

Synthesis and Antibody Recognition of Cyclic Epitope Peptides, Together with Their Dimer and Conjugated Derivatives Based on Residues 9–22 of Herpes Simplex Virus Type 1 Glycoprotein D

Annamária Jakab,[†] Gitta Schlosser,[‡] Matty Feijlbrief,[§] Sytske Welling-Wester,[§] Marilena Manea,^{||} Miquel Vila-Perello,[⊥] David Andreu,[⊥] Ferenc Hudecz,^{†,‡} and Gábor Mező^{*†}

Research Group of Peptide Chemistry, Hungarian Academy of Sciences, and Department of Organic Chemistry, Eötvös L. University, P.O. Box 32, 1518 Budapest 112, Hungary, Laboratory for Medical Microbiology, Universiteit Groningen, 9713 GZ Groningen, The Netherlands, Laboratory of Analytical Chemistry and Biopolymer Structure Analysis, and Zukunftskolleg, University of Konstanz, 78457 Konstanz, Germany, and Department of Experimental and Health Sciences, Pompeu Fabra University, 08028 Barcelona, Spain. Received July 29, 2008; Revised Manuscript Received February 3, 2009

The synthesis of new cyclic peptides comprising the 9–22 epitope ⁹LKMADPNRFRGKDL²² sequence derived from HSV gD-1 is reported. In addition, we describe procedures for the preparation of cyclic peptide dimers and conjugates with an oligo(tyrosine) derivative carrier. The binding of a monoclonal antibody, Mab A16, to the synthesized compounds was determined by enzyme-linked immunosorbent assay. It was demonstrated that cyclization decreased the binding activity of the antibody to the epitope. However, dimerization and conjugation could significantly increase the binding capacity of the cyclic epitope peptides. The attachment site in dimers and conjugates, as well as the topology of the construct, had a significant influence on the antibody recognition, while replacement of Met in position 11 by Nle had no marked effect.

INTRODUCTION

The application in human therapy of conventional vaccines consisting of either killed or live attenuated disease-causing organisms has some limitations such as the loss of efficacy due to the genetic variation of many viruses; difficulties in the production and storage of many vaccine preparations; and biohazard in both production and use of vaccines against lethal infections. To overcome these drawbacks, synthetic peptide-based vaccines have been developed; and they have advantages of being selective, chemically defined, and safe and can be prepared in large quantities (1, 2). However, it is known that the administration of a synthetic epitope peptide rarely elicits an appropriate immune response. Peptide epitopes are generally short and are not able to adopt the same conformation as in the native protein; in addition, they are subjected to fast *in vivo* degradation by proteases (3). In many cases, antibodies to synthetic peptides react strongly with the homologous peptides, but weakly or not at all with the original protein or the intact infectious agents (4). Several approaches have been developed in order to modify the immunorecognition of synthetic epitope peptides such as (i) incorporation in the same construct of both B- and helper T-cell epitopes (5); (ii) cyclization (6); (iii) conjugation of multiple copies of epitopes to synthetic or natural carriers (7); and (iv) dimerization or controlled polymerization of the peptide epitope (8, 9).

Herpes simplex virus, with two closely related serotypes (HSV-1 and HSV-2), is one of the most common infectious agents in humans. Glycoprotein D (gD) represents a major immunogenic component of the virion envelope (10), able to induce high titers of virus-neutralizing antibodies (11, 12). The *N*-terminal region of HSV gD-1, comprising residues 1–21 of mature gD, is known to be a strong antigenic site, and peptides from this domain are able to induce both B- and T-cell responses (13). In addition, gD is required for viral entry into the cells. gD can bind to at least three distinct cellular receptor proteins. These three receptors are herpes virus entry mediator (HVEM), nectin 1 and 2, and a modified form of heparan sulfate. The *N*-terminal domain of gD is the binding site to the HVEM receptor. The crystal structure of the gD-HveA complex revealed that, upon binding to the cellular receptor HVEM, the flexible *N*-terminal region of gD is folded into a fixed hairpin structure that contains the residues of gD required for binding to HVEM. Monoclonal antibody (Mab) A16 can inhibit the binding of gD to the cellular receptor HVEM. A16 monoclonal antibody was also used for selection of peptides from a random peptide library (RPL) displayed on the surface of a filamentous bacteriophage. The selected RPL-derived peptide and peptide 9–19 of gD-1 had approximately the same affinity for Mab A16. Immunization of mice with the phage-derived RPL-peptide as well as the gD-1 derived peptide (after conjugation to BSA) protected against a challenge with a lethal dose of herpes simplex virus type 1 (14).

Peptide 9–21 (LKMADPNRFRGKD) was shown to be the optimal epitope of the *N*-terminal part of gD for Mab A16, in which amino acids ¹³Asp, ¹⁶Arg, and ¹⁷Phe are essential for Mab A16 recognition (15). The 9–21 peptide exhibited good antibody recognition in linear form, either free or conjugated to carriers. It was further demonstrated that the peptide could adopt a β -turn-like structure at residues ¹⁴Pro and ¹⁵Asn under appropriate conditions (16–18), leading to the hypothesis that cyclization might stabilize the bioactive conformation of the peptide and thus increase its antibody recognition. Cyclization might also mimic the fixed hairpin structure of gD needed for

* Address correspondence to Gábor Mező, Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, 1518 Budapest 112, P.O. Box 32, Hungary. E-mail: gmezo@elte.hu; Tel.: (+36)-1-209-0555/1433; Fax: (+36)-1-372-2620.

[†] Research Group of Peptide Chemistry, Hungarian Academy of Sciences.

[‡] Department of Organic Chemistry, Eötvös L. University.

[§] Universiteit Groningen.

^{||} University of Konstanz.

[⊥] Pompeu Fabra University.

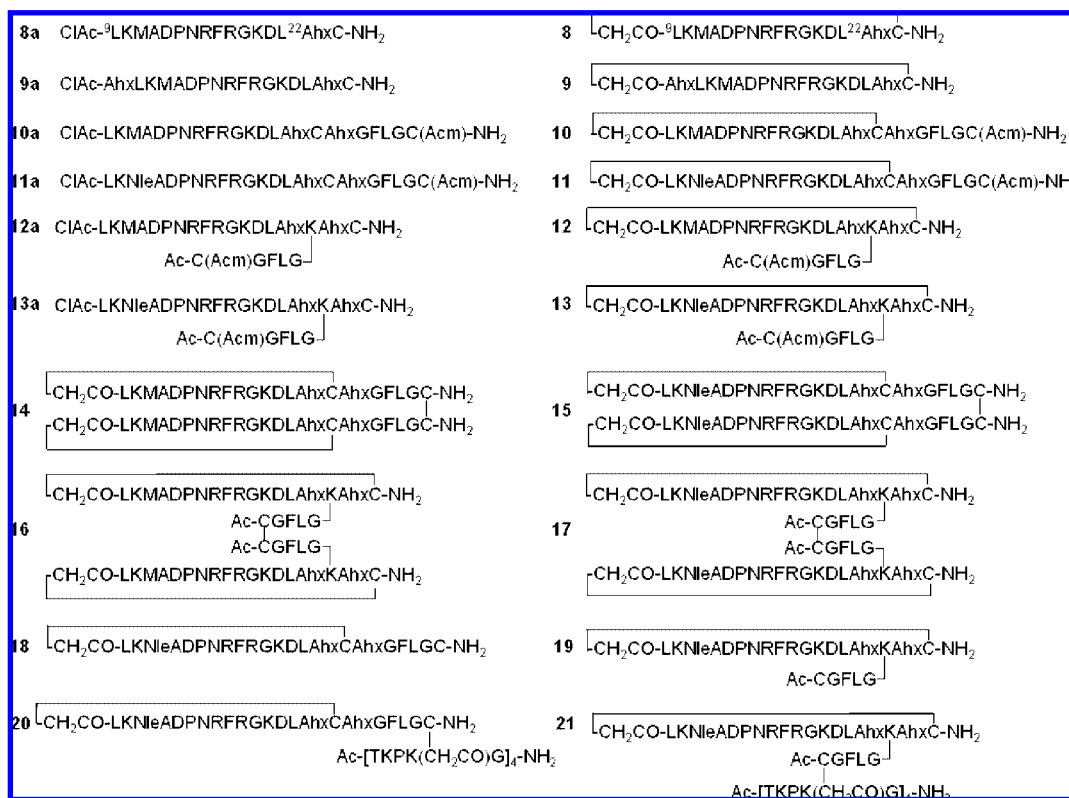


Figure 1. Chemical structure of cyclic epitope peptides (8–13, 18, and 19) derived from HSV gD-1, their precursors (8a–13a), dimer (14–17), and conjugated (20, 21) derivatives.

binding to the HVEM receptor. In addition, cyclization might enhance the stability of the peptide against enzymatic degradation. However, for peptide 9–21 no effective cyclic version has yet been described (19).

In previous studies, we demonstrated that replacement of ^{11}Met in peptide 9–22 by homocysteine (Hcy) or cysteine (Cys) and replacement of ^{18}Arg by Lys made cyclization possible through a thioether bond between a chloroacetyl group at the ϵ -amino group of Lys and the thiol group of Cys or Hcy. The cyclic peptides (H-LK[XADPNRFK(CH₂CO)]GKDL-NH₂, where X = Cys or Hcy) thus obtained bound to the Mab A16 with three magnitudes lower efficiency than the linear analogues (19) and the Hcy cyclic peptide was more potent in competition ELISA than the Cys-containing version. In another study (20), the conjugation of linear (^{11}Nle)-9–22 peptides (replacement of Met by norleucine (Nle) in position 11 is permitted (21)) to carriers such as MAP-type lysine dendrimer (22), sequential oligopeptide carrier (SOC) (23), oligotuftsins derivative (OT20) (24), branched-chain SAK polypeptide with polylysine backbone (25), or the widely used protein keyhole limpet hemocyanine (KLH) (26) was performed. Results showed that the conjugates bound to Mab A16 by 1 order of magnitude more strongly than the linear epitope peptides, regardless of the carrier used. The disulfide dimers of peptide 9–22, either C- or N-terminally linked, also showed improved binding compared to the linear peptide, similarly to peptide 9–22 conjugated to oligotuftsins (27).

In line with the above-mentioned results, we describe here a new series of linear, cyclic, dimeric, and conjugated derivatives of the HSV gD-1 9–22 epitope. Large ring-size cyclic peptides (16 instead of 8 residues) derived from 9–22 epitope peptide of HSV gD-1 and their dimeric versions, as well as conjugates

to OT20 carrier, were prepared, and their antigenicity was determined by enzyme-linked immunosorbent assay using Mab A16.

EXPERIMENTAL PROCEDURES

Materials. All amino acid derivatives were purchased from Senn Chemicals AG (Dielsdorf, Switzerland) or NovaBiochem (Läufelfingen, Switzerland). Rink-amide MBHA and 4-methylbenzhydrylamine (MBHA) resins were from NovaBiochem. Coupling agents (*N,N'*-diisopropylcarbodiimide (DIPCDI), 1-hydroxybenzotriazole (HOBt), *N*-ethyl-diisopropylamine (DIPEA)), cleavage reagents (trifluoroacetic acid (TFA), hydrogen fluoride (HF), piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)), and scavengers (triisopropylsilane (TIS), *m*-cresol, *p*-thiocresol) were from Fluka (Buchs, Switzerland) or SDS (Peypin, France), except for *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), which was from Matrix Innovation (Montreal, Canada). Tallyum(III) trifluoroacetate (Tl(tfa)₃) and 1,4-DL-dithiothreitol (DTT) were Fluka products, while acetic anhydride (Ac₂O) was purchased from Reanal (Budapest, Hungary). Solvents (dichloromethane (DCM), *N,N*-dimethylformamide (DMF), diethyl ether, methanol (MeOH), and acetic acid (AcOH)) for synthesis were obtained from SDS and Reanal. Acetonitrile for HPLC was from SDS or Sigma (Sigma-Aldrich Kft., Budapest, Hungary). Peptides 1–7 were synthesized as previously reported (19, 20, 27).

Synthesis of Linear Precursor Peptides (Peptides 8a–11a; Figure 1). Linear precursor peptides 8a–11a were synthesized by solid-phase methodology on Rink amide MBHA resin using an ABI 433A synthesizer (FAST-MOC, 0.1 mmol scale chemistry). Eight molar excess of Fmoc-amino acids were used in HBTU-HOBt-DIPEA-mediated coupling reactions. Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(O^tBu)-OH, and Fmoc-Asn(Trt)-OH were applied as side chain protected deriva-

Scheme 1. Outline of the Synthesis of Cyclic Epitope Peptides with Spacer in the Branch

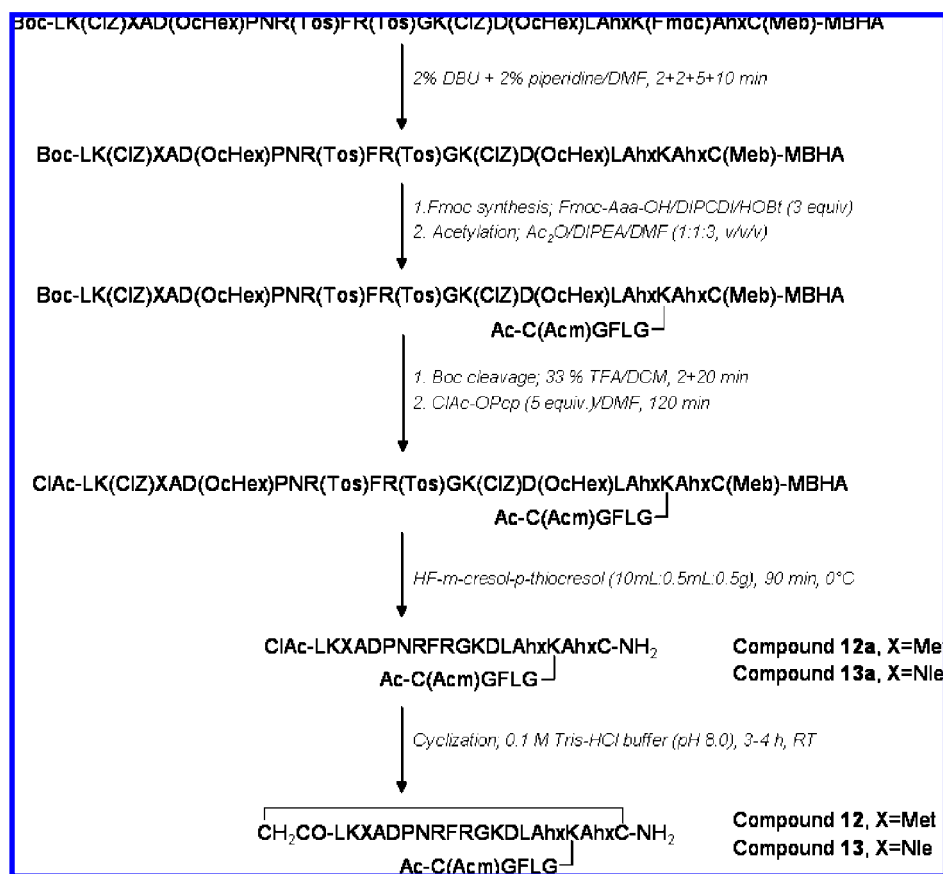


Table 1. ESI-MS Characteristics and RP-HPLC Retention Times of Derivatives of the Epitope Peptide 9–22 of HSV gD-1

| compound ^a | ESI-MS ^b | | HPLC ^c R _t [min] |
|-----------------------|---------------------|---------|---|
| | calcd | exp | |
| 8 | 1914.99 | 1914.8 | 24.1 |
| 9 | 2028.08 | 2028.3 | 24.8 |
| 10 | 2578.12 | 2578.0 | 27.3 |
| 11 | 2560.08 | 2560.1 | 27.7 |
| 12 | 2748.33 | 2748.2 | 27.5 |
| 13 | 2730.29 | 2730.0 | 27.8 |
| 14 | 5012.07 | 5012.4 | 30.1 |
| 15 | 4976.00 | 4976.0 | 30.4 |
| 16 | 5352.50 | 5352.1 | 29.1 |
| 17 | 5316.42 | 5315.8 | 29.4 |
| 18 | 2489.05 | 2489.2 | 29.3 |
| 19 | 2659.22 | 2658.6 | 28.4 |
| 20 | 12222.25 | 12222.3 | 28.8 |
| 21 | 12902.52 | 12902.2 | 27.8 |

^a The numbers of the compounds correspond to the structures presented in Figure 1. ^b Experimental MWs were calculated from the multiple charged ions measured by a Bruker Daltonics Esquire 3000+ mass spectrometer. ^c RP-HPLC conditions: Column, Phenomenex Jupiter C₁₈ (250 × 4.6 mm, 5 μm, 300 Å); gradient, 0 min 0% B; 5 min 0% B; 50 min 90% B; eluents, 0.1% TFA in water (A) and 0.1% TFA in acetonitrile–water (80:20, v/v) (B); flow rate, 1 mL/min; detection, λ = 214 nm.

tives. The side chain of the Cys residue involved in the cyclization was protected by a trityl group (Trt). In peptides **10a** and **11a**, the thiol group of the additional (C-terminal) Cys used for dimerization or conjugation was protected by acetylamidomethyl group (Acm). After chain assembly and removal of the N-terminal Fmoc protecting group, the free amino terminus was chloroacetylated with 5 equiv of chloroacetic acid pentachlorophenyl ester (ClAc-OPcp) (**20**). The peptides were cleaved from the resin with TFA-TIS-water (95:2.5:2.5, v/v/v)

at room temperature for 2 h. The crude peptides were precipitated with chilled anhydrous diethyl ether, isolated by centrifugation, redissolved in 10% acetic acid, and freeze-dried. Prior to cyclization, they were purified by semipreparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS.

Synthesis of Branched Precursor Peptides (Peptides 12a and 13a; Figure 1 and Scheme 1). The branched peptides were prepared on MBHA resin (0.62 mmol/g) by manual SPPS applying a mixed Boc-Fmoc strategy. Linear peptides with Met or Nle in position 11, elongated with the Ahx-Lys-Ahx-Cys tetrapeptide (where Ahx is ε-aminohexanoic acid) at the C-terminus, were built up by Boc chemistry using Boc-Arg(Tos)-OH, Boc-Lys(CIZ)-OH, Boc-Asp(OcHex)-OH, and Boc-Cys(Meb)-OH as side chain protected derivatives. The ε-amino group of Lys in the C-terminal extension as a future branching site was blocked with an Fmoc group. The protocol of the Boc synthetic cycle was the following: (i) deprotection with 40% TFA/DCM (2 + 20 min); (ii) DCM washing (5 × 0.5 min); (iii) neutralization with 5% DIPEA/DCM (4 × 1 min); (iv) DCM washing (4 × 0.5 min); (v) coupling of 3 equiv Boc-amino acid derivative/DIPCDI/HOBt in DCM-DMF 4:1 (v/v) solvent mixture (60 min); (vi) DMF washing (2 × 0.5 min); (vii) DCM washing (2 × 0.5 min); and (viii) monitoring by ninhydrin (**28**) or isatin assay (**29**). After completion of the synthesis of the linear peptide and prior to the Boc cleavage from the N-terminus, the peptide was further developed in the branch by Fmoc strategy. Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, and Fmoc-Cys(Acm)-OH were built in step by step, manually. The protocol of Fmoc chemistry was the following: (i) DMF washing (3 × 1 min); deprotection with 2% DBU + 2% piperidine in DMF (4 times for 2 + 2 + 5 + 10 min); (iii) DMF washing (8 × 0.5 min); (iv) coupling of 3 equiv Fmoc-amino acid derivative-DIPCDI-HOBt in DMF; (v) DMF washing (3 × 0.5 min); (vi) DCM washing (2 × 0.5 min);

Scheme 2. Outline of the Synthesis of Dimer and Conjugated Derivatives of Cyclic Epitope Peptide of 9–22 Sequence of HSV gD-1

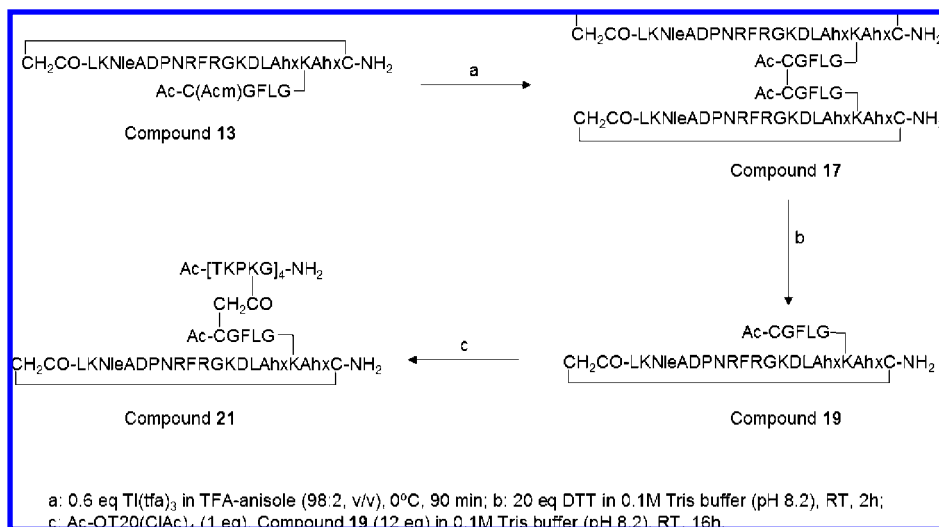


Table 2. Binding of Derivatives of Epitope Peptide 9–22 of HSV gD-1 to Mab A16

| code ^a | compound | competition ELISA ^b |
|-------------------|--|--------------------------------|
| 1 ^d | H-LKMADPNRFRGKDL-NH ₂ | 4.2 |
| 2 ^d | H-LKNleADPNRFRGKDL-NH ₂ | 3.4 ^e ± 1.8 |
| 3 ^e | H-LK[CADPNRFK(CH ₂ CO)]GKDL-NH ₂ | 7900 |
| 4 ^e | H-LK[HcyADPNRFK(CH ₂ CO)]GKDL-NH ₂ | 2300 |
| 5 ^d | H-[TKPK(H-LKNleADPNRFRGKDL{CH ₂ CO}-NH ₂)G] ₄ -NH ₂ | 0.72 |
| 6 ^d | {H-LKNleADPNRFRGKDL-NH ₂ } ₂ | 0.74 |
| 7 ^f | {H-CLKNleADPNRFRGKDL-NH ₂ } ₂ | 0.66 |
| 8 | c[CH ₂ CO-LKMADPNRFRGKDLAhxC]-NH ₂ | 28.6 |
| 9 | c[CH ₂ CO-AhxLKMADPNRFRGKDLAhxC]-NH ₂ | 28.0 |
| 10 | c[CH ₂ CO-LKMADPNRFRGKDLAhxC]AhxGFLGC(Acm)-NH ₂ | 79.1 |
| 11 | c[CH ₂ CO-LKNleADPNRFRGKDLAhxC]AhxGFLGC(Acm)-NH ₂ | 75.6 |
| 12 | c[CH ₂ CO-LKMADPNRFRGKDLAhxK(Ac-C(Acm)GFLG)AhxC]-NH ₂ | 88.8 |
| 13 | c[CH ₂ CO-LKNleADPNRFRGKDLAhxK(Ac-C(Acm)GFLG)AhxC]-NH ₂ | 92.3 |
| 14 | {c[CH ₂ CO-LKMADPNRFRGKDLAhxC]AhxGFLGC-NH ₂ } ₂ | 11.8 |
| 15 | {c[CH ₂ CO-LKNleADPNRFRGKDLAhxC]AhxGFLGC-NH ₂ } ₂ | 11.1 |
| 16 | {c[CH ₂ CO-LKMADPNRFRGKDLAhxK(Ac-CGFLG)AhxC]-NH ₂ } ₂ | 57.7 |
| 17 | {c[CH ₂ CO-LKNleADPNRFRGKDLAhxK(Ac-CGFLG)AhxC]-NH ₂ } ₂ | 41.4 |
| 18 | c[CH ₂ CO-LKNleADPNRFRGKDLAhxC]AhxGFLGC-NH ₂ | nt |
| 19 | c[CH ₂ CO-LKNleADPNRFRGKDLAhxK(Ac-CGFLG)AhxC]-NH ₂ | nt |
| 20 | Ac-[TKPK(18-CH ₂ CO)G] ₄ -NH ₂ | 249.3 |
| 21 | Ac-[TKPK(19-CH ₂ CO)G] ₄ -NH ₂ | 7.2 |

^a Compound numbers in the Table correspond to the compounds presented in Figure 1. ^b Pmol of the compound required to obtain a residual OD₄₉₀ of 1.0 at a coat of peptide 9–22 (Arg), after preincubation of serial dilutions of the compounds and Mab A16 (at “optimal” dilution). nt; not tested. ^c Peptide (¹⁵Nle) 9–22 (code 2) was included in all the competition ELISA assays performed. The amount required to obtain an OD₄₉₀ of 1.00 was 3.4 ± 1.8 pmol. ^d Ref 20. ^e Ref 19. ^f Ref 27.

and (vii) monitoring by ninhydrin assay. In the end, the Fmoc group was detached and the *N*-terminus of the branch was acetylated with Ac₂O/DIPEA/DMF (1:1:3, v/v/v) mixture. Finally, the Boc group was removed, and the free α-amino group was chloroacetylated with 5 equiv ClAc-OPcp in DMF. The peptide was cleaved from the dried resin simultaneously with the removal of the side chain protecting groups with liquid HF in the presence of *m*-cresol and *p*-thiocresol as scavengers (10 mL HF, 0.5 mL *m*-cresol, and 0.5 g *p*-thiocresol) at 0 °C for 90 min. The crude product was precipitated with dry diethyl ether. The solid material was filtered off, washed three times with diethyl ether, and then dissolved in 10% acetic acid prior to freeze-drying. The target peptide was obtained after purification by semipreparative RP-HPLC and characterized by analytical HPLC and mass spectrometry (MS) before cyclization.

Synthesis of Cyclic Epitope Peptides (Compounds 8–13; Figure 1 and Scheme 1). Cyclization of chloroacetylated linear epitope peptides by thioether bond formation was performed in 0.1 M Tris-HCl buffer, pH 8.0. The lyophilized solid material was added to the buffer solution (20 mL, 5 mg/mL final peptide concentration, in each case) in portions over 1 h. Then, the

reaction was continued in a closed tube at RT for 3 h, and the cyclization reaction was monitored by analytical RP-HPLC. The reaction mixture was acidified with TFA and purified directly by semipreparative RP-HPLC. The yield of the cyclization was over 90% for each construct. The compounds were characterized by analytical RP-HPLC and ESI-MS (Table 1).

Dimerization of Cyclic Epitope Peptides (Compounds 14–17; Figure 1 and Scheme 2). Intermolecular disulfide bond formation was performed with $\text{Ti}(\text{tfa})_3$ in TFA (30). The cleavage mixture contained 98% TFA, 2% anisole as a scavenger, and 0.6 equiv $\text{Ti}(\text{tfa})_3$ (calculated for the cyclic epitope peptide). This mixture was cooled to 0 °C, and then the cyclic epitope peptide was dissolved at a concentration of 10 mg/mL. The reaction mixture was stirred at 0 °C for 90 min, and then the product was precipitated with dry diethyl ether, centrifuged, redissolved in 10% acetic acid, and freeze-dried. The crude peptide was purified by semipreparative RP-HPLC and characterized by analytical RP-HPLC and ESI-MS. Yields of the Met containing peptides were in the 50–55% range, while the Nle containing compounds were prepared with slightly better yields (58–65%).

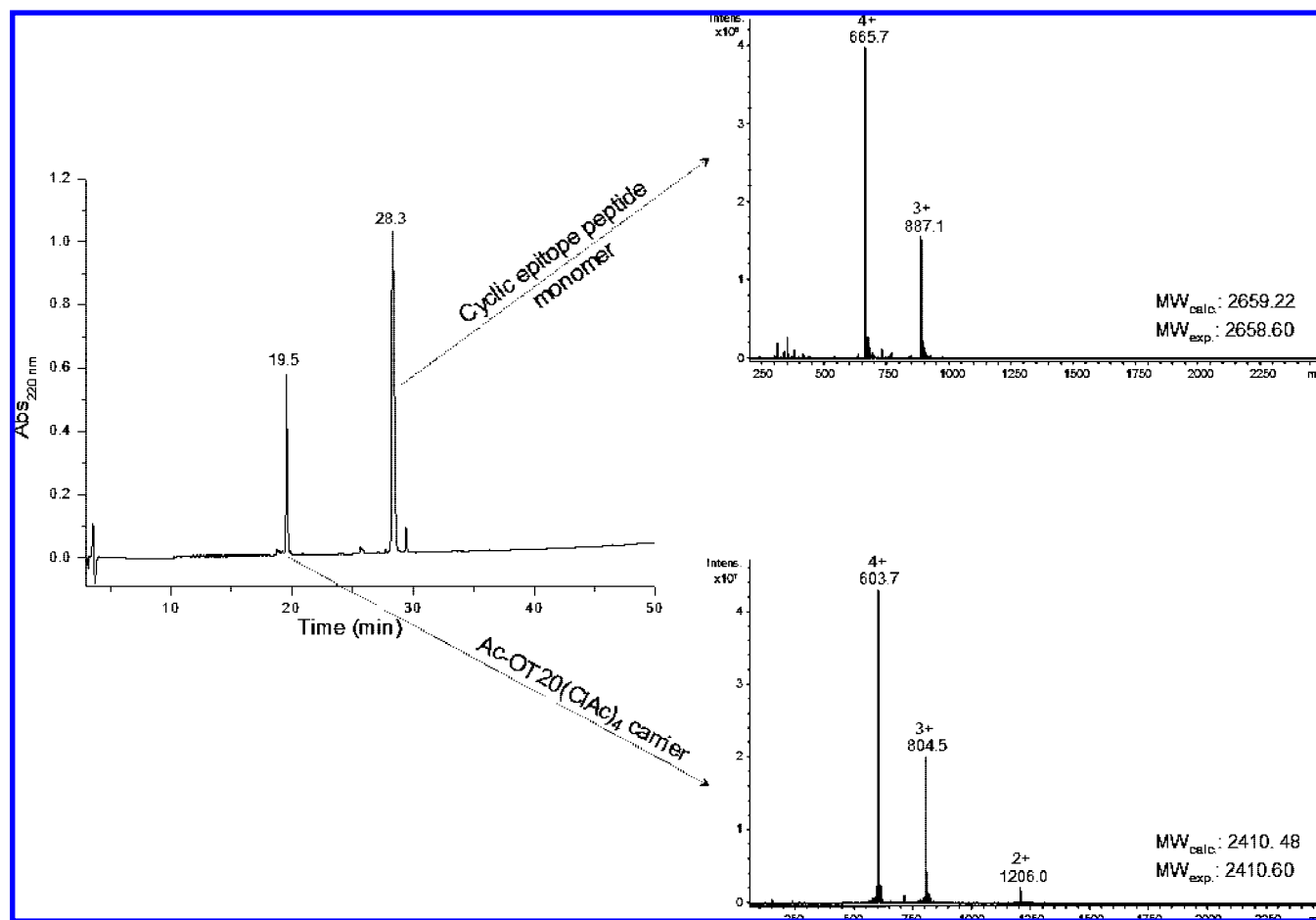


Figure 2. Conjugation of Ac-OT20(ClAc)₄ carrier with c[CH₂COLKNleADPNRFRGKDLAhxK(Ac-CGFLG)AhxC]-NH₂ (**19**) epitope peptide followed by RP-HPLC and mass spectrometry: 5 min reaction time.

Conjugation of Cyclic Epitope Peptides to Chloroacetylated Oligotuftsins Derivative (Compounds **20 and **21**; Figure 1 and Scheme 2).** Chloroacetylated oligotuftsins derivative (4 mg, 1.6 μ mol) was dissolved in 0.1 M Tris-HCl buffer (pH 8.2) at a concentration of 1 mg/mL. The cyclic epitope peptide with the free SH-group (compounds **18** and **19** prepared by the reduction of dimer peptides **15** and **17**, respectively, Scheme 2) was added to the solution in small portions over an 8 to 12 h period. A 3-fold excess of epitope peptide derivative (calculated for each chloroacetyl group) was necessary for the complete alkylation reaction. The conjugation was monitored by RP-HPLC. As soon as only the conjugate with four copies of the epitope was observed beside the cyclic epitope peptide and its dimer, the reaction was terminated by acidification and then the mixture was purified by semipreparative RP-HPLC. The conjugate was characterized by analytical RP-HPLC and ESI-MS. The yield of the conjugation was in the range 70–75%.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). Analytical RP-HPLC was performed with a Knauer system (H. Knauer, Bad Homburg, Germany) using a Phenomenex Jupiter C₁₈ column (250 \times 4.6 mm I.D.) with a 5 μ m silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, v/v)) was used at a flow rate of 1 mL/min at room temperature. Peaks were detected at $\lambda = 214$ nm. The crude products were purified on a semipreparative Phenomenex Jupiter C₁₈ column (250 \times 10 mm I.D.) with 10 μ m silica (300 Å pore size) (Torrance, CA). The flow rate was 4 mL/min. The same eluents with a linear gradient of 15–70% B over 40 min were applied.

Electrospray Ionization Mass Spectrometry (ESI-MS). Electrospray ionization mass spectrometry was performed with a Bruker Daltonics Esquire 3000+ mass spectrometer (Bremen, Germany), operating in continuous sample injection mode at 4 μ L/min flow rate. Samples were dissolved either in a mixture of acetonitrile–water (1:1, v/v) or 50% MeOH, 1% AcOH in water. Mass spectra were recorded in positive ion mode in the m/z 50–3000 range.

Enzyme-Linked Immunosorbent Assay (ELISA). Throughout the ELISA studies, culture medium of the hybridoma cell line Mab A16 was used. The preparation of Mab A16 has been described earlier (21). Mab A16 was isolated after immunization of mice with extracts of HSV-1 infected cells. Hybridomas reactive only with HSV were isolated. The isolated hybridomas were screened for reactivity with HSV specific proteins and for unspecific reactivity according to the standard procedures for monoclonal antibodies. Mab A16 is reactive with glycoprotein D. It is a conformation-independent monoclonal antibody, classified together with Mabs LP14, ID3 as a group VII monoclonal antibody of gD (21). The core residues of the epitope of group VII Mabs is located within residues 9–19 of mature gD.

The competition ELISA was performed as previously described (15). Briefly, dilution series of linear peptide (¹¹Nle)-9–22 of gD and the different constructs of the epitope peptide were preincubated for 2 h at room temperature with “optimal” dilutions of Mab A16. After preincubation, the residual binding capacity of Mab A16 in the preincubation mixture was determined by ELISA in microtiter plate wells (Greiner Labortechnik, Germany) coated with the linear peptide (¹¹Nle)-9–22 (0.5 μ g/well). As standard, a serial dilution of the peptide

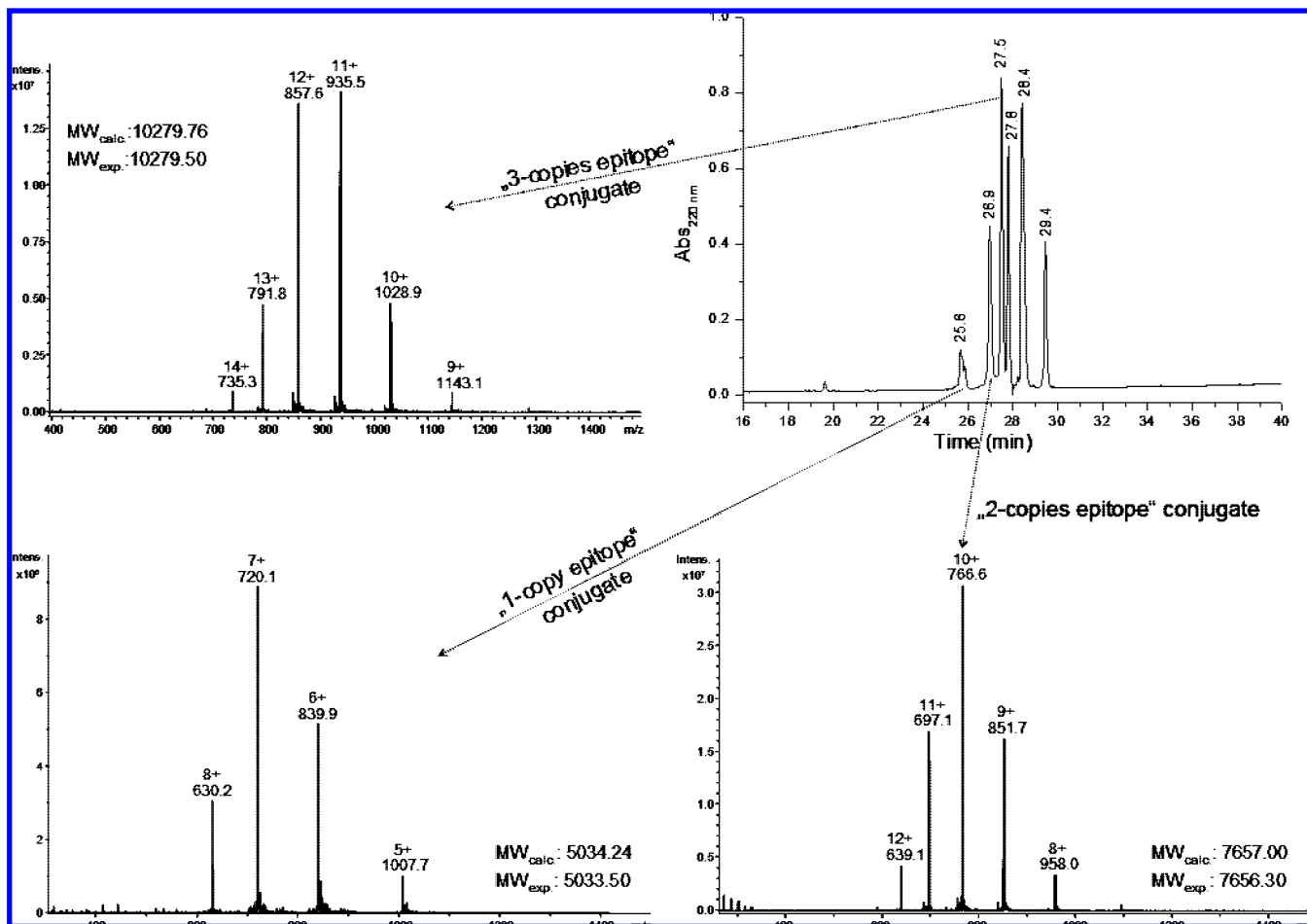


Figure 3. Conjugation of Ac-OT20(CIAc)₄ carrier with c[CH₂COLKNleADPNRFRGKDLAhxK(Ac-CGFLG)AhxC]-NH₂ (**19**) epitope peptide followed by RP-HPLC and mass spectrometry: 3 h reaction time.

(¹¹Nle)-9–22 with a fixed dilution of Mab A16 was always included: approximately 3 pmol peptide (¹¹Nle)-9–22 per 100 μ L in the preincubation mixture resulted in a residual Mab A16, which gave an optical density of 1.0 at 490 nm (OD₄₉₀) on the coated peptide (¹¹Nle)-9–22. The relative amount of the epitope peptide constructs in the preincubation mixture required to give an OD₄₉₀ of 1.0 was estimated graphically from plots of OD vs peptide concentrations. Data represent the average of at least three independent experiments for each compound.

RESULTS AND DISCUSSION

Here, we report on the synthesis, chemical characterization, and antibody binding of a new set of antigens derived from the sequence 9–22 of HSV gD-1. We investigated whether dimerization of the cyclic peptides and conjugation to an oligofucosin derivative carrier influenced the binding to Mab A16 as compared to the monomeric form. Peptide 9–22, containing the sequence of 9–19 recognized by monoclonal antibody A16, was used. It is known that the binding activity of peptide 9–21 is higher than that of 9–19 and the addition of Leu at position 22 has no significant effect on antibody binding, but it is important to avoid the potential succinimide formation of a C-terminal Asp-amide or Asp-Ahx. For both the cyclization and the conjugation, we employed the thioether bond formation, which is known to provide a chemically and biologically stable linkage and allows the use of unprotected precursors (31). The fast intramolecular (cyclization) or intermolecular (conjugation) reaction between the haloacetyl group (in our experiments, chloroacetyl group) and the thiol group of Cys under slightly

alkaline conditions provided the appropriate products with good yields and no significant side reactions.

Synthesis of Cyclic Derivatives of 9–22 Epitope Peptide of HSV gD-1. The 9–22 epitope peptide was elongated with ϵ -aminohexanoic acid (Ahx) either at the C-terminus of peptide **8a** or at both termini of the 9–22 sequence as in peptide **9a** (Figure 1). The cyclization of the peptides occurred between the chloroacetylated N-terminus and the thiol group of Cys attached to the C-terminus (compounds **8a** and **9a**). Thus, cyclic peptides **8** and **9** were obtained with a ring size of 55 and 62 atoms, respectively. These two cyclic derivatives were prepared with Met in position 11. However, peptides designed for dimerization and conjugation as well were synthesized either with Met (**10a**) or Nle (**11a**) in position 11 in order to analyze the effect of substitution of Met by Nle on the antibody recognition of the compounds.

Linear peptides **10a** and **11a**, similarly to peptides **8a** and **9a**, were synthesized by Fmoc chemistry, while their branched derivatives **12a** and **13a** were prepared by applying a mixed methodology of Boc and Fmoc chemistries (Scheme 1). In this case, the linear part containing the 9–22 epitope was built up using Boc amino acid derivatives, while for the synthesis of the branch, Fmoc strategy was utilized. Chloroacetylation of the N-terminus of the peptides was achieved by the use of chloroacetic acid pentachlorophenyl ester which provided products of higher purity than the chloroacetic anhydride. In the case of peptide **10a**, incorporation of a bromoacetyl (BrAc) moiety using bromoacetic anhydride was also attempted (data not shown). However, after cleavage of the peptide from the

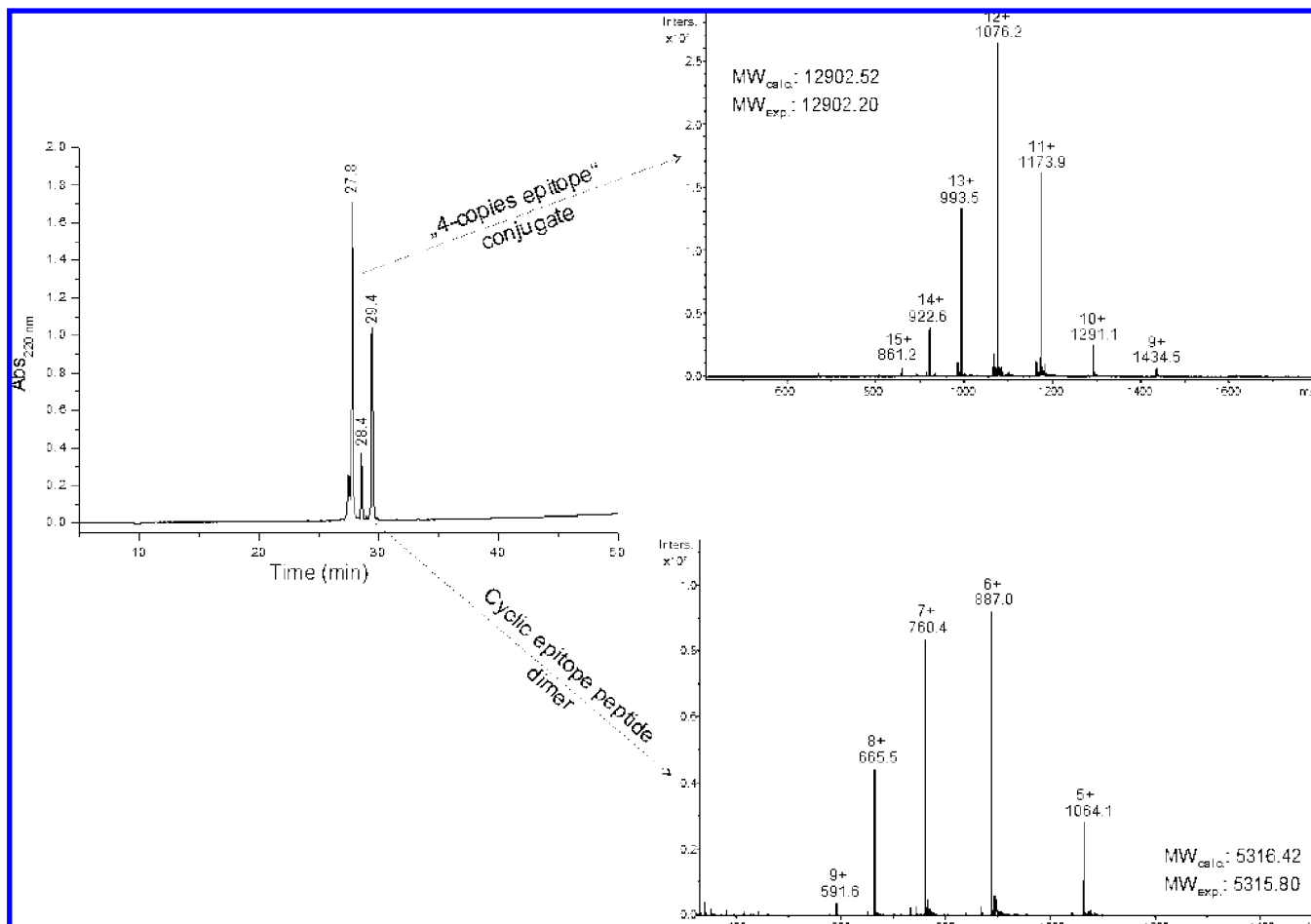


Figure 4. Conjugation of Ac-OT20(CIAc)₄ carrier with c[CH₂ COLKNIeADPNRFRGKDLAhxK(Ac-CGFLG)AhxC]-NH₂ (**19**) epitope peptide followed by RP-HPLC and mass spectrometry: 24 h reaction time.

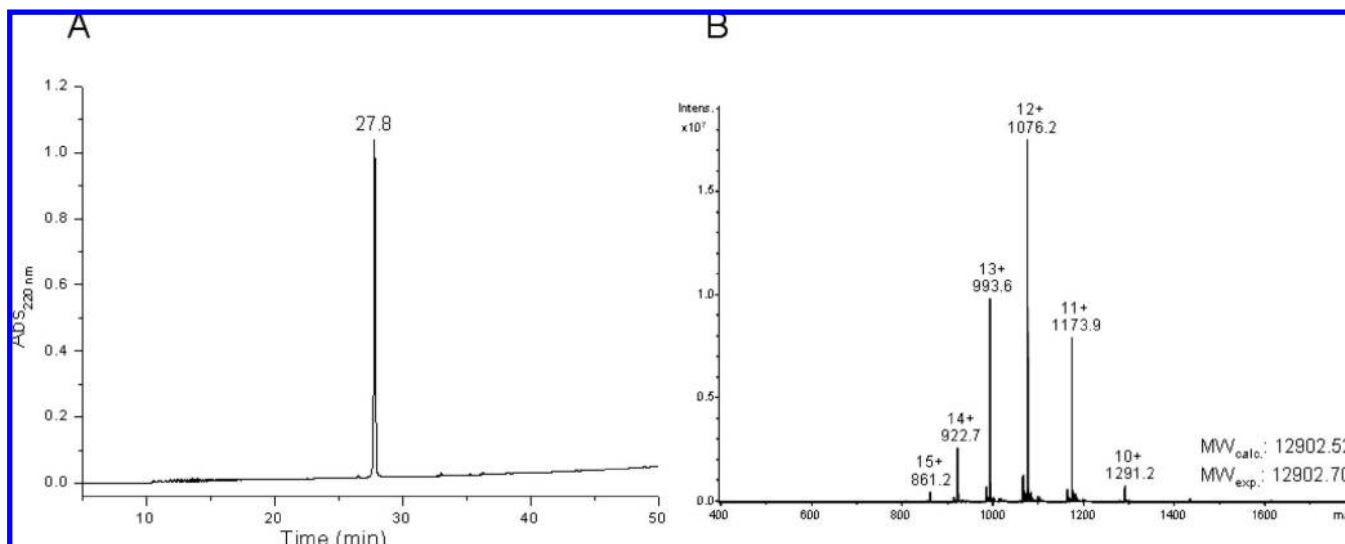


Figure 5. RP-HPLC profile (A) and ESI-ion trap mass spectrum (B) of purified conjugate **21**.

resin, a very complex crude product was detected by RP-HPLC. It was not stable even in freeze-dried form; a large number of byproducts were observed probably due to the reactivity of the BrAc group toward nucleophilic groups in the peptide (not only the thiol group) as well as to cross-linkage of peptide chains. On the basis of this observation, only chloroacetylated peptides were used in the further studies. In the linear peptide **10a**, the sequence at the *N*-terminal part was identical to the sequence

in the peptide **8a**. The *C*-terminus was elongated by a hexapeptide consisting of an enzyme-labile sequence GFLG (32), with Cys and Ahx as spacer units. This Cys residue protected by AcM, which is stable under the acidic cleavage conditions as well as under the conditions of the cyclization procedure, provided the conjugation site in the dimerization reaction. In peptide **11a**, which is similar to peptide **10a**, Met was replaced with Nle. The cyclization of peptides **10a** and **11a** via thioether

bond formation yielded compounds **10** and **11**, respectively, each with a 55-atom ring. In linear peptides **12a** (^{11}Met) and **13a** (^{11}Nle), the Ahx-Lys-Ahx-Cys sequence was added to the C-terminus of the 9–22 epitope peptide. The C-terminal Cys was involved in a thioether cyclization with the N-terminal ClAc-group that yielded a 65-atom ring, while an additional Cys residue to be used for conjugation was included in an enzyme-labile (Ac-CGFLG) pentapeptide chain branching on the ϵ -amino group of the Lys near the C-terminus. The α -amino group of the Cys in this branch sequence was acetylated in order to prevent the fast formation of undesired disulfide bridge (33). Cyclization of peptides **12a** and **13a** provided **12** and **13**, respectively, with good yields. Compounds **10–13** were dimerized using $\text{Ti}(\text{tfa})_3$ in a TFA solution. The disulfide bond formation was carried out at 0 °C for 90 min using 0.6 equiv of $\text{Ti}(\text{tfa})_3/\text{Cys}(\text{Ac})$. Dimerization of compounds **10** and **12** with Met at residue 11 gave slightly lower yields, but no significant amount of methionine sulfoxide was detected. Cyclic dimers (compounds **14–17**) were isolated with fairly good yields (50–60%). Because the binding studies with Mab A16 (Table 2) indicated no significant differences between the recognition of cyclic dimer epitopes containing either ^{11}Met (11.8 pmol/100 μL (compound **14**) and 57.7 pmol/100 μL (compound **16**)) or ^{11}Nle (11.1 pmol/100 μL (compound **15**) and 41.4 pmol/100 μL (compound **17**)), only the Nle derivatives were used for further conjugation experiments. The dimers (compounds **15** and **17**) were reduced to their monomers with DTT (Scheme 2). The Cys connected to the spacer sequence (compounds **18** and **19**) was the conjugation site of the cyclic peptides in their coupling to the chloroacetylated oligo tuftsin derivative carrier (Ac-[TKPK(ClAc)G] $_4$ -NH $_2$). The conjugation was carried out in 0.1 M Tris-HCl buffer at pH 8.2 and monitored by RP-HPLC (Figures 2–4). After attachment of four copies of the cyclic epitope peptides to the carrier, the reaction was terminated by acidifying the solution with TFA. The conjugates (compounds **20** and **21**) were purified by semi-preparative RP-HPLC. The excess of Cys peptides was recovered after reduction with DTT. All cyclic peptides, their dimers, and conjugated derivatives were characterized by analytical RP-HPLC and ESI-MS (Figure 5). Data are summarized in Table 1.

Binding of Cyclic Derivatives of 9–22 Epitope Peptide of HSV gD-1 to Mab A16. Direct ELISA studies are often hampered by the different coating properties of the peptide constructs, which makes binding determination somehow unreliable. In our case, none of the dimers and conjugates of the cyclic peptides (compounds **14–17**) were able to bind to the ELISA plates. Therefore, competition ELISA was used to compare the antibody recognition of the compounds. Mab A16, which recognizes the 9–22 sequence in native gD (9), was used in this experiment. In a competition ELISA, only the binding properties of the compounds with Mab A16 in solution are measured, which gives more information on the binding properties of the peptide constructs.

Effect of Cyclization on Mab A16 Binding. No significant differences in binding were detected for various constructs with either Met or Nle at position 11 [79.1 (compound **10**, Met 11) vs 75.6 (compound **11**, Nle 11) and 88.0 (compound **12**, Met 11) vs 92.3 (compound **13**, Nle 11) pmol/100 μL]. This is in agreement with our previous results concerning linear peptides **1** and **2** (Table 2). Comparison between 16- and 8-residue cyclic peptides (e.g., **8** and **9** vs **3** and **4**, Table 2) showed that the larger ring clearly improved the antibody binding (79- and 273-fold, respectively). Whether this was due to less amino acids in the 8-residue cyclic peptides (compounds **8** and **9**) or to the modification of Arg at residue 18, it cannot be concluded. The cyclic peptides with hexapeptide spacer at the C-terminus

(compounds **10**, **11**) or the branched ones (compounds **12**, **13**) had an approximately 2–3-fold lower binding activity to the Mab A16 than **8** and **9**. The binding activities of compounds **10** and **11** with the hexapeptide spacers were only slightly better than the binding of branched ones (compounds **12**, **13**).

Effect of Dimerization on Mab A16 Binding. Dimerization of the cyclic peptides **10**, **11**, **12**, and **13** resulting in compounds **14**, **15**, **16**, and **17**, respectively, increased the binding activity to Mab A16 in all cases. However, the disulfide bond formation at the C-terminus of the peptides (compounds **14**, **15**) led to higher antibody recognition of the cyclic dimer peptides than in the case of dimerization through the branching (compounds **16** and **17**). Both the Met and Nle containing derivatives (compounds **14** and **15**; 11.8 and 11.1 pmol/100 μL , respectively) had a higher binding activity to Mab A16 than the monomeric cyclic peptide without spacer sequences (compounds **8**, **9**; 28.6 and 28.0 pmol/100 μL , respectively). The binding capacity of dimers (57.7 (compound **16**) and 41.4 (compound **17**) pmol/100 μL), which had a disulfide bond between the branches, was better than that of their cyclic monomers (88.8 (compound **12**) and 92.3 (compound **13**) pmol/100 μL).

Effect of Conjugation on Mab A16 Binding. The binding data of the conjugates (compounds **20** and **21**) to the Mab A16 were surprising. In case of the conjugate (compound **20**) with the cyclic peptide containing a spacer and a Cys at the C-terminus (compound **18**) as the conjugation site, the binding affinity was dramatically decreased as compared to the monomer or dimer having the identical cyclic peptide (249.3 vs 75.6 (compound **11**) pmol/100 μL). In contrast, the other conjugate (compound **21**) in which the cyclic peptide was attached to the carrier through the branch showed excellent binding activity to the Mab A16 (7.2 vs 92.3 (compound **13**) pmol/100 μL). This binding capacity is comparable to the results of the linear epitope peptides as measured earlier (epitope construct **1** and **2**; Table 2). However, it is still 1 order of magnitude weaker than the binding of the OT20 conjugate containing the (^{11}Nle)-9–22C linear epitope peptide (**5**) and that of simple dimers of the linear peptide (**6**, **7**) (0.72, 0.74, and 0.66 pmol/100 μL , respectively; Table 2) (25).

CONCLUSION

The synthesis of cyclic forms of the epitope peptide 9–22 of gD-1, their dimers, and conjugates was carried out in this study. Dimers of cyclic peptides (without branches) and the cyclic peptide conjugated to an oligo tuftsin derivative through the peptide spacer in the branch showed the highest binding activity to the Mab A16. Although a large variety of cyclic peptides and dimers of cyclic peptides were synthesized, a simple linear peptide 9–22 conjugated to OT20 and dimers of the linear peptide had the highest binding activity to the Mab A16. We hypothesize that only an accurate conformation of the cyclic constructs may result in higher binding activity than a linear construct with a potential to adopt the appropriate conformation. The strong binding activities of the dimers of the linear peptide are interesting. These results might be explained by binding of one peptide dimer molecule to both binding sites of one IgG or by cross-linking of IgG molecules. Mab A16 is directed against the native gD, as it is present in a cell infected by HSV. Therefore, another possible explanation of the strong binding of the dimer peptide 9–22 could be that gD in the native form during the virus infection has a dimer structure (34–36). We expected higher binding activity by cyclization of the epitope peptide 9–22, but our results showed that this was not the case. In addition, cyclization might enhance the stability of the peptide against enzymatic degradation. According to other results (37, 38) by our group, one can assume that conjugated cyclic peptides might have increased stability

in human serum compared to their linear versions. For this reason, in addition to compound **5** containing the linear epitope in the conjugate, compound **21** in which cyclic epitope was conjugated to OT20 carrier on its branch was selected for further development for the investigation of their immune response inducing potential. The results of these studies will be reported elsewhere.

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