Pergamon

Tetrahedron Letters 42 (2001) 3779–3782

TETRAHEDRON LETTERS

Glycosylated diazeniumdiolates: a novel class of enzyme-activated nitric oxide donors

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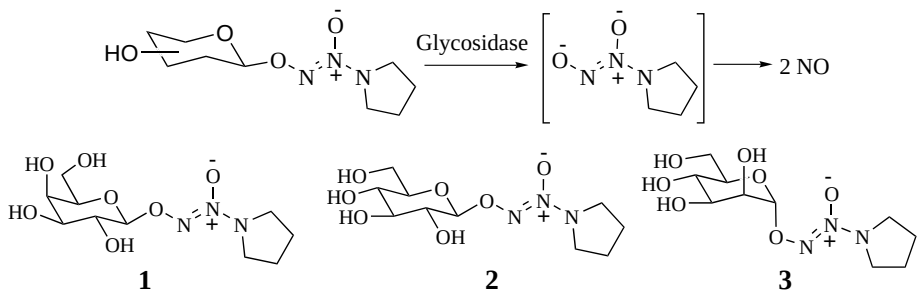
Received 2 April 2001; accepted 9 April 2001

Abstract—Synthetic procedures have been developed to attach the nitric oxide releasing diazeniumdiolate functional groups $[N(O)NO]^-$ to a carbohydrate unit. These glycosylated diazeniumdiolates exhibited significantly improved stability as compared to their parent diazeniumdiolate salts, yet they could readily release nitric oxide upon activation by glycosidases. Preliminary antitumor screen assay demonstrated that this class of compounds had antitumor activity. © 2001 Published by Elsevier Science Ltd.

Diazeniumdiolates are mainly salts containing the anionic $[N(O)NO]^-$ structural unit, and they can spontaneously release nitric oxide (NO) under physiological conditions (pH 7.4, 37°C) with a range of half lives from a few seconds to several days.[1–4] It has been shown that this class of NO donors could display vasorelaxant,[5] antithrombotic,[6] cytostatic,[7] and genotoxic[8] activities. However, in order to target a specific tissue with those compounds, careful protection at the terminal oxygen (O^2) is necessary. A number of different O^2-protected diazeniumdiolates have been reported by Keefer's group.[9–11] For example, O^2-vinyl diazeniumdiolates were reported to be liver-selective and activated by hepatic cytochrome P450,[10] whereas O^2-acetoxymethyl diazeniumdiolates were shown to be esterase-sensitive and to exhibit significant antileukemia activity.[12] Recently, we have reported a series of peptide–diazeniumdiolate conjugates which could be activated by α-chymotrypsin or prostate specific antigen.[13]

In this work we wish to report another series of enzyme-activated compounds: glycosylated diazeniumdiolates, which would release NO in the presence of certain glycosidase (Scheme 1). Moreover, we have demonstrated that sugar conjugated *S*-nitroso-*N*-acetylpenicillamine (SNAP) can be accumulated preferentially inside cells according to the expression of specific sugar transporters.[14–16] For example, Glu-2-SNAP was found to be 5000-fold more potent than SNAP in killing A2780S human ovarian cancer cells.[16] The enhanced cytotoxicity can be explained by the expression of GLUT-1 transporter in the cancer cells. Therefore, it is expected that glycosylated diazeniumdiolates could be easily transported into the cells because of the presence of sugar transporters in the cell membranes.

Synthesis: Three glycosylated diazeniumdiolates (**1**–**3**) have been obtained by anchoring the pyrrolidinyl dia-



Scheme 1. Glycosylated diazeniumdiolates derived from D-galactose (**1**), D-glucose (**2**) and D-mannose (**3**).

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PII: S0040-4039(01)00614-1

zoniumdiolate anion to D-galactose, D-glucose and D-mannose. They could be prepared from the coupling of acetylated glycopyranosyl bromide with sodium salt of pyrrolidinyl diazoniumdiolate, followed by deprotection with sodium methoxide in methanol (Scheme 2). It usually took several days to complete the glycosylation reaction, with only one anomer obtained. For example, the β-anomers **4** and **5** were obtained from 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, respectively. However, only α-anomer **6** was obtained from 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide. It is also interesting to note that the acetyl group at C2 was removed during the glycosylation reaction, forming tri-*O*-acetyl mannosyl diazeniumdiolate **6**. The steric configuration of the anomeric carbon was assigned by the $^{3}J_{H\text{-}H}$ coupling constants and further confirmed by NOESY analysis.

Stability: The solid glycosylated diazeniumdiolates are very stable, no decomposition was detected even if the compounds were let stand in the air for one month at room temperature. Unlike their parent diazeniumdiolate salts, these compounds were also very stable in neutral or acidic aqueous media. No change was observed after several hours as determined by UV–vis spectrophotometric experiments. However, these compounds decompose readily after the addition of certain glycosidases. When a solution of **1** (0.12 mM) at pH 5.6 was treated with β-D-galactosidase (13 mg/mL), its characteristic chromophore at 256 nm disappeared slowly with respect to time (Fig. 1). A half-life was obtained as 6.0 min by assuming pseudo-first-order kinetics. Similarly, the half lives of **2** and **3** were determined as 24.1 and 7.7 min, respectively.

NO measurements: The enzymatic hydrolysis of the compounds was also examined by NO measurements. The amount of NO generated could be quantitatively measured with an electrochemical ISO-NO Mark-II isolated nitric oxide meter (World Precision Instruments, Inc. Sarasota, Florida). In the experiment, each substrate was dissolved in a buffer solution (acetate, pH 5.6) to a final concentration of 0.33 mM. After a baseline was recorded, about 100 μL of an aqueous solution of glycosidase was injected into the substrate via a syringe and the change of the signals was recorded afterwards. Three different glycosidases, i.e. β-glucosidase (G-0395), β-galactosidase (G-5160) and α-mannosidase (M-7257), were purchased from Sigma and used without further purification. As shown in Fig. 2, the glycosylated diazeniumdiolates were stable in the buffer solution (acetate, pH 5.6), whereas substantial amount of NO would be generated after the addition of glycosidase.

Biological activity: The antitumor activity of compounds **2** and **5** were conducted by a soft-agar colony-formation disk-diffusion assay,[17] where each compound was simultaneously tested against solid tumor (mouse Panc-03 and Colon-38, human 15 and 116), leukemia cells (L1210), and normal cells (fibroblast). Both compounds exhibited broad-spectrum cytotoxicity against cancer lines, and the per-acetylated compound **5** appeared to be 10 times more potent than **2**.

Typical synthetic procedure: 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide (4.03 g, 9.8 mmol) was dissolved in 50 mL of anhydrous acetonitrile, to which was added 1.5 equivalents of sodium pyrrolidinyl diaze-

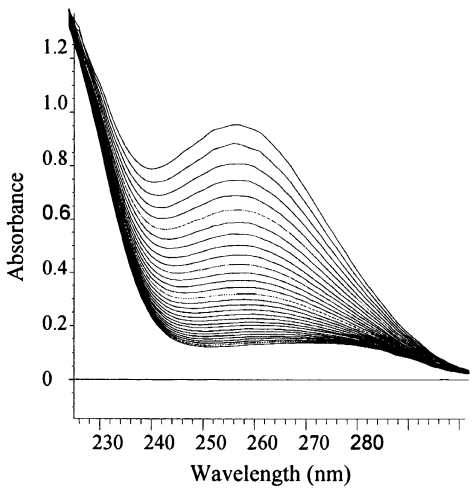
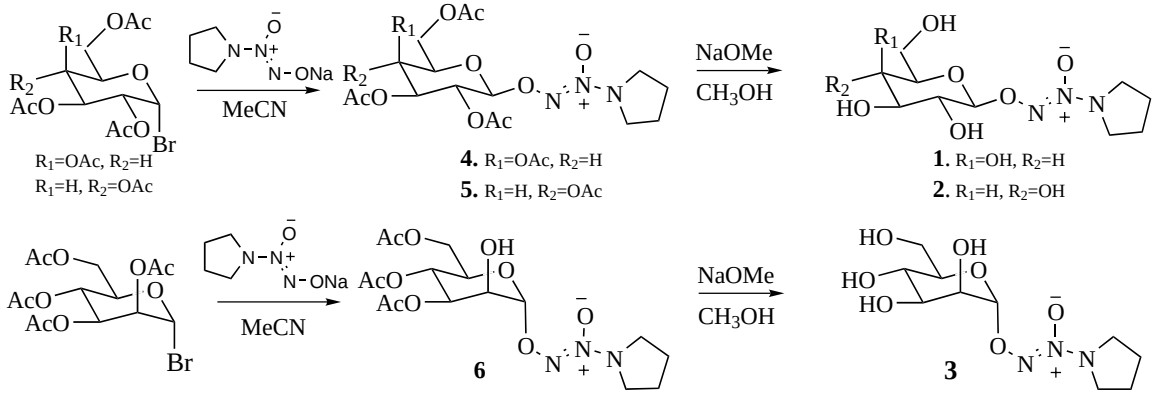


Figure 1. UV–vis spectral changes versus time after the addition of galactosidase (13 mg/mL) to a solution of **1** (0.12 mM) at pH 5.6.



Scheme 2. Synthesis of glycosylated diazeniumdiolates (**1**–**3**).

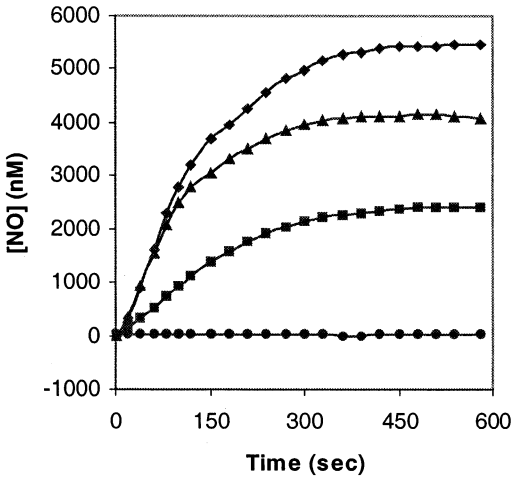


Figure 2. Enzymatic release of NO from an aqueous solution of substrate (0.33 mM, pH 5.6) in the presence and absence of its corresponding glycosidase (◆) **1**+4.8 μg/mL of β-D-galactosidase; (▲) **3**+2.0 μg/mL of α-D-mannosidase; (■) **2**+38.0 μg/mL of β-D-glucosidase; (●) **1** only.

niumdiolate (2.25 g, 14.7 mmol). The reaction mixture was stirred for 2 days at room temperature until starting material was totally consumed as indicated by TLC. Then the mixture was concentrated, and the residue was dissolved in 50 mL of ethyl acetate. The organic solution was washed with water (50 mL×3) and dried over anhydrous Na_2SO_4. After removal of the solvent, the crude glycosylation product was purified by silica gel column chromatography eluted with a solvent mixture of ethyl acetate and hexane. The product **4** (3.12 g, 69%) was obtained as a colorless syrup.

Compound **4** (0.326 g, 0.71 mmol) was dissolved in 15 mL of anhydrous methanol, to which was added sodium methoxide until pH about 8–9. The reaction mixture was stirred for 2 h at room temperature. After silica gel column chromatography eluted with a solvent mixture of methylene chloride and methanol, compound **1** (160 mg, 77%) was obtained as a white solid.

Characterization: O^2-β-Galactopyranosyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**1**): ^{1}H NMR (400 MHz, CD_3OD): δ 4.93–4.90 (d, 1H, H-1), 3.86 (d, 1H, J=2.4 Hz, H-4), 3.77 (dd, 1H, J=9.7, 8.1 Hz, H-2), 3.76–3.69 (m, 2H, H-6), 3.64–3.59 (m, 1H, H-5), 3.59–3.54 (m, 4H, H-1′ and H-4′), 3.54 (dd, 1H, J=9.7, 2.4 Hz, H-3), 1.99–1.94 (m, 4H, H-2′ and H-3′); ^{13}C NMR (100 MHz, CD_3OD): δ 104.19 (C-1), 76.12 (C-3), 73.61 (C-5), 69.26 (C-2), 68.83 (C-4), 61.10 (C-6), 50.69 (C-1′ and C-4′), 22.64 (C-2′ and C-3′). UV λ_{max} (pH 5.6, ε): 256 nm (8.39 mM^{-1} cm^{-1}); MS (ESI): m/z 316 $[M+Na]^+$, 609 $[2M+Na]^+$. HRMS (FAB) calcd for $C_{10}H_{19}N_3NaO_7$ $[M+Na]^+$: 316.1121. Found: 316.1132.

O^2-β-D-Glucopyranosyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**2**): ^{1}H NMR (500 MHz, CD_3OD): δ 4.97 (d, 1H, J=7.6 Hz, H-1), 3.85 (d, 1H, J=12.7 Hz, H-6), 3.68 (dd, 1H, J=11.9, 5.1 Hz, H-6), 3.60–3.50 (m, 4H, H-1′ and H-4′), 3.44 (dd, 1H, J=7.6, 2.5 Hz, H-2), 3.44 (m, 1H, H-4), 3.38–3.32 (m, 2H, H-5 and H-3), 2.00–1.90 (m, 4H, H-2′ and H-3′); ^{13}C NMR (CD_3OD, 125 MHz): δ 103.54 (C-1), 77.33 (C-5), 76.61 (C-2), 71.92 (C-4), 69.80 (C-3), 61.24 (C-6), 50.72 (C-1′ and C-4′), 22.66 (C-2′ and C-3′). UV λ_{max} (pH 5.6, ε): 255 nm (7.84 mM^{-1} cm^{-1}); MS (ESI): m/z 316 $[M+Na]^+$, 609 $[2M+Na]^+$. HRMS (FAB) calcd for $C_{10}H_{19}N_3NaO_7$ $[M+Na]^+$: 316.1121. Found: 316.1125.

O^2-α-D-Mannopyranosyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**3**): ^{1}H NMR (500 MHZ, CD_3OD): δ 5.44 (d, 1H, J=2.0 Hz, H-1), 3.99 (dd, 1H, J=3.0, 2.0 Hz, H-2), 3.81–3.78 (m, 1H, H-6), 3.79 (dd, 1H, J=9.6, 3.0 Hz, H-3), 3.73–3.69 (m, 1H, H-6), 3.69 (t, 1H, J=9.6 Hz, H-4), 3.60–3.56 (m, 1H, H-5), 3.57–3.53 (4H, H-1′ and H-4′), 1.98–1.94 (m, 4H, H-2′ and H-3′); ^{13}C NMR (125 MHz, CD_3OD): δ 101.88 (C-1), 74.92 (C-5), 71.10 (C-3), 69.28 (C-2), 66.95 (C-4), 61.43 (C-6), 50.73 (C-1′ and C-4′), 22.64 (C-2′ and C-3′). UV λ_{max} (pH 5.6, ε): 256 nm (8.00 mM^{-1} cm^{-1}); MS (ESI): m/z 316 $[M+Na]^+$, 609 $[2M+Na]^+$. HRMS (FAB) calcd for $C_{10}H_{19}N_3NaO_7$ $[M+Na]^+$: 316.1121. Found: 316.1112.

O^2-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl) 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**4**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.47 (dd, 1H, J=10.1, 8.6 Hz, H-2), 5.36 (dd, 1H, J=3.6, 1.0 Hz, H-4), 5.09 (d, 1H, J=8.6 Hz, H-1), 5.04 (dd, 1H, J=10.1, 3.6 Hz, H-3), 4.14 (dd, 1H, J=15.7, 6.6 Hz, H-6), 4.11 (dd, 1H, J=15.7, 7.1 Hz, H-6), 3.99 (ddd, 1H, J=7.1, 6.6, 1.0 Hz, H-5), 3.60–3.51 (m, 4H, H-1′ and H-4′), 2.11 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.95 (s, 3H, CH_3), 1.94–1.91 (m, 4H, H-2′ and H-3′); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.62 (C=O), 170.41 (C=O), 170.32 (C=O), 169.34 (C=O), 101.05 (C-1), 71.46 (C-5), 71.28 (C-3), 67.04 (C-2), 66.94 (C-4), 61.32 (C-6), 50.82 (C-1′ and C-4′), 23.22 (C-2′ and C-3′), 20.91 (CH_3), 20.88 (CH_3), 20.82 (CH_3), 20.75 (CH_3). MS (ESI): m/z 484 $[M+Na]^+$, 945 $[2M+Na]^+$.

O^2-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl) 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (**5**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.34 (dd, 1H, J=8.0, 9.5 Hz, H-2), 5.26 (t, 1H, J=9.5 Hz, H-3), 5.16 (d, 1H, J=8.0 Hz, H-1), 5.12 (dd, 1H, J=10.0, 9.5 Hz, H-4), 4.26 (dd, 1H, J=12.5, 4.5 Hz, H-6), 4.15 (dd, 1H, J=12.5, 2.5 Hz, H-6), 3.80 (ddd, 1H, J=10.0, 4.5, 2.5 Hz, H-5), 3.60–3.55 (m, 4H, H-1′ and H-4′), 2.07 (s, 3H, CH_3), 2.02 (s, 6H, $2CH_3$), 2.00 (s, 3H, CH_3), 1.98–1.94 (m, 4H, H-2′ and H-3′); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.64 (C=O), 170.17 (C=O), 169.34 (C=O), 169.07 (C=O), 100.25 (C-1), 72.88 (C-3), 72.47 (C-5), 69.12 (C-2), 67.87 (C-4), 61.72 (C-6), 50.58 (C-1′ and C-4′), 23.00 (C-2′ and C-3′), 20.69 (CH_3), 20.60 (CH_3), 20.54 ($2CH_3$). MS (ESI): m/z 484 $[M+Na]^+$, 945 $[2M+Na]^+$.

Acknowledgements

The authors would like to thank Dr. Thomas H. Corbett in the Barbara Ann Karmanos Cancer Institute for antitumor assay. This work is generously supported by a research grant from NIH (GM 54074).

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