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Sialated Diazeniumdiolate: A New Sialidase-Activated Nitric Oxide Donor

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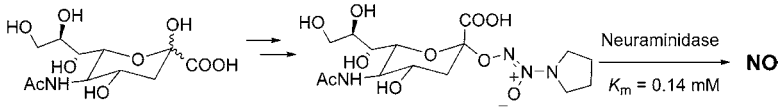
ABSTRACT



A new sialated diazeniumdiolate has been synthesized, and the glycosylation product was exclusively an α anomer. This new nitric oxide donor exhibited significantly improved stability as compared to its parent diazeniumdiolate salts, and it could be efficiently hydrolyzed by neuraminidase to release nitric oxide with a K_m of 0.14 mM. The sialic acid–NO conjugate would be a valuable prodrug that targets NO to influenza viruses.

Nitric oxide (NO) is a gaseous molecule that serves as a mediator of many physiological events.[1] To utilize this active small molecule in therapy and research, NO is usually released from a stable prodrug form, which is also known as an NO donor.[2] Design and synthesis of site-specific NO donors are current tasks in our laboratory. We have attached NO releasing moieties to carriers, which can be recognized by certain targets. We reported several classes of enzyme-activated NO donors previously. For example, the peptide−diazeniumdiolate conjugates were synthesized by the attachment of a diazeniumdiolate to the substrates of prostate specific antigen (PSA), and they were demonstrated to release NO upon the activation by PSA.[3] In addition, the conjugates of cephalosporin with 3-morpholinosydnonimine (SIN-1) release NO in the presence of β-lactamase.[4] It was also found that the diazeniumdiolate functional group could be attached to the anomeric position of monosaccharide, and those glycosylated diazeniumdialates could be hydrolyzed by glycosidases.[5] Here, we report the development of a new sialated diazeniumdiolate which promises to be utilized as an NO donor that targets influenza viruses.

Diazeniumdiolates (formerly known as NONOates) are important NO donors in biomedical research.[6] The major feature of this class of NO donors is that they can spontaneously release NO under physiological conditions with a range of half-lives from a few seconds to several days. Since O^2-alkylated *N*-diazeniumdiolates are stable compounds, many O^2-substituted diazeniumdiolates have been prepared which would decompose under a variety of conditions to regenerate the unsubstituted NO releasing moieties.[7] In our ongoing research, we focused on the development of O^2-protected diazeniumliolates.

The cytotoxicity of NO to viruses is an exciting area which can potentially provide better understanding of human immunity to viral infections as well as provide more information on antiviral drug designs.[8] The influenza virus is especially attractive to 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 influenza virus to bind and cut the terminal sialic acid, respectively. Neuraminidase (a type of sialidase)

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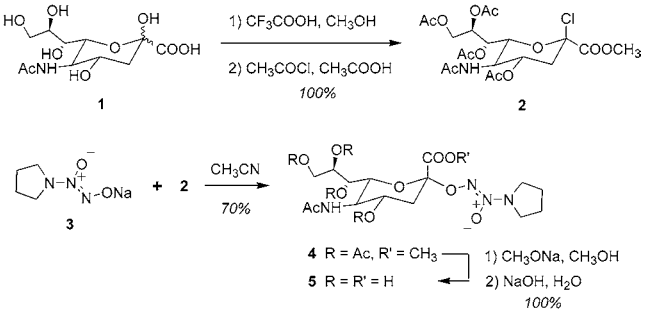
can catalyze the hydrolysis of the α-*O*-glycosidic linkage of *N*-acetylneuraminic acid (NeuAc, **1**) connected to a variety of aglycons on the cellular surface.[10] We have tried to synthesize *N*-acetylneuraminic acid−NO donor conjugates as novel NO prodrugs targeting influenza viruses. If these NO donors could bind and be hydrolyzed by influenza neuraminidases, the NO will be released around viruses and obtain the targeting effect. Our proposed synthetic NeuAc−NO conjugates can also be used as probes to selectively deliver NO to viruses. Such conjugates may also be used to test a neuraminidase-activated prodrug design for antiviral therapy.

PYRRO/NO (**3**), which has a half-life about 3 s, was chosen as a representative of diazeniumdiolates.[11] *N*-Acetylneuraminyl chloride (**2**), which was prepared from *N*-acetylneuraminic acid (**1**), was utilized as the sialadation donor. Acetonitrile was used as the solvent for the reaction, since it is an excellent solvent to obtain the α-anomer. Compound **2** was stirred with **3** in anhydrous acetonitrile at room temperature. The reaction was monitored by TLC. After 2 days, the starting material totally disappeared and there was one product shown on TLC. After column chromatography on silica gel, the α-anomer of sialyl diazeniumdiolate (**4**) was obtained in 70% yield (Scheme 1). Since the

Scheme 1



α-anomer is thermodynamically disfavored and there is no suitable neighboring participation at C-3, it would be difficult to control the anomeric selectivity. However, the result came out successfully. The reason might be the formation of *β*-acetonitrilium ion as an intermediate which provides predominantly α-sialosides.[12] Then deprotection of the acetyl and ester groups afforded the first sialated diazeniumdiolate (**5**). The anomeric configuration was determined as α by the chemical shift of H_{3e} (2.70 ppm).[13]

This novel NO donor (**5**) was water-soluble and very stable in the solid state. It could be stored at room temperature without any decomposition for months. The stability of this novel NO donor was tested in aqueous solutions with different pH. Compound **5** was very stable in both neutral and basic (pH 10, NaOH) solution and did not decompose within several days, while in acidic (pH 1, HCl) solution it showed a half-life about 19.6 h. Additionally, this compound decomposed readily after addition of neuraminidase (EC 3.2.1.18, from *Clostridium perfringens*, Sigma). The decomposition went steadily, as determined by the decay of the absorbance at 255 nm (Figure 1). The enzymatic kinetics

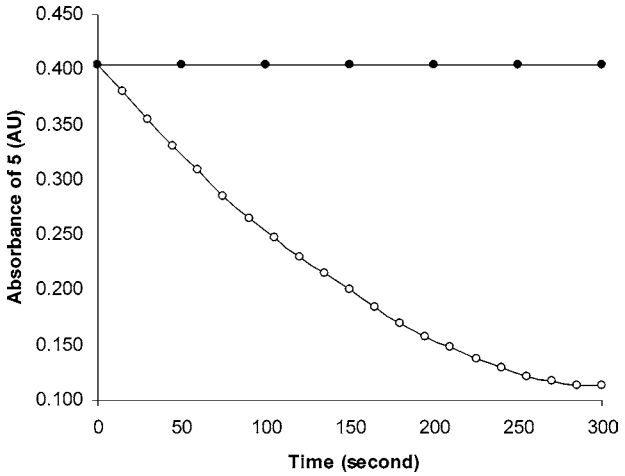


Figure 1. UV absorbance changing of **5** in the presence (○) and in the absence (●) of neuraminidase. Neuraminidase (0.05 mg/mL) and **5** (0.15 mM) were incubated in 150 mM phosphate buffer, pH 7.4, 37 °C. UV was measured at 255 nm.

were measured on a UV−vis spectrophotometer fitted with an electrically thermostated cell block. The Michaelis constant (K_m) was determined from the Lineweaver−Burk plot. The K_m (0.14 mM) was smaller than K_m values (0.6−1.6 mM) for oligosaccharides as substrates,[14] which indicated the binding between **5** and neuraminidase was stronger than the natural sialosides. The k_{cat} was calculated as 1.92 s^{-1}, revealing that the enzymatic hydrolysis was efficient.

The enzymatic decomposition of **5** was also examined by NO measurement. The amount of NO generated could be measured with an electrochemical ISO-NO Mark-II isolated nitric oxide meter (World Precision Instruments, Inc. Sarasota, Florida). As shown in Figure 2, **5** was stable in the buffer solution (pH 5.0), whereas a substantial amount of NO was generated after the addition of neuraminidase. The NO released from PYRRO/NO could not be shown in Figure 2, since its half-life is only 2.8 s, and the NO signal disappeared within 30 s. In aerobic solution, NO is released but it is consumed by the NO/O_2 reaction and dissipates by diffusion from the solutions. This is the reason that only a small amount of NO was detected. In addition, the Griess method[15] also showed the formation of nitrite ion from the enzymatic decomposition of **5**, because the NO was oxidized

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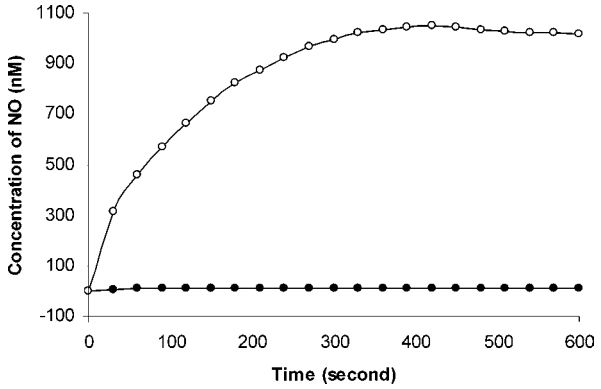


Figure 2. Time course of NO release from **5** in the presence (○) and in the absence (●) of neuraminidase. Neuraminidase (0.05 mg/mL) and **5** (0.2 mM) were incubated in 100 mM acetate buffer, pH 5.0, 25 °C.

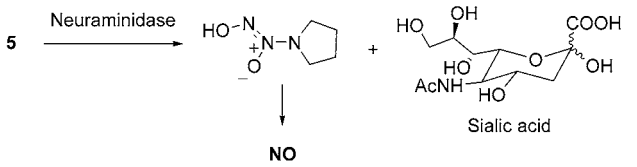
to nitrite by oxygen from air through a cascade of reactions[16] (Table 1). At pH 7, nitrite formation from **5** was comparable with that of **3** after 0.5 h at 37 °C, while no nitrite was formed from **5** without the enzyme. In addition, **5** could also slowly release NO in acidic solution. The two assays confirmed the decomposing pathway of **5** (Scheme 2). Neuraminidase first hydrolyzes the glycosidic bond, and then the diazenium diolate automatically decomposes to release NO.

Table 1. Nitrite Formation from **5** and **3**[a]

substrate (23 μM)	[NO_2^-] (μM)
3	5.6
5	negligible
5 + neuraminidase	4.1
5 in HCl (pH 1)	0.2

[a] The amount of nitrite released was measured using the Griess method. Neuraminidase (0.05 mg/mL) and **5** (23 μM) were incubated for 0.5 h in 150 mM phosphate buffer, pH 7.4, 37 °C. UV absorbance was measured at 548 nm.

Scheme 2. NO-Releasing Mechanism of **5**



Since sialic acids and enzymes that metabolize them have been implicated in the pathogenicity of a number of infectious microorganisms,[17] this class of NO donors would have great potential in biomedical applications. Further development of this class of novel NO donors is currently underway.

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Supporting Information Available: Synthetic procedures and characterization data for compounds **2**−**5** and enzyme assay methodology. This material is available free of charge via the Internet at http://pubs.acs.org.

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