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Solid Phase Synthesis of Glycopeptide Dendrimers with Tn Antigenic Structure and their Biological Activities. Part I

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Abstract: Multiple antigenic peptides containing dimeric Tn antigen [Ac-(Tn)$_2$-γ-Abu]$_4$-(Lys-X)$_2$-Lys-β-Ala (**V**: X = 0; **VIII**: X = γ-Abu) and [Ac-(Tn)$_2$-γ-Abu]$_8$-(Lys-X)$_4$-(Lys-X)$_2$-Lys-β-Ala (**XI**: X = 0; **XIV**: X = γ-Abu), immobilized on biocompatible Tenta Gel S NH_2 support were prepared by SPPS. Rosetting tests of **V**, **VIII**, **XI** and **XIV** showed positive reactions with anti-Tn (DAKO) and Tn+ erythrocytes, with anti-Tn/A (BRIC 66) and Tn+ and A erythrocytes, other combinations were negative. In all the animals immunized with **XIV**, we found a remarkable increase in the level of anti-Tn (titre 2000–64000, score 105–167) and no change of anti-A levels (titre 8, score 13–17). Neither non-immune nor immune sera showed any reactivity with T^+, Cad^+ and blood group O erythrocytes. Immunized mice did not exhibit any sign of adverse reaction to the administered conjugates. Biological activities were correlated with molecular modelling and molecular dynamic calculations. The biological activities of these synthetic Tn antigen conjugates (good availability for the immunological interactions, highly specific immunogenicity, good biological tolerance) together with their precise chemical characterization seem to be a promising approach to preparation of anti-tumour vaccine and affinity purification of anti-Tn antibodies. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid phase peptide synthesis (SPPS); glycopeptide dendrimers; multiple antigen glycopeptide systems (MAGs); Tn antigens; tumour associated antigens; molecular modelling

INTRODUCTION

Cell-surface carbohydrates undergo dramatic changes as a consequence of malignant transformation. The alteration results from either incomplete glycosylation or neoglycosylation of tumour cells leading to an accumulation of precursors or neostructures. Over the past decade studies with specific monoclonal antibodies have identified a number of tumour-associated carbohydrate antigens [1] expressed on glycolipids, glycoproteins, or both. The antigens identified exclusively on glycoproteins are derived from the precursors of O-linked carbohydrate chains and include Tn (GalNAcα1→O-Ser/Thr) [2–5], sialosyl-Tn (NeuNAcα2→6GalNAcα1→O-Ser/Thr) [6] and T (Galβ1→3GalNAcα1→O-Ser/Thr) [4,7] antigens. Among the large number of known tumour-associated carbohydrate antigens Tn and sialosyl-Tn antigens are the most specific to human cancer, and the most restrictive in their expression on normal cells and tissues.

Tn, sialosyl-Tn, and T antigens are expressed in carcinoma-associated mucins [4,5,8–11]. Mucins are highly O-glycosylated, high molecular weight glycoproteins expressed on endodermal epithelial cells, particularly those showing glandular secretory activity. The Tn structure in normal cells is

Abbreviations: AAA, amino acid analysis; DCM, dichloromethane; DIEA, diisopropylethylamine; DMAP, *N*,*N*-dimethyl-4-aminopyridine; MAPs, multiple antigen peptide system; RT, retention time; TG, Tenta Gel.

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cryptic since it is further glycosylated to construct complex O-linked glycans of mucin-type glycoproteins, whereas in most human carcinomas this cryptic structure is exposed at the surface due to an incomplete synthesis of carbohydrate chains. Increased expression of Tn and sialosyl-Tn antigens has been correlated with tumour aggressiveness and a poor prognosis in a number of epithelial tumours [5,8]. Tn antigen is expressed in over 70% of breast, lung, colon, and stomach carcinomas [4,5]. Tn antigens were found also on gp160 and gp120 of human immunodeficiency virus (HIV) and *in vitro* neutralization effect of anti-Tn antibodies was shown [12].

The ultimate goal of studies devoted to Tn antigen is the design and development of a cancer vaccine using chemically well-characterized synthetic antigens as immunogens [5,6,13,14]. For example cancer vaccine based on Tn antigen will be of particular interest because of the potential usefulness in treating metastatic breast cancer [15–17]. In spite of the striking development of gene techniques, biotechnological production of glycopeptides or glycoproteins is still limited. Consequently, chemical synthesis is the best way of supplying these compounds in large amounts and defined structures. Chemical syntheses of tumour antigens are therefore of utmost importance for the study and diagnostics of tumour processes and for the preparation of anti-tumour vaccines with the aim to suppress tumour growth [18–20].

Syntheses of monomeric Tn antigen [21] and dimeric $(Tn)_2$ antigen were described [18,19,22,23]. The syntheses are multistep and use D-galactose as a starting material. The chemistry of glycopeptides in general and of O-glycopeptides especially, was reviewed [24–27] and will not be discussed here in more detail.

Synthetic antigens are generally poor immunogens. This shortcoming has been, partially at least, overcome by the use of conjugates of synthetic antigens with immunogenic protein carriers. Although the use of protein carriers is relatively effective in the production of antibodies, these conjugates are ambiguous in chemical composition and structure. Since most carbohydrate–protein interactions are usually of low affinities, targeting cell surface carbohydrate receptors or inhibiting host infections by pathogens are of limited success with single carbohydrate residues. Multivalent glycoconjugates can override the low affinity of carbohydrate–protein interactions [28].

In order to solve this problem, Tam [29] described a new approach based on branched oligolysine core called Multiple Antigen Peptide (MAP). Later the term peptide dendrimer [30,31] was used too. The prepared MAP is then used for biological tests either still bound to the resin [32] (with deprotected amino acid side-chains) or in a free soluble form (i.e. deprotected and detached from the resin used). Purification of MAPs has been described [30,31,33] and HPLC-pure compounds fully characterized by amino acid analysis, NMR, mass spectrometry, etc. were prepared. The biological activities [34] and chemical syntheses [35] of MAPs have been reviewed.

Two areas of MAP application can be distinguished: (1) as a highly efficient immunogen and (2) as an efficient antigen for detection in immunoassays. Immunization with MAPs offers many advantages in comparison with the classical carrier–peptide construct approach. In the MAPs, most of the molecule (sometimes more than 90%) consists of the peptide antigen in contrast to the carrier–peptide conjugate where the peptide, in general, forms only a small part of the construct. Probable immunodominant or even suppressor determinants of the carrier protein can be also circumvented by using a MAP. Moreover MAPs are well defined compounds and the amount of peptide anchored on a MAP is exactly known, in contrast to the peptide dose administered by a peptide–carrier conjugate. All of these characteristics make MAPs extremely useful for application as an immunogen or as a synthetic vaccine. The MAPs are also very useful as antigens in solid phase immunoassays (ELISA) because coating to polymer surfaces leads to more efficient epitope exposition than that obtained from free low molecular weight peptides. On the basis of the aforementioned data we decided to use MAPs containing Tn determinants for our immunological studies. The aim of our study was the chemical synthesis of MAPs with bound Tn antigen having different dendrimer valency. We decided to use biocompatible Tenta Gel resin as a non-detachable support for our MAPs, because it is known from confocal laser microscopy and NMR spectroscopy studies, that the structure of Tenta Gel beads enables penetration of high molecular weight compounds to the core of the beads [36]. The immunochemical aim was to study immunogenicity and antibody binding capacity of Tn-MAPs in relation with their chemical structure.

MATERIALS AND METHODS

General Procedures

Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter at 23°C. Analytical RP HPLC was performed with a Spectra-Physics 8700 apparatus (Darmstadt, Germany) equipped with a column (250 × 4 mm) filled with Separon SGX-RPS, particle size 10 μm (Tessek, Prague). The chromatography was performed in a gradient system of methanol–water (2%/min, both phases contained 0.1% TFA) starting with 20% of methanol. Flow rate 1.0 mL/min, detection at 220 nm. Preparative RP HPLC was performed with a Knauer apparatus (Bad Homburg, Germany) equipped with a column (250 × 10 mm) filled with Separon SGX-RPS, particle size 10 μm (Tessek, Prague). The preparative chromatography was performed in a gradient of methanol–water (1%/min, both phases contained 0.1% TFA). Positive-ion FAB spectra were recorded on a ZAB-EQ mass spectrometer (VG Analytical, Manchester, UK), with an Ion-Tech gun and glycerol or glycerol + thioglycerol matrix.

Amino acid analyses were carried out on a BIOCHROM-20 PHARMACIA Amino Acid Analyzer (Biotech, UK) by an unmodified program. Under these conditions $GalNH_2$ is eluted in the position of Phe, but Phe was not present in our samples. Samples were hydrolysed in 6 N HCl at 110°C for 20 or 72 h or in 4 N HCl at 110°C for 8 h.

Tn^+ erythrocytes were obtained from Red Cross BTS in Brisbane, Australia (donor DIN 4496511) and from Gamma Biologicals Inc., Houston, TX (donor C.L.), Cad^+ erythrocytes from Mackay Memorial Hospital, Taipei (donor 852-4630376), all through the SCARF International Exchange Group (Gamma Biologicals). T^+ erythrocytes were prepared from normal erythrocytes after digestion with neuraminidase (Sigma). Normal erythrocytes of all blood groups (A, B, O) were from regular blood donors of the Transfusion Department of the Institute of Hematology, Prague. Tn, Cad and T antigens were confirmed with reactions with the Lectin kit (Gamma Biologicals). Monoclonal antibodies were IgM anti-Tn (DAKO-HB-Tnl, DAKO, DK), anti-Tn/A (BRIC 66, IBGRL Research Products, UK) and anti-A (Birma-l, Gamma Biologicals). A rosetting test was done as incubation of Ac-$(Tn)_2$-containing MAPs **V**, **VIII**, **XI** and **XIV** (see Table 3) and analogous structures **IV**, **VII**, **X** and **XIII** (without Tn antigen as a negative standard, respectively) with different antibodies followed with washing and incubation with Tn^+ and blood group A red cells and final microscopic observation of the beads. Inhibition was compared after 1 h incubation of monoclonal anti-Tn with equal amounts of **V**, **VIII**, **XI**, **XIV**, **X** and **XIII**. Evaluations of inhibition and immunization studies were done by agglutination in the standard tube method.

For the immunization **XIV** and **XIII** with/without antibodies were suspended in phosphate buffered saline and sonicated with a probe sonicator (diameter 4 mm). Five female balb/c mice, 4–6 weeks old, were immunized with **XIV** (E1–E5), two with **XIII** without Tn antigen as a negative control (C1–C2). Five doses of 300 μg of conjugate were administered subcutaneously (without adjuvans) at *ca.* 3 week intervals. Sera and spleen cells were collected after the fifth immunization.

Molecular Modelling and MM and MD Calculations

The initial structures were modelled *de novo* by the Builder module of the InsightII program package (MSI) and then optimized by molecular mechanics. The steepest descent method was used for optimization of modelled structures by molecular mechanics (MM) with a criterion of convergence 0.1 followed by approximately 5000 steps of the conjugate gradient method with criterion of convergence 0.001. The models were then soaked in a precomputed box of water and minimized again before applying molecular dynamics. The dynamics of compounds were carried out both *in vacuo* and in water, with neutral side-chains. Dynamics were run at room temperature for 30 ps with equilibration at 300 K for 2 ps with the AMBER force field implemented in the Discover95 module of the InsightII environment. The calculations were carried out with the side-chains in their uncharged states. All non-restrained MD simulations employed the Verlet velocity integration algorithm with a 1 fs time step.

The resulting trajectory was analysed for transition between conformational states. Minimization was performed periodically during the trajectory to characterize the conformational states being sampled. The whole trajectory was divided into ten conformational groups based on the similarity between structures and finally minimized. A set of ten different conformers was obtained and classified with respect to their potential energy. Only the lowest energy conformers were taken into account for structural comparison between different classes of

Table 1 List of Prepared Resin Bound Peptides and Glycopeptides

Compound number	Compound
III	[Boc-Lys(Boc)]$_2$-Lys-β-Ala-NH-TG
IV	[Ac-Lys(Ac)]$_2$-Lys-β-Ala-NH-TG
V	[Ac-(Tn)$_2$-γ-Abu]$_4$-Lys$_2$-Lys-β-Ala-NH-TG
VI	[Boc-Lys(Boc)-γ-Abu]$_2$-Lys-β-Ala-NH-TG
VII	[Ac-Lys(Ac)-γ-Abu]$_2$-Lys-β-Ala-NH-TG
VIII	[Ac-(Tn)$_2$-γ-Abu]$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG
IX	[Boc-Lys(Boc)]$_4$-Lys$_2$-Lys-β-Ala-NH-TG
X	[Ac-Lys(Ac)]$_4$-Lys$_2$-Lys-β-Ala-NH-TG
XI	[Ac-(Tn)$_2$-γ-Abu]$_8$-Lys$_4$-Lys$_2$-Lys-β-Ala-NH-TG
XII	[Boc-Lys(Boc)-γ-Abu]$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG
XIII	[Ac-Lys(Ac)-γ-Abu]$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG
XIV	[Ac-(Tn)$_2$-γ-Abu]$_8$-(Lys-γ-Abu)$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG

compounds. Solvent was not included in the systems with the Tn antigens, because the interest was not in the conformation of compounds in solution, but rather in conformers bound to their receptor. For this purpose only the simulation *in vacuo* was chosen in these cases.

Peptide Synthesis

All Boc-amino acids were obtained from Bachem (Bubendorf, Switzerland). Peptide synthesis was performed manually by the solid phase method [37] with Boc/Bzl strategy. Protected amino acids used: Boc-Lys(Boc)-OH, Boc-γ-Abu-OH and Boc-β-Ala-OH. The solid support was Tenta Gel S NH_2 (Rapp Polymer, Tuebingen, Germany), S 30902, 90 μm, 0.28 or 0.25 mmol NH_2/g. The synthetic protocol was: (1) DCM, 1 × 30 s; (2) 55% TFA in DCM, 5 + 25 min; (3) DCM, 4 × 1 min; (4) 10% DIEA in DCM, 2 × 2 min; (5) DCM, 2 × 1 min; (6) DMF, 1 × 1 min; (7) 3-fold molar excess of Boc-AA, HOBt/DCC, preactivation in minimum of DCM–DMF 9:1 (v/v) for 20 min at room temperature. The precipitated dicyclohexylurea was filtered off, washed with a small amount of DCM and the filtrate concentrated *in vacuo*, dissolved in DMF to an approximate concentration of 0.2 mol/L and added to the resin. After 60 min the Kaiser test [38] was done. When positive, DMAP was added and the reaction continued for another 2 h. When the Kaiser test was positive again, solvents were filtered off, the resin was neutralized, washed twice with DMF and the coupling step was repeated with 3-fold molar excess of BOP reagent and Boc-AA and 6-fold excess of DIEA in DMF; (8) DMF, 1 × 1 min; (9) DCM, 3 × 1 min.

During the synthesis of octavalent dendrimers, starting at the third Lys level and next amino acids, the condensation reactions were forced to completion by elevated temperature (50°C). When the Kaiser test was still positive, we used 'magic mixture' [39] and sonication.

At the end of the synthesis, the Boc group was cleaved off by treatment with TFA, the resin was washed with DCM, neutralized as described above and acetylated with Ac_2O–pyridine (1:2, v/v, 10-fold molar excess) for 30 min. The peptide resin was then thoroughly washed with DMF, DCM, ethanol and ether (four times each) and dried.

Before the deprotection of O-acetyl protecting groups, the resin bound dendrimer was washed by DMF, DCM and absolute MeOH (4 × each). The O-acetyl groups were deprotected by treatment with $NH_2NH_2 \cdot H_2O$ (15 mL) in MeOH (100 mL) for 2 × 1 h in accordance with Reference [40], with the exception of a shorter reaction time. Then the resin was washed five times with DMF, ethanol and ether, respectively. The prepared resin peptides and glycopeptides are given in Table 1 and their amino acid analyses are given in Table 2.

H-Ser(3,4,6-tri-O-Ac-D-GalNAc-1-α)-OH (I)

Hydrogenolysis of Z-Ser(3,4,6-tri-O-Ac-D-GalNAc-1-α)-OBzl [18,19,21] (10.24 g, 15.55 mmol) was done in 500 mL of methanol with 2 g of 10% Pd/C as catalyst. After 6 h stirring at ambient temperature the catalyst was filtered off, washed with methanol and the filtrate was concentrated *in vacuo* to approximately 50 mL. Diethyl ether (50 mL) and petroleum ether (50 mL) were added. Crystallization at 4°C overnight afforded 6.59 g (97.6%) of product,

Table 2 Amino Acid Analyses of Peptide and Glycopeptide Dendrimers Prepared (Values for Ser and $GalNH_2$ were Corrected for the Decomposition During Hydrolysis, see Experimental Part)

Compound	β-Ala	Lys	γ-Abu	Ser	$GalNH_2$
IV[a]	1 (1)	2.8 (3)	–	–	–
V[a]	1 (1)	2.8 (3)	3.7 (4)	7.6 (8)	7.9 (8)
V[b]	1 (1)	2.8 (3)	3.8 (4)	7.8 (8)	8.1 (8)
VII[a]	1 (1)	2.8 (3)	1.8 (2)	–	–
VIII[c]	1 (1)	2.8 (3)	5.9 (6)	7.8 (8)	8.2 (8)
VIII[a]	1 (1)	2.7 (3)	5.8 (6)	7.6 (8)	7.8 (8)
VIII[b]	1 (1)	2.8 (3)	5.9 (6)	7.8 (8)	7.9 (8)
X[a]	1 (1)	6.9 (7)	–	–	–
XI[a]	1 (1)	6.9 (7)	6.6 (8)	12.9 (16)	12.5 (16)
XI[b]	1 (1)	6.9 (7)	6.9 (8)	12.6 (16)	12.4 (16)
XIII[a]	1 (1)	7.4 (7)	5.7 (6)	–	–
XIV[a]	1 (1)	6.8 (7)	12.5 (14)	12.5 (16)	12.4 (16)
XIV[b]	1 (1)	6.8 (7)	12.5 (14)	12.4 (16)	12.2 (16)
XIV[c]	1 (1)	6.7 (7)	12.4 (14)	12.8 (16)	12.3 (16)

[a] 20 h hydrolysis in 6 N HCl.
[b] 72 h hydrolysis in 6 N HCl.
[c] 8 h hydrolysis in 4 N HCl.

m.p. 175–177°C (decomposition), $[\alpha]_D$ +99° (c 0.5, MeOH). Reference [21]: m.p. 177°C (decomposition), $[\alpha]_D$ +93° (c 0.5, MeOH); Reference [18]: m.p. 174–175°C, $[\alpha]_D$ +88° (c 0.1, MeOH). The product is homogeneous on TLC (Silufol, Kavalier, Czech Republic) in *n*-butanol/AcOH/H_2O (4:1:1, v/v/v) with R_f 0.2 (detection by ninhydrin or heating). The starting compound has R_f 0.75 (detection by UV or heating). HPLC of the product: purity 96%, RT 12.62 min. For $C_{17}H_{26}N_2O_{11}$ calc. monoisotopic m.w. 434.15; FAB MS (MeOH) found: 435.1 $[M+H]^+$ (100%), 457.1 $[M+Na]^+$ (35%) and fragmentation ion 330.1 [sugar oxonium ion]$^+$ (70%).

Study of Acidic Hydrolysis and Decomposition of I. The analysis was done from two samples, twice in any case. The values found differed in all cases for less than ±10%. The results for hydrolysis done 8 h in 4 N HCl at 110°C: The average value found for Ser was 82.9% and for $GalNH_2$ 83.9% of theory. The ratio of Ser to $GalNH_2$ was 1:1.01. For 20 h hydrolysis in 6 N HCl at 110°C the average value found for Ser was 73.6% and for $GalNH_2$ 48.2% of theory. The ratio of Ser to $GalNH_2$ was 1:0.65. For 72 h hydrolysis in 6 N HCl at 110°C the average value found for Ser was 63.5% and for $GalNH_2$ 7.4% of theory. The ratio of Ser to $GalNH_2$ was 1:0.12. These values were used for corrections of amino acid analysis data of all glycopeptides at the given conditions.

Boc-Ser(3,4,6-tri-O-Ac-D-GalNAc-l-α)-OH (II)

A solution of **I** (2 g, 4.6 mmol) in methanol (80 mL) containing DIEA (0.81 mL, 4.6 mmol) was treated with Boc_2O (1.10 mL, 4.64 mmol) at ambient temperature for 2 h. When the reaction was finished (TLC control), the solvents were evaporated *in vacuo* to dryness and the residue was triturated by petroleum ether (2 × 100 mL). Purification by RP-HPLC yielded **II** (2.1 g, 85.4%) as a colorless foam: $[\alpha]_D$ +107° (c 1.5, acetone). Literature data: Reference [18]: $[\alpha]_D$ +105° (c 1.5, acetone). For $C_{22}H_{34}N_2O_{13}$ calculated monoisotopic m.w. 534.21, FAB MS (MeOH) found $[M+H]^+$ 535.20.

RESULTS AND DISCUSSION

As a building block for incorporation of the Tn antigen we used Boc-Ser(3,4,6-tri-O-Ac-D-GalNAc-1-α)-OH (**II**) [18]. The synthesis was performed starting from Z-Ser(3,4,6-tri-O-Ac-D-GalNAc-1-α)-OBzl [18,19,21]. Catalytic hydrogenation afforded H-Ser(3,4,6-tri-O-Ac-D-GalNAc-1-α)-OH (**I**) [18,21]. **I** was transformed by Boc_2O to **II** and this protected building block was used for SPPS.

High efficiency of chain assembly and steric accessibility of the growing peptide anchored on a carrier play a key role in successful peptide synthesis in solid phase. The problem is even more pro-

nounced with clustered peptide chains, e.g. cascade branched MAPs. Tam and Lu [41] described coupling difficulties associated with interchain clustering and phase transition during MAP synthesis. We supposed that the insertion of a suitable spacer (e.g. β-Ala [42] or γ-Abu) into the branching lysine structure could have a positive effect on both the synthesis and also on immunological properties. We decided to evaluate the use of γ-Abu as a spacer and tried to compare its influence on coupling efficiency during synthesis and also to immunological activity.

Therefore dendrimers **V**, **VIII**, **XI**, and **XIV** (Table 1) were prepared. As negative standards for immunological tests we synthesized also the corresponding structures without Tn antigen, i.e. **IV**, **VII**, **X** and **XIII**. Due to the advantageous properties of Tenta Gel (e.g. mechanical stability, swelling in many solvents, biocompatibility) we used Tenta Gel S NH_2 for synthesis of the polymer-supported Tn antigen–MAPs conjugate.

Both tetrameric and octameric MAPs were assembled by conventional solid-phase methodology using Boc chemistry. The couplings were performed with DCC/HOBt or BOP/HOBt activation, DMF was used as solvent. The completness of reactions was checked by qualitative ninhydrin test [38]. After attachment of the β-alanyl spacer on the TG resin the lysine core was constructed by coupling successively either two levels of Boc-Lys(Boc)-OH, providing four amino groups or three levels of the Lys derivative providing eight amino groups. The lysine core was first elongated by γ-Abu as a spacer [18] and then twice by Tn antigen building block **II**. During the syntheses of tetravalent MAPs with γ-Abu inserted into the lysine core and even more during the syntheses of octavalent MAPs (both with and without γ-Abu inserted into the lysine core) we met with coupling difficulties associated with interchain clustering and phase transition [41]. Improvements were observed using aprotic polar solvent mixtures as well as sonication under elevated coupling temperature to 50°C for 30 min. Amino acid analyses of prepared peptides and glycopeptides are given in Table 2.

After completion of the synthesis, the Boc groups were split off by 50% TFA/DCM and amino groups were acetylated (Ac_2O/DMF) yielding TG bound peptides **IV**, **VII**, **X** and **XIII** (without Tn antigen). Deprotection of O-acetyl groups from Tn-MAPs was done by NH_2–$NH_2 \cdot H_2O$ [40]. The resin bound glycopeptides **V**, **VIII**, **XI** and **XIV** were used for biological studies.

Because the TG-resin used in this paper was a non-cleavable one and therefore purification of the final product was not possible, the attaining of maximum coupling yield was of paramount importance. In order to evaluate the synthetic efficiency of peptides and glycopeptides which remain fixed to the resin it was necessary to elaborate reliable conditions for amino acid analysis including estimation of acidic decomposition of Ser and $GalNH_2$ and their separation in the presence of Lys, β-Ala and γ-Abu. Therefore compound **I** was hydrolysed under three different conditions: the analyses were determined from two samples, twice in any case. The values found differed in all cases by less than 10% (for details see Materials and Methods). The results of 8 h hydrolysis in 4 N HCl at 110°C, 20 h hydrolysis in 6 N HCl at 110°C and 72 h hydrolysis in 6 N HCl at 110°C were used for corrections of amino acid analysis data of all glycopeptides at the given conditions.

From the aforementioned data it follows that the molar amount of found $GalNH_2$ was lower in every case than for Ser and with stronger hydrolysis conditions it lowers. All amino acid analysis data were therefore corrected by the previously mentioned decomposition factors for the given condition. Basak *et al.* [33] described that for MAPs only 72 h hydrolysis gave reliable results of AAA but according to our results the obtained values depend on the composition of peptide or glycopeptide bound to MAP. Longer hydrolysis releases more stable amino acids (e.g. Lys, β-Ala, and γ-Abu) but destroys the unstable ones like Ser and $GalNH_2$. Using the corrections for acidic decomposition, we found both 20 and 72 h hydrolysis fully comparable. On the basis of AAA data we found that the composition of all peptide dendrimers **IV**, **VII**, **X** and **XIII** (without Tn antigen) and both tetravalent Tn-MAPs **V** and **VIII** was in accord with their structure. AAA of octavalent Tn-MAPs **XI** and **XIV** has shown good results for the oligolysine core but the incorporation of the determinant Ac-$(Tn)_2$-γ-Abu was only roughly 80% of theory. These values did not improve with prolonged or repeated couplings.

A rosetting test of all Tn antigen conjugates **V**, **VIII**, **XI** and **XIV** showed positive reactions with anti-Tn (DAKO) and Tn^+ erythrocytes, with anti-Tn/A (BRIC 66) and Tn^+ and A erythrocytes, other combinations were negative (Table 3 and Plate 1).

Results of the rosetting test showed good accessibility of Tn antigen on the surface of the Tenta Gel beads for the antibody binding which is important for the utility of the conjugate in affinity purification

Table 3 Results of Rosetting Tests

MoAb	Anti-Tn (DAKO)		Anti-Tn/A (BRIC)		Anti-A (Gamma)	
RBCs	Tn^+	A	Tn^+	A	Tn^+	A
IV	−	−	−	−	−	−
V	+	−	+	+	−	−
VII	−	−	−	−	−	−
VIII	+	−	+	+	−	−
X	−	−	−	−	−	−
XI	+	−	+	+	−	−
XIII	−	−	−	−	−	−
XIV	+	−	+	+	−	−

of antibodies. The cross-reactivity of **V**, **VIII**, **XI** and **XIV** with blood group A antigen was shown to be related to the unique specifity of the anti-Tn/A antibody (BRIC 66) and not to the antigen itself.

Immunized mice did not exhibit any sign of adverse reaction to the administered conjugates. We detected low levels of anti-A (titre 4–8, score 7–14) and anti-Tn (titre 32–64, score 24–27) in the sera of non-immunized animals (M1) and those immunized with **XIII**. In all the animals immunized with **XIV** we found a remarkable increase in the level of anti-Tn (titre 2000–64000, score 105–167) and no change of anti-A levels (titre 8, score 13–17) (Figure 1). Neither non-immune nor immune sera showed any reactivity with T^+, Cad^+ and blood group O erythrocytes.

The immunization with **XIV** was very effective (100–1000 × increase of the titre of agglutination reaction) and highly specific for the Tn antigen (no influence on reactions with related structures of T, Cad and blood group A antigens).

The biological activities of the synthetic Tn-antigen conjugates **V**, **VIII**, **XI** and **XIV** (good availability for the immunological interactions, highly specific

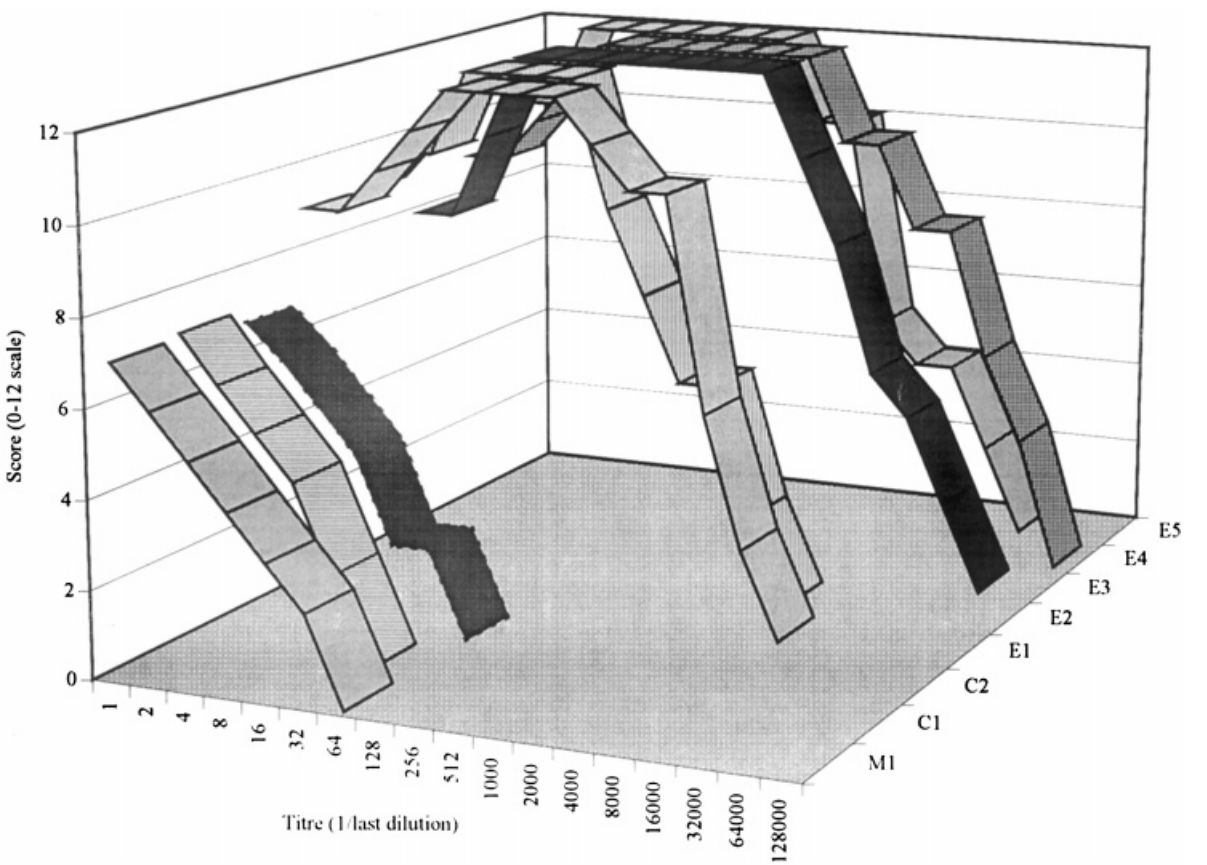

Figure 1 Titre of anti-Tn antibodies obtained in immunization study with **XIV**. (**XIII** was used as negative control). Titre (1/last dilution giving positive reaction) and score (values 0–12 assigned to each reaction based on observed strength of agglutination). M1, serum of non-immunized mice; C1–C2, sera of mice immunized with **XIII** (without Tn antigen); E1–E5, sera of mice immunized with **XIV** (containing Tn antigen).

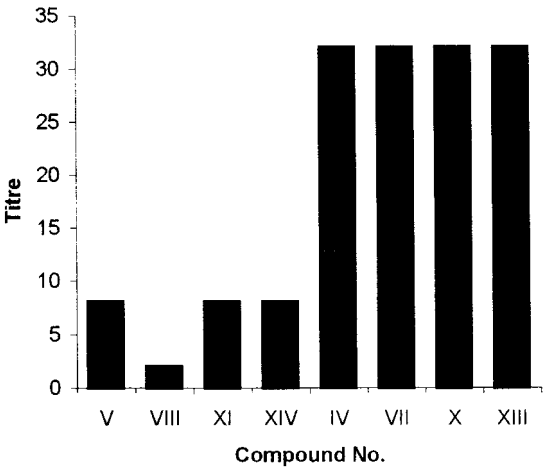

Figure 2 Titre (1/last dilution giving positive reaction) of agglutination reaction of Tn^+ red blood cells with monoclonal anti-Tn antibody (DAKO) after inhibition with conjugates with Tn antigen (**V**, **VIII**, **XI** and **XIV**) compared with negative controls (**IV**, **VII**, **X** and **XIII**). Inhibition: 100 μg of beads were incubated for 1 h at room temperature with 100 μL of antibody containing solution. The supernatant was then tested in agglutination test (1 h incubation with Tn^+ red blood cells at room temperature, centrifugation at $1000 \times g$ for 15 s and reading).

immunogenicity, good biological tolerance) together with their defined chemical structure seem to be a promising approach to the development of anti-tumour vaccine and affinity purification of anti-Tn antibodies. For the purification of anti-Tn antibodies, conjugate **VIII** seems to be the best from the compounds tested (Figure 2). The titre (l/last dilution giving positive reaction) of agglutination reaction of Tn^+ red blood cells with monoclonal anti-Tn antibody (DAKO) after incubation with conjugates with Tn antigen (**V**, **VIII**, **XI** and **XIV**) showed decreased values (**VIII** is the best inhibitor), while incubation with conjugates without Tn antigen (**IV**, **VII**, **X** and **XIII**) did not affect the antibody reactivity. The experiments in animals challenged by Tn-expressing tumour cells will be performed in order to evaluate suspect protective effect of immunization with synthetic Tn antigen conjugate. Biological activities of conjugates **V**, **VIII**, **XI** and **XIV** will be published in more detail elsewhere [43]. During the preparation of our manuscript, Bay *et al.* [28] published synthesis and biological activities of MAPs with monomeric Tn antigen in soluble form.

For the study of the spacer influence we modelled eight different compounds (**V**, **VIII**, **XI**, **XIV**, (γ-Abu)$_4$-(Lys)$_2$-Lys-β-Ala-NH-TG (**XV**), (γ-Abu)$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG (**XVI**), (γ-Abu)$_8$-(Lys)$_4$-(Lys)$_2$-Lys-β-Ala-NH-TG (**XVII**) and (γ-Abu)$_8$-(Lys-γ-Abu)$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG (**XVIII**)). The insertion of the γ-Abu into the Lys branched peptides resulted in more compact conformers in all computationally studied structures in comparison with the ones without the insertion (Plate 2). From the structural analysis it is obvious that insertion of γ-Abu brings a conformational flexibility allowing extensive hydrogen bond network and thus makes the final structure more tightly packed. This feature is most obvious in structures **XV** (Plate 2(a)) and **XVI** (Plate 2(b)). Prolongation of the structures by adding another Lys or γ-Abu-Lys decreases the effect of differences in terms of structure compactness. There are two implications for the chemical synthesis and the biological activity of the compounds studied. The compactness of dendrimers with inserted γ-Abu means that their termini are less accessible for further branching and the sterical barriers would be probably the reason for their more difficult synthesis. On the other hand, this compactness enables a better orientation of the termini with Tn antigens for their interactions in immunological recognition. Distribution of the termini over the structure is suitable for interaction of the Tn antigens with antibodies and slightly favors the compounds with inserted γ-Abu over the unmodified ones (Plate 3). The figure shows compounds **V** (Plate 3(a)) and **VIII** (Plate 3(b)) in their lowest potential energy states and illustrates the above mentioned conclusions. We do not present structures **XI** and **XIV** for which the conclusions are the same as above.

CONCLUSIONS

Our results show that the dimeric Tn antigen Ac-(Tn)$_2$ structures bound to oligolysine cores immobilized on Tenta Gel resin are accessible for interactions with antibodies and are able to produce anti-Tn antibodies in immunization experiments. The insertion of γ-Abu into the oligolysine core improved the immunological properties of our constructs and these results are supported by molecular modelling studies. On the other hand, the synthesis of such constructs is more difficult due to the tendency of the core to fold into more compact and less accessible structures.

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