D-Configuration of Serine Is Crucial in Maintaining the Phalloidin-like Conformation of Viroisin

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ABSTRACT: NMR studies have revealed that the conformation of the monocyclic viroisin is dissimilar to that of the corresponding monocyclic derivative of phalloidin, dethiophalloidin, but has much similarity with the conformation of the bicyclic phalloidin. Obviously, one of three structural features found exclusively in the virotoxins is able to compensate for the conformational strain that in the bicyclic phallotoxins maintains the toxic conformation. Synthetic work on virotoxin analogues has shown that both the additional hydroxy group in *allo*-hydroxyproline and the methylsulfonyl moiety in the 2′-position of tryptophan are unlikely to represent the structural element in question, leaving the D-serine moiety as the supposed key element. In this study we asked whether it is the hydroxy group of this amino acid or its D-configuration that is responsible for the effect. We synthesized four viroisin analogues and submitted them to conformational analysis by NMR as well as to an actin binding assay. While the rotating-frame nuclear Overhauser effect (ROESY) spectra of the analogues with L-configured amino acids showed several sets of signals, indicating the existence of conformers interconverting more slowly than the NMR time scale, the spectra of the analogues with D-configured amino acids showed only one set of signals. Remarkably, the two viroisin analogues with D-serine and D-alanine also had distinctly higher affinities for filamentous actin than their L-configured counterparts, suggesting that the high biological activity may be correlated with the absence of multiple and slowly interconverting conformers. Anyhow, D-configuration of serine is the structural element that maintains the phalloidin-like structure, while the hydroxy group does not contribute to conformational stability but is likely to be in contact with the actin surface.

Virotoxins are a family of cyclic peptides discovered in the toadstool *Amanita virosa* (*1*). Although this white Amanita species shares with the green species (*Amanita phalloides*) several toxic peptides such as α-amanitin and most of the phallotoxins, virotoxins were found in *A. virosa* only. The toxins bind to filamentous actin with an affinity comparable to that of phallotoxins and displace [^{3}H]demethylphalloidin from its binding site (*1*).

The structure of the virotoxins is related to that of phallotoxins; however, three of the seven amino acids of the virotoxins differ from the corresponding amino acids in phallotoxins (Figure 1, Table 1). In position 3, the L-cysteine residue that in the phallotoxins forms a thioether bridge with tryptophan in position 6 is replaced by a D-configured serine. In position 4, the *allo*-4-hydroxyproline residue is further hydroxylated to 3,4-dihydroxyproline, with the two hydroxy groups being in *trans* positions (*2*). Finally, tryptophan in position 6, which in the phallotoxins is part of the thioether bridge, is replaced by a 2′-(methylsulfonyl)tryptophan. The most striking difference in the two structures is, however, that virotoxins are monocyclic in nature, while phallotoxins are bicyclic.

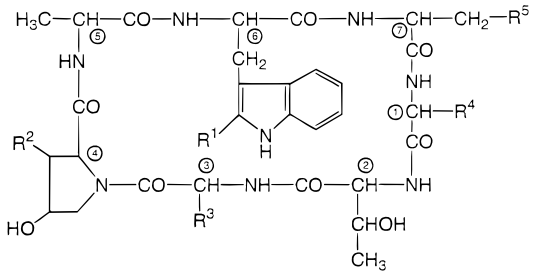

FIGURE 1: Structure of viroisin, dethiophalloidin, and their analogues. (For residues R^1−R^5 see Table 1.)

Monocyclic derivatives of phalloidin, as for example dethiophalloidin, prepared from phalloidin by reductive removal of the sulfur atom by Raney-Ni, lack almost all biological activity. A possible explanation for this was found by NMR studies of Kobayashi et al. (*3*), who showed that the bicyclic phalloidin exists as a single conformer, while the monocyclic dethiophalloidin is a population of conformers. It was therefore thought that the existence of only one distinct conformation might be a prerequisite for biological activity. This idea was substantiated by the finding that viroisin, although monocyclic in nature, likewise existed as a single conformer. Moreover, the conformation of viroisin as seen from NMR had similarities with that of phalloidin:

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Table 1: Structures of Viroisin, Dethiophalloidin, and Their Analogues Together with Their Actin Affinities Related on Viroisin = 1.0 or (α-Hyp[4])Ala[1]Leu[7]viroisin = 1.0

	name	R^1	R^2	R^3	R^4	R^5	affinity to actin viroisin = 1.0	affinity to actin analogue Ia = 1.0	references
I	viroisin	CH_3SO_2	OH	CH_2OH (D-)	$CH(CH_3)_2$	$C(OH)(CH_2OH)_2$	1.0		*3*
Ia	(α-Hyp[4])Ala[1]Leu[7]viroisin	CH_3SO_2	H	CH_2OH (D-)	CH_3	$CH(CH_3)_2$	0.2	1.0	*2*
Ib	(α-Hyp[4])Ala[1]Leu[7](D-Ala[3])viroisin	CH_3SO_2	H	CH_3 (D-)	CH_3	$CH(CH_3)_2$	0.076	0.38	
Ic	(α-Hyp[4])Ala[1]Leu[7]Ser[3]viroisin	CH_3SO_2	H	CH_2OH (L-)	CH_3	$CH(CH_3)_2$	0.005	0.03	
Id	(α-Hyp[4])Ala[1]Leu[7]Ala[3]viroisin	CH_3SO_2	H	CH_3 (L-)	CH_3	$CH(CH_3)_2$	<0.001	<0.005	
IIa	dethiophalloidin	H	H	CH_3 (L-)	CH_3	$C(OH)(CH_2OH)_2$	<0.001	<0.005	*18*
IIb	2′-(methylsulfonyl)dethiophalloidin	CH_3SO_2	H	CH_3 (L-)	CH_3	$C(OH)(CH_2OH)_2$	<0.001	<0.005	Buku (unpublished)

Although the two structures were not completely superimposable, they showed close proximity of the indole nucleus of tryptophan 6 to the methyl group of alanine 5. In both peptides the side chains of these alanine residues are subjected to the ring current effect of the indole as seen from the methyl signals shifted to upfield positions in the 1D NMR spectra. While in the phallotoxin series the close proximity of the two side chains is believed to be achieved by a conformational strain in the bicyclic peptide, this structural element is absent in the virotoxins, and the question was asked, which of the structural details compensates for the missing conformational strain, i.e., helps to maintain a phalloidin-like conformation in the monocyclic peptides?

One of the structural elements in the virotoxins that could play this role is the methylsulfonyl substituent in 2′-position of tryptophan (Figure 1, Table 1). However, this moiety is certainly not the feature sought because introduction of this group into dethiophalloidin did not restore biological activity (A. Buku, unpublished results). Similarily, the additional hydroxy group in the β-position of proline 4 is unlikely to have such a function, because a synthetic virotoxin analogue (*4*), in which *allo*-hydroxyproline was inserted instead of 3,4-dihydroxyproline (for reasons of easier synthesis), had an affinity for actin only ca. 5 times less than viroisin, suggesting that also this hydroxy group cannot be the key structure necessary to maintain the toxic conformation in the monocyclic peptide. Such considerations left the D-serine moiety as the only candidate for the conformation-stabilizing function. It remained open, however, whether the additional hydroxy group or the D-configuration of this building block was responsible.

To answer this question we compared, by NMR as well as by an actin affinity assay, compound Ia (Figure 1, Table 1), a viroidin analogue with D-serine in position 3 and with high biological activity, with three newly synthesized analogues (compounds Ib, Ic, and Id), in which the D-serine residue in position 3 was replaced by D-alanine, L-serine, or L-alanine, respectively. (The latter compound, Id, is almost identical in structure with dethiophalloidin, because the L-configuration of the former cysteine residue in position 3 is conserved when the alanine residue is formed.)

MATERIALS AND METHODS

Amino acids, chemicals, and solvents were of analytical grade. Identity and purity of all peptides was checked by amino acid analysis, ^{1}H NMR, FAB-MS, and TLC. Analysis by TLC was performed on silica (Merck 60 F 254) with the following solvent systems: chloroform/methanol (95:5), A; chloroform/methanol/water (65:25:4), B; diethyl ether/hexane (8:2), C; ethyl acetate/acetic acid (95:5), D; chloroform/methanol (1:1), E. The spots were identified by iodine vapor.

Synthesis of Peptides. The viroidin analogues investigated in this study were synthesized as shown in Figure 2. Boc-(methylthio)tryptophan was prepared essentially as described by Savige et al. (*5*). To 20 mL of cooled TFA at −30 °C was added Hpi (10 mmol) and sodium methanethiolate (20 mmol). The reaction vessel was sealed and the reaction mixture was vigorously stirred for 3 h at room temperature. After evaporation of the solvent in vacuo, the residue was dissolved in a mixture of dioxane/H_2O (1:1), and di-*tert*-butyl dicarbonate (10 mmol) and 2 mM NaOH solution (10 mL) were added. After stirring for 2 h the mixture was acidified to pH 2−3 with $KHSO_4$, extracted with ethyl acetate, and washed with saturated NaCl. Peptide derivatives 1, 5, 7, and 8a−d were prepared by the mixed anhydride method with yields of 46−100%. To have the serine hydroxy group protected during the cyclization reaction, the Boc group was exchanged for Ddz (*6*) at the stage of peptide 2. Amino groups of intermediate peptides 7, 8a−d, and 9a−d were protected by the Z-group, which was removed by H_2/PdC. Condensation of the threonine residue to the tetrapeptides 9a−d was carried out by DCC/HOBt coupling, with yields of 60−75%. The Ddz-protected N-terminal tripeptides and the OBg-protected C-terminal tetrapeptides were coupled via the HBTU reaction (*7*) to give 10a−d with yields of 65−80%. Deprotection of the linear heptapeptides 10a−d and cyclization are described below.

Deprotection and Cyclization of the Linear Heptapeptides. For removal of the OBg group, to the solution of 0.2 mmol of the protected linear heptapeptide 10a−d in 4 mL of DMF was added the solution of 0.4 mmol of K_2CO_3 in 2 mL of water. After stirring overnight at room temperature, the reaction mixture, diluted with 10 mL of water, was extracted with 2 × 50 mL of ether. The aqueous phase was acidified with 0.5 M $KHSO_4$ to pH 3 and extracted with 3 × 50 mL of chloroform. The organic layer was washed with water, dried with Na_2SO_4, and evaporated in vacuo. For removal of the Ddz group, the residue was dissolved in a solution of 0.15 mL of TFA (2 mmol) in 4 mL of CH_2Cl_2, and kept at room temperature for 2 h. The precipitate obtained by

[1] Abbreviations: MA, mixed anhydride method; iBCCl, isobutyloxycarbonyl chloride; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; Ddz, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl; HOBT, hydroxybenztriazole; OBg, *N*-benzhydrylglycolamide ester; DIEA, *N*-ethyldiisopropylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid; TEA, triethylamine; NMM, *N*-methylmorpholine; TLC, thin-layer chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance; DQF−COSY, two-dimensional double quantum-filtered coherence transfer spectroscopy; TOCSY, total coherence-transfer spectroscopy; DIPSI-2, decoupling in the presence of scalar interactions, method 2; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; FAB-MS, fast atom bombardment mass spectroscopy.

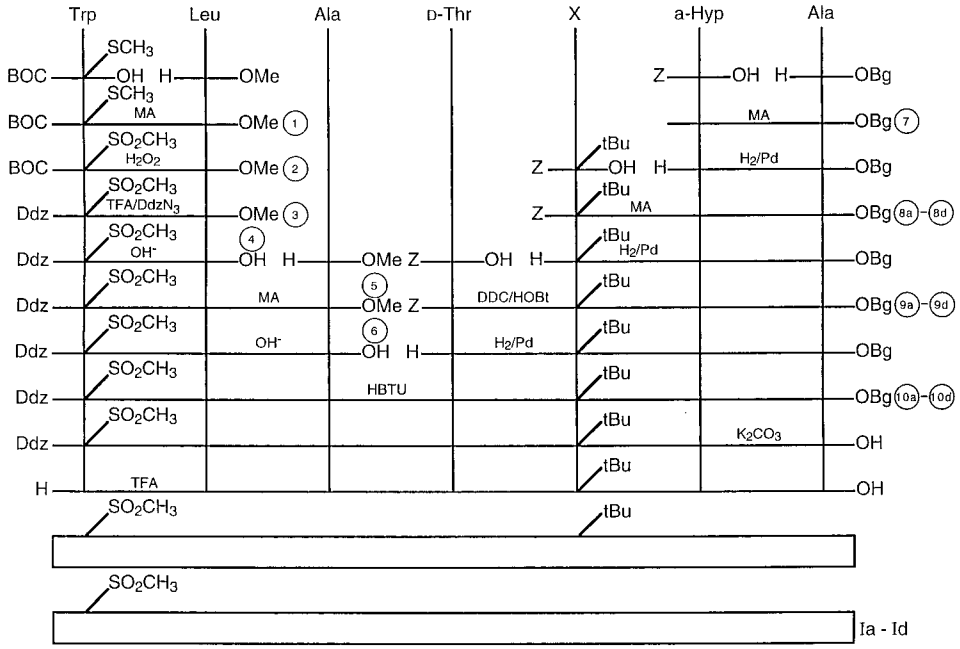

FIGURE 2: Synthesis scheme of linear precursor peptides of the viroisin analogues Ia−d. Ia, X = D-Ser; Ib, X = D-Ala; Ic, X = L-Ser; Id, X = L-Ala.

addition of 50 mL of ether was filtered, washed with ether, and dried.

To the solution of 0.2 mmol of deprotected linear heptapeptide trifluoroacetates in 200 mL of DMF was added 103 mg of HBTU (0.24 mmol) and 0.1 mL of DIEA (0.6 mmol). After stirring overnight at room temperature the solvent was evaporated in vacuo, and the residues, dissolved in chloroform, were washed with aqueous 0.5 M $KHSO_4$, saturated $NaHCO_3$ solution, and water, dried with Na_2SO_4, and evaporated in vacuo. Purification of viroidin analogues was achieved by preparative HPLC chromatography on a C_{18} RP column eluted by a linear gradient of H_2O/0.1% TFA and CH_3CN/0.1% TFA. Viroidin analogues containing serine residues in position 3 (Ia and Ib) were obtained from the respective tBu-protected compounds by treatment with 1 mL of TFA for 1 h at room temperature. After evaporation of the solvent, the products were repurified by preparative HPLC chromatography, adopting the procedure described above. Yields of cyclization: 19% (Ia), 32% (Ib), 13% (Ic), and 16% (Ic). FAB $(M + 2H)^+$: 822 (Ia and Ic) and 806 (Ib and Id). R_f in solvent A: 0.45 (Ia, Ic) and 0.7 (Ib, Id).

NMR Spectroscopy. Samples of Ia−d were prepared by dissolving 1 mg of lyophilized peptides in 0.2 mL of 90% H_2O/10% D_2O, pH 2.8. The pH value was adjusted by the addition of minute amounts of NaOH or HCl. NMR experiments were performed with a Bruker DMX500 spectrometer at 301 K with ^1H resonance frequency of 500.13 MHz. The reference for all spectra was sodium-3-trimethylsilyl propionate-d_4 at 0 ppm for H_2O. All 2D spectra were recorded in the pure absorption mode according to the time-proportional phase incrementation method with spectral widths of 7000 Hz. For identification of the spin systems, DQF−COSY (*8*) and TOCSY (*9*) with the DIPSI-2 sequence (*10*) at a mixing time of 79 ms on a spin-locking field of 9 kHz were used. ROESY (*11*) spectra were recorded at a mixing time of 200 ms with a spin-locking field of 3.3 kHz.

Solvent suppression for the ROESY and TOCSY was achieved by use of the WATERGATE sequence (*12*). The typical data size was 2048 points in *t*2 and 512 points in *t*1, which was zero-filled into 1024 points. Prior to Fourier transformation, a squared cosine-bell weighting function was multiplied to the *t*1 and *t*2 directions.

Actin Affinity Assay. Affinity for actin was measured from the release of a tritiated phallotoxin, [^3H]demethylphalloidin, from its F-actin complex through replacement by increasing concentrations of viroisin, or viroisin analogues. F-Actin was prepared from rabbit muscle by the procedure of Spudich and Watt (*13*) and used as a 5×10^{-5} M solution of G-actin adjusted by spectrophotometry ($\epsilon_{290} = 26\,460\ M^{-1}\ cm^{-1}$) in a buffer of 2 mM Tris-HCl, 0.1 mM ATP, and 0.2 mM Ca^{2+}, pH 7.8. To this solution was added 1 equiv of the tritiated phallotoxin, and the polymerization was started by the addition of 3 mM $MgCl_2$. After polymerization for 30 min at room temperature, the actin complex was diluted to a concentration of 1.1×10^{-6} M with the above buffer. To 0.5 mL aliquots were added 50 μL of a dilution series of the virotoxin to be tested, in final concentrations of 10^{-4}−10^{-7} M. After 1 h of incubation at room temperature, 180 μL aliquots were centrifuged in a 42.2 Ti rotor (Beckman) for 50 min at 200000g, and aliquots of 20 μL were counted.

RESULTS

Synthesis of Viroisin Analogues. From the structure−activity relationship in the phallotoxin and virotoxin series, several building blocks present in native viroisin (Figure 1, Table 1) were known to be of minor significance for biological activity, as, for example, the β-branched L-valine in position 1, which in the present study was replaced by L-alanine, promising easier handling during synthesis. Similarily, in position 4, the 3,4,-dihydroxylated L-proline with its three chiral centers was replaced by L-*allo*-hydroxyproline, which is much easier to obtain but still shares with the dihydroxylated proline the *trans*-configuration of the OH at the γ−C atom. Finally, dihydroxylated leucine in position 7 was replaced by leucine, since both OH groups are un-

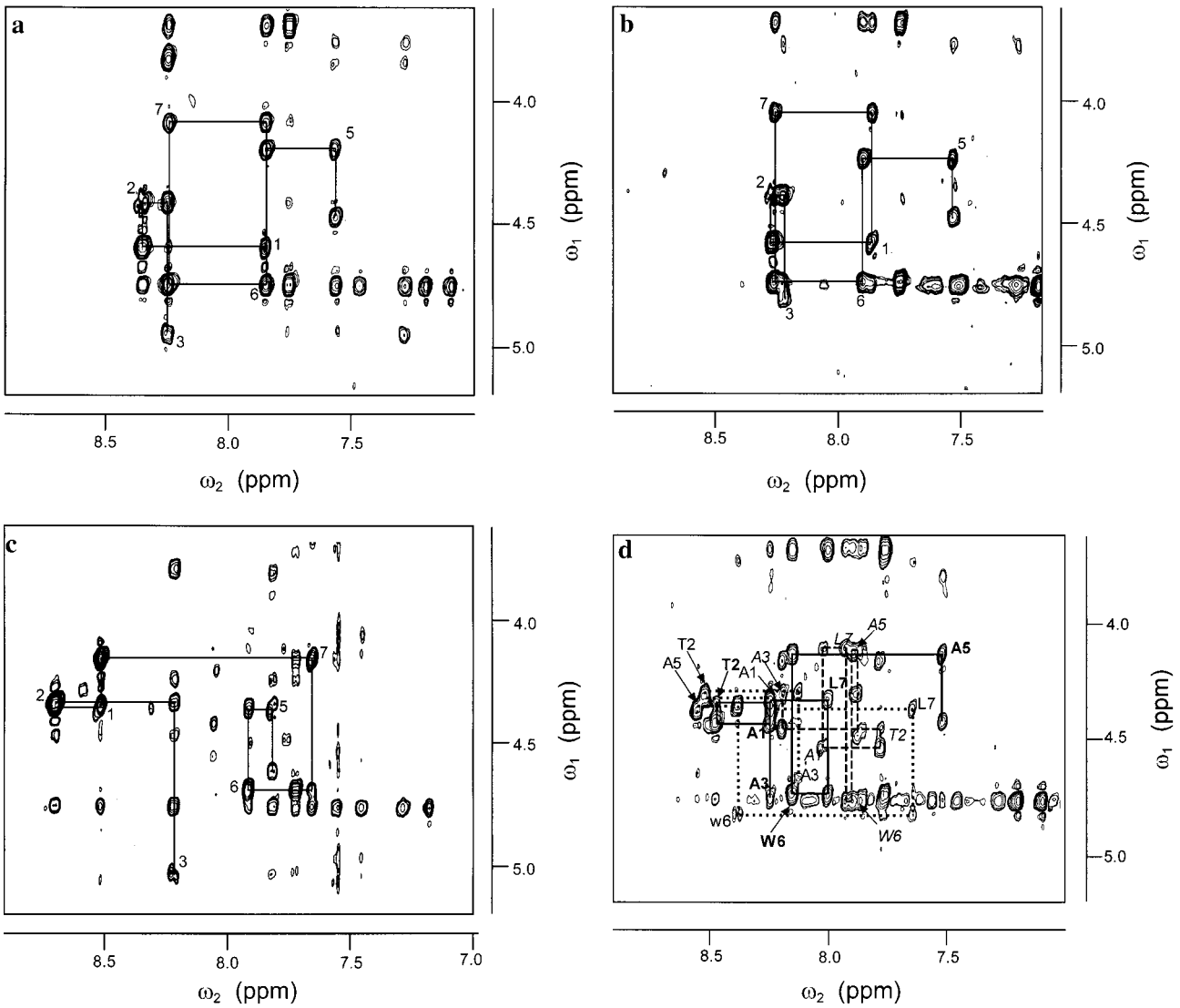

FIGURE 3: $H_N-C_\alpha H$ regions of ROESY spectra for Ia−d (panels a−d) in 90% H_2O/10% D_2O, at pH 2.8 and 301 K. Solid lines indicate sequential assignment, with residue numbers indicated at the intraresidual cross-peaks. In panel d, dotted lines indicate conformer minor1, and broken lines indicate conformer minor2. Residue numbers for major, minor1, and minor2 conformers are shown in boldface, plain, and italic type, respectively, at the intraresidual ROE cross-peaks.

important for biological activity and one of the two hydroxy groups, that in γ-position, destabilizes the adjacent amide bond in acidic medium. All together, these replacements resulted in the analogue Ia (Figure 1, Table 1), a virotoxin analogue already synthesized by Kahl and Wieland (*4*) and reported to have an affinity for actin only 5 times lower than that of native viroisin. This analogue (Ia) was used as a reference compound for three further analogues, in which the amino acid in position 4, D-serine, was changed into D-alanine (analogue Ib); L-serine (analogue Ic); or L-alanine (analogue Id). From the physical and biological properties of these three analogues, as related to the properties of Ia, we were able to decide whether it is the presence of the OH group, or the D-configuration, that is crucial for the stability of conformation and for biological activity.

In analogy to the procedure reported (*4*), synthesis of Ia and its analogues was performed in homogeneous phase, although with a different strategy. Thus, the methylsulfonyl group was introduced into tryptophan at an early stage and not in the last step. Further, the heptapeptide was condensed from a tripeptide and a tetrapeptide, instead of stepwise elongation, and HBTU was used for the cyclization reaction instead of the MA method. Details of the synthesis are shown in Figure 2.

NMR Studies. The conformations of the viroisin analogues Ia−d were studied by 2D NMR in aqueous solution. As shown by the sequential assignment pattern in the ROESY spectra of Ia and Ib (Figure 3a,b), the spectra of these two analogues with D-configured amino acids in position 3 look very similar to each other. In addition, the chemical shifts of backbone proton signals of Ia were quite similar to those of viroisin determined previously (*3*). While most of the chemical shift differences were not significant (below the range of $\pm 0.1-0.2$ ppm), allowing us to consider similar conformations for Ia and viroisin, some signals exhibited medium differences (in the range of $\pm 0.2-0.3$ ppm), presumably due to the fact that Ia and viroisin differ in three side chains. Still, these differences were smaller than those of the major conformers of Ic and Id; see Table 2. In contrast, the sequential assignment pattern of Ic (Figure 3c) was completely dissimilar to that of Ia and showed the coexistence of two conformations at a ratio of 10:1. For the major

Table 2: Chemical Shifts of Ia−d in 90% H_2O/10% D_2O at pH 2.8 and 301 K[a]

residue no.	Ia[b]		Ib		Ic-major		Id-major		Id-minor1		Id-minor2	
	NH	Cα	NH	Cα	NH	Cα	NH	Cα	NH	Cα	NH	Cα
1	7.86 (−0.12)	4.60 (−0.22)	7.90 (−0.04)	4.61 (−0.01)	8.55 (−0.69)	4.37 (0.23)	8.27 (−0.41)	4.44 (0.16)	8.26 (−0.40)	4.32 (0.28)	8.04 (−0.18)	4.54 (0.06)
2	8.35 (−0.01)	4.42 (−0.01)	8.31 (0.04)	4.42 (0.00)	8.74 (−0.29)	4.35 (0.07)	8.50 (−0.15)	4.35 (0.07)	8.54 (−0.19)	4.28 (0.14)	7.79 (0.56)	4.46 (−0.04)
3	8.26 (−0.02)	4.94 (−0.02)	8.26 (0.00)	4.84 (0.10)	8.25 (0.01)	5.05 (0.11)	8.26 (0.00)	4.73 (0.11)	8.14 (0.12)	4.68 (0.26)	8.21 (0.05)	4.31 (0.63)
4		4.51 (0.23)		4.56 (−0.05)		4.61 (−0.10)		4.41 (0.10)		*c*		*c*
5	7.56 (−0.05)	4.19 (−0.01)	7.56 (0.00)	4.26 (−0.07)	7.82 (−0.26)	4.36 (−0.17)	7.16 (0.40)	4.13 (0.06)	8.58 (−1.02)	4.36 (−0.17)	7.89 (−0.33)	4.12 (0.07)
6	7.84 (0.31)	4.75 (0.15)	7.88 (−0.04)	4.77 (−0.02)	7.95 (−0.11)	4.72 (0.03)	8.17 (−0.33)	4.76 (0.01)	8.41 (−0.57)	4.84 (−0.09)	7.91 (−0.07)	4.79 (0.04)
7	8.24 (0.12)	4.09 (−0.01)	8.30 (0.02)	4.07 (0.02)	7.69 (0.55)	4.18 (0.09)	8.01 (0.23)	4.33 (−0.24)	7.66 (0.58)	4.37 (−0.28)	7.95 (0.29)	4.09 (0.06)

[a] Chemical shifts are given in parts per million. The major conformer of analogue Ic and the three conformers of Id, assigned Id-major, Id-minor1, and Id-minor2, (ratio 6:4:3) were used. [b] In columns under Ia, the numbers in parentheses represent chemical shift differences between analogue Ia and Viroisin (taken from ref 3). In the other columns, the numbers in parentheses indicate chemical shift differences between analogue Ia and these conformers. [c] Signals have not been determined.

conformer, chemical shift differences compared to Ia (Table 2) reached values of up to 0.69, indicating that the major conformer differed significantly from the conformation of Ia. Analogue Id existed, as illustrated in Figure 3d, even as a mixture of three conformers (ratio of 6:4:3). In agreement with the ROESY spectra, chemical shift differences in all conformers of Id, as compared to Ia, were as large as 1.0 ppm, suggesting that also the conformers present in this population were different from Ia. To exclude that the signals obtained from Id originated from byproducts eventually formed during NMR measurement, Id was recovered and subjected to HPLC. The analysis revealed a purity of >95% (not shown), indicating that the three compounds seen in NMR indeed represented conformers of Id. In the ROESY spectra of both Ic and Id several positive cross-peaks appeared between the major and minor signals, indicating that the major and minor conformers interconvert slowly on an NMR time scale (data not shown). In total, the NMR study gave evidence that several slowly interconverting conformers are coexisting in the two, nonactive analogues containing L-configured serine or alanine.

Actin Binding. Affinity to actin was measured as the capacity of the analogues to displace [^{3}H]demethylphalloidin from its binding site on filamentous actin. This assay is based on separation of free and bound [^{3}H]demethylphalloidin by ultracentrifugation, i.e., a rapid procedure (*2h*), which is superior to the equilibrium dialysis procedure (*16h*) used in the previous study (*4*). Viroisin (or phalloidin, which has virtually the same affinity to actin) was used as internal standard in all assays. Relative affinity values were calculated from that concentration of the viroisin analogue able to replace 50% of bound [^{3}H]demethylphalloidin, related to the concentration of phalloidin, or viroisin that was required to achieve the same effect. Relative affinity values of the four viroisin analogues are compiled in Table 1.

We confirmed the results of Kahl and Wieland (*4*), who found that the analogue Ia, lacking one OH in the proline residue of position 4, has an affinity for actin ca. 5 times lower than that of viroisin. By omitting also the OH of D-serine, the affinity for actin dropped further by a factor of ca. 3. Different from this, a change in the configuration of the amino acid in position 3 (L-serine for D-serine or L-alanine for D-alanine) diminished the affinity for actin by a factor of ca. 40 or >200, respectively. This indicates that it is the configuration of the building block in position 3 rather than the presence or absence of the OH group that determines the biological activity.

DISCUSSION

The present study is based on previous work in which we identified, on the atomic level, structural elements in the cyclopeptide α-amanitin involved in binding the toxin to an amatoxin-specific monoclonal antibody (*14*). For this aim, the affinities to the immunoglobulin of ca. 20 chemically modified amatoxins were measured and related to the affinity of the mother compound, α-amanitin. Provided that only one structural feature was changed at a time and that the chemical modification had not altered backbone conformation of the amatoxin, the decrease in affinity to the immunoglobulin could be directly correlated to the structural change in the analogue. By this method, we showed, for example, that

exchange of the phenolic OH for H in the 6′-OH tryptophan side chain decreased the affinity by a factor of ca. 2, while the corresponding exchange of the γ-positioned OH in the isoleucine side chain caused a decrease in affinity by a factor of 8. From such factors we calculated changes in free binding energy ($-\Delta\Delta G°$), equal to 2 and 5 kJ/mol, respectively, representing the contributions of these OH groups to the overall binding energy (*15*), most probably via hydrogen bonds with the protein. We found that in most cases chemical modifications did not cause changes in the overall shape of the molecule, as proved by circular dichroism. In a few cases, however, as for example the aldo derivative of methylated α-amantin, circular dichroism was different from that of α-amanitin, indicating that a conformational change had occurred. Concomitantly with such conformational changes, the affinity to the monoclonal antibody dropped by a factor of 10^4. Thus we could clearly distinguish between chemical modifications leaving the backbone conformation intact, and others that changed the overall conformation. For the latter, the loss of activity was in all cases much more pronounced or total.

Such classification of chemical modifications, with and without effect on backbone conformation, was possible also in the present study on structure−activity relationship of virotoxins. Here, we took advantage of 2D NMR measurements to examine the backbone conformations of the four viroisin analogues. We first ascertained that for analogue Ia the chemical shifts for the HN and $C_\alpha H$ signals were very similar to those of viroisin, indicating that the three structural modifications made in analogue Ia had not caused significant changes in the backbone conformation. This confirmed previous studies in the phallotoxin family, where two of these modifications, replacement of dihydroxyleucine/leucine and replacement of valine/alanine, were shown by circular dichroism measurements to be without effect on conformation. They likewise did not change actin affinity indicating that the structural elements concerned were also not involved in actin binding. The third modification made in analogue Ia, the insertion of *allo*-hydroxyproline for dihyroxyproline, was also without effect on conformation but caused a decrease in actin affinity by a factor of 5. In context with the grossly unchanged conformation, this moderate decrease in actin affinity strongly suggested that the second hydroxy group of the proline moiety is involved in actin binding. Since the amount of binding energy corresponding to a factor of 5 is in the range determined for hydroxy groups of amanitin being involved in protein binding, we conclude that also the second hydroxy group of dihydroxyproline is part of a hydrogen bond between viroisin and actin.

For the analogue Ib, differing from Ia in the exchange of D-serine for D-alanine, the loss in actin affinity corresponded to a factor of 3, as compared to Ia. Since the effects on conformation caused by this exchange were likewise negligible (Figure 3a,b), the loss in binding energy is also in this case a consequence of the OH/H exchange and, considering the amount of energy, presumably attributable to the loss of another hydrogen bond. Accordingly, we can assume that also the hydroxy group of D-serine is in contact with the actin surface.

In contrast to Ib, analogue Ic differed from Ia by a factor of 40 in actin affinity, an amount that is beyond the range of an OH/H exchange. In agreement with this, the NMR spectrum of Ic differed significantly from those of Ia and Ib, indicating that change of configuration from D to L had caused a change in backbone conformation. Moreover, Ic obviously existed as a mixture of two interchanging conformers with an equilibrium at a ratio of 10:1. As concluded from the pattern of the ROESY spectra, not even the major conformation of Ic is identical with the conformation of Ia. A similar and even stronger effect on the cross-peak pattern of the ROESY spectrum was seen for the analogue Id, where L-alanine was inserted into this position. Here, the NMR data demonstrated the existence of at least three conformers at a ratio of 6:4:3. Analogue Id is identical in structure with dethiophalloidin, except for the two hydroxy groups in leucine 7, which are not essential for actin binding, and, like dethiophalloidin, showed an affinity to actin that was below the limit of detection (Table 1).

From the fact that multiple conformers of Ic and Id can be distinguished in NMR, we conclude that interconversion occurs at rates slower than the NMR time scale. Kobayashi et al. (*3*) studied such slow conformational changes for dethiophalloidin and showed that the major conformer of this inactive phalloidin analogue was *cis*-configured at proline 4. This observation opens the possibility that also in the conformer populations of the analogues Ic and Id the slow conformational transitions reflect *cis*/*trans*-interconversions at proline 4. Unfortunately, we could not assign ROE cross-peaks in the region of $C_\alpha H-C_\alpha H$ in order to determine the *cis*/*trans* state of these conformers because of severe overlapping and weak intensities of the signals in the ROESY spectra. Therefore, forthcoming studies must show whether the slow conformational interconversions observed for these cyclopeptides really represent *cis*/*trans* isomerizations.

The two analogues showing the slow interconversion of conformers are also the two analogues with the lowest affinity in the actin binding assay. It is tempting to suggest that there may exist some relationship between the slow conformational transitions and the low biological activity. In the special case of the viroisin analogues there is indeed a close connection between an apparent single conformation and the high affinity for actin, since these properties are restricted to the two analogues containing the D-configured amino acids. However, these are only two examples, and for the general validity of such a correlation further examples supporting this idea must be found. It has been speculated (*1*) that biosynthesis of virotoxins may start from a phalloidin-like precursor molecule and that methylation of the thioether bridge, followed by β-elimination of the sulfonium moiety, might result in the formation of a dehydroalanine residue in position 3, which, on the addition of water, could develop into L- or D-serine. As it appears, it was not by chance that in this process, if it really occurs, the D-configuration was favored over the L-configuration, which, as we now know, is associated with multiple and slowly interconverting conformations and strongly reduced activity.

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