Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 297–302
Published online 23 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.723

JOURNAL OF PEPTIDE SCIENCE

Circular dichroic properties of the tyrosine residues in tetrazole analogues of opioid peptides

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Received 20 May 2005; Accepted 29 July 2005

Abstract: CD studies on tetrazole analogues of opioid peptides show that peptides sharing the same *N*-terminal sequence, H-TyrΨ[CN_4]Gly-, give very large Cotton effects of the Tyr side chain in the near-UV region. CD spectra of five such peptides: H-TyrΨ[CN_4]Gly-Gly-Phe-Leu-OH (**I**), H-TyrΨ[CN_4]Gly-Phe-Pro-Gly-Pro-Ile-NH_2 (**II**), H-TyrΨ[CN_4]Gly-Phe-Pro-NH_2 (**III**), H-TyrΨ[CN_4]Gly-Phe-Gly-Tyr-Pro-Ser-NH_2 (**IV**), and H-TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (**V**), and two others for comparison: H-Tyr-GlyΨ[CN_4]Gly-Phe-Leu-OH (**VI**) and H-TyrΨ[CN_4]Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (**VII**), were measured in methanol, 2,2,2-trifluoroethanol, and water at different pH values. The spectra show that the conformations of the Tyr^1 residue in peptides **I**–**V** are very similar in all solvents used but differ distinctly from those observed for **VI** and **VII**. Strong Tyr bands in the aromatic region result probably from the rigid structure of the common *N*-terminal part of peptides **I**–**V**. These bands are weaker for **IV**, which maybe due to the presence of a second Tyr residue in that peptide, giving an opposite contribution to the CD spectrum as that arising from Tyr [1]. It seems that the rigid structure of the *N*-terminal part of **I**–**V** results from the interaction of the Tyr^1 side chain and the tetrazole ring. The CD bands of the Tyr residues of **VI** and **VII** are much smaller than those of **I**–**V** in all solvents, except **VII** in trifluoroethanol (TFE) where Tyr bands comparable in intensity to those of **I**–**V** are observed. This spectral property may derive from the same sign contribution of both Tyr residues of **VII** to the CD spectrum. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: circular dichroism; opioid peptides; peptide conformation; tetrazole analogues of peptides; tyrosine

INTRODUCTION

In 1981, Marshall *et al.* [1] proposed the 1,5-disubstituted tetrazole ring system Ψ[CN_4] as a mimetic of the *cis* peptide bond. Subsequent investigations on the model compounds [2] showed that 88% of the conformers accessible to a dipeptide with the *cis* peptide bond are also available to its tetrazole analogue. Because of the structural properties of the tetrazole ring, a natural site for its substitution in peptides is the X–Pro bond, where the *cis–trans* isomerization is possible, and other peptide bonds experimentally found to be in the *cis* conformation. The tetrazole ring has been incorporated into biologically active peptides like a dopamine receptor modulator [3], deaminooxytocin [4], bradykinin [5], a somatostatin hexapeptide [6], and a peptide substrate of HIV protease [7]. Except the somatostatin hexapeptide, all other tetrazole analogues show greatly diminished biological activity, which suggests that the *cis* conformer of the appropriate amide bond is not part of the biologically active conformation, or that the steric hindrance of the tetrazole ring precludes binding and potential recognition of the substitution sites through hydrogen bonding.

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Recently, we have been studying the conformational preferences of tetrazole analogues of opioid peptides by CD spectroscopy. Analogues of enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) [8], casomorphin (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) [9], morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2) [10], dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) [11], and deltorphin-I (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) [12] were investigated. In the course of these studies, we observed unusually large CD bands of the Tyr side chain in the near-UV region, corresponding to the ^1L_b electronic transition [13,14], for the following analogues:

H-TyrΨ[CN_4]Gly-Gly-Phe-Leu-OH (**I**), analogue of enkephalin
H-TyrΨ[CN_4]Gly-Phe-Pro-Gly-Pro-Ile-NH_2 (**II**), analogue of casomorphin
H-TyrΨ[CN_4]Gly-Phe-Pro-NH_2 (**III**), analogue of morphiceptin
H-TyrΨ[CN_4]Gly-Phe-Gly-Tyr-Pro-Ser-NH_2 (**IV**), analogue of dermorphin
H-TyrΨ[CN_4]Gly-Phe-Asp-Val-Val-Gly-NH_2 (**V**), analogue of deltorphin-I

To the best of our knowledge, these are the first examples of Tyr residues in linear peptides giving such large Cotton effects in the aromatic region. For comparison, we also present the results obtained for two other analogues:

H-Tyr-GlyΨ[CN_4]Gly-Phe-Leu-OH (**VI**), analogue of enkephalin

H-TyrΨ[CN_4]Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (**VII**), analogue of dermorphin

for which large Tyr CD bands in that region were not observed. Analogues **VI** and **VII** were chosen because of a different position of the tetrazole ring in the peptide chain as compared with **I**–**V** and a very small difference in the sequence (Ala replacing Gly) as compared with **IV**, respectively. It can be seen that analogues **I**–**V** share the same *N*-terminal part H-TyrΨ[CN_4]Gly- (Scheme 1). In this paper, we present the results of CD studies on the tetrazole analogues **I**–**VII** in methanol, 2,2,2-trifluoroethanol (TFE), and water at different pH values. This is a preliminary report where we describe the CD results only and put forward a working hypothesis on what the origin of the unusually large Tyr CD bands might be.

EXPERIMENTAL

Syntheses of peptides will be published elsewhere.

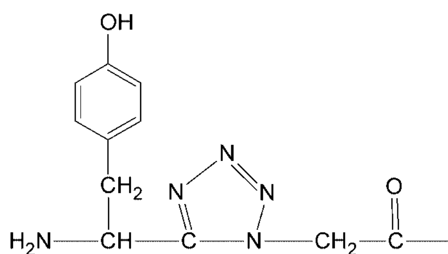
Circular Dichroism Measurements

CD spectra were recorded at room temperature on a Jasco J-600 spectropolarimeter (Tokyo, Japan). The spectra were measured in methanol, TFE, and water at pH 2, 7, and 11. Concentrations of the solutions ranged from 0.13 to 0.28 mg/ml (2×10^{-4}–4.8×10^{-4} M). Cell pathlength of 1 cm was used. Each spectrum is the average of at least four scans. The data are presented as total molar ellipticity [θ].

RESULTS AND DISCUSSION

The CD spectra of peptides **I**–**VII** represent the contributions of the aromatic residues only. We checked with a model compound, HCl.H-ProΨ[CN_4]Bu^t, that the tetrazole chromophore does not give any CD band in the absorption spectrum in the near-UV region.

The CD spectra in the near-UV region of **I**–**VII** in methanol are presented in Figure 1. The overall shape of the spectra of **I**–**V** is very similar, the only difference being in their intensities. Their striking feature is the very large intensity of the bands in the region of the ^1L_b Tyr electronic transition, markedly exceeding that of the bands of linear peptides [14,15–20]. They show a positive band at 275–278 nm and a distinct shoulder at about 284 nm. The largest Tyr band is observed for **V** (2788 deg cm^2/dmol) and slightly smaller for **I** (2633 deg cm^2/dmol). The intensities of Tyr bands in the spectra of the other compounds decrease by a comparable value in the order **III** (2395 deg cm^2/dmol), **II** (2064 deg cm^2/dmol), and **IV** (1730 deg cm^2/dmol). The signals of the Tyr residues in **VI** and **VII** are remarkably smaller. They appear between 270 and 295 nm and are positive and negative, respectively. The intensities of these bands are in the range −265/+195 deg cm^2/dmol. We also measured the CD spectra of unmodified Leu-enkephalin, casomorphin, and morphiceptin in methanol (not shown) and found the Tyr residues to give CD bands in the near-UV region of intensity from −393 to +422 deg cm^2/dmol. On the short-wavelength tail of the Tyr maxima are superimposed the bands or shoulders corresponding to the ^1L_b transition of the Phe residue [13,21], at 266–267, 260–261, and 254 nm. The Phe maxima are negative for all of the peptides studied. They are most pronounced for **VII** due to a lower Tyr background.



Scheme 1

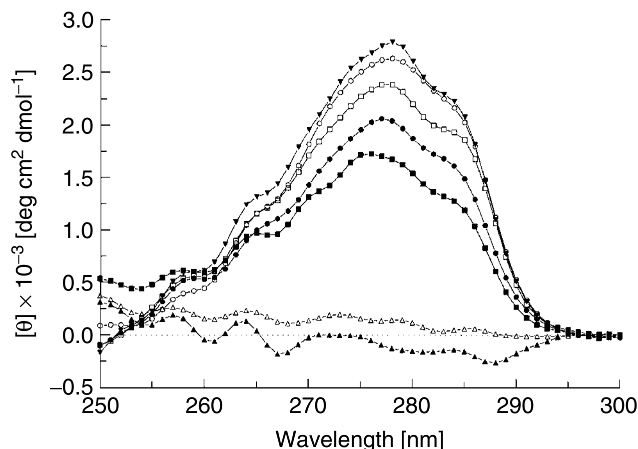


Figure 1 CD spectra of **I**–**VII** in MeOH: (○) **I**; (●) **II**; (□) **III**; (■) **IV**; (▼) **V**; (△) **VI**; (▲) **VII**.

The spectra in TFE are shown in Figure 2. The spectra of **I**–**V** are very similar to each other and much less differentiated with regard to their intensity when compared to methanol. Their shapes are very similar to those observed in methanol; however, the maxima of **I**–**V** are smaller in TFE and they are slightly shifted to higher wavelengths. In the Tyr absorption region, the spectra of **I**–**V** consist of positive bands at 273–274 nm and shoulders or bands at 278–279 nm. The most intense bands are present in the spectra of **I**, **II**, and **V**, and the bands of **III** and **IV** are somewhat smaller. The spectrum of **VI** shows positive Tyr bands slightly larger than those in methanol but with a much poorer fine-structure. On the other hand, the negative Tyr bands of **VII** are much larger than in methanol and their maxima are shifted to shorter wavelengths. The

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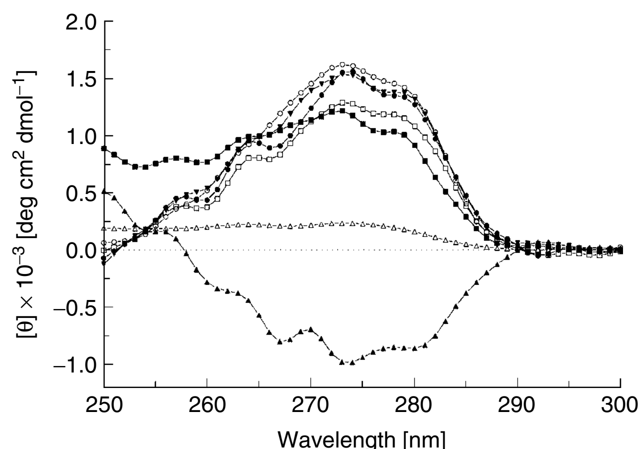

Figure 2 CD spectra of **I–VII** in TFE: (○) **I**; (●) **II**; (□) **III**; (■) **IV**; (▼) **V**; (△) **VI**; (▲) **VII**.

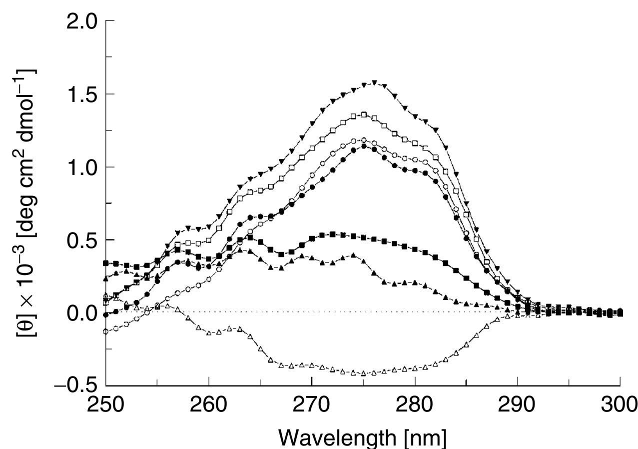

Figure 3 CD spectra of **I–VII** in water at pH 7: (○) **I**; (●) **II**; (□) **III**; (■) **IV**; (▼) **V**; (△) **VI**; (▲) **VII**.

maxima corresponding to the Phe absorptions of **I–V** and **VII** are similar to those in methanol. They can be seen as bands or shoulders superimposed on the Tyr absorption and they are all negative. In the case of **VI**, very poorly visible negative Phe bands are observed.

In water (Figure 3), the spectra of **I–III** and **V** in the Tyr absorption region are similar to those in TFE. They show intense positive bands and shoulders at 275–276 and 280–281 nm, respectively. A large difference instead is observed for peptide **IV**. A CD band and a shoulder of that peptide are located at 272 and 282 nm respectively, and their intensity is markedly decreased and not much larger than that of **VI** and **VII**. The smaller intensity of the CD bands observed for peptide **IV** in water is consistent with the CD spectra measured in methanol and TFE (Figures 1 and 2). In these two solvents, especially in methanol, peptide **IV** exhibits the smallest Tyr bands among **I–V**. The smallest intensity of the Tyr bands of **IV** as compared with **I–III** and **V** in all solvents studied may result from

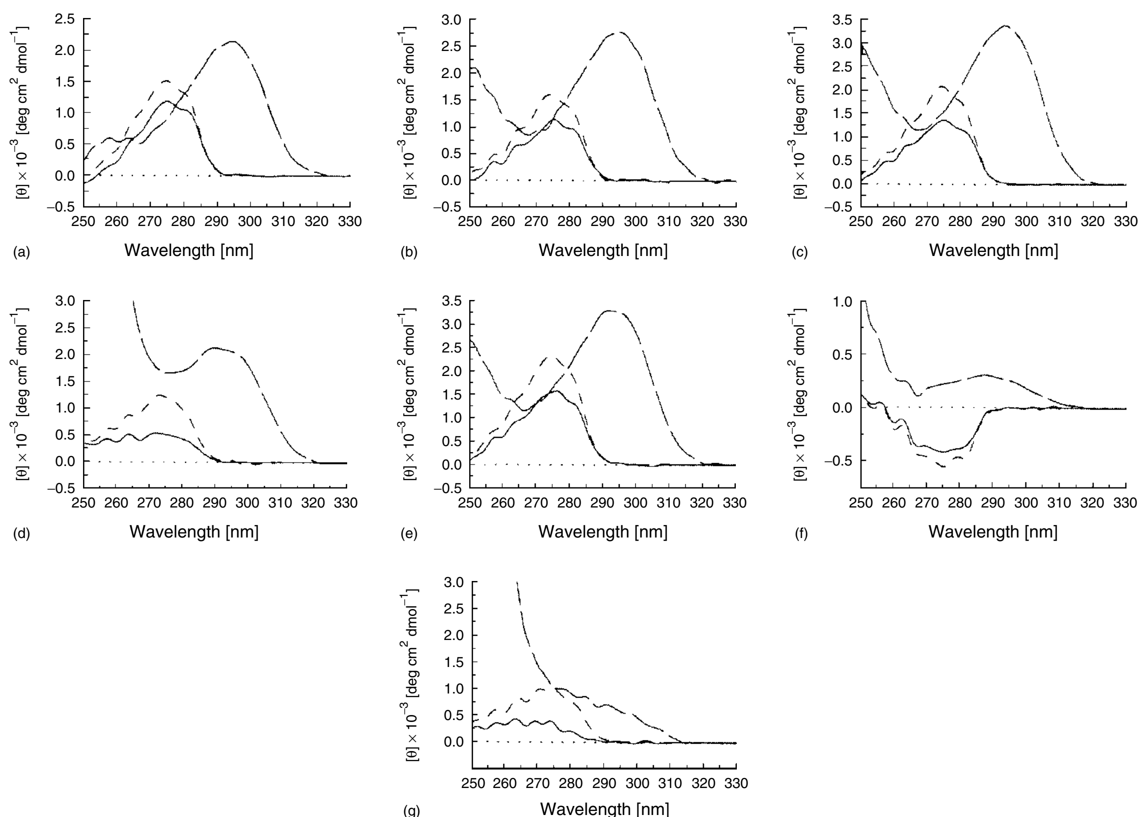

Figure 4 CD spectra of **I–VII** in water at pH 2 (- - - -), 7 (———), and 11 (— —): (a) **I**; (b) **II**; (c) **III**; (d) **IV**; (e) **V**; (f) **VI**; (g) **VII**.

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the presence of two Tyr residues at positions 1 and 5. The two residues may give opposite contributions to the CD spectrum. The shapes of the Phe bands of **I**–**VII** in water are rather similar.

In Figure 4, the pH dependence of the CD spectra of analogues **I**–**VII** is presented. In the Tyr absorption region, a very similar CD pattern was observed for peptides **I**–**V** (Figures 4a–e). At pH 2, the band at 275 nm increases markedly in its intensity. At pH 11, it moves to 291–295 nm and simultaneously a twofold or larger increase of its intensity is observed. The spectra of **I**–**III** and **V** are quite similar. Like in methanol and TFE, the intensities of the Tyr bands of **IV** at each pH value are smaller than those observed for **I**–**III** and **V**. A pattern of pH dependence close to **IV** was observed for **VII** (Figure 4g), the only difference being a very similar intensity of the Tyr bands at pH 2 and 11. This similarity is probably a result of the close resemblance of the *N*-terminal parts of peptides **IV** and **VII** and the presence of two Tyr residues in their sequences. A similar pH dependence of CD spectra of the Tyr side chain has been found for free Tyr and its derivatives with a free amino group, i.e. tyrosine ethyl ester, tyrosine amide, and H-Tyr-Ala-OH [15,19]. For these compounds, a positive band at 270–275 nm has been observed in acidic and neutral solution. The intensity of this band for tyrosine amide and H-Tyr-Ala-OH, on going from acidic to neutral solution, is the same and slightly increases, respectively [19]. At basic pH, the band of both compounds shifts to 293 nm and increases in intensity, though considerably less than in the case of **I**–**V**. A similar effect has been observed for tyrosine ethyl ester [15]. A significant difference between the model compounds studied [15,19] and **I**–**V** is a much larger intensity of the Tyr bands of the tetrazole analogues.

A different pattern of pH dependence was observed for **VI** (Figure 4f). In this case, negative Tyr bands with the maximum at 275 nm are observed in acidic and neutral solution. On going to basic pH, a sign reversal and a red shift to 280–310 nm are seen. This result is surprising, for such a pH dependence of CD spectra has been observed for the Tyr residue with its *N*-terminus blocked, i.e. *N*-acetyl-tyrosine ethyl ester, *N*-acetyl-tyrosine amide, *N*-acetyl-tyrosine, *N*-Ac-Tyr-Ala-OH, and di- and tripeptides with the Tyr residue at positions 2 and 3, respectively [15,19]. For these compounds, the band at 270–275 nm is negative for acidic and neutral solutions, and it changes its sign to positive and moves to 290–295 nm at basic pH.

A similarity of the CD spectra of the Tyr residues in **I**–**V** in each of the solvents used (Figures 1–3) suggests that conformations of their side chains are very much alike. They are independent of the conformation of the rest of the molecule and are characterized by very large CD bands in the near-UV region. Comparison of sequences of analogues **I**–**V** shows that they have a common feature, i.e. the *N*-terminal sequence H-TyrΨ[CN_4]Gly-. The CD spectra of **I**–**VII** and other tetrazole analogues [unpublished data] indicate that this very sequence is responsible for the large Tyr CD bands. Apart from the differences in their intensities, the shapes of the Tyr CD bands of **I**–**V** do not exhibit any solvent dependence, which shows that the conformations of the Tyr[1] side chain in methanol, TFE, and water are very similar and stable. The smallest intensity of the Tyr bands of **IV** as compared with **I**–**III** and **V** in all solvents studied may result from the presence of two Tyr residues at positions 1 and 5. The two residues may give the opposite contributions to the CD spectrum. Very large Tyr bands in the CD spectra of analogues **I**–**V** suggest a significant limitation of the conformational freedom of the Tyr side chain at position 1. Smaller intensities of the Tyr bands of **I**–**V** in TFE (Figure 2) and water (Figure 3) as compared with methanol (Figure 1) suggest a greater conformational freedom of the Tyr residues in these two solvents. In the case of TFE, this result is somewhat surprising in view of the well-known structure-promoting properties of this solvent [22,23].

Substantially decreased intensities of the Tyr bands in the spectra of **VI** and **VII** in methanol (Figure 1) show that the *N*-terminal Tyr residue in each of these two peptides has a larger conformational freedom as compared with that of **I**–**V**. A much poorer fine-structure of the positive Tyr bands of **VI** in TFE (Figure 2) than in methanol indicates that the *N*-terminal part of that analogue is very flexible. A significantly increased intensity of the negative Tyr bands of **VII** in TFE (Figure 2) as compared with that in methanol maybe due to the same sign contribution of its two Tyr residues in TFE. This result may also show that **VII** is more ordered in TFE than in methanol. Reversal of the sign of the Tyr bands of **VI** and **VII** on going from TFE to water (Figure 3) shows that there is a change in the conformation around the Tyr residues in both peptides. However, the CD contribution of the Tyr[1] residue in **VII** is overlapped by that of a second Tyr residue at position 5. As a consequence, despite various shapes and opposite signs of the Tyr bands in the spectra of **VI** and **VII** in all solvents, it cannot be taken for granted that the conformations of the *N*-terminal Tyr residues in these two analogues are different.

The intensities of the Phe CD bands of **VI** and **VII** in methanol (Figure 1) are comparable to those of the Tyr residues in these peptides, whereas the Tyr bands are larger than those of Phe in TFE (Figure 2) and water (Figure 3). The studies on *N*-acetylaromatic amino acid amides [16] have shown that Cotton effects of the Phe derivative in the aromatic region are distinctly smaller than those for Tyr. It suggests that in methanol the Phe side chains in **VI** and **VII** are more conformationally restricted than those of Tyr residues.

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The CD spectra of analogues **I**–**V** in methanol, TFE, and water suggest a severe limitation of the conformational freedom of the Tyr side chain at position 1. This limitation maybe brought about by the short-range interaction of the Tyr side chain and the tetrazole ring. A hydrogen bond between the Tyr^1 phenolic group and other residues in the peptide chain of **I**–**V** should be also taken into account in this connection. Comparison of the CD spectra of **I**–**VII** and many other tetrazole analogues [21] in the aromatic region shows that unusually large Tyr bands appear only for those peptides in which the tetrazole ring is located next to the tyrosine residue and is followed by Gly. The mutual spatial location of the hydroxyl group of Tyr^1 and the tetrazole ring makes a hydrogen bond between them impossible. Still, an interaction between the Tyr aromatic and tetrazole rings is possible. In the case of **VI**, such an interaction is not possible because of a larger distance between the two rings. It is not present in **VII** either, probably because of the substitution of Gly by Ala.

To check how different ionization states of Tyr^1 and the tetrazole ring affect this hypothetical interaction, the pH dependence of the CD spectra of the peptides studied was examined. Larger intensities of the Tyr aromatic bands of **I**–**V** (Figure 4a–e) at pH 2 as compared with those at neutral pH maybe due to the protonation of the tetrazole ring. In the case of the unsubstituted tetrazole, CH_2N_4, the protonation occurs at pH 4.9 [24]. Hence, one can expect that at pH 2, the tetrazole ring in the peptides studied would be protonated. The difference in intensity of the Tyr bands between the neutral and acidic pH values suggests a greater rigidification of the Tyr side chain in the acidic solution. A possible explanation for this effect maybe the interaction of the positively charged tetrazole ring and the π-electrons of the Tyr aromatic ring. It could support the assumption that there is an interaction between the Tyr side chain and the tetrazole ring in **I**–**V**. A similar pattern of spectral changes can also be seen for peptide **VII** (Figure 4g), in which the tetrazole ring is located next to the Tyr residue. In the case of **VI** (Figure 4f), the changes observed in the CD spectra are very different. This finding could lend additional support for Tyr$\cdots$tetrazole interactions in **I**–**V** because in peptide **VI** the Tyr aromatic and tetrazole rings are further apart. At basic pH, a very large increase in intensity of the Tyr bands in peptides **I**–**V** is probably caused by a much more rigid conformation of the Tyr residue. The spectra of peptides **VI** and **VII** show that ionization of the Tyr phenolic group alone cannot evoke such changes. It is difficult at present to say what the mechanism of that additional rigidification of the Tyr side chain at basic pH is. It may result from a hydrogen bond formed by the phenolate anion acting as acceptor of an amide proton from another residue in the peptide chain. Though a hydrogen bond of the Tyr^1 side chain with other residues of **I**–**V** is possible, limiting its conformational freedom, the CD spectra of **I**–**VII** and other tetrazole analogues suggest that presence of the $\Psi[CN_4]$Gly- moiety next to the Tyr^1 residue is of primary importance for large Tyr bands to appear.

CONCLUSIONS

It was found that tetrazole analogues of opioid peptides with the same *N*-terminal sequence H-Tyr$\Psi[CN_4]$Gly- exhibit unusually large Tyr CD bands in the aromatic region. The CD spectra show that conformations of the Tyr^1 side chains in these analogues are very similar and rigid, and they show little solvent dependence. Such large Tyr CD bands are not observed for other tetrazole analogues in which the H-Tyr$\Psi[CN_4]$Gly- sequence is not present. They are also distinctly more intense than the bands observed generally for the Tyr residue in peptides. A working hypothesis was put forward that large Tyr CD bands result from the interactions of the Tyr aromatic and tetrazole rings. Participation of the hydrogen bond of the Tyr^1 OH group with other residues in the peptide chain in the stabilization of the Tyr^1 side chain is also possible, though it seems to be of minor importance. Changes of pH to 2 or 11 do not increase the conformational freedom of the Tyr^1 side chains. On the contrary, the Tyr CD bands in acidic and especially basic solutions become distinctly larger than at neutral pH. Further studies on peptides **I**–**V** and their analogues are planned, which should give more detailed information on the conformation of the H-Tyr$\Psi[CN_4]$Gly- segment and on the origin of the unusually large Tyr CD bands for peptides **I**–**V**.

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