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New Glycosidase Activated Nitric Oxide Donors: Glycose and 3-Morphorlinosydnonimine Conjugates

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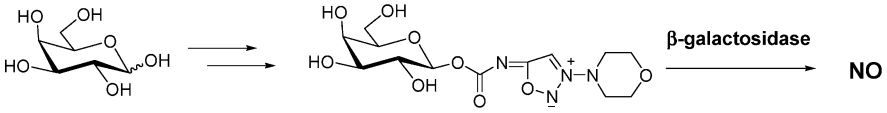

To achieve site specific delivery of nitric oxide (NO), a new class of glycosidase activated NO donors has been developed. Glucose, galactose, and *N*-acetylneuraminic acid were covalently coupled to 3-morphorlinosydnonimine (SIN-1), a mesoionic heterocyclic NO donor, via a carbamate linkage at the anomeric position. The β-glycosides were successfully prepared for these conjugates, while the α-glycosidic compounds were very unstable. The new stable sugar-NO conjugates could release NO in the presence of glycosidases. Such NO prodrugs may be used as enzyme activated NO donors in biomedical research.

Introduction

Nitric oxide (NO) is a small gaseous molecule that serves as a mediator of many physiological events.[1] NO can be easily converted into a variety of reactive nitrogen species (RNS), such as dinitrogen trioxide (N_2O_3), nitrogen dioxide (NO_2), and the peroxynitrite anion ($ONOO^-$).[2] While these RNS are highly reactive, their reactivity can be utilized to achieve beneficial results. It has been reported that sufficient generation of RNS can lead to cellular dysfunction and cytotoxicity.[3] By controlling the generation of a RNS to a localized area in which it is desirable to kill cells, such as in a solid tumor, the reactivity of the RNS can be used to achieve a desired result.

To achieve site-specific delivery of NO, spontaneous NO releasing agents can be converted into stable prodrugs by attaching them to carriers, which can be specially recognized by certain targets. We have designed and synthesized novel NO donors by taking advantage of the role of carbohydrates in cell recognition and internalization processes, since sugar derivatives can be delivered by hexose transporters on the cell membrane.[4] Two types of carbohydrate-linked NO donors have been developed in our group. The first type is the sugar-*S*-nitrosothiol. For example, Glyco-SNAP-1 (**1**) is a water soluble and stable NO donor, which showed superior cytotoxicity in vitro and is commercially available for biological research.[5] The second type is the sugar-NONOate (diazeniumdiolates are used instead of NONOates in recent nomenclature). For example, glucose-NONOate (**2**) could release NO by the action of glycosidases.[6] Our previous studies also showed that carbohydrates could stabilize the NO releasing moieties. The sugar-linked approach could be a good solution for the instability that most NO donors suffer from. Here we report new glycosyl carbamate linked NO donors (**3**). The carbohydrate moiety ensures that the NO donor compound is inactive until it encounters an appropriate activating enzyme, such as a glycosidase, and also increases the water solubility. We are interested in the development of carbamate-linked sugars, because this linkage is stable in aqueous solution and could be hydrolyzed by glycosidases[7] and no other toxic groups are released from the linkage portion of the molecule.

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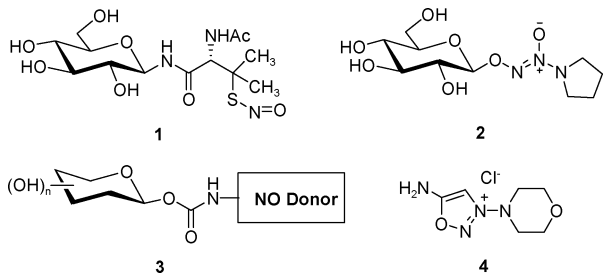

FIGURE 1. Structures of sugar-NO conjugates and SIN-1

The cytotoxicity of RNS against viruses is an exciting area in which we can potentially gain better understanding of the human immunity to viral infection and discover more information on antiviral drug design.[8] The influenza virus is especially attracting us since the replication of influenza A and B viruses could be severely impaired by NO donors.[9] Hemagglutinin (HA) and neuraminidase are embedded in the lipid bilayer of the influenza virus to bind and cut the terminal sialic acid. Therefore we also synthesized an *N*-acetylneuraminic acid-NO donor conjugate as an NO prodrug targeting the influenza virus. If this kind of prodrug is able to bind and be hydrolyzed by influenza neuraminidases, the NO will be released around the viruses and obtain the targeting effect. Our proposed synthetic sialic acid-NO conjugates can also be used as probes to selectively deliver RNS to the virus. Such conjugates may also be used to test an idea of neuraminidase activated prodrug design for antiviral therapy.

3-Morphorlinosydnonimine (SIN-1) (**4**) was first chosen as a model NO donor moiety.[10] SIN-1 is a vasodilator and is believed to release NO nonenzymatically. At physiological or alkaline pH, SIN-1 undergoes rapid hydrolysis to the ring-open form from which the NO radical is released. The activation of SIN-1 also produces the superoxide anion, which combines with NO to produce the highly reactive peroxynitrite anion. This reactive species can damage biomolecules and induce cytotoxicity.[11]

Results and Discussion

SIN-1 (**4**) as one of the starting materials was synthesized from 4-aminomorpholine by a two-step transformation following literature procedures.[12] Glucose and galactose derivatives (**6a** and **6b**) with unprotected anomeric hydroxyl groups were obtained by treating peracetylated glycoses with benzylamine in THF at an ambient temperature.[13] After purification by silica gel column chromatography, the glycose was coupled with 1 equiv of 4-nitrophenyl chloroformate in the presence of 3 equiv of triethylamine in methylene chloride at 0 °C, thereby producing 4-nitrophenyl (peracetyl-D-glycosyl)carbonate (**7a** and **7b**) as α/β mixtures (molar ratio 1: 3). The α/β mixtures could be separated in the following step. The coupling reaction of SIN-1 with protected glycosyl carbonate was carried out at room temperature in anhydrous pyridine.[14] The crude product was purified by silica gel chromatography and the anomeric mixtures of the products were separated. For this step, since SIN-1 itself was unstable in the solution, the best yield obtained was 40%. Then the acetyl protecting groups were removed in anhydrous methyl alcohol with sodium methoxide as a catalyst. This deprotection step required several hours at room temperature before the reaction mixture was neutralized with a strong acidic resin (Amberlyst 15 ion-exchange resin). Complete assignment of the chemical shifts of **9a** and **9b** was based on the 2D COSY and HMQC NMR spectra. The β configuration at the anomeric postion was also confirmed by the coupling constant (7.5 and 8.0 Hz) between H_1 and H_2. The deprotection of the α anomers of **8a** and **8b** failed. NMR showed that the heterocyclic ring decomposed. It seems that α conjugates were unstable after removal of acetyl protecting groups.

Applying our strategy to other heterocyclic NO donors, more carbohydrate and NO donor conjugates could be developed. Mesoionic oxatriazole-5-imines (GEAs) are structurally isosteric to sydnonimines. They are potent anti-platelet, fibrinolytic, thrombolytic, and bronchiectatic agents.[15] We have synthesized one member of this type of NO donors, GEA 3162 (**10**), according to a reported procedure.[16] Coupling of the GEA 3162 with 4-nitrophenyl (2,3,4,6-tetra-O-acetyl-α/β-D-galactopyranosyl)carbonate (**7b**) following a previously established method gave the desired Gal-GEA3162 conjugates (**11**) in fair yields (Scheme 2). However, deprotection of acetyl groups caused the decomposition of the oxatriazole ring.

The synthesis of sialic acid/SIN-1 conjugate was slightly different from that of other monosaccharides due to the one additioanl carboxyl at the anomeric carbon (Scheme 3). After the protection of carboxyl and hydroxyl groups with methyl ester and acetyl groups, the free anomeric OH form of sialic acid (**13**)[17] was coupled with 4-nitrophenyl chloroformate in the presence of triethylamine to give **14**. The following reaction with SIN-1 failed in the previously described condition. Optimization of the reaction conditions showed that this reaction worked better in aqueous THF solution, which could give the protected sialic acid/SIN-1 conjugate (**15**) in 40% yield. The anomeric configurations of **14** and **15** could not be determined by NMR until the deprotected compound was obtained. The following two steps of deprotection afforded the sialic acid/SIN-1 conjugate (**16**) successfully. The anomeric configuration of the final product was determined as a β-sialoside, since the chemical shift of H_{3e}

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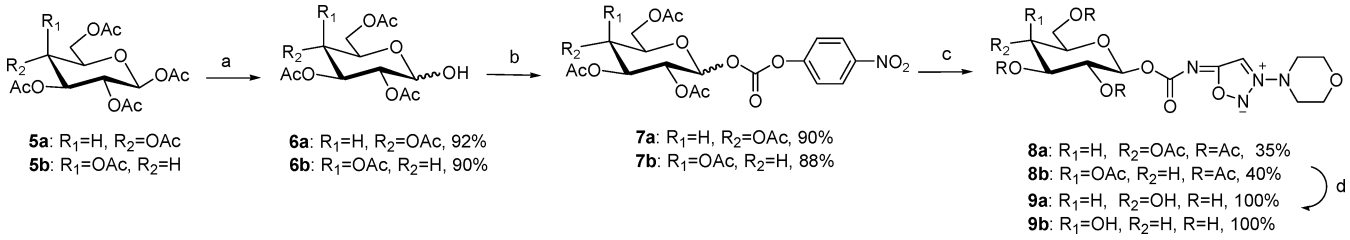
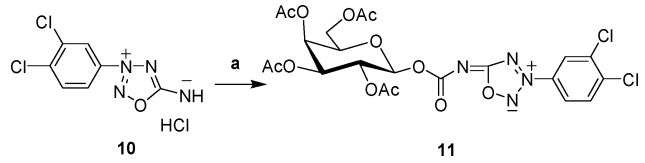
SCHEME 1. Synthesis of Glucose, Galactose, and SIN-1 Conjugates[a]



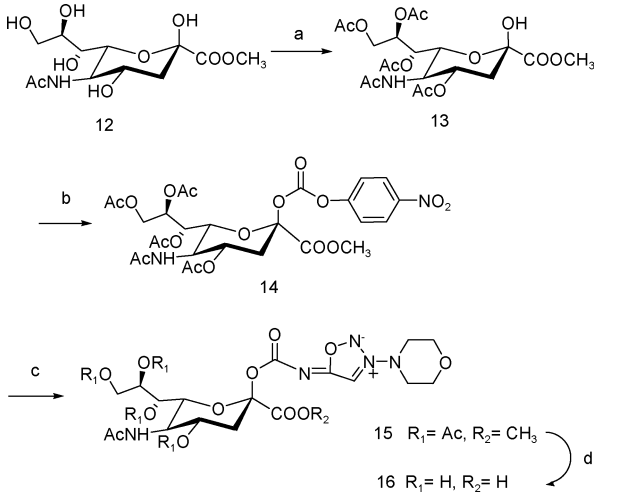
[a] Reagents and conditions: (a) $BnNH_2$, THF, rt, 30 h; (b) *p*-$NO_2C_6H_4OCOCl$, NEt_3, CH_2Cl_2, rt, 4.5 h; (c) **4**, pyridine, rt, 12 h; (d) NaOMe, MeOH, rt, 3 h.

SCHEME 2. Synthesis of Galactose and GEA 3126 Conjugate[a]



[a] Reagents and conditions: (a) **7b**, pyridine, rt, 12 h (31%).

SCHEME 3. Synthesis of Sialic Acid and SIN-1 Conjugate[a]



[a] Reagents and conditions: (a) Ac_2O, $HClO_4$, 2 h, 75%; (b) *p*-$NO_2C_6H_4OCOCl$, NEt_3, CH_2Cl_2, 4.5 h, 64%; (c) **4**, $NaHCO_3$, THF−H_2O, 10 h, 40%; (d) $NaOCH_3$, CH_3OH, 2 h then NaOH, H_2O. 10 h, 68%.

(2.28 ppm) filled in the range of 2.32 ± 0.08 ppm according to the general rule.[18] There was no α anomers obtained after column chromatography. The reason may be the anomeric effect, which made the thermodynamically favored β anomer dominant in the products. It was shown that this β-sialoside was not the substrate of the neuraminidase, which hydrolyzes the α-sialosides.

To test our design, the decomposition of **9b** by β-galactosidase was detected by the decay of the absorbance at 307 nm (Figure 2), which is the characteristic of the NO donating heterocycle. The enzymatic hydrolysis was measured on a UV−vis spectrophotometer fitted with an electrically thermostated cell block. The half-life of **9b** with β-galactosidase was estimated to be 0.8 h, while **9b** was fairly stable in the buffer without the enzyme. It was noticed that **9b** decomposed very slowly in the UV spectrophotometer. The reason may be that UV causes

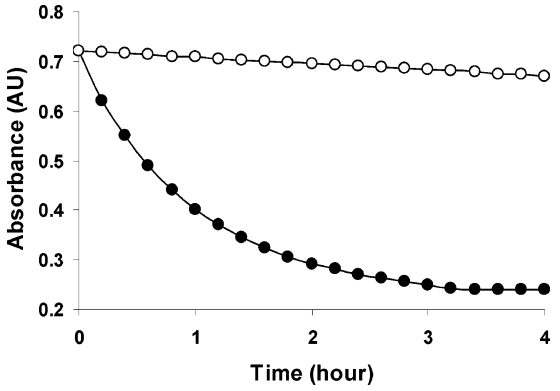


FIGURE 2. The UV absorbance changing of **9b**. **9b** (0.04 mM) was incubated in 150 mM phosphate buffer at pH 7.4 and 37 °C in the presence (●) and in the absence (○) of β-galactosidase (0.05 mg/mL). UV was measured at 307 nm.

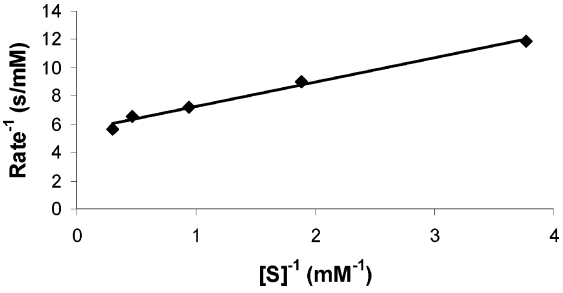


FIGURE 3. Lineweaver−Burk plot of **9a** as the substrate of β-glucosidase. β-Glucosidase (0.5 mg/mL) and substrate ([S]) **9a** (0.1, 0.2, 0.4, 0.8, 1.25 mg/mL) were incubated in 150 mM Tris-CF_3COOH buffer for 10 s at pH 7.30.

the decomposition of the SIN-1. To study the details of enzymatic kinetics, both **9a** and **9b** were incubated with β-glucosidase and β-galactosidase, respectively, and then the solutions were analyzed by LC-MS (Shimadzu, 2010A). The kinetic data were obtained by the Lineweaver−Burk plots for **9a** (Figure 3) and **9b** (Figure 4). K_m, V_{max}, and k_{cat} are listed in Table 1. The K_m for both substrates is within the ranges of known substrates (from about 0.05 to 85 mM). The turnover numbers (k_{cat}) are relatively large in the range of known substrates. It indicates that both **9a** and **9b** are good substrates of the correspondent glycosidases when enzymes are fully saturated with substrates. At low substrate concentrations, the hydrolysis is still efficient, since k_{cat}/K_M is also large. From the kinetic data, we can see that glycosidases showed good catalytic efficiency for this type of carbohydrate conjugates with carbamate linkages.

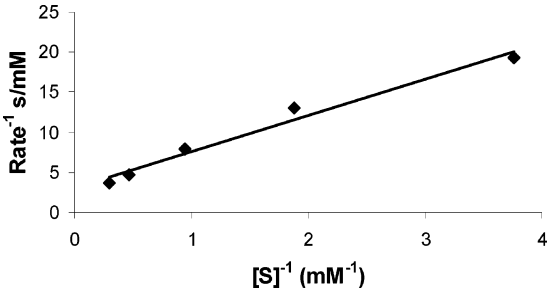


FIGURE 4. Lineweaver−Burk plot of **9b** as the substrate of β-galactosidase. β-Galactosidase (0.5 mg/mL) and substrate ([S]) **9b** (0.1, 0.2, 0.4, 0.8, 1.25 mg/mL) were incubated in 150 mM Tris-CF_3COOH buffer for 10 s at pH 7.30.

TABLE 1. The Kinetic Parameters of 9a/9b as Substrates of Glycosidases

substrate	K_M (mM)	V_{max} (mM/s)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)
9a	0.311	0.181	2.75×10^5	8.87×10^5
9b	1.47	0.327	6.54×10^5	4.45×10^5

TABLE 2. Nitrite Concentration in the Incubation of 9a/9b with or without the Glycosidases[a]

substrate (23 μM)	$[NO_2^-]/\mu M$
SIN-1	3.1
9a	negligible
9a + β-glucosidase	2.4
9b	0.3
9b + β-galactosidase	2.8

[a] The amount of nitrite released was measured by using the Griess method. Glycosidases (0.05 mg/mL) and **9a** or **9b** (23 μM) were incubated for 17 h in 150 mM phosphate buffer at pH 7.4 and 37 °C. UV absorbance was measured at 548 nm.

The NO releasing ability of **9a** and **9b** in the presence and absence of glycosidases was evaluated by measuring the formation of nitrite ion, since nitrite ion is generated from the oxidation of NO radical by oxygen. The nitrite (NO_2^-) anion in solution was quantified by the Griess method.[19] The results are summarized in Table 2. Both **9a** and **9b** were relatively stable under physiological conditions without enzymes. The trace amount of nitrite formed might come from the photodecomposition of the heterocycle.[20] In contrast, SIN-1 decomposed quite easily in the aqueous solution. Compounds **9a** and **9b** generated comparable amounts of NO_2^- compared to SIN-1 only in the presence of glycosidases.

The hydrolysis of the glycosides was also studied by monitoring the NO release with an Electrochemical ISO-NO marker II isolated Nitric Oxide Meter manufactured by World Precision Instruments, Inc. NO was not detected when **9a** or **9b** was incubated in aqueous solution at neutral pH, even in the presence of glycosidases. This is presumed to be because NO/O_2^- reacted so rapidly that NO is consumed. Yet, when 0.3 mg/mL of superoxide dismutase (SOD) was present, NO formation was observed (Figure 5). This property is the same as that for

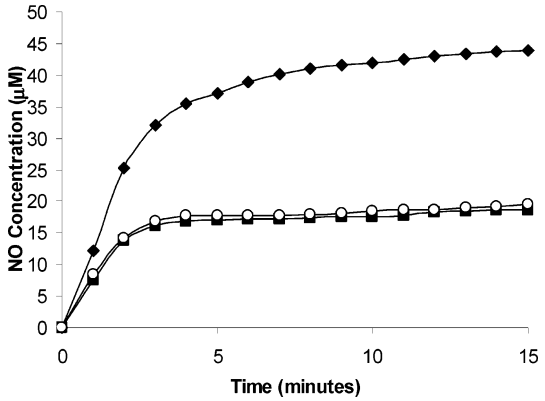


FIGURE 5. Measurement of NO profiles of **9a**, **9b**, and SIN-1: (◆) SIN-1 (100 μM) and SOD (0.3 mg/mL) were incubated, (■) **9a** (100 μM) and SOD (0.3 mg/mL) were incubated with β-glucosidase (0.005 mg/mL), and (○) **9b** (100 μM) and SOD (0.3 mg/mL) with β-galacosidase (0.005 mg/mL) were incubated, all in 150 mM phosphate buffer at pH 7.4 and 37 °C.

SIN-1. A possible decomposition pathway of **9b** is proposed in Scheme 4. β-Galactosidase first hydrolyzes the glycosidic linkage of **9b** to generate SIN-1. At the physiological condition, SIN-1 undergoes rapid hydrolysis to form the ring-opened product, SIN-1C. NO radical is released in this process.[21] Stoichiometric amounts of superoxide anions ($^{\bullet}O_2^-$) are formed as a result of oxygen reduction. Since NO is known to react with $^{\bullet}O_2^-$ at an almost diffusion-controlled rate,[22] peroxynitrite ($OONO^-$) production is inevitable. Since most of the NO released from both glycosides was converted to peroxynitrite, which could isomerize to nitrate (NO_3^-), only a small amount of nitrite was detected by Griess assay. Peroxynitrite is a well-known active species that can cause damage to biomolecules.[11]

Conclusions

In summary, we have prepared a new class of glycosidase dependent NO donors, glycosyl carbamate of nitric oxide donors. SIN-1 as an NO releasing moiety was coupled with glucose, glactose, and sialic acid via well-defined chemical steps. The carbohydrate−SIN-1 conjugates are much more stable than SIN-1 itself. We also found that glycosyl carbamate of NO donors could be hydrolyzed by glycosidases and form NO. Such prodrugs may find applications in biological research and Antibody-Directed Prodrug Therapy (ADEPT).

Experimental Section

2,3,4,6-Tetra-*O*-D-glucopyranose (6a). A solution of pentaacetate-D-glucose (3.9 g, 10.0 mmol) and $BnNH_2$ (1.2 mL, 11.0 mmol) in THF (45 mL) was stirred at ambient temperature for 30 h. The solvent was removed and the residue was dissolved in CH_2Cl_2 (100 mL), then washed with 1 N HCl (100 mL × 2) and water (100 mL) successively. The organic layer was concentrated and chromatographed on silica gel with EtOAc/hexane (2:1−1:1), giving **6a** (3.2 g, 9.2 mmol, 92%) as

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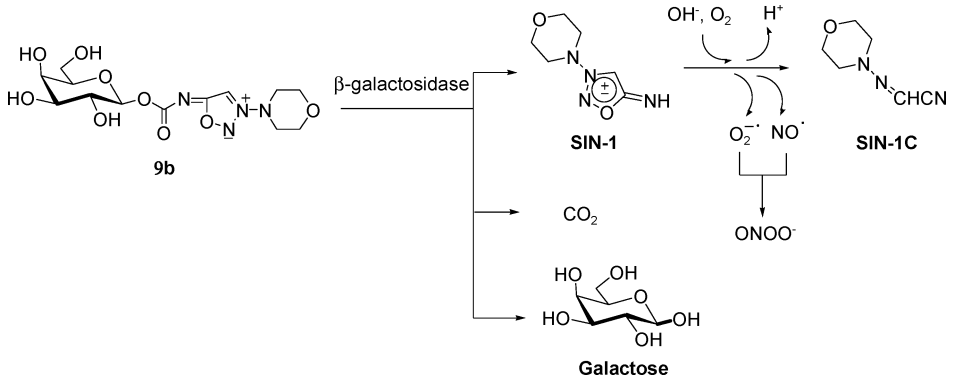
SCHEME 4. Proposed NO Releasing from 9b



a 3:1 (α/β) mixture of anomers as judged by NMR. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.41 (d, 1H, J = 5.6 Hz, H-1 of β-anomer), 3.89 (d, 1H, J = 3.2 Hz, H-1 of α-anomer); ^{13}C NMR (100 MHz, $CDCl_3$) δ 96.0, 90.3 (C-1).

4-Nitrophenyl (2,3,4,6-Tetraacetyl-D-glucopyranosyl)-carbonate (7a).[14] To a cooled solution (0 °C) of **6a** (2.48 g, 7.13 mmol) in anhydrous CH_2Cl_2 (50 mL) were successively added 4-nitrophenyl chloroformate (1.72 g, 8.53 mmol) and triethylamine (2.96 mL, 21.3 mmol). After being stirred for 4.5 h at room temperature, the crude mixture was diluted with another portion of CH_2Cl_2 (50 mL) and then washed with brine and water. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 then concentrated. After purification by silica gel chromatography with EtOAc/hexane (1:2) as an eluent, **7a** (3.28 g, 6.39 mmol, 90%) was obtained as a 1:3 (α/β) mixture of anomers as judged by ^{1}H NMR. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.28 (d, 1H, J = 3.0 Hz, H-1 of α-anomer), 5.66 (d, 1H, J = 8.0 Hz, H-1 of β-anomer); ^{13}C NMR (125 MHz, $CDCl_3$) δ 95.8, 94.1 (C-1); ESI-MS m/z 536 $[M + Na]^+$, 1049 $[2M + Na]^+$.

***N*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)carbonyl-3- morpholinosydnonimine (8a).** To a solution of **7a** (1.53 g, 2.98 mmol) in anhydrous pyridine (20 mL) was added **4** (678 mg, 3.28 mmol). The reaction mixture was stirred overnight and then the solvent was removed in vacuo to give a sticky oil. The residue was purified by silica gel chromatography with EtOAc/CH_2Cl_2 (1:1) as an eluent. **8a** (568 mg, yield 35%) and the α-anomer (164 mg, 0.301 mmol, yield 10%) were obtained as foams. **8a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 (s, 1H, H-4′), 5.72 (d, 1H, J = 8.5 Hz, H-1), 5.26 (t, 1H, J = 9.0 Hz, H-3), 5.20∼5.10 (m, 2H, H-4 and H-2), 4.25 (dd, 1H, J = 12.5, 4.0 Hz, H-6), 4.12 (dd, 1H, J = 12.0, 2.0 Hz, H-6), 3.97 (t, 4H, J = 5.0 Hz, H-2″ and H-6″), 3.85 (dd, 1H, J = 4.0, 2.0 Hz, H-5), 3.54 (t, 4H, J = 5.0 Hz, H-3″ and H-5″), 2.06−1.98 (m, 12H, $COCH_3$ × 4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3 (C-carbamate), 170.7 (C═O of Ac), 170.2 (C═O of Ac), 169.4 (C═O of Ac × 2), 158.3 (C-5′), 99.8 (C-4′), 93.3 (C-1), 73.1 (C-3), 72.2 (C-5), 70.4 (C-2), 67.8 (C-4), 65.4 (C-2″ and C-6″), 61.5 (C-6), 54.5 (C-3″ and C-5″), 20.7 (CH_3 of Ac × 2), 20.6 (CH_3 of Ac × 2); ESI-MS m/z 545 $[M + H]^+$, 567 $[M + Na]^+$, 1089 $[2M + H]^+$, 1111 $[2M + Na]^+$; HRMS calcd for $C_{21}H_{28}N_4O_{13}Na$ ($M + Na^+$) m/z 567.1545, found m/z 567.1564. α-Anomer: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (s, 1H, H-4′), 6.31 (d, 1H, J = 3.5 Hz, H-1), 5.63 (t, 1H, J = 10.0 Hz, H-3), 5.15 (t, 1H, J = 10.0 Hz, H-4), 5.06 (dd, 1H, J = 10.0, 4.0 Hz, H-2), 4.28−4.26 (m, 2H, H-5 and H-6), 4.10−4.00 (m, 1H, H-6), 3.97 (t, 4H, J = 5.0 Hz, H-2″ and H-6″), 3.54 (t, 4H, J = 5.0 Hz, H-3″ and H-5″), 2.10−1.97 (m, 12H, $COCH_3$ × 4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.7 (C-carbamate), 170.7 (C═O of Ac), 170.0 (C═O of Ac), 169.8 (C═O of Ac), 169.6 (C═O of Ac), 159.2 (C-5′), 99.7 (C-4′), 90.1 (C-1), 70.1 (C-3), 69.6 (C-2), 69.0 (C-5), 68.0 (C-4), 65.4 (C-2″ and C-6″), 61.5 (C-6), 54.6 (C-3″ and C-5″), 20.7 (CH_3 of Ac), 20.6 (CH_3 of Ac × 2), 20.6 (CH_3 of Ac); ESI-MS m/z 545 $[M + H]^+$, 567 $[M + Na]^+$, 1089 $[2M + H]^+$, 1111 $[2M + Na]^+$.

***N*-(β-D-Glucopyranosyl)carbonyl-3-morpholinosydnonimine (9a).** To a solution of **8a** (15 mg, 27.5 mmol) in anhydrous methanol (5 mL) was added $NaOCH_3$ to adjust the solution pH value to 8−9. The reaction mixture was stirred at room temperature for several hours and then strong acid ion-exchange resin was added to neutralize the reaction mixture. After removing the solvent in vacuo, the residue was purified by silica gel chromatography with CH_2Cl_2/CH_3OH (4:1) as an eluent. **9a** (10 mg, 26.6 mmol) was obtained as a colorless syrup in quantitative yield. UV 307 nm; ^{1}H NMR (500 MHz, CD_3OD) δ 8.14 (s, 1H, H-4′), 5.39 (d, 1H, J = 7.5 Hz, H-1), 3.95 (t, 4H, J = 5.0 Hz, H-6″ and H-2″), 3.83 (dd, 1H, J = 11.5, 2.0 Hz, H-6), 3.66 (dd, 1H, J = 12.0, 5.0 Hz, H-6), 3.62 (t, 4H, H-3″ and H-5″), 3.40 (t, 1H, J = 8.5 Hz, H-3), 3.38−3.32 (m, 2H, H-5 and H-4), 3.32∼3.29 (m, 1H, H-2); ^{13}C NMR (125 MHz, CD_3OD) δ 169.0 (carbamate), 101.4 (C-4′), 97.4 (C-1), 78.6, 78.0, 74.0, 71.2 (C-2,3,4,5), 65.6 (C-2″ and C-6″), 62.5 (C-6), 55.5 (C-3″ and C-5″); ESI-MS m/z 377 $[M + H]^+$, 399 $[M + Na]^+$, 753 $[2M + H]^+$, 775 $[2M + Na]^+$; ESI-HRMS calcd for $C_{13}H_{20}N_4O_9Na$ ($M + Na^+$) m/z 399.1122, found m/z 399.1120.

2,3,4,6-Tetra-*O*-D-galactopyranose (6b). 6b was prepared from **5b** and obtained as foams (92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50−5.30 (m, 3H), 5.20−5.00 (m, 2H), 4.69 (d, 0.25H, J = 6.6 Hz), 4.43 (m, 1H), 4.10−4.00 (m, 3H), 4.00−3.90 (m, 0.25H), 3.00−2.70 (m, 1H), 2.13, 2.11, 2.06, 2.02, 2.01, 1.96 (s, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 95.8, 90.4 (C-1).

4-Nitrophenyl (2,3,4,6-Tetraacetyl-D-galactopyranosyl-)carbonate (7b).[14] **7b** was prepared from **6b** and obtained as foams (88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.33 (d, 1H, J = 3.0 Hz, H-1 of α-anomer), 5.63 (d, 1H, J = 8.0 Hz, H-1 of β-anomer); ^{13}C NMR (125 MHz, $CDCl_3$) δ 96.3, 94.8 (C-1).

***N*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)carbonyl-3-morpholinosydnonimine (8b). 8b** was prepared from **7b** and obtained as foams (40%). **8b**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 (s, 1H, H-4′), 5.69 (d, 1H, J = 8.5 Hz, H-1), 5.41 (d, 1H, J = 3.5 Hz, H-4), 5.36 (dd, 1H, J = 10.0, 8.5 Hz, H-2), 5.09 (dd, 1H, J = 10.0, 3.5 Hz, H-3), 4.14∼4.09 (m, 2H, H-6 and H-6), 4.09−4.04 (m, 1H, H-5), 3.97 (t, 4H, J = 5.0 Hz, H-2″ and H-6″), 3.55 (m, 4H, H-3″ and H-5″), 2.15−1.96 (m, 12H, $COCH_3$ × 4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.2 (C-carbamate), 170.3 (C═O of Ac), 170.2 (C═O of Ac), 170.1 (C═O of Ac), 169.4 (C═O of Ac), 158.2 (C-5′), 100.0 (C-4′), 93.8 (C-1), 71.1 (C-3 and C-5), 67.9 (C-2), 66.9 (C-4), 65.9 (C-2″ and C-6″), 60.9 (C-6), 54.5 (C-5″ and C-4″), 20.8 (CH_3 of Ac), 20.6 (CH_3 of Ac × 2), 20.5 (CH_3 of Ac); ESI-MS m/z 545 $[M + H]^+$, 567 $[M + Na]^+$, 1089 $[2M + H]^+$, 1111 $[2M + Na]^+$; HRMS calcd for $C_{21}H_{28}N_4O_{13}Na$ ($M + Na^+$) m/z 567.1545, found m/z 567.1518. α-Anomer (12%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (s, 1H, H-4′), 6.35 (d, 1H, J = 3.5 Hz, H-1), 5.52−5.50 (m, 2H, H-3 and H-4), 5.29 (dd, 1H, J = 11.0, 3.5 Hz, H-2), 4.44 (t, 1H, J = 7.0 Hz, H-5), 4.15−4.00 (m, 2H, H-6 and H-6), 4.10−4.00 (m, 1H, H-6), 3.97 (t, 4H, J = 5.0 Hz, H-2″ and H-6″), 3.53 (t, 4H, J = 5.0 Hz, H-3″ and H-5″), 2.14−1.94 (m, 12H, Ac × 4); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.7 (C-carbamate), 170.3 (C═O of Ac), 170.3 (C═O of Ac), 170.1 (C═O of Ac), 169.7 (C═O of Ac), 159.4 (C-5′), 99.6 (C-4′), 90.7 (C-1), 68.1 (C-5), 67.7 (C-3), 67.5 (C-4), 66.9 (C-2), 65.3 (C-2″ and C-6″), 61.2 (C-6), 54.6

(C-3″ and C-5″), 20.7 (CH_3 of Ac), 20.6 (CH_3 of Ac), 20.6 (CH_3 of Ac × 2); ESI-MS *m/z* 545 $[M + H]^+$, 567 $[M + Na]^+$, 1089 $[2M + H]^+$, 1111 $[2M + Na]^+$.

***N*-(*β*-D-Galactopyranosyl)carbonyl-3-morpholinosydnonimine (9b).** *9b* was prepared from **8b** and obtained as a syrup (100%). UV 307 nm; ^{1}H NMR (500 MHz, CD_3OD) δ 8.16 (s, 1H, H-4′), 5.35 (d, 1H, J = 8.0 Hz, H-1), 3.96 (t, 4H, J = 5.0 Hz, H-6″ and H-2″), 3.88 (d, 1H, J = 3.0 Hz, H-4), 3.74−3.66 (m, 3H, H-6, H-6 and H-2), 3.65−3.60 (m, 5H, H-5, H-3″ and H-5″), 3.56−3.52 (m, 1H, H-3); ^{13}C NMR (125 MHz, CD_3OD) δ 175.3 (C-carbamate), 160.6 (C-5′), 101.5 (C-4′), 98.1 (C-1), 77.4 (C-5), 74.9 (C-3), 71.3 (C-2), 71.1 (C-4), 66.6 (C-2″ and C-6″), 62.3 (C-6), 55.4 (C-3″ and C-5″); ESI-MS *m/z* 377 $[M + H]^+$, 399 $[M + Na]^+$, 775 $[2M + Na]^+$; ESI-HRMS calcd for $C_{13}H_{20}N_4O_9Na$ $(M + Na^+)$ *m/z* 399.1122, found *m/z* 399.1129.

***N*-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl)carbonyl-3-(3,4-dichlorophenyl)-1,2,3,4-oxatriazole-5-imine (11).** **11** was prepared from **10** and **7b** and obtained as a red powder (31%). **11**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (d, 1H, J = 2.5 Hz), 8.10 (dd, J = 8.5, 2.5 Hz), 7.80 (d, 1H, J = 8.5 Hz), 5.73 (d, 1H, J = 8.5 Hz, H-1), 5.41−5.38 (m, 2H, H-4 and H-2), 5.09 (dd, 1H, J = 10.0, 3.5 Hz, H-3), 4.14∼4.06 (m, 2H, H-6, H-5), 2.13, 2.01, 1.99, 1.97 (4 OAc); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.8, 170.6, 170.4, 170.3, 169.5 (C═O of Ac), 157.0, 140.2, 135.6, 132.4, 123.7, 120.8, 94.3 (C-1), 71.7 (C-3), 71.4 (C-5), 68.0 (C-2), 67.1 (C-4), 61.2 (C-6), 21.0, 20.9, 20.8 (CH_3 of Ac); MS (ESI) *m/z* 627 $[M + Na]^+$, 1231 $[2M + Na]^+$; HRMS calcd for $C_{22}H_{22}Cl_2N_4O_{12}Na$ $(M + Na^+)$ *m/z* 627.0503, found *m/z* 627.0488. α-Anomer (26%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (d, 1H, J = 2.5 Hz), 8.10 (dd, J = 9.0, 2.5 Hz), 7.80 (d, 1H, J = 9.0 Hz), 6.41 (d, 1H, J = 3.5 Hz, H-1), 5.52−5.48 (m, 2H, H-3 and H-4), 5.37 (dd, 1H, J = 11.0, 3.5 Hz, H-2), 4.47 (t, 1H, J = 6.5 Hz, H-5), 4.11 (m, 2H, H-6), 2.16, 2.04, 2.01, 1.99 (4Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 209.6, 170.4, 170.2, 170.1, 170.0 (C═O of Ac), 156.7, 140.1, 135.4, 132.3, 132.1, 123.4, 120.5, 91.4 (C-1), 68.6 (C-5), 67.6 (C-3), 67.6 (C-4), 66.7 (C-2), 61.3 (C-6), 20.7, 20.7, 20.6 (CH_3 of Ac); MS (ESI) *m/z* 627 $[M + Na]^+$, 1231 $[2M + Na]^+$.

Methyl 5-Acetamido-3,5-dideoxy-*β*-D-galacto-2-nonulopyranosonate (12).[23] *N*-Actylneuraminic acid (800 mg, 2.6 mmol) was dissolved in dry methanol (100 mL) and CF_3COOH (0.2 mL) was added. The reaction mixture was stirred at room temperature for 3 days until the solution became clear. Then the solvent was removed under reduced pressure and the residue coevaporated twice with dry methanol (5 mL). The product was used without purification.

Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-*β*-D-glycero-D-galacto-2-nonulopyranosonate (13).[17] To a stirred, warmed (40 °C) solution of acetic anhydride (2.88 g, 2.7 mL) and aqueous 60% perchlorice acid (20 μL) was added **12** (2.6 mmol) during 30 min in small portions. The mixture was stirred for 2 h at 40 °C, then cooled to room temperature, diluted with cold water (40 mL), saturated with ammonium chloride, and extracted with dichloromethane (3 × 100 mL). The combined extracts were washed with saturated aqueous sodium hydrogencarbonate (40 mL) and water (40 mL), dried ($MgSO_4$), and concentrated to give a white solid. Crystallization from ethyl acetate−hexane gave **2** (1.00 g, 75%) as white needles, mp 147−148 °C; ^{1}H NMR (400 MHz, $CDCl_3$), δ 5.79 (m, 1H, NH), 5.36 (m, 1H), 5.26−5.16 (m, 2H), 4.65 (br, 1H), 4.52 (dd, 1H, J = 12 Hz), 4.21−4.12 (m, 2H), 4.02 (dd, J = 12 Hz, 8 Hz), 3.85 (s, 3H), 2.28−2.16 (m, 2H), 2.14, 2.10, 2.02, 2.01, and 1.89 (5 s, 15H); ^{13}C NMR (100 MHz, $CDCl_3$), 171.2 (2×), 170.5 (2×), 170.4, 169.3, 95.1, 71.6, 71.3, 69.5, 68.3, 62.8, 53.7, 49.6, 36.3, 23.4, 21.3, 21.1 (2×), 21.0 (2×); ESI-MS 514.2 $(M + Na^+)$.

Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-2-(4-nitrophenoxycarbonyloxy)-3,5-dideoxy-*β*-D-glycero-D-galacto-2-nonulopyranosonate (14). To a cooled solution (0 °C) of **13** (400 mg, 0.815 mmol) in anhydrous CH_2Cl_2 (8 mL) was successively added 4-nitrophenyl chloroformate (197 mg, 0.978 mmol) and triethylamine (247 mg, 2.45 mmol). After being stirred for 4.5 h at room temperature, the crude mixture was diluted with CH_2Cl_2 (50 mL). Column chromatography with ethyl acetate afforded **14** (342 mg, 64%) as a white syrup. ^{1}H NMR (500 MHz, CD_3OD) δ 8.34 (d, 2H, J = 9 Hz), 7.52 (d, 2H, J = 9 Hz), 5.41 (dd, 1H, J = 6.5, 2 Hz), 5.24 (td, 1H, J = 11, 5 Hz), 5.17 (td, 1H, J = 6, 2.5 Hz), 4.44 (dd, 1H, J = 13, 2.5 Hz), 4.34 (dd, 1H, J = 10.5, 2 Hz), 4.11 (t, 1H, J = 10.5 Hz), 4.09 (dd, 1H, J = 12, 7 Hz), 3.83 (s, 3H), 2.68 (dd, 1H, J = 13.5, 5 Hz), 2.12 (s, 3H), 2.03−1.99 (m, 1H), 2.01 (s, 3H), 2.00 (s, 3H), 1.97 (s, 3H), 1.88 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.3, 171.1, 170.6, 170.4, 170.3, 165.8, 155.2, 149.8, 146.1, 125.3, 122.0, 100.8, 73.1, 70.4, 68.4, 67.7, 62.0, 53.0, 48.5, 35.6, 21.6, 19.7, 19.6, 19.5, 19.4; ESI-MS 679 $(M + Na^+)$, 695 $(M + K^+)$; HRMS calcd for $C_{27}H_{32}N_2O_{17}Na$ $(M + Na^+)$ *m/z* 679.1593, found *m/z* 679.1589.

***N*-[Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-*β*-D-glycero-D-galacto-2-nonulopyranosyl)onate]carbonyl-3-morpholinosydnonimine (15).** To a suspension of **4** (1.68 mmol, 350 mg) in water (3 mL) was added $NaHCO_3$ (1.85 mmol, 155 mg) with ice-cooling and stirring. After 10 min a solution of **14** (1.4 mmol, 900 mg) in THF (5 mL) was added dropwise with stirring. The mixture was further stirred overnight. Then THF was removed in vacuo with subsequent extraction with ethyl acetate (10 mL) 3 times. The extract was applied on column chromatography ($CHCl_3$:CH_3OH, 20:1). The product **4** was collected as white foam (400 mg) (40%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.12 (s, 1H), 5.38 (dd, 1H, J = 6.5, 2 Hz), 5.22 (td, 1H, J = 11, 5 Hz), 5.10 (td, 1H, J = 6, 2.5 Hz), 4.43 (dd, 1H, J = 13, 2.5 Hz), 4.14 (dd, 1H, J = 10.5, 2 Hz), 4.11−4.02 (m, 2H), 3.95 (t, 4H, J = 5 Hz), 3.74 (s, 3H), 3.63 (t, 4H, J = 5 Hz), 2.56 (dd, 1H, J = 13.5, 5 Hz), 2.09 (s, 3H), 2.06−2.01 (m, 1H), 1.98 (s, 6H), 1.90 (s, 3H), 1.88 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 174.4, 172.2, 171.2, 170.6, 170.3, 168.2, 157.7, 100.8, 96.9, 71.6, 70.1, 69.4, 67.4, 65.3, 61.6, 54.2, 52.,1, 48.7, 36.4, 21.5, 19.6 (2×), 19.5 (2×); ESI-MS 688 $(M + H^+)$, 710 $(M + Na^+)$; HRMS calcd for $C_{27}H_{37}N_5O_{16}Na$ $(M + Na^+)$ *m/z* 710.2128, found *m/z* 710.2160.

***N*-(5-Acetamido-3,5-dideoxy-*β*-D-glycero-D-galacto-2-nonulopyranosyl)carbonyl-3-morpholinosydnonimine (16).** Compound **15** (0.29 mmol, 200 mg) was dissolved in methanol (30 mL) and 0.1 M $NaOCH_3$ in methanol (0.68 mL) was added. The mixture was stirred for 2 h at room temperature. Strong acid ion-exchange resin was added to neutralize the base. The resin was filtered off and twice washed with methanol. The solvent was concentrated in vacuo and the residue was dissolved in 0.1 M NaOH (70 mL). The solution was stirred overnight. After neutralization with acidic resin, the solution was concentrated and loaded on C-18 silica gel. Washing with water gave product as an off-white powder (100 mg, 68%). ^{1}H NMR (500 MHz, D_2O) δ 7.91 (s, 1H), 3.95 (td, 1H, J = 10, 5 Hz), 3.83 (t, 4H, J = 5 Hz), 3.79−3.76 (m, 1H), 3.69 (d, 1H, J = 10.5 Hz), 3.63 (m, 1H), 3.55 (dd, 1H, J = 12.5, 3 Hz), 3.47 (t, 4H, J = 5 Hz), 3.44 (d, 1H, J = 8 Hz), 3.36 (dd, 1H, J = 12, 6 Hz), 2.28 (dd, 1H, J = 13.5, 5 Hz), 1.87 (s, 3H), 1.60 (t, 1H, J = 13 Hz); ^{13}C NMR (125 MHz, D_2O), δ 174.9, 174.6, 173.8, 158.7, 101.2, 98.9, 72.2, 71.3, 68.0, 66.9, 65.4, 63.0, 53.9, 51.7, 39.3, 22.2; ESI-MS 506 $(M + H^+)$, 528 $(M + Na^+)$, 544 $(M + K^+)$; HR-MS calcd for $C_{18}H_{27}N_5O_{12}Na$ $(M + Na^+)$ *m/z* 528.1548, found *m/z* 528.1551.

Measurement of Enzymatic Kinetics. Six **9a** or **9b** solutions of concentrations from 0.2 to 5 μM (0.5 mL) were incubated for 10 s with 10 μL of β-glucosidase or β-galactosidase (50 mg/mL), respectively. Then the solutions were injected into HPLC for analysis. The concentrations of substrates were quantified by the areas of the corresponding peaks. The reaction rates and concentrations of substrates were treated with the classical Lineweaver−Burk plot to obtain K_m, V_{max}, and k_{cat}. The final data were the average of three repetitive experiments.

(23) Bandgar, B. P.; Hartmann, M.; Schmid, W.; Zbiral, E. *Justus Liebigs Ann. Chem.* **1990**, 1185.

Measurement of Nitrite Formation. The stock solution of **9b** (10 mM) was diluted to 0.023 mM with phosphate buffer (pH 7.4). Next the solution of β-galactosidase (E.C. 3.2.1.23, from Aspergillus Oryzae) in phosphate buffer (pH 7.4) was added, and the mixture was incubated at 37 °C for about 17 h before the addition of Griess reagent [150 μL of 1% sulfanilamide in 1 M HCl and 150 μL of 0.1% *N*-(1-naphthyl)-ethylenediamine in 1 M HCl]. The absorbance of the solution was then measured at 548 nm. Calibration curves were made from known concentrations of aqueous $NaNO_2$ in the same buffer solution so as to determine the amount of NO_2^- formed after the reactions.

Measurement of Nitric Oxide. NO measurement was carried out with an Electro-chemical ISO-NO Mark II Isolated Nitric Oxide Meter (World Precision Instruments, Inc.). The calibration plot was obtained by measurements of standard NO solutions with current changes. For the assay, **9a**, superoxide dismutase (SOD) (0.3 mg/mL), and β-glucosidase (0.005 mg/mL) (E.C. 3.2.1.21) were incubated in pH 7.4 phosphate buffer. The current changes were recorded every minute.

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Supporting Information Available: Copies of NMR spectra of synthesized compound **4**, **6** through **11**, and **13** through **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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