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# Conformational Mimicry. 3. Synthesis and Incorporation of 1,5-Disubstituted Tetrazole Dipeptide Analogues into Peptides with Preservation of Chiral Integrity: Bradykinin†

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New synthetic procedures for preparing 1,5-disubstituted tetrazole dipeptide analogues, which are a conformational mimic of the cis amide bond, and incorporating these analogues into longer peptides, such as bradykinin, while maintaining chiral integrity are presented. The simple addition of the organic base, quinoline, to the reaction with $PCl_5$ when generating an imidoyl chloride from the amide was effective in reducing racemization of the N-terminal amino acid residue of the protected tetrazole dipeptide to minimal levels. The resulting tetrazole dipeptide is quite sensitive to base, and the normal procedures of solid-phase synthesis for neutralization were sufficient to cause racemization of the $\alpha$ carbon on the C-terminal side of the tetrazole ring. The use of Z for amino protection and benzyl ester for carboxyl protection with differential removal of the Z group by HBr/HOAc has proven a practical route to a wide variety of tetrazole dipeptides. Immediate acylation of the tetrazole dipeptide with a Boc amino acid was necessary to prevent formation of the diketopiperazine, which is favored because of the cis conformation of the amide bond surrogate. Three bradykinin analogues, [L-Pro$^2\psi$[$CN_4$]-L-Ala$^3$]-BK, [L-Ala$^6\psi$[$CN_4$]-L-Ala$^7$]-BK, and [L-Ala$^6\psi$[$CN_4$]-D-Ala$^7$]-BK, in which the peptide bond of a proline residue was replaced with the tetrazole surrogate, were prepared to illustrate the synthetic procedures. The availability of these procedures should increase the use of the tetrazole dipeptide analogue in molecular recognition studies.

## Introduction

In natural peptides, *N*-methyl amino acids may play a special role because of their proclivity for cis–trans isomerism of the amide bond. Numerous peptides with important biological activity, such as cyclosporin and didemnin, contain *N*-methyl amino acids. Proline occupies a special role among those amino acids incorporated into proteins by normal biochemical pathways, as it is the only residue which leads to an N-alkyl amide bond when incorporated into a protein. Over 10% of proline residues in protein crystal structures have been found with cis amide bonds. Cis–trans isomerism of the N-alkyl amide bond involving the amino group can readily be observed[1] in the NMR of proline and *N*-methyl amino acid containing peptides. Recently, Bairaktari et al.[2] have reported that the amide bond between an Ile and Lys in the linear peptide, bombolitin, has the cis conformation when bound to phospholipid micelles. In the case of angiotensin and thyroliberin (TRH) analogues, the quantity of cis isomer in aqueous solution was correlated[3] with the biological activity. This suggested that the cis isomer might be the one bound to the receptor and responsible for the observed biological activity. Brandl and Deber[4] have proposed that cis–trans isomerism of proline residues might play a role in transduction of transmembrane proteins. Others have suggested that this interconversion may be responsible for many of the slow kinetic events seen in enzyme reactions and protein folding. The immunosuppressive drugs, cyclosporin A and FK506, used to prevent organ rejection during surgical transplantation, both inhibit different enzymes which function as peptidyl-proline cis–trans isomerases, or rotamases.[5,6] Synthetic replacement of the amide bond with a surrogate which would lock the conformation either cis or trans would address its role in molecular recognition. While the cis olefinic group might appear an ideal cis amide bond mimic, isomerization of the cis $\beta,\gamma$-unsaturated carbonyl system to the more stable $\alpha,\beta$-unsaturated system has prevented this approach.[7] Marshall et al.[8] proposed the 1,5-disubstituted tetrazole ring system, $\Psi$[$CN_4$], as a peptide bond surrogate for the cis amide bond in order to lock the dipeptide analogue into a geometry corresponding to the cis isomer. Conformational analysis has shown that peptides containing the tetrazole cis amide bond surrogate can assume most of the conformations available to the parent compound.[9,10] Initial synthetic procedures resulted in racemization of one or both chiral centers adjacent to the tetrazole ring.[8,11] The difficulties in the preparation and use of this dipeptide analogue as reported by Yu and Johnson[11] have been overcome. This paper reports new synthetic procedures which allow the preparation of peptide analogues from dipeptides and incorporation into longer peptides with maintenance of chiral integrity. Because of the high percentage of proline residues in the nonapeptide bradykinin (BK), analogues of this compound were chosen to illustrate the synthetic procedure. Preliminary results for the incorporation of tetrazole dipeptide analogues into biologically active peptides, such as thyroliberin (TRH)[8], somatostatin,[12] and bradykinin[13] have been reported. A

† The first two papers in this series are refs 9 and 10.
‡ Institute of Organic Chemistry.

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**Table I. Comparison of Synthetic Methods for Tetrazole Dipeptides**

| peptide | procedure 1[a,b] yield (%) | procedure 1[a,b] $[\alpha]_D$ | procedure 2[a,b] yield (%) | procedure 2[a,b] $[\alpha]_D$ |
|---|---|---|---|---|
| Z-ambo-ala$\psi$[$CN_4$]-Ala-OBzl (**5**) | 42 | - | na | na |
| Z-L-Ala$\psi$[$CN_4$]-L-Ala-OBzl (**5a**) | 20 | -48.8 | 25 | -51.2 |
| Z-D-Ala$\psi$[$CN_4$]-L-Ala-OBzl (**5b**) | 18 | -56.5 | - | - |
| Z-ambo-Phe$\psi$[$CN_4$]-Ala-OBzl (**6**) | 49 | - | na | na |
| Z-L-Phe$\psi$[$CN_4$]-L-Ala-OBzl (**6a**) | 23 | -60.8 | 62 | -61.2 |
| Z-D-Phe$\psi$[$CN_4$]-L-Ala-OBzl (**6b**) | 18 | -43.0 | 59 | -42.7 |
| Z-ambo-Pro$\Psi$[$CN_4$]-Ala-OBzl (**7**) | 84 | - | na | na |
| Z-L-Pro$\Psi$[$CN_4$]-L-Ala-OBzl (**7a**) | 36 | -15.5 | 68 | -15.9 |
| Z-D-Pro$\Psi$[$CN_4$]-Ala-OBzl (**7b**) | 40 | -76.0 | - | - |
| Z-ambo-Phe$\Psi$[$CN_4$]-Leu-OBzl (**8**) | 78 | - | na | na |
| Z-L-Phe$\Psi$[$CN_4$]-L-Leu-OBzl (**8a**) | 34 | -52.1 | 52 | -53.9 |
| Z-D-Phe$\Psi$[$CN_4$]-L-Leu-OBzl (**8b**) | 30 | -33.3 | - | - |

[a] Procedure 1: (a) $PCl_5/CHCl_3$, (b) $HN_3$. Procedure 2: (a) $PCl_5$/quinoline (1:2)/$CHCl_3$, (b) $HN_3$. [b] na = not applicable; - = not determined.

**Table II. Comparison of Epimerization for Tetrazole Dipeptides**

| peptide | $PCl_5$ alone L-L $R_T$ | L-L % | D-L $R_T$ | D-L % | with quinoline L-L % | with quinoline D-L % |
|---|---|---|---|---|---|---|
| Z-Pro$\Psi$[$CN_4$]-Ala-OBzl | 12.9 | 49 | 13.4 | 51 | 96 | 4 |
| Z-Phe$\Psi$[$CN_4$]-Ala-OBzl | 18.3 | 53 | 18.6 | 47 | 97 | 3 |
| Z-Ala$\Psi$[$CN_4$]-Ala-OBzl | 11.4 | 42 | 12.0 | 58 | 95 | 5 |

tetrazole analogue of deaminooxytocin was prepared by Lebl et al.[14] in which Leu$\Psi$[$CN_4$]-Gly-$NH_2$ was synthesized by a different synthetic route in which the performed Z-Leu-tetrazole was alkylated with methyl bromoacetate.[15]

## Results and Discussion

The conversion of the protected dipeptide into a protected tetrazole dipeptide with retention of the chiral integrity of the starting material requires special experimental conditions. In our initial experiments[8] and in those reported by Yu and Johnson,[11] racemization of one or both chiral centers was observed. The use of $PCl_5/HN_3$ to convert the amide bond to the tetrazole via the imidoyl chloride leads to racemization of the amino acid N-terminal to the tetrazole ring supposedly by the mechanism proposed by Yu and Johnson.[11] The simple addition of the organic base, quinoline, to the reaction with $PCl_5$ when generating an imidoyl chloride from the amide (Scheme I) as first suggested by Hirai et al.[16] was effective in preventing racemization of the N-terminal amino acid residue of the protected tetrazole dipeptide (Table I). Only small, but still significant, amounts of the undesirable epimer were obtained when the quinoline procedure was used as shown for three examples in Table II. In an effort to optimize the yields and minimize epimerization, several bases besides quinoline were investigated for one dipeptide as shown in Table III. Bases with $pK_a$'s varying between 4.9 and 7.4 were effective in suppressing epimerization (with the unexplained exception of (dimethylamino)-pyridine), but only the use of collidine was slightly superior

**Scheme I. Possible Epimerization Pathways**

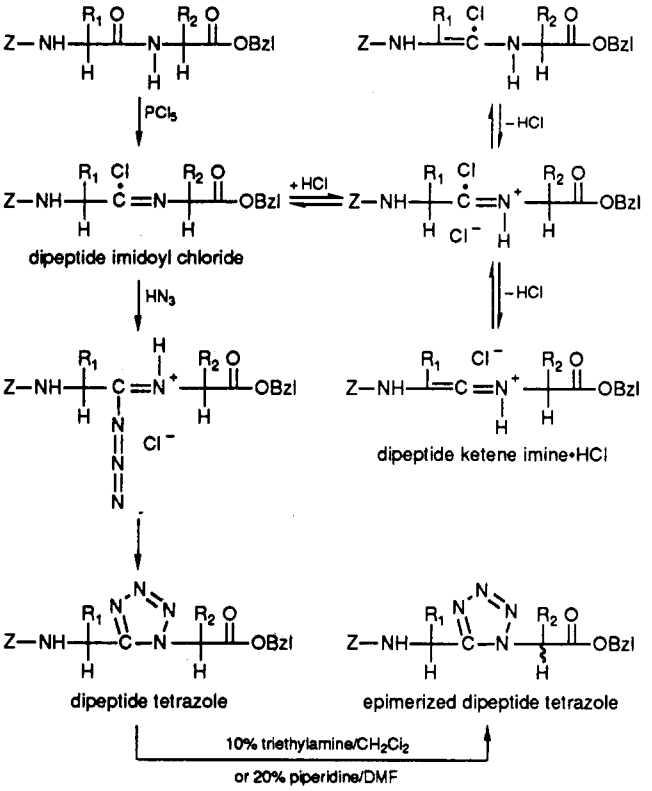


**Table III. Effect of Base on Epimerization and Yield of Z-Pro$\Psi$[$CN_4$]-Ala-OBzl**

| base | $pK_a$[a] | L-L (%) | D-L (%) | overall yield (%) |
|---|---|---|---|---|
| quinoline | 4.9 | 96.5 | 3.5 | 68 |
| triethylamine | 10.72 | no product | | |
| pyridine | 5.25 | 96.5 | 3.5 | 37 |
| (dimethylamino)pyridine | 6.09 | no product | | |
| collidine | 7.43 | 95.4 | 4.6 | 80 |
| quinaldine | 5.83 | 95.5 | 4.5 | 51 |
| 2,4-lutidine | 6.99 | 92.2 | 7.8 | 9.5 |
| *N*,*N*-dimethylaniline | 5.15 | 100 | - | 8.1 |

[a] From Lange's Handbook of Chemistry.

in yield to that obtained with quinoline.

Yu and Johnson[11] reported that epimerization did not occur at the stage of the imidoyl chloride. Addition of quinoline after formation of the imidoyl chloride and prior to the addition of hydrogen azide failed, however, to suppress epimerization (data not shown). This implied that either the complex of quinoline with $PCl_5$ had altered the mechanism of the reaction with hydrogen azide or that the base was suppressing epimerization at the imidoyl chloride stage. A possible role of a complex between hydrogen azide and the amine with enhanced reactivity as recently reported by Saito et al.[17] was also ruled out by this observation. We decided to reinvestigate epimerization during the preparation of the imidoyl chloride. Using the conditions as described by Yu and Johnson[11] and the dipeptide Z-Pro-Phe-OBzl, epimerization was observed with formation of Z-D-Pro-Phe-OBzl after the imidoyl chloride was quenched with water. To further substantiate this observation, Z-Pro-Leu-OMe, the peptide reported by Yu and Johnson[11] to be recovered unchanged after the addition of water to the imidoyl chloride, was prepared along with a standard of Z-D-Pro-Leu-OMe for comparison.

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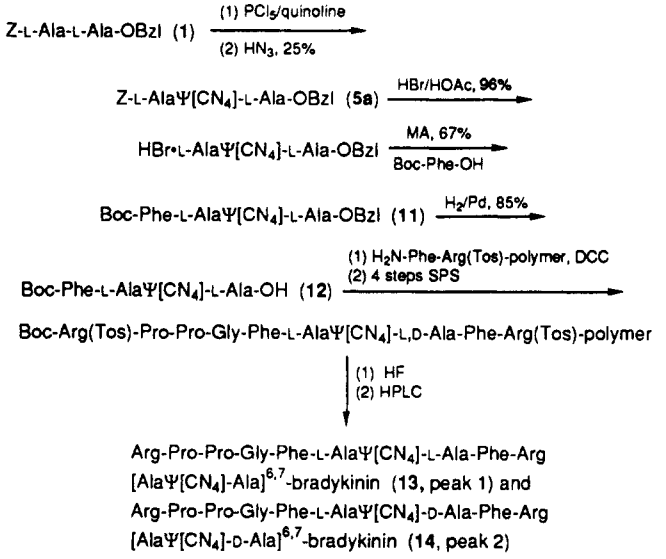
Treatment of Z-Pro-Leu-OMe with $PCl_5$ in dry benzene for 1 h followed by the addition of water gave almost equivalent concentrations of the two epimers as revealed by HPLC in a solvent system selected to separate the two. These results suggest that epimerization occurs during the preparation of the imidoyl chloride and that the more elaborate mechanisms for epimerization of the N-terminal $\alpha$-proton as suggested by Yu and Johnson[11] are not necessary. One may postulate that reversible elimination of the $\alpha$-proton of the N-terminal residue, which is more acidic as the imidoyl chloride·HCl, to give the eneamine, or ketene imine, may be responsible for epimerization which could be buffered by the presence of an organic base of appropriate $pK_a$ (Scheme I). Further studies to elucidate the detailed mechanism of epimerization of the imidoyl chloride and the role of quinoline and other organic bases (Table III) are necessary, however, before any firm conclusions can be drawn.

The resulting tetrazole dipeptide is quite sensitive to base, however, and special protecting schemes had to be invoked in order to maintain its chiral integrity during peptide synthesis. In fact, the normal procedures of solid-phase synthesis for neutralization (10% triethylamine in methylene chloride) or protecting-group removal in the case of FMOC protection (20% piperidine in DMF) were sufficient to cause racemization of the $\alpha$ carbon on the C-terminal side of the tetrazole ring (data not shown). This lability to base required the use of acidolytic protecting groups. The use of Z for amino protection and benzyl ester for carboxyl protection with differential removal of the Z group by HBr/HOAc has proven a practical route to a wide variety of tetrazole dipeptides (Zabrocki et al., unpublished results). Immediate acylation of the tetrazole dipeptide with a Boc amino acid was necessary to prevent formation of the diketopiperazine, which is favored because of the cis conformation of the amide bond surrogate.

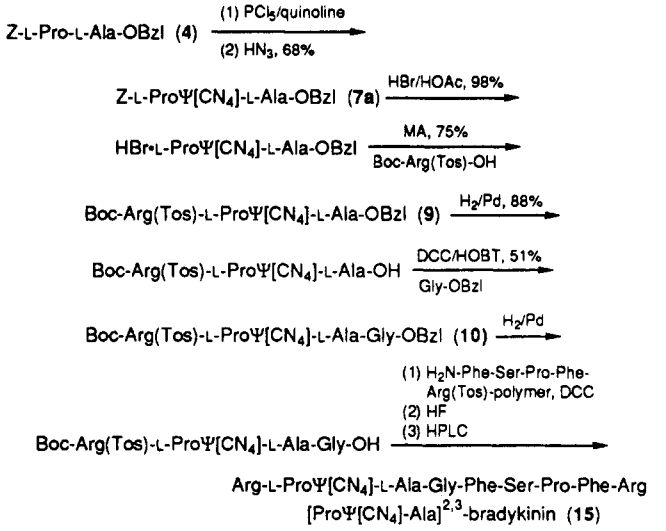
The normal route (procedure 1) to an epimerized product with subsequent separation of two diastereomeric products is compared (Table I) with the $PCl_5$/quinoline method (procedure 2), which preserves the chiral integrity of the starting material for four dipeptides. Yields of the desired tetrazole analogue are improved with procedure 2 as compared with the more laborious procedure 1. Yu and Johnson[11] reported no tetrazole formation when the N-terminal amino acid was either alanine or glycine. While the yields with glycine peptides are exceptionally low, alanine-containing peptides can be converted to tetrazole peptides without difficulty. As examples of the preparation of tetrazole dipeptide analogues and their incorporation into peptides, we report the synthesis of Z-L-Ala$\psi$-[$CN_4$]-L-Ala-OBzl in 25% yield and Z-L-Pro$\psi$[$CN_4$]-L-Ala-OBzl in 68% yield. Single crystals of Z-Pro$\Psi$-[$CN_4$]-Ala-OBzl were grown from an ethyl acetate/petroleum ether mixture by slow evaporation, and the structure was solved by direct methods[10] which showed that the chiral centers of the Pro and Ala $\alpha$-carbons had identical chirality. The most remarkable feature of this structure is the similarity of the tetrazole ring system to that observed[9] in the diketopiperazine, *c*-[L-Phe$\Psi$[$CN_4$]-L-Ala], and the linear tripeptide, Pro-Leu$\Psi$[$CN_4$]-Gly-$NH_2$.[15]

**Synthesis of [L-Ala$^6\psi$[$CN_4$]-L-Ala$^7$]-BK and [L-Ala$^6\psi$[$CN_4$]-D-Ala$^7$]-BK** (Scheme II). For use of Z-L-Ala$\psi$[$CN_4$]-L-Ala-OBzl as an analogue of Ser$^6$-Pro$^7$ and incorporation at position 6-7 of bradykinin, the Z group of Z-L-Ala$\psi$[$CN_4$]-L-Ala-OBzl was removed with HBr/AcOH and Boc-Phe coupled using isobutyl chloroformate to give Boc-Phe-L-Ala$\psi$[$CN_4$]-L-Ala-OBzl. Hydrogenolysis gave the tripeptide corresponding to Phe$^5$-Ser$^6$-Pro$^7$ of the

**Scheme II**




**Scheme III**




[Ala$^{6,7}$]-BK analogue. This was incorporated by solid-phase synthesis to give both [L-Ala$^6\psi$[$CN_4$]-L-Ala$^7$]-BK and [L-Ala$^6\psi$[$CN_4$]-D-Ala$^7$]-BK after HF and purification. Epimerization of the Z-L-Ala$\psi$[$CN_4$]-L-Ala-OBzl (**5a**) to give an equimolar mixture of Z-L-Ala$\psi$[$CN_4$]-L-Ala-OBzl and Z-L-Ala$\psi$[$CN_4$]-D-Ala-OBzl occurs (see Experimental Section) on prolonged exposure (>3 h) to 10% triethylamine in methylene chloride (used for neutralization of the amine during solid-phase peptide synthesis) and presumably occurred during the subsequent elongation of the bradykinin analogue by solid-phase synthesis. Basic conditions used for removal of FMOC groups (20% piperidine in DMF) were even more effective (>20 min exposure, see Experimental Section) in epimerizing the C-terminal amino acid of the dipeptide unit and argues against the use of FMOC chemistry with tetrazole dipeptides.

**Synthesis of [L-Pro$^2\psi$[$CN_4$]-L-Ala$^3$]-BK** (Scheme III). Z-L-Pro$\psi$[$CN_4$]-L-Ala-OBzl as an analogue of Pro$^2$-Pro$^3$ was incorporated at position 2-3 of bradykinin. Boc-Arg-(Tos)-Pro$\psi$[$CN_4$]-Ala-Gly-OBzl was prepared by removal of the Z group of Z-L-Pro$\psi$[$CN_4$]-L-Ala-OBzl with HBr/AcOH and Boc-Arg(Tos) coupled using isobutyl chloroformate, followed by hydrogenolysis of the C-terminal benzyl ester and coupling with Gly-OBzl using DCC/HOBT. After hydrogenolysis, the tetrapeptide was cou-

Table IV. Biological Activity of Bradykinin Analogues

| | % bradykinin rat uterus | $IC_{50}$ binding assay |
|---|---|---|
| bradykinin | 100 | $1.8 \times 10^{-10}$ |
| [L-Ala$^6\psi$[CN$_4$]-L-Ala$^7$]-BK | <0.1 | $3 \times 10^{-6}$ |
| [L-Ala$^6\psi$[CN$_4$]-D-Ala$^7$]-BK | <0.1 | inactive |
| [L-Pro$^2\psi$[CN$_4$]-L-Ala$^3$]-BK | <0.1 | $3 \times 10^{-6}$ |
| [Ala$^3$]-BK | 100[17,18] | - |
| [MeA$^7$]-BK[a] | 67[19] | - |

[a] MeA = $\alpha$-methylalanine or $\alpha$-aminoisobutyric acid (Aib).

pled to Phe-Ser-Pro-Phe-Arg(Tos)-polymer. Only one product, [L-Pro$^2\psi$[CN$_4$]-L-Ala$^3$]-BK, was found after HF cleavage and purification by HPLC.

**Biological Activity of BK Analogues.** The three BK analogues were assayed on isolated rat uterus by the protocol described by Marshall et al.[18] for angiotensin II and in binding assays to bovine brain membranes as shown in Table IV. The lack of activity for the three analogues implies that the cis conformer of either Pro$^2$ or Pro$^7$ is not recognized at the bradykinin B2 receptor. An alternative explanation would be that the cis conformer of bradykinin is essential for recognition with the cis amide bond playing a key role, perhaps as a hydrogen-bond acceptor. The tetrazole modification would preclude interaction with the receptor because of increased bulk. The fact that both [Ala$^3$]-BK[19,20] and [MeA$^7$]-BK[21] (MeA = $\alpha$-methylalanine or $\alpha$-aminoisobutyric acid (Aib)) have substantial activity would argue that an *N*-methyl amino acid residue and its accompanying propensity for cis–trans isomerism in either position 3 or 7 is not essential for recognition. The likelihood of the cis amide conformer in these two analogues is, therefore, sufficiently low that the lack of biological activity obtained with the tetrazole analogues is not surprising. Receptor recognition of the trans isomer of proline at both positions 3 and 7 is consistent with the data. London et al.[22] have suggested that the *trans*-Ser$^6$-Pro$^7$ amide bond conformer is the one recognized at the receptor based a correlation between the enhanced cis conformer content of [Gly$^6$]-BK and its lower potency.

These results demonstrate that the tetrazole surrogate for the cis amide bond can be prepared from protected dipeptides with retention of the starting configuration of the amino acids by use of the $PCl_5$/quinoline procedure. Use of acidolytic protecting groups allows extension of the dipeptide at the N-terminus with Boc-amino acids followed by chain extension at the C-terminus without epimerization of the amino acid residue adjacent to the tetrazole ring if basic conditions are avoided. With appropriate selection of sidechain protecting groups, this approach should be applicable to most dipeptides, with the possible exception of those containing glycine where side reactions reduce the synthetic yields. An alternative route[15] to tetrazoles with glycine as the C-terminal amino acid has been demonstrated in the preparation of Leu$\Psi$[CN$_4$]-Gly-NH$_2$ for use in an oxytocin analogue by Lebl et al.[14] Tetrazole modifications of amide bonds have been used in an HIV protease substrate,[23] TRH analogues,[8] and enkephalin analogues (Zabrocki et al., unpublished results), as well as a cyclic hexapeptide analogue of somatostatin.[12] The availability of this synthetic route to chirally pure tetrazoles and methods for incorporation into peptides should increase the use of this conformational mimic of the cis-amide bond in studies of molecular recognition.

## Experimental Section

**General Methods.** Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured in a 1-dm cell on a Perkin-Elmer polarimeter (Model 271) at 589 nm (Na D line). Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). $^1$H NMR spectra were recorded on a Varian XL 300 spectrometer at 300 MHz. Splitting multiplicities are given as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) in $CDCl_3$ or DMSO-$d_6$; *J* values are given in Hz. $^{13}$C NMR spectroscopy was performed on the same spectrometer at 75 MHz. When either $CDCl_3$ or DMSO-$d_6$ was used as solvent, they also served as internal standards at 77.0 or 39.5 ppm, respectively. FAB mass spectra were recorded on a Finnigan 3300 spectrometer equipped with a capillaritron gas gun from PHRASOR Scientific (Duarte, CA). For TLC, 250-nm silica gel GF precoated uniplates (Analtech) were used with the following solvent systems: A = dichloromethane/acetone (3:1); B = dichloromethane/methanol (10:1); C = chloroform/methanol/$CH_3COOH$ (9:1:0.5); D = dichloromethane/methanol/$H_2O$ (14:6:1); E = dichloromethane/acetone (7:1); F = hexane/ethyl acetate (1:1); G = hexane/ethyl acetate (3:1); H = hexane/ethyl acetate (4:1); I = benzene/ethyl acetate (20:1). TLC plates were developed with either iodine or chlorine followed by starch/KI spray. For flash chromatography, silica gel 60 (Merck) was used with the solvent system given in the text. HPLC was performed on a Beckman 110A instrument using an 8SI 10-$\mu$m Radial-PAK cartridge and (a) ethyl acetate/hexane (1:1) or (b) ethyl acetate/hexane (1:2), at a flow rate of 1 mL/min, or (c) on a Spectra-Physics instrument with an SP8800 ternary pump, using a Vydec $C_{18}$ column, 0.46 × 25 cm, particle size 5 $\mu$m, at a flow rate of 1 mL/min and solvents (A) 0.05% trifluoroacetic acid in $H_2O$ and (B) 0.033% trifluoroacetic acid in acetonitrile/$H_2O$ (90:10).

**Preparation of the Benzyloxycarbonyl-Protected Dipeptides 1–4.** The benzyloxycarbonyl-protected dipeptides were prepared by using the mixed anhydride procedure of Anderson et al.[24] with isobutyl chloroformate.

**Z-Ala-Ala-OBzl (1)**: yield 96.3%; mp = 139–140 °C (lit.[25] mp = 138 °C); $[\alpha]^{25}_D$ −54.9° (*c* 1, MeOH); $^{13}$C NMR ($CDCl_3$) $\delta$ 18.2, 18.6 (Ala$\beta$C), 48.2, 50.4 (Ala$\alpha$C), 67.0, 67.2 ($CH_2$Ph), 128.00, 128.16, 128.18, 128.47, 128.53, 128.60, 135.25, 136.18 (Z and OBzl Ph), 155.89 (Z C=O), 171.79, 172.49 (Ala C=O). Anal. Calcd for $C_{21}H_{24}N_2O_5$: C, 65.60; H, 6.29; N, 7.29. Found: C, 65.73; H, 6.37; N, 7.28.

**Z-Phe-Ala-OBzl (2)**: yield 93.5%; mp = 132–133 °C; $[\alpha]^{25}_D$ −24.8° (*c* 0.62, MeOH); $^{13}$C NMR ($CDCl_3$) $\delta$ 18.27 (Ala$\beta$C) 38.47 (Phe$\beta$C), 48.26 (Ala$\alpha$C), 55.99 (Phe$\alpha$C), 67.06, 67.16 (Z and OBzl $CH_2$Ph), 127.07, 128.03, 128.16, 128.19, 128.49, 128.52, 128.63, 128.67, 129.32, 135.24, 136.15 (Z, Phe and OBzl Ph), 155.90 (Z C=O), 170.31, 172.17 (Ala and Phe C=O). Anal. Calcd for $C_{27}H_{28}N_2O_5$: C, 70.41; H, 6.13; N, 6.08. Found: C, 70.08; H, 6.23; N, 5.95.

**Z-Phe-Leu-OBzl (3)**: yield 77.6%; mp = 99.5–101.5 °C; $[\alpha]^{25}_D$ −27.1° (*c* 1, MeOH); $^{13}$C NMR ($CDCl_3$) $\delta$ 21.88, 22.66 (Leu$\delta$C), 24.67 (Leu$\delta$C), 38.28 (Phe$\beta$C), 41.41 (Leu$\beta$C), 50.93 (Phe$\alpha$C), 67.04, 67.16 (Z and OBzl $CH_2$Ph), 127.03, 128.03, 128.20, 128.23, 128.43, 128.53, 128.60, 128.66, 129.36, 135.29, 136.1, 136.21 (Z, Phe and OBzl Ph), 155.80 (Z C=O), 170.50, 172.15 (Ala and Phe C=O). Anal. Calcd for $C_{30}H_{34}N_2O_5$: C, 71.69; H, 6.82; N, 5.58. Found: C, 71.56; H, 7.04; N, 5.60.

**Z-Pro-Ala-OBzl (4)**: yield 73.8%; mp = 94–95 °C; $[\alpha]^{25}_D$ −84.6° (*c* 1, MeOH); $^{13}$C NMR ($CDCl_3$) $\delta$ 18.29 (Ala$\beta$C) 24.71 (Pro$\gamma$C) 31.09 (Pro$\beta$C), 47.12 (Pro$\delta$C), 48.38 (Ala$\alpha$C), 60.46

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(Proαc), 67.13, 67.38 (Z and OBzl $CH_2Ph$), 128.11, 128.37, 128.46, 128.60, 135.34, 136.40 (Z and OBzl Ph), 155.95 (Z C=O), 170.21, 172.48 (Ala and Pro C=O). Anal. Calcd for $C_{23}H_{26}N_2O_5$: C, 67.30; H, 6.39; N, 6.83. Found: C, 67.16; H, 6.48; N, 6.79.

**General Procedure for the Preparation of the Tetrazole Dipeptides as the Mixture of Two Diastereoisomers. Procedure 1.** To a stirred suspension of protected dipeptide ester (1 mmol) in dry benzene (5 mL) was added crystalline $PCl_5$ (1 mmol). A clear solution was formed after a few min; however, the stirring was continued for 45 min, and a benzene solution of hydrazoic acid was added (3 mL). The reaction mixture was stirred at room temperature for 2 h before being diluted with benzene (30 mL) and washed with 1 N $NaHCO_3$ (3 × 15 mL), $H_2O$ (2 × 15 mL), and saturated NaCl solution (15 mL). When the dried ($Na_2SO_4$) benzene solution was evaporated and the residue was purified by flash chromatography, the tetrazole derivative was isolated as a mixture of two diastereoisomers.

**Z-ambo-Ala-ψ[$CN_4$]-Ala-OBzl (5).** Purification of the crude reaction product by flash chromatography (solvent system dichloromethane/acetone (30:1), v/v) yielded an oily product as a mixture of two tetrazole derivatives **5a** and **5b** (42%). They were separated from one another by flash chromatography (solvent system hexane/ethyl acetate (4:1), v/v).

**5a**: yield 20.5%; mp = 141–142 °C; $[\alpha]^{25}_D$ −48.8° (*c* 0.5, MeOH); TLC $R_f$(H) = 0.1; HPLC (a) $t_R$ = 7.9 min, (c) $t_R$ = 11.34 min, gradient 45–90% in 40 min; FABMS *m/e* 410 ($MH^+$), calcd for $C_{21}H_{23}O_4N_5$ 409; $^1H$ NMR ($CDCl_3$) δ 1.55 (d, *J* = 7.0, 3 H, $Ala_1$ βCH3), 1.98 (d, *J* = 7.2, 3 H, $Ala_2$ βCH3), 4.96–5.08 (dd over m, 3 H, $CH_2Ph$ and $Ala_1$αCH), 5.2 (dd, 2 H, $CH_2Ph$), 5.6 (d, *J* = 8.19, 1 H, NH), 5.71 (q, *J* = 7.2, 1 H, $Ala_2$ αCH), 7.2–7.4 (m, 10 H, Ph); $^{13}C$ NMR ($CDCl_3$) δ 16.17, 19.15 (Alaβc), 40.72, 55.66 (Alaαc), 67.28, 68.26 ($CH_2Ph$), 127.88, 128.20, 128.42, 128.60, 128.69, 134.23, 135.53 (Z and OBzl Ph), 155.66, 156.63 ($CN_4$ and Z C=O), 167.89 (Ala C=O). Anal. Calcd for $C_{21}H_{23}O_4N_5$: C, 61.60; H, 5.66; N, 17.11. Found: C, 61.77; H, 5.86; N, 17.09.

**5b**: yield 18.3%; mp = 86–87 °C; $[\alpha]^{25}_D$ −56.5° (*c* 1, MeOH); TLC $R_f$(H) = 0.17; HPLC (a) $t_R$ = 6.4 min, (c) $t_R$ = 11.93 min, gradient 45–90% in 40 min; FABMS *m/e* 410 ($MH^+$), calcd for $C_{21}H_{23}O_4N_5$ 409; $^1H$ NMR ($CDCl_3$) δ 1.68 (d, *J* = 6.7, 3 H, $Ala_1$βCH3), 1.96 (d, *J* = 7.2, 3 H, $Ala_2$βCH3), 4.90–5.11 (m, 4 H, $CH_2Ph$), 5.18 (m, 1 H, $Ala_1$αCH), 5.31 (d, *J* = 8.9, 1 H, NH), 5.52 (q, *J* = 7.3, 1 H, $Ala_2$αCH), 7.1–7.4 (m, 10 H, Ph); $^{13}C$ NMR ($CDCl_3$) δ 17.22, 19.95 (Ala βC), 41.07, 56.03 (Ala αC), 67.33, 68.01 ($CH_2Ph$), 127.94, 128.25, 128.46, 128.55, 134.37, 135.57 (Z and OBzl Ph), 155.38, 155.82 ($CN_4$ and Z C=O), 167.85 (Ala C=O). Anal. Calcd for $C_{21}H_{23}O_4N_5$: C, 61.60; H, 5.66; N, 17.11. Found: C, 61.56; H, 5.66; N, 17.12.

**Z-ambo-Phe-ψ [$CN_4$]-Ala-OBzl (6).** Purification of the crude reaction product by flash chromatography (solvent system dichloromethane/acetone (60:1), v/v) yielded (49.5%) an oily tetrazole derivative as a mixture of two diastereomers **6a** and **6b**.

**6a** was crystallized from the mixture with ethyl acetate/petroleum ether: yield 22.7%; mp = 144–145 °C; $[\alpha]^{25}_D$ −60.8° (*c* 0.5, MeOH); TLC $R_f$(G) = 0.38; HPLC (b) $t_R$ = 8.2 min, (c) $t_R$ = 18.08 min, gradient 45–90% in 40 min; FABMS *m/e* 486 ($MH^+$), calcd for $C_{27}H_{27}O_4N_5$ 485; $^1H$ NMR ($CDCl_3$) δ 1.92 (d, *J* = 7.2, 3 H, Alaβ$CH_3$), 3.28 (d, *J* = 7.3, 2 H, Pheβ$CH_2$), 4.89–5.15 (m, 5 H, Z and OBzl $CH_2$ Ph and Pheαch), 5.62 (q, *J* = 7.1, 1 H, Alaαch), 5.69 (d, *J* = 8.4, 1 H, NH), 7.04–7.35 (m, 15 H, Z, Phe, and OBzl Ph); $^{13}C$ NMR ($CDCl_2$) δ 16.44 (Alaβc), 39.23 (Pheβc), 46.40 (Pheαc), 55.75 (Alaαc), 67.35, 68.17 (Z and OBzl $CH_2Ph$), 127.24, 127.91, 128.22, 128.31, 128.54, 128.61, 128.74, 129.17, 129.24, 134.49, 135.41, 135.64 (Z, Phe, and OBzl Ph), 155.84, 155.94 ($CN_4$ and Z C=O), 167.82 (Ala C=O). Anal. Calcd for $C_{27}H_{27}O_4N_5$: C, 66.79; H, 5.61; N, 14.43. Found: C, 66.55; H, 5.80; N, 14.29.

**6b** was isolated by flash chromatography (solvent system hexane/ethyl acetate (3:1), v/v) as an oil: yield 17.9%; $[\alpha]^{25}_D$ −43.0° (*c* 0.8, MeOH); TLC $R_f$(G) = 0.41; HPLC (b) $t_R$ = 7.5 min, (c) $t_R$ = 18.38 min, gradient 45–90% in 40 min; FABMS *m/e* 486 ($MH^+$) calcd for $C_{27}H_{27}O_4N_5$ 485; $^1H$ NMR ($CDCl_3$) δ 1.51 (d, *J* = 7.3, 3 H, Alaβ$CH_3$), 3.21–3.43 (m, 2 H, Pheβ$CH_2$), 4.70 (q, *J* = 7.2, 1 H, Alaαch), 4.91 (d, 2 H, $CH_2$ Ph), 4.92–5.1 (broad d, 2 H, $CH_2$ Ph), 5.15–5.25 (m, 1 H, Pheαch), 5.68 (d, *J* = 8.9, 1 H, NH), 6.9–7.4 (m, 15 H, Z, Phe, and OBzl Ph); $^{13}C$ NMR ($CDCl_3$) δ 16.46 (Alaβc), 41.37 (Pheβc), 47.52 (Pheαc), 55.68 (Alaαc), 67.27, 68.07 ($CH_2Ph$), 127.38, 127.85, 128.11, 128.46, 128.54, 128.88, 129.15, 134.39, 135.19, 135.80 (Z, Phe, and OBzl Ph), 155.10, 155.34 ($CN_4$ and Z C=O), 167.38 (Ala C=O). Anal. Calcd for $C_{27}H_{27}O_4N_5$: C, 66.79; H, 5.61; N, 14.43. Found: C, 67.02; H, 5.89; N, 14.70.

**Z-ambo-Pro-ψ[$CN_4$]-Ala-OBzl (7).** Purification of the crude reaction product by flash chromatography (solvent system dichloromethane/acetone (30:1), v/v) yielded (84%) an oily tetrazole derivative as a mixture of two diastereomers **7a** and **7b**.

**7a** was crystallized from the mixture with ethyl acetate/petroleum ether: yield 35.8%; mp = 97–98 °C; $[\alpha]^{25}_D$ −15.5° (*c* 1, MeOH), TLC $R_f$(F) = 0.55; HPLC (a) $t_R$ = 12.9 min, (c) $t_R$ = 12.49 min, gradient 45–90% in 40 min; FABMS *m/e* 436 ($MH^+$), calcd for $C_{23}H_{25}O_4N_5$ 435; $^1H$ NMR ($CDCl_3$) δ 2.06 and 1.90–2.63 (d over m, *J* = 7.2, 7 H, Alaβ$CH_3$ and Pro β, γ$CH_2$), 3.48–3.69 (m, 2 H, Proδ$CH_2$), 4.75–5.29 (m, 5 H, $CH_2Ph$ and Proαch), 5.93 (q, *J* = 7.2, 1 H, Alaαch), 7.2–7.5 (m, 10 H, Z and OBzl Ph); $^{13}C$ NMR ($CDCl_3$) δ 16.05 (Alaβc), 24.72 (Proγc), 31.14 (Proβc), 46.45 (Proδc), 50.05 (Proαc), 55.63 (Alaαc), 67.13, 67.97 ($CH_2Ph$), 127.50, 127.90, 128.07, 128.17, 128.26, 128.48, 128.53, 134.32, 135.88 (Z and OBzl Ph), 154.81, 156.27 ($CN_4$ and Z C=O), 168.21 (Ala C=O). Anal. Calcd for $C_{23}H_{25}O_4N_5$: C, 63.47; H, 5.79; N, 16.08. Found: C, 63.32; H, 6.00; N, 16.13.

**7b** was isolated by flash chromatography (solvent system dichloromethane/acetone (30:1), v/v) as an oil: yield, 39.7%; $[\alpha]^{25}_D$ −76.0° (*c* 1.6, MeOH); TLC $R_f$(F) = 0.69; HPLC (a) $t_R$ = 8.2 min, (c) $t_R$ = 13.0 min, gradient 45–90% in 40 min; FABMS *m/e* 436 ($MH^+$), calcd for $C_{23}H_{25}O_4N_5$ 435; $^1H$ NMR ($CDCl_3$) δ 1.96 and 1.90–2.38 (d over m, *J* = 6.9, 7 H, Alaβ$CH_3$ and Proβ,γ$CH_2$), 3.50–3.70 (m, 2 H, Proδ$CH_2$), 4.9–5.28 (m, 5 H, $CH_2Ph$ and Proαch), 5.80 (q, *J* = 7.2, 1 H, Alaαch), 7.2–7.5 (m, 10 H, Z and OBzl Ph); $^{13}C$ NMR ($CDCl_3$) δ 17.55 (Alaβc), 24.43 (Proγc), 31.45 (Proβc), 46.56 (Proδc), 51.04 (Proαc), 56.27 (Alaαc), 67.29, 67.84 ($CH_2Ph$), 127.67, 127.85, 128.02, 128.25, 128.38, 128.49, 128.71, 134.74, 136.14 (Z and OBzl Ph), 155.91, 155.96 ($CN_4$ and Z C=O), 169.95 (Ala C=O). Anal. Calcd for $C_{23}H_{25}O_4N_5$: C, 63.47; H, 5.79; N, 16.08. Found: C, 63.22; H, 6.08; N, 15.82.

**Z-ambo-Phe-ψ[$CN_4$]-Leu-OBzl (8).** Purification of the crude reaction product by flash chromatography (solvent system hexane/ethyl acetate (4:1), v/v) yielded (77.8%) an oily tetrazole derivative as a mixture of two diastereoisomers **8a** and **8b**. They were separated from one another by flash chromatography (solvent system benzene/ethyl acetate (20:1), v/v).

**8a**: oil; yield 33.8%; $[\alpha]^{25}_D$ −52.1° (*c* 1.1, MeOH); TLC $R_f$(I) = 0.29; FABMS *m/e* ($MH^+$), calcd for $C_{30}H_{33}O_4N_5$ 527; $^1H$ NMR ($CDCl_3$) δ 0.85 (dd, *J* = 6.3, 5.7, 6 H, Leuδch3), 1.38–1.48 (m, 1 H, Leuγch), 2.10–2.50 (m, 2 H, Leuβ$CH_2$), 3.24 (d, *J* = 8.6, 2 H, Pheβ$CH_2$), 4.97, 5.04 (dd and s, 4 H, Z and Bzl $CH_2Ph$), 5.20 (dd, *J* = 7.4, 8.8, 1 H, Pheαch), 5.49 (dd, *J* = 4.9, 9.9, 1 H, Leuαch), 5.52 (d, *J* = 8.8, 1 H, NH), 7.0–7.4 (m, 15 H, Z, Phe, and OBzl Ph); $^{13}C$ NMR ($CDCl_3$) δ 21.32, 22.64 (Leuδc), 24.80 (Leuγc), 38.88 (Pheβc), 39.72 (Leuβc), 46.40 (Pheαc), 58.72 (Leuαc), 67.32, 68.16 ($CH_2Ph$), 127.10, 127.87, 128.18, 128.41, 128.63, 129.17, 134.35, 135.21 (Z, Phe, and OBzl Ph), 155.59, 155.95 ($CN_4$ and Z C=O), 167.50 (Leu C=O). Anal. Calcd for $C_{30}H_{33}O_4N_5$: C, 68.29; H, 6.30; N, 13.27. Found: C, 68.15; H, 6.51; N, 13.06.

**8b**: oil; yield 30.1%; $[\alpha]^{25}_D$ −33.3° (*c* 1, MeOH); TLC $R_f$(I) = 0.33; FABMS *m/e* 528 ($MH^+$), calcd for $C_{30}H_{33}O_4N_5$ 527; $^1H$ NMR ($CDCl_3$) δ 0.12 (d, *J* = 6.5, 3 H, Leuδ$CH_3$), 0.70 (d, *J* = 6.5, 3 H, Leuδ$CH_3$), 0.90–1.05 (m, 1 H, Leuγch, 1.96–2.10 (m, 1 H, Leuβ$CH_2$) 2.25–2.36 (m, 1 H, Leuβ$CH_2$), 3.28 (dd, *J* = 6.8, 13.4, 1 H, Pheβ$CH_2$), 3.45 (dd, *J* = 8.4, 13.2, 1 H, Pheβ$CH_2$), 4.81 (d, *J* = 12.3, 1 H, $CH_2Ph$), 4.98 (d, *J* = 12.3, 1 H, $CH_2Ph$), 5.05 (d, *J* = 1.5, 2 H, $CH_2Ph$), 5.19–5.28 (m, 2 H, Pheαch and Alaαch), 5.50 (d, *J* = 9.4, 1 H, NH); 7.1–7.35 (m, 15 H, Z, OBzl, and Phe Ph); $^{13}C$ NMR ($CDCl_3$) δ 21.16, 22.53 (Leuδc), 24.57 (Leuγc), 39.23 (Pheβc), 40.25 (Leuβc), 46.99 (Pheαc), 58.97 (Leuαc), 67.22, 67.94 ($CH_2Ph$), 127.20, 127.81, 127.94, 128.16, 128.65, 129.26, 134.52, 135.35, 135.90 (Z, Phe, and OBzl Ph) 155.42, 155.56 ($CN_4$ and Z C=O), 167.76 (Leu C=O). Anal. Calcd for $C_{30}H_{33}O_4N_5$: C, 68.29, H, 6.30; N, 13.27. Found: C, 68.30; H, 6.50; N, 13.14.

**General Procedure for the Preparation of Tetrazole Dipeptides with the Desired Stereochemistry. Procedure 2.** To a stirred solution of $PCl_5$ (1 mmol) in chloroform (5 mL) was added quinoline (2 mmol) at room temperature (a white precipitate formed). The mixture was stirred for 20 min before the

crystalline dipeptide (1 mmol) was added in portions with stirring at such a rate that the temperature stayed below 20 °C. After 30 min at 20 °C (a clear solution had formed), a benzene solution of hydrazoic acid (3 mL) was added. The reaction mixture was stirred at room temperature for 1 h before being evaporated. The crude residue was partitioned between ethyl acetate and water (30 mL of each). The organic layer was washed with 1 N HCl (2 × 15 mL), 1 N $NaHCO_3$ (2 × 15 mL), $H_2O$ (2 × 15 mL), and saturated NaCl solution (30 mL). When the dried ($Na_2SO_4$) ethyl acetate solution was evaporated and the residue purified by flash chromatography, the tetrazole derivative was isolated as only one stereoisomer.

**Z-L-Ala-ψ[$CN_4$]-L-Ala-OBzl (5a).** After **5a** was separated from unreacted starting material by flash chromatography (solvent system dichloromethane/acetone (30:1), v/v), it was isolated (24.9%) as white crystals: mp = 142–143 °C; $[\alpha]^{25}_D$ −51.2° (*c* 1, MeOH); TLC $R_f$(H) = 0.1; HPLC (a) $t_R$ = 7.9 min; FABMS *m/e* 410 ($MH^+$), calcd for $C_{21}H_{23}O_4N_5$ 409.

**Z-L-Phe-ψ[$CN_4$]-L-Ala-OBzl (6a).** After **6a** was separated from unreacted starting dipeptide ester by flash chromatography (solvent system dichloromethane/acetone (60:1), v/v), it was isolated (62%) as white crystals: mp = 145–146 °C; $[\alpha]^{25}_D$ −61.2° (*c* 1, MeOH); TLC $R_f$(G) = 0.38; HPLC (b) $t_R$ = 8.2 min; FABMS *m/e* 486 ($MH^+$), calcd for $C_{27}H_{27}O_4N_5$ 485.

**Z-D-Phe-ψ[$CN_4$]-L-Ala-OBzl (6b).** After **6b** was separated from unreacted starting dipeptide ester by flash chromatography (solvent system dichloromethane/acetone (60:1), v/v), it was isolated as an oil (58.7%); $[\alpha]^{25}_D$ −42.7° (*c* 0.5, MeOH); TLC $R_f$(G) 0.41; HPLC (b) $t_R$ = 7.5 min; FABMS *m/e* 486 ($MH^+$), calcd for $C_{27}H_{27}O_4N_5$ 485.

**Z-L-Pro-ψ[$CN_4$]-L-Ala-OBzl (7a).** After **7a** was separated from unreacted starting material by flash chromatography (solvent system dichloromethane/acetone (30:1), v/v), it was isolated as white crystals (68.3%): mp = 97.5–98 °C; $[\alpha]^{25}_D$ −15.9° (*c* 0.5, MeOH); TLC $R_f$(F) = 0.55; HPLC (a) $t_R$ = 12.9 min; FABMS *m/e* 436 ($MH^+$); calcd for $C_{23}H_{25}O_4N_5$ 435.

**Z-L-Phe-ψ[$CN_4$]-L-Leu-OBzl (8a).** After **8a** was separated from unreacted starting material by flash chromatography (solvent system hexane/ethyl acetate (4:1), v/v), it was isolated as an oil (51.8%); $[\alpha]^{25}_D$ −53.9° (*c* 1.5, MeOH); TLC $R_f$(I) = 0.29; FABMS *m/e* 528 ($MH^+$), calcd for $C_{30}H_{33}O_4N_5$ 527.

**Boc-Arg(Tos)-Pro-ψ[$CN_4$]-Ala-OBzl (9). (a) Removal of Z from Dipeptide 7a.** A solution of 970 mg (2 mmol) of **7a** in 1 mL of acetic acid was treated (stirring) with 5 mL of 30% solution of HBr in acetic acid. After 20 min at room temperature, the solution was poured into 50 mL of ether (precooled to −10 °C) with vigorous stirring. To the resulting precipitate was added 20 mL of petroleum ether (30–60 °C), and the mixture was allowed to stand 15 min at 0 °C and then filtered. The solid was washed two times with 1:1 ether/petroleum ether and dried in vacuo to give 748 mg (98%) of dipeptide HBr salt, a nonhygroscopic solid: mp = 152–153 °C; $[\alpha]^{25}_D$ −44.8° (*c* 1, MeOH); FABMS *m/e* 302 ($MH^+$), calcd for $C_{15}H_{19}O_2N_5$ 301.

**(b) Coupling with Boc-Arg(Tos)-OH.** A solution of 813 mg (1.9 mmol) of Boc-Arg(Tos)-OH in 5 mL of dichloromethane/DMF (1:1) was cooled to −15 °C and treated with 0.21 mL (1.9 mmol) of *N*-methylmorpholine, followed by 0.26 mL (1.9 mmol) of isobutyl chloroformate. The mixture was stirred for 10 min; then 732 mg (1.9 mmol) of solid HBr·HN-Pro-ψ[$CN_4$]-Ala-OBzl was introduced, followed by addition of 0.21 mL (1.9 mmol) of *N*-methylmorpholine at such a rate that the temperature stayed below −10 °C. After being stirred 1 h at −10 °C, the mixture was warmed up slowly to room temperature and stirred overnight. The solvents were removed in vacuo. The residue was taken up in ethyl acetate (50 mL) and washed with 1 N $NaHSO_4$ (3 × 20 mL), 1 N $NaHCO_3$ (3 × 20 mL), water (2 × 20 mL), and saturated NaCl solution (20 mL). Then the dried ($Na_2SO_4$) ethyl acetate solution was evaporated and the residue purified by flash chromatography (solvent system dichloromethane/acetone (3:1), v/v). The tripeptide derivative (1.01 g, 75%) was isolated as an amorphous powder: $[\alpha]^{25}_D$ +2.0° (*c* 1, MeOH); TLC $R_f$(A) = 0.35, $R_f$(B) = 0.48; FABMS *m/e* 712 ($MH^+$), calcd for $C_{33}H_{45}O_7N_9S$ 711; $^1H$ NMR ($CDCl_3$) δ 1.40, 1.2–1.7 (s over m, 12 H, $BocCH_3$ and Arg$\beta,\gamma CH_2$), 1.95 (d, *J* = 7.4, 3 H, Ala$\beta CH_3$), 2.36 (s, 3 H, $TosCH_3$), 3.02–3.20 (m, 2 H, Arg$\delta CH_2$), 3.60–3.78 (m, 2 H, Pro$\delta CH_2$), 4.28–4.40 (m, 1 H, Arg$\alpha$CH), 5.15–5.28 (m, 3 H, $CH_2$Ph and Pro$\alpha$CH), 5.70 (q, *J* = 7.4, 1 H, Ala$\alpha$CH), 6.40 (broad s, 2 H, NH), 7.20 (d, 2 H, TosPh), 7.25–7.38 (m, 5 H, OBzlPh), 7.74 (d, 2 H, TosPh); $^{13}C$ NMR ($CDCl_3$) δ 17.43 (Ala$\beta$C), 21.45 (tosCH3), 23.81 (Arg$\gamma$C), 25.01 (Pro$\gamma$C), 28.37 ($BocCH_3$), 29.28 (Arg$\beta$C), 30.94 (Pro$\beta$C), 40.48 (Arg$\delta$C), 47.03 (Pro$\delta$C), 50.64, 51.15 (Arg and Pro$\alpha$C), 56.69 (Ala$\alpha$C), 68.24 ($CH_2$Ph), 79.96 (BocC), 125.91, 128.19, 128.55, 128.60, 128.98, 134.49, 140.93 (Tos and Bzl Ph), 155.3, 156.21, 156.67 ($CN_4$, Boc C=O, Arg$\delta$C), 168.07, 170.93 (Arg and Ala C=O). Anal. Calcd for $C_{33}H_{45}O_7N_9S$: C, 55.68; H, 6.37; N, 17.71; S, 4.50. Found: C, 55.26; H, 6.74; N, 17.30; S, 4.59.

**Boc-Arg(Tos)-Pro-ψ[$CH_4$]-Ala-Gly-OBzl (10). (a) Hydrogenolysis of Tripeptide 9 Benzyl Ester.** A solution of 1.01 g (1.4 mmol) of tripeptide benzyl ester **9** in 20 mL of ethanol and a few drops of acetic acid was hydrogenated overnight in the presence of 250 mg 10% Pd/C. The filtered solution was evaporated and the residue taken up in a small amount of ethyl acetate and sufficient 1 N $NaHCO_3$ solution (1:9). The aqueous phase was acidified with solid sodium bisulfate to pH = 2.5 and the chromatographically pure (TLC) tripeptide acid was extracted with ethyl acetate (3 × 30 mL). When the dried ($Na_2SO_4$) ethyl acetate solution was evaporated, the product (766 mg, 88%) was isolated as a glassy powder: $[\alpha]^{25}_D$ −7.9° (*c* 1, MeOH); TLC $R_f$(C) = 0.54, $R_f$(D) = 0.88; FABMS *m/e* 622 ($MH^+$), calcd for $C_{26}H_{39}O_7N_9S$ 621; $^1H$ NMR ($CDCl_3$) δ 1.41, 1.40–1.60 (s over m, 11 H, Boc $CH_3$ and Arg$\gamma CH_2$), 1.95, 1.8–2.0 (d over m, *J* = 7.3(d), 5 H, Ala$\beta CH_3$ and Arg$\beta CH_2$), 2.14–2.32 (m, 4 H, Pro$\beta,\gamma CH_2$), 2.38 (s, 3 H, $TosCH_3$), 2.9–3.1 (m, 2 H, Arg$\delta CH_2$), 3.6–3.8 (m, 4 H*, Pro$\delta CH_2$), 4.22–4.32 (m, 1 H, Arg$\alpha$CH), 5.16–5.24 (m, 1 H, Pro$\alpha$CH), 5.72–5.84 (m, 2 H*, Ala$\alpha$CH), 7.14–7.40 (broad m, 6 H*, TosPh and COOH), 7.67 (d, *J* = 7.83, 2 H, TosPh) (*presumed impurity in sample); $^{13}C$ NMR ($CDCl_3$) δ 17.82 (Ala$\beta$C), 21.58 (Tos $CH_3$), 24.53 (Arg$\gamma$C), 25.11 (Pro$\gamma$C), 28.48 ($BocCH_3$), 28.92 (Arg$\beta$C), 30.61 (Pro$\beta$C), 40.72 (Arg$\delta$C), 47.26 (Pro$\delta$C), 49.94, 51.89 (Arg and Pro $\alpha$C), 56.16 (Ala$\alpha$C), 80.23 (BocCq), 126.03, 129.28 (TosPh), 155.64, 156.21, 156.60 ($CN_4$, Boc C=O, and Arg$\delta$C); 170.28, 171.13 (Arg and Ala C=O). Anal. Calcd for $C_{26}H_{39}O_7N_9S$: C, 50.23; H, 6.32; N, 20.28; S, 5.16. Found: C, 49.60; H, 6.77; N, 20.13; S, 4.05.

**(b) Coupling with Gly-OBzl.** The tripeptide acid (621 mg, 1 mmol) was activated with HOBT (135 mg, 1 mmol) and DCC (206 mg, 1 mmol) in DMF (5 mL) at 0 °C. After 30 min, a solution of glycine benzyl ester *p*-toluenesulfonate (337 mg, 1 mmol) and *N*-methylmorpholine (0.11 mL, 1 mmol) was added at 0 °C. After the solution was stirred overnight at room temperature, the dicyclohexylurea was filtered off and the solvent removed in vacuo. The residue was taken up in ethyl acetate (50 mL) and washed with 1 N $NaHSO_4$ (3 × 20 mL), 1 N $NaHCO_3$ (3 × 20 mL), water (2 × 20 mL), and saturated NaCl solution (20 mL). When the dried ($Na_2SO_4$) ethyl acetate solution was evaporated and the residue purified by flash chromatography (solvent system dichloromethane/acetone (3:1), v/v) the tetrapeptide derivative (395 mg, 51%) was isolated as a glassy powder: $[\alpha]^{25}_D$ −8.9° (*c* 1, MeOH); TLC $R_f$(A) = 0.20, $R_f$(B) = 0.54; FABMS *m/e* 769 ($MH^+$), calcd for $C_{35}H_{48}O_8N_{10}S$ 768; $^1H$ NMR ($CDCl_3$) δ 1.43 (s, 9 H, $BocCH_3$), 1.3–1.7 (m, 4 H, Arg$\beta,\gamma CH_2$), 1.8 (d, *J* = 7.0, 3 H, Ala$\beta CH_3$), 2.37, 2.08–2.42 (s over m, 7 H, $TosCH_3$ and Pro$\beta,\gamma CH_2$), 3.05–3.2 (m, 2 H, Arg$\delta CH_2$), 3.65–3.84 (m, 2 H, Pro$\delta CH_2$), 4.09–4.15 (m, 2 H, Gly$\alpha CH_2$), 4.39–4.48 (m, 1 H, Arg$\alpha$CH), 5.12 (s, 2 H, $OBzlCH_2$), 5.15–5.20 (m, 1 H, Pro$\alpha$CH), 5.50 (q, *J* = 7.2, 1 H, Ala$\alpha$CH), 6.44 (bs, 2 H, NH), 7.21 (d, 2 H, TosPh), 7.28–7.36 (m, 5 H, OBzlPh), 7.76 (d, *J* = 8.1, 2 H, TosPh), 7.87 (t, *J* = 4.88, 1 H, NH). Anal. Calcd for $C_{35}H_{48}O_8N_{10}S$: C, 54.67; H, 6.29; N, 18.22; S, 4.17. Found: C, 54.17; H, 6.61; N, 17.71; S, 4.58.

**Boc-Phe-Ala-ψ[$CN_4$]-Ala-OBzl (11). (a) Removal of Z from Dipeptide 5a.** A solution of 586 mg (1.43 mmol) of **5a** in 1 mL of acetic acid was treated (stirring) with 3.5 mL of 30% solution of HBr in acetic acid. After 20 min at room temperature, the solution was poured into 30 mL of ether (precooled to −10 °C), with vigorous stirring. The oily hydrobromide precipitated, and the upper phase was discarded. The oil was washed with ether (3 × 20 mL) and dried in vacuo over KOH to give 490 mg (96.3%) of dipeptide HBr salt as a very hygroscopic glass: FABMS *m/e* 276, calcd for $C_{13}H_{17}O_2N_5$ 275.

**(b) Coupling with Boc-Phe-OH.** Boc-Phe-OH (358 mg, 1.35 mmol) was coupled with HBr salt of $H_2N$-Ala-ψ[$CN_4$]-Ala-OBzl

(480 mg, 1.35 mmol) using isobutyl chloroformate as described above for **9**. Crystallization of crude material from ethyl acetate/petroleum ether yielded 560 mg (66.6%) of white crystals: mp = 159–160 °C; $[\alpha]^{25}_D$ −26.6° (*c* 0.5, MeOH); TLC $R_f$(E) = 0.67, $R_f$(F) = 0.45; FABMS *m/e* 523 ($MH^+$), calcd for $C_{27}H_{34}O_5N_6$ 522; $^1H$ NMR ($CDCl_3$) δ 1.37 (s, 9 H, $BocCH_3$), 1.59 (d, *J* = 6.9, 3 H, $Ala_2\beta CH_3$), 1.91 (d, *J* = 7.3, 3 H, $Ala_3\beta CH_3$), 2.95 (d, *J* = 6.7, 2 H, $Phe\beta CH_2$), 4.16–4.28 (m, 1 H, $Phe\alpha CH$), 5.20 (m, 2 H, $CH_2Ph$), 5.35–5.48 (m, 1 H, $Ala_2\alpha CH$), 5.56 (q, *J* = 7.3, 1 H, $Ala_3\alpha CH$), 6.78 (d, 1 H, NH), 7.0–7.4 (m, 11 H, Phe and OBzl Ph and NH); $^{13}C$ NMR ($CDCl_3$) δ 17.47, 19.69 ($Ala\beta C$), 28.23 ($BocCH_3$), 37.66 ($Phe\beta C$), 38.77 ($Ala\alpha C$), 55.49, 56.10 (Phe and $Ala\alpha C$), 68.21 ($CH_2Ph$), 80.60 (BocCq), 126.94, 128.17, 128.57, 129.07, 134.48, 136.08 (Z and Phe Ph), 155.00, 155.52 ($CN_4$ and Boc C=O), 167.68, 170.83 (Phe and Ala C=O). Anal. Calcd for $C_{27}H_{34}O_5N_6$: C, 62.05; H, 6.56; N, 16.08. Found: C, 62.27; H, 6.63; N, 16.11.

**Boc-Phe-Ala-ψ[$CN_4$]-Ala-OH (12).** Tripeptide **11** (243 mg, 0.46 mmol) was hydrogenated with 100 mg of Pd/C as described above for **9** to yield 208 mg (84.9%) of crystalline material: mp 186–187 °C; $[\alpha]^{25}_D$ −24.2° (*c* 0.5, MeOH); TLC $R_f$(B) = 0.33; FABMS *m/e* 433, calcd for $C_{20}H_{28}O_5N_6$ 432; $^1H$ NMR (DMSO) δ 1.28 (s, 9 H, $BocCH_3$), 1.55 (d, *J* = 6.8, 3 H, $Ala_2\beta CH_2$), 1.76 (d, *J* = 7.3, 3 H, $Ala_3\beta CH_3$), 2.59–2.83 (m, 2 H, $Phe\beta CH_2$), 4.01–4.19 (m, 1 H, $Phe\alpha CH$), 5.45–5.55 (m, 1 H, $Ala_2CH$), 5.62 (q, *J* = 7.3, 1 H, $Ala_3\alpha CH$), 6.96 (d, *J* = 8.7, 1 H, NH), 7.1–7.5 (m, 5 H, PhePh), 8.75 (d, *J* = 8.6, 1 H, NH); $^{13}C$ NMR (DMSO) δ 17.29, 19.12 ($Ala\beta C$), 28.14 ($BocCH_3$), 37.10 ($Phe\beta C$), 38.05 ($Ala_2\alpha C$), 55.09, 55.69 ($Phe\alpha CH$ and $Ala_3\alpha CH$), 77.87 (BocCq), 125.97, 127.78, 129.08, 138.12 (PhePh), 155.16, 156.20 ($CN_4$ and Boc C=O), 169.70, 171.51 (Phe and Ala C=O). Anal. Calcd for $C_{20}H_{28}O_5N_6$: C, 55.54; H, 6.53; N, 19.43. Found: C, 55.71; H, 6.62; N, 19.36.

**Epimerization Test during Imidoyl Chloride Formation: (A) Reaction of the Dipeptide, Z-D-Pro-Phe-OBzl, with $PCl_5$.** A 486-mg (1-mM) portion of Z-D-Pro-Phe-OBzl in dry benzene (10 mL) was reacted with crystalline $PCl_5$ (210 mg, 1 mM). A clear solution was formed, and the reaction was stirred at rt for 1 h when it was quenched by the addition of water (10 mL) while stirring was continued for 40 min. After the addition of 50 mL of benzene, the organic layer was separated and washed with 1 N $NaHCO_3$ (3 × 15 mL), water (2 × 15 mL), and saturated salt solution (15 mL). The dried ($Na_2SO_4$) benzene solution was evaporated and the product isolated as an oil. Attempts to find an HPLC or TLC system which separated authentic Z-D-Pro-Phe-OBzl and Z-Pro-Phe-OBzl were not successful, but separation of Boc-Leu-D-Pro-Phe-OBzl and Boc-Leu-Pro-Phe-OBzl was demonstrated.

Therefore, the reaction mixture obtained above was treated with 3 mL of 36% HBr in acetic acid prior to isolation as HBr·*ambo*-Pro-Phe-OBzl. The mixed anhydride procedure was used to add Z-Leu as follows: 249 mg (1 mM) of Boc-Leu-OH·$H_2O$ was dissolved in 5 mL of DMF/$CH_2Cl_2$ (1:1) followed by addition of 111 μL of *N*-methylmorpholine. After the solution was cooled to 20 °C, 135 μL (1 mM) of isobutyl chloroformate was added and the reaction mixture was stirred for 15 min. HBr·*ambo*-Pro-Phe-OBzl was added followed by 110 μL of *N*-methylmorpholine. The reaction was stirred for 20 min at −10 °C followed by 2 h at rt. After evaporation of the solvents, the reaction mixture was dissolved in ethyl acetate (30 mL) and washed with 1 N $NaHCO_3$ (3 × 15 mL), 1 N $KHSO_4$ (3 × 15 mL), water (2 × 15 mL), and saturated NaCl solution (15 mL). When the dried ($Na_2SO_4$) solution had been evaporated, the product was isolated as an oily mixture of the two diastereoisomers, Boc-Leu-D-Pro-Phe-OBzl and Boc-Leu-Pro-Phe-OBzl as identified by TLC of authentic standards.

TLC hexane/ethyl acetate (1:1 vol): $R_f$ Boc-Leu-D-Pro-Phe-OBzl = 0.34; $R_f$ Boc-Leu-Pro-Phe-OBzl = 0.41.

**(B) Reaction of the Dipeptide, Z-Pro-Leu-OMe, with $PCl_5$.** To a stirred solution of Z-Pro-Leu-OMe (795 mg, 2.11 mM) in dry benzene (10 mL) was added crystalline $PCl_5$ (444 mg, 2.11 mM). A clear solution was formed, and the reaction was stirred at rt for 45 min when it was quenched by the addition of water (10 mL) while stirring was continued for 1 h. After the addition of 30 mL of benzene, the organic layer was separated and washed with 1 N $NaHCO_3$ (3 × 20 mL), water (2 × 20 mL), and saturated salt solution (20 mL). The dried ($Na_2SO_4$) benzene solution was evaporated and the product (727 mg) isolated as a mixture of the two diastereomers (L-L:D-L = 57:43). The relationship of the two diastereomers in the reaction product was determined using authentic samples of Z-Pro-Leu-OMe and Z-D-Pro-Leu-OMe by HPLC using a Vydac $C_{18}$ column and a gradient of 30–80% B over 40 min.

Elution times: authentic Z-Pro-Leu-OMe = 16.66 min; authentic Z-D-Pro-Leu-OMe = 17.58 min; mixture of authentic diastereomers = 16.8 and 17.2 min; reaction mixture = 17.07 and 17.47 min.

**Racemization Test for Z-Ala-ψ[$CN_4$]-Ala-OBzl (5a).** **5a** (150 mg) was dissolved in 2 mL of the 10% solution of triethylamine in methylene chloride. After being stirred 8 h at room temperature, the solution was diluted with 20 mL of methylene chloride and washed repeatedly with 1 N HCl solution (2 × 10 mL), water (2 × 10 mL), and saturated NaCl solution (10 mL) and then dried over $Na_2SO_4$. Filtration and concentration in vacuo gave the tetrazole derivative (134 mg) as a mixture of two diastereoisomers. They were separated from one another by flash chromatography (solvent system, hexane/ethyl acetate (4:1), v.v).

**Z-L-Ala-ψ[$CN_4$]-D-Ala-OBzl (5c):** 84 mg oil; $[\alpha]^{25}_D$ −53.1° (*c* 1.67, MeOH); $R_f$(H) = 0.17.

**Z-L-Ala-ψ[$CN_4$]-L-Ala-OBzl (5a):** 40.5 mg; mp = 140–141 °C; $[\alpha]^{25}_D$ −46.8° (*C* 0.78, MeOH); $R_f$(H) = 0.1.

The equilibrium state was reached in 2 h 40 min (5c:5a = 1:2) as monitored by TLC. The racemization reaction in 20% piperidine in DMF was even faster, and equilibrium was reached after 20 min.

**Solid-Phase Synthesis of Bradykinin Analogues Using Tetrazole Peptides.** The tetrazole peptides were incorporated into the bradykinin sequence using conventional solid-phase peptide synthesis. One gram of Boc-Arg(Tos)-benzyl ester resin (0.4 mmol/g) was extended by the following synthetic cycle: $CH_2Cl_2$, 3 × 2 min; 50% TFA/$CH_2Cl_2$, 5 min and 25 min; $CH_2Cl_2$, 3 × 2 min; 10% TEA/$CH_2Cl_2$, 5 min and 10 min; $CH_2Cl_2$, 3 × 2 min; coupling, 3 equiv of Boc-AA and 3 equiv of DCC in $CH_2Cl_2$; second coupling, 3 equiv of Boc-AA and 3 equiv of DCC in DMF. After the addition of Boc-Phe, the polymer was divided into two halves.

**[AlaΨ[$CN_4$]-Ala]$^{6,7}$-bradykinin (13) and [AlaΨ[$CN_4$]-D-Ala]$^{6,7}$-bradykinin (14).** Boc-Phe-Ala-ψ[$CN_4$]-Ala-OH (**12**) was then added to Phe-Arg(Tos)-polymer using DCC/HOBT in DMF. The bradykinin sequence was completed by the successive additions of Boc-Gly, Boc-Pro, Boc-Pro, and Boc-Arg(Tos) using the solid-phase protocol. Protected peptide resin (720 mg) was cleaved with 10 mL of HF/anisole (9:1) for 1 h at 0 °C to yield 150 mg of crude product. Part of the crude peptide was purified on a Vydac $C_{18}$ semipreparative column (10 × 250 mm) using the following solvents: A = $H_2O$ (0.1% TFA); B = 90% acetonitrile/$H_2O$ (0.1% TFA) with a gradient of 15–30% B in 40 min. Two compounds were isolated in the ratio of 2:1. Peak 1 was assigned to [AlaΨ[CN]-Ala]$^{6,7}$-bradykinin (**13**) and peak 2 to [AlaΨ[$CN_4$]-D-Ala]$^{6,7}$-bradykinin (**14**) based, in part, on their relative abundance and the epimerization experiments cited previously. The isolated peptides were characterized on analytical HPLC (Vydac $C_{18}$) 15–40% B in 25 min, $t_R$ (1) = 15.1 min and $t_R$ (2) = 15.9 min. The two peptides gave the same molecular ion, $MH^+$ = 1043, calcd for $C_{48}H_{70}O_9N_{18}$ 1042. Amino acid analysis. Peak 1: Arg, 2.30; Gly, 1.00; Phe, 2.03; Ala, 0.81; and Pro, 2.03. Peak 2: Arg, 2.09; Gly, 1.00; Phe, 1.98; Ala, 0.83; and Pro, 1.88.

Peak 1: $^{13}C$ NMR ($D_2O$) δ 15.85, 16.51 (Ala βC), 21.79, 23.01 (Arg γC), 23.36, 23.43 (Pro γC), 25.67 (Arg βC), 26.80, 26.99 (Pro βC), 28.05 (Arg βC), 35.53, 35.90 (Phe βC), 38.19 ($Ala^6$ αC), 39.12, 39.27 (Arg δC), 41.02 (Gly αC), 46.47, 46.75 (Pro δC), 50.09, 50.86 (Arg αC), 53.35, 54.12 (Phe αC), 55.46 ($Ala^7$ αC), 57.83, 59.31 (Pro αC), 125.93, 125.99, 127.46, 127.79, 127.84, 134.59, 134.64 (Phe Ph), 155.18, 155.22 (Arg δC), 155.55 (CN4), 166.34, 167.57, 169.55, 170.34, 170.75, 170.91, 173.28, 174.41 (C=O).

**[ProΨ[$CN_4$]-Ala]$^{2,3}$-bradykinin (15).** The peptide sequence Phe-Ser-Pro-Phe-Arg(Tos) was established on the Merrifield polymer. Boc-Arg(Tos)-Pro-ψ[$CN_4$]-Ala-Gly-OBzl (**10**) was hydrogenated to give the free acid, Boc-Arg(Tos)-Pro-ψ[$CN_4$]-Ala-Gly-OH and then coupled to the peptide polymer (150 mg) using DCC/HOBT in DMF. The peptide was then cleaved from the polymer (200 mg) with 10 mL of HF/anisole (9:1) for 1 h at 0 °C. The crude yield was 58 mg, part of which was purified on a Vydac $C_{18}$ semipreparative column using the same conditions as above

for the other bradykinin analogues. Only one compound was isolated with a $t_R$ = 15.3 min and an $MH^+$ = 1059, calcd for $C_{48}H_{70}O_{10}N_{18}$ 1058. Amino acid analysis: Arg, 2.37; Gly, 1.00; Phe, 2.37; Ser, 0.90; Ala, 0.36; and Pro, 1.41: $^{13}C$ NMR ($D_2O$) $\delta$ 15.79 (Ala $\beta$C), 21.78, 22.98 (Arg $\gamma$C), 23.03, 23.42 (Pro $\gamma$C), 25.63 (Arg $\beta$C), 26.91, 28.00 (Pro $\beta$C), 29.46 (Arg $\beta$C), 35.53, 36.03 (Phe $\beta$C), 39.15, 39.33 (Arg $\delta$C), 41.44 (Gly $\alpha$C), 46.51, 46.81 (Pro $\delta$C), 50.09, 50.19 (Arg $\alpha$C, Pro$^2$ $\alpha$C), 51.58 (Ser $\beta$C), 51.57 (Arg $\alpha$C), 53.56, 53.62 (Phe $\alpha$C), 55.91 (Ala$^3$ $\alpha$C), 59.33, 59.83 (Pro$^7$ $\alpha$C, Ser $\alpha$C), 125.99, 126.02, 127.51, 127.58, 127.94, 128.08, 134.91, 135.02 (Phe Ph), 155.54, 155.57 (Arg $\delta$C, CN4), 166.88, 168.43, 168.66, 169.21, 171.32, 171.43, 172.34, 174.05 (C=O).

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# Ab Initio Study of the Conrotatory Ring Opening of Phospha- and Azacyclobutenes. 1. Monophospha- and Monoazacyclobutenes

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The allowed conrotatory ring-opening reactions of 1,2-dihydrophosphete (1), 2,3-dihydrophosphete (2), 1,2-dihydroazete (3), and 3,4-dihydroazete (4) were examined at the HF/6-31G* and MP2/6-31G* levels. Reasonable activation barriers were obtained only with the inclusion of electron correlation; however, geometry optimization at MP2 did not significantly change the geometry from those obtained at the HF level. The opening of the dihydrophosphetes is thermoneutral or slightly endothermic, while the opening of the dihydroazetes is exothermic. The calculated activation barriers for the opening of 2 and 4 are 40.76 and 37.08 kcal mol$^{-1}$, respectively. The opening of 1 and 3 can occur via two diastereomeric pathways. Inward rotation of the heteroatom lone pair is favored for both systems; the lower barrier is 24.59 kcal mol$^{-1}$ for 1 and 29.76 kcal mol$^{-1}$ for 2. The differences in these reactions are compared and explained in terms of ring strain and orbital interactions.

The electrocyclic ring opening of cyclobutene to give 1,3-butadiene has garnered a great deal of experimental[1-4] and theoretical[5-14] interest. Orbital symmetry rules demand a thermal conrotatory ring opening.[15] The experimental activation energy[1,2] is 32.9 ± 0.5 kcal mol$^{-1}$, and the heat of reaction[3] is −11.4 kcal mol$^{-1}$. Spellmeyer and Houk[13] have surveyed this reaction at a variety of theoretical levels. Calculations at the uncorrelated level overestimate the activation barrier by about 10 kcal mol$^{-1}$, but inclusion of correlation at the MP2 level lowers the barrier so that is is only a couple of kcal mol$^{-1}$ above the experimental value. Houk and co-workers have also explored the effect of substituent groups on the activation barrier and the stereocontrol of the ring opening.[10,12,16-18]

In comparison, very little work has been published concerning the ring opening of dihydroazetes, due primarily to the instability of this system. Neiman[19] and Snyder[20] have argued, using HMO and semiempirical calculations, that azacyclobutenes should undergo thermal conrotatory ring openings to give 1-aza- and 2-aza-1,3-butadiene. Guillemin, Denis, and Lablache-Combier[21] were

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