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Locally Constrained Tyrosine Analogues with Restricted Side Chain Dynamics

Ding Jiao, K. C. Russell and Victor J. Hruby*

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

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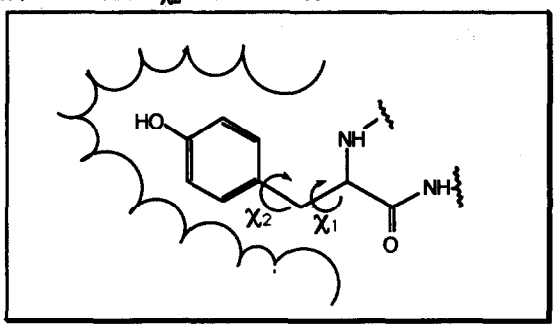
Abstract: While investigating synthetic locally constrained analogues of amino acids, we have found that a series of tyrosine analogues, such as the 2',6'-dimethyl-β-methyl-tyrosines, exhibit restricted rotations about their C^{β}-C^{γ} bonds which can be detected in the ^{1}H-NMR spectra of these compounds. The dynamic properties of these tyrosine analogues and their synthetic intermediates are described in this paper. The potential use of these tyrosine analogues in the study of the roles of side chain dynamics played in peptide-receptor interactions and protein functions also is discussed.

INTRODUCTION

During the past decade tremendous progresses have been made in conformational studies of bioactive peptides especially using the conformational constraint approach,[1,2] which was developed to reduce peptide flexibility in order to more critically assess the relationships of peptide conformations in terms of their interactions with receptors/acceptors.[3,4] One obvious physical consequence of conformational constraint is limiting the number of accessible conformational states of the molecule. This helps to alleviate the conformation averaging problem associated with short linear peptides which usually have a vast number of low energy conformations, and makes it feasible to study peptide conformation in solution by modern NMR spectroscopic techniques, and further to correlate the conformational information to the "bioactive conformation" of the peptide. Such information is critical for the development of peptide mimetics. From another point of view, conformational constraints also influence the dynamic behavior of peptides, *i.e.*, they reduce the rate and magnitude of molecular motions. Although the use of conformational constraints for studying the conformational properties of peptides has been widely practiced, application of the same concept for studying the dynamic properties of peptides has not been explored as extensively. Nonetheless, the dynamic properties of proteins and peptides is clearly an important aspect of their biological properties and have been the subject of considerable interest especially from a theoretical perspective.[5]

One example in protein and peptide dynamics is the side chain rotation of aromatic amino acid residues. It was observed nearly twenty years ago that the aromatic side chains of tyrosine and phenylalanine in the interior of proteins undergo restricted rotation (lifetime $\tau = 10^{-2} \sim 10^{-3}$ sec) around their C^{β}-C^{γ} bonds due to the severe steric constraints imposed by the dense packing of surrounding atoms.[6-10] It was shown from the study of bovine pancreatic trypsin inhibitor (BPTI) that a tyrosine residue in the interior of the protein is subjected to large

constraints in terms of its χ_1 and χ_2 dihedral angles. Essentially only two conformations are allowed for the aromatic ring, $\chi_2 \approx \pm 90°$.[9] In both functional forms of the filamentous bacteriophage fd coat protein, slow 180° ring flips of the tyrosine side chains about χ_2 were observed.[11]



Similar restricted sidechain rotation also occurs in bioactive peptides. In methionine-enkephalin, a slow rotational motion rate of the order of ~400 sec^{-1} was observed for the tyrosine ring of this linear pentapeptide.[12] It also is clear that the sidechain conformation and dynamics have great impact on the recognition processes of these peptides by their receptors/acceptors and the activation processes of the receptors and in peptide-carrier protein interactions. An interesting example is from the interaction of a neurohypophyseal hormone oxytocin with its carrier protein, neurophysin. The binding between oxytocin and neurophysin have different modes. The position-2 tyrosine ring in oxytocin undergoes fast rotation in its free solution form. When it is bound to neurophysin, the rate of its tyrosine ring flip becomes 10^2 s^{-1} or 10^4~10^8 s^{-1} depending on the mode of binding.[13] In one binding mode a slower ring flip motion (10^2 s^{-1}) was observed, which is comparable to that observed in the interior of proteins. Thus it was concluded that the Tyr^2 side chain in oxytocin was buried in the binding pocket of the receptor/acceptor. Since the aromatic ring in position-2 of neurohypophyseal hormones is essential for interaction with neurophysin proteins,[14,15] the peptide-protein complex may make the position-2 tyrosine ring experience the same packing force as occurs in the interior of proteins.

Incorporating unnatural amino acids and amino acid surrogates into peptides by chemical methods has long been used to study structure-function relationships.[1-3,16] More recently advances in protein synthesis by chemical methods[17] and by genetic engineering[18-21] have made it possible to incorporate unnatural amino acids into proteins for structure-function studies. In principle this approach also can be used to study dynamic features of peptides or proteins that affect in their biological activities. For example, one could incorporate synthetic amino acid analogues with built-in dynamic features and examine the consequent changes of properties in terms of their chemical/physical and biological effects.

As part of our studies of structure-activity relationships of proteins and peptides, we have began a systematic examination of the use of a series of unusual amino acids with their side chains constrained. This family includes a series of β-methyl and aromatic mono- or dimethyl substituted phenylalanine and tyrosine analogues that has provided some interesting features both in their conformational properties and in the biological properties of their resulting peptides.[22-24] Among serveral factors introduced by these synthetic amino acid analogues, such as side chain conformational constraint, increased molecular size and higher lipophilicity, restricted ring flipping motion on the time scale of ^1H NMR chemical shift[25,26] observed in a set of tyrosine

analogues with substitutions at β-position and on the aromatic ring is particularly interesting. Because they can be potentially useful tools for investigating the dynamic properties of aromatic side chains in proteins and peptides. We present herein a detailed analysis of the dynamic properties of the tyrosine analogues and their synthetic intermediates (Table 1), and further provide some understanding of the nature of such restricted rotations.

RESULTS AND DISCUSSION

In the ^{1}H-NMR spectra of the compounds examined in this study (Table 1) except for compounds **5**, **6** and **11**, the 2' and 6' methyl groups exhibited two distinct single peaks around 2.3 ppm at room temperature (Figure 1). For some of these compounds, e.g., compounds **1** ~ **4**, the 3' and 5' aromatic protons also showed different chemical shifts. On increasing the temperature, these pairs of distinct resonance signals, especially the 2' and 6' methyl groups move closer and coalesce at certain temperatures then become sharper single peaks with a further increase of temperature. This process is best illustrated by a stack plot of spectra for compound **1** at different temperatures (Figure 1). For compounds **5**, **6** and **11**, only single peaks were observed for the aromatic methyl groups in their NMR spectra at room temperature, whether the aromatic ring was mono or bis-methylated. No obvious line broadening was detected for these compounds in CH_3OH-d_6 solvent even at -70°C.

The observation of different chemical shifts for the 2' and 6'-methyl groups is obviously due to the anisotropy of their chemical environments because they are diastereotopic.[27] However the methyl groups in compound **5** were observed as a single peak in its NMR spectrum caused by a fast rotation around the single bonds at room temperature. Restricted rotation around the χ_2 angle instead of the χ_1 angle appears to be the reason the two aromatic methyl groups show chemical shift nonequivalence. In the NMR spectra, any significant slow rotation around the χ_1 angle on this time scale would show its effects on the chemical shifts of β-protons, β-methyls or even more remote protons in the molecules. In fact, over the range of temperatures no significant changes were observed for all other protons except for the two aromatic methyls where restricted rotations occur. This provides evidence that the rotation around the χ_1 angle is much faster than the rotation around the χ_2 angle. Thus the sole consequence of this restricted rotation is the appearance of chemical shift nonequivalence for the 2',6'-methyl protons and/or 3',5'-aromatic protons. The exchange between these two methyl groups or two protons will not introduce any different shielding effects to their surrounding nuclei. This will lead to an apparent restricted rotation round the χ_2 angle instead of the χ_1 angle, although this does not necessarily exclude that during the rotation round the χ_2 angle, molecular motions around the χ_1 angle may also be involved at a different time scale.

Entered in Table 1 are the coalescence temperatures (T_c), the separation of the chemical shifts ($\Delta\nu$) of the two 2', 6' methyl groups (at room temperature), the free energies of activation ($\Delta G^{\neq}$) and the lifetimes (τ) at coalescence temperatures estimated by equations (1) and (2) shown in experimental section. The observed coalescence temperatures (300~370°K or 27~100°C) and their free energies of activation $\Delta G^{\neq}$ (14~20 kcal/mol) fall into the same range of values as those found for 2', 6'-disubstituted phenyl alkanes.[28] However, their free energies of activation vary with the nature of the aliphatic chain and the number of substituents on the aromatic ring.

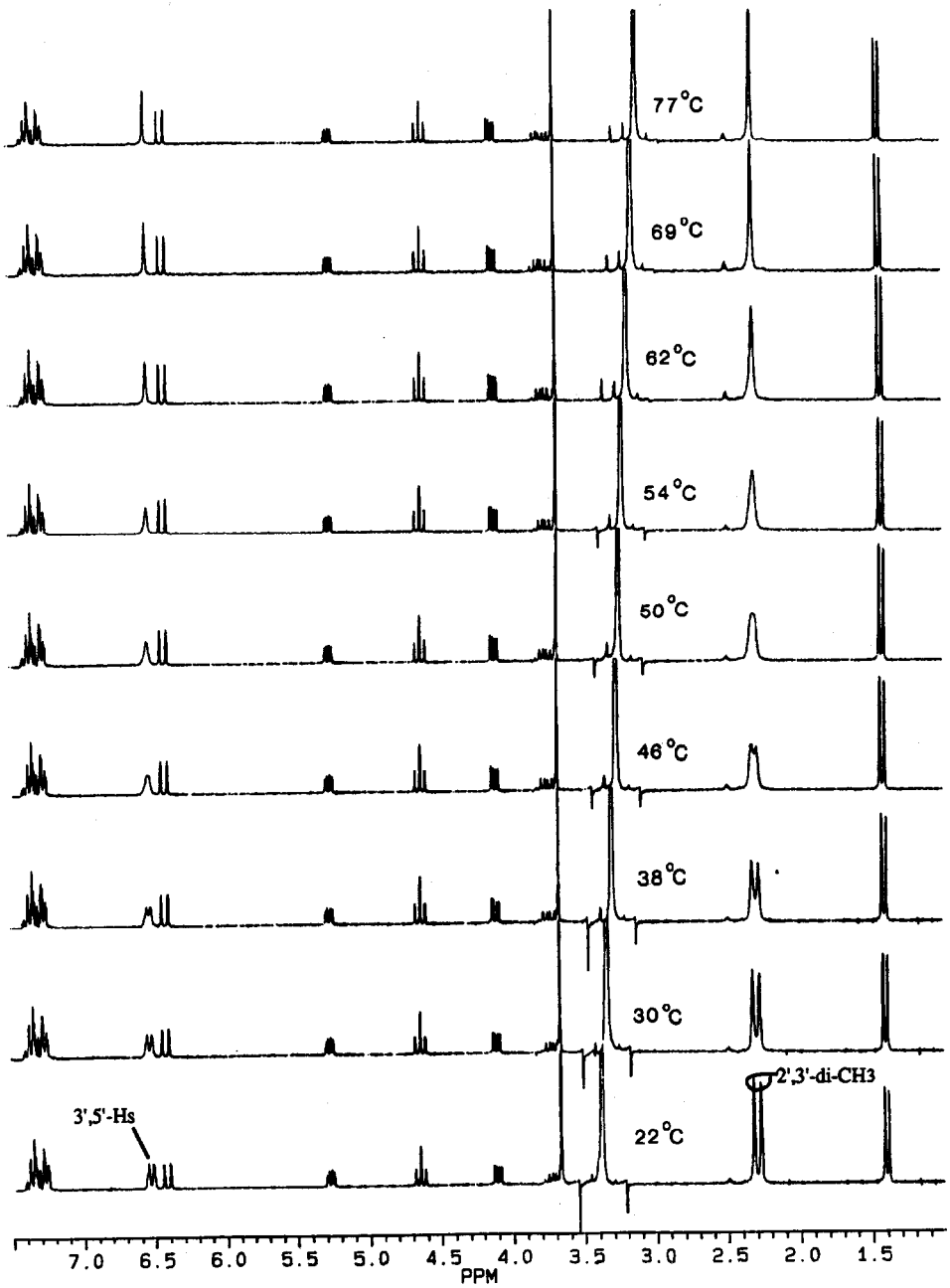
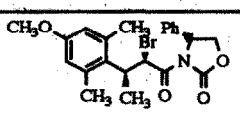
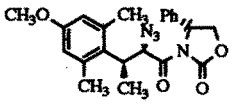
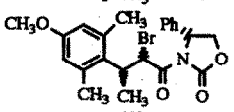
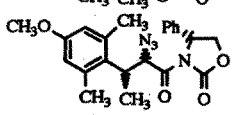
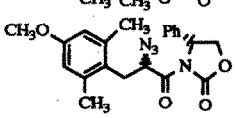
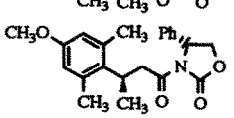
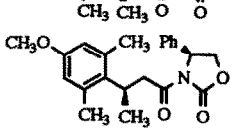
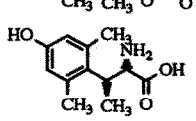
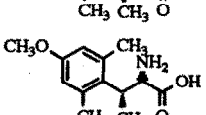
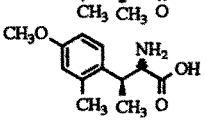


Figure 1. Proton NMR spectra of compound **1** in DMSO-d6 at different temperatures, showing the coalescence (at 50°C) of the two aromatic methyl resonances at ~2.3 ppm.

Table 1. Proton Chemical Shift Differences (Δν) for the 2',6'-methyl Groups at Room Temperature, Coalescence Temperatures (T_c), Lifetimes (τ) and Free Energies of Activation ($\Delta G^{\neq}_c$).

Number	Structures	$\Delta G^{\neq}_c$ (kcal/mol)	T_c (K)	Δν (Hz)	τ (sec^{-1})
1		16.8	323	13.1	3.4×10^{-2}
2		20.1	373	5.5	8.2×10^{-2}
3		17.0	351	86.8	5.2×10^{-3}
4		14.2	315	119.2	3.8×10^{-3}
5		-[a]	-[a]	-[a]	-[a]
6		-[a]	-[a]	-[a]	-[a]
7		15.2	301	25.0	1.8×10^{-2}
8		15.1	305	41.8	1.1×10^{-2}
9		15.6[b]	301[b]	12.8[b]	3.2×10^{-2}[b]
10		16.2	313	13.8	3.4×10^{-2}
11		-[a]	-[a]	-[a]	-[a]

[a] No slow exchange was observed at room temperature and -70°C in CH_3OH-d_6.

[b] Measured in CH_3OH-d_6 solvent.

The coexistence of both β-methyl group and 2',6'-dimethyl groups appears to be necessary to create high energy barriers observed in this series of compounds (compounds **1-4, 7-10** in Table 1). In these molecues the space between the C^{β}-C^{γ} bonds is extremely crowded by these methyl groups ((a) and (b) of Figure 2). Therefore, the observed large rotational barrier can be attributed to the Van der Waals (*vdw*) interactions among these methyl groups during rotation around the χ_2 angle. If this is the case, removing one of these three methyl groups would substantially relieve the local *vdw* constraint between the C^{β}-C^{γ} bond in these compounds and thus reduce the observed high rotational barriers. Actually this effect was shown clearly in compounds **5, 6** and **11** (Table 1, also (c) in Figure 2). Although rotation around χ_2 angle for compounds **5, 6** and **11** is not slow enough to be observed on the NMR time scale used in this study, they were expected to have a slower rotation rate in comparison with an unsubstituted tyrosine. Their conformational and dynamic properties await further studies. However an upper limit of a possible restricted rotation can be estimated. Assuming that compound **6** has a similar magnitude of $\Delta\nu$ as compound **4** since the only difference between them is the number of aromatic methyl groups, it can be estimated that the ring flip of the compound **6** has a life time shorter than 3.8×10^{-3} sec at -70°C. This would give a upper limit of the free energy of activation of 9.0 Kcal/mol at this temperature.

Although we focused on the substituents close to the C^{β}-C^{γ} bonds in the above discussion, the rest of the molecule also contributes significantly to the apparent restricted rotational motion around the χ_2 angles. For instance, compounds **1** and **2** are both in the (2R, 3S) configurations, compound **2** with an azide group at α-position of the C=O has a significantly higher rotational barrier around χ_2 angle than compound **1.** However, in compounds **3** and **4**, both in the (2S, 3R) configurations, the trend is the opposite of the above. In this case, compound **3** with a bromine group at the α-position of the C=O has a higher rotational barrier than compound **4.** This may indicate that both the nature and orientation of the substituents at the α-position have significant impact on this rotation process. On the other hand, in compounds **7** and **8**, where substitutions at the α-position are absent, the different configurations at position-4 in the 2-oxazolidinone moiety appears to have less impact on the barrier. In compounds **9** and **10**, it seems that with the same configuration at α position, a β-(S)-methyl led to a slightly larger barrier.

In summary, all of the compounds examined in this study except **5, 6** and **11** showed restricted rotation around the χ_2 angle with a rate comparable to that of tyrosine or phenylalanine in the interior of proteins or in peptide-protein complexes. Although the nature of these restricted rotations are the same, their origins are quite different. In proteins or peptide-protein complexes, the motion of aromatic rings are hindered by atoms close to them in space. These atoms may be from nearby amino acid residues or even remote residues in the same or different molecules. In the constrained tyrosine analogues studied here the effect is rather local. As discussed above, the major contribution comes from the *vdw* interaction between the β-methyl and the 2' or 6'-methyl groups in the same amino acid side chain. This local effect is so great that it has a comparable magnitude with the global effect formed by large proteins or peptides. Meanwhile, the α-substituents which corresponds to the N-terminal in a peptide chain also have a significant impact on the apparent restricted rotation around the χ_2 angles. Based on the tyrosine analogues and their synthetic intermediates examined in this study, it seems that the N-terminal substitution may have a larger effect on the observed rotational process than the C-terminal substitution, although this needs to be further investigated in appropriate peptide analogues. These contributions to the rotation barrier (e.g., $\Delta\Delta G^{\neq} = 2 \sim 4$ kcal/mol) would certainly be large enough to significantly modify the conformation and dynamics of a protein or a peptide, and undoubtedly this will affect its biological activities.

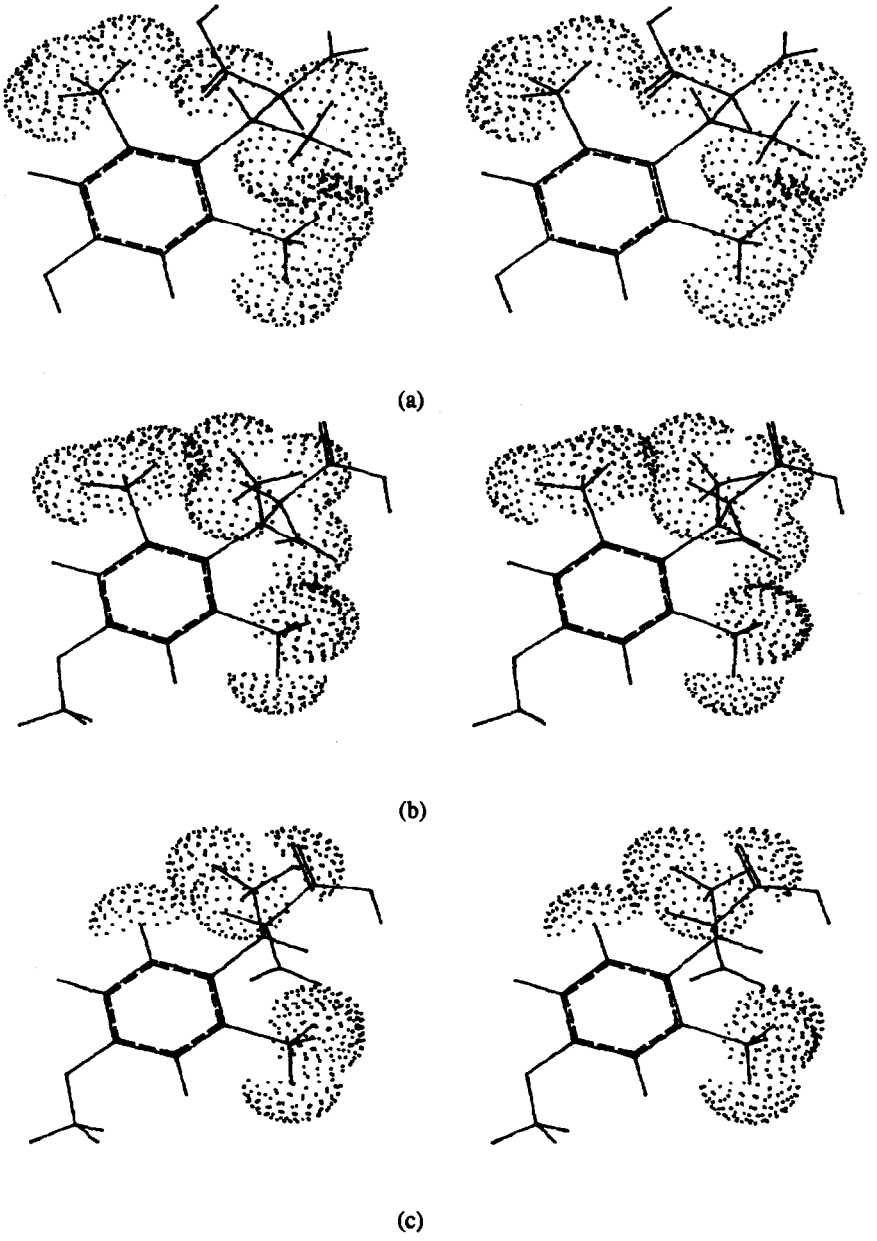


Figure 2. Stereo views of compounds 9 (a), 10 (b) and 11 (c), showing the *vdw* surfaces for the β-methyl, β-proton and 2', 6'-dimethyl groups.

One may speculate that if such a constrained amino acid is incorporated into a protein, *e.g.*, BPTI, a combination of the local and the global effects would make the ring flipping motion nearly impossible! It would be interesting to see the structural and functional consequences of such a modification. In any case the application of these amino acids to biologically active peptides, especially conformationally constrained peptides should provide a powerful tool for examining the effect of local side chain conformational constraints on a wide variety of biologically active peptides and provide further insights into the topographical and the dynamic requirements for peptide and peptidomimetic design.

EXPERIMENTAL SECTION

Description of the NMR experiments. The samples used in this study were asymmetrically synthesized and characterized previously.[22(b),29] They were dissolved in DMSO-d_6 (Aldrich, 99.9% D) or CH_3OH-d_4 (Aldrich, 99.8% D). Nitrogen gas was bubbled through the sample solution for fifteen minutes before use. All ^{1}H-NMR experiments were performed on a Bruker AM-250 NMR spectrometer equipped with an Aspect 3000 computer. The probe temperature was controlled by the variable temperature unit on the spectrometer and calibrated with a thermal couple in a 5-mm NMR sample tube. The precision of the temperature control was ± 1° K. All spectra were recorded with spectral width of 2500 Hz, 16K data points (zero filled to 32K before FT).

A series of ^{1}H-NMR spectra at various temperatures were recorded for each compound in Table 1. Normally coalescence of the two peaks corresponding to the two aromatic methyl groups can be observed with an increase of temperature (e.g., see Figure 1). For compounds **5**, **6** and **11**, the ^{1}H-NMR spectra at lower temperatures up to -70°C were recorded in CH_3OH-d_4 solution. Detailed discussions of dynamic NMR (DNMR) and its applications in chemistry can be found in several books and a large body of literature.[30-32] In this study, however, only the free energy of activation $\Delta G^{\neq}$ and lifetime τ of the exchange processes at coalescence temperature T_c were calculated according to the well known equations for the two-site model:[28,33]

$$\tau_c = \frac{1}{k_c} = \frac{\sqrt{2}}{\pi \Delta \nu} \qquad (1)$$

$$\Delta G_c^{\neq} = 4.57\, T_c \{ 9.97 + \log_{10} (T_c/\Delta \nu) \} \text{ cal/mol} \qquad (2)$$

Where $\Delta\nu$ is the difference of chemical shifts of the two sites in the absence of exchange (here it is approximated by the difference of chemical shifts at room temperature).

Description of the modeling. The molecular models in Figure 2 were constructed by using a molecular modeling package SYBYL 5.2 (Tripos Associates, Inc., St. Louis, MO) on a Silicon Graphics workstation IRIS (Silicon Graphics, Inc., Mountain View, CA). The geometry of the natural tyrosine, retrieved from the built-in amino acid database of the SYBYL, was used as a template to build the three analogues shown in Figure 2. The standard Tripos force field[34] in vacuum with the exclusion of electrostatic interactions was used in energy minimization to obtain the final structures of these molecules.[35] The *Van der Waals (VdW)* surfaces were calculated with the built-in routine in SYBYL.

ACKNOWLEDGEMENTS

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35. All three staggered conformations around the χ_2 angles of these compounds were minimized by using given energy minimization method. The structures shown in Figure 2 are low energy conformers. These models only show those conformations with minimum *vdw* interactions.