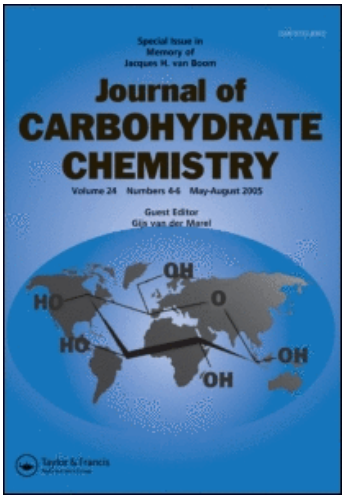

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Improved Preparation of β-d-ManNAc-(1→4)-d-Glc and β-d-TalNAc-(1→4)-d-Glc Disaccharides and Evaluation of Their Activating Properties on the Natural Killer Cells NKR-P1 and CD69 Receptors

Emanuele Attolino[a]; Filippo Bonaccorsi[a]; Giorgio Catelani[a]; Felicia D'Andrea[a]; Karel Křenek[b]; Karel Bezouška[bc]; Vladimir Křen[b]

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Improved Preparation of β-D-ManNAc-(1→4)-D-Glc and β-D-TalNAc-(1→4)-D-Glc Disaccharides and Evaluation of Their Activating Properties on the Natural Killer Cells NKR-P1 and CD69 Receptors

Emanuele Attolino,[1] Filippo Bonaccorsi,[1] Giorgio Catelani,[1] Felicia D'Andrea,[1] Karel Křenek,[2] Karel Bezouška,[2,3] and Vladimir Křen[2]

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The synthetic access of either β-D-ManNAc-(1→4)-D-Glc (**5**) is β-D-TalNAc-(1→4)-D-Glc (**6**) disaccharides has been effectively improved with respect to previous syntheses (J. Carbohydr. Chem. **2000**, *19*, 79–91 and **2004**, *23*, 179–190), optimizing the preparation of suitably protected 4-*O*-(2-acetamido-2-deoxy-3,4-*O*-isopropylidene-β-D-talopyranosyl)-2,3:5,6-di-*O*-isopropylidene-*aldehydo*-D-glucose dimethyl acetal derivatives obtained by complete stereoselective $LiAlH_4$ reduction of new 2′-oximino precursors

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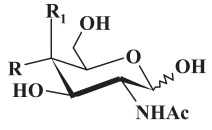
derived from lactose. The affinity of the disaccharides **5** and **6** toward the natural killer cell NKR-P1 and CD69 receptors has been evaluated and discussed.

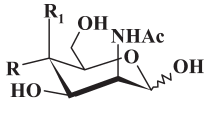
Keywords β-D-ManNac-(1→4)-D-Glc, β-D-TalNAc-(1→4)-D-Glc, NK cells, NKR-P1 receptor, 2-Oximino-β-D-*lyxo*-hexopyranosides, Reductions

INTRODUCTION

2-Amino-2-deoxyhexoses constitute an important class of monosaccharides present as *N*-acetamido derivatives in several kinds of naturally occurring biologically active compounds.[1] The most important members of the family are certainly *N*-acetyl-D-glucosamine (**1**) and its C-4 epimer, *N*-acetyl-D-galactosamine (**2**), both displaying an equatorially oriented acetamido group at C-2. In particular, they are constituent of different types of glycoconjugates (glycolipids, lypopolysaccharides, and glycoproteins),[2] glycosaminoglycans (heparin, dermatan, chondroitin, and hyaluronic acid),[3] and blood group determinants.[4] Furthermore, they play significant roles in several processes involving cellular recognition.[5] Much less distributed in nature are the two analog aminosugars carrying an axially disposed C-2 acetamido group: *N*-acetyl-D-mannosamine (**3**) and *N*-acetyl-D-talosamine (**4**). In particular, *N*-acetyl-D-mannosamine is a constituent of the capsular polysaccharide repeating unit of some pathogens[6,7] and of the natural spacer unit linking teichoic acids to the peptidoglycan chain in some Gram-positive bacteria.[8] *N*-acetyl-D-talosamine has been recognized only as a minor constituent of ovine and bovine cartilage.[9]



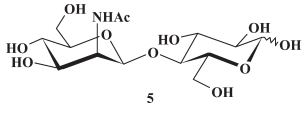
1: R = OH, R_1 = H
2: R = H, R_1 = OH



3: R = OH, R_1 = H
4: R = H, R_1 = OH

An interesting common biological role of the four hexosamines **1**–**4** as agonists of the major activating receptors of rat natural killer cells, NKR-P1, was established from our previous studies.[10,11] In this contest, a systematic study of the structural requirements of the recombinant soluble dimeric form of NKR-P1 for its optimal carbohydrate ligands was made,[11] showing that (a) the affinity of *N*-acetyl-D-hexosamines is in the order ManNAc > GalNAc > GlcNAc >> TalNAc; (b) the orientation of the anomeric substituent is very important, showing, in all cases, an increase of the affinity of about two orders of magnitude when it is fixed as β-pyranoside bond; and (c) the preferred interglycosidic connection, in the case of di- and oligosaccharides, is the

β-(1→4) one. It could be noted that an apparent lack of structurally related regularity is present in the affinity for the NKR-P1 receptor of the four hexosamines **1**–**4**. In fact, while in the C(2)-NHAc equatorial couple GlcNAc/GalNAc (**1**/**2**) the most active by one order of magnitude is the second one displaying an axial C-4 hydroxyl group, in the C-4 epimeric couple of C(2)-NHAc axially oriented ManNAc/TalNAc (**3**/**4**) is the first one having the C-4 hydroxyl group equatorially disposed that is by far the most active by three orders of magnitude. This apparently anomalous behavior was explained considering that TalNAc (**4**) in aqueous solutions occurs mainly in its furanose forms[12] and supposing that only pyranose forms are suitable for binding.[11] In order to demonstrate this hypothesis, it was necessary to test some ManNAc and TalNAc β-pyranosides, for which synthesis through common glycosidation methods, however, is difficult because of the general problems encountered in the construction of β-1,2-*cis* hexosamine pyranosides.[13,14] Recently in this context new stereoselective methods have been proposed for the preparation of β-1,2-cis hexosamine glycosides based on stereoselective manipulations of β-D-galactopyranoside. Starting from lactose, the two title disaccharides (**5** and **6**) have been prepared avoiding the difficult glycosidation steps.[15,16]



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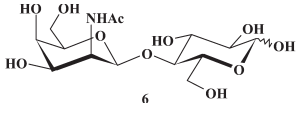

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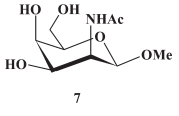

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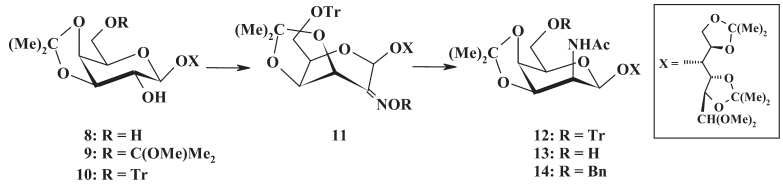
We present here the results concerning the optimization of our previous approaches to both disaccharides **5** and **6**, defining an effective improvement on the preparation of some intermediates. The evaluation of **5** and **6** and of the β-methyl D-talosamino monosaccharide **7**[17] as activators of two natural killer cell receptors is also presented and discussed.

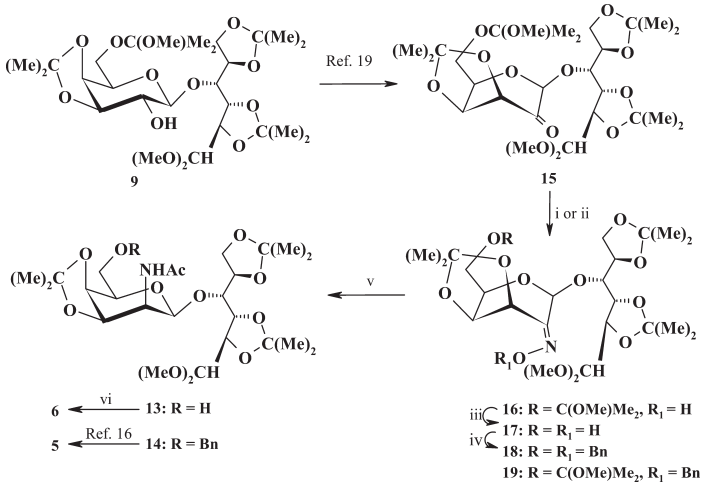
RESULTS AND DISCUSSION

Our previous syntheses of **5**[15] and **6**[16] were based on the preparation, respectively, of protected talosamine intermediates **12** and **14** obtained by stereoselective hydride reduction of 6′-*O*-trityl-2′-oximino precursors (**11**) in turn prepared from the triacetonide **8**, directly obtained in good yield from lactose.[18] However, when we considered the repreparation of **5** and **6**, it appeared evident that the major drawback of our approach was in the use of the trityl group for the temporary protection of OH-6′. The modest yield (45%) reported[18] for the preparation of **10** by tritylation of **8** was the first problem, further complicated by the change of the protection from the 6′-*O*-trityl derivative **12**, to the acid-stable benzyl one (**14**, Sch. 1) needed[15] for the synthesis of the β-D-ManNAc-containing disaccharide **5**.



Scheme 1

We considered a promising alternative using as starting material the poliacetonated derivative **9**, obtained in good yield directly from lactose.[18] Recently compound **9** has been transformed in nearly quantitative yield[19] into the 2′-ulo derivative **15** (Sch. 2) saving the 6′-*O*-methoxyisopropyl (MIP) as a useful temporary protecting group. The MIP group proved to be stable also under the oximation condition proposed by Tsuda and coworkers[20] ($NH_2OH \cdot HCl$ and Na_2CO_3 in MeOH), if the base was added before the hydroxylamino hydrochloride reactant. In fact, 6′-*O*-protected 2′-oximino derivative **16** (Sch. 2) was obtained in an excellent 96% yield as the sole (E) diastereoisomer, because of the kinetic control operating in the basic reaction conditions. The selective removal of the MIP group (10:1 MeOH-H_20 + 0.5% AcOH) gave (E)-**17** in nearly quantitative yield and, after standard benzylation, the 6′-*O*-benzyl-*O*-benzyloximino derivative (E)-**18** in 82% yield. Interestingly, when the preparation of (E)-**18** from **15** was performed without any purification of intermediates (E)-**16** and (E)-**17**, an overall 88% yield was obtained over three steps.

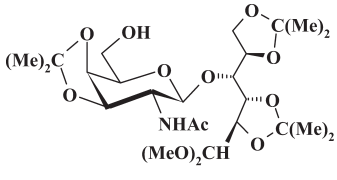


Scheme 2: Reagents and conditions. i: $NH_2OH \cdot HCl$, Na_2CO_3 (**16**,96% yield); ii: $NH_2OBn \cdot HCl$, Na_2CO_3 (**19**,94% yield); iii: MeOH-H_20 10:1 + 0.5% AcOH(96% yield); iv: KOH, BnBr, THF, (82% yield); v: $LiAlH_4$, Et_2O then Ac_2O, MeOH; vi: 80%aq AcOH, 80°C(98%).

The oximino to acetamido group transformation was performed through classical manipulations by reduction with $LiAlH_4$ in Et_2O followed by acetylation with Ac_2O in MeOH. The hydride reduction was performed on the oximino derivatives **16**–**19** to check the role of the protection pattern on the reduction stereoselectivity.

As reported in Table 1, the diastereoselectivity of the reduction was sensibly dependent on the presence of free hydroxyl groups on the oximes, leading in the case of the fully protected *O*-benzyloxime **18** to the formation of the *N*-acetyl-D-talosamino disaccharide **14**, isolated as the sole diastereoisomer in an excellent 85% yield. The disaccharide **5** was then obtained from **14** in 53% yield as previously described.[15] This new synthesis of **5** from **9** in a 35% overall yield is thus definitely more efficient than that previously reported, yielding **5** in an overall poor 13% yield starting from **8**.

The prevalent formation of the *talo* derivatives **13** and **14** in all reductions is evidently due to the intermolecular hydride attack mostly on the α-face of the oximino double bond. This selectivity is easily explained taking into account the synergistic effects of the steric hindrance exerted by the 3′,4′-acetonide and of the stereoanomeric effect.[21] The formation of the *galacto* diastereoisomer **20** in a 22% yield from **17** (having both 6′-OH and N-OH groups free) and in 11% yield from **16** (having the 6′OH protected as MIP group and the oximino OH group free) can be reasonably attributed to an intramolecular hydride transfer on the β-face of the C=N bond from intermediate alkoxyaluminium hydride species.



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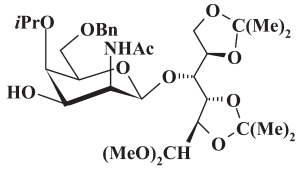

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It was clear that the 6′-OMIP protecting group was removed during the *N*-acetylation step because of the acid reaction conditions. Considering our

Table 1: Stereoselectivity of the $LiAlH_4$ reductions of oximino derivatives **16**–**19**.

Compound	Products (isolated yield)	*talo*/*galacto* ratio
16	**13** (62%) + **20** (11%)	70/30
17	**13** (53%) + **20** (22%)	85/15
18	**14** (85%)	only *talo*
19	**13** (84%)	only *talo*

new achievements in the use of MIP temporary protection and that derivative **13** can yield the completely deprotected talosamino disaccharide **6** through acid hydrolysis, avoiding, thus, the use of the trityl derivative **12**, the formation of oximino derivative **19** was planned. Its synthesis was performed by treatment of **15** with *O*-benzylhydroxylamine hydrochloride and Na_2CO_3 in MeOH (94% yield). Compound **19** was then transformed in **13** through the $LiAlH_4$ reduction followed by *N*-acetylation. Also in this case the *talo* derivative **13** was isolated as the sole diastereoisomer in a very good yield (84%). The completely deprotected disaccharide **6** was finally obtained from **13** in 98% yield through mild acid hydrolysis of **13** (80% aq AcOH at 80°C). In this way the synthesis of **6** was effectively improved from the reported[16] 31% overall yield starting from the polyacetonide **8** to the present 81% from the mixed acetonide **9**.

As a marginal observation, the unexpected 4′-*O*-isopropyl by-product **21** was obtained (7% isolated yield) during the reduction of (E)-**18** in a run conducted on a rather large scale, for which a long reaction time (8 d) was required for the complete disappearance of the starting oxime. A similar reductive opening was previously observed in the analogous reduction of some β-L-*arabino* acetonides with chloroalane[22] but, to the best of our knowledge, to date it has not been observed using $LiAlH_4$. The presence in the reaction medium of traces of AlH_3 in equilibrium with $LiAlH_4$ could provide an explanation of this unprecedented result.

We have tested the potencies of the prepared oligosaccharides to inhibit the binding of two natural killer cell receptors, NKR-P1A and CD69, to their high-affinity ligand, $GlcNAc_{23}BSA$ neoglycoprotein (Table 2). While none of the newly prepared compounds proved to be a good inhibitor for CD69, both ManNAc and the corresponding disaccharide display quite high affinities for NKR-P1 receptor, for which ManNAc is the highest-affinity monosaccharide ligand.[11]

It is a rather intriguing question why just the β-ManNAc residue is one of the strongest ligands for the NK-cells receptor. This residue can hardly be found on the surface of somatic cells or on the surface of transformed cells (tumors).[10] The only documented occurrence of the ManNAc in mammals is its involvement in the sialic acid biosynthesis (5-neuraminic acid aldolase).[23]

Table 2: Affinity of carbohydrate ligands to two NK-cell activation receptors, NKR-P1A (rat) and CD69 (human), expressed in the logarithmic scale ($-\log IC_{50}$).

Compound	NKR-P1	CD69
TalNAc-β-OMe (**7**)	6.7	5.0
TalNAc-β(1→4)Glc (**6**)	6.7	4.5
ManNAc-β(1→4)Glc (**5**)	7.4	3.2
ManNAc (**3**)	8.0	3.0
GlcNAc (**1**)	6.7	5.0

Data are average values from three independent experiments.

Nevertheless, 2-acetamido-2-deoxy-D-mannopyranose (ManNAc) is a frequently occurring glycosyl residue in a number of bacterial capsular polysaccharides and lipopolysaccharides (e.g., *Haemophilus influenzae* and *Streptococcus pneumoniae*).[6,7,24,25] In the Gram-positive bacteria, such as *Staphylococcus aureus* H and *Bacillus subtilis*, the β-ManNAc residue is a component of the "linkage unit" attaching teichoic acids to the peptidoglycan.[26] ManNAc is probably the strongest monosaccharidic ligand for the natural killer cell activating protein NKR-P1,[11] and some ManNAc-containing saccharides (e.g., GlcNAc-ManNAc) were found to be strong immunostimulants.[27]

In the early stages of their evolution NK cells had as a major role defense against bacterial invaders. At that stage detection of ManNAc was plausibly of the utmost importance for the recognition of bacterial infection as this saccharide was a clear-cut signal of the foreign-body invader. At present, pathogenicity of some bacterial strains occurring in their R-forms (pathogenic—contain ManNAc) and S-forms (nonpathogenic—lower-content ManNAc) is related partly to the content of ManNAc. The β-ManNAc units have potent implication in the virulence and pathogenicity of some bacteria (e.g., *S. pneumoniae* 19F and 19A).[24,25] It was shown that the presence of mannosamine derivatives can cause modification of the cell wall synthesis and some changes in mucin production.[28–30]

At present the major role of the NK cells is the first defense barrier against tumors and virally infected cells. Thus, the ability to recognize activation by ManNAc seems to be an evolutionary rudiment; however, it could be efficiently used in the activation of these cells in the glycomimetics.

Another interesting feature of our disaccharide is its intrinsic stability *in vivo*. When another disaccharide with high activation potency toward NK cells is used [e.g. β-GalNAc-(1→4)-GlcNAc or β-GlcNAc-(1→4)-GlcNAc], it is very quickly decomposed by the exoglycosidase action of β-*N*-acetylhexosaminidase or lysozyme present in the body fluids. However, respective enzyme for the hydrolysis of the β-ManNAc moiety does not exist. This was tested also by respective chromogenic substrate 4-nitrophenyl 2-acetamido-2-deoxy-β-D-mannopyranoside.[31] Therefore, our structures are virtually "glycomimetics" having both high biological activity and also high stability *in vivo*.

EXPERIMENTAL

General Methods

Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 20 ± 2°C. NMR were recorded with a Bruker AC 200 instrument

operating at 200.13 MHz (^{1}H) and 50.33 (^{13}C) and with a Bruker Avance II 250 spectrometer operating at 250.15 and 62.9 MHz for ^{1}H and ^{13}C, respectively, using Me_4Si as internal reference. Assignments were made, when possible, with the aid of DEPT, HETCOR, and COSY experiments. In the case of mixtures, assignments were made by referring to the differences in the peak intensities. All reactions were followed by TLC on Kieselgel 60 F_{254} with detection by UV light and/or with ethanolic 10% phosphomolybdic or sulphuric acid, and heating. Kieselgel 60 (E. Merck, 70–230 and 230–400 mesh, respectively) was used for column and flash chromatography. Solvents were dried and purified by distillation according to standard procedure, and stored over 4 Å molecular sieves activated for at least 24 h at 200°C. $MgSO_4$ was used as the drying agent for solutions.

*4-O-[2-Deoxy-3,4-O-isopropylidene-6-O-(1-methoxy-1-methylethyl)-2-oximino-β-D-lyxo-hexopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal [(E)-**16**]*

A solution of the crude uloside **15** (6.50 g, 11.2 mmol), obtained from **9**,[19] in MeOH (300 mL) was treated with solid Na_2CO_3 (2.70 g, 25.4 mmol), stirred for 30 min at rt, and $NH_2OH \cdot HCl$ (1.80 g, 25.8 mmol) was added. The reaction mixture was heated to reflux and stirred until TLC (3:7 hexane-EtOAc) showed the complete disappearance of the starting material (4 h) and the formation of a new product with R_f 0.50. MeOH was removed at reduced pressure and the residue was partitioned between CH_2Cl_2 (150 mL) and satd aq $NaHCO_3$ (70 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 40 mL) and the organic phases were collected, dried, filtered, and concentrated under diminished pressure to give a crude residue (6.35 g, 96% yield) constituted (^{1}H and ^{13}C NMR) almost exclusively by the (E) diastereoisomer of the title compound. Flash chromatography (2:3 hexane-EtOAc + 0.1% Et_3N) gave an analytical sample of (E)-**16** as a clear syrup; R_f 0.50 (3:7 hexane-EtOAc); $[\alpha]_D$ −20.3 (*c* 1.1, $CDCl_3$); ^{1}H NMR (250 MHz, CD_3CN): δ 5.46 (dd, 1H, $J_{1',3'} = 0.7$ Hz, $J_{3',4'} = 7.9$ Hz, H-3′), 5.40 (d, 1H, H-1′), 4.46 (dd, 1H, $J_{1,2} = 6.2$ Hz, $J_{2,3} = 7.6$ Hz, H-2), 4.34 (d, 1H, H-1), 4.33 (dd, 1H, $J_{4',5'} = 1.3$ Hz, H-4′), 4.17 (m, 1H, H-5), 4.06 (dd, 1H, $J_{3,4} = 1.3$ Hz, H-3), 4.02 (dd, 1H, $J_{5,6b} = 6.0$ Hz, $J_{6a,6b} = 8.4$ Hz, H-6b), 3.97 (dd, 1H, $J_{5,6a} = 6.2$ Hz, H-6a), 3.87 (dd, 1H, $J_{4,5} = 6.2$ Hz, H-4), 3.45 (m, 3H, H-5′, H-6′a, H6′b), 3.38, 3.37 (2s, each 3H, 2 × OMe-1), 3.15 [s, 3H, C(*OMe*)Me_2], 1.45, 1.39, 1.33, 1.31, 1.30, 1.29 [6s, each 3H, 3 × C*Me*$_2$),1.28 [s, 6H, C(OMe)*Me*$_2$]; ^{13}C NMR (62.9 MHz, CD_3CN): δ 151.8 (C-2′), 111.3, 110.6, 109.3 (3 × *C*Me_2), 106.3 (C-1), 100.9 [*C*(OMe)Me_2], 100.6 (C-1′), 78.7 (C-3), 77.7, 77.6 (C-4, C-5), 75.9 (C-2), 73.9 (C-4′), 72.0 (C-5′), 66.9 (C-6), 65.4 (C-3′), 60.6 (C-6′), 56.1, 53.7 (2 × O*Me*-1), 48.9 [C(*OMe*)Me_2], 27.6, 27.2, 27.0, 26.6, 25.9, 24.9 (3 × C*Me*$_2$), 24.8, 24.7 [C(OMe)*Me*$_2$]. Anal. Calcd for $C_{27}H_{47}NO_{13}$: C, 54.63; H, 7.98; N, 2.36; found C, 54.45; H, 8.03; N, 2.38.

*4-O-[2-Deoxy-3,4-O-isopropylidene-2-oximino-β-D-lyxo-hexopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal [(E)-**17**]*

A solution of (E)-**16** (1.48 g, 2.50 mmol) in 10:1 MeOH-H_2O mixture (15 mL) was treated with acetic acid (70 μL) and heated to 50°C while stirring. After 2 h TLC analysis (3:7 hexane-EtOAc) revealed the complete disappearance of the starting material. The solution was then allowed to cool to rt and coevaporated with toluene (4 × 20 mL) under diminished pressure. Flash chromatography purification (2:3 hexane-EtOAc) of the crude residue (1.42 g) afforded pure (E)-**17** (1.25 g, 96% yield) as a white solid foam; R_f 0.32 (3:7 hexane-EtOAc); $[\alpha]_D$ −2.0 (*c* 0.9, $CHCl_3$); ^{1}H NMR (250 MHz, CD_3CN): δ 5.46 (d, 1H, $J_{3',4'}$ = 7.9 Hz, H-3′), 5.31 (s, 1H, H-1′), 4.63 (dd, 1H, $J_{1,2}$ = 6.6 Hz, $J_{2,3}$ 7.9 Hz, H-2), 4.37 (d, 1H, H-1), 4.26 (dd, 1H, $J_{4',5'}$ = 1.8 Hz, H-4′), 4.19 (m, 1H, H-5), 4.05 (dd, 1H, $J_{5,6b}$ = 6.0 Hz, $J_{6a,6b}$ = 8.6 Hz, H-6b), 4.03 (dd, 1H, $J_{3,4}$ = 1.4 Hz, H-3), 3.99 (dd, 1H, $J_{5,6a}$ = 6.3 Hz, H-6a), 3.90 (dd, 1H, $J_{4,5}$ = 6.4 Hz, H-4), 3.66 (dd, 1H, $J_{5',6'b}$ = 8.1 Hz, $J_{6'a,6'b}$ = 11.6 Hz, H-6′b), 3.55 (dd, 1H, $J_{5',6'a}$ = 4.4 Hz, H-6′a), 3.39 (m, 1H, H-5′), 3.40, 3.38 (2s, each 3H, 2 × OMe), 3.10 (bs, 1H, OH-6), 2.70 (bs, 1H, N-OH), 1.47, 1.43, 1.39, 1.36, 1.32, 1.30 (6s, each 3H, 3 × C*Me*$_2$); ^{13}C NMR (62.9 MHz, CD_3CN): δ 151.4 (C-2′), 111.3, 110.2, 109.5 (3 × *C*Me_2), 107.2 (C-1), 100.9 (C-1′), 78.6 (C-3), 78.2 (C-4), 77.6 (C-5), 75.7 (C-2), 74.0 (C-5′), 73.5 (C-4′), 66.7 (C-6), 66.0 (C-3′), 61.8 (C-6′), 56.9, 53.8 (2 × O*Me*), 27.4, 27.1, 27.0, 26.8, 25.9, 24.9 (3 × C*Me*$_2$). Anal. Calcd for $C_{23}H_{39}NO_{12}$: C, 52.97; H, 7.54; N, 2.69. Found C, 52.95; H, 7.56; N, 2.65.

When a crude sample of (E)-**16** obtained from **15** (12.5 g, 21.6 mmol) as reported above was directly de-*O*-metoxyisopropylidenated, pure (E)-**17** (10.7 g, 20.5 mmol) was obtained after flash chromatography in a 95% yield over two steps from **15**.

*4-O-[6-O-Benzyl-2-O-benzyloximino-2-deoxy-3,4-O-isopropylidene-β-D-lyxo-hexopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal [(E)-**18**]*

A solution of (E)-**17** (2.00 g, 3.83 mmol) in THF + 0.5% water (20 mL) was treated with powdered KOH (1.72 g, 30.7 mmol) and 18-crown-6 (50.6 mg, 0.19 mmol), and the resulting mixture was vigorously stirred at rt for 30 min and BnBr (2.62 g, 15.3 mmol) was added. The reaction mixture was stirred at rt until TLC analysis (1:1 hexane-EtOAc) revealed the complete disappearance of the starting material (4 h, R_f 0.15) and the formation of a major faster-moving product (R_f 0.44). MeOH (20 mL) was added and the mixture was further stirred for 10 min. Solvents were removed under reduced pressure, the residue was dissolved in CH_2Cl_2 (30 mL) and washed with brine (15 mL), and the aq phase was further extracted with CH_2Cl_2 (4 × 30 mL). The collected organic phases were concentrated under diminished pressure and the crude

residue purified by flash chromatography (4:1 hexane-EtOAc) gave the title compound (E)-**18** (2.20 g, 82% yield), as a clear syrup; R_f 0.44 (1:1 hexane-EtOAc); $[\alpha]_D$ −20.6 (*c* 0.9, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$): δ 7.38–7.26 (m, 10H, Ar-H), 5.49 (d, 1H, $J_{3',4'} = 7.6$ Hz, H-3′), 5.48 (s, 1H, H-1′), 5.19, 5.02 (AB system, 2H, $J_{A,B} = 12.7$ Hz, CH_2Ph), 4.63 (dd, 1H, $J_{1,2} = 6.6$ Hz, $J_{2,3} = 6.8$ Hz, H-2), 4.56, 4.49 (AB system, 2H, $J_{A,B} = 11.9$ Hz, CH_2Ph), 4.37 (d, 1H, H-1), 4.31 (dd, 1H, $J_{4',5'} = 1.2$ Hz, H-4′), 4.24 (m, 1H, H-5), 4.15–3.93 (m, 4H, H-3, H-4, H-6a, H-6b), 3.68 (m, 1H, H-6′b), 3.62 (dd, 1H, $J_{5',6'a} = 6.0$ Hz, $J_{6'a,6'b} = 9.5$ Hz, H-6′a), 3.50 (m, 1H, H-5′), 3.35, 3.34 (2s, each 3H, 2 × OMe), 1.53, 1.42, 1.38, 1.37, 1.34, 1.33 (6s, each 3H, 3 × CMe_2); ^{13}C NMR (50 MHz, $CDCl_3$): δ 150.3 (C-2′), 137.9, 137.0 (Ar-C), 128.2–127.5 (Ar-CH), 110.2, 110.0, 108.2 (3 × *C*Me_2), 104.8 (C-1), 99.5 (C-1′), 78.0, 77.4, 76.4, 76.3 (C-2, C-3, C-4, C-5), 76.8, 73.4 (2 × *C*H_2Ph), 73.1 (C-4′), 71.0 (C-5′), 68.8 (C-6′), 65.7 (C-6), 65.1 (C-3′), 55.0, 52.0 (2 × OMe), 27.3, 26.6, 26.5, 26.1, 25.6, 24.7 (3 × CMe_2). Anal. Calcd for $C_{37}H_{51}NO_{12}$ (701.82): C, 63.32; H, 7.32; N, 2.00. Found C, 63.35; H, 7.33; N, 1.96.

When the preparation of (E)-**18** was performed starting from **15** (13.1, 22.5 mmol), without any purification of the intermediates (E)-**16** and (E)-**17**, an overall 88% yield (13.9 g, 19.8 mmol) was obtained.

*4-O-[2-O-benzyloximino-2-deoxy-3,4-O-isopropylidene-6-O-(1-methoxy-1-methylethyl)-β-D-lyxo-hexopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal [(E)-**19**]*

Crude ketone **15** (3.50 g, 6.0 mmol) was treated with Na_2CO_3 (1.45 g, 13.7 mmol) and *O*-benzylhydroxylamine hydrochloride (1.92 g, 12.0) mmol) in MeOH (150 mL) as described above for the preparation of (E)-**16**. After flash chromatography of the reaction product (2:1 hexane-EtOAc), the title compound (E)-**19** was isolated (3.84 g, 94% yield) as a clear syrup having R_f 0.49 (1:1 hexane-EtOAc); $[\alpha]_D$ −17.9 (*c* 0.9, $CHCl_3$); ^1H NMR (250 MHz, CD_3CN): δ 7.40–7.33 (m, 5H, Ar-H), 5.42 (s, 1H, H-1′), 5.40 (d, 1H, $J_{3',4'} = 7.8$ Hz, H-3′), 5.18, 5.13 (AB system, 2H, $J_{A,B} = 12.4$ Hz, CH_2Ph), 4.48 (dd, 1H, $J_{1,2} = 6.3$ Hz, $J_{2,3} = 7.6$ Hz, H-2), 4.34 (d, 1H, H-1), 4.33 (dd, 1H, $J_{4',5'} = 1.3$ Hz, H-4′), 4.15 (dt, 1H, $J_{5,6a} = J_{5,6b} = 6.1$ Hz, $J_{4,5}$ 5.9 Hz, H-5), 4.06 (dd, 1H, $J_{3,4} = 1.4$ Hz, H-3), 4.02 (dd, 1H, $J_{6a,6b} = 8.6$ Hz, H-6b), 3.93 (dd, 1H, H-6a), 3.88 (dd, 1H, H-4), 3.53–3.45 (m, 3H, H-5′, H-6′a, H-6′b), 3.36, 3.35 (2s, each 3H, 2 × OMe-1), 3.14 [s, 3H, C(*OMe*)Me_2], 1.43, 1.37, 1.30, 1.29, 1.28, 1.27 (6s, each 3H, 3 × CMe_2), 1.31 [s, 6H, C(OMe)Me_2]; ^{13}C NMR (62.9 MHz, CD_3CN): δ 152.0 (C-2′), 138.4 (Ar-C), 129.4, 129.3, 129.0 (Ar-CH), 111.4, 110.6, 109.2 (3 × *C*Me_2), 100.8 [*C*(OMe)Me_2], 106.3 (C-1), 100.2 (C-1′), 78.7 (C-3), 77.8 (C-5), 77.6 (C-4), 77.5 (*C*H_2Ph), 75.8 (C-2), 73.8 (C-4′), 72.0 (C-5′), 66.8 (C-6), 66.3 (C-3′), 60.5 (C-6′), 56.0, 53.6 (2 × OMe-1), 48.8 [C(*OMe*)Me_2], 27.5, 27.2, 27.1, 26.7, 25.8, 25.0 (3 × CMe_2), 24.7, 24.6

[C(OMe)*Me*$_2$]. Anal. Calcd for $C_{34}H_{53}NO_{13}$ (683.80): C, 59.72; H, 7.81; N, 2.05. Found C, 59.68; H, 7.83; N, 2.03.

General Procedure for the Reduction of Oximino Derivatives ***16–19***

A solution of the pertinent oximino derivative (**16–19**, 1.0 mmol) in dry Et_2O (10 mL) was slowly added (15 min) at rt to a stirred suspension of $LiAlH_4$ (275 mg, 7.00 mmol) in dry Et_2O (10 mL). The reaction mixture was heated to reflux and stirred for the time specified for every reaction. The reaction mixtures were quenched by treatment, in order, with H_2O (0.3 mL), 10% aq NaOH (0.4 mL), and H_2O (0.3 mL). The white granular precipitate was filtered and repeatedly washed with Et_2O, and the collected ethereal extracts were concentrated at reduced pressure. The crude residue was dissolved in MeOH (12 mL), treated at rt with Ac_2O (2 mL), and left to react overnight. The reaction mixture was coevaporated with toluene (4 × 10 mL) under diminished pressure and the crude residue subjected to flash chromatographic purification.

*Reduction of (E)-****17***

A sample of (E)-**17** (937 mg, 1.80 mmol) was submitted to reduction (8 h) according to the general protocol described above, giving after flash chromatography (93:7 CH_2Cl_2-^{i}PrOH) the following products.

*4-O-[2-acetamido-2-deoxy-3,4-O-isopropylidene-β-D-talopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal (****13****)*

White solid (522 mg, 53% yield); R_f 0.40 (93:7 CH_2Cl_2-^{i}PrOH); physical properties and NMR data are identical to those of a previously described sample.[16]

*4-O-[2-acetamido-2-deoxy-3,4-O-isopropylidene-β-D-galactopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal (****20****)*

White solid foam (215 mg, 22% yield); R_f 0.15 (93:7 CH_2Cl_2-^{i}PrOH); $[\alpha]_D$ + 22.2 (*c* 0.95, $CHCl_3$); ^{1}H NMR (250 MHz, CD_3CN): δ 6.50 (d, 1H, $J_{2',NH}$ = 9.0 Hz, NH), 4.56 (d, 1H, $J_{1',2'}$ = 8.6 Hz, H-1′), 4.45 (dd, 1H, $J_{1,2}$ = 6.7 Hz, $J_{2,3}$ 7.5 Hz, H-2), 4.34 (d, 1H, H-1), 4.19 (dd, 1H, $J_{2',3'}$ = 8.3 Hz, $J_{3',4'}$ = 5.2 Hz, H-3′), 4.17 (m, 1H, H-5), 4.04 (dd, 1H, $J_{4',5'}$ = 1.8 Hz, H-4′), 4.00 (dd, 1H, $J_{3,4}$ = 1.4 Hz, H-3), 3.90 (m, 2H, H-6a, H-6b), 3.80 (m, 1H, H-4), 3.78 (m, 1H, H-5′), 3.67 (m, 1H, H-2′), 3.59 (m, 2H, H-6′a, H-6′b), 3.40 (s, 6H, 2 × OMe), 1.87 (s, 3H, *Me*CO), 1.43, 1.39, 1.31, 1.30, 1.28, 1.26 (6s, each 3H, 3 × C*Me*$_2$); ^{13}C NMR (62.9 MHz, CD_3CN): δ 171.3 (CO), 110.5, 110.4, 108.9 (3 × *C*Me$_2$), 107.8 (C-1), 102.1 (C-1′), 78.8 (C-3), 77.7, 77.6 (C-3′, C-5), 76.5, 76.3 (C-5′, C-4), 75.0 (C-2), 74.0 (C-4′), 66.1 (C-6), 62.6 (C-6′), 57.5, 54.9 (2 × O*Me*), 55.5 (C-2′), 28.4, 27.5, 27.0, 26.8, 26.6, 25.1 (3 × C*Me*$_2$), 23.4 (*Me*CO). Anal. Calcd for $C_{25}H_{43}NO_{12}$: C, 54.63; H, 7.89; N, 2.55. Found C, 54.55; H, 7.85; N, 2.50.

*Reduction of (E)-**16***

The treatment of (E)-**16** (1.00 g, 1.68 mmol) for 8 h, according to the general protocol described above, gave after flash chromatography (93:7 CH_2Cl_2-^{i}PrOH) two pure samples of **13** (575 mg, 62% yield) and **20** (101 mg, 11% yield).

*Reduction of (E)-**18***

Treatment of (E)-**18** (10.6 g, 15.1 mmol) for 72 h, according to the general protocol described above, gave after flash chromatography (3:7 hexane-EtOAc) **14** (8.21 g, 85% yield), having physical properties and NMR data identical to those of the previously described compound.[16]

In a run conducted on a double amount of (E)-**18**, the reduction rate was sensibly reduced requiring 8 d for the complete disappearance of the starting oxime. In this case, after chromatography, compound **14** was isolated in 74% yield and a slower moving by-product was isolated in 7% yield and identified as **21**.

*4-O-[2-acetamido-6-O-benzyl-2-deoxy-4-O-isopropyl-β-D-talopyranosyl]-2,3:5,6-di-O-isopropylidene-aldehydo-D-glucose Dimethyl Acetal (**21**)*

Thick syrup; R_f 0.14 (85:15 CH_2Cl_2-Me_2CO); $[\alpha]_D = -46$ (c 1.0, $CHCl_3$); ^{1}H NMR (250 MHz, CD_3CN): δ 7.25–7.15 (m, 5H, Ar-H), 6.61 (d, 1H, $J_{2',NH} = 9.7$ Hz, NH), 4.69 (d, 1H, $J_{1',2'} = 1.7$ Hz, H-1′), 4.50 (s, 2H, CH_2Ph), 4.40 (m, 1H, H-2′), 4.37 (d, 1H, $J_{1,2} = 6.2$ Hz, H-1), 4.31 (dd, 1H, $J_{2,3} = 6.7$ Hz, H-2), 4.14 (app. sextet, 1H, $J_{5,6a} = J_{5,6b} = 6.7$ Hz, H-5), 3.98 (dd, 1H, $J_{3,4} = 1.5$ Hz, H-3), 3.92 (m, 2H, H-6a, H-6b), 3.88 (dd, 1H, $J_{4,5} = 4.0$ Hz, H-4), 3.65 (m, 4H, H-4′, H-5′, H-6′a, H-6′b), 3.55 (m, 2H, H-3′, OCHMe$_2$), 3.31, 3.29 (s, each 3H, 2 × OMe), 1.84 (s, 3H, MeCO), 1.38, 1.29, 1.28, 1.27 (4s, each 3H, 2 × CMe_2), 1.19, 1.10 (2d, each 3H, $J = 6.1$ Hz, CHMe_2); ^{13}C NMR (62.9 MHz, CD_3CN): δ 170.8 (CO), 139.3 (Ar-C), 129.3–128.6 (Ar-CH), 110.9, 108.7 (2 × CMe$_2$), 106.1 (C-1), 101.7 (C-1′), 78.8 (C-3), 78.4 (C-5), 76.6 (C-4), 76.3 (C-2), 74.9, 74.7, 74.3 (C-4′, C-5′, OCHMe$_2$),), 73.8 (CH_2Ph), 69.3 (C-3′), 69.2 (C-6′), 66.1 (C-6), 55.9, 54.0 (2 × OMe), 53.8 (C-2′), 27.7, 27.0, 26.8, 25.8 (2 × CMe_2), 24.0, 23.0 (CHMe_2), 22.4 (MeCO). Anal. Calcd for $C_{32}H_{51}NO_{12}$: C, 59.89; H 8.01; N, 2.18. Found C, 59.84; H, 7.99; N, 2.17.

*Reduction of (E)-**19***

Treatment of (E)-**19** (1.83 g, 2.68 mmol) for 70 h, according to the general protocol described above, gave after flash chromatography (3:7 hexane-EtOAc) **13** (1.25 g, 84% yield), identical to the sample obtained above.

*4-O-(2-acetamido-2-deoxy-β-D-talopyranosyl)-α,β-D-glucopyranose (**6**)*

A solution of **13** (388 mg, 0.70 mmol) in 80% aq AcOH (15 mL) was warmed at 80°C and stirred for 5 h, when TLC analysis (1:1 $CHCl_3$-MeOH) revealed the complete disappearance of the starting material. The solution was concentrated at diminished pressure and coevaporated with toluene (4 × 15 mL).

The residue was triturated three times with CH_2Cl_2 to give an amorphous white solid (263 mg, 98% yield) identical (^{13}C NMR) to the sample previously prepared by us.[15]

Biological Tests

Compounds **1**, **3**, and **5**–**7** were tested for their affinity toward two representative NK-cell activation receptors, NKR-P1A and CD69 proteins. NKR-391 protein, the major activation receptor of rat NK cells, was expressed and purified as described previously.[10,11] CD69CWTY protein that contained the soluble ligand-binding domain of the earliest activation receptor of lymphocytes and NK cells, CD69 antigen, was prepared as described earlier.[32] The proteins were purified from inclusion bodies that were dissolved in 6 M guanidin-HCl and 100 mM DTT, and refolded by dropwise addition into a hundred times larger volume of 50 mM Tris-HCl buffer pH 8.5 containing 0.4M arginine, redox buffer (10 mM cysteamine and 1 mM cystamine), 1 mM PMSF, 1 μM leupeptine, and 1 μM pepstatin. The folded protein was dialyzed against low salt buffer, and purified by a combination of ion exchange chromatography, reversed-phase separation, and gel filtration as described previously.[32] The identity and homogeneity of the proteins was verified using SDS-PAGE under both reducing and nonreducing conditions, N-terminal sequencing (10 cycles of automated Edman degradation), and MALDI mass spectrometry, as described previously.[32] Moreover, Fourier transform–ion cyclotron resonance mass spectrometry was employed (APEX-Q, Bruker Daltonics, Bremen, Germany).[33] The proteins were radiolabeled with $Na^{125}I$ using Iodogen (Pierce, Rockville, IL, USA). Binding and inhibition assays were performed as described previously.[11] Briefly, 96-well polyvinylchloride microplates (Titertek Immuno Assay-Plate, ICN Flow, Irvine, Scotland) were coated overnight at 4°C with $GlcNAc_{23}BSA$ (50 μL, Sigma) in PBS buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM NaN_3). Plates were blocked with 1% BSA in PBS for 2 h at 4°C, incubated with the concentration of the radiolabeled protein corresponding to half of the saturating amount and various dilutions of the inhibitors (total reaction volume 100 μL), washed three times with PBS, and drained. Scintillation solution (100 μL) was added, and the radioactivity in the individual wells was counted by β-counting (Microbeta, Wallac, Turku, Finland). All experiments were performed in duplicates and the inhibition degree was calculated with regard to the wells containing no inhibitor.

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