Sialic Acids

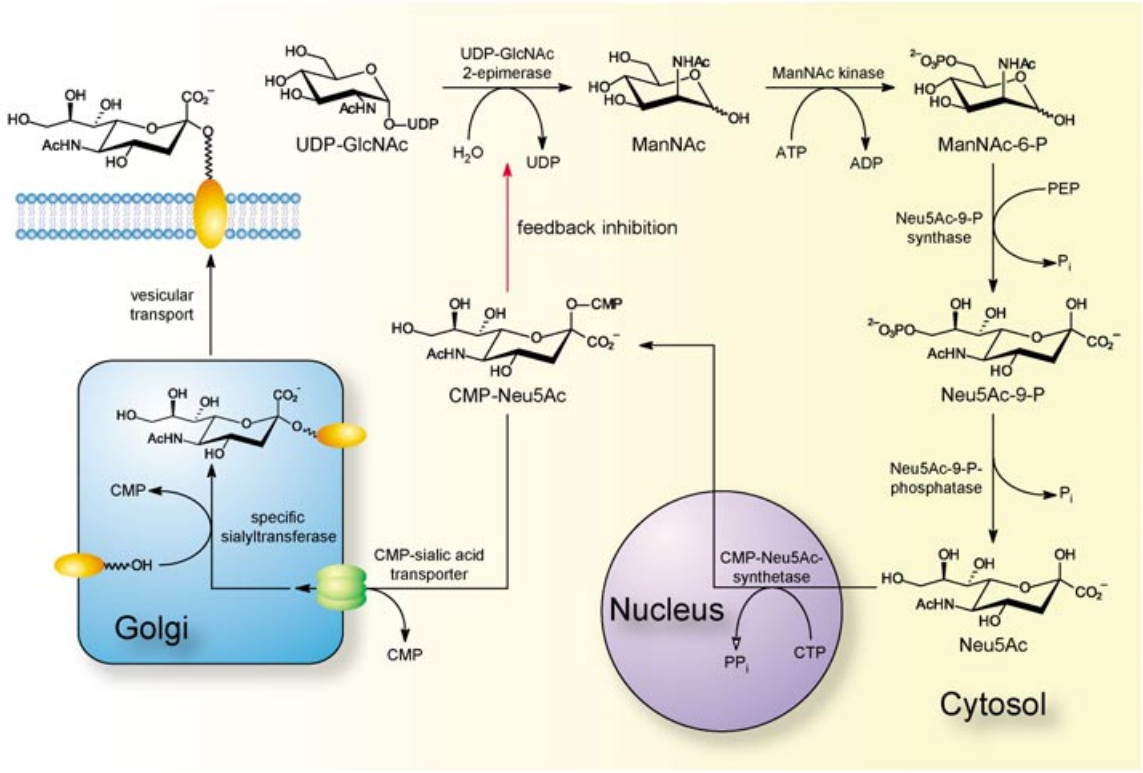
Synthesis and Biochemical Properties of Reversible Inhibitors of UDP-*N*-Acetylglucosamine 2-Epimerase

Samy Al-Rawi, Stephan Hinderlich, Werner Reutter, and Athanassios Giannis**

Sialic acids comprise a family of α-keto acids with a backbone skeleton consisting of nine carbon atoms. *N*-Acetylneuraminic acid (Neu5Ac or NANA; see Scheme 1) is the most common sialic acid and also the biosynthetic precursor of almost all known 50 members of this class.[1] Due to their exposed position at the distal end of oligosaccharide chains, sialic acids contribute to the biological properties of glyco-

[*] Dr. S. Hinderlich, Prof. Dr. W. Reutter
Charité – Universitätsmedizin Berlin
Campus Benjamin Franklin
Institut für Molekularbiologie und Biochemie
Arnimallee 22, 14195 Berlin-Dahlem (Germany)
Fax: (+49) 30-8445-1541
E-mail: stephan.hinderlich@charite.de
Dipl.-Chem. S. Al-Rawi, Prof. Dr. A. Giannis
Institut für Organische Chemie
Universität Leipzig
Johannisallee 29, 04103 Leipzig (Germany)
Fax: (+49) 341-973-6599
E-mail: giannis@chemie.uni-leipzig.de

  DOI: 10.1002/anie.200453863



Scheme 1. Biosynthesis of Neu5Ac and incorporation on cell-surface glycoconjugates.

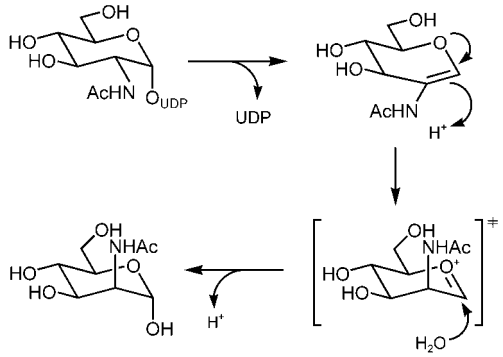
conjugate structures in a significant manner. Glycoconjugates containing sialic acid play an essential role in a variety of fundamental physiological and pathological processes,[2] for example, embryogenesis, organogenesis, immune defense, migration and homing of leucocytes, and metastasis of neoplastic cells as well as inflammation reactions and invasion of pathogens in cells. Many tumors bear a large amount of sialic acids like a protective shield on their cell surface. The most prominent example of sialic acid mediated cell–virus interaction is the infection by the influenza virus. The invasion of cells, as well as the release of newly formed viruses is mediated by sialic acid containing structures on the host cell surface. Inhibitors of influenza sialidase, the virus-releasing enzyme, are currently applied in antiviral therapy (Zanamivir, Oseltamivir).[3] Although all steps of Neu5Ac biosynthesis have been known for a long time,[4] only a limited number of inhibitors have been developed.[5,6] In this paper we report the conception, synthesis, and biochemical properties of a potent reversible inhibitor of UDP-*N*-acetylglucosamine (GlcNAc) 2-epimerase (UDP = uridinediphosphate, GlcNAc = *N*-acetylglucosamine), the key enzyme in Neu5Ac biosynthesis.

Biosynthesis of Neu5Ac begins with the epimerization of UDP-GlcNAc under concomitant release of UDP, followed by phosphorylation of the formed *N*-acetylmannosamine (ManNAc) at the 6-hydroxy functionality. Both steps are catalyzed by the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNac kinase (Scheme 1).[7,8] This key enzyme is regulated in a complex manner.[7–10] Lymphocytes that show a deficiency in activitiy of this enzyme are not capable of synthesizing sialic acids by themselves and show serious defects in sialic acid dependent functions.[11] Inactivation through targeted mutagenesis in mice causes hyposialylation of the embryos and induces early lethality on day 8.5 of embryonic development.[12] These results emphasize the importance of UDP-GlcNAc 2-epimerase in the biosynthesis of *N*-acetylneuraminic acid and make this enzyme an interesting target for the development of effective inhibitors.

The homologous mammalian UDP-GlcNAc 2-epimerase has recently become available in a recombinant form and does not differ in mechanism[13] from the bacterial[14] enzyme. Both enzymes first generate 2-acetamidoglucal as an intermediate after *anti*-elimination of UDP. Positional isotope exchange experiments showed that the reaction proceeds by C–O bond cleavage at the anomeric position. By monitoring the reaction with coupled NMR experiments, we were able to determine that the subsequent addition of water occurs from the bottom side of the 2-acetamidoglucal. Thus, the released ManNAc is the α-anomer (Scheme 2). The oxocarbenium intermediate might represent the transition state of the addition of water on the 2-acetamidoglucal.

Because stable analogues of the transition state are known to be bound much more tightly to the active site of the enzyme than analogues of the substrate in its ground state,[15] we considered the synthesis of iminosugars **2**–**4** (Figure 1) as potential inhibitors of UDP-GlcNAc 2-epimerase. At physiological pH such iminosugars are protonated and they should consequently be good analogues of the proposed transition state.

The bicyclic oxazolidinylpiperidine of type **9** (Scheme 3) served as the central, chiral building block in the preparation



Scheme 2. Proposed reaction mechanism of mammalian UDP-GlcNAc 2-epimerase and postulated transition state for the *syn*-addition of water at the 2-acetamidoglucal.

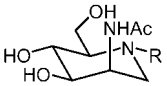
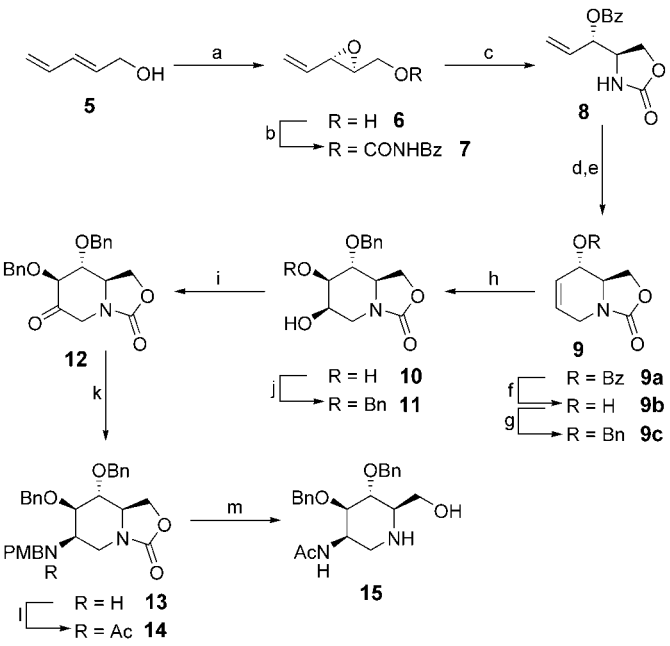


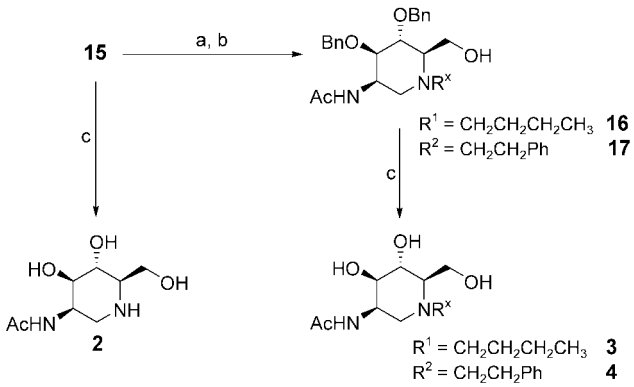
Figure 1. Transition-state analogues of UDP-GlcNAc 2-epimerase. **2**: R = H, **3**: R = *n*Bu, and **4**: R = CH_2CH_2Ph.

of the 1-deoxyiminosugar. According to the previous work by Martin et al.,[16] the doubly unsaturated alcohol **5** was stereoselectively epoxidized under Sharpless conditions.[17] Subsequent conversion of the (2*S*,3*S*)-epoxyalcohol **6** with benzoylisocyanate gave the benzoylcarbamate **7**. Regioselective opening of the epoxide under mild conditions in a biphasic system lead to an intramolecular migration of the benzoyl group to afford the substituted oxazolidinone **8**. The compound was first allylated at the ring nitrogen then subjected to a ring-closing metathesis to give the bicyclic derivate **9a** in almost quantitative yield. With regard to the following synthetic steps, it was necessary to convert the ester group of the piperidine ring into a benzyl ether. Compound **9c** was synthesized from **9a** in two steps. By using catalytic amounts of potassium osmate and *N*-methylmorpholine-*N*-oxide (NMMO) as the cooxidant in a mixture of *t*BuOH/water, we converted compound **9c** into the corresponding diol. In this reaction no acceptable diastereomeric excess could be achieved; however, with $(DHQD)_2Phal$ as chiral auxiliary[18] and $K_3[Fe(CN)_6]$ as cooxidant, bishydroxylation of compound **10** resulted with nearly complete diastereoselectivity in very good yields. The structure was determined by NOE and COSY experiments.

Reaction of compound **10** with dibutyltin oxide gave as intermediate the corresponding dibutyltin ketal, which was converted into compound **11** using benzyl bromide in the presence of tetrabutylammonium bromide[19] with a regioselectivity of 3.5:1. Swern oxidation of **11** yielded ketone **12**. Reductive amination of this derivative with *p*-methoxybenzylamine and sodium triacetoxyborohydride resulted in compound **13**. Acetylation of compound **13** gave the amide **14** in good yield. Oxidative cleavage of the *p*-methoxybenzyl group in the presence of cerium(IV) ammonium nitrate (CAN) followed by opening of the oxazolidinone ring with barium hydroxide afforded compound **15**. After cleavage of the protecting groups of compound **15** by hydrogenolysis, target structure **2** was finally formed (Scheme 4).[20] For subsequent application of the inhibitors in cellular systems and for the



Scheme 3. Synthesis of the protected iminosugar **15**: a) $Ti(O\mathit{i}Pr)_4$, L-(+)-DIPT, *t*BuOOH, CH_2Cl_2, 3-Å molecular sieves, −15 °C, 57%; b) BzNCO, Et_2O, RT, 84%; c) K_2CO_3, $(C_8H_{17})_3CH_3NCl$, CH_2Cl_2/H_2O, RT, 84%; d) NaH, allyl bromide, DMF, 0 °C→RT, 81%; e) Grubbs catalyst, toluene, RT, 99%; f) K_2CO_3, MeOH, RT, 85%; g) NaH, BnBr, DMF, 0 °C→RT, 91%; h) $(DHQD)_2Phal$, K_2OsO_4, K_2CO_3, $K_3[Fe(CN)_6]$, $CH_3SO_2NH_2$, *t*BuOH/H_2O, RT, 90%; i) 1. $n Bu_2SnO$, toluene, reflux; 2. BnBr, Bu_4NBr, 100 °C, 69%; j) DMSO, $(COCl)_2$, DIPEA, CH_2Cl_2, −70 °C→RT, 86%; k) 4-methoxybenzylamine, AcOH, $Na(OAc)_3BH$, 1,2-dichloroethane, RT, 78%; l) pyridine, DMAP, AcCl, 0 °C→RT, 86%; m) 1. CAN, CH_3CN/H_2O, RT; 2. $Ba(OH)_2\times 8H_2O$, EtOH/H_2O, reflux, 63%. Bz = benzoyl, Bn = benzyl, CAN = ceric ammonium nitrate, $(DHQD)_2Phal$ = dihydroquinidine 1,4-phthalazinediyl ether, DIPEA = *N*,*N*-diisopropylethylamine, DIPT = diisopropyl tartrate, DMAP = 4-dimethylaminopyridine, PMB = *p*-methoxybenzyl.



Scheme 4. Derivatization and deprotection: a) Butyraldehyde, AcOH, $Na(OAc)_3BH$, 1,2-dichloroethane, RT, 95%; b) phenylacetaldehyde, AcOH, $NaCNBH_3$, CH_3CN, RT, 85%; c) H_2, Pd/C, HCl, EtOH, RT, **2**: 87%, **3**: 90%, **4**: 90%.

determination of new structure–activity relationships, it could be helpful to synthesize more lipophilic N-alkylated derivatives like compounds **3** and **4**. The synthesis succeeded without any problems by reductive amination of **15** in the

presence of the corresponding aldehydes and gave derivatives **16** and **17**, which provided compounds **3** and **4** after hydrogenolysis (Scheme 4).

In tests with standard enzyme preparations[21] compounds **2**–**4** did not show any inhibition of UDP-GlcNAc 2-epimerase activity in either concentrations equimolar to (1 mM) or in concentrations up to four times higher of that of the substrate UDP-GlcNAc. These results show that the 1,2-dideoxy-2-acetamidomannojirimycin derivatives, if they are effective inhibitors, are not able to reach the active site of the enzyme, perhaps because they are blocked by UDP-GlcNAc or UDP, which is used in standard preparations to stabilize the enzyme. Further experiments were then performed using enzyme preparations containing no UDP.[21] Preincubation of these samples in the presence of the inhibitors **2**–**4** led to clear inhibition of enzyme activity (up to 70%). When 0.1 mM UDP was added to the enzyme samples before addition of the inhibitors and then preincubation, no significant inhibition (under 10%) was observed. Furthermore, no inhibition was achieved when UDP-free enzyme preparations were used in the enzyme activity experiments without any preincubation, that is, enzyme, inhibitor, and UDP-GlcNAc were mixed simultaneously. These experiments show that the synthesized compounds are readily able to bind to the active site of the enzyme, if it is not blocked by UDP and/or by UDP-GlcNAc.

In order to compare the effectiveness of the inhibitors **2**–**4**, they were tested under various concentrations (Figure 2). All three compounds examined inhibited the activity of UDP-GlcNAc 2-epimerase in a concentration-dependent manner. Inhibition of 50% activity was observed at a concentration of 0.5 mM. Concentrations of **2** and **3** up to 2 mM in the preincubation sample induced almost complete inhibition, while treatment with **4** resulted in a remaining activity of nearly 10%. The presence of UDP in the preincubation samples prevented enzyme inhibition in almost all experiments. Addition of UDP and/or UDP-GlcNAc after an incubation of 20 min had nearly no effect on the inhibition by **2**–**4**. These data suggest that the inhibitors are bound very tightly by the enzyme as soon as they have reached the active site. This observation supports the hypothesis that transition-state analogues are bound much more tightly by the enzyme than the physiological substrate. Although the binding constants of the inhibitors were not determined in these experiments, they should be lower than the binding constants of UDP and UDP-GlcNAc, since the subsequent addition of both substrates does not displace the inhibitors.[22] The K_m values of UDP-GlcNAc and UDP are about 10 μM; thus the K_i values of the inhibitors should be lower.

The specificity of the inhibitors was determined by the lack of inhibition of the natural sugars ManNAc and GlcNAc, which were tested under the same conditions as the inhibitors. Neither the activity of the ManNAc kinase of the bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase was influenced by the inhibitors nor was its hexameric oligomeric structure affected, which serves as an indicator of structural integrity.[7] By comparing the inhibitors it seems to be noteworthy to mention that the ring nitrogen can be substituted with hydrophobic residues without affecting inhibitory potency. This might be important for subsequent applications in cellular systems, since hydrophobic compounds permeate the plasma membrane much more easily. The application of these inhibitors, for which we propose the name nanastatines, opens up the possibility of better understanding the role of *N*-acetylneuraminic acid containing conjugates in physiological and pathological processes.

Received: January 27, 2004
Revised: May 19, 2004 [Z53863]

Keywords: biosynthesis · carbohydrates · epimerases · inhibitors · sialic acids

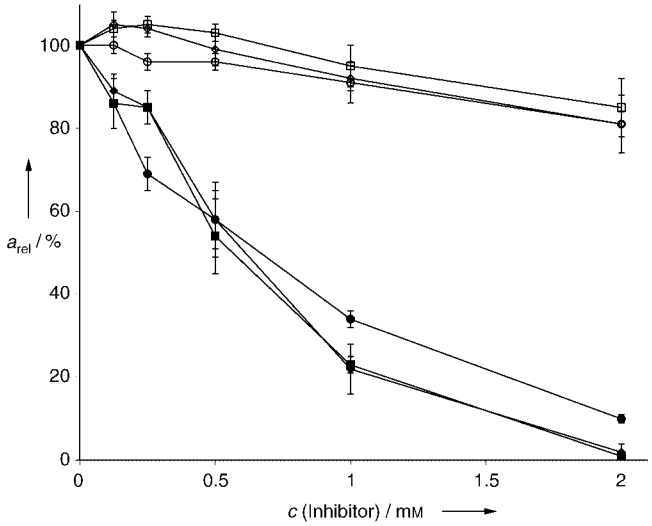


Figure 2. Inhibition of UDP-GlcNAc 2-epimerase by compounds **2**–**4**. ◆: **2**, ◇: **2** + UDP, ■: **3**, □: **3** + UDP, ●: **4**, ○: **4** + UDP. The enzyme was preincubated with the inhibitors for 20 min at 37°C in the absence or presence of 0.1 mM UDP and subsequently used in the activity assay. Noted concentrations refer to the final volume in the activity assay. Data shown above represent mean values ± standard deviation of three independent experiments.

[1] T. Angata, A. Varki, *Chem. Rev.* **2002**, *102*, 439.
[2] S. Kelm, R. Schauer, *Int. Rev. Cytol.* **1997**, *175*, 137.
[3] M. J. Kiefel, M. von Itzstein, *Chem. Rev.* **2002**, *102*, 471.
[4] a) A. P. Corfield, R. Schauer in *Sialic Acids—Chemistry, Metabolism and Function* (Ed.: R. Schauer), Springer, Vienna, **1982**, p. 195; b) A. Varki, *Glycobiology* **1993**, *3*, 97.
[5] R. Zeitler, A. Giannis, S. Danneschewski, E. Henk, T. Henk, C. Bauer, W. Reutter, K. Sandhoff, *Eur. J. Biochem.* **1992**, *204*, 1165.
[6] a) A. Blume, H. Chen, W. Reutter, R. R. Schmidt, S. Hinderlich, *FEBS Lett.* **2002**, *521*, 127; b) F. Stolz, M. Reiner, A. Blume, W. Reutter, R. R. Schmidt, *J. Org. Chem.* **2004**, *69*, 665–679.
[7] S. Hinderlich, R. Stäsche, R. Zeitler, W. Reutter, *J. Biol. Chem.* **1997**, *272*, 24313.
[8] R. Stäsche, S. Hinderlich, C. Weise, K. Effertz, L. Lucka, P. Moormann, W. Reutter, *J. Biol. Chem.* **1997**, *272*, 24319.
[9] S. Kornfeld, R. Kornfeld, E. F. Neufeld, P. J. O'Brien, *Proc. Natl. Acad. Sci. USA* **1964**, *61*, 371.
[10] R. Horstkorte, S. Nöhring, K. Danker, K. Effertz, W. Reutter, L. Lucka, *FEBS Lett.* **2000**, *470*, 315.
[11] O. T. Keppler, S. Hinderlich, J. Langner, R. Schwartz-Albiez, W. Reutter, M. Pawlita, *Science* **1999**, *284*, 1372.

[12] M. Schwarzkopf, K.-P. Knobeloch, E. Rohde, S. Hinderlich, N. Wiechens, L. Lucka, I. Horak, W. Reutter, R. Horstkorte, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5267.
[13] W. K. Chou, S. Hinderlich, W. Reutter, M. E. Tanner, *J. Am. Chem. Soc.* **2003**, *125*, 2455.
[14] P. M. Morgan, R. F. Sala, M. E. Tanner, *J. Am. Chem. Soc.* **1997**, *119*, 10269.
[15] L. Pauling, *Chem. Eng. News* **1946**, *24*, 1375.
[16] R. Martin, A. Moyano, M. A. Pericas, A. Riera, *Org. Lett.* **2000**, *2*, 93.
[17] Y. Gao, J. M. Klunder, R. M. Hanson, H. Masamune, S. Y. Ko, K. B. Sharpless, *J. Am. Chem. Soc.* **1987**, *109*, 5765.
[18] K. B. Sharpless, W. Amberg, Y. L. Bennani, G. A. Crispino, J. Hartung, K. S. Jeong, H. L. Kwong, K. Morikawa, Z. M. Wang, D. Xu, X. L. Zhang, *J. Org. Chem.* **1992**, *57*, 2768.
[19] H. Böshagen, F. R. Heiker, A. M. Schüller, *Carbohydr. Res.* **1987**, *164*, 141.
[20] Compound **2** was already synthesized earlier using a chemoenzymatic strategy as well as starting from suitable functionalized carbohydrate structures and tested as an inhibitor for a wide range of glycosidases. However, no inhibitory effect was detected. a) G. W. Fleet, L. E. Fellows, P. W. Smith, *Tetrahedron* **1987**, *43*, 979; b) T. Kajimoto, K. K. C. Liu, R. L. Pederson, Z. Zhong, Y. Ichikawa, J. A. Porco, Jr., C. H. Wong, *J. Am. Chem. Soc.* **1991**, *113*, 6187.
[21] Recombinant UDP-GlcNAc-2-epimerase from insect (Sf9) cells was purified as described by A. Blume, D. Ghaderi, V. Liebig, S. Hinderlich, P. Donner, W. Reutter, L. Lucka, *Protein Expression Purif.* **2004**, in press. For purification of UDP-free preparations the samples were applied to a PD-10 column (Amersham) and the column was eluted with 10 mM NaH_2PO_4 (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiotreitol. UDP-GlcNAc 2-epimerase assays contained in a final volume 200 μL 45 mM NaH_2PO_4 (pH 7.5), 10 mM $MgCl_2$, 1 mM UDP-GlcNAc, 0.2 mM NADH, 2 mM phospho*enol*pyruvat, 2 U pyruvat kinase, 2 U lactate dehydrogenase, 5 μg purified UDP-GlcNAc 2-epimerase/ManNAc kinase, and indicated concentrations of inhibitors. The samples were incubated for 20 min at 37 °C and quenched by addition of 800 μL 10 mM EDTA. Subsequently the extinction was determined at 340 nm. Preincubation samples contained 5 μg purified UDP-GlcNAc 2-epimerase/ManNAc kinase and variable amounts of inhibitors in a final volume of 20 μL. The samples were incubated for 20 min at 37 °C, and enzyme reaction was started by addition of remaining components in a final volume of 200 μL. Inhibitor concentrations noted in the text refer to the final volume.
[22] In this context it might be interesting to use chimeric compounds, which consist of the iminosugars described here and specific parts of UDP-GlcNAc. Currently we are synthesizing such derivatives.

