cyclo(*β*-Asp-*β*^{3}-hVal-*β*^{3}-hLys) – Solid-Phase Synthesis and Solution Structure of a Water Soluble *β*-Tripeptide

Preliminary Communication

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The H_2O-soluble cyclic β^3-tripeptide cyclo(β-Asp-β^3-hVal-β^3-hLys) (**4**) was obtained by on-resin cyclization of the side-chain-anchored β-peptide **3** (*Scheme*). In aqueous solution, **4** adopts a structure with uniformly oriented amide bonds and all side chains in lateral positions (*Fig. 3*).

Introduction. – In recent years, the investigation of β-peptides has gained considerable attention (for a review, see [1a]). Consisting of β-amino acids, and thereby differing only by the presence of one additional methylene group from the parent natural α-amino acids, these derivatives are cornerstones in the 'foldamer' research field (for a review, see [1b]). Most studies on these backbone-modified oligomers reported so far have concentrated on mainly two goals. One objective has been to increase the knowledge about the secondary structures that these non-natural oligomers are able to form in solution and in the solid state. Pioneering studies of *Seebach*, *Gellman*, and others have shown that β-peptides can exist in a variety of conformations such as helices, β-sheet-like strands, and other secondary structures [1c]. Other work has focused more on creating biologically active β-peptides, especially after it was found that they are stable to proteolytic degradation *in vitro* [2] and *in vivo* [3]. These efforts resulted, *e.g.*, in the synthesis of inhibitors of cholesterol absorption [4], as well as a number of compounds with antibacterial activity (see, *e.g.*, [5]). The majority of these results were achieved by using linear β-peptides, while only a few studies concerning their cyclic analogues are available.

The limited number of studies on cyclic β-peptides is unfortunate, in particular when considering that natural products comprising cyclic peptides often display unique biological activities. Moreover, cyclic β-peptides have been shown to form self-assembled tubular structures, *i.e.*, nanotubes [6], and some derivatives possess antiproliferative [7] and somatostatin-like activity [8][9]. We believe that the main reason for the limited number of reports on cyclic β-peptides is related to the low solubility of the intermediates involved during solution-phase synthesis [9][10]. Only a few examples on cyclic β-peptides, where the side-chain-protecting groups could be removed and a water-soluble peptide isolated, can be found in the literature [9][11]. In these cases, cumbersome procedures, like the use of chaotropic salts (*e.g.*, LiCl), had to be used to bring the protected peptide into solution for final deprotection.

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To overcome this limitation, and thus open the field for more detailed physical and biological studies on cyclic derivatives of β-peptides, we decided to focus our attention on finding a general and convenient synthetic entry to these molecular entities. One possibility to avoid the solubility problem would be to cyclize the linear β-peptide while still attached to a resin, and thus take advantage of the pseudodilution effect of a resin with a low level of substitution. We started our investigations on this methodology by the preparation of a simple cyclic β-tripeptide and present our results herein.

Synthesis. – On-resin cyclization of cyclic head-to-tail peptides can be accomplished either by anchoring the side-chain or the backbone to the solid phase. Backbone anchoring may be considered more general, as it allows peptides without functionalized side chains to be prepared[2]); nevertheless, we chose to focus on the use of side-chain anchoring, as we expect that biologically active peptides (or peptides with other interesting properties) must contain functionalized side chains to make them H_2O-soluble. Thus, our general strategy for the synthesis of the β-tripeptide cyclo(β-D-Asp-β^3-hVal-β^3-hLys) is based on work by *Trzeciak* and *Bannwarth* [12] and *Albericio* and co-workers [13], who introduced the allyl group as a three-dimensional orthogonal protecting strategy in solid-phase peptide synthesis of cyclic peptides. The trifunctional, diprotected amino acid Fmoc-D-Asp(OAl)-OH[3]) was selected for attachment to the resin. This derivative can be regarded as a β-amino acid with the analogous spatial arrangement of the substituents at the chiral center as β^3-amino acids derived from the *Arndt–Eistert* homologation of natural L-α-amino acids. After standard Fmoc peptide synthesis, the selective cleavage of the allyl group, subsequent on-resin cyclization, and cleavage from the resin should yield the desired product.

TentaGel-S-PHB resin[4]) **1** (0.29 mmol/g) was chosen for the solid-phase synthesis as this resin combines good swelling properties with a low level of substitution. Fmoc-D-Asp(OAl)-OH was anchored to the resin with EDC[3]) (→**2**) (*Scheme*), furnishing somewhat varying substitution levels (between 62% and 93% based on Fmoc UV spectrophotometry). The two β^3-amino acids used were synthesized by *Arndt–Eistert* homologation of the corresponding α-amino acid, following the procedure developed by *Seebach* and co-workers [15a] and later improved by *Seewald* and co-workers [15b]. Successive coupling of Fmoc-β^3-hVal-OH and Fmoc-β^3-hLys(Boc)-OH to the D-Asp(OAl)-resin **2** was achieved by standard Fmoc peptide synthesis protocols: Fmoc deprotection was carried out with 2% DBU/2% piperidin in DMF (5 × 5 min), and the subsequent amino acid coupling with HBTU/HOBt and $^i Pr_2NEt$ for 3 h (completeness controlled by the TNBS test)[3]). This reaction sequence yielded the linear, fully protected β-tripeptide **3** on the solid support. Removal of the allyl protecting group was

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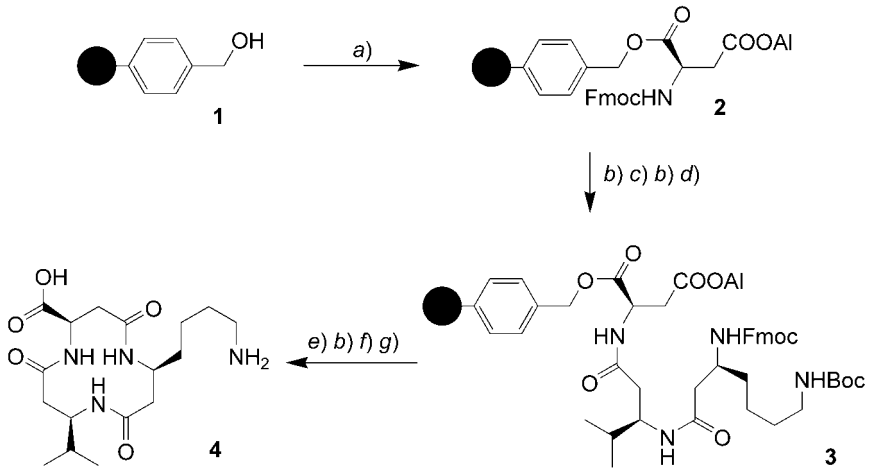
[2]) *Royo et al.* [14] have reported the synthesis of a cyclic β-tetrapeptide using backbone anchoring. In this case, no chiral proteinogenic amino acids were used.

[3]) Abbreviations: Al = Allyl, Boc = (*tert*-butoxy)carbonyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DMAP = *N*,*N*-dimethylpyridine-4-amine, EDC = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, Fmoc = (9*H*-fluoren-9-ylmethoxy)carbonyl, HBTU = 2-(1*H*-benzotriazol-1-yl-)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxy-1*H*-benzotriazole, NMM = 4-methylmorpholine, TNBS = 2,4,6-trinitrobenzenesulfonic acid.

[4]) This polyethyleneglycol-based resin contains a *Wang* linker.

achieved with [$Pd(PPh_3)_4$] following a method created by *Bloomberg et al.* [16]. The subsequent Fmoc cleavage furnished the precursor for the on-resin cyclization, which was carried out with HBTU/HOBt and $^iPr_2NEt$. The TNBS test again showed completion of the reaction after 3 h. Treatment of the resin with CF_3COOH resulted in Boc deprotection and simultaneous liberation of the crude cyclic product. Purification by reversed-phase HPLC gave the title compound **4** in an overall yield of 50% based on anchored Fmoc-D-Asp(OAl)-OH[5]). This peptide, containing one basic and one acidic function is extremely H_2O-soluble in the whole pH range.

Scheme[3])



a) Fmoc-D-Asp(OAl)-OH, EDC, DMAP, DMF, 18 h. *b*) DBU, piperidine, DMF, 5 × 5 min. *c*) Fmoc-β^3-hVal-OH, HBTU, HOBt, $^iPr_2NEt$, DMF, 3 h. *d*) Fmoc-β^3-hLys(Boc)-OH, HBTU, HOBt, $^iPr_2NEt$, DMF, 3 h. *e*) [$Pd(PPh_3)_4$] DMSO, THF, 0.5N HCl, NMM, 4 h. *f*) HBTU, HOBt, $^iPr_2NEt$, DMF, 3 h. *g*) CF_3COOH, $^iPr_3SiH$, H_2O, 2 h.

NMR Investigations. – To the best of our knowledge, the only NMR structure reported for a cyclic β-tripeptide so far is that of cyclo(β^3-hGlu)$_3$ by *Gademann* and *Seebach* in 1999 [11]. This molecule was shown to adopt a C_3-symmetrical structure in D_2O, with all side chains occupying lateral positions at the trilactam ring. Due to the high symmetry, only dihedral angle constraints could be used for calculating a representative solution-phase structure of this peptide.

The amide region of the ^{1}H-NMR spectra of **4** at 500 MHz is shown in *Fig. 1*. Although there are small signals from an alternative conformation in MeOH solution, this molecule clearly exists in one predominant conformation in both MeOH and H_2O. A H_2O/D_2O 9:1 mixture was chosen as solvent for all subsequent NMR spectroscopic studies reported here[6]). Solvent suppression was accomplished using the WET presaturation sequence [17]. The large ^3J(NH,H–C(β)) coupling constants (10.1, 9.7,

[5]) Based on our experience, the reported methodology typically provides *all*-β^3-cyclotripeptides in > 50% yields, while the yields for longer cyclo-β^3-peptides are lower (*ca.* 20%).

[6]) As stated above, the peptide was extremely H_2O-soluble at all pH values investigated (pH 1–10). An acidic pH was chosen for the NMR analysis due to enhanced signal dispersion (see also *Fig. 4*).

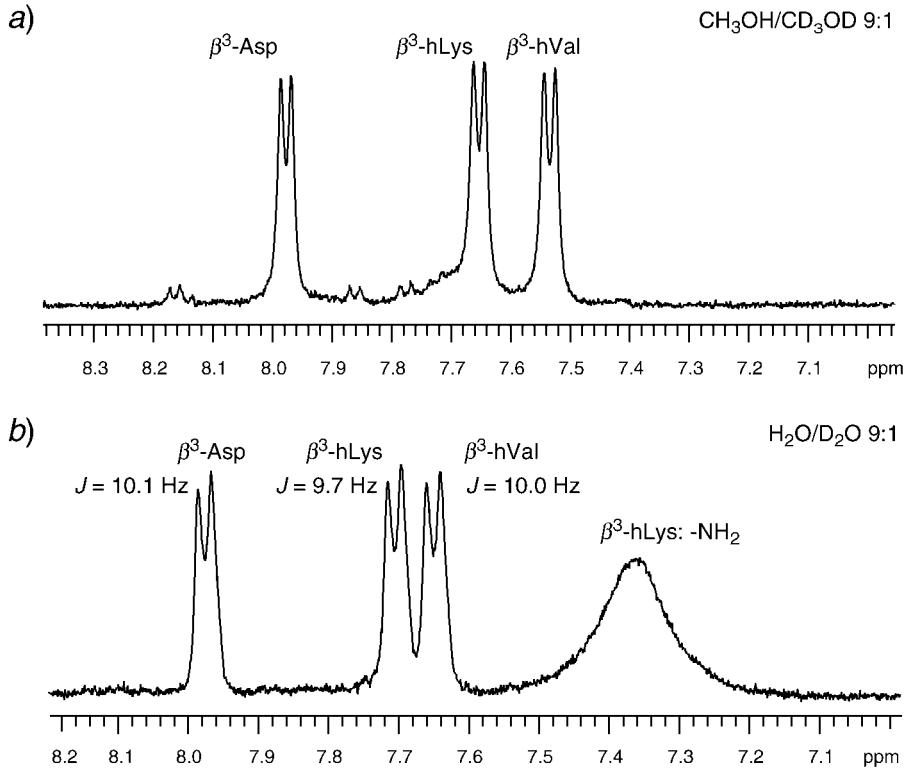


Fig. 1. *Section of the* ^{1}*H-NMR spectrum* (500 MHz) *of* **4** *in* a) *MeOH* (25°, 25 mM) *and* b) H_2O (25°, 25 mM, pH 2.5)

and 10.0 Hz) clearly indicate an *anti* arrangement of these protons in each amino acid residue. Although the scalar $^3J(H-C(\beta),H_{ax/lat}-C(\alpha))$ coupling constants could not be directly measured from the spectra, the *J*-doubling method [18] and a P.E.-COSY experiment [19] allowed us to identify one larger and one smaller ^3J-coupling constant for each residue[7]).

The preference for a structure where all amide NH protons are oriented on one face of the ring is also seen in the ROESY [20] spectrum (300 ms mixing time) shown in *Fig. 2*. Here, only inter- and intra-residue NOEs between the amide NH protons and the axial protons at C(α) are visible. Likewise, the inter-residue NOEs between H–C(β) and $H_{ax/lat}$–C(α) are much stronger for the lateral H–C(α), as compared to the axial proton, supporting a *gauche* orientation of these protons around the C(β)–C(α) bonds (data not shown). It should be stated that the very weak intra-residue NOEs observed between NH and H–C(β) (*Fig. 2*) contradicts the structure supported by the other NOEs and shows that there may indeed be dynamics going on in

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[7]) We hesitate to make use of these coupling constants in the structure calculation as their precise determination would require analysis of higher-order spectra. Further, the signal originating from the H–C(β) of β-Asp is hidden below the suppressed H_2O-signal.

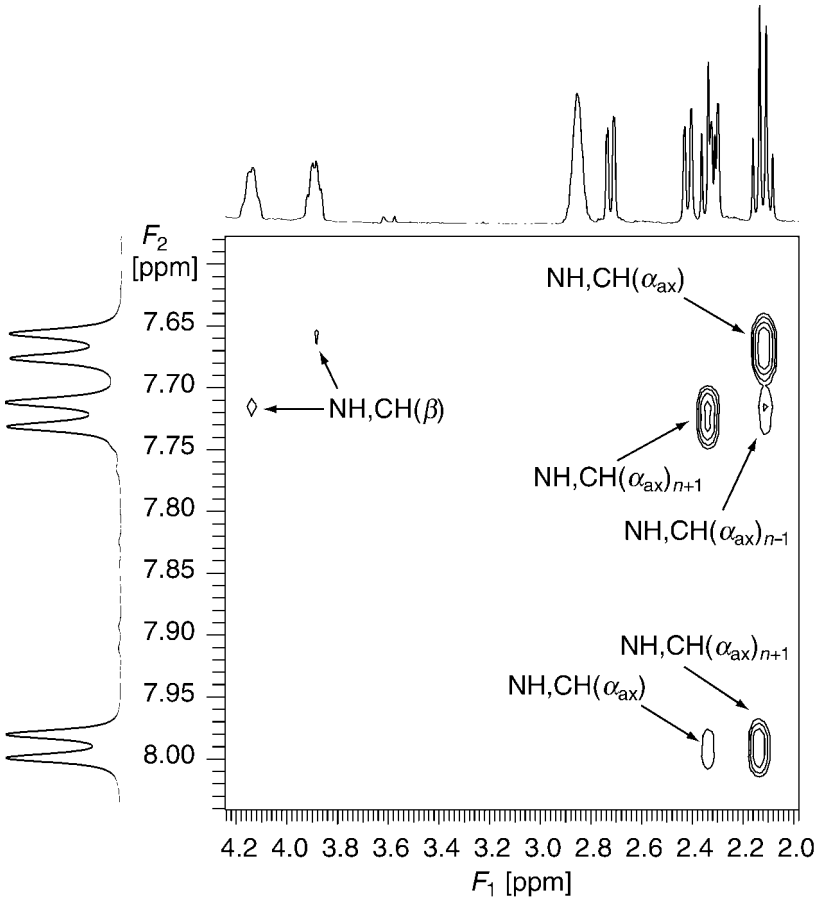

Fig. 2. *Section of the ROESY spectrum* (500 MHz; mixing 300 ms) *of* **4** *in* H_2O (25°, 25 mM, pH 2.5). $CH(\alpha_{ax}) = H_{ax}-C(\alpha)$.

this small ring[8]). A collection of 15 NOEs (6 inter-residual, 9 intra-residual) were used as restraints for a Monte Carlo conformational search with the Macromodel program [21][9]). A superposition of the ten lowest conformations resulting from this calculation is shown in *Fig. 3*.

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[8]) The molecule is monomeric under the conditions used for 2D-NMR analysis, as verified by diffusion measurements (3–44 mM) and amide temperature-coefficient measurements. Only one set of signals, with similar $J(NH,H-C(\beta))$ coupling constants, are observed upon heating the sample to 80°. These observations suggest that one conformer is clearly more stable than the others.

[9]) The Monte Carlo search (20000 steps) followed by PR conjugate gradient minimization (max. 1000 iterations) was done with the program Macromodel, Vs. 7.0. The OPLS-AA all-atom force field and the 'general born solvent accessible' (GB/SA) surface area method was used in all calculations. The number of torsion angles allowed to vary during each Monte Carlo step ranged from 1 to $n-1$ where n equals the total number of rotatable bonds. Amide bonds were fixed in the *trans* configuration. Conformational constraints derived from ROESY cross-peaks were introduced by using the CDIS command (strong, 2.5 ± 1 Å; medium, 4 ± 1 Å; weak, 5 ± 1 Å).

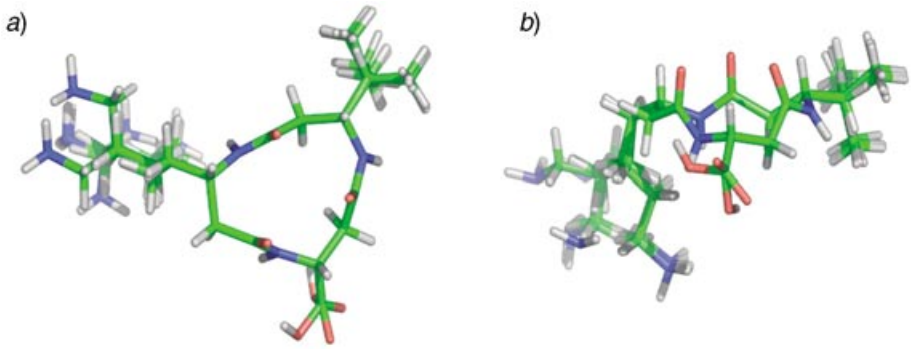

Fig. 3. *Representative structure of* **4** *in aqueous solution*. A superposition of the ten lowest conformations resulting from NOE-restrained conformational searching is shown *a*) from the top and *b*) from the side.

In H_2O solution, the cyclic β-tripeptide **4** adopts a structure with all amide bonds uniformly oriented, and all side chains in lateral positions. The amide bond of β-D-Asp is not completely perpendicular to the plane of the ring in this structure. This twist is possibly due to a H-bond between the COOH side chain and the C=O of the amide at low pH. To test this hypothesis, a pH titration was performed. As seen in *Fig. 4*, the signal for NH of β-D-Asp drifts considerably upfield with increased pH. This drift may

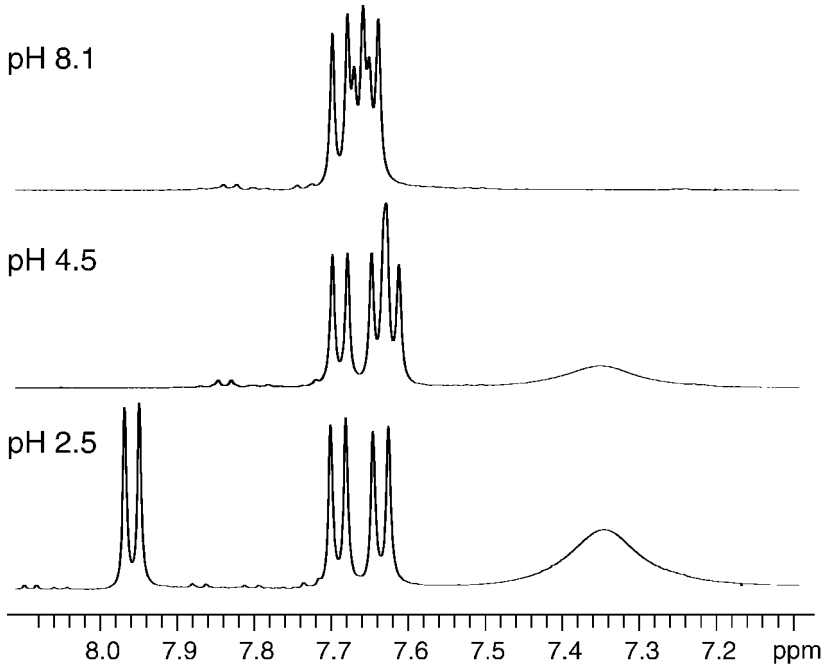

Fig. 4. *Section of the* ^{1}*H-NMR spectra* (500 MHz) *of* **4** in H_2O (25°, 25 mM) *at different pH values.* pH Measurements were done directly in the NMR tube with a special electrode, and the pH was adjusted by addition of 1M HCl and 1M NaOH.

simply reflect the ionization of the β-D-Asp side chain; however, the observed association of all NH signals could also be related to the adoption of a more 'C_3-symmetric' arrangement of the peptide backbone. Although it is difficult to distinguish these possibilities experimentally, this observation implies that β-D-Asp may be an interesting residue for fine-tuning conformational preference in peptide design.

In summary, on-resin cyclization of side-chain-anchored β-peptides can be efficiently used for the synthesis of shorter cyclic β^3-peptides, as demonstrated by the synthesis of a cyclo-β-tripeptide. In H_2O solution, this peptide adopts a structure with uniformly oriented amide bonds and all side chains in lateral positions. We believe that this methodology overcomes many of the problems previously associated with the synthesis of cyclic β-peptides. Hopefully, the increased availability of H_2O-soluble cyclic β-peptides paves the way for more detailed physical and biomedical studies of these interesting molecules.

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