Synthesis and Two-Dimensional ^1H and ^{13}C NMR Investigations of an Inhibitor of Hematopoietic Stem Cell Proliferation Ac-Ser-Asp-Lys-Pro-OH

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The solid phase synthesis of the inhibitor of hematopoietic stem cell proliferation, Ac-Ser-Asp-Lys-Pro-OH, and its derivative Ac-Ala-Asp-Lys-Pro-OH is described. ^1H and ^{13}C NMR investigations demonstrate that both peptides show no preferred conformation in water solution. Both peptides exist in a Pro-*cis-trans* equilibrium ratio of 9 (*trans*) : 1 (*cis*). Thymosin β_4 is believed to be the precursor molecule of the tetrapeptide Ac-SDKP. The attachment of the random coil tetrapeptide to a rigid helical fragment could facilitate its *in vivo* enzymatic cleavage.

Introduction

From frozen fetal calf bone marrow a peptide was recently isolated and characterized (Ac-Ser-Asp-Lys-Pro-OH), which inhibits at a dose of

100 ng/mouse the proliferation of bone marrow stem cells [1]. The peptide protects mice against lethal doses of 1 β -D-arabinofuranosyl-cytosine, used in man as an S-phase-specific cytotoxic agent

Fig. 1. Amino acid sequences of thymosin β_4 and β_9 ; the N-terminal tetrapeptide sequences are underlined.

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Abbreviations:

A: alanine; Ac: acetyl; Boc: *tert*-butoxycarbonyl; BOP: benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; Bu': *tert*-butyl; D: aspartic acid; DCC: dicyclohexylcarbodiimide; DCM: dichloromethane; DCU: dicyclohexylurea; DIEA: N-ethyl-diisopropylamine; DMAP: 4-dimethylaminopyridine; DMF: dimethylformamide; DQF: double quantum filter; E. COSY: exclusive correlation spectroscopy; Fmoc: 9-fluorenylmethoxycarbonyl; HMQC: heteronuclear multiple quantum correlation spectroscopy; HOBt: 1-hydroxybenzotriazole; HOSu: N-hydroxysuccinimide; HPLC: high performance liquid chromatography; K: lysine; P: proline; PSA: preformed symmetrical anhydride; 4QF: four quantum filter; S: serine; TBAF: tetrabutylammonium fluoride; TFA: trifluoroacetic acid.

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and might become important in cancer therapy [2]. This tetrapeptide is identical with the N-terminal part of thymosin β_4 , isolated and structurally determined in 1982 [3] (Fig. 1).

Though thymosins have been isolated from many tissues and species [4–11], the occurrence of $\text{T}\beta_4$ in hematopoietic stem cells was demonstrated only recently and also the cleavage of Ac-Ser-Asp-Lys-Pro-OH from $\text{T}\beta_4$ by an unknown enzyme of these cells [12]. Therefore, there exists strong evidence for the hypothesis that $\text{T}\beta_4$ is the precursor molecule of the inhibitor Ac-Ser-Asp-Lys-Pro-OH. It seems also worthwhile mentioning that in many mammalian tissues $\text{T}\beta_4$ is often associated with a second highly homologous peptide of the β -thymosin family: *e.g.* from calf tissues $\text{T}\beta_9$ was isolated to a smaller amount com-

pared to $\text{T}\beta_4$ [13]. If $\text{T}\beta_9$ is also a precursor molecule for short-chain peptide factors of biological relevance is under current investigation by our group. In connection with our research activities on the biological function of thymosins respectively their fragments several syntheses of corresponding peptide sequences [14–18], bioactivity tests [19], development of antibodies [20–22], and NMR studies [23] have been undertaken by our groups. In this context we describe here the solid phase syntheses of $\text{Ac-Ser(Ala)-Asp-Lys-Pro-OH}$ and the results of the two-dimensional ^1H and ^{13}C NMR investigations on these peptides.

Results

Synthesis of *Ac-SDKP* and *Ac-ADKP*

Both peptides were synthesized by the continuous flow solid phase method on p-hydroxymethylphenoxymethyl-polydimethylacrylamide

resin (0.1 mmol/g) using a semiautomatic synthesizer. The C-terminal Pro was attached to the resin by the PSA method. Capping of the remaining free hydroxyl groups was achieved by acetic anhydride and pyridine (1/1; tenfold excess). For the couplings N^α -Fmoc amino acids with *tert*-butyl side chain protection (threefold excess) and the BOP/HOBt-method were employed. Deblocking of the N^α -functions was performed by 20% piperidine in DMF with the exception of the Fmoc-Lys(Boc)-Pro-resin for which a 0.02 M TBAF [24] in DMF solution was used. In this way a substantial suppress of the diketopiperazine formation in this step was achieved. Acetylation of the N-terminal residue was carried out by acetic anhydride/pyridine (1/1; tenfold excess in DCM). Treatment of the acetylated resin with TFA/ H_2O (95/5; 2 h) resulted in crude *Ac-SDKP* respectively *Ac-ADKP* (yield 75%), and both peptides were then purified by preparative HPLC. The results of amino acid ana-

Fig. 2. Aliphatic part of the DQF COSY of 50 mM *Ac-Ala-Asp-Lys-Pro* in H_2O (820 experiments, 144 scans) (': *cis*-Pro conformation).

Fig. 3. ^1H NMR spectrum of 50 mM Ac-Ala-Asp-Lys-Pro in H_2O (128 scans).

lyses and FAB-MS of the purified products were in good agreement with the expected values.

NMR measurements

Complete assignment of proton signals was obtained with DQF COSY spectra (Figs 2 and 4). ^{13}C chemical shifts of all protonated carbons were determined with the HMQC spectra (Fig. 5). Quarternary carbons could not be assigned in the one-dimensional spectra with the exception of C-1 of Ala and Ser (Figs 6 and 7). Both peptides exist in a Pro-*cis-trans* equilibrium ratio of 9 (*trans*) : 1 (*cis*) as determined by integration of several resolved peaks of the one-dimensional ^1H NMR spectra (Figs 3, 4). $J_{\text{NH}-\alpha}$ coupling constants were obtained from the COSY spectra and exhibit the expected random coil values of 7.3 Hz. Only the

Fig. 4. ^1H NMR spectrum of 40 mM Ac-Ser-Asp-Lys-Pro in D_2O (128 scans).

Fig. 5. ^1H - ^{13}C HMQC of 40 mM Ac-Ser-Asp-Lys-Pro in D_2O (512 experiments, 64 scans).

coupling constants $J_{\text{NH}-\alpha}$ of the Lys residues in both *cis*-Pro peptides have larger values (9.0 Hz). The coupling between the C^α proton of Pro and the C^β proton is larger than the coupling between the C^α proton and the $\text{C}^{\beta'}$ proton. The latter one

can be stereospecifically assigned to be in *cis* position related to the C^α proton of Pro. The determination of further $J_{\text{H}-\text{H}}$ coupling constants was not possible due to the strong coupling effects (see Tables I and II).

Fig. 6. Proton broad band decoupled ^{13}C NMR spectrum of 40 mM Ac-Ser-Asp-Lys-Pro in D_2O (350 scans, relaxation delay: 5 s).

Fig. 7. Proton broad band decoupled ^{13}C NMR spectrum of 50 mM Ac-Ala-Asp-Lys-Pro in H_2O (350 scans, relaxation delay: 5 s).

	Ala	Asp	Lys	Pro	Pro +	Lys +
NH	8.26	8.46	8.14			7.95
C $^\alpha$ H	4.27	4.68	4.63	4.43	4.61	4.53
C $^\beta$ H	1.37	2.92	1.84	2.33	2.36	1.74
C $^\beta$ ' H		2.82	1.75	2.03	2.23	1.68
C $^{\gamma\gamma'}$ H			1.46	2.05	1.97/1.88	(1.33)
C $^{\delta\delta'}$ H			1.71	3.80/3.68	3.61/3.49	
C $^\epsilon$ H ₃			3.01			
Ac/NH ₃ ⁺	2.03		7.49			
C $^\alpha$	51.0	51.1	52.4	60.8		(52.0)
C $^\beta$	17.5	36.4	30.9	29.9	(32.1)	(32.2)
C $^\gamma$			22.7	25.6		
C $^\delta$			27.5	48.7	48.1	
C $^\epsilon$ /AcCH ₃	22.7		40.5			
C'=O	176.3					

Table I. ^1H and ^{13}C chemical shifts of Ac-Ala-Asp-Lys-Pro (+ : *cis*-Pro conformation: values in brackets are not unambiguously assigned because of the low intensities of the corresponding peaks).

	Ser	Asp	Lys	Pro	Pro +	Lys +
NH	8.31	8.53	8.16			7.98
C $^\alpha$ H	4.43	4.74	4.64	4.43	4.61	4.53
C $^\beta$ H	3.86	2.90	1.84	2.33	2.36	(1.74)
C $^\beta$ ' H	3.86	2.86	1.75	2.03	2.23	(1.68)
C $^{\gamma\gamma'}$ H			1.47	2.04	1.97/1.88	(1.33)
C $^{\delta\delta'}$ H			1.71	3.80/3.67	3.61/3.48	
C $^\epsilon$ H ₃			3.01			
Ac/NH ₃ ⁺	2.07		7.52			
C $^\alpha$	56.8	51.2	52.4	60.9		52.1
C $^\beta$	62.2	36.5	30.9	29.9	32.2	(32.3)
C $^\gamma$			22.7	25.6	23.1	
C $^\delta$			27.5	48.7	48.1	
C $^\epsilon$ /AcCH ₃	22.9		40.5			
C'=O	173.1					

Table II. ^1H and ^{13}C chemical shifts of Ac-Ser-Asp-Lys-Pro (+ : *cis*-Pro conformation: values in brackets are not unambiguously assigned because of the low intensities of the corresponding peaks).

Discussion

Nearly all ^1H chemical shifts show only small deviations from the random coil values of amino acid residues reported by Wüthrich [25]. Only the C $^\alpha$ proton signal of the *trans*-Pro peptide is strongly downfield shifted, as reported in reference [26] for a heat-unfolded protein. The NH-proton of Lys appears to be upfield shifted to about the same amount. In the ^{13}C NMR spectra the resonances of C $^\alpha$ and C $^\beta$ of Asp and C $^\alpha$ of Lys show strong deviations from the random coil values, caused by the low pH value of the medium and the neighbouring Pro residue. Based on these data and the coupling constants there is strong evidence for both peptides to adopt random coil conformation. According to our investigations the signal assignment of the C $^\beta$ proton of Pro in reference [1] is incorrect,

we suggest this resonance to be caused by the C $^\beta$ proton of the Lys residue. Based on pH-dependent ^{13}C chemical shift values of lysylglycine the former published and also quoted [27–29] signal assignments [30] of the C $_\beta$ and C $_\delta$ resonances from lysine should be reversed [31, 32]. This is clearly indicated by our HMQC spectra of Ac-Ser(Ala)-Asp-Lys-Pro which show two definitely resolved peaks for the C $^\beta$ protons in connectivity with C $^\beta$ and only one peak for the two identical C $^\delta$ protons in connectivity with C $^\delta$ of Lys. In the precursor molecule of Ac-S-D-K-P, thymosin β_4 , the random coil tetrapeptide is attached to a rigid helical segment and there exists strong evidence that the peptide bond between both fragments of different secondary structure facilitates the enzymatic cleavage of the inhibitory factor (Fig. 8).

Fig. 8. Stereoscopic picture of the three-dimensional conformation of thymosin β_4 according to reference [23]. (yellow: backbone of residues 5 to 43; red: side chains of residues 5 to 43 without hydrogen atoms; blue: residues 1 to 4 (Ac-S-D-K-P) with hydrogen atoms).

Experimental

For the solid phase synthesis the semiautomatic peptide synthesizer BIOLYNX 4175 (Pharmacia-LKB; Freiburg) was used. For ion spray mass spectrometry a Sciex-API-III (Sciex; Toronto) and for FAB mass spectrometry a MAT 711A (Varian; Bremen) instrument were applied. The amino acid analyses were performed with an HPLC-system BT 8100 of Eppendorf-Biotronik (Maital) using the method as described in reference [18]. Pepsyn KA (0.1 mmol/g) was purchased from Milligen (Millipore; Eschborn, Germany). All protected amino acids were products of Bachem (Heidelberg, Germany). DMF (Lab. grade), acetic anhydride and pyridine (p.a. grade) were obtained from Merck (Darmstadt, Germany), piperidine from Merck-Schuchardt (Darmstadt), BOP, HOBt, DCC, DMAP, TBAF and DIEA from Fluka (Neu-Ulm, Germany) and TFA (p.A. grade) from Aldrich (Steinheim, Germany). The HPLC device consisted of a 7125 Rheodyne (Berkeley, USA) sample injection system, a Gynkotek (Germering, Germany) high precision pump model 480, a Gynkotek gradient former model 2500, a C-R6A Chromatopact recorder (Eppendorf-Biotronik, Maital, Germany) and an UV detector BT 3030 (Eppendorf-Biotronik, Maital).

For the preparative HPLC a Nucleosil 7 C₁₈ column (L: 250 mm, I.D.: 10 mm, 7 μm ; Macherey-Nagel, Düren, Germany) and as eluents: A) 0.058% TFA in H₂O and B) CH₃CN/H₂O/TFA (400/600/0.5) were used. The preparative HPLC was performed using a linear gradient program starting from 95% A and 5% of B (1 min), followed by a linear gradient of B (5–95%) over 30 min (flow rate: 2.5 ml/min). The eluted material is detected at a wavelength of $\lambda = 214$ nm.

Synthesis of Ac-Ser-Asp-Lys-Pro-OH

1.5 g Pepsyn KA resin (0.1 mmol/g) was swollen for 1/2 h in DMF. A solution of 154.7 mg (0.75 mmol) of DCC in 1 ml abs. DCM was added to a solution of 506 mg (1.5 mmol) Fmoc-Pro-OH in 3 ml abs. DCM at 0 °C. The reaction mixture was stirred for 45 min, the precipitated DCU was filtered and the filtrate evaporated to dryness under reduced pressure at room temperature. The residue was taken up in 2 ml DMF and added to the swollen resin. 18.32 mg (0.15 mmol) DMAP in 0.7 ml DMF were added and the suspension kept for 24 h at room temperature with occasional swirling. The Fmoc-Pro-resin was then filtered, washed twice with DMF (10 ml), glacial acetic

acid (10 ml), isopropanol (10 ml), glacial acetic acid (10 ml), isopropanol (10 ml) and finally with abs. ether (3 times; 10 ml) and dried *in vacuo*. The spectrometric determination of the loading of the resin indicated that 0.066 mmol/g of Fmoc-Pro-OH were bound to the resin (83%). Capping of the remaining OH groups of the resin was achieved by treatment with 141 μl acetic anhydride and 121 μl pyridine in DCM for 90 min. The resin was then filtered, washed three times with CH_2Cl_2 (10 ml) and ether (10 ml) and dried *in vacuo*. Thereafter the Fmoc-Pro-resin was suspended for 1/2 h in DMF and transferred to the reaction column (150 $\times$ 10 mm) of the continuous flow synthesizer. The following steps were then performed: 1) deblocking of the N^α -function by treatment with 20% piperidine in DMF (10 min; flow rate: 3.5 ml/min); 2) washing with DMF (10 min; flow rate 3.5 ml/min); 3) loading of the next amino acids as follows: 0.45 mmol of Fmoc-Lys(Boc)-OH (211 mg), 0.45 mmol HOBt (61 mg) and 0.45 mmol BOP (199 mg) were dissolved in 1.8 ml DMF and transferred into the loading pot of the synthesizer; for each protected amino acid then 154 μl DIEA were added and the loading pot mixture was pumped into the reaction column (pumping rate: 2 ml/min); 4), coupling was achieved by recirculating the reaction mixture through the column for 90 min (flow rate 3.5 ml/min) and 5) removal of the excess of protected amino acid, coupling reagents and soluble coupling products by washing with DMF (10 min; flow rate 3.5 ml/min). The mentioned synthetic cycle was repeated for the following amino acids sequentially: Fmoc-Asp(OBu^t)-OH (185 mg) and Fmoc-Ser(Bu^t)-OH (173 mg) with one exception for the deblocking of the N^α function of Fmoc-Lys(Boc)-Pro-resin (step 1): In this case the 20% piperidine/DMF reagent was replaced by a 0.02 M TBAF/DMF solution (20 min; flow rate: 3.5 ml/min). After completion of the chain elongation the protected peptide resin was subjected to steps 1) and 2), taken out from the column, washed three times with 10 ml abs. ether and dried *in vacuo*. Then, acetylation of the N-terminal amino acid residue was performed by treatment of the peptide resin with 141 μl acetic anhydride and 121 μl pyridine in abs. DCM for 2 h. The acetylated peptide resin was filtered, washed three times with 10 ml abs. DCM and abs. ether and dried *in vacuo*. For the removal of the acid-labile side chain protecting groups and the simultaneous removal of the peptide from the resin the acetylated peptide resin was suspended in a mixture of 16 ml TFA and 840 μl H_2O for 2 h and then the resin removed by filtration. The resin was

washed twice with 5 ml TFA and the filtrate evaporated to dryness under reduced pressure at room temperature and dissolved in 1.5 ml glacial acetic acid. This solution was transferred into a centrifugation tube and the peptide precipitated by addition of 7 ml abs. ether. After centrifugation for 5 min (50%) the supernatant was discarded, the pellet resuspended in 7 ml ether and the above described procedure repeated twice. The obtained peptide was then dried *in vacuo*, dissolved in H_2O (1.5 ml) and lyophilized to yield 45.6 mg (75%) of crude Ac-Ser-Asp-Lys-Pro-OH. After preparative HPLC purification of the crude product 35 mg (57% total yield) of analytically pure Ac-Ser-Asp-Lys-Pro-OH were collected. The obtained values of the amino acid analysis are: Ser 0.9 (1), Asp 0.8 (1), Lys 1 (1). The FAB mass spectrum of the product showed the expected m/z value ($m/z = 488 (\text{M}+1)^+$; calculated mass: 487.2 (M^+)).

Synthesis of Ac-Ala-Asp-Lys-Pro-OH

The synthesis was performed in an analogous manner as described before. Only Fmoc-Ser(Bu^t)-OH was replaced by Fmoc-Ala-OH (140.3 mg) in the last coupling step. 56 mg (79%) of crude Ac-Ala-Asp-Lys-Pro-OH were obtained which, after purification by preparative HPLC, yielded 45 mg (63% total yield) of analytically pure product. The obtained values of the amino acid analysis are: Ala 1.1 (1), Asp 0.8 (1), Lys 1 (1). The ion spray mass spectrum showed the correct mass ($m/z = 471.8$) of the synthesized peptide (calculated mass: 471.2).

NMR measurements

The samples for the NMR investigations were 40 mM solutions of Ac-Ser-Asp-Lys-Pro respectively 50 mM of Ac-Ala-Asp-Lys-Pro in H_2O or D_2O (pH of 2.9). All NMR measurements were carried out at 500 MHz on a Bruker AM-500 spectrometer at 300 K. All two-dimensional spectra were recorded in the pure-phase absorption mode by using the time proportional phase incrementation methods. The following spectra were recorded for Ac-Ser-Asp-Lys-Pro: 4QF-E.COSY [33] in H_2O with the phases of the first two pulses of 0° , 45° , 90° , 135° , 180° , 225° , 270° , 315° with a relative weighting of 10, -7, 2, -1, 2, -1, 2, -7 and presaturation of the water signal, one-dimensional ^1H NMR spectra in H_2O and D_2O , one-dimensional ^{13}C NMR spectra with a relaxation delay of 5 s to allow a comparison of signal intensities, and inverse ^1H - ^{13}C inverse HMQC [34] in D_2O to get a better signal to noise ratio

compared to normal HMQC (the used pulse sequence was: $90^\circ \times ^1\text{H} - 1/2J - 90^\circ \times ^{13}\text{C} - t_1/2 - 180^\circ \times ^1\text{H} - t_1/2 - 90^\circ \times ^{13}\text{C} - 1/2J$ - acquisition in the ^1H channel and GARP decoupling in the ^{13}C channel, so that there exists no splitting of the cross-peaks due to J_{CH} coupling). The following

spectra were recorded from Ac-Ala-Asp-Lys-Pro : ^1H 2QF-COSY in H_2O with presaturation of the water signal, one-dimensional ^1H and ^{13}C NMR spectra in H_2O , and HMQC inverse in H_2O with presaturation of the water signal.

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