NMR-Structural Investigations of a β^3-Dodecapeptide with Proteinogenic Side Chains in Methanol and in Aqueous Solutions

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The structural properties of an all-β^3-dodecapeptide with the sequence H-β-HLys(N^{ε}-CO$(CH_2)_3$-S–Acm)-β-HPhe-β-HTyr-β-HLeu-β-HLys-β-HSer-β-HLys-β-HPhe-β-HSer-β-HVal-β-HLys-β-HAla-OH (**1**) have been studied by two-dimensional homonuclear ^{1}H-NMR and by CD spectroscopy. In MeOH solution, high-resolution NMR spectroscopy showed that the β-dodecapeptide forms an (*M*)-3_{14}-helix, and the CD spectrum corresponds to the pattern expected for an (*M*)-3_{14}-helical secondary structure. In aqueous solution, however, the peptide adopts a predominantly extended conformation without regular secondary-structure elements, which is in agreement with the absence of the characteristic trough near 215 nm in the CD spectrum. The NMR and CD measurements with solutions of **1** in MeOH containing 3M urea further indicated that the peptide retains the regular secondary structural elements under these conditions, whereas, after addition of 40% (*v*/*v*) H_2O to the MeOH solution, the large ^{1}H-chemical-shift dispersion indicative of a defined spatial peptide fold was lost. The β^3-dodecapeptide is – so far – the longest β-peptide shown to adopt a regular (*M*)-3_{14}-helix conformation in an organic solvent. The observation that the structure of this long β^3-peptide is not maintained in aqueous solution indicates that the (*M*)-3_{14}-fold is primarily stabilized by short-range interactions.

Introduction. – Helices are ubiquitous in proteins, with the α-helix [1][2] as the most common secondary structural motif. The right-handed α-helix is characterized by 3.6 residues per turn, which corresponds to a pitch of 5.4 Å (1.5 Å per residue), and H-bonds between C=O of residue i and NH of residue $i+4$ forming 13-membered H-bonded rings. The mean values of all residues for the backbone dihedral angles (ϕ,ψ) are *ca.* $-60°$ and $-50°$. In proteins, α-helices vary considerably in length, with an average of 10–12 residues; this is also the chain length required for α-peptides to form stable helices in polar protic organic solvents, such as MeOH or trifluoroethanol [3].

In contrast, small β- and γ-peptides with as few as six amino acid residues fold into turns, helices, and sheet-like structures, analogous to the secondary structures of proteins [4–9]. In addition, these compounds are resistant to proteolytic degradation by common proteases and peptidases [10–12], indicating that they might be useful as peptidomimetics for pharmaceutical applications. Indeed, first biologically active β-peptides have been synthesized and demonstrated to mimic α-peptidic hormones [13–15], to inhibit cholesterol uptake [16], and to possess antimicrobial [17–20] and

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[1]) Part of the projected Ph.D. thesis of *M. R.*, ETH-Zürich, 2002.

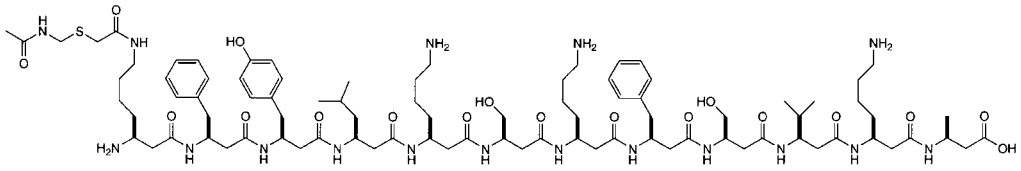
[2]) Part of the Ph.D. thesis of *J. S.*, ETH-Zürich, No. 14298, 2001.

antiproliferative properties [21]. Furthermore, it was shown that certain β-peptides are able to penetrate the cell membrane and accumulate in the cytosol and nucleus [22][23].

β-Peptides consisting solely of β^3-amino acids, which are derived from the natural L-amino acids, have been shown to fold into left handed 3_{14}-helical structures (three residues per turn and 14-membered H-bonded rings between the amide H-atom of residue i and the carbonyl O-atom of residue $i+2$) in organic and aqueous solutions [24–32].

However, longer β^3-peptides have as yet not been structurally characterized other than by CD measurements [20][33–36][3]).

In the present study, we examined the structure of β^3-dodecapeptide **1** with seven different proteinogenic side chains in MeOH and aqueous solutions by means of CD spectroscopy and high-resolution NMR techniques. β-Peptide **1** was prepared by solid-phase synthesis according to the improved procedure described previously [34][38].



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Results and Discussion. – *CD-Spectroscopic Measurements.* Circular dichroism (CD) spectroscopy is a useful method for analyzing α-peptidic structures [39]. For certain β-peptides, CD spectra have been correlated with secondary structures[4]). It has been established by numerous CD measurements and corresponding NMR investigations that β-peptides consisting of L-β^3-amino acids exhibit a characteristic CD pattern with a negative *Cotton* effect near 215 nm, zero-crossing between 205 and 210 nm, and a positive peak near 200 nm, a pattern that was assigned to a left-handed (*M*) 3_{14}-helical structure [41][42]. The β-peptide **1** exhibits the CD spectrum shown in *Fig. 1,a*, and, thus, should be present in the 3_{14}-helical arrangement in MeOH.

However, by switching to H_2O as the solvent, the CD spectrum shows a pattern with a single maximum at *ca.* 200 nm at pH 3.5, 7, and, with a lower intensity, also at pH 11 (*Fig. 1,b*). Additional spectra have been recorded in the presence of helix-destabilizing urea and in acidic solution. The spectra were measured in MeOH solution with increasing amounts of urea (0.1–3M) or $MeSO_3H$ (0–75%) (*Fig. 1,c* and *d*). Interestingly, in the case of urea addition, the trough at 215 nm remains with unchanged intensity, even in the presence of 3M urea[5]). A different behavior is observed upon addition of $MeSO_3H$[6]): the CD curve changes to a pattern with a single maximum at *ca.* 200 nm, similar to the curves observed with aqueous solutions (*Fig. 1,d*).

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[3]) *Gellman* and co-workers reported on NMR-structural investigations of cyclic constrained octapeptides consisting of *trans*-ACHC amino acids [37].

[4]) For review articles in which CD spectra of β-peptides are discussed, see [6][40].

[5]) Self-absorption by urea in the short-wavelength range 'closes the window' in the CD spectrum under these conditions.

[6]) Upon addition of $MeSO_3H$ to a solution of the polymer H-β-HLys(Cbz)-OH in 75% $(CF_3)_2CHOH$/25% H_2O, a similar effect on the CD spectrum has been observed [43].

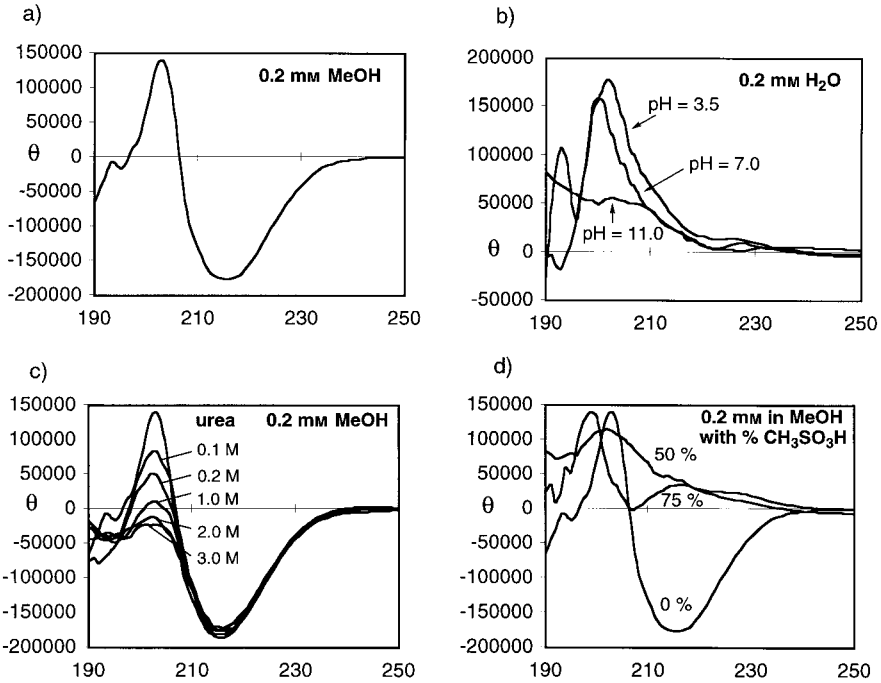

Fig. 1. *CD Spectra of the β^3-dodecapeptide* **1** *in various solutions. a*) In MeOH: the negative *Cotton* effect between 210 and 220 nm is considered to be characteristic of a 3_{14}-helical structure; *b*) in buffered H_2O solution: the CD spectra show a pattern with a single maximum at *ca.* 200 nm at pH 3.5 and 7, but with a lower intensity at pH 11 (which might indicate a change of secondary structure); *c*) the trough at 215 nm remains at the same intensity, even in the presence of 3M urea, indicating that there is no considerable change in the 3_{14}-helical structure; *d*) by addition of $MeSO_3H$, the CD pattern becomes similar to those observed in aqueous solution. The spectra were recorded at room temperature, at a concentration of 0.2 mM. Molar ellipticity $[\theta]$ in 10 deg · cm^2 · mol^{-1}.

The conclusions from the CD measurements would be: *i*) there is a helix structure of **1** in MeOH, but not in H_2O; *ii*) 3M urea is not able to cause unfolding of the helix; and *iii*) $MeSO_3H$ destroys the helical structure. To see whether this is true, we have examined the β^3-dodecapeptide **1** by NMR spectroscopy.

NMR Investigation. First NMR-structural investigations of the β^3-dodecapeptide **1** were carried out in MeOH solution. The presence of a regular secondary structure was indicated by a large dispersion of the chemical shifts of the backbone amide H-atoms (*Table 1* and *Fig. 2,a*), as well as by the observation of different values for the scalar coupling constants $^3J(H-C^\beta, H_{ax}-C^\alpha)$ and $^3J(H-C^\beta, H_{la}-C^\alpha)$[7]) for the pairs of diastereotopic protons of the backbone CH_2 groups. In the resulting well-resolved spectra, the amino-acid spin systems were identified by 2D [^{1}H,^{1}H]-2QF-COSY and 2D [^{1}H,^{1}H]-TOCSY measurements, and, for some residues, [^{13}C,^{1}H]-HSQC and 2D

[7]) The superscripts and subscripts α, β, *ax*, and la refer to the C-atoms in the 2- and 3-positions of the β-amino acid residues, and to backbone H-atoms in axial (parallel to the helix axis) and lateral orientations (perpendicular to the helix axis), respectively.

[^{1}H,^{1}H]-NOESY spectra were used in addition. The sequence-specific assignments were established by $d_{\alpha N}(i, i+1)$ and $d_{NN}(i, i+1)$ sequential NOEs by adapting the procedures for α-amino acid peptides [44].

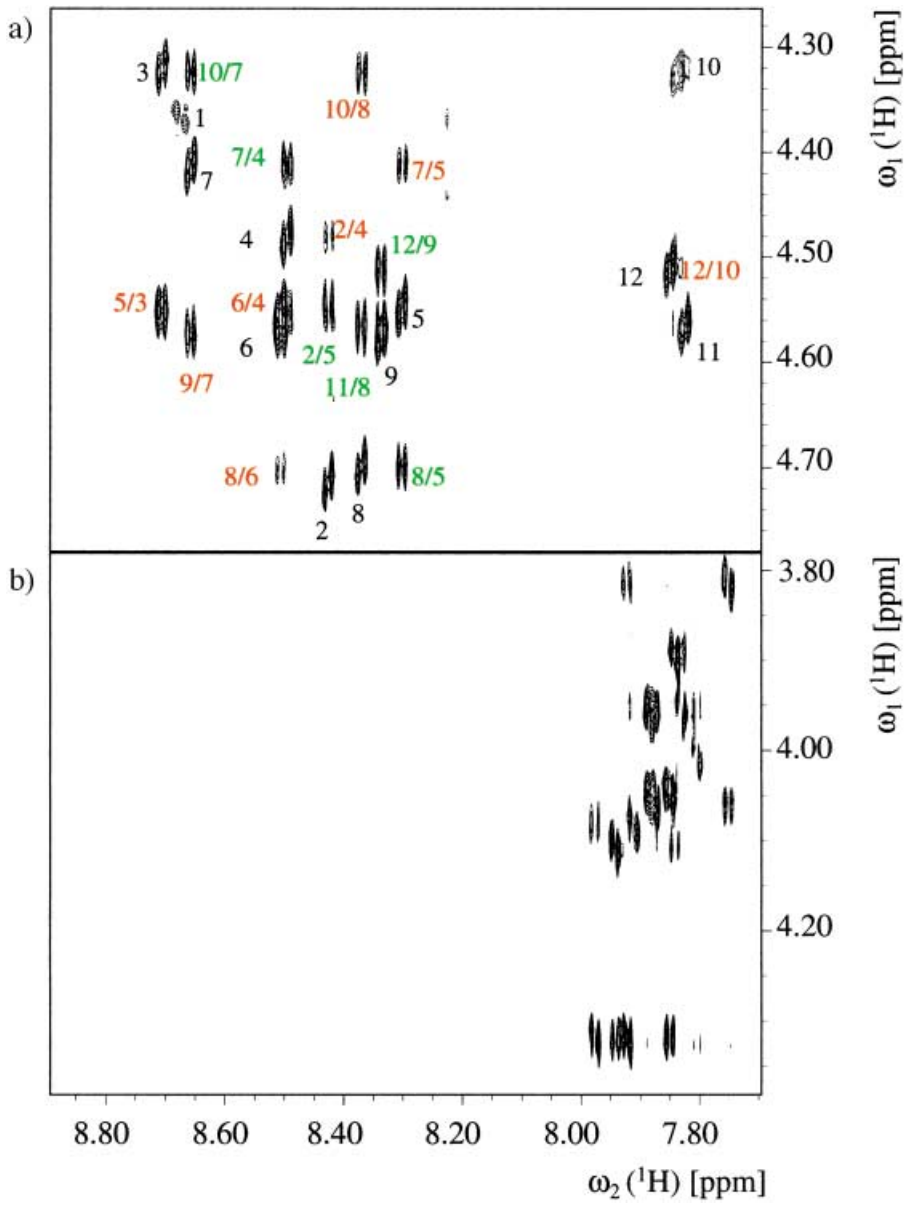

Fig. 2. *Region of the 2D [^{1}H,^{1}H]-NOESY spectra containing the fingerprint of the amide H/β-H cross-peaks of the β^3-dodecapeptide* **1** *in MeOH solution* (a) *and in aqueous solution* (b). The spectra were recorded at 800 MHz and 293 K. In *a*, intraresidual $d_{N\beta}$ cross-peaks are indicated in black numbers, $d_{N\beta}(i, i+2)$ medium-range NOEs are indicated in red, and $d_{N\beta}(i, i+3)$ medium-range NOEs in green.

Table 1. *^{1}H-NMR Chemical Shifts* [ppm] *Relative to DSS of the β^3-Dodecapeptide* **1** *in MeOH Solution*

	H^N	H^{α}_{ax}	H^{α}_{la}	H^{β}	H^{γ}	Others
β^3–Lys 1	–	2.57	2.83	3.51	1.74, 1.68	δCH_2 1.55, 1.44; ζCH_2 3.24; ηNH_2 8.26
β^3–Phe 2	8.41	2.42	2.57	4.71	2.83	εH 7.27, 7.27; ζH 7.29, 7.29
β^3–Tyr 3	8.72	2.31	2.69	4.33	2.78, 2.54	εH 7.03, 7.03; ζH 6.68, 6.68
β^3–Leu 4	8.49	2.47	2.68	4.48	1.41, 1.33	δCH_2 1.57; ζCH_3 0.90, 0.94
β^3–Lys 5	8.31	2.58	2.93	4.55	1.71	δCH_2 1.56; ζCH_2 2.85
β^3–Ser 6	8.49	2.58	2.92	4.56	3.57, 3.61	
β^3–Lys 7	8.66	2.40	2.85	4.41	1.57, 1.66	δCH_2 1.48
β^3–Phe 8	8.37	2.44	2.79	4.70	2.86	εH 7.23, 7.23; ζH 7.27, 7.27
β^3–Ser 9	8.34	2.52	2.72	4.57	3.54	
β^3–Val 10	7.84	2.42	2.52	4.33	1.80	δCH_3 0.98
β^3–Lys 11	7.83	2.35	2.54	4.57	1.65	δCH_2 1.50; ζCH_2 2.87
β^3–Ala 12	7.85	2.46	2.53	4.51	1.15	

In the region of the NOESY spectrum containing cross-peaks between amide-H-atoms and C^{β}–H, most of the residues show two cross-peaks to other residues of the peptide (*Fig. 2*,*a*). These are the medium-range $d_{N\beta}(i, i+2)$ and $d_{N\beta}(i, i+3)$ NOEs, which are characteristic for a (*M*)-3_{14}-helix-like fold [31].

Calculation of the complete three-dimensional structure was performed with the program DYANA [45]. The NOE intensities were calibrated with the tools of this program, and yielded an input of 151 NOE upper-distance limits (*Table 2*).

Table 2. *Input of Conformational Constraints and Statistics of the Structure Calculation for the β^3-Dodecapeptide* **1** with the Program DYANA

Quantity	Value
NOE upper-distance limits	151
Intraresidual	98
Sequential	25
Medium-range	28
Long-range	0
Residual target function value [Å^2]	0.036 ± 0.002
rmsd to the mean [Å]	
N, C(β), C(α), and C′ (1–12)	0.24 ± 0.06
All heavy atoms (1–12)	0.96 ± 0.20
Residual NOE violation	
Number >0.1 Å	0
Maximum [Å]	0.094 ± 0.001

The final calculation was started with 100 randomized conformers, and a bundle of the 20 best DYANA conformers is used to represent the NMR structure. The program MOLMOL [46] was used for structure analysis and for preparing the drawings of molecular models (*Fig. 3*). The NMR structure of the β^3-dodecapeptide **1** shows a well-defined, regular (*M*)-3_{14}-helix fold, with a mean pitch of 5.2 Å, 3.0 amino acids per turn

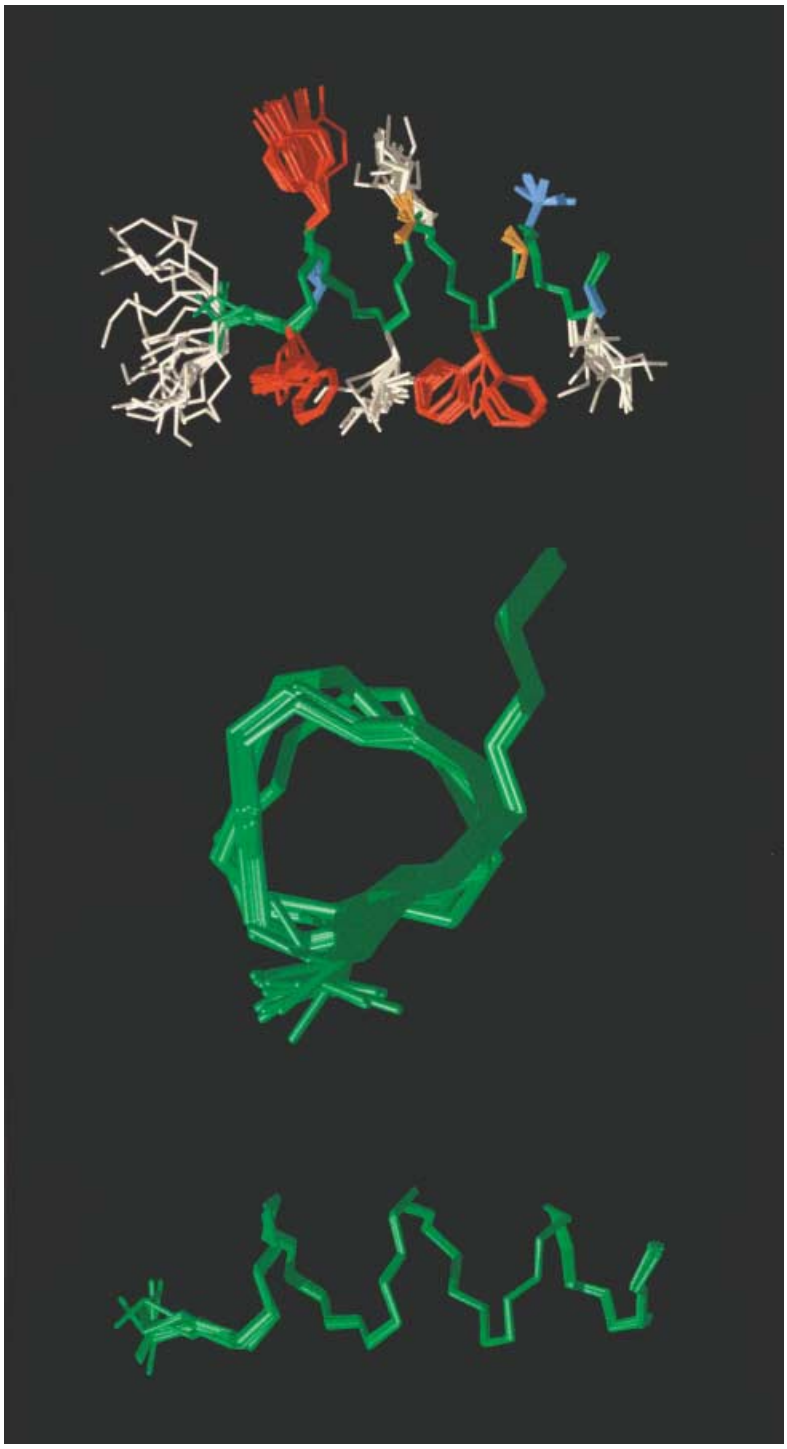

Fig. 3. *NMR Structure of the β³-dodecapeptide* **1** *in MeOH solution*. From left to right: side view of the backbone of the complete structure; view of the backbone in the direction along the helix axis; side view of an all-heavy-atom presentation; the $N^{\varepsilon}CO(CH_2)_3$-S-Acm protecting group is not shown. Each drawing represents a bundle of 20 DYANA conformers, which have been superimposed for best fit of the backbone heavy atoms N, C^{β}, C^{α}, and C' (carbonyl C-atom) of the residues 1–12.

and a radius of 2.4 Å (*Fig. 3*). With the exception of the *N*-terminal protecting group and the long side chains of the β^3–Lys residues 5, 7, and 11, the side chains of the individual conformers also superimposed fairly tightly, indicating that the β^3-dodecapeptide in MeOH solution adopts a well-defined overall conformation. For the aromatic residues β^3–Phe 2 and β^3–Phe 8, two different arrangements of the side chain appear to be compatible with the input data (*Fig. 3*).

The NMR spectra of the peptide in H_2O solution (*Fig. 2,b*) were analyzed along the same lines as described for the MeOH solution. Besides the intraresidual NOEs, only sequential $d_{N\beta}(i, i+1)$ NOEs were observed. The chemical-shift dispersion of the amide-H-atoms is very limited, indicating an absence of regular secondary structure. To determine the transition from a 3_{14} helix in MeOH to an extended nonregular secondary structure in H_2O, a titration of β^3-dodecapeptide **1** in MeOH solution with H_2O was performed. The titration experiment produced the following NMR spectral changes (*Fig. 4*): *i*) the amide-H-atom chemical shifts undergo a gradual change to higher field, and *ii*) the large dispersion of the amide-H-atom chemical shifts is lost. A detailed analysis revealed that these changes do not occur in a concerted manner for all residues. The amide-H-atom resonances of the residues β^3–Val 10, β^3–Lys 11, and β^3–Ala 12 are actually shifted downfield until a H_2O concentration of 30% (*v*/*v*) is reached, and, upon further addition of H_2O, these resonances shift towards higher field. The amide-H-atom resonance of β^3–Lys 5 is insensitive to H_2O concentrations up to 20% (*v*/*v*), but, at higher H_2O concentration, it is also shifted upfield. The resonances of all the other residues shift monotonously towards higher field upon addition of H_2O.

The CD spectra measured in MeOH solution and in MeOH solution containing 3M urea indicated that there is no significant change of the secondary structure. Hence, the β^3-dodecapeptide **1** was analyzed by ^{1}H-NMR spectroscopy in MeOH solution containing 3M urea. The similarity of the chemical-shift dispersion in the two spectra of *Fig. 5* indicates that the peptide largely retains the regular secondary structure after addition of 3M urea to the MeOH solution. In particular, in both spectra, three amide-H-atom-resonance peaks of the three C-terminal residues are in the spectral region 7.7–8.0 ppm, three resonances of the amide-H-atoms of the residues 1, 3, and 7 are in the region 8.5–8.7 ppm, and most of the other resonances are located between these two groups of lines.

Conclusions. – For the first time, we have analyzed a longer β^3-peptide (12 residues) with various functionalized side chains in organic and aqueous solutions by high-resolution NMR techniques. In MeOH solutions, the NMR structure of the β^3-dodecapeptide **1** consists of a regular 3_{14}-(*M*) helix, which is also fully compatible with the CD measurements. Similar to α-peptide helices, the intensity of the *Cotton* effect increases as the helix is lengthened[8]). However, in aqueous solution, the peptide adopts an extended conformation without regular secondary structural elements[9]).

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[8]) The mean residue ellipticity of the trough at 215 nm is -15000 deg cm^2 mol^{-1} for the β^3-dodecapeptide, compared to *ca.* -8500 deg cm^2 mol^{-1} for a β^3-hexapeptide [42].

[9]) It was shown that β-peptide helices can be stabilized by electrostatic interactions of salt-bridge-forming side chains [32][36] or by the introduction of disulfide bridges [30].

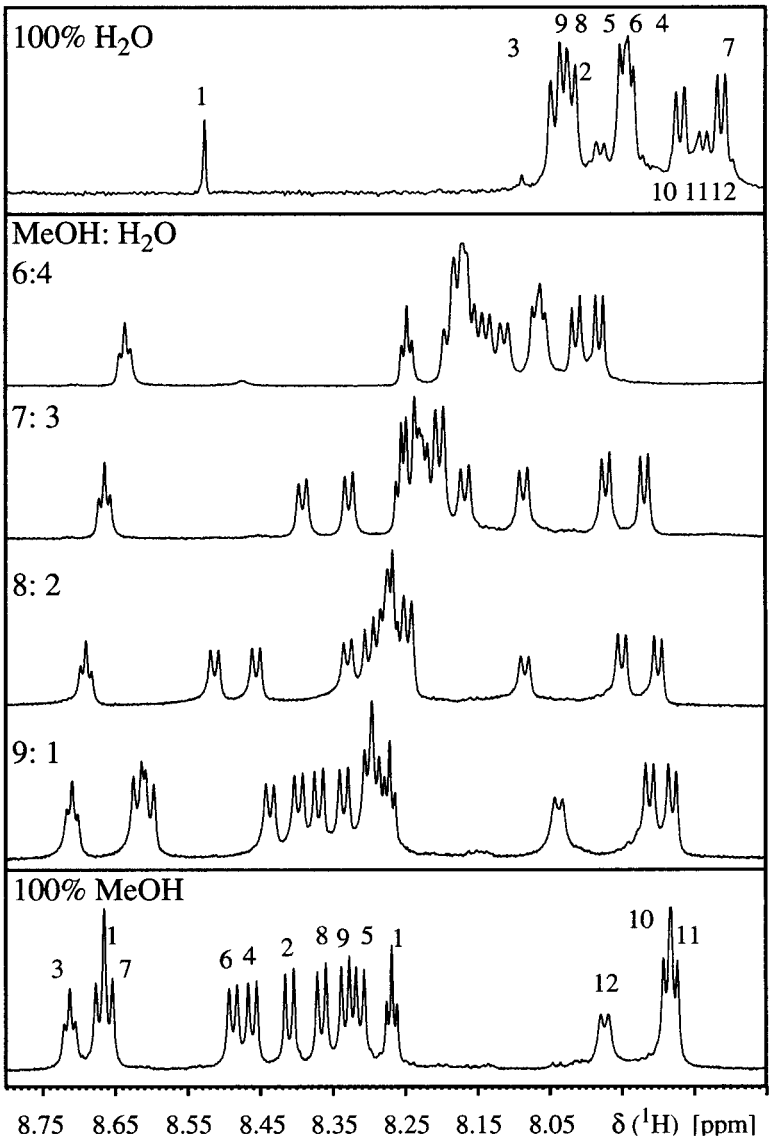

Fig. 4. *Titration of a MeOH solution of the β^3-dodecapeptide* **1** *with H_2O.* Sequence-specific assignments of the amide H-atoms are indicated by the numbers in the spectra at the top and the bottom of the figure. The solvent composition is indicated on the left end of each spectrum.

This could not be predicted form the CD spectra, because previous CD and NMR investigations taught us that a single maximum at *ca.* 200 nm can also be correlated with a hairpin structure [47] or a *12/10* helix [48][49]. The titration studies with the β^3-dodecapeptide **1** in MeOH with H_2O show a gradual transition of amide-H-atom

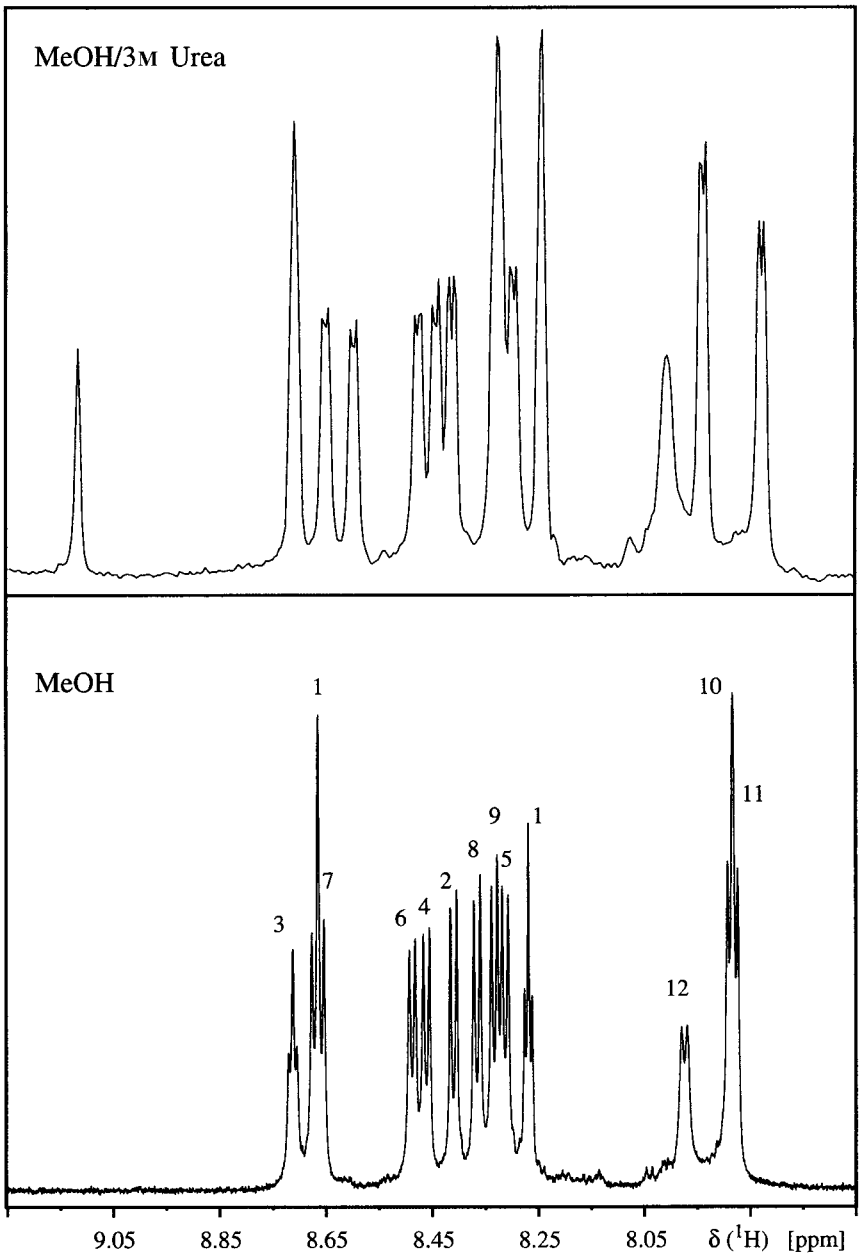

Fig. 5. *Comparison of the amide H-atom region of the β³-dodecapeptide* **1** *in MeOH solution and in MeOH solution containing 3M urea*

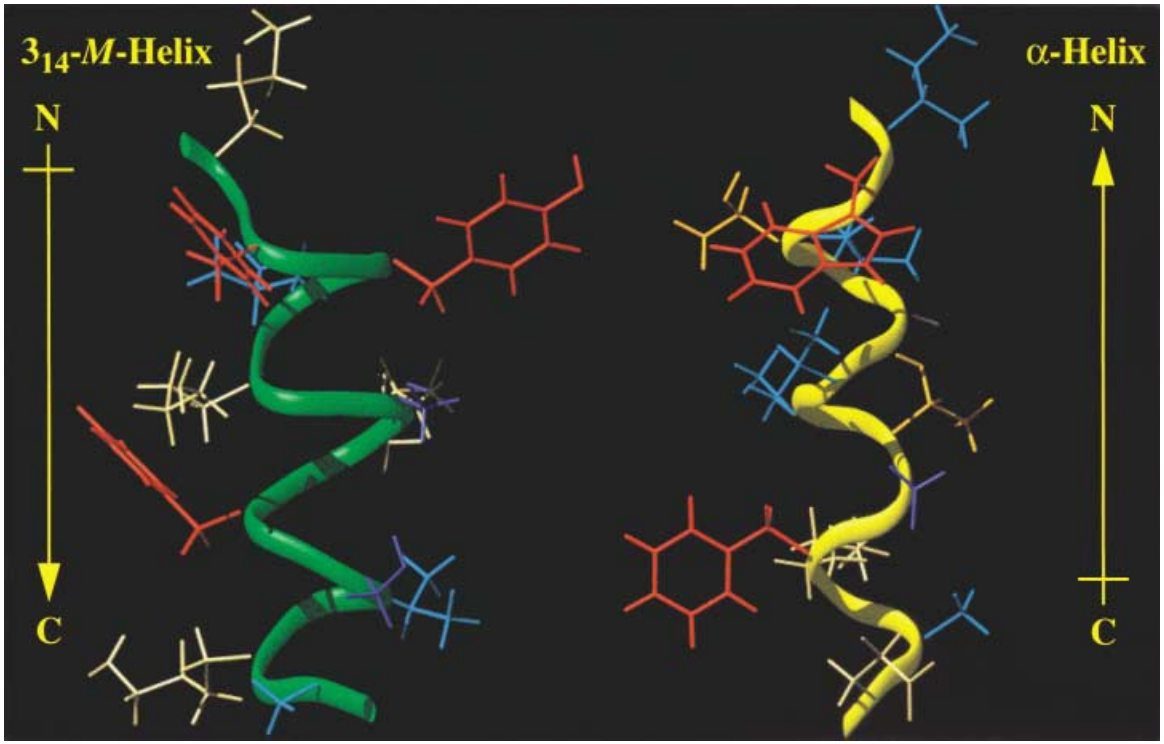

Fig. 6. *Visual comparison of a (*M*)-3_{14} helix with an α-(*P*)helix*. The (*M*)-3_{14} helix is represented by the β^3-dodecapeptide **1**, and the 3.6_{13} or α-helix is formed by the residues 109–121 in the A form of the pheromone-binding protein of *Bombyx mori* [51].

chemical shifts from helical to unordered structure in the spectra[10]) (*Fig. 4*), indicating that the regular 3_{14}-helix is primarily stabilized by short-range interactions. The observation that the amide-H-atom resonance of β^3-Lys 5 is not changing with H_2O concentrations up to 20% (*v*/*v*), while the terminal residues are more sensitive, may indicate that the transition from the folded to the unfolded state occurs through various partially folded intermediates; similar behavior was observed in thermochemical studies with a β-heptapeptide [27]. A comparative analysis of a 12-residue α-peptidic 3.6_{13} helix[11]) with the 3_{14} helix of the β^3-dodecapeptide (*Fig. 6*) nicely illustrates the different handedness of the two helices (right-handed *vs.* left-handed), visualizes the different polarities of the two helices with respect to their C- and N-termini, and shows that the two helices have similar values for the pitch (4.3 *vs.* 4.7 Å) and radius (2.1 *vs.* 2.4 Å). Previously, it has been pointed out that the positioning of the side chains differs in the two helices [4][8].

Experimental Part

CD Spectra: Jasco j-710 spectropolarimeter, from 190 to 250 nm at r.t. in 1-mm rectangular cells. The optical system was flushed with N_2 at a flow rate of *ca.* 10 l/min; parameters band width 1.0 nm, resolution 0.2 – 1.0 nm, sensitivity 100 mdeg, response 0.5 s, speed 50 nm/min, 5 accumulations. All spectra were corrected for the corresponding solvent spectrum. Peptide concentration 0.2 mM. The molar ellipticity $[\theta]$ in 10 deg · cm^2 · mol^{-1} (λ in nm) is calculated for the corresponding peptide (not normalized), taking into account the mass of CF_3COOH for each free amino group. Smoothing was done by *Jasco* software. Solvents: MeOH (HPLC grade), aq. buffers: pH 3.5: 0.1M AcOK/AcOH, pH 7.0: 0.1M KH_2PO_4/K_2HPO_4; pH 11: 0.05M $NaHCO_3$/NaOH.

NMR Spectra: All spectra were measured at a temp. of 293 K on a 800-MHz *Bruker Avance* spectrometer. Solvent suppression was achieved by presaturation. In the TOCSY experiments, the heating effects were compensated for. Chemical shifts were referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) (*Table 1*). The NMR sample consisted of 500 µl of a 4 mM soln. of the β^3-dodecapeptide **1** in CD_3OH (99.5 atom-% D, *Armar*, CH-Döttingen). For measurements in aq. soln., the peptide was lyophilized from the MeOH soln. and dissolved in 95% H_2O/5% D_2O. Other experiments started with a polypeptide soln. in 100% MeOH, to which H_2O was added in steps of 10% (*v*/*v*) up to a final H_2O content of 40% (*v*/*v*). A spectrum was also measured in MeOH soln. containing 3M urea. The following spectra were used for the resonance assignments: 2D-[^{1}H,^{1}H]-TOCSY [52] (data size 4096 · 400 complex points, $t_{1max} = 55$ ms, $t_{2max} = 560$ ms, $t_{mix} = 100$ ms); 2D-[^{1}H,^{1}H]-NOESY [53] (4096 · 500 complex points, $t_{1max} = 69$ ms, $t_{2max} = 560$ ms, $t_{mix} = 450$ ms); 2D-[^{1}H,^{1}H]-2QF-COSY [54] (4096 · 500 complex points, $t_{1max} = 69$ ms, $t_{2max} = 560$ ms); 2D-[^{13}C,^{1}H]-HSQC [55] at natural isotope abundance (2048 · 400 complex points, $t_{1max} = 27$ ms, $t_{2max} = 180$ ms). Based on NOE buildup measurements, $t_{mix} = 150$ ms was used in a 2D-[^{1}H,^{1}H]-NOESY spectrum to collect the input for the structure determination (4096 · 500 complex points, $t_{1max} = 69$ ms, $t_{2max} = 560$ ms).

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10) Similar observations have been made for α-helices in attempts to identify intermediates of folding in the refolding process of denatured peptides and proteins [50].

11) Residues 109 – 121 in the A-form of the pheromone-binding protein of *Bombyx mori* [51].

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