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subunits of the dimer and that a lysine might serve as an "arm" to transfer the aldehyde equivalent as its imine between the two subsites. We note that results of biochemical characterization[4] and in vitro complementation studies[19] on isolated mutant subunits do not provide support for this hypothesis in its simplest form and that catalysis is more readily explained by a single active site oxidizing both the alcohol substrate and a structurally similar thiohemiacetal, hemiacetal, carbinolamine, or hydrate.

(19) Lee, S.-Y.; Grubmeyer, C. T. *J. Bacteriol.* **1987,** *169,* 3938–3944.

Our knowledge of aldehyde oxidations is rather poor, being based on GAPDH, which uses a thiohemiacetal, and enzymes like glucose-6-phosphate dehydrogenase that employ substrates in which the aldehyde group is present as a readily oxidized derivative. Explorations of the chemistry of HDH may reveal new paradigms for NAD-linked enzymatic oxidation.

An Unexpected Cis Peptide Bond in the Minor Conformation of a Cyclic Hexapeptide Containing Only Secondary Amide Bonds[†]

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Abstract: The conformation of the cyclic hexapeptide *cyclo*(-D-Ala[1]-Phe[2]-Val[3]-Lys(Z)[4]-Trp[5]-Phe[6]-) [VDA008], a very potent inhibitor of the bile-acid transport system in hepatocytes, was investigated in hexadeuteriodimethyl sulfoxide solution by NMR spectroscopy and restraint MD calculations. Surprisingly, this peptide exhibits two conformations (94:6) in slow exchange on the NMR time scale at room temperature. Chemical exchange between both isomers was proven by 2D-ROESY and 2D-NOESY spectra. The dominant conformation has all-trans peptide bonds forming a βII,βII' backbone conformation and preferred side chain orientations similar to that of the analogue *cyclo*(-D-Pro-Phe-Thr-Lys(Z)-Trp-Phe-), 008, whereas the minor conformation has a cis peptide bond between Lys(Z)[4] and Trp[5] forming a βVI turn about these residues. The conformational analyses of the backbones of both conformers are based on the temperature dependences of H_N chemical shifts, on scalar coupling constants, and on quantitative evaluation of ROEs and NOEs for restraint MD calculations. The populations of the side chain rotamers are derived from quantitative evaluation of homonuclear coupling constants and qualitative evaluation of heteronuclear coupling constants. For the MD calculations in vacuo, the side chains are fixed in the dominant rotamers.

Introduction

The cyclic hexapeptide *cyclo*(-D-Ala[1]-Phe[2]-Val[3]-Lys(Z)[4]-Trp[5]-Phe[6]-) [VDA008] was synthesized in the course of structure-activity studies of a number of cyclic hexapeptides, originally derived from somatostatin and antamanide, as inhibitors of a hepatocytic transport system.[1] Usually only one amino acid was replaced at a time, but a comparison of various analogues suggested that replacement of D-Pro[1] in "008"[1h] by D-Ala and Thr[3] by Val would be advantageous. In fact, VDA008 turned out to be the most potent cyclic hexapeptide in the biological tests, exceeding the activity of 008 by more than an order of magnitude.[2]

During NMR studies of this peptide cross-peaks, unequivocally indicating a slow chemical exchange between two conformers (relative population 94:6), were found in ROESY and NOESY spectra. This study revealed that the conformers are trans–cis isomers about the Lys(Z)[4]–Trp[5] peptide bond. Cis–trans isomerism about the amide bond was the first problem studied by dynamic NMR spectroscopy.[3,4] It has been shown that the barrier of amide bond rotation is in the order of 80 kJ/mol.[5–9] In secondary N-alkylamides the trans configuration is generally strongly preferred in solution;[5–9] cis configurations have so far only been observed in N-methylacetamide (1–3%),[10] ortho-substituted

trans, *Z*   cis, *E*
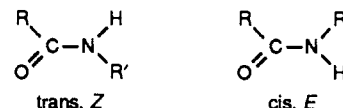
acetanilides,[11] strained cyclic structures,[12] and in a small protein in micellar solution.[13] On the contrary, the occurrence of cis-

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[†]Abbreviations: E. COSY, exclusive correlated spectroscopy; DEPT-H,-C-COSY, distorsionless enhancement by polarization transfer heteronuclear correlated spectroscopy; DQF-H,H-COSY, double quantum filtered proton correlated spectroscopy; H,C-COLOC, heteronuclear correlation via long-range couplings; NOE, nuclear Overhauser effect/enhancement; NOESY-nuclear Overhauser and exchange spectroscopy; ROE, rotating frame NOE; ROESY, rotating frame Overhauser and exchange spectroscopy, TOCSY, total correlation spectroscopy; Boc *tert*-butoxycarbonyl; Z, benzyloxycarbonyl.

(1) (a) Kessler, H.; Bernd, M.; Damm, I. *Tetrahedron Lett.* **1983,** *23,* 4685–4688. (b) Kessler, H.; Eiermann, V. *Tetrahedron Lett.* **1983,** *23,* 4689–4692. (c) Kessler, H.; Bernd, M.; Kogler, H.; Zarbock, J.; Sørensen, O. W.; Bodenhausen, G.; Ernst, R. R. *J. Am. Chem. Soc.* **1983,** *105,* 6944–6952. (d) Ziegler, K.; Frimmer, M.; Kessler, H.; Damm, I.; Eiermann, V.; Koll, S.; Zarbock, J. *Biochim. Biophys. Acta* **1985,** *845,* 86–93. (e) Kessler, H.; Klein, M.; Müller, A.; Wagner, K.; Bats, J. W.; Ziegler, K.; Frimmer, M. *Angew. Chem., Int. Ed. Engl.* **1986,** *25,* 997–999. (f) Kessler, H.; Gehrke, M.; Haupt, A.; Klein, M.; Müller, A.; Wagner, K. *Klin. Wochenschr.* **1986,** *64,* 74–78. (g) Usadel, K. H.; Kessler, H.; Rohr, G.; Kusterer, K.; Palitzsch, K. D.; Schwedes, U. *Somatostatin;* Reichlin, S., Ed.; Plenum Publishing Corp.: New York, 1987; pp 193–200. (h) Kessler, H.; Bats, J. W.; Griesinger, C.; Koll, S.; Will, M.; Wagner, K. *J. Am. Chem. Soc.* **1988,** *110,* 1033–1049. (i) Kessler, H.; Gemmecker, G.; Haupt, A.; Klein, M.; Wagner, K.; Will, M. *Tetrahedron* **1988,** *44,* 745–759. (j) Kessler, H.; Klein, M.; Wagner, K. *Int. J. Peptide Protein Res.* **1988,** *31,* 481–498. (k) Kessler, H.; Haupt, A.; Schudok, M.; Ziegler, K.; Frimmer, M. *Int. J. Peptide Protein Res.* **1988,** *32,* 183–193. (l) Kessler, H.; Gemmecker, G.; Haupt, A.; Lautz, J.; Will, M. *NMR Spectroscopy in Drug Research;* Alfred Benzon Symposium 26; Jaroszewski, J. W., Schaumburg, K., Kofod, H., Eds.; Munksgaard: Copenhagen, Denmark, 1988; pp 138–152. (m) Kessler, H.; Haupt, A.; Will, M. *Computer-Aided Drug Design;* Perun, T. J., Propst, C. L., Eds.; Marcel Dekker, New York, 1989; pp 461–484.
(2) Kessler, H.; Schudok, M.; Haupt, A. *Peptides 1988;* Jung, G., Bayer, E., Eds.; de Gruyter: Berlin, FRG, 1989; pp 664–666.
(3) Phillips, W. D. *J. Chem. Phys.* **1955,** *23,* 1363–1364.
(4) Gutowski, H. S.; Holm, C. H. *J. Chem. Phys.* **1956,** *25,* 1228–1234.
(5) Stewart, W. E.; Siddall, T. H. *Chem. Rev.* **1970,** *70,* 517–551.
(6) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1970,** *9,* 219–235.

cyclo(-D-Ala[1]-Phe[2]-Val[3]-Lys(Z)[4]-Trp[5]-Phe[6]-)
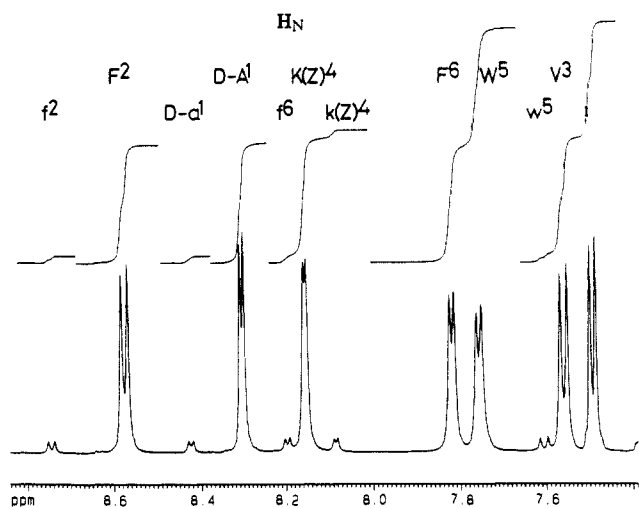
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Figure 1. Amide proton region of the 600-MHz 1D spectrum of cyclo-(-D-Ala-Phe-Val-Lys(Z)-Trp-Phe-) [VDA008] in Me_2SO-d_6 solution at 300 K. The amide proton of the valine of the minor populated conformer (v^3-H_N) is buried under the aromatic signals at 7.32 ppm (cf. Figure 2).

peptide bonds in proline-containing peptides[14] or peptides with other N-alkylated amino acids,[15] is a general phenomenon. It is notable that Boc-protected amino acids and oligopeptides usually exhibit both trans and cis conformations about the urethan bond,[16] as the size of the carbonyl oxygen and the alkoxy group is comparable.

In general, steric interactions between R and R' lead to a preference for the trans conformation in secondary amides. Cis isomers can only be observed in peptides if there is some sterical strain associated with the trans isomer. Therefore the occurrence of an additional conformer (in an amount of 6%) in the cyclic hexapeptide VDA008 was a surprising finding. Hence, careful conformational analyses were carried out for both conformers. To our knowledge the cis isomer of a non-alkylated amide bond has so far not been observed in weakly strained cyclic peptides or in linear peptides. However, the lack of a systematic search for such minor isomers in equilibrium, which requires an extremely careful analysis of high-resolution spectra, and the misinterpretation of spurious signals as impurities might be the reason for that.

Synthesis

The linear precursor of VDA008 was prepared by solid-phase synthesis[17-19] by using the classical Merrifield technique with the Boc-protective group strategy as previously described.[1h,20]
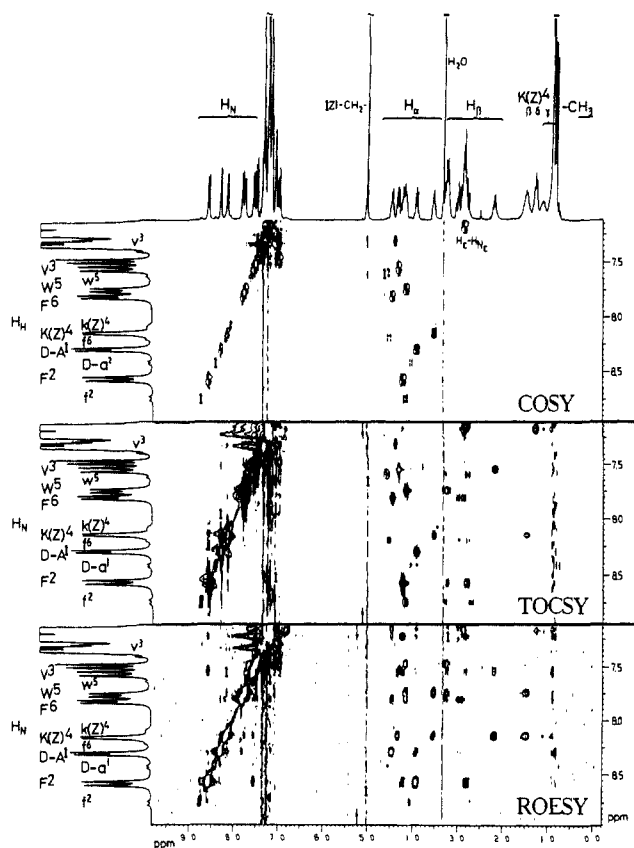

Figure 2. 250-MHz proton spectra of cyclo(-D-Ala-Phe-Val-Lys(Z)-Trp-Phe-) [VDA008] in Me_2SO-d_6 solution at 300 K. (COSY) Part of the DQF-H,H-COSY spectrum. Only the lowest of the negative levels is plotted. Five out of six H_N,H_α cross-peaks of the minor populated conformer can clearly be seen. The lysine cross-peak is rather weak. (TOCSY) Part of the TOCSY spectrum. Only positive levels are plotted. The position of the carrier is marked with arrows. (ROESY) Part of the ROESY spectrum. Positive and negative levels are plotted without distinction.

However, methanol was used for washing instead of dimethylformamide. The peptide was synthesized as Boc-Trp-Phe-D-Ala-Phe-Val-Lys(Z)-O-R on the hydroxymethylated resin (R). Cleavage of the linear peptide was achieved with hydrazine,[17] and the N-terminal Boc-protective group was removed with neat trifluoroacetic acid. Cyclization was achieved via the azide by the method of Medzihradszky.[18] The cyclic peptide was finally purified by HPLC.

NMR Measurements

Conformational Analysis of VDA008. The VDA008 is very soluble in dimethyl sulfoxide but sparingly soluble in other common NMR solvents. The conformational analysis was therefore carried out in dimethyl sulfoxide. The spectrum of the H_N-region, where splittings between both conformations are best separated, is shown in Figure 1. Two distinct signal sets are found, and the relative ratio remains unchanged at concentrations from 3 to 64 mM (at 300 K, see Supplementary Material). Proof that the minor signals resulted from a second conformation in slow exchanging equilibrium was provided from the following: (i) On warming, the small signals broadened and finally disappeared. This process was totally reversible. (ii) In the 250-MHz ROESY spectrum[21-23] (Figure 4b), exchange signals (positive contours with respect to the diagonal) were clearly observable between pairs of small and large signals in addition to the usual ROESY cross-peaks (negative contours) within each conformation. (iii) In the 500-MHz

(7) Sutherland, I. O. *Ann. Rep. NMR* **1971**, *4*, 71.

(8) Jackman, L. M. *Dynamic Nuclear Magnetic Resonance Spectroscopy*; Jackman, L. M., Cotton, F. A., Eds.; Academic Press: New York, 1975; pp 203–252.

(9) Oki, M. *Application of Dynamic NMR Spectroscopy to Organic Chemistry*; VCH Verlagsgesellschaft: Weinheim, FRG, 1985; pp 4377.

(10) Drakenberg, T.; Forsén, G. *J. Chem. Soc., Chem. Commun.* **1971**, 1404–1405.

(11) Kessler, H.; Rieker, A. *Liebigs Ann. Chem.* **1967**, *708*, 57–68.

(12) (a) Dale, J.; Titlestad, K. *Tetrahedron Lett.* **1978**, *4*, 379–380. Rothe, M.; Fähnle, M.; Wermuth, S. *Peptides 1984*; Ragnersson, U., Ed.; Almqvist and Wiksell: Stockholm, Sweden, 1984; pp 573–576. (b) Mierke, D. F.; Yamazaki, T.; Said-Nejad, O. E.; Felder, E. R.; Goodman, M. *J. Am. Chem. Soc.* **1989**, *111*, 6847–6849.

(13) Bairaktari, E.; Mierke, D. F.; Mammi, S.; Peggion, E. *Second Naples Workshop on Bioactive Peptides*; Capri, Italy, May 21–23, 1990.

(14) Dorman, D. E.; Bovey, F. A. *J. Org. Chem.* **1973**, *38*, 2379–2383.

(15) See e.g. in Cyclosporin.

(16) Branik, M.; Kessler, H. *Chem. Ber.* **1975**, *108*, 2176–2188, 2722–2727.

(17) Barany, G.; Merrifield, R. B. *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol. II, pp 1–284.

(18) Klausner, Y. S.; Bodanszky, M. *Synthesis* **1974**, 549–559.

(19) Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Peptide Protein Res.* **1987**, *30*, 705–739.

(20) Kessler, H.; Haupt, A.; Frimmer, M.; Ziegler, K. *Int. J. Peptide Protein Res.* **1987**, *29*, 621–628.

(21) Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811–813.

(22) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207–213.

(23) Kessler, H.; Griesinger, C.; Kerssebaum, R.; Wagner, K.; Ernst, R. R. *J. Am. Chem. Soc.* **1987**, *109*, 607–609.

Table I. ^{1}H NMR Chemical Shifts[a] (δ) of VDA008 in Me$_2$SO-d_6 Solution at 300 K

amino acid	proton	populated conformer major	populated conformer minor
D-Ala^1	H$_N$	8.31	8.43
	H$_\alpha$	3.93	4.05
	H$_\beta$	0.84	0.75
Phe^2	H$_N$	8.58	8.75
	H$_\alpha$	4.23	4.17
	H$_{\beta1}$	3.24$_{pro-S}$	3.28
	H$_{\beta2}$	2.81$_{pro-R}$	2.72
Val^3	H$_N$	7.56^b	7.32
	H$_\alpha$	4.33	4.41
	H$_\beta$	2.19	2.11
	H$_{\gamma1}$	0.90^b	0.78
	H$_{\gamma2}$		0.85
Lys(Z)^4	H$_N$	8.16	8.09
	H$_\alpha$	3.54	3.47
	H$_{\beta1}$	1.52	
	H$_{\beta2}$	1.28	0.95^b
	H$_{\gamma1}$	1.12	c
	H$_{\gamma2}$	0.93	c
	H$_\delta$	1.28^b	c
	H$_\epsilon$	2.88^b	c
	H$_{N\epsilon}$	7.19	c
	Z$_{-CH_2-}$	5.02	c
Trp^5	H$_N$	7.75	7.61
	H$_\alpha$	4.16	4.59
	H$_{\beta1}$	3.30$_{pro-R}$	3.30
	H$_{\beta2}$	3.22$_{pro-S}$	2.81
	indole-H$_N$	10.79	10.85
	indole-H$_2$	7.07	c
	indole-H$_7$	7.33	c
Phe^6	H$_N$	7.82	8.20
	H$_\alpha$	4.47	4.54
	H$_{\beta1}$	3.01$_{pro-R}$	3.01
	H$_{\beta2}$	2.90$_{pro-S}$	2.80

[a]Chemical shifts of the 64 mM sample in ppm relative to Me$_2$SO-d_5 = 2.50 ppm. ^{b}Degenerated proton signals. ^{c}Not determined.

NOESY spectrum[24] (Figure 4c), again exchange peaks were seen besides the NOEM cross-peaks (all positive with respect to the diagonal).

In order to distinguish the amino acids of the major and minor populated conformer, the one- and three-letter abbrevations of the amino acids of the minor conformer are not capitalized.

Assignment of the NMR Spectra. All amino acid residues of the major conformer were identified in the DQF-H,H-COSY spectrum[25,26] according to their characteristic pattern of chemical shifts[27,28] (Table I). From the TOCSY spectrum[29,30] (Figures 2 and 5) all amino acids, except lysine of the minor populated conformer, could be identified.

The distinction of the three aromatic amino acids of the major conformer, which exhibit identical spin patterns in the DQF-H,H-COSY spectrum and the TOCSY spectrum, was made by sequence analysis with an H,C-COLOC spectrum[31] (Figure 3). Due to the low population of only 6%, this was not possible for the minor conformer. The cross-peak between the D-Ala^1 β-protons and a ^{13}C signal at 171.95 ppm unequivocally assigned the D-Ala^1 carbonyl carbon. Hence, the amide proton coupling to this carbonyl carbon must be the Phe^2-H$_N$ proton. Two α-

(24) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546–4553.
(25) Piantini, U.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.
(26) Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 458–479.
(27) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
(28) Kessler, H.; Bermel, W. *NMR in Stereochemical Analysis*; Takeuchi, Y., Marchand, A. P., Eds.; VCH Publishers, Inc.: Deerfield Beach, 1986; pp 179–205.
(29) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521–528.
(30) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360.
(31) Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H. R. *J. Magn. Reson.* **1984**, *57*, 331–340.
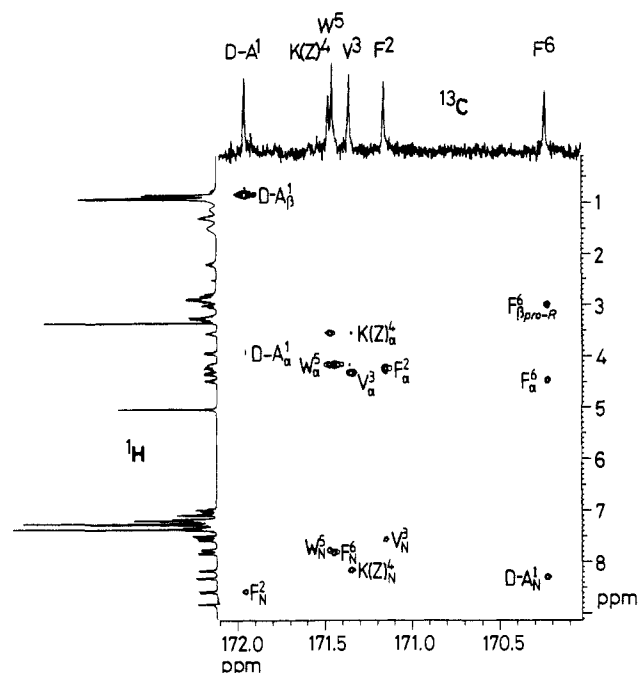
Table II. ^{13}C Chemical Shifts[a] (δ) of the Major Populated Conformer of VDA008 in Me$_2$SO-d_6 Solution at 300 K

	D-Ala^1	Phe^2	Val^3	Lys(Z)^4	Trp^5	Phe^6
C'	172.95	171.16	171.35	171.47	171.45	170.24
C$_\alpha$	49.45	55.18	57.21	55.95	55.65	54.99
C$_\beta$	16.09	36.59	35.93	30.82	26.37	37.90
C$_\gamma$			17.79	22.75		
			19.62			
C$_\delta$				30.07		
C$_\epsilon$				40.19		
Z$_{-CH_2-}$				65.33		

[a]Chemical shifts in ppm relative to Me$_2$SO-d_6 = 39.5 ppm.

Table III. $^3J_{H,H}$ Coupling Constants[a] of VDA008 and Populations of the Side-Chain Rotamers of Phe^2, Val^3, Trp^5, and Phe^6

	$^3J_{H_\alpha,H_N}$,^{b} Hz	$^3J_{H_\alpha,H_{\beta1}}$,^{c} Hz	$^3J_{H_\alpha,H_{\beta2}}$,^{c} Hz	P_I,^{d} %	P_{II},^{d} %	P_{III},^{d} %
D-Ala^1	5.4					
D-ala^1	6.2					
Phe^2	8.7	3.5$_{pro-S}$	11.5$_{pro-R}$	81	8	11
phe^2	8.6	3.1	12.0	(85	4)^e	10
Val^3	9.4	6.0		31^f		
val^3	9.3	4.5		17^f		
Lys(Z)^4	4.3	g	g			
lys(Z)^4	5.2	g	g			
Trp^5	7.3	10.2$_{pro-R}$^{h}	4.3$_{pro-S}$^{h}	69	16	15
trp^5	10.7	3.4	11.2	(79	7)^e	14
Phe^6	6.4	5.7$_{pro-R}$^{h}	8.4$_{pro-S}$^{h}	28	53	19
phe^6	6.6	4.9	9.5	(20	63)^e	17

[a]Values in Hz ±0.1 Hz. The noncapitalized three-letter abbreviations are the amino acids of the minor populated conformer. ^{b}Obtained from 1D spectra of VDA008. ^{c}Obtained from a complementary E.COSY spectrum at 600 MHz. ^{d}I: $\chi_1 = -60°$. II: $\chi_1 = 180°$. III: $\chi_1 = +60°$. ^{e}As the prochiral protons are not assigned, populations I and II cannot be distinguished. ^{f}Only the population of rotamer II can be calculated. ^{g}Not determined. ^{h}Not weakly coupled.

Table IV. Temperature Dependence[a] of the Amide Proton Chemical Shifts in $-\Delta\delta/\Delta T$ (ppb/K)

	D-Ala^1	Phe^2	Val^3	Lys(Z)^4	Trp^5	Phe^6
major pop. conformer	5.6	5.4	2.5	2.8	4.0^b	2.1
minor pop. conformer	5.1	5.0	1.0^c	3.1	5.8^c	1.5

[a]Determined in the range of 300 to 340 K. ^{b}The temperature gradient of the indole-H$_N$ is 2.9 ppb/K. ^{c}Determined with the aid of a 250-MHz TOCSY spectrum at 320 K.

protons couple to the carbonyl at 171.47 ppm; one is the Lys-(Z)^4-H$_\alpha$ and the other is the Trp^5-H$_\alpha$. If two α-protons couple to the same carbonyl carbon, it has to be the one between them. The carbonyl is therefore Lys(Z)^4-C'. The cross-peak between the amide proton of Trp^5 and the carbonyl carbon of Lys(Z)^4 is proof of the successful cyclization. The tryptophan α-proton shows a cross-peak not only to the lysine carbonyl but also to its own carbonyl carbon at 171.45 ppm. Another amide proton coupling to this carbonyl is Phe^6-H$_N$. The amide protons of the nonaromatic amino acids exhibit cross-peaks to the carbonyl carbons of the preceding amino acids.

The spin systems of the aromatic amino acids of the minor conformer can be assigned via the exchange peaks in the ROE-SY[21-23] and NOESY[23] spectra (Figure 4). In the ROESY spectrum five pairs of cross-peaks were found within the H$_N$-region having the same sign as the diagonal, thus indicating chemical exchange between the two conformers. As the amide proton of the valine of the minor conformer is buried under the aromatic signals and the corresponding signal of the major conformer is very close to the aromatic signals, no cross-peak due to exchange for these signals could be identified. In the NOESY spectrum (Figure 4c), nuclear Overhauser effects and exchange peaks cannot be distinguished by their sign. However, as the 500-MHz NOESY spectrum is much better resolved than the 250-MHz ROESY spectrum, the cross-peaks due to exchange can be seen more clearly. Additionally, peaks due to exchange can be found within the α-proton region of these spectra (Figure 6). Hence it is

cyclo(-D-Ala¹-Phe²-Val³-Lys(Z)⁴-Trp⁵-Phe⁶-)

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Figure 3. H,C-COLOC spectrum of the carbonyl region of *cyclo*(-D-Ala-Phe-Val-Lys(Z)-Trp-Phe-) [VDA008] in Me₂SO-d₆ solution at 300 K (¹H ≙ 300 MHz, power spectrum). The annotations indicate the protons that couple to the respective carbonyl carbons whose assignment is given above. The indole-H_N was folded back for optimization of the resolution in the proton dimension.

obvious that the minor conformer is *not* an impurity.

The chemical shifts of the aliphatic carbons of the major populated conformer (Table II) were extracted from a DEPT-H,C-COSY spectrum[32-35] (see supplementary material). It is remarkable that the valine γ-protons are degenerate whereas the corresponding carbon atoms have a chemical shift difference of almost 2 ppm.

Extraction of Conformationally Relevant NMR Parameters. Important parameters for conformational analysis include scalar coupling constants of vicinal protons from which dihedral angles can be derived via Karplus equations.[36-39] Distances between protons can be derived from dipolar proton–proton couplings. Besides these parameters, temperature dependencies of amide proton chemical shifts (Table IV) indicate the orientation of these protons.[38]

The H_α, H_N coupling constants were extracted directly from a 1D spectrum after resolution enhancement (Table III). Equivalent information was derived from the H_α, H_N region of the DQF-H,H-COSY spectrum (see supplementary material), which was especially helpful in the case of the minor conformer.

For the determination of the populations of the side chain rotamers, the H_β, H_α coupling constants are needed.[40] From a 600-MHz E.COSY spectrum[41,42] these coupling constants could easily be extracted for the aromatic amino acids of *both* conformers

(32) Levitt, M. H.; Sørensen, O. W.; Ernst, R. R. *Chem. Phys. Lett.* **1983**, *94*, 540–544.

(33) Bendall, M. R.; Pegg, D. T. *J. Magn. Reson.* **1983**, *53*, 144–148.

(34) Pegg, D. T.; Bendall, M. R. *J. Magn. Reson.* **1983**, *55*, 114–127.

(35) Nakashima, T. T.; John, B. K.; McClung, R. E. D. *J. Magn. Reson.* **1984**, *59*, 124–131.

(36) Karplus, M. J. *J. Chem. Phys.* **1959**, *30*, 11–15.

(37) Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 41–81.

(38) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512–523.

(39) Pardi, A.; Billeter, M.; Wüthrich, K. *J. Mol. Biol.* **1984**, *180*, 741–751.

(40) Pachler, K. G. R. *Spectrochim. Acta* **1963**, *19*, 2085–2092; **1964**, *20*, 581–587.

(41) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1985**, *107*, 6394–6396.

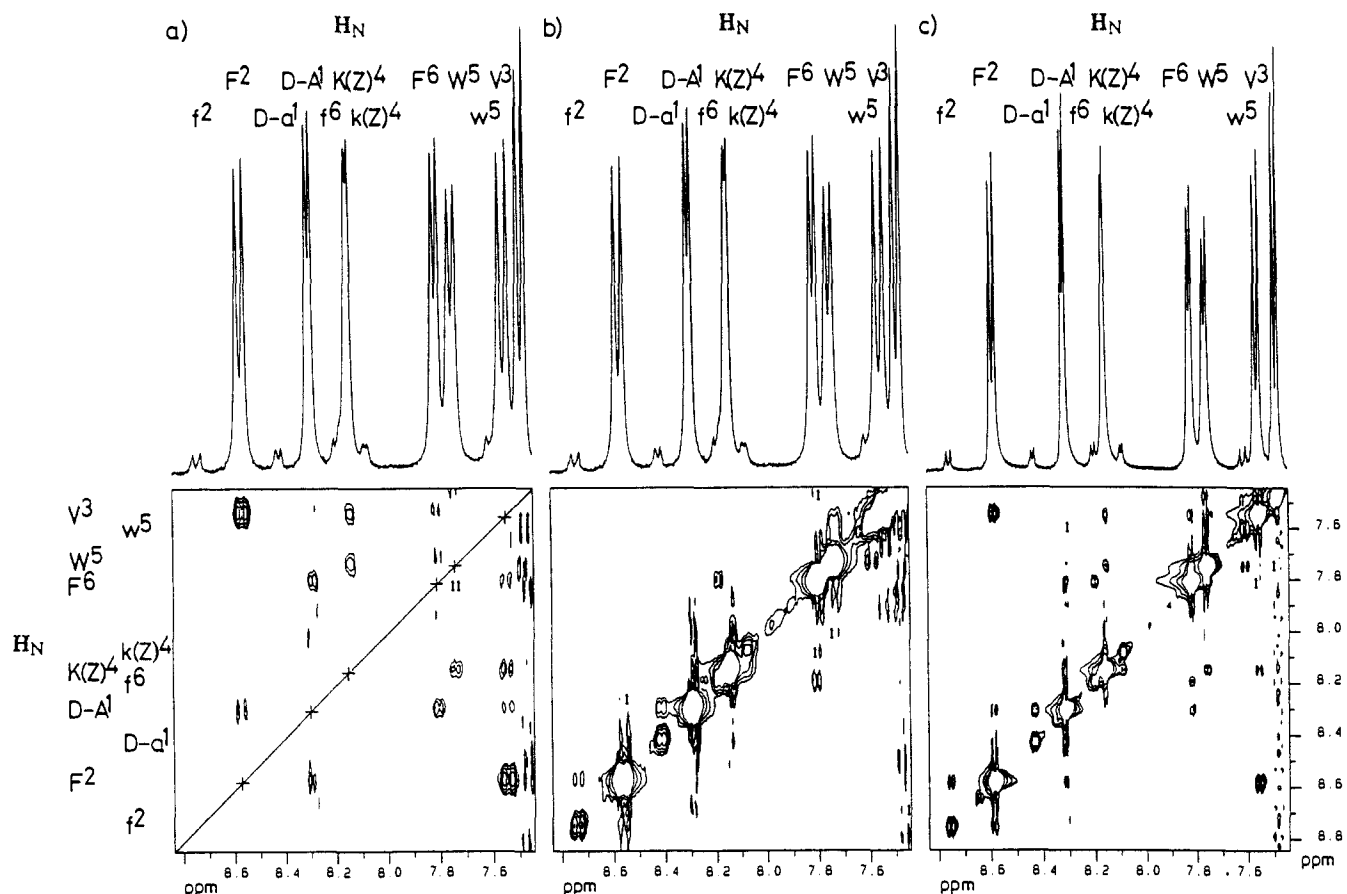(42) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Chem. Phys.* **1986**, *85*, 6837–6852.

Table V. Comparison of Proton–Proton Distances Obtained from ROESY and NOESY Spectra with Those from the MD Calculation (for the Major Populated Conformer of VDA008)

no.	involved protons		$d(H,H)^{exp}$, pm	$d(H,H)^{MD}$, pm	Δ
1.	D-Ala¹-H_N	D-Ala¹-H_α	260	278	18
2.	D-Ala¹-H_N	D-Ala¹-H_β	334ᵃ	269	(−65)ᵇ
3.	D-Ala¹-H_N	Phe⁶-H_N	335	367	32
4.	D-Ala¹-H_N	Phe⁶-H_α	217	226	9
5.	D-Ala¹-H_α	Phe²-H_N	207	209	2
6.	Phe²-H_N	Phe²-H_α	275	279	4
7.	Phe²-H_N	Val³-H_N	237	260	23
8.	Phe²-H_α	Val³-H_N	282	345	63
9.	Val³-H_N	Val³-H_α	279	289	10
10.	Val³-H_N	Val³-H_β	308	363	55
11.	Val³-H_α	Val³-H_β	250	250	0
12.	Val³-H_α	Lys⁴-H_N	224	243	19
13.	Val³-H_β	Lys⁴-H_N	234	241	7
14.	Lys⁴-H_N	Lys⁴-H_α	247	263	16
15.	Lys⁴-H_α	Trp⁵-H_N	224	210	−14
16.	Trp⁵-H_N	Trp⁵-H_α	228	208	−20
17.	Trp⁵-H_α	Phe⁶-H_N	293	344	51
18.	Phe⁶-H_N	Phe⁶-H_α	291	293	2

ᵃ100 pm were added to the determined "distance" for taking into account the methyl group.[48,49] ᵇNo restraint violation.

Table VI. Hydrogen Bondsᵃ Occurring during 40-ps Restraint Molecular Dynamics Calculation of the Major Populated Conformer of VDA008

donor	acceptor	$d(H\cdots A)$,ᵇ pm	θ,ᶜ deg	pop. %	type
D-Ala¹-H_N	Trp⁵-CO	229	138	25	
Phe²-H_N	Phe⁶-CO	222	125	2	
Val³-H_N	Phe⁶-CO	217	156	78	βII'
Phe⁶-H_N	Val³-CO	210	141	42	βII
Phe⁶-H_N	Lys⁴-CO	206	144	81	γ
Phe⁶-H_N	{ Val³-CO	218	133 }ᵈ	20	
	{ Lys⁴-CO	218	133 }		

ᵃDefinition of hydrogen bonds: the H⋯A distance is shorter than 250 pm and the D—H⋯A angle θ is greater than 120°. ᵇDistance between donor proton and acceptor oxygen. ᶜThe D—H⋯A angle. ᵈBifurcated hydrogen bridge.

Table VII. Comparison of Proton–Proton Distances Obtained from ROESY and NOESY Spectra with Those from the MD Calculation (for the Minor Populated Conformer of VDA008)

no.	involved protons		$d(H,H)^{exp}$, pm	$d(H,H)^{MD}$, pm	Δ
1.	D-ala¹-H_N	D-ala¹-H_β	345ᵃ	268	(−77)ᵇ
2.	D-ala¹-H_N	phe⁶-H_α	210	227	17
3.	D-ala¹-H_α	phe²-H_N	209	211	2
4.	val³-H_α	val³-H_β	232	250	18
5.	val³-H_α	lys⁴-H_N	224	230	6
6.	val³-H_β	lys⁴-H_N	246	330	84
7.	lys⁴-H_N	lys⁴-H_α	282	274	−8
8.	lys⁴-H_α	trp⁵-H_α	191	159	−32
9.	trp⁵-H_α	phe⁶-H_N	254	265	11

ᵃ100 pm were added to the determined "distance" for taking into account the methyl group.[48,49] ᵇNo restraint violation.

(see supplementary material) (Table III). The H_β, H_α -coupling constants of the valine residues could be extracted from the 1D and the E.COSY spectra.

For the determination of proton–proton distances, two ROESY spectra with 50 and 100 ms mixing time, respectively, were recorded at 250 MHz and, in addition, a NOESY spectrum at 500 MHz with a mixing time of 200 ms was recorded. The ROESY spectra were used to determine relative buildup rates in a completely iterative way. All three spectra were analyzed in terms of the linear approximation of the two-spin approximation, where simply the volume intergral of a cross-peak is proportional to d^{-6}. The distances were calibrated as follows for the major conformer: often the indole-H_N, indole-H₇ cross-peak of tryptophan (distance 282 pm) is used for calibration. However, this distance is fairly

Figure 4. Region of the amide protons of the ROESY (a and b) and NOESY spectrum (c), respectively, of *cyclo*(-D-Ala-Phe-Val-Lys(Z)-Trp-Phe-) [VDA008] in Me_2SO-d_6 solution at 300 K. (a) Negative levels of the 250-MHz ROESY spectrum. The cross-peaks show rotating frame Overhauser effects (ROEs). The 1D spectrum was also recorded at 250 MHz. (b) Positive levels of the 250-MHz ROESY spectrum. The diagonal and the cross-peaks due to exchange are visible. (c) Positive levels of the 500-MHz NOESY spectrum. The negative levels do not contain cross-peaks. The 1D spectrum was also recorced at 500 MHz.

long and therefore the volume integral of the cross-peak is not as accurate as the volume integral of a much stronger cross-peak associated with a shorter distance. Therefore an alternative approach was used here. The cross-peak between the β-protons of phenylalanine 2 is strong in the NOESY spectrum and corresponds to a distance of 176 pm. With these data the D-Ala[1]-H_α to Phe[2]-H_N distance was determined to be 207 pm, which is reasonable. This distance was then used for calibration. The indole-H_N-H_7 distance was correctly calculated on this basis within experimental error. The resulting distances are given in Tables V and VII.

Conformational Analysis of the Major Populated Conformer

Starting Conformation of the Backbone. As VDA008 is an analogue of 008 the conformations of both *could* be similar. The temperature dependencies of the amide protons are not *strongly* different from each other within VDA008 (Table IV). Nevertheless, the amide protons of Val[3] and Phe[6] show the typically small temperature dependency for amino acids in position *i* + 3 of β turns. Thus a β,β structure as in 008 was likely. The temperature dependencies of the other H_N protons support this.

In a βII or βII' turn the $H_\alpha{}^{i+1}$-$H_N{}^{i+2}$ proton–proton distance is short (about 210 pm), whereas this distance is much longer in a βI or βI' turn (approximately 340 pm). In both ROESY and NOESY spectra very intense cross-peaks were found between D-Ala[1]-H_α and Phe[2]-H_N and also between Lys(Z)[4]-H_α and Trp[5]-H_N. Henceforth, the major populated conformer adopts a βII',βII structure with Phe[6] and Val[3] in position *i* of the turn, respectively. In the H,C-COLOC spectrum (Figure 3), a cross-peak between Trp[5]-H_α and Lys(Z)[4]-C' is found. This α-proton to carbonyl cross-peak can only be observed if both atoms are antiperiplanar, as in the case of a βII turn. On the contrary the corresponding region of 008 was found to exist as a βI turn.[1h] This difference can be explained as a result of the hydrogen bond

between the amide proton of tryptophan and the hydroxyl oxygen of threonine, thus yielding a βI turn in 008.

Side-Chain Conformations. In peptides side chains are usually much more flexible than the backbone. Nevertheless, it is possible to identify preferred orientations. For this it is reasonable to assume that the three staggered rotamers, with respect to the torsion angle χ_1 about the C_α-C_β bond, are dominantly populated. From the vicinal H_α-H_β coupling constants of amino acids with two β-protons, the populations of these rotamers can be calculated according to Pachler[40] (Table III). The required assignment of the prochiral β-protons can be derived with the aid of the H,-C-COLOC spectrum (Figure 3).[1h,43,44]

As valine has only a single β-proton, the populations of the side-chain rotamers cannot be determined directly by this procedure.[1h,43] The population of the rotamer in which the α- and β-proton are antiperiplanar (χ_1 = 180°) is 31%. The strong ROE or NOE between the Val[3]-H_β and Lys(Z)[4]-H_N and the absence of a Val[3]-H_β-C' cross-peak in the H,C-COLOC spectrum indicate that the dominant conformation is with the amide nitrogen and the β-proton antiperiplanar to each other (χ_1 = -60°).

Refinement of the Conformation by MD Calculation. The proton–proton distances, determined from ROESY and NOESY spectra, have been used in constrained molecular dynamics (MD) calculations in vacuo with use of the GROMOS program[45] for the refinement of the structure. The determined distances restrain the conformation more or less to that found in solution.[46–48] As

(43) Kessler, H.; Griesinger, C.; Wagner, K. *J. Am. Chem. Soc.* **1987**, *109*, 6927–6933.

(44) Anders, U.; Gemmecker, G.; Kessler, H.; Griesinger, C. *Fresenius, Z. Anal. Chem.* **1987**, *327*, 72–73.

(45) Gronigen Molecular Simulation (GROMOS), Biomos B.V., Groningen (1987), W.F. van Gunsteren and H.J.C. Berendsen, Nijenborgh, 16, NL 9747 AG Groningen.
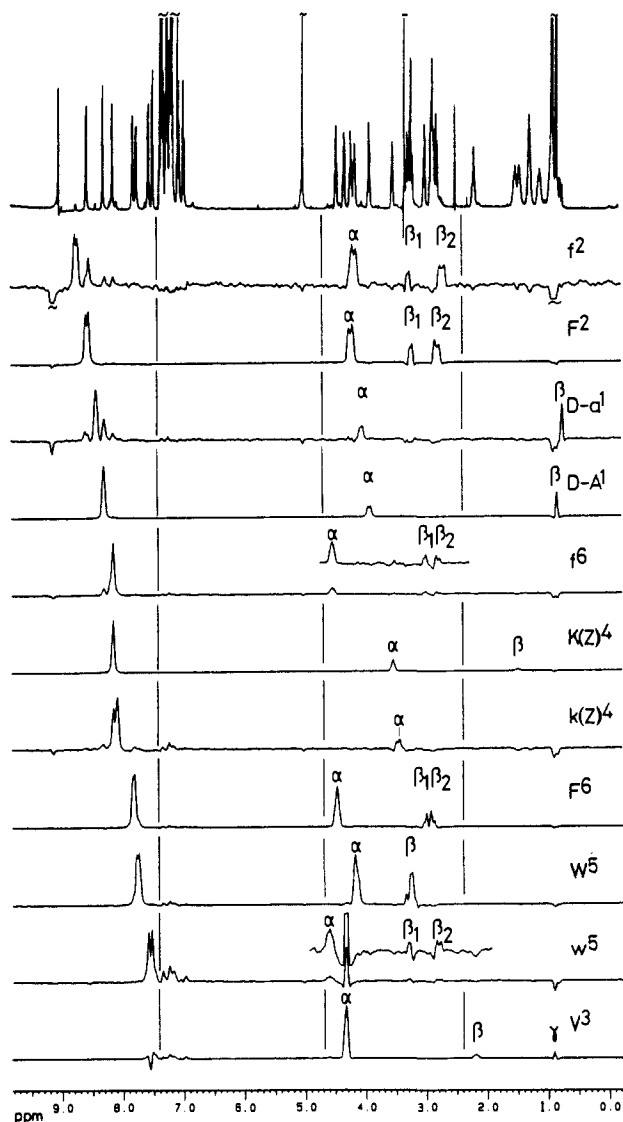
cyclo(-D-Ala¹-Phe²-Val³-Lys(Z)⁴-Trp⁵-Phe⁶-)

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Figure 5. 500-MHz 1D proton spectrum of *cyclo*(-D-Ala-Phe-Val-Lys-(Z)-Trp-Phe-) [VDA008] in Me₂SO-d_6 solution at 300 K (on top) and sums of traces of the 250-MHz TOCSY spectrum (Figure 2) over the amide proton signals of the indicated amino acids along F_1. The traces are scaled arbitrarily. As val³-H_N is buried under the aromatic signals, a summation is worthless along F_1 due to the strong t_1 noise.

Figure 6. Region of the α-protons of the ROESY (a) and NOESY spectra (b) of *cyclo*(-D-Ala-Phe-Val-Lys(Z)-Trp-Phe-) [VDA008] in Me₂SO-d_6 solution at 300 K. The asterisks indicate an unknown impurity. (a) 250-MHz ROESY spectrum. Positive and negative levels are plotted without distinction. The 1D spectrum was recorded under identical conditions. (b) Positive levels of the 500-MHz NOESY spectrum. The negative levels do not contain cross-peaks. The 1D spectrum is recorded at 500 MHz.

charges are partially shielded by the solvent, the charges of amide protons exposed to the solvent (cf. Table IV) were reduced for the calculation in vacuo to take account of solvation effects.[1h] In this way formation of additional intramolecular hydrogen bridges is suppressed: the charge of Phe⁶-H_N ($-\Delta\delta/\Delta T$ = 2.1 ppb/K) was not reduced while that of Ala¹-H_N ($-\Delta\delta/\Delta T$ = 5.6 ppb/K) was reduced to 25% (cf. Table IV). The effective charges of the other amide protons were interpolated linearly.[1h]

The Z-protection group was not included in the MD calculations; the effective charge of the Lys⁴-$H_{N'}$ was reduced to 25% to minimize artificial effects of the side chain on the backbone. Certainly, solvent effects are also important for the conformation of the backbone and especially the side chains. However, the solvent effects are mimicked by the experimental restrains used in the MD calculations. Hence we assume that the influence of the omission of the Z-protection group is minor.
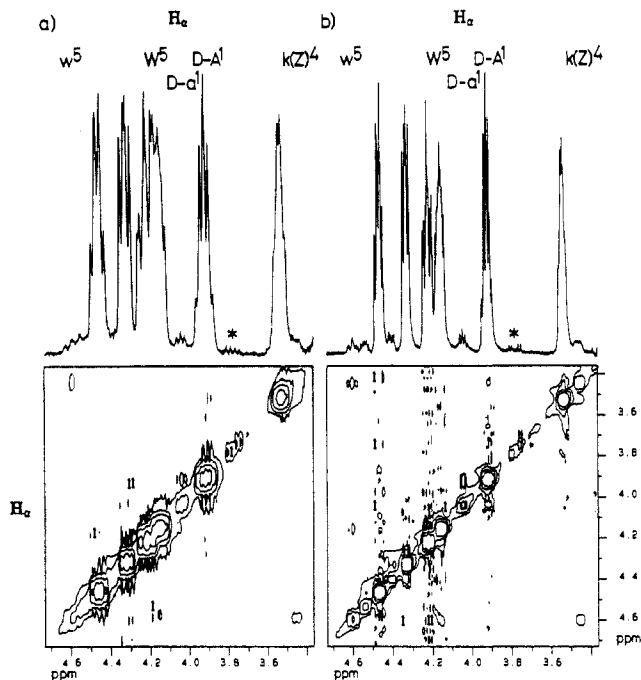
(46) Lautz, J.; Kessler, H.; Boelens, R.; Kaptein, R.; van Gunsteren, W. F. *Int. J. Peptide Protein Res.* **1987**, *30*, 404–414.

(47) Lautz, J.; Kessler, H.; Kaptein, R.; van Gunsteren, W. F. *J. Comp. Aided Mol. Design* **1987**, *1*, 219–241.

(48) van Gunsteren, W. F.; Boelens, R.; Kaptein, R.; Scheek, R. M.; Zuiderweg, E. R. P. *Molecular Dynamics and Protein Structure*; Hermans, J., Ed.; Polycrystal Book Service: Western Springs, IL, 1985; pp 92–99.
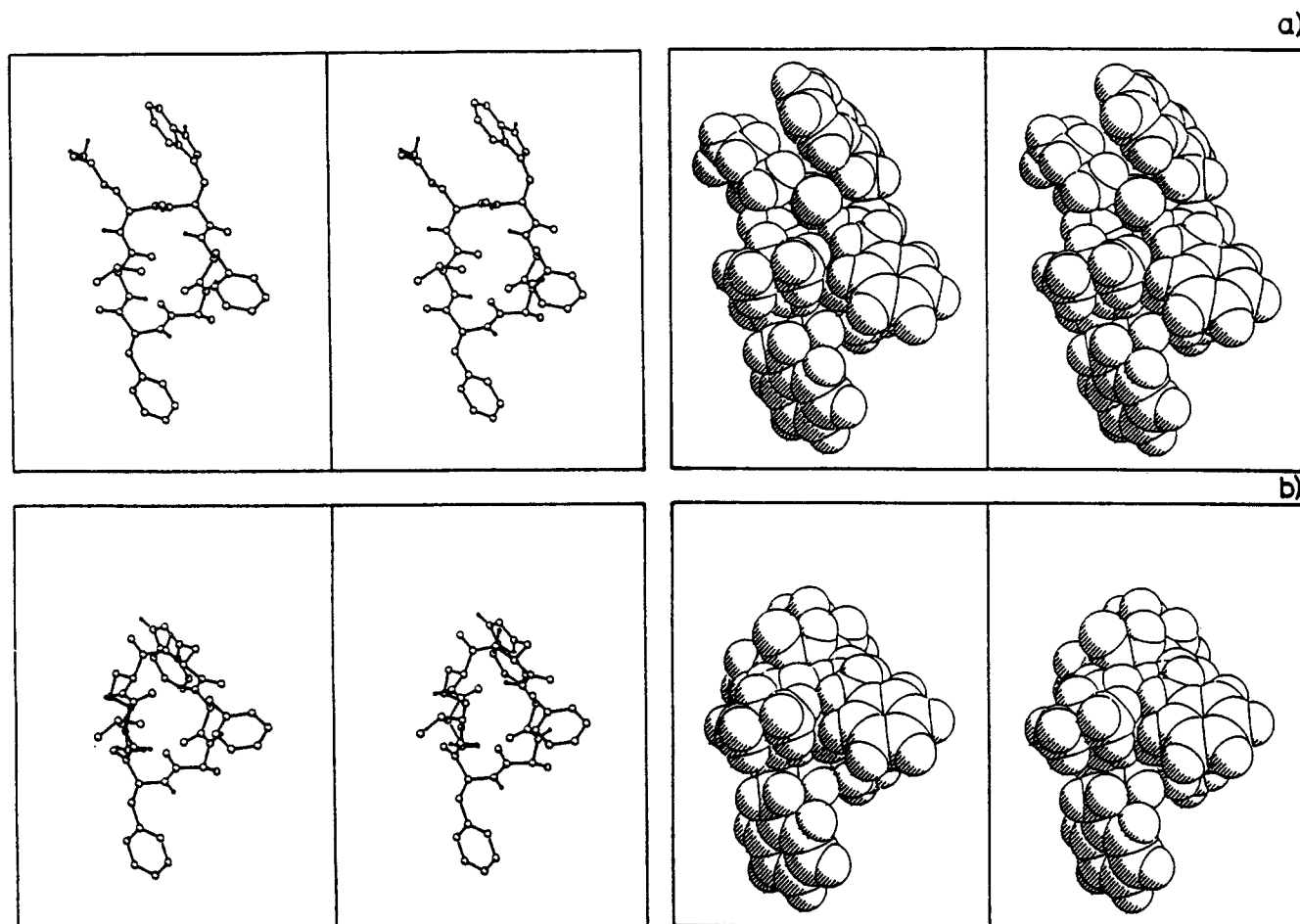
Table VIII. Hydrogen Bonds*[a]* Occurring during 40-ps Restraint Molecular Dynamics Calculation of the Minor Populated Conformer of VDA008

donor	acceptor	d(H···A),*[b]* pm	θ,*[c]* deg	pop., %	type
ala¹-H_N	trp⁵-CO	232	134	17	
phe²-H_N	phe⁶-CO	223	125	1	
val³-H_N	phe⁶-CO	219	149	**65**	*β*II′
lys⁴-H_N	phe²-CO	227	135	14	
phe⁶-H_N	val³-CO	189	153	**94**	*β*VI

[a] Definition of hydrogen bonds: the H···A distance is shorter than 250 pm and the D—H···A angle is greater than 120°. *[b]* Distance between donor proton and acceptor oxygen. *[c]* The D—H···A angle. *[d]* Bifurcated hydrogen bridge.

The basic starting structure was that of 008.[1h] The side chains of Phe², Trp⁵, and Phe⁶ were kept in the experimentally determined dominantly populated conformations by dihedral constrains. If the side chains are unconstrained, according to the experimental conformational equilibrium, they often adopt minor populated conformations in the calculation: If molecular dynamics calculations in vacuo would be carried out without constraints, the peptide would adopt the maximum folded conformation, maximizing van der Waals interactions.[1h] The usual constraints between Lys(Z)⁴-$H_α$, Trp⁵-H_N, and Trp⁵-$H_α$ (cf. Experimental Section) were not strong enough to change the *β*I loop of 008 into the *β*II loop of VDA008. Hence, ten-times higher force constances were applied during energy minimization and 10 ps molecular dynamics simulation to yield the appropriate starting structure.

Subsequently the MD calculation was carried out as outlined in the Experimental Section. The resulting averaged structure over 40 ps trajectory of restraint MD (cf. Experimental Section) is shown in Figure 7 (above) and the results are given in Tables V, VI, and IX. The experimental NMR data are well enough represented in the calculation.

The total potential energy was 229 kJ/mol on average. The averaged difference of the distance constraints of all 18 distances was 9 pm with a total restrain energy of 18 kJ/mol. The larger deviations are found in cases where ROEs or NOEs were less

a)

b)

Figure 7. Mean conformations of cyclo(-D-Ala-Phe-Val-Lys-Trp-Phe-) [VDA008] without the Z-protection group, obtained by restraint MD calculations: (above) Major conformer (94% populated in Me$_2$SO-d_6 solution); (below) minor conformer (6% populated). The protons bound to carbon are not shown here.

Table IX. Comparison of Backbone Angles of the Averaged MD Structures and the Experimental Angles

amino acid	$\varphi^{exp,a}$ deg	$\varphi^{MD,b}$ deg	ψ^{MD}	ω^{MD}
D-Ala^1	75	64 (11)	-106 (10)	-179 (6)
Phe^2	-98	-69 (12)	-31 (13)	173 (7)
Val^3	-105	-106 (15)	162 (9)	179 (7)
Lys^4	-68	-42 (13)	117 (12)	-176 (7)
Trp^5	77	75 (11)	-35 (20)	-178 (7)
Phe^6	-81	-110 (20)	83 (11)	173 (7)
D-ala^1	80	63 (10)	-114 (10)	178 (6)
phe^2	-97	-65 (14)	-39 (12)	176 (7)
val^3	-104	-95 (14)	115 (40)	-172 (9)
lys^4	-74	-63 (27)	120 (10)	3 (14)
trp^5	-120^c	-111 (12)	49 (30)	174 (8)
phe^6	-83, -158^d	-120 (30)	85 (12)	-173 (10)

^{a}The φ angle determined via the Karplus relation that is closest to the value determined by MD calculation. ^{b}RMS fluctuations (averaged over 40 ps). ^{c}The $^3J_{H_N,H_\alpha}$ coupling constant is 10.7 Hz and is thus larger than the maximum value in the Karplus relation. ^{d}Both values are possible according to the MD calculation.

precise. No distance constraints were used for the side chains of the aromatic amino acids as ROEs or NOEs between the backbone and protons of rotating side chains cannot be well transformed into distances due to the d^{-6} dependency between Overhauser effects and distances. The average dihedral restrain energy was 4 kJ/mol for those three aromatic side chains.

The proposed βII' turn, with Phe^6 in position i, was populated at 78% during the MD simulation (Table VI). Within this turn, a hydrogen bond between Phe^2-H$_N$ and Phe^6-CO is found at 2%. The backbone angles in this part of the peptide are in good accordance with the experimental values (Table IX) and close to

the values for an ideal βII' turn.

In the other hemisphere of the peptide, the postulated βII turn with Val^3 in position i is only populated at 42%. A γ turn with Lys^4 in position j is dominating (81% populated). Additionally, a bifurcated hydrogen bond is found here. The amide proton of Phe^6 bridges to Val^3 and Lys^4 carbonyl oxygens (20% populated). Comparison of the angles found by MD and those for ideal turns reveals that the ψ angle of Trp^5 is just between that of a βII and a γ turn (Figure 8).

Besides these turns another loop between Ala^1-H$_N$ and Trp^5-CO is populated at 25%. This bridge forms due to a folding of the molecule along an axis running through amino acids Val^3 and Phe^6.

Conformational Analysis of the Minor Populated Conformer

Backbone Conformation. The temperature dependencies of the amide proton chemical shifts in the minor populated conformer are stronger differentiated than those of the major conformer (Table IV). The amide protons of val^3 and phe^6 show the lowest values ($-\Delta\delta/\Delta T = 1.0$ and 1.5 ppb/K, respectively), typical of an internal orientation. The amide proton of trp^5 is clearly externally orientated ($-\Delta\delta/\Delta T = 5.8$ ppb/K). In the ROESY[21-23] and NOESY[24] spectra (Figure 6) strong Overhauser effects are found between D-ala^1-H$_\alpha$ and phe^2-H$_N$ and between lys(Z)^4-H$_\alpha$ and trp^5-H$_\alpha$. According to these findings, the minor populated conformer has a βII',βVI structure with phe^6 and val^3 in position i, respectively. In the βVI turn the cis peptide bond occurs between lysine and tryptophan, otherwise the rather strong Overhauser effect between the respective α-protons could not be explained. All H$_N$,H$_\alpha$ coupling constants in the minor populated conformer are very similar to those of the major conformer except for tryptophan (Table III). In the trp^5 this coupling is 10.7 Hz, indicating that the amide and α-protons are antiperiplanar. In fact, this value is even larger than the maximum of the usual

Karplus relations,[37,39] which are derived from trans configurated amide bonds. Nevertheless, there is no doubt about the anti-periplanar orientation of these two protons.

Side-Chain Conformations. The H_α, H_β coupling constants of the three aromatic amino acids of the major and the minor populated conformer are similar; the maximum difference is 1.1 Hz between corresponding amino acids (Table III). One feature is remarkable: the larger α,β-coupling constant in tryptophan of the dominating conformer is to the low-field β_1-proton but in the minor conformer to the high-field β_2-proton. However, chemical shifts cannot be used to distinguish *pro-R* and *pro-S* β-protons.[1h,43] Thus only the relative populations of the rotamer type III (χ_1 = +60°) can be determined without ambiguity. However, the small differences between the coupling constants of the two conformers of the cyclic peptide suggest that the dominant side-chain conformations could be similar. In the case of valine the α,β-coupling constants differ by 1.5 Hz, and in the minor conformer the rotamer in which α- and β-protons are antiperiplanar (χ_1 = 180°) is significantly less populated (17% instead of 31%).

Refinement of the Conformation by MD Calculation. Conformational refinement by molecular dynamics simulations is nowadays routinely done especially for small cyclic peptides. However, structural refinement of the minor (only 6% populated) conformer was not a trivial task. Because of the low population, only short proton–proton distances gave rise to detectable cross-peaks in the ROESY and NOESY spectra. The volume integrals of these cross-peaks have a considerable uncertainty due to the noise in the spectra. Additionally, errors due to exchange of magnetization cannot always be excluded. Nevertheless, the basic conformation is very much determined by the cis peptide bond as showed up during this refinement.

Similar to the conformational refinement of the major populated conformer, the effective charges of the amide protons where reduced according to the temperature dependences of the chemical shifts. The effective charge of val³-H_N ($-\Delta\delta/\Delta T$ = 1.0 ppb/K) (Table IV) was retained, that of trp⁵-H_N ($-\Delta\delta/\Delta T$ = 5.8 ppb/K) was reduced to a fourth, and all others were interpolated.

The conformationally relevant NMR parameters showed a structural similarity between the two conformers in one part of the molecule. Both conformers exhibit a βII' turn with phenylalanine 6 in position i. However, the other hemisphere is basically different as the lys(Z)⁴-trp⁵ peptide bond is cis configurated in the 6% populated conformer. In order to get an appropriate starting structure, the force constant of the lys⁴-H_α-trp⁵-H_α distance constraint was increased by one order of magnitude. An MD simulation over a few picoseconds yielded a starting structure that was basically compatible with the NMR parameters. Subsequently a MD simulation was carried out as outlined for the major populated conformer. As the distance information was not as accurate as for the 94% populated conformer, the force constants of the distance pseudopotential were reduced to only 500 kJ·mol⁻¹·nm⁻¹ (cf. Experimental Section). Additionally, the side chains were not fixed as the major populated rotamers could not be determined unambiguously. Nevertheless, it was checked after the MD simulation that the adopted orientation was not in contradiction with the experimental results.

The crucial step in structural refinement of NMR derived structures is the calibration of volume integrals to distances. As none of the conventional calibration methods could be applied here, the distances were set as follows. A strong Overhauser effect between ala¹-H_α and phe²-H_N and the temperature dependences of the chemical shifts of the amide protons in that region indicated a βII' turn here. The H_α-H_N distance should be about 210 pm and cannot be shorter than 205 pm. The strong ROE and NOE between lysine and tryptophan α-protons is typical for a βVI turn. Similar turns are found in peptides[50] and proteins, usually with proline in position i + 2; in protein structures, investigated by
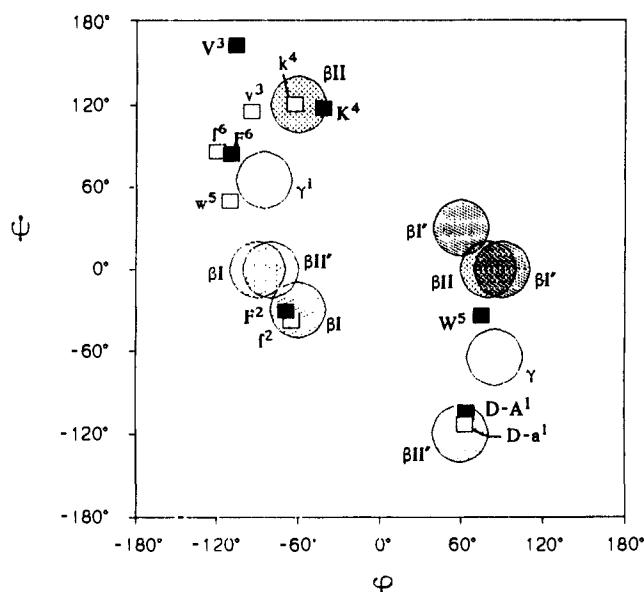

Figure 8. Ramachandran diagram of the averaged structures determined by MD calculations of the two conformers of VDA008. The capital letters indicate the amino acids of the major populated conformer while the others indicate those of the minor populated conformer. The circles show the areas of ideal turns (±20°).

X-ray, the H_α-H_α distances are about 191 pm (±10 pm approximately).[51,52] Both conditions could be fulfilled as shown in Table VII.

The average total potential energy of the minor populated conformer was 221 kJ/mol, thus rather close to the value of the major populated conformer. However, as the two simulations could not be carried out under identical conditions, the results cannot be compared directly. The few distance constrains available were basically satisfied in the MD simulation (Table VII). The lys⁴-H_α-trp⁵-H_α distance is only 159 pm, which is a rather short distance. The deviations of the distance constraints were 10 pm on average with a total restrain energy of 19 kJ/mol.

In this conformer two hydrogen bridges are dominant (Table VIII): one is between phe⁶-H_N and val³-CO within the βVI turn (94% populated) and the other is between val³-H_N and phe⁶-CO within the βII' turn (65% populated). In analogy with the major populated conformer, a hydrogen bridge between ala¹-H_N and trp⁵-CO occurred. Also the corresponding hydrogen bridge is found between lys⁴-H_N and phe²-CO. The backbone angles of the βII' turn are close to those of an ideal turn (Figure 8, cf. Table IX). The βVI turn is determined by the cis peptide bond between the amino acids i + 1 and i + 2, but no "ideal angles" are available. The experimental φ angles and those found during the MD simulation are in reasonable agreement (Table IX). The side-chain conformations are identical with those of the major populated conformer. Though the structure is not defined precisely, the simulation satisfies the NMR parameters well enough to be a good approximation of the conformation in solution.

Comparison of the Two Conformations. In the case of proline containing cyclic hexapeptides the conformers with cis and trans configurated Xaa-Pro peptide bonds are usually basically different.[1j,43] This becomes apparent if one simply compares the temperature dependencies of the chemical shifts of the amide protons: major changes indicate a reorientation of the hydrogen bonds. The reason for reorientation is that proline often prefers the i + 1 position of a β-turn (L-Pro, βI; D-Pro, βII'). However, a cis peptide bond favors a βVI turn with L-Pro in the i + 2 position, thus a shift of the hydrogen bonding in the cyclic peptide occurs.[1j] The conformational parameters of both conformers of VDA008 do not indicate that (eg. Table IV). Obviously the

(49) Wüthrich, K.; Billeter, M.; Braun, W. *J. Mol. Biol.* **1983**, *169*, 949–961.

(50) Kessler, H.; Bernd, M.; Kogler, H.; Zarbock, J.; Sørensen, O.; Bodenhausen, G.; Ernst, R. R. *J. Am. Chem. Soc.* **1983**, *105*, 6944–6952.

(51) Chou, P. Y.; Fasman, G. D. *J. Mol. Biol.* **1977**, *115*, 135–175.

(52) Némethy, G.; Sheraga, H. A. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 320–327.

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D-amino acid has a strong tendency to be in position $i + 1$ of βII' turn.

Comparison of the backbone angles of both conformers shows good agreement within the βII' turn region (Table IX). In the Ramachandran diagram (Figure 8) it is easier to visualize which amino acids have similar φ/ψ angle combinations. Additionally, angles of various ideal turns are given (differently hatched circles). One finds that D-A^1 and F^2 have angles very close to those of an ideal βII' turn (not to be confused with the area of a βI turn). Further K^4 and W^5 have angles close to that of an ideal βII turn; but W^5 is at position $j + 1$ of a γ turn as well. Figure 8 shows the large difference between the φ/ψ angles of tryptophan in the major and minor populated conformations. Valine also exhibits a distinct difference. The prominent cis configurated lysine-tryptophan peptide bond can be seen in Figure 7 (below). Comparison of various analogue peptides[1i,53] indicates that the valine in position 3 is responsible for that trans-cis isomerization. The H_α,H_β coupling constant of valine is significantly smaller in the minor populated conformer than in the major populated conformer (Table III). Hence the steric environment must be different in both conformers. The side-chain conformation of valine 3 shown here need not be the dominant conformation in solution. Apparently the rather bulky side chain of valine has a strong influence on the overall conformation. However, one should keep in mind that the solvent and even traces of water in a sample probably do have an influence on the equilibrium between the conformers. This property has been demonstrated with peptides that are soluble in more than one solvent.[54]

Experimental Section

Synthetic Methods. Boc-Trp-Phe-D-Ala-Phe-Val-Lys(Z)-N₂H₃ (M_r **1045**). Esterification of Boc-Lys(Z)-OCs to chloromethylated polystyrene-1% divinylbenzene[55] yielded a resin with a capacity of 0.8 mmol of amino acid/g. Then 5.5 g (4.4 mmol) of resin were used for solid-phase synthesis, which was carried out with a manual shaking apparatus. Boc cleavage proceeded within 30 min through a single treatment with 5 mL of trifluoroacetic acid and 0.25 mL of methane sulfonic acid in 40 mL of dichloromethane. After neutralization of the amino trifluoroacetic acid salt with excess diisopropylethylamine in dichloromethane, each coupling was performed with 10 mmol of Boc-amino acid, 10 mmol of dicyclohexylcarbodiimide, and 10 mL of 1-hydroxybenzotriazole in 45 mL of dichloromethane; the coupling time was 2 h. For coupling of Boc-Trp-OH, diisopropylcarbodiimide was used instead of dicyclohexylcarbodiimide. After several washing steps, hydrazinolysis was carried out overnight in the shaking vessel by using 5 mL of hydrazine in 50 mL of dimethylacetamide. The resin was then filtered off and washed well with dimethylacetamide, and the combined solutions were evaporated in vacuo. Precipitation of the product occurred by addition of water: yield after drying, 3.68 g (3.5 mmol, 80% of theory); mp 140–143 °C; [α]²⁰_D –18.5 (c = 1, dimethylformamide); single spots in TLC; correct ¹H NMR spectrum.

H-Trp-Phe-D-Ala-Phe-Val-Lys(Z)-N₂H₃·2(trifluoroacetic acid) (M_r **1173**). Final Boc cleavage was achieved by treatment of 2.0 g of Boc-Trp-Phe-D-Ala-Phe-Val-Lys(Z)-N₂H₃ (1.91 mmol) with 9 mL of neat trifluoroacetic acid for 30 min. Precipitation of the product occurred by addition of ether: yield, 2.17 g (1.85 mmol, 97% of theory); mp 152–154 °C; [α]²⁰_D –4.1 (c = 1, methanol); single spots in TLC; correct ¹H NMR spectra.

cyclo(-Trp-Phe-D-Ala-Phe-Val-Lys(Z)-) (M_r **913**). For azide cyclization, 1.27 g of the deprotected peptide (1.08 mmol) was treated with 0.44 mL of concentrated HCl and 0.22 mL of isopentyl nitrite in 5 mL of dimethylformamide for 120 min at –30 to –15 °C; this solution was then diluted with 1 L of cold dimethylformamide and kept for 5 days at 4 °C after addition of 1.30 mL of diisopropylethylamine. Evaporation of the solvent was then followed by treatment with mixed bed ion exchange resin and gel chromatography on sephadex LH 20. Cyclization yield, 0.80 g (0.87 mmol, 81% of theory); final purification by HPLC (Nucleosil C 18, 5 μm, 16 × 250 mm, UV 254 nm, flow rate 5 mL/min, 60% acetonitrile/water); mp 126–130 °C; [α]²⁰_D –51.0 (c = 0.5, methanol); TLC, R_f n-butanol/acetic acid/H₂O (3:1:1) 0.91, ethyl acetate/CHCl₃/methanol (4:2:1) 0.35, CHCl₃/methanol/acetic acid (80:20:3)

0.88, ethyl acetate/n-butanol/pyridine/H₂O (20:10:3:5) 0.93; FAB-MS, 914 (M⁺ + H).

NMR Methods. Proton and carbon spectra were recorded on Bruker spectrometers (AM 250, WH 270, AM 500, AM 600) and processed on Bruker work stations with Aspect 1000 or X32 computers. For the proton spectra a degassed 64 mM sample of VDA008 in a 5-mm tube was used if not otherwise stated. For the heteronuclear experiments a nondegassed 60 mM sample in a 10-mm tube was used. All spectra were recorded at 300 K.

DQF-H,H-COSY (250 MHz):[25,26] pulse sequence $\pi/2_\varphi$–t_1–$\pi/2_\varphi$–Δ_1–$\pi/2$–acq.; 610 experiments of 2 dummy-scans and 48 scans each, relaxation delay 1.5 s, acquisition time for one scan 0.8 s, $\Delta_1 = 4$ μs, complex size 4K, spectral width in F_2 and F_1 2564 Hz, zerofilling in F_1 to 2K, apodization in both dimensions with squared sinebell shifted by $\pi/3$.

TOCSY (250 MHz):[29,30] pulse sequence $\pi/2$–t_1–MLEV-17–acq.; 580 experiments of 2 dummy-scans and 56 scans each, relaxation delay 1.5 s, acquisition time for one scan 0.8 s, 5 kHz MLEV-17 spin-lock with 30.6 ms mixing time, complex size 4K, spectral width in F_2 and F_1 2564 Hz, zerofilling in F_1 to 2K, apodization in both dimensions and squared sinebell shifted by $\pi/2$.

ROESY (250 MHz):[21-23] pulse sequence $\pi/2$–t_1–(β–τ)$_n$–acq.; 750 experiments of 2 dummy-scans and 56 scans each, relaxation delay 1.8 s, acquisition time for one scan 0.7 s, 2 kHz spin-lock with 100 ms mixing time, $\beta = 13°$, complex size 4K, spectral width in F_2 and F_1 3145 Hz, zerofilling in F_1 to 2K, apodization with squared sinebell shifted by $\pi/3$ (F_1) and $\pi/2$ (F_2).

NOESY (500 MHz):[24] pulse sequence $\pi/2$–t_1–$\pi/2$–Δ_1–$\pi/2$–acq.; 700 experiments of 2 dummy-scans and 32 scans each, relaxation delay 2.5 s, acquisition time for one scan 0.4 s, 200 ms mixing time with a maximum of ±20 ms random variation, complex size 4K, spectral width in F_2 and F_1 5050 Hz, zerofilling in F_1 to 2K, apodization with squared sinebell shifted by $\pi/2$ (F_1) and $\pi/3$ (F_2).

H,C-COLOC (300 MHz):[31] carbonyl carbon resonances observed; pulse sequence $\pi/2$(¹H)–t_1/2–π(¹H,¹³C)–[Δ_1–t_1/2]–$\pi/2$(¹H,¹³C)–Δ_2–acq.(¹³C,¹H=CPD); 150 experiments of 2 dummy-scans and 128 scans each, relaxation delay 2.2 s, acquisition time for one scan 1.2 s, $\Delta_1 = 25$ ms, $\Delta_2 = 30$ ms, complex size 0.5K, spectral width in F_2 206 Hz and in F_1 3030 Hz, zerofilling in F_1 and F_2 to 2K, apodization in F_2 with line broadening of 2 Hz and in F_1 with unshifted sinebell, power spectrum.

Molecular Dynamics Calculations. The program used was GROMOS.[45] The distances were used in an unsymmetrical pseudopotential as constraints.[48] The force constant for the upper distance range of the pseudopotential was $K_{dc} = 2000$ kJ·mol⁻¹·nm⁻², and it was twice as large for the lower range (for the minor conformer a fourth of these values were used). The MD calculation was carried out over 50 ps in 0.02-ps steps. During the first picosecond strong coupling to a thermal bath of 1000 K was applied (temperature relaxation time = 0.01 ps). Afterwards, the temperature was lowered stepwise to 300 K and the thermal coupling reduced (temperature relaxation time 0.1 ps). These first 10 ps were used for equilibration of the molecule. The following 40 ps were used for averaging. The averaged structure was relaxed by two energy minimization steps (EM).

Registry No. Boc-Lys(z)-OCs, 77236-12-5; Boc-Trp-OH, 13139-14-5; Boc-Val-OH, 13734-41-3; Boc-Phe-OH, 13734-34-4; Boc-D-Ala-OH, 7764-95-6; Boc-Trp-Phe-D-Ala-Phe-Val-Lys(z)-N₂H₃, 127819-50-5; H-Trp-Phe-D-Ala-Phe-Val-Lys(z)-N₂H₃·2TFA, 127819-52-7; cyclo(-Trp-Phe-D-Ala-Phe-Val-Lys(z)-), 127819-53-8.

Supplementary Material Available: DEPT-H,C-COSY spectrum of the aliphatic region, the H_α,H_N region of a DQF-H,H-COSY spectrum showing cross-peaks of the minor conformer in detail, the H_β,H_α region of a complementary E.COSY showing cross-peaks of the minor conformer as well, amide regions of three 1D spectra of samples of different concentrations of VDA008, and the averaged coordinates of the two conformers (10 pages). Ordering information is given on any current masthead page.

(53) Kessler, H.; Haupt, A.; Klein, M.; Wagner, K. *Peptides* **1986**; Theodoropoulos, D., Ed.; Walter de Gruyter: Berlin, FRG, 1987; pp 327–330.

(54) Kopple, K. D.; Sarkar, S. K.; Giacometti, G. *Biopolymers* **1981**, *20*, 1291–1303.

(55) Gisin, B. F. *Helv. Chim. Acta* **1973**, *56*, 1476–1482.