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Synthesis of protected peptides from the human IgG1 hinge region on PEG support using disulfide bond synthons and alkaline or enzymatic detachment

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Abstract—Peptides from the hinge region of human IgG1 are potential defined carriers of synthetic immunogens. Their synthesis was carried out on the soluble support—PEG-OMe 2000 using disulfide bond synthons. The loading capacity of the polymer was increased twofold by anchoring the lysine as a simple branching unit. The amino acid leucine was used as an enzymatically cleavable linker. Peptide detachment from the polymer was performed either by classical ester bond saponification or newly by peptide bond cleavage catalyzed by thermolysin in water.

Selected sequences from native proteins have been found as potential carriers of antigenic peptides with defined structures. For example, the central part of the protein from the surface region of human immunoglobulin IgG1 is occupied by a dimeric sequence, in which both linear parts in parallel alignment are connected by two disulfide bridges. The abundance of proline residues enables this rigid structure to act as a swivel point for the flexible part of the hinge region[1] that may well represent a suitable core peptide for the design and synthesis of various conjugates with regard to their immunochemical applications.

The hexadecapeptide **1** from the hinge region of IgG1 was used as a carrier of antibodies against peptides related to gastrin[2,3] and was designed to be a structurally defined carrier of antigenic determinants of potential synthetic vaccines. A synthesis of this peptide has been already described using a classical approach in solution.[4,5] The sulfhydryl groups of cysteine residues were selectively protected to form, upon successive oxidation, a dimeric structure with parallel alignment only. Without such protection, random oxidation afforded over 80% of the parallel dimer, which could be purified by chromatography.

Our alternative synthetic procedure is aimed towards bis-N-protected structures from the hinge region of IgG1, which could be used in segment condensation with a variety of antigenic peptides. We herein describe the synthesis of bis-N-protected dimeric bis-disulfide peptides with unambiguous parallel alignment of both the peptide chains (analogues of peptide **1** in Fig. 1) on polyethylene glycol (PEG).[6–8] This soluble polymer support offers certain advantages towards insoluble carriers with respect to analysis and monitoring and, most importantly, to the establishment of homogenous conditions. PEGs are soluble in many, mostly polar solvents (including water) and are insoluble in a few non-polar solvents, for example, diethyl ether. Similar to solution methods, the protected peptide-PEG conjugate can be analyzed after each coupling step after its precipitation from solution with diethyl ether and filtration to remove impurities.

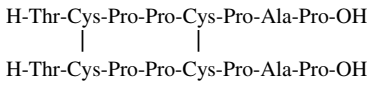

Figure 1. Primary structure of peptide 225-232/225′-232′ (**1**) from the hinge region of human IgG1.

Keywords: Hinge peptide; Disulfide synthon; PEG; Thermolysin; Defined carrier of immunogens.
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To control growth of the peptide chain efficiently, we chose PEG 2000 mono-methyl ether (PEG-OMe 2000), which bears hydroxyl groups on one side of the polymer only as the support (Fig. 2). To ensure that both the peptide chains are assembled in the natural parallel alignment and to avoid the necessity of orthogonal side-chain protection of cysteine residues, we performed the syntheses directly with the disulfide bond synthon, bis-*N*-Boc-cystine-bis-pentafluorophenyl ester.[9,10] The same protection and activation sequence was also used for threonine coupling in the last step of the syntheses. Other amino acids were coupled using symmetric anhydrides of Boc-leucine, Boc-alanine and Boc-proline. All coupling reactions, the deprotection with TFA and neutralization with DIEA were carried out in CH_2Cl_2. Besides the direct loading of the first amino acid leucine to the polymer, in an alternative synthetic route, N^{α},N^{ε}-Boc-Lys-OH was coupled with the polymer first and after N^{α},N^{ε}-deprotection, both the amino groups of this dendrimeric unit were acylated with the corresponding derivative of leucine and then the synthesis continued as before.[11,12] This strategy doubled the loading capacity of the polymer with regard to direct loading of leucine. This amino acid was introduced to the C-terminus of the corresponding peptides as an enzymatically cleavable linker. Successful detachment[13] of the peptides from the polymer was carried out either, classically by hydrolysis of the ester bonds with 0.1 M NaOH at room temperature for 30 min to obtain peptides **2** and **3** or newly, by thermolysin catalyzed cleavage of the peptide bond Ala-Leu in water, at room temperature for 4 h, resulting in peptide **4**. Yields of over 95% for each of the peptides were obtained. Preparative HPLC was used to purify[14] the peptides **2**–**4**.

In summary, bis-N-protected dimeric peptides **2**–**4** in parallel alignment (Table 1) were prepared by step-wise synthesis on soluble PEG-OMe 2000 as the hinge region segments suitable for further condensation with a variety of peptide epitopes. The disulfide bond synthon

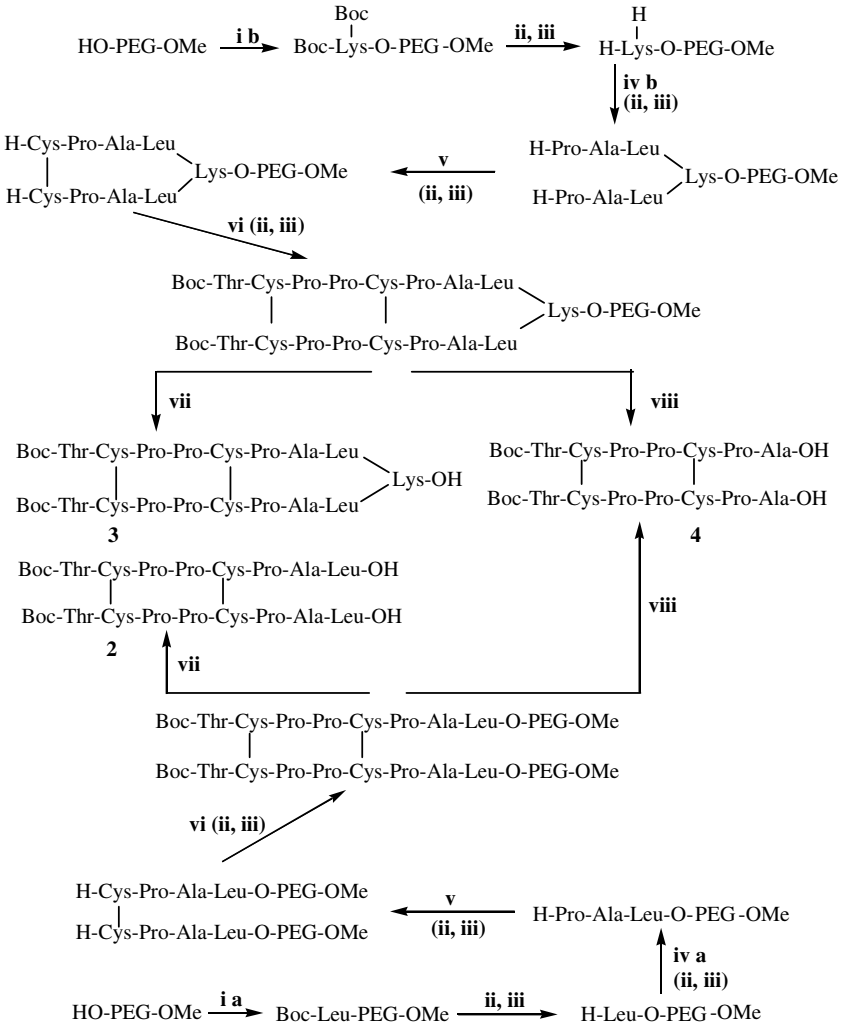

Figure 2. Synthesis of peptide derivatives **2**–**4** from the hinge region of human IgG1 on PEG-OMe 2000. Reagents and conditions: (**i a**) Boc-Leu-OH or (**i b**) Boc-Lys(Boc)-OH, DCC/DMAP in CH_2Cl_2, 4 h; (**ii**) 50% TFA/DCM 0 °C, 30 min; (**iii**) 40% DIEA/CH_2Cl_2; (**iv a**) elongation by $(Boc\text{-}Ala)_2O$ and $(Boc\text{-}Pro)_2O$ or (**iv b**) assembly of the bis-peptide chain by $(Boc\text{-}Leu)_2O$, $(Boc\text{-}Ala)_2O$ and $(Boc\text{-}Pro)_2O$ in CH_2Cl_2/DIEA, 2 h; (**v**) coupling of $(Boc\text{-}Cys\text{-}OPfp)_2$ in CH_2Cl_2/DIEA 2 h; (**vi**) elongation by $(Boc\text{-}Pro)_2O$, $(Boc\text{-}Cys\text{-}OPfp)_2$ and Boc-Thr-OPfp in CH_2Cl_2/DIEA, 2 h; (**vii**) 0.1 M NaOH 30 min, yield > 95%; (**viii**) thermolysin in water 4 h, RT, yield > 95%.

Table 1. Analytical data of dimeric peptides **2**, **3** and **4** from the hinge region of IgG1

Compound	Formula[a]	HPLC[b]	Amino acid composition[c]					
	MW/M^+		Cys	Pro	Ala	Thr	Leu	Lys
2	$C_{78}H_{124}N_{16}O_{24}S_4$ 1798.3/1797.8	21.68	1.98	3.02	1.00	0.98	1.02	—
3	$C_{84}H_{134}N_{18}O_{24}S_4$ 1908.5/1907.9	20.40	3.89	5.89	2.02	1.95	2.03	1.00
4[d]	$C_{66}H_{102}N_{14}O_{22}S_4$ 1572.0/1571.6	15.37	2.01	2.97	1.00	0.96	—	—

[a] Determined by FAB analysis.

[b] Retention time in minutes, 25 × 0.4 cm column, 5 μm (LiChrospher WP-300, Merck Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm, gradient 5–95% CH_3CN in 0.05% aqueous TFA, 40 min.

[c] Amino acid analyses were performed on a Biochrom 20 instrument (Pharmacia, Sweden). The samples were hydrolyzed with 6 M HCl containing 3% phenol for 20 h at 110 °C. Cystine was determined as the cysteic acid after treatment of the hydrolyzed sample with performic acid.

[d] Electrophoresis was carried out on Whatman 3MM (20 V/cm) paper in 6% acetic acid (pH 2.4) and in pyridine–acetate buffer (pH 5.7) for 1 h. Prior to electrophoresis the sample was treated with TFA for 30 min; $E_{2.4}{}^{Gly}$ 0.77, $E_{5.7}{}^{His}$ 0.51.

derived from cystine was used to build the parallel dimer peptide sequences unambiguously and to avoid classical random oxidation of cysteine residues in monomeric sequences. The introduction of a lysine residue doubled the loading capacity of the PEG polymer with respect to direct introduction of leucine, which was used to replace the C-terminal proline residue in the original structure of the hinge peptide **1** as an enzymatically cleavable linker. As a result, detachment of peptide **4** by thermolysin catalyzed cleavage of the peptide bond Ala-Leu, proceeded stereospecifically and in nearly quantitative yield and is comparable with the detachment by mild saponification of the ester bond between the peptide and polymer (peptides **2** and **3**).

Acknowledgements

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References and notes

1. Burton, D. R. *Mol. Immunol.* **1985**, *22*, 161–206.
2. Wűnsch, E.; Moroder, L.; Gőhring-Romani, S.; Musiol, H.-J.; Gőhring, W.; Scharf, R. *Int. J. Pept. Protein Res.* **1991**, *37*, 61–71.
3. Wűnsch, E.; Moroder, L.; Hűbener, G.; Musiol, H.-J.; Von Grűnigen, R.; Gőhring, W.; Scharf, R.; Schneider, C. H. *Int. J. Pept. Protein Res.* **1991**, *37*, 90–102.
4. Wünsch, E.; Moroder, L.; Gőhring-Romani, S.; Musiol, H.-J.; Gőhring, W.; Bovermann, G. *Int. J. Pept. Protein Res.* **1988**, *32*, 368–383.
5. Moroder, L.; Hűbener, G.; Gőhring-Romani, S.; Gőhring, W.; Musiol, H.-J.; Wűnsch, E. *Tetrahedron* **1990**, *46*, 3305–3314.
6. Mutter, M.; Bayer, E.. In *The Peptides, Analysis, Synthesis, Biology, Part A*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, London, 1980; Vol. 2, pp 285–332.
7. Harris, J. M. In *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Application*; Harris, J. M., Ed.; Plenum Press: New York, 1992.
8. Zhao, X.; Harris, J. M. *ACS Symp. Ser.* **1997**, *680*, 458–460.
9. Roth, G. A. *J. Org. Chem.* **1995**, *60*, 8105–8109.
10. Kisfaludy, L.; Lőw, M.; Nyéki, O.; Szirtes, T.; Schőn, I. *Justus Liebigs Ann. Chem.* **1973**, 1421–1429.
11. Loading of the first amino acid: To a stirred mixture of PEG-OMe 2000 (5 g), Boc-amino acid (2.8 mmol) and 4-dimethylaminopyridine (0.76 mg; 0.62 mmol) in CH_2Cl_2 (40 ml), a solution of DCC (0.62 g; 3 mmol) in CH_2Cl_2 (5 ml) was added at −20 °C. The temperature was allowed to increase to room temperature and after 2 h the precipitated dicyclohexylurea was filtered off. The filtrate was concentrated to 25 ml in vacuo and then poured into cold diethyl ether (200 ml) to precipitate the product, which was filtered off and dried under vacuum for 5 h at room temperature.
12. Elongation of the peptide chain: Boc-amino acid-PEG-OMe 2000 (5 g; 5 mmol, based on the terminal amino group) was stirred with a TFA–CH_2Cl_2 (1:1) mixture (30 ml) for 30 min at room temperature. After evaporation, the residue was triturated with diethyl ether (3 × 30 ml), dissolved in CH_2Cl_2 (50 ml) and the solution stirred with a Boc-amino acid symmetric anhydride (20 mmol, 4 M excess) or *N*-Boc-threonine pentafluorophenyl ester (7.5 mmol, 1.5 M excess) at pH 8 adjusted using DIEA. In the case of bis-*N*-Boc-cystine-bis-penta fluorophenyl ester, only 1 equiv (1.8 g; 2.5 mmol) was added to the reaction mixture in several portions. The progress of the couplings was monitored by chloranil test,[15] HPLC and electrophoresis. The reaction mixtures were worked up after 2–4 h as described in Ref. 11.
13. Cleavage the of peptides from the polymer: (A) A mixture of bis-Boc-peptide-PEG-OMe 2000 (3.5 g) and 0.1 M NaOH (20 ml) was stirred for 30 min at room temperature, then neutralized with 0.1 M HCl (20 ml) and lyophilized. (B) N^{α}-(Boc-peptide)-N^{ε}-(Boc-peptide)-Lys-O-PEG-OMe 2000 (2.0 g) was treated with thermolysin (2 mg) in water (10 ml) at room temperature for 4 h. The peptide that precipitated was then separated by filtration.
14. Purification: Vydac RP-18 (The Separations Group, Hesperia CA, USA) 25 × 2.2 cm column, 10 μm, flow rate 7 ml/min, detection at 220 nm using a 0–100% gradient of CH_3CN in 0.05% aqueous TFA, 120 min.
15. Christensen, T. In *Peptides, Structure and Biological Function*; Gross, E., Meienhofer, J., Eds.; Proc. 6th Am. Peptide Symp.; Pierce Chemical Company: Rockford, IL, USA, 1979, pp 385–388.