DOI: 10.1002/adsc.201000008

# Multi-Gram Synthesis of a Hyaluronic Acid Subunit and Synthesis of Fully Protected Oligomers

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Received: January 5, 2010; Revised: September 11, 2010; Published online: October 7, 2010

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201000008.

**Abstract:** Fully protected tetra-, hexa- and octasaccharides of hyaluronic acid were synthesized on a scale of several 100 mg up to gram quantities using allyl (methyl 2-*O*-benzoyl-3-*O*-benzyl-4-*O*-levulinoyl-*β*-D-glucopyranosyluronate)-(1→3)-4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-trichloroacetamido-*β*-D-glucopyranoside as a key building block. This disaccharide was subjected to deprotection, then glycosylation *via* the trichloroacetimidate method was employed to achieve the formation of the oligosaccharides.

**Keywords:** carbohydrates; glycosaminoglycans; glycosylation; hyaluronic acid; oligosaccharides

## Introduction

Hyaluronic acid (HA), first isolated from the bovine vitreous body,[1] is a linear, high molecular weight (up to $2\times10^4$ kDa) glycosaminoglycan assembled from β-1,3-linked-2-acetamido-2-deoxy-D-glucose-β-(1→4)-D-glucuronic acid repeating units (Figure 1). As an extracellular matrix component, HA is involved in a wide variety of biological processes, such as cell migration, proliferation, adhesion, recognition,[2] tumor invasion[3] and tumor inhibition.[4] Degradation of HA during inflammation and tumor growth results in the accumulation of fragments of HA (sHA).[5] These fragments are potent activators of angiogenesis,[5] dendritic cells[6] and the expression of matrix metalloproteases by tumors.[7]

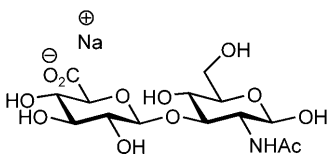


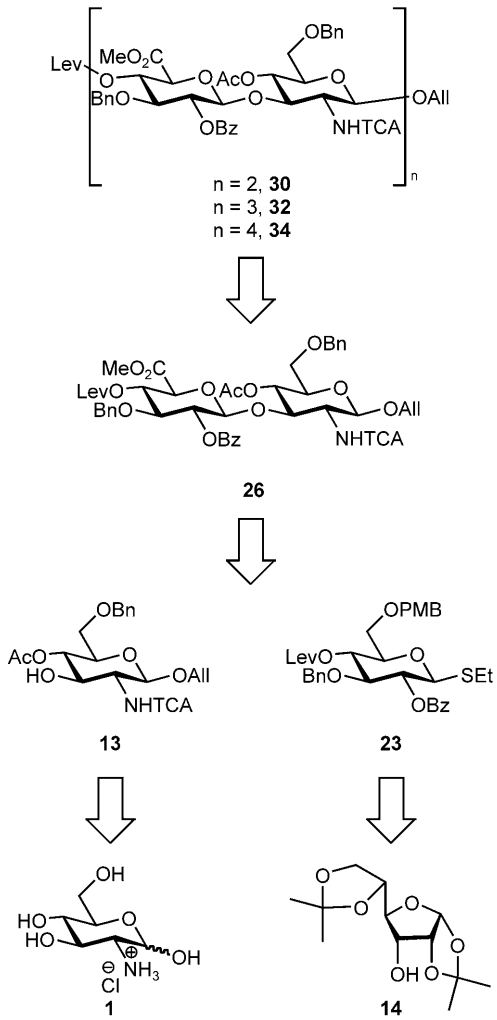

**Figure 1.** Hyaluronic acid disaccharide repeating unit.

sHA is commonly prepared by partial enzymatic degradation of HA followed by fractionation using size exclusion chromatography. Heterogeneous mixtures of sHA are often isolated by this method. However, the size of sHA is critical for its biological activity, so it would be highly desirable to produce defined lengths of sHA through organic synthesis. Organic synthesis would also avoid potential problems associated with lipopolysaccharide contamination of sHA preparations.

Since the pioneering work[8] of Jeanloz et al. in 1964, several research groups have developed the synthesis of disaccharide subunits[9] but only three groups have recently reported the synthesis of oligosaccharides.[10] Here we report the multigram scale-up of the disaccharide unit and the synthesis of fully protected tetra-, hexa- and octasaccharides.

Starting from D-glucosamine hydrochloride **1** and 1,2,4,6-diisopropylidene-D-glucofuranose **14** we synthesized the disaccharide **26**, originally proposed by Seeberger et al. as a key building block,[9b] on a multigram scale. The key step in our synthesis is the glycosylation of glucosamine acceptor **13** employing the glycosyl donor **23** using *N*-iodosuccinimide and catalytic amounts of TMSOTf. The formed disaccharide, a key intermediate, was further elaborated by selective




**Scheme 1.** Retrosynthesis of oligosaccharides **30**, **32** and **34** *via* the disaccharide acceptor **26**.

deprotection and subsequent glycosylation *via* the trichloroacetimidate method. (Scheme 1).

## Result and Discussion

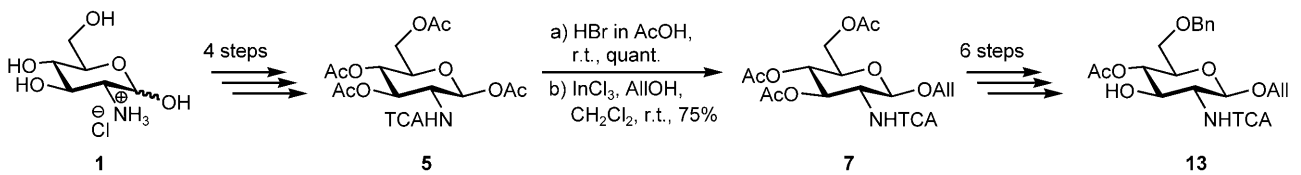
### Synthesis of D-Glucosamine Acceptor 14

In our strategy, the glucosamine building block bears three orthogonal *O*-protecting groups at the positions C-1, C-4 and C-6, respectively, and the 2-amino group has to be protected with a trichloroacetyl group[9a] since glycosylation of the ***N***-acetylated glucosamine derivative does not proceed with high yields.[11] In principle, tetraacetate **5** could be prepared using a two-step procedure as described in the literature,[9a] where the glucosamine hydrochloride **1** was first selectively acetylated at the amino group. However, the obtained *N*-trichloroacetylglucosamine was difficult to recrystallize and the yields for large-scale preparation were rather poor (<20%). We therefore decided to prepare the *N*-trichloroacetylglucosamine using a four-step sequence.[12] Tetraacetate **5** was prepared in this way with an overall yield of 51% (4 steps from **1**) on a 0.1 mol scale. The glycosyl bromide **7** was formed by the action of 33% HBr in acetic acid, and subsequent glycosylation with allyl alcohol and indium chloride[13] as activating reagent. Formation of the β-glycoside **7** proceeded with very good selectivity (α/β, 1:13). The obtained mixture of anomers could be separated by column chromatography. The use of tin tetrachloride was also tested as an activating reagent at this stage but the reaction proceeded unselectively and a 1:1 mixture of α/β anomers was obtained. A further 6 steps (see Supporting Information) were necessary for selective protection of the OH-4 and OH-6 and freeing of the OH-3. With this route the glycosyl acceptor **13** was synthesized with a 22% yield over twelve steps in multigram quantities (Scheme 2).

### Synthesis of D-Thioglucoside Donor 23

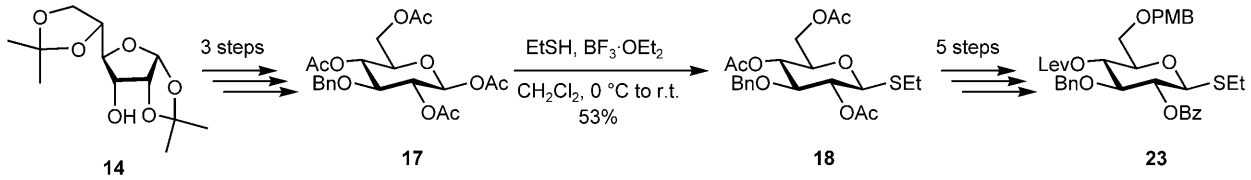
The synthesis of donor **23** is depicted in Scheme 3. Due to the incompatibility of the glucuronic methyl ester moiety with many reactions, we chose to start with a glucose derivative and to oxidize the C-6 position at a late stage of the synthesis. 1,2,4,6-tetra-*O*-acetyl-3-*O*-benzyl-β-D-glucopyranose **17** was elaborated in a three-step sequence[15] from 1,2,4,6-diisopropylidene-D-glucofuranose **14**. This sequence could be performed on an 80 mmol scale with good yields (75–83%).

The glucose derivative **17** was further converted into thioglycoside **22** using a procedure described by Nilsson et al.[16] Thioglycoside **18** was formed using ethanethiol and boron trifluoride etherate as promoter. The glycosylation proceeded with moderate selec-




**Scheme 2.** Synthesis of the glycoside acceptor **13**.

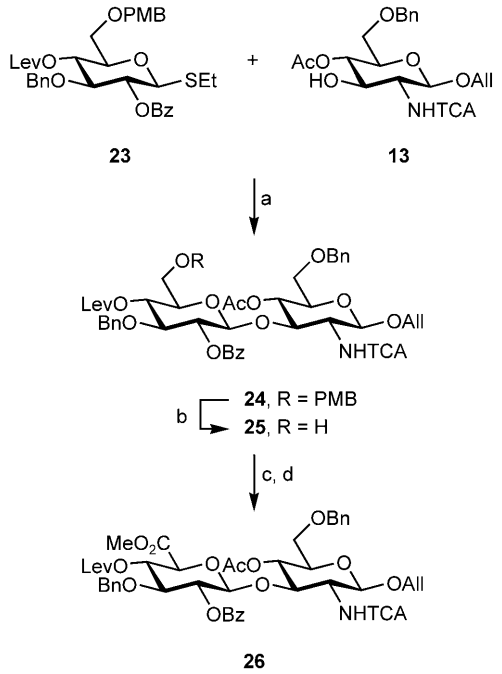



**Scheme 3.** Synthesis of the thioglycoside donor **23**.

tivity (α/β, 1:2.5) and 53% yields for the desired β-product. A further 5 steps (see Supporting Information) were needed to selectively protect the OH-2, OH-4 and OH-6. With this route the thioglycoside donor **23** was synthesized with a 26% yield over ten steps and on a 10-gram scale.

## Synthesis of Key Intermediate 26

Glycosyl acceptor **13** and thioglycoside donor **23** were reacted with *N*-iodosuccinimide and catalytic amounts of trimethysilyl trifluoromethansulfonate (Scheme 4).[17] The reaction was fast and only minor amounts of by-products were formed. The use of methyl trifluoromethansulfonate[18] as promoter was also tested but longer reaction times (about 20 h) and concomitant deprotection of the *p*-methoxybenzyl (PMB) group was observed, resulting in lower yields and the formation of by-products. This observation was strongly dependent on the quality of the reagent and may have been caused by traces of trifluoromethansulfonic acid in the reagent. The PMB protecting group of disaccharide **24** was then removed with cerium ammonium nitrate (CAN) and the resulting primary alcohol **25** was oxidized with periodic acid and a catalytic amount of chromium trioxide, as developed by Seeberger et al.[9b] This reaction proceeded smoothly without the formation of by-products but only with partial conversion. The remaining starting material could be recovered. The carboxylic acid intermediate was directly converted into the methyl ester with a solution of diazomethane in diethyl ether. Disaccharide building block **26** was obtained with a yield of 45% over four steps on a multigram scale.




**Scheme 4.** Synthesis of the disaccharide **26**. *Reaction conditions:* a) NIS, cat. TMSOTf, $CH_2Cl_2$, −30 °C, 72%; b) CAN, $CH_2Cl_2$, room temperature; c) $H_5IO_4$, cat. $CrO_3$, $CH_3CN$/$H_2O$, 0 °C; d) diazomethane, $CH_2Cl_2$, room temperature, 62% (3 steps).

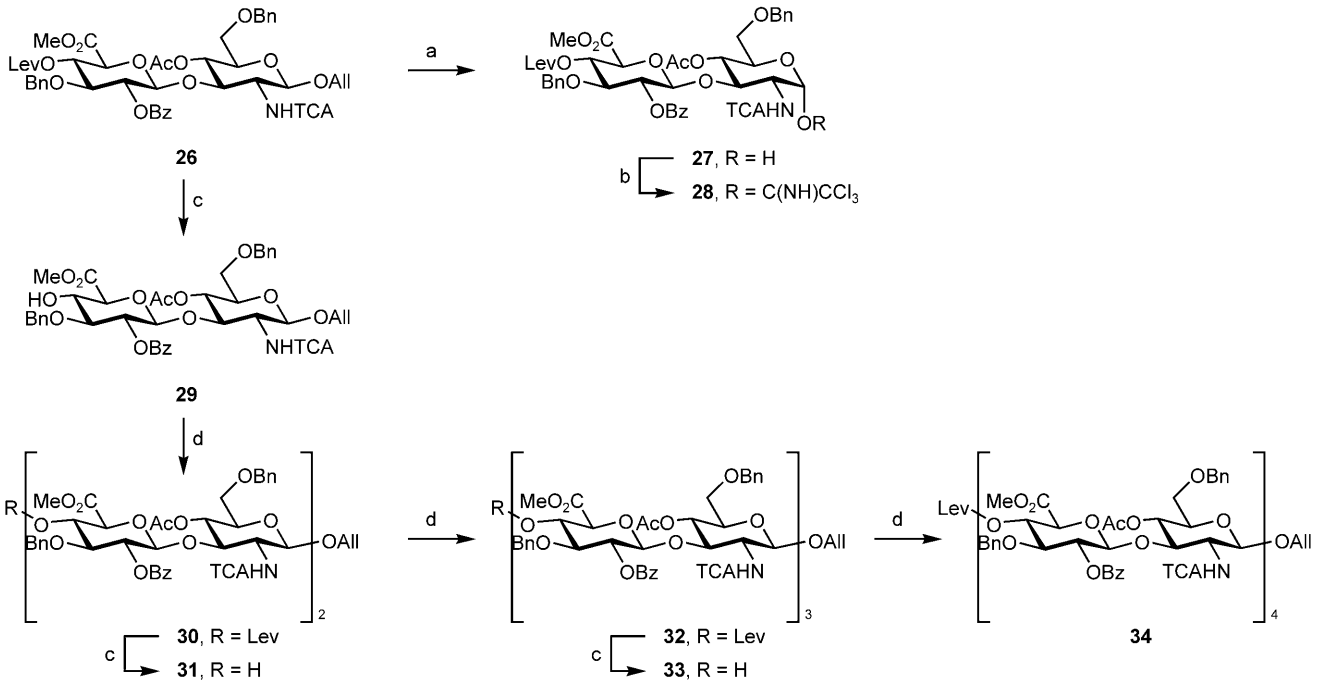
## Synthesis of Oligomers 30, 32 and 34

For the further glycosylations of building block **26** to tetra-, hexa- and octasaccharides **30**, **32** and **34** respectively, the trichloroacetimidate method[19] was used (Scheme 5). On one hand the allyl protecting group of **26** was removed with palladium chloride[20] and sodium acetate in wet acetic acid to give the hemiacetal **27**, from which the trichloroacetimidate donor **28** was formed by the use of trichloroacetonitrile and catalytic amounts of DBU as a base with 45% yields over two steps. On the other hand the levulinoyl group of **26** was cleaved with hydrazine acetate to give the disaccharide acceptor **29** in 90% yields. After glycosylation of **29** with **28**, fully protected tetrasaccharide **30** was obtained in 51% yield in amounts of up to 1.5 g (Scheme 5). Trichloroacetimidate donor **28** was also coupled with the deprotected tetrasaccharide **31** and hexasaccharide **33** to give hexasaccharide **32** and octasaccharide **34** with 45% and 32% yields over the two steps, respectively.

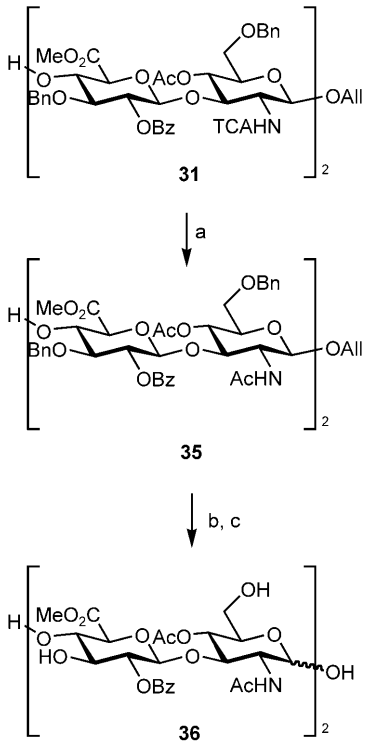
## Deprotection of Dimer 31

Deprotection was performed on the tetrasaccharide **31** as a model for higher oligomers (Scheme 6). First, the TCA group had to be changed into an acetyl group. We explored an indirect transformation in which the TCA group was cleaved with sodium meth-




**Scheme 5.** Synthesis of the tetra- **30**, hexa- **32** and octasaccharides **34**. *Reaction conditions:* a) $PdCl_2$, NaOAc, AcOH/$H_2O$, room temperature, 79%; b) $Cl_3CCN$, cat. DBU, $CH_2Cl_2$, 0 °C, 52%; c) $N_2H_4$·AcOH, $CH_2Cl_2$/MeOH, room temperature, 90%; d) **28**, cat. TMSOTf, $CH_2Cl_2$, 0 °C, 32–51%.




**Scheme 6.** Deprotection of dimer **31**. *Reaction conditions:* a) Zn/AcOH, 1,4-dioxane, room temperature, 15%; b) $PdCl_2$, NaOAc, AcOH/$H_2O$, room tempeature; c) $H_2$, cat. $Pd(OH)_2$/C, MeOH, room temperature, (37%, 2 steps).

anolate and acetylated with acetic anhydride. However, both the one-pot and the two-step procedures led to decomposition of **31**. Importantly, the reduction with the couple Zn/AcOH in 1,4-dioxane gave rise to the formation of the dimer **35** but only in poor yields (20%). Since the reaction was very slow at room temperature, we also tried different approaches. First, we performed this step at 50 °C but unfortunately more decomposition products were formed, which caused a significant drop in yields. The direct reduction of the trichloromethyl group into a methyl group with tributyltin hydride and catalytic amounts of AIBN as a radical starter under reflux conditions also resulted in the decomposition of the tetrasaccharide **31**. In contrast, the reduction of the TCA group of the disaccharide **26** under the same conditions led to a rapid conversion in 78% yields. During the deprotection of tetrasaccharide **31** we observed in MALDI mass spectrometry a decomposition product in the range of a disaccharide subunit. This allowed us to conclude that the TCA group was reduced but also the β-1,4 glycosyl bond of the tetrasaccharide was cleaved. The β-1,4 glycosyl is quite labile and the high temperatures might cause its cleavage. We tried to perform this reaction at lower temperatures of 70 °C but unfortunately, also in this case we observed decomposition to disaccharides subunits. Further optimization studies are ongoing to improve the yields of the reduction. Next, the allyl group and benzyl groups were cleaved respectively with $PdCl_2$ in a mixture of acetic acid and

water and subsequently with catalytic amount of $Pd(OH)_2$ on charcoal in the presence of hydrogen to give **36**.

## Conclusions

Herein we describe the synthesis of the modular disaccharide subunit of hyaluronic acid on a multi-gram scale, as well as the synthesis of the corresponding, fully protected, tetra-, hexa- and octamers on a scale of several 100 mg. The first deprotection strategy was demonstrated on the tetrasaccharide and gave promising results to achieve the full deprotection.

## Experimental Section

### General Remarks

All reactions were performed in oven-dried glassware under an argon atmosphere. Solvents and chemicals used were purchased from commercial suppliers. Solvents were dried under standard conditions. All materials were used without further purification. Thin-layer chromatography (TLC) was carried out on silica gel plates (silicagel 60, F254, Merck) with detection by UV and visualized by Seebach solution. Purification was performed with preparative chromatography using normal-phase silica gel (silica gel 60, 230–400 mesh, Merck). IR spectra were recorded on a Bruker IFS88. Absorption is reported as the ν value in $cm^{-1}$. $^1H$ NMR and $^{13}C$ NMR were recorded on a Bruker AM 400 spectrometer. Chemical shifts are reported as $\delta$ values (ppm). High-resolution mass spectra (HRMS) were determined on a Finnigan MAT90.

Detailed experimental procedures and characterization data for compounds **2–34** are given in the Supporting Information.

### Deprotection of Dimer 31

**Allyl (methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-(4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→4)-(methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-4-O-acetyl-6-O-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (35):** Allyl (methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-(4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl)-(1→4)-(methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranoside (**31**) (0.20 g, 0.13 mmol) was dissolved in 1,4-dioxane (5 mL) and AcOH (0.37 mL, 50 equiv.) and activated zinc powder (0.42 g, 50 equiv.) were added. The reaction mixture was stirred at room temperature until complete conversion (TLC/MALDI control). In those cases where the reaction was not completed, again the same amounts of AcOH and activated zinc powder were added. This procedure was repeated several times until complete conversion. After completion, the reaction mixture was diluted with water and neutralized with saturated sodium bicarbonate solution. The aqueous phase was extracted with THF (3×10 mL). The combined organic phases were collected, dried over sodium sulfate and evaporated to yield compound **35**. The crude product was purified by preparative HPLC (yield: 26 mg, 15%). MS-MALDI for $C_{79}H_{88}N_2O_{27}$ [M+Na]: $m/z=1519.8$

**Methyl 2-*O*-benzoyl-β-D-glucopyranosyluronate)-(1→3)-(4-*O*-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→4)-(methyl 2-*O*-benzoyl-β-D-glucopyranosyluronate)-(1→3)-4-*O*-acetyl-2-deoxy-2-acetamido-β-D-glucopyranoside (36):** Allyl (methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-(4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→4)-(methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-acetamido-β-D-gluco-pyranoside (**35**) (0.015 g, 0.01 mmol) was dissolved in AcOH (1 mL). Water (0.2 mL) was added, followed by addition of NaOAc (0.0125 g, 0.15 mmol) and $PdCl_2$ (0.0035 g, 0.02 mmol), and the mixture was stirred for 12 h at room temperature. The reaction mixture was diluted with EtOAc and water, and aqueous $NaHCO_3$ was added until neutralization. The aqueous layer was extracted with EtOAc (3×10 mL), dried over $Na_2SO_4$, filtered and concentrated under vacuum to yield the product **36**. The crude product was used without further purification in subsequent reaction. MS-MALDI: for $C_{76}H_{84}N_2O_{27}$ [M+Na]: $m/z=1479.8$.

Crude methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-(4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→4)-(methyl 2-*O*-benzoyl-3-*O*-benzyl-β-D-glucopyranosyluronate)-(1→3)-4-*O*-acetyl-6-*O*-benzyl-2-deoxy-2-acetamido-β-D-glucopyranoside (**36**) was subsequently dissolved in MeOH (1 mL) and catalytic amounts of $Pd(OH)_2$ were added. The reaction mixture was stirred at room temperature under a hydrogen atmosphere (hydrogen balloon) overnight. After completion, the catalyst was filtered and washed with MeOH. The filtrate was evaporated to give the product **36**. The crude product was purified by preparative HPLC (4 mg, 37% over 2 steps). MS-MALDI measurement for $C_{48}H_{60}N_2O_{27}$ [M+Na]: $m/z=$ 1119.4.

## Acknowledgements

*This work was supported by a grant to JPS from the Deutsche Forschungsgemeinschaft under the auspices of the Schwerpunkt Program SPP1190 (tumor-vessel interface).*

## References

[1] K. Meyer, J. Palmer, *J. Biol. Chem.* **1934**, *107*, 629–634.
[2] T. C. Laurent, J. R. E. Fraser, *FASEB J.* **1992**, *6*, 2397–2404.
[3] C. B. Knudson, W. Knudson, *FASEB J.* **1993**, *7*, 1233–1241.
[4] C. Zeng, B. P. Toole, S. D. Kinney, J-W. Kuo, I. Stamenkovic, *Int. J. Cancer* **1998**, *77*, 396–401.
[5] R. Stern, A. A. Asari, K. N. Sugahara, *Eur. J. Cell Biol.* **2006**, *85*, 699–715.

[6] C. Teermer, F. Benedix, J. Sleeman, C. Fieber, U. Voith, T. Ahrens, K. Miyake, M. Freundenberg, C. Galanos, J. C. Simon, *J. Exp. Med.* **2002**, *1*, 99–111.
[7] C. Fieber, P. Baumann, R. Vallon, C. Termeer, J. Simon, M. Hofmann, P. Angel, P. Herrlich, J. P. Sleeman, *J. Cell Sci.* **2004**, *117*, 359–367.
[8] H. M. Flowers, R. W. Jeanloz, *Biochemistry* **1964**, *3*, 123–125.
[9] a) G. Blatter, J.-M. Beau, J.-C. Jacquinet, *Carbohydr. Res.* **1994**, *260*, 189–202; b) E. R. Palmicci, P. H. Seeberger *Tetrahedron* **2004**, *60*, 7755–7766; c) Y. Zeng, Z. Wang, D. Whitfield, X. Huang *J. Org. Chem.* **2008**, *73*, 7952–7962.
[10] a) T. M. Shlaghek, T. K. Hyppönen, T. Ogawa, J. P. Kamerling, J. F. G. Vliegenthart, *Tetrahedron Lett.* **1993**, *34*, 7939–7942; b) G. Blatter, J.-C. Jacquinet, *Carbohydr. Res.* **1996**, *288*, 109–125; c) J. Dinkelaar, J. D. C. Codée, L. J. van den Bos, H. S. Overkleeft, G. A. van der Marel, *J. Org. Chem.* **2007**, *72*, 5737–5742; d) J. Dinkelaar, H. Gold, H. S. Overkleeft , J. D. C. Codée, G. A. van der Marel, *J. Org. Chem.* **2009**, *74*, 2408–4216; e) L. Huang, X. Huang, *Chem. Eur. J.* **2007**, *13*, 529–540; f) for a recent addition: X. Lu, M. N. Kamat, L. Huang, X. Huang, *J. Org. Chem.* **2009**, *74*, 7608–7617.
[11] S. E. Zurabyan, T. S. Antonenko, A. Ya. Khorlin *Carbohydr. Res.* **1970**, *15*, 21–27.
[12] a) M. Bergman, L. Zervas, *Ber. Dtsch. Chem. Ges.* **1931**, *64*, 975–980; b) D. J. Silva, H. Wang, N. M. Allanson, R. K. Jain, M. J. Sofia, *J. Org. Chem.* **1999**, *64*, 5926–5929.
[13] D. Mukherjee, P. K. Ray, U. S. Chowdhury, *Tetrahedron* **2001**, *57*, 7701–7704.
[14] M. P. DenNinno, J. B. Etienne, K. C. Duplantier, *Tetrahedron Lett.* **1995**, *36*, 669–672.
[15] S. David, A. Malleron, C. Dini, *Carbohydr. Res.* **1989**, *188*, 193–200.
[16] M. Nilsson, C.-M. Svahn, J. Westman, *Carbohydr. Res.* **1993**, *246*, 161–172.
[17] P. K. Mandal, A. K. Misra, *Tetrahedron* **2008**, *64*, 8685–8691.
[18] H. Lonn, *Carbohydr. Res.* **1985**, *139*, 105–113.
[19] a) R. R. Schmidt, W. Kinzy, *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21–123; b) G. Grundler, R. R. Schmidt, *Carbohydr. Res.* **1985**, *##136-145##135*, 203–218.
[20] a) T. Ogawa, S. Nakabayashi, *Carbohydr. Res.* **1981**, *93*, C1–C5; b) R. Boss, R. Sheffold, *Angew. Chem.* **1976**, *88*, 578–579.