Conformational Properties of the Unnatural Amino Acid β-Methylphenylalanine in a Linear Octapeptide System; Correlations of ^{13}C-NMR Chemical Shifts with the Side-Chain Stereochemistry of These Amino Acid Residues

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Conformational properties of the four stereoisomers ([2*S*,3*S*], [2*S*,3*R*], [2*R*,3*S*], and [2*R*,3*R*]) of a synthetic amino acid, β-methylphenylanaline (β-MePhe), in a bioactive octapeptide sequence of CCK, H-Asp^1-Tyr^2-β-MePhe^3-Gly^4-Trp^5-Nle^6-Asp^7-Phe^8-NH_2, have been studied by using ^{1}H and ^{13}C-2D NMR spectroscopy. β-MePhe^3 residues introduce significant perturbations to the side-chain conformations. On the basis of the rotamer populations determined by a combination of homonuclear and heteronuclear vicinal coupling constants, each of the four different stereoisomers of β-MePhe residues virtually eliminates one of the three staggered side-chain conformations, trans for (2*S*,3*S*)- and (2*R*,3*R*)-β-MePhe, gauche(+) for (2*S*,3*R*)-β-MePhe, and gauche(−) for (2*R*,3*S*)-β-MePhe, respectively. It also was revealed that the side-chain rotamer populations of the Tyr^2 residue are influenced by different configurations of the β-carbon in the adjacent β-MePhe^3 residues. An empirical correlation between the ^{13}C chemical shifts of the β-CH_3 and the stereochemistry of β-methylphenylalanine side chains has been established, i.e., the δ_C of the β-MePhe in (2*S*,3*S*)- and (2*R*,3*R*)-isomers is at lower field by ca. 3 ppm relative to those in (2*S*,3*R*)- and (2*R*,3*S*)-isomers. This correlation can be rationalized on the basis of the γ-substituent effect in ^{13}C-NMR chemical shift, and it may become a useful probe for side-chain conformations of similar molecules. Furthermore, these β-methylphenylalanine amino acids will provide useful side-chain conformational constraints in peptide and mimetic design.

In the studies of peptide–receptors interactions, peptide conformation has played a central role.[1-5] Applications of various conformational constraints have become a common practice in studying the possible conformational properties of peptide ligands required by the receptor for binding and activation.[4,5] A series of synthetic analogues of the natural amino acid phenylalanine, β-methylphenylalanines (β-MePhe),[6,7] have been incorporated into several bioactive peptide systems, including angiotensin II,[8] cholecystokinin (CCK),[9] enkephalin,[10] oxytocin,[11] and somatostatin.[12] In addition to the larger molecular size and lipophilicity introduced by the β-methyl groups, it also is apparent that the β-methyl groups increase steric interactions with surrounding groups especially the side chains of these amino acid residues. Significant modifications in the biological activities of the synthetic peptide analogues with β-MePhe have been obtained, including increased antagonist activity,[8] diverse binding potency,[11] increased selectivity for different receptor types or subtypes,[9,10] and elucidation of topographic features of peptide ligands in terms of receptor recognition.[9-13] However, there does not appear to be a complete account of the conformational behavior of β-methylphenylalanines in peptide systems. This makes it difficult to reliably correlate the conformations of the synthetic β-MePhe-containing peptide analogues with observed bioactivities. In a recent study of cyclic somatostatin analogues containing β-MePhe, Huang and co-workers[12] have determined populations for one of the three side-chain rotamers. The population distribution among the other two rotamers still remain unknown. Furthermore, it is probable that the distribution of the side-chain population depends on the backbone conformation and thereby different linear and cyclic peptide systems might exhibit different patterns. This information, however, is crucial for understanding the structural or conformational basis of the peptides for their different abilities to bind to biological receptors and for further peptide and mimetic design.[14] Therefore, we

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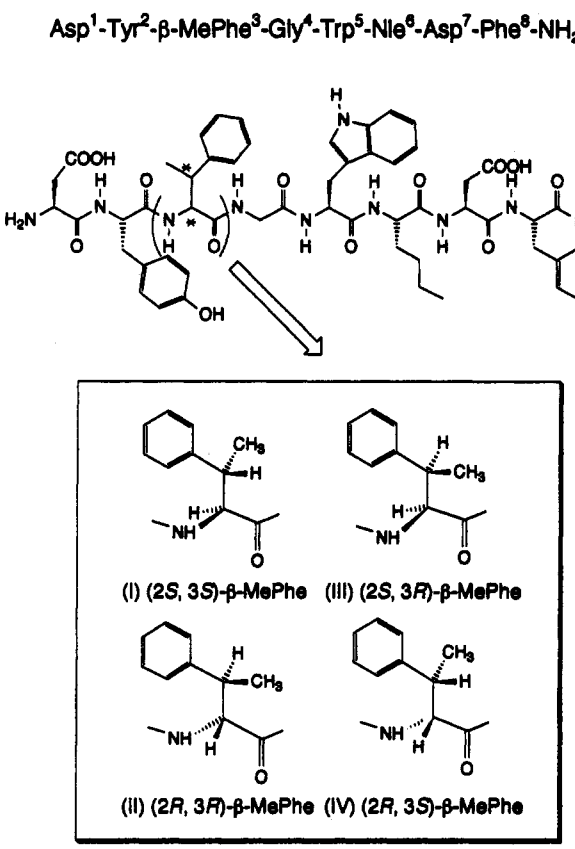

Figure 1. Structure of peptides I–IV.

present here a complete ^1H and ^{13}C NMR conformational study of a linear model peptide system containing all four isomers of β-MePhe in the same position.

In previous studies we have shown[15] that z-filtered homonuclear and heteronuclear relay NMR spectroscopy can provide a means to evaluate conformationally important vicinal coupling constants that can be used to help evaluate side-chain conformations of β-methylphenylalanine in the peptides studied. In this study we greatly expand these studies using several 2D-NMR methods to show that the introduction of a β-methyl group in the different stereoisomers of the β-MePhe residues produces a stronger constraint on the side-chain conformation than on the peptide backbone conformation; (b) that the distributions of the side-chain rotamer populations in these β-MePhe residues are significantly different from the statistical distribution of the side-chain conformation of the Phe residues in proteins and peptides; and (c) that an empirical correlation exists between the ^{13}C chemical shifts of the β-CH_3 and the stereochemistry of the side chains, which can be rationalized by the γ-substituent effect in the ^{13}C-NMR chemical shifts.

The linear model peptide system chosen is a group of CCK octapeptide analogues: H-Asp-Tyr-β-MePhe-Gly-Trp-Nle-Asp-Phe-NH_2, in which all four stereoisomers of β-MePhe ([2*S*,3*S*]-, [2*S*,3*R*]-, [2*R*,3*S*]- and [2*R*,3*R*]-β-MePhe (Figure 1)) have been placed in position 3. Due to multiple biological functions of CCK,[16] there is increasing interest in the therapeutic potential of CCK for the treatment of a variety of gastrointestinal, neuropsychiatric disorders, and some CCK-related CNS disorders.[17] It was found in previous studies that the incorporation of β-methylphenylalanine[7] greatly modifies the binding affinity and selectivity for CCK-A and/or CCK-B receptors.[9] Therefore, it was assumed that conformational constraint effects in the side chain introduced by the β-methyl group may play an important part in presetting the side-chain conformations or topography of the peptides for interaction with the binding surfaces of the CCK-receptors. This series of β-MePhe-containing analogues provides a good model system for examining the conformational behavior of these side-chain-constrained phenylalanine analogues in a linear peptide sequence.

NMR Strategy

Due to the vast number of multidimensional and multinuclear NMR techniques[18] available, practical applications of these techniques for peptide systems often require a careful choice of relevant experiments for the particular research problem. In our conformational studies of peptides, the goal is to obtain all relevant structural NMR parameters, including ^1H- and ^{13}C-NMR chemical shifts, homonuclear and heteronuclear coupling constants, and NOE/ROE interactions. In order to achieve such a goal in minimum time, we have adopted a strategy that uses a combination of three 2D-homonuclear and heteronuclear correlation experiments for each peptide.

^1H and ^{13}C spectral assignments in this work have been obtained by using two homonuclear and one heteronuclear 2D correlation experiments. The homonuclear correlation experiments include a z-filtered TOCSY[19–22] and a dipolar correlated ROESY[23–25] or NOESY[26,27] experiment. All proton spin systems of individual amino acid residues of peptides I–IV were identified by means of the TOCSY experiments. The sequential assignments[27] of the resonances rely on the detection of inter-residue dipolar interactions between NH(i + 1) and H_α(i) protons using ROESY experiment. The conformationally important homonuclear coupling constants were obtained with an accuracy of 0.3 Hz from the highly digitized 1D traces of the z-filtered TOCSY spectra. Figures 2 and 3 show respectively the z-filtered TOCSY spectrum and the expanded fingerprint (NH/H_α) region of the ROESY spectrum for the peptide [(2*S*,3*R*)-β-MePhe³, Nle⁶]CCK-8 (**III**). Even with these small linear peptides, the proton assignments based exclusively on homonuclear techniques were not complete because of severe resonance overlap in the NH and H_α region of the 2D-TOCSY and ROESY spectra. To resolve these ambiguities, the inverse z-filtered heteronuclear coupled HSQC-TOCSY[29,30] method was

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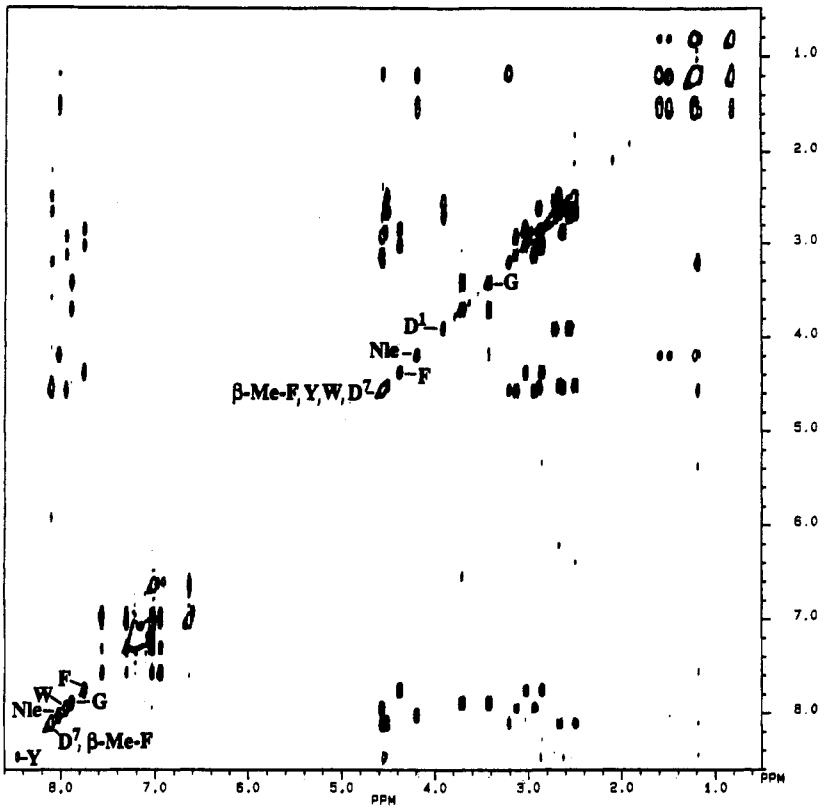

Figure 2. Phase-sensitive z-filtered ^{1}H-TOCSY spectrum of **III**. The experimental conditions are given in Experimental Section. For the assignment of resonances the one-letter notations of amino acids were used.

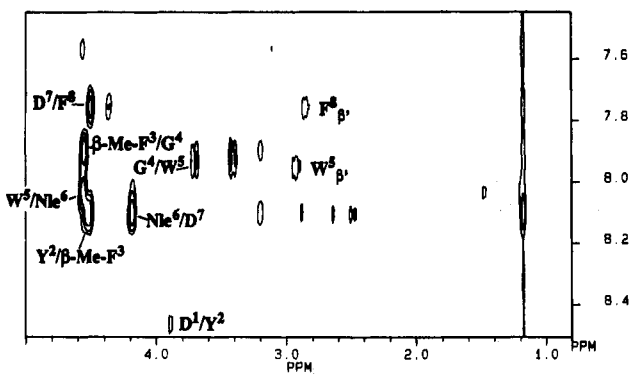

Figure 3. Expanded fingerprint region of ROESY spectrum of **III**. Beside the sequential assignment, the intraresidue ROEs observed between the NH and β′ protons of tryptophan-5 and phenylalanine-8 residues are also demonstrated.

used, taking advantage of the large chemical shift dispersion of the carbon resonances. The proton-detected carbon-coupled heteronuclear TOCSY experiment offers several advantages. First of all, proton detection gains considerably in sensitivity in comparison with ^{13}C detection. Furthermore, since there is no carbon decoupling during acquisition, direct one-bond correlation peaks can be easily identified by their characteristic doublet structure with $^1J_{CH}$ splitting, allowing an unambiguous assignment of protonated carbons. The z-filter inserted after the TOCSY magnetization transfer step provides pure absorption phase data making feasible an easy evaluation of both one-bond and long-range heteronuclear coupling constants.[29] Since the experiment does not require any long delays (that usually have to be applied when measurement of small long-range heteronuclear coupling is considered), the magnetization loss due to T_2 relaxation is negligible. The efficient magnetization transfer through the large one-bond proton–carbon coupling also contributes to the high sensitivity of this experiment. The phase-sensitive carbon-coupled HSQC-TOCSY spectrum for **III** is depicted in Figure 4.

Results and Discussion

The ^{1}H and ^{13}C spectral assignments for the four peptide analogues (**I**, **II**, **III**, and **IV**) were made utilizing the three 2D NMR experiments (see Experimental Section for detail) mentioned above. The ^{1}H-NMR parameters, such as chemical shifts (δ_H) of amide and aliphatic protons, intraresidue geminal (^2J) and vicinal (^3J) homonuclear coupling constants, and temperature coefficients of amide protons ($\Delta\delta/\Delta T$, ppb/K), of these peptides are included in Table 1. The ^{13}C chemical shifts of the aliphatic carbons are summarized in Table 2. The ROE patterns obtained from ROESY experiment for molecule **III** are shown in Figure 3. In the same figure a sequential assignment based on ROESY and TOCSY spectra is demonstrated. The other three peptides have virtually identical patterns with **III** because of their strong structural homology.

Backbone Conformations. For the identification of the secondary structure of a peptide sequence, medium and/or long-range NOE/ROE interactions among protons of different amino acid residues have often been used. In the present study, however, no specific secondary structure elements can be identified based on the ROE interactions

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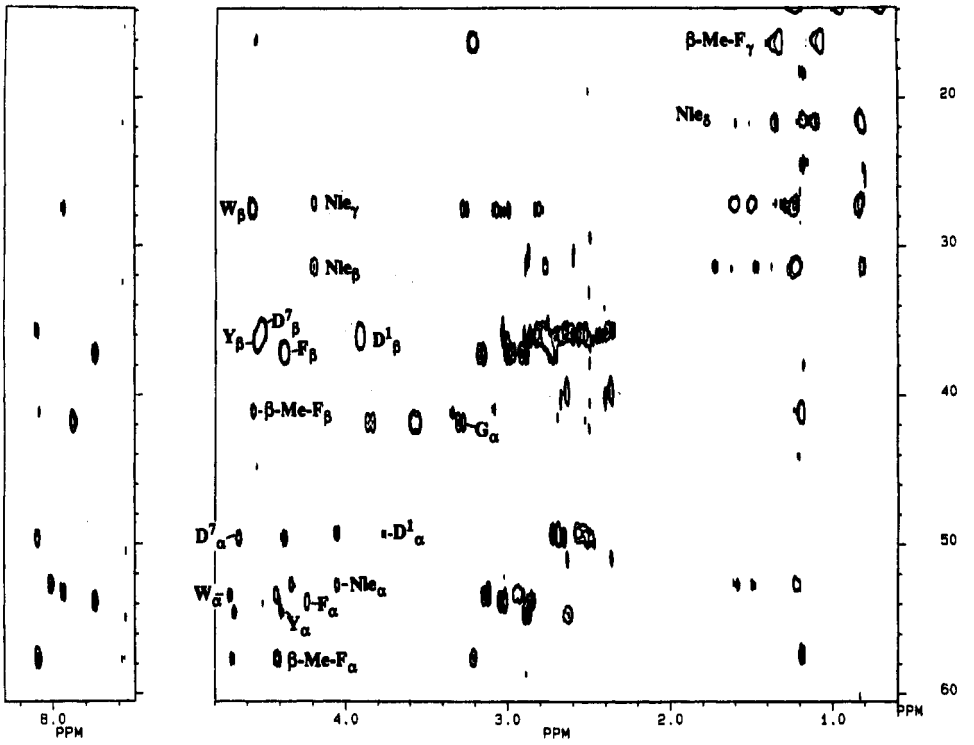

Figure 4. Carbon-coupled z-filtered HSQC-TOCSY spectrum for **III**. The assignments of carbon resonances are given at the corresponding carbon traces.

Table 1. ^{1}H Chemical Shifts (δ in ppm) and Coupling Constants (J in hertz) for I–IV (T 310 K, DMSO-d_6)

	NH[a]				H[b]				Hβ[c]				Hγ			
residue	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
					3.88	3.86	3.90	3.85	2.66 β	2.78 β	2.71 β	2.74 β				
Asp[1]					3.6	2.8	2.7	2.6	2.50 β	2.54 β	2.55 β	2.51 β				
					8.9	9.3	9.3	8.9	17.3	17.9	17.3	17.6				
	8.36	8.26	8.46	8.37	4.30	4.31	4.54	4.58	2.72 β	2.17 β	2.87 β	2.72 β				
Tyr	6.3	8.0	7.6	7.3	5.1	3.3	3.0	4.6	2.49 β	1.98 β	2.61 β	2.38 β				
	(4.7)	(3.6)	(4.5)	(4.2)	9.3	9.9	9.9	9.6	13.9	14.6	13.6	14.3				
β-Me-Phe	7.81	8.26	8.10	8.27	4.53	4.59	4.55	4.60	3.08	2.99	3.20	3.28	1.14	1.15	1.18	1.17
	8.9	9.4	8.6	9.1	8.8	9.2	7.1	6.3	6.7	6.8	7.1	7.0				
	(8.9)	(10.5)	(7.8)	(8.2)												
	8.21	8.37	7.88	8.10	3.73	3.89	3.71	3.63								
Gly	5.6	6.0	6.0	6.0		3.62	3.42									
	(7.3)	(8.4)	(6.7)	(7.9)		16.6	16.6									
	7.99	8.06	7.94	7.95	4.61	4.63	4.56	4.58	3.15 β	3.15 β	3.13 β	3.13 β				
Trp	8.0	8.0	8.0	8.0	4.8	4.6	4.6	4.6	2.97 β	2.95 β	2.93 β	2.93 β				
	(6.1)	(5.6)	(5.9)	(6.1)	8.6	9.2	8.6	8.6	14.8	14.6	14.6	14.6				
	8.04	8.07	8.02	8.02	4.19	4.22	4.19	4.19	1.59 β	1.61 β	1.58 β	1.59 β	1.20-	1.25-	1.25-	1.25-
Nle*	7.9	8.0	8.0	7.7	7.3	6.9	6.9	7.3	1.49 β	1.50 β	1.48 β	1.48 β	1.15	1.19	1.12	1.15
	(8.8)	(8.7)	(8.4)	(8.1)	6.9	6.9	6.9	7.3					($\gamma\delta$)	($\gamma\delta$)	($\gamma\delta$)	($\gamma\delta$)
	8.11	8.14	8.10	8.11	4.51	4.53	4.51	4.51	2.67 β	2.68 β	2.66 β	2.66 β				
Asp[7]	7.6	8.0	8.0	7.6	7.0	6.6	6.6	6.3	2.50 β	2.51 β	2.49 β	2.50 β				
	(6.8)	(6.5)	(6.6)	(6.3)	7.0	7.6	7.3	7.0	16.6	16.6	16.3	16.6				
	7.75	7.76	7.75	7.74	4.38	4.39	4.37	4.38	3.03 β	3.04 β	3.02 β	3.03 β				
Phe	8.3	8.3	8.0	8.0	4.9	4.9	5.3	5.3	2.85 β	2.86 β	2.85 β	2.85 β				
	(6.5)	(6.1)	(5.8)	(5.8)	8.3	8.0	8.6	8.6	13.9	14.0	13.9	13.9				

[a] Values in each column for I–IV are δ_H(NH), $^3J_{NH\alpha}$, and temperature coefficients of NH protons (–ppb/K) which are given in brackets. [b] Values in each column for I–IV are δ_H(Hα), $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta}$, except for Gly where they are $\delta_H(\alpha)$, $\delta_H(\alpha')$, and $^2J_{\alpha\alpha'}$. [c] Values in each column for I–IV are δ_H(Hβ), δ_H(Hβ'), and $^2J_{\beta\beta'}$ except for β-MePhe where they are δ_H(Hβ) and $^3J_{\beta\gamma}$. [d] Chemical shifts of Nle-H$_\omega$ for I, 0.81 ppm; II, 0.83 ppm; III, 0.81 ppm; and IV, 0.82 ppm.

observed for these peptides. The large temperature dependence (larger than –3.5 ppb/K, see Table 1) indicates that all amide protons in these peptides are exposed to solvent. Therefore, considerable conformational flexibility of these linear peptides in DMSO-d_6 solutions can be assumed from the lack of any nonsequential inter-residue ROE interactions and from the large temperature coefficients.[32]

NMR chemical shifts also are sensitive measures to the electronic environments of nuclei. In the present work, the structural homology among these peptides provides an opportunity to compare their ^{1}H and ^{13}C chemical shifts to identify the differences if any among the four peptides. It is noted that all ^{1}H resonances of the amino acid residues that are separated from the β-MePhe residue by at least one amino acid residue are identical within experimental error for all four peptides (Table 1). Significant differences in ^{1}H chemical shifts (0.1–0.6 ppm) of the tyrosine-2 (Tyr^2), β-methylphenylalanine-3 (β-MePhe^3), and glycine-4 (Gly^4) residues are observed for their NHs, Hαs, and Hβs among

Table 2. ^{13}C Chemical Shift Data of Aliphatic Carbons for I–IV (T = 310 K, DMSO-d_6, δ in ppm)

	C_α				C_β				C_γ				C_δ			
residue	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
Asp[1]	49.7	48.9	49.7	49.5	36.9	35.9	36.2	36.4								
Tyr	54.6	55.1	54.4	54.2	37.0	36.7	36.5	37.2								
β-Me-Phe	58.5	57.8	57.8	57.4	41.8	42.8	41.6	41.2	18.7	18.9	16.3	15.4				
Gly	42.6	42.3	42.3	42.2												
Trp	53.6	53.6	53.6	53.2	27.8	28.1	28.6	27.9								
Nle*	52.9	52.9	53.1	52.7	31.5	32.0	32.0	31.8	27.6	27.7	27.6	27.2	22.2	22.4	21.9	22.5
Asp[7]	50.2	49.9	49.9	50.0	36.4	36.4	36.0	36.0								
Phe	53.9	53.9	54.1	54.0	37.9	37.9	37.9	37.7								

* Chemical shifts of Nle-C_ω for **I**, 14.1 ppm; **II**, 14.0 ppm; **III**, 13.9 ppm; and **IV**, 14.2 ppm.

Table 3. Rotamer Populations[a] (in %) of Amino Acid Residues with One or Two β-Protons for I–IV

	I			II			III			IV		
residue	P_{g-}	P_t	P_{g+}	P_{g-}	P_t	P_{g+}	P_{g-}	P_t	P_{g+}	P_{g-}	P_t	P_{g+}
Asp[1]	57	9	34	61	2	37	61	1	38	57	0	43
Tyr	61	23	16	66	6	28	66	4	30	64	18	18
Trp	55	20	25	60	18	22	55	18	27	55	18	27
Asp[7]	40	40	20	45	36	19	43	36	21	40	34	26
Phe[8]	52	21	27	49	21	30	55	25	20	55	25	20
β-Me-Phe[b]	56	4	40	39	1	60	47	41	12	2	34	64

[a] Rotamer populations were calculated from the $^3J_{H\alpha H\beta}$ coupling constants using the Pachler equations: $P_{g-} = (J_{H\alpha H\beta(Pro\text{-}R)} - {}^{sc}J_{H\alpha H\beta})/({}^{ap}J_{H\alpha H\beta} - {}^{sc}J_{H\alpha H\beta})$, $P_t = (J_{H\alpha H\beta(Pro\text{-}S)} - {}^{sc}J_{H\alpha H\beta})/({}^{ap}J_{H\alpha H\beta} - {}^{sc}J_{H\alpha H\beta})$, where the following values ${}^{ap}J_{H\alpha H\beta}$ = 13.6 Hz and ${}^{sc}J_{H\alpha H\beta}$ = 2.6 Hz were used. The stereospecific assignment of β-protons were deduced from the ROE patterns, see the text for detail. [b] Rotamer populations of β-MePhe residues were derived from the measured $J_{H\alpha H\beta}$ and $J_{H\alpha C\gamma}$ coupling constants using the following equations: $J_{H\alpha H\beta} = P^{ap}J_{H\alpha H\beta} + (1-P){}^{sc}J_{H\alpha H\beta}$ and $J_{H\alpha C\gamma} = P^{ap}J_{H\alpha C\gamma} + (1-P){}^{sc}J_{H\alpha C\gamma}$, where P^{ap} and P are rotamer populations corresponding to the antiperiplanar arrangements of the relevant spins. Furthermore, ${}^{ap}J_{H\alpha C\gamma}$ = 8.5 Hz and ${}^{sc}J_{H\alpha C\gamma}$ = 1.4 Hz were used. The experimental $J_{H\alpha H\beta}$ coupling constants are from Table 1 and $J_{H\alpha C\gamma}$ coupling constants for peptides I–IV are 1.7, 1.5, 4.7, and 6.0 Hz respectively, which were obtained from the z-filtered relay experiments.[28]

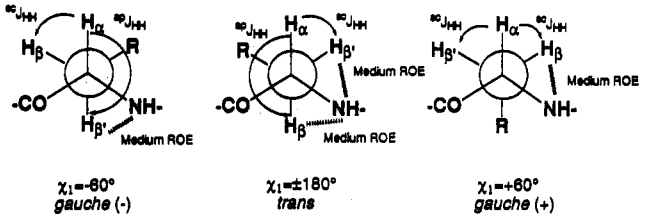

Figure 5. Newman projection of three staggered conformations for an amino acid residue with two β-protons. Characteristic J-coupling and NOE patterns for each conformer also are depicted.

these four peptides (Table 1). The NH resonances of the β-MePhe residues in peptides **II** and **IV** (with (2*R*,3*R*)-β-MePhe and (2*R*,3*S*)-β-MePhe, respectively) appear at lower field by 0.1–0.4 ppm than those in peptides **I** and **III** (with (2*S*,3*S*)-β-MePhe and (2*S*,3*R*)-β-MePhe, respectively). A different situation occurs in the ^1H resonances of the Tyr[2] NHs and the Gly[4] NHs. Similarly, some variations in the ^1H chemical shifts of α- and β-protons also have been observed in these amino acid residues of the peptides. Do these ^1H chemical shift variations result from backbone conformational changes imposed by different side-chain constraints? Or are they simply the consequences of the ^1H chemical shift anisotropy due to the conformational change of the β-MePhe side chains? These questions can be addressed on the basis of further considerations of the vicinal homonuclear coupling constants[32] and ^{13}C chemical shifts.[34] First, the coupling constants ($^3J_{H\alpha NH}$) of Tyr[2], β-MePhe[3], and Gly[4] for the four different peptides are quite similar within experimental error (Table 1). Small differences of the J values are consistent with the conservation of the local backbone conformations. Further, the ^{13}C-NMR chemical shifts[34] are more sensitive and specific for conformational changes because of their larger chemical shift range and the relatively small anisotropic chemical shielding effects induced by unsaturated functional groups, such as aromatic groups and C=O double bonds. In this model system only minor chemical shift variations (less than 1 ppm) were found in some of the Cα and Cβ carbons in aspartic acid-1 (Asp[1]), Tyr[2], and β-MePhe[3] residues among the four peptides (Table 2). For the rest of the amino acid residues, the ^{13}C NMR resonances have almost identical chemical shifts for the four different analogues. This suggests that there is little perturbation of the global backbone conformation by the different stereoisomers of β-MePhe in this linear peptide system. However, some minor local perturbation to the backbone conformation around the β-MePhe[3] residues may exist, which is consistent with the observed small ^{13}C-NMR chemical shift variations. Therefore, a large portion of the observed ^1H chemical shift variations of Tyr[2], β-MePhe[3], and Gly[4] in the four peptide molecules are probably caused by the anisotropic shielding effect of the aromatic rings in different orientations, especially that of the β-MePhe residues, since different stereoisomers of β-MePhe introduce different side-chain conformation preferences in each of these peptides as is evident from Table 3 and the following discussion.

Side-Chain Conformations. Stereospecific assignments of β-CH_2 protons on the basis of homonuclear coupling constants and NOEs have been discussed by Wagner and co-workers.[35,36] A simplified version using both $^3J_{\alpha\beta}$ and intraresidue ROEs of the amino acid residues, Asp[1], Tyr[2], tryptophan-5 (Trp[5]), aspartic acid-7 (Asp[7]), and phenylalanine-8 (Phe[8]) are used here in determining side-chain conformations. For the Tyr[2], Trp[5], and Phe[8]

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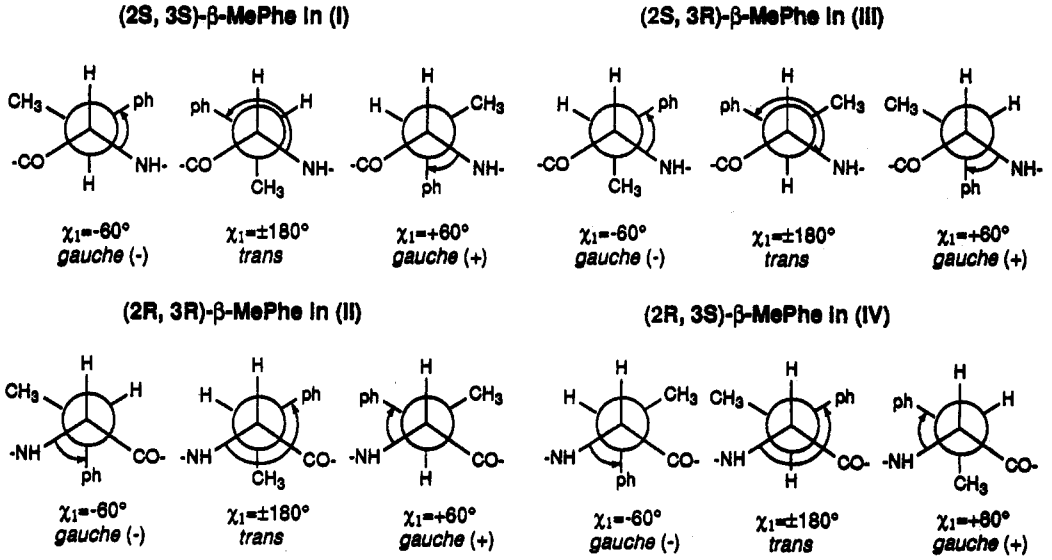

Figure 6. Three staggered conformations for each of four stereoisomers of β-methylphenylalanine.

residues in these peptides, the obtained coupling constants, one large $J_{\alpha\beta'}$ = 8–10 Hz and one small $J_{\alpha\beta}$ = 3–5 Hz, are typical for either the gauche(−) or trans conformer. The strong intraresidue ROE contacts observed between the NH and only one of the β-protons (β′ high-field, pro-*R*) (Figure 3), however, suggest the predominance of gauche-(−) conformations (Figure 5). In the case of Asp[7], both gauche(−) and trans conformations have similar contributions to the rotamer populations. In brief, the Newman projections of the three staggered side-chain conformations with their characteristic ROE and *J*-coupling patterns are depicted in Figure 5. The rotamer populations calculated from the measured homonuclear coupling constants using the Pachler equations[25,37,38] are given in Table 3. Evaluation of side-chain conformations of amino acids with only one β-proton (β-MePhe[3]) requires the quantitative determination of heteronuclear vicinal coupling constants[31] along with the homonuclear vicinal coupling constants. It has been recently demonstrated[29] that the combined use of z-filtered homonuclear and heteronuclear relay spectroscopy provides a facile and sensitive means for the measurement of these conformationally important heteronuclear coupling constants. Comparison of the corresponding multiple widths (e.g. H_α and $H_\alpha(^{13}C_\gamma)$ of β-MePhe[3]) in the z-filtered homonuclear and heteronuclear relay spectra provides the required three-bond $^3J_{H\alpha C\gamma}$ heteronuclear coupling constants.[29] The measured heteronuclear long-range coupling constants and the calculated rotamer populations of β-MePhe[3] for each peptide are summarized in Table 3. The Newman projections of the four stereoisomers of β-MePhe residues and the three staggered side-chain conformations are depicted in Figure 6. The intraresidue ROEs observed between the NH and CH_3 protons or H_α and β-CH_3 protons of β-MePhe residue also are consistent with the side-chain conformations deduced from the coupling constants.

On the basis of the experimentally determined side-chain rotamer populations shown in Table 3, several pertinent points can be discussed with regard to the conformational impacts that β-MePhe residues introduced into these peptides. One immediate question is the kind of conformational restriction effects that these β-MePhe residues have on the χ_1 angles in comparison with the Phe residues. The bias imposed by β-MePhe residues in this peptide system is apparent in comparison with the statistical rotamer distribution of the L-phenylalanine in peptides and proteins.[39] This point can also be substantiated by a comparison of β-MePhe[3] residues with Phe[8] within these peptides. The previous discussion in this paper indicated that the conformations of the C-terminal tetrapeptide, Trp-Nle-Asp-Phe-NH_2, are not disturbed by different stereoisomers of β-MePhe[3]. It also is noted that the influence of side-chain rotamer populations from the peptide sequence is insignificant in this model system because similar distributions of the side-chain rotamer populations for Trp[5] and Phe[8] were observed in all four peptides. Thus we can simply compare the side-chain rotamer populations of the β-MePhe[3] residues with those of Phe[8]. Peptides **I** and **III** both have an (*S*)-configuration at their α-carbons, which is the same as that for L-phenylalanine. From Table 3, it is clear that the (2*S*,3*S*)-β-MePhe residue leads to a virtual elimination of the trans conformation, and at the same time increases the populations for the gauche(−) and gauche(+) rotamers in comparison with those found for the Phe[8] residue in peptide **I**. On the other hand, (2*S*,3*R*)-β-MePhe residue reduces the population of the gauche(+) rotamer and increases the populations of the trans conformation with respect to the Phe[8] residue in peptide **III**. Now it becomes obvious that the introduction of a methyl group at the β-position of the L-phenylalanine residue does not change its preference for the gauche(−) conformation. Instead, the (2*S*,3*S*)-isomer also favors gauche(+) and (2*S*,3*R*)-isomer favors trans in addition to the gauche(−), respectively. For the D-Phe residue, introduction of the (*S*)-β-CH_3 and the (*R*)-β-CH_3 favor trans and gauche(−) rotamers, respectively, in addition to their common preference for the gauche(+) conformation. Thus, the intraresidue side-chain conformational constraint affected by β-MePhe residues is clearly demonstrated. Furthermore, due to the small energy differences among the different rotamers,

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conformational analysis based on simple steric considerations may not always succeed in these molecules.[15]

In this model system, a significant change in the side-chain populations was observed for the Tyr² residue which is adjacent to the β-MePhe³ residue in the peptide sequences. For example, the trans-rotamer populations in [(2S,3S)-β-MePhe³, Nle⁶]CCK-8(NS) (I) and [(2R,3S)-β-MePhe³, Nle⁶]CCK-8(NS) (IV) are 23 and 18%, respectively. In [(2R,3R)-β-MePhe³, Nle⁶]CCK-8(NS) (II) and [(2S,3R)-β-MePhe³, Nle⁶]CCK-8(NS) (III), however, the trans-rotamer populations are reduced significantly to 6 and 4%, respectively. As a consequence, the gauche-(+) rotamers in the latter two analogues become more abundant. Furthermore, this variation in the rotamer populations of the Tyr² appears to be correlated with the configuration of the β-carbons of their neighboring β-methylphenylalanine residues, i.e., relatively higher trans populations in Tyr² correspond to the (3S)-configurations of the β-MePhe³ residues. Presumably, the β-CH_3 in (3R)-isomers may impose stronger steric interactions with the aromatic ring of Tyr² in its trans side-chain conformation, which in turn raise the relative energy of the trans conformations in the Tyr² residue when the β-MePhe³ is (2S,3R) and (2R,3R). However, such an effect was not observed for other amino acid residues further away in the peptide sequence.

Empirical Correlation of ^{13}C Chemical Shifts with the β-Methylphenylalanine Side-Chain Stereochemistry. Consistent with the fact that the introduction of different stereoisomers of β-MePhe into the CCK-8(NS) peptide system primarily influences the side-chain conformation at that position, a rather large chemical shift variation (ca. 3 ppm) was found for the β-CH_3 carbons in the different stereoisomers of β-MePhe-containing analogues, which is in contrast to the much smaller chemical shift variations for other carbons observed in these peptides. Also, these β-CH_3 carbon chemical shifts are well correlated with the stereochemistry of the β-MePhe residues, *i.e.*, the low-field chemical shifts at 18.7 and 18.9 ppm are associated with *erythro*-β-MePhe ((2S,3S)- and (2R,3R)-isomers in peptides I and II), and the relatively higher field chemical shifts of 16.3 and 15.4 ppm are associated with *threo*-β-MePhe ((2S,3R)- and (2R,3S)-isomers in peptides III and IV, respectively) (Table 2). The strong correlation between the ^{13}C-NMR chemical shifts and stereochemistry observed here can be rationalized on the basis of the conformational analysis of β-methylphenylalanine side chains described above and the well-known conformational dependence of the γ-substituent effect in ^{13}C chemical shifts.[40]

Recently, on the basis of ab initio IGLO calculations,[41] an angular dependence of the γ-substituent effect has been formulated and also was found in the syn/anti dependence of ^{13}C chemical shifts in peptide bonds.[42] This angular dependence can be applied in the present work to explain the experimental observations. By comparing the side-chain rotamers in each of the four stereoisomers of β-MePhe³ substituted analogues (Figure 6), it was found that the trans rotamers in III and IV are approximately

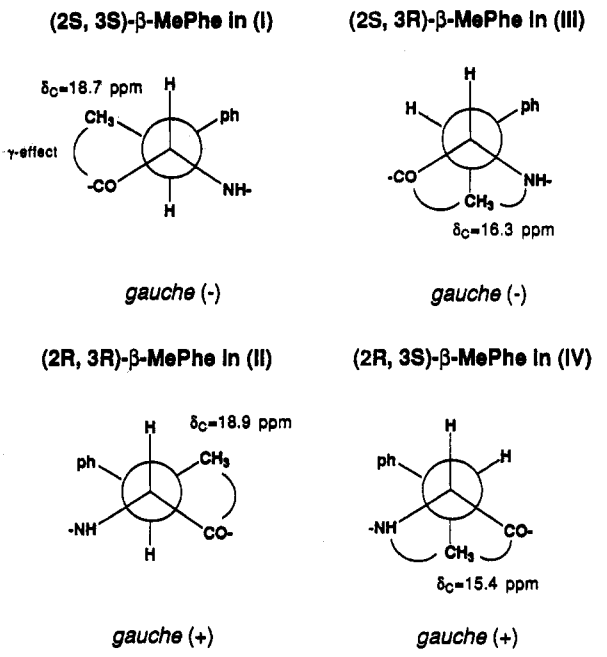

Figure 7. An empirical correlation of the γ-substituent effect with the stereochemistry of the β-methylphenylalanine residues.

equivalent to the gauche(+) rotamer in I and the gauche-(−) in II, respectively, in terms of the γ-substituent effect experienced by the β-CH_3 carbons from the NH groups. Also these equivalent rotamers have the same population, about 40%. They would contribute equally to the shielding of the β-CH_3 carbons and thus can be eliminated from further considerations for the observed differences in ^{13}C chemical shifts. What is left in III and IV, therefore is the gauche(−) and the gauche(+) conformers, respectively (Figure 7), in which the β-CH_3 carbons experience strong γ-effects from both the CO and the NH groups. In contrast, the rotamers with such a strong γ-substituent effect are the least populated trans conformers in I and II. Their most populated rotamers are the gauche(−) in I and the gauche(+) in II. In these rotamers, the β-CH_3 carbons are only subject to one strong γ-substituent effect from the CO group, which should be substantially smaller than the sum of the two strong γ-substituent effects occurring in III and IV. Therefore, the observed upfield shifts of β-CH_3 carbons by 3 ppm in III and IV relative to those in I and II can be qualitatively rationalized.

Conclusions

On the basis of available NMR parameters, a complete conformational analysis was performed on the side-chain conformations of most residues in the four peptides, particularly for the β-MePhe³ residues. Each of the four different stereoisomers of the β-methylphenylalanine have two rather than one heavily populated side-chain rotamer. Compared with the side-chain rotamer populations of the unconstrained phenylalanine-8, these β-MePhe-containing analogues virtually eliminate one of the rotamers accessible by the phenylalanine residue. The different stereoisomers of the β-MePhe³ residues may impose perturbations on the side-chain and backbone conformations of its neighboring amino acid residues, but have little effect on the side-chain and backbone conformations of remote (further than one residue away) residues in the same peptide sequence. A strong correlation of ^{13}C chemical shifts of

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the β-CH_3 carbons and the stereochemistry of β-methylphenylalanine residues was uncovered and was rationalized in terms of the angular-dependent γ-substituent effect in ^{13}C-NMR chemical shifts. In this regard, a similar conformational bias for the side-chain conformational properties was observed in the distribution of rotamers of β-MePhe residues in the β-MePhe[4] analogues of a cyclic pentapeptide, H-Tyr[1]-D-Pen[2]-Gly[3]-Phe[4]-D-Pen[5]-OH (DPDPE),[43] and Huang et al.[12] observed an identical bias in a somatostatin analogue. This suggests that these observed trends for β-MePhe should be applicable to a wide variety of peptide sequences unless a severe steric effect is imposed. However, one must always proceed with caution, and this underlines the importance of a reliable, rapid, and quantitative analysis of the side-chain conformations such as that reported here. In this regard, the empirical correlations between the β-CH_3 ^{13}C chemical shifts and the stereochemistry also may provide an additional useful probe for the side-chain conformations of β-methylphenylalanine residues in peptides.

Experimental Section

All NMR parameters used in the present study have been derived from 1D and 2D experiments performed at 310 K with a 500 MHz NMR spectrometer equipped with a 5-mm inverse probe head. The peptides I–IV have been synthesized via the solid-phase method and have been fully characterized by FAB-MS, amino acid analysis, RP-HPLC, and NMR.[44] Peptide samples were dissolved in DMSO-d_6 (100% D, Aldrich) at a concentration of 18 mg/0.4 mL.

The z-filtered homonuclear TOCSY spectra were recorded using a repetition delay of 1.0 s between the subsequent transients, and the isotropic mixing times were set at 60 ms; 64 transients were accumulated for each of 256 experiments; 4096 data points were acquired in the acquisition dimension, and the spectral width was 5435 Hz. Quadrature detection in the F_1 dimension was achieved by TPPI.[45] With zero-filling in both F_1 and F_2, multiplication with a cosine square function was performed prior to 2D Fourier transformation. For evaluation of coupling constants, a digital resolution of 0.3 Hz/point was achieved by inverse Fourier transformation, zero-filling, and back transformation of selected traces.

ROESY experiments were carried out in inverse configuration using the decoupler for ^1H pulsing. Decoupler power was attenuated to give a 90° pulse of 80 μs. The duration of the CW spin-lock pulse was 180 ms; 256 experiments with 16 transients each were carried out.

The phase-sensitive z-filtered ^{13}C-coupled HSQC-TOCSY[29] spectra were obtained by carrying out 256 experiments of 224 scans each. A repetition delay of 0.7 s was used between the subsequent transients. The spectral width in the ^1H dimension was 5435 Hz and in the ^{13}C dimension was 15722 Hz; 4096 data points were acquired in the acquisition dimension. Spin-lock pulses with durations of 3 and 4 ms were employed to suppress the undesired ^1H–^{12}C magnetization. The durations of ^1H and ^{13}C 90° hard pulses were 13 and 15 μs, respectively. For the MLEV-17 mixing period, the decoupler was attenuated to give a proton 90° pulse of 25 μs. Following the MLEV-17 mixing sequence with a duration of 60 ms, a z-filter consisting of two 90° pulses separated by a randomly varied delay was inserted to provide pure absorption-phase spectra. The heteronuclear long-range coupling constants were obtained by comparison of the corresponding multiple widths in the z-filtered ^1H-TOCSY and HSQC-TOCSY spectra.[29]

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