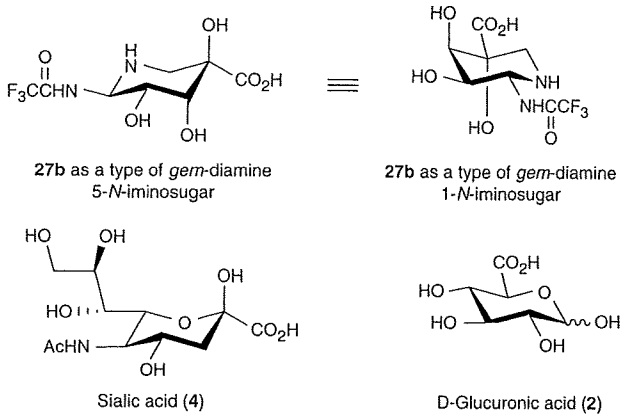


Figure 6 Structural similarity of **27b** to sialic acid (**4**) as *gem*-diamine 5-*N*-iminosugar and to D-glucuronic acid (**2**) as *gem*-diamine 1-*N*-iminosugar.

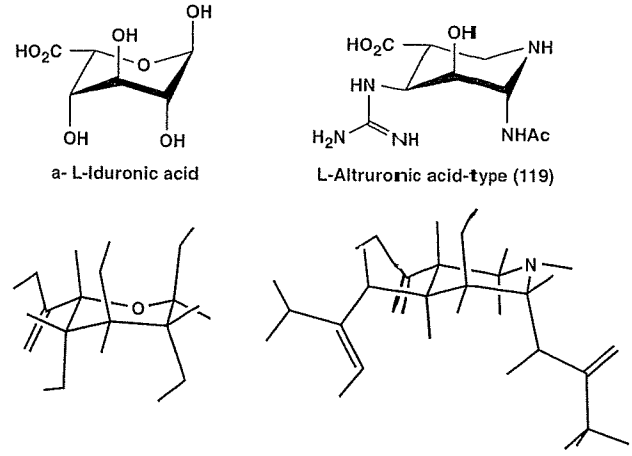


Figure 7 PM3/MOPAC-optimized structures of L-iduronic acid and **119**.

Table 6 Inhibition of experimental pulmonary metastasis of the B16BL6 by *in vitro* treatment with D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars in mice

Compound	*Dose ($\mu g\,ml^{-1}$)*	*Inhibition of metastasis (%)*
Saline (0.9%)	0	0
27b	10	11.9
	30	75.0
	50	80.5**
	100	90.4**
32	10	48.5
	30	61.9
	50	90.8**
106	10	26
	30	29.6
	50	67.3*
107	10	59.1*
	30	74.2*
	50	87.1*

The B16BL6 cells were cultured with or without compounds in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum for 3 days.
The cells were harvested with 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) solution from culture dishes and washed twice with phosphate-buffered saline (PBS). The cell suspension (1×10^5) in PBS were implanted intravenously (i.v.) into the tail vein of BDF1 mice. Fourteen days later, the mice were autopsied and the numbers of pulmonary tumor nodules were counted.
*$P<0.01$; **$P<0.001$.

Table 7 Inhibition of experimental metastasis of the B16BL6 by *in vitro* treatment with L-altruronic acid-type *gem*-diamine 1-*N*-iminosugars in mice

Compound	*Dose ($\mu g\,ml^{-1}$)*	*Inhibition of metastasis (%)*
Saline (0.9%)	0	0
116	10	0
	30	12.1
	50	44.3*
119	10	40.1*
	30	91***
	50	97***
130	10	3.8
	30	38.1**
	50	75.5***
133	10	14.1
	30	58.8***
	50	81.0***

The B16BL6 cells were cultured with **116** and **119** for 3 days and with **130** and **133** for 1 day in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum. The cells were harvested with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) solution. The cells (1×10^5) in 0.1 ml of divalent cation-free Dulbecco's phosphate-buffered saline were collected and injected intravenously (i.v.) into the tail vein of BDF1 mice. Fourteen days later, the mice were autopsied and the pulmonary tumor colonies were counted.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.

Table 8 Inhibitory effect of D-galacturonic acid-type 2-trifluoroacetamide 1-*N*-iminosugar 32 on the spontaneous lung metastasis of 3LL cells in mice

Compound	*Administered dose ($mg\,kg^{-1}$)×days*	*Inhibition of metastasis (%)*
Saline (0.9%)	0×5	0
32	10×5	5.1
	50×5	23.5
	100×5	57.1*

Five female C57BL/6 mice per group inoculated with 3LL cells (1×10^6) by intra-footpad injection were administered intravenously (i.v.) with **32** for 5 days starting on the day of the surgical excision of primary tumors on day 9. Mice were killed 10 days after tumor excision.
*$P<0.01$.

differentiation of normal esophageal epithelium through nuclear translocation and nuclear HS cleavage and has an important role in the development of normal esophageal epithelium.

Inhibition of microglial cell migration

Very recently, microglia, the resident macrophages in the brain, have been found to express heparanase mRNA and protein, which can degrade the glycan chain of HSPGs.[48] Heparanase activity is correlated with the *in vitro* transmigration ability of microglia through an artificial basement membrane (BM)/extracellular matrix (ECM) containing HSPGs. D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugar **32** inhibits this process in a dose-dependent manner.[48] The transmigration of microglia through BM/ECM appears to be associated with the degradation of HSPG, and this is also inhibited dose dependently by **32**. The results suggest the involvement of heparanase in the migration or invasion of microglia or brain macrophages across the BM around the brain vasculature.

Table 9 Inhibition of invasive activity of tumor cells by 32

Experimental	Treatment ($\mu g ml^{-1}$)	Tumor cell line	Inhibition
1	0	3LL	0
	100	3LL	72.4*
	200	3LL	80.1*
2	0	B16BL6	0
	100	B16BL6	29.1
	300	B16BL6	64.1*

Tumor cells were cultured in the presence of **32** for 72 h (B16BL6) or 15 h (3LL). Numbers of cells that invaded the reconstituted basement membrane Matrigel in 6 h (Experiment 1) or 3 h (Experiment 2) were counted. A laminin coated under the filter surface was used as a cell attractant.
*$P<0.05$.

Table 10 Inhibition of invasive activity of B16BL6 cell by 119 and 133

Experimental	Compound	Treatment ($\mu g ml^{-1}$)	Inhibition %
1	**119**	0	0
		200	44.8*
		300	58.9**
2	**133**	0	0
		100	44.4
		200	61.1***
		300	63.9*

The cells were cultured with **119** and **133** for 72 and 24 h, respectively. Numbers of invaded cells on the lower surface of the Matrigel/laminin-coated filters in 3 h (Experiment 1) or 6 h (Experiment 2) were counted.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.

THERAPEUTIC POTENTIALS

Tumor metastasis

Recent biochemical studies have shown that cellular function and phenotype are highly influenced by HSPGs of ECM, and that the enzymatic degradation of ECM is involved in fundamental biological phenomena, including angiogenesis and cancer metastasis.[49–53] Proteolytic enzymes (heparanase and matrix metalloproteinases) secreted by tumor cells are capable of degrading ECM and BM components, and their activities are closely related to the metastasis potential of malignant cells.[54–60]

The inhibitory effects of uronic acid-type *gem*-diamine 1-*N*-iminosugars that have inhibitory activities against *exo*-uronidase, heparanase, sulfotransferase and sialyltransferase were also evaluated on tumor metastasis using the experimental and spontaneous pulmonary metastasis in mice.

D-galacturonic acid-type *gem*-diamine 1-*N*-iminosugars **27b**, **32**, **106** and **107** that have inhibitory activities against *exo*-uronidase and heparanase significantly suppress in a dose-dependent manner the number of colonies of pulmonary metastasis of B16BL6 cells in the experimental metastasis (Table 6).[6,7,26,27] Of these, 2-trifluoroacetamide **32** inhibits pulmonary metastasis most potently.

L-altruronic acid-type *gem*-diamine 1-*N*-iminosugars **116**, **119**, **130** and **133** that have inhibitory activity against HS 2-*O*-ST also reduce remarkably in a dose-dependent manner the pulmonary colonization of B16BL6 cells in the experimental metastasis (Table 7).[28] Of these, 2-acetamido-4-guanidino-1-*N*- iminosugar **119** inhibits the experimental metastasis very strongly.

As shown in Table 8, the inhibition of spontaneous lung metastasis in mice by the intravenous (i.v.) injection of **32** is more noticeable.[6,61] Compound **32** shows 57% inhibition of metastasis by the administration of 100 mg kg^{-1} per day for 5 days.

Table 11 Inhibitory activity (IC_{50} (M)) of 3-episiastatin B (160) and DDNA (161) against influenza virus *N*-acetylneuraminidase

	Influenza virus neuraminidase		
Compound	A/FM/1/47 (H1N1)	A/Kayano/57 (H2N2)	B/Lee/40
3-Episiastatin B	7.4×10^{-5}	$>1.0\times10^{-5}$ (25.6)	4.2×10^{-5}
DDNA	$<1.0\times10^{-5}$ (93.2)	2.9×10^{-5}	4.9×10^{-5}

Abbreviations: IC_{50}, half maximal inhibitory concentration;
( ): inhibition (%) at 1.0×10^{-5} M.
Neuraminidase inhibition assay was carried out using the method of Aminoff.[71,72]

Table 12 Inhibition (%) of 3-episiastatin B (160) and DDNA (161) against influenza virus A/FM/1/47 (H1N1) infection in MDCK cells

	Plaque forming units (PFU)			Stained area		
Compound	40 μM	20 μM	10 μM	40 μM	20 μM	10 μM
3-Episiastatin B	88.9	55.5	35.6	97.1	87.2	64.1
DDNA	100	100	89.6	100	100	98.7

Plaque assay was carried out using the modified method of Schulman and Palese.[71,73]

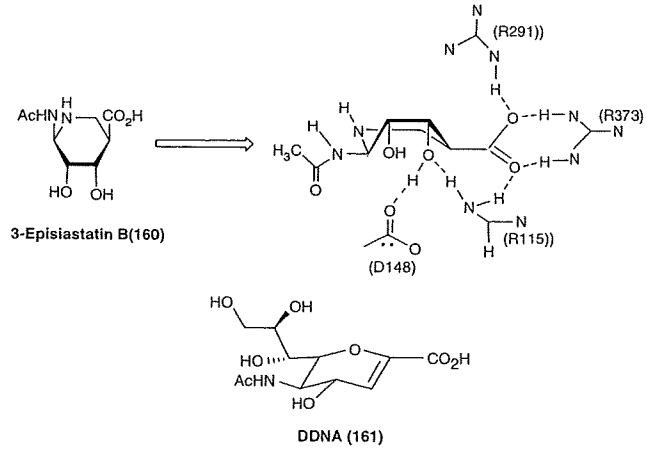


Figure 8 BIOCES[E] /AMBER minimized structure of 3-episiastatin B (**160**) in a pocket of active site residues of the crystal structure of influenza virus B/Beijin/1/87 and structure of DDNA (**161**).

On the other hand, D-galacturonic acid-type **32** and L-altruronic acid-types **119** and **133** inhibit in a dose-dependent manner the transmigration of B16BL6 and 3LL cells through the reconstituted BM (Matrigel) by *in vitro* treatment (Tables 9 and 10).[8,61]

Gem-diamine 1-*N*-iminosugars related to D-glucuronic and L-iduronic acids markedly inhibit the experimentally induced lung metastasis of B16BL6 and/or 3LL cells, and also the spontaneous lung metastasis of 3LL cells after i.v. administration. D-uronic acid-type iminosugars inhibit tumor heparanase activity, an effect that probably results from their resemblance to D-glucuronic acid as a substrate for tumor heparanase. L-uronic acid-type iminosugars inhibit HS 2-*O*-ST activity, an effect that probably results from their resemblance to L-iduronic acid as a substrate for HS 2-*O*-ST. Furthermore, *gem*-diamine 1-*N*-iminosugars prevent the transmigration of B16BL6 and/or 3LL cells through the reconstituted BM with no cytotoxicity. These results suggest that the anti-metastatic effect of the iminosugars may be due to their anti-invasive rather than their

anti-proliferative activities. It is likely that *gem*-diamine 1-*N*-iminosugars related to D-glucuronic and L-iduronic acids act as the mimic of respective uronic acids in the metabolism of ECM and/or BM involved in tumor metastasis. These iminosugars seem to modify the cell surface glycoconjugates of tumor cells simultaneously, thereby altering the cell properties involved in cellular recognition and adhesion.

Influenza virus infection

Some of the uronic acid-type *gem*-diamine 1-*N*-iminosugars could also mimic sialic acid (**4**) in the sialidase (*N*-acetyneuraminidase) reaction as an alternative type of *gem*-diamine 5-*N*-iminosugar such as siastatin B (**1**) (Figure 1).

Two integral membrane glycoproteins, hemagglutinin (HA) and NA, of the influenza virus were proved to have important roles at the beginning of infection and during the spread of the infection,[61–66] respectively, and it has been postulated that the inhibitors of HA and NA should have antiviral properties. NA is a glycosidase that cleaves the α-ketosidic bond linking the terminal sialic acid to the adjacent oligosaccharide residues of glycoproteins and glycolipids.[67,68] In 1992, the binding modes of sialic acid to NAs of the influenza virus B/Beijin/1/87 and A/Tokyo/3/67 were clarified to involve the characteristic α-boat conformation.[69,70]

3-Episiastatin B (**160**) shows specific potent inhibitory activities against influenza virus NAs and the influenza virus infection in the MDK cell *in vitro* (Tables 11 and 12).[71] Its activity is almost comparable with that of DDNA **161**, a standard inhibitor. The lowest energy boat conformer of **160** obtained by molecular modeling using PM3/MOPAC is superimposed onto the α-boat conformer of **4** in a pocket of the active site residue of the crystal structure of influenza virus B/Beijin/1/87 NA complex with **4** by a docking experiment using BIOCES/AMBER[6] (Figure 8). Compound **160** was shown to be a possible lead compound for anti-influenza virus agents.

Lysosomal storage disease

Lysosomal storage disease, in which specific enzymes of glycoconjugate degradation are deficient, is an inherited storage disorder characterized by the accumulation of partially degraded molecules in lysosomes, eventually resulting in cell, tissue and organ dysfunctions.[74] The strategies for overcoming the deficit in enzyme capacity is to provide an endogenous supply of completely functional enzymes by direct infusion or by cellular replacement with cells capable of secreting enzymes (bone marrow replacement) or by gene delivery.[75–77] An alternative to enzyme replacement is to reduce substrate influx to the lysosome by inhibiting the synthesis of glycoconjugates. This strategy has been called substrate reduction therapy.[78–80] By balancing the rate of glycoconjugate synthesis with the impaired rate of glycoconjugate breakdown, the substrate influx–efflux should be regulated to rates that do not lead to storage.

The enzyme deficient in Hunter's syndrome (MPS II) is iduronate 2-*O*-sulfatase, which functions by removing a 2-*O*-sulfate group from the iduronic acid unit of HS.[81–83] As mentioned above in the section 'Total synthetic route to *gem*-diamine 1-*N*-iminosugars,' compounds **119** and **159** inhibit strongly recombinant iduronate 2-*O*-ST over 80% at 25 μM.[43] Therefore, a partial inhibition of iduronate 2-*O*-ST by these iminosugars would reduce the build-up of the sulfated iduronic acid of HS in cells.

On the other hand, an alternative strategy, chemical chaperon therapy has been proposed for lysosomal storage disease, on the basis of a paradoxical phenomenon that states that an exogenous competitive inhibitor of low molecular weight stabilizes the target mutant protein and restores its catalytic activity as a molecular chaperon.[84–87] A competitive inhibitor binds to a misfolded mutant protein as a molecular chaperon in the endoplasmic reticulum/ Golgi apparatus of the cell, resulting in the formation of a stable complex at neutral pH and transport of the catalytically active enzyme to lysosomes, in which the complex dissociates under acidic conditions and the mutant enzyme remains stabilized and functional. Some iminosugars have shown remarkable efficacy for chemical chaperon therapy of Fabry and Gaucher's disease in clinical trials.[88] *Gem*-diamine 1-*N*-iminosugars have proven to be highly potent and specific competitive inhibitors against glycosidases, glycosyltransferases and sulfotransferases. These facts suggest that *gem*-diamine 1-*N*-iminosugars would be reasonable candidates for chemical chaperon therapy in lysosomal storage diseases, and that they are in principle applicable to all types of lysosomal storage diseases.

The main advantage of these therapies is the potential ability of the inhibitors as small molecules to cross the blood–brain barrier (BBB) and elicit a favorable response in the central nervous system (CNS). The difficulties in delivering proteins (enzymes) or genes to the CNS are not apparent using a small molecule that can cross the BBB easily. The therapy using iminosugars has the potential to prevent and/or reverse the effects of lysosomal storage disease both in the body and in the brain.

CLOSING REMARKS

This article describes our current progress in the chemical, biochemical and therapeutic potential of *gem*-diamine 1-*N*-iminosugars, a new family of glycomimetics, with a nitrogen atom in place of the anomeric carbon. Mechanistically, the protonated form of new glycomimetics may act as a mimic of a glycopyranosy cation and/or the transition state formed during enzymatic glycosidic hydrolysis. New inhibitors that mimic the charge at the anomeric position of the transition state have proven to be potent and specific inhibitors of various kinds of glycosidases.

New inhibitors that affect some metabolic enzymes of glycoconjugates have been found to participate in tumor metastasis. Uronic acid-type *gem*-diamine 1-*N*-iminosugars certainly contribute to the study regarding the involvement of carbohydrates in malignant cell movement and seem to be a promising new drug candidate for cancer chemotherapy. The *N*-acetylneuraminic acid-type iminosugar has shown potency against influenza virus infection, indicating a possible drug candidate that inhibits NA. It is also likely that *gem*-diamine 1-*N*-iminosugar, a new family of glycomimetics, is a reasonable drug candidate for chemical chaperon therapy and/or substrate reduction therapy in lysosomal storage disorder.

Iminosugars have proven to be a rich source of therapeutic drug candidates in the past several years and have thus become the special focus of research attention. Of these, *gem*-diamine 1-*N*-iminosugars 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.

ACKNOWLEDGEMENTS

A part of this work was supported by the Sumiki-Umezawa Memorial Award Research Grants from the Japan Antibiotic Research Association, by MPS Research Grants from the National MPS Society, USA and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (KAKENHI 14370761).

1 Compain, P. & Martin, O. R. ed. *Iminosugars: From Synthesis to Therapeutic Applications* 1–467 (John Wiley & Sons, West Sussex, (2007)).

2 Asano, N. Naturally occurring iminosugars and related compounds: structure, distribution, and biological activity. *Curr. Top. Med. Chem.* **3**, 471–484 (2003).
3 Inouye, S., Tsuruoka, T. & Niida, T. The structure of nojirimycin, a piperidinose sugar antibiotic. *J. Antibiot.* **19**, 288–292 (1966).
4 Cipolla, L., La Ferla, B. & Nicotra, F. General methods for iminosugar synthesis. *Curr. Top. Med. Chem.* **3**, 485–511 (2003).
5 Umezawa, H. *et al.* Purification and characterization of sialidase inhibitor, siastatin, produced by *Streptomyces*. *J. Antibiot.* **27**, 963–969 (1974).
6 Nishimura, Y. Synthesis and transformation of siastatin B, a novel glycosidase inhibitor, directed toward new drugs for viral infection and tumor metastasis in *Studies in Natural Products Chemistry* Vol. 16 (ed. Atta-ur-Rahman), 75–121 (Elsevier, Amsterdam, (1995)).
7 Nishimura, Y., Kudo, T., Kondo, S. & Takeuchi, T. Totally synthetic analogues of siastatin B. III. Trifluoroacetamide analogues having potent inhibitory activity for tumor metastasis. *J. Antibiot.* **47**, 101–107 (1994).
8 Nishimura, Y., Satoh, T., Kudo, T., Kondo, S. & Takeuchi, T. Synthesis and activity of 1-*N*-iminosugar inhibitors, siastatin B analogues for α-*N*-acetylgalactosaminnidase and β-*N*-acetylglucosaminidase. *Bioorg. Med. Chem.* **4**, 91–96 (1996).
9 McNaught, A. D. Nomenclature of carbohydrates. *Pure Appl. Chem.* **68**, 1919–2008 (1996)In the past many years, the incorrect terms 'azasugar' has been used by chemists refer to structures where oxygen is replaced by nitrogen. Use of the terms 'aza' should be restricted when carbon is replaced by nitrogen in the IUPAC rules of carbohydrate nomenclature. Thus, the 1-N-iminosugar described in this review is a true 1-aza-carbasugar. However, the terms '1-N-iminosugar' is expedientially used for easy discrimination from the old incorrect azasugar in this review.
10 Jespersen, T. M. *et al.* Isofagomine, a potent, new glycosidae inhibitor. *Angew. Chem. Int. Ed. Engl.* **33**, 1778–1779 (1994).
11 Lillelund, V. H., Jensen, H. H., Liang, X. & Bols, M. Recent developments of transition-state analogue glycosidase inhibitors non-natural product origin. *Chem. Rev.* **102**, 515–553 (2002).
12 Butter, T. D. Iminosugar inhibitors for substrate reduction therapy for the lysosomal glycosphingolipidoses in *Iminosugars: From Synthesis to Therapeutic Applications* (eds. Compain, P. & Martin, O.R.) 249–268 (John Wiley & Sons, West Sussex, (2007)).
13 Look, G. C., Fotsh, C. H. & Wong, C.-H. Enzyme-catalyzed organic synthesis: practical routes to aza sugars and their analogs for use as glycoprocessing inhibitors. *Acc. Chem. Res.* **26**, 182–190 (1993).
14 Ganem, B. Inhibitors of carbohydrate-processing enzymes: design and synthesis of sugar-shaped heterocycles. *Acc. Chem. Res.* **29**, 340–347 (1996).
15 Hoos, R., Vasella, A., Rupitz, K. & Withers, S. G. D-glyconhydroximolactams strongly inhibit α-glycosidases. *Carbohydr. Res.* **298**, 291–298 (1997).
16 Nishimura, Y., Wang, W., Kondo, S., Aoyagi, T. & Umezawa, H. Siastatin B, a potent sialidase inhibitor: the total synthesis and absolute configuration. *J. Am. Chem. Soc.* **110**, 7249–7250 (1988).
17 Nishimura, Y., Wang, W., Kudo, T. & Kondo, S. Total synthesis and absolute configuration of siastatin B, neuraminidase inhibitor. *Bull. Chem. Soc. Jpn* **65**, 978–986 (1992).
18 Walker, T. E. Hogenkamp HPC. A new synthesis of L-ribofuranose derivatives. *Carbohyd. Res.* **32**, 413–417 (1974).
19 Mitsunobu, O. The use of diethyl azodicarboxylate and triphenylphosphine in synthesis and transformation of natural products. *Synthesis* 1–28 (1981).
20 Kudo, T., Nishimura, Y., Kondo, S. & Takeuchi, T. Totally synthetic analogues of siastatin B. I. Optically active 2-acetamidopiperidine derivatives. *J. Antibiot.* **45**, 954–962 (1992).
21 Nishimura, Y., Kudo, T., Kondo, S. & Takeuchi, T. Totally synthetic analogues of siastatin B. II. Optically active piperidine derivatives having trifluoroacetamide and hydroxyacetamide groups at C-2. *J. Antibiot.* **45**, 963–970 (1992).
22 Nishimura, Y. *et al.* Flexible synthesis and biological activity of uronic acid-type 1-*N*-iminosugars: a new family of glycosidase inhibitors. *J. Org. Chem.* **65**, 2–11 (2000).
23 Morgenlie, S. Synthesis of di-*O*-isopropylidene derivatives of L-fructose. *Carbohycl. Res.* **107**, 137–141 (1982).
24 Nishimura, Y., Shitara, E. & Takeuchi, T. Enantioselective synthesis of a new family of α-L-fucosidase inhibitors. *Tetrahedron Lett.* **40**, 2351–2354 (1999).
25 Shitara, E., Nishimura, Y., Kojima, F. & Takeuchi, T. *Gem*-diamine 1-*N*-iminosugars of L-fucose-type, the extremely potent L-fucosidase inhibitors. *Bioorg. Med. Chem.* **8**, 343–352 (2000).
26 Satoh, T., Nishimura, Y., Kondo, S. & Takeuchi, T. A practical synthesis of (3*S*,4*S*,5*R*,6*R*)-4,5-dihydroxy-6-(trifluoroacetamido)piperidine-3-carboxylic acid having antimetastatic activity in mice from siastatin B. *Carbohydr. Res.* **286**, 173–178 (1996).
27 Satoh, T. *et al.* Synthesis and antimetastatic activity of 6-trichloroacetamido and 6-guanidino analogues of siastatin B. *J. Antibiot.* **49**, 321–325 (1996).
28 Nishimura, Y. *et al.* The first L-iduronic acid-type 1-*N*-iminosugars having inhibitory activity of experimental metastasis. *J. Am. Chem. Soc.* **118**, 3051–3052 (1996).
29 Nishimura, Y. *et al.* Synthesis and antimetastatic activity of L-iduronic acid-type 1-*N*-iminosugars. *J. Med. Chem.* **40**, 2626–2633 (1997).
30 Nishimura, Y., Satoh, T., Kudo, T., Kondo, S. & Takeuchi, T. Synthesis and activity of 1-*N*-iminosugar inhibitors, siastatin B analogues for α-*N*-acetylgalactosaminidase and β-*N*-acetylglucosaminidase. *Bioorg. Med. Chem.* **4**, 91–96 (1996).
31 Shitara, E., Nishimura, Y., Kojima, F. & Takeuchi, T. A facile synthesis of D-galactose-type *gem*-diamine 1-*N*-iminosugr: a new family of galactosidase inhibitor. *J. Antibiot.* **52**, 348–350 (1999).
32 Shitara, E., Nishimura, Y., Kojima, F. & Takeuchi, T. A facile synthesis of D-glucose-type *gem*-diamine 1-*N*-iminosugars: a new family of glucosidase inhibitors. *Bioorg. Med. Chem.* **7**, 1241–1246 (1999).
33 Nishimura, Y. Glycosidase and glycosyltransferase inhibitors in *Studies in Natural Products Chemistry* Vol. 10 (ed. Atta-ur-Rahman), 495–583 (Elsevier, Amsterdam, (1992)).
34 Stütz, A. D. ed. *Iminosugars as Glycosidase Inhibitors: Nojirimycin and Beyond* 1–412 (Wiley-VCH, New York, (1999)).
35 Martin, O. R. & Compain, P. (ed.) Iminosugars: recent insights into their bioactivity and potential as therapeutic agents. *Curr. Top. Med. Chem.* **3**, 471–591 (2003).
36 Nishimura, Y. *Gem*-diamine 1-*N*-iminosugars and related iminosugars, candidate of therapeutic agents for tumor metastasis. *Curr. Top. Med. Chem.* **3**, 575–591 (2003).
37 Nishimura, Y. *Gem*-diamine 1-*N*-iminosugars, a new family of glycosidase inhibitors: synthesis and biological activity. *Heterocycles* **67**, 461–488 (2006).
38 Nishimura, Y. Iminosugar-based antitumoral agents in *Iminosugars: From Synthesis to Therapeutic Applications* (ed. Compain, P. & Martin, O.R.) 269–294 (John Wiley & Sons, West Sussex, (2007)).
39 Kondo, K., Adachi, H., Shitara, E., Kojima, F. & Nishimura, Y. Glycosidase inhibitors of *gem*-diamine 1-*N*-iminosugars: Structures in media of enzyme assays. *Bioorg. Med. Chem.* **9**, 1091–1095 (2001).
40 Lopez, O. & Bols, M. Isofagomine, noeuromycin and other 1-azasugars, iminosugar-related glycosidase inhibitors in *Iminosugars: From Synthesis to Therapeutic Applications* (ed. Compain, P. & Martin, O.R.) 131–151 John Wiley & Sons, West Sussex (2007).
41 Toyoshima, M. & Nakajima, M. Human heparanase. *J. Biol. Chem.* **274**, 24153–24160 (1999).
42 Tsuruoka, T., Tsuji, T., Nojiri, H., Holmes, H. & Hakomori, S. Selection of a mutant cell line based on differential expression of glycosphingolipid, utilizing anti-lactosylceramide antibody and complement. *J. Biol. Chem.* **268**, 2211—2216 (1993).
43 Brown, J. R., Nishimura, Y. & Esko, J. D. Synthesis and biological evaluation of *gem*-diamine 1-*N*-iminosugars related to L-iduronic acid as inhibitors of heparan sulfate 2-O-sulfotransferase. *Bioorg. Med. Chem. Lett.* **16**, 532–536 (2006).
44 Esko, J. D. & Lindahl, U. Molecular diversity of heparan sulfate. *J. Clin. Invest.* **108**, 169–173 (2001).
45 Ohkawa, T. *et al.* Localization of heparanase in esophageal cancer cells: respective roles in prognosis and differentiation. *Lab. Invest.* **84**, 1289–1304 (2004).
46 Nobuhisa, T. *et al.* Emergence of nuclear heparanase induces differentiation of human mammary cancer cells. *Biochem. Biophys. Res. Commun.* **331**, 175–180 (2005).
47 Kobayashi, M. *et al.* Heparanase regulates esophageal keratinocyte differentiation through nuclear translocation and heparan sulfate cleavage. *Differentiation* **74**, 235–243 (2006).
48 Takahashi, H. *et al.* Involvement of heparanase in migration of microglial cells. *Biochim. Biophys. Acta* **1780**, 709–715 (2008).
49 Esko, J. D. & Selleck, S. B. Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu. Rev. Biochem.* **71**, 435–471 (2002).
50 Turnbull, J., Powell, A. & Guimond, S. Heparan sulfate: decoding a dynamic multifunctional cell regulator. *Trends Cell Biol.* **11**, 75–82 (2001).
51 Bernfield, M. *et al.* Function of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* **68**, 729–777 (1999).
52 Iozzo, R. V. Matrix proteoglycans: from molecular design to cellular function. *Annu. Rev. Biochem.* **67**, 609–652 (1998).
53 Wight, T. N., Kinsella, M. G. & Qwarnstrom, E. E. The role of proteoglycans in cell adhesion, migration and proliferation. *Curr. Opin. Cell Biol.* **4**, 739–810 (1992).
54 Ilan, N., Elkin, M. & Vlodavsky, I. Regulation, function and clinical significance if heparanase in cancer metastasis and angiogenesis. *Int. J. Biochem. Cell Biol.* **38**, 2018–2039 (2006).
55 Goldshmidt, O. *et al.* Cell surface expression and secretion of heparanase markedly promote tumor angiogenesis and metastasis. *Proc. Natl Acad. Sci. USA* **99**, 10031–10036 (2002).
56 Vlodavsky, I. *et al.* Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. *Nat. Med.* **5**, 793–802 (1999).
57 Hulett, M. D., Freemen, C., Hamdorf, B. J., Baker, R. T., Harris, M. J. & Parish, C. R. Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. *Nat. Med.* **5**, 803–809 (1999).
58 Nakajima, M., Morikawa, K., Fabra, A., Bucana, C. D. & Fidler, I. J. Influence of organ environment on extracellular matrix degradation activity and metastasis of human colon carcinoma cells. *J. Natl Cancer Inst.* **82**, 1890–1898 (1990).
59 Woolley, D. E. Collagenolytic mechanisms in tumor cell invasion. *Cancer Metastasis Rev.* **3**, 361–372 (1984).
60 Sloane, B. F. & Honn, K. V. Cystein proteinase and metastasis. *Cancer Metastasis Rev.* **3**, 249–263 (1984).
61 Nishimura, Y. *et al.* Effect on spontaneous metastasis of mouse lung carcinoma by a trifluoroacetamide analogues of siastatin B. *J. Antibiot.* **47**, 840–842 (1994).
62 Paulson, J. C. Interactions of animal viruses with cell surface receptors in *The Receptors* Vol. 2 (ed. Conn, P.M.) 131–219 (Academic Press, New York, (1985)).
63 Wiley, D. C. & Skehel, J. J. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* **56**, 365–394 (1987).
64 Weis, W. *et al.* Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333**, 426–431 (1988).
65 Klenk, H. D. & Rott, R. The molecular biology of influenza virus pathogenicity. *Adv. Virus Res.* **34**, 247–281 (1988).
66 Colman, P. & Ward, C. W. Structure and diversity of influenza virus neuraminidase. *Curr. Top. Microbiol. Immunol.* **114**, 177–255 (1985).

67 Gottschalk, A. Neuraminidase: the specific enzyme of influenza virus and Vibrio cholera. *Biochem. Biophys. Acta* **23**, 645–646 (1957).
68 Schauer, R. Sialic acid and their role as biological masks. *Trends Biochem. Sci.* **10**, 357–360 (1985).
69 Burmeister, W. P., Ruigrok, R. W. H. & Cusack, S. The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.* **11**, 49–56 (1992).
70 Varghese, J. N. & Colman, P. M. Three-dimensional structure of the neuraminidase of influenza virus A/Tokyo/3/67 at 2.2 Å resolution. *J. Mol. Biol.* **221**, 473–486 (1991).
71 Nishimura, Y. *et al.* Synthesis of 3-episiastatin B analogues having anti-influenza virus activity. *J Antibiot.* **46**, 1883–1889 (1993).
72 Aminoff, D. Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**, 384–392 (1961).
73 Schulman, J. L. & Palese, P. Susceptibility of different strains of influenza A virus to the inhibitory effect of 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA). *Virology* **63**, 98–104 (1975).
74 Winchester, B., Vellodi, A. & Young, E. The molecular basis of lysosomal storage diseases and their treatment. *Biochem. Soc. Trans.* **28**, 150–154 (2000).
75 Schiffmann, R. & Brady, R. O. New prospect for the treatment of lysosomal storage diseases. *Drugs* **62**, 733–742 (2002).
76 Ellinwood, N. M., Vite, C. H. & Haskins, M. E. Gene therapy for lysosomal storage diseases: the lessons and promise of animal models. *J. Gene. Med.* **6**, 481–506 (2004).
77 Eto, Y., Shen, J. S., Meng, X. L. & Ohashi, T. Treatment of lysosomal storage disorders: cell therapy and gene therapy. *J. Inherit. Metab. Dis.* **27**, 411–415 (2004).
78 Butters, T. D., Raymond, D. A. & Platt, F. M. Inhibition of glycosphingolipid: application to lysosomal storage disorder. *Chem. Rev.* **100**, 4683–4969 (2000).
79 Platt, F. M. *et al.* Inhibition of substrate synthesis as a strategy for glycolipids lysosomal storage disease therapy. *J. Inherit. Metab. Dis.* **24**, 275–290 (2001).
80 Cox, T. *et al.* Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT918) to decrease substrate biosynthesis. *Lancet* **355**, 1481–1485 (2000).
81 Wraith, J. E. *et al.* Mucopolysaccharidosis type II (Hunter syndrome): a clinical review and recommendation for treatment in the era of enzyme replacement therapy. *Eur. J. Pediatr.* **167**, 267–277 (2008).
82 Hunter, C. A. A rare disease in two brothers. *Proc. R. Soc. Med.* **10**, 104–116 (1917).
83 Wilson, P. J. *et al.* Hunter syndrome: isolation of an iduronate-2-sulfatase cDNA clone and analysis of patient DNA. *Proc. Natl Acad. Sci. USA* **87**, 8531–8535 (1990).
84 Sawkar, A. R. *et al.* Chemical chaperones increase the cellular activity of N370S β-glucosidase: a therapeutic strategy for Gaucher disease. *Proc. Natl Acad. Sci. USA* **99**, 1528–15433 (2002).
85 Matsuda, J. *et al.* Chemical chaperon therapy for brain pathology in G(M_1)-gangliosidosis. *Proc. Natl Acad. Sci. USA* **100**, 15912–15917 (2003).
86 Perlmuntter, D. H. Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. *Pediatr. Res.* **52**, 832–836 (2002).
87 Suzuki, Y., Ogawa, S. & Sakakibara, Y. Chaperone therapy for neuronopathic lysosomal disease: competitive inhibitors as chemical chaperones for enhancement of mutant enzyme activities. *Perspectives in Medicinal Chemistry* **3**, 7–19 (2009).
88 Fan, J. Q. Iminosugars as active-site-specific chaperones for the treatment of lysosomal storage disorders in *Iminosugars: From Synthesis to Therapeutic Applications* (eds. Compain, P. & Martin, O.R.) 225–247 (John Wiley & Sons, West Sussex, (2007)).

Carbohydrate Research 344 (2009) 2088–2092

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

5a-Carba-glycopyranoside primers: potential building blocks for biocombinatorial synthesis of glycosphingolipid analogues

Hiroshi Aoyama, Seiichiro Ogawa *, Toshinori Sato *

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

ARTICLE INFO

Article history:
Received 21 December 2008
Received in revised form 29 May 2009
Accepted 22 June 2009
Available online 26 June 2009

Keywords:
Carba sugars
Biocombinatorial synthesis
Carba glycosides
Saccharide primers

ABSTRACT

Three ether-linked alkyl 5a-carba-glycopyranosides **1b,d,** and **5b**, and 5a′-carba-lactoside **7b** were examined as potent primers in mouse B16 melanoma cells for their feasibility as building blocks for oligosaccharide biosynthesis. Uptake by B16 cells was first observed for all carba-glycoside primers, and, especially, the 5a-carba-sugar analogues of *N*-acetyl-β-D-glucosaminide **1b** and β-D-glucoside **1d** were shown to produce two-to-four-fold larger amounts of glycosylated products than the corresponding true sugar primers **1a** and **1c**. The carba glycoside uptake by cells resulted in β-galactosylation and subsequent sialylation of the incorporated galactose residues, giving rise to glycosylated products **3b** and **3d** having similar glycan structures as the ganglioside GM3. According to efficient uptake in cells, in addition to stability of the ether-linked pseudo-reducing ends of the oligosaccharides that formed, the carba glycoside primers have been demonstrated to be versatile building blocks for these biocombinatorial syntheses of glycolipid oligosaccharide mimetics. On the other hand, uptake for 5a-carba-galactopyranoside residue was found to be decreased by one-third for dodecyl 5a-carba-β-D-galactopyranoside **5b**. Observation of similar levels for 5a′-carba-β-lactoside **7b** under both cellular and cell-free conditions suggested that enzymes are likely to recognize the pyranose oxygen atom.

Biosynthesis of glycolipids, glycoproteins, and glycosaminoglucans is initiated by the presence of primary precursors to which glycose residues are transferred for elongation of glycosyl chains. Therefore, organic compounds mimicking such precursors may be utilized for biosynthetic processes. Initially, glycosyl primers stimulated interest as competitive inhibitors in biosynthesis of glycans. In 1973, Suzuki and co-workers[1] described that *p*-nitrophenyl β-xyloside incorporated into cells initiated elongation of glycosaminyl gylcan chains, whereas biosynthesis of glycosaminyl glycans on cell surfaces was inhibited. Thereby, xylosides were shown to act as competitive inhibitors of the Ser-*O*-xyloside in normal biosynthesis of glycans. Subsequently, benzyl α-GalNAc was demonstrated to be an inhibitor of genesis of mutin-type glycoproteins and polylactosamine chains in CHO cells.[2] On the other hand, Galβ1→4GlcNAcβ-*O*-CH_2Napht (NM) and GlcNAcβ1→4Galβ-NM were demonstrated to function as primers for elongation of sialyl Lewis X oligosaccharide chains.[3,4] In 1997, lactosylceramide was recognized to be a common intermediate in glycosphingolipid synthesis.[5] In addition, administration of certain lactose derivatives to PC12 cells was found to stimulate transport of galactose residues,[6] affording Gb3 type oligosaccharides that were subsequently excreted to the extracellular environment. From such observations, sugar primers became considered applicable for priming production of new types of glycans, rather than as inhibitors of glycan biosynthesis. Furthermore, it was proposed that effective combinations of sugar primers and cell types could be utilized for construction of a novel oligosaccharide library.[7] A number of dodecyl glycosides, α- and β-lactosides, β-galactoside, *N*-acetyl-β-glucosaminide, and *N*-acetyl-β-lactosaminide are known to act as substrate primers[8,9] for elongation of specific oligosaccharide chains when administered into B16 mouse melanoma cells. In contrast the α-galactoside and the structurally related α- and β-glucosides have been found not to be sialylated.[8] The β-glucoside primer exhibited cytotoxicity. Thus, a terminal galactose moiety that is linked to the adjacent saccharide or aglycone unit should have been thought essential for glycosylation by cellular enzyme. However, octyl β-glucoside was later been demonstrated to act as substrate to give sialylated oligosaccharides,[10] revealing that the aglycone structure may be crucial as potent primers.

Carba sugars are important sugar mimics[11] that have attracted attention for the development of new bioactive compounds. We have extensively investigated elaboration of glycosidase[12] and glycosyltransferase inhibitors,[13] and probes for certain glycose substrates.[14,15] In this paper, we describe application of carba glycosyl primers for biocombinatorial synthesis. The reducing ends of carba oligosaccharides formed in cells cannot be hydrolyzed by glycosidases owing to their ether-linked pseudo-glycosidic bonds, so that these priming moieties of oligosaccharide chains are

* Corresponding authors. Tel.: +81 45 566 1771; fax: +81 45 566 1447.
E-mail address: sato@bio.keio.ac.jp (T. Sato).

0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2009.06.029

biochemically very stable in cells, allowing us to effectively isolate products subsequent to incubation.

Based on the preceding experimental results[8,9] on true sugar glycoside primers, attempts were made to choose three dodecyl carba glycosides **1b**, **5b**, and **7b**, and octyl carba glucoside **1d** as primers and were introduced into mouse B16 melanoma cells. Dodecyl *N*-acetyl-5a-carba-β-D-glucosaminide[16] (**1b**, Scheme 1) was first administered to estimate elongation of oligosaccharide chains by determining components of product fractions with high-performance thin-layer chromatography (HPTLC).

Comparison of the respective products formed by incorporation of the true sugar primer **1a** and the carba sugar primer **1b** is a convenient approach for the rough estimation of ongoing processes. The latter case, in addition to two products **2b** and **3b** with similar HPTLC R_f values to those of **2a** and **3a**, formation of new components **3b'** and **4b** was observed (Table 1). Their assigned structures, evidenced by R_f value, demonstrated that carba sugar primer **1b** could be incorporated effectively to form a series of new carba oligosaccharides. In fact, the components corresponding to **3b'** and **4b** were not detected in the product mixture obtained on incubation with **1a**.

In the previous paper, the structures of **2a** and **3a** derived from **1a** in B16 melanoma cells were deduced to be Galβ1→4GlcNAc-C12 and NeuNAcα2→3Galβ1→4GlcNAc-C12, respectively.[9] The structures of **2b** and **3b** derived from **1b** were inferred to have the same sugar elongation as **2a** and **3a**. Four HPTLC bands obtained on incorporation of carba sugar primer **1b** (Scheme 1) were subjected to TLC blotting for isolation of components, and mass spectrometric analyses were carried out using a MALDI-TOFMS instrument (Table 2). Compound **2b** was observed to have *m/z* 550.99, pointing to a structure composed of **1b** coupled with one hexose unit. From previously generated knowledge on the biosynthesis of oligosaccharides in B16 cells, a carba disaccharide might comprise **1b** with a D-galactose residue bonded via a β-(1→4)-linkage. Compound **3b** was indicated to possess *m/z* 863.47 and 885.31, consistent with the carba trisaccharide **2b** bonded to NeuNAc via an α-(2→3)-linkage.

The amount of compound **3b'** was insufficient to carry out a MALDI-TOFMS analysis, but considering the HPTLC R_f values, it ap-

Table 1
Carba glycolipids produced by adding carba sugar primer **1a** and **1b** to mouse B16 melanoma cells incubated for 24 h

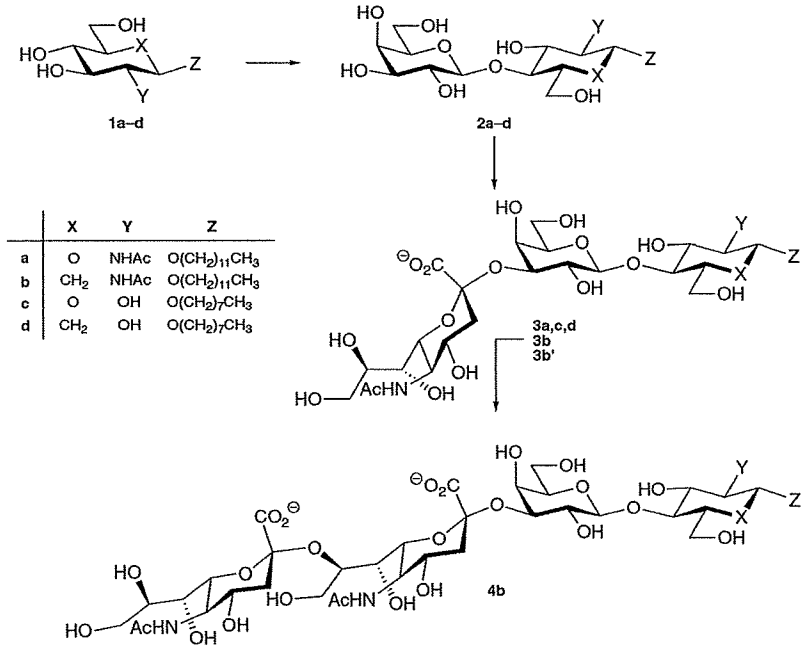
Compounds	R_f
1a	0.86
2a	0.61
3a	0.43
1b	0.78
2b	0.62
3b	0.43
3b'	0.40
4b	0.37

Glycolipid analogues were separated by HPTLC, then analyzed by coloring with resorcinol–aq HCl and orcinol–H_2SO_4.

Table 2
MALDI-TOFMS data for biocombinatorial products

Primer	Products	MALDI-TOF MS	
		Calcd (*m/z*)	Found (*m/z*)
1b	**2b**	550.36[a] $[M+H]^+$	550.99
	3b	863.44[a] $[M+Na]^+$	863.47
		885.42[a] $[M-H+2Na]^+$	885.31
	4b	1198.49[a] $[M-2H+3Na]^+$	1199.67
1d	**2d**	453.27[a] $[M-H]^+$	453.58
	3d	742.35[b] $[M-H]^+$	742.65
5b	**6b**	636.36[b] $[M-H]^+$	636.62
7b	**8b**	798.41[b] $[M-H]^+$	798.16

[a] Positive-ion mode.
[b] Negative-ion mode.



Scheme 1. Biocombinatorial synthesis of GM3 analogues **3b** and **3d** after uptake of carba sugar primers **1b** and **1d** into mouse B16 melanoma cells. Compound **3b'**: positional isomer with regard to the sialyl moiety.

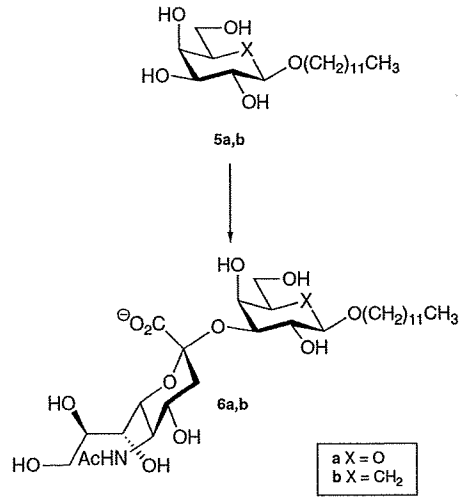
peared to be an isomer of **3b**, with the NeuNAc residue bonded differently in terms of type and/or position.

Compound **4b** showed *m/z* 1199.67, indicating the presence of one additional sialyl residue compared to that of **3b**.

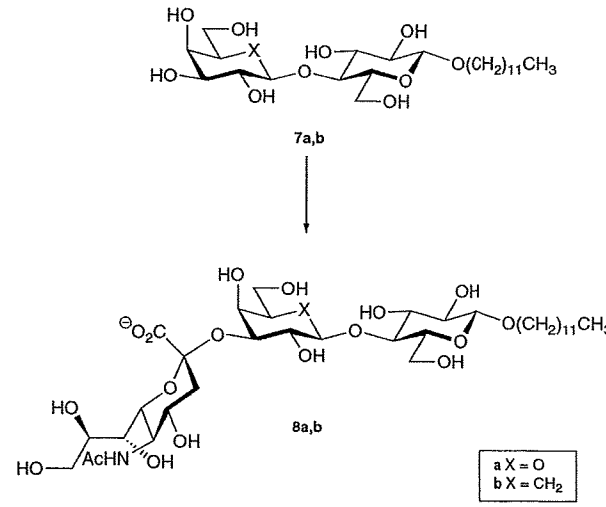
A similar experiment in which carba sugar primers[16] **1d**, **5b**, and **7b** (Schemes 1–3) were administered to B16 melanoma cells was carried out. Since dodecyl β-D-glucopyranoside is known to be toxic,[13] the corresponding octyl derivatives **1c** and **1d** were chosen as the primers. Two products **2d** and **3d** formed after uptake of **1d** were characterized, and a standard structural analysis demonstrated them to be the lactose and GM3 type disaccharides, respectively, which is in line with the experimental results observed for **1c**. Sialylgalactose type and GM3 type oligosaccharides formed from **5b** and **7b** were assigned the structures **6b** and **8b**, respectively. Enzymatic degradation of **8b** by use of α-(2→3)-neuraminidase was shown to produce **7b** to give additional evidence for the proposed structure. The structure of **8a** was determined to be GM3 type according to the literature.[8]

In order to determine product ratios of the respective products formed by incorporation of true and carba sugar primers, HPTLC was performed by spraying resorcinol and orcinol–H_2SO_4 reagents, and relative amounts of each component were measured calorimetrically by a densitometer. Figures 1–3 show ratios with the amount of products obtained by incorporation of the true sugar primer as the unit. Carba oligosaccharides **2b** and **3b** derived by incorporation of **1b** were shown to be produced in one-half to three to four times the quantities as compared with the corresponding compounds obtained from the true sugar primer **1a** (Fig. 1).

All four tested carba sugar primers[16] **1b**, **1d**, **5b**, and **7b** became incorporated into cells and initiated elongation of oligosaccharide chains. It is noteworthy that production of new oligosaccharides initiated by the primers **1b** and **1d** was much higher than the corresponding true sugar primers **1a** and **1c** (Figs. 1 and 2). In addition, two new oligosaccharides **3b′** and **4b** were formed in the case of **1b**, which were lacking under similar conditions when the true primer **1a** was applied. These results might be explained by assuming that primers can play roles as toxic substances, but no such toxic effects were apparent. Therefore, the good yields of the oligosaccharides might be attributed to their biochemical stability in cells and ready transport into the Golgi complex, thereby improving the production and isolation of the desired compounds.



Scheme 2. Biocombinatorial synthesis of NeuAc(α2-3)5aCGalβ-$C_{12}H_{25}$ (**6b**) after uptake of carbagalactoside primer **5b** into mouse B16 melanoma cells.



Scheme 3. Biocombinatorial synthesis of GM3 analogue **9b** after uptake of carbalactoside primer **8b** into mouse B16 melanoma cells.

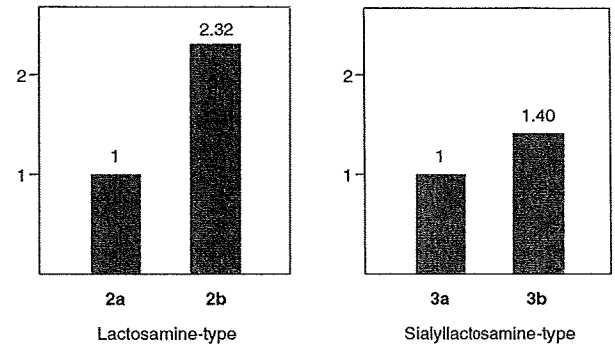


Figure 1. Production ratios of lactosamine-type and sialyl-lactosamine-type oligosaccharides, derived from the *N*-acetyl glucosamine-type primers **1a**,**b** incubated for 24 h in mouse B16 melanoma cells. Compounds **2a** and **3a** were assigned the value of 1.

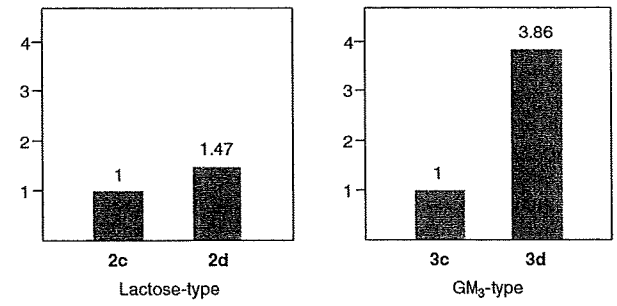


Figure 2. Production ratios of lactose-type and GM3-type oligosaccharides derived from the true and carba sugar glucoside-type primers **1c**,**d** incubated for 24 h in mouse B16 melanoma cells. Compounds **2c** and **3c** were assigned the value of 1.

The mouse B16 melanoma cell line is well known to express high levels of GM3. Vedralova et al.[17] noted that all examples tested expressed low levels of GM3. Accordingly, compound **4b** was assigned a pseudotetrasaccharide structure containing one additional NeuNAc residue attached at C-8″. It has yet to be

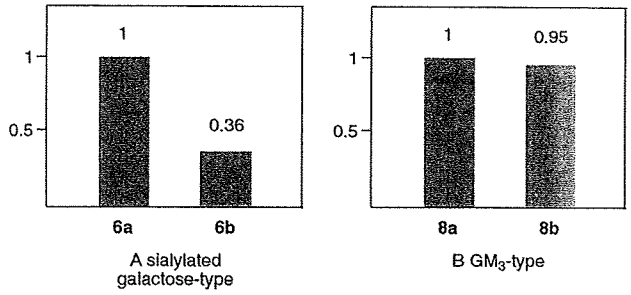


Figure 3. Production ratios of sialylated galactose-type oligosaccharides and GM3-type oligosaccharides, derived from the galactose-type **5a,b** and lactose-type primers **7a,b** incubated for 24 h in mouse B16 melanoma cells. Compounds **6a** and **8a** were assigned the value of 1.

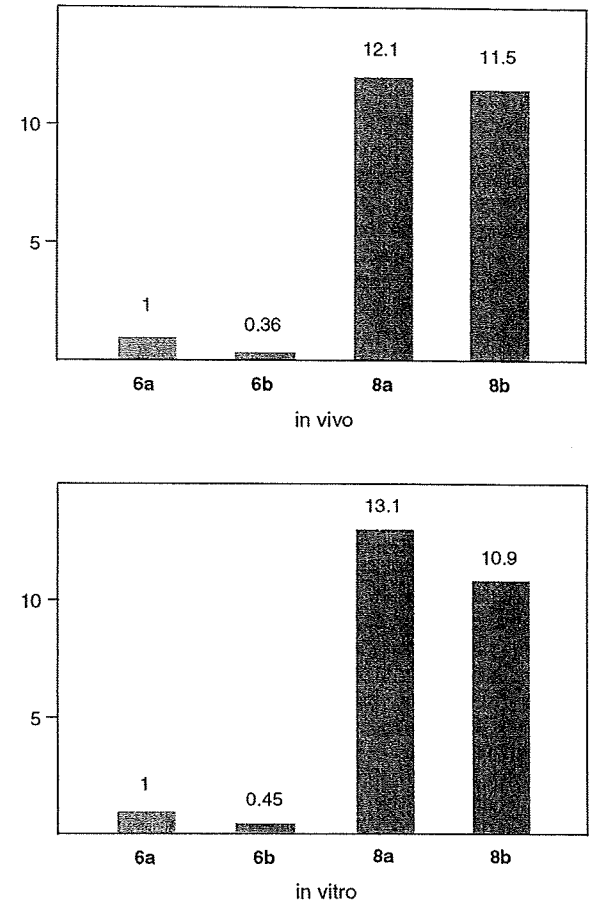


Figure 4. Comparison of sialylated products **6a,b** and **8a,b** derived from the respective galactoside-type **5a,b** and lactoside-type primers **7a,b**, respectively, from in vivo and in vitro experiments. The amounts of compound **6a** were assigned the value of 1.

confirmed by which cellular biosynthetic routes for glycoproteins or glycolipids the primers could be incorporated.

Production of the sialyl galactoside **6b** from carba sugar primer **5b** was decreased appreciably compared to that obtained from the true sugar primer **5a** (Fig. 3, left). In contrast, sialyl lactoside **8b** was produced at a similar level to that of **8a** from the lactoside-type primer **7a** (Fig. 3, right), so that the sialyl transferase appeared to recognize the substrate galactoside ring oxygen atom was with important roles for lactoside molecules. The transferase was therefore considered to recognize both galactose and glucose residues of lactoside primers **7a,b**.

Human milk α-(1→3/4)-fucosyltransferase seemed to recognize not the ring oxygen atom of the carbagalactose (CGal) residue of CGal β-(1→3)-GlcNAc, but that of CGal β1→4GlcNAc.[15] Therefore, the α-(2→3)-sialyltransferase (mouse B16 melanoma cells) may recognize pyranose oxygen atoms.

When cells were homogenized in order to destroy the localization of the enzyme and other factors involved in in vivo reaction, primer incubation demonstrated that, under both in vivo and in vitro conditions, relationships of primers and products are similar (Fig. 4). Thus the amounts of products should be due to enzyme recognition. However, this would not clear the case for each primer regarding ease of production of oligosaccharides. Biochemical stability of the products in cells seemed to be more reflective of the present results.

Under cell-free conditions, both sugar and carba sugar primers, and the lactoside primer could be more readily sialylated at the galactoside moiety than at a single galactoside primer. Mouse B16 melanoma sialyltransferase can therefore be considered capable of recognizing both galactose and glucose residues of the lactoside substrate **7a**.

On the other hand, with incubation of *N*-acetylglucosaminide and glucoside type primers **1b** and **1c**, recognition of the ring oxygen atoms may have influenced the glycosylation reaction, and the unhydrolyzable features of the carba oligosaccharides could be advantageous for production initiated by the carba sugar primers **1b** and **1d**.

1. Experimental

1.1. General

Mouse B16 melanoma cells were obtained from Riken Cell Bank (Tsukuba, Japan). Dulbecco modified Eagles's Medium (DMEM) was from ICN Biomedicals, Inc., DMEM and Ham F12 (1:1), and fetal bovine serum (FBS) were from Gibco BRL. Insulin and transferrin were from Wako Pure Chemical, Tokyo, SepPak C_{18} cartridges were from Waters, and HPTLC (Silica Gel 60) and preparative TLC plates were from E. Merck, Darmstadt, Germany. The carba glycoside primers were dissolved in Me_2SO to an initial concentration of 50 mM. MALDI mass spectra were recorded on a Voyager DE™ Bio-Specrometry™ Workstation (Perseptive Biosystems) with a 2,5-dihydroxybenzoic acid (DHB) matrix. All incubation experiments were carried out in triplicate in order to examine the reproducibility of values. α-(2→3)-Neuraminidase (480706, *Macrobdella decora*, Ricombinant) was obtained from Calbiochem.

1.2. Saccharide-chain elongation reactions in vivo

1.2.1. Cell culture

Mouse B16 melanoma cells were cultured in 1:1 DMEM–F12 supplemented with 10% fetal bovine serum (FBS). Cells were detached through application of 0.25% trypsin–EDTA, passaged every three days and maintained in a humidified atmosphere of 5% CO_2 air at 37 °C.

1.2.2. Incubation of B16 melanoma cells with carba glycoside primers

Inocula of 3.0×10^6 cells were seeded into 100-mm culture dishes containing 7 mL of medium and incubated for 48 h. This was followed by washing with TI-DF without phenol red (1:1 DMEM–Hams F12 containing 30 nM SeO_2, 5 μg/mL transferrin,

and 50 μg/mL insulin) to remove the serum, and cells were incubated with 50 μM of the primers for 48 h at 37 °C. After incubation, the culture media were collected and the cells were washed with PBS (−), harvested with 0.25% EDTA in PBS (−), and centrifuged at 1000 rpm for 10 min. The lipids were extracted from the culture medium with 2:1 $CHCl_3$–MeOH, then with 7:11:2 $CHCl_3$–2-propanol–water, and purified using a SepPak C_{18} cartridge. Lipids from the culture medium fractions were analyzed by HPTLC with 5:4:1 $CHCl_3$–MeOH–0.2% aq $CaCl_2$ as the developing solvent. The plates were sprayed with resorcinol–aq HCl and orcinol–H_2SO_4 reagent, and then heated to detect the separated glycolipids, which were later quantified using a densitometer (CS 9000, Shimadzu Seisakusho CO.).

1.2.3. TLC blotting

The glycolipids were developed on HPTLC plates, and spots were visualized by spraying with Primulin reagent. After being dipped in a mixture of 2-propanol–MeOH–0.2% aq $CaCl_2$ 40:7:20 2-PrOH- for 20 min., each plate was covered in turn with a PVDF sheet (ATTO), a PTFE sheet (ATTO), and glass fiber, and the glycolipids were plotted on the PVDF sheet with a TLC Thermal plotter (AC-5970, ATTO) for 30 s at 180 °C. The desired glycolipid fractions were cut out and extracted successively with MeOH (1 mL) and 2:1 $CHCl_3$–MeOH (1 mL).

1.3. Saccharide-chain elongation reactions in vitro

1.3.1. Extraction of the Golgi fractions from melanoma B16 cells

Confluent B16 cell cultures obtained from forty dishes (100 mmϕ) were centrifuged for 5 min., the supernatants were removed, and the precipitates were washed with PBS (−) before centrifugation of the mixture once again. Precipitated cells were collected and suspended in 1 mM DTT–10 mM Tris–HCl buffer, containing 1.5 mM $MgCl_2$ and 10 mM KCl.

1.3.2. Enzymatic reactions in vitro

To each Golgi fraction in a centrifuge tube were added the primer (0.5 mM) and CMP–NeuAc (600 μg), together with a cacodyl acid buffer solution (100 μL, pH 6.5) [5 mM $MgCl_2$, 1 mM triton X-100 (Sigma)], and the mixture was incubated for 3 h at 37 °C. The reaction was quenched by addition of 1 mL of Milli-Q water, and the glycolipids were extracted using a Sep-Pak C_{18} cartridge (Waters) with MeOH (4 mL) and 2:1 $CHCl_3$–MeOH (5 mL).

2. Enzymatic degradation

Compound **8b** obtained from the HPTLC analysis was treated with α-(2→3)-neuraminidase (10 mU) in 50 mM NaOAc buffer solution (pH 5.5) containing sodium taurodeoxycholate (1 mg/mL) at 37 °C overnight. The reaction was quenched by addition of water, and the products were developed over HPTLC.

Acknowledgments

The authors wish to thank Professor Tatsuya Yamagata (Shenyang Pharmaceutical University, China) for helpful discussions and Ms. Miki Kanto for her assistance in preparing the manuscript.

References

1. Okayama, M.; Kimata, K.; Suzuki, S. *J. Biochem.* **1973**, *74*, 1069–1073.
2. Kuan, S. F.; Burd, J. C.; Basbaum, C.; Kim, Y. S. *J. Biol. Chem.* **1989**, *264*, 19271–19277.
3. Sarker, A. T.; Fritz, T. A.; Taylor, W. H.; Esko, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3323–3327.
4. Sarker, A. T.; Rostand, K. S.; Jain, R. K.; Matta, K. L.; Esko, J. D. *J. Biol. Chem.* **1997**, *272*, 25608–25616.
5. Miura, Y.; Arai, T.; Yamagata, T. *Carbohydr. Res.* **1996**, *289*, 193–199; Miura, Y.; Yamagata, T. *Biochem. Biophys. Res. Commun.* **1997**, *241*, 698–703; Kasuya, M. C. Z.; Wang, L. X.; Lee, Y. C.; Mitsuki, M.; Nakajima, H.; Miura, Y.; Sato, T.; Hatanaka, K.; Yamagata, S.; Yamagata, T. *Carbohydr. Res.* **2000**, *329*, 755–763.
6. Nakajima, H.; Miura, Y.; Yamagata, T. *J. Biochem.* **1998**, *124*, 148–156.
7. Sato, T.; Hatanaka, K.; Hashimoto, H.; Yamagata, T. *Trends Glycosci. Glycotechnol.* **2007**, *19*, 1–17.
8. Kasuya, M. C. Z.; Ikeda, M.; Hashimoto, K.; Sato, T.; Hatanaka, K. *J. Carbohydr. Chem.* **2005**, *24*, 705–715.
9. Sato, T.; Takashiba, M.; Hayashi, R.; Zhu, X.; Yamagata, T. *Carbohydr. Res.* **2008**, *343*, 831–838.
10. Sato, T.; Takashiba, M.; Yamagata, T., unpublished.
11. For reviews, see: Suami, T.; Ogawa, S. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 21–90; Ogawa, S. *Trends Glycosci. Glycotechnol.* **2004**, *16*, 33–53; Ogawa, S.; Kanto, M.; Suzuki, Y. *Mini-Rev. Med. Chem.* **2007**, *7*, 679–691; Arjona, O.; Goméz, A. M.; Lopéz, J. C.; Plumét, J. *Chem. Rev.* **2007**, *107*, 1919–2036.
12. Ogawa, S.; Ashiura, M.; Uchida, C.; Watanabe, S.; Yamazaki, C.; Yamagishi, K.; Inokuchi, J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 929–932; Ogawa, S.; Kobayashi, Y.; Kabayama, K.; Jimbo, M.; Inokuchi, J. *Bioorg. Med. Chem.* **1998**, *6*, 1955–1962; Ogawa, S.; Sekura, R.; Maruyama, A.; Yuasa, H.; Hashimoto, H. *Eur. J. Org. Chem.* **2000**, 2089–2093; Ogawa, S.; Matsunaga, K. Y.; Suzuki, Y. *Bioorg. Med. Chem.* **2002**, *10*, 1967–1972.
13. Yuasa, H.; Palcic, M. M.; Hindsgaul, O. *Can. J. Chem.* **1995**, *73*, 2190–2195; Okazaki, K.; Nishigaki, S.; Ishizuka, F. *Org. Biomol. Chem.* **2003**, *1*, 2229–2230.
14. Kitaoka, M.; Ogawa, S.; Taniguchi, H. *Carbohydr. Res.* **1993**, *247*, 355–359; Ogawa, S.; Furuya, T.; Tsunoda, H.; Hindsgaul, O.; Stangiel, K.; Palcic, M. M. *Carbohydr. Res.* **1995**, *271*, 197–205; Kajihara, Y.; Hashimoto, H.; Ogawa, S. *Carbohydr. Res.* **2000**, *323*, 44–48.
15. Ogawa, S.; Matsunaga, N.; Li, H.; Palcic, M. M. *Eur. J. Org. Chem.* **1999**, 631–642.
16. Ogawa, S.; Aoyama, H.; Sato, T. *Carbohydr. Res.* **2002**, *337*, 1979–1992.
17. Vedralova, E.; Borovansky, J.; Hach, P. *Melanoma Res.* **1995**, *5*, 87–92.

Tetrahedron: *Asymmetry* 20 (2009) 2105–2111

Contents lists available at ScienceDirect

Tetrahedron: *Asymmetry*

journal homepage: www.elsevier.com/locate/tetasy

Enzyme-catalyzed resolution of 3,8-dioxatricyclo[3.2.1.$0^{2,4}$]octane-6-carboxylic esters and the application to the synthesis of 3-epishikimic acid

Manabu Hamada [a], Yoshikazu Inami [b], Yasuhito Nagai [c], Toshinori Higashi [b], Mitsuru Shoji [b], Seiichiro Ogawa [d], Kazuo Umezawa [a], Takeshi Sugai [b,*]

[a] *Department of Applied Chemistry, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*
[b] *Faculty of Pharmacy, Keio University, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan*
[c] *Department of Chemistry, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*
[d] *Department of Bioscience and Bioinformatics, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*

ARTICLE INFO

Article history:
Received 14 June 2009
Accepted 6 July 2009
Available online 6 October 2009

ABSTRACT

3,8-Dioxatricyclo[3.2.1.$0^{2,4}$]octane-6-carboxylic acid, whose racemic form is readily available on a large scale, is a versatile starting material for the synthesis of carbasugars and carbocyclic biologically active natural products. In this study, the enzyme-catalyzed kinetic resolution was attempted on a variety of corresponding carboxylic esters. The hydrophobic and hydrophilic properties of ester substituents greatly affected the rate of reaction and the enantioselectivity. Hydrolysis of the corresponding 2′-chloroethyl ester with pig liver esterase worked well in a highly enantioselective manner ($E = 116$) to give the hydrolyzate (90.6% ee) and unreacted ester recovery (99.4% ee). The hydrolyzate is a precursor for (−)-oseltamivir phosphate, and a route to (3*S*,4*S*,5*R*)-(−)-3-epishikimic acid was developed from the recovered ester.

1. Introduction

3,8-*endo*-Dioxatricyclo[3.2.1.$0^{2,4}$]octane-6-carboxylate (acid or ester) **1** is a polyoxygenated cyclohexenecarboxylate with many controlled stereocenters which has been developed as a starting material for carbasugars, including carba-Neu5Ac and carba-KDO as shown in Scheme 1.[1] In the synthesis of epoxyquinols and related compounds, Hayashi et al. have explored new entries. An efficient selective ring-opening reaction was a key step to provide cyclohexenecarboxylate.[2,3] The racemic form of **1** is available in large quantity.[1,6] So far, however, the supply of the enantiomerically pure form has been rather limited, except for asymmetric Diels–Alder reactions controlled by chiral auxiliary[4,5] or tedious preferential crystallization of the diastereomeric salts of a certain precursor.[6]

Herein, we report the kinetic resolution by the action of hydrolytic enzymes on esters **1b–g** (Scheme 1). The hydrolysis of carboxylic esters **1** has a clear and significant advantage, for the large-scale enantiomeric resolution. In this approach, the hydrolyzates, carboxylic acids, and the unreacted recoveries, the esters, are separable by only extractive workup under properly adjusted pH conditions. If these attempts are successful, the unreacted recoveries, the esters would be converted to (3*S*,4*S*,5*R*)-(−)-3-epishikimic acid **2**.

2. Results and discussion

The preparation of racemic esters **1b–g** is shown in Scheme 2. The Diels–Alder reaction between furan and acrylic acid, and the subsequent iodolactonization of the crude *endo*–*exo* mixture (8:2) yielded racemic iodolactone **5**.[1] The hydrolysis of the lactone followed by the direct alkylation of the resulting carboxylate provided esters **1b–g**.

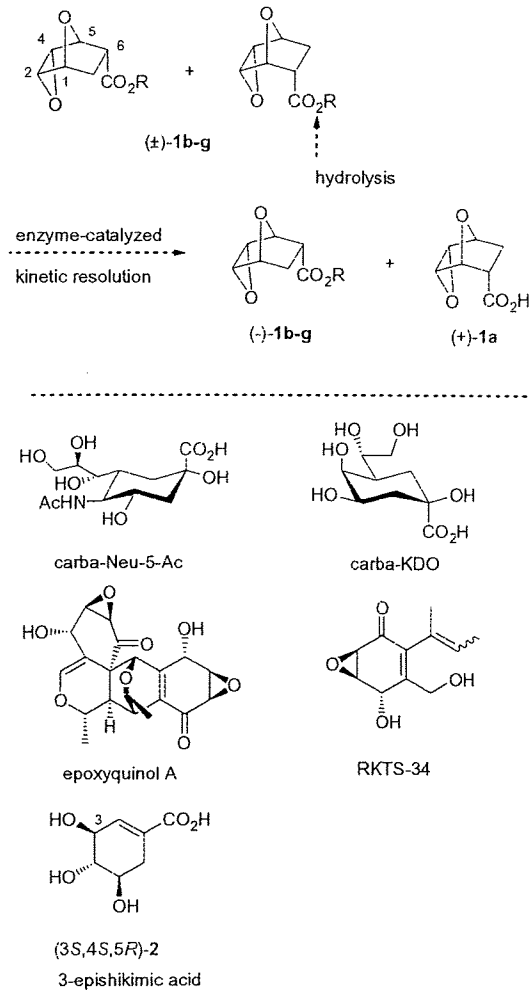
Our first candidate was the ethyl ester **1b**, as it showed a lower melting point (mp 52–53 °C) than the corresponding methyl ester **1c** (mp 75 °C). The lower melting point of the crystalline substrate has been observed to be advantageous for enzyme-catalyzed hydrolysis under aqueous conditions.[7] So far, attempts at the kinetic resolution of the similar *endo*-carboxylic acid **3**, by the action of hydrolytic enzymes on the corresponding ester, have only resulted in moderate enantioselectivity.[8]

Our substrate **1b** reacted poorly with many kinds of hydrolytic enzymes, probably due to high steric hindrance around the *endo*-oriented ester moiety. Among the proteases (*Rhizopus* sp., XP-415; *A. melleus*, XP-488, Nagase ChemteX Co.), lipases (*Candida rugosa*, Meito OF; *Candida antarctica*, Roche diagnostics, Chirazyme L-2), and esterases (*Klebsiella oxytoca*, SNSM-87, Nagase ChemteX Co.; pig liver esterase, Sigma), only pig liver esterase (PLE) showed substantial hydrolysis (Scheme 3). However, the conversion and enantioselectivity (*E*-value) were as low as 33% and 11%, respectively, as shown in entry 1, Table 1.

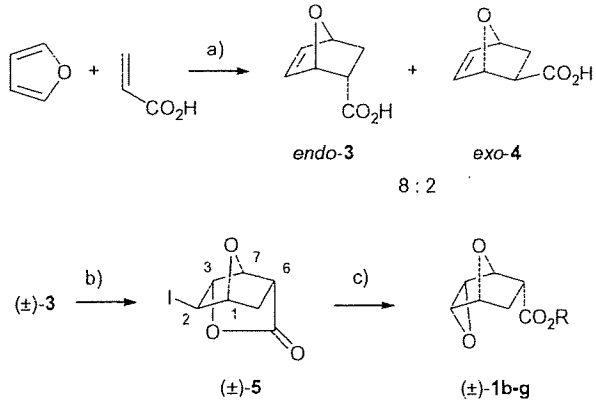
The decrease in bulkiness from ethyl to methyl **1c** (entry 2) only resulted in lower selectivity ($E = 7$). We then introduced an

* Corresponding author. Tel./fax: +81 3 5400 2665.
E-mail address: sugai-tk@pha.keio.ac.jp (T. Sugai).

0957-4166/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2009.07.049

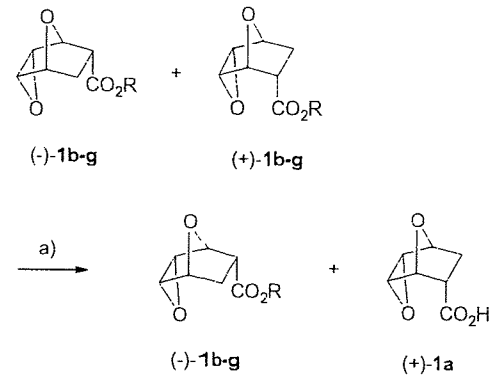


Scheme 1. Enzyme-catalyzed kinetic resolution of 3,8-dioxatricyclo[3.2.1.$0^{2,4}$]-octane-6-carboxylic ester 1 and utilization thereof.



Scheme 2. Preparation of *endo*-epoxy esters. Reagents and conditions: (a) see Ref. 6. The ratio between *endo*- and *exo*-adducts (8:2) was determined by ^{1}H NMR; (b) $NaHCO_3$, H_2O/I_2, THF, rt, 24 h (quant.); (c) KOH, DMF, 40 °C, 24 h/RI, 50 °C, 6 h (yields: see Section 4).

electron-withdrawing group on the ester moiety, expecting that the rate of the 'fast' (1*S*,2*R*,4*S*,5*R*,6*S*)-isomer would be enhanced, so that the enantioselectivity becomes higher. Our first attempt



Scheme 3. Enzyme-catalyzed hydrolysis of *endo*-epoxyesters. Reagents and conditions: (a) pig liver esterase (Sigma E2884), rt, 24 h.

Table 1
PLE-catalyzed hydrolysis of (±)-**1b–g**[a]

Entry	Substrate	R	Conv. (%)	*E*-value
1	**1b**	CH_2CH_3	33	11
2	**1c**	CH_3	32	7
3	**1d**	CH_2Cl	NA	NA
4	**1e**	CH_2CH_2Cl	51	78
5	**1f**	CH_2CONH_2	26	5
6	**1g**	CH_2CF_3	50	9

[a] For reaction conditions and evaluation of enantioselectivity, see Section 4.

to directly replace one hydrogen atom with chlorine **1d** (entry 3) turned out to be unsuccessful, because **1d** was too unstable. The introduction of 2-chloroethyl[9] (**1e**, entry 4) and carbamylmethyl[10] **1f** (entry 5) groups brought about contrasting effects. The former showed a great increase in selectivity ($E = 78$) and the conversion reached nearly 50%.

This result suggested that the reactivity of the 'fast' isomer was enhanced, this is well supported by applying an empirical model of the catalytic site of PLE as proposed by Jones (Fig. 1, top).[11] The tricyclic skeleton of the substrate occupies a large hydrophobic pocket (L). In turn, the 2-chloroethyl group fits a small hydrophobic site (S) of the model so that the attack by the hydroxyl group of a serine residue easily takes place. The ^{1}H NMR spectra of **1e** prompted us to hypothesize that an extended conformation is advantageous to fit in the hydrophobic site of PLE. The two geminal protons of Ha and Hb (Fig. 1, top) were not equivalent (Ha: 4.44 ppm, Hb: 4.32 ppm, $\Delta\delta$ 0.12 ppm), and one of them would be located in the eclipsed position of the ester carbonyl group make the chloroethyl group fit into the hydrophobic site (S) (Fig. 1, top).

On the other hand, the lower selectivity in the carbamylmethyl ester **1f** (entry 5, $E = 5$), as well as the lower conversion, means that the 'fast' isomer is not preferable in an enzyme-catalyzed reaction. The ^{1}H NMR measurement suggested that **1f** has a different conformation from **1e**, as judged by a smaller $\Delta\delta$ value between Hc and Hd (Fig. 1, bottom) (Hc: 4.69 ppm, Hd: 4.62 ppm, $\Delta\delta$ 0.07 ppm). When **1f** in this conformation occupies the catalytic site, a carbamylmethyl group fits the hydrophilic site, while the ester carbonyl group is far away from the nucleophilic serine hydroxyl group (Fig. 1, bottom). The crooked conformation of **1f** is supported by the fact that **1f** is prone to epimerization to an *exo*-ester, when under treatment with base followed by an intramolecular delivery of protons from $CONH_2$ group. In contrast, β-elimination exclusively occurs very quickly in **1e** even under low temperatures, as described later.

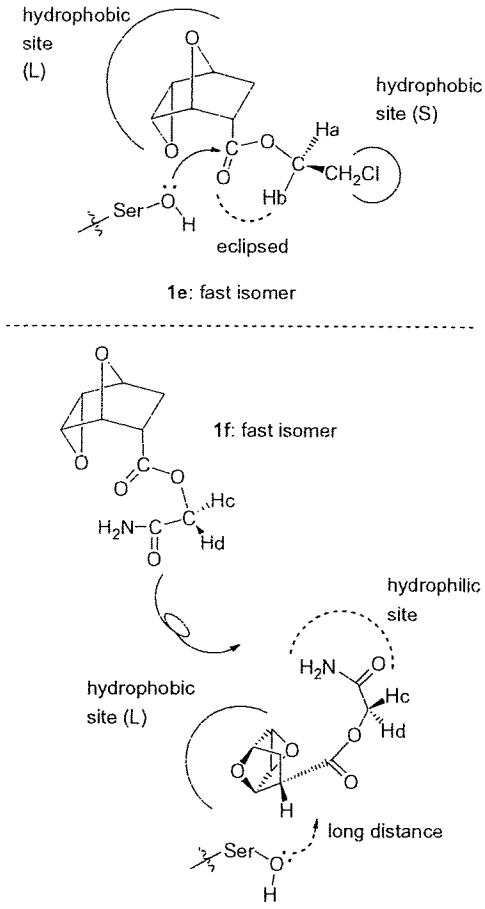
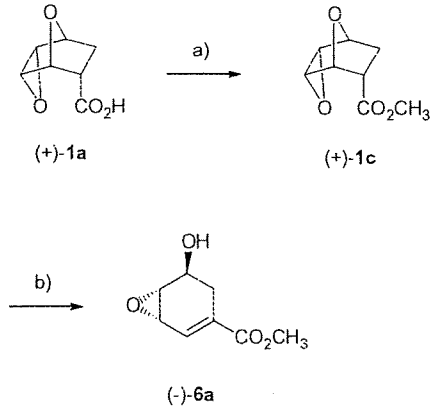


Figure 1. Behavior of 2-chloroethyl **1e** and carbamylmethyl **1f** esters in Jones' empirical catalytic site model of PLE.

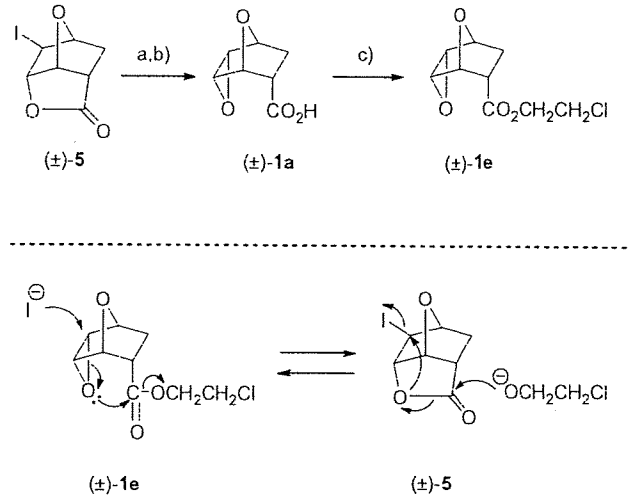
Based on these observations, the more electron-withdrawing and hydrophobic trifluoromethylester **1g** was prepared with the aim of obtaining improved results. However, **1g** was again substantially unstable even in neutral buffer solution. The lower enantioselectivity ($E = 9$) was probably due to enzyme-uncatalyzed spontaneous hydrolysis.

The absolute configuration of the 'fast' isomer in **1e** was determined as follows in Scheme 4. Esterification of (+)-acid **1a**, the hydrolyzate, followed by β-elimination[2] of the resulting ester **1c** using LHMDS gave (−)-**6a**. The absolute configuration of this sample was unambiguously determined to be (1*S*,5*S*,6*R*) by the minus sign of the rotation value compared with literature data.[2] This result is consistent with the stereochemical preference of the 'fast' isomer being similar to that in the case of the ester of *endo*-**3** (Scheme 2).[8]



Scheme 4. Absolute configuration of (+)-**1a**. Reagents and conditions: (a) Cs_2CO_3, MeI, DMF, 50 °C, 24 h (59%); (b) LHMDS, THF, −78 °C, 1 h (70%).

Since the combination of highly enantioselective catalyst and substrate was optimized, the next task was the establishment of suitable reaction conditions for the large-scale preparation of (±)-**1e** from iodolactone (±)-**5**. The conventional method for ester synthesis is the hydrolysis of iodolactone to give the salt of acid **1a** with concomitant epoxide ring formation, and subsequent treatment with an alkylating agent in one pot (Scheme 5).



Scheme 5. Preparation of 2-chloroethyl ester **1e**. Reagents and conditions: (a) KOH, EtOH, 70 °C, 5 h; (b) 1 M HCl (quant.); (c) $ClCH_2CH_2X$, base, additive. For the conditions, see Table 2.

Esterification with 1-chloro-2-iodoethane smoothly proceeded to give **1e** in 80% yield (Table 2, entry 1). However, this mixed dihalide is quite expensive and not suitable for large-scale preparation. The replacement of this iodide with inexpensive 1,2-dichloroethane resulted in only a 33% yield (Table 2, entry 2). Next, KI (4 equiv) was added with the expectation of the in situ formation of iodide, which caused the yield to drop to 3% (Table 2, entry 3). By analysis of the by-products, iodolactone **5** appeared again during the alkylation, even after confirmation of completion of the hydrolysis in the first step (Scheme 5 and **5**→**1a**). At this stage we became aware that the presence of iodide ions in the reaction mixture has a deleterious effect, as the direct attack of an iodide ion opens the epoxide ring of 2-chloroethyl ester itself and the reaction reaches an equilibrium between iodolatone **5** and 2-chloroethyl ester **1e** (Scheme 5, bottom). Thus we switched the procedure into the following conditions. Acid **1a** was extracted after acidic workup, and then it was separately incubated with 1,2-

Table 2
Alkylation of free carboxylic acid (±)-**1a**

Entry	Base	Alkylating agent X (equiv)	Additive	Yield (%)
1	KOH	I (1.5)	None	80
2	KOH	Cl (3.0)	None	33
3	KOH	Cl (3.0)	KI	3
4	K_2CO_3	Cl (4.0)	None	71

dichloroethane and K_2CO_3. Under these iodide-free conditions, the reaction was successful and a 71% yield was recorded (Table 2, entry 4). In the case of scaled-up conditions, the iodide-free potassium salt of **1a** became accessible via an alternative method by careful observation during hydrolysis. When iodolactone **5** was hydrolyzed in ethanol, first, a precipitate appeared. This was proven by NMR analysis to be a potassium salt of iodohydrin carboxylic, an intermediate acid. If the hydrolysis was continued under prolonged heating at 70 °C, the precipitate disappeared. The resulting potassium salt and all of the by-products were soluble in ethanol. Then the reaction mixture was dried onto silica gel, and elution with methanol provided the desired carboxylate salt. Most of the contaminant, especially inorganic salts such as KI, was removed by adsorption on silica gel.

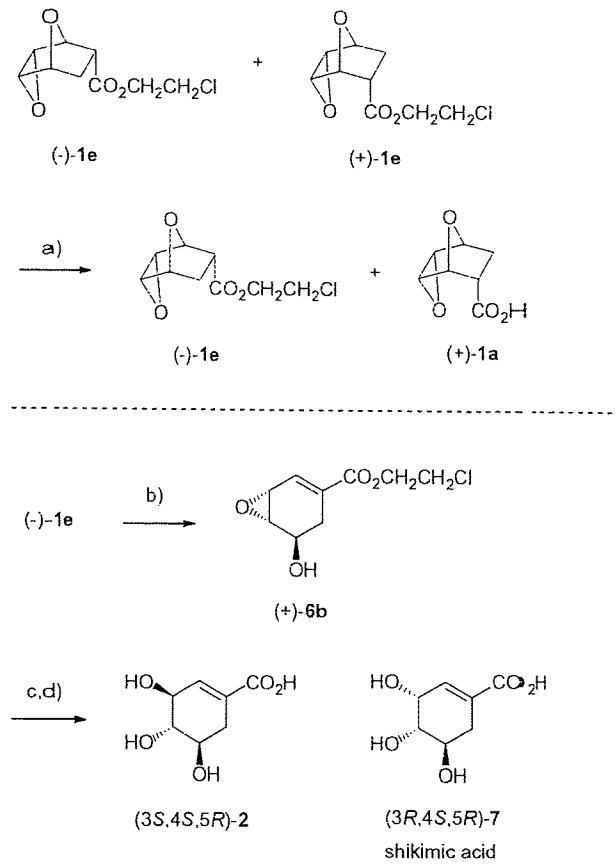
The PLE catalyzed hydrolysis in the scaled-up experiment proceeded in a reproducible manner, and (+)-**1a** (54.7% yield) and (−)-**1e** (42.7% yield) were obtained with only an extractive work-up separation. In this case, both ees of (+)-**1a** (90.6% ee) and (−)-**1e** (99.4% ee) were unambiguously determined (see Section 4) ($E = 116$). The simple recrystallization of acid (+)-**1a** from EtOAc enhanced the enantiomeric excess to 96.4%. It is noteworthy that acid (+)-**1a** has been reported as the starting material for oseltamivir phosphate by Terashima and Ujita.[12]

Our product, epoxyester **1e**, has the same level of oxygen functionality in suitable positions as shikimic acid and related compounds. Naturally occurring shikimic acid (3*R*,4*S*,5*R*)-**7** is essential for the industrial synthesis of (−)-oseltamivir phosphate.[13,14] The process for fermentative production has already been established,[15,16] as (3*R*,4*S*,5*R*)-**7** is a biosynthetic key intermediate for the well-known shikimate pathway. An epimeric form, (3*S*,4*S*,5*R*)-**2** (3-epishikimic acid), has recently gained attention as the starting material for vitamin D_3 precursors,[17] the synthon of natural products,[18,19] and as a template for combinatorial synthesis.[20] The availability of this epi-form, however, is very low due to no direct biosynthetic pathway. An attempted acid-catalyzed epimerization under harsh conditions only results in a stereoisomeric mixture with parent (3*R*,4*S*,5*R*)-shikimic acid **7**, accompanied with the dehydrated 4-hydroxybenzoic acid.[18] Otherwise, a tedious multi-step conversion was required, involving selective protection of 4,5-*trans* diol and inversion at C-3.[19]

The above-mentioned situation prompted us to establish a route from (−)-**1e** to epishikimate (3*S*,4*S*,5*R*)-**2**. Epoxy ester (−)-**1e** was submitted to LHMDS-mediated β-elimination[2] to give (+)-**6b** (Scheme 6). It is noteworthy that the desired reaction occurred in as high as 94% yield without any damage on the 2-chloroethyl ester, which would also suffer from β-elimination. For the transformation of **6b** to (3*S*,4*S*,5*R*)-**2**, the electron-withdrawing property of the chloroethyl group was quite advantageous. The alkaline hydrolysis of **6b** proceeded very smoothly, and the following stereoselective epoxide ring opening[21] gave (3*S*,4*S*,5*R*)-**2a** (79.7%); $[\alpha]_D^{25} = -33.1$ (*c* 0.34, H_2O) {lit.[21] $[\alpha]_D = -31.0$ (*c* 0.1, H_2O)}; whose spectroscopic data coincided with those reported previously.[21]

3. Conclusion

Based on the pig liver esterase-catalyzed kinetic resolution of 2-chloroethyl 3,8-dioxatricyclo[3.2.1.$0^{2,4}$]octane-6-carboxylate **1e**, an expeditious route for polyhydroxylated cyclohexenoids has been established. The design of the substrate structure was supported by conformational analysis and fitness in an enzyme catalytic site model. The reaction conditions for the synthesis of optimized substrate, excluding the formation of by-products necessary to simplify the workup procedure, which is indispensable for preparative-scale synthesis have been elucidated. 3-Epishikimic acid should be a more promising starting material in organic synthesis following our establishment of a scalable supply.



Scheme 6. Derivation of hydrolyzate to (3*S*,4*S*,5*R*)-3-epishikimic acid **2a**. Reagents and conditions: (a) pig liver esterase, 0.2 M phosphate buffer (pH 7.0), [42.7% for (1*R*,2*S*,4*R*,5*S*,6*R*)-(−)-**1e** (99.4% ee)], [54.7% for (1*S*,2*R*,4*S*,5*R*,6*S*)-(+)-**1a** (90.6% ee)]; (b) LHMDS, THF, −78 °C, 1 h, (96%); (c) KOH, THF, H_2O, 50 °C, 1 h; (d) TFA, H_2O, 50 °C, 3 h (79.7%).

4. Experimental

4.1. Materials and methods

Merck Silica Gel 60 F_{254} thin-layer plates (1.05744, 0.5 mm thickness) and Silica Gel 60 (spherical and neutral; 100–210 μm, 37560-79) from Kanto Chemical Co. were used for preparative thin-layer chromatography and column chromatography, respectively. The commercial PLE preparation was purchased from Sigma.

4.2. Analytical methods

All melting points are uncorrected. IR spectra were measured as thin films for oils or ATR for solid on a Jeol FT-IR SPX60 spectrometer. ^{1}H NMR spectra were measured in $CDCl_3$ or D_2O at 270 MHz on a Jeol JNM EX-270 or at 400 MHz on a Jeol JNM GX-400 spectrometer or at 400 MHz on a VARIAN 400-MR spectrometer, and ^{13}C NMR spectra were measured in $CDCl_3$ or D_2O at 100 MHz on a Jeol GX-400 spectrometer or at 100 MHz on a VARIAN 400-MR spectrometer. High resolution mass spectra were recorded on a Jeol JMS-700 MStation spectrometer. HPLC data were recorded on Jasco MD-2010 multi-channel detectors and SHIMADZU SPD-M20A diode array detector. Optical rotation values were recorded on a Jasco P-1010 polarimeter. Silica Gel 60 (spherical, 100–210 μm, 37558-79) of Kanto Chemical Co. was used for column chromatography. Preparative TLC was performed with E. Merck Silica Gel 60 F_{256} plates (0.5 mm thickness, No. 5744).

4.3. Screening of hydrolytic enzymes

The screening of hydrolytic enzymes were performed as follows. A 2 mL sample tube was charged with an appropriate amount of racemic ethyl ester **1b** (10.0 mg) and potassium phosphate buffer (0.2 M, 0.25 mL, pH 7.0) at room temperature for 24 h in the presence of several lipases and protease at an amount of 80–100 mg/mL of phosphate buffer, in the case of pig liver esterase 0.2 mg/mL of phosphate buffer. The progress of the reaction was monitored by TLC analysis [silica gel, developed with hexane–EtOAc (1:1)]. The reaction mixture was quenched with citric acid to pH 2, and extracted with EtOAc. The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. Among the seven enzymes tested, only pig liver esterase showed the progress of hydrolysis.

4.3.1. (±)-7-*endo*-Oxabicyclo[2.2.1]hept-5-carboxylic acid 3

The known procedure[5] was slightly modified in regard to the reaction temperature. Furan (250 mL) and acrylic acid (250 mL) were mixed and kept for 6 days at room temperature, for 17 days at 4 °C, and then for 28 days at 7 °C. The precipitated solids were recovered by filtration to give carboxylic acid (±)-**3** (41.7 g, *endo:exo* = 8:2) as a colorless solid, mp 95–96 °C, lit.[6] mp 97–100 °C. To the above mentioned filtrate was added furan and acrylic acid and kept for one month at 7 °C to give another crop of crystal. The NMR spectrum was identical with that reported previously.[1,6]

4.3.2. (±)-(1*R**,2*R**,3*R**,6*R**,7*S**)-2-Iodo-4,8-dioxatricyclo[4.2.1.0^{3,7}]-nonan-5-one 5

To a solution of the acid (±)-**3** (20.0 g, 142 mmol) in $NaHCO_3$ aq solution (300 mL) was added dropwise a solution of I_2 (40.0 g, 157 mmol) in THF (80 mL) under ice-cooling, and the mixture was stirred for 68 h at room temperature. To the mixture was added saturated $Na_2S_2O_3$ aq solution, and the precipitates were collected in filtration to give crude iodolactone (±)-**5** (25.1 g). The filtration was extracted with EtOAc (three times). The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo, to give another crop of iodolactone (±)-**5** (2.00 g) as the sample for NMR measurement. Its NMR spectrum was identical with that reported previously.[1] The combined yield of above-mentioned (±)-**5** (27.0 g) was 71%, and this was employed for the next step without further purification.

4.3.3. (±)-3,8-Dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylic acid 1a

To a solution of iodolactone (±)-**5** (1.01 g, 3.80 mmol) in DMF (15 mL) was added KOH (0.54 g, 9.98 mmol) and stirred for 24 h at room temperature. After removal of water in vacuo, the residue was added 1 M HCl to pH 2. The mixture was extracted with EtOAc (10 times), and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (6:1) to afford carboxylic acid (±)-**1a** (193 mg, 33.5%) as a colorless solid, mp 153–154 °C. Its NMR spectrum was identical with that reported previously.[12]

4.3.4. Ethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1b

To a solution of iodolactone (±)-**5** (0.51 g, 1.87 mmol) in DMF (10 mL) was added with KOH (0.37 g, 6.60 mmol) and stirred for 24 h at room temperature. After removal of water in vacuo, the residue was dissolved anhydrous DMF. The mixture was added EtI (0.96 g, 6.56 mmol) at 40 °C, and stirred for 6 h. After removal of volatile materials in vacuo, the reaction was quenched with NH_4Cl aq solution, and extracted with EtOAc (three times). The combined organic phases were washed with brine and dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography with hexane–EtOAc (2:1) to afford ethyl ester (±)-**1b** (316 mg, 91.8%) as a colorless solid, mp 52—53 °C. Its NMR spectrum was identical with that reported previously.[12]

4.3.5. Methyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1c

In a similar manner as described for **1b**, a solution of iodolactone (±)-**5** (0.80 g, 3.01 mmol) in DMF (10 mL) was treated with KOH (0.40 g, 7.13 mmol) and MeI (1.28 g, 6.56 mmol) to give methyl ester (±)-**1c** (390 mg, 78.3%) as a colorless solid; mp 75 °C. Its NMR spectrum was identical with that reported previously.[2]

4.3.6. 2-Chloroethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1e

In a similar manner as described for **1b**, a solution of iodolactone (±)-**5** (266 mg, 1.00 mmol) in DMF (3 mL) was treated with KOH (132 mg, 2.35 mmol) and $ClCH_2CH_2I$ (300 mg, 1.58 mmol), to give 2-chloroethyl ester (±)-**1e** (171 mg, 78.5%) as a colorless oil: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.97 (1H, ddd, J = 4.9, 11.3, 11.3 Hz, H-7$_{exo}$), 2.07 (1H, dd, J = 4.9, 11.3 Hz, H-7$_{endo}$), 2.93 (1H, dt, J = 4.9, 11.3 Hz, H-6), 3.69 (2H, t, J = 5.7 Hz, CH_2Cl), 4.01 (1H, dd, J = 2.4, 4.3 Hz, H-4), 4.11 (1H, dd, J = 2.2, 4.3 Hz, H-2), 4.32 (1H, dt, J = 5.7, 11.3 Hz, C*H*HCH$_2$Cl, Ha in Fig. 1), 4.44 (1H, dt, J = 5.7, 11.3 Hz, CH*H*CH$_2$Cl, Hb in Fig. 1), 4.50 (1H, dt, J = 2.2, 4.9 Hz, H-5), 4.70 (1H, dt, J = 2.2, 4.9 Hz, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.5, 41.8, 44.7, 64.1, 66.6, 66.7, 77.5, 78.2, 171.0; IR ν_{max} 3006, 2962, 2358, 1732, 1449, 1334, 1207, 1176, 883 cm^{-1}. Anal. Calcd for $C_9H_{11}ClO_4$: C, 49.44; H, 5.07. Found: C, 49.19; H, 5.04.

This ester was also able to be prepared by the action of $ClCH_2CH_2Cl$. To a solution of the acid (±)-**1a** (15.6 mg, 0.10 mmol) in anhydrous DMF was added K_2CO_3 (55.2 mg, 0.40 mmol) and $ClCH_2CH_2Cl$ (59.4 mg, 0.60 mmol), and the mixture was stirred at 40 °C for 24 h. The same workup as above provided (±)-**1e** (16.5 mg, 75.7%).

4.3.7. Scaled-up and preparative synthesis of 1e

A solution of iodolactone (±)-**5** (3.20 g, 12.0 mmol) in EtOH (20 mL) was added KOH (2.00 g, 35.6 mmol) and the mixture was stirred for 5 h at 70 °C. After removal of volatile materials in vacuo, the residue was re-dissolved in MeOH. To the mixture was added silica gel (50 g), and stirred for 30 min. After concentration in vacuo, the residual solid was charged on a glass column, and that was eluted with ethanol to give carboxylic acid (±)-**1a** (86.4 mg) as a colorless solid. Further elution with MeOH afforded potassium salt (±)-**1a** (2.40 g).

To a solution of the above potassium salt (±)-**1a** (2.07 g) in DMF (10 mL) was added $ClCH_2CH_2Cl$ (5.28 g, 53.4 mmol), and the mixture was stirred at 60 °C for 24 h. The same workup provided (±)-**1e** (1.55 g, 71.1%).

4.3.8. Carbamylmethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]octane-6-carboxylate 1f

In a similar manner as described for **1b**, a solution of iodolactone (±)-**5** (0.80 g, 3.01 mmol) in DMF (5 mL) was treated with KOH (0.40 g, 7.13 mmol) and $ClCH_2CONH_2$ (0.84 g, 8.98 mmol), gave carbamylmethyl ester (±)-**1f** (423 mg, 65.9%) as a colorless solid. Further purification by recrystallization from EtOAc afforded (±)-**1f**: mp 131.0–133.0 °C: ^1H NMR (400 MHz, $CDCl_3$): δ = 2.03 (1H, ddd, J = 4.6, 11.2, 11.6 Hz, H-7$_{exo}$), 2.09 (1H, dd, J = 4.3, 11.6 Hz, H-7$_{endo}$), 3.02 (1H, dt, J = 4.6, 11.2 Hz, H-6), 4.11 (1H, dd, J = 1.9, 4.4 Hz, H-4), 4.18 (1H, dd, J = 2.2, 4.4 Hz, H-2), 4.55 (1H, dt, J = 2.2, 4.6 Hz, H-5), 4.62 (1H, d, J = 15.6 Hz, C*H*HCONH$_2$, Hc in Fig. 1), 4.69 (1H, d, J = 15.6 Hz, CH*H*CONH$_2$, Hd in Fig. 1), 4.77 (1H, dt, J = 1.9, 4.4 Hz, H-1), 5.81 (1H, br s, NH_2), 6.69 (1H, br s,

NH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.6, 44.7, 62.8, 67.0, 67.7, 77.4, 78.1, 169.9, 170.4; IR ν_{max} 3400, 3190, 2958, 1753, 1680, 1417, 1306, 1197 cm^{-1}. Anal. Calcd for $C_9H_{11}NO_5$: C, 50.70; H, 5.20; N, 6.57. Found: C, 50.53; H, 5.15; N, 6.47.

4.3.9. 2,2,2-Trifluoroethyl (±)-3,8-dioxatricyclo[3.2.1.0^{2,4}]-octane-6-carboxylate 1g

A mixture of carboxylic acid (±)-**1a** (156 mg, 1.00 mmol), DMAP (245 mg, 2.00 mmol), EDC-Cl (384 mg, 2.00 mmol), CF_3CH_2OH (150 mg, 1.50 mmol), and triethylamine (202 mg, 2.00 mmol) in DMF (1 mL) was stirred at room temperature under argon. The reaction was monitored by silica gel TLC, developed with hexane–EtOAc (1:4). After stirring for 10 h at room temperature, the mixture was quenched by the addition of EtOAc–water. The organic materials were extracted with EtOAc, and the combined organic phases were washed with brine and dried over Na_2SO_4. The organic phase was concentrated in vacuo. The residue was purified by silica gel column chromatography with hexane–EtOAc (1:1) to afford trifluoroethyl ester (±)-**1g** (192 mg, 80.6%) as a colorless oil; ^1H NMR (270 MHz, $CDCl_3$): δ = 1.93 (1H, ddd, J = 4.6, 11.3, 11.6 Hz, H-7$_{exo}$), 2.04 (1H, dd, J = 4.3, 11.6 Hz, H-7$_{endo}$), 2.94 (1H, dt, J = 4.6, 11.3 Hz, H-6), 3.98 (1H, dd, J = 2.4, 4.6 Hz, H-4), 4.05 (1H, dd, J = 2.2, 4.6 Hz, H-2), 4.44 (1H, dddd, J = 8.4, 12.7 Hz, CH_2CF_3), 4.53 (1H, dt, J = 1.9, 4.9 Hz, H-5), 4.58 (1H, dddd, J = 8.4, 12.7 Hz, CH_2CF_3), 4.69 (1H, dt, J = 1.9, 4.9 Hz, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.5, 44.4, 60.2, 60.6, 66.5, 66.6, 77.4, 77.5, 121.5, 124.3, 169.7; IR ν_{max} 3010. 2969, 2368, 2337, 1747, 1411,1276, 1155, 879 cm^{-1}. HRMS (EI): calcd for $C_9H_9F_3O_4$: [M$^+$]: 238.0453; found: m/z = 238.0453.

4.3.10. PLE-catalyzed hydrolysis of esters 1b–1g

The hydrolysis of each substrate was carried out under the same conditions as described for the screening of enzymes with ethyl ester **1b**. The E-value of the each substrate was uniformly calculated from the conversion and ee(P) as follows. The conversion was determined by ^1H NMR analysis of crude reaction mixture. Ee(P) was determined by the HPLC analysis at the stage of **6a**, after methylation of hydrolyzate and following β-elimination as described later.

4.3.11. PLE-catalyzed hydrolysis of 2-chloroethyl ester 1e

To a stirred solution of 2-chloroethyl ester (±)-**1e** (373.3 mg, 1.71 mmol) in a phosphate buffer (0.2 M, pH 7.0; 8.5 mL), PLE (Sigma, E2884, 850 μL) was added and the mixture was stirred for 24 h at room temperature. The reaction was quenched with 1 M HCl to pH 2, and extracted with EtOAc (10 times). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo, and the ratio between unreacted recovery **1e** and hydrolyzate **1a** was determined by ^1H NMR measurement. The above mentioned crude mixture was washed with saturated $NaHCO_3$ aq solution. The organic layer was washed with brine and dried over Na_2SO_4, concentrated in vacuo to give (−)-**1e** (159.3 mg, 0.73 mmol) as the unreacted recovery. The aqueous layer was acidified to pH 3 and extracted with EtOAc (10 times). The extract was dried over Na_2SO_4 and concentrated in vacuo to give (+)-**1a** (145.4 mg, 0.93 mmol, mp 107–108 °C). These samples were employed for the next step without further purification.

Ester (−)-**1e**: $[\alpha]_D^{23}$ = −5.3 (c 1.02, $CHCl_3$), 99.4% ee as shown below. Its IR and NMR spectra were in good accordance with those of racemic sample. Acid (+)-**1a**: $[\alpha]_D^{23}$ = +11.7 (c 1.00, MeOH), 90.6% ee as shown below. This was further purified by recrystallization from EtOAc to give (+)-**1a** (93.6 mg, 71%, mp 114–115 °C) $[\alpha]_D^{23}$ = −14.4 (c 0.75, MeOH). The sample obtained by recrystallization as above (15.0 mg) was treated with CH_2N_2 to give (+)-**1c** (15.6 mg, 96%); mp 63–64 °C, $[\alpha]_D^{23}$ = +11.7 (c 0.75, MeOH). This was further converted to (−)-**6a** (11.1 mg, 74%, 95.6% ee); $[\alpha]_D^{23}$ = −207 (c 0.55, MeOH). HPLC analysis was performed in the same manner: t_R (min) = 15.1 (97.8%), 33.1 (2.2%).

Further recrystallization provided a sample of (+)-**1a** (49% recovery, mp 112–113 °C), $[\alpha]_D^{23}$ = +15.8 (c 0.76, MeOH). This sample was revealed to be 96.4% ee by the HPLC analysis at the subsequent stage of **6a** as below, and we concluded that the enantiomeric excess of the acid **1a** reaches constant value by repetition of the recrystallization from EtOAc.

The scaled-up experiment by applying (±)-**1e** (1.00 g, 4.59 mmol) worked well in a reproducible manner to give (−)-**1e**: (230 mg, 22.9%) $[\alpha]_D^{24}$ = −5.3 (c 1.00, $CHCl_3$); 99.7% ee after derivatization to **6b** and its HPLC anlalysis. Acid (+)-**1a**: (428 mg, 59.8%) $[\alpha]_D^{24}$ = +11.0 (c 1.00, MeOH); 77.3% ee by HPLC analysis of corresponding **6a**.

4.3.12. Methyl (1S,2R,4S,5R,6S)-(+)-3,8-dioxatricyclo[3.2.1.0^{2,4}]-octane-6-carboxylate 1c

To a solution of the acid (+)-**1a** (30.6 mg, 0.20 mmol) as above in anhydrous DMF was added Cs_2CO_3 (163 mg, 0.50 mmol) and CH_3I (85.1 mg, 0.60 mmol). The mixture was stirred at 50 °C for 24 h. After concentration to dryness in vacuo, the residue was extracted with EtOAc (three times), and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC with hexane–EtOAc (1:1) to afford methyl ester (+)-**1c** (20.1 mg, 59%) as a colorless solid. Mp 67–68 °C, $[\alpha]_D^{23}$ = +11.7 (c 0.57, MeOH). Its IR and NMR spectra were identical with that of the authentic specimen.[2]

4.3.13. Methyl (1S,5S,6R)-(−)-5-hydroxy-7-oxabicyclo[4.1.0]-hept-2-en-3-carboxylate 6a

To a solution of lithium hexamethyldisilazide [$(TMS)_2NLi$, 0.20 mL, 0.20 mmol] was added in THF (0.20 mL) at −78 °C. To a solution of methyl ester (−)-**1c** (20.1 mg, 0.13 mmol) in THF (0.20 mL) was added the LHMDS solution above dropwise at −78 °C, and the mixture was stirred for 1 h at that temperature. The reaction was quenched with saturated NH_4Cl aq solution, and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC with hexane–EtOAc (1:1) to afford methyl ester (−)-**6a** (14.0 mg, 70%, 90.6% ee) as a colorless solid. $[\alpha]_D^{23}$ = −200 (c 0.70, MeOH) [lit.:[2] $[\alpha]_D$ = +213 (c 0.56, MeOH), for (1R,5R,6S)-**6a**]. The product (−)-**6a** was analyzed by HPLC [column, Daicel Chiralcel OD-H, 0.46 cm × 25 cm; hexane–2-propanol (5:1); flow rate 0.5 mL/min]: t_R (min) = 15.1 (95.3%), 33.1 (4.7%).

Enantiomerically enriched acid (+)-**1a** (15.6 mg, 0.10 mmol) by recrystallization in twice was treated with CH_2N_2 to give (+)-**1c** (15.7 mg, 89%); mp 63–64 °C, $[\alpha]_D^{23}$ = +11.7 (c 0.75, MeOH). This was converted to (−)-**6a** (11.1 mg, 74%, 96.4% ee); $[\alpha]_D^{23}$ = −207 (c 0.55, MeOH). HPLC analysis was performed in the same manner: t_R (min) = 15.1 (98.2%), 33.1 (1.8%).

4.3.14. 2-Chloroethyl (1R,5R,6S)-(+)-5-hydroxy-7-oxabicyclo-[4.1.0]hept-2-en-3-carboxylate 6b

In a similar manner as described for (±)-**6b**, 2-chloroethyl ester (−)-**1e** (47.2 mg, 0.21 mmol) in THF (0.30 mL) was added with a solution of lithium hexamethyldisilazide [$(TMS)_2NLi$, 0.31 mL, 0.31 mmol] in THF (0.30 mL), gave (+)-**6b** (36.4 mg, 77%, 99.4% ee: $[\alpha]_D^{23}$ = +233 (c 1.08, MeOH); The product (+)-**6b** was analyzed by HPLC analysis [column, Daicel Chiralcel OD-H, 0.46 cm × 25 cm; hexane–2-propanol (5:1); flow rate 0.5 mL/min]: t_R (min) = 18.0 (0.3%), 38.0 (99.7%); ^1H NMR (400 MHz, $CDCl_3$): δ = 2.32 (1H, ddd, J = 3.3, 5.2, 17.6 Hz, H-6β), 2.80 (1H, dt, J = 2.1, 17.6 Hz, H-6α), 3.48 (1H, t, J = 3.9 Hz, H-3), 3.57 (1H, ddd, J = 2.1, 2.8, 3.9 Hz, H-4), 3.69 (2H, t, J = 5.7 Hz, CH_2Cl), 4.38 (2H, t, J = 5.7 Hz, CO_2CH_2), 4.57 (1H, br m, H-5), 7.19 (1H, dd, J = 3.3, 3.9 Hz, H-2); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.3, 41.5, 46.2, 56.1, 63.5, 64.5, 130.3, 134.4, 165.5; IR ν_{max} 3425, 2964, 1709, 1641, 1417, 1392, 1250,

1099, 918 cm^{-1}. Anal. Calcd for $C_9H_{11}ClO_4$: C, 49.44; H, 5.07. Found: C, 49.43; H, 5.36.

4.3.15. (3*S*,4*S*,5*R*)-(−)-3,4,5-Trihydroxy-1-cyclohexene-1-carboxylic acid 2

To a solution of (+)-**6b** (55.0 mg, 0.25 mmol) in THF and water (1:1, 4 mL) was added KOH (21.2 mg, 0.38 mmol). After stirring for 1 h at 50 °C, the mixture was neutralized with 1 M HCl to pH 3, and concentrated in vacuo. The solid was dissolved in water (1 mL) and trifluoroacetic acid (400 μL, 5.39 mmol) was added to the solution with stirring. The mixture was stirred for 3 h at 50 °C. The reaction mixture was concentrated in vacuo to remove volatile materials. The residue was purified by silica gel column chromatography with $CHCl_3$–MeOH (10:1) to afford carboxylic acid (−)-**2**: $[\alpha]_D^{25} = -33.1$ (*c* 0.34, H_2O) [lit.:[20] $[\alpha]_D = -31.0$ (*c* 0.1, H_2O)]; ^1H NMR (400 MHz, D_2O): $\delta = 2.06$ (1H, dddd, $J = 2.8$, 4.0, 10.0, 16.8 Hz, H-6 β), 2.61 (1H, ddd, $J = 1.6$, 6.0, 16.8 Hz, H-6α), 3.33 (1H, dd, $J = 8.4$, 10.0 Hz, H-4), 3.62 (1H, dt, $J = 6.0$, 10.0, 10.0 Hz, H-5), 4.11 (1H, dddd, $J = 1.6$, 2.4, 4.0, 8.4 Hz, H-3), 6.51 (1H, dd, $J = 2.4$, 2.8 Hz, H-2); ^{13}C NMR (100 MHz, D_2O): $\delta = 31.7$, 68.6, 71.5, 76.2, 128.2, 139.2, 169.7; IR ν_{max} 3261, 1556, 1409, 1072 cm^{-1}. Its ^1H NMR spectrum was identical with that reported previously.[21] As this product **2** is a trihydroxy acid and shows highly hydrophilic property and is susceptible to an irreversible adsorption on silica gel, the yield was estimated to be 79.7%, at the stage just before the final purification, based on ^1H NMR with an internal standard [methyl β-D-glucoside, Tokyo Kasei Co., M709, analytically pure grade, standard signal at $\delta = 4.23$ (1H, d, $J = 7.6$ Hz)].

Acknowledgements

The authors thank Professor Kaoru Nakamura of Institute for Chemical Research, Kyoto University, for his valuable discussion and encouragement on this study. Meito Sangyo Co. Ltd and Nagase ChemteX Co. were acknowledged with thanks, for generous gift of enzymes. This work was supported both by a Grant-in-Aid for Scientific Research (No. 18580106) and 'High-Tech Research Center' Project for Private Universities: matching fund subsidy 2006–2011 from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and acknowledged with thanks.

References

1. Ogawa, S.; Yoshikawa, M.; Taki, T.; Yokoi, S.; Chida, N. *Carbohydr. Res.* **1995**, *269*, 53–78.
2. Shoji, M.; Imai, H.; Mukaida, M.; Sakai, K.; Kakeya, H.; Osada, H.; Hayashi, Y. *J. Org. Chem.* **2005**, *70*, 79–91 and references cited therein.
3. Kakeya, H.; Miyake, Y.; Shoji, M.; Kishida, S.; Hayashi, Y.; Kataoka, T.; Osada, H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3743–3746.
4. Evans, D. A.; Barnes, D. M. *Tetrahedron Lett.* **1997**, *38*, 57–58.
5. Sarakinos, G.; Corey, E. J. *Org. Lett.* **1999**, *1*, 1741–1744.
6. Ogawa, S.; Iwasawa, Y.; Nose, T.; Suami, T.; Ohba, S.; Ito, M.; Saito, Y. *J. Chem. Soc., Perkin Trans. 1* **1985**, 903–906 and references cited therein.
7. Kurokawa, M.; Sugai, T. *Bull. Chem. Soc. Jpn.* **2004**, *77*, 1021–1025.
8. Tran, C. H.; Crout, D. H. G. *J. Chem. Soc., Perkin Trans. 1* **1998**, 1065–1068.
9. Fotakopoulou, I.; Barbayianni, E.; Constantinou-Kokotou, V.; Bornscheuer, U. T.; Kokotos, G. *J. Org. Chem.* **2007**, *72*, 782–786 and references cited therein.
10. Hirose, Y.; Kariya, K.; Sasaki, I.; Kurono, Y.; Achiwa, K. *Tetrahedron Lett.* **1993**, *34*, 5915–5918.
11. Provencher, L.; Jones, J. B. *J. Org. Chem.* **1994**, *59*, 2729–2732.
12. Terashima, S.; Ujita, K.; Kuwayama, T.; Sugioka, T.; Shimizu, K.; Kanehira, K. WO 2000-JP8348 20001127 (CA 135:76775), 2001. They prepared racemic oseltamivir phosphate from (±)-**1a**.
13. Abrecht, S.; Harrington, P.; Iding, H.; Karpf, M.; Trussardi, R.; Wirz, B.; Zutter, U. *Chimia* **2004**, *58*, 621–629.
14. Shibasaki, M.; Kanai, M. *Eur. J. Org. Chem.* **2008**, 1839–1850 and references cited therein.
15. Darths, K. M.; Knop, D. R.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, *121*, 1603–1604.
16. Adachi, O.; Ano, Y.; Toyama, T.; Matsushita, K. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 2579–2582.
17. Sánchez-Abella, L.; Fernández, S.; Verstuyf, A.; Verlinden, L.; Ferrero, M.; Gotor, V. *Bioorg. Med. Chem.* **2007**, *15*, 4193–4202.
18. Zhang, Y.; Liu, A.; Ye, Z. G.; Lin, J.; Xu, L. Z.; Yang, S. L. *Chem. Pharm. Bull.* **2006**, *54*, 1459–1461.
19. Huang, J.; Chen, F.-E. *Helv. Chim. Acta* **2007**, *90*, 1366–1372.
20. Tan, D. S.; Foley, M. A.; Shair, M. D.; Schreiber, S. L. *J. Am. Chem. Soc.* **1998**, *120*, 8565–8566.
21. Brettle, R.; Cross, R.; Frederickson, M.; Haslam, E.; MacBeath, F. S.; Davies, G. M. *Tetrahedron* **1996**, *52*, 10547–10556.

Current Topics in Medicinal Chemistry, 2009, *9*, 58-75

Design and Synthesis of 5a-Carbaglycopyranosylamime Glycosidase Inhibitors

Seiichiro Ogawa* and Miki Kanto

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Hiyoshi, Kohoku-ku, Yokohama, 223-8522 Japan

Abstract: 5a-Carba-α-D-glucopyranosylamine, validamine, and analogous compounds valienamine and valiolamine, have proved to be important lead compounds for development of clinically useful medicines, including the very strong α-glucosidase inhibitor, voglibose, *N*-(1,3-dihydroxyprop-2-yl)valiolamine, now used widely as a clinically important antidiabetic agent. In this review, we describe recent advances in development of glycosidase inhibitors on the basis of the ground-state mimics of the postulated glycopyranosyl cation, considered to be formed during hydrolysis of glycopyranosides, and introduce a new type of highly potent α-fucosidase inhibitor, 5a-carba-α-L-fucopyranosylamine, α-fuco validamine. Interestingly, the corresponding β-anomer, and in particular its D-enantiomer, has been shown to possess very strong cross-inhibitory activity toward β-galactosidase and β-glucosidase. Structure and inhibitory activity relationships concerning these α,β-fuco derivatives, as well as parent α,β-galacto validamines are discussed here with reference to our results.

1. INTRODUCTION

Acarbose (**1**) [1] and synthetic voglibose (**2**) [2,3], potent and specific α-glucosidase inhibitors, are clinically important for control of diabetes. These carbasugars [4] are carbocyclic analogues of glycofuranoses and pyranoses. The naturally occurring carbasugar 5a-carba-α-D-glucopyranosylamine (validamine, **4α**) [5] and some related compounds, 5,5a-unsaturated (valienamine, **5**) [6], 5-hydroxyl (valiolamine, **6**) [5], and 5a-hydroxyl derivatives (hydroxyvalidamine, **7**) [7], were first isolated from fermentation broth of the antibiotic validamycin A (**3**) [8] and then characterized as active components from the degradation products of **3** and its homologues. Their structures were fully established on the basis of spectroscopic data and total syntheses. Subsequently, **5** and its 5,5a-epoxy derivative **8** [9] were found to be components of the α-amylase inhibitors acarbose (**1**) and NS-504, the oxidized homologue of **1**, respectively, Fig. (**1**).

These carbaglycosylamines themselves possess more or less inhibitory activity toward α-glucosidases. Their activity is likely to be attributable to their structures mimicking the ground- and/or transition-state glucopyanosyl cations postulated to be formed during hydrolysis of α-glucopyranosides. Extensive efforts have been made for development of new type α-glucosidase inhibitors, leading to the discovery of very potent compound, voglibose [**2**, *N*-(1,3-dihydroxyprop-2-yl)valiolamine].

By analogy with the structural features of **4α** and **5**, some carbaglycosylamines, structurally related to the naturally occurring hexopyranoses involved in cell-surface oligosaccharide chains, might be valuable targets for exploration. In particular, 5a-carba-glucosyl (**4α,β**), galactosyl (**9α,β**), mannosyl (**10α,β**), and fucosylamines (**11α,β**), and *N*-acetyl-2-amino-2-deoxy-6a-carbaneuraminic acid (**12α,β**) could provide lead compounds for development of potent inhibitors active against the corresponding glycohydrolases, Fig. (**2**).

2. CARBAGLYCOSYLAMINE GLYCOSIDASE INHIBITORS

2-1. Naturally Occurring Glycosidase Inhibitors: Validamycins and Acarbose

Agricultural antibiotic validamycins A-H have been widely used to control sheath bright disease of rice plants. The major and most active validamycin A (**3**) is the 5a,5a'-dicarbatrisaccharide, composed of validoxylamine A and β-linked D-glucopyranose. Trehalase inhibitory activity is due to the N-linked dicarbadisaccharide core mimicking the transition-state glycopyranosyl cation assumed to be formed during hydrolysis of αα-trehalose. In contrast, acarbose (**1**) is a carbatetrasaccharide, containing valienamine N-linked to C-4 of the 4,6-dideoxy-α-D-glucopyranose residue. The structural features of this carba-oligosaccharide core are very similar to the non-reducing end of amylose type α (1-4) D-glucan, and it's α-glucosidase inhibitory activity is attributable to mimics of the transition-state glycosyl cation thought to be formed on activation by α-glucosidase. In both cases, biological activity is apparently due to stereospecificity for certain glycosidases, and the structural core provided by the unsaturated carbaglycosylamine **5** has been shown to play important roles in their biological features.

Recent progress and increase in the scope of unsaturated carbaglycosylamine glycosidase inhibitors was reviewed [10] in a Mini-Review of Medicinal Chemistry. Here, we would like to summarize developments with regard to inhibitors related to the ground-state mimic validamine.

*Address correspondence to this author at the Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Hiyoshi, Kohoku-ku, Yokohama, 223-8522 Japan; Tel: +81 (45)-566-1788; Fax: +81 (45)-566-1789; E-mail: sogawa379@ybb.ne.jp

1568-0266/09 $55.00+.00

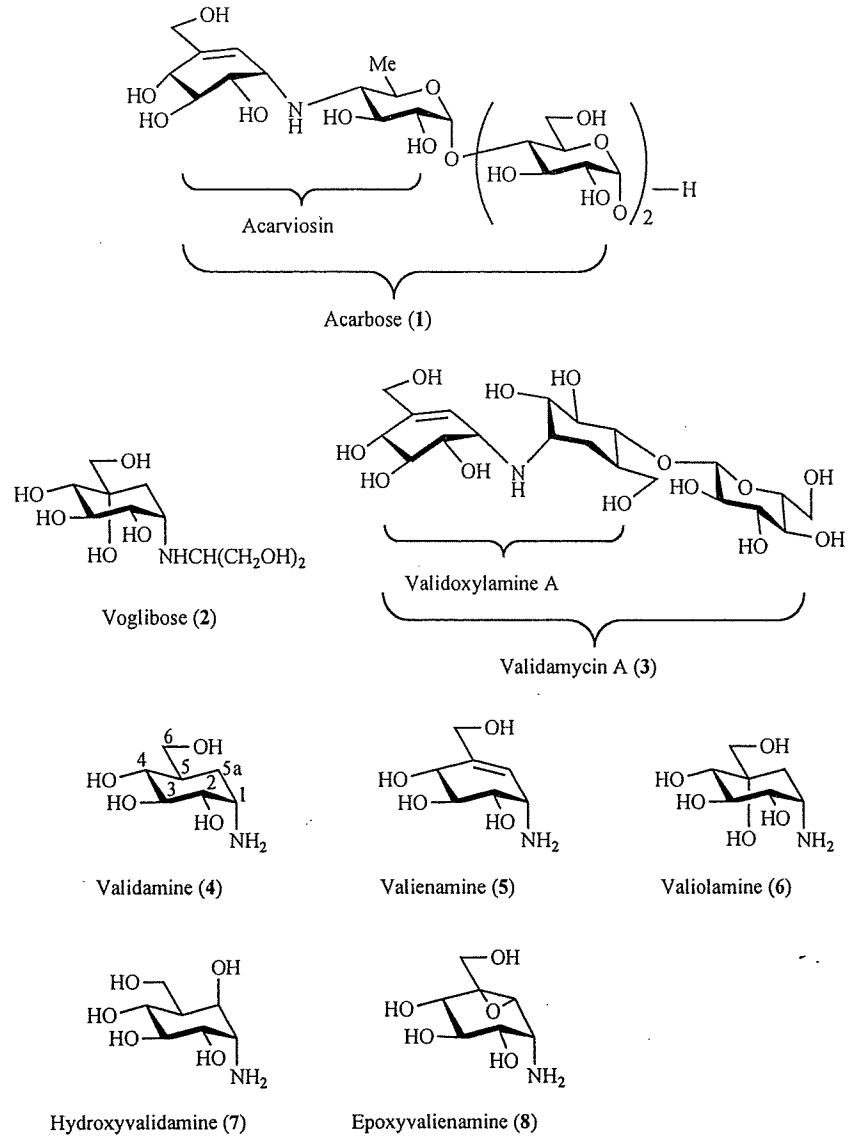


Fig. (1). Acarbose, validamycin A, and validamine, and related compounds derived from antibiotics and glycosidase inhibitors.

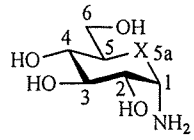
2–2. Synthesis of 5a-Carbaglycosylamines

We initially attempted to prepare 5a-carbasugars by incorporation of hydroxymethyl branches into the cyclitol rings, leading to the synthesis of two new 5a-carbahexoses with α-*altro* and β-*galacto* configurations [11]. However, owing to the practical difficulty in obtaining optically active compounds, we soon established that production of a large quantity of precursors and ready optical resolution to chiral carbasugars would be indispensable for further developments in carbasugar chemistry and biochemistry.

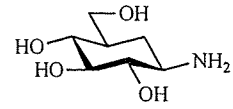
For this purpose, it appeared attractive to establish systematic routes to carbasugars, starting from readily available 1,4-anhydro-5a-carba-α-glucopyranose, the *endo*-adduct **13** of furan and acrylic acid. When ready formation of crystalline *endo*-adduct **13** from a reaction mixture was first observed [12], we soon realized that this compound might become a most versatile precursor for preparation of various kinds of carbasugars and derivatives.

We here briefly detail the systematic synthetic routes to several useful intermediates in Figs. (**3-5**). All preparative processing was elaborated using common reagents under conventional conditions as simply as possible [13,14]. Although optical resolution is difficult to overcome in any preparative design of carbasugars, optically pure samples of **13** could be obtained with readily available (*R*)- and (*S*)-phenylethylamines.

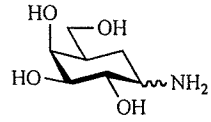
In synthetic carbohydrate chemistry, naturally abundant D-glucose, D-galactose, and D-mannose, for example, have effectively been applied for preparation of D-series sugar derivatives. However, elaboration of the corresponding L-series sugars is always difficult in practice. Since in our carbasugar synthesis, both enantiomers of **13** could be readily provided pure [15], optically pure compounds could



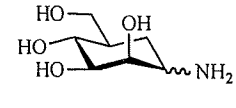
α-D-Glucopyranosylamine: X = O
Validamine (4α): X = CH_2
5a-Carba-α-D-glucopyranosylamine



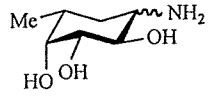
β-Validamine (4b)
5a-Carba-β-D-glucopyranosylamine



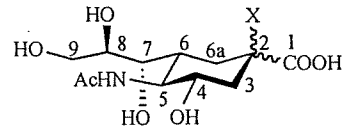
Galactovalidamine (9α,β)
5a-Carba-α,β-D-galactopyranosylamine



Mannovalidamine (10α,β)
5a-Carba-α,β-D-mannopyranosylamine

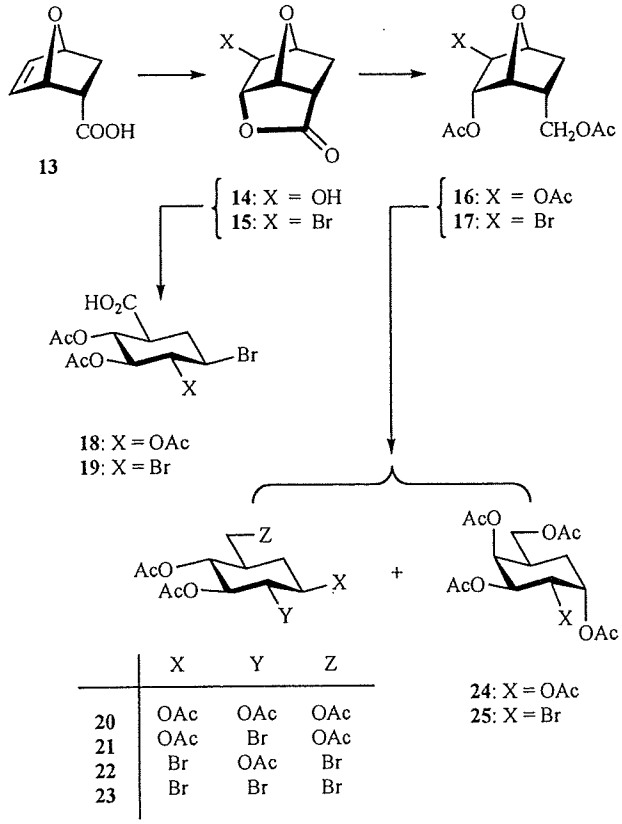


Fucovalidamine (11α,β)
5a-Carba-α,β-L-fucopyranosylamine



N-Acetyl-6a-carbaneuraminic acid: X = OH
2-Amino-2-deoxy derivative (12α,β): X = NH_2

Fig. (2). Validamine and related carbaglycosylamines of biological interest.



	X	Y	Z
20	OAc	OAc	OAc
21	OAc	Br	OAc
22	Br	OAc	Br
23	Br	Br	Br

Fig. (3). Several synthetic precursors derived from the *endo*-adduct of furan and acrylic acid. Unless otherwise noted, for convenience, the formulae only depict only one of the respective enantiomers throughout in this article.

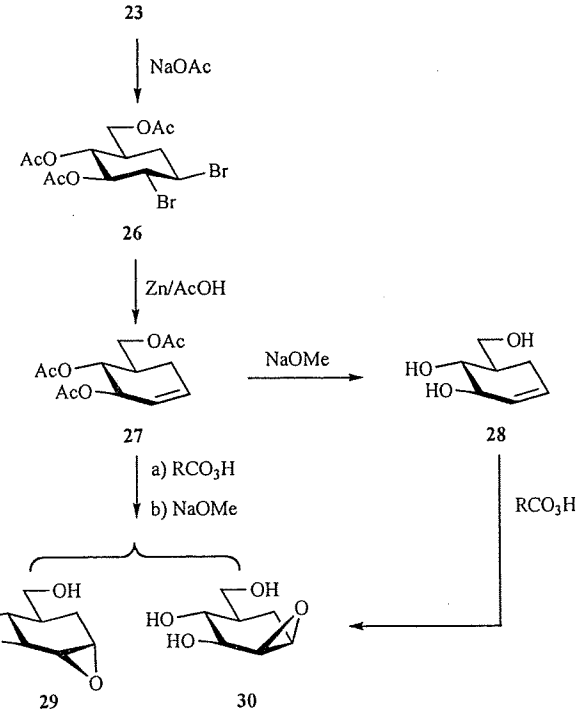


Fig. (4). Preparation of synthetically useful 1,2-anhydro-5a-carba-α-gluco and β-mannopyranoses.

be obtained when preparative routes for the racemates were established.