[b]	
V	14.3 ± 3.2[a]	
IId	18.0[b]	
IVd	32.0[b]	2700 ± 600[a]
H-Dmt-Tic-OH		1.9 ± 0.6[a]
c[Dmt-Tic]		1500[c]
BNTX		1.7 ± 0.1[a]
DADLE		3.1 ± 0.3[a]
DAGO		168.2 ± 0.3[a]

[a] Values are mean ± S.E. of three independent experiments each performed in duplicate.
[b] Values are average of two independent experiments performed in duplicate.
[c] Reference [25].

derived K_i value for inhibition of [^{3}H]-naltrindole binding by compound **IVd** was 2.7 ± 0.6 μM ($n = 3$).

As mentioned in the 'Introduction', the minimum energy conformers of both H-Tyr-Tic-NH_2 and H-Dmt-Tic-NH_2 (T1b+ and C1b+, respectively, for *trans* and *cis* isomers) have a shape consistent with that of rigid δ-selective opioids [2,6]. The results of the biological activity (Table 2) show that all peptides with Tyr analogues different from Dmt undergo a drastic decrease of activity. This behaviour can be interpreted in terms of (i) a change in the global conformation of the peptide; (ii) a less effective interaction of the side chain of the Tyr analogue with the corresponding hydrophobic pocket in the receptor; (iii) a combination of both. It is possible to discriminate among these possibilities by means of an appropriate conformational analysis.

We performed a molecular mechanics (MM) study for each of the analogues listed in Table 1. The natural starting points for judging the relative stability of conformers of all dipeptide analogues are the conformational analyses previously performed on H-Tyr-Tic-NH_2 [2,3] and H-Dmt-Tic-NH_2 [6]. The fact that the peptides of the present work have a free carboxyl *C*-terminal group *in lieu* of the amide did not represent a problem since the results of the previous conformational analyses could easily be transferred to H-Tyr-Tic-OH, H-Dmt-Tic-OH [2] and even to longer peptides [3]. Nonetheless, we have performed a new conformational analysis of H-Tyr-Tic-OH and H-Dmt-Tic-OH. In the analyses of these two peptides, the search was limited to conformers characterized by either a *cis* or *trans* peptide bond and with the two main boat conformations (called b+ and b−, with reference to the $g+$ or $g-$ value of Tic χ^1) of the Tic ring. All combinations of backbone torsion angles with three values of Tyr χ^1 ($g+$, $g-$, t) were explored and subjected to unrestrained minimization. If one limits the analysis to the conformers whose energy lies within 5 kcal/mol from the absolute minimum of the search, *cis* and *trans* conformers are of comparable energy, albeit with a prevalence of the *cis* conformers. The structures comprised within 5 kcal/mol from the absolute minimum were compared to a rigid mould, MeNTI, a δ-selective opioid antagonist [19,20] by fitting the carbon atoms of the corresponding aromatic rings. Once again, only conformers C1b+ and T1b+ emerged from this selection [2].

Figure 2 shows the overlay of conformers C1b+ and T1b+ of H-Tyr-Tic-OH with the corresponding conformers of H-Dmt-Tic-NH_2 [6]. The overall shapes of the peptides are similar to that of rigid moulds (*vide infra*), lending strong support to our proposal [2,3] that the arrangement of the two aromatic rings of Dmt and Tic is consistent with specific δ-receptor requirements. It also noteworthy that all positions around the aromatic ring of Tyr seem far away from the atoms of the Tic moiety, particularly in the case of C1b+. Accordingly, ring substitutions should not affect too much the global shape of the peptide.

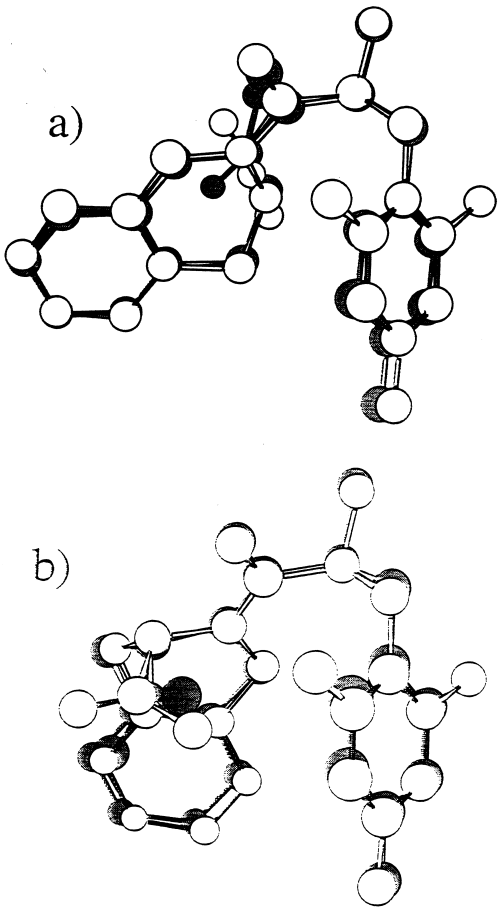


Figure 2 Overlay of the ball and stick molecular models of conformers C1b+ and T1b+ of H-Tyr-Tic-OH (a, atoms shown as grey balls) with the corresponding conformers of H-Dmt-Tic-NH_2 (b, atoms shown as white balls).

The situation with the newly synthesized H-nMeTyr-Tic-OH peptides is very similar. A preliminary grid search on each peptide showed that only two conformers, corresponding to C1b+ and T1b+, among the minimum energy structures have sufficient resemblance to rigid moulds. Table 3 summarizes the relevant conformational parameters and relative energies of the minimum energy conformers of H-nMeTyr-Tic-OH peptides. All

Table 3 Minimum Energy Conformations of H-nMeTyr-L-Tic-OH

	ΔE (kcal)	χ_1 [1a]	χ_1 [2b]	ψ_1
T1b+				
H-Tyr-Tic-OH	2.80	−175	81	136
H-Dmt-Tic-OH	4.44	−174	73	138
I	4.29	−175	86	140
II	1.26	−175	86	140
III	3.08	−176	86	140
IV	1.84	−176	88	139
V	2.08	−176	88	139
C1b+				
H-Tyr-Tic-OH	1.42	−179	88	145
H-Dmt-Tic-OH	**2.78**	−173	83	151
I	2.26	−171	−101	149
II	0	−174	89	145
III	1.70	−174	89	145
IV	0.52	−173	145	90
V	0.84	−175	144	90
c[Dmt-Tic]	7.327	*t*		
IId	4.857	*t*		
IVd	5.247	*t*		

[a] Torsion angle about C^{α}–C^{β}: χ^1.
[b] Torsion angle about C^{β}–C^{γ}: χ^2.

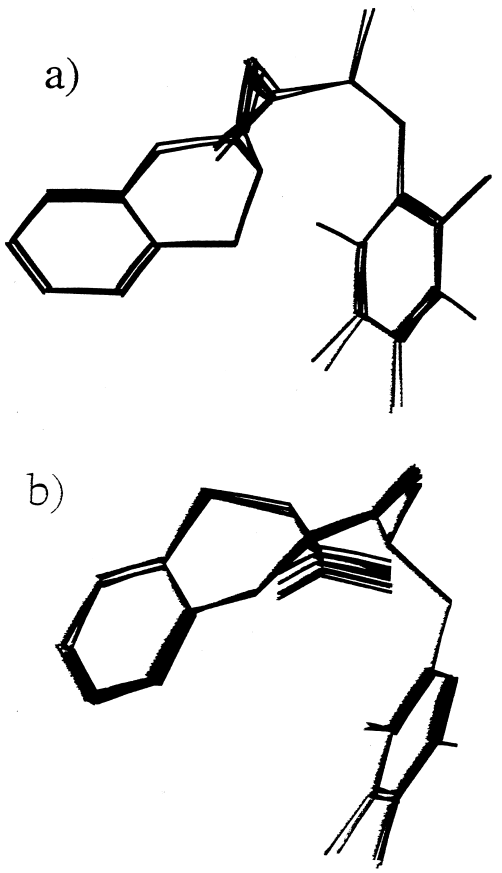


Figure 3 Superposition of wireframe models of C1b+ (a) and T1b+ (b) conformers of peptides **I**–**V**.

analogues have minimum energy conformers of comparable stability to (or even higher than) that of the parent peptide (H-Dmt-Tic-OH). Indeed, the absolute minimum is that of conformer C1b+ of the peptide **II**. Figure 3 shows the superposition of all C1b+ and T1b+ conformers. Even widely different substitutions on the aromatic ring of the first residue have little influence on the backbone conformation. Thus, the global shape is little influenced by the substitutions, whereas the observed energy differences mainly reflect a different number of van der Waals contributions.

However, the relative antagonist activities (Table 2) of the analogues does not parallel their relative stabilities (Table 3). The only peptide with δ-opioid receptor affinity comparable to that of the parent antagonist (H-Dmt-Tic-OH) is peptide **III**. Figure 4 shows the superposition of the molecular models of conformers C1b+ of peptides **II** and **III**. The model of MeNTI, a typical rigid antagonist, is also shown for comparison. Peptide **II** is the absolute minimum of Table 3 while peptide **III** is the analogue with the highest antagonist activity. Data of Table 2 suggest that methyl groups in positions 2′,6′ (i.e. those corresponding to Dmt) would induce stronger binding, whereas their presence in all other positions is detrimental. It is not surprising that substitution of the hydroxyl group with a methyl is even more detrimental.

For comparison, we studied also two cyclic analogues, namely **IId** and **IVd**. These compounds are representative of chargeless antagonists. The antagonism of linear Tyr/Dmt-Tic peptides may be interpreted as an indication that the *N*-terminal Tyr-Tic sequence behaves as an effective 'message' for δ-selective antagonism, either by shifting the basic nitrogen from its natural position [21–23] or by preventing a conformational change at the receptor [24]. That is, peptide antagonists have bioactive conformations that either prevent the proper interaction of the basic centre with the corresponding anionic site of the receptor or simply block the access to agonists without inducing an allosteric conformational change that can trigger the transmission of a message. The last hypothesis was substantiated by the synthesis of the Dmt-Tic

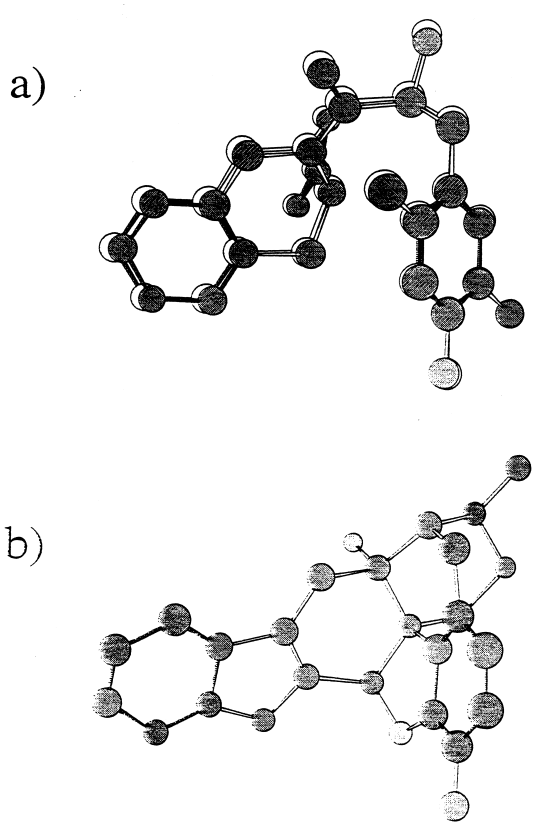


Figure 4 Comparison of the superposition of the ball and stick molecular models of conformers C1b+ of peptides **II** and **III** (a) and of the ball and stick molecular model of a rigid antagonist, MeNTI (b). Peptide **II** is coloured in violet, peptide **III** is coloured in yellow. The model of MeNTI is shown with green carbons, red oxygens and blue nitrogens.

diketopiperazine, the first, partially rigid, opioid antagonist bearing no charge [25]. The cyclic analogue reported in this paper (**IVd**), like *c*[Dmt-Tic], has an activity one order of magnitude lower than that of typical linear antagonists. We also found that its conformation is similar to that of *c*[Dmt-Tic]. Thus, the lower activity of compound **IVd**, with respect to *c*[Dmt-Tic], may be due to detrimental substitutions on the first aromatic ring. Owing to the intrinsically low activity of the cyclic analogues, it is not possible to draw more definitive conclusions, but it is very interesting to find that the model of chargeless antagonist is also consistent in less favourable cases.

CONCLUSIONS

Opioid peptide antagonists characterized by either Tyr-Tic or Dmt-Tic in the *N*-terminal part show a difference in activity that can only be explained assuming that the side chain of Dmt fits a hydrophobic cavity of the receptor very tightly and precisely. We have investigated the specificity of this fit by systematic changes of the number and position of substituents (methyl and hydroxyl groups) on the aromatic ring of Tyr in dipeptides of general formula H-nMeTyr-Tic-OH. Mono- and disubstitutions different from 2′,6′- invariably lead to catastrophic decreases of activity. The only substitution compatible with retention of substantial antagonism is 2′-methyl. MM calculations show that the intrinsic conformational properties of the analogues are not significantly affected by the small changes on the aromatic ring. The only possible interpretation of the biological data and conformational tendencies is that the steric requirements of the hydrophobic pocket hosting the nMeTyr side chain are very strict. Accordingly, it is possible to use the shape of the different side chains to map the hydrophobic cavity of the receptor. The combination of sharp activity decrease following the introduction of any substitution in positions *ortho* to the hydroxyl group and the concomitant retention of activity with even a single methyl group in *meta* position indicates that the receptor cavity that hosts the side chain of the first residue has a funnel shape whose tip coincides with the hydroxyl group.

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