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Selective Inhibition of β-1,4- and α-1,3-Galactosyltransferases: Donor Sugar-Nucleotide Based Approach

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Abstract—A combined rational and library approach was used to identify bisphosphonates ($IC_{50}=20\,\mu M$) and galactose type 1-*N*-iminosugar ($IC_{50}=45\,\mu M$) as novel motifs for selective inhibition of β-1,4-galactosyltransferase (β-1,4-GalT) and α-1,3-galactosyltransferase (α-1,3-GalT), respectively. Our results demonstrate that, though these two galactosyltransferases both utilize the same donor sugar-nucleotide (UDP-Gal), the difference in their mechanisms can be utilized to design donor sugar or nucleotide analogues with inhibitory activities selective for only one of the galactosyltransferases. Investigation of β-1,4-GalT inhibition using UDP-2-deoxy-2-fluorogalactose (UDP-2-F-Gal), UDP, and bisphosphonates, also led to the observation of metal dependent inhibition of β-1,4-GalT. These observations and the novel inhibitor motifs identified in this study pave the way for the design and identification of even more potent and selective galactosyltransferase inhibitors.

Cell surface carbohydrates serve as key elements in various molecular recognition processes, including bacterial and viral infections, cell adhesion in inflammation and metastasis, immune response, differentiation, development, regulation and many other intercellular communication and signal transduction events.[1] Selective inhibition of the enzymes, such as glycosyltransferases, that are involved in the synthesis of cell-surface oligosaccharides is thus of interest as it may lead to the development of new therapeutic agents. To date, however, only limited success has been achieved in developing inhibitors of glycosyltransferases.[2–9]

There are several reasons that development of glycosyltransferase inhibitors has been difficult: (1) lack of structural data for glycosyltransferases makes it difficult to design structure-based inhibitors; (2) the transition state of the enzyme reaction includes many components (nucleotide, metal, acceptor sugar and donor sugar) and is thus complex and difficult to mimic; (3) glycosyltransferases bind their substrates weakly, with K_m usually in the mM range; and (4) lack of an efficient, sensitive and convenient method for the analysis of glycosyltransferase inhibition makes it difficult to use 'combinatorial' approaches to screen libraries.

This paper describes a combined rational and library approach, that utilizes our knowledge of the transition state of galactosyltransferase reactions and a rapid electrospray mass spectrometry assay,[10] to identify selective inhibitors of β-1,4- and α-1,3-galactosyltransferase that are based on the donor sugar-nucleotide substrate UDP-Gal. Also described is a metal dependence of β-1,4-galactosyltransferase inhibition.

Of the two galactosyltransferases chosen for our study, β-1,4-galactosyltransferase (β-1,4-GalT) is the more extensively studied and characterized.[11–17] β-1,4-GalT is an enzyme that transfers galactose from uridine-5′-diphosphogalactose (UDP-Gal) to the 4-OH of terminal *N*-acetylglucosamine (GlcNAc) residues. This galactosyltransferase is of biological and medicinal interest as it is involved in the biosynthesis of many cell surface oligosaccharide structures such as blood group antigens and sialyl Lewis X.[18,19] Increase or decrease in β-1,4-GalT activity has been associated with disease states such as

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arthritis[20–23] and cancer.[24] Interestingly, β-1,4-GalT can also act as a cell surface receptor[25–30] and is known to be important for fertilization and development in mammals.[31–33] The proposed transition state of the reaction catalyzed by β-1,4-GalT is shown in Figure 1. In the acceptor sugar portion, there is a base that abstracts a proton and increases the nucleophilicity of the 4-hydroxyl group.[4] In the donor sugar portion, there is a substantial sp^2 character[3,14] at the anomeric center. In the nucleotide portion, there is a new negative charge developed on the pyrophosphate portion. To date, however, there has been no X-ray crystal structure of this enzyme reported.

Compared to β-1,4-GalT, α-1,3-galactosyltransferase (α-1,3-GalT) has been less studied.[34–36] α-1,3-GalT is similar to β-1,4-GalT in that it transfers galactose from UDP-Gal to an acceptor sugar, but is different in the acceptor sugar specificity, and in the anomeric configuration of the newly formed galactosidic bond. There is considerable interest in this enzyme as its products, Galα1-3Galβ1-4GlcNAc and related structures, are the major cause of hyperacute xenotransplant rejections, and selective inhibitors against this enzyme may find use in xenotransplantation.[37] Though little has been reported concerning the mechanism of this enzyme, a distinction from β-1,4-GalT is that α-1,3-GalT is a 'retaining' glycosyltransferase, where the anomeric configuration of the galactose moiety transferred is alpha in both the substrate sugar nucleotide and in the product oligosaccharide, whereas β-1,4-GalT is an 'inverting' glycosyltransferase where the anomeric configuration of the galactose moiety transferred is inverted from alpha in the substrate sugar-nucleotide to beta in the product oligosaccharide. The 'retaining' nature of α-1,3-GalT implies a two-step mechanism: a double displacement reaction or an SN1 reaction with front attack by the acceptor (Fig. 2).

Identification of bisphosphonates as β-1,4-galactosyltransferase inhibitors

Based on the proposed transition state of β-1,4-GalT, a series of compounds were screened as inhibitors using our recently developed electrospray mass spectrometry assay[10,38] as well as conventional radioactive assays.

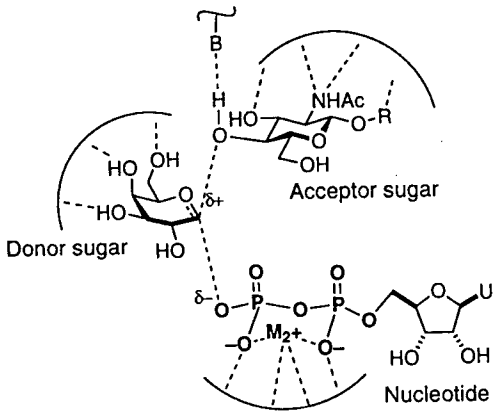


Figure 1. A possible transition state structure of the β-1,4-galactosyltransferase reaction.

Fifty-six potential inhibitors were screened using electrospray mass spectrometry (Fig. 3). These compounds were chosen due to their similarity with either the donor sugar portion, the nucleotide portion, or both, in the proposed reaction transition state.

Since β-1,4-GalT reactions are thought to proceed through transition states similar to those of the glycosidase reactions, with substantial sp^2 character developed at the anomeric center,[3,14] we started our screening efforts by assaying various imino sugars (**1–7**) that are known to be potent transition-state analogue inhibitors of glycosidases.[38,39] However, consistent with previous results,[14] none of the simple iminocyclitols showed potent inhibition against β-1,4-GalT. Next, since uridine-5′-diphosphate (UDP, **8**) is a known potent product inhibitor, various nucleotide containing compounds (**8–38**) were tested.[13,18] The results suggest that the pyrophosphate portion is crucial for inhibition as none of the monophosphates, including uridine-5′-monophosphate (UMP, **20**), showed significant inhibition. An interesting result was that thymidine-5′-diphosphate (TDP, **13**) and 2′-deoxy-UDP (**12**) which lack the 2′-hydroxyl group showed markedly decreased inhibition compared to UDP. The attachment of any moiety to UDP also resulted in decreased inhibition (**32–38**) except in the case of UDP-2-deoxy-2-fluoro-galactose (UDP-2-F-Gal, **31**).[3] However, an even simpler version of the nucleotide portion—pyrophosphate (**40**) and methylene diphosphonate (**39**)—showed surprisingly effective as inhibitors against β-1,4-GalT. Intrigued by this result, other simple diacids or bidentate compounds (**41–56**) were also tested for inhibition; however, no potent inhibitors were found.

From the above structure–activity relationship studies and knowledge about the transition state, we propose that: (1) UDP-2-F-Gal (**31**) is a good inhibitor because it mimics well the donor-sugar-nucleotide in the ground state and the F group may be involved in a H-bonding interaction with some residue in the binding site. In addition, the F group strengthens the glycosidic bond and slows down the cleavage through an SN1 process. (2) UDP (**8**) is a good inhibitor not only because it is a partial structure of the ground state of the donor sugar nucleotide, UDP-Gal, but also because its extra negative charge mimics the negative charge build-up on the nucleotide portion in the transition state. Thus, attachment of anything to UDP (**8**), which results in one less charge, decreases binding unless it is a good galactose mimic.[5] The uridine portion of UDP (**8**) is more important for specificity because TDP (**13**), dUDP (**12**) and UDP-dialdehyde (**9**) all show much weaker inhibition. (3) The good inhibition by pyrophosphate (**40**) and methylene diphosphonate (**39**) may be because they possess an extra charge which can mimic the negative charge build-up in the transition state (Fig. 1). They also do not contain any moiety that may interfere with the enzyme binding.

Based on this screening, methylene diphosphonate derivatives (bisphosphonates) were identified as new inhibitors of β-1,4-GalT (Table 1). These compounds have several advantages: (1) they mimic the transition state of

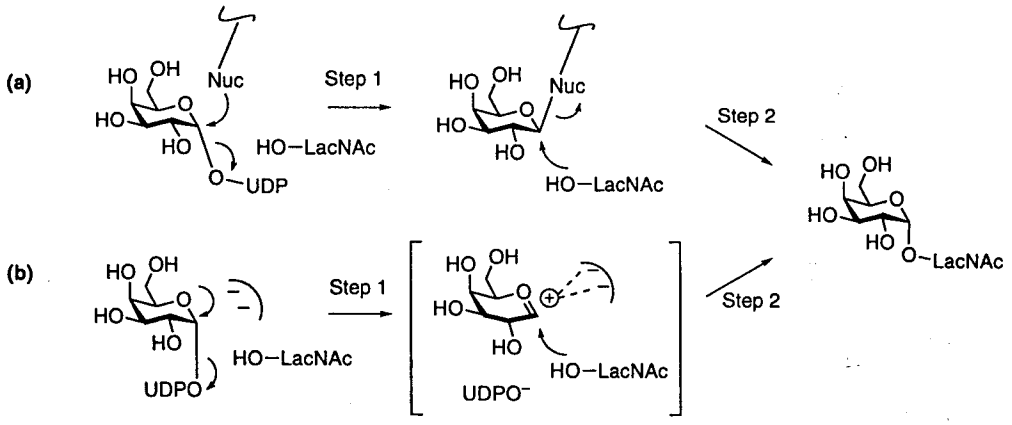


Figure 2. Possible mechanisms for the reaction catalysed by α-1,3-galactosyltransferase. (a) Double inversion mechanism; (b) galactosyl cation mechanism.

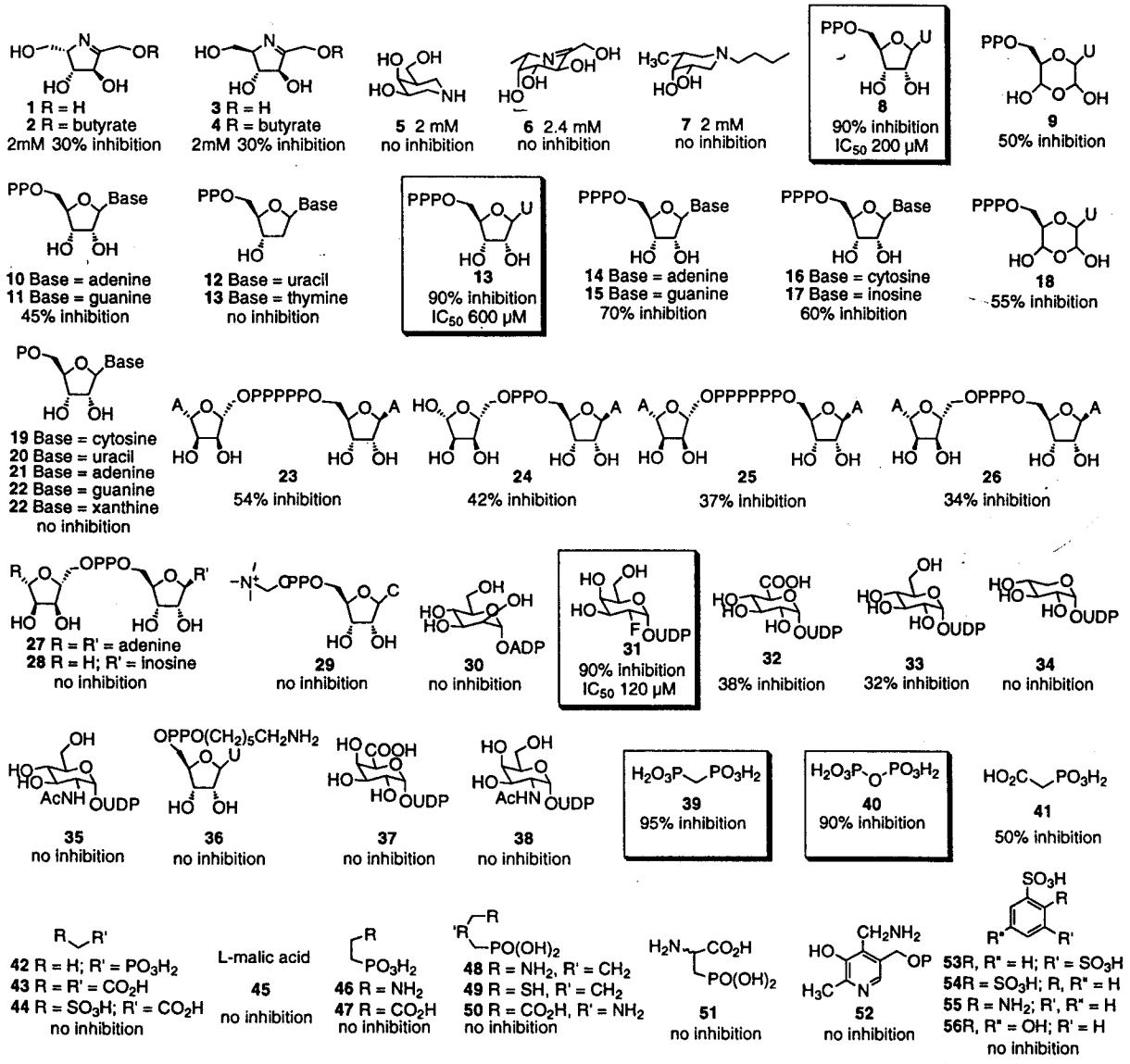
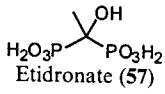
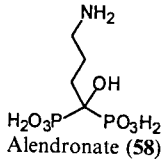
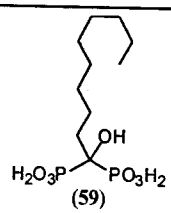
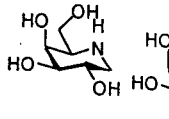
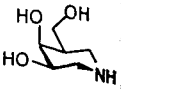


Figure 3. Potential inhibitors of β-1,4-galactosyltransferase were screened using electrospray mass spectrometry (% inhibition at 1 mM inhibitor unless otherwise stated). The better inhibitors are boxed.

Table 1. Effect of metal ions on the K_m of the substrate uridine-5′-diphosphogalactose (UDP-Gal) and on the inhibition of β-1,4-galactosyltransferase (β-1,4-GalT) and α-1,3-galactosyltransferase (α-1,3-GalT). IC_{50} in μM (K_i in μM)

		UDP-Gal[a]	UDP-2-F-Gal (31)	UDP (8)	 Etidronate (57)	 Alendronate (58)	 (59)		
β-1,4-GalT	1 mM Mn^{2+}	25 ± 4	(39 ± 9)	–	–	–	–	–	–
	2 mM Mn^{2+}	9 ± 2	(10 ± 2)	–	–	–	–	–	–
	5 mM Mn^{2+}	7 ± 1	(7 ± 1)	–	–	–	–	–	–
	10 mM Mn^{2+}	3 ± 0.6	7 (2 ± 0.3)	25	NI[b]	NI[b]	489	NI[b]	NI[b]
	Metal mixture[c]	38 ± 0.3	202	27	49	35 (51 ± 4)	20	–	–
α-1,3-GalT	10 mM Mn^{2+}	17 ± 3	245	13	NI[b]	NI[b]	NI[b]	NI[b]	48 (75 ± 3.6)
	Metal mixture[c]	51 ± 5	501	15	NI[b]	NI[b]	NI[b]	NI[b]	50

[a] K_m in μM.
[b] Less than 30% inhibition at 800 μM.
[c] Mixture of divalent cations at physiologically relevant concentrations (4 mM Ca^{2+}, 4 mM Mg^{2+}, 80 μM Zn^{2+}).

the nucleotide portion (Fig. 1); (2) they are easy to synthesize and derivatize; (3) they are physiologically stable, and (4) they are known to have low toxicity, as they are clinically used drugs, and are orally bioavailable.[40–46]

Metal dependent inhibition of β-1,4-galactosyltransferase

UDP-2F-Gal (**31**), UDP (**8**), and three bisphosphonates, **57**, **58**, and **59** were subjected to detailed inhibition studies that led to the discovery of metal dependent inhibition of β-1,4-GalT (Table 1). The metal dependence of β-1,4-GalT *activity* is well documented.[13,47–50] Many glycosyltransferases, though not all, are activated by millimolar concentrations of divalent metal ions, of which Mn^{2+} is the most commonly used and often the most efficient.[51,52] In 1971, it was observed that the apparent K_m of UDP-Gal for β-1,4-GalT decreased with increase in manganese concentration.[48] There has, however, been no studies concerning the effect of metal ions on the *inhibition* of galactosyltransferase. We found that the K_i for UDP-2-F-Gal (**31**) decreased with increased manganese concentration, a trend similar to the decrease in the K_m of the substrate UDP-Gal (Table 1). The K_i of UDP-2-F-Gal (**31**), reported to be 149 μM previously under conditions with low concentrations of manganese,[3] became 3 μM under conditions identical to that used by Hashimoto,[2] where high concentrations of manganese and bovine serum albumin (BSA) were used.

Though we observed an increase in inhibitory potency at higher manganese concentrations, we were concerned with the high manganese concentration used (10 mM) in these assays. Since the initial report of manganese activation of galactosyltransferase in 1966,[47] the most often used condition for mechanistic studies,[11–14,16,17,48,53–55] for synthetic reactions,[18] and for inhibition studies[2–8] of β-1,4-GalT has been to have millimolar concentrations of Mn^{2+}. However, the manganese concentration used in these 'optimal' conditions exceeds the likely physiological concentration of Mn^{2+} by 10,000–100,000 times. The kinetic and inhibition data obtained under such conditions may thus be physiologically irrelevant. Several studies have addressed the question of what the physiological 'metal activator'[49,51,56–58] might be and have shown that millimolar manganese is not an absolute requirement. β-1,4-GalT has two metal binding sites that need to be occupied for optimal activity (K_m of 20 μM and 400 μM for manganese),[49] and though manganese is the only metal that can satisfy both sites, there are many species that can satisfy one of the two metal binding sites.[49,51,56–58]

In order to obtain inhibition data that may be more related to physiological conditions, UDP-2-F-Gal (**31**) was tested for inhibition under a 'metal mixture' condition in which a combination of metals were used at their physiologically relevant concentrations.[59] We found that the inhibitory activity of UDP-2-F-Gal (**31**) became much weaker under this condition (Table 1). Next, the inhibition of UDP (**8**) was compared under the 10 mM manganese condition and the physiological metal mixture condition. UDP (**8**) displayed similar inhibition potencies under either condition (Table 1). Finally, we compared the inhibition by the bisphosphonate inhibitors under the two assay conditions. Bisphosphonates actually are better inhibitors under the physiological metal mixture condition compared to the high manganese concentration condition (Table 1). We observed that UDP-2-F-Gal (**31**) was the most potent and bisphosphonates were the weakest inhibitor at high manganese concentration and that bisphosphonates were the most potent and UDP-2-F-Gal (**31**) the weakest inhibitor under the physiological metal mixture condition.

For compound **58**, a widely used drug for treatment of bone disease, a more detailed inhibition study was performed. The inhibition could not be fitted to simple pseudo first-order kinetics due to a less inhibitory phase at lower concentrations and a higher inhibition phase at

higher concentrations. The less inhibitory phase, however, displayed a competitive inhibition pattern (K_i = 51 μM) against UDP-Gal (K_m = 38 μM), consistent with bisphosphonate **58** binding in the UDP-Gal binding site of the enzyme (Fig. 4). Though the mechanism of metal-dependent changes in the inhibition of β-1,4-GalT is unclear at the moment, enhancement of inhibition by specific metal-mediated binding has been described for other enzyme-inhibitor systems[60] and the decrease in the K_m of UDP-Gal with increasing manganese concentration has been suggested to be due to additional manganese-mediated binding.[50] Such metal mediated binding may also be important in the metal dependent inhibition of β-1,4-GalT by UDP-2-F-Gal (**31**) and the bisphosphonate inhibitors (**57–58**). In any case, the metal dependence of β-1,4-GalT is an important factor that must be considered carefully when evaluating the inhibition data.

Identification of galactose-type 1-*N*-iminosugar as an inhibitor of α-1,3-galactosyltransferase

Inhibition studies with α-1,3-GalT were first performed using the potent inhibitors identified for β-1,4-GalT, namely, UDP-2-F-Gal (**31**), UDP (**8**) and bisphosphonates **57**, **58**, and **59** (Table 1). Though α-1,3-GalT and β-1,4-GalT both utilize the same donor sugar-nucleotide (UDP-Gal) and both are inhibited substantially by UDP (**8**), UDP-2-F-Gal (**31**) and bisphosphonates **57–59** showed less inhibitory activity against α-1,3-GalT. It turned out that UDP-2-F-Gal (**31**) and bisphosphonates **57–59** were more selective inhibitors of β-1,4-GalT, perhaps due to some subtle differences in binding. We note that UDP-2-F-Gal (**31**) reactivity was monitored by electrospray mass spectrometry and thin layer chromatography and confirmed not to be a substrate for α-1,3-GalT.

Next, we tested iminocyclitols **5** and **60** as inhibitors against α-1,3-GalT. Though these compounds had shown poor inhibitory activity against β-1,4-GalT, they were considered to be promising as inhibitors against α-1,3-GalT because of the difference in mechanism (Figs 1 and 2). α-1,3-GalT is expected to contain either a nucleophile that forms a covalent galactosyl-enzyme intermediate (Fig. 2a) or some positive charge stabilizing groups to stabilize the galactosyl-cation intermediate (Fig. 2b); the enzyme in either case may form favorable contacts with a properly placed imino-group. Indeed, we observed a good inhibition with the galactose-type 1-*N*-iminosugar (**5**) which gave an IC_{50} of 45–48 μM, though iminocyclitol **60** showed no inhibition up to 2 mM. Compound **5** is the most potent iminocyclitol inhibitor of galactosyltransferases known to date. A more detailed study of inhibition by compound **5** revealed it to be a noncompetitive inhibitor against the donor sugar (UDP-Gal) and the acceptor sugar (LacNAcOMe) with K_i of 75 ± 3.6 and 35 ± 1.6 μM, respectively (Fig. 6).

It is surprising that **5** is not a competitive inhibitor against UDP-Gal; however, it should be noted that, while non-competitive inhibition is often interpreted as the result of an inhibitor binding to a site independent of the substrate site, this mode of inhibition is commonly observed in multisubstrate enzyme reactions where dead-end complexes can be formed.[61,62] An amine-containing acceptor sugar analogue inhibitor against α-1,3-GalT has previously been reported, which also displayed a non-competitive inhibition pattern, instead of the expected competitive inhibition pattern, with respect to the acceptor sugar.[63] To obtain a better idea of the mode of inhibition, product inhibition studies were also performed with UDP and it was shown to be a competitive inhibitor (6.5 ± 0.8 μM) against UDP-Cal and a non-competitive inhibitor (16 ± 0.8 μM) against LacNAcβOMe. Inhibition by the trisaccharide product (Galα1,3Galβ1,4GlcNAcOH) was also observed, though it was too weak (30% inhibition at 5 mM) for detailed inhibition analysis. Incubation of

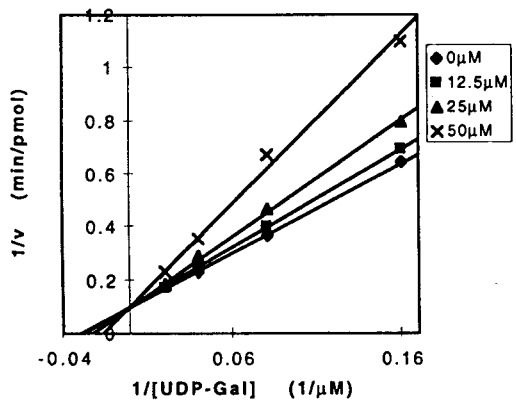
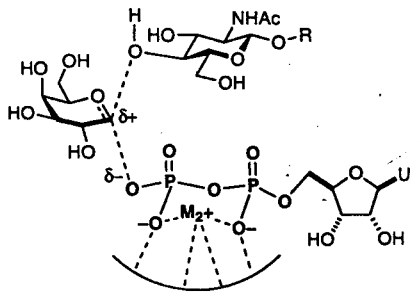


Figure 4. Lineweaver–Burk plot of the inhibition of β-1,4-galactosyltransferase by bisphosphonate **58**.



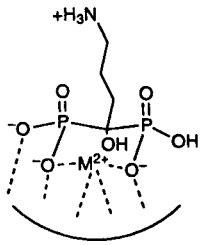


Figure 5. A proposed transition-state structure of β-1,4-galactosyltransferase reaction and a proposed binding mode of alendronate (**58**).

UDP-Gal and α-1,3-GalT without acceptor sugar gave only negligible activity, excluding the possibility of a ping-pong mechanism. Though binding of iminocyclitol **5** to a site independent of the substrate site on the enzyme cannot be excluded, the inhibition patterns observed can be explained as **5** binding to the substrate binding site of an enzyme with Bi Bi mechanism in both the substrate free state and the UDP and/or LacNAc-βOMe bound states.

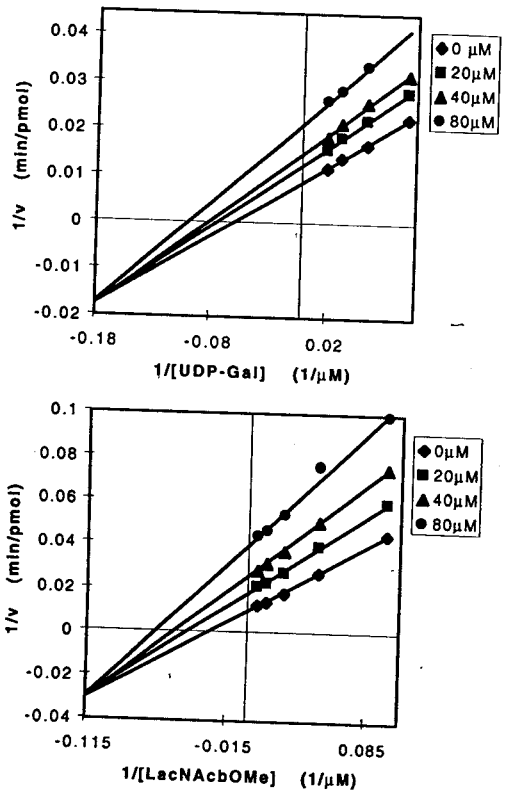


Figure 6. Lineweaver–Burk plots of the inhibition of α-1,3-galactosyltransferase with galactose type 1-*N*-iminosugar (**5**).

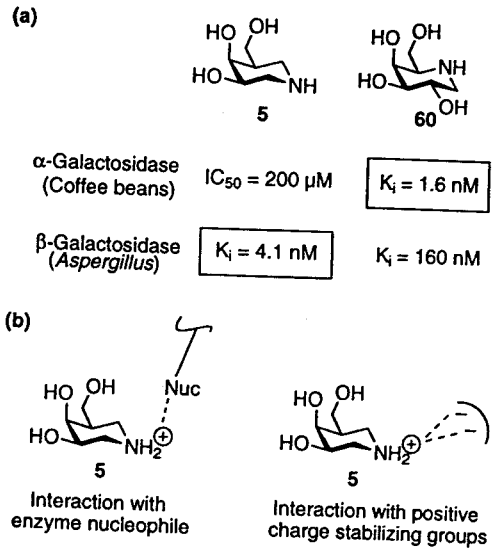


Figure 7. (a) Comparison of the inhibition of **5** and **60** for α- and β-galactosidases and (b) possible interactions between **5** and α-1,3-GalT.

The large difference in the inhibition of α-1,3-GalT by the two iminocyclitols **5** and **60** is interesting. The selectivity in the inhibition of galactosidases has recently been reported, showing that **5** is a potent inhibitor of β-galactosidase whereas **60** is a more potent inhibitor of α-galactosidase (Fig. 7). The selectivity in the inhibition of galactosidases presumably arises from the different positions of the imino-group.[39] The imino-group of **5** being more favorably positioned to interact with the positive-charge stabilizing groups of β-galactosidase whereas the imino-group of **60** is more favorably positioned to interact with positive-charge stabilizing groups of α-galactosidase. The potent inhibition of α-1,3-GalT by iminocyclitol **5** but not by **60** is probably due also to the more favorable positioning of the protonated imino group to interact with the enzyme nucleophile or positive charge stabilizing groups present in α-1,3-GalT (Fig. 7).

Conclusion

Using a combination of rational design and rapid screening, we have identified bisphosphonates and galactose-type 1-*N*-iminosugar as novel inhibitor motifs for β-1,4-galactosyltransferase and α-1,3-galactosyltransferase, respectively. Though the design of these inhibitors is based on the common substrate, UDP-Gal, these inhibitors are selective for their respective galactosyltransferases, perhaps due to differences in the mechanism of the two enzymes. The results also demonstrate that mass spectrometry is a useful tool for identifying novel glycosyltransferase inhibitors. We also observed the metal-dependent inhibition of β-1,4-galactosyltransferase. Efforts are underway to develop more potent and selective galactosyltransferase inhibitors using these relatively simple inhibitor motifs.

Experimental

General. Recombinant bovine β-1,4-galactosyltransferase was purchased from Calbiochem and recombinant α-1,3-galactosyltransferase was expressed in *Escherichia coli* (A. Sepp and R. I. Lechler). *N*-Acetylglucosamine, UDP-galactose, bovine serum albumin, all nucleotides and acidic compounds screened in the mass spectral assay, Dowex 1X8 (200–400 mesh), $MnCl_2$, $CaCl_2$, $ZnCl_2$ were purchased from Sigma or Aldrich. Preparation of compounds **1–4**, **6**, **31** has been described previously.[3,38] Preparation of compounds **5** and **7** has been described previously.[39] Bisphosphonate **57** was purchased from TCI America. Bisphosphonate **58** was prepared according to known procedures.[64] Compound **59** was prepared in a manner similar to the preparation of **58** (see below). Uridine 5′-diphospho-[galactose-6-^{3}H] was purchased from Amersham Life Science. Scinti-Verse I scintillation cocktail and $MgCl_2$ was purchased from Fisher Scientific. The scintillation counter used was the Packard Tri-Carb 2100 TR. Analytical thin layer chromatography was performed using silica gel 60 F_{254} pre-coated glass plates (Merck); compound spots

were visualized by quenching of fluorescence and/or by charring after treatment with cerium molybdophosphate.

Screening of inhibitors using electrospray mass spectrometry. Performed according to procedures described previously.[10,38] Reaction mixtures contained GlcNAcβOBn (1 mM), $MnCl_2$ (1 mM), β-1,4-galactosyltransferase (0.6 mU), UDP-Gal (0.4 mM) and inhibitor in a pH 7.4, 20 mM HEPES buffer. The individual reactions were carried out on a 50 μL scale in a 0.5 mL Eppendorf tube at 25 °C for 20 min and then quenched by adding 400 μL MeOH followed by 4 μL of LacNAcβSPh[65] (1 mM aq solution) as an internal standard. The reaction mixtures were directly injected into the PE SCIEX API100 electrospray mass spectrometer via the HP 1090 HPLC autosampler.

(1-Hydroxydecylidene)bisphosphonic acid disodium salt (59). Analogous to literature procedure,[64] to a solution of 1-decanoic acid (1.53 g, 12 mmol) and phosphorous acid (1.07 g, 13 mmol) in methanesulfonic acid (7 mL) at 65 °C was added dropwise PCl_3 (2.27 mL, 26 mmol) and the resulting mixture was stirred for 24 h at the same temperature. The reaction mixture was cooled to room temperature (rt), then poured into ice-cold water (15 mL) and the flask was rinsed with cold water (7 mL). The combined aqueous mixture was stirred for 5 h at 95 °C, then cooled to rt. The solution was adjusted to pH 5 with 50% NaOH and cooled in an ice bath to afford a white precipitate. The precipitate was collected on a filter, washed with cold water (2×10 mL) and cold methanol (2×10 mL), and then dried in vacuo yielding 2.65 g of white solid product (61%). MS (FAB) *m/z* 339 (M–Na), 317 (MH^+–2Na); ^1H NMR (NaOD/D_2O) δ 1.85–1.73 (2H, m), 1.51–1.37 (2H, m), 1.23–1.20 (12H, m), 0.789 (3H, t); ^{13}C NMR (NaOD/D_2O) δ 79.24 (t, $J_{c\text{-}p}$ = 536 Hz), 39.68, 33.67, 32.96, 31.61 (2C), 31.01, 26.85, 24.50, 15.88; 31P NMR (NaOD/D_2O) δ 20. Anal. calcd for $C_{10}H_{22}Na_2O_7P_2$: C, 33.16; H 6.12. Found: C, 33.27; H, 5.86.

IC_{50} measurements of inhibitors against β-1,4-galactosyltransferase. For measuring IC_{50} under reaction conditions using physiologically relevant metal mixture, assays contained 4 mM $CaCl_2$, 4 mM $MgCl_2$, 80 μM $ZnCl_2$, 0.034 milliunit of β-1,4-galactosyltransferase, UDP-[6-^{3}H]-galactose (35 μM), GlcNAcβOMe (0.5 mM), inhibitor (12–1000 μM), bovine serum albumin (1 mg/mL), and 50 mM HEPES buffer (pH 7.4) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were halted by the addition of 0.3 mL of deionized water and immediately applying it to a pipette column of Dowex 1X8 (1 mL). The pipette was washed with an additional 0.9 mL of water (3×0.3 mL). The flow-through and column washes were collected in 11 mL of ScintiVerse I scintillation cocktail. A control reaction without enzyme was used to establish the background count. The IC_{50} was determined by plotting 1/v versus inhibitor concentration.

For measuring IC_{50} under reaction conditions using 10 mM manganese, assays contained 10 mM $MnCl_2$, 0.01 milliunit of β-1,4-galactosyltransferase, UDP-[6-^{3}H]-galactose (5 μM), GlcNAcβOMe, (0.5 mM), inhibitor (1–1000 μM), bovine serum albumin (1 mg/mL), and 50 mM HEPES buffer (pH 7.4) in a total assay volume of 0.05 mL. Reaction temperature, work up, etc are as described above.

IC_{50} measurements of bisphosphonate inhibitors and UDP-2F-gal against α-1,3-galactosyltransferase. For measuring IC_{50} under reaction conditions using physiologically relevant metal mixture, assays contained 4 mM $CaCl_2$, 4 mM $MgCl_2$, 80 μM $ZnCl_2$, α-1,3-galactosyltransferase (0.0006 mg/mL), UDP-[6-^{3}H]-galactose (60 μM), LacNAcβOMe (0.1 mM), inhibitor (2–1000 μM), bovine serum albumin (1 mg/mL), and 50 mM MES buffer (pH 6.0) in a total assay volume of 0.05 mL. Reaction temperature, work up, etc are as described for β-1,4-galactosyltransferase.

For measuring IC_{50} under reaction conditions using 10 mM manganese, assays contained 10 mM $MnCl_2$, α-1,3-galactosyltransferase (0.0003 mg/mL), UDP-[6-^{3}H]-galactose (25 μM), LacNAcβOMe (0.1 mM), inhibitor (2–1000 μM), bovine serum albumin (1 mg/mL), and 50 mM MES buffer (pH 6.0) in a total assay volume of 0.05 mL. Reaction temperature, work up, etc are as described for β-1,4-galactosyltransferase.

Determination of the K_i of 58 against β-1,4-galactosyltransferase. Assays contained 4 mM $CaCl_2$, 4 mM $MgCl_2$, 80 μM $ZnCl_2$, 0.034 milliunit of galactosyltransferase, UDP-[6-^{3}H]-galactose (13 to 60 μM), GlcNAcβOMe (0.5 mM), inhibitor (0 to 60 μM), bovine serum albumin (1 mg/mL), and 50 mM HEPES buffer (pH 7.4) in a total assay volume of 0.05 mL. Reaction temperature, work up, etc are as described above. Double reciprocal plot analysis of galactosyltransferase activity as a function of **58** and UDP-Gal showed a competitive inhibition pattern. K_i for **58** was determined to be 51 ± 4 μM (K_m = 38 ± 3 μM for UDP-Gal) with a nonlinear, least-square fit of the data to the kinetic equation for competitive inhibition.

Determination of the K_i of UDP-2-F-Gal (31) against β-1,4-galactosyltransferase. Assays contained $MnCl_2$ (1–10 mM), 0.034 milliunit of galactosyltransferase, UDP-[6-^{3}H]-galactose (1–100 μM), GlcNAcβOMe (3 mM), inhibitor (0–100 μM), bovine serum albumin (1 mg/mL), and 50 mM HEPES buffer (pH 7.4) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were worked up and data analyzed as described above.

Determination of K_is of inhibitors against α-1,3-galactosyltransferase. To obtain K_is against UDP-Gal, assays contained $MnCl_2$ (10 mM), galactosyltransferase (0.0003 mg), UDP-[6-^{3}H]-galactose (10–50 μM), LacNAcβOMe (100 μM), inhibitor (0–80 μM), bovine serum albumin (1 mg/mL), and 50 mM MES buffer (pH 6.0) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were worked up as described above, inhibition pattern determined by reciprocal plot analysis, and K_i determined by a least-square fit of the data to an appropriate kinetic equation.

To obtain K_is against LacNAcβOMe, assays contained $MnCl_2$ (10 mM), galactosyltransferase (0.0003 mg), UDP-[6-^{3}H]-galactose (50 μM), LacNAcβOMe (10–160 μM), inhibitor (0–80 μM), bovine serum albumin (1 mg/mL), and 50 mM MES buffer (pH 6.0) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were worked up and data analyzed as described above.

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References

1. Varki, A. *Glycobiology* **1993**, *3*, 97.
2. Hashimoto, H.; Endo, T.; Kajihara, Y. *J. Org. Chem.* **1997**, *62*, 1914.
3. Hayashi, T.; Murray, B. W.; Wang, R.; Wong, C.-H. *Bioorg. Med. Chem.* **1997**, *5*, 497.
4. Hindsgaul, O.; Kaur, K. J.; Srivastava, G.; Blaszczyk-Thurin, M.; Crawley, S. C.; Heerze, L. D.; Palcic, M. M. *J. Biol. Chem.* **1991**, *266*, 17858.
5. Endo, T.; Kajihara, Y.; Kodama, H.; Hashimoto, H. *Bioorg. Med. Chem.* **1996**, *4*, 1939.
6. Vaghefi, M. M.; Bernacki, R. J.; Dalley, K.; Wilson, B. E.; Robins, R. K. *J. Med. Chem.* **1987**, *30*, 1383.
7. Vaghefi, M. M.; Bernacki, R. J.; Hennen, W. J.; Robins, R. K. *J. Med. Chem.* **1987**, *30*, 1391.
8. Wang, R.; Steensma, D. H.; Takaoka, Y.; Yun, J. W.; Kajimoto, T.; Wong, C.-H. *Bioorg. Med. Chem.* **1997**, *5*, 661.
9. Yuasa, H.; Palcic, M. M.; Hindsgaul, O. *Can. J. Chem.* **1995**, *73*, 2190.
10. Wu, J.; Takayama, S.; Wong, C.-H.; Siuzdak, G. *Chem. & Biol.* **1997**, *4*, 653.
11. Bell, J. E.; Beyer, T. A.; Hill, R. L. *J. Biol. Chem.* **1976**, *251*, 3003.
12. Ebner, K. E.; Magee, S. C. In *Lactose synthetase: α-lactalbumin and β-(1–4) galactosyltransferase*; Ebner, K.E., Magee, S. C., Ed.; Academic: New York, 1975; Vol. 2, pp 137–179.
13. Khatra, B. S.; Herries, D. G.; Brew, K. *Eur. J. Biochem.* **1974**, *44*, 537.
14. Kim, S. C.; Singh, A. N.; Raushel, F. M. *Arch. Biochem. Biophys.* **1988**, *267*, 54.
15. O'Keeffe, E. T.; Hill, R. L.; Bell, J. E. *Biochemistry* **1980**, *19*, 4954.
16. Takase, K.; Ebner, K. E. *Curr. Top. Cell Regul.* **1984**, *24*, 51.
17. Trayer, I. P.; Hill, R. L. *J. Biol. Chem.* **1971**, *246*, 6666.
18. Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9283.
19. Ram, B. P.; Munjal, D. D. *CRC Crit. Rev. Biochem.* **1985**, *17*, 257.
20. Alavi, A.; Axford, J. *Glycoconj. J.* **1995**, *12*, 206.
21. Furukawa, K.; Matsuta, K.; Takeuchi, F.; Kosuge, F.; Miyamoto, T.; Kobata, A. *Int. Immun.* **1990**, *2*, 105.
22. Richard, M.; Vignon, E.; Peschard, M. J.; Boquet, P.; Carret, J. P.; Loisot, P. *Biochem. Int.* **1990**, *22*, 535.
23. Endo, T.; Furukawa, K. In *Rheumatoid arthritis and serum IgG*; Endo, T., Furukawa, K., Eds.; Elsevier: Amsterdam, 1996; Vol. 30, pp 277–290.
24. Krishnaraj, R.; Saat, Y. A. *Cancer Res.* **1985**, *45*, 3615.
25. Barcellow-Hoff, M. G. *Exp. Cell Res.* **1992**, *201*, 225.
26. Bayna, R. M.; Shaper, J. G.; Shur, B. D. *Cell* **1988**, 145.
27. Lopez, L. C.; Youakim, A.; Evans, S. C.; Shur, B. D *Biol. Chem.* **1991**, *266*, 15984.
28. Russo, R. N.; Shaper, N. L.; Shaper, J. L. *J. Biol. Ch* **1990**, *265*, 3324.
29. Riopelle, R. J.; Dow, K. E. *Brain Res.* **1991**, *541*, 265.
30. Evans, S. C.; Youakim, A.; Shur, B. D. *BioEssays* **19** *17*, 261.
31. Gong, X.; Dubois, D. H.; Miller, D. J.; Shur, B. D. *ence* **1995**, *269*, 1718.
32. Larson, J. L.; Miller, D. J. *Biol. Reprod.* **1997**, *57*, 442.
33. McLeskey, S. B.; Dowds, C.; Carballada, R.; White R.; Saling, P. M. *Int. Rev. Cytol.* **1998**, *177*, 57.
34. Sujino, K.; Malet, C.; Hindsgaul, O.; Palcic, M. M. *C bohydr. Res.* **1998**, *305*, 483.
35. Henion, T. R.; Macher, B. A.; Anaraki, F.; Galifi, *Glycobiology* **1994**, *4*, 193.
36. Blanken, W. M.; Van den Eijnden, D. H. *J. Biol. Ch* **1985**, *260*, 12927.
37. Parker, W.; Saadi, S.; Lin, S. S.; Holzknecht, Z. E.; B tos, M.; Platt, J. L. *Immunol. Today* **1996**, *17*, 373.
38. Takayama, S.; Martin, R.; Wu, J.; Laslo, K.; Qiao, Siuzdak, G.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 81
39. Ichikawa, Y.; Igarashi, Y.; Ichikawa, M.; Suhara, Y. *Am. Chem. Soc.* **1998**, *120*, 3007.
40. Sato, M.; Grasser, W.; Endo, N.; Akins, R.; Simmons, Thompson, D. D.; Golub, E.; Rodan, G. A. *J. Clin. Inve* **1991**, *88*, 2095.
41. Rodan, G. A.; Fleisch, H. A. *J. Clin. Invest.* **1996**, 2692–2696.
42. Schmidt, A.; Rutledge, S. J.; Endo, N.; Opas, E. Tanaka, H.; Wesolowski, G.; Leu, C. T.; Huang, Z.; Ram chandaran, C.; Rodan, S. B.; Rodan, G. A. *Proc. Natl. Acc Sci. USA* **1996**, *93*, 3068–3073.
43. Nugent, R. A.; Schlachter, S. T.; Murphy, M.; Dun C. J.; Staite, N. D.; Galinet, L. A.; Shields, S. K.; Wu, H.; Aspa D. G.; Richard, K. A. *J. Med. Chem.* **1994**, *37*, 4449–4454.
44. Lin, J. H.; Chen, L.-W.; DeLuna, F. A. *J. Pharm. S* **1994**, *83*, 1741–1746.
45. Atack, J. R.; Prior, A. M.; Fletcher, S. R.; Quirk, K Mckernan, R.; Ragan, C. I. *J. Pharm. Exp. Ther.* **1994**, *27* 70–76.
46. Dunn, C. J.; Galinet, L. A.; Wu, H.; Nugent, R. A Schlachter, S. T.; Staite, N. D.; Aspar, D. G.; Elliott, G. A Essani, N. A.; Rohloff, N. A.; Smith, R. J. *J. Pharm. Ex Ther.* **1993**, *266*, 1691–1698.
47. Babad, H.; Hassid, W. Z. *J. Biol. Chem.* **1966**, *241*, 2672 2678.
48. Morrison, J. F.; Ebner, K. E. *J. Biol. Chem.* **1971**, *24* 3977–3984.
49. Powell, J. T.; Brew, K. *J. Biol. Chem.* **1976**, *251*, 3645 3652.
50. Tsopanakis, A. D.; Herries, D. G. *Eur. J. Biochem.* **197** *83*, 179–188.
51. Navaratnam, N.; Virk, S. S.; Ward, S.; Kuhn, N. J. *Bi chem. J.* **1986**, *239*, 423–433.
52. Murray, B. W.; Takayama, S.; Schultz, J.; Wong, C.-H *Biochemistry* **1996**, *35*, 11183–11195.
53. Andree, P. J.; Berliner, L. J. *Biochemistry* **1980**, *19*, 929 934.
54. Morrison, J. F.; Ebner, K. E. *J. Biol. Chem.* **1971**, *24* 3985–3991.
55. Morrison, J. F.; Ebner, K. E. *J. Biol. Chem.* **1971**, *24* 3992–3998.
56. Navaratnam, N.; Ward, S.; Fisher, C.; Kuhn, N. L; Keen J. N.; Findlay, J. B. C. *Eur. J. Biochem.* **1988**, *171*, 623–629.
57. Kuhn, N. J.; Ward, S.; Leong, W. S. *Eur. J. Biochem* **1991**, *195*, 243–250.

58. Kuhn, N. J.; Stankiewica, M.; Ward, S. *Biochem. Soc. Trans.* **1992**, *20*, 714–716.
59. Altman, P. L.; Dittmer, D. S. *Biological Data Book*; *2nd Edition*; Federation of American Societies for Experimental Biology: Bethesda, Maryland, 1974; Vol. III.
60. Katz, B. A.; Clark, J. M.; Finer-Moore, J. S.; Jenkins, T. E.; Johnson, C. R.; Ross, M. J.; Luong, C.; Moore, W. R.; Stroud, R. M. *Nature* **1998**, *391*, 608–612.
61. Cleland, W. W. *The Enzymes* **1970**, *2*, 1–65.
62. Todhunter, J. A. *Methods Enzymol.* **1979**, *63*, 383–481.
63. Helland, A.-C.; Hindsgaul, O.; Palcic, M. M.; Stults, C. L. M.; Macher, B. A. *Carbohydr. Res.* **1995**, *276*, 91–98.
64. Kieczykowski, G. R.; Jobson, R. B.; Melillo, D. G.; Reinhold, D. F.; Grenda, V. J.; Shinkai, I. *J. Org. Chem.* **1995**, *60*, 8310–8312.
65. Takayama, S.; Shimazaki, M.; Qiao, L.; Wong, C.-H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1123–1126.