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ORIGINAL ARTICLE

Synthetic study on cystinyl peptides using solution and solid phase methodology: human IgG1 hinge region

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Abstract Synthetic study on cystinyl peptides using solution and solid phase methodology was carried out with the central hinge region of immunoglobulin IgG1. In the solid phase synthesis of hexadecapeptide **1c**, the time necessary for the formation of disulfide bonds between linear precursors was shortened four times by the action of pure oxygen in buffered solution, in comparison with air oxidation. The product was thus obtained devoid of impurities from side reactions. In the preparation of the shortened *bis*-cystinyl analogs **2k** and **3d** of the natural hexadecapeptide **1c**, both the classical and polyethylene glycol (PEG6000) solution methods were utilized using a disulfide synthon (Boc-Cys-OPfp)$_2$ to obtain peptide chains in a natural parallel alignment. In the PEG6000 strategy, lysine as a linker on both sides of the polymer was attached to enhance the loading capacity. The leucine residue, instead of proline one, was introduced to the carboxy terminus to facilitate a specific enzymatic cleavage of the peptides from PEG6000 by thermolysine.

Keywords Hinge region · Immunoglobulin · Prion protein · Solution synthesis · Solid phase synthesis · PEG · Disulfide synthon · Enzymatic cleavage · Thermolysin

The nomenclature and symbols of amino acids follow the recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature (1984) Eur J Biochem 138:9–37.

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Abbreviations

AA	Amino acid analysis
AcOH	Acetic acid
ACN	Acetonitrile
BGE	Background electrolyte
Boc	*Tert*-butoxycarbonyl
(Boc-Cys-OPfp)$_2$	*Bis-tert*-butoxycarbonylcystine *bis*-pentafluorophenyl ester
Cl-Trt-PS	Chlorotrityl-polystyrene resin
CZE	Capillary zonal electrophoresis
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	*N*,*N*′-diisopropylcarbodiimide.
DIEA	*N*,*N*-diisopropylethylamine
DMAP	4-(*N*,*N*-dimethylamino)-pyridine
DMF	*N*,*N*-dimethylformamide
EDT	1,2-Ethanedithiol
Et_3N	Triethylamine
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
ESI MS	Electro spray ionization mass spectrometry
Fmoc	[(Fluoren-1-yl)methoxy]carbonyl
HOBt	1-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
iProOH	Isopropanol
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight
MeOH	Methanol
OSu	Succinimide ester

PEG	Polyethylene glycol
OPfp	Pentafluorophenyl ester
tBu	*Tert*-butyl
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TIS	Tri-isopropylsilane
Trt	Triphenylmethyl

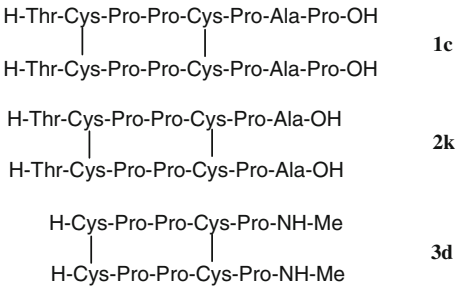

Fig. 1 Primary structure of hinge peptide 225–232/225′–232′ (**1c**), truncated analogs 225–231/225′–231′ (**2k**) and methyl amide 226–230/226′–230′ (**3d**) from hinge region of human IgG1

Introduction

Disulfide bonds in peptides and proteins are biologically important structural features that stabilize an overall shape of various biomolecules, such as peptide hormones (Schröder and Lübke 1966), antimicrobial peptides (Lehrer et al. 1993; Schneider et al. 2005) and venoms (Habermann 1972, 1984; Olivera et al. 1990). They also participate in a structure of the cocaine- and amphetamine-regulated transcript (Douglass et al. 1995; Maixnerová et al. 2007) in the protein chains inside immunoglobulin structures (Burton 1985; Zalipsky 1995) and in meta-stable forms of prion proteins (Prusiner 1998; Legname et al. 2004). Prions occur in the form of different strains that can possess conformation states triggering the aggregation and misfolding of the prion proteins leading to different forms of neuropathogenic diseases (Horiuchi and Caughey 1999). In these conformation stages, the disulfide bridges may play a significant role as important parts of the covalently assembled protein structure.

For our study, we have chosen the sequence from the central part of native immunoglobulin protein IgG1, which was designed to function as a potential carrier of antigenic peptides with defined structure (Wünsch et al. 1988) and reported as an acceptor of antibodies against peptides related to gastrin (Wunsch et al. 1991a, b). This proline-rich peptide (**1a**) and its analogs (**2k**, **3d**) contain dimeric sequences in a parallel alignment connected by disulfide bridges (Fig. 1). The proline residues determine the rigid structure to act as a swivel point for the flexible part of the hinge region.

We considered it interesting to inspect this molecule also with respect to the presence of the cystine structure in the prion proteins, in which the disulfide bond changes might influence their conformation and biological function. First, we focused our attention on the disulfide bonds, regarding the ease of their formation as well as the high yields and purity of the peptides investigated.

The synthesis of the peptide **1c** was originally carried out by a classical approach in solution (Wünsch et al. 1988; Moroder et al. 1990). The sulfhydryl groups of cysteine residues were selectively protected to form, upon successive oxidation, a dimeric structure with a parallel alignment only. Without protection, a random oxidation by air oxygen took place affording parallel dimer in the yield of over 80%, which could be purified by chromatography.

In our preliminary communication (Niederhafner et al. 2006), we described the synthesis of *bis-N*-protected dimeric *bis*-disulfide peptides of the hinge sequence with parallel alignment of both peptide chains on polyethylene glycol monomethyl ether (PEG2000-OMe).

In the current study, we examined three methods for the preparation (Figs. 2, 3, 4) of hinge structures related to the above-mentioned hexadecapeptide, which were supposed to be utilized later as defined carriers of peptide epitopes and also as tools in secondary structure studies.

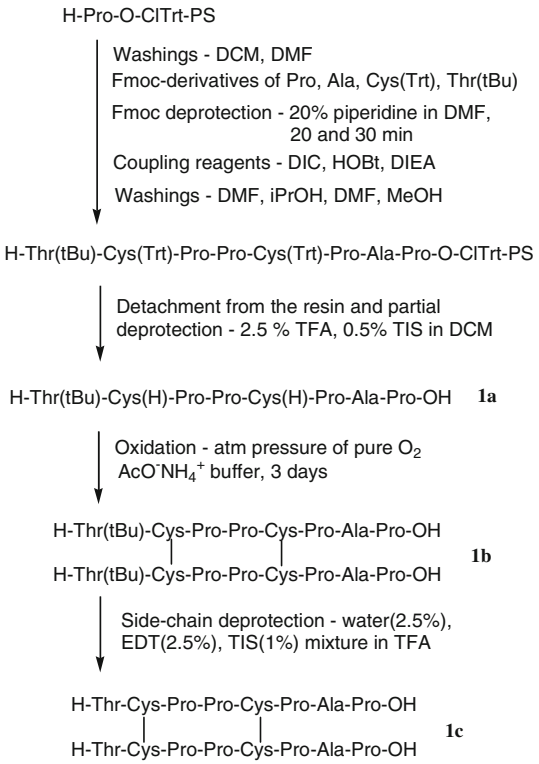

Fig. 2 Synthetic scheme for the preparation of the hinge peptide **1c**

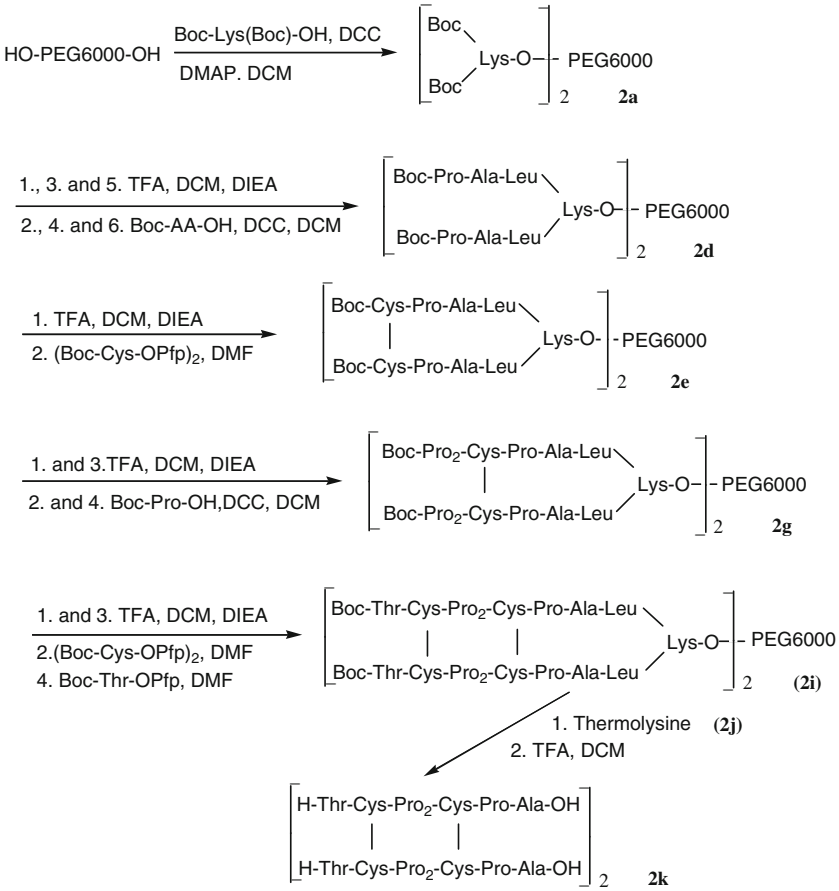

Fig. 3 Synthetic scheme for the preparation of the hinge peptide analog **2k**

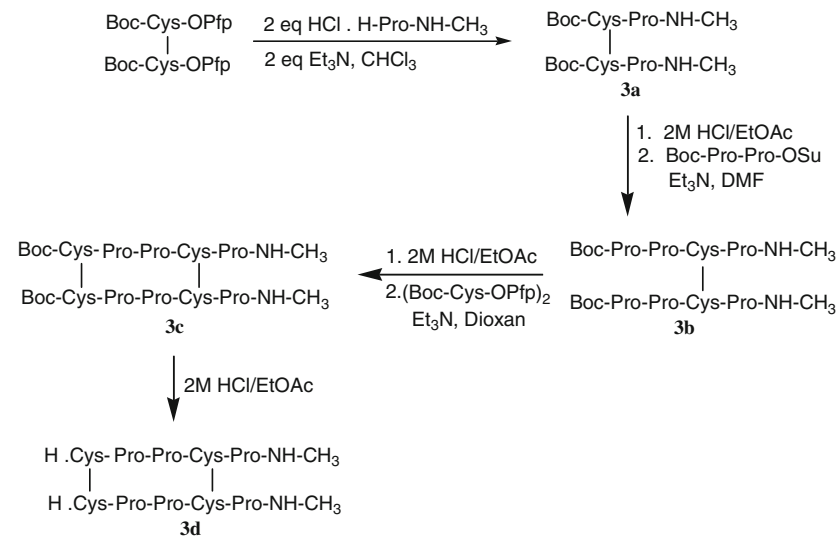

Fig. 4 Synthetic scheme for the preparation of the hinge peptide analog **3d**

Materials and methods

General methodologies

The solvents ACN, DCM and DMF and the cleaving reagent TFA were purchased from Fluka (Switzerland). The 2-chlorotrityl chloride resin (substitution 1.2 mmol/g), PEG6000 (0.33 mmol OH/g), DIC, DCC, HOBt and reagents for amino acid couplings were purchased from NovaBiochem (Switzerland), and the protected amino acids from Iris Comp. (Germany). The solvents were evaporated in vacuo on a rotary evaporator (bath temperature, 30°C); DMF was evaporated at 30°C and 150 Pa. The products were dried in a vacuum dry box (Salvis AG, Emmenbrűcke-Luzern, Switzerland) at room

temperature for 16 h. Electrophoresis was carried out in a modified moist chamber apparatus (Durrum 1950) on Whatman No. 3 MM paper at 20 V/cm using 6% aqueous acetic acid (pH 2.5) and pyridine acetate buffer (pH 5.7) as the electrolytes for 1.5 h. Electrophoretic mobilities, $E_{2.4}^{Gly}$, $E_{2.4}^{His}$ and $E_{5.7}^{His}$, respectively, were expressed as the distance ratios of the compound and the reference amino acids Gly and His from the start line. Dried papers were stained with a 1% solution of ninhydrine in ethanol or with the chloranile reagent (Christensen 1979). The samples for amino acid analysis were hydrolyzed with 6 M HCl at 110°C for 20 h in the presence of phenol; cysteine was estimated as the cysteic acid after oxidation of the sample with peroxide. The amino acid analyses were carried out on Biochrom-20 amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England). Elemental analyses were performed in Analytical Laboratories of the Institute of Organic Chemistry and Biochemistry, CAS, v.v.i., Prague, Czech Republic. Molecular weights of the compounds prepared were determined by mass spectrometry using either an ESI technique (Agilent 5975B MSD) from Agilent Technologies, USA, or MALDI-TOF spectra, recorded on mass spectrometer Lasermat (Finnigan, USA) with Lasermat 2000 program. For HPLC, a Spectra Physics SP 8800 pump with a TSP Chrom Jet SP4290 integrator and Spectra 100 UV detector were used. Analytical HPLC was carried out on a Supelco RP-18 Discovery 15 × 0.4 cm column, 5 µm (Supelco-Sigma–Aldrich, Czech Republic) or on a Vydac RP-18, 25 × 0.4 cm, 5 µm (Separation Groups, Hesperia, USA), with a flow rate of 1 mL/min at 220 nm, using a 0–100% gradient of ACN in 0.05% aqueous TFA over 40 min. The purification by the semipreparative HPLC was carried out on a Vydac RP-18, 25 × 1 cm, 10 µm column (Separation Groups, Hesperia, USA) of flow rate 3 mL/min and detection at 220 nm, using a 5–50% gradient of ACN in 0.05% TFA over 120 min unless stated otherwise.

NMR structure determination

The NMR spectra were measured on Bruker AVANCE-600 (^{1}H at 600.13 MHz; ^{13}C at 150.90 MHz) spectrometer in d_6-DMSO at 30°C and referenced to the solvent using relations δ_H(DMSO) = 2.50 and δ_C(DMSO) 39.70. The chemical shifts were determined from one-dimensional ^{1}H- and ^{13}C-NMR spectra. Two-dimensional homonuclear spectra (2D-^{1}H,^{1}H-COSY, 2D-^{1}H,^{1}H-TOCSY and 2D-^{1}H,^{1}H-ROESY) and heteronuclear spectra (2D-^{1}H,^{13}C-HSQC and 2D-^{1}H,^{13}C-HMBC) were used for structural assignment of hydrogen and carbon signals.

Capillary electrophoresis (CZE)

Capillary electrophoresis (CZE) analyses were performed in a home-made apparatus for high-performance capillary electrophoresis (Kašička et al. 1999) equipped with internally untreated fused silica capillary with an outer polyimide coating (i.d. 50 µm, o.d. 200 µm, total length 300 mm, effective length 190 mm from injection end to the detector and UV-absorption photometric detector at 206 nm). The peptides were dissolved in the background electrolyte (BGE) or in water in the concentration range of 0.5–1.1 mg/mL and analyzed as cations in acidic BGE I (100 mM H_3PO_4, 50 mM Tris, pH 2.25) or as anions in weakly alkaline BGE II (40 mM Tris, 40 mM tricine, pH 8.1). The nanoliter sample volumes were introduced hydrodynamically into the capillary, with the pneumatically induced overpressure (500–700 Pa) for 2–10 s. The applied voltage was 10.0 kV (anode at the injection end of the capillary), while the electric current was 50–56.0 µA in BGE I and 7–8 µA in BGE II, at an ambient temperature of 22–24°C. The purity degree was quantified by the relative peak height (area), i.e., peak height (area) of the main component was related to the sum of heights (areas) of all peaks present on electropherogram (Šolínová et al. 2004).

Synthesis of hinge peptide **1c** on insoluble polymer (Fig. 2)

A solution of Fmoc-Pro-OH (0.61 g; 1.56 mmol) in a DCM (20 mL)-DMF (1.1 mL) mixture was treated with Cl-Trt-PS (2 g; 1.2 mmol of Cl^-/g of the resin) in the presence of DIEA (0.36 mL for 5 min and 0.67 mL for 2 h). Then, the reaction was quenched on adding MeOH (2 mL) for 10 min (Barlos et al. 1991a, b). The resin was filtered off, washed with 10 mL portions each of DCM (3×), DMF (2×), iPrOH (2×), DMF (2×), iPrOH (2×), MeOH (2×) and Et_2O (2×) and dried in a desiccator. The Pro substitution 0.43 mmol/g of the resin was calculated as the average of the values determined by (a) weight increase, (b) measurement of the dibenzofulvene–piperidine complex absorption after cleavage of the Fmoc protection group by 20% piperidine in DMF (2 × 10 mL) for 20 and 30 min, and (c) by AA. After this Fmoc deprotection, one half of H-Pro-resin was sequentially acylated with eight equivalents (3.44 mmol) of Fmoc-Ala-OH (1.08 g), Fmoc-Pro-OH (1.16 g), Fmoc-Cys(Trt)-OH (2.02 g), twice Fmoc-Pro-OH (2 × 1.16 g), Fmoc-Cys(Trt)-OH (2.02 g) and Fmoc-Thr(tBu)-OH (1.36 g), which were activated by the HOBt (0.51 g; 3.79 mmol)—DIC (0.74 g; 3.57 mmol)—DIEA (0.32 mL; 1.8 mmol) mixture in DMF (10 mL). After each coupling, the Fmoc group was removed by 20% piperidine in DMF as before. The side chain-protected octapeptide resin was washed, after the last Fmoc deprotection, with 10 mL of DMF (5×), MeOH (4×) and Et_2O (4×) and finally dried in a desiccator. After swelling in DCM (3 × 10 mL), the resin was stirred with a 2.5% TFA–0.5% TIS in DCM (20 mL) mixture for 2 × 90 min at room temperature to detach the partially protected peptide **1a** from the resin, which was subsequently

filtered off and washed with 50% AcOH (3 × 10 mL). The collected DCM and AcOH solutions were evaporated to dryness in vacuo, the residue was dissolved in water (30 mL) and this solution was freeze-dried to yield the above intermediate (150 mg; 0.18 mmol; 42%) that was purified by semipreparative HPLC on the Vydac RP-18 column (25 × 2.5 cm at 220 nm), using a flow rate of 8 mL/min and a gradient of 0–50% ACN in 0.05% TFA, 120 min. The pure **1a** was characterized by analytical HPLC as described in the general part of "Materials and methods" section (retention time, 14.43 min) and by CZE in acidic BGE I (4.78 min). AA: Ala 1.00; Thr 0.96; Pro 3.82; Cys(SO_3) 2.03. MS ESI (*m/z*): for $C_{37}H_{60}N_2O_{10}S_2$ was calculated at 841.07/840.39, but found 841.40 $(M + H)^+$.

The **1a** (76 mg; 0.09 mmol) was dissolved in a degassed 0.1 mM ammonium acetate buffer of pH 6.8 (60 mL) and then the pure oxygen from a rubber container was introduced into the solution at room temperature until the starting monomer disappeared (3 days), as monitored by HPLC and chloranile test. Then, the solution was lyophilized repeatedly with gradient HPLC water to remove the buffer salt. The product **1b** (75 mg; 0.045 mmol) was characterized by analytical HPLC (retention time 13.43 min), using the column and conditions as above, and by CZE in acidic BGE I (4.37 min). In MALDI-TOF (*z/m*), for $C_{74}H_{116}N_{16}O_2S_4$ calculated value was 1,678.20/1,676.74, while the value found was 1,678.2 $(M + H)^+$.

The final deprotection of dimer **1b** (74 mg; 0.044 mmol) was carried out by its treatment with a TFA (4.7 mL)–H_2O (125 µL)–EDT (125 µL)–TIS (50 µL) mixture at room temperature for 2 h. The reaction mixture was then evaporated, the residue triturated successively with EtOAc (3 × 10 mL) and Et_2O (3 × 10 mL) and finally dissolved in 10% AcOH (20 mL). The solution was freeze-dried to yield the hinge peptide **1c**, which was purified by preparative HPLC under the conditions described for **1a** with a yield of 65 mg (0.042 mmol; total yield 39%). It was characterized by analytical HPLC (retention time, 11.91 min) and by CZE in acidic BGE I (5.14 min). MS ESI (*m/z*): for $C_{66}H_{100}N_{16}O_{20}S_4$ the calculated value was 1,565.88/1,564.62; the value found was 1,565.5 $(M + H)^+$. AA: Ala 1.00; Thr 0.94; Pro 3.79; Cys(SO_3) 2.02. For ^{1}H- and ^{13}C-NMR, see Table 2 in the electronic Supplementary data.

Synthesis of hinge peptide analog **2i** using the PEG6000 soluble polymer (Fig. 3)

To a stirred solution of Boc-Lys(Boc)-OH (3.0 g; 8.65 mmol) in DCM (25 mL) cooled to −15°C, a solution of DCC (0.9 g; 4.3 mmol) in DCM was added. After ambient temperature was reached, the precipitated dicyclohexylurea was filtered off and washed with DCM. The filtrate, containing symmetric anhydride [Boc-Lys(Boc)-]$_2$O was poured to a stirred solution of PEG6000 (5.0 g; 0.33 mmol/g) in DCM (25 mL). To this mixture, DMAP (48 mg; 0.4 mmol) as a catalyst was added at 0°C and the stirring was continued for 5 h at room temperature. This mixture was concentrated in vacuo and the remaining solution poured to cold Et_2O (200 mL) with stirring for 10 min. The precipitated product **2a** (Fig. 3) was filtered off and washed with Et_2O to provide a yield of 5.3 g (95%). The loading was determined as follows (Šebestík et al. 2005): 5 mg of the product was treated with a 1:1 TFA/DCM mixture for 1 h, then the solution was evaporated to dryness and dissolved in DCM. Paper electrophoresis checking was then done in 6% AcOH (1 h); the paper was dried, stained with a solution of $Co(NO_3)_2$ and NH_4SCN in water and immediately digitalized (Mustec BearPaw 2400 TA scanner). A complete esterification (0.31 mmol/g) was observed. The product **2a** (5.1 g; 1.68 mmol) was dissolved in a TFA (10 mL)–DCM (10 mL) mixture, and the entire content was evaporated to dryness after 90 min. The formed trifluoroacetate was repeatedly co-evaporated with benzene (2 × 20 mL) and then, gradually, in accordance with the peptide sequence, treated with symmetric anhydrides (7.4 mmol) of Boc-Leu-OH (3.31 g), Boc-Ala-OH (2.79 g) and Boc-Pro-OH (3.18 g) prepared by the reaction with DCC (1.53 g; 7.4 mmol) in DCM (40 mL) at pH 7 in situ and with (Boc-Cys-OPfp)$_2$ (2.63 g; 3.4 mmol) and Boc-Thr-OPfp (2.85 g; 7.4 mmol) in the presence of DIEA at pH 7 in DMF (20 mL) or DMF–DCM mixture (20 mL), depending on the solubility of the growing peptide-PEG6000-peptide product, for 60 min. The ninhydrine (Kaiser et al. 1970) and, in the case of Pro coupling, the chloranile (Christensen 1979) tests with the paper electrophoresis were carried out to determine the progress in the coupling (for electrophoresis data see Table 1). Also, the analytical HPLC of the Boc-peptide samples of **2b–2i** (Fig. 3) detached from the PEG carrier was performed. To 20 mg of Boc-peptide-PEG6000-peptide-Boc, 0.1 M NaOH (1.5 mL) was added. After 30 min, the reaction mixture was neutralized to pH 7 by 0.1 M HCl and the first part applied to analytical HPLC column. The second part of the Boc-peptide solution was concentrated in vacuo, lyophilized and purified by HPLC to obtain the product for AA and ESI MS measurements. In the HPLC analysis, a perturbation of the PEG polymer absorbance could be eliminated at the wavelength of 222 nm. Analytical data of the protected peptides **2b–2i** detached from the PEG6000 carrier are listed in Table 1.

Enzymatic detachment of *N,N*-protected tetradecapeptide **2j** from the PEG6000 carrier

The (Boc-octapeptide)$_2$ -Lys-PEG6000-Lys-(octapeptide-Boc)$_2$ **2i** (3.0 g; substitution 0.3 mmol/g) was dissolved in

Table 1 Analytical data of compounds **2b–2j** after their detachment from PEG6000 by 0.1 M NaOH

Compound	Formula[a] Calc./found (*m/z*)	HPLC RT (min)[b]	Amino acid analysis[c]						Electrophoresis[d]		
			K	L	A	P	C	T	$E_{2.4}^{Gly}$	$E_{2.4}^{His}$	$E_{5.7}^{His}$
2b, Boc-LK-OH | Boc-L	$C_{28}H_{52}O_8N_4$ 572.4/573.4	24.4/8.9[e]	1.00	1.91					0.709	0.404	0.625
2c, Boc-ALK-OH | Boc-AL	$C_{34}H_{62}O_{10}N_6$ 714.5/715.3	24.1/11.8[e]	1.00	1.94	1.95				0.683	0.389	0.603
2d, Boc-PALK-OH | Boc-PAL	$C_{44}H_{76}O_{12}N_8$ 908.6/909.6	24.1/13.5[e]	1.00	1.89	1.89	1.88			0.660	0.376	0.583
2e, Boc-CPALK-OH | | Boc-CPAL	$C_{50}H_{84}O_{14}N_{10}S_2$ 1,112.6/1,113.6	22.4/14.2[e]	1.00	1.96	1.88	1.98	1.85		0.639	0.364	0.564
2f, Boc-PCPALK-OH | | Boc-PCPAL	$C_{60}H_{98}O_{16}N_{12}S_2$ 1,306.7/1,328.9 ($+Na^+$)	22.0/15.8[e]	1.00	2.05	1.92	4.03	1.83		0.620	0.353	0.547
2g, Boc-PPCPALK-OH | | Boc-PPCPAL	$C_{70}H_{112}O_{18}N_{14}S_2$ 1,500.8/1,501.7	23.9/16.7[e]	1.00	2.06	2.11	5.96	1.87		0.602	0.343	0.532
2h, Boc-CPPCPALK-OH | | | Boc-CPPCPAL	$C_{76}H_{120}O_{20}N_{16}S_4$ 1,704.8/1,705.7	21.3/15.3[e]	1.00	2.03	1.97	5.82	4.12		0.585	0.335	0.519
2i, Boc-TCPPCPALK-OH | | | Boc-TCPPCPAL	$C_{84}H_{134}O_{24}N_{18}S_4$ 1,906.9/1,907.8	20.4/15.4[e]	1.00	2.00	1.99	5.75	3.92	2.01	0.569	0.324	0.503
2j, Boc-TCPPCPA-OH | | Boc-TCPPCPA-OH	$C_{66}H_{102}O_{22}N_{14}S_4$ 1,570.6/1,571.5	17.4/11.4[e]	0.00	0.00	1.00	3.08	1.91	0.97			

[a] Determined with FAB MS technique onZAB-EQ mass spectrometer, VG Analytical, Manchester, UK

[b] For HPLC, a TSP instrument with an SP 8800 pump, an SP 4290 integrator, TSP Spectra 100 UV detector and 5 μm Supelco 15 × 0.4 cm column, 20 min gradient 0–50% ACN in 0.05% TFA were used

[c] The samples for amino acid analysis were hydrolyzed with 6 M HCl at 110°C for 20 h in the presence of phenol; cystine was determined as an 1/2 cysteine. The amino acid analyses were carried out on Biochrom-20 amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England)

[d] Electrophoresis of *bis*-peptide-PEG6000 after Boc removal by TFA was carried out in a moist chamber apparatus on Whatman No. 3 MM paper at 20 V/cm using 6% aqueous acetic acid (pH 2.5) and pyridine acetate buffer (pH 5.7) as the electrolytes for 1.5 h. Electrophoretic mobilities $E_{2.4}^{Gly}$, $E_{2.4}^{His}$ and $E_{5.7}^{His}$, respectively, were expressed as the distance ratios of the compound and the reference amino acids Gly and His from the start line

[e] HPLC peak retention time of deprotected peptide (without Boc group)

water (15 mL) and thermolysin (1 mg) was added. The reaction mixture was stirred overnight at room temperature and then the solution was dialyzed using cellulose membrane tubing MWCO 3500 against water, with a yield of over 90%. Monitoring of the cleavage was done by HPLC analysis as described above. After detachment of the protected *bis*-peptide **2j** from its precursor **2i** by thermolysin (Fig. 3, Table 1), a 43% total yield (calculated to the starting Lys-PEG6000-Lys) was obtained.

Hinge tetradecapeptide **2k**

N,N-protected tetradecapeptide **2j** (0.16 g; 0.1 mmol) was treated with a TFA (10 mL)–DCM (5 mL) mixture for 30 min

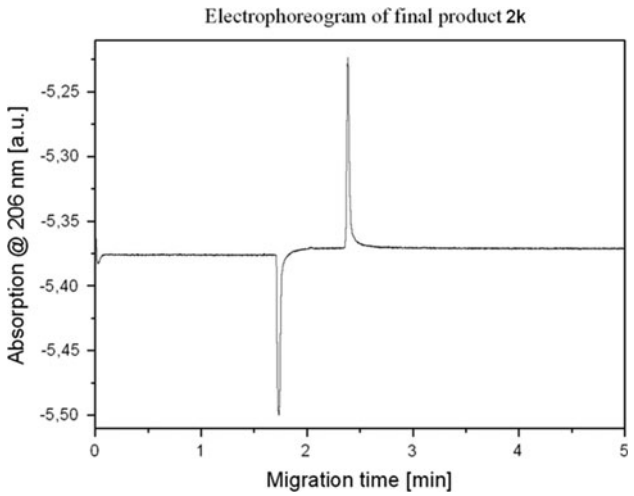

Fig. 5 Electrophoretogram of the hinge peptide derivative **2k** (peak on the *right*) in alkaline BGE II (water peak is on the *left*)

at room temperature. The reaction mixture was partly concentrated in vacuo and poured into a threefold excess of cold Et_2O. The precipitated product was filtered off after 10 min and washed with Et_2O, yielding 0.13 g (0.95 mmol; 42% total yield) of **2k**. Analytical HPLC showed a peak of 11.45 min of retention time. MS ESI (*m/z*): for $C_{56}H_{86}N_{14}O_{18}S_4$ the calculated value was 1,370.1, and the value found was 1,370.9 $(M + H)^+$. The amino acid composition was the same as in the protected peptide **2j** (Table 1). The NMR data of **2k** are provided in Table 3 in the electronic Supplementary data; the results of CZE in alkaline BGE II are in Fig. 5.

Synthesis of hinge decapeptide *bis*-methyl amide **3d** in solution (Fig. 4)

*N,N′-tert-butoxycarbonyl-cystinyl-bis-proline methylamide (**3a**)*

$(Boc\text{-}Cys\text{-}OPfp)_2$ (3.6 g; 4.7 mmol) and Et_3N (1.32 mL; 9.5 mmol) were added to a solution of proline methylamide hydrochloride (1.56 g; 9.5 mmol) in $CHCl_3$ (90 mL) at 0°C and the reaction mixture was stirred for 5 h at room temperature. The reaction mixture was evaporated to dryness, the residue was dissolved in EtOAc (100 mL) and the solution washed with 5% aqueous solution of $NaHCO_3$ (3 × 50 mL) and concentrated solution of $NaHSO_4$ (50 mL). After evaporation of the solvent, the voluminous gel obtained was dissolved in 2-propanol (10 mL) and after evaporation to dryness, **3a** crystallized from a 2-propanol-*tert*-butyl-methyl ether-*n*-pentane mixture with a yield of 2.56 g (82%). M.p., 153–156°C. For $C_{28}H_{48}N_6O_8S_2$ (660.86), calculated value was 50.89%C, 7.32%H, 12.72%N, 9.70%S; values found were: 51.08%C, 7.29%H, 12.57%N, 9.89%S. MS ESI (*m/z*): 661.2 $(M + H)^+$. AA: Pro 1.00, ½ Cys 0.98.

Bis-N-tert-butoxycarbonyl-prolyl-prolyl-cystinyl-*bis*-proline methylamide (**3b**)

Boc protecting groups of compound **3a** (2.5 g; 3.8 mmol) were cleaved by 2 M HCl in EtOAc (8.2 mL) to obtain *bis*-hydrochloride (1.96 g; 3.67 mmol), which was allowed to react with Boc-Pro-Pro-OSu (3.02 g; 7.4 mmol) (Wünsch et al. 1988) in the presence of Et_3N (1.03 mL; 7.4 mmol) in DMF (15 mL) at 0°C. The temperature of the mixture was let to rise to 20°C during 12 h. Then the reaction mixture was evaporated to dryness, the residue was dissolved in water (100 mL) and the solution washed with EtOAc (3 × 20 mL). The aqueous phase was then applied onto the top of IRA-410 (15 mL) ion exchanger column in OH^- cycle. After evaporation of water, the product **3b** (3.12 g, 78%) was obtained as a solid gel. The HPLC peak retention time was 19.7 min, under a gradient of 0–100% ACN in 0.05% TFA, 40 min and flow of 1 mL/min at 222 nm, using a Vydac C-18 column 25 × 0.4 cm. MS ESI (*m/z*): for $C_{48}H_{76}N_{10}O_{12}S_2$ (1,049.13) the value found was 1,049.1 $(M + H)^+$. AA: Pro 3.00, ½ Cys 1.04.

*N,N′-tert-butoxycarbonyl-cystinyl-bis-prolyl-prolyl-cystinyl-bis-proline methylamide (**3c**)*

Boc protecting groups of compound **3b** (3.1 g; 2.9 mmol) were cleaved by 2 M HCl in EtOAc (6.4 mL) to yield the corresponding *bis*-tetrapeptide methyl amide *bis*-hydrochloride (1.92 g; 2.08 mmol). To this compound in DMF (200 mL) the $(Boc\text{-}Cys\text{-}OPfp)_2$ (1.62 g; 2.1 mmol) in dioxane (50 mL) and Et_3N (0.33 mL; 2.3 mmol) were added with stirring for 18 h at room temperature. After 24 h of stirring, the reaction mixture was evaporated to dryness, the residue was dissolved in water (100 mL) and the entire content was filtered through IRA-410 (20 mL) ion exchanger column in OH^- cycle. After evaporation of water, the product **3c** was obtained as a solid gel with a yield of 1.6 g (44%). HPLC peak retention times (97% purity) were 17.1 min using a Vydac C-18, 15.6 min using a Vydac C-8 and 19.5 min using a Supelco HSF5 column and conditions as described in HPLC of the compound **3b**. MS ESI (*m/z*): for $C_{54}H_{84}N_{12}O_{14}S_4$ the calculated value was 1,253.6/1,252.2; the value found was 1,253.2 $(M + H)^+$, 1,275.2 $(M + Na)^+$. AA: Pro 3.00, ½ Cys 1.96. ^{1}H-and ^{13}C-NMR data are provided in Table 4 in the electronic Supplementary data.

*Cystinyl-bis-prolyl-prolyl-cystinyl-bis-proline methylamide (**3d**)*

The protected peptide **3c** (0.61 g; 0.49 mmol) was treated with a 2 M HCl–EtOAc (1.1 mL) mixture to obtain the corresponding *bis*-pentapeptide methyl amide *bis*-hydrochloride (0.24 g; 0.42 mmol). The reaction mixture was evaporated to dryness, and the residue was dissolved in

water (30 mL) and applied onto the top of the Dowex 50 × 2 (10 mL) ion exchanger column. First, the chloride ions were eluted with water and then the peptide followed upon elution with 3% NH_4OH (20 mL). This solution was concentrated under vacuum and lyophilized to obtain the compound **3d** (0.35 g, 68%) with HPLC peak retention times 9.35 min (Vydac C-18), 8.02 min (Vydac C-8) and 10.4 min (Supelco HSF5) using the conditions described in the HPLC of the compound **3b**. MS ESI (*m/z*): for $C_{44}H_{68}N_{12}O_{10}S_4$ the calculated value was 1,053.4/1,052.4; the value found was 1,053.4 $(M + H)^+$, 1,275.2 $(M + Na)^+$. Calculated to the starting $(Boc\text{-}Cys\text{-}OPfp)_2$, an 18% total yield was obtained.

Results and discussion

Three synthetic methods were utilized in the preparation of peptides based on hinge structures from the central region of immunoglobulin IgG1.

First, we prepared the full sequence of hexadecapeptide **1c** by using the solid phase methodology with 2-chlorotrityl polystyrene-divinylbenzene resin and Fmoc chemistry (Fig. 2). This procedure enabled a quick preparation of the corresponding fully protected octapeptide polymer, from which the octapeptide **1a** with side chain-protected Thr residue was obtained. The free sulfhydryl groups of both the Cys residues were consequently oxidized to create the intermolecular disulfide bonds between two parallel peptide chains in the hexadecapeptide **1b**. With the intention to eliminate possible side products in the reaction mixture and to enable an easy workup and purification of the disulfide products after oxidation, we excluded the utilization of some common reagents, such as ferricyanide, iodine, silver oxide or DMSO. In connection with this, we decided to follow the "pure" original air oxidation (49% of oxygen in a mixture, cf.) described by Wünsch et al. (1988), which we also had fair experiences with (a series of our oxytocin analogs synthesized in the past; Hlaváček and Jošt 1985). However, to increase the amount of oxygen in the reaction mixture (up to 100%), we simply opted for pure O_2 for oxidation. It was introduced into the solution of **1a** in 0.1 M ammonium acetate buffer at pH 6.8. This modification shortened the time necessary for the disulfide bond formation by fourfold in comparison with air oxidation. Along with this, the amount of impurities and by-products was significantly reduced. Finally, the deprotection of the Thr residue side chain by a TFA–EDT–TIS–water mixture provided the hinge peptide **1c**, which was further easily purified by HPLC.

The only published conformational analyses of the hinge peptide were based on the combined use of the NMR experiments in DMSO and on restricted molecular dynamics calculations (Bovermann et al. 1989; Moroder et al. 1988; Wünsch and Bovermann 1988). Later, vibrational circular spectra (VCD) and Raman optical activity (ROA) signal measurements (vibrations associated with the disulfide bridges) were evaluated (Baumruk et al. 2005; Kapitán et al. 2005, 2006) and a polyproline II helical conformation was designed for hinge structures. Our NMR study of compounds **1c**, **2k** and **3c** provided complete structural assignment of hydrogen and carbon atoms (see Tables 2–4 in electronic Supplementary data), temperature coefficients of amide NH protons and relaxation times T_1 of carbon atoms. The NOE contacts were observed only between neighboring amino acid residues (sequential NOEs), but it should be noticed that possible NOE contacts between structurally identical residues of parallel peptide chains cannot be detected due to symmetry equivalence. All amide bonds (including those tertiary X-Pro amide bonds) are present in *trans*-configuration. The values of the temperature coefficients of amide NH protons do not give any evidence for strong intramolecular hydrogen bonding and the ^{13}C relaxation times T_1 indicate only slightly higher mobility of acyclic part of hinge peptide in comparison with its cyclic part. An investigation of ROA signal potential for the detection and chirality assignment of the S–S group is under way.

A permanent interest in utilization of soluble polymers such as polyethylene glycols (Bayer and Mutter 1972; Mutter and Bayer 1980; Harris 1992; Zhao et al. 1997; Sedlák 2005; Zalipsky et al. 1984) in the peptide synthesis stimulated our search for another type of suitable material for assembling the hinge structures. In addition to the use of PEG2000OMe, which was described in our previous preliminary communication (Niederhafner et al. 2006), we have introduced PEG6000 now. It features a good solubility in the polar solvents and insolubility in the non-polar ones, an optimal ratio of molecular weight to loading capacity and a good crystallization ability of corresponding intermediates (Veronese 2001; Benaglia et al. 1998; Fishman et al. 2003). This polymer has two accessible hydroxyl groups as the attachment sites on both sides for a simultaneous attachment of the amino acid residues of growing peptide chains (Fig. 3). Using this carrier, we synthesized the protected truncated peptide **2i**, with omitted C-terminal proline, which was replaced by leucine, as an enzymatically cleavable linker. The PEG6000 has enabled the initial coupling with $N^{\alpha},N^{\varepsilon}$-Boc-Lys-OH (**2a**) and, after $N^{\alpha},N^{\varepsilon}$-deprotection, the acylation of both amino groups by the next amino acid (leucine). This strategy doubled the loading capacity of the polymer and evidently enabled both the growing peptide branches to adopt a conformation, which facilitated their simultaneous acylation by cystine derivative $(Boc\text{-}Cys\text{-}OPfp)_2$ (Kisfaludy et al. 1973; Kisfaludy and Schön 1983) as shown in Fig. 3. Only one

equivalent of this disulfide bond synthon per 2 amino groups thus provided both peptide chains, which were assembled in the natural parallel alignment. In this way, the orthogonal side chains protection of cysteine residues (Wünsch et al. 1988) could be eliminated. The spatial arrangement, as well as the higher dilution used, favored the desired reaction pathway over oligomerization or "double single" acylation. The leucine was introduced instead of proline to both of the lysine amino groups as an enzymatically cleavable linker. In each step of the synthesis, a sample of the growing peptide was taken for analysis (Table 1). After the peptide chains (**2i**) on both sides of PEG6000 were assembled, they were easily detached by thermolysin-catalyzed hydrolysis of the peptide bond on the amino side of Leu residue under mild conditions and with a high yield of **2j** (>90%), also due to the presence of Lys residue increasing the distance of the cleavage site from the carrier. After a final N terminus of **2j** deprotection, the resulting **2k** could be simply purified by preparative HPLC.

Unfortunately, this method suffers from non-specificity of thermolysin, which splits the peptide backbone also at the amino ends of the Ile, Trp and Phe. Therefore, such amino acids cannot be present in the peptide sequence.

Similar to hinge analog **2k**, we employed the above-mentioned disulfide synthon in the solution synthesis of methyl amides **3c** and **3d** (Fig. 4). We introduced this synthon directly into the growing peptide using again the $(\text{Boc-Cys-OPfp})_2$, which was reactive enough to acylate the starting proline methylamide in DMF solution. Thus, we obtained the protected peptide methyl amide **3a**. The synthesis continued with two proline residues added subsequently to both amino groups of the cystine peptide methyl amide via symmetrical anhydrides of the corresponding Boc derivatives (**3b**). The same cystine derivative as above was used to complete the synthesis of the above-mentioned *N*-protected decapeptide **3c**. The orthogonal side chain protection of the cysteine residues could be therefore avoided even in the solution synthesis. After HPLC purification, one part of **3c** was used as a tool for the conformation investigation of the hinge peptide central part by ROA measurement (Kapitán et al. 2005), while the second part was treated with a TFA–EtOAc mixture to yield the target peptide **3d** (Table 4).

Conclusion

In this study, we have checked three synthetic methodologies to achieve our goal to simplify the preparation of peptides containing disulfide bonds on the hinge peptide model structures. We proved that the cystine synthon [$(\text{Boc-Cys-OPfp})_2$], which was employed in our earlier synthesis on PEG2000OMe (Niederhafner et al. 2006), is also applicable in the synthesis on PEG6000 with binding sites on both sides of this polymer. A dendrimeric unit of lysine was introduced to these positions to double the binding capacity and to improve the space fit for simultaneous acylation of the peptide chains by the $(\text{Boc-Cys-OPfp})_2$. The PEG6000 carrier also facilitated the thermolysine-catalyzed detachment of the tetradecapeptide **2j** from the polymer in high yield. The cystine derivative was even applicable to the solution synthesis, as demonstrated in the preparation of the shortened decapeptide methyl amide analog **3d**. On the other hand, the solid phase synthesis could be successfully applied only to the preparation of monomeric octapeptide of the hinge sequence. Due to sterical hindrance, a space in the solid polystyrene carrier does not appear suitable for the proper attachment of the *bis*-Boc-cystine to the growing peptide chains. Fortunately, the random oxidation of the sulfhydryl groups in Cys residues by pure oxygen in ammonium acetate buffer shortened fourfold the time necessary to create both disulfide bonds in the parallel alignment, in comparison with alternative oxidation performed by air oxygen. The higher total yield was obtained in the synthesis of the target compound **2k**, performed on the soluble carrier (PEG6000, 42%) when compared to the synthesis of the target compound **1c**, carried out on the solid carrier (polystyrene, 39%). However, the total yield of only 18% was obtained in the classical solution synthesis of the target compound **3d**. The synthesis on the PEG6000 enabled larger quantities of the material to be obtained in one run, in comparison with the solid phase synthesis. In summing up our findings, we suggest that the results of this study might be of practical importance in applications aimed at the preparation of related peptides and protein moieties containing the cystine structures, with the preference for the soluble PEG6000 carrier.

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