AMBER9.[61] The computed binding free energy was –20.08 (kcal/mol) at pH 7.

Fourth, we calculated the effect of low pH in the lysosome on the binding affinity between the β-galactosidase and NOEV molecules. The low pH effect was represented as protonation of charged residues estimated by PROPKA.[62] The computed binding free energy at pH 5 was –18.06 (kcal/mol); higher than that at pH 7. This result indicates that affinity between β-galactosidase and NOEV is weakened at pH 5 compared with that at pH 7. Consequently, we concluded that (1) the enzyme-NOEV complex has lower free energy than the unbound enzyme, and (2) protonation of an active site residue causes free energy change consistent with the chemical chaperone hypothesis.

Conclusion

This new therapeutic strategy (chaperone therapy) is in principle applicable to all lysosomal diseases, if a specific compound is developed for each enzyme in question. We have already confirmed the effect in Fabry disease, G_{M1}-gangliosidosis, and Gaucher disease. Other related diseases also are currently studied by other investigators.[63,64] Theoretically this principle can be applied to all other lysosomal diseases. Furthermore, there may well be other genetic diseases to be considered, if molecular pathology in somatic cells has been clarified in detail. We hope studies in this direction will disclose a new aspect of molecular therapy for inherited metabolic diseases with central nervous system involvement in the near future.

Acknowledgments

This research was supported by grants from the Ministry of Education, Culture, Science, Sports, and Technology of Japan (13680918, 14207106), and the Ministry of Health, Labour and Welfare of Japan (H10-No-006, H14-Kokoro-017, H17-Kokoro-019). We thank all collaborators contributing to this research project for the past 16 years.

Abbreviations

NOEV, *N*-octyl-4-epi-β-valienamine; NOV, *N*-octyl-β-valienamine; DGN, 1-deoxygalactonojirimycin; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; LAH, lithium aluminum hydride; KO, knockout; Tg, transgenic.

Disclosure

The authors report no conflicts of interest.

References

1. De Duve C, Wattiaux R. Functions of lysosomes. *Annu Rev Physiol.* 1966;28:435–92.
2. Hers HG. Inborn lysosomal diseasese. *Gastroenterology.* 1965;48:625–33.
3. Brady RO. Emerging strategies for the treatment of hereditary metabolic storage disorders. *Rejuvenation Res.* 2006;9:237–344.
4. Suzuki Y, Nanba E, Matsuda J, Higaki K, Oshima A. β-Galactosidase deficiency (β-galactosidosis): G_{M1}-Gangliosidosis and Morquio B disease. In *The Online Metabolic and Molecular Bases of Inherited Disease* Chapter 151, Valle D, Beaudet AL, Vogelstein B, Kinzler KW, Antonarakis SF, Ballabio A. eds. New York, McGraw-Hill, 2008; p. 1–101, http://www.ommbid.com/.
5. Suzuki Y, Crocker AC, Suzuki K. G_{M1}-gangliosidosis. Correlation of clinical and biochemical data. *Arch Neurol.* 1971;24:58–64.
6. Suzuki Y, Nakamura N, Fukuoka K. G_{M1}-gangliosidosis: accumulation of ganglioside G_{M1} in cultured skin fibroblasts and correlation with clinical types. *Hum Genet.* 1978;43:127–31.
7. Suzuki Y, Furukawa T, Hoogeveen A, Verheijen F, Galjaard H. Adult type G_{M1}-gangliosidosis: a complementation study on somatic cell hybrids. *Brain Dev.* 1979;1:83–6.
8. Ko YM, Yamanaka T, Umeda M, Suzuki Y. Effects of thiol protease inhibitors on intracellular degradation of exogenous β-galactosidase in cultured human skin fibroblasts. *Exp Cell Res.* 1983;148:525–9.
9. Nanba E, Tsuji A, Omura K, Suzuki Y. G_{M1}-gangliosidosis: abnormalities in biosynthesis and early processing of β-galactosidase in fibroblasts. *Biochem Biophys Res Commun.* 1988;152:794–800.
10. Oshima A, Tsuji A, Nagao Y, Sakuraba H, Suzuki Y. Cloning, sequencing, and expression of cDNA for human β-galactosidase. *Biochem Biophys Res Commun.* 1988;157:238–44.
11. Eng CM, Ioannou YA, Desnick RJ. α-Galactosidase A Deficiency: Fabry Disease. In *The Online Metabolic and Molecular Bases of Inherited Disease* Valle D, Beaudet AL, Vogelstein B, Kinzler KW, Antonarakis SF, Ballabio A. eds. New York, McGraw-Hill, p. <http://www.ommbid.com/>. 2008
12. Okumiya T, Ishii S, Takenaka T, et al. Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. *Biochem Biophys Res Commun.* 1995;214:1219–24.
13. Fan JQ, Asano N, Suzuki Y, Ishii S. Chemical chaperone therapy for Fabry disease, a genetic disorder. *Glycoconjugate J.* 1999;16:S156.
14. Matsuda J, Suzuki O, Oshima A, et al. Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. *Proc Natl Acad Sci U S A.* 2003;100:15912–7.
15. Lin H, Sugimoto Y, Ohsaki Y, et al. *N*-octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. *Biochim Biophys Acta.* 2004;1689:219–28.
16. Iwasaki H, Watanabe H, Iida M, et al. Fibroblast screening for chaperone therapy in β-galactosidosis. *Brain Dev.* 2006;28:482–6.
17. Suami T, Ogawa S. Chemistry of carba-sugars (pseudo-sugars) and their derivatives. *Adv Carbohydr Chem Biochem.* 1990;48:21–90.
18. Ogawa S. Synthesis studies on glycosidase inhibitors composed of 5a-carba-sugars. In: *Carbohydrate Mimics*, Y. Chapleur, ed. Weinheim, Wiley-VCH, 1998. p. 87–106.
19. Ogawa S. Design and synthesis of carba-sugars of biological interest. *Trends Glycosci Glycotechnol.* 2004;16:33–53.
20. McCasland GE, Stanley F, Lois JD. Alicyclic carbohydrates. XXIX. The synthesis of a pseudo-hexose (2,3,4,5-tetrahydroxycyclohexane-methanol). *J Org Chem.* 1966;31:1516–21.
21. Miller TW, Arison BH, Albers-Schonberg G. Isolation of a cyclic antibiotic: 2,3,4,5-tetrahydroxycyclohexanemethanol. *Biotech Bioeng.* 1973;15:1075–80.
22. Suami T, Ogawa S, Toyokuni T. Sweet tasting pseudo-sugars. *Chem Lett.* 1983;611–12.

23. Iwasa T, Yamamoto H, Shibata M. Studies on validamycins, new antibiotics. I. Streptomyces hygroscopicus var. limoneus nov. var., validamycin-producing organism. *Jpn J Antibiot.* 1970;23:595–602.
24. Horii S, Iwasa T, Mizuta E, Kameda Y. Studies on validamycins, new antibiotics. VI. Validamine, hydroxyvalidamine and validatol, new cyclitols. *J Antibiot.* 1971;24:59–63.
25. Kameda Y, Horii S. The unsaturated cyclitol part of the new antibiotics, the validamycins. *J Chem Socy Chem Commun.* 1972;746–7.
26. Schmidt DD, Frommer W, Junge B, et al. α-Glucosidase inhibitors. New complex oligosaccharides of microbial origin. *Naturwissenschaften.* 1977;64:535–36.
27. Junge B, Heiker FR, Kurz J, Muller L, Schmidt DD. Untersuchungen zur struktur des α-D-glucosdiase inhibitors acarbose. *Carbohydrate Res.* 1984;128:235–68.
28. Horii S, Fukase H, Matsuo T, Kameda Y, Asano N, Matsui K. Synthesis and α-D-glucosidase inhibitory activity of N-substituted valiolamine derivatives as potential oral antidiabetic agents. *J Med Chem.* 1986;29:1038–46.
29. Fukase H, Horii S. Synthesis of valiolamine and its N-substituted derivatives AO-128, validoxylamine-G, and validamycin-G via branched-chain inosose derivatives. *J Org Chem.* 1992;57:3651–8.
30. Ogawa S, Suetsugu M, Toyokuni T, Suami T. Synthesis of DL-6'-epivalidoxylamine A. *Nippon Kagaku Kaishi.* 1982;1721–6.
31. Ogawa S, Uchida C, Shibata Y. Alternative synthesis and enzyme-inhibitory activity of methyl 1'-epiacarviosin and its 6-hydroxy analog. *Carbohydr Res.* 1992;223:279–86.
32. Lockhoff O. Glycolipids as immunomodulators: synthesis and properties. *Angew Chem, Internatl Ed English.* 1991;30:1611–20.
33. Tsunoda H, Ogawa S. Synthesis of some 5a-carbaglycosylamides, glycolipid analogs of biological interests. *Liebigs Annal Chem.* 1994;103–7.
34. Tsunoda H, Ogawa S. Synthesis of 5a-carba-β-D-glycosylceramide analogs linked by imino, ether and sulfide bridges. *Liebigs Annal Chem.* 1995;267–77.
35. Tsunoda H, Inokuchi J, Yamagishi K, Ogawa S. Synthesis of glycosylceramide analogs composed of imino-linked unsaturated 5a-carbaglycosyl residues: potent and specific gluco- and galactocerebrosidase inhibitors. *Liebigs Annal Chem.* 1995;279–84.
36. Ogawa S, Ashiura M, Uchida C, et al. Synthesis of potent β-D-glucocerebrosidase inhibitors: *N*-alkyl-β-valienamines. *Bioorg Med Chem Lett.* 1996;6:929–32.
37. Ogawa S, Sakata Y, Ito N, et al. Convenient synthesis and evaluation of glycosidase inhibitory activity of α- and β-galactose-type valienamines, and some N-alkyl derivatives. *Bioorg Med Chem.* 2004;12: 995–1002.
38. Ogawa S, Matsunaga YK, Suzuki Y. Chemical modification of the β-glucocerebrosidase inhibitor *N*-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-O-(β-D-galactopyranosyl) derivatives. *Bioorg Med Chem.* 2002;10:1967–72.
39. Arjona O, Gomez AM, Lopez JC, Plumet J. Synthesis and conformational and biological aspects of carbasugars. *Chem Rev.* 2007;107: 1919–2036.
40. Ogawa S, Iwasawa Y, Nose T, et al. Total synthesis of (+)-(1,2,3/4,5)-2,3,4,5-tetrahydroxycyclohexane-1-methanol and (+)-(1,3/2,4,5)-5-amino-2,3,4-trihydroxycyclohexane-1-methanol [(+)-validamine]. X-ray crystal structure of 3-(+)-2-*exo*-bromo-4,8-dioxatricyclo[4.2.1.$0^{3,7}$]nonan-5-one. *J Chem Soc Perkin Trans.* 1985;1:903–6.
41. Ogawa S, Kobayashi Y, Kabayama K, Jimbo M, Inokuchi J. Chemical modification of β-glucocerebrosidase inhibitor *N*-octyl-β-valienamine: synthesis and biological evaluation of N-alkanoyl and N-alkyl derivatives. *Bioorg Med Chem.* 1998;6:1955–62.
42. Ogawa S, Asada M, Ooki Y, Mori M, Itoh M, Korenaga T. Design and synthesis of glycosidase inhibitor 5-amino-1,2,3,4-cyclohexanetetrol derivatives from (−)-*vibo*-quercitol. *Bioorg Med Chem.* 2005;13: 4306–14.
43. Ogawa S, Kanto M. Synthesis of valiolamine and some precursors for bioactive carbaglycosylamines from (−)-*vibo*-quercitol produced by biogenesis of myo-inositol. *J Nat Prod.* 2007;70:493–7.
44. Ogawa S, Kanto M, Suzuki Y. Development and medical application of unsaturated carbaglycosylamine glycosidase inhibitors. *Mini Rev Med Chem.* 2007;7:679–91.
45. Yoshida K, Oshima A, Shimmoto M, et al. Human β-galactosidase gene mutations in G_{M1}-gangliosidosis: a common mutation among Japanese adult/chronic cases. *Am J Hum Genet.* 1991;49:435–42.
46. Oshima A, Yoshida K, Shimmoto M, Fukuhara Y, Sakuraba H, Suzuki Y. Human β-galactosidase gene mutations in Morquio B disease. *Am J Hum Genet.* 1991;49:1091–3.
47. Schiffmann R, Brady RO. New prospects for the treatment of lysosomal storage diseases. *Drugs.* 2002;62:733–42.
48. Samoylova TI, Martin DR, Morrison NE, et al. Generation and characterization of recombinant feline β-galactosidase for preclinical enzyme replacement therapy studies in G_{M1}-gangliosidosis. *Metab Brain Dis.* 2008;23:161–73.
49. Suzuki Y. β-galactosidase deficiency: an approach to chaperone therapy. *J Inherit Metab Dis.* 2006;29:471–6.
50. Ishii S, Kase R, Sakuraba H, Suzuki Y. Characterization of a mutant α-galactosidase gene product for the late-onset cardiac form of Fabry disease. *Biochem Biophys Res Commun.* 1993;197:1585–9.
51. Frustaci A, Chimenti C, Ricci R, et al. Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. *N Engl J Med.* 2001;345:25–32.
52. Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med.* 1999;5:112–5.
53. Tominaga L, Ogawa Y, Taniguchi M, et al. Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. *Brain Dev.* 2001;23:284–7.
54. Conzelmann E, Sandhoff K. Partial enzyme deficiencies: residual activities and the development of neurological disorders. *Dev Neurosci.* 1983;6:58–71.
55. Suzuki Y, Ichinomiya S, Kurosawa M, et al. Chemical chaperone therapy: clinical effect in murine G_{M1}-gangliosidosis. *Ann Neurol.* 2007;62:671–5.
56. Matsuda J, Suzuki O, Oshima A, et al. β-Galactosidase-deficient mouse as an animal model for G_{M1}-gangliosidosis. *Glycoconjugate J.* 1997;14:729–36.
57. Ichinomiya S, Watanabe H, Maruyama K, et al. Motor and reflex testing in G_{M1}-gangliosidosis model mice. *Brain Dev.* 2007;29:210–6.
58. Ginalski K, Elofsson A, Fischer D, Rychlewski L. 3D-Jury: a simple approach to improve protein structure predictions. *Bioinformatics.* 2003;19:1015–8.
59. Kajan L, Rychlewski L. Evaluation of 3D-Jury on CASP7 models. *BMC Bioinformatics.* 2007;8:304.
60. Morris GM, Goodsell DS, Halliday RS, et al. Automated docking using a Lamarckian genetic algorithm and emprical binding free energy function. *J Comput Chem.* 1998;19:1639–62.
61. Case DA, Cheatham-III-TE, Darden T, et al. The Amber biomolecular simulations programs. *J Comput Chem.* 2005;26:1668–88.
62. Li H, Robertson AD, Jensen JH. Very fast empirical prediction and rationalization of protein Pk_a values. *Proteins.* 2005;61:704–21.
63. Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S β-glucosidase: a therapeutic strategy for Gaucher disease. *Proc Natl Acad Sci U S A.* 2002;99:15428–33.
64. Tropak MB, Reid SP, Guiral M, Withers SG, Mahuran D. Pharmacological enhancement of β-hexosaminidase activity in fibroblasts from adult Tay-Sachs and Sandhoff Patients. *J Biol Chem.* 2004;279:13478–87.

CHEMBIOCHEM

DOI: 10.1002/cbic.200900442

Chaperone Activity of Bicyclic Nojirimycin Analogues for Gaucher Mutations in Comparison with *N*-(*n*-nonyl)-Deoxynojirimycin

Zhuo Luan,[a] Katsumi Higaki,[b] Matilde Aguilar-Moncayo,[c] Haruaki Ninomiya,[a] Kousaku Ohno,*[a] M. Isabel García-Moreno,[c] Carmen Ortiz Mellet,*[c] José M. García Fernández,*[d] and Yoshiyuki Suzuki*[e]

Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations of lysosomal β-glucosidase (acid β-Glu, β-glucocerebrosidase); these mutations result in protein misfolding. Some inhibitors of this enzyme, such as the iminosugar glucomimetic *N*-(*n*-nonyl)-1-deoxynojirimycin (NN-DNJ), are known to bind to the active site and stabilize the proper folding for the catalytic form, acting as "chemical chaperones" that facilitate transport and maturation of acid β-Glu. Recently, bicyclic nojirimycin (NJ) analogues with structure of sp^2 iminosugars were found to behave as very selective, competitive inhibitors of the lysosomal β-Glu. We have now evaluated the glycosidase inhibitory profile of a series of six compounds within this family, namely 5-*N*,6-*O*-(*N'*-octyliminomethylidene-NJ (NOI-NJ), the 6-thio and 6-amino-6-deoxy derivatives (6S-NOI-NJ and 6N-NOI-NJ) and the corresponding galactonojirimycin (GNJ) counterparts (NOI-GNJ, 6S-NOI-GNJ and 6N-NOI-GNJ), against commercial as well as lysosomal glycosidases. The chaperone effects of four selected candidates (NOI-NJ, 6S-NOI-NJ, 6N-NOI-NJ, and 6S-NOI-GNJ) were further evaluated in GD fibroblasts with various acid β-Glu mutations. The compounds showed enzyme enhancement on human fibroblasts with N188S, G202R, F213I or N370S mutations. The chaperone effects of the sp^2 iminosugar were generally stronger than those observed for NN-DNJ; this suggests that these compounds are promising candidates for clinical treatment of GD patients with a broad range of β-Glu mutations, especially for neuronopathic forms of Gaucher disease.

Introduction

Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations in the gene encoding for acid β-glucosidase (acid β-Glu; β-glucocerebrosidase; EC 3.2.1.45).[1–3] Such mutations give rise to significant protein misfolding effects during translation in the endoplasmic reticulum followed by a reduction in enzyme trafficking to the lysosome.[4] The defective activity of lysosomal β-Glu results in progressive accumulation of glucosylceramide in macrophages; this often leads to hepatosplenomegaly, anemia, bone lesions and respiratory failure, and sometimes central nervous system (CNS) involvement. Patients without neurological symptoms are classified as type 1, whereas those with neurological symptoms are classified into type 2 (acute infantile form) and type 3 (juvenile form). At present, there are two established therapeutic strategies for GD: enzyme replacement[5,6] and substrate reduction therapies.[7] Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu,[8] whereas substrate reduction has been realized by oral administration of *N*-(*n*-butyl)deoxynojirimycin (NB-DNJ, Zavesca), which inhibits glucosyltransferase[9] and decreases substrate biosynthesis.[10] Both therapies have been proven to be effective for visceral, hematologic and skeletal abnormalities.[11–13] However, the efficacy of these therapies for neurological manifestations is limited.[14–17] Bone marrow transplantation can also

[a] Z. Luan, Dr. H. Ninomiya, Prof. K. Ohno
Division of Child Neurology, Institute of Neurological Sciences
Faculty of Medicine, Tottori University
Nishi-cho, Yonago 683-8504 (Japan)
Fax: (+81) 8593-86779
E-mail: ohno@med.tottori-u.ac.jp

[b] Prof. K. Higaki
Division of Functional Genomics,
Research Center for Bioscience and Technology
Faculty of Medicine, Tottori University
86 Nishi-mach, Yonago 683-8503 (Japan)

[c] M. Aguilar-Moncayo, Dr. M. I. García-Moreno, Prof. C. Ortiz Mellet
Departamento de Química Orgánica,
Facultad de Química, Universidad de Sevilla
Profesor García González 1, 41012 Sevilla (Spain)
Fax: (+34) 954624960
E-mail: mellet@us.es

[d] Prof. J. M. García Fernández
Instituto de Investigaciones Químicas, CSIC–Universidad de Sevilla
Américo Vespucio 49, Isla de la Cartuja, 41092 Sevilla (Spain)
Fax: (+34) 954489559
E-mail: jogarcia@iiq.csic.es

[e] Prof. Y. Suzuki
International University of Health and Welfare, Graduate School
2600-1 Kita Kanemaru, Otawara 324-8501 (Japan)
Fax: +(81) 2872-43229
E-mail: SuzukiY@iuhw.ac.jp

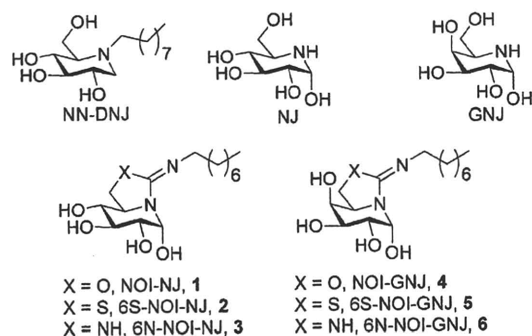
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900442

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

reverse the disease, but thus far gene therapy strategies have been unsuccessful.

It has been recently found that compounds that act as inhibitors of acid β-Glu can also stabilize the properly folded structure of the enzyme; thus, this renders them suitable for a third treatment paradigm, namely chaperone therapy, in which active-site-directed inhibitors stabilize mutant forms of the enzyme as they pass through the secretory pathway.[18–25] Thus, iminosugar-type glycomimetics with β-glucocerebrosidase inhibitory activity, such as *N*-(*n*-nonyl)-deoxynojirimycin (NN-DNJ, Figure 1, below), have also shown high promise as chemical chaperones for the treatment of Gaucher disease, as they are able to increase the enzyme activity of N370S and G202R mutants.[26,27] However, NN-DNJ and related iminosugars generally behave as broad range glycosidase inhibitors and simultaneously inhibit α- and β-glucosidases; this might represent a problem for clinical uses due to unwanted side effects.[28]

Recently we found that bicyclic sugar-shaped compounds that incorporate a bridgehead nitrogen atom with substantial sp^2 character (sp^2 iminosugars) behave as competitive inhibitors of glycosidases with tuneable selectivity patterns.[29–35] Interestingly, sp^2-iminosugar analogues of the natural reducing alkaloid nojirimycin (NJ) bearing lipophilic substituents, such as 5-*N*,6-*O*-(*N*'-octyliminomethylidene)nojirimycin (NOI-NJ, **1**) or its 6-thio derivative (6S-NOI-NJ, **2**; Scheme 1), are anomeric-specific inhibitors of β-glucosidases, including the lysosomal acid β-Glu associated with Gaucher disease.[36] X-ray and isothermal titration calorimetry (ITC) studies on the corresponding complexes with β-glucosidase from the microorganism *Thermotoga maritima* (*Tm*GH1),[37] a glycosidase that belongs to the same clan GH-A that human acid β-Glu in the CAZy classification,[38] indicated a binding mode analogous to that of classical iminosugars. Formation of the enzyme–inhibitor complex was, however, entropically driven, a thermodynamic signature that is unique for this family of sugar mimics. Further X-ray structural studies on the interaction of the 6-amino-6-deoxy-5,6-di-*N*-(*N*'-octyliminomethylidene)nojirimycin analogue (6N-NOI-NJ, **3**; Scheme 1) with recombinant human β-glucocerebrosidase confirmed a binding mode analogous to that previously encountered for NN-DNJ,[39] with additional interactions involving the pseudoanomeric hydroxyl group. The rigid bicyclic framework imposes a restricted orientation of the hydrophobic substituent that is probably responsible for the unprecedented anomeric selectivity.



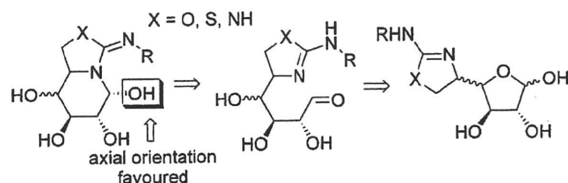
Scheme 1. Chemical structures of *N*-(*n*-nonyl)-deoxynojirimycin (NN-DNJ) and the bicyclic nojirimycin (**1–3**) and galactonojirimycin analogues (**4–6**) prepared in this work. The structures of the parent natural iminosugars nojirimycin (NJ) and galactonojirimycin (GNJ) are also depicted.

The above results warranted further investigation on the potential of bicyclic-NJ analogues as chemical chaperones for mutant GD-associated β-Glu. Here we report a full account of the molecular diversity-oriented approach disclosed for the preparation of this type of sp^2 iminosugars. It allows systematic modifications at the bicyclic skeleton and at the configurational profile. In this work we have focused on derivatives of NJ and of its C-4 epimer (galactonojirimycin, GNJ) bearing an *N*'-octyl substituent, because these structural features appeared most favourable for strong inhibitory activity towards commercial β-glucosidases in preliminary assays. The synthetic strategy is purposely conceived to allow further optimization by acting on the nature of the *N*'-substituent and/or structural modifications at other regions of the molecule.[35] Compounds **1–3** and their GNJ counterparts NOI-GNJ (**4**), 6S-NOI-GNJ (**5**) and 6N-NOI-GNJ (**6**) have been, thus, prepared and evaluated against lysosomal human glycosidases (Figure 1, below). Selected candidates were further investigated as chemical chaperones for Gaucher mutations in comparison with NN-DNJ.

Results

Synthesis

The synthesis of sp^2 iminosugars relies in the capability of nitrogen atoms in pseudoamide functionalities (cyclic isourea, isothiourea and guanidine in the case of the present work) to participate in intramolecular nucleophilic addition reactions to the masked aldehyde group of monosaccharides through the open-chain form (Scheme 2). The resulting hemiaminal derivatives are characterized by a very strong anomeric effect, which determines the axial orientation of the anomeric hydroxyl group and imparts stability. As a result, sp^2 iminosugars exhibit conformational and configurational stability in aqueous solutions; this behaviour is significantly different to that encountered in classical iminosugars such as NJ or GNJ.



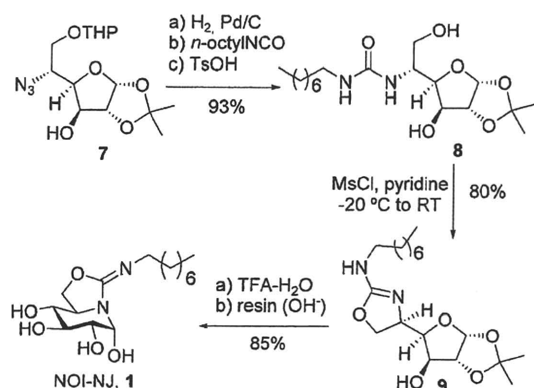
Scheme 2. Retrosynthesis of bicyclic NJ and GNJ derivatives.

We have developed a divergent strategy to introduce molecular diversity in the bicyclic sp^2-iminosugar structure with a relatively low synthetic cost; this strategy involves several key steps: 1) the construction of a five-membered heterocycle between positions C-5 (*N*-substituted) and C-6 (*O*-, *S*- or *N*-substi-

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

tuted for oxazoline, thiazoline and imidazoline derivatives, respectively) in a hexofuranose template and 2) subsequent furanose→piperidine rearrangement of the transient pseudo-C-nucleoside derivative to zip-up the bicyclic skeleton (Scheme 2). In the case of bicyclic NJ analogues, D-*gluco*-configured precursors are requested. The known 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose (**7**),[40] readily accessible from commercial D-glucofuranurono-6,3-lactone, was chosen as a pivotal synthetic intermediate from which **1–3** can be obtained in a limited number of steps.[37,39] The optimized reaction sequence leading to **1** is discussed hereinafter to illustrate the methodology because it represents a significant improvement with respect to the previously reported preparation.[32]

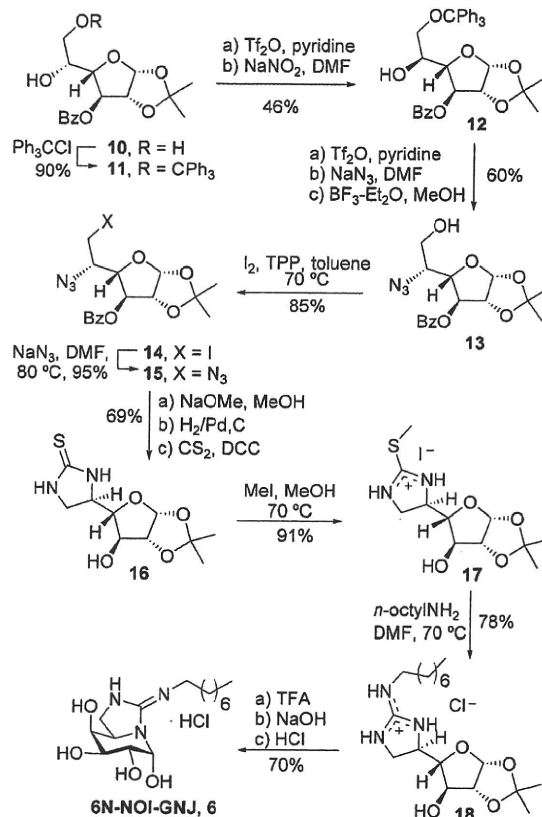
Reduction of the azido group in **7** by catalytic hydrogenation, coupling of the resulting amine with octyl isocyanate and hydrolysis of the tetrahydropyranyl (THP) group with *p*-toluenesulfonic acid (PTSA) afforded the *N'*-octylurea adduct **8** in 93% overall yield (Scheme 3). To close the oxazoline ring, the



Scheme 3. Synthesis of 5-*N*,6-*O*-(*N'*-octyliminomethylidene)nojirimycin (NOI-NJ, **1**).

neighbour primary OH group was transformed into the corresponding methanesulfonate (mesylate) ester, which underwent spontaneous nucleophilic displacement by the urea carbonyl oxygen (→**9**, 80% yield). This reaction pathway was found to be more convenient to build the 2-amino-1,3-oxazoline moiety than the previously reported synthesis through a relatively unstable carbodiimide intermediate,[32] particularly when scaling up the preparation over one gram. Further acid-promoted hydrolysis of the acetal protecting group with TFA–water led to the corresponding reducing glucofuranose derivative which, after repeated co-evaporation of the acid and final neutralization with basic ion-exchange resin, afforded the target *N'*-octyliminomethylidene-NJ derivative **1**.

The synthesis of the C-4 epimers of compounds **1** and **2**, namely the cyclic analogues of GNJ **4** and **5**, followed a similar general approach starting from 3-*O*-acetyl-5-azido-5-deoxy-1,2-*O*-isopropylidene-β-D-galactofuranose[33] as a common intermediate.[37] Attempts to access the previously unreported cyclic guanidine GNJ derivative **6** through the introduction of a second azido group at C-6 in this scaffold by following a strategy similar to that previously demonstrated for the preparation of the cyclic guanidine NJ analogue **3**, were problematic, however. Mesylation, tosylation or trifluorometanesulfonylation (triflation) of the primary hydroxyl was unsuccessful. To avoid acetyl migration under sulfonylation conditions, the 3-*O*-benzoyl derivative **10** was considered as starting material (Scheme 4).[41] The first azido group was introduced by trityla-



Scheme 4. Synthesis of 6-amino-6-deoxy-5,6-di-*N*-(*N'*-octyliminomethylidene)galactonojirimycin (6N-NOI-GNJ, **6**).

tion at O-6 (→**11**) and double inversion at C-5 through a reaction sequence that involves triflation, S_N2 displacement with sodium nitrite and hydrolysis of the resulting nitrite ester to give the D-*altro* derivative **12** (46%). A new triflation cycle followed by nucleophilic displacement by azide anion and removal of the transient trityl protecting group by treatment with boron trifluoride–diethyl ether complex afforded the D-*galacto* hydroxy-azide **13** in 60% overall yield. Deceivingly, sulfonylation of O-6 in **13** was likewise unsuccessful. Alternatively, Garegg's iodination methodology[42] (iodine/triphenylphosphine (TPP)/imidazole/toluene) was attempted. Conditions were optimized to prevent concomitant Staudinger reaction[43] of TPP with the already installed azido group by preforming the active phosphonium species; this yielded the 6-iodo derivative **14** in 85% yield. We were delighted to confirm that compound **14** underwent displacement by sodium azide (→**15**) in almost

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

quantitative yield. Reduction and thiocarbonylation of the resulting diamine with carbon disulfide-dicyclohexylcarbodiimide (DCC) afforded the corresponding imidazolidine-2-thione **16** (69% yield), which was transformed into the requested cyclic guanidine by treatment with methyl iodide (→**17**, 91%) and displacement of the methylthio group with *n*-octylamine (→**18**). Deprotection of the acetal functionality and treatment with sodium hydroxide gave the target 6-amino-6-deoxy-5,6-di-*N*-(*N*′-octyliminomethylidene)-GNJ derivative, characterized as the corresponding hydrochloride salt **6**, in 70% yield.

Glycosidase inhibition profiling

Compounds **1–6** were first assayed against a panel of commercial glycosidases with differing substrate specificity (Table 1). Strong inhibitory activity, with inhibition constant (K_i) values in the low µM to nM range, was observed exclusively for enzymes that hydrolyze β-D-glucopyranosides, even in the case of compounds **4–6**, which have structural resemblance to D-galactose. Although β-glucosidases are frequently not very exigent regarding configuration at the position equivalent to C-4 in the natural substrates,[44] the higher inhibition potency of **4–6** as compared with **1–3** for the enzymes from almonds and bovine liver (about one order of magnitude), belonging to the same clan GH-A that human acid β-Glu, is remarkable and prompted us to retain the galactose mimics for further studies in spite of their a priori mismatching configuration. When evaluated against the human enzyme, the GNJ derivatives also showed strong inhibitory activity (Table 1), though 1.2 to 4-fold weaker than the corresponding bicyclic NJ analogues. No apparent chaperone activity on mutant Gaucher disease-associated acid β-Glu mutants was observed, however, for compounds **4** and **6**.

Table 1. Inhibitory activities (K_i, µM, for the enzymes from almonds, bovine liver and *Penicillium decumbes*; IC_{50}, µM, for the human enzyme) for bicyclic NJ (**1–3**) and GNJ derivatives (**4–6**).[a]

Enzyme[b]	1	2	3	4	5	6
β-glucosidase (almond)	1.9	0.76	0.42	0.019	0.023	0.16
β-glucosidase (bovine liver, cytosolic)	2.7	3.7	35	0.052	0.042	0.04
naringinase (*P. decumbes*)	0.84	0.23	0.18	54	37	44
acid β-Glu (human)	5.6	3.5	4.0	80.8	7.1	5.2

[a] The inhibition was competitive in all cases; errors in K_i values are ±10%. [b] No inhibition detected at 1 mM for α-glucosidase (yeast), α-galactosidase (green coffee), β-galactosidase (*E. coli*), isomaltase (yeast), amyloglucosidase (*Aspergillus niger*), α-mannosidase (Jack beans) and β-mannosidase (*Helix pomatia*). Compounds **1–3** inhibited trehalase (pig kidney) with K_i values in the range 13-180 µM).

To test the selectivity profile of sp² iminosugars towards lysosomal glycosidases, we next checked the effects of the cyclic NJ derivatives **1–3** and the GNJ derivative **5** on lysosomal enzyme activities in lysates from normal human fibroblasts (Table 2). Compounds **1–3**, which have a configurational pattern that matches that of D-glucose, showed strong inhibition of acid β-Glu and no or low inhibition of other lysosomal enzymes, such as α-glucosidase, α-galactosidase, β-galactosidase and β-hexosaminidase. Surprisingly, compound **5**, formally a mimic of D-galactose, was a much stronger inhibitor of acid β-Glu than of lysosomal β-galactosidase. In stark contrast, the iminosugar NN-DNJ showed no anomer specificity (Table 2), and behaved as a strong inhibitor of both lysosomal α-glucosidase and acid β-Glu, in agreement with literature reports.[24–26]

Table 2. Inhibitory activities (IC_{50}, µM) on lysosomal human α-glucosidase (α-Glu), β-glucosidase (β-Glu), α-galactosidase (α-Gal), β-galactosidase (β-Gal) and β-hexosaminidase (β-Hex) in lysates from human control fibroblasts for bicyclic NJ (**1-3**) and GNJ derivatives (**5**) in comparison with NN-DNJ.[a]

Enzyme	1	2	3	5	NN-DNJ
α-Glu	n.i.[b]	n.i.	n.i.	n.i.	4.4
β-Glu	3.8	1.3	14.4	1.4	0.9
α-Gal	n.i.	n.i.	n.i.	n.i.	n.i.
β-Gal	>100	n.i.	n.i.	>100	n.i.
β-Hex	>100	>100	n.i.	>100	n.i.

[a] Each data point represents the mean of triplicate determinations obtained in a single experiment. [b] n.i., no inhibition detected at 0.1 mM.

We further studied the inhibitory activity of these compounds on mutant acid β-Glu fibroblasts including F213I/F213I, N370S/N370S and L444P/L444P in addition to normal fibroblasts (Table 3). As a general trend, the IC_{50} values on F213I/F213I and L444P/L444P mutants were similar to those measured on normal acid β-Glu, whereas those on the N370S/N370S mutant were about one order of magnitude higher (Table 3).

Table 3. Inhibitory activities (IC_{50}, µM) on lysosomal human β-glucosidase (β-Glu) in lysates from human normal fibroblasts (H37) and F213I/F213I, N370S/N370S, L444P/L444P mutant fibroblasts for bicyclic NJ (**1-3**) and GNJ derivatives (**5**) at pH 5.2 and 7.0, in comparison with NN-DNJ.[a]

Fibroblasts	1	2	3	5	NN-DNJ
H37 (pH 5.2)	3.8	1.3	14.4	1.4	0.88
H37 (pH 7.0)	0.58	0.28	1.7	0.45	0.54
F213I/F213I (pH 5.2)	8.7	0.56	19.4	4.7	0.57
F213I/F213I (pH 7.0)	0.10	0.128	1.0	0.28	0.20
N370S/N370S (pH 5.2)	30.2	12.4	279	28.9	4.6
N370S/N370S (pH 7.0)	2.6	0.26	2.8	3.7	0.62
L444P/L444P (pH 5.2)	5.1	0.9	24.5	5.2	0.78
L444P/L444P (pH 7.0)	0.62	0.18	0.91	0.36	0.46

[a] Each value represents the mean of three independent determinations.

Ideal chaperones should bind to the β-Glu in the ER for assisting folding and trafficking and dissociate in the lysosome. Compounds that exhibit higher inhibitory activity at the neutral pH of ER than at the acidic pH of the lysosome are, therefore, better chemical chaperone candidates. Data collected in Table 3 indicate that the sp² iminosugars **1–3** and **5** are about one order of magnitude stronger inhibitors of β-Glu at pH 7.0 than at pH 5.2 for the normal enzyme and all the assayed mutants. In the case of the iminosugar NN-DNJ this feature was

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

only observed for the N370S/N370S β-Glu mutant. A similar dependence of the inhibitory activity from the pH for this particular mutant has been previously reported for other compounds, including the 1-azasugar isofagomine,[45] which is currently in phase II clinical trials for chaperone-mediated therapy of type 1 Gaucher disease.[4]

Chaperone activity and cytotoxicity in human fibroblasts

In our initial experiments, all the bicyclic sp^2 iminosugars prepared in this work (**1–6**) were incubated with GD fibroblasts for four days followed by in situ cellular assay for acid β-Glu activity. Only compounds **1–3** and **5** exhibited obvious chaperone activities and were further profiled (Figure 1). To evaluate the enzyme activity enhancements, three control cell lines (H8, H22 and H37; See Supporting Information) and seven lines of GD cells (F213I/F213I, G202R/L444P, N188S/G193W, N370S/N370S, F213/L444P, L444P/RecNcil, L444P/L444P) were cultured with the individual inhibitors, as well as with NN-DNJ, at various concentrations (0, 0.3, 1, 3, 10 and 30 μM) for four days. The in situ cell lysosomal β-Glu assay was then performed. For F213I/F213I mutant cells, treatment with low (0.3 and 1 μM) and high (3-30 μM) concentrations of bicyclic sp^2 iminosugars resulted in 40–50% and 70–100% increase of acid β-Glu activity respectively. NN-DNJ only showed a 50% increase at low concentrations. In the case of the G202R/L444P mutation, treatment with low and high concentrations of bicyclic sp^2 iminosugars resulted in 60–75% and 125–175% increases of the acid β-Glu activity respectively, while NN-DNJ showed no effect. In N188S/G193W mutant cells, treatment with low and high concentrations of the bicyclic sp^2 iminosugars resulted in 15–20% and 25–45% increases of the acid β-Glu activity respectively. NN-DNJ showed 55% (low) and 25% (high) increases. In N370S/N370S mutant cells, treatment with low and high concentrations of bicyclic sp^2 iminosugars led to 60% and 40–165% enhancements of the acid β-Glu activity, respectively. NN-DNJ showed 240% (low) and 300% (high) increases. In F213I/L444P mutant cells, treatment with low and high concentrations of bicyclic sp^2 iminosugars resulted in 30–40% and 40–120% increases of the lysosomal β-Glu activities, respectively, while NN-DNJ showed no effect. None of the assayed compounds, including NN-DNJ, had an effect in the L444P/RecNcil and L444P/L444P mutant cells and the three control cell lines (Figure 1).

An additional ten day time-course analysis of chaperone activities was carried out at the optimal inhibitor concentration chosen from the results presented above, namely 30 μM for compounds **1–3** and **5** and 1 μM for NN-DNJ (Figure 2). For the F213I/F213I and N370S/N370S cells, the acid β-Glu activity increased in a time-dependent manner in the presence of the bicyclic sp^2 iminosugars, reached a peak on days 3 to 5, then decreased slightly and came to a plateau at about 40–100% increase in the last few days. The effect of NN-DNJ was more pronounced in N370S/N370S mutants, but had a much lower effect in F213I/F213I cells. When cells were deprived of the chemical chaperones on day 4, the activity gradually decreased to the basal level within one to three days (Figure 2). No effects were observed in the L444P/L444P

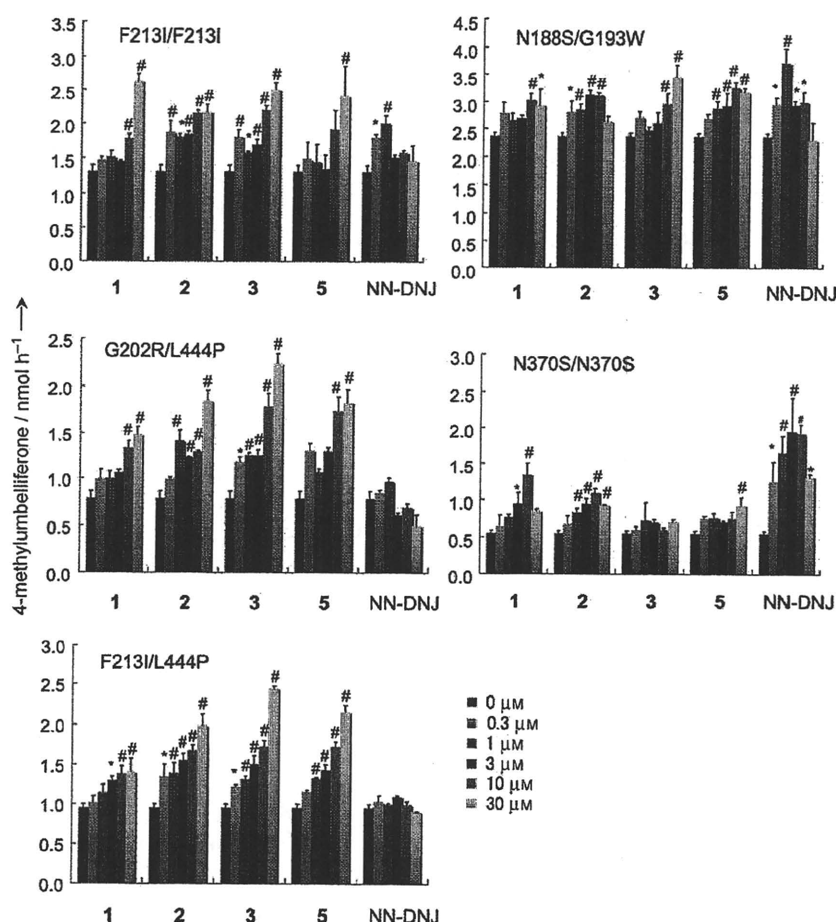


Figure 1. Chaperone activities of bicyclic NJ (**1–3**) and GNJ derivatives (**5**) and NN-DNJ on mutant β-Glu in fibroblasts (in situ cell enzyme assay). Cells were cultured for four days in the absence or presence of increasing concentrations of the compounds. Lysosomal β-Glu activity was estimated in intact cells as described in the Experimental Section. Each bar represents the mean ±S.E.M. of 3 determinations each done in triplicate. *$p<0.05$ significant, #$p<0.01$ highly significant, statistically different from the values in the absence of the compound (t-test). None of the assayed compounds, including NN-DNJ, had an effect in the L444P/RecNcil and L444P/L444P mutant cells and the three control cell lines (see Figure S1 in the Supporting Information).

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

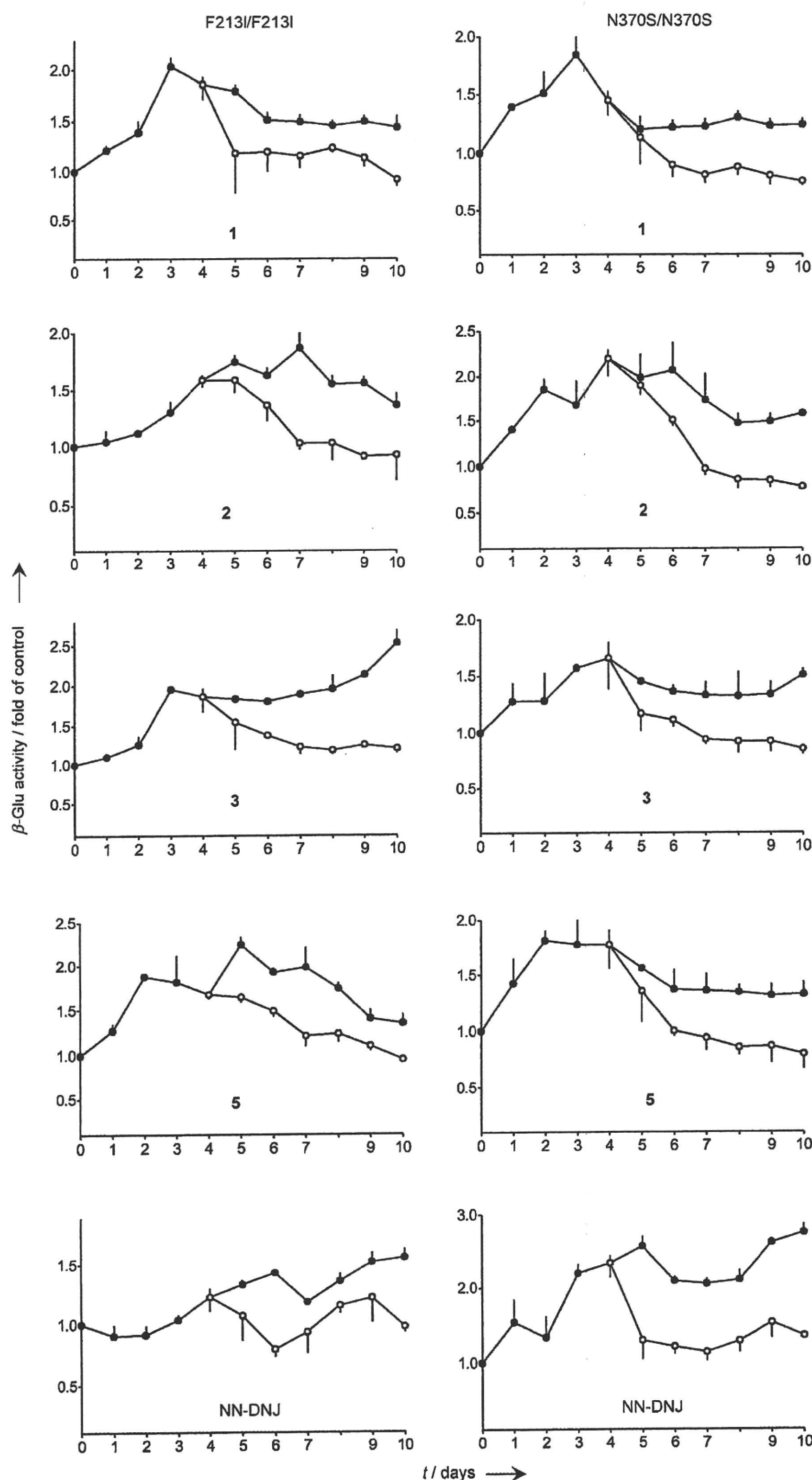


Figure 2. Chaperone activities of bicyclic NJ (1–3) and GNJ derivatives (5) and NN-DNJ on mutant β-Glu in fibroblasts (time course). Cells were cultured in the presence of the optimal concentration of each compound (30 μM for 1–3 and 5; 1 μM for NN-DNJ) of the corresponding bicyclic nojirimycin for up to 10 days (●). A subset of cells was cultured with chaperones for four days, washed and further cultured without the drug for six days (○). β-Glu activity in cells was determined at the indicated time in triplicate.

cell line for any of the assayed compounds. In the normal H37 cells, β-Glu activity seemed to increase slightly in the presence of compounds **1–3** and **5** during the first few days, then it dropped back to the basal level (see Supporting Information, Figure 2S). No change in the presence of NN-DNJ was observed.

To evaluate the cytotoxicity of compounds **1–3** and **5**, we cultured normal and F213I/F213I, N370S/N370S and L444P/L444P mutant human fibroblasts in the presence of various concentrations of each sp^2 iminosugar (0, 0.3, 1, 3, 10 and 30 μM) for four days, and then assayed the cell viabilities. NN-DNJ was included in this study for comparative purposes. The results showed that the viabilities of all four fibroblast types were unchanged after incubation with the inhibitors, even at the maximum concentration of 30 μM.

Discussion

Chemical chaperone therapy is a promising approach for the treatment of lysosomal storage disorders because of its potential for simple oral administration, penetration of the blood–brain barrier and low cost. Chemical chaperone activity in Gaucher disease (GD) mutants has been reported for several *N*-substituted derivatives of the carbasugar β-valienamine[21,46] and of the iminosugar 1-deoxynojirimycin (DNJ),[26,27,47,48] among which the *N*-(*n*-nonyl) derivative (NN-DNJ). Recently, we found that bicyclic sp^2-iminosugar analogues of the parent alkaloid nojirimycin (NJ), with structure of 5-*N*,6-*X*-(*N'*-alkyliminomethylidene)nojirimycin (X represents O, S or N) behaved as very selective competitive inhibitors of β-glucosidases, including human β-glucocerebrosidase. Preliminary X-ray structural studies of the corresponding enzyme-inhibitor complexes sup-

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

ported that these compounds bind at the active site of β-glucosidases in a manner that is analogous to that previously encountered for iminosugars such as NN-DNJ.[49] The remarkable selectivity for β-Glu was ascribed to the rigidity of the bicyclic skeleton.[39] Since the synthesis of sp^2 iminosugars is readily amenable to molecular diversity-oriented strategies and biological activity optimization, evaluation of their potential as chemical chaperones for the treatment of Gaucher disease was very appealing.

Prior to this work, a broad screening of bicyclic sp^2-iminosugar structures with different configurational patterns and *N'*-substituents was carried out against a panel of commercial glycosidases.[36] Compounds with hydroxylation profiles analogous to D-glucose (NJ derivatives) or D-galactose (4-*epi*-NJ; galactonojirimycin, GNJ derivatives) and bearing long chain linear *N'*-alkyl substituents were found to be promising candidates in view of their strong and selective inhibition of clan GH-A β-glucosidases to which human acid β-Glu belongs. On those grounds, the *N'*-octyliminomethylidene NJ and GNJ derivatives **1–3** and **4–6**, respectively, were initially considered for additional studies. In a next set of experiments, the bicyclic NJ derivatives **1–3** were found to enhance the activity of several GD β-glucocerebrosidase mutants, whereas compound **5** was the only GNJ analogue that exhibited obvious chaperone activities.

The chaperone effect of compounds **1–3** and **5** was further evaluated and compared with the effect of NN-DNJ in human GD cells by using an in situ cell enzyme assay. The bicyclic sp^2 iminosugars exhibited chaperone activities in five of seven GD cell lines assayed, namely the F213I/F213I, N188S/G193W, G202R/L444P, N370S/N370S and F213I/L444P mutations, whereas they were ineffective in cells with the L444P/L444P and L444P/RecNcil mutations.

The rationale behind the selection of sp^2-iminosugar glycomimetics as chemical chaperone candidates was based on the generally accepted theory that effective competitive inhibitors are more likely to show a potent chaperone effect. This is particularly true for compounds that are more potent β-Glu inhibitors at neutral than at acidic pH values, which emulate the scenarios encountered in the ER and the lysosome, respectively. Our results indicate, however, that the chaperone activity is not directly related to the inhibitory efficiency. Thus, compounds **1–3** and **5**, as well as NN-DNJ, exhibited good inhibitory properties on human normal acid β-Glu and no toxicity in fibroblasts at the range of concentrations tested. They also inhibited the F213I/F213I, N370S/N370S and L444P/L444P mutant β-Glu proteins, with IC_{50} values that showed mutation dependence. Good chaperone activities were achieved on the N370S/N370S mutant in spite of IC_{50} values that were five- to 20-fold higher than those measured on normal and other mutant acid β-Glu. In the case of the L444P/L444P and F213I/F213I mutant acid β-Glu, the IC_{50} values were similar to those measured for the normal enzyme, but the compounds showed no effect on the L444P/L444P whereas they exhibited potent chaperone activity on the F213I/F213I mutant enzyme. Altogether, these results underline that the chaperone activity of a given compound does not solely rely on its binding affinity towards the target enzyme, but that properties such as membrane permeability, metabolism, intracellular localization and the structure of the protein upon binding to the chaperone, among others, might play a decisive role.

No strong variations in the β-glucosidase inhibitory activity were encountered between bicyclic sp^2 iminosugars differing in the endocyclic heteroatom at the five-membered ring in the bicyclic skeleton (O for **1**, S for **2** and **5** or N for **3**). However, the cyclic guanidine derivative **3** was found to be a tenfold weaker inhibitor as compared with the cyclic isourea (**1**) or the cyclic isothiourea derivatives (**2** and **5**) in cases in which the acid β-Glu activities were determined in lysates from human normal and mutant fibroblasts in the presence of increasing concentrations of the compounds. Previous structural and thermodynamic studies suggested that desolvation of the inhibitor probably represents an important contribution to the binding free energy in these family of glycomimetics,[37] a process that is expected to be less favourable for the more basic guanidine functionality. Nevertheless, these differences were not paralleled in the chaperone experiments. Thus, while compound **2** was a 40-fold stronger inhibitor of the F213I/F213I mutant acid β-Glu than **3**, the enzyme activity enhancement achieved with the later compound was slightly higher. It seems that the ratio between the binding affinity to β-Glu in the ER and the lysosome, which can be considered to be proportional to the corresponding ratio of the IC_{50} values at 7.0 and 5.2, is a more critical parameter than the individual inhibition potency at either of those cellular compartments. The fact that NN-DNJ is a less efficient chemical chaperone as compared with the sp^2 iminosugars for the F213I/F213I mutant is also consistent with this observation.

The primary goal of this work was the evaluation of the potential of sp^2 iminosugars as chemical chaperones for a variety of Gaucher mutations in comparison with the classical iminosugar NN-DNJ, already in preclinical studies.[4] It must be stressed, however, that there is an increasing number of reports on new chaperones for GD and that a broader comparative evaluation of the therapeutic potential for different structures would be highly desirable. Synthetic cost and pharmacokinetic properties are also critical questions regarding drug development. The hydrophilic 1-azasugar isofagomine, a specific and potent β-Glu inhibitor (IC_{50} 50 nM), is a molecule of reference at this respect. It can increase the N370S β-Glu activity about two- to threefold by enhancing its cellular folding and trafficking.[45,50,51] Recently, Zheng et al.[52] used quantitative high-throughput screening (qHTS) to find three structural series of potent, selective, nonsugar β-Glu inhibitors. Three compounds from these series, with IC_{50} values 0.031, 0.103 and 0.33 μM, respectively, increased β-Glu activity by 40–90% in N370S mutant cells. By HTS of the 50000-compound Maybridge library, Tropak et al.[23] identified two noncarbohydrate-based inhibitory compounds with IC_{50} values of 5 and 8 μM respectively, which can increase β-Glu activity 50–150% in N370S or F213I GD fibroblasts. This results are, actually, quite similar to ours (40–100% increase for F213I and 40–165% increase for N370S).

Studies on genotype–phenotype relationships in human GD patients have shown that except for the N370S mutations, which are exclusively associated with type 1, the nonneurono-

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

pathic form of GD, the other three mutations with positive responses to the bicyclic NJ derivatives **1–3** and **5** (N188S, G202R, F213I), can be associated with types 2 or 3, neuronopathic forms of the disease,[53–55] which are not responsive to enzyme replacement therapy because the recombinant enzyme cannot cross the blood–brain barrier. We propose the sugar mimics in this study mainly for chemical chaperone therapy of neuronopathic forms of Gaucher disease. Preliminary confocal microscopy studies carried out with a fluorescently labeled derivative of the bicyclic NJ derivative **1** indicated enhanced trafficking of the mutant protein to the lysosome in Gaucher patient cells. Compound **1** also evidenced good properties regarding oral availability and ability to enhance the β-Glu activity in tissues, including brain, as well as the lack of acute toxicity at high doses in normal mice (all procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Tottori University; data not shown).

As far as we are aware, no glycomimetic-type chaperones, including isofagomine and the sp^2 iminosugars here described, have been reported for the L444P GD mutation. This mutation is not located in the catalytic domain of β-Glu and, consequently, is not sensitive to active site-directed chemical chaperones.[27,50] Mu et al.[56] found that inhibition of L-type Ca^{2+} channels by diltiazem or verapamil can partially restore the L444P mutant β-Glu homeostasis in GD fibroblasts, and they suggested that this effect might be due to a modest up-regulation of a subset of intrinsic molecular chaperones which are essential for the maintenance of cellular protein homeostasis. Recent results by Rigat and Mahuran pointed to a classical glucocerebrosidase chemical chaperone behaviour for diltiazem.[57] However, neither diltiazem nor verapamil were found to enhance mutant enzyme activity in homozygous L444P Gaucher cells. A better understanding of the mechanism of action of intrinsic molecular chaperones would provide us clues to develop nonactive site directed chaperones to broaden the potential of chaperone therapy for GD.

Conclusions

In this study, we have developed a general approach for the synthesis of bicyclic sp^2-iminosugar glycosidase inhibitors that is very well-suited for molecular diversity-oriented strategies. Higher inhibitory selectivities towards β-glucosidase, including the lysosomal human β-glucocerebrosidase, as compared with the structurally related iminosugar NN-DNJ have been achieved. Interestingly, sp^2 iminosugars also were more efficient as chemical chaperones than NN-DNJ for some Gaucher disease mutations. Differences in chaperone activity seem to be related, though not solely, to variations on binding affinity of the glycomimetic to the mutant protein at neutral and acidic pH, a situation that emulates the environment at the ER and the lysosome, respectively.

The body of results presented here stresses the necessity of a thorough mutation-based profiling of chemical chaperones to evaluate their therapeutic potential. The future of chaperone therapy for the treatment of GD and other lysosomal storage disorders depends on our capacity to enlarge the battery of compounds that can promote mutant glycosidase activity enhancements in a highly specific and efficient manner, including active site- and nonactive site-directed chemical chaperones. The development of flexible synthetic strategies suitable for the generation of focused libraries of glycomimetics is therefore mandatory. Physicians could then choose the most suitable chaperone for an individual patient upon his/her mutation. When using active-site chaperones to treat GD, determination of the therapeutically useful doses is critical because high doses would inhibit the acid β-Glu activity heavily and might aggravate the symptoms of GD. While very strong inhibitors can be effective at low doses, moderate inhibitors would allow the use of higher doses. Rather than a very potent inhibitory potency, an ideal active-site chaperone should have a high ratio between the chaperone and inhibition activities.

We have previously demonstrated the effectiveness of chemical chaperone therapy in G_{M1}-gangliosidosis,[58,59] Gaucher disease[20,21] and Fabry's disease.[60] Notably, we recently found that chaperone therapy can prevent neurological deterioration in a G_{M1}-gangliosidosis model mouse expressing R201C mutant human β-galactosidase.[61] In the case of the bicyclic NJ derivatives studied in this work our findings in cultured cells, although indicative of potent chaperone effects for specific GD mutations, do not allow us to predict their pharmacological chaperone efficiency in whole animals and, hence, which structure has the best therapeutic value. We are currently developing a transgenic mouse model that lacks the endogenous wild-type enzyme and expresses instead a mutant human acid β-Glu. The in vivo results will be reported in due curse.

Experimental Section

Materials and methods: The sp^2 iminosugars **1–6** were synthesized in our laboratories and their purity was confirmed by spectroscopic techniques and combustion analysis. For the preparation of **2–5** the previously reported reaction sequences were followed with slight modification.[37,39] For compound **1**,[32] an advantageous route has been developed, while compound **6** has not been previously reported. NN-DNJ was obtained from United States Biological (Marblehead, MA, USA). Stock solutions of the compounds were prepared in H_2O at 3 mM and stored at −20 °C. Reagents and solvents were purchased from commercial sources and used without further purification. Optical rotations were measured at 22 °C in 1 cm or 1 dm tubes. ^1H (and ^{13}C) NMR spectra were recorded at 300 (75.5) MHz. 2D COSY and HMQC experiments were carried out to assist in signal assignment. TLC was performed with E. Merck precoated TLC plates, silica gel 30F-245, with visualization by UV light and by charring with H_2SO_4 (10 %) or cerium(IV) sulfate (0.2 % *w/v*)/ammonium molybdate (5 % *w/v*) in H_2SO_4 (2 M) or ninhydrin (0.1 %) in EtOH. Column chromatography was carried out with Silica Gel 60 (E. Merck, 230–400 mesh). In the FABMS spectra, the primary beam consisted of Xe atoms with a maximum energy of 8 keV. The samples were dissolved in *m*-nitrobenzyl alcohol or thioglycerol as the matrixes and the positive ions were separated and accelerated over a potential of 7 keV. NaI was added as cationizing agent. Dulbecco's Modified Eagle's Medium (DMEM) and foetal

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

bovine serum (FBS) were obtained from Life Technologies Inc. (Rockville, MD, USA).

5-Deoxy-1,2-*O*-isopropylidene-5-(*N*′-octylureido)-α-D-glucofuranose (8): A solution of 5-azido-5-deoxy-1,2-*O*-isopropylidene-6-*O*-tetrahydropyranyl-α-D-glucofuranose[40] (**7**, 3.0 g, 9.1 mol) in MeOH (50 mL) was hydrogenated at atmospheric pressure for 1 h using Pd (10%)/C (900 mg total) as catalyst. The suspension was filtered through Celite and concentrated. The resulting residue was dissolved in MeOH (100 mL), octyl isocyanate (2.5 mL, 1.0 equiv) was added and the reaction mixture was stirred at RT for 12 h. After conventional work up, the crude material was dissolved in CH_2Cl_2/MeOH (1:1, 120 mL) and *p*-toluenesulfonic acid (2.6 mmol) was added. The reaction mixture was stirred for 2 h at RT, diluted with CH_2Cl_2 (60 mL), washed with saturated, aqueous $NaHCO_3$ (2× 50 mL), dried ($MgSO_4$) and concentrated. The resulting residue was purified by column chromatography (EtOAc/petroleum ether 1:1 → 2:1). Yield: 4.06 g (93%); R_f=0.45 (EtOAc/petroleum ether 3:1); $[\alpha]_D$=+41.7 (*c*=1.2 in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ=5.85 (d, $J_{1,2}$=3.6 Hz, 1H; H-1), 5.80 (brs, 1H; OH), 5.33 (d, $J_{5,NH}$=8.1 Hz, 1H; NH), 5.00 (t, $^3J_{NH,CH_2}$=5.4 Hz, 1H; N′H), 4.51 (d, 1H; H-2), 4.02 (d, $J_{3,4}$=1.8 Hz, 1H; H-3), 3.96 (dd, $J_{4,5}$=10.2 Hz, 1H; H-4), 3.92 (dd, $J_{6a,6b}$=11.2 Hz, $J_{5,6a}$=2.6 Hz, 1H; H-6a), 4.84 (m, 1H; H-5), 3.67 (dd, $J_{5,6b}$=2.6 Hz, 1H; H-6b), 3.07 (m, 2H; CH_2N), 1.43, 1.25 (2s, 6H; CMe_2), 1.42 (m, 2H; CH_2CH_2N), 1.21 (m, 10H; CH_2), 0.81 (t, $^3J_{H,H}$= 6.4 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=159.1 (CO), 111.5 (CMe_2), 105.1 (C-1), 84.6 (C-2), 80.3 (C-4), 74.1 (C-3), 62.3 (C-6), 49.2 (C-5), 40.7 (CH_2N), 31.8, 30.1, 29.3, 29.2, (CH_2), 26.9, 26.7 (CMe_2), 26.0, 22.7 (CH_2), 14.1 (CH_3); FABMS: *m/z* 397 $[M+Na]^+$, 375 $[M+H]^+$; HRCIMS: calcd for $C_{18}H_{35}N_2O_6$: 375.2495, found 375.2487.

5-Amino-5-deoxy-1,2-*O*-isopropylidene-5-*N*,6-*O*-(*N*′-octyliminomethylidene)-α-D-glucofuranose (9): Methanesulfonic chloride (0.38 mL, 4.85 mmol, 1.1 equiv) was added to a solution of the corresponding ureido derivative **8** (1.65 g, 4.41 mmol) in pyridine (44 mL) at −20 °C, under Ar atmosphere. The reaction mixture was stirred for 12 h and allowed to warm to RT. Then, the solvent was removed under reduced pressure and the resulting residue was coevaporated several times with toluene. The residue was dissolved in CH_2Cl_2, filtered, concentrated, and purified by column chromatography (EtOAc/EtOH 20:1 and EtOAc/EtOH/H_2O 45:5:3). Yield: 1.26 g (80%); $[\alpha]_D$=+11.3 (*c*=1.5, CH_2Cl_2); R_f=0.25 (EtOAc/EtOH/H_2O 45:5:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ=6.63 (brs, 1H; OH), 5.86 (d, $J_{1,2}$=3.5 Hz, 1H; H-1), 4.62 (m, 1H; H-6a), 4.51 (d, 1H; H-2), 4.49 (dd, $J_{6a,6b}$=9.8 Hz, $J_{5,6a}$=3.4 Hz, 1H; H-6a), 4.37 (m, 1H; H-5), 4.33 (d, $J_{3,4}$=3.1 Hz, 1H; H-3), 4.02 (dd, $J_{4,5}$=8.3 Hz, 1H; H-4), 3.18 (t, $^3J_{H,H}$=7.3 Hz, 2H; CH_2N), 1.51 (m, 2H; CH_2CH_2N), 1.42, 1.24 (2s, 6H; CMe_2), 1.23 (m, 10H; CH_2), 0.81 (t, $^3J_{H,H}$=6.9 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=161.9 (CN), 111.9 (CMe_2), 105.5 (C-1), 85.6 (C-2), 82.5 (C-4), 73.6 (C-3), 70.7 (C-6), 56.1 (C-5), 42.9 (CH_2N), 31.7, 29.4, 29.2, 29.1 (CH_2), 27.0, 26.6 (CMe_2), 22.6 (CH_2CH_3), 14.1 (CH_3); CIMS: *m/z* 357 $[M+H]^+$; HRCIMS: calcd. for $C_{18}H_{33}N_2O_5$: 357.2389, found 357.2389.

5-*N*,6-*O*-(*N*′-Octyliminomethylidene)nojirimycin (NOI-NJ, 1): The D-glucofuranose precursor **9** (1.09 g, 3.06 mmol) was treated with TFA/H_2O (9:1, 10 mL) for 2 min at 0 °C, concentrated under reduced pressure, coevaporated several times with water, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography (MeCN→MeCN/H_2O 50:1→MeCN/H_2O 10:1) to give **1** (823 mg, 85%) as an amorphous solid. R_f=0.39 (MeCN/H_2O/NH_4OH 10:1:1); $[\alpha]_D$=−5.0 (*c*=1.0 in H_2O); ^{1}H NMR (500 MHz, D_2O): δ=5.74 (d, $J_{1,2}$=3.8 Hz, 1H; H-1), 5.22 (t, $J_{6a,6b}$= $J_{5,6a}$=8.9 Hz, 1H; H-6a), 4.90 (t, $J_{5,6b}$=8.9 Hz, 1H; H-6b), 4.45 (dt, $J_{4,5}$=9.5 Hz, 1H; H-5), 3.95 (t, $J_{2,3}$=$J_{3,4}$=9.5 Hz, 1H; H-3), 3.83 (dd, 1H; H-2), 3.80 (t, 1H; H-4), 3.57 (td, $^2J_{H,H}$=7.1 Hz, $^3J_{H,H}$=4.2 Hz, 2H; CH_2N), 1.53 (m, 2H; CH_2CH_2N), 1.24 (m, 10H; 5 CH_2), 0.87 (t, 3H; CH_3); ^{13}C NMR (75.5 MHz, D_2O): δ=158.7 (CN), 74.9 (C-1), 73.9 (C-2), 73.0 (C-4), 71.9 (C-3), 70.9 (C-6), 56.2 (C-5), 43.2 (CH_2N), 31.2, 28.4, 28.2, 25.8, (5CH_2), 28.3 (CH_2CH_2N), 22.1 (CH_2CH_3), 13.5 (CH_3). FABMS: *m/z* 317 $[M+H]^+$; elemental analysis calcd (%) for $C_{15}H_{28}N_2O_5$: C 56.94, H 8.92, N 8.85; found: C 56.67, H 8.88, N 8.74.

3-*O*-Benzoyl-1,2-*O*-isopropylidene-6-*O*-trityl-α-D-galactofuranose (11): Trityl chloride (6.5 g, 23 mmol, 1.5 equiv) and DMAP (376 mg, 3.1 mmol, 0.2 equiv) were added to a solution of 3-*O*-benzoyl-1,2-*O*-isopropylidene-α-D-galactofuranose[41] (**10**, 5 g, 15.4 mmol) in CH_2Cl_2 (9.5 mL) and pyridine (25 mL). The solution was stirred at 40 °C under Ar for 18 h, then poured into ice water (10 mL) and the aqueous phase was extracted with CH_2Cl_2 (2×25 mL). The combined organic extracts were washed with iced aqueous AcOH (10%, 2×15 mL), saturated aqueous $NaHCO_3$ (15 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (EtOAc/petroleum ether 1:4→1:3) to give **11** (7.8 g, 90%). R_f=0.35 (EtOAc/petroleum ether 1:3); $[\alpha]_D$=14.6 (*c*= 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ=8.00–7.16 (m, 20H; Ph), 5.96 (d, $J_{1,2}$=4.0 Hz, 1H; H-1), 5.32 (d, $J_{3,4}$=2.5 Hz, 1H; H-3), 4.73 (d, 1H; H-2), 4.41 (dd, $J_{4,5}$=6.5 Hz, 1H; H-4), 4.04 (m, 1H; H-5), 3.31 (dd, $J_{6a,6b}$=10.0 Hz, $J_{5,6a}$=5.0 Hz, 1H; H-6a), 3.27 (dd, $J_{5,6b}$= 5.5 Hz, 1H; H-6b), 2.69 (d, $J_{OH,5}$=5.0 Hz, 1H; OH), 1.57, 1.32 (2s, 6H; CMe_2); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ=165.5 (CO), 143.8–127.0 (Ph), 113.1 (CMe_2), 105.5 (C-1), 86.9 (CPh_3), 86.1 (C-4), 85.3 (C-2), 78.7 (C-3), 69.9 (C-5), 64.9 (C-6), 26.8, 26.1 (CMe_2); IR (KBr): ν_{max}= 3412, 3047, 2991, 1720, 1633, 1490, 1379, 1267, 1069 cm^{-1}; FABMS: *m/z* 589 $[M+Na]^+$; elemental analysis calcd (%) for $C_{35}H_{34}O_7$: C 74.19, H 6.05; found: C 73.92, H 5.84.

3-*O*-Benzoyl-1,2-*O*-isopropylidene-6-*O*-trityl-β-L-altrofuranose (12): Pyridine (1.38 mL) and trifluoromethanesulfonic anhydride (2.16 mL, 13.1 mmol) were added to a solution of **11** (4.93 g, 8.72 mmol) in CH_2Cl_2 (23 mL) at −25 °C under nitrogen. The reaction mixture was allowed to reach RT and further stirred for 30 min. The mixture was diluted with CH_2Cl_2 (15 mL), washed with iced saturated aqueous $NaHCO_3$ (15 mL), dried ($MgSO_4$), and concentrated. The resulting triflate ester was dissolved in DMF (22 mL), $NaNO_2$ (2.71 g, 39.3 mmol) was added and the reaction mixture was stirred at RT under Ar for 18 h. The solvent was removed under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (15 mL) and washed with water (8 mL). The organic extract was dried ($MgSO_4$), concentrated and the residue was purified by column chromatography (EtOAc/toluene 1:20→1:10) to give **12** (2.24 g, 46%); R_f=0.64 (EtOAc/toluene 1:10); $[\alpha]_D$ −6.7 (*c*=1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ=8.07–7.18 (m, 20H; Ph), 6.00 (d, $J_{1,2}$=3.9 Hz, 1H; H-1), 5.68 (d, $J_{3,4}$=1.3 Hz, 1H; H-3), 4.61 (d, 1H; H-2), 4.42 (dd, $J_{4,5}$=8.7 Hz, 1H; H-4), 4.18 (m, 1H; H-5), 3.46 (dd, $J_{6a,6b}$=9.5 Hz, $J_{5,6a}$=3.7 Hz, 1H; H-6a), 3.40 (dd, $J_{5,6b}$=5.5 Hz, 1H; H-6b), 2.74 (d, $J_{OH,5}$=5.4 Hz, 1H; OH), 1.58, 1.36 (2s, 6H; CMe_2); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ=165.6 (CO), 143.8–125.3 (Ph), 112.7 (CMe_2), 106.0 (C-1), 86.9 (CPh_3), 85.9 (C-4), 85.0 (C-2), 78.3 (C-3), 70.4 (C-5), 64.2 (C-6), 26.8, 25.9 (CMe_2); IR (KBr) ν_{max}=3524, 3059, 2988, 1723, 1601, 1480, 1379, 1267, 1092 cm^{-1}; FABMS: *m/z* 589 $[M+Na]^+$; elemental analysis calcd (%) for $C_{35}H_{34}O_7$: C 74.19, H 6.05; found: C 74.19, H 5.81.

5-Azido-3-*O*-benzoyl-5-deoxy-1,2-*O*-isopropylidene-α-D-galactofuranose (13): Pyridine (0.65 mL) and trifluoromethanesulfonic anhydride (0.91 mL, 5.54 mmol) were added to a solution of **12** (1.86 g, 3.69 mmol) in CH_2Cl_2 (11 mL) at −25 °C under nitrogen. The reaction mixture was allowed to reach RT and further stirred for 30 min. The mixture was diluted with CH_2Cl_2 (10 mL), washed with

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

iced saturated aqueous $NaHCO_3$ (10 mL), dried ($MgSO_4$), and concentrated. The resulting triflate ester was dissolved in DMF (16 mL), NaN_3 (1.2 g, 18.5 mmol) was added and the reaction mixture was stirred at RT under Ar for 18 h. The solvent was removed under diminished pressure and the resulting residue was dissolved in CH_2Cl_2 (15 mL) and washed with water (10 mL). The organic extract was dried ($MgSO_4$) and evaporated. The resulting product was dissolved in CH_2Cl_2 (27 mL) at 0 °C and BF_3/Et_2O (1.08 mL) and MeOH (5.4 mL) were added. The reaction mixture was allowed to reach RT and stirred for 2 h, diluted with CH_2Cl_2 (10 mL), then washed with saturated aqueous $NaHCO_3$ (2×8 mL), dried ($MgSO_4$), and concentrated. The resulting residue was purified by column chromatography (EtOAc/petroleum ether 1:5→1:2) to give **13** (771 mg, 60%); R_f 0.45 (EtOAc/petroleum ether1:2): $[\alpha]_D=-23.9$ ($c=0.7$ in CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): $\delta=8.06$–7.45 (m, 5H; Ph), 6.10 (d, $J_{1,2}=4.0$ Hz, 1H; H-1), 5.39 (d, $J_{3,4}=1.7$ Hz, 1H; H-3), 4.88 (d, 1H; H-2), 4.33 (dd, $J_{4,5}=9.2$ Hz, 1H; H-4), 3.95 (m, 2H, H-5; H-6a), 3.86 (dd, $J_{6a,6b}=12.0$ Hz, $J_{5,6b}=5.6$ Hz, 1H; H-6b), 2.60 (brs, 1H; OH), 1.65, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta=166.1$ (CO), 133.9–128.6 (Ph), 113.2 (CMe_2), 105.8 (C-1), 86.3 (C-4), 84.4 (C-2), 78.2 (C-3), 63.5 (C-5), 62.7 (C-6), 26.7, 25.8 (CMe_2); IR (KBr) $\nu_{max}=$ 3507, 3031, 2988, 2101, 1723, 1381, 1269, 1113 cm^{-1}; FABMS: m/z 372 $[M+Na]^+$; elemental analysis calcd (%) for $C_{16}H_{19}N_3O_6$: C 55.01, H, 5.48, N 12.03; found: C 54.96, H 5.38, N 11.71.

5-Azido-3-*O*-benzoyl-5,6-dideoxy-6-iodo-1,2-*O*-isopropylidene-α-D-galactofuranose (14): I_2 (1.4 g, 5.52 mmol, 2.0 equiv) was slowly added to a solution of PPh_3 (868 mg, 3.31 mmol, 1.2 equiv) and imidazole (470 mg, 6.9 mmol, 2.5 equiv) in dry toluene (29 mL) heated at 50 °C. After stirring for 30 min, a solution of **13** (963 mg, 2.76 mmol) in toluene (9.7 mL) was added dropwise and the reaction mixture was stirred at 70 °C for 2 h. The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (1:4 EtOAc/petroleum ether) to give **14** (1.06 g, 84%); $R_f=0.56$ (EtOAc/petroleum ether 1:3); $[\alpha]_D=-12.6$ ($c=1.0$ in CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): $\delta=8.07$–7.28 (m, 5H; Ph), 6.05 (d, $J_{1,2}=4.0$ Hz, 1H; H-1), 5.34 (d, $J_{3,4}=2.6$ Hz, 1H; H-3), 4.85 (d, 1H; H-2), 4.21 (dd, $J_{4,5}=7.8$ Hz, 1H; H-4), 3.98 (ddd, $J_{5,6b}=$ 8.1 Hz, $J_{5,6a}=4.2$ Hz, 1H; H-5), 3.54 (dd, $J_{6a,6b}=10.8$ Hz, 1H; H-6a), 3.23 (dd, 1H; H-6b), 1.66, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta=165.6$ (CO), 133.8–128.6 (Ph), 113.6 (CMe_2), 105.6 (C-1), 87.2 (C-4), 84.8 (C-2), 78.0 (C-3), 63.5 (C-5), 26.8, 26.0 (CMe_2), 3.4 (C-6); IR (KBr) $\nu_{max}=3031$, 2936, 2113, 1724, 1602, 1472, 1376, 1263, 1111, 1024 cm^{-1}; FABMS: m/z 444 $[M-15]^+$; elemental analysis calcd (%) for $C_{16}H_{18}IN_3O_5$: C 41.85, H 3.95; N 9.15; found: C 41.72, H 3.90, N 9.01.

5,6-Diazido-3-*O*-benzoyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-galactofuranose (15): NaN_3 (224 mg, 3.44 mmol) was added to a solution of **14** (316 mg, 0.69 mmol) in DMF (7 mL), and the reaction mixture was stirred at 80 °C under Ar for 12 h. The solvent was removed under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (20 mL) and washed with water (10 mL). The organic extract was dried ($MgSO_4$), filtered and concentrated. The resulting residue was purified by column chromatography (EtOAc/petroleum ether 1:4) to give **26** (258 mg, 95%); $R_f=0.52$ (EtOAc/petroleum ether 1:3); $[\alpha]_D=-38.4$ ($c=1.0$ in CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$): $\delta=8.06$–7.46 (m, 5H; Ph), 6.07 (d, $J_{1,2}=4.0$ Hz, 1H; H-1), 5.33 (d, $J_{3,4}=2.3$ Hz, 1H; H-3), 4.86 (d, 1H; H-2), 4.19 (dd, $J_{4,5}=8.4$ Hz, 1H; H-4), 4.00 (ddd, $J_{5,6b}=7.4$ Hz, $J_{5,6a}=4.0$ Hz, 1H; H-5), 3.67 (dd, $J_{6a,6b}=13.0$ Hz, 1H; H-6a), 3.47 (dd, 1H; H-6b), 1.66, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta=165.6$ (CO), 133.8–128.6 (Ph), 113.5 (CMe_2), 105.7 (C-1), 85.7 (C-4), 84.6 (C-2), 77.8 (C-3), 62.0 (C-5), 51.8 (C-6), 26.8, 25.9 (CMe_2); IR (KBr) $\nu_{max}=$ 3030, 2989, 2102, 1723, 1602, 1450, 1374, 1265, 1112, 1024 cm^{-1}; FABMS: m/z 359 $[M-15]^+$; elemental analysis calcd (%) for $C_{16}H_{18}N_6O_5$: C 51.33, H, 4.85, N 22.45, found: C 51.09, H 4.65, N, 21.99.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-galactofuranose 5,6-(cyclic thiourea) (16): Methanolic NaMeO (1 M, 0.1 equiv per mol of acetate) was added to a solution of **15** (258 mg, 0.69 mmol) in dry MeOH (6 mL). The reaction mixture was stirred at RT for 1 h, then neutralized with Amberlite IRA-120 (H^+) ion-exchange resin, concentrated, and the resulting residue was purified by column chromatography using EtOAc/petroleum ether 1:1 as eluent. A solution of the resulting solid (166 mg, 0.61 mmol) in MeOH (3.3 mL) was hydrogenated at atmospheric pressure for 1 h by using 10% Pd/C (117 mg) as catalyst. The suspension was filtered through Celite and concentrated to give the corresponding diamine that was used in the next step without further purification. CS_2 (0.3 mL, 4.88 mmol, 8 equiv) and DCC (138 mg, 0.67 mmol, 1.1 equiv) were added to a solution of the diamine derivative in CH_2Cl_2 (7 mL). The reaction mixture was stirred for 1 h and then concentrated. The resulting residue was purified by column chromatography (CH_2Cl_2/MeOH 30:1→10:1) to give **16** (124 mg, 69%); $R_f=0.44$ (CH_2Cl_2/MeOH 10:1); $[\alpha]_D=-123.8$ ($c=1.0$ in MeOH); ^1H NMR (300 MHz, CD_3OD): $\delta=5.88$ (d, $J_{1,2}=3.7$ Hz, 1H; H-1), 4.51 (d, 1H; H-2), 4.21 (ddd, $J_{5,6a}=10.1$ Hz, $J_{4,5}=9.1$ Hz, $J_{5,6b}=6.4$ Hz, 1H; H-5), 4.00 (d, $J_{3,4}=2.2$ Hz, 1H; H-3), 3.86 (dd, 1H; H-4), 3.76 (t, $J_{6a,6b}=10.1$ Hz, 1H; H-6a), 3.47 (dd, 1H; H-6b), 1.66, 1.39 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta=183.6$ (CS), 113.9 (CMe_2), 107.1 (C-1), 90.3 (C-4), 88.4 (C-2), 76.5 (C-3), 59.9 (C-5), 47.7 (C-6), 27.3, 26.2 (CMe_2); IR (KBr) $\nu_{max}=3379$, 2989, 1634, 1510, 1381, 1292, 1198, 1068 cm^{-1}; UV (CH_2Cl_2) 242 nm (ε_{mM} 16.8); FABMS: m/z 261 $[M+H]^+$; elemental analysis calcd (%) for $C_{10}H_{16}N_2O_4S$: C 46.14, H 6.20, N, 10.76; found: C 45.98; H 6.26, N 10.54.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-galactofuranose 5,6-(cyclic *S*-methylthiouronium) iodide (17): A solution of **16** (127 mg, 0.49 mmol) and methyl iodide (153 μL, 5 equiv) in MeOH (5 mL) was heated under reflux (70 °C) during 2 h and concentrated. The resulting residue was purified by column chromatography using 70:10:1 CH_2Cl_2/MeOH-H_2O as eluent to give **17** (181 mg, 91%); $R_f=0.36$ (CH_2Cl_2/MeOH 40:10:1); $[\alpha]_D=-97.8$ ($c=$ 1.0 in MeOH); ^1H NMR (300 MHz, CD_3OD): $\delta=5.91$ (d, $J_{1,2}=3.7$ Hz, 1H; H-1), 4.55 (m, 1H; H-5), 4.54 (d, 1H; H-2), 4.10 (t, $J_{6a,6b}=J_{5,6a}=$ 11.0 Hz, 1H; H-6a), 4.08 (d, $J_{3,4}=2.7$ Hz, 1H; H-3), 3.94 (dd, $J_{4,5}=$ 8.7 Hz, 1H; H-4), 3.77 (dd, $J_{5,6b}=6.8$ Hz, 1H; H-6b), 2.66 (s, 3H; SMe), 1.50, 1.31 (2s, 6H; CMe_2); ^{13}C NMR (75.5 MHz, CD_3OD): $\delta=$ 172.8 (SCN), 114.1 (CMe_2), 107.1 (C-1), 89.5 (C-4), 88.4 (C-2), 76.2 (C-3), 61.3 (C-5), 49.2 (C-6), 27.4, 26.3 (CMe_2), 14.0 (SMe); IR (KBr) $\nu_{max}=3365$, 2936, 1554, 1378, 1215, 1026 cm^{-1}; FABMS: m/z 297 $[M-I+Na]^+$, 275 $[M-I]^+$); elemental analysis calcd (%) for $C_{11}H_{19}IN_2O_4S$: C 32.84, H 4.76, N 6.96; found: C 32.81, H 4.72, N 6.82.

5,6-Diamino-5,6-dideoxy-1,2-*O*-isopropylidene-5,6-di-*N*-(*N*′-octyliminomethylidene)-α-D-galactofuranose hydrochloride (18): A solution of **17** (181 mg, 0.45 mmol) and *n*-octylamine, (1.5 equiv) in DMF (10 mL) was heated at 70 °C under Ar during 18 h and concentrated. The resulting residue was purified by column chromatography (CH_2Cl_2/MeOH/H_2O 90:10:1 → 80:10:1) and freeze dried from an aqueous HCl solution (pH 5) to give **18** (138 mg, 78%), $R_f=0.38$ (CH_2Cl_2/MeOH/H_2O 70:10:1); $[\alpha]_D=-27.3$ ($c=1.0$ in MeOH); ^1H NMR (300 MHz, CD_3OD): $\delta=5.93$ (d, $J_{1,2}=3.7$ Hz, 1H; H-1), 4.57 (d, 1H; H-2), 4.31 (m, 1H; H-5), 4.08 (d, $J_{3,4}=2.5$ Hz, 1H; H-3), 3.89 (dd, $J_{4,5}=9.0$ Hz, 1H; H-4), 3.87 (t, $J_{6a,6b}=J_{5,6a}=10.0$ Hz, 1H; H-6a), 3.23 (t, $J_{5,6b}=10.0$ Hz, 1H; H-6b), 3.23 (t, $^3J_{H,H}=7.1$ Hz, 2H;

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

CH_2N), 1.60 (m, 2H; CH_2CH_2N), 1.53, 1.34 (2s, 6H; CMe_2), 1.35 (m, 10H; CH_2), 0.92 (t, $^3J_{H,H}$=7.0 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, CD_3OD): δ=160.4 (CN), 114.0 (CMe_2), 107.1 (C-1), 89.9 (C-4), 88.4 (C-2), 76.3 (C-3), 58.1 (C-5), 46.1 (C-6), 43.9 (CH_2N), 32.9, 30.3, 30.2, 30.1 (CH_2), 27.6 (CH_2), 27.4, 26.2 (CMe_2), 23.6 (CH_2CH_3), 14.4 (CH_3); IR (KBr) ν_{max}=3401, 2924, 1673, 1592, 1462, 1378, 1215, 1015 cm^{-1}; FABMS: m/z 356 $[M+H]^+$; elemental analysis calcd (%) for $C_{18}H_{34}ClN_3O_4{\cdot}H_2O$: C 52.74, H 8.85, N 10.25; found: C 52.61, H 8.62, N 10.24.

6-Amino-6-deoxy-5,6-di-N-(N′-octyliminomethylidene)galactonojirimycin hydrochloride (6N-NOI-GNJ, 6): A solution of **18** (0.41 mmol) in 90% TFA–H_2O (1.7 mL) was stirred at 0°C for 1 h, concentrated under reduced pressure, coevaporated several times with water, treated with NaOH 0.1 N until it reached pH 8, subjected to column chromatography ($MeCN/H_2O/NH_4OH$ 4:1:1), then to GPC (Sephadex G-10, 1:1 $MeOH/H_2O$) and finally freeze dried from an aqueous HCl solution (pH 5) to give **6** (101 mg, 70%) as an amorphous solid. R_f=0.33 ($MeCN/H_2O/NH_4OH$ 4:1:1); $[\alpha]_D$=−1.9 (c=1.0, H_2O); $[\alpha]_{578}$=−3.9 (c=1.0 in H_2O); ^{1}H NMR (500 MHz, D_2O): δ=5.42 (d, $J_{1,2}$=4.0 Hz, 1H; H-1), 4.28 (t, $J_{5,6a}$=$J_{5,6b}$=9.8 Hz, 1H; H-5), 4.01 (brs, 1H; H-4), 3.85 (dd, $J_{2,3}$=10.3 Hz, $J_{3,4}$=2.8 Hz, 1H; H-3), 3.77 (dd, 1H; H-2), 3.72 (t, $J_{6a,6b}$=9.8 Hz, 1H; H-6a), 3.59 (t, 1H; H-6b), 3.18 (t, $^3J_{H,H}$=7.0 Hz, 2H; CH_2N), 1.53 (m, 2H; CH_2CH_2N), 1.23 (m, 10H; CH_2), 0.80 (t, $^3J_{H,H}$=7.0 Hz, 3H; CH_3); ^{13}C NMR (125.7 MHz, D_2O): δ=155.9 (CN), 74.1 (C-1), 69.2 (C-3), 68.1 (C-4), 67.8 (C-2), 55.7 (C-5), 42.9 (CH_2N), 41.7 (C-6), 31.0, 28.3, 28.2, 28.0, 25.7 (CH_2), 22.0 (CH_2CH_3), 13.4 (CH_3); FABMS: m/z 316 $[M+H-Cl]^+$); elemental analysis calcd (%) for $C_{15}H_{30}ClN_3O_4{\cdot}H_2O$: C 48.71, H 8.72, N 11.36; found: C 48.84, H 8.72, N 11.25.

General procedure for the inhibition assay against the commercial enzymes: Inhibitory potencies were determined by spectrophotometrically measuring the residual hydrolytic activities of the glycosidases against the respective o- (for cytosolic β-glucosidase/β-galactosidase from bovine liver and β-galactosidase from *E. coli*) or p-nitrophenyl α- or β-D-glycopyranoside or α,α′-trehalose (for trehalase), in the presence of the corresponding guanidine derivative. Each assay was performed in phosphate buffer at the optimal pH for each enzyme. The K_M values for the different glycosidases used in the tests and the corresponding working pHs are listed herein: α-glucosidase (yeast), K_M=0.35 mM (pH 6.8); isomaltase (yeast) K_M=1.0 mM (pH 6.8), β-glucosidase (almonds), K_M=3.5 mM (pH 7.3); β-glucosidase/β-galactosidase (bovine liver), K_M=2.0 mM (pH 7.3); β-galactosidase (*E. coli*), K_M=0.12 mM (pH 7.3); α-galactosidase (coffee beans), K_M=2.0 mM (pH 6.8); trehalase (pig kidney), K_M=4.0 mM (pH 6.2); naringinase (*Penicillium decumbens*), K_M=2.7 mM (pH 6.8); α-mannosidase (Jack bean), K_M=2.0 mM (pH 5.5); β-mannosidase (*Helix pomatia*), K_M=0.57 mM (pH 5.5). The reactions were initiated by addition of enzyme to a solution of the substrate in the absence or presence of various concentrations of inhibitor. After the mixture was incubated for 10–30 min at 37°C or 55°C the reaction was quenched by addition of Na_2CO_3 (1 M) or a solution of Glc-Trinder (Sigma, for trehalase). The absorbance of the resulting mixture was determined at 405 nm or 505 nm. The K_i value and enzyme inhibition mode were determined from the slope of Lineweaver–Burk plots and double reciprocal analysis. Representative examples of the Lineweaver–Burk plots, with typical profiles for competitive inhibition mode, are shown in the Supporting Information (Figure 19S-22S).

Cell cultures: Human skin fibroblasts were cultured in DMEM/FBS (10%) at 37°C in a humidified atmosphere containing CO_2 (5%). For fibroblasts, we used three control cell lines (H8, H22, and H37) and seven lines of GD cells. Six cell lines carried β-Glu mutations of F213I/F213I, G202R/L444P, N188S/G193W, F213 L444P, L444P/RecNcil and L444P/L444P. These cells were from Japanese patients. The other line of GD cells that carried the N370S homozygous mutation was from Caucasian patients (a gift from Prof. C. Kaneski and R. O. Brady). Culture medium was replaced every 2 days with fresh media supplemented with or without compounds at the indicated concentrations. In all cases, informed, signed consent was obtained from either the patient or next of kin.

In vitro enzyme assay: Lysosomal enzyme activities in cell lysates were determined as described.[58,62–64] Briefly, cells were scraped into ice-cold H_2O (10^6 cells mL^{-1}) and lysed by sonication. Insoluble materials were removed by centrifugation at 15000 rpm for 5 min and protein concentrations were determined with a BCA microprotein assay kit (Pierce). The lysates (10 μL) were incubated at 37°C with the substrate solution (20 μL) in citrate buffer (0.1 M, pH 4.5). The substrates were 4-methylumbelliferone-conjugated α-D-glucopyranoside (for α-glucosidase), α-D-galactopyranoside (for α-galactosidase), β-D-galactopyranoside (for β-galactosidase), and N-acetyl-β-D-glucosaminide (for β-hexosaminidase). β-Glu activities in cell lysates were determined by using 4-methylumbelliferone-conjugated β-D-glucopyranoside as a substrate. The lysates (10 μL) were incubated at 37°C with the substrate solution (20 μL) in citrate buffer (0.1 M, pH 5.2 or pH 7), supplemented with sodium taurocholate (0.8% w/v). The reactions were terminated by adding glycine sodium hydroxide buffer (0.2 mL, 0.2 M, pH 10.7). The liberated 4-methylumbelliferone was measured with a Perkin–Elmer Luminescence Spectrometer (λ_{ex}: 340 nm; λ_{em}: 460 nm). One unit of enzyme activity was defined as nmol of 4-methylumbelliferone released per hour and normalized for the amount of protein contained in the lysates.

In situ cell enzyme assay: β-Glu activities in live cells were estimated by the methods described by Sawkar et al.[26] with modification. Briefly, cells in 96-well assay plates were treated with compounds for 4 days. After washing with PBS, the cells were incubated in PBS (8 μL) and acetate buffer (8 μL, 0.2 M, pH 4.0). The reaction was started by addition 4-methylumbelliferyl-β-D-glucoside (10 μL, 5 mM), followed by incubation at 37°C for 1 h. The reaction was stopped by lysing the cells by the addition of glycine buffer (200 μL, 0.2 M, pH 10.7) and the liberated 4-methylumbelliferone was quantified. Every experiment was performed in parallel with cells that had been preincubated with or without conduritol B epoxide (0.5 mM, CBE, Toronto Research Chemicals, Canada) for 1 h. The CBE-sensitive component was ascribed to lysosomal β-Glu, whereas the CBE-insensitive component was ascribed to nonlysosomal β-Glu.

Cytotoxicity assay: The cytotoxicity assay was performed by using the colorimetric assay reagent TetraColor One (Seikagaku, Tokyo, Japan),[65] according to the manufacturer's instructions. Fibroblasts were seeded on a 96-well assay plate at a density of 3.0×10^4 cells mL^{-1} medium and incubated for 4 days with chaperones. Then, the TetraColor One reagent (10 μL) was added to each well, and cells were further incubated for 2 h. The absorbance at 450 nm was then measured with a reference wavelength at 630 nm in the microplate reader. Measurements were repeated in triplicate and then averaged for each sample.

Acknowledgements

This research was supported by grants from the Ministry of Education, Culture, Science, Sports, and Technology of Japan (20390297, 13680918, 14207106), the Ministry of Health, Labour

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

and Welfare of Japan (H14-Kokoro-017, H17-Kokoro-019, H20-Kokoro-022), the Spanish Ministerio de Ciencia e Innovación (contract numbers CTQ2006-15515-C02-01/BQU and CTQ2007-61180/PPQ) and the Junta de Andalucía (Project P08-FQM-03711). M.A.-M. is a FPU fellow.

Keywords: Gaucher disease · glucocerebrosidase · glucosidase · imino sugars · inhibitors · medicinal chemistry

[1] E. Beutler, G. A. Grabowski in *The Metabolic and Molecular Bases of Inherited Disease* (Eds.: C. R. Scriver, W. S. Sly, B. Childs, A. L. Beaudet), McGraw-Hill, New York, **2001**, pp. 2641–2670.
[2] M. Jmoudiak, A. H. Futerman, *Br. J. Haematol.* **2005**, *129*, 178–188.
[3] A. H. Futerman, G. van Meer, *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 554–565.
[4] T. D. Butters, *Curr. Opin. Chem. Biol.* **2007**, *11*, 412–418.
[5] E. Beutler, *PLoS Med.* **2004**, *1*, e21.
[6] D. Aviezer, E. Brill-Almon, Y. Shaaltiel, S. Hashmueli, D. Bartfeld, S. Mizrachi, Y. Liberman, A. Freeman, A. Zimran, E. Galun, *PLoS ONE* **2009**, *4*, e4792.
[7] T. D. Butters, R. A. Dwek, F. M. Platt, *Glycobiology* **2005**, *15*, 43R–52R.
[8] N. W. Barton, R. O. Brady, J. M. Dambrosia, A. M. Di Bisceglie, S. H. Doppelt, S. C. Hill, H. J. Mankin, G. J. Murray, R. I. Parker, C. E. Argoff, R. P. Grewal, K. T. Yu, *N. Engl. J. Med.* **1991**, *324*, 1464–1470.
[9] F. M. Platt, G. R. Neises, R. A. Dwek, T. D. Butters, *J. Biol. Chem.* **1994**, *269*, 27 108–27 114.
[10] T. Cox, R. Lachmann, C. Hollak, J. Aerts, S. van Weely, M. Hrebicek, F. Platt, T. Butters, R. Dwek, C. Moyses, I. Gow, D. Elstein, A. Zimran, *Lancet* **2000**, *355*, 1481–1485.
[11] G. A. Grabowski, N. Leslie, R. Wenstrup, *Blood Rev.* **1998**, *12*, 115–133.
[12] F. M. Platt, M. Jeyakumar, U. Andersson, D. A. Priestman, R. A. Dwek, T. D. Butters, *J. Inherited Metab. Dis.* **2001**, *24*, 275–290.
[13] R. Schiffmann, M. P. Heyes, J. M. Aerts, J. M. Dambrosia, M. C. Patterson, T. DeGraba, C. C. Parker, G. C. Zirzow, K. Oliver, G. Tedeschi, R. O. Brady, N. W. Barton, C. Nagineni, C. R. Kaneski, G. J. Murray, J. J. Higgins, A. Tournay, T. K. Banerjee, C. Kreps, L. J. C. Scott, M. A. McKee, K. Crutchfield, K. Frei, *Ann. Neurol.* **1997**, *42*, 613–621.
[14] C. A. Prows, N. Sánchez, C. Daugherty, G. A. Grabowski, *Am. J. Med. Genet.* **1997**, *71*, 16–21.
[15] M. Aoki, Y. Takahashi, Y. Miwa, S. Iida, K. Sukegawa, T. Horai, T. Orii, N. Kondo, *Eur. J. Pediatr.* **2001**, *160*, 63–64.
[16] J. M. Aerts, C. E. Hollak, R. G. Boot, J. E. Groener, M. Maas, *J. Inherited Metab. Dis.* **2006**, *29*, 449–456.
[17] I. Ron, M. Horowitz, *Hum. Mol. Genet.* **2005**, *14*, 2387–2398.
[18] Z. Q. Yu, A. R. Sawkar, J. W. Kelly, *FEBS J.* **2007**, *274*, 4944–4950.
[19] J. Q. Fan, *Trends Pharmacol. Sci.* **2003**, *24*, 355–360.
[20] H. Lin, Y. Sugimoto, Y. Ohsaki, H. Ninomiya, A. Oka, M. Taniguchi, H. Ida, Y. Eto, S. Ogawa, Y. Matsuzaki, M. Sawa, T. Inoue, K. Higaki, E. Nanba, K. Ohno, Y. Suzuki, *Biochim. Biophys. Acta Mol. Basis Dis.* **2004**, *1689*, 219–228.
[21] K. Lei, H. Ninomiya, M. Suzuki, T. Inoue, M. Sawa, M. Iida, H. Ida, Y. Eto, S. Ogawa, K. Ohno, Y. Suzuki, *Biochim. Biophys. Acta Mol. Basis Dis.* **2007**, *1772*, 587–596.
[22] G. J. Kornhaber, M. B. Tropak, G. H. Maegawa, S. J. Tuske, S. J. Coales, D. J. Mahuran, Y. Hamuro, *ChemBioChem* **2008**, *9*, 2643–2649.
[23] M. B. Tropak, G. J. Kornhaber, B. A. Rigat, G. H. Maegawa, J. D. Buttner, J. E. Blanchard, C. Murphy, S. J. Tuske, S. J. Coales, Y. Hamuro, E. D. Brown, D. J. Mahuran, *ChemBioChem* **2008**, *9*, 2650–2652.
[24] P. Compain, O. R. Martin, C. Boucheron, G. Godin, L. Yu, K. Ikeda, N. Asano, *ChemBioChem* **2006**, *7*, 1356–1359.
[25] L. Yu, K. Ikeda, A. Kato, I. Adachi, G. Godin, P. Compain, O. R. Martin, N. Asano, *Bioorg. Med. Chem.* **2006**, *14*, 7736–7744.
[26] A. R. Sawkar, W. C. Cheng, E. Beutler, C.-H. Wong, W. E. Balch, J. W. Kelly, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15428–15433.
[27] A. R. Sawkar, S. L. Adamski-Werner, W. C. Cheng, C.-H. Wong, E. Beutler, K. P. Zimmer, J. W. Kelly, *Chem. Biol.* **2005**, *12*, 1235–1244.
[28] For a recent monograph, see *Iminosugars: From Synthesis to Therapeutic Applications* (Eds.: P. Compain, O. R. Martin), Wiley-VCH, Weinheim, **2007**.
[29] J. L. Jiménez Blanco, V. M. Díaz Pérez, C. Ortiz Mellet, J. Fuentes, J. M. García Fernández, *Chem. Commun.* **1997**, 1969–1970.
[30] V. M. Díaz Pérez, M. I. García-Moreno, C. Ortiz Mellet, J. Fuentes, J. C. Díaz Arribas, F. J. Cañada, J. M. García Fernández, *J. Org. Chem.* **2000**, *65*, 136–143.
[31] M. I. García-Moreno, P. Díaz-Pérez, C. Ortiz Mellet, J. M. García Fernández, *Chem. Commun.* **2002**, 848–849.
[32] M. I. García-Moreno, P. Díaz-Pérez, C. Ortiz Mellet, J. M. García Fernández, *J. Org. Chem.* **2003**, *68*, 8890–8901.
[33] P. Díaz-Pérez, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, *Eur. J. Org. Chem.* **2005**, 2903–2913.
[34] M. Aguilar, P. Díaz-Pérez, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, *J. Org. Chem.* **2008**, *73*, 1995–1998.
[35] E. Sánchez-Fernández, R. Rísquez-Cuadro, M. Aguilar-Moncayo, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, *Org. Lett.* **2009**, *11*, 3306–3309.
[36] J. M. García Fernández, C. Ortiz Mellet, M. I. García-Moreno, M. Aguilar-Moncayo, Y. Suzuki, K. Ohno, ES Patent P200802988.
[37] M. Aguilar-Moncayo, T. M. Gloster, J. P. Turkenburg, M. I. García-Moreno, C. Ortiz Mellet, G. J. Davies, J. M. García Fernández, *Org. Biomol. Chem.* **2009**, *7*, 2738–2747.
[38] B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, *Nucleic Acids Res.* **2009**, *37*, D233–D238.
[39] B. Brumshtein, M. Aguilar-Moncayo, M. I. García-Moreno, C. Ortiz Mellet, J. M. García Fernández, I. Silman, Y. Shaaltiel, D. Aviezer, J. L. Sussman, A. H. Futerman, *ChemBioChem* **2009**, *10*, 1480–1485.
[40] K. Dax, B. Caigg, V. Grassberger, B. Kölblinger, A. E. Stütz, *J. Carbohydr. Chem.* **1990**, *9*, 479–499.
[41] H. Wang, J. A. Ning, *J. Org. Chem.* **2003**, *68*, 2521–2524.
[42] P. J. Garegg, B. Samuelsson, *J. Chem. Soc. Perkin Trans. 1* **1980**, 2866–2869.
[43] For a review, see P. Molina, M. J. Vilaplana, *Synthesis* **1994**, 1197–1218.
[44] G. Legler, S. Pohl, *Carbohydr. Res.* **1986**, *155*, 119–129.
[45] R. A. Steet, S. Chung, B. Wustman, A. Powe, H. Do, S. A. Kornfeld, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13813–13818.
[46] S. Ogawa, M. Ashiura, C. Uchida, S. Watanabe, C. Yamazaki, K. Yamagishi, J. Inokuchi, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 929–932.
[47] Z. Q. Yu, A. R. Sawkar, L. J. Whalen, C.-H. Wong, J. W. Kelly, *J. Med. Chem.* **2007**, *50*, 94–100.
[48] G.-N. Wang, G. Reinkensmeier, S.-W. Zhang, J. Zhou, L.-R. Zhang, L.-H. Zhang, T. D. Butters, X.-S. Ye, *J. Med. Chem.* **2009**, *52*, 3146–3149.
[49] H. Dvir, M. Harel, A. A. McCarthy, L. Toker, I. Silman, A. H. Futerman, J. L. Sussman, *EMBO Rep.* **2003**, *4*, 704–709.
[50] H. H. Chang, N. Asano, S. Ishii, Y. Ichikawa, J. Q. Fan, *FEBS J.* **2006**, *273*, 4082–4092.
[51] R. Steet, S. Chung, W. S. Lee, C. W. Pine, H. Do, S. Kornfeld, *Biochem Pharmacol.* **2007**, *73*, 1376–1383.
[52] W. Zheng, J. Padia, D. J. Urban, A. Jadhav, O. Goker-Alpan, A. Simeonov, E. Goldin, D. Auld, M .E. LaMarca, J. Inglese, C. P. Austin, E. Sidransky, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 13192–13197.
[53] Y. Eto, H. Ida, *Neurochem. Res.* **1999**, *24*, 207–211.
[54] K. P. Zimmer, P. le Coutre, H. M. Aerts, K. Harzer, M. Fukuda, J. S. O'Brien, H. Y. Naim, *J. Pathol.* **1999**, *188*, 407–414.
[55] E. Beutler, T. Gelbart, D. Balicki, A. Demina, J. Adusumalli, *Proc. Assoc. Am. Physicians* **1996**, *108*, 179–184.
[56] T. W. Mu, D. M. Fowler, J. W. Kelly, *PLoS Biol.* **2008**, *6*, e26.
[57] B. Rigat, D. Mahuran, *Mol. Genet. Metab.* **2009**, *96*, 225–232.
[58] J. Matsuda, O. Suzuki, A. Oshima, Y. Yamamoto, A. Noguchi, K. Takimoto, M. Itoh, Y. Matsuzaki, Y. Yasuda, S. Ogawa, Y. Sakata, E. Nanba, K. Higaki, Y. Ogawa, L. Tominaga, K. Ohno, H. Iwasaki, H. Watanabe, R. O. Brady, Y. Suzuki, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15912–15917.
[59] H. Iwasaki, H. Watanabe, M. Iida, S. Ogawa, M. Tabe, K. Higaki, E. Nanba, Y. Suzuki, *Brain Dev.* **2006**, *28*, 482–486.
[60] J. Fan, S. Ishii, N. Asano, Y. Suzuki, *Nat. Med.* **1999**, *5*, 112–115.
[61] Y. Suzuki, S. Ichinomiya, M. Kurosawa, M. Ohkubo, H. Watanabe, H. Iwasaki, J. Matsuda, Y. Noguchi, K. Takimoto, M. Itoh, M. Tabe, M. Iida, T. Kubo, S. Ogawa, E. Nanba, K. Higaki, K. Ohno, R. O. Brady, *Ann. Neurol.* **2007**, *62*, 671–675.
[62] A. M. Vaccaro, M. Muscillo, M. Tatti, R. Salvioli, E. Gallozzi, K. Suzuki, *Clin. Biochem.* **1987**, *20*, 429–433.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[63] S. Ichisaka, K. Ohno, I. Yuasa, E. Nanba, H. Sakuraba, Y. Suzuki, *Brain Dev.* **1998**, *20*, 302–306.
[64] Y. Suzuki, A. Tsuji, K. Omura, G. Nakamura, S. Awa, M. Kroos, A. J. Reuser, *Clin. Genet.* **1988**, *33*, 376–385.
[65] K. Kawasaki, A. Nishio, H. Nakamura, K. Uchida, T. Fukui, M. Ohana, H. Yoshizawa, S. Ohashi, H. Tamaki, M. Matsuura, M. Asada, T. Nishi, H. Nakase, S. Toyokuni, W. Liu, J. Yodoi, K. Okazaki, T. Chiba, *Lab Instrum.* **2005**, *85*, 1104–1117.

Received: July 16, 2009
Published online on October 14, 2009

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

The Journal of Antibiotics (2009) 62, 407–423
© 2009 Japan Antibiotics Research Association All rights reserved
0021-8820/09 $32.00
www.nature.com/ja

1908

REVIEW ARTICLE

Gem-diamine 1-*N*-iminosugars as versatile glycomimetics: synthesis, biological activity and therapeutic potential

Yoshio Nishimura

Iminosugars, which carry a basic nitrogen in the carbohydrate ring, have attracted increasing interest as new glycomimetics. *Gem*-diamine 1-*N*-iminosugars, a new class of iminosugars, have a nitrogen atom in place of the anomeric carbon. Various kinds of 1-*N*-iminosugars have been synthesized from glyconolactones as a chiral source in a totally stereospecific manner and/or by the convergent strategy from siastatin B, a secondary metabolite of *Streptomyces*. The protonated form of 1-*N*-iminosugar mimics the charge at the anomeric position in the transition state of enzymatic glycosidic hydrolysis, resulting in a strong and specific inhibition of glycosidases and glycosyltransferases. They have been recently recognized as a new source of therapeutic drug candidates in a wide range of diseases associated with the carbohydrate metabolism of glycoconjugates, such as tumor metastasis, influenza virus infection, lysosomal storage disorder and so forth.
The Journal of Antibiotics (2009) **62,** 407–423; doi:10.1038/ja.2009.53; published online 3 July 2009

Keywords: glycosidase inhibitor; heparanase inhibitor; influenza virus infection; lysosomal storage disease; 1-*N*-iminosugar; siastatin B; tumor metastasis

INTRODUCTION

Iminosugars, which are carbohydrate analogs that most frequently carry the nitrogen atom at the position of the endocyclic oxygen, form the most attractive class of glycomimetics reported so far.[1] Several types of iminosugars have been discovered from natural sources[1,2] since nojirimycin[3] was first isolated as an antibiotic from *Streptomyces* culture in 1966. Many more have also been synthesized on the basis of enzymatic glycoside biosynthesis.[1,4] Of late, they have gained remarkable importance not only as molecular tools to unravel the manner in which glycoconjugates regulate various biological functions, but also as new therapeutic agents in a wide range of diseases associated with the metabolism of carbohydrates.

In 1974, siastatin B (**1**), an unusual iminosugar, was isolated as an inhibitor against neuraminidases (NAs) from *Streptomyces* culture.[5] Siastatin B (**1**) also inhibits β-D-glucuronidase and *N*-acetyl-β-D-glucosaminidase. We recognized from biological activity that siastatin B (**1**) structurally resembles D-glucuronic acid (**2**) and *N*-acetyl-D-glucosamine (**3**), as well as *N*-acetylneuraminic acid (**4**) (Figure 1). It is distinct from the known glycosidase inhibitors, such as nojirimycin, that contain a nitrogen atom in place of the ring oxygen. In the course of our study on siastatin B (**1**), we proposed a new class of glycosidase inhibitors, *gem*-diamine 1-*N*-iminosugars[6–8] (*gem*-diamine 1-aza-carbasugar in IUPAC nomenclature,[9] cyclic methanediamine monosaccharide, **5**) in which the anomeric carbon atom is replaced by nitrogen. We hypothesized that the protonated form of *gem*-diamine 1-*N*-iminosugar **6** may mimic the putative glycopyranosyl cation **7** that was formed during enzymatic glycosidic hydrolysis (Figure 2). This turned out to be the case and led to new findings of highly potent and specific inhibitors of glycosidases and glycosyltransferases, as well as potential therapeutics for tumor metastasis and so forth. On the other hand, the synthetic isofagomine (**8**), another type of 1-iminosugar, was developed by Bols and colleagues in 1994.[10] The isofagomine type 1 iminosugars showed a potent inhibition of their corresponding β-glycosidase.[11] These findings suggest that 1-iminosugars might provide another alternative to the development of therapeutic agents based on the inhibitors of metabolism of glycoconjugates different from the common iminosugars, such as Zavesca (*N*-*n*-butyl-1-deoxynojirimycin)[12] used for the treatment of Gaucher's disease.

This review describes our current progress in the chemistry, biochemistry and pharmacology of *gem*-diamine 1-*N*-iminosugars.

SYNTHESIS

Various types of iminosugar inhibitors, such as polyfunctional piperidines and pyrrolidines, have been designed on the basis of a flattened, half-chair oxocarbenium ion-like transition state in the reaction catalyzed by glycosidases.[13–15] They are all carbohydrate mimics in which the ring oxygen is replaced by nitrogen. On the other hand, 1-*N*-iminosugars have a unique structure with a nitrogen atom in place of the anomeric carbon atom. *Gem*-diamine 1-*N*-iminosugars

Bioactive Molecules Research Group, Microbial Chemistry Research Center, Kamiosaki, Shinagawa-ku, Tokyo, Japan
Correspondence: Professor Y Nishimura, Microbial Chemistry Research Center, 3-14-23, Kamiosak, Shinagawa-ku, Tokyo, 141-0021, Japan.
E-mail: nishimuray@bikaken.or.jp
Received 22 May 2009; accepted 22 May 2009; published online 3 July 2009

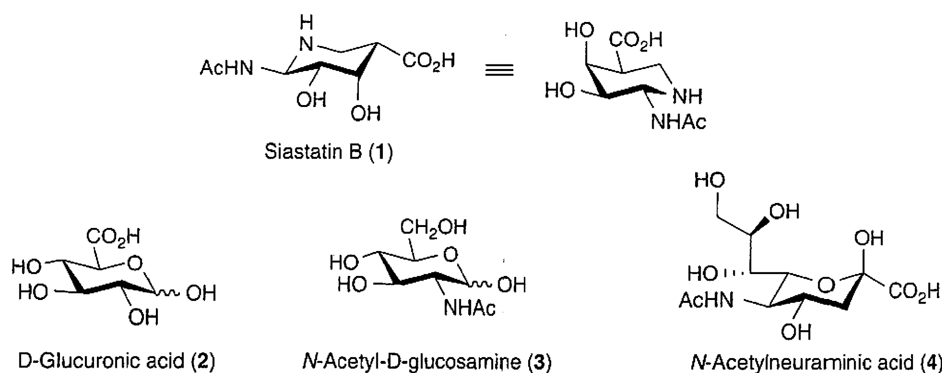


Figure 1 Structural resemblance of siastatin B (1) to D-glucuronic acid (2), *N*-acetyl-D-glucosamine (3) and *N*-acetylneuraminic acid (4).

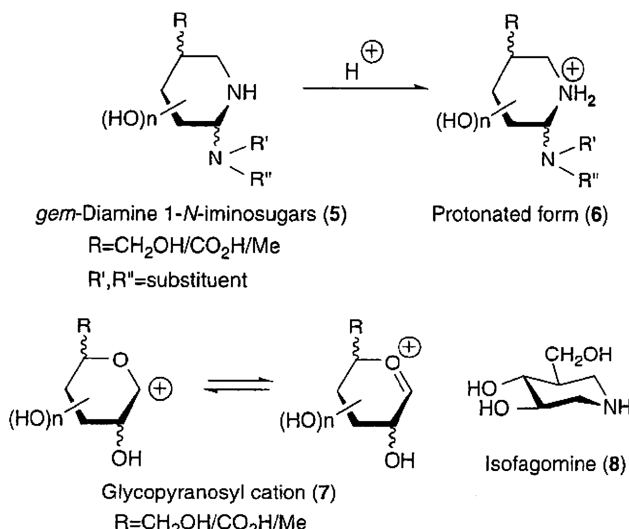


Figure 2 General structures of *gem*-diamine 1-*N*-iminosugars (5), its protonated form (6), glycopyranosyl cation (7), the putative intermediate of enzymatic glycosidic hydrolysis and isofagomine (8), another type of 1-iminosugar.

have an especially unusual structure possessing the continuous –CH(OH)–CH(OH)–CH–(NHR)–NH–CH2–CH(CH2OH/COOH)– in a framework. Their multi-functionalized structures with many asymmetric centers in a small molecule and fascinating biological activities have attracted intensive synthetic interest. As the interest in this class of glycomimetics comprised analogs of both D- and L-sugars, we have developed flexible divergent strategies applicable to a wide range of *gem*-diamine 1-*N*-iminosugars using glyconolactones as chiral substrates. Efficient and convenient synthetic methodologies of *gem*-diamine 1-*N*-iminosugars were also developed from natural siastatin B. These convergent strategies using natural siastatin B could be useful and practical for drug development.

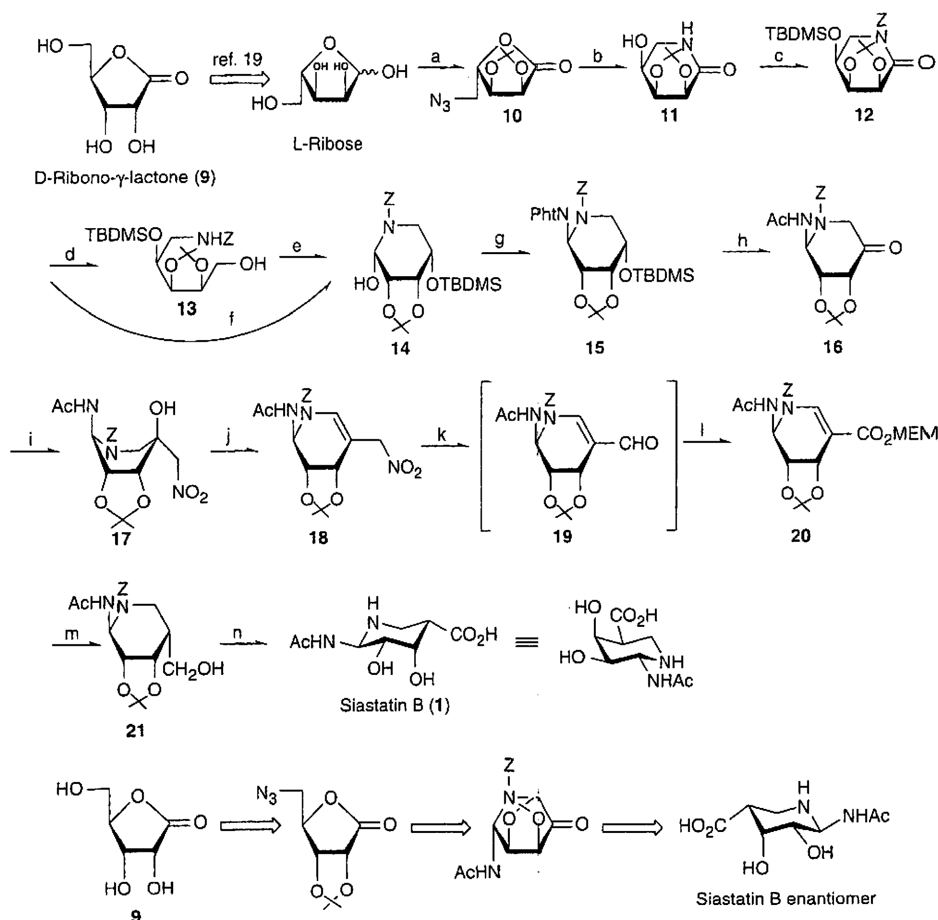
Total synthetic route to *gem*-diamine 1-*N*-iminosugars

The chiron strategy using D-ribono-γ-lactone (9) was first adapted for the total synthesis of siastatin B (1) and its enantiomer.[16,17] D-galacturonic acid-type 2-acetamido-1-*N*-iminosugar was synthesized in a totally stereospecific and enantiospecific manner as shown in Scheme 1.[18] The strategy involves the formation of the cyclic methanediamine using the Mitsunobu reaction[19] on an aminal (14→15) and the stereospecific introduction of a carboxylic acid group into a ketone (16→21) as the key steps. The synthesis of the key intermediate, lactam 11, commenced with L-ribose, which was transformed into azido-L-ribonolactone 10 by the protection of 2,3-diol, azide formation and oxidation. Hydrogenation of the azide group of 10 resulted in crystalline 11 with ring expansion. Stereospecific introduction of the hydroxyl group at the C-2 position was best achieved by hydride reduction of the protected lactam 12 followed by the Swern oxidation to yield aminal 14. One-step stereospecific transformation of 12 into 14 was also efficiently achieved by the reduction of L-selectride in tetrahydrofuran. The Mitsunobu reaction with phthalimide in dimethylformamide was proved to quantitatively yield the desired cyclic methanediamine 15. Replacement of the amino substituent, removal of the *tert*-butyldimethylsilyl (TBDMS) group and oxidation to 16 were carried out straightforwardly. Condensation of 16 with nitromethane was found to proceed smoothly to quantitatively yield 17 as a single stereoisomer. The endocyclic nitro olefin 18 was effectively derived from 17 by acetylation followed by base-catalyzed elimination of the acetoxy group. The crucial intermediate, carboxylate 20, was obtained through α,β-unsaturated aldehyde 19 generated by simply warming in pyridine. Transformation of 20 into siastatin B (1) was best achieved by the following three-step sequences: stereoselective reduction to the α,β-saturated hydroxymethyl compound 21, oxidation and removal of protecting groups. The antipode of 1 was also synthesized from D-ribono-1,4-lactam using the above-mentioned method. Thus, the total synthesis also elucidated the absolute configuration of siastatin B (1) as the (3*S*,4*S*,5*R*,6*R*) isomer.

The strategy of total synthesis of siastatin B (1) is applicable to a wide range of D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars (Schemes 2 and 3).[20,21]

Syntheses of D-galacturonic acid-type 2-acetamido- and trifluoroacetamido-1-*N*-iminosugars (27a and 27b) having a hydroxyl group at the C-5 position and their antipodes 27c and 27d are achieved in a straightforward manner. The nitromethane condensation of the ketones 23a and 23b stereospecifically proceeded to afford adducts 24a and 24b. The *S*-configuration at position C-5 was clarified by an X-ray crystallographic analysis of the antipode of 23a. Successive sequences of catalytic reduction, ninhydrin oxidation of the resultant aminomethyl group, oxidation of the resultant aldehyde with sodium chlorite and removal of the protecting groups afforded the final products. The antipodes 27c and 27d were also synthesized starting from D-ribono-1,4-lactone using similar methods that are mentioned above.

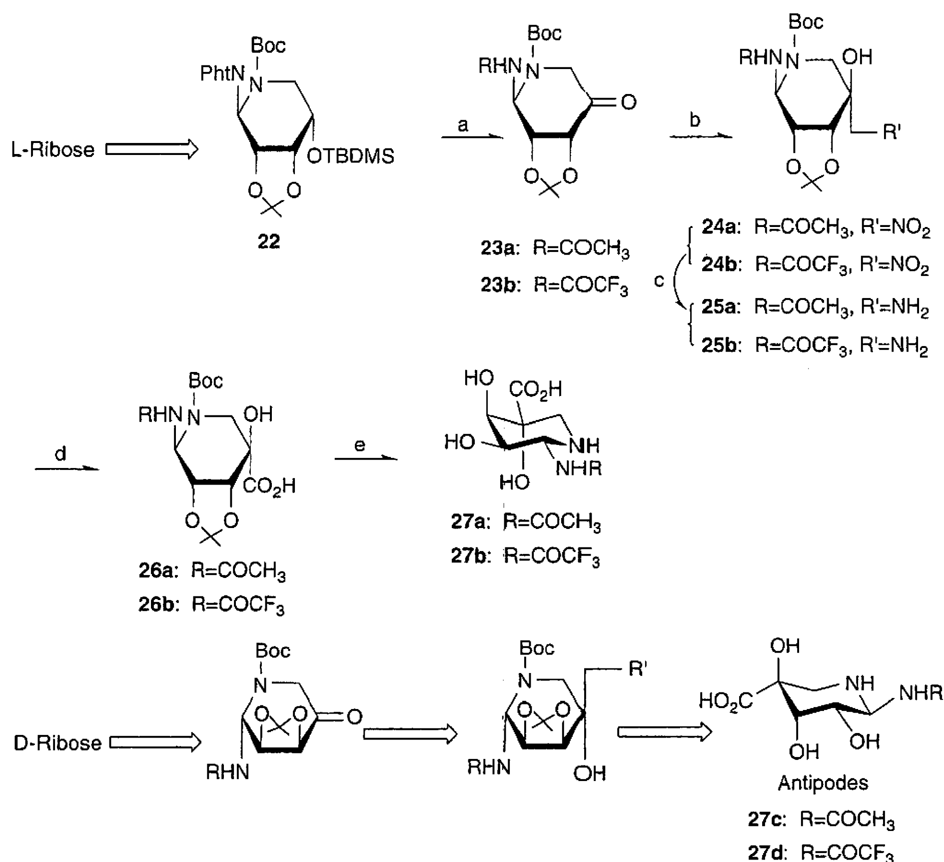
An alternative route from the ketone 23b to D-galacturonic acid-type 2-trifluoroactamido-1-*N*-iminosugar 32 was also developed



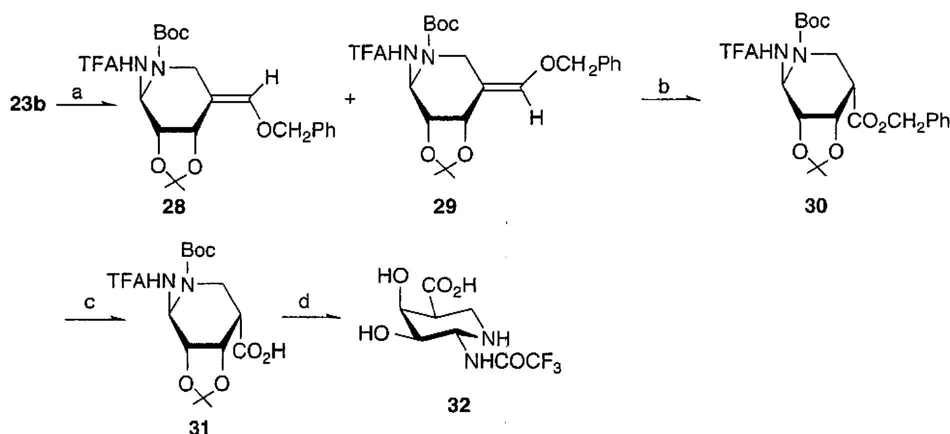
Scheme 1 Reagents and conditions: (a) *p*-TsOH, Me_2CO; MsCl, py; NaN_3, DMSO; CrO_3/py, CH_2Cl_2 (89%); (b) H_2, Raney Ni, MeOH (88%); (c) TBDMSCl, imidazole, DMF; ZCl, NaH, DMF (99%); (d) $NaBH_4$, EtOH (96%); (e) Swern oxidation (88%); (f) L-selectride, THF (88%); (g) phthalimide, Ph_3P, DEAD, DMF (100%); (h) $H_2N{\cdot}NH_2$, MeOH; Ac_2O, py; *n*-Bu_4NF, THF; RuO_4, CH_2Cl_2 (99%); (i) $MeNO_2$, NaH, DME (100%); (j) Ac_2O, *p*-TsOH; K_2CO_3, PhH (100%); (k) py, 38 °C, 1 week (80%); (l) $NaClO_2$–NaH_2PO_4, $CH_3CH{=}CMe_2$, H_2O-*t*-BuOH; MEMCl, *i*-Pr_2NEt, CH_2Cl_2 (55%); (m) $NaBH_4$, THF–CF_3CH_2OH (75%); (n) PDC, DMF; H_2, 10% Pd/C, MeOH; 1 M HCl, then Dowex 50W X_4 (H^+) eluted with 2% NH_4OH (66%).

using the Wacker process oxidation of the enol ethers **28** and **29** as a key step. The Wacker process oxidation stereospecifically proceeded to yield carboxylate **30** as a sole product. The transformation of **30** into **32** was unexceptional.

A flexible synthetic route to four *gem*-diamine 1-*N*-iminosugars of D- and L-uronic acid type (D-glucuronic, D-mannuronic, L-iduronic and L-guluronic acid) from L-galactono-1,4-lactone was also developed in an enantiodivergent manner through a sequence involving as the key steps (1) the formation of *gem*-diamine 1-*N*-iminopyranose ring by the Mitsunobu reaction of an aminal (**44**→**45**, **46**) and (2) the flexible introduction of a carboxylic acid group by the Wittig reaction on a ketone, followed by hydroboration and oxidation, as well as the Sharpless oxidation (**45** and **46**→**47**, **48** and **55**, **56**) (Schemes 4 and 5).[22,23] The diastereoselective construction of amino and carboxylic acid substituents at positions C-2 and C-5, respectively, on the versatile aminal **44** led to the formation of four enantiomerically pure stereoisomers (**51**, **54**, **61** and **66**). The Wittig reaction on the ketone **37** derived from L-galactono-1,4-lactone resulted in the methylene derivative **38**, which was converted into the diol **39**. The monoalcohol **40** was successfully obtained by the Luche reduction of the labile aldehyde generated by the periodate oxidation of **39**. Conversion of the hydroxyl group of **40** to the azide group was best achieved from the corresponding sulfonate by one-pot reaction *in situ*. Hydrogenation of the azide group of **41** with sodium hydrogentelluride (NaTeH) was found to proceed preferentially without any effect on the reduction of the methylene group. The pivotal intermediate, aminal **44** was obtained as an epimeric mixture by the removal of a TBDMS group and the Swern oxidation. The Mitsunobu reaction with phthalimide afforded both desired epimers of iminophthalimides **45** and **46** in a 3:1 ratio. The absolute stereochemistry and a boat conformer of **45** were clarified by an X-ray crystallographic analysis. Another epimer **46** was assigned its stereochemistry and boat conformation by the hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) spectrum. Hydroboration of **45** followed by oxidation efficiently yielded the D-*gluco* isomer **47** and the L-*idulo* isomer **48** in a 2:9 ratio. Hydrazinolysis of **47** and conventional trifluoroacetylation furnished the trifluoroacetamide **49**. The ruthenium tetraoxide-catalyzed Sharpless oxidation effectively yielded the carboxylic acid **50**. Removal of the protecting groups of **50** resulted in D-glucuronic acid-type 2-trifluoroacetamido-1-*N*-iminosugar **51**. The same sequences of reactions also successfully resulted in L-iduronic acid-type 2-trifluoroacetamido-1-*N*-iminosugar **54** from **48**. The ^{1}H-NMR spectrum of **51** showed the $^4C_1$-conformation, whereas the ^{1}H-NMR spectrum of **54** indicated the boat conformation. On the other hand, D-mannuronic



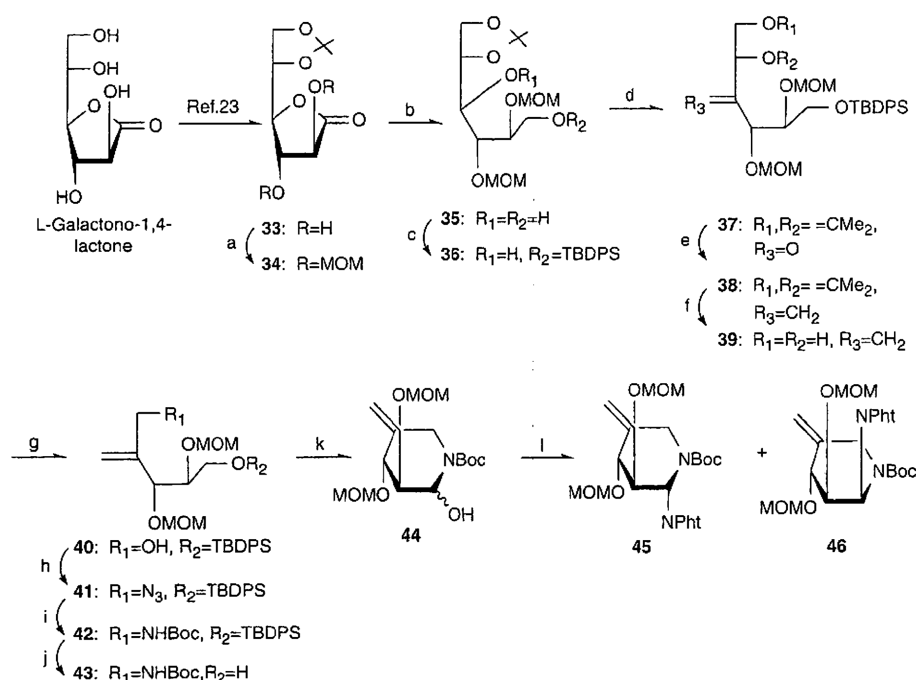
Scheme 2 Reagents and conditions: (a) $H_2N{\cdot}NH_2$, MeOH; Ac_2O, py (or CF_3CO_2Et, *i*-Pr_2NEt, DMF): *n*-Bu_4NF, THF; RuO_4, CH_2Cl_2 (81 and 91%); (b) CH_3NO_2, NaH, DME (69 and 74%); (c) H_2, Raney Ni, MeOH (100 and 98%); (d) ninhydrin, $NaHCO_3$, $MeOH/H_2O$; $NaClO_2$, NaH_2PO_4, $MeCH{=}CMe_2$, *t*-$BuOH/H_2O$ (38 and 43%); (e) 4 M HCl/dioxane (92 and 96%).



Scheme 3 Reagents and conditions: (a) $PhCH_2OCH_2PPh_3Cl$, PhLi, THF (48%); (b) $PdCl_2$, CuCl, O_2, DMF/H_2O (46%); (c) H_2, Pd/C, EtOAc (92%); (d) 4 M HCl/dioxane (96%).

acid-type and L-guluronic acid-type 1-*N*-iminosugars **61** and **66** were prepared in a straightforward manner by a similar sequence of structure transformation, except for the protection of the hydroxyl groups of **55** and **56** before hydrazinolysis of the phthalimide group for improvement in yield. The ^{1}H-NMR spectra of **61** and **66** showed the boat and $^1C_4$-conformations, respectively.

An enantioselective synthesis of L-fucose-type *gem*-diamine 1-*N*-iminosugars from D-ribono-γ-lactone was developed that used the Mitsunobu reaction on an aminal in the *gem*-diamine 1-*N*-iminopyranose ring formation (**74**→**75**) and a stereospecific reduction of an *exo*-methylene group to form the correct configuration of L-fucose (**75**→**76**) (Scheme 6).[24,25] The synthesis of the pivotal intermediate,



Scheme 4 Reagents and conditions: (a) $MeOCH_2Cl$, *n*-Bu_4NI, *i*-Pr_2NEt, 70°C, 81%; (b) $LiAlH_4$, THF, 100%; (c) *t*-$Bu(Ph_2)SiCl$, *i*-Pr_2NEt, DMAP, CH_2Cl_2, 99.7%; (d) Dess–Martin periodinane, CH_2Cl_2, 93%; (e) Ph_3PMeBr, *n*-BuLi, THF, −78°C, 96%; (f) 80% AcOH, rt, 99%; (g) $NaIO_4$, $MeCN/H_2O$; $NaBH_4$, $CeCl_3$, MeOH, 88%; (h) MsCl, py; NaN_3, DMF, 88.7%; (i) Te, $NaBH_4$, EtOH; $(t\text{-}BuCO)_2O$, *i*-Pr_2NEt, DMF, 88%; (j) *n*-Bu_4NF, THF, 100%; (k) $(COCl)_2$, DMSO, CH_2Cl_2, 93%; (l) PPh_3, DEAD, phthalimide, DMF, **45**: 61.4%; **46**: 20%.

aminal **74** began with the known lactam **67**. Transformation of **67** into the diol **70** was unexceptional. The Dess–Martin oxidation of **70** followed by the Wittig reaction with methylenetriphenylphosphorane yielded the *exo*-methylene **72**. Removal of a protecting group of **72** and the Swern oxidation resulted in the desired aminal **74** as a sole product. The Mitsunobu reaction of **74** with phthalimide efficiently afforded the iminophthalimide **75**. The catalytic hydrogenation of **75** yielded the desired product **76**, its epimer **77** and the rearranged derivative **78** in a ratio of 15:1:3. Compound **78** was also successfully converted into the desired **76** on the same hydrogenation. The expected stereochemistry and a boat conformation of **76** were clarified by an X-ray crystallographic analysis. Hydrazinolysis of **76** yielded the amine **79**, which was transformed into the acetamide **80**, the trifluoroacetamide **81** and the trichloroacetamide **82**. Removal of the protecting groups resulted in L-fucose-type 2-acetamido, 2-trifluoroacetamido and 2-trichloroacetamido-1-*N*-iminosugars **83**, **84** and **85**. Other L-fucose-type 1-*N*-iminosugars **86**, **87** and **88** were also obtained from the intermediates **76**, **77** and **75**, respectively, by conventional transformation. The ^{1}H-NMR spectra of **83**, **84**, **85** and **86** showed $^1C_4$-conformations.

Intermediates prepared during the total synthetic route to uronic acid-type *gem*-diamine 1-*N*-iminosugars are also available for the synthesis of various kinds of glycose and glycosamine-type *gem*-diamine 1-*N*-iminoisugars (Scheme 7).[22]

Semi-synthetic route to *gem*-diamine 1-*N*-iminosugars

Natural siastatin B (**1**) can also serve as a starting material in a simple and easy route to D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars (Scheme 8).[26,27] Transketalization using chlorotrimethylsilane successfully proceeded to yield the ketal **94**. A sequence of esterification, hydride reduction and hydrazinolysis efficiently afforded the amino alcohol **97**, which was converted into the trifluoroacetamide **98**. The ruthenium-catalyzed Sharpless oxidation followed by the removal of the protecting group resulted in the desired product **32**. 2-Trichloroacetamido, guanidino and phthaloyl analogs **106**, **107** and **108** were also prepared using similar methods.

Configurational inversion of the carboxyl group of siastatin B (**1**) leads to *gem*-diamine 1-*N*-iminosugars corresponding to L-sugars.[28,29] The intramolecular Michael addition of *O*-imidate to the α,β-unsaturated ester through *cis* oxiamination[30] (Overman rearrangement, **110**→**111**) as a key step effectively yielded L-uronic acid-type *gem*-diamine 1-*N*-iminosugars (Schemes 9 and 10). The α,β-unsaturated ester **110** readily available from siastatin B (**1**) smoothly underwent *cis* oxiamination through the conjugate addition of the intermediate imidate anion to result in the desired oxazoline **111** in a good yield and a trace amount of its epimer. Hydrolysis of **111** afforded the trichloroacetamides **112** and **113**, which were converted into the amines **114** and **115**, respectively, on reductive cleavage of the trichloroacetamide group. Removal of the protecting groups of **114** and **115** resulted in L-alturonic acid-type and L-mannuronic acid-type 2-acetamido-1-*N*-iminosugars **116** and **117**, respectively. Another type of L-alturonic acid-type 2-acetamido-1-*N*-iminosugar **119** with a guanidine group was also obtained by the conventional method. The ^{1}H-NMR spectra of **116**, **117** and **119** showed $^1C_4$-conformations. 2-Trifluoroactamide analogs **130** and **133** were also prepared by a similar sequence of reactions using the α,β-unsaturated ester **123** readily available from **97**.

Siastatin B (**1**) has the correct configuration corresponding to D-galactose- and D-galactosamine-type gem-diamine 1-*N*-iminosugars. Therefore, the various kinds of D-glycose and D-glycosamine-type *gem*-diamine 1-*N*-iminosugars could be obtainable by a semi-synthetic method starting from **1** (Schemes 11 and 12)[30,31]