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High-Field 2D NMR Spectroscopy of Amanitin Isomers

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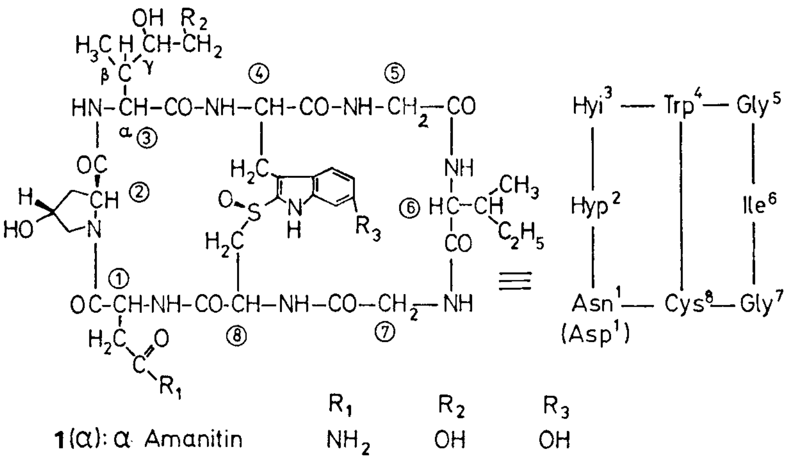
The ^{1}H, ^{13}C and ^{15}N NMR spectra of α-, β- and γ-amanitins and amaninamide were recorded at 11.7 T and analysed by various two-dimensional (2D) NMR spectroscopic techniques. All diastereotopic protons were assigned, and the side-chain conformations and the conformation of the hydroxyproline residue were determined. The observed ^{15}N chemical shifts are caused by non-bonded interactions of the peptide nitrogens. ^{1}H, ^{13}C and ^{15}N data are complementary to each other and show that the conformation of β-amanitin is slightly different from that of the other isomers, in accordance with the lower toxicity. The NOESY and carbonyl ^{13}C–^{1}H chemical shift diagrams confirm the closeness of the conformations of amanitins in DMSO solution to that of β-amanitin in the crystalline state.

KEY WORDS Amanitin isomers ^{1}H, ^{13}C, ^{15}N 2D NMR Conformational analysis Biological activity

INTRODUCTION

The α-, β- and γ-isomers of the bicyclic octapeptide amanitin (1–3) and amaninamide (4) contain eight L-amino acids (*S* configuration of the α-carbon atoms of the amino acid residues, except for Cys^8), including the unusual γ-hydroxyisoleucine (in 3) γ,δ-dihydroxyisoleucine (Hyi^3 in 1, 2 and 4) and 6-hydroxytryptophan (in 1–3). The transannular bridge in 1–4 is formed between the Cys^8 and Trp^4 via the cysteine sulphoxide group (Fig. 1).

The α-isomer 1 is the most poisonous toxin of the mushroom *Amanita phalloides*. Its toxicity is caused by inhibition of protein synthesis mainly in the kidney and liver at concentrations as low as 10^{-9} M.[1] The high specificity of α-amanitin to all RNA polymerases II of vertebrate origin is the main reason for its popularity in various studies in molecular biology.[2] Modification of the amino acid side-chains in amatoxins shows[3] that removal of the bicyclic bridge by Raney nickel reduction results in an entirely non-toxic product. Various modifications of hydroxylated isoleucine indicate that for high toxicity it is essential to have a four-carbon chain branched at the β-position and the presence of at least one hydroxy group. Substitution of hydroxyproline by proline results in a significant loss of toxicity, but etherification of hydroxytryptophan has no effect on toxicity. The (*R*)-sulphoxide group is dispensible because the corresponding sulphide and sulphone are also highly toxic. However, the reversal of configuration at the sulphoxide group from *R* to *S*



	R₁	R₂	R₃
1(α): α-Amanitin	NH_2	OH	OH
2(β): β-Amanitin	OH	OH	OH
3(γ): γ-Amanitin	NH_2	H	OH
4(αH): Amaninamide	NH_2	OH	H

Figure 1. Structural formulae of the studied amanitin isomers.

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results in a more than an 8-fold reduction in toxicity. Therefore, comparative investigations of the physical properties of amanitin isomers are an important step in the elucidation of their mechanism of action at the molecular level.

X-ray data have been published only for the β-isomer[4] and there are two reports on the ^1H NMR spectroscopy of the α-isomer at 220 and 360 MHz.[5,6] Both of the reports on NMR spectroscopy demonstrate the similarity of the α-amanitin conformation in solution to that of β-amanitin in the crystalline state. However, at 220 MHz[5] the assignment of the two glycine residues was possible only with the aid of x-ray data. At 360 MHz[6] the use of selective decoupling and nuclear Overhauser effect measurements resulted in a more complete picture of the chemical shifts and interproton coupling constants. Comparison of the data obtained at 220 and 360 MHz accentuates the advantages of high polarizing magnetic fields in oligopeptide studies. At 360 MHz twenty J(HH) values were obtained, but at 220 MHz only ten J(HH) values were reported.[5] The use of an even stronger magnetic field allows us to obtain additional data not only on the conformational state of the main polypeptide chain, but also on the side-chain conformations of individual amino acid residues. The increased sensitivity provides the possibility of obtaining the NMR parameters of two additional important nuclei, ^{13}C and ^{15}N, at the natural abundance of these isotopes. The availability of x-ray data on **2** and ^1H NMR data at lower magnetic fields forms a reliable basis for the NMR study of amanitin isomers at a magnetic field of 11.7 T.

EXPERIMENTAL

Amanitin isomers used for the NMR measurements were isolated from the mushrooms *Amanita phalloides* (α, β, γ) or *Amanita virosa* (amaninamide), and 4-hydroxy-L-proline was purchased from Calbiochem. NMR spectra were obtained at 313 K from *ca* 0.08 M solutions of **1**–**4** in DMSO-d_6; for ^1H 5 mm o.d. NMR tubes and for ^{13}C and ^{15}N 10 mm o.d. NMR tubes were used (total sample volume *ca* 2 ml). All experiments were performed on a Bruker AM-500 NMR spectrometer (resonance frequencies for ^1H 500.13 MHz, for ^{13}C 125.77 MHz and for ^{15}N 50.68 MHz). ^1H and ^{13}C chemical shifts were measured from internal DMSO ($\delta^1H = 2.500$ ppm, $\delta^{13}C = 39.5$ ppm) and ^{15}N chemical shifts were referenced to the NH_3 scale[7] by comparison of the ^{15}N NMR spectra with the external reference, 90% formamide solution in DMSO-d_6 ($\delta^{15}N = 112.9$ ppm). Various two-dimensional experiments[8] were performed using the standard pulse programmes of the AM-500 spectrometer.

RESULTS AND DISCUSSION

^1H NMR spectroscopy

At 11.7 T nearly all amino acid residues of **1**–**4** give first-order spectra in DMSO. The only exceptions are H-γ and H-β in Ile.[6] Individual amino acid residues can be simply assigned from a homonuclear 2D chemical shift correlation experiment via spin–spin couplings between the protons (COSY).[8] Most of the coupling constants were measured from the single resonance spectra. A J-resolved 2D diagram was used for checking the few overlapping multiplets. An iterative calculation by the Bruker PANIC program was used for the determination of the coupling constants in the side-chain of Ile.[6] The two coupling constants measured from the signals of the amide hydrogens of the two glycine residues (Gly^5 and Gly^7) can be used without any modification owing to the large differences in the chemical shifts of the diastereotopic α-protons. For example, the difference in the chemical shifts of the α-protons in **1** for Gly^5 equals 454 Hz, and the error in the determination of the two coupling constants from the amide proton signals exceeds 0.1 Hz only when $\Delta\delta$ H-α is less than 100 Hz. Therefore, there is no need to use the sum of two ^3J(HNCH) values[9] for the conformational analysis at the magnetic field of 11.7 T. The ^1H chemical shifts and coupling constants of **1**–**4** are given in the Tables 1 and 2, respectively, together with earlier literature data on **1**.[5,6] The present data show that at 500 MHz the chemical shifts of all non-equivalent protons and all their vicinal and geminal coupling constants can be determined unambiguously. Small differences from an earlier study[6] on **1** are the result of the different concentrations used. Of the 39 chemical shifts only six differ by more than 0.02 ppm. The most essential differences from Ref. 5 in the coupling constants occur in ^3J(HNCH) for Asn^1 and the observation of two ^3J(HNCH) coupling constants for Gly^5 (9.3 and 1.8 Hz).

The availability of reliable geometric data[4] for **2** allows the assignment of diastereotopic hydrogens and a detailed discussion of the amino acid side-chain geometries. The diastereotopic hydrogens on the main-chain α-carbon atoms of Gly^5 and Gly^7 can be immediately assigned from the Newman projections of these amino acid residues along the N—C-α bonds (Fig. 2). The pro-S protons of Gly^5 and Gly^7 are shifted to lower fields owing to their proximity to the carbonyl groups of neighbouring amino acid residues. The larger chemical shift corresponds to a smaller dihedral angle between H and CO (Fig. 2). The regularity of the occurrence of signals from the pro-S protons of glycine residues at lower fields and their larger ^3J(HNCH) values have also been pointed out earlier in other studies.[10,11]

For Asp^1 in **2** one obtains two similar values of ^3J(HH) (5.0 and 3.0 Hz), in accordance with the dihedral angles along the C-α—C-β bond from the x-ray study (Fig. 2). At the same time, in aspartic acid or asparagine there are two different coupling constants $^3J(\alpha\beta)$ 8.8 and 8.0 Hz (to pro-S) and 3.8 and 4.0 Hz (to pro-R),[12] which, according to Pachler,[13] lead to the conclusion that there is a considerable share (*ca* 50%) of the conformation with *trans*-oriented carbonyl groups. The small difference in the $^3J(\alpha\beta)$ values does not allow an unambiguous assignment of the pro-S and pro-R hydrogen atoms in **1**–**4**. However, this can be done from the analysis of the 2D carbonyl carbon–^1H chemical shift correlation diagram (see below), where a strong correlation peak appears only with the low-field β-

Table 1. ^1H **chemical shifts of amino acid residues of amanitin isomers in dimethyl sulphoxide**[a]

Amino acid residue	Proton	Chemical shift, δ (ppm)					
		220 MHz[5]	α 360 MHz[6]	500 MHz	αH, 500 MHz	β 500 MHz	γ, 500 MHz
Asn^1 (Asp^1)	NH	8.41	8.411	8.416	8.422	8.395	8.468
	α	4.68	4.636	4.657	4.671	4.560	4.655
	pro-*S*β		2.950	2.962	2.981	2.889	2.956
	pro-*R*β	3.48	3.5	3.503	3.521	3.141	3.503
	NH_2		8.317	8.303	8.273	—	8.400
			7.511	7.505	7.505	—	7.602
Hyp^2	α	4.28	4.278	4.286	4.311	4.304	4.275
	pro-*S*β	1.86	1.848	1.864	1.883	1.805	1.800
	pro-*R*β	2.16	2.189	2.208	2.222	2.213	2.222
	γ		4.372	4.384	4.395	4.356	4.375
	pro-*S*δ		~3.8	3.801	3.821	3.707	3.801
	pro-*R*δ			3.732	3.729	3.675	3.721
Hyi^3	NH	7.83	7.775	7.781	7.743	8.361	7.862
	α	4.40	4.426	4.437	4.473	4.346	4.270
	β	2.16	2.113	2.131	2.152	2.329	1.990
	γ		3.522	3.482	3.499	3.363	3.659
	pro-*S*δ		~3.3	3.334	3.369	3.399	—
	pro-*R*δ			3.294	3.312	3.257	—
	CH_3	0.87	0.867	0.876	0.900	0.811	0.832
							0.934
Trp^4	NH	7.92	7.834	7.835	7.839	7.901	7.950
	α	4.95	4.917	4.953	5.046	5.078	4.863
	pro-*S*β	2.72	2.739	2.751	2.799	2.917	2.714
	pro-*R*β	~3.1	3.198	3.205	3.277	3.037	3.258
	H-4		7.428	7.437	7.674	7.400	7.452
	H-5		6.594	6.605	7.076	6.590	6.599
	H-6		—	—	7.190	—	—
	H-7		6.747	6.759	7.410	6.740	6.755
	NH (indole)		11.181	11.269	11.529	11.137	11.177
Gly^5	NH	8.00	7.943	7.945	7.949	7.976	7.900
	pro-*S*α	4.28	4.306	4.291	4.294	4.231	4.321
	pro-Rα	3.40	3.389	3.383	3.395	3.441	3.344
Ile^6	NH	8.54	8.443	8.440	8.391	8.475	8.429
	α	3.68	3.665	3.672	3.682	3.687	3.661
	β	1.57	1.556	1.560	1.589	1.577	1.574
	pro-*S*γ		1.500	1.532	1.540	1.535	1.536
	pro-*R*γ		1.111	1.120	1.131	1.117	1.118
	γ-CH_3		0.797	0.801	0.811	0.797	0.800
	δ-CH_3		0.825	0.827	0.835	0.825	0.828
Gly^7	NH	8.78	8.691	8.695	8.648	8.713	8.688
	pro-*S*α	3.93	3.904	3.913	3.921	3.905	3.909
	pro-*R*α	3.40	3.444	3.425	3.435	3.437	3.416
Cys^8	NH	8.33	8.250	8.258	8.270	8.215	8.304
	α	~4.95	4.944	4.956	5.008	4.922	4.947
	pro-*S*β	3.01	2.958	2.984	3.012	2.977	2.962
	pro-*R*β		3.081	3.088	3.165	3.061	3.088

[a] Our assignment.

proton of Asp^1 or Asn^1 owing to its *trans* orientation to the peptide carbonyl. Hence the $^3J(\alpha\beta)$ values in Asn^1 or Asp^1 of **1**–**4** differ strongly from those in aspartic acid or in aspargine, but the pro-*R* proton in both cases is shifted to lower fields. The differential shielding of the β-protons of Asn^1 in **1**, **3** and **4** (0.54 ppm) is more than twice that found in linear tetrapeptides ($\Delta\delta = 0.22$ ppm).[14]

The coupling constants of Hyp^2 in **1**–**4** should be compared with those of 4-hydroxyproline. In order to obtain all the coupling constants of 4-hydroxyproline, its ^1H spectrum in D_2O solution was remeasured at 500 MHz. A nearly first-order spectrum was obtained, which is simple to analyse compared with the earlier studies at lower frequencies.[15–17] Comparison with the earlier measurements demonstrates that at 500 MHz three additional coupling constants can be determined (Table 3). Of the 15 possible coupling constants of this six-spin system only three have absolute values of less than 0.2 Hz. With the consideration of the three additional coupling constants, agreement with the earlier sets of data is satisfactory. The similar values of the measured coupling constants of Hyp^2 in **1**–**4** and 4-hydroxyproline, and their correspondence to the dihedral angles in β-amanitin (Table 3) prove the so-called C_s—$C_{\gamma exo}$ conformation[18] of Hyp^2 where C-γ protrudes

Table 2. Absolute values of ^1H–^1H spin–spin coupling constants (Hz) in amanitin isomers[a]

Amino acid residue	Isomer	α–NH	α–β pro-*S*β	α–β pro-*R*β	γ–β pro-*S*β γCH-β	γ–β pro-*R*β γCH_3-β	γ–δ pro-*S*δ	γ–δ pro-*R*δ
Asn[1]	α	3.0 (2.2, <2)	3.7 (4.0)	3.9 (3.3)	—		—	
(Asp[1])	β	3.7	5.0	3.0	—		—	
	γ	3.1	3.7	4.4	—		—	
Hyp[2 b]	α	—	11.7	6.9	3.3	1.0	3.3	<1
	β	—	11.4	7.1	3.6	1.0	3.2	<1
	γ	—	11.6	7.4	3.7	1.0	3.3	<1
Hyi[3]	α	9.6 (9.9; 9)	6.7 (6.6; 6.0)		6.4	7.1 (6.6)	4.0	6.8
	β	9.8	4.3		8.2	7.1	3.5	6.0
	γ	10.1	8.5			7.0	6.4	
Trp[4]	α	7.7 (7.7; 7.5)	12.3 (12.6)	7.4 (7.1)	—		—	
	β	9.4	12.2	7.4	—		—	
	γ	7.0	12.0	7.4	—		—	
Gly[5]	α	9.3 (9.3; 9) 1.8 (0; ~0)	—		—		—	
	β	9.1 1.9	—		—		—	
	γ	9.4 1.9	—		—		—	
Ile[6]	α	3.6 (3.3; 3)	7.8 (7.7; 7.5)		3.8	8.3 6.7	7.3	
	β	3.7	8.1		3.6	8.4 6.8	7.4	
	γ	3.6	7.7		3.7	8.4 6.8	7.4	
Gly[7]	α	7.4 (7.4; 7) 5.2 (5.0; ~3.5)	—		—		—	
	β	7.5 5.0	—		—		—	
	γ	7.6 5.1	—		—		—	
Cys[8]	α	10.3 (10.0; 11)	4.4	13.1 (13.2)	—		—	
	β	10.3	4.6	13.1	—		—	
	γ	10.2	4.5	13.2	—		—	

[a] Values in parentheses refer to published data. The first figures are from Ref. 6 and the second figures from Ref. 5. Geminal coupling constants in Hz: α–α Gly[5], 18.4 (α), 18.6 (β), 18.5 (γ); Gly[7], 17.4 (α, γ), 17.3 (β); β–β Asn[1], 16.3 (α), 16.8 (β), 16.1 (γ); Hyp[2], 12.8 (α), 13.0 (β), 12.9 (γ); Trp[4], 15.0 (α), 15.3 (β, γ); Cys[8], 13.3 (α, β), 13.2 (γ); γ–γ Ile[6], 13.3 (α, γ), 13.5 (β); δ–δ Hyp[2], 11.3 (α, γ), 11.5 (β); Hyi[3], 11.0 (α), 10.5 (β). The values for the αH isomer are within 0.1 Hz of those of the α-isomer.

[b] ^4J(pro-*R*δ, pro-*R*β) = 1.0 Hz in all isomers.

out from the nearly planar remaining part of the five-membered ring and the OH group has a pseudo-axial orientation (Table 3).

In Hyi[3] and Ile[6] the diastereotopic protons appear correspondingly at the δ- and γ-carbon atoms. The ^3J couplings of the diastereotopic γ-hydrogens with H-β of Ile[6] are different (8.3–8.4 and 3.6–3.8 Hz), in accordance with the x-ray analysis, from which the pro-*S* proton has a *gauche* and the pro-*R* proton a *trans* orientation to H-β. From this it follows that the pro-*R* proton resonates at a higher field (Fig. 2). A more complicated situation arises with Hyi[3] in **1**, **2** and **4** owing to the absence of definite data on the conformation of the OH group at the δ-carbon atom.[4] Consideration of the similar values of the two $^3J(\gamma\delta)$ coupling constants and the possible formation of an intramolecular hydrogen bond between the OH groups leads to the conformation with *gauche* OH groups (Fig. 2). In this conformation the pro-*S* δ hydrogen is closer to the CH_3 group of Hyi[3]. Observation of a cross-peak in the NOESY diagram of γ-CH_3 with the low-field δ-proton was used for the assignment of the diastereotopic δ-protons of Hyi[3] in **1**, **2** and **4**.

The shortest bridge in the bicyclic isomers **1**–**4** is formed by Trp[4] and Cys[8]. The assignment of the diastereotopic β-hydrogen atoms in these two residues is simple on the basis of x-ray data and the large differences in $^3J(\alpha\beta)$ (Fig. 2).

The above examples show that consideration of x-ray data and the coupling constants from the NMR spectra can lead to the assignment of diastereotopic hydrogens. Otherwise, this is possible only by complicated stereospecific labelling of diastereotopic protons by deuterium.[12]

In Ref. 6 much important data for the assignment of several amino acid residues resulted from the observed nuclear Overhauser effects between various hydrogen atoms. A more effective method for obtaining the same information is two-dimensional shift-correlated NMR spectroscopy via interproton cross-relaxation (NOESY).[19] The main advantage of NOESY is not the possibility of obtaining information on interproton

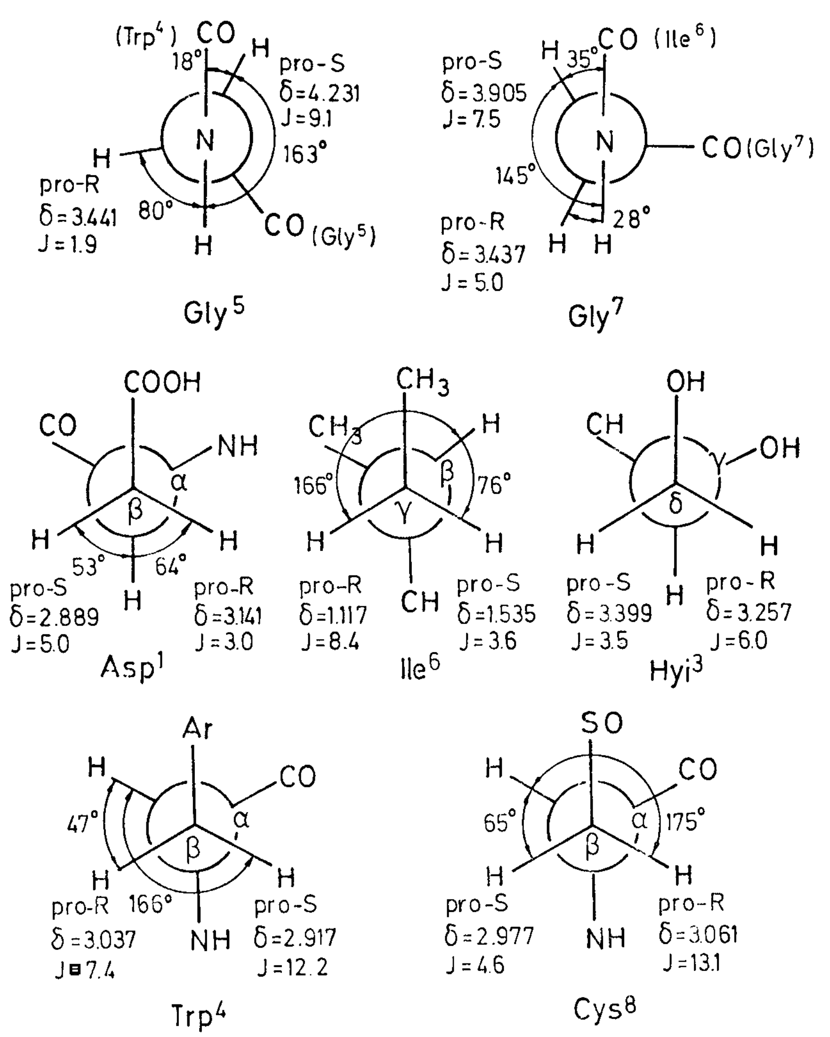

Figure 2. Newman projections of characteristic bonds of β-amanitin, showing the spatial arrangement of diastereotopic hydrogen atoms. Dihedral angles are given on the basis of coordinates from Ref. 4.

nuclear Overhauser effects from a single experiment, but the fact that it does not suffer from the problem of restricted selectivity inherent in the irradiation of overcrowded spectral regions. By varying the mixing time one can accentuate cross-peaks according to the interproton distances.

All four measured isomers of amanitin gave similar NOESY diagrams. The NOESY diagrams for the α-isomer clearly show its advantages over traditional NOE measurements.[6] With a mixing time of 0.3 s one can immediately see all four cross peaks reported in Ref. 6 using the traditional NOE method (there is a misprint

Table 3. Comparison of the absolute values of J(HH) spin–spin coupling constants of 4-hydroxyproline and Hyp^2 in amanitin isomers

	4-Hydroxyproline				Amanitin isomer				
J(HH)[a]	60 MHz[15]	90 MHz[16]	250 MHz[17]	500 MHz	α-	αH-	β-	γ-	Dihedral angle (°)[b]
$^2J(\beta\beta)$	14.06	14.21	14.06	14.09	12.8	12.9	13.0	12.9	
$^2J(\delta\delta)$	12.69	12.63	12.69	12.64	11.3	11.3	11.5	11.3	
$^3J(\alpha, \text{pro-}S\beta)$	10.44	10.11	10.44	10.10	11.7	11.7	11.4	11.6	150
$^3J(\alpha, \text{pro-}R\beta)$	7.66	7.95	7.66	8.02	6.9	7.0	7.1	7.4	30
$^3J(\gamma, \text{pro-}S\beta)$	4.31	4.33	4.31	4.49	3.3	3.4	3.6	3.7	43
$^3J(\gamma, \text{pro-}R\beta)$	1.41	1.70	1.41	1.83	1.0	1.0	1.0	1.0	80
$^3J(\gamma, \text{pro-}S\delta)$	4.09	3.77	4.09	3.91	3.3	3.2	3.2	3.3	34
$^3J(\gamma, \text{pro-}R\delta)$	1.22	1.47	1.22	1.54					86
$^4J(\alpha\gamma)$		0.60	0.70	0.70					
$^4J(\alpha, \text{pro-}S\delta)$				0.35					
$^4J(\text{pro-}R\beta, \text{pro-}R\delta)$	1.66	2.22	1.60	2.00	1.0	1.0	1.0	1.0	
$^4J(\text{pro-}S\beta, \text{pro-}S\delta)$				0.50					

[a] $|^4J|_{\alpha,\ \text{pro-}R\delta;\ \text{pro-}S\beta,\ \text{pro-}R\delta;\ \text{pro-}R\beta,\ \text{pro-}S\delta} < 0.2$ Hz.
[b] From the x-ray study of the β-isomer.[4]

in Ref. 6; Gly^{7}NH–Ile^{6}NH should read Gly^{7}NH–Ile^{6}H-α). In the 2D plot of **1**, numerous other useful cross-peaks appear which can be correlated well with the x-ray data: Asn^{1}NH–Cys^{8}pro-H-β (distance 2.02 Å) [the cross-peaks with pro-*S* H-β (3.61 Å) is missing]; Ile^{6}NH–Gly^{5}H-α (cross-peaks with both H-α protons at distances of 2.45 and 2.59 Å); Cys^{8}NH–Cys^{8}H-β (two cross-peaks, the stronger one with pro-*R*H-β (2.72 and 3.10 Å); Trp^{4}NH–Trp^{4}pro-*S*H-β (2.41 Å, with pro-*R*H-β 3.16 Å); Asn^{1}H-α–Hyp^{2}pro-*R*H-δ (2.25 Å, with pro *S*H-δ 3.35 Å);Asn^{1}pro-*S*H-β–Hyp^{2}H-δ (two cross-peaks, 2.18 and 2.52 Å). Extra attention should be paid to the cross-peaks between the NH protons. This region of the NOESY diagram for **1** is given in Fig. 3. In addition to the trivial cross-peak between the NH$_2$ protons of Asn^1, several cross-peaks appear between the NH protons of adjacent amino acid residues: Asn^1–Cys^8 (distance 2.86 Å); Hyi^3–Trp^4 (2.45 Å); Trp^4–Gly^5 (2.26 Å); Gly^7–Cys^8 (2.59 Å). Cross-peaks between the NH groups of Gly^5–Ile^6 (4.42 Å) and Ile^6–Gly^7 (4.60 Å) are missing owing to the longer inter-proton distances. Instead we obtain a cross-peak between the NH groups of Asn^1 and Gly^5, which proves the rudiments of the α-helix structure of amanitin[4] due to the intramolecular hydrogen bond between Asn^1 (Asp^1) and Gly^5, the distance between the NH protons being 2.32 Å. With a longer mixing time of 0.5 s one obtains additional cross-peaks between the more distant protons. For example, Asn^{1}NH is shown to be additionally coupled to the NH groups of Trp^4 (3.37 Å) and Hyi^3 (4.67 Å). In this connection, the high selectivity of NOESY compared with the traditional method should once again be emphasized, if only with the examples of cross-peaks between the NH groups of Trp^4–Gly^5 and Hyi^3–Trp^4 (Fig. 3).

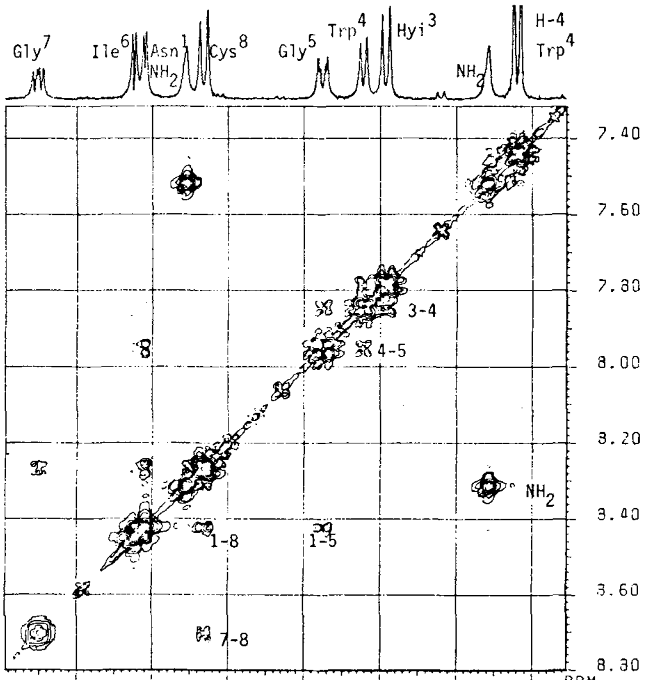

Figure 3. Part of the NOESY diagram of α-amanitin, showing cross-peaks between the amide hydrogen atoms. Mixing time = 0.3 s.

The ^{1}H chemical shifts, coupling constants and NOESY cross-peaks of **1–4** confirm the similarity of their conformations to that of the β-isomer in the crystalline state. However, there are still some definite differences in the chemical shifts and coupling constants of the isomers; these must be caused by conformational peculiarities of these octapeptides. For comparison purposes, the α-isomer is taken as a basis. The loss of a hydroxy group from the Trp^4 residue (αH isomer **4**) leads only to local effects on the indole ring chemical shifts because the 6-OH group points outwards from the bicyclic frame-work and has no close contacts with the remaining parts of the molecule. Small differences also occur in going from the α- to the γ-isomer by replacement of the δ-OH group in Hyi^3 with an H atom. In addition to the shift changes in Hyi^3 itself, only the chemical shift of the NH proton of Trp^4 is changed by more than 0.1 ppm, accompanied by a 0.7 Hz decrease in ^3J(HNCH). Therefore, the α-, α-H- and γ-isomers must have very similar conformations in solution. Replacement of the NH$_2$ group of Asn^1 in **1** by an OH group results in chemical shift changes of more than 0.1 ppm in three amino acid residues, Asp^1, Hyi^3 and Trp^4. Compared with the replacement of Asn by Asp in linear tetrapeptides,[14] different and even opposite trends are observed on going from the α- to the β-isomer. The Asn protons are all shifted to lower field in amanitin, but to high field in linear tetrapeptides. A 4-fold larger change is observed in amanitins for the pro-*R*β proton. The most remarkable shift is for the NH proton of Hyi^3, which is shifted by *ca* 0.6 ppm to lower field in **2**. This large downfield shift must be caused by hydrogen bonding between Asp^1 and the NH group of Hyi^3, the distance between one of the oxygen atoms of Asp^1 and the amido proton of Hyi^3 being 2.62 Å from x-ray data.[4] This hydrogen bond is not listed among the four shortest intramolecular bonds in β-amanitin,[4] but is described by Shoham as being of importance in amatoxins.[3] The variation of this hydrogen bond is responsible for the different conformations of the α- and β-isomers. The largest differences in the coupling constants of **1** and **2** also occur in Asn^1 (Asp^1), Hyi^3 and Trp^4. Most remarkable is the 1.7 Hz larger value for ^3J(HNCH) of Trp^4 in **2** compared with 1. For α-amanitin, the only two ϕ angles calculated from ^3J(HNCH) values and differing significantly from the β-amanitin x-ray values were those for Hyi^3 and Trp^4.[6] Using the ^3J(HNCH) value of Trp^4 in β-amanitin gives a value of $-105°$ for the ϕ angle, which is within experimental error of that determined by x-ray analysis ($-109°$).[6] Therefore, the calculated ϕ value of α-amanitin ($-90°$) certainly reflects the differences in conformations of the α-, αH-, γ- and β-isomers. The bridge geometry between the Trp^4 and Cys^8 is altered owing to the longer hydrogen bond between Asn^1 and Hyi^3 in the α-, αH- and γ-isomers. This effect must also be reflected in the ^{13}C and ^{15}N NMR data.

^{13}C NMR spectra

The ^{13}C NMR spectra of amanitin isomers immediately reveal the presence of Ile (methyl groups at 10.6 and 10.7; 14.7 and 14.8 ppm), Hyp (CHOH at 68.5 and 68.7 ppm), hydroxytryptophan and two glycines on the basis of the observed characteristic chemical shifts. However, the published data on the chemical shifts of amino acid residues[20] give only approximate values for isomers **1**–**4**. The same holds true for the free amino acids as models for **1**–**4**.[14] Therefore, heteronuclear 2D ^1H–^{13}C chemical shift correlation experiments via $^1J(CH)$ were performed in order to complete the assignments of all isomers. The method is effective owing to the large and similar values of $^1J(CH)$ (about 130 Hz). The alternative use of selective decoupling from the assigned protons is complicated by the presence of diastereotopic methylene hydrogens in all the amino acid residues of **1**–**4**. The observed large deviations of several chemical shifts (Table 4) from those in model linear pentapeptides[20] must be caused by different non-bonded interactions in amanitins. The lack of data for all amino acid residues in model linear peptides[20] and their conformations makes a detailed analysis of the carbon chemical shifts of **1**–**4** very speculative.

Carbonyl carbons of peptide bonds form the link between the different amino acid residues and can be an independent source of information for the determination of the amino acid connectivities of polypeptide chains. All nine carbonyls in **1**–**4** are separated and give signals in the range 166.7–172.9 ppm. These chemical shifts do not provide the possibility of an unambiguous assignment to individual residues and do not allow the determination of the structures of neighbouring amino acid residues. It has been shown that selective irradiation of the protons of NH groups and α-protons can be used for the determination of connectivities between the amino acid residues.[21] The observed effects are sometimes too weak to allow unambiguous assignments. The use of selective π-pulses on the ^{13}C satellites of α-protons with the recording of carbonyl carbon signals[22] is complicated by the necessity of determining the exact resonance frequencies of selective pulses. The most versatile method is again the 2D ^1H–carbonyl carbon chemical shift correlation via the $J(CH)$ long-range coupling constants. Wynants *et al.*[23] have used the 2D correlation between the α-hydrogen and carbonyl carbon chemical shifts for this purpose. A modification

Table 4. Comparison of the ^{13}C chemical shifts of the amino acid residues in amanitin isomers and in linear peptides (in DMSO)

Amino acid residue	Carbon atom	Amanitin isomer				
		α	αH	β	γ	Polypeptides[a]
Asn^1	α	50.8	51.0	5.14	50.8	49.3
(Asp^1)	β	33.5	33.6	36.6	33.4	37.1
	γ	172.6	172.9	172.1	172.7	171.2
	CO	170.7	170.8	171.7	170.4	170.3
Hyp^2	α	61.9	62.1	61.5	61.7	60.9
	β	37.4	37.5	37.8	37.6	38.8
	γ	68.7	68.9	68.5	68.5	71.2
	δ	55.8	55.9	55.9	55.7	54.4
	CO	170.2	170.5	171.7	170.1	174.5
Hyi^3	α	55.2	55.4	55.8	55.9	
	β	38.0	38.1	37.8	41.2	
	CH_3	13.4	13.5	14.6	11.3	
	γ	72.2	72.4	71.7	65.8	
	δ	63.3	63.5	63.9	18.3	
	CO	170.7	171.0	171.0	170.4	
Trp^4	α	53.0	52.9	52.5	53.1	53.1
	β	28.6	28.8	28.6	28.4	27.9
	C-2′	129.8	132.5	129.7	129.8	120.5
	C-3′	111.6	111.3	111.9	111.4	109.7
	C-3′a	120.7	128.0	120.7	120.7	127.2
	C-4′	122.2	121.6	122.0	122.2	117.9
	C-5′	110.7	119.4	110.5	110.5	123.5
	C-6′	154.6	123.3	154.6	154.6	118.2
	C-7′	96.7	112.3	96.6	96.6	111.0
	C-7′a	138.8	137.4	138.8	138.8	135.9
	CO	170.1	170.3	170.0	170.0	171.2
Gly^5	α	41.2	41.3	41.3	41.0	42.1
	CO	170.3	170.6	170.2	170.2	168.7
Ile^6	α	59.2	59.4	59.1	59.1	56.2
	β	34.6	34.7	34.5	34.5	37.1
	γ-CH_3	14.8	14.7	14.7	14.7	15.1
	CH_2	25.2	25.2	25.1	25.1	24.2
	δ-CH_3	10.7	10.6	10.6	10.6	11.0
	CO	171.6	171.8	171.6	171.4	170.6
Gly^7	α	42.4	42.5	42.3	42.3	42.1
	CO	167.8	168.1	167.6	167.7	168.7
Cys^8	α	50.1	50.2	49.9	50.0	51.6
	β	58.9	59.0	58.7	58.8	40.3
	CO	167.1	167.3	166.7	167.1	169.1

[a] Data from Refs 14 and 20, except for Hyp^2. The reported shifts for Hyp^2 are hydroxyproline chemical shifts in a D_2O–DMSO mixture.

(COLOC) of the traditional pulse scheme for correlation via long-range J couplings has been proposed.[24] From the assigned proton chemical shifts one obtains by these methods the assignments of carbonyl carbons and connectivities between the amino acid residues. The need for long delays within the pulse sequencing owing to the small values of the coupling constants has a strong impact on the signal-to-noise ratios obtained. Large differences between the long-range coupling constants can result in the missing of some cross-peaks between the carbonyl and α-protons. Not all cross-peaks between the carbonyl carbons and α-protons are seen even in the case of simple tetrapeptides.[23] Therefore, a more universal approach includes all long-range couplings of the carbonyl carbons with the NH, α- and β-protons of the amino acid residues. The results of these correlations are shown in Table 5, where the relative intensities of the various cross-peaks are given together with the dihedral angles from the x-ray data for the cross-peaks corresponding to ^3J(NH). These data give unambiguous assignments for all carbonyl carbons and determine the connectivities of the amino acid residues. However, not all cross-peaks with the α-hydrogens of the same residue [^2J(CH)] or the neighbouring residue [^3J(CH)] are seen, as was proposed in Ref. 23, and therefore correlation with all protons is needed. As a rule, the cross-peaks via ^3J(CH) appear from the protons having suitable dihedral angles for stronger vicinal spin–spin coupling (Table 5). This also explains the large differences in the intensities of the cross-peaks from diastereotopic protons. Hence the $^1H-^{13}CO$ chemical shift correlation diagram also contains additional information about the conformation of the peptide framework. One has to keep in mind the fact that the intensity of a cross-peak is not only the function of a coupling constant value between the carbonyl carbon and a particular proton, but also a function of the whole coupling network.[25]

The correlation experiments by the traditional method and by the COLOC pulse sequence[24] with 40 ms delay times complement each other, accentuating some cross-peaks in different ways.

The assigned ^{13}C chemical shift variations are in accordance with a different conformation of the β-isomer in comparison with the α-, αH- and γ-isomers. Much larger variations occur on going from Asn[1] of the α-, αH- and γ-isomers to Asp[1] of the β-isomer than in linear tetrapeptides. In addition, there are detectable differences in the carbonyl carbon chemical shifts of the Cys[8] over six bonds (0.4 ppm) and, even more remarkable, on Hyp[2] over eight bonds (0.9 ppm). The Hyp[2] carbonyl is a neighbour to the NH group of Hyi[3] which has, from ^1H data, a much stronger hydrogen bond to Asp[1] in the β-isomer compared with that of the α-isomer. This intramolecular hydrogen bond is also reflected in the differential shielding of the α-carbon atoms of Hyi[3] in the α- and β-isomers (0.6 ppm). Changes in the chemical shifts of the αH- and γ-isomers compared with the α-isomer have only a local nature.

The bicyclic rigid structure of amanitin isomers is confirmed by the spin–lattice relaxation times of the carbon atoms of α-amanitin (Table 6). Even the presence of two OH groups in Hyi[3] does not cause any noticeable effects on the segmental mobility characteristic of alcohols. An intramolecular hydrogen bond between the OH groups of Hyi[3] may be responsible for this fact. The rotational diffusion time, τ_r, can be calculated from the measured T_1 values. Its value for α-amanitin at 313 K in DMSO solution is approximately 0.5 ns, which corresponds to the values measured in several oligopeptides.[20]

^{15}N NMR spectra

^{15}N NMR spectra with the low natural abundance of

Table 5. Determination of connectivities of amino acid residues in β-amanitin from 2D $^1H-^{13}CO$ chemical shift correlation diagrams

Amino acid residue	δ_{CO}	Relative intensities of cross-peaks and dihedral angles θ[a]							
		$^2J(OC^nCH)$	$^2J(OC^nN^{n+1}H)$	$^3J(OCCCH)$	θ (°)	$^3J(OCCNH)$	θ (°)	$^3J(OC^nN^{n+1}CH)$	θ (°)
Asp[1] COOH	172.11	++	o	+++	176	o		o	
		++	o						
Asp[1]	171.68	++	o	+++	176	++	8	–	53
					66			–	28
								–	92
Hyp[2]	171.14	+	+	+	35	o		–	37
				–	85				
Hyi[3]	170.98	+++	++	–	158	–	100	–	4
Trp[4]	170.04	–	+	+++	160	–	71	–	18
				–	81			–	99
Gly[5]	170.21	+++	+++	o		–	41	–	61
		++							
Ile[6]	171.63	+	++	–	68	+	121	+++	152
								+	35
Gly[7]	167.64	+++	+	o		–	93	+	4
		++							
Cys[8]	166.67	+	+++	+++	178	–	59	–	52
				–	58	–			

[a] Intensities of cross-peaks are given in the following relative units: o, cross-peak impossible; –, cross-peak not observed; +, weak cross-peak; ++, intermediate cross-peak; +++, strong cross-peak. The θ angles are calculated from coordinates given in Ref. 4.

Table 6. Spin–lattice relaxation times of ^{13}C and amide protons of α-amanitin in DMSO at 313 K

Amino acid residue	nT_1 (ms)						
	C=O	C-α T_1	C-α τ_R[a] (ns)	C-β	C-γ	C-δ	NH
Asn[1]	960	250	0.45	250	920 ($CONH_2$)	—	890
Hyp[2]	900	250	0.45	260	250	270	—
Hyi[3]	870	240	0.53	250	300 (CHOH) 1280 (CH_3)	420	810
Trp[4]	890	230	0.64	250	—	—	750
Gly[5]	930	300	0.27	—	—	—	900
Ile[6]	870	260	0.40	280	420 (CH_2) 1530 (CH_3)	2904	750
Gly[7]	930	260	0.40	—	—	—	870
Cys[8]	830	230	0.64	260	—	—	970

[a] r_{C-H} = 1.1 Å.

this isotope (1.04×10^{-3}) have been recorded only for a few oligopeptides. The most effective method by ^{15}N detection for this purpose is the INEPT technique without decoupling[26] or with decoupling[27] from protons. The recording of signals from the proline residues is much harder to achieve owing to the lack of a proton connected to the nitrogen atom. Polarization transfer from the non-bonded-to-nitrogen protons with non-selective ^1H pulses does not usually provide a high enough signal-to-noise ratio. The method using selective ^1H pulses seems to be more advantageous,[28] but it needs at least one separate time-consuming experiment for each proline residue.

The values of $^1J(NH)$ (Table 7) indicate that the proposed limits of $^1J(NH)$ for the determination of *cis* and *trans* isomers of peptide bonds[9] [$^1J(NH)$ *cis* = 89.3–91.1 Hz and $^1J(NH)$ *trans* = 92.0–94.5 Hz] have to be modified to an even smaller gap between the two ranges. From the x-ray data this angle ω for Trp[4] in **1** equals 172°,[4] and $^1J(NH)$ = 91.5 Hz (Table 7) corresponds to this peptide bond with a *trans* conformation.

At this stage it is useful to compare the chemical shift ranges of various nuclei in the polypeptide chain of amanitins: H-α 1.7 ppm, amide protons 0.93 ppm, carbonyl carbons 5.0 ppm, amide nitrogens (without Hyp[2]) 25.1 ppm. The chemical shifts of the ^{15}N nuclei are most sensitive to the polypeptide chain structure and its conformation. The available scarce data on ^{15}N chemical shifts in polypeptide chains, however, do not contain any useful generalizations which can be connected with their structural and conformational peculiarities.

For the assignment of the ^{15}N signals to definite amino acid residues the 2D ^1H–^{15}N chemical shifts correlation diagrams via $^1J(NH)$ were measured (Fig. 4). The use for this purpose of the ^{15}N chemical shifts of model compounds does not lead to reasonable results.[29] The amide nitrogens of glycine residues usually give signals at higher fields, which is connected with the smaller number of β-substituents to nitrogen. The data in Table 7, however, show that the NH resonances of Hyi[3] are shifted to a higher field than those of Gly[7]. The large differences in the shieldings of ^{15}N from Gly[7]

Table 7. ^{15}N chemical shifts of amino acid residues in amanitin isomers

Amino acid residue	NH_3[a] α	NH_3[a] β	NH_3[a] γ	NH_3[a] αH	$^1J(NH)$, α
Asn[1] (Asp[1]) NH	111.9	115.4	111.7	111.9	∼92[b]
(Asp[1]) NH_2	119.6	—	120.8	119.4	89.3
Hyi[3]	108.5	106.2	108.5	108.5	93.1
Trp[4] NH	118.0	118.5	118.0	118.1	91.5
Trp[4] indole	124.0	124.6	124.1	126.0	99.1
Gly[5]	97.9	99.6	97.2	98.4	93.0
Ile[6]	121.2	122.3	121.1	121.1	92.5
Gly[7]	111.5	112.3	111.4	111.5	92.8
Cys[8]	113.5	114.5	113.6	113.7	∼92[b]

[a] Measured from external 90% formamide in DMSO (δ_{NH_3} = 112.9 ppm).

[b] Approximate values owing to overlapping multiplets.

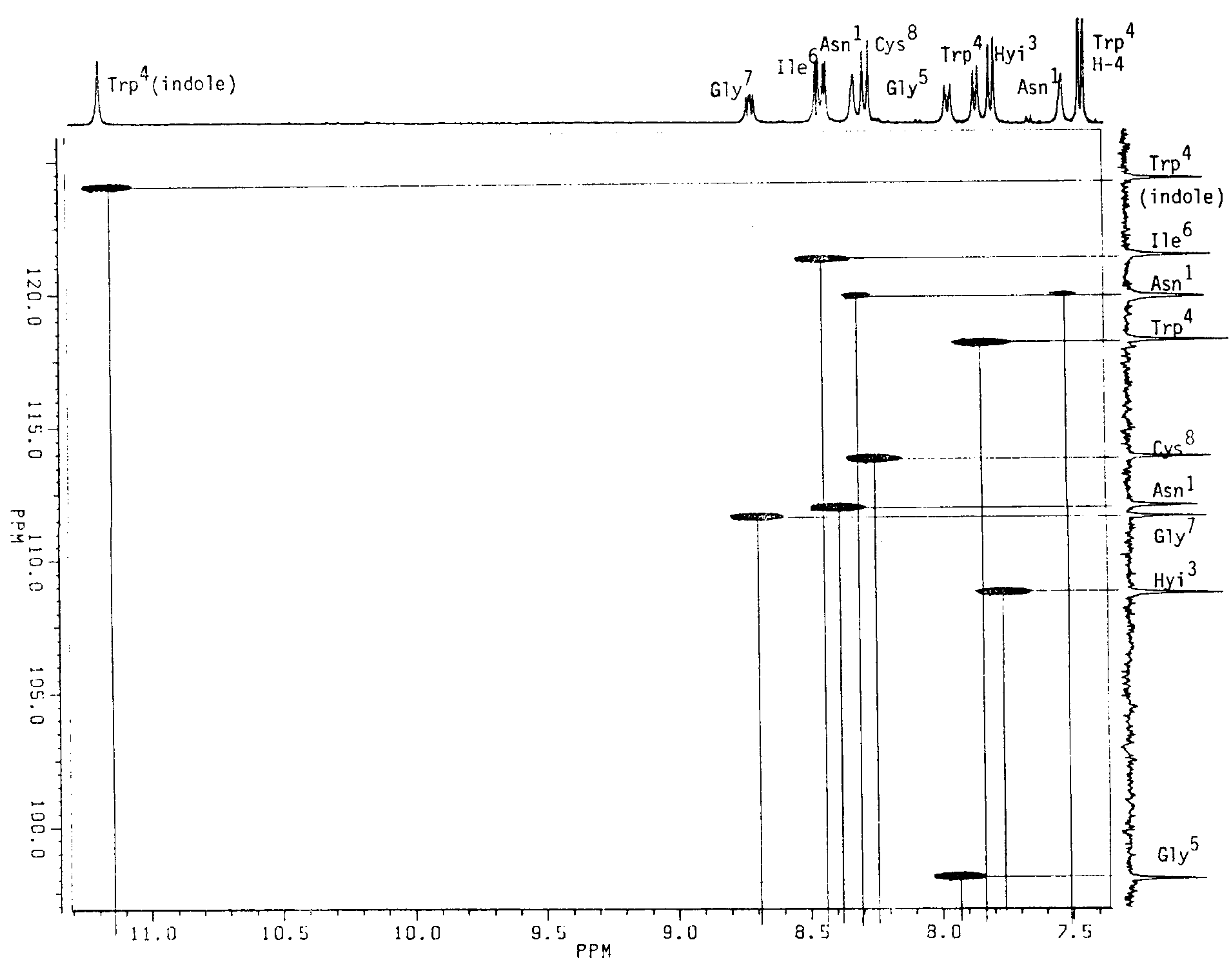

Figure 4. ^1H–^{15}N chemical shift correlation of α-amanitin.

and Gly^5 may be caused by eclipsing of the NH group of Gly^5 by its carbonyl oxygen (dihedral angle only 7°).[4] At the same time the NH group of Gly^7 is *trans* oriented to carbonyl oxygen ($\theta = 178°$). It is tempting to connect the observed ^{15}N chemical shifts in amide groups with non-bonded interactions.

Torsional angles ψ (which determine the dihedral angles between the amide nitrogens in a polypeptide chain) have various values from eclipsing (ψ_7) to *trans* orientations of the amide nitrogens. Exclusion from the discussion of Gly^5 and Gly^7 with one β-substituent less than at other residues, the terminal NH_2 group of Asn^1 in **1**, **3** and **4** and the indole NH of Trp^4 leaves five NH groups. Of these, Hyi^3 and Asn^1 (Asp^1) both have four *gauche* interactions with atoms heavier than H [Hyi^3 with NH of Trp^4, N of Hyp^2, Ch$_3$ of Hyi^3 and C-β of Hyp^2; Asn^1 (Asp^1) with NH of Cys^8, =O of Asn^1 (Asp^1), C of Cys^8 and the carbonyl carbon of Asn^1 (Asp^1)]. Cys^8 has three *gauche* interactions [with NH of Asn^1 (Asp^1), NH of Gly^7 and with the carbonyl oxygen of Cys^8], and Trp^4 and Ile^6 both have two *gauche* interactions (Trp^4 with the NH groups of Hyi^3 and Gly^5; Ile^6 with its carbonyl oxygen and C-γ). Increasing the number of *gauche* interactions must shift the ^{15}N resonance to higher field, which is observed experimentally in the case of amanitin isomers. This crude explanation of the observed chemical shifts does not take into account deviations from the ideal *gauche* conformations with the dihedral angles of 60°, and ignores the supposedly different values of γ effects from various heteroatoms. The influence of intramolecular hydrogen bonds with the participation of NH groups from Asn^1, Gly^5 and Cys^8 should also be taken into account.[4] It is believed that hydrogen bonds cause low-field ^{15}N NMR shifts.[29,30] However, comparison of the ^{15}N chemical shifts of the NH groups of Hyi^3 in the α- and β-isomers reveals a long-range interaction with Asp^1 in the β-isomer, with a high-field shift of 2.3 ppm due to stronger hydrogen bonding. Of the two glycine residues, Gly^5 has a strong intramolecular hydrogen bond responsible for the helix-like structure of amanitins, also confirmed by the NOESY diagrams. However, Gly^5 amide nitrogens are shifted by 12.7–14.2 ppm to higher (!) field.

Mutual comparison of the ^{15}N chemical shifts of amanitin isomers again accentuates the difference of the β-isomer conformation from all others. The specific interaction between Asp^1 and Hyi^3 is reflected in a low-field shift of 1 NH by 3.5 ppm and a high-field shift of 3 NH by 2.3 ppm. Therefore, non-bonded interactions with the amide nitrogens compared with the hydrogen bonds must have a much more pronounced influence on ^{15}N shielding.

The ^{1}H, ^{13}C and ^{15}N NMR results are all in accordance with a general similarity of the conformations of the measured amanitin isomers. Small but definite peculiarities of the β-isomer conformation are observed owing to a different hydrogen-bonding of the Hyi^3 NH with the Asp^1 side-chain carbonyl oxygen atom. A weaker hydrogen bond in the α-, αH- and γ-isomers results in a lower value of the Trp^4 ϕ angle, which modifies the rudimentary character of the α-helix inherent in all amanitin isomers. The lower toxicity of the β-isomer compared with the α-isomer ($LD_{50} = 0.97$ and 0.39 mg kg^{-1}, respectively[31]) may be connected with this modification of conformation. At the same time the α-, αH- and γ-isomers have, from the NMR data, the same conformation for the bicyclic framework and similar toxicities. It seems that the biological activity of the amanitins is connected with a definite conformation of the macromolecule, defined by intramolecular hydrogen bonds and by the presence of OH groups at Hyp^2 and Hyi^3, but not at Trp^4. Further investigations on the structure of enzyme–inhibitor complexes must give conclusive answers to the problem

CONCLUSIONS

The application of various two-dimensional NMR techniques at a high magnetic field of 11.7 T to the bicyclic octapeptide amanitin isomers shows the advantages of high magnetic fields. The analysis of the ^{1}H NMR spectra is greatly simplified, and the diastereotopic methylene protons can be assigned from consideration of chemical shifts and spin–spin coupling constants.

The correlation of proton chemical shifts via the interproton cross-relaxation provides information for the identification of individual amino acid residues, their connectivities and the conformation of the whole macromolecule. Correlation of carbonyl carbon chemical shifts via ^2J(CH) and ^3J(CH) with the proton chemical shifts confirms the connectivities of the amino acid residues and gives useful data about the dihedral angles of polypeptide chain atoms. The large dispersion of the amide ^{15}N chemical shifts is caused predominantly by non-bonded interactions, and the amide nitrogen chemical shifts can be used for conformational analysis of oligopeptides.

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