Fluorescent Labelled Thiourea-Bridged Glycodendrons

ARTICLE *in* CHEMBIOCHEM · APRIL 2004

Impact Factor: 3.09 · DOI: 10.1002/cbic.200300669 · Source: PubMed

Fluorescent Labelled Thiourea-Bridged Glycodendrons

Pavel Krist,[a] Luca Vannucci,[a] Marek Kuzma,[a] Petr Man,[a, c] Kashinath Sadalapure,[b] Anupama Patel,[b] Karel Bezouška,[a, c] Milan Pospíšil,[a] Ladislav Petruš,[d] Thisbe K. Lindhorst,[b] and Vladimír Křen*[a]

GlcNAc-coated glycodendrimers, which are polyvalent glycomimetics, display strong in vitro affinity for the rat natural killer cell protein-1A (NKR-P1A), a C-type lectin-like receptor of natural killer (NK) cells in rats, humans and some strains of mice. Administration of these compounds in vivo results in a substantial increase in the antitumour activity with involvement of the natural cell immunity. To clarify the in vitro and in vivo fate of these molecules, we synthesized labelled glycodendron analogues of the previously studied glycodendrimers. Labelling with fluorescent tags enabled the localization of the glycodendrons in white blood cells, tumours and other tissues by using different imaging techniques such as fluorescence and confocal microscopy. These studies are useful for probing the mechanism of action and fate of artificial ligands and the cell receptors involved.

Introduction

Carbohydrates can serve as structural components of natural products, energy sources or key elements in various biomolecular recognition phenomena. Carbohydrate-mediated signalling is especially important during bacterial and viral infections, cell–cell adhesion in inflammation and metastases implantation, tissue differentiation, development and the regulation of many other inter- and intracellular communication and signal transduction events.[1–5] The molecules involved are characterized by a wide complexity, which contributes to their diversity and biological activity. For example, multibranched structures are commonly expressed on the surface of many types of cancer cells as a result of aberrant glycosylation.[6]

Glycobiology research is often aimed at the development and synthesis of glycomimetics that are able to modulate the above-mentioned processes. As with putative physiological ligands for receptor structures, these artificial molecules are expected to influence the cell functions and can help to clarify the properties and mechanisms of action of complex carbohydrates in living systems.[7–10] The synthesis of glycomimetics is usually less difiicult than classical natural oligosaccharide synthesis. Therefore, larger amounts of product and standardized quality of preparation can be obtained. These products have defined structure, which facilitates controlled structural variations.[11] Glycomimetics with non-natural glycosidic linkages (e.g. *C*-glycosidic, thiourea bridges etc.)[12–16] are resistant to the action of hydrolases, which is crucial for their in vivo applications. In most cases the natural structures are replaced by artificial multivalent scaffolds terminating with carbohydrate moieties.[17]

Multivalent neoglycoconjugates are also gaining importance in basic research. Their structures are designed to mimic saccharidic components of animal or bacterial cell walls and to amplify interactions of weak ligands (e.g. carbohydrate–protein interactions).[18–20]

We recently determined optimum carbohydrate ligands for the recombinant soluble dimeric form of rat natural killer cell protein-1A (NKR-P1A). NKR-P1 belongs to a family of lectin-like receptors expressed mainly on natural killer (NK) cells and cytotoxic T lymphocytes. The isoform A is involved in activation of cell cytotoxicity against tumour cells.[21] The physiological ligands of these receptors have not yet been unambiguously identified. Different monosaccharides, oligosaccharides and sugar derivatives were tested for their affinity for the recombinant isoform A[22,23] to define molecules and structures able to trigger activation of the NK cells. By using direct binding experiments, four carbohydrate binding sites were identified in the binding groove of the NKR-P1A. 2-Acetamido-2-deoxy-D-glucose (GlcNAc) had one strong, one intermediate, and two weak binding sites. The single high-affinity binding site for 2-acetamido-2-deoxy-D-galactopyranose (GalNAc) was different from that for 2-acetamido-2-deoxy-D-mannopyranose (ManNAc).[24] GlcNAc is a very common monosaccharide in nature and is present in many glycostructures (e.g. chitin and chitooligomers), is crucial for protein and lipid glycosylation, and has a relevant role in the syntheses of the aberrant gly-

[a] *Dr. P. Krist, Dr. L. Vannucci, M. Kuzma, P. Man, Dr. K. Bezouška, Prof. M. Pospíšil, Prof. V. Křen*
Institute of Microbiology, Academy of Sciences of the Czech Republic
Vídeňská 1083, 142 20 Prague 4 (Czech Republic)
Fax: (+420) 2-9644-2509
E-mail: kren@biomed.cas.cz

[b] *Dr. K. Sadalapure, Dr. A. Patel, Prof. T. K. Lindhorst*
Institute of Organic Chemistry, Christiana Albertina University
Otto-Hahn-Platz 3, 24098 Kiel (Germany)

[c] *P. Man, Dr. K. Bezouška*
Department of Biochemistry, Faculty of Science
Charles University Prague, Hlavova 8, 128 40 Praha 2 (Czech Republic)

[d] *Prof. L. Petruš*
Institute of Chemistry, Slovak Academy of Sciences
Dúbravská cesta 9, 842 38 Bratislava (Slovakia)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 DOI: 10.1002/cbic.200300669

cosylated molecules on cancer cell surfaces (multiantennary structures). Hence, GlcNAc was chosen for use in the production of glycomimetics similar to natural multivalent glycoconjugates. Moreover, GlcNAc is commercially available at a low cost.[25]

Further studies, during which different kinds of multivalent dendritic molecules were evaluated, led to the selection of the GlcNAc-coated octavalent glycodendrimers (PAMAM–$GlcNAc_8$), which are based on a first-generation polyamidoamine (Starburst®) scaffold with GlcNAc terminal substitution, that had displayed a very high affinity for the receptor tested.[26–28] These well-defined multivalent neoglycoconjugates were able to strongly stimulate an antitumour immune response in animal cancer models in vivo, thereby implying both innate and acquired immunity.[29,30] Such a result underlined the importance of the multivalency effect in biological systems and a possible role as artificial ligands for lectin-like receptors (e.g. NKR-P1) in vivo.

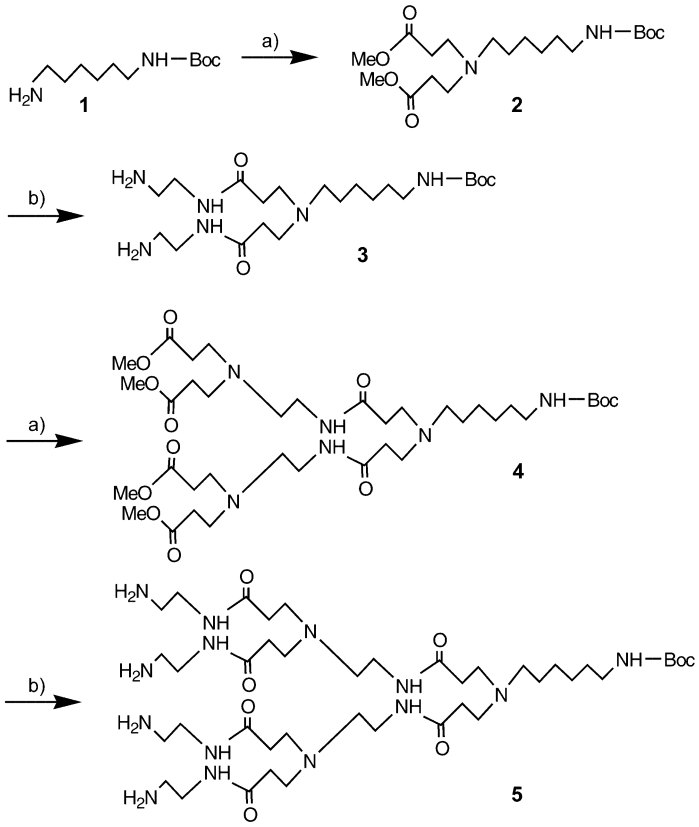
To assist biological research into the fates of glycodendrimers in vivo and glycodendrons at the cellular level, labelled glycodendrons were synthesized.[31–35] The structure of PAMAM–$GlcNAc_8$ does not allow direct linking to a fluorescent label (e.g. fluorescein) without structural changes. Therefore, we decided to synthesize a 0.5 generation PAMAM derivative which preserved the terminal tetravalent core of the original molecule (PAMAM–$GlcNAc_4$) but allowed the coupling to a fluorescent dye.

In this paper we describe the synthesis of the fluorescein-labelled PAMAM–$GlcNAc_4$ and examples of their application in glycobiology.

Results

Preparation of fluorescent labelled polyvalent glycoconjugates

One amino group of 1,6-hexanediamine was selectively protected with a *tert*-butoxycarbonyl (Boc) group.[36] The other amino group was condensed with methyl acrylate in a conjugate addition, and the resulting methyl ester was cleaved with ethylene diamine. These two steps were consecutively repeated to yield a molecule carrying four free terminal amino groups. In each step the products obtained (**2**–**4**) were further reacted without any purification (Scheme 1). The structural assignments were confirmed by NMR experiments. NMR data for compounds **1**–**5** are given in Tables 1 and 2.



Scheme 1. *a) Methyl acrylate, MeOH, 24–48 h, 4 °C; b) ethylene diamine, MeOH, 24–48 h, 4 °C.*

Table 1. *^1H NMR chemical shifts [ppm] of compounds **1**–**5**.*

	1		2		3		4		5	
(CH_3)C	1.447	s	1.453	s	1.450	s	1.450	s	1.452	s
H-1	3.050	t	3.048	t	3.042	t	3.043	t	3.043	t
H-2	1.54[a,b]	m	1.48[b]	m	1.49[b]	m	1.49[b]	m	1.49[b]	m
H-3	1.50[a,b]	m	1.272–	m	1.290–	m	1.289–	m	1.293–	m
H-4	1.287–	m	1.389	m	1.400	m	1.404	m	1.401	m
H-5	1.423[a]	m	1.47[b]	m	1.51[b]	m	1.51[b]	m	1.50[b]	m
H-6	2.718	t	2.440	t	2.505	t	2.504	m	2.483	t
H-1′	–	–	2.773	t	2.807	t	2.808	t	2.781	m
H-2′	–	–	2.471	t	2.436	t	2.400	t	2.512	m
O-CH_3	–	–	3.678	s	–	–	–	–	–	–
H-1″	–	–	–	–	3.405	t	3.275	t	3.280	t
H-2″	–	–	–	–	2.957	t	2.579	t	2.601	t
H-1‴	–	–	–	–	–	–	2.795	t	2.815	t
H-2‴	–	–	–	–	–	–	2.483	t	2.390	t
O-CH_3	–	–	–	–	–	–	3.690	s	–	–
H-1⁗	–	–	–	–	–	–	–	–	3.289	t
H-2⁗	–	–	–	–	–	–	–	–	2.774	t

[a] Might be interchanged. [b] HMQC readouts.

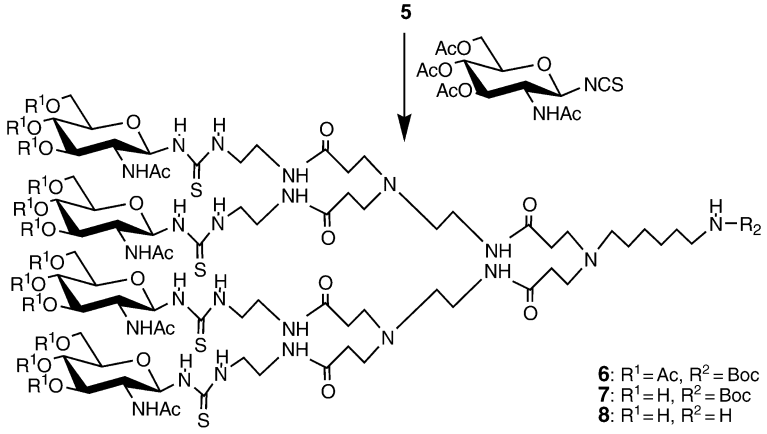
Table 2. ^{13}C NMR chemical shifts [ppm] of compounds **1–5**.

	1		2		3		4		5	
$(CH_3)_3C$	29.16	q	29.13	q	29.12	q	29.12	q	29.13	q
$(CH_3)_3C$	79.87	s	79.97	s	80.07	s	80.04	s	80.07	s
OCONH	158.43	s	158.69	s	158.81	s	158.74	s	158.80	s
C-1	41.38	t	41.59	t	41.71	t	41.63	t	41.69	t
C-2	27.59[a]	t	31.24	t	31.25	t	31.28	t	31.33	t
C-3	27.65[a]	t	28.34[a]	t	28.04[a]	t	28.10[a]	t	28.16[a]	t
C-4	30.97[a]	t	28.01[a]	t	28.51[a]	t	28.59[a]	t	28.26[a]	t
C-5	32.57[a]	t	28.34[a]	t	27.90	t	28.23	t	28.63[a]	t
C-6	42.14	t	55.03	t	54.81	t	54.78	t	54.78	t
C-1′	–	–	50.66	t	51.07	t	51.16	t	51.20	t
C-2′	–	–	33.50	t	34.58	t	34.69	t	34.77	t
*C*OO	–	–	174.93	s	–	–	–	–	–	–
OCH_3	–	–	52.32	q	–	–	–	–	–	–
*C*ONH	–	–	–	–	176.03	s	175.02	s	175.22	s
C-1″	–	–	–	–	40.52	t	38.77	t	38.96	t
C-2″	–	–	–	–	40.71	t	54.11	t	53.87	t
C-1‴	–	–	–	–	–	–	50.82	t	51.52	t
C-2‴	–	–	–	–	–	–	33.91	t	35.15	t
*C*OO	–	–	–	–	–	–	175.02	s	–	–
OCH_3	–	–	–	–	–	–	52.43	q	–	–
*C*ONH	–	–	–	–	–	–	–	–	175.57	s
C-1⁗	–	–	–	–	–	–	–	–	43.01	t
C-2⁗	–	–	–	–	–	–	–	–	42.29	t

[a] Might be interchanged.

The four terminal amino groups of the product **5** were derivatized with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate,[37] and the resulting tetravalent glycocluster **6** was purified by silica gel flash chromatography.

Glycocluster **6** was deacetylated to afford **7**, and the Boc group was then hydrolysed with 30% trifluoroacetic acid (TFA) in water to give **8**. Final purification was performed on a Sephadex LH-20 column with MeOH as eluent (Scheme 2).



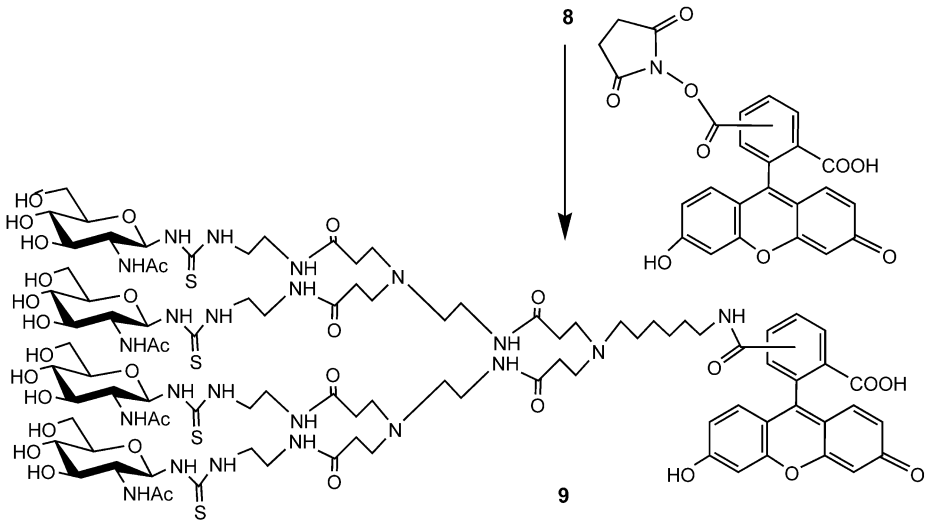
Scheme 2. *1st step (**5**→**6**): $CHCl_3$, RT, 24 h; 2nd step (**6**→**7**): NaOMe/MeOH, RT, 2 h; 3rd step (**7**→**8**): 30% TFA/H_2O, 6 h, 4 °C.*

Labelling of the free amino group of **8** was performed in *N*,*N*-dimethylformamide (DMF) (Schemes 3 and 4). It was important to use an excess (1.5 equiv) of the NCS-activated label to achieve high yields. Unreacted fluorescent label could be removed by extraction, and the yields were 75% and 87% for **9** and **10**, respectively. Structural assignments were supported by NMR experiments. NMR data for compounds **6**–**10** are given in Tables 3 and 4.

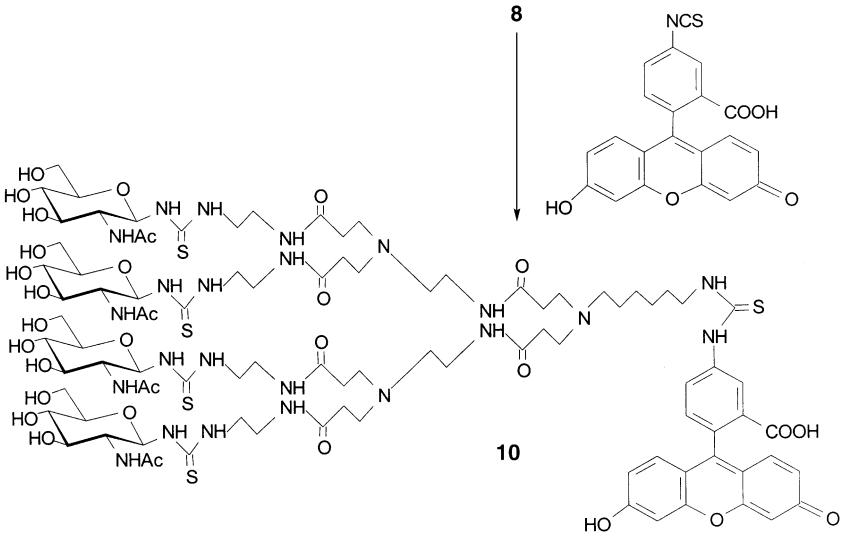
The fluoresceinylated glycodendrons **9** and **10** were suitable for tracing the fate of the compounds in vitro (Figure 1) and in vivo (Figure 2).

Figure 1 shows two examples of lymphatic cell populations: the first one (A-B) is a rat lymphoma cell line (RNK-16). It is characterized by a very high expression of the NKR-P1A receptor with a high affinity for hexosamines. As is obvious from plate B, all the cells give positive reaction with the labelled dendrimers. When observing a mixed population of mononuclear peripheral blood cells (Figure 1, C-D), only large granular cells were labelled by the fluorescein-bound PAMAM–$GlcNAc_4$. These data, which need further confirmation, suggest a possible selectivity of the GlcNAc dendrimers for the NKR-P1A-positive lymphocytes.

When injected in vivo (intracardiac injection into a mouse), the fluorescein-labelled PAMAM–$GlcNAc_4$ distribution in the mouse tissues was localized mainly in the liver, kidney, spleen and cancer tissues. As shown in Figure 2, at an equivalent dose, the fluorescence distribution in the spleen appeared to be more selective and organized after the administration of la-



Scheme 3. *Labelling of tetravalent glycodendron* ***8*** *with FLUOS (5-(and 6-)-carboxyfluorescein* N*-succinimidyl ester).*



Scheme 4. *Labelling of tetravalent glycodendron* ***8*** *with FITC (fluorescein 5-isothiocyanate).*

As in the synthesis of PAMAM glycodendrimers, thiourea bridging was used to attach the β-GlcNAc units to free amino groups. An orthogonally protected amino group was coupled to a fluorescent label [5- and 6-carboxyfluorescein *N*-succinimidyl ester (FLUOS) or fluorescein 5-isothiocyanate (FITC)] after deprotection.

The synthesis of the tetravalent dendron with one free amino group was accomplished in seven reaction steps in an overall yield of 22%. The possibility of labelling this molecule (e.g. with a fluorescent tag) makes it a suitable tool for functional ligand–receptor interaction studies where multivalency is required.

When used for in vivo experiments, such glycodendrons enabled us to trace them in blood cells and tissues by imaging techniques (fluorescence and confocal microscopy). In fact, they were stable in body fluids, as demonstrated by their capacity to reach and be taken up by singular cells (in lymphatic organs, blood and tumours). Their capability to target certain cell types (NKR-P1-positive cells, heavily glycosylated tumour cells) is especially evident when we compare the different distributions of the fluorescence after administration of labelled dendrons or free fluorescein (Figures 1 and 2). The glycodendrons maintained in vivo their multivalency effects in this system, as shown by the induction of an increased expression of CD69 (early activation marker) on splenocytes 24 h after parenteral administration in tumour-bearing mice,[30] albeit to a lower extent compared with the analogous glycodendrimer with double the amount of sugar moieties (PAMAM–$GlcNAc_8$).[30]

belled dendrimers (A), whilst the free dye stained all the structures nonspecifically (B).

Discussion

In vitro and in vivo biological assays with PAMAM dendrimers of various multiplicities revealed that a minimum degree of carbohydrate clustering was required to elicit a good reactivity or biological response. Therefore, in this paper we have described the synthesis of tetravalent glycodendrons with a free amino group that is available for subsequent derivatization, for example, labelling with a fluorescent dye. To obtain a sufficient stability of these neoglycoconjugates it was necessary to avoid the GlcNAc-β-*O*-glycosidic linkage, which is susceptible to the hydrolytic attack of β-*N*-acetylhexosaminidases and lysozyme present in the plasma and other body fluids.

Conclusion

In conclusion, the fluorescein-labelled PAMAM–$GlcNAc_4$ glycodendrons described in this paper appear to be very useful molecules for the investigation of biological mechanisms and for tracing the fate of artificial ligands and the cells that they target.

Table 3. ^{1}H NMR chemical shifts of compounds **6–10**.

	6		8		9		10	
(CH_3)C	1.454	s	–	–				
H-1	3.050	t	2.886	dd	3.25[a], 3.20[a]	m	3.34[a]	m
H-2	1.49	m	1.644	m	1.47[a], 1.38[a]	m	1.393	m
H-3	1.296–	m	1.309–	m	1.156	m	1.154	m
H-4	1.404		1.468		1.233	m		
H-5	1.50	m	1.524	m	1.49[a], 1.42[a]	m	1.494	m
H-6	2.504	m	2.504	dd	2.848, 2.752	m	2.90[a]	m
H-1′	2.815	m	2.811	m	3.09[a], 3.00[a]	m	3.17[a]	m
H-2′	2.426	m	2.424	m	2.48[a], 2.38[a]	m	2.516	t
H-1″	3.309	m	3.309	m	3.09[a], 3.02[a]	m	3.190	t
H-2″	2.617	t	2.614	t	2.44[a]	m	2.705	t
H-1‴	2.826	t	2.824	t	2.624	t	2.850	t
H-2‴	2.410	t	2.406	t	2.162	t	2.293	t
H-1⁗	3.41[a]	m	3.42	m	3.32–	m	3.32–	m
H-2⁗	3.60–3.77[a]	m	3.60–3.77[a]	m	3.50[a]		3.54[a]	
sugar moiety								
H-1	5.381	br s	5.364	br s	5.242	br m	5.253	br m
H-2	3.873	t	3.865	t	3.618	t	3.622	m
H-3	3.553	dd	3.542	dd	3.404	dd	3.409	m
H-4	3.42[a]	m	3.41[a]	m	3.235	m	3.242	m
H-5	3.44[a]	m	3.43[a]	m		m	3.292	m
H-6	3.921, 3.749	2 dd	3.739, 3.913	2 dd	3.650, 3.514	2 dd	3.652, 3.519	dd
CH_3-2	2.017	s	2.015	s	1.758, 1.748	s	1.754	s
CH_3	2.041	s	–	–	–	–	–	–
fluorescent moiety								
					6.346	ddd	6.442	m
					6.387	m	7.005	m
					6.423	s	7.319	m
					6.429	s	7.576	m
					6.905	m		
					7.175	d		
					7.512	d		
					7.747	m		
					7.809	dd		
					8.032	d		

[a] HMQC readout.

Experimental Section

NMR spectroscopy: NMR spectra were recorded on a Varian ^{UNITY}*INOVA*-400 MHz spectrometer (399.90 MHz for ^{1}H, 100.56 MHz for ^{13}C) in CD_3OD or D_2O at 303 K. Chemical shifts were referenced to the residual solvent signal (CD_3OD: $\delta_H = 3.330$, $\delta_C = 49.30$) or internal acetone (D_2O: $\delta_H = 2.030$, $\delta_C = 30.50$). The assignments were based on COSY, HMQC and HMBC experiments performed by using the manufacturer's software. Missing correlations in HMBC spectra of compounds **9** and **10** prevented a rigorous structural proof and the chemical shift determinations of carbonyls. Doubling of some signals observed with **9** was due to the presence of regioisomers in the starting material. Carbon chemical shifts were obtained from HMQC.

MALDI-TOF mass spectrometry: A 10 mg mL^{-1} solution of ferulic acid (Sigma) or α-cyano-4-hydroxycinnamic acid (Sigma) in aqueous 30% acetonitrile/0.1% TFA was used as a MALDI matrix. A 2 μL aliquot of sample and the matrix solution (2 μL) were premixed in a tube; 0.5 μL of the mixture was placed on the sample target and allowed to dry at the ambient temperature. Positive ion MALDI-TOF mass spectra were measured on a Bruker BIFLEX II mass spectrometer (Bruker–Daltonics) equipped with a SCOUT 26 sample inlet, a griddles delayed extraction ion source and a nitrogen laser (337 nm) (Laser Science). The instrument was operated in the reflectron mode, and the ion acceleration voltage was set to 19 kV. Spectra were calibrated externally by using $[M+H]^+$ peaks of peptide standards MRFA (Sigma) (m/z = 524.26) or angiotensin I (Bachem) (m/z = 1296.69).

Chromatography: Flash chromatography was performed on Silica Gel 60 (40–63 μm, Merck). Thin-layer chromatography was run on precoated Silica Gel 60 F_{254} aluminium sheets (Merck); detection was performed by using UV light (254 nm) and charring with 5% H_2SO_4 in EtOH. Gel chromatography was performed on Sephadex LH-20 columns (100×2.5–5 cm) eluted with methanol.

Synthesis of the 0.5 generation tetravalent dendron with one free terminal amino group (Schemes 1 and 2): A solution of the corresponding partially protected amine **1**[36] or **3** in absolute MeOH was added dropwise at 0 °C under argon to a stirred solution of methyl acrylate (5 equiv per amino group) in absolute MeOH. The sealed flask was stored in a fridge (4 °C) and the flask was occasionally shaken. After TLC (CH_2Cl_2/MeOH, 9:1) had shown the complete disappearance of the starting amine (typically after 24–48 h), the unreacted methyl acrylate was evaporated in vacuo to yield **2** or **4**. A solution of ethylene diamine (5 equiv per methyl ester group) in absolute MeOH was added dropwise at 0 °C under argon to a solution of the corresponding ester **2** or **4** in absolute MeOH, and the above procedure was repeated.

***N*-(6-(*N*,*N*-Bis-2-(methoxycarbonyl)ethyl)aminohexyl)carbamic acid *tert*-butyl ester (2)**: Prepared from **1** (6.19 g, 28 mmol) in MeOH (60 mL) and methyl acrylate (12.8 mL, 140 mmol) in MeOH (40 mL) according to the above procedure to give **2** (10.9 g, 98%). MALDI MS: m/z calcd for $C_{19}H_{36}N_2O_6$ ($[M+H]^+$): 389.3; found: 389.1.

***N*-(6-(*N*,*N*-Bis-2-((2-aminoethyl)aminocarbonyl)ethyl)aminohexyl)-carbamic acid *tert*-butyl ester (3)**: Prepared from **2** (10.5 g, 28 mmol) in MeOH (100 mL) and ethylene diamine (18.7 mL, 280 mmol) in MeOH (50 mL) according to the above procedure to give **3** (11.8 g, 98%). MALDI MS: m/z calcd for $C_{21}H_{44}N_6O_4$ ($[M+H]^+$): 445.3; found: 445.2.

***N*-(6-(*N*,*N*-Bis-2-((2-*N*,*N*-bis-2-((methoxycarbonyl)ethyl)aminoethyl)aminocarbonyl)ethyl))aminohexyl)carbamic acid *tert*-butyl ester (4)**: Prepared from **3** (11.5 g, 26 mmol) in MeOH (100 mL) and methyl acrylate (23.2 mL, 260 mmol) in MeOH (60 mL) according to the above procedure to give **4** (18.1 g, 89%). MALDI MS: m/z calcd for $C_{37}H_{68}N_6O_{12}$ ($[M+H]^+$): 789.5; found: 790.0.

Table 4. ^{13}C *NMR chemical shifts of compounds* ***6–10****.*

	6		8		9[a]		10[a]	
$(CH_3)_3C$	29.18	q	–	–	–	–	–	–
$(CH_3)_3C$	80.14	s	–	–	–	–	–	–
OCONH	158.81	s	–	–	–	–	–	–
C-1	41.70	t	41.66	t	40.0	t	44.9	t
C-2	31.33	t	30.32	t	28.2	t	28.1	t
C-3	28.17	t	27.88	t	25.7	t	25.8	t
C-4	28.21	t	28.47	t				
C-5	28.66	t	28.20	t	23.8	t	23.6	t
C-6	54.81	t	54.73	t	53.2	t	53.3	t
C-1′	51.15	t	51.15	t	49.7	t	49.8	t
C-2′	34.78	t	34.82	t	29.9, 29.7	2t	29.4	t
COO	–	–	–	–	–	–	–	–
OCH_3	–	–	–	–	–	–	–	–
CONH	175.28	s	175.30	s	n.d.[b]	–	n.d.	–
C-1″	38.99	t	39.00	t	36.7, 36.8	2t	36.1	t
C-2″	53.76	t	53.76	t	51.5	t	52.0	t
C-1‴	51.42	t	51.39	t	49.3	t	49.7	t
C-2‴	35.14	t	35.14	t	32.4	t	31.2	t
COO	–	–	–	–	–	–	–	–
OCH_3	–	–	–	–	–	–	–	–
CONH	175.72	s	175.75	s	n.d.	–	n.d	–
C-1⁗	40.19	t	40.22	t	43.8	t	43.9	t
C-2⁗	45.68	t	45.76	t				
C=S	185.53	s	185.55	s	–	s	n.d.	–
NHCO	–	–	–	–	n.d.		–	–
sugar moiety								
C-1	85.01	d	85.00	d	n.d.	d	n.d.	d
C-2	56.49	d	56.50	d	54.7	d	54.7	d
C-3	76.48	d	76.45	d	74.2	d	74.2	d
C-4	72.38	d	72.40	d	69.9	d	70.0	d
C-5	79.68	d	79.70	d	77.5	d	77.4	d
C-6	63.08	t	63.10	t	61.0	t	60.9	t
CO-2	174.95	s	174.99	s	n.d.	–	n.d.	–
CO-3	n.d.	–	–	–	–	–	–	
CO-4	n.d.	–	–	–	–	–	–	
CO-6	n.d.	–	–	–	–	–	–	
CH_3-2	23.30	q	23.24	q	22.3	q	22.3	q
CH_3	20.77	q	–	–	–	–	–	–
fluorescent moiety								
					104.0	d	103.8	d
					123.0	d	122.3	d
					127.6	d	124.8	d
					128.3	d	125.6	d
					128.8	d	131.7	d
					129.1	d	131.9	d
					130.8	d		
					131.7	d		

[a] All carbon chemical shifts are HMQC readouts. [b] Not determined.

***N*-(6-(*N*,*N*-Bis-2-((2-*N*,*N*-bis-2-((2-aminoethyl)aminocarbonyl)ethyl)aminocarbonyl)ethyl)aminohexyl)carbamic acid *tert*-butyl ester (5)**: Prepared from **4** (3 g, 3.8 mmol) in MeOH (50 mL) and ethylene diamine (5 mL, 76 mmol) in MeOH (10 mL) to give **5** (2.9 g, 85%). MALDI MS: *m/z* calcd for $C_{41}H_{84}N_{14}O_8$ ($[M+H]^+$): 901.7; found: 902.1.

***N*-(6-(*N*,*N*-Bis-2-({2-*N*,*N*-bis-2-((2-(3-(2-deoxy-2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)thioureido)ethyl)aminocarbonyl)ethyl)aminocarbonyl)ethyl)aminohexyl)carbamic acid *tert*-butyl ester (6)**: A solution of the selectively protected tetraamine **5** (0.8 g, 0.9 mmol) in $CHCl_3$ (60 mL) was added dropwise to a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate (2.5 g, 6.4 mmol) in $CHCl_3$ (80 mL). The mixture was stirred at room temperature for 24 h. The solvent was removed by evaporation and the residue was purified on silica gel (MeOH/acetone, 9:1) to afford **6** (1.41 g, 65%). MALDI MS: *m/z* calcd for $C_{101}H_{164}N_{22}O_{40}S_4$ ($[M+H]^+$): 2454.0; found: 2453.5.

***N*-(6-(*N*,*N*-Bis-2-((2-*N*,*N*-bis-2-((2-(3-(2-deoxy-2-acetamido-β-D-glucopyranosyl)thioureido)ethyl)aminocarbonyl)ethyl)aminocarbonyl)ethyl)aminohexyl)carbamic acid *tert*-butyl ester (7)**: NaOMe (2 M solution in methanol) was added to the solution of **6** (1.4 g, 0.57 mmol) in methanol (30 mL) to give approximately pH 9. The mixture was stirred for 2 h until the starting material **6** disappeared on TLC (MeOH/acetone, 9:1). The reaction mixture was diluted with water (20 mL) and neutralized with Dowex 50W-X2 (H^+ form), filtered and concentrated to give **7** (0.91 g, 81% yield). MALDI MS: *m/z* calcd for $C_{77}H_{140}N_{22}O_{28}S_4$ ($[M+H]^+$): 1949.9; found: 1949.8.

6-(*N*,*N*-Bis-2-((2-*N*,*N*-bis-2-((2-(3-(2-deoxy-2-acetamido-β-D-glucopyranosyl)thioureido)ethyl)aminocarbonyl)ethyl)aminocarbonyl)ethyl)aminohexylamine (8): TFA (4 mL, 30% aqueous solution) was added to a solution of the tetravalent cluster **7** (800 mg, 0.41 mmol) in water (10 mL) at 4 °C. The mixture was allowed to warm to room temperature. The reaction was monitored by TLC (isopropanol/NH_3(aq)/water, 7:1:2) and was complete after 6 h. The mixture was evaporated to half its initial volume and then neutralized with a saturated solution of $NaHCO_3$. The residue was lyophilized and purified by gel chromatography on Sephadex LH-20 (mobile phase MeOH) to give **8** (454 mg, 60%). MALDI MS: *m/z* calcd for $C_{72}H_{132}N_{22}O_{26}S_4$ ($[M+H]^+$): 1849.9; found: 1849.7.

General procedure for the preparation of fluorescent labelled PAMAM glycodendrons (Schemes 3 and 4): The tetravalent dendron **8** (1 equiv) in DMF was added dropwise to a solution of fluorescent label (FLUOS or FITC) (1.5 equiv) in dry DMF. The reaction mixture was monitored by TLC (isopropanol/NH_3(aq)/water, 7:1:2) and was stirred in the dark over night at 20 °C. The solvent was evaporated, and the residue was diluted with water and extensively washed with EtOAc. The water phase was lyophilized and puri-

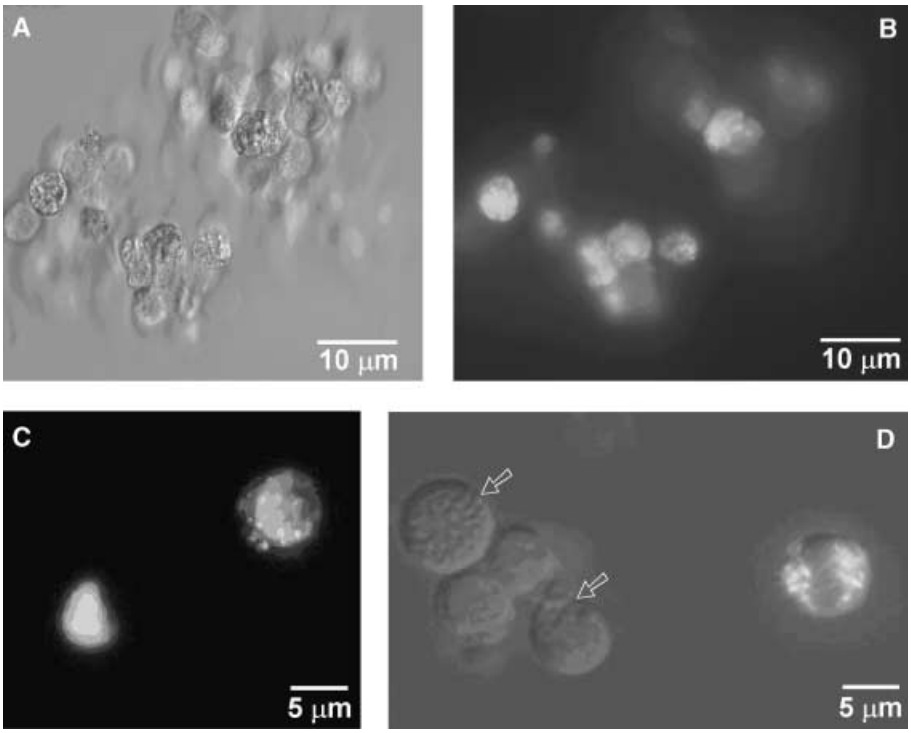


Figure 1. *Examples of the fluorescein-linked PAMAM–$GlcNAc_4$ dendron uptake by mononuclear cells expressing lectin-like receptors (NK cells, cytotoxic cells). A-B) RNK16 (NK cell lymphoma), a cell line highly expressing the NKR-P1A receptors. All the cells were fluorescence-positive after a 15 min incubation with the fluorescein-linked dendrons: A) contrast phase; B) fluorescence. Controls with an unmodified fluorescein had shown a partial or negative staining depending on the length of the incubation period (not shown). C–D) Mononuclear peripheral blood cells. In particular, the two photographs show a superficial binding (C) and an intracellular uptake (D) of the fluorescein-labelled PAMAM–$GlcNAc_4$ by large granular cells. Cells lacking the morphological characteristics of large granular cells (arrows) appear negative even when granulated (D, the image was obtained by a merge of the contrast phase and fluorescence images).*

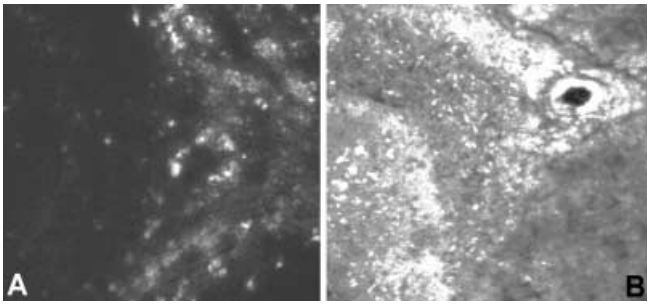


Figure 2. *Mouse spleen fresh section. The observation was performed 90 min after intracardiac injection of the fluorescein-labelled PAMAM-$GlcNAc_4$ (A) and free dye (B), at an equivalent dose, into a mouse. The distribution of the fluorescence appeared more selective when labelled glycodendrons were used relative to the wider nonspecific staining produce by the free dye (confocal microscopy, 20× magnification).*

fied by gel chromatography on Sephadex LH-20 (mobile phase MeOH) to give the fluorescent labelled tetravalent cluster **9** or **10**.

Fluorescein-5 (and 6) -carb-(6-(*N*,*N*-bis-2-((2-*N*,*N*-bis-2-((2-(3-(2-deoxy-2-acetamido-β-D-glucopyranosyl)thioureido)ethyl)aminocarbonyl)ethyl)aminocarbonyl)ethyl)aminohexyl)amide (9): Prepared from **8** (19 mg, 0.01 mmol) in DMF (5 mL) and FLUOS (7.4 mg, 0.015 mmol) in DMF (5 mL) according to the general procedure to give **9** (17 mg, 75%). MALDI MS: *m/z* calcd for $C_{93}H_{142}N_{22}O_{32}S_4$ ($[M+H]^+$): 2207.9; found: 2208.0.

5-(3-(6-(*N*,*N*-Bis-2-((2-*N*,*N*-bis-2-((2-(3-(2-deoxy-2-acetamido-β-D-glucopyranosyl)thioureido)ethyl)aminocarbonyl)ethyl)aminocarbonyl)ethyl)aminohexyl)thioureido)fluorescein (10): Prepared from **8** (19 mg, 0.01 mmol) in DMF (5 mL) and FITC (6 mg, 0.015 mmol) in DMF (5 mL) according to the general procedure to give **10** (20 mg, 87%). MALDI MS: *m/z* calcd for $C_{93}H_{143}N_{23}O_{31}S_5$ ($[M+H]^+$): 2238.9; found: 2239.1.

Fluorescence microscopy: Mononuclear peripheral blood cells were prepared by Ficoll–Hypac 1083 density gradient separation, then incubated for 5 min (Figure 1C) and 15 min (Figure 1B and D) in the presence of fluorescein-labelled PAMAM–$GlcNAc_4$, then fixed in paraformaldehyde (3.7%) and washed three times with culture medium without serum. Fluorescence microscopy and digital image acquisition and analysis were performed with an Olympus BX60 fluorescence microscope (Olympus Optical Co.), Olympus U-TV1X camera and digital imaging microscope system AnalySIS (Soft Imaging System, GmbH), with appropriate filters for fluorescein.[30]

Confocal microscopy: Mice, under general anaesthesia, were intracardially given either fluorescein-labelled PAMAM–$GlcNAc_4$ (0.0015 mg) or an equivalent dose of free fluorescent dye. After 90 min animals were sacrificed, and organ samples were taken to evaluate the fluorescence distribution in the organism. Spleen was harvested and frozen in nitrogen. Frozen sections (4-μm thickness) were prepared and immediately evaluated. Thick slices of tissue were freshly examined by confocal microscopy with a Bio-Rad MRC600 confocal system and Confocal Assistant Version 4.02 software for image acquisition and elaboration (Bio-Rad Laboratories

and Todd Clark Brelje, USA), connected to a Nikon Diaphot inverted microscope acquisition system (Nikon, Japan).[30]

Acknowledgements

We gratefully acknowledged support from the Volkswagen Foundation, the Grant Agency of the Czech Republic (Grant Nos. 203/01/1018 and 312/98/KO34), the COST Chemistry Action D13 (MSMT OC D13.50), institutional research concept (Inst. Microbiol., Prague) AV0Z5020903, grant A7020006 from the Grant Agency of the Academy of Sciences of the Czech Republic, and grant MSM113100001 from the Ministry of Education of the Czech Republic.

Keywords: anticancer activity · dendrimers · glycoconjugates · glycomimetics · natural killer cells

[1] S. Takayama, C. H. Wong, *Curr. Org. Chem.* **1997**, *1*, 109 – 126.
[2] A. Varki, *Glycobiology* **1993**, *3*, 97 – 130.
[3] Y. C. Lee, R. T. Lee, *Acc. Chem. Biol.* **1995**, *28*, 321 – 327.
[4] S. Roseman, *J. Biol. Chem.* **2001**, *276*, 41 527 – 41 542.
[5] K. A. Karlsson, *Curr. Opin. Struct. Biol.* **1995**, *5*, 622 – 635.
[6] J. W. Dennis, M. Granovsky, C. E. Warren, *Biochim. Biophys. Acta* **1999**, *6*, 21 – 34.
[7] T. K. Lindhorst, *Chem. unserer Zeit* **2000**, *34*, 38 – 52 (CAN 132.318947).
[8] R. A. Dwek, T. D. Butters, *Chem. Rev.* **2002**, *102*, 283 – 284.
[9] P. Nangia-Makker, J. Conklin, V. Hogan, A. Raz, *Trends Mol. Med.* **2002**, *8*, 187 – 192.
[10] I. Marchal, G. Golfier, O. Dugas, M. Majed, *Biochimie* **2003**, *85*, 75 – 81
[11] R. Roy in *Carbohydrate Chemistry* (Ed.: G. J. Boons), Chapman and Hall, UK, **1998**, pp. 243 – 321.
[12] C. O. Mellet, J. Defaye, J. M. García Fernández, *Chem. Eur. J.* **2002**, *8*, 1982 – 1990.
[13] S. André, R. J. Pieters, I. Vrasidas, H. Kaltner, I. Kuwabara, F. T. Liu, R. M. J. Liskamp, H. J. Gabius, *ChemBioChem* **2001**, *2*, 822 – 830.
[14] I. Vrasidas, N. J. de Mol, R. M. J. Liskamp, R. J. Pieters, *Eur. J. Org. Chem.* **2001**, 4685 – 4692.
[15] F. Sansone, E. Chierici, A. Casnati, R. Ungaro, *Org. Biomol. Chem.* **2003**, *10*, 1802 – 1809.
[16] F. Burkhart, M. Hoffmann, H. Kessler, *Angew. Chem.* **1997**, *109*, 1240 – 1241; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1191 – 1192.
[17] N. Röckendorf, T. K. Lindhost, *Top. Curr. Chem.* **2001**, *217*, 201 – 238.
[18] M. J. Cloninger, *Curr. Opin. Chem. Biol.* **2002**, *6*, 742 – 748.
[19] W. B. Turnbull, J. F. Stoddart, *Rev. Mol. Biotechnol.* **2002**, *90*, 231 – 255.
[20] K. Bezouška, *Rev. Mol. Biotechnol.* **2002**, *90*, 269 – 290.
[21] J. C. Ryan, E. C. Niemi, M. C. Nakamura, W. E. Seaman, *J. Exp. Med.* **1995**, *181*, 1911 – 1915.
[22] P. Krist, E. Herkommerová-Rajnochová, J. Rauvolfová, T. Semenuk, P. Vavrušková, J. Pavlícek, K. Bezouška, L. Petruš, V. Křen, *Biochem. Biophys. Res. Commun.* **2001**, *287*, 11 – 20.
[23] P. Krist, M. Kuzma, I. F. Pelyvás, P. Simerská, V. Křen, *Collect. Czech. Chem. Commun.* **2003**, *68*, 801 – 811.
[24] O. Plíhal, P. Byrtusová, J. Pavlíček, L. Mihok, R. Ettrich, P. Man, P. Pompach, V. Havlíček, L. Hušáková, K. Bezouška, *Czech. Chem. Commun.* **2004**, in press.
[25] T. Semenuk, P. Krist, J. Pavlícek, K. Bezouška, M. Kuzma, P. Novák, V. Křen, *Glycoconjugate J.* **2001**, *18*, 817 – 826.
[26] K. Bezouška, V. Křen, C. Kieburg, T. K. Lindhorst, *FEBS Lett.* **1998**, *426*, 243 – 247.
[27] T. K. Lindhorst, C. Kieburg, *Angew. Chem.* **1996**, *108*, 2083 – 2086; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1953 – 1956.
[28] C. W. von der Lieth, M. Frank, T. K. Lindhorst, *Rev. Mol. Biotechnol.* **2002**, *90*, 311 – 337.
[29] M. Pospíšil, L. Vannucci, A. Fišerová, K. Krausová, O. Horváth, V. Křen, F. Mosca, K. Sadalapure, T. K. Lindhorst, K. Bezouška, *Adv. Exp. Med. Biol.* **2001**, *495*, 343 – 347.
[30] L. Vannucci, A. Fišerová, K. Sadalapure, T. K. Lindhorst, M. Kuldová, P. Rossmann, O. Horváth, V. Křen, P. Krist, K. Bezouška, M. Luptovcová, F. Mosca, M. Pospíšil, *Int. J. Oncology* **2003**, *23*, 285 – 296.
[31] R. Roy, M. G. Baek, *Rev. Mol. Biotechnol.* **2002**, *90*, 291 – 309.
[32] R. Roy, J. M. Kim, *Tetrahedron* **2003**, *59*, 3881 – 3893.
[33] R. Roy, M. G. Baek, K. Rittenhouse-Olson, *J. Am. Chem. Soc.* **2001**, *123*, 1809 – 1816.
[34] C. Grandjean, C. Rommens, H. Gras-Masse, O. Melnyk, *Tetrahedron Lett.* **1999**, *40*, 7235 – 7238.
[35] W. B. Turnbull, J. F. Stoddart, *Rev. Mol. Biotechnol.* **2002**, *90*, 231 – 255.
[36] A. P. Krapcho, C. S. Kueli, *Synth. Commun.* **1990**, *20*, 2559 – 2564.
[37] M. J. Camarasa, P. Fernandez-Resa, M. Garcia-Lopes, F. G. De Las Heras, P. P. Mendez-Castrillon, A. San Felix, *Synthesis* **1984**, *6*, 509 – 510.

Received: May 20, 2003
Revised: December 8, 2003 [F 669]

