Spontaneous Diketopiperazine Formation via End-to-End Cyclization of a Nonactivated Linear Tripeptide: An Unusual Chemical Reaction

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Abstract: The δ-opioid antagonist H-Tyr-TicΨ[CH_2-NH]Phe-OH (TIP[Ψ], Tic = tetrahydroisoquinoline-3-carboxylic acid) was shown to undergo *spontaneous* formation of *N*-(Mti)-Phe-Tyr diketopiperazine (Mti = [(3′*S*)-tetrahydroisoquinolinyl]methyl) in DMSO and MeOH but not in aqueous solution or DMSO:H_2O solvent mixtures which were at least 20% water. The reaction mechanism proposed for the diketopiperazine formation involves amide bond formation between the N and C terminal groups of the nonactivated linear tripeptide, followed by transannular nucleophilic attack of the Tyr carbonyl group by the secondary amine. Molecular mechanics calculations carried out on TIP[Ψ] revealed low-energy structures available to the molecule in which the carboxyl and amino terminal functions are within a distance suitable for nucleophilic attack (2.8 Å). The rate of diketopiperazine formation was shown to be dependent on both sample pH and nucleophilicity of the terminal peptide amino group. Reaction intermediates or additional compounds in tautomeric equilibria with the diketopiperazine were not observed during the HPLC analyses or NMR investigations. The diketopiperazine obtained from TIP[Ψ] was isolated and structurally characterized by ^{1}H NMR spectroscopy in conjunction with molecular modeling. According to ^{1}H NMR coupling constants, several orientations of the side chains around the diketopiperazine ring are possible. Large chemical shift displacements from expected values observed for the Tyr β and Mti methylene protons are explained in terms of ring current effects manifested by aromatic interactions which stabilize the low-energy structures. The calculated diketopiperazine ring structure is slightly puckered out of plane by −5° to −15°.

Introduction

Intramolecular reactions involving linear tripeptides have been the subject of numerous investigations during the past several years.[1-5] Most interesting are the cyclic tripeptides which form a special class of intramolecular reaction products characterized by their unusual chemistry, structure, and conformational properties.[5-9] They are usually formed by direct end-to-end ring closure of the *activated* linear precursors or by independent synthesis via N-protected aminoacyl diketopiperazines.[10] Historically, attempts at synthesizing these highly strained nine-membered rings from *activated* linear tripeptides have usually resulted in the formation of higher cyclic peptides.[11] The few cyclotripeptides known to be stable incorporate either tertiary amides or alternatively lactone bonds in place of peptide bonds at the three junctions of the ring.[7,12]

From a structural point of view, neither an all *trans* nor a two *trans* and one *cis* arrangement is geometrically possible for cyclotripeptides.[12] The most favorable backbone conformation is characterized by a 3-fold symmetry with the C_α carbon atoms situated on one side of a plane defined by the N atoms and the CO carbons on the other side. Symmetric cyclotripeptides adopting this crown configuration include, for example, cyclo-(L-Pro_3), cyclo-(L-Hyp-L-Pro_2), and cyclo-(L-Sar_3) and involve an all *cis* arrangement of the peptide units although asymmetric structures with nonplanar *cis* and/or *trans* peptide bonds have also been reported.[6,9,12,13] Most interesting is the *cis–cis–trans* arrangement of the peptide units which introduces the possibility for a nine-membered ring containing one standard amino acid. However, homodetic and heterodetic cyclotripeptides possessing even a single secondary amide bond have shown a general tendency toward cyclodimerization reactions or intramolecular interactions leading to more stable tautomeric forms.[14-16] In 1973, Rothe et al. proposed that cyclic tripeptides containing a secondary amide bond exist in a tautomeric equilibrium with their corresponding cyclols and aminoacyl diketopiperazines.[17] It was suggested that the equilibrium favors the diketopiperazine since an unstrained 6-membered ring is formed in place of the nine-membered one in this case.

In this paper, we report an unusual example of diketopiperazine formation from a linear pseudotripeptide which occurs *spontane-*

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ously in DMSO. To the best of our knowledge, the cyclization mechanism involving a condensation reaction between the terminal groups of the *nonactivated* linear pseudotripeptide H-Tyr-Tic-Ψ[CH_2-NH]Phe-OH (TIP[Ψ]) followed by a transannular nucleophilic attack from the secondary amine to the Tyr carbonyl is the first reported reaction of its kind.

The observed instability of TIP[Ψ] in DMSO came as a surprise since incorporation of a reduced peptide bond between Tic[2] and Phe[3] of the highly potent and selective opioid δ-antagonist H-Tyr-Tic-Phe-OH (TIP) was originally intended to prevent Tyr-Tic diketopiperazine formation and concomitant Tic–Phe peptide bond cleavage that had previously been found to occur spontaneously in DMSO and MeOH.[4] In comparison with its parent peptide, TIP[Ψ] showed increased δ-opioid antagonist potency and further improved δ-receptor selectivity.[18] These findings prompted a conformational investigation in order to determine structural properties of TIP[Ψ] which may be responsible for its improved opioid activity profile. Evidence of monomeric cyclization discovered during the NMR investigations of TIP[Ψ] in DMSO represented an unexpected but welcome surprise in that the reaction pathway leading to formation of a stable diketopiperazine is highly unusual from both chemistry and energy points of view.

Results and Discussion

Chemical Stability of TIP[Ψ]. Initial evidence of tripeptide cyclization surfaced during the NMR spectroscopic investigations of TIP[Ψ] in DMSO (5 mM, pH = 6.9). Within 1 h of sample preparation, a new set of resonances emerged, suggesting formation of a new compound. By contrast, samples of the same peptide dissolved in H_2O (5 mM) at two different pH levels (5.3 and 9.3) were found to be completely stable. ^1H NMR spectra of TIP[Ψ] in H_2O showed no signs of chemical degradation or conformational change even 6 months after sample preparation.

An HPLC analysis of TIP[Ψ] dissolved in DMSO was carried out to determine the kinetics of chemical degradation. In accordance with NMR conditions, sample concentrations were kept at approximately 5 mM although preparation proceeded without further adjustment of the sample pH. This resulted in an initial sample pH of 9.1 where the terminal and reduced bond amine groups exist in the deprotonated form.[19] Sample injections administered at approximately 1 h intervals following dissolution revealed a gradual disappearance of the parent compound peak accompanied by the emergence of a new peak which eluted 2 min later. The reaction was essentially complete after 48 h as indicated by the detection of a single chromatographic peak at the degradation product retention time 2 days after the initial run.

A follow-up study involved monitoring the time-dependent change of the ^1H NMR spectrum of a TIP[Ψ]–DMSO (5 mM) sample at pH 9.1. As expected, complete conversion of the original peptide to a new product was realized after 48 h (Figure 1). In order to test the importance of sample pH for TIP[Ψ] stability, the experiment was repeated on a more acidic (pH = 5.5) TIP[Ψ]–DMSO (5 mM) NMR sample. Interestingly, there were no signs of structural change in the ^1H NMR spectrum acquired for this sample during the 3 weeks of observation. This strongly suggests that the protonated form of TIP[Ψ] is much more resistant to chemical degradation in DMSO than the deprotonated form.

Chemical stability of TIP[Ψ] in methanol was also determined by HPLC analysis. Chromatograms of TIP[Ψ] dissolved in MeOH (pH = 6.9) followed a pattern similar to that observed for DMSO although on a much slower time scale. Degradation reached completion after 1 month in this case. The fact that no additional peaks appeared in the TIP[Ψ]–MeOH chromatograms during the entire 1 month of study is convincing evidence that the degradation reaction proceeds spontaneously to completion without the formation of stable intermediates.

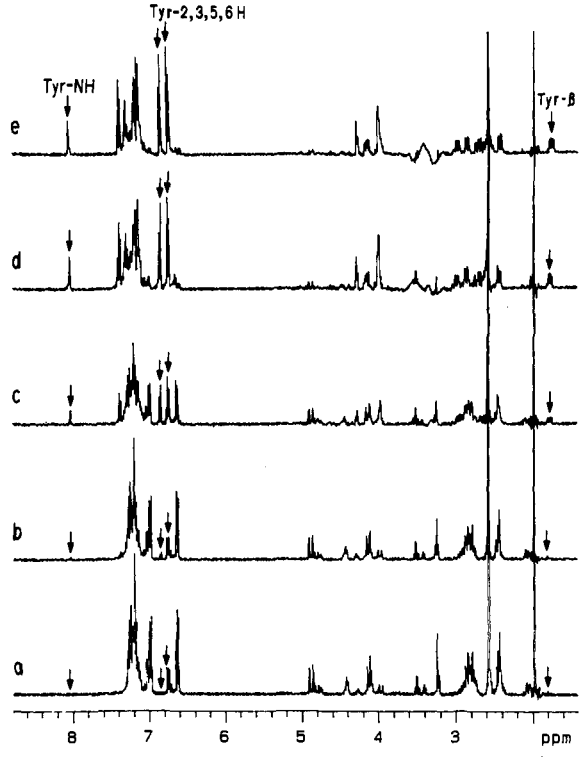

Figure 1. ^1H NMR spectra of H-Tyr-TicΨ[CH_2-NH]Phe-OH in DMSO-d_6 (pH 9.1) recorded immediately after sample preparation (a) and 1 h (b), 6 h (c), 24 h (d), and 48 h (e) later. Peaks corresponding to the Tyr protons of *N*-(Mti)-Phe-Tyr diketopiperazine are identified with arrows.

Other systems tested were TIP[Ψ] dissolved in mixtures of DMSO and H_2O (DMSO:H_2O of 1:9, 1:2, 1:1, and 4:1). Results of the study revealed the tripeptide to be stable for all solvent ratios used, suggesting that TIP[Ψ] is closely solvated by water molecules in DMSO/H_2O mixtures containing only 20% H_2O by volume.

For comparison, the stabilities of two additional TIP[Ψ]-related peptides (H-Tyr-TicΨ[CH_2-NH]Phe-Phe-OH(TIPP[Ψ])[18] and Tyr(NMe)-TicΨ[CH_2-NH]Phe-OH) dissolved in DMSO (pH 9.1) were also monitored by HPLC over several days. There was no evidence of degradation product formation in any of the solvent systems described above for the pseudotetrapeptide TIPP[Ψ] even 1 month after dissolution. However, HPLC analysis of the N-methylated tripeptide dissolved in DMSO did reveal the formation of a stable product albeit at a much slower rate than that observed for TIP[Ψ]. Only 15% of the starting material had converted to its final product after 1 month in this case. The fact that both TIP[Ψ] and its N-methylated analog convert to reaction products which elute 2 min later than their respective parent peptides suggests that both TIP[Ψ] and T(NMe)IP[Ψ] may convert to similar species in DMSO. The extra stability imposed on TIP[Ψ] through N-methylation may be attributed to a reduction in nucleophilic strength upon going from a primary to a secondary amine and possibly also to steric factors. It can be speculated that conformational and/or steric reasons are likely responsible for the resistance of the tetrapeptide TIPP[Ψ] to degradation in DMSO. A graphical summary of the HPLC kinetics for TIP[Ψ] dissolved in DMSO (pH 9.1) and MeOH (pH 6.9) is provided in Figure 2.

According to the HPLC chromatograms of TIP[Ψ] in DMSO and MeOH acquired at regular intervals, the preferred reaction pathway for TIP[Ψ] degradation does not involve stable inter-

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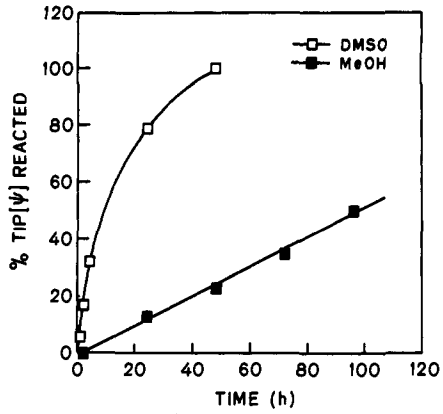


Figure 2. Rate of *N*-(Mti)-Phe-Tyr diketopiperazine formation in DMSO (pH 9.1) and MeOH (pH 6.9) as determined from HPLC kinetic analyses.

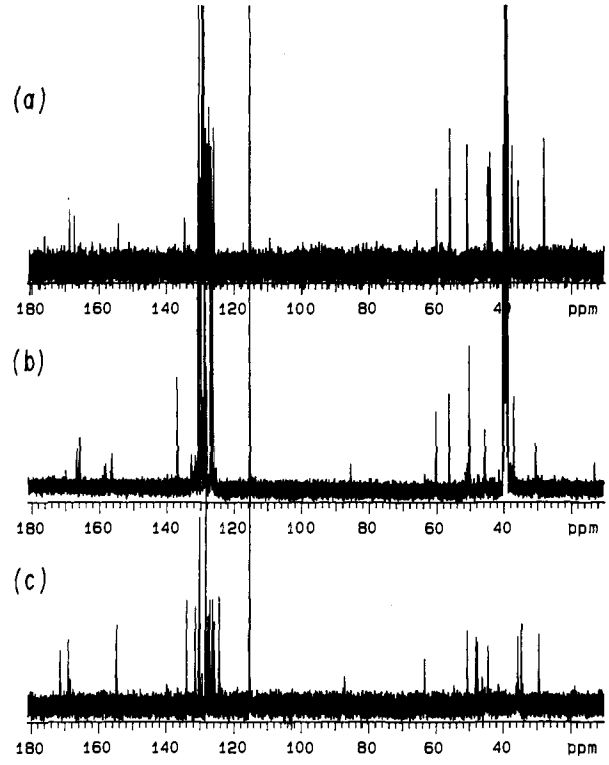


Figure 3. ^{13}C NMR spectra of (a) purified *N*-(Mti)-Phe-Tyr diketopiperazine in H_2O (pH 3.5), (b) H-Tyr-TicΨ[CH_2-NH]Phe-OH in DMSO-d_6 (pH 9.1) acquired for 48 h with recording initiated immediately after sample preparation, and (c) H-Tyr-TicΨ[CH_2-NH]Phe-OH in H_2O (pH 3.5).

mediates. However, it is possible that intermediary compounds were not present in solution each time a chromatogram was collected and consequently reaction intermediates were not detected. Thus an alternative method was required to obtain a more detailed picture of the sequence of reactions involved in the chemical conversion of the pseudotripeptide TIP[Ψ] to a stable product. Acquisition of a ^{13}C NMR spectrum of TIP[Ψ] dissolved in DMSO at pH 9.1 acquired over a period of 48 h and started immediately after sample preparation provided a reliable way of continuously monitoring the reaction events. The resulting ^{13}C spectrum is shown in Figure 3b. For comparison, ^{13}C NMR spectra of the purified reaction product dissolved in H_2O and a linear precursor dissolved in H_2O are shown in Figure 3, parts a and c, respectively. Visual comparison of the three spectra in Figure 3 did not reveal any obvious new compounds. This result supports conclusions drawn from the HPLC kinetic experiments which suggest a highly spontaneous reaction mechanism for degradation of TIP[Ψ] in DMSO and MeOH.

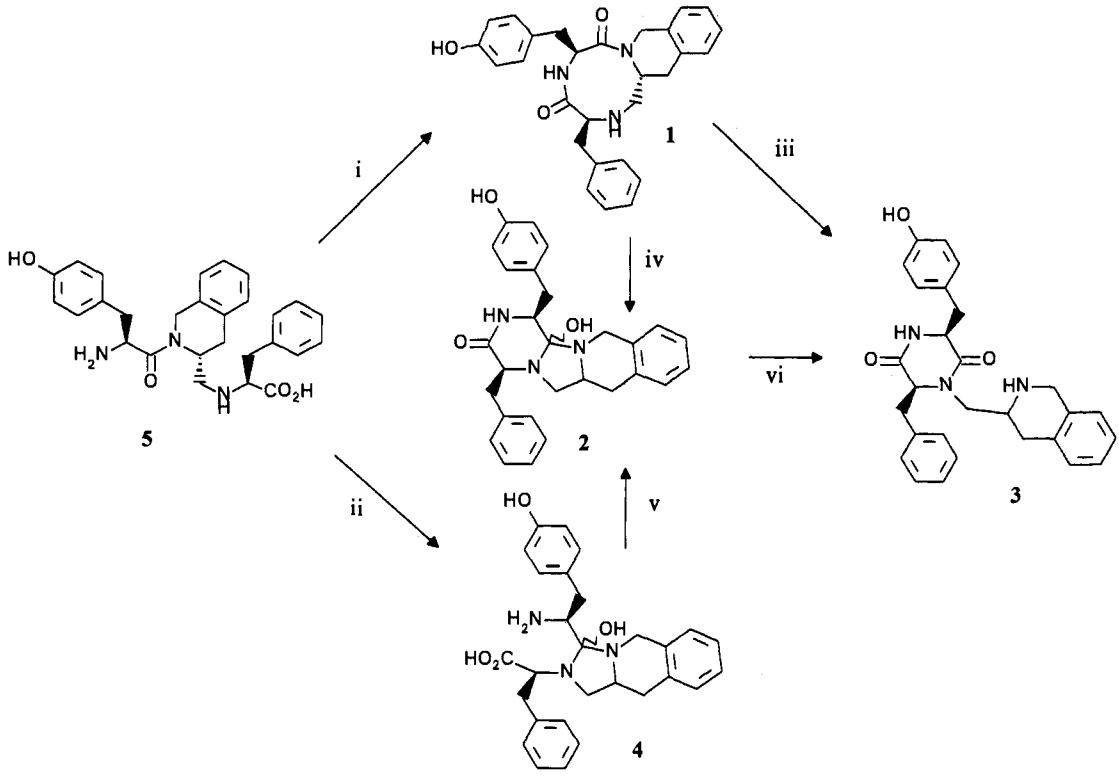
Chemical Composition of the TIP[Ψ] Degradation Product. The single product resulting from the degradation of TIP[Ψ] in DMSO was purified by semipreparative HPLC for the purpose of identifying its structure. On the basis of HPLC, TLC, FAB-MS, and NMR data, a stable diketopiperazine (*N*-[[(3′*S*)-tetrahydroisoquinolinyl]methyl]-Phe-Tyr diketopiperazine) was assigned to the reaction product structure. The molecular weight of the compound determined by FAB mass spectrometry provided the first convincing evidence of ring formation. A molecular species was detected at *m/e* 456 compared to *m/e* 474 for the parent peptide TIP[Ψ], suggesting a loss of one water molecule. On the basis of the usual tautomeric equilibria expected for cyclic products of linear tripeptides in polar solvents,[2,17] there were three possible species which fit the observed molecular weight (**1**, **2**, and **3** in Scheme 1 which will be discussed in detail later). The ^{13}C spectrum of the TIP[Ψ] reaction product in H_2O (Figure 3a) was particularly informative in that a singlet at ~96 ppm, characteristic of a quaternary carbon bonded to three heteroatoms, was not observed. This property was sufficient evidence to rule out **2** as a possible candidate structure. Previous studies indicate that a linear tripeptide does not form a stable 9-membered ring unless it is able to accommodate at least two *cis* peptide bonds.[12] However, TIP[Ψ] contains a Tic residue in the second position which is an N-alkylated amino acid and, therefore, can be expected to engage in a *cis* peptide bond in a significant population of the conformational ensemble. Furthermore, the increased backbone flexibility resulting from introduction of the reduced peptide bond between the Tic and Phe residues also allows for a spatial arrangement of nuclei analogous to that of a *cis* peptide bond. Thus, the possibility of a nine-membered ring could not be eliminated, although it is expected that if a cyclic tripeptide (**1** in Scheme 1) was initially formed it would quickly give rise to the *N*-(Mti)-Phe-Tyr diketopiperazine (Mti = [(3′*S*)-tetrahydroisoquinolinyl]methyl) since an unstrained 6-membered ring is formed in this case. Additional evidence of higher molecular weight compounds was not apparent in the FAB-MS spectrum, thus ruling out the possibility of cyclopolymerization.

Another important clue was the apparent pH dependence of the degradation rate determined from the proton NMR studies. Assuming that the chemical conversion of TIP[Ψ] proceeds via an S_N2 mechanism, it is expected that an increase in sample pH would result in a higher rate of chemical reaction. The fact that the time-dependent change in the ^1H NMR spectrum of TIP[Ψ] in DMSO was rapid at pH 9.1 but did not occur at pH 5.5 is strong supporting evidence that the terminal amino group and/or reduced peptide bond amine of TIP[Ψ] is involved in its chemical conversion.

Further supporting evidence of peptide cyclization was obtained from TLC analyses of the parent peptide and purified product. When sprayed with ninhydrin, the two products showed single spots for the expected amine groups, confirming the purity of the compounds. According to the determined R_f values, the reaction product is a less polar molecule than TIP[Ψ], presumably due to the absence of free amine and carboxylic functions which are present in the parent peptide. In addition, a strong purple hue characteristic of a primary amine was evident in the TIP[Ψ] chromatogram but not in the other. This observation alone confirms that the N-terminal amine is disappearing during the degradation process. By contrast, inspection of the reaction product chromatogram revealed a single yellow spot which is typical for a secondary amine. Since both candidate structures (**1** and **3** in Scheme 1) contain one secondary amine group, additional experiments were required to unequivocally establish the product as either the 9-membered cyclic tripeptide or the diketopiperazine structure.

Assignment of the TIP[Ψ] Reaction Product to *N*-[[(3′*S*)-Tetrahydroisoquinolinyl]methyl]-Phe-Tyr Diketopiperazine by NMR Spectroscopy. NMR measurements were made on the

Scheme 1. Possible Reaction Pathways for the Cyclization of H-Tyr-TicΨ[CH_2-NH]Phe-OH



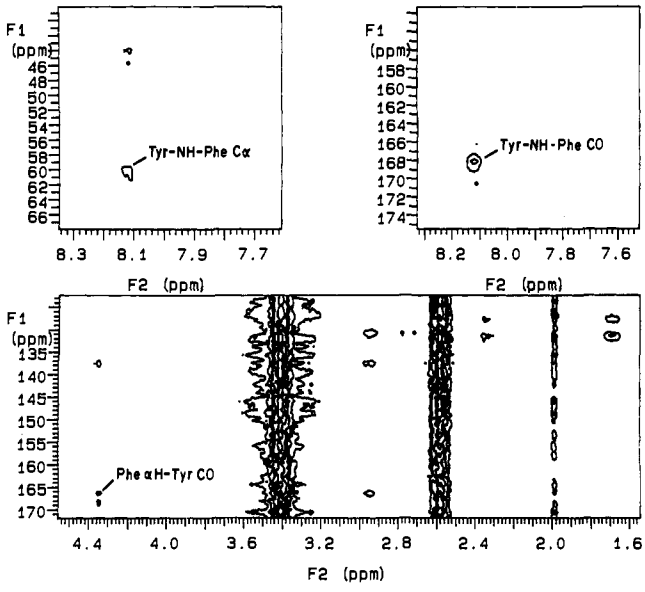


Figure 4. ^1H-detected, ^1H–^{13}C multiple-bond, multiple-quantum correlation (HMBC) spectrum of *N*-(Mti)-Phe-Tyr diketopiperazine in DMSO-d_6. Marked cross peaks are those which confirm a peptide bond between Tyr and Phe.

reaction product in order to confirm its structural identity and to determine its conformation. Experiments were carried out using three different solvent systems (DMSO, H_2O, and DMSO/D_2O (80:20)) in order to maximize the available structural information. The ^1H NMR spectra in DMSO and H_2O were consistent with the molecule possessing a peptide bond between the N-terminal and C-terminal functions of TIP[Ψ]. A sharp doublet was observed at approximately 8.0 ppm in both cases which we assigned to the Tyr NH on the basis of ROESY experiments. This observation strongly supports formation of a peptide bond between Tyr and Phe since the intrinsic exchange rate of terminal amine protons with the solvent usually renders these protons unobservable on the NMR time scale.[19]

Through-bond and dipolar connectivities were established using a combination of homonuclear and heteronuclear 2D NMR experiments. In cases where proton spectral overlap created ambiguities in chemical shift and/or ROE assignments, further resolution was achieved by careful adjustment of the sample pH or by using a selective 1D TOCSY experiment.[20] Spin system patterns displayed in TOCSY and ROESY[21] spectra fit well with those of the proposed cyclic compounds.

Heteronuclear multiple quantum coherence NMR techniques[22,23] served to further confirm the existence of a peptide bond between Tyr and Phe in the TIP[Ψ] reaction product. Proton chemical shifts were first assigned to their attached ^{13}C resonances by examination of an HMQC[22] spectrum of the reaction product dissolved in DMSO. Long-range ^{13}C–^1H connectivities were then obtained from a ^{13}C HMBC[23] spectrum acquired for the same sample. As expected, ^{13}C–^1H crosspeaks were observed between the NH of Tyr and the carbonyl C of Phe, between the Tyr NH and Phe α carbon, and between the Tyr CO carbon and α proton of Phe, thus unambiguously confirming a covalent link between the two ends of TIP[Ψ] (Figure 4). Each of these through-bond correlations would only be observed if the two nuclei involved were separated by five bonds or less, a structural feature that is not realized by the linear tripeptide.

Despite the establishment of a peptide bond between Tyr and Phe of TIP[Ψ], there remained some uncertainty regarding the true chemical structure of the reaction product. On the basis of all the studies discussed so far in addition to the fact that no intermediate products or indications of tautomeric equilibria were observed during any of the NMR experiments or HPLC analyses, the structure of the unknown compound had to be either **1** or **3**. Unfortunately, NMR techniques which establish through-bond connectivities in a molecule were not capable of solving the problem since similar spectra would necessarily be observed for structures **1** and **3**. Thus, relative shifts of proton resonances with changing

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pH were used to assign the unknown compound as the *N*-(Mti)-Phe-Tyr diketopiperazine. When a protonated species in solution is successively deprotonated by addition of a base, ^{1}H nuclei close to the deprotonating site show a significant upfield displacement. Furthermore, the extent of chemical shift displacement for a particular proton diminishes as the number of bonds separating it from the protonated nucleus increases.[19] When the sample pH of the reaction product in D_2O was increased from 4.0 (protonated amine) to 9.3 (deprotonated amine) three proton resonances showed significant upfield displacements. These were, in order of decreasing displacement, the tetrahydroisoquinoline (Tiq) 3-proton (a 0.4 ppm shift), the Tiq 1 protons (a 0.3 ppm shift), and the protons of the exocyclic methylene group adjacent to C-3 of Tiq (a 0.1 ppm shift) (see Figure 6 for atomic numbering). In addition, the Phe αH chemical shift remained stationary during the pH titration. Without question, this result assigns the diketopiperazine (**3** in Scheme 1) to the cyclic product structure, since the Phe α proton would show a significant migration at elevated pH if we were dealing with ring structure **1**.

In order to determine the preferred cyclization mechanism responsible for converting TIP[Ψ] to *N*-(Mti)-Phe-Tyr diketopiperazine, several different pathways had to be considered. These are outlined in Scheme 1. Among the various routes, there are two reactions which can serve as the first step in the cyclization of TIP[Ψ]: (i) end-to-end ring closure of the linear precursor (step i in Scheme 1) and (ii) closure of the 5-membered ring aminal through nucleophilic addition of the phenylalanine NH to the tyrosine carbonyl (step ii in Scheme 1). HPLC and ^{13}C NMR kinetic experiments did not provide important clues to the reaction mechanism since no intermediates were observed. This dilemma is further compounded by the fact that both 9-membered cyclic tripeptides and their tautomeric cyclols are usually unstable in solution.[3,10,11,24] This eliminates the possibility of assigning the cyclization pathway based on stability of reaction intermediates since all intermediary species in Scheme 1 are likely to be short-lived, thus making their detection difficult. A solution to the problem was obtained by repeating the TIP[Ψ] cyclization reaction under slightly different reaction conditions. It was anticipated that addition of a coupling reagent to TIP[Ψ] in DMF would lead to a substantial increase in the reaction rate provided that the rate-determining step involves amide bond formation between the peptide end groups (step i in Scheme 1). This is a reasonable assumption since nucleophilic attack from a primary amine to a nonactivated carboxyl group under basic conditions is extremely difficult owing to the nature of the leaving group (OH^-). As expected, when 1 equiv of DIC (DIC = 1,3-diisopropylcarbodiimide) was initially added to the TIP[Ψ]/DMF solution, complete conversion to the same stable end product was observed after a reaction time of only 1.5 h. It is unlikely that addition of a coupling reagent to the reaction mixture would produce a similar increase in the rate of diketopiperazine formation if a cyclol was initially formed (step ii in Scheme 1) since in this case nucleophilic addition from the Tyr NH to the Phe carboxyl (step v in Scheme 1) would necessarily occur undetectably fast even in the absence of a coupling reagent. In addition, monitoring the ^{13}C spectrum of TIP[Ψ] in DMSO during the course of its chemical conversion did not reveal a signal at ~96 ppm typical of a cyclolic carbon. These observations strongly support a cyclization mechanism driven by step i in Scheme 1. Assuming, however, that the mechanism for diketopiperazine formation proceeds via route i, it is not possible to unambiguously state whether the cyclization involves a simultaneous nucleophilic attack from the reduced peptide bond amine and terminal amino group to their respective targets or whether an unstable nine-membered ring forms first followed by a transannular attack from the reduced peptide bond amine to the Tyr carbonyl. If the latter situation were true then the second step would have to occur very rapidly since no intermediate products were observed in any of the experimental studies.

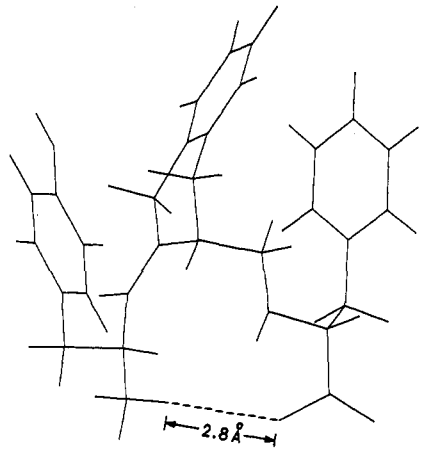

Figure 5. Lowest-energy conformer of TIP[Ψ] containing a *cis* Tyr–Tic peptide bond.

The proposed two-step reaction mechanism according to which the linear tripeptide is first converted to a highly unstable nine-membered ring structure and then is further transformed to a 2-N-substituted diketopiperazine is an exciting discovery in that, to the best of our knowledge, synthetic routes leading to cyclic tripeptides have always required activated precursors for cyclization to occur.[2,5,10] With this in mind, one might expect the existence of a low-energy structure accessible to linear TIP[Ψ] in which the terminal amino and carboxyl functions are unusually close in space. Thus, a theoretical conformational analysis of TIP[Ψ] was carried out independently in order to examine possible low-energy structures accessible to the molecule. A molecular mechanics investigation of TIP[Ψ] in its deprotonated state revealed a low-energy conformer which contained geometric properties suitable for an intramolecular end-to-end cyclization reaction (Figure 5). Interestingly, this particular structure corresponded to the lowest-energy conformer in which TIP[Ψ] assumed a *cis* Tyr–Tic peptide bond. It seems fair then to assume that the conformationally correct precursor for TIP[Ψ] cyclization is already highly populated in solution.

Conformational Analysis of *N*-[[(3′*S*)-Tetrahydroisoquinolinyl]-methyl]-Phe-Tyr Diketopiperazine. The proton spectral assignments of *N*-(Mti)-Phe-Tyr diketopiperazine at pH 3.5 are shown in Table 1. Corresponding calculated side chain rotamer populations and diagnostic $J_{\alpha\beta}$ coupling constants[25] are given in Table 2. Several features of these data are noteworthy. First and foremost is the striking consistency among chemical shifts and coupling constant values determined for the *N*-(Mti)-Phe-Tyr diketopiperazine in the three solvent systems used. This strongly suggests that the conformation or the conformational equilibrium is unaltered by variations in solvent conditions. A number of protons are found to resonate at chemical shifts atypical of a flexible peptide.[19] Specifically, the β and 2,6 protons of Tyr were shifted upfield from their normal values, indicating that these protons are situated in an area above or below the face of an aromatic ring.[19,26] Conversely, local fields outside the confines of an aromatic ring were likely responsible for the lower field chemical shift displacement observed for the Mti methylene protons.[19,26] Finally, measurement of the Tyr $J_{NH-C\alpha H}$ coupling constant consistently gave a value around 3.5 Hz which, within experimental error, translates to a torsion angle ϕ_1 between 6° and 10°.[27] The diketopiperazine ring thus exhibits a slightly out

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Table 1. Chemical Shifts for *N*-(Mti)-Phe-Tyr Diketopiperazine in (a) H_2O[a] at 21 °C, (b) DMSO[b] at 21 °C, (c) DMSO/D_2O[b] (80:20) at 21 °C, and (d) DMSO/D_2O[b] (80:20) at −5 °C

residue	HN, ppm	Hα, ppm	Hβ, ppm	other, ppm
Tyr				
a	8.18	4.21	1.40, 2.56	2,6H, 6.96; 3,5H, 6.88
b	8.12	3.97	1.65, 2.30	2,6H, 6.82; 3,5H, 6.75
c		3.95	1.50, 2.23	2,6H, 6.81; 3,5H, 6.73
d		3.94	1.27, 2.14	2,6H, 6.78; 3,5H, 6.72
Mti				
a		3.94		4,4'H, 2.99, 3.14; 1,1'H, 4.39
				5H, 7.29; 9H, 7.23; c,dH, 3.39, 4.48
b		3.95		4,4'H, 2.92, 3.04; 1,1'H, 4.33
				5H, 7.27; 8H, 7.30; c,dH, 3.27, 4.39
c		3.82		4,4'H, 2.87, 3.02; 1,1'H, 4.29
				5H, 7.26; 8H, 7.32; c,dH, 3.25, 4.34
d		3.86		4,4'H, 2.77, 3.01; 1,1'H, 4.30
				5H, 7.26; 8H, 7.32; c,dH, 3.32, 4.38
Phe				
a		4.44	2.77, 3.02	2,6H, 7.15; 3,5H, 7.46
		4.35	2.76, 2.94	2,6H, 16; 3,5H, 7.38
		4.33	2.71, 2.97	2,6H, 7.11; 3,5H, 7.40
		4.38	2.77, 3.01	2,6H, 7.12; 3,5H, 7.41

[a] Spectral reference: H_2O, 4.8 ppm. [b] Spectral reference: DMSO, 2.57 ppm. Protons cH and dH refer to the exocyclic methylene protons in the Mti substituent.

Table 2. $^3J_{\alpha\beta}$ Coupling Constants from NMR Spectra and Corresponding Side Chain Rotamer Populations[a] for *N*-(Mti)-Phe-Tyr Diketopiperazine in (a) D_2O, (b) DMSO, and (c) DMSO/D_2O (80:20) at 21 °C

residue	$^3J_{\alpha\beta}$, Hz[b]	% g⁻ and/or t[c]	% g⁺
Tyr			
a	4.25, 8.65	15, 55	30
b	4.67, 7.54	19, 45	36
c	4.95, 7.81	21, 47	32
Mti			
a	4.97, —	21 g⁻	79
b	4.00, —	13 g⁻	87
c	4.77, —	20 g⁻	80
Phe			
a	4.55, 5.28	18, 24	58
b	4.19, 5.48	14, 26	60
c	4.25, 5.20	15, 24	61

[a] Populations calculated according to Pachler.[40] [b] — indicates $J_{\alpha\beta} <$ 3 Hz. [c] Prochirality of β protons not assigned.

of plane puckering probably due to steric factors introduced by its bulky aromatic substituents.[28]

Side chain rotamer populations determined from the $J_{\alpha\beta}$ coupling constants[25] (Table 2) also provided some interesting insight into the solution structural features of the 2-N-substituted Tyr-Phe diketopiperazine. Quite unexpectedly, one low and one intermediate value of $J_{\alpha\beta}$ was measured for the Phe and Tyr residues, indicating that the side chains of Phe and Tyr assume a g⁺ configuration in a significant population of the conformational ensemble. This is unusual since the g⁺ staggered state represents the least favorable orientation from an energetics point of view.[29] The fact that no extreme values for $J_{\alpha\beta}$ were observed, however, indicates that other side chain orientations are also possible albeit with lower probability in the case of Phe and equal probability in the case of Tyr (Table 2).

ROESY spectra acquired for the cyclic peptide revealed common dipolar connectivities for the molecule in the three solvent systems used, thus confirming that variation of the solvent conditions had little effect on the conformation or the conformational equilibrium. Among the visible ROEs, there were a number of cited interresidue dipolar contacts which served to better define the molecular tertiary structure. These are defined in Table 3.

Table 3. Intensity of Interresidue ROEs (T = 21 °C) and NOEs (T = −5 °C) Observed for 2-*N*-(Mti)-Phe-Tyr Diketopierazine[a,b]

NOE	DMSO T = 21 °C	D_2O T = 21 °C	DMSO/D_2O (80:20) T = 21 °C	DMSO/D_2O (80:20) T = 5 °C
Mti-Phe $d_{\alpha\text{-}\alpha}$	w	w	w	*
Mti-Phe $d_{c,d\text{-}\alpha}$	m-w	m-s	m-s	*
Mti-Phe $d_{c,d\text{-}\beta}$	m	m-w	m	m
Mti-Phe $d_{c,d\text{-}2,6}$	m	m	m	m-s
Tyr-Phe $d_{2,6\text{-}3,5}$	m-w	w	m-w	m-w
Tyr-Phe $d_{2,6\text{-}2,6}$	*	*	m-w	*
Tyr-Phe $d_{3,5\text{-}2,6}$	*	*	m-w	*
Tyr-Phe $d_{\beta\text{-}2,6}$	w	*	w	w
Tyr-Phe $d_{\beta\text{-}3,5}$	w	*	*	*
Tyr-Phe $d_{NH\text{-}\alpha}$	w	w	*	*

[a] Weak (w), medium (m), or strong (s). [b] Asterisks indicate that a dipolar cross peak could not be assigned due to spectral overlap or solvent–proton fast exchange.

In an attempt to discover additional dipolar interactions, a NOESY spectrum was acquired for **3** in the cryogenic solvent mixture DMSO/D_2O (80:20) at −5 °C. It was necessary to lower the temperature in order to decrease the molecular tumbling rate since compounds of this size usually have correlation times which fall into the null region of normal NOESY experiments.[30,31] The optimal mixing time was determined from an NOE buildup curve of the Tyr 2,6 protons obtained by irradiating the Tyr 3,5 protons in a selective 1D NOESY experiment.[32] Unfortunately, no new NOEs were found although the low-temperature NOESY spectrum did display the maximum number of dipolar connectivities previously observed in the ROESY spectra acquired for *N*-(Mti)-Phe-Tyr diketopiperazine dissolved in the various solvent systems. Thus, the dramatic increase in viscosity in going from 20 to −5 °C (2.5 to 10 cP)[33] had no effect on the conformation or conformational equilibrium of *N*-(Mti)-Phe-Tyr diketopiperazine.

Since the *N*-(Mti)-Phe-Tyr diketopiperazine is characterized by a rigid six-membered ring surrounded exclusively by bulky aromatic groups, it is possible that its solution conformation may contain one or more aromatic interactions which help to stabilize the molecule. Consequently, molecular modeling studies were performed on the 2-N-substituted Tyr-Phe diketopiperazine using a molecular mechanics approach in order to better define its conformation. Several conformers were generated, and these were further screened for compatibility with the above NMR data. Taken as a group, 16 structures with energies less than 2.7 kcal/mol higher than that of the lowest-energy conformer fully explained the observed ring current effects, side chain rotamer populations, and nuclear Overhauser enhancements. Molecular torsion angles and energies for the 16 conformers are tabulated in Table 4. For reference purposes, Figure 6 shows the molecular structure of *N*-(Mti)-Phe-Tyr diketopiperazine together with atomic numbering and defined torsion angles.

A particularly striking conformational property found among the 16 low-energy structures concerns the preferred geometry of interaction between the Tyr and Phe aromatic rings. In all cases where an aromatic interaction occurred between these two residues, perpendicular stacking was favored over parallel stacking. This is not surprising since theoretical calculations have shown

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Table 4. Lowest-Energy Conformations of *N*-(Mti)-Phe-Tyr Diketopiperazine[a]

no.	energy, kcal/mol	ϕ_1, deg	Ψ_1, deg	ω_1, deg	ϕ_2, deg	Ψ_2, deg	ω_2, deg	χ_{11}, deg	χ_{21}, deg	Z_1,[b] deg	Z_2, deg	Z_3, deg	Z_4, deg
1	4.34	10.7	−9.6	−5.6	18.9	−17.9	3.2	66.1	−68.0	−72.6	−179.5	57.4	151.5
2	4.89	24.6	−24.1	−1.2	25.2	−24.8	−0.7	175.1	−67.1	−74.9	−179.2	57.8	151.5
3	5.58	23.4	−22.7	−1.7	25.0	−24.6	0.0	−51.6	−65.0	−75.1	−179.5	58.0	151.3
4	5.74	10.3	−10.1	−4.2	17.6	−17.5	3.4	64.4	−166.8	−72.9	−176.1	57.8	151.2
5	5.79	9.8	−6.9	−10.1	−23.0	−20.4	3.9	64.5	−60.5	−69.2	−175.5	−48.8	−163.5
6	6.03	8.5	−8.2	−6.2	19.1	−18.9	5.0	66.2	−172.5	−70.3	−175.4	−49.3	−162.6
7	6.26	21.1	−19.4	−5.5	27.6	−26.0	1.3	−177.9	−58.7	−70.6	−175.5	−49.3	−162.6
8	6.34	11.5	−10.6	−3.1	15.4	−14.6	0.9	−171.3	64.6	−69.3	−175.2	−49.2	−162.6
9	6.48	11.9	−9.1	−5.5	16.8	−14.2	−0.3	−58.0	63.9	−69.7	−175.5	−49.1	−162.7
10	6.61	22.1	21.1	−4.8	28.6	−27.7	2.0	−49.0	−48.6	−71.6	−176.2	−49.2	−162.6
11	6.62	23.8	−23.2	−1.9	25.5	−24.6	−0.4	175.2	−177.0	−74.1	−176.2	57.0	152.0
12	6.65	23.6	−22.4	−2.0	26.2	−25.2	−0.3	175.4	176.0	−72.8	−176.4	−49.1	−162.7
13	6.75	11.6	−10.8	−3.6	16.1	−15.2	1.0	−171.1	64.3	−69.5	−177.1	55.7	153.2
14	6.78	22.9	−23.5	−1.3	24.2	−24.2	−0.1	−52.5	−168.9	−74.7	−176.0	57.6	151.5
15	6.86	12.2	−9.2	−5.9	17.5	−14.8	−0.2	−57.5	63.3	−69.7	−177.4	55.7	153.2
16	7.00	21.4	−19.8	−4.0	25.3	−23.9	0.1	−58.5	178.6	−72.4	−176.2	−49.2	−162.7

[a] Subscript 1 assigned to Tyr; subscript 2 assigned to Phe. [b] For *Z* angle definitions, see Figure 6.

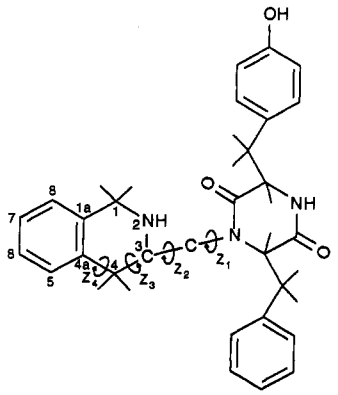

Figure 6. Structural representation of the *N*-(Mti)-Phe-Tyr diketopiperazine. Numbering of the atoms of the tetrahydroisoquinoline ring is provided for reference purposes, and torsional angles Z_1, Z_2, Z_3, and Z_4 for **3** are defined.

that a 90° interplanar dihedral angle between two interacting aromatic rings is energetically more favorable than the corresponding 0° interaction by about 1 kcal/mol.[39,35]

Within this set of lowest-energy structures, individual members exhibited considerable variation in the χ_1 angles but not in the backbone torsional angles. Furthermore, the configurations of the peptide bonds within the calculated ring structure match those suggested by NMR results, i.e. a slightly puckered out of plane geometry, as indicated by the low Tyr $J_{NH-C\alpha H}$ coupling constant (3.5 Hz). It is also interesting to note that several of the 16 low-energy structures have the Phe or Tyr side chain in a g^+ orientation. This is an encouraging result since the Tyr and Phe rotamer populations calculated from the $J_{\alpha\beta}$ coupling constants were found to be biased toward the g^+ staggered state (Table 2).

The main structural characteristic that distinguishes one low-energy conformer from another is the orientation of the Phe and Tyr side chains. Careful inspection of individual structures reveals that the entire set of NMR results is satisfied if one considers numerous combinations of Tyr and Phe rotamer states. Oddly enough though, the two individual conformations which best fit the NMR data have energies weighted toward the high end of the allowed values (structures 13 and 15). These two structures are about 2.5 kcal/mol higher in energy than the lowest-energy conformer. Visual inspection of conformers 13 and 15 reveals that the Tyr and Phe rings are oriented in a perpendicular configuration with the rings interacting in a cogwheel fashion in the case of structure 15 and an edge–ring–face fashion in the case of conformer 13.[36] This would explain the observed upfield shift of the β and C2, -3, -5, and -6 ring protons of Tyr (Table 1), since the aromatic ring current originating from the Phe residue creates a magnetic anisotropy at these protons.[28] Conversely, the Mti aromatic ring does not engage in an aromatic interaction with either Tyr or Phe. However, the exocyclic methylene protons of Mti are located within a 2.5 Å distance outside the confines of the Phe ring. This structural property would explain the dramatic upfield shift observed for these protons in the ^{1}H NMR spectrum. The most important feature common to both structures 13 and 15 which is absent in the remaining low-energy structures is a g^+ configuration of the Phe and Mti side chains. This particular combination of rotamer states is the only one which manifests an overall conformation completely compatible with the obtained NMR data. In addition to being consistent with observed ring current effects and calculated rotamer populations, conformers 13 and 15 satisfy the entire set of long-range ROEs determined for *N*-(Mti)-Phe-Tyr diketopiperazine (Table 3). These NMR signals are important in that they characterize internuclear distances up to approximately 4.5 Å. The main feature which distinguishes structure 13 from 15 is the orientation of the Tyr side chain. In the case of structure 15, Tyr adopts a g^- staggered state, while structure 13 is characterized by a *trans* Tyr configuration. Interchanging the Tyr side chain configuration between the t and g^- states does not alter the molecule's compatibility with the above-mentioned NMR results and does not produce a significant change in energy.

By comparing a different pair of conformers to structures 13 and 15 (structures 8 and 9 in Table 4) which differ from the former only in the configuration of the Mti side chain (g^- as opposed to g^+ as defined by the Z_3 angle in Figure 6), one notices many similarities. In fact, it is likely that structures 8 and 9 are also populated in the conformational ensemble since most of the NMR parameters are satisfied by these two conformers as well. Calculation of rotamer populations for the Mti side chain, however, indicates a high probability of finding Mti in a g^+ configuration (80% compared to 20% for the g^- state). On the basis of this result, one would expect structures 13 and 15 to be more populated in solution than structures 8 and 9.

Another structure that warrants attention is the lowest-energy conformer (structure 1 in Table 4). Although the backbone structure is unaltered, the Phe side chain in this conformation adopts a g^- configuration, thus forcing the Phe and Mti aromatic rings to be close to one another. Furthermore, the Mti exocyclic methylene protons are situated between the Mti and Phe aromatic rings but shifted slightly outside the ring boundaries. This property again supports the upfield shift observed for these protons in the ^{1}H NMR spectrum. Also interesting to note is the g^+ configuration of the Tyr side chain. On the basis of the $J_{\alpha\beta}$

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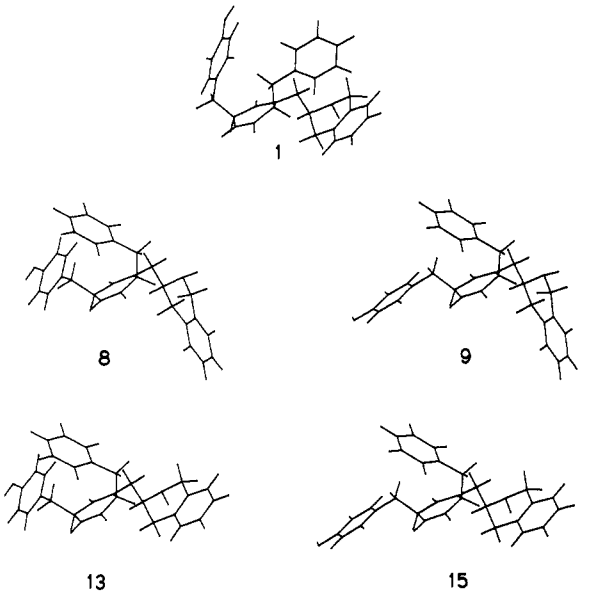

Figure 7. Low-energy conformations of compound 3. Numbering is taken from Table 4.

coupling constant, Tyr is expected to assume a g^+ staggered state in approximately 35% of the conformational ensemble. ROEs satisfied by conformer 1 include the majority of ROEs tabulated in Table 3 with the exception of the dipolar contact expected between the Tyr β and Phe aromatic ring protons. In addition, an aromatic ring current is not experienced by the Tyr β protons, as would be expected in structure 1. However, the fact that many experimental parameters are in very good agreement with structure 1 leads one to believe that structure 1 may also exist in solution.

Proceeding through the list of structures in Table 4, one finds that conformers 4 and 6 are also viable solution structures although they are expected to exist in a much lower proportion owing to their reduced compatibility with experimental data. The nine remaining low-energy conformers tabulated in Table 4 are expected to occur with a very low probability if at all. Not only are these structures inconsistent with NMR results, but they all posses a ϕ_1 torsion angle greater than 20°. If any of these nine remaining structures were significantly populated in the conformational ensemble, one would observe a larger Tyr $J_{NH-C\alpha H}$ coupling constant, i.e. 5 Hz as opposed to the 3.5 Hz determined. The low-energy structures obtained for *N*-[[(3′*S*)-tetrahydroisoquinolinyl]methyl]-Phe-Tyr diketopiperazine (structures 1, 8, 9, 13, and 15) are shown in Figure 7.

Another aspect of *N*-(Mti)-Phe-Tyr diketopiperazine which demanded attention was the likelihood of finding a low-energy conformer in which both Tyr and Phe assumed a g^+ side chain orientation. It is not unreasonable to anticipate a structure of this nature since $J_{\alpha\beta}$ coupling constants indicate a high g^+ population for both residues. Interestingly, though, conformations in which both the Tyr and Phe side chains assume a g^+ orientation were found to be sterically forbidden since in this situation the Tyr and Phe aromatic rings would necessarily occupy the same volume in three-dimensional space.

In the discussion of the conformational properties describing the 2-N-substituted diketopiperazine, as determined by NMR in conjunction with molecular modeling, focus has been primarily on the orientations of the Phe and Tyr side chains. However, there is an additional interesting property concerning the Mti substituent which surfaced during the investigations. The Z_1 torsion angle (see Figure 6) does not change among all 16 low-energy conformes listed in Table 4. Since a high degree of flexibility is expected at the junction of the Mti side chain and the diketopiperazine ring, it seems peculiar that a single value of the Z_1 angle could meet the low-energy requirements of the diketopiperazine. In fact, higher energy calculated conformers (not shown) exhibited a significantly greater range of Z_1 torsional angles. However, these were rejected on the basis of the internuclear distance measurements. NMR results consistently revealed a dipolar contact of less than 4.5 Å between the Phe α and Mti exocyclic methylene protons. Conformers within 2.7 kcal/mol of the lowest-energy structure clearly meet this criterion while structures above the 2.7 kcal/mol cutoff usually fail to satisfy the short Phe αH–Mti CH_2 distance requirement. Steric and/or aromatic interaction factors are likely responsible for the decreased molecular stability implemented by allowing Z_1 to assume many different values.

It should be mentioned that molecular modeling investigations were also carried out on cyclic TIP[Ψ]. Although several low-energy structures were obtained for this molecule, the minimum energy value exceeded that of the corresponding lowest-energy structure obtained for the 2-N-substituted diketopiperazine by 10 kcal/mol. This explains the rapid transannular attack across the nine-membered ring following TIP[Ψ] cyclization which is clearly favored from a thermodynamics point of view.

Experimental Section

General Methods. Thin layer chromatography (TLC) was performed on precoated silica gel plates 60F-254 (E. Merck) in the following solvent systems: (A) *n*-Bu-OH/AcOH/H_2O (4:1:1) and (B) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). Peptides were visualized with UV and ninhydrin. The HPLC system used for peptide purification, purity control, and kinetics experiments was a Gold (Beckman) chromatograph, consisting of a programmable solvent module and a diode array detector module 168. Reversed-phase HPLC was carried out using a gradient made up from two solvents: (A) 0.1% TFA/H_2O and (B) 0.1% TFA/acetonitrile. The analytical applications were performed on a Vydac 218-TP column (250 × 4.6 mm) with a linear gradient of 15–40% B in 30 min, at a flow rate of 1.0 mL/min. Absorption was measured at both 216 and 280 nm. Molecular weights of the compounds were determined by FAB mass spectrometry on a MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Drs. M. Evans and M. Bertrand, Department of Chemistry, University of Montreal).

Peptide Synthesis. The tripeptide TIP[Ψ] was synthesized as previously described.[18] The synthesis of Tyr(NMe)-TicΨ[CH_2-NH]Phe-OH followed a series of steps analogous to those involved in the preparation of TIP[Ψ] except that Tyr(NMe) was substituted for Tyr in the final coupling step. Preparation of the *N*-(Mti)-Phe-Tyr diketopiperazine involved dissolving 5 mg of purified TIP[Ψ] in 1000 μL of DMSO at pH 9.1 and then allowing the solution to stand for 48 h. The crude product was obtained in solid form through evaporation of the solvent. For the activated TIP[Ψ] cyclization reaction, 1 equiv of NMM (*N*-methylmorpholine) and 1 equiv of DIC were added to 4.72 mg of TIP[Ψ] in 1000 μL of DMF. The reaction mixture was allowed to stand for several hours, during which time *N*-(Mti)-Phe-Tyr diketopiperazine formation was monitored by HPLC.

Crude *N*-(Mti)-Phe-Tyr diketopiperazine was purified by reversed-phase chromatography on a semipreparative Vydac C18 column (250 × 10 mm), with a linear gradient of 15–40% acetonitrile in 0.1% TFA at a flow rate of 2.5 mL/min. The HPLC conditions for the purification of Tyr(NMe)-TicΨ[CH_2-NH]Phe-OH were identical to those used in the case of TIP[Ψ].[18] The purity of the peptides was checked by thin layer chromatography (TLC) and by analytical RP-HPLC. Molecular weights were confirmed by FAB-MS. TIP[Ψ]: HPLC k' 2.91; TLC R_f(A) 0.25, R_f(B) 0.66; FAB-MS m/e 474 (M^+). *N*-(Mti)-Phe-Tyr diketopiperazine: HPLC k' 4.78; TLC R_f(A) 0.44, R_f(B) 0.86; FAB-MS m/e 456 (M^+). H-Tyr(NMe)-TicΨ[CH_2-NH]Phe-OH: HPLC k' 2.94; TLC R_f(A) 0.61, R_f(B) 0.64; FAB-MS m/e 488 (M^+).

NMR Sample Preparation. Weighed amounts of approximately 3 mg of the purified peptides *N*-[[(3′*S*)-tetrahydroisoquinolinyl]methyl]-Phe-Tyr diketopiperazine and H-Tyr-TicΨ[CH_2-NH]Phe-OH were first dissolved in 500 μL of D_2O and then lyophilized. The dried samples were then redissolved in 700 μL of either H_2O/D_2O (6.5:0.5), DMSO-d_6, or DMSO-d_6/D_2O (80:20). Following dissolution, sample pHs were adjusted to the required value with dilute DCl and NaOD.

NMR Data Acquisition and Processing. All NMR experiments were carried out on a Varian VXR-400 S spectrometer interfaced to a Sun

3/160 computer. Sequence specific resonance assignments were made using a combination of DQF-COSY, TOCSY, ROESY, and HMQC 2D NMR experiments. The non-protonated ^{13}C resonances were assigned by examination of a 1D ^{13}C spectrum. ROESY spectra were acquired using several mixing times ranging from 100 to 400 ms. An NOE mixing time of 200 ms was employed for the low-temperature (-5 °C) NOESY experiment. Unless otherwise stated, all experiments were conducted at 21 °C. 2D FID matrices were typically 2048 by 512 points with spectral widths of 4000 Hz in each dimension. Fourier transformation of the 2D data sets was accompanied by application of cosine squared window functions in F1 and F2. In the case of aqueous samples, solvent suppression was achieved by selective irradiation during the relaxation delay.

Coupling constants were obtained directly from finely digitized 1D spectra by measuring the peak-to-peak separation of well-resolved α–β proton quartets. When necessary, spectral resolution was enhanced with the aid of a deconvolution routine provided by the Varian VNMR1 software and by Lorenzian–Gaussian apodization. In cases of severe spectral overlap, a 1D TOCSY experiment, employing a DANTE-Z pulse train for selective excitation,[37] was used to separate overlapping spin multiplets.

Molecular Modeling. All calculations were performed using the molecular modeling software SYBYL (Tripos Associates, St. Louis, MO) on a VAXstation 3500. Molecules were viewed on an Evans and Sutherland PS330 computer graphics display terminal. The standard SYBYL force field was employed for energy calculations,[38] and a dielectric constant of 78 was chosen to simulate an aqueous environment. A stepwise procedure was employed to determine low-energy conformations of both the linear tripeptide TIP[Ψ] and structure **1** using previously described methodology with minor modifications.[18,39]

For structure **3**, a similar procedure was adopted. First the diketopiperazine ring structure was constructed and energy-minimized. The side chains of Tyr and Phe and the Mti group were added, and the resulting exocyclic rotatable bonds were subjected to a systematic grid search routine using a 30° increment. Conformations obtained were grouped into low-energy families on the basis of the similarity of their dihedral angles, and the lowest-energy member of each low-energy family was extensively minimized. Torsion angles for the 16 lowest-energy structures obtained are listed in Table 4.

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