Tetrahedron Vol 44, No. 3, pp 745 to 759, 1988
Printed in Great Britain

0040-4020/88 $3.00 + .00
Pergamon Journals Ltd

ALANINE CONTAINING ANALOGUES OF CYCLO(-D-PRO-PHE-THR-LYS(Z)-TRP-PHE-) - CONFORMATIONALLY CONTROLLED STRUCTURE-ACTIVITY-RELATIONSHIPS -

H. Kessler, G. Gemmecker, A. Haupt, M. Klein, K. Wagner, and M. Will

Institut für Organische Chemie, J.W. Goethe-Universität,
Niederurseler Hang, D-6000 Frankfurt 50, Federal Republic of Germany.

(*Received in USA* 19 *July* 1987)

Abstract

Six analogues of the cyclic hexapeptide cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-Phe-) (**1**) were synthesized in which each amino acid was substituted by an alanine or D-alanine residue, respectively. Their conformation was determined by two-dimensional NMR-spectroscopy and refined by restraint molecular dynamics calculations (MD). The backbone conformation of five of these derivatives is identical to the parent peptide **1**. Only the peptide in which the Thr residue is substituted by an Ala residue shows a change of the backbone in the Ala-Lys-Trp-Phe region, which is obviously induced by the lack of the Thr hydroxyl group. This hydroxyl group takes part in strong intramolecular hydrogen bonds in **1**. However, the population of the side chain rotamers is almost identical in all analogues. Thus, the comparison of the activities in the inhibition of the cholate uptake of isolated hepatocytes with the conformations of these peptides leads to the result that the aromatic side chains are important to achieve high activity.

INTRODUCTION

It is a major goal of drug research to find correlations between the structure of a compound and its biological activity (1). Application of these correlations, perhaps yielding the active site of a drug, could lead to compounds with higher receptor selectivity and, as a result, fewer unwanted side reactions. A great number of procedures have been developed for quantitative structure-activity-relationships (QSAR) of small organic molecules, taking into account kinetic, lipophilic, and steric parameters (2). It has to be emphasized at this point that the mentioned parameters represent more or less global properties of the discussed drugs instead of describing local features. These are e.g. partial conformational changes, which can be induced by the various constitutional modifications. This inherent disadvantage of conventional QSAR becomes even more evident in the case of peptidic drugs. Lipophilic parameters for each kind of amino acid have been calculated and determined experimentally by different authors (3) but have not found widespread application. As a result, correlations between structure and biological activity of a peptide hormone are usually carried out by substituting amino acid residues, most commonly by alanine (4). As this procedure induces constitutional changes as well as conformational changes, it is difficult to differentiate between both effects. For an efficient structure-activity-relationship it is therefore essential to study these effects under conformational control, most preferably with the help of NMR spectroscopy. While X-ray analysis only provides a picture of the molecule in the crystalline state, modern NMR spectroscopy with subsequent computerized

structural refinement (e.g. Molecular Dynamics (MD) calculations) (5) often yields the predominant conformation in solution.

In the present article we describe the application of *conformationally controlled structure-activity-relationships* on the cyclic hexapeptide cyclo(-D-Pro[1]-Phe[2]-Thr[3]-Lys[4](Z)-Trp[5]-Phe[6]-) (**1**). This compound contains the retro sequence of the amino acid residues 7-11 of the peptide hormone somatostatin (6) bridged by D-proline. Somatostatin is known to protect tissues and organs against intoxication by various cell toxins (7) such as ethanol (8), cysteamin (9), and others (10). In addition, somatostatin prevents the formation of phalloidin induced protrusions on the membrane of hepatocytes (11) through the inhibition of the hepatocellular uptake of phalloidin and cholate (12). With respect to this effect, cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-Phe-) (**1**) is about 70 times more active than somatostatin (13,14). In order to define the contribution of each amino acid to the biological activity, we synthesized analogues of **1** in which each residue 2 to 6 was replaced successively by alanine; the D-proline was replaced by D-alanine.

SYNTHESIS

The general synthetic scheme for all these cyclic peptides involved the azide cyclization (15) of linear precursors, which were synthesized via Merrifield solid phase technique (16). The side chain functionality of lysine was protected with the benzyloxycarbonyl group. Threonine was used without side chain protection. Tryptophan was introduced as the last residue into the growing peptide chain to avoid degradation caused by the instability of this particular amino acid. The linear peptides were removed from resin as Boc-protected hydrazides on treatment with hydrazine. Cyclization was always performed in 10^{-3} molar solution in DMF after removal of the N-terminal tert.-butoxycarbonyl group with conc. trifluoro acetic acid. The protected cyclic hexapeptides were treated with mixed bed ion exchange resin to remove linear fragments and purified by gel filtration chromatography on a Sephadex LH 20 column. The analytical data of all analogues are given in the experimental section.

CONFORMATIONAL STUDIES

The identification of the ^{1}H resonances was achieved by correlated spectroscopy (H,H-COSY) (17). The ^{13}C signals were assigned by H,C-COSY (18), inverse H,C-COSY (19) or in some cases by comparison with the extensively studied parent peptide (20) (see Table 2). Tryptophan was assigned by the high-field shift of its C^δ-resonance (21). The discrimination of the Phe residues was done by sequencing the peptides either via heteronuclear long range couplings to the carbonyl carbons (22), obtainable from H,C-COLOC experiments (23), or via NOE effects (24,25).

*Table 1. ^{1}H chemical shift values [ppm], $^3J(NH,H^\alpha)$, $^3J(H^\alpha,H^\beta)$ coupling constants [Hz], and temperature dependency of the NH chemical shift values [-Δppb/K] of cyclo(-D-Pro[1]-Phe[2]-Thr[3]-Lys[4](Z)-Trp[5]-Phe[6]-) (**1**) and its alanine containing analogues **2** - **7** (H^β = highfield β-proton; $H^{\beta'}$ = lowfield β-proton). (cyclo(-D-Ala-Phe-Thr-Lys(Z)-Trp-Phe-) = **2**, cyclo(-D-Pro-Ala-Thr-Lys(Z)-Trp-Phe-) = **3**, cyclo(-D-Pro-Phe-Ala-Lys(Z)-Trp-Phe-) = **4**, cyclo(-D-Pro-Phe-Thr-Ala-Trp-Phe-) = **5**, cyclo(-D-Pro-Phe-Thr-Lys(Z)-Ala-Phe-) = **6**, cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-Ala-) = **7**.)*

Amino acid residue in position 1

Peptid	NH	H^{α}	H^{β}	H^{γ}	H^{δ}	-ppb/K	J(NH,H^{α})
1		4.11	1.44	1.26	2.52		
			1.62	1.78	3.36		
2	8.60	3.84	0.78			3.7	4.8
3		4.14	1.29	1.30	2.60		
			1.77	1.85	3.40		
4		4.06	1.41	1.31	2.66		
			1.60	1.78	3.32		
5		4.13	1.46	1.28	2.57		
			1.64	1.81	3.37		
6		4.09	1.45	1.28	2.56		
			1.61	1.79	3.35		
7		4.23	1.57	1.83	3.50		
			1.90	2.03	3.66		

Amino acid residue in position 2

Peptid	NH	H^{α}	H^{β}	$H^{\beta'}$	-ppb/K	J(NH,H^{α})	J(H^{α},H^{β})	J(H^{α},$H^{\beta'}$)
1	8.81	4.32	2.77	3.33	7.0	8.9	11.5	3.1
2	8.72	4.31	2.77	3.29	5.5	8.9	11.8	3.1
3	8.73	4.17	1.28		4.4	8.2		
4	8.76	4.22	2.72	3.22	4.8	9.0	11.8	3.0
5	8.80	4.34	2.79	3.33	5.2	8.9	11.6	3.0
6	8.71	4.32	2.78	3.34		9.6	9.5	5.2
7	8.77	4.40	2.78	3.36	4.9	9.6	10.5	4.4

Amino acid residue in position 3

Peptid	NH	H^{α}	H^{β}	H^{γ}	-ppb/K	J(NH,H^{α})	J(H^{α},H^{β})
1	7.86	4.61	4.45	1.24	3.3	9.7	3.5
2	7.69	4.57	4.41	1.22	2.0	9.7	3.8
3	7.79	4.53	4.41	1.19	2.2	9.6	3.8
4	7.97	4.43	1.40		1.8	9.0	
5	7.86	4.57	4.44	1.23	2.4	9.7	
6	7.84	4.60	4.42	1.18	2.3	10.4	3.7
7	7.92	4.65	4.45	1.28	3.4	10.4	3.7

Amino acid residue in position 4

Peptid	NH	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ϵ}	-ppb/K	J(H^{α},H^{β})
1	8.12	3.60	1.22	0.89	1.23	2.80	4.3	3.4
2	8.14	3.61	1.31	0.95	1.21	2.84	3.4	3.9
3	8.10	3.60	1.25	0.92	1.18	2.83	3.4	3.7
4	8.00	3.53	1.30/1.45	0.8/1.05	1.22	2.83	3.4	4.6
5	8.30	3.70	0.93				3.6	3.2
6	8.07	3.72	1.63	1.28	1.41	2.99	2.9	4.4
7	8.17	3.65	1.26	0.91	1.24	2.82	3.6	4.4

Amino acid residue in position 5

Peptid	NH	H^{α}	H^{β}	$H^{\beta'}$	-ppb/K	J(NH,H^{α})	J(H^{α},H^{β})	J(H^{α},$H^{\beta'}$)
1	7.32	4.40	2.93	3.20	3.0	8.9	10.9	4.2
2	7.26	4.35	2.94	3.15	1.5	8.6	10.8	4.3
3	7.26	4.42	2.93	3.19	1.8	8.8	10.8	4.0
4	7.68	4.37	3.20	3.28	3.8	8.1	10.0	4.2
5	7.38	4.38	2.98	3.20	2.2	9.0	10.6	4.0
6	7.45	4.13	1.24		1.7	8.7		
7	7.30	4.36	2.91	3.14	1.9		10.5	4.7

Amino acid residue in position 6

Peptid	NH	H^{α}	H^{β}	$H^{\beta'}$	-ppb/K	J(NH,H^{α})	J(H^{α},H^{β})	J(H^{α},$H^{\beta'}$)
1	7.08	4.62	2.76	3.26	1.0	5.8	9.0	4.5
2	7.03	4.42	2.78	3.20	0.5	5.4	8.6	5.0
3	7.16	4.64	2.76	3.27	0.9	5.4	9.4	4.3
4	7.23	4.64	2.73	3.12	0.6	6.6	9.3	4.4
5	7.10	4.64	2.78	3.26	0.8	5.6	9.0	4.4
6	7.03	4.57	2.72	3.17	0.6	5.6	8.5	4.5
7	7.03	4.49	1.25		0.6	6.4		

*Table 2. ^{13}C chemical shift values [ppm] for 1 and alanine containing analogues (except 2). *: signals were not assigned. The resonances of 3 were assigned via an inverse H,C-COSY spectrum.*

Amino acid residue in position 1

Peptid	C'	C^{α}	C^{β}	C'	C^{δ}
1	171.9	60.3	28.2	24.8	47.1
3	*	60.1	28.2	24.5	47.0
4	171.7	59.9	28.4	24.7	47.0
5	171.6	60.0	27.9	24.5	46.9
6	*	60.0	28.0	24.6	46.8
7	*	60.0	28.1	24.9	46.9

Amino acid residue in position 2 — Amino acid residue in position 3

Peptid	C'	C^{α}	C^{β}	C'	C^{α}	C^{β}	C'
1	171.4	54.6	35.9	170.5	56.8	68.9	19.6
3	*	48.0	16.8	*	56.7	68.6	19.3
4	170.9	54.7	36.0	172.6	47.2	18.5	
5	171.2	54.3	35.7	170.0	56.6	68.6	19.3
6	*	54.4	35.8	*	56.7	68.3	19.3
7	*	55.2	35.8	*	56.6	68.8	19.4

Amino acid residue in position 4

Peptid	C'	C^{α}	C^{β}	C^{γ}	C^{δ}	C^{ε}	C^{ζ}
1	171.3	55.6	29.9	22.4	29.4	40.1	156.3
3	*	55.3	29.7	22.2	29.1	39.9	*
4	172.2	56.5	30.2	22.7	29.5	39.7	156.3
5	171.9	50.8	16.0				
6	*	55.6	30.0	22.8	29.1	39.5	156.1
7	*	55.3	29.8	22.2	29.2	39.5	156.1

Amino acid residue in position 5 Amino acid residue in position 6

Peptid	C'	C^{α}	C^{β}	C'	C^{α}	C^{β}
1	171.4	55.2	28.2	169.5	53.5	38.3
3	*	54.8	27.9	*	53.3	
4	171.5	54.3	26.9	168.3	53.4	38.5
5	171.1	54.9	28.0	169.3	53.2	38.0
6	*	49.5	18.3	*	53.1	37.7
7	*	54.1	28.1	*	47.7	17.7

The conformational analysis of the peptidic backbone is based on the temperature dependency of the NH chemical shift values, the $^3J(NH,H^{\alpha})$ coupling constants, and NOE effects (26). Due to the weak intensity of the NOE signals in molecules of this size, these NOEs were determined in the rotating frame (27) by using the pulsed variant of ROESY with small flip angles to suppress J-contributions (28). Volume integration of these rotating frame NOE (ROE) cross peaks was performed to get proton-proton distances. It is essential to use an offset correction for the quantitative ROE evaluation (29). As an example, we will discuss here the conformational analysis of the (Ala)-derivative 4. This compound was chosen because its NMR parameters slightly deviate from the other cyclic peptides described here (see Table 1). The latter ones exhibit in all respects, including quantitative ROE values, a very close analogy to the parent peptide 1 (20).

The corresponding distances for the ROEs of cyclo(-D-Pro-Phe-Ala-Lys(Z)-Trp-Phe-) (4) are given in Table 3. The transformation of relative integral values into absolute distances requires at least one known distance for calibration. In this case, the distance between the NH and the H_7 proton in the indole ring system of the tryptophan residue (282 pm) was selected (20).

All NMR data, including the $-\Delta\delta/\Delta T$ values of the NH protons, suggest a backbone conformation including two internal hydrogen bridges with the NH protons of Phe^6 and Ala^3 as hydrogen donors. We therefore used the X-ray coordinates of cyclo(-D-Pro-Phe-Thr-Phe-Trp-Phe-) (20) as starting point for the refinement procedure by MD calculations.

26 proton-proton distances from ROE data were used as restraints during the calculation (30, 31). At first, the starting conformation obtained from X-ray analysis was refined by performing 400 conjugate gradient energy minimization (EM) steps (32). In addition restrained MD calculations over 10 ps were used to get further refinement of the conformation. These computer simulation technique yields the possibility to scan the constitutional space at a given temperature. Energy barriers in the range of kT (2.5 kJ at 300 K) can be surmounted in a reasonable time span. It has been shown previously that this procedure can result in a defined structure independent from the starting structure (33).

Table 3. Proton-proton distances obtained from the ROESY spectrum of **4** *recorded at 300 MHz spectrometer frequency in DMSO solution at 300 K, mixing time: 0.2 s, flip angle 17°.*

No.	Involved protons		distance [pm]	No.	Involved protons		distance [pm]
1	Phe^2-NH	D-Pro-H^α	201	14	Trp-NH	Lys-H^α	243
2	Phe^2-NH	Phe^2-H^α	267	15	Trp-NH	Lys-$H^{\beta l)}$	350
3	Phe^2-NH	Phe^2-$H^{\beta pro\text{-}R}$	249	16	Trp-NH	Trp-H^α	244
4	Phe^2-NH	Ala-NH	265	17	Trp-NH	Trp-$H^{\beta pro\text{-}R}$	292
5	Ala-NH	D-Pro-H^α	348	18	Phe^6-NH	D-Pro-H^α	310
6	Ala-NH	Phe^2-H^α	280	19	Phe^6-NH	Ala-NH	323
7	Ala-NH	Ala-H^α	253	20	Phe^6-NH	Ala-H^β	305
8	Ala-NH	Ala-H^β	228	21	Phe^6-NH	Trp-H^α	290
9	Lys-NH	Ala-H^α	220	22	Phe^6-NH	Phe^6-H^α	251
10	Lys-NH	Lys-H^α	261	23	Phe^6-NH	Phe^6-$H^{\beta pro\text{-}R}$	283
11	Lys-NH	Lys-$H^{\beta l)}$	267	24	Phe^6-NH	Phe^6-$H^{\beta pro\text{-}S}$	230
12	Lys-NH	Lys-$H^{\beta h)}$	300	25	Phe^6-H^α	D-Pro-$H^{\delta l)}$	227
13	Lys-NH	Trp-NH	332	26	Phe^6-H^α	D-Pro-$H^{\delta h)}$	220

[l)] and [h)] indicate low- and highfield diastereotopic protons.

Table 4. Hydrogen bonds[a)] in **4** *averaged over 40 ps molecular dynamics run.*

Donor	Acceptor	r(H-A)[b)] [pm]	Θ[c)] [°]	population [%]
Ala^3-NH	Phe^6-CO	217	151.9	56.2
Phe^6-NH	Lys^4-CO	198	146.4	79.3
Phe^6-NH	Ala^3-CO	217	146.1	9.3

[a)] Definition of hydrogen bridges: Donor-H Acceptor is shorter than 300 pm and the corresponding Θ angle is greater than 120° degrees. [b)] Distance between XH-Donor and Y-Acceptor. [c)] Θ angle between (Donor-H Acceptor).

All calculations were performed in vacuo, but solution conditions were simulated by the use of the experimental proton-proton distance restraints obtained from the ROE measurements. The restraints were built in as an additional harmonic potential. In the beginning a strong force constant (K_{dc} = 4000 kJ·mol^{-1}·nm^{-2}) was applied to force the molecule into the correct conformation, the temperature bath being set to 1000 K. After 1 ps of calculation the force constants K_{dc} and the temperature were decreased to 2000 kJ·mol^{-1}·nm^{-2} and 300 K. The molecule was allowed to relax for 9 ps, and the following 40 ps MD run was evaluated to get a refined 3D-structure. The proton-proton distances were also well represented by the simulation: the averaged distance violation was only 15 pm. The time averaged structure of **4** is shown in Figure 1.

The probability of hydrogen bonding in the MD run corresponds well with the observed temperature dependence of the NH chemical shifts. Hydrogen bonding is no explicit input of the MD calculation, but it is a result of the calculation if one defines certain distances and angles as a "bond" (Table 4).

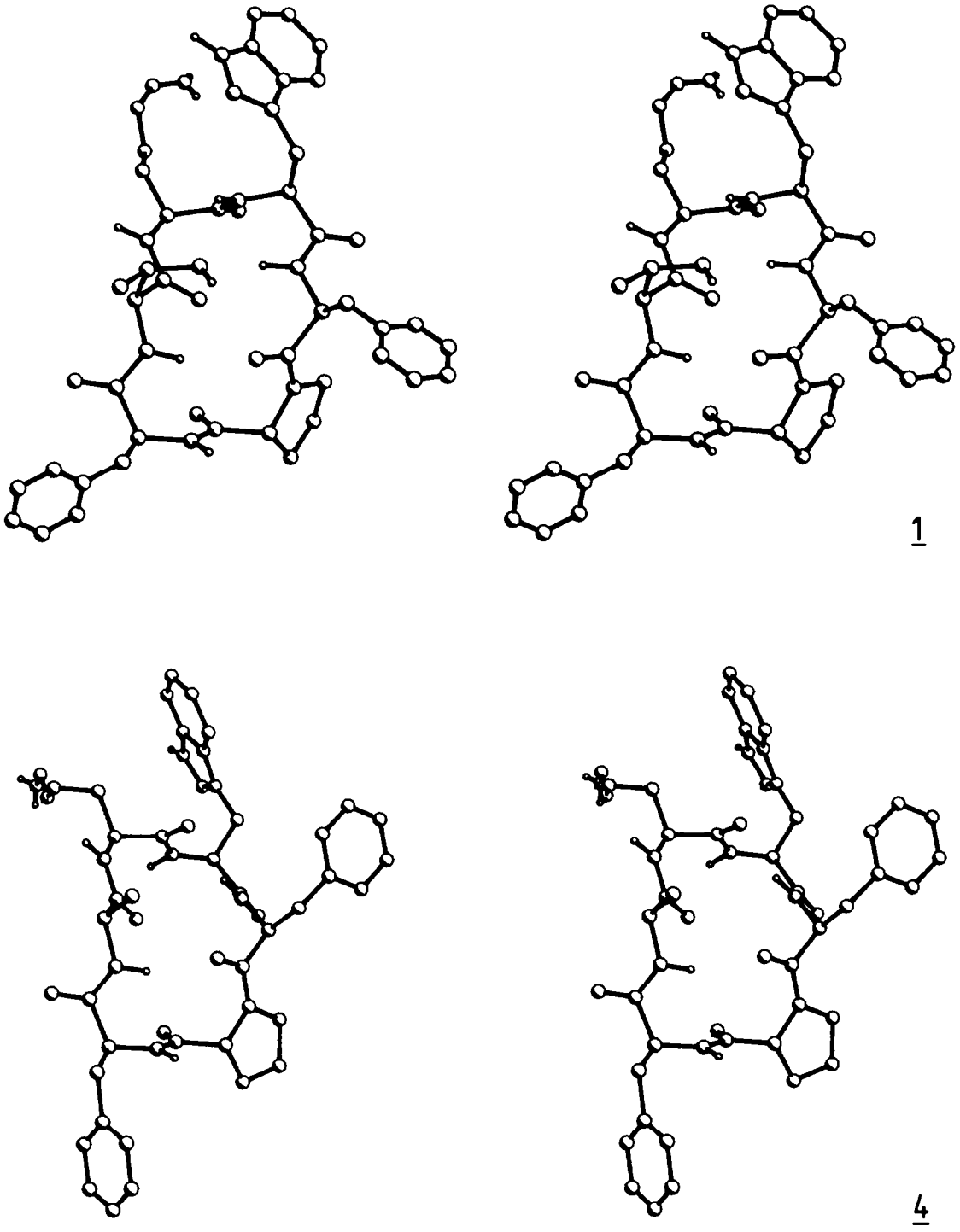

Fig. 1. Backbone conformations of cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-Phe-) (1) and cyclo(-D-Pro-Phe-Ala-Lys(Z)-Trp-Phe-) (4) averaged over 40 ps restraint MD calculations in vacuo. Please note that the conformation of the side chains (except for Thr) may differ considerably from those shown in the figure (see text).

The side chains can rotate more or less during the calculations. In cases where NOEs to stereospecifically assigned β-protons are included in the calculation the most probable side chain conformation results. On the other hand, calculations in vacuo tend to emphasize hydrophobic and Coulomb interactions, and side chains are folded over the backbone. This has been found in many of our calculations in vacuo, e.g. for the side chain of MeBmt in cyclosporin (34) and also for **1** (20). To our opinion, side chain rotamer populations about χ_1 (C^α-C^β bond) can only be derived unambiguously from coupling constants (35,36,37).

The corresponding H^α,$H^{\beta pro\text{-}S}$ and H^α,$H^{\beta pro\text{-}R}$ coupling constants were extracted from phase-sensitive double quantum filtered H,H-COSY spectra by application of the DISCO technique (38). The discrimination between the two rotamers with $\chi_1 = -60°$ and $\chi_1 = 180°$ was achieved by using the different heteronuclear couplings of the two β-protons to the carbonyl carbon. In conformation I ($\chi_1 = -60°$) both β-protons show only a small coupling to the carbonyl carbon due to their synclinal orientation, whereas in conformation II ($\chi_1 = 180°$) there is a large antiperiplanar coupling between the *pro-R* β-proton and the carbonyl carbon. The differentiation between both alternative conformations can easily be done by evaluation of the intensity of the H^β,C' cross peak in a routinely performed H,C-COLOC spectrum (36,37). According to this method, Phe^6 adopts conformation II, while Phe^2 and Trp^5 prefer conformation I.

The same procedure was used for **1** and **4**, for the other derivatives only proton coupling constants were used and the assignment of the β-protons was done by comparison by **1**. This seems reliable to us because all ^1H- and ^{13}C-parameters exhibit so close similarity that an interchange of the β-protons is highly improbable. All peptides **1** - **7** show similar side chain conformations in the corresponding residues.

In conclusion, the results show that the influence of the alanine substitutions on the peptide conformation can be neglected. An exception is present in the Thr^3 substituted compound where a conformational change in the Lys-Trp substructure is observed. This is due to the fact that the threonine hydroxyl group was found to take part in intramolecular hydrogen bonds (described in detail for **1** (20)) thus stabilizing a structure as shown in Fig. 1. In **1** we found a strong hydrogen bond between Trp^5-NH and Thr-OH forcing this NH slightly above the backbone plane. The missing of this hydroxyl group leads to a twisting of the Lys-Trp peptide bond, thus forming a γ-turn between Phe^6-NH and Lys^4-CO (see Figure 1.).

Substitution of D-Pro by D-Ala has no influence on the conformation. This is not surprising as a peptide sequence with a D-amino acid has a strong tendency to form a βII'-loop with the D-configurated residue in position i + 1.

STRUCTURE IN H_2O

In order to check the solvent's influence on the conformation in solution, additional NMR measurements of the analogues **3** and **4** were carried out in aqueous solution. In both peptides the ε-amino function of Lys^4 had been deprotected to achieve sufficient solubility in water.

The two cyclic peptides gave ^1H-NMR spectra very similar to those of the corresponding Z-protected analogues in DMSO solution. A COSY spectrum in D_2O provided a complete identification of all proton spin systems except for the connectivity to the NH-protons. The AMXY systems of the aromatic amino acids could not be differentiated. The identification of the Trp^5 spin system resulted from homonuclear long range coupling of the Trp^5 β-protons to the aromatic protons of the indole system which gave rise to cross peaks in a COSY spectrum optimized for small couplings. It was not possible to differentiate between Phe^2 and Phe^6 in analogue **4**, because the concentration in the

sample was to low to detect NOE effects or to aquire heteronuclear 2D spectra. However, from a comparison of the NMR parameters in DMSO and water it seems quite reasonable to assign the Phe residues as shown in Table 5.

Table. 5. Comparison of the NMR parameters of 3 *and* 4 *in DMSO* (upright letters) *and water (italic letters).*

Amino acid residue in position 1

Peptid	NH	H^{α}	H^{β}	H^{γ}	H^{δ}
3		4.14	1.29/1.77	1.30/1.85	2.60/3.40
		4.21	*1.88/2.05*	*1.50/1.93*	*2.93/3.49*
4		4.06	1.41/1.60	1.31/1.78	2.66/3.32
		4.08	*1.56/1.83*	*1.42/1.78*	*2.77/3.42*

Amino acid residue in position 2

Peptid	NH	H^{α}	H^{β}	-ppb/K	$^3J(NH,H^{\alpha})$	$^3J(H^{\alpha},H^{\beta})$
3	8.73	4.17	1.28	4.4	8.2	
	8.83	*4.38*	*1.45*	*8.9*	*7.7*	
4	8.76	4.22	2.72/3.22	4.8	9.0	12.0/3.7
	8.53	*4.57*	*2.89/3.33*	*7.4*	*8.1*	*10.5/4.3*

Amino acid residue in position 3

Peptid	NH	H^{α}	H^{β}	H^{γ}	-ppb/K	$^3J(NH,H^{\alpha})$	$^3J(H^{\alpha},H^{\beta})$
3	7.79	4.53	4.41	1.19	2.2	9.6	3.8
	8.22	*4.66*	*4.44*	*1.26*	*3.6*	*10.2*	*3.8*
4	7.97	4.43	1.40		1.8	9.0	
	8.08	*4.48*	*1.23*		*2.0*	*8.7*	

Amino acid residue in position 4

Peptid	NH	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ϵ}	-ppb/K	$^3J(NH,H^{\alpha})$
3	8.1	3.60	1.25	0.92	1.18	2.83	3.4	3.7
	8.25	*3.79*	*1.13/1.33*	*0.68*	*1.32*	*2.65*	*6.9*	*3.5*
4	8.0	3.53	1.30/1.45	0.93	1.22	2.83	3.4	4.6
	7.98	*3.76*	*1.37/1.54*	*0.93/0.78*	*1.42*	*2.75*	*5.8*	*3.4*

Amino acid residue in position 5

Peptid	NH	H^{α}	H^{β}	-ppb/K	$^3J(NH,H^{\alpha}$	$^3J(H^{\alpha},H^{\beta})$
3	7.26	4.42	2.93/3.19	1.8	8.8	10.8/4.0
	7.44	*4.70*	*3.06/3.44*	*4.2*	*	*10.8/4.3*
4	7.68	4.37	3.16/3.28	3.8	8.1	10.0/4.4
	7.58	*4.76*	*3.25/3.38*	*5.8*	*8.7*	*8.6/5.0*

Amino acid residue in position 6

Peptid	NH	H^{α}	H^{β}	-ppb/K	$^3J(NH,H^{\alpha})$	$^3J(H^{\alpha},H^{\beta})$
3	7.16	4.64	2.76/3.27	0.9	5.4	9.4/4.3
	7.17	*4.84*	*2.95/3.30*	*1.9*	*	*8.8/4.3*
4	7.23	4.54	2.73/3.12	0.6	6.6	9.3/4.4
	7.01	*4.79*	*2.79/3.23*	*1.6*	*5.8*	*8.9/5.2*

The assignment of the exchangeable amide protons of Phe[2], Thr[3], and Lys[4] was based on a phase sensitive COSY spectrum in H_2O, which was recorded with solvent suppression by rf irradiation. However, the H^{α} signals of Trp[5] and Phe[6] were very close to the water resonance and therefore also saturated by the rf irradiation during t_1. The resulting extinction of the NH,H^{α} cross peaks at $\omega_1 = \omega(H^{\alpha})$ made the assignment of the amide protons of Trp[5] and Phe[6] impossible, since the corresponding cross peaks at $\omega_2 = \omega(H^{\alpha})$ disappeared in the t_1 noise of the nearby solvent signal.

Fortunately, a 2D ^{1}H-INADEQUATE experiment does not suffer from such extinction phenomena, because no net magnetization of the H^{α} is required. The pulse sequence allows a magnetization transfer from the NH-proton via NH,H^{α} double quantum coherence during t_1 back to the NH-proton, where it can be detected without interference with the residual H_2O signal, giving rise to a cross-peak at $\omega_1 = \omega(NH) + \omega(H^{\alpha})$ and $\omega_2 = \omega(NH)$.

This method led to a straightforward assignment of the amide protons of all residues in both cyclic hexapeptides. The relation of the solvent shielding of the single amino acid positions in the peptide remains almost constant (although the temperature coefficients of the NH chemical shifts in water are about twice as large as in DMSO). The same is true for the chemical shift values of the NH protons. The coupling constants $^3J(NH,H^{\alpha})$, $^3J(H^{\alpha},H^{\beta\text{high field}})$ and $^3J(H^{\alpha},H^{\beta\text{low field}})$ are nearly identical in both solvents. It was not possible to measure NOE effects in water, but a comparison of all parameters from Table 5 shows that both peptides under investigation obviously adopt a very similar conformation in water and DMSO. Because of the great similarity between the analogues discussed in this paper, the same behaviour in aqueous solution can be expected for all of them.

BIOLOGICAL ACTIVITIES

Inhibition of phalloidin and cholate uptake in isolated hepatocytes are strongly correlated. Therefore, the latter test is sufficient for the determination of the biological activities and was applied to the six analogues. The results are summarized in Table 6.

Table 6. Inhibition of cholate uptake by the alanine-derivatives of **1**

No.	COMPOUND	CD_{50} (μM)
1	cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-Phe-)	1.5
2	cyclo(-**D-Ala**-Phe-Thr-Lys(Z)-Trp-Phe-)	1.0
3	cyclo(-D-Pro-**Ala**-Thr-Lys(Z)-Trp-Phe-)	5.0
4	cyclo(-D-Pro-Phe-**Ala**-Lys(Z)-Trp-Phe-)	0.5
5	cyclo(-D-Pro-Phe-Thr-Ala -Trp-Phe-)	4.0
6	cyclo(-D-Pro-Phe-Thr-Lys(Z)-**Ala**-Phe-)	7.0
7	cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-**Ala**-)	3.7

CD_{50} is the concentration resulting in a 50% inhibition of cholate uptake.

CONCLUSIONS

The identical conformations of the peptides **1** - **7** and the independence of the main conformational features from the nature of the solvent represent an ideal condition to determine the influence of each amino acid on biological activity in terms of steric and lipophilic effects.

Changes in biological activity due to the alanine substitutions were surprisingly low: The most active and the least active compound for example do only differ in one order of magnitude concerning cholate uptake inhibition. However, the following conclusions may be drawn: substitution of each of the aromatic amino acid residues by alanine leads to a substantial decrease of biological activity. This emphasizes the importance of these amino acids for the cytoprotective effect. It is already known for the lysine position that large aromatic modifications like the Z-group have an increasing influence on the biological activity. Therefore, substitution of this residue by alanine also yields the expected low activity.

ACKNOWLEDGEMENTS

We thank Prof. M. Frimmer and Dr. K. Ziegler (University of Giessen) for the performance of the biological testing. G.G. thanks the *Studienstiftung d. Deutschen Volkes* for a grant. The authors are indepted to M. Gehrke for introduction in the use of personal computers. We greatfully acknowledge financial support by the *Deutsche Forschungsgemeinschaft*, the *Bundesministerium für Forschung und Technologie* and the *Fonds der Chemischen Industrie*.

EXPERIMENTAL SECTION

Synthesis

The linear Boc-protected hexapeptide hydrazides were synthesized and cyclized as described before. Melting points were determined on a Kofler melting point apparatus. The values are uncorrected. Optical activity was measured by determining the optical rotations at 589.3 nm corresponding to the sodium D line with a Perkin Elmer polarimeter model 141. The purity of the compounds was assessed by t.l.c. using silica gel plates with fluorescent indicator (254 nm, Riedel de Haën) with the following solvent systems:

A	n-butanol/acetic acid/methanol	3 : 1 : 1
B	chloroform/methanol/acetic acid	95 : 5 : 3
C	chloroform/methanol/acetic acid	80 : 20 : 3.

Column chromatography was done via gel filtration using Sephadex LH 20 (Pharmacia Fine Chemicals).

Boc-Trp-Phe-D-Ala-Phe-Thr-Lys(Z)-$NHNH_2$

yield: 1.94 g (1.85 mmol) with 2.5 g hydroxymethylated resin, m.p.: 112-113°C, t.l.c.: R_f(A) 0.79, R_f(B) 0.20, R_f(C) 0.74, $[\alpha]^{20}$: -0.38° (c = 0.264, DMF).

cyclo(-D-Ala-Phe-Thr-Lys(Z)-Trp-Phe-) (2)

1.8 mmol linear Boc-protected hexapeptide hydrazide were cyclized. yield: 350 mg (0.383 mmol, 21.3%), m.p.: 133-136°, t.l.c.: R_f(B) 0.19, R_f(C) 1.0, $[\alpha]^{20}$: -16.0° (c = 0.181, DMF), FAB-MS: 916 $(M+H)^+$.

Boc-Trp-Phe-D-Pro-Ala-Thr-Lys(Z)-NHNH$_2$

yield: 1.2 g (1.2 mmol) with 2.5 g hydroxymethylated resin, m.p.: 97-101°C, t.l.c.: R_f(A) 0.84, R_f(B) 0.22, R_f(C) 0.82, $[\alpha]^{20}$: -0.0° (c = 0.04, DMF).

cyclo(-D-Pro-Ala-Thr-Lys(Z)-Trp-Phe-) (3)

1.1 mmol linear Boc-protected hexapeptide hydrazide were cyclized. yield: 195 mg (0.23 mmol, 20.5%), m.p.: 148-150°C, t.l.c.: R_f(B) 0.05, R_f(C) 0.65, $[\alpha]^{20}$: -29.3° (c = 0.092, methanol), FAB-MS: 865 $(M+H)^+$.

Boc-Trp-Phe-D-Pro-Phe-Ala-Lys(Z)-NHNH$_2$

yield: 2.2 g (2.1 mmol) with 3 g hydroxymethylated resin, m.p.: 123-128°C, t.l.c.: R_f(A) 0.78, R_f(B) 0.61, R_f(C) 0.87, $[\alpha]^{20}$: -19.2° (c = 1, methanol).

cyclo(-D-Pro-Phe-Ala-Lys(Z)-Trp-Phe-) (4)

1 mmol linear Boc-protected hexapeptide hydrazide were cyclized. yield: 285 mg (0.74 mmol, 74%), m.p.: >200°C, t.l.c.: R_f(A) 0.82, R_f(B) 0.45, R_f(C) 0.85, $[\alpha]^{20}$: -40° (c = 0.25, methanol), FAB-MS: 911 $(M+H)^+$.

Boc-Trp-Phe-D-Pro-Phe-Thr-Ala-NHNH$_2$

yield: 1.8 g (2.0 mmol) with 3 g hydroxymethylated resin, m.p.: 190-200°C, t.l.c.: R_f(A) 0.75, R_f(B) 0.47, R_f(C) 0.82, $[\alpha]^{20}$: -14.2° (c = 0.3, methanol).

cyclo(-D-Pro-Phe-Thr-Ala-Trp-Phe-) (5)

1 mmol linear Boc-protected hexapeptide hydrazide were cyclized. yield: 674 mg (0.384 mmol, 38%), m.p.: > 250°C, t.l.c.: R_f(A) 0.71, R_f(B) 0.29, R_f(C) 0.78, $[\alpha]^{20}$: -35.8° (c = 0.2, methanol), FAB-MS: 750 $(M+H)^+$.

Boc-Phe-D-Pro-Phe-Thr-Lys(Z)-Ala-NHNH$_2$

yield: 1.76 g (1.81 mmol) with 3.0 g hydroxymethylated resin, m.p.: 165-168°C, t.l.c.: R_f($CHCL_3$/methanol 9:1) 0.41, $[\alpha]^{20}$: 2.9° (c = 0.95, DMF).

cyclo(-D-Pro-Phe-Thr-Lys(Z)-Ala-Phe-) (6)

1.5 mmol linear Boc-protected hexapeptide hydrazide were cyclized. yield: 800 mg (0.993 mmol, 66%), m.p.: 146-148°C, t.l.c.: R_f(B) 0.37.

Boc-Trp-Ala-D-Pro-Phe-Thr-Lys(Z)-NHNH$_2$

yield: 1.00 g (0.99 mmol) with 3.0 g hydroxymethylated resin, m.p.: 108-111°C, t.l.c.: R_f(B) 0.12, $[\alpha]^{20}$: 5.1° (c = 0.95, DMF).

cyclo(-D-Pro-Phe-Thr-Lys(Z)-Trp-Ala-) (7)

0.8 mmol linear Boc-protected hexapeptide hydrazide were cyclized. yield: 290 mg (0.300 mmol, 37%), m.p.: 160-163°C, t.l.c.: R_f(A) 0.69, R_f(B) 0.13.

NMR experimental parameters

The NMR experimental parameters are shown exemplarily for the measurements of cyclo(-D-Pro-Phe-Ala-Lys(Z)-Trp-Phe-) (4) performed on a 300 MHz spectrometer (Bruker AM 300) equipped with an Aspect 1000 data station. The proton measurements were done with a degassed 15 mg sample dissolved in 0.5 ml DMSO, the heteronuclear experiments were performed with a 72 mg sample dissolved in 2.5 ml DMSO.

DQF-COSY (300 MHz): $\pi/2$, t_1, $\pi/2$, Δ_1, $\pi/2$, acquisition.

1024 experiments of 16 scans each, relaxation delay 1.5 s, Δ_1 = 4 μs, size 4 K, spectral width in F2 and F1 3400 Hz, zerofilling to 8 K in F2 and to 2 K in F1, apodization in both dimensions with squared shifted sinebell by $\pi/4$.

ROESY (300 MHz): $\pi/2$, t_1, $(\beta\text{-}\tau)_n$, acquisition.

800 experiments of 16 scans each, relaxation delay 2.5 s, β = 2.5 μs, n = 7272, total mixing time 200 ms, size 4 K, spectral width in F2 and F1 4000 Hz, zerofilling in F2 to 8 K and in F1 to 4 K, squared cosine multiplication in both dimensions, automatic baseline correction with Bruker software.

H,C-COSY (300 MHz): $\pi/2(^1H)$, $t_1/2$, π (^{13}C), $t_1/2$, Δ_1, $\pi/2$ (^1H, ^{13}C), Δ_2, acq.(^{13}C, ^1H-BB).

192 experiments of 128 scans each, relaxation delay 1.5 s, Δ_1 = 3.7 ms, Δ_2 = 2.5 ms, size 4 K, spectral width in F2 4500 Hz, in F1 1600 Hz, zerofilling to 8 K in F2, exponential multiplication (linebroadening of 1.2 Hz), zerofilling to 0.5 K in F1, squared cosine multiplication.

H,C-COLOC (300 MHz): $\pi/2$ (^1H), $t_1/2$, π (^1H, ^{13}C), Δ_1-$t_1/2$, $\pi/2$ (^1H, ^{13}C), Δ_2, acq.(^{13}C, ^1H-BB).

128 experiments of 256 scans each, relaxation delay 1.5 s, Δ_1 = 25 ms, Δ_2 = 30 ms, size 1 K, spectral width in F2 600 Hz, in F1 2900 Hz, zerofilling to 2 K in F2, exponential multiplication (linebroadening of 1.2 Hz), zerofilling to 0.5 K in F1, sinebell multiplication.

NMR measurements in water

6 mg samples of the Lys(Z) deprotected cyclic peptides were dissolved in 0.5 ml of D_2O/10% CD_3COOD, TSP (sodium salt of 3-(trimethylsilyl)-propionic acid-d_4) was added as reference substance. The amide protons were assigned by 2D H,H-INADEQUATE spectra of a solution of 6 mg of peptide in 0.5 ml H_2O/10% CD_3COOD/TSP. Oxygen was removed by bubbling N_2 through the solutions.

H,H-INADEQUATE (300 MHz): $\pi/2$, Δ_1, π, Δ_1, $\pi/2$, t_1, 120°, acquisition.

512 experiments of 64 scans each, relaxation delay 1.2 s, Δ_1 = 15 ms, size 4K, spectral width in F2 and F1 3500 Hz, solvent saturation by CW irradiation during relaxation delay (20 mW), Δ_1 and t_1 (2 mW), zerofilling to 2K in F1, apodization in both dimensions with squared shifted sinebell by $\pi/4$.

MD calculation conditions

All calculations were performed on a VAX 8300 using the GROMOS package. Initial velocities were taken from a Maxwell distribution at 1000 K and after the overall translational and rotational motion of the molecule was stopped, the MD simulation over 10 ps was started. A temperature of 1000 K and a strong coupling to a thermal bath (temperature relaxation time = 0.001 ps) were applied during the first picosecond. In addition a weak force constant (K_{dc} = 4000 kJ·mol^{-1}·nm^{-2}) was used. In the next 9 ps the temperature was decreased to 300 K and the temperature relaxation time was stepwise increased to 0.01 ps. Furthermore, a force constant of (K_{dc} = 2000 kJ·mol^{-1}·nm^{-2}) was applied. The MD time step was 0.002 ps, this step size is possible by using the SHAKE routine. The time span from 10 ps to 50 ps was analyzed.

References

1. Conformational Directed Drug Design, Vida, J.A.; Gordon, M.; (Ed.), ACS Symposium Series, Washington D.C. 1983.

2. Chemische Struktur und biologische Aktivität von Wirkstoffen, Seydel, J.K.; Schaper, K.-J.; Verlag Chemie, Weinheim, 1979

3. QSAR and Strategies in the Design of Bioactive Compounds, Seydel, J.K.; Proceedings of the 5th European Symposium on Qualitative-Structure-Activity Relationships, Verlag Chemie, Bad Segeberg, 1984

4. Nutt, R.; in "Peptides", Proceedings of the 10th American Peptide Symposium, Marshall, G. (ed.); Pierce Chem. Comp., Rockford, Ill., in press

5. Molecular Dynamics and Protein Structure, Hermans, J.; (Ed.), Polycrystal Book Service, Western Springs, Ilinois, USA, 1985

6. Brazeau, P.; Vale, W.; Burgus,R.; Ling, N.; Butcher, M.; Rivier, J.; Guillemin, R.; Science, **1973**,*179*,77-79

7. Usadel, K.H.; Kessler, H.; Rohr, G.; Kusterer, K.; Palitzsch, K.D.; Schwedes, U.; Klin. Wochenschr. **1986**,*64*,59-63

8. Kusterer, K.; Rohr, G.; Schwedes, U.; Usadel, K.H.; Szabo, S.; Klin.Wochenschr. **1986**,*64*,97-99

9. Palitzsch, K.D.; Schuler, H.; Schwedes, U.; Usadel, K.H.; Klin. Wochenschr. **1986**,*64*,93-96

10. Limberg, B.; Kommerell, B.; Klin. Wochenschr. **1986**,*64*,64-65

11. Rao, G.S.; Lemoch, H.; Kessler, H.; Damm, I.; Eiermann, V.; Koll, S.; Zarbock, J.; Usadel, K.H.; Klin. Wochenschr. **1986**,*64*,79-86

12. Ziegler, K.; Frimmer, M.; Klin. Wochenschr. **1986**,*64*,87-89

13. Ziegler, K.; Frimmer, M.; Kessler, H.; Damm, I.; Eiermann, V.; Koll, S.; Zarbock, J.; Biochim. Biophys. Acta **1985**,*845*,86-93

14. Kessler, H.; Klein, M.; Müller, A.; Wagner, K.; Bats, J.W.; Frimmer, M.; Ziegler, K.; Angew. Chem. *98*,1030-1031, Int. Ed. Engl. **1986**,*25*,997-999

15. Klausner, Y.S.; Bodanszky, M.; Synthesis **1974**,549

16. Kessler, H.; Bernd, M.; Liebigs Ann. Chem. **1985**,1145-1167

17. Aue, W.P.; Bartholdi, E.; Ernst, R.R.; J. Magn. Reson. **1976**,*64*,2229-2246

18. Bax, A.; Freeman, R.; Morris, G.A.; J. Magn. Reson. **1981**,*42*,164-168

19. Bax, A.; Summers, M.F.; J. Am. Chem. Soc. **1986**,*108*,2093-2094

20. Kessler, H.; Bats, J.W.; Griesinger, C.; Koll, S.; Will, M.; Wagner, K.; J. Am. Chem. Soc., submitted for

publication

21.Kessler, H.; Bernd, M.; Kogler, H.; Zarbock, J.; Sørensen, O.W.; Bodenhausen, G.; Ernst, R.R.; J. Am. Chem. Soc. **1983**,*105*,6944-6952

22.Hansen, P.E.; Feeney, J.; Roberts, G.C.K.; J. Magn. Reson. **1975**,*17*,249-261

23.Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H.R.; J. Magn.Reson. **1984**,*57*,331 -336

24.Kuo, M.C.; Gibbons, W.A.; J. Biol. Chem. **1979**,*254*,6278

25.NMR of Proteins and Nucleic Acids, Wüthrich, K.; John Wiley and Sons, 1986

26.Kessler, H.; Ang. Chem. Int. Ed. **1982**,*94*,512-523

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

28.Kessler, H.; Griesinger, C.; Kerssebaum, R.; Wagner, K.; Ernst, R.R.; J. Am. Chem. Soc. **1987**,*109*,607-609

29.Griesinger, C.; Ernst, R.R.; J. Magn. Reson., in press

30.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, Illinois, USA, **1985**,92-99

31.Clore, G.M.; Gronenborn, A.M.; Brunger, A.T.; Karplus, M.; J. Mol.Biol. **1985**,*186*,435-455

32.van Gunsteren, W.F.; Karplus, M.; J.Comp.Chem. **1980**,*7*,266-274

33.Lautz, J.; Kessler, H.; Boelens, R.; Kaptein, R.; van Gunsteren, W.F.; Int. J. Computer Aided Mol. Design; in press

34.Loosli, H.R.; Kessler, H.; Oschkinat, H.; Weber, H.P.; Petcher, T.J.; Widmer, A.; Helv. Chim. Acta **1985**,*68*,682-703

35.Pachler, K.G.R.; Spectrochim. Acta **1964**,*20*,581-587

36.Kessler, H.; Griesinger, C.; Wagner, K.; J. Am. Chem. Soc.; in press

37.Anders, U.; Gemmecker, G.; Kessler, H.; Griesinger, C.; Fresenius Z. Anal. Chem. **1987**,*327*,72-73

38.Kessler, H.; Müller, A.; Oschkinat, H.; Magn. Reson. Chem. **1985**,*23*,844-852