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Glycosylated PROLI/NO Derivatives as Nitric Oxide Prodrugs

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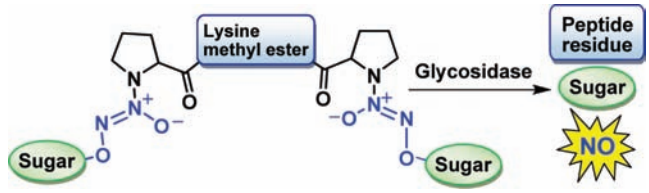
ABSTRACT



GlcNAc-PROLI/NO prodrugs that are activated by *N*-acetylglucosaminidase to release nitric oxide (NO) are described. A classical acid−amine coupling is used to bifunctionalize these PROLI/NO prodrugs, which on activation generate up to 4 mol of NO, a peptide residue, and an *N*-acetylglucosamine residue. Many of the prodrugs synthesized are efficient sources of intracellular NO.

Nitric oxide (NO) plays a vital role in many physiological processes such as blood pressure regulation, platelet aggregation, immune response, and neurotransmission.[1] Hence, NO-releasing drug development is emerging as a promising area.[2] Among different NO donors, diazeniumdiolate anions are reliable and efficient sources of NO. These compounds under physiological conditions release up to 2 mol equiv of NO, with well-defined half-lives varying from 2 s to 20 h.[3] Diazeniumdiolate anions are often transformed to O^2-substituted derivatives (diazeniumdiolate prodrugs), which upon activation by a metabolic trigger or hydrolysis generate NO through the intermediacy of the parent anion. This prodrug approach helps in site-directed delivery of NO. The literature is replete with examples of diazeniumdiolate prodrugs that are activated by glutathione,[4] cytochrome P450,[5] and esterases.[6] Although many of these prodrugs have significant biological activities and efficiently release NO on activation, they also form secondary amines and cleaved protecting groups that may not always be biocompatible and nontoxic (Figure 1).

Our goal was to design, synthesize, and evaluate diazeniumdiolate prodrugs that can be metabolically activated to efficiently release NO and whose degradation products are normal mammalian metabolites. These prodrugs could have potential applications in targeting NO for therapeutic benefit and could also be excellent research tools to study NO-dependent signaling mechanisms.

O^2-Glycosylated diazeniumdiolates[7−9] (Figure 1, R = sugar residue), upon activation by corresponding glycosidases, produce NO, a sugar, and a secondary amine. For example, O^2-glycosylated PYRRO/NO compounds would

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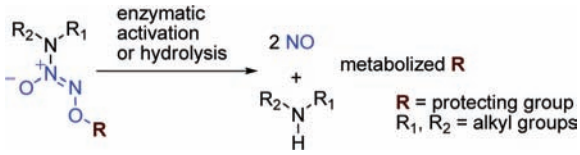

Figure 1. Diazeniumdiolate prodrugs releasing NO and potentially nonbiocompatible metabolites on activation.

generate NO, the cleaved sugar unit, and pyrrolidine (Figure 2). While the glycoside protecting group released should not

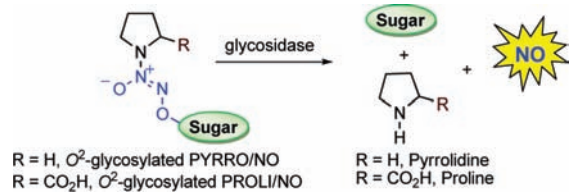

Figure 2. O^2-Glycosylated PYRRO/NO, O^2-glycosylated PROLI/NO, and their corresponding metabolites on activation.

be toxic to the host, the secondary amine (pyrrolidine) generated may have toxic effects.

The diazeniumdiolate of proline (PROLI/NO),[10] on decomposition, forms NO and proline, which is a natural amino acid and biocompatible metabolite. Thus, O^2-glycosylated PROLI/NO prodrugs on degradation would form proline, NO, and a sugar (Figure 2). Morever, PROLI/NO has a carboxylic acid functional group that can be synthetically manipulated for future drug development, including increased payload of NO.

Glucose was the first choice among all possible sugar protective groups due its status as an inexpensive and commercially available substrate for glycosylation. Thus, O^2-glucosylated-PROLI/NO peracetate was synthesized using a reported protocol.[11] Interestingly, the literature suggests that esters of PROLI/NO prodrugs have better cell permeability than their free carboxylic acid counterparts.[12] The cell permeabilities are determined by comparing intracellular NO release by the compounds using the nitric oxide-sensitive fluorophore, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate).[13] Accordingly, the O^2-glucosylated-PROLI/NO peracetate was transformed to its methyl ester, which was further deacetylated (see Supporting Information for details). However, to our surprise, O^2-glucosylated-PROLI/NO peracetate,[11] its methyl ester, and O^2-glucosylated-PROLI/NO methyl ester (deacetylated derivative) did not show a significant amount of intracellular NO release by DAF-FM assay (data in Supporting Information). This indicated that either the prodrugs were not cell-permeable or they were not activated intracellularly to release NO during the course of the experiment. Thus, our strategy to develop cell-permeable and biocompatible nitric oxide donors had to be revised. A recent report from our group demonstrated that *N*-acetylglucosamine (GlcNAc)-protected diazeniumdiolate prodrugs are activated by their corresponding *N*-acetylglucosaminidase.[9] Therefore, we envisaged GlcNAc-PROLI/NO and its analogues as cell-permeable and biocompatible NO donor candidates.

The synthesis of GlcNAc-PROLI/NO began with the treatment of an acetone solution of 1-chloro-*N*-acetylglucosamine triacetate to the previously reported compound **1**[11] to give **2** in 38% yield (Scheme 1). The primary alcohol was oxidized by a modified Sharpless protocol (ruthenium trichloride and sodium periodate) to afford GlcNAc-PROLI/NO peracetate **3** in 49% yield. The deacetylation of **3** using 3 equiv of sodium methoxide in methanol gave GlcNAc-PROLI/NO **4**. Both the compounds **3** and **4** were converted to their methyl esters **5** and **6**, respectively, using diazomethane in ether in good yields (Scheme 1).

The next task was to bifunctionalize the PROLI/NO prodrug, with the aim of generating up to 4 mol of NO, thus doubling the theoretical payload. The classical carboxylic acid and amine coupling was the first choice to be explored. There are several coupling agents commercially available to execute such coupling reactions.[14] The coupling of **3** and ethylenediamine was accomplished with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′- tetramethyluronium hexafluorophosphate (HATU), 1- hydroxy-7-azabenzotriazole (HOAt), and Hünig's base (DIPEA) to afford **7** in 55% yield. Compound **7** was deacetylated in 62% yield to afford **8** (Scheme 1). Furthermore, the ethylenediamine, a non-natural amine linker, was replaced with the naturally occurring diamine, L-lysine. The carboxylic acid in lysine could be advantageous as a synthetic handle for further drug development.[11] The desired bivalent peracetate **9** was synthesized by reaction between acid **3** and lysine methyl ester dihydrochloride using the protocol developed for compound **7**. Removal of the acetyl protection of **9** furnished the bis-PROLI/NO with lysine methyl ester linker **10** in 69% yield (Scheme 1). The methyl ester may generate methanol which can be toxic to the cells. However, the methyl ester derivatives can be replaced by the ethyl counterparts, to overcome any methanol-related toxicity in future investigations.

To demonstrate the generality of this approach, we replaced the PROLI/NO moiety of these prodrugs by SARCO/NO (Figure 1, $R_1 = CH_2CO_2H$; $R_2 = Me$; $R = Na$).

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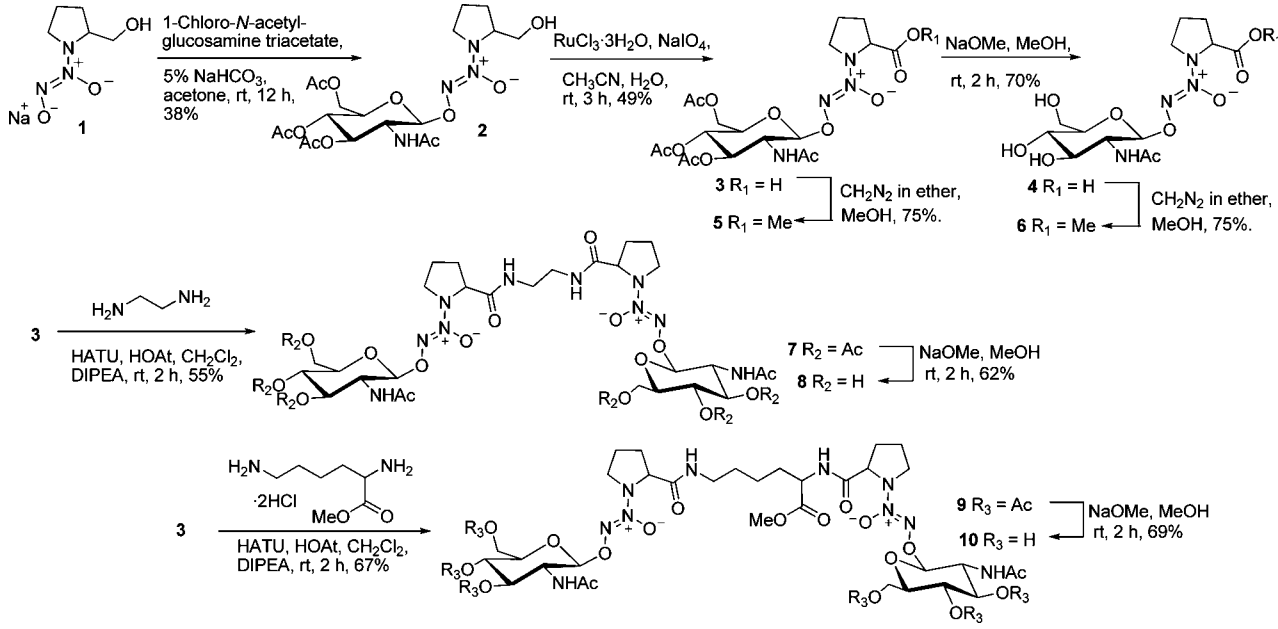
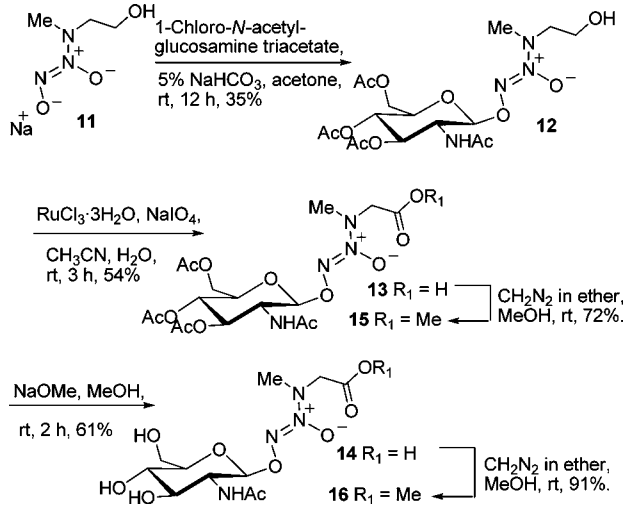
Scheme 1. Synthesis of GlcNAc-PROLI/NO Derivatives



GlcNAc-SARCO/NO, when metabolized, would produce sarcosine (*N*-methyl glycine) along with GlcNAc and NO. Sarcosine is a nontoxic amino acid found in muscles and other tissues and is often used in manufacturing biodegradable surfactants and toothpaste.[15] The synthesis began with **11**[16] and involved reaction conditions similar to those of their PROLI/NO counterparts resulting in comparable yields (Scheme 2). Efforts are underway to synthesize the bis-GlcNAc-SARCO/NO derivatives with different linkers.

Scheme 2. Synthesis of GlcNAc-SARCO/NO Compounds



The *N*-acetylglucosaminidase-activated NO release was determined by chemiluminescence assay. Compounds **4**, **6**, **14**, and **16** are expected to release 2 mol of NO per mole of compound. Compound **6** displayed quantitative NO release over the period of experiment (ca. 60 min), whereas the compounds **4**, **14**, and **16** displayed between 50% and 75% NO-release (Table 1). The bivalent compounds **8** and **10** are expected to generate 4 mol of NO. Compounds **8** and **10** released 88% and 82%, respectively, of NO on activation by *N*-acetylglucosaminidase. Thus, the chemiluminescence assay using commercially available *N*-acetylglucosaminidase is a proof of concept for the hypothesis of enzymatic activation to release NO.[17]

Table 1. Extent of NO Generation on Hydrolysis of Compounds under Catalysis by *N*-Acetylglucosaminidase Isolated from Jack Bean

compound	NO released (mol/mol)	% NO
4	1.31	66[a]
6	2.00	100[a]
14	1.00	50[a]
16	1.49	75[a]
8	3.51	88[b]
10	3.28	82[b]

[a] 2 mol of NO equals 100% yield. [b] 4 mol of NO equals 100% yield.

The ability of these prodrugs to deliver NO intracellularly was determined by DAF-FM assay on normal human skin

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(17) Concentration of the activating enzyme has an important role in NO yield. However, it is difficult to determine exactly which enzyme, including concentration, is involved for the activation of these prodrugs inside the cell. Hence, the chemiluminescence assay and intracellular NO-release by DAF-FM assay may have different NO yields.

fibroblast BJ-5ta cells. Most of the compounds synthesized were tested for DAF-FM activity to evaluate their intracellular NO release. Some of the compounds displayed significant NO release over the control, highlighting their potential to deliver intracellular NO (Figure 3). However, a few

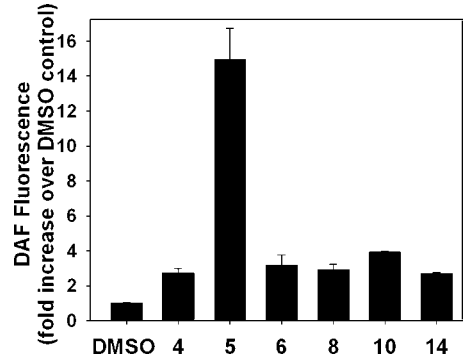

Figure 3. Levels of intracellular nitric oxide formation upon treatment of BJ-5ta cells with compounds (100 μM final concentration) and DMSO (control) as determined by DAF-FM diacetate fluorescence study.

compounds displayed DAF fluorescence comparable to that of the control (data for these compounds are not shown in Figure 3), indicating that either these compounds are not cell permeable or the cells are not equipped to activate these compounds to release NO during the course of the experiment. The intracellular NO release during the course of the experiment by compound **5** was noteworthy and may be the result of its very high cell permeability and/or fast hydrolysis to release NO. Efforts are underway to reveal the mechanistic origin of this high intracellular NO release by compound **5**.

Thus, a series of novel GlcNAc-PROLI/NO compounds were synthesized. The synthetic strategy developed was successfully extended to prepare the SARCO/NO analogues and can, in principle, be further extended to tether these compounds to a variety of dendrimers, fluorescent tags, or peptides for future drug development. These GlcNAc compounds upon activation by jack bean enzyme efficiently released NO. The bis-PROLI/NO conjugates doubled NO payload accompanied by plausibly biocompatible metabolites. The DAF-FM assay conducted on normal cells demonstrated cell permeability and their ability to generate intracellular NO. Two previously reported O^2-glycosylated diazeniumdiolate prodrugs are lead antileishmanial compounds.[9] Efforts are underway to arrange further biological screening of the new Glc-NAc compounds.

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Supporting Information Available: Preparative and cell culture procedures, analytical data, and NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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