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Synthetic *N*-Acetyl-D-glucosamine Based Fully Branched Tetrasaccharide, a Mimetic of the Endogenous Ligand for CD69, Activates CD69$^+$ Killer Lymphocytes upon Dimerization via a Hydrophilic Flexible Linker

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On the basis of the highly branched ovomucoid-type undecasaccharide that had been shown previously to be an endogenous ligand for CD69 leukocyte receptor, a systematic investigation of smaller oligosaccharide mimetics was performed based on linear and branched *N*-acetyl-D-hexosamine homooligomers prepared synthetically using hitherto unexplored reaction schemes. The systematic structure−activity studies revealed the tetrasaccharide GlcNAcβ1−3(GlcNAcβ1−4)(GlcNAcβ1−6)GlcNAc (compound **52**) and its α-benzyl derivative **49** as the best ligand for CD69 with IC_{50} as high as 10^{-9} M. This compound thus approaches the affinity of the classical high-affinity neoglycoprotein ligand $GlcNAc_{23}BSA$. Compound **68**, GlcNAc tetrasaccharide **52** dimerized through a hydrophilic flexible linker, turned out to be effective in activating CD69$^+$ lymphocytes. It also proved efficient in enhancing natural killing in vitro, decreasing the growth of tumors in vivo, and activating the CD69$^+$ tumor infiltrating lymphocytes examined ex vivo. This compound is thus a candidate for carbohydrate-based immunomodulators with promising antitumor potential.

Introduction

In view of the recent increasing incidence of pathological states characterized by secondary immune deficiencies in the general population, considerable attention has been given to the investigation of effective ways to modulate the activities of the individual components of the immune system. Natural killer (NK[a]) cells, which form 5−10% of mononuclear cells in the blood, have many unique immunological activities and thus provide an obvious target for immune activation. NK cells play a crucial role in innate immunity, as they are characterized by fast and strong cytolytic response against tumor or virally infected cells. They also have the ability to release cytokines and chemokines mediating inflammatory responses and to influence hematopoiesis and the adaptive immune response.[1−3] Despite their role in immune defense mechanisms, major questions regarding their therapeutical potential remained unanswered.[4,5] The ability of NK cells to discriminate between normal and tumor or virally infected cells is now much better understood because of the identification of various surface NK receptors contributing to the process of NK cell activation or inactivation.[4−6] The activation and triggering of natural killing are under the control of a complex signaling machinery to which many receptors, components of the cell surface "receptor zipper", are known to contribute.[7] In this study, we focused our attention on a group of calcium-dependent animal lectins with binding affinity for carbohydrate structures. The complex saccharide structures are involved in many biologically important signal transduction processes, and thus, they play a key role in molecular recognition events contributing to cell−cell, cell−bacteria, and cell−virus interactions.[8−10] The lectin receptors are able to recognize oligosaccharide structures present on the surface of tumor cells and initiate their lysis by cells of the immune system.[11,12] We are interested in two NK cell lectin activation receptors, rat NKR-P1 and human CD69, unique for their ability to distinguish between closely related carbohydrate structures and to recognize the *N*-acetyl-D-hexosamines (HexNAc) in both gluco and galacto configurations.[13,14] Carbohydrates interact with these lectins over an extensive surface area, but the structure and position of the oligosaccharide binding sites are unique for each of the two receptors. Rat NKR-P1 has a binding groove that accommodates the linear oligosaccharides,[15] whereas sugar-binding sites in human CD69 are at three separate locations, and thus branched carbohydrates seem to be preferred.[16,17] Our previous systematic study of the activating lectin receptor NKR-P1

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[a]Abbreviations: 3N, GlcNAcβ1−3GlcNAc; 6N, GlcNAcβ1−6GlcNAc; A, GalNAc; BN, GlcNAcβ1−3(GlcNAcβ1−4)GlcNAc; CB, GlcNAcβ1−4GlcNAc; CT, GlcNAcβ1−4GlcNAcβ1−4GlcNAc; HexNAc, *N*-acetyl-D-hexosamine; ION, ionomycin; LAC, lactose dimer; mAb, monoclonal antibody; N, GlcNAc; NG, $GlcNAc_{23}BSA$ neoglycoprotein; NK, natural killer; OM, [GlcNAcβ1−2(GlcNAcβ1−4)(GlcNAcβ1−6)Manα1−6]-[GlcNAcβ1−2(GlcNAcβ1−4)Manα13][GlcNAcβ1−4]-Manβ1−4GlcNAcβ1−4GlcNAc.

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Chart 1. Structures of the Synthesized HexNAc Based Oligosaccharides Used in the Study

First series

GlcNAc	GlcNAcβ1-3GlcNAc	GlcNAcβ1-4GlcNAc	GlcNAcβ1-4GlcNAcβ1-4GlcNAc
N	3N	CB	CT
GalNAc	GalNAcβ1-3GalNAc	GalNAcβ1-4GalNAc	GalNAcβ1-4GalNAcβ1-4GalNAc
A	**37**	**21**	**32**

Second series

GlcNAcβ1-3(GlcNAcβ1-4)GlcNAc

BN

GalNAcβ1-3(GalNAcβ1-4)GalNAc

61

Third series

GlcNAcβ1-6GlcNAc	GlcNAcβ1-3(GlcNAcβ1-6)GlcNAc
6N	**53**
GlcNAcβ1-4(GlcNAcβ1-6)GlcNAc	GlcNAcβ1-3(GlcNAcβ1-4)(GlcNAcβ1-6)GlcNAc
54	**52**

High affinity natural

[GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6][GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3]

[GlcNAcβ1-4]Manβ1-4GlcNAcβ1-4GlcNAc OM

High affinity artificial

$GlcNAc_{23}$BSA neoglycoprotein

NG

demonstrated the binding hierarchy of HexNAc type oligosaccharides. The binding affinity increases in the group of monosaccharides from GlcNAc and GalNAc to ManNAc and in the order of chitooligomers ([-β-D-GlcNAc(1→4)]$_n$) with elongation of the oligosaccharide chain up to four sugar units. In linear oligosaccharides of the *N*-acetylglucosamine type, the β(1→4) glycosidic bond is preferred over β(1→6)- and β(1→3)-linked regioisomers of chitobiose ([-β-D-Glc*p*NAc(1→4)]$_2$) showing lower binding affinity.[18,19]

In the case of the human CD69 receptor, the physiological ligands are not yet known. Using recombinant CD69 protein, we have previously identified three separate binding sites for GlcNAc in the monomeric unit of this receptor.[20] Moreover, we have shown that the complex pentaantennary bisecting undecasaccharide from egg white glycoprotein ovomucoid with the structure [GlcNAcβ1−2(GlcNAcβ1−4)(GlcNAcβ1−6)-Manα1−6][GlcNAcβ1−2(GlcNAcβ1−4)Manα13][GlcNAcβ1−4]Manβ1−4GlcNAcβ1−4GlcNAc (OM, Chart 1), is one of the best ligands of natural origin identified so far.[17] The affinity of this natural oligosaccharide for CD69 is in the low nanomolar range, thus competing successfully with the artificial high affinity ligand, GlcNAcBSA neoglycoprotein (NG, Chart 1). This natural structure has thus provided an important paradigm for the development of smaller synthetic oligosaccharide mimetics that would be accessible for large scale synthesis and further potential use for in vivo experimental tumor therapies. The total chemical synthesis of complex-type N-linked oligosaccharides identical to natural ones has been achieved,[21] but this involves a number of chemical protection/deprotection steps and remains a time-consuming and costly task. Additional approaches have been developed to supply the glycobiology community with a sufficient amount of pure complex oligosaccharides, but these procedures have not yet been sufficiently adapted for robust and inexpensive synthesis.[9,18] Yet another possibility is represented by de novo chemical syntheses of branched homooligosaccharides, which might afford new oligosaccharides rarely occurring in nature in pure form and in sufficient amounts.[22,23] Recently, we synthesized the branched homotrisaccharide β-D-GlcNAc-(1→3)[β-D-GlcNAc-(1→4)]-D-GlcNAc and found that its binding affinity for NK cell lectin receptors competed successfully with oligosaccharides of much greater complexity.[19]

Here we aimed to identify oligosaccharide ligands useful for the modulation of activities of NK cells through their surface receptors, NKR-P1 and CD69.[11,17] We focused on assessing the binding affinity of NKR-P1 receptor for linear homooligosaccharides composed of β-linked HexNAc with several types of glycosidic bonds. In the case of CD69, we tried to select a small size oligosaccharide composed of highly branched HexNAc that would mimic the natural oligosaccharide from ovomucoid described above.[17] For this purpose we synthesized and biologically tested a group of model oligosaccharides: (a) linear homooligosaccharides of *N*-acetylgalactosamine type, i.e., [-β-D-GalNAc(1→4)]$_2$ (**21**), [-β-D-GalNAc(1→4)]$_3$ (**32**), and [-β-D-GalNAc(1→3)]$_2$ (**37**); (b)

branched homooligosaccharides of *N*-acetyl-D-glucosamine type, i.e., triantennary tetrasaccharide β-D-GlcNAc-(1→3)-[β-D-GlcNAc-(1→6)]-[β-D-GlcNAc-(1→4)]-D-GlcNAc (**52**) and biantennary trisaccharides β-D-GlcNAc-(1→3)-[β-D-GlcNAc-(1→6)]-D-GlcNAc (**53**) and β-D-GlcNAc-(1→4)-[β-D-GlcNAc-(1→6)]-D-GlcNAc (**54**); and (c) biantennary homooligosaccharide of *N*-acetyl-D-galactosamine type, β-D-GalNAc-(1→3)-[β-D-GalNAc(1→4)]-D-GalNAc (**61**). Dimerization of oligosaccharide **52**, which proved to be one of the best ligands for human CD69 using well established chemistry used in peptide and protein chemistry,[24] provided a series of compounds of which **68** proved to be an efficient activators of the effector cells of the immune system when tested both in vitro and in vivo. Thus, the ability of the dimerized oligosaccharide **68** to efficiently activate cells of the immune system parallels that found previously for the dimerized peptide ligand for CD69.[24]

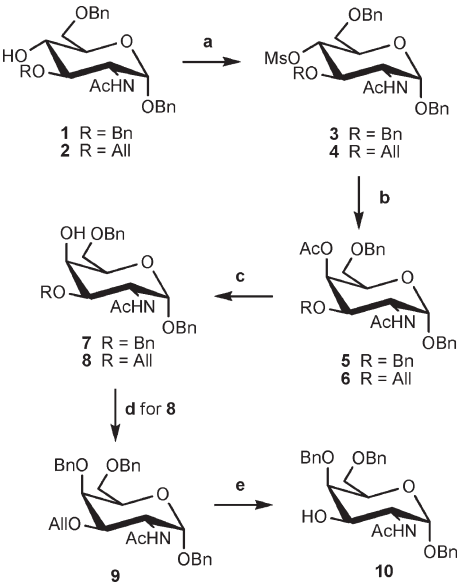
Results

We prepared three series of carbohydrate ligands for NK cell receptors NKR-P1 and CD69 (Chart 1). The first series consisted of linear GalNAc based oligosaccharides **37**, **21**, and **32** used together with commercially available (chitobiose, CB; chitotriose, CT) and previously described (3N, ref 19) oligosaccharides in the GlcNAc (N) format. The second series contained biantennary GalNAc based oligosaccharide **61** intended to provide an equivalent to the corresponding GlcNAc based oligosaccharide BN described previously.[19] The third, most important, series was prepared (based on the results of biological tests with two previous series) only in the GlcNAc (N) format and included the biantennary oligosaccharides **53** and **54** constituting (together with BN) the complete series of trisaccharide GlcNAc (N) isomers, as well as the fully branched triantennary tetrasaccharide **52**.

Chemistry. In the synthesis of the target oligosaccharides, we were often confronted with sterically hindered systems, especially in the case of highly branched structures and oligosaccharide structures consisting of *N*-acetylgalactosamine units. In general, the axially oriented C(4)−OH group on a galactopyranose skeleton is the least reactive secondary OH group.[25] To overcome this fact, the phthalimide glycosylation method was applied. This procedure is considered to be one of the most efficient 1,2-trans-stereoselective glycosylation processes for low-reactive secondary OH groups.[26] Nevertheless, we recently observed reduced stereoselectivity of this method when very low reactive aglycons were used.[27]

The parallel approach enabling simultaneous glycosylation of several hydroxyl groups of the glycosyl acceptor was employed for the preparation of branched oligosaccharides.[19] In addition, a silver perchlorate promoted glycosylation procedure was applied using glycosyl bromides as glycosyl donors in the presence of silver carbonate. This was selected because of relatively good efficiency and stereoselectivity in the case of low reactive, axially oriented OH(4) groups in comparison to other standard glycosylation methods.[27] Silver carbonate acts as a scavenger of perchloric acid and was used instead of a commonly used organic base, which can inhibit glycosylation reaction.[28,29] Appropriate glycosyl donors and glycosyl acceptors of galacto configuration were obtained via inversion of the configuration at the C(4) carbon of the corresponding synthons with gluco configuration by nucleophilic displacement of the mesyl group by sodium acetate.[27]

Scheme 1[a]



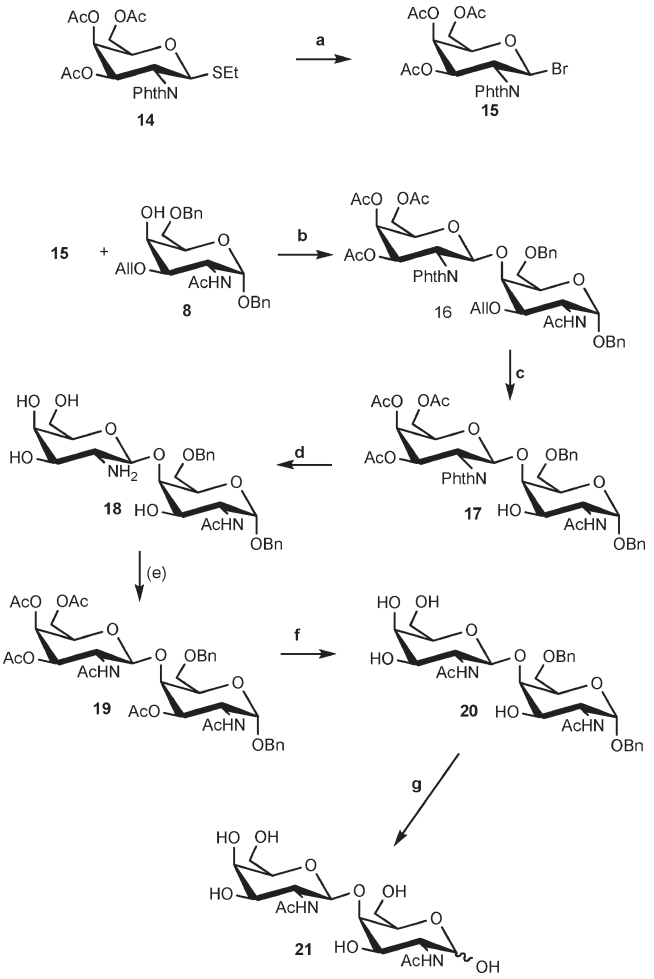
[a] Reagents and conditions: (a) MsCl, pyridine, room temp; (b) NaOAc, DMSO, 130 °C; (c) MeONa, MeOH, room temp; (d) BnBr, NaH, DMF, room temp; (e) $(Ph_3P)_3RhCl$, toluene−EtOH−H_2O, reflux, and then HCOOH, reflux.

Synthesis of Linear Oligosaccharides of the D-Galactosamine Type. In contrast to the large number of studies devoted to the synthesis of linear oligosaccharides with β(1→4)-linked 2-amino-2-deoxy-D-glucopyranose units, efficient and practical methods for the synthesis of analogous oligosaccharides with β(1→4)-linked 2-amino-2-deoxy-D-galactopyranose units remain scarce.[26] This unsatisfactory situation was apparently due to problems with formation of a β(1→4) glycosidic bond between two D-galactosamine units. Only few reports on disaccharide syntheses of this type have been published so far, and these used a glycosyl acceptor having the azido group as the masked amino function at the C(2) position and 2-deoxy-2-phthalimido-D-galactopyranosyl bromide as the glycosyl donor.[30,31]

Our primary goal in the synthesis of the target β(1→4)- and β(1→3)-linked linear oligosaccharides of the D-galactosamine type (**21**, **32**, and **37**) was to prepare appropriate glycosyl acceptors **7**, **8**, **10** (Scheme 1), and **13**. Compound **7** was obtained from benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-4-*O*-methanesulfonyl-α-D-glucopyranoside[32] (**3**) upon treatment with anhydrous sodium acetate in dimethyl sulfoxide at 130 °C to give the galacto-derivative **5**, which afforded **7** by Zemplén deacetylation. The same synthetic approach was applied for the preparation of compound **8** starting from benzyl 2-acetamido-3-*O*-allyl-6-*O*-benzyl-2-deoxy-α-D-glucopyranoside[28] (**2**). O-Benzylation of **8** and subsequent deallylation of compound **9** so obtained by catalytic isomerization of the protecting allyl group to prop-1-enyl group using Wilkinson's catalyst $(Ph_3P)_3RhCl$, followed by acid hydrolysis, led to glycosyl acceptor **10**. Benzyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-galactopyranoside (**13**) was prepared from ethyl 4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-galactopyranoside[27] (**11**) in two steps using a procedure described by Westerlind et al.[33]

The use of 3,6-di-*O*-benzylderivative **7** as a starting glycosyl acceptor in preparation of the target β(1→4)-linked oligosaccharides **21** and **32** was not successful. Glycosylation

Scheme 2[a]



[a] Reagents and conditions: (a) Br_2, CH_2Cl_2, 0 °C; (b) $AgClO_4$, Ag_2CO_3, CH_2Cl_2, −15 °C; (c) $(Ph_3P)_3RhCl$, toluene−EtOH−H_2O, reflux, and then HCOOH, reflux; (d) $BuNH_2$, MeOH, reflux; (e) Ac_2O, pyridine, room temp; (f) MeONa, MeOH, room temp; (g) H_2, Pd/C, AcOH−H_2O, room temp.

of **7** with 4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-D-galactopyranosyl bromide[27] (**22**) promoted by silver perchlorate in the presence of silver carbonate gave a complex mixture of reaction products. Any attempts to isolate the expected disaccharide benzyl 4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-galactopyranoside failed.

The disaccharide **21** was successfully prepared when the less sterically hindered 3-*O*-allyl derivative **8**, as glycosyl acceptor, and glycosyl bromide **15** protected with less bulky and more electronegative *O*-acetyl groups as glycosyl donor were used at the glycosylation step (Scheme 2). Their reaction under the same conditions afforded the required β(1→4)-linked disaccharide **16** in a yield of 37%. The glycosyl donor **15** described by Nilsson et al.[34] was prepared by an alternative way, i.e., from ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-galactopyranoside[35] (**14**) in reaction with bromine.

Deallylation of the protected disaccharide **16** using the aforementioned procedure gave compound **17**. The alkali-labile protecting groups were removed by treatment with *n*-butylamine in boiling methanol, and the obtained amine **18** was peracetylated by reaction with acetic anhydride in pyridine to give **19**. Zemplén O-deacetylation of **19** yielded benzyl glycoside **20**. Hydrogenolysis of benzyl groups over Pd/C catalyst afforded the target unprotected β(1→4)-linked disaccharide **21**.

The problem of effectively synthesizing β(1→4)-linked galactosamine type oligosaccharides was solved by employing compound **13** as the starting glycosyl acceptor (Scheme 3). Compound **13**, bearing a phthalimido group at position C(2) instead of an acetamido group, was chosen to decrease the negative influence of intermolecular H-bonds between the OH group and the NH group of the amide function in the glycosylation process.[36−38] Glycosylation of **13** with glycosyl bromide **22** under the above-described conditions gave the required disaccharide **23** in a satisfactory yield of 48%. Furthermore, 4-*O*-acetyl-1,5-anhydro-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-D-lyxo-hex-1-enitol (**24**) and 4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimi-do-β-D-galactopyranoside (**25**) were isolated as the reaction side products, i.e., the products of elimination and cross-reaction of a glycosyl donor.[39,40] Formation of glycal and symmetrical disaccharide with the β head to head glycosidic bond similar to that found in β,β-trehalose from glycosyl donors of 2-deoxy-2-phthalimido-D-glucopyranose type has been described.[39,40]

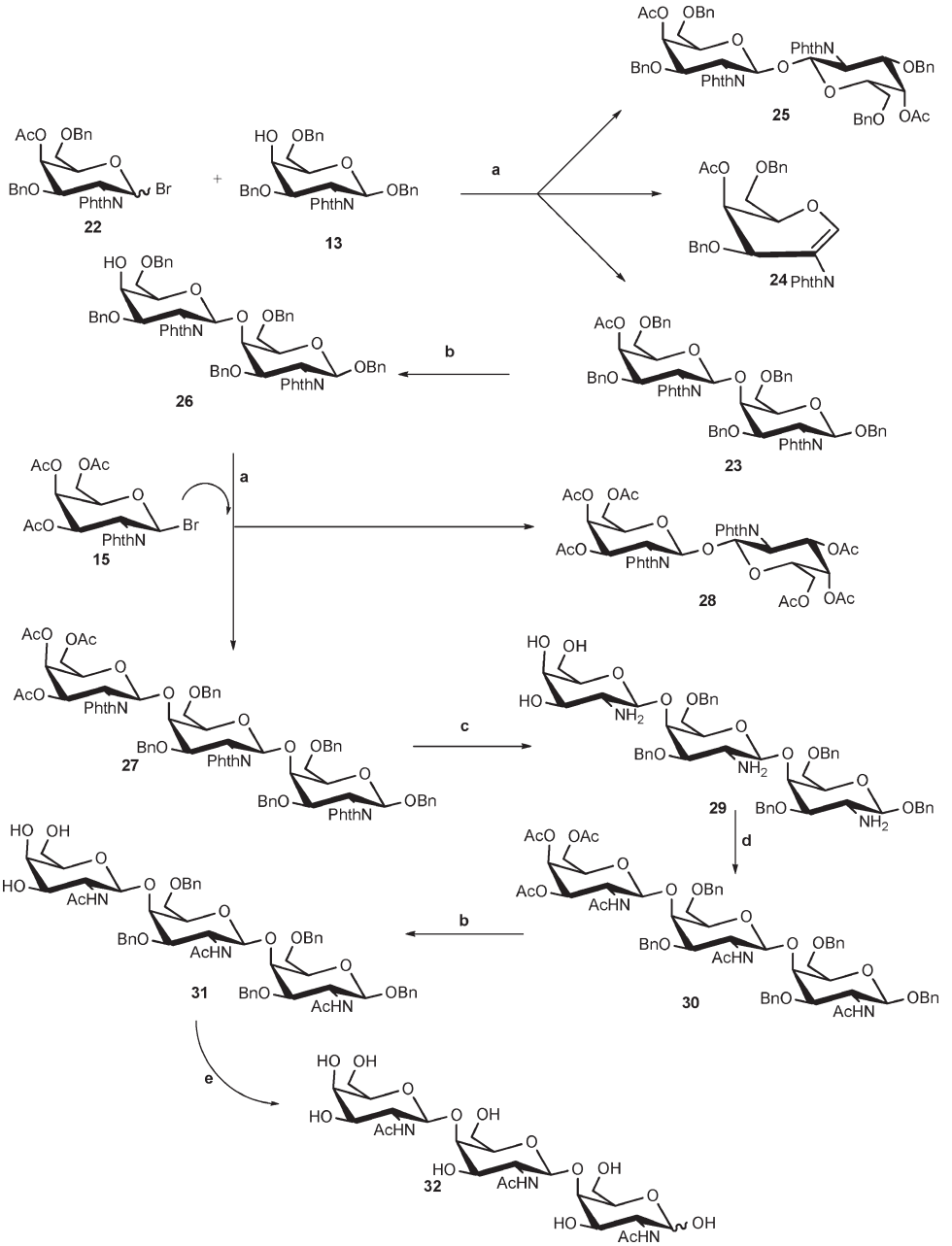
Zemplén deacetylation of disaccharide **23** followed by glycosylation of the obtained compound **26** with glycosyl donor **15** afforded trisaccharide **27** in an overall yield of 51%. Furthermore, the β,β-trehalose type product **28** was also isolated.[19] The alkali-labile protecting groups were removed by treatment with hydrazine hydrate in boiling ethanol, and the obtained crude amine **29** was acetylated by acetic anhydride in pyridine to give compound **30**. Zemplén deacetylation of **30** gave benzyl glycoside **31**, and its benzyl groups were subsequently hydrogenolysed over Pd/C catalyst to give the target unprotected trisaccharide **32**.

For the preparation of β(1→3)-linked galactosamine disaccharide **37** (Scheme 4) a synthetic approach analogous to the one used for the synthesis of linear oligosaccharides containing β(1→4)-linked *N*-acetyl-D-galactosamine was employed. Silver perchlorate in the presence of silver carbonate promoted glycosylation of glycosyl acceptor **10** with galactopyranosyl bromide **15** to provide β(1→3)-linked disaccharide **33** in a good yield of 70%. This was then deprotected to obtain the final disaccharide **37**.

Synthesis of Branched Oligosaccharides of the D-Glucosamine Type. Synthesis of the target triantennary tetrasaccharide **52** and biantennary trisaccharides **53** and **54** of D-glucosamine type was carried out starting from multiple glycosylation of the glycosyl acceptor benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**39**) (Scheme 5). Glycosylation of **39** with 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[28] (**38**), promoted with silver perchlorate in the presence of silver carbonate, afforded the tetrasaccharide **40** (15%) and deletion trisaccharides **41** (8%) and **42** (17%). The obtained oligosaccharides were converted to unprotected oligosaccharides **52**, **53**, and **54** as described above.

Synthesis of Branched Trisaccharide of the D-Galactosamine Type. Similar to the synthesis of branched oligosaccharides containing D-glucosamine units, the approach based on multiple glycosylation was applied as well for D-galactosamine type oligosaccharides. The glycosyl acceptor benzyl 2-acetamido-6-*O*-benzyl-2-deoxy-α-D-galactopyranoside (**55**),

Scheme 3[a]



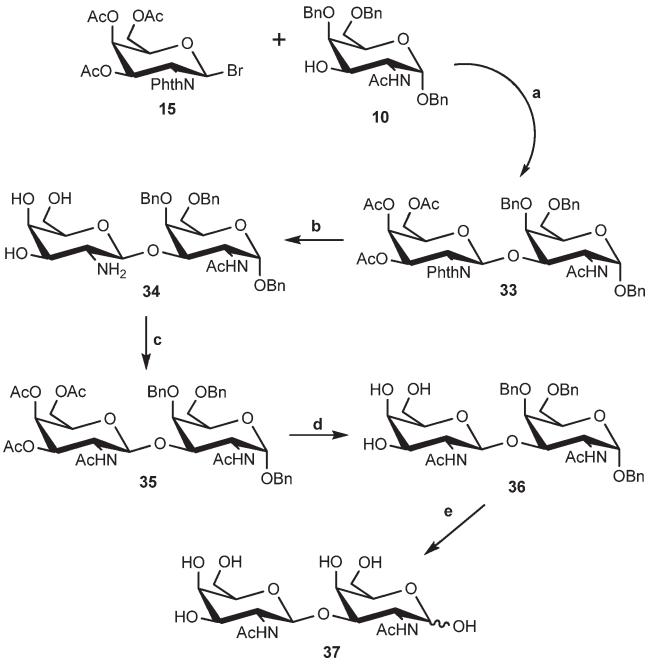
[a] Reagents and conditions: (a) $AgClO_4$, Ag_2CO_3, CH_2Cl_2, −15 °C; (b) MeONa, MeOH, room temp; (c) $N_2H_4.H_2O$, EtOH, reflux; (d) Ac_2O, pyridine, room temp; (e) H_2, Pd/C, AcOH - H_2O, room temp.

obtained by deallylation of compound **8**, was glycosylated by 3 molar excess of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-*β*-D-galactopyranosyl bromide[34] (**15**), employing promotion by silver perchlorate in the presence of silver carbonate (Scheme 6). This reaction afforded not only branched trisaccharide **57** (9%) but also disaccharide **56** (13%) as an undesired side product resulting from the lower reactivity of the axial C(4)−OH group of the glycosyl acceptor. After deprotection as above we obtained the target biantennary disaccharide **61**.

Biological Evaluation. In order to evaluate the binding affinity of the synthesized oligosaccharides for the target NK cell receptors, we started with the recombinant rat NKR-P1A receptor shown previously[13,18] to have high affinity for GlcNAc monosaccharide and related compounds. Microtiter plates were coated with high affinity ligand $GlcNAc_{23}BSA$ neoglycoprotein, and individual compounds were tested as inhibitors of binding of the soluble radiolabeled receptor to these plates. Results shown in Figure 1 indicate that the synthesized compounds were mostly average or poor ligands compared to the GlcNAc control. Here, in the linear GlcNAc/GalNAc series, the $\beta1-4$ linkage is preferred to other ($\beta1-3$ or $\beta1-6$) linkages (Figure 1A). Branching of the oligosaccharides resulted in significant decrease in the inhibitory potencies independently of the series used (Figure 1B and Figure 1C).

More interesting results were obtained when the synthesized compounds were tested as inhibitors of binding of another lymphocyte receptor, human CD69, to its high affinity $GlcNAc_{23}BSA$ ligands.[14,20] This receptor has been

Scheme 4[a]



[a] Reagents and conditions: (a) $AgClO_4$, Ag_2CO_3, CH_2Cl_2, −15 °C; (b) $N_2H_4.H_2O$, EtOH, reflux; (c) Ac_2O, pyridine, room temp; (d) MeONa, MeOH, room temp; (e) H_2, Pd/C, AcOH−H_2O, room temp.

shown previously[16,20] to contain multiple binding sites for GlcNAc in its molecule and thus to prefer branched *N*-acetyl-D-hexosamine sequences to linear ones.[17] Indeed, only minor differences have been found in the linear GlcNAc/GalNAc series compared to the GlcNAc monosaccharide control (Figure 1D). However, a notable hierarchical increase in inhibitory potencies has been found in the branched GlcNAc/GalNAc series (Figure 1E). This increase has been considerably more profound in the GlcNAc series compared to the GalNAc series (Figure 1E).

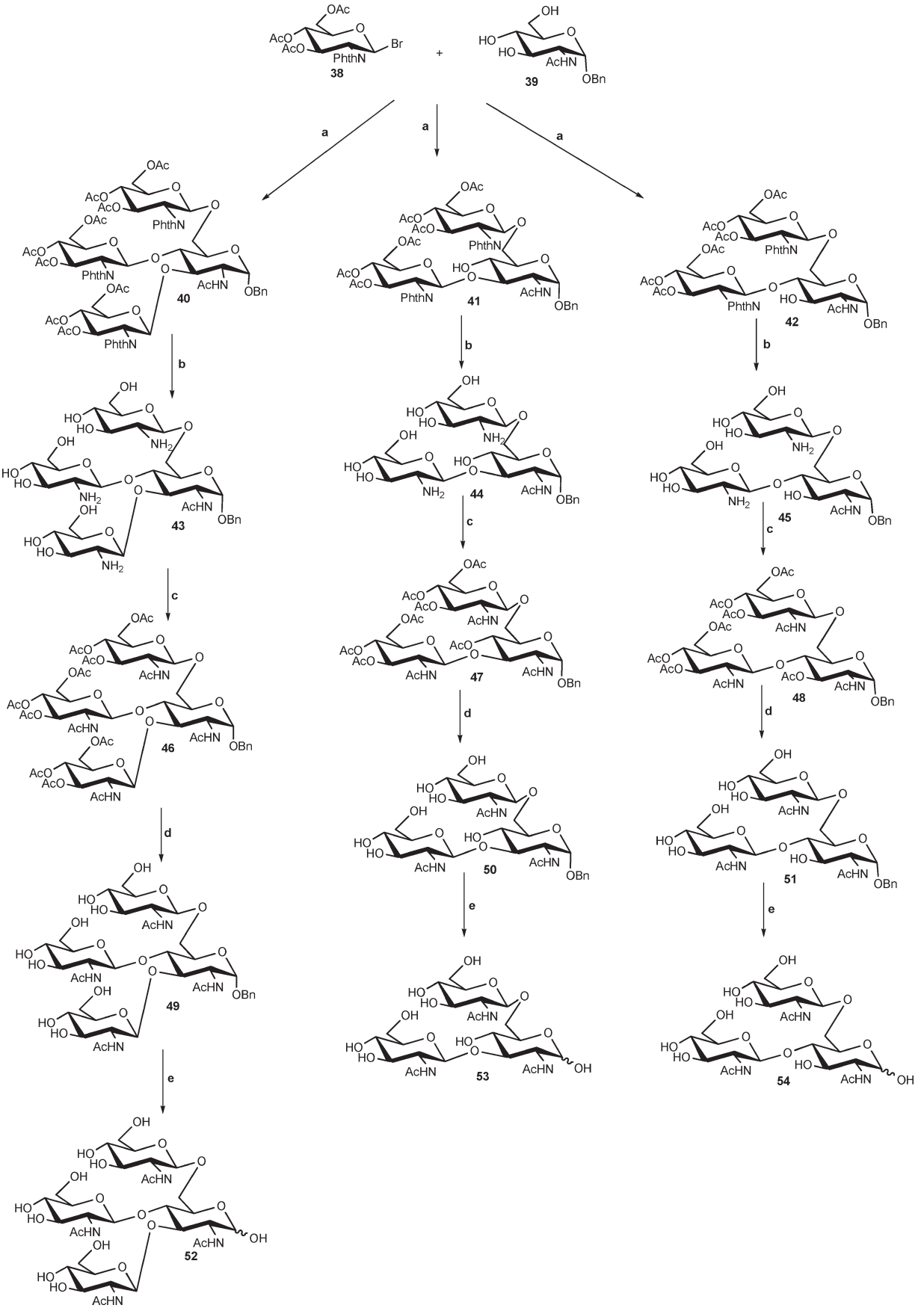
On the basis of these findings, detailed structure−activity studies were performed in the branched GlcNAc series. Here, an even more developed hierarchy of the gradually increasing affinities could be seen (Figure 1F). The trisaccharide BN described previously[19] has already attained 10 times better inhibitory potency compared to the GlcNAc control. The newly synthesized compounds **53** and **54** have reached 100 times and 1000 times greater inhibitory activities, respectively. The small, fully branched tetrasaccharide **52** was an even better inhibitor, achieving 10 000 times better inhibitory activity than the GlcNAc control. Its corresponding α-benzyl derivative was even 10 times more efficient, attaining the potency of the much more complex natural oligosaccharide OM, and it was a 100 000 times more potent inhibitor compared to the *N*-acetyl-D-glucosamine monosaccharide (Figure 1F). Thus, the tetrasaccharide **52** has been selected for further work as an efficient mimetic of a much larger ovomucoid undecasaccharide (OM, Chart 1) described previously as natural ligand,[17] as well as of the classical artificial high affinity ligand, GlcNAc neoglycoprotein (NG, Chart 1) .[14]

Direct Binding Assay for 52 and 49. The high affinity interaction of **49** with the soluble recombinant lymphocyte receptor CD69 was further confirmed using several techniques allowing us to observe the binding directly. Compound **49** appeared to be particularly suitable for these studies, since its anomeric configuration is fixed in the α-position through the benzyl residue. The initial confirmation of high affinity binding of **49** to recombinant human CD69 was provided by NMR titration (Figure 2A). Evaluation of the binding curve revealed a complete disappearance of the acetate signals due to specific binding until the saturation point corresponding to one bound molecule of the oligosaccharide per receptor subunit, after which a linear increase of the free (unbound) ligand was observed. Evaluation of the binding curve also provided a quantitative estimation for the value of K_d in the nanomolar range, which is in complete agreement with the inhibition data. Two additional binding techniques were employed. Direct binding assay using ^{3}H-labeled tetrasaccharide **52** allowed us to establish the details of the binding parameters: there was one high affinity binding site per receptor subunit (two sites per receptor dimer) with $K_d = 3.2 \times 10^{-9}$ M (Figure 2B). Moreover, the binding to the dimeric receptor displayed the same cooperativity reported previously for the high affinity binding site for GlcNAc with the Hill coefficient approaching the maximal theoretical value for a two-subunit protein ($h_{theor} = 2$, $h_{expt.} = 1.98$).[41] The third direct binding assay based on tryptophan fluorescence quenching was performed using both the dimeric CD69 receptor[42] and its monomeric form obtained by site-directed mutagenesis.[41] This arrangement allowed us to confirm the dependence of both affinity and ligand binding cooperativity on the dimeric arrangement of the receptor: the K_d value dropped from 3.4×10^{-9} to 1.12×10^{-7} M, and only binding according to the single-site model could be observed for the monomeric protein (Hill's coefficients were 1.94 and 1.05 for the dimeric and for the monomeric protein, respectively; Figure 2C and D).

Dimerized 52 Efficiently Precipitates Soluble CD69 Depending on Linker Chemistry. High affinity binding of **52** to CD69 shown by both inhibition and direct binding tests indicated that this oligosaccharide might represent a suitable minimum mimetic of the complex physiological ligand for the receptor. In order to activate CD69$^+$ immune cells through an engagement of this antigen, however, the ligand mimetic must be present in a multivalent (at least bivalent) form. To achieve efficient dimerization of the tetrasaccharide **52**, we have adopted the standard chemistry used for peptide and protein cross-linking[24] and elaborated it for efficient activation and coupling of oligosaccharides activated as β-glycosylamines (Scheme 7).[43] The entire procedure involves a conversion of the reducing oligosaccharide **52** into the corresponding β-glycosylamine **62**, followed by reaction with thiophosgene to produce the reactive isothiocyanate **63**. This compound is then coupled to a series of linear aliphatic diamines to produce the dimeric tetrasaccharides **64**−**67** having a linker of varying length defined by two to eight methylene groups. The synthesized and purified GlcNAc tetrasaccharide dimers were evaluated for their abilities to precipitate soluble CD69 protein in a process that resembles the cross-linking of the cellular form of the receptor. While the monomeric tetrasaccharide **52** was not active in this test, the dimeric compounds **64**−**67** proved to be positive (Figure 3A). The best activity was achieved for compound **65** bearing the butyl aliphatic linker.

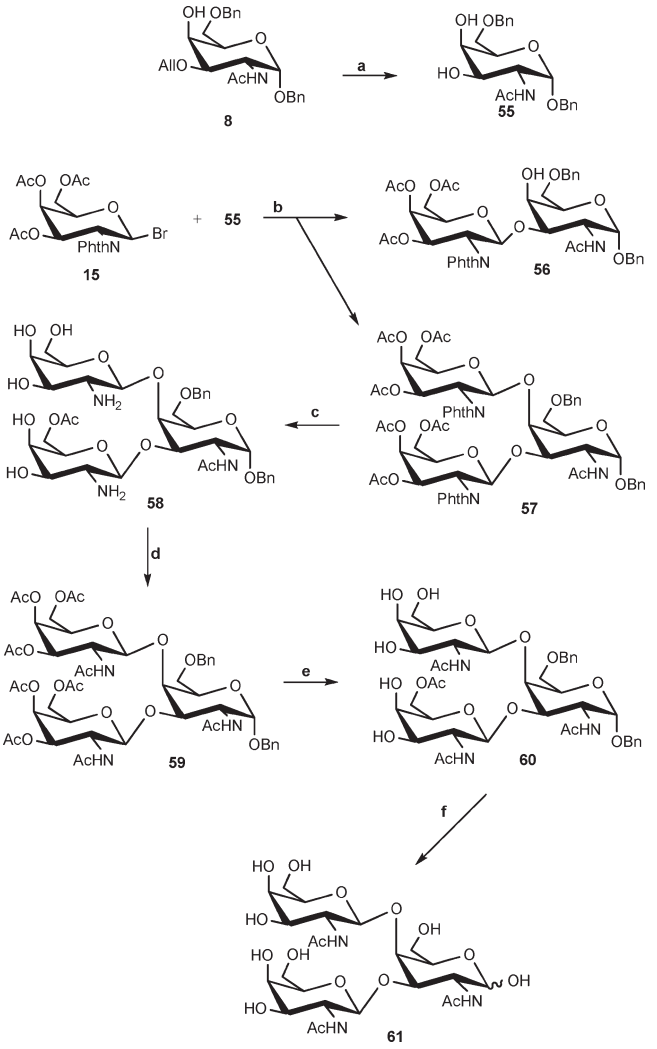
Recently published work has provided a clear indication that not only the length but also the chemical nature of a linker, such as its hydrophobicity and flexibility, may significantly influence the outcome of an interaction of a bivalent artificial ligand with a corresponding receptor.[44]

Scheme 5[a]



[a] Reagents and conditions: (a) $AgClO_4$, Ag_2CO_3, CH_2Cl_2, −15 °C; (b) $N_2H_4 \cdot H_2O$, EtOH, reflux; (c) Ac_2O, pyridine, room temp; (d) MeONa, MeOH, room temp; (e) H_2, Pd/C, AcOH−H_2O, room temp.

Scheme 6[a]



[a] Reagents and conditions: (a) $(Ph_3P)_3RhCl$, toluene−EtOH−H_2O, reflux, and then HCOOH, reflux; (b) $AgClO_4$, Ag_2CO_3, CH_2Cl_2, −15 °C; (c) $N_2H_4.H_2O$, EtOH, reflux; (d) Ac_2O, pyridine, room temp; (e) MeONa, MeOH, room temp; (f) H_2, Pd/C, AcOH−H_2O, room temp.

Therefore, we took the dimeric tetrasaccharide **65** as the lead compound and prepared three additional similar compounds, **68**, **69**, and **70**, differing in the hydrophobicity and flexibility of the linker based on the starting divalent amine (Scheme 8) used. Compounds **65**, **68**, **69**, and **70** thus all had the optimal length of the four-carbon linker, albeit in four different chemical variants: hydrophobic flexible, hydrophilic flexible, hydrophobic rigid, and hydrophilic rigid, respectively. These compounds, after synthesis and purification, were again tested in the receptor precipitation assay. The results show that optimal activity was achieved for compound **68** containing the hydrophilic flexible linker (Figure 3B). This activity was comparable to that of the previously described lactose-di-*N*-acetyl dimer.[45] The original lead compound **65** with the hydrophilic flexible linker was also quite active, but compounds **69** and **70** with the rigid linker were much less active.

Dimerized Tetrasaccharides 68 Activate Lymphocytes Expressing High Levels of CD69. We decided to verify our conclusions concerning the optimal immunostimulating compound further using $CD69^{high}$ lymphocytes containing the cellular form of the receptor in its natural dimeric and

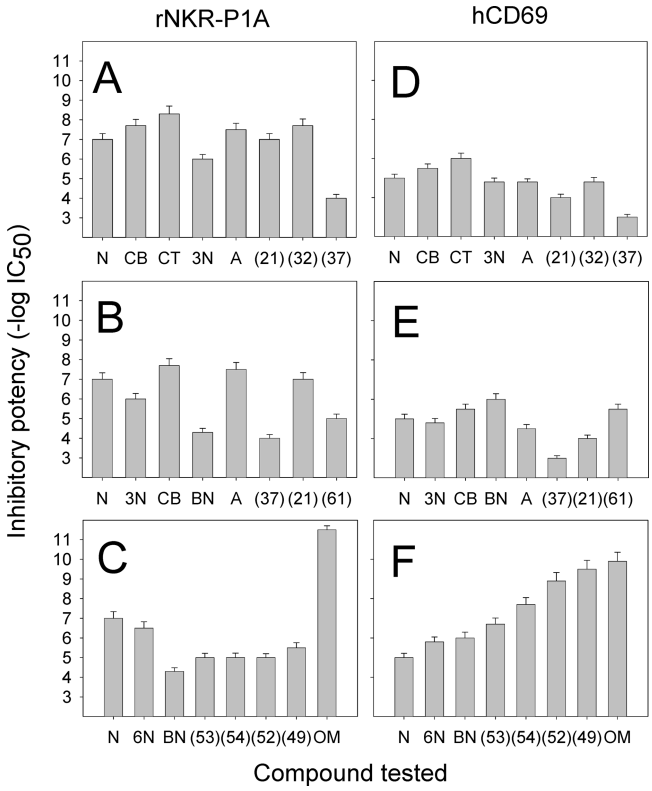


Figure 1. Biological testing of the synthesized HexNAc based oligosaccharides using inhibition assay. Indicated compounds were tested as the inhibitors of binding of the radiolabeled rNKR-P1A (left) or hCD69 (right) to the high affinity $GlcNAc_{23}BSA$ ligand. From the complete inhibition curves, $IC_{\%}$ values were calculated. Shown are the mean ± SD from at least three independent experiments.

fully glycosylated form.[46] Such verification is essential because the activities using the soluble and the cellular forms of the immune receptors may differ significantly.[17] We used the standard cellular activation assays based on the production of inositol phosphates and monitoring of intracellular calcium concentrations.[11] We isolated $CD69^{low}$ (<5% surface expression) and $CD69^{high}$ (>30% surface expression) lymphocytes from human peripheral blood mononuclear cells. Both monomeric tetrasaccharide **52** and four dimeric tetrasaccharides described above (compounds **65**, **68**, **69**, and **70**) were dissolved in PBS for testing as activators of these cellular populations and were compared to the effect of PBS only (negative control), or of specific monoclonal antibodies against CD69 as well as the *N*-acetyllactosamine disaccharide described previously[41,45] to be a potent activator of $CD69^+$ lymphocytes (positive controls). All tested compounds were added at 10-fold molar excess over the estimated amount of surface CD69, as well as over the measured K_d concentration. The inositol phosphate production results show that very little activation of $CD69^{low}$ lymphocytes occurred despite the presence of a small amount of $CD69^+$ cells in this population (Table 1). On the other hand, the dimerized (although not the monomeric) tetrasaccharides **65**−**70** all had notable effects on the production of inositol phosphates with the best compound (**68**) exerting effects comparable to those of the positive controls, monoclonal antibody, and *N*-acetyllactosamine disaccharide (Table 1). The effect of the tested compounds on an increase in intracellular calcium levels followed the effects on inositol phosphates production. In $CD69^{low}$ cellular population only the

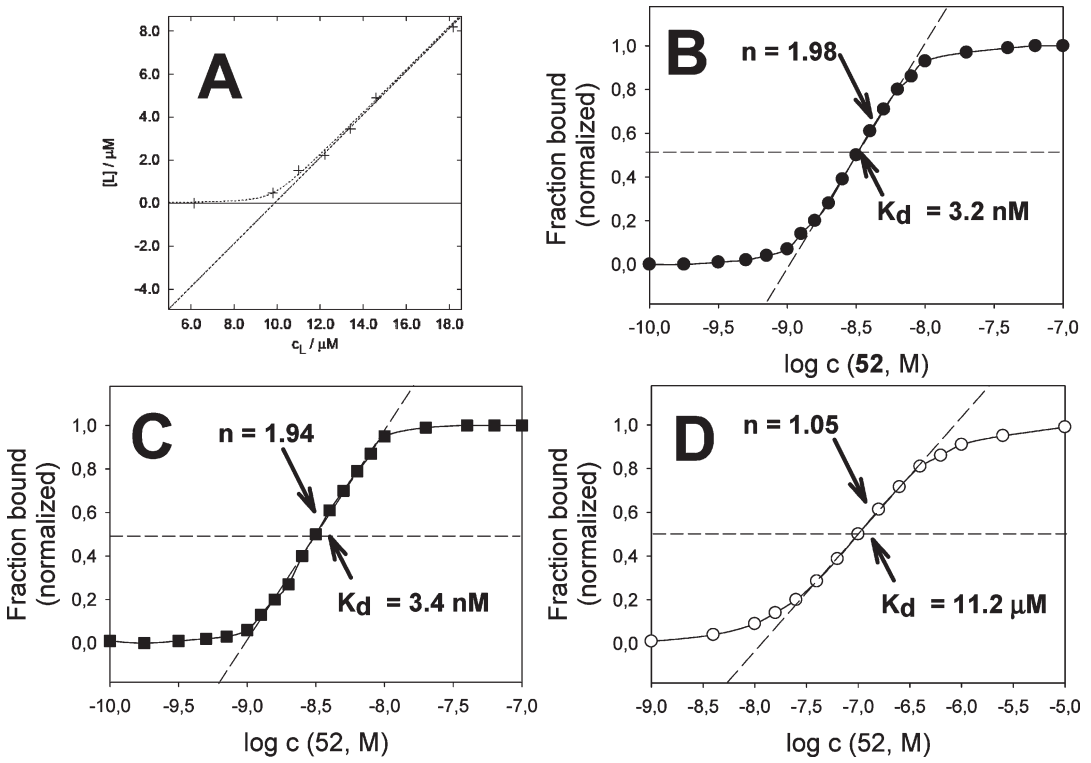
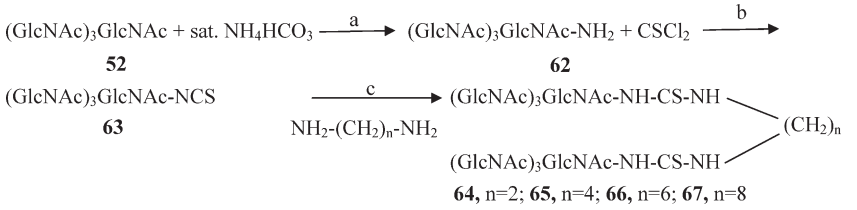


Figure 2. Direct binding of compound **52** to human CD69 receptor: (A) NMR titration of 10 μM soluble CD69 with **49**; (B) binding of ^{3}H-labeled **52** to CD69 followed by equilibrium dialysis; (C, D) binding of **52** to the dimeric and monomeric form of CD69 followed by tryptophan fluorescence quenching.

Scheme 7[a]



[a] Reagents and conditions: (a) sat. aq NH_4HCO_3, 30 °C, 7 days; (b) $CSCl_2$, $NaHCO_3$, acetone−H_2O; (c) 2.4 equiv of **63** per 1 equiv of diamine, CH_2Cl_2, room temp, 2 days.

addition of ionomycin resulted in a significant increase in intracellular calcium (Figure 4A). In CD69^{high} population, all four dimeric tetrasaccharides were active, and the activity of compound **68** was comparable with that of monoclonal antibody positive control. Testing the compounds further at 10 times higher concentrations did not increase their activity (data not shown).

Dimerized Tetrasaccharide 68 Increased the Killing of Tumor Cell Lines in Vitro. To estimate the efficiency of the synthesized compounds in enhancing the antitumor potential of the immune system, we have first tested their effects in the standard short-term (4 h) cytotoxicity assays. Compound **68** and the lead compound **65** increased significantly the killing of human erythroleukemic cell line K562, a standard target cell line known to be sensitive for natural killing. The effect of compound **65** was comparable to that of the two monoclonal antibodies against CD69 used as positive controls, while compound **68** had even much higher effect enhancing the efficiency of natural killing about 4.5 times under the given experimental conditions in vitro (Figure 5A). Moreover, compound **68** was also active in the case of NK resistant tumor cell line RAJI (Figure 5B) in the situation where other compounds or monoclonal antibodies used as positive controls had little effect. Under these experimental conditions compound **68** enhanced natural killing about 3.5 times (Figure 5B).

Dimerized Tetrasaccharides 68 Suppresses the Growth of Experimental Tumors and Activates Tumor Infiltrating Lymphocytes. An initial assessment of the antitumor efficacy of the synthesized compounds was performed using the experimental model of mouse B16 melanoma using a low metastasis variant.[47] In this assay, compound **68** was most efficient decreasing the size of the tumors at day 26 and day 30 (Table 2). Compounds **65** and **70** also had some effects seen at day 30 after the injection of tumor cells. Interestingly, the two monoclonal antibodies against CD69 and the dimerized *N*-acetyllactosamine had very little effect in this assay (Table 2). In order to assess the possible effects of the dimerized tetrasaccharides on the immune system, a large number of immune system parameters must be monitored. However, one of the most important parameter is the activity of killer cells operating inside the tumors (especially tumor infiltrating lymphocytes and dendritic cells). In order to estimate this parameter, we performed cytotoxicity assay

using tumor (melanoma) infiltrating lymphocytes isolated from animals treated with the individual compounds and assayed after isolation of these cells ex vivo. Notably, compound **68** was the only compound among the tested set that remained effective in this assay using both B16 melanoma and NK resistant P815 mastocytoma (Figure 6A and Figure 6B, respectively). Compound **65** also had a statistically significant effect but only in the case of the B16 melanoma (Figure 6A). We have initially tested the effect of the injection of 1 μmol (approximately 2 mg) of the individual compounds, a dose that was much smaller compared to the tested antibodies that had to be injected in 10 mg amounts. While increasing the amounts of the tested compounds had very little benefit, injecting 0.1 μmol amounts had very similar effects, and 0.01 μmol amounts gave no effect.

Discussion

Although the physiological ligand for the widespread leukocyte activation marker and triggering receptor CD69 has not been identified, calcium and certain *N*-acetyl-D-hexosamines have been shown previously to be specific ligands for this receptor.[14,17] Direct binding assays and molecular modeling studies revealed the existence of three binding sites for GlcNAc in the CD69 molecule, one of which represents the high affinity binding site with an estimated value of K_d of 63 μM.[20] Later, by use of a new construct for recombinant

Table 1. Activation of Purified CD69^{low} and CD69^{high} NK Cells Using the Dimeric HexNAc Based Oligosaccharides

cell	control	Mab	LAC	**52**	**65**	**68**	**69**	**70**
CD69^{low}	60[a]	58[a]	72[a]	55[a]	70[a]	65[a]	56[a]	52[a]
	82[b]	77[b]	75[b]	65[b]	71[b]	78[b]	72[b]	75[b]
CD69^{high}	58[a]	1252[a]	1123[a]	58[a]	750[a]	1310[a]	520[a]	720[a]
	76[b]	1420[b]	1374[b]	60[b]	820[b]	1455[b]	610[b]	800[b]

[a] Amount of insP2 was measured 4 min after the addition of individual compounds. [b] Amount of insP3 (cpm) was measured 2 min after the addition of individual compounds.

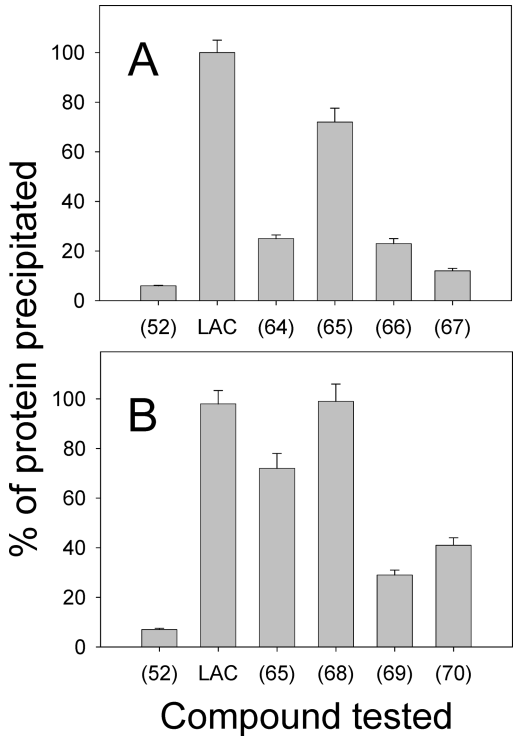


Figure 3. Precipitation of soluble recombinant CD69 by equimolar amounts of the tested ligand: (A) monomeric and dimerized **52** with linker of different length; (B) monomeric and dimerized **52** with linker having various chemical properties (hydrophobicity and rigidity). LAC designates the lactose-di-*N*-acetyl dimer described previously.[45]

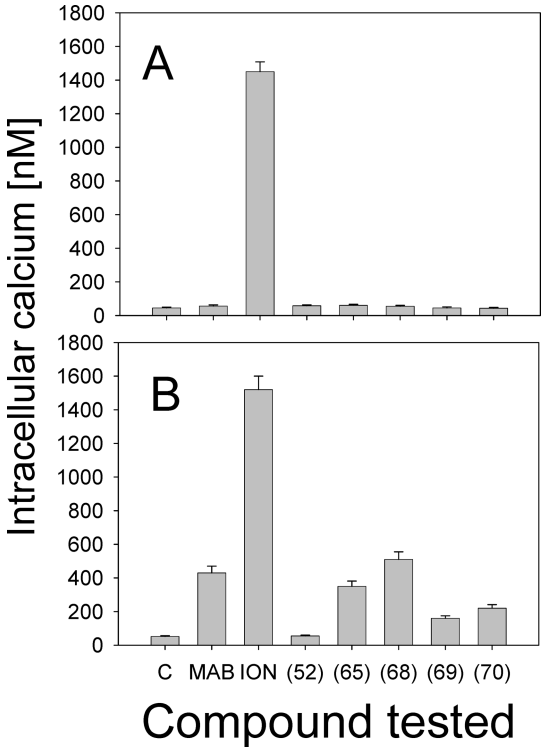
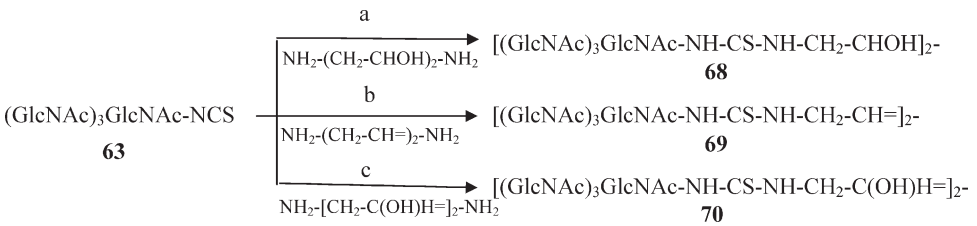


Figure 4. Monitoring of intracellular calcium 3 min after the addition of tested compounds was performed in CD69^{low} (A) and CD69^{high} (B) lymphocytes. ION is the ionomycin positive control. All changes in (B) were statistically significant at $p \leq 0.01$ compared to the PBS control (C). Values for the tested compounds and ionomycin were taken from the complete curves at 3 and 11 min, respectively.

Scheme 8[a]



[a] Reagents and conditions: (a) 1 equiv of 1,4-butanediamine-2,3-diol per 2.4 equiv of **63**, CH_2Cl_2, room temp, 2 days; (b) 1 equiv of 1,4-butanediamine-2-en per 2.4 equiv of **63**, CH_2Cl_2, room temp, 2 days; (c) 1 equiv of 1,4-butanediamine-2,3-diol-2-en, CH_2Cl_2, room temp, 2 days.

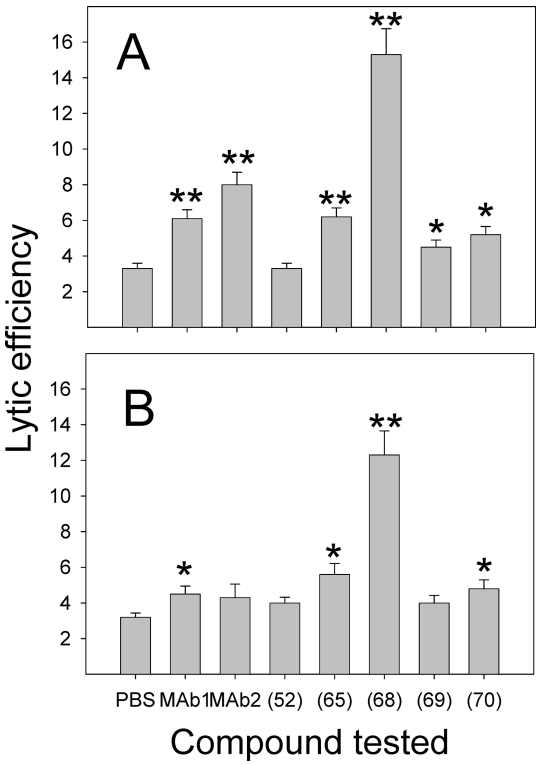


Figure 5. Natural killing assays in the presence of the tested compounds using sensitive human cell line K562 (A) and resistant human cell line RAJI (B). Statistical significance of changes compared to PBS control is marked by asterisks as described in Experimental Section.

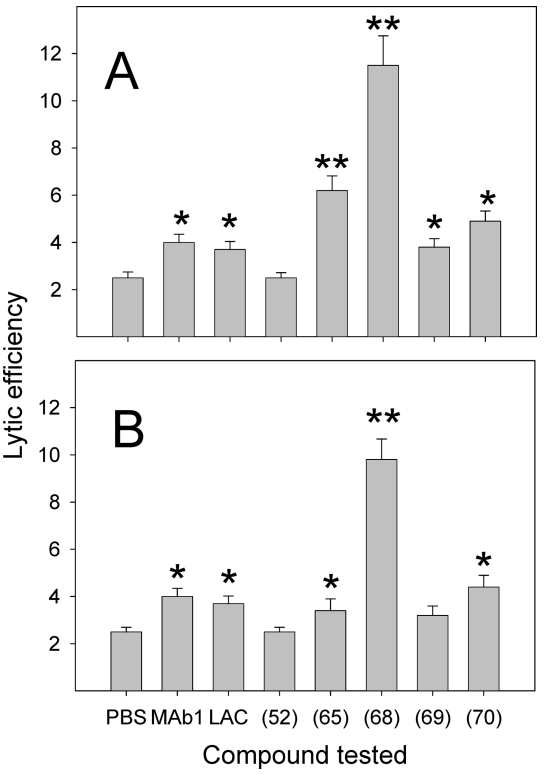


Figure 6. Natural killing of tumor infiltrating lymphocytes isolated from mice treated with the indicated compounds. Killing of B16 melanoma (A) and NK resistant mastocytoma P815 (B) targets is shown. Statistical significance of changes compared to PBS control is marked by asterisks as described in Experimental Section.

Table 2. Initial Assessment of the Antitumor Properties of the Synthesized Compounds Using the Mouse B16S Melanoma Model

	tumor size (cm^2)	
compd	day 26	day 30
PBS	0.6 ± 0.2	1.3 ± 0.3
MAb1	0.6 ± 0.1	1.1 ± 0.2
MAb2	0.4 ± 0.1	0.8 ± 0.2
LAC	0.4 ± 0.1	0.7 ± 0.1
65	0.5 ± 0.1	0.8 ± 0.2
68	0.1 ± 0.1	0.2 ± 0.1
70	0.6 ± 0.2	0.8 ± 0.2

expression of CD69 optimized to have a high physical and biochemical stability,[42] direct binding assays including NMR titrations, equilibrium dialysis, and fluorescence quenching measurements revealed that binding of GlcNAc to the dimeric CD69 protein proceeded in a cooperative fashion with K_d = 0.4 μM.[41] However, after dissociation into the monomeric subunits when no cooperativity in carbohydrate binding could occur, the value of K_d dropped to about 16 μM using the optimized monomeric protein.[41] Recently, high affinity physiological ligand for CD69 has been identified among the highly branched ovomucoid type oligosaccharides.[17] In particular, the pentabranched undecasaccharide having a structure [GlcNAcβ1−2(GlcNAcβ1−4)(GlcNAcβ1−6)Manα1−6][GlcNAcβ1−2(GlcNAcβ1−4)Manα1−3][GlcNAcβ1−4]Manβ1−4GlcNAcβ1−4GlcNAc turned out to be an efficient ligand for CD69 with K_d in the low nanomolar range. This ligand may represent a target structure for $CD69^+$ NK cells, since it could be detected at the surface of NK sensitive targets cell lines and could be induced by stress in NK resistant targets as well.[17] This undecasaccharide may thus represent a suitable initial compound targeting killer lymphocytes including natural killer cells to tumor sites and keeping the killer lymphocytes active in the inhibiting microenvironment of the tumors. Since this undecasaccharide turned out to be available only in small amounts after complicated isolation from biological material[17] and since the total chemical synthesis of such complex oligosaccharide was shown to be very complicated,[21] considerable experimental efforts have been aimed toward the synthesis and identification of smaller oligosaccharide mimetics.

Here we describe one approach toward the development of efficient oligosaccharide mimetic for CD69 based on the synthesis of *N*-acetyl-D-hexosamine-based homooligosaccharides and their dimerization through several types of chemical linkers in order to prepare bivalent compounds suitable for CD69 cross-linking. The initial screening of GlcNAc and GalNAc based homooligosaccharides, both linear and branched, identified the fully branched tetrasaccharide **52** as the best ligand for CD69 with affinity in the nanomolar range, thus approaching the affinity of the much more complex ovomucoid derived undecasaccharide and that of the classical high affinity ligand, $GlcNAc_{23}BSA$ neoglycoprotein. The α-benzyl derivative of this compound was an even better inhibitor, indicating the possibility of additional hydrophobic interaction near the carbohydrate recognition site of CD69.[18] This tetrasaccharide was then used as a lead for the development of an efficient dimerization protocol and optimalization of the linker used for dimerization (length, hydrophobicity, rigidity). The previously developed assay measuring the amount of soluble CD69 precipitated after interaction with the bivalent ligand[45] has been used to provide a simple test of

binding efficiency of the dimerized GlcNAc tetrasaccharides. From these tests, dimerized tetrasaccharide **68** bearing a hydrophilic flexible linker emerged as the most promising compound. Further biological evaluation revealed this compound to be effective in cellular activation of $CD69^{high}$ NK cells, which led to the ability to enhance natural killing in vitro, to decrease the rate of tumor growth in vivo, and to keep the tumor infiltrating lymphocytes activated even in aggressive tumor microenvironment, as revealed after ex vivo examination of their cytotoxicity. Moreover, this compound compared favorably with other reagents cross-linking the CD69 target receptor; it was active in much smaller concentration than the specific monoclonal antibodies against this receptor, and most probably it would also be much less immunogenic.

The exact role of CD69 antigen in NK cell biology is not fully understood. First, CD69 is expressed on many leukocyte subsets[48] and thus cannot be considered as an NK cell specific receptor. Second, the work by Sanchez-Madrid and colleagues using $CD69^{-/-}$ mice provided a clear indication for the negative role of this antigen in tumor cell killing, since the mice lacking surface expression of CD69 were more resistant to tumors.[49] On the other hand, the work of Moretta et al. emphasized that monoclonal antibodies (and perhaps ligands) that are strongly bound to CD69 can activate CD69 positive cells even in the absence of other (antigen dependent) proliferation pathways.[50] Our present work provides a strong support for such a possibility and advocates the dimerized tetrasaccharide **68** as a strong candidate for further systematic experiments looking at the details of its efficacy in other experimental tumor models and the details of the molecular mechanisms of its action.

Conclusions

We prepared efficient mimetics of natural oligosaccharide ligands for CD69, a widespread receptor triggering leukocyte activation. Homooligomeric fully branched GlcNAc tetrasaccharide **52** proved to be the most efficient ligand and could be used as a lead for the development of effective activators of $CD69^{+}$ NK cells. Compound **68**, GlcNAc tetrasaccharide dimerized through a hydrophilic flexible linker, turned out to be active in enhancing natural killing in vitro, decreasing the growth of tumors in vivo, and increasing cytotoxic activity of tumor infiltrating lymphocytes examined ex vivo. This compound thus represents a strong candidate for an efficient carbohydrate-based immunomodulator with a promising antitumor potential.

Experimental Section

General Chemistry. All reagents and solvents were purchased from Sigma-Aldrich and used as received. Analytical samples were dried at 6.5 Pa and 25 °C for 8 h. Melting points were determined with a Kofler apparatus and are uncorrected. Optical rotations were measured on Rudolph Research Analytical AUTOPOL IV polarimeter at 20 °C. $[\alpha]_D$ values are given in $deg \cdot cm^3 \cdot g^{-1}$. Elemental analyses were performed using a PerkinElmer 2400 series II CHNS/O elemental analyzer. IR spectra were recorded on a Bruker Equinox 55 FTIR spectrometer, and wavenumbers are given in cm^{-1}. NMR spectra were recorded using Bruker Avance spectrometer at 500.1 MHz (^1H) and 125.8 MHz (^{13}C) in $CDCl_3$, using TMS as an internal standard for ^1H NMR spectra and $CDCl_3$ as a standard for ^{13}C NMR spectra. Chemical shifts are given in ppm (δ scale) and coupling constants (J) in Hz. For unambiguous assignment of signals in ^{13}C NMR spectra, the heterocorrelated 2D NMR spectra were measured by the HSQC technique[51] if necessary. Positive-ion FAB mass spectra were acquired on a BEqG geometry mass spectrometer ZAB-EQ (VG Analytical). Complex mixtures were analyzed using LCQ mass spectrometric detector (Finnigan) coupled to a HPLC system for LC/MS applications, equipped with an ion-trap analyzer. +APCI ionization was used for recording spectra. TLC was carried out on Merck aluminum sheets silica gel 60 F254, and column chromatography was carried out on Fluka silica gel 60 (40−63 μm). Analytical RP HPLC was performed using Waters Alliance HPLC system (PDA 996 detector) equipped with a column (150 mm × 3.9 mm) filled with Nova-Pak C18 (4 μm, Waters). Purity of all synthesized compounds was determined to be ≥95% by RP HPLC using a water−methanol gradient. Preparative RP HPLC was performed with a Knauer system equipped with a column (250 mm × 25 mm) filled with LiChrosorb RP-18 (5 μm, Merck).

2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-galactopyranose (21). Hydrogenolysis of compound **20** (50 mg, 0.08 mmol) according to general procedure D afforded 25 mg (74%) of an α/β-anomeric mixture of compound **21**. $[\alpha]_D$ −98 (*c* 0.2, H_2O). FAB MS calcd for $C_{16}H_{28}N_2O_{11}$ 424.2, found *m/z* 425.1 $[M + H]^+$. For $C_{16}H_{28}N_2O_{11}$ (424.4): calcd 45.28% C, 6.65% H, 6.60% N; found 45.13% C, 6.76% H, 6.51% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-galactopyranose (32). Hydrogenolysis of compound **31** (85 mg, 0.08 mmol) according to general procedure D afforded 42 mg (85%) of an α/β-anomeric mixture of compound **32**. $[\alpha]_D$ +10 (*c* 0.3, H_2O). MS EI calcd for $C_{24}H_{41}N_3O_{16}$ 627.2, found *m/z* 650.5 $[M + Na]^+$. For $C_{24}H_{41}N_3O_{16}$ (627.6): calcd 45.93% C, 6.58% H, 6.70% N; found 46.08% C, 6.53% H, 6.73% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose (37). Hydrogenolysis of compound **36** (100 mg, 0.14 mmol) according to general procedure D afforded 30 mg (50%) of an α/β-anomeric mixture of compound **37**. $[\alpha]_D$ +56 (*c* 0.1, H_2O). FAB MS calcd for $C_{16}H_{28}N_2O_{11}$ 424.2, found *m/z* 425 $[M + H]^+$. For $C_{16}H_{28}N_2O_{11}$ (424.4): calcd 45.28% C, 6.65% H, 6.60% N; found 45.13% C, 6.76% H, 6.48% N. For NMR spectra see Supporting Information.

Benzyl-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)]-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-α-D-glucopyranoside (49). Zemplén O-deacetylation of compound **46** (225 mg, 0.17 mmol) according to general procedure A followed by RP HPLC in in water afforded 132 mg (83%) of compound **49**. $[\alpha]_D$ −41 (*c* 0.2, H_2O). MS EI calcd for $C_{39}H_{60}N_4O_{21}$ 920.4, found *m/z* 943.4 $[M + Na]^+$. For $C_{39}H_{60}N_4O_{21}$ (920.9): calcd 50.86% C, 6.57% H, 6.08% N; found 50.98% C, 6.64% H, 5.96% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)]-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-D-glucopyranose (52). Hydrogenolysis of compound **49** (25 mg, 0.03 mmol) according to general procedure D afforded 17 mg (66%) of an α/β-anomeric mixture of compound **52**. $[\alpha]_D$ −25 (*c* 0.2, H_2O). FAB MS calcd for $C_{32}H_{54}N_4O_{21}$ 830.3, found *m/z* 853.5 $[M + Na]^+$. For $C_{32}H_{54}N_4O_{21}$ (830.8): calcd 46.26% C, 6.55% H, 6.74% N; found 46.09% C, 6.53% H, 6.78% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-D-glucopyranose (53). Hydrogenolysis of compound **50** (90 mg, 0.13 mmol) according to general procedure D afforded 2 mg (15%) of an α/β-anomeric mixture of compound **53**. $[\alpha]_D$ −93 (*c* 0.1, H_2O). MS EI calcd for $C_{24}H_{41}N_3O_{16}$ 627.2, found *m/z* 650.3 $[M + Na]^+$. For $C_{24}H_{41}N_3O_{16}$ (627.6): calcd 45.93% C, 6.58% H, 6.70% N; found 45.89% C, 6.53% H, 6.78% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-D-glucopyranose (54). Hydrogenolysis of compound **51** (230 mg, 0.32 mmol) according to general procedure D afforded 133 mg (66%) of an α/β-anomeric mixture of compound **54**. $[\alpha]_D$ −65 (*c* 0.2, H_2O). FAB MS calcd for $C_{24}H_{41}N_3O_{16}$ 627.2, found *m*/*z* 628 $[M + H]^+$. For $C_{24}H_{41}N_3O_{16}$ (627.6): calcd 45.93% C, 6.58% H, 6.70% N; found 45.79% C, 6.73% H, 6.59% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→3)-[2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)]-2-acetamido-2-deoxy-D-galactopyranose (61). Hydrogenolysis of compound **60** (28 mg, 0.32 mmol) according to general procedure D afforded 14 mg (64%) of an α/β-anomeric mixture of compound **54**. $[\alpha]_D$ +25.77 (*c* 0.3, H_2O). FAB MS calcd for $C_{24}H_{41}N_3O_{16}$ 627.2, found *m*/*z* 628 $[M + H]^+$. For $C_{24}H_{41}N_3O_{16}$ (627.6): calcd 45.93% C, 6.58% H, 6.70% N; found 45.89% C, 6.63% H, 6.78% N. For NMR spectra see Supporting Information.

2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)]-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-D-glucopyranose Thiourea Dimerized through Hydrophobic Linkers (64−70). Compound **52** (24 mg) was incubated in saturated aqueous NH_4HCO_3 (10 mL) at 30 °C for 7 days.[52] The bulk of NH_4HCO_3 was removed by lyophilization. Dowex 50W 1X2 H^+ (Fluka) was added to a solution of the solid sugar amine in water until the pH of the mixture dropped to 4. The resin with absorbed glycosylamines was filtered off and washed with ice cold water. Pure product was eluted with 2 M NH_3 in MeOH, affording 20.6 mg of the corresponding β-glycosylamine **62** (86%). This compound was dissolved in 2 mL of water and added dropwise within 3 min at room temperature to a stirred mixture of 400 μL of $CSCl_2$ and 200 mg of $NaHCO_3$ in 2 mL of acetone. After 45 min, water (2 mL) was added and acetone was removed in vacuo at 30 °C. Unreacted $CSCl_2$ was extracted with chloroform, and the resulting solution of glycosyl isothiocyanate **63** was lyophilized and immediately used in the subsequent coupling reaction. A solution of **63** (2.4 equiv) in dichloromethane was slowly added to the corresponding diamine (1 equiv) in dichloromethane. After 24 h at room temperature the reaction mixture was purified by column chromatography on silica gel (EtOAc−MeOH, 9:1). Thereafter, the compounds were purified by chromatography on BioGel P2 (BioRad) as described previously.[53] The yields of the individual compounds were 2.1 mg (66%) for **64**, 1.8 mg (62%) for **65**, 2.0 mg (65%) for **66**, 1.7 mg (58%) for **67**, 1.5 mg (50%) for **68**, 1.8 mg (60%) for **69**, and 2.1 mg (67%) for **70**. Analytical data for **64**: ^{1}H NMR 8.14 d (NH), 5.07 d (1H, *J* = 3.0, H-1), 4.82 d (1H, *J* = 8.3, H-1′), 4.75 d (1H, *J* = 8.3, H-1′), 4.70 d (1H, *J* = 8.4, H-1″), 4.68 d (1H, *J* = 8.4, H-1″), 4.68 d (1H, *J* = 8.4, H-1), 4.59 d (1H, *J* = 8.5, H-1‴), 4.58 d (1H, *J* = 8.5, H-1‴), 4.26 m (1H, H-2), 4.06 m (2H, H-2, 3), 3.65−3.86 m (3H, H-2, 2′, 2″), 3.47−4.07 m (18H, 3′, 3″, 3‴, 4′, 4″, 4‴, 5′, 5″, 5‴, 6′, 6″, 6‴), 2.07 m (CH_2). FAB MS calcd for $C_{68}H_{114}N_{12}O_{40}S_2$ 1802.3, found *m*/*z* 1825.3 $[M + Na]^+$. For $C_{68}H_{114}N_{12}O_{40}S_2$ (1802.3): calcd 45.27% C, 6.32% H, 9.32% N; 3.55% S; found 45.32% C, 6.43% H, 9.28% N, 3.32% S. For NMR spectra see Supporting Information. Analytical data for compounds **65**−**70** are given in the Supporting Information.

Binding and Inhibition Experiments. Soluble dimeric rat NKR-P1A and soluble dimeric human CD69 were expressed in *Escherichia coli*, and purified essentially as described previously.[13,42] These proteins were radioiodinated as reported,[13] with carrier-free Na^{125}I (Amersham) to a specific activity of 10^7 cpm per μg of protein. Binding and inhibition assays were performed as described previously[13] with minor modifications. Briefly, 96-well poly(vinyl chloride) microplates (Titertek Immuno Assay-Plate, ICN Flow, Irvine, U.K.) were coated overnight at 4 °C with 50 μL of Glc*p*NAc$_{17}$BSA (10 μg/mL, Sigma) in TBS + C buffer (10 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM NaN_3). Plates were blocked with 1% BSA (Sigma) in TBS + C for 2 h at 4 °C and incubated with ^{125}I-NKR358 corresponding to half of the saturation amount and the indicated dilutions of the tested compounds in a total reaction volume of 100 μL. Plates were washed three times with TBS + C, drained, and dried, and 100 μL of a scintillation solution was added to each well. Radioactivity in wells was determined in a β-counter Microbeta (Wallac). All experiments were performed in duplicate. The results are average values from duplicate experiments within the range indicated by error bars.

NMR Titrations. All NMR experiments were run at 300 K in a Bruker Avance 600 MHz spectrometer equipped with a cryogenic H/C/N TCI probehead. ^{1}H−^{15}N HSQC spectra of 0.3 mM [^{15}N]-labeled wild-type CD69 protein were used as a routine check of protein folding and stability. The sample buffer consisted of 10 mM MES, pH 5.8, with 49 mM NaCl, 1 mM NaN_3, and 10% D_2O. During NMR titration, a 0.1 mM solution of the unlabeled wild-type CD69 protein was titrated. In an initial experiment, aliquots of the GlcNAc ligand corresponding to 25%, 50%, 75%, 100%, 200%, and 500% of saturation were added and signals of the free GlcNAc ligand were observed at 2.2 ppm in the 1D proton spectra and used for the estimation of the free ligand concentration. In a separate experiment aimed at estimating the binding constant, smaller ligand additions were used as equivalence was approached. The protein was titrated to 75% of the estimated number of binding sites, after which the amount of ligand was increased in increments of 5% of the estimated number of binding sites until the equivalence point was reached. All spectra were processed using the software NMRPIPE.[54] The dissociation constant K_d, defined as $K_d = (c_p - c_L + [L])[L]/(c_L - [L])$, was obtained by a nonlinear fitting of the [L] vs c_L titration curves (Figure 1A). Volume changes during titration were accounted for.

Equilibrium Dialysis. Oligosaccharide **52** was labeled using NaB^3H_4 (specific activity of 500 GBq/mmol) and was prepared as described previously[20] and diluted with the unlabeled compound according to the required specific activity. To set up equilibrium dialysis experiments, a rotating apparatus with glass blocks containing separate sealable chambers with external access was used as described previously (*5*). Then 200 μL aliquots of 0.1 μM solutions of CD69 proteins in 10 mM MES, pH 5.8, with 49 mM NaCl and 1 mM NaN_3 were incubated with varying amounts of ligand at 278 or 300 K for 48 h. After equilibration, 100 μL aliquots were withdrawn from the control and from the protein-containing chambers. The total ligand concentration was determined by liquid scintillation, and the bound ligand was calculated as the difference between the amount of GlcNAc in the chamber containing the protein and the control chamber. The results were calculated and plotted according to Scatchard as described previously.[11]

Tryptophan Fluorescence Quenching. Tryptophan fluorescence quenching experiments were performed according to the described methodology[51] with minor modifications. In initial experiments, 100 nmolar aliquots of CD69 protein were pipetted into multiple wells of a UV Star plate (Greiner, Germany) and mixed with 10-fold serial dilutions of the GlcNAc ligand. Incubation proceeded for 1 h at room temperature, and then the fluorescence of tryptophane residues was measured in duplicate wells using the bottom fluorescence measurements on a Safire2 plate reader (Tecan, Austria) with the following settings: λ_{ex} = 275 nm, λ_{em} = 350 nm, excitation and emission slits were set to 5 and 20, respectively, and the fluorescence gain was manually set to 66. When the lowest concentration of ligand that still caused the quenching of tryptophane fluorescence was found, detailed dilutions of the ligand by 10% saturation steps were performed, and the concentrations of free and bound ligand were calculated as described previously.[55]

Precipitation Assays with the Soluble CD69 Receptor. Each ligand was dissolved in water at 20 nM corresponding to 10-fold the K_d value. The ^{125}I-labeled protein (20 nM, 50 μL)[13] was

added to each sample (50 μL) in 96-well microtiter plates. Mixtures were incubated at 4 °C for 30 min, and then a 20% (v/v) solution of PEG 8000 was added (100 μL). The mixture was left to precipitate for 1 h at 4 °C. After centrifugation (10 min, 4 °C, 1800g_{av}), the supernatant was carefully removed and a 10% (v/v) solution of PEG 8000 (100 μL) was added. This procedure was repeated three times to wash the precipitate. After additional centrifugation and supernatant removal, the precipitates were dried overnight at 37 °C.

Preparation of CD69^{low} and CD69^{high} Lymphocytes. Peripheral blood mononuclear cells were obtained from standard blood fraction enriched in leukocytes (buffy coats from the local blood transfusion service) after dilution with RPMI1640 medium and centrifugation over Ficoll-Paque. Cells were incubated overnight in complete RPMI1640 in plastic cell culture dishes to allow the adherent cells to attach. Collected nonadherent fraction of PBMC (N-PBMC) contained mostly lymphocytes (T, B, and NK cells). Lymphocytes from donors expressing less than 5% of CD69 were designated CD69^{low}. Lymphocytes from donors with more then 20% CD69 positive cells were further activated by incubation at a density of 2×10^6 cells/mL in complete RPMI1640 medium for 4 h with PMA (50 ng/mL) and ionomycin (500 ng/mL). This procedure increased the surface expression of CD69 to 75−85%, as analyzed by flow cytometry using monoclonal antibody against CD69 labeled with phycoerythrin. Such lymphocytes were designated as CD69$^{high.}$

Inositol Phosphate Production. [^{3}H]Inositol phosphates were separated and quantified by the methods described previously.[11] Incorporation of [^{3}H]inositol into phospholipid was achieved by incubating human CD69^{low} or CD69^{high} lymphocytes obtained as described above (10^7 cells/ml) with 100 μL of [^{3}H]inositol (1.48 TBq/mol, 37 MBq/ml; GE Healthcare) for 3 h at 37 °C, followed by extensive washing, and resuspension at 10^8 cells/mL. An amount of 50 μL of this suspension containing 5×10^6 cells in complete RPMI with 10 mM Hepes, pH 7.4, was mixed with 50 μL of the tested compounds (60 nM), and the mixture was incubated at 37 °C for indicated times. Antibodies were added in saturating concentrations (10 μg/mL). Reaction was stopped by rapid transfer of the reaction mixture to 100 μL of 10% trichloroacetic acid. Reaction was neutralized by the addition of 50 μL of triethylamine, and 20 μL of 50% aqueous slurry of Dowex 1X8, 100−200 mesh (Sigma), in formate form was added. The supernatant was collected, and inositol bisphosphates and inositol trisphosphates were eluted by the addition of 50 μL of 0.3 and 0.6 M ammonium formate, pH 7.0, respectively. The eluant was dried in thin-walled 96-well plate, and the radioactivity was counted in 100 μL of biodegradable counting scintillant (GE Healthcare) using the Microbeta counter (Wallac).

Monitoring of Intracellular Calcium. Human CD69^{low} or CD69^{high} lymphocytes were loaded with the calcium-sensitive fluor Indo-1 by incubating $l0^7$ cells/mL with 5 pM Indo-1AM (Molecular Probes, Eugene, OR) in complete RPMI with 25 pM 2-ME at 37 °C. Cells were washed twice and resuspended at 5×10^6 cells/mL in medium. The fluorescence of the cell suspension was monitored with a Safire2 spectrofluorimeter by using an excitation wavelength of 349 nm and emission wavelength of 410 nm. The setting of the instrument was calibrated for each experiment by lysing the Indo-1-loaded cells with Triton X-100 (0.07%) for maximum fluorescence. The minimum fluorescence was determined after the addition of 10 mM EGTA and sufficient Tris base to raise the pH to >8.3. Intracellular calcium concentration was calculated using the formula $[Ca^{2+}]$ (nmoles) = $250[(F - F_{min})/(F_{max} - F)]$ where F is the measured fluorescence and 250 (nM) is the dissociation constant of Indo-1. Signaling was measured in the absence of extracellular calcium in medium containing 1 mM EGTA. Tested compounds were added in 2 min by rapid mixing of 10 μL of 300 nM solutions of these compounds with 90 μL of cellular suspensions, and monitoring continued for an additional 10 min. At 10 min after the beginning of the experiment (8 min after the addition of compounds), ionomycin (Sigma) was added to a final concentration of 1 μM. Antibodies were added in saturating concentrations (10 μg/mL).

Natural Killing. The standard ^{51}Cr release test was performed as described previously.[11] Briefly, 10^4 chromium-labeled target cells in 100 μL of complete RPMI 1640 were mixed in triplicate with 150 nM solutions of the tested compounds, or control compounds, in 50 μL of RPMI 1640 in round-bottomed 96-well plates. Antibodies were added in saturating concentrations (10 μg/mL). Thereafter, the appropriate amount of effector cells (CD69^{high} lymphocytes obtained by Ficoll−Isopaque separation;[12] see above) was added to 100 μL of complete RPMI 1640, and the plate was incubated at 37 °C for 3 h. Then 50 mL of 1% Triton X-100 was added into the maxima release wells, and the incubation continued for another 1 h. Plates were cooled on ice bath, and 100 μL of the supernatant was used for radioactivity measurements. The percentage of specific lysis was calculated using the formula % = [(exp − spont)/(max − spont)] × 100, where exp is the counts in experimental wells, spont is the counts in wells containing medium instead of the effector cells, and max is the counts in wells containing 1% Triton X-100. Complete killing curves were constructed, from which the lytic unit counts for individual experiments were calculated. Lytic efficiency was defined as the inverse of the lytic unit count.

Animal Tumor Therapies. Young (6−8 week old) female C57BL/6 mice were purchased from Charles River (Montreal, Quebec, Canada) and handled under the guidelines of Institute Animal Care Protocol. Mice were shaved in the right flank area and were given injections sc with 2.5×10^4 viable B16F1 cells (low metastasis variant, ref 56) in a final volume of 100 μL of PBS. Ten days after the injection of tumor cells, 1 μmol of the tested compound, or 10 mg of mAb, was injected. Tumor growth was followed by Vernier caliper measurement every other day from day 7 after injection. All of the experiments included 10 mice/group. Tumor area was calculated according to the formula $A = (ab)/2$, where a is the largest superficial diameter and b is the smallest superficial diameter. The tumor infiltrating lymphocytes were isolated from animals at day 30,[45] and their cytolytic activity was measured as described above using the appropriate mouse tumor cells as targets.

Statistical Analyses. Statistical analyses were calculated by Student's t test. P values of ≤0.05 were considered as significant (*, $p \leq 0.05$; **, $p \leq 0.01$).

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Supporting Information Available: Synthesis and analytical data of compounds **4**−**10**, **15**−**20**, **23**−**31**, **33**−**36**, **40**−**48**, **50**, **51**, **55**−**60** and analytical data for target compounds **21**, **32**, **37**, **49**, **52**−**54**, **61**, and **65**−**70**. This material is available free of charge via the Internet at http://pubs.acs.org.

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