Examination of a New Cross-Linker in Monoclonal Antibody Reaction Against the C-Terminus of Tobacco Mosaic Virus Coat Protein

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Dedicated to Professor Dr. Ernst Bayer on the occasion of his 60th birthday

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Monoclonal Antibody, Synthetic Antigen, Enzyme-Linked Immunosorbent Assay, Tobacco Mosaic Virus Protein, Cross-Linking Reagent

The C-terminal tetrapeptide Gly—Pro—Ala—Thr of TMV coat protein was linked to poly-(L-lysine) of 37,300 and 80,000 daltons and to human serum albumin using different ratios of tetrapeptide to carrier. The new hydrazidosuccinyl HSc cross-linker effected the specific amino terminal attachment of the tetrapeptide by azide coupling. The synthetic peptide and its conjugates were controlled by various methods including \(_{13}C\) NMR and CD. Using a conjugate containing 74 tetrapeptide residues linked to poly(L-lysine) of 80,000 daltons for screening of monoclonal antibody by ELISA, 5.8 fmol of peptide were detectable.

The serological and biological properties of the monoclonal antibody were assayed by indirect ELISA and by quantitative determination of virus neutralization. This neutralization could be reversed by the poly(L-lysine)-tetrapeptide conjugate but not by the free tetrapeptide.

The C-terminal region of the coat protein of TMV was discovered to be antigenic \([1, 2]\) by use of polyclonal antibodies and conjugated synthetic peptides of a sequence of three to six amino acids. Recently the C-terminal decapetide has also been found to bind to anti-TMV monoclonal antibodies \([3, 4]\). Further reports on anti-TMV monoclonal antibodies \([5]\) indicated the steady interest in this system. In this study TMV has also been chosen as a model since the sequence of many strains is known and various attempts to characterize the antigenic structure have been made with polyclonal antibodies. Partially protected C-terminal sequences of TMV coat protein have been synthesized by Pettit \textit{et al.} \([6]\), however, no immunological assays were described. For the analysis of the fine structure of an antigenic region or an epitope, monoclonal antibodies and unequivocally characterized synthetic peptides of the corresponding sequence are highly desirable tools \([7]\). In this context it should be emphasized that larger amounts of the C-terminal TMV tetrapeptide and derivatives were prepared by conventional fragment condensation in order to ensure their purity by various analytical methods including \(_{13}C\) NMR. This is a prerequisite for reproducible and quantitative immunological studies. Purity is not always guaranteed for longer HPLC-purified peptides (>15 residues) made by the solid phase method.

From 138 characterized antibody clones \([8]\) directed against TMV epitopes, the one (No. 95) reacting with the C-terminal region was selected for this study because of its efficiency in neutralizing virus infectivity \([9]\).

The tetrapeptide was conjugated to different carrier molecules in varying molar ratios and the immunological reactivity of the conjugates compared. The peptide conjugates most reactive in the ELISA were selected for screening with the purpose to find a monoclonal antibody specific for the C-terminal epitope of TMV coat protein.

In contrast to former studies, by employing a systematic series of conjugates consisting of different ratios of poly(L-lysine) to tetrapeptide, it was intended to improve the detection limit for defined antibodies in the ELISA. The immunological reactivity of this monoclonal antibody was investigated with the synthetic peptide as well as with the corre-

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\textbf{Abbreviations:} The names of synthetic peptides were abbreviated according to the IUPAC-IUB rules (Eur. J. Biochem. 138, 9–37 (1984)); ATTC, American Type Culture Collection, Rockville, MD; BSA, bovine serum albumine; FAB-MS, fast atom bombardment-mass spectrum; HSA, human serum albumine; HSc, hydrazido succinyl; ELISA, Enzyme-Linked Immunosorbent Assay; Sc, succinyl; STP, synthetic tetrapeptide Gly—Pro—Ala—Thr; TMV, Tobacco Mosaic Virus.

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sponding native sequence on the assembled virus and with the isolated coat protein of different related TMV strains.

The potency of the monoclonal antibody to neutralize the infectivity of the virus was assayed on tobacco plants. For the first time quantitative investigations with regard to plant virus neutralization were carried out.

**Experimental Part**

**Immunological assays**

**Virus**

Tobacco Mosaic Virus strains *vulgare, dahlemense* (Max-Planck-Institut für Virusforschung, Tübingen, FRG) and Holmes’ Ribgrass (ATCC, ePV 46) were propagated on *Nicotiana tabacum* cv Samsun and purified [10] including isopycnic centrifugation in CsCl. The coat protein was isolated according to the method described by Fraenkel-Conrat [11] and the monomers [12] used in ELISA.

**Monoclonal antibody**

The monoclonal antibodies were purified from the culture supernatant of hybridoma clones by ammonium sulfate precipitation [13] and subsequent chromatography on protein A-Sepharose [14]. Hybridomas were generated by fusion of the spleen cells of TMV *vulgare* hyperimmunized STU-mice with NS-1 myeloma cells [8]. Monoclonal antibody 95 was determined to be of IgG2a isotype.

**Enzyme-linked immunosorbent assay**

The indirect ELISA [15] was conducted (Table I) for three purposes: 1. quantitative determination of the immunological reactivity of polyclonal antibodies with the synthesized tetrapeptide, both free and coupled to poly(L-lysine) or HSA, 2. determination of a monoclonal antibody binding specifically to this tetrapeptide as well as to the virus, 3. determination of the immunological cross-reactivity of this monoclonal antibody with related TMV strains and their isolated coat protein.

The ELISA procedure (Table I) involved polystyrene micro-ELISA plates (Dynatech, 129 B), two antiglobulins, goat anti-rabbit IgG and rabbit antimouse IgG, each labelled with alkaline phosphatase (Zymed Laboratories, California, USA), tablets of p-nitrophenyl phosphate (Sigma) as enzyme substrate and buffers described by Clark and Adams [16]. In some experiments glycine buffer (Boehringer, Mannheim, FRG) substituted the diethanolamine substrate buffer. After coating, the non-occupied sites on the solid phase were blocked with BSA [17].

For screening of monoclonal antibodies the microtiter plates were coated with 2.8 μg peptide/ml (1.62 nmol/well) of the poly(L-lysine) linked with 74 peptides, and with 1.0 μg peptide/ml (0.58 nmol/well) of HSA linked with 16–18 peptides.

**Assessment of viral infectivity**

The infectivity of the purified TMV *vulgare* was assayed on *Nicotiana tabacum* cv Xanthi nc and also the infectivity of the virus after incubation overnight with the tetrapeptide-specific monoclonal antibody. The treatment followed the method described for polyclonal antisera [18].

**Chemical synthesis and analysis**

**Chemicals**

HSA (66,000 daltons) was from Behring (Marburg, FRG), poly(L-lysine) (37,300 daltons) from Calbiochem and poly(L-lysine) (80,000 daltons) from Sigma. All reagents and solvents (p.a. grade) were from E. Merck. The protected amino acids were synthesized according to published procedures [19].

<table>
<thead>
<tr>
<th>ELISA steps</th>
<th>Concentration/dilution</th>
<th>Volume/well [μl]</th>
<th>Incubation [h]</th>
<th>Incubation [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>peptide conjugates</td>
<td>10⁻⁷–10⁻¹⁵ mol/ml</td>
<td>200</td>
<td>3</td>
<td>37</td>
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<tr>
<td>viral antigens</td>
<td>10 μg/ml</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Blocking:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA/saline</td>
<td>1%</td>
<td>300</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Mouse monoclonal antibodies</td>
<td>1–30 μg/ml</td>
<td>200</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Rabbit polyclonal antibodies</td>
<td>1–10 μg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme-linked antiglobulins</td>
<td>1:500</td>
<td>200</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td>Substrate</td>
<td>1 mg/ml</td>
<td>300</td>
<td>5 min–2 h</td>
<td>37</td>
</tr>
</tbody>
</table>

Table I. Indirect ELISA procedure.
Chromatography and analyses

R<sub>f</sub> values were determined in saturated glass chambers (Camag) on silica gel plates Si 60 F<sub>254</sub> (Merck, No. 5714) at 21 °C using the following solvent systems (v/v): A = chloroform/methanol/acetic acid/water (65:25:3:4); B = chloroform/methanol/acetic acid (55:5:1); C = chloroform/methanol (95:5); D = chloroform/methanol/water/conc. ammonia (65:35:4:3); E = chloroform/methanol/acetic acid/water (35:45:3:4); F = 1-butanol/acetic acid/water (2:1:1); G = isopropanol/acetic acid/water (3:1:1). For detection we used ninhydrin, chlorine/4,4'-bis(dimethylamino) diphenylmethane (TDM) and Barton’s reagent (for hydrazides). Amino acid analyses were made from hydrolysates (6 N HCl, 110 °C, 24 h; addition of 2.5% (v/v) thioglycolic acid) on the amino acid analyzer LC 6000 E (Biotronik). Melting points were determined according to Tottoli and are not corrected.

Spectra

<sup>13</sup>C NMR spectra were recorded on the Bruker NMR spectrometers WP-80 (20.115 MHz) and WM-400 (100.62 MHz) using proton decoupling and J-modulated spin-echo experiments. The chemical shift values, δ (ppm), refer to external tetramethylsilane with δ = 0 ppm and have an error of ±0.1 ppm. Circular dichroism spectra were recorded on the dichrograph CD 185 (Roussel-Jouan). The data are calculated as molar circular dichroic ellipticity per amino acid residue [θ]<sub>M</sub> without taking into account the cross-linker and the tetrapeptide moieties linked to the ε-amino groups of poly(L-lysine). Mass spectra were recorded on a mass spectrometer MAT 311 A (Varian) using the fast atom (Argon) bombardment (FAB) method with ion source temperature of 30 °C.

Synthesis of peptides

Tetrapeptide and conjugates were synthesized according to the scheme shown in Fig. 1.

N<sup>α</sup>-tert-Butyloxy carbonyl-prolyl-alanine methyl ester

Chilled methyl isobutylchloroformate (110 mmol, 10.85 ml) was added dropwise to Boc—Pro—OH (110 mmol, 23.65 g) and N-methylmorpholine (110 mmol, 12.2 ml) in dimethylformamide (45 ml) at −20 °C. After 15 min a chilled solution of HCl×H—Ala—OMe (97 mmol, 13.5 g) and N-methylmorpholine (97 mmol, 10.85 ml) in dimethylformamide (50 ml) was added dropwise at −15 to −13 °C. After 30 min at −10 °C the mixture was allowed to warm up to room temperature and the solvent was removed in vacuo. The oily residue was washed in ethyl acetate (120 ml) with 5% aqueous solutions of KHCO<sub>3</sub>, KHSO<sub>4</sub> and saturated NaCl solution (3×50 ml of each). After drying over Na<sub>2</sub>SO<sub>4</sub> and evaporation of the solvent the dipeptide Boc—Pro—Ala—OMe was crystallized from ethyl acetate/light petroleum (b.p. 30–50 °C) to give colourless prisms. Yield 19.8 g (69%); m.p. 78 °C; R<sub>f</sub> (A) 0.82, R<sub>f</sub> (B) 0.65, R<sub>f</sub> (C) 0.54. Amino acid analysis: Pro 1.00, Ala 1.01.

\[
C_{14}H_{25}N_{3}O_{5} (300.35)
\]

Calcd C 46.66 H 8.00 N 9.33,

Found C 46.12 H 8.60 N 9.23.

N<sup>α</sup>-Benzyl oxy carbonyl-glycyl-prolyl-alanine methyl ester

N,N′-Dicyclohexylcarbodiimide (57 mmol, 11.7 g) was added to Z—Gly—OH (57 mmol, 11.9 g) and 1-hydroxybenzotriazol (71 mmol, 8.8 g) in dimethylformamide (40 ml) at 0 °C. After 15 min H—Pro—Ala—OMe (57 mmol, 11.4 g) in dichloromethane (25 ml) was added. After 90 min at 0 °C and 5 h at room temperature the dicyclohexylurea was filtered off and the solvents removed in vacuo. The tripeptide Z—Gly—Pro—Ala—OMe was chromatographically pure crystals as described above for Boc—Pro—Ala—OMe. Yield 17.8 g (93%); R<sub>f</sub> (A) 0.61.

\[
N^\alpha\text{-Benzyl oxy carbonyl-glycyl-prolyl-alanine methyl ester}
\]

N,N′-Dicyclohexylcarbodiimide (57 mmol, 11.7 g) was added to Z—Gly—OH (57 mmol, 11.9 g) and 1-hydroxybenzotriazol (71 mmol, 8.8 g) in dimethylformamide (40 ml) at 0 °C. After 15 min H—Pro—Ala—OMe (57 mmol, 11.4 g) in dichloromethane (25 ml) was added. After 90 min at 0 °C and 5 h at room temperature the dicyclohexylurea was filtered off and the solvents removed in vacuo. The tripeptide Z—Gly—Pro—Ala—OMe was chromatographically pure crystals as described above for Boc—Pro—Ala—OMe. Yield 17.8 g (79%); m.p. 148 °C; R<sub>f</sub> (A) 0.85; R<sub>f</sub> (B) 0.55; R<sub>f</sub> (C) 0.4. Amino acid analysis: Pro 0.99, Ala 1.00, Gly 1.00.

\[
C_{16}H_{26}N_{3}O_{6} (391.44)
\]

Calcd C 58.30 H 6.43 N 10.73,

Found C 59.52 H 6.12 N 9.96.
**N’-Benzyloxycarbonyl-glycyl-prolyl-alanine**

Z—Gly—Pro—Ala—OMe (43 mmol, 16.8 g) was saponified in methanol (70 ml) by addition of 0.5 N NaOH (120 ml) within 30 min. After neutralization and removal of methanol the tripeptide acid was extracted at pH 3 with ethyl acetate. After washing with saturated NaCl solution the organic phase was concentrated in vacuo, and the colourless, chromatographically uniform Z—Gly—Pro—Ala—OH crystallized by addition of light petroleum. Yield 13.4 g (82%); m.p. 145 °C; \( R_f \) (A) 6.68; \( R_f \) (B) 0.26; \( R_f \) (D) 0.54; \( R_f \) (F) 0.81.

\[ C_{18}H_{23}N_3O_6 \] (377.39)
Calcd C 57.28 H 6.14 N 11.13, 
Found C 57.63 H 5.82 N 11.03.

**N’-Benzyloxycarbonyl-glycyl-prolyl-alanyl-O-tert-butyl-threonine tert-butyl ester**

N,N’-Dicyclohexylcarbodiimide (35.2 mmol, 7.2 g) was added to Z—Gly—Pro—Ala—OH (35.2 mmol, 13.3 g) and 1-hydroxybenzotriazole (35.2 mmol, 4.7 g) in dimethylformamide/dichloromethane (3:1, v/v) at 0 °C. After 20 min H—Thr(Bu’)—OBu’ (40.6 mmol, 9.4 g) was added, after 12 h stirring at room temperature the N,N’-dicyclohexyl urea was filtered off (see below), and the tetrapeptide (9.6 g) was isolated as described for the dipeptide Boc—Pro—Ala—OMe. The product crystallized spontaneously from methanol/ether (1:2, v/v) (15 ml) by addition of light petroleum. From the N,N’-dicyclohexyl urea a second fraction (4.68 g) was isolated by extraction with methanol, evaporation and washing the ethyl acetate solution as described. Total yield 14.28 g (67%); m.p. 118 °C; \( R_f \) (A) 0.84; \( R_f \) (C) 0.72; \( R_f \) (D) 0.86; \([\alpha]_D^{20} = -93.7 ^\circ \text{C} \) \( (c=1, \text{methanol}) \). Amino acid analysis: Ala 0.97, Pro 1.00, Gly 0.98, Thr 0.99.

\[ C_{30}H_{46}N_4O_8 \] (590.72)
Calcd C 60.99 H 7.84 N 9.48, 
Found C 61.75 H 7.11 N 9.30.
Glycyl-prolyl-allyl-O-tert-butyl-threonyl-tert-butyl ester

Z-Gly-Pro-Ala-Thr(Bu')-OBu' (3.38 mmol, 2 g) was hydrogenated in methanol/water (9:1, v/v) (30 ml) in the presence of 120 mg Pd charcoal (Fluka). After 30 min the solvent was removed, and the residue stirred in diethyl ether. The colourless precipitated tetrapeptide ester was used immediately for the next step. Yield 1.4 g (92%); \( R_f \) (A) 0.46; \( R_f \) (D) 0.80; \( R_f \) (E) 0.71.

\[ C_{27}H_{40}N_{4}O_{8} (456.58) \]
Calcld C 55.00 H 8.05 N 12.53,
Found C 55.41 H 8.19 N 12.67.

Glycyl-prolyl-allyl-threonine trifluoroacetate

H-Gly-Pro-Ala-Thr(Bu')-OBu' (1.4 g, 3.07 mmol) was deprotected in trifluoroacetic acid/dichloromethane (1:1, v/v) (3 ml) at room temperature. After 3 h the solvents were removed in vacuo, and the oily residue was given dropwise to stirred, chilled diethyl ether. The tetrapeptide trifluoroacetate was isolated as a colourless, hygroscopic product, which was chromatographically uniform. Yield 1.28 g (91%); \( R_f \) (D) 0.13; \( R_f \) (E) 0.14; \( R_f \) (F) 0.22.

Amino acid analysis: Ala 0.98, Pro 1.01, Gly 1.02, Thr 1.00.

\[ C_{18}H_{25}N_{4}O_{8}F_{3} (458.39) \]
Calcld C 41.92 H 5.49 N 12.22,
Found C 40.90 H 5.03 N 11.91.

Glycyl-prolyl-allyl-threonine hydrochloride

H-Gly-Pro-Ala-Thr-OBu' (1.0 g, 2.18 mmol) was dissolved in 6 ml water and stirred with ion exchange resin DOWEX 1x4 (Cl-) (0.5 g). After 15 min the resin was filtered off, and the aqueous solution was lyophilized. Yield 0.78 g (94%); m.p. 95–100 °C; \( R_f \) (D) 0.13; \( R_f \) (E) 0.14; \( R_f \) (F) 0.22; \( R_f \) (G) 0.3. \(^{13}C\) NMR: Table II; CI-D: Fig. 3; FAB-MS: calc. 380.82 (M+), found 381 (M+H)\(^+\).

\[ C_{14}H_{25}N_{4}O_{6}Cl (380.82) \]
Calcld C 44.15 H 6.61 N 14.71,
Found C 44.84 H 6.91 N 13.82.

\( N^\alpha-(N^\alpha\text{-tert-Butyloxycarbonyl-hydrazido})-\text{succinyl-glycyl-prolyl-allyl-O-tert-butyl-threonine-tert-butyl ester} \)

\( N^\alpha\text{-tert-Butyloxycarbonyl-succinic acid monohydra-} \]

dride (Boc-HSC-OH) [21] (670 mg, 2.88 mmol), and H-Gly-Pro-Ala-Thr(Bu')-OBu' (1.2 g, 2.63 mmol) in dichloromethane (20 ml) was added to 1-hydroxybenzotriazole (500 mg, 0.3 mmol) in 3 ml dimethylformamide. At 0 °C, N,N'-dicyclohexylcarbodiimide (590 mg, 2.88 mmol) was added. After 12 h stirring at room temperature the dicyclohexylurea was filtered off, the solvents were removed in vacuo, and the product isolated as described for dipeptide Boc-Pro-Ala-OMe. Repeated dissolution in ether followed by evaporation gave a dry and colourless material. Yield 1.51 g (79%). The product was at least 95% pure according to thin-layer chromatography and NMR. Impurities were removed by gel chromatography on Sephadex LH 20 with chloroform/methanol (1:1, v/v). Yield 163 mg (68%); m.p. 71 °C (decomp.); \( R_f \) (A) 0.77; \( R_f \) (C) 0.25; \( R_f \) (D) 0.91; \( R_f \) (E) 0.89. The gas chromatographic test for racemization [20] revealed 0.4% D-Ala, 0.9% D-Pro, 0.3% D-Thr (not corrected for racemization due to hydrolysis). The succinic acid derivative was eluted between the Gly and Pro peaks. \(^{13}C\) NMR: Table II.

\[ C_{13}H_{34}N_{6}O_{10} (670.80) \]
Calcld C 55.50 H 8.05 N 12.53,
Found C 55.41 H 8.19 N 12.67.

**Preparation of conjugates**

Conc. HCl (90 μl, 1.08 mmol, 6-fold excess) was added to a solution of HSc-Gly-Pro-Ala-Thr (180 μmol) in 1–2 ml dimethylformamide. After addition of a 14% NaNO\(_2\) solution (270 μmol, 133 μl, 1.5 fold excess) at −15 °C, the mixture was stirred for 5 min. The pH was adjusted to 7–8 at −25 °C. Containing the azide ready for coupling, to this mixture the following polymers were added:

a) Human serum albumine dissolved in PBS (pH 7.2). After 24 h at 4 °C the slightly yellow solution was dialyzed for 2 days against 0.01 N acetic acid and 1 day against distilled water and finally freeze-dried.

b) Poly(L-lysine) × HBr (37,300 and 80,000 daltons) dissolved in water. The pH was adjusted to 9
Table II. Assignments of the $^{13}$C NMR signals of the synthetic tetrapeptide, derivatives and conjugates with poly(L-lysine).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>STP×HCl</th>
<th>HSc-1STP×HCl</th>
<th>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{170}$×HCl</th>
<th>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{380}$</th>
<th>Boc→HSc-1STP</th>
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</thead>
<tbody>
<tr>
<td>Ala—C$_{a}$</td>
<td>19.0</td>
<td>18.8</td>
<td>19.1; 19.2</td>
<td>19.1</td>
<td>17.7</td>
</tr>
<tr>
<td>Thr—C$_{y}$</td>
<td>21.3</td>
<td>21.2</td>
<td>21.7; 21.9</td>
<td>21.6</td>
<td>20.5</td>
</tr>
<tr>
<td>Lys—C$_{y}$</td>
<td>26.8</td>
<td>26.8</td>
<td>25.1 (broad)</td>
<td>25.2 (broad)</td>
<td>24.6</td>
</tr>
<tr>
<td>Pro—C$_{y}$</td>
<td>27.1</td>
<td>27.1</td>
<td>27.1</td>
<td>27.1</td>
<td>27.1</td>
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<tr>
<td>Sc—CH$_{2}$</td>
<td>30.6</td>
<td>29.1</td>
<td>30.6</td>
<td>29.5</td>
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<td>Lys—C$_{t}$</td>
<td>31.7</td>
<td>33.3 (bound)</td>
<td>33.3</td>
<td>31.0</td>
<td>31.0</td>
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<tr>
<td>Pro—C$_{a}$</td>
<td>32.1</td>
<td>32.1</td>
<td>32.1</td>
<td>29.4</td>
<td>29.4</td>
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<tr>
<td>Lys—C$_{a}$</td>
<td>33.8; 33.6</td>
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<tr>
<td>Lys—C</td>
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<td>41.9</td>
<td>41.7 (free)</td>
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<td>Lys—C$_{a}$</td>
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<td>44.4</td>
<td>44.4</td>
<td>42.0</td>
<td>42.0</td>
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<tr>
<td>Pro—C</td>
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<td>49.7</td>
<td>49.7</td>
<td>46.4</td>
<td>46.4</td>
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<tr>
<td>Ala—C$_{a}$</td>
<td>52.3</td>
<td>52.1</td>
<td>52.3; 52.1</td>
<td>48.9</td>
<td>48.9</td>
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<tr>
<td>Lys—C$_{a}$</td>
<td>60.4</td>
<td>61.6</td>
<td>62.7</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Pro—C</td>
<td>62.8</td>
<td>63.0; 62.7</td>
<td>61.7 (broad)</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Thr—C$_{a}$</td>
<td>69.9</td>
<td>69.7; 70.7</td>
<td>69.0</td>
<td>67.0</td>
<td>67.0</td>
</tr>
</tbody>
</table>

C=O

Lys—CO | 179.0; 177.5 | 177.6; 177.5 | 177.6; 177.5 | 177.6; 177.5 | 177.6; 177.5 |
Sc—CO | 176.4 | 177.0; 176.8 | 177.0; 176.8 | 177.0; 176.8 | 177.0; 176.8 |
Sc—CO$_{2}$ | 175.2 | 176.5; 176.2 | 176.5(2); 171.8 | 172.6 | 172.6 |
Gly—CO | 175.9 | 176.1; 176.0 | 176.5(2); 171.8 | 172.1 | 172.1 |
Pro—CO | 177.8 | 173.3; 172.0 | 173.3; 172.0 | 171.2 | 171.2 |
Ala—CO | 176.2 | 171.9 | 169.4 | 169.4 | 169.4 |
Thr—CO | 168.1 | 171.7 | 168.3 | 168.3 | 168.3 |

Protecting Groups

Boc(CO) | 155.6 |
Boc(q) | 81.0 |
Boc(CH$_{3}$) | 28.0 |
OBu'(q) | 81.7 |
OBu'(CH$_{3}$) | 28.5 |
tBu'(q) | 78.4 |
tBu'(CH$_{3}$) | 27.9 |

a Conjugate is containing uncoupled Sc-STP; b solvent.

Table III. Poly(L-lysine)-tetrapeptide-conjugates using HSc-group for crosslinking.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Lys/STP$^{a}$</th>
<th>Lys/STP$^{b}$ (AA)</th>
<th>Loading with STP$^{c}$</th>
<th>Excess of STP$^{d}$</th>
<th>Percentage of STP$^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{180}$</td>
<td>1:2.2</td>
<td>1:1</td>
<td>100</td>
<td>2.2</td>
<td>45</td>
</tr>
<tr>
<td>Poly(L-Lys)$<em>{180}$→(Sc-1STP)$</em>{180}$</td>
<td>1:0.55</td>
<td>1:0.39</td>
<td>50</td>
<td>1.42</td>
<td>71</td>
</tr>
<tr>
<td>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{170}$</td>
<td>1:0.63</td>
<td>1:0.45</td>
<td>45</td>
<td>1.36</td>
<td>74</td>
</tr>
<tr>
<td>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{180}$</td>
<td>1:0.54</td>
<td>1:0.42</td>
<td>38</td>
<td>1.28</td>
<td>77</td>
</tr>
<tr>
<td>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{170}$</td>
<td>1:0.22</td>
<td>1:0.19</td>
<td>19</td>
<td>1.11</td>
<td>90</td>
</tr>
<tr>
<td>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{380}$</td>
<td>1:0.18</td>
<td>1:0.17</td>
<td>17</td>
<td>1.05</td>
<td>95</td>
</tr>
<tr>
<td>Poly(L-Lys)$<em>{380}$→(Sc-1STP)$</em>{32}$</td>
<td>1:0.09</td>
<td>1:0.08</td>
<td>8</td>
<td>1.04</td>
<td>96</td>
</tr>
</tbody>
</table>

Sc = Succinyl; STP = synthetic tetrapeptide.

a Molar ratio of available Lys-e-amino groups to HSc-tetrapeptide used; b experimental ratio as determined by amino acid analysis (AA) per cent; c loading of poly(L-lysine); d molar excess of tetrapeptide used related to experimentally obtained loading; e percentage of peptide coupled to polylysine.
Table IV. Molecular masses and content of synthetic tetrapeptide (STP) in synthesized conjugates.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Molecular mass</th>
<th>Amount of bound STP/mg conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg STP</td>
</tr>
<tr>
<td>Poly(L-Lys)&lt;sub&gt;380&lt;/sub&gt;−(Sc-STP)&lt;sub&gt;170&lt;/sub&gt; • HCl</td>
<td>129,000</td>
<td>0.46</td>
</tr>
<tr>
<td>Poly(L-Lys)&lt;sub&gt;380&lt;/sub&gt;−(Sc-STP)&lt;sub&gt;74&lt;/sub&gt; • HCl</td>
<td>91,000</td>
<td>0.28</td>
</tr>
<tr>
<td>Poly(L-Lys)&lt;sub&gt;380&lt;/sub&gt;−(Sc-STP)&lt;sub&gt;34&lt;/sub&gt; • HCl</td>
<td>87,000</td>
<td>0.25</td>
</tr>
<tr>
<td>Poly(L-Lys)&lt;sub&gt;380&lt;/sub&gt;−(Sc-STP)&lt;sub&gt;32&lt;/sub&gt; • HCl</td>
<td>75,000</td>
<td>0.147</td>
</tr>
<tr>
<td>Poly(L-Lys)&lt;sub&gt;180&lt;/sub&gt;−(Sc-STP)&lt;sub&gt;90&lt;/sub&gt; • HCl</td>
<td>65,000</td>
<td>0.48</td>
</tr>
<tr>
<td>Poly(L-Lys)&lt;sub&gt;180&lt;/sub&gt;−(Sc-STP)&lt;sub&gt;70&lt;/sub&gt; • HCl</td>
<td>57,000</td>
<td>0.42</td>
</tr>
<tr>
<td>HSA−(Sc-STP)&lt;sub&gt;16−8&lt;/sub&gt;</td>
<td>70,000</td>
<td>0.04</td>
</tr>
<tr>
<td>HSA−(Sc-STP)&lt;sub&gt;16−18&lt;/sub&gt;</td>
<td>74,000</td>
<td>0.083</td>
</tr>
</tbody>
</table>

using N-ethylpiperidine. After 24 h at 4 °C under control of pH 9, the solution was acidified to pH 2 with 1 N HCl, subsequently dialyzed and freeze-dried as described for the HSA-conjugates. All conjugates were synthesized using the lysine/tetrapeptide ratios listed in Table III. The peptide content and the resulting molecular mass of the conjugates are summarized in Table IV.

Results

Chemical synthesis of the C-terminal tetrapeptide and conjugates

Following the conventional synthetic methods [19] the C-terminal antigenic determinant was built up according to the scheme outlined in Fig. 1. Deblocking with 1.2 N HCl/CH$_3$COOH should not be used, because of possible O-acetylation [22]. Each intermediate and the final products were purified to homogeneity and unequivocally characterized by $^{13}$C NMR, FAB-MS and common analytical methods. Gas chromatography of the N-pentafluoropropionyl-amino acid-n-propyl esters on a chiral phase [20] revealed no racemization.

In order to provide specifically the N-terminal linkage of the tetrapeptide to polymers we used the new cross-linker tert-butyloxycarbonyl-hydrazido-succinic acid (Boc−HSc−OH) [21]. The crosslinker was coupled with 1-hydroxybenzotriazole/N,N'-dicyclohexylcarbodiimide to the tetrapeptide ester to give Boc−HSc−Gly−Pro−Ala−Thr(Bu')−OBu'. Trifluoroacetolytic cleavage yielded the free hydrazide HSc-Gly−Pro−Ala−Thr×CF$_3$COOH ready for azide coupling [23]. The peptide crosslinker is the key product for the preparation of various conjugates with poly(L-lysine) (37,300 and 80,000 daltons) and serum albumine. The reaction times were about 24 h at 4 °C, and the work-up procedure consisted simply of a dialysis and gel-chromatography on Sephadex G 10 in 0.05 M acetic acid.

Depending on the molar ratio of HSc-peptide to the polymers we obtained the conjugates listed in Table IV. Low loading values up to about 20% of available lysine amino groups can be obtained quantitatively by application of the intended amount of HSc-linked antigen. Higher loadings required only a relatively low excess of HSc-linked antigen, e.g. only a two-fold excess for total loading. In the case of coupling to HSA carrier we obtained similar results.

The coupling method with the new crosslinker is recommendable for the reproducible preparation of well defined polymer-antigen conjugates. As outlined in the following, the conjugates were analyzed also by $^{13}$C NMR spectroscopy and circular dichroism, and the results were discussed in context with their antigenic reactivity.

$^{13}$C NMR spectroscopy and circular dichroism

Because of excellent solubilities spectra of the tetrapeptide, and spectra of the tetrapeptide with the crosslinker and the conjugates could be recorded in aqueous solutions. All $^{13}$C NMR signals are assignable by comparison with published data [24] and by J-modulated spin-echo experiments (Table II). The identity of the synthetic products is unequivocally demonstrated in Figs. 2 and 3. No O-acetylation of threonine is detectable. The signals of the crosslinker are easily detectable at 176.4/175.2 ppm (carbonyl) and 30.6/31.7 ppm (methylene). Due to a short dialysis time one poly(L-lysine) conjugate contained succinylated tetrapeptide (Sc-TP) which is evident by appearance of double peaks. Due to a considerable content of $\alpha$-helical conformation in the conjugates loaded with 380 and 170 peptide residues
Fig. 2. a) $^{13}$C NMR spectrum of the conjugate poly(L-Lys)$_{380}$-(Sc-Gly-Pro-Ala-Thr)$_{170}$ in $^2$H$_2$O (100.1 MHz; with J-modulated spin-echo spectrum, above), containing uncoupled succinyl-tetrapeptide (Sc-STP);
b) $^{13}$C NMR spectrum of the conjugate poly(L-Lys)$_{380}$-(Sc-Gly-Pro-Ala-Thr)$_{380}$ in $^2$H$_2$O (100.1 MHz).
Sharpening of Lys$-\text{C}_\alpha$ signals occurs in the random coil conformation at pH 7 [25, 26]. The splitting of the Lys$-\text{C}_\alpha$ signal in partially loaded poly(L-lysine)-tetrapeptide conjugates can be used to estimate roughly the loading of the polymer. Circular dichroism spectra of the free tetrapeptide in water at pH = 2 and 11.5 indicate an almost pH-independent conformation. The prolyl residue is common to all C-termini of various TMV strains. The monoclonal antibody 95 reacts equally well with all strains which reflects the common recognition of this turn. Due to the increasing loading with tetrapeptide the Cotton effect at 207 nm shows a steady increase and a blue shift (Fig. 3). However, at the maximal loading with about 380 tetrapeptide residues we still observe some helical content, both under alkaline and acidic conditions. Thus, a pH-dependent conformational change to random coil as known from unsubstituted poly(L-lysine) does not take place [27]. The spectral changes of the tetrapeptide conjugates with human serum albumine are much less significant indicating the retention of conformation upon binding different amounts of antigens. In conclusion it can be stated that the new crosslinker turned out to be a versatile and efficient tool for the reproducible attachment of an antigen.

**Binding studies with synthetic tetrapeptide conjugates**

The synthetic C-terminal tetrapeptide Gly-Pro-Ala-Thr of TMV coat protein can bind to polyclonal antibodies raised against native TMV (Table V).

Table V. Detection limit of synthetic tetrapeptide conjugates in the indirect ELISA. The detection limit with polyclonal rabbit antibodies directed against TMV *vulgare* was determined by assessment of the immunological reactivity within a 10-fold dilution series of each conjugate, the free synthetic tetrapeptide and the control compounds. This was measured at A 405 nm after 60 min of substrate incubation in glycine buffer. Tetrapeptide concentrations resulting in absorbance values ≥0.1 were considered as detection end-points.

<table>
<thead>
<tr>
<th>Tetrapeptide$^a$ conjugates</th>
<th>Absorbance$^b$ 405 nm</th>
<th>Tetrapeptide concentration [g/ml]</th>
<th>[mol/well]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free STP$^c$</td>
<td>0.064</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$5.8 \times 10^{-8}$</td>
</tr>
<tr>
<td>Sc-STP$^c$</td>
<td>0.413</td>
<td>$8.0 \times 10^{-5}$</td>
<td>$4.6 \times 10^{-8}$</td>
</tr>
<tr>
<td>HSA : (Sc-STP)$_{6-28}$</td>
<td>0.104</td>
<td>$4.9 \times 10^{-10}$</td>
<td>$2.8 \times 10^{-13}$</td>
</tr>
<tr>
<td>HSA : (Sc-STP)$_{16-18}$</td>
<td>0.353</td>
<td>$1.0 \times 10^{-10}$</td>
<td>$5.8 \times 10^{-14}$</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{360}$ : (Sc-STP)$</em>{74}$</td>
<td>0.343</td>
<td>$2.8 \times 10^{-11}$</td>
<td>$1.6 \times 10^{-14}$</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$ : (Sc-STP)$</em>{380}$</td>
<td>0.204</td>
<td>$4.8 \times 10^{-11}$</td>
<td>$2.8 \times 10^{-14}$</td>
</tr>
</tbody>
</table>

$^a$ No immunological reactivity could be detected with non-conjugated carrier molecules (human serum albumin (HSA), polylysine (poly(Lys)$_{380}$) and free threonine, the C-terminal amino acid of TMV *vulgare* coat protein (Table VIII), in concentrations up to $1 \times 10^{-4}$ g/ml; $^b$ each value represents the mean of 8 measurements; $^c$ STP, synthetic tetrapeptide; Sc, cross-linker; Sc-STP, cross-linked synthetic tetrapeptide.
In the indirect ELISA using polyclonal antibodies (Table V) the detection limit of the free peptide is about 5 to 6 orders of magnitude less than that of the peptide conjugated to a carrier. A detection limit of the peptide as low as 16 fmol was obtained using poly(L-Lys)$_{380}$-(Sc-TP)$_{74}$ of a loading of one fifth of the available lysine residues. HSA is a somewhat less highly loadable carrier since there are only 18 lysine residues available for conjugation thus allowing a detection of not less than 58 fmol of the peptide (Table V). The indirect ELISA was chosen because of a sensitivity superior to that of the direct double antibody sandwich ELISA by 3 to 4 orders of magnitude using the same quantity of reagents (data not shown).

The immunological reactivity of various tetrapeptide conjugates depends on the size of the poly(L-lysine) molecule provided they carry about the same amount of peptides (Table V). Obviously, the peptides are more available for binding when coupled to a larger carrier molecule, thus reducing steric hindrance in the reaction with antibodies. In order to optimize the sensitivity of detection of the immunological reactivity of peptides conjugated to poly(L-lysine), conjugates in different molar ratios were produced (Table IV). Furthermore, in the indirect ELISA the diethanolamine substrate buffer was used which enhanced the enzymatic activity of the alkaline phosphatase, and thus the sensitivity could be increased almost 3 fold. With all the molar proportions tested, the detection limit was found to be 5.8 fmol tetrapeptide (Table VI), considering absorbance values 0.1 as detection end-points. Again, the sensitivity of the direct ELISA was inferior to that of the indirect ELISA by 3 orders of magnitude (data not shown).

With the most efficient ELISA and peptide conjugates (HSA$_{18}$: (Sc-TP)$_{16-18}$ and poly(L-Lys)$_{380}$: (Sc-TP)$_{74}$) monoclonal antibody 95 was found to react specifically with the synthetic tetrapeptide as well as with the native TMV _vulgare_ and with its isolated coat protein monomers (Fig. 4). Thus, this epitope is exposed on the surface of both, the assembled virion and also the isolated coat protein monomers. Comparative data (Fig. 4) indicate

![Fig. 4. Reactivity of monoclonal antibody 95 with native TMV _vulgare_ (○—○), TMV coat protein monomers (●—●) and the synthetic tetrapeptide Gly–Pro–Ala–Thr homologous to the C-terminal end poly(L-Lys)$_{380}$–(Sc-TP)$_{4}$ (▲—▲). The absorbance values of the indirect ELISA (Table I) were measured after 2 h of substrate incubation at 37 °C and expressed on the basis of 10 μg/ml vins for coating. Each value represents the mean of 6 separate measurements.](image)

**Table VI. Immunological reactivity of polylysine-linked synthetic tetrapeptide in the indirect ELISA.** The immunological reactivity (A 405 nm) was assessed with rabbit polyclonal antibodies directed against native TMV _vulgare_. This was measured after 60 min of substrate incubation in diethanolamine substrate buffer.

<table>
<thead>
<tr>
<th>Tetrapeptide$^a$ conjugates</th>
<th>Concentration of linked tetrapeptides [fmol/well]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>580</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$: (Sc-TP)$</em>{380}$</td>
<td>2.000$^c$</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$: (Sc-TP)$</em>{370}$</td>
<td>2.000</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$: (Sc-TP)$</em>{36}$</td>
<td>2.000</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$: (Sc-TP)$</em>{32}$</td>
<td>2.000</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$: (Sc-TP)$</em>{30}$</td>
<td>2.000</td>
</tr>
<tr>
<td>Poly(Lys)$<em>{380}$: (Sc-TP)$</em>{30}$</td>
<td>2.000</td>
</tr>
</tbody>
</table>

$^a$ No immunological reactivity could be detected with non-conjugated carrier molecules (poly(Lys)$_{380}$, polylysine; Sc-TP, cross-linked synthetic tetrapeptide).$^b$ Each value represents the mean of 8 measurements.

---
that the synthetic peptide coupled to poly(L-lysine) is approximately ten times more efficient in binding the monoclonal antibody, than are the native virus or its isolated coat protein monomers.

Monoclonal antibody 95 binds not only to TMV vulgaris but with comparable strength to the assembled particle and the coat protein monomers of the TMV strains dahlemense and Holmes' Ribgrass as well (Table VII). This indicates a common C-terminal epitope regardless of one or even two amino acid substitutions (Table VIII).

The preincubation of TMV with monoclonal antibody 95 before the bioassay of the virus on tobacco plants neutralized the infectivity almost entirely. The amount of neutralization depended on the proportion of virus particles added to a constant amount of antibody molecules (Table IX). Inhibition of virus infectivity by binding of monoclonal antibody 95 was specifically reduced by more than 50% when the antibody was preincubated with a 45-fold excess per antibody binding site of the synthetic peptide linked to poly(L-lysine). By addition of the respective concentration of the free tetrapeptide no capacity to reduce the monoclonal antibody-mediated neutralization could be observed.

Using the synthetic tetrapeptide coupled to Sepharose 4B antibodies specific for the C-terminal epitope of TMV coat protein could be purified by affinity chromatography. Monoclonal antibodies from ascitic fluids or hybridoma culture supernatants and polyclonal, monospecific antibodies from rabbit and mouse antisera were eluted successfully from the immunoabsorbent with 0.1 M HCl, pH 2.5.

### Table VII. Immunological reactivity of monoclonal antibody 95 with various TMV strains and their isolated coat protein monomers in the indirect ELISA. TMV strains and their coat protein monomers in concentrations of 10 µg/ml were used for coating of microtiter plates and the indirect ELISA performed as described (Table I). Binding of affinity purified monoclonal antibody (10 µg/ml), expressed as absorbance values, was measured after incubation of substrate for 1 h at 37 °C.

<table>
<thead>
<tr>
<th>TMV strains and their coat protein</th>
<th>Immunological reactivity (A405 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vulgaris</td>
<td>0.222</td>
</tr>
<tr>
<td>Vulgaris protein</td>
<td>0.215</td>
</tr>
<tr>
<td>Dahlemense</td>
<td>0.184</td>
</tr>
<tr>
<td>Dahlemense protein</td>
<td>0.218</td>
</tr>
<tr>
<td>Holmes' Ribgrass</td>
<td>0.176</td>
</tr>
<tr>
<td>Holmes' Ribgrass protein</td>
<td>0.206</td>
</tr>
</tbody>
</table>

### Table VIII. C-Terminal amino acid sequence of TMV strains.

<table>
<thead>
<tr>
<th>Virus strains</th>
<th>C-Terminal amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMV vulgaris</td>
<td>Gly-Leu-Val-Trp-Thr-Ser</td>
</tr>
<tr>
<td>TMV dahlemense</td>
<td>Gly-Leu-Val-Trp-Thr-Ser</td>
</tr>
<tr>
<td>TMV Holmes' Ribgrass</td>
<td>Leu-Pro-Trp-Thr-Ser</td>
</tr>
</tbody>
</table>

According to Wittmann-Liebold & Wittmann [24].

### Table IX. Neutralization of TMV infectivity with monoclonal and polyclonal antibodies.

<table>
<thead>
<tr>
<th>Purified antibody sample</th>
<th>Inhibition of infectivity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1×10⁻⁴ g/ml]</td>
<td>TMV concentration [g/ml]</td>
</tr>
<tr>
<td></td>
<td>1×10⁻⁶</td>
</tr>
<tr>
<td>MCA 95</td>
<td>93.6</td>
</tr>
<tr>
<td>Monospecific polyclonal rabbit antibodies against MTV</td>
<td>93.1</td>
</tr>
<tr>
<td>Polyclonal antibodies of non-immunized rabbits</td>
<td>35.2</td>
</tr>
<tr>
<td>Ratio molecules antibody: TMV</td>
<td>2.67×10²:1</td>
</tr>
</tbody>
</table>

* Inhibition of infectivity (%) = 100 - lesions/half-leaf (TMV + antibody) / lesions/half-leaf (TMV) × 100.

Each value is based on the mean of lesion counts on 15 half-leaves of Xanthi tobacco.
Discussion

Using a synthetic antigenic determinant and a monoclonal antibody possessing the binding site specific for this determinant the corresponding region on the native virus, an epitope was determined and thus confirmed [2]. By screening with the tetrapeptide a monoclonal antibody (95) was found, which reacted with the native epitope (Fig. 4) exposed on both the assembled virus and isolated coat protein monomers. Due to the relatively free mobility of the C-terminal end of proteins the structural resemblance between this native protein segment and the synthesized and conjugated C-terminal peptide is high [29]. One may explain the about ten times higher reactivity of the tetrapeptide conjugate (Fig. 4) by an even better accessibility to the monoclonal antibody.

X-ray crystallographic analysis of the coat protein monomer [30, 31] and investigations using TMV-specific antibodies were used to locate eight epitopes [1, 32–34]. Attempts to determine the antigenic structure of the C-terminal region using polyclonal antibodies and synthetic peptides conjugated to p-amino benzoic acid have been reported by Anderer and Stroebel [2] who postulated an epitope within the six C-terminal amino acids. Indeed, a sequence of four to five amino acids has been described to be sufficient for defining an epitope [35, 36].

With the selected monoclonal antibody 95 a cross-reacting epitope, common to three related TMV strains with different C-terminal sequences (Table VIII) was recognized (Table VII). Another example of cross-reactivity of monoclonal antibodies tolerating even multiple amino acid exchanges within the sequence 103–112 of TMV coat protein has been reported recently [4]. The binding of monoclonal antibody 95 to all three strains of TMV (crossreactivity) may be attributed to comparable polarity and size of the exchanged amino acids and the proposed [2, 33] flexible reversed turn of the C-terminal region. The turn inducing proline residue seems to be crucial in determining the antigen-combining structure and thereby the binding with the monoclonal antibody. However, more detailed conclusions would require a repetition of this study using the synthetic tetrapeptides Ala–Pro–Ala–Ser (TMV dahlemense) and Ala–Pro–Ala–Thr (TMV Holmes' Ribgrass) as well.

A biological function within the process of virus infection can be ascribed to this C-terminal epitope. Infectivity of TMV vulgare was reduced by 75–94% depending on the molar ratio of virus and monoclonal antibody (Table IX). The specificity of this neutralization of infectivity could be verified by blocking the monoclonal antibody by pretreatment with the synthetic epitope linked to poly(L-lysine). No blocking of neutralization could be observed using the free synthetic tetrapeptide, presumably because of lower affinity for the antibody. An excess of antibodies over the theoretical amount of 2130 antibody binding sites on each TMV particle was adjusted to obtain quantitative results. The C-terminal tetrapeptide was discerned as a critical region for TMV neutralization because of the specific blocking by monoclonal antibody 95. This is in accordance with results reported with polyclonal antisera raised against synthetic peptides of the C-terminal region [18, 36]. The hypothesis of stabilization of the viral capsid by the binding of specific antibodies which thus inhibit the liberation of RNA ("uncoating") was supported by the strong binding of the monoclonal antibody to the C-terminal epitope (Fig. 4) and the subsequent inhibition of infectivity (Table IX). Other monoclonal antibodies, directed against other epitopes on TMV also caused inhibition of infectivity, but not to the extent as did No. 95 [9]; furthermore, their respective epitopes were not yet available in a synthesized form.

The knowledge of the antigenic topography and function of epitopes exposed on viral coats will be advantageous for improvement of immunological diagnosis [37], serotyping and epidemiological studies of plant viruses. For the majority of plant viruses, the topography of their coat protein is not yet known. Prediction methods will reveal exposed potentially immunogenic sequences. By application of the corresponding synthetic peptides, suitably coupled to a carrier for screening the respective monoclonal antibodies, antigenic structures of such plant viruses may be determined. Another advantage of synthetic epitopes of plant viruses is their application in affinity chromatography, immobilized on a gel, for the purification of the respective monoclonal antibody from hybridoma cultures or polyclonal antibodies with predetermined specificity from antisera.

We would like to thank Mrs. G. Eggers-Schumacher for excellent and persistent technical assistance.
New Cross-Linker Tested in Antigen-Antibody Reactions

Note added in proof: Recently, Dietzgen and Zaitlin, Virology 155, 262–266 (1986) demonstrated the cross-reactivity of MCA 95 with the large subunit of the host enzyme ribulose-1,5-bisphosphate carboxylase. The reactivity of MCA 95 with TMV strains dahlemense and Holmes’ Ribgrass (Table VII) is unaffected by this finding, since purified virus preparations did not contain detectable amounts of this enzyme.