Conjugation of Epitope Peptides with SH Group to Branched Chain Polymeric Polypeptides via Cys(Npys)

ARTICLE *in* BIOCONJUGATE CHEMISTRY · JULY 2000

Impact Factor: 4.51 · DOI: 10.1021/bc990116o · Source: PubMed

4 AUTHORS, INCLUDING:

Gabor Mezo
Eötvös Loránd University
131 PUBLICATIONS **1,530** CITATIONS
SEE PROFILE

David Andreu
University Pompeu Fabra
270 PUBLICATIONS **7,957** CITATIONS
SEE PROFILE

Ferenc Hudecz
Eötvös Loránd University
274 PUBLICATIONS **3,276** CITATIONS
SEE PROFILE

Conjugation of Epitope Peptides with SH Group to Branched Chain Polymeric Polypeptides via Cys(Npys)

Gábor Mezö, Nikolett Mihala, David Andreu,† and Ferenc Hudecz*

Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, H-1518, Budapest, Hungary, and Department of Organic Chemistry, University of Barcelona, Barcelona, Spain.
Received September 18, 1999; Revised Manuscript Received March 21, 2000

Since bioconjugates may play an important role as therapeutics in the future, the development of new and effective conjugation strategies is necessary. For the attachment of peptide-like molecules to carriers, there are two main coupling methods involving amide or disulfide bonds. Conjugation through an amide bond can be achieved in several well-defined ways known from peptide chemistry. However, the formation of disulfide bridges between cysteine-containing peptides and carrier molecules still has some problems. In this paper, we describe a novel approach in which the carrier polypeptide is modified by 3-nitro-2-pyridinesulfenyl (Npys)-protected cysteine and this derivative has been applied for conjugation of Cys-containing epitope peptides with poly(L-lysine)-based branched polypeptides. Considering the stability of Npys group in the presence of pentafluorophenol, Boc-Cys(Npys)-OPfp dervivative was selected for introduction to the N-terminal of branches of polypeptides backbone. The branches of the polymers were built up from oligo(DL-alanine) (poly[Lys(DL-Ala$_m$)], AK) and elongated by an optically active amino acid [poly[Lys(X$_i$-DL-Ala$_m$)], XAK]. We found that the nature of X (Glu, Ser, Thr) has great influence on the incorporation of the protected cysteine residue. Herpes simplex virus and adenovirus epitope peptides were conjugated to Boc-Cys(Npys)-modified polypeptides. Results indicate that the incorporation of epitope peptides depends on the number of Npys group on the polymers as well as on the presence/absence of Boc-protecting group on the Cys residue. This new class of Cys(Npys)-derivatized branched polypeptides is stable for a couple of months and suitable for effective preparation of epitope peptide conjugates possessing increased water solubility.

INTRODUCTION

The combination of macromolecules and small bioactive molecules has a wide range of applications, particularly in the development of polymeric therapeutics (*1*−*3*). In accordance with the strategy invented by Ringsdorf, both drug and targeting moieties are attached to the same carrier (*4*). In immunology, protein carriers are frequently applied to induce immune responses against covalently attached low molecular weight peptide epitopes, for monoclonal antibody production or for synthetic vaccine construction (*5*, *6)*. It has been also demonstrated that the effective synthetic vaccine must contain both B- and T-cell epitope sequences. These constructs usually consist of two different epitope peptides and a vehicle (e.g., macromolecule, liposome, and nanoparticle).

Ideally, the unambiguous conjugation of two different epitope peptides to a macromolecular carrier requires two unrelated coupling methods. The two most commonly used procedures for conjugation are based on amide and disulfide formation. There are many well-defined coupling methods for the attachment of biological active peptides to carriers through amide bonds (water-soluble carbodiimide (*7*), active ester (*8*), and BOP[1] (*9*). The conjugation via disulfide bridge is usually carried out by using heterobifunctional reagents such as SPDP (*10*) (Figure 1). However, the application of amino/thiol-type bifunctional compounds raises some problems. First of all, these molecules are usually connected to the carrier through amide bonds, which diminish the number of available amino functions on the macromolecule. In addition, this type of bifunctional reagent involves the introduction of a hydrophobic spacer moiety, which often decreases the water solubility of the parent macromolecule. Finally, the disulfide formation reaction between the activated thiol of the carrier and the cysteine-containing epitope peptide usually proceeds at neutral or slightly alkaline pH, which can give rise to side reactions such as oxidation (dimerization) of the HS-peptide.

An alternative strategy for disulfide-based conjugation is the application of Cys(Npys) as a coupling agent. The

* To whom correspondence should be addressed. Fax: 36-1-372 2620. Phone: 36-1-372 2828. E-mail: hudecz@szerves.chem.elte.hu.

† Department of Organic Chemistry.

[1] Abbreviations: Acp, E-aminocaproic acid; Boc, *tert*-butyloxycarbonyl; Bom, benzyoxymethyl; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; BrZ, 2-bromobenzyl-oxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; cHex, cyclohexyl; ClZ, 2-chloro-benzyloxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, *N*, *N*′-dicyclohexylurea; DIEA, *N*,*N*′-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; $\overline{DP}_n$, *number average ratio of polymerization; DTT, dithiothreitol; FAB-MS, fast-atom bombardment mass spectrometry; HATU, 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HSV gD, Herpes simplex virus glycoprotein D; KLH, keyhole limpet hemocyanin; MBHA, 4-methylbenzhydrylamine; MBS*, *N*-(3-maleimido-benzoyloxy)-succinimide; Meb, 4-methylbenzyl; MeOH, methanol; Npys, 3-nitro-2-pyridinesulphenyl; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)-propionate; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluor-acetic acid; TLC, thin-layer chromatography; Tos, tosyl; UV, ultraviolet.

10.1021/bc990116o CCC: $19.00
Published on Web 06/10/2000

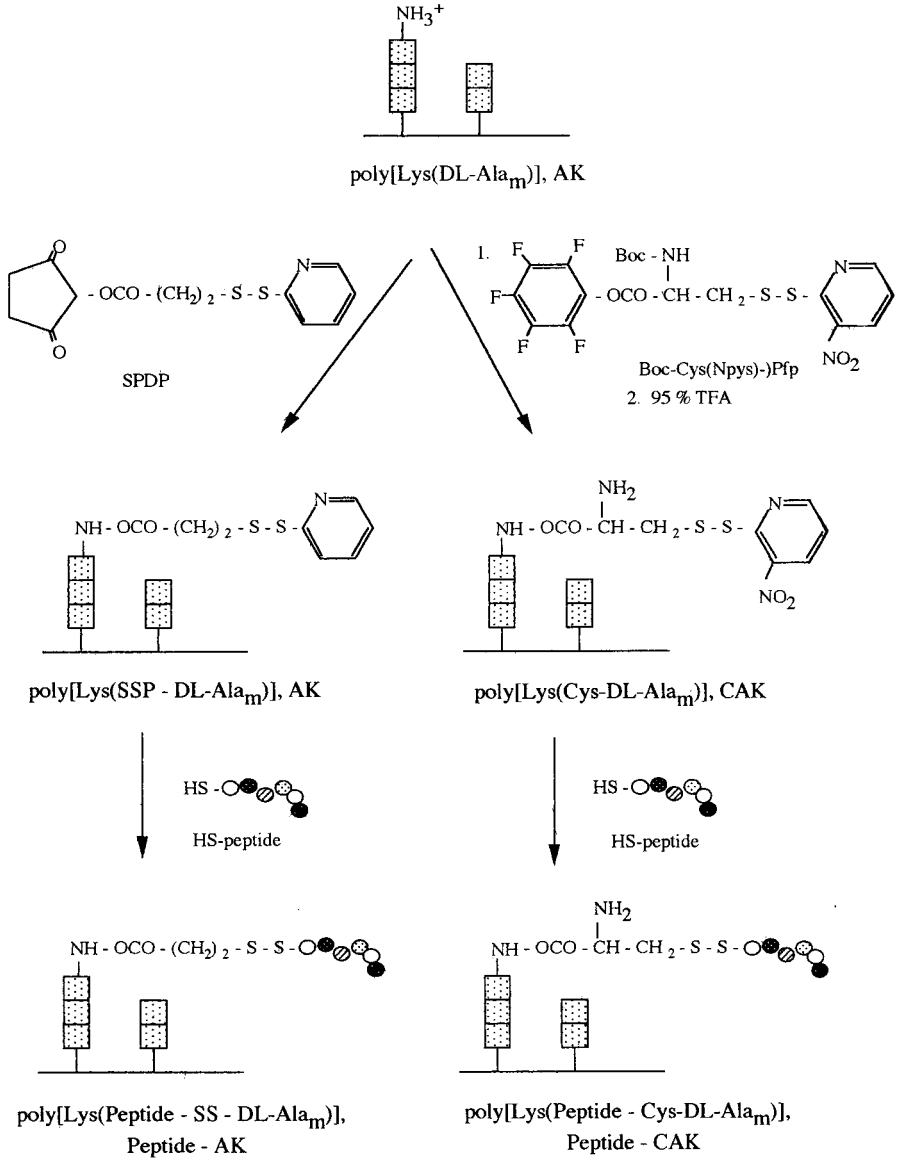

Figure 1. Outline of the synthesis of HS-oligopeptide conjugates with branched polypeptides by introduction disulpide bonds. Branched polypeptides were modified with heterobifunctional coupling reagent, SPDP or Cys(Npys).

Npys (3-nitro-2-pyridinesulfenyl) group was originally proposed as a protection for amino and/or hydroxyl functions during solid-phase peptide synthesis (*11*). The greatest potential of Npys, however, has gradually been realized to lie in the side-chain protection of Cys (*12*). Indeed, the most attractive feature of Npys is its dual character as an S-protecting and S-activating group.

Epitope peptides modified with Cys(Npys) residue at their C- or N-terminal were successfully conjugated to partially reduced BSA and KLH (*13*−*15*). The coupling procedure between the Cys(Npys) peptide and the free SH groups of the protein was carried out under acidic conditions (pH 4.5−7.0) to prevent the oxidation of the peptide and/or refolding of the reduced proteins. The aim of our studies reported here was the development of a new approach in which the macromolecular partner contains Cys(Npys) residues (Figure 2) and this derivative is conjugated with SH-oligopeptides. This strategy outlined in Figure 1 possesses several advantages: (i) the attachment of Cys(Npys) will not alter the number of amino groups on the macromolecule; (ii) it will allow direct peptide-carrier bonding without introduction of spacer entity and it will therefore preserve the solubility of the carrier; (iii) it will yield increased solubility since the optimal pH of this conjugation procedure is acidic (pH 4.5−7.0), and oxidation-related side reactions are suppressed.

In our laboratory, branched chain polymeric polypeptides with poly[L-Lys] backbone are used as macromolecular carriers (*16, 17*). The side chains of these polymers consist of a short oligo(DL-Ala) chain (3−6 amino acid residue) and an optically active amino acid residue at the N- or C-terminal part of the branches (poly[Lys(X_i-DL-Ala$_m$)] (XAK) and poly[Lys(DL-Ala$_m$-X_i] (AXK), where $i \approx 1$ and $m \approx 3$−6). These macromolecules were used earlier for the synthesis of epitope peptide conjugates to be used as antigens for the specific and sensitive detection of mucin1 glycoprotein-specific antibodies (*18*) and of *Mycobacterium tuberculosis* protein sensitized T cells (*19*). Branched polypeptide conjugates of epitope peptides derived from glycoprotein D of HSV have shown promising results as immunogens for vaccination of mice against lethal dose of HSV infection (*20*). For some of the artificial antigens described above we have utilized SPDP for the incorporation of Cys-peptide (*18*).

Here we present data on an alternative approach by utilizing Cys(Npys) residues on the side chains of four different XAK types branched chain polypeptides. Ap-

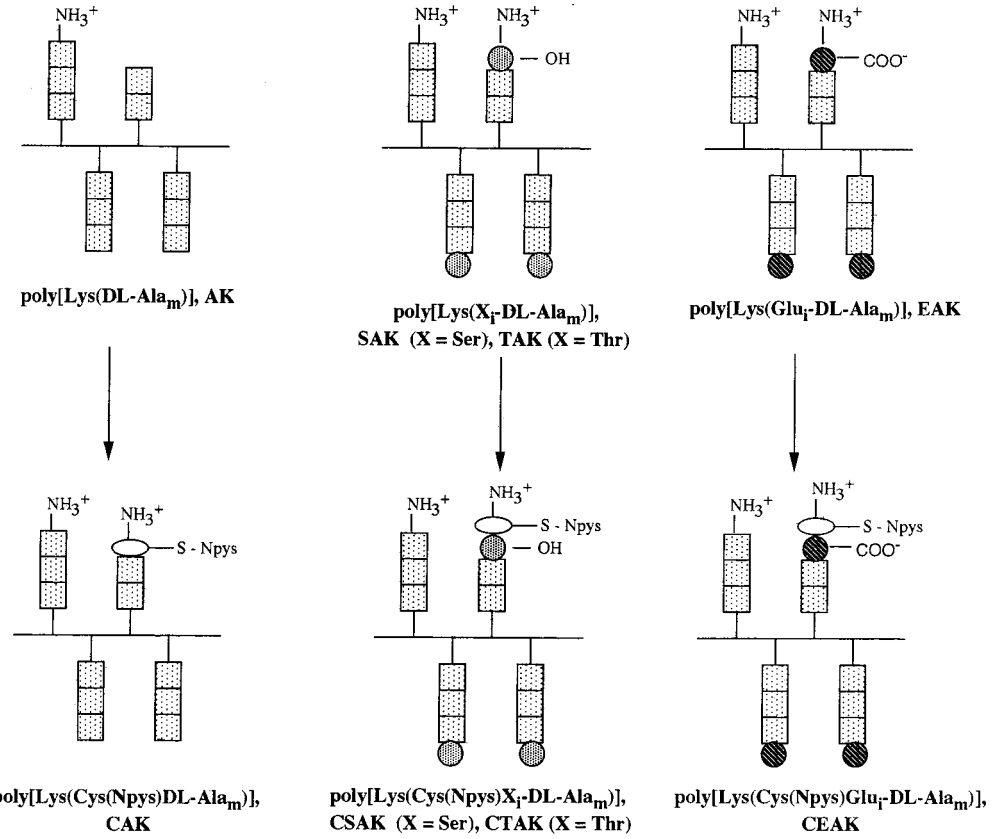

Figure 2. Schematic presentation of branched polypeptides used in this study.

propriate synthetic conditions developed for this methodology as well as the results of the synthesis of various conjugates applying Cys(Npys)-modified macromolecules and HS-peptide epitopes of viral origin are described. For this we have selected epitopes from glycoprotein D (gD) of Herpes simplex virus type 1 (^{9}LKNleADPNRFRGKDL^{22} and ^{276}SALLEDPVG^{284}) (*21*−*23*) and from adenovirus proteins AD2 (^{309}THLSYKPGKGDE^{320} and ^{399}NQAVDSYDPD^{408}) and AD41 (^{148}FIGTNINKDNGI^{159}) (*24*). In addition, we report for the first time on the preparation of a prototype conjugate in which two epitopes of gD type 1 joined by a dipeptide Acp-Cys spacer (where Acp is ϵ-amino caproic acid) are attached through the SH function of a single peptide to the Cys(Npys)-modified branched polypeptide (Figure 3).

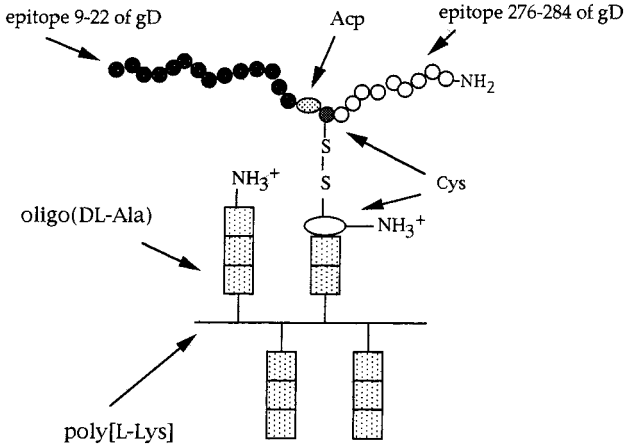

Figure 3. Schematic structure of a conjugate in which a single peptide containing epitopes corresponding to 9−22 and to 276−284 regions of glycoprotein D of HSV-1 is connected with carrier molecule. Two epitopes are joined by an Acp−Cys dipeptide spacer and the SH function of the peptide was used for conjugation with Cys(Npys) modified branched polypeptide.

EXPERIMENTAL PROCEDURES

Abbreviations used in this paper follow the rules of the IUPAC−IUB Commission of Biochemical Nomenclature (*25*) in accordance with the recommended nomenclature of graft polymers (*26*).[1]

Synthesis of Polymer Polypeptides. Branched polypeptides were synthesized as previously described (*16*, *17*, *27*). Briefly, poly[Lys] was prepared by the polymerization of N^{α}-carboxy-N^{ϵ}-benzyloxycarbonyl-lysine anhydride. After cleavage of the protecting groups poly[Lys(DL-Ala$_m$)] (AK) was prepared by grafting of short oligomeric DL-Ala side chains onto the ϵ-amino groups of poly[Lys]. Benzyloxycarbonyl-protected amino acids derivatives (*Z*-Ser-OPcp, *Z*-Thr-OPcp, and *Z*-Glu(OBzl)-OPcp) were coupled to the end of the side chains of AK by HOBt catalyzed active ester method (*28*). Blocking groups were removed completely with HBr in glacial acetic acid, as confirmed by UV spectroscopy at 254 nm resulted in poly[Lys(X$_i$-DL-Ala$_m$)] (XAK) polymers (where $i \leq 1$ and $m \approx 3-6$, X = Ser, Thr, or Glu). The composition of polypeptides was determined by amino acid analysis. The size and molecular weight of polymers were calculated from the amino acid composition and the sedimentation analysis of polylysine (*29*).

Synthesis of Epitope Peptides. Peptides (Table 1) were synthesized on MBHA resin (0.5 g, 1.1 mmol/g capacity) manually by Boc/Bzl strategy. The following protected amino acids were used: Asp(OcHex), Glu(OcHex), Thr(Bzl), Ser(Bzl), Tyr(BrZ), Lys(ClZ), Arg(Tos), His(Bom), and Cys(Meb). The protocol of the solid-phase synthesis was the following: (i) deprotection with 33% TFA/DCM (2 + 20 min); (ii) DCM washing (5 × 0.5 min); (iii) neutralization with 10% DIEA/DCM (3 × 1 min); (iv) DCM washing (4 × 0.5 min); (v) coupling 3 equiv of amino

Table 1. Characterization of Peptides Corresponding to Epitope(s) of HSV gD-1 or Adenovirus AD2, AD41 Proteins

amino acid sequence (code)	amino acid analysis found (calculated)	t_r (min)[a]	molar mass $[M + H]^+$
H-CSALLEDPVG-NH_2 HSV gD-1, Cys-276−284)	Asp, 0.95 (1); Ser, 0.90 (1); Glu, 1.12 (1); Pro, 0.99 (1); Gly, 1.12 (1); Ala, 1.00 (1); Val, 0.90 (1); Leu, 2.00 (2)	12.6[a]	1002.3 (1002.4)
H-LKNleADPNRFRGKDL-Acp-CSALLEDPVG-NH_2 (HSV gD-1, [Nle^{11}]-9−22-Acp-Cys-276−284)	Asx, 3.92 (4); Ser, 0.96 (1); Glu, 1.06 (1); Pro, 1.95 (2); Gly, 2.08 (2); Ala, 2.04 (2); Val, 1.00 (1); Nle, 1.00 (1); Leu, 4.04 (4); Phe, 1.00 (1); Lys, 2.08 (2); Arg, 1.96 (2)	16.4[a]	2740.0 (2740.2)
H-CTHLSZKPGKGDE-NH_2 (AD-2, Cys-309−320)	Asp, 0.98 (1); Thr, 0.97 (1); Ser, 0.96 (1); Glu, 1.05 (1); Pro, 1.01 (1); Gly, 2.04 (2); Leu, 1.00 (1); Tyr, 0.93 (1); His, 0.98 (1); Lys, 2.06 (2)	9.5[b]	1434.6 (1434.7)
H-CNQAVDSZDVD-NH_2 (AD-2, Cys-399−408)	Asx, 3.95 (4); Ser, 0.95 (1); Glu, 1.05 (1); Pro, 1.03 (1); Ala, 1.00 (1); Val, 0.97 (1); Tyr, 0.94 (1)	11.8[c]	1425.8 (1425.6)
H-CFIGTNINKDNGI-NH_2 (AD-41, Cys-148−159)	Asx, 3.94 (4); Thr, 0.96 (1); Gly, 2.02 (2); Ile, 3.03 (3); Phe, 1.01 (1); Lys, 1.02 (1)	17.9[c]	1407.7 (1407.5)

[a] Column: Phenomenex Jupiter C18 (250 × 4.6 mm, 10 μm, 300 Å). Eluent: (A) 0.1% TFA/water, (B) 0.1% TFA/AcN-water (80:20 v/v). Gradient: 0 min, 15% B; 5 min, 15% B; 35 min, 75%B. [b] Column: Vydac C4 (250 × 4.6 mm, 5 μm, 300 Å). Eluent: (A) 0.1% TFA/water, (B) 0.1% TFA/AcN-water (80:20 v/v). Gradient: 0 min, 10% B; 5 min, 10% B; 35 min, 50% B. [c] Column: Knaurt Eurosil Bioselect (250 × 4.0 mm, 5 μm, 300 Å). Eluent: (A) 0.1% TFA/water, (B) 0.1% TFA/AcN. Gradient: 0 min, 5% B; 25 min, 50%B. [d] Asx: Asn and Asp measured as aspartic acid.

acid−DCC−HOBt in DCM-DMF 4:1 or 1:4 mixture depends on the solubility of Boc-amino acid derivatives (60 min); (vi) DMF washing (1 × 0.5 min); (vii) DCM washing (2 × 0.5 min); (viii) ninhydrine assay. The peptides were cleaved from the resin by the aid of liquid HF in the presence of *p*-cresol and DTT (HF-*p*-cresol-DTT= 10 mL:1 g:0.1 g) for 1.5 h at 0 °C. The crude products were purified by HPLC using Delta Pak C_{18} semipreparative column (30 × 1.9 cm, 10 μm). The purified peptides were analyzed by HPLC, amino acid analysis and FAB-MS (Table 1).

Synthesis of Boc-Cys(Npys)-OPfp. A total of 500 mg (1.33 mmol) of Boc-Cys(Npys)-OH (Mw 375) and 245 mg (1.33 mmol) pentafluorophenol were dissolved in 5 mL of DCM, then 274 mg (1.33 mmol) of DCC dissolved in 2 mL of DCM was added to the solution. The reaction mixture was stirred in an ice bath for 15 min then for 2 h at RT. The reaction was followed by TLC. After cooling, the solution to 0 °C the precipitated DCU was filtered out, the solvent was evaporated, and the product was recrystallized from methanol. Yield 594 mg (83%) (Mw 541). Melting point: 96−99 °C. R_f = 0.65 (chloroform-methanol = 3:1); R_f = 0.92 (ethyl acetate). Optical rotation: $[\alpha]_D = -62.2$ (c = 1, DMF).

Synthesis of Branched Polypeptides with Cys-(Npys) Residues. The synthesis of poly[Lys(Cys-(Npys)$_i$-DL-Ala$_m$)] (CAK) is described in details. A total of 20 mg (57 μmol) of poly[Lys(DL-$Ala_{3.11}$)] (AK, $\overline{DP}_n$ = *66*, $\overline{Mw}_{monomer}$ 349) was dissolved in 0.5 mL d.i. water, and the solution was diluted with 3 mL of DMF. Equivalent amount (31 mg, 57 μmol) of Boc-Cys(Npys)-OPfp dissolved in 2 mL of DMF was added to the solution. The reaction mixture was stirred overnight at RT. The solvent was removed in vacuo (30 °C), and the remaining product was treated with ether (containing 10% DCM) several times for washing out the unreacted ester and the resulted pentafluorophenol. Yield: 35 mg. Boc-protection was removed in 5 mL of TFA-water (95:5, v/v) at 0 °C for 1h in the presence or absence of 5% *p*-cresol or thioanisole. The solution was concentrated in vacuo, then the remaining material was dissolved in water and dialyzed versus 0.1% acetic acid solution for 2 days followed by liophylization (yield 27 mg). The average degree of substitution was calculated from the UV absorption of Npys group at 350 nm (ϵ = 3930 M^{-1} cm^{-1} in DMF). The data were also corrected by peptide content of amino acid analysis measured from the same solution.

Other Cys(Npys) containing polymers were synthesized in the same way using poly[Lys(Ser$_{1.0}$-DL-Ala$_{3.1}$)] (SAK, $\overline{DP}_n$ = 66, $\overline{Mw}_{monomer}$ 428), poly[Lys(Glu$_{0.9}$-DL-Ala$_{3.1}$)] (EAK, $\overline{DP}_n$ = 66, $\overline{Mw}_{monomer}$ 465), or poly[Lys(Thr$_{1.0}$-DL-Ala$_{3.1}$)] (TAK, $\overline{DP}_n$ = 60, $\overline{Mw}_{monomer}$ 450). The starting molar ratio of Boc-Cys(Npys)-OPfp and the polymers as well as the average degree of incorporation to the branches are summarized in Table 2.

Conjugation of Epitope Peptides to Branched Polypeptides with Cys(Npys). Branched polypeptides containing Boc-Cys(Npys) (Boc-CAK or Boc-CSAK) were dissolved in DMF (A) or in DMF-0.06M phosphate buffer (pH 5.5) = 75:25 (v/v) mixture (B). Alternatively polymers without Boc protecting groups (CAK, CSAK) were dissolved in the 0.06 M phosphate buffer solution (C). A range of 1.2−1.4 equiv (calculated for the Npys content of the polymers) of Cys-epitope peptide dissolved in the same solvent was added to the polymer solution. The final concentration of the reaction mixture was 1 mg/mL for the polymer component. The mixtures were stirred for 4 h at RT. Then the solutions were filled in Visking tubes (cut off 8000−12000) and dialyzed for 2 days against 0.1% acetic acid. The conjugates containing Boc-protecting groups were deblocked with 95% TFA and then dialyzed and freeze-dried. In case of the presence of UV band at λ = 350 nm Npys groups were reacted with the 1.2 equiv excess of cysteine in 0.06 M phosphate buffer (pH 5.5) followed by dialysis and freeze-drying. The average degree of substitution of conjugates was calculated from amino acid composition and it is presented in Table 3.

The conjugate containing two epitopes joined by Acp-Cys spacer was prepared as follows. Boc-deprotected poly-[Lys(Cys$_{0.24}$-Ser$_{0.9}$-DL-Ala$_{3.1}$)] trifluoroacetate salt (10 mg, 16.5 μmol) was dissolved in 10 mL of 0.06 M phosphate buffer (pH 5.5). A total of 1.35 equiv amount of H-LKNleADPNRFRGKDL-Acp-CSALLEDPVG-NH_2 (14.8 mg, 5.4 μmol) calculated to the Npys content of polymer was added to the reaction mixture. The solution was stirred for 4 h at RT. The solution was filled in Visking tube (cut off 8000−12000) and dialyzed for 2 days against

Table 2. Incorporation of Boc-Cys(Npys) Residues into Branched Polypeptides

polymers	Boc-Cys(Npys)-Opfp/ polymer ratio (mol/mol)	amount of polymer (mg)	amount of Cys residue (mg)	average degree of substitiuon (%)[a]	
				I	II
poly[Lys(DL-$Ala_{3.1}$)] (AK)	0.25	20	7.8	26	27
	0.5	20	15.5	42	46
	0.75	20	23.3	65	73
	1.00	20	31.0	74	100
	1.50	20	46.5	75	nd
poly[Lys($Ser_{0.9}$-DL-$Ala_{3.1}$)] (SAK)	0.25	20	6.3	16	17
	0.50	20	12.7	24	24
	0.75	20	19.0	26	27
	1.00	20	25.3	28	27
	1.50	20	38.0	28	nd
poly[Lys($Glu_{0.9}$-DL-$Ala_{3.1}$)] (EAK)	0.50	20	11.7	37	40
	1.00	20	23.3	41	48
	1.50	20	35.0	42	54
poly[Lys($Thr_{1.0}$-DL-$Ala_{3.1}$)] (TAK)	1.00	10	12.0	11	nd

[a] Determined by UV analysis (I) and corrected with amino acid composition (II).

Table 3. Preparation and Characterization of Epitope Peptide-Branched Polypeptide Conjugates

polymer	Cys(Npys) substitution[a] (%)	amount of polymer (mg)	Cys(Npys) contant of polymer (mmol)	solvent	peptide	amount of peptide[b] (mg)	avarage substitution[c] (%)
Boc-CAK	100	5.0	7.1	A	H-CSALLEDPVG-NH_2	8.5	7
Boc-CAK	100	5.0	7.1	A	H-CFIGTNINKDNGI-NH_2	12.1	6
Boc-CAK	100	5.0	7.1	A	H-CTHLSYKPGKGDE-NH_2	15.1	6
CAK	27	5.0	2.2	C	H-CSALLEDPVG-NH_2	2.7	16
CAK	46	5.0	3.8	C	H-CSALLEDPVG-NH_2	4.6	42
CAK	100	5.0	8.3	C	H-CSALLEDPVG-NH_2	9.9	52
CAK	27	10.0	4.4	C	H-LKNleADPNRFRGKDL-Acp-CSALLEDPVG-NH2	14.8	26
Boc-CSAK	24	5.0	1.9	A	H-CSALLEDPVG-NH_2	2.3	18
Boc-CSAK	24	5.0	1.9	B	H-CSALLEDPVG-NH_2	2.3	25
CSAK	24	5.0	2.0	C	H-CNQAVDSYDPD- NH_2	2.8	20
CSAK	24	5.0	2.0	C	H-CSALLEDPVG-NH_2	2.4	21
CSAK	24	10.0	4.0	C	H-LKNleADPNRFRGKDL-Acp-CSALLEDPVG-NH_2	14.8	20

[a] Determined by UV analysis and corrected with amino acid composition. [b] Corresponding to 1.2 μmol of peptide. [c] Determined by amino acid analysis.

0.1% acetic acid. No Npys content was detected by UV spectroscopy in the conjugate after liophylization. The yield was 15 mg (86%). According to the amino acid analysis, 20% of side chains of branched polypeptide contained the bifunctional epitope peptide.

Analytical Methods. The amino acid composition of branched polypeptides, epitope peptides, and their conjugates was determined by amino acid analysis using a Beckman (Fullerton, CA) model 6300 amino acid analyzer. Prior to the analysis, samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h. Fast-atom bombardment mass spectrometry measurements were carried out on a Fisons (U.K.) VG-ZA-2SEQ tandem mass spectrometer equipped with a Cs^+ ion gun (30 keV). The peptide samples were dissolved in DMSO and mixed with glycerol matrix. Analytical HPLC was performed using reversed-phase columns on Knauer apparatus (H. Knauer, Bad Homburg, Germany). The instrument was assembled from two HPLC Pump 64, a Variable Wavelength Monitor, and functionalized by HPLC Software/Hardware Package Version 2.21 A. The types of columns, gradients, and solvents are shown in Table 1.

RESULTS AND DISCUSSION

The synthesis and simplified structure of branched chain polypeptides containing Cys(Npys) residue at the N-terminal of the side chains are outlined in Figures 1 and 2. These compounds were prepared for conjugation with HS-peptides comprising one or co-linearly two epitopes (Figure 3.). Prior to the incorporation of cysteine residue to the polymers, the stability of Npys group was studied in the presence of pentafluorophenol. Due to the contradictory results reported on the sensitivity of Npys group in the presence of HOBt during solid-phase peptide synthesis (*15*, *30*), we have attempted to avoid the in situ HOBt-catalyzed active ester method for the introduction of Boc-Cys(Npys) on the polypeptide branches. An alternative active ester approach was developed using Boc-Cys(Npys)-OPfp (*31*) prepared from Boc-Cys(Npys)-OH and pentafluorophenol by DCC condensation (*32*) and characterized. The reaction between Boc-Cys(Npys)-OPfp and the α-amino groups of the N-terminal amino acids of branched polypeptide yield pentafluorophenol as byproduct. Considering this, we have initiated a model experiment in which Boc-Cys(Npys)-OH was kept in DMF-water (9:1, v/v) mixture in the absence or presence of pentafluorophenol. This solvent is the preferred mixture for the coupling of protected amino acids to the branched polymers (*17*). On the basis of HPLC analysis of the solutions, we found no changes in the profile of Boc-Cys-(Npys)-OH for 1 day at RT (data not shown). These results indicate that the Npys group is stable under conditions applicable for Boc-Cys(Npys)-OPfp modification of polymeric polypeptides.

In the next set of experiments, the protected and activated cysteine residue was reacted with the branched polymers in the molar ratio of 0.25−1.5 mol/mol. The

average degree of substitution values is summarized in Table 2. These values were calculated from the UV absorption of the Npys chromophore containing polymers at $\lambda = 350$ nm using ϵ determined under conditions used for analysis. These values were corrected by the conjugate content of the same solution measured by amino acid analysis. The difference between these figures might be due to the water content of freeze-dried samples. The data of Table 2. indicate that the increase of input Boc-Cys(Npys)-OPfp/polymer ratio resulted in higher substitution in case of AK polymer. The application of 1:1 molar ratio was sufficient to achieve almost complete incorporation of Boc-Cys(Npys). Similar tendency was observed with EAK containing Glu at the end of the branches. But in this case, only 54% of the side chains could be modified even at 1.5 mol/mol input value. In contrast, the reaction between Boc-Cys(NPys)-OPfp and SAK resulted in only a small elevation of averge degree of substitution after increasing the input ratio from 0.25 to 0.75, and no further changes could be observed at 1.0 mol/mol ratio. Even more limited modification was detected with TAK polymer at the same input value. On the basis of the highest substitution figures achieved in these experiments, the following order could be established: AK (100%) > EAK (54%) > SAK (27%). The fairly low values for EAK and SAK could be indicative for the presence of noncovalent interactions (e.g., ionic- and H-bond) between side chains of the polymers. In case of EAK, the ionic forces between the α-amino group of the side-chain terminal Glu and the γ-COOH group of the neighboring, one could produce protonated amino groups and prevent the effective coupling of Boc-Cys(Npys)-OPfp to the side chains. The conformational properties, namely the tendency to form ordered structure of Ser-containing polymer indicated the presence of H-bond interaction between the side-chain terminals (*27*). In case of the other OH-amino acid containing polymer (TAK) where the incorporation of Boc-Cys(Npys) residues might be hindered not only by H-bonds but also by the bulky side chain of threonine.

Addition of MeOH to the reaction mixture or substitution of DMF by MeOH decreased the average degree of substitution of polymers studied. In case of SAK, these values were 25% in MeOH-DMF-water (45%−45%−10%) and 22% in MeOH-water (90%−10%) at 1:1 input molar ratio.

Epitope peptides elongated by Cys at their N-terminal were conjugated either to Boc-Cys(Npys)-polymers or to NH_2−Cys(Npys)-polymers. In the latter case, the N-protecting group was removed with 95% TFA/water in the presence or absence of scavangers. We found that the addition of 5% *p*-cresol or thioanisole to the cleavage mixture had no influence on the disulfide bond between SH of Cys and Npys moiety. Similarly no changes in the UV spectrum of the polymer could be observed after prolonged (2 days) dialysis of the N-deprotected polymer in 0.1% acetic acid solution. It should be noted that decrease of absorbance was detected when the dialysis was performed against distilled water.

For the conjugation of epitope peptides of HSV gD-1 (H-^{276}SALLEDPVG^{284}-NH_2) and two adenovirus proteins [H-^{148}FIGTNINKDNGI^{159}-NH_2 (AD41), H-^{309}THLSYK-PGKGDE^{320}-NH_2 (AD2) and H-^{399}NQAVDSYDPD^{408}-NH_2 (AD2)], two Cys(Npys)-polymers were selected. Both compounds (CAK and CSAK) were used in their Boc-protected forms as well as with free NH_2 groups at the end of the side chains, and the average degree of SH-peptide substitution was compared (Table 3). In the first set of experiments, polymer polypeptides with Boc-protecting groups were dissolved in DMF and reacted with SH-epitope peptides. In case of CAK, the average degree of substitution observed with three peptides of different composition and sequence was very low (6−7%). This indicates that the introduction of SH-peptides might not be favored in Boc-CAK containing Boc-Cys(Npys) residue at all of the side-chain terminal (100% substitution). In case of CSAK polymer, the SH-peptide incorporation was significantly higher in DMF (18%) or in DMF-0.06 M phosphate buffer (pH 5.5) mixture (25%). It should be noted that Boc-CSAK possesses Boc-Cys(Npys) moiety only on 24% of the branches. The comparison of data obtained with Boc-CAK and Boc-CSAK suggests that the steric hindrance caused by the presence of the large number of Boc-Cys(Npys) at the end of the side chains might prevent the incorporation of SH-peptides into polymers.

In case of CSAK containing no Boc-blocking groups, the average degree of SH-peptide incorporation was in the same range as compared to Boc-CSAK (Table 3). However, much higher average degree of substitution could be achieved with CAK polypeptide (16−52%) than with that of Boc-CAK (6−7%). These data underline that the removal of Boc-protecting group from a highly Boc-Cys(Npys) modified polypeptide (Boc-CAK vs CAK) could improve the disulfide exchange reaction between the polymer and SH-peptides. At the same time, there are no significant differences in reactivity of Cys(Npys)-polymers with or without Boc-group, when the average degree of Cys(Npys) content of the polymer is relatively modest (Boc-CSAK vs CSAK) (Figure 2).

We have also prepared a conjugate in which a 25-residue peptide is attached to Cys(Npys) polymers (Figure 2). The peptide was designed to combine two different epitopes of gD-1 HSV (^{9}LKNleADPNRFRGKDL^{22} and ^{276}SALLEDPVG^{284}) by a dipeptide spacer containing ϵ-aminocaproic acid residue (Acp) and Cys. In this dipeptide sequence, Acp connected the N^{α}-amino goup of C^{276}SALLEDPVG^{284} with the C^{α} -carboxy group of Leu residue in peptide ^{9}LKNleADPNRFRGKDL^{22}. Thiol group of Cys in the inner part of hybrid peptide containing two epitopes was used for conjugation to branched polypeptide. It has been demonstrated that the 9−21 sequence contains overlapping T- and B-cell epitopes (*21*, *22*), while 276−284 represents a B-cell epitope of the same protein (*21*). Immunization with protein (KLH or BSA) conjugates of peptide 8−23 (*33*) or Nle^{11}-substituted peptide 9−21 (*34*) produced high titer antibody response with virus neutralizing activity. We have also demonstrated that branched chain polypeptide conjugates either of peptide 276−284 or peptide 1−23 resulted in minimum 50% survival of mice infected by lethal dose of HSV virion (*23*, *35*). On the basis of these considerations, the 25-mer peptide was coupled in 0.06 M phosphate buffer to unprotected form of CAK and CSAK polymers containing 27 and 24% of Cys(Npys), respectively. In these cases, the average degree of substitution of branched polymers with the 25-mer peptide having Cys residue in central position was 26% in case of CAK and 20% in case of CSAK (Table 3). These data show that the use of AK- and SAK-based polymers with relatively low Cys(Npys) content (24−27%) results in epitope peptide conjugates in which almost all Npys group of the polymer could be replaced by the reaction of SH-peptides.

CONCLUSION

In this paper, a new method is described for the conjugation of branched polypeptides with cysteine con-

taining oligopeptides via Cys(Npys). We found that the efficacy of the incorporation of Cys(Npys) residue to the polymers using Boc-Cys(Npys)-OPfp depends mainly on the identity of the branch terminating amino acid, but the input molar ratio is also important. Bulky side chain of the N-terminal amino acid in the branches and/or its ability to participate in noncovalent interactions (ionic-, H-bond) decrease the incorporation of cysteine residue into the polymers. Cys(Npys)-modified polymers react effectively with cysteine-containing oligopeptides in a disulfide exchange reaction. However, the average degree of substitution was not increased significantly by elevation of the number of Cys(Npys) residue on the polymers. This suggests that determination of the optimal density of the reacting, namely Cys(Npys) group on the branched polypeptide is a crucial factor for the design and preparation of conjugates. Procedure described in this communication provides a new effective route for the conjugation through disulfide bond, which does not decrease the solubility of the carriers or the conjugates. In addition, our data suggest that this synthetic route is suitable for incorporation not only small oligopeptides with N-terminal SH groups but also relatively large peptide (25-mer) possessing Cys residue at the central region of the peptide.

ACKNOWLEDGMENT

These studies were supported by grants from the Hungarian Research Fund (OTKA T-14964 and T-03838), from the Hungarian Spanish Intergovernmental Program (5/1998) and from the Hungarian Ministry of Culture (MKM No. 0101/1997)

LITERATURE CITED

(1) Poznansky, M. J., and Cleland, L. G. (1980) Biological macromolecules as carriers of drugs and enzymes. In *Drug Delivery Systems* (R. L. Juliano, Ed.) pp 253−315, Oxford University Press, U.K..

(2) Seymour, L. W. (1992) Passive tumor targeting of soluble macromolecules and drug conjugates. *CRC Crit. Rev. Ther. Drug. Carrier Sys. 9,* 135−187.

(3) Maeda, H., and Matsumura, Y. (1989) Tumorotropic and lymphotropic principles of macromolecular drugs. *CRC Crit. Rev. Ther. Drug Carrier Sys. 6,* 193−210.

(4) Ringsdorf, H. (1975) Structure and properties of pharmacologically active polymers. *J. Polym. Sci. Polymer Symp. 51,* 135−153.

(5) Müller, G. M., Shapira, M., and Arnon, R. (1982) Anti-influenza response achived by immunization with a synthetic conjugates. *Proc. Natl. Acad. Sci. U.S.A. 79,* 5042−5046.

(6) Neurath, A. R., Kent, S. B. H., and Strick, N. (1982) Specificity of antibodies elicited by a synthetic peptide having a sequence in common fragment of virus protein the Hepatitis B surface antigen. *Proc. Natl. Acad. Sci. U.S.A. 79,* 7871−7875.

(7) Dean, C., Claassen, E., Gerritse, K., Zegers, N. D., and Boersma, W. J. A. (1990) A novel carbodiimide coupling method for synthetic peptides. *J. Immunol. Methods 129,* 119−125.

(8) Atassi, M. Z., Kazim, A. L., and Sakata, S. (1981) High yield coupling of peptides to protein carriers. *Biochim. Biophys. Acta 670,* 300−302.

(9) Mezö, G., Mezö, I., Pimm, M. V., Kajtár, J., Seprödi, A., Teplán, I., Kovács, M., Vincze, B., Pályi, I., Idei, M., Szekerke, M., and Hudecz, F. (1996) Synthesis, conformation, biodistribution and hormon related in vitro antitumor effect of GnRH antagonist branched polypeptide conjugate. *Bioconjugate Chem. 7,* 642−650.

(10) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein−protein conjugation. N−Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem. J. 173,* 723−728.

(11) Matsueda, R., and Walter, R. (1980) 3-nitro-2-piridine-sulfenyl (Npys) group. *Int. J. Pept. Protein Res. 16,* 392−401.

(12) Matsueda, R., Kimura, T., Kaiser, E. T., and Matsueda, G. R. (1981) 3-nitro-2-pyridinesulfenyl group for protection and activation of the thiol function of cystein. *Chem. Lett.* 737−740.

(13) Drijfhout, J. W., Perdijk, E. W., Weijer, W. J., and Bloemhoff, W. (1988) Controlled peptide−protein conjugation by means of 3-nitro-2-pyridinesulfenyl protection-activation. *Int. J. Pept. Protein Res. 32,* 161−166.

(14) Ponsati, B., Giralt, E., and Andreu, D. (1989) A synthetic strategy for simultaneous purification-conjugation of antigenic peptides. *Anal. Biochem. 11,* 389−395.

(15) Albericio, F., Andreu, D., Giralt, E., Navalpotro, C., Pedroso, E., Ponsati, B., and Ruiz-Gayo M. (1989) Use of the Npys thiol protection in solid-phase peptide synthesis. *Int. J. Pept. Protein Res. 34,* 124−128.

(16) Hudecz, F., and Szekerke, M. (1984) Synthesis of new branched polypeptides with poly(lysine) backbone. *Collect. Czech. Chem. Commun. 50,* 103−113.

(17) Mezö, G., Kajtár, J., Hudecz, F., and Szekerke, M. (1993) Carrier design: Conformational studies of amino acid (X) and oligopeptide (X-DL-Ala_m) substituted poly(L-lysine). *Biopolymers 33,* 873−885.

(18) Hudecz, F., and Price, M. R. (1992) Monoclonal antibody binding to peptide epitopes conjugated to synthetic branched polypeptide carriers. Influence of the carrier upon antibody recognition. *J. Immunol. Methods 147,* 201−210.

(19) Wilkinson, K. A., Vordermeier, M. H., Wilkinson, R., Iványi, J., and Hudecz, F. (1998) Synthesis and in vitro T cell immunogenicity of conjugates with dual specificities: attachment of epitope peptides of 16 kDa and 38 kDa proteins from M.tuberculosis to branched polypeptide. *Bioconjugate Chem. 9,* 539−547.

(20) Hudecz, F., Hilbert, Á., Mezö, G., Kajtár, J., and Rajnavölgyi, É. (1994) B-cell epitopes in Herpes simplex Virus-(HSV-1) glycoprotein D (gD). In *Synthetic peptides in the search for B-and T-cell epitopes* (Rajnavölgyi, É., Ed.) pp 157−169, R. G. Landes Company, Austin.

(21) Dietzschold, B., Eisenberg, R. J., Ponce deLeon, M., Golub, E., Hudecz, F., Varrichio, A., and Cohen, G. H. (1984) Fine structure analysis of type-specific and type common antigens sites of Herpes simplex virus glycoprotein D. *J. Virol. 52,* 431−435.

(22) Heber-Katz, E., Hollósi, M., Dietzschold, B., Hudecz, F., and Fasman, G. (1985) The T cell response to the glycoprotein D of the Herpes simplex virus: The significance of antigen conformation. *J. Immunol. 135,* 1385−1390.

(23) Hudecz, F., Hilbert, Á., Mezö, G., Mucsi, I., Kajtár, J., Bösze, Sz., Kurucz, I., and Rajnavölgyi, É. (1993) Epitope mapping of 273−284 region of HSV glycoprotein D by synthetic branched polypeptide carrier conjugates. *Pept. Res. 6,* 263−271.

(24) Ádám, É., Nász, I., Hudecz, F., Lengyel, A., Mezö, G., and Dobay, O. (1998) Characterisation of intertype specific epitopes on adenovirus hexone. *Arch. Virol. 142,* 1669−1682.

(25) IUPAC−IUB Commission on Biochemical Nomenclature. (1972) *Biochem. J. 127,* 753−756.

(26) IUPAC−IUB Commission on Biochemical Nomenclature. (1984) *Eur. J. Biochem. 138,* 9−37.

(27) Mezö, G., Kajtár, J., Nagy, I., Szekerke, M., and Hudecz, F. (1997) Carrier design: Synthesis and conformational studies of poly(L-lysine) based branched polypeptides with hydroxyl groups in the side chains. *Biopolymers 42,* 719−730.

(28) Mezö, G., Votavova, H., Hudecz, F., Kajtár, J., Sponar, J., and Szekerke, M. (1988) Conformation of branched polypeptides: The influence of DL-alanine oligomer spacers in the side chains. *Collect. Czech. Chem. Commun. 53,* 2843−2853.

(29) Hudecz, F., Kovács, P., Kutassi-Kovács, S., and Kajtár, J. (1984) GPC, CD and sedimentation analysis of poly-Lys and branched chain poly-Lys-poly-DL-Ala polypeptides. *Colloid Polym. Sci. 262,* 208−212.

(30) Matsueda, R., Higashida, S., Albericio, F., and Andreu, D. (1992) Compatibility of the S−(3-nitro-2-pyridinesulfenyl) protecting group with DCC/HOBt coupling chemistry. *Pept. Res. 5,* 262−264.

(31) Simmonds, R. G., Tupper, D. E., and Harris, J. R. (1994) Synthesis of disulfide-bridged fragments of omega-conotoxins GVIA and MVIIA. Use of Npys as a protecting/activating group for cysteine in Fmoc syntheses. *Int. J. Pept. Protein Res. 43*, 363−366.

(32) Kisfaludy, L., and Schön, I. (1983) Preparation of pentafluorophenyl esters of 9-fluoromethyloxycarbonyl amino acids for peptide synthesis. *Synthesis 1983*, 572−574.

(33) Dietzschold, B., Heber-Katz, E., Hudecz, F., Hollósi, M., Fasman, G., Eisenberg, R. J., and Cohen, G. H. (1985) In *Vaccines 85* (R. A. Lerner, R. M. Chanock, and F. Brown, Eds.) pp 227−234, Cold Spring Harbor Laboratory, Plainview, NY.

(34) Weijer, W. J., Drijfhout, J. W., Geerligs, H. J., Bloomhoff, W., Feijlbrief, M., Bos, C. A., Hoogerhout, P., Kerling, K. E. T., Popken-Boer, T., Slopsema, K., Wilterdrink, J. B., Welling, G. W., and Welling-Wester, S. (1988) Antibodies against synthetic peptides of Herpes simplex virus type-1 glycoprotein D and their capability to neutralize viral infectivity in vitro. *J. Virol. 62*, 501−510.

(35) Hilbert, Á., Hudecz, F., Mezö, G., Mucsi, I., Kajtár, J., Kurucz, I., Gergely, J., and Rajnavölgyi, É. (1994) The influence of branched polypeptide carriers on the immunogenicity of predicted epitopes of HSV-1 glycoprotein D. *Scand. J. Immunol. 40*, 609−617.

BC990116O